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Short communication

Sensitivity of *Fusarium verticillioides* isolates from rice to a novel cyanoacrylate fungicide

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ABSTRACT

The baseline sensitivity of *Fusarium verticillioides* to the novel cyanoacrylate fungicide 2-cyano-3-amino-3-phenylancryic acetate (experimental code JS399-19) was established, and its impact on the germination of rice seeds and efficacy in controlling rice bakanae disease by seed treatment were evaluated. The EC_{50} values for JS399-19 inhibiting mycelial growth of wild-type *F. verticillioides* isolates ranged from 0.162 to 0.559 μg/ml (a 3.5-fold difference between the lowest and highest value), and the average (±SE) EC_{50} value was 0.316 ± 0.072 μg/ml. The sensitivity curve was unimodal over the sensitive range and the sensitivity data can be used as a baseline for monitoring any future changes in sensitivity to JS399-19 by *F. verticillioides* populations. Germination of healthy seeds was greater than 77% for all the selected cultivars, and was unaffected by treatment with JS399-19. Treatment of infected seeds (harvested from diseased plants) with all concentrations of JS399-19 in this study significantly decreased the abnormal elongation and damping-off of seedlings in plastic bottles in the greenhouse, indicating that JS399-19 has excellent efficacy in controlling rice bakanae disease, and was safe to rice seeds. It could be used as a disinfectant of rice seeds infected by *F. verticillioides*. The results also suggested that the *F. verticillioides* isolates exhibited little sensitivity shift to this fungicide after a single application. If properly applied, JS399-19 might be used as a major fungicide or a good companion fungicide for the control of rice bakanae disease in China.

1. Introduction

Fusarium verticillioides Nirenberg (formerly known as *Fusarium moniliforme*, teleomorph: *Gibberella fujikuroi*) is an important pathogen that infects a wide range of crops all over the world. It is a complex species and has been divided into four mating groups, A, B, C and D (Hsieh et al., 1977). Group C is confined to rice while the major hosts of the other groups are sugarcane, pine, rye, wheat, asparagus, corn or sorghum (Puhalla and Spieth, 1983). A recent research has reclassified the different fungi responsible for rice bakanae disease as three different *Fusarium* species in the *G. fujikuroi* mating complex (Amatulli et al., 2010). *F. verticillioides* is also considered a major pathogen of the Gramineae, particularly in tropical and subtropical regions, where it causes economic losses as high as 50%, depending on the crop (Webster and Gunnell, 1992). The pathogenicity of this fungus is due to its ability to produce large quantities of a plant growth hormone, gibberellic acid (GA_3), which is produced commercially as an agrochemical (Jacklin et al., 2000). Field infection of rice (*Oryza sativa* Linnaeus) by *F. verticillioides* causes the disease known as bakanae disease (Shurtleff, 1980; Ou, 1985; Webster and Gunnell, 1992; Klittich and Leslie, 1988, 1989), which is characterized by the appearance of tall thin plants that obviously overgrow their uninfected counterparts. It is also a seed-transmitted pathogen and reduces germination by causing seed decay, damping-off and seedling blight (Kini et al., 2002). In addition to causing rice bakanae disease, *F. verticillioides* infection may also result in food safety problems for humans and animals as the fungus produces mycotoxins which cannot easily be removed or detoxified from grains (Kini et al., 2002).

In China, benzimidazole fungicides (carbendazim and benomyl) and ergosterol biosynthesis inhibitors (prochloraz and triflumizole) have been widely used to treat rice seeds. However, in the 1980s, *F. verticillioides* isolates resistant to benzimidazoles appeared (Tateishi and Chida, 2000). Although ergosterol biosynthesis

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inhibitors like prochloraz and triflumizole, which are applied as rice seed disinfectants, are effective against benzimidazole-resistant isolates, prochloraz is always photolytic, and isolates with resistance to triflumizole were found concomitantly with reduced efficacy of triflumizole (Tateishi and Chida, 2000).

JS399-19 (development code number for 2-cyano-3-amino-3-phenylancryic acetate) (Fig. 1), is a novel cyanoacrylate fungicide discovered and patented by the Jiangsu Branch of National Pesticide Research & Development South Center of China. According to some previous studies, this new fungicide exhibited specific activity against fungal plant pathogens of the genus Fusarium in which it strongly interferes with mycelial growth and showed potential activity against *F. verticillioides* (Li et al., 2008). Meanwhile, this fungicide is under registration for the control of this disease. Resistance to fungicides is an important problem that must be managed. Part of this management requires monitoring of resistance, and monitoring of resistance requires determination of the baseline sensitivity of the fungal pathogen, i.e., the sensitive of fungal populations that have not been exposed to the fungicide. The first objective of this study was to establish the baseline sensitivity of *F. verticillioides* to JS399-19; this was done by determining the *in vitro* response profiles of *F. verticillioides* field populations to the fungicide. The second objective was to evaluate the impact of JS399-19 on the germination of rice seeds and its efficacy in controlling rice bakanae disease by seed treatment.

2. Materials and methods

2.1. Isolation of F. verticillioides from rice plant

Potato sucrose agar (PSA) was used in isolating and storing the tested isolates. Rice samples were collected from two commercial rice fields, Nanjing and Yangzhou, Jiangsu Province, where the fungicide JS399-19 had never been used. These regions have a humid, subtropical climate. The samples were collected in 2007 and 2008. The fields were more than 100 km apart. At each location a $5.0 \times 5.0\ m^2$ plot was chosen for sampling. More than 50 plants with the symptoms of bakanae disease were randomly collected in each field, air-dried, and stored at −20 °C. In these fields, thiram + carbendazim and carbendazim were used as seed disinfectants. The isolation of *F. verticillioides* from rice was performed according to a previous study (Tateishi and Chida, 2000). These isolates were identified to be *F. verticillioides* according to the previous study in Nanjing Agricultural University (Amatulli et al., 2010), and subsequently, a total of 60 single-spore isolates were obtained.

2.2. Determination of sensitivity to fungicide JS399-19

JS399-19 in technical grade (95.5%) was dissolved in methanol to 10 mg/ml for stock solution, which was diluted into media for testing inhibition of mycelial growth. Autoclaved PSA was amended with JS399-19 to obtain final concentrations of 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 μg/ml, by adding appropriate volumes of the stock solution into the medium before solidification. In all cases (including nonamended control plates), the final methanol concentrations were the same. The 60 single-spore isolates were cultured on PSA Petri dishes (9 cm in diameter). Each concentration was replicated for three plates and tests were repeated twice. Mycelial plugs, 5 mm in diameter, were cut from the margin of actively growing colonies of 3-day-old cultures and one plug was placed in the center of a 9-cm culture Petri dish with the mycelium in contact with the medium amended with JS399-19. Then, the fungicide-amended and fungicide-free media were incubated at 25 °C. The average radius (cm) was measured after 3 to 5 days of incubation for each treatment. Then the colony area was calculated and expressed as percentage growth inhibition (=1 − (the mean colony area on amended media divided by the mean colony area on nonamended media) × 100). The median effective concentration (EC_{50}) values for each isolate were calculated by regressing percentage growth inhibition against the log of fungicide concentration as described previously (Chen et al., 2008).

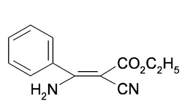

Fig. 1. Chemical structure of fungicide JS399-19.

2.3. Effect on germination of rice seeds and efficacy of seed treatment

An SC formulation (25% a.i.) of JS399-19 was used and diluted to 25 μg/ml, 12.5 μg/ml, 8.33 μg/ml and 6.25 μg/ml with water. The seeds of the cultivars Xinliangyou 6, Fengliangyou 4, Liangyou 6326 and Huiliangyou 6 were used for the seed germination test. The seeds of each cultivar were soaked in the solutions for 72 h with over 100 seeds per concentration at 25 °C and each concentration was replicated three times. The volumetric ratio of the fungicide solution against rice seeds was 1:1. Then the germination percentage was calculated by the formula: germination percentage = number of germinated seeds/total number of seeds tested × 100%. The seed germination test was repeated twice. Analysis of variance was performed by using the SAS GLM (SAS Institute, Inc., Cary, NC) procedure, and the Fisher's least significant difference (LSD) test was used to determine significant difference on the germination ratio between the treated and untreated seeds.

Thirty isolates were arbitrarily selected to test the efficacy of JS399-19 in controlling rice bakanae disease. The isolates were cultured in 5% sterile mung bean broth for shake culture at 150 rpm at 25 °C for 7 days. The conidia were collected and suspended in sterile water to 1.0×10^6/ml. The rice plants (cv. fengliangyou 4 growing in pots containing sterile soil in the greenhouse) were artificially inoculated at the flowering stage with the conidial suspension. Then plants were then kept in a greenhouse (27–38 °C, 50%–70% relative humidity, natural light) until the seeds were harvested. The seeds were dipped in the test solutions for 72 h at 30 °C as described earlier. The volumetric ratio of seeds to water in the water soaking was 1:1. The seeds were then sown on sterile soil in 8 × 15 cm plastic bottles (five seeds per bottle) and incubated at 30 °C for 3 days. Each combination of isolate and fungicide concentration (plus controls of healthy seeds and seeds without fungicide treatment) was represented by more than 20 replicate bottles. The plants were then kept in the greenhouse until the third-leaf stage. The severity of rice bakanae disease was evaluated by determining the number of seedlings that were abnormally elongated or that exhibited symptoms of damping-off as described previously (Tateishi and Chida, 2000).

2.4. Sensitivity of the F. verticillioides isolates to fungicide JS399-19 after seed treatment

After the seed treatment experiments, the rice samples with the symptoms of bakanae disease, which had been treated by fungicide JS399-19 were collected and 30 single-spore *F. verticillioides* isolates were identified and obtained as described above from each seed treatment experiment. Sensitivity of these isolates was determined by evaluation the EC_{50} values of the fungicide against these isolates as described above.