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Defense Reactions by *Clonostachys Rosea* in Tomatoes against *Botrystis Cinerea*

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Abstract. Tomato gray mold disease, caused by *Botrytis cinerea*, is a serious disease of tomato production. *Clonostachys rosea* is an antagonistic microorganism to *Botrytis cinerea*. The reactions of *C.rosea* on the control of gray mold disease in tomato leaves were investigated in this study. To investigate the reactions of *C.rosea* in inducing resistance to tomato plants, four treatments, including *B.cinerea* treatment (treatment 1), *C.rosea* treatment (treatment 2), *C.rosea* and *B.cinerea* treatment (treatment 3) and water (control) were applied on tomato leaves. The result obtained revealed that the *C.rosea* treatment stimulated the activity of the defense related enzymes: Phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO) and peroxidase (POD); and the treatment 3 reduced the incidence and severity of the gray mold. This study indicates that *C.rosea* treatment can enhance the resistance of tomato plants to gray mold; also it is able to evoke enzymes defenses activities in tomato plants infected by *B.cinerea*.

Keywords: *Botrytis cinerea, Clonostachys rosea*, defence enzymes.

1. Introduction

C.rosea is an antagonistic fungus plant pathogens widely present in the soil for the growth of plants, and can produce a series of antibacterial metabolites. It a biocontrol agent that is used to combat and prevent phytopathogenic fungi attacks because of its ability to involve many factors and diverse modes of action. It has been tested successfully as a biological control agent against divergent fungal plant pathogens [1] and [2]. Many isolates of *C.rosea* are highly efficient antagonists against several plant pathogenic fungi, as previous reports have shown in the controls of *B.cinerea* in strawberry, raspberry, and tomato [1].

The cultivated tomato *Lycopersicon esculentum* is an herbaceous plant cultivated around the world. However, tomato crops may be susceptible to damages due to attacks by fungal diseases, bacterial or viral diseases. The gray mold disease caused by *Botrytis cinerea* is one of its most eminent prevalent and destructive diseases. The gray mould in the crop can be controlled by using synthetic fungicides, but the use of fungicides has led to environmental pollution and possible side-effects on human health, as well as the development of resistance to common fungicides [3]. A natural method, safe and effective to the disease control without the use of chemicals exist, specially the use of antagonistic microorganisms.

Certain researchers have showed that peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) are key enzymes conferring disease resistance in plants [4]. In this study, activities of POD, PPO and PAL in leaves of tomato plants treated with *Clonostachys rosea* were analyzed.

2. Materials and Methods

2.1. Plant Materials

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The tomato plants used in this study are homozygous 704 f. Seeds was propagated in the horticultural experimental station of the Northeast Agricultural University.

2.2. Pathogen Culture

Strain antagonist *Clonostachys rosea Gliocladium* was isolated from soil of a lawn in the suburbs of Jilin (North East China) and gray mold of tomato, *C.rosea* was cultured on potato dextrose agar (PDA) for 21 days at 21 ℃.

Botrytis *cinerea* was isolated from infected tomato plants growing in a greenhouse and cultured on potato dextrose agar (PDA) for 15 days at 28 ℃. The spores were suspended in a mixture of distilled water added to 0.01% Tween glucose and 0.01mol/L KH_2PO_4 (pH = 5) 6.7mmol/L and the concentration of spores was adjusted to 10^7 spores $ml^{-1.}$

2.3. Fungal Treatments and Infection

The fungal treatment was conducted by spraying (10 ml/plant). Tomato leaves were washed with sterile distilled water and dried on filter paper and was then treated with sterilized spores. The experiment contains three different types of treatments with a control: treatment 1 is the tomato leaves treated with *B.cinerea* only, treatment 2 is the tomato leaves treated with *C.rosea* only and treatment 3 is the tomato leaves treated first with *B. cinerea*, and 4 hours after the same plants were treated for a second time with conidia and mycelia of *C.rosea* and the control plants were treated with distilled water only. Every experiment was subjected to three replications.

2.4. Disease Assessment

After spraying, tomato plants were immediately transferred to an air-tight plastic bag to maintain a high relative humidity and incubated at 25 ℃. The determination of the activity related to the defense was made by samples of tomato leaves with each treatment administrated for 32 hours at an interval of 4 hours. Treated leaf samples were taken to study the enzymatic activity.

The severity of the symptoms of gray mould disease was evaluated by calculation of the index on a scale of 0 to 5 [4] and [5] , where 0 indicates no necrosis, leaf area is completely healthy; 1= less than 5% of the leaf area are with necrotic symptoms; 2= less than 15% of the leaf area is with necrotic symptoms; 3= less than 25% of the leaf area is with necrotic symptoms; 4= less than 50% of leaf area show necrotic symptoms; 5= more than 50% of leaf area is covered with *Botrytis* symptoms. The formula used to calculate the index of the disease is as follows:

$$DI = \left(\sum i \times j / n \times k\right) \times 100\% \qquad (1)$$

In which i = infection class, j = the number of leaves scored for that infection class, n = the total number of plants in the replicate, and k = the highest infection class.

2.5. Enzyme Activity Assay PAL, PPO and POD

For enzyme assays, fresh leaves tissues were collected at different times after treatment. All enzyme extract procedures were conducted at 4 ℃.

For PAL, 0.5 g of the tissue was ground and mixed with 1ml extracting buffer [0.2 M boric acid buffer containing 10% (w/v) polyviny polypyrolidone (PVPP), 1 mM EDTA, and 50 mM β -mercaptoethanol, pH 8.8], 300 μ l of the extract was incubated with 1 ml 0.02 M L-phenylalanine and 2 ml of the PAL extracting buffer at 24 ℃ for 2 min, and absorbance at 290 nm was measured in an ultraviolet spectrophotometer [6].

For PPO, 1g of the tissue were ground with 5 ml of 100 mM sodium phosphate buffer (pH 6.4) containing 0.2 g of PVPP, homogenized and centrifuged at 12,000 × g at 4 ℃ for 30 min, and the supernatant was collected, extract (100 μl) was incubated with 2 ml 0.05 M phosphate buffer (pH 7.0) and 0.5 ml of 0.5 M catechol at 24 ℃ for 2 min, and the absorbance at 398 nm was measured with an ultraviolet spectrophotometer [7].

For POD, 1g of the tissue were ground with 5 ml of 100 mM sodium phosphate buffer (pH 6.4) containing 0.2 g of PVPP, homogenized and centrifuged at 12,000 × g at 4 ℃ for 30 min, and the supernatant

was collected, POD activity was determined using guaiacol as substrate. The reaction mixture consisted of 0.1 ml of crude extract and 2 ml of guaiacol (8 mM, in 100 mM sodium phosphate buffer, pH 6.4), incubated for 30 min at 30 ℃. The increase in absorbance at 460 nm was measured after 1 ml H_2O_2 (24 mM) was added [8].

3. Results

3.1. Effect of *C.rosea* on Tomato Gray Mould

The per cent disease index was calculated using the disease score card for *B.cinerea*. It was observed that, the per cent disease index of the tomato leaves treated with *C.rosea* and then inoculated with *B.cinerea* was significantly lesser when compared to the leaves treated only with *B.cinerea* (Fig. 1). *C.rosea* treatment can enhanced the resistance of tomato plants to *B. cinerea* infection.

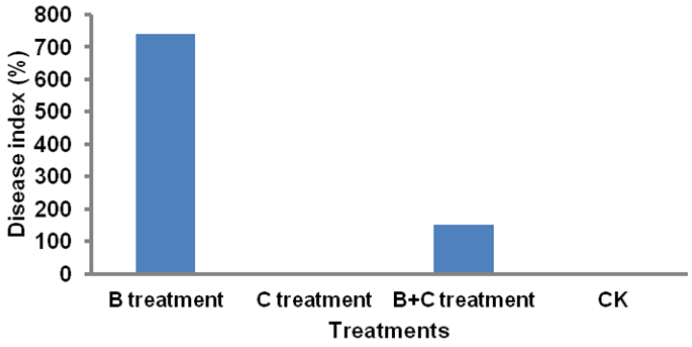

Fig. 1: Effects of C.rosea on gray mold caused by B. cinerea on tomato leaves

3.2. *C.rosea* Treatment Increased Activities of Defense Enzymes in Leaves

PPO and guaiacol peroxidase (POD) showed an increase in activity within the first few four hours after the treatment was applied. This increase was especially significant in the leaves treated only with antagonism agent and in the leaves treated with *C.rosea* and then inoculated with pathogen. The peak POD accumulation was detected at 16h after treatment in the leaves treated with *C.rosea* and then inoculated with pathogen, and the peak PPO accumulation was detected at 20h also in treatment 3 before gradually decreasing out (Fig. 2 B and Fig. 2C).

Although PAL activity was slowly increased to eight hours after treatment, a maximum peak was observed after 20h especially in leaves treated with *C.rosea* and then inoculated with pathogen, this accumulation was followed by a gradual decreasing (Fig. 2 A).

4. Discussion

The *C. Rosea* success as a bio control agent is believed to involve many factors and diverse modes of action [9]. Our results revealed that application of *C.rosea* significantly increased the expression level of defense enzymes activity (Fig. 2). Observing the expression level of the activity of defence enzymes, all three treatments presents the higher level of activity at 20h, this is the same for PAL and POD, so the higher protection against *B.cinerea* can occur at 20 h for PAL and POD and at 16h for PPO.

PAL is a key enzyme of the phenylpropanoid pathway and takes part in the synthesis of phenolic compounds, phytoalexin and lignin [10]. PPO participates in the oxidation of many types of phenolic compounds leading to the production of quinones that are extremely toxic to several pathogens [11]. POD is generally considered important in host resistance mechanisms as it catalyzes the last step of lignin biosynthesis [12]. These substances are associated with the process of local disease defence [13]. Comparing the expression level of defense enzymes activity in this study between the three different types of treatments, it was revealed that the application of treatment 3 performed best.

In conclusion the application of *C.rosea* can confer protection on tomato plants from *B.cinerea* invasion.

C.rosea treatment can enhance the resistance of tomato plants to *B.cinerea* infection; and also able to evoke enzymes defenses activities in tomato plants infected by *B.cinerea*.

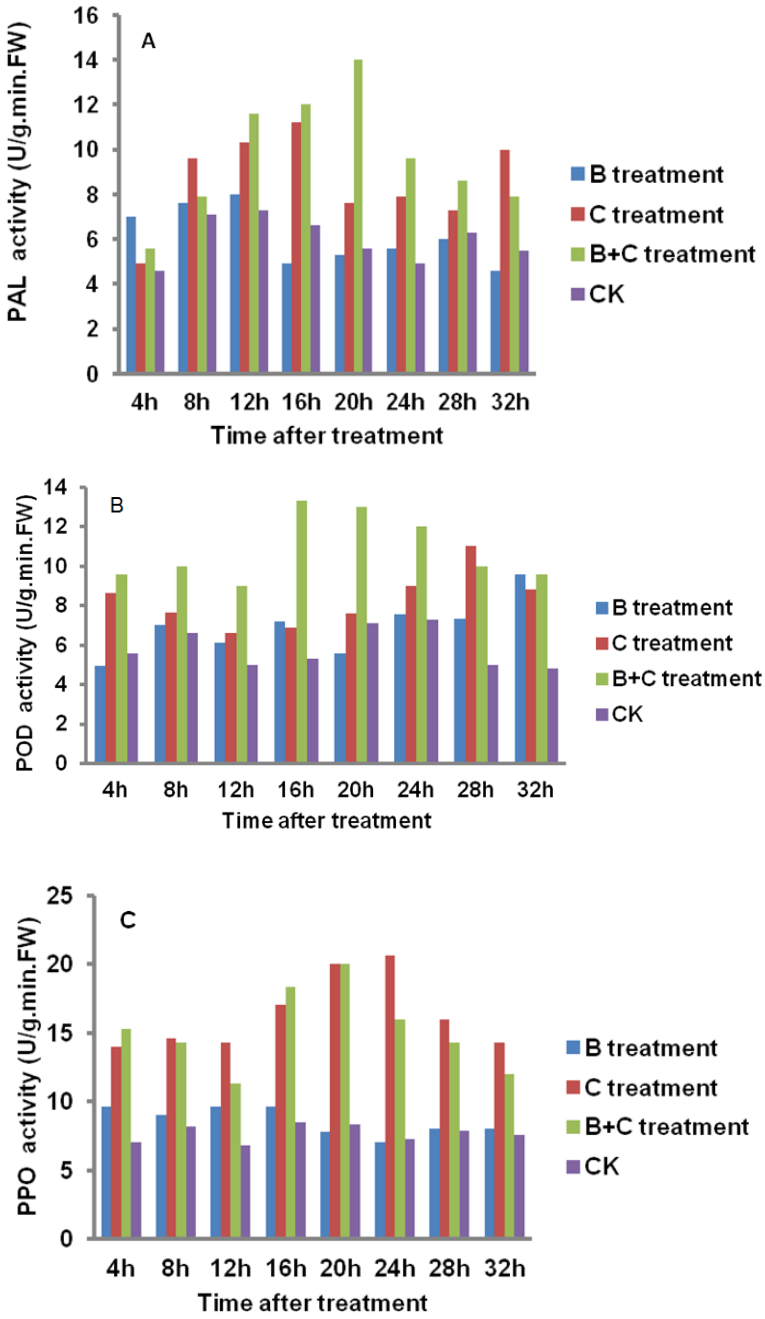

Fig. 2: Activity of defense enzymes in leaves of tomato treated with *B.cinerea*, *C.rosea*, *C.rosea* + *B.cinerea*, and distilled water.

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