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Sensitivity, resistance stability, and cross-resistance of *Plasmopara viticola* to four different fungicides

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ABSTRACT

Baseline sensitivity to fungicides was determined in 105 *Plasmopara viticola* isolates using the floating leaf disk test. The mean EC_{50} values were 0.372 ± 0.104, 0.604 ± 0.215, 0.306 ± 0.101, and 0.922 ± 0.209 μg mL^{-1} for fluxapyroxad, benthiavalicarb-isopropyl, ametoctradin, and famoxadone, respectively, which we regarded as the baseline sensitivity to the four fungicides. Resistant mutants can be generated using a median effective concentration or a minimum inhibitory concentration to fluxapyroxad, benthiavalicarb-isopropyl, ametoctradin, and famoxadone respectively, but the resistance stability of resistant mutants from the minimum inhibitory concentration (MIC) was significantly lower than that from the median effective concentration (EC_{50}). In brief, high resistant mutants obtained was at the higher concentration, the resistance stability of the mutants recovered was more quickly. But lower resistant mutants obtained was at the lower concentration, the resistance stability of the mutants recovered was slower. Even if the resistance level of the mutants is low, it is more capable of stably heritable. These data indicate that the highest dosage is not used, resistance will still develop. In a correlation analysis, no cross-resistance to each other of these four fungicides was observed.

1. Introduction

Downy mildew caused by *Plasmopara viticola* which is a native species of northern France is a major disease of grape seen worldwide. Grapevine downy mildew (*P. viticola*) affects all of the important cultivars for vine and grape production. Depending on the epidemic pressure and cultural practices, production losses of up to 100% can occur (Batovska et al., 2008; Wong et al., 2001). Grape downy mildew occurs in almost all areas of China. According to a recent survey, the total viticultural area in China was 799,000 hm^2 in 2014, and the incidence of grape downy mildew is >70% in some areas. At present, production mainly depends on chemical pesticides to control downy mildew that include metalaxyl, cymoxanil, azoxystrobin, and mandipropamid, among other compounds, although some of them are risky because of the resistance or potential resistance of *P. viticola*. A fungicide with high efficiency, broad spectrum, and high absorption is required to better control grape downy mildew.

Monitoring and managing the development of field resistance to fungicides in the pathogen population requires establishing the baseline sensitivity of the pathogen population. Simultaneously, studies examining the development of resistance are needed. Fluxapyroxad is a carboxylic acid amide fungicide that inhibits mitochondrial respiration. Benthiavalicarb-isopropyl is an amino acid derivative fungicide that inhibits cell wall synthesis. Ametoctradin is a triazolopyrimidine fungicide that binds to the submicron site of respiratory complex III in fungi. Famoxadone is an oxazolidinedione fungicide that inhibits mitochondrial electron transport. At present, no study has described the sensitivity or cross-resistance of *P. viticola* to these four fungicides. Baseline sensitivity to fungicides can be used as the basis for evaluation of resistance level. The resistance level variation will guide the dosage and the application frequency of fungicides in the field. Cross resistance results will guide chemicals fungicides exchange and rotation. Such studies would be valuable to develop effective and

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sustainable management programs.

The objectives of this study were to (i) determine the baseline sensitivity of *P. viticola* to fluxapyroxad, benthiavalicarb-isopropyl, ametoctradin, and famoxadone; (ii) select mutants resistant to the aforementioned four fungicides by adaptation; (iii) examine the resistance stability of resistant mutants; and (iv) determine whether cross-resistance exists between fluxapyroxad, benthiavalicarb-isopropyl, ametoctradin, and famoxadone.

2. Materials and methods

2.1. Materials

2.1.1. P. viticola sampling and collection

A total of 105 *P. viticola* isolates were collected from 49 orchards in different regions of Hebei, Henan, Beijing, Tianjin, Shanxi, and Shandong provinces of China between 2013 and 2014. Two or three typical isolates were collected from each orchards and the number of isolates were determined according to the size of the orchard. The orchards interval of *P. viticola* collected from more than 40 km. Each isolate was collected between June and July during the growing season. All the isolates were obtained from infected grape leaves showing the typical symptoms of downy mildew. Infected grape leaves were obtained from grape plants growing in different districts where no fluxapyroxad, benthiavalicarb-isopropyl, ametoctradin, or famoxadone had been applied previously. Those isolates were used to establish the baseline sensitivity of *P. viticola* to fluxapyroxad, benthiavalicarb-isopropyl, ametoctradin, and famoxadone.

The isolates were collected as follows: fresh leaves with *P. viticola* were put into an insulation box with ice and brought to the laboratory. Old *P. viticola* was washed away with distilled water, which was controlled by a throat sprayer with a QWJ-150 air compressor at 0.08 MPa. The washed grape leaves were placed on a wet filter paper in Petri dishes at 18 °C with a 16:8 h light:dark photoperiod for 24 h (Brent and Hollomon, 2007), after which a large amount of fresh sporangia emerged. Sporangia were washed with distilled water, centrifuged twice at 2000 rpm for 5 min, and resuspended at 1×10^5–1.5×10^5 sporangia mL^{-1} with distilled water and incubated at 4 °C for 15 min for inoculation. The sporangia concentration was measured and adjusted using a hemocytometer (Gobbin et al., 2003).

2.1.2. Plant material and P. viticola culture

The tests were performed with grapevine leaves from the Cabernet Sauvignon cultivar. Grapevine plants (*Vitis vinifera* cv. Cabernet Sauvignon) were propagated from woodcuttings in a greenhouse (Corio-Costet et al., 2011). Strict measures were undertaken to avoid cross-contamination, and these isolates were maintained by weekly transfers to detached grape leaves on wet filter paper in Petri dishes at 18 °C with a 16:8-h light:dark photoperiod (Chabane et al., 1993). Sporangia suspensions were prepared by flooding actively sporulating lesions with distilled water at a rate of 1×10^5–1.5×10^5 sporangia mL^{-1} and incubating at 4 °C for 15 min, followed by 15 min of incubation at room temperature. All of the experimentation on foliar disks was performed with grapevine leaves from 2-year-old plants (four to six leaves) (Steimetz et al., 2012).

2.1.3. Fungicide

The technical-grade fungicides fluxapyroxad, benthiavalicarb-isopropyl, ametoctradin, and famoxadone were provided by Shandong United Pesticide Industry Co., Ltd. (China), Hubei Jianyuan Chemical Co., Ltd. (China), BASF SE (China), and Jiangsu Rudong Zhongyi Chemical Co., Ltd (Yancheng, Jiangsu, China), respectively.

The baseline sensitivities of 105 *P. viticola* isolates were determined by the aforementioned floating leaf disk assay (Genet et al., 1997; Schwinn and Sozzi, 1982). Briefly, different technical-grade concentrations of fungicides were dissolved in acetone. Stock solutions of 1000 μg mL^{-1} of the active ingredient were prepared for the different fungicides and stored at 4 °C in the dark. The stock solutions were serially diluted with double-distilled water containing 0.05 μg mL^{-1} of Tween 20. The final concentrations of the different fungicides were adjusted to 0, 0.01, 0.05, 0.10, 0.50, 1.00, 5.00, or 10.00 μg mL^{-1}.

2.2. Methods

2.2.1. Sensitivity of P. viticola to fluxapyroxad, benthiavalicarb-isopropyl, ametoctradin, and famoxadone

Of each concentration of fungicide solution, 20 mL was added to separate Petri dishes. Leaf disks (15 mm diameter) of the second leaf from the tip (four to six leaves) were cut from healthy 2-year-old greenhouse-grown grape plants using a cork borer. They were then randomly placed in 9 cm-diameter Petri dishes (15 leaf disks per dish) to float on the fungicide solution with their adaxial surfaces facing upward (Genet et al., 1997). There were 45 leaf disks and three replicates for every concentration of the different fungicides. Fresh sporangia from *P. viticola* were harvested from diseased grape leaves into cold distilled water (4 °C). Leaf disks were inoculated by placing one droplet (10 mL) of inoculum (1×10^5 sporangia mL^{-1}) on the center of the adaxial side of each leaf disk (Wang et al., 2006). Dishes containing leaf disks were incubated at 18 °C for 24 h in a growth chamber in the dark for infection to occur. Subsequently, the dishes were maintained at 18 °C in a 16:8-h light:dark photoperiod for disease development. Mildew development on each leaf disk was recorded 7 days after inoculation using the following scale: 0 = no visible mildew development, 1 = 1–5%, 3 = 6–10%, 5 = 11–25%, 7 = 26–50%, and 9 > 50% of disk surface covered with mildew. Disease severity (DS) was calculated as [(9A t 7B t 5C t 3D t E)/15F] × 100, in which A, B, C, D, and E represent the number of leaf disks corresponding to a value of 9, 7, 5, 3, and 1 for mildew development, respectively, and F is the total number of leaf disks assessed. The percentage (%) of sporangia inhibition by each fungicide concentration was calculated as [(DS of untreated leaf disks − DS on treated leaf disks)/DS of untreated leaf disks × 100]. The median effective concentration for 50% inhibition (EC_{50}) for each isolate was calculated by a linear regression analysis between the value for sporangia inhibition percentage and the log of fungicide concentration using DPS software (v. 6.55). The experiments were conducted in triplicate for each isolate.

2.2.2. Development of resistant isolates by adaptation or selection of adapted resistant isolates

The EC_{50} value for the six wild isolates was lower than the average EC_{50} observed in Section 2.2.1 measured for the four fungicides respectively. The wild isolates can be used as parent isolates of any one of fungicides respectively. By using detached leaves, the minimum inhibitory concentration (MIC) and the median effective concentration (EC_{50}) of sporangia for any one of the four fungicides were determined respectively(Qiao, 2009). Sporangia suspensions (1×10^5 sporangia mL^{-1}) were prepared for each of the six wild-type isolates and sprayed onto grape leaves treated with each different fungicide at increasing concentrations (0.10, 0.50, 1.00, 5.00, 10.00, 20.00, 50.00 μg mL^{-1}). The same sporangia suspensions were also sprayed onto fungicide-free leaves as a control. The inoculated leaves were incubated on moist filter papers in Petri dishes at 18 °C using a 16:8 h light:dark light culture box for 7 days.