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Research article

Trichoderma as a biocontrol agent for damping-off and its impact on cucumber biochemical alterations

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Abstract
Cucumber plants are vulnerable to infection by soil borne fungal diseases. Fungicides control cucumber diseases but pose health risks from pesticide residues. Native biocontrol agent strains suppressing plant pathogens while promoting plant growth and maintaining soil health. This study aimed to isolate and identify indigenous *Trichoderma* species and evaluate their efficacy as cucumber seed dressing for controlling damping-off caused by *Rhizoctonia solani*, *Fusarium solani*, *Macrophomina phaseolina*, and *Agroathelia rolfsii*. *Trichoderma afroharzianum* demonstrated the highest linear growth inhibition (81.3%) against *M. phaseolina*, while *Trichoderma asperellum* showed 75.0% inhibition against *F. solani*. Seed dressing with these biocontrol agents have significantly reduced damping-off incidence, increased plant survival, and enhanced the accumulation of phenolic compounds, including total phenols. It has also boosted peroxidase, polyphenoloxidase, and carboxymethyl cellulase activities. Furthermore, *T. afroharzianum* improved chlorophyll and carotenoid levels, promoting photosynthesis and disease resistance. The two *Trichoderma* species exhibited potential as novel biocontrol agents with antifungal and plant growth-promoting properties.

Keywords
T. afroharzianum, *T. asperellum*, defense-related enzymes, seed dressing

Introduction

Cucumber (*Cucumis sativus* L.) belongs to the family Cucurbitaceae, is one of the most common vegetables worldwide (Kaur and Sharma, 2021). Egypt uses cucumbers for both local consumption and exportation. Cucumber contains a high nutritional value (Szalay, 2017). In Egypt, cucumbers were produced under open field conditions and were recently considered as one of the main greenhouse-cultivated vegetables

Many soil borne fungal diseases, such as *Pythium* spp., *Fusarium solani* (Mart.) Sacc, *Rhizoctonia solani* Kühn, *Agroathelia rolfsii* Sacc Redhead & S-T. Mullineux, *Macrophomina phaseolina* (Tassi) Goid, and the fungal-like *Phytophthora* spp., can infect cucumber plants and cause economic losses, including damping-off, root rot, and wilt (Mahdy et al., 2011; Mohamed and Hasan, 2018; Mohammed, 2023). Both *R. solani* and *F. solani* have a wide host range and are capable of

resisting extreme environmental conditions and causing damping-off in greenhouses and open fields (Al-Fadhal et al., 2019). *Macrophomina phaseolina* is a non-specialized fungus, attacking more than 500 host species in more than 100 families throughout the world (Purkayastha et al., 2006) causing seedling death and root-rot of cucumber plants (El-Mougy et al., 2012; Khaleel et al., 2020). Soil borne diseases are among the most dangerous diseases that may cause losses in production and large economic costs.

Different strategies can be employed to control these diseases, such as the use of biocontrol agents, chemicals that induce resistance, fungicides, and plant extracts. Biological control of plant pathogens has become important in plant disease management. The long-term benefits of *Trichoderma* in integrated pest management systems could lead to a shift towards more sustainable agricultural practices. Therefore, the isolation and evaluation of new isolates are needed.

Trichoderma afroharzianum has demonstrated strong biocontrol potential against *Fusarium culmorum*, a key pathogen responsible for fusarium crown rot and head blight in wheat. Both in vitro and in vivo studies revealed their capacity to suppress mycelial growth and decrease disease severity, resulting in higher yields in wheat cultivars (Bouanaka et al., 2021). *Trichoderma afroharzianum* has shown great promise as a biocontrol agent challenges remain regarding its application in diverse agricultural settings, particularly concerning the potential for resistance development in target pathogens. Further research is required to optimize its use in various crops and conditions. *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg can act as a biofertilizer, promoting nutrient availability and plant growth under stress conditions (Asghar et al., 2024). Moreover, it effectively suppresses plant pathogens like *Agroathelia rolfsii*, demonstrating its potential in disease management (Sutthisa et al., 2024). This species exhibited significant growth promotion in tomato plants, enhancing resistance to soil borne diseases (Sehim et al., 2023). This study aimed to evaluate the efficacy of *T. afroharzianum* and *T. asperellum* for the management of cucumber damping-off, both in vitro and in vivo, and their impact on cucumber biochemical changes.

Materials and Methods

Isolation and identification of fungi associated with damping-off symptoms and pathogenicity test

Infected cucumber roots exhibiting damping-off and root rot symptoms were collected from commercial greenhouses in Giza (El-Ayat County) and Bani-Sweif (Bani-Sweif County) governorates, for the high impact of cucumber diseases in the region.

The infected root samples (n = 10) were thoroughly washed with tap water to eliminate any soil particles, then cut into small pieces (5 mm). These pieces were dis-infested by soaking in a 1% sodium hypochlorite solution for 2 min, washed three times with sterilized water, and finally the root tissues were dried between sterilized filter papers and transferred to petri dishes containing potato dextrose agar medium (PDA) (Khaleel et al., 2020; Mohammed, 2023). The identification was carried out based on their morphological characteristics according to the standard descriptions by Booth (1971) and Domsch et al. (1980). The identified fungi were subsequently transferred to slants containing PDA medium and stored at 5 ± 1 °C as stock cultures. The pathogenicity test was conducted under greenhouse conditions. A clay-sand mixture (1:1 w:w) was sterilized with 5% formalin, covered for 15 days, and left for an additional week for formalin evaporation. The disinfected soil was inoculated with the fungal inoculum at 3%. Fungal isolates were cultured individually on a sand-barley medium (1:3 w:w) for 15 days at 25 ± 2 °C (Abdel-Kader et al., 2012). Pots contained sterilized soil amended

with the same amount of autoclaved sand-barley medium only served as negative control (n. Control). The pots were watered for one week before sowing. Then 10 seeds cv. Beit-Alpha (Delta for Agencies and Trade company), per pot, with three replicates per treatment were used. Percentages of pre- and post-emergence damping-off and survived plants were determined at 15, 30 and 45 days after sowing, respectively, using the formulas outlined by Ahmed and Zyton (2016).

Isolation, and identification of the potential antagonistic Trichoderma

Rhizosphere from apparently healthy cucumber plants, grown in a field severely infected with damping-off and root rot, was used to isolate the antagonist microorganisms. The serial dilution technique was employed to isolate native antagonistic *Trichoderma* species (Naveenkumar et al., 2011; Hassan et al., 2021). The fungal cultures of *Trichoderma* spp. were selected, isolated, and purified using the hyphal tip method, and subsequently identified based on cultural and microscopic morphological characteristics (Gilman, 1957; Rifai, 1969; Booth, 1971).

Molecular identification of the potential antagonistic Trichoderma *isolates*

Genomic DNA extraction was carried out using the Gene JET Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific Baltics, UAB, Lithuania), following the manufacturer's instructions. PCR amplification of the extracted DNA was performed in a 50 µL reaction mixture (White et al., 1990). The amplification was carried out on DNA Internal Transcribed Spacer (ITS) region with ITS primers (ITS1F TCCGTAGGTGAACCTGCGG, ITS4R TCCTCCGCTTATTGATATGC; Haque et al., 2020) and on the Translational Elongation Factor 1-α (Tef1-α) with specific primer (TEF1-728F CATCGAGAAGTTCGAGAAGG, TEF1-986R TACTTGAAGGAACCCTTACC; Cai et al., 2022). Specific amplicons were sequenced at Macrogen, Inc., Seoul, Korea (Gupta, 2019).

Phylogenetic Analysis

ITS and TEF1-α sequences of *Trichoderma* strains were individually compared with other *Trichoderma* species and related genera available in the National Center for Biotechnology Information (NCBI) GeneBank (http://www.ncbi.nlm.nih.gov/BLAST). The consensus sequences obtained, were analyzed using the neighbor-joining method and multiple sequence alignments with the Clustal W method in MEGA 11 software. A bootstrap tree was generated with 1000 replicates to represent the evolutionary history of the taxa analyzed. The percentage of taxa clustered together in the bootstrap test is shown next to the branches (Haque et al., 2020; Mazrou et al., 2020; Heflish et al., 2021).

In vitro evaluation of Trichoderma *isolates as potential antagonistic microorganisms*

The antagonistic effect of *Trichoderma* isolates was evaluated against the pathogenic fungi responsible for cucumber damping-off, including *Rhizoctonia solani*, *Fusarium solani*, *Macrophomina phaseolina*, and *Agroathelia rolfsii*, using the dual culture technique on PDA medium (Rahman et al., 2009; Kumari et al., 2024), with slight modifications. A 5 mm mycelial plug of each *Trichoderma* isolate was inoculated into 100 mL flasks, each containing 60 mL of autoclaved liquid gliotoxin fermentation medium (GFM) (Mergawy et al., 2022). The flasks were incubated at 25 ± 2 °C for 10 days. The *Trichoderma* cultures were then blended, and five concentrations were prepared: 1×10^7, 2.5×10^7, 5×10^7, 1×10^8, and 2×10^8 CFU mL^{-1} for each isolate. Each Petri dish (90 mm in diameter) containing PDA medium was inoculated on one side with a 5 mm diameter disk of the desired pathogenic fungus, and a hole (created using a cork borer) on the opposite side was inoculated

with 200 µL of the tested *Trichoderma* concentration. Three replicates were performed for each concentration. Negative control, consisting in pathogen alone was set up in order to estimate the growth without any interference. The percentage of mycelial growth reduction of the pathogenic fungi was calculated using the formula described by Singh et al. (2021) as follows:
Growth inhibition (%) = ((growth of pathogen in control - growth of pathogen in treatment)/growth of pathogen in control) × 100

Effect of the antagonistic Trichoderma *isolates on cucumber damping-off under greenhouse conditions*
The two tested *Trichoderma* isolates were evaluated individually for their efficacy against cucumber damping-off. The isolates were cultured in sterilized liquid gliotoxin fermentation medium (GFM) (Ali et al., 2012). After incubation, the mycelial mat and broth of the *Trichoderma* isolates were blended together. The spore suspension of each isolate was used at three different concentrations: 0.5×10^9, 1×10^9, and 2×10^9 CFU g^{-1} as seeds treatment. Tween 20 (5%) was added to the suspension. Cucumber seeds were sterilized with 3% sodium hypochlorite, for two minutes, followed by three washes with sterilized water and allowed to dry before being treated with 5% Arabic gum (Ali, 2021). The seeds were dipped in the suspension of *Trichoderma* isolate for 1 min, air dried, and finally sown in pots. Treatments consisted of seeds treated with Arabic gum only, sown in soil infested with the tested fungi served as control. Ten seeds sown per pot, three pots were used for each treatment. Pre- and post-emergence damping-off and survival rates of the plants were determined as described previously.

Effect of Trichoderma *isolates on biochemical changes in cucumber cv. beit-alpha.*
Cucumber leaves were collected from plants raised from seeds dipped in high concentrations of the bio control agent, individually, 45 days after sowing, with the same leaf growth stage. Plants raised from seeds dipped in Arabic gum only, grown in infested soil served as the positive control (C + *R. solani*; C + *F. solani*; C + *M. phaseolina* and C + *A. rolfsii*). Plants raised from seeds dipped in Arabic gum only, grown in non-infested soil served as the negative control (n. Control). These leaves were used to evaluate the activity of chlorophyll, carotenoid, phenolic compounds and enzymes.

Determination of free, conjugated, and total Phenols
Total and free phenolic compounds were quantified using the Folin-Ciocalteu reagent method, as outlined by Gomaa et al. (2016). The reaction products were assessed through spectrophotometric analysis at a wavelength of 520 nm, with catechol serving as the standard reference. The measurements were conducted using a UV–VIS spectrophotometer (Spectronic 601, Milton Roy, U.S.A). The conjugated phenols were determined by subtracting the value of free phenols from the total phenols.

Preparation of enzyme extracts and assay methods
Crude enzyme extracts for the assays were prepared according to Aluko and Ogbadu (1986). Enzyme activity was assayed using a Spectronic 601 spectrophotometer.

Peroxidase enzyme assay
Peroxidase (PO) activity was assessed using pyrogallol as a substrate, following the modified method of Chance and Maehly (1955) as described by Falade et al. (2019). Briefly, 0.1 mL of the diluted

extract was mixed with phosphate buffer containing pyrogallol (pH 6.0). After thorough mixing, hydrogen peroxide H_2O_2 (0.5%) was added to the solution. The change in absorbance of the reaction mixture was measured at a wavelength of 420 nm over a period of 10 s to 1 min.

Polyphenol oxidase enzyme assay

Polyphenol oxidase (PPO) activity was measured using the method described by Arnnok et al. (2010). The crude enzyme extract was mixed with phosphate buffer, and the sample was adjusted to zero absorbance. Subsequently, 0.5 mL of 0.01 M catechol in 0.1 M phosphate buffer was added to the mixture, and the final volume was adjusted to 3 ml with distilled water. The change in absorbance of the reaction mixture was measured at 495 nm at 10-second intervals for up to 1 min.

Carboxymethyl cellulase enzyme assay

Carboxymethyl cellulase (CMCase) activity was determined by measuring the release of reducing sugars during the degradation of carboxymethyl cellulose (CMC). Briefly, 0.5 mL of the enzyme supernatant was mixed with 0.5 mL of a 1% CMC solution in acetate buffer. The reaction mixture was then incubated at 50 °C for 30 min to ensure the release of sugars at a constant rate. The reaction was terminated by adding DNS reagent, followed by heating the mixture to develop colour. The absorbance was measured at 540 nm using a spectrophotometer, as described by Miller (1959).

Determination of chlorophyll

The ARNON method, as described by Gu et al. (2016), was employed to determine the chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid content in plant leaves. The absorbance of the chlorophyll extract was recorded at 645 nm, 663 nm, and 470 nm using a UV-VIS spectrophotometer (Spectronic 601).

Statistical analysis

Data collected were analyzed using R Statistical Software (v.4.4.2; R Core Team, 2024). Analysis of variance (ANOVA) was performed and the differences between the mean values of various treatments were compared by Tukey´s multiple range test at $\alpha = 0.05$.

Results

Isolation and identification of fungi associated with damping-off symptoms and pathogenicity test

From rotted cucumber roots numerous fungi were isolated, based on cultural characteristics, growth patterns, and the morphological features of hyphae and spores observed under a microscope it was allowed to identify *R. solani*, *F. solani*, *M. phaseolina*, and *A. rolfsii*. The pathogenicity test of *F. solani* (4 isolates) and *R. solani* (1 isolate), *M. phaseolina* (1 isolate) and *A. rolfsii* (1 isolate) was carried out (Table1). The soil infested with *R. solani* and *A. rolfsii* exhibited the highest rates of pre- and post-emergency damping-off, resulting in a survival rate of 50% for the plants. However, the soil infested by *M. phaseolina* has shown 55% survival rate for the plants. The *F. solani*-1, *F. solani*-3 and *F. solani*-4 isolates have shown low percentages in pre-and post-emergency damping-off (Table1). Meanwhile, *F. solani*-2 isolate did not show any damping-off symptoms. Depending on the obtained results isolates of *R. solani*, *F. solani*-1, *M. phaseolina* and *A. rolfsii* were chosen to carry out the subsequent experiments.

Table 1 –Pathogenicity test of the isolated fungi at 15, 30 and 45 days after sowing (cv. Beit- Alpha cucumber).

The tested fungi	Damping-off (%)			Survived plants (%, 45 d)
	Pre-emergence (15 d)	Post-emergence (30 d)	Total	
R. solani	45[a]	5[b]	50	50
F. solani-1	35[c]	0[e]	35	65
F. solani-2	0[f]	0[e]	0	100
F. solani-3	10[e]	0[e]	10	90
F. solani-4	20[d]	0[e]	20	80
M. phaseolaina	45[a]	0[e]	45	55
A. rolfsii	40[b]	10[a]	50	50
n. control	0[f]	0[e]	0	100

Molecular identification of the potential antagonistic Trichoderma *isolates*

The ITS region of two *Trichoderma* isolates (TAM1 and TAM2), was analyzed and aligned against homologous sequences retrieved from the NCBI database. The sequences of these isolates were submitted to the GenBank database, with accession numbers PQ302270 and PQ303663 for TAM1 and TAM2, respectively. Fungal isolate TAM1 was grouped within a distinct clade with several *Trichoderma* species, including *Trichoderma atrobrunneum* F.B. Rocha, P. Chaverri & Jaklitsch (strain CBS548.92, NR137298, with 99.66% identity), *Trichoderma lentiforme* (Rehm) P. Chaverri, Samuels & F.B. Rocha (strain CBS100542, (NR144868, with 99.63% identity), *T. afroharzianum* (strain CBS124620, NR137304, with 99.62% identity), and *Trichoderma vermifimicola* Jing Z. Sun & X.Z. Liu (strain HMAS248255, NR171951, with 99.66% identity) (Supplementary Fig. S1). In contrast, TAM2 was grouped within a distinct clade with *T. asperellum* (strain CBS433.97, NR130668, with 99.57% identity) (Supplementary Fig. S1). Additionally, sequence analysis of the translation elongation factor 1-alpha (Tef1α) gene for both isolates was performed. The sequences of TAM1 and TAM2 were also deposited in the GenBank database, with accession numbers PQ389509 and PQ389508, respectively. For TAM1, the Tef1α sequence showed 99.67% similarity with *T. afroharzianum* GJS 04-186 (FJ463301) (Supplementary Fig. S2).

In contrast, TAM2 was grouped within a distinct clade, closely related to *T. asperellum* CBS 433.97 and *Trichoderma kunmingense* Z.F. Yu & J.Y. Li (strain YMF1.02659), with a 100% identity with *T. asperellum* CBS 433.97 (Supplementary Fig. S2). These findings suggest that the two isolates, TAM1 and TAM2, occupy different phylogenetic positions within the *Trichoderma* spp. TAM1 was identified as *T. afroharzianum* and TAM2 as *T. asperellum*

In vitro evaluation of Trichoderma *species against pathogenic fungi*

Trichoderma afroharzianum and *T. asperellum* had a significant effect on reducing the linear growth of the *R. solani*, *F. solani*, *M. phaseolina* and *A. rolfsii* (Table 2 and Supplementary Figs S3 and S4). There was a positive relationship between the concentration of both *Trichoderma* isolates and the linear growth inhibition of the four tested fungi. The highest inhibition level was shown for *M. phaseolina*, being 81.3% at 2×10^8 CFU mL^{-1} concentration of *T. afroharzianum*, while being 72.5% at the same concentration of *T. asperellum*. The linear growth inhibition of *F. solani* was 72.5 and 75% at 2×10^8 CFU ml^{-1} of *T. afroharzianum* and *T. asperellum*, respectively. In addition, the growth inhibition of *A. rolfsii* was 69.4 and 66.3% at 2×10^8 CFU mL^{-1} of *T. afroharzianum* and *T.*

asperellum, respectively. Meanwhile, the linear growth of *R. solani* was inhibited by the same level (63.8%) at 2 × 10^8 CFU mL^{-1} for both *Trichoderma* isolates.

Table 2 – Effect of different concentrations of antagonistic *T. afroharzianum* and *T. asperellum* on the linear growth (mm) and inhibition percentage of the tested pathogenic fungi, *in vitro*.

Pathogen	Biocontrol agent Conc. CFU mL^{-1}									
	T. afroharzianum									
	1x10^7		**2.5x10^7**		**5x10^7**		**1x10^8**		**2x10^8**	
	#LG (mm)	***In (%)**	**#LG (mm)**	***In (%)**	**#LG (mm)**	***In (%)**	**#LG (mm)**	***In (%)**	**#LG (mm)**	***In (%)**
R. solani	37.3^{abc}	53.7	34.0^{cde}	57.5	33.0^{cdef}	58.8	32.0^{def}	60.0	29.0^{fgh}	63.8
F. solani	25.0^{hij}	68.8	24.0^{ijk}	70.0	23.0^{ijk}	71.3	23.0^{ijk}	71.3	22.0^{jk}	72.5
M. phasolina	32.0^{def}	60.0	25.7^{ghij}	68.1	23.7^{ijk}	73.8	20.3^{k}	74.6	15.0^{l}	81.3
A. rolfisii	37.3^{abc}	53.1	35.3^{bcd}	55.6	35.0^{cd}	56.3	30.0^{efg}	62.5	24.3^{ijk}	69.4
	T. asperellum									
R. solani	35.0^{cd}	56.3	35.0^{cd}	56.3	34.0^{cde}	57.5	32.0^{def}	60.0	29.0^{fgh}	63.8
F. solani	30.0^{efg}	62.5	26.0^{ghij}	67.5	25.0^{hij}	68.8	25.0^{hij}	68.8	20.0^{k}	75.0
M. phasolina	35.0^{cd}	56.3	30.0^{efg}	62.5	30.0^{efg}	62.5	23.3^{ijk}	70.6	22.0^{jk}	72.5
A. rolfisii	40.0^{a}	50.0	39.7^{ab}	50.5	35.0^{cd}	56.3	30.0^{efg}	62.5	27.0^{ghi}	66.3

Mean of three replicates; LG = Linear Growth
* In (Inhibition%) = [(control-treatment)/control] × 100
Means followed by different letter (s) are significantly different according to Tukey's multiple range test at α = 0.05

Effect of the two Trichoderma *isolates against damping-off under greenhouse conditions*

Data presented in Tables 3 and 4 reveal that there was significant reduction in the incidence of damping-off with considerable increase in the average of survived plants when cucumber seeds (cv. Beit-Alpha) were treated with *T. afroharzianum* and *T. asperellum* at three different concentrations (0.5 × 10^9, 1 × 10^9, 2 × 10^9 CFU g^{-1} seed) compared to control treatment. As the applied dose used increased, the percentages of damping-off were decreased, while the number of surviving plants increased. In addition, *F. solani* was the highly affected by both biocontrol agents (BCA) and *M. phaseolina* was the least affected one.

Data presented in Table 3 revealed that, cucumber seeds treated with *T. afroharzianum* at a concentration of 2 × 10^9 CFU g^{-1} exhibited a significant reduction in the incidence of total damping-off in pots infested with *F. solani* vs control (10.0% vs 43.4%), *R. solani* (16.7% vs 53.3%), and *A. rolfsii* (26.7% vs 56.7%), respectively with noticeable increase in the percentages of survived plants, being 33.4, 36.6 and 30%, respectively. Meanwhile, pots infested with *M. phaseolina* recorded 40.0% total damping-off, with 3.4% increase in the percentages of surviving plants. In addition, cucumber seeds treated with *T. afroharzianum* recorded percentages of post-emergence damping-off lower than the percentages of pre-emergence damping-off.

Results presented in Table 4 show that cucumber seeds treated with *T. asperellum* at the concentration of 2 × 10^9 CFU g^{-1} exhibited a significant reduction in the incidence of damping-off in pots infested with *F. solani* vs control (10.0 % vs 43.3%), and *R. solani* vs control (16.7% vs 53.3%) with increasing the percentages of surviving plants, being 33.3% and 36.6%, respectively. Meanwhile, the percent of total damping-off in pots infested with *M. phaseolina* vs control (33.3 % vs 43.3), and *A. rolfsii* vs control (33.3 % vs 56.7%) with increase in the percentages of survived plants, being 10.0 and 23.4% respectively. In addition, cucumber seeds treated with *T. asperellum* recorded percent of the post-emergence damping-off lower than the percentages of pre-emergence damping-off.

Effect of seed treatments with biocontrol agents (BCA) on some enzymes and phenolic compounds in leaves of cucumber plants grown in soil infested with the causals of damping-off

Data in Fig. 1 show the effect of cucumber seed dressing with bio-agents (*T. afroharzianum* and *T. asperellum*) then planted in soil infested with the tested pathogenic fungi individually, on the phenolic compounds content (total, free and conjugate). The impact of using a high concentration (2×10^9 CFU g^{-1}) of biocontol agents on the accumulation of total phenols was clear in plants treated with *T. afroharzianum*, grown in soil infested with *F. solani* and *A. rolfsii* (Fig. 1a) compared with positive control, C+ *F. solani* and C+ *A. rolfsii*, the values of total phenolic compounds reached 13.92 and 10.52 mg g^{-1} respectively. Meanwhile, they were 9.85 mg g^{-1} for both when treated with *T. asperellum* (Fig. 1b) compared with positive control C+ *F. solani* and C+ *A. rolfsii*, being 6.83 and 7.86 mg g^{-1}, respectively.

The accumulation of conjugate phenols was clear in the treatment with *T. afroharzianum* in soil infested with *F. solani* and *A. rolfsii*, reached 7.82 and 5.16 mg g^{-1}, respectively (Fig. 1a). Meantime, *T. asperellum* treatments were 3.74 and 4.58 mg g^{-1} respectively (Fig. 1b), compared with positive control1.49 and 1.73 mg g^{-1}, respectively. In the meantime, the levels of free phenolic compounds showed a slight increase in this respect. All treatments demonstrated a rise in the levels of total, free and conjugate phenols compared with negative control (n. Control), being 4.72, 3.56, 1.16 mg g^{-1}, respectively.

All treatments showed an increase in the levels of peroxidase (PO) enzyme compared with positive control (Fig. 2). Cucumber plant treated with *T. afroharzianum* grown in infested soil with *M. phaseolina* and *F. solani* recorded 58.55 and 37.17 units mL^{-1}, respectively, compared with positive control, being 9.98 and 12.98 units mL^{-1}, respectively (Fig. 2a). The increase of PO enzyme showed the highest value by treatment with *T. afroharzianum* than *T. asperellum* in general (Fig. 2a). All treatments showed an increase in the levels of peroxidase (PO) enzyme compared with negative control (n. Control), being 14.8 units mL^{-1}. The same trend was obtained in the level of polyphenoloxidase enzyme (PPO) where the highest values were in *T. afroharzianum* treatment grown in infested soil with *M. phaseolina*, being 3.83 units ml^{-1} followed by soil infested with *F. solani*, being 3.18 units mL^{-1} (Fig. 2b). Meanwhile, treatment with *T. asperellum* grown in infested soil with *M. phaseolina* recorded 2.46 units mL^{-1}, which higher than infested control, being 0.96 units mL^{-1} (Fig. 2b).

All treatments showed an increase in the levels of carboxymethyl cellulase enzyme (CMCase) compared with positive control (Fig. 2c). Cucumber plants raised from seeds, dressed with *T. asperellum*, grown in infested soil with *M. phaseolina* recorded 16.18 µg reduced sugars g^{-1}, followed by *R. solani,* being 14.94 µg reduced sugars g^{-1} compared with positive control of *M. phaseolina* and *R. solani,* being 5.35, 6.53 µg reduced sugars g^{-1}, respectively. The increase of CMCase enzyme showed the highest value by treatment with *T. asperellum* than *T. afroharzianum* in general.

Effect of seed treatments with biocontrol agents (BCA)on chlorophyll content and carotenoids in leaves of cucumber plants grown in soil infested with the causals of damping-off

Data presented in Table 5 indicate the effect of cucumber seed dressing with *T. afroharzianum* and *T. asperellum* individually, on the chlorophyll and carotenoid content in cucumber leaves, when these seeds were planted in a soil infested with the tested pathogenic fungi.

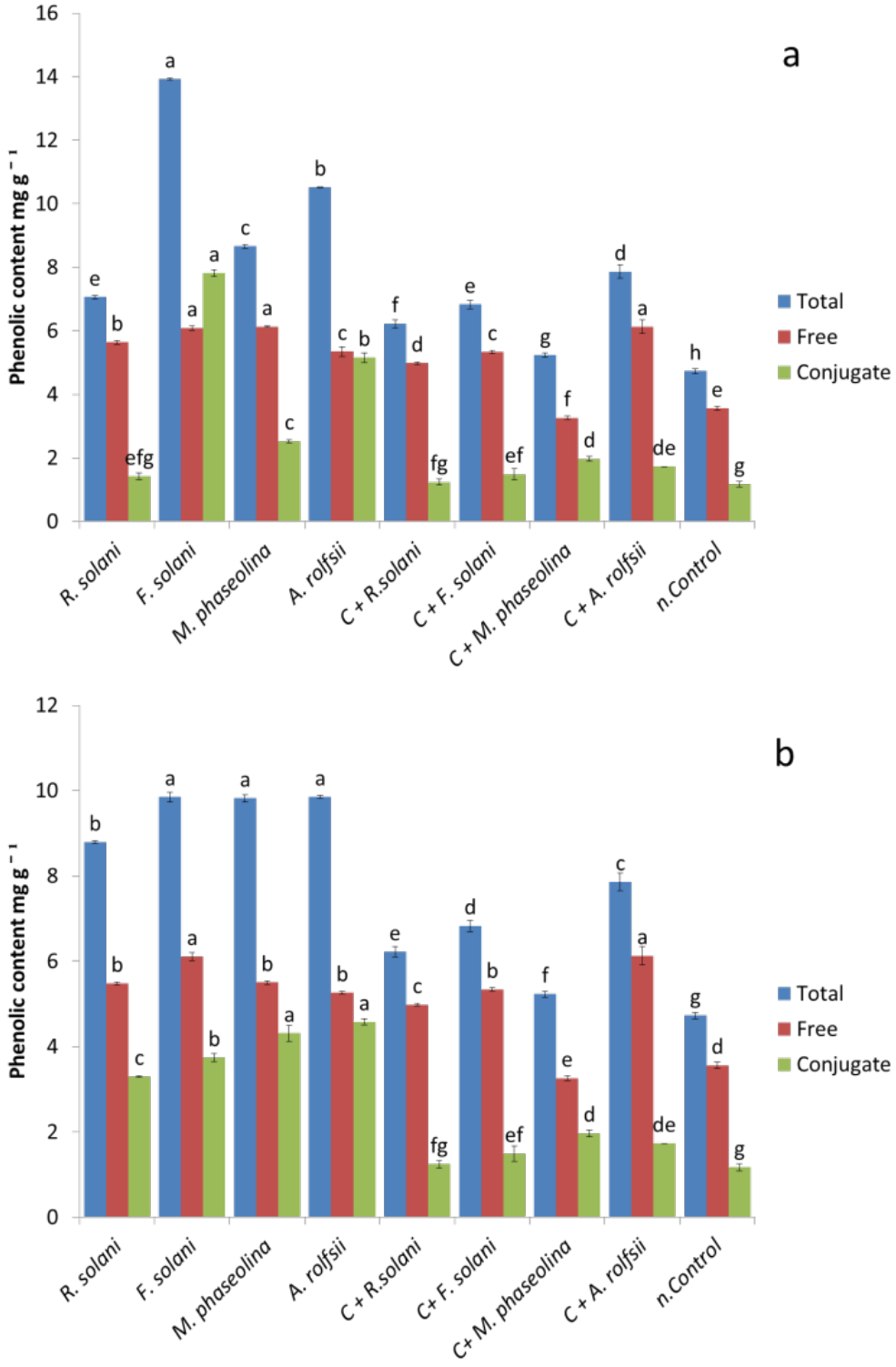

Fig. 1 – Phenolic compounds content (mg g^{-1}) of cucumber plants raised from seeds dressed with *T. afroharzianum* (a), *T. asperellum* (b), in the presence of pathogens compared with positive control (grown in soil infested with C+ *R. solani*, C+ *F. solani*, C+ *M. phaseolina* and C+ *A. rolfsii*) and negative control, n. Control (grown in non-infested soil).

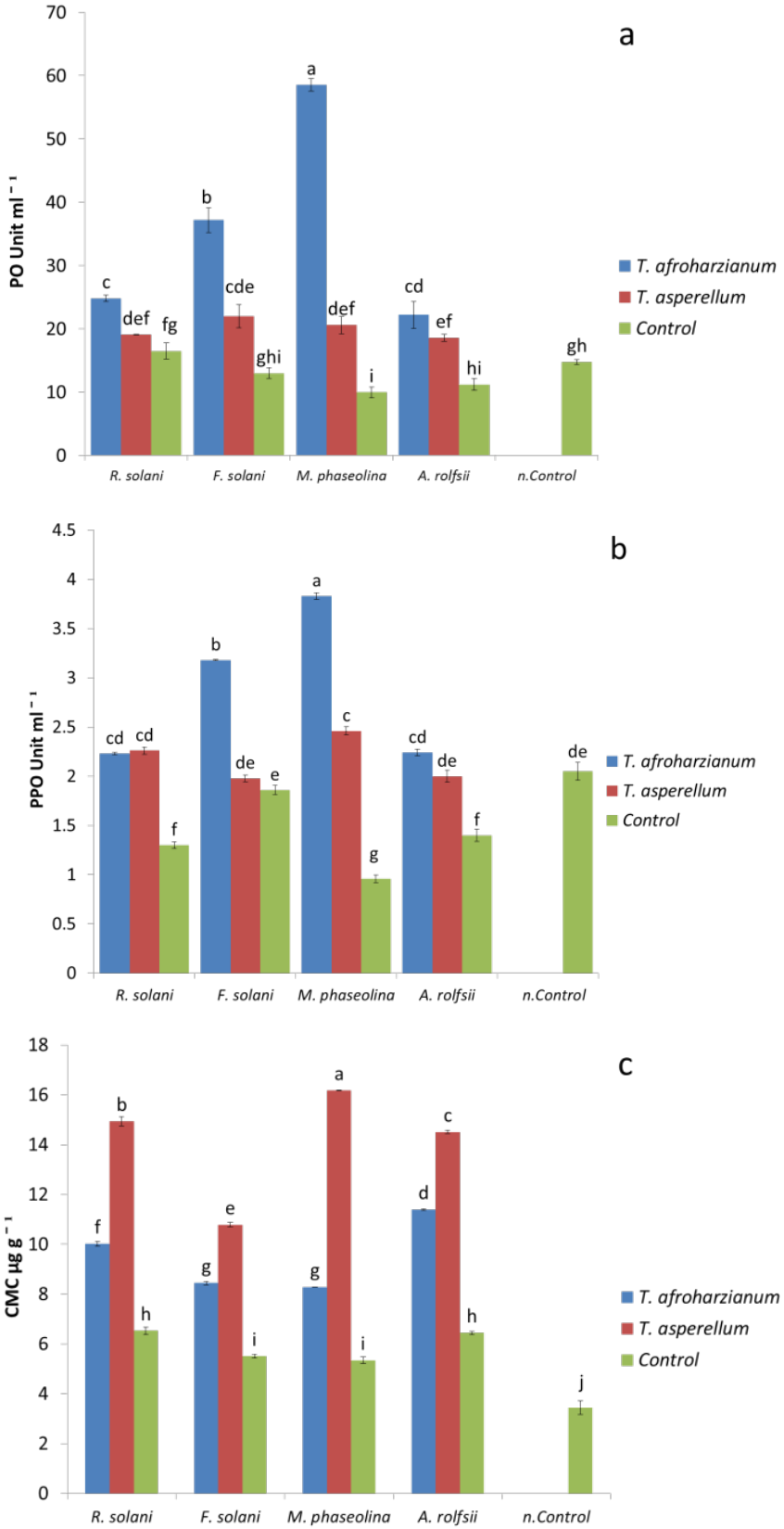

Fig. 2 – Peroxidase activity (PO) a, Polyphenol-oxidase activity (PPO) b, and Carboxymethyl cellulase (CMCase) c, in cucumber leaves raised from seeds dressed with *T. afroharzianum*, and *T. asperellum*, in the presence of pathogens compared with positive control, (grown in soil infested with *R. solani*, *F. solani*, *M. phaseolina* and *A. rolfsii*) and negative control, n. Control (grown in non-infested soil).

Table 3 – Effect of *T. afroharzianum* as cucumber seed soaking on incidence of damping off disease under greenhouse conditions.

Pathogens	10^9 CFUs g^{-1}	Damping-off (%)			Survived plants (%)	Increase in survived plants (%)
		Pre-emergence	Post-emergence	Total		
R. solani	Control	33.3^{abc}	20.0^{ab}	53.3	46.7	---
	0.5	20.0^{abc}	23.3^{a}	43.3	56.7	10.0
	1	16.7^{abc}	23.3^{a}	40.0	60.0	13.3
	2	16.7^{abc}	10.0^{ab}	16.7	83.3	36.6
F. solani	Control	36.7^{abc}	6.7^{ab}	43.4	56.6	---
	0.5	33.3^{abc}	3.3^{ab}	36.6	63.3	6.7
	1	10.0^{bc}	13.3^{ab}	23.3	76.7	20.1
	2	3.3^{c}	6.7^{ab}	10.0	90.0	33.4
M. phaseolina	Control	43.3^{ab}	0.0^{b}	43.3	56.7	---
	0.5	43.3^{ab}	0.0^{b}	43.3	56.7	0.0
	1	43.3^{ab}	0.0^{b}	43.3	56.7	0.0
	2	40.0^{ab}	0.0^{b}	40.0	60.0	3.4
A. rolfsii	Control	50.0^{a}	6.7^{bc}	56.7	43.3	---
	0.5	50.0^{a}	3.3^{ab}	53.3	56.7	13.4
	1	46.7^{a}	3.3^{ab}	50.0	50.0	6.7
	2	26.7^{abc}	0.0^{b}	26.7	73.3	30.0

Means followed by different letter (s) are significantly different according to Tukey's multiple range test at α = 0.05

Table 4 – Effect of *T. asperellum* as cucumber seed soaking on incidence of cucumber damping-off under greenhouse conditions.

Pathogens	10^9 CFUs g^{-1}	Damping- off (%)			Survived plants (%)	Increase in survived plants (%)
		Pre-emergence	Post-emergence	Total		
R. solani	Control	33.3^{abcde}	20.0^{abc}	53.3	46.7	---
	0.5	16.7^{cdef}	30.0^{a}	46.7	53.3	6.6
	1	20.0^{bcdef}	23.3^{ab}	43.3	56.7	10.0
	2	6.7^{ef}	10.0^{abc}	16.7	83.3	36.6
F. solani	Control	43.3^{abc}	0.0^{c}	43.3	56.7	---
	0.5	30.0^{abcdef}	6.7^{bc}	36.7	63.3	6.6
	1	10.0^{def}	16.7^{abc}	26.7	73.3	16.6
	2	3.3^{f}	6.7^{bc}	10.0	90.0	33.3
M. phaseolina	Control	43.3^{abc}	0.0^{c}	43.3	56.7	---
	0.5	43.3^{abc}	0.0^{c}	43.3	56.7	0.0
	1	36.7^{abcd}	0.0^{c}	36.7	63.3	6.6
	2	33.3^{abcde}	0.0^{c}	33.3	66.7	10.0
A. rolfsii	Control	50.0^{a}	6.7^{bc}	56.7	43.3	---
	0.5	46.7^{ab}	0.0^{c}	46.7	53.3	10.0
	1	40.0^{abc}	0.0^{c}	40.0	60.0	16.7
	2	33.3^{abcde}	0.0^{c}	33.3	66.7	23.4

Means followed by different letter (s) are significantly different according to Tukey's multiple range test at α = 0.05

Both bio control agent treatments enhanced chlorophyll a, b and total content as well as carotenoid content in cucumber leaves compared with the control. Cucumber leaves of plants raised from seeds dressed with *T. afroharzianum*, grown in infested soil with *R. solani*, resulted in the highest of chlorophyll a (0.245 mg g^{-1}), b (0.201 mg g^{-1}) and total content (0.446 mg g^{-1}) as well as for carotenoid contents (0.039 mg g^{-1} fresh weight). followed by cucumber leaves of plants raised from seeds dressed with *T. afroharzianum* grown in infested soil with *A. rolfsii*, being 0.167, 0.141, 0.308 and 0.030 mg g^{-1} fresh weight, followed by cucumber leaves of plants raised from seeds dressed with *T. asperellum* grown in infested soil with *A. rolfsii,* being 0.148, 0.136, 0.284 and 0.019 mg g^{-1} fresh weight, respectively. Meanwhile, cucumber leaves of plants raised from seeds dressed with *T. asperellum* grown in infested soil with *R. solani* and *F. solani* resulted in the lowest figures of chlorophyll a, b, and total content as well as carotenoids.

Table 5 – Effect of seed treatment with biocontrol agents on chlorophyll and carotenoids content in the leaves of cucumber plants grown in soil infested with the tested pathogens, 45 days after sowing.

Treatments	**Chlorophyll (mg g^{-1})**			**Carotenoids (mg g^{-1})**
	Chl. a	**Chl. b**	**Total**	
Biocontrol agents + Pathogens				
T. afroharzianum + *R. solani*	0.245^{a}	0.201^{a}	0.446^{a}	0.039^{a}
T. afroharzianum + *F. solani*	0.095^{ef}	0.086^{d}	0.181^{e}	0.016^{c}
T. afroharzianum + *M. phaseolina*	0.066^{g}	0.070^{def}	0.135^{g}	0.020^{c}
T. afroharzianum + *A. rolfsii*	0.167^{b}	0.141^{b}	0.308^{b}	0.030^{b}
T. asperellum + *R. solani*	0.050^{h}	0.053^{gh}	0.103^{h}	0.013^{ed}
T. asperellum + *F. solani*	0.034^{i}	0.047^{h}	0.081^{i}	0.005^{e}
T. asperellum + *M. phaseolina*	0.109^{d}	0.116^{c}	0.225^{d}	0.014^{cd}
T. asperellum + *A. rolfsii*	0.148^{c}	0.136^{b}	0.284^{c}	0.019^{c}
Control				
CK, *R. solani*	0.102^{de}	0.082^{de}	0.184^{e}	0.007^{de}
CK, *F. solani*	0.089^{f}	0.065^{fg}	0.154^{f}	0.008^{de}
CK, *M. phaseolina*	0.063^{g}	0.055^{fgh}	0.118^{h}	0.008^{de}
CK, *A. rolfsii*	0.089^{f}	0.067^{efg}	0.156^{f}	0.007^{de}
Non-infested (n. Control)	0.067^{g}	0.046^{h}	0.113^{h}	0.007^{de}

Means followed by different letter (s) are significantly different according to Tukey's multiple range test at $\alpha = 0.05$

Discussion

Cucumber damping-off presents significant challenges to the Egyptian agricultural industry, leading to substantial economic losses. This disease adversely impacts seed and seedling viability, which can subsequently lead to a reduction in crop yields. The restricted cucumber availability on the market can have repercussions on rising prices, impacting both consumers and producers. The isolation trial yielded several fungal pathogens (*R. solani*, *F. solani*, *M. phaseolina* and *A. rolfsii*), which were associated with damping-off and root-rot symptoms. These findings align with those reported by Hassan et al (2021) who documented, *R. solani*, *F. oxysporum*, *F. solani*, *A. rolfsii*, *M. phaseolina*, and *Phytophthora* spp., as primary related to cucumber damping-off and root rot. These pathogens can severely hinder seed germination and plant establishment, particularly under cool and wet weather conditions. The pathogenicity test of the isolated fungi has been confirmed aligning with the earlier observations reported by Al-Fadhal et al. (2019) and has demonstrated variable inhibition of seed germination by *F. solani, R. solani, A. rolfsii, M. phaseolina*.

The enzyme production by *R. solani* (e.g., cutinase, cellulase and protease) and by *A. rolfsii* (e.g., oxalic acid and cellulase) was highlighted as key mechanisms impacting germination (Punja

and Damiani, 1996). Additionally, *M. phaseolina* has been implicated in severe damping-off cases, confirming similar findings by Nasreen et al. (2009).

Native antagonistic *Trichoderma* spp. were isolated and identified by morphological characters as *T. afroharzianum* and *T. asperellum*.

The TAM1 isolate was placed within a distinct clade with several *Trichoderma* species, when phylogenetic analysis was conducted using ITS region. Moreover, the Tef1α barcode had confirmed identities with 99.67% similarity to *T. afroharzianum*. Phylogenetic analysis using the Tef1α region for the TAM2 isolate placed it within a distinct clade with *T. asperellum* strain CBS 433.97 and *T. kunmingense* strain YMF1.02659, showing 100% similarity with *T. asperellum*. Moreover, the ITS barcode recorded 99.57% identities with to *T. asperellum* (CBS 433.97) strain, GenBank accession NR130668, and this is in agreement with Ismaiel et al. (2022).

The effectiveness of *Trichoderma* species, in inhibiting the linear growth of the tested fungal pathogens was evaluated. The concentration of biocontrol agents (BCA) was found to significantly influence their efficacy, with higher concentrations generally leading to increase in the linear growth inhibition. This suggests that optimizing biocontrol agent concentration is crucial for effective biocontrol strategies. *T. afroharzianum* and *T. asperellum* have demonstrated varying levels of inhibition against the tested pathogens. *T. asperellum* has been identified as a highly effective biocontrol agent, as evidenced by its capacity to inhibit the linear expansion of phytopathogenic organisms (Shanmugaraj et al., 2023; Elshahawy and Marrez, 2023). *Trichoderma asperellum* was found to possess significant enzymatic activities, including cellulase, chitinase, and protease, these enzymes likely contribute to its ability to suppress *Fusarium* growth and enhance its effectiveness as a biocontrol, explaining its effect against the tested fungi. Moreover, the percentage of growth inhibition of the tested pathogens by *T. afroharzianum* was attributed to the secretion of chitinase and β-1,3 glucanase, which led to the analysis of the cell wall (Bouanaka et al., 2021).

Seed dressing with biocontrol agents (BCA)is an important practice for promoting healthy plant growth, reducing the use of chemical inputs, enhancing soil health, and offering sustainable disease management. This approach plays a vital role in integrated pest management strategies and helps ensure the long-term productivity and the health of agricultural systems (Sujatha et al., 2024). In line with this, the seed dressing application with *T. afroharzianum* or *T. asperellum* at the highest concentration of 2×10^9 CFU g^{-1}seed, each alone, has been shown to pointedly enhance plant survival rates compared to control. This beneficial effect is attributed to several mechanisms through which *Trichoderma* operates, including disease suppression and growth promotion.

The mechanisms that contribute to *Trichoderma*-mediated plant disease suppression include competitive interactions in the rhizosphere with other microorganisms, the induction of systemic resistance in plants against diseases, mycoparasitism characterized by the secretion of fungal cell wall-degrading enzymes, solubilization of inorganic plant nutrients (El-Hassan et al., 2013), and the biosynthesis of antimicrobial secondary metabolites (Samolski et al., 2009; Vieira et al., 2013). Moreover, it not only suppress the proliferation and reproduction of pathogenic fungi but also encourages the advancement of intrinsic defensive systems in crops, thus aiding in the acquisition of local or systemic disease resistance, which was compliant with subsequent results.

The impact of applying biocontrol agents to cucumber seeds in the presence of pathogenic fungi on certain enzymes and phenolic compounds was observed *in vivo*, under pathogenic fungal invasion and during the interaction between *Trichoderma* spp. and cucumber plants, phenolic compounds are

synthesized as part of the plant's defence strategy, helping to reinforcing cell walls against pathogen invasion (Kaur et al., 2024; Tariq and Ahmed, 2024). The results reported here indicated that cucumber treated with *Trichoderma* spp. increased total phenol contents, these results agree with those reported by several authors (Yan and Khan, 2021; Guo et al., 2023). Therefore, elevated total phenolic content and induction of enzymes involved in phenyl propanoid metabolism occur in cucumber plants following treatment with biocontrol agents. The implications of phenolic compound changes on the overall plant defence response in cucumber seedlings treated with biocontrol agents and exposed to pathogenic fungi are noteworthy. These changes enhance the plant's ability to resist infections. The results revealed the impact of using a high concentration of the tested biocontrol agent on the accumulation of total and conjugate phenols, which was more prominent in cases of infection with *F. solani* and *A. rolfsii* compared with infested control. Which were compliant with Yang et al. (2024) who reported that cucumber seedlings exhibit increased levels of phenolic acids and flavonoids in response to Fusarium infection. Numerous studies have demonstrated the beneficial effects of a higher level of phenolic compounds (Daayf et al., 2012; Kulbat, 2016; Kumar et al., 2020; Lian et al., 2023). Additionally, it was shown that adding biocontrol agents (BCA) to plants induced the production of phenol compound as a defense mechanism against infections (Gangwar and Sinha, 2014; Ragab et al., 2015; Awad-Allah et al., 2022). Phenolic compounds appear to mitigate disease progression through various mechanisms, which include the suppression of extracellular fungal enzymes (such as cellulases, pectinases, laccase, and xylanase), the inhibition of oxidative phosphorylation in fungi, the deprivation of essential nutrients (via metal complication and protein insolubilization), the inhibition of both spore germination and the mycelial proliferation of various pathogenic fungi, as well as the manifestation of antioxidant activities within plant tissues (Chérif et al., 2007).

All treatments showed an increase in the levels of peroxidase and polyphenol oxidase enzymes compared with infested control, these enzymes play a crucial role in plant defence against diseases. Peroxidase plays a role in respiration, growth control, photosynthesis, cell wall lignification, and substrate oxidation (Srivastava, 1987; Natalie et al., 2020). The polyphenol oxidase (PPO) enzyme converts phenols into extremely toxic quinones that appear to be harmful to plant pathogen, hence promoting disease resistance. It has been connected to defence systems against plant diseases. Jukanti (2017) and El Nahas et al. (2019) found that the application of fungicides and biocide *T. asperellum* individually or together caused increases in the activity of polyphenol oxidase (PPO).

Trichoderma species are widely recognized for their multiple methods of combating plant diseases, which include mycoparasitism, nutrient competition, the generation of antifungal metabolites, and the development of systemic resistance in plants. A crucial aspect of their mycoparasitic activity is the release of cellulase enzymes, which is pivotal in enhancing plant defenses against pathogens (Harman et al., 2004; Kamel et al., 2017; Mahmoud et al., 2023). Through biocontrol mechanisms against diseases, the cellulases produced by *Trichoderma* spp. are used in agricultural operations to improve soil health and enhance plant growth (Saravanakumar et al., 2016). *Trichoderma* cellulases have the ability to directly interact with plant roots through biochemical reactions that enhance plant growth and disease resistance (Hermosa et al., 2012). Studies have shown that *Trichoderma*-colonized roots have increased levels of enzymes that scavenge reactive oxygen species (ROS), decreasing oxidative stress in the event of a pathogen attack. In addition, *Trichoderma* may modify the structure of roots, producing stronger root systems resistant to fungal attack (Lang

and Chen, 2023). This was particularly evident with *T. asperellum*, which exhibited high levels of cellulase production.

Photosynthetic pigments, such as chlorophyll (Chl) a, Chl b, and carotenoids, are essential for plant processes. Their concentrations serve as significant indicators of a plant's physiological condition and can be used to gauge photosynthetic activity (Su et al., 2024). Both bio-agent treatments enhanced chlorophyll a, b and total content as well as carotenoids content in cucumber leaves compared with the control. cucumber leaves of plants raised from seeds dipped in *T. afroharzianum*, grown in infested soil with *R. solani* resulted in the highest figures, followed cucumber leaves of plants raised from seeds dipped in *T. afroharzianum*, grown in infested soil with *A. rolfsii* followed by cucumber leaves of plants raised from seeds dipped in *T. asperellum*, grown also in infested soil with *A. rolfsii*. These treatments not only improved chlorophyll and carotenoid content but also contributed to overall plant health and resistance to pathogens. The potential for bio control agents to improve plant health and productivity remains a significant area of interest for sustainable agriculture. *Trichoderma afroharzianum* and *T. asperellum* exhibit distinct mechanisms of action in combating soil-borne diseases. *Trichoderma afroharzianum* primarily induces the production of phenolic compounds and enhances the activity of peroxidase and polyphenol oxidase enzymes, contributing to plant defence responses. In contrast, *T. asperellum* stimulates the synthesis of carboxymethyl cellulase enzyme, which not only helps in disease suppression but also acts as a biofertilizer. This facilitates nutrient availability and supports plant growth under stress conditions.

Conclusions

This study identified four main fungi associated with damping-off in cucumbers: *R. solani*, *F. solani*, *M. phaseolina*, and *A. rolfsii*. Among them, *R. solani* and *A. rolfsii* caused the highest levels of damping-off, while certain isolates of *F. solani* showed lower pathogenicity. Two *Trichoderma* species, *T. afroharzianum* and *T. asperellum*, were molecularly identified and found to effectively inhibit the growth of these pathogens *in vitro*, with the highest inhibition against *M. phaseolina*. In greenhouse trials, both biocontrol agents (BCA) significantly reduced damping-off and increased plant survival, with *T. afroharzianum* being more effective against *F. solani, R. solani*, and *A. rolfsii*, while *T. asperellum* was more effective against *F. solani* and *R. solani*. These biocontrol agents (BCA) also enhanced plant defence mechanisms, shown by increased enzyme activity and phenolic compounds. Additionally, *T. afroharzianum* and *T. asperellum* improved chlorophyll and carotenoid levels, promoting plant health. Overall, both species are promising candidates for controlling damping-off in cucumbers. Future research should focus on the long-term effects of combined treatments on soil health and the development of resistance in pathogens.

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