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Action of selenium against *Sclerotinia sclerotiorum*: Damaging membrane system and interfering with metabolism

Wei Jia, Chengxiao Hu, Jiajia Ming, Yuanyuan Zhao, Juan Xin, Xuecheng Sun, Xiaohu Zhao*

Hubei Provincial Engineering Laboratory for New Fertilizers / Research Center of Trace Elements /Key Laboratory of Arable Land Conservation (Middle and Lower Reaches of Yangtze River), Ministry of Agriculture / College of Resources and Environment, Huazhong Agricultural University, Wuhan 430070, China

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ABSTRACT

Selenium (Se) in soil is beneficial for environmental stress tolerance of plants, and it has widespread toxic effects on pathogens. Based on the fact that Se significantly inhibited the growth of *Sclerotinia sclerotiorum*, we set experiments with different concentrations of Se to investigate the action of Se against *S. sclerotiorum* in this study. The results showed that Se ($> 0.5\ mg\ L^{-1}$) changed the morphology of *S. sclerotiorum* mycelia, and higher Se concentrations severely damaged mycelial structures. Fourier transform infrared spectroscopy (FTIR) analysis indicated that Se treatment induced the chemical composition of mycelia with much abundance of functional groups such as alcohols, ketones, ammonium and esters, and $0.5\ mg\ L^{-1}$ Se maximized their concentrations. Under Se treatments, the electrical conductivity of mycelia increased in a time-dependent manner, and osmolyte concentrations of mycelia increased as well. Se supplementation significantly reduced polymethylgalacturonase (PMG) and carboxymethylcellulase (Cx) activities, which protecting plants from infection, and increased the energy expenditure in *S. sclerotiorum*. Combined action of Se damage on membrane system, osmoregulation, reduction of cell wall degrading enzymes activities and improvement of energy expenditure resulted in the inhibition of *S. sclerotiorum* growth. Findings in this study provided evidences for using Se as a potential fungicide to control *S. sclerotiorum*.

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a broad spectrum necrotrophic phytopathogen that infects over 400 plant species including amounts of important vegetables and crops worldwide, such as oilseed rape, tomato, potato, soybean and sunflower [1, 2]. This soil-borne fungus mainly infects stems, leaves and flowers in the form of sclerotia, which resides in soil for many years and then germinates and forms mycelia on plant surfaces once under proper temperature and humidity [3, 4]. Carpogenic germination of sclerotia can release many air-borne ascospores, which are difficult to control [5]. The complex pathogenesis of *S. sclerotiorum* makes it one of the most economically damaging pathogens [6]. To control *S. sclerotiorum*, amounts of measures were developed [7, 8], and chemical fungicide was considered as the main control method. However, extensive use of chemical fungicides lead to negative effects on the environment, such as soil water and soil quality [9, 10]. The development of an efficient, safe and environment-friendly control method to prevent and control *S. sclerotiorum* is therefore important.

Selenium (Se) is an essential trace element in both humans and animals because of its presence in antioxidant defence systems [11]. While its essential role in higher plants remains ambiguous and unresolved, all plants can assimilate Se and accumulate it in their organs [12]. In addition, traces of Se promote the growth of many plant species such as wheat and lettuce [13–15]. Mounting evidences have demonstrated that Se reduced the reactive oxygen species production and lipid peroxidation levels, as well as increased glutathione peroxidase activity and free proline concentrations in plant cells [16, 17]. Furthermore, it was confirmed that Se could enhance the resistance to adverse effects on plants by removing excessive free radicals in plants and alleviate stress from the environment [18–21]. Increasing evidences have suggested that Se could help plants defend against varieties of fungal pathogens [22]. For example, it is reported that Se protected *B. juncea* from fungal infections from species including *A. brassicicola* and *Fusarium*sp [23]. Se controlled *Bacillus cereus* and might be used as an effective alternative insecticide to avert biofilm formation by pathogens [24]. Additionally, Se inhibited spore germination, germ tube elongation and mycelial spread of *P. expansum* in a culture medium [25], and the presence of $10\ mg\ L^{-1}$ Se in the medium inhibited the growth of *Phanerochaete chrysosporium* and substrate consumption [26], and

* Corresponding author.
E-mail address: xhzhao@mail.hzau.edu.cn (X. Zhao).

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24 mg L^{-1} Se significantly inhibited spore germination of fungal pathogen and effectively controlled gray mold in harvested tomato fruit [20].

Due to the beneficial role, increasing application of Se was used in soil for producing Se-enriched food and for improving plant resistance against environmental stress [27–29]. In addition, Se was also used as fertilizers in soil to enhance the interaction of Se and pathogenic microorganisms such as *S. sclerotiorum*. Results of our previous study indicated that exogenous Se inhibited sclerotia germination and the mycelial growth of *S. sclerotiorum* [30]. However, the mechanisms of Se influence on *S. sclerotiorum* are poorly defined, and it would obviously be of great interest to explore multidimensional action of selenium against *S. sclerotiorum*, which not only provide helpful information for developing new methods to control *S. sclerotiorum*, but also suggest another approach of Se as a potential fungicide for crop protection.

Therefore, this study was conducted to determine the growth inhibition of Se on *S. sclerotiorum*. The specific objectives were to (1) examine Se effect on the membrane system of *S. sclerotiorum*, such as the membrane component and electrical conductivity, and (2) investigate the modulation of Se on the metabolism of inclusions in *S. sclerotiorum*, such as cell wall degrading enzyme (CWDE) activities and osmolytes. Besides, energy metabolism of *S. sclerotiorum* needs to be studied as well.

2. Materials and methods

2.1. Pathogens and chemicals

S. sclerotiorum (JZJL-13) used in this study was obtained from the Key Laboratory of Crop Disease Monitoring and Safety Control, College of Plant Science & Technology, Huazhong Agricultural University.

Potato dextrose agar (PDA) medium: 200 g of potato, 20 g of sucrose, 20 g of agar, and 1000 mL of deionized water, natural pH. Potato dextrose broth (PDB) medium: 200 g of potato, 20 g of sucrose and 1000 mL of deionized water, natural pH. All the media and dishes were autoclaved at 121 °C for 20 min.

Action of Se on *S. sclerotiorum* growth was determined by poisoned culture medium technique. The experiment was designed completely random and five Se treatments (Se_0, $Se_{0.5}$, Se_5, Se_{20} and Se_{50}) with five Se concentrations (0, 0.5, 5, 20 and 50 mg L^{-1}) were arranged. Se concentrations in this study were designed according to the result of our previous study [30] as well as others' study [20, 23–26]. Sodium selenite was used as Se source in the present study.

2.2. Scanning electron microscopy (SEM) observation of mycelial morphology

Based on the result of our previous study, samples under treatments of Se_0, $Se_{0.5}$ and Se_{20} which carried out with PDA, were selected for SEM observation in this present study. Cover glasses were oblique into PDA in order to obtain *S. sclerotiorum* mycelia samples. Here, the SEM observation was carried on according to the method of Di et al. [31] with some slight modifications. In brief, cover glasses were cut into small pieces (side length $<$ 3 mm) and placed in tubes containing 2.5% (wt/vol) glutaraldehyde solution in sodium phosphate buffer (0.05 M, pH 7.0) at 4 °C for 24 h, and then washed three times in 0.05 M sodium phosphate buffer (each time for 10 min). After their dehydration in an ethanol gradient, samples were dried at room temperature and sputter coated with Au in a sputter coater (JFC-1600; NTC, Japan) and were observed with SEM (JSM-6390/LV; NTC, Japan).

2.3. Fourier transform infrared spectroscopy (FTIR) of membrane compositions

The membrane components from samples of three treatments (Se_0, $Se_{0.5}$ and Se_{20}) were analyzed by FTIR spectroscopy using potassium bromide pellet method according to Gad et al. [32]. In brief, after being cultivated in PDA medium, mycelial plugs (5 mm in diameter) were transferred into 50 mL PDB medium and incubated at 25 °C for 3 days. After incubation, 1 mg freeze-dried mycelia mixed with 100 mg dry spectroscopic grade potassium bromide (KBr) were ground with agate mortar, and then pressed into a disc. FTIR spectra were obtained by scanning from 4000 to 400 cm^{-1} at 4 cm^{-1} resolution using VERTEX 70 (Bruker, Germany). Each spectrum consisted of 32 scans in total. All spectra were collected against a background of air to remove interference of CO_2 and H_2O. Each sample contained three replicates and was exhibited as the average spectrum.

2.4. Electrical conductivity assay

The electrical conductivity of the mycelia was measured by the method of Firoz et al. [7]. Cultivated in PDA medium for 3 days, mycelial plugs (5 mm in diameter) from fresh edges of three-day-old colonies were transferred into 50 mL PDB medium and incubated at 25 °C for 7 days. Mycelia were selected to centrifuge at 4000 rpm for 10 min and washed three times with sterilized water. Then 3 g of mycelia was placed into tubes containing 10 mL Se solutions with different concentrations (0, 0.5, 5, 20 and 50 mg L^{-1}). The electrical conductivity of the solution was measured by electrical conductivity meter (DDS-307A, Shanghai Leici Instrument Inc) at 0, 15, 30, 120, 180, 300 and 360 min, respectively. Three replicates were performed for each treatment.

2.5. Analysis of osmolytes

For the soluble protein concentration measurement, mycelial plugs (5 mm in diameter) from fresh edges of three-day-old colonies in PDA medium were transferred into 50 mL PDB medium with different concentrations of Se (0, 0.5, 5, 20 and 50 mg L^{-1}), and then incubated at 25 °C for 7 days. After incubation, mycelia were washed three times with sterilized water. Next, 1 g of freeze-dried mycelia fixed with 5 mL Tris-HCl buffer solution (0.05 M, pH 7.5, 4 °C) was ground with liquid nitrogen and then centrifuged at 4 °C, 10000 rpm for 20 min. The supernatant was collected for soluble protein concentration assays, which followed by the Coomassie brilliant G-250 method of Bradford [33]. The absorbance at 595 nm was spectrophotometrically read. Bovine serum albumin (BSA) protein was used as standard for soluble protein concentration assays.

Reducing sugar concentration was determined by the 3,5-dinitrosalicylic acid colorimetric method of Zhao et al. [34]. Mycelia plugs from PDA were incubated in PDB medium for 7 days, for determination of reducing sugar concentration, a 0.1 mL aliquot of mycelia supernatant was pipetted to a tube and 2 mL of 3,5-dinitrosalicylic acid was added to incubate in boiling water for 5 min, then cooled to room temperature. The absorbance at 540 nm was read and glucose was used as the standard to measure reducing sugar concentration. Pyruvate concentration was assayed by the standard curve method according to Crowther et al. [35]. In brief, 0.1 mL mycelia supernatant was mixed with 0.5 mL of 2.5 mM 2,4-dinitrophenylhydrazine and incubated for 10 min at 37 °C in a water bath. After cooling to room temperature, 4 mL of 0.4 M NaOH was added in the mixture, and the absorbance at 520 nm was then measured immediately with a UV-5200 spectrophotometer (Shanghai) to determine pyruvate concentration. Sodium pyruvate was used as standard. Three replicates were performed for each treatment.

2.6. Analysis of CWDE activities

Mycelial plugs (5 mm in diameter) from fresh edges of three-day-old colonies in PDA medium were transferred into 50 mL PDB medium and incubated at 25 °C for 7 days with different concentrations of Se (0, 0.5, 5, 20 and 50 mg L^{-1}). 1 g selected mycelia was washed three times with sterilized water, freeze-dried and ground into power with liquid