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ORIGINAL PAPER

Evaluation of Different Biological Treatments on Control of Charcoal-Rot Incidence on *Zea mays* Caused by *Macrophomina phaseolina*

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ABSTRACT

Macrophomina phaseolina is one of the primary diseases influencing maize productivity, whether qualitatively or quantitatively. During the progress of this work the antagonistic activities of *Trichoderma* spp, bacteria, and novel vermicompost compared to fungicide in were used order to manage charcoal root in maize. According to all tested treatments, the novel vermicompost (V1) and *T. asperellum* (T2) were the best treatments enhanced the plant's survival rate by 86.67%. while both of *T. harzianum* (T4) and *Pantoea* sp. (B1) enhanced plant survival by 80.0 %, respectively, in comparison to the control. The enzyme activity of polyphenoloxidase, chitinase and glucanase was increased with all treatments after 14 and 45 days. In two locations, vermicompost, *T. asperellum*, and *Pantoea* sp. were the most effective treatments for maize stalk sugar and proline content. The maximum NPK content was recorded with *T. asperellum* (T2) followed by *T. harzianum* (T4), *Ps. stutzeri*, *Pantoea* sp., and vermicompost compared to the control. Therefore, the tested biocontrol agents and vermicompost showed antagonistic behavior against plant pathogens and help plants to grow by resisting directly or by enhancing their natural defenses which consider one of the main bio-control mechanisms. Our work tries to reducing fungicides and using integrated control as approaches method to achieve sustainable agriculture.
Keywords: *T. asperellum*, *T. harzianum*, *Pseudomonas stutzeri*, *Pantoea* sp, vermicompost, polyphenoloxidase, chitinase, Glucanase enzyme, Charcoal-Rot and *Zea mays*.

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INTRODUCTION

In Egypt, maize plays a significant role in the rural economy and means of subsistence. On 3.4762 million feddans, produced 7.50 million tons. (Salama, 2019 and FAOSTAT, 2022). However, growing this crop is challenging because of its vulnerability to infection by various diseases. *Macrophomina phaseolina* is a very devastating soil-borne fungus that causes the Corn Stalk Rot (CSR) and is considered one of the worst diseases which leading to significant losses in maize yield. *M. phaseolina's* primary trait is wilting, premature drying discoloration of nodal tissues, discoloration and tilted cobs and producing a type of sclerotia in maize stalk and clog xylem arteries, hence preventing water absorption in general. *M. phaseolina* secretes a variety of toxins and cell wall-degrading enzymes (CWDEs), which directly contribute to disease pathogenesis and progression (Chaudhary *et al.*, 2022). The fungus requires high temperatures and low soil moisture to grow and develop profusely. The control processes become more difficult when they expand higher and show symptoms close to the crop's blossoming stage (Verma *et al.*, 2023). What makes control of the disease even more difficult that there is no approved fungicides on the market (Khan and Javaid, 2022) not to mention that, the use of most chemical fungicides as seed coatings was unable to successfully control CSR. Moreover, fungicide use has the potential to worsen environmental concerns related to toxicity (Harlapur *et al.,* 2023). Therefore, the use of biocontrol agents (BCAs) capable of colonizing the rhizosphere could constitute a potential disease management strategy for maize cultivation (Khan and Javaid, 2022). The only approach to manage the disease is through cultural techniques such tillage, mixed cropping, and changing the sowing date to reduce plant stress (Singh *et al.*, 2022). Besides that, using of integrated approaches can be used alternative to accomplish the goals of sustainable agriculture, minimize the external inputs of chemical fertilizers and pesticides, and maximize the yield by maintaining soil health (Bhowmick *et al.,* 2023).

During agricultural practices of crops, some beneficial fungi, such as *Trichoderma* spp, may be able to lower the density of pathogens. In agriculture, *Trichoderma* spp are a promising long-term bioagents that work against soil- and seeds-borne diseases like *M. phaseolina*. Numerous strategies are employed by this fungus, such as enhanced plant tolerance to abiotic stressors, pathogen competition, mycoparasitism, antibiosis, and activation of the pathogen defensive system. It also promotes plant growth. It has the capacity to create a variety of biochemical compounds, including siderophores that may facilitate biocontrol activities and both volatile and nonvolatile molecules (Rubayet and Bhuiyan, 2022; Joshi *et al.*, 2022). Saleh *et al.* (2022) clarified the effects of different *Trichoderma* segregates on *Alternaria solani*. Results showed that *T. harzianum* followed by *T. hamatum* diminished parasitic development or illness seriousness of *A. solani*, and enhanced the potato plant's development boundaries. Additionally, one of the factors that will play a major role in the control of potato early blight is the acceptance of foundational guard instruments with *T. harzianum*. Moreover, Abou-Zeid *et al.*, (2018a), mentioned that *T. harzianum* was found to be the most efficient in reducing tomato wilt. Moreover, Bacteria are used as biocontrol agents, and the excretions they produce help plants thrive. Antibiotics, cyanide, siderophores, and chitinases can all induce resistance action. Also, beneficial microorganisms that are also used display antagonistic behavior against phytopathogens (Joshi *et al*., 2022 and Mehmood *et al.*, 2023). According to Joshi *et al.* (2022) biocontrol agents (BCAs) and such as species of *Bacillus*, *Pseudomonas*, *Serratia*, *Burkholderia* and *Trichoderma* and mycorrhizal fungi have been shown to exhibit strong inhibitory effect against *M. phaseolina*. Furthermore, generate a diverse array of natural metabolites to directly resisting or by strengthening their natural defenses is one of its main bio-control mechanisms, *Pantoea agglomerans* ENA1 which possesses the capacity to significantly negatively impact *M. phaseolina* by reducing its mycelial growth by up to 89%, decreasing the pathogen population in the soil, increasing host-plant weight gain, and decreasing the host tissues' microsclerotial coating (Vasebi *et al.*, 2015 and Duchateau *et al.*, 2024). According to this approach, Pal *et al.*, 2001 found that, maize charcoal and root rots caused by *M. phaseolina* can be inhibited by bacterium *Pseudomonas* sp., due to many characteristics including phosphate solubilization, nitrogen fixation, and the synthesis of organic acids and IAA that encourage plant development. The bacterium also produced siderophores, HCN, and antifungal medicines. *M. phaseolina*-related disease was inhibited by this isolate, *Pseudomonas* sp. While, Khan and Javaid, (2022) concluded that, *P. stutzeri* has strong qualities that promoted plant growth and inhibited the charcoal rot caused by *M. phaseolina*, thus it is considered an efficient bio-fungicide.

On the other hand, adding more organic matter and increasing the amount of soil fertility by utilized vermicompost can improve the physical and biological characteristics of the soil. Vermicompost is increasingly used worldwide in sustainable agriculture as an environmentally safe and soil-appropriate biofertilizer. Vermicompost has a positive effect on disease prevention because of its level of stabilization, which is well-known to affect plant disease caused by soil-borne pathogens (Millner *et al.*, 2004; Noble and Coventry, 2005; El-Demerdash *et al.*, 2017). According to Ali *et al.* (2023) applying a mixture of novel vermicompost to soil infested with *Fusarium oxysporum* reduced the wilt incidence of maize crops. Also, adding vermicompost to the maize root led to an increase in the activity of peroxidase and polyphenol oxidase.

Therefore, the aim of this research was to minimize the applied fungicides and to, increase the use of integrated approaches as an alternative to achieve the sustainable agriculture.

MATERIALS AND METHODS

1- Plant materials

Seeds of maize 324 (*Zea mays* L.) white hybrids three-way cross (TWC 324) were obtained from the Field Crop Research Institute, ARC, Giza, Egypt. In order to disinfect the seeds, they were immersed in a 5% sodium hypochlorite solution for 3 min.

2- Isolation, purification and identification of the associated fungi

Isolation trials were conducted at Maize and Sugar Crop Diseases Department, Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza, Egypt, from the basal portion showing the charcoal symptoms rot of the infected corn stem segments collected randomly from different regions of Qalyobiya, Kafr-El Sheikh and Giza governorates. The infected samples were first surface washed under running tap water to remove dirt, sand. Then, flamed knife was used to cut both diseased and healthy samples, which were then, surface sterilized with 70% alcohol, rinsed with sterile distilled water and cultivated on PDA in 9cm diameter Petri plates, 4 pieces/plate, and maintained at 25±2°C for 5 days. The fungus was identified as described by Santosh et al., (2022) and then purified on single microsclerotia cultures and identified under microscope.

3- Pathogenicity tests of *M. phaseolina* isolates

The pathogenicity of seven isolates of *M. phaseolina* was determined using maize seeds water agar (15 g/l) in 9cm diam. Petri -1plates. The plates were inoculated in the center with 0.5 cm diam. taken from the margin of isolates colony, on PDA of 5 days old, and maintained at 25±2°C for two days. Seeds of maize were surface sterilized with 2% sodium hypochlorite for 2 min, washed with sterilized distilled water let dry on filter paper and cultivated on water agar in petri plates at 1 cm of the plate margin. Seeds were cultivated on uninoculated plates as control four replicates of each treatment, 10 seeds/plates were used and maintained at 25±2°C. The percentages of rotten seeds and dead seedling. Before and after emergence were calculated after 7 and 14 days of cultivation. according to Al-Juboory and Al-Jarah, (2020).

4-Isolation of *Trichoderma* spp

Soil rhizosphere samples of selected healthy plants, collected from naturally heavily infested fields representing five governorates (Behaira, Gharbiya, Kafr-El Sheikh, Qaloubiya and Giza) for isolation different antagonistic microorganisms. *Trichoderma* isolates were identified based on colony morphology and spores according to (Aneja, 2003 and El Komy *et al.*, 2015).

5-Bacterial strains

Isolates of *Pantoea* sp. HP2-MG738254 (B1) and *Pseudomonas stutzeri* H2-MG738255 (B2) as bio-agents were kindly supported by Dr. Kandil, Agricultural Microbiology Department, Soils, Water and Environment Research Institute (SWERI), Agricultural Research Center (ARC), Giza.

Egypt. These bacterial strains were isolated and identified by Kandil *et al.* (2018).

6-Novel vermicompost preparation

Novel vermicompost was prepared according to Amer *et al.*, (2022) and Ali *et al.*, (2023) and, where it contains 2.80, 0.23, and 0.60, % NPK, respectively, and 1.78, 15.06, 11.90 and 47.00 % Fe, Mn, Zn, and Cu, respectively pH and EC value were 8 and 5.20. In this experiment, 3 types of vermicompost (V) were used, (V1) consisted 25% vermicompost and 75% Agricultural Soil (Agri-Soil), (V2) consisted of 50% vermicompost and 50% agri-soil, and (V3) consisted 75% vermicompost and 25% agri-soil.

7-Laboratory Experiment

7-1 Effect of antagonistic microorganisms on mycelial growth of *M. phaseolina in vitro.*

The antifungal efficacy of ten *Trichoderma* isolates, *Pantoea* sp and *Ps. stutzeri* antagonists was tested by dual culture technique (Shrivastava *et al.*, 2017 and Santosh *et al.*, 2022) against the most aggressive isolated *M. phaseolina* (No.1) on PDA medium. The efficacy of the antagonistic organisms against *M. phaseolina* was rated based on the inhibition zone observed. The following formula was used to determine the percent inhibition over control:

$$I = \frac{C - T}{C} * 100$$

Where:

I= percentage of fungal growth inhibition.

C= Fungal growth of control (Pathogen alone).

T= Fungal growth of treatment (Pathogen against the antagonist).

7-2 Biocontrol agents tests on bacteria

- **Qualitative study of Chitinolytic activity by bacterial strain**

For qualitative determination of chitinase enzyme, the change in petri dishes from yellow to red test was recorded as an indication of chitinolytic according the method explained by Monreal and Reese, (1969).

- **Hydrogen cyanide (HCN) determination**

For the production of HCN, bacterial cultures were streaked overnight on Luria-Bertani (LB) plates that had 4.4 g/L of glycine. According to Lorck, (1948), the filter paper's color changing from yellow to brown was noted as a sign of cyanogenic production.

- **Intrinsic Antibiotic Resistance**

Six antibiotics e.g. Ampicillin10mg, Chloramphenicol 30mg, Kanamycin 30mg, Azithromycin 15mg, Colistin 10mg and Gentamycin 10mg were used to estimate the antibiotic resistance of bacterial strains according to Quinn *et al.* (1994).

8-Greenhouse Experiment

8-1 Effect of bioagents, Vermicompost and fungicide against *M. phaseolina* on Maize planted under greenhouse conditions.

Pot experiments were carried out in an open greenhouse during the summer of 2022 at the Plant Pathology Institute, Agricultural Research Centre, Giza, Egypt. The inoculum was prepared according to Srinivas *et al.* (2017) and Santosh *et al.* (2022).The experiment was carried out to study the antagonistic activity of the most effective four *Trichoderma* spp using spore suspensions (10^7spore/ml) of the fungi, two bacteria cell suspensions (10^8cfu cell/ml), three concentrations of novel vermicompost according to Ali *et al.* (2023) moreover, mineral fertilization (fertilizers recommended for maize) and fungicide Maxim (50cm^3/100 kg seed), maize seeds were soaked one hour in suspensions of some different treatments then grown in pots containing soil infested with the most aggressive *M. phaseolina* (No.1) according to Abou-Zeid *et al.*, (2016) Swamy *et al.*, (2018) Santosh *et al.*, (2022). To determine the impact of disease incidence, the previous treatments were set up with five replications for each.

The following formula was used to determine the percentage of infected plants after 60 days from planting according to Darwesh and El shahawy (2023):

$$\text{Charcoal rot infection (\%)} = \frac{\text{number of infected plants}}{\text{total number of examined plants}} \times 100$$

8-2 Determination of oxidative enzymes and hydrolytic enzymes in treated maize roots *in vivo*.

Samples of maize (*Zea mays* L.) plants representing the most effective treatments *Trichoderma* spp., *Pantoea* sp. and *Ps. stutzeri*, Vermicompost, mineral fertilization and Maxim fungicide. In addition, untreated plants as control were chosen for assaying the different oxidative enzymes (polyphenoloxidase) and hydrolytic enzymes (chitinase and ß-1, 3-glucanase). The collected roots from each treatment at 14 and 45 days were homogenized immediately using liquid nitrogen (Ojha and Chatterjee, 2012). The crude extract was used to estimate the polyphenoloxidase, chitinase and Gloconise activities (Anand *et al.*, 2007).

a) Polyphenoloxidase activity (PPO): Activity of PPO was determined according to Abou-Zeid *et al.* (2018b).
b) Chitinase activity: Chitinase activity was assayed according to Abou-Zeid *et al.* (2018b).
c) ß-1,3-glucanase activity: ß-1,3-glucanase activity was assayed as described by Abou-Zeid *et al.* (2018b).

9-Molecular characterization of most effective Trichoderma isolates (T2 and T4)

The most effective *Trichoderma* isolates were identified by sequence in GenBank. DNA was extracted using the Dellaporta procedure for genomic DNA isolation (Dellaporta *et al.*, 1983). The internal transcribed spacer region (ITS) of rRNA was sequenced and amplified using primer ITS4 and ITS5 (White *et al.*, 1990). The PCR reaction was carried out in a 25 µL reaction volume with 10 µL of PCR Master Mix (amaROnePCR, GeneDirex, Inc.), 11 µL of ddH_2O, 1.5 µL of each primer, and 1 µL of template DNA. Sequencing Service in Seoul, Korea. The PCR amplification conditions were carried out following Haouhach *et al.* (2020). To assign taxonomy, the BLASTn algorithm was performed using the NCBI GenBank database, comparing the queries to type specimens.

10-Field Experiment

The experimental area was designed randomly in clay soil in two governorates, e.g. Giza and Kafr-Elshikh from June to October 2023 the soil of the experimental layout is well known with high contamination with charcoal-rot inoculum and a fungus population to determine the impact of *T. asperellum, T. harzianum, Pantoea* sp, *Ps. stutzeri*, Vermicompost, and the fungicide Maxim on maize charcoal-rot disease incidence and crop yield. The experimental area was divided into plots (2x3 m). Seeds at a rate of 20 kg fed^{-1} were sown into rows 2.5 m long and 30 cm apart and covered with a thin layer of soil before irrigation. The diseases assessments were taken after 45-50 days at the tassel emergence stage of the plant.

The experiment was set up in a randomized block design with eight treatments and three replications. Maize (TWC, 324) was sown in holes (about 2seeds\hole) and seedlings were thinned 7 days after emergence. Traditional maize cultivation practices were followed, including hoeing, weeding, inorganic fertilizer, and insect management. The best treatments (*T. asperellum, T. harzianum, Pantoea* sp, *Pseudomonas stutzeri*) and fungicide Maxim were applied to maize seeds before planting, but Vermicomposting and mineral fertilization (fertilizers recommended for maize) were added to the soil during cultivation (Zeller *et al.*, 2002). The following formula was mentioned before in greenhouse. The percentage was determined of infested plants after 90 days from planting according to Darwesh and Elshahawy (2023)**.** Ten plants were chosen at random from each plot at harvest to establish the following parameters: plant morphological characteristics, including plant height, cob length, ear weight, ear length, dry weight the number of grains per cob, and thousand-grain weight (MTZ) were recorded. The plant's growth and development phase spanned 178 days, measured from the day of sowing until the day of harvesting.

- **Determination of sugar concentrations and proline**

The dried samples were ground into a powder and mixed with 80% ethanol before being heated for 30 minutes at 70°C and centrifuged for 10 minutes at 8000 g and 4°C. After collecting the supernatant and repeatedly extracting the residue with 80% ethanol, all collecting supernatants were mixed. After 30 minutes of incubation in an 80°C water bath, a 1mL sample of the filtrate was combined with 5mL of anthrone reagent and placed in a boiling water bath. Samples were read at 620 nm with spectrophotometer. The blank consisted of 5ml of the reagent and 2.5 ml of water and the color was clear blue green. Following the procedure outlined by McCready *et al.* (1950).

The ninhydrin colorimetric method was used to determine the proline (Du *et al.*, 2019). The 0.5 g of maize stalk tissue was crushed in 5mL of 3% aqueous sulfo salicylic acid, allowed to boil for 10 minutes, and after that filtered out. After mixing 2:2:2 with acid-ninhydrin, glacial acetic acid, and the filtrate, the test tube was placed in a water bath at 100 °C for 30 minutes. Then, adding 4mL of toluene to the extract, the absorbance (520 nm) was measured. Using the L-proline standard curve, the proline content was calculated and expressed as micrograms of proline per gram of fresh plant weight (µg/g FW).

- **Plant analysis**

Plants were subjected to the recommended agricultural practices done during the growing season of 2023. Plants were sampled after 45 days from planting and analyzed, ground, and digested. Determination of plant N, P and K contents was carried out as described by Van Schouwenburg (1968).

11-Statistical analysis

According to the methodology provided by Snedecor *et al.*, (1989), statistical analysis was performed using the F-test for significance at p 0.05 and computing least significant difference (LSD) test, values to distinguish means in distinct statistical groups.

RESULTS

1- Isolation, purification and identification of the isolated fungi from rotted maize stalks

Data in Table (1) show that seven isolates were obtained from naturally infested maize stalks showing stalk rot symptoms collected from different governorates e.g. Qalyobiya, Kafr-El Sheikh and Giza. *M. phaseolina* was considered the most important pathogen because its widespread and its ability to infect maize plants in many situations. The isolated fungi were differed in their morphological characters.

Table1. Isolated fungi from rotted maize stalks collected from three governorates

Governorates	Locations	Isolates of fungi *A	*B	*C	*D
Qalyobiya	Toukh	+	-	+	-
	Kaha	-	+	+	+
	Miet-Kinana	+	+	+	+
Kafr-El Sheikh	Qleen	+	-	+	+
	Sakha	-	+	+	-
Giza	Giza	-	+	+	-
	Badrasheen	+	-	+	+

Note: A) *F. oxysporum*, B) *Aspergillus* spp., C) *M. phaseolina., and* D) *Fusarium.* sp

2- Pathogenicity test

The most tested isolates of *M. phaseolina* were found to be active and caused root rot and seedling death at 7 days after seeds sowing on PDA. It has been found that *M. phaseolina* isolates No.1and 3 recorded the highest infection to maize seedlings compared with other isolates after 7 days on PDA. While isolates No. 2 and 7 were not able to show any infection after 7 days such as control (Table, 2).

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Table 2. Pathogenicity test of seven *M. phaseolia* isolates collected from different locations on maize seeds after 7 and 14 days from sowing on PDA.

Locations	Isolate No. of *M. phaseolina*	Percent death		Control (Percent death)	
		7 days	14 days	7days	14days
Toukh	1	98	100	0	0
Kaha	2	0	88	0	0
Miet-Kinana	3	98	100	0	0
Qleen	4	94	100	0	0
Sakha	5	93	100	0	0
Giza	6	16	100	0	0
Badrasheen	7	0	89	0	0
L.S.D at 5%		1.758	3.517	-	-

3- Isolation and identification of *Trichoderma* spp

Ten *Trichoderma* isolates were purified and identified according to their morphological features by using light microscope at the Unit of Identification of Microorganisms, Plant Pathology Research Institute, ARC, Giza, Egypt according to (Aneja, 2003 and El Komy *et al.*, 2015). Fungal isolates were maintained on PDA medium and kept in a refrigerator at 6°C, as shown in Table (3).

Table 3. Sources of *Trichoderma* isolates

No.	Code	Isolates	Location
1	T1	*Trichoderma* sp.	Kafr-El Sheikh
2	T2	*Trichoderma* sp.	Giza
3	T3	*Trichoderma* sp.	Kafr-El Sheikh
4	T4	*Trichoderma* sp.	Giza
5	T5	*Trichoderma* sp.	Qaloubiya
6	T6	*Trichoderma* sp.	Qaloubiya
7	T7	*Trichoderma* sp.	Kafr-El Sheikh
8	T8	*Trichoderma* sp.	Gharbiya
9	T9	*Trichoderma* sp.	Gharbiya
10	T10	*Trichoderma* sp.	Giza

4- Effect of antagonistic microorganisms on mycelial growth of *M. phaseolina* in vitro.

The tested *Trichoderma* isolates (10) ,*Pantoea* sp and *Ps. stutzeri* were able to decrease the mycelial linear growth of *M. phaseolina* compared with the control. Results in Table (4) and Figure (1) reveal that T2 was significantly the most effective bioagent which recorded (83.33%) reduction in mycelium growth followed by T4, T1, T6 and T8, respectively without significant differences compared to control. Also, *Pantoea* sp and *Ps. stutzeri* recorded (66.66 and 61.11%) in comparison with the control, (Figure, 2).

Table 4. Effect of different *Trichoderma* isolates and bacterial strains on the linear growth of *M. phaseolina* on PDA medium.

Isolates No.	Governorates	Locations	*M. phaseolina*	
			Linear growth (cm)	Reduction (%)
T1	Kafr-El Sheikh	Sakha	2.15	76.11
T2	Kafr-El Sheikh	Sakha	1.5	83.33
T3	Kafr-El Sheikh	Qleen	3.5	61.11
T4	Qualubiya	Kaha	2.0	77.77
T5	Qualubiya	Kaha	2.75	69.44
T6	Qualubiya	Kaha	2.25	75.00
T7	Qualubiya	Kaha	3.1	65.55
T8	Garbiya	Tanta	2.25	75.00
T9	Garbiya	Gemaiza	2.85	68.33
T10	Giza	Badrasheen	3.0	66.66
Pantoea sp (B1)	-	-	3.0	66.66
Ps. Stutzeri (B2)	-	-	3.5	61.11
Control			9	0
L.S.D at 5%			0.455	2.395

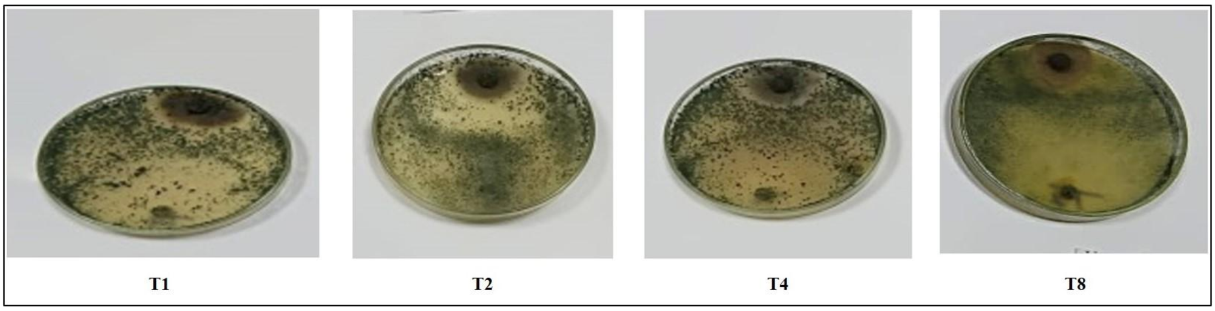

Figure 1. Antagonistic activity of *Trichoderma* isolates against *M. phaseolina***.**

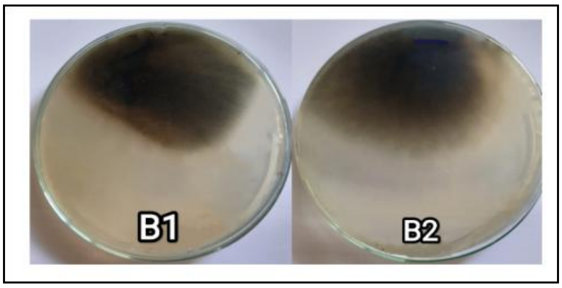

Figure2. Antagonistic activity of *Pantoea* sp (B1) and *Ps. stutzeri* (B2) against *M. phaseolina*.

5- Effect of the most effective bioagent isolates and vermicompost, fungicides, against *M. phaseolina*, under greenhouse conditions

Under greenhouse conditions, the most effective antagonistic *Trichoderma* isolates, *Pantoea* sp ,*Ps. stutzeri* and vermicompost compared to fungicide Maxim were investigated to compare their control effect against charcoal rot on maize. Results in Table (5) show that T2 and vermicompost (V1) were the most effective for controlling maize charcoal root, being 13.33% followed by T4 and *Pantoea* sp which recorded 20.0 %, compared to control.

Table 5. Effect of fungicide maxim, bioagents and vermicompost on maize plants grown under greenhouse conditions

Treatments	Charcoal-rot infection	Plant survival (%)
T1	33.34	66.66
T2	13.33	86.67
T4	20.0	80.0
T8	33.34	66.66
***Pantoea* sp**	20.0	80.0
Ps. Stutzeri	26.67	73.33
V1	13.33	86.67
V2	60.00	40.0
V3	80.00	20.00
Mineral fertilization	40.0	60.0
Fungicide Maxim	6.67	93.33
Control, infested soil	100	0.0
Control un-infested soil	0	100
L.S.D at 5%	1.224	0.595

6- Effect of bioagents and vermicompost on enzymes activity in stalks of corn planted in infested soil by charcoal rot:

The activities of oxidative enzymes, polyphenoloxidase, and a hydrolytic enzyme (chitinase and ß-1, 3-glucanase) were determined after 14 and 45 days with *M. phaseolina* after soaking in 4 *Trichoderma* spp., *Pantoea* sp, *Pseudomonas stutzeri* and vermicompost as well as infested and uninfested control.

6-1 Polyphenoloxidase activity

Data in Table (6) indicate that the maximum increase in polyphenoloxidase activity was recorded at 14 days in infested maize stalks treated with T4 and T2 isolates, followed by *Pantoea* sp and V1 each alone. Meanwhile, the maximum increase was recorded in 45 days old maize stalks treated with T4 isolate followed by B1 treatment.,

Table 6. Effect of fungicide Maxim, bioagents and vermicompost on polyphenoloxidase activity at 14 and 45 days after treatment under greenhouse conditions.

Treatments	Enzyme activity	
	14 days	**45 days**
T1	0.507	0.402
T2	1.084	0.945
T4	1.117	1.045
T8	0.974	0.878
***Pantoea* sp**	1. 052	1.001
Ps. stutzeri	1.020	0.919
V1	1.041	0.996
V2	0.886	0.970
V3	0.702	0.779
Mineral fertilization	1.009	0.914
Fungicide Maxim	0.900	0.664
Control infested soil	0.650	0.508
Control infested soil	0.429	0.302
L.S.D at 5%	0.158	0.031

6-2 Determination of chitinase activity

Chitinase activity was determined at 14 and 45 days in treated maize plants, Data in Table (7) reveal that all tested treatments increased the chitinase activity after 45 days more than after14 days. The best increase in chitinase activity was recorded due to using bioagents T4, *Pantoea* sp, T2 isolates and V1after 14 days and 45 days.

6-3 Determination of ß-1, 3-glucanase activity

Data in Table (8) indicate that all treatments increased ß-1,3-glucanase activity in 45 days treated maize stalks more than 14 days. In this respect, the highest activity of ß-1, 3-glucanase was recorded at 45 days with T4 and *Pantoea* sp. followed by T2 and V1 treatments compared with other treatments. On the other hand, V1 treatment recorded the highest activity at 14 days.

Table 7. Effect of fungicide Maxim, bioagents and vermicompost on chitinase activity at 14 and 45 days after treatment under greenhouse conditions

Treatments	Enzyme activity	
	14 days	45 days
T1	0.037	0.078
T2	1.988	2.090
T4	2.402	2.624
T8	0.990	1.120
***Pantoea* sp**	1.872	2.291
Ps. Stutzeri	0.810	1.350
V1	1.547	1.969
V2	0.265	1.451
V3	0.075	0.190
Mineral fertilization	0.137	1.046
Fungicide Maxim	0.198	1.046
Control infested Soil	0.078	0.639
Control uninfested soil	0.023	0.037
L.S.D at 5%	0.034	0.049

Table 8. Effect of fungicide Maxim, bioagents and vermicompost on ß-1, 3-glucanase activity at 14 and 45 days after treatment under greenhouse conditions

Treatments	Enzyme activity	
	14 days	45 days
T1	0.051	0.073
T2	1.068	1.857
T4	1.100	1.987
T8	0.987	1.266
***Pantoea* sp**	1.087	1.954
Ps. stutzeri	0.990	1.501
V1	1.204	1.581
V2	0.157	1.060
V3	0.094	0.702
Mineral fertilization	0.054	0.530
Fungicide Maxim	0.414	0.788
Control infested soil	0.229	0.996
Control uninfested soil	0.014	0.370
L.S.D at 5%	0.019	0.033

7- Molecular characterization of most effective Trichoderma isolates (T2 and T4)

The most effective *Trichoderma* isolates were identified by sequence in GenBank as *T. asperellum* for T2 isolate with accession No. OR911936 and *T. harzianum* for T4 isolate with accession No. MZ681867.

8- Qualitative study of bacterial biocontrol agents

8-1 The chitinase activity screening process

Chitinase activity assays were carried out on approved *Pantoea* sp. and *Ps. stutzeri* to secrete chitinase for biocontrol use. Adding chitin to a solid media and then watching as halos form around the colonies

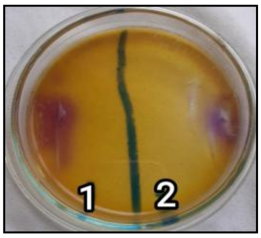

Figure 3. Showing *Pantoea* sp (B1) and *Ps. Stutzeri* (B2) ability to produce the chitinase enzyme.

as a result of chitin degradation are some of the most basic techniques. Figure (3) shows the change of yellow color to red color which is a positive result of the ability of bacteria to produce the chitinase enzyme.

8-2 Qualitative analysis of hydrogen cyanide (HCN) synthesis

Results show that both *Pantoea* sp. and *Ps. stutzeri* can release hydrogen cyanide. The positive reaction indicated that the formation of HCN was authenticated by color change from yellow to dark brown after incubation was considered as microbial production of HCN (Figure, 4).

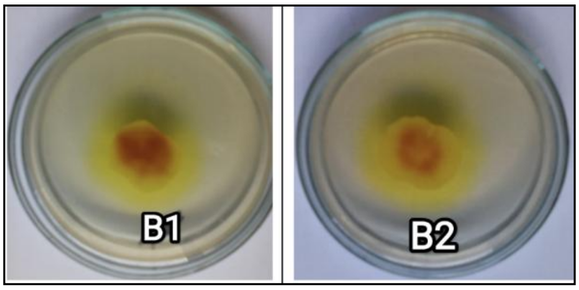

Figure 4. Showing the ability of *Pantoea* sp (B1) and *Ps. Stutzeri* (B2) to produce HCN.

8-3 Intrinsic antibiotic resistance by the bacterial strains

Data presented in Table (9) and Figure (5) show that, both bacterial strains were resistance to 10 µg Colistin. The growth with the medium containing Azithromycin showed a growth inhibition measuring 19 mm was developed. *Ps. stutzeri* seemed to be antibiotic sensitive as their growth was suppressed by all the examined antibiotics regardless of Colistin. *Pantoea* sp. was Colistin- and Ampicillin- resistant while *Pantoea* sp. a gentamycin-resistant bacterial strain with growth inhibition zone of 12 mm diameter. The overall pattern was shown by these antibiotics: azithromycin > kanamycin> chloramphenicol = gentamycin > ampicillin = colistin.

Table 9. Antibiotic resistance by the bacterial strains.

Tested antibiotics	Disc. Conc.	Diameter of the growth inhibition zone(mm)	
		Pantoea. sp.	*Ps. stutzeri*
Ampicillin	10 µg	12 (R)	19 (S)
Chloramphenicol	30 µg	14 (I)	12 (I)
Kanamycin	30 µg	15 (I)	22 (S)
Azithromycin	15 µg	18(S)	19 (S)
Colistin	10 µg	6 (R)	0 (R)
Gentamycin	10 µg	11(R)	17 (S)

(R): Resistant, (I): Intermediate, (S): Susceptible.

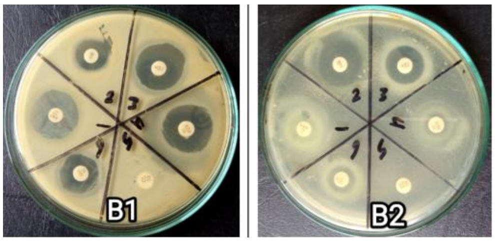

Figure 5. Showing *Pantoea* sp (B1) and *Ps. Stutzeri* (B2) ability to antibiotic resistance.

9-Field experiment

Previous experiments, *invitro* and in greenhouse showed that *T. asperellum*, *T. harzianum*, *Pantoea* sp., *Ps. stutzeri* and V1 were the most effective treatments against stalk rot pathogens. So, this experiment aimed to control maize charcoal rot during crop season in the field. Data presented in Tables (10) and (11) clearly show that all tested treatments significantly decreased the infection by charcoal rot compared to the untreated plants (control) at different locations in the same season. The highest reduction of disease incidence with *T. asperellum* and vermicompost V1 followed by *Pantoea* sp. treatments for two locations were recorded (15, 15 and 17%), (15, 15 and 22%), respectively, at 2023 growing season. In general, Maxim treatment was significantly the most effective in comparison with where disease incidence recoded 13.54 and 14%, respectively, other treatments in both Giza and Sakha locations.

On the other hand, the reduction in charcoal rot was reflected on the produced yield, which was affected with seed treatment. Seeds previously treated with each of V1, *T. asperellum* and *Pantoea* sp. had the highest ear weight (g), ear length (cm), seeds dry weight (100g) and plant height (cm) at Giza location, which recorded (348.5, 322.3 and 305.8g), (22.7, 22.15 and 21.25cm), (40.22, 38.24 and 36.86g) and (288.25,268 and 248.25cm), respectively. While at Sakha location the same treatments recorded that (387.2, 384.9 and 377.1g), (23, 22.3 and 21.5cm), (42.10, 37.69 and 36.69g) and (297,274 and 252cm), respectively compared to the control. Also, Figure (7) shows characteristics of maize under all tested treatments.

Table 10. Efficacy of treating maize seeds with bioagents, vermicompost and mineral fertilizers on infection %, Sakha, Giza, 2023.

Treatments	**Incidence%**		**Efficacy**	
	Giza	**Sakha**	**Giza**	**Sakha**
***Trichoderma asperellum*(T2)**	15	15	75	65.90
***Trichoderma harzianum*(T4)**	20	25	66.67	43.18
***Pantoea* sp**	17	22	71.67	50
Ps. Stutzeri	19	23	71.67	47.72
Vermicompost (V1)	15	15	75	65.90
Mineral fertilization	25	27	58.33	38.63
Fungicide Maxim	13.54	14	77.43	68.18
Control	60	44	0	0
L.S.D at 5%	**1.221**	**0.791**	**1.743**	**4.013**

Table 11. Efficacy of treating maize seeds with bioagents, vermicompost and mineral fertilizers on growth parameters, Sakha, Giza, 2023.

Treatments	**Ear weight (g)**		**Ear length (cm)**		**Seeds dry weight (100g)**		**Plant Height (cm)**	
	Giza	**Sakha**	**Giza**	**Sakha**	**Giza**	**Sakha**	**Giza**	**Sakha**
Trichoderma asperellum	322.3	384.9	22.15	22.3	38.24	37.69	268	274
Trichoderma	277.5	322.5	20.95	20.92	32.90	33.81	239.1	242
***Pantoea* sp.**	305.8	377.1	21.25	21.5	36.86	36.69	248.25	252
Pseudomonas stutzeri	284.1	336.8	20.4	21.5	35.83	34.15	240	238
Vermicompost (V1)	348.5	387.2	22.7	23	40.22	42.10	288.25	297
Mineral fertilization	286.8	357.3	21.1	19	36.76	36.0	244.14	231
Fungicide Maxim	261.2	250	20	20	30	32.9	250	248
Control	253	253	15.75	15	33.45	32.90	225.95	235
L.S.D at 5%	**6.91**	**4.65**	**2.84**	**2.2**	**3.47**	**1.33**	**2.21**	**3.56**

9-1 Estimation of Sugar and proline concentrations

Generally, the sugar content was increased due to using all treatments (Table, 12). At Giza location, the maximum sugar content in fresh stalk biomass was found with V1, *T. asperellum*, and *Pantoea* sp. Corresponding values were 211, 207 and 194.8 mg\g, respectively compared to the control. While at Sakha location, V1and *T. asperellum*, showed the maximum sugar content of fresh stalk followed by *Pantoea* sp., being 212, 212, and 205 mg\g. On the other hand, higher proline content was recorded with V1, *T. asperellum* and *Pantoea* sp. (600, 570, and 550) at Giza location (Table 12). While at Sakha location, higher proline content was observed in plants previously treated with V1, *T. asperellum* followed by *Pantoea* sp. at (620, 600 and 556µg) while, the control plants showed the minimum proline content. This means that V1 is the best treatment followed by *T. asperellum* for both sugar and proline contents in maize plants grown in the two locations.

Figure 6. The infected stalk rot (charcoal-rot) under all tested treatments

Figure7. Ear of maize under all tested treatments

Table 12. Effect of the tested treatments on sugar and proline concentrations in maize plants growing in two locations during, 2023

Treatments	Total sugar content (mg\ g-		Total proline content (µg/g	
	Giza	Sakha	Giza	Sakha
T. asperellum (T2)	207	212	570	600
T. harzianum (T4)	186	188	486	500
***Pantoea* sp.**	194.8	205	550	556
Ps. stutzeri	162	170	476	450
Vermicompost (V1)	211	212	600	620
Mineral fertilization	121	138	374	400
Fungicide Maxim	189	187	243	242
Control	65	71	250	300
L.S.D at 5%	**7.38**	**5.35**	**3.58**	**8.37**

9-2 Plant analysis

Results in Table (13) show that a maximum of total N_2-content at Giza location was recorded due to using each of T2, T4 and *Pantoea* sp. treatments compared to mineral fertilization (recommended NPK). same way, the results of the plant content of phosphorus and potassium were in the same ordering direction for the same treatments compared with the control Also,at Sakha location the same trend was appeared also in the same way for the content of plant nutrients.

Table 13. Effect of the tested treatments on NPK content in maize shoot dry biomass, 2023

Treatments	N_2 content (mg plant^{-1})		P content (mg plant^{-1})		K content (mg plant^{-1})	
	Giza	Sakha	Giza	Sakha	Giza	Sakha
Trichoderma asperellum (T2)	797.33	871.67	123	130	305	343.67
Trichoderma harzianum (T4)	789	865	107.33	111.67	237.67	246.67
***Pantoea* sp.**	769.67	669.67	112.33	125.33	363.33	288
Ps. stutzeri	625.67	528.33	78	75.67	277.33	309.33
Vermicompost (V1)	539.33	576.67	84	99.67	197	219
Mineral fertilization	702.33	624.67	140.33	144	199.33	188
Fungicide Maxim	416.67	540	98	96.33	157.67	162
Control	369.33	462.67	93.33	85.67	135.33	139.33
L.S.D at 5%	**268.7**	**127.98**	**30.8**	**13.12**	**78.2**	**58.48**

DISCUSSION

According to the findings, the seven isolates of *M. phaseolina* of the present investigation obtained from three different governorates were capable to infect maize plants causing charcoal and stalk rot diseases. These results are consistent with the results of Ashraf *et al.* (2015) and Rashid *et al.* (2021), who noted that charcoal rot of maize is caused by *M. phaseolina* and is considered an important challenge in the global production of maize seeds Majumdar *et al.* (1996), confirmed the antagonistic property between *Trichoderma* spp. against *M. phaseolina*. Moreover, *T. harzianum* has the ability to inhibit the formation of microsclerotia of *M. phaseolina*. These findings corroborated our findings, which showed that, the two isolates, *T. asperellum* (T2) and *T. harzianum* (T4) were by far the most potent bioagents.

Greenhouse testing for antagonism of these isolates with the pathogen in concern, under strict disease conditions, the forecited experimental results are in agreement with those as stated through Patil *et al.* (2003), who mentioned that seed treatment with *Trichoderma* sp (4 g/kg seed) along with castor and neem cake, furrow application at 250 kg/ha, 15 days before sowing, gave effective control of stalk rot diseases and gave better cost-benefit ratios. In addition, many studies indicated that *Trichoderma* spp. had been demonstrated as efficient for the control of *M. phaseolina* in melon, maize, eggplant, sorghum, and chickpea (Valiente *et al.*, 2008; Ramezani, 2008; Larralde-Corona *et al.*, 2008; Manjunatha *et al.*, 2013). Furthermore, Martínez-Salgado *et al.* (2021), found that several isolates Rhizospheric *Trichoderma* were quite efficient in decreasing the diseases, *Macrophomina* sp. incidence and promoting host plant growth traits. On the other hand, Soltan *et al.* (2022), found that the ten bacterial isolates obtained from vermicompost had an *in vitro* antagonistic effect against *Fusarium solani*, *Fusarium* spp., *M. phaseolina* and *Rhizoctonia solani*. Lakhran *et al.* (2018) reported that organic manure tested reduced root rot incidence of chickpeas significantly over control.

Results concerning polyphenoloxidase activity at 14 and 45 days are consistent with studies by Li *et al.* (2003) and Mohammadi and Kzami (2002) who found that resistance to maize stalk rot is significantly correlated with the enzymatic activities of peroxidase and polyphenoloxidase. Furthermore, the pith senescence in the stalk may cause the sugar content to decrease. Thus, following physiological maturity, a decrease in stalk activity, moisture, and soluble sugar may lead to a fall in disease resistance, making the plant more vulnerable to the infection by stalk rot. Abou-Zeid *et al.* (2018b) stated that the oxidative potential of H_2O_2 aids in the production of lignin through the conversion of O-dihydroxyphenols to toxic O-quinones by polyphenoloxidase and the peroxidase-mediated crosslinking of structural proteins that are abundant in phytoalexin biosynthesis and proline.

As for the chitinase activity, the outcomes agree with the conclusions from (El-Khallal, 2007; Latha *et al.,* 2009; Abd-El-Khair *et al.,* 2011; Seo *et al.,* 2012; Surekha *et al.,* 2014), wherein the authors reported that the used bioagents such as *Trichoderma* spp., *Bacillus* spp., *Pseudomonas* spp., and *Serratia marcescens* have a significant role in the defense mechanisms of bean plants against pathogen infection. Chitinase activity was increased considerably with the increase in the inoculation period. The highest value of enzyme activity was recorded up to the 45 days after treating corn plants These results are in line with Seo *et al.* (2012) Wang *et al.* (2013) Surekha *et al.* (2014) who emphasized that chitinases hydrolyze chitin which is major component of fungal cell walls, leading to direct inhibition of growth of several fungi. Chitosan affects various physiological responses like plant immunity, defense mechanisms involving various enzymes such as, phenylalanine ammonium lyase, polyphenoloxidase, tyrosine ammonialyase and antioxidant enzymes (Zhang *et al.,* 2011).

Additionally, field results showed the important role of the treatments in improving the growth of the treated plants, both in terms of quantity and quality, that verified those obtained by Lombardi *et al.* (2020), who noted that *Trichoderma* spp. can decompose organic soil matter, increase soil nutrient supply, improve crop photosynthetic efficiency, improve plant height, stem diameter, and other agronomic traits, and increase production. *Trichoderma* spp. can also produce plant growth stimulators, such as indoleacetic acid (IAA), to promote the development and growth of plant roots by secreting phytase and ferritin to help plants absorb. Moreover, tomato and strawberry productivity and quality were significantly increased when vermicompost containing plant outgrowth-promoting bacteria was added, as reported by Mahadeen, (2009) and Ruiz and Salas Sanjuan, (2022). In addition, NPK content was increased in maize plants when inoculated with *Ps. stutzeri*, these results are in harmony with those obtained by Mufti and Bano (2019). PGPR improved the macronutrient availability in the infested soil's rhizosphere. Suman *et al.* (2020) reported that *Pantoea* inoculation on maize and wheat as test crops in a glasshouse improved plant biometric parameters and was the most efficient in enhancing plant growth compared to recommended NPK fertilized controls.

On the other hand, Sehrawat *et al.* (2022) mentioned that certain soil bacteria, algae, fungi, plants, and insects possess what is called cyanogenic bacteria, have the ability to produce hydrogen cyanide (HCN), which plays an important role in inhibiting the growth of various pathogenic fungi, weeds, insects, termites, and nematodes and their role in biocontrol activity in a variety of plants. That agrees with our results and those recoded by Spence *et al.* (2014) who found that *P. agglomerans* strain effectively inhibited growth and reduced appressoria formation of the fungal pathogen *M. oryzae* through HCN in rice plants.

The results also showed a positive effect of the treatments on increasing the sugar and proline content compared to the control. These results are confirmed with Xue *et al.* (2016), who established that during infection, stalk rots pathogens need an energy source. Since lignin and cellulose, two structural carbohydrates found in maize stalks, are hard to separate, the soluble sugar content of the stalk is largely utilized as energy during the infection process. Furthermore, the soluble sugar content and physiological activity of the stalk are linked to the resistance of maize plants to stalk rot (Anderson and White, 1994).

According to Du *et al.* (2019), the higher water and soluble sugar contents in maize make for a higher resistance to stalk rot. In the case of proline content estimation, the outcomes were found to agree with Mansour, (2000); Zeng and Zhang, (2010). who stated that proline, also, referred to as a hydroxyl radical scavenger, acts as an energy source for plants. However, they also mentioned that proline can cause the accumulation of proline in plants as a result of both biotic and abiotic stressors. Moreover, seeds treated with a binary mix with both strains showed the greatest increase in proline content under both stressed and unstressed conditions.

Our findings unequivocally demonstrate that the *Trichoderma* agent has benefits for managing soil-borne disease such as stalk rots which are present during the entire infection growth period. This was most likely made possible by the root colonization effect that *Trichoderma* mycelia produce over the course of the plant growth period (Harman *et al.*, 2004). On the other side, the experiment's outcomes showed a decreasing in the disease, and this was consistent with the results by Mufti and Bano (2019) who found that disease suppression was substantially linked with plant defense and antioxidant enzymes. In parallel with the present work, Mufti and Bano (2019) pointed out that *P. stutzeri* caused a significant decrease in the charcoal rot disease severity index by inducing linear increases in the activities of peroxidase, catalase, phenylalanine ammonia-lyase, superoxide dismutase, and polyphenoloxidase in addition to greater concentration of soluble proteins and leaf proline. The amount of dissolved copper and zinc in vermicompost is sufficient for the development and growth of plants, as copper is a micronutrient. Moreover, copper is a component of many enzymes, such as cytochrome oxidase, polyphenoloxidase, and ascorbic acid oxidase. Consequently, adding copper to all planting methods greatly decreased the risk of root rot diseases and improved the quantity and quality of produce. Therefore, copper prevented most of root rot fungi from growing, sporulating, and/or producing sclerotia (Wang *et al.*, 2013; Zhang *et al.*, 2011and Ali *et al.*, 2023).

CONCLUSION

One of the primary bio-control agent strategies is the antagonistic behavior of the biocontrol agents and novel vermicompost used against plant diseases, which aids in plant growth by directly opposing or strengthening natural defenses. As a result, biological control and vermicompost are safer for the environment and can progressively replace fungicides in managing stem rot (charcoal rot). Use of biocontrol agents as a sustainable, eco-friendly, and effective alternative to controlling phytopathogenic.

Author contributions

M. A. A. & H. T. E. & E. A. conceived the presented idea. H. T. E & H.B. &R.A.&H. F. contributed to sample preparation. All authors conceived the presented idea, developed the theory, performed the computations, and conducted the experiments. H. T. E & H.B. & R.A. & M. A. A. analyzed all treatment-related soil diseases. E. A. & H. F. conceived and planned the experiments. H. T. E & H.B & E. A. provided critical feedback and helped shape the research, analysis, and manuscript. Also, all authors discussed the results and contributed to the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

All the authors declared that they have no competing interests.

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