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Research Article

Evaluation of potential antifungal activity of secondary metabolites produced by *Trichoderma harzianum* against Alternaria blight disease of Cumin

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Abstract: This study was aimed to identify secondary metabolites produced by native *Trichoderma harzianum* isolates and investigate the effect of ethyl acetate (EtOAc) extract of constituents extracted on seed germination and control of Alternaria blight disease. The phytochemical constituents of EtOAc extract of *T. harzianum* were identified by gas chromatography-mass spectrometry techniques. Also, the effects of foliar application, seed and soil treatments of EtOAc extract and its constituents: benzoic acid, palmitic acid and diisooctyl phthalate, on Alternaria blight disease index was investigated. All *T. harzianum* isolates were able to significantly reduce the mycelial growth of *A. alternata* by producing volatile and non-volatile metabolites. The major constituents of the EtOAc extract were 2-phenylethanol (14.36%), palmitic acid (12.07%), diisooctyl phthalate (11.23%), which have antifungal effects against *A. alternata*. The lowest values of minimum inhibitory concentration (MIC) of the EtOAc extract and its main constituents were in the range of 1,044-3,970 µg. mL^{-1}. The MIC value of the benzoic acid (1,044 µg. mL^{-1}) against *A. alternata* was lower than iprodione-carbendazim (1,391 µg. mL^{-1}) and mancozeb (1,600 µg. mL^{-1}). Combining benzoic acid with diisooctyl phthalate induced a synergistic activity against *A. alternata* and in combination with palmitic acid caused an additive effect. Seed treatment with EtOAc extract and/or benzoic acid significantly reduced the development of Alternaria blight disease of Cumin compared to foliar and soil applications. The seed treatment with EtOAc extract and palmitic acid significantly improves the seed germination by 11.34% and 9.57%, respectively. The findings provide new perspectives on the effect of the secondary metabolites produced by native *T. harzianum* isolates on the quality characteristics of seeds and the rate of soil-borne and seed-borne diseases progression caused by *A. alternata*.

Keywords: Benzoic acid, Biological control, Germination, Secondary metabolites, Seed quality

Introduction

The Cumin *Cuminum cyminum* L. belongs to the Apiaceae family, and its seeds are rich in secondary metabolites with antioxidant properties. Cumin growth and production are seriously affected by abiotic and biotic stresses. Among the biotic constraints, Alternaria blight

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disease caused by *Alternaria burnsii* Uppal, Patel & Kamat and *A. alternata* (Fr.) Keissl., has been a severe problem in cumin-growing areas of the world, which after the flowering stage affects cumin plant and often results in lower seed yield and quality (Wadud *et al*., 2021). Both *Alternaria* spp. are soil-borne pathogens. They can be seed-transmitted and have been reported in seeds from Cumin. The seed-borne Alternaria are naturally occurring on or within seed, which can result in infection of radicles, reduction of vigor, germination capacity, and blight of seedlings (Khaledi *et al*., 2021; Piri *et al*., 2019).

Several management strategies have been suggested to control Alternaria blight of Cumin, such as using certified and healthy seeds and seed and foliar treatment with synthetic fungicides (Didwania *et al*., 2019). The *Trichoderma* species can, as biological control agents, induce a combination of antagonistic mechanisms, such as antibiosis through the production of volatile (e.g. terpenes) and non-volatile (e.g. trichotoxin and trichodermin) metabolites, mycoparasitism with the production of cell wall-degrading enzymes (e.g. cellulase, chitinase, protease, and glucanase), competition for nutrients and space in colonization sites, induction of resistance in plants through the production and secretion of elicitor molecules (Silva *et al*., 2019). In addition to biocontrol activities, *Trichoderma* spp. can promote plant growth through production of secondary metabolites, morphological and biochemical changes in the host plants, solubilization and sequestration of inorganic nutrients, rhizosphere change, regulation and induction of growth factors (Nieto-Jacobo *et al*., 2017). Various studies have been performed to identify and evaluate the effect of secondary metabolites of *Trichoderma* species (Zeilinger *et al*., 2016; Khan *et al*., 2020). There are few studies investigating the impact that trichoderma-derived natural products have in the biological control of soil and/or seed-borne fungal diseases on various plants (Kubicek *et al*., 2001). Antifungal potential of Trichoderma secondary metabolites is supposed to be associated with high levels of palmitic acid and benzoic acid (Ahluwalia *et al*., 2015; Dini *et al*., 2021).

The seed is one of the most critical inputs of agricultural products, and its quality and health can play an important role in achieving potential real yield (Piri *et al*., 2019). Seed priming is a pre-sowing treatment using suitable materials, including biocontrol agents and their produced secondary metabolites, plant growth regulating agents, nutrients, and chemicals. Biopriming, a sub-category of seed priming, involves inoculation of seed with beneficial microorganisms. Seed priming can improve seed germination, early seedling growth, and establishment under biotic and abiotic stress conditions (Bennett and Whipps, 2008). In an experiment, Tabatabaei and Shakeri (2014) reported that seed priming significantly increases seed germination, root and shoot length, and seedling weight of Cumin. Seed priming with polyethylene glycol improves seed germination and seedling quality of Cumin under temperature and water stress (Rahimi, 2013).

Despite the economic importance of diseases in cumin production, little information is available about the effective and suitable fungicides that can prevent yield losses without causing harmful effects on plants and the environment. Therefore, the main objectives of this study were to (i) screen and select effective native *T. harzianum* isolates against *A. alternata*, (ii) identify and evaluate the antifungal potential of trichoderma secondary metabolites, and (iii) evaluate the effect of seed biopriming, soil treatment and foliar application by EtOAc extract and compounds of benzoic acid, palmitic acid and diisooctyl phthalate, as its main constituent, on decreasing progress of the Alternaria blight disease on cumin plants in greenhouse conditions.

Materials and Methods

Antagonistic and phytopathogenic fungal isolates

Ten isolates of *Trichoderma harzianum* (obtained from the culture collection of Phytopathology

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Laboratory in Ferdowsi University of Mashhad, Iran) were used in this study (Bagheri Azghandi, 2013). The phytopathogenic fungus, isolate *Alternaria alternata* AA14 (Khaledi *et al*., 2021), isolated from infested cumin seed in Mashhad region (Razavi Khorasan province, Iran) was obtained from Seed Health Laboratory in Seed and Plant Certification and Registration Institute, Karaj, Iran.

Plant material and seed health test

Seed of native cumin population obtained from the Seed and Plant Improvement Institute of Karaj, Iran, was used in this study. Before starting the experiment, seed viability was monitored by the tetrazolium (2, 3, 5-triphenyl tetrazolium chloride) test (seed viability = 70.75 %) as a rapid method to replace germination-based assessments.

Antagonistic activity of *Trichoderma harzianum* isolates

The antagonistic effect of all *T. harzianum* isolates was evaluated individually against *A. alternata* by employing the dual culture technique described by Coskuntuna and Özer (2008). The inhibition percentage of mycelial growth was calculated using a formula described previously (Karličić *et al*., 2021).

Influence of metabolites on mycelial growth inhibition

Seven selected *T. harzianum* isolates based on the mycelial growth inhibition assay against *A. alternata* were evaluated for the possibility of producing volatile inhibitory substances *in vitro* conditions following the modified methods of Raut *et al*. (2014). The production of non-volatile substances by the *T. harzianum* isolates against the test pathogen was studied using the method described by Lee *et al*. (2015). As control, the petri plates were inoculated with only *A. alternata*. Each assay was performed in quadruple and then repeated twice. The percent inhibition of mycelial growth of *A. alternata* by volatile and non-volatile compounds was calculated using a formula as described previously (Raut *et al*., 2014).

Extraction of metabolites production

Three 5-mm diameter plugs from isolate *T. harzianum* TH7 were separately inoculated into 5 L conical flasks containing 1 L of sterile liquid broth containing 7.0 g KH_2PO_4, 2.0 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 1.0 g NH_2SO_4, 0.6 g yeast extract and 10 g glucose in 1 L of distilled water and pH 7.2 ± 0.2 as described by Ahluwalia *et al*. (2015). The suspension cultures were incubated for 28 days at 25 ± 1 ˚C. Fungal mycelium was removed by filtration under vacuum using Whatman filter paper. According to Bae *et al*. (2015), 200 mL of ethyl acetate (EtOAc) was added to 400 mL of crude cultural filtrate of fungal isolate, mixed well for 10 min, and kept until two phases got separated. By using a separating funnel, the upper layer of the solvent, which contained the extracted compounds, was separated. The extracted compounds were concentrated and dried using a rotary vacuum evaporator at 36 ˚C to get a red-brown residue. The crude extract was then dissolved in dimethyl sulphoxide (DMSO) at 10 mg. mL^{-1} of concentration and was filtered through 0.22 µm syringe filter before injecting into Gas chromatography-mass spectrometry and the secondary metabolites were kept at -20 ˚C until they were needed for bioassays.

Gas chromatography-mass spectrometry analysis

Secondary metabolites extracted from the isolate *T. harzianum* TH7 were performed with an Agilent 7809A gas chromatograph (Agilent Technologies) coupled to a Triple-Axis detector (Agilent 5975C), using a HP-5 MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm) and helium as a carrier gas at a flow rate of 1 $ml.min^{-1}$. The injector and detector temperature settings were 250 and 280 ˚C, respectively. The column oven temperature was set initially at 50 ˚C for 5 min, followed by a ramp of 4 $˚C.min^{-1}$ until the temperature reached 260 ˚C, which was held for 3 min. The injection volume was found to be 1 µL, and was done in a split-less mode. The mass spectrometer was obtained by electronic impact at 70 eV. The identification of spectra was performed by using the data

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obtained from the NIST (National Institute of Standards and Technology) and WILEY libraries through a comparison of standard mass spectra. Most of the compounds produced by *T. harzianum* were identified using retention times of their reference standard (Shahiri Tabarestani *et al*., 2017).

Determination of effective inhibitory concentrations

The EtOAc extract prepared from the isolate *T. harzianum* TH7 and its main constituents were examined for antifungal activity against *A. alternata*. The standard broth microdilution method was employed in order to determine the minimum inhibitory concentration (MIC) and Inhibitory concentration 50 (IC_{50}) (Plodpai *et al*., 2013). The MIC values were defined as the lowest concentration of EtOAc extract and its main constituents that completely prevented visible fungal growth. IC_{50} (concentration that produces a 50% inhibitory effect) values were graphically calculated from the dose-response curves based on measurements at various concentrations.

Fungitoxicity of EtOAc extract vs chemical fungicides

The efficacy of EtOAc extract was compared with some common fungicides, such as Iprodione + carbendazim (Rovral-TS® [WP 52.5 %]) and Mancozeb (Dithane M-45® [WP 80 %]) by mixing with culture medium assay as described by Abd-El-Khair and El-Gamal Nadia (2011).

Identification of synergistic effects between EtOAc extract constituents

The microdilution checkerboard method, according to Turgis *et al*. (2012), was carried out on 96-well plates to evaluate the synergistic effects of EtOAc extract constituents (benzoic acid, palmitic acid, and diisooctyl phthalate). A fractional inhibitory concentration index (FICI) of the dual combination of EtOAc extract constituents was calculated by using the following formula:

$$FICI = FIC\ A + FIC\ B = \frac{\text{MIC A combined}}{\text{MIC A alone}} + \frac{\text{MIC B combined}}{\text{MIC B alone}}$$

Interaction of the combination of two substances were defined as a synergistic effect if the FICI was ≤ 0.5, additive if $0.5 < FICI < 1$, indifferent if $1 < FICI \leq 4$, and antagonistic if $FICI > 4$ (Gutierrez *et al*., 2008).

Effects of EtOAc extract and its main constituents

Seed biopriming with different treatments was performed using the method described by Entesari *et al*. (2013). Before applying different treatments, cumin seeds were surface-sterilized in 1% sodium hypochlorite solution for 3 min, and rinsed twice in sterile distilled water. The spore suspension of *T. harzianum* isolate was prepared at a concentration of 10^5 spores.ml^{-1} by using the serial dilution method. Carboxymethyl cellulose (CMC) at a concentration of 1% was used to stick the fungal suspension and different compositions to the seeds. Seed biopriming by EtOAc extract and compounds of benzoic acid, palmitic acid, and diisooctyl phthalate at various concentrations ($1 \times IC_{50}$; $0.1 \times IC_{50}$; $0.01 \times IC_{50}$) were obtained by suspending in distilled water and surfactant mixture of 1% CMC with 0.05% Tween-20. Seeds were soaked in each treatment for 5 min before sowing in soil. The soil used in this experiment was a combination of sterilized peat moss, vermiculite, and perlite at a ratio of 2: 1: 1 (v/v/v). For sterilization, the soil was autoclaved at 121 ˚C for a minimum of 20 min at 100 kPa (15 psi) on 2 successive days.

For soil treatment, the surface-sterilized seeds were incubated for five days on a wet sterile filter paper in petri dishes at 25 ± 1 ˚C on distilled water-soaked filter papers. Then, the seeds were each sown in 15 cm-diameter plastic pots. Cumin seedlings were inoculated by spore suspension (10^5 spores.ml^{-1}) of *T. harzianum* at the rate of 100 ml.pot^{-1}. Inoculation of cumin plants was carried out by 10 mL of spore suspension (10^5 spores.ml^{-1}) of *A. alternata* which was sprayed onto the leaves of seedlings. For foliar spray treatment, EtOAc extract and compounds at concentrations mentioned above were sprayed on plants until run-off at two days

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post inoculation (dpi). Inoculated plants were kept for three weeks in the greenhouse at 25 ± 3 ˚C; 16/8 h light/dark photoperiod and irrigated when needed. Four replicate plants were inoculated in a completely randomized design, and the experiment was repeated two times. In all cases, when disease symptoms developed, the pathogen was re-isolated from infected plants. Disease severity was estimated at 21 dpi using a 0-6 disease scale (Pryor and Gilbertson, 2002), and the disease index (DI) was calculated (Khaledi *et al*., 2021). The percentage of disease decrease was calculated using a formula described previously (Plodpai *et al*., 2013).

Statistical analysis

All experiments were set up in a completely randomized design with four replicates and conducted twice. The data were analyzed by one-way analysis of variance (ANOVA) and comparison of means was carried out using Tukey's tests at the level $P < 0.05$. Statistical analysis was performed using SAS software (version 9.2; SAS Institute, Cary, NC, USA).

Results

Effect of *T. harzianum* isolates on mycelial growth of *A. alternata* in vitro

The comparison of the data obtained from the dual culture test showed that all *T. harzianum* isolates inhibited the mycelial growth of *A. alternata* from 13.97% to 65.92%. The highest level of inhibition belonged to TH7, the lowest to TH15 (Fig. 1). Five isolates of *T. harzianum* (TH5, TH7, TH13, TH17, and TH20), which showed the highest levels of inhibiting the pathogen growth in the dual culture test, were used for determining the capability of producing volatile and non-volatile metabolites. The results indicated that *T. harzianum* isolates apparently produced volatile and non-volatile metabolites that suppressed the mycelial growth of the *A. alternata in vitro*. (Fig. 2) and statistically significant difference was observed among all *T. harzianum* isolates tested for the effect of volatile and non-volatile metabolites (Fig. 2). The isolate TH7 had the highest inhibitory effect of volatile metabolites and culture filtrates with 91.53% and 69.88%, respectively (Fig. 2).

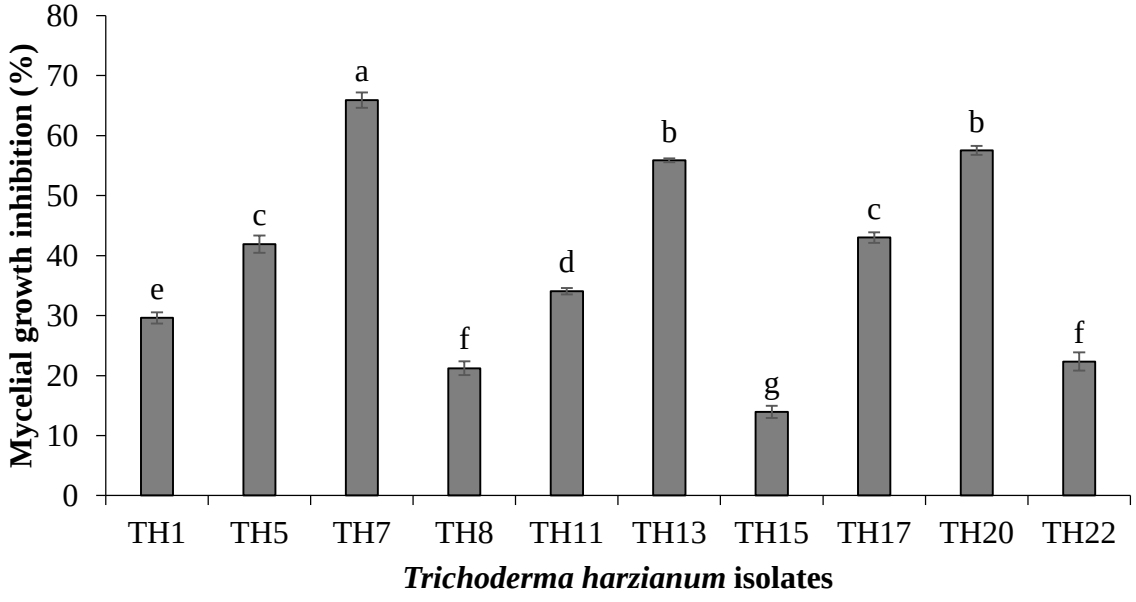

Figure 1 *In vitro* screening of *Trichoderma harzianum* isolates against *Alternaria alternata* by dual culture test (14 days post inoculation). Different letters indicate significant differences according to Tukey's test at the level $p < 0.05$. The bars indicate standard errors (SE).

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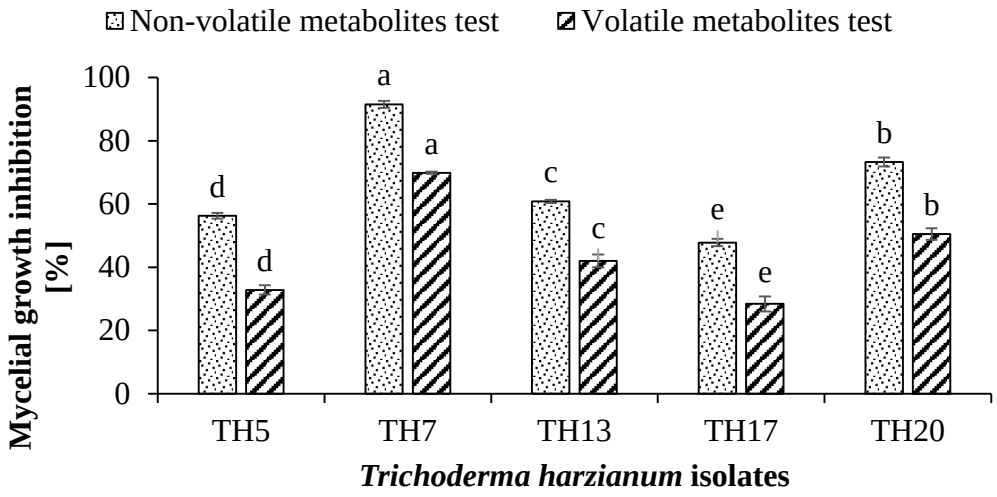

Figure 2 Effect of volatile and non-volatile metabolites of *Trichoderma harzianum* isolates on mycelial growth of *Alternaria alternata*. Different letters indicate significant differences according to Tukey's test at the level $p < 0.05$. The bars indicate standard errors (SE).

Chemical composition of volatile metabolite of the *Trichoderma* EtOAc extract

The chemical composition *Trichoderma* EtOAc extract, as determined by GC-MS analysis is shown in Table 1. Fifteen volatile metabolite compounds were identified and isolated from the *T. harzianum* TH7, constituting about 85.38% of the EtOAc extract. The main volatile metabolites were 2-phenylethanol (14.36%), palmitic acid (12.07%), undecane (11.63%), diisooctyl phthalate (11.23%), benzoic acid (7.21%), 6-amyl-α-pyrone (6.53%), dibutyl phthalate (3.36%), dl-limonene (2.48%), 1,3-dimethylbenzene (3.59%), tetracosane (2.72%), tricosane (2.66%), 1-hexadecanol (2.20%), dihexyl phthalate (1.79), 3,5-di-tert-butylphenol (1.53%) and eicosane (1.02%).

Inhibitory effect of EtOAc extract and its main constituents on mycelial growth

The results of percentage inhibition of mycelial growth of *A. alternata* by EtOAc extract and its main constituents at 2,000 $\mu g.mL^{-1}$ concentration are presented in Fig. 3. Comparing the data obtained from volatile metabolites produced by *T. harzianum* TH7 at 2,000 $\mu g.mL^{-1}$ concentration revealed that all compounds tested were not capable of inhibiting mycelial growth of *A. alternata* (Fig. 3). The compounds 1,3-dimethylbenzene, undecane, 6-amyl-α-pyrone, 3,5-di-tert-butylphenol, 1-hexadecanol, dihexyl phthalate and tetracosane did not have any effect on growth of *A. alternata*, whereas the EtOAc extract and compounds of 2-phenylethanol, dl-limonene, benzoic acid, palmitic acid, dibutyl phthalate, eicosane, tricosane and diisooctyl phthalate inhibited mycelial growth of *A. alternata* from 55.25% to 100% (Fig. 3). The *A. alternata* pathogen did not show any visible mycelial growth in the presence of EtOAc extract, benzoic acid, palmitic acid and diisooctyl phthalate compounds at 2,000 $\mu g.mL^{-1}$ concentration as presented in Fig. 3.

Thus, according to the results of this test, EtOAc extract and compounds of 2-phenylethanol, dl-limonene, benzoic acid, palmitic acid, dibutyl phthalate, eicosane, tricosane and diisooctyl phthalate, which reduced the growth of this phytopathogen were selected and used for determination of the IC_{50} and MIC. Minimum inhibitory concentration (MIC) and inhibitory concentration 50 (IC_{50}) values of EtOAc extract and main constituents produced by *T. harzianum* TH7 with antifungal properties were determined and are shown in Table 2. Different values of MIC for treatments against the growth of *A. alternata* were observed. The MIC values for the EtOAc extract and its main constituents ranged between

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1,044 and 3,970 µg. mL^{-1}. The lowest levels of MIC and IC_{50} were obtained for benzoic acid against *A. alternata* among the EtOAc extract and other compounds tested. The lowest MIC value was related to benzoic acid with 1,044 µg. mL^{-1}. In addition, the lowest and highest IC_{50} values for benzoic acid and dibutyl phthalate were 643 µg.mL^{-1} and 2,002 µg.mL^{-1} (Table 2). The MICs of synthetic fungicides including Iprodione-carbendazi and Mancozeb against *A. alternata* were found to be 1,391 and 1,600 µg.mL^{-1}, respectively, which were lower than that of the benzoic acid compound (Table 2).

The effect of different concentrations of EtOAc extract and compounds of benzoic acid, palmitic acid and diisooctyl phthalate on mycelial growth of *A. alternata* are shown in Fig. 4. The treatments inhibited the growth of pathogen in a dose-dependent manner. The 1 × MIC and 1 × IC50 concentrations of each EtOAc extract and compounds of benzoic acid, palmitic acid and diisooctyl phthalate were equally effective against *A. alternata* without significant differences (Fig. 4). Low level of antifungal activity was observed for EtOAc extract and other compounds at 0.01 × MIC concentration against *A. alternata*. But, at 0.01 × IC_{50} concentration, the treatments did not inhibit the fungal growth (Fig. 4).

Inductive effect of EtOAc extract and its main constituents on seed germination

The results of seed germination tests as affected by EtOAc extract and its main constituents are presented in Fig. 5. Investigating effect of seed treatment on germination and seedling growth revealed that compounds of 1,3-dimethylbenzene, undecane, 3,5-di-tert-butylphenol, 1-hexadecanol, dihexyl phthalate, tetracosane, 2-phenylethanol, dl-limonene, eicosane and diisooctyl phthalate had no effect on germination, whereas the seed treatment with EtOAc extract and compounds of palmitic acid, benzoic acid, dibutyl phthalate, 6-amyl-α-pyrone and tricosane significantly improves germination percentage from 0.35% to 11.34% (Fig. 5). Seed treatment with chemical fungicides had influence on seed germination (Fig. 5). The results showed that the seed treatment with Iprodione-Carbendazim and Mancozeb could successfully enhance seed germination to 6.02% and 4.61%, respectively, which were lower than that of EtOAc extract and palmitic acid compound (Fig. 5). Based on the results of inhibitory effect of fungal growth and inductive effect on seed germination, the compounds of benzoic acid, palmitic acid and diisooctyl phthalate were selected and used for additional experiments.

Table 1 Main volatile metabolite content of *Trichoderma harzianum* isolate TH7 as identified by gas chromatography-mass spectrometry (GC-MS) analysis.

Compound name	Molecular formula	Molecular weight (g.mol^{-1})	RT (min)	Composition [%]
1,3-Dimethylbenzene	C_8H_{10}	106.16	10.785	3.59
2-Phenylethanol	$C_8H_{10}O$	122.16	11.352	14.36
DL-Limonene	$C_{10}H_{16}$	136.23	12.718	2.48
Undecane	$C_{11}H_{24}$	156.31	23.943	11.63
6-Amyl-α-pyrone	$C_{10}H_{14}O_2$	166.22	13.527	6.53
3,5-Di-tert-butylphenol	$C_{14}H_{22}O$	206.32	19.568	1.53
1-Hexadecanol	$C_{16}H_{34}O$	242.44	15.135	2.20
Benzoic acid	$C_{15}H_{22}O_3$	250.33	36.691	7.21
Palmitic acid	$C_{16}H_{32}O_2$	256.42	18.428	12.07
Dibutyl phthalate	$C_{16}H_{22}O_4$	278.34	21.853	3.36
Eicosane	$C_{20}H_{42}$	282.50	23.641	1.02
Tricosane	$C_{23}H_{48}$	324.63	36.769	2.66
Dihexyl phthalate	$C_{20}H_{30}O_4$	334.45	21.225	1.79
Tetracosane	$C_{24}H_{50}$	338.65	39.541	2.72
Diisooctyl phthalate	$C_{24}H_{38}O_4$	390.60	31.353	11.23
Total		-	-	85.38

* Only compounds with quality match scores >75 are listed.

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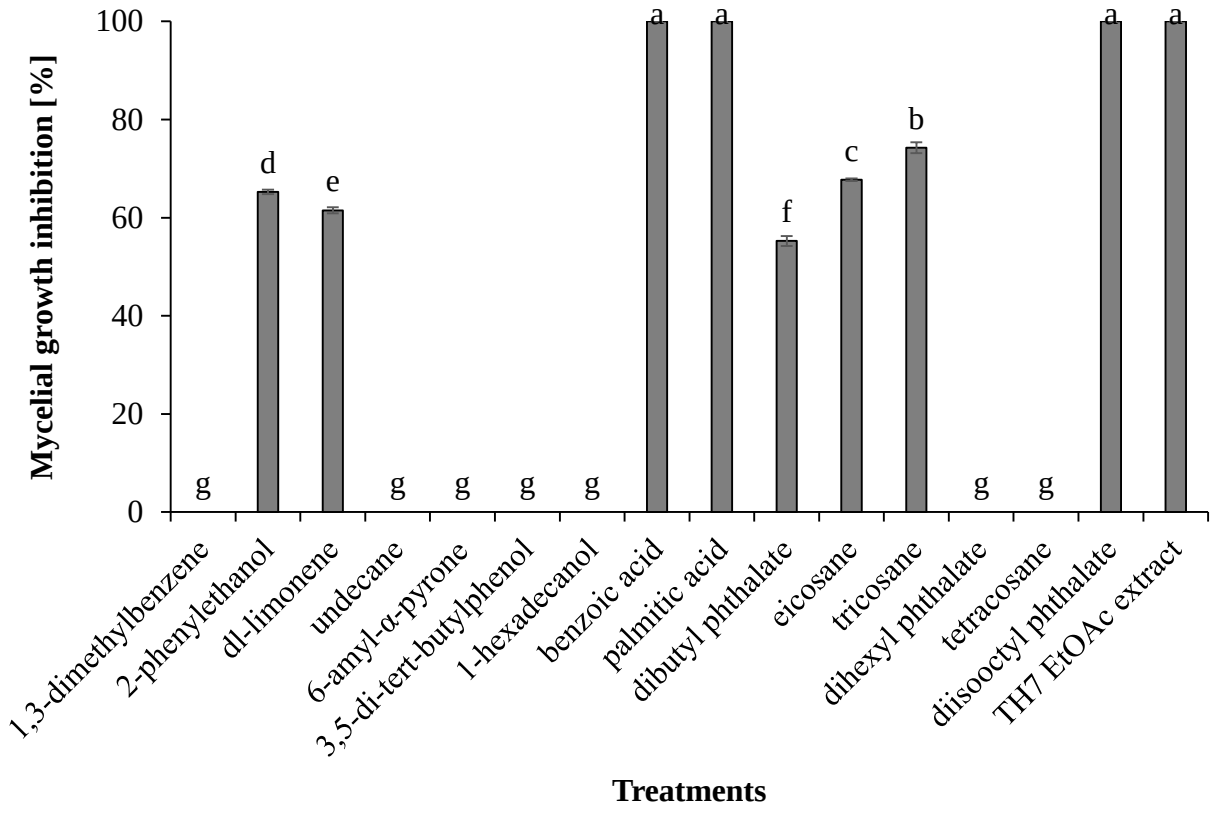

Figure 3 Effect of EtOAc extract and main constituents produced by *Trichoderma harzianum* TH7 at 2,000 µg. mL^{-1} concentration on mycelial growth of *Alternaria alternata*. Different letters indicate significant differences according to Tukey's test at the level $P < 0.05$. The bars indicate standard errors (SE).

Table 2 *In vitro* antifungal activity of EtOAc extract and main constituents produced by *Trichoderma harzianum* TH7 compared to synthetic fungicides against mycelial growth of *Alternaria alternate.*

Treatments	MIC [µg.mL^{-1}]	IC_{50} [µg.mL^{-1}]
Trichoderma harzianum isolate		
EtOAc extract TH7	1896 d	1014 d
Compounds		
2-phenylethanol	2745 i	1432 h
dl-limonene	2980 j	1714 i
benzoic acid	1044 a	643 a
palmitic acid	1995 e	1085 e
dibutyl phthalate	3970 k	2002 j
eicosane	2557 h	1348 g
tricosane	2500 g	1287 f
diisooctyl phthalate	2462 f	1346 g
Fungicides		
Iprodione-carbendazi	1391 b	679 b
Mancozeb	1600 c	789 c

* MIC: minimum inhibitory concentration; IC_{50}: inhibitory concentration with 50% inhibitory effect on the fungal growth. The results are means ± standard errors of four replications.
* Means within a column indicated by the same letter were not significantly different according to Tukey's test at the level $P < 0.05$.

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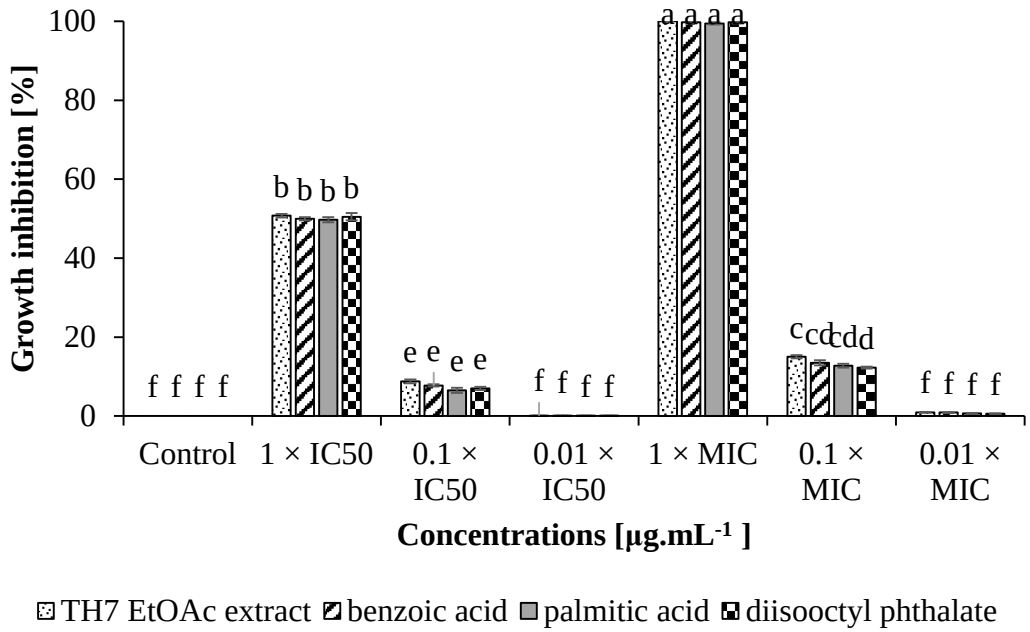

Figure 4 Effects of different concentrations of essential oil and its main constituents on the mycelial growth ± SE of *Alternaria alternata*. Different letters indicate significant differences according to Tukey's test at the level $p < 0.05$. MIC: minimum inhibitory concentration; IC_{50}: inhibitory concentration with 50% inhibitory effect on the fungal growth.

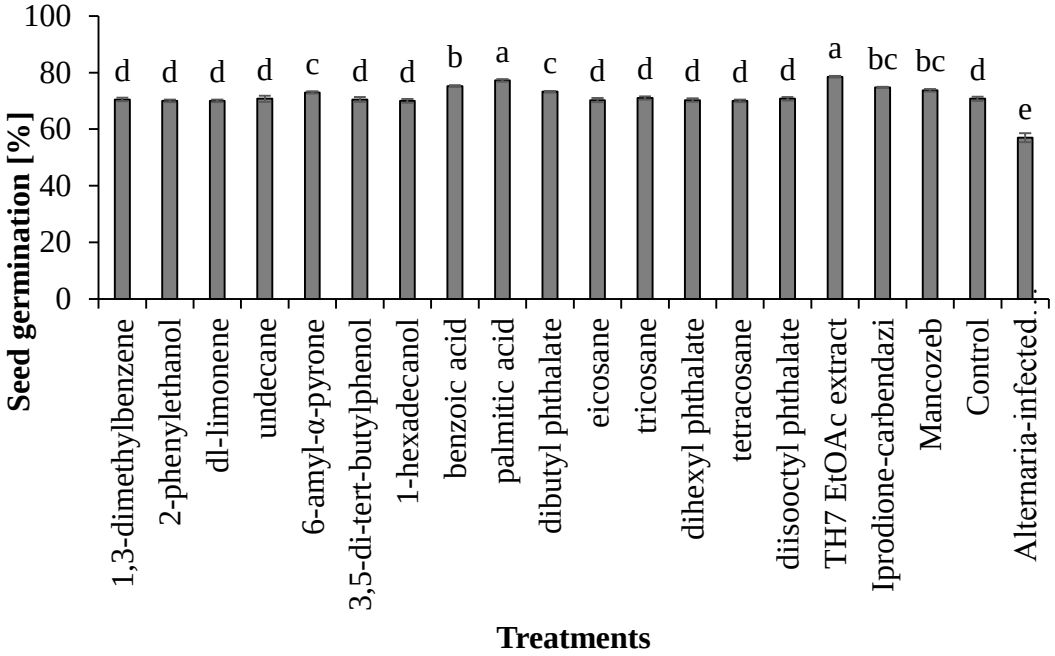

Figure 5 Effect of EtOAc extract and main constituents produced by *Trichoderma harzianum* TH7 on seed germination. Different letters indicate significant differences according to Tukey's test at the level $P < 0.05$. The bars indicate standard errors (SE).

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Synergy assay

To investigate *in vitro* synergistic interactions of combinations of benzoic acid, palmitic acid and diisooctyl phthalate a microdilution checkerboard method was used. According to the obtained results, synergistic effects between benzoic acid × palmitic acid, benzoic acid × diisooctyl phthalate, and palmitic acid × diisooctyl phthalate was observed, and no antagonistic effect was found between the tested constituents. The highest level of synergistic effect was related to a combination of benzoic acid × diisooctyl phthalate with 0.278 FIC index (Table 3).

Efficiency of EtOAc extract and main constituents produced by *T. harzianum*

The data presented in Table 4 indicate that seed biopriming, soil treatment and foliar application with EtOAc extract and main constituents produced by *T. harzianum* TH7 reduced the development of Alternaria blight of Cumin caused by *A. alternata*. Disease index caused by this pathogen on Cumin significantly decreased with seed biopriming by EtOAc extract or other compounds at 1 × IC50 concentration followed by 0.1 and 0.01 × IC50 concentrations. The results showed that higher levels of suppression were obtained for EtOAc extract than for other treatments (Table 4). The EtOAc extract showed the highest antifungal efficacy against *A. alternata*, which could be associated with benzoic acid and palmitic acid as their main constituents. Similar results for Alternaria blight disease were obtained in the experiments using soil treatment and foliar spray. No phytotoxicity on the cumin shoots and leaves at the low concentrations of EtOAc extract and studied compounds was observed in this research. On the whole, highest suppression efficacy in the disease index of Alternaria blight of Cumin was observed using the EtOAc extract, followed by compounds of benzoic acid, palmitic acid, and diisooctyl phthalate (Table 4).

Table 3 The fractional inhibitory concentration index (FICI) of main EtOAc extract constituents with antifungal properties against *Alternaria alternata*.

Compounds	FICI	Activity
benzoic acid × palmitic acid	0.617 b	additive
benzoic acid × diisooctyl phthalate	0.278 a	synergistic
palmitic acid × diisooctyl phthalate	2.045 c	indifferent

* Means within a column indicated by the same letter were not significantly different according to Tukey's test at the level $P < 0.05$.

Table 4 Efficiency of foliar spray, seed and soil treatment using EtOAc extract and main constituents produced by *Trichoderma harzianum* TH7 to control Alternaria blight disease caused by *Alternaria alternata* under greenhouse conditions.

Treatment	Application type	Disease index	Suppression efficacy [%]
Untreated control	seed treatment	86.25 ± 0.47 a	-
	soil treatment	85.00 ± 0.41 a	-
	foliar spray	85.25 ± 0.25 a	-
TH7 EtOAc extract (1 × IC_{50})	seed treatment	56.00 ± 0.91 h	35.06 ± 1.11 a
	soil treatment	70.50 ± 0.64 g	17.06 ± 0.59 a
	foliar spray	76.50 ± 0.48 g	9.97 ± 0.35 a
TH7 EtOAc extract (0.1 × IC_{50})	seed treatment	70.50 ± 0.28 f	18.26 ± 0.21 c
	soil treatment	78.00 ± 0.71 e	8.23 ± 0.50 c
	foliar spray	82.75 ± 0.25 d	4.69 ± 0.01 c
TH7 EtOAc extract (0.01 × IC_{50})	seed treatment	79.25 ± 0.25 d	7.53 ± 0.29 e
	soil treatment	80.00 ± 0.41 d	5.88 ± 0.03 d
	foliar spray	83.75 ± 0.48 bc	1.76 ± 0.34 e
benzoic acid (1 × IC_{50})	seed treatment	67.75 ± 0.85 g	21.42 ± 1.42 b
	soil treatment	75.00 ± 0.41 f	11.76 ± 0.45 b
	foliar spray	79.25 ± 0.25 d	7.03 ± 0.02 b

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Table 4 Continued

Treatment	Application type	Disease index	Suppression efficacy [%]
benzoic acid (0.1 × IC_{50})	seed treatment	76.75 ± 0.48 e	11.01 ± 0.71 d
	soil treatment	78.75 ± 0.48 d	7.35 ± 0.29 cd
	foliar spray	81.25 ± 0.25 e	2.93 ± 0.33 d
benzoic acid (0.01 × IC_{50})	seed treatment	81.75 ± 0.25 c	5.21 ± 0.31 f
	soil treatment	81.50 ± 0.64 c	4.10 ± 1.11 e
	foliar spray	84.50 ± 0.29 ab	0.87 ± 0.29 f
palmitic acid (1 × IC_{50})	seed treatment	75.75 ± 0.75 e	12.17 ± 0.74 d
	soil treatment	79.75 ± 0.25 d	6.17 ± 0.26 d
	foliar spray	81.50 ± 0.50 e	4.40 ± 0.30 c
palmitic acid (0.1 × IC_{50})	seed treatment	81.75 ± 0.63 c	5.22 ± 0.34 f
	soil treatment	81.75 ± 0.25 c	3.81 ± 0.55 e
	foliar spray	83.25 ± 0.25 cd	2.35 ± 0.01 de
palmitic acid (0.01 × IC_{50})	seed treatment	82.75 ± 0.25 c	4.05 ± 0.73 f
	soil treatment	83.50 ± 0.29 b	1.76 ± 0.33 fg
	foliar spray	84.75 ± 0.25 a	0.58 ± 0.33 f
diisooctyl phthalate (1 × IC_{50})	seed treatment	77.00 ± 0.58 e	10.72 ± 0.30 d
	soil treatment	82.00 ± 0.41 c	3.51 ± 0.94 ef
	foliar spray	83.50 ± 0.29 cd	2.05 ± 0.29 e
diisooctyl phthalate (0.1 × IC_{50})	seed treatment	82.00 ± 0.41 c	4.92 ± 0.27 f
	soil treatment	83.75 ± 0.48 ab	1.46 ± 0.87 g
	foliar spray	84.75 ± 0.25 a	0.58 ± 0.24 f
diisooctyl phthalate (0.01 × IC_{50})	seed treatment	84.50 ± 0.29 b	2.02 ± 0.54 g
	soil treatment	84.25 ± 0.25 ab	0.87 ± 0.56 g
	foliar spray	85.00 ± 0.00 a	0.29 ± 0.29 f

* IC_{50}: inhibitory concentration with 50% inhibitory effect on the fungal growth. The results are means ± standard errors of four replications.
* Means within a column indicated by the same letter(s) were not significantly different according to Tukey's test at the level $P < 0.05$.

Discussion

In the present study, the biocontrol capability of EtOAc extract and the main constituents produced by *T. harzianum* against *A. alternata* was investigated using *in vitro* and in *vivo* assays. Furthermore, this is the first report on the effect of seed biopriming, soil treatment, and foliar spray of EtOAc extract and its main constituent compounds, benzoic acid, palmitic acid, and diisooctyl phthalate, on Alternaria blight disease progress.

The results showed reduced seed germination and unequal seedling emergence of Cumin associated with infection by *A. alternata*. Similar results were reported by Kumar (2005), who observed that the percentage germination of cumin seeds infected by fungi decreased with germination and vigor indices. Khaledi *et al*. (2021) reported that germination and vigor indices were decreased due to increased *Alternaria*-infected seeds, which is supported by our results.

The main metabolites were identified and isolated from the *T. harzianum* TH7, including 2-phenylethanol, palmitic acid, undecane, diisooctyl phthalate, benzoic acid, and 6-amyl-α-pyrone, which are similar to findings of Yassin *et al*. (2021) and Siddiquee *et al*. (2012). The EtOAc extract from *T. harzianum* TH7 and compounds of 2-phenylethanol, dl-limonene, benzoic acid, palmitic acid, dibutyl phthalate, eicosane, tricosane, and diisooctyl phthalate indicated antifungal activity. These results were similar to results reported by other investigators. Heflish *et al*. (2020) reported that the benzoic acid has an inhibitory effect against seed-borne pathogens such as *A. alternata*, *Penicilliun citrinum* and *Aspergillus flavus*. Benzoic acid reduced the growth rate of *A. alternata* and *Alternaria citri in vitro* (Embaby *et al*., 2013). Palmitic acid reduced the mycelial growth of

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Alternaria solani, *Colletotrichum lagenarium*, and *Fusarium oxysporum in vitro* (Liu *et al*., 2008). Lykholat *et al*. (2021) showed that the diisooctyl phthalate as the main constituent of ethyl acetate extract produced by *Penicillium* sp. was responsible for its antifungal activity against A. *alternata*. Al-Maawali *et al*. (2021) showed that the tricosane produced by *Meyerozyma guilliermondii* might be involved in the suppression of *A. alternata*.

Our study revealed that seed treatment with EtOAc extract and compounds of palmitic acid, benzoic acid, dibutyl phthalate, 6-amyl-α-pyrone, and tricosane had a significant influence on germination and seedling emergence of Cumin. This is following the results obtained by Ding *et al*. (2019), who reported that palmitic acid can reduce the incidence of plant diseases and promote the growth of seedlings. The application of benzoic acid significantly affected seed germination and growth parameters and could help improve seedling health and vigor (Dawood *et al*., 2019). Seed priming with *T. harzianum* positively affects seed germination under osmotic stress (Mastouri *et al*., 2010). Gao *et al*. (2017) inferred that the dibutyl phthalate inhibits seed germination, root and shoot elongation of wheat seedlings. Khaledi and Assareh (2021) reported that seed treatment with Iprodione-Carbendazim and Mancozeb fungicides significantly influenced germination and vigor indices, which agrees with our observations.

Investigating the synergistic effects of benzoic acid, palmitic acid, and diisooctyl phthalate revealed that the combination of benzoic acid with diisooctyl phthalate induced synergistic activity against *A. alternata* and in combination with palmitic acid caused an additive effect. The indifferent effect was observed in the combination of diisooctyl phthalate and palmitic acid. Wang *et al*. (2010) reported that the interactions between compounds of palmitic acid and benzoic acid had additive effect. A combination of rich extracts of diisooctyl phthalate with benzoic acid exhibited superior and synergistic effects compared to individual extracts (Khalid *et al*., 2022). Using EtOAc extract and/or a combination of constituents produced by *T. harzianum* TH7 could reduce the effective dose of constituents and expand the antifungal spectrum.

The effect of antifungal activity was previously reported for the volatile and non-volatile metabolites released by *T. harzianum* against *A. alternata* (Gveroska and Ziberoski, 2012; Shafique *et al*., 2019), which agrees with our observations. Our results indicate that the level of volatile compounds of antifungal activity was higher than non-volatile compounds produced by *T. harzianum* and testing their antifungal efficacy against *A. alternata.* So, it may be probable that most of the antifungal activity of the EtOAc extract from *T. harzianum* TH7 was due to its volatile compounds. Antifungal activity of 2-phenylethanol, dl-limonene, benzoic acid, palmitic acid, dibutyl phthalate, eicosane, tricosane, and diisooctyl phthalate increased with increasing their concentration. The minimum concentration of the EtOAc extract from *T. harzianum* TH7 and its main constituents required to inhibit mycelial growth of *A. alternata* differed.

In this study, the EtOAc extract and compounds of benzoic acid, palmitic acid, and dibutyl phthalate had the best inhibitory effects on the mycelia growth of *A. alternata* with MIC value of less than 2,500 µg. mL^{-1} *in vitro*. These findings were in accord with the results obtained by Pundir and Jain (2010), Yoon *et al*. (2012) and Yuyama *et al*. (2020). Investigating fungistatic and/or fungicidal effects of the EtOAc extract and its main constituents showed that EtOAc extract and compounds of benzoic acid, palmitic acid, and diisooctyl phthalate, had fungistatic activity against *A. alternata*. The IC_{50} and MIC values obtained for benzoic acid were considerably lower than the values obtained for the synthetic fungicides tested. This is a novel finding, suggesting that the EtOAc extract and benzoic acid might be used as a powerful biological agent instead of synthetic fungicides and would be more effective in reducing Alternaria blight disease progress. Investigations on mechanisms of Alternaria blight disease

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suppression by *T. harzianum* have suggested that the volatile and non-volatile metabolites secreted may affect the pathogen directly and also while promoting host plant growth parameters, causing induced activation of defense responses in cumin plants, leading to a reduction of disease progress. This is in accordance with previous reports on the effects of *Trichoderma* on host plant growth improvement and control of plant pathogens (Silva *et al*., 2019; Alfiky and Weisskopf, 2021). In this study, we used three methods of seed biopriming, soil treatment, and foliar spray with EtOAc extract and compounds of benzoic acid, palmitic acid, and dibutyl phthalate to evaluate Alternaria blight disease control. The results of the greenhouse experiment showed that using EtOAc extract and compounds of benzoic acid, palmitic acid, and dibutyl phthalate as a foliar spray, soil, and seed treatment was effective in reducing Alternaria blight of Cumin caused by *A. alternata* in a dose-dependent manner.

In most cases, seed biopriming and soil treatment were more effective in disease suppression than foliar spray. One of the reasons for this result might be the possibility that the EtOAc extract and some of its constituent ingredients improve seed germination, seedling emergence, and growth parameters of Cumin. Seed biopriming seems to lead to higher induction of defense responses in cumin plants, thus further leading to a reduction of disease progress than soil treatment and foliar spray. Similar results were obtained by Nehela *et al*. (2021), who reported that the application of benzoic acid significantly reduced early blight disease of tomato and promoted plant growth parameters such as plant height, and fresh and dry weights under greenhouse conditions. Moreover, it seems that pre-sowing seed biopriming leads to higher induction of plant defense mechanisms. This finding is in accord with observations of Ge *et al*. (2020), who demonstrated seeds treated with dibutyl phthalate significantly affect the germination, growth of the vegetables and antioxidant defense system. The application of palmitic acid significantly alleviated wilt disease by inducing positive plant-microbial interactions and promoted the growth of watermelon (Ma *et al*., 2021). Ahsan *et al*. (2017) reported that the eicosane and dibutyl phthalate as bioactive compounds were effective for control of target spot disease.

Our research is the first to provide evidence for the efficacy of bioactive compounds produced by *T. harzianum* against Alternaria blight disease of Cumin *in vivo*. The EtOAc extract was more effective in decreasing the disease index of *A. alternata* on Cumin than compounds of benzoic acid, palmitic acid and dibutyl phthalate. EtOAc extract and compounds of benzoic acid and palmitic acid decreased the disease index of *A. alternata* on Cumin and may represent new alternative disease management strategies. The findings of these results are supported by the studies of Nehela *et al*. (2021), who demonstrated the potential application of benzoic acid and its hydroxylated derivatives as a sustainable alternative control strategy for early blight disease. Likewise, exogenous application of benzoic acid significantly reduced the disease severity and disease incidence of rice brown spot (Shabana *et al*., 2008). The findings of this study confirm that the high antifungal activity and growth-promoting characteristics of EtOAc extract increased activity that can be attributed to the functional activity of each of the components and their interaction. The volatile and non-volatile metabolites produced by *Trichoderma* are a rich source of bioactive compounds, which have been reported to have various antifungal properties and promoted plant growth at the early stage (Lee *et al*., 2016; Jangir *et al*., 2021), which are in agreement with our results.

This research showed that EtOAc extract and some of its constituent ingredients could decrease the progress of diseases caused by *A. alternata* in Cumin. In conclusion, EtOAc extract and benzoic acid could be applied as an alternative to synthetic fungicides to control *A. alternata*. These results suggest that the EtOAc extract of *T. harzianum* after suitable formulation could control Alternaria blight of Cumin caused by *A. alternata*.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Formal consent

For this type of study formal consent is not required.

Human participants

This paper does not contain any studies with human participants performed by any of the authors.

Informed consent

Additional informed consent was obtained from all individual participants for whom identifying information is included in this paper.

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ارزیابی فعالیت ضدقارچی متابولیت‌های ثانویه تولید شده توسط *Trichoderma harzianum* علیه بیماری سوختگی آلترناریایی زیره سبز

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چکیده: این مطالعه با هدف شناسایی متابولیت‌های ثانویه تولید شده توسط جدایه‌های بومی *T. harzianum* و بررسی تأثیر عصاره اتیل‌استات (EtOAc) و ترکیبات استخراج شده روی جوانه‌زنی بذر و مهار بیماری سوختگی آلترناریایی می‌باشد. ترکیبات فیتوشیمیایی عصاره EtOAc با استفاده از روش کروماتوگرافی گازی متصل به طیف‌سنج جرمی شناسایی شدند. هم‌چنین اثرات محلول‌پاشی، تیمارهای بذر و خاک با عصاره EtOAc و ترکیبات آن، بنزوئیک‌اسید، پالمیتیک اسید و دی‌ایزواکتیل فتالات روی شاخص بیماری سوختگی آلترناریایی مورد بررسی قرار گرفت. تمامی جدایه‌ها قادر به کاهش رشد میسلیومی قارچ بودند. ترکیبات اصلی شناسایی شده در عصاره EtOAc شامل ۲-فنیل اتانول (۱۴/۳٦ درصد)، پالمیتیک اسید (۱۲/۰۷ درصد)، دی‌ایزواکتیل فتالات (۱۱/۲۳ درصد) بودند، که دارای اثرات ضدقارچی هستند. کم‌ترین مقادیر حداقل غلظت‌های مهارکنندگی عصاره EtOAc و ترکیبات اصلی آن علیه *A. alternata* در محدوده ۳۹۷۰-۱۰٤٤ میکروگرم در میلی‌لیتر متغیر می‌باشد. میزان حداقل غلظت مهارکنندگی بنزوئیک‌اسید (۱۰۴۴ میکروگرم در میلی‌لیتر) کم‌تر از ایپرودیون-کاربندازیم (۱۳۹۱ میکروگرم در میلی‌لیتر) و مانکوزب (۱٦۰۰ میکروگرم در میلی-لیتر) بود. ترکیب بنزوئیک اسید با دی‌ایزواکتیل‌فتالات موجب فعالیت سینرژیستی و در ترکیب با پالمیتیک اسید موجب فعالیت افزایشی علیه *A. alternata* شد. تیمار بذر با عصاره EtOAc و/یا اسید بنزوئیک در مقایسه با تیمارهای محلول‌پاشی و خاک به‌طور قابل‌توجهی موجب کاهش بیماری سوختگی آلترناریایی زیره سبز شد. تیمار بذر با عصاره EtOAc و پالمیتیک اسید به‌طور قابل‌توجهی روی جوانه‌زنی بذر تأثیر گذاشته و سطح آن را به‌ترتیب ۱۱/۳٤ درصد و ۹/۵۷ درصد بهبود می‌بخشد. یافته‌های این پژوهش دیدگاه‌های جدیدی را درباره تأثیر متابولیت‌های ثانویه تولید شده توسط جدایه‌های بومی *T. harzianum* روی خصوصیات کیفی بذر و میزان پیشرفت بیماری‌های خاکزاد و بذرزاد ناشی از *A. alternata* ارائه می‌دهد.

واژگان کلیدی: اسید بنزوئیک، مهارزیستی، جوانه‌زنی، متابولیت‌های ثانویه، کیفیت بذر

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