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Toxicity and biochemical action of the antibiotic fungicide tetramycin on *Colletotrichum scovillei*

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ABSTRACT

Tetramycin, a novel polyene macrolide antibiotic, has strong activity against a broad spectrum of fungi and may have potential uses in future agricultural applications. Thus, the antifungal activity and biochemical action of tetramycin on *Colletotrichum scovillei* were investigated in this study. The experimental results indicated that tetramycin had strong inhibitory activity against the mycelial growth, spore germination and germ tube elongation of *C. scovillei*. The baseline sensitivity curves were unimodal, with mean EC_{50} values of 1.98 ± 0.078 μg/mL and 0.003 ± 0.005 μg/mL for mycelial growth and spore germination inhibition, respectively. Tetramycin also inhibited the germination of spores and formation of appressoria. After tetramycin treatment, the edge of the mycelial diaphragm showed protuberances, with decreased offshoots at the top. Additionally, disruption of the membrane was detected through an increase in membrane permeability, leakage of sugars and a reduction in the ergosterol content. Tetramycin effectively controlled *C. scovillei* on detached pepper fruits. These results will contribute to our evaluation of the potential of tetramycin for successful management of pepper anthracnose and to our understanding of the possible biochemical action of tetramycin against *C. scovillei*.

1. Introduction

Pepper (*Capsicum* spp.) is an important vegetable crop throughout the world [1]. China is the second largest producer and exporter of pepper, with a yield of > 28 M tons [1]. Anthracnose caused by *Colletotrichum* spp. is one of the most destructive diseases in pepper production [2,3]. Recently, the *C. acutatum* species complex was identified as a common and aggressive fungal pathogen that can cause serious losses of yield and quality for multiple important economic crops, such as strawberry, grapevine, mango and apple [4–7]. *C. scovillei*, which belongs to the *C. acutatum* species complex, is also economically significant [8]. Anthracnose was reported to cause 40% of the annual pepper yield loss in China [9].

C. scovillei infection initiates when the conidia tightly adhere to the surface of pepper fruits and produce germ tubes. The germ tubes then form appressoria and penetrate the fruits cuticle by a penetration peg. After penetration, the pathogen establishes itself by producing highly differentiated infectious hyphae, which result in anthracnose lesions. Under favorable conditions, an abundance of conidia are produced on the lesions, which serve as secondary inocula and allow the spread of anthracnose. Fungicides with high activity against *C. scovillei* secondary infection may prevent anthracnose dispersal in the field.

Control of pepper anthracnose relies heavily on commercial fungicides because agricultural practices and biological measures only provide limited relief against this disease [10]. However, chemical management of pepper anthracnose is a challenge because only a few chemical classes are available for the control of anthracnose disease [4,11,12]. Triazole fungicides have excellent inhibitory activity against *Colletotrichum* species *in vitro*; however, the high risk of phytotoxicity has limited their use [13]. Furthermore, the *C. acutatum* species complex was shown to be inherently insensitive to benomyl and other benzimidazole fungicides compared with other *Colletotrichum* species [14,15]. Therefore, there is an urgent need to develop safe and effective compounds for the successful management of pepper anthracnose.

Tetramycin, produced by *Streptomyces hygrospinosus* var. *Beijingensis*, is a polyene macrolide antibiotic [16,17]. Tetramycin exhibits strong inhibitory activity against ascomycetes, basidiomycetes, and adelomycetes [18]. Recently, tetramycin was registered for the control of rice and fruit diseases in China [19,20]. As an antibiotic fungicide, tetramycin could increase the disease resistance of plants by inducing the activity of phenylalanine ammonia lyase (PAL), peroxidase (POD), and polyphenol oxidase (PPO) [21]. Our preliminary study indicated that tetramycin showed strong fungicidal activity against *C. scovillei in vitro* and may be a potential alternative for controlling pepper

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anthracnose. Elucidation of the inhibitory activity and possible biochemical action is important for sustainable use of compounds for the management of disease. Tetramycin has not been registered for the control of pepper anthracnose. Establishing the baseline sensitivity of *C. scovillei* to tetramycin prior to its legal use will be helpful.

The objectives of this study were to investigate the antifungal activity and biochemical action of tetramycin on *C. scovillei*, to establish the baseline sensitivity of *C. scovillei* to tetramycin and to evaluate the protective and curative *activities* of tetramycin against *C. scovillei* on detached pepper fruits.

2. Materials and methods

2.1. Isolates, fungicides and media

The fungal pathogen *C. scovillei* was isolated from infected pepper fruits that were collected in 2015 from Jining, Heze, Jinan, Weifang, Dezhou, and Tai'an in Shandong Province. In each region, the sample locations were at least 20 km from each other, and no tetramycin and other antibiotics had been applied. The tested strains were isolated according to the standard procedures [22]. A total of 185 single-spore cultures were maintained on potato dextrose agar (PDA) slants at 4 °C. These isolates were identified by colony morphology, conidial shape and multi-locus phylogeny analysis (the internal transcribed spacers, glyceraldehyde-3-phosphate, actin, beta-tubulin, calmodulin, and chitin synthase 1) [23]. Two *C. scovillei* isolates (QB3 and WL5) were randomly selected from all tested isolates to evaluate the antifungal activity and biochemical action of tetramycin.

Formulated tetramycin (0.3% AS; Liaoning Wkioc Bioengineering Co., Ltd., China; PD20160345) and chlorothalonil (40% SC; Rainbow Chemical, China; PD20132036) were used to evaluate the protective and curative activities.

PDA was prepared with 200 g potato, 20 g agar, and 20 g dextrose per liter of distilled water. Potato dextrose broth (PDB) was prepared with 200 g potato and 20 g dextrose per liter of distilled water. Water agar (WA) was prepared with 20 g dextrose and 10 g agar per liter of distilled water.

2.2. Effects of tetramycin on different developmental stages of C. scovillei

The effects of tetramycin on mycelial growth was measured as described by Myresiotis et al. [24]. Mycelial plugs (5 mm in diameter) cut from the active margins of 6-day-old colonies were transferred to PDA plates with a range of tetramycin concentrations: 0, 0.2, 0.4, 0.8, 1.6, 2, 4, 6 and 8 μg/mL. PDA media amended with acetone was used as the control. After incubation for 6 days at 25 °C in the dark, the colony diameters were measured in two perpendicular directions. Each treatment had four replicate plates, and the experiment was repeated twice.

The tetramycin-induced suppression of spore germination and germ tube elongation was determined according to Amiri et al. [25]. Spores were produced on PDA plates after 6 days of incubation at 25 °C in the dark. The spore suspensions were prepared with sterile distilled water to yield a final concentration of 1×10^5 spores mL^{-1}. Next, spore suspensions (100 μL) were spread onto 9 cm WA plates containing 0, 0.0005, 0.001, 0.0015, 0.002, 0.004, 0.006, 0.009 and 0.012 μg/mL of tetramycin. After incubation for 10 h at 25 °C in the dark, spore germination and germ tube elongation were examined. A spore was considered to be germinated when germ tube length was over half the length of the short radius of the spore. Germination was quantified by counting 200 spores per plate for each tested fungicide concentration. The germ tube lengths of 20 germinated spores per plate were measured microscopically. All experiments were repeated twice with four replicate plates.

2.3. Impact of tetramycin on secondary infection of C. scovillei

2.3.1. Effects on sporulation, spore attachment, germination and appressorium formation

Sporulation was evaluated using the protocol of a previous study [26]. Mycelial plugs (5 mm in diameter) cut from the margins of 6-day-old colonies were transferred to PDA plates with tetramycin at concentrations of 0, 1.25 and 2.5 μg/mL. After incubation for 6 days at 25 °C in the dark, the area of mycelial growth was calculated, and spores were scraped from the surface of the mycelium using 10 mL of sterile distilled water. Spore suspensions were filtered through three layers of sterile gauze and centrifuged at 10,000 rpm for 10 min. Spores were resuspended in 5 mL of sterile water. The number of spores per cm^2 of mycelium was determined under microscope with a hemocytometer.

A previous method reported by Chaky et al. [27] was used as a reference to determine the spore attachment. Spores were collected as described above to yield a final concentration of 1×10^4 spores mL^{-1}. Spore suspensions (50 μL) were transferred to a 96-well plate. After standing for 30 min, the plate was vortexed (Genie Vortex-2) at 500 rpm for 30 s. The ability of spores to attach onto the plastic surface was defined as the percentage of the spores remaining after vortexing.

Spore germination and appressorium formation were evaluated using a method described in a previous study [28]. Spores were obtained as described above to yield a final concentration of 1×10^5 spores mL^{-1}. A quantity of 100 μL of spore suspensions was spread on 9 cm water agar plates. After incubation for 10 h at 25 °C in the dark, spore germination was determined under an optical microscope. For analysis of appressorium formation, the plates were incubated in the dark for 48 h. Unless otherwise stated, all experiments conducted in Section 2.3.1 were repeated twice with four replicates.

2.3.2. Effect on the infection efficiency of the spores

The methods described by Kunova et al. [26] were used to evaluate the infection efficiency of the spores on detached pepper fruits. Spore suspensions (1×10^5 spores mL^{-1}) of tetramycin-treated or untreated isolates were collected as described in Section 2.3.1. Simila-sized green pepper fruits (cv. Huangxian 7318) were collected from an experimental greenhouse that had never been exposed to fungicides. Fruits were rinsed three times with sterile distilled water and air-dried for 1 h. Next, these pepper fruits were transferred into plastic boxes (16 cm × 10 cm × 6 cm) that were lined with three-layer wet gauze (saturated with sterile distilled water) to maintain high humidity. The middle of each pepper fruit surface was inoculated with 10 μL of the spore suspension [29]. Following inoculation, the fruits were incubated in a growth chamber (25 °C; 12 h photoperiod; 85% relative humidity) for 10 days. Infection efficiency of each treatment was determined by measuring the lesion area and infection rate [30]. Each treatment had twenty replicate pepper fruits, and the experiment was repeated twice.

2.4. Baseline sensitivity of C. scovillei to tetramycin

The sensitivity of 185 *C. scovillei* isolates to tetramycin was determined by mycelial growth and spore germination using the procedures described in Section 2.2. For the mycelial growth assay, mycelial plugs were transferred to fresh PDA plates with 0, 0.4, 0.8, 1.6, 2, 4 and 8 μg/mL of tetramycin. In the spore germination assay, the tested concentrations of tetramycin were 0, 0.001, 0.0015, 0.002, 0.004, 0.006, and 0.009 μg/mL. The experiment was performed twice with four replicate plates. The EC_{50} value for each isolate was calculated by regression analysis.

2.5. Protective and curative activities of tetramycin against C. scovillei on detached pepper fruits

For determination of the efficacy of tetramycin against *C. scovillei* on