Biol Res 42: 297-304, 2009

Growth of *in vitro Fusarium oxysporum* f. sp. *niveum* in chemically defined media amended with gallic acid

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ABSTRACT

Gallic acid was artificially added to the media to grow *Fusarium oxysporum* f.sp.*niveum* to investigate its effect on the pathogenic fungus. Results indicate that gallic acid inhibited the growth of *F. oxysporum* f.sp.*niveum*. The colony diameter, the conidia germinating rate and the conidia yield were reduced by 5.7-22.9%%, 35.8-55.6% and 38.9-62.2% respectively. However, the virulence factors by the fungus were stimulated. The activity of pectinase, proteinase and cellulase increased by 12.3-627.8%, 11.8-41.2% and 0.5-325.0% respectively, while the activity of amylase increased slightly. The results suggest that gallic acid repressed growth but facilitated the relative pathogenicity of invading pathogens.

Key terms: Allelopathy; Gallic acid;*Fusarium oxysporum* f. sp. *niveum*; Plant-microbe interaction; Virulent factors.

1. INTRODUCTION

Watermelon production in the long-term monoculture system has been limited due to frequent occurrence of fusarial wilt by *Fusarium. oxysporum* f. sp. *niveum*, which is very difficult to remove from soil once it has been introduced (Booth 1971; Wu *et al*. 2007a,b). It is the most important soilborne pathogen limiting watermelon production in many areas of the world (Martyn 1996; Wu *et al* 2007a, b).

Fusarium species are a genus of harmful fungi that cause vascular disease in plants, such as watermelon, cucumber, tomato, pepper, muskmelon, bean and cotton (Armstrong and Armstrong 1981; Gordon *et al*. 1997; Nelson 1981). Most of them are toxigenic and produce deleterious secondary metabolites (Marasas *et al* 1984), damaging host plants through penetration of hyphae into host vascular tissues, secretion of hydrolytic enzymes related to pathogenesis, mycotoxin production and cellular apoptosis of host plant cells in the progression of the infection (Gaumann 1957; Abbas *et al*. 1997; Bacon *et al*. 1996; Pavlovkin *et al*. 2004).

Much study has been focused on the effect of *F. oxysporum* f.sp.*niveum* on host plants. Little attention has been given to the effect of the host plant on the pathogen. It has been found that many kinds of root exudates and decaying residues (decomposing litter) are phytotoxic as the result of allelochemicals contained in root exudates and decaying residues that have been isolated and identified as organic acids, especially phenolic acids, such as cinnamic, vanillic, coumaric, and ferulic acid (Lee *et al*. 2006; Hao *et al*. 2006; Ohno *et al*. 2001; Yu *et al*. 2003). Phenolic acids frequently occur in plants, such as hydroxycinnamic acids ester-linked to

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Received: September 30, 2008. In Revised form: June 14, 2009. Accepted: July 8, 2009.

polysaccarides and large amounts of simple phenolic acids are released during the aerobic catabolism of lignin (Kuwahara 1980; Toms & Wood 1970). The effects of root exudates and decaying residues on methanogenic microbial communities on rice or maize roots and in the rhizosphere (Lu *et al* 2004; Baudoin *et al*. 2003), bacterial community composition (Landi *et al*. 2006; Sturz 2006), microbial biomass (Sung *et al*. 2006), stimulation of ginseng root exudates on growth of *Phytophthora cactorum* and *Pythium irregulare* (Nicol *et al*. 2003), rhizobacterial populations (Nehl *et al*. 1997; Sturz and Christie 2003) and fungal species abundantly rich in the desert (Mandeel 2002) have been studied.

Some studies have found that root exudates might initiate and manipulate biological and physical interactions between roots and soil organisms, and thus play an active role in root-microbe communication (Bais *et al*. 2004). However, the role of specific compounds from root exudates or decaying plant residues in plant-microbe interaction is almost unknown, though artificially applied chemicals, such as ferulic, caffeic and vanillic acid added to the soil to test for the effects of phenolic acids on microbial biomass and populations had been discussed (Sparling *et al*. 1981; Blum 1997).

Higher plants synthesize various secondary metabolites including tannic acid and its derivatives, such as ellagic or gallic acid (Lin *et al*. 2004). Gallic acid (3,4,5-trihydroxybenzoic acid,) may be considered as one of the simplest models for natural organic matter (Quici *et al*. 2008) and is one of the organic acids found in plants that exhibit activity against the bacteria *Sallmonella typhi* and *Staphycococcus aureus* and other pathogenic microbes (Chanwitheesuk *et al*. 2007; Hasegawa *et al*. 2008). It is a precursor of lignin in cell walls and can be hydrolyzed from lignin by tannase (Trevino-Cueto *et al*. 2007). It has been widely studied as an antioxidant (Lin *et al*. 2004; Chanwitheesuk *et al*. 2007; Hasegawa *et al*. 2008; Soong and Barlow 2006; Abdelwahed *et al*. 2007). However, little is known about the immediate effect of gallic acid on *F. oxysporum* f.sp.*niveum*, i.e. relationships between gallic acid and *F. oxysporum* f. sp. *niveum*.

Our purpose of this study was to investigate the potential effect of artificially applied gallic acid on the growth and virulence factors by *Fusarium oxysporum* f. sp. *niveum* during plant-microbe interactions to evaluate potential risk of gallic acid-driven pathogens to host plants and humans.

2. METHODS AND MATERIALS

2.1 Pathogen strains and chemicals

Fusarium oxysporum *f.sp*. niveum *(FON) was obtained from the Laboratory of Plant-Microbe Interactions, Nanjing Agricultural University, China. Gallic acid and the other main chemicals used in the experiment were obtained from the Sigma Co. (St. Louis, MO, USA).*

2.2 Measurement of FON colony growth

A 5-mm agar plug taken from a 7-day-old PDA (potato dextrose agar) culture was inoculated into the center of a plate and incubated at 28 ºC for 7days(d). Colony diameter was measured in three directions on each plate after incubation for 3 and 7 d.

2.3 Assessment of conidial germination

To determine the effect of gallic 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 at 28 ºC for 7d. The broth was filtered to collect conidia. Conidial suspension was diluted to ≤200 conidia per millimeter with sterile distilled water. Some 0.1 ml of the diluted suspension was spread on plates and incubated at 28 ºC for 3d. The number of colonies was counted daily.

2.4 Determination of sporulation

Sporulation was determined following the growth of *Fusarium oxysporum* f.sp.*niveum* (as described above) in Bilay and Joffe's

medium (Booth, 1971), with minor modifications (4.0 g CMC-Na instead of 15 g CMC, pH adjusted to 4 with 2 mol L^{-1} HCl). After incubation for 7d, 0.1 ml of culture broth, diluted to 10^{-5} - 10^{-7}, was spread onto PDA. Plates were incubated at 28 ºC in the dark for 4 d, after which colonies were counted and converted to the number of conidia in a liquid culture.

2.5 Measurement of biomass production and enzyme activity

Fusarium oxysporum f.sp.*niveum* was grown in 100 ml conical flasks filled with 30 ml potato dextrose broth adjusted to pH 4.5 with 2 mol. L^{-1} HCl and was inoculated with a 5-mm agar plug taken from a 7-day-old PDA culture. Cultures were incubated in a shaker (170 rpm) at 28ºC for 7d. Fungal biomass (dry weight) was determined after filtration and drying at 80ºC 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 (1974). 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 by the DNS method (Silva *et al*. 2005). One unit of enzyme activity was defined as the amount of β-galacturonic acid hydrolyzed from pectin per minute under the assay conditions. Cellulase activity was also determined by the DNS method (Berlin *et al*. 2005). One unit of cellulase activity was defined as the amount of enzyme that produced 1μmol of reduced sugar per minute under the above assay condition. Total amylase activity was assayed by the DNS method (Murado *et al*. 1997). 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.

2.6 Extraction and assay of mycotoxin

Mycotoxin production (mainly fusaric acid) was determined following growth in Richard's medium (Gaumann 1957), as described above, but with a 12 h photoperiod under fluorescent light for 35 d. Broth was acidified to pH 2 with 2 mol L^{-1} HCl, mixed with an equal volume of ethyl acetate, vigorously shaken for 2 min, allowed to settle for 30 min and the organic phase removed. After repeating this procedure 5 times, the organic phase was centrifuged for 10 min at 5000g. The supernatant was dried and condensed at ≤ 40ºC. The dried residue was redissolved in 5 ml of ethyl acetate and the OD_{268} (Matsui and Smith 1988) was determined by UV spectrophotometry (UV-120-02 spectrophotometer Shimadzu, Japan).

2.7 Experimental design and statistical analysis of data

Based on our preliminary experiments, studies were carried out using five concentrations of gallic acid: 0, 50, 100, 200, and 400 mg. L^{-1}. The control was 0 mg. L^{-1} of gallic acid, but with 2 ml of sterilized methyl alcohol-ethyl acetate instead of gallic acid because gallic acid dissolves in a mixture of methyl alcohol-ethyl acetate. Gallic acid solution was filter-sterilized by a 0.22 µm pore membrane (Millipore). Data were analyzed by Microsoft Excel™. The values were represented as the mean of three replicates (mean±SD) for each treatment. One way ANOVA was used and comparison of means were conducted when significant difference was tested by Duncan's multi range LSD at $p<0.05$.with SPSS version 11.5 (SPSS Co., IL.USA).

3. RESULTS

3.1 Effect of gallic acid on biomass and colony growth

Biomass of *Fusarium oxysporum* f.sp.*niveum* was significantly stimulated by gallic acid in a liquid culture, but inhibited its colony growth on PDA plates. Compared to the untreated control, the dry weight of mycelia increased by 70.0-121.0% (Fig. 1), the colony diameter decreased by 5.7-22.9% (Fig. 2).

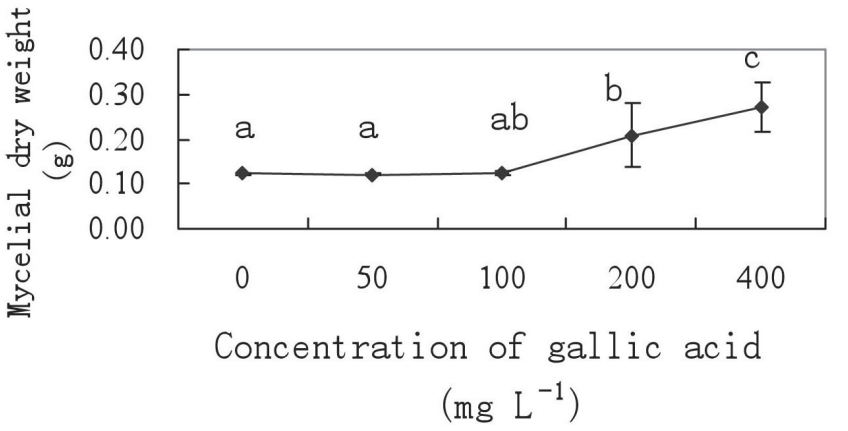

Figure 1: Effect of gallic acid on biomass of *Fusarium oxysporum* f. sp. *niveum*. Bars indicate standard deviation of three replicates. (F=0.465,n=4,p<0.05)

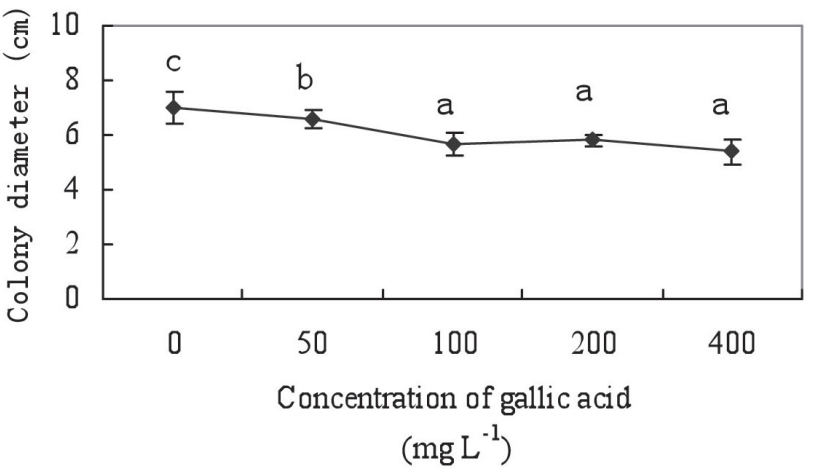

Figure 2: Effect of gallic acid on colony growth of *Fusarium oxysporum* f. sp. *niveum*. Bars indicate standard deviation of three replicates. (F=1.536,n=4,p<0.05)

3.2 Effect of gallic acid on conidia germination and sporulation

Conidia germination on a plate and conidia formation in a liquid culture was significantly inhibited. The number of germinating conidia was reduced by 35.8-55.6% (Fig. 3), while the number of conidia produced declined by 38.9-62.2% (Fig. 4).

3.3 Effect of gallic acid on mycotoxin production

Mycotoxin production of *Fusarium oxysporum* f.sp. *niveum* in a liquid culture was significantly inhibited by gallic acid in a concentration-dependent manner. The yield of mycotoxin was decreased by 2.4-59.8% compared with control (Fig. 5).

3.4 Effect of gallic acid on the activities of enzymes related to pathogenesis

Gallic acid stimulated the activity of pathogenic enzymes in the present study. Pectinase activity increased by 12.3-627.8% in liquid culture (Fig. 6a). A substantial increase of the proteinase activity was observed with treatment by ferulic acid. The proteinase activity initially increased at concentrations of 0-400 mg L^{-1}, with a rise of 11.8-41.2% but decreased at higher

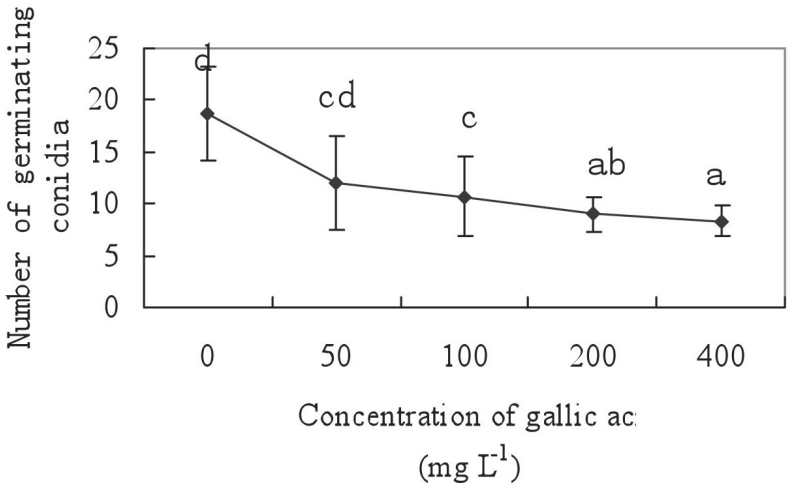

Figure 3: Effect of gallic acid on conidia germination of *Fusarium oxysporum* f. sp. *niveum*. Bars indicate standard deviation of three replicates. (F=5.846,n=4,p<0.05)

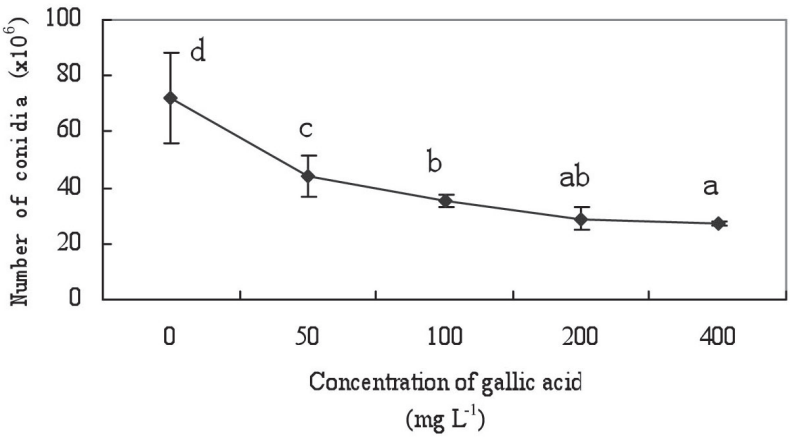

Figure 4: Effect of gallic acid on sporulation of *Fusarium oxysporum* f. sp. *niveum* in a liquid culture. Bars indicate standard deviation of three replicates. (F=11.365,n=4,p<0.05)

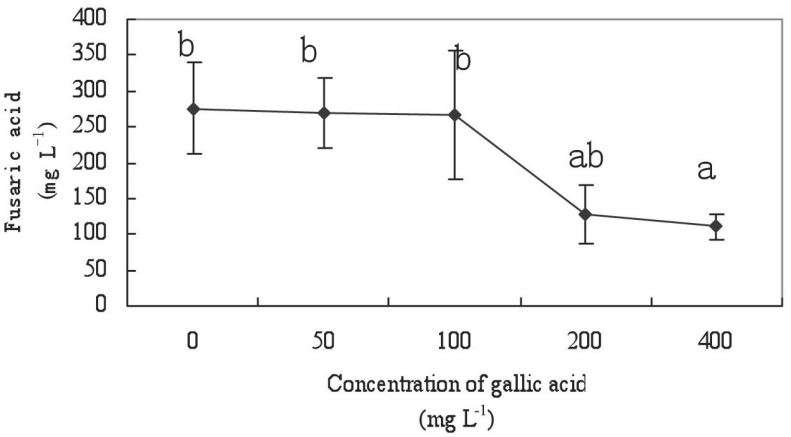

Figure 5: Effect of gallic acid on mycotoxin production of *Fusarium oxysporum* f. sp. *niveum*. Bars indicate standard deviation of three replicates. (F=18.938,n=4,p<0.05)

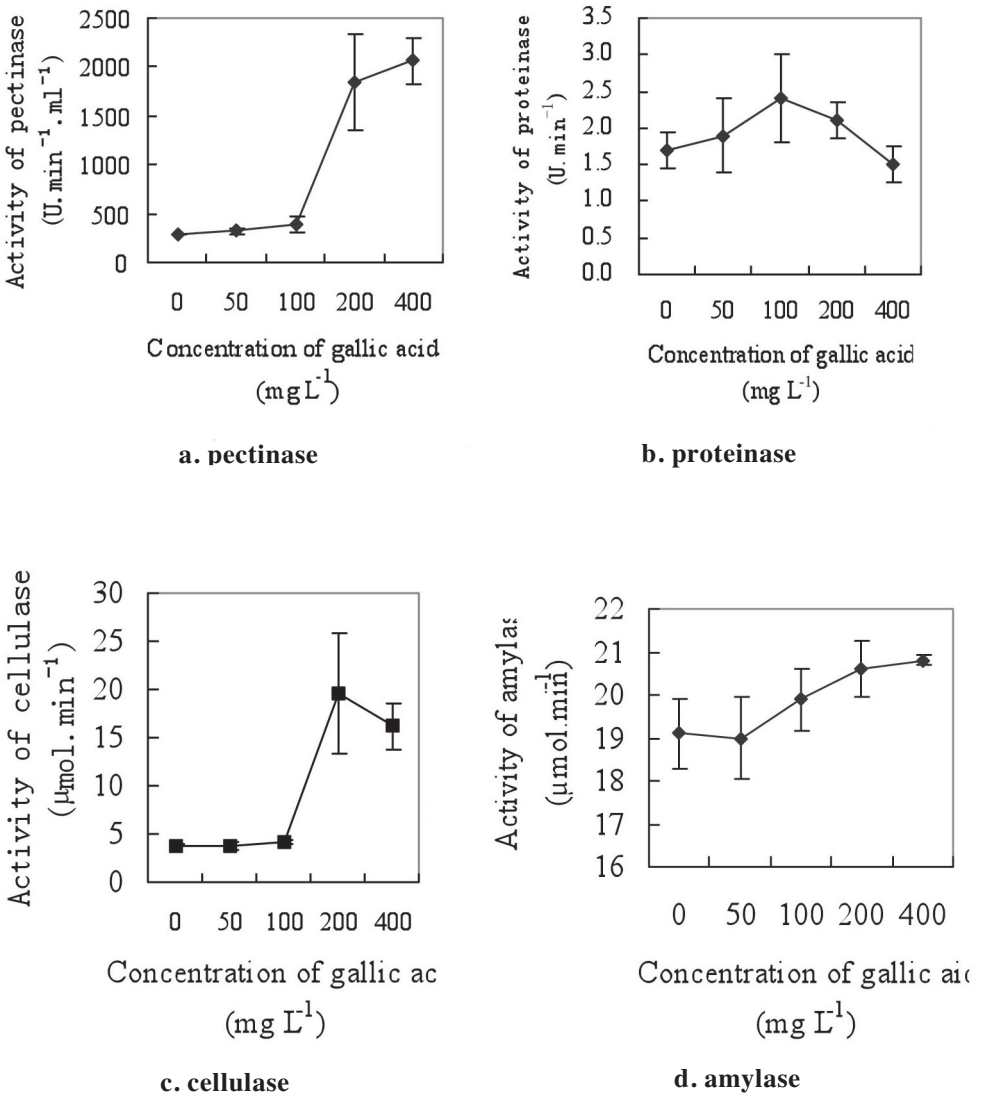

Figure 6: Effect of gallic acid on activities of different hydrolytic enzymes by *Fusarium oxysporum* f. sp. *niveum* in a liquid culture. Bars indicate standard deviation of three replicates.

concentration of gallic acid (Fig. 6b). Stimulation of cellulase activity was obtained with increasing concentrations of gallic acid, with activity increasing by 0.5-325.0% (Fig. 6c). A slight increase of cellulase activity was found (Fig. 6d).

4. DISCUSSION

Gallic acid is a normal metabolite found in plants, which is often detected in the root exudates and plant-grown soil (Wu *et al*. 2007a,b). There are some relationships between gallic acid and soil microorganisms and pathogens that affect the soil microbial community and ecological functions.

In the present study, results showed that the mycelial growth of *Fusarium oxysporum* f.sp. *niveum* in plates was inhibited by gallic acid in a concentration-dependent manner, with the colony diameter decreasing by 5.7-22.9% compared to the control (Fig. 2). However, the mycelial growth of *F. oxysporum* f.sp. *niveum* in a liquid culture was stimulated, with the mycelial dry weight increasing by

70.0-121.1% (Fig. 1). This has also been confirmed by the result that the mycelial growth of the *F. oxysporum* f. sp. *albedinis* is inhibited by cell wall-bound phenolics in date palm roots (Elmondafar & Boustani, 2001). This was consistent with coumaric and cinnamic acid inhibition of hyphal growth of *F. oyxsporum* f.sp. *niveum* (Wu *et al*. 2007a, b). However, the results are in disagreement with the absence of inhibition of *Fusarium* spp by gallic acid from *Caesalpinia mimosoides* Lamk (Chanwitheesuk *et al*. 2007).

The difference in mycelial stimulation or inhibition between plate and liquid culture results from the physiological mechanisms under the two culture conditions.

In the current study, gallic acid dramatically stimulated conidia germination and formation of the fungus. The conidia germinating rate and the yield of conidia were reduced by 35.8-55.6% and 38.9-62.2% respectively (Fig. 3). This was not in accordance with the coumaric acid inhibition of *F. oxysporum* f. sp. *niveum* (Wu *et al*. 2007a). Though gallic acid stimulated the biomass of *F. oxysporum* f.sp. *niveum* in a liquid culture, eventually the growth of the fungus was inhibited due to the simultaneous inhibition of conidia germination and formation.

Furthermore, gallic acid affected the virulence factors and pathogenesis of the fungus. Mycotoxin production was heavily restricted by gallic acid in a concentration-dependent manner (Fig. 5). A fall of mycotoxin production implied a reduction of fungus virulence to the host. This was confirmed by the report that the mycotoxin production of *F. oxysporum* f. sp. *niveum* was suppressed by coumaric acid (Wu *et al*. 2007). As a powerful defense, mycotoxin is produced when FON invade watermelon or other plants, which is an important pathogenic factor causing plant wilting (Gaumann 1957). Moderate fusaric acid (a fusarilal mycotoxin) doses induce apoptosis in saffron, while high fusaric acid doses stimulate necrosis (Leili & Behboodi 2006). However, gallic acid increased pathogenic enzyme activities. In this study, the activity of pectinase, proteinase, and cellulase increased by 12.3-627.8%, 11.8-41.2% and 0.5-325.0% respectively (Fig. 6), while the activity of amylase was slightly elevated. This indicates that gallic acid enhances the virulence of *F. oxysporum* f. sp. *niveum* against the host. This is in accordance with cinnamic acid increasing pathogenic enzyme activity by *F. oxysporum* f. sp. *niveum* (Wu *et al*. 2007b). Other virulence factors equally important for FON are the enzymes related to pathogenesis. Pectinases and cellulases of phytopathogenic fungi stimulate the infection process in many plant diseases. They facilitate the penetration of the fungus into the plant by the hydrolytic cleavage of polymers (pectic substances, cellulose), which constitute the plant cell walls (Fuchs *et al*. 1965)**.** Fusarial fungi damage host plants through penetration of hyphae into host vascular tissues, secretion of hydrolytic enzymes related to pathogenesis, and mycotoxin production in the progression of the infection (Fuchs *et al*. 1965).

5. CONCLUSIONS

Gallic acid inhibited *Fusarium oxysporum* f. sp. *niveum* in this investigation, which might be responsible for host resistance against the pathogen during biotic stress.

ACKNOWLEDGEMENTS

This work has been financially supported by the Jiangsu Key Lab for Solid Organic Waste Utilization (2007001).

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