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Biological Control of *Botrytis Cinerea* in Tomato Leaves

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Abstract. *Clonostachys rosea* (*C.rosea*) is known to induce resistance against a number of plant diseases; it is an antagonistic microorganism to *Botrytis cinerea* (*B.cinerea*). The effects of *C.rosea* on the control of gray mold disease caused by *B.cinerea* in tomato leaves were examined in this study. To examine the reactions of *C.rosea* in inducing resistance in tomato plants, three treatments, including *B.cinerea* treatment (treatment B), *C.rosea* treatment (treatment C), *C.rosea* and *B.cinerea* treatment (treatment C+B) and water (control), to be applied on tomato leaves were set up. The results indicated that the *C.rosea* treatment stimulated the activity of the superoxide dismutase (SOD), the nitric oxide (NO) and hydrogen peroxide (H_2O_2), while the treatment (C+B) reduced the incidence and severity of gray mold. These results indicate that *C.rosea* treatment has the potential to control gray mold of tomato plants and it can induce the activities of SOD, NO and H_2O_2 in tomato leaves infected with *B.cinerea*.

Keywords: *Botrytis cinerea*, *Clonostachys rosea*, hydrogen peroxide, nitric oxide, resistance, superoxide.

1. Introduction

Botrytis cinerea (*B.cinerea*) is a phytopathogenic fungus capable of infecting more than 200 plant species [1]. *B.cinerea* which causes gray mold, is capable of infecting plant tissue and causing serious pre- and post-harvest diseases, in agronomically important crops and harvested commodities, such as grapevine, tomato, strawberry, cucumber, pear, cherry, strawberry , eggplant , carrots, lettuce, and peppers, etc. *B.cinerea* conidia are ubiquitous in the air and can be transported by wind over long distances before infecting the next host [1].

Tomato (*Solanum lycopersicum*) crops are susceptible to *B.cinerea* although some cultivars show some quantitative resistance. Control of gray mold in the crop relies on spraying systemic fungicides and/or the application of biocontrol agents, but the Continuous use of fungicides has faced two major obstacles: increase in public concern regarding contamination of perishables with fungicidal residues, and proliferation of resistance in the pathogen populations [2]. The biocontrol agents are a natural, safe, and effective alternative to the control of the disease without the use of fungicides. *Clonostachys rosea* (*C.rosea*) suppresses development and sporulation potential of *B.cinerea* in plants through nutrient competition, hyperparasitism, competitive colonization of senescing and dead tissues, and other control modes [3] and [4]. Treatment with *C.rosea* conferred enhanced resistance against *B.cinerea* in *Rhizoctonia solani* on tobacco [5], and as an entomopathogenic fungus of insects *Oncometopia tucumana* and *Sonesimia grossa* [6].

Superoxide dismutase (SOD) catalyzes two molecules of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2), and its role is to maintain a steady level of H_2O_2 and O_2 in the cell state. Nitric

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oxide (NO) and H_2O_2 play an important role in the response of plants against pathogenic agents. In this study, activities of SOD, NO and H_2O_2 in leaves of tomato plants treated with *C.rosea* were analyzed.

2. Materials and Methods

2.1. Plant Materials

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 Inoculums

Strain antagonist *C.rosea Gliocladium* was isolated from soil of a lawn in the suburbs of Jilin (North East China) and gray mold of tomato, and was cultured on potato dextrose agar (PDA) for 21 days at 21 ℃. *B.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 distilled water to which was added 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 (1.0 x 10^5spores/ml). Tomato leaves were washed with sterile distilled water, dried on filter paper before been treated with the sterilized spores. Besides the control, three different types of treatments were applied: treatment B is the tomato leaves treated only with *B.cinerea*, treatment C is the tomato leaves treated only with *C.rosea*, and treatment (C+B) is the tomato leaves treated first with *C.rosea*, and 4 hours after the same plants were treated for a second time with conidia and mycelia of *B.cinerea*, 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 sampling of tomato leaves with each treatment administrated at an interval of 4 hours up to 32 hours. Treated leaf samples were taken to study the enzymatic activity and the expression of the second messenger.

The severity of the symptoms of gray mould disease was evaluated by calculation of the index on a scale of 0 to 5 on the basis of the Murray method with modifications [7], where 0 indicates no necrosis, here leaf area is completely healthy; 1= less than 5% of the leaf area are with symptoms; 2= less than 15% of the leaf area is with symptoms; 3= less than 25% of the leaf area is with symptoms; 4= less than 50% of leaf area; 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:

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

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. Assays of Superoxide Dismutase (SOD)

The activity of superoxide dismutase (SOD) was assayed by measuring its ability to inhibit the photochemical reduction of NBT following Beauchamp procedure with minor modifications [8]. A small amount (0.2 g) of the leaf tissue was homogenized at 0 ℃ - 4 ℃ in 1.5 ml of 50 mM phosphate buffer, pH 7.8. The homogenate was centrifuged at 15000 × g for 15 minutes and the supernatant obtained was used as enzyme extract, a volume of 3 ml of our reaction mixture contained 2.5 ml of 13μM methionine, 0.15 ml of 13 μM riboflavin, 0.25 ml of 75 μM NBT and 0.05 ml of 50 mM phosphate buffer (pH 7.8), and 0.05 ml of enzyme extract. Instead of NBT, the mixture contained a phosphate buffer to serve as control. Riboflavin was added last and the reaction was initiated by placing the tubes under 4000 lx light for 20 minutes. The absorbance was read at 560 nm. The volume of enzyme extract corresponding to 50% inhibition of the reaction was considered to be one enzyme unit.

2.6. Assays of Nitric Oxide (NO) and Hydrogen Peroxide (H2O2)

The extraction of nitrite was performed by the procedure described by [9]. Nitrite content in the filtrates was determined by the Griess reagent, the absorbance of the solution was measured at 548 nm.

The quantification of hydrogen peroxide (H_2O_2) in extracts from tomato leaves was measured by monitoring the absorbance of a titanium peroxide complex at 410 nm, following the methods of [10].

3. Results

3.1. Effect of *C.rosea* Treatment on Tomato Gray Mold

Results show that, the per cent disease index of the tomato leaves treated with *C.rosea* 4h before the inoculation was significantly lesser when compared to the leaves treated only with *B.cinerea* (Fig. 1). *C.rosea* treatment had significantly protective and curative effect against gray mould in the tomato leaves.

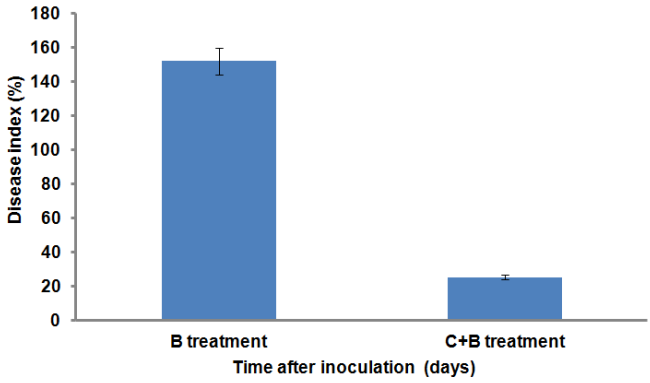

Fig. 1: Effects of *C.*rosea on gray mold caused by *B.cinerea* on tomato leaves.

3.2. Effect of *C.rosea* Treatment On Activities of SOD, H_2O_2 and NO in Tomato Leaves

SOD showed exponential growth in activity. This exponential growth was especially significant in the leaves treated only with antagonism agent and in the leaves treated with *C.rosea* and then inoculated with pathogen, but a maximum peak was observed after 24h especially in the leaves treated with *C.rosea* and then inoculated with pathogen (Fig. 2).

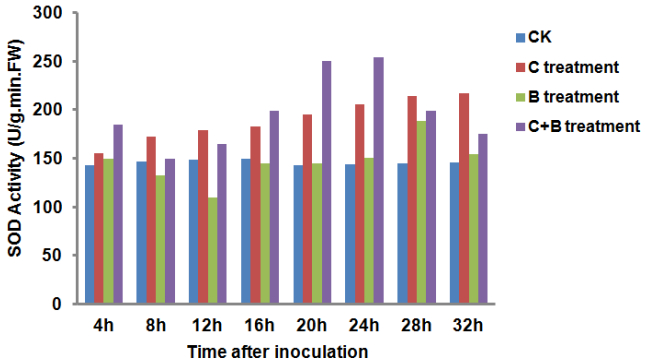

Fig. 2: Activity of SOD enzymes in leaves of tomato treated with *B.cinerea*, *C.rosea*, *C.rosea* + *B.cinerea* and distilled water.

NO showed an increase in activity in the first few four hours after the treatments were applied. This increase was especially significant in the leaves treated only with antagonism agent, but a maximum peak was observed after 24h especially in the leaves treated with *C.rosea* and then inoculated with pathogen (Fig. 3).

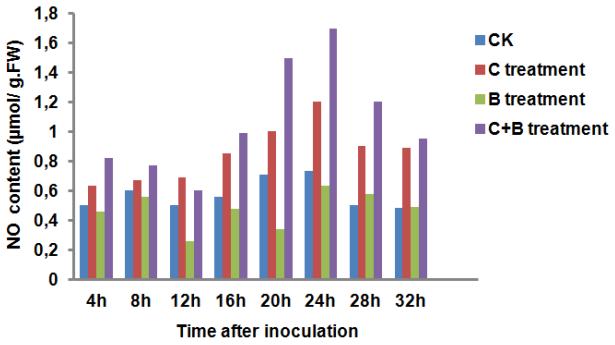

Fig. 3: Content of NO in leaves of tomato treated with *B.cinerea*, *C.rosea*, *C.rosea*+*B.cinerea* and distilled water.

H_2O_2 activity was unstable with an increase in activity in the first few four hours after the treatment. This increase was especially observed in the leaves treated only with antagonism agent, but a maximum peak was observed after 24h especially in the leaves treated with *C.rosea* and then inoculated with pathogen, this accumulation was followed by a gradual decreasing (Fig. 4).

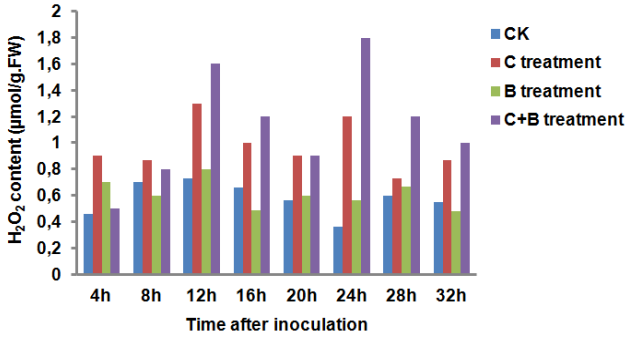

Fig. 4: Content of H_2O_2 in leaves of tomato treated with *B.cinerea*, *C.rosea* + *B.cinerea* and distilled water.

4. Discussion

Certain studies have shown that *C.rosea* is an effective biofungicide for management of gray mold, caused by *B.cinerea* [3], [11]. Results obtained in this experiment further supports previous research results that the production of spores of *C.rosea* is effective in the control of *B.cinerea* on tomato leaves (Fig. 2-Fig. 4). Observing the expression level of the activity of SOD, NO and H_2O_2, all three treatments presented the highest level of activity after 24h, hence the spores of *C.rosea* can ensure a better protection against *B.cinerea* after 24h.

SOD is an antioxidant enzyme that induces a response to environmental stress in tomato [12]. NO and H_2O_2 have been shown to be important signalling molecules that participate in the regulation of several physiological processes [13]. Comparing the expression level of SOD, NO and H_2O_2 in this study among the three different types of treatments, treatment 3 was observed to perform best. So *C.rosea* can produce signals that respond to the protection of tomato plants against *B.cinerea*.

In conclusion, *C.rosea* can control gray mold disease caused by *B.cinerea* in the tomato plant evoking the activity of SOD, NO and H_2O_2; also it can ensure a resistance against *B.cinerea*.

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