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Synergistic effect of the combined bio-fungicides ε-poly-L-lysine and chitooligosaccharide in controlling grey mould (*Botrytis cinerea*) in tomatoes

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ABSTRACT

The antifungal properties and the induction of resistance by ε-poly-L-lysine (ε-PL) and chitooligosaccharide (COS) were examined to find an alternative to synthetic fungicides currently used in the control of the devastating fungal pathogen *Botrytis cinerea*, the causal agent of grey mould disease of tomatoes. As presented herein, this combined treatment (200 mg/L ε-PL + 400 mg/L COS) was found to have optimal *in vitro* antifungal activities, achieving an inhibition rate of 90.22%. *In vivo* assays with these combined bio-fungicides, under greenhouse conditions using susceptible tomato plants, demonstrated good protection against severe grey mould. In field tests, the combined bio-fungicides had a control effect of up to 66.67% against tomato grey mould. To elucidate the mechanisms of the combined bio-fungicide-induced resistance in the tomato, plants were subjected to three treatments: 1) inoculation with *B. cinerea* after spraying with 200 mg/L ε-PL alone, 2) inoculation with the combined bio-fungicides, and 3) inoculation with 400 mg/L COS alone. Compared to the control (sterile water), increases in salicylic acid (SA) and jasmonic acid (JA) levels and increased phenylalanine ammonia lyase (PAL), peroxidase (POD), and superoxide dismutase (SOD) activities were observed. Catalase (CAT) activity and abscisic acid (ABA) and gibberellin (GA) levels decreased, particularly in the combined bio-fungicide-treated plants. Altogether, these findings reveal that the combined bio-fungicides (200 mg/L ε-PL + 400 mg/L COS) should be an excellent biocontrol agent candidate that combines direct antifungal activity against *B. cinerea* with plant resistance.

1. Introduction

Botrytis cinerea, the causal agent of grey mould, causes large economic losses worldwide, including on a variety of soft fruits, vegetables and flowers, and especially greenhouse-grown tomatoes (*Lycopersicon esculentum*). In addition to having a broad range of hosts, this pathogen produces large numbers of spores and is able to survive in a dormant state in soil. *B. cinerea* infects the leaves, stems, flowers, and fruits of plants *via* multiple strategies, including the secretion of cell wall-degrading enzymes, phytotoxic metabolites, and cell death elicitors (Huang et al., 2012). A high relative humidity, free moisture on plant surfaces and moderate temperatures are considered the most important environmental factors that promote infection by *B. cinerea* (O'Neill et al., 1997). Consequently, tomato production loss due to *B. cinerea* can reach 20%, and under the worst conditions, tomato production could be reduced by 40–50% (Zhao et al., 2011). Due to a lack of resistant tomato germplasm, no effective *B. cinerea*-resistant tomato cultivars have been bred to date. Currently, chemical control remains the main method to reduce tomato grey mould in greenhouses. However, the indiscriminate use of chemical agents may result in problems such as the development of pathogen resistance in addition to leaving toxic residues in fruit and posing potential risks to the environment, human and animal health (Nakajima and Akutsu, 2014). The development of pesticide-resistant strains, which would eliminate the use of pesticides, is a goal of considerable interest within the framework of a sustainable, economically profitable agriculture. Consequently, these issues require innovative alternative control methods to successfully increase agricultural productivity and meet future food demands in a sustainable manner (Luna, 2016).

ε-Poly-L-lysine (ε-PL) is a homo-poly-amino acid characterized by the peptide bond between the carboxyl and α-amino groups of L-lysine. It is composed of a series of 25–35 L–lysine monomers and has a molecular weight of around 5000 Da. Shima and Sakai (1977) found ε-PL to be one of the Dragendorff-positive compounds in the culture filtrate of an actinomycete, *Streptomyces albulus* 346, isolated from soil. ε-PL exhibits a broad spectrum of inhibitory activities against Gram-positive and negative bacteria, fungi, yeasts, and bacteriophages (Chang et al., 2010; Shima et al., 1984). The antimicrobial activity of ε-PL occurs as a

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physical action on the cytoplasmic membrane, and antimicrobial mechanism of ε-PL being an electrostatic interaction with the microbial cell surface, followed by disorganization of the membrane and abnormal distribution of the cytoplasm ultimately leading to physiological damage of the microbial cells (Yamanaka and Hamano, 2010). Due to its efficacy and safety, ε-PL has GRAS status (Generally Recognized As Safe) in a variety of food applications and is nontoxic to eukaryotic cells in chronic and acute animal studies using high dosages (Hiraki et al., 2003). However, ε-PL can be subject to rapid depletion after initial application and lose activity quickly (Bi et al., 2016). Studies have been carried out to improve the effectiveness of ε-PL, such as through combination with organic acids (Geornaras and Sofos, 2005) and its covalent immobilization to multi-walled carbon nanotubes for constructing a nanocomposite with enhanced antibacterial activity (Zhou and Qi, 2011).

The attraction of concertedly using multiple preparations to control the same pathogen is that the likelihood of selection for resistance to each individual preservative is greatly reduced. In recent years, all-natural methods for controlling grey mould have become increasingly applicable and are quickly becoming very important in plant protection. Thus, we chose to investigate any possible synergies between ε-PL and chitooligosaccharide, two all-natural substances with antimicrobial properties.

Chitooligosaccharides (COS) are the degraded products of chitosan or chitin, which have been produced recently by several methods such as enzymatic and acidic hydrolysis (Kim et al., 2003). Generally, the molecular weights of COS are 10 kDa or less. The formation of polyelectrolyte complexes between COS and negatively charged groups on the cell surface directly interferes with the growth and normal physiological functions of fungi, suggesting that the charge distribution of COS has a correlation with its antifungal activity (Hirano and Nagao, 1989). Plant-origin COS have been shown to elicit defence responses and to induce protection against *B. cinerea* in grapevine, cucumber plants and tomato fruits (Aziz et al., 2006; Badawy and Rabea, 2009; Ben-Shalom et al., 2003).

Plants have evolved a series of effective defence mechanisms against pathogen invasion that result in highly coordinated sequential changes at the cellular level, including altered levels of signaling molecules. These signaling molecules are involved in plant defence mechanisms and include phytohormones such as salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), indole acetic acid (IAA), and gibberellin (GA). These molecules trigger changes in the expression of defence genes, resulting in metabolic alterations that increase plant defence responses (Mai and Kinga, 2014; Quazi et al., 2015). Additionally, defence enzymes play pivotal roles in host plant resistance against pathogen invasion, and reductions in disease severity in stressed plants have been attributed to changes in the activities of defence enzymes (Binutu and Cordell, 2000; Marrs, 1996), such as phenylalanine ammonia lyase (PAL), catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD).

The objectives of this study were to study the synergistic action of the combined substances (ε-PL and COS) against *B. cinerea* by (a) evaluating the antifungal activity of the combined (ε-PL and COS) *in vitro* and *in vivo*, (b) investigating the mechanism by which changes in signaling molecules (SA, JA, ABA, IAA, GA) and defence enzyme (CAT, PAL, POD, SOD) activity induce resistance. Our study will contribute to understanding the mechanisms of *B. cinerea* biocontrol by the combined bio-fungicides (ε-PL and COS) in an effort to further improve resistance to grey mould disease in the tomato.

2. Materials and methods

2.1. Fungal isolation and storage

Botrytis cinerea strain B05.10 was provided by the Laboratory of Plant Pathology, College of Plant Protection, Huazhong Agricultural University, Wuhan, China. *Valsa mali*, *Alternaria solani*, *Pseudocercospora fijiensis*, *Fusarium oxysporum*, *Rhizoctonia cerealis*, *Gloeosporium musarum*, *Sclerotinia sclerotiorum*, *Cercospora fagopyri*, *Sclerotium cepivorum*, *Glomerella cingulata*, *Cochliobolus sativus* and *B. squamosa* were kindly provided by College of Plant Protection, Northwest A&F University, Shaanxi province, China. These pathogens were cultured on potato dextrose agar (PDA) medium.

2.2. Preparation of spore suspensions

B. cinerea spore suspensions were prepared using spores collected from a one-week pathogen culture suspended in sterile water supplemented with 0.3% Tween-80. The spore suspension was filtered to adjust the concentration of the suspension to 1.0×10^6 conidia/mL using a sterile sieve. The conidial spore concentration was measured using a Bürker chamber.

2.3. Synergistic effect of combined treatment on the mycelial growth of B. cinerea

The antifungal activity of the combined substances (ε-PL and COS) was assessed by the radial growth test on PDA. The COS solutions at four different concentrations (2×10^2, 4×10^2, 8×10^2, and 1.6×10^3 mg/L) were prepared with sterile water supplemented with 0.3% Tween 80. Two experiments were performed. Experiment 1 measured the mycelial growth of *B. cinerea* after treatment with different concentrations of COS. Experiment 2 measured the mycelial growth of *B. cinerea* after treatment with the combined substances ε-PL (2×10^2 mg/L) and different concentrations of COS. Different solutions were mixed with sterile PDA to prepare PDA mediums. PDA solutions were poured into 90 mm Petri dishes, and 5 mm plugs of *B. cinerea*, collected in the outer areas of active cultures, were inoculated onto the prepared plates. Three replicate plates for the fungus were incubated with a regular photoperiod of 12 h light/12 h dark at 23 °C. Procymidone (80% wettable powder, WP, Shaanxi Meibang Agrochemical Co., Ltd.) was the positive control. Control plates containing the medium mixed with sterile water (10%, by volume) were included. After incubation for 4 days, the diameter of mycelium growth of the fungus (mm) in both treated (T) and control (C) Petri dishes was measured in perpendicular directions until the fungus growth in the control dishes was almost complete. The percentage of growth inhibition (I) was calculated using the formula:

$$I\,(\%) = [(C - T)/(C - C_0)] \times 100.$$

C_0 means the diameter of the test fungus agar discs (5 mm).

Similarly, the antifungal activity of the combined substances (200 mg/L ε-PL + 400 mg/L COS) against twelve fungal species was calculated by contact assay based on mycelial growth inhibition using PDA medium. Different control plates containing the medium mixed with sterile water (10%, by volume) were included for each species. On the basis of the optimum growth habit of different species, the plates were incubated at 22–28 °C for 3–5 days until the fungus growth in the control dishes was almost complete. The percentage of growth inhibition was calculated using the above formula. This experiment was replicated three times.

2.4. The combined treatment induction to resistance in the pot experiments

Tomato seeds (*L. esculentum*, Oulong) were obtained from the College of Plant Protection, Northwest A&F University, Shaanxi, China. Seeds were surface sterilized with 3% sodium hypochlorite for 3 min and immediately rinsed with sterile distilled water three times. Each seed was planted in a 15 cm diameter pot filled with sterile soil in the greenhouse. At the 5–6 leaf stage, different bio-fungicides were sprayed onto the leaves 3 days prior to inoculation by *B. cinerea* B05.10 mycelium discs (0.5 cm diameter) (Sun et al., 2017). Each treatment consisted of three replicates, with six potted plants sprayed with 40 mL bio-fungicide each replicate. The greenhouse temperature was maintained

at 23 °C, and the relative humidity was between 90% and 95%. The size of lesions was measured on the 3rd day after inoculation (Cheng et al., 2014). These experiments were repeated three times. The following treatments were investigated: (A) ε-PL (200 mg/L); (B) COS (400 mg/L); (C) ε-PL (200 mg/L) + COS (400 mg/L); (D) procymidone (positive control); and (E) distilled water (negative control).

2.5. Control effect of combined treatment against B. cinerea in the field

The experiment with five treatments, (A) ε-PL (200 mg/L); (B) COS (400 mg/L); (C) ε-PL (200 mg/L) + COS (400 mg/L); (D) procymidone (positive control); and (E) tap water (negative control) were carried out from January 2016 to May 2017 in the field (Yangling, China). This field experiment adopted a random block design with four replicates. The day and night temperatures and relative humidity were between 20 and 25 °C and 14–18 °C and 70%–85% RH. Each treatment was composed of three 90 m^2 (6 m × 15 m) plots. Oulong, a susceptible tomato line, was planted with row and plant spaces of 40 cm and mulched with plastic film. Tomato leaves were sprayed evenly with the different test agents and the spraying quantity was 18 L per 90 m^2 tomato plants. All of the sprayings were conducted at the same time.

Disease development was recorded on the 7th day after each application. Disease severity on leaves was scored using the following scale: 0 = no diseased leaves; 1 = 0–5% of leaves covered with lesions; 3 = leaves with infection lesions making up 6–10%; 5 = leaves with infection lesions making up 11%–20%; 7 = leaves with infection lesions making up 21%–40%; and 9 = leaves with infection lesions making up 41%–100%. The disease index was calculated using the following equations:

$$\text{Disease index (DI)} = [\Sigma(\text{the number of diseased plant leaves at a disease score} \times \text{the disease score})/(\text{total plant leaves investigated} \times 9)] \times 100\%.$$

Following the protocol enacted by the Bioassay Lab, Institute for the Control of Agrochemicals, Ministry of Agriculture, China (1993), the control effects were calculated using the following formula:

$$E = [(C - T)/C] \times 100$$

where E is the control effect (%), C is the DIs of tap water to the control plot, and T is the DIs of the chemical application in the test plot.

2.6. Determination of SA, JA, ABA, IAA, and GA contents

The assays for measuring SA, JA, ABA, IAA, and GA levels are described by Cui et al. (2015). Tomato leaves from pot experiments were used for the phytohormone assays. Different bio-fungicides or water was applied 3 days prior to *B. cinerea* spores inoculation. In total, we analyzed four conditions: sprayed with sterile water (control), sprayed with 200 mg/L ε-PL, sprayed with combined bio-fungicides (200 mg/L ε-PL + 400 mg/L COS), and sprayed with 400 mg/L COS. Leaves treated with the chemicals were then separately collected 0, 12, 24, and 48 h after inoculation according to Gong et al. (2017), with some modification. 0.6 g of leaves was ground into a powder in liquid nitrogen and extracted over 12 h using 20 mL of 80% cold aqueous methanol (< 0 °C) in the dark at 4 °C. This extract was then centrifuged at 5,000 × *g* at 4 °C for 15 min. The supernatant was retained. The residue was extracted three times using fresh methanol. The total methanolic extract was collected by washing three times with 0.6% acetic acid followed by concentration at 40 °C in a rotary evaporator. These solutions were prepared in a 2 mL volumetric flask. Samples were filtered through a 0.45 μm membrane and analyzed using HPLC (Waters, USA).

2.7. Defence-related enzyme (CAT, PAL, POD, and SOD) activity assays

To explore the physiological mechanisms of the combined-enhanced plant disease resistance, we studied the effects of different bio-fungicides on the defence-related enzyme (CAT, PAL, POD, and SOD) activities in tomato leaves. Tomato leaves on pot experiments were used for defence-related enzyme assays. Different bio-fungicides were applied 3 days prior to *B. cinerea* spores or water inoculation. In total, we analyzed four conditions: sprayed with sterile water (control), sprayed with 200 mg/L ε-PL, sprayed with combined bio-fungicides (200 mg/L ε-PL + 400 mg/L COS), and sprayed with 400 mg/L COS. Leaves treated with these chemicals were then separately collected 0, 6, 12, 24, and 48 h after inoculation according to Lu et al. (2013), with some modification. The superoxide dismutase (SOD) activity was determined using the nitroblue tetrazolium (NBT) photochemical reduction method described by Giannopolitis and Ries (1977). The inhibition rate of NBT reduction was determined at 560 nm. The peroxidase (POD) activity was determined using the guaiacol colorimetric method described by Cakmak and Marschner (1992). The kinetic change of OD 470 in 1 min was used to calculate the enzymatic reaction rate. The catalase (CAT) activity was determined using the method described by Beers and Sizer (1952). The kinetic changes of OD_{240} in 1 min were used to calculate the enzymatic reaction rate. The phenylalanine ammonia lyase (PAL) activity was determined using the method described by Brueske (1980).

2.8. Data processing

Statistical analyses were performed using SAS (ver. 9.2). All measurements made at specific times were compared using Student's *t*-test at $P < 0.05$.

3. Results

3.1. Synergistic effect of combined treatment on B. cinerea

The inhibition rate was markedly increased following the combined treatment (200 mg/L ε-PL + 400 mg/L COS) compared with the ε-PL used alone (Table 1). Four days after inoculation with *B. cinerea*, single bio-fungicide COS significantly inhibited mycelial growth ($P < 0.05$) at all concentrations. However, the combined treatment showed a stronger inhibition effect than a single COS at the same concentration. COS could increase the inhibitory effect of ε-PL against *B. cinerea* and the synergistic effect of the combined treatment significantly increased with the increase of its COS concentration. The combined treatment (200 mg/L ε-PL and ≥ 400 mg/L COS) obviously inhibited the mycelial growth, but this inhibition did not differ significantly from procymidone ($P > 0.05$). The inhibition ratio of the combined treatment (200 mg/L ε-PL + 400 mg/L COS) reached 90.22%. Based on these

Table 1
Mycelial growth (mm) of *B. cinerea* on PDA media supplemented with ε-PL and COS.

Compound fungicide concentration (mg/L)		Growth (mm)	Inhibition rate (%)
ε-PL	COS		
0	200	69.00 ± 1.26^{b}	16.53 ± 1.64^{d}
0	400	61.17 ± 3.77^{b}	26.74 ± 4.92^{d}
0	800	18.50 ± 0.50^{d}	82.39 ± 0.65^{b}
0	1600	18.67 ± 2.0^{d}	82.17 ± 2.72^{b}
200	0	35.00 ± 4.04^{c}	60.87 ± 5.27^{c}
200	200	18.33 ± 1.86^{d}	82.61 ± 2.42^{b}
200	400	12.50 ± 0.76^{de}	90.22 ± 1.00^{ab}
200	800	11.75 ± 2.18^{de}	91.20 ± 2.85^{ab}
200	1600	6.00 ± 0.00^{e}	98.70 ± 0.00^{a}
80% procymidone		6.00 ± 0.00^{e}	98.70 ± 0.00^{a}
Sterile water (control)		81.67 ± 1.67^{a}	0.00 ± 0.00^{e}

Colony diameters were measured 4 days after inoculation. In the table, values are mean ± *SE*. In the same columns, different superscript letters indicate significant differences at $P < 0.05$ using Duncan's test.

Table 2
Antifungal spectrum of combined treatment (200 mg/L ε-PL + 400 mg/L COS).

Pathogenic fungi	Growth (mm)	Inhibition rate (%)
Valsa mali	37.83 ± 2.17	58.96 ± 2.71^{d}
Alternaria solani	13.17 ± 1.31	88.44 ± 1.85^{ab}
Pseudocercospora fijiensis	33.50 ± 1.04	62.13 ± 1.38^{d}
Fusarium oxysporum	49.33 ± 2.05	44.23 ± 2.58^{e}
Rhizoctonia cerealis	42.83 ± 1.92	46.96 ± 2.69^{e}
Gloeosporium musarum	24.67 ± 1.45	74.35 ± 1.89^{c}
Sclerotinia sclerotiorum	9.67 ± 2.17	94.17 ± 2.71^{a}
Cercospora fagopyri	14.75 ± 0.00	86.41 ± 0.00^{b}
Sclerotium cepivorum	11.50 ± 0.75	91.78 ± 0.95^{ab}
Glomerella cingulata	60.58 ± 3.86	23.86 ± 5.28^{f}
Cochliobolus sativus	19.58 ± 0.55	77.96 ± 0.83^{c}
Botrytis squamosa	8.70 ± 0.13	90.33 ± 2.67^{ab}

Colony diameter was measured when each fungus growth in the control dishes was almost complete. Different control plates containing the medium mixed with sterile water (10%, by volume) were included for each species. In the table, values are mean ± *SE*. In the same columns, different superscript letters indicate significant differences at $P < 0.05$ by Duncan's test.

results, the combined treatment (200 mg/L ε-PL + 400 mg/L COS) was selected for further testing of its control efficiency *in vivo* and its mechanism of action.

3.2. Antifungal spectrum of combined treatment ε-PL and COS

The antifungal activity of the combined treatment (200 mg/L ε-PL + 400 mg/L COS) was estimated using various plant pathogenic fungi, including *Valsa mali*, *Alternaria solani*, *Pseudocercospora fijiensis*, *Fusarium oxysporum*, *Rhizoctonia cerealis*, *Gloeosporium musarum*, *Sclerotinia sclerotiorum*, *Cercospora fagopyri*, *Sclerotium cepivorum*, *Glomerella cingulata*, *Cochliobolus sativus* and *B. squamosa*. The combined treatment exerted a potent antifungal activity against plant pathogenic fungi and is therefore a broad spectrum antifungal formulation (Table 2). This combined treatment exhibited high antifungal activity against *B. squamosa*, *Sclerotinia sclerotiorum*, and *Sclerotium cepivorum*, with inhibition rates of 90.33%, 94.17%, and 91.78%, respectively ($P < 0.05$). Additionally, a varying inhibition effect was achieved in other fungi.

3.3. The treatments induce resistance in tomato plants

As shown in Table 3, on the 3rd day after spraying the combined bio-fungicides (200 mg/L ε-PL + 400 mg/L COS) onto plants, the lesion size on tomato plants was less compared with the control plants. The control efficiency was quantified at approximately 60%, but this inhibition did not differ significantly from procymidone ($P > 0.05$). After spraying ε-PL or COS, the control efficiencies were found to gradually decrease to 34.37% and 13.79%, respectively.

Table 3
Control efficiencies of ε-PL and COS through resistance induction in the pot experiment.

Treatment	Lesion size (mm^2)	Control efficiency (%)
A	212.49 ± 5.60^{c}	34.37 ± 2.00^{b}
B	270.26 ± 2.74^{b}	13.79 ± 0.98^{c}
C	139.32 ± 5.47^{d}	60.44 ± 1.95^{a}
D	126.13 ± 1.88^{e}	59.17 ± 2.08^{a}
E	308.98 ± 2.67^{a}	0.00 ± 0.00^{d}

A Single bio-fungicide ε-PL (200 mg/L).
B Single bio-fungicide COS (400 mg/L).
C Combined bio-fungicides (200 mg/L ε-PL + 400 mg/L COS).
D 80% procymidone.
E Sterile water (control).
In the table, values are mean ± *SE*. In the same columns, different superscript letters indicate significant differences at $P < 0.05$ by Duncan's test.

Table 4
Control efficiencies of ε-PL and COS on tomato grey mould in the field.

Treatment	DI			Control efficiency (%)
	Before spraying	7 days after spraying	Increment	
A	2.10 ± 0.77	4.73 ± 1.50	2.63 ± 1.03	35.22 ± 1.33b
B	1.22 ± 1.23	4.12 ± 1.64	2.90 ± 0.23	28.57 ± 1.20c
C	1.67 ± 1.29	2.89 ± 1.16	1.22 ± 0.82	66.67 ± 2.60a
D	1.53 ± 0.51	2.77 ± 1.03	1.24 ± 0.66	65.17 ± 2.39a
E	2.22 ± 1.71	6.28 ± 1.99	4.06 ± 1.52	0.00 ± 0.00d

A Single bio-fungicide ε-PL (200 mg/L).
B Single bio-fungicide COS (400 mg/L).
C Combined bio-fungicides (200 mg/L ε-PL + 400 mg/L COS).
D 80% procymidone.
E Sterile water (control).
In the table, values are mean ± *SE*. In the same columns, different superscript letters indicate significant differences at $P < 0.05$ by Duncan's test.

3.4. Ability of treatments to reduce grey mould in the field

The combined bio-fungicides (200 mg/L ε-PL + 400 mg/L COS) and procymidone significantly reduced tomato grey mould in the field. Application of the combined bio-fungicides significantly decreased DI in comparison with the sterile water control (Table 4). The combined bio-fungicides and procymidone had significant control efficiencies of 66.67% and 65.17% ($P < 0.05$) respectively, and there was no mathematically significant difference between the combined application and procymidone.

3.5. The influence of treatments on phytohormone content in the tomato

The SA content in the control changed little, but higher levels of SA were observed in treatments by the single bio-fungicide ε-PL and the combined bio-fungicides. In treatment by the single bio-fungicide ε-PL, rapid SA production was observed with a maximum at 12 h. The SA content decreased rapidly by three treatments, reaching a minimum at 24 h. In treatment with 200 mg/L ε-PL + 400 mg/L COS, the SA content reached its highest level of 149.2 $\mu g \cdot g^{-1}$ at 48 h and was > 2 times that the control group (Fig. 1A).

The maximum JA content, an increase of 193.2% compared with the control plants, occurred in treatment by 200 mg/L ε-PL + 400 mg/L COS at 12 h (Fig. 1B). The JA content decreased thereafter. Similarly, the JA content peaked at the 12 h after inoculation in treatment by the single bio-fungicides ε-PL and COS, and the peaks were 1.37 and 1.98 times that in control group, respectively.

The ABA content in the control changed little, but a lower level of ABA was observed in treatment by the combined bio-fungicides (Fig. 1C). In treatment by 200 mg/L ε-PL + 400 mg/L COS, the ABA content was lowest at 12 h, with a minimum level 50.1% lower than that of the control. After the peak, large declines were observed in treatment by the single bio-fungicide COS.

The GA content began to decline at 0 h and reached its minimum value at 12 h in treatment by the single bio-fungicide ε-PL, and we then observed a peak at 24 h. In treatment with 200 mg/L ε-PL + 400 mg/L COS, the GA content peaked at the 12 h after inoculation, and this peak was 0.91 times that the control group (Fig. 1D). As time went on, the GA content gradually decreased in the treatment by the single bio-fungicide COS.

The concentration of IAA in the control remained broadly steady throughout the treatment period. The IAA content was stable and higher in the control group than in the treatment by the single bio-fungicide ε-PL. At 48 h, the IAA content was highest in the 200 mg/L ε-PL + 400 mg/L COS treatment (Fig. 1E). In treatment with the single

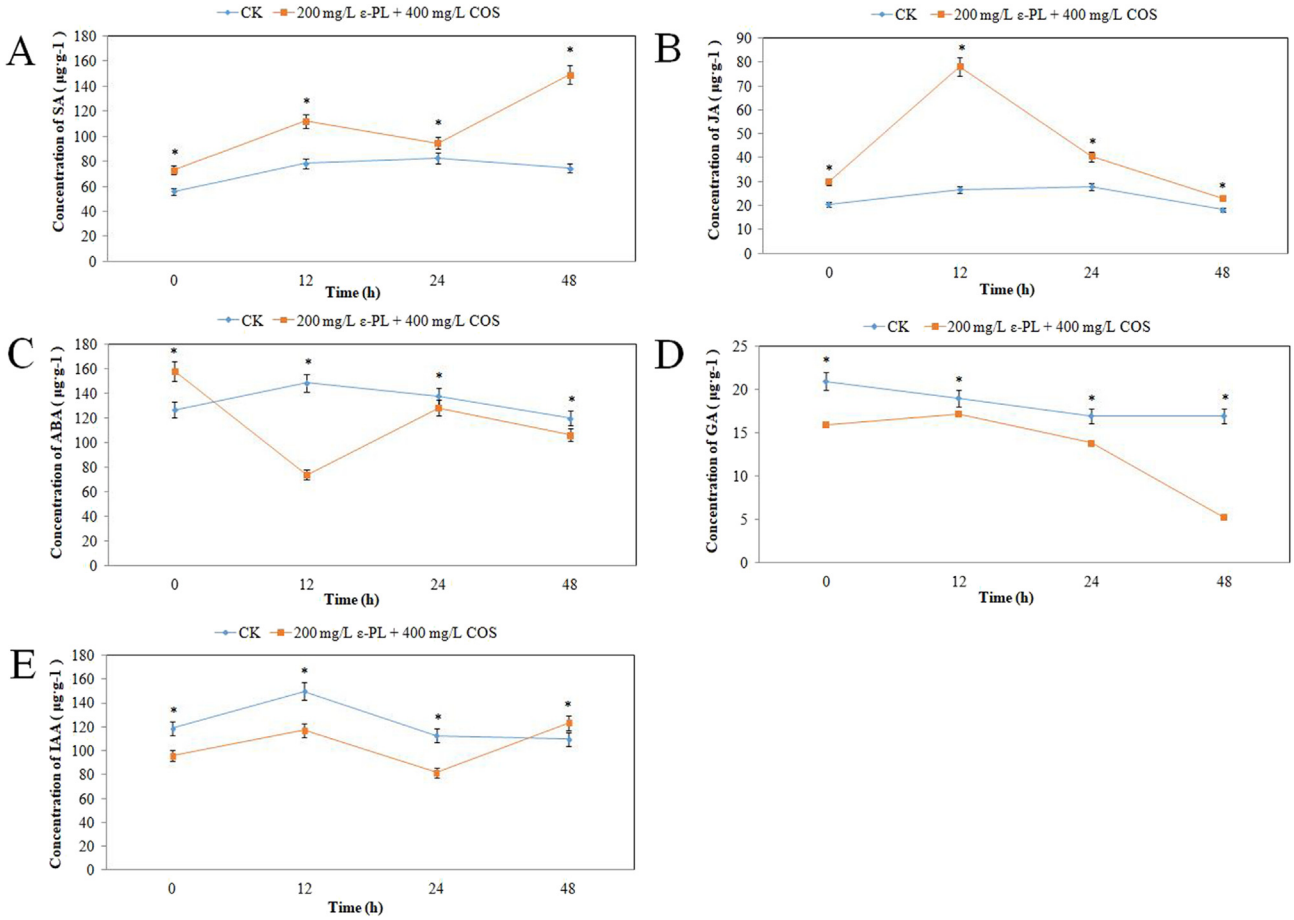

Fig. 1. The effect of combined bio-fungicides (200 mg/L ε-PL + 400 mg/L COS) and sterile water (control) on phytohormone levels in tomatoes. The combined bio-fungicides or water was applied 3 days prior to *B. cinerea* spores inoculation (10^7 cfu spores mL^{-1}). Tomato leaves were treated with the chemicals then stored at 25 °C and 95% RH for 0, 12, 24, and 48 h before extractions were obtained. Error bars represent the standard deviation across three independent replicates. Significant differences between the treatments and control were analyzed by ANOVA, and the results are represented by * ($P < 0.05$). A: Changes in SA levels in tomato leaves subjected to different treatments. B: Changes in JA levels in tomato leaves subjected to different treatments. C: Changes in ABA levels in tomato leaves subjected to different treatments. D: Changes in GA levels in tomato leaves subjected to different treatments. E: Changes in IAA levels in tomato leaves subjected to different treatments.

bio-fungicide COS, the IAA content did not change significantly but included a small peak at 12 h.

3.6. Changes in defence-related enzyme activity

CAT activity exhibited a sharp decrease after treatment by 200 mg/L ε-PL + 400 mg/L COS and reached the lowest enzyme activity at 12 h, at a level 0.41-fold lower than in the control (Fig. 2A). After 12 h, CAT activity significantly increased in treatment by the single bio-fungicide ε-PL, and reached its highest level, 1.81 times that in the control group at 24 h ($P < 0.05$).

Compared to the control, PAL activity increased from the beginning of the monitoring period up to 48 h in all treatments (Fig. 2B). Before 48 h, PAL activity was higher in the treatment by 200 mg/L ε-PL + 400 mg/L COS than in all other treatments, and reached a peak at 12 h. Additionally, PAL activity was higher with treatment by the single bio-fungicide COS than in treatments by the single bio-fungicide ε-PL before 24 h; PAL activity was subsequently higher in treatments by the single bio-fungicide ε-PL, but both treatments exhibited similar patterns of change thereafter.

Compared to the control, POD activities in the treatments increased to different levels after 0 h. Exhibiting large changes, POD activity reached its peak in treatments by 200 mg/L ε-PL + 400 mg/L COS at 24 h, and this peak was > 2 times that of the control group (Fig. 2C). Treatment by the single bio-fungicide COS exhibited a minimum value at 12 h, and increased slightly to exceed the control level.

SOD activity increased similarly at a gradual rate in all treatments before 24 h, reaching a peak at 12 h and subsequently declining. The SOD content peaked at 12 h after inoculation in treatments with 200 mg/L ε-PL + 400 mg/L COS, and that peak was 1.09 times that in the control group (Fig. 2D). However, SOD activity was higher in the control group than in treatments by the single bio-fungicides COS and ε-PL at 12 h.

4. Discussion

Although *B. cinerea* can be inhibited by a ε-PL dose of up to 1200 mg/L (Sun et al., 2017), ε-PL can be subject to rapid depletion after initial application and lose activity quickly (Bi et al., 2016). Therefore, reducing the ε-PL dose for an inhibition of *B. cinerea* through a combined treatment is important. The combined treatment can contribute to a reduction of grey mould and reduce the dosage or use of fungicides for disease control (Cia et al., 2007). In this study, the inhibition rate of combined bio-fungicides (200 mg/L ε-PL + 400 mg/L COS) against *B. cinerea* was investigated, and as demonstrated, was observed to have a higher specific activity than the single bio-fungicides ε-PL and COS, or similarly tested compounds (Muñoz and Moret, 2010; Najjar et al., 2009). The antimicrobial activity of ε-PL is electrostatic

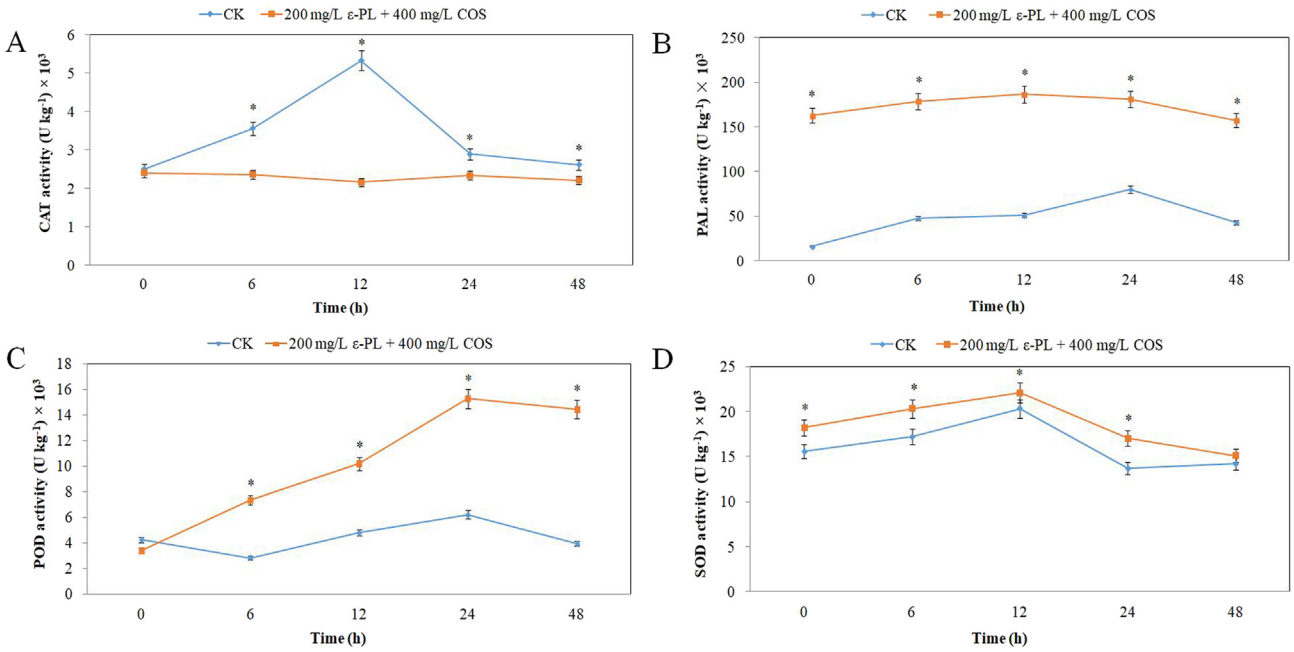

Fig. 2. The effect of combined bio-fungicides (200 mg/L ε-PL + 400 mg/L COS) and sterile water (control) on the activities of defence-related enzymes in tomatoes. The combined bio-fungicides or water was applied 3 days prior to *B. cinerea* spores inoculation (10^7 cfu spores mL^{-1}). Tomato leaves were treated with the chemicals then stored at 25 °C and 95% RH for 0, 6, 12, 24, and 48 h before extractions were obtained. Error bars represent the standard deviation for three independent replicates. Significant differences between inoculations and control were analyzed by ANOVA, and the results are represented by * ($P < 0.05$). A: Changes in CAT activity in tomato leaves subjected to different treatments. B: Changes in PAL activity in tomato leaves subjected to different treatments. C: Changes in POD activity in tomato leaves subjected to different treatments. D: Changes in SOD activity in tomato leaves subjected to different treatments.

interaction with the microbial cell surface (Yamanaka and Hamano, 2010). The formation of polyelectrolyte complexes between COS and negatively charged groups on the cell surface interferes with the growth and normal physiological functions of fungi (Hirano and Nagao, 1989). In this study, our results demonstrate that the combined bio-fungicides (200 mg/L ε-PL + 400 mg/L COS) could effectively control tomato grey mould caused by *B. cinerea*, and in a process that we hypothesize is the result of the induction of disorganization of the membrane and abnormal distribution of the cytoplasm, resulting in physiological damage to *B. cinerea* hyphae. Additionally, our study shows that these combined bio-fungicides have a broad antagonistic spectrum, as they inhibited the mycelial growth of 12 species of plant pathogenic fungi. This work provides evidence that the effective control of tomato grey mould can be achieved by using multiple antifungal agents with different mechanisms of action, which in turn is likely to provide synergistic action of combined substances against *B. cinerea* (Leistner, 2000). Thus, these combined bio-fungicides can be good alternative biological resources for biocontrol of tomato grey mould.

The majority of work initiated so far has concentrated on the effects of combined fungicides on inhibition of fungal mycelial growth under *in vitro* conditions (Ettayebi et al., 2000; Liu et al., 2015). Unlike such *in vitro* studies, very few studies have been conducted under *in vivo* conditions to show fungicidal properties of ε-PL and COS against *B. cinerea*. The application of bio-fungicide on the tomato leaves 3 days before fungal inoculation had a significant control of *B. cinerea* by inducing resistance (Sun et al., 2017), so this method for inoculation was chosen. Unique to this study, our findings highlighted that the combined bio-fungicides (200 mg/L ε-PL + 400 mg/L COS) against tomato grey mould could likely serve as a preventive measure in potted experiments. And the control efficiency of these combined bio-fungicides was significantly better than achieved by the single bio-fungicides ε-PL or COS. It is logical that combined bio-fungicides with different modes or sites of activity achieve higher antifungal efficiency while significantly diminishing the likelihood that the pathogen will develop resistance to one or more antifungal agents (Leistner, 2000). Further studies on these combined bio-fungicides are necessary to fully elucidate the defence-associated signaling processes. Additionally, both the combined bio-fungicides and procymidone had significant control efficiencies of this disease in the field. These results are consistent with the report of Xu et al. (2016). There are many factors, such as temperature, humidity, pH and microorganisms, which consistently impact the efficacy of the combined bio-fungicides in the field experiments. So some problems remain that need to be addressed, such as the best period for preventive spraying, environmental behavior of the combined bio-fungicides and the control efficiency of the combined bio-fungicides in different climatic zones.

In this study, we observed significant changes in SA, JA, GA, and ABA levels, though IAA levels decreased only marginally, in the treatment groups. SA, JA, GA, and ABA are likely involved in the development of resistance to *B. cinerea* in tomatoes, whereas IAA may not be involved or may only have a small role in the process.

Priming of defence is not only induced by biotic stimuli but also by abiotic agents including a variety of chemicals (Conrath et al., 2015). As a master regulator, SA plays an important role in systemic resistance as well as plant-microbial interactions (Lan et al., 2013; Li and Zou, 2017). In our study, the SA content in the control changed little, but higher levels of SA were observed in treatments with the single bio-fungicide ε-PL and with combined bio-fungicides. It appears that SA has a regulatory role in activating biochemical pathways associated with tolerance mechanisms (Sticher et al., 1997). The important role of SA in protecting plants is probably its ability to induce expression of genes coding not only for PR-proteins but also for the extension gene, as found in *Arabidopsis* (Merkouropoulos et al., 1999).

In this study, we observed that within 12 h of treatment, the content of JA was higher in tomato plants treated with the combined bio-fungicides than in tomatoes treated only with ε-PL or COS, suggesting that the combined bio-fungicide induction leads to increased accumulation of JA, and the production of a stronger defence response against pathogens, which could effectively prevent pathogenic infection or reduce disease severity (Thuleau et al., 2013). We hypothesize that the

intervention or production of JA may induce the expression of several defence-related genes in plants, such as genes encoding PAL, PR-10/chitinase, β-1, 3-glucanase and others (Mouekouba et al., 2014).

GA regulates all aspects of the life history of plants, from seed germination to vegetative growth and flowering (Ritchie and Gilroy, 1998). In the current study, tomato leaves infected with *B. cinerea* exhibited a higher GA level than did the other treatment groups, which suggests that GA, associated with the natural fungicide ε-PL or COS treatment, may participate in providing resistance against this disease pathogen. This result is in agreement with the results of Robert-Seilaniantz et al. (2011), who demonstrated that GA had an opposite effect on resistance and that plants with high levels of GA are more susceptible to pathogen infection.

ABA is known for its protective effect, as it stimulates stomatal closure in order to minimize water loss, and it mediates stress damage to plants through activation of many stress-responsive genes, which collectively increase the plant's stress tolerance (Aroca et al., 2008; Herrera-Medina et al., 2007). In this study, compared with the control group, the combined bio-fungicides significantly reduced ABA generation after 12 h, suggesting that ABA plays an indirect and negative role in managing *B. cinerea* infection. Several studies have suggested that the growth inhibitor ABA negatively regulates the defence system against biotic stress (Liu and Kracher, 2015). The ABA content was higher at 24 h in treatment by ε-PL or COS compared to treatment with 200 mg/L ε-PL + 400 mg/L COS, perhaps because ε-PL and COS are involved in inducing the expression of genes that function as negative regulators of ABA biosynthesis. Interestingly, several lines of evidence indicate that this immune-suppressive effect of ABA is due at least in part to suppression of SA-mediated defenses that normally serve to limit pathogen growth (Xu et al., 2013).

IAA is an essential hormone that improves different defence responses, thus protecting plants from stress conditions. IAA may induce or suppress expression of specific genes associated with disease resistance (Zhang et al., 2011). In the present study, tomato plants treated with the combined bio-fungicides exhibited a lower level of IAA at 24 h than did plants treated with water, and we attribute this decrease to IAA's role in inducing resistance against *B. cinerea*. Our current findings confirm the previous reports of Blanco-Ulate et al. (2013), who demonstrated that the ABA-deficient mutant sitiens could increase tomato resistance to *B. cinerea*. On the basis of these results, we suggest that SA, JA, GA, IAA, and ABA may be involved in the defence signaling of the combined bio-fungicides that influence tomato resistance to *B. cinerea*.

Defence enzymes are involved in the biosynthesis of signaling molecules. We observed that the combined bio-fungicides could effectively inhibit grey mould formation and significantly change CAT, PAL, POD, and SOD enzyme activities in tomato plants.

CAT is vital for maintaining the balance of active oxygen, and converts H_2O_2 to H_2O and O_2^-. The increase in SA content may have resulted in elevated H_2O_2 levels by inhibiting the activity of plant CAT, and higher H_2O_2 levels may have contributed to the inducement or activation of other biochemical processes related to plant disease resistance (Chen et al., 1993). In this study, high elicitor-induced resistance could efficiently decrease CAT activity, and we revealed lower CAT activity in tomatoes treated with the combined bio-fungicides than in the other treatments. These results are in agreement with those of Ciepiela (1989). Gong et al. (2017) observed lower CAT activity in the *B. cinerea* treatments, with increases in SA content and H_2O_2 levels at the initial stage of infection. These results suggest that the combined bio-fungicides may be an effective inhibitor of CAT activity for combating *B. cinerea*.

PAL is a key enzyme of the phenylpropanoid pathway for producing phenylpropanoids and phenols, and SA can also be synthesized *via* this pathway (Wang et al., 2011). In this study, the treated tomato plants exhibited an increase in PAL activity, particularly when treated with 200 mg/L ε-PL + 400 mg/L COS. These results match those obtained by Gong et al. (2017) who reported a marked increase in PAL activity in tomatoes infected with *Clonostachys rosea*. PAL levels increased in treatments by single ε-PL or COS but remained lower than those in treatment by 200 mg/L ε-PL + 400 mg/L COS, suggesting that PAL is involved in inducing disease resistance in tomatoes. It has also been reported that plants may excite their maximum energy for self-defence when an inducer is provided before inoculation (Ahmad et al., 2014). Hence, the resistance of tomato to grey mould disease may be strengthened by the combined bio-fungicide application.

POD, a group of heme-containing glycosylated proteins, are known to be induced by various types of stresses including pathogen infection (Sasaki et al., 2004). Su et al. (2014) illustrated that POD is involved in the MAPKs pathway, which contributes to the resistance of tomato fruit to invasion and expansion of *B. cinerea* infections. SOD is the major antioxidative enzyme in plants (Apel and Hirt, 2004). Castoria et al. (2003) found that a higher antagonistic activity of *Cryptococcus laurentii* LS-28 against *B. cinerea* was associated with greater resistance to ROS-generated oxidative stress in apple fruit wounds. These enzymes are involved in many plant functions including the generation of ROS during plant defence response (Bindschedler et al., 2006; Martinez et al., 1998) and pathogen resistance (Johrde and Schweizer, 2008). Unique to this study, tomato plants treated with the combined bio-fungicides exhibited higher levels of POD activity at 24 h after inoculation than did plants with other treatments, and we attribute this increase to POD's role in inducing resistance against *B. cinerea*. Interestingly, SOD activity increased similarly at a gradual rate in all treatments before 24 h, reaching a peak at 12 h and subsequently declining. It seems that SOD accumulation is the earlier defence activation signal which might be attributed to the biological effect of the combined bio-fungicides and their components causing an oxidative burst by accumulation of ROS (Singh et al., 2006), mainly produced by SOD. In fact, the ROS have various effects on plant defence responses, including defensive gene activation, as well as defensive compound induction (Levine et al., 1994). Such results can be justified by the fact that POD and SOD activities may be generally considered important in host resistance of tomatoes to *B. cinerea*.

In conclusion, we evaluated antifungal activity of the combined bio-fungicides (200 mg/L ε-PL + 400 mg/L COS) to detect new natural agents to be used as combined bio-fungicides against *B. cinerea*. This combined bio-fungicide has been shown to reduce growth of *B. cinerea* on solid media (*in vitro*) and to control infection of tomatoes by *B. cinerea* (*in vivo*). Since ε-PL and COS exhibit the characteristics of water solubility, biodegradability, stability and low toxicity (Yoshida and Nagasawa, 2003), the possibility of developing this combined bio-fungicide for use in crop protection may be an attractive venture. However, further studies need to be conducted to evaluate the cost and efficacy of the combined bio-fungicide on wide range of diseases in commercial greenhouses. Additionally, this study confirmed that the combined bio-fungicide induces resistance to *B. cinerea* in tomatoes. Although some clear differences were observed, the protein expression profiles were similar in all treatments. Thus, it can be concluded that the mechanisms of action differ in the various treatments but share some common disease-resistance pathways.

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