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Sensitivity of *Botryosphaeria dothidea* from apple to tebuconazole in China

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ABSTRACT

The white rot disease caused by *Botryosphaeria dothidea* threatens apple production in Bohai bay area and along the Yellow River of China, where disease control is largely dependent on fungicides such as tebuconazole. A total of 146 isolates of *B. dothidea* obtained from different apple orchards in six provinces were tested for their sensitivity to tebuconazole, carbendazim and iprodione. The EC_{50} values of all tested isolates for tebuconazole were from 0.035 to 1.415 μg/mL. The broad range of EC_{50} values of tebuconazole suggests an obvious variation among the 146 isolates. Isolate HB13 (EC_{50} = 1.415 μg/mL) showed reduced sensitivity to tebuconazole, with an EC_{50} value significantly higher than those of the other 145 isolates tested. The low sensitivity of HB13 was stable after 15 generations, and this isolate showed similar pathogenicity as susceptible strains. EC_{50} correlation analysis indicates no cross resistance between tebuconazole and carbendazim and iprodione. Field efficacy trials showed that tebuconazole remains very effective for apple white rot control in China.

1. Introduction

Botryosphaeria dothidea caused white rot (WR) disease of apple is widely distributed in apple growing regions of temperate climate worldwide (Tang et al., 2012). In China, this disease has recently become one of the most severe apple diseases in Bohai Bay area (Shangdong and Liaoning provinces) and along the Yellow River (Henan, Hebei, Shanxi, and Shaanxi provinces) where summer temperature and rainfall are relatively high (Guo et al., 2009).

For many years, scheduled fungicide applications have been an effective tool for managing WR in apple orchards. Fungicides mancozeb and thiram are effective for preventing WR but their applications are limited to early spring because they can cause phytotoxicity (brown spots on young and mature fruits) when applied at flowering stage (Fan et al., 2013). Therefore, use of systemic fungicides is usually necessary for WR control. Systemic fungicides such as carbendazim and thiophanate-methyl had provided excellent control of WR. However, the effectiveness of these fungicides has been threatened by the emergence of resistant pathogen populations in the field (Li et al., 2009; Ma et al., 2000; Yang and Liu, 2002; Wang et al., 2010).

Tebuconazole, a member of the sterol demethylation inhibitor (DMI) fungicides, is effective against various plant pathogenic fungi (Lin et al., 2009; Su et al., 2010) and has been widely used for the control of WR in China for more than 10 years (Wang et al., 2010). So far there has been no field failure for the control of *B. dothidea* by tebuconazole reported in China, while field evolved resistance to DMIs has been reported in many other fungi, including *Botryotinia fuckeliana*, *Rhynchosporium secalis*, *Monilinia fructicola*, *Mycosphaerella fijiensis*, *Podosphaera xanthii*, *Rhynchosporium secalis*, *Cercospora beticola*, and *Sclerotinia homoeocarpa* (Golembiewski et al., 1995; Leroux et al., 1999; Locke and Philips, 1995; McGrath and Shishk, 2001; Karaoglanidis and Thanassoulopoulos, 2003; Robbertse et al., 2001; Romero and Sutton, 1997; Yoshimura et al., 2004). Thus it is necessary to survey field *B. dothidea* sensitivity to tebuconazole and to initiate monitoring programs for detecting significant changes in pathogen sensitivity that may lead to loss of disease control.

The objectives of this study were to survey tebuconazole sensitivity in *B. dothidea* in major apple producing regions of China. The stability and virulence of surveyed isolates with the lowest and

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the highest tebuconazole sensitivity were evaluated in the laboratory. Finally, cross-resistance patterns between tebuconazole, carbendazim and iprodione were investigated, and field efficacies of these three fungicides were compared.

2. Materials and methods

2.1. Fungicides

Technical grade tebuconazole (97.5% a.i.) was provided by Bayer AG Inc., China. Technical grade carbendazim (97.0%) was provided by Shandong Huayang Technology Co., Ltd. Technical grade iprodione (95.0%) was provided by Jiangsu Xinyi Agrochemical Co., Ltd. Tebuconazole and iprodione were dissolved in 100% acetone. Carbendazim was dissolved in 1% hydrochloric acid containing 0.2% (v/v) of Tween-80 (Jiangsu Xinyi Agrochemical Co., Ltd.). All stock solutions were made at a concentration of 10 mg/mL and stored in a refrigerator at 4 °C before use. Subsequent dilutions were made with sterile distilled water.

2.2. Fungal pathogens

One hundred and forty six single-spore isolates of *B. dothidea* were collected from apple orchards in major producing provinces of China between August and October of 2009 and 2010. These included 35 isolates from Shandong, 22 isolates from Liaoning, 25 isolates from Henan, 24 isolates from Shanxi, 22 isolates from Hebei, and 18 isolates from Shaanxi province. The sampled orchards had a history of more than three consecutive years of applications of tebuconazole and were at least 20 km apart from each other.

Twenty apple fruits with WR symptoms were randomly collected from each orchard and placed in paper bags and stored at 4 °C before pathogen isolation. Isolation and purification were performed following the procedure by Fan et al. (2013). Briefly, diseased tissue plugs (0.5 by 0.5 cm) were surface sterilized by soaking in 0.1% sodium hypochlorite water solution for 4 min and rinsed with sterile distilled water three times, each for 30 s. The cleaned tissue plugs were placed in potato dextrose agar (PDA) dishes and incubated for 4 days at 28 °C in total darkness. Pure cultures were further transferred to PDA. Mycelial plugs cut from the edge of the colonies on PDA were kept either in 15% glycerol at −80 °C for long-term storage or in sterile water at 4 °C for short-term storage (<6 months) before testing.

2.3. Measurement of *B. dothidea* sensitivity (EC_{50})

B. dothidea sensitivity to fungicides was assessed based on inhibition of mycelial growth. 7-mm-diameter agar disks from the margin of a 4-day-old colony were placed at the center of a series of petri dishes (9 cm diameter) containing PDA amended with various concentrations of fungicides. Mycelial growth was quantified after 3 days of incubation at 28 °C in darkness by measuring two perpendicular diameters of the growth ring in each plate, and taking the average (the diameter of the original plug subtracted). There were four replicates for each concentration, and the whole tests were performed twice.

The concentrations of tebuconazole tested were 0, 0.15, 0.30, 0.60, 1.20, and 2.40 μg ai/mL. To evaluate cross resistance, sensitivity to carbendazim and iprodione was also measured. The concentrations of carbendazim were 0, 0.1, 0.2, 0.4, and 0.8 μg ai/mL, and 0, 0.0625, 0.125, 0.25, 0.5 and 1.0 μg ai/mL for iprodione. In all cases (including non-amended control), the final acetone concentrations were adjusted to 0.1% (v/v). Fungicides were added to PDA when the agar had cooled to approximately 50 °C after autoclaving.

2.4. Stability evaluation of tebuconazole sensitivity

In vitro stability of low tebuconazole sensitivity was investigated by sub-culturing HB13, an isolate with the lowest sensitivity identified by the survey, on fungicide-free PDA for 15 generations. At the 7th and 15th generation, tebuconazole EC_{50} was determined. The whole test was repeated twice.

HB13 isolate was also grown on fungicide-free apple fruits for 4 generations and EC_{50} of tebuconazole were determined for each generation. Briefly, apples (Fuji) were surface-disinfested for 4 min in 0.1% sodium hypochlorite solution, rinsed three times with sterile distilled water, and air dried. The disinfested apples were inoculated with mycelial plugs of HB13, and incubated at 28 °C for 3 days. Re-isolation was performed using the decayed fruits and the resulting mycelia were used for next generation inoculation and EC_{50} determination. Ten apples were inoculated at each generation. The inoculated fruits were placed on trays kept in plastic containers with a shallow layer of water (about 5 cm) at the bottom to maintain high relative humidity.

2.5. Pathogenicity of isolates with low and high tebuconazole sensitivity

The reduced sensitivity isolate HB13 (RS^T) and four susceptible isolates (S^T) (HB7, MY3, LN5 and SX10 with the highest tebuconazole sensitivity surveyed) were tested for pathogenicity on apple shoots and fruits. New shoots on 2-year-old apple (*Malus domestica* 'Fuji') trees in an orchard (36°11′N, 111°08′E, Tai'an, Shandong Province) were inoculated during May to July in 2010 and in 2011. Wounds (2 mm in diameter and 2 mm deep) were created on selected shoots with a disinfested nail (with 75% alcohol). A mycelium plug (5 mm in diameter) cut from the margin of a 5-day-old culture was placed on the wound and then covered with a sterile, moist cotton ball. The inoculation site was then wrapped with a piece of Parafilm to maintain high humidity. The lesion size was measured 5 days after inoculation. Four inoculation sites were installed on a given shoot and there were five shoots for each isolate as replicates. The same number of shoots was similarly inoculated with PDA plugs without fungus as controls (Liu et al., 2013).

Pathogenicity on detached apple fruits was tested in the laboratory (Johnson et al., 1997). Mature apple (Fuji) fruits were washed, surface disinfested with 3.5% NaClO for 5 min, rinsed three times with sterile distilled water, and air-dried. Two wounds per fruit (1.5 mm in diameter and 2 mm deep) were made with a sterilized nail. Four mycelial plugs (5 mm in diameter) cut from the margin of a 5-day-old culture were placed on the surface of wounded fruits. Fruits inoculated with sterile PDA plugs were used as controls. All inoculated apples were placed on trays in plastic containers with a shallow layer of water (about 5 cm) at the bottom to create high relative humidity at 25 °C. At 5 days after inoculation the number of decayed fruits was recorded and lesion diameters were measured. For each isolate, there were 4 replications of 10 apples each. The entire experiment was repeated twice.

2.6. Field efficacy trials

Two orchards with 20-year old trees of Fuji variety in Qufu (35°59′N, 116°98′E) and Feicheng (36°24′N, 116°76′E) cities of Shandong province were selected for field efficacy testing in 2010 and 2011. The two orchards were known to be naturally infested with *B. dothidea*. Each of the orchards was divided into 24 plots of two trees each with the adjacent trees as the buffer. A completely randomized design was used with six foliar spray treatments and four replicates applied using a Jacto Heavy-Duty HD400 sprayer