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Induction and mechanism of cucumber resistance to anthracnose induced by *Pieris rapae* extract

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Abstract *Pieris rapae* extract was sprayed on the surface of cucumber leaf to determine the induction of resistance to anthracnose. The enzyme activities of peroxidase (POD) and polyphenoloxidase (PPO) were detected on cucumber leaves after *P. rapae* extract induction and pathogen challenge. The results showed that the disease index of cucumber anthracnose was significantly decreased after the cucumber was induced with the *P. rapae* extract at a concentration of $5.0 \text{ mg}\cdot\text{mL}^{-1}$. The POD and PPO activities in foliar-applied *P. rapae* extract without pathogen inoculation (PETO) or with pathogen inoculation (PETI) were relatively higher than those with no-*P. rapae* extract treatment and without pathogen inoculation (CONO) or with pathogen inoculation (CONI), respectively. The results suggested that the increased levels of POD and PPO activities in PETO and PETI play an important role in the induction of resistance to cucumber anthracnose.

Keywords *Pieris rapae* extract, *Colletotrichum orbiculare*, induced resistance, POD, PPO

1 Introduction

Cucumber anthracnose, caused by *Colletotrichum orbiculare* (Berk & Mont.) Arx (*C. lagenarium* (Pass.) Ellis & Halst.), is one of the most destructive diseases of cucurbits. It occurs all over the world where cucumber is grown.

Received October 26, 2007; accepted November 22, 2007

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The symptoms appear on leaves, stems, and fruits. Defoliation caused by stem and foliar infections could result in reductions in bulk yield (Amin and Ullasa, 1981). Outbreaks of anthracnose could result in total losses in some fields (Latin, 1993).

At present, the control of anthracnose mainly relies on applying protective fungicides. However, the disadvantages of chemical fungicides have become more and more obvious. Consequently, more and more researchers have focused on creating new and safe measures for disease control. The activators of plant-induced resistance have opened an avenue for control of the disease. The pathogen, glycoprotein, polysaccharide, salicylic acid, oxalic acid, phosphate, and various pesticides have been implicated as inducers. There have also been several published reports concerning the effect of induced resistance on cucurbits and other hosts. Silicon (Si) was involved in the increased resistance of cucumber to downy mildew by enhancing the antifungal activity of infected leaves (Fawe et al., 1998). The synthetic chemicals Benzothiadiazole (BTH) has been used for induction of systemic acquired resistance (SAR) in wheat, bean, soybean and barley against fungal and bacterial pathogens (Dann and Deverall, 1995). After the first leaf of cucumber was inoculated with a bacterial suspension of *Pseudomonas syringae* pv. *syringae* or *P. syringae* pv. *lachryman*, systematic induced resistance to cucumber anthracnose was observed in cucumber plants (Smith et al., 1991). Recently, it was found that rapidly synthesized C-glycosyl flavonoid phytoalexins, known as cucumerins, accumulated at the sites of fungal penetration within Milsana-elicited cucumber plant (McNally et al., 2004).

The objectives of this study were to investigate the effect of *P. rapae* extract applied as foliar spray for protecting cucumber against *C. orbiculare*, and to demonstrate the relationship between the degree of resistance induced by *P. rapae* extract and the change of the relative enzyme activity. For this purpose, biochemical methods were used to analyze the change of peroxidase (POD) and polyphenoloxidase (PPO) activities.

2 Materials and methods

2.1 Plant production

The seeds of cucumber (*Cucumis sativus*) cv. Shandongmici were directly soaked in 50 mL of distilled water at 50°C. After 10 to 15 min, they were wrapped in moist gauze at 30°C in darkness until germination. Germinated seeds were carefully removed from the gauze and transferred to 10 cm flowerpots filled with a sterilized soil mixture of nutritive soil-vermiculite-perlite (3:1:1) at a density of two seedlings per pot. Seedlings were placed in a climate-controlled growth chamber at temperatures of 24°C (day) and 18°C (night) with 16 hours of light daily.

2.2 Fungal culture and growth conditions

The strain of *C. orbiculare* was cultured on potato dextrose agar (PDA) at 25°C. Conidia were obtained by gently scraping cultures incubated for 7 days and washed in distilled water supplemented with 0.05% Tween-20. They were filtered through a double layer of gauze. The conidial suspension was adjusted to a concentration of 5×10^5 conidia·mL⁻¹ with the aid of a hemacytometer.

2.3 Preparation of *P. rapae* extract

The mature *P. rapae* was air-dried naturally. It was ground to a fine powder. Twenty-five grams of *P. rapae* powder was soaked in 250 mL of 80% ethanol and shaking incubated at 28°C for 12 hours. The extract was filtered and centrifuged at 4500 r·min⁻¹ for 20 min. The supernatant was evaporated under reduced pressure to dryness at a temperature of 52°C. The residue was dissolved in 25 mL distilled water containing 0.05% Tween-20 and stored at 4°C in darkness.

2.4 Plant treatment with *P. rapae* extract and fungal pathogen inoculation

The concentrations of *P. rapae* extract were 2.5, 5.0, 10.0, 20.0 and 100.0 mg·mL⁻¹. They were sprayed onto the fully expanded first true leaves of four-week-old cucumber plants. The upper leaves were protected with plastic bags when systemic resistance was detected. Each treatment had thirty replicates. Control plants were sprayed with distilled water containing 0.05% Tween-20. Three days after induction, a challenge inoculation was imposed to examine the effect of subsequent fungal attack and activities of disease resistance-related enzymes. The conidial suspension of *C. orbiculare* (5×10^5 conidia·mL⁻¹) was inoculated onto the first and second leaves (20 drops per leaf, 10 µL per drop) with a micropipette. Thereafter plants were immediately covered with plastic bags for 24 hours to prevent moisture from evaporating. Disease

evaluation was conducted 15 days after challenge inoculation. Anthracnosed leaves were scored with a rating (*r*) of 0, 1, 3, 5, 7 or 9, denoting proportions of disease-affected parts over the whole leaf area of 0%, <5%, 6%–10%, 11%–25%, 26%–50% and >50%, respectively. Disease index was calculated according to the equation:

$$\text{Disease index} = \left[\sum (r n_r) / 9 N_t \right] \times 100$$

where *r* = rating value, *n* = number of disease leaves with a rating of *r*, and *N_t* = total number of leaves tested.

2.5 Inhibition of *P. rapae* extract to *C. orbiculare*

The *C. orbiculare* was assessed for mycelium growth and conidium germination on non-amended and amended medium with *P. rapae* extract. To assay the rate of mycelium growth, the *P. rapae* extract was added into PDA medium melted at 50°C to 5.0 mg·mL⁻¹. The plates were inoculated in the center with a 6-mm diameter plug of mycelium removed from the actively growing edge of the culture previously grown for 5 days at 25°C in the darkness. Radial growth was then measured. Each treatment contained 3 replicates and the test was performed three times.

To test the percentage of inhibition of the conidium germination, a drop of conidial suspension (5×10^5 conidia·mL⁻¹) was placed onto the surface of non-amended water agar (WA) and WA amended with *P. rapae* extract to 5.0 mg·mL⁻¹. After 12 hours of inoculation at 25°C, 300 conidia per plate were examined for germination rate. The conidia were considered germinated if the length of the germ tube was greater than the width of the conidia. The experiment was conducted three times with three replicates per treatment and data were subjected to pooled analysis of variance (ANOVA). Means were compared using Fisher's least significant difference.

2.6 Measurement of POD and PPO activity

The first true leaves of four-week-old cucumber plants—including (I) no-*P. rapae* extract treatment without pathogen inoculation (CONO); (II) no-*P. rapae* extract treatment but with pathogen inoculation (CONI); (III) foliar-applied *P. rapae* extract without pathogen inoculation (PETO); and (IV) foliar-applied *P. rapae* extract with pathogen inoculation (PETI)—were separately sampled from each treatment (0.5 g of fresh weight) starting 24 hours after pretreatment and subsequently at an interval of 7 days. The samples were stored immediately at -20°C until extraction. Enzyme extraction and POD activity measurements were performed according to Zou (2001) with minor modifications. Absorbance of the crude enzyme solution was measured at 470 nm with a spectrophotometer. Enzyme activity was expressed as the change

in absorbance per minute per gram fresh weight of leaves ($\Delta\text{OD}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$). As the control, distilled water substituted for H_2O_2 . The level of PPO activity was quantified within the same extraction that tested POD activity according to the protocol developed by Li et al. (1991). Enzyme measurement experiments were performed three times. Each time three samples were studied per treatment for each enzyme. Data collected at each sampling date following foliage applications were analyzed separately by ANOVA. Fisher's least significant difference test was used for the mean comparison.

3 Results

3.1 Effect of *P. rapae* extract on inducing cucumber resistance to anthracnose

Foliar application of *P. rapae* extract expressed a significant effect for suppressing subsequent fungal attack (Table 1). For all but the highest concentration tested, the pretreatment of cucumber seedling with *P. rapae* extract reduced the disease index significantly compared to non-treatment control (mean disease index of 51.4 and 56.9). More significantly, the disease index went down to the lowest rate (22.9 and 26.7) at the treatment concentration of $5.0\text{ mg}\cdot\text{mL}^{-1}$ of *P. rapae* extract. From the result, it was obvious that the *P. rapae* extract could enhance the local and systemic resistance of cucumber against anthracnose.

Table 1 Effect of *P. rapae* extract on inducing cucumber resistance against anthracnose

| treatment/ $\text{mg}\cdot\text{mL}^{-1}$ | challenge inoculation/disease index | |
|---|-------------------------------------|--------------------|
| | local induction | systemic induction |
| 2.5 | 35.8b | 44.4b |
| 5.0 | 22.9e | 26.7e |
| 10.0 | 26.8d | 36.2d |
| 20.0 | 29.5c | 40.0c |
| 100.0 | 50.2a | 56.3a |
| CK | 51.4a | 56.9a |

Note: Means within a column followed by the same letter were not significantly different according to Fisher's least significant difference test, $P = 0.05$. The same below.

The *P. rapae* extract treatment did not inhibit mycelia growth of *C. orbiculare* on petri dishes. The colony diameter of *C. orbiculare* on the PDA medium amended with *P. rapae* extract at concentration of $5\text{ mg}\cdot\text{mL}^{-1}$ (80.3 mm) was significantly larger than that on the non-amended PDA control (78.2 mm). In addition, the percentage of conidia germination on WA medium amended with *P. rapae* extract (86.8%) was slightly less than that on the non-amended WA (88.2%), but no significant difference was detected.

3.2 Analysis of POD and PPO enzymatic activity

Induced resistance is typically related to the activity of plant enzymes. The POD activities of CONO, PETO, CONI and PETI plant extract changed at different levels. According to Fig. 1a, the curves of POD activities in CONO and PETO plants were calm \rightarrow rise \rightarrow drop, with peak activity occurring on the 5th day. The POD activity of the PETO plant was higher than that of the CONO plant, notably with an increment of up to 154.8% on the fourth day after *P. rapae* extract treatment. However, the peaks of POD activity in CONI and PETI plants occurred on the 5th and 7th day. In addition, enzyme activity was significantly higher in the PETI plant than in the PETO plant during the period of challenge inoculation.

Figure 1b showed the changes of PPO activity of the CONO, PETO, CONI and PETI plant extracts. In CONO and PETO plants, the curve of PPO activity was drop \rightarrow rise \rightarrow drop, with peak activity occurring on the 1st and the 4th day. However, the peaks of the CONI plant and PETI plant only appeared on the 4th day after induction with an increase of 127.5% and 203.9%, respectively, compared with the CONO and PETO plants. In addition, the increased PPO activity in CONI and PETI plants was very obvious on the 4th day and remained at a high level until the 7th day.

4 Discussion

This was the first report of induced resistance to anthracnose elicited by *P. rapae* extract in cucumber. Although the nature of the *P. rapae* extract has not been identified, the inherent compound possessed basic characteristics as an elicitor that reduces cucumber damage from *C. orbiculare*. It agrees with previous reports that foliar-applied Si significantly suppressed downy mildew (Bowen et al., 1992). The *P. rapae* extract could inhibit the conidium germination of *C. orbicular* but not the mycelium growth in vitro at the concentration used in this study. In a case similar to that of *P. rapae* extract, localized resistance against *Helminthosporium* (= *Cochliobolus*) *carbonum* race 1 induced in maize by prior inoculation with race 2 was associated with the production of unidentified compounds that reversibly inhibited conidial germination (Cantone and Dunkle, 1990). However, many reports exist of the opposite, such as BTH having no effect on fungal conidium germination (Chen et al., 1998). This was possibly due to a difference in the concentrations of the inducer and the pathogen tested, suggesting that the activity of the inducer could differ from one plant-pathogen system to another according to the dose used.

In this study, we found that foliar-applied *P. rapae* extract could stimulate POD and PPO activities, confirming that *P. rapae* extract could elicit a host response. In

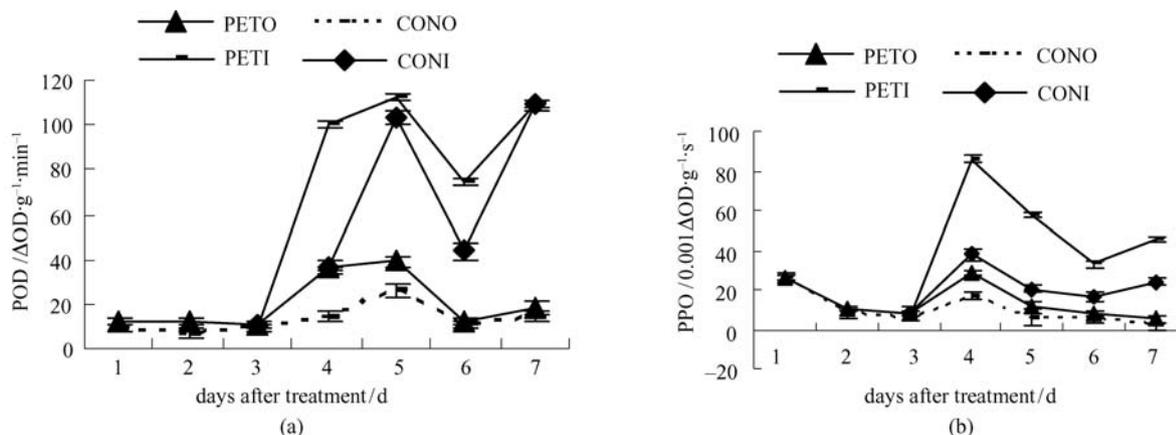


Fig. 1 The activity of POD and PPO in the 1st true leaf after *P. rapae* extract treatment and pathogen-challenge.

addition to being directly antifungal, POD and PPO were involved in the formation of lignin or lignin-containing papillae at infection sites and in the germination of toxic radicals that might function as antimicrobial agents during the earliest defensive response or oxidative burst. The PETO and PETI plants significantly increased POD and PPO activity compared with the CONO and CONI plants. Furthermore, POD and PPO activity levels induced in CONO and PETO plants were relatively low compared with the levels in CONI and PETI plants. The fact that the elevated POD and PPO activity was associated with induced resistance by *P. rapae* extract provides a biochemical marker for further studies on *P. rapae* extract-plant interactions.

Acknowledgements This research was supported by the Natural Science Foundation of Hebei Province, China (No. 300081) and the Youth Foundation of Agricultural University of Hebei, China. The authors are grateful to Dr Wang Yuanchao from the Laboratory of Fungus and Molecular Biology at Nanjing Agricultural University for providing the strain of *C. orbiculare*, as well as to Dr Cao Keqiang from the Bio-control Center of Plant Diseases and Pests of Hebei Province for instrumental assistance.

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