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The effect of potassium phosphite on PR genes expression and the phenylpropanoid pathway in cucumber (*Cucumis sativus*) plants inoculated with *Pseudoperonospora cubensis*

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ABSTRACT

In the present study, the impact of potassium phosphite (KPhi) was investigated at molecular and biochemical levels in response to *Pseudoperonospora cubensis* infection in cucumber plants. Real-time PCR was employed to explore the differential expression of defense genes against *P. cubensis*. The highest expression for *thaumatin-like protein* (*TLP*), *Ribosome-inactivating protein* (*RIP*) and *Defensin* genes was observed in pre-inoculation plants at 96, 72 and 48 h, respectively. These findings revealed the involvement of these genes in the defense response of cucumber leaves after KPhi treatment and pathogen inoculation. At the biochemical level, more induction in the contents of some end-products of phenylpropanoid pathway, such as phytoalexin, phenolic component, flavonoid and anthocyanin as well as phenylalanine ammonia-lyase (PAL) enzymatic activity was detected in pre-inoculation plants compared to post-inoculation plants at all-time points. Data suggest that KPhi primes rapid and robust response in plants against infection via activation of defense responses. The negative effects of *P. cubensis* on cucumber plants could be considerably mitigated by KPhi application before infection.

1. Introduction

Pseudoperonospora cubensis is one of the most destructive pathogens in cucumber plants. Researchers have focused on alleviating the damage caused by plant pathogens, and the application of fungicide is one the most effective ways to control infection. Therefore, activating defense responses through the use of chemical and bio-based elicitors should be an alternative method for improving plant tolerance to biotic stresses (Eshraghi et al., 2011).

During the interaction between plants and pathogens, plant defense responses exhibit some main features including PR (pathogenesis-related) protein accumulation (Shetty et al., 2009), lignification of cell walls of tissues, and involvement of the phenylpropanoid pathway in plant defense (Somssich and Hahlbrock, 1998). Thaumatin-like protein (TLP) is a type of PR proteins that exhibit antifungal activity and increase plant disease resistance (Wang et al., 2011). Ribosome-inactivating proteins (RIP) are another type of PR proteins that participates in antifungal activity. Plant RIPs with specific modifications of 28 s rRNA inhibit protein synthesis. RIPs have a broad range of antimicrobial activities, including antifungal and antibacterial effects (Hey et al., 1995). PR-12 known as Defensin is another antifungal protein with membrane permeabilization properties (Terras et al., 1992).

Plant and pathogen interaction, has received considerable attention due to the synthesis of secondary metabolites such as lignin, phenols and phytoalexin, which add mechanical rigidity and strength to the cell wall and provide barriers to pathogen infection. During these protection processes, key enzymes in the phenylpropanoid pathway, such as phenylalanine ammonia lyase (PAL), play important roles (Bednarek et al., 2005).

Among different secondary metabolites, phenolic and flavonoid compounds have protective activities with antioxidant and free radical scavenging properties in plants (Williams et al., 2004). Anthocyanin, as another product of the phenylpropanoid pathway, is usually associated with an increase in PAL activity and has biological roles including protection against many different pathogens, scavenging of free radicals and anti-oxidative activity (Kliebenstein, 2004).

In agriculture, potassium phosphite (KPhi) is a fertilizer and activator of natural resistance or systemic resistance (Guest and Bompeix, 1990). A previous study has shown that KPhi is effective against *P. cubensis* and translocates in both xylem and phloem tissues (Silva et al., 2011). KPhi acts on the pathogen by stimulating host defense responses to inhibit pathogen growth and ultimately, to alter pathogen

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metabolism. KPhi can also induce the accumulation of defense molecules (Eshraghi et al., 2011) such as phytoalexin which is produced as defensive chemical for disease resistance and for overcoming pathogen attack (Holland and O'Keefe, 2010).

The objective of this study was to elucidate the role of KPhi in priming defense responses in the *P. cubensis*–cucumber pathosystem, with an emphasis on the expression of pathogenesis-related genes and various signaling pathways that participate in plant defense.

2. Materials and methods

2.1. Plant materials

The study was performed at the laboratories in Genetics and Agricultural Biotechnology Institute of Tabarestan, Sari, Iran. The Isfahan native *Cucumis sativus* L. seeds were obtained from Pakan Bazr Co., Isfahan, Iran and disinfected in 70% ethanol for 5 min. Seeds were washed by distilled water, dried and sown in pots, filled with sterile soil mix in the greenhouse. The soil had been pre-moistened with distilled water in a growth cabinet (with a 16 h light/8 h dark photoperiod, temperature 24–27 °C and 70% humidity).

Experiment was performed on 4 week old seedlings with emergence of secondary true leaves. Plants were divided into 5 groups (each group consisted of 6 pots and each pot contained one plant) and different treatments were performed as follows: 1. The leaves were sprayed with 0 g L^{-1} KPhi (distilled water) (Control). 2. The leaves were solely treated with 2 g L^{-1} KPhi three times a day for one day. Four days after KPhi treatment, leaves were harvested at 24, 48, 72 and 96 h (KPhi). 3. The leaves were treated with 2 g L^{-1} KPhi three times a day for one day. Four days after KPhi application, *P. cubensis* inoculation was performed and eight days after inoculation, leaves were harvested at 24, 48, 72 and 96 h (Pre- inoculation). 4. Eight days after inoculation of leaves by *P. cubensis*, leaves were treated with 2 g L^{-1} KPhi three times a day for one day. Four days later, leaves were harvested at 24, 48, 72 and 96 h (Post-inoculation). 5. The leaves were solely inoculated with *P. cubensis* and after eight days were harvested at 24, 48, 72 and 96 h (*P. cubensis*).

2.2. KPhi stock solution preparation

A stock solution of filter-sterilized KPhi (pH 5, adjusted with KOH) was freshly prepared from phosphorous acid (AppliChem, Darmstadt, Germany). KPhi was applied to the plants as a foliar spray at the concentration of 2 g L^{-1} (Mofidnakhaei et al., 2016).

2.3. P. cubensis culture and plant inoculation

P. cubensis spores were aseptically produced on freshly grown cucumber leaves using the method described by Waffa (2002) in Sari Agriculture University. The spore density was determined using a bright line haemocytometer and adjusted to a concentration of 4.5×10^5 spores mL^{-1} using sterile distilled water.

P. cubensis inoculation was carried out as follows: leaves were sprayed by *P. cubensis* (with concentration of 4.5×10^5 mL^{-1} under controlled condition) three times a day for two days. Then, transferred to a growth cabinet with a 10 h photoperiod at 27 ± 2 °C to allow lesions to develop. Disease symptoms were observed as macroscopically visible necrotic spots. First, necrotic yellow spots appeared on the upper leaf surfaces, spreading from the marginal area to the central leaf surface followed by a change from yellow spots to a dried grayish leaf. The inoculated leaf underwent defoliation and the entire plant eventually became necrotic (Fig. 1).

The samples were collected in aluminum foils and stored at −80 °C for molecular and biochemical assessments at different time points (24, 48, 72 and 96 h), as mentioned specifically for each assessment.

Fig. 1. Representative cucumber plant leaf showing the symptoms caused by *P. cubensis* pathogen after two weeks of inoculation.

2.4. RNA extraction and first-strand cDNA synthesis

The secondary true leaves (100 mg fresh weight) were frozen in liquid nitrogen and stored at −80 °C. TRIzol reagent (Invitrogen, USA) was used for RNA extraction according to the manufacturer's instructions. The RNA concentration was determined by measuring the absorbance at 260 nm, and its intensity was visualized on 1% agarose gel. Approximately 2 µg of total RNA was further treated with DNaseI and used for first-strand cDNA synthesis using oligo (dT) primers, 10 mM dNTPs, and reverse transcriptase according to the manufacturer's instructions (Fermentas).

2.5. Real-time PCR conditions

Aliquots of the cDNA were used as template for real-time PCR analysis with SYBR Green Real-time PCR Master Mix (Thermo Scientific). For RT-PCR analysis, gene-specific primers were designed using Bio Edit 7.0.9.0 and Oligo Explorer V1.4 Software (Table 1). *Actin* was used as an internal control. For real-time PCR, the following program was used: 3 min at 95 °C, denaturation at 95 °C for 25 s, annealing at 60 °C for 20 s and extension at 72 °C for 25 s for 40 cycles. To check the amplified product, a melting curve analysis was performed. To determine the relative gene induction levels, the ΔCT method was used (Livak and Schmittgen, 2001).

2.6. Determination of phytoalexin

Phytoalexin were extracted according to the modified method of Andreu et al. (2001). Leaves (1 g fresh weight) were mixed by chloroform/acetic acid/methanol (50:5:45 v/v/v, 10 mL) using a homogenizer. The homogenate was kept overnight at room temperature and was then filtered. Chloroform and 0.2 M acetic acid were added in equal volumes to the container. The mixture was shaken, and two layers were separated. The chloroform layer containing phytoalexin was evaporated to dryness. The dried sample was resolved in 1 mL hexane and 2 mL sulfuric acid, and then agitated and centrifuged at 1000 rpm for 30 min. After 20 min, the absorbance of the red layer was recorded at 500 nm using a spectrophotometer (Biochrom WPA Biowave II). Phytoalexin content was calculated as µg g^{-1} FW.

2.7. PAL activity analysis

Preparation of PAL enzyme extract: to prepare the crude enzyme extract, 0.1 g leaf tissue was homogenized in 15 mL of 0.05 M phosphate buffer (phosphate buffer, pH = 7). The homogenate was centrifuged at 10,000 *g* for 15 min. The supernatant was used for further analysis. The extraction was conducted at 4 °C. PAL enzymatic activity was measured essentially according to the modified method of Hahlbrock and Grisebach (1979). PAL activity measurements were based on the formation of cinnamic acid at 290 nm. Approximately 0.1 mL PAL enzyme extract was mixed with 0.3 mL L-phenylalanine (50 mM). Phosphate buffer (0.05 M) was added to the reaction mixture (3 mL). After the reaction mixture was incubated at 30 °C for 15 min,