MAGYAR AGRÁR- ÉS ÉLETTUDOMÁNYI EGYETEM

Combined application of biocontrol microbes and Funneliformis mosseae increases phenolic compound and resistance to grey mold infection on okra plants and increases Glutathione S-transferases (GST) enzyme activity and plant growth

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1 - INTRODUCTION

The use of microbial control agents as alternatives to synthetic pesticides has emerged as a significant strategy for managing insect pests and plant diseases, offering outcomes comparable to those of chemical pesticides. This shift towards biological control agents and biofertilizers necessitates persuasive evidence and practical guidance to encourage adoption, thereby reducing reliance on harmful chemicals. Symbiotic interactions between microorganisms and crop plants have demonstrated the potential to enhance plant tolerance to pathogens and to foster optimal growth and development (Kour et al., 2020). Arbuscular mycorrhizal fungi (AMF) play a pivotal role in promoting environmentally sustainable agriculture, with approximately 72% of vascular plants associated with them. AMF aid in nutrient absorption, promote litter decomposition, and produce compounds that are beneficial for plant health, contributing to ecosystem biogeochemical processes. Additionally, incorporating Trichoderma spp. into compost extracts enhances biocontrol activity, further supporting the effectiveness of biocontrol measures (Poveda and Eugui, 2022). *Trichoderma asperellum*, *Aureobasidium pullulans*, and *Streptomyces griseoviridis* are among the biological agents that have shown promise in enhancing plant resistance to pathogens and promoting growth (Subramaniam, Arumugam and Rajendran, 2016; Guzmán-Guzmán et al., 2019; Zin and Badaluddin, 2020). These microorganisms modify the rhizosphere microbiome, produce bioactive compounds, and establish beneficial relationships with host plants, thereby contributing to improved plant health and stress management.Tomatoes, okra, and sweet potatoes, which are essential crops, face challenges from various stressors, necessitating sustainable production systems.

This problem stems from the necessity of reducing reliance on synthetic fertilizers and pesticides, aligning with the principles of sustainable agriculture (Vega, 2018). Understanding and leveraging plant-microbe interactions can lead to sustainable agricultural solutions that enhance plant development, mitigate infections, and increase resilience to environmental stressors, thereby addressing the multifaceted challenges facing global agriculture (Sambangi, Srinivas, & Gopalakrishnan, 2021). A multidisciplinary approach holds

significant potential for devising environmentally sustainable and effective strategies to address these challenges.

In conclusion, the combined application of biocontrol microbes and AMF represents a sustainable approach to agriculture by enhancing plant resistance to pathogens, increasing phenolic compound levels, and promoting plant growth. This integrated approach offers potential benefits to agricultural and ecosystem health.

Research objectives :

1- Determination of the effect of microbial biocontrol agents (BCA) on the efficacy of mycorrhiza inoculation in okra, tomato and sweet potato,

2- Testing and comparison of the effect of combined BCA and mycorrhiza inoculation on the vegetative growth and yield of three vegetable species,

3- Testing the effect of combined treatments on the enzyme (GST) activity of okra is related to disease resistance.

4-Testing the effect of the combined treatment and HPLC analysis of okra fruits indicated a noticeable increase in the concentration of phenolic compounds, which was attributed to the interaction between antagonist agents and mycorrhiza.

2 - MATERIALS AND METHODS

2.1 Microbes used

1. Arbuscular mycorrhiza (AM) were identified as *Funneliformis mosseae* (Glomerales and Glomeraceae).
2. *Trichoderma asperellum* strain T34 is a filamentous commercial product.
3. *Aureobasidium pullulans (AUREPU)*, a yeast-like fungus
4. *Streptomyces strain K61*, previously *known as S. griseoviridis*

2.2 Test plants

The three cultivars used in this experiment were as follows.

1. The seeds of okra (Betera), scientifically identified as *Abelmoschus esculentus* L. (Moench), were obtained from Agrimax.
2. The Tomato cultivar, Moneymaker, was identified as *Solanum lycopersicum* L
3. The sweet potato (*Ipomoea batatas* (L.) Lam.)

2.3 In vivo experiment

Soil type: sandy soil mixed with soil horticulture (3/1) (v/v); pots containing 2 kg of dry sandy soil mixed with the soil horticulture with one plant per pot. The pots were placed in glasshouse benches in a completely randomized design for each treatment.The experiment set up 12 replacats of tomato plants, 11 replacats of okra plants, and one sex replicat of sweet potato plants.

2.3.1 Botrytis infection of okra

Botrytis cinerea was obtained from naturally infected okra fruit (naturally infected) grown in a greenhouse. Detached okra plant infections were carried out using leaves of comparable age and size (leaves at nodes 3 and 4, counted from the top), selected randomly from the okra plant. To study the pathogenic effects of *B. cinerea* on okra plants, mycelium discs with PDA were placed at the base of the leaves or flowers of okra plants.

2.3.2 Soil inoculation

1. Arbuscular mycorrhiza fungi (*Funneliformis mosseae*)

Arbuscular mycorrhizae were introduced into okra and tomato plants through a meticulous inoculation process involving the addition of (1 g) of mycorrhizal spores per plant. Inoculation was performed by incorporating spores into the top 10 cm of soil adjacent to the root rhizosphere of okra plants. The plant is characterized by the presence of nine or more unfolded leaves on its main shoot, as illustrated in Figure 10. These experimental conditions were meticulously maintained within the controlled environment of a greenhouse, where temperatures ranged between (18-35) °C. This greenhouse setting ensures an ideal milieu for the growth and observation of plants, fostering an environment that closely mimics their natural habitat and facilitates the systematic exploration of the impacts of arbuscular mycorrhiza on plant development and responses under regulated conditions.

*2.**Trichoderma asperellum* **strain (T34)**: was cultured on PDA medium at 25 C (room temperature) for a week; after that a suspension of *T. asperellum* spores was added (20 ml) 9*10^7 spoors to the top of the soil around the plant equally to all the treatments after 20 days of inoculation with mycorrhiza during vaccination the treatments nine or more leaves on the main shoot unfolded of the plant.

*3.**Aureobasidum pullulans*** was grown on a PDA medium at 25 ^{o}C (room temperature) for a week; after that added suspension of *A. pullulans* spores (25 ml), 22*10^7 spoors to the top of the soil around the plant equally to all the treatments after 20 days of inoculation with mycorrhiza during vaccination the treatments nine or more leaves on the main shoot unfolded of the plant.

*4.**Streptomyces griseoviridis*** **(K61)** was cultured on nutrient agar medium at 25 °C (room temperature) for eight days and the suspension was added to the top of the soil around the plant equally to all treatments. After treatment with mycorrhiza, 20 ml of spore solution containing 22,48*107 spores was applied to each plant until 25 ml was utilized.

2.4 Evaluation of Botrytis infection (gray mold)

Within 24 h of infection, Botrytis cinerea onset was observed, with distinct symptoms. Disease progression was tracked by documenting plant symptoms three times during the infection using a standardized evaluation method. This method quantifies the extent of infection using a numerical scale as follows: 1 for leaf infection, 2 for both leaf and stem involvement, and 3 for stem death. The effect of Botrytis cinerea infection was systematically assessed.

2.5 Evaluations of plant growth

i. Plant height

To assess the height of the okra plant, a ruler was used from the base of the stem at the soil level to the apex of the plant, usually the tip of the apical bud. This process, repeated four times over a 10-day interval, is essential for precise monitoring of okra growth.

ii. Fruit weight measurement

The yields of okra and tomatoes can be measured in terms of the quantity of produce harvested per treatment, which is typically expressed in grams. In the case of okra fruit, the yield was collected during the three weeks of fruit production and fruit weighing each time. However, the tomato yield was harvested at the end of the season.

iii. Tuber weight of sweet potato

To accurately determine the weight of the sweet potato roots, a standardized methodology was employed. Sweet potatoes were harvested from the designated plots, typically when they reached maturity, which was typically approximately 100 days post-planting

iv. Okra and tomato plants dry weight measurement

Dry weight was determined by leaving the plant samples in the laboratory for 4-6 weeks, employing controlled drying methods until a constant weight was achieved. This process eliminated moisture from the samples, provided an accurate biomass assessment, and mitigated water content fluctuations.

2.6 Determination of root colonization

1- *Trichoderma asperellum* strain *34* in tree plants(okra, tomato and sweet potato)

Root samples were collected after the plants were inoculated with mycorrhiza species, and the samples were introduced into the top 10 cm of soil near the roots of the plant (Okra, Tomato, and sweet potato) rhizospheres. They were inoculated with the following microbial agents: *T. asperellum* strain 34. After three weeks, root samples were examined to investigate the cooperation of *T. asperellum*. At room temperature, chopped roots were placed on PDA in Petri dishes (9-inch diameter). After two days, Trichoderma colonization of the roots was examined by accounting for Trichoderma.

2- Mycorrhiza (*Funneliformis mosseae)*

Plant roots were randomly sampled to identify mycorrhizal colonization in the roots of the plants (Okra, Tomato, Sweet potato). Internal and external mycorrhizal mycelia in the roots were evaluated by taking the roots to the laboratory and staining them.

Arbuscular mycorrhiza (*Funneliformis mosseae*) in the roots of the okra plant were stained before microscopic examination to facilitate the assessment of root colonization(Phillips and Hayman 1970).

2.6.1 Glutathione-S-Transferase (GST) activity

Plant leaves (the third leaf from the top of the plant, which was healthy) collected from the field experiment were placed in aluminum foil immediately following collection and in liquid nitrogen until transfer to the laboratory, and then placed in a resealable plastic bag and held at approximately -80 C to prevent leaf stress and enzyme change concentration.

Based on the Sigma-Alddrich protocol of Glutathione-S-Transferase (GST.N CS0410) assay kit and Habig, et al. 1974 used to measure GST activity in the plant.

2.6.2 Polyphenol content measurement

1. Sampling

To conduct phenol analysis, the okra fruit was physically harvested by handpicking it every two–three days, commencing with the commencement of the flowering and fruiting periods. It was then preserved in a biotechnology laboratory for MATE at -80 °C.

2. HPLC determination of phenol compounds in okra

Three hundred milligrams (300 mg) of lyophilized (freeze-dried) whole okra fruit were crushed in a crucible mortar in the presence of 1-2 grams of quartz sand. 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 (RK-165-BH Bendelin Sonorex, 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, Poland). The supernatant was decanted 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 and 1% orthophosphoric acid and finally purified through a 0.45µ, 25 mm Cellulose acetate syringe filter before injection into the HPLC apparatus.

HPLC instrument and conditions

A Chromaster Hitachi HPLC instrument containing a Model 5160 gradient pump, Model 5260 autosampler, Model 5310 column oven, and Model 5430 diode-array detector was used with parad_KB0_2dat software for operation and data processing.

Phenolic compounds were separated on an Ascentis phosphor-conditioned C18 phase (C18-PCP, Supelco, USA) with a gradient elution of 1% ortho-phosphoric acid (A) and acetonitrile (B) according to a recently developed protocol (under publication).

3 -RESULTS

4.1 Effect of arbuscular mycorrhiza (*Funneliformis mosseae)* and antagonists treatment on Botrytis infection okra plant

This study aimed to evaluate the effectiveness of microbial inoculants, specifically *S. griseoviridis, T. asperellum,* and *A. pullulans*, as biological control agents (MBCA), against *B. cinerea* infection in okra plants. It was found that the volatiles produced by T. griseoviridis and *A. pullulans* inhibit the growth of B. cinerea. The results revealed significant differences in okra plant resistance to *B. cinerea* infection among various treatments. The combination of arbuscular mycorrhizal fungi (AMF) with *T. asperellum* (M+T34), AMF with A. pullulans (M+Ap), and mycorrhiza with *S. griseoviridis* (M+Sg) showed the highest levels of plant tolerance, reducing infection rates to 89%, 89%, and 75%, respectively, compared to the control group. Combining AMF with *T. asperellum*, *S. griseoviridis*, and *A. pullulans* significantly enhanced plant tolerance to *B. cinerea* infection, which can be attributed to mechanisms that promote systemic plant resistance Effects of mycorrhiza and antagonists on plant growth.

4.1.1 Effect plant height:

1- okra plant height

okra plant height measurements combined with AMF with *T. asperellum* (M+T34), AMF with *S. griseoviridis* (M+Sg), and AMF with *A. pullulans* (M+AP) exhibited significantly greater plant heights than the control treatment. Conversely, treatments with *T. asperellum* (T34), AMF alone (M), and *S. griseoviridis* (Sg) showed a slight increase in plant height compared with the control condition. To the best of our knowledge, this is the first study to combine biological agents with AMF. The increases in plant height were (M+Sg)=52%, (M+AP)=42.5%, T34=15%, M=11%, and AP=11%. Hence, the combined treatments significantly increased plant height, whereas treatments with antagonistic agents alone slightly increased plant height.

2- Tomato plant height.

tomato plant height combination treatments involving arbuscular mycorrhiza and other treatments. The introduction of antagonist agents yielded diverse

effects, as evidenced by the mycorrhiza (*F. mosseae*) (M) alone or in combination with biological agents: M + *T. asperellum* (M+T34), M + *S. griseoviridis* (M+Sg), and M + *A. pullulans* (M+Ap). Additionally, the *T. asperellum* (T34) treatment displayed a slight enhancement in plant height compared to the control treatment.

3- Sweet potato plant shoot length

Treatment of sweet potato plants with arbuscular mycorrhiza (*F. mosseae*) alone (M) or in combination with *S. griseoviridis* (M + Sg) increased shoot length relative to the control treatment. These findings imply that synergistic application of arbuscular mycorrhiza (*F. mosseae*) and *T. asperellum* (34) may be effective in fostering sweet potato growth. Moreover, the utilization of arbuscular mycorrhiza (*F. mosseae*) alone and in combination with *S. griseoviridis* also had favorable effects on plant growth.

4.1.2 Effect on the Yield:

Okra yield : The data obtained from the experimental trials revealed discernible patterns in the yield weight of okra plants subjected to Mycorrhiza (*F. mosseae*) inoculation and combination with biological agents: Mycorrhiza (*F. mosseae)* (M), *T. asperellum* T34 (T34), *A. pullulans* (Ap), *S. griseoviridis* K61 (Sg), and combination of Mycorrhiza (*F. mosseae*) with biological agents. (M+T 34), (M+A p), and (M+S g). Specifically, the weight of the okra fruits ranged from combined to single treatments, M+T34 = 75%, M+Sg =70%, and M+Ap = 53%, (Sg) = 43%, (M) = 41% (T34) = 39%. The results offer M+T34, M+Sg, Sg, M, T34, and M+Ap insights into the potential influence of mycorrhizal associations and antagonist agents on the overall okra yield. combinations and individual treatments significantly increased okra yield compared to the control.

Tomato yield: This study investigated the effects of combining various microbes with mycorrhiza (*F. mosseae*) (M) treatment on okra plant yield. The combinations examined were M+T34 (*T. asperellum*), M+Sg (*S. griseoviridis*), and M+Ap (*A. pullulans*). The results indicated significant variability in okra fruit weight across the treatments. M+Ap, M+T34, and M+Sg, respectively, showed significantly increased yields compared to the control, as well as with individual treatments (M and T34), showing improvement in tomato yield. However, Ap and Sg did not show any yield.

Sweet potato yield: The results depicted in Figure 28 provide insights into the variations in yield observed across various treatments administered to sweet potato plants, particularly when arbuscular mycorrhizal fungi (F. mosseae) were incorporated with biological agents. Notably, the combined treatment (M + Ap) and (M + T34)alone significantly increased yield, and the application of mycorrhiza alone exhibited the most substantial and statistically significant enhancements in tuber yield. Furthermore, treatments involving combinations such as (M + T34), T34 alone, Ap alone, and (M + Sg) also resulted in heightened tuber weight compared to the control group.

4.1.3 Effect on dry weight

I- **Upper part dry weight of okra plants**: The effect of biological agents combined with mycorrhiza on the biomass of the upper part of okra plants with discernible dry weight outcomes. In this study, the synergistic interplay between arbuscular mycorrhizal fungi (*F. mosseae*) (M) and host plants, coupled with three distinct microbial agents, evoked varied levels of dry weight biomass augmentation in the upper portions of okra plants. Specifically, the synergistic combination treatments of arbuscular mycorrhiza with A. pullulans (M+Ap = 82%) and *T. asperellum* (M+T34 = 77%) showed significant differences in dry weight biomass compared to the control group. Similarly, individual treatments with mycorrhiza (M = 84%) and *T. asperellum* alone (T34 = 43%) increased the biomass dry weight. However, the combination treatment (M + S g = 28%) slightly increased the dry weight compared to the control.

II - Upper part dry weight of tomato plants: The experimental trial results showed observable trends in the dry weight of the tomato plants. The results show the difference between the treatment of the vegetative dry weight of the tomato plants that arbuscular mycorrhiza (*F. mosseae)* combined with the biological agents and treatments alone (M), M+AP, M+T34, and M+Sg frequently showed significant differences compared to the control. The microbial agent treatments (T34, Sg, and Ap) did not affect tomato yield compared to the control.

III- Root dry weight measurements of okra: plantThe results illustrate significant differences in root dry mass among various treatment modalities applied to okra plants, particularly those involving combinations of arbuscular mycorrhizal fungi (AMF), *F. mosseae*, with biological agents. Treatment of

mycorrhizal fungi with microbial agents, namely AMF +*A. pullulans* (M + AP), AMF + *T. asperellum* strain 34 (M + T34), AMF alone, and AMF + *S. griseoviridis* (M + Sg), resulted in increased root dry weight compared with treatments consisting solely of microbial agents or the control group. Notably, the inclusion of microbial agents in the T34 treatment slightly amplified the disparity in root dry mass, whereas no significant differences in root dry weight were observed between the Ap and Sg treatments.

VI- Root dry weight of tomato plants: The results demonstrated the difference in root dry weight between various treatments of tomato plants in conjunction with arbuscular mycorrhiza (*F. mosseae)* and microbial agents. The combined treatments of arbuscular mycorrhiza with *A. pullulans* (M + AP), arbuscular mycorrhiza and *T. asperellum* strain 34 (M + T34), and arbuscular mycorrhiza and *S. griseoviridis* (M + Sg) exhibited higher root dry weights than the control group. Treatment with arbuscular mycorrhiza alone (M) also resulted in a significant increase in root dry weight. Conversely, treatments of T34, Ap, and Sg with biological agents resulted in no significant differences in root dry weight.

4.1.4 Trichoderma asperellum colonization in okra, tomato root, and sweet potato

The data acquired from the root colonization evaluation revealed the presence and spatial distribution of *T. asperellum* (T34) in tomato roots. Colonization density was meticulously quantified, thereby delineating the prevalence and colonization of roots and the percentage of symbiotic associations between *T. asperellum* (T34) and the plant root system. Observations of root colonization on PDA media have provided valuable insights into the spatial distribution of (T34) hyphae across distinct root zones of plants. The results showed the behavior of T34 with arbuscular mycorrhizal fungi (*F. mosseae*)., underscoring their ability to effectively colonize the root system, thereby fortifying plant growth, bolstering plant defenses, and fostering mutualistic relationships conducive to enhanced plant health and productivity.

4.1.5 Effect on dry weight

Root colonization by the mycorrhizal fungi *Funneliformis mosseae* (AMF) plays a crucial role in promoting biomass growth in food crops (Alam, Choudhury and Mridha, 2023), and the extent of root colonization by AMF is

indicative of the strength of the symbiotic relationship between the fungi and the plant. In this study, we examined the colonization of okra roots treated with AMF alone and in combination with biological agents (*T. asperellum*, *A. pullulans*, and *S. griseoviridis*) (T34, Ap, and Sg) (Figure 38). Our laboratory investigations revealed varying levels of root colonization under different treatments. The M+Sg treatment showed the highest colonization rate (85.16 %), followed by the M treatment (84.68 %). Conversely, the M+T34 and M+Ap treatments exhibited lower colonization rates of 61.18% and 60.92%, respectively. Significant increases in AMF root colonization were observed with M and M+Sg treatments compared with the other microorganisms. Additionally, simultaneous infection with *T. asperellum*, *A. pullulans,*and *S. griseoviridis* led to a dramatic decrease in AMF root colonization levels, potentially due to the suppression of AM parasitism by T34 and Ap (Wang et al., 2023).

4.2 Effect enzymes and antagonist treatment on the Glutathione-S-Transferase (GST) activity of okra plant

The observed variations in GST activity among the different microbial treatments suggested distinct influences on the antioxidative responses of okra plants. Mycorrhiza (*Funneliformis mosseae*) (M) treatment exhibits the highest GST activity. The combination of mycorrhiza with Trichoderma (M+T34) and *A. pullulans* (M+Ap) demonstrated noteworthy changes in GST activity, suggesting potential synergistic effects on the antioxidant capacity of the plant. Conversely, the combination of Mycorrhiza with *S. griseoviridis* (M+Sg) exhibited lower GST activity than mycorrhiza alone. The statistical analysis showed a difference between the enzyme activity of the okra and tomato plant treatments, showing that the combination and single treatments (M, Ap, T34, M+T34, Sg, and M+Ap) were highly significant compared with the Ctral and M+Sg treatments.

4.3 Effect of mycorrhiza and antagonist treatments on the polyphenol content of okra plants

4.3.1 **Kaempferol Derivatives**.

One of the important objectives of the present study was to investigate the effect of mycorrhiza alone and combined with microbes on the polyphenol content in okra fruits. The combination of arbuscular mycorrhiza with other

microorganisms in different treatments (T34, M, Sg, M+Sg, M+T34, and M+Ap) caused a significant increase in the level of okra fruit phenols compared to the control treatment. The results presented in Figure 44 demonstrate the effectiveness of various treatments on kaempferol content in okra fruit, in conjunction with arbuscular mycorrhiza and microbial agents. The results showed that the combination of mycorrhiza and microbe agents increased the percentage of fruit phenols (kaempferol derivatives) in okra. Inoculation of the plant with T. asperellum (T34), mycorrhizal fungi (M), Sg, M+Sg, M+T34, M+Ap, and Ap increased the content of kaempferol by 38%,29%, **27%**, 22%, 20**%**, 17% and14% respectively, increased the rate of okra fruit kaempferol, and was highly significant compared with the control.

4.3.2 Coumaric acid and its Derivatives.

The results demonstrate the response of coumaric acid derivatives to various treatments. These important polyphenolic compounds were present in okra fruits in two free and coumaroyl-hexosides. The treatments of M + Sg and M + AP showed an increase in the content of the two derivatives compared to the control group. Highly significant increases in coumaric acid and coumaroyl hexoside were recorded in the M+AP and M+S g treatments, respectively. It seems that the two treatments differ in their effect on the glycosylation of coumaric acid in okra fruits. Compared with the control, other treatments either decreased or had no significant effect on the level of coumaric acid derivatives. The results showed that inoculation with microbes increased the levels of coumaroyl derivatives by 43%, 22%, 12%, 9%, and 7% with M+Ap, M+Sg, M, Sg, and Ap, respectively. According to Ti et al. (2006), coumaric acid percentage enhancement has been found to be 29% with mycorrhizal fungi (M) and 27% with S. griseoviridis (Sg), who reported that microbes increased their concentration in the plants when infected. For *T. asperellum* and *A. pullulans*, no significant effect or enhancement of coumaric acid in the okra plant was observed.

4.3.3 Chlorogenic acid-catechin-glucoside

distinct patterns in the accumulation of chlorogenic acid-catechin-glucoside phenols in okra fruit under the influence of mycorrhiza and antagonistic agents. Mycorrhiza (M), *T. asperellum strain* T34, *A. pullulans*, *S. griseoviridis* K61 treatment and combined mycorrhiza with microbe agents: Mycorrhiza sp+ *T.*

asperellum strain T34 (M+T34), Mycorrhiza sp + A. pullulans (M+Ap)., Mycorrhiza sp+ Streptomyces griseoviridis (M+Sg). The highest level of the dimer was recorded for the mycorrhizal treatment. The biotic treatments increased by 163%, 116%, 86%,79%63%, 29% and 5% with M, M+T34, M+Sg, Sg, M+Ap, T34, and Ap the level of such polyphenol respectively. Thus, microbes with different abilities to induce plant immunity (M) and (M+T34) have significant roles in increasing the concentration of chlorogenic acid compared to the control, but M+Sg, Sg, M+Ap, and T34 exhibited no significant changes.

4.3.4 Sinapoyl feruloyl derivative

Sinapoyl malate is synthesized within the epidermal layer of leaves.(Kaling et al., 2015).. This antioxidative function is particularly crucial in the epidermal layer, where plants are exposed to environmental stressors such as ultraviolet (UV) radiation (Vink et al., 2023). Treatment with mycorrhiza alone or in combination with microbial agents promoted the biosynthetic pathways of sinapic acid derivatives compared to the control treatment, which was identified as a dimer of sinapoyl-feruloyl. The highest increase in the content of such dimers was observed in the fruits of okra treated with mycorrhiza combined with Ap.

4.3.5 Di- caffeolyl quinic acid

The synergistic effect of the combined application of mycorrhiza and microbial agents on di-caffeoylquinic acid levels in okra fruits was depicted. Except for the Sg and M+Sg treatments, all other treatments showed significantly higher di-caffeoylquinic acid content in okra fruits. The highest increase in the concentration of polyphenol glucoside was found in M, M+T34, and M+Ap, with no significant variation between them in their impact on di-caffeoylquinic acid. The Sg-type bacterial insulation had a particularly remarkable negative impact on the metabolic pathway of such polyphenols, most probably by partial inhibition of the enzymes involved in the biosynthesis of di-caffeoylquinic acid. The combination of Sg and mycorrhiza slightly moderated its negative effect on di-caffeoylquinic acid formation in okra fruits.

4.3.6 Quercitin-3-diglucoside

Quercetin was found in the extract of the whole Okra fruit to exist in quercetin-3-o- glucoside, quercetin- di-glucoside, and Quercitin-3-O-(melanoyl) glucoside. Effects of the different treatments on the quantity of quercetin derivatives. Interestingly, Ap 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 Ap (M + Ap) yielded fruits with a significantly higher level of quercetin 3-diglucoside compared to other treatments, but not as much as Ap alone.

5 -DISCUSSION

The objective of this study was to evaluate the efficacy of utilizing MBCA (*S. griseoviridis*, *T. asperellum*, and *A. pullulans*), which are biological agents, to mitigate the occurrence of *Botrytis cinerea* infections in okra plants. Volatiles produced by *S. griseoviridis* have been observed to induce vacuolation and degradation of the mycelial surface of *B. cinerea*, consequently impeding conidial germination (Bello *et al.*, 2022). Similarly, A. pullulans emits volatiles that induce rough cell surfaces with numerous outgrowths in *B. cinerea* (Don *et al.*, 2021).

The results of the experimental investigation underscored the impact of microbial inoculants on enhancing the resistance of okra plants to *B. cinerea* infection. Specifically, the combined treatment with biological agents and arbuscular mycorrhiza (*Funneliformis mosseae*) (M+T34, M+Ap, and M+Sg), as well as the synergistic pairing of arbuscular mycorrhiza and *S. griseoviridis* (M+Sg), demonstrated a significant increase in plant tolerance to *B. cinerea* infection when compared with the control group. The enhanced plant tolerance can be attributed to several mechanisms that promote systemic plant resistance and suppress pathogen growth. These mechanisms include an increase in phenolic compounds and GST enzyme activity, which are known to enhance plant tolerance to biotic and abiotic stresses

This study investigated the effects of individual and combined microbial treatments on the growth parameters of okra, tomato, and sweet potato plants. A symbiotic relationship between arbuscular mycorrhizal fungi and *T.*

asperellum was observed to enhance nutrient uptake and provide protection against microbial agents, which is consistent with previous studies. Similarly, combined treatments of Mycorrhiza with S. griseoviridis (M+Sg) and A. pullulans (M+Ap) significantly increased okra plant height, indicating a synergistic effect on growth attributed to the secondary metabolism of pullulans and nutrient uptake enhancement by mycorrhiza, consistent with previous studies (Wachowska et al., 2016; Di Francesco et al., 2021). Furthermore, this study revealed the efficacy of novel combinations, such as Mycorrhiza with A. pullulans and S. griseoviridis, in increasing okra plant height, underscoring the potential synergistic effects of these combinations under field conditions.

Individual microbial treatments, including T34, arbuscular mycorrhizal fungi (*Funneliformis mosseae),* and *A. pullulans*, exhibited modest increases in okra plant height compared to the control, albeit not statistically significant, which is consistent with previous findings (Guzmán-Guzmán et al., 2023; Wahab et al., 2023). However, further investigation of the effect of microbes on mycorrhizal colonization in okra roots is warranted to elucidate the mechanisms underlying the observed growth enhancement.

In the context of environmentally sustainable farming, the integration of beneficial microorganisms such as *T. asperellum*, Mycorrhiza, *A. pullulans*, and *S. griseoviridis* has garnered attention. Notably, the application of antagonist agents alongside arbuscular mycorrhiza led to diverse effects on tomato plant height, with certain treatments exhibiting significant enhancements compared to the controls. Additionally, the initial growth of mycorrhiza-treated plants, either alone or in combination with other beneficial microbes, had a positive effect on plant height, suggesting their utility in agricultural practice without inhibiting plant growth.

Moreover, examination of sweet potato plants revealed significant improvements in yield and shoot length with the application of microbial treatments, particularly when combined with arbuscular mycorrhizal fungi (*F. mosseae*). Various microbial combinations exhibited diverse effects on sweet potato growth parameters, indicating the importance of strain selection and microbial interactions. Differences in yield among identical treatments may stem from optimal microbial temperatures or resistance to mycorrhiza among other factors. Despite these variations, treatments involving microbial mixtures

consistently outperformed the untreated controls, underscoring their potential for enhancing crop productivity.

This study explored the impact of combining arbuscular mycorrhizal fungi AMF (*Funneliformis mosseae):* (M) with various biological agents on the dry weight biomass of okra and tomato plants. In the case of okra, distinct outcomes were observed, with varying levels of dry weight biomass enhancement resulting from symbiotic interactions between AMF and microbial agents. Combinations, such as M + T. *asperellum* (T34) and M + A. *pullulans* (Ap) exhibited significant enhancements in dry weight biomass (77% and 82% increase, respectively), whereas M alone also led to a notable increase (84%). However, treatment with T34 (43%) and M + S. *griseoviridis* (Sg) (28%) showed a slight improvement in dry weight. Previous studies have reported similar results, suggesting that these biological agents possess the unique capabilities to enhance nutrient uptake, promote plant growth, and improve resistance to environmental stress. Additionally, the stability of root hyphae and expansion of the mycorrhizal network contribute to alterations in root biomass, as indicated by (Zhang *et al.* (2020) and Liang *et al.* (2021). Field experiments conducted in this study confirmed that inoculation with M and biological agents significantly increased nutrient availability, stimulated plant development, and enhanced biomass accumulation, ultimately leading to increased plant height, basal diameter, and fibrous root extension in both okra and tomato plants.

Similarly, in the case of tomato plants, the integration of beneficial microorganisms, including *T. asperellum, A. pullulans*, and *S. griseoviridis*, has gained attention for sustainable farming. Extensive research has been conducted to understand the interactions and repercussions of these microorganisms on tomato plants. Figures indicate that combinations of Mycorrhiza with *T. asperellum, A. pullulans*, and *S. griseoviridis* have variable effects on promoting plant growth and dry weight. Moreover, the use of different microbial strains combined with mycorrhiza may result in varying effects on tomato plant dry weight, with the initial biomass of mycorrhiza-treated plants being significantly higher regardless of the presence of other beneficial microbes. Importantly, none of the treatments inhibited plant growth, suggesting that integration of mycorrhiza and selected biological agents into crop production systems is safe and beneficial.

This text is best suited to the discussion section of academic papers. It discusses the collaborative effects of two biocontrol agents, AMF (M) and *T. asperellum* (T34), on the T34 root colonization dynamics of various plant species, namely Okra (*Abelmoschus esculentus*), Tomato (*Solanum lycopersicum*), and Sweet potato (*Ipomoea batatas* (L.) Lam). This study aimed to elucidate the interactive influence of these beneficial symbionts on root systems, focusing on quantifiable measures such as colonization density, distribution patterns, and the synergistic impact on host plants.

We conducted an in vitro assay to test T34 colonization in the roots of arbuscular mycorrhizal fungi AMF (*Funneliformis mosseae)* (M)-treated okra, tomato, and sweet potato plants and assessed the mycoparasitic ability of T34. The results demonstrated active colonization of all roots tested, whether treated with AM alone or in combination with T34. This finding corroborates that reported by (Sain, Dewasi and Singh, 2023). Additionally, the study found that the combination of M and T34 did not significantly affect each other's efficacy in inducing and protecting the host plant, consistent with the results observed by (Alam, Choudhury, and Mridha (2023) and TANUI (2023), who highlighted the role of Trichoderma spp. as an opportunistic fungus symbiont that aids in disease suppression and benefits the host plant. M facilitates the delivery of minerals to plant roots and provides photosynthetic products to the fungi.

These findings underscore the potential of optimizing biocontrol strategies by harnessing the synergistic benefits of Mycorrhiza and Trichoderma in root colonization. Future research should delve into the molecular mechanisms underlying this collaboration, emphasizing the development of tailored approaches to enhance plant growth, resilience, and overall productivity across diverse agricultural settings.

Based on our results, we examined the effects of the combination of biological agents with arbuscular mycorrhiza fungi (*Funneliformis mosseae):* AMF on AMF root colonization in okra, tomato, and sweet potato plants. The root colonization of okra plants combined with arbuscular mycorrhizal fungi (AMF), biological agents (T34, Ap, Sg), and AMF alone was assessed in this study. The investigation revealed varying levels of root colonization across different treatments. Notably, the combination treatment of AMF with *Streptomyces griseoviridis* (M+Sg) resulted in the highest colonization rate of 85.16%, followed by AMF treatment alone at 84.68%. Conversely, colonization rates

decreased notably in treatments involving simultaneous infection with *T. asperellum* (T34) and A. pullulans (Ap), with rates of 61.18% for M+T34 and 60.92% for M+Ap, suggesting that AMF parasitism can be suppressed by these microorganisms. Previous studies have documented the biocontrol mechanisms of T34 and Ap strains, which could account for the observed reduction in colonization(Wang *et al.*, 2023).

The biological control properties of A. pullulans have been extensively studied, with proposed mechanisms including augmentation of host defenses, resource competition, and production of antifungal volatile organic compounds(Yalage Don *et al.*, 2020; Iqbal *et al.*, 2023; Podgórska-Kryszczuk, 2023). Similarly, Trichoderma spp. exhibit antagonistic processes such as secondary metabolite production, parasitism, and pathogen inhibition, potentially influencing mycorrhizal colonization levels (Khuong *et al.*, 2023).

In the case of tomato and sweet potato plants, the effectiveness of symbiotic associations in enhancing resistance or tolerance may vary depending on the AMF symbionts and other microorganisms involved. AMF colonization levels were not significantly affected by Trichoderma or Streptomyces treatments, although minor differences were observed. Notably, mycorrhiza-only treatments reduced the presence of Trichoderma fungi, suggesting potential interactions between these microorganisms in the rhizosphere.

We present findings regarding the modulation of glutathione-S-transferase (GST) activity in okra plants by various microbial treatments. (GST) are a crucial enzyme involved in conferring antimicrobial resistance to host plants and mitigating oxidative stress by synergistically removing lipid hydroperoxides as antioxidants (Gullner *et al.* 2018; Zhang *et al.* 2023). Our investigation revealed that the combination of arbuscular mycorrhizal fungi (*Funneliformis mosseae)* (AMF) with biological agents significantly increased GST activity in okra plants compared to the control treatments. Distinct GST activity patterns were observed in the individual microbial treatments. Notably, treatment with arbuscular mycorrhiza (*Funneliformis mosseae)* (M) and Mycorrhiza+Aureobasidium (M+Ap) demonstrated potential effects on GST activity, suggesting their role in enhancing the antioxidative response in okra fruits. Conversely, treatment with *T. asperellum* (T34), *S. griseoverdis* (Sg), and *A. pullulans* (Ap) may exert varying effects on GST activity.

Understanding the modulation of GST activity by microbial treatment has significant implications in enhancing the ability of plants to counteract oxidative stress and environmental challenges. In conclusion, this study provides valuable insights into the modulation of GST activity in okra using various microbial treatments. The observed variations underscore the potential role of arbuscular mycorrhizal fungi (*F. mosseae)* M and M+Ap combinations in influencing the antioxidative response of plants, thereby enriching our understanding of the interplay between microbial treatments and biochemical pathways in plants.

Plants employ diverse strategies to defend against pests, with one notable mechanism being the de novo synthesis of specific compounds such as phenolics. These phenolics are known for their significance in maintaining plant health, and numerous studies have affirmed their heightened production in response to insect feeding, pathogen infection, or colonization by beneficial microorganisms (Wallis and Galarneau, 2020). In addition, phenolic compounds and flavonoids are potential substitutes for bioactive agents in the pharmaceutical and medicinal industries to promote human health and prevent and cure various diseases. (Sun and Shahrajabian, 2023).

During the preharvest, harvest, and postharvest steps, plants and their products are exposed to several biotic factors, including microorganisms, among which bacteria, fungi, and viruses are the most important and can influence the secondary metabolic pathways of plants and, therefore, the production of bioactive compounds (Zeng et al., 2013). Microorganisms can act in selected ways during plant developmental stages and in different anatomical parts (Vivanco et al., 2005). Arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) are the most studied microbial groups in plant roots (Alfonso and Galán 2006). In general, the increased concentration of secondary metabolites in plant roots, leaves, and fruits is related to the defense response of plants to microorganism colonization (Suzuki et al., 2014; Toussaint, 2007).

Different microorganisms differ in their mechanisms of inducing or promoting the biosynthesis of phenolic compounds in plants, and some reports indicate that some microorganisms promote the absorption of phosphorus by plants, which activates the methylerythritol phosphate pathway that significantly affects secondary metabolite production (Carretero-Paulet et al., 2006).

However, studies have indicated that some microorganisms can be used as antagonists or to induce secondary metabolites to defend fruits and vegetables (fresh or processed) against deteriorative microorganisms (Terry and Joyce, 2004). Biotic factors include a more sophisticated interaction between plant biochemistry and physiology (Briskin, 2000). It can be assumed that biotic effects are related to plant interactions with microorganisms or plant physiological aspects such as phenology and ontogeny (Pavarini et al., 2012; Ochoa-Velasco et al., 2017). The strong induction of selected phenolic metabolites has been attributed to the presence of nitrogen and subsequent nitrogen deficit in phenolic metabolites and physiology. Flavones increased but flavonols decreased in response to lack of nitrogen (Kováčik and Klejdus, 2014)

6 -CONCLUSIONS

1- co-cultivation experiments involving T. asperellum, S. griseoviridis K61, and A. pullulans with arbuscular mycorrhiza did not have a detrimental effect on the reciprocal characteristics of microbial agents.

2- Inoculation of plants with a combination of antagonist agents and mycorrhiza is instrumental in enhancing plant growth and yield in cultivating tomato, okra, and sweet potato.

3- Furthermore, investigation of the interaction between antagonist agents and mycorrhiza revealed a significant improvement in gray mold resistance.

4- Additionally, assessment of GST enzyme activity in okra leaves revealed a pronounced elevation, particularly in glutathione S-transferase (GST), which increases the resistance of the plant to biotic and abiotic stresses.

5-HPLC analysis of okra fruits indicated a noticeable increase in the concentration of phenolic compounds, which was attributed to the interaction between the antagonist agents and mycorrhiza.

7 -NEW SCIENTIFIC RESULTS

1- asperellum treatment had no inhibitory effects on mycorrhizal root colonization. However, Streptomyces and Aureobasidium treatment slightly reduced the mycorrhiza colonisation

2- The combined application of biocontrol microbes and mycorrhiza increased okra resistance to gray mold infection compared to treatment with antagonists or mycorrhiza alone. In addition, these latter treatments reduced gray mold infection.

3- The combined treatments increased the growth of the vegetative parts and resulted higher yield both alone and in combinations

4- Glutathion-S-transferase activity was increased by each treatment, either alone or in combination.

5- The concentration of some phenolic compounds in okra fruits were increased in some combinations

8 -SUMMARY

Sustainable plant production necessitates minimizing the utilization of synthetic chemicals for plant nourishment and protection. Recent advancements highlight the role of microbial consortia interacting with mycorrhizal symbiosis to bolster plant growth, resilience, and productivity. Particularly, the collaborative action of *Trichoderma asperellum strain 34*, *Aureobasidium pullulans*, and *Streptomyces griseoviridis K61* with arbuscular mycorrhizal fungi shows promise for sustainable agriculture. Integrating microbial agents, including bacteria and fungi, with mycorrhiza confers various benefits such as enhanced phenolic compound concentration, promotion of plant growth, resilience, and resistance, leading to overall improved plant performance and increased yield.

This synthesis of microbial interactions with mycorrhiza introduces a novel strategy for optimizing plant-microbe associations, thereby fostering resilient plant ecosystems. Despite the presence of antagonists, mycorrhizal root colonization remained unaffected. Additionally, while mycorrhiza treatment slightly reduced the occurrence of *T. asperellum, A. pullulans*, and *S. griseoviridis* on the plants, it did not inhibit the colonization of roots by the applied Trichoderma strain.These findings underscore the potential for natural resource synergies between beneficial arbuscular mycorrhizal associations and microorganism agents to optimize reproductive parameters, offering valuable insights into sustainable agricultural practices focused on enhancing crop yield and environmental sustainability. This study contributes significantly to the scientific understanding of the interactive relationships between mycorrhiza and antagonistic agents in enhancing plant growth.

9 -RELATED PUBLICATIONS

- Yousif, A. I. A., Almuslimawi, A., Turóczi, G., Kiss, J., Kovács, A., & Körösi, K. (2023). Efficacy of some plant resistance inducers against several sunflower downy mildew (Plasmopara halstedii (Farl.) Berl. et de Toni) isolates. Acta Biologica Szegediensis, 67(1), 75-86.
- Alaa Abdulkadhim A. Almuslimawi 1,2, Borbála Kuchár 1, Susana Estefania Araujo Navas 1, György Turóczi 2, Katalin Posta 1 * (2024). Combined application of biocontrol microorganisms and arbuscular mycorrhizal fungus on plant growth and yield of tomato (Solanum lycopersicum L.). Acta Agronomy, xxxx.
- Alaa Abdulkadhim A. Almuslimaw , Yousif, A. I. A Lívia László, Alhassani Leith, György Turóczi, Katalin Posta (2024). Impact of beneficial soil microorganisms on okra plants polyphenol components. Acta Agronomy, xxxx

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Podgórska-Kryszczuk, I. (2023) 'Biological Control of Aspergillus flavus by the Yeast'.