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Treatment With Wheat Root Exudates and Soil Microorganisms From Wheat/Watermelon Companion Cropping Can Induce Watermelon Disease Resistance Against *Fusarium oxysporum* f. sp. *niveum*

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Abstract

Companion cropping with wheat (*Triticum aestivum* L.) can enhance watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] wilt disease resistance against *Fusarium oxysporum* f. sp. *niveum*. However, the mechanism of resistance induction remains unknown. In this study, the effects of microbial community dynamics and the interactions between wheat and watermelon plants, particularly the effect of wheat root exudates on watermelon resistance against *F. oxysporum* f. sp. *niveum*, were examined using a plant-soil feedback trial and plant tissue culture approach. The plant-soil feedback trial showed that treating watermelon with soil from wheat/watermelon companion cropping decreased watermelon wilt disease incidence and severity, increased lignin biosynthesis- and defense-related gene expression, and increased β-1,3-glucanase activity in watermelon roots. Furthermore, soil microbes can contribute to increasing disease resistance in watermelon plants. Tissue culture experiments showed that both exogenous addition of wheat root exudates and companion cropping with wheat increased host defense gene expression, lignin and total phenols, and increased β-1,3-glucanase activity in watermelon roots. In conclusion, both root exudates from wheat and the related soil microorganisms in a wheat/watermelon companion cropping system played critical roles in enhancing resistance to watermelon wilt disease induced by *F. oxysporum* f. sp. *niveum*.

Companion cropping is a form of intercropping in which certain plants are planted for their beneficial effects on the main crop (Jungers et al. 2015) rather than for harvest. Intercropping and companion cropping can alleviate soilborne plant diseases, as has been observed with rice/watermelon intercropping (Ren et al. 2008), watermelon/wheat companion cropping (Xu et al. 2015b), maize/soybean intercropping (Gao et al. 2014), Chinese chive/banana intercropping (Zhang et al. 2013), and potato onion/tomato companion cropping (Fu et al. 2015). The mechanisms by which intercropping and companion cropping suppress crop diseases are complicated and involve root exudates, soil microorganisms, and induced systemic resistance (Bakker et al. 2003; Ding et al. 2015; Li et al. 2014a, 2015).

Several studies have shown that interspecies interactions can enhance plant resistance to pathogen-induced disease, especially via inducing defense-related gene expression (Gao et al. 2014; Yang et al. 2014). Induced defense responses involve many defense mechanisms, including phytoalexin synthesis and activation of numerous defense-related genes that encode pathogenesis-related (PR) proteins (Maldonado-Bonilla et al. 2008). Phenylalanine ammonia lyase (PAL), the key enzyme in the phenylpropanoid pathway, converts phenylalanine to cinnamic acid, a precursor for salicylic acid (SA) and lignin biosynthesis (Koukol and Conn 1961). The chitinase (*CHI*) gene encodes hydrolytic enzymes and proteins related to plant defense responses (Zvirin et al. 2010). Similarly, plant defensins (PDFs), such as *ClPDF2.1* and *ClPDF2.4*, play important roles in disease resistance, particularly against fungi (Zhang et al. 2015). Therefore, upregulation of defense-related genes is an important component of disease resistance. Evidence suggests that accumulation of hydrolytic enzymes, such as β-1,3-glucanase (PR2), is associated with induced resistance in corn to *Fusarium moniliforme* infection (Cordero et al. 1994). In addition, phytoalexins (Hasegawa et al. 2010), lignin, and hydroxyproline-rich glycoprotein substances (Chang et al. 2008) accumulated to high levels in pathogen-infected plants. Therefore, genes associated with the lignin synthesis pathway play important roles in enhancing plant resistance to pathogens.

Several studies have shown that root exudates secreted from plants, consisting of ions, enzymes, mucilage, and primary and secondary metabolites, might exert inductive effects on surrounding plants (Bertin et al. 2003; De-la-Pena et al. 2008; Wen et al. 2007). For example, in plots with maize/pepper intercropping, root exudates from pepper plants act as inducers and improve the ability of maize to defend itself against southern corn leaf blight caused by *Bipolaris maydis* (Ding et al. 2015; Yang et al. 2014). Different compositions of root exudates from host plants can induce varying disease resistance responses in their corresponding companion plants. For instance, exogenous addition of catechin, a root exudate component from the invasive weed *Centaurea maculosa* (spotted knapweed), can improve *Arabidopsis* species resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and increase *PR1* gene expression (Prithiviraj et al. 2007). Moreover, exogenous addition of SA can induce tomato and soybean plant resistance to *F. oxysporum* f. sp. *lycopersici* and *Botrytis cinerea*, respectively (De Meyer et al. 1999; Mandal et al. 2009).

"Plant-soil feedback" refers to the effects of changed soil microbial composition and abundance on the growth, development, and health of successor crops (Gao et al. 2017; van de Voorde et al. 2012; Zhou et al. 2017, 2018), as illustrated by previous findings that alterations in the soil microbial community can affect plant growth and resistance to biotic and abiotic stresses. For example,

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intercropping tomato with leek results in a 20% higher arbuscular mycorrhizal colonization rate compared with nonintercropped tomato. In addition, this association decreased disease severity caused by *F. oxysporum* f. sp. *lycopersici* (Hage-Ahmed et al. 2013). Furthermore, peanut/*Atractylodes lancea* intercropping significantly increased soil microorganism biodiversity and reduced the incidence of damping-off and root rot diseases at the seedling and maturation stages, respectively (Li et al. 2014b). Many beneficial microorganisms can induce systemic resistance in plants, such as plant growth-promoting rhizobacteria and mycorrhizal fungi (Carvalhais et al. 2013, 2014, 2015; Tucci et al. 2011; Verhagen et al. 2010). Changes in the soil microorganisms in intercropping systems can thus induce systemic resistance in plants. However, little attention has been paid to the relationships between changes in microbial communities and induced plant disease resistance.

Our previous studies showed that wheat/watermelon companion cropping is characterized by a decreased incidence of Fusarium wilt in watermelon and an increased resistance to *F. oxysporum* f. sp. *niveum* (Xu et al. 2015a). We hypothesized that wheat root exudates and the associated alterations in the soil microbial community might be responsible for the increased disease resistance of watermelon observed in wheat/watermelon companion cropping. In this study, we aimed to (i) examine the effects of soil microorganisms on watermelon Fusarium wilt and watermelon disease resistance through a plant-soil feedback trial; (ii) investigate the effects of companion cropping with wheat on the induction of disease resistance in watermelon under microorganism-free conditions; and (iii) evaluate the effects of wheat root exudates on induced disease resistance in watermelon. This study will deepen our understanding of the mechanisms through which companion cropping with wheat can increase watermelon disease resistance to Fusarium wilt.

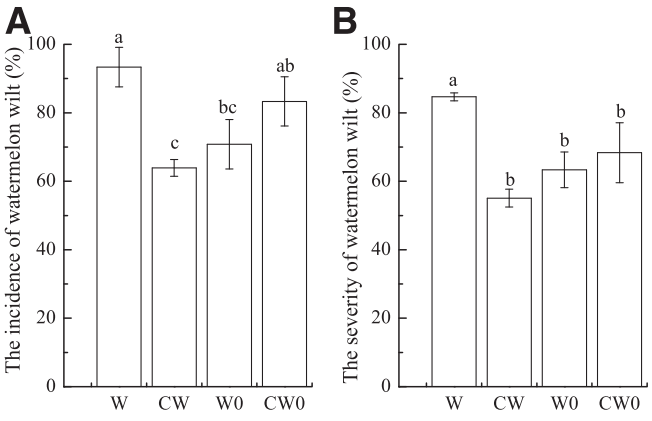

Fig. 1. A, Incidence and **B,** severity of watermelon wilt 10 days after inoculation with *Fusarium oxysporum* f. sp. *niveum*. Values (mean ± SD) with different letters above the bars are significantly different at $P < 0.05$ (one-way analysis of variance). Disease incidence was defined as the percentage of wilted plants. Disease severity was defined as the sum of the disease grade of each plant divided by the highest possible total score, expressed as a percentage. W = monoculture watermelon soil mixture, CW = wheat/watermelon companion cropping soil mixture, W0 = sterile monoculture watermelon soil mixture, and CW0 = sterile wheat/watermelon companion cropping soil mixture.

Materials and Methods

Plant materials and fungal culture. Wheat variety D123 was provided by the Vegetable Physiological Ecology Laboratory of the Northeast Agricultural University Department of Horticulture (Harbin, China) (45°41′N, 126°37′E). Seeds of the watermelon cultivar Heimeiren, which is susceptible to *F. oxysporum* f. sp. *niveum*, were purchased from the Nongyou Seed Company (Xiamen, China). *F. oxysporum* f. sp. *niveum* 1 strain (Xu et al. 2015b) was provided by the Vegetable Physiological Ecology Laboratory of the Northeast Agricultural University Department of Horticulture. The strain was cultured on potato dextrose agar (PDA) at 28°C in the dark for 7 days. A 6-mm plug from a PDA plate was placed into 300 ml of potato dextrose broth in a flask and placed on a rotary shaker at 200 rpm. After 5 days, fungal spores were collected and adjusted to 10^6 conidia/ml with sterile water.

Experimental design and methods. *Experiment 1: Plant-soil feedback trial.* Watermelon seedlings at the four-leaf stage were transplanted into plastic pots (20 × 17 cm) filled with soil collected from a field in which watermelon plants had been continuously cropped for many years. The soil had a sandy loam texture, contained 3.64% organic matter, 139 mg kg^{-1} available N, 271 mg kg^{-1} Olsen P, and 223 mg kg^{-1} available K. The electrical conductance (EC) was 0.68 mS cm^{-1} (1:2.5, wt/vol), and the pH was 6.91 (1:2.5, wt/vol). Five days after transplantation, 30 wheat seeds were sown 5 to 7 cm from each watermelon seedling. Watermelon monoculture was used as a control (CK). All plants were grown in a plant growth chamber with an 18-h/6-h light/dark cycle and a temperature cycle of 28/18°C. Soil samples from the wheat/watermelon and monoculture watermelon pots were collected 20 days after transplantation and used for the plant-soil feedback experiment.

Soil collected from the open field (depth to 15 cm) was autoclaved three times at 121°C for 30 min at intervals of 24 h to eliminate the influence of microorganisms in the soil (Gao et al. 2017). This sterile soil was used as a mixing partner with soil samples collected from wheat/watermelon and monoculture watermelon pots at a ratio of 94:6 (wt/wt) (Brinkman et al. 2010; Zhou et al. 2017); all treatments were mixed in the same manner. Each treatment was mixed separately in autoclave bags and maintained at a water holding capacity of approximately 65%. The bags of soil were incubated at 25°C in the dark for 3 days (van de Voorde et al. 2012). Each of 468 plastic pots was filled with approximately 300 g of the mixture, and 117 randomly arranged pots were subjected to each of the following four treatments: sterile soil mixed with (i) fresh monoculture watermelon soil (W), (ii) sterile monoculture watermelon soil (W0), (iii) fresh wheat/watermelon companion cropping soil (CW), and (iv) sterile wheat/watermelon companion cropping soil (CW0). Mixing with sterile soil as a negative control was used to avoid the influence of nutrients from the soil. Three replicates of each treatment condition were included.

Table 1. Genes and primers used in quantitative real-time polymerase chain reaction analysis[z]

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
ClPDF2.1	ATGAAGTTCTTTTCCGCTGC	TCAAACGCAGTGCTTTGTGCAGAAG
ClPDF2.4	ATGAAGTTTCTTTTTCAGCTGC	TCAAACGCAGTGCTTTGTG
PAL	GTGCCACTTCCCATAGAAGAAC	GGAGTGTGTTGATTCTCACCAA
CHI	CTGAATTCTTGGAGTCAGTGGA	ACGCCTTGCTCCATAACATAAC
18S rRNA	AGCAAGCCTACGCTCTGT	CTGGTCGGCATCGTTTAT
DAHPS	ATTCGTGATACCTTCCGTGTTC	CTGCCATTCTTCCTACCTTGAT
CCOMT	CGAGTTTGGGTTGCCATTTAT	CTCCACCTATCTTCACGAGTTTTTA
CCR	AGGAACCGTCAGAAATCCAGAG	CGAGAAGATCCGCTTTATGCA
POD	AGTGGGTGGGTTGACCTTTCT	ATCACAAAGGGCTTCTCCAAA

[z] 18S rRNA was used as an internal control. *PDF* = plant defensin, *PAL* = phenylalanine ammonia lyase, *CHI* = chitinase, *DAHPS* = 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, *CCOMT* = caffeoyl-coenzyme A *O*-methyltransferase, *CCR* = cinnamoyl-coenzyme A reductase, and *POD* = peroxidase.

Watermelon seeds were surface sterilized by soaking in 2.5% sodium hypochlorite solution for 10 min and then thoroughly rinsed with sterile water to remove the sodium hypochlorite. Surface-sterilized seeds of wheat and watermelon were placed on sterile filter paper at 28°C for 2 days, and the germinated watermelon seeds were then transferred to sterile soil. Seven days later, watermelon seedlings with two cotyledons were transplanted into the 468 pots, and the pots were moved into a plant growth chamber with a 16-h/8-h light/dark photoperiod and a temperature cycle of 28°C (light) and 18°C (dark). After 10 days, inoculation with *F. oxysporum* f. sp. *niveum* was carried out using the root irrigation method (Xu et al. 2015b). Briefly, approximately 30-ml aliquots of 10^6 conidia/ml were irrigated into the root zone of each watermelon plant. In total, 36 randomly selected plants were harvested for roots prior to inoculation with *F. oxysporum* f. sp. *niveum* and at 1 and 3 days after inoculation (3 plants × 4 treatments × 3 time periods), and this harvest process was repeated three times (108 plants all together). The roots were immediately frozen in liquid nitrogen and stored at −80°C until the lignin, total phenols, β-1,3-glucanase activity, and quantitative real-time polymerase chain reaction (qRT-PCR) analyses were performed. Ten days after inoculation, the remaining 360 plants were harvested (4 treatments × 30 pots × 3 replicates) to assess disease severity and incidence. Thirty of the 360 plants were used for each replicate sample, and each treatment had three replicates. Wilt incidence was calculated as the percentage of the number of wilted plants compared to the total number of plants as follows (Wu et al. 2009):

$$\text{Incidence of watermelon wilt} = \frac{\text{Number of wilted plants}}{\text{Number of total plants}} \times 100\%$$

Disease severity was calculated as follows based on symptom severity of each individual plant, which was rated using a previously described scale (Harvás et al. 1997):

$$\text{Severity of watermelon wilt} = \frac{\sum \text{Number of plants} \times \text{Level of wilt disease}}{\text{Total number of plants} \times \text{Highest level of wilt disease}} \times 100\%$$

Experiment 2: Effect of wheat/watermelon companion cropping on resistance of watermelon to F. oxysporum *f. sp.* niveum *under microorganism-free conditions.* Watermelon seeds were soaked in water for 10 h, surface sterilized by spraying with a 70% ethanol solution, washed with sterile water, soaked in a 0.1% mercuric chloride solution for 5 min, and rinsed four times with sterile water. Wheat seeds were pretreated as previously described with certain modifications (Huang et al. 2003). Briefly, wheat seeds were surface sterilized by soaking in 70% ethanol and 2.5% sodium hypochlorite solutions for 2.5 and 7.5 min, respectively, and rinsed four times with sterile water. Subsequently, the surface-sterilized wheat seeds were completely submerged in sterile water for 5 h for pregermination and then surface sterilized once more by soaking in a 0.1% mercuric chloride solution for 20 min and rinsing four times with sterile water. The surface-sterilized wheat and watermelon seeds were placed on sterile filter paper in separate dishes and incubated at 28°C in a growth chamber for 2 days.

The germinated watermelon seeds were sown in 100 ml of half-strength Murashige and Skoog (1962) solid medium in glass beakers (one seed per beaker) under microbe-free conditions. The seeds were cultured in a controlled environmental growth cabinet with an

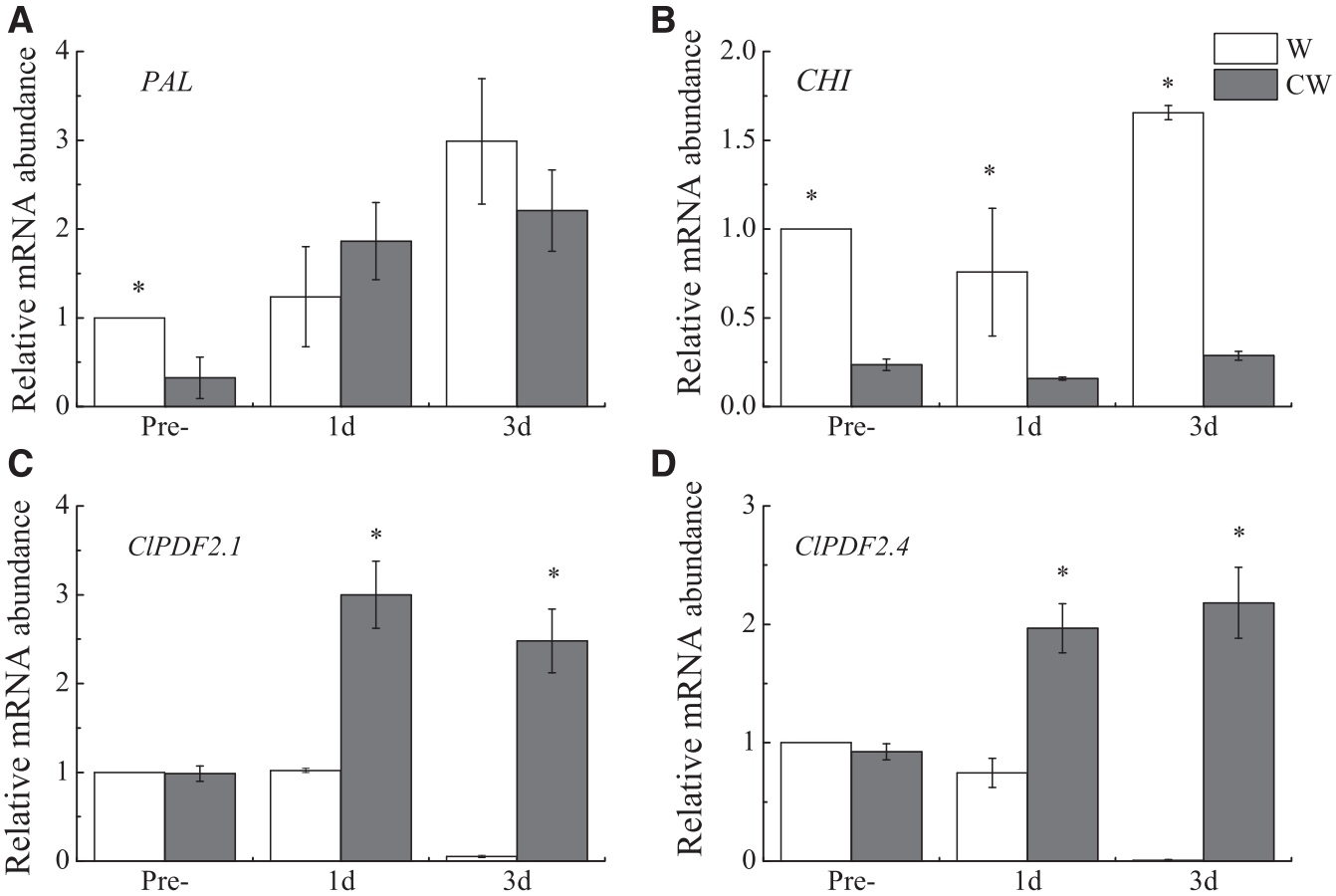

Fig. 2. Relative mRNA expression levels of defense-related genes after inoculation with *Fusarium oxysporum* f. sp. *niveum*. **A,** Phenylalanine ammonia lyase (*PAL*). **B,** Chitinase (*CHI*). **C,** Plant defensin *ClPDF2.1*. **D,** Plant defensin *ClPDF2.4*. Values (mean ± SD) with an asterisk above the bars are significantly different within each harvest interval (by the independent sample *t* test at $P < 0.05$). Watermelon roots were harvested before inoculation with *F. oxysporum* f. sp. *niveum* (Pre) and at 1 and 3 days afterward. W = monoculture watermelon soil mixture and CW = wheat/watermelon companion cropping soil mixture.

18-h/6-h light/dark cycle and a 28/18°C temperature cycle. Once the watermelon seedlings had two true leaves, 10 surface-sterilized wheat seeds were sown around each watermelon seedling at a distance of 1 cm (CW); watermelon seedlings grown alone were used as a control (W). After 10 days, the watermelon seedlings were inoculated with a conidial suspension of *F. oxysporum* f. sp. *niveum* (5×10^6 conidia/beaker) via the root irrigation method. Immediately after inoculation and then at 12 and 24 h (2 treatments × 3 time periods × 3 beakers), 18 randomly selected watermelon seedlings were harvested for roots and this root harvest process was repeated three times (54 plants in total). Subsequently, the roots were wrapped in tinfoil, immediately frozen in liquid nitrogen, and stored at −80°C. These samples were subjected to analyses of lignin, total phenols, β-1,3-glucanase activity, and qRT-PCR. Three replicates of each treatment were included, and each replicate consisted of the roots of three seedlings.

Experiment 3: Effect of exogenous addition of wheat root exudates on resistance of watermelon to F. oxysporum *f. sp.* niveum. Wheat seeds were grown in a 0.5 × 1-m tray with unsterile field soil in a conventional system. After 17 days, the roots were harvested for the collecting of root exudates, which was performed according to a previously described method with some modifications (Li et al. 2013; Ren et al. 2008; Vranova et al. 2013). The roots were removed from the soil, cleaned by washing with tap water, and further washed three times with deionized water. The cleaned seedling roots were placed in a beaker with deionized water, and the beaker was covered with tinfoil to ensure that the roots were not exposed to light and to thus prevent degradation of root exudates. The covered beaker was placed in a lighted plant growth chamber for 6 h at 28°C. The roots were then removed and weighed, and the concentration of the root exudates was adjusted with deionized water to 1 g of fresh root per 10 ml of exudate solution (Hao et al. 2010). Millipore filters (0.22 μm) were used to remove microorganisms from the root exudates.

The seed sowing method and the plant growth environment were the same as in experiment 2. The three treatments were as follows: (i) addition of 5 ml of root exudates (R), (ii) addition of 5 ml of 1 mM SA (used as a positive control), and (iii) addition of 5 ml of sterile deionized water (served as a control, CK). The exogenous additions of water, SA, and root exudates were performed twice at 24-h intervals. After 2 days, the watermelon plants were inoculated with a conidial suspension of *F. oxysporum* f. sp. *niveum* (5×10^6 conidia/beaker) as described in experiment 2. Then, 12 and 24 h after inoculation, 27 randomly selected watermelon seedlings (3 treatments × 3 plants per treatment × 3 time points) were harvested for roots, and this harvest process was replicated three times (81 plants in total). Roots were immediately frozen in liquid nitrogen and stored at −80°C for further analyses of lignin and total phenols, β-1,3-glucanase activity, and qRT-PCR.

Determination of β-1,3-glucanase activity in watermelon roots. Glucanase activity was evaluated according to a previously described method with some modifications (Ren et al. 2016). Briefly, 0.5 g of

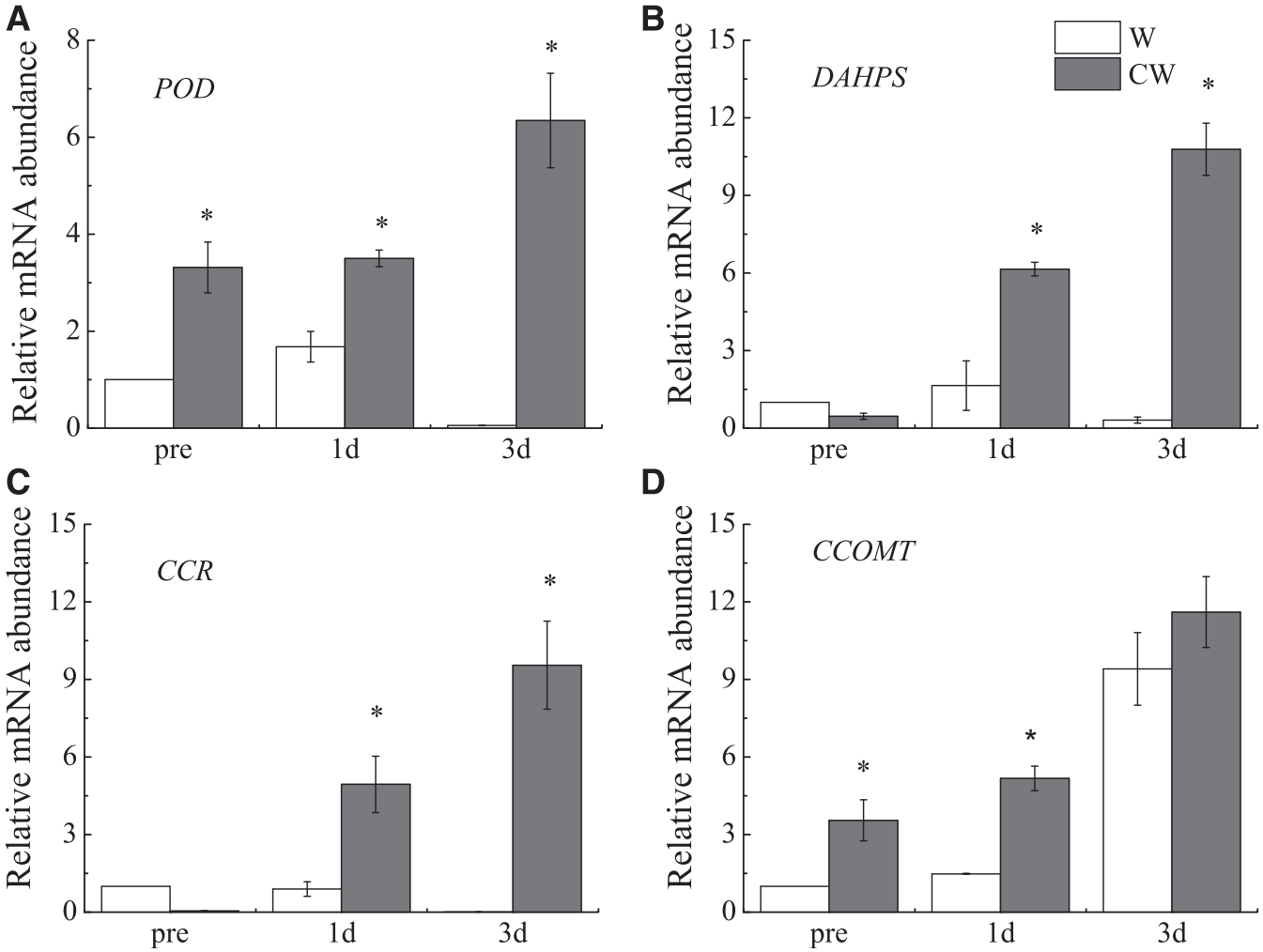

Fig. 3. Relative mRNA expression levels of genes related to lignin synthesis after inoculation with *Fusarium oxysporum* f. sp. *niveum*. **A,** Peroxidase (*POD*). **B,** 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase (*DAHPS*). **C,** Cinnamoyl-coenzyme A reductase (*CCR*). **D,** Caffeoyl-coenzyme A *O*-methyltransferase (*CCOMT*). Values (mean ± SD) with an asterisk above the bars are significantly different within each harvest interval (by the independent sample *t* test at $P < 0.05$). Watermelon roots were harvested before inoculation with *F. oxysporum* f. sp. *niveum* (Pre) and at 1 and 3 days afterward. W = monoculture watermelon soil mixture and CW = wheat/watermelon companion cropping soil mixture.

watermelon root was cut into small pieces and placed into a precooled mortar in ice with approximately 5 ml of sodium acetate buffer (pH 5.0) and ground to obtain a homogenate. Ground samples were transferred to new Eppendorf tubes and centrifuged at 12,000 rpm for 15 min, and the supernatant was maintained at −20°C for further assessment of β-1,3-glucanase activity using the 3,5-dinitrosalicylic acid method. One unit of enzyme activity was defined as 1 g of fresh tissue that catalyzes laminarin to produce 1 μg of glucose per minute at 37°C (Bucheli et al. 1985).

Determination of lignin and total phenols in watermelon roots. Frozen watermelon roots were placed into a precooled mortar and quickly ground into a fine powder with a pestle. Approximately 0.1 g of powder was transferred to an Eppendorf tube with liquid nitrogen, and 1.5 ml of 80% methanol was added. The root samples were then homogenized and extracted overnight in a rotary shaker (150 rpm) at room temperature. The Eppendorf tubes were covered with tinfoil to protect the homogenate solution from light-induced oxidation. The solution was centrifuged at 12,000 × *g* for 5 min, and the supernatant and residues were maintained at −20°C for further determination of total phenols and lignin. Total phenols were assayed as described by Zieslin and Ben-Zaken (1993) and expressed as milligrams per gram of fresh root tissue with catechol as a standard. Lignin was assayed as described previously (Xu et al. 2015b) with lignin alkali as a standard and the concentration of lignin thioglycolic acid derivatives was expressed as milligrams per gram of fresh root tissue.

qRT-PCR. The total RNA from watermelon roots was extracted using the TRIzol method as described by Pattemore (2014) and the total RNA concentration was determined using an Ultra Micro ultraviolet spectrophotometer. Five microliters of total RNA was used for the synthesis of first-strand cDNA using a TIAN Script RT kit (Tiangen, China). The qRT-PCR assays were performed on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The 20-μl reaction volume contained the template (0.6 μl of cDNA), 0.3 μl of each primer, 9 μl of Real Master Mix (SYBR Green) (Tiangen), and 9.8 μl of RNase-free ddH_2O. Sterile water was used as a negative control, and all samples were analyzed in triplicate. The reaction procedure was as follows: 95°C for 5 min, and 40 cycles of 95°C for 50 s, 58°C for 30 s, and extension at 72°C for 40 s. After the last cycle, the samples were subjected to an additional final extension at 72°C for 5 min (Abdin et al. 2011). The expression levels of four previously reported defense-related genes and four lignin synthesis-related genes were analyzed, including the PDF-like genes *ClPDF2.1* and *ClPDF2.4* (reported by Zhang et al. 2015), the *CHI* and *PAL* genes, and the lignin synthesis-related genes (reported by Lü et al. 2011) 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (*DAHPS*), cinnamoyl-coenzyme A reductase (*CCR*), caffeoyl-coenzyme A *O*-methyltransferase (*CCOMT*), and peroxidase (*POD*). The genes and related primers used in the qRT-PCR assay are listed in Table 1. Primers for the *PAL*, *CHI*, *ClPDF2.1*, and *ClPDF2.4* genes were taken from Zhang et al. (2015), and primers for the *18S rRNA*, *DAHPS*, *CCR*, *CCOMT*, and *POD* genes were designed by our laboratory. The 18S rRNA gene was used as an internal gene to calculate the mRNA expression level using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis. Total phenols and lignin, β-1,3-glucanase activity, and gene expression data were analyzed using SPSS 16.0 analysis software (SPSS Inc., Chicago, IL) with one-way analysis of variance and *t* tests. Significance was set at $P = 0.05$. All data are shown as the mean ± standard deviation.

Results

Incidence and severity of watermelon wilt after soil mixing with wheat/watermelon and monoculture watermelon soil (experiment 1). Ten days after inoculation with *F. oxysporum* f. sp. *niveum*, plants grown in soil mixed with wheat/watermelon companion cropping soil (CW) showed significantly lower disease incidence compared with plants in soil mixed with monoculture watermelon soil (W) (Fig. 1A). Plants in soil mixed with the sterile monoculture watermelon soil (W0) exhibited no differences compared with soil mixed with sterile wheat/watermelon companion cropping soil (CW0). The disease severity trend was similar to that for incidence among all treatments, and 10 days after inoculation with *F. oxysporum* f. sp. *niveum*, the severity obtained with the CW treatment was significantly lower compared with the W treatment (Fig. 1B).

Effect of soil biota on lignin biosynthesis-related gene expression, total phenols and lignin, and β-1,3-glucanase activity (experiment 1). *PAL* gene expression levels showed no significant differences among the treatments (Fig. 2A). After inoculation with *F. oxysporum* f. sp. *niveum*, a lower relative expression of the *CHI* gene in the roots was obtained with the wheat/watermelon companion cropping soil mixture treatment (CW) compared with the monoculture watermelon soil mixture treatment (W) (Fig. 2B). Similar trends were obtained for the expression of PDF-like genes *ClPDF2.1* and *ClPDF2.4*. Before inoculation with *F. oxysporum* f. sp. *niveum*, PDF-like genes *ClPDF2.1* and *ClPDF2.4* showed no significant differences in gene expression between the CW and W treatment (Fig. 2C and D). After inoculation with *F. oxysporum* f. sp. *niveum*, higher

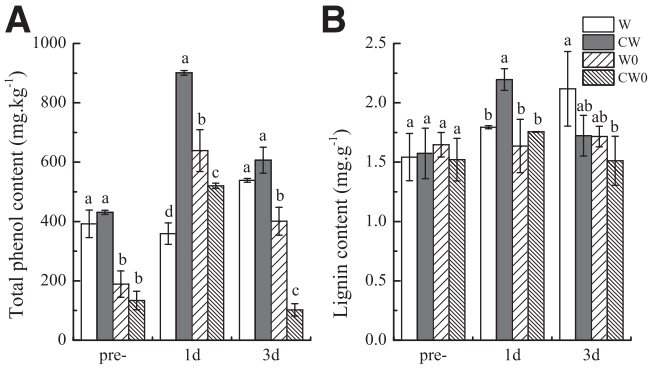

Fig. 4. A, Total phenol content and **B,** lignin content at different days after inoculation with *Fusarium oxysporum* f. sp. *niveum*. Values (mean ± SD) with different letters above the bars are significantly different within each harvest interval at $P < 0.05$ (one-way analysis of variance). Watermelon roots were harvested before inoculation with *F. oxysporum* f. sp. *niveum* (Pre) and at 1 and 3 days afterward. W = monoculture watermelon soil mixture, CW = wheat/watermelon companion cropping soil mixture, W0 = sterile monoculture watermelon soil mixture, and CW0 = sterile wheat/watermelon companion cropping soil mixture.

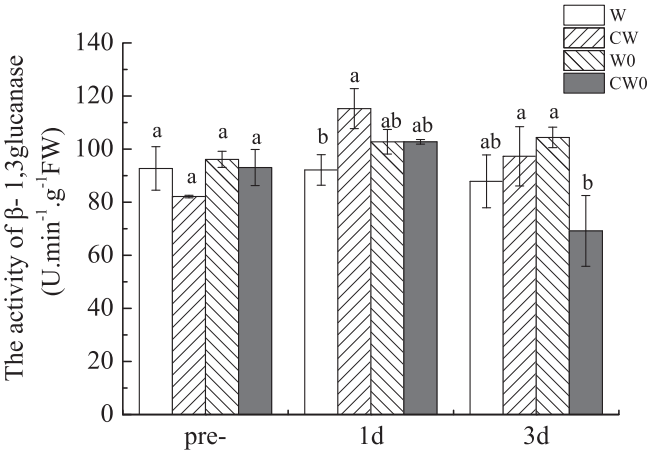

Fig. 5. Activity of β-1,3-glucanase at different days after inoculation with *Fusarium oxysporum* f. sp. *niveum*. Values (mean ± SD) with different letters above the bars are significantly different within each harvest interval at $P < 0.05$ (one-way analysis of variance). Watermelon roots were harvested before inoculation with *F. oxysporum* f. sp. *niveum* (Pre) and at 1 and 3 days afterward. FW = fresh weight, W = monoculture watermelon soil mixture, CW = wheat/watermelon companion cropping soil mixture, W0 = sterile monoculture watermelon soil mixture, and CW0 = sterile wheat/watermelon companion cropping soil mixture.

expression of these genes was obtained with the CW treatment compared with the W treatment (Fig. 2C and D).

We also analyzed expression of lignin synthesis-related genes in watermelon roots. Before inoculation with *F. oxysporum* f. sp. *niveum*, expression of the *DAHPS* and *CCR* genes showed no significant differences among the treatments. After inoculation with *F. oxysporum* f. sp. *niveum*, the expression levels of these genes were significantly upregulated in the CW treatment compared with the W treatment (Fig. 3B and C). Before inoculation with *F. oxysporum* f. sp. *niveum* and at 1 and 3 days afterward, *POD* gene expression was increased in the CW treatment, and the *POD* gene transcript levels obtained with this treatment were 3.3-, 2.2-, and 12.6-fold higher at these time points compared with the control treatment (Fig. 3A). Before and 1 day after inoculation with *F. oxysporum* f. sp. *niveum*, the *CCOMT* gene expression level also increased in the CW treatment. Specifically, this treatment yielded 3.5- and 3.3-fold higher *CCOMT* transcript levels compared with the control treatment before and after pathogen infection, respectively. However, 3 days after inoculation, *CCOMT* expression exhibited no significant differences between the W and CW treatments (Fig. 3D).

Before inoculation with *F. oxysporum* f. sp. *niveum*, total phenols in watermelon roots showed no significant differences between the W and CW treatments. However, total phenols obtained with these treatments were higher compared with the W0 and CW0 treatments. One day after inoculation, the highest total phenols were obtained with the CW treatment, followed by W0 treatment and then by the CW0 and W treatments. Three days after inoculation, there were no significant differences in total phenols between the W and CW treatments, but total phenols in the W0 and CW0 treatments were significantly lower compared with the W and CW treatments (Fig. 4A). The tendency obtained for lignin in watermelon roots was similar to that for total phenols. Before inoculation with *F. oxysporum* f. sp. *niveum*, lignin was not significantly different among the watermelon roots in any of the treatments. One day after inoculation, the lignin obtained with the CW treatment was higher compared with the other treatments. Three days after inoculation, no significant differences were found between the W and CW treatments or between the W0 and CW0 treatments (Fig. 4B).

The activity of β-1,3-glucanase in watermelon roots before inoculation with *F. oxysporum* f. sp. *niveum* exhibited no significant differences between the treatments. However, 1 day after inoculation, the β-1,3-glucanase activity obtained with the CW treatment was significantly higher compared with the W treatment (Fig. 5). In addition, the β-1,3-glucanase activity obtained with the W0 treatment did not differ compared with the CW0 treatment. Three days after inoculation, the β-1,3-glucanase activity showed no significant differences between the CW and W treatment.

Change in total phenols and lignin, β-1,3-glucanase activity, and defense-related gene expression responses to *F. oxysporum* f. sp. *niveum* in wheat/watermelon companion cropping (experiment 2). When watermelon plants were companion cropped with wheat plants for 0, 12, and 24 h, the relative expression level of

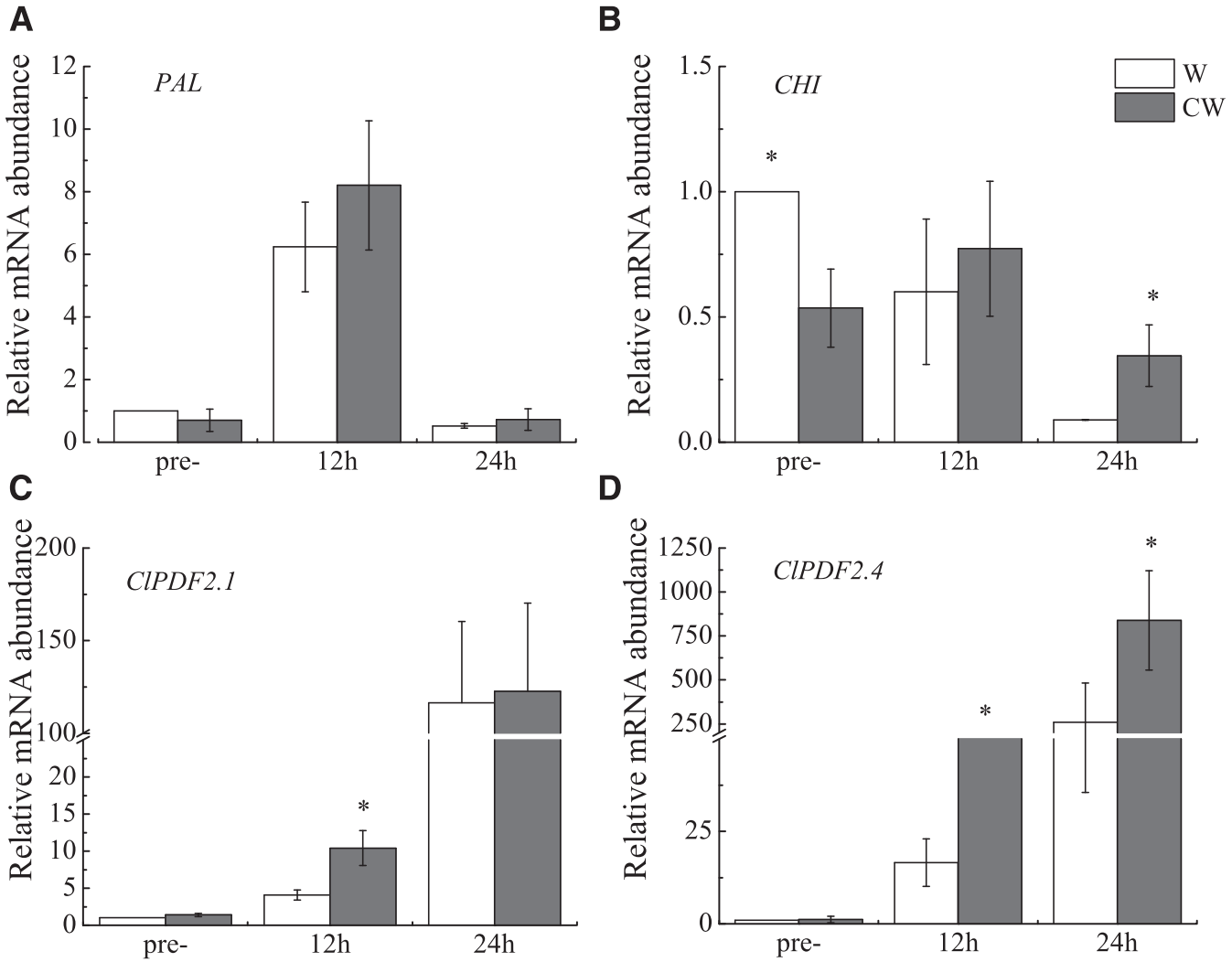

Fig. 6. Relative mRNA expression levels of defense-related genes after companion cropping with wheat. **A,** Phenylalanine ammonia lyase (*PAL*). **B,** Chitinase (*CHI*). **C,** Plant defensin *ClPDF2.1*. **D,** Plant defensin *ClPDF2.4*. Values (mean ± SD) with an asterisk above the bars are significantly different within each harvest interval (by the independent sample *t* test at $P < 0.05$). Watermelon roots were harvested before inoculation with *Fusarium oxysporum* f. sp. *niveum* (Pre) and at 12 and 24 h afterward. W = monoculture watermelon and CW = wheat/watermelon companion cropping.

PAL in watermelon roots obtained with the wheat/watermelon companion cropping treatment (CW) did not differ compared with the monoculture watermelon treatment (W) (Fig. 6A). The relative *CHI* expression level obtained with the CW treatment was lower before inoculation with *F. oxysporum* f. sp. *niveum*. However, 12 h after inoculation, *CHI* expression showed no significant differences between the treatments. *CHI* expression was significantly higher in the CW treatment 24 h after inoculation compared with the W treatment (Fig. 6B).

After inoculation with *F. oxysporum* f. sp. *niveum*, the *ClPDF2.1* and *ClPDF2.4* gene expression level significantly increased in the CW treatment compared with the W treatment (Fig. 6C and D). *ClPDF2.1* expression was significantly increased 12 h after inoculation in the CW treatment. In addition, *ClPDF2.4* expression was significantly upregulated at 12 and 24 h after inoculation.

Before inoculation with *F. oxysporum* f. sp. *niveum*, no significant differences between the W and CW treatments were observed for total phenols and lignin; however, total phenols and lignin were significantly higher in the CW treatment compared with the W treatment at 12 h after inoculation (Fig. 7). Before inoculation with *F. oxysporum* f. sp. *niveum*, the β-1,3-glucanase activity obtained with the CW treatment was significantly higher compared with the W treatment (Fig. 8). Twelve hours after inoculation, decreased β-1,3-glucanase activity was observed with the CW treatment compared with the W treatment, and 24 h after inoculation, no significant differences in β-1,3-glucanase activity were observed between the two treatments.

Effect of wheat root exudates on expression of defense-related genes, total phenols and lignin, and β-1,3-glucanase activity (experiment 3). *PAL* expression was induced at 12 and 24 h after inoculation with *F. oxysporum* f. sp. *niveum* (Fig. 9A), and the transcript levels obtained with treatments with exogenous addition of wheat root exudates (R) and SA were 30- and 12-fold higher compared with the control treatment. Expression of *CHI* was similar to expression of *PAL* (Fig. 9B). Before inoculation with *F. oxysporum* f. sp. *niveum*, *CHI* expression was not significantly different between the SA and R treatments and the control treatment. In contrast, 12 h after inoculation, the *CHI* transcript levels obtained with the R and SA treatments were 18- and 4-fold higher than the control treatment; 24 h after inoculation, *CHI* gene expression was significantly upregulated in the R-treated roots compared with the control-treated roots. Expression of *ClPDF2.1* and *ClPDF2.4* genes showed a similar trend. Before inoculation, the transcript levels of these two genes in the R and SA treatments were not significantly different compared with the control treatment. Twelve hours after inoculation, *ClPDF2.1* and *ClPDF2.4* gene expression levels were significantly increased with the R treatment, and the extent of this increase was even greater than that obtained with the SA treatment. Surprisingly, 24 h after inoculation, the expression levels of these two genes were significantly lower with the R treatment compared with the SA treatment (Fig. 9C and D).

Before inoculation with *F. oxysporum* f. sp. *niveum*, total phenols and lignin were significantly higher in both the R and SA treatments than in the controls (Fig. 10). However, 12 h after inoculation, total phenols and lignin obtained with both the R and SA treatments did not differ compared with the control treatment (Fig. 10). Twenty-four hours after inoculation, total phenol content was significantly higher in both the R and SA treatments. Likewise, significantly increased lignin content was obtained with the R treatment, whereas the SA treatment did not exert similar effects (Fig. 10).

The R and SA treatments increased β-1,3-glucanase activity in watermelon roots prior to inoculation with *F. oxysporum* f. sp. *niveum* (Fig. 11). Twelve hours after inoculation, the β-1,3-glucanase activity was not significantly different between these treatments. However, 24 h after inoculation, the β-1,3-glucanase activity in the R-treated watermelon roots was significantly increased compared with the control roots (Fig. 11).

Discussion

Several studies have shown that intercropping is an effective practice for controlling soilborne plant diseases, and recent studies have shown that interactions between plant species in intercropping (and companion cropping) systems can induce disease resistance (Gao et al. 2014; Hao et al. 2010; Ren et al. 2008). Our previous study also showed that a watermelon/wheat companion cropping system exhibits a decreased incidence of watermelon Fusarium wilt and increased disease resistance (Xu et al. 2015b).

Many beneficial microorganisms in soil can induce systemic resistance in plants (Van Wees et al. 2008; Verhagen et al. 2010). For example, root exudates of wheat, which contain benzoxazinoids, attract *Pseudomonas* spp. to the rhizosphere and improve plant development and health (Berendsen et al. 2012). Our previous study showed that the soil microbe community was significantly altered after companion cropping with wheat, and the observed alterations include increased relative abundances of Actinobacteria and Gemmatimonadetes and decreased *F. oxysporum* f. sp. *niveum* populations in the watermelon rhizosphere (Xu et al. 2015c). Therefore, we predicted that the soil microorganisms in a watermelon/wheat

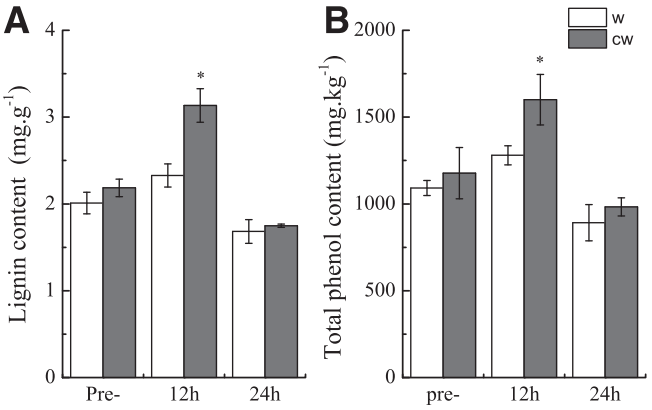

Fig. 7. A, Lignin content and **B,** total phenol content in companion cropping with wheat at different times after inoculation with *Fusarium oxysporum* f. sp. *niveum*. Values (mean ± SD) with an asterisk above the bars are significantly different within each harvest interval (by the independent sample *t* test at $P < 0.05$). Watermelon roots were harvested before inoculation with *F. oxysporum* f. sp. *niveum* (Pre) and at 12 and 24 h afterward. W = monoculture watermelon and CW = wheat/watermelon companion cropping.

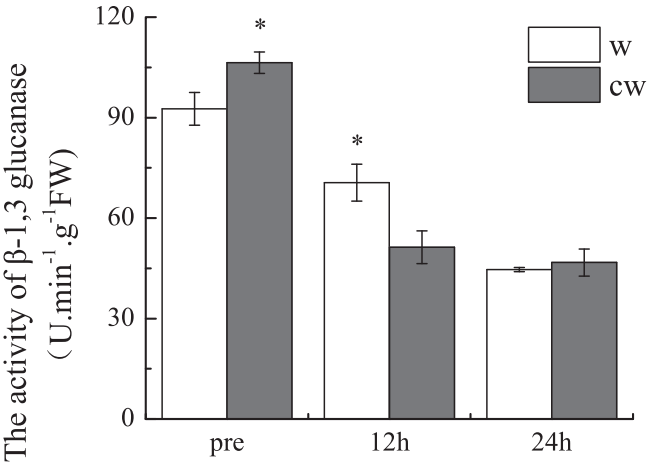

Fig. 8. Activity of β-1,3-glucanase in companion cropping with wheat at different times after inoculation with *Fusarium oxysporum* f. sp. *niveum*. Values (mean ± SD) with an asterisk above the bars are significantly different within each harvest interval (by the independent sample *t* test at $P < 0.05$). Watermelon roots were harvested before inoculation with *F. oxysporum* f. sp. *niveum* (Pre) and at 12 and 24 h afterward. FW = fresh weight, W = monoculture watermelon, and CW = wheat/watermelon companion cropping.

companion cropping system can induce resistance of watermelon to Fusarium wilt and tested this hypothesis through a plant-soil feedback trial. The disease severity and incidence of watermelon wilt obtained with the CW treatment was reduced compared with the W treatment (Fig. 1). However, no significant differences were found between the CW0 treatment and W0 treatment (Fig. 1). Together, these results indicate that the microorganisms in the watermelon/wheat companion cropping system play a role in decreasing disease severity and incidence. However, disease severity and incidence of watermelon wilt obtained with the W treatment were increased compared with the W0 treatment, potentially because the environment formed by the microbes in the W treatment was more suitable for *F. oxysporum* f. sp. *niveum* colonization and infection. Further study showed that the expression levels of genes related to lignin and phytoalexin synthesis were increased with the CW treatment (Figs. 2 and 3). These data were consistent with those reported by Lü et al. (2011), who showed that resistance of watermelon was correlated with the upregulation of genes related to the lignin synthetic pathway. Furthermore, the results obtained in the present study showed that total phenols and lignin were significantly increased (Fig. 4), which can be positively correlated with plant disease resistance in previous studies (El Modafar and El Boustani 2001; Lü et al. 2011), suggesting that soil microbes contribute to induced resistance in watermelon plants. Further study is needed to identify the specific microorganisms responsible for this effect and design strategies for manipulating these microorganisms to further increase plant disease resistance.

Several studies have revealed that exogenous addition of root exudates can induce disease resistance in plants (Ding et al. 2015; Ren et al. 2016), but whether wheat root exudates can induce resistance in watermelon has not been tested. Therefore, tissue culture experiments involving the wheat/watermelon companion system and addition of exogenous wheat root exudates were conducted. In tissue culture, the expression of defense-related genes (Fig. 6), total phenol and lignin content (Fig. 7), and β-1,3-glucanase activity increased significantly after treatment (Fig. 8). Similarly, the wheat root exudate experiment revealed that treatment with root exudates from wheat seedlings upregulated defense-related gene expression and significantly increased total phenols, lignin, and β-1,3-glucanase activity (Figs. 9, 10, and 11). These results suggest that root exudates from wheat seedlings were responsible for increased disease resistance in watermelon plants. The same phenomenon was reported in a maize/pepper intercropping system, where the levels of defense-related gene expression levels in both the roots and shoots of maize were increased by root exudates from neighboring pepper plants, which eventually led to an increase in maize disease resistance to *B. maydis* (Ding et al. 2015). In addition, a previous study showed that addition of exogenous coumaric acid from rice root exudates upregulated the expression of *CIPR3* and increased β-1,3-glucanase activity in watermelon roots, enhancing watermelon resistance to *F. oxysporum* f. sp. *niveum* in the watermelon/rice intercropping system (Ren et al. 2016). Root exudates of wheat contain phenolic acids, such as coumaric and ferulic acids (Wu et al. 2001); however, the

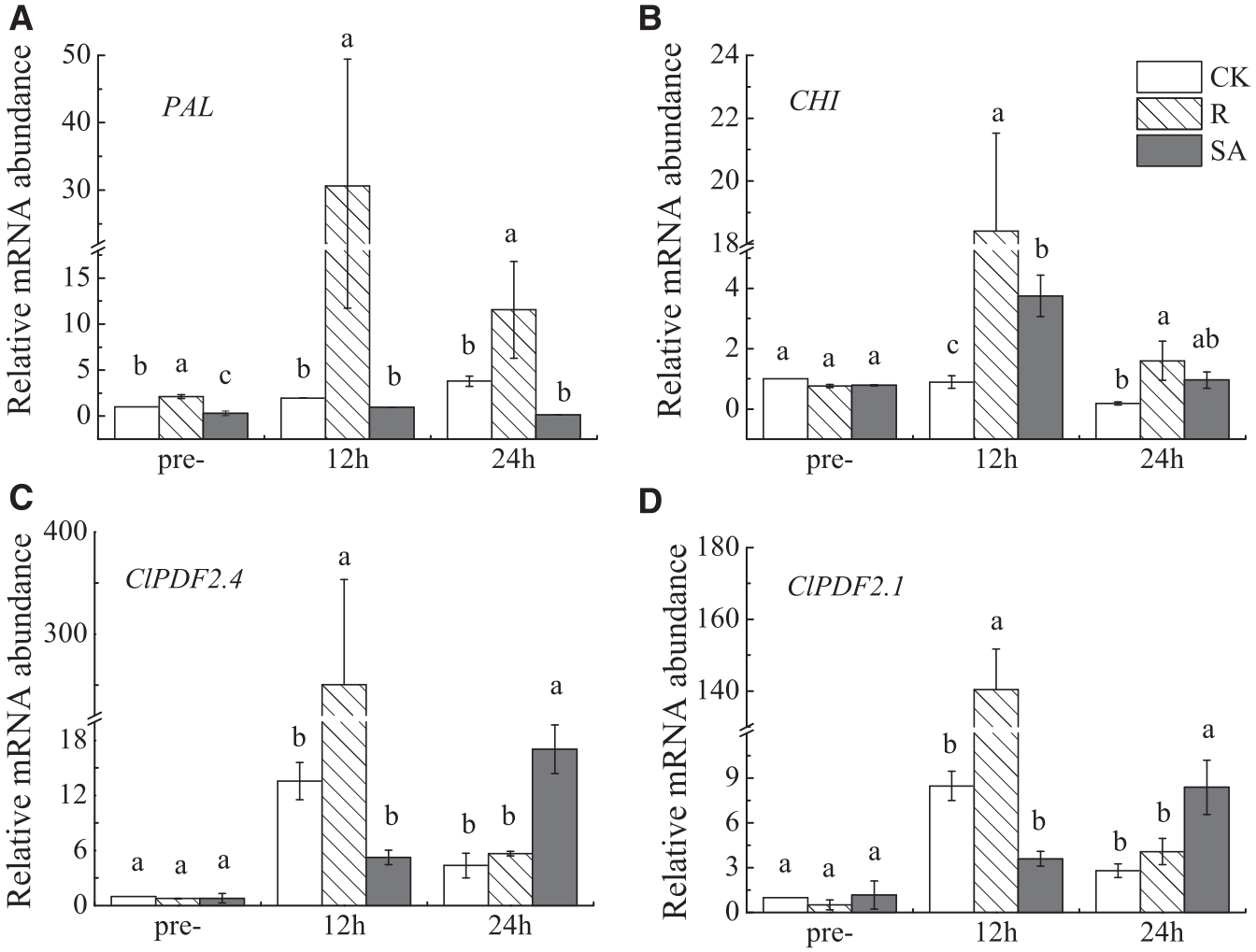

Fig. 9. Relative mRNA expression levels of defense-related genes after the exogenous addition of root exudates. **A,** Phenylalanine ammonia lyase (*PAL*). **B,** Chitinase (*CHI*). **C,** Plant defensin *ClPDF2.1*. **D,** Plant defensin ClPDF2.4. Values (mean ± SD) with different letters above the bars are significantly different within each harvest interval (by the independent sample *t* test at $P < 0.05$). Watermelon roots were harvested before inoculation with *Fusarium oxysporum* f. sp. *niveum* (Pre) and at 12 and 24 h afterward. CK = addition of sterile deionized water, R = addition of wheat root exudates, and SA = addition of salicylic acid.

importance of these compounds in disease resistance has yet to be determined.

Plant β-1,3-glucanase degrades fungal cell walls (Cachinero et al. 1996). In this study, β-1,3-glucanase activity was increased before and 24 h after inoculation with *F. oxysporum* f. sp. *niveum* in wheat/watermelon companion cropping and after the addition of wheat root exudates (Figs. 8 and 11), consistent with the results of previous studies (Xu et al. 2015a). An increase in β-1,3-glucanase activity in watermelon roots was shown previously to increase degradation of *F. oxysporum* f. sp. *niveum* cell walls and effectively prevent pathogen infection (Wróbel-Kwiatkowska et al. 2004). Thus, it is possible that wheat root exudates play an important role in inhibiting *F. oxysporum* f. sp. *niveum* growth and enhancing watermelon resistance to *F. oxysporum* f. sp. *niveum*. However, in the plant-soil feedback experiment, β-1,3-glucanase activity was increased 1 day after inoculation, whereas increased activity was not observed prior to inoculation with *F. oxysporum* f. sp. *niveum* (Fig. 5). Both wheat companion and wheat cover crop cultivation treatments were shown previously to decrease the relative abundance of soilborne pathogens such as *Fusarium* spp. and increase the relative abundance of plant growth-promoting rhizobacteria such as Gemmatimonadetes (Gao et al. 2017; Xu et al. 2015c).

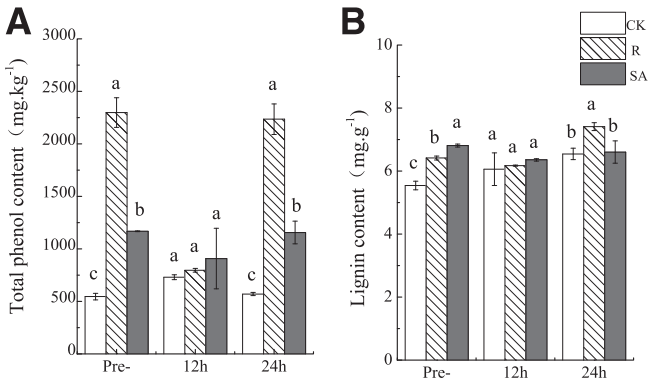

Fig. 10. A, Total phenol content and **B,** lignin content at different times after inoculation with *Fusarium oxysporum* f. sp. *niveum*. Values (mean ± SD) with different letters above the bars are significantly different within each harvest interval at $P < 0.05$ (one-way analysis of variance). Watermelon roots were harvested before inoculation with *F. oxysporum* f. sp. *niveum* (Pre) and at 12 and 24 h afterward. CK = addition of sterile deionized water, R = addition of wheat root exudates, and SA = addition of salicylic acid.

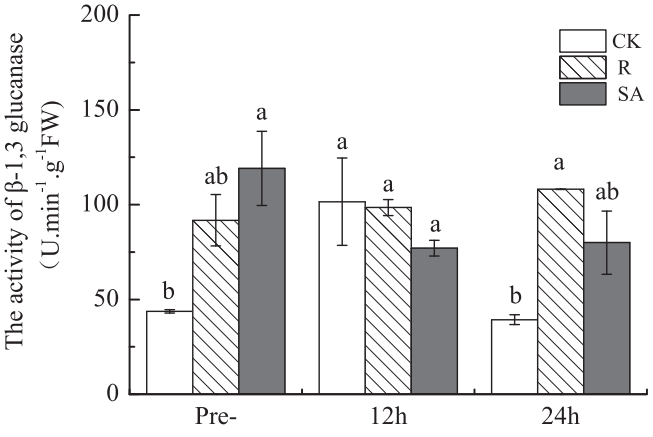

Fig. 11. Activity of β-1,3-glucanase at different times after inoculation with *Fusarium oxysporum* f. sp. *niveum*. Values (mean ± SD) with different letters above the bars are significantly different within each harvest interval at $P < 0.05$ (one-way analysis of variance). Watermelon roots were harvested before inoculation with *F. oxysporum* f. sp. *niveum* (Pre) and at 12 and 24 h afterward. FW = fresh weight, CK = addition of sterile deionized water, R = addition of wheat root exudates, and SA = addition of salicylic acid.

Other researchers have shown that the defense related genes such as *PDF1.2a*, *PAL*, and *CHI* are upregulated after pathogen infection and/or associated with host plants (Zhang et al. 2015; Zvirin et al. 2010). In this study, the expression levels of *ClPDF* genes were significantly upregulated in both the plant-soil feedback and tissue culture experiments (Figs. 2, 6, and 9), indicating that the soil biota and root exudates induced resistance. This effect might be related to the *ClPDF* metabolism and biosynthesis pathway. The exogenous addition of wheat root exudates upregulated *CHI* and *PAL* gene expression (Fig. 9A and B), whereas these genes were not upregulated in the wheat companion cropping experiment (Fig. 6A and B). This result may be related to unequal quantities of root secretions in the medium and root exudates collected in natural environments (Vranova et al. 2013). Moreover, the effect of soil microorganisms on root exudates in natural environments cannot be ignored (Hage-Ahmed et al. 2013) and should be investigated in future studies. Additionally, the concentration of root exudates added to the culture medium might not have been equal to that in the soil; thus, whether the concentration of root exudates affects disease resistance must also be further investigated. In conclusion, our results provide empirical evidence that both wheat root exudates and soil microorganisms are responsible for inducing disease resistance against *F. oxysporum* f. sp. *niveum* as observed through soil biota-mediated plant-soil feedback and tissue culture experiments. However, which components play more important roles contributing to the induced resistance needs further investigation.

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