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Biological Control of Three Fungal Diseases in Strawberry (*Fragaria* × *ananassa*) with Arbuscular Mycorrhizal Fungi

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Abstract: Similar to many other plant-based products, strawberries are susceptible to fungal diseases caused by various pathogen groups. In recent years, efforts have been made to combat these diseases using biological control methods, particularly the application of arbuscular mycorrhizal fungi (AMF). This study aimed to determine the effects of AMF (*Funneliformis mosseae* (Fm) and *Gigaspora margarita* (Gm)) on *Rhizoctonia fragariae* (Rf), *Fusarium oxysporum* (Fo), and *Alternaria alternata* (Aa), which are major pathogens for strawberry. The results showed that the effects of AMF on disease severity and plant growth varied depending on the pathogens involved. Rf caused the highest disease severity, followed by Fo and Aa, but all AMF treatments significantly reduced the disease severity compared to control treatments. The study also found that the specific AMF species and their combinations influenced plant growth responses under different pathogenic conditions. Different AMF treatments resulted in varying increases in plant fresh weight, dry weight, and length, depending on the pathogen. Moreover, the application of AMF led to increased levels of total phenolic content, antioxidant activity, and phosphorus content in pathogen-infected plants compared to control treatments. Fm was more efficient than Gm in increasing these biochemical parameters. The levels of root colonization by AMF were similar among different AMF treatments, but the effects on fungal spore density varied depending on the pathogen. Some AMF treatments increased fungal spore density, while others did not show significant differences. In conclusion, our research sheds light on the differential effects of AMF species on disease severity, plant growth, and biochemical parameters in strawberry plants facing diverse pathogens. These findings underscore the potential benefits of AMF in disease management, as they reduce disease severity and bolster plant growth and defense mechanisms.

Keywords: biological control; phytopathogens; management of phytopathogens; increase plant tolerance

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1. Introduction

Strawberry, belonging to the Rosaceae family and the *Fragaria* genus, is a fruit that is grown worldwide with high market and nutritional value. The hybrid *Fragaria* × *ananassa* is the most cultivated species thanks to its fruit characteristics [1]. It is an agriculturally preferred plant due to its features such as rich species diversity, rapid reproduction, and fruiting in one season [2]. Strawberries are also considered a healthy food as they are rich in beneficial compounds such as sugar and minerals [3,4]. This fruit is also a rich source of many bioactive compounds and antioxidants, e.g., vitamin C (ascorbic acid), flavonoids, tannins, and phenols [5]. Strawberry is mostly grown in the USA, Russia, Spain, Türkiye, and Canada [6]. Türkiye has an important potential for strawberry cultivation with its suitable climate and soil structure [7]. Mersin is the leading place in strawberry

production, followed by Aydın, Antalya, and Bursa, respectively [8]. In Spain, the main area of production is located in the province of Huelva, in the southwest of the country.

However, there are some factors that limit strawberry production and yield. Among the various strawberry diseases, black root rot is the most widespread in most strawberry-growing countries [9,10], and is caused by various biotic and abiotic factors. Among the pathogens causing the black root rot complex, the most common ones include *Rhizoctonia fragariae* Husain & W.E. McKeen, *Rhizoctonia solani* Kühn, *Idriella lunata* P.E. Nelson & K. Wilh., *Fusarium* spp., *Pythium* spp., *Cylindrocarpon* spp., *Verticillium* spp., *Alternaria* spp., and *Aspergillus* spp., of which most are soil-borne factors [11,12]. Among these, *Fusarium oxysporum*, *Rhizoctonia fragariae*, and *Alternaria alternata* have especially been reported as highly virulent in different geographic regions of the world [9,10,13–20].

These pathogens, single or in combination, fill the vascular system of strawberries, resulting in the prevention of nutrient and water uptake and causing root blackening, root death, and a loss of the vitality and productivity of the plant [21–23]. Soil-borne fungi can cause this pathogenicity [24,25]. Even when they cannot find a suitable host, they can survive latent in the soil for a long time [22]. Therefore, finding a sustainable solution to the pathogens that cause black root rot is gaining worldwide importance [26,27]. The use of cultural methods, resistant varieties, and some biological control agents can reduce the damage to some extent [28–30]. Among these biological control organisms, arbuscular mycorrhizal fungi (AMF), which are important micro-organism groups in the rhizosphere [31], provide very important advantages to the plant in terms of both plant growth and resistance [32].

Sustainable, environmentally friendly, and ecological methods of biological control increase soil activity and plant growth and provide resistance against pathogens [33–36]. The symbiosis between plant roots and some soil fungi is defined as "mycorrhiza" [37]. While these fungi take some organic matter and carbohydrates from the plant, they enable the plants to better absorb some macro- and micronutrients, especially phosphorus [38]. Thus, both benefit from each other in certain conditions [39,40]. The positive effect of AM symbiosis on plant tolerance against biotic and abiotic stresses is well known, even though the involvement of certain proteins of fungal origin is not fully understood [41].

Therefore, the importance of bio-control agents in plant–pathogen–environment interaction is a hot topic in agronomy. In this study, biological control elements that can be effective against the fungal pathogens *Rhizoctonia fragariae*, *Fusarium oxysporum*, and *Alternaria alternata* were determined and their effects on preventing the effects of disease on plant yield were investigated. We hypothesized that the presence of AMF could increase plant tolerance to these pathogens and thus reduce the detrimental effects of diseases on plant growth. The aim of our research was to test this hypothesis and shed light on the mechanisms by which AMF influence plant–pathogen interactions and contribute to the overall health of the plant.

2. Materials and Methods

2.1. Materials

In this study, *Fusarium oxysporum* (FOYYU13), *Rhizoctonia fragariae* (AG-A, RSYYU3), and *Alternaria alternata* (AAYYU5) isolates, which were previously isolated from strawberries and found to be virulent, were used. These isolates were kept at +4 °C in mycological culture stocks of Van YYU Faculty of Agriculture, Department of Plant Protection. The study was carried out using young isolates developed in PDA (potato dextrose agar) medium. In biological control studies, two arbuscular mycorrhizal fungi (AMF) species (*Funneliformis mosseae* FMYYU1 and *Gigaspora margarita* GMYYU2) and their mixture (*F. mosseae* + *G. margarita*) were used. These were provided by the mycology culture collection at the Van YYU Faculty of Agriculture, Department of Plant Protection. For the pathogenicity test, Frigo strawberry seedlings (*Fragaria* × *ananassa* cv. Fern) with 2 leaves were obtained from a commercial production company (YALTIR

Agricultural Products INC., Adana, Türkiye) and planted. A mixture of sterilized peat and perlite (1/1, *w*/*w*) was used as the seedling growing medium. AMF was applied before planting and seedling roots were cut at 5 cm length and planted [42]. Pathogen inoculations were started 5 days after planting. Irrigation was carried out according to the needs of the plants.

2.2. AMF Application

An amount of 10 g (150 spores per 1g soil) of AMF isolates was inoculated into the rhizosphere (approximately 10 cm depth) of the pots (1.5 kg) with seedlings. Sterilized sand (1 h autoclaved at 121 °C) was used in the seedling beds in control treatments instead of AMF isolates.

2.3. Pathogen Inoculation

Rhizoctonia fragariae isolate inoculum was grown on wheat (*Triticum aestivum* L.) seeds (10 g) in a twice-autoclaved 250 mL Erlenmeyer flask and inoculated with 2 disks of agar–mycelium plugs (1 cm diameter) [13]. Wheat seeds were colonized with the isolate for four weeks at 25 °C. Each pot was inoculated with 7 wheat seeds colonized with fungal isolates and non-inoculated sterilized seeds as the control treatments. *Fusarium oxysporum* isolate was incubated in PDA at 25 °C for 7 days. The spore suspension was prepared in sterile distilled water from the developing fungal cultures and the concentration was adjusted to 10^6 conidia/mL using a hemocytometer. After planting the seedlings (2 leaves Frigo seedlings), 10 mL of *Fusarium oxysporum* inoculum suspension was applied by embedding in the soil. The control plants received sterile water. *Alternaria alternata* isolate was incubated in PDA at 25 °C for 7 days. A 50 mL inoculum suspension containing 10^6 conidia/mL was prepared from the developing culture, as previously mentioned, and inoculated by spraying on the plants. Sterilized water was applied to control plants [43–45].

2.4. Determination of the Antagonist Effects against Pathogens

In the study, 16 × 18 cm plastic pots that could hold 1.5 kg of mixture were used. Plants were grown in a growth chamber at 25 °C, 60–70% humidity, and 12 h photoperiod with a light intensity of 6000 lux. The experiment was set up with 4 replications for each treatment group. The plants were fertilized three times with Hoagland nutrient solution (about 5 mL per plant; pH 6.2; composition: KNO_3 303.3 mg L^{-1}, $Ca(NO_3)_2{\cdot}4H_2O$ 472 mg L^{-1}, $NH_4H_2PO_4$ 115 mg L^{-1}, $MgSO_4{\cdot}H_2O$ 69.2 mg L^{-1}, KCI 1.8 mg L^{-1}, H_3BO_3 0.8 mg L^{-1}, $MnSO{\cdot}H_2O$ 0.1 mg L^{-1}, $CuSO_4{\cdot}5H_2O$ 0.1 mg L^{-1}, and H_2MoO_4 0.1 mg L^{-1}). After 8 weeks, the plants were harvested and their morphological parameters, total phenol content, antioxidant activity, and phosphorus content were measured. In addition, root and soil samples were taken from the AMF-inoculated plants for root colonization and spore counting, respectively.

2.4.1. Estimation of Disease Scale and Severity

The plants infected with *R. fragariae* were evaluated on a 0–4 scale, in which 0 = healthy plant, 1 = localized tissue discoloration without necrosis, 2 = nearly complete root necrosis and partially restricted root length, 3 = root rot and root length severely restricted, and 4 = dead plant [46]. The plants infected with *F. oxysporum* were evaluated on a 0–5 scale. The disease development was rated using the following disease index: 0 = no symptoms, 1 = 1–2 leaves rolled and yellowed, 2 = all leaves rolled and deformed, 3 = chlorosis and early plant wilting, 4 = necrosis and entire plant wilting, and 5 = dead [45]. The plants infected with *A. alternata* were evaluated on a 0–4 scale (0 = no symptoms, 1 = 25% symptoms of *A. alternata*, 2 = 50% symptoms of *A. alternata*, 3 = 75% symptoms of *A. alternata*, and 4 = dead leaves). After the evaluation was carried out according to the scales, the disease severity (DS) was calculated using the Tawsend–Hauberger formula [47]. Re-isolation of pathogenic fungi was performed by taking

samples from the roots and leaves of the strawberry plants for which disease evaluations were performed.

$$DS\ (\%) = [\sum(S \times L)/(M \times S_{max})] \times 100$$

where S represents scale value, L represents the number of plants (plant leaves) evaluated in the scale, M represents the total number of plants (plant leaves), and S_{max} represents the highest scale value.

2.4.2. Determination of Plant Growth Parameters

The length and fresh weight of the plants in each replication were determined after harvesting and then the samples were dried in an oven at 70 °C for 48 h to determine the dry weight [48].

2.4.3. Determination of Total Phenol Content and Antioxidant Activity

Here, 1 g of the 4th compound leaf from the top of each fully developed plant was taken, 5 mL of methanol was added, and this mixture was homogenized with a homogenizer (Ultra-Turrax T20 Basic, IKA Works, Inc., Wilmington, NC, USA) at medium speed for 2 min. Then, these samples were centrifuged at 10,000 rpm for 10 min and extracts were prepared from the liquid portion in approximately 2 mL and stored in a −80 °C refrigerator. The Folin–Ciocalteu colorimetric technique was used to determine the total phenol content [49]. The Folin- Ciocalteu colorimetric technique was used to determine the total phenol content [48]. An amount of 400 µL of distilled water and 150 µL of Folin–Ciocalteu solution were added to 150 µL of the extract, followed by 300 µL of 20% sodium carbonate (Na_2CO_3) (Merck 1.06392.1000, Darmstadt, Germany), and left for 30 min in darkness. A spectrophotometer (VarianBio 100, Australia) was used to measure the absorbance of the solutions at 725.0 and 700.0 nm. The antioxidant activity of the same extracts was evaluated using the FRAP (Ferric Reducing Antioxidant Power) technique [50], in which 150 µL of the extract was mixed with 2850 µL of the FRAP working solution and stored at 25 °C for 30 min in darkness. For the FRAP working solution, three preliminary solutions should be prepared. In the first one, 1.55 g of acetate buffer was mixed with 0.25 L of distilled water followed by adding glacial acetic acid up to 0.5 L. For the second solution, 100 mL of diluted HCl (40 mM) was prepared followed by adding 50 mL TPTZ (tetrazolium red (2,3,5-Triphenyltetrazolium chloride). In the third solution, 0.270 g of ferric chloride (2 mM) was added to 50 mL of distilled water. In the final working solution, 250 mL of the first solution and 25 mL of the second and third solutions were mixed and kept in a water bath at 37 °C for 30 min. The total antioxidant activity was measured at 593.0 nm using a spectrophotometer (VarianBio 100, Australia).

2.4.4. Determination of Phosphorus Content

The vanadomolybo-phosphoric yellow technique was used to quantify total phosphorus [51]. The dried green leaf samples were ground and 0.5 g was placed in crucibles and subjected to pre-incineration by adding 1 mL ethyl alcohol (Merck 818,760, Germany). The samples were then incinerated in a muffle furnace and treated with 4 mL hydrochloric acid solution (Merck 1.05590.2500, Germany) and incubated at 90 °C for 15 min. The samples were filtered into balloon jugs, filled with distilled water to 50 mL, and transferred to extract containers. From these samples, 1 mL was taken and transferred to glass tubes and 4 mL of distilled water was added. After these procedures, 1 mL of Barton's solution was added to these tubes and 4 mL of pure water was added again to make up 10 mL. The formation of a yellow color was observed for about 15 min and then P was read from this preparation in a spectrophotometer (Jenway 6505 UV/vis, London, UK) set to 430 nm wavelength.

2.4.5. Determination of AMF Activity

Determination of Mycorrhizal Dependence

Dry weights of all plants with and without AMF were determined after an 8-week growth period. Then, the mycorrhizal dependence (MD) index was determined according to the results of plant dry weight using the following formula [52]:

$$MD\ (\%) = [(A - B)/A] \times 100$$

where A represents the dry weight of plants inoculated with AMF and B represents the dry weight of plants without AMF.

Determination of AMF Colonization Rate

Root cleaning and staining were used to determine the extent to which the AM fungus colonized the roots of the host plant [53]. Then, the AM fungus was measured with the grid line intersect method [54]. Root pieces (0.5–1 cm) were washed in distilled water and treated in 10% KOH (Merck 1.05012.1000, Germany) for 24 h and 10% HCl (Merck 1.00312.2500, Germany) for 6 h. Root sections were then stained with 0.05% Lactophenol blue (Merck 1.13741.0100, Germany) and lactoglycerol was used to clean them 2–3 times at 6 h intervals. The fungal colonization and structures were observed using a stereomicroscope (10×, Leica, Wetzlar, Germany).

Determination of AMF Spore Density in Soil

The technique of wet sieving and centrifugation with sucrose solution (55%) [55] was used to separate fungal spores from soil samples. A total of 10 g of rhizospheres was mixed with 100 mL distilled water for 1 min using a magnetic stirrer. The mixture was passed through two sieves, first 80 µm and then 45 µm. The resulting liquid was centrifuged at 4000 rpm for 5 min and the supernatant was poured back into the 45 µm sieve. The sieve contents were thoroughly mixed with 55% sucrose solution and centrifuged at 2000 rpm for 2 min. The resulting supernatants were transferred to a 45 µm sieve and washed thoroughly with tap water to remove sucrose, which was then counted using a stereomicroscope.

2.5. Statistical Analyses

The experiment was designed as a factorial experiment based on a completely random design (CRD) with four replicates. The factors were pathogens (Fo, Rf, and Aa) and AM fungal species (Fm and Gm). The studied treatments are presented in Table 1.

Table 1. The non-pathogen- and pathogen-inoculated treatments studied in the experiment.

Treatments			
Non-Pathogen	**Pathogen**		
	R. fragariae	***F. oxysporum***	***A. alternata***
NC	Rf	Fo	Aa
Gm	Rf + Gm	Fo + Gm	Aa + Gm
Fm	Rf + Fm	Fo + Fm	Aa + Fm
Gm + Fm	Rf + Gm + Fm	Fo + Gm + Fm	Aa + Gm + Fm

All measured data were recorded, subjected to normality tests, and transformed ($\sqrt{x}$, logx, Box–Cox method) if necessary. Then, an analysis of variance (ANOVA) was performed on processed data using SPSS (SPSS statistics program, Ver.21.0, SPSS Inc., Chicago, IL, USA) software. The means were compared using Duncan's multiple range test (DMRT) at $p < 0.05$ and $p < 0.01$. The comparison of similar treatments in pathogenic and non-pathogenic conditions was carried out using Student's t-test analysis at $p < 0.05$ and $p < 0.01$.

3. Results

3.1. The Effect of AMF Treatments on the Disease Severity

The effects of AMF (*Funneliformis mosseae* (Fm) and *Gigaspora margarita* (Gm)) treatments on disease-causing agents (*Rhizoctonia fragariae* (Rf), *Fusarium oxysporum* (Fo), and *Alternaria alternata* (Aa)) showed that there were significant differences among the applied treatments in the disease severity index ($p < 0.01$, Table 2). While the disease severity was the highest among all the pathogen applications, *R. fragariae* was followed by *F. oxysporum* and *A. alternata*, respectively. It was determined that the Gm and Fm applications had significant effects on the disease severity index of all fungal diseases compared with control treatments. The dual inoculation with AM fungal species (Fm + Gm) was placed in the same statistical group as the others.

Table 2. Effect of AMF applications on *Rhizoctonia fragariae*, *Fusarium oxysporum*, and *Alternaria alternata*.

Treatments	Disease Severity (%)		
	***R. fragariae* (Rf)**	***F. oxysporum* (Fo)**	***A. alternata* (Aa)**
PC	81.25 ± 11.96 b*	50.00 ± 5.77 b	42.50 ± 7.50 b
Gm	20.00 ± 0.01 a	30.00 ± 5.77 a	20.00 ± 0.01 a
Fm	20.00 ± 0.01 a	25.00 ± 0.01 a	20.00 ± 0.01 a
Gm + Fm	20.00 ± 0.01 a	25.00 ± 0.01 a	20.00 ± 0.01 a

PC: positive control, Gm: *Gigaspora margarita*, Fm: *Funneliformis mosseae*. * Values with the same letter in each column are not significantly different based on Duncan's multiple range test at $p < 0.01$.

3.2. Effect of AMF Species Treatments on Growth in Ro-, Fo-, and Aa-Infected Plants

The effects of AMF species treatments on the growth of Ro-, Fo-, and Aa-infected plants are presented in Table 3. Differences were observed in the growth of the Rf-infected plants according to the treatments. It was determined that the Rf + Gm + Fm treatments significantly increased plant fresh and dry weight and length, and the values were statistically significant. Additionally, the Rf + Fm and Rf + Gm treatments gave values close to each other. It was determined that all applications gave higher values compared to Rf in terms of plant growth parameters and they were in different statistical groups.

Table 3. Effect of AMF species treatments on growth of non-infected plants or plants infected with *R. fragariae, F. oxysporum*, and *A. alternata.* NC: negative control, Gm: *Gigaspora margarita*, Fm: *Funneliformis mosseae*, Rf: *Rhizoctonia fragariae,* Fo: *Fusarium oxysporum*, and Aa: *Alternaria alternata.* *: Values with the same letter in each column are not significantly different based on Duncan's multiple range test at $p < 0.05$.

Treatments		Plant Fresh Weight (g)	Plant Dry Weight (g)	Plant Length (cm)
Non-pathogen	NC	6.05 ± 0.68 c*	1.30 ± 0.12 c	23.00 ± 0.16 c
	Gm	13.06 ± 0.38 a	3.49 ± 0.21 a	37.25 ± 0.47 a
	Fm	8.17 ± 0.52 b	2.51 ± 0.10 b	38.50 ± 0.95 a
	Gm + Fm	8.34 ± 0.75 b	1.87 ± 0.18 bc	29.25 ± 2.28 b
R. fragariae	Rf	3.86 ± 0.27 c	0.51 ± 0.01 c	10.75 ± 1.25 d
	Rf + Gm	7.79 ± 0.23 b	1.50 ± 0.09 b	31.50 ± 2.46 b
	Rf + Fm	7.30 ± 0.25 b	1.40 ± 0.07 b	26.25 ± 1.54 c
	Rf + Gm + Fm	10.50 ± 1.31 a	2.86 ± 0.07 a	34.50 ± 1.75 a
F. oxysporum	Fo	4.75 ± 0.29 c	1.07 ± 0.33 b	28.50 ± 3.70 c
	Fo + Gm	9.41 ± 1.10 b	2.25 ± 0.28 a	38.25 ± 2.01 a
	Fo + Fm	12.27 ± 0.92 a	2.71 ± 0.06 a	35.75 ± 1.93 a
	Fo + Gm + Fm	11.08 ± 0.52 a	2.70 ± 0.16 a	34.50 ± 1.44 b
A. alternata	Aa	4.10 ± 0.59 c	1.08 ± 0.11 c	27.75 ± 2.05 c
	Aa + Gm	9.96 ± 0.26 b	2.18 ± 0.15 b	32.75 ± 1.31 b
	Aa + Fm	12.55 ± 0.87 ab	2.41 ± 0.12 ab	37.50 ± 1.75 ab
	Aa + Gm + Fm	13.13 ± 1.15 a	2.68 ± 0.19 a	41.00 ± 1.47 a

There are significant differences among treatments for Fo-infected-plant growth. It was found that plant growth was decreased significantly in Fo-infected plants, while other treatments increased parameters ($p < 0.05$). The Fo + Gm + Fm treatment increased the plant fresh weight by 133%, the plant dry weight by 152%, and the plant length by 22% compared to Fo (Table 3). In *F. oxysporum* treatment, Fm was more effective than Gm in terms of plant fresh weight and plant dry weight, while the opposite was the case for plant length.

The effects of different AM fungal species (Fm and Gm) on Aa-infected-plant growth parameters differed among applications. The use of different treatments increased all plant growth parameters in Aa-inoculated plants. Moreover, all plant growth parameters were similarly increased in Aa-infected plants treated with Gm + Fm and Gm + Fm + Sc. It was also determined that Aa + Gm + Fm interaction increased plant growth parameters compared to single (Aa) and binary (Aa + Gm or Aa + Fm) combinations (Table 3). Despite the presence of the Aa pathogen (Aa + Gm + Fm), the plant fresh weight increased by 57%, and the plant dry weight increased by 43% compared to the non-pathogen group (Gm + Fm).

The results of the pairwise comparison of infected and non-infected plants are presented in Table 4. There are significant differences between negative control (non-inoculated) and Rf-inoculated plants in terms of PFW (plant fresh weight) as well as PDW (plant dry weight) parameters, while plant length was not changed significantly. When Rf + Gm was compared with Gm and Rf + Gm + Fm was compared with Gm + Fm, there was no significant difference in all parameters, while Rf + Fm treatment had a significant effect on all parameters compared to Fm treatment alone. A comparison of Fo- and Aa-infected and uninfected (negative control) plants showed significant differences in all plant growth parameters. Furthermore, significant differences were observed in PFW and PDW, but not in plant length, when comparing Gm with Gm + Fo, Fm with Fm + Fo, and Gm + Fm with Gm + Fm and Gm + Fm + Fo treatments. The plant growth parameters showed significant differences in most treatments when Aa-inoculated and uninoculated plants were compared. AM fungal species appear to have different effects on the growth of plants infected with different pathogens. The results showed that Fm and Gm had significant effects on plant growth in plants infected with Rf and Fo, respectively. On the other hand, AM fungal species (Gm and Fm) and their combinations (Gm + Fm) had significant effects on most of the growth parameters in Aa-infected plants.

Table 4. Comparison of the effects of AMF species on growth of non-infected plants or plants infected with *R. fragariae*, *F. oxysporum* and *A. alternata*. *: Significant at $p < 0.05$. **: Significant at $p < 0.01$; ns: Not significant.

Treatment Comparison	Student's *t*-Test		
	Plant Fresh Weight (g)	Plant Dry Weight (g)	Plant Length (cm)
Nc/Rf	19.05 **	6.76 **	8.59 **
Gm/Rf + Gm	60.47 **	34.33 **	9.05 **
Fm/Rf + Fm	11.43 **	14.46 **	7.80 **
Gm + Fm/Rf + Gm + Fm	−14.85 **	−13.48 **	−4.38 **
Nc/Fo	9.42 **	2.18 ns	−4.23 **
Gm/Fo + Gm	60.47 **	34.33 **	9.05 **
Fm/Fo + Fm	−52.46 **	−3.29 *	1.20 ns
Gm + Fm/Fo + Gm + Fm	−43.58 **	−11.96 **	−6.41 **
Nc/Aa	21.69 **	2.73 *	−3.54 *
Gm/Aa + Gm	44.64 **	24.50 **	5.21 **
Fm/Aa + Fm	−27.54 **	0.84 ns	1.60 ns
Gm + Fm/Aa + Gm + Fm	−49.68 **	−10.37 **	−13.21 **

3.3. Effect of AMF Species Treatments on Total Phenolic Content, Antioxidant Activity, and Total Phosphorus Content in Ro-, Fo-, and Aa-Infected Plants

The effects of AMF species treatments on total phenolic content, antioxidant activity, and total phosphorus content in Ro-, Fo-, and Aa-infected plants are presented in Table 5. It was determined that total phenolic content, antioxidant activity, and total phosphorus content in the applications with non-pathogenic mycorrhizae were statistically different compared to the control. The positive control (single pathogen) values increased compared to the negative control. It was determined that all of these values increased in pathogenic and mycorrhizal applications and gave statistically different values.

Table 5. Effect of AMF species treatments on total phenolic content, antioxidant activity, and total phosphorus content in non-infected plants and plants infected with *R. fragariae, F. oxysporum*, and *A. alternata.* NC: negative control, Gm: *Gigaspora margarita*, Fm: *Funneliformis mosseae*, Rf: *Rhizoctonia fragariae,* Fo: *Fusarium oxysporum*, and Aa: *Alternaria alternata.* *: Values with the same letter in each column are not significantly different based on Duncan's multiple range test at at $p < 0.05$.

Treatments		Total Phenolic Content (725 nm) (mg ga/100 g)	Total Phenolic Content (700 nm) (mg ga/100 g)	Total Antioxidant Content (593 nm) (µmol TE/g)	P Content (ppm)
Non-pathogen	NC	6.00 ± 1.01 c*	6.00 ± 0.99 c	1.44 ± 0.11 b	2731.24 ± 172.01 c
	Gm	16.60 ± 0.89 b	16.00 ± 0.96 b	7.95 ± 1.65 a	4038.87 ± 118.88 b
	Fm	19.83 ± 2.31 a	19.70 ± 2.34 a	8.90 ± 1.23 a	4172.23 ± 103.52 b
	Gm + Fm	18.10 ± 2.04 a	18.00 ± 2.03 a	9.26 ± 0.85 a	4892.87 ± 319.69 a
R. fragariae	Rf	16.30 ± 1.46 b	16.23 ± 1.44 b	10.36 ± 0.27 b	3196.79 ± 154.36 c
	Rf + Gm	18.33 ± 0.34 a	18.36 ± 0.35 a	10.59 ± 0.09 a	4532.94 ± 167.56 b
	Rf + Fm	19.03 ± 0.53 a	19.00 ± 0.46 a	10.40 ± 1.03 a	4735.77 ± 194.66 b
	Rf + Gm + Fm	18.23 ± 0.71 a	18.06 ± 0.65 a	10.45 ± 0.30 a	5018.33 ± 152.85 a
F. oxysporum	Fo	10.80 ± 0.76 b	10.70 ± 0.77 b	10.07 ± 0.19 b	3089.94 ± 193.31 c
	Fo + Gm	22.36 ± 0.40 a	22.40 ± 0.44 a	13.41 ± 0.32 a	4428.51 ± 201.05 b
	Fo + Fm	25.86 ± 1.25 a	25.76 ± 1.28 a	13.57 ± 0.26 a	4255.40 ± 289.61 b
	Fo + Gm + Fm	21.23 ± 1.82 a	21.26 ± 1.86 a	13.84 ± 0.29 a	4940.94 ± 173.92 a
A. alternata	Aa	17.63 ± 1.06 c	17.56 ± 1.01 c	9.02 ± 0.39 c	3611.20 ± 225.27 c
	Aa + Gm	21.06 ± 0.28 b	21.13 ± 0.27 b	12.73 ± 0.47 bc	3760.45 ± 203.44 b
	Aa + Fm	22.93 ± 0.14 b	23.16 ± 0.30 b	12.87 ± 0.22 bc	3823.94 ± 263.82 b
	Aa + Gm + Fm	25.90 ± 0.97 a	25.76 ± 0.97 a	14.75 ± 0.81 b	4855.79 ± 325.27 a

The pairwise comparison results of infected and non-infected plants are presented in Table 6. In the non-pathogenic treatments, Fm and Gm significantly ($p < 0.05$) increased total phenol, antioxidant, and phosphorus contents compared to Nc treatments, both alone and in the dual inoculation (Gm + Fm). No significant difference was found between AM fungal species (Gm and Fm) and their dual inoculation (Gm + Fm) in increasing phenol content. As for the antioxidant content, the use of AM fungal species (Fm, Gm, and Gm + Fm) increased this parameter. The highest phosphorus content was observed in the Gm + Fm treatment. The use of AM fungal species alone (Gm or Fm) also increased this parameter, but the dual inoculation (Gm + Fm) was lower than the combination. There was no significant difference between AM cultivars in terms of increasing P content. Under pathogen-inoculated conditions, the highest total phenol content of 59.61 was obtained from Fo + Fm and the highest phosphorus content of 8.24 from Aa + Gm treatments. This means that the combination of AM species with pathogens can increase the parameters studied in host plants. In plants inoculated with all pathogens (Rf, Fo, and Aa), Fm was more effective than Gm in increasing the studied parameters. However, these effects were more pronounced in plants infected with Rf but not in plants inoculated with Fo.

Table 6. Comparison of the effects of AMF species treatments on phenol, antioxidant, and phosphorus contents in non-infected plants and plants infected with *R. fragariae, F. oxysporum*, and *A. alternata.* *: significant at $p < 0.05$; **: significant at $p < 0.01$; ns: not significant.

Treatment Comparison	Total Phenolic Content (725 nm) Student's *t*-Test	Total Phenolic Content (700 nm) Student's *t*-Test	Total Antioxidant Content (593 nm) Student's *t*-test	P Content Student's *t*-Test
Nc/Rf	−126.45 **	−125.46 **	−80.80 **	−8.87 *
Gm/Rf + Gm	−9.14 **	−10,14 **	−24.01 **	−10.03 *
Fm/Rf + Fm	6.94 *	8.56 *	−9.55 **	−7.37 **
Gm + Fm/Rf + Gm + Fm	−2.07 ns	−0.68 ns	−11.62 **	−1.95 ns
Nc/Fo	−51.03 **	−49.93 **	−98.77 **	−8.83 *
Gm/Fo + Gm	−9.13 **	−10.14 **	−24.01 **	−11.69 **
Fm/Fo + Fm	−45.45 **	59.61 **	−30.36 **	−1.58 ns
Gm + Fm/Fo + Gm + Fm	−50.61 **	−52.76 **	−38.17 **	−0.68 ns
Nc/Aa	−107.20 **	−109.85 **	−72.05 **	−38.11 **
Gm/Aa + Gm	−23.37 **	21.71 **	−35.90 **	8.24 **
Fm/Aa + Fm	−27.20 **	−45.71 **	−24.81 **	3.17 *
Gm + Fm/Aa + Gm + Fm	−184.94 **	−208.06 **	−29.79 **	0.29 ns

There were significant differences ($p < 0.05$ and $p < 0.01$) between the negative controls (uninoculated) and the Rf-, Fo-, and Aa-inoculated plants in all parameters examined. Comparing the treatments with and without inoculation of pathogens, single (Gm and Fm) and double inoculation (Gm + Fm) of AM fungal species had significant effects on phenol and antioxidant contents. Moreover, the use of each AM species (Gm or Fm) had no effect on the phosphorus content of plants in either condition (with or without pathogens), but this parameter was significantly affected in the double treatments (Gm + Fm). The results showed that AM species had significant effects on pathogen-infected plants.

3.4. Evaluation of AMF Root Colonization, Spore Density, and Mycorrhizal Dependence in Ro-, Fo-, and Aa-Infected Plants

The effects of AMF species on AMF root colonization, spore density, and mycorrhizal dependence in plants infected with Ro, Fo, and Aa are presented in Table 7.

Table 7. Effect of AMF species treatments on mycorrhizal root colonization, spore density, and mycorrhizal dependence in non-infected plans and plants infected with *R. fragariae, F. oxysporum*, and *A. alternata.* Gm: *Gigaspora margarita*, Fm: *Funneliformis mosseae*, Rf: *Rhizoctonia fragariae,* Fo: *Fusarium oxysporum*, Aa: *Alternaria alternata.* *: values with the same letter in each column are not significantly different based on Duncan's multiple range test at at $p < 0.05$.

Treatments		Mycorrhizal Root Colonization (%)	Spore Density (spores/g)	Mycorrhizal Dependency
Non-Pathogen	Gm	63.38 ± 2.28 a	52.35 ± 1.56 a	+62.75
	Fm	64.17 ± 2.60 a	53.05 ± 1.20 a	+48.20
	Gm + Fm	57.33 ± 1.72 a	46.45 ± 1.78 a	+30.48
R. fragariae	Rf + Gm	50.10 ± 2.16 a	41.26 ± 1.68 a	+66.00
	Rf + Fm	55.66 ± 3.67 a	38.80 ± 1.37 a	+63.57
	Rf + Gm + Fm	53.84 ± 1.76 a	39.00 ± 1.49 a	+82.16
F. oxysporum	Fo + Gm	55.63 ± 2.62 a	33.07 ± 1.27 b*	+52.44
	Fo + Fm	57.68 ± 1.03 a	62.92 ± 4.26 a	+60.51
	Fo + Gm + Fm	55.12 ± 2.39 a	32.25 ± 0.65 b	+60.37
A. alternata	Aa + Gm	56.50 ± 1.11 a	31.47 ± 1.06 b	+50.45
	Aa + Fm	57.80 ± 2.17 a	31.52 ± 0.91 b	+55.18
	Aa + Gm + Fm	50.31 ± 1.60 a	40.27 ± 2.81 a	+59.70

Under non-pathogen conditions, the highest amount of mycorrhizal root colonization was observed in the Gm, Fm, and Gm + Fm treatments with no significant difference ($p < 0.05$). This means that there was no significant difference in root colonization between AM fungal species (Gm and Fm) and their dual-inoculation treatment (Gm + Fm). Considering soil spore density, the highest and lowest values were obtained in Fm and Gm treatments (53.05 and 32.25 spores/g), respectively. Moreover, the effect of dual inoculation with AM fungi (Gm + Fm; 46.45 spores/g) was lower than that of the Fm (53.05 spores/g) and Gm (52.35 spores/g) treatments. In this case, although the Fm treatment was higher than Gm, it was statistically insignificant ($p > 0.05$).

The effects of treatments on the studied parameters were different in plants infected with pathogens. In plants inoculated with Rf, Fo, and Aa, the highest amounts of mycorrhizal colonization indices were observed in AM fungal species treatments (Gm, Fm, and Gm + Fm) with different pathogens (Rf, Fo, and Aa), without significant differences, while the lowest amount was obtained in the Aa + Gm + Fm and Rf + Fm treatments with pathogens (50.31% and 50.10%, respectively). This means that there was no significant difference in root colonization between AM species and the dual-inoculation treatment (Gm + Fm). Regarding the parameter of fungal spore density, the treatments had different effects. In Rf-infected plants, the highest values were observed in the Rf + Gm and Rf + Gm + Fm treatments (41.26 and 39.00 spores/g), followed by Rf + Fm (32.80 spores/g) without significant differences. This means that the *G. margarita* species had a greater effect on the spore density parameter than *F. mosseae*. In Fo-infected plants, the highest spore density was obtained in the Fo + Fm treatment (62.92 spores/g). No significant difference was found in the Fo + Gm and Fo + Gm + Fm treatments. In this case, *F. mosseae* had a greater effect on the spore density parameter than *G. margarita*. However, in Aa-infected plants, no significant difference was found between AM fungal species on the spore density parameter (31.47 spores/g in Aa + Gm and 31.52 spores/g in Aa + Fm treatments, respectively), which was significantly lower than in the double inoculation (40.27 spores/g).

The mycorrhizal dependence results are consistent with the mycorrhizal colonization index. In all plants studied (non-pathogen-infected and pathogen-infected plants), the highest mycorrhizal dependence values (+82.16, +66.00, +63.57, and +62.75) were observed in the Rf + Gm + Fm, Rf + Fm, Fo + Fm, and Gm treatments, respectively. In the mycorrhizal dependence parameter, *F. mosseae* was more effective than *G. margarita* in the Aa and Fo treatments, while the opposite was the case in the Rf and non-pathogen groups.

4. Discussion

This study showed the differential effects of two different AMF (*Funneliformis mosseae* (Fm) and *Gigaspora margarita* (Gm)) on the disease severity, growth, and biochemical parameters of strawberry plants infected with three major strawberry pathogens (*Rhizoctonia fragariae* (Rf), *Fusarium oxysporum* (Fo), and *Alternaria alternata* (Aa)).

Among the pathogens, Rf caused the highest disease severity, followed by Fo and Aa. However, all AMF treatments, including both individual species and the dual inoculation of Fm and Gm, significantly reduced the disease severity index compared to control treatments. This suggests that the presence of AMF can effectively mitigate the severity of fungal diseases, but to a different extent depending on the AMF and the pathogen. The symbiotic AM fungus causes important physiological changes in the plants after penetration, and also under disease conditions. The protective effect of AMF species on root diseases may be due to a better P uptake in mycorrhizal plants [56] or competition for plant colonization. Some researchers have reported that mycorrhiza had a positive effect on the disease severity and plant growth of *Rhizoctonia solani* in strawberries [57,58]. It has been reported that AM fungal species applied to strawberries reduced the pathogenicity of *R. fragariae* [59]. In an experiment with shrubs, it was determined that mycorrhiza application both suppressed the *A. alternata* pathogen and encouraged the development of plants [60]. In another study, *F. mosseae* and *Trichoderma harzianum* were used against the

A. alternata in cucumber and a combination of bioagents had a positive synergistic effect on the reduction in disease severity [61]. The effects of AMF treatments on plant growth parameters varied depending on the pathogen. In Rf-infected plants, the Rf + Gm + Fm treatment showed significant increases in plant fresh weight, dry weight, and length, while the Rf + Fm and Rf + Gm treatments exhibited similar values. In Fo-infected plants, all treatments resulted in increased plant growth parameters compared to Fo alone, with the Fo + Gm + Fm treatment showing the highest increases. Notably, Fm was more effective than Gm in improving fresh weight and dry weight, whereas Gm was more effective in enhancing plant length. Similarly, in Aa-infected plants, all treatments increased plant growth parameters, with the highest increases observed in the Aa + Gm + Fm treatment. These findings suggest that the specific AMF species and their combinations can influence plant growth responses under different pathogenic conditions. In another study, it was found that *G. intraradices* and *G. etunicatum* AMF species applied to strawberry increased leaf area, dry weight, and shoot length [62]. Likewise, it is reported that mycorrhiza had a positive effect on plant growth [63]. The AMF species treatments also had significant effects on total phenolic content, antioxidant activity, and total phosphorus content in pathogen-infected plants. The application of AMF, whether individually or in combination, led to increased values of these parameters compared to control treatments. Again, we observed that the specific effects varied depending on the pathogen and the treatment. For instance, in Fo-infected plants, the Fo + Fm treatment resulted in the highest total phenolic content, while the Aa + Gm treatment showed the highest phosphorus content. Another interesting outcome of our study is related to the content of antioxidants. Phenolic compounds and related antioxidants may be altered in the presence of plant pathogens [64]. In this study, we have determined that the total amount of phenolic substances and antioxidant activity differed among the combinations with or without pathogens. Fm had more efficiency than Gm with respect to increasing these parameters in plants inoculated with all pathogens (Rf, Fo, and Aa). Our results indicate that AMF can positively influence the biochemical and nutritional composition of plants (i.e., higher antioxidant content) and enhance their defense mechanisms against pathogens. In some studies, it was stated that AMF application significantly increased the total amount of phenolic substances compared to controls, while the application of pathogens decreased it [65,66]. AMF application increased the total amount of phenolic substances, antioxidant activity, and P content in tomato plants contaminated with *A. solani,* while this situation also affected some biochemical events in the plants and activated the defense mechanisms of the plants against the pathogens [67]. Our results showed that using a single AM species (Gm or Fm) had no effect on the phosphorus content of plants, but when used together (Gm + Fm) the phosphorous content was significantly affected. AMF species can effectively uptake many macro- and micronutrients (zinc, copper, manganese, iron, calcium, potassium, nitrogen, etc.), especially P [39,58,68–72]. Root colonization and fungal spore density were also assessed in this study. The levels of root colonization were similar among the different AMF species treatments and the dual-inoculation treatment, indicating no significant differences in the extent of root colonization by Fm and Gm. However, the effects on fungal spore density varied depending on the pathogen. In Rf-infected plants, the highest spore density was observed in the Rf + Gm and Rf + Gm + Fm treatments, while in Fo-infected plants, the Fo + Fm treatment exhibited the highest spore density. No significant differences were found in spore density between the AMF species treatments in Aa-infected plants. These findings suggest that AMF species can influence fungal spore production differently depending on the pathogen. In this study, the percentage of AMF root colonization was slightly lower in the diseased plants than in the non-diseased plants. In previous studies, it was determined that AM fungi were affected by various hosts and different pathogens and root colonization was reduced [56,58,66,67,71–74]. There are some studies that report no correlation between mycorrhizal colonization and disease reduction in host plants [75,76], but the results presented in this report show that this is not the case in strawberry. Mycorrhizal dependence, which reflects the reliance of plants on AMF, was also evaluated. The results

indicated that Fm was more effective than Gm in increasing mycorrhizal dependence in Aa and Fo treatments, while Gm was more effective in Rf and non-pathogen treatments. This suggests that the specific AMF species can vary in their ability to establish mycorrhizal associations and contribute to plant growth and development depending on the pathogen present. In the current context of climate change, growing conditions are changing for natural environments and cultivable land [77]. This will change pathogen dynamics and extension. Therefore, the use of strategies like the ones described in the present report, that able to cope with pathogens and maintain yield and are based on natural products, is of paramount importance [78], as they are suitable for both organic and conventional farming.

5. Conclusions

The conclusions drawn from this study offer valuable insights into the effects of different AM fungal species on plant resistance against common strawberry pathogens. The combination of biocontrol factors, specifically the use of both *Gigaspora margarita* (Gm) and *Funneliformis mosseae* (Fm), demonstrated the potential to enhance plant growth parameters even when plants were under the stress of pathogen infection. Furthermore, an increase in both AMF root colonization and soil spore density was observed in treatments involving Fm, Gm, and their combination. It is noteworthy that mycorrhizal dependence was found to be compatible with mycorrhizal colonization, emphasizing the importance of these beneficial symbiotic relationships in plant health. This study revealed that the effects of AMF species on disease severity, growth, and biochemical parameters in strawberry plants depended on the specific treatment applied and whether the plants were infected with pathogens or not. Additionally, the research highlighted the potential for designing novel biological formulations to cope with biotic stress and maintain yield in crop production. The combination of AMF species, as described in this study, can be a valuable tool for sustainable food production in both conventional and organic farming systems. Taken all together, our study indicates that the use of AMF alone or in combination is a good strategy against the effect of pathogens in strawberry and to increase its antioxidant and phenolic content, but its effectiveness varies depending on the pathogen and the AMF, so it must be evaluated on a case-by-case basis.

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