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Article

Antifungal Activity and Action Mode of Cuminic acid from the seed of *Cuminum cyminum*. L against *Fusarium oxysporum* f. sp. *Niveum* (FON) on watermelon

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Abstract: Watermelon fusarium wilt caused by *Fusarium oxysporum* f.sp. *niveum* (FON) is a destructive soil-borne disease throughout the world leading to serious economic losses and limit watermelon production. Cuminic acid, extracted from the seed of *Cuminum cyminum* L., belongs to benzoic acid analogues. In this study, the median effective concentration (EC_{50}) values for cuminic acid in inhibiting mycelial growth of FON was 22.53μg/mL. After treatment with cuminic acid, mycelial morphology was seriously influenced; cell membrane permeability and glycerol content were increased markedly, but pigment and mycotoxin (mainly fusaric acid) were significantly decreased. Synthesis genes of bikaverin and fusaric acid both were down regulated compared with the control confirmed by quantitative RT-PCR. In greenhouse experiments, cuminic acid at all concentrations displayed significant bioactivities against FON. Importantly, significant enhancement of activities of SOD, POD, CAT and decrease of MDA content after cuminic acid treatment in watermelon leaves were observed in vivo. These indicated that cuminic acid not only showed high antifungal activity, but also could enhance the self-defense system of the host plant. Above all, cuminic acid showed the potential as a biofungicide to control FON.

Keywords: *Fusarium oxysporum* f. sp. *Niveum*; p-isopropyl benzoic acid; Biofungicide; Disease management

1. Introduction

Watermelon is one of the most important fruit worldwide. In china, watermelon cultivation has been increased year by year due to high comparative economic value and increasing consumption, but it is susceptible to fusarium wilt disease w in continuously monocropping systems [1]. Watermelon fusarium wilt caused by *Fusarium oxysporum* f.sp. *niveum* (FON) is a destructive soil-borne disease throughout the world leading to serious economic losses and limit watermelon production [2].

Importantly, FON is difficult to be eliminated from soil. Laboratory studies has reported that three biological forms of *F. oxysporum* survived unchanged morphologically for 11 or more years [3]. More than 50% of Fusarium species are toxigenic and produce harmful secondary metabolites(SM), such as the pigments fusarubins and bikaverin [3], as well as the mycotoxins, fumonisins, fusarins [4], and fusaric acid [5, 6]. In the progression of the infection, fusarium species damage host plants through intrusion of hyphae into host vascular system, secretion of hydrolytic enzymes and mycotoxin which lead to watermelon root and stem necrotic, cellular apoptosis, foliar wilting and then death in a few weeks [7, 8].

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Due to FON can survive for several years in soil as chlamydospores and many hosts are symptomless [9], fusarium wilt is difficult to control. Just because of this, although traditional crop rotations are an effective strategy to control FON [10]. For many other pathogens, the application of fungicide was the common and successful method for disease management. However, the application of fungicide should be phased out because of the increasing attention of environmental and human health and the development of fungicide resistance [9, 11]. Some experiments have documented that fungicide has drastic effects on the soil biota and most cause a decline in soil fertility [12]. Consequently, alternative control strategies of this disease would be useful and urgent in reducing health hazard, environments damage and the pollution potential [13]. Biofungicides may be an attractive alternative method for controlling this disease.

Biofungicides are living organisms (plants, microscopic animals such as nematodes, and microorganisms, including bacteria, fungi and viruses) or natural products derived from these organisms, that are used to suppress pest populations and pathogens [14]. Firstly, many researches have been reported that using nonpathogenic *Fusarium* spp. could control Fusarium wilts [15]. Secondly, some antagonistic strains showed high bioactivities against Fusarium wilt, such as *Trichoderma* spp. [16], *Bacillus* spp. [17] and *Aspergillus* spp [18]. Thirdly, plant extracts or phytochemicals, such as essential oils, sterides, phenolic acids and alkaloids had good antifungal activities. For example, it has been reported that essential oils from pepper, cassia tree, mustard and clove could suppress disease development caused by *F. oxysporum* f. sp *melonis* on muskmelon and reduce the population density of pathogen in greenhouse experiments [19]. Wu et al found many benzoic acid analogues such as gallic acid, ferulic acid and p-hydroxybenzoic acid both strongly inhibited FON growth [20-22].

Cuminic acid (p-isopropyl benzoic acid), isolated from the seed of *Cuminum cyminum*. L [23], belongs to the chemical groups of benzoic acid [24]. In previous study, it has been reported that cuminic acid possessed good inhibition to several plant pathogens, such as *Sclerotinia sclerotiorum*, *Phytophthora capsici*, *Rhizoctonia cerealis*, and *Fusarium oxysporum*. EC_{50} values of cuminic acid against mycelial growth of *P.capsic* and *S. sclerotiorum* were only 19.7 μg/mL and 7.3 μg/mL, respectively[25], which were lower than the EC_{50} value of other benzoic acid derivatives in previous report[20-22]. In pot experiment, after the application of cuminic acid at 1000μg/mL, control efficacies of over 60% against *P.capsic* and *S. sclerotiorum* were obtained, which was comparable with the efficacy of metalaxyl (250 μg/mL)[24] and procymidone (100 μg/mL) [25].

Considering the broad-spectrum and significantly antifungal activity of cuminic acid and the difficult management of fusarium wilt, it's necessary to evaluate cuminic acid as a potential biopesticide to control fusarium wilt on watermelon. The objectives of this research were to: (a) determine the effect of cuminic acid on FON colony growth, (b) evaluate the effect of cuminic acid on the morphological and physiological characteristics of FON, (c) in greenhouse experiments, test the efficacy of cuminic acid against FON in watermelon plant, and study the effect of cuminic acid on the antioxidant defensive enzymes in watermelon plant subjected to fusarium wilt. (d). examine the effect of cuminic acid on differences in transcript levels for FON genes associated with the biosynthesis of fusaric acid and pigment by quantitative RT-PCR method.

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2. Results

2.1. Effect of cuminic acid on FON colony growth

The effects of various concentrations of cuminic acid on the mycelial growth of FON are shown in Table. 1, and cuminic acid were found to inhibit the mycelial growth of cuminic acid in a dose-dependent manner. Mycelial growth of FON was strongly inhibited by cuminic acid at a relative low concentration of 25μg/mL. Based on log-transformation analysis, EC_{30} EC_{50} and EC_{70} values were calculated as 5.6, 22.53 and 91.3 μg/mL, respectively.

Table 1. The effect of cuminic on FON colony growth

Compounds	Regression equation (Y =)	EC_{50} (μg·mL-1)	Confidence interval of EC_{50} (P<0.05)	$\chi 2$
Cuminic acid	3.83+0.86X	22.53	17.85-25.96	4.83

Note: Data represents the mean value of triplication. The EC_{50} was assessed based on log-transformation analysis. Y: Probit-inhibition (%); X: log-dose.

2.2. Effect of cuminic acid on mycelial morphology of FON

A clear effect of the cuminic acid on mycelia morphology of FON was observed (Fig. 1). After 7 days' incubation, treatment with cuminic acid at the EC_{50}, the color of mycelia was visible lighter than control (Fig. 1a, d) in PDA plates. While the mycelia of the control were natural, uniseriate and uniform (Fig. 1b, c) in the mirror by SEM. For strains amended with cuminic acid at the EC50, mycelia were severely deformed, twining and clustered (Fig. 1d, e).

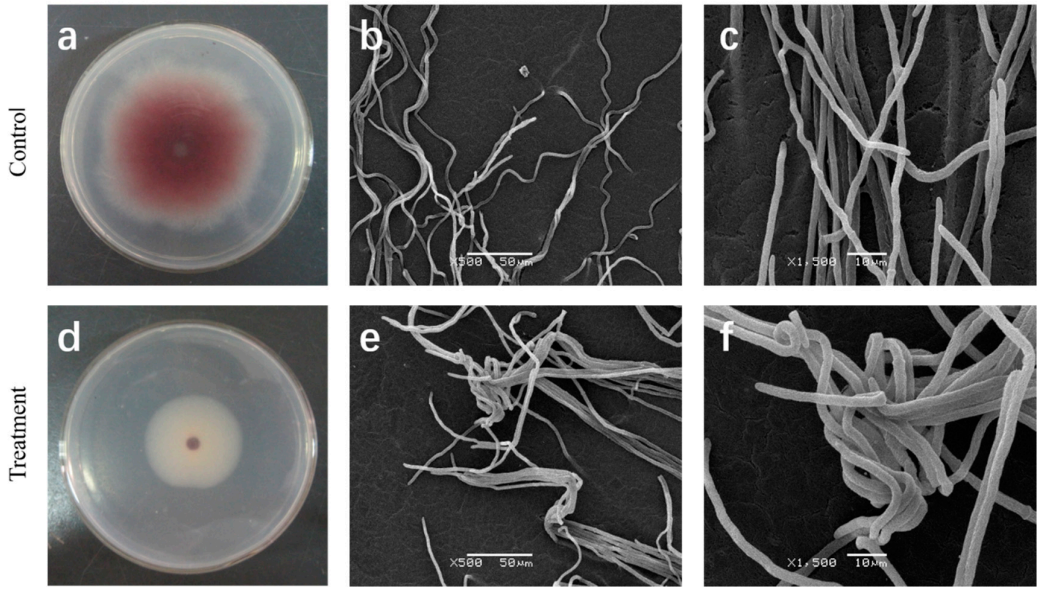

Figure 1. Effect of cuminic acid on mycelia morphology of FON. (c, d, e): Untreated plates; (a, b, c): Plates treated with cuminic acid at EC_{50} value (22.53 μg/mL). Values are means and standard errors.

2.3. Effect of cuminic acid on cell membrane permeability of FON

To confirm the membrane-disruption effects of cuminic acid on the hyphal cells, the relative conductivity of the mycelia treated with cuminic acid were determined. As shown Fig. 2, the relative

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conductivity of the mycelia treated with cuminic acid increased gradually during incubation, being about 45.78% higher than that of control after 120min incubation.

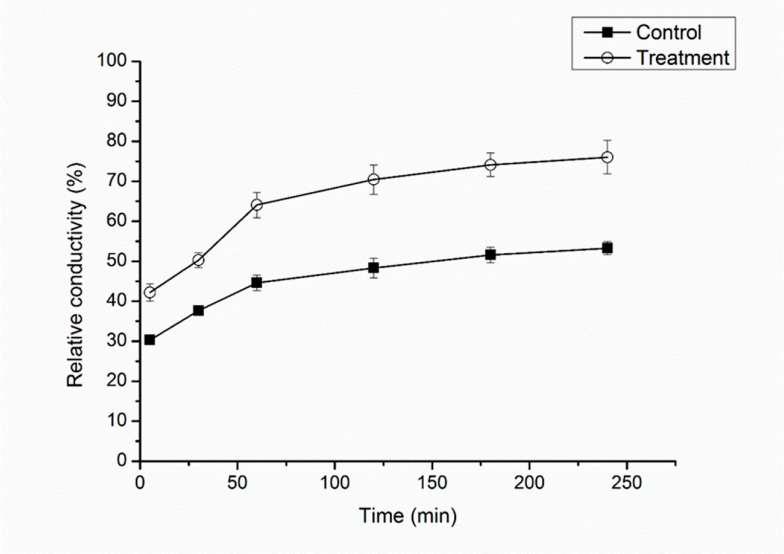

Figure 2. Mycelial relative conductivity of FON with or without cuminic acid treatment at the concentration of EC_{50} value (22.53 μg/mL). Values are means and standard errors.

2.4. Glycerol content of mycelia

After treated with cuminic acid, the content of glycerol was always significantly higher than the control without cuminic acid treatment (Fig. 3). With the concentration increasing, the glycerol content of mycelia was increased over time. The glycerol contents of three concentrations cuminic acid (EC_{30}, EC_{50} and EC_{70}) significantly increased by 79.3%, 313.56% and 631.57%.

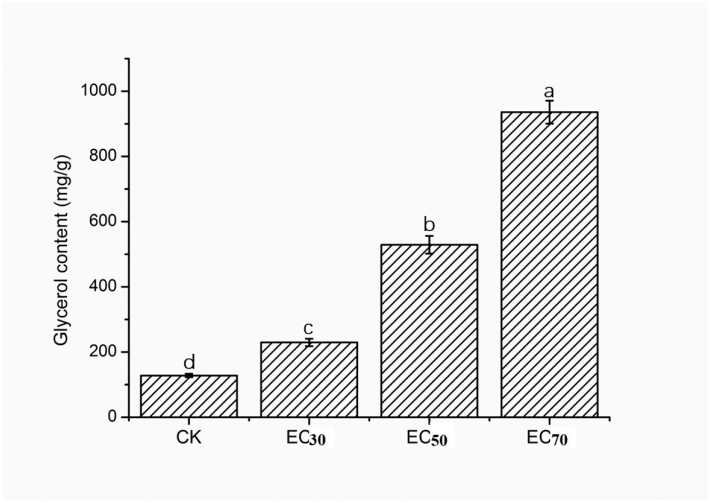

Figure 3. Glycerol content of mycelia of FON with or without cuminic acid treatment at concentrations of EC_{30}(5.6 μg/mL), EC_{50}(22.53 μg/mL) and EC_{70}(91.3 μg/mL). Bars denote the stand error of three experiments. Data represents means of three replications with standard deviation. Data (means ± SD, n=3) followed by the same letters in the row show no significant differences (small letters, $P<0.05$).

2.5. Mycotoxin concentration of FON in liquid culture

Mycotoxin production (mainly fusaric acid) production of FON in PDB was suppressed by

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cuminic acid treatment in a concentration dependent manner. Significant suppression was found even at the lower concentration of EC_{50} value. The mycotoxin concentration was decreased by 24.57-66.22% compared with control (Fig.4).

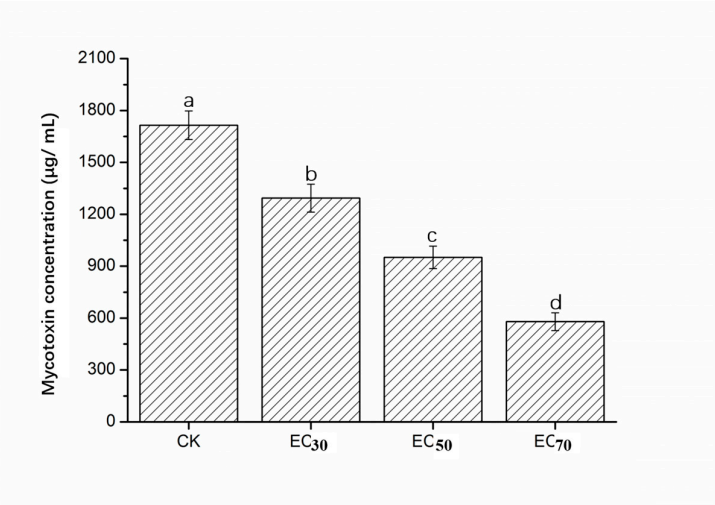

Figure 4. Mycotoxin production (mainly fusaric acid) concentration in FON with cuminic acid treatments at concentrations of EC_{30}(5.6 μg/mL), EC_{50}(22.53 μg/mL) and EC_{70}(91.3 μg/mL) in liquid culture. Bars denote the stand error of three experiments. Data represents means of three replications with standard deviation. Data (means ± SD, n=3) followed by the same letters in the row show no significant differences (small letters, $P<0.05$).

2.6. Greenhouse experiments

The effect of cuminic on FON was evaluated under greenhouse conditions (Table. 2). Our experiment demonstrated that cuminic acid at all concentrations has a significantly suppression effect on FON. In plants under cuminic acid at 2000 μg/mL obtained 21.5% disease index and 74.5% efficacy, which was no significant difference with the carbendazim at 1000μg/mL. However, in plants under cuminic acid at 1000 μg/mL, the disease index and efficacy were 38.8% and 54.5%, which was lower than under carbendazim at 1000 μg/mL.

Table 2. Effect of cuminic acid on control of Fusarium wilt on watermelon

Compounds	Concentrations (μg mL-1)	Disease index (%)	Efficacy (%)
Cuminic acid	1000μg/mL	38.8±2.5b	54.5±2.3b
	2000μg/mL	21.4±1.51c	74.5±1.5a
	3000μg/mL	24.8±1.15c	71.9±1.22a
Carbendazim	1000μg/mL	23.2±1.18c	72.8±1.4a
Water	___	85.5±3.5a	___

Note: Results are the means of 10 watermelon plants and from two independent experiments. Means followed by the same letters were not significant differences according to LSD ($P = 0.05$)

2.7. Assay of defense enzyme activities and malondialdehyde (MDA) content

The activities of SOD, POD and CAT are shown in Fig.5a-c and the content of MDA are shown

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in Fig.5d. Activities of SOD, POD, CAT under cuminic acid treatment in watermelon leaves were enhanced in comparison with control except for cuminic acid treatment at 4000 μg/mL in POD activities. SOD and POD activities experienced the trend in all the plants, the highest enzyme activity was found in treatment cuminc acid at 1000 μg/mL, which correspond to 43.65%(Fig. 5a) and 27.87%(Fig. 5b) increase compared with control. As for CAT activity, the highest enzyme activity was found in treatment cuminc acid at 2000 μg/mL, which correspond to 59.55%(Fig. 5c) increase compared to control. However, MDA content decreased steadily in all the samples during the whole experimental period with the increased concentration of cuminic acid.

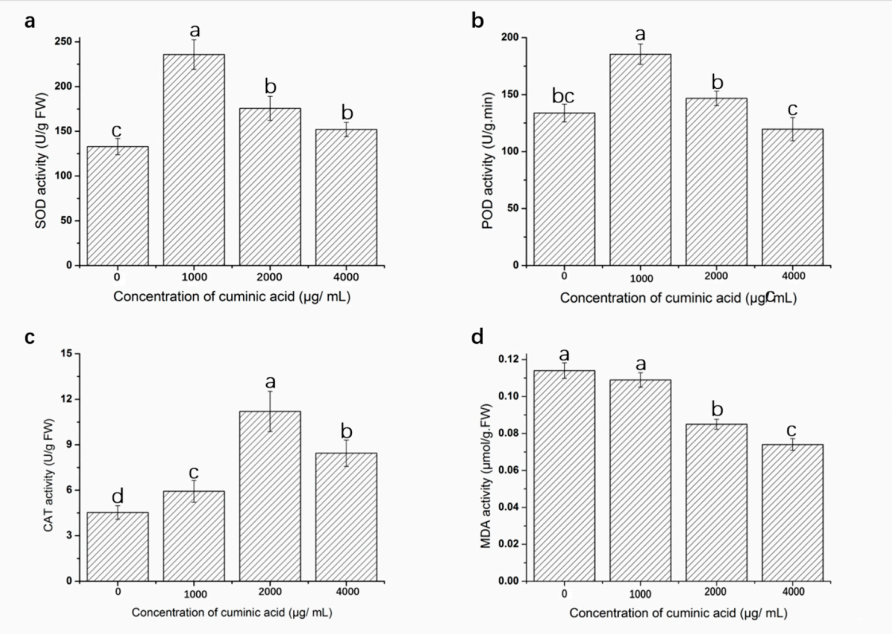

Figure 5. SOD(a), POD(b) and CAT(c)activities and MDA activity (d) of the watermelon leaves treated with cuminic acid at 0, 1000, 2000 and 3000 μg/mL, respectively. Data represents means of three replications with standard deviation. Data (means ± SD, n=3) followed by the same letters in the row show no significant differences (small letters, $P<0.05$).

2.8. Quantitative RT-PCR

To confirm whether the biosynthesis of fusaric acid and pigment in FON would be affected by cuminic acid, expression of genes (Table. 3) including synthesis of bikaverin (Bike1, Bike2 and Bike3), fusaric acid (FUB1, FUB2, FUB3 and FUB4) and components of a velvet-like complex (Lae1 and Vel1) were quantified. Relative to expression in the wild-type strain (Fig. 6), synthesis genes of bikaverin (Bik1, 2 and 3) and fusaric acid (FUB1,2,3 and 4) both exhibited decreased expression compared with the internal control. Expression of FUB3, FUB4 and Bike2 were about 0.88, 0.77 and 0.46 folds relative to the internal control. However, genes of components of a velvet-like complex (Lae1 and Vel1) exhibited significantly increased expression were 1.95 and 1.37 folds.

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170 **Table 3. qRT-PCR primers applied in this study**

Gene name	Accession number	Primer	Sequence (5′-3′)
Bike1	AJ278141	Forward	CGGTATCTGTGGTGGTGTC
		Reverse	TCGGGAGGTGATGTTGTG
Bike2	AM229668	Forward	TGCCTGCTCCACAGTCTACG
		Reverse	GCCAATCTTGACCGCCAC
Bike3	AM229667	Forward	CGCCAAAGTCATCAAGGA
		Reverse	AGGCTCAGGCACCACAAA
FUB1	FFUJ_02105	Forward	ACTTCGCCTCGTCATCTC
		Reverse	GAACCCAGCATCAAACTTAT
FUB4	FFUJ_02108	Forward	CACCCTTGCTCATCACAG
		Reverse	CGTAAAAATATCCTTCCGAATAATC
FUB2	FFUJ_02106	Forward	GCCAACTGCTGTCACTAT
		Reverse	TTCCGAGGTGGAGATTAG
FUB3	FFUJ_02107	Forward	CCCGATACACCATACCCT
		Reverse	CCAACTTCTTGCCGTGAG
Lae1	FVEG_00539	Forward	TATTGGTACGGGCACAGG
		Reverse	GGCATAAAGCCAGGAGGA
Vel1	FN548142	Forward	CTACTAAGGAGGAAAGGGACT
		Reverse	TCCATCAAACCAGGAAACT
Related actin gene	Foxq13729	Forward	GAGGGACCGCTCTCGTCGT
		Reverse	GGAGATCCAGACTGCCGCTCAG

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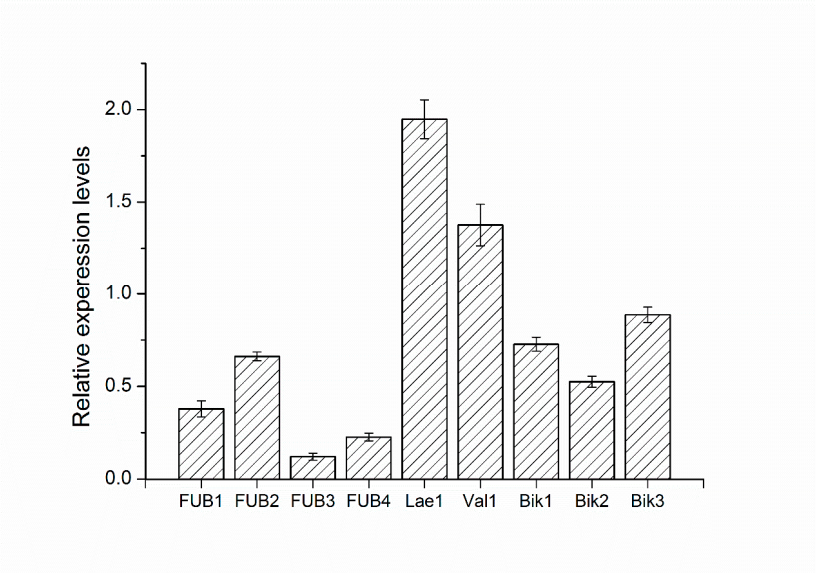

Figure 6. ene expression level of synthesis of fusaric acid (FUB1,2,3 and 4) and bikaverin (Bik1, 2 and 3), and components of a velvet-like complex (Lae1 and Vel1) relative to without treatment cuminic acid. Values are the means ± standard error (SE) of three repeated experiments.

3. Discussion

In previous study, cuminic acid and cuminic aldegyde as major bioactive constituents of *C. cyminum* seed was reported possess broad-spectrum antifungal activities [23, 25]. Cuminic acid as a representative chemical of benzoic acid group, also exhibited a significantly antifungal activity and enhanced the defense capacity of plants against *Phytophthora capsica* [24]. This study is focused on the biochemistry and physiology alterations in *Fusarium oxysporum* f.sp. *niveum* mediated by cumunic acid, and confirms that this chemical as a potential biofungicide has a value of development and utilization.

In the current study, results showed that the growth of FON was strongly inhibited by cuminic acid in a concentration-dependent manner, with a EC_{50} values of 22.53μg/mL. Cuminc acid exhibited a significant higher antifungal activity in PDA plates compared with other chemicals of benzoic acid group, such as cinnamic acid [26], gallic acid [20] and sinapic acid [8]. Interestingly, we found that the color of mycelia in the strains treated with cuminic acid at the EC_{50} in PDA plates was visible lighter than that in control, and mycelial would be abnormal by SEM. In addition, the cell membrane permeability and glycerol content were significantly enhanced, which was consistent with cuminic acid against *Phytophthora capsica* [24], which indicated that the mechanism of cuminic acid against plant pathogens might be through damaging the mycelial structure and inducing the intracellular plasma leakage.

Mycotoxin (mainly fusaric acid) production is widely distributed among the whole *Fusarium* species [27], particularly pathogenic strains of *F. oxysporum*. And it is an important pathogenic factor causing wilt diseases in various plants, such as watermelon, tomato and cucumber. Importantly, the increased virulence to host with the increase of mycotoxin production by *F. oxysporum* [28]. In the initiation of infection and symptom development, the toxins produced by pathogens was a pathogenicity determinant in FON [29]. In the current study, a significant reduction of mycotoxin was observed after treatment with cuminic acid, indicating that cuminic acid could reduce the pathogenicity of FON by inhibiting the secretion of mycotoxins (mainly fusaric acid). According to reduction of pigment and fusaric acid production, we selected 9 genes associated with the biosynthesis of fusaric acid [5, 30] and pigment [3] to determine whether FON treatment with cuminic acid would affect the biosynthesis of fusaric acid and pigment by quantitative RT-PCR. Synthesis genes of bikaverin (Bik1, Bik2 and Bik3) and fusaric acid (FUB1, FUB2, FUB3 and FUB4)

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both were down regulated compared with the control, which was consistent with previous study. Previous study has documented that genes of components of a velvet-like complex (Lae1 and Vel1) are participated in biosynthesis and modulate the expression of fusaric acid [3, 5]. However, these significantly overexpressed genes in this study still need to be further studied.

In greenhouse experiment, cuminic acid at all concentrations has a significantly suppression effect on FON. Treatment with cuminic acid at 2000 μg/mL, the disease index and efficacy were no significant difference with the carbendazim at 1000μg/mL, indicating that cuminic acid has significantly antifungal activities against FON and possesses potential as a biofungicide.

Due to Reactive Oxygen Species (ROS) are harmful to several cellular components and they can cause lipid peroxidation and induce membrane injury, then result in cell senescence [31]. Moreover, antioxidant enzymes such as SOD, POD, CAT play crucial roles in suppressive oxidative stress. When ROS increases, SOD directly catalyzes O^{2-} into H_2O_2. Then H_2O_2 is converted to water and oxygen by CAT [32], while POD decomposes H_2O_2 into H_2O and O_2. Meanwhile, the POD enzyme participates in the construction, rigidification and lignification of cell walls, which protects plant tissues from damage [33]. In addition, the high MDA reflects the higher production of H_2O_2 and ROS [34]. In the present study, activities of SOD, POD, CAT under cuminic acid treatment in watermelon leaves were significantly enhanced in comparison with control. Correspondingly, the decreased MDA content in cuminic acid treated was observed. These data clearly suggested that cuminic acid could prevent FON development and reduce the level of lipid peroxidation through a mechanism involved activation of antioxidant defensive enzymes.

In conclusion, cuminic acid has a high inhibition effect in the mycelial growth of FON and watermelon plant. Although this work needs further study to entirely understand the mode of action by cuminic acid against FON, we concluded that cuminic acid used in this study could be developed as a promising biofungicide.

4. Materials and Methods

2.1 Pathogen strains and fungicides

Fusarium oxysporum f.sp. *niveum* were collected from infected watermelon plant and maintained on Potato dextrose agar (PDA) [24] medium, which were provided by the Laboratory of Research and Development Center of Biorational Pesticide, Northwest A & F University.

Cuminic acid (98%) and carbendazim (98.0%) used in the experiment were purchased from the Sigma Co. (St. Louis, Mo, USA).

2.2 Effect of cuminic acid on FON colony growth

The effect of cuminic acid on colony growth was determined as following: PDA media were amended with a series of cuminic acid at the finally used concentrations of 0, 3.125, 6.25, 12.5, 25, 50 and 100μg/mL. A 5-mm mycelial plug taken from the leading edge of 7-day-old colonies was inoculated into the center of the amended PDA medium. Plate was incubated in a growth chamber at 28℃ for 7 days, colony diameter was determined by measuring the average of two perpendicular directions on each plate. According to previous studies [24], the EC_{50} values were calculated by regressing percentage growth inhibition against the log of cuminic acid concentration. Each concentration with three replicates was conducted thrice.

2.3 Effect of cuminic acid on mycelial morphology of FON

Mycelia plugs cut from the margin of 7-day-old colony were placed on PDA plates containing cuminic acid at the EC_{50}(22.5μg/mL) for inhibition of mycelial growth. Control was plates without cuminic acid. After 7 days at 28□, the margin of medium area (10 mm×10 mm) was placed on slide glass. High-resolution images of mycelial morphology changes in cuminic acid treated samples were obtained by scanning electron microscope (SEM, JSM-6360LV, Japan) [35]. Three replicates were processed and the experiment was repeated twice.

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252 *2.4 Effect of cuminic acid on cell membrane permeability of FON*

253 Mycelial cell membrane permeability was expressed as the relative conductivity. Ten mycelial
254 plugs were added into 250-mL flasks containing 100 mL of potato dextrose broth (PDB). The flasks
255 were shaken at 180 rpm and 28℃ for 5days, partial flasks were amended with cuminic acid at the
256 EC_{50}(22.5μg/mL). Control was flasks without cuminic acid. The flasks were continued to shake for 2
257 days, mycelia were collected by filtration through filter paper, per sample (0.5 g mycelia) was
258 suspended in 20 mL of distilled water. By a conductivity meter (CON510 Eutech/Oakton,
259 Singapore), conductivity of the treated water was measured after 5, 30, 60, 120, 180, 240min. After
260 240 min, final conductivity was determined by mycelia were boiled for 5 min to completely kill the
261 tissues and release all electrolytes and cooled to 25□. The experiment with three replicates was
262 repeated three times. The relative conductivity was calculated as following formula [36]:

263 $$\text{Relative conductivity} = \text{Conductivity at different times} / \text{Final conductivity} \times 100\%$$

264 *2.5 Glycerol content of mycelia*

265 Glycerol content was determined using the described method [37] with minor modification. A
266 standard curve for glycerol was obtained according to the described method. The mycelia of FON
267 strain was prepared as described above. In addition, partial flasks were amended with cuminic acid
268 at the EC_{30}(5.6μg/mL), EC_{50} (22.5μg/mL) and EC_{70}(91.3μg/mL). 0.5g mycelia of per sample were
269 rubbed with a freezed pestle and a mortar. The sample was washed thrice with autoclaved distilled
270 water and transferred to 10-mL centrifuge tubes. The volume for each sample was adjusted to 10 ml
271 with water and ground. According to the standard curve, glycerol content of the sample was
272 calculated. Each treatment was processed with three replicates, and the test was repeated three
273 times.

274 *2.6 Mycotoxin conerntration of mycelia*

275 The content of mycotoxin production (mainly fusaric acid) was determined as described by Wu
276 et al [26]. A standard curve was prepared with standard of fusaric acid (Sigma, St Louis, Mo, USA).
277 Ten mycelial plugs were added into 250-mL flasks containing 100 mL PDB. The flasks were shaken
278 at 150 rpm and 28□for 10 days, partial flasks were amended with cuminic acid at the EC_{30}, EC_{50} and
279 EC_{70}, Control was flasks without cuminic acid. The flasks were continued to shake for 4 days, the
280 culture filtrate was collected after filtration. The culture filtrate acidified to pH 2 with 2M HCL and
281 added an equal volume of ethyl acetate， shaken with sudden force for 1min, placed for 30min and
282 then collected organic phase in a new tube. Above procedure was repeated three times. The organic
283 phase was centrifuged at 4000 rpm for 15min and the supernatant was collected and dried. The dried
284 residue was redissolved with ethyl acetate to 5 ml. By UV spectrophotometry (UV-5100
285 spectrophotometer Yuan Xi, Shanghai, China), the OD268 was measured. Each treatment was
286 processed with three replicates, and the test was repeated three times.

287 *2.7 Preparation of FON inoculum and the watermelon seedlings*

288 Ten mycelial plugs were added into 250-mL flasks containing 100 mL PDB. The flasks were
289 shaken at 150 rpm and 28□for 7-10 days, depending on experiments. The spore suspensions were
290 filtered and adjusted to 1×106 cfu/ml with a hemacytometer.
291 The watermelon seeds were surface disinfected in sodium hypochlorite (5%, w/v) for 5 min,
292 washed twice with sterile water and then germinated in a 9cm diameter sterile plates containing wet
293 filter paper. The germinated seeds were sown into each nursery cups (4 cm diameter, 6 cm high)
294 containing a sterilized
295 mixture of nursery soil, organic manure and sand (2:1:1, w/w). The seedlings were grown in
296 greenhouse (natural light at 32/18□ (day/night) and 50-70% humidity with). Seeding were watered
297 when needed. Watermelon seedlings (two cotyledon period stage) transplanted into pots (10 cm
298 diameter, 15 cm high) containing enough sterilized mixture of nursery soil. The seedings (two true
299 leaves stage) were used for all experiments.

2.8 Greenhouse experiments

Experiments were completely randomized designs with five treatments. The five treatments were as follows: water, cuminic acid at 1000, 2000 and 4000μg/mL, and carbendazim at 1000μg/mL. 10 ml of treatment were poured into the plant root when 10 ml of FON spore suspension (106 cfu/ml) was inoculated. During the procedure of treatment, plant roots were injured by minor vulnerable cuts. After 3 weeks of treatment, 10 watermelon plants per treatment examined disease severity was measured according to Rojan et al [38]. The disease index and efficacy were calculated according to Zhao et al [39]. Ten plants per treatment were applied and the experiment was repeated twice.

2.9 Assay of defense enzymes activities and malondialdehyde (MDA) content

Watermelon leaves cut from the plants treated with cuminic acid in the above section were collected on ice. 3g leaf per sample were homogenized and suspended in 8 ml of 0.5 Mm phosphate buffer, pH 7.8, containing 0.2 mM EDTA and 2% PVPP and centrifuged 100000 rpm for 20 at 4□ and the resulting supernatants were directly used for assay. POD (peroxidase) and SOD (superoxide dismutase) activities were determined by the methods of Garcia-Limones et al [40]. CAT(catalase) activity was carried out following the procedures described by Sun et al [41]. As for MAD content, the assay mixture consisted of 5% trichloroacetic acid (TCA) and 0.6% thiobarbituric acid (TBA). And MDA concentration was carried out according to the methods described by Heath and Packer [2]. Five leaves per treatment were used and the experiment was conducted twice.

2.10 Quantitative RT-PCR.

Quantitative RT-PCR was carried out in FON to examine differences in transcript levels for genes associated with the biosynthesis of fusaric acid [5, 30] and pigment [3]. The mycelia of FON strain was prepared as described the method of 2.4. Total RNA was isolated from mycelia of FON strain using the RNA extraction kit (Takara, Dalian) according to the manufacturer's protocol. First-strand cDNA was generated from RNA using the Prime Script RT reagent kit (Takara, Dalian). In this study, actin gene was set as the internal control, and all applied primers for qRT-PCR were listed in Table.3. qRT-PCR was carried out in a 20μL reaction mixtures containing 12 μl SYBR Premix Ex Taq II (Takara, Dalian), 0.8 μL of each primer and 1.6 μL templated DNA. All quantitative RT-PCRs were performed with an CFX96TM real-time detection system (Bio-Rad, Hercules, CA, U.S.A). Each sample was run twice from three independent biological experiments. The results were calculated according to the $2^{-\Delta\Delta Ct}$ method [42].

2.11 Statistical analysis

In this study, data from repeated experiments were combined for analysis， owning to variances between experiments were homogeneous. All data were processed and analyzed using SPSS 14.0 (SPSS Inc., Chicago, IL) according to previous studies [24]. When ANOVAs were significant ($p = 0.05$), means were separated with Fisher's least significant difference (LSD)

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