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Research Article

Biocontrol of *Fusarium* wilt disease in pepper plant by plant growth promoting *Penicillium expansum* and *Trichoderma harzianum*

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Abstract

Plant growth promoting fungi (PGPF) were employed in the present study to biocontrol Fusarium wilt disease in pepper plants. Two of the five fungal isolates were chosen based on biochemical characteristics such as their production of hydrocyanic acid, siderophores, and IAA, phosphate solubilization, and in vitro antifungal activities. The most potent fungal isolates were identified as *Penicillium expansum* (*P. expansum*) and *Trichoderma harzianum* (*T. harzianum*). Using GC-MS, it was found that PGPF extracts contain compounds with antifungal activity, antioxidants, and plant growth stimulators. The combined effect of *T. harzianum* and *P. expansum* increased the protection against fusarial wilt by (76.74%), followed by *T. harzianum* by (50%), then *P. expansum* by (17.64%). Significant improvement because of using the mixture (*T. harzianum* and *P. expansum*) showed an increase in shoot length (59.4%), root length (129%), and number of leaves (52.6%). Chlorophyll A and B levels in infected plants were consistently raised by 28.71% and 67.58%, respectively; as a result of application the mixture (*T. harzianum* and *P. expansum*). Also, there was an increase in soluble proteins and carbohydrates in infected plants treated with (*T. harzianum*) by 25.42% and 31.78% over untreated infected plants, respectively. It could be recommended that the use of targeted PGPF strains, especially a mixture of *T. harzianum* and *P. expansum* could be commercially used as therapeutic nutrients against Fusarium wilt of pepper plants.

Keywords: *Fusarium*; biochemical defense; *Trichoderma*; *Penicillium*; primary metabolites; POD; PPO

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Introduction

Fusarium is a soil-born fungus and one of the most dangerous pathogens that causes vascular wilt in many fruit and vegetable crops leading to annual severe economic losses (Cardoso *et al*., 2007; Attia *et al*., 2016). The pathogen *Fusarium oxysporum* significantly inhibits pepper plant's growth and alters its physiological characteristics, which consequentially results in a significant loss in yield quantity and quality (Abdelaziz *et al*., 2021; Abdelaziz *et al*., 2022; Abdelaziz *et al*., 2023). The fungus infects plant's root, and once it is inside, it blocks vessels that cuts off water and nutrients transport (Li *et al*., 2022). The enzymes secreted by *F. oxysporum* may work to decompose the inner wall of the carrier vessels consisting of cellulose and pectin (Recorbet *et al*., 2003), so these materials act as plugs that close the carrier vessels and impede the rise of water (Castillejo *et al*., 2015). *Fusarium* also secretes mycotoxins (fusaric acid), that damage the carrier vessels (Doohan and Zhou, 2017; El-Batal *et al*., 2023). Thus, it is important to control *Fusarium oxysporum*. Because of ecological restrictions correlated to the usage of synthetic chemicals, the use of biological control has been encouraged (Attia *et al*., 2022; Hashem *et al*., 2023). Biological control depends on the use of microorganisms to significantly reduce growth of pathogenic parasites, especially those living in soil to reduce their harmful effect (Attia *et al*., 2022; Daigham *et al*., 2023).

Pepper is one of the extremely vital vegetables in the world including Egypt. It is extensively cultured in different periods throughout the year in open fields and under glasshouse conditions (Olatunji and Afolayan 2018). Pepper has great health properties, as it has antibacterial, anti-cancer and analgesic activity and it involves in controlling blood sugar, and reducing fat and cholesterol (Pundir *et al*., 2016). It riches in the antioxidants flavonoids, lutein, carotenoids and cryptoxanthin which protect the body from infections prevent diseases and reduce the incidence of heart disease (Azlan *et al*., 2022). These bioactive metabolites responsible for defending themselves against various stress factors that can be activated by external spraying with biotic and abiotic stimuli (Attia *et al*., 2016). In this regard, many reports have proven the effectiveness of microorganisms in inducing plant resistance against diseases and improving growth and productivity (Abdelaziz *et al*., 2022; Abdelaziz *et al*., 2023). Plant Growth Promoter Fungi (PGPF) is being used by researchers to boost plant defense and immunity as well as promote mineral uptake from the soil (Badawy *et al*., 2021). Microorganisms obtained from normal plant rhizosphers are useful not only against pathogens, but also as a biofertilizer agent (Abdelaziz *et al*., 2023).Use of PGPR provides multiple potential pathways toward fugal soil borne disorders such as the generation of fungi harmful compounds as Siderophores creation (Daigham *et al*., 2023), HCN, fungal cell wall damaging enzymes, concurrence for vital nutrients as space, conflict as break down the morphology of mycelium, reducing the mycelia development and break down fungal cell walls, plant growth promotion by plant growth regulators as IAA, auxins, cytokines, riboflavin and vitamins (Abdelaziz *et al*., 2023). In the present study, PGPF's capabilities to counteract *Fusarium* (*F. oxysporum*) and to produce plant growth-stimulating biochemicals were evaluated. The novelty of this study is the use of soil plant growth promoting fungi (PGPF) that stimulate plant growth and able to inhibit the harmful effects of *F. oxysporum* wilt by improving plant resistance and supporting immune responses.

Materials and Methods

Isolation and identification of PGPF

Soil samples were collected from the botanical garden of the Faculty of Science, Al-Azhar University Soil samples (Latitude: 30° 03' 15.48" N., longitude; 31° 19' 12.75" E.). The soil samples were collected from different sites at a depth of 5-20 cm, and all samples were mixed before being collected in clean plastic bags). One gram was placed directly on sterilized solidified potato dextrose agar media (PDA) (Sigma Aldrich,

Germany) accompanied with chloramphenicol (0.2 mg/L) (with three replicates). The cultivated Petri dishes were incubated for 5-7 days at 28 ± 2 °C (Abd Alhakim *et al*., 2022). PGPF isolates were identified depending on their macroscopic and microscopic features including colony surface color, and texture. Colony growth rate and pigmentation were also examined according to manual keys (Castle *et al.*, 1998; Gams and Bissett, 2002; Cardoso *et al*., 2007). Then culture characteristics were investigated using Nikon stereo-zoom binocular microscope. A light microscope was used for studying x20 and x40 objectives lens (Khalil *et al*., 2015). The internal transcribed spacer (ITS) allowed for molecular identification. The generated sequence was submitted to 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 11 software.

Biochemical traits

PGPF isolates ability to create hydrocyanic acid (HCN) was checked by Trivedi *et al*. (2008). HCN production is indicated by the appearance of a light brown to dark brown colour. The efficacy of (IAA) production is done using the colorimetric technique (Leveau and Lindow, 2005). Using the $FeCl_3$ test, fungal isolates were evaluated qualitatively for the presence of siderophores in their culture filtrates according to Attia *et al*. (2023). The capability of isolates to solubilize phosphate was tested by the method described by (Rezzonico *et al*., 2007).

Bioactive compounds extraction

Potato dextrose broth (PDB) (Sigma Aldrich, Germany) was used for grown PGPF isolates at 27 °C ± 2 for 21 days under static conditions. Then fungal filtrates were extracted using ethyl acetate (EtOAc) (1:1). The (EtOAc) layer was removed from the aqueous level using a separating funnel. Then was evaporated at 40 – 45 °C using a rotary evaporator (Heidolph VV2001, Germany) (Sharaf *et al*., 2022).

(GC–MS) investigation

According to Passari *et al*. (2017), Hashem *et al*. (2023). Metabolites of fungal extract were examined, enumerated, and identified using GC-MS. When compared to known chemicals spectrum kept in the WILEY 09 (Wiley, New York, NY, USA) and NIST 11 libraries, the identified components' spectra were identified. Additionally, the chemical formula, molecular weight, and name of any compounds found were identified.

Source of the fungal pathogen

F. oxysporum RCMB (008 001) was obtained RCMB, Al-Azhar University, then it was recognized through a pathogenicity test according to Hibar *et al*. (2007). The inoculum was made using the method of Büttner *et al*. (2004).

Antifungal activity in vitro

Antifungal activity of PGPF was carried out using agar well diffusion method, PDA plates were inoculated with 100 µL of the FI (1.5×10^7 CFU/mL) and dispersed on the prepared media's surface using a clean cotton swab. 8 mm diameter Agar cups were cut from the pre-inoculated plates using a sterilized corkborer. The antifungal control utilized for the fungi was fluconazole at 25 µg/mL (Bioanalyse, Ankara, Turkey); 100 microliters of the 5 PGPF crude extracts at 1 mg/mL were added to the agar cup. To allow for diffusion, all plates were held at 4 °C for a period of two hours. The plates were then incubated for 3 days at 28 ◦C. The sizes of the zones of inhibition were measured and noted after incubation (Khattab *et al*., 2022).

In vivo experiment (greenhouse experiment)

Experimental design

Pepper (*Capsicum annuum* L. 'Reda F1 hybrid') seedlings planted in plastic pots (25 cm width and 20 cm length), each pot contains 10 seedlings, and three biological replicates were performed. The pots were kept in the greenhouse at a temperature of 22 °C during the daylight hours and 18 °C at the nighttime, with a relative humidity of 70-85%. The capability of PGPF to stimulate biochemical resistance against *Fusarium* infection in pepper plants was assessed using plastic pots (25 × 20 cm). After planting of seedlings in the pots, seedlings were infected with *F. oxysporum* (10^7 spores mL), with the exception of the healthy control pot *(Abdelaziz et al*., 2022). PGPF isolates were applied through foliar application one week before *Fusarium* infection. Seedlings were planted in 8 groups as following: (T1) Healthy, (T2) Infected, (T3) Healthy + *T. harzianum*, (T4) Healthy + *P. expansum*, (T5) Healthy + *(P. expansum* + *T. harzianum*), (T6) Infected + *T. harzianum*, (T7) Infected + *P. expansum*, (T8) Infected + (*P. expansum* + *T. harzianum*). After 45 days following sowing, morphological and biochemical signals from plant samples were analyzed, and the progression of the disease was measured, with the purpose of evaluating plant resistance.

Disease index and protection

Sowing method was used for infection, then after 45 d the disease symptoms were after observed. Score consisting of five classes, as described in (Attia *et al*., 2016), the disease index and plant protection were evaluated. Protection percentage was calculated according to the following equation:

$$\text{Protection percentage \%} = \frac{A-B}{A} \text{ X100}$$

A = PDI in infected control plants

B = PDI in infected plants treated with (*PGPF*).

Determination of pigments

To determine photosynthetic pigments in fresh pepper new leaves using the method described by (Cohen-Bazire *et al*., 1966) , chlorophyll a, chlorophyll b, and carotenoids were extracted, and the green color was assessed spectrophotometrically at 665, 649, and 470 nm.

Determination of total soluble carbohydrates and proteins

The amount of soluble sugar that was present in the dried shoot was determined using the method described by (Attia *et al*., 2021), and the blue-green color that was produced was evaluated using a wavelength of 620 nm. On the other hand, total soluble proteins were determined using Folin's reagent according to method used by Lowry *et al*. (1951). The content of MDA in fresh tomato leaf was measured according to (Hu *et al*., 2004). The H_2O_2 content of fresh tomato leaf was measured as stated by (Mukherjee and Choudhuri 1983).

Isozyme electrophoresis

Peroxidase (POD) and polyphenol oxidase (PPO) isozymes were determined Trivedi *et al.* (2008), while polyphenol oxidase (PPO) isozymes were recorded using the method (Knegt and Bruinsma, 1973).

Statistical analysis

The pilot results were evaluated using a one-way analysis of variance. While mean differences were determined using the least significant difference (L.S.D.) at a level of probability of 5.0% with the help of Co State software.

Results

Screening of PGPF isolates according to promoting properties

Current results showed that all PGPF isolates produce IAA especially our F3 which produce IAA (227.36 µg/mL) followed by F5 (129.8 µg/mL), then F1, F2, and F4 (Table 1). all fungal strains could solubilize tricalcium phosphate and make it bioavailable especially F3 which gave (533.47 µg/mL) followed by F5 (504.86 µg/mL) (Table 1). The capacity of the five PGPF to produce HCN and siderophores and solubilize phosphates varied as in (Table 1). According to biochemical characterization of our 5 isolates, the best isolates were F5 (+++) and F3 (++) (Table 1). The best isolates to produce HCN were F5 and F2 (+++), followed by isolate F3 (++).

Table 1. Biochemical characterization of tested PGPF ability of PGPF to produce IAA, siderophores, and HCN production and to solubilize phosphate

PGPF	IAA µg/mL	Phosphate solubilization µg/mL	Siderophores	HCN
F1	42.20 ± 0.21^{d}	443.17± 0.07^{d}	+	+
F2	118.66 ± 0.12^{c}	456.54± 0.05^{c}	+++	+++
F3	227.36 ± 1.65^{a}	533.47±0.14 ^{a}	++	++
F4	13.66 ± 0.22 ^{c}	346.64± 0.07 ^{c}	++	+
F5	129.8 ± 0.13^{b}	504.86 ± 0.11^{b}	+++	+++

Data presented as means ± SD (n=3). Data LSD test at P ≤ 0.05. Different letters represent significant differences according to one way-ANOVA. Siderophores – The development of yellow to orange halo around the colonies was noted as positive for siderophore production. (+): light yellow, (++) dark yellow, and (+++) orange. HCN - HCN synthesis was indicated by the change in colour from light brown to dark brown. (+): light brown, (++) brown, and (+++) dark brown.

Identification of the most potent PGPF

According to the biochemical characterization (Table 1), the most potent PGPFs, were F3 and F5. Thus, we identified both isolates by morphological and molecular methods. F3 was identified as *P. expansum* Its fungal colonies on PDA after 3 days at 28 °C, showed low severely velutinous, mycelium white, conidia in modest numbers, cloudy green, reverse pale at the margins. Conidiophores were borne from subsurface hyphae, and mono verticillate phialides ampulliform. Furthermore, F3 isolate was confirmed molecularly as *P. expansum* and then deposited in NCBI under accession number ON678165.1 (Figure 1). Regarding the second isolate, F5 was morphologically identified as *T. harzianum* Fungal colonies after 7 days, at 28 °C on PDA showed whitish green mycelia, floccose, dense, white Smooth surface, orange reverse colony, branched conidiophore, form loose tufts which arise in ring zone, color Moderate, ellipsoidal conidia shape smooth and sub-globose. F5 isolate was also molecularly confirmed as *T. harzianum* and then it was deposited in NCBI under accession number ON678151.1 (Figure 1).

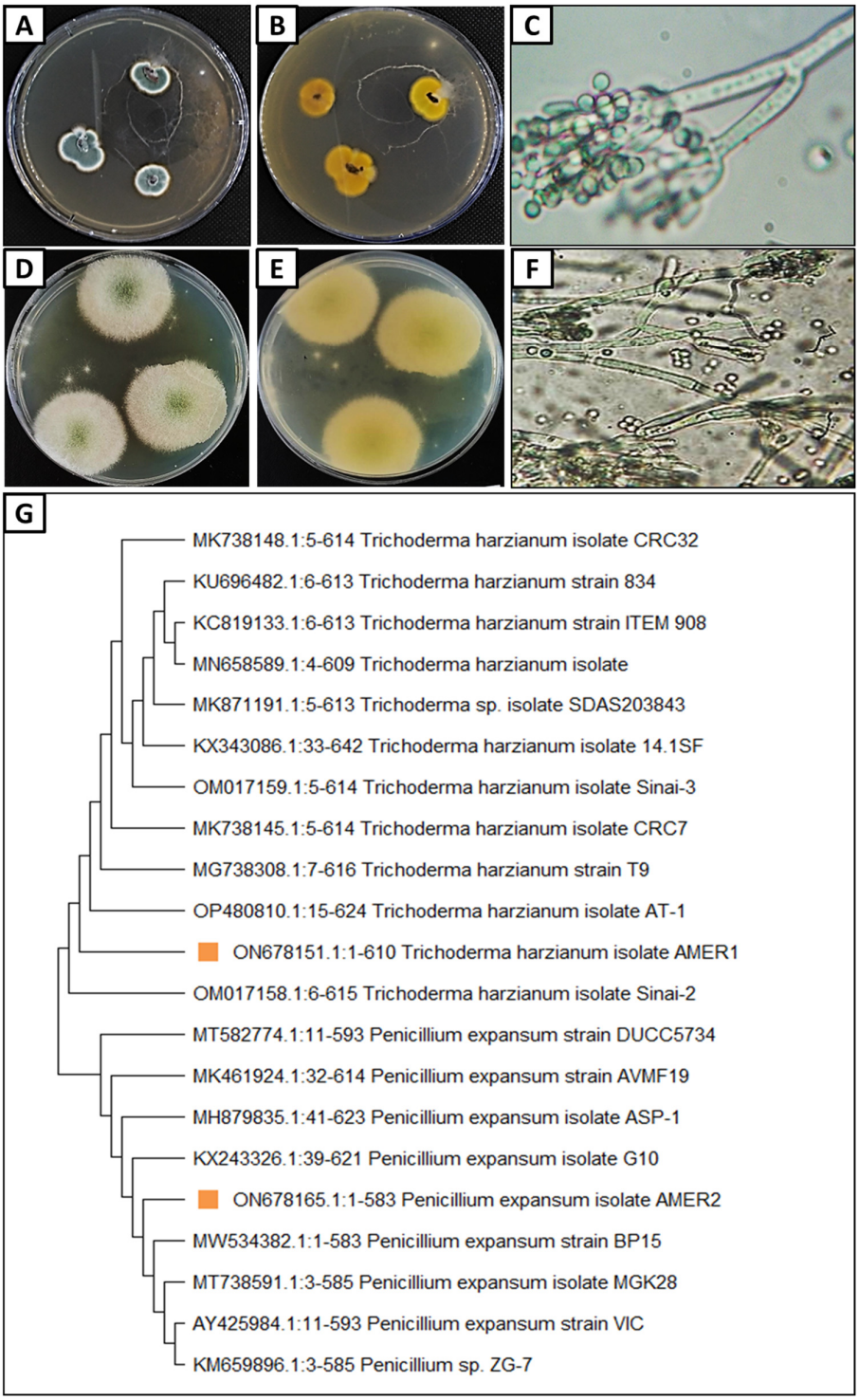

Figure 1. Characters of *P. expansum* (A-C); A= Surface colony, B= Reverse colony, C= Conidiophores, and conidia (a light microscope was used for the study using a magnification power 800 x), and characters of *T. harzianum* (D-F); D= Surface colony, E= Reverse colony, and F= Conidiophores, and conidia (a light microscope was used for the study using a magnification power 800 x), and G= phylogenetic tree of both *P. expansum* and *T. harzianum*.

GC–MS analysis

Our results showed that the average yield of the extract is about 11 and 10 compounds respectively (Figure 2, Table 2). The recognized complexes were grouped into 3 collections as the following; major (representing up to 10 % including 9-octadecenoic acid, 9-octadecenoic acid, methyl ester, and n-Hexadecanoic acid), minor (between 10% and 1% including hexadecanoic acid, methyl ester, 9,12.octadecadienoic acid (z,z)-, methyl ester, 10-octadecenoic acid, methyl ester and 13-docosenoic acid, methyl ester) and trace mixtures (fewer than 1%). The fusarial activity of PGPF extracts may be related to the major and minor components (Table 2).

Table 2. *T. harzianum* and *P. expansum* GC-MS results

No.	Compound name	RT (min)	Peak area % T1	Peak area % T2	Activity	References
1	Hexadecanoic acid, methyl ester	26.39	5.74	6.89	Antioxidant and antimicrobial.	(Albergon *et al*., 1980)
2	n-Hexadecanoic acid	28.06	10.48	10.25	Antioxidant and pesticide	(Krishnamoorthy and Subramaniam, 2014)
3	9,12-Octadecadienoic acid (z,z)-, methyl ester	29.50	2.84	3.25	Antihistaminic and hypocholesterolemic	(Ganesh and Mohankumar, 2017)
4	9-Octadecenoic acid, methyl ester	29.63	14.83	13.19	Antimicrobial	(Hussein *et al*., 2016)
5	10-Octadecenoic acid, methyl ester	29.74	2.39	2.30	Nematicides, insectifuge, and antieczemic,	(Hashem *et al*., 2022)
6	Octadecanoic acid methyl ester	30.09	2.55	2.48	Antimicrobial, anticancer, and diuretic	(Hussein *et al*., 2016)
7	9-Octadecenoic acid	31.21	50.70	21.30	Antibacterial and antifungal	(Krishnamoorthy and Subramaniam, 2014)
8	Octadecanoic acid	31.52	6.31	23.87	Antioxidant and antimicrobial	(Kumari *et al*., 2019)
9	Oleic acid	33.03	0.55	6.71	Anti-inflammatory and Preservative	(Sreekumar *et al*., 2014)
10	13-Docosenoic acid, methyl ester	36.22	1.89	2.58	Anticancer	(Paudel and Pant, 2017)
11	Dodecanoic acid	47.81	1.72	-	Antimicrobial	(Özçelik *et al*., 2005)

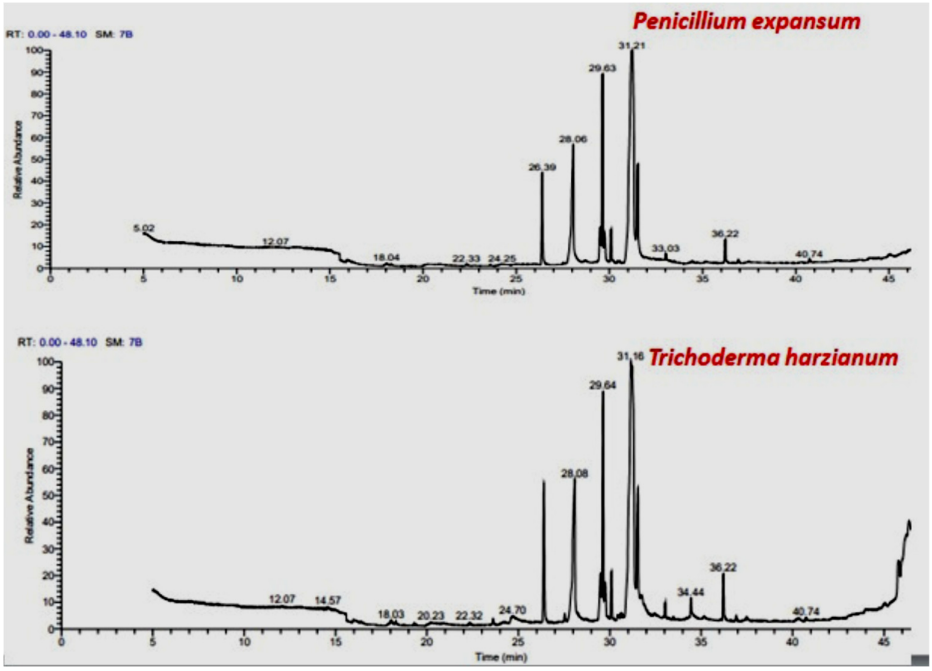

Figure 2. GC–MS chromatogram of *T. harzianum* and *P. expansum*

Antifungal activity of PGPF

F. oxysporum induces vascular wilt disease with severe damage to the pepper plant. Thus, antifungal activity of the five isolated PGPF were assayed against *F. oxysporum* (Table 3, Figure 3). The presented data indicate the isolates *T. harzianum* and *P. expansum* have the largest activity with inhibition zone 18.6 and 21.6 mm respectively.

Table 3. Antifungal activity of PGPF isolates

PGPF code	Diameter of inhibition zone (mm)
F1	16.1 ± 0.6^{d}
F2	17.6 ± 0.3^{c}
F3	18.6 ± 0.6^{b}
F4	13 ± 0.5^{e}
F5	21.6 ± 0.8^{a}
Control (DMSO)	0

Key: F3: *P. expansum*, F5: *T. harzianum*, F1 = Isolate 1 ; F2=Isolate 2;F3=Isolate 3;F4=Isolate 4 and F5=Isolate 5

Effect of PGPF treatments on disease index of infected pepper plants

Pepper plants that were exposed to *F. oxysporum* experienced typical wilt symptoms with DI of 85% (Table 4). When (*T. harzianum* and *P. expansum*) filtrate was applied, plants were significantly more resistant to the damaging effects of *F. oxysporum* wilt than untreated pepper plants. The treatment with (*T. harzianum* and *P. expansum*) showed lowest disease index and highest protection against *F. oxysporum* by (20% and 76.74%), followed by *T. harzianum* by (42.5% and 50%), then *P. expansum* by (70% and 17.64%).

Table 4. Effect of (*T. harzianum* and *P. expansum*) on disease index and protection

Treatment	Disease symptoms Classes					DI (disease index) (%)	Protection (%)
	0	1	2	3	4		
Control Infected	0	0	2	2	6	85	0
Infected + *T. harzianum*	3	2	2	1	2	42.5	50
Infected + *P. expansum*	0	2	3	0	5	70	17.64
Infected + *T. harzianum* and *P. expansum*	6	2	0	2	0	20	76.74

PGPF stimulated growth and biochemical defense system pepper plants

Morphological parameters

Evidence of pathological infestation showed highly significant decreases in all morphological parameters. There were significant decreases in shoot length, root length, and the number of leaves of infected plants by 36.11%, 70.01%, and 47.53% when being compared with control healthy plants, respectively (Figure 3). On the other hand, the efficiency of (*T. harzianum* and *P. expansum*) strains has been exploited to reduce the adverse effects either through the ability to inhibit fungus growth or to induce plant immunity. It is evident that growth measurements were significantly affected by (*T. harzianum* and *P. expansum*) (Figure 3). On the other hand, the application of *T. harzianum* and *P. expansum* successfully recovered the loss of shoot length (59.4%), root length (129%) and the number of leaves (52.6 %) of infected plants in comparison to the infected control plants.

Photosynthetic pigments

The levels of chlorophyll a and b in plants infected with *F. oxysporum* were dramatically lowered by 32.59% and 38.89%, respectively, according to the results shown in Figure 4. In contrast, compared to the healthy control, the infection increased the carotenoid levels in the pepper plants. However, as compared to infected control plants, the application of PGPF (*T. harzianum* and *P. expansum*) filtrate dramatically boosted the levels of carotenoids in infected plants. The results showed that the most efficient way to raise the levels of chlorophyll a and b in healthy plants by 28.71% and 67.58%, respectively, was to apply (*T. harzianum* and *P. expansum*) Like this, it was discovered that applying (*T. harzianum* and *P. expansum*) was the most efficient way to increase the levels of carotenoids (96.87%) and chlorophyll a and b (25.54% and 59.54%, respectively) in infected plants.

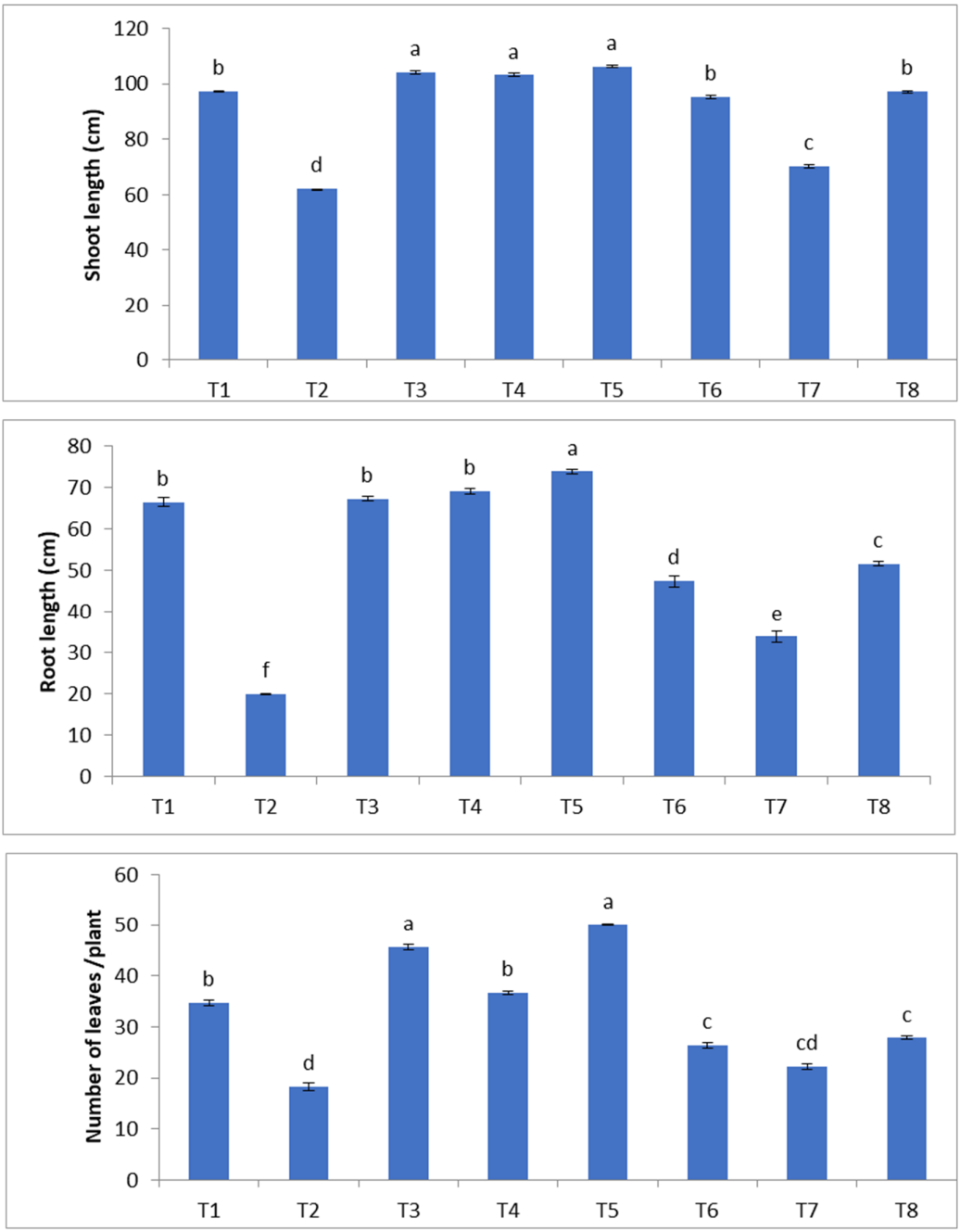

Figure 3. Effect of PGPF on morphological criteria of *fusarial* infected pepper plant. T1 = Healthy control; T2 = Infected control; T3 = Healthy + *T. harzianum*; T4 = Healthy + *P. expansum*; T5 = Healthy + *(T. harzianum* and *P. expansum)*; T6 = Infected + *T. harzianum*); T7 = Infected + *P. expansum*; T8 = Infected + *(T. harzianum* and *P. expansum).* Data presented as means ± SD (n=3). Data LSD test at $P \leq 0.05$.

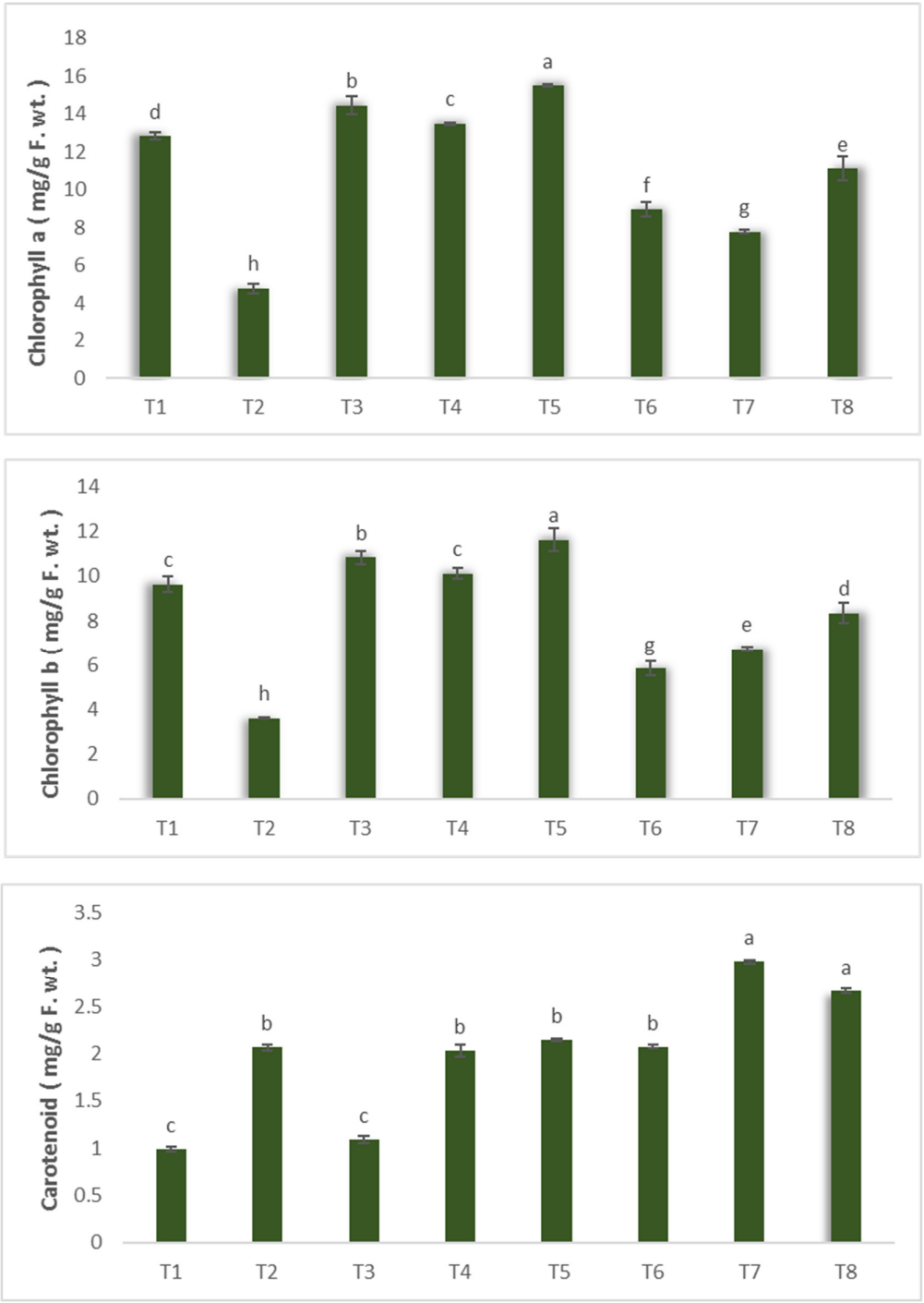

Figure 4. Effect of PGPF on chlorophyll a, chlorophyll b, and carotenoid of pepper plants. T1 = Healthy control; T2 = Infected control; T3 = Healthy + *T. harzianum*; T4 = Healthy + *P. expansum*; T5 = Healthy + *(T. harzianum* and *P. expansum)*; T6 = Infected + *T. harzianum*); T7 = Infected + *P. expansum*; T8 = Infected + *(T. harzianum* and *P. expansum).* Data presented as means ± SD (n=3). Data LSD test at P ≤ 0.05.

Metabolic responses

The carbohydrate content of the plant is greatly affected by the fungal infection, as is the case with the treatments used to control plant disease. infected plants exhibited failure in soluble proteins and sugars by 32.61% and 35.83%. In comparison to infected control, the use of *T. harzianum* and *P. expansum* alone or in combination enhanced the quantity of soluble carbohydrates and proteins in infected pepper plants (Figure 5). The maximum increase of proteins in infected pepper plants was detected in *T. harzianum* by 23.90 but the utmost recovery increase of soluble carbohydrates was perceived in the mixture of *T. harzianum* and *P. expansum* by 31.7% over Fusarium-infected plants. Application PGPF (*T. harzianum* and *P. expansum*) separately or in combination on healthy plants increased the levels of soluble carbohydrates and proteins compared to healthy control plants (Figure 5).

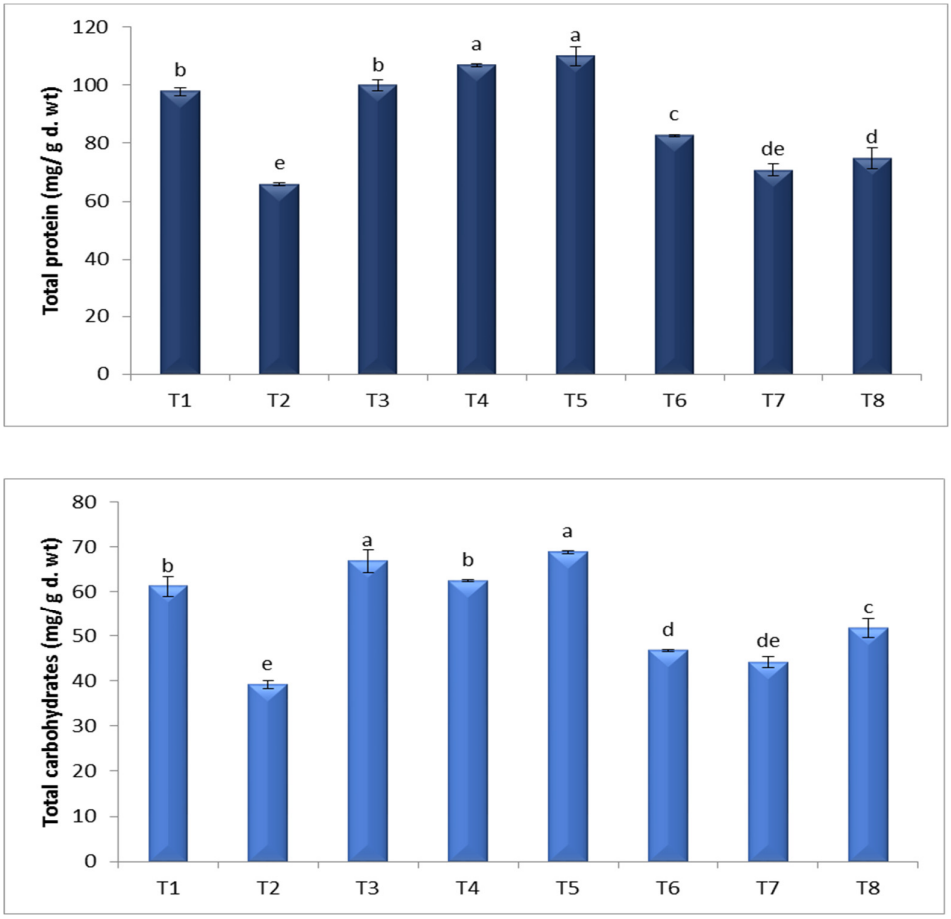

Figure 5. Effect of PGPF on total protein and carbohydrates of pepper plants. T1 = Healthy control; T2 = Infected control; T3 = Healthy + *T. harzianum*; T4 = Healthy + *P. expansum*; T5 = Healthy + *(T. harzianum* and *P. expansum)*; T6 = Infected + *T. harzianum*); T7 = Infected + *P. expansum*; T8 = Infected + *(T. harzianum* and *P. expansum).* Data presented as means ± SD (n=3). Data LSD test at $P \leq 0.05$.

Stress biomarkers

Pepper plants grown under fusarial infection had higher levels of MDA and H_2O_2 compared to healthy controls (Figure 6). Application of PGPF (*T. harzianum* and *P. expansum*) individual or combination to infected plants played a pivotal role in minimizing the level of MDA and H_2O_2 compared with the plants

exposed to infected only (Figure 6). The maximum decrease of of MDA in infected pepper plants was detected in *P. expansum* by (33.1%), while the maximum decline of H_2O_2 in infected pepper plants was detected in combination of (*T. harzianum* and *P. expansum*) by (38.6%). Under normal conditions pepper plants treated with PGPF (*T. harzianum* and *P. expansum*) individual or combination showed decreasing in the level of MDA and H_2O_2 respectively, as compared with that of untreated control.

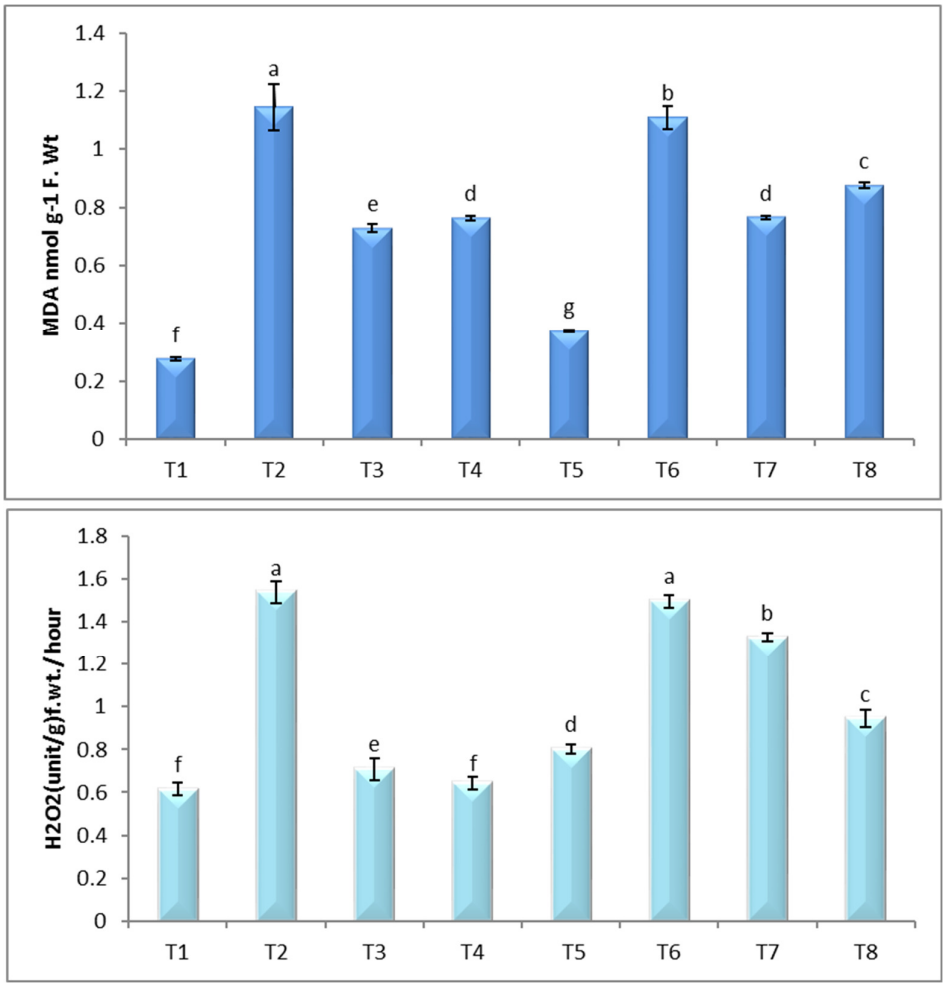

Figure 6. Effect of PGPF on MDA and H_2O_2 of pepper plants. T1 = Healthy control; T2 = Infected control; T3 = Healthy + *T. harzianum*; T4 = Healthy + *P. expansum*; T5 = Healthy + *(T. harzianum* and *P. expansum)*; T6 = Infected + *T. harzianum*); T7 = Infected + *P. expansum*; T8 = Infected + *(T. harzianum* and *P. expansum).* Data presented as means ± SD (n=3). Data LSD test at $P \leq 0.05$.

Antioxidant isozymes

Five POD isozymes were seen on native PAGE in (Figure 6) at Rf (0.1, 0.5, 0.6, 0.7, and 0.8). The POD in infected plants displayed five bands, including one intermediate band and four substantially dense bands. The least amount of POD was expressed in healthy control plants. Application of tested PGPF individually or combined on infected pepper plants induced the level of POD isozymes. The highest increase in POD expression was achieved using a mixture of *T. harzianum* and *P. expansum* which noted (Figure 7). PPO isozymes of pepper plant presented 5 PPO bands at Rf (0.1, 0.5, 0.6, 0.7, and 0. 8) (Figure 7). Untreated infected plants were highly overexpressed PPO isozymes revealing 5 bands including one moderate and 4 greatly dense

bands when compared to control healthy pepper plants that expressed 3 pale bands. The maximum increase of PPO was expressed in response to the use of a mixture of *T. harzianum* and *P. expansum* that recorded 3 highly dense bands and 2 moderate bands.

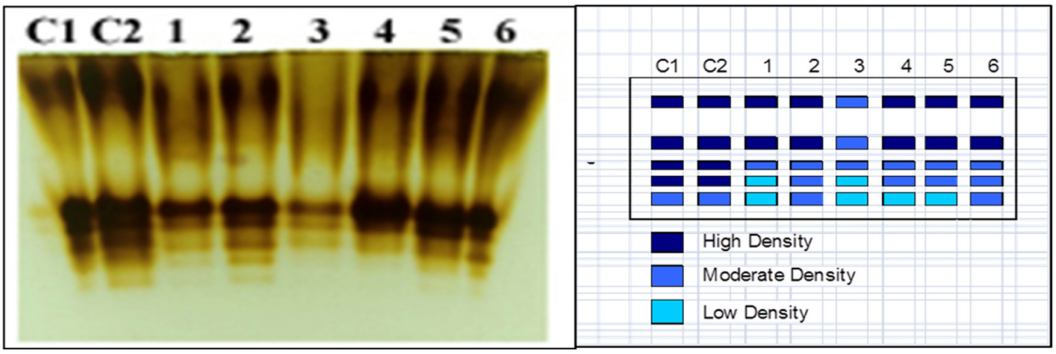

Figure 7. shows the impact of PGPF on the infected pepper plants on POD isozymes. C1= Healthy control; C2 = Infected control; 1 = Healthy + *T. harzianum*; 2 = Healthy + *P. expansum*; 3 = Healthy + *(T. harzianum* and *P. expansum)*; 4 = Infected + *T. harzianum*); 5 = Infected + *P. expansum*; 6 = Infected + *(T. harzianum* and *P. expansum).*

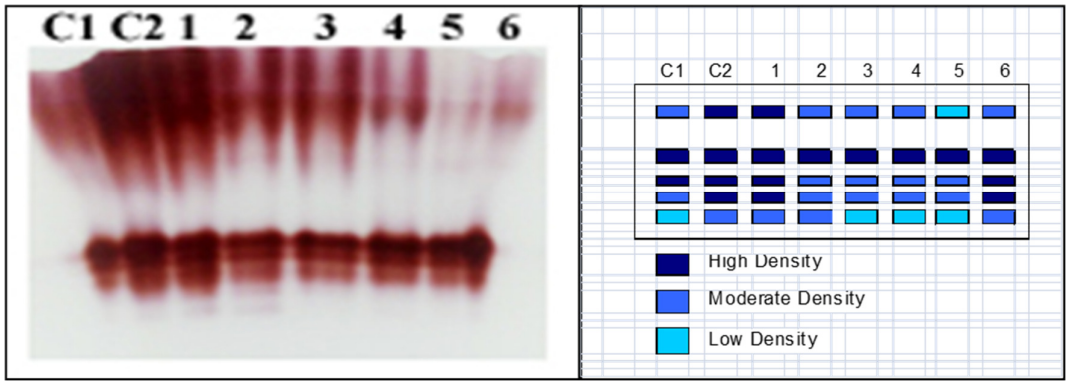

Figure 8. shows the impact of PGPF on the infected pepper plants on PPO isozymes. C1= Healthy control; C2 = Infected control; 1 = Healthy + *T. harzianum*; 2 = Healthy + *P. expansum*; 3 = Healthy + *(T. harzianum* and *P. expansum)*; 4 = Infected + *T. harzianum*); 5 = Infected + *P. expansum*; 6 = Infected + *(T. harzianum* and *P. expansum).*

Discussion

Scientists focused on reducing the risk of plant diseases by using biotic and abiotic inducers to stimulate plant physiological immunity and pathogen resistance (Eastburn *et al*., 2011; Elbasuney *et al.*, 2022). Reducing disease symptoms and severity of infection is strong and clear evidence of resistance to disease. Current results showed that all PGPF isolates produce IAA. In this regard, PGPF's ability to improve plant growth is depending on their capability to produce growth promoting phytohormones such as IAA (McSteen, 2010; Hashem *et al*., 2023). In this study, all fungal strains could solubilize tricalcium phosphate and make it bioavailable especially F3 which gave (533.47 µg/mL) followed by F5 (504.86 µg/mL). Additionally, our fungal isolates could produce siderophores Siderophore are synthesize low-molecular-weight iron-chelating molecules (Duca *et al*., 2014), where iron plays a direct role in plant proliferation and development. Previous studies also confirm that fungi ability to dissolve the organophosphates will play a role in promoting plant health (Walpola and Yoon 2012;

Igiehon and Babalola, 2017) and in promoting plant growth (Jangir *et al*., 2018). HCN has been established as a biological resistance agent, due to its severe injuriousness to pathogens. In the current study, PGPF (F1-F5) were screened according to their potency to produce IAA phytohormone is regulating the key processes of cell development and division, especially shoot and root prolongation ant it is improving all plant vigor (Sehrawat *et al*., 2022). The capacity of the five PGPF to produce HCN and siderophores and solubilize phosphates varied as in (Table 1). According to biochemical characterization of our 5 isolates, the best isolates were F5 (+++) and F3 (++) (Table 1). The best isolates to produce HCN were F5 and F2 (+++), followed by isolate F3 (++). In this regard, many findings show the significance of fungi Which produces siderophores and HCN in resisting pathogens as safe and eco-friendly alternatives (Samada and Tambunan, 2020). HCN is a molecule that is involved in a variety of biological processes and, in addition to being highly successful in making plants resistant to diseases, it possesses antifungal properties (Ramette *et al*., 2003).

Our findings supported a prior study that isolated *T. harzianum* and *P. expansum* from cultivated soils, whether they were cultivated organically or conventionally (Khalil *et al*., 2015). Overall, we hypothesized that *T. harzianum* and *P. expansum* have a significant role in enhancing soil qualities and boosting the yield of crops.

In the line with our results, many scientific reported that these mixtures were present in the metabolite PGPF (Kaul *et al*., 2012). The presence of the fatty acid octadecanoic acid has an effective effect on controlling *Fusarium* fungus because it can damages *Fusarium* DNA (Khan and Javaid, 2022). Additionally, the presence of antioxidants such hexadecanoic acid and hexadecanoic acid methyl ester can aid pepper plants recover from the negative effects of FI by enhancing plant resilience and the activity of the antioxidant enzymes that activate it.

The presented data indicate the isolates *T. harzianum* and *P. expansum* have the largest activity with inhibition zone 18.6 and 21.6 mm respectively. According to our hypothesis, the active substances HCN, 9-octadecenoic acid, methyl ester, octadecanoic acid, and dodecanoic acid were what provide extracts of *T. harzianum* and *P. expansum* their antifungal properties (Küçük and Kivanç, 2004; El-Hasan *et al*., 2009; Garrigues *et al*., 2018; Gandía *et al*., 2020). *F. oxysporum* was susceptible to *T. harzianum* treatment through mycoparasitism activity. In addition to its antibiosis and competition for nutrients and space, *T. harzianum* has additional ability to penetrating and lysing the Fusarial mycelium (Yassin *et al*., 2021).

The first guide to governing the occurrence of resistance in plants against the pathogen is a Disease Index (Roux *et al*., 2014; Attia *et al*., 2022). Pepper plants that were exposed to fusarial infection experienced typical wilt symptoms with DI of 85% This study shows that applying both (*T. harzianum* and *P. expansum*) causes resistance which reduced the disease severity percentage and provided a high protection against the pathogen infection (Farrag *et al*., 2017). In this regard, *T. harzianum* and *P. expansum* can inhibit the growth of the *F. oxysporum* mycellium directly through antibiosis or by entirely lysing the macro-, microconidia, and chlamidospores of the *F. oxysporum* (Eke *et al*., 2021). Evidence of pathological infestation showed highly significant decreases in all morphological parameters. These results are consistent with many studies (Herrera-Téllez *et al*., 2019; Al-Surhanee, 2022), where *F. oxysporum* can cause vascular obstruction, additionally, loss of typical physiological roots, such as ion uptake and water uptake (Bishop and Cooper, 1983; Muche and Yemata, 2022; Patra *et al*., 2022; Saad *et al*., 2023). The severe deficiency in the morphological parameters as a result of the fungal infection may be also explained by the oxidative explosion in the cells and accumulation of reactive oxygen species (ROS), causing growth hormone disorders (Abd Alhakim *et al*., 2022; Elbasuney *et al*., 2022). On the other hand, the efficiency of (*T. harzianum* and *P. expansum*) strains has been exploited to reduce the adverse effects either through the ability to inhibit fungus growth or to induce plant immunity. It is evident that growth measurements were significantly affected by (*T. harzianum* and *P. expansum*). On the other hand, the application of *T. harzianum* and *P. expansum* successfully recovered the loss of shoot length (59.4%), root length (129%) and the number of leaves (52.6%) of infected plants in comparison to the infected control plants. In this context, Th is one of the promising organisms as a biostimulant that is not only an anti-

pathogen but is rich in essential amino acids and antioxidants (Areeshi, 2022; Rakkammal *et al*., 2022). The production of IAA, siderophore, hydrogen cyanide, and a high ability to dissolve phosphates by *T. harzianum* and *P. expansum*, significantly contributed to the resistance against the *F. oxysporum* (Attia *et al*., 2022). *T. harzianum* and *P. expansum* are also known for their ability to improve supply plant with the nutrients as (N_2, P, K) necessary to carry out the vital processes (Martínez-Medina *et al*., 2014).

Reduced photosynthesis was caused by the plants' diminished capacity to absorb sunlight as a result of the drop in chlorophyll levels. The increase in ROS after the *F. oxysporum* may be partially to blame for the decrease in chlorophyll levels. However, as compared to infected control plants, the application of PGPF (*T. harzianum* and *P. expansum*) filtrate dramatically boosted the levels of carotenoids in infected plants. The application of *T. harzianum* to infected plants enhanced their morphological features, photosynthetic pigments, total phenols, and antioxidant enzyme activities, according to data from a study by (Abdelaziz *et al*., 2022). The improved carotenoids brought about by greater ROS scavenging may have offered protection to the photosynthetic process (Sampath-Wiley *et al*., 2008). The increase in the protein content in the plant, or the presence of pathogenic-related proteins, is considered one of the most important indicators of the severity of the disease (Attia *et al*., 2022). The carbohydrate content of the plant is greatly affected by the fungal infection, as is the case with the treatments used to control plant disease. This is due to the direct relationship between the effect of the pathogenic fungus on the content of the pigments and, consequently, how this affects the content of carbohydrates (Attia *et al*., 2022). In order to capture free radicals, protect cells from oxidation, and provide plant cells with energy, the buildup of (proteins and carbohydrates) is a crucial biological activity (Gill and Tuteja, 2010; Nicolás *et al*., 2014; Abdelaziz *et al*., 2022). The aforementioned increases in contents of total protein and carbohydrates are in a correlation with reduction in contents of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2)(Chattha, Hassan *et al*., 2022).

POD isozyme activity break down H_2O_2 to H_2O (Rios-Gonzalez *et al*., 2002). Isozymes are one of the key control mechanisms for cellular protection against versus infection (Harb *et al*., 2010; Akladious *et al*., 2019; El-Fawy *et al*., 2021). Induction of these isozymes plays a vital role in cellular defense against oxidative stress (El-Beltagi *et al*., 2010; Contreras-Zentella *et al*., 2022; Zulfiqar and Ashraf, 2022).

Conclusions

The objective of the current study was to isolate plant-growth-promoting fungi from Rhizosphere soil and to characterize their ability to synthesis HCN, IAA, and siderophores, to solubilize phosphate. The results indicated that *T. harzianum* and *P. expansum* have antifungal activity against *F. oxysporum* through create fungicidal chemicals such HCN. Mover, an in vivo study confirmed that *T. harzianum* and *P. expansum* can lessen the harmful effects of Fusarium wilt on pepper plants. The treatment with (*T. harzianum* and *P. expansum*) showed lowest disease index and highest protection against *F. oxysporum* by 20% and 76.74%. In general, all growth parameters improved as protein, carbohydrate, and antioxidant intake increased. Thus, the mixture of *T. harzianum* and *P. expansum* could be applied as safe, effective bio fungicide and therapeutic nutrients.

Authors' Contributions

Conceptualization, A. M. A., M. H. S., and M. S. A. Methodology, A. M. A., M. H. S., M. M. N., A. H. H., F. A. M., M. N. A., M. A. Z. and M. S. A. Software, A. M. A K.A. A. and M. S. A..; Formal analysis, A. M. A., and M. S. A. Investigation, A. M. A., M. H. S., M. M. N., A.A.Al., H.A.E. and M. S. A. Resources, A.M.A.

and M.S.A.; Data Curation, A.M.A., M. M. N., 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. M. A., K.A. A., M. H. S., M. M. N., A.H.H., A.A.Al., H.A.E. and M. S. A..; Supervision, A. M. A. 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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