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Effects of Exogenously applied palmitic acid on systemic resistance against *Fusarium oxysporum* f.sp.*niveum* in watermelon (*Citrullus lanatus* (Thunb.) Matsum. et Nakai)

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ABSTRACT

We studied the effects of exogenously applied palmitic acid (PA) on reactive oxygen species (ROS) production, defense-related enzyme activity and malondialdehyde (MDA) content of roots and analyzed the response of defense-related genes in *Fusarium oxysporum* f.sp.*niveum* (FON) inoculated watermelon (*Citrullus lanatus* (Thunb.) Matsum. et Nakai). Our results showed that as compared to control, PA reduced the morbidity of *Fusarium* wilt by 33.3 % and disease severity by 53.2 %, increased the level of ROS of watermelon roots at early infection stage and the activity of defense related enzymes, and decreased the MDA content of watermelon roots. Moreover, PA up-regulated the expression of disease defense-related genes. These results suggested that PA promoted the accumulation of ROS and up-regulated the transcript level of defense-related genes and eventually induced the systemic resistance in watermelon against FON.

Keywords: Antioxidant enzymes, *Citrullus lanatus* (Thunb.) Matsum. et Nakai, defense-related genes, fatty acids, *Fusarium oxysporum* f. sp. *Niveum*, reactive oxygen species, watermelon.

INTRODUCTION

Watermelon (*Citrullus lanatus* (Thunb.) Matsum. et Nakai) is one of the most popular fruits around the world, however, its continuous monoculture has led to huge losses in its production due to increase in soil-borne diseases such as *Fusarium* wilt, which seriously hindered the development of watermelon industry. *Fusarium oxysporum* f.sp. *niveum* (FON), a semi-biotrophic pathogen causing *Fusarium* wilt, is difficult to control due to its several physiological races and the strong bioenergy of spores (9,40).

Plants synthesize variety of fatty acids (FAs) and most of them induce systemic resistance in plants against several pathogens. However, the efficiency of FAs and mechanism of induced systemic resistance is still unknown. Fatty acids (FAs) synthesized by plants, are involved in many biochemical processes [cell membrane construction, and energy and carbon source storage (35,37)]. FAs and their derivatives also act as important intracellular signaling molecules in plants, against biotic and abiotic stresses. They act synergistically or antagonistically with variety of plant hormones and regulate plant defence responses (36,40). In addition, some specific FAs and their derivatives transmit the signals between plants and microorganisms, which induces gene expression in plants and alter their metabolic processes (28). Sumayo *et al*. (31) showed that the *Ochrobactrum lupine* KUDC1013, a plant-promoting bacterium, can produce linoleic acid that induce the systemic resistance in tobacco. Palmitic acid (PA) is an important member of FAs family, which works synergistically with some signaling molecules such as azelaic acid and

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*Correspondence author, [1]Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (Northeast Region), Ministry of Agriculture and Rural Affairs, Northeast Agricultural University, Changjiang 600, Xiangfang, Harbin 150030, China jasmonic acid, and plays an important role to regulate the system resistance (28). The accumulation of PA and sphingolipids of dihydroxy long chain base in plant intracellular can stimulate programmed cell death and induce systemic resistance (3,15). Besides, PA derivative 16-hydroxypalmitate can increase the level of H_2O_2 and disease-related protein expression in *Arabidopsis* (24). These studies indicate the PA could be an efficient component in induced systemic resistance in watermelon against lethal *Fusarium* wilt.

Our previous study found that PA from wheat root exudates promotes the growth of watermelon and alleviate the *Fusarium* wilt (4,22). However, the mechanism is not clear. We hypothesize that PA can directly or indirectly induce systemic resistance by promoting accumulation of ROS and up-regulating the disease defense-related genes against FON in watermelon. Hence, we studied the effects of exogenously applied PA on specific reactive oxygen species (ROS) molecules, defense enzyme activity and malondialdehyde (MDA) content. To better understand the watermelon systemic defense against FON, we analyzed the expression of signal regulatory genes: *WRKY1* and *NPR1* and defense genes *PR1* and *PR2* using qRT-PCR. This study aimed to investigate in-depth the mechanism of PA inducing systemic resistance against FON in watermelon.

MATERIALS AND METHODS

Fusarium oxysporum f. sp. *Niveum* was isolated from the infected roots of watermelon from the Horticulture Experimental Centre, College of Horticulture, Northeast Agricultural University, Harbin, China (45°74' N, 126°73' E), and identified as the physiological race no.1. The strains were preserved for long-term in glycerol with mass fraction of 20% at -80 °C. FON was cultured in liquid for 7 days at 28 °C and 150 rpm, and then the mycelium was filtered. The filtrate was centrifuged at 1400×g for 10 min and the spores were resuspended with sterile distilled water for inoculation (19).

Soil collection

Soil used was sandy loam collected from upper layer (0-15 cm) of a field of unplanted watermelons at the Horticulture Experimental Center in College of Horticulture, Northeast Agricultural University, Harbin, China. Soil was sieved (<5 mm) to remove plant residues and bulk stones and then transported to the greenhouse. The physicochemical properties of the soil, [determined as per Zhou *et al.* (43)], were: organic matter, 48.25 $g \cdot kg^{-1}$; inorganic N, 202.92 $g \cdot kg^{-1}$; available P, 130.80 $mg \cdot kg^{-1}$; available K, 154.67 $mg \cdot kg^{-1}$; EC, 0.50 $mS\ cm^{-1}$(1:2.5, w/v); pH,7.07(1:5, w/v).

PA solution preparation

PA were added to sterilized distilled water and pH was adjusted to 7 using 0.1 M of sodium hydroxide solution, because pH can affect soil microbial communities (7), and sodium hydroxide can also help PA to get dissolved. The solution was added to 0.1 % (v/v) Tween 20 at 70 °C for 30 min for better dissolution.

Pot experiment

The pot experiment was conducted from August to October 2019 in controlled climate chamber, Horticultural Experimental Centre, Northeast Agricultural University, Harbin, China. Seeds of watermelon (*Citrullus lanatus* (Thunb.) Matsum. et Nakai) cultivar ‘Zaojiao8424’ were obtained from seeds company Zhongke Maohua, Heibei, China. Watermelon seeds were soaked for 20 min in 2.5 % (v/v) sodium hypochlorite solution for disinfection, washed and again soaked in sterilized distilled water at room temperature for 12 h, then cultured in incubator at 30 °C for 24 h. Seeds were germinated in incubator and 2-leaved seedlings were transplanted in the pots (9 x 9 x 12 cms) containing 250 g soil each. Each pot contained one seedling. Sterilized soil was applied as control.

The soil was sterilized thrice at 121 °C for 30 min at 24 h intervals to remove microorganisms (10). There were two treatments i.e. PA applied at 0 mM and 1.0 mM per kg soil. To dissolve PA, 0.1% (v/v) of Tween 20 was used. The watermelon seedlings were grown in climate chamber (28 °C day/18 °C night, 16 h light/8 h dark, and relative humidity 60-80 %). Soil moisture content was maintained with sterilized distilled water (about 60 % of its water holding capacity). After 7 days of treatment, the roots of watermelon were inoculated with 10 ml FON suspension of 1×10^7 conidia ml^{-1} (39).

The roots were randomly harvested at 0, 1, 3 and 5 days after treatment application and 1 day after inoculating with FON. Roots were immediately washed with sterilized distilled water, frozen in liquid nitrogen and stored at -80 °C until qRT-PCR analyses was performed. The roots were randomly harvested at 0, 1, 3, 5 and 7 days after FON inoculation. These were stored at -80 °C to determine the changes in ROS, defense enzyme activity and MDA content. Twenty days after inoculation with FON, other 120 plants (2 treatments×20 pots×3 replicates) were used to assess the morbidity and disease severity as per Chu *et al.* (4).

Determination of ROS

H_2O_2: The root tissues (0.5 g) were grounded with pre-cooled acetone (1.5 ml) in liquid nitrogen to obtain homogenous extract, it was transferred to Eppendorf tube and centrifuged at 5000 rpm at 4°C for 5 min. The supernatant was used to determine the H_2O_2 as per Ferguson *et al.* (6).

O_2^-: Watermelon root tissues (0.5 g) were grounded with liquid nitrogen and 10 ml of precooled phosphate buffer [50 mM, pH 7.8, containing 1mM ethane diaminetetraacetic acid (EDTA) and 2 % (w/v) polyvinyl polypyrrolidone (PVPP)]. It was used to obtain the homogenous extract, which was transferred to Eppendorf tube and centrifuged at 12000 rpm at 4 °C for 20 min. The supernatant was used to determine the O^{2-} formation rate as per the standard curve of $NaNO_2$ reagent as per Li *et al.* (11).

Determination of defense enzyme activity

The method used to determine the $O_2$was to extract the crude enzyme solution from watermelon roots. The extracted solution was used to determine the activity of superoxide dismutase (SOD), peroxidase (POD), polyphenol oxidase (PPO), L-phenylalanine ammonia-lyase (PAL) and the content of soluble protein.

SOD: SOD activity was determined using the slightly modified nitrogen blue tetrazole (NBT) photo-reduction method as per El-Amery*et al.* (5). The absorption was measured at 560 nm, and SOD activity unit was expressed as inhibiting the photoreduction of NBT by 50 %.

POD: POD activity was determined using this enzyme to catalyze the oxidation of guaiacol by hydrogen peroxide as per Xu *et al.* (38). The produced quinones absorbance was measured at 470 nm and POD activity unit was expressed with increase absorption by 0.01 per min.

PPO: PPO activity was determined using the slightly modified enzyme oxidation method of catechol as per Xu *et al.* (41). The resulting quinones were determined by colorimetry at 410 nm, and PPO active units were defined to increase absorption by 0.01 per min.

PAL: PAL activity was determined using this enzyme to convert L- phenylalanine to trans cinnamic acid as per Lu *et al.* (14). Sodium borate buffer solution (20 mM, pH 8.8) was used to measure the absorbance at 290 nm. PAL activity unit was defined to increase the absorbance by 0.01 per h.

Soluble protein: The content of soluble protein was determined using method of Coomassie brilliant blue G-250 as per Li *et al.* (12). The absorbance of resulting protein-pigmented conjugates was measured at 595 nm, and the content of soluble protein was calculation using a standard curve.

MDA measurement

MDA content was assessed using thiobarbituric acid colorimetric method as per Ohkawa *et al.* (20). In brief, root tissues (0.2 g) were grounded with 10 % (w/v) trichloroacetic acid (1.6 ml) in a precooled mortar to obtain a homogenous extract, which was transferred to Eppendorf tube and centrifuged at 12000 rpm for 10 min. The supernatant was transferred to another new centrifuge tube and 0.67 % (w/v) thiobarbital of equal volume was added. After 30 min of boiling on water bath, the absorbance was measured at 450 nm, 532nm and 600nm.

Quantitative real-time (qRT- PCR) analysis

The total RNA from watermelon roots was extracted with TRIzol method as per Pattemore (25). An Ultra Micro Ultraviolet Spectrophotometer was used to determine the total RNA concentration. A total of 6μl of RNA was used for the synthesis of first-strand cDNA using a TIAN Script RT kit (Tiangen, China) following the manufacturer's instructions. The qRT-PCR assay was performed on an iQ5 Real-Time PCR Detecting System (Bio-Rad, USA). The 20μl reaction volume contained: diluted cDNA template (50-100 ng ul^{-1}), 1μl; each primer, 0.5 ul; 2×Real Master Mix (SYBR Green) (TaKaRa, China), 10ul; RNase-free dd H_2O, 8μl. Sterile water was used as negative control and all samples contained three technical replicates. The reaction procedure was as follows: 95°C for 2 min, 35 cycles of 50 s at 95°C, 30 s at 58 °C and extension 40 s at 72°C. The expression levels of four key genes (*WRKY1*, *NPR1*, *PR1* and *PR2*) of salicylic acid (SA) signaling pathways were analyzed in qRT-PCR using specific primer sets (Table 1) (15). The 18S rRNA gene was used as an internal gene to calculate the mRNA expression level using the $2^{-\Delta\Delta Ct}$ method (10,13).

Table 1. The primers of the five genesinq RT-PCR

Genes name	Primers name	5'-3'	Reference
18srRNA	*18srRNA*-F	AGCAAGCCTACGCTCTGT	Li *et al.* (9)
	18srRNA-R	CTGGTCGGCATCGTTTAT	
WRKY1	*WRKY1*-F	CCAGAGGCTAAGAGATGGCG	Lv *et al.* (15)
	WRKY1-R	GTGGTTTGGACGACGACTCT	
NPR1	*NPR1*-F	TGCAATGCGTAAGGACCCAT	
	NPR1-R	AGTCCGACCATCCAGAGTCA	
PR1	*PR1*-F	ATGCCAACCAACGCATCAAC	
	PR1-R	TGGCCAACCCCATGCAATAT	
PR2	*PR2*-F	CCGTCAACTGTGGTCACTGA	
	PR2-R	CTCCGCCAACCTTCTCCAAT	

Statistical analysis

Statistical analysis was done using SPSS software (IBM SPSS 19, NY, USA). ANOVA and Duncan's test ($P<0.05$) were used to analyze the differences among treatment means. All values were expressed as mean ± standard error.

RESULTS AND DISCUSSION

Morbidity and disease severity of *Fusarium* wilt

Our results showed that the watermelon in CK treatment had severe leaf yellowing wilt and poor growth than in PA treatment (Fig. 1). The PA treatment significantly decreased the morbidity and the disease the severity of *Fusarium* wilt in watermelon by 33.3 % and 53.2 %, respectively (Fig. 2) (*P*< 0.05). This suggested that PA can alleviate the *Fusarium* wilt, which agrees with findings of Chu *et al.* (4) who suggested that PA decreased the morbidity and disease severity of *Fusarium* wilt of watermelon in continuous monoculture.

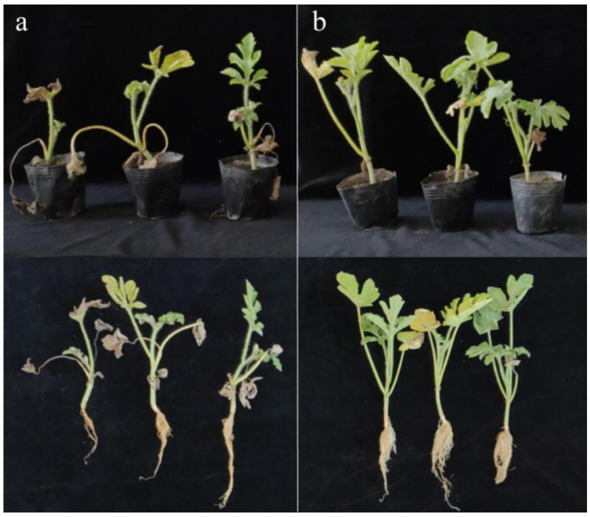

Figure 1. Potted watermelons plants 20 days after inoculation with FON in the greenhouse. Plants in ‘a’ untreated with PA were in the middle period of wilt disease. Plants in ‘b’ treated with 1.0 mM PA developed in good health.

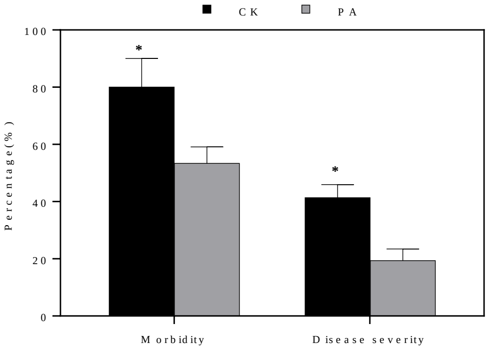

Figure 2. Effects of PA treatment on morbidity and disease severity of watermelon roots after inoculation with FON. One asterisk values (means ± SE, n = 3) with significant differences within the same group (*P*<0.05, Duncan’s multiple range test).

Oxidative stress and enzyme activity of watermelon roots

The ROS of O_2^- and H_2O_2can trigger multiple biotic or abiotic activators, that directly killed fungi and also act as signaling molecules to induce the systemic resistance, and plays important roles in early stages of plant defense responses (1,33,36). In our results, the levels of O_2^- and H_2O_2were immediately increased after inoculation with FON and then fluctuated (Fig. 3). These results indicated that the levels of O_2^- and H_2O_2were closely related to pathogen invasion and watermelon has undergone through complex physiological changes against pathogens. Moreover, compared to CK treatment, the levels of O_2^- and H_2O_2were significantly enhanced by exogenously applied PA at 0,1,3 days after inoculation with FON (Fig. 3). This suggested that exogenously applied PA increased the levels of O_2^- and H_2O_2, which may be the reason that PA enhanced the systemic resistance of watermelon against FON (P<0.05). Notably, some studies have confirmed that exogenously applied16-hydroxy PA can increase the level of H_2O_2 in Arabidopsis leaves and induces the systemic resistance in plants, which may be related to the enhanced level of ROS in watermelon with exogenously applied PA that strengthens the plant defence (24).

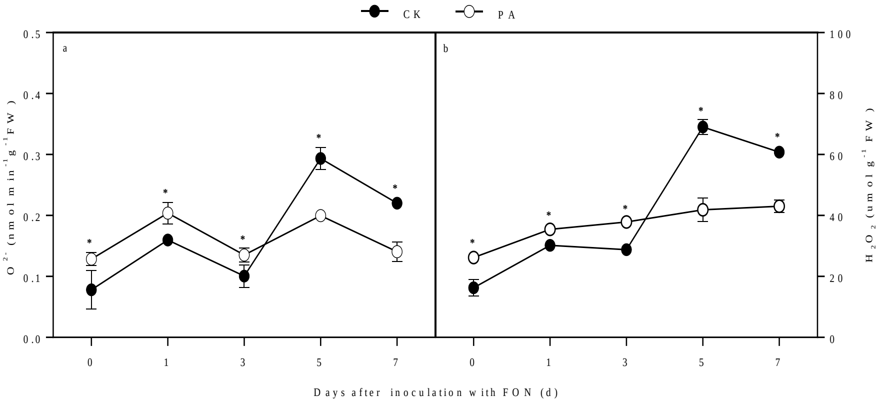

Figure 3. Effects of PA treatment on the levels of O_2^- (a) and H_2O_2 (b) of watermelon roots after inoculation with FON. One asterisk values (means ± SE, n = 3) with significant differences within the same group (P<0.05, Duncan's multiple range test).

Excessive levels of O_2^- and H_2O_2 can lead to lipid peroxidation, destruction of cell membrane integrity and toxicity to cells (34). Therefore, optimizing the levels of O_2^- and H_2O_2 is important to decrease the oxidative stress in plants. In our results, the activity of SOD was significantly increased at 0, 3, 5 and 7 days after inoculation with FON, and the activities of POD, PPO and PAL were significantly elevated at 0,1,5 and 7 days after inoculation than CK treatment (Fig. 4) (P< 0.05). At 5 days after inoculation, the activity of SOD, POD, PPO and PAL were increased1.2, 1.7, 1.5 and 1.7 times, respectively, in PA than CK treatment (Fig. 3). We also found that the levels of O_2^- and H_2O_2were significantly lower and were slightly increased in PA treatment than in CK treatment (Fig. 3) (P<0.05). Likewise, similar phenomenon was observed by Sun *et al.* (34). This might be because the antioxidant enzymes, such as SOD and POD, plays critical role in alleviating the oxidative stress. In an outbreak, the O_2^-in plant can be converted into $H_2O_2$by SOD, and H_2O_2 can be decomposed by POD through the co-substrate pathway (33,36). POD, PAL and PPO are the key enzymes for the synthesis of substances such as lignin and phenols, which are associated with plant resistance (32). Previous studies have shown that FAs can influence the phenolic metabolism and activate the plant defence enzymes (17,21). Therefore, ROS signaling mediated by exogenously applied PA may play an important role in watermelon resistance to FON.

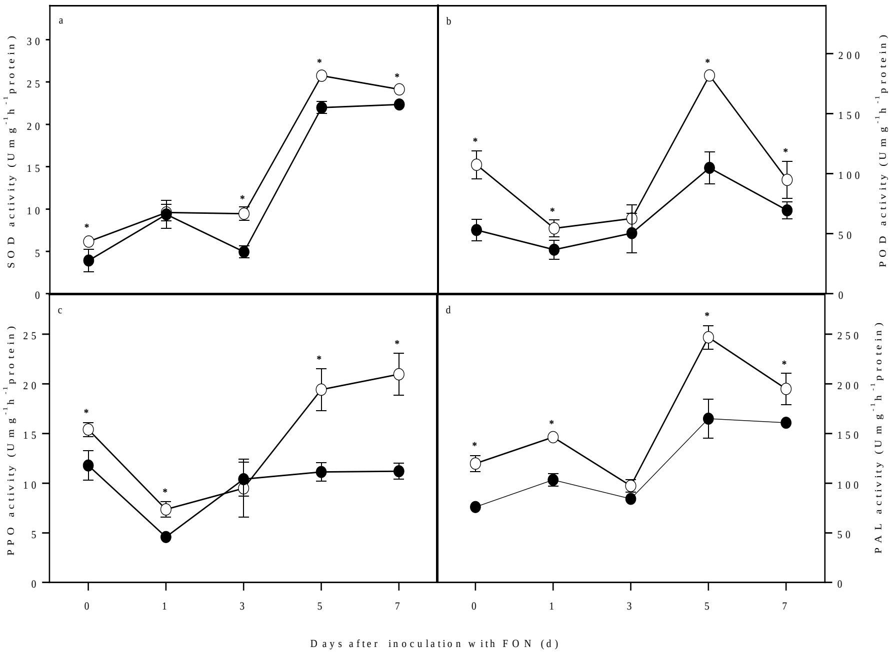

Figure 4. Effects of PA treatment on the activities of SOD (a), POD (b), PPO (c) and PAL (d) of watermelon roots after inoculation with FON. One asterisk values (means ± SE, n = 3) with significant differences within the same group (*P*<0.05, Duncan's multiple range test).

Lipid peroxidation

MDA is the decomposition product of polyunsaturated FAs and its level indicates the lipid peroxidation and the excessive MDA can damage the membrane and cause tissue necrosis (29). In our study, the content of MDA of watermelon roots accumulated rapidly after inoculation with FON and the MDA content in PA treatment was significantly reduced at 3, 5 and 7 days after inoculation (Fig. 5) (*P*< 0.05). This suggests that PA treatment can increase the activity of antioxidant enzymes of watermelon to prevent the cell membranes from being damaged by oxidation and maintain the normal operation of cells.

Gene expression

Plants have developed SA-mediated disease-resistant pathways to combat semi-biotrophic pathogens (2). The up-regulation of PRgenes such as *PR1* and *PR2*are associated with the resistance for *etr1-1* mutant plants of *Arabidopsis* against *Fusarium oxysporum* f. sp. *Raphani* (23). *NPR1* gene is the most important gene in SA signal transduction (18). The over-expression of *NPR1* enhanced the cotton resistance to *Fusarium oxysporum* f. sp.*vasinfectum* (8). The binding of *WRKY* genes to W boxes located in *NPR1*promoter region can promote *R* gene expression (42). Over-expressing *WRKY1* enhanced the tomato resistance to *Alternaria solani* (27). Therefore, we further analyzed the response of SA signal regulation and defense related genes to PA and FON.

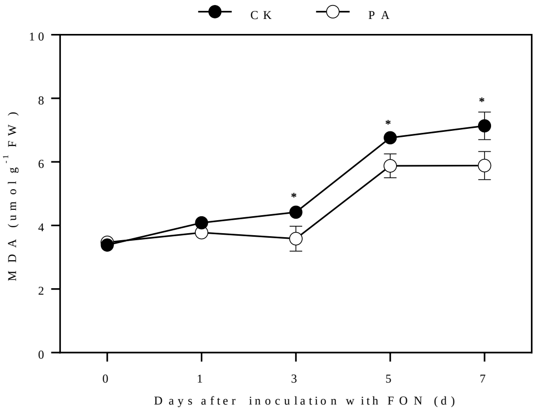

Figure 5. Effects of PA treatment on MDA content of watermelon roots after inoculation with FON. One asterisk values (means ± SE, n = 3) with significant differences within the same group (P<0.05, Duncan's multiple range test).

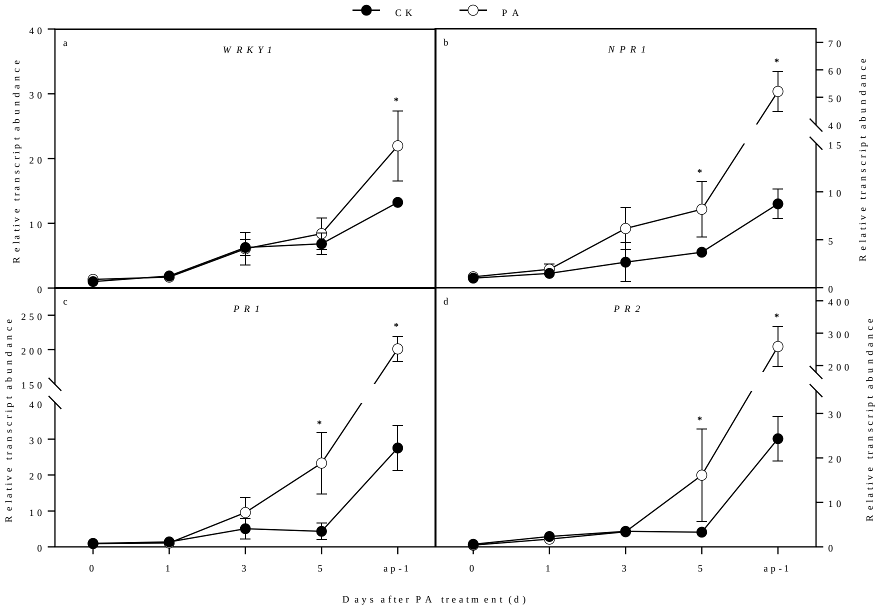

Figure 6. Effects of PA treatment on the gene expression analysis of *WKRY1* (a), *NPR1* (b), *PR1*(c) and *PR2* (d) of watermelon roots after treating with PA, ap-1 indicates one day after inoculation with FON. One asterisk values (means ± SE, n = 3) with significant differences within the same group (P<0.05, Duncan's multiple range test).

Our results showed that the relative transcript abundances of *NPR1*, *PR1* and *PR2* were significantly increased 5-days after treatment with PA (Fig. 6) (P< 0.05). Meanwhile, the relative transcript abundances of *WRKY1*, *NPR1*, *PR1* and *PR2* in treatment were significantly increased by 1.7, 6.0, 7.3 and 10.6 times, respectively at 1 day after FON inoculation (P<0.05). In previous study, SA synthesis and SA signal-regulated gene expression were enhanced in wheat-watermelon intercropping systems, which induced watermelon systemic resistance to FON (16). Therefore, PA may promote the accumulation of SA and then further induce a series of defense responses, which improve the systemic resistance of watermelon.

CONCLUSIONS

Our study suggested that exogenously applied PA enhanced the level of ROS in watermelon roots at early infection stage and the activity of related defense enzymes, reduced the MDA content. Moreover, exogenously applied PA enhanced the disease-related defense-responsive gene expressions. Conclusively, these findings improved our understandings of the mechanism, how PA could induce systemic resistance in watermelon against FON by changing the defense genes expressions and certain biochemical processes.

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