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Allelochemical *p*-hydroxybenzoic acid inhibits root growth *via* regulating ROS accumulation in cucumber (*Cucumis sativus* L.)

HUANG Cheng-zhen[1], XU Lei[1], Sun Jin-jing[2], ZHANG Zhong-hua[2], FU Mei-lan[3], TENG Hui-ying[3], YI Ke-ke[1]

[1] *Key Laboratory of Plant Nutrition and Fertilizer, Ministry of Agriculture and Rural Affairs/Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 10081, P.R.China*

[2] *Key Laboratory of Biology and Genetic Improvement of Horticultural Crops of the Ministry of Agriculture, Sino-Dutch Joint Laboratory of Horticultural Genomics, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 10081, P.R.China*

[3] *The Semi-arid Agriculture Engineering & Technology Research Center of China, Hebei 050051, P.R.China*

Abstract

Allelopathy is prevalent in agricultural ecosystems and mediated by plant-derived secondary metabolites (allelochemicals). Allelochemicals are released by donor plants and affect the root growth and development of receptor plants. Allelopathy is responsible for the continuous cropping obstacles in cucumber (*Cucumis sativus* L.). *p*-Hydroxybenzoic acid (pHBA), an autotoxin from root exudates of cucumber, has been proposed to be an important allelopathic chemical. However, the molecular mechanism by which pHBA affect root growth and development in cucumber is unknown. Here, we found that pHBA treatment suppressed root growth of cucumber by reducing the meristem activity and cell length. This root growth defect is caused by reduced reactive oxygen species (ROS) accumulation in root tips. After pHBA treatment, the expression levels of several ROS-scavenging-related genes were increased, including peroxidase (*POD*), catalase (*CAT*) and metallothionein (*MT*). Moreover, exogenously application of salicylhydroxamate (SHAM), a peroxidase inhibitor, can partially restore the pHBA treatment induced root growth inhibition. Furthermore, we found that there is natural variation for the inhibitory effect of pHBA on root growth. We also showed that pHBA treatment could maintain higher level of ROS accumulated in the pHBA less sensitive cucumber than that in the pHBA-sensitive cucumber. These results suggest that pHBA inhibits root growth by reducing root tip ROS level in cucumber.

Keywords: allelopathy, cucumber, reactive oxygen species, natural variation

1. Introduction

Many crops, including cucumber (*Cucumis sativus* L.), are often cultivated repeatedly in the same soil in intensive agriculture. Continuous monocropping often results in severely poor growth and a crop yield decline. Allelopathy has been believed to be responsible for the continuous agricultural obstacle faced by many crops, like maize

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Correspondence XU Lei, Tel: +86-10-82108643, E-mail: xuleisy@zju.edu.cn

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(*Zea mays* L.), mungbean (*Vigna radiata* L. Wilczek), carrot (*Daucus carota* L. *cv*. Saint Valery), and cucumber (*Cucumis sativus* L.) (Yu and Matsui 1997; Abenavoli *et al*. 2003; Abrahim *et al*. 2003; Vishwajith *et al*. 2017). Phenolic compounds are a class of important and common plant allelochemicals in ecosystems. It was found that cucumber growth was inhibited by its own root extracts and root exudates, but improved by removal of these substances from the rhizosphere (Yu and Matsui 1997; Yu *et al*. 2003). *p*-Hydroxybenzoic acid (pHBA) is the most abundant phenolic compound in the field soil samples (Whitehead 1964; Yu and Matsui 1994). When cucumber plants have been continuously cropped for three years, pHBA is one of the main phenolic compounds in rhizospheric soil (Zhou *et al*. 2012). pHBA could directly affect the activities of metabolic enzymes of glycolysis and the oxidative pentose phosphate pathway to restrain seed germination and root growth (Muscolo *et al*. 2001; Eng-Kiat *et al*. 2002). It has been demonstrated that pHBA strongly inhibit ion uptake, transpiration and photosynthesis, and also resulted in DNA and proteins damage in plants (Yu and Matsui 1997; Yu *et al*. 2003; Zhang *et al*. 2010; Chen *et al*. 2015).

Plant root system is crucial for plant survival and performs a wide range of functions, such as water or nutrients acquisition, structural support of above-ground parts and sensing rapidly changing soil environment. As plant roots are directly exposed to soil allelochemicals, the root growth defect may be the primary and potentially decisive phytotoxic response to allelochemical stress. However, the molecule mechanism by which pHBA regulating root growth in cucumber is still unclear.

Reactive oxygen species (ROS), containing H_2O_2 and $O_2^{-}\cdot$, are normally generated as by-products of many metabolic processes. They also play important signaling roles in all living organisms. ROS are involved in many biological processes in plants, such as systemic acquired resistance, stomatal closure, programmed cell death, germination and root development (Grant and Loake 2000; Schopfer and Frahry 2001; Ren *et al*. 2002; Bindschedler *et al*. 2006; Li *et al*. 2007; Torres 2009).

Recent reports showed that ROS play an important role in root development (Xu *et al*. 2017; Zhang *et al*. 2018). In plants, root tips represent a zone of active ROS production (Liszkay *et al*. 2004). In *Arabidopsis*, $O_2^{-}\cdot$ was predominantly located in the meristem zone, whereas H_2O_2 accumulated in the differentiation zone. Differences in $O_2^{-}\cdot$ and H_2O_2 accumulation in the root tip significantly affected root growth and differentiation (Julia *et al*. 2003; Christophe *et al*. 2007). Glutathione reductase 2, a glutathione biosynthetic enzyme, is essential for maintaining root growth and meristem activity by regulating glutathione redox status (Yu *et al*. 2013). Salicylic acid (SA) modulates root meristem activity through promoting ROS accumulation in the root tip of rice (Xu *et al*. 2017). Environmental stresses arising from the rhizosphere also affect the accumulation of ROS in the roots of plant. For example, mechanical damage, drought, soil salinization and nutrient deficiency can lead to alteration in ROS levels in the root tip, which in turn affects root development and growth direction (Hernández *et al*. 1995; Sánchez-Fernández *et al*. 1997; Proietti *et al*. 2013). However, our knowledge about the relationship between ROS and pHBA in root development during allelopathy is very limited.

Here, we found that pHBA treatment inhibits root growth by reducing the meristem activity and cell elongation in cucumber. Further physiological analysis showed that pHBA inhibits root growth by decreasing the accumulation of H_2O_2 and $O_2^{-}\cdot$ *via* increasing expression of redox and ROS-scavenging-related genes in the root tips. We also found that cucumber can have considerable natural variation in response to pHBA allelopathy. This variation might be due to the different abilities for the maintaining of ROS accumulation in cucumber root tip.

2. Materials and methods

2.1. Plant materials and growth conditions

Cucumber (*Cucumis sativus* L.) seeds were germinated on moist plates at 28°C for 2 d. Hydroponic experiments were conducted using the 1/10 Hoagland nutrient solution, which contained 5 mmol L^{-1} KNO_3, 5 mmol L^{-1} $Ca(NO_3)_2$, 1 mmol L^{-1} $NH_4H_2PO_4$, 2 mmol L^{-1} $MgSO_4$, 10 μmol L^{-1} $MnSO_4$, 50 μmol L^{-1} H_3BO_3, 0.7 μmol L^{-1} $ZnSO_4$, 0.2 μmol L^{-1} $CuSO_4$, 0.01 μmol L^{-1} $(NH_4)_6Mo_7O_{24}$ and 70 μmol L^{-1} Fe-EDTA-Na_2. The pH of the solution was adjusted to 7.1. All plants were grown in a greenhouse with a 12-h-day (28°C)/12-h-night (21°C) photoperiod, ~200 μmol m^{-2} s^{-1} photon density, and ~65% humidity. For the exogenous chemicals' treatment, stock solution for pHBA (100 mmol L^{-1}), salicylhydroxamate (SHAM, 50 mmol L^{-1}) and diphenyleneiodonium (DPI, 5 μmol L^{-1}) were freshly prepared. After germination, the cucumber seeds were exposed to different chemicals treatment, pHBA (0.5 mmol L^{-1}), SHAM (20 μmol L^{-1}) and DPI (0.2 μmol L^{-1}). The ascorbic acid (0.5 mmol L^{-1}) was added to the culture solution directly.

2.2. Root length measurement

For root elongation assays, 5-day-old cucumber seedlings grown on mock medium and medium containing 0.5 mmol L^{-1} pHBA were scored. To measure the length of root meristem and cortical cells, the roots were immersed in clearing solution (8 g of chloral hydrate, 2 mL of water and

1 mL of glycerol) in 2-mL tubes for 2 weeks. DIC images were taken under a microscope (LEICA DM6 B). The root meristem and cortical cell sizes were measured by imageJ.

2.3. NBT and HPF staining

Five-day-old cucumber seedlings grown on mock medium and medium containing different chemicals were stained for 20 min in a solution of 2 mmol L^{-1} nitro blue tetrazolium (NBT) or 5 μmol L^{-1} 3'-(ρ-hydroxyphenyl) fluorescein (HPF) in 20 mmol L^{-1} phosphate buffer (pH 6.1). The reaction was stopped by transferring the seedlings to distilled water. Root tips were imaged under bright-field illumination for NBT staining or green fluorescent protein (GFP) channel for HPF staining using a Leica DM6 B microscope (Leica, Germany). The intensity of NBT and HPF staining was quantified using ImageJ software.

2.4. RNA isolation and qRT-PCR

Total RNA was isolated from primary root tips (~5 mm) of 5-day-old mock or pHBA treated cucumber. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Germany). Reverse transcription was performed using 2 μg of total RNA and M-MuLV reverse transcriptase (NEB) according to the manufacturer's instructions. Real-time quantitative PCR (qRT-PCR) was performed using the Roche SYBR Green I Kit (Roche, Switzerland) on a LightCycler480 machine (Roche) according to the manufacturer's instructions. Three technical replicates were performed per gene within an experiment. Three biological replicates were performed. The cucumber *α-tubulin* gene was used as an internal control. The primers used for qRT-PCR are listed in Appendix A.

2.5. Statistics

Data were evaluated by one-way ANOVA and the means were compared by Duncan's test. Different letters on the histograms indicate the statistically significant differences at $P<0.05$.

3. Results

3.1. The inhibitory effect of pHBA on root growth

To reveal the role of pHBA on the cucumber root growth, we treated cucumber (*C. sativus cv*. Zhongnong 16) with different concentrations of pHBA for five days. As shown in Fig. 1-A and B, pHBA treatments could inhibit root growth in a dosage-dependent manner. Exogenous application of 0.5 mmol L^{-1} pHBA significantly suppressed root growth. As the pHBA concentrations increased, root elongation was more severely inhibited (Fig. 1-A and B). To characterize the root growth defects after pHBA treatment, we compared the root growth rates between mock and pHBA-treated cucumber and found that the pHBA-mediated root growth inhibitory was due to a decrease in growth rate rather than an early cessation of root growth (Fig. 1-C). We chose 0.5 mmol L^{-1} pHBA as the allelopathic stress to perform subsequent experiments.

Measurement of the root meristem length showed that the meristem size of pHBA-treated plants was smaller compared to that of the untreated plants (Fig. 1-D). The root mature cortical cell length of pHBA-treated plants was also slightly shorter than that of untreated cucumber (Fig. 1-E). We further examined the expression levels of the cell cycle-related genes (*CDKB1;2*, *CYCB1;1* and *CYCB2;4*) in pHBA-treated plants and found that the expression levels of these genes were significantly lower than those in untreated cucumbers (Fig. 1-F). These results indicated that the pHBA treatment inhibit root growth by reducing meristem activity and cell elongation ability.

3.2. pHBA treatment reduce the accumulations of H_2O_2 and $O_2^{-}\cdot$ in the root tips

To investigate whether ROS levels were altered in cucumber after pHBA treatment, we measured ROS levels in cucumber root tip using HPF and NBT staining for H_2O_2 and $O_2^{-}\cdot$, respectively. pHBA treatment reduced the H_2O_2 and $O_2^{-}\cdot$ levels in the root tips based on the lower intensity of HPF fluorescences and weaker NBT staining compared with those of untreated roots (Fig. 2-A–D). Given that ROS play an important role in regulating root meristem activity, we hypothesized that decreased ROS accumulation after pHBA treatment in cucumber root tips leads to a reduction in root growth. To test this hypothesis, we first treated cucumber seedlings with NADPH oxidase (a major source of ROS production) inhibitor DPI and the antioxidants ascorbic acid (Vc, the primary water-soluble antioxidants in plants) (Gill and Tuteja 2010; Zhang *et al*. 2018). The addition of DPI and Vc led to a reduction in both ROS (H_2O_2 and $O_2^{-}\cdot$) accumulation in the root tip and root growth (Fig. 3-A–F). These results indicate that the reduced ROS levels in cucumber roots inhibit root growth. Furthermore, application of DPI or Vc to pHBA treated plants further inhibited ROS accumulation and root growth (Fig. 3-A–F), supporting the hypothesis that reduced ROS levels in root tips is responsible for the root growth defect in pHBA-treated cucumber plants.

To investigate the molecular mechanism by which pHBA regulated ROS accumulation, we analyzed the expression levels of ROS-scavenging-related genes upon

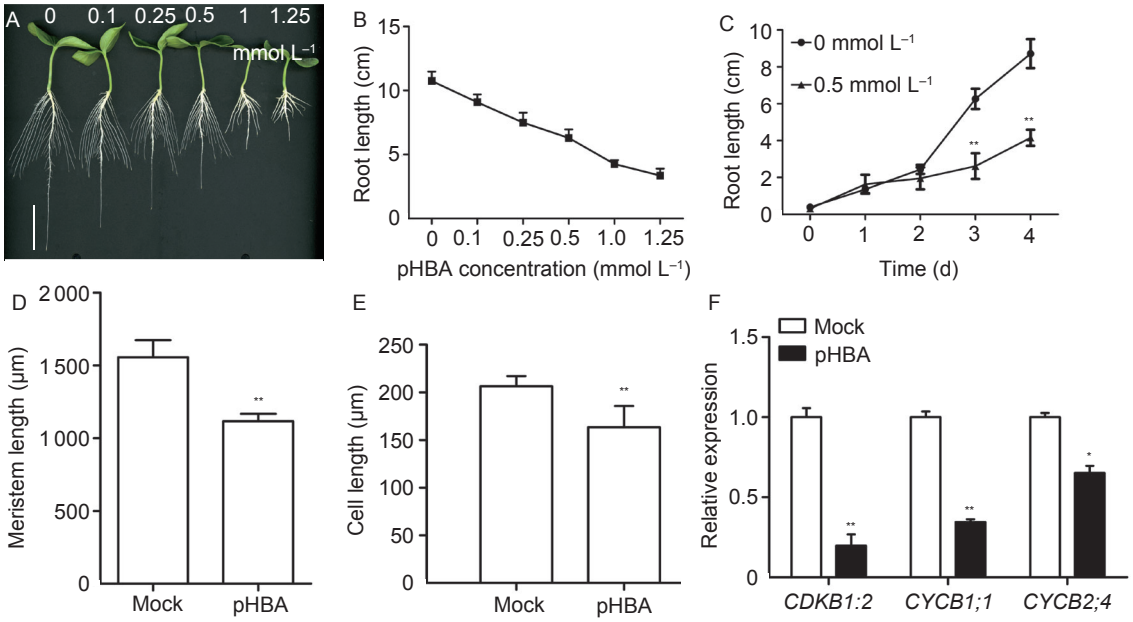

Fig. 1 *p*-Hydroxybenzoic acid (pHBA) stress inhibits root growth by reducing meristem activity and cell length. A and B, dose effect of pHBA on root growth. The cucumber seeds after germination were exposed to different concentrations of pHBA for 5 days. Bar=3 cm. Error bars represent SD (*n*=10). C, time course of primary root length of 4-day-old cucumber seedlings. Error bars represent SD (*n*=10). D and E, meristem length (from the quiescent center to the start of the elongation zone) and longitudinal cell length of cortical cells in the mature region of 5-day-old cucumber seedlings, respectively. Error bars represent SD (*n*=20 in D and *n*=30 in E). F, relative expression of *Cyclin* genes (*CDKB1;2*, *CYCB1;1* and *CYCB2;4*) in root tips of 5-day-old cucumber seedlings. The expression level was compared with that in the cucumber grown on mock medium. Total RNA was collected from the 5-mm root apex of seedlings. Three biological replicates were performed. Cucumber seedlings were grown on mock medium and medium supplemented with 0.5 mmol L^{-1} pHBA in C–F. The asterisks in C–F indicate a significant difference (*, P<0.05; **, P<0.01, by Student's *t* test).

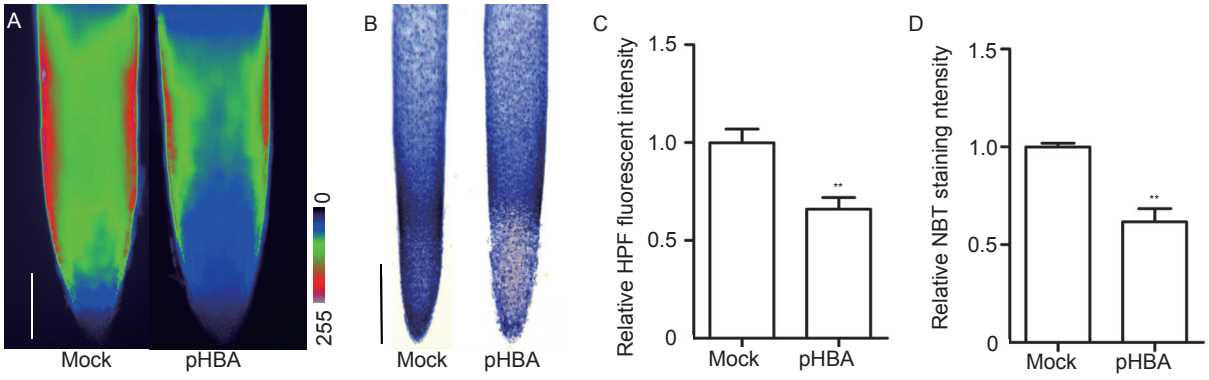

Fig. 2 Effects of *p*-hydroxybenzoic acid (pHBA) on H_2O_2 and O_2^{-}· accumulation in roots of 5-day-old cucumbers grown on mock medium and medium supplemented 0.5 mmol L^{-1} pHBA. A, representative pseudo-color images of roots stained with 3'-(ρ-hydroxyphenyl) fluorescein (HPF) to detect H_2O_2. The gradation of colors reflects the intensity of fluorescence. Bar=250 μm. B, O_2^{-}· accumulation revealed by nitro blue tetrazolium (NBT) staining. Bar=1 mm. C and D, quantification of HPF fluorescence and NBT staining intensity, respectively. Error bars represent SD (*n*=8). The intensity was compared with that in the cucumber grown on mock medium. The asterisks in C and D indicate a significant difference (**, P<0.01, by Student's *t* test).

pHBA treatment. We analyzed the expression level of peroxidase (*POD*) that display higher expression level in the cucumber root based on RNA-seq data (unpublished). qRT-PCR analysis showed that seven *POD* genes were upregulated upon pHBA treatment (Fig. 4-A). The expression levels of catalase (*CAT*), metallothionein (*MT*) and superoxide dismutase (*SOD*), also slightly increased after pHBA treatment (Fig. 4-B and C). We also analyzed

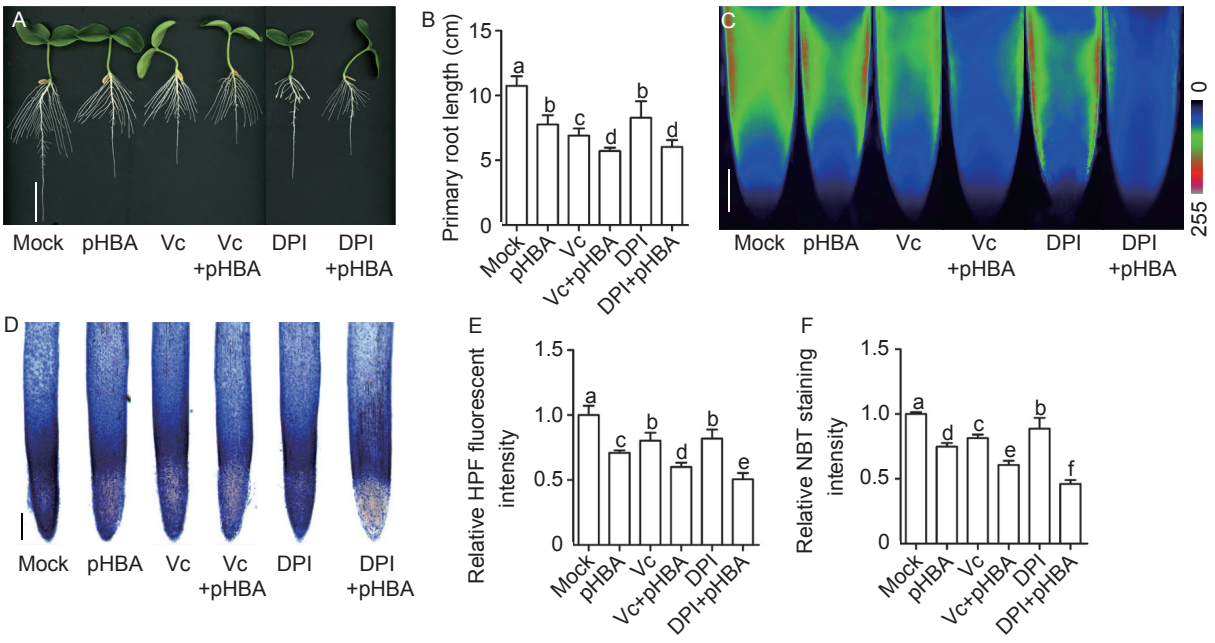

Fig. 3 Effects of *p*-hydroxybenzoic acid (pHBA) and reactive oxygen species (ROS) scavengers or metabolism inhibitors on root growth and ROS accumulation. A, root phenotype of 5-day-old cucumber under different treatments. Mock, 0 mol L^{-1}; pHBA, 0.5 mmol L^{-1}; Vc, ascorbic acid (0.5 mmol L^{-1}); DPI, diphenylene iodonium (0.2 μmol L^{-1}). Bar=3 cm. B, primary root length of 5-day-old cucumber under different treatments. Error bars represent SD (*n*=10). C, pseudo-color images of 5-day-old roots stained with 3'-(ρ-hydroxyphenyl) fluorescein (HPF) for H_2O_2 in cucumber under different treatments. The gradation of colors reflects the intensity of fluorescence. Bar=250 μm. D, O_2^{-}· accumulation revealed by nitro blue tetrazolium (NBT) staining. Bar=1 mm. E and F, quantification of HPF fluorescence and NBT staining intensity, respectively. Error bars represent SD (*n*=8). The intensity was compared with that in the cucumber grown on mock medium. Different letters in B, E and F indicate a significant difference (*P*<0.05, by Duncan's test).

the expression levels of *NADPH* oxidase genes, catalyzing the production of O_2^{-}·. However, our expression analysis showed that none of the *NADPH oxidase* genes displayed reduced expression after pHBA treatment (Appendix B). Therefore, pHBA treatment reduces ROS levels in the root tip likely through modulating the expression level of ROS-scavenging-related genes. We speculated that increased peroxidase activity may be partially responsible for the observed phenotype after pHBA treatment. We found that exogenously application of SHAM, a peroxidase inhibitor (Tsukagoshi *et al*. 2011), partially rescued the short root phenotype caused by pHBA treatment by increasing the root length by ~20.7% (Fig. 5-A and B). Application of SHAM also increased ROS levels in the root tip of pHBA-treated plants (Fig. 5-C–F). These results suggest that root growth defect caused by pHBA treatment was partially due to the evaluated peroxidase activity, which reduced the ROS accumulation in the root tips.

3.3. Inhibitory effect of *p*-hydroxybenzoic acid on root growth in different cucumber accessions

To understand the nature variation in pHBA responsiveness and to identify the potential mechanisms responsible for adaptation to allelopathy, we investigated the root growth of 48 cucumber accessions (Ren *et al*. 2009) after pHBA treatment and revealed a wide range of natural variation in pHBA responsiveness in different accessions (Appendix C). We further performed allelopathic stress on different cucumber accessions, the Zhongnong 16 (ZN16) and Hexin 25 (HX25), because the inhibitory effect of pHBA on roots growth in HX25 was significantly lower than that of ZN16 (Fig. 6-A and B). Consistent with the different root growth responses, pHBA treatment repressed the expression of the cell cycle genes (*CDKB1;2*, *CYCB1;1* and *CYCB2;4*) in the root tips of ZN16, whereas the expression of these genes in the HX25 was only slightly inhibited (Fig. 6-C).

We have demonstrated that the root growth defect after pHBA treatment in the cucumber is partially caused by the evaluated expression of ROS-scavenging-related genes. We further determined the expression of ROS-scavenging-related genes in both ZN16 and HX25. qRT-PCR analysis showed that although the exogenous application of pHBA can induce the expression of *POD*, *CAT* and *MT* genes in both ZN16 and HX25, the transcript levels in HX25 were significantly lower than those in ZN16 (Fig. 6-D and E).

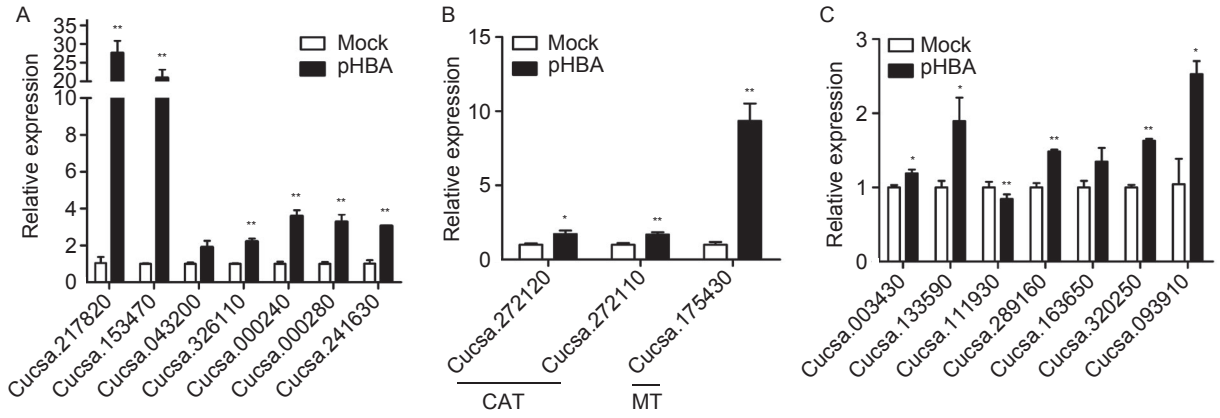

Fig. 4 *p*-Hydroxybenzoic acid (pHBA) alter the expression of reactive oxygen species (ROS) scavenging-related genes in the root tip of 5-day-old cucumber grown on mock medium and medium supplemented with 0.5 mmol L^{-1} pHBA. A, relative expression of peroxidase (*POD*) genes. B, relative expression of catalase (*CAT*) and metallothionein (*MT*) genes. C, relative expression of superoxide dismutase (*SOD*) genes. The expression level of each gene was compared with that in the cucumber grown on mock medium. Total RNA was collected from the 5-mm root apex of seedlings. Error bars represent SD (n=3). The asterisks (*, P<0.05; **, P<0.01, by Student's t test) indicate a significant difference between cucumbers grown on mock medium and medium supplemented with 0.5 mmol L^{-1} pHBA.

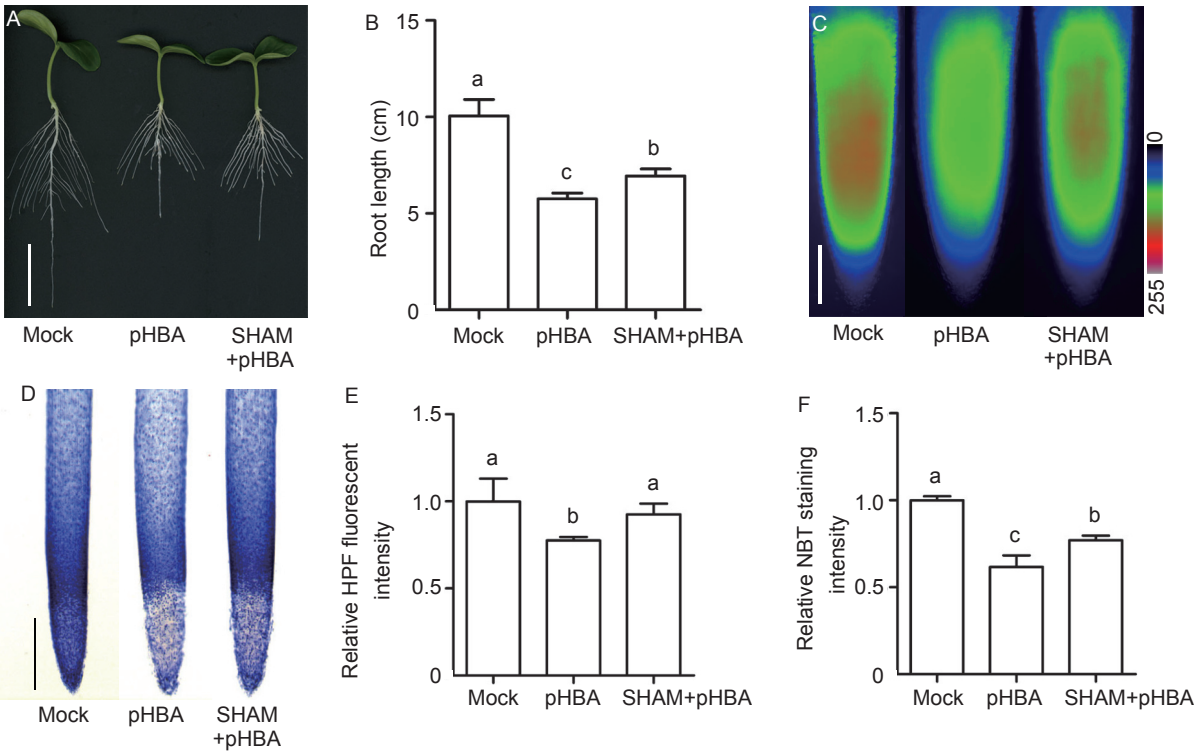

Fig. 5 Effects of *p*-hydroxybenzoic acid (pHBA) and salicylhydroxamate (SHAM) on root growth and reactive oxygen species (ROS) accumulation of 5-day-old cucumber under different treatments. Mock, 0 mol L^{-1}; pHBA, 0.5 mmol L^{-1}; SHAM, 20 μmol L^{-1}. A, root phenotype. Bar=3 cm. B, primary root length. Error bars represent SD (n=10). C, pseudo-color images of 5-day-old roots stained with 3'-(ρ-hydroxyphenyl) fluorescein (HPF) for H_2O_2. The gradation of colors reflects the intensity of fluorescence. Bar=250 μm. D, $O_2^{\cdot-}$ accumulation revealed by nitro blue tetrazolium (NBT) staining. Bar=1 mm. E and F, quantification of HPF fluorescence and NBT staining intensity, respectively. Error bars represent SD (n=8). The intensity was compared with that in the cucumber grown on mock medium. Different letters in B, E and F indicate a significant difference (P<0.05, by Duncan's test).

However, *SOD* genes showed a similar expression pattern in HX25 and ZN16 in the mock and pHBA-treated plants (Fig. 6-F). Consistent with the lower expression levels of *POD*, *CAT* and *MT* genes in HX25, it accumulated higher

levels of H_2O_2 in the root tip than ZN16 after pHBA treatment (Fig. 7-A–D). These results suggest that ROS-scavenging capacity paly a crucial role in modulating root growth during allelopathic stress in cucumber.

4. Discussion

ROS are important signaling molecules in regulating root growth (Bartosz 1997). In *Arabidopsis*, UPB1 regulated the expression of peroxidase genes and consequently affected the accumulation of superoxide and hydrogen peroxide in the root tip (Tsukagoshi *et al*. 2011). Recent study showed that salicylic acid regulates root meristem activity through maintaining sufficient levels of ROS by suppressing the expression of redox and ROS-scavenging-related genes in rice roots (Xu *et al*. 2017). Thus, ROS may function as common signaling molecules for root growth in different plants. Our study showed that ROS also play a crucial role in regulating root growth in cucumber, and the reduced ROS levels in cucumber roots inhibit root growth.

Allelopathy cause a series of ecological and economic problems, such as declines in crop yield and the invasion of exotic plant species (Bais *et al*. 2003; Huang *et al*. 2013). Cucumber could release allelochemicals into the rhizosphere causing replant failure (Yu *et al*. 2003). However, the molecular mechanism by which allelochemical stress negatively affects root growth is still unclear in cucumber. Our results showed that pHBA inhibited the root growth in cucumber due to the reductions in the meristem activity and inhibition of root cell elongation. pHBA treatment could induce the expression of ROS-scavenging-related genes, including *POD*, *SOD*, *CAT* and *MT*, and in turn decrease ROS accumulation in the cucumber root tip. POD is known as H_2O_2-reducing enzymes that can oxidize or polymerize various hydrogen donors while converting H_2O_2 into water (Asada 1992). SOD catalyzes the conversion of $O_2^{-}\cdot$ to H_2O_2 in many organelles (Gill and Tuteja 2010). CAT and MT catalyze the decomposition of H_2O_2 in plants (Scandalios

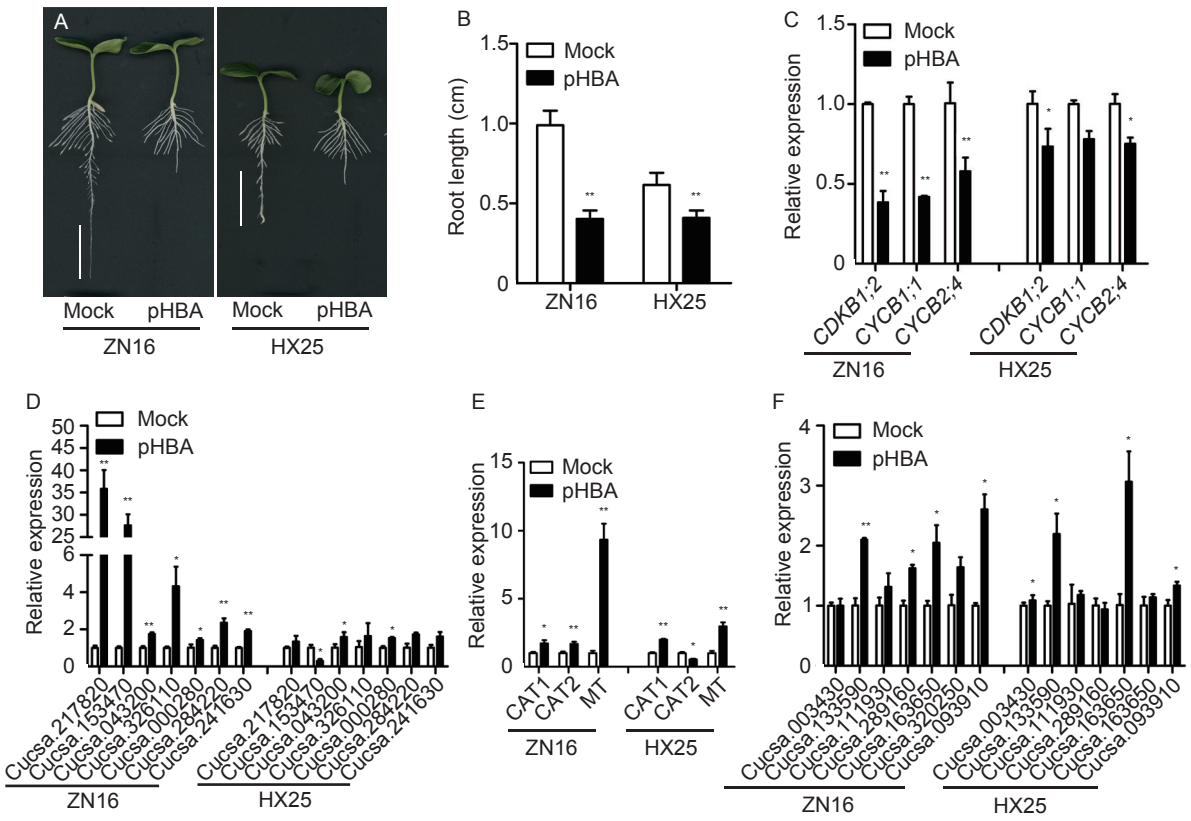

Fig. 6 Effect of *p*-hydroxybenzoic acid (pHBA) on different cucumber accessions Zhongnong 16 (ZN16) and Hexin 25 (HX25) grown on mock medium and medium supplemented with 0.5 mmol L^{-1} pHBA for 5 days. A, phenotype of ZN16 and HX25. Bar=3 cm. B, primary root length of ZN16 and HX25. Error bars represent SD (n=10). C, relative expression of *Cyclin* genes (*CDKB1;2*, *CYCB1;1* and *CYCB2;4*). D, relative expression of peroxidase (*POD*) genes. E, relative expression of catalase (*CAT*) and metallothionein (*MT*) genes. F, relative expression of superoxide dismutase (*SOD*) genes. The expression level of each gene was compared with that in cucumber grown on mock medium. Total RNA was collected from the 5-mm root apex of seedlings. Error bars represent SD (n=3). The asterisks in B–F indicate a significant difference between cucumbers grown on mock medium and medium supplemented with 0.5 mmol L^{-1} pHBA (*, P<0.05; **, P<0.01, by Student's t test).

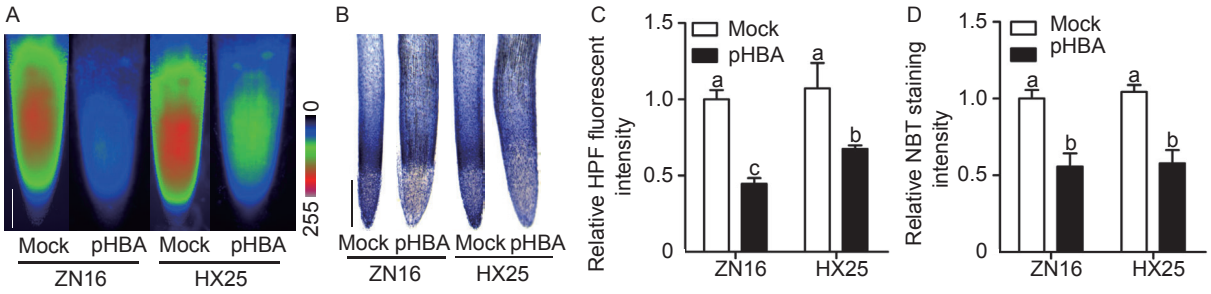

Fig. 7 Effects of *p*-hydroxybenzoic acid (pHBA) on H_2O_2 and $O_2^{-}\cdot$ accumulation in the root of 5-day-old Zhongnong 16 (ZN16) and Hexin 25 (HX25) grown on mock medium and medium supplemented 0.5 mmol L^{-1} pHBA. A, representative pseudo-color images of roots stained with 3'-(ρ-hydroxyphenyl) fluorescein (HPF) to detect H_2O_2. The gradation of colors reflects the intensity of fluorescence. Bar=250 μm. B, $O_2^{-}\cdot$ accumulation revealed by nitro blue tetrazolium (NBT) staining. Bar=1 mm. C and D, quantification of HPF fluorescence and NBT staining intensity, respectively. Error bars represent SD (n=8). The intensity was compared with that in ZN16 grown on mock medium. Different letters indicate a significant difference (P<0.05, by Duncan's test). All the treatments were performed three times.

et al. 1994; Hassinen *et al*. 2011). Consistent with the increased expression of *POD* genes after pHBA treatment, exogenously application of SHAM (a peroxidase inhibitor) could partially rescue the short root phenotype caused by pHBA treatment. Previous studies have also showed that pHBA treatment could increase the activities of antioxidant enzymes, including POD and SOD in plants (Yu *et al*. 2003; Zhang *et al*. 2012). These results suggest that pHBA inhibits root growth by reducing ROS accumulation in cucumber through activating antioxidant system. Several studies have shown that allelochemicals inhibited root growth and development. For example, ferulic acid (FA) released by roots altered H_2O_2 and $O_2^{-}\cdot$ levels, inhibiting the growth and development of cucumber seedlings (Blum and Dalton 1985; Zhang *et al*. 2015). The benzoic acid (BA) treatments significantly increased ROS levels in the root meristem, elongation and mature zones leading to the inhibition of primary root elongation (Zhang *et al*. 2018). Guan *et al*. (2014) have reported that pHBA induced the oxidative burst in roots resulting in a significant increase in NO and ROS levels in *Arabidopsis* and proposed that both NO and H_2O_2 are important signals that mediate *Arabidopsis* response to the allelopathic chemical pHBA. During this process H_2O_2 may work upstream of the NO signal. These studies suggest that ROS may act as a common signaling molecular in response to different allelochemicals. Moreover, other signaling pathways may be also involved in pHBA mediated root growth inhibition. Recent study showed that ROS, auxin and ethylene signaling pathway contribute to allelochemical benzoic acid mediated root growth inhibition (Zhang *et al*. 2018). Rapid changes in ROS homeostasis are among the earliest symptoms following fluctuations in environmental conditions. In *Arabidopsis*, pHBA rapidly induced the generation of H_2O_2 after 10 s of pHBA treatment, which reached a maximum level after 60 s of treatment (Guan *et al*. 2014). ROS level increased in the first 12 h after BA treatment and then decreased with the increase of BA exposure time (Zhang *et al*. 2018). Our results showed that long-time (5 days) pHBA treatment could reduce the ROS accumulation. We speculate that short-time pHBA treatment induced H_2O_2 acts as the signaling to trigger the downstream response. However, long-time pHBA treatment activated the expression of ROS-scavenging-related genes, which led to reduced ROS accumulation.

It has been suggested that the inhibitory effects of allelochemical varied in different plant species (Zhang *et al*. 2018). In the present study, we aimed to gain insight into the genetic basis of the ability to adapt to allelopathy in naturally occurring cucumber accessions. We studied the phenotypic variations among 48 cucumber accessions after pHBA treatment. This study shows that plants can have considerable natural variation in their response to pHBA treatment. Furthermore, pHBA less sensitive cucumber showed lower expression levels of ROS-scavenging-related genes and accumulated higher levels of H_2O_2 in the root tip than pHBA-sensitive cucumber after pHBA treatment. By mining this natural variation using genome-wide association study (GWAS) in future study, we will be able to identify a number of candidate genes involved in allelopathic response. It can be instrumental in future cucumber breeding for allelopathy resistance traits.

5. Conclusion

In the present study, we found that pHBA treatment significantly inhibited cucumber root growth through decreasing root meristem activity and reducing root cell length. Further physiological and genetic data showed that pHBA inhibited root growth by decreasing the accumulation of H_2O_2 and $O_2^{-}\cdot$ *via* increasing the expression of ROS-

scavenging-related genes. In conclusion, this work further clarified the role of ROS in response to allelopathic chemical in cucumber. Besides, the study indicated that the inhibitory effects of allelochemical varied in different accessions. It will help us to breed new cucumber varieties more resistant to allelopathic stress.

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Appendices associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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