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Growth responses of *in vitro Fusarium oxysporum* f. sp. *niveum* to external supply of tannic acid

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Abstract: *Allelochemicals released from root exudates or decaying residues of plants play diversified roles in ecological interactions of plant-pathogen. The objective of this work was to evaluate the allelopathic effect of an externally supplied tannic acid on soil-borne in vitro Fusarium oxysporum f.sp.niveum. Results showed that the tannic acid decreased the growth of the fungus up to 9.5% at 800 mg l^{-1}. Conidial germination was reduced by 52.3% in comparison with the control. However, sporulation and mycotoxin production by the fungus were stimulated. The activity of pectinase and proteinase were initially increased and finally decreased with increase in concentrations of tannic acid. Tannic acid served as an ecological allelochemical, repressing the growth of the pathogen*

Key words: *Allelopathy, Fusarium oxysporum f. sp. niveum, Plant-microbe interaction, Virulent factors*

Introduction

Fusarium oxysporum f. sp. *niveum*, a pathogenic fungus responsible for watermelon fusarium wilt, is difficult to remove from soil. Fusarium wilt severely affacts watermelon production particularly in the long-term monoculture system. It is the most important soilborne pathogen limiting watermelon production in many areas of the world (Martyn, 1996).

More than 50% of isolates of the known *Fusarium* species are toxigenic and produce deleterious secondary metabolites (Marasas *et al.*, 1984). They damage host plants through penetration of hyphae into the 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 (Bacon *et al.*, 1996; Pavlovkin *et al.*, 2004).

Fusarium oxysporum f.sp.*niveum* has long been studied as a plant pathogen but the effect of host plant on the pathogen in ecological plant-microbe interactions is little documented. It is known that root exudates and decaying residues (decomposing litter) are phytotoxic due to the presence of allelochemicals identified as organic acids, especially phenolic acids, such as cinnamic, vanillic, coumaric, and ferulic acid (Ohno *et al.*, 2001; Lee *et al.*, 2006; Hao *et al.,* 2006). Phenolic acids such as hydroxycinnamic acids ester-linked to polysaccarides frequently occur in plants and large amounts of simple phenolic acids, such as ferulic, p-comaric and syringic acids, are released during the aerobic catabolism of lignin (Toms and Wood, 1970; Kuwahara, 1980). Root exudates and decaying residues have been studied for their effect on methanogenic microbial communities on rice and maize roots in the rhizosphere (Baudoin *et al.*, 2003; Lu *et al.*, 2004), bacterial community composition (Landi *et al.*, 2006; Sturz, 2006), microbial biomass (Sung *et al.*, 2006), rhizobacterial populations (Nehl *et al.*, 1997) and fungal species (Mandeel, 2002; Nicol *et al.*, 2003).

Root exudates may initiate and manipulate biological and physical interactions between roots and soil organisms, and thus play active role in root-microbe communication (Bais *et al.*, 2004). However, the effect of specific compounds from root exudates or decaying plant residues on specific pathogens are hardly known, though the effect of artificially applied chemicals, such as ferulic, caffeic and vanillic acid added to the soil on microbial biomass and populations have been worked out (Sparling *et al.*, 1981; Blum and Shafer, 1988; Blum, 1997).

Tannic acid, chemically called penta-m-digallolyl glucose, is ordinarily used as a synonym for tannin. It occurs widely in root exudates, decaying plant residues and soil and is distributed throughout the plant kingdom. The anti-oxidant activity of tannic acid was demonstrated by its effect on hydroxyl radical and singlet oxygen mediated cleavage of plasmid DNA; tannic acid provided the maximum protection against cleavage (Khan *et al.*, 2000). Fungi (*Phaeomoniella chlamydospora, Togninia minimaand Fomitiporia mediterranea*) are able to live in media containing tannic acid as the sole carbon source and to convert tannic acid by secretion of tannase (Bruno and Sparapano, 2006). So we can presume tannic acid may stimulate fungal growth.

The mycelial growth of *F. oxysporum* f.sp.*albedinis* is inhibited by the cell wall-bound phenolics in resistant cultivars of date palm roots (Elmondafar and Boustani, 2001). However, little information is available on the influence of tannic acid on *Fusarium oxysporum* f.sp.*niveum.*

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This study was undertaken with an aim to test the ecologically allelopathic role of externally supplied tannic acid during the host-pathogen interactions, and to investigate whether there can be some relationship between application of tannic acid and the virulence factors of *Fusarium oxysporum* f.sp.*niveum*.

Materials and Methods

Pathogen strains and chemicals: *Fusarium oxysporum* f.sp. *niveum* was isolated from infected watermelon in a greenhouse plot, by the Laboratory of Microbiology, Nanjing University of Information Science and Technology, China. Tannic acid and other chemicals used in the experiment were obtained from Sigma Co. (St. Louis, MO, USA).

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

To determine the effect of tannic 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°C for 7d. The broth was filtered to collect conidia. Conidial suspension was diluted to $\leq$200 conidia mm^{-1} with sterile distilled water. A0.1 ml of the diluted suspension was spread on plates and incubated at 28°C for 3d. The number of colonies was counted daily.

Sporulation was determined in Bilay and Joffe's medium (Booth, 1971) with minor modifications (4.0 g CMC-Na, carboxyl methyl cellulose) 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°C in the dark for 4 d, after which colonies were counted and converted to the number of conidia in a liquid culture.

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 28°C for 7d. Fungal biomass (dry weight) was determined after filtration and drying at 80°C for 12 hr, 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.

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 hr 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 5000 g. The supernatant was dried and condensed at $\leq$40°C. 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).

Statistical analysis: Based on our preliminary experiments, studies were carried out using five concentrations of tannic acid: 0, 100,200, 400 and 800 mg l^{-1}. The control was 2 ml of sterilized ethyl acetate instead of tannic acid. Tannic 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.

Results and Discussion

Biomass and colony growth: Biomass (dry weight of mycelia) of the test species was strongly inhibited by tannic acid in a liquid culture. The dry weight of mycelia was decreased by 9.5% compared with the control, at the highest (800 mg l^{-1}) concentration (Fig. 1). However, colony growth was slightly stimulated at low (100-200 mg l^{-1}) concentrations, while there was a little inhibition at high (400-800 mg l^{-1}) concentrations.

Conidia germination and sporulation: Conidia germination on a solid plate was suppressed at both low (100-200 mg l^{-1}) and high (400-800 mg l^{-1}) concentrations in a concentraion-dependant manner, showing a 52.3% reduction with highest (800 mg l^{-1}) concentration, as compared with control (Fig. 2). However, an overall stimulation of conidia formation was found in a liquid culture. Much more increase of condial yield at lower (100-200 mg l^{-1}) concentraions was obtained than at higher (400-800 mg l^{-1}) ones.

Mycotoxin production: Production of mycotoxin by *Fusarium oxysporum* f.sp. *niveum* in liquid culture was stimulated, up to 53.9%, by tannic acid at low (100-400 mg l^{-1}) concentrations comapred with the control, while a decreased yield of mycotoxin was observed at higher concentration (Fig. 3).

Activities of enzymes related to pathogenesis: The activity of pectinase was stimulated at low concentrations of tannic acid (100-200 mg l^{-1}) in a liquid culture, while it was depressed at a high

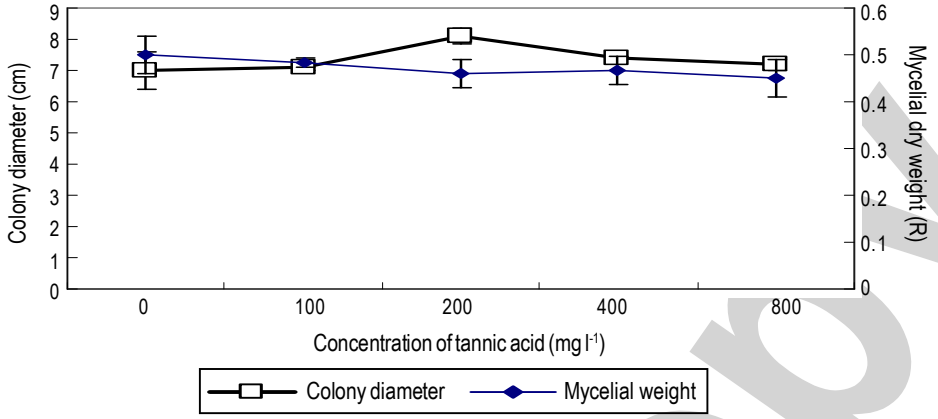

Fig. 1: Effect of tannic acid on biomass and colony diameter of *Fusarium oxysporum* f. sp. *niveum* on PDA. Bars mean standard deviation of these replicates

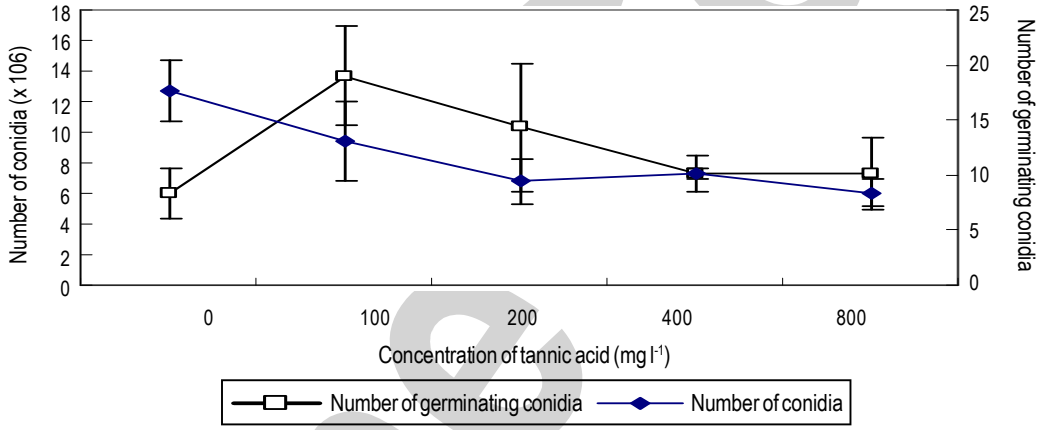

Fig. 2: Effect of tannillic acid on conidial formation and germination of *Fusarium oxysporum* f. sp. *niveum*. Bars mean standard deviation of three replicates

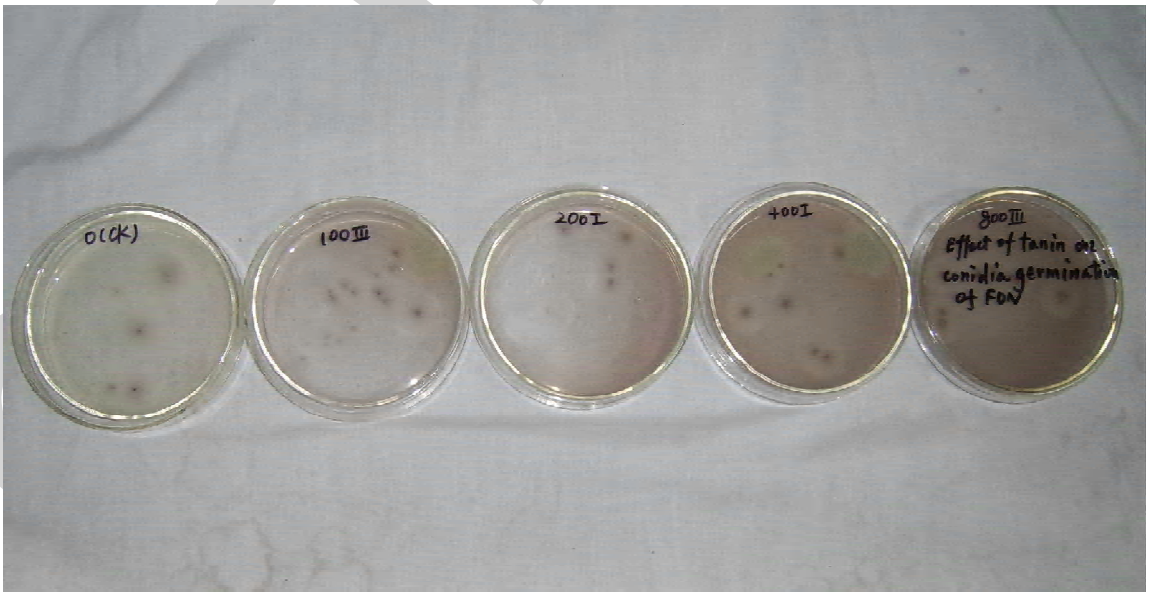

Fig. 3: Effect of tannillic acid on conidia germination of *Fusarium oxysporum* f.sp.*niveum* on the plates, from left to right 0,100,200,400 and 800 mg l^{-1} tannic acid

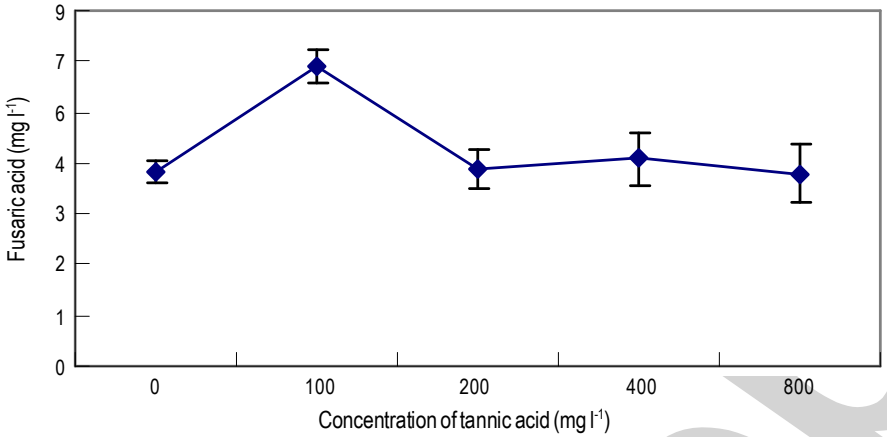

Fig. 4: Different effect of tannic acid on mycotoxin production on *Fusarium oxysporum* f. sp. *niveum*. Bars mean standard deviation of three replicates

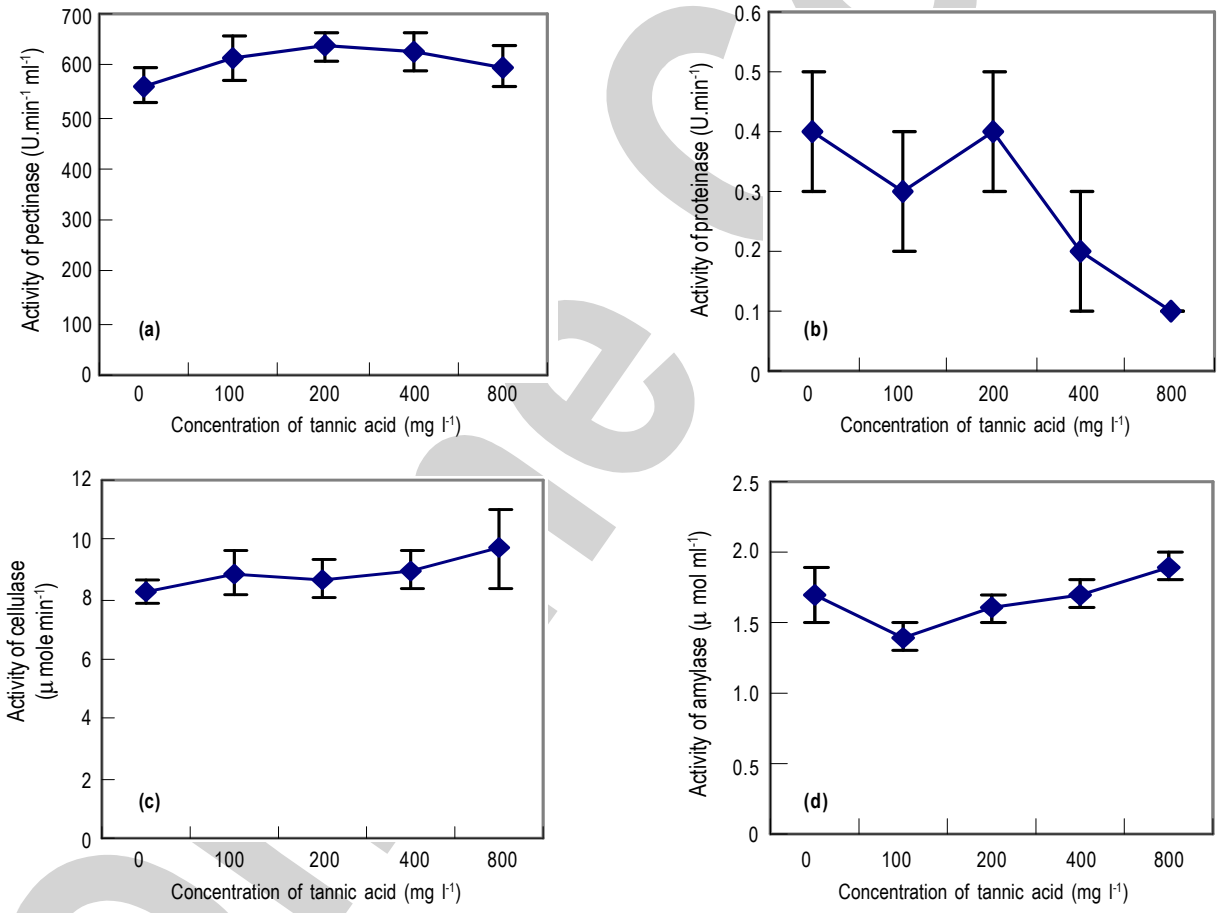

Fig. 5: Different effect of tannic acid on activities of different pathogenically hydrolytic enzymes by *Fusarium oxysporum* f. sp. *niveum* in a liquid culture. Bars mean standard deviation of three replicates

(400-800 mg l^{-1}) concentration (Fig. 4a). Substantial reduction of the proteinase activity was observed with treatment by tannic acid. The proteinase activity declined by 75% at concentrations of 800 mg l^{-1} compared with the control (Fig. 4b). Stimulation of cellulase activity was obtained, with a rise of activity by 16.9% at the highest (800 mg l^{-1}) concentration (Fig. 4c). A decrease of cellulase activity was found with low concentrations of tannic acid (100-200 mg l^{-1}), while increased at higher concentrations ranging from 400-800 mg l^{-1} (Fig. 4d).

In the current study, results showed that the growth of *Fusarium oxysporum* f.sp. *niveum* was inhibited by tannic acid in a

concentration-dependent manner, with the biomass in a liquid culture decreasing by 9.5% at the highest concentration of 800 mg l^{-1}. This is in agreement with the mycelial growth of the *F. oxysporum* f. sp. *albedinis* is inhibited by cell wall-bound phenolics in resistant cultivars of date palm roots (Elmondafar and Boustani, 2001). This was consistent to the decreased growth of the fungi *Phaeomoniella chlamydospora, Togninia minima and Fomitiporia mediterranea* by tannic acid at both state and liquid culture, with growth decreasing 9-76% (Bruno and Sparapano, 2006). And it was also in agreement with the report that artificially applied pure phenolic acids (p-hydroxybenzoic, ferulic, caffeic and vanillic acid) added to soil stimulate the growth of phenolic acid degrading organisms (Sparling *et al*., 1981). Results demonstrated that sporulation was stimulated by tannic acid in a liquid culture (Fig. 2). However, Conidial germination was depressed by tannic acid at both low and high concentrations ranging from 100 to 800 mg l^{-1} with germination rate reduced 52.3% (Fig. 2). Differring effect of tannic acid on FON growth, conidial formation and germination might contribute to their diversified mechanism and process, as some reported tannic acid inhibits free radical formation and 2-deoxyribose damage by chelating iron ions to display the antioxidant effects (Andrade *et al.*, 2006).

As a powerful defense, mycotoxin is produced when FON invade watermelon or other plants, which is an important pathogenic factor causing plant wilting. Mycotoxin is a well-known phytotoxin produced by several *Fusarium* species, particularly pathogenic strains of *F.* oxysporum, the cause of wilt diseases in a great variety of plants, such as watermelon, cucumber, tomato, beans and cotton (Gaumann, 1957). Mycotoxin is a wilt toxin on tomato plants infected with *F. oxysporum* f. sp. *lycopersici,* and the toxic concentration needed to cause wilting is 150 mg l^{-1} (David, 1969). The toxins produced by pathogens are primary determinants of pathogenesis, when they act as the key element in the initiation of infection and symptom development. They are secondary determinants when they only modify the symptoms, even if they do so intensively (Lepoivre, 2003). Moderate fusaric acid (a fusarilal mycotoxin) doses induce apoptosis in saffron while high fusaric acid doses stimulate necrosis (Leili and Behboodi, 2006). In the present investigation, stimulation of mycotoxin production was found at the lowest concentration of tannic acid, whereas reduction of mycotoxin was observed at high concentration (Fig. 3). Theoretically, the increased mycotoxin would lead to risk of plant to pathogen. However, in this work, the decreased growth and conidial germination of the fungus reduced and offset the effect of the increased mycotoxin by tannic acid on host.

Other virulent factors equally important for FON are the enzymes associated with 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; Booth, 1971). In this paper, pectinase activity was elevated by tannic acid at a low concentration (200 mg l^{-1}), while it was repressed at high concentrations (400-800 mg l^{-1}). Proteinase activity was inhibited with increasing concentration of tannic acid, with a fall of 75% at the concentration of 800 mg l^{-1}. An increase of cellulase activity was obtained. Initial decrease of amylase activity was then followed increase of amylase. The findings was similar to the reported result that tannase activity produced by *Paecilomyces variotii* is increased at 12% tannic acid but decreased at 20% at liquid culture (Battestin and Macedo, 2007). However, the tannic acid concentration inside the actual plant is generally not so high, it is necessary to conduct further studies under the actual field conditions particularly in vivo study. Modification of concentration of tannic acid in conjugated with other fungitoxic agent would be suggested to control fusarium wilt disease in the process of ecologically allelochemical interactions of plant-microbe.

In a summary, tannic acid inhibited the growth and conidial germination of *Fusarium oxysporum* f.sp. *niveum*, although stimulated virulence factors at higher concentration, which is much beyond the actual plant concentration. Genetic modification of watermelon allolechemicals may hopefully improve watermelon resistance to fusarial wilt.

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