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Evaluation of the effectiveness of some mycorrhizal fungi isolates against charcoal rot disease

Waleed Khalid Ahmed^{1*} , Hutaf A. A. Alsalim², Ashwaq Talip Mohammed¹ and Hiba Mohammed Youssef³

Abstract

Background The sunflower plants are attacked by serious seed and soil-borne pathogens including charcoal rot disease that caused by *Macrophomina phaseolina*. This disease has serious damages to sunflower crop. This study aimed to assess the efficacy of Arbuscular mycorrhizal fungus against charcoal rot disease as fungicide alternative.

Results Morphological and molecular identification was done, using universal primers for molecular identification. Finally, a greenhouse experiment was conducted, and the length and weight of the plant shoot and root as well as disease incidence and severity percentages were estimated for the treatments infected with the *M. phaseolina* pathogen. The results showed that molecular identification indicates their subordination to *Claroideoglossum etunicatum* and *Funneliformis mosseae*. The greenhouse experiment showed that combined inoculation treatments (*C. etunicatum* + *F. mosseae* and *C. etunicatum* + *F. mosseae* + *M. phaseolina*) had the highest averages of length and weight of the plant shoot and root in the non-infected and pathogen-infected (*M. phaseolina*) treatments. *F. mosseae* and *F. mosseae* + *M. phaseolina* treatments had better effects on plant growth. *M. phaseolina* pathogen had suppression effects than the *C. etunicatum* inoculum (*C. etunicatum* and *C. etunicatum* + *M. phaseolina* treatments). The disease incidence and severity percentages decreased significantly in the inoculated plants than in non-inoculated plants. *F. mosseae* inoculum showed more efficiency in reducing DI and DS than *C. etunicatum* inoculum, while adding both, resulting in an extra significant reduction. The combined inoculation, which functions as a biofungicide and a biofertilizer, presented a viable biocontrol technique for crop protection.

Conclusions Such the AM fungi treatments are considered good and biological safe. The symbiotic relationship of AM fungi with plants can also be utilized to gain successful management of agricultural against soil-borne diseases for getting rid of the negative effects of pesticides.

Keywords *Funneliformis mosseae*, *Claroideoglossum etunicatum*, *Macrophomina phaseolina*, Arbuscular mycorrhiza, Biological control, Molecular diagnosis

Background

The sunflower (*Helianthus annuus*) is one of the most important oil seed crops as it contains high-quality edible oil (40–50% by weight) and protein (15–21% by weight) (Haj Sghaier et al. 2023). Diseases are serious threats to the sunflower crop all over the world. Charcoal rot caused by *Macrophomina phaseolina* is of prime importance in reducing crop yield, especially in arid regions of the world. It has been estimated that the disease can cause an average annual loss of 12% in yield, from nearly

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12 million hectares of world land (Ijaz et al. 2013). *Macrophomina phaseolina* is an important soil-borne pathogen that infects many economically important crops such as sunflower, maize, cotton, soybean, and sesame (Adel-Kaderet et al. 2010). Chemical fungicides are used to control the *M. phaseolina* pathogen. However, these chemicals are not eco-friendly; hence, an alternative method is needed to manage this disease. Recently, soil-borne diseases have been controlled by utilizing certain beneficial microorganisms that are indigenous to the plants' rhizosphere (Kumar et al. 2014). Various microorganisms have been used effectively, such as *Bacillus* spp., *Trichoderma* species, and Arbuscular mycorrhizal (AM) fungi, as biological control agents for several pathogens (Weng et al. 2022). These bioinsecticides provide a better option than chemicals due to their safety for humans and the environment and lower cost (Adekunle et al. 2001).

AM fungi, which are widely spread in soil environments, can develop symbioses with more than 80% of terrestrial higher plants and enhance the plants' nutrition (Weng et al. 2022). The fungi have been broadly used as biological control agents to control numerous phytopathogenic fungi (Lin et al. 2021). AM fungi can organize secondary metabolite formation in the host plants by altering the plant roots' morphology or anatomical structure, developing the rhizosphere environment characteristics (chemical and physical), competing with the pathogens for infection space and photosynthetic products, and activating defense systems and disease resistance in plants (Aseel et al. 2019). According to Sudhasha (2020), the chemical balance of mycorrhizae inhibits growth and reproduction of pathogenic fungi. They found out that *G. intraradices* prevented the growth of *F. oxysporum*. Aguk et al. (2018) found that the disease incidence and index of *Ralstonia solanacearum* treatments inoculated with *G. mossie* and *G. rhizogenes* were reduced by 49.8 and 9.7%, respectively, than the treatment not inoculated with AM fungi (the control).

As a result, the employment of AM fungi is regarded as a technique in sunflower plant improvement. It was necessary to use AM fungi that were adapted to the region's environmental conditions. Thus, the study's goal was to isolate and identify AM fungi and assess their efficacy on sunflower plant growth and the incidence of charcoal rot.

Methods

Soil samples

An extensive field survey was carried out (in August 2021) to randomly collect the sunflower (*Helianthus annuus*) rhizospheric soil samples. About 500 g of 30 rhizospheric soil samples near the root zone (depth 15–20 cm) was collected in sterile polyethylene bags. The soil

samples were transferred to the laboratory in an icebox, where they were kept at 4 °C until use.

Monosporal culture

A variety of techniques can be used to separate monosporal cultures from the soil. The wet-sieving and decanting technique was used to isolate Arbuscular mycorrhiza (AM) fungi spores (Gerdemann and Nicolson 1963). After filtration, spores of different sizes were picked up, while observing under a microscope. Then, they were observed under a microscope after being mixed with a drop of lactophenol blue (phenol, lactic acid, aniline blue dye, and glycerol) on clean slides.

Microscopic identification

Spores' morphology serves as the basis for the identification of AM fungi because the hyphae and the organs, like vesicles and arbuscules, are not species-specific. AM fungal spores were observed microscopically, and identifications, at the genus level, were performed by examining diagnostic characteristics such as spore size, shape, color, wall, and type of hyphal attachment (Oehl et al. 2011).

Molecular identification

The primers

The forward (ITS1 F: 5' - TCCGTAGGTGAACCTGCG G-3') and reverse (ITS4 R: 5' TCCTCCGCTTATTGA TATGC-3') primers used for AM molecular identification (White et al. 1990), based on amplifying the genomic DNA region of the fungi utilizing a thermal cycler (PCR-system 9700; Applied Biosystems), were supplied by Macrogen Company/Korea.

Extracting of DNA

DNA was extracted from AM spores (morphologically identical) by crushing the spores with 40 µl of Tris-EDTA (TE) and heating them at 95 °C for 10 min in the presence of 10 µl of Chelex-100 (Dong and Zhao 2006).

PCR technique

AM fungi species were identified using the PCR technique and the universal primers ITS1 F and ITS4 R. The PCRs were achieved in a total volume of 25 µl; 5 µl DNA, 5 µl of Taq PCR PreMix (Intron, Korea), 1 µl of each primer (10 pmol), and then, distilled water was added into the tube (13 µl). The thermal cycler was programmed as shown in Table 1. The results were obtained by the software provided with the device. Electrophoresis was performed to examine the amplification products on a 1.5% agarose gel stained with ethidium bromide (EtBr).

Table 1 PCR program for ITS gene detection of the studied fungi

| Steps | Temperature (°C) | Time (M:S) | No. of cycle |
|----------------------|------------------|------------|--------------|
| Initial denaturation | 95 | 03:00 | 1 |
| Denaturation | 95 | 00:45 | 35 |
| Annealing | 58 | 00:45 | |
| Extension | 72 | 00:45 | 1 |
| Final incubation | 72 | 07:00 | |

Collection of *Macrophomina phaseolina*

An intensive survey was conducted (during the summer of 2021) on the incidence of charcoal rot in different locations of sunflower fields. Twenty samples of sunflower plants showing typical symptoms of charcoal rot were collected from the various hosts. The roots were transferred to the laboratory, rinsed carefully with tap water, surface-sterilized with 0.1% sodium-hypochlorite solution, and washed three times with sterilized distilled water before being cut into small pieces and air-dried. The root pieces were incubated on PDA (potato dextrose agar) Petri plates in the dark for seven days at 28 ± 2 °C. The arisen fungal colonies (*M. phaseolina*) were examined and then maintained as pure cultures on PDA slants, using the single hyphal tip technique (Singh and Srivastava 1988). Finally, *M. phaseolina* fungal were identified by the PCR technique and the primer pair ITS5 (5'-TCCGTAGGTGAACCTGCG -3') and ITS4 (5'-TCCTCCGCTTATTGATATGC -3') based on amplifying the genomic DNA region of the fungi (Integrated DNA Technologies company, USA) (White et al. 1990).

Pot culture

Sorghum (*Sorghum halepense* L.) and alfalfa (*Medicago sativa* L.) host species were used as trap plants for the mass production of soil-based AM spores inocula (Kapoor et al. 2007). Sterilized pots were loaded with 3 kg of soilrite: soil (1:3) sterilized mixture and then covered with 100 spores of AM inoculum per 3 kg of soilrite, for both *C. etunicatum* and *F. mosseae* isolates separately. Fungi species have an inoculum density of 100–105 spores per 3 kg of soil. Sorghum and alfalfa seeds were sanitized with HgCl₂ (0.01%) for two min and then washed three times with sterilized distilled water before being transferred to the pots. Twenty-four pots were used: 12 pots for each plant species of which the six ones were for each fungus species. The pots were kept in a greenhouse under controlled conditions for 60 days. Of the nine plants, three were chosen

Table 2 The properties of greenhouse experiment soil

| Properties | Soil contents |
|---|-----------------|
| pH (1:1) | 7.5 |
| Electric conductivity (EC) (1:1) (ds m ⁻¹) | 2.67 |
| Cation exchange capacity (CEC) (cmol kg ⁻¹) | 16 |
| Nitrogen (mg kg ⁻¹) | 2.12 |
| Phosphorus (mg kg ⁻¹) | 10.3 |
| Organic matter (g kg ⁻¹) | 1.11 |
| Calcium phosphate (g kg ⁻¹) | 140 |
| Calcium carbonate (g kg ⁻¹) | 157 |
| Sand (g kg ⁻¹) | 510 |
| Silt (g kg ⁻¹) | 290 |
| Clay (g kg ⁻¹) | 200 |
| Texture | Sandy clay loam |

at random and pulled from each pot. Three plants were chosen at random and pulled from each pot. Root samples were washed with sterile water, then sterilized with sodium hypochlorite (NaOCl, 2%) for 5 min, and rewashed with sterilized distilled water. After staining with Trypan blue (Philips and Hayman 1970), the samples were cut into 1-cm segments, and AM colonization was investigated according to the following equation:

$$\text{Colonization\%} = \frac{\text{Number of AM positive segment}}{\text{Number of the total segment}} \times 100$$

Greenhouse experiment

Soil preparation

The soil was collected from the fields, sieved through 2mm after air-drying, and then autoclaved for 1 h at 121 °C (Bashan et al. 1995). The properties of the soil are presented in Table 2.

Seeds preparation

Sunflower (*Helianthus annuus*) seeds, provided by Ebaa agriculture research center/Iraq, were sterilized with sodium-hypochlorite solution (4%) for 10 min and then washed with sterile distilled water (Younesikelaki et al. 2016).

Inoculums preparation

Mass-multiplied AM fungi species that were obtained by pot culture were used as inoculums. One hundred grams of roots plus soil, which contained 100 spores per 1 g, was used as inoculums for *C. etunicatum* and *F. mosseae* treatments. *M. phaseolina* isolate was grown on local millet seeds, *Panicum miliaceum* L. The seeds were washed well and moistened for 6 h and then dried at laboratory

temperature. One hundred grams of millet seeds was placed in a 250-ml flask, a little water was added, and it was autoclaved. After cooling, the flasks were inoculated with a *M. phaseolina* isolate and incubated at 25 ± 2 °C for 10 days, with shaking every 2–3 (Dewan and Sivasithanparam 1988).

Greenhouse pots preparation and cultivation

Sterile pots were filled with 8 kg of autoclaved soil. Sunflower seeds were planted (10 seeds per pot) after applying 100 g of mycorrhizal inoculum at 3–4 cm depth. Pathogen inoculum was inoculated around the plants' roots after 20 days of germination. The experiment was carried out in a complete random design (CRD) with three replicates and the eight treatments listed below: C (Control, no inoculum or pathogen), C.e (*C. etunicatum* inoculum), F.m (*F. mosseae* inoculum), C.e+F.m (*C. etunicatum* and *F. mosseae* inoculums), M.p (*M. phaseolina* pathogen), C.e+M.p (*C. etunicatum* inoculum and *M. phaseolina* pathogen), F.m+M.p (*F. mosseae* inoculum and *M. phaseolina* pathogen), and C.e+F.m+M.p (*C. etunicatum*, *F. mosseae* inoculums, and *M. phaseolina* pathogen). NPK fertilizer (200 kg hec^{-1}) was added to pots, which were arranged randomly inside a green house and irrigated with sterilized distilled water.

Plant growth assessment

Plant growth was assessed after 45 days from germination by estimating the length and weight of each plant shoot and root. Air-drying plant shoots were crushed for digestion (Gresser and Parsons 1979), and the concentrations of calcium (Ca), potassium (K), and magnesium (Mg) were determined using a flame photometer, while phosphorus (P) was determined using a spectrophotometer (Tandon 1995).

Disease incidence and disease severity

Percentages of disease incidence (DI) and disease severity (DS) were calculated for the treatments infected with the *M. phaseolina* pathogen. DI, the infected leaves percentage, is calculated according to the following formula:

$$\text{DI}(\%) = \frac{\text{Number of infected plants}}{\text{Number of observed plants}} \times 100$$

DS was evaluated on a 5-degree scale (from 1 to 5) according to symptom severity, wilt, develop a gray-black discoloration, external discoloration on the basal part of the stem, and depth 1=healthy, 2=lesion on stem only, 3=25% plant symptomatic, 4=26–50% plant

symptomatic and $5 \geq 50\%$ of the plant symptomatic (Shokes et al. 1996) as presented in the following formula:

$$\text{DS}(\%) = \frac{\sum (\text{scale} \times \text{number of infected plants}) \times 100}{4 \times \text{total number of plants}}$$

Statistical analysis

The experiment was carried out in a complete random design (CRD) in vitro condition, and data were analyzed for variance (ANOVA) using the SAS software version (9.1). The means were compared using a 0.05 least significant difference (LSD).

Results

Microscopic identification

Sunflower rhizosphere soil from the Al-Alam area, in Saladin governorate-Iraq, showed the presence of various types of AM spore populations. Spores' morphology serves as the basis for the identification of AM fungi because the hyphae and the organs, like vesicles and arbuscules, are not species-specific. AM fungal species were identified at the genus level after their spores were isolated from soil samples and adopted the taxonomic key mentioned in Oehl et al. (2011). The morphological identification showed that the spores were related to two species after matching its characteristics with the key, which depends on the spore's color, size, external shape, contents, number of walls, thickness of the walls, and type of hyphal attachment.

The results showed that 17 (56.7%) samples seemed to be related to *Funneliformis* spp. and 13 (43.3%) samples seemed to be related to *Claroideoglomu* spp. Spores collected from soil often deteriorate, which is why they can be used only for tentative identification at the genus level. Therefore, molecular identification was done for the most abundant and similar in morphological for spores obtained from the 30 samples. The spores of mycorrhizal fungi spread in many soils, but there is a difference in the species and number of spores due to the type of host plant, the genus of the fungus itself, the physical, chemical, and fertility properties of the soil (Lynch 2011).

Molecular identification of AM fungi

In general, the ITS region assay is a commonly used method for the diagnosis of fungi, AM fungal spores were classified, and typical spores were chosen. The DNA was extracted from spores, and then, PCR analysis was performed. An approximately 650 bp of ITS DNA fragment was successfully amplified from AM fungi species (Fig. 1). AM fungi species showed similarity rate, which

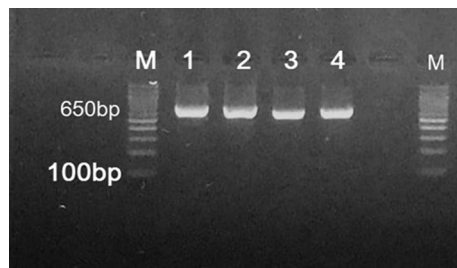


Fig. 1 Agarose gel electrophoresis image that shows PCR product analysis of AM fungi by (ITS1- ITS4) primers. Lanes: M, 100 bp ladder marker, (1 and 2) *Funneliformis mosseae* (3 and 4) *Claroideoglomus etunicatum* the band size 650 bp

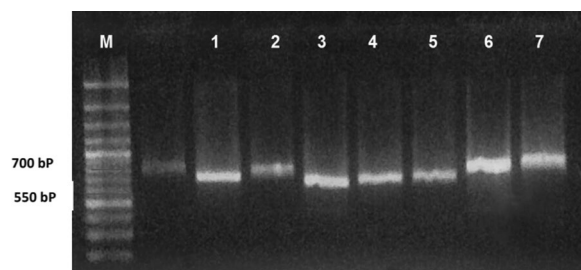


Fig. 2 Agarose gel electrophoresis image that shows PCR product analysis of *Macrophomina phaseolina* by (ITS4-ITS5) primers, the band size 550 to 700 bp

was 100% with the *F. mosseae* and *C. etunicatum*. *F. mosseae* and *C. etunicatum* isolates were recorded at the gene bank, National Center Biotechnology Information (NCBI), No: OP615192.1 and OP620675.1, respectively.

Molecular identification of *Macrophomina phaseolina*

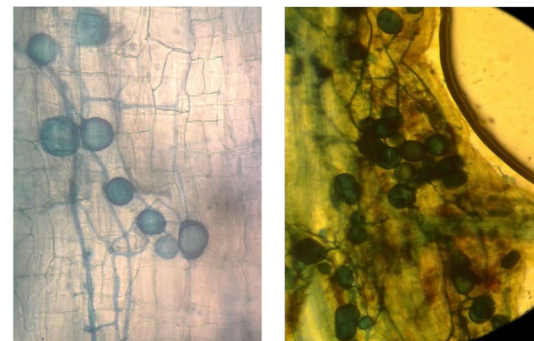
The collected fungal strains from sunflower plants showing typical symptoms of charcoal rot were identified as *M. phaseolina* by the production of several individual sclerotia on the Petri plates containing PDA. The DNA was extracted from spore samples, and then, PCR analysis was performed. An approximately 550 to 700 bp of ITS DNA fragment was successfully amplified from all *M. phaseolina* (Fig. 2). *M. phaseolina* isolate was recorded at the gene bank, NCBI, No: OQ215734.1.

Pot culture

The percentages of *F. mosseae* spores' colonization were higher than those of *C. etunicatum* spores in both Sorghum and alfalfa host plants. The colonization of *F. mosseae* spores was 100% and 95% in Sorghum and alfalfa host plants, respectively, while the colonization was 80% and 70% for *C. etunicatum* spores in Sorghum and alfalfa host plants, respectively (Table 3). Sorghum plants showed higher *C. etunicatum* and *F. mosseae*

Table 3 The colonization percentage and spores number of *Claroideoglomus etunicatum* and *Funneliformis mosseae* in the Sorghum and alfalfa host plants

| Genus | Host plant | Colonization (%) |
|----------------------|------------|------------------|
| <i>C. etunicatum</i> | Sorghum | 80 |
| | Alfalfa | 70 |
| <i>F. mosseae</i> | Sorghum | 100 |
| | Alfalfa | 95 |



(A) *Claroideoglomus etunicatum* (B) *Funneliformis mosseae*

Fig. 3 AM fungi colonizing spores in the Sorghum host plant; A *Claroideoglomus etunicatum* and B *Funneliformis mosseae*

colonizations than alfalfa plants, suggesting that the Sorghum host species is the preferable plant trap for the mass production of soil-based AM spore inocula. AS shown in Fig. 3, *F. mosseae* and *C. etunicatum* fungi colonizing spores in the Sorghum host plant. The colonization of AM fungi and the subsequent production of spores depend on the host type and increase with the infection period (Suada et al. 2018). Studies discovered that mycorrhizal colonization and spore density did not significantly correlate with soil pH, Mg, Ca, Al, K, or organic matter levels, but only with P content. The highest colonization rates take place at low or absent P fertilization levels (Santos et al. 2017).

Greenhouse experiment

The greenhouse experiment treatments are depicted in Figs. 4 and 5 as illustrated; shoot length (A), root length (B), shoot fresh weight (C), and root fresh weight (D) of sunflower plants after 45 days from germination.

Shoot length

The average of sunflower shoot length after 45 days from germination recorded a significantly the highest value when both *C. etunicatum* and *F. mosseae* inoculums were added (C.e+F.m), which was 102.25 cm. The addition of *F. mosseae* and *C. etunicatum* separately (F.m and C.e



Fig. 4 The greenhouse experiment treatments

treatments) resulted in a significant increase in the shoot length (93.25 and 91.75 cm, respectively) than the control treatment (80 cm). The effects of the two AM fungal species did not differ significantly. In the treatments of *M. phaseolina* pathogen infection, both AM fungal species (F.m + M.p and C.e + M.p treatments) reduced the effect of the pathogen on the plant shoot length significantly, with significant superiority to *F. mosseae*, while the treatment of adding both *C. etunicatum* and *F. mosseae* inoculums (C.e + F.m + M.p), which gave an average of 91 cm, showed the most significant effect on the pathogen suppression.

Root length

The highest length of roots was recorded by C.e + F.m treatment (13.96 cm), followed by F.m (13.92 cm), and then C.e treatments (12.63 cm) with non-significant differences, but the three treatments showed a significant increase from the control (C) treatment (11.16 cm). In the pathogen-infected (*M. phaseolina*) treatments, the binary inoculation treatment (C.e + F.m + M.p) showed the highest root length (12.48 cm), which differs significantly from the single inoculation. The F.m + M.P treatment (11.02 cm) had greater significant effect on pathogen suppression than the C.e + M.P treatment (8.87 cm), but both treatments increased plant root length significantly as compared to M.p treatments (5.98 cm).

Shoot fresh weight

The C.e + F.m. treatment recorded the highest average of shoot fresh weight (79.1 g), followed by the F.m. (77.21

g) and C.e. (76.42 g) treatments with non-significant differences. These three treatments showed a significant increase from the control (C) treatment (64.1 g). The (C.e + F.m + M.p) treatment (64.42 g) showed a maximum shoot fresh weight in the infected treatments, followed by the F.m + M.P treatment (62.23 g), with non-significant differences, and the (C.e + M.p) treatment (49.98 g), with a significant difference. Nevertheless, when compared to the MP treatment (35.84 g), these three inoculation treatments resulted in a significant increase in shoot fresh weight.

Root fresh weight

The combined inoculation treatment (C.e + F.m) had the highest average of fresh root weight (5.88 g), which was significantly different from the other treatments. Single inoculation (C.e and F.m treatments) showed a significant increase in the fresh root weight (5.21 and 5.3 g) than the control (C) treatment (4.45 g), whereas in the pathogen infection treatments, (C.e + F.m + M.p) and (F.m + M.p) treatments (5.4 and 5.08 g, respectively) revealed a significant increase in root fresh weight from the rest treatments, followed by (C.e + M.p) treatment (3.89 g), which also showed a significant increase from M.p treatment (3.07 g).

Ca, K, Mg, and P concentrations

The concentrations of Ca, K, and Mg were determined in the plant shoots after 45 days from sunflower germination (Fig. 6). The results showed that the combined inoculation treatment (C.e + F.m) recorded the highest

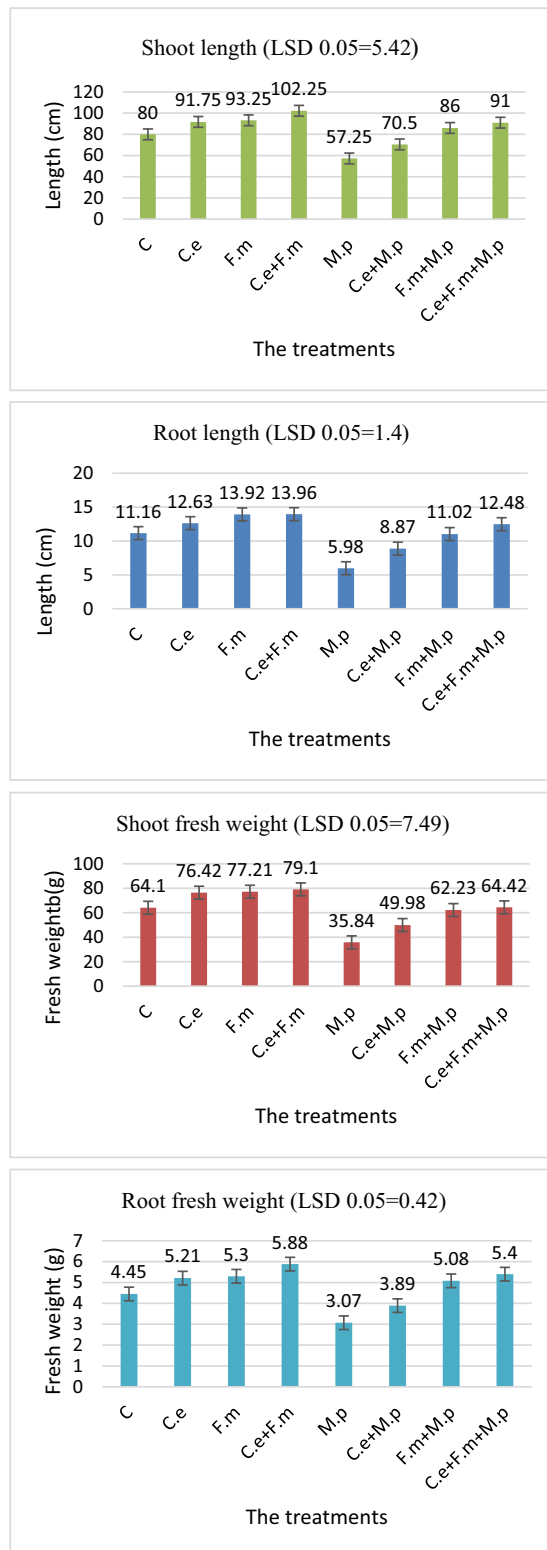


Fig. 5 Sunflower shoot length (cm) (A), root length (cm) (B), shoot fresh weight (g) (C), and root fresh weight (g) (D) after 45 days from germination. The treatments: C (Control, no inoculum or pathogen), C.e (*Claroideoglomus etunicatum* inoculum), F.m (*Funneliformis mosseae* inoculum), C.e + F.m (*C. etunicatum* + *F. mosseae* inoculums), M.p (*Macrophomina phaseolina*), C.e + M.p (*C. etunicatum* inoculum + *M. phaseolina* pathogen), F.m + M.p (*F. mosseae* inoculum + *M. phaseolina* pathogen), and C.e + F.m + M.p (*C. etunicatum* inoculum + *F. mosseae* inoculum + *M. phaseolina* pathogen)

average of these element concentrations, followed by the F.m treatment, then C.e, with a significant difference between them and a significant increase than the control treatment (C). In the pathogen-infected treatments, the combined inoculation by (C.e + F.m + M.p) showed a significant increase at the concentrations of all the tested elements. But the significance of the single inoculation's treatments varied. The (F.m + M.p and C.e + M.p) treatments had significantly decreased Ca concentration values than the (C.e + F.m + M.p) treatment, with a significant difference between them and a significant increase than the M.p treatment. K concentration increased significantly in the (C.e + F.m + M.p) treatment. (F.m + M.p) treatment showed a significant increase from M.p treatment but a non-significant increase from C.e + M.p treatment, which in turn showed a non-significant increase from M.p treatment. Mg concentration recorded a significant increase value in (C.e + F.m + M.p) treatment, followed by (F.m + M.p) then (C.e + M.p) treatments that showed a significant difference between them, while (C.e + M.p) treatment revealed a non-significant difference from M.p treatment. The concentration of P recorded a significant increase in the combined treatment (C.e + F.m + M.p). The treatments (F.m + M.p and C.e + M.p) did not show a significant difference between them, but they differed significantly from the M.p treatment, and the better effect of *F. mosseae* inoculum on plant growth and *M. phaseolina* pathogen suppression.

Disease incidence and severity

AM fungal inoculated plants were less influenced by the presence of *M. phaseolina* pathogene, as a result of the *F. mosseae* and *C. etunicatum* fungal promoting roles in the plant growth enhancement (Fig. 7). The disease incidence percentage decreased significantly in the inoculated plants than in the non-inoculated plants (M.p treatment), which was 93.00%. *F. mosseae* (51.40%) had more effect than *C. etunicatum* inoculum

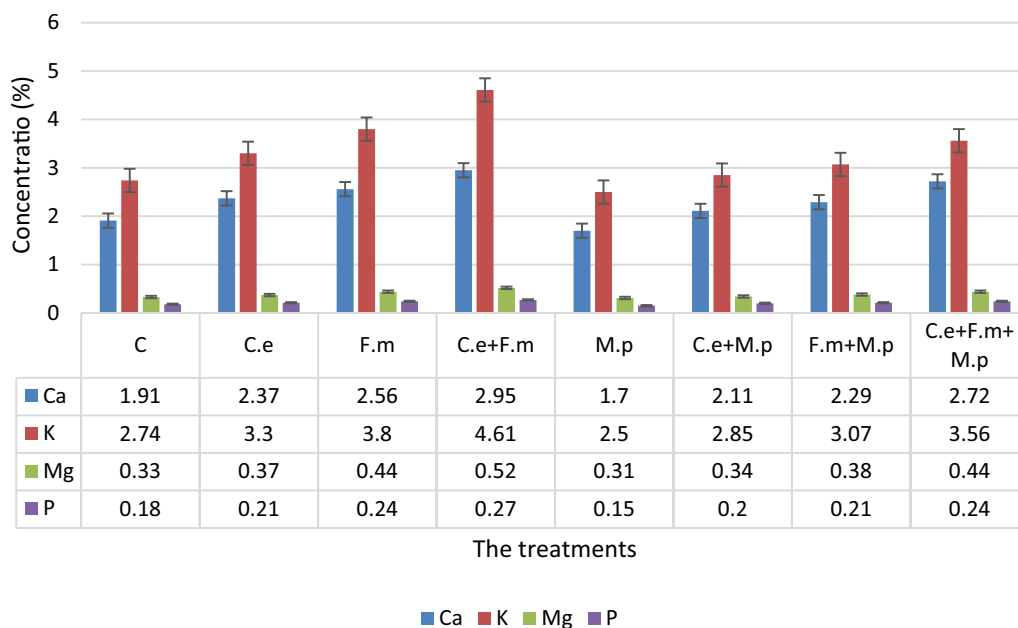


Fig. 6 The concentrations of Ca, K, Mg, and P (%) in sunflower plants after 45 days from germination. The treatments: C (Control, no inoculum or pathogen), C.e (*Claroideoglomus etunicatum* inoculum), F.m (*Funneliformis mosseae* inoculum), C.e + F.m (*C. etunicatum* + *F. mosseae* inoculums), M.p (*Macrophomina phaseolina*), C.e + M.p (*C. etunicatum* inoculum + *M. phaseolina* pathogen), F.m + M.p (*F. mosseae* inoculum + *M. phaseolina* pathogen), and C.e + F.m + M.p (*C. etunicatum* inoculum + *F. mosseae* inoculum + *M. phaseolina* pathogen). LSD 0.05 values: for Ca = 0.16, K = 0.45, Mg = 0.04, and P = 0.02

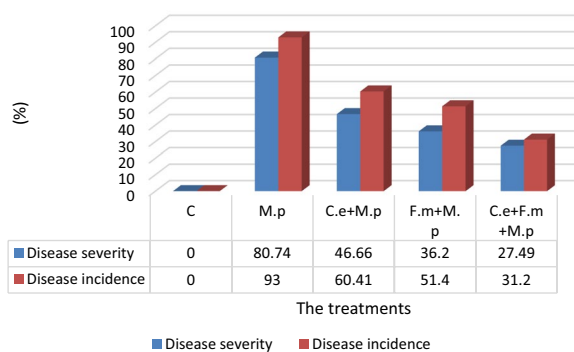


Fig. 7 The percentage of disease severity and disease incidence in the *Macrophomina phaseolina* pathogen-infected sunflower plants. The treatments: M.p (*Macrophomina phaseolina*), (C.e + M.p) (*C. etunicatum* inoculum + *M. phaseolina* pathogen), F.m + M.p (*F. mosseae* inoculum + *M. phaseolina* pathogen), and (C.e + F.m + M.p) (*C. etunicatum* inoculum + *F. mosseae* inoculum + *M. phaseolina* pathogen). LSD 0.05 values: for disease severity = 5.42 and disease incidence = 6.35

(60.41%), and adding them both (C.e + F.m + M.p treatment) resulted in the most efficacy (31.22%). The disease severity percentage was significantly reduced by adding AM fungal inocula, which was 80.74% in the non-inoculated plants. More reduction was gained by adding the two strains in the treatment C. e. + F. m. + M. p. (27.49%), followed by adding the strain *F.*

mosseae (F.m + M.p treatment), which was 36.20%, and then *C. etunicatum* inoculum (C.e + M.p treatment), which was 46.66%. AM fungi colonization aids in the reduction of disease caused by charcoal rot; *F. mosseae* and *C. etunicatum* were effective in improving the tolerance traits and disease resistance of the sunflower plant.

Discussion

DNA barcoding is so useful for diagnosis of fungal types. The internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene bunch are utilized as the fungal barcode and the major advantage of using ITS is making it possible to amplify the gene from small amounts of biological specimens (Abarna and Vishnupriya 2022). The molecular identification was carried out by DNA barcoding using the ITS region sequencing. The ITS rDNA sequences were compared with those in the databases using NCBI-BLAST. AM fungi and the pathogenic fungus, *M. phaseolina*, were identified using DNA barcoding with an identity of 100%. The AM fungi have great influences, directly on enhance health plant and on the life on land. Therefore, molecular identification of AM fungi is becoming more crucial in scientific research and application. Depend molecular identification for AM fungi on the rRNA genes (Thilagar et al. 2018). In this study, the presence of the two types of AM

fungi (*F. mosseae* and *C. etunicatum*) was determined by PCR and followed by sequencing and recorded at the gene bank, National Center Biotechnology Information (NCBI). The previous results showed that the inoculation of plants with AM fungi, particularly *F. mosseae*, affected positively the length and weight of the vegetative plant parts and the roots. The presence of AM fungi inoculation reduced the pathogenic fungus's negative effect on the length and weight of the shoots and roots. The establishment of AM fungi in the host plant roots primarily reduced diseases caused by soil-borne pathogens (Dehne 1982), which could be attributed to AM fungi interacting with plant pathogenic organisms and improving the plants' defenses and resistance to *M. phaseolina*. Generally, in both non-infected and infected plants, combined inoculation treatments performed best. Numerous studies reported an increase in plant growth because of AM fungi colonization, arguing that increased nutrient and water uptake resulted in increased hormonal balance or photosynthetic production, resulting in increased plant biomass. Inoculating wheat, rice, and maize plants with AM fungi had a significant effect on their height, weight, and root colonization because this fungus may serve as a plant's natural fertilizer or biofertilizer (Bholay et al. 2018). The medicinal plant *Solanum viarum* that was colonized with AM fungi showed increased growth, plant height, and dry biomass, mainly due to an increase in the supply of phosphorus (Selvaraj et al. 2011).

Huang et al. (2020) assessed the effect of five AM fungi species on walnut plant growth after 3 months of inoculation and found that *Diversispora spurca*-, *Glomus etunicatum*-, and *Glomus mosseae*-inoculated plants showed higher root length, surface volume, and area than non-inoculated plants. Also, *D. spurca*, *Acaulospora scrobiculata*, *G. etunicatum*, and *G. mosseae* significantly increased leaf transpiration, photosynthesis rate, and stomatal conductivity, while reducing leaf temperature and intracellular CO₂ concentrations. Consequently, they concluded that the positive role of AM fungi on walnuts was dependent on the fungus species.

These findings exhibited the potential contribution of mycorrhizal fungi in getting nutrients available, particularly immobile phosphorus, in the soil. The nutrient content analysis of sunflower indicated that *F. mosseae* had greater potential effect than *C. etunicatum*. Combined inoculation treatments revealed the highest concentrations of Ca, K, Mg, and P in the healthy and infected plants, but they decreased in the infected plants as compared to the healthy plants.

AM fungus colonization can improve a plant's ability to utilize soil nutrients, particularly Ca, K, Mg, P, N, and Mn, and, as a result, increase plant growth (Weng et al.

2022). The increase in nutrient absorption area brought on by the action of widespread mycorrhizal hyphae in soils contributes to the increase in growth parameters carried on by mycorrhizal symbioses (Tarraf et al. 2017). Enhancements of plant growth and the uptake of nutrient by AM fungi inoculation were earlier elucidated by many researchers (Weng et al. 2022). P enhancement may be related to the organic acids and phosphate released by AM fungi (Nafady et al. 2019).

The colonization of AM fungi in plant roots increased their resistance to pathogenic microorganisms and decreased several diseases caused by soil-pathogenic organisms (Oyewole et al. 2017). Sunflower plants inoculated with AM fungi showed less damage from the pathogenic fungus. This signifies defense against pathogen attack on the root tissues, which may be due to competition between AM fungi and *M. phaseolina* for nutrients and space (Nafady et al. 2019).

Obtained results confirm a decrease in disease incidence and severity; this decrease may be due to competition for space and photosynthates between the AM fungus and the pathogen in the host plant, which is similar to the finding of Doley and Jite (2013). The AM fungus's effect on disease incidence and severity may be attributed to the fact that the MF affects the physiology of the host plant and positively activates the plant's resistance by creating or inducing a type of mechanical barrier against the invading pathogen (Wendimu 2022).

Conclusions

The symbiotic relationship of AM fungi with plants can be utilized to gain successful management of agricultural soils because of its significance in improving plant growth by increasing nutrient uptake and providing disease protection. The concept here is to use AM fungi as a natural, harmless, and effective alternative to pesticides and fertilizers. Sunflower plants inoculated with AM fungi showed remarkable improvements in comparison with healthy non-inoculated plants. In addition, with the help of AM fungi inoculations, the harmful effects of the *M. phaseolina* pathogen can be reduced in pathogenic-infected plants.

Abbreviations

| | |
|-----|------------------------------------|
| AM | Arbuscular mycorrhizal |
| M.P | <i>Macrophomina Phaseolina</i> |
| C.e | <i>Claroideoglossum etunicatum</i> |
| F.m | <i>Funneliformis mosseae</i> |
| DI | Disease incidence |
| DS | Disease severity |
| SAS | Statistical analysis software |

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Author contributions

WK, AT and HM collected the samples (mycorrhizal fungi and pathogen), microscopic and molecular identification, conduct and design of experiment in greenhouse of and the acquisition, analysis, interpretation of data; WK and HA have drafted the work or substantively revised it were a major contributor in writing the manuscript.

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