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Pyraclostrobin sensitivity of baseline and fungicide exposed isolates of *Pyrenophora tritici-repentis*

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ABSTRACT

Tan spot, caused by *Pyrenophora tritici-repentis*, is an important disease of wheat (*Triticum aestivum* L.) in the United States (U.S). Wheat growers sometimes rely on fungicide applications to manage foliar diseases. Pyraclostrobin is one of the recommended quinone outside inhibitor (QoI) fungicides and is commonly used to manage tan spot on wheat. Although *P. tritici-repentis* isolates has been reported as insensitive to QoI fungicides in Europe, little is known regarding the sensitivity of the *P. tritici-repentis* population to fungicides in the U.S. Twenty-eight isolates collected prior to 1997 and 136 isolates collected from pyraclostrobin treated fields in North Dakota were collected in 2007 and 2009 to determine if *P. tritici-repentis* isolates can use alternative respiration in the presence or absence of salicylhydroxamic acid (SHAM) *in vitro* and to determine the effective fungicide concentration that inhibited conidia germination by 50% (EC_{50}). Six of 10 baseline isolates of *P. tritici-repentis* assayed had significantly higher ($P \leq 0.05$) EC_{50} values when SHAM was not added to agar medium amended with pyraclostrobin, confirming that the fungus can utilize alternative respiration to overcome QoI toxicity *in vitro*. EC_{50} values of the baseline isolates ranged from 0.0012 to 0.0024 μg/ml (mean EC_{50} value of 0.0017 μg/ml and standard deviation is ±0.00039 μg/ml) while EC_{50} values of the 136 fungicide exposed isolates ranged from 0.0013 μg/ml to 0.0027 μg/ml (mean EC_{50} value of 0.0017 μg/ml and standard deviation is ±0.00030). These results indicate that the field exposed isolates have not shifted to reduced sensitivity to pyraclostrobin and that the baseline sensitivity values can be useful for a fungicide resistance monitoring program for *P. tritici-repentis* in the U.S.

1. Introduction

Tan spot, caused by *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis*), is one of the major foliar diseases of wheat worldwide. This disease can cause yield losses of up to 50% in favorable weather conditions (Rees and Platz, 1983; Shabber and Bockus, 1988). The shift from conventional tillage to minimal tillage has increased the incidence of tan spot (Kohli et al., 1992; Mehta and Gaudencio, 1991) and the application of fungicides reduces *P. tritici-repentis* epidemics (Adee and Pfender, 1989).

Natural fungicide derivatives such as strobilurin A and oudmansin A, produced by *Strobilurus*, *Mycena* and *Oudemansiella* species inspired the discovery of quinone outside inhibitor (QoI) fungicides (Kraiczy et al., 1996). QoI fungicides affect a protein produced from a mitochondrial gene rather than a nuclear gene. QoIs hinder electron transfer between cytochrome *b* and cytochrome *c1*, which significantly reduces ATP production (Bartlett et al., 2002). QoIs have a high risk of resistance evolution since they are single-site inhibitors (Fungicide Resistance Action Committee, 2010). A few years after registration of QoI fungicides for cereal crops, fungicide insensitivity was reported in wheat pathogens such as *Blumeria graminis* f. sp. *tritici*, *Microdochium nivale*, *Microdochium majus*, and *Mycosphaerella graminicola* (Amand et al., 2003; Sierotzki et al., 2000; Walker et al., 2009). In addition, several other fungal pathogens such as *Botrytis cinerea*, *Alternaria alternata*, *Colletotrichum graminicola*, *Pyricularia grisea*, *Pythium aphanidermatum*, *Podosphaera fusca*, *Pyrenophora teres* and *Pseudoperonospora cubensis* have also reported to have resistance to QoIs (Avila-Adame et al., 2003; Banno et al., 2009; Gisi et al., 2002; Ishii et al., 2001; Kim et al., 2003; Ma et al., 2003; Sierotzki et al., 2007; 2000). Insensitivity in all of these fungal pathogens occurred due to one of two codon changes in the cytochrome *b* (cyt *b*) gene. Two mutations are common: glycine to alanine at amino-acid position 143 (G143A) (Heaney et al., 2000; Sierotzki et al., 2007) and phenylalanine to leucine at amino-acid position 129 (F129L) (Kim et al., 2003; Pasche et al., 2005; Sierotzki et al., 2007). The G143A mutation results in 1000–1300 fold higher EC_{50} values

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in *P. grisea*,whereas, the F129L mutation results in 30–140 fold higher EC_{50} values in *P. grisea* (Kim et al., 2003). Other fungal pathogens also have higher magnitude of shift in sensitivity in the presence of G143A mutation and lower magnitude of shift in sensitivity in the presence of F129L mutation (Pasche et al., 2005; Sierotzki et al., 2007). Isolates of *Phaeosphaeria nodorum* had been found with high EC_{50} values without G143A substitution (Blixt et al., 2009). This explains possible association with alternative respiration pathway and mechanism of resistance has been found to reduce efficacy of QoI fungicides in certain fungi *in vitro* (Wise et al., 2008; Ziogas et al., 1997). The plant mitochondrial alternative oxidase (AOX), a ubiquinone oxidase, provides for a limited level of coupled electron transport resulting in enough ATP production for the fungus to survive in presence of strobilurins *in vitro* (Wood and Hollomon, 2003). Salicylhydroxamic acid (SHAM) has been reported and used to inhibit the activity of the AOX (Sierotzki et al., 2007; Vincelli and Dixon, 2002; Ziogas et al., 1997).

In 2003, pyraclostrobin (Headline, BASF Corporation, Research Triangle Park, NC) received a section 3 registration from the United States (U.S.) Environmental Protection Agency (EPA) and was recommended for use on wheat to manage tan spot disease. Although Headline has become the second most used fungicide for control of tan spot in North Dakota (Zollinger et al., 2009), sensitivity of *P. tritici-repentis* populations to pyraclostrobin has not been measured. Implementation of a monitoring program could be used to detect any significant changes in *P. tritici-repentis* populations in wheat and to assess the effectiveness of resistance management strategies. The main objectives of this study were to i) examine the effect of SHAM amended pyraclostrobin and SHAM non-amended pyraclostrobin in water agar medium on conidia germination, ii) investigate the baseline sensitivity of *P. tritici-repentis* isolates to pyraclostrobin, and iii) assess sensitivity of pyraclostrobin exposed *P. tritici-repentis* isolates to pyraclostrobin.

2. Materials and methods

2.1. Collection and isolation of P. tritici-repentis isolates

A total of 28 baseline isolates were collected prior to registration of any QoIs in North Dakota (ND). Among these, 27 isolates were collected from ND, and one isolate was collected from South Dakota before 1997 (Table 1). In addition, 136 pyraclostrobin fungicide exposed isolates were collected in 2007 and 2009. Among these, 74 fungicide exposed isolates were collected from a research plot at Lisbon, ND in 2007 while 62 isolates were collected from a research plot at Fargo, ND in 2009 (Table 1). These isolates were from fields that were sprayed with one application of recommended pyraclostrobin per season at both locations (Ransom and McMullen, 2008; personal communication); however, pyraclostrobin has been used in ND for more than 7 years (Ransom and McMullen, 2008; personal communication). Isolates collected in 2007 were isolated by Dr. S. Ali. Leaf samples collected in 2009 were from the pyraclostrobin exposed field and brought to the lab. Tan spot infected-leaf samples were cut into small segments and surface sterilized with approximately 1.5% sodium hypochlorite (diluted from 6% commercial Clorox) for 5–10 s and rinsed with sterile distilled water twice. The leaf segments were placed on two layers of sterile Whatman # 1 filter paper in Petri dishes (100 × 15 mm) and were kept for a 24 h light and 24 h dark cycle to induce spore production. A single spore was collected with an isolating needle and placed on V-8 potato dextrose agar (150 ml V-8 juice, 10 g PDA, 10 g agar, 3 g CaCO3, and 850 ml distilled water). Mycelia were cut using 5-mm diameter cork borer, dried and stored in −80 °C.

Table 1
Pyrenophora tritici-repentis isolates were collected from 1970's to 2009. Isolates collected before 1997 were considered baseline isolates.

Year collected	Location	No. of isolates tested
1970's	South Dakota	1
1980's	North Dakota	2
1990	North Dakota	3
1992	North Dakota	13
1995	North Dakota	5
1996	North Dakota	4
2007	Lisbon, North Dakota	74
2009	Fargo, North Dakota	62

2.2. Effect of SHAM on conidia germination

All experimental procedures were as described previously (Pasche et al., 2004). To determine the effect of SHAM (Alfa Aesar, Ward Hill, MA) on conidia germination, a stock solution (100,000 μg/ml) was prepared by adding 0.1 g of SHAM to 1 ml of 99.9% methanol. The mixture was warmed at 37 °C for approximately 10 min to completely dissolve the SHAM. SHAM amended (100 μg/ml) and non-amended 2% water agar medium was prepared and methanol concentration was maintained at 0.1% (Pasche et al., 2004). Ten isolates (Ptr 86-4, Ptr 92 145-2-4, Ptr 92 115-2-3, Ptr 92 148-1-1, Ptr 92 119-2-3, Ptr 1A, Ptr 90 DL-22, Ptr 92 120-2-2, Ptr 4b, and Ptr 90 P7d) were randomly selected from the baseline isolates using the RAND function from Microsoft Excel 2007 (Microsoft Corp. Redmond, WA). The spore concentration of each isolate was adjusted to 4000 spores/ml and 300 μl of the spore suspension was uniformly spread on Petri dishes (65 × 15 mm). The Petri plates were incubated for 6 h in continuous light at 21C and a total of 50 spores per isolate were counted to estimate the percentage of germinating spores. At least one germ tube with the same length as the spore or more than one germ tube, irrespective of germ tube length, was used as a criterion for conidium germination as previously described (Pasche et al., 2004). Germination of the 10 isolates was recorded for the two replicated Petri dishes in each of two experiments. Experiments were conducted in completely randomized design (CRD) and data were analyzed separately for each experiment. The F-test was used to determine if both experiments could be combined. During the statistical analysis, $P = 0.05$ was considered as the level of significance.

2.3. Effect of pyraclostrobin on conidia germination in the presence and absence of SHAM

To determine if alternative respiration was induced in the absence of SHAM, the same 10 isolates assayed for sensitivity to pyraclostrobin in the presence and absence of SHAM in 2% water agar medium. A stock solution of 10,000 μg/ml pyraclostrobin (95% a. i.) was prepared by dissolving 10.5 mg pyraclostrobin into 1 ml of acetone. After 6 h of incubation, a total of 50 conidia were assessed for germination. Conidia germination was evaluated on 2% water agar amended with 0, 0.001, 0.002, 0.004, 0.008 and 0.0016 μg/ml of pyraclostrobin. Conidia germination was first converted to percentage germination and then percent inhibition of conidia germination was calculated to compare with the untreated control using the following formula: 100-[(% germination of fungicide amended) ÷ (mean % germination of non-amended)] (Wise et al., 2009). The linear interpolation method was used to determine fungicide concentration which inhibited conidia germination by 50% of the control and was termed as EC_{50} value. Each isolate was tested in two replicates in CRD and the experiment was repeated. Proc ANOVA procedure of Statistical Analysis System (Version 9.2, SAS Institute Inc., Cary, NC) and Proc TTEST were used to compare EC_{50} values of each isolate on SHAM amended and SHAM non-amended water agar.