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Essential oil of *Syringa oblata* Lindl. as a potential biocontrol agent against tobacco brown spot caused by *Alternaria alternata*

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ABSTRACT

Alternaria alternata causes serious brown spot disease in tobacco. To effectively control the disease, we tested the antifungal effects of the essential oil from the buds of *Syringa oblata* Lindl. Twelve different compounds were identified by gas chromatography–mass spectrometry. The major constituents were eugenol (40.426%), eugenol acetate (28.784%), β-caryophyllene (21.989%), and α-caryophyllene (3.456%). Among these, the most active antifungal component was eugenol, exhibiting complete inhibition of mycelial growth. The minimum inhibitory concentration of eugenol against *A. alternata* was 150 μg/mL, and the minimum fungicidal concentration was 250 μg/mL. The effect of eugenol on the surface morphology and membrane integrity of *A. alternata* was studied by scanning electron microscopy (SEM) and fluorescent microscopy (FSM). SEM observations revealed shriveled hyphae, while FSM observations revealed a disrupted membrane in eugenol-treated samples. Further experiments confirmed that eugenol activated a membrane-active mechanism that increased the membrane permeability, as evidenced by extracellular conductivity measurements. Under field conditions, an *in vivo* assay indicated that eugenol emulsion was effective in protecting tobacco from brown spot infection. This study suggests that eugenol has the potential to be used as a fungicide to control tobacco brown spot disease caused by *A. alternata*.

1. Introduction

Tobacco diseases caused by pathogenic fungi, which result in considerable losses to the tobacco yield, are the most important issues faced by the tobacco industry and farmers (Bai et al., 2016). Tobacco brown spot, caused by *Alternaria alternata*, has been reported as the major fungal disease endangering tobacco production (Hou et al., 2016). In China, the annual incidence area of tobacco brown spot can reach 100,000 hm^2, resulting in a loss of one billion yuans, according to recent statistical data (Liu et al., 2009).

A. alternata causes small yellowish-brown round spots at the initial stage of the disease and infects a larger area of tobacco leaves later, which results in reduction in the yield and quality (Cheng et al., 2011). Traditionally, the most effective method to control this disease in tobacco has been the use of synthetic chemical fungicides such as dimethachlon (Chen et al., 2017). However, this may result in a chemical residue in tobacco products because the disease mainly occurs at the late growth stage. Moreover, chemical strategies have now been discouraged because of growing concerns over human toxicity, antifungal resistance, and environmental problems (Ribes et al., 2017). Therefore, much attention has been paid to a search for new, safe, and biodegradable natural fungicides as substitutes for conventional fungicides (Tao et al., 2014; Wang et al., 2015).

Natural fungicides can be obtained from various sources, including plants, animals, and microorganisms. Among them, plant-derived products have been reported to possess antifungal activities against a wide range of fungi (Ribes et al., 2017). Essential oils originating from medicinal plants are among such agents that exhibit various antifungal activities against fungal pathogens (Kacem et al., 2016; Said et al., 2016). Our previous study has revealed that the *S. oblata* essential oil and its derived component eugenol exhibited strong activities against *Ralstonia solanacearum* (Bai et al., 2016), suggesting its potential usefulness as an antifungal agent. However, there is no literature regarding the application of essential oils and their derived components against tobacco brown spot caused by *A. alternata*.

Therefore, the aim of the present study was to investigate the

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fungicidal activity of essential oils from *S. oblata* and their main components against *A. alternata* using gas chromatography–mass spectrometry (GC–MS) results. Effects of eugenol, the major constituent derived from the essential oil, were studied on the mycelial morphology and cell membrane integrity by scanning electron microscopy (SEM) and fluorescent microscopy (FSM), respectively. Extracellular conductivity was assessed to further confirm the cell membrane damage caused by eugenol. *In vivo* application tests were performed to evaluate the ability of eugenol emulsions to control tobacco brown spot caused by *A. alternata* under field conditions.

2. Materials and methods

2.1. Materials

Syringa oblata Lindl., purchased from the Beijing Tongrentong Pharmacy (Qingdao City, China), was powdered in a blender and stored in sealed paper bags for further experiments. *A. alternata* was isolated from infected tobacco plants at the Key Laboratory of Tobacco and authenticated by Professor Fenglong Wang of Pest Integrated Management Key Laboratory of China Tobacco, Tobacco Research Institute of Chinese Academy of Agricultural Sciences. The fungus was routinely cultured on potato dextrose agar (PDA) consisting of an extract of boiled potatoes, 200 mL; dextrose, 20 g; agar, 20 g; and deionized water, 800 mL, and stored at 4 °C in a refrigerator (Yu et al., 2007). All reagents used in this study were of analytical grade. Propidium iodide (PI) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of A. alternata spores and S. oblate extracts

Briefly, the test *A. alternata* was cultured on PDA plates for 7 days at 28 °C. Then, the plates were flooded with 5 mL sterile 0.1% Tween-20 and gently scraped with a sterile transfer pipette to obtain a spore suspension. The spore concentration was adjusted to 10^5 colony-forming units/mL. *S. oblata* extracts were obtained as described previously (Bai et al., 2016) with some modifications. First, the herb was dried at 60 °C, crushed, filtered through a 0.425-mm sieve, and extracted using 95% ethanol in a microwave for 90 s. The ethanolic extract was filtered, and the residue was re-extracted using 95% ethanol at room temperature for 5 days. Then, the extracts were combined and concentrated under vacuum. The final extract was dissolved with methanol at a concentration of 100 g/L and stored at 4 °C.

2.3. GC–MS analysis

Diluted samples were analyzed by GC–MS according to the method reported by Bai et al. (2016). The initial temperature was set at 40 °C for 5 min, then gradually increased to 240 °C at a rate of 20 °C·min^{-1}, and held at 240 °C for another 5 min. The injection temperature was 250 °C, with the helium carrier gas rate of 1.0 mL/min at a split ratio of 20:1, and the injection volume was 1.0 μL. Electron ionization was used as the ion source, and the ionization energy was set at 70 eV. The sector mass analyzer was set to a range from 30 to 300 amu. The quadrupole temperature was 150 °C, and the ion source temperature was 230 °C. The composition of samples was determined and quantitative analyses of each component were performed by matching their retention times and mass spectra with the standard mass spectra from the NIST08 database provided by the software of the GC–MS system.

2.4. Antifungal effects against A. alternata

Antifungal activities of the main components were tested against *A. alternata* using the Oxford cup method (Yu et al., 2009). From the results of GC–MS, the main components of the extracts were chosen for antifungal tests. Dimethyl sulfoxide (DMSO; 10%) was added to the extracts to the required concentration, and then the solution was mixed with PDA medium to produce a final extract concentration of 10 g/L. Control plates were incubated following the same procedure, using equal volumes of 10% DMSO in place of samples. Fungal mycelia were inoculated into the center of a plate, followed by incubation at 28 ± 2 °C. All tests were performed in triplicate, and the efficacy of each treatment was evaluated by measuring the diameter of each colony:

$$\text{Mycelium inhibition}(\%) = [(Dc-Dt)/Dt] \times 100$$

where Dc (cm) is the mean colony diameter in the control, and Dt (cm) is the mean colony diameter in the treatment.

2.5. Determination of the minimum inhibitory concentration and minimum fungicidal concentration

The minimum inhibitory concentration (MIC) of eugenol for *A. alternata* was determined as follows. First, *A. alternata* was grown on a PDA plate for 4 days until it covered the plate. Then, *A. alternata* was harvested using a 5-mm sterilized puncher along the edges of the colonies, and the fungal blocks were placed in the center of PDA plates containing 25, 50, 100, 150, 200, 250, 300, 350, 450, and 500 μg/mL eugenol, followed by agitation on a rotary shaker at 150 rpm for 20 s and incubation at 28 °C for 48 h. MIC was defined as the lowest concentration of eugenol required for growth inhibition of *A. alternata*. To determine the minimum fungicidal concentration (MFC), samples from complete growth inhibition plates were subcultured on PDA plates at 28 °C for 3 days. MFC was regarded as the lowest eugenol concentration that allowed no growth on the plates (Abbaszadeh et al., 2014). Each treatment was repeated in triplicate.

2.6. Scanning electron microscopy and fluorescent microscopy

Spore suspensions of *A. alternata* were obtained from 3-day-old cultures by adding 5 mL of normal saline to the plates and using a sterile L-shaped spreader to free spores. The spore suspension of *A. alternata* (100 μL) was added to PDA medium and incubated at 28 °C with shaking at 120 rpm for 3 days. Three-day-old fungal cultures on PDA, treated with eugenol, were used for SEM. About 5 × 10 mm segments were cut out from the plates and washed three times in normal saline. Then, the mycelium was fixed with 2.5% glutaraldehyde, which was diluted with 0.1 mol/L phosphate buffer (pH 7.0), for 2 h at 4 °C. After fixation, the samples were dehydrated in an ethanol series (30%, 50%, 70%, and 100%) for 30 min each. The samples were then dried in liquid CO_2 and viewed under a scanning electron microscope operated at 20 kV and a magnification of 2000×. Membrane permeability was assayed following the method of Yu et al. (2015) with some modifications. *A. alternata* was treated with eugenol at a concentration of 1 × MIC. After 2 h, mycelium was sampled from the treatment and control and washed with sodium phosphate buffer. Mycelium was then fixed with 70% ethanol at 4 °C and washed with phosphate-buffered saline twice. The mycelium was stained with PI at a concentration of 1 μg/mL for 30 min in the dark, and the samples were observed under a fluorescent microscope. The fields of view were chosen randomly from each cover slip, and all experiments were repeated three times.

2.7. Measurement of extracellular conductivity

The permeability of the membrane was expressed as extracellular conductivity and tested according to the method described by Tian et al. (2015) using a DDSJ-308A conductivity meter (Shanghai Precision Scientific Instrument Co., Ltd., Shanghai, China) with some modifications. First, 100-μL aliquots of an *A. alternata* suspension (approximately 1.0 × 10^5 spores/mL) were inoculated in a medium and shaken at 120 rpm at 24 °C for 4 days. After the incubation, 3 g of mycelium was washed with distilled water three times and added to