UNIVERSIDADE FEDERAL DE VIÇOSA

SHEILA MARIA PEREIRA DE ANDRADE

PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF THE RESISTANCE TO QUINONE OUTSIDE INHIBITORS IN *Fusarium graminearum*

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“*Somewhere, something incredible is waiting to be discovered.”*

(Carl Sagan)

ABSTRACT

ANDRADE, Sheila Maria Pereira de, M.Sc., Universidade Federal de Viçosa, May, 2021. **Phenotypic and molecular characterization of the resistance to quinone outside inhibitors in *Fusarium graminearum***. Adviser: Emerson Medeiros Del Ponte.

Fusarium Head Blight (FHB) is a fungal disease of small grains caused mainly by *Fusarium graminearum*. In Brazil, farmers rely on fungicides to minimize yield losses caused by a complex of foliar and floral diseases, as well as to reduce mycotoxin contamination due to FHB. Usually, a range of active ingredients are used in isolation or dual premixes that include a triazole (DMI) and a quinone outside inhibitor (QoI) fungicides. Comprehensive information on the sensitivity/resistance of *F. graminearum* to fungicides is available only for DMIs, while for QoI, data are scarce and controversial. A total of 225 strains were studied in this work and they were split into two geographical subcollections: one from Rio Grande do Sul (RS), 125 strains, and other from Paraná (PR), 100 strains. The *in vitro* sensitivity of *F. graminearum* isolates was assessed through the conidia germination assay and tested concentrations for azoxystrobin were 0, 0.001, 0.01, 0.1, 1,10 and 100 μg mL^{-1}, while for pyraclostrobin were 0, 0.05, 0.5, 1.0, 5.0 and 10 μg mL^{-1}. Effective concentration leading to 50% inhibition (EC_{50}) of conidial germination was obtained based on three-parameter Weibull function. Isolates, in which the EC_{50} was not previously determined, were screened by discriminatory doses (DD) for both fungicides. Molecular analysis of the *cytb* gene was performed and nine isolates were selected and sequenced. The median EC_{50} value for azoxystrobin (n = 25) was 2.20 μg mL^{-1} in the PR collection and 4.04 μg mL^{-1} in the RS collection. For pyraclostrobin (n = 50), the median EC_{50} was 0.28 μg mL^{-1} in the PR collection and 0.24 μg mL^{-1} in the RS collection. These median values are greater than the maximum EC_{50} reported in previous works. Shapiro-Wilk test indicated that EC_{50} values were normally distributed (P = 0.23), and no significant difference was observed among the collections. A comparison between the two fungicides showed significant differences between them and pyraclostrobin was more fungitoxic than azoxystrobin. There was no significant correlation between the EC_{50} values of the two fungicides. Based on

the DD, 50% of the strains analysed were classified as less sensitive to azoxystrobin in the PR collection (n = 75) and 28% in the RS collection (n = 100). For pyraclostrobin, 33% of the strains were classified as less sensitive in the PR collection and 18.8% in the RS collection. We also observed an increase in the relative frequency of less sensitivity isolates between five years (2007-2011) in the RS collection. Sequence alignments showed no point mutation in any hot spot (F129L, G137R, G143A) even in the isolates with highly EC_{50}. In conclusion, we are able to set a sensitivity profile for *F. graminearum* populations, however the resistance mechanism associated with QoI fungicides remains unclear. Our study suggested a shift towards less sensitive populations and further studies shall be conducted for monitoring more contemporary isolates from these regions, both *in vitro* and *in vivo* experiments, as well as testing other mechanisms of resistance.

Keywords. Fusarium Head Blight. Strobilurin. *Gibberella zeae. Triticum aestivum*

RESUMO

ANDRADE, Sheila Maria Pereira de, M.Sc., Universidade Federal de Viçosa, maio de 2021. **Caracterização fenotípica e molecular da resistência à inibidores da quinona externa em *Fusarium graminearum***. Orientador: Emerson Medeiros Del Ponte.

A giberela do trigo é uma doença fúngica de pequenos grãos causada principalmente por *Fusarium graminearum*. No Brasil, os agricultores contam com fungicidas para minimizar as perdas de produtividade causadas por um complexo de doenças foliares e florais, bem como para reduzir a contaminação por micotoxinas devido à giberela. Normalmente, uma variedade de ingredientes ativos é usado isoladamente ou em pré-misturas que incluem um fungicida triazol (DMI) e um inibidor externo de quinona (QoI). Informações abrangentes sobre a sensibilidade/resistência de *F. graminearum* a fungicidas estão disponíveis apenas para DMIs, enquanto para QoI, os dados são escassos e controversos. Neste trabalho, foram estudados 225 isolados, os quais foram divididos em duas subcoleções geográficas: uma do Rio Grande do Sul (RS), 125 isolados, e outra do Paraná (PR), 100 isolados. A sensibilidade *in vitro* de isolados de *F. graminearum* foi avaliada por meio do teste de germinação de conídios e as concentrações testadas para azoxistrobina foram 0, 0,001, 0,01, 0,1, 1,10 e 100 μg mL^{-1}, enquanto para piraclostrobina foram 0, 0,05, 0,5, 1,0, 5,0 e 10 μg mL^{-1}. A concentração efetiva levando a 50% de inibição (EC_{50}) da germinação de conídios foi obtida com base na função de Weibull de três parâmetros. Os isolados, nos quais a EC_{50} não foi determinada previamente, foram selecionados por doses discriminatórias (DD) para ambos os fungicidas. A análise molecular do gene cytb foi realizada e nove isolados foram selecionados e sequenciados. O valor mediano de EC_{50} para azoxistrobina (n = 25) foi de 2,20 μg mL^{-1} na coleção PR e 4,04 μg mL^{-1} na coleção RS. Para a piraclostrobina (n = 50), a EC_{50} mediana foi de 0,28 μg mL^{-1} na coleção PR e 0,24 μg mL^{-1} na coleção RS. Esses valores medianos são maiores do que os valores máximo de EC_{50} previamente relatados. O teste de Shapiro-Wilk indicou que os valores de EC_{50} apresentaram distribuição normal (P = 0,23), não sendo observada diferença significativa entre as coleções. Uma comparação entre os dois fungicidas

mostrou diferenças significativas entre eles e, a piraclostrobina foi mais fungitóxica do que a azoxistrobina. Não houve correlação significativa entre os valores de EC_{50} dos dois fungicidas. Com base na DD, 50% (n = 75) dos isolados analisados foram classificadas como menos sensíveis à azoxistrobina na coleção PR e 28% na coleção RS. Para a piraclostrobina, 33% (n = 100) das cepas foram classificadas como menos sensíveis na coleção PR e 18,8% na coleção RS. Observamos também um aumento na frequência relativa de isolados de menor sensibilidade entre cinco anos (2007-2011) na coleção RS. Os alinhamentos de sequência não mostraram nenhuma mutação em nenhum *hot spot* (F129L, G137R, G143A), mesmo nos isolados com altos valores de EC_{50}. Em conclusão, nós definimos um perfil de sensibilidade para populações de *F. graminearum*, no entanto, o mecanismo de resistência associado aos fungicidas QoI permanece incerto. Nosso estudo sugere uma mudança para populações menos sensíveis e novos estudos devem ser conduzidos monitorando isolados mais contemporâneos, tanto em experimentos *in vitro* quanto *in vivo*, bem como testando outros mecanismos de resistência.

Palavras-chave: Giberela. Estrobilurina. *Gibberella zeae*. *Triticum aestivum.*

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INTRODUCTION

Fusarium head blight (FHB) is a fungal disease of small grains caused mainly by a handful of species of the *Fusarium graminearum* species complex (FGSC) (O'Donnell et al. 2004; Aoki et al. 2012). In the temperate and subtropical regions of the Americas, *Fusarium graminearum* sensu stricto (hereafter *F. graminearum* or Fgra) is the main pathogen, including Brazil. However, *F. meridionale*, another FGSC member, increases in importance in Paraná state, Brazil, where maize is a major crop and also a host for this species (Del Ponte et al. 2015; Pereira et al. 2021).

FHB is known to reduce yield loss, and disease severity may vary according to year and wheat-growing region. In Brazil, for example, yield losses ranged from 3.0 to 25% during the last decades (Paul et al. 2008, 2018; Duffeck et al. 2020). In addition to quantitative losses, the disease reduces grain quality, which is a significant concern due to the presence of mycotoxins such as those of the trichothecene and zearalenone classes, which are both harmful for humans and animals, and have been reported in surveys conducted in the country (Del Ponte et al. 2011; Pagnussatt et al. 2014; Santana et al. 2016, 2017; Duffeck et al. 2017).

F. graminearum is a potent trichothecene producer, particularly deoxynivalenol (DON) and smaller amounts of its acetylated derivatives (3AcDON, 15AcDON), although some are able to produce nivalenol (NIV) (Ward et al. 2008; Schmale et al. 2011; Del Ponte et al. 2013; Nicolli et al. 2015). FHB symptoms and DON are generally positively correlated and management approaches should focus on both disease control and mycotoxin reduction (Paul et al. 2008; Wegulo et al. 2011, 2015).

The occurrence and intensity of FHB epidemics are driven mainly by humid conditions whenever airborne inoculum is present during anthesis (McMullen et al. 2012). In general, the primary inoculum is constituted of the sexual spores (ascospores) originated from crop residues. Assexually-produced spores (macroconidia) also contribute to a smaller extent of infections and are the main source used in artificial inoculations. Fungus proliferation and intracellular growth is associated with the increase of mycotoxins production, and it is well known that DON is a virulence factor (Jansen et al. 2005;Fernando et al. 2021). Management practices such as tillage and previous crop used in rotation are important factors driving epidemics, depending on the region (Dill-Macky and Jones 2000; McMullen et al. 2012; Del Ponte et al. 2015).

Disease control is best achieved through the use of fungicides sprays and less susceptible cultivars (Willyerd et al. 2012; McMullen et al. 2012). To date, there are no commercial wheat cultivars in Brazil with a good level of resistance to control FHB without the need of fungicides (Mendes et al. 2018; Santana et al. 2020). Therefore growers rely mostly on fungicide applications for which the rate of success is variable (Machado et al. 2017; Feksa et al. 2019; Barro et al. 2020). Consequently, legislative measures, such as the establishment of maximum tolerance limits for *Fusarium* mycotoxins have been implemented in several countries, including Brazil (Miller et al. 2014; Cheli et al. 2014; ANVISA 2017).

Globally, the triazole chemical class (DMIs), such as tebuconazole, metconazole and prothioconazole has been shown the best results against FHB, even though they have shown variable efficacy among the active ingredients, cultivars and environmental conditions (Paul et al. 2008; McMullen et al. 2012; Machado et al. 2017). In China, carbendazim, a benzimidazole chemical class, have

been vastly applied but in recent years efficacy has decreased resulting in a failure of control (Yuan and Zhou 2005; Liu et al. 2019). Quinone-outside inhibitors (QoI) fungicides, known as strobilurin chemical class have also been tested and used to manage FHB (Duan et al. 2020).

Quinone outside inhibitor (QoIs) fungicides (FRAC group 11) were introduced in the market in 1996. Nowadays they are extensively used in agriculture, accounting 30% of the global market (Ishii and Hollomon 2015; Duan et al. 2020). This class inhibits the mitochondrial respiration due to the block of Q_o site (outer quinol oxidation site) of cytochrome bc1 complex. Subsequently the fungal cells have a lack of ATP due to blocking of electron flow. Highly energy dependent stages such as spore germination and zoospore movement are extremely dependent on mitochondrial respiration (Bartlett et al. 2002; Ellner 2005; Ishii and Hollomon 2015).

Nine chemical classes can be distinguished from the QoI group. Between them, methoxy-acrylates, methoxy-carbamates and oximino-acetates are the most applied groups, and they comprise azoxystrobin, pyraclostrobin and trifloxystrobin respectively. These fungicides share a common mode of action but their spectra and intrinsic levels of biological activity are different (Fernández-Ortuño and Torés 2008; Ishii and Hollomon 2015).

According to FRAC (Fungicide Resistance Action Committee; www.frac.info), the strobilurin class presents a single-site mode action, which is classified as medium to high risk for resistance development. Many cases of QoI resistance have been reported in different plant pathogens and some mutations are possibly involved in reducing binding of Qo inhibitors (Stevenson et al. 2004; Pasche et al. 2004; Rosenzweig et al. 2008). Consequently, studies for monitoring the temporal and

regional variation in fungicide resistance are important to identify risk factors related to management that could contribute to the decline of sensitivity due to selection for resistance (Chen et al. 2012).

In many fungi, resistance to strobilurins is caused by mutations affecting the amino acid positions 129, 137 and 143 in the mitochondrial cytochrome b gene (*cytb*) (Gisi et al. 2002; Kim et al. 2003; Fernández-Ortuño and Torés 2008). The change of a phenylalanine to leucine at position 129 (F129L) is close to the heme bL and interferes with the toxophore of QoI fungicides. The change of a glycine to an arginine at the position 137 (G137R), also mediates moderate resistance but still not well understood and its minor importance. Meanwhile, a stronger resistance is observed when a change of a glycine to an alanine at position 143 (G143A) in the *cytb* gene occurs. It leads to an interference with the linker part of QoI molecules resulting in strikingly reduced binding (Kim et al. 2003; Ishii and Hollomon 2015).

Isolates carrying the F129L are usually controlled by the recommended field levels of QoI, while isolates carrying the G143A are always associated with the failure in disease control (Fernández-Ortuño and Torés 2008). The cost of resistance to QoI fungicides seems to vary with environmental conditions, but generally fitness penalty is assumed to have a stronger effect in isolates with F129L mutation when compared with isolates carrying the G143A mutation (Ishii and Hollomon 2015).

Cytochrome b is a membrane protein belonging to the core of the mitochondrial bc_1 complex (complex III) in the respiratory chain. The gene responsible for the encode of the cytochrome b is the cytochrome b (*cytb*) gene and it is located in the mitochondrial genome (Brandt and Trumpower 1994). Exon/intron organization is an important element for prediction of resistance risk for QoI

fungicides, and point mutation on the hot spot 143 only occurs if there is no group 1-like intron after the codon. For example, *M. grisea, M. graminicola* and *P. viticola* are some pathogens whose resistance to QoI fungicides caused by G143A is known. On the other hand, all rusts and *A. solani* this mutation have never been reported. This can be explained because these pathogens have an intron after the codon and a mutation at the exon/intron boundary could strongly affect the splicing process, leading a deficient cytochrome b and individuals carrying this substitutions would not survive (Grasso et al. 2006). To date, for *F. graminearum* there is no organization of the coding part (exon) and non-coding part (intron).

In addition to the commonly known point mutations, other resistance mechanisms to QoI fungicides have already been described. According to Wood and Hollomon (2003), an alternative respiration route could represent an essential pathway in the transition of sensitivity to resistant isolates to QoI fungicides. This alternative route is sustained by alternative oxidase (AOX), which rescues the electron transfer surrounding the inhibitory site of QoI. Another mechanism is through the efflux transporters which prevent the accumulation of toxic compounds inside the fungi cells. ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) are examples of efflux pumps. This condition was reported under field and *in vitro* conditions (Reimann and Deising 2005; Fernández-Ortuño and Torés 2008).

Overall, anti-resistance strategies should be incorporated as a management approach by the farmers and it should include practices that cover all species in all ranges of sensitivity. In this case the implementation of some techniques as appropriate dosage, timing intervals and mixing fungicides has been shown to be efficient in reducing the frequency of resistant isolates (Fernández-Ortuño and Torés 2008; Ishii and Hollomon 2015). For example, in *Plasmopara viticola* a decrease of

mutation frequency has been reported after significant reductions in QoI applications and this recovery of sensitivity suggests that resistant phenotypes in these populations are less competitive than the sensitive ones (Genet et al. 2006).

In Brazil, fungicide programs play an important role not only to control FHB, but other fungal leaf diseases such as tan spot (*Pyrenophora tritici-repentis*), brown spot (*Bipolaris sorokiniana*) and leaf rust (*Puccinia triticina*) (Feksa et al. 2019). It is common practice in Brazil to apply fungicide premix, which contains both a triazole and strobilurin chemical fungicides. This appears controversial in view of reports that showed that strobilurin fungicides can stimulate DON production by up-regulating the expression of *Tri5* and *Tri6* genes and increasing the acetil-CoA production (Magan et al. 2002; Duan et al. 2020). However, technical and economic benefits from their use especially when mixed with DMI have been reported (Paul et al. 2018; Feksa et al. 2019; Barro et al. 2020).

Globally, data about the resistance in *F. graminearum* strains to QoI fungicides are scarce and controversial (Audenaert et al. 2010; Avozani et al. 2014; Duan et al. 2020). According to a study conducted by Audenaert et al. (2010), azoxystrobin did not affect the conidial germination of the wild-type strain 8/1 in any time at any dilution. Therefore they point that *F. graminearum* is highly resistant to this type of fungicide. Conversely, Duan (2020) tested six QoIs, including azoxystrobin and pyraclostrobin, and reported an excellent antifungal effect on spore germination and mycelial growth of 32 strains. They classified these fungicides as potentially useful for controlling FHB.

Dubos et al. (2011) tested trifloxystrobin against 55 isolates of *F. graminearum* from different countries, years and chemotypes. That fungicide was unable to reduce

fungal growth; hence the EC_{20} (concentration reducing fungal growth by 20%) was calculated instead of EC_{50}. Even though the isolates expressed a similar level of resistance, no point mutation was found when an *in silico* analysis was performed in the position 129 and 143 on the PH-1 strain. The authors suggested that *F. graminearum* is naturally resistant to trifloxystrobin and other QoIs, even though this species is not listed among the plant pathogenic organisms resistant to disease control agents (FRAC 2020). Therefore results on the effectiveness of strobilurins against *Fusarium* spp. are inconsistent and remain uncertain.

Due to the importance of chemical control for managing FHB in Brazil, that includes the use of QoI fungicides in commercial premixes, it is urgent to characterize the sensitivity of *F. graminearum* to this chemical group. To date, just one study was conducted in the country and used 10 FGSC strains from Rio Grande do Sul (RS) and Paraná (PR) states to characterize their EC_{50} (Avozani et al. 2014). In our study, we tested the hypothesis that the sensitivity to QoIs in *F. graminearum* populations are variable in space and time given the differences in management practices and the more intensive use of fungicides over the years, which may be selecting for QoI resistance via genetic mutation.

The objectives of this study were: i) to characterize the sensitivity of *F. graminearum* that cause Fusarium head blight of wheat in Brazil to two most used QoI fungicides (azoxystrobin and pyraclostrobin); and ii) to detect the presence of known point mutations associated with these fungicides.

MATERIAL AND METHODS

Strain collections

A total of 225 strains were studied in this work. They were split into two geographical subcollections: RS (125 strains) and PR (100 strains). The selected strains for both collections were chosen to represent samples for a range of years and locations.The RS collection was obtained from 2007 to 2011 at 26 municipalities**.** The PR collection was obtained from seven years (between 2011 to 2020) at 10 municipalities. Their spatial distribution, number of isolates per year are shown in Fig 1.

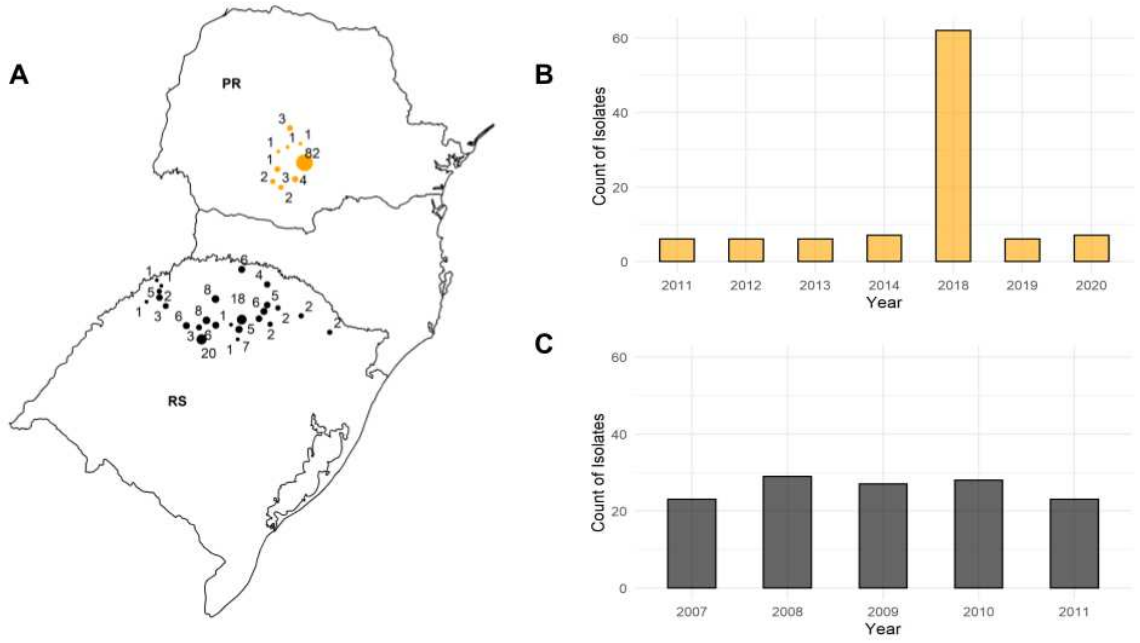

Fig 1. In (A) location and number of *Fusarium graminearum* strains by municipality obtained from blighted grains or spikes of wheat grown in Rio Grande do Sul (RS), n =125 and Paraná (PR) states, n =100, Brazil. (B) Number of isolates per year in the PR collection and (C) RS collection.

Mycelium production and DNA extraction

The purified strains from monosporic culturing were reactivated in Petri dishes on PDA (Potato- Dextrose- Agar) with chloramphenicol and streptomycin at 50 µg ml^{-1} for 5-6 days. After that, for mycelium production, they were transferred to another plate for 1 week.

The DNA extraction was performed as previously described by Farman et al. (2017) with some adaptations. The mycelium of the isolates with 7 days of growth were scraped and added in eppendorf with two beads and it was suspended in 1.0 ml of Lysis buffer. The cells were disrupted using the tissuelyser II (Qiagen) and placed in a 65°C water bath for 30 minutes. Chloroform/isoamyl alcohol (24:1, 0.7 ml) were added and mixed by flicking the tube a few times. The suspension was placed back in the water bath for an additional 30 min, with the tube being inverted several times after 15 min to resuspend the mycelium. The material was subsequently centrifuged for 15 min at 14000 rpm to separate the aqueous and organic phases. The supernatant was carefully pipetted into another tube and the DNA was precipitated by adding 0,45 ml of isopropanol. After inverting the tube several times, the tube was immediately centrifuged at 1400 rpm for 10 min to form the DNA pellet. The supernatant was rinsed with 70% ethanol and then dried on the benchtop. At the end the pellet was dissolved in 100 µl of Tris-EDTA (TE) containing 2µl RNase A (1µg mL^{-1}) and stored at -4ºC. DNA samples were quantified and adjusted to 50 µg mL^{-1}, using a Nanodrop 2000 (Thermo Scientific).

Species identification

All but the strains from PR (2018, 2019 and 2020) were identified in previous studies using sequencing or multilocus genotype (MLGT) (Astolfi et al. 2012; Del Ponte et al.

2015; Pereira et al. 2021). The unidentified strains from PR state were assayed using the Fg16F/Fg16R, which allow to differentiate between *F. graminearum (< 500 pb)* and *F. meridionale* (> 500 *pb*) according to the band product size (Nicholson et al. 1998; Hafez et al. 2020). Sequence and product size of the primers can be assessed in Table 1.

PCR reactions were performed in 25µl volumes containing 12.5µl of MasterMix (Promega), 1µl of each primer and 9.5µl of ultrapure water. Amplifications were performed on PCR thermocycler with cycle conditions as follows: 94°C for 8 min, followed by 30 cycles of 94°C for 1.5 min, 60°C for 1 min, 72°C for 2 min, and a final extension of 5 min at 72°C. In all reactions a previously identified positive control for known strains were added (*F. graminearum*, *F. meridionale*, *F. cortaderiae* and *F. austroamericanum*), as well a previously identified negative control (*F. avenaceum*). The PCR products were separated by electrophoresis using a 2.5% agarose gel and visualized under UV 360 nm light.

Fungicide sensitivity in vitro assays

Determination of EC_{50}

The two QoIs evaluated were azoxystrobin (Amistar®, Syngenta) and pyraclostrobin (Comet®, BASF). The active ingredients were diluted in sterile distilled water until the solution reached a concentration of 1000 µg mL^{-1} (stock solution). For azoxystrobin the tested concentrations were: 0, 0.001, 0.01, 0.1, 1,10 and 100 µg mL^{-1}, while for pyraclostrobin the tested concentrations were: 0, 0.05, 0.5, 1.0, 5.0 and 10 µg mL^{-1}. Salicylhydroxamic acid (SHAM), a characteristic inhibitor of an alternative respiration route (AOX), was dissolved in methanol and added in each fungicide concentration to reach the final concentration of 100 µg mL^{-1} (Duan et al. 2012).

For these assays, for azoxystrobin, a subcollection of 25 strains were used, being 12 from PR (2011 to 2014) and the rest from RS state (2007 to 2011), within each, the year was represented by approximately three strains. Meanwhile, for pyraclostrobin, a subcollection of 50 strains were used, being 25 from PR (2011 to 2014) and the rest from RS state (2007 to 2011), within each, the year was represented by approximately five strains.

The sensitivity of *F. graminearum* isolates was assessed through the conidia germination assay. The germination test was conducted using a standard method for this fungicide group, the glass drop technique (Dhingra and Sinclair 1995). The isolates were activated on PDA (Potato-Dextrose-Agar), on which a mycelial plug was transferred. For inoculum production, after 7 days, the mycelium was washed and transferred to SNA plates with streptomycin at 50 μg ml^{-1}, and incubated at 25°C under 16/8 h (light/dark) for 7 days. Then, plates were washed with sterilized water plus tween 20 (0.01%) and gelatin (6%). The gelatin was used to help hold the droplets and it does not influence conidia germination. Conidial suspension was calibrated at the final concentration of $1x10^5$ conidia per ml.

Droplets of 30μl of conidial suspension were transferred on a glass adding a 30μl fungicide solution corresponding to each fungicide and each final concentration and twice of the volume of the stock solution was added to avoid dilution. The glass was placed in a plastic box (11x113.5cm) with a moistened paper towel to maintain the humidity around 90%. The box was placed in darkness at 25°C for 7 hours as described by Duan et al. (2020). Two replicates were done for each isolate and the experiment was repeated once.

Under microscope light, the number of germinated macroconidia (a conidium in which the germination tube grows to at least half the length of the conidium was counted as germinated) was subsequently obtained. Fifty macroconidia were evaluated for each replication.

Statistical analysis

The ‘drc’ package (Ritz et al. 2015) was used to select the best fit nonlinear model for ten isolates from each collection for each fungicide. The ‘mselect’ function was used to determine the best fit nonlinear model for conidial germination. The nonlinear models, log-logistic models, Weibull models and Brain-Cousens hormesis models were tested. The best-fitting model was determined based on the lowest Akaike’s Information Criteria (AIC) value. Subsequently, for all collections and fungicides, effective concentration leading to 50% inhibition of conidial germination (EC_{50}) was obtained based on three-parameter Weibull function through the ‘ec50estimator’ package (Alves 2020). All data from two experiments were combined for each fungicide.

A Shapiro-Wilk test was used to assess normality of EC_{50} values. A t-test was used to test whether the sensitivity of isolates differed between the two collections and the two fungicides. Spearman correlation analysis ($\alpha = 0.05$) for the relationship between the EC_{50} values of pyraclostrobin and azoxystrobin was used to detect possible cross-resistance. All analyses were conducted in the R statistical computing environment (R Core Team 2020).

Discriminatory dose for screening sensitivity

A discriminatory dose (DD) is defined as the one capable of classifying the isolates as less sensitive or sensitive to fungicides according to previous EC_{50} assays (Russell 2004; Lehner et al. 2015). In our work, DD for azoxystrobin and pyraclostrobin was chosen based on the slightly above of the median value of EC_{50} for 25 and 50 strains, respectively. For this assay, 175 strains, for which the EC_{50} was not previously determined, were screened using the DDs for each fungicide. Of all, 75 isolates (mostly of the isolates collected in 2018) belonged to the PR collection and 100 strains (approximately 20 isolates per year) belonged to the RS collection.

Droplets of 30µl of conidial suspension were transferred on a glass adding 30µl of the DD of each fungicide in addition to the control (no fungicide). The glass was placed in a plastic box in the same conditions described in the conidia germination assay. Under the microscope light, the number of germinated macroconidia (a conidium in which the germination tube grows to at least half the length of the conidium was counted as germinated) was subsequently obtained. Fifty macroconidia were evaluated per replication. Three replicates were done for each isolate and the whole experiment was repeated once.

A generalized linear model with ‘glm’ function was fitted to the germinated conidia from the discriminatory dose trials. In the literature, isolates are considered less sensitive (or resistant) when the number of germinated conidia at the discriminatory dose exceeded by 50% the number of germinated conidia in the control (Ishii et al. 2009). However, in our study, we calculated the 95% confidence intervals for the parameter fitted to the model in order to classify the isolates (α = 0,05). When the lower interval was higher than 0.5, the isolate was classified less

sensitive, otherwise, they were classified as sensitive. The data from two experiments were combined for each fungicide.

Molecular characterization of resistance

Cytochrome b gene structure

Sequences of genomic DNA of *F. graminearum* were assessed on the European Nucleotide Archive (ENA) and the genome sequence of the isolate CML3066 was used as a model (Wood, et al. 2020). The ENA accession number is LT222057. The coding sequence was aligned with the genome sequence and the exon/intron organization was tagged with the Geneious prime® software version 2021.0.1 (Kearse et al. 2012).

Primer design and amplification of cytochrome b gene fragment

Field resistance to strobilurins in phytopathogenic fungi are associated with *cytb* gene mutations. The most common point mutation occurs at the target spot 143, 137 and 129. Based on the organization of the *cytb* gene described above and the size of the exon-intron between the target spots, two pairs of primers were designed. The first one tagging the target spot 129 located at the Exon-2 and the second one tagging the target spots 137 and 143 located at the Exon-3. In this way, primers forward and reverse were designed to cover the entire exon region corresponding to each target spot. Oligonucleotide primers were designed with the program Primer3 version 0.5 available online (Koressaar and Remm 2007). The specificity of the primers was first tested *in silico* by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and then one PCR product from one isolate was sequenced and aligned with the isolate CML3066. All the information of the primers can be assessed on Table 1.

Table 1. Information about Polymerase Chain Reaction (PCR) primers used in this study.

Target	Point mutation	Primers Name	Primer Sequence (bp)	PCR product (bp)
FGSC	-	Fg16-F	CTCCGGATATGTTGCGTCAA	500-600
	-	Fg16-R	GGTAGGTATCCGACATGGCAA	
Cytb/ Exon-2	129	Fg129-F	AAATGGGTGGTAATCCCTACAA	346
		Fg129-R	TACGTGGGAACGAAGTCTCC	
Cytb/ Exon-3	137/143	Fg143-F	TGGGTTAGCTTTAGCTTGCTT	313
		Fg143-R	CATTGCGCCGTACATTAAAA	

Polymerase chain reaction (PCR) were performed using the primers Fg129-F and Fg129-R; Fg143-F and Fg143-R. PCR reactions were performed in 25µl volumes containing 12.5µl of MasterMix (Promega), 1µl of each primer and 9.5µl of ultrapure water. Amplifications were performed on PCR thermocycler with cycle conditions as follows: 95°C for 2.5 min, followed by 40 cycles of 94°C for 30 s, 52°C for 1 min, 72°C for 1.5 min, and a final extension of 8.5 min at 72°C. The PCR products were separated by electrophoresis using a 1% agarose gel and visualized under UV 360 nm light.

Sequencing and bioinformatics analysis

Nine isolates were chosen in order to select the entire range of sensitivities (sensitive, moderate and less sensitive) based on the EC_{50} values and the discriminatory doses established in this work. The PCR products of each isolate and target spot were sent to ACTGene (Rio Grande do Sul, Brazil). They were purified using ExoSAP-ITTM kit and then the DNA sequencing was performed on AB 3500

Genetic Analyzer. To obtain a contig sequence of each isolate for each target spot, sequence data were first analyzed with SeqAssem program version 07/2008. Then, all the sequences were imported to Geneious prime® software and the partial cyt*b* sequences of each isolate for each target spot were aligned with the sequence of the CML3066 isolate. The tool translation (genetic code: transl_table4) was used for detection of mutations at the target spots. A transition from G to C at codon 428 (G143A) shows a higher level or complete resistance, while a transversion from C to A at codon 387 (F129L) and G to R at the codon 411 (G137R) shows a partial or moderate resistance (Kim et al. 2003; Ishii et al. 2007).

RESULTS

EC_{50} determination

The EC_{50} values for azoxystrobin in 25 *F. graminearum* isolates ranged from 0.26 to 268.53 µg mL^{-1} in the PR collection and from 0.33 to 329.71 µg mL^{-1} in the RS collection (Table 2; Fig. 2A). EC_{50} values were not normally distributed (P = 0.02), and statistical analyses showed no significant difference between the collections (Table 2). For pyraclostrobin (n = 50 isolates), the EC_{50} ranged from 0.12 to 0.66 µg mL^{-1} in the PR collection and from 0.028 to 1.13 µg mL^{-1} in the RS collection (Table 2; Fig. 2B). Shapiro-Wilk test indicated that EC_{50} values were normally distributed (P = 0.23). In addition, EC_{50} values were statistically analysed among the collections and no significant difference was observed (Table 2).

A comparison between the two fungicides was also analysed. EC_{50} values for azoxystrobin EC_{50} values ranged from 0.26 to 329.71 µg mL^{-1} while for pyraclostrobin ranged from 0.028 to 1.13 µg mL^{-1} (Table 2). EC_{50} values, comparing the two

fungicides, were not normally distributed (*P* = 2.217e-13) and statistical analyses showed significant differences between the fungicides (Table 2). Summary statistics of the strains can be assessed on Table 2. All EC_{50} values from each isolate, collection and fungicide can be assessed on the Supplementary Table1 and Supplementary Table 2.

Table 2. Summary of pyraclostrobin and azoxystrobin sensitivity range and distribution of *Fusarium graminearum* strains used in this study.

State	Fungicide	n	Min	Lower quartile	Median	Upper quartile	Max	*P*-value[z]
PR	Azoxystrobin	25	0.26	0.59	2.20	5.87	268.53	0.875
RS	Azoxystrobin	25	0.33	0.56	4.04	9.98	329.71	
PR	Pyraclostrobin	50	0.12	0.19	0.28	0.43	0.66	0.742
RS	Pyraclostrobin	50	0.028	0.16	0.24	0.45	1.13	
All	Azoxystrobin	25	0.26	0.56	2.94	9.95	329.71	<0.001
	Pyraclostrobin	25	0.03	0.16	0.39	0.51	1.13	

For each interaction, median, lower quartile and upper quartile were calculated from tested isolates based on EC_{50} values.

[z] P-value >0.05 suggests no significant differences according to anova test for normal data and glm models for non-normal data.

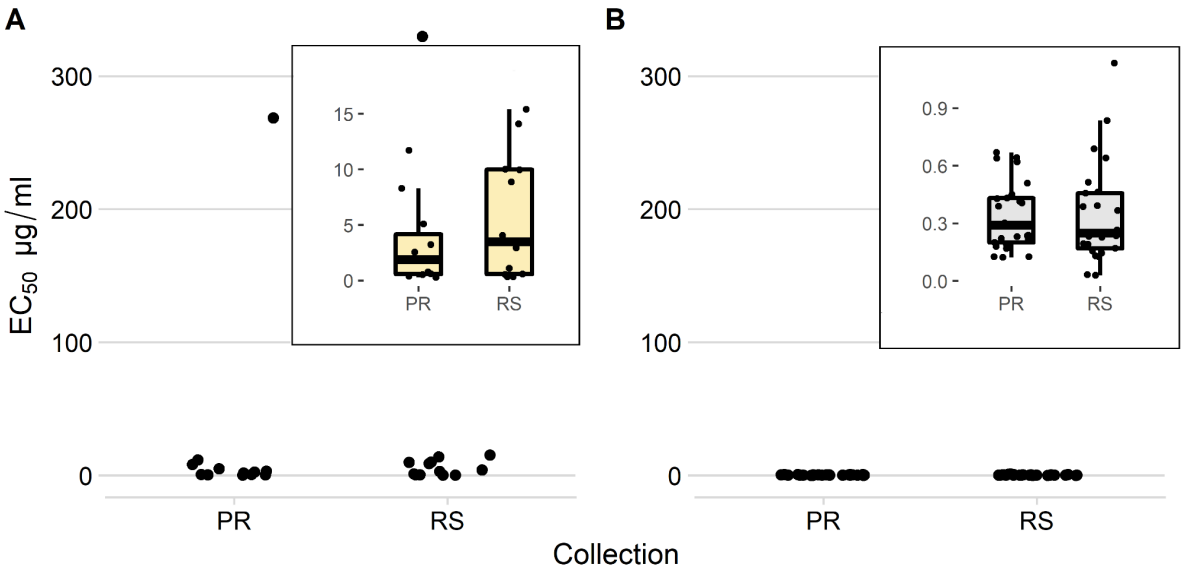

Fig 2. Distribution of the effective concentration of azoxystrobin (A) and pyraclostrobin (B) that reduced 50% of the conidia germination (EC_{50}) of *Fusarium graminearum* isolates for both subcollections. The boxplots embedded show the distribution without the isolates in which EC_{50} was bigger than 100 µg mL^{-1}. The thick horizontal line inside the box represents the median, the lines at the top and bottom of the boxes represent the 75 and 25% percentiles of the data, respectively. The individual dots represent the mean value for each strain. The points outside that range indicate outliers.

According to the correlation analysis of the transformed data (log), the EC_{50} values determined for pyraclostrobin and azoxystrobin were not significantly associated (n = 25; r = - 0.13; P = 0.53) and no cross-resistance could then be suggested (Fig. S1).

Sensitivity based on the discriminatory dosis

We defined the concentrations of 0.5 µg mL^{-1} and 5 µg mL^{-1}, which are slightly above the median for all strains within each fungicide, as discriminatory doses for

pyraclostrobin and azoxystrobin, respectively. Overall, for azoxystrobin 50.01% of the strains analysed were classified as less sensitive in the PR collection and 28.05% in the RS collection (Fig. 3A). For pyraclostrobin, 33.00% of the strains were classified as less sensitive in the PR collection and 18.77% in the RS collection (Fig. 3B). The relative frequency of less sensitive isolates were generally higher for azoxystrobin than pyraclostrobin in both collections (Fig. 3).

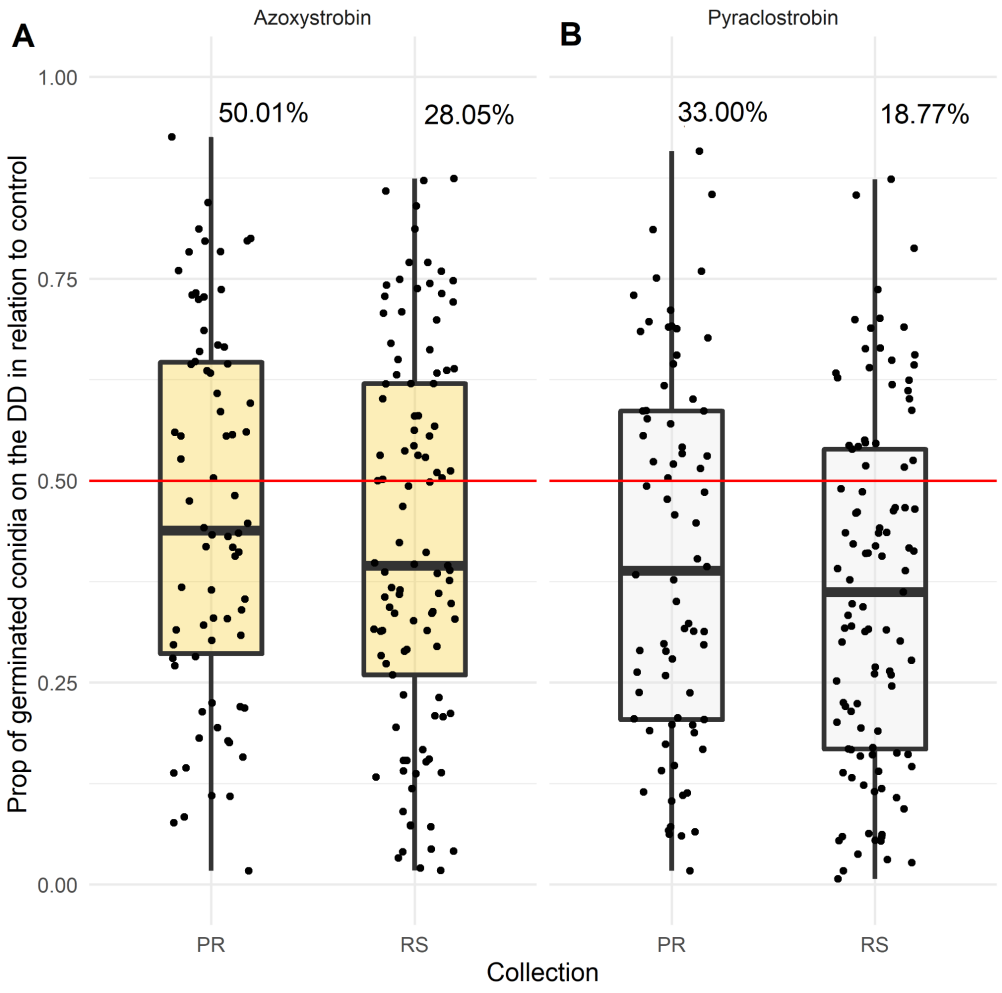

Fig 3. Box-plot of the proportion of the germinated conidia at the discriminatory dose of *Fusarium graminearum* isolates for PR collection (n = 75 strains) and RS collection (n = 100 strains). In (A) for azoxystrobin and in (B) for pyraclostrobin. The individual dots represent the

mean value for each strain. The red line represents 50% of germination in relation to control. The values above show the relative frequency of less sensitive isolates according to the lower interval (LL IC 95 > 0.5) in each collection for each fungicide.

For the RS collection, we also were able to compare the sensitivity over five years. An increase in the relative frequency of less sensitivity isolates could be observed over time. For azoxystrobin, a total increase of 37.63% of less sensitivity isolates were detected (Fig. 4A), while for pyraclostrobin, an increase of 42.36% was reported (Fig. 4B), both between the years 2007-2011. The relative frequency for each year can be assessed in Fig. 4.

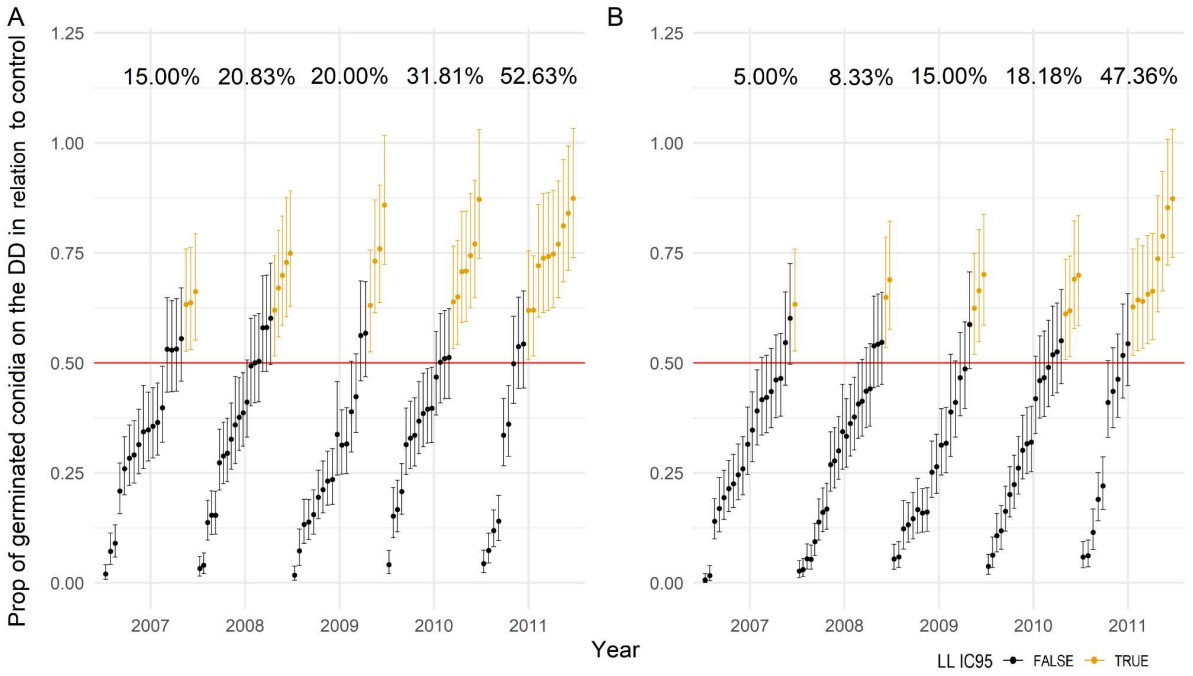

Fig 4. Proportion of germinated conidia on the discriminatory dose over the years for *Fusarium graminearum* isolates from RS collection, in the presence of azoxystrobin (A) and in the presence of pyraclostrobin (B). The red line represents 50% of germination in relation to control, and isolates in orange are considered less sensitive (LL; Lower Limit of the CI95% > 0.5). The values above show the relative frequency of less sensitive isolates over the years.

Molecular analysis of *cytb* gene

Cytb gene structure

Five introns and six exons characterize the *cytb* of *F. graminearum* (Fig 5). The first hot spot comprises the point mutation F129L, it is located on the exon 2, while the second hot spot comprises the point mutations G137R and G143A it is located on the exon 3. Between these two hot spots there is an intron of 2.243 bp long. Consequently, two primers had to be designed to cover these two regions. The exon-intron organization is an important piece of information that helps to predict the mutation on the hot spot 143. According to the structure of *cytb* of *F. graminearum* isolates there is no intron directly after the G143A codon, and therefore there is a possibility of mutations in the position 143.

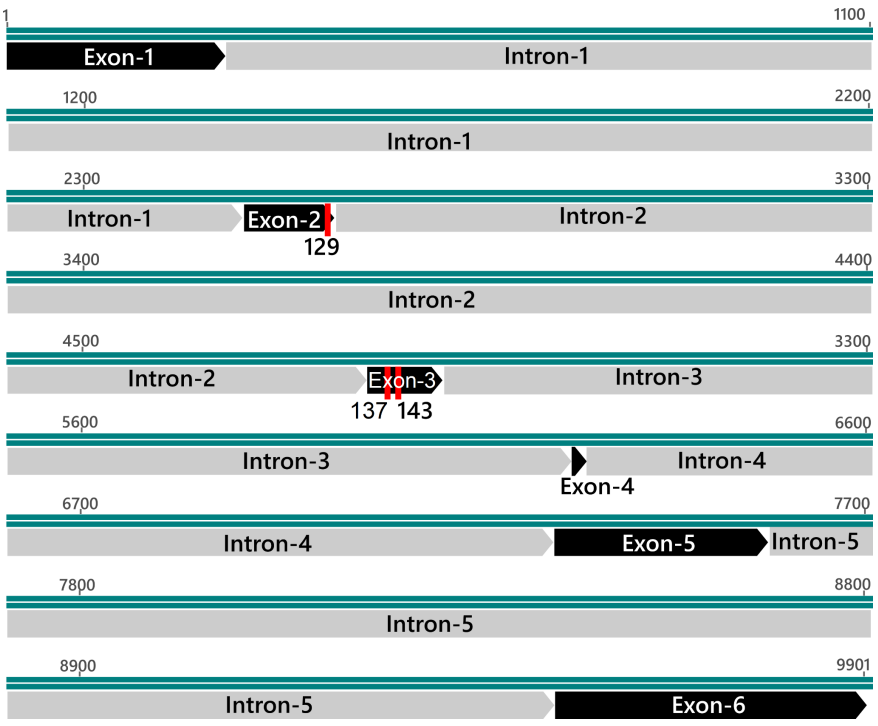

Fig 5. Exon/intron junctions in the *cytb* gene of CML 3066 *Fusarium graminearum* isolate. European Nucleotide Archive accession number of the sequence used is LT222057. The first

hot spot 129 (first red line) is located in the Exon-2, and the second and third hot spots 137 and 143 (second and third red line) are located in the Exon-3.

Sequence analysis of the *cytb* gene

A 346 *cytb* fragment was amplified for each isolate using the Fg129 primer (P1) where is located the hot spot 129, while a 313 *cytb* fragment was amplified for each isolate using the Fg143 primer (P2) where is located the hot spot 137 and 143. Based on the sequence alignments no point mutation could be found in any hot spot analysed (Fig. 6).

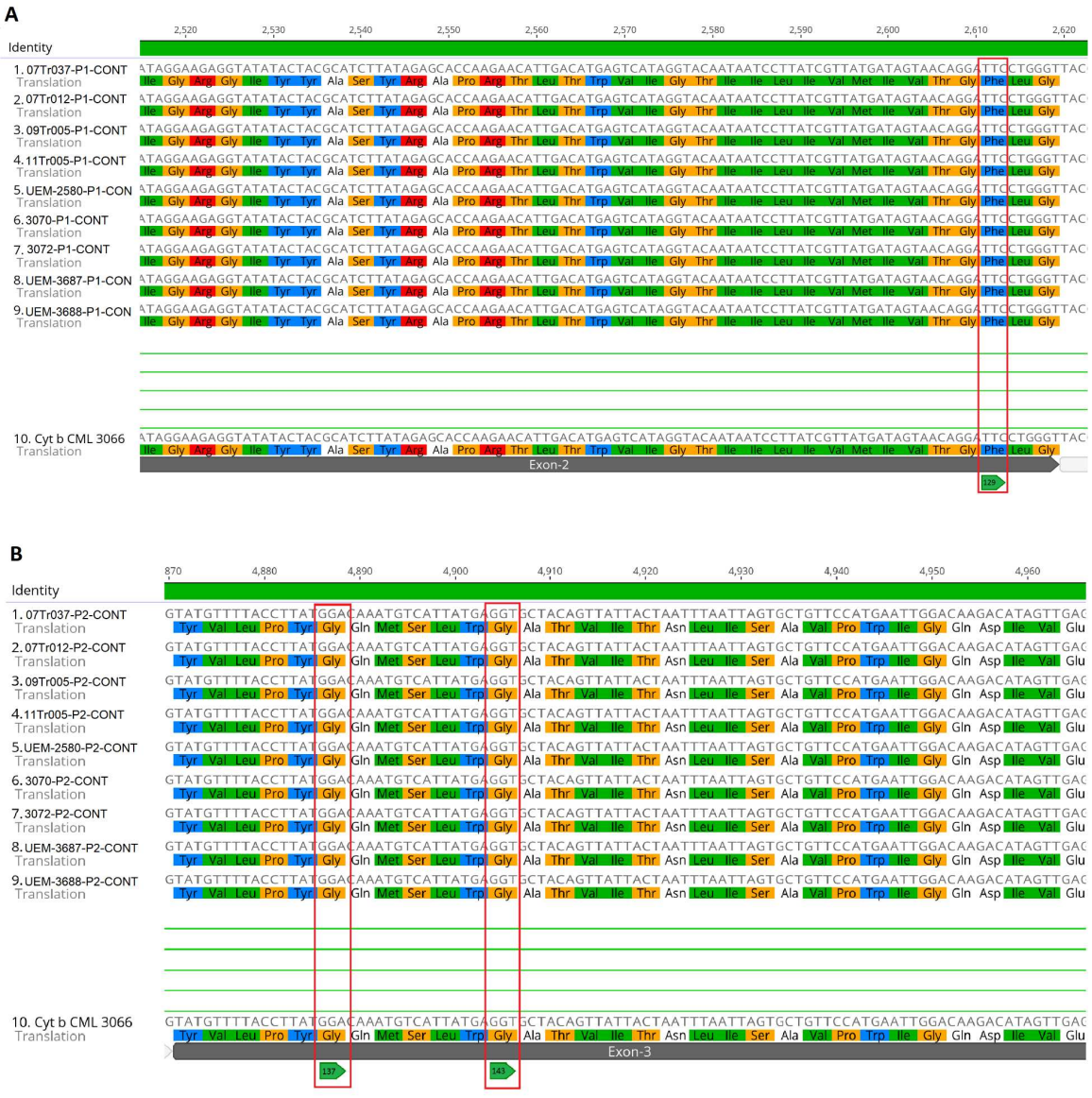

Fig 6. Alignment of amino acid sequences of part of the *cytb* gene of nine *Fusarium graminearum* isolates. The first red box (A) is showing the amino acid position 129, while the second and third red box (B) is showing the amino acid position 137 and 143 respectively. A change of phenylalanine to leucine at position 129 (F129L) or a change of glycine to an arginine at the position 137 (G137R) indicates isolates with moderate resistance. While a change of a glycine to an alanine at position 143 (G143A) indicates isolates with a stronger resistance. The sequences 1 to 9 are isolates tested in this work and the sequence number 10 were downloaded from European Nucleotide Archive, accession number LT222057.

DISCUSSION

In this study we characterized the sensitivity to azoxystrobin and pyraclostrobin for more than two hundreds of *F. graminearum* strains that cause FHB in wheat and barley in Brazil. Our results showed a significant range of sensitivity, and tests based on the discriminatory doses suggested an increase of less sensitivity isolates over the years for both fungicides within each of the two geographic regions. This is the first study to establish a sensitivity profile for these fungicides and link molecular and phenotypic analysis. None of the common point mutations could be associated with the less sensitive isolates, including the two strains that exhibited extremely high EC_{50} values.

Even though QoI fungicides are widely used for the control of plant diseases worldwide (Ishii and Hollomon 2015; Duan et al. 2020), data on the efficacy and sensitivity of the pathogen to this group for FHB control is scarce and controversial. Previous studies reported the *in vitro* fungicidal activity of pyraclostrobin and azoxystrobin against *F. graminearum.* In China, for example, a recent work with 32 isolates showed that EC_{50} values ranging from 0.2065 to 1.8422 μg mL^{-1} for azoxystrobin and 0.0379 to 0.1953 μg mL^{-1} for pyraclostrobin (Duan et al. 2020). In

Brazil, Avozani et al. (2014), studied ten isolates (five from Rio Grande do Sul and five from Paraná) and reported EC_{50} values for pyraclostrobin ranging from 0.06 to 0.15 μg mL^{-1}. Conversely, according to Audernaert et al. (2010) azoxystrobin did not influence conidial germination of a wild-type strain of *F. graminearum* in any time at field dose and in any dilution (1/10, 1/100 and 1/1000).

Overall, the range of EC_{50} values reported in this work were greater than the previous studies (Avozani et al. 2014; Duan et al. 2020). The only work with Brazilians strains was conducted with a small number of isolates (n = 10), and even so the median EC_{50} values obtained in our work, 0.28 μg mL^{-1} for PR collection (50 strains) and 0.24 μg mL^{-1} for RS collection (50 strains), were two times greater than the median values, 0.09 μg mL^{-1} in PR collection and 0.10 μg mL^{-1} for RS collection, reported by Avozani et al. (2014).

For reference, we measured the EC_{50} for an isolate that has no point mutation, a sensitivity genotype. CML3066 is an isolate from Rio Grande do Sul collected in 2009, which had its genome sequenced and it also was used as a model in molecular analysis of this work (Wood, et al. 2020). For azoxystrobin the EC_{50} was 0.3370 μg mL^{-1} while for pyraclostrobin was 0.1409 μg mL^{-1}. Such values suggest that the isolates tested in this work represent all ranges of sensitivity with lower and higher values compared to CML 3066. Interestingly, our results show an increase in the EC_{50} values suggesting a shift towards phenotypically less sensitivity populations.

We found that pyraclostrobin was, on average, seven times more fungitoxic than azoxystrobin, and consequently, potentially more effective against FHB. A similar result was found when pyraclostrobin was compared to azoxystrobin against C*ercospora* spp. isolates, and even when pyraclostrobin was compared to trifloxystrobin against *F. graminearum* isolates (Avozani et al. 2014; de Mello et al.

2021). In *Alternaria solani,* studies showed that this pathogen was 10 times more sensitive to pyraclostrobin than azoxystrobin and the authors attributed it to a greater intrinsic activity of pyraclostrobin (Pasche et al. 2004).

Quinone outside inhibitors fungicides have a high specificity mode of action which promote the selection of resistant strains after prolonged periods of pesticide use (Ishii and Hollomon 2015). These fungicides have been extensively used since the mid-1990's and azoxystrobin was one of the first molecules applied, which also may explain the decrease in its fungitoxic action. In Brazil, for FHB control, two fungicide applications are recommended, however, in fields with a high-risk and history of the disease three applications have been applied to reach control and decrease DON concentrations (Cunha et al. 2014; Santana et al. 2016). The consecutive applications may lead to high selection pressure resulting in reduction of sensitivity over time in the RS collection.

Dubos et al. (2011) suggested that *F. graminearum* populations in their study were naturally resistant towards trifloxystrobin. To support that the authors worked with a collection of 55 strains from different countries, between 1969 and 2009, and all isolates, including isolates collected before the market introduction of strobilurins, expressed a similar level of resistance. Contradictory, our results showed isolates with all ranges of sensitivity which is corroborated with the study of Avozani et al. (2014) and Duan et al. (2020). Therefore, our findings provide additional evidence that *F. graminearum* is not naturally resistant to QoI's.

Resistance mechanisms to single-site inhibitors are widely studied and they are important to understand the emergence of resistance isolates in field populations (Lucas et al. 2015). Molecular aspects related to QoI resistance sites has been

intensively studied in plant pathogens, however this is not the only mechanism and previous studies found that QoI resistance of some plant pathogens are not linked to typical mutations (Kim et al. 2003; Fernández-Ortuño and Torés 2008; Ishii et al. 2009; Lucas et al. 2015; de Mello et al. 2021).

For *F. graminearum*, to date, this is the first report of molecular analysis of the *cytb* gene. According to the exon-intron organization all point mutations could be predicted (Grasso et al. 2006). However in none of the nine isolates sequenced, no point mutations were found in any target spot even in the isolates with the greatest EC_{50} values as 11Tr005 and UEM-3688. It is important to mention that the EC_{50} tests were repeated once and the results were very similar. Prior to this work, just an *in silico* analysis was performed in the resistant isolates, and even with high EC_{20} value no modification was found in any target spots of PH-1 (Dubos et al. 2011). Therefore, the authors suggested that resistance must be caused by factors other than the already known target mutations.

According to Ishii and Hollomon (2015) and Lucas et al. (2015) alternative respiration route, reduction of intracellular fungicide concentration by enzymatic degradation or secretion by efflux transporters are some reported examples of other mechanisms which also may lead to resistant populations. In our study, alternative respiration routes could not be attributed to the less sensitivity isolates since an inhibitor of alternative oxidase was used (SHAM). Field resistance to QoI and DMIs fungicides has already been reported in isolates of *Pyrenophora tritici-repentis* due to efflux transporters. This system has the capacity to secrete antifungals compounds and prevent the accumulation in fungitoxic concentrations inside fungal cells (Reimann and Deising 2005). Therefore other mechanisms should be more investigated.

Ishii and Hollomon (2015), defined resistance as "*a field-based problem recognised by a decline in fungicide performance, to which growers may often respond by increasing dose rate and/or treatment frequency*". Although molecular assays have been used to monitor resistance in pathogen populations, it does not mean that these isolates will cause disease control problems in the field. Consequently further studies should be conducted testing these *in vitro* resistant isolates at field levels and also test whether mycotoxin production is affected by this shift.

In conclusion, we were able to find *F. graminearum* isolates possessing a wide range of sensitivity (including highly resistant isolates), however the resistance mechanism associated with QoI remains unclear. The determination of a sensitivity profile for these populations and the characterization of the *cytb* gene was an important step to establish a sensitivity profile for subsequent monitoring and implementation of management strategies. Our study suggested a shift towards less sensitive populations and further studies shall be conducted monitoring contemporary isolates from these regions, both *in vitro* and *in vivo* experiments, as well as testing other mechanisms of resistance.

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SUPPLEMENTARY

Tables

Table S1: Effective concentration of pyraclostrobin in which spore germination is inhibited by 50% (EC_{50}) for *Fusarium graminearum* isolates from Rio Grande do Sul and Paraná.

EC_{50} (µg pyraclostrobin mL^{-1})[x]			
Collection RS		**Collection PR**	
Work Code	**Estimate (±SD)[y]**	**Work Code**	**Estimate (±SD)**
07Tr037	0.0285 ± (0.006)	UEM-2580	0.3882 ± (0.071)
07Tr012	0.5128 ± (0.018)	UEM-2628	0.2296 ± (0.034)
07Tr013	1.1339 ± (0.169)	UEM-2665	0.4280 ± (0.043)
07Tr322	0.1904 ± (0.062)	UEM-2687	0.4314 ± (0.060)
07Tr323	0.3869 ± (0.055)	UEM-2703	0.1677 ± (0.013)
08Tr003	0.2349 ± (0.048)	UEM-2705	0.4141 ± (0.048)
08Tr004	0.1936 ± (0.030)	3066	0.6684 ± (0.032)
08Tr005	0.2656 ± (0.0192)	3070	0.1249 ± (0.042)
08Tr012	0.4570 ± (0.027)	3071	0.1229 ± (0.018)
08Tr013	0.4622 ± (0.023)	3072	0.4494 ± (0.071)
09Tr005	0.3653 ± (0.053)	3279	0.5088 ± (0.039)
09Tr006	0.2490 ± (0.020)	3178	0.2898 ± (0.051)

09Tr019	0.1299 ± (0.028)	UEM-3687	0.2203 ± (0.018)
09Tr020	0.1549 ± (0.011)	UEM-3688	0.6387 ± (0.077)
09Tr021	0.1699 ± (0.027)	UEM-3696	0.2384 ± (0.092)
10Tr001	0.2262 ± (0.022)	UEM-3697	0.6199 ± (0.092)
10Tr002	0.0322 ± (0.009)	3393	0.6416 ± (0.038)
10Tr003	0.3910 ± (0.028)	3396	0.4053 ± (0.111)
10Tr042	0.2528 ± (0.084)	3705	0.2346 ± (0.085)
10Tr045	0.2320 ± (0.021)	3710	0.3017 ± (0.059)
11Tr005	0.6398 ± (0.028)	3711	0.1260 ± (0.032)
11Tr006	0.1448 ± (0.011)	3712	0.1868 ± (0.024)
11Tr007	0.1278 ± (0.014)	3713	0.2188 ± (0.037)
11Tr074	0.8348 ± (0.069)	3720	0.1996 ± (0.027)
11Tr075	0.6874 ± (0.065)	3708	0.1788 ± (0.020)
Mean EC_{50}	0.3440 ± (0.260)	**Mean EC_{50}**	0.3374 ± (0.289)

[x] EC_{50} assays were conducted following the procedures described on Estimation of EC_{50} .

[y] Average of four replicates (combining two independent assays). SD= standard deviation.

Table S2: Effective concentration of azoxystrobin in which spore germination is inhibited by 50% (EC_{50}) for *Fusarium graminearum* isolates from Rio Grande do Sul and Paraná.

EC_{50} (µg azoxystrobin mL^{-1})[x]			
Collection RS		**Collection PR**	
Work Code	**Estimate (±SD)[y]**	**Work Code**	**Estimate (±SD)**
07Tr037	15.3972 ± (6.1165)	UEM-2580	8.2703 ± (3.7457)
07Tr012	8.8559 ± (6.1335)	UEM-2703	0.6131 ± (0.1532)
07Tr013	0.3346 ± (0.1953)	UEM-2705	2.5306 ± (0.5389)
08Tr003	1.0944 ± (0.5358)	3066	3.2252 ± (1.7508)
08Tr013	2.9323 ± (1.6314)	3070	0.2594 ± (0.2047)
09Tr005	9.9565 ± (2.6226)	3072	11.7019 ± (6.0114)
09Tr019	14.0673 ± (8.2339)	UEM-3687	5.0744 ± (2.1538)
10Tr002	9.9872 ± (4.2738)	UEM-3688	268.53 ± (394.81)
10Tr003	0.5540 ± (0.2222)	3396	0.5257 ± (0.0986)
11Tr005	329.71 ± (245.08)	3705	0.7588 ± (0.2756)
11Tr007	0.5677 ± (0.1958)	3710	1.8818 ± (0.3927)
11Tr074	0.3312 ± (0.4634)	3711	0.3724 ± (0.1214)
11Tr075	4.0419 ± (2.5893)	-	-
Mean EC_{50}	30.6027 ± (90.0354)	**Mean EC_{50}**	25.3122 ± (76.6779)

[x] EC_{50} assays were conducted following the procedures described on Estimation of EC_{50}.

[y] Average of four replicates (combining two independent assays). SD= standard deviation.

Figures

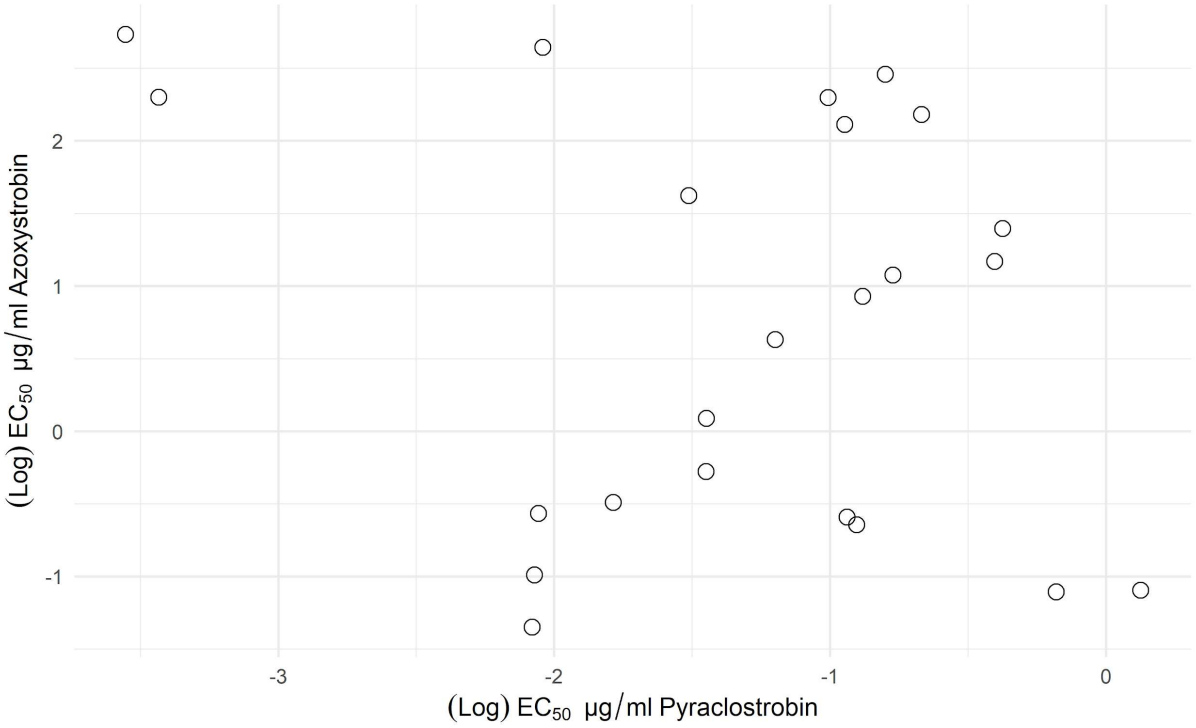

Fig. S1. Relationship between the log of values of the effective concentration of pyraclostrobin and azoxystrobin, which reduces the conidia germination of 25 isolates of *Fusarium graminearum* by 50% (EC_{50}) from the RS and PR collections.