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RESISTÊNCIA A FUNGICIDAS E VARIABILIDADE GENÉTICA DE

Magnaporthe oryzae EM LAVOURAS DE ARROZ NO BRASIL.

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Tese apresentada à Universidade de Brasília como requisito parcial para a obtenção do título de Doutor em Fitopatologia pelo Programa de Pós-graduação em Fitopatologia

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"Não importa o que a vida fez de você,

importa o que você fez do que a vida fez de você"

(Jean-Paul Sartre)

À minha família.

Dedico.

AGRADECIMENTOS

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Chapter 3

Occurrence of idiomorphs MAT1-1 and MAT-2 of *Magnaporthe oryzae* in the state of Tocantins: sexual recombination of the blast fungus is at play in Brazilian rice fields?

RESUMO GERAL

A brusone causada pelo fungo *Magnaporthe oryzae* é a doença mais destrutiva à cultura do arroz no Brasil. O patógeno está amplamente distribuído em todas regiões produtoras e causa sérios prejuízos pela redução da produtividade da cultura. O controle da brusone é realizado pelo uso intensivo de fungicidas uma vez que cultivares resistentes apresentam baixa durabilidade devido a alta plasticidade genética do patógeno. Contudo, o controle químico têm-se mostrado ineficaz e as aplicações têm sido intensificadas a fim de se obter um controle satisfatório. Na busca pela compreensão do problema e no intuito de buscar o melhor manejo da doença, o objetivou-se caracterizar as populações de *M. oryzae* quanto a sensibilidade aos principais fungicidas (Azoxystrobina, Triciclazol, Trifloxistrobina + Tebuconazol e Tebuconazol) utilizados para o controle da doença no Brasil. Em um primeiro estudo, utilizando uma coleção de 322 isolados, dois cenários, compostos por duas coleções geográficas de isolados do patógeno, foram definidos como sendo um com menor frequencia de aplicações (menor pressão de doença, coleção SUL) e em um ambiente com aplicações (coleção TO). Em nosso, uma coleção composta por 60 isolados, do estado de Goiás, coletados ao longo de 25 anos (1989-2014) foi investigada para tentar identificar a mudança da sensibilidade de *M. oryzae* a fungicidas no tempo. Finalmente, um terceiro estudo foi conduzido para determinar a distribuição dos genes idiomorfos mating types no estado do Tocantins. Dos isolados dos cenários de alta e baixa aplicação de fungicidas, 72/322 (22%) do estado do Tocantins form identificados com a mutação G143A (resistência a QoI) pelo método de digestão com enzimas de restrição. O sequenciamento do gene cyt b de uma subamostra de 110 isolados (30 SUL e 80 TO) confirmou a mutação G143A para 68 isolados (85%) do TO, enquanto os demais 12 isolados tinham genótipo sensível. Todos os 30 isolados do SUL

exibiram genótipo sensível. Foi encontrada uma significativa perda de adaptabilidade na maioria dos isolados de *M. oryzae* resistentes a QoIs, quando comparados ao isolado selvagem. Foi observada uma progressiva redução da sensibilidade a Azoxystrobina pelo aumento gradual da CE_{50} com o passar dos anos, sendo que seis isolados mutantes G143A foram identificados no ano de 2014. Foram identificados, pela primeira vez no Brasil, isolados de *M. oryzae* portadores do gene MAT1-1 associados ao arroz no estado do TO (sete isolados). A partir do conjunto de resultados observados aqui, estratégias anti-resistência devem ser adotadas imediatamente para evitar um aumento de isolados resistentes nas populações de *M. oryzae*. Especificamente, sugere-se a suspensão do uso de alguns princípios ativos, levando em consideração também a resistência cruzada.

Palavras- chave: brusone, genética de populações, 'fitness'.

THESIS ABSTRACT

Leaf and panicle blast, caused by the ascomycete *Magnaporthe oryzae* is the most destructive disease of the rice crop in Brazil. The pathogen is widely distributed among all rice growing regions and causes severe grain yield losses. Since the commercial resistant cultivars are short-lived, due to the high genetic plasticity of the pathogen, rice blast management is mainly achieved by the intensive use of fungicides. Yet, chemical control by means of synthetic fungicides is not entirely effective and the frequency of applications has increased progressively, in order to achieve satisfactory levels of control. To better understand failures in fungicide efficacy and improve disease management, the sensitivity and resistance of *M. oryzae* populations to most used fungicides in Brazil (Azoxystrobin, Tricyclazole, Trifloxystrobin + Tebuconazole and Tebuconazole) were analyzed. In a first study, two distinct scenarios, made of two geographic collections of isolates which represented one with less frequent fungicide applications (low disease pressure, collection SUL) with one with more frequent fungicide applications (high disease pressure, collection TO). Another study was carried out using 60 isolates collected over a 25-year period (1989-2014) in order to investigate temporal changes in sensitivity of *M. oryzae* to the same set of fungicides. Finally, a third study aimed to determine the distribution of idiomorphic genes for mating types in the State of Tocantins, Brazil. Out of 322 isolates in the first study, 72 (22%) isolates from Tocantins were detected carrying the G143A mutantion (resistance to QoI) by restriction enzyme analysis. Sequencing of the cyt b gene for a subsample of 110 isolates (30 SUL e 80 TO) confirmed mutation G143A for 68 (85%)TO isolates, while the remaining 12 isolates were wild type. All 30 isolates of the collection SUL had the sensitive genotype. A

measurable loss of fitness was found amongst the most resistant strains to QoIs in contrast with wild-type isolates. A progressive loss in sensitivity to Azoxystrobin was detected along the entire period, as depicted by the gradual rise in CE_{50}, and by the finding of six G143A mutants, collected in 2014. For the first time, *M .oryzae* rice isolates (7 strains) carrying the MAT1-1 gene are reported in Brazil, all originated from of TO.

Key-words: rice blast, population genetics, fitness.

GENERAL INTRODUCTION

Brazil is the largest producer of rice outside Asia (Gazzola et al., 2009), with an estimated harvest of 11 million tons, in approximately 2 million hectares (CONAB, 2018). Rice yields in both the irrigated and upland planting systems are affected by the occurence of biotic diseases, especially leaf and panicle blast caused by the fungus *Magnaporthe oryzae* (Ascomycota). Of worldwide distribution, levels of rice blast damage to crop yield are driven by host resistance and the environmental conditions (Prabhu et al., 2002).

Blast is typically a polycyclic disease, sensu Vanderplank (1963). The primary inoculum may originate from infected seeds, plant debris remaining in the field, and spores dispersed by the wind from adjacent blasted rice fields. The pathogenic phase begins by conidium adhesion onto the rice leaves, followed by conidium germination, germtube elongation, appressorium formation and penetration of leaf tissues. The first symptoms, small brown necrotic lesions with gray or whitish center, are visible 3 to 4 days after the adhesion of the conidium to the host leaf, in susceptible cultivars. As disease progresses, lesions coalesce leading to the death of the leaf tissue and, eventually, of the whole plant (Prabhu et al., 2006). Disease progress is influenced by edaphoclimatic factors. Temperatures between 20 and 30 °C, relative humidity above 90%, excess of organic matter and either excess or deficiency in nitrogen favors epidemic development in susceptible hosts.

Disease management should integrate the use of resistant cultivars, cultural practices and fungicides. Nevertheless, for most growers, chemical control has been the main or only available choice in Brazil (Filippi et al., 1999; Balardin, 2003). The number of fungicide applications varies according to the planting system and prevailing climatic conditions: in the South of Brazil, farmers usually spray one to three times, during rice cycle, while in the

Northern and Mid-Western regions, farmers perform from five to nine applications duringthe wholw rice growing cycle.

Among the fungicides, strobilurins, formulated either solely or in mixtures with other fungicides are the most used in Brazil during the last xx years (Maciel, 2011; Maciel et al., 2014). A known potential problem associated with the intensive use fungicides is the emergence of a population that is less sensitive or resistant to the chemicals. Like other living organisms, fungi are able to adapt to environmental pressures (e.g. fungicide) and the new population prevail in the evironment. Resistance is generally expressed in terms of the proportion of resistant individuals (isolates) in the total population. The factor or degree of resistance can also be calculated, which is expressed by the ratio of EC50 in the resistant and sensitive strains (Zambolim et al., 2007). Until the 1970s, due to the predominance of multi-site (action mode) fungicides in the market, cases of resistant genotypes of plant pathogens to fungicides were rare, limited to about 10 genera of fungi. However, as early as 1980, 35 genera of fungicide-resistant fungi have been reported, mainly to single-site action mode (Staub, 1991). With the advent of systemic fungicides, with single (specific) modes of action, which are now the majority of the a.i. in the market, the problem resurged. The application of a systemic (single-site) fungicide increases the chance for selection of resistant microbial cells, while eliminating the sensitive ones. Over the years, with the repeated and intensive use of these fungicides, many cases have reported of fungal species that have acquired resistance to the products. Nevertheless, in spite of the literature reports of resistance of fungi to different groups of fungicides, there is almost no information about the resistance of *Magnaporthe oryzae* to fungicide in rice populations.

The specificity of the systemic fungicides increases the risk of fungicide resistance (Rodrigues et al., 2007; Zambolim et al., 2007). In the past two decades, information was

gained on the exact amino acid changes in the target proteins that cause resistance to different fungicide groups, such as the benzimidazoles, the demethylation inhibitors (DMI) and the strobilurins (Davidse & Ishii et al., 2001; Delye et al., 1998; Gisi et al., 2000). Davidse & Ishii (1995) reported high levels of resistance to strobilurins and similar fungicides in various fungal species. Ghini & Kimati (2000) reported that strobilurins interfere in mitochondrial respiration, by blocking electron transfer made by cytochrome bc1 complex. Therefore, It is possible that cross-resistance occur for fungicides belonging to the same chemical group. One practical example of cross-resistance is found among two benzimidazoles, carbendazim and methyl thiophanate, both used for control of citrus diseases: cross-resistance against both a.i. was found in populations of *Botrytis cinerea*. For strobilurins (QoI), cross-resistance may occur for azoxystrobin, trifloxystrobin and pyraclostrobin (Oliveira et al., 2015; Kim et al., 2003). This information is important for the establishment of anti-resistance strategies (Rodrigues et al., 2007). Soon after the commercial introduction of QoI in 1996, resistant isolates of *Podospora fusca*, the causal agent of cucurbit powdery mildew, were detected in many parts of the world (Fernández-Ortuño et al., 2006). In most of the reports of resistance, a single change in cytochrome b occurred, changing the amino acid guanine at position 143 to alanine (G143A). Two resistant isolates with the same type of mutation were found in *P. fusca*. In regions where resistant population is prevalent, use of QoI was reassessed, and the management needed to be adjusted to avoid spread of resistance, including the ban of QoI alone for the control of powdery mildews as a whole (Fernández-Ortuño et al., 2006).

In another work, *Botrytis cinerea* mutants with moderate to high resistance to piraclostrobin (QoI), another inhibitor of the cytochrome bc1 electron transporter mitochondrial complex, were selected after chemical mutagenesis and selection in medium containing pyraclostrobin (Markoglou et al, 2006). The authors performed molecular

studies and observed that the *B. cinera* mutants compared to the wild parental isolate had either one of two point changes: a glycine exchange by serine at position 143 (G143S), found in the isolate with high phenotypic resistance, or the exchange of amino acid phenylalanine by leucine at position 129 (F129L). In biochemical studies of strobilurin-resistant isolates, an alternative respiratory mechanism was observed, which is now considered a biochemical mechanism of resistance. This was the first report suggesting the genetic and biochemical potential for the development of resistance by field isolates of *B. cinera* to quinone-inhibiting fungicides (Markoglou et al., 2006).

Magnaporthe grisea mutants resistant to azoxystrobin were generated and characterized in a study by Avila-Adame & Köller (2003). Two types of genotypes were characterized with mutations at site of action 143, either G143A or G143S. The mutations were stable even when the resistant mutants were grown in culture media for several cycles in the absence of azoxystrobin. The authors observed that, an alternative respiration mechanism facilitated the residual growth of *M. grisea* mycelium and contributed to the generation of spontaneous and stable mutation in cytochrome b, which can withstand high doses of azoxystrobin. However, although the alteration in respiration mechanism is a mechanism of resistance of the fungus, Avila-Adame & Köller (2003) observed that, in practice, such alternative respiration mechanisms are not related to the resistance of *M. grisea* to the fungicide *in plant*.

In summary, the substitution of three amino acid, in cytochrome b genes of plant pathogens seem to govern resistance to quinone inhibitory fungicides (Sierotzki et al., 2000a; 2002b; Gisi et al., 2002; Avila-Adame & Köller, 2003; Malandrakis et al., 2006; Markoglou et al., 2006; Sierotzki et al., 2007): (i) Change of glycine by alanine at position 143: G143A; (ii) Change of phenylalanine by leucine at position 129: F129L; and, (iii) Change of glycine by arginine at position 137: G137R. According to some authors

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(Sierotzki et al., 2000a, 2000b; Gisi et al., 2000) all G143A, G137R and F129L mutations are based on the single nucleotide polymorphism located in the cytochrome b gene and the qualitative selection process occurs in a single step. Based on the knowledge of resistance factors, the association with G143A, G137R and F129L generates different factors, in the majority of cases between 5-15% and in a few cases above 50%. Resistance factors related to G143A, in most cases, reach values greater than 100, meaning that these isolates express high resistance, or complete resistance. Isolates with G137R and F129L mutations express moderate (partial) resistance, more controlled by the use of quinone inhibitory fungicides. Most recently, Castroagudín et al. (2015) in a study carried out in Brazil, with 325 *Magnaporthe oryzae* isolates from wheat and other grasses, identified 90% of azoxystrobin resistant wheat isolates carrying the G143A mutation in four haplotypes.

Another important factor to consider is the adaptability of these resistant mutant populations, when compared to the sensitive, wild type populations, and its effect on the development of rice blast epidemics. Adaptability, or fitness, is the ability of a fungus strain to reproduce and survive, when compared to other strains under the same conditions (Zambolim et al., 2007). Plant pathogens that have developed resistance to a chemical control molecule often have their competitiveness reduced against sensitive isolates, when the chemical agent is absent. Sensitive isolates may have faster mycelial growth *in vitro*, produce larger number of conidia (both *in vitro* and *in planta*), or cause greater number of lesions, faster growing lesions, or shorter latency periods *in planta*. Resistant isolates in some pathosystems may have reduced aggressiveness and impaired competitive ability (Araújo et al., 2012; Sanoamuang & Gaunt, 1995). However, considering other pathosystems, there are instances where no adaptive cost was detected due to the acquisition of resistance to fungicides (Café-Filho & Ristaino, 2008; Corio-Costet et al., 2011). A study by Ma & Uddin (2009) evaluating the competitive ability of a *M. oryzae*

mutant demonstrated that the wild *M. oryzae* strain had a competitive advantage over the mutant strain QoI within the tested environment, thus indicating that there likely is an adaptive cost for the blast pathogen to acquire resistance to fungicide.

Although resistance to fungicides has been reported for many other pathosystems worldwide, the occurrence and distribution of *M. oryzae* rice isolates resistant to fungicides has never been examined in Brazil. Based on the above, the objective of this study was to investigate the sensitivity of rice-infecting populations *M. oryzae* populations to the main fungicides commonly used to control blast in Brazil, and to estimate the adaptive cost associated with resistance.

Literature cited

Araújo E.R., Pereira R.C., Ferreira M.A.S.V., Quezado-duval A.M., Café-filho A.C. (2012) Sensitivity of *Xanthomonas* causing tomato bacterial spot to copper and stremptomycin and in vivo infra-specific competitive ability in *Xanthomonas perforans* resistant and sensitive to copper. Journal of Plant Pathology 94: 79-87.

Avila-adame C., Olaya G., Koller W. (2003) Characterization of *Colletotrichum graminicola* isolates resistant to strobilurin related QoI fungicides. Plant Disease 87:1426-1443.

Avila-adame C., Koller W. (2003) Characterization of spontaneous mutants of *Magnaporthe grisea* expressing stable resistance to the Qo-inhibiting fungicide azoxystrobin. Current Genetics 42: 332–338.

Balardin, R. S (2003). Doenças do arroz. Santa Maria. 59 p.

Café-Filho A.C., Ristaino J.B (2008) Fitness of isolates of *Phytophthora capsici* resistant to mefenoxam from squash and pepper fields in North Carolina. Plant Disease 92: 1439-1443.

Castroagudín, C. L., Ceresini, P. C., Oliveira, S. C., Reges, J. T. A., Maciel, J. L. N., bonato, A. L. V., Dorigan, A. F., and Mcdonald, B. A (2015). Resistance to QoI fungicides is widespread in Brazilian populations of the wheat blast pathogen *Magnaporthe oryzae*. Phytopathology 105:284-294.

CONAB (2018). 2° Levantamento de grãos-Safra 2017/18. Disponível em: http://www.conab.gov.br/OlalaCMS/uploads/arquivos/15_11_16_15_18_26_safras_nov_2 018.pdf. Acesso em: 10 de janeiro de 2018.

Corio-Costet M.F., Dufour M.C., Cigna J., Abadie P., Chen W.J (2011). Diversity and fitness of *Plasmopara vitícola* isolates resistant to QoI fungicides. European Journal of Plant Pathology 129: 315-329.

Delye, C., Laigret, F., Corio-Costet, M.F. (1998) PCR cloning and detection of point mutations in the eburicol 14 alphademethylase (CYP51) gene from *Erysiphe graminis* f.sp. hordei, a "recalcitrant" fungus. Current Genetics 34: 399-403.

Fernandez-ortûno D., Pérez-García A., López-Ruiz F., Romero D., Vicente A.,Torés J.A. (2006) Occurrence and distribution of resistance to qoi fungicides in populations of *Podosphaera fusca* in south central spain. European journal of plant pathology 115: 222, 2006.

Filippi, M. C., Prabhu, A. S., Araujo, L. G. & Faria, J. C. (2002) Genetic diversity andvirulence pattern in field populations of *Pyricularia grisea* from rice cultivar Metica-1. Pesquisa Agropecuária Brasileira 37.

Filippi, M.C.; Prabhu A.S. & Levy, M. (1999) Differential Compatibility of *Pyricularia grisea* isolates with some Brazilian irrigated rice cultivars. Fitopatologia Brasileira 24: 447-450.

Gazzola, R; Wander, A. E; Souza, G. D. S. E. (2009) Comércio internacional de arroz. Anais, 6° Congresso Brasileiro de Arroz Irrigado. Porto Alegre.

Gisi U., Chin K.M., Knapora G., Farber R.K., Mohr U., Parisi S., Sierotski H., Steinfild B. (2000) Recent developments in elucidating modes of resistance to phenylamide, DMI and strobilurin fungicides. Crop Protection 19: 863-872.

Ghini, R.; Kimati, H. (2000). Resistência de fungos a fungicidas. 1ª edição. Jaguariúna: Embrapa Meio Ambiente, 78p.

Ishii, H.; Fraaije, B.A.; Sugyama, T.; Noguchi, K.; Nishimura, K.; Takeda, T.; Amano, T.; Hollomon, D.W. (2001) Occurrence and molecular characterization of strobilurin resistance in cucumber powdery mildew and downy mildew. Phytopathology, 91: 1166-1171.

Kim, Y.S.; Dixon, P.; Vincelli, P.; Farman, M.L. (2003) Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial cytochrome b gene. Phytopathology 93: 891-900.

Maciel, J. L. N. (2011) *Magnaporthe oryzae*, the blast pathogen: Current status and options for its control. Plant Science Review 1: 233-240.

Maciel, J. L. N., Ceresini, P. C., Castroagudin, V. L., Kema, G. H. J., and McDonald, B. A. (2014) Population structure and pathotype diversity of the wheat blast pathogen *Magnaporthe oryzae* 25 years after its emergence in Brazil. Phytopathology 104:95-107

Malandrakis A.A., Markoglou A.N., Nikou D.C., Vontas J.G., Ziogas B.N. (2006) Biological and molecular characterization of laboratory mutants of *Cercospora beticola* resistant to QoI inhibitors. European Journal of Plant Pathology 116: 155–166.

Markoglou A.N., Malandrakis A.A., Vitoratos A.G., Ziogas B.N. (2006) Characterization of laboratory mutants of *Botrytis cinerea* resistant to QoI Fungicides. European Journal of Plant Pathology 115: 149–162.

Oliveira, S.C.; Castroagudin, V.L.; Maciel, J.L.N.; Pereira, D.A.S.; Ceresini, P.C. (2015) Resistência cruzada aos fungicidas IQo azoxistrobina e piraclostrobina no patógeno da brusone do trigo *Pyricularia oryzae* no Brasil. Summa Phytopathologica, v.41, n.4, p.298-304.

Prabhu A. S., Filippi M. C., Araújo L. G. (2002) Pathotype diversity of *Pyricularia grisea* from improved upland rice cultivars in experimental plots. Fitopatologia Brasileira 27: 468-473. 19

Prabhu, A.S. & Filippi, M.C. (2006) Brusone em arroz: controle genético, progresso e perspectivas. Santo Antônio de Goiás GO. Embrapa Arroz e Feijão.

Rodrigues M.B.C., Andreote F.D., Spósito M.B., Aguillar-Vildoso C.I., Araújo W.L., Pizzirani-Kleiner, A.A. (2007) Resistência a benzimidazóis por *Guignardia citricarpa*. Pesquisa Agropecuária Brasileira, v.42, n.3, p.323-327, 2007.

Sanoamuang N., Gaunt R.E., (1995) Persistence and fitness of carbendazim- and dicarboximide-resistant isolates of *Monilinia fructicola* (Wint.) Honey in flowers, shoots and fruits of stone fruit. Plant Pathology 44: 448-457.

Sierotzki H., Frey R., Wullschleger J., Palermo S., Karlim S., Godwin J., Gisi U. (2007) Cytochrome b gene sequence and structure of *Pyrenophora teres* and *P. tritici-repentis* and implications for QoI resistance. Pest Management Science 63: 225-233.

Sierotzki H., Parisi S., Steinfeld U., Tenzer I., Poirey S., Gisi U. (2000a) Mode of resistance to respiration inhibitors at the cytochrome bc1 complex of *Mycosphaerella fijiensis*. Pest Management Science 56: 833-841.

Sierotzki H., Wullschleger J., Gisi U. (2000b) Point-mutation in cytochrome b gene conferring resistance to strobilurin fungicides in *Erysiphe graminis f. sp. tritici* field isolates. Pesticide Biochemistry and Physiology 68: 107-112.

Sierotzki H., Pavic L., Hugelshofer U., Stanger C., Cleere S., Windass J., Gisi U. (2005) Population dynamics of *Mycosphaerella graminicola* in response to selection by different fungicides. In: International Reinhardsbrunn Symposium, Agroconcept, 14, 2005, Bonn. Proceedings, Bonn: Verlag.

Zambolim L., Venâncio S.V., Oliveira S.H.F. (2007) Manejo da Resistência de Fungos a Fungicidas. Visconde do Rio Branco: Suprema Gráfica e Editora, 168p.

Chapter 1

Fungicide sensitivy, detection the G143A point mutation associated a QoI´S and competitive fitness of *Magnaporthe oryzae* populations from Brazil

Abstract

Magnaporthe oryzae, the cause of the leaf and panicle blast, is the most damaging pathogen of commercial rice crops worldwide, which can cause severe yield losses. Disease management is based mainly on the application of fungicides; however, the sensitivity of the pathogen populations to the main fungicides is largely unknown. More or less sensitive phenotypes were identified when exposed to most used fungicides for disease control in Brazil such as azoxystrobin, tricyclazole, trifloxystrobin + tebucunazole and tebuconazole. Genotypes that were resistance to azoxystrobin, based on the detection of G143A mutation, were found in 90% of the TO state isolates. The EC_{50} value for these isolates were $>10\mu g.\ L^{-1}$. Most of the resistant isolates was less fit than the sensitive ones when inoculated *in planta*. The proportion of resistant and sensitive isolates in mixed cultures (80R:20S and 20R:80S**)** was kept constant in the presence of the fungicide after four generations. Theoretically, mutants with multiple resistances to active ingredients in different fungicide groups may also arise. However, the fitness costs associated with resistance to more than one active ingredient may impair competitive abilities of such mutants even further.

Key words: Resistance, estrobilurin, rice blast, *Oryza sativa.*

Introduction

Rice blast, caused by the ascomycetous fungus *Magnaporthe oryzae* (Herbet) Barr. [anamorph *Pyricularia oryzae* (Cook) Sacc.], is chiefly managed using single (systemic) and multi-site (protectant) fungicides. The quinone outside inhibitor (QoI) fungicides, known as strobilurins, have proven effective in controlling rice blast (Soares et al., 2014). QoI fungicides target the quinol-oxidizing (Qo) site of the mitochondrial enzyme cytochrome b. Binding of these chemicals at the Qo site blocks electron transport, thereby inhibiting respiration (Bartlett et al., 2000). Development of resistance to one QoI in *M. oryzae* was first detected for azoxystrobin in the year 2000 (Vincelli, 2000), and the finding was attributed to the intensive and repetitive use of the fungicide at the same location. In the development of resistance in *M. oryzae*, two point mutations in cytochrome b of mitochondria, G143A and F129L, are known to occur at the target site (Kim et al., 2003). The G143A mutation reportedly carries a high level of resistance, which renders the fungicide completely ineffective. A much lower level of resistance is carried by the F129L mutation, which, in many cases, does not affect field performance of QoI fungicides (Fungicide Resistance Action Committee, www.frac.info).

To date, experimental work on resistance in *M. oryzae* to QoIs has focused on the molecular and biochemical aspects of the fungicide resistance and very little is known about the dynamics of resistance at the population level (Ma & Uddin, 2009). Field resistance to QoI fungicides has been observed in Europe and Asia for other pathogens causing diseases in wheat (*Blumeria graminis* f. sp. *tritici*) (Sierotzki et al., 2000b), banana (*Mycosphaerella fijiensis*) (Sierotzki et al., 2000a), grape (*Plasmopara viticola*) (Gisi et al., 2000), barley (*Blumeria graminis* f. sp. *hordei*) (Heaney et al., 2000), and apple (*Venturia inaequalis*) (Steinfeld et al., 2002). In Brazil, Castroagudin et al. (2015) reported a widespread distribution of QoI resistance in *M. oryzae* populations sampled from wheat

fields and other poaceous hosts across central and southern Brazil and studied the evolution of the cytochrome b (cyt b) gene. Sequence analysis of the cyt b gene in these populations distinguished nine haplotypes, with four haplotypes carrying the G143A mutation associated with QoI resistance and two haplotypes from isolates sampled from wheat and other poaceous hosts. They found that the frequency of the G143A mutation in the wheat-infecting population increased from 36% in 2005 to 90% in 2012.

Two mechanisms of resistance to QoI fungicides have been reported. In the first mechanism, the mutation of the cytochrome b gene results in peptide sequence changes that prevent fungicide binding. A second resistance mechanism, which functions *in vitro*, has been identified in a number of phytopathogenic fungi, including *Magnaporthe grisea* (Yukioka et al., 1997, Yukioka et al., 1998). This involves the activity of an alternative oxidase (AO) enzyme, which oxidizes ubiquinone while reducing oxygen to water. This bypasses the QoI-induced block in the electron transport chain allowing mycelial growth (Siedow et al., 2000). In *Magnaporthe grisea*, the AO gene is expressed *in vitro* in response to oxidative stress; however, AO transcription is repressed *in planta*, which thus does not affect QoI efficay for controlling phytopathogenic fungi, including rice blast pathogen (Yukioka et al., 1998).

The dynamics of the competition between fungicide-resistant and -sensitive strains determines whether or not resistance becomes established in the pathogen population (Leung et al., 1988, Thornbury & Farman, 2000). In recent years, empirical studies demonstrated variable outcomes of such competition. In a study on the dynamics of carbendazim resistance in eyespot fungi [(*Tapesia yallundae* Wallwork & Spooner and *T. acuformis* (Boerema, R. Pieters & Hamers) Crous] on winter wheat, a stable coexistence of about 50% of carbendazim-resistant and sensitive strains on carbendazim treated plots between 1987 and 2000 was observed (Farman, M. L. 2002). However, in a study on

Botrytis cinerea Pers. affecting geranium, the dicarboximide-resistant isolate completely replaced the sensitive strain after two applications of the a.i. Vinclozolin whereas the resistant isolate prevalence remained at the initial level of 0.02% of the population in the absence of the same a.i. (Tamura et al., 1999). Competiveness studies between QoI-resistant and sensitive strains were conducted for other pathosystems. For instance, in a study on powdery mildew of wheat, caused by *B. graminis* f. sp. *tritici*, the initial 10% QoI-resistant population of the pathogen reportedly increased to 20 to 35% of the total population and remained steady on untreated host tissue over three generations (Vincelli & Dixon, 2001). In addition, *Plasmopara viticola*, the oomycete causing grape downy mildew, displayed a progressive decrease in sensitivity to QoI, to almost full resistance after four generations (Genet et al., 2006). However, the resistant population gradually reverted to full sensitivity after consecutive transfers in untreated plants, as the resistant phenotypes seem to lose competitiveness against sensitive ones. In contrast, the QoI-resistant population of failed to increase in the pathogen population in a cycling experiment (Heaney et al., 2000).

The outcomes of competition between resistant and wild-type strains are largely determined by their respective fitness in the absence of fungicide (Ishii et al., 2001; Kachroo et al., 1994; Thornbury & Farman, 2000; Uddin, 1999). Studies on QoI resistance mutations in cytochrome b of *Saccharomyces cerevisiae* revealed that most mutations were concurrent with functionally impaired mitochondria which have reduced electron flow through the cytochrome bc1 complex (Siedow et al., 2000); therefore, it has been suspected that QoI-resistant strains may suffer from fitness penalties. A laboratory G143A mutant of *Venturia inaequalis* reverted to a sensitive stage in the absence of fungicide, which is also an indication of such fitness impairments (Zheng et al., 2000). Further evidence of the fitness penalty was observed for a G143A mutant of *Ustilago maydis*, which growth in

liquid culture and infection of corn plants were adversely affected by this mutation (Ziogas et al., 2002). However, the G143A mutation does not hamper fitness of all pathogens. For example, no fitness penalty was found in a spontaneous G143A mutant of *B. graminis* (Chin et al., 2000; Heaney et al., 2000). Laboratory-selected G143A and G143S mutants of *M. grisea*, pathogenic on goosegrass (*Eleusine indica*), weeping lovegrass (*Eragrostis curvula*), and barley (*Hordeum vulgare*), neither showed differences in the saprophytic stage parameters or the pathogenic development compared with a wildtype strain (Avila-Adame & Köller, 2003). On the other hand, Ma & Uddin (2009) assessed the relative fitness and competitive ability of a field-collected azoxystrobin-resistant G143A mutant by comparing it with a wild-type strain on detached perennial ryegrass (*Lolium perenne*) blades. They demonstrated that the wild-type strain of *M. oryzae* had a competitive advantage over the mutant within the environment tested. However, a systematic study, involving a large collection of field isolates to determine whether there are fitness penalties for the G143A resistant field mutants in the rice blast pathosystem, is not yet available.

Considering the importance of resistance management in the early stages of resistance development, it is important to understand the dynamics of a small portion of resistant mutants of *M. oryzae* in a pathogen population in the absence and presence of commonly used fungicides for rice blast control. This has not been studied worldwide or in Brazil, which has a sizable area planted with rice every year (*c*. 2.3 million ha), and is the world´s largest grower outside Asia (http://www.fao.org/faostat/en/#data). Therefore, this study was undertaken to (1) measure the *in vitro* sensitivity of Brazilian populations of *M. oryzae* to different fungicides; (2) describe the occurrence and distribution of QoI resistant populations in Brazil; (3) evaluate the fitness of G143A mutants of *M. oryzae* in rice plants, relative to sensitive isolates, and; (4) determine the competitive relationship

between resistant and wild type strains in whole plants of rice in the absence of the QoI fungicide.

Materials and Methods

M. oryzae **isolates.** A collection of 322 *M.oryzae* isolates were obtained from rice plants with blast symptoms in fields from the southern Brazilian states of Rio Grande do Sul, RS (n=147) and Santa Catarina, SC (n=77) and the northern state of Tocantins, TO (n=98) during the planting seasons of years 2013 to 2016. The states of RS and SC are at coordinates 27-31° lat. S, in the subtropical zone (Köppen class Cwa), while TO is at 10-11° lat. S, with Köppen climate class Aw (tropical wet savanna). These states account for approximately 90% of total rice production in Brazil (CONAB, 2018). Monosporic isolates were obtained according to methodology described by D'Ávila et al. (2016). Monosporic cultures were further grown on filter paper and stored as described elsewhere (Valent et al. 1986).

In vitro **tests for QoI sensitivity.** QoI sensitivity was assessed for all the 322 isolates. Fungal colonies for stock cultures were grown on potato dextrose agar (PDA) containing both chloramphenicol and streptomycin at 50 μg ml^{-1} (PDA+) and incubated at 25°C and 12-h photoperiod. Fungicide active ingredients (a.i.) azoxystrobin (Priori®), tricyclazole (Bim®), trifloxystrobin + tebuconazole (Nativo®) and tebuconazole (Folicur®) were diluted 100-fold in deionized water to produce stock solutions. Test media were prepared by cooling PDA+ to 55°C and then adding 0.5 mM salicylhydroxamic acid (SHAM) (required to suppress the alternative oxidase pathway) and the fungicide stock solution to 100 μg ml^{-1}. For colony growth assays, 5-mm mycelial disks were removed from the edge of 3-day-old colonies and transferred onto PDA+ plates with or without Azoxystrobin.

Four isolates were tested on each Petri plate and each plate was replicated three times. Growth was measured after incubation at 25°C for 6 days, subtracting the original mycelial disk diameter (5 mm) from each measurement. Colony growth was represented by the relative growth rate (RGR) in the presence of fungicide relative to the colony size of the isolate on the control plates using the formula: $100 \times$ (mean colony diameter on PDA+ amended with fungicide)/(mean colony diameter in nonamended PDA+). The experiment was arranged in a complete randomized design, with 4 replicates. A representative subgroup of 75 isolates was selected to repeat the experiment. Isolate Guy 11 [sensitive to QoI (QoI-S), Leung et al., 1988] was included in all assays to serve as the sensitive control. Analysis of variance (ANOVA) was performed with SPSS (Statistic 21). For separation of means, the Scott-Knott test was applied, with Assistat software, v. 7.7 beta (Federal University of Campina Grande, Paraíba, Brazil).

Conidial germination and appressorium formation in the presence of azoxystrobin was preliminarily assessed with a 10 µl water suspension of conidia (10^5 conidia/mL) amended with each fungicide at a concentration of 10.0 µg mL^{-1}. Suspensions were plated in hydrophobic surfaces in Petri dishes. Moist filter paper was used for keeping humidity high. Plates were sealed with parafilm® and incubated for 24 h at 28°C. One-hundred conidia were counted for each of three replicates, and the number of germinated conidia and of appressoria formed were recorded. Conidia were counted as germinated when the germination tube was larger than 50% the size of the conidium. The relative germination rate (RGE) was calculated for each replicate as $100 \times$ percent germination in the presence of fungicide divided by mean percent germination of that isolate in the respective fungicide-free control. In all tests, conidial germination in fungicide-free controls was 85 to 95%. Following these preliminary assays, serial dilutions were determined for a sub-set

of arbitrarily selected baseline isolates and putative resistant isolates, for the determination of the half maximum effective concentration (EC_{50}), as follows:

Isolates were first tested in a series of 10-fold dilutions of fungicides on PDA+ with SHAM; concentrations used for each fungicide ranged from 0.001 to 10 µg/ml for baseline isolates and from 0.1 to 100 µg/ml for the presumed resistant isolates. From these preliminary evaluations using the 10-fold dilution series, ranges of concentrations appropriate for testing each group of isolates were identified. Germination then was determined for each isolate over a threefold dilution series within those ranges. For isolates that were either fully germinated or ungerminated over a given threefold dilution series, tests were repeated with threefold dilutions over a different range of concentrations. RGEs for selected baseline isolates and for all suspect resistant isolates were determined at least twice in completely separate tests. Data were analyzed by analysis of variance following a completely randomized design. To determine the EC_{50} value for each fungicide, RGE was regressed against $\log_{10}$ [fungicide]. EC_{50} was determined by solving the regression equation for $\log_{10}$[fungicide] at RGE = 50.

Extraction of genomic DNA and PCR amplification of cytochrome b gene. Genomic DNA of all isolates was extracted as described by Delaportta (1983), with slight modifications. DNA samples were quantified and adjusted to 80 µg/mL, using Nanodrop. Primers PgCytb-F1 (5'-AGTCCTAGT GTAATGGAAGC-3') and PgCytb-R1 (5'-3-ATCTTCAACGTG TTTAGCACC') were employed to amplify one internal fragment of cytochrome b gene by PCR (Kim et al., 2003). PCR reactions performed using a commercial kit, following the manufacturer´s instructions (Qiagen Inc., Valencia, CA). Amplication was as follows: initial denaturation at 95 ° C for 5 min; followed by 35 cycles

of 95 $^\circ$ C for 45 s, 55 $^\circ$ C for 45 s, and 72 $^\circ$ C for 45 s; final extension was at 72 $^\circ$ C for 8 min.

Screening isolates for mutants in the cytochrome b gene. The G143A mutation creates an *Fnu*4HI restriction site (…GGTGC… to …GCTGC…), while F129L eliminates a *Sty*I restriction site (…CCTAGG… to …CATAGG…) [Kim et al., 2003]. Screening for each mutation was carried out using *Fnu*4HI and *Sty*I restriction enzymes, for cleavable amplified polymorphic sequence (CAPS) analysis of PCR products. PCR products (5 μl) were digested with each enzyme and the restriction fragments were resolved by electrophoresis in 4% agarose gels using a Kilobase Plus® size marker (Invitrogen, Carlsbad, CA).

Fitness assays for the G143A mutant isolates. Fitness studies *in planta* were conducted in the tropical lowland rice variety IRGA 424. Twenty-day-old rice plants were inoculated with 3 x 10^5 conidia/mL from each of eight G143A mutant isolates. One sensitive isolate (Guy 11) was also included as a representative wild-type (wt) isolate, and the following three disease variables were determined: (i) *Latent period*. Latent period is the time elapsed between spore deposition and the beginning of sporulation. Here, latent period is operationally defined as the time, in days, required to observing 50% sporulating lesions (LP50). Leaves were observed twice a day starting 3 DAI (days after inoculation). This was estimated by linear interpolation of the number of sporulating lesions counted between successive assessments. (ii) *Infection efficiency*. Infection efficiency was estimated by counting the total number of lesions in one leaf blade, 9 DAI. (iii) *Sporulation intensity*. Sporulation intensity was estimated by collecting a portion of 10 cm^2 green leaf segment of all lesions produced at each treatment, which was transferred to a centrifuge tube

containing 1 ml of 0.025% Tween20 solution. Tubes were shaken with a Vortex® shaker (model: VM-2000, Digisystem Laboratory Instruments Inc., Taiwan) at full speed for 10 s, to dislodge the conidia. The resulting conidial suspension was counted with a haemocytometer at 640 X magnification. Assessments were made at *c*. 5 DAI. The mean number of spores over two counts was computed for each lesion. (iv) *Leaf blast severity*. Leaf blast severity was measured 9 DAI in plants or not treated with QoI fungicide azoxystrobin, and kept in controlled glasshouse conditions under high humidity. Severity, or the proportion of leaf area infectd, was assessed visually by a same rater with the aid of a standard area diagram (Nótteghem, 1981).

Competitiveness and stability of G143A mutant isolates. To determine the competitive ability and the stability of G143A mutants during successive life cycles of the fungus *in planta*, conidial suspensions (3×10^5 conidia ml^{-1}) of resistant (R) and sensitive (S) isolates were concomitantly inoculated on azoxystrobin-treated or nontreated leaves of IRGA 424 in three different ratios: 80R:20S, 20R:80S, and 50R:50S. The number of conidia produced on symptomatic leaves was assessed after every disease cycle, and the experiment was completed after four cycles. The experiment was conducted in glasshouse conditions, on 21 day old plants at inoculation, and each treatment was replicated four times, in complete randomized design. The same procedures for conidial transfer and molecular analysis described above were employed here after each disease cycle. Disease cycles were estimated to last 10 days.

Sequencing and characterization of an internal fragment of the Cyt b gene. PCR products of a subsample of 110 isolates (68 to TO and 42 to SUL) were purified and sequenced at Macrogen Inc. (Seoul, South Korea), using reaction kit ABI Prism BigDye

Terminator Cycle Sequencing Ready in the 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were visualized and aligned with the Geneious R 6.7.1 (Biomatters, New Zealand) software. Partial sequences of the cytb gene were manually screened for the presence of mutations kown to confer resistance to QoIs: one change from G to C in codon 428 (G143A) or from C to A in codon 387 (F129L) (Kim et al., 2003).

Results

***In vitro* tests for fungicide sensitivity**. *M. oryzae* isolates differed quantitatively in response to the presence of fungicides in culture media. The response of isolates all fungicides showed that, while most of isolates grew in the presence of the fungicide, sensitivity levels varied as measured by RGR (P< 0.0001) (Figure 1). Given the geographic distance and the climatic and ecologic differences among the southern and northern rice-growing regions, the data were examined separately for each region. While all isolates from northern states were highly resistant to this a.i., with relative growths rates ranging from 76.21 to 99.4%, the respective range among southern isolates was 44.28 to 98.89%. Relative growth rates (RGR) in azoxystrobin-ammended media were more pronounced: while RGR among the southern isolates varied from 8.5 to 46.43%, northern isolates had RGRs from 45.39 to 80.15%. As a whole, resistance to tebuconazole appeared to be more widespread among northern Brazilian populations of *M. oryzae* (RGRs 38.95-85.71%), contrasting with the more sensitive southern isolates (9.78-37.21%). Regarding the mixture trifloxystrobin + tebuconazole, TO isolates showed RGRs of 29.5 to 74.5%, that were not strikingly different from the southern isolates (RGRs of 2.89 - 24.28%). Effective concentrations (EC_{50}) for azoxystrobin, determined using a subset of 20 arbitrarily selected baseline and resistant isolates using data from RGRs, were: $EC_{50} = 0.0101$ ($\pm$ 0.027) to

0.0979 (± 0.063) µg/mL for the isolates of southern isolates, and always greater than 10.0 µg/mL for the northern isolates of Tocantins state (Table 1).

Results from the conidial germination and appressorium formation studies of southern and northern isolates are shown separately in Figure 2. In all fungicide pairwise comparisons, the collection of southern isolates was more sensitive than the collection of northern isolates (Figure 2). Azoxystrobin was the least effective fungicide in inhibiting conidial germination and appressorium formation, and many isolates were insensitive to this a.i. Isolates were comparatively more sensitive to tryciclazol, with RGEs of 18.5-26.3% and most sensitive to tebuconazole and trifloxystrobin + tebuconazole. Appressorium formation and conidial germination trends were similar, with smaller values for appressorium formation, since not all germinated conidia formed appressoria.

Sporulation varied widely *in vitro* among mutant and isolate Guy 11, ranging from 98-103 conidia/mL for the sensitive isolate Guy 11 and resistant isolate 5.1 TO, respectively to 18 to 74 x 10^2 conidia/mL for the remaining seven resistant strains (Figure 3).

Detection of mutants in the cytochrome b gene. Seventy-two isolates, all from the northern region (corresponding to 73.5% of the northern collection) were positive for the G143A mutation in the cyt b gene. No representative isolate of the southern population (n=224) presented either the G143A or the F129L mutation. Mutation F129L was not found in any of isolated studied (n=322).

Fitness comparison. Latent period (Lp) varied from 120 to 168 h across the isolates. However, resistant isolates usually had larger Lps than the wide-type, sensitive isolate Guy 11 (120 h, Figure 4). Number of lesions per 10 cm of leaf also varied broadly, with highest

numbers observed in Guy 11 and 5.1 TO (18.2 and 17.2 respectively), to 2.0-11.7 lesions for the rest of the resistant strains (Fig. 5). In the same manner, the number of spores per lesion (sporulation intensity, Figure 5) varied from 78.4 and 77.5 in the Guy 11 and 5.1 TO, respectively, to much lower values in the resistant strains (6.75 to 53.4 spores per lesion). While severity of symptoms caused by the mutant strains was barely affected in the presence of azoxystrobin, the wt isolate was completely unable to cause any blast symptoms in plant at this condition (Figure A,B). On the other hand, in the absence of the a.i. most resistant mutants caused significantly less disease *in planta* in comparison with the sensitive isolate Guy 11 (35% blasted leaves, Figure 6A). However, some mutant isolates, namely 5.1 TO, 2.1 TO and 2.6 TO, resistant to azoxystrobin, were able to cause blast severity values reaching 25 to 35 %. The other five resistant mutant isolates had their aggressiveness significantly reduced (less than 9% blasted leaves).

Competitive ability and stability of G143A mutant isolates *in planta*. In the absence of azoxystrobin, in two out of three instances (80R:20S and 20R:80S), the resistant mutants' relative proportion in the mixed population *in planta* progressively decreased for four disease cycles (Figure 7A). Conversely, in the presence of azoxystrobin, the relative proportion of resistant and sensitive individuals in the mixed population remained fairly constant for four generations (Figure 7B).

The G143A allele was widely distributed in genotypes. A *cyt b* fragment was amplified from 110 isolates of *M. oryzae*. *Cyt b* sequences distinguished nine haplotypes, two (H1 to H2) encoding the G143A mutation associated with QoI resistance. No haplotypes contained the F129A mutation. The H1 haplotype was found in 38.18 % of the tested isolates,

including 30 isolates from SUL and 12 isolates from TO. The QoI resistance H2 haplotype was found in 61.81% of the isolates, including 68 isolates from TO (Table 2).

Discussion

This is the first in-depth study describing the occurrence and generalized distribution of resistance to fungicides in the Brazilian field populations of *M. oryzae* on rice. Our data suggest various levels of sensitivity to a DMI fungicides (inhibiting sterol biosynthesis, tebuconazole), fungicides inhibiting melanin synthesis (tricyclazole), as well as among QoI fungicides inhibiting respiration (azoxystrobin, trifloxystrobin). A striking difference was detected among populations from two widely separated geographical and ecological regions: while more than 90% of the northern isolates carried the G143A mutation for QoI resistance, no mutant isolate was found in the southern region. Nevertheless, isolates from the southern regions had quantitatively different phenotypic patterns against fungicides. The differences observed can be explained by the scope of the working collection, which comprised many years in different environments and rice cultivars. Mainly, the differences may be ascribed to the intensive use of particular fungicides, which exerted a selection pressure in each specific scenario. Particularly, the contrast among the southern and northern populations is probably due to the fact that while standard growing practices in the south include two to three fungicide applications per season, in the state of TO, an average of 10 applications are done. Some growers have reported up to 13 applications per planting season (personal communication???). The central-north region of Brazil has been described as a hot-spot for *M. oryzae* diversity (Dias Neto et al., 2010), and its climatic conditions are extremely favorable to the blast in the rainy season. Therefore, excessive fungicide applications in that

region seems to be effectively selecting resistant strains to most used, mainly the QoI fungicides, which are of higher risk of resistance.

Few fungicide efficacy studies for rice blast are available in Brazil. One study in the southern state of SC, reported adequate blast control with two applications of commercial mixtures of triazoles and strobirulins (Scheuermann & Eberhardt, 2011). However, Maciel et al (2011) found that, even if the disease was somewhat reduced with triazol and strobirulin mixtures, chemical control was not sufficient for the treated plots achieve valuable commercial yields. Similar results were found by Pagani et al (2014) for wheat blast, an emerging disease, caused by the same pathogen. A recent study by Castroagudín et al (2015) in wheat and other *Poaceae* found a high frequency and generalized distribution of *M. oryzae* haplotypes resistant to QoIs, carrying the G143A mutation among these populations. This finding may be taken as evidence that resistance to azoxystrobin emerged following various independent events (i.e., by intraspecific parallel evolution), and that QoI-R haplotypes in southern Brazil had distinct origins.

Acquisition of resistance to antimicrobial compounds may reduce biological fitness of plant pathogens, but there are also examples of low fitness costs or even cost-free examples in several pathosystems (Kang and Park, 2010; Luciani et al., 2009; Lilley and Bailey, 1997). In some instances, such as in the genus *Phytophthora*, isolates resistant to metalaxyl/mefenoxam were at least as competitive as sensitive ones, in the absence of the a.i. (Hu et al., 2008; Café-Filho & Ristaino, 2008; Kadish et al., 1990). In addition, Corio-Costet et al (2010) did not find any costs of resistance among *Plasmopara viticola*, resistant to QoI fungicides. However, in other pathosystems, when selection pressure is removed, resistant isolates are disadvantaged over the sensitive ones (Sanoamuang and Gaunt, 1995; Webber, 1988; Araújo et al. 2012). Mikaberidze and contributors (2013) proposed that the adaptive costs of fungicide resistance are the key parameters determining

the result of competition among resistant and sensitive strains of a pathogen, in order to evaluate the utility of fungicide mixtures. If adaptive cost is low or null, then the combination of a high-risk a.i. in a fungicide mixture may select the target population against sensitivity to this a.i., and eventually, the a.i. turns inefficient. Contrarily, if the cost of adaptation to insensivity is significant, withdraw of the a.i. from the mixture, followed by re-introduction of the same a.i. after a period of time, may be a feasible alternative, in the hope that resistant strains will return to minimal levels. Of course, the rate at which a population with a preponderance of resistant individuals will revert to a majority of sensitive ones will depend on the type and magnitude of the cost of resistance. The cost of resistance to benomyl for example was observed to be notoriously low, and populations of many pathogens mutant for tubulin persisted almost indefinitely, rending the alternation/rotation of this fungicide not practicable.

Widespread presence of resistant isolates in field populations is cause of concern. Rice blast causes up to 30 % (Prabhu et al., 2003) yield reduction of this crop, which is a staple crop in Brazil. Clearly, the present practices of chemical management employed are not sustainable in the short run. However, the many evidences of fitness penalties among resistant mutants detected in this study offer a hint to improved fungicide management practices, in the form of active ingredient rotations. Alternations of fungicides with different modes of actions may prevent, and rotations may possibly reverse, the build-up of resistance in the rice blast pathosystem. Indeed, the longer Lps, lower number of conidia, lower infection efficiencies (as measured by number of lesions), lower reproduction, and overall reduced aggressiveness, indicate that mutants' fitness is indeed reduced. These evidences were corroborated in the competition studies, when the frequency of resistant mutants *in planta* was, in two out of three cases, progressively reduced. Certainly, one of the mutants (namely isolate 5.1 TO) seemed to avoid hefty fitness penalties, and more

studies of such isolates are warranted, in order to explain possible mechanisms of that avoid or compensate fitness penalties.

The most direct action to be taken to manage the resistance levels found in this study would be the removal of selection pressure for QoI resistance among *M. oryzae* populations (Fernández-Ortuño et al., 2008; Gi et al., 2002). Our data on fitness costs, and the consequent impairment of the competitive ability of G143A mutant isolates *in planta* strongly suggest that most resistant isolates may not be stable in the absence of selection pressure. Then, it is likely that populations of *M. oryzae* could revert to being chiefly susceptible to QoIs or, possibly, the other fungicide groups (Hobbelen; Paveley; Van Der Bosch, 2014). Contrary evidence as to the adaptive costs for *M. oryzae* resistance against QoIs was provided by Adame & koller (2009), who did not report any fitness costs with mutations G143A or G143S. No reversion to sensitivity was observed after three consecutive cycles in the absence of Azoxystrobin. Forcelini et al. (2017) investigated the relative fitness and competitive ability of QoI-resistant and -sensitive *C. acutatum* isolates. A fitness comparison study did not indicate any difference between resistant and sensitive isolates in aggressiveness, spore production, and mycelial growth at different temperatures. Additionally, in the absence of selection pressure, resistant and sensitive isolates were equally competitive. Cultivation of QoI-resistant and QoI-sensitive isolates for four culture cycles *in vitro* in the absence of azoxystrobin showed that QoI resistance was stable. In contrast, we observed a loss of adaptability in most isolates, which may be due to the great genetic and pathogenic diversity of the *M. oryzae* species, evidenced by many authors (Maciel et al., 2004; Castroagudín et al., 2015; al., 2016) associated to characteristics of the environment where it was sampled, type of crop, source of origin, areas with high disease pressure in epidemic years more propitious to recombination processes due to the high reproduction rates of the pathogen. In such a scenario, where the *status quo ante* cannot be

achieved by withdrawing fungicide use, only strategies employed before the rise of resistant populations could be employed. Among those are the use of mixtures of low-risk and high-risk active ingredients, to prevent or delay the rise of insensitive mutants (Hobbelen; Paveley; Van Der Bosch, 2014). Theoretically, mutants with multiple resistances to active ingredients in different fungicide groups may also arise. However, the versatility costs associated to resistance to more than one active ingredient may impair competitive abilities of such mutants even further.

Literature Cited

Avila-Adame, C., and Koller, W. 2002. Disruption of the alternative oxidase gene in *Magnaporthe grisea* and its impact on host infection. Mol. PlantMicrobe Interact. 15:493-500.

Avila-Adame, C., and Koller, W. 2003. Characterization of spontaneous mutants of *Magnaporthe grisea* expressing stable resistance to the Qo-inhibiting fungicide azoxystrobin. Curr. Genet. 42:332-338.

Avila-Adame, C., and Koller, W. 2003. Impact of alternative respiration and target-site mutations on responses of germinating conidia of *Magnaporthe grisea* to Qo-inhibiting fungicides. Pest Manage. Sci. 59:303-309.

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., and Parr-Dobrzanski, B. 2000. The strobilurin fungicides. Pest Manage. Sci. 58:649-662.

Castroagudín, V. L., Ceresini, P. C., de Oliveira, S. C., Reges, J. T. A., Maciel, J. L. N., Bonato, A. L. V., Dorigan, A. F., and McDonald, B. A. 2015. Resistance to QoI fungicides is widespread in Brazilian populations of the wheat blast pathogen *Magnaporthe oryzae*. Phytopathology 105:284-294.

Café-Filho, A. C. & Ristaino, J. B. 2008. Fitness of isolates of Phytophthora capsici resistant to Mefenoxam from Squash na pepper fields in North Carolina. Plant Disease, v.92, n.10, p.1439-1443.

Chin, K. M., Chavaillaz, D., Kaesbohrer, M., Staub, T., and Felsenstein, F. G. 2001. Characterizing resistance risk of *Erysiphe graminis* f.sp. *tritici* to strobilurins. Crop Prot. 20:87-96.

CONAB, 2018. Acompanhamento de safra brasileira de grãos. Available at: www.conab.gov.br. Accessed on , february 2018.

D'Ávila, L.S., Lehner, M.S.,Filippi, M.C.C., Scheuermann, K. K., Del Ponte, E. M. 2016. Genetic structure and mating type analysis of the Pyricularia oryzae population causing widespread epidemics in southern Brazil. Tropical plant pathology DOI: 10.1007/s40858-016-0101-9.

Dellaporta, S.L.; Wood, J.; Hicks, J.B. 1983. A plant DNA minipreparation version II. Plant Mol Biol Report 1:19–21.

Dias Neto, J.J., Santos, G.R., Silva, L.M.A., Cunha, A.C.R., Rangel, P.H.N., & Ferreira, M.E., 2010. Hot spots for diversity of *Magnaporthe oryzae* physiological races in irrigated rice fields in Brazil. Pesquisa Agropecuaria Brasileira 45: 252–260.

FAO. 2014. Food and agriculture organization of the United Nations. Available at: http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor. Accessed on december, 2017.

Farman, M. L. 2002. *Pyricularia grisea* isolates causing gray leaf spot on perennial ryegrass (*Lolium perenne*) in the United States: Relationship to *P. grisea* isolates from other host plants. Phytopathology 92: 245-254.

Fernández-Ortuño, D.; Torés, J.A.; Vicente, A.; Pérez-García, A. 2008. Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. International Microbiology, v.11, n.1, p.1- 10.

Forcelini, B.B.; Rebello, C. S.;Wang, N.Y.; Peres, N.A. 2018. Fitness, competitive ability, and mutation stability of isolates of Colletotrichum acutatum from strawberry resitant to QoI fungicides.Phytopathology 13: doi: 10,1094/PHYTO-09-17-0296-R

Genet, J. L, Jaworka, G. de Paris, F. 2006. Effect of dose rate and mixtures of fungicides on selection for QoI resistance in populations of Plasmopara vitícola. Pest Management Science 62(2):188-94.

Gisi, U., Sierotzki, H., and McCaffery, A. 2002. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. Pest Manage. Sci. 58:859-867.

Ghatak A., Willocquet L., Savary S., Kumar J. 2013. Variability in Aggressiveness of Rice Blast (Magnaporthe oryzae) Isolates Originating from Rice Leave and Necks: A Case of Pathogen Specialization? PLoS ONE 8(6): e66180. doi:10.1371/journal.pone.0066180

Heaney, S., Hall, A., Davies, S., and Olaya, G. 2000. Resistance to fungicides in the QolSTAR cross-resistance group: Current perspectives. Proc. 2000 BCPC Conf. Pests Dis. 2:755-762.

Hobbelen, P. H., Paveley, N. D., and van der Bosch, F. 2014. The emergence of resistance to fungicides. PLoS One 9:e91910. doi: 91910.91371/journal.pone.0091910

Ishii, H., Fraaije, B. A., Noguchi, K., Nishimura, K., Takeda, T., Amano, T., and Holloman, D. W. 2001. Occurrence and molecular characterization of strobilurin resistance in cucumber powdery mildew and downy mildew. Phytopathology 91:1166-1171.

Kachroo, P. K., Chattoo, B. B., and Leong, S. A. 1994. Pot2, an inverted repeat transposon from *Magnaporthe grisea.* Mol. Gen. Genet. 245:339-348.

Kadish, D.; Grinberger, M.; Cohen, Y. 1990. Fitness of metalaxyl-sensitive and metalaxyl-resistant isolates of Phytophthora infestans on suseptible and resistant potato cultivars. Phytopathology 80: 200-205.

Kang, Y.S. & Park, W. Trade-off between antibiotic resistance and biological fitness in Acinetobacter sp. Strain DR1. 2010. Enviromental Microbiology.vol.12,n.4,p.1304-1318.

Kim, Y. S., Dixon, E. W., Vincelli, P., and Farman, M. L. 2003. Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by

mutations in the mitochondrial cytochrome b gene. Phytopathology 93:891-900.

Kunova, A.; Pizzatti, C.; Cortesi, P. 2012. Impact of tricyclazole and azoxystrobin on growth, sporulation and secondary infection of the rice blast fungus, Magnaporthe oryzae. Pest Management Science, Malden, v. 69, n. 2, p. 278-284. Disponível em: <http://onlinelibrary.wiley.com/doi/10.1002/ps.3386/full>. Acesso em: 15 abr. 2014.

Leung, H.; Borromeo, E.S.; Bernardo, M.A.; Nottéguem, J.L. 1988. Genetic analysis of virulence in the rice blast fungus Magnaporthe grisea. Phytopathology, Saint Paul, v.78, p.1227-1233.

Lilley, A.K., Bailey, M.J. 1997. Impacto f plasmid Pqbr103 acquisition and carriage on the Phytosphere Fitness of Pseudomonas fluorenscens SBW25: Burden and Benefit Appl. Environ.Microbiol. vol.63, n.4, p.1584-1587.

Luciani, F.; Sisson, S.A., Jiang, H., Francis, A. R., Tanaka, M.M. 2009. The epidemiological Fitness cost of drug resistance in Mycobacterium tuberculosis. PNAS, vol.106, n.34, p.14711-14715.

Ma, B.; Uddin, W.; Olaya, G. 2009. Baseline and non-baseline sensitivity of Magnaporthe oryzae isolates from perennial ryegrass to azoxystrobin in the northeastern United States. Canadian Journal of Plant Pathology, Canadá, v. 31, n. 1, p. 57-64.

Maciel, J. L. N. 2011. Magnaporthe oryzae, the blast pathogen: current status and options for its control. CABI Reviews - Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources, Oxfordshire, v. 6, n. 50, p. 1-8.

Maciel, J. L. N., Ceresini, P. C., Castroagudin, V. L., Zala, M., Kema, G. H. J., and McDonald, B. A. 2014. Population structure and pathotype diversity of the wheat blast pathogen *Magnaporthe oryzae* 25 years after its emergence in Brazil. Phytopathology 104:95-107.

Mikaberidze, A.; Mcdonald, B. A.; Bonhoeffer, S. 2014. Can high-risk fungicides be used in mixtures without selecting for fungicide resistance? Phytopathology, Palo Alto, v. 104, n. 4, p. 324-331.

Notteghem, J.L. 1981. Cooperative experiment on horizontal resistance to rice blast. In: International Rice Research Institute (Los Baños, Filipinas). Blast and upland rice: report and recommendations from the meeting for international collaboration in upland rice improvement, p. 43-51.

Ou, S. H. 1985. Blast. In: OU, S. H. Rice diseases. 2. ed. Wallingford: CAB International, p. 109-201.

Pagani, A. P. S.; Dianese, A. C.; Café-Filho, A. C. 2014. Management of wheat blast with synthetic fungicides, partial resistance and silicate and phosphite minerals. Phytoparasitica, vol.42, n. 2, p.609-617.

Prabhu A. S., Araújo L. G., Faustina C., Berni R. F. 2003. Estimativa de danos causados pela brusone na produtividade de arroz de terras altas. Pesq. agropec. bras., Brasília, v. 38, n. 9, p. 1045-1051.

Sierotzki, H., Parisi, S., Steinfeld, U., Tenzer, I., Poirey, S., and Gisi, U. 2000a. Mode of resistance to respiration inhibitors at the cytochrome bc1 enzyme complex of *Mycosphaerella fijiensis* field isolates. Pest Manage. Sci. 56:833-841.

Sierotzki, H., Wullschleger, J., and Gisi, U. 2000b. Point mutation in cytochrome b gene conferring resistance to strobilurin fungicides in *Erysiphe graminis* f. sp. *tritici* field isolates. Pestic. Biochem. Physiol. 68:107-112.

Scheuermann, K. K.; Eberhardt, D.S. 2011. Avaliação de fungicidas para o controle da brusone de panícula na cultura do arroz irrigado. Revista de Ciências Agroveterinárias, Lages, v.10, n.1, p. 23-28.

Siedow, J. N., and Umbach, A. L. 2000. The mitochondrial cyanideresistant oxidase: Structural conservation amid regulatory diversity. Biochem. Biophys. Acta 1459:432-439.

Steinfeld, U., Sierotzki, H., Parisi, S., and Gisi, U. 2002 Comparison of resistance mechanisms to strobilurin fungicides in *Venturia inaequalis*. Pages 167-176 in: Modern Fungicides and Antifungal Compounds III. H.

W. Dehne, U. Gisi, P. E. Kuck, H. Russell, and H. Lyr, eds. AgroConcept, Bonn, Germany.

Soares et al. 2014. Brazilian Journal of Applied Technology for Agricultural Science, Guarapuava-PR, v.7, n.2, p.109-119.

Tamura, H., Mizutani, A., Yukioka, H., Miki, N., Ohba, K., and Masuko, M. 1999. Effect of the methoxyiminoacetamide fungicide, SSF 129, on respiratory activity of Botrytis cinerea. Pestic. Sci. 55:681-686.

Thornbury, D. W., and Farman, M. L. 2000. Re-use of nylon membranes for radioactive hybridizations. Biotechniques 29:1250-1254.

Yukioka, H., Inagaki, S., Tanaka, R., Katoh, K., Miki, N., Mizutani, A., and Masuko, M. 1998. Transcriptional activation of the alternative oxidase gene of the fungus Magnaporthe grisea by a respiratory-inhibiting fungicide and hydrogen peroxide. Biochem. Biophys. Acta 1442:161169. 28.

Yukioka, H., Tanaka, R., Inagaki, S., Katoh, K., Miki, N., Mizutani, A., and Masuko, M. 1997. Mutants of the phytopathogenic fungus Magnaporthe grisea deficient in alternative, cyanide-resistant, respiration. Fungal Genet. Biol. 22:221-228. 29.

Vincelli, P., and Dixon, E. 2001. Resistance to QoI (strobilurin-like) fungicides in isolates of *Pyricularia grisea* from perennial ryegrass. Plant Disease, 86:235-240.

Uddin, W. 1999. Gray leaf spot "blasts" U.S. golf course turf. Golf Course Manage. 67:52-56.

Valent B, Crawford MS, Weaver CG, Chumley FG (1986) Genetic studies of fertility and pathogenicity in Magnaporthe grisea (Pyricularia oryzae). Iowa State J Res 60:569–594

Vincelli, P. 2000. Fungicidal control of gray leaf spot. Golf Course Manage. 68:68-74.

Vincelli, P., and Dixon, E. 2001. Resistance to QoI (strobilurin-like) fungicides in isolates of *Pyricularia grisea* from perennial ryegrass. Plant Dis. 86:235-240

Ziogas, B. N., Baldwin, B. C., and Young, J. E. 1997. Alternative respiration: A biochemical mechanism of resistance to azoxystrobin (ICIA5504) in Septoria tritici. Pestic. Sci. 50:28-34.

Zheng, D., Olaya, G., and Koller, W. 2000. Characterization of laboratory mutants of Venturia inaequalis resistant to the strobilurin-related fungicide kresoxim-methyl. Curr. Genet. 38:148-155. 30.

Figure Legend

Figure 1. *In vitro* relative mycelial growth rates (RGR) of *Magnaporthe oryzae* in PDA ammended with different fungicide active ingredients at 100 µg.mL^{-1}. Upper graph: Average RGR of 224 isolates from the southern states of RS and SC. Lower graph: Average RGR of 98 isolates from the state of TO.

Figure 2. *In vitro* percentage of conidial germination (RGE, dark gray bar) and percentage of conidia that formed appresorium (light gray bar) by 224 southern and 98 northern isolates of *Magnaporthe oryzae* in PDA amended with 10 µg.mL^{-1} each of four fungicide active principles.

Figure 3. Number of conidia (conidia x10^2 10/mL) produced *in vitro* by *Magnaporthe oryzae* isolates carrying the G143A mutation for QoI resistance and wt isolate Guy11.

Figure 4. Latency period (hours) of *Magnaporthe oryzae* isolates carrying the G143A mutation for QoI resistance and wt isolate Guy11 on rice cultivar IRGA 424, untreated with fungicide.

Figure 5. Infection efficiency (number of lesions per leaf) and sporulation intensity (number of spores in 10 cm leaf) of *Magnaporthe oryzae* isolates carrying the G143A mutation for QoI resistance and wt isolate Guy11 on rice cultivar IRGA 424, untreated with fungicide.

Figure 6. Rice leaf blast severity in percentage of necrotic leaf area, caused by *Magnaporthe oryzae* isolates carrying the G143A mutation for QoI resistance and one wt isolate (Guy11), on rice cultivar IRGA 424 untreated with fungicide (A) or on plants previously sprayed with Azoxystrobin (250 g.L^{-1}) (B).

Figure 7. Frequency of *Magnaporthe oryzae* isolates carrying the G143A mutation for resistance to QoI fungicides inoculated initially at different Resistant (R) to Sensitive (S) proportions (80R:320S, 50R:50S and 20R:80R), during four disease cycles. Untreated with fungicide (A) or on plants previously sprayed with Azoxystrobin (250 g.L^{-1}) (B).

Table 1: Effective concentration at which spore germination is inhibited by 50% (EC_{50}) for a representative sample of quinone-outside inhibitor (QoI) Azoxystrobin by Brazilian southern and northern of the rice blast pathogen *Magnaporthe oryzae*[x]

EC_{50} (µg azoxystrobin mL^{-1})[y]			
Southern isolates		Northern isolates	
Isolate	Average (±SD)[z]	Isolate	Average (±SD)
1 SUL	0.0419 ± (0.021)	1.1 TO	>10
2 SUL	0.0751 ± (0.034)	1.2 TO	>10
3 SUL	0.0523 ± (0.042)	1.3 TO	>10
4 SUL	0.0912 ± (0.031)	1.4 TO	>10
5 SUL	0.0543 ± (0.022)	1.5 TO	>10
6 SUL	0.0789 ± (0.067)	2.2 TO	>10
7 SUL	0.0614 ± (0.054)	3.1 TO	>10
8 SUL	0.0782 ± (0.058)	3.2 TO	>10
9 SUL	0.0595 ± (0.044)	3.3 TO	>10
10 SUL	0.0601 ± (0.012)	3.4 TO	>10
11 SUL	0.0618 ± (0.053)	7.10 TO	>10
12 SUL	0.0101 ± (0.033)	7.11 TO	>10
13 SUL	0.0829 ± (0.075)	7.12 TO	>10
14 SUL	0.0876 ± (0.048)	7.13 TO	>10
15 SUL	0.0947 ± (0.090)	7.14 TO	>10
16 SUL	0.0975 ± (0.019)	7.15 TO	>10
17 SUL	0.0632 ± (0. 018)	8.1 TO	>10
18 SUL	0.0653 ± (0.031)	8.2 TO	>10
19 SUL	0.0767 ± (0.027)	8.3 TO	>10
20 SUL	0.0979 ± (0.088)	8.4 TO	>10
Mean EC_{50}	0.0606 ± (0.0171)	…	>10

[x] EC_{50} assays were conducted following the same general procedures of the in vitro QoI-sensitive assay.

[y] All QoI-sensitive isolates tested had *cyt b* haplotype H1, while the QoI resistant isolates had *cyt b* haplotype H2.

[z] Average of three replicates (combining two independent assays with four replicates each). SD = standard deviation.

Table 2: Polymorphic sites in the sequence of the cytochrome b (*cyt b*) gene from 110

isolates of *Magnaporthe oryzae* from Brazil[x]

Genotype	NCBI Number	Haplótypes	N	Sequence
QoI-S	AY245424	Wild Type		T T T T A T C C C G G T A G G T C T T G T T T C T T T T A C T C T C T A T T T A C
QoI-R	AY245426	Mutation F129L		. . . . A . . . . A . . . .
	AY245427	Mutation G143A		. . . . C . . . . A . . . .
H1 (QoI-S)	-		42 (30 SUL/12 TO)	. . . . A . . . .
H2 (QoI-R)	-		68 (TO**)**	. . . . C . . . . A . . . .

[x] The first three sequences were downloaded from the NCBI database as references (Kim et al. 2003; 21):

Rice wild-type QoI sensitive (QoI-S) allele from *M. oryzae* isolates recovered from rice (AY245424); F129L

QoI resistance (QoI-R) reference sequence AY245426 showing the C-to-A transversion at codon 129; G143L

QoI-R reference sequence AY245427 showing the G-to-C transition codon 143. Shaded

rectangular boxes indicate the position F129L and G143A mutations of the *cyt b* sequence. Frequencies of

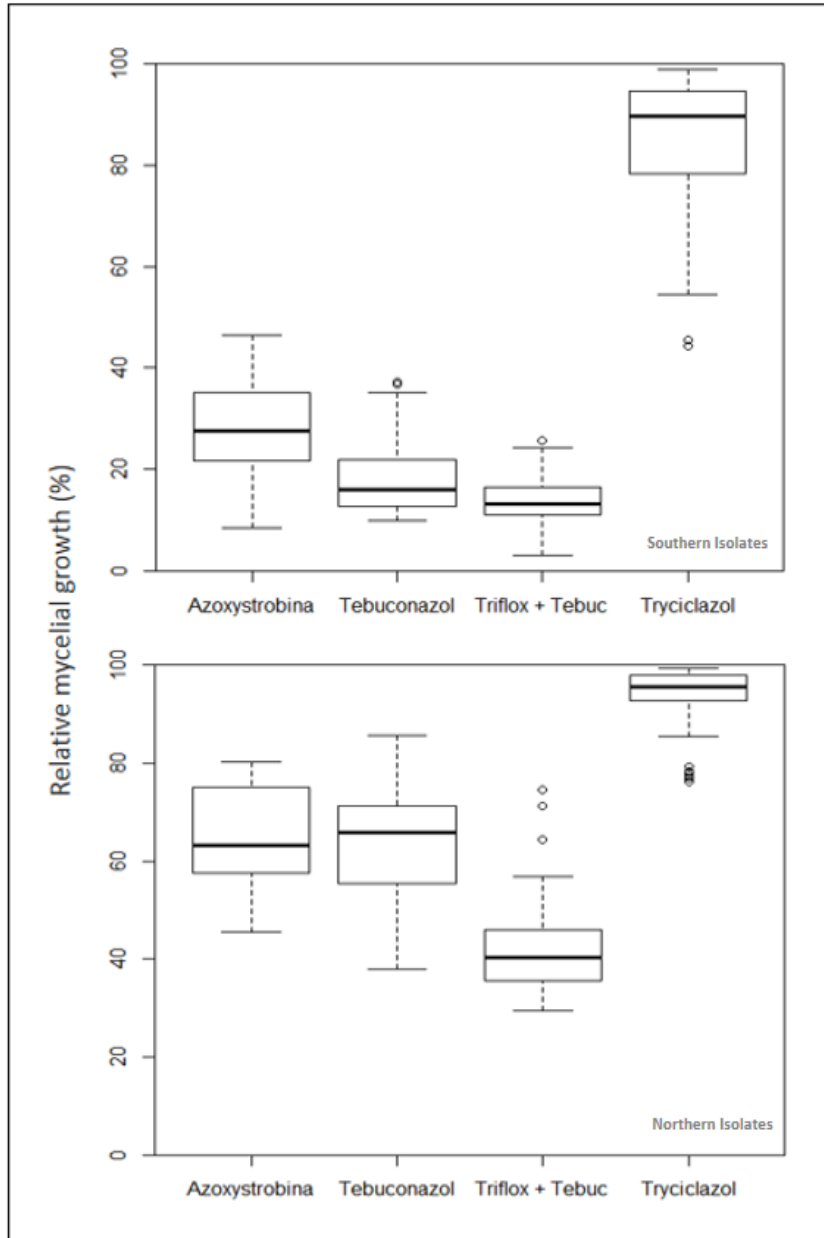each haplotype are also indicated. N:number of isolates.
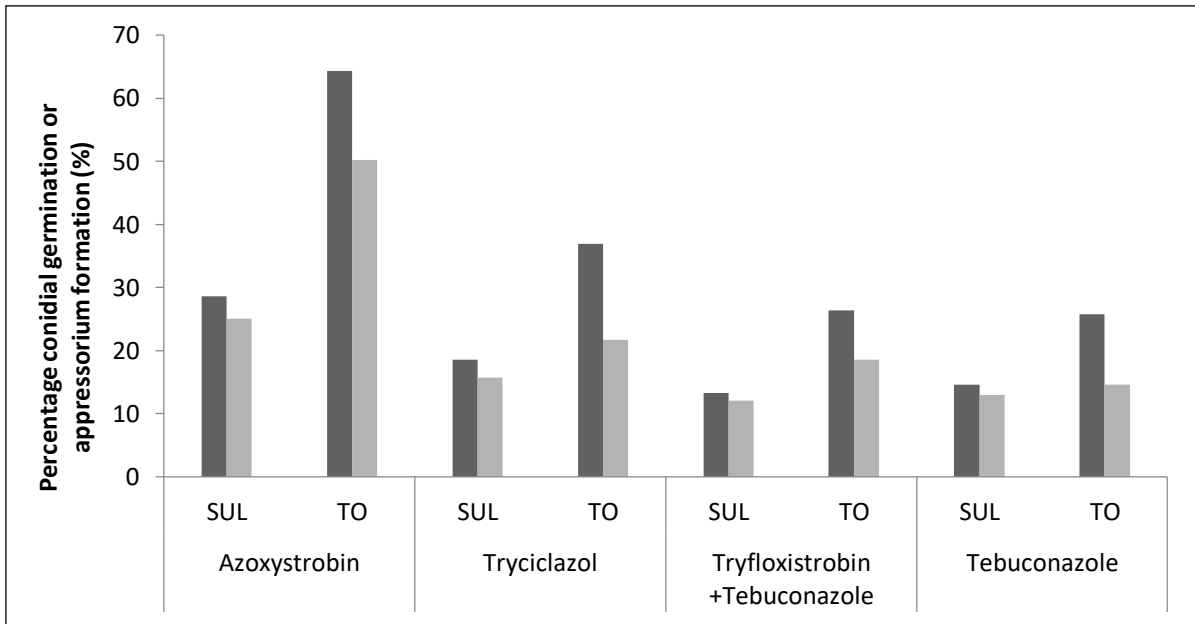
Figure 1. D'Ávila et al
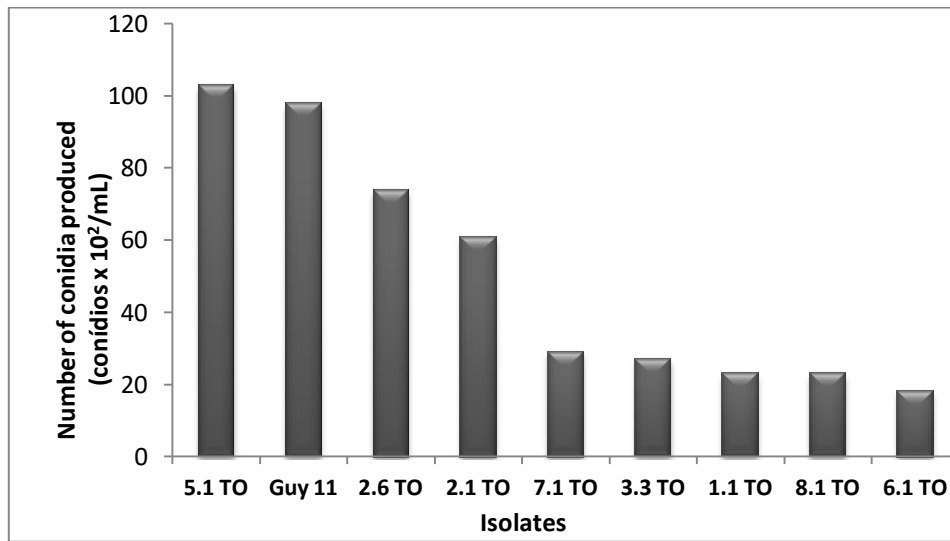
Figure 2. D'Ávila et al
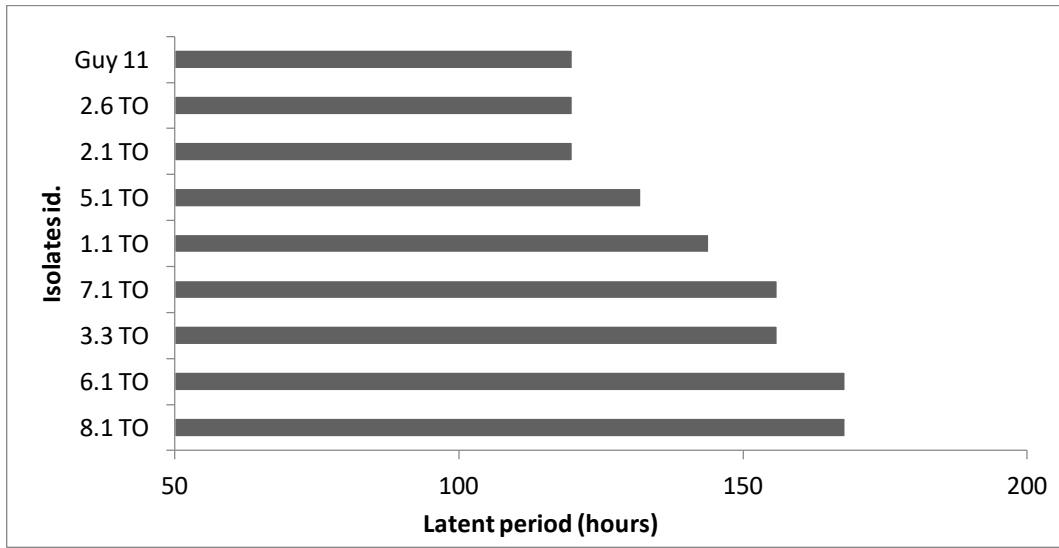
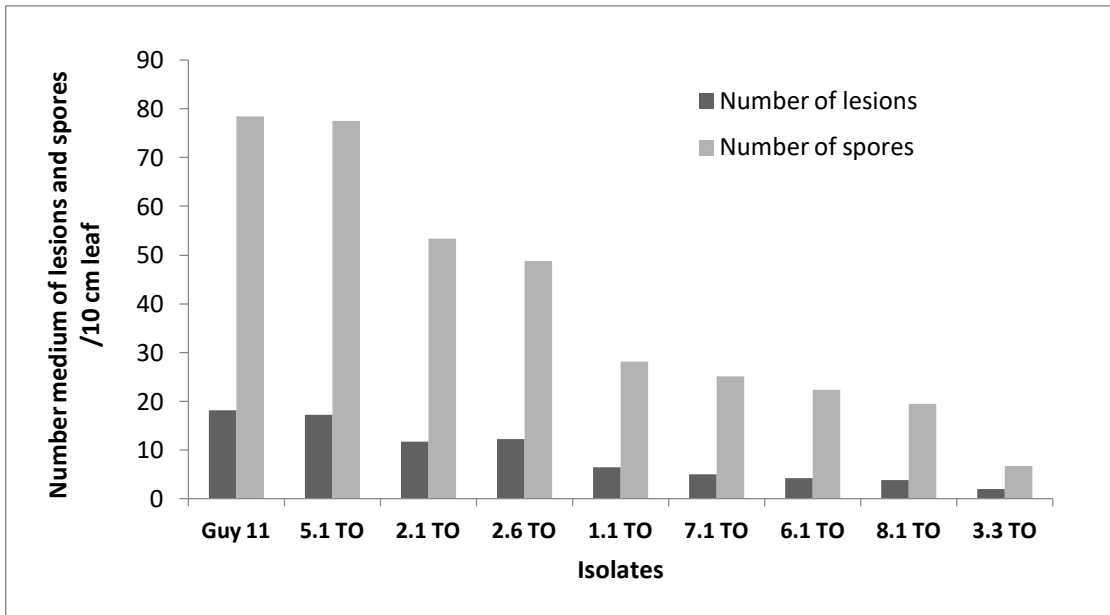**Figure 3. D'Ávila et al**

Figure 4. D'Ávila et al
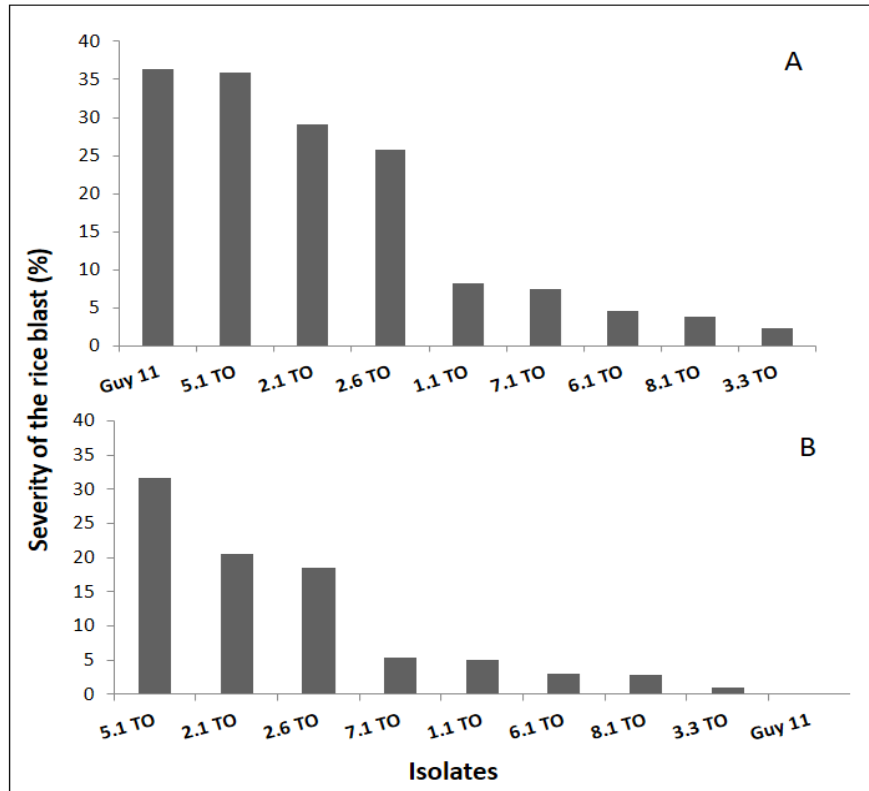
Figure 5. D'Ávila et al
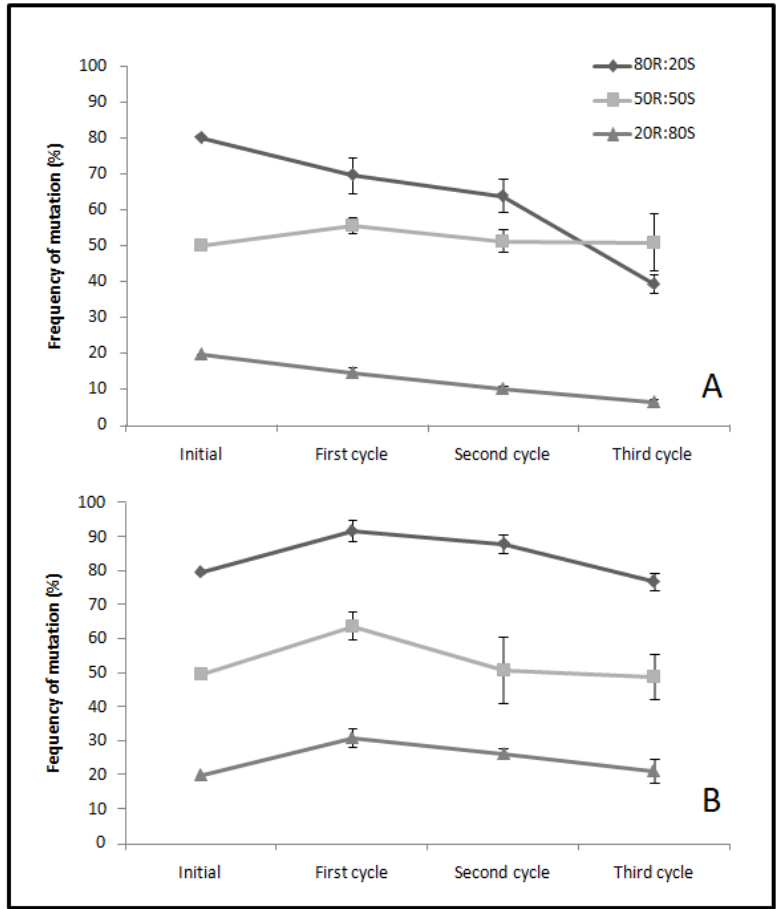
Figure 6. D'Ávila et al

Figure 7. D'Ávila et al

Chapter 2

Sensitivity of *Magnaporthe oryzae* populations to fungicides over a 25-year time frame in Brazil

Abstract

The changes in fungicide resistance frequency of the rice blast fungus *Magnaporthe oryzae* were monitored by 60 isolates collected over a period of 25 years in rice fields. Initially, the *in vitro* sensitivity of all isolates was measured in the presence of fungicides azoxystrobyn, tryciclazole, trifloxystrobin+tebuconazole and tebuconazole. Over the 25 year collection period, a gradual rise in the EC_{50} estimates for mycelial growth sensitivity was observed for all fungicides, but most strikingly for Azoxystrobin. The older isolates are much more sensitive to the fungicides tested when compared to the contemporary collection of isolates. Sequencing of the amplified cyt *b* fragment distinguished two haplotypes, H1 and H2. Haplotype H1 (six isolates) contained the G to C transversion at codon 143 (resulting in change G143A), linked to the resistance phenotype QoI-R. haplotype H2 (40 isolates), gathered the isolates sensitive to QoI. anti-resistance strategies should be immediately adopted to avoid an increase of resistant isolates in *Magnaporthe oryzae* populations.

Key words: Chemical control, EC_{50}, G143A.

Introduction

Rice (*Oryza sativa* L.) is one of the main staple crops cultivated worldwide. Although it is predominantly grown in Asia, it has also been cultivated in Brazil, which is the largest

rice grower outside of Asia (FAO, 2016), estimated at 12.3 million tonnes in a planted area of approximately 1.94 million hectares (CONAB, 2018).

Magnaporthe oryzae B.C. Couch is the causal agent of rice blast disease, one of the most widespread and destructive diseases of rice worldwide (Liu et al., 2010). *M. oryzae* can infect rice in all the developmental stages, causing leaf or panicle blast, and, more economically important, neck or panicle blast. Depending on the environmental conditions combined, cultivar susceptibility level, rate of nitrogen fertilization and date of sowing, rice blast can cause significant damage, from 10% to almost 100% yield loss (Baldacci & Picco, 1948).

Blast management is based on integrated tactics involving resistant cultivars, cultural practices and fungicides, which have been considered the most economical and sustainable way to control the disease (Filippi *et al*., 1999; Balardin, 2003; Teng *et al*., 1991). However, the high genetic diversity of *M. oryzae*, including a high variability in pathogenicity, has resulted in frequent breakdowns of several cultivars´ genetic resistances founded on specific host-pathogen interactions (complete resistance) (Ou, 1985; Zeigler & Correa, 2000; Prabhu & Filippi, 2006). In Brazil, rice fields are usually large, and most of the time planted with the one single cultivar, in a monoculture system, a condition conducive to the fast and drastic genetic resistance breakdown. This situation causes the farmer to follow a fungicide calendar, irrespective of the presence or absence of plant infections. In this context, the use of chemical control has been highlighted as the main method to be employed for control, often prompting abusive chemical control practices.

Based on official Brazilian state regulations, there are 43 registered fungicides for rice blast control (Agrofit, 2017). Although the most used molecules are DMI's (sterol demethylation inhibitors), QoI's (inhibitors of mitochondrial respiration) and others with specific sites of action, as the inhibitors of melanin biosynthesis (MBI's) (Scheuermann &

Eberhardt, 2011) are also used. MBIs fungicides are divided into two subgroups, depending on which part of the melanin biosynthesis pathway it will effect, either dehydration (MBI-D) or reduction (MBI-R) of enzymes involved in melanin biosynthesis, these active ingredients (a.i.) were expected to have a low risk of resistance development (Sawada et al., 2004). However, shortly after their introduction into commercial use, MBI-D resistant strains emerged, due to single point mutation in the SDH gene (Kaku et al., 2003; Takagaki et al., 2004; Yamaguchi et al., 2002).

Sterol demethylation inhibitors (DMIs) were introduced in the late 1960s as agricultural active ingredients and have become the largest and most important group of modern systemic fungicides. Despite their site-specific mode of action, resistance to DMIs under practice conditions was initially considered to be rather unlikely (Fuchs and Drandarevski, 1976). However, after almost three decades later their introduction, there have been published reports of reduced sensitivity or even field resistance to DMIs in various plant pathogen populations (De Waard et al., 1986; Brown and Wolfe, 1991; Kendall et al., 1993; Koller et al., 1995; Steva and Cazenave, 1996; Romero and Sutton, 1997). The DMIs inhibit the ergosterol biosynthesis by inhibiting the demethylation of precursor sterols at position 14, in a reaction catalyzed by Cyp51. Sterol 14a-demethylase (Cyp51) is a key enzyme in the ergosterol biosynthesis pathway. Ergosterol depletion, coupled with the accumulation of methylated sterol precursors, has been affects both membrane integrity and the function of membrane-bound proteins, resulting in inhibition of fungal growth (Lees et al., 1995; Ji et al., 2000; Ruge et al., 2005). Reports indicate that in European countries, instances of resistance to DMI fungicides were mainly due to changes in the cyp51 gene. (Leroux & Walker, 2011).

The QoI's fungicides Azoxystrobin, Kresoxim-methyl and Trifloxystrobin all carry a group of mitochondrial respiration inhibitors, derived from naturally occurring

strobilurins. Specifically, QoI´s inhibits mitochondrial respiration by blocking the transference of an electron to the Qo site from the cytochrome *bc1* complex, located at the inner mitochondrial membrane, thereby preventing ATP formation and disrupting the energy cycle of the fungus (Bartlett et al., 2002). Similarly to MBI-D fungicides, many instances of plant pathogen resistance to QoIs have been detected shortly after their introduction, including *M. oryzae* from rice and the closely related *M. oryzae* subgroup, pathogenic to turfgrass, goosegrass, barley and wheat (Avila-Adame & Koller, 2003; Ishii, 2006; Vincelli & Dixon, 2002; Castroagúdin et al., 2015). These strobilurin-resistant strains tolerate high doses of these a.i.'s due to three different amino acid substitutions, detected in the cytochrome *b* target site (Kim et al., 2003).

MBI-D fungicides were used in Asia since 1998, when the emergence of resistance among plant pathogens, caused their use to be discontinued. Subsequently, the proportion of the MBI-D-resistant subpopulation on the total *M. oryzae* population was quickly reduced, which may enable its reintroduction in the crop fields (Suzuki et al., 2010). On the other hand, MBI-R a.i.´s, such as Tricyclazole, Pyroquilon or Phtalide, target two types of hydroxynaphthalene reductases, enzymes involved in melanin biosynthesis (Ishii,2006; Kaku et al., 2003; Kurahashi, 2001; Thompson et al., 1997; Thompson et al., 2000). Surprisingly, even after 30 years of their use in some countries, no resistance has been observed in the field to MBI-R fungicides (Skamnioti & Gurr, 2009).

For the durable management of rice blast, detailed and accurate information about the sensitivity of *M. oryzae* populations for a.i's in use, and the monitoring for fungicide-resistant strains are needed, particularly for molecules at risk of resistance applied repeatedly over many years. In Brazil, there have been no studies monitoring the sensitivity of *M. oryzae* populations associated with the rice crop to fungicides, especially on a long-term basis. Therefore, the objective of this work was to examine the sensitivity

of isolates to the fungicides of the tricyclazole, triazole and especially the QoI group collected over a period of 25 years.

Material and Methods

Origin, purification and isolate preservation. The study was conducted on group of 60 monosporic isolates, collected on plants of cv. BRS primavera, presenting typical blast symptoms, over 25 years, from the 1989 to the 2014 cropping seasons in the state of Goiás, Brazil. This collection is named the "Primavera Collection", a subset of the Embrapa Rice and Beans Fungi and Functional Microorganisms Collection. These isolates are stored at -80°C (Valent et al., 1986) in paper envelopes or 2-mL cryogenic tubes previously sterilized, and are grown on fragments of filter paper. They were transferred to Petri plates containing BDA (dextrose-potato-agar) culture medium to be reactivated and compose a work collection (Table 1).

In vitro *M. oryzae* sensitivity to commercial fungicides

Fungicide stock solutions. Priori® (250 g/L Azoxystrobin), Bim® (750 d/Kg Tricyclazole), Folicur® (200 g/L Tebuconazole) and Nativo® (100 g/L Trifloxystrobin + 200 g/L Tebuconazole) fungicides were diluted 100-fold in deionized water to produce a stock solution.

Mycelial growth. The assay culture medium was prepared by cooling the BDA at 55°C and then adding 0.5 mM salicylhydroxamic acid (SHAM) (required to suppress the alternative oxidase route) for all treatments, including the control (Ma et al., 2009). The stock solutions of Azoxystrobin, Tricyclazole, Tebuconazole and Trifloxystrobin + Tebuconazole were added and homogenized to BDA to a final concentration of 100

μg.mL^{-1}. The fungicides were not added to the control. Isolates were cultured in BDA containing chloramphenicol (5ug / ml) and incubated at 25 °C in a 12 h photoperiod for 5 days before the establishment of the assay. Five mm mycelial discs were transferred to Petri plates containing culture medium with or without the addition of fungicides.

Colony growth was evaluated 7 days after incubation at 25 °C. Colony diameter was measured with a pachymeter. Colony growth for each isolate was converted into relative growth (CR), by comparing the size of the colonies in the presence of the fungicides with the size of the colonies in the control plates by the formula: CR = 100 x (mean of colony diameter growth in the presence of fungicide) / (mean of colony diameter growth without fungicide). Experiment was in completely randomized design with four replicates. The CR values were submitted to variance analyses (ANOVA) performed with the SPSS (Statistic 21) software. For mean separations, the Scott-Knott test was applied with the Assistat version 7.7 beta software (Federal University of Campina Grande, Paraíba, Brazil).

Conidia germination and apressorium formation. Conidial suspensions of the *M. oryzae* isolates were prepared according to Sena et al. (2013). The final concentration was adjusted to 10^5 conidia / mL using a Neubauer-type haemocytometer and a light microscope. Droplets of 30 μl containing conidia, suspended in a solution of water and the a.i.'s Azoxystrobin, Tricyclazole, Tebuconazole and Trifloxystrobin + Tebuconazole, were dispensed on a hydrophobic artificial surface, conditioned in a humid chamber. Plates were then sealed and incubated for 24 h at 28 °C. The drops contained the final concentrations of each fungicide of 100.0 μg mL^{-1}. One hundred conidia were evaluated for each replication. The Relative Germination (RG) was calculated by the formula RG = 100 × conidial germination in the presence of the a.i. / conidial germination of each isolate in the

absence of a.i. (%). Experiment was in a completely randomized design with four replicates. RG values were analyzed by ANOVA method and mean separation for the Scott-Knott test.

EC$_{50}$ estimates. The EC$_{50}$ values for mycelial growth were estimated for a subsample composed of 20 isolates. The assay was set as previously described (mycelial growth), with some adjustments. The isolates were transferred and cultured in Petri dishes containing SHAM (0.5 mM) and Azoxystrobin, Tricyclazole, Tebuconazole and Trifloxystrobin + Tebuconazole at concentrations of 0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.25, 2.5, 5.0, 7.0, and 10.0 μg mL-1 of each respective a.i. Five-mm disks of each of four isolates were arranged equidistantly in a single plate, with four replicates for each treatment. This experiment was conducted in a completely randomized design. To determine the EC50 value for each fungicide, the RGR (relative growth rate) was regressed against log$_{10}$ [fungicide]. EC50 was determined by solving the regression equation for log$_{10}$[fungicide] at RGR = 50.

Molecular analysis

Genomic DNA extraction and amplification of the cytochrome b gene by PCR for the detection of mutation conditioned by QoI's. DNA from all isolates was extracted by the Delaportta method (1983) with some modifications and quantified in Nanodrop (Nanodrop 2000 Thermo Scientific) and the concentration was adjusted to 30 μg / mL. An internal fragment from cytochrome *b* was amplified by polymerase chain reaction (PCR) with the primers PgCytb-F1 (5'-AGTCCTAGT GTAATGGAAGC-3') and PgCytb-R1 (5'-3-ATCTTCAACGTG TTTAGCACC') described previously (Kim et al., 2003). PCR

reactions were performed in 25μl volumes containing 30ng of DNA, 0.1μM of each primer, 80μM of each dNTP, 2.5mM MgCl₂, 2.5μl of 10x PCR reaction buffer (Sigma-Aldrich), and 0.1 U of Taq polymerase (Sigma-Aldrich). Amplification was performed on a PCR thermocycler with cycle conditions as follows: initial denaturation at 95°C for 5 min; followed by 35 cycles of 95°C for 45 sec, 55°C for 45 sec, and 72°C for 45 sec; with a final extent of 72 °C for 8 min (Kim *et al*., 2003).

Restriction enzymes digestion of cytochrome b gene fragment. The G143A mutation creates a Fnu4HI restriction site (GGTGC to GCT GC), while the F129L mutation causes the elimination of a Styl restriction site (CCTAGG to CATAGG). Thus, we used Fnu4HI and StyI for cleavable analysis of the amplified polymorphic sequence (CAPS) of PCR products. PCR product (5ul) is digested with each of the enzymes and the restriction fragments were separated by electrophoresis in 1.5 and 4% agarose gel using a marker according to the sizes of fragments to be amplified (1KB Invitrogen marker).

DNA Sequencing of internal region of cytochrome B gene fragment. The PCR product from a sub-sample composed of 46 isolates were purified using the ABI Prism BigDye Terminator Cycle Sequencing Ready reaction kit and shipped to Macrogen Inc. (Seoul, South Korea) where DNA sequencing was performed on an automated ABI 3730xl sequencer (Applied Biosystems , Foster City, CA, USA). Sequencing data were analyzed with the Geneious R 6.7.1 software (Biomatters, New Zealand). Partial cyt *b* sequences were manually analyzed for the detection of mutations: a transition from G to C at codon 428 (G143A) or a transversion from C to A at codon 387 (F129L) (KIM et al., 2003).

Results

Mycelial growth. In general, lower mycelial growth sensitivity was observed for Azoxystrobin, when compared to the other fungicides, with CR ranging from 0 to 76.03% (average=49.95). In the presence of Tricyclazole, CR ranged from 12.5 to 58.41% (average=35.88) and in the presence of the mixture Trifloxystrobin + Tebuconazole, CR ranged from 0 to 69.86% (average=24.78), and finally from 0 to 78.87% (average=26.66) in the presence of tebuconazole. The maximum values were considered outliers (Figure 1).

Conidia germination and appressorium formation. Isolates also showed lower germination and appressorium formation sensitivity to Azoxystrobin (the relative germination mean was 29,64% and appressorium formation was 26.1%) in isolates collected in recent years and in isolates previously colleted (the relative germination mean was 13.9 % and appressorium formation was 9.2%) (Fig. 2). Inhibition of conidium germination and appressorium formation was less evident in the presence of the other fungicides. The relative germination/appressorium formation was 18.56% and 6.3%, respectively, for the Triciclazole, 8.35% and 7.59 %, respectively for Trifloxystrobin + Tebuconazole and 7.89% and 5.61%, respectively for Tebuconazole (Figure 2). Figure 3 represents conidial germination in the presence of Azoxystrobin (100 μg. ml^{-1}), for 60 *M. oryzae* isolates arranged form left to right in order of the year of collection from 1989 to 2014. Clearly, conidial germination raised gradually and steadily, over the 25-year time span studied (Figure 3).

EC$_{50}$ estimates. Over the 25 year collection period, a gradual rise in the EC$_{50}$ estimates for mycelial growth sensitivity was observed for all fungicides, but most strikingly for

Azoxystrobin. The older isolates are much more sensitive to the fungicides tested when compared to the more current isolates (Fig. 4).

Detection of mutant isolates by restriction enzyme digestion. From all 60 isolates tested, six isolates were shown to carry the G143A mutation (Py 11125, Py11127, Py 11128, Py 11129, PY 11141 and Py 11142). All mutant isolates (G143A) were sampled in the year 2014. None of the isolates of Primavera Collection presented F129L mutation (Fig. 5).

Sequencing analyzes. All 46 isolates analysed showed amplification of a fragment of approximately 700 bp. Sequencing of the amplified cyt *b* fragment distinguished two haplotypes, H1 and H2. Haplotype H1 (six isolates) contained the G to C transversion at codon 143 (resulting in change G143A), linked to the resistance phenotype QoI-R. haplotype H2 (40 isolates), gathered the isolates sensitive to QoI (QoI-S, Table 2). None of the haplotypes showed the transversion from C to A at codon 129 (F129L mutation). Haplotype H2 was the predominant haplotype, thus confirming the data obtained by restriction enzyme analysis. No new mutations were detected. The mutation observed in the six isolates of the H1 haplotype, confirmed by sequencing data. It is noteworthy that these G143A QoI mutants also showed higher EC_{50}s for the other Tebuconazole fungicides and to the mixture Trifloxystrobin + Tebuconazole. In figure 6 we can observe the difference between the sensitive and mutant QoI isolates in the presence and absence of the fungicide azoxystrobin (100 μg ml^{-1}). In this case, showing the mutation-related phenotypic adaptation to QoIs.

Discussion

This is the first long-term study on the sensitivity to fungicides in *M. oryzae* populations collected from rice fields in Brazil. Among the molecules evaluated, we only investigated, at the molecular level, the isolates that showed resistance to QoI's. The progressive reduction of sensitivity among the isolates that were collected in different years from the same rice cultivar, in the same geographic area was clear. Among the tests carried out, the results with Azoxystrobin highlighted the increase of resistant isolates, with individuals 70% phenotypically more resistant to this a.i. (Table 1). Response of isolates to each a.i. was tested separately since the mechanisms of action of each group are different. Nevertheless, it is interesting to verify that the isolates carrying the QoI mutation also presented lower sensitivity to the other fungicide groups.

The mycelial growth test has been widely used to measure the *M. oryzae* sensitivity to fungicides, and it´s correlation with genetic resistance to the QoI's has been reported earlier by others (Castroagudín et al., 2015; Kim et al., 2003).

For conidial germination, isolates were least sensitive to Azoxystrobin, followed by Tricyclazole, then to Tebuconazole and to the mixture Trifloxystrobin + Tebuconazole. All data indicate a gradual increase of tolerance to all fungicides, from 1989 to 2014 (Table 1). There was an increase in the number of applications of the same molecule over time. We observed in our study that resistance to azoxystrobin began to emerge in 1997 and grows very, uninterruptedly after 2003, in Brazil the QoIs fungicides were introduced in the 1990s (Zambolim et al., 2010), we notice that a few years after its introduction in the market already it is observed a lower sensitivity of M. oryzae to these molecules. The percentage of appressoria formed followed the same tendency, except for Tricyclazole. For this fungicide, the isolates had a greater reduction in the inhibition of

appressorium formation than in the reduction of conidial germination and mycelial growth, which is most probably due to the mode of action of this MBI fungicide, which inhibits melanin biosynthesis. Melanin is an essential molecule present in the cell wall of fungal appressoria and is essential for the pathogenicity of *M. oryzae*. In the absence of it, the fungus can't penetrate directly into the host, necessitating injury to the infection (Butler & Day, 1998). In addition, isolate responses to different doses of Azoxystrobin indicated that while the older (collected from1989 to 2006) and sensitive individuals had a mean of 0.05 μg / mL for EC_{50}, the younger isolates (2014) had a EC_{50} of >10.0 μg / mL, considered high for the individuals who were resistant to QoIs (Kim et al., 2003, Castroagudín et al., 2015).

Overall, considering the progressive increase of EC_{50} values observed for Azoxystrobin, but also for all the other fungicides tested, the reduction of the sensitivity over time is the result of the high selection pressure exerted by consecutive years of QoI applications among the practices adopted for the management of rice diseases. In the specific case of rice blast control on cv. Primavera, for 20 years the chemical spraying was basically performed with the application of Tricyclazole, DMI's and QoI's. QoI fungicides have been used since the 1990s to control fungal diseases in most cultivated crops, as well as to control rice blast, a disease that has occurred for at least 100 years in Brazil (Prabhu & Filippi, 2006). Among the fungicides from this group, the first one to be extensively applied in the Brazilian fields was Azoxystrobin, first singly, and later in mixtures, mainly with DMIs. Currently, QoIs are still widely used in mixtures with other groups, even after numerous reports of QoI-resistant individuals (FRAC, 2018) among phytopathogenic fungi species.

In addition to the choice of the chemical group of the fungicide molecule, the plant stage at application, the number of applications, and other variables, are also factors

that affect the rate of selection of less sensitive individuals. Field applications of fungicides on cv. Primavera are usually concentrated between the first10 days of the vegetative stage and 20 first days of the reproductive stage. In spite of the preventive applications, the contact of the pathogen with the host, as an initial inoculum, starts in the seed and / or in the early vegetative phase. Thus, there is time for the completion of several disease cycles in this susceptible cultivar, thus providing the possibility of the selection of less sensitive individuals (Silva et al., 2011). Indeed, in ideal conditions, one monocycle may take as little as four days in this pathosystem (Prabhu et al., 2009). The number of applications that, notably, increased over the years in cv. Primavera, also exacerbates selection as the fungicide a.i.´s are made pervasive during the crop cycle. A susceptible cultivar, in a favorable environment (average temperature of 28 °C and high humidity in the cropping season in Goiás), lead to the increase in the number of fungicide applications, usually with the same mode of action. These findings may serve as an alert to other rice producing regions in Brazil. In order to delay the emergence of resistant isolates, other sustainable strategies should be adopted concomitantly with the chemical control, such as balanced fertilization, plant spacing and the use of partial and vertical resistance (Filippi & Prabhu, 1997). The understanding of *M. oryzae* population variability, is also an important factor to enable a better management of rice blast at the regional level in a more adequate and efficient way (Kawasaki, 2013).

The variability among EC_{50} values was much higher for the Azoxystrobin than for the Triazole or the mixture. Recently, isolates of *M. oryzae* resistant to Azoxystrobin were detected under field conditions in Japan (Araki et al., 2005; FRAC, 2017; Zhang et al., 2009). The sensitivity of a subgroup of *M. oryzae* occurring on wheat to this a.i. was also lower in Brazil (Castroagudin et al., 2015), and the same was found on isolates of perennial ryegrass in the United States, because of cytochrome b gene mutation (Kim et

al., 2003, Vincelli & Dixon, 2002). On the other hand, there have been so far no reports on *M. oryzae* populations resistant to Tricyclazole in the literature. Indeed, Zhang et al. (2006) tried to obtain stable Triciclazole-resistant isolates in the laboratory with no success and concluded that a slightly reduced sensitivity to Tricyclazole was conveyed lower physical fitness, reduced conidiogenesis and pathogenicity. The reduction of the efficiency of Tricyclazole to control rice blast in the field has been observed in China, but after a detailed analysis of these isolates, the presence of resistant individuals was not confirmed (Zhang et al., 2009).

Resistance to QoIs seems to evolve faster when compared to the other fungicide groups. Although all tested fungicides are systemic monosites, each mode of action is different, which may have influenced the emergence and prevalence of resistance in the studied population. Possibly, resistance to QoIs is higher and more prevalent in the population due to the nature of its mutation and / or perhaps because of the low adaptive cost (low fitness penalties) associated to it (Ma & Uddin, 2009). QoI resistant isolates survive and compete with sensitive ones. On the other hand, resistance to Tricyclazole is probably associated to the loss of some essential function, decreasing the resistant individual competitiveness in the agricultural environment.

Another aspect to consider is the interference of genetic variability mechanisms in heritability and perpetuation of cytochrome b mutation in the progeny. Among the mechanisms of genetic variability in *M. oryzae*, assexual reproduction is the one most reported, since the sexual phase in nature has not yet been observed in regions outside of the origin center of the rice plant (Saleh et al., 2012). These mutants reproduce rapidly as clones, making the shift from sensitive to resistant stable. In Italy, Kunova et al. (2014) did not identify any isolates resistant to Tricyclazole and Azoxystrobin, even in the experimental population. However, they identified 20 strains that were probably resistant

to Azoxystrobin, with a resistance factor equal to 2, without the mutations F129 or G137 in the cytochrome b gene, known to confer resistance factors between 5 and 15 (Kim et al. al., 2003). Resistance factor is calculated by dividing the EC_{50} value of the resistant population by the EC_{50} value of the wild population. This index measures the proportion of resistance to fungicides, that is, how many times a suspected population is more resilient than its wild population. (FRAC, 2018). We found in our study that a high number of individuals had lower sensitivity to Azoxystrobin, however, we did not identify mutations in the cytochrome b gene. In our study we observed resistance factors of 237 for Azoxystrobin, 42 for trifloxystrobin + tebuconazole and 27 for tebuconazole, comparing the 5 oldest isolates with the 6 isolates with the G143A mutation.

We must consider that the selection pressure over a long time frame, directly and gradually governs the emergence of resistant populations and, on the other hand, that the two mutations so far described (F129L or G143A) lead to high levels of resistance to the QoI group, so that individuals with medium resistance will not be detected molecularly. Additionally, the isolates tested are from a tropical environment, high disease pressure and from a hot spot for genetic variability of *M. oryzae* (Gonçalves et al., 2016; Dos Anjos et al., 2009). The complex ecology and epidemiology of this pathossystem demand a high level of biological monitoring for the sustainable management of even efficient fungicide molecules.

Additionally, the lower sensitivity observed in this study may be linked to another factor, with origin still unknown, demonstrated by Kunova et al. (2014). A mutation not detected or related to another region of the *M. oryzae* genome is a phenotypic adaptation to the fungicide. Regarding the DMI group, specifically the Tebuconazole, we have not yet found any information about *M. oryzae* isolates resistant to triazoles collected from rice fields.

Resistance to DMI's has been reported in Europe, Central and South America for a diverse group of phytopathogens, such as *Blumeria jaapi* (Ma, Z. et al.,2006; Proffer et al., 2006), *Blumeria graminis* (Delye & Corio., 2000; Wyand & Brown., 2005), *Monilinia fructicola* (Luo & Schnabel.,2008; Bean et al.,2009), *Penicillium digitatum* (Nakaune et al., 1998; Hamamoto et al., 2001; Zhao et al., 2007), *Erysiphe graminis* (Tosa & Tada, 1990), *Uncinula necator* (Delye et al., 1997), *Venturia inaequalis* (Schnabel & Jones, 2001), *Fusarium asiaticum* and *Fusarium graminearum.* (Yin et al., 2009) and *Mycosphaerella graminicola* (Leroux et al., 2007). Among these, the largest amount of information about the emergence of fungicide resistance is found on *M. graminicola*. To date, 22 alterations in different amino acids (substitutions and deletions) have been detected in the cyp51 gene in Western European populations. Other studies detected a large variation in the sensitivity to DMI fungicides in populations (Zhan et al., 2006; Stergiopoulos et al., 2003) with differences of up to 40 times among isolates from the same country. According to Leroux, Albertini (2011) and Leroux et al. (2007), several amino acid substitutions were found in *M. graminicola* field isolates, which expressed different levels of sensitivity to DMI fungicides. This suggests a more complex case of genetic fungicide resistance. Our data indicate a possible phenotypic resistance to this group, which should be confirmed in future studies by molecular analysis of this population. Thus, based on our data, resistance to strobilurins is probably the main factor that explains the lack of efficacy associated with the use of QoI fungicides for rice blast management (Cruz et al., 2010; Duveiller and Hodson; Tiedmann, 2010).

Another important aspect observed was a lower sensitivity of the isolates to the Azoxystrobin + Trifloxystrobin mixture, a strong suggestion of cross-resistance to the active ingredients of the same group. A previous work with *Magnaporthe grisea* from wheat plants andother Poaceae showed that cross-resistance does occur, however, for

Trifloxystrobin, the resistance index is lower when compared to Azoxystrobin (Oliveira et al., 2015). Cases of cross-resistance among QoI fungicides have been reported for several other pathossystems in which resistance had been detected. In *Mycosphaerella fijiensis*, causing black banana Sigatoka, cross-resistance of field isolates was observed among all QoI inhibitors, including Trifloxystrobin, Azoxystrobin, Famoxadone, Strobilurins B and Myxothiazol (Sierotzki et al., 2000). In *Microdochium nivale* and *M. majus* cereal pathogens, positive cross-resistance was observed among all QoIs tested in the study, and others: Azoxystrobin, Trifloxystrobin, Creoximetil, Picoxystrobin and Famoxadone (Walker et al., 2009). In cucumber production areas in Japan, Ishii et al. (2001) reported cross-resistance between Azoxystrobin and Crezoxymethyl for powdery mildew (*Podosphaera fusca*) and mildew (*Pseudoperonospora cubensis*).

Despite the high risk of resistance emergence attributed to QoI fungicides (FRAC, 2013), questionable arguments indicated that it was difficult to predict the time period for the development of QoI resistance, since this would depend on numerous factors associated with the complexity of each pathossystem. The following factors were cited as triggers: a) the inherent capacity of phytopathogenic fungi populations to develop resistance (genetic variability); b) the biology of the pathogen (especially its rate of reproduction); c) the magnitude of the selection pressure (directly related to the number of fungicide applications in the crop); d) the frequency of adopting anti-fungicide resistance strategies; e) the quality of the crop cover in the application of the fungicide; and f) the rate of degradation of the fungicide (which is dependent on local climatic variations), (MA; Uddin; Olaya, 2009). However, despite the alleged unpredictability, there have been numerous reports of QoI resistance in the last decade (Kim et al., 2003).

This study has contemplated a situation where the majority of these factors were probably at play, possibly aggravating and accelerating the emergence of less sensitive QoI populations in the field. In extreme situations, for example, for wheat powdery mildew, evidence was found of resistance development within two years of commercial use (Sierotzki; Wullschleger; Gisi, 2000).

In the specific case of *M. oryzae* and rice, we detected six isolates from the 2014 season carrying the G143A mutation responsible for resistance to QoIs, presenting a Resistant Factor of 500. This mutation selected isolates 500 times morem resistant to QoIs fungicides. Furthermore, a correlation between sensitivity *in vitro* and the rise of the mutation has been observed. While we cannot use only the phenotypic results to classify the resistance class to a certain fungicide group, the results of this study are clear to show that a progressive and constant reduction in the sensitivity to the product with the passage of time took place. The lower sensitivity of *M. oryzae* populations (phenotypic and / or presence of mutations) to fungicides is conditioned by many factors intrinsic to fungal biology, environment, disease pressure and fungicide application, mechanisms of variability, stability and adaptive cost among others. Further studies must be carried out in order to elucidate these dynamics, mainly regarding the genetics of resistance to MBI's and DMI's.

In conclusion, anti-resistance strategies should be immediately adopted to avoid an increase of resistant isolates in *Magnaporthe oryzae* populations. Specifically, suspend use of some active principles, taking into account also cross resistance. The adoption of additional sustainable practices, such as optimum management of fertilizers, soil and water management, partial and complete host genetic resistance, as well as the integration of biological and alternative control methods, all need to be combined in order reduce our dependence on chemical fungicides for blast control.

Literature Cited

Anjos, Gil R. Santos, Justino J. Dias Neto, Wilson F. Oliveira & Manoel D. Castro Neto. Identificação de raças fisiológicas de Magnaporthe grisea em áreas de arroz irrigado no Estado do Tocantins Tropical Plant Pathology 34 (3) May - June 2009.

Araki, Y., Sugihara, M., Sawada, H., Fujimoto, H., and Masuko, M. 2005. Monitoring of the sensitivity of Magnaporthe grisea to Metominostrobin 2001-2003: no emergence of resistant strains and no mutations at codon 143 or 129 of the cytochrome b gene. J. Pestic. Sci. 30:203-208

Avila-Adame, C., and Koller, W. 2002. Disruption of the alternative oxidase gene in Magnaporthe grisea and its impact on host infection. Mol. PlantMicrobe Interact. 15:493-500. 4.

Avila-Adame, C., and Koller, W. 2003. Characterization of spontaneous mutants of Magnaporthe grisea expressing stable resistance to the Qo-inhibiting fungicide azoxystrobin. Curr. Genet. 42:332-338. 5.

AGROFIT. Sistema de agrotóxicos fitossanitários. Available at: http://www.agricultura.gov.br/assuntos/insumos-agropecuarios/insumos-agricolas/agrotoxicos/agrofitAccessed on, 2018.

Baldacci, E., and Picco, D. 1948. Osservazioni sulle malttie del riso durante gli anni 1946 e 1947. Risocultura 36:73-77.

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., and Parr-Dobrzanski, B. 2002. The strobilurin fungicides. Pest Manage. Sci. 58:649-662

Balardin, R.S. Doenças do arroz. Santa Maria. 59p. Orium. 2003.

Bean, T.P., Cools, H.J., Lucas, J.A., Hawkins, N.D., Ward, J.L., Shaw, M.W., Fraaije, B.A.,2009. Sterol content analysis suggests altered eburicol 14a-demethylase (CYP51) activity in isolates of Mycosphaerella graminicola adapted to azole fungicides. FEMS Microbiol. Lett. 296, 266–273.

Brown, J. K. M. & Wolfe, M.S. Levels of resistance of Erysiphe graminis f.sp. hordei to the systemic fungicide triadimenol. 1991.Netherlands Journal of Plant Pathology, V.97,N.4,P.251-263.

Castroagudín, V. L., Ceresini, P. C., de Oliveira, S. C., Reges, J. T. A., Maciel, J. L. N., Bonato, A. L. V., Dorigan, A. F., and McDonald, B. A. 2015. Resistance to QoI fungicides is widespread in Brazilian populations of the wheat blast pathogen Magnaporthe oryzae. Phytopathology 105:284-294.

CONAB, 2018. Acompanhamento de safra brasileira de grãos. Available at: www.conab.gov.br. Accessed on February, 2018.

Cruz, M. F. A. et al. Resistência parcial à brusone de genótipos de trigo comum e sintético nos estádios de planta jovem e de planta adulta. Tropical Plant Pathology, Brasília, v. 35, p. 24-31, 2010.

De waard, M.A., Kipp, E.M.C., Horn, N.M. & Van Nistelrooy, J.G.M. 1986. Variation in sensibility to fungicides which inhibit ergosterol biosynthesis in wheat powdery mildew. Netherlands Journal of Plant Pathology 92:21-32.

Delye, C., Bousset, L., Corio-Costet, M.F., 1998. PCR cloning and detection of point mutations in the eburicol 14a-demethylase (CYP51) gene from Erysiphe graminis f. sp. hordei, a ''recalcitrant" fungus. Curr. Genet 34, 399–403.

Duveiller, E.; Hodson, D.; Tiedmann, A. V. Wheat blast caused by magnaporthe grisea: a reality and new challenge for wheat research. In: international wheat conference, 8., 2010, St. Petersburg. Abstracts... St. Petersburg: Vavilov Research Institute of Plant Industry, 2010, p. 247-248.

FAO. 2014. Food and agriculture organization of the United Nations. Available at: http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor. Accessed on december, 2017.

Fuchs, A. and Drandarevski, C.A. 1976. The likelihood of development of resistance to systemic fungicides which inhibit ergosterol biosynthesis. Netherlands Journal of Plant Pathology, vol.82, n.2, p.85-87.

Filippi, M.C., Prabhu, A.S., 1997. Integrated effect of host plant resistance and fungicidal seed treatment on rice blast control. Brazil Plant Dis., 81:351-355.

Filippi, M.C., Prabhu, A.S., Levy, M. 1999. Differential compatibility of Pyricularia grisea isolates with some Brazilian irrigated rice cultivars. Fitopatologia Brasileira: 24:447-450.

FRAC. 2006.Fungicide Resistance Action Committee. Mutations associated with QoI-resistance (Status 12/2006). www.frac.info.

Gonçalves FJ, Filippi MCC, Silva Lobo VL, Araújo LG, Silva GB, Guedes Coelho AS, Prabhu AS (2016) Polymorphism detection by microsatellite markers in a Magnaporthe oryzae population from different geographical areas of Brazil. J Phytopathol. doi:10.1111/jph.12485.

Hamamoto, H., Hasegawa, K., Nakaune, R., Lee, Y.J., Akutsu, K., Hibi, T., 2001. PCRbased detection of sterol demethylation inhibitor-resistant strains of Penicillium digitatum. Pest Manag. Sci. 57, 839–843.

Ishii, H. 2006. Impact of fungicide resistance in plant pathogens on crop disease control and agricultural environment. Jpn. Agric. Res. Q. 40:205. 22.

Ji, H., Zhang, W., Zhou, Y., Zhang, M., Zhu, J., Song, Y., Lu, J., Zhu, J., 2000. A threedimensional model of lanosterol 14a-demethylase of Candida albicans and its interaction with azole antifungals. J. Med. Chem. 43, 2493–2505.

Jin, L.-H., Chen, C.-J., Wang, J.-X., Chen, Y., and Zhou, M.-G. 2009. Activity of azoxystrobin and SHAM to four phytopathogens. Agric. Sci. China 8:835-842. 23.

Kaku, K., Takagaki, M., Shimizu, T., and Nagayama, K. 2003. Diagnosis of dehydratase inhibitors in melanin biosynthesis inhibitor (MBI-D) resistance by primer-introduced restriction enzyme analysis in scytalone dehydratase gene of Magnaporthe grisea. Pest Manage. Sci. 59:843-846.

Kawasaki, S. 2013. Interaction with Rice and Control: Proceedings of the 3rd International Rice Blast Conference.Springer Science & Business Media. p. 302.

Kendall SJ, Hollomon DW, Cooke LR and Jones DR (1993) Changes in Sensitivity to DMI Fungicides in Rhynchosporium secalis. Crop Protection 12: 357–362.

Kim, Y. S., Dixon, E. W., Vincelli, P., and Farman, M. L. 2003. Field resistance to strobilurin (QoI) fungicides in Pyricularia grisea caused by mutations in the mitochondrial cytochrome b gene. Phytopathology 93:891-900.

Koller W, Parker DM and Reynolds KL (1991) Baseline sensitivities ¨ of Venturia inaequalis to sterol demethylation inhibitors. Plant Dis. 75: 726–728

Kunova, A., Pizzatti, C., and Cortesi, P. 2013. Impact of tricyclazole and azoxystrobin on growth, sporulation and secondary infection of the rice blast fungus, Magnaporthe oryzae. Pest Manage. Sci. 69:278-284. 26.

Kurahashi, Y. 2001. Melanin biosynthesis inhibitors (MBIs) for control of rice blast. Pestic. Outlook 12:32-35.

Lees, N.D., Skaggs, B., Kirsch, D.R., Bard, M., 1995. Cloning of the late genes in the ergosterol biosynthetic pathway of Saccharomyces cerevisiae – a review. Lipids 30, 221–226.

Leroux, P., Albertini, C., Gautier, A., Gredt, M., Walker, A.S., 2007. Mutations in the CYP51 gene correlated with changes in sensitivity to sterol 14a-demethylation inhibitors in field isolates of Mycosphaerella graminicola. Pest Manag. Sci. 63,688–698.

Leroux, P. et al. Mutations in the CYP51 gene correlated with changes in sensitivity to sterol 14α-demethylation inhibitors in field isolates of mycosphaerella graminicola. Pest Management Science, Sussex, v. 63, n. 7, p. 688-698, 2007.

Liu, J., Wang, X., Mitchell, T., Hu, Y., Liu, X., Dai, L., and Wang, G. L. 2010. Recent progress and understanding of the molecular mechanisms of the rice-Magnaporthe oryzae interaction. Mol. Plant Pathol. 11:419-427.

Luo, C.X., Schnabel, G., 2008. The cytochrome P450 lanosterol 14a-demethylase gene is a demethylation inhibitor fungicide resistance determinant in Monilinia fructicola field isolates from Georgia. Appl. Environ. Microbiol. 74, 359–366.

Ma, B., Uddin, W., and Olaya, G. 2009. Baseline and non-baseline sensitivity of Magnaporthe oryzae isolates from perennial ryegrass to azoxystrobin in the northeastern United States. Can. J. Plant Pathol. 31:57-64.

Nakaune, R., Adachi, K., Nawata, O., Tomiyama, M., Akutsu, K., Hibi, T., 1998. A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus Penicillium digitatum. Appl. Environ. Microbiol. 64, 3983–3988.

Oliveira, S.C.; Castroagudin, V.L.; Maciel, J.L.N.; Pereira, D.A.S.; Ceresini, P.C. 2015. Resistência cruzada aos fungicidas IQo azoxistrobina e piraclostrobina no patógeno da brusone do trigo Pyricularia oryzae no Brasil. Summa Phytopathologica, v.41, n.4, p.298-304.

OU, S. H. 1985. Rice Diseases, 2nd ed. Commonwealth Agricultural Bureaux International, Kew, UK, p.380.

Prabhu A.S, Filippi M. C. 2006. Brusone em arroz: controle genético, progresso e perspectivas. Santo Antônio de Goiás GO. Embrapa Arroz e Feijão.

Proffer, T.J., Berardi, R., Ma, Z., Nugent, J.E., Ehret, G.R., McManus, P.S., Jones, A.L., Sundin, G.W., 2006. Occurrence, distribution, and polymerase chain reactionbased detection of resistance to sterol demethylation inhibitor fungicides in populations of Blumeriella jaapii in Michigan. Phytopathology 96, 709–717.

Romero RA, Sutton TB, 1997. Sensitivity of Mycosphaerella fijiensis, causal agent of Black Sigatoka of banana, to propiconazole. Phytopathology 87, 96–100.

Ruge, E., Korting, H.C., Borelli, C., 2005. Current state of three-dimensional characterisation of antifungal targets and its use for molecular modelling in drug design. Int. J. Antimicrobiol. Ag. 26, 427–441.

Saleh, D., Xu P., Shen Y, Li C.Y, Adreit H, Milazzo J, Ravign V., Bazin E., Notteghem J.L., Fournier E. 2012. Sex at the origin: an Asian population of the rice blast fungus Magnaporthe oryzae reproduces sexually. Molecular Ecology 21: 1330–1344.

Schnabel, G., Jones, A.L., 2001. The 14a-demethylase (CYP51A1) gene is overexpressed in Venturia inaequalis strains resistant to myclobutanil. Phytopathology 91, 102–110.

Sierotzki, H.; Wullschleger, J.; Gisi, U. Point mutation in cytochrome b gene conferring resistance to strobilurin fungicides in Erysiphe graminis f. sp tritici feld isolates. Pesticide Biochemistry and Physiology, New York, v.68, p.107-112, 2000.

Skamnioti, P., and Gurr, S. J. 2009. Against the grain: Safeguarding rice from rice blast disease. Trends Biotechnol. 27:141-150.

Scheuermann, K, K & Eberhardt, D. S. 2011. Avaliação de fungicidas para o controle da brusone de panícula na cultura do arroz irrigado. Revista de Ciências Agroveterinárias. Lages, v.10, n.1, p. 23-28.

Silva G.B, Prabhu A.S, Filippi M.C.C., Trindade M.G., Araújo L.G., Zambolim L. 2009. Genetic and phenotypic diversity of Magnaporthe oryzae from leaves and panicles of rice in commercial fields in the State of Goiás, Brazil. Tropical Plant Pathology 34:77-86.

Silva, G. B. et al. Use of local rice cultivars as additional differentials to identify pathotypes of Pyricularia oryzae. Bragantia, São Paulo, v. 70, p. 860-868, 2011.

Steva H, Cazenave C, 1996. Evolution of grape powdery mildew insensitivity to DMI fungicides. In: Proceedings of the British Crop Protection Conference – Pests and Diseases 1996. Farman, UK: BCPC, 725–30.

Sawada, H., Sugihara, M., Takagaki, M., and Nagayama, K. 2004. Monitoring and characterization of Magnaporthe grisea isolates with decreased sensitivity to scytalone dehydratase inhibitors. Pest Manage. Sci. 60:777-785.

Sierotzki, H.; Wullschleger, J.; Gisi, U. Point mutation in cytochrome b gene conferring resistance to strobilurin fungicides in Erysiphe graminis f. sp tritici feld isolates. Pesticide Biochemistry and Physiology, New York, v.68, p.107-112, 2000.

Stergiopoulos, I. et al. 2003. Multiple mechanisms account for variation in base-line sensitivity to azole fungicides in field isolates of mycosphaerella graminicola. Pest Management Science, Sussex, v. 59, p. 1333–1343.

Suzuki, F., Yamaguchi, J., and Koba, A. 2010. Changes in fungicide resistance frequency and population structure of Pyricularia oryzae after discontinuance of MBI-D fungicides. Plant Dis. 94:329-334.

Takagaki, M., Kaku, K., Watanabe, S., Kawai, K., Shimizu, T., Sawada, H., Kumakura, K., and Nagayama, K. 2004. Mechanism of resistance to carpropamid in Magnaporthe grisea. Pest Manage. Sci. 60:921-926.

Thompson, J. E., Basarab, G. S., Andersson, A., Lindqvist, Y., and Jordan, D. B. 1997. Trihydroxynaphthalene reductase from Magnaporthe grisea: Realization of an active center inhibitor and elucidation of the kinetic mechanism. Biochemistry-US 36:1852-1860.

Thompson, J. E., Fahnestock, S., Farrall, L., Liao, D.-I., Valent, B., and Jordan, D. B. 2000. The second naphthol reductase of fungal melanin biosynthesis in Magnaporthe grisea. J. Biol. Chem. 275:34867-34872.

Valent B., Crawford M.S., Weaver C.G., Chumley F.G. 1986. Genetic studies of fertility and pathogenicity in Magnaporthe grisea (Pyricularia oryzae). Iowa State J Res 60:569–594

Vincelli, P., and Dixon, E. 2002. Resistance to Q(o)I (strobilurin-like) fungicides in isolates of Pyricularia grisea from perennial ryegrass. Plant Dis. 86:235-240.

Walker, A.S., Auclair, C., Gredt, M., Leroux, P. First occurrence of resistance to strobilurin fungicides in Microdochium nivale and Microdochium majus from French naturally infected wheat grains. Pest Management Science, West Sussex, v.65, p.906-915, 2009.

Wyand, R.A., Brown, J.K., 2005. Sequence variation in the CYP51 gene of Blumeria graminis associated with resistance to sterol demethylase inhibiting fungicides. Fungal Genet. Biol. 42, 726–735.

Yin, Y., Liu, X., Li, B., Ma, Z., 2009. Characterization of sterol demethylation inhibitorresistant isolates of Fusarium asiaticum and F. Graminearum collected from wheat in China. Phytopathology 99, 487–497.

Yamaguchi, J., Kuchiki, F., Hirayae, K., and So, K. 2002. Decreased effect of carpropamid for rice blast control in the west north area of Saga Prefecture in 2001. Jpn. J. Phytopathol. 68:261.

Zeigler, R.S. and Correa, F.J. 2000. Applying Magnaporthe grisea population analyses for durable rice blast resistance. 2000. APSnet Features. Online. doi: 10.1094/APSnetFeature-2000-0700A.

Zhan, J.; Stefanato, F. L.; McDonald, B. A. Selection for increased cyproconazole tolerance in Mycosphaerella graminicola through local adaptation and in response to host resistance. Molecular Plant Pathology, London, v. 7, n. 4, p. 259-268, 2006.

Zhang, C. Q., Zhu, G. N., Ma, Z. H., and Zhou, M. G. 2006. Isolation, characterization and preliminary genetic analysis of laboratory tricyclazoleresistant mutants of the rice blast fungus, Magnaporthe grisea. J. Phytopathol. 154:392-397.

Zhang, C.-Q., Huang, X., Wang, J.-X., and Zhoub, M.-G. 2009. Resistance development in rice blast disease caused by Magnaporthe grisea to tricyclazole. Pestic. Biochem. Physiol. 94:43-47

Zhao, L., Liu, D., Zhang, Q., Zhang, S., Wan, J., Xiao, W., 2007. Expression and homology modeling of sterol 14a-demethylase from Penicillium digitatum. FEMS Microbiol. Lett. 277, 37–43.

Figure legends

Figure 1: Boxplot distribution of in vitro sensitivity of *Magnaporthe oryzae* isolates to the quinone-outside inhibitor (QoI) fungicide azoxystrobin (AZO), sterol demethylation inhibitors (DMI) fungicide tebuconazole (TEB), melanine biosynthesis inhibitor (BMI) fungicide tricyclazole (TRI) and misture commercial trifloxystrobin + tebuconazole (TRI +TEB). Relative growth was based on the ratio of the diametric growth of an isolate after 5 days of incubation on potato dextrose agar containing both chloramphenicol and streptomycin amended or not with fungicides at 100 μg ml^{-1}. Contain 60 rice-derived isolates sampled in 1989 at 2014 in Brazilian fields.

Figure 2: Relative percentage of germination of conidia and appressorium formation in vitro sensitivity of 60 *Magnaporthe oryzae* isolates to the quinone-outside inhibitor (QoI) fungicide azoxystrobin, sterol demethylation inhibitors (DMI) fungicide tebuconazole, melanine biosynthesis inhibitor (BMI) fungicide tricyclazole and misture commercial trifloxystrobin + tebuconazole. The drops contained final concentrations of each fungicide (100.0 μg mL^{-1}). The Relative Germination (GR) was calculated by the formula GR = 100 × conidial germination average in the presence of fungicide (%) /conidial germination average of each isolate in the absence of fungicide (%). Means were calculated and variance analyzed (ANOVA) following a completely randomized experimental design.

Figure 3: Percentage of conidia germination of 60 *Magnaporthe oryzae* isolates in the presence of Azoxystrobin (100 μg. ml^{-1}), quantified after 24 hours incubated at 25 ° C and RH = 100%. Isolates arranged in order of year of collection (1989 to 2014).

Figure 4: Effective dose that inhibits 50 % mycelial growth (DE_{50}) in 20 *Magnaporthe oryzae* isolates estimated for assay was installed as previously described (Inhibition of mycelial growth) with some modifications. The isolates were transferred and cultured in Petri dishes containing SHAM (0.5 mM) and the azoxystrobin, tricyclazole, tebuconazole and trifloxystrobin + tebuconazole fungicides. The isolates were arranged in ascending order of collection year.

Figure 5. An internal fragment from cytochrome *b* was amplified by polymerase chain reaction (PCR) with the primers PgCytb-F1 (5'-AGTCCTAGT GTAATGGAAGC-3') and PgCytb-R1 (5'-3-ATCTTCAACGTG TTTAGCACC') described previously (Kim et al., 2003). *Fnu4HI* enzyme digestion *Fnu4HI* for detection of the G143A mutation. S (isolate sensitive) and R (isolate resistant / mutant).

Figure 6: In vitro sensitivity of *Magnaporthe oryzae* isolates to the quinone-outside inhibitor (QoI) fungicide azoxystrobin. **A.** Four isolates of *M. oryzae* growing in potato dextrose agar containing both chloramphenicol and streptomycin (PDA+) medium amended with azoxystrobin at 100 μg ml^{-1} and 0.5 mM salicylhydroxamic acid (SHAM).(1.3 QoI resistant) and (2.4 QoI sensitive). **B.** Isolates of *M. oryzae* growing in PDA+ medium amended without and with azoxystrobin at 100 μg ml^{-1} and 0.5 mM SHAM in the repeat experimente. Isolate Py 268 without (1) and with (2) azoxystrobin (QoI sensitive), and isolate Py1128 without (3) and with (4) azoxystrobin (QoI resistant).

Table 1: Origin, year and phenotypic characterization of *Magnaporthe oryzae* for resistance to quinone-outside inhibitor (QoI), Sterol demethylation inhibitors (DMI) and melanine biosynthesis inhibitors (BMI) fungicides according to an in vitro sensitivity assay[w]

			Average relative growth (mm)[y]				
	Population[x]		Azoxystrobin	Tebuconazole	Tricyclazole	Trifloxystrobin+Tebuconazole	*cyt b* Haplotype[z]
PY 175	1989	Sto Antônio De Goiás-GO	0.00 a	0.00 a	15.60 a	0.00 a	H1
PY 176	1989	Sto Antônio De Goiás-GO	0.00 a	0.00 a	16.59 a	0.00 a	H1
PY 198	1990	Sto Antônio De Goiás-GO	0.00 a	0.00 a	18.28 a	0.00 a	H1
PY 204	1993	Sto Antônio De Goiás-GO	0.00 a	0.00 a	17.12 a	0.00 a	H1
PY 268	1993	Sto Antônio De Goiás-GO	0.00 a	0.00 a	16.43 a	0.00 a	H1
PY 1300	1997	Sto Antônio De Goiás-GO	21.03 b	34.87 d	23.33 b	28.57 c	H1
PY 1302	1997	Sto Antônio De Goiás-GO	22.64 b	38.23 d	21.33 b	34.28 c	H1
PY 1303	1997	Sto Antônio De Goiás-GO	22.87 b	32.96 d	27.66 b	28.57 c	-
PY 1304	1997	Sto Antônio De Goiás-GO	22.95 b	37.03 d	23.33 b	28.57 c	-
PY 1305	1997	Sto Antônio De Goiás-GO	23.02 b	35.68 d	29.33 b	31.42 d	H1
PY 2376	2000	Sto Antônio De Goiás-GO	23.46 b	34.65 d	29.33 b	32.55 d	H1
PY 2377	2000	Sto Antônio De Goiás-GO	23.74 b	36.87 d	29.33 b	28.57 c	H1
PY 2378	2000	Sto Antônio De Goiás-GO	33.91 c	35.23 d	32.10 c	31.40 d	-
PY 2379	2001	Sto Antônio De Goiás-GO	33.98 c	30.40 d	32.03 c	14.28 b	H1
PY 2380	2001	Sto Antônio De Goiás-GO	34.05 c	26.55 c	32.85 c	14.28 b	H1
PY 2637	2001	Sto Antônio De Goiás-GO	34.18 c	24.78 c	31.04 c	14.28 b	-
PY 2638	2001	Sto Antônio De Goiás-GO	34.23 c	23.65 c	33.23 c	14.28 b	-
PY 2639	2001	Sto Antônio De Goiás-GO	35.47 c	19.76 b	33.13 c	32.42 d	-
PY 2640	2001	Sto Antônio De Goiás-GO	35.68 c	23.19 c	38.54 c	28.57 c	-
PY 4056	2002	Sto Antônio De Goiás-GO	35.85 c	24.76 c	37.12 c	33.50 d	H1
PY 4057	2002	Sto Antônio De Goiás-GO	36.08 c	28.23 c	33.08 c	33.50 d	H1
PY 4058	2002	Sto Antônio De Goiás-GO	36.27 c	18.65 b	29.45 b	14.28 b	-
PY 4059	2002	Sto Antônio De Goiás-GO	38.04 c	27.63 c	37.19 c	22.85 c	-
PY 4060	2002	Sto Antônio De Goiás-GO	39.23 c	22.14 c	35.2 c	14.28 b	-
PY 6029	2003	Sto Antônio De Goiás-GO	39.35 c	23.76 c	37.33 c	14.28 b	H1
PY 6030	2003	Ceres-GO	39.73 c	22.83 c	38.45 c	28.57 c	H1
PY 6031	2003	Ceres-GO	40.80 d	21.76 c	37.03 c	27.14 c	-
PY 6032	2003	Ceres-GO	45.67 d	11.54 b	35.46 c	28.57 c	-
PY 6033	2003	Ceres-GO	46.78 d	13.48 b	34.71 c	28.57 c	-
PY 8045	2005	Sto Antônio De Goiás-GO	46.93 d	12.15 b	38.00 c	22.85 c	H1
PY 8046	2005	Sto Antônio De Goiás-GO	47.41 d	11.78 b	39.30 c	22.85 c	H1
PY 8047	2005	Sto Antônio De Goiás-GO	47.65 d	10.65 b	39.00 c	22.85 c	-
PY 8048	2005	Sto Antônio De Goiás-GO	47.83 d	13.75 b	38.50 c	22.85 c	H1
PY 8049	2005	Sto Antônio De Goiás-GO	47.98 d	12.06 b	39.10 c	20.01 c	H1
PY 9086	2006	Sto Antônio De Goiás-GO	51.35 e	11.94 b	39.15 c	22.85 c	H1
PY 9087	2006	Sto Antônio De Goiás-GO	51.85 e	12.48 b	37.33 c	20.00 c	H1
PY 9088	2006	Sto Antônio De Goiás-GO	51.74 e	34.29 d	37.50 c	20.00 c	H1
PY 9090	2006	Sto Antônio De Goiás-GO	51.82 e	21.76 c	38.06 c	22.85 c	H1
PY 9890	2009	Goiânia -GO	52.89 e	11.54 b	38.32 c	22.85 c	H1
PY 9910	2009	Goiânia -GO	53.27 e	13.48 b	38.80 c	20.20 c	H1
PY 9911	2009	Goiânia -GO	55.40 e	12.15 b	38.60 c	22.85 c	H1
PY 10649	2011	Sto Antônio De Goiás-GO	55.72 e	11.78 b	39.20 c	28.57 c	H1
PY 10652	2011	Sto Antônio De Goiás-GO	55.87 e	10.65 b	39.50 c	28.57 c	H1
PY 10655	2011	Sto Antônio De Goiás-GO	55.91 e	13.75 b	38.60 c	28.57 c	H1
PY 10657	2011	Sto Antônio De Goiás-GO	57.05 e	17.65 b	37.90 c	28.57 c	H1
PY 10781	2011	Sto Antônio De Goiás-GO	67.45 f	15.99 b	38.10 c	22.85 c	H1
PY 10811	2011	Sto Antônio De Goiás-GO	67.82 f	15.98 b	37.50 c	22.85 c	H1
PY 10812	2011	Sto Antônio De Goiás-GO	68.01 f	15.67 b	36.80 c	21.42 c	H1
PY 11003	2012	Goiânia -GO	68.34 f	14.32 b	40.33 d	22.85 c	H1
PY 11046	2012	Goiânia -GO	68.70 f	57.56 e	41.79 d	22.85 c	H1
PY 11047	2012	Goiânia -GO	69.40 f	57.94 e	42.66 d	21.42 c	H1
PY 11048	2012	Goiânia -GO	70.12 f	58.06 e	45.18 d	28.32 c	H1
PY 11050	2012	Goiânia -GO	70.67 f	57.39 e	45.90 d	27.89 c	H1
PY 11051	2012	Goiânia -GO	71.24 f	57.50 e	46.60 d	25.32 c	H1
PY 11125	2014	Goiânia -GO	72.38 g	57.56 e	46.70 d	24.94 c	H2
PY 11127	2014	Goiânia -GO	73.71 g	57.94 e	48.90 d	48.86 e	H2
PY 11128	2014	Goiânia -GO	74.39 g	58.06 e	49.23 d	49.62 e	H2
PY 11129	2014	Goiânia -GO	74.42 g	58.39 e	49.90 d	49.75 e	H2
PY 11141	2014	Goiânia -GO	74.76 g	58.45 e	55.70 e	49.78 e	H2
PY 11142	2014	Goiânia -GO	76.03 g	78.87 f	58.41 e	69.86 f	H2
$F_{Isolates}$			93.82	71.49	201.5	70.3	$p \leq 0.0001$

Table 2: Polymorphic sites in the sequence of the cytochrome b (*cyt b*) gene from 46 isolates of *Magnaporthe oryzae* from Brazil[a]

Genotype	NCBI Number	Haplótypes	N	Sequence
QoI-S	AY245424	Wild Type		TTTTATCCCGGTAGGTCTTGTTTCTTTTACT CTCTATTTAC
QoI-R	AY245426	Mutation F129L		..........A............................A.........
	AY245427	Mutation G143A		...............C.......................A.........
H1 (QoI-S)	-		40	......................................A.........
H2 (QoI-R)	-		6	.............C........................A.........

[a]The first three sequences were downloaded from the National Center for Biotechnology Information database as references (KIM et al., 2003): rice wild-type quinone-outside inhibitor-sensitive (QoI-S) allele from M. oryzae isolates recovered from rice (AY245424); F129L QoI-resistant (QoI-R) reference sequence AY245426 recovered de *Lolium perene* showing the C-to-A transversion at codon 129; and G143L QoI-R reference sequence AY245427 recovered de *Lolium perene* showing the G-to-C transition codon 143.
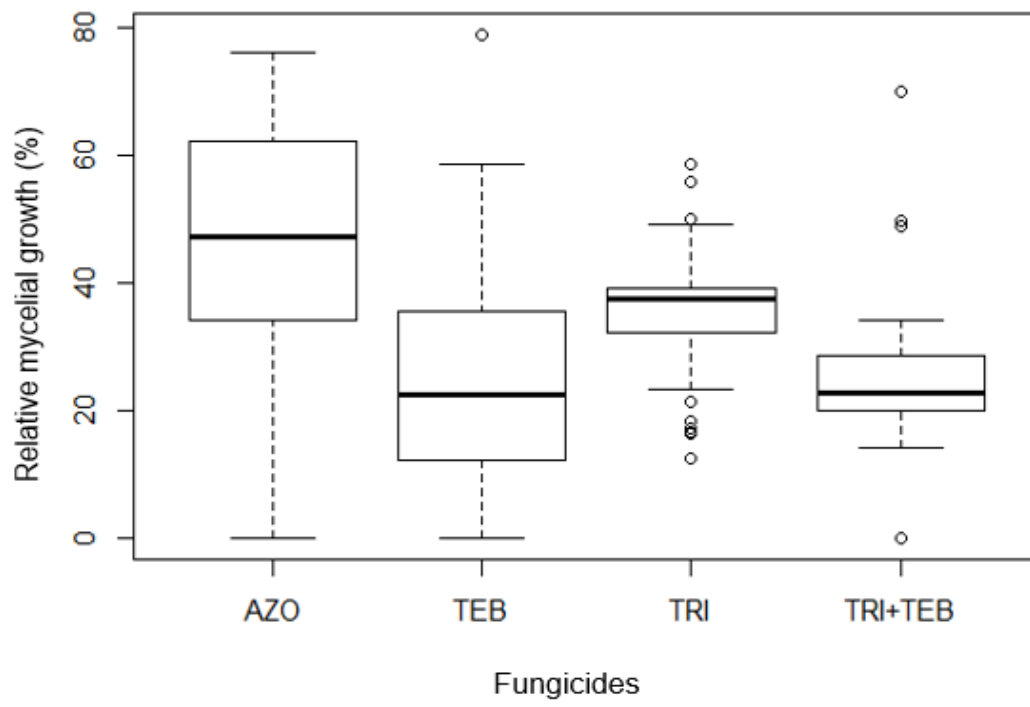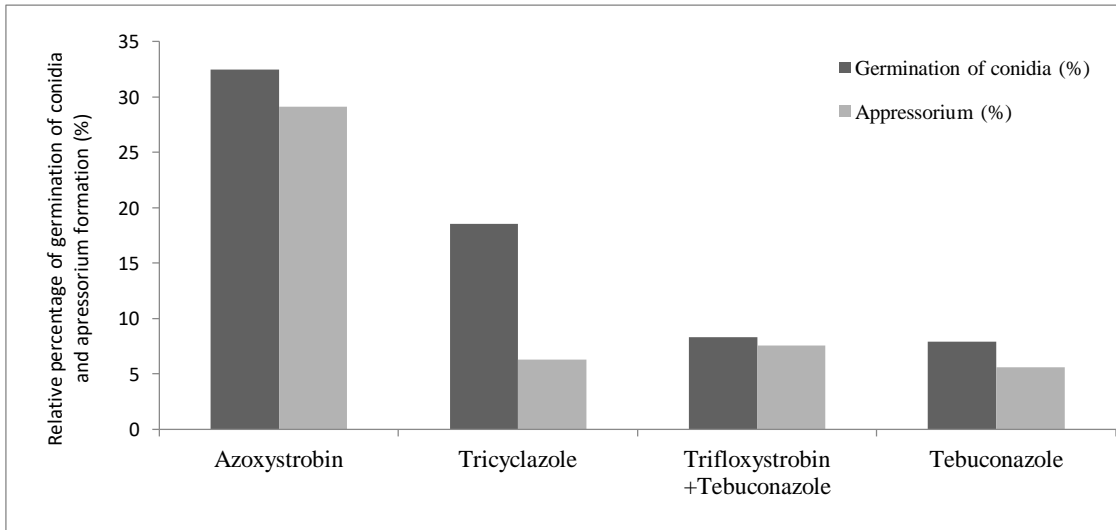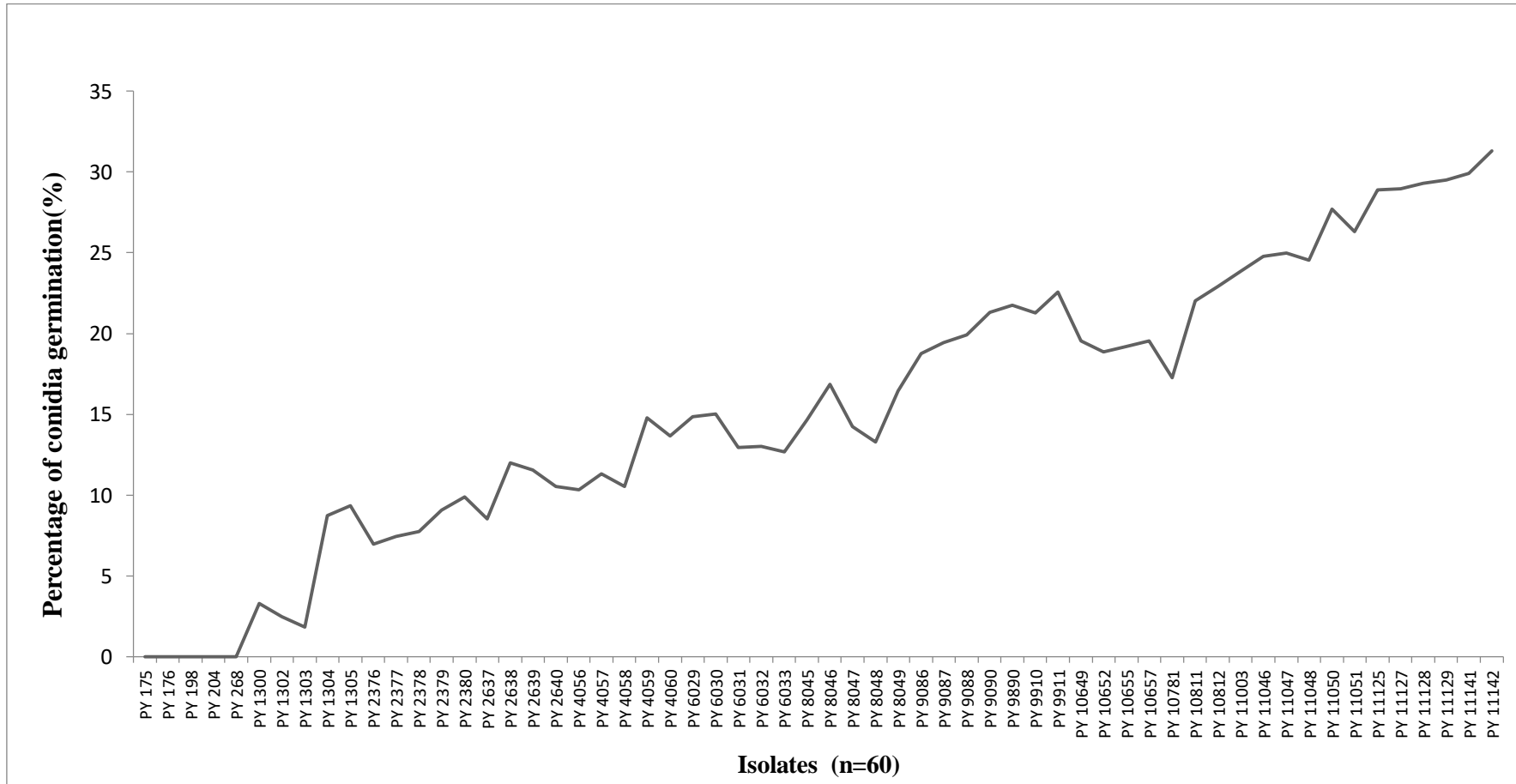
Figure 1. D'Ávila et al

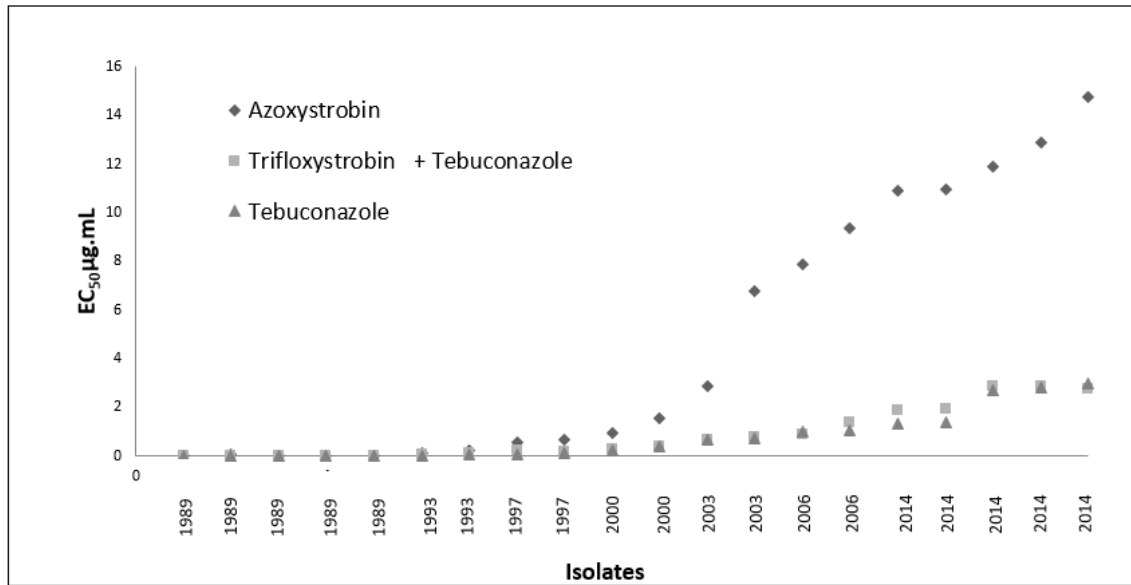**Figure 2. D'Ávila et al**

Figure 3. D'Ávila et al
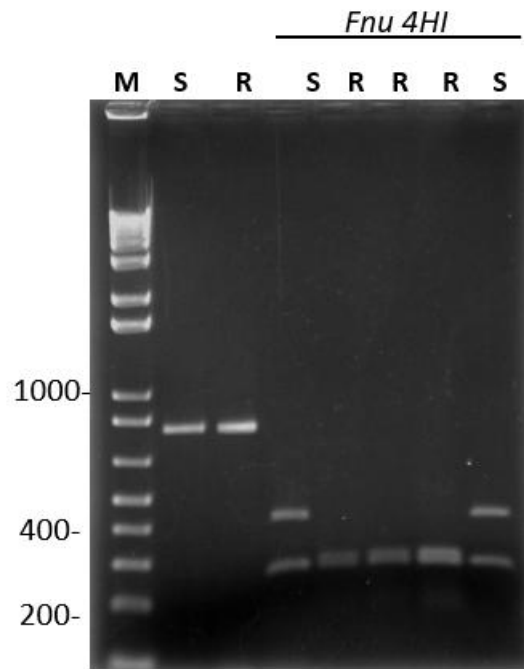
Figure 4. D'Ávila et al
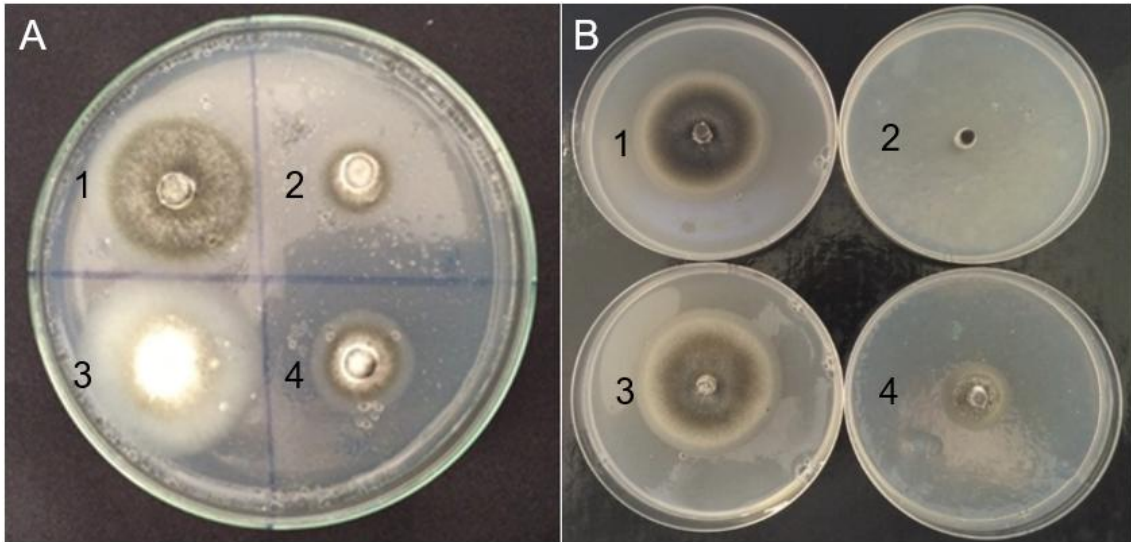
Figure 5. D'Ávila et al

Figure 6. D'Ávila et al

Chapter 3

Occurrence of Idiomorphs MAT1-1 and MAT-2 of *Magnaporthe oryzae* in the state of Tocantins: sexual recombination of the blast fungus is at play in Brazilian rice fields?

 Abstract

Blast is a major disease of rice in Brazil, the largest rice-producing country outside Asia. This study a total of 98 *M. oryzae* isolates were obtained, from TO State. A single amplicon matching the MAT1-2 idiomorphic was produced for the 91 isolates and 7 isolates MAT1-1 idiomorphic. This is the first study to detect isolates of MAT1-1 idiomorph associated with rice in Brazil. Although a low frequency is found, we are faced with a hypothesis of possible occurrence of sexual reproduction or at least recognizing a condition of high genetic variability of M. oryzae, this possibility has direct implications for blast management in Brazil, as transmission and prevalence of the fungicide resistance characteristic in the field populations, making it even more difficult to control the disease.
Key Words: sexual reproduction, mating types, genetic variability.

Introduction

Rice (*Oryza sativa* L.) is a staple crop in several major countries and stands out as the third most produced grain in the world. Brazil is ranked among the top ten largest rice-producing countries and is the major producer outside Asia with a total production estimated from 11 to 12 million tons (CONAB, 2018). Rice blast is caused by the fungus *Magnaporthe oryzae* Couch [anamorph *Pyricularia oryzae* Cavara, formerly known as *P. grisea* (Cook) Sacc.], also is considered the most destructive rice disease causing serious rice yield losses around the globe.

Magnaporthe oryzae is notable for expressing a large number of pathotypes, often undetected in a given population. However, when new blast resistant rice cultivars are introduced (Prabhu et al., 2006) new pathotypes are soon detected in the field, eventhough it is so farbelieved that *M. oryzae* reproduction is asexual (clonal reproduction). The high variability of the fungus contributes to the frequent loss of resistance in rice cultivars, and the changes in agro-ecosystems also contributes to the phenomenon of 'host change' (Couch & Kohn, 2002; Couch et al., 2005). The work of Couch et al. (2005) points to evidence that isolates pathogenic to rice emerged from *Setaria* isolates in China, followed by specialization in weeds common in rice fields. Stuckenbrok & Mcdonald (2008) suggest that the same process occurred leading to the emergence of wheat blast in southern region, in Brazil. There is evidence that sexual reproduction is the main source of variability of *M. oryzae* (Moreira et al., 2015).

The recognition of sexually compatible individuals is regulated by mating type (Turgeon, B. G., 1998) and pheromone (Shen et al., 1999) genes. From the matching between compatible individuals, *in vitro* or in senescent plants tissues (Hayashi et al., 1997) long-necked black perithecia develop, containing unitunicated asci with eight tri-septate ascospores inside. Although this observation leads to the belief that the teleomorph occurs in cultural remains, some issues remains unclear. There are few studies about the role of sexual reproduction in the genetic structure of *M. oryzae* populations, as well as in the reproductive biology of the pathogen involving morphogenesis and genetic regulation. The locus for mating type (MAT) genes in *M. oryzae*, is called Mat1, located on chromosome three. This locus has two idiomorphs, Mat1-1, which encodes the transcripts Mat1-1-1, Mat1-1-2 and Mat1-1-3, and Mat1-2, which encodes the transcripts Mat1-2-1 and Mat1 -2-2 (Kanamori et al., 2007). The MAT1-1-3 and MAT1-2-2 genes have, respectively, the ORFs MAT1-1-3a and MAT1-1-3b, and MAT1-2-2a and MAT1-2-2b,

determined by alternative splicing (Kanamori et al., 2007). The predicted protein from Mat1-1-1 transcript includes an α-box motif, which is conserved in many Ascomycota fungi (Turgeon, B.G, 1998). This gene defines the idiomorph Mat1-1, and evidences support that its α protein is a transcription factor that binds to DNA via the conserved α domain. Its corresponding gene in *Saccharomyces cerevisae* Meyen ex E.C. Hansen is MATα1p, a transcriptional co-activator essential for the expression of mating type-specific genes as well as pheromones and pheromone receptors (Johnson, A.D.,1995). On the other hand, MAT1-2-1, characteristic of idiomorph Mat1-2 (Turgeon, B.G, 1998), predicts protein that contains a DNA binding GAM-box motif and is conserved in many Ascomycotas. The HMG (High Mobility Group) domain presents DNA affinity normally found in non-histone chromosomal proteins and transcription factors.

Sexual recombination in *M. oryzae* was considered extremely rare because rice isolates collected in the field are considered of low sexual fertility (Kato & Yamaguchi, 1982; Nottéghem & Silué, 1992). In addition, mating type studies have shown that only one mating type predominates in a particular rice region (Kato & Yamaguchi, 1982, Yaegashi & Yamada, 1986). However, there are reports about the presence of highly fertile and hermaphrodite isolates and isolates carrying both mating types, in regions close to the center of rice origin suggesting that sexual recombination may contribute to genetic variability of this fungus (Saleh et al., 2014). Despite the occurrence of both fertile idiomorphs, the structures of sexual reproduction, (perithecia), were not found under natural conditions. However, the same artificially manipulated idiomorphs successfully produced perithecia (Hayashi et al., 1997, Kumar, 1999; Mekwatanakarn et al., 1999).

Studies, about pairing compatibility and sexual characteristics have been carried out worldwide, with the objective of investigate the natural occurrence of perithecia, but until very recently, the sexual structure has not been found in natural conditions. Without this

evidence it is still hard to explain the genetic variability of this pathogen in rice fields. Nevertheless, the search for this evidence needs to be continuous, since natural occurrence of the sexual reproduction would direct impact rice blast management in the field, especially on the resistance durability of rice cultivars as well as the management of fungicide.

The distribution of idiomorphic types is an indicative that may aid in determining the existence of the sexual phase in the field, or, at least, of its predisposition to it. A single gene with two idiomorphs, MAT1-1 and MAT1-2, controls paring between compatible types in *M. oryzae*, an essential phase for sexual reproduction cycle to occur and results in progenies of both M-mating types in equal proportion (Noteguem & Silué, 1992). Therefore, if the proportion of mating types seems to be in equilibrium, there is a high possibility that sexual reproduction is going on in the population. The mechanisms of variability, as well as the occurrence of sexual recombination of a pathogen direct influence the inheritance and distribution of a characteristic into the progeny, such as the distribution of fungicide resistant among individuals in the population. For example, in the case of resistance to QoIs the main mechanism is asexual (Gisi et al., 2002). The difficulties raised by migrations and / or selective effects, due to differences in fitness between the two mating types, may have led to the establishment of a single mating type in invaded areas, imposing an obligatory asexual mode of reproduction on pathogen populations outside from the center of origin (Saleh et al., 2012).

Thus, the objective of this project was to detect if both mating types are present among *M. oryzae* isolates collected from commercial rice fields, in Tocantins state, a "hot spot" for blast disease.

Materials and Methods

Origin of the isolates

In all, 98 *M. oryzae* isolates were obtained from rice plants with blast symptoms in fields collected in the northern Brazilian state of Tocantins, TO (n=98) during the planting seasons of years 2015 to 2016. The TO is at 10-11° lat. S, with Köppen climate class Aw (tropical wet savanna). Monosporic isolates were obtained according to methodology described by D'Ávila et al. (2016). Monosporic cultures were further grown on filter paper and stored as described elsewhere (Valent et al. 1986).

DNA extraction and mating type characterization

DNA from isolates grown on PDA for seven days was extracted by adapting the method described in Dellaporta (1983). The modification was the inclusion of repeated washings with 70% ethanol at the end of the extraction process. The dried DNA was re-suspended in water and its concentration determined using a Nanodrop spectrophotometer (Nanodrop 2000 Thermo Scientific) and adjusted to 30 ng.μL^{-1}.

The frequencies of MAT1-1 and MAT1-2 idiomorphs were assessed by amplicons produced after PCR using the primers MAT1-1F, MAT1-1R, MAT1-2F and MAT1-2R (Takan et al. 2012). PCR was performed in a final volume of 10 µl using the QIAGEN Multiplex PCR Kit as described by the manufacturer (Qiagen Inc.). The 10 µl of PCR product was homogenized with four microliters of BlueJuice™ Gel Loading Buffer (Invitrogen) and subjected to electrophoresis in 1.5% agarose gel in 1 × TBE buffer. Fragments were compared with a 100 bp DNA ladder (Invitrogen) and scored as either MAT1-1 (960 bp) or MAT1-2 (802 bp) (Takan et al. 2012). DNA of isolates KA-3

(MAT1-1) and GUY 11 (MAT1-2) were used as control because its mating idiomorphs had been previously determined (Leung et al. 1988).

Results

From different rice fields, a subsample of no more than 12 isolates per field was studied, totaling 98 isolates. A single amplicon matched with MAT1-2 idiomorph in 91 isolates and seven isolates matched the MAT1-1 idiomorph (Figure 1). Among the seven isolates identified as MAT1-1, five of them had the cytocrome b gene sequenced and did not show the presence of mutation, although all seven isolates were phenotypically less sensitive to all four fungicides tested (Azoxystrobin, tryciclazol, trifloxystrobin+tebuconazole and tebuconazole).

Discussion

This is the first report on the occurrence of *M. oryzae* isolates collected from commercial rice fields identified as MAT1-1 in Brazil (figure 1). Previous studies evaluating the distribution of mating types demonstrated the predominance of MAT1-1 in South America (except in Brazil), 100% MAT1-1 in Europe, MAT1-2 in Asia, while African isolates showed a balance of mating types, which characterizes the possible occurrence of sexual reproduction in that continent (Yamaguchi et al., 2000). In a more recent study, Saleh et al. (2012) analyzed the global geographic distribution of mating types of 3,800 *M. oryzae* isolates. Both types of mating were found in most regions of the world, with the exception of the Mediterranean region (MAT1-1 only). In Brazil, a recent study conducted by Peixoto, L.F. (2014) analyzed the frequency of mating types from a sample of 208 isolates, collected from different domestic rice production regions. They found that 100% of the individuals presented the idiomorph MAT1-2. Subsequently,

D'Ávila et al. (2016) also observed 100% MAT1-2 in a population sample of 224 isolates from southern in Brazil (RS and SC states).

In contrast to the above results, among the 98 isolates evaluated in this study, 7.15% were identified as the idiomorph MAT1-1. Although a low frequency of MAT1-2 was observed, we are facing the first necessary requirement for pairing and recombination, raising the hypothesis of possible occurrence of sexual reproduction, or at least recognizing a high genetic variability for this characteristic in *M. oryzae*. It is well known that the presence of both idiomorphs is not the only issue for sexual reproduction, other characteristics are necessary to complete a sexual cycle such as sexuality (male, female, hermaphrodite) and fertility (capacity to form complete and viable perithecia) of the isolates, as well as the production of viable ascospores. In addition, all these characteristics are genetically expressed and segregate independently. The complexity of events for the combination of these characteristics, accompanied by high rates of independent mutations can lead to the sterility of individuals, thus directly implicating the occurrence of recombination in *M. oryzae* (Leslie & Klein, 1996).

Many studies around the globe used matching assays to identify sexual compatibility. However, *in vitro* pairing is often not appropriate for detecting mating types, since in order to reveal the result, it is dependent on other characteristics such as sexuality and fertility of field isolates, which may influence the rate capacity / crossing ability (Galbieri & Urashima, 2008). Low crossing rates may thus reveal a false, underestimated result as to the identity of the idiomorph. This may explain the difficulty encountered in obtaining perithecium formation *in vitro*. Even in the presence of opposing and fertile mating types, there are limitations to the occurrence of natural events under controlled environment, such as sexual reproduction. Therefore, it is not possible to rule out the possibility that when in a native environment, sexual recombination does occur, when in

the presence of opposite idiomorphs. In addition, although the detected frequency of opposing idiomorphs is low in *M. oryzae* populations from rice plants, we should consider the possibility of match to occurring in secondary host plants in the field, where we commonly find symptomatic blast (Maciel et al., 2014) plants. It is known that isolates from rice have the capacity to infect other grasses but isolates from other grasses do not infect rice. This adaptive characteristic may confer a competitive advantage on these individuals (Couch et al., 2005).

In population genetics studies it is common to use geographic criteria to define subpopulations. When geographic subpopulations were analyzed, the genetic profiles were similar and there was weak genetic differentiation among them, ruling out the geography as factor shaping the population. It is interesting to note that the MAT1-1 isolates were detected within a single field, and that the ratio of MATI-1 to MAT1-2 was 1:3. This ratio indicates the co-habitation of these two compatible types. This result is unprecedented and opposed to many reports found in the literature.

According to Saleh et al., 2014, in Asia, the center of origin of *M. oryzae*, there are two *M. oryzae* diversity centers, both located on the Himalayan mountain ranges: one in southern China-Laos-North of Thailand and the other at west of Nepal. Sexual reproduction is thought to have persisted only in southern China-Laos-North of Thailand, identified as the possible center of origin of all *M. oryzae* populations of rice, and the origin of most *M oryzae* migrations to other continents. Corroborating with previous results, the same authors identified three genetic groups worldwide. They suggested that clonal populations found outside Asia originated from clonal Asian populations that existed before the worldwide migrations, and that the absence of sexual reproduction in all areas outside Asia can be explained by migrations of originally native clonal populations. It should be noted that this study did not count with isolates from Brazil, which is the

largest rice grower outside the Asian continent (Malavolta, 2009). Scheuermann et al. (2012), analyzing data on genetic diversity in different countries, observed that some sites presented low genetic diversity with considerable pathogenic diversity, while in other sites presented had a greater genetic and pathogenic diversity. Thus, they suggested that different mechanisms act in order to generate genetic variability in *M. oryzae*. Gonçalves et al. (2016) tracking the genetic variability of *M. oryzae* in Brazil used 18 microsatellite markers (SSR) and found 19 genetic populations representative of the Brazilian rice producing regions. The authors indicated that there are grouping trends determined by the isolate and the environment.

Previous studies in Brazil (Dávila et al., 2016; Peixoto, L. F, 2014) detected the presence of only one idiomorph, MAT1-2 in the studied populations, excluding the possibility of sexual reproduction. These populations presented pathogenic variability and each of them presented the predominance of one pathotype group (IA, IB and IH), with predominance of a single pathotype in each environment sampled. Therefore, the genetic variability and pathogenic of *M. oryzae* in each of those environments tested did not depend on the recombination of genetically distinct individuals. Therefore, the results of this study, that refer to the presence of opposing mating type idiomorphs in the same region (TO), as those referring to high genetic and pathogenic variability (D'Ávila et al., 2016; Gonçalves et al., 2016), bothsuggest that the high genetic diversity observed may be conditioned perhaps by different mechanisms of reproduction. Several authors, in an attempt to explain the high genetic variability of *M. oryzae*, consider that the mechanisms of variability involved in a given population are sexual recombination, parasexuality, and the occurrence of mutations (Kistler & Miao, 1992). Parasexual recombination represents an alternative to the sexual cycle, when sexual cycle rarely occurs (Zeigler, 1998). Zeigler et al. (1997) observed the formation of anastomose among isolates from the field. They

observed it in Petri dishes, where the parental colonies meet each other, with the identification of characteristics common to both parental, indicating the occurrence of recombination. This finding is an indication that parasexual recombination is occurring in nature, and possible responsible for the detection of new pathotypes. In *M. oryzae*, pathogenic diversity is monitored, traditionally, through the detection of physiological races or pathotypes, that is, the combinations of the reaction of eight differentiating host genotypes, after spray inoculation of conidia of a given isolate. In addition, the analyses of a given population with molecular markers, can infer genetic diversity in an attempt to cluster the similar individuals, according to their genetic profile. This high phenotypic and genetic variability of *M. oryzae* may have an impact on the natural selection and prevalence of individuals less sensitive to fungicides. Thus, because of the supposed different reproduction mechanisms in *M. oryzae* populations, it is possible to observe the distribution of resistant individuals in natural populations, under field conditions. Resistance to QoI'S, which inhibit cytochrome bc1 gene, is a characteristic encoded by mitochondrial genetic material. There are several differences between the pattern of nuclear (Mendelian) and cytoplasmic (extra-nuclear, non-Mendelian) inheritance. In the Mendelian pattern, recombination and allele pairs are independently, whereas segregation of mitochondrial genes always occurs during mitotic cell division (clonal reproduction).

Inheritance of resistance to QoI segregates as 0:1 proportion in one single progeny, and 1:1 proportion at the population level. The rearrangement in mitochondrial genomes does not affect metabolic processes and, therefore, there may be no cost for adaptation (fitness). Alternatively, this rearrangement may not be deeply expressive or lethal, but it may still interfere with some processes such as cellular aging (Gisi et al., 2002). The evolution of resistance to QoI fungicides, at the individual level, probably, depends on the onset of mitotic events such as mutation rate and repair mechanisms. However, the

evolution of QoI resistance at the population level becomes the result of recurrent mutation, recombination and migration of the pathogen, as well as the selection process imposed by the fungicide (Gisi et al., 2002).

Based on our results, resistance to QoIs showed no relation to the occurrence of opposing mating type idiomorphs, since the MAT1-1 individuals did not present mutations conditioned by QoIs. It was evident that the assexual recombination is the main *M. oryzae* mechanism of reproduction; the prevalence of individuals resistant to fungicide QoI is the result of its clonal process of reproduction in the populations; the low frequency of the isolates sensitive to the QoI and MAT1-1 may be related to assexual recombination. However, they may also perpetuate the fungicide resistance detected in individuals MAT1-2, through a Mendelian pattern of inheritance, if the sexual cycle is completed; MAT1-2 individuals, on becoming resistant, may have lost competitive advantage over MAT1-1 individuals, who may possibly remain in alternative hosts.

Although the occurrence of sexual reproduction in M. oryzae from rice fields is only infered, presence of both idiomorphs may help to explain the challenges for the management of rice blast in Brazil, which is increasingly difficult, due to the high variability of this pathogen and the dependence of disease management in chemical control, with products with the same mechanism of action.

Literature Cited

CONAB, 2018. Acompanhamento de safra brasileira de grãos. Available at: www.conab.gov.br. Accessed on February, 2018.

Consolo, V. F., et al. "Mating-Type Distribution and Fertility Status in Magnaporthe Grisea Populations from Argentina." Mycopathologia, vol. 160, no. 4, 2005, pp. 285–290., doi:10.1007/s11046-005-4333-3.

Couch, B.C.; Kohn, M.L. 2002. A multilocus gene genealogy concordant with host preference indicates segregation of a new species, Magnaporthe oryzae, from M. Grisea. Mycologia, New York, v.94, n.4, p. 683–693.

Couch, B.C.; Fudal, I.; Lebrun, M.-H.;Tharreau, D.; Valent, B.; van Kim, P.; Nottéghem, J.; Kohn, L.M. 2005. Origins of Host-Specifc Populations of the Blast Pathogen Magnaporthe oryzae in Crop Domestication with Subsequent Expansion of Pandemic Clones on Rice and Weeds of Rice. Genetics, Bethesda, v.170, p.613-630.

D'Ávila, L.S., Lehner, M.S.,Filippi, M.C.C., Scheuermann, K. K., Del Ponte, E. M. 2016. Genetic structure and mating type analysis of the Pyricularia oryzae population causing widespread epidemics in southern Brazil. Tropical plant pathology DOI: 10.1007/s40858-016-0101-9.

Dellaporta, S.L.; Wood, J.; Hicks, J.B. 1983. A plant DNA minipreparation version II. Plant Mol Biol Report 1:19–21.

Galbieri, R.; Urashima, A.S. 2008. Caracterização, compatibilidade e ocorrência de reprodução sexual entre isolados de Pyricularia grisea de diferenteshospedeiros. Summa Phytopathologica, Botucatu, v.34, n.1, p.22-28.

Gisi U, Sierotzki H, Cook A and McCaffery A. 2002. Mechanisms influencingthe evolution of resistance to Qo inhibitor fungicides. Pest Manag Sci 58:859–867.

Gonçalves, F.J., Filippi, M.C.C., Silva Lobo, V.L., Araújo, L.G., Silva, G.B., Guedes Coelho, A.S., Prabhu, A.S. 2016. Polymorphism detection by microsatellite markers in a Magnaporthe oryzae population from different geographical areas of Brazil. J Phytopathol. doi:10.1111 /jph.12485.

Hayashi, N.; Li, Y.C.; Li, J.L.; Naito, H. 1997. In vitro production on rice plants of perithecia of Magnaporthe grisea from Yunnan, China. Mycological Research, Cambridge, v.101, n.11, p.1308-1310.

Johnson, A.D. 1995. Molecular mechanisms of cell-type determination in budding yeast. Current Opinion in Genetics & Development, Baltimore, v.5, p.552-558.

Kanamori, M.; Kato, H.; Yasuda, N.; Koizumi, S.; Peever, T. L.; Kamakura, T.; Teraoka, T.; Arie, T. 2007. Novel mating type-dependent transcripts at the mating type locus in Magnaporthe oryzae. Gene, Orlando-Amsterdam, v.403, p.6-17.

Kato, H.; Yamaguchi, T.; Nishihara, N. The Perfect State of Pyricularia oryzae Cav. in Culture. Annals of the Phytopathological Society of Japan, Tokyo, v.42, p.507-510, 1976.

Kistler, H.C., Mião, V.P.1992. New modes os genetic Chang in filamentous fungi. Annual Rewiew Phytopathology, Palo Alto, v.30, n.1, p.131-152.

Kumar J, Nelson RJ, Zeigler RS. 1999. Population structure and dynamics of Magnaporthe grisea in the Indian Himalayas. Genetics 152: 971–984.

Leung, H., Borromeo, E.S., Bernardo, M.A, Nottéghem, J.L. 1998. Genetic analysis of virulence in the rice blast fungus Magnaporthe grisea. Phytopathology, Saint Paul, v.78, p.1227-1233.

Maciel, J. L. N., Ceresini, P. C., Castroagudin, V. L., Kema, G. H. J., and McDonald, B. A. 2014. Population structure and pathotype diversity of the wheat blast pathogen *Magnaporthe oryzae* 25 years after its emergence in Brazil. Phytopathology 104:95-107.

Mekwatanakarn, P., Kositralana, K., Phromraksa, T., Zeigler, R.S.1999. Sexually fertile Magnaporthe grisea rice pathogens in Thailand. Plant Disease, Saint Paul, v.83, p.939-946.

Moreira, S.I.; Ceresini, P.C.; Alves, E. 2015. Reprodução Sexuada em Pyricularia oryzae. Summa Phytopathologica, v.41, n.3, p.175-182.

Nottéguem, J.l., Silué, D. 1992. Distribution of mating type alleles in Magnaporthe grisea populations pathogenic on rice. Phytopathology, Saint Paul, v.82, p.421-424.

Onaga, G., Wydra, K., Koopmann, B., Ser´ e, Y., and von Tiedemann, A. 2015. ´ Population structure, pathogenicity, and mating type distribution of Magnaporthe oryzae isolates from East Africa. Phytopathology 105:1137-1145.

Peixoto LF (2014) Identificação da compatibilidade, sexualidade, fertilidade e AVR1-CO39 em populações de Magnaporthe aryzae coletadas em lavouras de arroz no Brasil: Universidade Federal de Goiás, MSc thesis.

Prabhu, A.S.; Filippi, M.C.; Silva, G.B. Dinâmica da População do Patógeno. In: Prabhu, A.S.; Filippi, M.C.; Silva, G.B. Brusone em arroz: Controle genético, progresso e perspectivas. Santo Antônio, de Goiás: Embrapa Arroz e Feijão, 2006. p. 388.

Saleh, D., Milazzo, J.L., Adreit, H., Fournier, E., Tharreau, D. 2014. South-East Asia is the center of origin, diversity and dispersion of the rice blast fungus, Magnaporthe oryzae. New Phytologist, 201:1440–1456 doi: 10.1111/nph.12627.

Saleh D, Xu P, Shen Y, Li CY, Adreit H, Milazzo J, Ravign e V, Bazin E, Nott eghem JL, Fournier E et al. 2012. Sex at the origin: an Asian population of the rice blast fungus Magnaporthe oryzae reproduces sexually. Molecular Ecology 21: 1330–1344.

Shen, W.C.; Bobrowicz, P.; Ebbole, D.J. 1999. Isolation of pheromoneprecursor genes of Magnaporthe grisea. Fungal Genetics and Biology, Amsterdam, v.27, p.253-263.

Stuckenbrok, E.H.; Mcdonald, B.A. 2008. The Origins of Plant Pathogens in Agro-ecosystems. Annual Review of Phytopathology, Palo Alto, v.46, p.75-100.

Takan JP, Chipili J, Muthumeenakshi S, Talbot NJ, Manyasa EO, Bandyopadhyay R, Sere Y, Nutsugah SK, Talhinhas P, Hossain M et al. 2012. Magnaporthe oryzae populations adapted to finger millet and rice exhibit distinctive patterns of genetic diversity, sexuality and host interaction. Molecular Biotechnologies 50: 145–158.

Turgeon, B.G. 1998. Application of mating type gene technology to problems in fungal biology. Annual Review of Phytopathology, Palo Alto, v.36, p.115-137.

Valent B, Crawford MS, Weaver CG, Chumley FG (1986) Genetic studies of fertility and pathogenicity in Magnaporthe grisea (Pyricularia oryzae). Iowa State J Res 60:569–594.

Yaegashi, H.; Yamada, M. 1986. Pathogenic race and mating type of Pyricularia grisea from Soviet Union, China Nepal, Thailand, Indonesia and Colombia. Development and Nuclear Behaviour in Pyricularia. Phytopathology, St. Paul, v.66, p.122-166.

Zeigler R.S. 1998. Recombination in Magnaporthe grisea. Annu Rev Phytopathol 36:249–275.

Zeigler, R.S.; Scott, R.P., Leung, H., Bordeos, A.A., Kumar, J., Nelson, R.J.1997. Evidence of parasexual exchange of dna in the rice blast fungus challenges its exclusive clonality. Phytopathology, St. Paul, v.87, p.284-294.
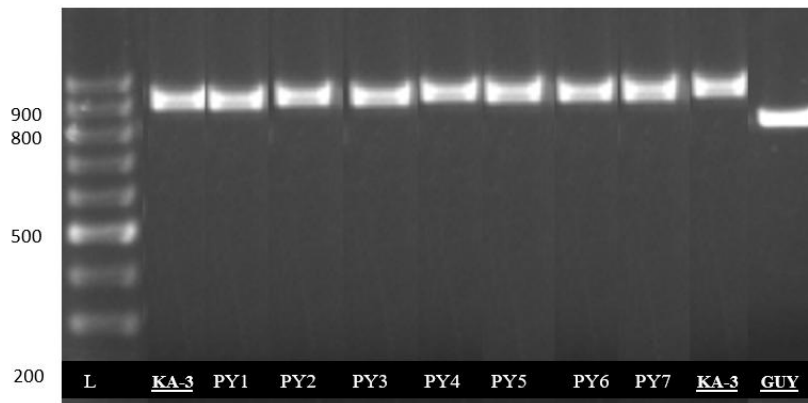
Figure 1: Polymerase Chain Reaction, on agarose gel, for identification of idiomorphs *Mating types* (*MAT1-1* ou *MAT1-2*). L: 100 bp DNA ladder (Invitrogen) and scored as either MAT1-1 (960 bp) or MAT1-2 (802 bp) (Takan et al. 2012). DNA of isolates KA-3 (MAT1-1) and GUY 11 (MAT1-2) were used as control because its mating idiomorphs had been previously determined (Leung et al. 1988). PY1, PY2, PY3, PY4, PY5, PY6 and PY7: Isolates MAT1-1 from TO.

Supporting information

Disease note

First report of the occurrence of isolates of *Magnaporthe oryzae* carrying the two idiomorphs MAT1-1 and MAT1-2 in Brazil.

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Magnaporthe oryzae is notable for expressing a large number of pathotypes, and new forms appear in the field when new resistant rice cultivars are introduced (Prabhu et al., 2009), eventhough it has so far been found that the rice blast fungus reproduction is assexual (clonal reproduction). Sexual recombination in *M. oryzae* was considered extremely rare because the perfect stage has never been found in nature, outside the center of origin of rice. Furthermore, rice isolates collected in the field presented low sexual fertility and studies on the distribution of compatible types showed that only one *mating type* predominates in a particular rice region (Saleh et al., 2012). We studieds 98 leaf isolates of *M. oryzae*, collected on commercial fields in the State of Tocantins (TO, Northern Brazil), in the 2015/2016 crop. DNA from isolates grown on PDA for seven days was extracted by adapting the method described in Dellaporta (1983), modified by the inclusion of repeated washings with 70% ethanol at the end of the extraction process. The dried DNA was re-suspended in water and its concentration determined using a Nanodrop spectrophotometer (Nanodrop 2000 Thermo Scientific) and adjusted to 30 ng.μL^{-1}. The frequencies of MAT1-1 and MAT1-2 idiomorphs were assessed by amplicons produced after PCR using the primers MAT1-1F, MAT1-1R, MAT1-2F and MAT1-2R (Takan et al.

2012). PCR was performed in a final volume of 10 µl using the QIAGEN Multiplex PCR Kit as described by the manufacturer (Qiagen Inc.). The 10 µl of PCR product was homogenized with four microliters of BlueJuice™ Gel Loading Buffer (Invitrogen) and subjected to electrophoresis in 1.5% agarose gel in 1 × TBE buffer. Fragments were compared with a 100 bp DNA ladder (Invitrogen) and scored as either MAT1-1 (960 bp) or MAT1-2 (802 bp) (Takan et al. 2012). DNA of isolates KA-3 (MAT1-1) and GUY 11 (MAT1-2) were used as controls because their mating idiomorphs had been previously determined (Leung et al. 1988). A total of 98 *M. oryzae* isolates were obtained, from a total of xxx fields from TO. The number of isolates varied per field, but generally it was lower than 12 isolates. A single amplicon matching the MAT1-2 idiomorph was produced for the 91 isolates, while seven isolates had the MAT1-1 idiomorph. This is the first study to detect *M. oryzae* rice isolates carrying the MAT1-1 idiomorph in Brazil. Even if found in a low frequency, the study shows that sexual reproduction is possible in the Northern rice producing regions in Brazil. The study also supports the conditions for high genetic variability in *M. oryzae* in Northern Brazil, similar to the one reported in rice center of origin.

References:

Prabhu et al., 2009.

Saleh et al., 2012.

Takan et al., 2012.

Leung et al., 1988.

GENERAL CONCLUSIONS

1. The population of *Magnaporthe oryzae* affecting rice in Brazil present levels of phenotypic sensitivity to fungicides QoI, DMI, MBI, but the isolates the northerm showed lower sensitivity compared to the southern region.

2. We identified that more than 70% of the Tocantins isolates are G143A mutants with high resistance to fungicides.

3. Analyzing the evolution of resistance in time we observe that a few years after (1997) the introduction of QoI´s in Brazil we have observed isolates of *M .oryzae* less sensitive to azoxystrobin.

4. Occurrence of Idiomorphs MAT1-1 and MAT-2 of *M. oryzae* in the state of Tocantins supports the conditions for high genetic variability in *M. oryzae* in Northern Brazil, similar to the one reported in rice center of origin.

5. Anti-resistance strategies should be immediately adopted to avoid an increase of resistant isolates in *M. oryzae* populations.