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Research Article

Biocontrol potential of endophytic *Trichoderma* sp. against the pathogenic fungus, *Alternaria alternata* that causes leaf spot in tomato plants

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ABSTRACT: This study was aimed at evaluating the two isolates of the bio-resistant fungi, *Trichoderma harzianum* and *T. koningii* in management of the fungus that causes *Alternaria alternata* leaf spot disease in tomato plants by inducing systemic resistance in the plant. It was demonstrated that two isolates of the pathogenic fungus, *A. alternata* could infect tomato seeds on water agar media. Isolate No. 1 was the most successful, with an infection rate of 63%, while isolate No. 2 achieved an infection rate of 56%. When employed in concentrations of (10, 20, or 30%) with PDA culture medium, bio-resistant fungus filtrate demonstrated suppression of the pathogenic fungus *A. alternata*, with the enhanced levels of inhibition with increasing concentration used. Oxacycloheptadec-8-en-2-one, compound 9,12-Octadecadienoic acid, methyl ester, and many more chemical compounds with the ability to inhibit fungi were discovered via the use of the GC-MS equipment to analyze the fungal infiltrates produced by *T. harzianum* and *T. koningii*. The bio-resistant fungi significantly lessened the severity of the infection caused by the pathogenic fungus, *A. alternata*, reaching a reduction of 33.81% during treatment with *T. harzianum* as opposed to injury of 56.855% in pathogen alone. The tomato leaves that were treated with *T. konhngii* produced the maximum phenolic content (0.56 mg/g).

KEYWORDS: *Alternaria alternata,* phenols*,* tomato plant, *Trichoderma harzianum, Trichoderma koningii*

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INTRODUCTION

Tomato plants are one of the widely grown vegetable crops and have great nutritional value. In terms of their nutritional makeup, tomato fruits are high in moisture (95%), carbs (3%), total fats (1%), and protein (1.2%). They also possess a variety of vitamins, such as vitamins A and C, as well as minerals like calcium, potassium, and sodium (Melfi *et al*., 2018). Both in greenhouses and in open cultivation systems, tomatoes are susceptible to a wide range of agricultural pests, including aphids, cutworms, and a number of diseases like powdery mildew, root rot, leaf spot, and wilting. One of the key pathogen affecting the plant is *Alternaria alternata* since it produces a variety of side effects, such as spots on the leaves, stem necrosis, and black mold on the fruits. Infection with the fungus, *A. alternata* also manifests both during and following harvest (Jabnoun-Khiareddine *et al*., 2016). Studies and research have focused on safer alternatives to chemical pesticides in order to tackle plant diseases because of the harm that they cause to the environment and human health. The two types of induced resistance are local induced resistance, which occurs at the point where the host and the pathogen overlap and are caused by the destruction of host cells surrounding the pathogen, and systemic resistance, which takes place elsewhere. Because of ethylene and jasmonic acid, the resistance products are different from the acquired systemic resistance proteins, which boost output and enhance plant growth (Matrood *et al*., 2022). *Trichoderma* sp. are fungi that induce systemic resistance by raising the activity of phenol and peroxidase enzymes in plants that have been exposed to it (Agrios, 1997). *Trichoderma koningii* is involved in strengthening the plant's defenses and making the defense elements more ready, resulting in a healthy and more resilient plant.

MATERIALS AND METHODS

Isolation and identification of the pathogen

Infected tomato leaves were collected from two different regions in the form of an olive-black spot and transported to the pathology lab of the College of Agriculture / University of Basra. There, they were cleaned with water to remove dirt and impurities before being cut into tiny pieces, each measuring 1 cm in size and surface sterilized. The surface

was treated for 3 mins with 3% sodium hypochlorite solution then washed with sterile water to remove any remaining sterilization solution, placed on Whatman No. 1 filter paper to dry, and then 2-3 small pieces were transferred to Petri dishes containing sterile PDA medium, where they were incubated at a temperature of 25 ± 2 °C for seven days (Poly and Srikanta, 2013).

Trichoderma sp. isolation and identification

Trichoderma isolate was obtained from soil rhizosphere samples collected from tomato-growing areas in Basrah - Iraq governorate. Using media, the antagonistic isolate was isolated using the serial dilution plate method by Matrood *et al.* (2021b). The resultant fungal colonies were purified using the hyphal tip separation procedure, and the fungus was kept on Potato Dextrose Agar (PDA) for further examination.

Testing the pathogenicity of the fungus, *Alternaria alternata*

The two isolated *A. alternata* pathogens were grown on W.A. medium and incubated at a temperature of 25 ± 2°C for three days. Then, sterilized tomato seeds were surface sterilized with 3% sodium hypochlorite (NaOCl) for three mins, and then washed in a circular motion with sterile distilled water. The seeds were then sown around the fungal colonies at ten seeds per plate in addition to the control treatment, by sowing the seeds on the same medium without fungus, the plates were incubated at 25 ± 2°C for 10 days, and the pathogenicity of the fungus *A. alternata* was calculated using a scale of 0 to 5 degrees (Matrood, 2018).

Degree description

0 Seeds intact.
1 Part of the seedling is colored brown with its connection to the fungus.
2 The fungus invades the seed coat, but the seedlings are intact.
3 The seed coat is free of fungus, but infected.
4 Seed cover and seedlings infected.
5 Seeds infected and not germinated.

The severity data were processed by McKinney's formula, which generates a numeric disease severity index (DSI): DSI (%) = (Σvn)/(NV)×100, where v represents the numeric value of the disease index scale, n is the number of plants assigned to the disease index scale, N is the total number of the plants and V is the numeric value of the highest disease index scale.

Isolation and assay of culture filtrates from *Trichoderma* sp.

Both the *Trichoderma* sp. were grown in Potato Dextrose Broth (PDB) liquid culture media. PDB was distributed in 500 ml glass flasks, each flask was inoculated with five tablets, each disk placed 0.5 cm from the border of the fungal colony growing on medium, and each flask was steam sterilized for 20 mins at 121°C and 15 pounds/ang2 of pressure. After cooling each flask was inoculated with five disks of *Trichoderma* sp. that were grown on PDA plates The flasks were then incubated for 14 days at a temperature of 25 ± 2°C while being shaken every third day. After that, Whatman-No.1 filter paper was used to filter the fungal culture The filtrate was then sterilized using a fine filter (Mllipore 0.22 m) and added to PDA medium for each fungus at a ratio of 10, 20, and 30% while taking into account the adjustments made to the agar ratios prior to sterilization. The filtrate-containing medium was poured into Petri plates, and once the medium solidified, a disk of the pathogenic fungus, *A. alternata* measuring 0.5 cm in diameter was placed in the center of each plate, with the control treatment containing the pathogenic fungus in its entirety in a filtrate-free PDA medium. The plates were incubated at 25 ± 2°C. Following treatment, when the pathogenic fungus had reached the edge of the control plate, the growth of the fungus was measured by averaging two orthogonal diameters that passed through the center of the plate.

Compounds present in the filtrate of bioresistant fungi and their identification by GCMS

The filtrate from the two fungi, *T. harzianum* and *T. koningii* were placed in Petri dishes and placed in the freezer for one day and then to the Freeze dryer, after which the samples were extracted using 10% ethanol. 0.2 ml of the extract was taken and injected into the GCMS device to read the materials and compounds present in the leachate.

Studying the biological effect of bio-resistant fungi in stimulating and encouraging the growth of tomato plants in a greenhouse

A fungal inoculum of *T. harzianum* and *T. koningii* at a rate of 1% w/w (fungus/soil) was mixed thoroughly with soil, then the soil was watered and left for three days with continuous watering, then tomato seedlings (4 weeks old) were planted in plastic pots before the flowering stage the treatments were sprayed with a suspension of spores of the pathogenic fungus *A. alternata*. Plant height and fresh and dry weight of the roots were calculated, and then the infection severity was calculated on a 5-point scale after flowering stage as detailed (Table 1). The results were represented in per cent based on control.

Estimation of the total content of phenols

One g of tomato leaves were crushed in a ceramic mortar with the addition of 10 ml of 80% methanol and continuous stirring for 15 mins at a temperature of 70 °C, then 1 ml of the filtrate was added to 5 ml of sterile distilled

Table 1. Injury severity scale

Class/point	Number of spots/leaf
0	No injury
1	1-3
2	4-6
3	7-9
4	Lower leaves die off

water containing 250 µl of Folin reagent in a sterilized glass tube The solution was incubated at a temperature of 25 °C for 30 mins. The optical absorption was estimated using a spectrophotometer at a wavelength of 725 nm. The amount of phenol was calculated on the basis of milligrams of phenols per gram of soft tissue (Meena *et al.*, 2008).

RESULTS AND DISCUSSION

Isolation and identification of the pathogenic fungus, *Alternaria alternata*

Two isolates of *A. alternata* were obtained from infected tomato leaves sourced from two different regions. The fungus colonies were distinguished by their upper surface being olive-black in color with a velvety texture and irregular in shape. When examined microscopically at (40x) power, the spore-bearing back was single and bore a series of small-sized spores. It is characterized by the presence of three to eight transverse septa with longitudinal septa. The spores were neckless or contained a short neck. Its dimensions ranged from 23.45 to 46.90 x 7.70 to 14.00 micrometers (Figure 1). Chen (2018) identified the fungus as *A. alternata* that infects tomato leaves and is characterized by an olive-black color.

Pathogenicity of isolated *Alternaria alternata* of the two isolates of *A. alternata*, isolate number 1 was isolated from the farms of the Svan region, and isolate number 2 was isolated from the farms of the Zubair region. When testing the pathogenic ability of the two isolates in W. A. medium (Table 2), isolate number 1 was more virulent as it infected 63% of the seeds compared to 56% for isolate No. 2.

The superiority of isolate No. 1 may be due to its high ability to secrete toxic compounds such as alternariol, alternic acid, and other compounds that contribute to the severity of plant disease (Matrood *et al.*, 2021). In light of these results, isolate No. 1 was selected for subsequent studies.

Trichoderma sp. fungi isolation and identification

The results obtained revealed that all fungi were recovered from all soil samples. The antagonistic fungi percentage isolated from soil ranged from 12.87% and 8.60%. Statistical analysis revealed a significant difference in fungal densities ($P < 0.05$). The highest fungal frequency was recorded for *Aspergillus niger* (12.87%), followed by *Trichoderma harzianum* (11.94%) followed by *T. koningii* (11.51%). However, the lowest concentration was recorded for *Cladosporium cladosporioides* with a percentage of 8.60% (Table 3).

Effect of filter-sterilized biofilms on *Alternaria alternata* growth in PDA medium

The results (Table 4) showed significant differences between the fungus *T. harzianum* and *T. koningii,* as the highest inhibition rate for the pathogenic fungus *A. alternata* of 61.71% was obtained with filtrate of *T. koningii* at a concentration of 30%, while the highest inhibition rate of the pathogenic fungus was 59.94% with filtrate of *T. harzianum*. *i*. At 20% concentration inhibition was 57.76% for filtrate of *T. koningii* and it was 55.05 % for *T. harzianum.* It was

Table 2. Infection intensity of two isolates of the pathogenic fungus *Alternaria alternata* in W.A medium on tomato seeds

Pathogen isolates	Severity of injury (%)
Isolate number 1	63
Isolate number 2	56

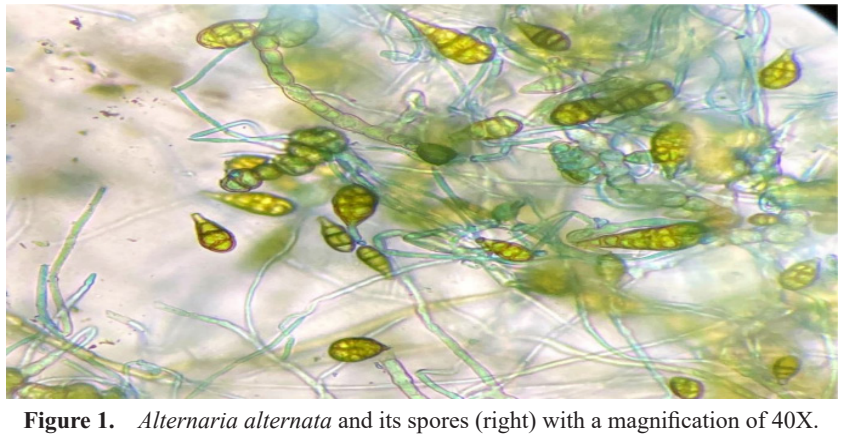

Figure 1. *Alternaria alternata* and its spores (right) with a magnification of 40X.

Table 3. Relative frequency (Rf) of the fungal species isolated from the whitefly adults

Fungal strain	Fungal families	Relative frequency (%)
Aspergillus niger	Trichocomaceae	12.87
A. carponrius	Trichocomaceae	11 .39
A. flavus	Trichocomaceae	10.82
A. oryzae	Trichocomaceae	10.41
Alternaria chlamydospora	Pleosporaceae	8.43
Cladosporium oxysporum	Cladosporiaceae	10.11
C. cladosporioides	Cladosporiaceae	8.60
Fusarium moniliformae	Nectriaceae	9.49
Trichoderma koningii	Trichocomaceae	11.51
T. harzianum	Trichocomaceae	11.94

Table 4. The effect of different concentrations of fungal biofilms on inhibiting the growth of the fungus *Alernaria alternata*

Fungi	Inhibition of fungal growth (%)		
	Leachate concentration		
	10 %	20 %	30 %
T. harzianum	43.41	55.05	59.94
T. koningii	50.58	57.76	61.71
LSD 0.01	for filtrate 3.88 for concentration 1.91		

observed that the percentage of inhibition increased with the increase in the filtrate used. Several studies indicated the efficiency of culture filtrates of *Trichoderma* sp. in inhibiting many plant pathogens, including *A. alternata*. This may be due to the fact that the filtrates of these fungi contain many substances that inhibit the growth of the pathogenic fungus (Deguzman *et al*., 1992).

Characterization of compounds present in culture filtrates of *Trichoderma harzianum* and *T. koningii* by GC-MS

GC-MS analysis showed that the culture filtrates of *T. harzianum* and *T. koningii* contained a variety of chemicals, including Oxacycloheptadec-8-en-2-one, which has strong inhibitory activity against infections (Table 5). This compound having inactivating effects on *Aspergillus*, *Mucor*, and *Fusarium* have been reported. Fatty acids are recognized to be anti-acids and suppressive to infections, and this was suggested by Zain (2009) as the chemical 9,12-Octadecadienoic acid, methyl ester, was discovered. Due to the food medium's high ability to inhibit the tested fungi and lack of spores, many pathogenic fungi, including *Penicillium expansum* and *Aspergillus flavus* are known to be inhibited. There is availability of limited information about plant genes involved in overcoming the effects of organic compounds released by the pathogen and also the genes involved in the release of the antagonistic fungi that favorably affect plant diameter and chlorophyll contents.

Biological effects of bio-resistant fungi on promoting and aiding the growth of tomato plants in a greenhouse

Tomato plants infected with the pathogenic fungus *A. alternata* had their infection severity decreased by the bio-resistant fungi utilized in the experiment. In *T. harzianum* + *A. alternata* treated disease severity was 33.81 % as compared to 56.85 % in pathogen treated whereas it was 41.90 % in *T. koningii* + *A. alternata* treated. Matrood (2018) obtained 81.33 % reduction with *T. harzianum.* Due to their ability to resist plant pathogenic fungi through a variety of mechanisms, including direct parasitism, competition for nutrients, increased availability of elements, production of toxins, and the production of enzymes like protease and chitinase, Trichoderma species rank among the most significant biological fungi for this purpose. Regarding the impact of bio-resistant fungus on the fresh and dry weights of the root system, we see a rise in both of these weights. Alternatively, it can be because the plant can take advantage of certain nutrients, like phosphorus and nitrogen, which are found in most plants' makeup. The results are in conformity with studies conducted by Matrood *et al.* (2021).

Impact of *Trichoderma* on overall phenol content

The phenol concentration was greater in the *T. koningii* treatment than in the pathogenic fungus treatment, reaching 0.56 mg/g wet weight of shoots (Figure 2). Many plant

Table 5. Compounds found in fungus filtrate *Trichoderma harzianum* and *T. koningii* by GC-MS

The name of the chemical compound	Its chemical formula	Molecular weight
Oxacycloheptadec-8-en-2-one	$C_{16}H_{32}O_2$	**256**
9,12-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	**294**
2-Pentenoic acid	$C_5H_8O_2$	**100**
2-Pentenoic acid	$C_5H_8O_2$	**100**
2-Nonenal	$C_9H_{16}O$	**140**
2-Undecenoic acid	$C_{11}H_{20}O_2$	**184**
Octadecanoic acid, docosyl ester	$C_{40}H_{80}O_2$	**592**

Table 6. Biological effect of bio-resistant fungi in stimulating and encouraging the growth of tomato plants in the greenhouse

Treatment	The severity of the injury (%)	Root total weight (g)	
		Fresh weight	Dry weight
Control	9.12	23.62	4.01
T. harzianum	11.87	31.71	5.21
T. koningii	9.12	29.04	4.99
A. alternata	56.85	21.94	3.86
T. harzianum + *A. alternata*	33.81	23.56	3.91
T. koningii + *A. alternata*	41.90	21.85	4.27
T. harzianum + *T. koningii*	11.96	33.40	5.16
LSD 0.05	9.62	4.11	0.93

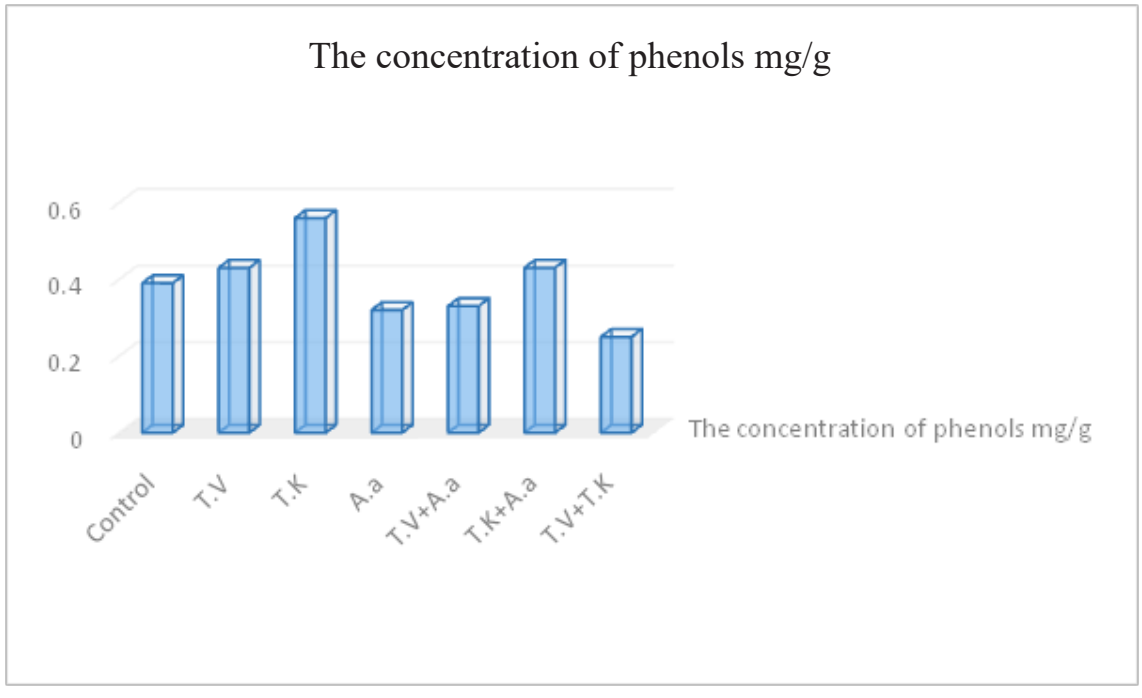

Figure 2. Effect of induction factors on total phenol content in tomato leaves.

diseases, whether they are found on the shoot or root system, may be effectively inhibited by the fungus *T. harzianum* and *T. koningii*. These illnesses include the root rot and leaf spot diseases of tomatoes, cucumbers, and peppers, which are brought on by a variety of fungi, such as *A. alternata* and *Rhizoctonia solani*. By promoting plant systemic resistance to *Fusarium solani* and *Macrophmina phaseolina*, the severity of infection with pathogenic fungus was reduced (Abdel-Kader *et al*., 2012). The amount of phenols in the plant may rise when *Trichoderma* sp. is used. The activity of the enzymes polyphenol oxidase and peroxidase, which operate as a beneficial first defense against the pathogen, increases in correlation with a rise in the concentration of total phenols. The conversion of phenols to quinones, which have higher toxicity to phenol-producing pathogens, is facilitated by an increase in the activity of the enzymes polyphenol oxidase and peroxidase.

CONCLUSION

The pathogenic fungus, *A. alternata* can be effectively controlled by *Trichoderma* species, whether in the laboratory or in the field, because the bio-resistant fungi possess many mechanisms against the pathogens, including parasitism, antagonism, and induction of systemic resistance in plants. It can enhance plant defenses by increasing the amount of phenols and nutrients in the plant. The pathogenic fungus, *T. koningii* had the greatest effect on fungal growth biomarkers.

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