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Cinnamic Acid Inhibits Growth but Stimulates Production of Pathogenesis Factors by *in Vitro* Cultures of *Fusarium oxysporum* f.sp. *niveum*

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Long-term monoculture of watermelon leads to frequent occurrence of watermelon fusarium wilt caused by *Fusarium oxysporum* f.sp. *niveum* (FON). Some allelochemicals contained in watermelon root exudates and decaying residues are possibly responsible for promoting the wilt disease. The purpose of this study was to evaluate the allelopathic effect of artificially applied cinnamic acid on FON. Results demonstrated that hyphal growth of FON was strongly inhibited by cinnamic acid. At the highest concentration of cinnamic acid, the biomass in liquid culture was decreased by 63.3%, while colony diameter, conidial germination on plates, and conidial production in liquid culture were completely inhibited. However, mycotoxin production and activity of phytopathogenic enzymes were greatly stimulated. Mycotoxin yield, pectinase activity, proteinase activity, cellulase activity, and amylase activity were increased by 490, 590, 760, 2006, and 27.0%, respectively. It was concluded that cinnamic acid dramatically stimulated mycotoxin production and activities of hydrolytic enzymes by FON but inhibited growth and germination of FON. The findings presented here indicate that cinnamic acid is involved in promoting watermelon fusarium wilt.

KEYWORDS: Biomass; cinnamic acid; *Fusarium oxysporum* f.sp. *niveum* (FON); mycotoxin production; pathogenic enzymes

INTRODUCTION

Watermelon production is highly remunerative. However, long-term monoculture of watermelon results in frequent incidence of watermelon fusarium wilt because of continuous accumulation of the pathogen, *Fusarium oxysporum* f.sp. *niveum* (FON), which is the causal agent of this disease. The pathogen is very difficult to eradicate from the soil once it has been introduced into watermelon fields. It causes severe economic losses and is the most important soilborne pathogen limiting watermelon production in many areas of the world (*31*).

Fusarium sp. is a genus of harmful fungi that cause vascular diseases of plants, such as watermelon, cucumber, tomato, pepper, muskmelon, bean, and cotton (*3, 21, 33, 37*). Fusarial fungi damage host plants through penetration of hyphae into host vascular tissues, secretion of hydrolytic enzymes, and mycotoxin production (*1, 2, 5, 12, 17, 20, 40*). More than 50% of the isolates of the known *Fusarium* species are toxigenic and produce deleterious secondary metabolites (*30*).

A lot of work has been performed on the effect of *Fusarium* sp. on watermelon and other plant hosts. These studies have been focused on how the pathogen invades and damages the host. Little attention has been paid to the effects of the host plant on the pathogen. In fact, invasion of the pathogen into the host plant is affected by root exudates and decaying residues of host plants. It is well-known that root exudates and decaying residues are phytotoxic because of some allelochemicals contained in them. These allelochemicals have been isolated and identified mainly as organic acids especially phenolic acids, such as cinnamic, vanillic, coumaric, and ferulic acid (*4, 22, 39, 53*). However, very little information is available on the allelopathic action of physiologically active components from root exudates or decaying residues of plants on specific microorganisms, although some research has been done on the effect of root exudates or decaying residues on microbial community structure. In particular, the effect of root exudates on the methanogenic microbial community of rice or maize roots and the rhizosphere (*7, 28*) as well as bacterial community composition (*27, 41, 46*) have been studied. The antibacterial activity of cinnamic acid against species of *Corynebacteria*, *Enterococci*, *Staphylococci*, and *Streptococci* (*35*) has also been reported. The effect of cinnamic acid on microbial biomass (*48*) has been studied. The stimulation of gingseng root exudates on growth of *Phytophthora cactorum* and *Pythium irregulare* (*38*) was comfirmed. The effect of root exudates on rhizobacterial populations (*36, 41, 47*) and fungal species abundance and richness in desert (*29*) have been extensively examined.

Growing evidence shows that root exudates might initiate and manipulate biological and physical interactions between roots

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and soil organisms and thus play an active role in root−microbe communication (*6*). Artificially applied chemicals, such as ferulic, caffeic, and vanillic acid, were added to soil to test an effect of phenolic acids on microbial populations and biomass (*9, 10, 45*). Cinnamic acid is a phytotoxic allelochemical produced in plant root exudates and is derived from the L-phenylalanine ammonia-lyase reaction inside the plant (*11, 53*). Growth inhibition and decrease of respiration of *Rhodotorula minuta*, a basidiomycete fungus by cinnamic acid and biodegradation of cinnamic acid by *Colletotrichum gloeosporoides* and inhibition of fungal 17 β-hydroxysteroid dehydrogenase from *Cochliobolus lunatus* by cinnamates has been reported (*18, 19, 23*). Very little work, if any, has been carried out to test the effect of cinnamic acid on FON.

The objective of this study was to assess the potential allelopathic role of artificially applied cinnamic acid in affecting the watermelon−fusarium interaction. This would be expected to help understand the mechanism of fusarial wilt diseases.

MATERIALS AND METHODS

Pathogen Strains and Chemicals. *F. oxysporum* f.sp. *niveum* (coded NJAUS-1) was isolated from infected watermelon by the Laboratory of Plant−Microbe Interactions, Nanjing Agricultural University, China. Cinnamic acid and the other main chemicals used in the experiment were obtained from Sigma Co. (St. Louis, MO).

Measurement of FON Growth. A 5 mm agar plug taken from a 7-day-old potato dextrose agar (PDA) culture was inoculated on the center of the plate and was incubated at 28 °C for 7 days. The colony diameter was measured in three directions on each plate after incubation for 3 and 7 days.

Assessment of Conidial Germination. To determine the effect of cinnamic acid on conidial germination, FON was grown in 2% water agar. A 5 mm agar plug taken from a 7-day-old PDA culture was inoculated in a liquid culture and incubated in 28 °C for 7 days. The broth was filtered to collect conidia. Conidial suspension was diluted to ≤1000 conidia per millimeter with sterile distilled water. A total of 0.1 mL of the diluted suspension was spread on plates and incubated at 28 °C for 3 days. The number of colonies was counted daily for 3 days.

Determination of Sporulation. Sporulation was determined following growth of FON (as described above) in Bilay and Joffe's medium (*12*) with minor modifications (4.0 g of sodium carboxymethyl cellulose instead of 15 g of carboxymethyl cellulose, pH adjusted to 4 with 2 mol L^{-1} HCl). After incubation for 7 days, 0.1 mL of culture broth, diluted to 10^{-5}−10^{-7}, was spread onto PDA. Plates were incubated at 28 °C in dark for 4 days, after which colonies were counted and converted to the number of conidia in a liquid culture.

Measurement of Biomass Production and Enzyme Activity. FON was grown in 100 mL conical flasks filled with 30 mL of potato dextrose broth adjusted to pH 4.5 with 2 mol L^{-1} HCl and inoculated with a 5 mm agar plug taken from a 7-day-old PDA culture. Cultures were incubated in a shaker (170 rpm) at 28 °C for 7 days. Fungal biomass (dry weight) was determined after filtration and drying at 80 °C for 12 h, when constant weight was achieved. Culture filtrate was used for enzyme assays.

Protease activity was assayed as described by Tseng and Mount (*50*). One unit of enzyme activity was defined as a 0.001 increase in absorbance per minute under the assay conditions. Pectinase activity (mainly polygalacturonase) was assayed using the 3,5-dinitrosalicylic acid (DNS) method (*43*). One unit of enzyme activity was defined as the amount of β-galacturonic acid hydrolyzed from pectin per minute under the assay condition. Cellulase activity was also determined by the DNS method (*8*). One unit cellulase activity was defined as the amount of enzyme that produced 1 μmol reduced sugar per minute under the above assay condition. Total amylase activity was assayed by DNS method (*34*). One unit of amylase activity was defined as the amount of enzyme that releases 1 mg of reducing sugars (glucose equivalents) per minute under the above assay conditions.

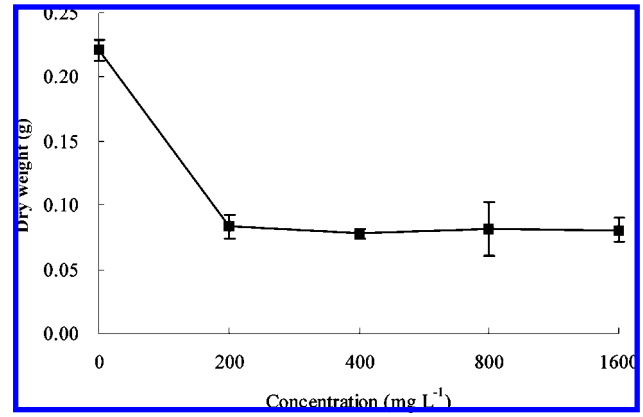

Figure 1. Effect of cinnamic acid on biomass of FON after 7 days of liquid culture. Bars represent SE.

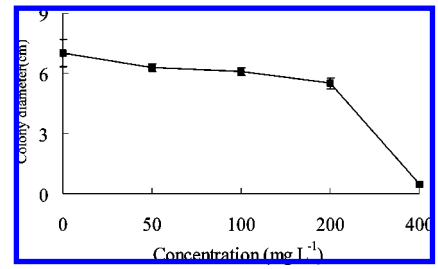

Figure 2. Effect of cinnamic acid on colony growth of FON for 7 days on PDA plates. Bars represent SE.

Extraction and Assay of Mycotoxin. For estimation of mycotoxin production, FON was grown in Richard's medium (*20*), as described above but with a 12 h photoperiod with fluorescent light for 35 days. Broth was acidified to pH 2 with 2 mol L^{-1} HCl, mixed with equal volume of ethyl acetate, vigorously shaken for 2 min, and settled for 30 min, and the organic phase was removed. After this procedure was repeated 5 times, the organic phase was centrifuged for 10 min at 5000 rpm. The supernatant was dried and condensed at ≤40 °C. The dried residue was redissolved in 5 mL of ethyl acetate, and the A_{268} was determined by UV spectrophotometry (UV-120-02 spectrophotometer, Shimadzu, Japan).

Experimental Design and Statistical Analysis of Data. On the basis of our preliminary experiments, studies were carried out using the following concentrations of cinnamic acid: 200, 400, 800, and 1600 mg L^{-1}. Controls were not treated with cinnamic acid but instead 2 mL of sterilized water. Cinnamic acid solution was filter-sterilized by a 0.22 μm of pore membrane (Millipore). Data were analyzed by Microsoft Excel. The values were represented as means of three replicates (mean ± SE) for each treatment. Here, SE means standard error.

RESULTS

Inhibition of Cinnamic Acid on Growth and Biomass of FON. Strong inhibition of FON growth by cinnamic acid was observed. From 200 mg L^{-1} of cinnamic acid, FON growth was strikingly suppressed. Dry weight of mycelia in treatments of 0–1600 mg L^{-1} was decreased from 0.22 ± 0.01 to 0.08 ± 0.01 g (**Figure 1**). Hyphal growth (colony diameter) of FON was inhibited by cinnamic acid in a concentration-dependent manner. The higher the concentration of cinnamic acid, the less the growth on the plate. The colony diameter was reduced from 7.0 ± 0.63 to 0.0 cm by treatments of 0–1600 mg L^{-1} (**Figure 2**).

Inhibitory Effect on Conidial Germination and Sporulation. Severe inhibition of conidial germination and sporulation by cinnamic acid was observed. This inhibition was concentration-dependent. Little inhibition of conidial germination was observed at low concentrations (200–400 mg L^{-1}), with greater

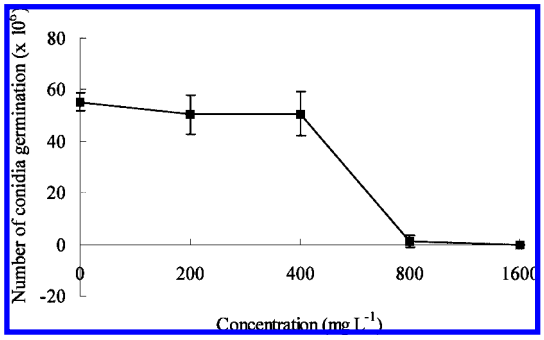

Figure 3. Effect of cinnamic acid on conidial germination of FON after growth on plates for 7 days. Bars represent SE.

inhibition at concentrations more than 800 mg L^{-1} and complete inhibition at 1600 mg L^{-1} on plate (**Figures 3** and **4**). The number of conidia formed in liquid culture in treatments of 0, 200, 400, 800, and 1600 mg L^{-1} was 76.4 ± 24.0 × 10^6, 5.8 ± 2.0 × 10^6, 0.9 ± 0.8 × 10^6, 0, and 0, respectively, which indicated full inhibition of sporulation at 800–1600 mg L^{-1} (**Figure 5**).

Stimulatory Effect on Mycotoxin Production. Mycotoxin yield of FON in liquid culture was increased by cinnamic acid treatment. The yields at different treatments (0–1600 mg L^{-1}) were elevated from 26.9 ± 4.9 to 132.5 ± 0.7 μg mL^{-1} (**Figure 6**).

Impact on Activity of Hydrolytic Enzymes. *Stimulation of Pectinase Activity.* A strong stimulation of pectinase activity in liquid culture by cinnamic acid was found. The activity at concentrations of 0–1600 mg L^{-1} was increased from 0.01 ± 0.000 to 0.052 ± 0.004 units mL^{-1} min^{-1} mL^{-1} (**Figure 7a**).

Stimulation of Proteinase Activity. Proteinase activity of FON in liquid culture was greatly stimulated by cinnamic acid in a concentration-dependent manner. The activity at concentrations of 0−1600 mg L^{-1} was increased from 0.42 ± 0.2 to 3.21 ± 1.2 units min^{-1} mL^{-1} (**Figure 7b**).

Stimulation of Cellulase Activity. A strong stimulation of cellulase activity in liquid culture by cinnamic acid was found. The activity was increased from 0.03 ± 0.01 (treatment without cinnamic acid) to 0.33 ± 0.06 μmol min^{-1} mL^{-1} (1600 mg L^{-1} cinnamic acid) with maximum activity in the treatment with 200 mg L^{-1} cinnamic acid (**Figure 7c**).

Stimulation of Amylase Activity. A strong stimulation of amylase activity in liquid culture by cinnamic acid was found. The activity increased from 3.7 ± 0.5 μmol min^{-1} mL^{-1} (treatment without cinnamic acid) with a maximum of 5.2 ± 0.9 μmol min^{-1} mL^{-1} at a concentration of 400 mg L^{-1} and, finally, 4.6 ± 0.1 μmol min^{-1} mL^{-1} at a concentration of 1600 mg L^{-1} (**Figure 7d**).

DISCUSSIONS

As an allelochemical to microorganisms, cinnamic acid may be characterized as having two main effects on the watermelon wilt pathogen. One is the inhibition of hyphal growth, sporulation, and conidia germination; another is the stimulation of mycotoxin production and enzymes related to pathogenesis.

Results showed that strong inhibition of hyphal growth and biomass was observed. Dry weight of mycelia at the highest concentration of cinnamic acid (1600 mg L^{-1}) in liquid culture was decreased by 63.3% (**Figure 1**). This is consistent with the observation that the mycelial growth of the *F. oxysporum* f.sp. *albedinis* is inhibited by cell wall-bound phenolics in resistant cultivars of date palm roots (*16*). Sporulation of FON was also greatly inhibited by cinnamic acid, particularly at concentrations of 800–1600 mg L^{-1}, wherein there is no conidial formation (**Figures 2**−**5**). Our results are also consistent with the observation that cinnamic acid at 1 g L^{-1} inhibits growth and reduces the respiration of *Rhodotorula* without killing the yeast (*19*). Also, the results are in accordance with other reports that growth of *Phytophthora parasitica* and *Pythium* sp. were strongly inhibited by cinnamic acid (*44, 49*). Also, it has been reported that incubation of *Neurospora crassa* for 24 h in liquid culture containing 200 mg L^{-1} of cinnamic acid reduces fungal mycelial growth by 94% as compared to growth without cinnamic acid (*42*). Use of cinnamic acid by soil microbes was a biodegradation of individual phenolic acids in soils (*10*). However, artificially applied phenolic acids (*p*-hydroxybenzoic, ferulic, caffeic, and vanillic acid) added to soil stimulate the growth of phenolic-acid-degrading organisms (*45*). We believe that effect of applied phenolic acids added to soil on microbial growth would have been affected by complicated soil environmental factors. This might be different with pure culture in a controlled environment in a laboratory study.

As a powerful weapon, mycotoxin production is observed when FON conquers watermelon and other plants. Mycotoxin is a fatal pathogenic factor causing plants to wilt. It is a well-known phytotoxin produced by several *Fusarium* sp., particularly pathogenic strains of *F. oxysporum*, which causes wilt diseases in a great variety of plants, such as watermelon, cucumber, tomato, beans, and cotton (*20*). Mycotoxin is a wilt toxin on tomato plants infected with *F. oxysporum* f.sp. *lycopersici*, and the toxic concentration needed to cause wilting is about 150 mg L^{-1} (*15*). Toxins produced by pathogens are primary determinants of pathogenesis when they act as the key elements in infection initiation and symptom development. They are secondary determinants when they only modify the symptoms (*26*). Moderate doses of fusaric acid (a fusarial mycotoxin) induce apoptosis in saffron, while high fusaric acid doses stimulate necrosis (*25*). In this study, cinnamic acid stimulated mycotoxin production by FON. Mycotoxin yield at the highest concentration of cinnamic acid was increased by 490% (**Figure 5**). Therefore, we believe that susceptibility of watermelon plant to FON would be associated with much more cinnamic acid formation in their root exudates and decaying residues. Stimulation of mycotoxin production by cinnamic acid leads to increased risk of attack by the pathogen and great damage to the plant.

Equally important phytopathogenic factors for FON are hydrolytic enzymes. Pectinases and cellulases of phytopathogenic fungi promote the infection process in many plant diseases. They facilitate the penetration of the fungus into the plant by a hydrolytic cleavage of the polymers (such as pectic substances, cellulose, etc.), which constitute important constituents of the plant cell walls (*17*). Fusarial fungi damage host plants through penetration of hyphae into host vascular tissues, secretion of hydrolytic enzymes, and mycotoxin production (*2, 12, 17*). In the present study, a great stimulation of hydrolytic enzyme activities was observed in liquid cultures of FON that are treated with cinnamic acid. Pectinase activity by FON at the highest concentration of cinnamic acid was increased by 590%. Proteinase activity (at the highest treated concentration) was elevated by 760%. Cellulase activity was increased by up to 2006% following treatment with cinnamic acid. Amylase activity was also increased following treatment with cinnamic acid. (parts **a**−**d** of **Figure 7**). Interestingly, cinnamic acid stimulated the activity of H^+-ATPase of *Saccharomyces cerevisiae* (*13*). This suggested that cinnamic acid contained in root exudates

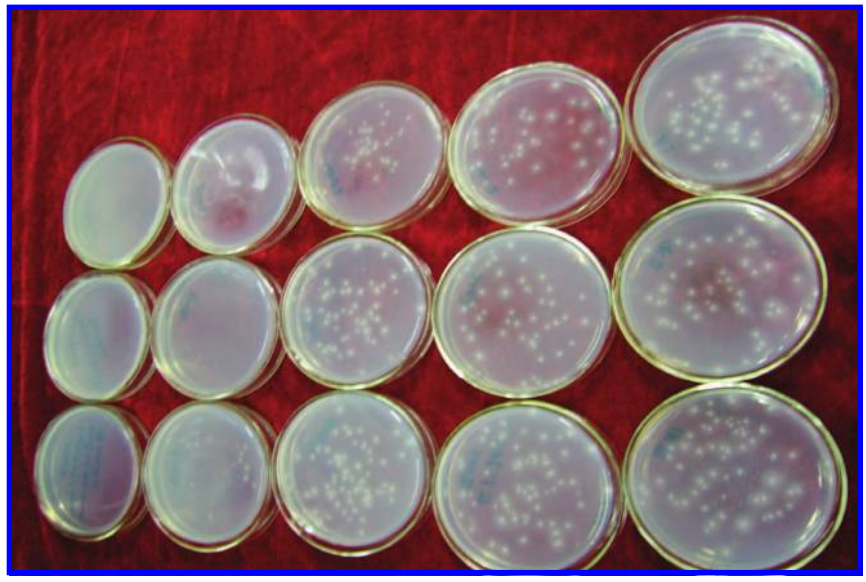

Figure 4. Inhibiton of FON conidial germination by cinnamic acid on plates. From left to right, the concentrations of cinnamic acid were 1600, 800, 400, 200, and 0 mg L^{-1}. Three replicates are represented with three plates in one column.

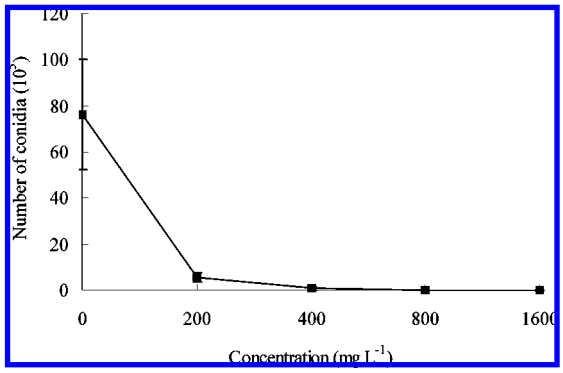

Figure 5. Effect of cinnamic acid on sporulation by FON after liquid culture for 7 days. Bars represent SE.

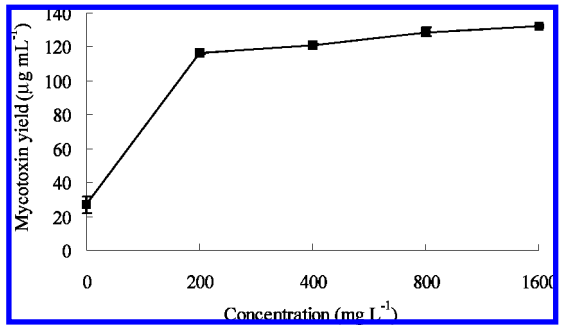

Figure 6. Effect of cinnamic acid on mycotoxin production by FON after liquid culture for 7 days. Bars represent SE.

of watermelon and other plants may promote wilt infection. The observation that cucumber fusarium wilt is stimulated by cinnamic acid (*52*) supports this idea. Our results show that cinnamic acid inhibits growth, sporulation, and conidia germination of FON but stimulates production of mycotoxin production and hydrolytic enzymes. However, some researchers reported that cinnamic acid in plants provides a natural protection against infection by pathogenic microorganisms (*14, 32*). The findings of this study indicate that cinnamic acid increases the risk of infection and plant damage by pathogenic microbes.

In summary, cinnamic acid is one of the compounds contained in root exudates and decaying residues of watermelon that is allelopathic to FON. The new findings demonstrate that hyphal

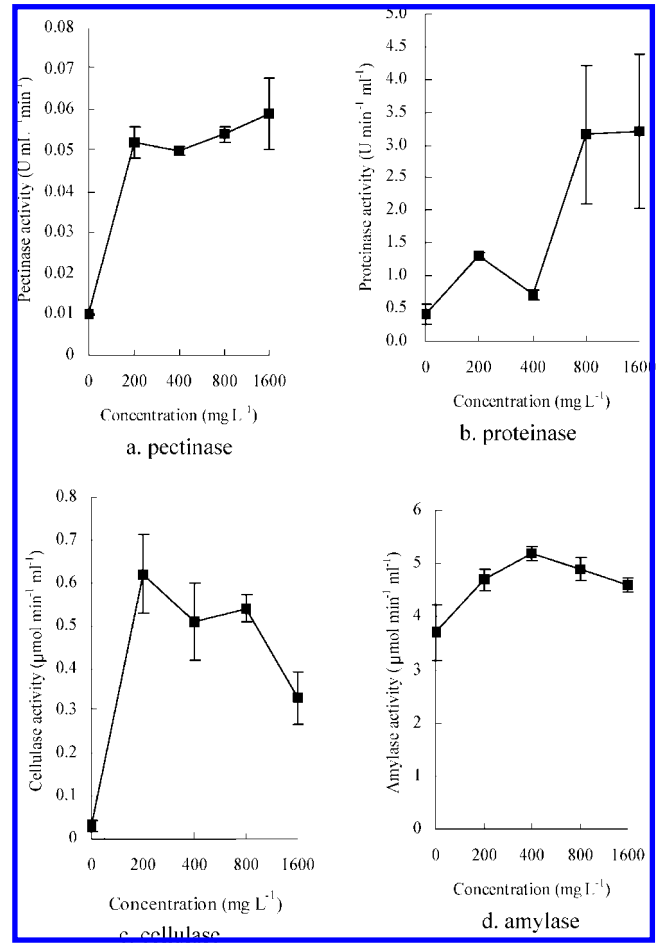

Figure 7. Effect of cinnamic acid at different concentrations on the production of enzymes related to pathogenesis by FON after liquid culture for 7 days. Bars represent SE.

growth of FON was strongly inhibited by cinnamic acid. A great increase in the production of mycotoxin and enzymes related to pathogenesis was observed following cinnamic acid treatment. There might be different mechanisms to explain the effect of cinnamic acid on hyphal growth and pathogenesis of FON. The cinnamic acid could be considered as a signal component in

the process of the interaction between the plant and the pathogen. Thus, we may also be able to explain the possible mechanism of watermelon fusarium wilt from the point view of allelopathic interactions. Genetic modification of cinnamic acid levels inside plants might be a way to improve plant resistance to FON.

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