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RESEARCH ARTICLE

Effect of *Trichoderma harzianum* Rifai and mycorrhizal fungi on total phenolics, peroxidase and glycoalkaloids in *Fusarium oxysporum* Schltdl. infected tomato plants

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Abstract

Pathogenicity tests of *Fusarium oxysporum* Schltdl. isolates on tomato (*Solanum lycopersicum* L.) demonstrated a significant capacity to induce seed rot and seedling damping-off under greenhouse conditions. The most virulent isolate, confirmed via polymerase chain reaction (PCR), was selected for further study. Inoculation with *Trichoderma harzianum* and mycorrhizal fungi substantially enhanced the total phenolic content and peroxidase (POX) enzyme activity in *F. oxysporum*-infected plants. Furthermore, high-performance liquid chromatography (HPLC) analysis revealed that these biological control agents significantly boosted the biosynthesis of key secondary metabolites, specifically the glycoalkaloids (GAs) α-tomatine, tomatidine and solanine. The interaction between *F. oxysporum* and *T. harzianum* resulted in the highest accumulation of these defense-related compounds compared to plants infected with the pathogen alone. These findings suggest that *T. harzianum* and mycorrhizal fungi activate the plant's systemic resistance by modulating physiological and biochemical defense mechanisms against Fusarium wilt.

Keywords: *Fusarium oxysporum*; HPLC; Iraq; mycorrhizal fungi; peroxidase; phenolics

Introduction

Tomato (*Solanum lycopersicum* L.) is a solanaceous species known for its richness in various mineral elements such as calcium, phosphorus and iron, as well as essential vitamins like vitamin A and C, fibres, sugars, proteins and citric acid (1). It is a major vegetable crop of economic importance worldwide (2). It is widely grown in many countries, including Iraq and Tunisia, as open-field or protected crops (3). The cultivated areas in Iraq reached 69686 dunums in 2018 with a production estimated at 133430 tons (4). Seedling damping-off or wilt may be induced by one or more fungal and pseudo-fungal pathogens; these agents may be of soil-borne or seed-borne origin, where the most important ones are affiliated with *Pythium* spp., *Fusarium* spp. and *Rhizoctonia solani* (5). Fusarium wilt of tomato, caused by *Fusarium oxysporum* f. sp. *Lycopersici* (*FOL*), is a devastating disease in major tomato-growing regions worldwide and has been reported in at least 32 countries (6). Disease symptoms include yellowing of the leaves, vascular discoloration, wilting, stunting and eventual death of the whole plant (7). The resistance of tomato cultivars to races 1 and 2 of *FOL*, which has long been adopted as an effective and safe alternative (8), was overcome by the emergence of race 3 in several countries (9). The use of beneficial microorganisms as biocontrol agents is gaining more attention. Among the most explored agents is the fungus *Trichoderma harzianum*, which has been shown as a potent agent employing various direct and indirect mechanisms of action while controlling target pathogens (10). These biocontrol agents are able to produce antibiotics leading to the inhibition of spore production (11), increase the availability of nutrients (12) and induce systemic resistance (13). Similarly, mycorrhizal fungi play a key role in protecting plants from infection, enhancing tolerance to abiotic stresses and improving nutritional status (14, 15). Despite the extensive research on *Trichoderma* and mycorrhizal fungi as biocontrol agents, a significant gap remains in understanding their specific role in modulating the biosynthesis of key secondary metabolites, particularly glycoalkaloids (GAs), during *F. oxysporum* infection. Most previous reports have focused on general growth promotion or enzymatic activities, whereas the synergistic effect of these fungi on chemical defense barriers like α tomatine and solanine remains poorly explored. Therefore, the present study fills this gap by investigating the effect of *T. harzianum* and mycorrhizal fungi on total phenolics and peroxidase (POX) enzyme activity, while specifically identifying and quantifying GAs in tomato leaf extracts. This approach aims to provide a deeper understanding of these fungi as more effective and environmentally safe alternatives for disease control.

Materials and Methods

Sample collection and isolation of fungi causing tomato vascular wilt disease (isolation and diagnosis)

Samples were taken from each greenhouse, they were collected from infected tomato plants that showed symptoms of infection.

The samples were kept in plastic bags and brought to the microbiology laboratory at the College of Agriculture, University of Karbala in order to isolate the pathogenic fungi. The tomato plants were grown in 8 greenhouses protected in some Iraqi provinces (4 provinces) which include (Baghdad, Najaf, Karbala and Babylon) during the autumn season 2021–2022. The infestation was determined by the general weakening of growth on infected tomato plants to determine the extent of the infestation, 48–50 tomato plants were taken from each greenhouse.

In the phenotypic and microscopic diagnosis, the standard method was adopted (7). The fungal isolates were grown separately by transferring a one cm diameter disc from the edge of a fresh fungal colony of a pure isolate to the middle of a Petri dish containing the nutritional medium of the fungal isolates. The same thing was repeated with all isolates and the dishes were kept in the dark at a temperature of 25 ± 2 ℃ for 5 days. After the end of the incubation period, the fungal cultures were examined, especially for the culture's color, edges, culture tissue and pigment development. The microscopic characteristics were studied using a light microscope and several magnification powers, depending on the shape of the fungal filament, the conidia, the phialids, the presence and shape of the large conidia, the number of septa, the terminal cell composition, the shape of the basal cell, the presence or absence of squamous spores or microconidia and their location. The most pathogenic isolate was obtained from among 50 isolates using the rotten seeds percentage and the percentage of seedling death. The physiological characterisation of FOL isolates was carried out using morphological and microscopic characteristics together with 3 differential varieties of tomato seeds, a local susceptible cultivar (serving as a susceptible control), '7002' (resistant to Race 1) and 'Aya' (resistant to both Races 1 and 2) (16). Seedlings at the two-leaf stage were inoculated using the root-dip method. The criteria for race identification were based on the disease responses at 21 days post-inoculation. The isolates were identified as Race 1 when they failed to infect the '7002' cultivar. Crucially, isolates that induced typical wilt symptoms and mortality on the 'Aya' cultivar were conclusively identified as Race 3, as they successfully overcame the resistance genes for Races 1 and 2. No isolates belonging to Race 2 were detected in this study, as all isolates that overcame Race 1 resistance also overcame the resistance in 'Aya', confirming they belong to Race 3.

Then, the polymerase chain reaction (PCR) technique was used at the ISCO Learning Centre, Baghdad, Iraq to molecularly confirm the identity of the isolate under study.

Experiment in the greenhouse

In this experiment, a mixture of soil-sand and peat moss was used in a ratio of 1:3. The soil and peat moss were sterilised. This experiment was carried out in plastic pots in the greenhouses of the College of Agriculture, University of Karbala, during the autumn season of 2023. The experimental design followed a randomized complete block design (RCBD) with 3 replicates for each isolate to ensure statistical accuracy where the soil and peat moss were mixed and sterilised using an autoclave device at a temperature of 121 ℃ and a pressure of 15 psi for 60 min. The next day, the sterilisation process was repeated under the same conditions mentioned previously, the sterilised soil was spread in plastic pots with a capacity of 3 kg. The pots were arranged in the greenhouse following a randomisation strategy to minimise environmental bias with 3 replicates for each isolate and the treatment without adding the pathogenic fungus was used as the control treatment, mycorrhizal fungi were added at a rate of 10/kg soil and the biological agent mycorrhizal was added to the soil before planting as for the pathogenic fungus, it was added to the soil at a rate of 1 g/kg soil. Sterilised tomato were sown at the rate of 10 seeds per pot. Environmental conditions within the greenhouse were monitored, with temperatures maintained around 28 ± 2 ℃ and plants were carefully watered as needed to ensure optimal growth and disease development.

The experiment was carried out by making 3 replicates per individual treatment. Pots were distributed in the plastic house according to the modern commercial design. It was completed as follows: -

Control treatment - without any fungus.

Treatment with *F. oxysporum* only.

Treatment with mycorrhizal and *F. oxysporum.*

During the experiment, the following analysis were performed: phenols and POX.

Measurement of induction of resistance

Phenols

A sample of 1 g of tomato leaves was crushed using a ceramic mortar by adding 10 mL of 80 % methanol, the obtained extract was heated for 30 min in a water bath at 70 ℃, then filtered through Whatman No. 1 filter paper. 1 mL of the extract was taken and add 5 mL of distilled water and 250 µL of standard Folin 1 reagent. The mixture was left until the blue colour appeared and the reading was done using a spectrophotometer at a wavelength of 725 nm.

Peroxidase (POX)

A sample of 1 g of tomato leaves removed for each treatment separately was crushed with 10 mL of phosphate buffer with a pH of 7 in a ceramic mortar. The homogenates were stored in 10 mL test tubes. The mixtures were subjected to centrifugation at a speed of 3000 rpm the supernatant were kept at 0 ℃ until the effectiveness of POX was measured. The activity of the POX enzyme was measured with the Guaiacol test.

The components of the reaction mixture consisted of:

1. Guaiacol solution with a concentration of 0.5 M, prepared by diluting 1.1 mL of Guaiacol in 250 mL of distilled water.
2. Hydrogen peroxide solution, H_2O_2, 0.02 M: prepared by diluting 0.56 mL of H_2O_2, concentration 30 %, in 50 mL of distilled water, prepared immediately.
3. Tris buffer solution was prepared by dissolving 1.211 g of Tris with 14.11 g of sodium chloride (NaCl) dissolved in distilled water. The volume was filled with distilled water to 250 mL to obtain a concentration of 0.04 M for Tris solution and 1 M for sodium chloride (NaCl). Using standard 1 N hydrochloric acid (HCl) to adjust pH from 9–7.5.
4. Reaction mixture: Solutions 1–3 were mixed with distilled water in a ratio of 1:1:1:7 respectively to obtain the reaction mixture.

The activity of the enzyme was measured using a spectrophotometer by placing a mixture of 3 mL of the reaction mixture and 0.2 mL of tomato leaf extract into the device (17). The change in absorbance was recorded at a wavelength of 420 nm at a temperature of 30 ℃.

The results were taken by recording the readings for 3 replicates of each treatment and the enzyme activity was calculated through the following equation:

$$\text{Enzyme activity/minute/g of wet texture} = \frac{\Delta A \times 3}{\Delta T}$$

where:

A = change in absorbance

T = time period of change in absorbance

Determination of GAs in leaves of tomato extracts by high-performance liquid chromatography (HPLC)

Quantitative estimation of α-tomatine, tomatidine, dihydrotomatidine, filotomatidine and solanine was carried out using an HPLC system (Green Rawabi Laboratory, Baghdad, Iraq) and the standard method was followed (18). The alkaloids were separated on a fast liquid chromatographic (FLC) column (Phenomenex C-18, 50 × 4.6 mm I.D., 3 µm particle size). The mobile phase consisted of 0.01 M phosphate buffer (pH 6.2) and acetonitrile (75:25 v/v) with a flow rate of 1.4 mL/min and UV detection. The HPLC method validation was performed according to standard protocols to ensure reliability. Calibration curves were constructed for each GA using five concentrations ranging from 10–100 µg/mL, demonstrating high linearity (R^2 < 0.99). The limit of detection (LOD) and limit of quantitation (LOQ) were determined based on signal-to-noise ratios of 3:1 and 10:1 respectively. For α-tomatine, the LOD was approximately 0.5 µg/mL and the LOQ was 1.5 µg/mL, ensuring sufficient sensitivity for detecting these secondary metabolites in tomato extracts.

Each standard was analysed at a concentration of 50 µg/mL to identify the sequence of the eluted materials α-tomatine, tomatidine, dihydrotomatidine, filotomatidine (Table 1) and solanine (Table 2).

Table 1. HPLC retention times and peak areas of α-tomatine, tomatidine, dihydrotomatidine, and filotomatidine in tomato leaf extracts at a concentration of 50 µg/mL

Seq.	Subjects	Retention time (min)	Area (uV)	Concentration 5 (µg/mL)
1	α-tomatine	2.762	65246	
2	Tomatidine	4.272	67908	
3	Dihydrotommatidine	5.353	94679	
4	Filotomatidine	6.427	76582	

Table 2. HPLC retention times and peak areas of solanine in tomato leaf extracts at a concentration of 50 µg/mL

Seq	Subject	Retention time (min)	Area (uV)	Concentration
1	Solanine	2.352	77865	50 µg/mL

High performance liquid chromatography (HPLC) separation of solanine from tomato leaves extract

The extract of alkaloids according to enclosed procedure were separated on fast liquid chromatographic (FLC) column, 3 µm particle size, phenomenex C-18 (50 x 4.6 mm I.D) column, mobile phase; were 0.01 M phosphate buffer, pH 6.2: acetonitrile (75:25, V/V) detection UV set at 330 nm flow rate 1.4 mL/min.

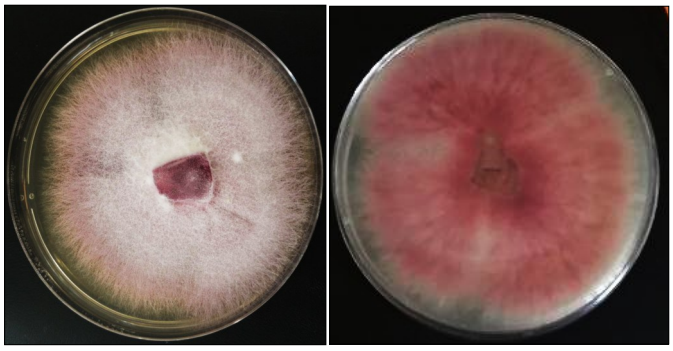
Statistical analysis

All experimental data were subjected to statistical analysis using statistical analysis system (SAS) software. The experimental design followed a factorial RCBD with 4 replications for each treatment. To determine the significance of the differences between treatment means, the least significant difference (LSD) test was employed at a probability level of 0.05. Furthermore, all essential statistical assumptions, including the normality of data and homogeneity of variance, were verified prior to the analysis of variance (ANOVA) to ensure the validity and reliability of the statistical results.

Results and Discussion

Morphological identification and pathogenicity of *F. oxysporum* isolates

Phenotypic diagnosis of isolate 39 for the current study showed that the colony of *F. oxysporum* appeared on the potato dextrose agar medium and was white/pink in colour in the centre and it grew rapidly (Fig. 1– 3). The diameter of the colony reached approximately 9 cm after 7 days of incubation at a temperature of 25–27 °C. Upon microscopic examination, the microconidia appeared. The color of its colonies on the back of the plate ranged from pink to dark pink. Conidia were observed to be small (microconidia), measuring 5.4–7.2 × 1.8 µm and they were oval or kidney-shaped and contained a single cell. The macroconidia had dimensions of 7.3–14 x 1.8–2.3 µm curved, thick-walled and contained 3–5 cells. Chlamydospores had dimensions of 1.8 x 3.6 µm and were spherical, with a thick wall to protect them from harsh conditions. Furthermore, the pathogenicity tests of the 45 fungal isolates demonstrated their ability to induce disease under greenhouse conditions. The most virulent isolate recorded the highest rate of seed rot (69 %) and seedling death (37.40 %), while the lowest isolate



(A) (B)

Fig. 1. Colony on potato dextrose agar. (A) surface; (B) reverse.

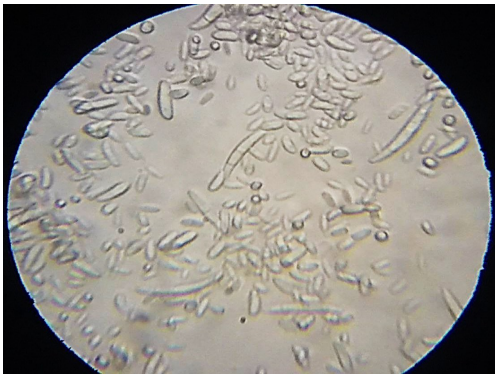


Fig. 2. *Fusarium oxysporum* various types of the conidia on potato dextrose agar.

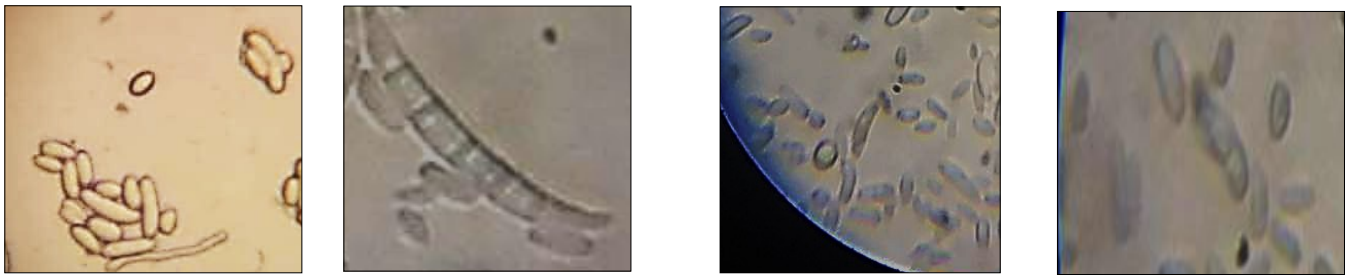


Fig. 3. *Fusarium oxysporum.* (A) microconidia; (B) macroconidia; (C) chlamydospores; (D) macroconidia two–septate.

recorded 9 % and 2.70 % respectively. The diagnosis was further confirmed by PCR.

The suppression of such virulent isolates in our study is attributed to the complex biochemical defense mechanisms triggered by biocontrol agents. The colonisation of tomato roots by *T. harzianum* and mycorrhizal fungi triggers a cascade of metabolic changes associated with induced systemic resistance (ISR). This biological interaction stimulates the phenylpropanoid metabolism, a major pathway for plant defense. Our results show that this stimulation leads to the accumulation of phenolic precursors which are essential for lignification, effectively reinforcing the root cell walls against *F. oxysporum* penetration. Furthermore, the HPLC analysis confirmed that these treatments significantly upregulated the biosynthesis of GAs. These compounds serve as potent natural antibiotics; their increased concentration represents a chemical barrier induced by the biocontrol agents. This synergistic effect between structural reinforcement (lignin) and chemical defense (GAs) explains the high efficacy of these fungi in suppressing Fusarium wilt.

Physiological characterisation of *FOL* isolates

The fungal isolates were distributed between 2 physiological strains, Race 1 and Race 3 no isolates belonging to strain 2 were detected, which is sensitive to the disease strain. There are 3 known strains of the fungus worldwide: strain 3, strain 1 and strain 2 (9). These strains differ in their behaviour towards fusarium wilt infection on tomatoes (19, 20). The importance and damage of Fusarium wilt on tomato, as well as the molecular identification of the two strains causing the disease, were mentioned.

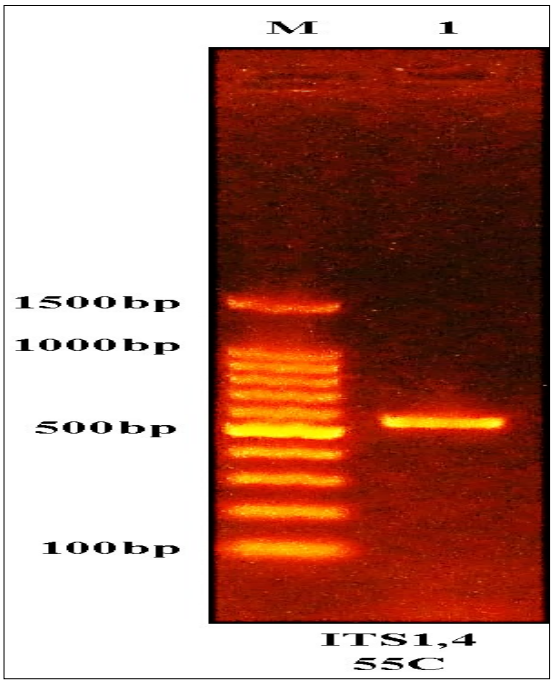


Fig. 4. Agarose gel electrophoresis of amplified PCR product (550bp).; Lane M: 1.5 Kbp DNA ladder marker; Lane 1: ITS region amplified bands.

Molecular identification of the fungus associated with Fusarium wilt of tomato in Iraq

Nucleotide sequence analysis of the PCR product from the fungus isolated in this study demonstrated that, using BLAST, this isolate was identified as a *F. oxysporum* (Fig. 4).

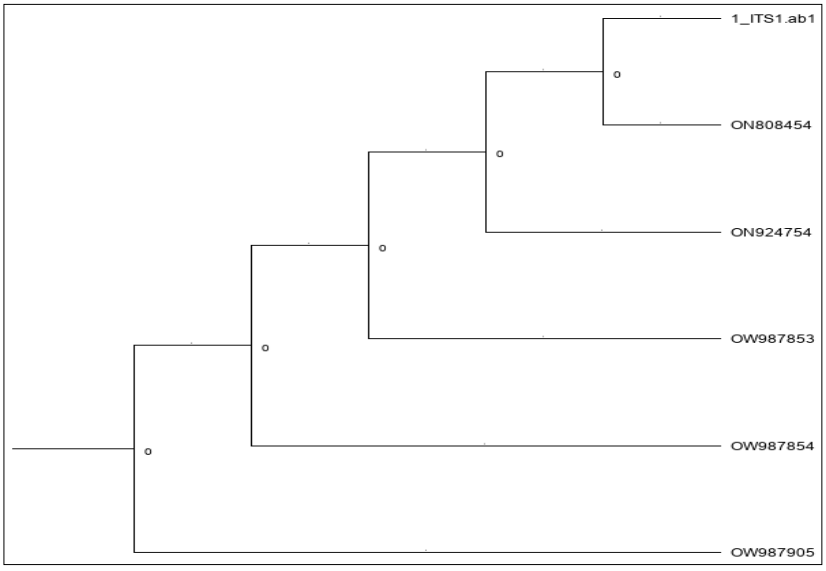


Fig. 5. Genetic tree for *Fusarium oxysporum* isolate No. 39.

A neighbor-joining tree shows the genetic relationship between the fungi isolated in this study from the tomato plant and other isolates of the same fungus that were previously registered in the National Center for Biotechnology Information (NCBI) (Fig. 5).

The important, necessary and precise role played by variations in the intergenic region (ITS) of rDNA is is particularly used by eukaryotes and prokaryotes to identify many microorganisms such as nematodes.

Effect of *T. harzianum* and mycorrhizal on the total phenolics and peroxidase (POX) enzyme of infection by *F. oxysporum*

Table 3. The effect of *Trichoderma harzianum* and mycorrhiza on the total content of phenols and peroxidase enzymes of infection by *Fusarium oxysporum* of tomato plants in the greenhouse

Treatment	Total phenols	Peroxidase (PO)
Control	3.025100	0.01233
FOL	3.040367	0.03600
T+ *FOL*	3.356200	0.37600
F+M	3.335667	0.21100
M+T+F	3.516100	0.36333
LSD 0.05	0.0003958	0.002657

tomato plants in the greenhouse

It is clear from the results of Table 3 that treatment with *T. harzianum* and mycorrhizal fungi caused an increase in the total content of phenols, as it reached in plants treated with the fungus *T. harzianum* + *FOL* and mycorrhizal+ *FOL* and *T. harzianum* + mycorrhizal+ *FOL*3.356200 and 3.335667 and 3.516100 mg/g of fresh weight respectively while the comparison was recorded for the pathogen *F .oxysporum* was 3.040367 mg/g wet weight. The total phenols content in the intact treatment reached 3.025100 mg/g of wet weight.

The emergence of varying degrees of resistance depends on the type of inducer around the sites of infection. The enzyme phenylalanine ammonia lyase (PAL) is the basis for the occurrence of activity or the construction of large quantities of it in diseased plant tissues, in addition to being the key in the production of the basic molecules used in the biosynthesis of most phenols, as well as phytoalexins and lignin. Phenol is involved in resistance to plant diseases and is concentrated in large quantities in diseased plants. Its structure and presence increase after infection occurs. It is found in healthy plants, but not in the same quantity as in infected plants, as it increases in the presence of the pathogen and the inducing factor as a result of stimulating acquired induced resistance, which begins by introducing the pathogen to the host plant Thus, the signal is sent to the tissues adjacent to and far from the recognition area, leading to the stimulation of plant genes (stimulation of resistance), suppression of the pathogen and increased activity of enzymes and phenols (21).

Phenolic compounds serve as natural pesticides and antibiotics, doubling as innate antioxidants distributed throughout all plant components. Studies have demonstrated a direct link between heightened concentrations of total phenolic compounds and enhanced antimicrobial efficacy (22). Findings demonstrate a positive correlation between elevated polyphenol levels and potent antimicrobial activity against E. coli (23). Although the numerical differences in total phenolic content among treatments may appear relatively small, they are biologically significant as they represent a state of "biochemical priming". In this study, these increases directly correlate with ISR, where even minor elevations in phenolic precursors provide the necessary substrates for defense-related enzymes like POX and polyphenol oxidase (PPO). This metabolic shift was closely associated with a significant reduction in Fusarium wilt severity and incidence, as the accumulated phenols contribute to cell wall lignification and the formation of toxic quinones, thereby creating a chemical and physical barrier that enhances the intensity of plant resistance against *F. oxysporum*.

The same table shows the effectiveness of the oxidase enzyme when treating tomato plants with the fungus *T. harzianum* + *FOL* and mycorrhizal+ *FOL* and *T. harzianum* + mycorrhizal+ *FOL* 0.37600 and 0.21100 and 0.36333 mg/g wet weight respectively while the value recorded for the pathogen *F. oxysporum* 0.03600 mg/g wet weight the total phenols content in the intact treatment reached 0.01233 mg/g of wet weight. Peroxidase is a mechanical defense enzyme. Its enzymatic activity increases the deposition of lignin in the cell wall through the process of lignification, which acts as a mechanical barrier that prevents the passage of the pathogen. The POX enzyme contributes to the formation of H_2O_2, which is an antifungal substance that directly inhibits the fungal growth of pathogens (24). Sometimes, the POX enzyme contributes to the production of active oxygen, which is considered one of the important factors for inducing the oxidative burst, which subsequently leads to the death of the infected cell, as well as the occurrence of a state of hypersensitivity in the areas adjacent to the infected area, which prevents the spread of the pathogen (25). In addition to its role in the process of oxidising phenols and converting them into compounds that are more toxic to the pathogen, it is also effective in making the plant more tolerant to unsuitable environmental factors (26, 27).

Determination of glycoalkaloids in tomato leaf extracts by high-performance liquid chromatography (HPLC)

HPLC interpretation and chromatographic profile

The HPLC chromatograms denote the chemical profile of secondary metabolites (GAs) in tomato leaves (Fig. 6, 7). Each peak in the chromatogram represents a specific compound, where the X-axis indicates the retention time (min) and the Y-axis indicates the absorbance intensity (mAU). The area and height of these peaks reflect the concentration of α-tomatine, tomatidine and solanine.

Calibration, linearity and analytical sensitivity

The sequence of the eluted material of the standard was as follows; each standard was at 25 µg/mL. The quantitative determination of GAs was performed based on external standard calibration curves for αtomatine and solanine, demonstrating high linearity ($R^2 > 0.99$). The LOD and LOQ were determined to ensure the sensitivity and reliability required for the identification and quantification of these secondary metabolites in tomato leaf extracts.

Impact of fungal interactions on glycoalkaloid production

The results of the chemical analysis revealed that the interaction between *F. oxysporum* + *T. harzianum* achieved the highest production of α-tomatine (27.122 ppm), tomatidine (34.644 ppm) and solanine (58.985 ppm). These compounds recorded retention times of 2.77, 4.272 and 2.337 min respectively. Compared to plants treated only with *F. oxysporum*, which achieved the lowest production of α-tomatine (12.577 ppm), tomatidine (19.671 ppm) and solanine (48.814 ppm), with retention times of 1.857, 2.763 and 2.360 min

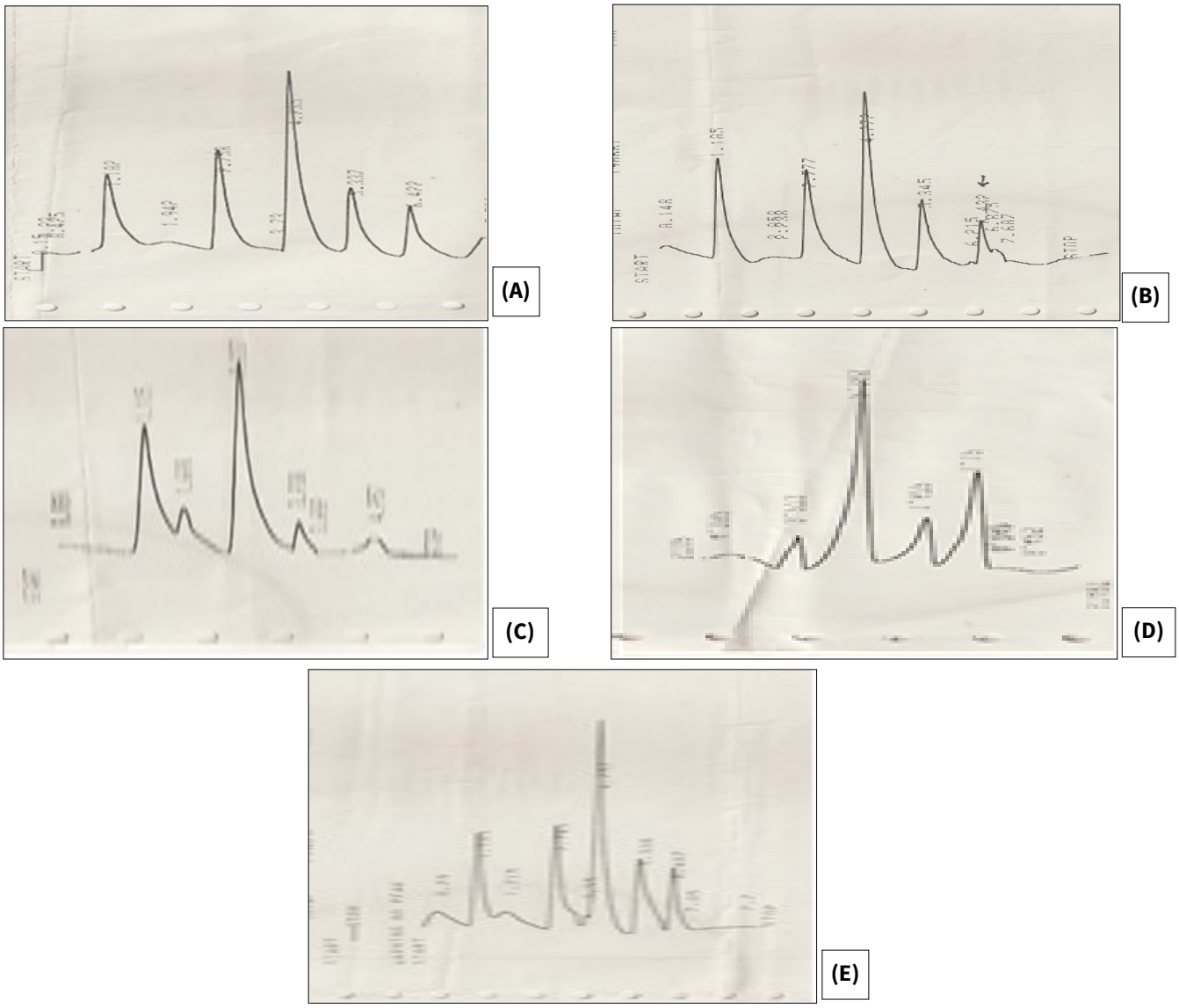


Fig. 6. High-performance liquid chromatography (HPLC) chromatograms of glycoalkaloids in tomato leaf extracts. (A) control; (B) *FOL*; (C) *FOL* + *Trichoderma harzianum*; (D) *FOL* + mycorrhizal fungi; (E) *FOL* + mycorrhizal + *Trichoderma harzianum.*

Table 4. Secondary metabolites (glycoalkaloids) in tomato leaves with HPLC retention times under control treatment

Seq.	Compound	Retention time	Con.
1	α –tomatine	2.758	20.024
2	Tomatidine	4.235	26.892
3	Dihydrotomatidine	5.337	12.919
4	Filo tomatidine	6.422	8.055

respectively.

Detailed chemical analysis of secondary metabolites by treatment

Group 1: Control treatment: For positive comparison, the presence of a number of compounds was detected using HPLC to determine the ratio of a number of compounds (Table 4 and Fig. 6i). Secondary metabolites found in the leaves of tomato plants; the percentage reached of α-tomatine was detected at a rate of 20.024 ppm. While the percentages of tomatidine and dihydrotomatidine and filotomatidine were 26.892 ppm and 12.919 ppm and 8.055 ppm respectively.

Table 5. Secondary metabolites (glycoalkaloids) in tomato leaves with corresponding HPLC retention times under *FOL* treatment

Seq.	Compound	Retention time	Con.
1	α -tomatine	1.857	12.577
2	Tomatidine	2.763	19.671
3	Dihydrotomatidine	4.278	27.638
4	Filotomatidine	5.358	17.133

Group 2: *FOL* treatment: For *FOL* the presence of a number of compounds was detected using HPLC to determine the ratio of a number of compounds (Table 5 and Fig. 6ii). Secondary metabolites found in the leaves of tomato plants, the percentage of α-tomatine was detected at a rate of 12.577 ppm. While the percentages of tomatidine and dihydrotomatidine and filotomatidine 19.671 ppm and 27.638 ppm and 17.133 ppm respectively.

Table 6. Secondary metabolites (glycoalkaloids) in tomato leaves with corresponding HPLC retention times under FOL + mycorrhiza treatment

Seq.	Compound	Retention time	Con.
1	α-tomatine	2.777	27.122
2	Tomatidine	4.272	34.640
3	Dihydrotomatidine	5.345	18.010
4	Filotomatidine	6.215	-

Group 3: *FOL* + mycorrhizal treatment: For *FOL* + mycorrhizal, the presence of a number of compounds was detected using HPLC to determine the ratio of a number of compounds (Table 6 and Fig. 6iii).

Secondary metabolites found in the leaves of tomato plants, the percentage of α-tomatine was detected at a rate of 27.122 ppm. While the percentage of tomatidine and dihydrotomatidine were 34.640 ppm and 18.010 ppm respectively. Filotomatidine was not

Table 7. Secondary metabolites (glycoalkaloids) in tomato leaves with corresponding HPLC retention times under *FOL* + Trichoderma harzianum treatment

Seq.	Compound	Retention time	Con.
1	α -tomatine	2.730	15.581
2	Tomatidine	4.245	29.237
3	Dihydrotomatidine	5.325	25.145
4	Filotomatidine	6.385	13.291

recorded.

Group 4: *FOL*+ *T. harzinum* treatment: For *FOL*+ *T. harzianum* the presence of a number of compounds was detected using a HPLC to determine the ratio of a number of compounds (Table 7 and Fig. 6iv). Secondary metabolites found in the leaves of tomato plants, the percentage of α-tomatine was detected at a rate of 15.581 ppm. While the percentages of tomatidine, dihydrotomatidine and filotomatidine were 29.237 ppm, 25.145 ppm and 13.291

Table 8. Secondary metabolites (glycoalkaloids) in tomato leaves with corresponding HPLC retention times under FOL + mycorrhiza + Trichoderma harzianum treatment

Seq.	Compound	Retention time	Con.
1	α –tomatine	2.743	20.969
2	Tomatidine	4.257	36.699
3	Dihydrotomatidine	5.338	18.131
4	Filotomatidine	6.407	9.556

respectively.

Group 5: Triple treatment : *FOL* + mycorrhizal + *T. harzianum*: For *FO*l+ mycorrhizal + *T. harzianum* the presence of a number of compounds was detected using a HPLC to determine the ratio of a

Table 9. Solanine in tomato leaves with corresponding HPLC retention times under control treatment

Seq.	Compound	Retention time	Con.
1	Solanine	2.358	51.320

number of compounds (Table 8 and Fig. 6v). Secondary metabolites found in the leaves of tomato plants, the percentage reached of α-Tomatine was detected at a rate of 20.969 ppm. While the percentage of tomatidine, dihydrotomatidine and filotomatidine were 36.699 , 18.131 ppm and 9.5561 ppm respectively.

Table 10. Solanine in tomato leaves with corresponding HPLC retention times under FOL treatment

Seq.	Compound	Retention time	Con.
1	Solanine	2.360	48.814

Quantitative determination of solanine across different conditions

Group 1: Control treatment: For control the presence of a number of compounds has been detected using a HPLC to determine the ratio of a number of compounds (Table 9 and Fig. 7i). Secondary metabolites found in the leaves of tomato plants, the percentage reached of solanine was detected at a rate of 51.320 ppm.

Table 11. Solanine in tomato leaves with corresponding HPLC retention times under *FOL* + Trichoderma harzianum treatment

Seq.	Compound	Retention time	Con.
1	Solanine	2.337	58.985

***Group 2: FOL* treatment**: For *FOL* the presence of a number of compounds was detected using a HPLC to determine the ratio of a number of compounds (Table 10 and Fig. 7ii). Secondary metabolites found in the leaves of tomato plants, the percentage of solanine was detected at a rate of 48.814 ppm.

Table 12. Solanine in tomato leaves with corresponding HPLC retention times under *FOL* + mycorrhiza treatment

Seq.	Compound	Retention time	Con.
1	Solanine	2.385	54.594

***Group 3: FOL+ T. harzianum* treatment**: For *FOL* + *T. harzinum* the presence of a number of compounds has been detected using a HPLC to determine the ratio of a number of compounds (Table 11 and Fig. 7iii). Secondary metabolites found in the leaves of tomato plants, the percentage reached of solanine was detected at a rate of 58.985 ppm.

Table 13. Solanine in tomato leaves with corresponding HPLC retention times under FOL + mycorrhiza + Trichoderma harzianum treatment

Seq.	Compound	Retention time	Con.
1	Solanine	2.368	53.999

***Group 4: FOL* + mycorrhizal treatment:** For *FOL* + mycorrhizal, the presence of a number of compounds was detected using a HPLC to determine the ratio of a number of compounds (Table 12 and Fig. 7iv). Secondary metabolites found in the leaves of tomato plants, the percentage of solanine was detected at a rate of 54.594 ppm.

***Group 5:* Triple treatment *FOL* + mycorrhizal+ *T. harzianum*:** For *FOL*+ mycorrhizal + *T. harzianum,* the presence of a number of compounds was detected using a HPLC to determine the ratio of a number of compounds (Table 13 and Fig. 7v). Secondary metabolites found in the leaves of tomato plants, the percentage of solanine was detected at a rate of 53.999 ppm.

Chemical structure and classification of tomato glycoalkaloids

Plants belonging to the *Solanum* genus (Solanaceae) are a common source of GAs. In tomatoes (*S. lycopersicum*), GAs are a type of steroidal glycosides containing nitrogen and they have an amphiphilic nature due to their dual structural components. The initial component, known as aglycones, is a hydrophobic 27-carbon cholestane skeleton with fused nitrogen in the F ring. The second part is a hydrophilic carbohydrate side chain that links to the 3-OH position. Naturally occurring GAs are called α-compounds. The sugar chains can undergo hydrolytic cleavage through enzymatic or chemical processes. Tomato plants produce GAs, namely α-tomatine and dehydrotomatine (28).

Biological activities and plant defense mechanisms

Tomato GAs exhibit diverse biological activities, defending against viruses, fungi, bacteria, inflammation and lowering cholesterol. Additionally, investigations into the impact of *Pseudomonas syringae* on phenolic extracts from tomato leaves have revealed that the extent of microbiological contamination influences the augmentation of phenolic compounds. This phenomenon serves as a responsive mechanism when the leaves come under attack from microorganisms (29).

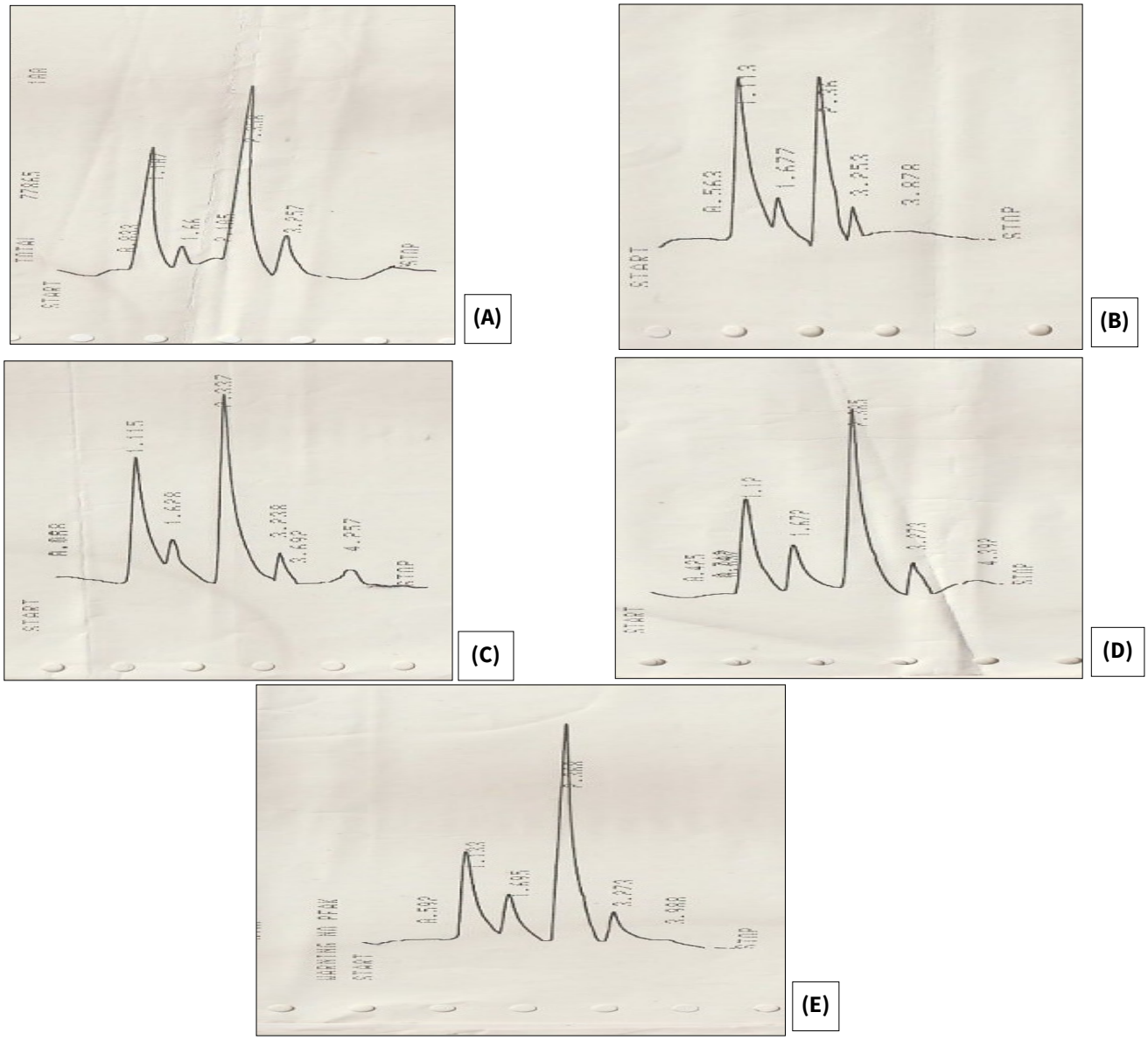


Fig. 7. High-performance liquid chromoatography (HPLC) chromatograms for solanine determination. (A) control; (B) *FOL*; (C) *FOL* + *Trichoderma harzianum*; (D) *FOL* + mycorrhizal fungi; (E) *FOL* + mycorrhizal + *Trichoderma harzianum.*

Glycoalkaloids detected in tomato, such as solanine have shown that that tomatine has a strong ability to bind with cholesterol (30). The production and breakdown of dehydrotomatine and tomatine in tomato (*S. lycopersicum*) leaves may be regulated by different genetic processes (31). Although GAs are not essential for plant growth and functionality, they play a crucial role in plant defense against pests and pathogens, GAs possess antimicrobial and pesticidal properties, GAs have demonstrated various degrees of toxicity dependent on concentration to a broad spectrum of organisms ranging from fungi to humans, causing a variety of damage at various levels of biological system (32).

Conclusion

Fusarium oxysporum has shown high pathogenicity in causing seed rot and seedling death in tomato plants. The isolated strains of *F. oxysporum* were distributed among 2 physiological races (Race 1 and Race 3) and no Race 2 was found, indicating the susceptibility of tomato plants to the first and third races. Treatment with the biofungus *T. harzianum* and mycorrhizal fungi increased the total phenolic content in tomato plants, indicating the activation of a natural plant defense mechanism to resist infection by *F. oxysporum*. There was also a significant increase in the level of the POX enzyme in plants infected with *F. oxysporum*, which confirms the role of these treatments in enhancing the plant's immune response.

Treatment with *T. harzianum* caused a significant increase in the production of defensive secondary metabolites (GAs) such as α-Tomatine, tomatidine and solanine in the leaves of tomato plants compared to infected plants alone. HPLC analysis enabled quantitative detection and retention time determination of secondary metabolites, demonstrating the effectiveness of *T. harzianum* treatment in stimulating the production of these defensive compounds in infected plants.

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Authors' contributions

RAAA carried out the experimental work and drafted the manuscript. ZAMA participated in the study design and performed the data analysis. AM participated in the coordination of the study and helped with manuscript revision. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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