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RESEARCH ARTICLE

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Salicylic acid induces the accumulation of defense-related enzymes in Whangkeumbae pear and protects from pear black spot

Abstract The ability of salicylic acid (SA) to induce disease resistance was studied with Whangkeumbae pears affected by *Alternaria kikuchiana* Tanaka. SA (0.02, 0.2 and 2 mmol·L^{-1} active ingredient) protected Whangkeumbae pear leaves from artificial infection when applied before inoculation. When the concentration of SA reached 0.2 mmol·L^{-1}, the disease-infected index was the lowest, and the rate of induced resistance reached 59.0%. The protection of Whangkeumbae pear leaves was associated with the activation of three defense-related enzymes, SOD, POD and PAL. Accumulation of three enzymes was induced locally in treated leaves and systemically. These results suggested that SA could induce systemic resistance in Whangkeumbae pear leaves by increasing defense-related compounds.

Keywords Whangkeumbae pear, *Alternaria kikuchiana* Tanaka, salicylic acid, induced resistance

1 Introduction

Pear black spot is an economically important pear disease worldwide (Wang et al., 2006). The disease is caused by the fungus *Alternaria kikuchiana* Tanaka, which infects the host through natural openings in leaves or wounds occurring in young organs (Rieko et al., 2002). To control the disease, lots of methods have been tried. The most common disease control practice for pear black spot is to prevent infection during the bloom period before wind-driven rain and pollinating insects spread the disease throughout the orchard. Chemical control measures include copper compounds sprayed in the whole orchard during the bloom period. However, in the development and use of chemical agents for crop disease control, considerable attention must be paid to the preservation of the global environment. Pear growers need new methods to prevent the severe losses when suffering from pear black spot.

Novel approaches to crop disease control include the use of chemicals that stimulate the plant natural defense mechanisms through a plant resistance phenomenon called "systemic acquired resistance" (SAR) (Lucas, 1999). Several chemical inducers are often used to induce SAR. They included salicylic acid (SA) (Willits and Ryals, 1998), 2,6-dichloroisonicotinic acid (INA) (Kessmann et al., 1994), 3-aminobutyric acid (BABA) (Olivieri et al., 2009) and benzothiadiazole compounds (BTHs) (Sparla et al., 2004). It has been reported that SA does not exhibit significant direct activity against phytopathogenic fungi, but induces plant resistance mechanism to defense disease (Hou et al., 2006; Li et al., 2006). Furthermore, as an environmentally-friendly plant activator, SA can induce SAR to disease and protect cucumber (Shi et al., 2004), tobacco (Park et al., 2007), pepper (Mao et al., 2004) and so on. However, whether or not SA has the ability to induce the resistance of Whangkeumbae pear leaves against pear black spot has not been reported so far. In the present study, the authors tested the control efficacy of SA on inhabiting pear black spot on Whangkeumbae pear leaves. Simultaneously, antioxidant enzyme activities were also analyzed.

2 Materials and methods

2.1 Biological materials and fungal strains

Whangkeumbae pear trees (*Pyrus pyrifolia* cv. Whangkeumbae) were selected from the orchard of Agricultural University of Hebei, and healthy young leaves of 5 to 10 days old were collected as the materials.

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The pathogenic fungus *A. kikuchiana* Tanaka was obtained from the College of Plant Protection, Agricultural University of Hebei. Fungal cultures were grown on solid potato dextrose agar (PDA) for 7 d at 25°C. Then conidia were washed with sterile water by centrifugation and suspended in 0.1% Tween solution. The conidial concentration was adjusted to about 2×10^5 conidia·mL^{-1}.

2.2 Methods

2.2.1 Plant protection experiments

SA was diluted into different concentrations (0.02, 0.2, 2 mmol·L^{-1}) and was sprayed on young leaves of Whangkeumbae pear with distilled water as a control. The branches were cut down and inserted into a conical flask with hydroponic culture at intervals (1, 2, 3, 4, 5, 6, 7, 10, 15 and 20 days after treatment). Then conidial suspension was brushed onto the leaves with a writing brush. The inoculated branches were incubated in the moist chamber at 20°C in the dark for 48 h and transferred to a phytotron and maintained at 25°C, with three replicates. In each experiment, 20 branches per treatment were used. Seven days later, the disease-infected index was calculated. The classification of black spot disease degrees was according to Fang (1998) (Table 1).

Table 1 The classification of black spot disease degrees

level	disease severity	represented number
1	no symptom	0
2	area with symptom 0–25%	1
3	area with symptom 26%–50%	2
4	area with symptom 51%–75%	3
5	area with symptom more than 75% or withered leaf	4

Young leaves of the test shoots were divided into left and right parts, and the quantity of each part was about three leaves. The left side of leaves was carefully brushed with 0.2 mmol·L^{-1} SA (SL) or distilled water as a control (CL), while the right side was smeared with nothing (SR and CR mean the right-side leaves on which neither SA nor water is brushed). And then, conidial suspension was sprayed onto all the leaves. Seven days after inoculation, disease index was calculated for the two parts separately. Twenty branches were used per treatment in three independent replicates.

2.2.2 Assays for enzymatic activities

In the first set of experiments, SA (0.2 mmol·L^{-1}) or distilled water (control) was sprayed onto young leaves of Whangkeumbae trees, and the treatment leaves were detached from the plants at 1, 2, 3, 4, 5, 6, 7, 10, 15 and 20 days, which were used for enzymatic activity assay. In the second set of experiments, 0.2 mmol·L^{-1} SA or water was sprayed onto the leaves 2 d before or 4 and 2 days after inoculation with *A. kikuchiana* Tanaka. Young leaves at the left side were carefully sprayed with SA (or water for control plants), while the right-side leaves were carefully protected by wrapping them with aluminium foil. The sprayed leaves and the right-side leaves were separately sampled for independent enzyme investigation. Each experiment was repeated for three times. The activities of superoxide dismutase (SOD) (Rao et al., 1996), peroxidase (POD) (Moerschbacher et al., 1998) and phenylalanine ammonia-lyase (PAL) (Mozzetti et al., 1995) were determined.

3 Results

3.1 Protection of Whangkeumbae pear leaves after SA treatment

Different delays between treatments and inoculation were 1–20 d, and the four doses tested (0, 0.02, 0.2, 2 mmol·L^{-1} active ingredient) were treated to the leaves (Fig. 1). The SA treatment could reduce the black spot disease on

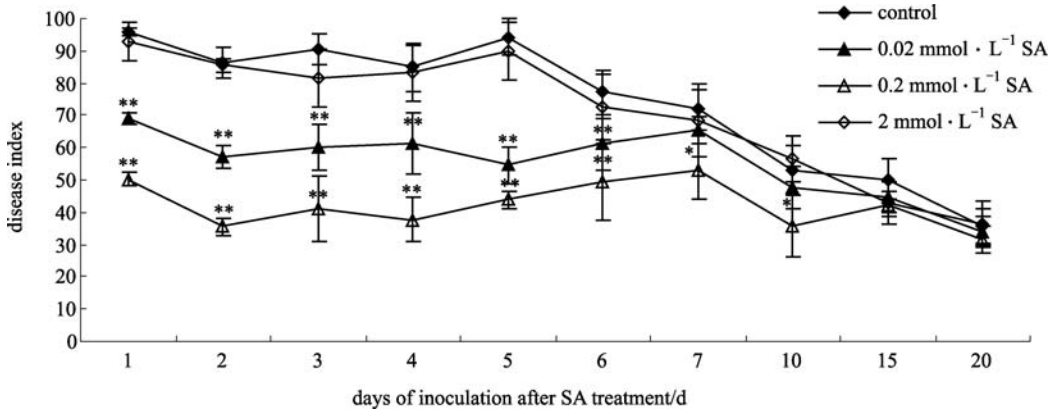

Fig. 1 Effect of pre-treatment SA before inoculation on disease index of Whangkeumbae pear leaves
Note: * denotes significance at 0.05 probability level; ** denotes significance at 0.01 probability level.

Whangkeumbae pear leaves, and with the time going, the effects of SA were decreasing. The SA treatment at 0.02 mmol·L^{-1} or 0.2 mmol·L^{-1} reduced the severity of disease throughout the measurement period, while the treatment with 0.2 mmol·L^{-1} SA was more effective in protecting against pear black spot. 0.2 mmol·L^{-1} SA could induce the resistance of Whangkeumbae pear leaves significantly, and the induced effect could last for 10 d. And a peak of significant protection, compared to control, was obtained in the plants treated at the 2nd day in terms of severity (−59.0%). There was no significant difference in disease index observed between the controls and 2 mmol·L^{-1} SA-treated leaves.

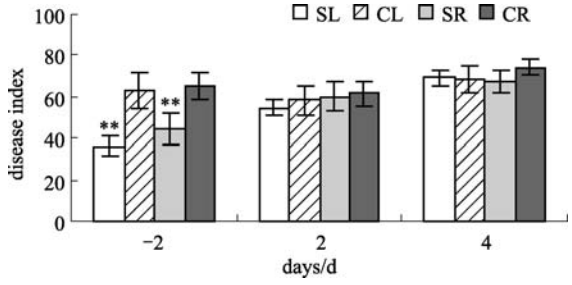

Fig. 2 Effect of SA solutions treatment before or after inoculation on the black spot disease index in the Whangkeumbae pear
Note: ** significance at 0.01 probability level. SL, CL, SR and CR represent the left-part leaves after treatment with 0.2 mmol·L^{-1} SA, the left-part leaves after treated with water, the right-part leaves after treatment with 0.2 mmol·L^{-1} SA and the right-part leaves after treatment with water, respectively.

3.2 Local and systemic resistance

The SA treatment decreased the disease index of both sides (left side and right), and the disease index decreased by 42.4% and 31.2%, respectively, compared to control (Fig. 2). It was said that the effects of SA delivered to the leaves of the right side started the expression of SAR. However, SA was not significantly effective to the leaves, with either 2 or 4 d after infection with *A. kikuchiana* Tanaka. It was suggested that SA could not remedy the leaves infected by the pathogen of black spot disease.

3.3 Correlation of protection with SOD, POD and PAL activities

Although 0.2 mmol·L^{-1} SA significantly alleviated the disease injury in Whangkeumbae pear leaves, further experiment was done to investigate this concentration. Young leaves sampled from Whangkeumbae pear followed by the same schedule of treatments as above were assayed for SOD, POD and PAL activities (Fig. 3). The results showed that activities of three enzymes were significantly higher in SA-treated leaf compared to the control sampling at 2–10 d. Changes in SOD activities showed a rise and

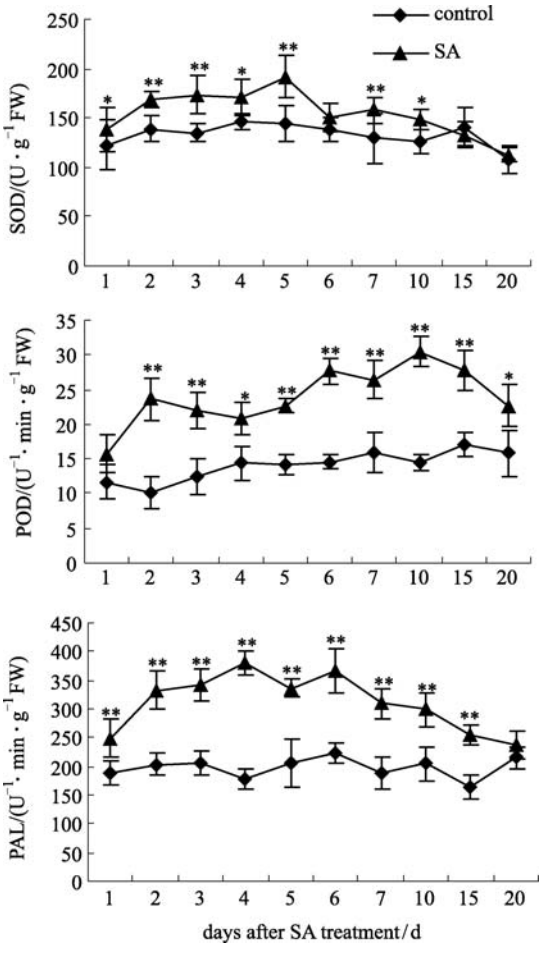

Fig. 3 Effects of exogenous SA on activities of SOD, POD and PAL in Whangkeumbae pear leaves
Note: * denotes significance at 0.05 probability level; ** denotes significance at 0.01 probability level.

then a slow decrease. It reached a peak value on day-5, and the activity of SOD was increased by 33.0% with respect to control. Furthermore, the activity of SOD was not significantly different from the control after 15 d.

The POD activity increased up to day-2 and then declined. A sharp increase in the activity of this enzyme was observed from day-4 to day-10. During the remaining period of measurement, the activity of POD decreased slowly. The activities of POD reached to a peak at day-10 (109.1% in respect to control).

The PAL activity increased up to day-4, but decreased gradually during the next 16 d. At the 4th day, the activity of PAL was increased by 112.7% compared to the control.

3.4 Local and systemic activation of SOD, POD and PAL

When SA treated Whangkeumbae pear leaves two days before inoculation with *A. kikuchiana* Tanaka, applications of SA induced a progressive and significant increase of all enzymes, not only in locally treated tissues but also in

right-side untreated leaves compared with control plants (Fig. 4). For POD and PAL, the level of induction was higher in treated leaves than that in the right-side untreated leaves. However, the SOD activity of untreated leaves was higher than that of the treated left leaves. When treating the infected leaves with SA, three enzymes only increased in locally treated tissues with respect to control, while there was no significant increase in the enzyme activity of the right untreated leaves. It was suggested that SA can not induce the expression of SAR in the infected leaves.

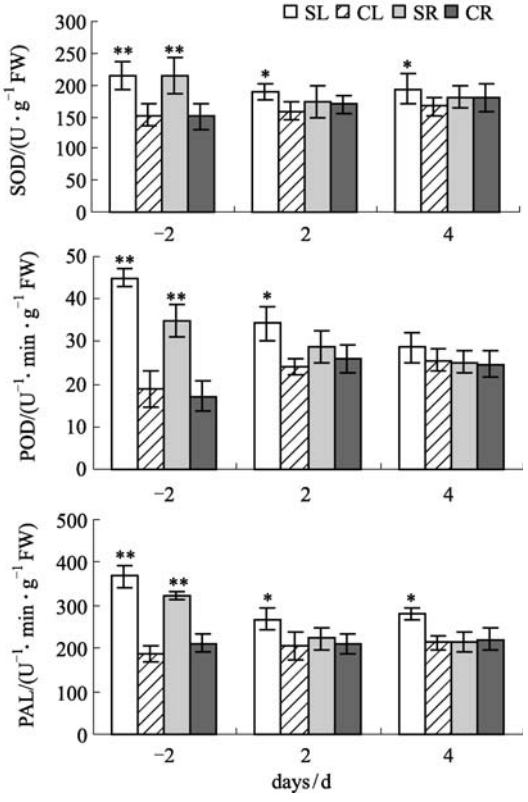

Fig. 4 SA treatment induced of SOD, POD and PAL in leaves of Whangkeumbae pear before or after inoculation on the pear black disease

Note: * denotes significance at 0.05 probability level; ** denotes significance at 0.01 probability level.

4 Discussion

SA provided a significant effect on controlling pear black spot after Whangkeumbae pear leaves infection. In the present study, it has been shown that the treatment with 0.2 mmol·L^{-1} SA was effective in alleviating pear black spot in Whangkeumbae pear leaves (Fig. 1). A lower concentration (0.02 mmol·L^{-1} SA) was ineffective, while 2 mmol·L^{-1} SA had no influence on the development of disease injure symptoms. Gu and Wang (2003) reported that 0.01 mmol·L^{-1} SA provided protection against *Curvularia lunate* Boed in turf-type fescue; however, when more than 0.01 mmol·L^{-1} SA was applied, negative effects were observed. When 10 mmol·L^{-1} SA was applied exogenously to maize, it induced expression of a set of defense genes, thus enhancing resistance against *Helminthosporium turcicum* Pass. (Yu et al., 1999). These results suggest that the effect of SA concentration on alleviating disease injure could vary with species and tissue types.

SA could protect not only the local treated leaves but also the untreated right leaves (Fig. 2). The protection was correlated with the activation of defense mechanisms, as indicated by the induction of three defense enzymes. Because the biological activator of SAR was not measured, we cannot confirm whether SA induces SAR in this plant species or not. However, since this chemical does not demonstrate any bacteriostatic or bactericidal effect (data not shown) and systemically activate three defense-related enzymes, these data provide a primary evidence that SA triggers systemic resistance in Whangkeumbae pear. In the infected leaves, the activity of enzymes was induced by SA (Fig. 4), but the disease index was not lower than that of the control (Fig. 2). It was implied that the metabolic pathway of Whangkeumbae pear was disrupted, and SA could not play the protective effect on the pear. The role of SA might be through many changes in physiological indicators to work. Therefore, in order to know the action principles of SA, more work has to be done.

From the data, the relationship between protection against infection and activation of defensive markers in treated leaves was clear. The protection against pear black spot was generally obtained when SA was applied two days before inoculation, and the sustained induction lasted for 10 d. Mao et al. (2004) believed that different times were needed for SA induction among different plants. The time of SA-induced resistance of rice to leaf blight was two days (Liu and Wang, 2000), and cucumber needs four days (Okuno et al., 1991).

Brisset et al. (2000) believed that the effect of SA against pathogen would depend upon the plant treated. It is likely that each species and perhaps even each cultivar may respond differently to chemical induction. To evaluate the effect of these chemicals, the defensive enzymes could be regarded as a valuable tool. Studies have shown that many antioxidant enzymes were related to plant defense response (Wang et al., 2005; Liang et al., 2006). Our results showed a significant local accumulation of these enzymes and a persistent tendency to their systemic accumulation. Therefore, the activity of defence-related enzymes could be inducted to defend pathogen attacks by SA.

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