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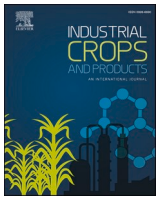

Inhibition efficacy of *Tetradium glabrifolium* fruit essential oil against *Phytophthora capsici* and potential mechanism

Bi Wang [a,1], Pirui Li [a,1], Jingjing Yang [a,b], Xuhong Yong [a], Min Yin [a], Yu Chen [a], Xu Feng [a,*], Qizhi Wang [a,*]

[a] Jiangsu Key Laboratory for the Research and Utilization of Plant Resources, The Jiangsu Provincial Platform for Conservation and Utilization of Agricultural Germplasm, Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing 210014, China
[b] Nanjing University of Chinese Medicine, Nanjing 210023, China

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ABSTRACT

Phytophthora capsici is a notorious pathogen capable of infecting various host plants and causing serious threats to agriculture worldwide. *Tetradium glabrifolium* fruit essential oil (TFO) is widely utilized in traditional Chinese medicine treatment, but its antimicrobial activity is unknown. In this study, we first investigated the inhibitory efficacy of TFO against *P. capsici* and found TFO strongly inhibited the development of phytophthora blight on pepper by *in vivo* inoculation assay. Second, we found TFO could significantly suppress vegetative and reproductive growth of *P. capsici in vitro*. Microscope observations indicated that TFO destroyed the mycelial morphology and cellular ultrastructure of *P. capsici*. Additionally, TFO could damage the cell membrane integrity of the pathogen, leading to cellular leakages of intercellular contents of *P. capsici*. Third, TFO could enhance the activities of defence-related enzymes in pepper fruits during pathogen infection. Finally, we identified the chemical composition of TFO by GC-MS analysis and demonstrated three abundant components (D-limonene, *β*-elemene, and 2-tridecanone) contributed to the antifungal activity of TFO. Together, out results revealed that TFO had outstanding inhibitory efficacy against *P. capsici*, and uncovered its potential antifungal mechanism. This study would propose the application of TFO as an efficient alternative approach for controlling phytophthora blight.

1. Introduction

Phytophthora capsici Leonian is a destructive soilborne pathogen in agriculture that causes substantial economic losses worldwide. It has been reported to attack more than 50 species of agriculturally important vegetables that includes solanaceous, cucurbitaceous, and fabaceous crops (Lamour et al., 2012; Kamoun et al., 2015). Typical symptoms caused by this pathogen includes stem rot, fruit rot, wilting, stunting, damping-off, as well as stem and leaf blight (Hausbeck and Lamour, 2004; Lamour and Kamoun, 2009). *P. capsici* has been an important pathogen limiting vegetable growth and leading to tremendous losses (Ölmez, 2006; Kamoun et al., 2015).

To date, the application of chemical fungicides remains the most effective measure to control phytophthora blight caused by *P. capsici* due to their efficiency and stability (Hausbeck and Lamour, 2004). However, prolonged usage of chemical fungicides may result in pathogen resistance and environment pollution (Keinath, 2007; Siegenthaler and Hansen, 2021). Therefore, it is urgently required to develop alternative fungicides to control phytophthora blight. Plant essential oils (EOs) are mixtures of volatile aromatic components, which are biosynthesized in various parts of plants. Many plant EOs are categorized as "generally recognized as safe (GRAS)" due to their ecofriendly properties by FDA, and many of them have effective antimicrobial activities (de Souza et al., 2009; An et al., 2019). Hence, in recent years, a variety of plant EOs were reported to be widely used to control plant diseases. For example, cinnamon oil and lemongrass oil were found to effectively suppress the growth of pomegranate pathogens *Botrytis* sp., *Penicillium* sp., and *Pilidiella granati* in pomegranate (Munhuweyi et al., 2017). More recently, (Kong et al., 2020) reported that tea tree oil effectively controlled the rot disease caused by *Aspergillus ochraceus* in grape.

Tetradium glabrifolium (Champ. ex Benth.) T.G. Hartley is a medium-sized shrub, which is widely distributed in most area of eastern Asia and

* Corresponding authors.
E-mail addresses: fengxucnbg@cnbg.net (X. Feng), wangqizhi@cnbg.net (Q. Wang).
[1] These authors contributed equally to this paper.

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north-eastern India. *T. glabrifolium* belongs to the Rutaceae family, *Tetradium* genus, although in the early years it had been assigned into *Euodia* genus (Wu et al., 2009). *T. glabrifolium* has been widely utilized in traditional Chinese medicine treatment, and its fruit is used as a substitute of traditional medicine for the treatment of stomachache and headache. Furthermore, leaves and roots of this plant are used in patients with cough, chronic ulcers of lower extremities, or burn and scald wounds in folklore (Liu et al., 2006). To date, no adverse health effect from the use of *T. glabrifolium* has been reported. *T. glabrifolium* fruit essential oil (TFO) is a mixture of volatile compounds derived from the fruit of *T. glabrifolium,* and the chemical composition of TFO has been investigated (Liu et al., 2014). Moreover, TFO and its three constituents 2-tridecanone, 2-undecanone, and D-limonene have been reported to exhibit strong larvicidal effects on Asian tiger mosquito (Liu et al., 2014). However, whether TFO has antimicrobial effects to phytopathogens has not been reported.

In the present study, our objectives were to 1) investigate the inhibitory efficacy of TFO against *P. capsici* on pepper; 2) assess the effects of TFO on vegetative and reproductive growth of *P. capsici in vitro*; 3) explore the action mechanism of TFO by microscopic observation, cell membrane integrity detection, and defence-related enzyme activity analysis; 4) clarify the chemical composition and main effective constituents of TFO. Our results may propose an efficient alternative approach for controlling phytophthora blight.

2. Materials and methods

2.1. Plant materials

Pepper (*Capsicum annuum* L. cv. Sujiao 5) plants at four- to six-leaf stage were obtained from vegetable fields in Nanjing, Jiangsu Province, China. Mature pepper fruit without any physical injuries or pathological diseases were harvested from the same vegetable fields. The whole plant, leaves, and fruit of pepper were immediately transported to our laboratory. Leaves and fruit which were uniform in size, color, and shape were selected, surface-sterilized with 2% NaOCl, and washed with sterile distilled water before use.

2.2. Pathogen

P. capsici (strain LT263) was kindly provided by Prof. Daolong Dou (Nanjing Agricultural University, China) and cultivated on V8-agar (10% V8 juice, 0.02% $CaCO_3$ and 1.5% agar) plates at 25 ℃.

2.3. TFO and chemicals

Fruits of *T. glabrifolium* were harvested from Jinhua, Zhejiang Province, China (29°16′ N, 120°13′ E) in August 2019. For TFO isolation, the fresh fruits were subjected to hydrodistillation for 3 h using a round-bottomed flask, and TFO was collected in a sealed glass bottle. After that, TFO was dried by anhydrous Na_2SO_4. Components of TFO were separated and identified by gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890 N gas chromatograph equipped with an Agilent 5973 N mass spectrometric detector, with a HP-5MS (30 m × 0.25 mm × 0.25 μm) capillary column (Wang et al., 2019). TFO was diluted with acetone according to the ratio of 1:100 (v/v). An aliquot of 1 μL diluted TFO at 10 g L^{-1} was injected in the 1:10 split mode. The MS was operated in the electron impact ionization mode. Spectra were scanned from 20 to 650 amu at the rate of 5 scans s^{-1}. The retention indices were determined in relation to a homologous series of *n*-alkanes (C_8-C_{24}) run under the same conditions. The volatile compounds were identified by comparing their mass spectra and retention indices with the National Institute of Standards and Technology library. The relative percentages of the main components were calculated based on the normalized GC peak areas. The experiment was carried out three times with similar results.

Five most abundant compounds of TFO were purchased (Table S1). D-limonene (purity > 99.0%), 2-undecanone (purity ≥ 99.5%), and *β*-elemene (purity = 95.0%) were purchased from Aladdin Biotechnology (Shanghai, China). Caryophyllene (purity ≥ 98.0%) and 2-tridecanone (purity ≥ 98.0%) were purchased from Sigma-Aldrich (St. Louis, USA). TFO and all these chemicals were dissolved in acetone at 10 g L^{-1} as stock solutions at 4 ℃ until use, and Tween-80 at final concentration of 0.1% was used to help dissolve TFO in water or V8 culture medium. All the other normal reagents were purchased from Aladdin Biotechnology (Shanghai, China).

2.4. In vivo inhibitory efficacy of TFO on P. capsici infection

The inhibitory efficacy of TFO against *P. capsici* infection on pepper leaves was determined according to (Wang et al., 2021). The stock solution of TFO was diluted to 50, 100, 200, and 500 mg L^{-1} in sterile distilled water containing 0.1% Tween-80. Detached pepper leaves were sprayed with TFO solutions at indicated concentrations until liquid flowed on the surface. Leaves sprayed with sterile distilled water or 200 mg L^{-1} of metalaxyl solution were used as controls. To determine protective activity, marginal mycelial plugs (diameter = 5 mm) of *P. capsici* were inoculated on these sprayed leaves at 24 h after treatment with TFO. To determine curative activity, another set of pepper leaves was inoculated with *P. capsici*; then, these inoculated leaves were sprayed with TFO at 24 h after inoculation. Inoculated leaves were incubated under long-day photoperiod for 4 d at 25 ℃. The lesion area produced by *P. capsici* were measured every 24 h after inoculation. Each treatment comprised three replications with at least ten pepper leaves.

The effect of TFO on phytophthora blight on pepper fruit was tested according to (Pei et al., 2020). Pepper fruits were wounded (wound = 2 mm wide × 3 mm deep) with a stainless needle at the equator. An aliquot of 5 μL TFO solutions at different concentrations (50, 100, 200, and 500 mg L^{-1}) was injected into each wound. Fruits injected with sterile distilled water or 200 mg L^{-1} of metalaxyl solution were used as controls. For protective activity, marginal mycelial plugs of *P. capsici* were inoculated on these injected fruits at 24 h after treatment with TFO. For curative activity, pepper fruits were inoculated with *P. capsici* for 24 h, then injected with TFO. Inoculated fruits were incubated at 25 ℃ for 4 d. The lesion area produced by *P. capsici* were also measured every 24 h after inoculation. Each treatment comprised three replications with at least 15 pepper fruits. The control efficacy = (lesion area of sterile distilled water treatment - lesion area of drug treatment)/lesion area of sterile distilled water treatment.

P. capsici is a soilborne disease, to further clarify the effect of TFO against *P. capsici* on pepper, root-drench experiment was performed according to (Reeves et al., 2013). Briefly, at about 3 cm from the root of pepper plants, a glass rod (diameter = 8 mm) was used to pierced a 3 cm deep hole. Then, an aliquot of 10 mL TFO solutions at different concentrations (500, 800, and 1000 mg L^{-1}) was injected into each hole. Pepper plants injected with sterile distilled water or 500 mg L^{-1} of metalaxyl solution were used as controls. To determine protective activity, 5 mL of 1×10^4 mL^{-1} spores were injected into the hole at 24 h after treatment with TFO. To determine curative activity, pepper plants were first inoculated with *P. capsici* for 24 h, then these inoculated plants were injected with TFO. Inoculated plants were incubated under long-day photoperiod for 14 d at 25 ℃. The disease severity was recorded using crown rot system scale from 0 to 5. A scale of 0 indicates a healthy plant; 1 = symptom of less than 10 mm on the stem; 2 = 10–20 mm; 3 = 20–30 mm; 4 = symptom of more than 30 mm on the stem; 5 = damping off (Fig. S1; modified from Chen et al., 2016). The disease index = ∑(scale × plant numbers). Each treatment had 20 replicates, and the experiment was repeated three times in the greenhouse. The control efficacy = (disease index of sterile distilled water treatment - disease index of drug treatment)/disease index of sterile distilled water treatment.

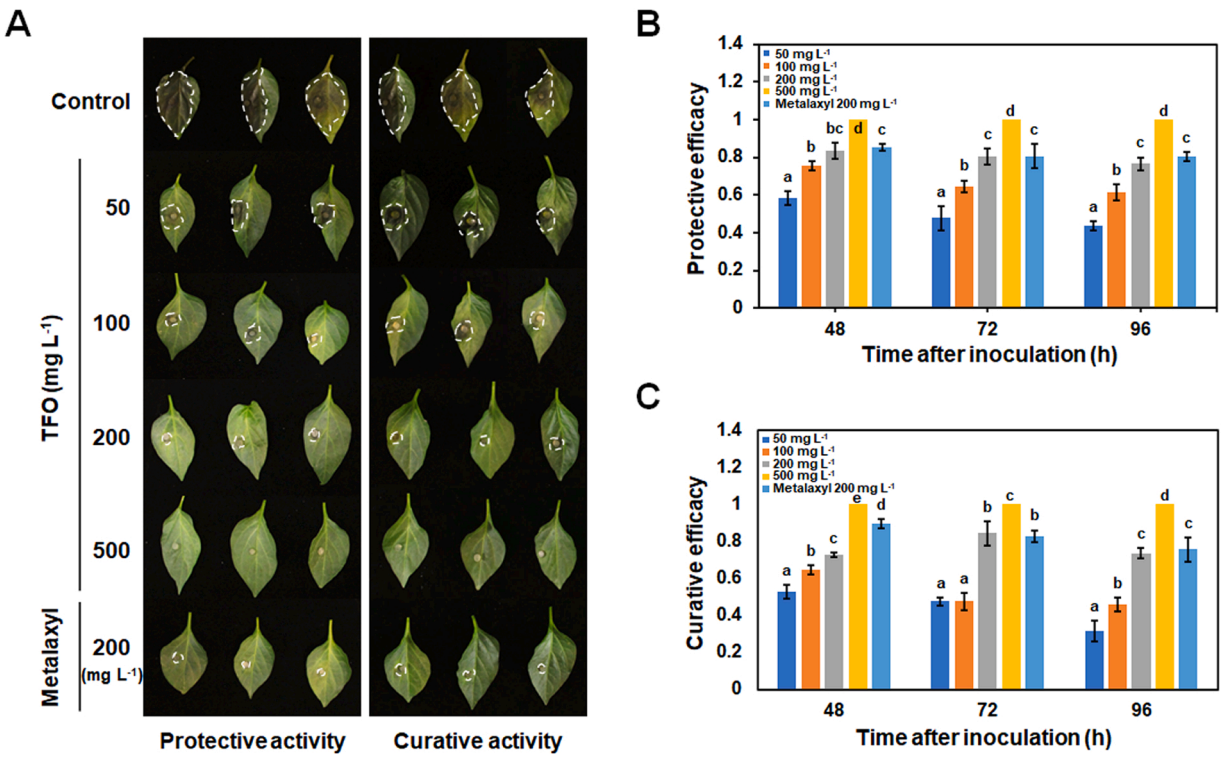

Fig. 1. Tetradium glabrifolium fruit essential oil (TFO) inhibited phytophthora blight on pepper leaves. Detached pepper leaves were inoculated with Phytophthora capsici before or after being sprayed with TFO solutions at indicated concentrations (50, 100, 200, and 500 mg L^{-1}) for 24 h. Leaves sprayed with sterile distilled water or metalaxyl solution were used as controls. Inoculated pepper leaves were placed at 25 ℃ under long-day photoperiod for 4 d. Disease symptoms on pepper leaves were photographed (A), protective and curative efficacies (B and C) were assessed every 24 h after inoculation. Each treatment comprised three replications and each replication contained at least ten pepper leaves. Vertical bars represent SE of the means. Different little letters over the columns show significant difference ($p < 0.05$).

2.5. In vitro inhibitory activity of TFO against P. capsici

The inhibitory effect of TFO on mycelial growth of *P. capsici* was tested according to (Wang et al., 2019). TFO at different concentrations (1.25, 2.5, 5, 10, and 20 mg L^{-1}) were added into V8-agar plates, respectively. Marginal mycelial plugs from *P. capsici* fresh cultures were placed onto each V8-agar plate and the plates were incubated at 25 ℃ for 4 d. The diameter of *P. capsici* colonies was measured every 24 h after inoculation.

The inhibitory effect of TFO on spore production and spore germination of *P. capsici* was determined as previously reported (Gao et al., 2016). For each treatment, ten fresh marginal mycelial plugs were cut and transferred into a plate which contains V8 liquid medium. After incubated at 25 ℃ for 2 d, the mycelia were washed with sterile distilled water. Then, the mycelia were resuspended in sterilized tap water which contains TFO at different concentrations (0, 2.5, 5, 10, and 20 mg L^{-1}) for sporangial formation. After incubated at 25 ℃ for another 12 h, the plates were transferred to a 4 ℃ refrigerator for 1 h, then placed to 25 ℃ again to release spores. The number of spores was calculated with a haemacytometer under an IX-71 optical microscope (Olympus, Tokyo, Japan). For the determination of spore germination, spore suspensions of *P. capsici* were first adjusted to 1×10^4 mL^{-1}, then these spore suspensions were transferred to V8 liquid medium which contains TFO at different concentrations (0, 2.5, 5, 10, and 20 mg L^{-1}). The cultures were incubated at 25 ℃ for up to 24 h, and the number of germinated spores of each treatment was counted at 2, 4, 6, 8, 10, 12, and 24 h after incubation with a haemacytometer under a microscope. Each treatment was replicated three times, and the experiment was performed in triplicate.

2.6. Optical microscope and Transmission electron microscope observations

Optical microscope (OM) observations of the mycelial morphology of *P. capsici* were performed according to (Wang et al., 2021). TFO at different concentrations (0, 5, and 10 mg L^{-1}) were added into V8-agar plates. Fresh marginal mycelial plugs of *P. capsici* were placed onto each V8-agar plate. After incubated at 25 ℃ for 4 d, the mycelial morphology of *P. capsici* was observed under an optical microscope.

Transmission electron microscope (TEM) observations were also carried out as described previously (Wang et al., 2021). Fresh marginal mycelial plugs of *P. capsici* were placed onto V8-agar plates amended with TFO at different concentrations (0, 5, and 10 mg L^{-1}). After incubation at 25 ℃ for 4 d, fresh marginal mycelial tips were cut and fixed in 0.1 M PBS (pH 7.2), containing 2.5% glutaraldehyde, for 4 h. Then, the samples were rinsed with 0.1 M PBS (pH 7.2), fixed with 0.1 M OsO_4 for 2 h, and dehydrated in a series of ethanol solutions. Afterwards, the samples were embedded in Epon 812 resin, cut into ultrathin sections, and collected with copper grids. After staining with uranyl acetate and lead citrate, the mycelial ultrastructure of *P. capsici* was observed under a Talos-F200C transmission electron microscope (Thermo Fisher Scientific, Waltham, USA). The experiment was carried out three times.

2.7. Detection of extracellular electrical conductivity and membrane integrity

The effect of TFO on the extracellular electrical conductivity was performed as reported by (Tian et al., 2016). For each treatment, ten fresh marginal mycelial plugs of *P. capsici* were cut and transferred into 100 mL of V8 liquid medium. After rotation at 175 rpm for 4 d at 25 ℃, the mycelia were harvested through a sterile gauze of double layer.

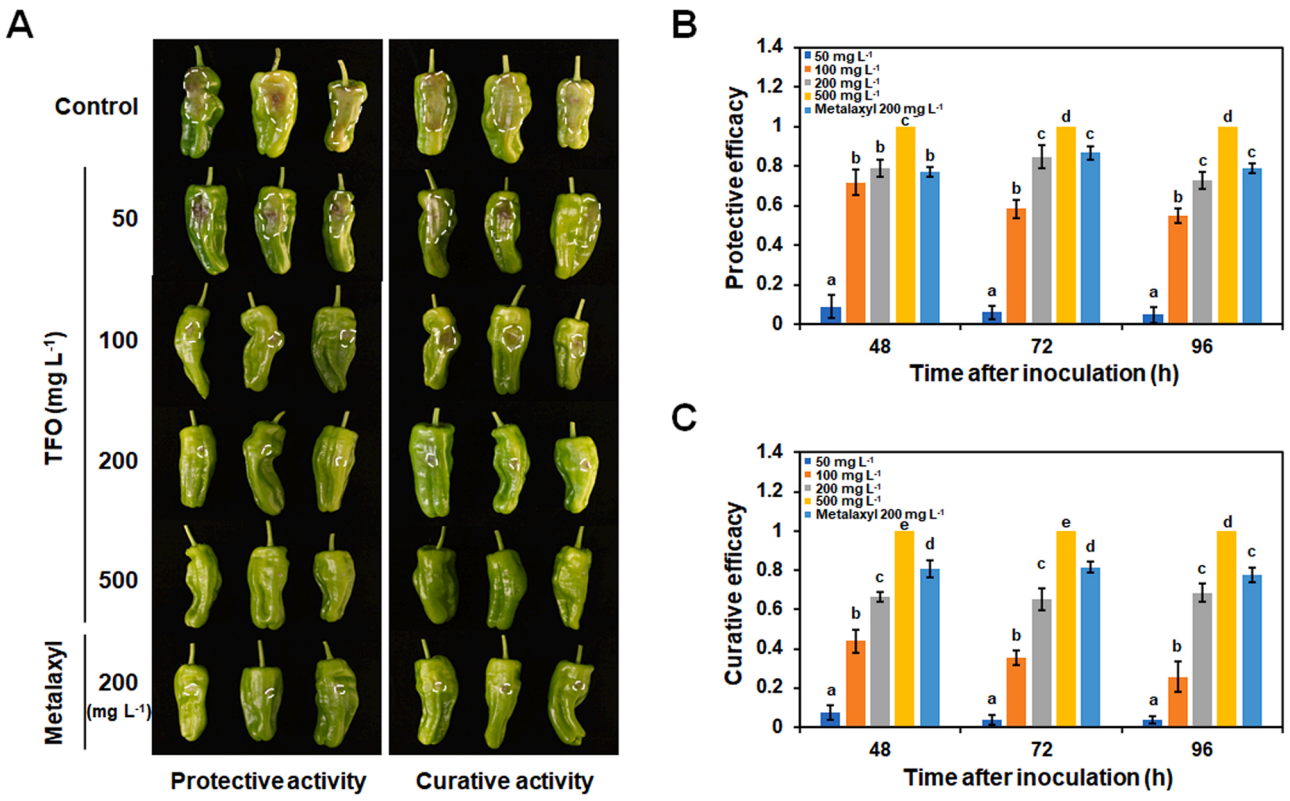

Fig. 2. TFO inhibited phytophthora blight on pepper fruit. Detached pepper fruits were inoculated with *P. capsici* before or after being injected with TFO solutions at different concentrations (50, 100, 200, and 500 mg L^{-1}) for 24 h. Fruits injected with sterile distilled water or metalaxyl solution were used as controls. Inoculated pepper fruits were placed at 25 ℃ for 4 d. Disease symptoms on pepper fruits were photographed (A), protective and curative efficacies (B and C) were assessed every 24 h after inoculation. Each treatment comprised three replications with at least 15 pepper fruits. Vertical bars represent SE of the means. Different little letters over the columns show significant difference ($p < 0.05$).

Table 1
The disease index and control efficacy of *Phytophthora capsici* on pepper plants when treated with *Tetradium glabrifolium* fruit essential oil (TFO). Each treatment had 20 replicates, and the experiment was repeated three times in the greenhouse. Different little letters over the columns show significant difference ($p < 0.05$).

Treatment (mg L^{-1})	Protective effects		Curative effects	
	Disease index	Control efficacy (%)	Disease index	Control efficacy (%)
Control	79.56 ± 3.86	–	81.02 ± 2.74	–
TFO (500)	54.13 ± 3.35	31.47 ± 4.24 a	58.68 ± 6.99	27.56 ± 7.63 a
TFO (800)	30.16 ± 2.84	61.82 ± 3.60 b	45.73 ± 3.05	43.54 ± 3.77 b
TFO (1000)	17.92 ± 2.13	77.32 ± 2.70c	29.06 ± 3.24	64.12 ± 3.99c
Metalaxyl (500)	15.41 ± 1.63	80.49 ± 2.06c	23.02 ± 1.94	70.05 ± 2.40c

Subsequently, the samples were resuspended in sterile distilled water containing TFO at different concentrations (0, 2.5, 5, 10, and 20 mg L^{-1}), and incubated at 25 ℃ for up to 3 h on a rotary shaker. The electrical conductivity of the samples was measured at 0, 10, 20, 40, 60, 80, 100, 120, 140, 160, and 180 min using a DDS-11A electric conductivity meter (Shanghai Leici Instrument Inc., Shanghai, China), respectively. After 180 min, all the samples were boiled and the final electric conductivity was measured. The relative conductivity (%) = electric conductivity/final electric conductivity × 100.

The effect of TFO on the membrane integrity was evaluated using propidium iodide (PI). Ten fresh marginal mycelial plugs of *P. capsici* were cut and transferred into V8 liquid medium. After incubated at 25 ℃ for 2 d, TFO at different concentrations (0, 5, and 10 mg L^{-1}) was added into the V8 liquid medium. After incubated at 25 ℃ for another 1 d, the mycelia were washed with sterile distilled water, then stained with 10 mg L^{-1} PI. Afterwards, the stained mycelia were washed with 50 mM PBS (pH 7.2), and observed with a LSM880 confocal microscope (Carl Zeiss, Jena, Germany) (Yang et al., 2019). The whole experiments were repeated three times.

2.8. Detection of cellular leakage

The cellular leakages of proteins and nucleic acids were measured as reported previously (Wang et al., 2021). The mycelia were obtained as described before. Afterwards, the samples were resuspended in sterile distilled water with TFO at different concentrations (0, 2.5, 5, 10, and 20 mg L^{-1}), and rotated at 175 rpm for 0, 2, 4, 6, or 8 h, respectively. The supernatants were collected by filtering through a sterile gauze of double layer, and the aqueous solutions were used for determination of the leakage of proteins and nucleic acids. The leakage of proteins in various treatments was quantified by the Coomassie brilliant blue staining method. The release of nucleic acids was measured by detecting the absorbance value at 260 nm (A_{260} nm). Each treatment was replicated three times, and the experiment was performed in triplicate.

2.9. Analysis of defence-related enzyme activity

Pepper fruits were wounded as described before and TFO solutions at final concentrations of 50 and 100 mg L^{-1} were injected into each wound. Fruits injected with sterile distilled water was used as the control. Inoculated fruits were incubated at 25 ℃ for 7 d. Fruit tissues surrounding the wounds were peeled using a stainless-steel knife at 0, 1, 2, 3, 4, 5, 6, and 7 d after incubation. Crude enzymes were extracted as previously reported (Zhao et al., 2020). Enzyme activities of CAT, PAL, POD, and PPO were measured in accordance with the methods described

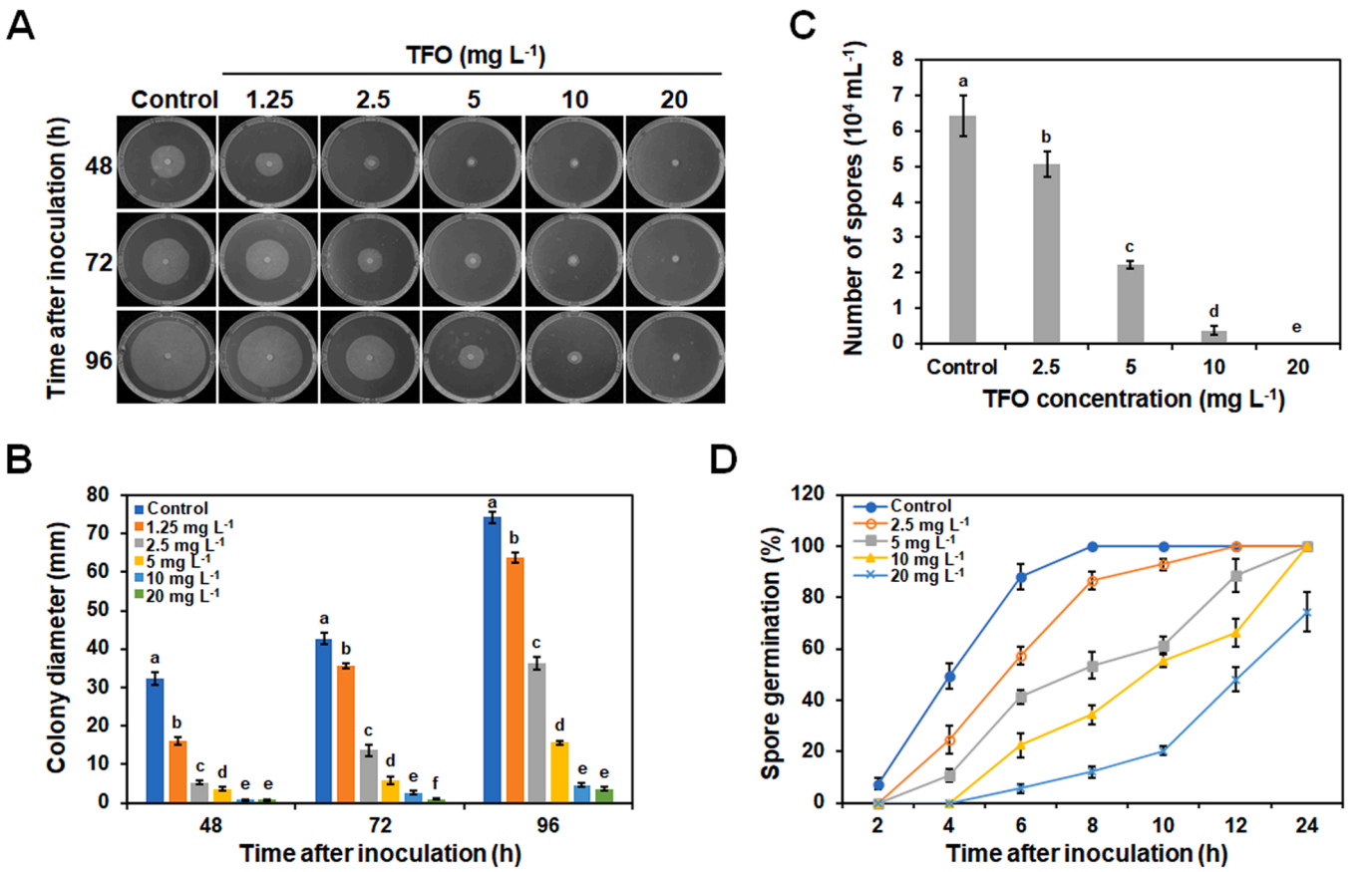

Fig. 3. TFO suppressed *P. capsici* growth *in vitro*. Colony morphology (A) and colony diameter (B) of *P. capsici* on the culture medium with TFO at indicated concentrations (1.25, 2.5, 5, 10, and 20 mg L^{-1}) were measured every 24 h. The effect of TFO with different concentrations (2.5, 5, 10, and 20 mg L^{-1}) on spore production (C) and spore germination (D) of *P. capsici* was assessed. The number of spores of each treatment was calculated with a haemacytometer and a microscope. The number of germinated spores of each treatment was counted at 2, 4, 6, 8, 10, 12, and 24 h after incubation. Each treatment was replicated three times, and the experiment was performed in triplicate. Bars represent SE. Different little letters over the columns show significant difference ($p < 0.05$).

before (Apaliya et al., 2017; Guo et al., 2019; Wang et al., 2018, 2019). Briefly, for CAT and PAL activities determination, crude enzyme solution was mixed with 0.25 M H_2O_2 or 10 mM phenylalanine, respectively, CAT and PAL activities were tested by measuring the A_{240} nm and A_{290} nm values. For POD activity measurement, crude enzyme solution was first mixed with 0.3% guaiacol. Afterwards, 0.3% H_2O_2 was added into the mixture and incubated at 30 ℃ for 6 min. POD activity was measured by detecting the A_{470} nm value. For PPO activity detection, crude enzyme solution was mixed with 0.25 M pyrocatechol. After incubated at 30 ℃ for 6 min, PPO activity was tested by measuring the A_{398} nm value. Finally, the enzyme activities were calculated based on an increase in absorbance of 0.01 unit per minute. The bioassay data were obtained from three independent biological replicates.

2.10. Statistical analysis

All these experiments in our study were repeated at least three times, and the corresponding values were showed as means ± standard errors. All data were analyzed using SPSS software (v14.0 SPSS Inc., Chicago, USA). Significant differences between treatments were examined using Fisher's least significant differences (LSD) tests at 95% confidence limit.

3. Results

3.1. TFO controlled phytophthora blight on pepper

The biocontrol effect of TFO against *P. capsici* was first performed on detached pepper leaves. Results revealed that TFO exhibited strong control efficacy on the development of phytophthora blight (Fig. 1). After inoculation for 4 d, the protective and curative efficacy values were 43.65% and 31.65%, 61.45% and 45.74%, 76.53% and 73.56%, respectively, after treating leaves with 50, 100, and 200 mg L^{-1} of TFO. When the TFO concentration rose to 500 mg L^{-1}, its protective and curative efficacy values reached 100% (Fig. 1B and C). Additionally, the protective and curative efficacy values of 200 mg L^{-1} of metalaxyl was 80.43% and 75.53%, which was roughly the same as that under 200 mg L^{-1} of TFO treatment (Fig. 1B and C).

To further explore the effect of TFO on phytophthora blight controlling, detached pepper fruits were injected with TFO and inoculated with *P. capsici*. Results showed that the protective and curative efficacy values were only 4.82% and 3.71% after treating with 50 mg $L^{-1}$of TFO, indicating that low concentration of TFO was ineffective against phytophthora blight on pepper fruit (Fig. 2). In contrast, the protective and curative efficacy values were increased significantly by the treatment of 100, 200, or 500 mg L^{-1} of TFO. After inoculation for 4 d, the protective and curative efficacy values were respectively 54.69% and 25.77%, 72.60% and 56.73% after treating with 100 and 200 mg L^{-1} of TFO (Fig. 2B and C). Similarly, the protective and curative efficacy values of 200 mg L^{-1} of metalaxyl was 78.75% and 77.69%, which was approximate to the treatment of 200 mg L^{-1} of TFO (Fig. 2B and C). Notably, when the TFO concentration rose to 500 mg L^{-1}, its protective and curative efficacy values reached 100% (Fig. 2B and C).

Since *P. capsici* is a destructive soilborne pathogen, systemic infection through the roots is highly likely. To further clarify the effect of TFO against *P. capsici* on pepper, root-drench experiment was performed. As shown in Table 1, TFO exhibited strong protective and curative effects

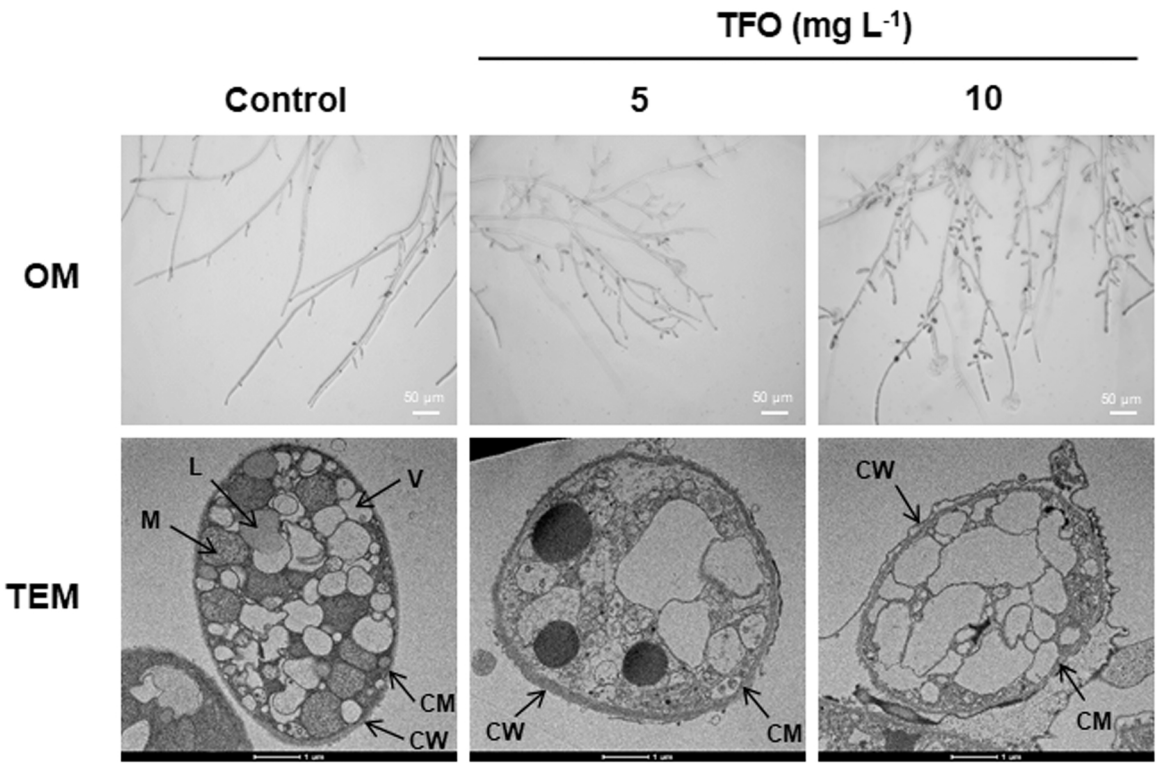

Fig. 4. TFO changed the mycelial morphology and ultrastructure of *P. capsici*. TFO at indicated concentrations (5 and 10 mg L^{-1}) were added into culture medium of *P. capsici*, respectively. After incubated at 25 °C for 4 d, the mycelial morphology and cellular ultrastructure was observed with an optical microscope and a transmission electron microscope, respectively. The experiment was carried out three times. The scale bars represent 50 μm and 1 μm, respectively. CM: cell membrane; CW: cell wall; M: mitochondria; V: vacuole; and L: lipidosome.

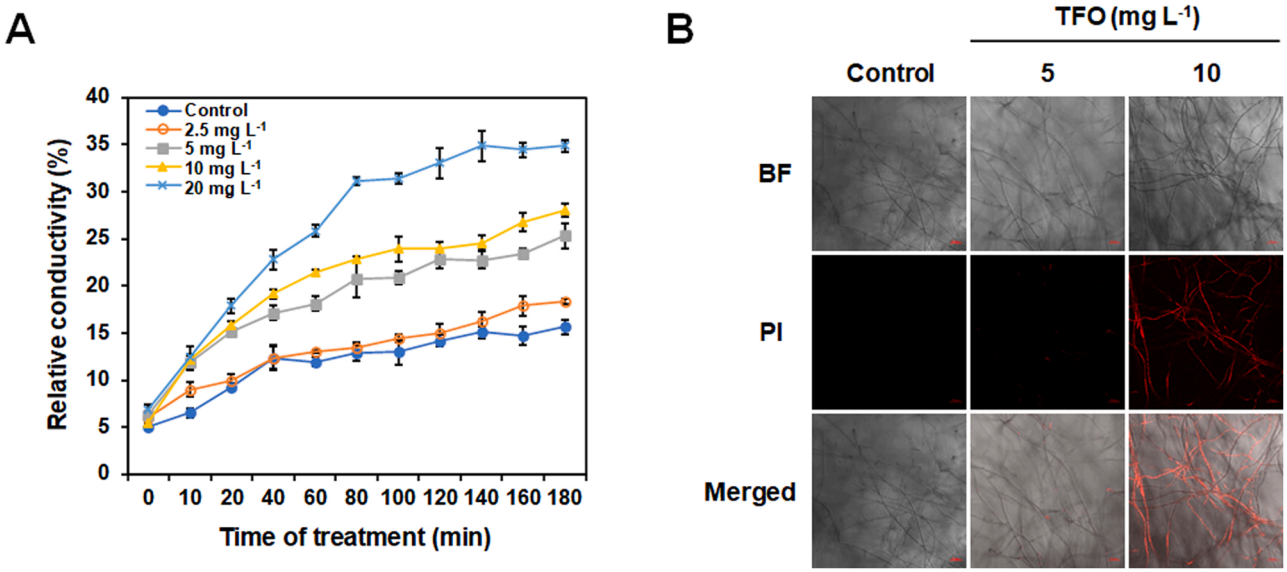

Fig. 5. TFO affected the cell membrane permeability and integrity of *P. capsici*. The extracellular electric conductivities of mycelia (A) were measured with TFO at indicated concentrations (2.5, 5, 10, and 20 mg L^{-1}) at 0, 10, 20, 40, 60, 80, 100, 120, 140, 160, and 180 min using an electric conductivity meter, respectively. The membrane integrity (B) was evaluated using propidium iodide (PI) staining. The mycelia were treated with TFO at indicated concentrations and stained with PI, which was followed by observations with a confocal microscope. The whole experiments were repeated three times. BF represents bright field, PI represents PI staining, the scale bar represents 50 μm.

on the systemic infection of *P. capsici*. The protective and curative efficacy values were 31.47% and 27.56%, 61.82% and 43.54%, 77.32% and 64.12%, respectively, after treating leaves with 500, 800, and 1000 mg L^{-1} of TFO (Table 1). Additionally, the protective and curative efficacy values of 500 mg L^{-1} of metalaxyl was 80.49% and 70.05%, which had no significant difference from that under 1000 mg L^{-1} of TFO treatment (Table 1). Consistent results on leaves, fruits, and whole plants of pepper suggested that TFO had the potential to control disease caused by *P. capsici*.

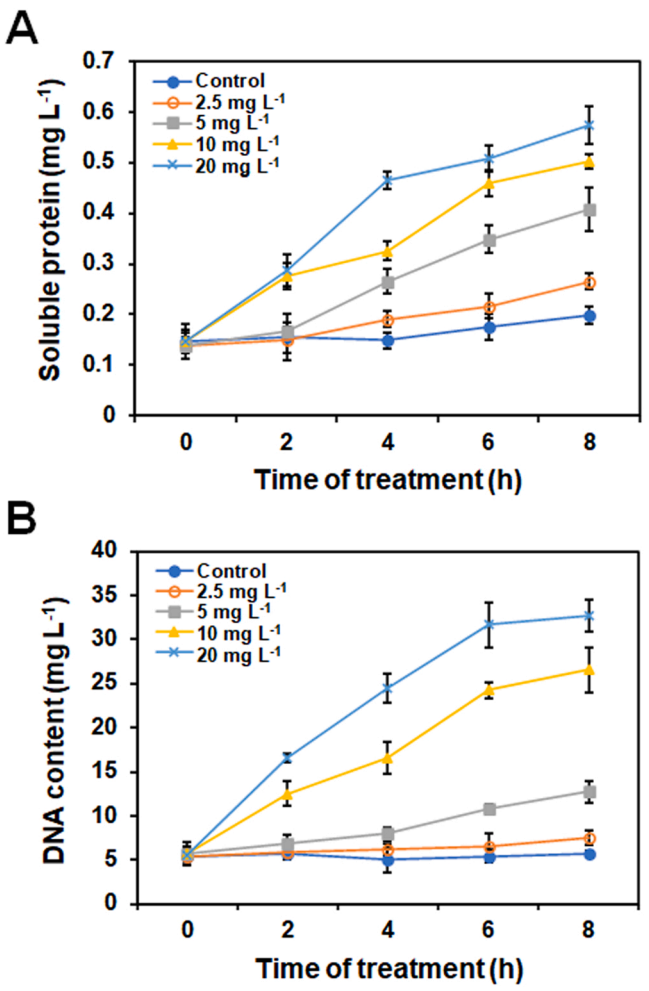

Fig. 6. TFO caused cellular leakages of *P. capsici*. Mycelia of *P. capsici* were resuspended in sterile distilled water amended with different concentrations (2.5, 5, 10, and 20 mg L^{-1}) of TFO. The leakage of soluble proteins (A) and nucleic acids (B) were measured by the Coomassie brilliant blue staining method and the absorbance value at 260 nm (A_{260} nm), respectively. Each treatment was replicated three times, and the experiment was performed in triplicate. The SE of means was represented by vertical bars.

3.2. TFO suppressed mycelial growth, spore production, and spore germination of P. capsici

Next, the inhibitory effect of TFO on mycelial growth of *P. capsici* was evaluated. As indicated in Fig. 3, mycelial of *P. capsici* grew continually throughout the incubation period, but the increase was effectively inhibited by TFO. The colony diameter of *P. capsici* was reduced by 14.13%, 51.12%, 78.92%, 93.72%, and 95.07%, respectively, after treating with 1.25, 2.5, 5, 10, and 20 mg L^{-1} of TFO for 4 d (Fig. 3A and B).

Furthermore, to explore the effect of TFO on secondary infection of *P. capsici*, spore production and spore germination were analyzed in the presence of TFO. The results revealed that TFO could significantly suppress spore production and spore germination of *P. capsici*. TFO treatment at the concentrations of 2.5, 5, and 10 mg L^{-1} decreased the number of spores by 21.12%, 65.39%, and 94.34%, respectively, and the spore production was completely inhibited when the concentration of TFO reached 20 mg L^{-1} (Fig. 3C). Similarly, the spore germination of *P. capsici* was also suppressed by TFO. The spore germination reached 100% at 12 h in the untreated control, whereas it took 24 h to reach 100% after treating with 2.5, 5, and 10 mg L^{-1} of TFO. Furthermore, the spore germination could not reach 100% when treated with 20 mg L^{-1} of TFO. Thus, our results demonstrated that TFO could inhibit both vegetative and reproductive growth of *P. capsici*.

3.3. TFO destroyed the mycelial morphology and cellular ultrastructure of P. capsici

OM observations were carried out to investigate the effect of TFO on mycelial morphology of *P. capsici*. The mycelial morphology of *P. capsici* appeared to be normal in untreated controls, whereas many abnormalities of mycelial morphology appeared after treating with TFO. The mycelia became swollen and contorted, the edge of the diaphragm showed protuberances, and the branches increased and became shorter (Fig. 4). Results of TEM observations further indicated that TFO treatment also changed the mycelial ultrastructure of *P. capsici*. Without TFO treatment, the structure of cell wall and membrane were uniform, and the cytoplasmic organelles were intact. However, these morphological structures destroyed in TFO treated groups. After treating with 5 mg L^{-1} of TFO, the cell wall and cell membrane of *P. capsici* mycelia became thinner, large vacuoles appeared, cytoplasm matrix decreased, and cytoplasmic organelles began to degrade. A higher concentration of TFO (10 mg L^{-1}) caused greater destructions included rupture of the cell wall and membrane, and absence of most cytoplasmic organelles (Fig. 4). Our observations indicated that TFO treatment resulted in the disruptions of cell wall, cell membrane and the internal structure of *P. capsici* mycelia, which might be responsible for cell death of this pathogen.

3.4. TFO damaged cell membrane of P. capsici

Extracellular electric conductivity assay was performed to test whether TFO could affect the cell membrane permeability of *P. capsici*. As indicated in Fig. 5A, in untreated control, the value of electric conductivity stayed at a low level throughout the incubation period, whereas the value increased over time when *P. capsici* mycelia were treated with TFO. Furthermore, the value of electric conductivity increased with increasing TFO concentrations.

We further explored the effect of TFO on membrane integrity using PI staining. PI is a fluorescent dye, which can enter into cell when cell membrane is severely damaged, therefore cell will be shown in red by PI staining when it lost its membrane integrity (Wu et al., 2013). As shown in Fig. 5B, *P. capsici* mycelia in untreated control showed no red fluorescence, indicating that they were intact. After treating with 5 mg L^{-1} of TFO for 1 d, most of *P. capsici* mycelia maintained cell membrane integrity. However, after treating with 10 mg L^{-1} of TFO, most of *P. capsici* mycelia lost their membrane integrity (Fig. 5B). Thus, based on these results, TFO could damage the cell membrane of *P. capsici*.

3.5. TFO induced cellular leakages of P. capsici

Cellular leakage is an important indicator of cell membrane damage. In view of the above results, we have demonstrated that TFO could destroy the integrity of *P. capsici* cell membrane. To corroborate these results, the effect of TFO on cellular leakage was determined. Results in Fig. 6 showed that there was almost no change in the leakages of soluble proteins and nucleic acids in untreated control. In contrast, the leakage from the mycelia of *P. capsici* significantly increased after treating with TFO. As shown in Fig. 6, leakages of soluble proteins and nucleic acids were observed at 2 h after TFO treatment, and the leakages were sustained over 8 h. Leakages of soluble proteins and nucleic acids were 3.89 and 5.98-fold compared to that of the untreated control, when the concentration of TFO reached 20 mg L^{-1}. These data indicated that TFO could induce cellular leakages from the mycelia of *P. capsici*.

3.6. TFO induced higher activities of defence-related enzymes in pepper

Next, we evaluated the effect of TFO on four defence-related enzymes in pepper fruit during infection. As shown in Fig. 7A, CAT activity

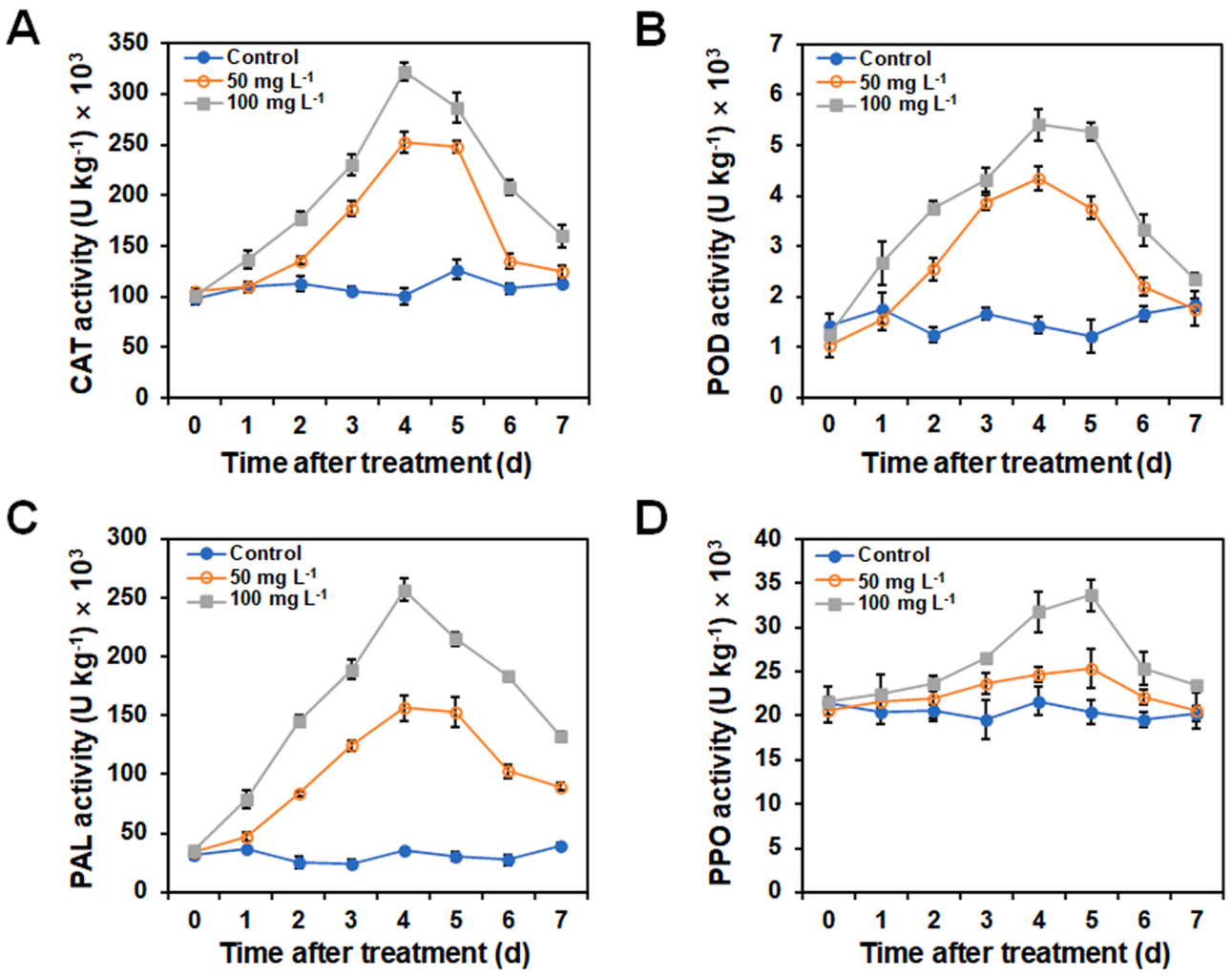

Fig. 7. TFO increased defence-related enzyme activities in pepper fruit. TFO solutions at indicated concentrations (50 and 100 mg L^{-1}) was injected into each wound, while sterile distilled water was used as the control. After incubated for 0, 1, 2, 3, 4, 5, 6, and 7 d, pepper tissue around wounds was collected and assayed for (A) CAT, (B) POD, (C) PAL, and (D) PPO activities. The bioassay data were obtained from three independent biological replicates. Vertical bars represent SE of the data.

was higher in TFO treated fruit than that in the untreated control. Over the whole period of 7 d, CAT activities gradually increased to the maximum value after treating with TFO for 4 d, and the peak value were 2.51 and 3.22-fold compared to that in the untreated control, respectively, under treatment with 50 and 100 mg L^{-1} of TFO (Fig. 7A). After 4 d, CAT activities reduced until the end of storage. Like CAT, the activities of POD and PAL were also increased and then reduced during the whole period in TFO treated fruit. The activities of POD and PAL reached peaks at 4 d after TFO treatment, which were 3.03, 3.78, 4.42, and 7.25-fold compared to that in the untreated control, respectively, under treatment with 50 and 100 mg L^{-1} of TFO (Fig. 7B and C). Notably, PPO activities slightly increased and reached peaks at 4 d after TFO 50 and 100 mg L^{-1} of TFO treatment, which were only 1.24 and 1.64-fold compared to that of the untreated control (Fig. 7D). Together, these results suggested that TFO could enhance the activities of defence-related enzymes in pepper, which contributed to the antagonism of phytophthora blight *in vivo*.

3.7. Chemical composition and main effective constituents of TFO

To clarify the chemical composition of TFO, we analyzed it by GC-MS. As shown in Fig. 8 and Table S2, a total of 15 compounds were identified, representing 71.71% constituents of TFO. Among them, 2-tridecanone (18.39%), caryophyllene (11.81%), 2-undecanone (9.98%), D-limonene (7.37%), and *β*-elemene (6.81%) were the most abundant compounds, which accounted for 54.36% of the total TFO.

The antifungal activities of EOs rely on their bioactive components. Subsequently, these five most abundant compounds from TFO were purchased and their antifungal activities were determined *in vitro* (Table S1). Results indicated that D-limonene exhibited the most significant antifungal activity, with 87.54% inhibition rate at 20 mg L^{-1}. Antifungal activities of *β*-elemene and 2-tridecanone were mediocre, with 62.45% and 51.21% inhibition rate at 20 mg L^{-1}, respectively. However, 2-undecanone and caryophyllene had no effect on the mycelial growth of *P. capsici* (Table 2). Therefore, D-limonene, *β*-elemene, and 2-tridecanone contributed to the antifungal activity of TFO.

4. Discussion

Phytophthora blight caused by *P. capsici*, is one of the major diseases of pepper throughout its whole growth period. Chemical fungicides are still conventionally utilized to control phytophthora blight due to their efficiency and stability, but long-term use of them has led to the increasing resistance of *P. capsici* and environmental pollution in recent years (Keinath, 2007; Siegenthaler and Hansen, 2021). Currently, plant EOs were reported to be promising alternatives due to their ecofriendly properties and effective antimicrobial activities (Burt, 2004; An et al., 2019). TFO is a mixture of volatile compounds which were

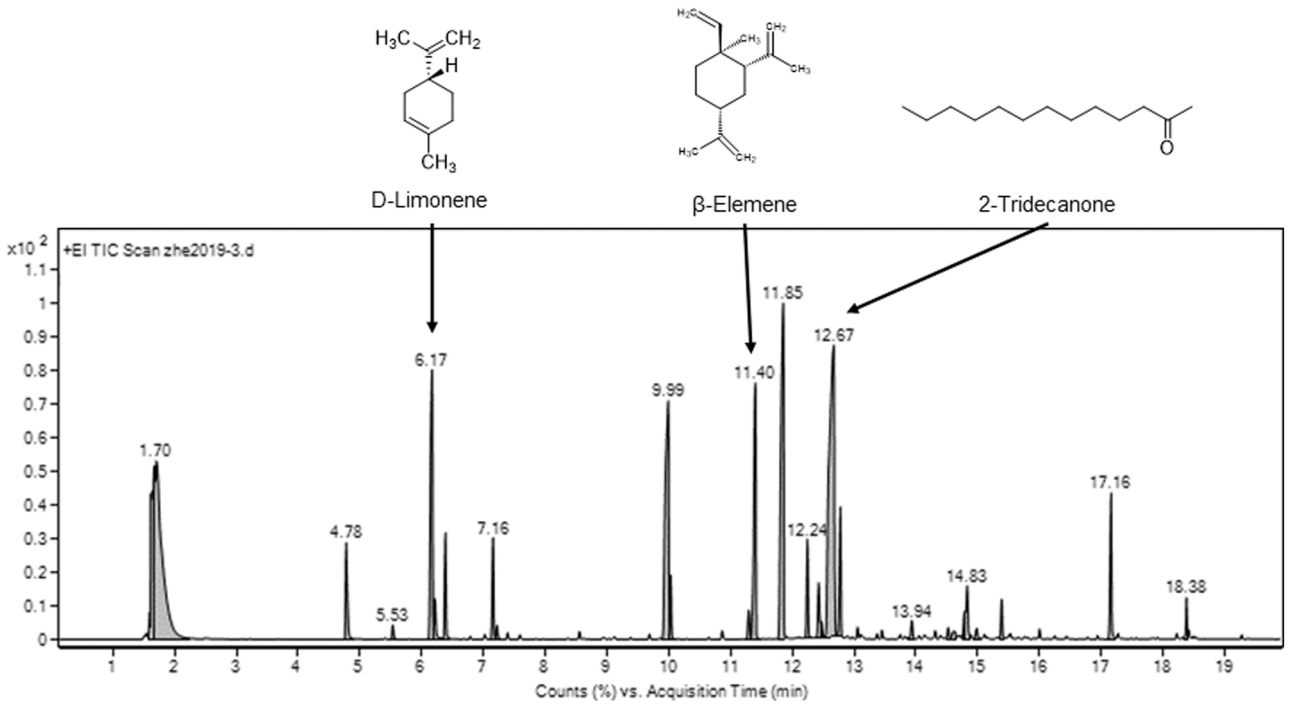

Fig. 8. Total ion chromatograms (TIC) of volatile compounds of TFO by GC-MS analysis. Three arrows indicated the components of TFO with the main antifungal activity, which were D-limonene, *β*-elemene and 2-tridecanone. The experiment was carried out three times with similar results.

Table 2
The antifungal activities of the five most abundant compounds from TFO against *P. capsici* at different concentrations. Each treatment was replicated three times, and the experiment was performed in triplicate.

Compound	Inhibition rates (%) at different concentrations				
	1.25 mg L^{-1}	2.5 mg L^{-1}	5 mg L^{-1}	10 mg L^{-1}	20 mg L^{-1}
D-Limonene	38.65 ± 3.52	58.44 ± 3.28	73.53 ± 4.87	81.31 ± 4.32	87.54 ± 4.37
2-Undecanone	0	0	3.57 ± 0.36	4.32 ± 0.78	0
β-Elemene	24.34 ± 2.64	25.22 ± 3.12	36.88 ± 4.90	43.65 ± 3.21	62.45 ± 3.75
Caryophyllene	0	0	0	1.25 ± 0.67	0
2-Tridecanone	26.55 ± 3.29	31.12 ± 2.75	41.23 ± 4.36	45.94 ± 2.84	51.21 ± 3.29

biosynthesized in the fruit of *T. glabrifolium*. Previous studies have shown that TFO and its three constituents 2-tridecanone, 2-undecanone, and D-limonene exhibited strong larvicidal effect on mosquitoes (Liu et al., 2014). However, the antimicrobial effects of TFO have not been reported. In the present study, we verified the inhibitory efficacy of TFO against *P. capsici*, which caused phytophthora blight on pepper.

To investigate whether the inhibition effect of TFO against phytophthora blight depended on its direct antifungal activity, we assessed the effect of TFO on mycelial growth of *P. capsici*. Results revealed that TFO significantly restricted the mycelial growth. Spores are important in secondary infection of *P. capsici* on hosts. Therefore, any fungicide that inhibit spore production and spore germination should be useful for controlling diseases caused by *P. capsici* (Matheron and Porchas, 2000). Here, we further determined the effect of TFO on spore production and spore germination of *P. capsici*. Results indicated that TFO could significantly suppress spore production and spore germination of *P. capsici*. In view of the above results, we concluded that TFO reduced the development of phytophthora blight by its direct inhibitory activity on mycelial and reproductive growth of the pathogen.

Subsequently, OM and TEM observations were performed to gain an insight into the morphological and ultrastructural damage induced by TFO. OM observations displayed that, after TFO treatment, the mycelia of *P. capsici* became swollen and contorted, and the branches increased and became shorter. Further TEM observations showed that TFO treatment resulted in the disruptions of cell wall, cell membrane and the internal structure of *P. capsici* mycelia. These observations suggested that cell membrane of *P. capsici* maybe the target of TFO. The membrane disruption of *P. capsici* was further confirmed by determination the extracellular electric conductivity and PI staining assay. As a result, we observed that the cell membrane integrity was seriously impaired in *P. capsici,* suggesting that cell membrane was the target of TFO.

Damaging of cell membrane usually led to the cellular leakages of soluble proteins as well as nucleic acids from the mycelia (Pei et al., 2020). Thus, we explored the effect of TFO on cellular leakage from *P. capsici* mycelia. As expected, the leakages of proteins and nucleic acids from *P. capsici* mycelia significantly increased after treating with TFO. Hence, these results indicated that TFO treatment damaged the cell membrane, caused the cellular leakage of contents, and led to cell death of *P. capsici*. Destroying cell membrane is a common strategy for some plant EOs. (Liu et al., 2017) reported that the oil extracts of *Eupatorium adenophorum* could disrupt the cell membrane of *P. capsici*. In addition, (Ren et al., 2018) have found that mint oil effectively controlled the gray mold in table grapes through inducing the disruption of cell membrane of *Botrytis cinerea*. More recently, our study on zedoary turmeric oil (ZTO) demonstrated that the inhibitory activity of ZTO against *P. capsici* depend on its capability of destroying the integrity of cell membrane \(Wang et al., 2019). In the current study, although we have concluded that TFO can destroy the cell membrane integrity, the specific action site on membrane needs to be further investigated.

CAT, POD, PAL, and PPO are defence-related enzymes involved in

disease resistance in plants. CAT and POD are antioxidant enzymes that help to remove excess intracellular H_2O_2 in different ways: CAT directly decomposes H_2O_2 and POD catalyzes H_2O_2 to H_2O and O_2 (Mittler et al., 2004; Vatansever et al., 2013). PAL is a key enzyme of phenylpropanoid metabolic pathway, which regulates the biosynthesis of many plant primary antifungal compounds, including flavonoids, phenolic compounds, and lignin (Ferrer et al., 2008). PPO is also an important enzyme which plays a critical role in plant defence mechanisms. PPO can catalyze phenolic compounds to quinone, which is more poisonous to invading pathogens (Chen et al., 2014). Thus, enzyme activities of PAL and PPO can contribute to disease resistance in plants. Many studies have demonstrated that plant EOs or their volatile compounds could prevent excessive ROS accumulation and stimulate antifungal compounds biosynthesis by increasing enzyme activities of these four defence-related enzymes. For example, (Wu et al., 2017) demonstrated that the PPO and PAL activities in citrus fruit are strongly induced by cinnamaldehyde treatment. In this study, the activities of these four enzymes were also examined. Similarly, though the activities of four enzymes were differentially affected by TFO, they all significantly increased in pepper. These results indicated that TFO induced a defence response in pepper.

The antifungal activities of plant EOs rely on their bioactive components. In this study, fifteen compounds were identified from TFO by GC-MS analysis. Among them, 2-tridecanone, caryophyllene, 2-undecanone, D-limonene, and β-elemene were the most abundant compounds, which accounted for more than 54% of the total EO. Similar to our results, 2-tridecanone, caryophyllene, 2-undecanone, D-limonene, and β-elemene also has been testified as the main components of TFO, though the content of the main components and whole composition of this oil are quite different (Liu et al., 2014). It is important to point out that, these differences may be caused by geographical differences and harvesting time (Firuzi et al., 2010). Subsequently, the antifungal activities against *P. capsici* of these five main components were tested. Results showed that, D-limonene, β-elemene, and 2-tridecanone contributed to the antifungal activity of TFO. In previous reports, D-limonene exhibited strong antifungal and antibacterial activities against several phytopathogens. For instance, (Ma et al., 2015) reported antifungal activity of limonene against *Sclerotinia sclerotiorum*. Later, (Hajian-Maleki et al., 2021) showed that D-limonene had strong antifungal activity against *Pectobacterium carotovorum*. However, this is the first report on the inhibitory activity of D-limonene against an oomycete pathogen. In addition, this is the first report about the antifungal activity of β-elemene and 2-tridecanone. More experiments are required to validate whether these compounds have synergistic effect on the inhibition of oomycete pathogens.

5. Conclusions

TFO is effective for controlling phytophthora blight of pepper by significantly suppressing vegetative and reproductive growth of *P. capsici* in a concentration dependent manner. TFO also causes damages of cell membrane, leakages of intracellular contents including proteins and nucleic acids of *P. capsici*. Additionally, TFO induces higher activities of defence-related enzymes in pepper. Our results imply that TFO has the potential to control phytophthora blight and the three abundant components (D-limonene, β-elemene, and 2-tridecanone) contribute to the antifungal activity of TFO.

CRediT authorship contribution statement

Bi Wang: Investigation, Writing – original draft. **Pirui Li:** Data curation, Writing – original draft. **Jingjing Yang:** Investigation. **Xuhong Yong:** Writing – review & editing. **Min Yin:** Validation. **Yu Chen:** Resources, Writing – review & editing. **Xu Feng:** Supervision, Validation. **Qizhi Wang:** Supervision, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2021.114310.

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