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Baseline sensitivity of QoI-resistant isolates of *Pyrenophora tritici-repentis* from Argentina to fenpicoxamid

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Abstract Tan spot (TS) of wheat caused by *Pyrenophora tritici-repentis* (*Ptr*) is an important disease worldwide. Premixes of quinone outside inhibitors (QoI) and demethylation inhibitors are the most frequently used fungicides for TS control in Argentina. Recently, QoI resistance was reported in *Ptr* populations in Argentina, where the prevalence and intensity of this disease has increased steadily over the past decades. Therefore, development of new tools and active ingredients (a.i.) for the management of this pathosystem are needed. Fenpicoxamid is a new fungicide that belongs to the quinone inside inhibitors. This a.i. shows no cross-resistance and strong efficacy against *Zymoseptoria tritici* strains resistant to other fungicide classes. Here we evaluated a total of 50 QoI-resistant *Ptr* isolates from Argentina for their sensitivity to fenpicoxamid. Different concentrations were tested in both in vitro (0, 0.01, 0.025, 0.05, 0.075, 0.1 and 1 μg/ml) and in vivo (0, 5, 10 and 20 μg/ml) bioassays. Fenpicoxamid strongly inhibited in vitro spore germination of all tested isolates (EC_{50} values ranged from 0.004 to 0.067 μg/ml). In greenhouse tests, fenpicoxamid significantly decreased TS leaf incidence at 10 ppm and the average spots per leaf at 20 ppm. For the first time, we report the high fungitoxicity and lack of cross-resistance of fenpicoxamid against QoI-resistant *Ptr* isolates. This is a promising new a.i. for controlling TS of wheat.

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Introduction

Tan spot (TS), caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechsler 1923 (*Ptr*), is a serious foliar disease of wheat not only in Argentina but also in other parts of the world (Carmona et al., 2006; Ciuffetti et al., 2014; De Wolf et al., 1998; Gamba et al., 2012; Kremneva et al., 2021; Moffat & Santana, 2018; Momeni et al., 2014; Oliver et al., 2008). TS causes chlorosis and tan-colored necrotic lesions on leaves, decreasing photosynthetic surface area and consequently reducing the amount of resources available for grain yield formation (number and weight of grains) (Rees et al., 1982). Considerable yield losses (up to 50% - 70%) have been documented due to the disease when susceptible wheat cultivars are planted (Kohli & Ackermann, 1992; Rees & Platz, 1983). Thus, TS is considered a major constraint to wheat productivity (Wegulo, 2011). The intensity of the disease will depend on the combination of various factors: the susceptibility of the wheat genotype planted, the pathogenicity / virulence of the predominant races of the pathogen in the planted area, the amount / density of initial inoculum available and environmental conditions throughout the growing season. Although numerous alternative hosts have been reported for *Ptr* (Ali & Francl, 2003; de Wolf et al., 1998), the most important sources of inoculum in agricultural

systems are infested stubble from previous growing seasons and infected seeds (Carmona et al., 2006).

When compared to other foliar diseases of wheat, TS is believed to be a recently expanding disease (Friesen et al., 2006). The first description of the typical symptoms caused by *Ptr* dates from the year 1941 (Barrus, 1942). Subsequently, TS started to gain importance gradually since the 1960s / 1970s until today, reaching a prominent place among wheat diseases worldwide (Oliver & Solomon, 2008). In Argentina, TS is among the three most important foliar diseases of wheat, with a continuously increasing intensity and prevalence. This constant increase may be due to at least four factors that have occurred concomitantly during the last 20 years: 1) the expansion of wheat cultivation under no-till and monoculture, which ensures the presence of sufficient inoculum in the stubble from a growing season to the following; 2) the spread of the pathogen over short and long distances through infected seeds (Carmona et al., 2006); 3) the difficulty in obtaining TS-resistant wheat cultivars to stop the spread of the disease in the field (Faris et al., 2013; Jecke et al., 2014), and 4) the appearance of quinone outside inhibitor (QoI)-resistant *Ptr* strains (Sautua & Carmona, 2021).

In Argentina, few TS-tolerant wheat varieties are currently commercially available (Alberione et al., 2021). Although crop rotation and the use of healthy seed (or efficiently treated with fungicide) can have a significant effect in reducing TS intensity (Carmona et al., 2006), currently, the use of foliar fungicides is one of the most frequently used tools for disease management. However, the frequent increase in the emergence and spread of new fungicide-resistant *Ptr* strains endangers not only the effective life of fungicide active ingredients (a.i.), but also the sustainability of wheat production. In general, the genetic variability and adaptability of a phytopathogen are very important aspects to consider when evaluating the success of controlling the disease it causes in a crop. *Ptr* produces three host-selective proteinaceous toxins and at least eight races of the pathogen are known to occur, which result in complex and constantly evolving pathogen populations (Ciuffetti et al., 2010; Friesen et al., 2006; Kader et al., 2021; Kamel et al., 2019; Kariyawasam et al., 2021; Pandelova et al., 2012). Furthermore, *Ptr* populations are the only ones that have been shown to have the ability to develop up to three different types of mutations (G143A, F129L and G137R) in the cytochrome *b* gene, which are related to QoI fungicide resistance (Sierotzki et al., 2007). Recently, FRAC reported that in Europe (Denmark, Hungary, Latvia and Poland) a high frequency of QoI-resistant *Ptr* strains has been found, indicating that the G143A mutation is now predominant (FRAC, 2020). As this mutation confers robust QoI-resistance to individual *Ptr* isolates, unlike the other two known ones, when the G143A allele frequency is high enough in a population, strobilurins become ineffective at the field level. In Argentina, Sautua and Carmona (2021) reported the G143A mutation for the first time in South American *Ptr* strains collected during 2014, 2016 and 2018 in different locations of the main wheat-producing areas of the Pampas region. The in vitro, in vivo and molecular studies confirmed the resistance in all the 82 isolates evaluated, determining that the resistance of *Ptr* to QoI is wide spread ().

The development of new molecules that can control resistant strains is deserving a high priority in fungicide companies. The process of developing and registering a new fungicide has changed significantly in recent years. In addition to the initial investment needed for the discovery and development, the costs to meet the environmental protection requirements, pesticide applicator safety and human health care have increased considerably. According to McDougall (2016), the current investment is around 250 to 300 million USD and the process can last on average more than 10 years. When a new fungicide marketed, two aspects are critical: 1) determining baseline fungicide sensitivities of the pathogen, and 2) establishing appropriate strategies to manage fungicide resistance, in order to maximize its effective life. New fungicide a.i. may have a previously known or a new mode of action (MoA), or they may even be fungicides that, although the MoA has already been reported, are applied to crops on which they have never been used. Recently, new molecules have been reported that, despite having a previously known MoA, manage to control strains resistant to this MoA. One example is the azole mefentrifluconazole, which shows high efficacy against demethylation inhibitor-resistant strains of *Zymoseptoria tritici* (*Zt*) and performs better in the field than other triazoles depending on the *Zt* field populations structure, which was influenced by azoles used previously in the area (Kiiker et al., 2021). Another example is metyltetraprole, a QoI that is capable of controlling strains of various pathogens resistant to strobilurins (e.g. G143A) (Matsuzaki et al., 2020,b; Suemoto et al., 2019). Fenpicoxamid is a

picolinamide, a new class of fungicide chemistry targeting the mitochondrial cytochrome bc_1 complex at the inner side of the mitochondrial membrane (Q_i site) (Young et al., 2018). This a.i. was derived from an antibiotic (UK-2A) that was isolated from the fermentation of the actynomycete *Streptomyces* sp. 517–02, and has strong antifungal activity against a wide range of fungal pathogens (Owen et al., 2017). Although the MoA of quinone inside inhibitors (QiI) is well known, until the arrival of fenpicoxamid only two QiI fungicides specific to oomycetes (e.g. *Phytophthora infestans*, *Plasmopara viticola*, etc.) (Leadbeater, 2015) were marketed: amisulbrom and cyazofamide (FRAC, 2014). For the first time, a new QiI, fenpicoxamid, displays an anti-ascomycete activity, which makes it suitable to control major wheat pathogens like *Zt*. This a.i. had never been previously tested for *Ptr* control.

Most of the worldwide fungicide sensitivity studies of wheat pathogens are conducted on *Zt*, causing *Septoria tritici* blotch (STB) (Birr et al., 2021; Garnault et al., 2021; Kiiker et al., 2021; Kildea et al., 2019). Some of the reasons for this are that this disease is the main problem in European wheat production and presents great difficulties for its control (Fones & Gurr, 2015; Torriani et al., 2015), in part due to the reports of *Zt* fungicide resistance (Birr et al., 2021; Cheval et al., 2017; Garnault et al., 2021; Hellin et al., 2021; Kiiker et al., 2021; Kildea et al., 2019). In contrast, resistance and sensitivity studies on *Ptr* are very scarce because TS is usually epidemiologically more important in non-European countries or regions. Contrary to what happens in Europe, in Argentina TS is the most important foliar disease of wheat along with stripe rust and leaf rust, while STB does not present epidemiologically important levels (Carmona et al., 2020; Sautua & Carmona, 2021). According to the information provided by FRAC (2021), there is an intensive monitoring program of the *Zt* sensitivity to fenpicoxamid, but there is no information related to the sensitivity of *Ptr* to this new fungicide. The specialized literature does not offer any study that includes *Ptr* as a target control through the use of fenpicoxamid. Likewise, a second-generation picolinamide fungicide inspired by UK-2A, florylpicoxamid, has recently been evaluated mainly for *Zt* control (Meyer et al., 2021; Yao et al., 2021). Given this lack of research for *Ptr*, and the urgent need to obtain reference studies for a resistance monitoring program in *Ptr* populations, the objective of this study was to evaluate and estimate the baseline sensitivity to fenpicoxamid of Argentine *Ptr* isolates resistant to QoI.

Materials and methods

Collection, isolation, and in vitro sensitivity of *Ptr* isolates to fenpicoxamid

From a collection of 82 single-spore *Ptr* isolates resistant to QoI that was previously obtained in Argentina (Sautua & Carmona, 2021), 50 isolates were randomly selected and tested in vitro against fenpicoxamid. The information of the isolates included in the present study is shown in Supplementary Table 1.

In vitro experiments were conducted as previously described by Sautua and Carmona (2021). Technical grade fenpicoxamid (98.7% a.i.) was provided by Corteva™ Inc. (Delaware, U.S.A.). The fungicide was dissolved in acetone to make a stock solution of 100 mg/ml. Conidial germination was assessed on 2% water agar amended with fenpicoxamid at 0, 0.01, 0.025, 0.05, 0.075, 0.1 and 1 μg/ml. Because bc_1 complex inhibitors can activate alternative oxidase (AOX) in fungi belonging to the Ascomycetes and pseudofungi (FRAC, 2014), the salicylhydroxamic acid (SHAM) was included in the tests at 100 μg/ml (as previously evaluated in Sautua & Carmona, 2021). The SHAM (99% a.i.; Alfa Aesar) was dissolved in 99% methanol to make a stock solution of 100 mg/ml. The acetone and methanol concentration did not exceed the non-toxic concentration of 0.1% (as previously evaluated in Sautua & Carmona, 2021). Each isolate was tested in two replicates and 200 conidia were examined in each 9 cm Petri dish to estimate the percentage of germinating spores. The methodology used in the spore germination inhibition assays is described in detail in Sautua and Carmona (2021). The percentage inhibition of conidial germination was estimated for each isolate as previously described. The experiment was repeated once (400 conidia evaluated in each run and 800 conidia in total by isolate). The fungicide 50% effective concentration (EC_{50}) at which conidial germination of *Ptr* is reduced by 50% compared to the nonamended water agar control, was estimated for each isolate using a nonlinear regression analysis with a log-logistic model, specifically a Weibull type I four-parameter model, as previously described in Sautua et al. (2020).

In planta assay

Tan spot control by fenpicoxamid (98.7% a.i.) was evaluated in a greenhouse bioassay. In vivo experiments were conducted as previously described by Sautua and Carmona (2021). Fenpicoxamid was applied at 0, 5, 10 and 20 μg/ml. A treatment with azoxystrobin (99.5% a.i.; Nova SA) at 25 μg/ml was included. Fungicides were applied preventively 24 h prior to inoculation. An uninoculated control was sprayed with water and 0.2% Tween 20. Wheat plants of the TS-susceptible variety DM Algarrobo were grown in pots arranged in a completely randomized design and for each treatment twenty plants (five per pot) were sprayed to run-off. After 24 h of treatment, plants were inoculated with a mixture of conidia from the isolates ARG_2018_005, ARG_2018_010, ARG_2018_018, ARG_2018_044, ARG_2018_050, ARG_2018_051, ARG_2018_055, ARG_2018_062, ARG_2018_070, ARG_2018_071, ARG_2018_083 and ARG_2018_086, by spraying a 50 ml conidial suspension of approximately 3000 conidia/ml. On the seventh day after inoculation, the plants were evaluated for TS symptoms intensity: (a) foliar incidence was estimated as the number of leaves with at least one spot with a chlorotic halo of 2–3 mm with respect to the total number of leaves, expressed as a percentage (Reis et al., 2016); (b) average number of spots per leaf was estimated in ten leaves taken at random from each treatment, counting the spots with a chlorotic halo greater than 2–3 mm and averaging the total of spots by the number of leaves evaluated (Reis et al., 2016). The experiment was repeated once. Data were analyzed using One-way ANOVA with Tukey's HSD post hoc test.

Results

Fenpicoxamid strongly inhibited spore germination after 8 h incubation (Fig. 1). All 50 isolates tested were sensitive to fenpicoxamid with EC_{50} values ranging from 0.004 to 0.067 μg/ml (Fig. 2, Supplementary Table 2). The average EC_{50} value of 0.033 μg/ml indicates that this a.i. is fungitoxic against QoI-resistant *Ptr* isolates carrying the G143A mutation in the mitochondrially encoded cytochrome *b* gene. We confirmed the lack of cross-resistance of fenpicoxamid against QoI-resistant *Ptr* isolates. The mean EC_{50} value of the baseline *Ptr* isolates from the USA to pyraclostrobin was 0.0017 mg/ml (Patel et al., 2012); and the mean EC_{50} value of four QoI-sensitive and three QoI-resistant *Zt* isolates to fenpicoxamid was 0.013 mg/ml and 0.0027, respectively (Young et al., 2018).

In vivo, fenpicoxamid significantly reduced the foliar incidence at 10 ppm (43.3% of TS control) and both the foliar incidence and the average number of spots per leaf at 20 ppm with 64.2% and 76.5% of TS control, respectively (Table 1). Azoxystrobin did not decrease the incidence nor the average number of spots per leaf with respect to the control without fungicide application (1.6% and 0% control, respectively).

Discussion

For the first time, we report on the fungitoxicity and lack of cross-resistance of fenpicoxamid against QoI-resistant *Ptr* isolates. Based on the in vitro and greenhouse trials presented here, fenpicoxamid is a promising new picolinamide a.i. for controlling TS of wheat. However, the fungicide dose and number of applications that maximize effective field control of TS must be adjusted and validated in field trials (Reis et al., 2015).

Accordingly, this new a.i. is potentially good to be added to fungicide mixtures within anti-resistance management programs and can be a fundamental tool for the control of *Ptr* populations resistant to QoI. Thus, including fenpicoxamid in fungicide mixtures could reduce the probability developing of resistance to existing MoA such as demethylation inhibitors (DMIs) and succinate dehydrogenase inhibitors (SDHIs). Among the possible partners, the following can be mentioned: prothioconazole, propiconazole and epoxiconazole, which have shown fungitoxicity against *Ptr* in preliminary studies in our laboratory (unpublished data). More research is required to determine the fungitoxicity of both the existing SDHIs and those that will soon be launched in the market, and of multisite fungicides.

Nevertheless, single-site fungicides such as QiIs, QoIs and SDHIs possess a high intrinsic risk to develop resistance development, mainly due to the emergence of spontaneous target-site resistance mutations in the phytopathogenic fungi (Hawkins & Fraaije, 2021). Fouché et al. (2022) recently reported on a potential mutation in the cytochrome *b* Q_i site as the most likely resistance mechanism towards fenpicoxamid in *Zt.* Therefore, fenpicoxamid must be carefully used respecting

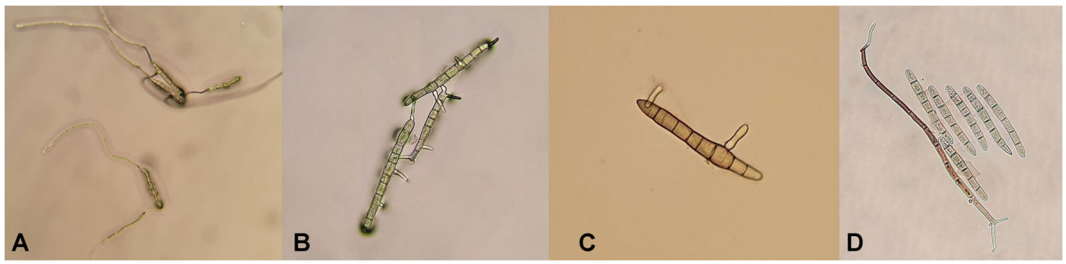

Fig. 1 Descriptive photographs of inhibition of QoI-resistant *Pyrenophora tritici-repentis* conidial germination at different concentrations of fenpicoxamid, taken with an optical microscope. A, non-amended control; B, fenpicoxamid at 0.05 μg/ml; C, fenpicoxamid at 0.075 μg/ml; D, fenpicoxamid at 0.1 μg/ml

appropriate anti-resistance management measures. This is extremely important to prolong its effective life (Corkley et al., 2022). Fungicide resistance management strategies mainly include the use of mixtures of effective fungicides with different MoA with no cross-resistance and not concerned by field resistance yet when possible, alternation of MoA, correct timing of applications according to disease development (as

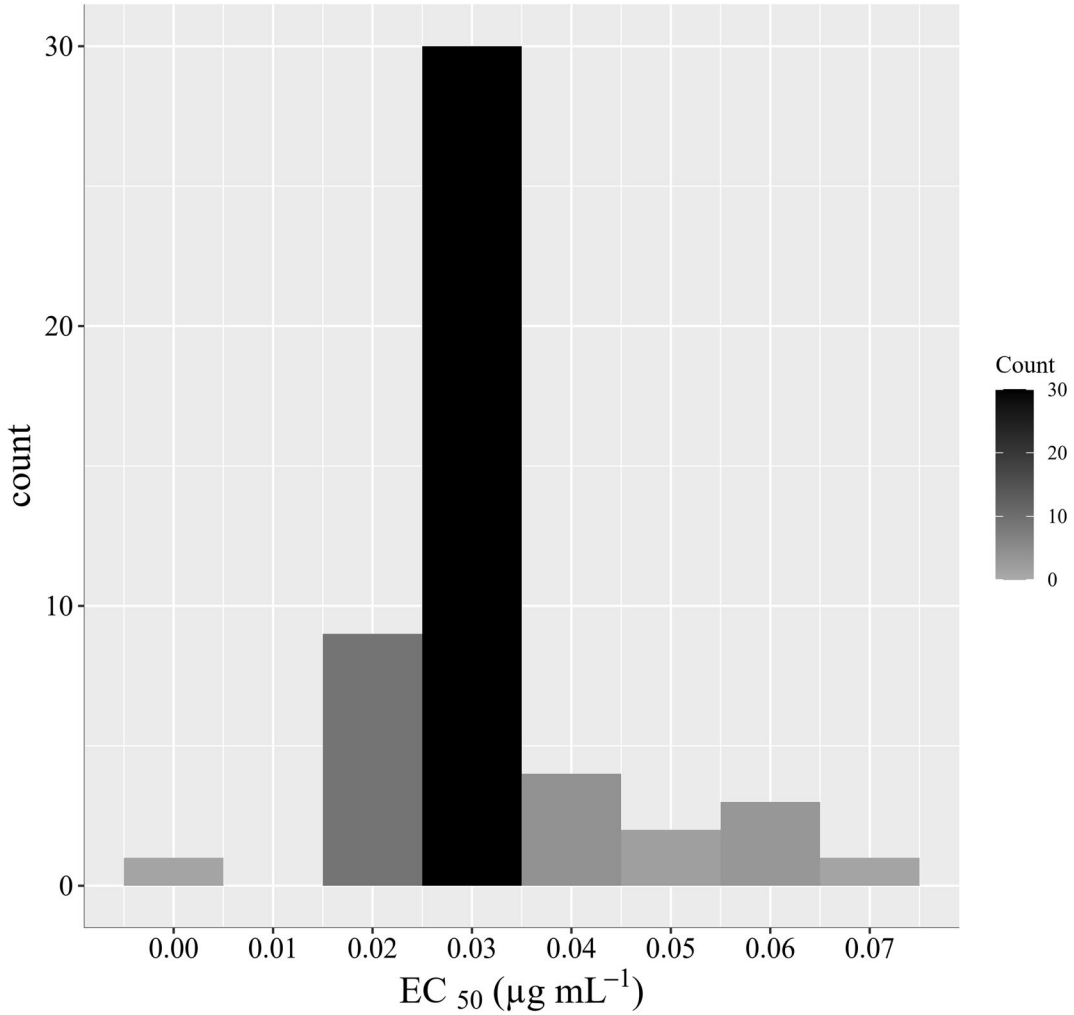

Fig. 2 Frequency distribution of effective fenpicoxamid concentrations that inhibited conidial germination by 50% (EC_{50}) for baseline QoI resistant *Pyrenophora tritici-repentis* isolates. Individual isolates are grouped in class intervals of 0.01 μg/ml

Table 1 Preventive control of tan spot of wheat on leaves of potted wheat cultivar DM Algarrobo in the greenhouse with various fungicide treatments. Plants were evaluated seven days after inoculation with a mixture of conidia from *Ptr* isolates resistant to QoI fungicides

Treatment	Incidence (%)		Incidence control (%)	Average spots per leaf		Spots per leaf control (%)
control	89.6	a	0	20.3	ab	0
azoxystrobin 25 ppm	88.2	a	1.6	20.5	a	0
fenpicoxamid 5 ppm	85.7	a	4.4	22.3	a	0
fenpicoxamid 10 ppm	50.9	b	43.3	11.6	bc	43
fenpicoxamid 20 ppm	32.1	c	64.2	4.8	cd	76.5

Significant differences were evaluated using ANOVA followed by Tukey's post hoc test. Means followed by the same letter are not significantly different ($P < 0.05$)

treating bigger infestations increases the risk of resistance selection), limit to one application of picolinamide per season and respecting full application dose according to labels (Brent & Hollomon, 2007; Carmona et al., 2018; Carmona et al., 2020; FRAC, 2019).

The potential benefit of determining the baseline sensitivity of fenpicoxamid prior to market launch in South America is of great value. This is particularly important for pathogens such as *Ptr* that had become resistant to the widely used QoIs. This will help improve risk assessment, monitoring programs and administration of this new class of fungicide MoA after market launch. The information generated here will serve as a baseline sensitivity of Argentine *Ptr* populations to fenpicoxamid and will help to monitor and identify any shifts in sensitivity over time. Likewise, the EC_{50} values found in this work could be useful as reference values for studies carried out in other wheat producing regions or countries where TS is an important disease.

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Data availability The isolates are available and deposited in the fungal culture collection at the Plant Pathology department of the School of Agriculture of the University of Buenos Aires (FAUBA).Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10658-022-02582-y.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval This manuscript did not involve any human participants, and/ or animals.

Informed consent All the authors certify that the work carried out in this research followed the principles of ethical and professional conduct have been followed. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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