Effects of *Fusarium graminearum* and *Fusarium poae* on disease parameters, grain quality and mycotoxin contamination in barley (Part II)

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ABSTRACT

BACKGROUND: Barley is one of the most important winter crops in the world, with multiple uses such as human consumption, animal feed and for the malting industry. This crop is affected by different diseases, such as *Fusarium* Head Blight (FHB), that causes losses in yield and quality. In the last years *F. graminearum* and *F. poae* were two of the most frequently isolated species in barley grains, so the aim of this study was to evaluate the interaction between these *Fusarium* species and the effects on disease parameters, grain quality and mycotoxin contamination on five barley genotypes under field conditions.

RESULTS: Statistical differences between *Fusarium* treatments for some parameters depending mainly on the year/genotype were found. The results showed that germination process was affected by both *Fusarium* species. As to grain quality and the different hordein fractions, it was observed that *F. graminearum* affects preferentially D and C-hordeins Different concentrations of nivalenol, deoxynivalenol and their acetylated derivatives (3-ADON and 15-ADON) were detected.

CONCLUSIONS: In the present work, no evidence of synergism between *F. graminearum* and *F. poae* were found regarding disease parameters and mycotoxin contamination. However, at least in the years with favorable climatic conditions to FHB development and depending on the barley genotype, a continuous monitoring is deemed

necessary to prevent the negative impact on protein composition and germinative parameters ***Keywords:*** *Fusarium*, barley, mycotoxins, disease parameters, grain quality, plant-pathogen interaction.

Introduction

Barley (*Hordeum vulgare* L.) is one of the most sown crops in the world due to its multiple uses such as human consumption, animal feed and for the malting industry. Barley grains are the major raw material used for brewing, being the malting barley the major input for the brewing industry. Within the commercial quality parameters for malting barley, the most important ones are germinative power (>95%), protein concentration (<12%) and screening percentage (>85% grains with a diameter greater than 2.5 millimeters).[1,2] In the last few years, the production of two-row barley has grown significantly and the main destination is the brewing industry, although the use of barley for animal feed is also increasing. The global barley production in 2017/2018 was about 142.97 million tons, with the US Department of Agriculture (USDA) estimating that the production for 2018/2019 will be about 147.57 million tons. The major barley producers are the European Union (59.09 million tons) and Russia (20.18 million tons).[3]

Regarding grain quality, a group of storage proteins highly abundant in cereal seeds is the prolamins, known as hordeins in barley and representing the major fraction of the endosperm storage proteins in grains. The type of protein stored influences malt extract regardless of grain protein concentration. Hordeins are classified into three groups: 1) high molecular weight (D-hordeins); 2) poor in sulfur (C-hordeins); 3) rich in

sulfur (B-hordeins). The D-hordeins have an approximate size of 100 kDa and represent less than 10% of the total seed content. The C-hordeins have a size of 55-70 kDa, do not contain cysteine residues, so they do not tend to form complexes with other proteins and their abundance is 10-20%. B-hordeins have a size of 36-44 kDa, are rich in cysteine residues, therefore they can form intra or interchain disulfide bridges and are the most abundant (75-80%).[4] The B-hordeins represent the primary factor affecting grain protein content, with a negative correlation between the B-hordein content and malt extract.[5]

Fusarium Head Blight (FHB) is one of the most devastating diseases that occur in barley in most areas of the world causing not only damage to crops but also serious economic losses. FHB is observed mainly in the regions with a warm and wet climate coinciding with the flowering stage of this cereal. Currently, *Fusarium graminearum* is the dominant species isolated worldwide but in the last years, *F. poae* has been commonly found by several researchers in diverse substrates such as barley and wheat[6] . FHB causes quantitative and qualitative damage to crops with a significant impact on yield and several functional parameters of grain related to malting and brewing quality, with beer gushing (violent overfoaming of beer) being the most infamous. In general, pronounced effects on germination, soluble nitrogen, free amino nitrogen, wort color, and β-glucan levels were reported and many of the changes likely resulted from enzymes produced by *Fusarium* spp.[1,6,7,8,9]However, the greatest concern for the use of barley infected with FHB has been the presence of mycotoxins.[1,7]

Fusarium has the capacity to produce trichothecenes, one of the most important groups of mycotoxins that can cause harmful effects on humans and animals through

ingestion of contaminated cereal grains. Variations in environmental conditions, crops and storage factors can influence the type and amount of mycotoxins produced by different *Fusarium* species.[10] *F. graminearum* has the capacity to produce a wide spectrum and quantity of mycotoxins, such as deoxynivalenol (DON), that can be acutely lethal when consumed in large amounts, and its acetylated derivatives 3-ADON and 15-ADON. DON has been found to carry through malting and brewing into finished beer and has been reported in commercial beers at levels of 0.30 to 569 µg/L.[1,11]. On the other hand, *F. poae* has special importance since it is the main *Fusarium* pathogen able to produce nivalenol (NIV), an important mycotoxin that in high concentrations can inhibit cell proliferation and produces cytotoxic effects on cells.[12] High NIV concentrations were found in commercial samples of beer reaching values of 2.40 ± 1.9 µg/L.[13] However, the European Scientific Committee on Food (SCF) has established limits for DON only, considering a tolerable daily intake of 1mg/kg body weight, while for NIV established a provisional limit value of 0.7 mg/kg body weight.[14]

It is known that the competition for resources between *Fusarium* species can produce more toxins under stress conditions, while in co-inoculations no evidence was found to support synergism between fungal isolates in causing visual symptoms[15]. The aim of this study was to evaluate the effects of the presence of *F. graminearum* and *F. poae* in terms of disease parameters, grain quality and mycotoxin contamination in two-row barley under field conditions.

Materials and methods

Fusarium isolates. A mixture of four isolates of *F. graminearum* with the ability to produce DON, 3-ADON and 15-ADON *in vitro* were selected for the production of the inoculum (isolates 3.4, 88.1, 92.2 and 129.1)[16] while another mixture of four isolates of *F. poae* (FP-TSa1b, FP-TBig1a, FP-TMa1a, and FP-TPC1a) based on the production of NIV *in vitro* were used.[17] The fungal inoculum was produced by placing individual agar plugs with mycelium and conidia onto liquid medium containing carboxymethyl cellulose (CMC) for *F. graminearum* and potato dextrose agar (PDA) in Petri dishes (90 mm) for *F. poae.* For *F. graminearum,* 50 mL of CMC medium were placed in 250 mL Erlenmeyer flask and shaken continuously for 10 days (100 rpm, 25 ± 2°C and darkness). For *F. poae* the time of incubation was 7 days at 25 ± 2°C under 12 h each of light and darkness. In this case, the conidial harvest was done by flooding the plates with 5 mL of sterilized distilled water (SDW) and dislodging the conidia with a bent glass rod. For both *Fusarium* species, the resulting suspension was filtered through cheesecloth and the conidial suspension was adjusted to 1×10^5 conidia/mL with a Neubauer hemacytometer.[18]For each *Fusarium* species, the final conidial suspension was prepared with equal parts of each of the four isolates. Tween 20 (0.05%) was added to the suspension as a surfactant.

Barley genotypes. Five genotypes of spring barley were evaluated: Scarlett (the genotype sown by most growers in Argentina, with excellent performance and malt quality), Shakira (second genotype in sowing area, for malting and with high yield potential), Andreia (new genotype, high screening percentage and malt quality), Scrabble

(barley genotype for malting and with high yield potential) and INTA 7302 (two-row barley for malting or forage use). These genotypes were chosen because their times to harvest and rates of development through phenological stages were similar.

Experimental design. Field assays were carried out on the experimental farm at the Faculty of Agronomy, Azul, Buenos Aires province, Argentina (36°49′41.4″ S, 59°53′11.6″ W). The soil is a typical Argiudoll and the following are the characteristics of this soil at the depth of 0-20 cm: texture=clay loam soil, pH=6.06 (1:2.5 in water), N-nitrate=7.10 kg N/ha (reflectometry), available P=26.50 ppm, organic matter=3.23%. The field trials were repeated in 2014, 2015, and 2016 growing seasons. Conventional tillage practices were made with a disc plow and harrow to a depth of 15 cm. Sowing dates ranged from 14 to 16 July in the three years because they were adjusted for the different genotypes to ensure uniformity in the timing of inoculation. Each plot size was 8 x 1.5 m and genotypes were sown at 350 seeds/m^2. The distance between plots was 1 m. Plants were grown in the absence of any nutritional or pest stress, without supplemental irrigation or fungicide treatments. Plants were fertilized with 150 kg N/ha, using urea (46% nitrogen) in split doses at sowing and Z2.3.[19] During crop cycle, insecticide (cypermethrin) were applied in the three years and weeds were removed by hand. Barley heads were inoculated when >50% of the plants having undergone fertilization (Z.49) according to Buerstmayr.[19,20] Conidial suspensions were applied until run-off using a hand-held garden sprayer (2 L), with adjustable brass nozzles. Plots were artificially inoculated by spraying 1L of spore suspension (250 mL in each subplot). For control treatment, SDW with Tween 20 (0.05%) was used to inoculate. The inoculum was

applied: A) in the absence of wind, to limit the drift of the inoculum to neighbouring plots; B) in the evening on preferably cloudy days with high relative humidity (>80%), to avoid the evaporation of the inoculum; C) keeping a distance between the nozzle and the spikes of around 5 cm, to avoid spore dispersion. Furthermore, the plot to be inoculated was temporarily isolated from adjacent plots by placing 1.60 m plastic panels on the three sides of the plot and removing the panels when the inoculation was finished. To check the possibility of contamination with other *Fusarium* spp., 100 grains/subplot were selected at random, superficially disinfected (70% ethanol for 2 min and 5% sodium hypochlorite for 2 min, then finally rinsed twice in SDW) and placed on PDA with 0.25 g of chloramphenicol and incubated for 7 days at 25 ± 2°C under 12 h each of light and darkness. *Fusarium* spp. were identified according to Leslie and Summerell.[21] The field experiment was a split-plot design with four blocks, where the 20 treatments (five genotypes by two levels -presence or absence- of each *Fusarium* species) were applied for each block. Each plot sown with a genotype was divided into four subplots which were randomly assigned to one of the four inoculation combinations: 1) with *F. graminearum* alone (FP_0FG_1), 2) with *F. poae* alone (FP_1FG_0), 3) with both pathogens (FP_1FG_1), 4) control without *Fusarium* species (FP_0FG_0). Temperature, relative humidity, and precipitation data (from inoculation to harvest in 2014, 2015, and 2016) were obtained from the National Meteorological Center Weather Station located 100 m from the experimental site and from the Regional Center of Agrometeorology (RCA).

Measurements. Visual disease assessment (incidence, severity, FHB Index) was conducted at 21 days post-inoculation by counting the number of symptomatic grains (lesions or bleaching of grains or glumes with a dark margin) of 40 spikes/plot selected at random according to Campbell and Lipps.[22] After physiological maturity the plots were harvested, threshed and cleaned manually. Grain yield per plot (g/m^2) was measured. Protein content and percentage of moisture were measured with a NIT analyzer with double-face monochromator (Agricheck, Bruins Instruments, Salem, NH, US). The grains were sieved and the percentage of grains retained on a 2.5-mm sieve (screening percentage) were recorded. The germinative energy and the germinative power of seeds were evaluated according to ISTA rules for seed testing.[23] The different fractions of hordeins were extracted from symptomatic/healthy grains by the method described by Salgado-Albarrán et al.[4] All proteins were separated by SDS-PAGE (T=13.5%). The gels were stained with 0.05% Coomassie Brilliant Blue R250 for 24 h, distained in TCA 12% for 48 h and finally washed in SWD for 24 h. The resulting gels were scanned and analyzed by using TotalLab (v1.10 demo) software to measure the intensity of the pixel as an abundance indicator. Background subtraction was applied to avoid the variability due to the staining process. The contents of total hordein and D, C, B hordein fractions were determined. The toxins found in the grain samples were analyzed. About 200 g of seeds were taken, reduced to 25 g using a grain divider (Cereal Tools®) and ground with a high speed disintegrator FW-110 (Arcano©; Pasteur Instrumental, Buenos Aires, Argentina).[24] Trichothecenes were extracted for 1 h at 300 rpm with 125 mL of acetonitrile:acetylacetate:water (50:41:9). The clean-up was performed with a column

packed with charcoal:alumina:celite (0.7:0.5:0.3) and dried in a rotary evaporator. Gas chromatography, with 63Ni electron capture detection Shimadzu Model GC17 (Shimadzu Corp., Kyoto, Japan) equipped with split/splitless injector and fitted with RX-5MS capillary column (25 m x 0.2 mm id), was used to detect and quantify trichothecenes. The detection limits were 0.02 µg/g for DON and its acetyl derivatives and 0.05 µg/g for NIV, while the quantification limits were 0.06 µg/g for DON and 0.15 µg/g for NIV. Standards of DON, 3-ADON, 15-ADON and NIV were purchased from SIGMA Chemical Company (St Louis, MO, USA). The presence of compounds was confirmed by Gas Chromatography-Mass spectrometer system (GC-MS QP 5050A, Shimadzu®) with Electron Impact (EI) mode (70 eV) as described by Alvarez et al.[25]

Statistical analysis. All the variables evaluated were analyzed using the software R (v.3.3.3, R Core Team 2018).[26] Due to the contrasting climatic conditions observed in the three growing seasons (mainly during the anthesis-harvest period), each year was evaluated separately. The main factor was the barley genotype and two levels (presence or absence) of each *Fusarium* species nested within the genotype, with 4 blocks for each combination of treatments. We used mixed-effects linear models, which allow nesting plots within blocks and subplots within plots. Data assumptions were verified graphically using plots of fitted values versus the residuals for homogeneity of variances and using normal Q-Q plots for normality of residuals. Furthermore, the Shapiro-Wilk test was used to check for normality of residuals. Protein concentration (PC), thousand kernel weight (TKW), grain yield (GY) and hordein fractions (D, C, and B-hordeins) were analyzed

using the lmer function (lmer→variable ~ *F. graminearum* * *F. poae* * barley genotype + (1|block/bigplot), data=barley) (lme4 package)[27] with normal distribution of error. For mycotoxins analysis (DON, 3-ADON, 15-ADON and NIV) log transformation was performed (lmer→log (mycotoxin+1) ~ *F. graminearum* * *F. poae* * barley genotype + (1|block/bigplot), data=barley). The variables incidence (I), severity (S), FHB Index, germinative energy (GE), germinative power (GP) and screening percentage (SP) were analyzed with a generalized linear mixed model (glmer→variable (cbind) ~ *F. graminearum* * *F. poae* *barley genotype + (1|block/bigplot), family = binomial (link = "logit"), data = barley) that considered the restrictions in the randomization and non normal errors with the function glmer (lme4 package).[27] In all cases, a full model including all interactions was analyzed and significance was tested with Type II Likelihood Ratio Test. Significant of effects were tested with the lsmeans function (emmeans package).[28] Results were reported as the mean ± standard error of the mean (SEM) for all the variables analyzed, except for mycotoxins contamination that was reported as mean ± SEM of the log transformed data.

Results

Climatic conditions. The environmental conditions in the three years were different particularly in the flowering stage. In 2014, the average temperature was the highest (18.22 ± 3.71°C) being the warmest and wettest year (209.60 mm, 67.24 ± 11.55% RH), favorable for growth conditions for *Fusarium* spp. In contrast, in 2015 and 2016 growing seasons the weather conditions were not optimal for the development of the

disease (Table 1). The spring of 2015 had the lowest minimum temperatures (9.88 ± 4.08 °C) with less rainfall than 2014 (144.60 mm, 68.88 ± 11.17% RH), while the spring of 2016 was the driest with low rainfall and less relative humidity (74.90 mm, 59.79 ± 13.07% RH) with moderate temperatures (17.62 ± 3.88 °C). Regarding historical precipitation for the anthesis-harvest period, in 2014 we registered an increase of 27%, while contrarily in 2015 and 2016 we observed a decrease in the precipitation (12% and 55%, respectively) (Table 1).

Disease parameters. Significant differences ($p<0.05$, n=80) were detected only for incidence and severity parameters in 2014, but not for 2015 and 2016 growing season (Fig. 1). In addition, all genotypes showed symptoms with all the isolates used as inoculum. Regarding incidence ($p=0.0055$, n=80), effects of the *Fusarium* treatments were observed only in 2014 (Fig. 1A) showing the highest values for FP_1FG_1 treatment (58.00 ± 6.00%) followed by FP_0FG_1 (50.00 ± 5.00%) and FP_1FG_0 (46 ± 6.00%). In the same way, severity was significantly differences ($p<0.0005$, n=800) in 2014 for *Fusarium* treatments (Fig. 1B), being the most affected FP_1FG_1 and FP_0FG_1 (3.00 ± 0.23%) followed by FP_0FG_1 (2.80 ± 0.23%) and FP_1FG_0 (2.00 ± 0.23%).

On the other hand, in 2015 only significant differences ($p<0.0010$, n=80) were observed among different genotypes for incidence, being Shakira (29.00 ± 3.00%), Andreia (23.00 ± 3.00%) and INTA 7302 (21.00 ± 3.00%) the most affected genotypes, while Scrabble (8.00 ± 2.00%) and Scarlett (6.00 ± 2.00%) showed the lowest symptoms (Fig. 1D). In the same way, for severity only significant differences ($p<0.0001$, n=800)

were found among the different genotypes being Shakira (5.80 ± 0.26%) the most affected genotype, while in Scrabble (0.30 ± 0.09%) the lowest values of severity were observed (Fig. 1E). Although the values were low, for FHB Index significant differences were observed (p=0.0012, n=80), being Shakira the most affected (2.30 ± 0.70), while Scrabble (0.10 ± 0.03) and Scarlett (0.10 ± 0.01) showed the lowest values (Fig. 1F).

Germination and grain quality parameters. Significant differences were observed only for *Fusarium* treatments in parameters such as GE, GP, and SP depending on the genotype, while there were no effects on PC (Table 2). For yield parameters as TKW and GY, there were only significant differences for genotype.

Regarding GE, in 2014 significant differences were found (p <0.0001, n=60) for *Fusarium* x genotype interaction, showing for FP_0FG_1 that Andreia was not affected (85.00 ± 3.00%), while Shakira (83.00 to 71.00%) was the most affected, with a decrease of 12.00% in relation to FP_0FG_0 . In contrast, in 2015 and 2016 growing seasons significant differences were found (p <0.0001, n=80) for the interaction of the FP_1FG_0 x genotype. In 2015, Scarlett was the least affected genotype reducing GE by 2.00% (94.00 to 92.00%) while Andreia was the most affected with a loss of 11.00% (65.00 to 54.00%). In addition, Scarlett remained the least affected genotype in 2016, losing 2.00% (94.00 to 92.00%), while INTA 7302 was the most affected genotype with a loss of 8.00% (93.00 to 85.00%).

On the other hand, GP was affected in 2014 by the FP_1FG_1 x genotype interaction (p=0.0048, n=60), being Andreia the least affected losing 2.00% (92.00 to 90.00%), while

a decrease of 6.00% was observed in INTA 7302 (90.00 to 84.00%). In addition, in 2015 and 2016 there were significant differences ($p < 0.0001$, $n=80$) for the FP_1FG_0 x genotype interaction. In 2015, Scarlett was the least affected genotype, reducing only by 2.00% (98.00 to 96.00%), while a reduction of 11.00% was observed in Andreia (83.00 to 72.00%). In the same way, in 2016 Scarlett was the least affected genotype losing only 2.00% (97.00 to 95.00%), while the greatest decreases (7.00%) were observed in INTA 7302 (97.00 to 90.00%).

Regarding SP, in 2014 only significant differences were observed for genotype, showing Shakira the highest values (96.75 ± 0.59%) and INTA 7302 the lowest values (90.30 ± 1.35%). On the other hand, in 2015 FP_1FG_1 x genotype interaction was observed, showing the highest values of SP for Shakira x FP_0FG_0 (97.55 ± 0.50 %) while the lowest values were reported for INTA 7302 x FP_1FG_1 (94.73 ± 0.76%). In 2016, *F. poae* x genotype interaction showed significant differences, being Shakira the genotype most affected, decreasing the SP in 0.62% respect to the control.

As to grain yield parameters such as TKW and GY, there were significant differences only for genotype. In contrast, for PC there were no significant differences in any of the years evaluated (Table 2). For TKW in 2014 and 2015, Scrabble had the heaviest grain (41.64 and 51.51 g, respectively) while the lower grain weights were found in Scarlett (34.69 and 42.27 g, respectively). In the same way, for GY the lowest values were reported in Scarlett (478.81 ± 32.75 g/m^2), while Andreia showed the highest grain yield (611.56 ± 32.75 g/m^2).

Protein composition. The analysis of protein fractions was performed only in 2014, due to the favorable conditions for FHB development. Significant differences were found for *Fusarium* treatments, showing degradation of the different hordeins fractions with respect to the control (Table 3, Fig. 2). For D-hordeins, the highest decrease in relative abundance were observed in FP_0FG_1 treatment decreasing by 83.41% compared to the control, while a decrease of 81.24% on average was observed for FP_1FG_0 and FP_1FG_1. In the same way, for C-hordeins the lowest values were observed in FP_0FG_1 decreasing by 82.35% with respect to FP_0FG_0, while for FP_1FG_0 and FP_1FG_1 a decrease of 79.46 % on average was reported. On the other hand, the greatest degradation in B-hordeins fraction was caused by FP_1FG_1 treatment, being 50.25% lower than the control, while a decrease of 39.07% on average was observed in FP_1FG_0 and FP_0FG_1 treatments. Regarding the total hordeins content, the lowest values of relative abundance were found in FP_1FG_1 treatment being 68.55% lower than FP_0FG_0, while for FP_1FG_0 and FP_0FG_1 a decrease of 66.66 % on average was observed.

Mycotoxin contamination. Regarding the possible antagonism/synergism interaction between the mycotoxins accumulation of *F. graminearum* and *F. poae* treatments, no significant differences were observed in all the years analyzed. In 2014, the highest mycotoxin value was observed in FP_0FG_1 treatment, with high concentrations of DON (6.12 ± 1.77 µg/g) and 15-ADON (1.21 ± 0.05 µg/g), while the 3-ADON and NIV production was higher in FP_1FG_1 treatment (21.60 ± 11.41 and 1.20 ± 0.06 µg/g, respectively). In 2015 and 2016, mycotoxin values were lower compared to 2014 and

NIV production was not detected. In 2015, the major amounts of mycotoxins were observed for FP_0FG_1 (DON: 1.53 ± 0.11 µg/g) and FP_1FG_1 treatments (3-ADON: 5.20± 1.29 µg/g;15-ADON: 1.41± 0.11 µg/g). In the same way, in 2016 the highest values of mycotoxins were registered in FP_0FG_1 treatment, with low concentrations of DON (1.02 ± 0.002µg/g) and 3-ADON (2.58 ± 0.30 µg/g), while for 15-ADON (1.41± 0.11 µg/g) the highest values were observed in FP_1FG_1 treatment. In terms of total mycotoxin amount, we found a higher concentration of 3-ADON over the years evaluated (Table 4).

Discussion

The experiment was carried out under field conditions, being the three years analyzed different from each other, mainly with respect to environmental conditions such as relative humidity, temperature, and accumulated rainfall during the period from anthesis to harvest. Our results showed significant differences between the *Fusarium* treatments in disease parameters only in the 2014 growing season, with warm temperatures and wet conditions that were favorable especially for the growth of *F. graminearum*. On the other hand, in 2015 and 2016 no statistical differences were observed in disease parameters, due to the lowest temperatures and the driest conditions at the flowering stage. Several authors found evidence indicating that environmental conditions play a key role in *Fusarium* spp.–host interactions. Moreover, the composition of *Fusarium* species changes according to the effects of different climatic factors on each growing season, mainly during the flowering stage.[29] Turner & Jennings[30] observed that

increasing humidity produced earlier disease development and a major incidence of the disease for all *Fusarium* species evaluated except to *F. poae*. The variations in the composition of the *Fusarium* species can be explained by the occurrence of different thermo-hygrometric conditions, and when the conditions were not favorable for the main causal agents of FHB such as *F. graminearum* and *F. culmorum*, other species as *F. poae* and *F. avenaceum* significantly increase their presence. In general, warm temperatures (around 28°C) and wet conditions are favorable for *F. graminearum* infection, while temperatures around 25°C and dry conditions are for *F. poae.*[15,31.] In addition, it is known that genotype plays an important role in FHB resistance. In our work, in 2015 different responses were observed to *Fusarium* spp. infection among different barley genotypes. Although the barley genotypes were chosen for their similar phenological stages, these results could be explained by slight differences in temporal and spatial flowering patterns, the different micro-environment and microclimatic conditions combined of each trial plot, and the possibility of resistance genes present in different barley genotypes.

Previously studies reported a decrease in kernel plumpness, low values of kernels larger than 2.5 mm in diameter and a slight increase in protein and total nitrogen content in inoculated barley grains.[7,32] In the current study, we observed *Fusarium* x genotype interaction for the screening percentage (2015 and 2016), while there were no significant differences among *Fusarium* treatments on protein concentration and thousand kernel weight. These effects showed a significant effect mainly of the genotype, being consistent with other works that describe a strong genetic and environmental influence on the differences in the parameters analyzed on barley genotypes.[33,34]

Regarding germinative parameters, several studies have revealed that the growth of *Fusarium* spp. may affect germination capacity and therefore malt characteristics. Furthermore, *Fusarium* spp. can produce other undetected proteinases during the infection, and can trigger the synthesis or activation of some barley proteinases that normally function during the germination process.[8] According to this, Schwarz *et al*. [32]reported a decrease from 42.00% to 32.00% in barley infected with *F. graminearum* and a decrease from 14.00% to 8.00% in treatments inoculated with *F. poae*. In the same way, Sarlin *et al*.[7] observed a reduction of 10.00% on average using a mixture of *Fusarium* species, while Oliveira *et al*. [35] found a decrease around 45.00% in germinative energy by inoculating with *F. culmorum*. Results observed in our work agree with those previously reported, showing that in 2014 highest effects on GE/GP were observed for *F. graminearum* treatment decreasing by 6.00% on average, while in 2015 and 2016 growing season *F. poae* affected both parameters depending on the genotype, reducing by 4.00% on average.

Fusarium spp. infection may lead to the production of different hydrolytic enzymes such as cutinases, proteinases, xylanases, and cellulases. These cell wall-degrading enzymes could play an important role in pathogenicity and are likely to be involved in the colonization of barley grains, being the proteinases the most important, therefore protein degradation can strongly affect the malting or brewing quality of the diseased grain.[8,32] In our work, we reported a slight trend in symptomatic barley grains showing that *F. graminearum* presence degraded a great amount of hordeins, around 83.41% and 82.35% in D-hordeins and C-hordeins, respectively. Furthermore, we

reported a decrease caused by *F. graminearum* x *F. poae* treatment (FPF_1G_1) of 50.25% in B-hordeins and a reduction of the total hordeins content in 68.55% respect to the control. Changes observed in hordeins fraction could be explained due to fungal proteases activity such as trypsin protease or serine protease that belongs to the exoproteome of *Fusarium* spp. known as protein-degrading enzymes.[8,9] Eggert *et al.* [36] found that the influence of *Fusarium* infection on naked barley reduced slightly the content of hordeins, while the albumins and globulins were not affected. In addition, Schwarz *et al.* [32] detected the higher proteinase, β-glucanase, and xylanase activity levels in barley samples artificially infected with *F. graminearum* and *F. poae* compared with the control, concluding that enzyme activity levels in barley samples were so high that they might affect the grain quality and therefore malt quality. Regarding the potential impact on malt quality, several authors have reported that heavy *Fusarium* spp. infection decreases β-glucan content and simultaneously increases soluble nitrogen, free amino nitrogen (FAN) and wort color, suggesting that *Fusarium* proteinases could degrade barley proteins already in the field or during malting and mashing.[7]

Based on the major values of incidence/severity in the field during 2014, we expected a higher mycotoxin concentration in this year compared to 2015 and 2016. Mycotoxin concentration varied greatly between *Fusarium* treatments, genotypes and years, observing the highest amount of DON in 2014 for *F. graminearum* treatment (Table 4). For DON, these values were above the maximum limits established by the European Commission for unprocessed grains (1.25 µg/g).[14] Moreover, we found an

important concentration of 3-ADON in the different years evaluated, which coincides with our results obtained in bread wheat.[37]

In some studies, the predominant toxin is NIV which is believed to be more toxic than DON or its acetylated derivatives, although the maximum limit for NIV has not been established yet.[38] The Scientific Committee on Food (SCF) has set limits only for DON, considering a tolerable daily intake of 1 mg/kg body weight, while for NIV, a provisional limit value of 0.7 mg/kg was established.[14]Stenglein *et al.*[39] found higher levels of NIV in barley genotypes inoculated under field conditions (ranging between 0.20 and 10.80 µg/g), while Nogueira *et al.* [40] found NIV in 29% of the barley samples evaluated (with an average concentration of 2.36 µg/g) under natural infection conditions. In the present study, we reported lower values that could be explained due to the climatic conditions (mainly temperature) during the flowering stage. These climatic conditions were not optimal to the development of *F. poae* and the subsequent NIV production. This is supported by Nazari *et al.* [31], who established that the optimum temperature for *F. poae* growth is 24.7 °C and for NIV production 27.5 °C.

Scarce information about *Fusarium* species interaction and their potential impact in mycotoxin production are available. Xu *et al.* [41] reported that there was no evidence to support synergism between fungal isolates in causing visual symptoms; thus suggesting the existence of competitive interactions that led to decrease in the fungal biomass (until 90% for weaker species) compared to single-isolate inoculations. Regarding mycotoxin contamination, reports in co-inoculation experiments indicate that the productivity increased considerably (in many cases exceeding 100-fold) suggesting that competition

resulted in greater production of trichothecene mycotoxins.[41] This effect on mycotoxin production in co-inoculations could be explained by competition between *Fusarium* species which produces more toxin under stress conditions as resources for competence.[15] In the present work, no significant differences were observed between *Fusarium* treatments. However, we found a major concentration of 3-ADON along the different years, despite the fact that the *F. graminearum* isolates used were DON, 3-ADON and 15-ADON producers. This prevalence of 3-ADON biosynthesis could be explained by the important role that plays some climatic parameters such as the temperature and other environmental conditions.[42] In accordance with this, Ramírez Albuquerque *et al*.[43] found under *in vitro* conditions a major production of DON acetylated derivatives, being the production of 3-ADON maximum at 25-30°C, while the production of 15-ADON is maximum at 10°C.

Conclusions

This is the first work under field conditions during three growing seasons evaluating the interaction between *F. graminearum* and *F. poae* on barley genotypes. We conclude that: 1) no evidence of synergism between *F. graminearum* and *F. poae* were found regarding disease parameters and mycotoxin contamination; 2) coinciding with previous works, different response patterns to *Fusarium* infection exists between barley genotypes, that could be useful for future genetic improvement; 3) depending on the genotype, the germination process was affected by both *Fusarium* species (*F. graminearum* > *F. poae*) which could affect the protein composition, the malt quality and

therefore the malting process4 However, at least in the years with favorable climatic conditions to FHB development and depending on the barley genotype, a continuous monitoring is deemed necessary to prevent the negative impact on protein composition and germinative parameters

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Conflict of interest

The authors declare no have conflict of interest.

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Table 1. Mean values and standard deviation of temperature, relative humidity (RH), and accumulated precipitation during 2014, 2015, and 2016 growing seasons (from inoculation to harvest)[a].

Year	Temperature (°C)			RH% (average)	Accumulated precipitation (mm)	Historical precipitation (mm)	% Precipitation Variation
	Average	Average minimum	Average maximum				
2014	18.22 ± 3.71	11.64 ± 3.52	25.19 ± 4.87	67.24 ± 11.55 %	209.60		**+27%**
2015	17.65 ± 3.06	9.88 ± 4.08	24.97 ± 3.75	66.88 ± 11.17 %	144.60	165.10	**-12%**
2016	17.62 ± 3.88	10.13 ± 4.10	25.10 ± 4.58	59.79 ± 13.07 %	74.90		**-55%**

[a]Weather station was located 100 m from the experimental site and data were taken every 30 min.

Table 2. Analysis of variance for different grain parameters. **GE**: germinative energy; **GP**: germinative power; **PC**: protein concentration; **SP**: screening percentage; **TKW**: thousand kernel weight; **d.f**.: degrees of freedom; **Chisq**: Type II Wald Chi-square tests.

Source	2014									
		GE		GP		PC		SP		TKW	
	d.f	Chisq	p-value	Chisq	p-value	Chisq	p-value	Chisq	p-value	Chisq	p-value
F. poae (FP)	1	0.10	0.75	1.81	0.18	0.18	0.67	0.023	0.87	0.0522	0.82
F. graminearum (FG)	1	11.47	<0.0001	16.41	<0.0001	0.14	0.71	0.30	0.58	0.7934	0.37
FP*FG	1	2.03	0.16	4.70	0.26	0.38	0.54	1.25	0.26	0.8556	0.36
Genotype (G)	4	2.26	0.69	5.25	0.03	3.64	0.46	20.02	0.0005	23.2228	<0.0001
FP x G	4	3.71	0.45	9.52	0.05	6.16	0.19	0.89	0.93	1.1394	0.89
FG x G	4	32.31	<0.0001	25.92	<0.0001	0.51	0.97	1.08	0.90	1.6520	0.80
FP x FG x G	4	4.19	0.38	14.91	0.01	4.84	0.31	3.54	0.47	3.9729	0.41
2015											
F. poae (FP)	1	0.17	0.68	5.24	0.02	2.43	0.12	0.05	0.83	2.0318	0.15
F. graminearum (FG)	1	1.17	0.28	0.53	0.47	0.81	0.37	0.42	0.52	1.6586	0.20
FP*FG	1	0.85	0.36	1.80	0.18	0.89	0.35	0.01	0.93	0.0400	0.84
Genotype (G)	4	21.76	<0.0001	186.60	<0.0001	9.42	0.05	8.09	0.09	151.3667	<0.0001
FP x G	4	34.19	<0.0001	49.82	<0.0001	3.27	0.51	0.68	0.95	3.2791	0.51
FG x G	4	2.77	0.60	2.35	0.67	2.86	0.58	0.95	0.92	2.0991	0.72
FP x FG x G	4	3.37	0.50	3.88	0.42	4.04	0.40	11.42	0.02	1.0364	0.90
2016											
F. poae (FP)	1	5.15	0.02	4.88	0.03	1.76	0.18	0.77	0.38	0.0021	0.96
F. graminearum (FG)	1	7.21	0.01	10.10	0.002	1.85	0.17	0.07	0.79	0.1604	0.69
FP*FG	1	19.83	<0.0001	24.93	<0.0001	0.07	0.79	2.99	0.08	0.7424	0.39

Genotype (G)	4	34.90	<0.0001	33.96	<0.0001	2.42	0.66	2.19	0.70	7.5830	0.11
FP x G	4	21.80	<0.0001	33.76	<0.0001	3.56	0.47	11.76	0.02	2.2611	0.69
FG x G	4	6.60	0.16	1.84	0.28	7.24	0.12	6.85	0.14	6.8474	0.14
FP x FG x G	4	4.98	0.2897	8.62	0.07	8.71	0.07	7.59	0.11	4.1841	0.38

Table 3. Analysis of variance for different grain quality parameters (d.f.: degrees of freedom; Chisq: Type II Wald Chi-square tests).

Source	2014							
	d.f	D-hordeins		C-hordeins		B-hordeins		Total hordeins content	
		Chisq	p-value	Chisq	p-value	Chisq	p-value	Chisq	p-value
F. poae (FP)	1	23.27	<0.0001	29.55	<0.0001	15.11	<0.0001	28.09	<0.0001
F. graminearum (FG)	1	25.92	<0.0001	34.31	<0.0001	12.40	0.0004	29.73	<0.0001
FP*FG	1	25.83	<0.0001	37.90	<0.0001	5.01	0.03	26.60	<0.0001
Genotype (G)	4	13.09	0.01	2.44	0.66	10.13	0.04	6.02	0.20
FP x G	4	7.01	0.14	0.93	0.92	8.24	0.08	3.48	0.48
FG x G	4	8.82	0.07	0.65	0.96	2.57	0.63	1.39	0.85
FP x FG x G	4	0.99	0.43	2.39	0.67	5.28	0.26	2.14	0.71
***Fusarium* Treatments**		**Mean ± SEM**		**Mean ± SEM**		**Mean ± SEM**		**Mean ± SEM**	
FP_1FG_0		6.24 ± 1.06 a		32.03 ± 4.75 a		57.69 ± 9.10 a		95.96 ± 12.40 a	
FP_0FG_1		5.51 ± 1.05 a		27.52 ± 5.89 a		60.14 ± 9.35 a		93.17 ± 15.15 a	
FP_1FG_1		6.22 ± 0.95 a		35.23 ± 9.15 a		49.10 ± 5.60 a		90.54 ± 14.26 a	
FP_0FG_0		33.21 ± 5.07 b		155.94 ± 7.91 b		98.70 ± 9.31 b		287.85 ± 17.89 b	

Table 4. Grain contamination with deoxynivalenol (DON), 3-acetyl deoxynivalenol (3-ADON), 15-acetyl deoxynivalenol (15-ADON) and nivalenol (NIV) in five barley genotypes during 2014, 2015 and 2016 growing seasons. Mean ± SEM values were from the log transformed data.

Year	Inoculation	DON (µg/g)	3-ADON (µg/g)	15-ADON (µg/g)	NIV (µg/g)
2014	*F. poae*	n.d.	n.d.	n.d.	1.18 ± 0.06
	F. graminearum	6.12 ± 1.77	14.60 ± 7.71	1.21 ± 0.05	n.d.
	F. poae / *F. graminearum*	4.69 ± 1.35	21.60 ± 11.41	1.18 ± 0.05	1.20 ± 0.06
	Control	1.87 ± 0.54	4.20 ± 2.22	n.d.	n.d.
2015	*F. poae*	n.d.	n.d.	1.29 ± 0.10	n.d.
	F. graminearum	1.53 ± 0.11	3.97 ± 0.98	1.39 ± 0.11	n.d.
	F. poae / *F. graminearum*	1.37 ± 0.09	5.20 ± 1.29	1.41 ± 0.11	n.d.
	Control	1.46 ± 0.10	3.74 ± 0.93	1.26 ± 0.10	n.d.
2016	*F. poae*	n.d.	n.d.	n.d.	n.d.
	F. graminearum	1.02 ± 0.002	2.58 ± 0.30	1.39 ± 0.11	n.d.
	F. poae / *F. graminearum*	1.02 ± 0.002	2.38 ± 0.28	1.41 ± 0.11	n.d.
	Control	n.d.	2.83 ± 0.34	n.d.	n.d.

*nd: non detected

Figure legends

Fig.1. Left: Incidence **(A)**, severity **(B)** and FHB Index **(C)** values for different treatments in 2014, 2015 and 2016. Right: Differences between genotypes for incidence **(D)**, severity **(E)** and FHB Index **(F)** in 2015 growing seasons. Treatments: *F. poae* (FP_1FG_0), *F. graminearum* (FP_0FG_1), both pathogens (FP_1FG_1) and control without *Fusarium* (FP_0FG_0). Mean ± SEM. Columns with different letters are statistically different according to Tukey´s test at $p \leq 0.05$.

Fig.2. Relative abundance and fractions of polyacrylamide gel (SDS-PAGE; T% = 13.5%) showing the pattern of D-hordeins, C-hordeins, and B-hordeins. Treatments: *F. poae* (FP_1FG_0), *F. graminearum* (FP_0FG_1), both pathogens (FP_1FG_1) and control without *Fusarium* (FP_0FG_0).