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Research Article

Application of *Rhizopus microsporus* and *Aspergillus oryzae* to enhance the defense capacity of eggplant seedlings against *Meloidogyne incognita*

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Abstract

Several phytopathogens attack eggplant, causing crop damage. One of the most destructive plant diseases, Root-Knot Nematode (RKN), causes significant damage to eggplant seedlings. Finding safe and effective biological alternatives to prevent eggplant root nematode disease, which significantly limits plant productivity, is the innovative aspect of this study. Six isolates of plant growth-promoting fungus (PGPF) were tested in the current work for improving biochemical defense and physio-biochemical performance in eggplant seedlings under the *Meloidogyne incognita* challenge. PGPF isolates were tested *in vitro* for some biochemical traits such as Siderophores and HCN production. Besides, the antagonistic efficacy of PGPF filtrates against *M. incognita* was tested in vitro. The best isolates capable of producing HCN were F5 and F3 respectively. Also, F5 followed by F3 exhibited the maximum mortality proportions of 74.20% and 60.35% mortality in nematode juveniles after 96 hours respectively. Moreover, F5 has the highest level of antioxidant activity, with IC_{50} 145 µg/mL followed by F3 with IC_{50} 350 µg/mL. thus, we identified F5 and F3 completely as *Rhizopus microsporus* (OQ291571.1 and *Aspergillus oryzae* OQ291572.1. Implementing *R. microsporus* and *A. oryzae* collectively *in vivo* study was the most successful therapy, limiting nematode recordings as 95.23%, 86.98%, 80.35%, 80%, and 68.78% reduction in females, galls, developmental stage, egg masses, second juveniles, respectively, in diseased seedlings. It could be suggested that the use of ethyl acetate extracts (EAE) of *A. oryzae* and *R. microsporus* might be commercially applied as a stimulator of eggplant and or anti-nematodes against *M. incognita*.

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Keywords: *Aspergillus oryzae*; biochemical defense; eggplant; fungi; nematicidal; *Rhizopus microspores*; root-knot nematode

Abbreviations: PGPF: plant growth-promoting fungus; RKN: Root-Knot nematode; *M. incognita: Meloidogyne incognita*; HCN: Hydrocyanic acid; EAE: ethyl acetate extracts; PDA: Potato Dextrose Agar; GC-MS: Gas Chromatography-Mass Spectroscopy

Introduction

Under the risk of weather alteration and the feast of plant pathogens, enhancement of crop productivity and avoiding the use of chemical pesticides is a significant issue for the agronomic production (Chakraborty and Newton, 2011; Delcour *et al*., 2015; Zaker, 2016). Nematodes are cylindrical or worm-shaped invertebrates that cannot be seen by the naked eye (Jonathan, 2009; Yuen *et al*., 2020). They are aquatic animals, so they live on the film of water around the soil grains, as their body must be covered by a thin membrane of water to remain alive (Tahseen, 2012). They cause much damage to the plant when infected with it, such as nodules and swellings on the roots of affected plants, yellowing, withering, and appearance of symptoms of nutrient deficiency (Ravichandra, 2014; Lima *et al*., 2017). Other injuries include stunting plants in some cases, reducing the purchasing value of lands contaminated with nematodes, which leads to a reduction in soil fertility and reduced productivity of its crops, spreading of some other diseases caused by other pathogens such as fungi and bacteria, as it facilitates their entry into the roots of plants causing injury to them and finally increasing production expenses due to high control costs, especially chemical control elements (Attia *et al*., 2021).

One of the most destructive plant nematode diseases disturbing vegetables and fruits is root galling disease caused by RKN (Rivard *et al*., 2010; Long *et al*., 2022). The following four types of root-knot nematodes (*M.arenaria, M. hapla, M. javanica and M. incognita*) are the main and most common types in agricultural lands, where they constitute about 95% of all RKN in the world (Tariq-Khan *et al*., 2017). RKN are the most destructive plant nematodes, and many plant pathologists consider them to be one of the five most important and most dangerous economic pathogens in the world *(*Onkendi *et al*., 2014*)*. This is due to several factors, the most important of which is it's rapid widespread throughout the world and its extensive host range, as well as its cooperation with other pathogens, especially fungi and bacteria, in causing many complex plant diseases that are hardest eliminating (Khan *et al*., 2020), in addition to its ability to break the resistance of plants to some other diseases or weaken plants and prepare them to infection with secondary pathogens that unable to cause injury alone (Fujimoto *et al*., 2011). The agronomic business faces significant challenges in increasing crop output and avoiding chemical pesticides because of the risk of climate change and the growth of infectious diseases (Ramakrishna *et al*., 2019). Environmental restrictions on the use of synthetic pesticides have rekindled interest in biocontrol that has nematicidal potential for M. incognita attack (Abd-Elgawad, 2020). It is agreed that plant immunity against pathogen infection within plants can be activated by external spraying of biotic and abiotic stimuli or inducers (Attia *et al*., 2016; Farrag *et al*., 2017; Abdelaziz *et al*., 2022a; Elbasuney *et al*., 2022; Khattab *et al*., 2022). Numerous studies focused on living microorganisms' application and their products as pesticides against biotic and abiotic plant stress (Badawy *et al*., 2021; Abdelaziz *et al*., 2022b; Attia *et al*., 2022b). In temperate and tropical climates around the world, eggplant is a crucial vegetable crop. Egypt ranks third in the world for producing eggplants, behind China and India, hence it is regarded as one of the country's key crops. Due to its high fiber content, low amounts of carbohydrates that regulate blood sugar and reduce the level of glucose, and phenols that inhibit the enzymes involved in diabetes, eggplant offers antioxidant properties that support heart health. In addition, eggplant is a rich source of phytonutrients such as vitamins that support human health, as well as calcium, magnesium, iron, and polyphenols. Moreover, the

study of plant physiology and genetics employs eggplant as a model plant (Sharma and Kaushik, 2021). Unfortunately, biotic diseases that affect the eggplant plant cause very significant crop losses, both in terms of quantity and quality (Albalawi *et al*., 2022; Attia *et al*., 2022c; Abdelaziz *et al*., 2023).

Utilizing bio-nutrients, plant growth-promoting microorganisms have successfully increased the therapeutic nutrition content of crops (Abdelaziz *et al*., 2022a). Agricultural soils have a very wide range of these microbial populations depending on the type of soil, and crops (Khalil *et al*., 2015). Ecofriendly biological agents can be used to develop the characteristics of the soil and produce enzymes that make it easier for plants to absorb nutrients and at the same time do not lead to ecological effluence to accomplish greater efficiency and produce more and higher value crops (Glick, 2012; Etesami and Maheshwari, 2018; Kour *et al*., 2020). Plant growth promoting fungi (PGPF) reported to reduce the harmful impacts of various nematodes (Verma *et al*., 2019) by eliciting induced plant systemic resistance. Herein, we are investigating the capabilities of PGPF to produce this plant growth promoting products that enhance plant development and control harmful impacts on the development of eggplant infected with *M. incognita*. The novelty of this study lies in reaching plant growth-stimulating fungi with a high ability to inhibit the growth of nematodes and their infective stages in the laboratory, as well as with a positive and effective effect to improve the physiological plant immunity against nematode infection. Our study opens the approach to an alternative and safe technique to plant growth promotion as well as induce resistance of eggplant to resist *M. incognita.*

Materials and Methods

Isolation and identification of PGPF

Rhizosphere eggplant cultivated soil from a plant garden (location: latitude, 30° 03' 15.48" N, longitude, 31° 19' 12.75" E) was collected. Soil samples (1 g) were placed directly on sterilized solidified PDA (Sigma Aldrich, Germany) enhanced with antibiotic chloramphenicol (0.2 mg/L) (Attia *et al*., 2022a; Hashem *et al*., 2021; Khalil *et al*., 2015). PGPF isolates were identified depending on their macroscopic and microscopic features (Suleiman *et al*., 2018a; Suleiman *et al*., 2018b; Saied *et al*., 2022; Soliman *et al.*, 2022) The colour, texture, colony development rate, and pigmentation of the colony's surface were studied. Then culture characteristics were examined by using a Nikon stereo-zoom binocular microscope. The light microscope that was used for studying was ×20 and ×40 objectives lens. The most potent PGPF isolates were recognized genetically using the ITS region. The fungal DNA was obtained by Quick-DNA Fungal Microprep Kit (Zymo Research; D6007), and identification was completed by ITS region according to (Abdelaziz *et al*., 2022b). The generated sequence was submitted to the National Centre for Biological Information (NCBI) database's BLAST algorithm to be searched for phylogenetic sequences that were surprisingly alike. The tree of phylogeny was developed using the neighbor-joining strategy in the Mega 5.0 program.

Biochemical traits of fungal isolates

Hydrocyanic acid (HCN) Production

The capability of the isolates to generate (HCN) was tested according to (Trivedi *et al*., 2008). On a PDA medium that had 4.4 g/l of glycine added as a supplement, fungal isolates were inoculated. Picrate carbonate solution was applied to Whatman paper discs. Each Petri dish has these discs on top of it. Plates were inverted, wrapped in Para film, and incubated at 27 °C for 4 days. The Whatman paper's colour (which develops from yellow-to-yellow orange to brown or brown colour) represents HCN production.

Indole acetic acid production

The efficacy of isolates for (IAA) production was determined by the colorimetric technique (Leveau and Lindow, 2005). The colorimetric method was used to evaluate the capacity of fungal isolates to generate

Indole Acetic Acid (IAA). After adding 500 µl of old fungal cultures, 100 mL of Potato Dextrose Broth (PDB), which contains 0.1% tryptophan, was cultured for five days in the dark at 30 °C. The fungi cultures underwent a 10-minute centrifugation at 10,000 rpm and 4 °C. Using a colorimetric technique, the indole-3-acetic acid (IAA) in the supernatants was calculated. Orthophosphoric acid, two drops, and Salkowski reagent were combined with taken supernatant (2 ml). The emergence of a pinkish-red hue denotes IAA production.

Siderophores production

The capability of the isolates to generate siderophores was tested according to (Sujatha and Ammani, 2013)**.** On PDB medium at 28 °C, the isolated fungi were cultivated for 14 days. 1 mL of fungal culture filtrate was mixed with 2 mL of 2% of ferric chloride to see how the yellow colour turned brown.

Bioactive compounds extraction

PGPF isolates were cultured in potato dextrose broth (PDB) (Sigma Aldrich, Germany) at 27 °C ± 2 °C for 21 days under static conditions. The fermentation broth was subjected to filtration under septic conditions to remove fungal mycelia. The culture filtrates of the isolated fungal endophytes were subjected to a double extraction process utilising ethyl acetate (EtOAc) in a 1:1 ratio. A volume of 100 mL from each filtrate was combined with an equal volume of ethyl acetate and subjected to vigorous agitation on a vortex shaker for a duration of 10 minutes. Following this, the mixture was allowed to settle for a period of 5 min, during which time two distinct and transparent layers were observed to form. The ethyl acetate (EtOAc) phase was successfully separated from the aqueous phase using a separating funnel. The organic phase that was obtained was subjected to evaporation under reduced pressure at a temperature range of 40-45 °C, utilizing a rotary evaporator (Heidolph VV2001, Germany). The fungal crude extract was stored at 4 °C until further experiments (Sharaf *et al*., 2022).

Gas Chromatography-Mass Spectroscopy (GC–MS) Analysis

According to (Rivero-Cruz *et al*., 2020) Using GC–MS, the components of isolate metabolites extracts were examined. When compared to the spectrum of known chemicals kept in the WILEY 09 (Wiley, New York, NY, USA) and NIST 11 libraries, the identified components' spectra were found to match**.** In a concise manner, the extract was solubilized in methanol, desiccated using anhydrous sodium sulphate, and subsequently passed through a syringe filter (0.45 m particle size) prior to injection. In order to conduct chromatographic analysis, a mass spectrometer (Trace GC Ultra-ISQ, Thermo Scientific, USA) was utilized. The initial column temperature was set at 70 °C, subsequently elevated to 280 °C with a 2-minute duration at a rate of 5 °C per minute, and ultimately raised to 300 °C at a rate of 10 °C per minute. By employing the mass spectra and retention time databases sourced from the Wiley 09 and NIST 11 libraries, the extract components were successfully identified and quantified.

Antioxidant (DPPH assay)

Antioxidant activity was investigated for fungal extracts via (2,2-diphenyl-1-picrylhydrazyl) method according to (Khalil *et al*., 2021). A solution of methanol containing DPPH at a concentration of 0.15% was combined with various concentrations (ranging from 7.81 to 1000 µg/ml) of the extract. Following a 10-minute incubation period, the absorbance of the resulting mixture was measured at a wavelength of 515 nm. The antioxidant activity was quantified by determining the IC_{50} value, which represents the concentration (expressed in µg/ml) required to inhibit 50% of the radicals. Ascorbic acid, commonly known as Vitamin C, was employed as the reference standard in the experiment. The quantification of scavenging activity was determined utilising the subsequent mathematical expression:

$$DPPH\ scavenging\ activity = \frac{\text{Control absorbance} - \text{Fungal absorbance}}{\text{Control absorbance}} \times 100$$

Eggplant seedlings

Eggplant seedlings (*Solanum melongena* L.), which are four weeks old, were obtained from the Egyptian agricultural research centre.

Source of pathogen

Nematode samples were brought from Nematode Department at Plant Diseases Institute. According to (Attia *et al*., 2021), nematode eggs were extracted.

Anti-nematode activity of PGPF

In vitro study

To investigate the fungal isolates' impact on nematode mortality in vitro, two concentrations (100% and 50%) of each isolate were associated with M. incognita juveniles isolated as in Figure 1. According to Oostenbrink (1960), the activity of each isolate was assessed as the percentage of the inactive nematodes **.** In sterile Petri plates with a diameter of 5.0 cm, 3.0 mL of freshly made juvenile suspension (containing approximately 20.0 juveniles per ml) and 3.0 mL of each treatment with their concentration were used. Each treatment was repeated three times in triplicate. In the end, distilled water was used as the control instead of the treatments. In contrast, all Petri dishes were incubated for varied lengths of time, such as 24, 48, 72, and 96 h, and the juveniles that were inactive were counted using a low-power stereomicroscope, after which the mortality % was calculated. The percentage of inactive nematodes served as a measure of the treatment's effectiveness. In order to determine the actual nematode juvenile mortality, the three states of the root knot nematode, including the second, third, and fourth juveniles (developing stage) and adult females, were also analysed according to Attia *et al*. (2021) and Ji *et al*. (2019).

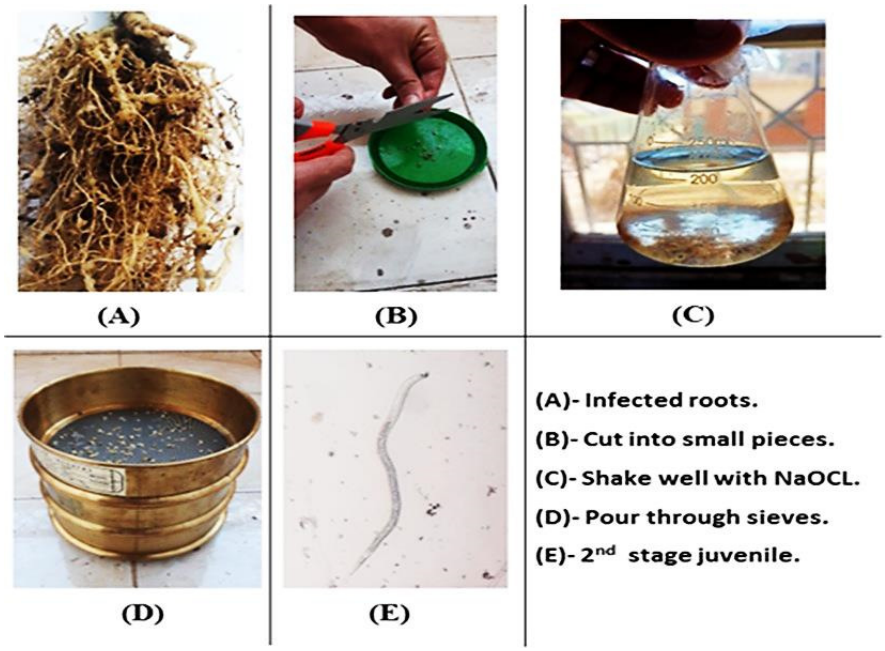

Figure 1. Steps of nematode 2nd stage juveniles' extraction

Pot experiment

In the greenhouse, 3000 J2 juveniles of *Meloidogyne incognita* were injected into 20 eggplant seedlings, then after eight days, the Pots were divided into eight groups and used in a completely random way. Each group of five seedlings received the following assignment: T1- Healthy control, T2- Infected Control (Nematode only), T3- Healthy + *R. microsporus*, T4- Healthy + *A. oryzae*, T5-Healthy + (*A. oryzae* and *R. microsporus*),

T6- Infected + *R. microsporus*, T7- Infected + *A. oryzae* and T8-Infected + (*A. oryzae and R. microsporus*). As 2 mL/plant in all treatments.

For plant resistance evaluation, photosynthetic pigments and biochemical signals from plant samples were analysed 45 days after sowing, and the disease was assayed.

The photosynthetic pigments were analysed to assess the levels of chlorophyll a, chlorophyll b, and carotenoids in fresh leaves (one leaf for each replicate) (Abdelaziz *et al*., 2022c; Cohen-Bazire *et al*., 1966; Strain and Svec, 1966). The sample was subsequently subjected to filtration using Whatman No. 1 filter paper, followed by spectrophotometric measurements at wavelengths of 665 nm, 649 nm, and 470 nm.

Free proline was established by the method of Bates *et al.* (1973). A total of 5 g of dried shoots were subjected to extraction using 10 ml of sulfosalicylic acid solution (3% concentration). Subsequently, 2 ml of the resulting extract were combined with 2 ml of ninhydrin acid and 2 ml of glacial acetic acid. This mixture was then subjected to boiling conditions for a duration of one hour, after which the reaction was promptly halted by the addition of ice. Finally, 4 mL of toluene was added to the mixture, and assayed at 520 nm.

Total phenolics were measured using the Dai *et al*. (1993)method. For one day, 1 g of dried pepper tissues were extracted in 10 mL of 80% ethanol. The filtrate was subsequently replenished to a volume of 50 mL using a solution consisting of 80% ethanol. After that, a total of 0.5 mL of the filtrate was thoroughly combined with an equal volume of Folin's reagent, followed by agitation for a duration of 3 min. Subsequently, 3 mL of distilled water and 1 mL of a saturated sodium carbonate solution were added to the mixture. The resulting solution was then subjected to detection at a wavelength of 725 nm.

Statistical analyses

One-way analysis of variance (ANOVA) was used to evaluate the pilot data. While using Co State software, means were compared the (L.S.D.) at a probability level of 5.0% (Brownlee, 1952; Snedecor and Cochran, 1982).

Results

Investigation of isolates according to promoting properties

The data presented in Table 1 showed that the best isolates to produce HCN were F5 (+++), followed by isolates F2 and F3 (++). The present results revealed all isolates (F1, F2, F3, F4, F5, and F6) can produce IAA Table 1. Also, data showed that F3 was the best isolate to give IAA (+++) followed by F5 and F2 (++), then F1 and F4. Based on the results of Table (1), it appears that all isolates have the efficiency to produce siderophores except for F6. The best isolates were F5 (+++), F3 and F4 (++).

Table 1. Screening of tested fungal isolates for HCN, IAA, and siderophores

Fungal isolates	IAA	Siderophores	HCN
F1	+	+	+
F2	++	+++	+++
F3	+++	++	++
F4	+	++	+
F5	++	+++	+++

The emergence of a pinkish-red hue denotes. IAA production was noted as positive for IAA production. (−) represents the negative reaction (+): dark yellow, (++) Pinkish and (+++) red. Siderophores -Development of yellow to orange halo around the colonies was noted as positive for siderophore production. (+): light yellow, (++) dark yellow and (+++) orange HCN -Development of light brown to dark brown color indicated HCN production. (+): light brown, (++) brown and (+++) dark brown.

Impact of fungal isolates on RKN (in vitro)

Root-Knot Nematode damages roots of worldwide crops leading to poor yield and huge financial losses annually. Our results in Figure 2 exhibited increasing in juvenile mortality by increasing the concentration and application period of fungal isolates. This was the case for all test fungal isolates, however, after isolates F3 and F5, the highest notable mortality rates of *M. incognita* 2nd juveniles were found. rather than isolates (F2, F4, F1 and F6) respectively. The largest mortality was attained when the application of F3 was recorded (74.2% and 64.38%) in ^{2nd} juveniles' mortality at concentrations (100% and 50%, respectively) after 96 hours of application. Additionally, the use of F5 led to the second-highest death percentages ever observed, with high mortality rates in nematode juveniles of 60.35 and 47.53% at concentrations of 100% and 50%, respectively, after 96 hours of administration. However, after 96 hours of treatment, F6 caused the lowest mortality ever observed (16.08% and 15.25%) in nematode juveniles at doses of 100% and 50%, respectively.

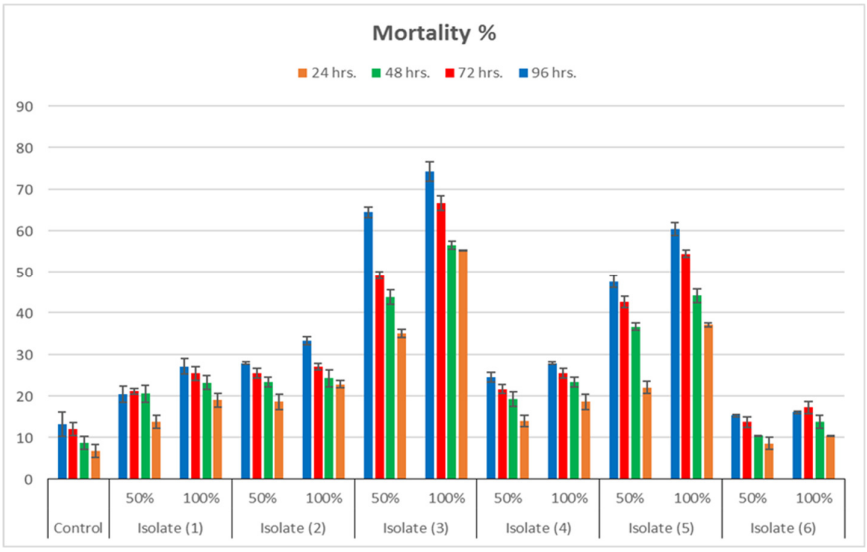

Figure 2. Influence of tested PGPF isolates on RKN *in vitro*

Identification of the most potent PGPF

Results indicated that the two fungi F3 and F5 were the maximum producers of plant growth promoting activity (HCN, IAA, and Siderophores). At the morphological level, the fungal isolate F3 was identified as *Aspergillus oryzae* (*A. oryzae*) and deposited in the gene bank (NCBI) as OQ291572.1 where colonies grow quickly, as seen in Figure 3, reaching 5 cm in diameter after 5 days at 28 °C ±2 on (PDA, intense yellowish green colonies (powdery). On the other side, F5 was identified as *Rhizopus microsporus,* and deposited in gene bank (NCBI) as OQ291571.1. where colonies grow after 3 days at 28 °C ±2 on PDA filling the whole Petri dish white mycelium rapidly becoming black reverse uncolored. Sporangiophore Pale brownish is borne in groups of three to five from clusters of rhizoids. Sporangia brown walls spherical, Columella roughly spherical Figure 4.

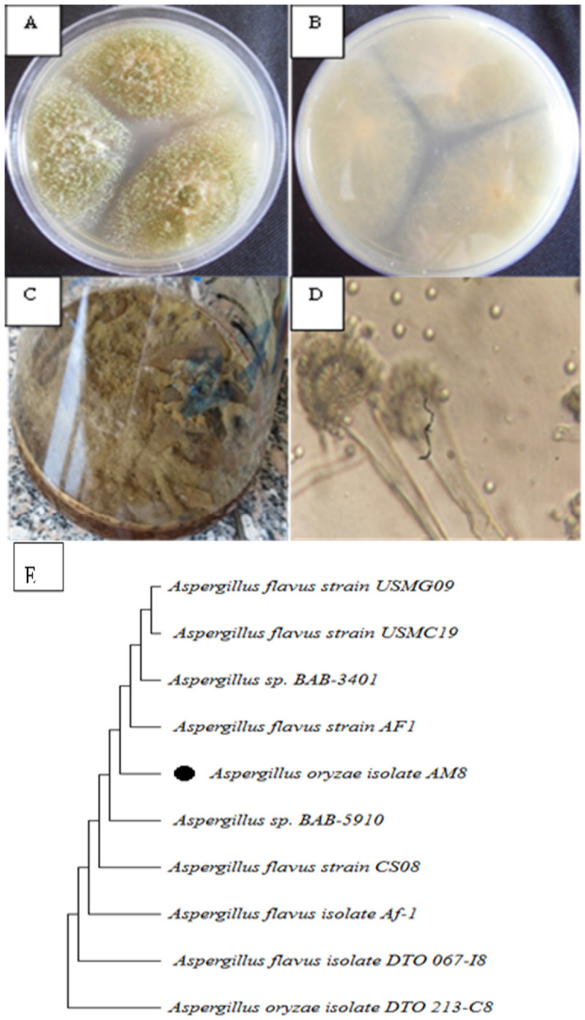

Figure 3. Morphological identification; Surface colony of Ao (A), reverse (B), growth on PDB medium and Conidiophore, conidia (400X) (D) and (E) phylogenetic tree of *Aspergillus oryzae*

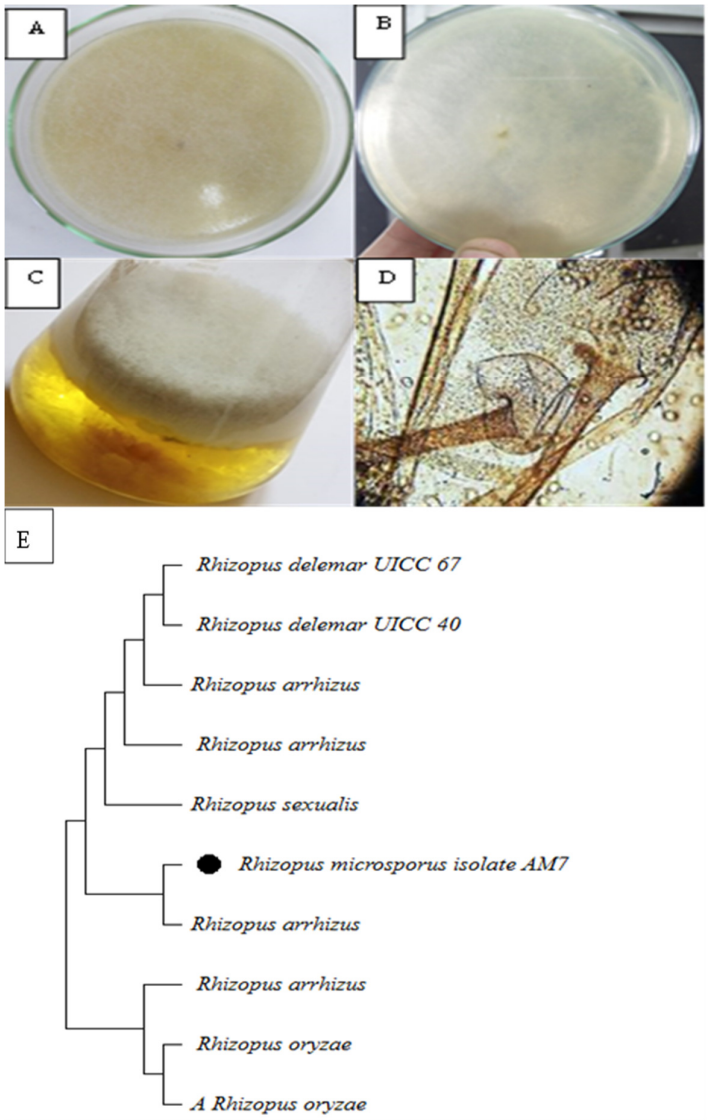

Figure 4. Surface colony of *Rhizopus microsporus* (A), reverse (B), growth on PDB medium, Sporangiophore and sporangium (400X) (D), and (E) phylogenetic tree

GC–MS analysis

The GC–MS of $CH_3COOC_2H_5$ crude extracts of the *A. oryzae* showed the presence of 13 major and minor compounds in the extracts of *A. oryzae*. The major compounds in the fungal extracts are diisooctyl phthalate, octadecanoic acid, methyl ester, docosane, hexadecanoic acid, methyl ester, isochiapin B, Oleic Acid, and heptacosane (Figure 5 and Table 2). On the other hand, the GC–MS of $CH_3COOC_2H_5$ crude extracts of the *R. microsporus* showed the presence of 9 major and minor compounds. The major compounds in the fungal extracts are 9-octadecenoic acid, methyl ester, azulen-2-ol, 1,4-dimethyl-7-(1-methylethyl), diisooctyl phthalate, benzofuran, 6-ethenyl-4,5,6,7-tetrahydro-3,6-dimethyl-5-isopropenyl, and hexadecanoic acid, methyl ester (Figure 5 and Table 3).

Table 2. The *A. oryzae* crude extracts' determined compounds via GC-MS

No.	Compound name	RT (min)	Peak area %	Activity	Reference
1	Hexadecanoic acid, methyl ester	26.25	7.16	Cancer enzyme inhibitors, antioxidants, antimicrobials, hemolytic, hemolytic, 5-alpha reductase inhibitors	(Albergoni *et al*., 1980)
2	Hexadecanoic acid	28.10	3.88	Antioxidant, nematicide, pesticide, and antiandrogenic properties	(Krishnamoorthy and Subramaniam, 2014)
3	9-Octadecenoic acid, methyl ester,	29.48	15	Antibacterial, anticancer, diuretic, and anti-inflammatory	(Hussein *et al*., 2016)
4	9,12-Octadecadienoic acid, methyl ester	30.30	1.43	Antihistaminic, hypocholesterolemic, and antieczemic.	(Ganesh and Mohankumar, 2017)
5	Oleic Acid	31.09	4.25	Antimicrobial	(Dilika *et al*., 2000)
6	Hexadecanol	32.77	1.88	Antioxidant	(Mishra and Sree, 2007)
7	Oxiraneundecanoic acid, 3-pentyl-, methyl ester, trans	33.33	1.32	anticancer nematicides, antimicrobial, anti-inflammatory, and antioxidant	(Sympli, 2021)
8	Docosane	34.38	10.85	antifungal and antibacterial	(Lammers *et al*., 2021)
9	Docosanoic acid, methyl ester	36.48	1.37	antioxidant	(Mehtab *et al*., 2018)
10	Diisooctyl phthalate	36.87	21.69	anticancer, Antimicrobial and antioxidant	(Zhang *et al*., 2018)
11	Octacosane	37.44	7.49	Antibacterial	(Khatua *et al*., 2016)
12	Isochiapin B	40.26	4.27	Anti-insect, antimicrobial, antioxidant, and anticancer properties	(Senthilkumar *et al*., 2012)
13	Heptacosane	42.89	4.15	Antimicrobial activity.	(Carev *et al*., 2023)

Table 3. The compounds that the GC-MS analysis of crude extracts of the *R. microsporus* revealed

No.	Compound name	RT (min)	Peak area %	Activity	Reference
1	Benzofuran, 6-ethenyl-4,5,6,7-tetrahydro-3,6-di methyl-5-isopropenyl	17.00	7.56	anti-oxidative, anti-tumor, anti-viral, and antibacterial	(Miao *et al*., 2019)
2	Selina-3,7(11)-diene	17.91	1.01	antimicrobial and antioxidant properties.	(Chao *et al*., 2005)
3	Azulen-2-ol, 1,4-dimethyl-7-(1-methyl ethyl)	20.43	8.02	antimicrobial	(Dahiya *et al*., 2021)
4	Hexadecanoic acid, methyl ester	26.34	7.35	Antioxidant, antimicrobial,	(Albergoni *et al*., 1980)
5	n-Hexadecanoic acid	28.31	3.93	Nematicide, Antioxidant, and pesticide	(Krishnamoorthy and Subramaniam, 2014)

6	9-Octadecenoic acid, methyl ester, (E)-	29.63	22.9	Antibacterial, anticancer, diuretic, and anti-inflammatory properties.	(Hussein *et al.*, 2016)
7	Nonanoic acid, 9-(o-propyl phenyl)-, methyl ester	30.38	1.70	Antimicrobial	(Sahin *et al.*, 2006)
8	Eicosanoic acid, methyl ester	33.38	1.14	Antifungal	(Pereira *et al.*, 2016)
9	Di isooctyl phthalate	36.90	7.66	Antimicrobial, anticancer, and antioxidant properties	(Zhang *et al.*, 2018)

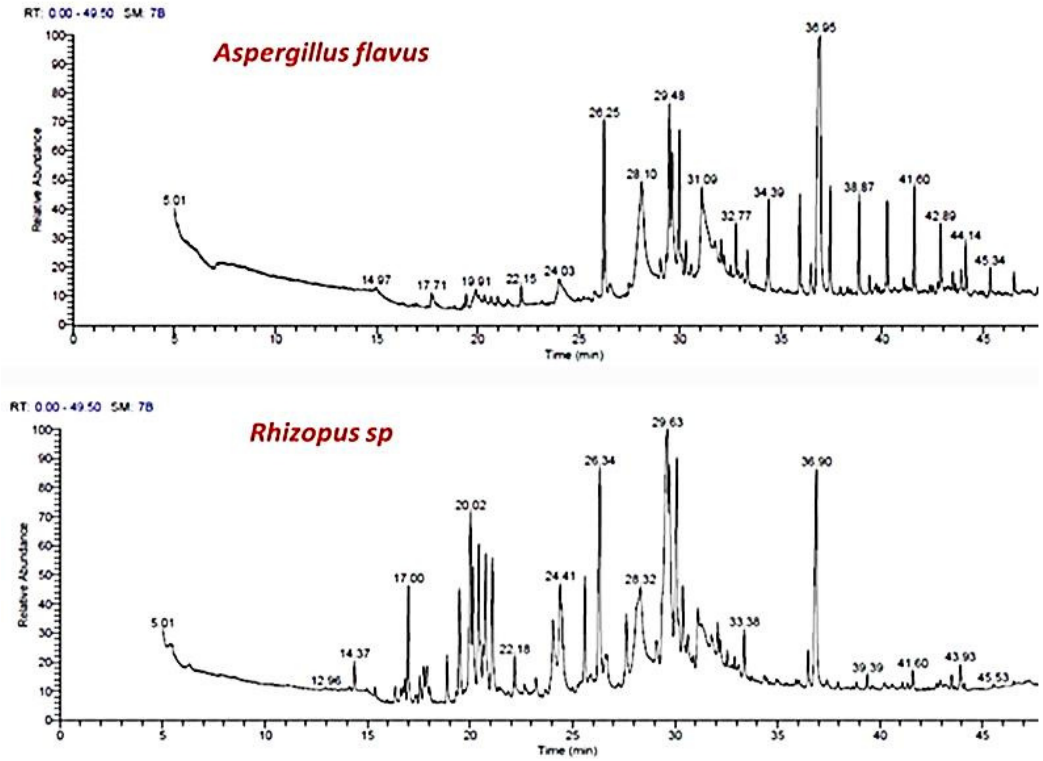

Figure 5. Ethyl acetate extracts of *A. oryzae* and *R. microsporus*, GC-MS chromatogram

Antioxidant activity

The antioxidant activity of *R. microsporus* and *A. oryzae* extracts at various concentrations (1000-7.81 g/mL) was specified using the DPPH method. Figure 6 shows *A. oryzae* antioxidant activity was the maximum among other fungal strains, Moreover, IC_{50} of *A. oryzae* was 145 µg/mL compared to Ascorbic acid (7.8 µg/mL) Also, IC_{50} of *R. microsporus* was 350 µg/mL.

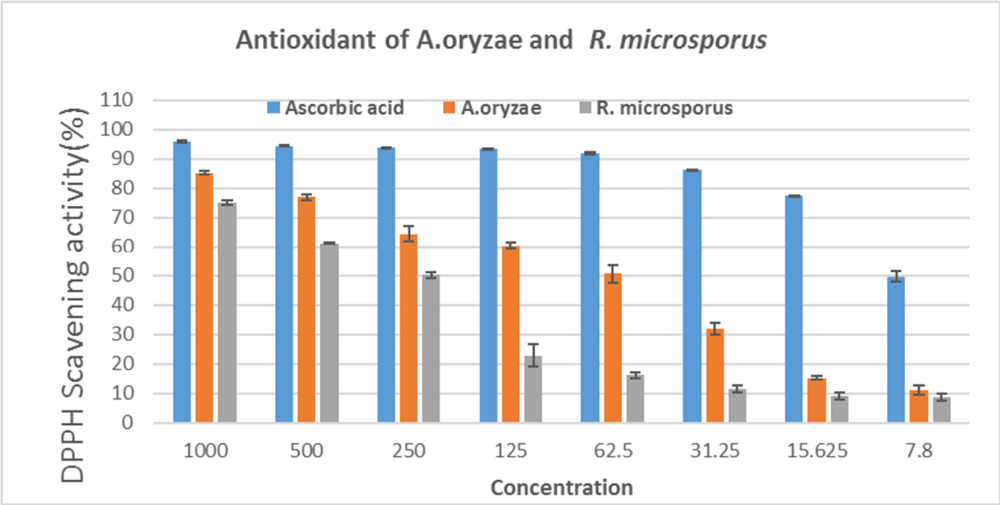

Figure 6. Figure 6. Antioxidant activity of) *A. oryzae* and *R. microsporus*) extracts using DPPH Scavenging activity (%)

Effect of Ao and Rm on juveniles under greenhouse conditions

Achieved results in Figure 7 indicated that, the application of tested treatments *R. microsporus* and *A. oryzae* individual and combination in nematode-infested eggplant seedlings significantly decreased all nematode parameters. These effects varied according to the different treatments. The most influential treatment-reducing nematode parameters was the combination between *R. microsporus* and *A. oryzae* recording 68.78%, 86.98%, 95.23%, 80%, and 80.35% reduction in 2nd juveniles, galls, females, egg masses and developmental stages. While *R. microsporus* recorded the second order in reducing 2nd juveniles, galls and developmental stages as 59.18%, 86.3%, and 50.92% respectively. When it comes to fewer females and egg masses, *A. oryzae* recorded the second order at 90.47 and 70%, respectively.

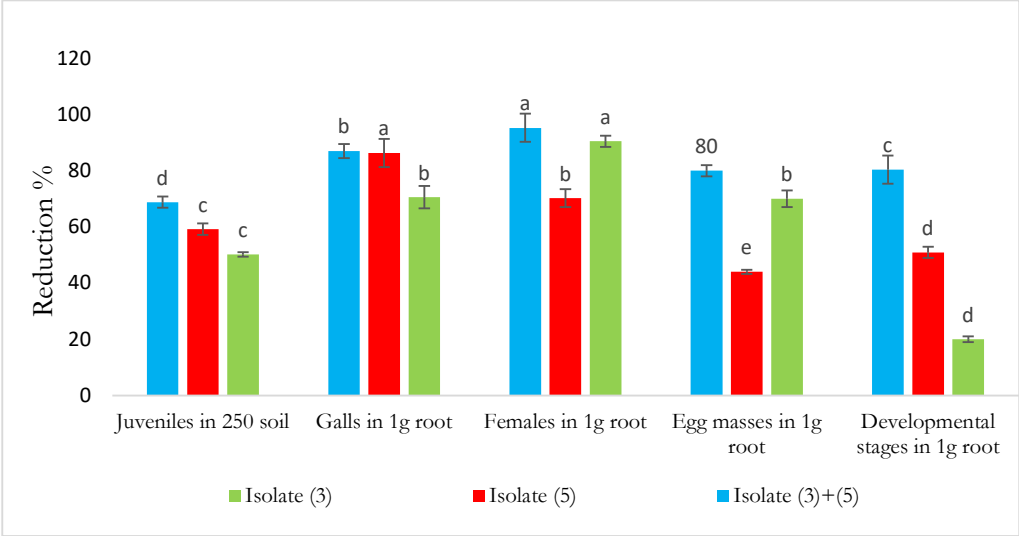

Figure 7. Effect of tested fungal isolates on nematode parameters in eggplant seedlings infested with *M. incognita* under greenhouse conditions

Effect of A. oryzae and R. microsporus on photosynthetic pigments

The experimental findings revealed a notable reduction in the levels of chlorophyll a and b within infected plants by *M. incognita*, as shown in Figure 8. Lower contents of carotenoids were observed in plants infected with M. incognita compared to the carotenoid levels detected in healthy control plants. In contrast, it was observed that in healthy plants subjected to the application of either *A. oryzae* or *R. microsporus*, or a combination a notable enhancing in comparison to the healthy control plants was detected. Uninoculated plants subjected to the application of both *A. oryzae* and *R. microsporus*, either individually or in combination, exhibited notable enhancements in the levels of chlorophyll (a) and (b), as well as carotenoid content (Figure 8). Furthermore, it has been observed that plants infected with *M. incognita* and subsequently treated with either *A. oryzae* or *R. microsporus* individually, as well as a combination of both, exhibit a substantial enhancing in the levels of chlorophyll (a) and (b) as well as carotenoid content when compared to plants solely infected with *M. incognita*. In terms of enhancing the contents of chlorophyll (a) and (b) as well as carotenoid, the treatment involving a combination of *A. oryzae* and *R. microsporus* exhibited the most potent effect. This effect was observed to be significantly greater when compared to the individual treatments involving *A. oryzae* and *R. microsporus*, as depicted in Figure 8.

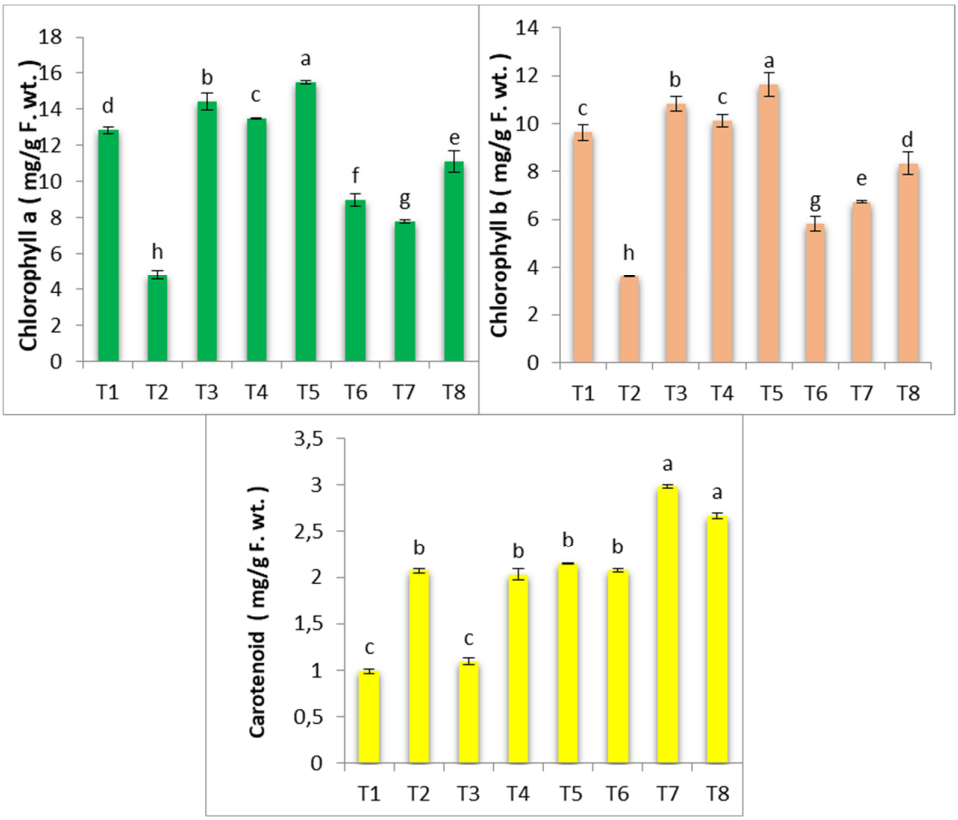

Figure 8. Effect of PGPF on pigments T1- Healthy control, T2- Infected Control, T3- H + *R. microsporus*, T4- H + *A. oryzae*, T5-H + (*A. oryzae* and *R. microsporus*), T6- I + *R. microsporus*, T7- I + *A. oryzae* and T8-I + (*A. oryzae* and *R. microsporus*). Data presented as means ± SD (n=3). Data LSD test at P ≤ 0.05

A. oryzae and R. microsporus' impact on the level of free proline and phenol

Data generated in Figures 9 and 10 showed that *M. incognita*, caused a marked significant increase in total phenols as well as free proline of the infected plants. Also, contents of total phenols as well as free proline

in *M. incognita* - infected plants were increased due to tested inducers (either individual or combination). Concerning the effect of (*A. oryzae* or *R. microsporus*) treatments on the challenged plants with *M. incognita*, it was found that (*R. microsporus*) show a significant increase in total phenols related to combination of (*A. oryzae* and *R. microsporus*) and came next (*A. oryzae*), respectively (Figure 9). Regarding the effect of (*A. oryzae* or *R. microsporus*) treatments on the challenged plants with *M. incognita*, it was found that combination of (*A. oryzae* and *R. microsporus*) show significant increase in free proline related to (*R. microsporus*) and came next (*A. oryzae*), respectively (Figure 10).

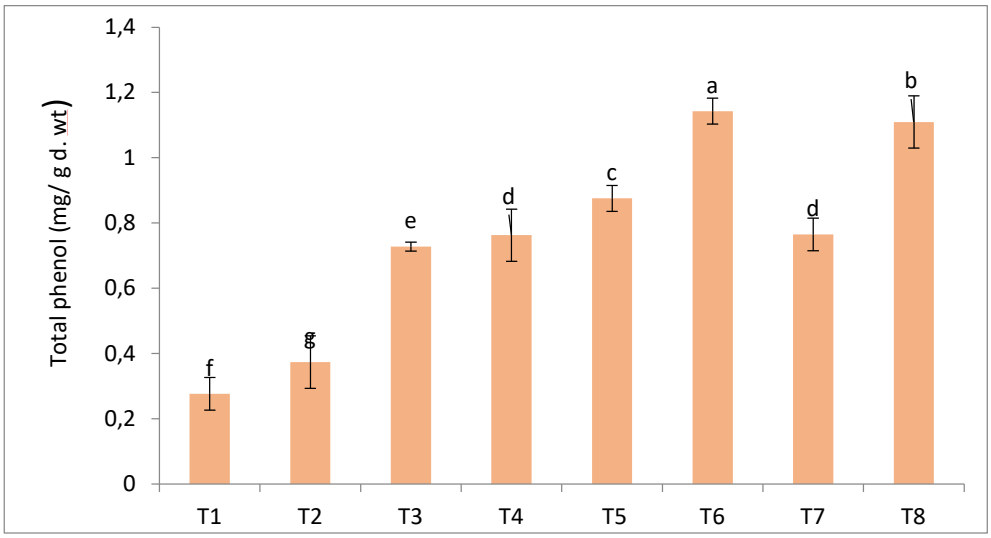

Figure 9. Effect of PGPF on total phenol T1- Healthy control, T2- Infected Control, T3- H + *R. microsporus*, T4- H + *A. oryzae*, T5-H + (*A. oryzae* and *R. microsporus*), T6- I + *R. microsporus*, T7- I + *A. oryzae* and T8-I + (*A. oryzae* and *R. microsporus*). Data presented as means ± SD (n=3). Data LSD test at P ≤ 0.05.

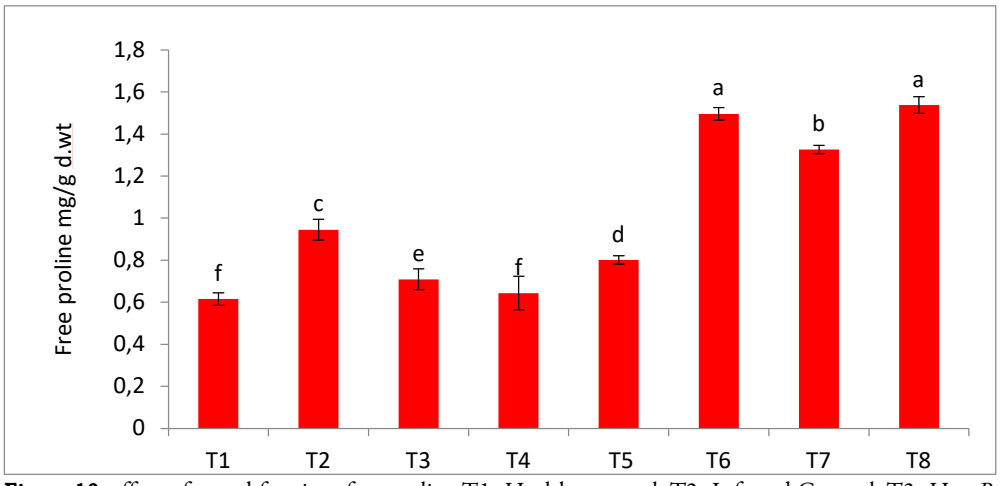

Figure 10. effect of tested fungi on free proline T1- Healthy control, T2- Infected Control, T3- H + *R. microsporus*, T4- H + *A. oryzae*, T5-H + (*A. oryzae* and *R. microsporus*), T6- I + *R. microsporus*, T7- I + *A. oryzae* and T8-I + (*A. oryzae* and *R. microsporus*). Data presented as means ± SD (n=3). Data LSD test at P ≤ 0.05.

Discussion

In the current data appeared that PGPF (F1-F6) were collected from eggplant cultivated soil; all PGPF were screened according to their potency to supply plant promoting substances including HCN, IAA, and Siderophores. The capacity of the six PGPF to produce HCN varied. According to the findings, isolates F5 (+++) and F2 and F3 (++) were the best at producing HCN. Previous study has shown that hydrogen cyanide is very harmful to biotic plant pathogens, indicating that it can be used as a highly effective chemical substance for combating plant diseases (Mishra *et al*., 2020a). In this context, several studies highlight the significance of naturally generating hydrogen cyanide (a very powerful approach to combat diseases) particularly from fungus, as a safe and environmentally acceptable alternative to chemically synthesized pesticides. because it is a very powerful approach to combat diseases (Elnahal *et al*., 2022; Samada and Tambunan, 2020). HCN is a compound that participates in many biological processes and has antifungal activity in addition to its effective role in inducing plant resistance against pathogens (Meena *et al*., 2020; Costa *et al*., 2022; Dimkić *et al*., 2022). HCN plays an effective role in inhibiting the respiration process in the cytochromes of pathogen cells (Ramette *et al*., 2003; Schippers *et al*., 1990).

IAA contributes to the main processes of development and division of cells, especially the elongation of shoot and root, which leads to the improvement of plants (Bhalerao *et al*., 2002; Li *et al*., 2018; Sehrawat *et al*., 2022). The present results revealed all isolates (F1, F2, F3, F4, F5, and F6) can produce IAA. Also, data showed that F3 was the best isolate to give IAA (+++) followed by F5 and F2 (++), then F1 and F4. Heavily studies proved that the organisms that stimulate plant growth are characterized by their ability to produce indole, which makes us explain the role of these organisms in improving plant growth *(Jasim et al., 2014; Kapadia et al*., 2022; McSteen, 2010; Scagliola *et al*., 2016; Singh, 2014). Plant cells directly depend on iron for growth and development, while some microbes produce siderophores, low-molecular-weight iron-chelating molecules (Duca *et al*., 2014; Swarnalatha *et al*., 2022). Based on the present data it appears that all isolates have the efficiency to produce siderophores except for F6. The best isolates were F5 (+++), F3 and F4 (++). Studies confirm the need for plants for phosphorous for improving the plant resistance against plant biotic stresses (Enebe and Babalola, 2018; Dowarah *et al*., 2021; Abd Alhakim *et al*., 2022).

RKN damages roots of worldwide crops leading to poor yield and huge financial losses annually. Our results exhibited increasing in juvenile mortality by increasing the concentration and application period of fungal isolates. The maximum mortality was observed where the application of F3 recorded (74.2% and 64.38%) in 2nd juveniles' death at concentrations (100% and 50%, respectively), after 96 hours of application. Additionally, the use of F5 led to the second-highest death percentages ever observed, with high mortality rates in nematode juveniles of 60.35 and 47.53% at concentrations of 100% and 50%, respectively, after 96 hours of management. Studies have shown that many fungi's culture filtrates have nematode-killing properties because they produce toxic compounds that impact nematode survival and hatching (Devi and Bora, 2018; Wa *et al*., 2021; Baazeem *et al*., 2022). Another mechanism is some other fungi produce volatile organic compounds and enzymes that can attack nematode cuticles (Ahmad *et al*., 2021; Deng *et al*., 2022; Diyapoglu *et al*., 2022). Finally, the different responses of the fungal isolates in our study could be due to the different nature of nematotoxins secreted by each of these isolates (Benttoumi *et al*., 2020; Kim, 2022; Sikder *et al*., 2022).

The GC-MS analysis provides a representative spectral output for each compound found in the analyzed samples. As a result, in recent years, GC-MS has gained widespread acceptance as a significant technological platform for describing bioactive molecules in both plant and non-plant species (Ms and Pushpa, 2017). The major compounds in the fungal extracts are belonging to fatty acid, fatty acid esters, tetrahydro furans, and sterols including diisooctyl phthalate, octadecanoic acid, methyl ester, docosane, hexadecanoic acid, methyl ester, isochiapin B, oleic acid, 9-octadecenoic acid, methyl ester, azulen-2-ol, 1,4-dimethyl-7-(1-methylethyl), Di isooctyl phthalate, benzofuran, 6-ethenyl-4,5,6,7-tetrahydro-3,6-dimethyl-5-isopropenyl, and hexadecanoic

acid, methyl ester heptacosane. Different studies reported that these compounds were extracted from microbial metabolite (Hussein *et al*., 2016; El-Fayoumy *et al*., 2021).

Antioxidants are capable of containing and balancing the free radicals that cause a variety of plant diseases (Pham-Huy *et al*., 2008; Engwa, 2018; Mishra *et al*., 2020b). The antioxidant activity of Rm and Ao extracts at various concentrations (1000-7.81 g/mL) was specified using DPPH method. The current data showed that Ao has maximum antioxidant activity among other fungal isolates. Moreover, IC_{50} of Ao was 145 µg/mL compared to Ascorbic acid (7.8 µg/mL) Also, IC_{50} of Rm was 350 µg/mL. Previous studies confirmed the potential antioxidant activity of fungal strains (Photolo *et al*., 2020; Punia *et al*., 2020; Ibrahim *et al*., 2021).

Our results indicated that, the most influential treatment reducing nematode parameters was the combination between Ao and Rm recording 68.78%, 86.98%, 95.23%, 80%, and 80.35% reduction in 2nd juveniles, galls, females, egg masses and developmental stages, respectively. Nematophagous fungi attack nematodes through different mechanisms; They can be classified according to their mode of action against nematodes in four groups: (a) toxin-producing fungi (de Freitas Soares *et al*., 2018); (b) nematode-trapping fungi (Zhang *et al*., 2020); (c) opportunistic or ovicidal fungi (de Freitas Soares *et al*., 2018); and (d) endozoic or endoparasitic fungi (Lawal *et al*., 2022). Fungi can attack nematode eggs, capture the second-stage juveniles and colonize females bodies of *Meloidogyne* spp. (Soliman *et al*., 2021). As it was demonstrated that the mortality of *M. incognita* ^{2nd} juveniles normally increases with increasing treatment concentration as well as application period, the ability of PGPF to reduce nematode numbers at the in vitro level. The most effective PGPF were identified morphologically and genetically as *Aspergillus oryzae* and *Rhizopus microsporus* and deposited in NCBI with accession numbers OQ291572.1 and OQ291571.1. Moreover, *in-vivo* results confirmed that application of isolates Ao and Rm individual and combination on nematode infested eggplant significantly decreased numbers of galls, egg masses and females. Finally, we recommend using of PGPF isolates Ao and Rm singly or combination as a promising biological nematicides agents a safe alternative to chemical pesticides in agricultural field.

Conclusions

The current study attempted to isolate fungi that enhance plant growth from Rhizosphere eggplant soil. The in vitro studies demonstrated the six fungi's capacity to produce siderophores, IAA, and HCN. Both *Aspergillus oryzae* OQ291572.1 and *Rhizopus microsporus* OQ291571.1 were shown to be the most efficient PGPF. several effective bioactive compounds with nematicidal activity were detected by GC-MS. Furthermore, the administration of *A. oryzae* and *R. microsporus*, both separately and together, dramatically reduced the quantities of galls, egg masses and females on nematode-infested eggplant, according to in-vivo results. Furthermore, the mortality of M. incognita 2nd juveniles increases typically with increasing treatment concentration as well as application period, which further supported the in vitro results that PGPF might reduce nematode numbers. Additionally, through the application of PGPF, chlorophyll, free proline, and phenol contents of nematode-infected plants' were improved. From these favorable findings we recommend employing these isolates as an efficient and secure nematicide rather than chemically produced nematocides.

Authors' Contributions

Conceptualization, A. M. A., M. H. S., and M. S. A. Methodology, A. M. A., M. H. S., A. H. H., M. M. N., A. H. H., M. S. O., A. E. T., M. M. A., Y. R. E., A. Y. M, and M. S. A. Software, A. M. A and M. S. A..; Formal analysis, A. M. A., G. E. D, and M. S. A. Investigation, A. M. A., M. H. S., M. M. N., A. A. Al., H.A.E. A.Y.M, and M. S. A. Resources, A.M.A., and M.S.A.; Data Curation, A.M.A., M. M. N., G.E.D, and M.S.A.;

Writing original draft preparation, A. M. A., M. H. S., M. M. N., and M. S. A.; Writing Review and Editing, A. Y. M, A. M. A., M. H. S., M. M. N., A. H. H., A. A. Al., H. A. E. G. E. D, and M. S. A..; Supervision, A. M. A. A.Y.M, and M. S.A.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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