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Fusarium graminearum infection during the reproductive stages of closed-flowering barley, grain deoxynivalenol contamination, and response to fungicide application

Abstract – The objective of this work was to investigate the influence of the reproductive stage of closed-flowering barley on *Fusarium graminearum* infection and to evaluate the efficacy of pre-inoculation fungicide application in controlling Fusarium head blight (FHB) and reducing grain contamination with the deoxynivalenol (DON) mycotoxin. Two experiments, in a completely randomized design with eight replicates, were conducted in a growth chamber at different temperatures. The statistical analysis was nonparametric, using the Kruskal-Wallis test. Potted plants of the BRS BRAU barley cultivar were inoculated by spraying a conidial suspension at the following three reproductive stages: anthesis halfway, grain at medium milk, and soft dough. The tebuconazole fungicide was sprayed 48 hours before pathogen inoculation. The FHB symptoms caused by the inoculation with *F. graminearum* at the medium milk stage are fully visible, although fungicide application before pathogen inoculation controls the disease, but mild at the anthesis halfway and soft dough stages. Inoculation at the three stages results in grain contamination with DON, which is reduced by pre-inoculation fungicide application.

Index terms: *Hordeum vulgare*, cleistogamous barley, mycotoxin, scab.

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Infecção por *Fusarium graminearum* durante as fases reprodutivas da cevada de florescimento fechado, contaminação dos grãos por desoxinivalenol e resposta à aplicação de fungicida

Resumo – O objetivo deste trabalho foi investigar a influência do estágio reprodutivo da cevada de florescimento fechado na infecção por *Fusarium graminearum* e avaliar a eficácia da aplicação pré-inoculação de fungicida no controle da fusariose da espiga e na redução da contaminação dos grãos pela micotoxina desoxinivalenol (DON). Dois experimentos, em delineamento completamente casualizado, com oito repetições, foram conduzidos em câmara de crescimento com diferentes temperaturas. A análise estatística foi não paramétrica, tendo-se utilizado o teste de Kruskal-Wallis. Plantas da cultivar BRS BRAU de cevada, em vasos, foram inoculadas por pulverização de suspensão de conídios, nos seguintes três estágios reprodutivos: metade da antese, grão leitoso e grão massa mole. O fungicida tebuconazol foi pulverizado 48 horas antes da inoculação do patógeno. Os sintomas de fusariose da espiga causados pela inoculação de *F. graminearum* no estágio de grão leitoso são claramente visíveis, mas são leves nos estágios de metade da antese e grão com massa mole. A inoculação nos três estágios resulta em

contaminação dos grãos com DON, que é reduzida com a aplicação de fungicida pré-inoculação.

Termos para indexação: *Hordeum vulgare*, cevada cleistógama, micotoxina, sarna.

Introduction

Barley (*Hordeum vulgare* L.) is one of the main cereal crops worldwide, ranking fourth, with 145.6 million tons produced in 2021, behind maize (*Zea mays* L.), rice (*Oryza sativa* L.), and wheat (*Triticum aestivum* L.) (FAO, 2023). In Brazil, barley reached a production of 425 thousand tons in this same year, cultivated over an area of approximately 111.5 thousand hectares (Conab, 2023), specifically in the Southern region of the country under the double cropping system in winter and spring, preceding summer-autumn crops such as soybean [*Glycine max* (L.) Merr.] and maize.

Barley grains are commonly used as livestock feed, raw materials for the brewing and distilling industries through the process of malting, and ingredients in various food products (OECD, 2015). In Brazil, the primary use of barley is for malt production in the beer industry, but the grains that fail to meet the quality standards for malt, as well as the residues from malt production, are used in animal feed (Mallmann et al., 2017).

Barley production worldwide (Wegulo et al., 2015) and in Brazil (Pereira & Chaves, 2021), however, is affected by fusarium head blight (FHB), a significant disease also known as scab. FHB in barley is caused by several *Fusarium* species, with the *Fusarium graminearum* species complex being predominant in many affected regions (Osborne & Stain, 2007; Wegulo et al., 2015), as the South of Brazil, where the *F. graminearum sensu strictu* 15-ADON genotype predominates (Pereira et al., 2021). The disease, in addition to causing direct yield losses that can reach 74% under favorable conditions for its progress, also reduces grain quality and causes the contamination of grains by mycotoxins, particularly deoxynivalenol (DON), the most significant one (Wegulo et al., 2015; Khodaei et al., 2021).

DON is produced by certain species of fungi, primarily by *Fusarium graminearum* Schw. and *Fusarium culmorum* (W.G. Sm.) Sacc., being considered a type B trichothecene mycotoxin, whose ingestion by humans can cause a range of health issues, such as diarrhea, vomiting, gastrointestinal inflammation, and even neurological symptoms (Yu & Pedroso, 2023). In livestock, the consumption of feed contaminated with DON can lead to a reduced feed intake, poor weight gain, and suppression of the immune system (Mishra et al., 2014; Wegulo et al., 2015; Yao & Long, 2020).

As the presence of DON in barley and its derivatives poses a substantial threat to human and animal health, many countries have established regulations to control the maximum permissible levels of this mycotoxin in barley grains and barley-derived products. In the European Union, for instance, the maximum allowable limit for DON in grains is 1,250 μg kg^{-1} (European Commission, 2006), whereas, in Brazil, the maximum limit for DON is 2,000 μg kg^{-1} in barley grains for subsequent processing and 1,000 μg kg^{-1} in hulled barley grains and malted barley (Anvisa, 2021).

For the control of FHB and DON, fungicides from the triazole group, including tebuconazole, have proven to be more effective than strobilurins or strobilurins mixed with triazoles (Paul et al., 2018; Feksa et al., 2019). An integrated management strategy for FHB and DON in barley also involves the use of closed-flowering cultivars (Wegulo et al., 2015).

Closed-flowering barley, also known as cleistogamous barley, is a natural variation of barley in which the palea and lemma remain tightly closed throughout the pollen-release period, a trait that promotes self-fertilization (Alqudah & Schnurbusch, 2017). When the barley spike is exposed to the environment during anthesis, it facilitates the entry of FHB-causing pathogen spores into the spike, which does not occur in closed-flowering barley, where the head is enclosed within the flag leaf sheath at the boot stage (Yoshida et al., 2008). Therefore, cleistogamy reduces the risk of pollen-mediated gene flow and provides a mechanism to escape FHB infection and DON contamination. Research has shown that closed-flowering barley cultivars are more resistant to FHB than chasmogamous cultivars (Yoshida et al., 2005, 2007).

The objective of this work was to investigate the influence of the reproductive stage of closed-flowering barley on *F. graminearum* infection and to evaluate the efficacy of pre-inoculation fungicide application in controlling FHB and reducing grain contamination with the DON mycotoxin.

Materials and Methods

The barley cultivar inoculated with *F. graminearum* was BRS BRAU, which is early maturing (132 days to maturity) and dwarf sized (76 cm tall), used for spring malting, characterized by closed flowering, and susceptible to FHB (Pereira & Minella, 2021).

From four to five barley plants, with three to four tillers each, were grown in 11 L capacity pots containing a 1:1 mixture of soil (Latossolo Vermelho, equivalent to an Oxisol) and vermiculite. Prior to sowing, 0.20 g N-P-K fertilizer (10-10-10) was added to each pot, followed by 0.10 g urea 25 days after seedling emergence. Irrigation was performed to a ensure sufficient water supply for a normal plant growth.

The used inoculum, the *F. graminearum sensu strictu* 15-ADON genotype, consisted of macroconidia produced by a combination of three previously identified isolates, i.e., UEM3976, UEM4074, and UEM4156 (Pereira et al., 2021). To achieve the mass production of the macroconidia, the isolates were cultured on 9.0 cm diameter Petri dishes containing Spezieller Nährstoffarmer agar medium (Leslie & Summerell, 2006). To stimulate sporulation, four filter paper fragments, each measuring 1.0 cm^2, were evenly placed on the surface of the culture medium. Each Petri dish was inoculated with four mycelial plugs, with a 5.0 mm diameter, arranged equidistantly; these plugs were taken from the edge of a 7 day old colony grown in potato-dextrose-agar medium, at 22±2°C, under a 12 hour photoperiod. To promote sporulation, the Petri dishes were then kept in an incubation chamber, at 22±2°C, under a 12 hour photoperiod for 14 days. Sterile water was then added to each Spezieller Nährstoffarmer plate, and the macroconidia were harvested by gently scraping the surface of the medium with a rubber spatula. The spore suspension was diluted with water to produce a suspension of 5×10^4 macroconidia per milliliter.

Two experiments were carried out. In the first, the potted plants were placed in a growth chamber at temperatures ranging from 20 to 25°C, and, in the second, from 18 to 22°C. In both cases, a 12 hour light/dark cycle was achieved using alternating 45 W 3200 K yellow LED lamps and 45 W 6500 K white LED lamps. Each experiment was repeated once.

The experimental design was completely randomized, with six treatments and eight replicates corresponding to a single pot. The treatments targeted pathogen inoculation at the following growth stages of barley: Z65, anthesis halfway (Zadoks et al., 1974), representing open flowering, when the head is enclosed within the flag leaf sheath; Z75, grain at medium milk; and Z85, soft dough. At each inoculation stage, a fungicide was applied pre-inoculation.

The applied fungicide was tebuconazole, formulated as an emulsifiable concentrate with 200 g of active ingredient (a.i.) per liter (Bayer S.A., São Paulo, SP, Brazil). Forty-eight hours before inoculation with the pathogen, the fungicide was applied 35 cm above the plant canopy at a rate equivalent to 190 g ha^{-1} a.i. (Feksa et al., 2019). The application was carried out using a CO_2-pressurized backpack and a handheld boom equipped with two 110.02 VP nozzles (Teejet Technologies, Spraying Systems Co., Springfield, IL, USA) at a rate of 200 L of the spray solution per hectare, with a spray pressure of 276 kPa, as adapted from the methods used by Yoshida et al. (2007). During the reproductive stages, 20 µL of the spore suspension at the aforementioned concentration was sprayed onto the plants of each pot. After inoculation, the pots were placed inside a mist-irrigated chamber, with 95% relative humidity, for 72 hours, at 22°C, under a 12 hour photoperiod, and, subsequently, maintained in a growth chamber under the same temperature and light conditions. Fourteen days after inoculation, FHB severity was evaluated by determining the overall percentage of symptomatic florets exhibiting premature whitening and a gray or brown discoloration of the spikelets. Upon reaching maturity at the Z92 stage, the barley heads were harvested and manually threshed, and the grains were placed in envelopes for storage, at -20°C, until the analysis of DON could be performed.

The percentage of control of FHB severity and DON contamination was defined according to D'Angelo et al. (2014), as follows:

$$\text{Control} = [(\bar{x}_{\text{untreated}} - \bar{x}_{\text{treated}})/ \bar{x}_{\text{untreated}}] \times 100$$

where $\bar{x}_{\text{untreated}}$ and $\bar{x}_{\text{treated}}$ represent the means of FHB severity (%) or DON concentration (µg kg^{-1}) for plants not treated or treated with the fungicide, respectively.

DON was quantified at the Central Laboratory of Cooperativa Agrária Agroindustrial, located in the municipality of Guarapuava, in the state of Paraná, Brazil, using the Acquity ultra-performance liquid

chromatography (UPLC)-mass spectrometry (MS)/MS system (Waters Corporation, Milford, MA, USA). Aliquots of 5.0 g of the milled samples from each replicate were extracted by shaking with 40 mL acetonitrile:water (80:20, v/v) using the HS501 laboratory shaker (IKA Werke, Staufen, Germany), at 200 rpm, for 2 hours. Subsequently, the samples were centrifuged, at 2,320 rpm, for 10 min using the Rotanta 460 R centrifuge (Hettich, Kirchlengern, Germany). The supernatant was filtered through a PVDF syringe filter, with a 0.2 µm and 13 mm pore size and diameter, respectively (Whatman, Merck KGaA, Darmstadt, Germany). The extract was diluted in ultrapure water, resulting in a 250 µL aliquot, being further diluted to 1,000 µL. Aliquots of 20 µL were injected into the UPLC-MS/MS system. The liquid chromatographic separation was performed using the Acquity UPLC C18 column, ID 50x2.1 mm, with a 1.7 µm length (Waters Corporation, Milford, MA, USA), adopting the following parameters: solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile; gradient, 10–90% solvent B over 10 min, 2 min retention of 90% solvent B, and 90–10% solvent B over 3 min; flow rate of 0.4 mL min^{-1}; and injection volume of 20 µL. The samples were analyzed using the Acquity UPLC system coupled to a triple quadrupole mass spectrometer equipped with an electrospray interface operated in the positive ion mode. After the DON analysis on UPLC, a table with the amount of deoxynivalenol in each treatment was generated, displaying the DON values in µg kg^{-1} (Almeida et al., 2016).

The descriptive analysis of the data was conducted using the boxplot function in the R software (R Core Team, 2022). Homogeneity and homoscedasticity of variances were evaluated using the Shapiro-Wilk and Bartlett tests, respectively. Because the assumptions of normality and homogeneity of variances were not met even after data transformation, the nonparametric Kruskal-Wallis test, at 5% probability, was performed using the R software (R Core Team, 2022). With the same software, Spearman's correlation coefficients between FHB severity and DON concentration in the grains were calculated.

Results and Discussion

The infection with *F. graminearum* led to FHB, whose severity ranged from 19.1 to 37.5% across the reproductive stages of cleistogamous barley. However, visible symptoms only occurred when inoculation was performed at the medium milk stage, when the head is enclosed within the flag leaf sheath and the awns are visible (Table 1). The symptoms were minimal at the anthesis halfway (when heads were unexposed) and the soft dough stages. The dispersion of FHB severity data in the first and second experiments is shown in Figure 1 A and B. The application of the fungicide before inoculation resulted in a 97% control of FHB in both experiments.

The level of DON contamination in the grains was not influenced by the reproductive stage in which *F. graminearum* inoculation was performed (Table 2). In addition, DON contamination was observed in all three reproductive stages, with values ranging from 2,404 to 3,883 µg kg^{-1} and from 1,510 to 2,035 µg kg^{-1} in the first and second experiments, respectively. The distribution of the DON contamination data in both experiments is shown in Figure 1 C and D. Overall, DON concentrations were higher in all treatments (except in one) in the first experiment, which could be attributed to temperature variations during pathogen incubation. Although the two experiments were

Table 1. Fusarium head blight (FHB) severity across three reproductive stages of the BRS BRAU cleistogamous barley (*Hordeum vulgare*) cultivar inoculated with *Fusarium graminearum*, without or with the application of the tebuconazole fungicide (190 g ha^{-1} a.i.) before pathogen inoculation, in two experiments carried out at 20–25 and 18–22°C, respectively, under a 12 hour photoperiod[1].

Reproductive stage	Fungicide	FHB severity (%)	
		Experiment 1	Experiment 2
Anthesis halfway[2]	Untreated	0.6b	0.2b
	Treated	0.4b	0.4b
Grain at medium milk[3]	Untreated	37.5a	19.1b
	Treated	0.9b	0.6a
Soft dough[4]	Untreated	0.4b	0.5b
	Treated	0.3b	0.5b

[1]Means followed by equal lowercase letters, in each column within each experiment, do not differ by the nonparametric Kruskal-Wallis test, at 5% probability. [2]Head enclosed within the flag leaf sheath and visible awns. [3]Head from halfway anthesis emerged to fully exposed. [4]Head fully exposed.

conducted under controlled conditions, temperatures were higher in the first experiment (20–25°C), when compared with the second (18–22°C). According to Osborne & Stein (2007), higher temperatures, such as those recorded in the first experiment, are more favorable for the development of FHB and the contamination of the grains with DON.

Fungicide application before inoculation significantly reduced the level of DON contamination in the grains, at 5% probability, in all three reproductive

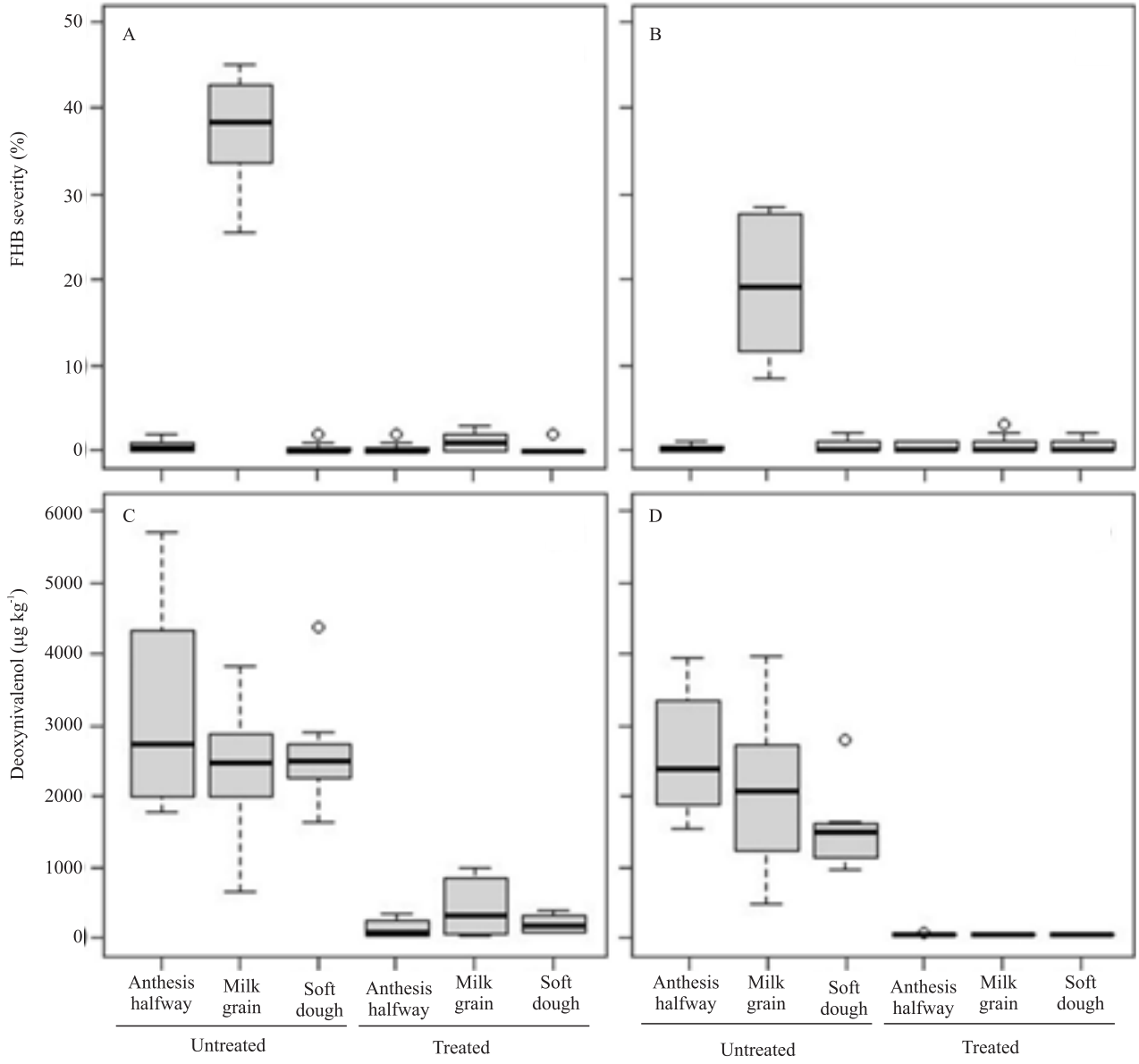

Figure 1. Box plot summarizing: the distribution of mean Fusarium head blight (FHB) severity in barley (*Hordeum vulgare*) plants in the first (A) and second (B) experiments; and the level of contamination with deoxynivalenol in the grains with the application of the tebuconazole fungicide (190 g ha^{-1} a.i.) before FHB inoculation in three reproductive stages in the first (C) and second (D) experiments carried out at 20–25 and 18–22°C, respectively, under a 12 hour photoperiod. The reproductive stages are: anthesis halfway, head enclosed within the flag leaf sheath and visible awns; medium milk grain, head from halfway anthesis emerged to fully exposed; and soft dough, head fully exposed. The solid line within the box represents the mean, whereas the top and bottom lines represent the 75th and 25th percentiles of the data, respectively. The vertical bars extending beyond the boxes represent the 10th and 90th percentiles, and the circles indicate outliers.

stages. The control of DON contamination across the reproductive stages was 78–96% in the first experiment and 94–96% in the second.

According to the obtained results, the reproductive stage of cleistogamous barley had a significant effect on *F. graminearum* infection and FHB symptoms, but not on DON contamination levels in the grains.

In the literature, under controlled and field conditions, closed-flowering barley varieties were resistant to the disease during anthesis, becoming susceptible afterwards (Yoshida et al., 2007, 2008). In two experiments, Yoshida et al. (2007) observed this susceptibility 10 days after anthesis, coinciding with the milk grain stage, but that open-flowering and chasmogamous cultivars were already susceptible during anthesis. These same authors found that the severity of FHB caused by pathogen inoculation at anthesis ranged from 15 to 20% in the first experiment and from 3.3 to 13.3% in the second, with higher levels, from 46.3 to 71.7%, in open-flowering cultivars. Contrastingly, no significant differences in DON accumulation in the grains were observed between times of pathogen application in the present work. However, the evaluated cultivars accumulated more DON when *F. graminearum* was inoculated 10 or 20 days after anthesis, corresponding to the milk grain and soft dough stages, respectively.

Yoshida et al. (2008) also reported a positive response of barley plants to fungicide application aimed at reducing DON contamination in the grains. In their study, maize kernels colonized by *F. graminearum* were used as an inoculum source, allowing of a natural inoculation in the experimental area through ascospores or conidia dispersed by wind and raindrops. The fungicide thiophanate-methyl was applied at the anthesis and late milk grain or soft dough stages, which reduced DON contamination in the grains from 21.3 to 10.7–12.1 μg kg^{-1} and from 4.6 to 2.0–2.6 μg kg^{-1} in untreated and treated plants, respectively, representing a control of 43.2–49.8 and 43.5–56.2% in two different experiments. Notably, the control rates observed in the present work, conducted in a controlled environment without repeated inoculation, were higher than those found by Yoshida et al. (2008), allowing of the reduction of DON in unprocessed grain samples to levels below the maximum limit of 2,000 and 1,250 μg kg^{-1} tolerated in barley by Brazilian regulations (Anvisa, 2018) and European Union countries (European Commission, 2006), respectively. This result may be related to the fact that the triazole fungicide used in the present study, tebuconazole, is one of the most effective choices for FHB control and DON reduction in wheat (Paul et al., 2018; Feksa et al., 2019). Therefore, the information presented here can also be considered in the chemical control strategies for FHB and DON in barley.

The obtained results are an indicative that the window of intervention through fungicide application is larger for DON contamination than for FHB severity, since the former ranges from the anthesis stage, when the barley spike is still enclosed within the flag leaf sheath containing flowers, to the soft dough stage, when the spike is fully exposed. The Spearman correlation coefficient between FHB severity and grain contamination with DON ranged from 0.31 to 0.37 (p=0.05), suggesting that, under the evaluated conditions, the measurement of severity had a limited predictive relevance for DON. Under field conditions, Mishra et al. (2014) found correlation coefficients ranging from 0.23 to 0.60, at 5% probability.

The findings of the present work are important for the development of FHB and DON management strategies that rely on fungicide applications, considering the

Table 2. Contamination of deoxynivalenol (DON) in the grains of the BRS BRAU cleistogamous barley (*Hordeum vulgare*) cultivar inoculated with *Fusarium graminearum* across three reproductive stages, without or with the application of the tebuconazole fungicide (190 g ha^{-1} a.i.) before pathogen inoculation, in two experiments carried out at 20–25 and 18–22°C, respectively, under a 12 hour photoperiod[1].

Reproductive stage	Fungicide	DON contamination (μg kg^{-1})	
		Experiment 1	Experiment 2
Anthesis halfway[2]	Untreated	3,883a	1,553a
	Treated	164b	68b
Grain at medium milk[3]	Untreated	2,024a	2,035a
	Treated	453b	75b
Soft dough[4]	Untreated	2,623b	1,510a
	Treated	227b	84b

[1]Means followed by equal lowercase letters, in each column within each experiment, do not differ by the nonparametric Kruskal-Wallis test, at 5% probability. [2]Head enclosed within the flag leaf sheath and visible awns. [3]Head from halfway anthesis emerged to fully exposed. [4]Head fully exposed.

more extensive optimal timeframe for this procedure when the inoculum persists until the soft dough stage. However, field work will be necessary to validate these results. Moreover, the obtained information can be used as a basis for research aimed at screening closed-flowering barley lines or cultivars with enhanced genetic resistance to FHB and DON. Therefore, a more resistant germplasm associated with timely fungicide applications may be the key to improve the management of FHB and DON in barley in Brazil.

Conclusions

1. The inoculation of *Fusarium graminearum* at the medium milk stage of barley (*Hordeum vulgare*) grains causes fully-visible Fusarium head blight symptoms, although the disease is controlled by the application of the tebuconazole fungicide before inoculation, whereas inoculation at the anthesis halfway and soft dough stages results in the development of minimal symptoms.

2. Pathogen inoculation at the anthesis halfway, medium milk, and soft dough reproductive stages results in grain contamination with deoxynivalenol, which is reduced by the application of the fungicide before inoculation.

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