SHORT COMMUNICATION / *COMUNICAÇÃO*

Sensitivity of *Fusarium graminearum* causing head blight of wheat in Brazil to tebuconazole and metconazole fungicides

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ABSTRACT

Fusarium head blight (FHB) of wheat, is caused by fungal populations belonging to the *Fusarium graminearum* species complex. Although triazole fungicides are widely used in FHB management, information about the sensitivity of *F. graminearum* populations to such fungicides is scarce, particularly in Brazil. This work aimed at determining the sensitivity of pathogenic populations of *F. graminearum* to metconazole and tebuconazole. Fungal isolates were obtained from diseased wheat heads collected in commercial fields at the northern region of Rio Grande do Sul state, Brazil, along the 2009 and 2010 growing seasons. Fifty isolates obtained from eleven locations were grown in media amended with increasing fungicide dosages and the effective concentration reducing mycelial growth to 50% (EC_{50}) was determined. The median of the EC_{50} values was 0.001 mg/L (<0.001-0.152 mg/L) and 0.037 mg/L (<0.001-0.324), for tebuconazole and metconazole, respectively. Cross-resistance was detected based on significance of correlation analysis (r_s = 0.46; P < 0.0001) between the log-transformed EC_{50} of the two triazoles. The mean EC_{50} for a group of ten isolates classified as "more sensitive" differed statistically from the ten isolates representing the "less sensitive" group. Nevertheless, these groups did not differ in terms of mycelial growth on PDA in the absence of fungicides. It was concluded that pathogenic populations of *F. graminearum* are sensitive to the triazoles at various levels due to the intrinsic characteristics of the isolates or to selection pressure by the increasing fungicide use.

Keywords: *Gibberella zeae*, *Triticum aestivum*, fungicide resistance, Fusarium head blight.

Fusarium graminearum species complex (*Fg* complex) comprises more than a dozen lineages or phylogenetic species (Sarver et al., 2011) and is the main causal agent of Fusarium head blight (FHB) of small grain in Brazil and around the world (Goswami and Kistler, 2005; Astolfi et al., 2012). In the Americas, *Fusarium graminearum sensu stricto* is the dominant species of the *Fg* complex associated with head blight of wheat (Alvarez et al., 2011; Schmale et al., 2011; Astolfi et al., 2012). The pathogen produces deoxynivalenol (DON), a major trichothecene mycotoxin that acts as a virulence factor (Proctor et al., 1995; Goswami and Kistler, 2005; Hallem-Adams et al., 2011) and may accumulate in grains at levels considered unsafe for both human and animal consumption (Pieters et al., 2002).

The most effective control measures employed to reduce FHB impact are the use resistant cultivars and fungicide application during flowering stage (Hollingsworth et al., 2008; Willyerd et al., 2012). Fungicides of the demethylation inhibitors (DMIs) group, which includes triazoles, inhibit the C-14 α-demethylation of 24-methylenedihydrolanosterol, a precursor of ergosterol in fungi (Brent, 1995). Tebuconazole, metconazole and prothioconazole are among the azole fungicides with higher efficacy in suppressing FHB symptoms and reducing mycotoxin levels in mature grain (Paul et al., 2008).

Currently, triazoles are the main fungicides used in FHB control in the major wheat producing countries (Paul et al., 2008; Becher et al., 2011).

In Europe and Asia, the increasing usage of triazoles in FHB management is suspected to have led to selection of less sensitive isolates of *F. graminearum* (Klix et al., 2007; Yin et al., 2009; Becher et al., 2010). Fungal resistance to triazoles is associated with mutations in the 14-α-demethylase (*CYP51*) gene and overexpression of the ATP-binding cassette transporters (Ma and Michailides, 2005; Liu et al., 2010). As discussed by Klix et al. (2007), the decline in the sensitivity to triazoles of *F. graminearum* owing to selection is not generally considered a key determinant of the relatively low fungicide efficacy against FHB under field conditions. However, it is well known from the literature that failures in disease control have been associated with the decline in sensitivity of other important pathogens such as *Monilinia fructicola* (Chen et al., 2012), *Venturia inaequalis* (Pfeufer & Ngugi, 2012) and *Mycosphaerella graminicola* (Leroux et al., 2007) due to indiscriminate use of triazoles.

Commercial mixtures that include a combination of two triazoles or a single triazole have shown the highest control efficacy in uniform trials to reduce FHB and DON levels (Paul et al., 2008). When multiple triazoles are used, it may be critical to assess cross-resistance when they have

similar mode of action (Karaoglanidis & Thanassoulopoulos, 2003; Klix et al., 2007). Hence, the determination of the sensitivity of the pathogenic population to the most used fungicides in disease management is a first step towards developing an anti-resistant strategy (Lu et al., 2012).

In Brazil, literature data on *F. graminearum* sensitivity to fungicides is scarce. Spolti et al. (2012) observed a distinct sensitivity to tebuconazole when comparing phenotypic traits of two pairs of isolates belonging to the *Fg* complex, each possessing a distinct trichothecene genotype; *F. meridionale* isolates possessing a nivalenol genotype were much more sensitive than the *F. graminearum* isolates possessing a deoxynivalenol genotype. The objective of this work was to determine the sensitivity to tebuconazole and metconazole in a regional population of *Fg* complex isolates obtained from symptomatic wheat heads and to check whether fitness costs and cross-resistance were involved.

In 2009 and 2010, commercial wheat fields at 11 municipalities in the northern wheat-growing regions of Rio Grande do Sul State, Brazil, were inspected at the time of milk and soft dough stages of grain development. In each field, wheat heads exhibiting FHB symptoms were randomly collected and taken to the laboratory. Although there was no information available about the history of fungicide spray on these fields, it was assumed that triazoles fungicides had been sprayed on the areas along the years since these products are generally used regularly for managing wheat disease in this region.

In the laboratory, symptomatic spikelets were excised from the heads and incubated under moist conditions at $25\pm2^{o}C$ during 48 hours. Fungal isolations were made from colonies resembling *F. graminearum*. Monosporic isolates were obtained from each individual wheat head. Isolates obtained from a single field were taken from distinct wheat heads. The isolates were identified to species using primer set Fg16F/Fg16R primer set, which is polymorphic for the *F. graminearum* species complex. Additionally, isolates were identified to trichothecene genotype - the two acetylates of deoxynivalenol (3-ADON and 15-ADON) and nivalenol (NIV) - using primers targeting portions of the *Tri*3 and *Tri*12 toxin genes. These identifications were made as described in previous studies (Scoz et al., 2009; Astolfi et al., 2012). A total of 50 isolates was identified and represented the regional *F. graminearum* complex population as defined in this study. The municipalities where the isolates were sampled and number of isolates from each municipality (n) were as follows: Carazinho (n=8), Coronel Barros (n=3), Coxilha (n=10), Cruz Alta (n=2), Ernestina (n=6), Lagoa Vermelha (n=1), Não-Me-Toque (n=5), Passo Fundo (n=4), Santa Bárbara do Sul (n=3), Tio Hugo (n=4) and Victor Graeff (n=4).

Fungicide sensitivity was quantified by measuring the radial growth of the cultures on PDA (potato dextrose agar) media (Becher et al., 2010; Tateishi et al., 2010) amended with increasing concentrations of tebuconazole

(Tebuconazole Nortox 200 EC, Nortox S.A.) or metconazole (Caramba 90, BASF S.A.) (0.0; 0.01; 0.05; 0.1; 1 and 10 mg/L). The fungicides were added to PDA media in three replicates each. After 5 days of incubation at 23°C in the dark, colony diameters were measured in two perpendicular directions and the original plug diameter was subtracted. Effective concentration leading to a reduction in mycelial growth was determined for each fungicide concentration relative to the check treatment (PDA not amended with the fungicides). The experiment was repeated once in time.

Effective concentration (mg/L) leading to a 50% reduction (EC_{50}) of mycelial growth was calculated on the basis of linear regression analysis of log-transformed fungicide efficiency and the log10 transformed fungicide concentrations (Becher et al., 2010). Bartlett test was used to assess variance homoscedasticity and the data from the two experiments were combined ($P < 0.05$) for analysis (Chen et al., 2012).

Cumulative frequency plots of the EC_{50} were produced and the median and mean values were calculated for each fungicide (Pfeufer & Ngugi, 2012). The isolates were split into two groups of ten isolates. One group was composed by the isolates with the highest EC_{50} and represented the "less sensitive" and ten with the lowest EC_{50} represented the "more sensitive" phenotypes (Pereira et al., 2012). The non-parametric Wilcoxon test was used to assess the difference between the two strain groups and between the two fungicides (Shapiro-Wilk test $P < 0,001$).

Mycelial growth in pure PDA medium, as described previously, was used to detect fitness costs associated with the decline in fungicide sensitivity. Significant difference between the two groups of ten isolates (the more and less sensitive groups) was assessed using t-test (Becher et al., 2010). Cross-resistance was evaluated by the significance of the Spearman correlation coefficient (r_s) between the log transformed EC_{50} values of tebuconazole and metconazole. All analyses were run in SAS software (SAS Institute Inc., Cary, NC, USA).

The mean EC_{50} of the *F. graminearum* populations was 0.0136 mg/L and 0.037 mg/L for tebuconazole and metconazole, respectively. The majority of the isolates possessed a DON genotype (15-ADON) and was identified as *F. graminearum sensu stricto*. Only two isolates possessed a NIV genotype and were identified as *F. meridionale*. For the latter, they showed very low EC_{50} values (0.002 mg/L and <0.0001 mg/L) and no statistical comparison in sensitivity was made between the two species due to the small number of isolates of *F. meridionale*.

Cumulative frequency plots for the distribution of the EC_{50} values for all isolates showed a much higher frequency towards the lower EC_{50} values for both fungicides (Figure 1A). The two triazoles differed significantly in their fungitoxicity based on the mean EC_{50} ($P < 0.001$). For tebuconazole, 92% of the isolates showed EC_{50} between 0.01-0.03 mg/L, with a maximum of 0.15 mg/L. For metconazole, 85% of the isolates had EC_{50} between 0.03-
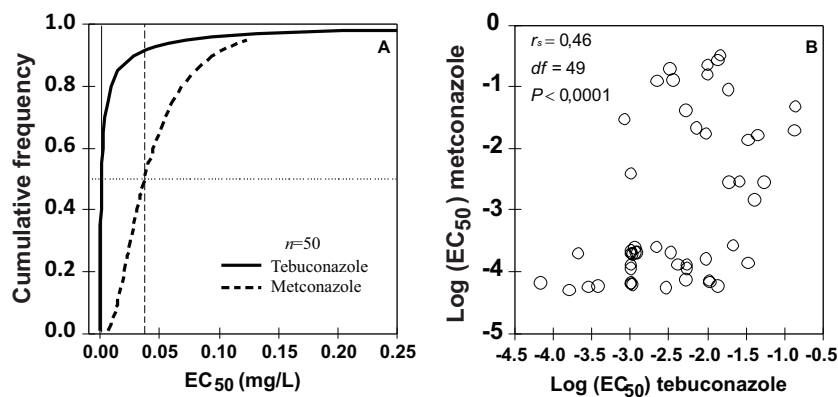
FIGURE 1 - Cumulative frequency of the effective concentration of tebuconazole and metconazole that reduces 50% of mycelial growth (EC_{50}) (**A**); and the relationship between the log-transformed values of EC_{50} of the two fungicides (**B**) for a series of 50 *Fusarium graminearum* complex isolates causing head blight of wheat originated from 11 municipalities in Rio Grande do Sul, Brazil, in the 2009 and 2010 growing seasons. Solid and dashed vertical lines in A represent the median for tebuconazole and metconazole, respectively.

0.08 mg/L, with a maximum of 0.33 mg/L (Figure 1A). A larger variability in EC_{50} values was observed at the upper limit, which determined higher variance in the sensitivity to metconazole (>MS) (Table 1). The median EC_{50} was 0.0011 mg/L and 0.0373 mg/mL for tebuconazole and metconazole, respectively, which translates into a resistance factor of 33 between these triazoles. Nevertheless, cross-resistance between these two triazoles was inferred based on the significance of the correlation analysis (r_s = 0.46, P < 0.0001) (Figure 1B).

The two groups of isolates classified as more or less sensitive differed significantly (P < 0.001) for both fungicides. The mean EC_{50} in the "more sensitive" group was 0.001 mg/L (± 0.0003) and 0.002 mg/L (± 0.0001) for tebuconazole and metconazole, respectively. The largest difference between the groups was observed for metconazole (resistance factor of 83). No difference in mycelial growth rate was observed between these groups, in the absence of fungicides (Table 2).

Although we could not to compare sensitivity at the species level because of the low number of isolates belonging to *F. meridionale*, the two isolates belonging to that species which possessed the NIV genotype, showed the lowest EC_{50} values. This corroborates with our previous findings for these genotypes (Spolti et al., 2012) and suggests that the dominant species in southern Brazil - *F. graminearum* (possessing a 15-ADON genotype) is relatively less sensitive to triazole than other species of the complex possessing a NIV genotype (Del Ponte, unpublished). Since our study was limited to two years and a relatively small sample, it will be instructive to further assess fungicide sensitivity in a larger set of isolates representing multiple species and toxigenic genotypes belonging to the complex.

The *F. graminearum* population, investigated here was less sensitive to metconazole than tebuconazole. Contrastingly, Klix et al. (2007), assessed fungicide sensitivity of *F. graminearum* isolates, based on the ascosporic germination rate, and observed a larger decline in sensitivity over time (1987 to 2004) for tebuconazole, followed by metconazole and prothioconazole. In China, Yin et al., (2009) assessed sensitivity of 40 *F. graminearum*

isolates to tebuconazole and found a EC_{50} mean of 0.248 mg/L (0.021–1.152 mg/L), which is much higher than those found in our study to both fungicides tested. However, in Japan, Tateishi et al. (2010) studied 101 isolates representing *F. graminearum* and *F. asiaticum* populations and found that 80% of isolates showed EC_{50} towards metconazole of less than 0.1mg/L, a level of sensitivity more similar to that found in the present study.

The sensitivity determined in this study constitutes a critical step for monitoring temporal and spatial changes in EC_{50} and identification of risk factors related to management practices leading to decline in sensitivity (Chen et al., 2012). The cross-resistance detected in our study suggests similarities in the mode of action between tebuconazole and metconazole (Karaoglanidis & Thanassoulopoulos, 2003). Cross-resistance may have implications in chemical control and the management strategy to avoid or minimize the risk of fungicide resistance in the population (Becher et al., 2011). In the United States, the highest FHB control efficacy was achieved with a commercial mixture of prothioconazole and tebuconazole, which may be due to a synergistic effect (Liu et al., 2011). Up to now, there are not a strong evidence that regular applications of single or mixed applications of triazoles is selecting less sensitive isolates more efficiently because of cross-resistance.

We observed that the more sensitive and less sensitive isolates did not differ in mycelial growth rate. This agrees with results of Becher et al. (2010) who did not observe changes in both mycelial growth and sporulation between resistant (induced by successive cultures on media amended with 10 mg/L of tebuconazole) and the respective wild isolate. These results are supported by the recent knowledge that sensitivity to triazoles is under the control of three paralogous genes (*cyp51A*, *cyp51B* e *cyp51C*), which are not related to mycelial growth (Liu et al., 2011).

In conclusion, *F. graminearum* complex isolates associated with FHB in Brazil are sensitive to triazoles at various levels due to the intrinsic characteristics of the isolates or to selection pressure by the increasing use of triazoles, especially metconazole, which is currently used solely or in commercial mixtures with strobilurin targeting multiple diseases of wheat in southern Brazil.

TABLE 1 - Effective concentration of triazol fungicides that reduces 50% of mycelial growth (EC_{50}) for a series of fifty *Fusarium graminearum* complex isolates associated with Fusarium head blight symptoms in wheat crops from eleven municipalities in northern region of the state of Rio Grande do Sul state, Brazil, in the 2009 and 2010 growing seasons

Fungicide	EC_{50} (mg/L)[x]	Standard error	I.C 95%[y]	Limit		Sum of squares
				Lower	Upper	
Tebuconazole	0.013 a	0.003	0.007	<0.001	0.152	0.036
Metconazole	0.036 b	0.011	0.022	<0.001	0.324	0.353

[x] Means followed by different letters in the column are significantly different according to Wilcoxon test (P<0,05).
[y] 95% confidence interval for EC_{50}.

TABLE 2 - Effective concentration of triazol fungicides that reduces 50% of mycelial growth (EC_{50}) and the radial mycelial growth rate in the absence of fungicide in PDA media for selected isolates of *F. graminearum* grouped by sensitivity levels and that were associated with Fusarium head blight symptoms in wheat crops from 11 municipalities in the northern region of the state of Rio Grande do Sul state, Brazil, 2009 and 2010 growing seasons

Sensitivity group[w]	EC_{50} (mg/L)[x]		Mycelial growth (mm/day)[z]
	Tebuconazole	Metconazole	
More sensitive (n = 10)	0.001 a (± 0.0003)	0.002 a (± 0.0001)	1.20^{ns}
Less sensitive (n = 10)	0.053 b (± 0.0395)	0.166 b (± 0.0894)	1.10
Resistance factor[y]	53	83	

[w] Each group was composed by ten highest (less sensitive) and the ten lowest (more sensitive) EC_{50} values for the 50 isolates.
[x] Mean (standard error) of EC_{50} values followed by different letters in the same column differ significantly according Wilcoxon test (P <0.05).
[y] Calculated by dividing EC_{50} of the less sensitive by the respectively more sensitive group.
[z] Daily mycelial growth rate from colonies in PDA without fungicides amended. Mean differences by t-test ($P > 0.05$).

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