Journal of Phytopathology and Pest Management 9(1): 14-31, 2022
pISSN: 2356-8577 eISSN: 2356-6507
Journal homepage: http://ppmj.net/

Chia charcoal rot disease and its management using certain bio-agents in Egypt

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Abstract

The current study was performed to control the charcoal stem rot disease caused by *Macrophomina phaseolina* in chia plants (*Salvia hispanica* L.). *Macrophomina phaseolina* was morphologically identified as the causal pathogen of charcoal stem rot on naturally infected chia plants showing typical symptoms of disease and obtained from Fayoum, Giza, and Menoufia governorates, Egypt in the 2021 cultivation season. Additionally, the molecular characterization of the causal pathogen revealed 99.65-100% identity with several isolates of the same species. The isolates obtained from all governorates under study resulted in pre- and post-emergence damping-off with significant variations. However, *M. phaseolina* isolated from Fayoum governorate recorded the highest percentage of charcoal stem rot. Moreover, the filtrate of the same isolate caused the highest percentages of wilted seedlings. The presence of yeast, amonium chloride and urea as nitrogen sources resulted in the loss of *M. phaseolina* mycelial color. The pigmented isolate of *M. phaseolina* (Fayoum isolate) exhibited a high virulence to chia plants in greenhouse compared to the non-pigmented one. The two bio-agents; *Trichoderma asperellum* and *Streptomyces rochei*, isolated from the rhizosphere soil of healthy chia plants, significantly inhibited *M. phaseolina* fungal growth in comparison with the control *in vitro*. In greenhouse experiment, the fungicide Tricyclazole was the most efficient application for reducing the incidence of disease as well as increasing the plant growth measurements, i.e., number of spikes per plant, number of branches and plant height, followed by the biocide New-Actino. The combined use of the bio-agents *T. asperellum* and *S. rochei* was greatly efficient in both decreasing the disease incidence and improving the plant growth parameters compared to individual use of each. The current study indicated the potential use of the biocide New-Actino, *T. asperellum* and *S. rochei* as fungicidal alternatives for controlling chia charcoal rot disease.

Keywords: chia, charcoal rot, *Macrophomina phaseolina*, *Trichoderma asperellum*, *Streptomyces rochei*.

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1. Introduction

Chia (*Salvia hispanica*), belonging to the family Lamiaceae, is cultivated for its edible seeds. Chia seeds, which are rich in omega-3 fatty acids and fibre, receive attention for their potential health advantages (Ayerza & Coates, 2011) and they are being cultivated for commercial use in many countries, including the US, Bolivia, Argentina, Australia, and Peru. In Egypt, chia farms had indications of charcoal stem rot, which reduced plant stand and vegetative development. The causal pathogen was found to be *Macrophomina phaseolina*, a soil-borne fungus that has several commercial hosts (Su et al., 2001). It is a dangerous disease that reduces chia yield (Nada, 2016; El-Kaed et al., 2021). Charcoal stem rot disease could result in a reduction of both seed quality and yield (Smith & Wyllie, 1999). Five hundred plant species belonging to about 100 families are affected by the global spread of *M. phaseolina*. Certain diseases incited by the pathogen include seedling blight and charcoal rot diseases (Ghosh et al., 2018; Dhingra & Sinclair, 1978). The fungal pathogen could significantly reduce the yields of crops like sorghum and soybean when temperatures are between 30–35 °C and soil moisture is lower than 60% (Kaur et al., 2012). In the worst-case situation, diseases that developed in the pre-emergence stage of groundnut cultivars have been known to cause 100% yield losses (Marquez et al., 2021). *Trichoderma* spp. is known to improve systemic acquired resistance and development in plants. They also effectively control several soil-borne fungal pathogens, such as *M. phaseolina* (El-Kaed et al., 2021). *Trichoderma* may exhibit biocontrol by using many antagonistic actions, *i.e.*, mycoparasitism, release of antibiotics, and nutrition competitiveness. The genus *Trichoderma* has several different species and is considered to be a plant growth-stimulating and promising biological control agent in several crops (Savazzini et al., 2009; Bai et al., 2008; Verma et al., 2007). The present study was performed to investigate the charcoal stem rot disease in chia plants incited by *M. phaseolina* under Egyptian conditions and the role of its black pigment in the virulence of this disease, as well as the evaluation of different fungicidal alternative means for controlling this disease under greenhouse conditions.

2. Materials and methods

2.1 Isolation and identification of the charcoal stem rot causal pathogen

Chia plants exhibiting the classic symptoms of naturally occurring infection with charcoal stem rot disease were obtained from Fayoum, Giza, and Menoufia governorates, Egypt in the 2021 season. Following a tap water wash and cutting into tiny pieces, the infected stems were treated with 2% sodium hypochlorite for 2 min for surface sterilization. They were then washed many times in sterilized distilled water and placed on potato dextrose agar (PDA) plates, followed by incubation for 7 days at 27±1°C. Based on the cultural and morphological characteristics of the causal pathogen, it was purified and identified according to Lakhran et al. (2018). For additional uses, purified cultures were placed on slants of PDA and stored at 5 °C.

2.2 Isolation and purification of the fungal biological agents

Rhizosphere soil samples of healthy chia plants cultivated in fields extensively infested with the causal agents of root-rot were obtained from different governorates, *i.e.*, Fayoum, Giza and Menoufia. As described by Abd-El-Moity (1976), one g of rhizosphere soil sample and 99 ml sterilized water were mixed in a glass bottle, followed by using an electric shaker for 1 h. The suspension was then diluted to 10^{-4} and 1 ml was added to Rose

Bengal medium (Johnson et al., 1960) to isolate the antagonistic fungi.

2.3 Isolation and purification of the actinomycetes biological agents

One gram of rhizosphere soil sample and 19 ml sterilized water were mixed in a flask to prepare a 1/20 dilution, followed by a shake in a rotary shaker (150 rpm). To avoid bacterial and fungal contamination, two drops of the aforementioned suspensions of each sample were then individually transferred to a 25 ml cylinder that contained 10 ml of a 1:140 dilution of phenol with water. Then, using the method outlined by Crook et al. (1950) and Waksman (1959), Petri dishes containing 15 ml of starch nitrate agar (SNA) medium (pH 7.2) were streaked with 0.1 ml of each soil suspension. For every sample, five plates were utilized, followed by incubation at 28°C and routinely checked. Colonies of actinomycetes were selected after 3-5 days according to their morphological parameters, and they were purified and grown on SNA medium.

2.4 Identification of the causal pathogen and bio-agent isolates based on molecular methods

The molecular characterization of *M. phaseolina*, *T. asperellum* and *S. rochei* isolates was performed at the Molecular Biology Research Unit, Assiut University. Potato sucrose agar (PSA) medium was used for the cultivation of *T. asperellum* and *M. phaseolina*, followed by incubation at 28°C for 5 days, as described by Pitt and Hocking (2009)**.** For *S. rochei* cultivation, 10 ml of nutrient broth medium in sterile test tubes were used, followed by incubation at 28°C for 48 h, as described by Zimbro et al. (2009). DNA extraction was carried out using Patho Gene-Spin DNA/RNA extraction kit (Intron Biotechnology Company, Korea). The primers ITS1 F (5'-TCCGTAGGTGAACCTGCGG-3'), and ITS4 R (5'-TCCTCCGCTTATTGAT ATGC -3') were used in polymerase chain reaction (PCR) to amplify ITS regions of the rRNA gene of *T. asperellum* and *M. phaseolina*. While the primers 27F (5'-AGAGTTTGATCC TGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify the rRNA gene of *S. rochei*, as described by (White *et al.*, 1990). The amplified products were purified before being sequenced with the same primers used in PCR amplification. The Basic Local Alignment Search Tool (BLAST) available on (NCBI) website was then used to analyze the obtained sequences. Phylogenetic tree construction was performed with MegAlign (DNA Star) software version 5.05.

2.5 Pathogenicity tests

Three *M. phaseolina* isolates from the governorates of Fayoum, Giza, and Menoufia were used in the pathogenicity test. Separate cultures of each isolate were made using autoclaved sorghum grain medium (100 ml water + 100 g sorghum + 50 g washed sand), which were kept at 27±1°C for 15 days. Before being used, sand-clay soil (1:1 w/w) was treated with a 5% formalin solution and allowed to dry for 14 days prior to usage. The tested isolates were mixed at a rate of 1% with the sterilized soil and put into 50-cm-diameter pots. Additionally, pots were left without infestation and used as uninoculated control. To promote the colonization of the tested fungal isolates, pots were watered until completely submerged one week prior to planting with 20 seeds/ pot. Four replicates were used for each tested isolate and control. Pre-emergence damping-off, post-emergence damping-off and charcoal stem rot incidence at 15, 30 and 60 days following planting,

respectively, were recorded as percentages using the following formula noted by Waller et al. (2002):

$$Disease\ incidence\ (\%) = \frac{Number\ of\ infected\ plants}{Total\ number\ of\ plants} \times 100$$

After re-isolating of the fungal isolates from the infected plants, they were compared to the initial isolates.

2.6 Effect of *M. phaseolina* cultural filtrates on chia seedlings

Three isolates of *M. phaseolina* from the governorates of Fayoum, Giza, and Menoufia were grown on Czapek's liquid medium. One hundred ml of media in a 250-ml conical flask was inoculated with a 5-mm-diameter disc of *M. phaseolina* isolate (7-day-old), and it was then incubated at 27 °C for two weeks. Flasks without fungal inoculation were used as a control. Cultures were sterilized with a Millipore syringe filter (0.45 µm) after being filtered by Whatman's No. 1 filter paper. Using deionized water, five concentrations of fungal filtrate for each isolate were made at rates of 20, 40, 60, 80, and 100%. Equal amounts of the sterilized fungal filtrates with their concentrations and the control medium (uninoculated) were then transferred to glass vials and planted with healthy chia seedlings (30-day-old), 5 seedlings for each, and kept under 12 h light and 12 h dark at 28-30 °C. For each treatment and control, three vials were used, and the percentage of wilted seedlings was recorded at 72 h after inoculation, as reported by Hassanin (2007).

2.7 Antagonistic effect of *S. rochei* and *T. asperellum* on *M. phaseolina* mycelial growth *in vitro*

In the current experiment, three isolates of *T. asperellum* (from Fayoum, Giza, and Menoufia governorates) and two isolates of *S. rochei* (from Fayoum and Giza governorates) as mentioned by Siddiqui et al. (2001) and Abd-El-Moity (1985), were evaluated for their antifungal effect on *M. phaseolina* isolate (from Fayoum governorate) mycelial growth *in vitro*. Starch Nitrate Agar (SNA) medium [soluble starch (20 g), K_2HPO_4 (1 g), KNO_3 (2g), $MgSO_4.7H_2O$ (0.5 g), NaCl (0.5 g), $CaCo_3$ (3 g), agar (20 g), $FeSO_4.7H_2O$ (0.001 g) and distilled water (1000 ml), (Waksman, 1959)] was used to study the antagonistic impact of *S. rochei* on *M. phaseolina*. On the other hand, Gliotoxin Fermentation Agar (GFA) medium [Dextrose (25 g), KH_2PO_4 (2 g), ammonium tartrate (2 g), $FeSO_4.7H_2O$ (0.1 g), agar (20 g), $MgSO_4.7H_2O$ (1 g) and distilled water (1000 ml), (Brian & Hemming, 1945)] was used to study the antagonistic impact of *T. asperellum* on *M. phaseolina*. Using the tested medium in each case, Petri plates (9-cm-diameter) were inoculated at one side with a disc of *M. phaseolina*, measuring 0.5 cm in diameter, which was obtained from the outer edges of a culture that was 7 days old on GFA medium. The opposite side of every plate, in consideration of the tested medium, was streaked with a loop from a 7-day-old *S. rochei* culture grown on starch nitrate broth or inoculated with a disc of *T. asperellum* (0.5-cm-diameter), which was obtained from a 4-day-old culture. As an untreated control, Petri dishes were inoculated with *M. phaseolina* only, which were replicated, as well as the treated ones, three times. Following incubation at 25°C and 27°C, in the case of the antagonistic study with *S. rochei* and *T. asperellum*, respectively, the percentage of the linear growth reduction of *M. phaseolina* in treated plates was calculated once the mycelial growth had completely covered the control plates medium surface, as follows:

$$\text{Linear growth reduction (\%)} = 100 - [G2 / G1 \times 100]$$

Where, G1= the mycelial growth diameter (mm) of *M. phaseolina* in control plates.
G2= the mycelial growth diameter (mm) of *M. phaseolina* in treated plates.

2.8 Effect of nitrogen sources on dark pigment production in *M. phaseolina* mycelium

In this experiment, five different sources of nitrogen were separately added to Czapek's medium, which contained [sucrose (30 g), $NaNO_3$ (2 g), $MgSO_4.7H_2O$ (0.01 g), KCl (0.5 g), agar (15 g), K_2HPO_4 (1 g) and distilled water (1000 ml)]. Nitrogen sources such as yeast, ammonium chloride, urea, peptone, and sodium nitrate were added to the medium individually, instead of $NaNO_3$, at a rate of 2 g/liter before sterilization. Medium of each nitrogen source, as well as medium without any nitrogen sources as a control, were poured into Petri dishes and 3 replicates were used for each. Using *M. phaseolina* discs selected from the outer edges of 7-day-old culture that was isolated from Fayoum governorate and grown on PDA media, dishes were inoculated and then incubated for 7 days at 27 °C. The morphological characters of the tested isolate were visionary examined, as mentioned by Ali et al. (2017).

2.9 Greenhouse experiments

2.9.1 Effect of the fungal dark pigment production on *M. phaseolina* aggressiveness on chia plants in greenhouse

In this investigation, both pigmented and non-pigmented colonies of *M. phaseolina* (Fayoum isolate) were cultivated on autoclaved Czapek's liquid medium supplemented by two distinct sources of nitrogen: sodium nitrate and yeast, for 15 days at 27°C. Before being used, sand-clay-peatmoss soil (1:1:1 w/w/w) was air dried for 14 days after being sterilized for one week with a 5% formalin solution. Following that, the soil was mixed separately with the pigmented isolates of *M. phaseolina* and the non-pigmented one at a 1% ratio (w/w) and it was put into 50-cm-diameter pots. For comparison, pots were left without fungal inoculation and used as a control. To promote fungal colonization, pots were watered until completely submerged one week prior to planting with 20 chia seeds/ pot, with four replicates for each treatment and control. The incidence of disease was recorded at 15, 30 and 60 days following planting as a percentage of pre-emergence damping-off, post-emergence damping-off and charcoal stem rot, respectively, using the following formula:

$$Disease\ incidence\ (\%) = \frac{Number\ of\ infected\ plants}{Total\ number\ of\ plants} \times 100$$

2.9.2 Controlling chia charcoal stems rot using different treatments under greenhouse conditions

Two biological agents individually or in combination, *T. asperellum* and *S. rochei* were prepared as a suspension at a rate of 20 ml/l water after being adjusted to a concentration of 30×10^6 cfu/ml, biocide: New-Actino (*Streptomyces griseorubes* and *Trichoderma hamatum*; produced at Central Laboratory of Organic Agriculture, Agricultural Research Center (ARC), Giza, Egypt) at a rate of 20 ml/l water and Fungicide: Tricyclazole WP [Common name: Tricyclazole. Chemical composition: 5-Methyl-1,2,4-triazolo{3,4-b}-(1,3) benzothiazole. Manufacture: Elanco Products Co., Div. of Eli Lilly and co.] at a rate of 1 g/L water **(**according to Hassanin, 2013**)** were evaluated for controlling chia charcoal stem rot disease incited by *M. phaseolina* (Fayoum governorate) in greenhouse. *Trichoderma asperellum* isolate (Fayoum governorate) was cultured in liquid Gliotoxin Fermentation Medium at 25°C for 11 days in total darkness, as described by Abd-El-Moity and Shatla (1981), while liquid starch medium

was used for growing *S. rochei* isolate (Fayoum governorate) at 30°C ±2 for 7 days. Treatments were applied two times as soil drench: at the sowing date and 15 days following the sowing. Pots without treatments were used as an untreated control and 20 chia seeds/pot (50-cm-diameter) were planted with four replicates for each treatment and control. Using the previously mentioned formula, pre-emergence damping-off, post-emergence damping-off and charcoal stem rot incidence were calculated as percentages at 15, 30, and 60 days after sowing, respectively. The plant growth measurements, *i.e.*, number of branches, plant height, as well as spike number/ plant were also recorded.

2.10 Statistical analysis

Experiments were carried out using a totally randomized design. Using SAS software, version 2004, data were subjected to statistical analysis of variance in accordance with methods described by Snedecor and Cochran (1980).

3. Results

3.1 Disease symptoms

As shown in Figure (1), charcoal stem rot disease symptoms on naturally infected chia plants begin on stems as little black spots that enlarge to cover the entire stem. Subsequently, the disease may cause significant losses in chia yield.

3.2 Molecular identification of the causal pathogen and bio-agent isolates

The amplified PCR products of DNA obtained from *M. phaseolina*, *T. asperellum* and *S. rochei* were submitted to GenBank after being sequenced using the selected primers with accession numbers (OP861486 for *M. phaseolina*, OP164570 for *T. asperellum* and OP164572 for *S. rochei*).

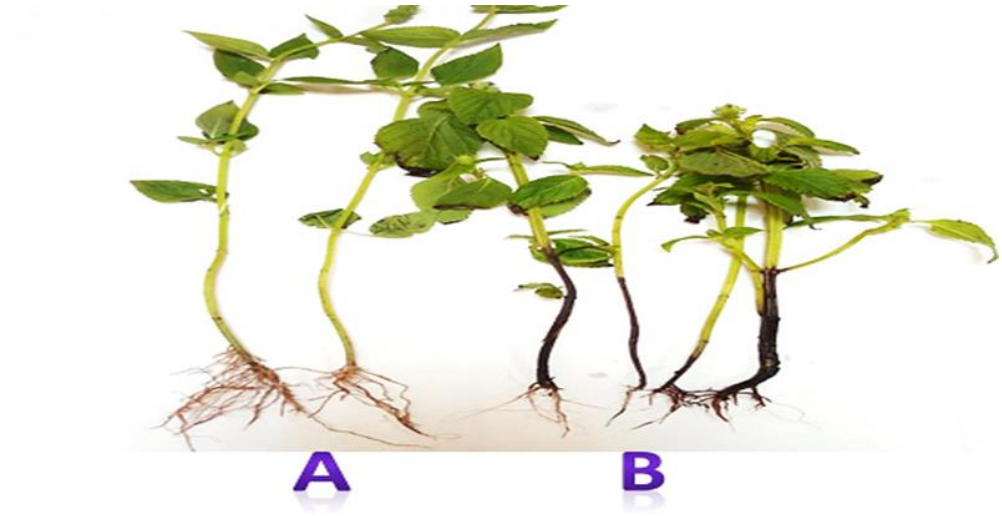

Figure 1: Naturally infected chia plants with charcoal stem rot disease compared to healthy plants. A (Healthy control plants) and B (infected plants).

Macrophomina phaseolina strain AUMC15577 showed 99.65-100% identity with several strains of the same species (Figure 2). The phylogenetic tree also showed *Phoma herbarum* as an outgroup strain. *Trichoderma asperellum* strain AUMC15576 showed

99.67-100% identity with several strains of the same species (Figure 3). *Streptomyces rochei* strain AUMC-B471 showed 99.93-100% identity with several strains of the same species (Figure 4). *Bacillus subtilis* is also included in the phylogenetic tree as an outgroup strain.

3.3 Pathogenicity tests

Table (1) presents the pathogenicity test of three isolates of *M. phaseolina*, which were isolated from naturally infected chia plants obtained from Fayoum, Giza and Menoufia governorates. All the tested isolates of *M. phaseolina* resulted in pre-emergence damping-off and post-emergence damping-off with significant variations. Nevertheless, Fayoum isolate showed the greatest percentage of charcoal stem rot (10%) at 60 days after planting, which subsequently decreased those of healthy survival plants to the lowest percentages (32.92%). In contrast, the lowest percentages of pre-emergence damping-off, post-emergence damping-off and charcoal stem rot (28.33 and 12.08% and 5.41%, respectively) resulted from *M. phaseolina* isolated from Menoufia governorate. Subsequently, it reduced the healthy survival plants to 54.18%.

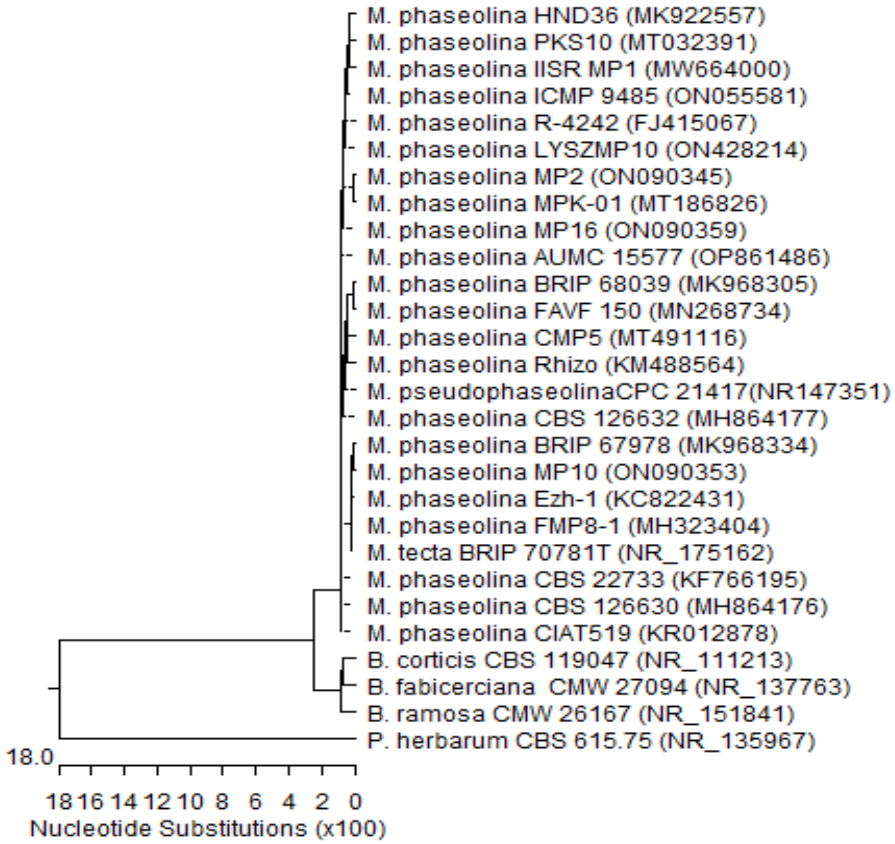

Figure 2: A phylogenetic tree based on ITS sequences of the rDNA of *M. phaseolina* strain AUMC15577 (Accession: OP861486) matched closely similar strains obtained from the GenBank. B (*Botryosphaeria*), M (*Macrophomina*) and P (*Phoma*).

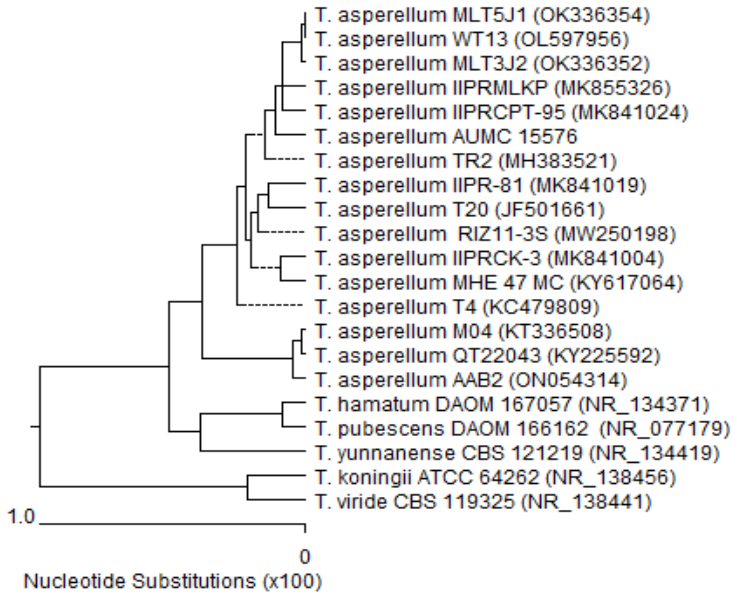

Figure 3: A phylogenetic tree based on ITS sequences of the rDNA of *T. asperellum* strain AUMC15576 (Accession: OP164570) matched closely similar strains obtained from the GenBank.

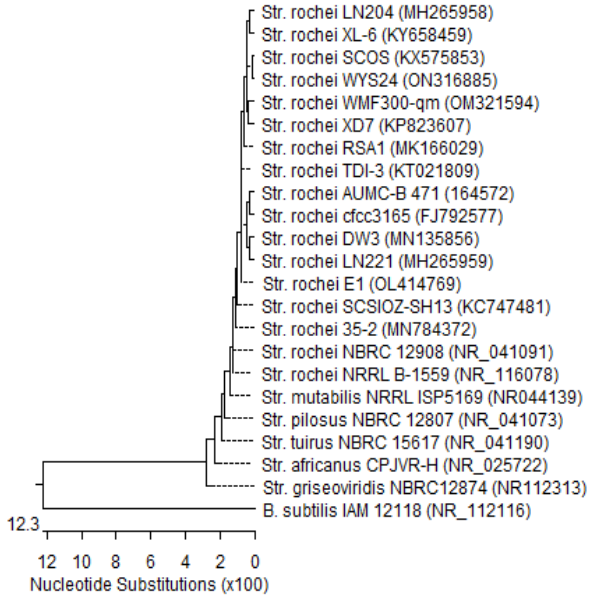

Figure 4: A phylogenetic tree based on 16S sequences of *S. rochei* strain AUMC-B471 (Accession: OP164572) matched closely similar strains obtained from the GenBank.

3.4 Effect of fungal filtrates on percentages of wilted chia seedlings

As shown in Figures (5 and 6), all the tested fungal filtrates increased the percentage of wilted seedlings after 72 h from treatment when compared to their controls. The tested concentrations of fungal filtrates were shown to be positively correlated with the percentages of wilted seedlings. In this regard, *M. phaseolina* filtrate of Fayoum isolate at concentrations of 80 and 100% recorded the highest percentages of wilted seedlings (100%) (Figure 5). Additionally, its lower

concentration (20%) increased the percentage of wilted seedlings (26.7%) compared to the control.

Table 1: Pre-, post-emergence damping-off and chia charcoal stem rot percentages incited by *M. phaseolina* at 15, 30 and 60 days after sowing, respectively.

Governorate	Disease incidence (%)			
	At seedling stage (15 and 30 days after sowing)		At maturity stage (60 days after sowing)	
	Pre-emergence	Post-emergence	Charcoal stem rot (%)	Survived plants (%)
Fayoum	30.00	27.08	10.00	32.92
Giza	33.33	15.41	8.30	42.96
Menoufia	28.33	12.08	5.41	54.18
Uninoculated control	0.0	0.0	0.0	100.0
L.S.D. at 5%	0.563	0.631	0.665	0.985

3.5 Antagonistic effect of *S. rochei* and *T. asperellum* on *M. phaseolina* mycelial growth *in vitro*

In this investigation, three isolates of *T. asperellum* (from Fayoum, Giza, and Menoufia governorates) and two isolates of *S. rochei* (from Fayoum and Giza governorates) were evaluated for their antagonistic effect on the *M. phaseolina* (Fayoum governorate) mycelial growth. All the tested isolates of *Trichoderma* and *Streptomyces* significantly inhibited the fungal growth of *M. phaseolina* compared to the untreated control.

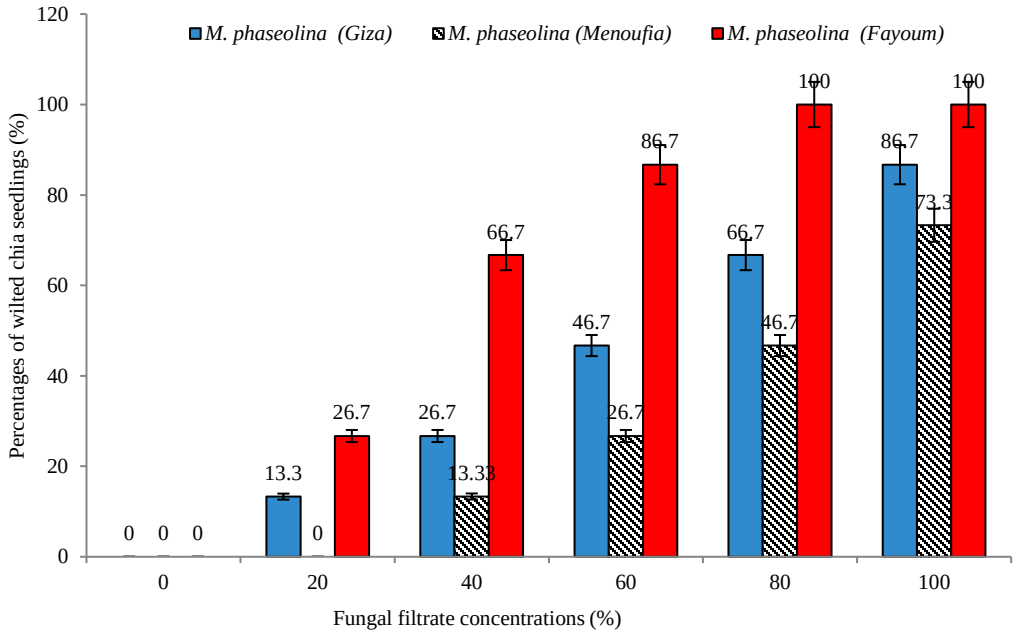

Figure 5: Effect of fungal filtrates of *M. phaseolina* isolates on percentages of wilted chia seedlings after 72 hours of inoculation.

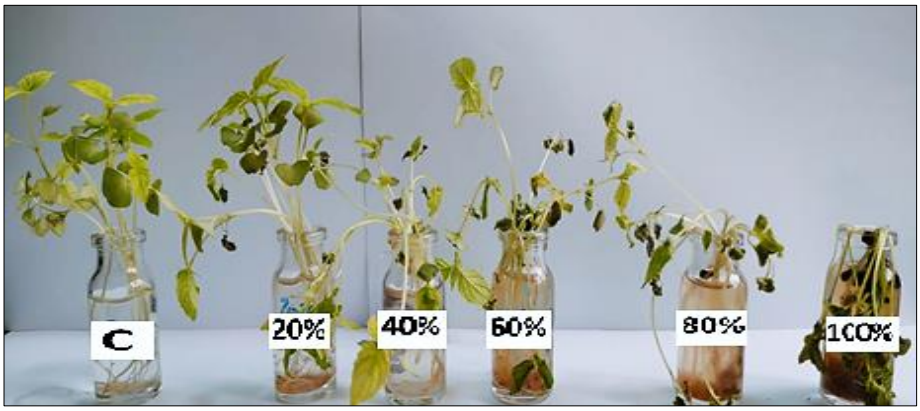

Figure 6: Effect of *M. phaseolina* cultural filtrates at various concentrations on chia seedlings compared to control.

The highest antagonistic effect was obtained by *T. asperellum* treatment (T1, isolated from Fayoum governorate), which recorded 60.1% reduction in linear growth of *M. phaseolina* (Table 2, Figure 7). Following that, *S. rochei* (ST1, isolated from Fayoum governorate) inhibited *M. phaseolina* linear growth to 54.9% (Table 2, Figure 8). On the contrary, *S. rochei* (ST2, isolated from Giza governorate) showed the least effect against *M. phaseolina*, with 35.8% inhibition in linear growth.

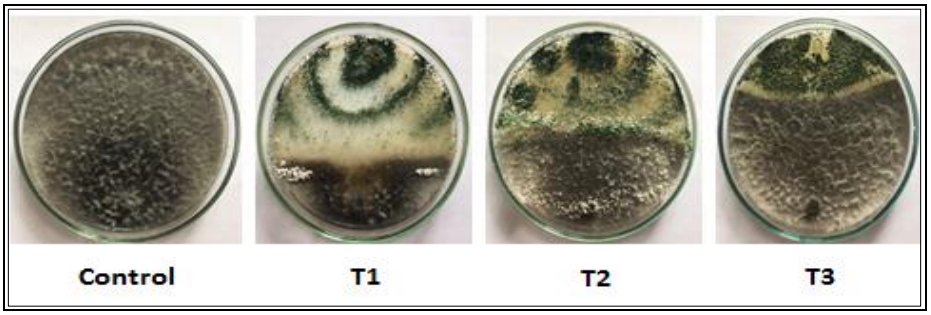

Figure 7: Antagonistic effect of *T. asperellum* on the growth of *M. phaseolina in vitro*. T1 (Fayoum isolate), T2 (Giza isolate) and T3 (Menoufia isolate).

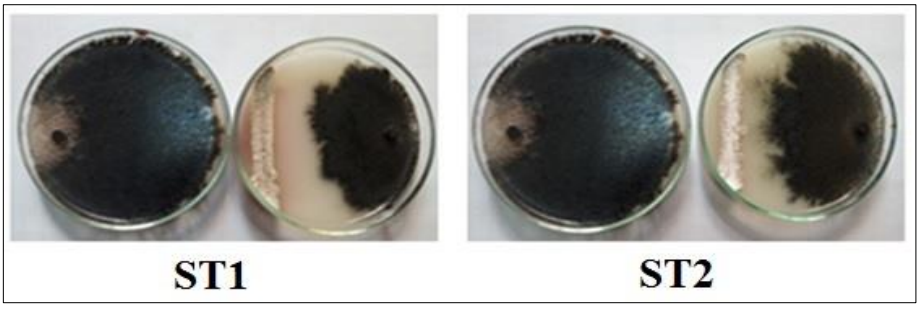

Figure 8: Antagonistic effect of *S. rochei* on growth of *M. phaseolina in vitro.* ST1 (Fayoum isolate) and ST2 (Giza isolate).

Table 2: Antagonistic effect of *T. asperellum* and *S. rochei* isolates on *M. phaseolina* mycelial growth.

Bio-agents	Reduction* in *M. phaseolina* mycelial growth (%)
Trichoderma asperellum (T1, Fayoum isolate)	60.1
T. asperellum (T2, Giza isolate)	50.3
T. asperellum (T3, Menoufia isolate)	48.1
Streptomyces rochei (ST 1, Fayoum isolate)	54.9
S. rochei (ST 2, Giza isolate)	35.8
Control	0.0
L.S.D. at 5%	0.958

*Reduction compared to the control treatment.

3.6 Effect of nitrogen sources on dark pigment production in *M. phaseolina* mycelium (Fayoum isolate)

Figure (9) shows the great impact of altering any of the nitrogen sources on the morphological characteristics of *M. phaseolina* mycelial growth including color and density. As shown, the presence of yeast, amonium chloride and urea resulted in the loss of *M. phaseolina* mycelial color.

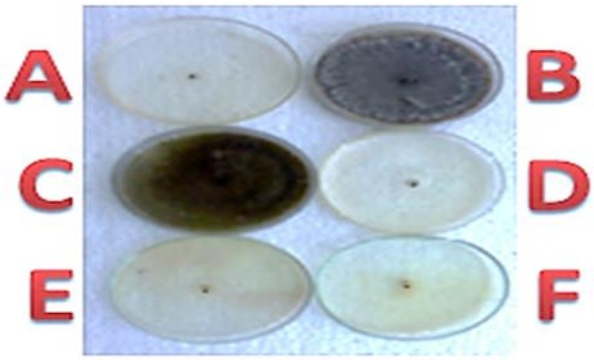

Figure 9: Effect of nitrogen sources on mycelium color of *M. phaseolina* (Fayoum isolate): A (Control), B (Pepton), C (Sodium nitrate), D (Urea), E (Amonium chloride) and F (Yeast).

3.7 Effect of the *M. phaseolina* pigment on the aggressiveness of *M. phaseolina* on chia plants in greenhouse

Table (3) shows that the pigmented isolate of *M. phaseolina* (Fayoum isolate) was higher than the non-pigmented one in their virulence to chia plants, as they showed the greatest percentages of pre-emergence damping-off and post-emergence damping-off, 36.67 and 13.33% at 15 and 30 days after sowing, respectively. Additionally, it showed the greatest percentage of charcoal stems rot (8.33%) at 60 days after planting, which subsequently decreased those of healthy survival plants to the lowest percentages (41.67%).

3.8 Using different treatments for controlling chia charcoal stem rot under greenhouse conditions

3.8.1 Effect on disease incidence

As shown in Table (4), all treatments were efficient in decreasing the disease incidence. However, the fungicide Tricyclazole was the best treatment for decreasing the disease incidence to the lowest percentage (1.25%), subsequently; it recorded the highest

percentage of survived plants (93.75%). Following that, the biocide New-Actino reduced the disease incidence to 5% and subsequently resulted in increases in the percentages of survived plants (88.75%). The obtained results demonstrate that using biological agent isolates in combination was more efficient in managing the disease than using individual ones. In this regard, *S. rochei* treatment recorded the highest percentage of pre-emergence damping-off, post-emergence damping-off and disease incidence (16.25, 17.50 and 11.25% respectively) compared to the other treatments, while the percentages decreased when it was applied in combination with *T. asperellum* (7.5, 6.25 and 8.75%, respectively). Generally, using any of the biological agents led to a significant reduction in pre-, post-emergence and charcoal stem rot incidence and subsequently resulted in increases in the percentages of survived plants compared to the control.

Table 3: Pre-, post-emergence damping-off and chia charcoal stem rot percentages caused by the pigmented and non-pigmented *M. phaseolina* isolates at 15, 30 and 60 days after planting, respectively.

Fungus	Pigmentation of the fungal cell wall	Damping-off (%)		Charcoal stem rot (%)	Survived plants (%)
		Pre-emergence	Post-emergence		
Macrophomina phaseolina (Fayoum)	Pigmented	36.67	13.33	8.33	41.67
	Non pigmented	8.33	3.33	3.33	85.01
Uninoculated control		0.00	0.00	0.00	100.00
L.S.D. at 5%		1.639	0.831	0.576	1.345

Table 4: Impact of different treatments on the incidence of chia charcoal rot under greenhouse conditions.

Treatments	Disease incidence (%)			
	At seedling stage (15 and 30 days after sowing)		At maturity stage (60 days after sowing)	
	Pre-emergence	Post-emergence	Charcoal stem rot (%)	Survived plants (%)
Trichoderma asperellum	10.00	13.75	10.00	66.25
Streptomyces rochei	16.25	17.50	11.25	55.00
T. asperellum + *S. rochei*	7.5	6.25	8.75	77.50
New-Actino	2.5	3.75	5.00	88.75
Tricyclazole	2.5	2.5	1.25	93.75
Untreated control	37.50	25.00	27.50	10.00
L.S.D. at 5%	0.828	0.982	0.946	0.995

3.8.2 Effect on plant growth parameters

Data in Table (5) shows that all tested treatments enhanced plant height, number of branches and number of spikes per plant in comparison with the untreated control. Nevertheless, Tricyclazole was the best treatment, which increased plant height (cm), no. of branches and number of spikes/plant to the highest percentages (52.25, 6.50 and 7.25, respectively). Following that, the biocide New-Actino increased the plant growth parameters to 46.00, 5.00 and 6.5, respectively. The combined use of the biological agents was better than the individual use for improving the plant growth parameters. In this regard, the treatment of *T. asperellum* increased the plant growth parameters to 36.75, 3.50 and 4.25, respectively, while the combined treatment of *T. asperellum* and *S. rochei* increased them to 40.75, 4.25 and 5.00, respectively.

Table 5: Impact of various treatments on chia vegetative growth.

Treatments	Plant height (cm)	Number of branches	Number of spikes/plant
Trichoderma asperellum	36.75	3.50	4.25
Streptomyces rochei	33.25	3.00	3.25
T. asperellum + S. rochei	40.75	4.25	5.00
New-Actino	46.00	5.00	6.50
Tricyclazole	52.25	6.50	7.25
Untreated control	25.75	1.25	1.75
L.S.D. at 5%	1.408	0.391	0.303

4. Discussion

In the current study, samples of naturally infected chia plants had classical symptoms of charcoal stem rot disease were collected from Fayoum, Giza, and Menoufia governorates. The disease symptoms begin on stems as little black spots that enlarge to cover the entire stem. The symptoms described by Misaka (2019) seem a lot like those of the infected plants. As mentioned by Kaur et al. (2012), the pathogen can spread from diseased roots to stems, blocking the vascular tissues in the tap root. It is additionally capable of spreading to seeds, decreasing germination and resulting in seedling rot. The causal pathogen's molecular characterization in the present research matched 99.65-100% with many strains of the same species. It has been shown that specific primers aimed at the ITS region may specifically identify a number of significant agricultural fungi, as reported by Edel et al. (2000) and Druzhinina et al. (2005). In comparison to the uninoculated control, the isolates from all governorates under study produced significant variations in pre- and post-emergence damping-off. Nonetheless, the highest percentage of charcoal stem rot was caused by *M. phaseolina* isolated from the Fayoum governorate. Furthermore, the highest percentage of wilted seedlings was induced by the filtrate of the same isolate. Melanin pigment may be the cause of *M. phaseolina*'s aggressiveness, as reported by Polak (1990), who mentioned that some soil fungi's melanin pigment is a key factor in determining their pathogenicity. Our obtained results are also in accordance with those mentioned by Nada (2016), who reported that *M. phaseolina* was among the most destructive fungi on chia plants. Our findings are consistent with El-Garhy's (1994) findings, which indicated that *M. phaseolina* culture filtrates produced browning and damaging of the veins in lentil leaf tissues in addition to necrotic areas. Our results reveal the significant effects of changing any of the nitrogen sources on the morphological characteristics of *M. phaseolina* mycelial growth, such as color and density. These findings indicate that variations in the media's nitrogen source and structure may have an impact on the fungal pathway. These results are in accordance with those mentioned by Pihet et al. (2009), who reported that sequencing of the genes related to the melanin production pathway revealed a genetic abnormality in the early stages of this pathway for *Aspergillus fumigatus* isolates. Also, our results are in accordance with those obtained by Ali et al. (2017). *In vitro* studies, the mycelial growth of *M. phaseolina* was significantly inhibited by the two bio-agents, *T. asperellum* and *S. rochei*, which were extracted from the rhizosphere soil samples of healthy chia plants. There is a clear variation in the antagonistic effect of the two tested bio-agents as expressed in the obtained results, which reveal that *T. asperellum* treatment (T1, isolated from Fayoum governorate) recorded the highest percentage reduction in *M. phaseolina* mycelial growth. The quantity and number of antifungal compounds that the bio-

agent produces might be the cause of these differences, as reported by Harman et al. (2004). According to Abd-El-Moity (1976) and Harman et al. (2004), *Trichoderma* spp. interacts with the pathogen by a variety of mechanisms, including mycoparasitism and the synthesis of trichodermin and gliotoxin. In several studies, Actinomycetes that produce chitinase enzyme have been used as biological control agents (Loqman et al., 2009; Prapagdee et al., 2008; Sharifi et al., 2007). In greenhouse experiment, the pigmented isolate of *M. phaseolina* was shown to be more virulent against chia plants than the non-pigmented isolate, as proven by its greatest pre- and post-emergence damping-off percentages as well as the incidence of charcoal stem rot. These results are supported by those obtained by Pihet et al. (2009), who noted that the fungal isolates with black pigments may be more virulent because they protect the fungus from host immune responses. Although other colors may occasionally occur, the majority of dark pigments in nature that are thought to be melanin are typically either black or brown in color, as reported by Cerenius and Söderhäll (2004). Our results are also in agreement with those reported by Ali et al. (2017). The most effective treatment for both decreasing the incidence of disease and improving the plant growth measurements in greenhouse was the fungicide Tricyclazole. The role of Tricyclazole 75%WP in disease resistance was studied by Ali et al. (2017), who mentioned that Tricyclazole 75%WP at 500 and 1000 ppm entirely suppressed the hyphal color of *M. phaseolina in vitro* study. The authors also proved the role of fungal black pigment in increasing *M. phaseolina* aggressiveness to cassia plants under greenhouse conditions. In another investigation by Wheeler et al. (2004), the authors mentioned that Tricyclazole 75% WP in PDA cultures blocked melanin synthesis by the *Monosporascus cannonballus* wild types. The biocide New-Actino treatment followed Tricyclazole for decreasing the incidence of disease and improving the plant growth measurements. As reported by Hajieghrari et al. (2008) and Poovendran et al. (2011), *Trichoderma* strains have a variety of biocontrol mechanisms, including mycoparasitism, competition, antibiosis, hyphal contacts, and enzyme release, to prevent infections caused by plant diseases. Additional reports also explain this impact by stating that *T. harzianum* secretes a variety of enzymes (chitinase, protease, β-1,3-glucanase, and cellulase) and antibiotics into the medium, where they break down the pathogen's cell wall and are essential for microparasitism (Ait-Lahsen et al., 2001; Lorito et al., 1993; Elad et al., 1982; Papavizas & Lumsden, 1980). *Streptomyces rochei*, belonging to the actinomycetales order, is a valuable source of bioactive secondary metabolites with industrial and commercial applications, as mentioned by El-Tarabily et al. (2000) and Bressan and Figueiredo (2005). According to Doumbou et al. (2001), actinomycetes are a source of bioactive compounds, organisms that promote plant development, and tools for bio-controlling plant diseases**.** Many antibiotics known to be effective against phytopathogenic fungi have been isolated from *Streptomyces* (Remsing et al., 2003; Rodríguez et al., 2002; Hwang et al., 2001; Kim et al., 1999). When compared to their separate applications, the combined use of the bio-agents *T. asperellum* and *S. rochei* significantly improved the plant growth parameters and reduced the disease in greenhouse experiment. These findings are in accordance with those mentioned by Ezziyyani et al. (2007), who reported that pepper root rot incited by *Phytophthora capsici* can be reduced more effectively by combining *T. harzianum* and *S. rochei* than by applying them alone. Also, Schmidt et al.

(2004) reported that the erratic nature of biological control must be lessened by the application of many biocontrol agents.

5. Conclusion

Macrophomina phaseolina was characterized, depending on morphological and molecular methods, as the causal agent of charcoal rot disease in naturally infected chia plants showing typical symptoms of disease and obtained from Fayoum, Giza, and Menoufia governorates in the 2021 cultivation season. Changing any of the nitrogen sources was found to be highly effective on the morphological characteristics of *M. phaseolina* mycelial growth, such as color and density, which subsequently influenced the isolate's aggressiveness against plants in greenhouse. The combined use of the bio-agents *T. asperellum* and *S. rochei*, isolated from the rhizosphere soil of healthy chia plants, was highly effective for both reducing the disease incidence and improving the plant growth parameters, as well as the biocide New-Actino, under greenhouse conditions.

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