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RESEARCH PAPER

The components of rice and watermelon root exudates and their effects on pathogenic fungus and watermelon defense

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ABSTRACT

Watermelon (*Citrullus lanatus*) is susceptible to wilt disease caused by the fungus *Fusarium oxysporum* f. sp *niveum* (FON). Intercropping management of watermelon/aerobic rice (*Oryza sativa*) alleviates watermelon wilt disease, because some unidentified component(s) in rice root exudates suppress FON sporulation and spore germination. Here, we show that the phenolic acid *p*-coumaric acid is present in rice root exudates only, and it inhibits FON spore germination and sporulation. We found that exogenously applied *p*-coumaric acid up-regulated the expression of *ClPR3* in roots, as well as increased chitinase activity in leaves. Furthermore, exogenously applied *p*-coumaric acid increased β-1,3-glucanase activity in watermelon roots. By contrast, we found that ferulic acid was secreted by watermelon roots, but not by rice roots, and that it stimulated spore germination and sporulation of FON. Exogenous application of ferulic acid down-regulated *ClPR3* expression and inhibited chitinase activity in watermelon leaves. Salicylic acid was detected in both watermelon and rice root exudates, which stimulated FON spore germination at low concentrations and suppressed spore germination at high concentrations. Exogenously applied salicylic acid did not alter *ClPR3* expression, but did increase chitinase and β-1,3-glucanase activities in watermelon leaves. Together, our results show that the root exudates of phenolic acids were different between rice and watermelon, which lead to their special ecological roles on pathogenic fungus and watermelon defense.

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Introduction

Watermelon (*Citrullus lanatus* (Trunb.) Matsum and Nakai), a popular fruit around the world, is a member of the gourd family (Cucurbitaceae). Watermelon plants are susceptible to wilt disease, which is caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *niveum* (FON), when grown under continuous monocropping management. Chemoattractants and chemorepellents in root exudates attract and repel soil-borne pathogens in the rhizosphere, respectively.[1] The quantity and composition of root exudates depends on the plant species and external biotic and abiotic factors.[2,3] Plant root exudates include amino acids, organic acids, and sugars, and secondary metabolites such as phenolic acids.[4] Most secondary metabolites have antioxidant properties or function as chemoattractant signals in the rhizosphere. Some secondary metabolites function as microbial growth promoters, whereas others, such as the phytoalexins, are synthesized in response to pathogen attack and inhibit microbial growth.[1] For example, *Zea mays* (maize) resistance to *Fusarium graminearum Schwabe* depends on the quantity of cell wall-bound phenolic acid present in silks.[5]

p-Coumaric acid (CA) is a phenolic acid that has antibacterial properties and inhibits *Escherichia coli* growth.[6,7] It is produced by plants in response to infections or wounding. CA is an active compound in medicinal plants and its derivatives, including indole, β-carbolines, norharman, and harman, protect the host organism from biotic and abiotic stresses.[8,9] Ferulic acid (FA), another phenolic acid widely found in the plant kingdom, also functions in plant resistance. *Hordeum vulgare* (barley) roots secrete CA and FA within 2 d of *Fusarium graminearum* inoculation, and CA and FA antagonize *Fusarium graminearum*.[10] The levels of both CA and FA were higher in the kernels of a susceptible variety of maize than in a resistant variety.[11] The FA secreted by *Pseudostellaria heterophylla* roots stimulated growth of the pathogen *Fusarium oxysporum* f. sp *heterophylla* and induced wilt disease.[12] FA is also reported to serve as a precursor of phenylpropanoid derivatives that have antimicrobial properties and improve plant defense.[13] Salicylic acid (SA) is a phenolic acid that functions as a signal of systemic acquired resistance (SAR) and regulates systemic plant immunity.[14-16] SA induces the expression of pathogenesis-related (PR) genes that encode proteins with antimicrobial activities.[17] Plants secrete SA from their roots, and an SA-deficient *Arabidopsis thaliana* mutant had altered microbial communities.[18] Exogenously applied SA improves plant resistance to pathogens, and the SAR signal transduction pathway reduces bacterial colonization.[18,19]

The accumulation of plant root exudates in the rhizosphere after monoculture causes rapid pathogen proliferation in

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replanted soil, which negatively impacts soil quality, crop health, and yield.[12,20] Watermelon wilt disease was found to be alleviated by intercropping with aerobic rice.[21] This alleviation was due to suppression of FON pathogen spore germination and sporulation by rice root exudates.[20,21] By contrast, watermelon root exudates stimulated spore germination and sporulation of FON.[20] The main obstacle for continuous watermelon monoculture is thought to be increased FON infection due to the stimulating properties of watermelon root exudates on sporulation and FON spore germination. However, the key substances in plant root exudates that suppress or stimulate FON growth in watermelon and rice have not been identified.

In this study, we aimed to (1) identify the different phenolic acids in the root exudates of watermelon and rice; (2) study the effects of the phenolic acids secreted from watermelon and rice roots on *Fusarium oxysporum f. sp. niveum*; and (3) investigate the effect of the phenolic acids in watermelon or rice root exudates on watermelon plant resistance.

Results

Rice and watermelon roots secrete different phenolic acids

We analyzed the phenolic acid content in rice and watermelon root exudates. We found that rice and watermelon roots secreted different types of phenolic acids. Rice roots released CA, SA, phthalic acid, and *p*-hydroxybenzoic acid, but not FA, whereas watermelon roots secreted FA, SA, phthalic acid, and p-hydroxybenzoic acid, but not CA. Therefore, CA is a specific component of rice root exudates, while FA is specific to watermelon exudates, and both watermelon and rice roots secrete SA, phthalic acid, and p-hydroxybenzoic acid (Table 1).

FON responses to different exogenously applied phenolic acids

Because CA, FA, SA, phthalic acid, and *p*-hydroxybenzoic acid were present in the root exudates of rice and watermelon roots, we next evaluated the effects of these phenolic acids on FON spore germination and sporulation (Experiment 2).

The effects of various phenolic acids on FON spore germination

The phenolic acids detected in rice or watermelon root exudates had different effects on FON spore germination. Exogenously applied CA, which was present in rice root exudates only, dramatically inhibited FON spore germination at concentrations of above 20 mg L^{-1}, in a dose-dependent manner. The inhibition rate reached 70.69% when 160 mg L^{-1} exogenous CA was added. It seems likely that the CA secreted from rice roots is one factor that inhibits FON spore germination in a rice/watermelon intercropping system and prevents watermelon wilt disease. Exogenous FA, which was specifically secreted by watermelon roots, stimulated FON spore germination at concentrations of above 40 mg L^{-1}. The increase in sporulation rate was as high as 113.12% of the untreated control rates when higher dosages of FA was added. Thus, FA stimulates FON spore germination. It therefore seems likely that FA is one of the mechanisms that stimulate watermelon wilt disease when the plants are subjected to continuous monocropping management. The effects of salicylic acid on FON sporulation differed with the concentration added; low concentrations (10–40 mg L^{-1}) stimulated FON spore germination, while high concentrations (80–100 mg L^{-1}) suppressed it. Exogenously applied phthalic acid did not affect FON spore germination. p-Hydroxybenzoic acid stimulated FON spore germination, but to a lesser extent than did FA (Table 2).

Table 1. Primers for half quantitative real-time polymerase chain reaction (RT-PCR).

Gene	Accession number	Primer sequence
ClPR3	DQ180495.1	F: 5′-GGTCCTCAACCTTGCCGGTCAC-3′ R: 5′-CACTGTCAAACGCCTTGCTCC-3′
18SrRNA		F: 5′-CGCAAATTACCCAATCCTGAC-3′ R: 5′-TTCGCAGTTGTTCGTCTTTCA-3′

Table 2. Phenolic acids in root exudates of rice and watermelon ($\mu g \cdot g^{-1}$ Root FW).

Phenolic acid	Rice	Watermelon
p-Coumaric acid	0.114	ND
Ferulic acid	ND	0.017
Salicylic acid	0.026	0.148
Phthalic acid	0.088	0.115
p-Hydroxybenzoic acid	0.073	0.031

Note: ND, not detected.

The effects of different phenolic acids on FON sporulation

We added different concentrations of CA, FA, and SA to Bilay's medium containing FON spores to evaluate the influence of the phenolic acids on FON sporulation. The phenolic acids present in rice and watermelon root exudates had different effects on FON sporulation. Exogenously applied CA dramatically inhibited FON sporulation, in a dose-dependent manner (from 5 mg L^{-1} to 160 mg L^{-1}). The inhibition rate reached 99.83% at a concentration of 160 mg L^{-1}. Exogenously applied FA stimulated FON sporulation at concentrations ranging from 5 mg L^{-1} to 160 mg L^{-1}, in a dose-dependent manner. The sporulation rate was 55.68% higher than that of the control when 160 mg L^{-1} of FA was added. Exogenously applied SA (160 mg L^{-1}) slightly inhibited FON sporulation, whereas exogenously applied phthalic acid stimulated FON sporulation when present at concentrations of above 80 mg L^{-1}. Exogenously applied *p*-hydroxybenzoic acid also stimulated FON sporulation and the effect was greatest in the presence of 10 mg L^{-1} p-hydroxybenzoic acid.

Effects of FON inoculation and exogenous phenolic acids on watermelon resistance to FON

In the first experiment, we demonstrated that CA was only present in rice root exudates and FA was only found in watermelon root exudatese showed that CA and FA dramatically inhibited and stimulated FON growth, respectively. SA, which was detected in both rice and watermelon root exudates, has both antimicrobial activity and serves as a SAR signal. We were

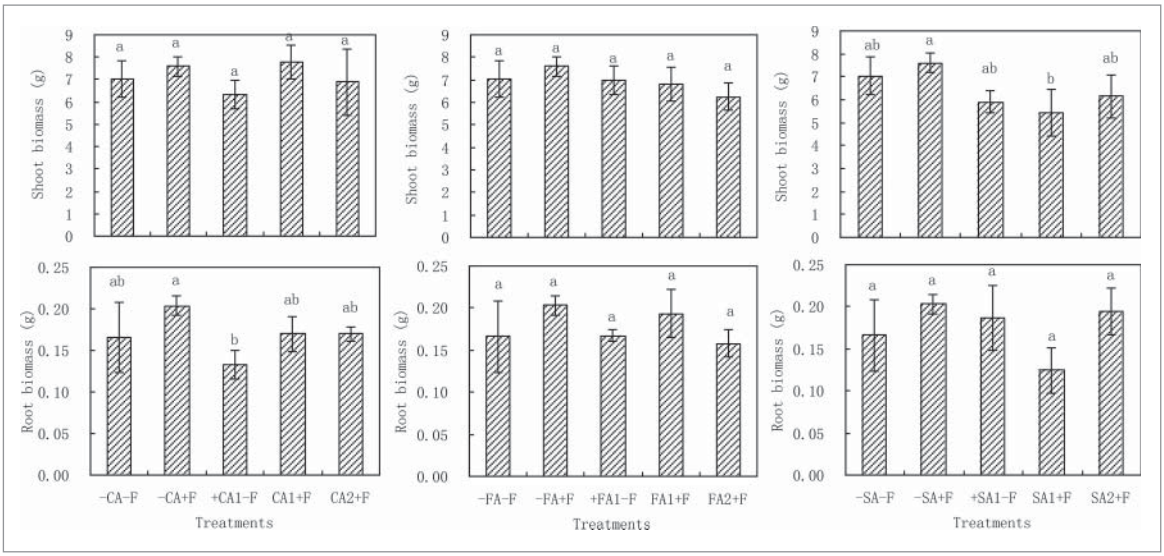

Figure 1. Effects of exogenously applied phenolic acids on watermelon growth. Upper row, shoot biomass; lower row, root biomass. CA, *p*-coumaric acid; FA, ferulic acid; SA, salicylic acid. +, represents present; and –, represents absence; 1 and 2 indicate the concentrations of the different phenolic acids, with 1 representing 30 mol L^{-1} for CA, FA, and SA; and 2 representing 100 mol L^{-1} for CA, FA, and SA; Vertical bars indicate the standard errors of the means. Bars labeled with the same lowercase letter were not significantly different (Tukey's test; $P \leq 0.05$).

interested in determining whether these phenolic acids had different effects on watermelon resistance to FON. We thus examined the effects of CA, FA, and SA on defense-related enzyme activity and on pathogenesis-related gene expression.

Effect of phenolic acid treatment on watermelon resistance to FON

FON inoculation did not affect shoot and root growth in watermelon. Furthermore, exogenous application of CA, FA, or SA did not influence shoot and root growth in watermelon. However, when plants were pre-treated with 30 μmol L^{-1} CA or with 30 μmol L^{-1} SA, FON inoculation suppressed shoot and root growth, respectively, in watermelon plants (Fig. 1).

The effect of FON inoculation and exogenous phenolic acid on chitinase activity in watermelon

FON inoculation enhanced chitinase activity in watermelon leaves in the 10 d after inoculation. Compared to the control treatment, exogenous application of 30 μmol L^{-1} CA did not affect chitinase activity, as evaluated by N-acetyl glucosamine method, in watermelon leaves, but enhanced chitinase activity in roots. Pretreatment with 100 μmol L^{-1} CA significantly increased chitinase activity in watermelon leaves infected with FON. Compared to the control treatment, FON inoculation significantly decreased chitinase activity in watermelon leaves pretreated with 100 μmol L^{-1} FA. The addition of SA enhanced chitinase activity in watermelon leaves regardless of whether or not the plants were inoculated with FON. Whereas the roots of watermelon plants pretreated with 30 μmol L^{-1} SA and then inoculated with FON exhibited reduced chitinase activity compared with the uninoculated control, those pretreated with 30 μmol L^{-1} SA did not.

The effects of FON inoculation and exogenous phenolic acids on β-1,3-glucanase activity in watermelon

β-1,3-Glucanase is a biological control enzyme that lyses the cell walls of pathogenic fungi, thereby protecting the plant. We found that FON inoculation inhibited β-1,3-glucanase activity in watermelon leaves, and that the addition of 100 μmol L^{-1} CA prevented this decrease in activity. FON inoculation alone did not affect β-1,3-glucanase activity in watermelon roots. However, exogenous application of 100 μ μmol L^{-1} CA increased β-1,3-glucanase activity in the roots of watermelon plants inoculated with FON. Therefore, CA enhances watermelon resistance. Exogenous FA decreased β-1,3-glucanase activity in watermelon leaves and roots, regardless of whether or not the plants had been inoculated with FON. Thus, exogenous FA likely impairs watermelon resistance by decreasing β-1,3-glucanase activity. Exogenous SA enhanced β-1,3-glucanase activity in watermelon leaves, regardless of whether or not the plants had been inoculated with FON. Both 30 μmol L^{-1} and 100 μM SA stimulated β-1,3-glucanase activity in the leaves of watermelon plants that had been inoculated with FON.

Effects of FON inoculation and exogenous phenolic acid application on ClPR3 gene expression

Chitinase, which is encoded by *ClPR3* in watermelon, degrades the cell walls of pathogenic fungi and thus serves as a plant defense-related enzyme. We found that *ClPR3* expression was downregulated in watermelon leaves inoculated with FON. Exogenous CA application prevented this decrease in *ClPR3* expression. By contrast, *ClPR3* gene expression in the roots of watermelon plants inoculated with FON was upregulated by the addition of 100 μM CA. *ClPR3* expression in the leaves of watermelon plants inoculated with FON was down-regulated in the

presence of 100 μmol L^{-1} exogenous FA. Furthermore, SA treatment up-regulated *ClPR3* expression in the roots of watermelon plants inoculated or not with FON. These results suggest that exogenously applied CA up-regulates *ClPR3* expression and thereby enhances watermelon resistance to wilt disease caused by FON inoculation. Exogenously applied FA downregulated *ClPR3* expression and decreased watermelon resistance to FON wilt disease, whereas *ClPR3* expression in watermelon roots was upregulated in response to exogenously applied SA.

Discussion

Phenolic acids in plant root exudates affect the microbes in the rhizosphere

Plant roots secrete enormous amounts of organic compounds into the surrounding soil during their growth.[3,22,23] These organic compounds include both low- and high-molecular weight compounds. Low-molecular weight compounds include phenolics, amino acids, organic acids, sugars, and secondary metabolites. These compounds not only serve as an energy source to soil microbes, but they are also allelopathic chemicals that modulate the microbial community in the rhizosphere. Root exudates therefore have a critical ecological impact on soil microbes and their distribution in the rhizosphere.[22] The composition of plant root exudates is affected by environmental factors, including microbes and chemical substances released from other plants.[3,23] Root exudates also modulate the microbial community by suppressing or stimulating the growth of specific types of microbes in the rhizosphere.[22,24] Microbial communities are influenced by root exudates, which alter the nutrient status of the soil, adhere to microbes, promote biofilm formation, or serve as chemo-stimulants that enhance microbial growth.[3,25-27] Bacterial communities in the rhizosphere were altered in *Arabidopsis thaliana* jasmonate pathway mutants (*myc2* and *med25*), due to changes in the composition of root exudates.[26]

The components present in root exudates depend on the plant species and cultivar, and on environmental conditions.[3,20] The plant root is exposed to both pathogenic and beneficial microbes in the soil and secretes chemicals that protect it from pathogens or stimulate the growth of beneficial microbes.[28,29] Maize exudates containing benzoxazinone increased the population density of *Pseudomonas putida* strains, which are beneficial to plants.[30] Phenolic acids are the main constituents of root exudates and they have an allelopathic effect on the microbial community in the rhizosphere. In addition, phenolic compounds in root exudates send specific signals to soil bacteria.[31] *Solanum lycopersicum (L.)* root exudates contain phenolic compounds that stimulate the germination of *Fusarium oxysporum* f. sp *lycopersici* (FOL) microconidia.[32] CA regulates the microbial community by decreasing the growth of *Fusarium oxysporum* f. sp. *cucumerinum* in the rhizosphere of *Cucumis sativus (L.)*.[33] Higher levels of CA were secreted by resistant cultivars than by susceptible cultivars of *Arachis hypogaea* (*L.*).[34] Rice root exudates had an allelopathic effect on the root growth of *Sagittaria montevidensis* (arrowhead) plants.[35] Watermelon did not secrete CA regardless of whether it had its own root system or was grafted onto bottle gourd root.[36] However, it released FA from its own root, but not when grafted onto bottle gourd root.[36] Furthermore, FA that was secreted by *Pseudostellaria heterophylla* roots also stimulated sporulation of the pathogen *Fusarium oxysporum* f. sp *heterophylla* and induced plant wilt disease.[12]

Previously, our study showed that watermelon wilt disease can be alleviated by rice/watermelon intercropping management.[21] FON is suppressed in vitro by root exudates.[20,21] In addition, exudates from watermelon roots stimulate FON spore germination and sporulation.[20] In this study, we demonstrated that rice and watermelon secreted different types of phenolic acids that either stimulated or inhibited FON infection. Rice roots released CA, but not FA, whereas watermelon roots secreted FA, but not CA. CA suppressed FON spore germination and sporulation, whereas FA stimulated FON spore germination and sporulation. It is likely that the CA secreted by rice roots is one of the factors that alleviate watermelon wilt disease during rice/watermelon intercropping management. Furthermore, the FA released by watermelon roots is likely one of the factors that renders watermelon susceptible to wilt disease during continuous monocropping management.

Phenolic acids in plant root exudates altered plant resistance to biotic stress

The SA signaling pathway regulates SAR when a plant is under pathogen attack. SAR is involved in the regulation of defense responses, which include the expression of pathogenesis-related (PR) proteins and defense enzymes.[37] Chitinases and glucanases are typical PR proteins that limit pathogen growth.[38] Chitinases hydrolyze poly-β-1,4-N-acetyl glucosamine (chitin). Since the cell wall of most fungi is mainly composed of chitin, chitinases produced by plants are an important defense mechanism against fungi.[39,40] Plant chitinases therefore serve as a safe and biodegradable biocontrol agent.[41] Overexpression of *OsCHI11* in rice reduced membrane damage in plant cells and enhanced sheath blight resistance.[42] Stimulation of the SA signaling pathway increased tobacco chitinase activity, thereby enhancing resistance to aphids.[38] The present study demonstrates that exogenous CA enhances chitinase expression in roots and relieves the inhibition of chitinase expression caused by *Fusarium* inoculation in leaves (Fig. 4). Furthermore, exogenous CA enhances chitinase activity in leaves and relieves the inhibition caused by *Fusarium* inoculation in roots (Fig. 2). Since CA is specifically secreted by rice roots and not by watermelon roots (Table 1), we conclude that the CA secreted by rice root enhances watermelon resistance in watermelon/rice intercropping. CA not only suppresses *Fusarium* sporulation and spore germination directly, but also enhances watermelon resistance to *Fusarium*. Exogenous FA (100 μM) down-regulates chitinase gene expression, and also decreases chitinase enzyme activity in watermelon leaves (Figs. 2 and 4). Since FA is secreted by watermelon roots, but not by rice roots, it is likely that FA secretion induces watermelon wilt disease in plants subjected to continuous monocropping management, by stimulating *Fusarium* growth (Tables 2 and 3) and inhibiting watermelon resistance (Figs. 2 and 4). SA is not only a signaling molecule in SAR, but also a phenolic acid with direct antimicrobial properties, and is secreted by both watermelon and rice plants (Table 1). Exogenous SA did not change *ClPR3* gene expression in the leaves and roots of watermelon, while it did

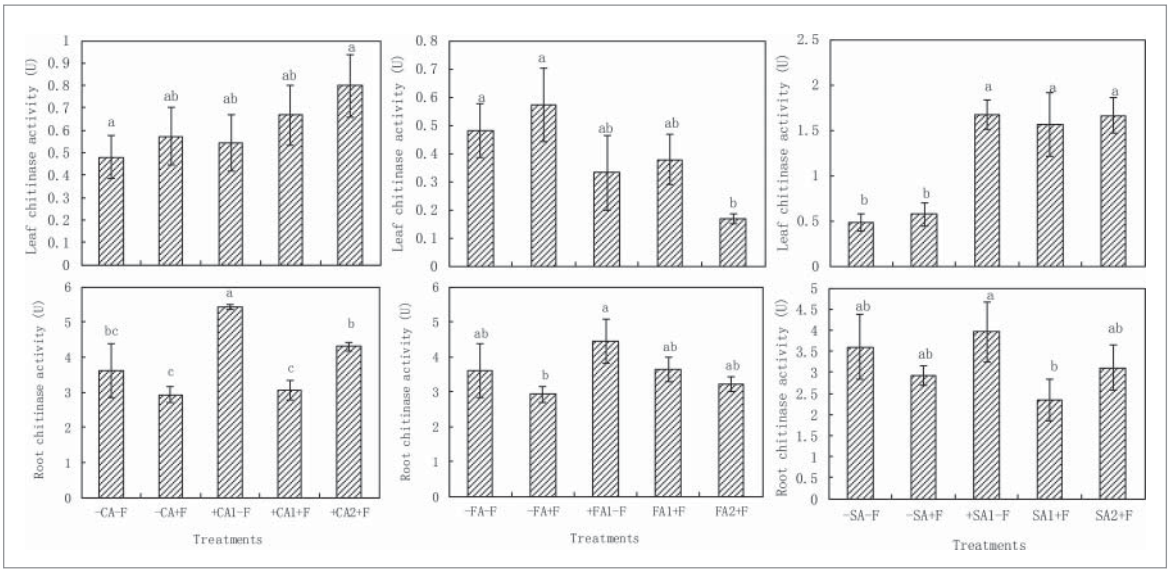

Figure 2. Effects of exogenously applied phenolic acids on chitinase enzyme activity in watermelon leaves and roots. CA, *p*-coumaric acid; FA, ferulic acid; SA, salicylic acid. +, represents present; and –, represents absence; 1 and 2 indicate the concentrations of the different phenolic acids, with 1 representing 30 mol L^{-1} for CA, FA, and SA; and 2 representing 100 mol L^{-1} for CA, FA and SA; Bars labeled with the same lowercase letter were not significantly different (Tukey's test at $P \leq 0.05$).

enhance chitinase enzyme activity in watermelon leaves (Fig. 2). High concentrations of SA suppressed FON spore germination, whereas low concentrations stimulated it (Table 2). Through this mechanism, SA enhances watermelon resistance.

β-1,3-Glucanase is an enzyme that lyses the cell walls of plant pathogenic fungi and can therefore be used as a biological control enzyme.[43] Enhanced β-1,3-glucanase activity in tobacco increased its resistance to aphids.[38] Expression of theβ-1,3-glucanase gene from *Trichoderma harzianum* in *Fragaria* × *ananassa* (strawberry) enhancesβ-1,3-glucanase activity and increases tolerance to crown rot diseases.[44] Moreover, it was shown that the increase in β-1,3-glucanase was mediated by the SA signaling pathway.[38] The present study demonstrates that 100 μM exogenous CA increasesβ-1,3-glucanase activity in watermelon roots and relieves its inhibition caused by FON inoculation in leaves. Exogenous FA, which is specifically secreted by watermelon roots, inhibits β-1,3-glucanase activity in leaves, whereas exogenous SA enhances β-1,3-glucanase activity in watermelon leaves (Fig. 3). We thus suggest that exogenous CA and SA enhance β-1,3-glucanase activity, thereby increasing the resistance of watermelon to pathogens, while exogenous FA inhibits β-1,3-glucanase activity and thus inhibits the resistance of watermelon.

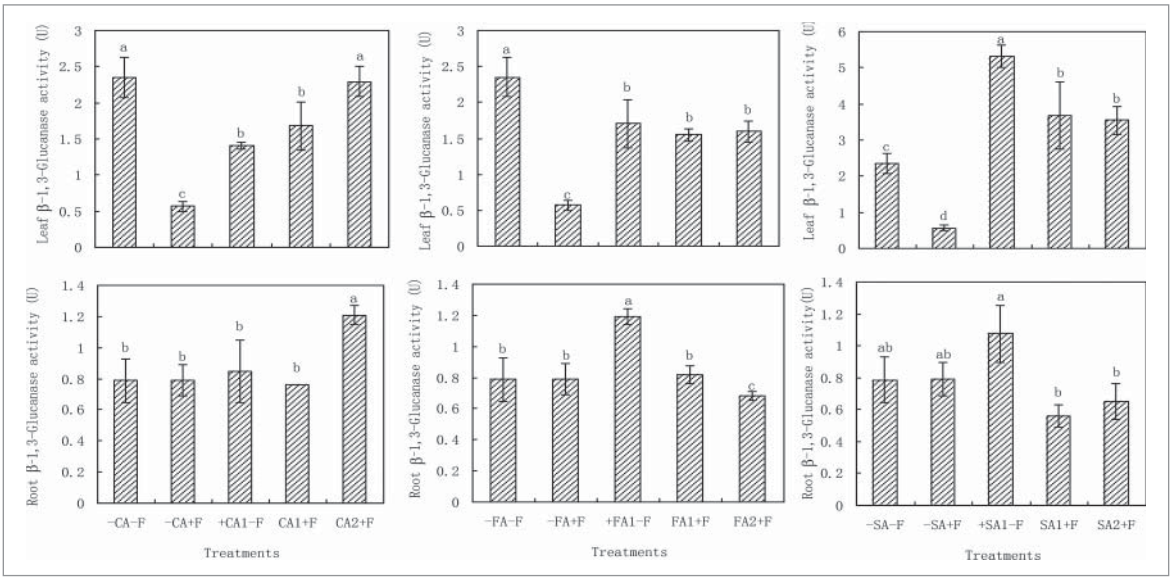

Figure 3. Effects of exogenously applied phenolic acid on β-1,3-glucanase activity by sugar reduction and cyanoacetamide method in watermelon leaves and roots. CA, *p*-coumaric acid; FA, ferulic acid; SA, salicylic acid. +, represents present; and –, represents absence; 1 and 2 indicate the concentrations of the different phenolic acids, 1 represents 30 mol L^{-1} for CA, FA, and SA; and 2 represents 100 mol L^{-1} for CA, FA and SA;. Vertical bars indicate the standard errors of the means. Bars labeled with the same lowercase letter were not significantly different (Tukey's test; $P \leq 0.05$).

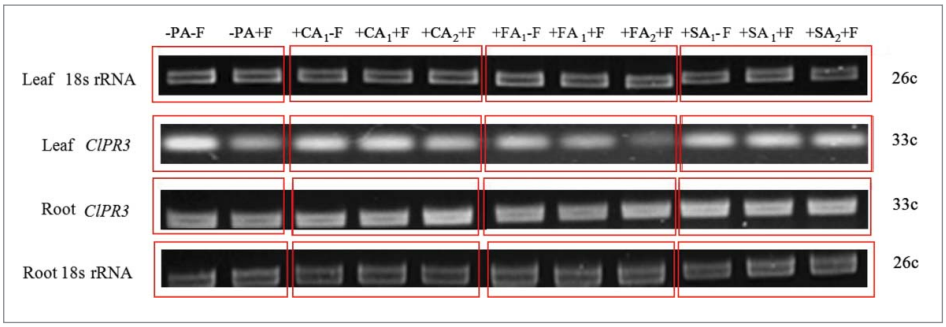

Figure 4. Effects of phenolic acids on *ClPR3* expression.-PA-F, application of neither phenolic acid nor FON; -PA+F, inoculation with FON alone; $+CA_1$-F, application of 30 μmol L^{-1} *CA* alone; $+CA_1$+F, application of 30 μmol L^{-1} CA and inoculation with FON; $+CA_2$+F, application of 100 μmol L^{-1} CA and inoculation with FON; $+FA_1$-F, application of 30 μmol L^{-1} ferulic acid alone; $+FA_1$+F, application of 30 μmol L^{-1} ferulic acid and inoculation with FON; $+FA_2$+F, application of 100 μmol L^{-1} ferulic acid and inoculation with FON; $+SA_1$-F, application of 30 μmol L^{-1} salicylic acid alone; $+SA_1$+F, application of 30 μmol L^{-1} salicylic acid and inoculation with FON; $+SA_2$+F, application of 100 μmol L^{-1} salicylic acid and inoculation with FON.

In conclusion, the root exudates of phenolic acids were different between rice and watermelon, which lead to their different ecological roles on pathogenic fungus and watermelon defense, which is one of the mechanisms of watermelon wilt disease alleviated by intercropping with aerobic rice.

Materials and methods

Materials

Watermelon (*C. lanatus* (Trunb.) Matsum and Nakai cv. Zaojia 84-24) was used in Experiment 1 and Experiment 3 (described below). Rice (*Oryza sativa L.* cv 4007) was used in Experiment 1. *Fusarium oxysporum* f. sp. *niveum* (coded NJAUS-1) was isolated from infected watermelon seedlings.[20] Spore suspensions of the pathogen were obtained from 14-day-old cultures on potato dextrose agar (PDA) medium cultivated at 28°C. The spores were collected by adding 10 mL of sterile water to the Petri dish and rubbing the PDA surface with a sterile L-shaped glass rod. The suspension was subsequently filtered through 4 layers of cheesecloth, and the concentration of spores was determined using a hemocytometer. Phenolic acids (including *p*-coumaric, ferulic, and salicylic acids) were purchased from Sigma.

Experimental design

Experiment 1. Analysis of phenolic acids secreted by rice and watermelon roots. Rice and watermelon seeds were sterilized and germinated. The sprouted seedlings were cultured hydroponically first in deionized water, then in 25% nutrient solution for 3 days, then in 50% nutrient solution for 3 days, and finally in 100% nutrient solution until harvest. Rice was grown in nutrient solution made according to specifications of the International Rice Research Institute (IRRI) and watermelon was grown in Hoagland's nutrient solution.[45] The seedlings were maintained under natural light and temperature conditions (30/19°C day/night) in a greenhouse and the relative humidity ranged from 80% to 95%. The watermelon cultivation system was bubbled with air to supply sufficient O_2 and to circulate nutrient solution. The pH of the nutrient solutions was measured daily and maintained at pH 5.5 and 6.8 for rice and watermelon, respectively. The nutrient solution was replaced every 3 d. Root exudates from rice seedlings were collected at the 4th leaf stage, when the rice plants had strong allelopathic potentials, whereas root exudates from watermelon seedlings were collected at the flowering stage, when the watermelon plants were susceptible to infection by *Fusarium oxysporum*. When collecting root exudates, the plants were gently removed from the nutrient solution and the roots were washed first with running tap water and then several times with deionized water. The seedlings were then placed in cups containing 300 mL of deionized water and the roots were submerged in the water. The cups were covered with a plastic lid to prevent contamination and both the cups and the lids were wrapped in black paper to avoid exposure to light. A drop of phosphoric acid was added to inhibit microbial activity. Each cup contained 6 rice seedlings or 2 watermelon seedlings. The root exudates were collected after 4 hours and then filtered through a 0.45 μm Millipore membrane.

Table 3. Effects of phenolic acids on spore germination of FON.

	p-Coumaric acid		Ferulic acid		Salicylic acid		Phthalic acid		*p*-Hydroxybenzoic acid	
Treatments	GN	IR(%)	GN	IR(%)	GN	IR(%)	GN	IR(%)	GN	IR(%)
0	43.5 ± 1.91a	—	35.3 ± 4.16d	—	43.3 ± 1.53b	—	78.3 ± 0.58a	—	73.3 ± 5.51b	—
5	39.5 ± 3.11a	−9.20	26.7 ± 3.06d	−24.53	47.7 ± 4.04ab	10.00	81.7 ± 2.89a	4.26	73.7 ± 3.51b	0.45
10	39.3 ± 4.43a	−9.77	30.0 ± 8.00d	−15.09	54.7 ± 4.73a	26.15	81.0 ± 7.81a	3.40	78.7 ± 7.77ab	7.27
20	32.5 ± 6.14b	−25.29	32.7 ± 2.31d	−7.55	53.5 ± 3.21a	23.46	80.3 ± 6.43a	2.55	83.5 ± 2.52a	13.86
40	32.5 ± 2.52b	−25.29	45.3 ± 5.03c	28.30	50.7 ± 6.35a	16.92	79.0 ± 3.61a	0.85	83.0 ± 3.00a	13.18
80	24.5 ± 5.07c	−43.68	64.7 ± 5.03b	83.02	17.3 ± 3.06c	−60.00	82.0 ± 4.58a	4.68	83.0 ± 3.61a	13.18
160	12.8 ± 3.10d	−70.69	75.3 ± 1.15a	113.21	14.3 ± 1.53c	−66.92	76.0 ± 1.00a	−2.98	87.0 ± 5.29a	18.64

Note: Treatments, the added concentration (mg kg^{-1}) of phenolic acids. GN, the number of germinating spores; IR, increase rate; the minus value means suppression rate, namely the effect is suppression. The unit of addition phenolic acids was mg L^{-1}.

The collected root exudates were lyophilized and stored at −20°C. The lyophilized root exudates were dissolved in deionized water and the phenolic acids were detected by high-performance liquid chromatography (HPLC; Agilent 1200, Germany).

Experiment 2. Evaluating the effects of exogenous p-coumaric acid, ferulic acid, and salicylic acid on FON spore germination and sporulation. Various concentrations of *p*-coumaric acid, ferulic acid, and salicylic acid (0, 5, 10, 20, 40, 80, and 160 mg·L^{-1}) were used to detect the response of FON to the 3 phenolic acids. As a control for the chemical treatment (0 mg·L^{-1}), an equal volume of distilled water was added. All three phenolic acids were filter-sterilized through a 0.22 μm Millipore membrane. Then the phenolic acids were added to steam-sterilized PDA medium to determine their effects on spore germination and Bilay's medium to determine their effects on sporulation. The effects of phenolic acids (CA, FA, or SA) on spore germination and sporulation of FON were described in terms of IR (increased rate). A negative value indicates suppression.

Increased rate (IR) (%) = (A − B) / B × 100 %. Where A represents the sporulation number or germinated spore number in the treated samples and B represents the corresponding values in the control.

Experiment 3. The effects of exogenously applied p-coumaric acid, ferulic acid, and salicylic acid on watermelon resistance. Exogenous CA, FA, and SA were applied to watermelon roots to mimic the effects of phenolic acids secreted by roots. Exogenous phenolic acids were added to watermelon roots to investigate the effects of allelopathic substances secreted from watermelon roots on disease resistance. Watermelon seeds were surface-sterilized with 5% (v/v) H_2O_2 for 30 min, washed with deionized water, and then sown in a seedling tray containing sterilized vermiculite. Fifteen days after emergence, watermelon seedlings were transplanted into pots (22 × 15, cm, diameter×height) containing sterilized vermiculite. Four seedlings were planted in each pot. The pots were irrigated with Hoagland's nutrient solution every 2 d throughout the experiment. Ten days after transplantation, phenolic acids were mixed with nutrient solution and added every 6 d.

A FON spore suspension (6.5 ×10^6 spores) was poured around the watermelon seedling roots 20 d after transplantation, and again 5 d later. The watermelon seedlings were harvested 5 d after the second FON inoculation and analyzed. For the control treatment, only sterilized water was used. The treatments were as follows: (1) control, without CA or *Fusarium* (−CA-F); (2) inoculation with *Fusarium* only (−CA+F); (3) the addition of 30 μM CA only (+CA-F); (4) the addition of 30 μM CA and inoculation with *Fusarium* (+CA$_1$+F); (5) the addition of 100 μM CA and inoculation with *Fusarium* (+CA$_2$+F). The same design was used for FA and SA treatments.

Determination methods

Phenolic acid analysis

Ten different phenolic acids were isolated and identified from watermelon and rice root exudates using HPLC (Agilent 1200, Germany).[20] The analytical conditions were as follows. Chromatographic column: XDB-C18 (4.6 mm × 250 mm); temperature of column: 40°C; flow velocity: 1.0 ml·min^{-1}; detector wavelength: 280 nm; and injection volume: 20 μL. Methanol (A) and 2% (v/v) acetic acid solution (pH 2.59) (B) were used as mobile phases with a gradient elution (B: 100% (0 min), 60% (22.5 min-27 min), 37% (40.5 min-42 min), 25% (52 min), 0% (55 min), and finally 0% (60 min). Standard phenolic acids used for HPLC analysis were gallic acid, CA, p-hydroxybenzoic acid, phthalic acid, vanillic acid, syringic acid, ferulic acid, benzoic acid, salicylic acid, and cinnamic acid. All chemicals were of high purity and the solvents used were HPLC spectral grade. Major peaks were identified by comparing the retention time with that of the matching standard.

Spore germination test

Spores (100 μL, 1 × 10^3 spores per mL) were germinated in Petri dishes containing 20 mL of agar medium, to which the indicated concentration of test compound had been added before gelling. Each treatment consisted of 3 replicates.

Sporulation test

To determine the sporulation rate of the pathogen, the pathogen was incubated in Bilay's medium and various concentrations of test compounds on a liquid shaker (120 rpm) at 28°C.[46] Liquid medium (20 mL) was inoculated with 100 μL spore suspension containing 2 × 10^5 spores·mL^{-1}. Each treatment consisted of 3 replicates.

Chitinase gene (ClPR3) expression

Modified RT-PCR was used to detect the expression of the pathogenesis-related (PR) gene *ClPR3*.[47] Total RNA was isolated from the fresh leaves or roots (100 mg) of treated watermelon using Trizol reagent according to the manufacturer's instructions (Invitrogen, USA). Two-step RT-PCR was carried out using a Maxima First Strand cDNA Synthesis Kit, following the manufacturer's instructions. cDNA was synthesized using isolated total RNA. Semi-quantitative PCR was performed with gene-specific primers. The gene encoding 18SrRNA was used as a control to normalize the data. *ClPR3* and 18SrRNA were amplified by 33 and 26 cycles, respectively. A list of primers used is shown in Table 4. Each experiment was performed in triplicate (Table 1).

Chitinase and β-1,3-glucanase activities

A chitinase activity assay described in Marina et al. (2011) was modified as follows. First, colloidal chitin was prepared.[47,48] Equal amounts of total protein from plants were incubated with colloidal chitin at 37°C for 1 h in a water bath. The reaction was terminated by adding 100 μL HCl (1 N) and incubating the samples on ice for 10 min. The mixture was centrifuged at 13,000 rpm for 10 min at 4°C to facilitate the precipitation of undigested substrate. The resulting product, N-acetyl glucosamine, was spectrophotometrically measured using the dinitrosalycylic acid (DNSA) method.[47] Heat-inactivated extracts, obtained by incubation in boiling water for 10 min, were used as the control. The amount of protein in the leaf or root extracts was measured by the Bradford method.[47] Three independent extractions were performed.

Table 4. Effects of phenolic acids secreted from rice or watermelon roots on sporulation of FON.

	p-Coumaric acid		Ferulic acid		Salicylic acid		Phthalic acid		*p*-Hydroxybenzoic acid	
Treatments	SN ($\times 10^5$)	IR(%)	SN ($\times 10^5$)	IR(%)	SN ($\times 10^5$)	IR(%)	SN ($\times 10^5$)	IR(%)	SN ($\times 10^5$)	IR(%)
0	57.7 ± 0.58a	—	61.7 ± 8.50cd	—	53.7 ± 3.21a	—	81.0 ± 7.00c	—	53.3 ± 4.93c	—
5	43.7 ± 1.15b	−24.28	50.0 ± 2.00e	−18.92	48.7 ± 0.58ab	−9.32	81.7 ± 5.51c	0.82	62.7 ± 6.03b	17.5
10	16.3 ± 1.15c	−71.68	56.0 ± 7.21de	−9.19	49.0 ± 1.00ab	−8.70	83.3 ± 2.31c	2.88	74.0 ± 5.29a	38.75
20	13.0 ± 1.00d	−77.46	67.3 ± 3.51bc	9.19	50.7 ± 6.43ab	−5.59	85.7 ± 4.93bc	5.76	66.0 ± 5.00ab	23.75
40	10.3 ± 1.53e	−82.08	72.7 ± 2.08b	17.84	50.0 ± 2.65ab	−6.83	88.3 ± 2.08bc	9.05	67.0 ± 3.00ab	25.63
80	2.0 ± 1.73f	−96.53	76.3 ± 6.66b	23.78	44.3 ± 5.13b	−17.39	91.3 ± 6.51ab	12.76	65.7 ± 2.08ab	23.13
160	0.1 ± 0.00g	−99.83	96.0 ± 3.61a	55.68	0.0 ± 0.00	−100	97.0 ± 1.00a	19.75	62.0 ± 5.29b	16.25

Note: Treatments, the added concentration (mg kg^{-1}) of phenolic acids. SN, the number of spores; IR, increase rate, the minus value means suppression rate, namely the effect is suppression. The unit of addition phenolic acids was mg L^{-1}.

β-1,3-glucanase activity was measured in the young expanding leaves and roots of 45-day-old watermelon seedlings. Frozen material (0.1 g) was ground to powder using liquid nitrogen and extracted with 1 ml of 50 mM acetate buffer at pH 5.5. Extracts were centrifuged at 20,000 g for 15 min at 4°C. The reaction mixture, containing 150 μl of 0.5% (w/v) laminarin in extraction buffer and 50 μl desalted extract, was incubated at 37°C for 20 h. Then, the amount of reduced sugars was estimated by the cyanoacetamide method using glucose as a standard.[47] Heat-inactivated extracts were used as a control. The amount of protein in leaf or root extracts was measured by the Bradford method. Three independent extractions were performed.[47]

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS 13.0, Inc., Chicago, USA). All data were expressed as means ± standard errors. Significant differences between treatments were evaluated using 2-factor ANOVA, followed by Tukey's test at the 5% probability level.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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