Effects of *Fusarium graminearum* and *Fusarium poae* on disease parameters, grain quality and mycotoxins contamination in bread wheat (Part I)

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Running title: Interaction between *F. graminearum* and *F. poae* in wheat

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ABSTRACT

BACKGROUND: Wheat is the most important winter crop in the world being affected by the presence of fungal mainly those belonging to the *Fusarium* genus. *Fusarium* Head Blight (FHB) is a serious disease that causes important economic damages and quantitative/qualitative losses, being *F. graminearum* and *F. poae* two of the most isolated species worldwide. The aim of this study was to evaluate the interaction between *F. graminearum* and *F. poae* and the effects on disease parameters, grain quality and mycotoxins contamination on five wheat genotypes under field conditions in three growing seasons.

RESULTS: Statistical differences between *Fusarium* treatments were found for disease parameters, grain quality and mycotoxins contamination during 2014/2015 growing season. High values of incidence (58.00 ± 8.00%), severity (6.28 ± 1.51%) and FHB index (4.72 ± 1.35) were observed for *F. graminearum*+*F. poae* treatment. Regarding grain quality, results showed that the degradation of different protein fractions depends on each *Fusarium* species: glutenins were degraded preferably by *F. graminearum* (-70.82%), gliadins were degraded preferably by *F. poae* (-29.42%), while both protein fractions were degraded when both *Fusarium* species were present (-60.91% and -16.51%, respectively). Significant differences were observed for mycotoxin contamination between genotypes, being Proteo the most affected (DON=12.01 ± 3.67 µg/g). In addition, we reported that 3-ADON predominated over 15-ADON in the three seasons evaluated.

CONCLUSIONS: Variations in plant-pathogen interaction (*Fusarium*-wheat pathosystem) should be considered at least in the years with favorable climatic conditions to FHB development, due to the potential impact of this disease on grain quality and mycotoxin contamination.

Keywords: *Fusarium*, wheat, mycotoxins, disease parameters, grain quality, plant-pathogen interaction.

Introduction

Wheat (*Triticum aestivum* L.) is the most important winter crop in the world and the production is aimed at export and local consumption. The wheat world production was about 758.38 million tons during 2017/2018 harvest, with the US Department of Agriculture (USDA) estimating that the production for 2018/2019 will be about 747.76 million tons. The main wheat producers during 2017/2018 harvest season were the European Union (151.58 million tons), China (129.77 millions of tons) and India (98.51 million tons)[1]. Wheat grains can be used for animal feed, although their main use is for flour production and baking. This is possible due to the viscoelastic properties of wheat dough that make it suitable for various bakery products, where gluten proteins play an important role in determining wheat baking quality. Thus, gliadins and glutenins represent 80-85% of the total protein in mature wheat grain[2].

One of the major diseases that occur in most of the cereal regions worldwide and causes serious economic damage in wheat is *Fusarium* Head Blight (FHB). The FHB is observed mainly in regions with warm and wet climate during the flowering stage of this cereal. This disease causes both quantitative and qualitative losses. Firstly, there is a reduction in yield, decreasing the thousand kernel weight (TKW), reducing the germination and vigor of the seeds. Secondly, FHB reduces the starch content of the grains and degrades different subunits of proteins, causing a lower baking quality along with the presence of harmful toxins for health[3]. Among *Fusarium* species causing FHB, *Fusarium graminearum* is the dominant species isolated worldwide but over the last years, *F. poae* has been found by several researchers in diverse substrates such as barley and wheat[34]. Covarelli *et al.*,[4] showed that *F. poae* increases its presence when the climatic conditions are not suitable for *F. graminearum* growth. At present, in Argentina and in different countries in the world, *F. graminearum* and *F. poae*

predominate over other *Fusarium* species[5]. However, it is not clear if both pathogens are more aggressive if they infect together or separately, and their potential damages to the crops.

The most important toxins produced by *Fusarium* spp. are trichothecenes (inhibitors of eukaryotic protein synthesis) that can cause adverse effects in humans and animals through ingestion of contaminated cereal grain. The type and amount of mycotoxins produced by a species vary on an annual basis, depending mainly on environmental, crop and storage factors[6]. *F. graminearum* has the capacity to produce a wide spectrum and quantity of toxins, especially deoxynivalenol (DON) and its acetylated derivatives (3-ADON and 15-ADON), which are associated with feed refusal, vomiting and suppressed immune functions[7]. Besides, *F. graminearum* has the ability to produce other micotoxins such as aurofusarin, fusarin C, nivalenol (NIV) and zearalenone, an important mycotoxin that can affect sexual reproduction causing hyperstrogism in monogastrics[7,8,]. On the other hand, *F. poae* is the main *Fusarium* pathogen able to produce high amounts of nivalenol, an important mycotoxin that inhibits cell proliferation and produces cytotoxic effects on cells[9]

Fusarium spp. has a significant effect on grain yield, causing losses of up to 70% in wheat[10]. Regarding grain quality, wheat grain proteins are composed by a fraction soluble in aqueous solutions (albumins and globulins), and a fraction insoluble (gliadins and glutenins) that are responsible for the gluten formation. Glutenins (GLU) form intra and intermolecular disulfide bonds and are classified into high molecular weight (HMW) and low molecular weight (LMW) subunits, while gliadins (GLI) form only intramolecular disulfide bonds and are divided into four structural types (α-, β-, γ- and ω-gliadins). *Fusarium* spp. infection and the consequent production of proteases by

the fungus lead to gluten degradation thus changing dough consistency and resistance to extension. This results in a loss of dough functionality and loaf volume[11].

Currently, there are not many works on wheat that evaluate the interaction of *Fusarium* species under field conditions, being scarce the information concerning about their potential impact on mycotoxin contamination[12,13]. Early colonization of the lemma by *F. poae* may promote subsequent colonization by *F. graminearum*[14]. Furthermore, the presence of NIV can synergize the toxicity of DON and its acetylated derivatives, thus increasing the potential risk in food safety[15]. In co-inoculations, the competence for resources between *Fusarium* species can produce more toxins under these stress conditions, although no evidence was found to support synergism between fungal isolates in causing visual symptoms[13,16]. However, it is unknown if synergism or interaction can occur between *F. graminearum* and *F. poae*. Therefore, the aim of this study was to evaluate the effects of *F. graminearum* and *F. poae* presence on disease parameters, grain quality and mycotoxin contamination in bread wheat.

Materials and methods

Fusarium isolates. In the present work, *F. graminearum*, *F. poae* and a mixture of *F. graminearum*+*F. poae*, were used as inoculum in the fields experiments. Regarding *F. poae* (FP_1FG_0), a mixture of four isolates of *F. poae* (FP-TSa1b, FP-TBig1a, FP-TMa1a, and FP-TPe1a) based on the production of NIV *in vitro* were used.[5] In this case, the fungal inoculum was produced by placing individual agar plugs with mycelium in Petri dishes (90 mm) with potato dextrose agar 2% (PDA). The time of incubation for *F. poae* was 7 days at 25 ± 2°C under 12 h each of light and darkness. The conidial harvest was done by flooding the plates with 5 ml of sterile distilled water

(SDW) and dislodging the conidia with a bent glass rod. Then, the resulting suspension was filtered through a cheesecloth, and the conidial suspension was adjusted to 1×10^5 conidia/ml with a Neubauer hemacytometer for each *F. poae* isolate[17]. 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.

On the other hand, for *F. graminearum* (FP_0FG_1) a mixture of four isolates with the ability to produce mainly 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)[18]. These isolates were cultured on PDA 2% for 7 days at room temperature. The *F. graminearum* macroconidial suspension was produced by transferring four PDA plugs (1 cm x 1 cm) of the established fungal culture to 50 ml of carboxymethylcellulose (CMC) broth (CMC 15 g, NH_4NO_3 1 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 0.5 g, yeast 1 g and H_2O 1 L) in 250 ml Erlenmeyer flask. The culture was incubated on a rotary shaker for 10 days (100 rpm, 25 ± 2°C and darkness). The conidial harvest was done by filtering the resulting suspension through a cheesecloth, adjusting the conidial suspension to 1×10^5 conidia/ml with a Neubauer hemacytometer for each *F. graminearum* isolate[17]. 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.

Finally, the inoculum composed of the mixture of *F. graminearum* and *F. poae* (FP_1FG_1) was prepared by mixing equal parts of the inoculums descripted above, with a final concentration of 1×10^5 conidia/ml. In addition, a mock inoculum prepared with SDW was used as control treatment (FP_0FG_0). ***Wheat genotypes.*** Five genotypes (G) of bread wheat were evaluated: Klein León (high yield, poor baking quality, and moderate susceptibility to FHB); Klein Nutria (high yield, high values of gluten and moderate susceptibility to FHB); Klein Proteo (good baking quality, high protein and gluten,

moderate susceptibility to FHB); Buck AGP Fast (good baking quality andlow susceptibility to FHB); and Buck Pleno (high yield, good baking quality and moderate susceptibility to FHB)[19,20]. These genotypes were chosen due to intermediate-short maturity cycle and the similarity of the time until the anthesis.

Experimental design. Field experiments were carried out in 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 experiments 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, to ensure uniformity in the timing of anthesis and 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 (insecticides and herbicides were applied as needed), without supplemental irrigation or fungicide treatments. Wheat heads were inoculated in mid-anthesis at Z.60-Z.65 and 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 in order 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 avoid cross contamination, the plastic panels were rinsed twice with SDW between *Fusarium* treatments and dried. Finally, to check the possibility of contamination with other *Fusarium* spp., 100 grains/plot 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 2% 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[8].

The field experiment was a split-plot design with four blocks, where the main plot was the wheat genotype, while the subplot was the *Fusarium* treatment. 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 of incidence (number of spikes with symptoms), severity (number of spikelets with symptoms per spike) and FHB Index (incidence*severity/100) was conducted at 21 days post-inoculation by counting the number of symptomatic spikelets (typical lesions or bleaching of spikelets) of 40

spikes/subplot selected at random[21]. After physiological maturity, each subplot was harvested manually and threshed using a wheat stationary tresher (Forti©)

Grain yield per subplot (g/m^2) was measured. Protein concentration, the percentage of moisture and percentage of wet gluten were measured with NIT analyzer with double-face monochromator (Agricheck®, Bruins Instruments, USA). The germinative energy and the germinative power of the seeds were evaluated in accordance with International Seed Testing Association (ISTA) protocols. Sodium dodecyl sulfate microsedimentation test (SDSS) was used to predict the gluten strength. To show the effects of each *Fusarium* species on each protein subunit during the three growing seasons evaluated, the glutenin (GLU) and gliadin (GLI) proteins were extracted by a sequential extraction method. 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 (trichloroacetic acid) 12% for 48 h and finally washed in SWD for 24 h. Also, the resulting gels were scanned and analyzed using TotalLab (v1.10) 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 GLI, GLU, HMW-GS (high molecular weight glutenin subunits), LMW-GS (low molecular weight glutenin subunits), ω-gliadins and α-β-γ-gliadins contents were evaluated. Also, the GLI/GLU ratio, the HMW-GS/LMW-GS ratio, and the ω-gli/α-β-γ-gli ratio were calculated.

For toxins analyses, during the harvest the grain samples were obtained from the five central furrows of the plot (1m x 1m), eliminating the two lateral furrows. To obtain a representative subsample, each sample obtained (around 300 to 500 g) were completely homogenized. Then, the samples were reduced successively using a grain divider (Cereal Tools®) until obtaining 25 g, and ground with a high speed disintegrator

FW-110 (Arcano©, Pasteur Instrumental). 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 Rotavap®. Gas chromatography, with 63Ni electron capture detection Shimadzu Model GC17, equipped with a split/splitless injector and fitted with RX-5MS capillary column (25 mm x 0.2 mm id), were used to detect and quantify trichothecenes[22]. The quantification limits were 0.06 µg/g for DON and 0.15 µg/g for NIV, while the detection limits were 0.02 µg/g for DON and its acetyl derivatives, and 0.05 µg/g for NIV. Standards used of DON, 15-ADON, 3-ADON, and NIV were from SIGMA Chemical Company (St Louis, MO, USA). Toxins present in treatments are refered to the grains that contain toxins after the specific treatment.

Statistical analysis. All the variables evaluated were analyzed using the software R (v.3.3.3, R Core Team 2018). 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 wheat genotype and two levels (presence or absence) of each *Fusarium* species nested within the genotype, with 4 blocks for each combination of treatments. Comparisons were performed using linear mixed models: A) for variables with a normal distribution of the error we used a linear mixed-effects model (lme); B) for variables with a non-normal distribution of the error we used a generalized linear mixed model (glm). *Fusarium* treatments and genotype were considered as fixed effects, while block and main-plot were assigned as random effects.

Among the variables evaluated, the following showed a normal distribution of the error: protein concentration (PC), SDS sedimentation test (SDSS), thousand kernel weight (TKW), grain yield (GY), wet gluten (WG), protein fractions (gliadins and glutenins) and concentration of toxins (DON, 3-ADON, 15-ADON and NIV). These

variables were analyzed using the lmer function (lme4 package)[23]. 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.

On the other hand, the following variables showed a non-normal distribution of the error: incidence (I), severity (S), FHB Index, germinative energy (GE) and germinative power (GP). These variables were analyzed using a generalized linear mixed model that considered the restrictions in the randomization and non normal errors with the function glmer (lme4 package)[23].Significance was tested with Type II Likelihood Ratio Test and tested with the lsmeans function (emmeans package)[24]. Results were reported as the mean ± standard error of the mean (SEM) and significance with α=0.05.

Results

Climatic Conditions. The environmental conditions in the three years were different particularly during the flowering stage (Fig. 1). 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). In contrast, in 2015 and 2016 growing seasons the weather conditions were not optimal for the development of the disease. 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).

Disease parameters. Analysis of the control plots indicated lower development of disease symptoms for each year compared to inoculated treatments. In 2014 the greatest level of visual symptoms was observed, while in 2015 and 2016 fewer symptoms were reported. All genotypes showed symptoms with all the *Fusarium* treatments used as inoculum. Significant differences ($p<0.05$, $n=80$) were detected for the different *Fusarium* treatments in incidence, severity and FHB Index depending on the year (Fig. 2). In 2014, FP_1FG_1 treatment show higher incidence values (58.00 ± 8.00%) with respect to FP_0FG_1 (52.00 ± 6.00%) and FP_1FG_0 (49.00 ± 6.00%), although there were no significant differences between *Fusarium* treatments. In addition, this trend coincided with higher values of severity and FHB Index in this growing season. On the other hand, no significant differences were observed for incidence and FHB Index in 2015 and 2016, being similar in terms of visualization of symptoms. For incidence, the highest incidence values were observed in FP_0FG_1 (30.00 ± 2.00% in 2015 and 28.00 ± 2.00% in 2016). Regarding the FHB Index, lower values were reported showing FP_0FG_1 the highest values (0.34 ± 0.09) in 2015, while in 2016 the highest values were observed for FP_1FG_1 (0.53 ± 0.17%). As to severity, no significant differences were observed in none of the years evaluated.

Germination and grain quality parameters. For GE and GP, no significant differences were observed in all the years, except for GE in 2015 between genotypes. In the same way, no effects of *Fusarium* treatments were observed in parameters such as

PC, SDSS and WG showing only significant differences ($p<0.05$, n=80) for genotype. However, for GY as in TKW, there were significant differences ($p<0.05$, n=80) only in 2015 for FP_0FG_1 x G interaction (Supp.Info 1).

For PC, genotypes Proteo (11.94 ± 0.23%) and Nutria (10.89 ± 0.19%) showed the highest values in all the years with respect to the rest of the genotypes. Regarding SDSS, the same tendency was observed in the three years, showing Pleno (109.97 ± 1.71 mm) and Proteo (97.34 ± 1.66 mm) the highest values of sedimentation. Wet gluten values also showed that Proteo (30.12 ± 0.55%) and Nutria (27.19 ± 0.49%) were the genotypes with the highest percentage in all the growing seasons analyzed.

For GY, interaction FP_0FG_1 x G were found in 2015, being Proteo the genotype most affected, decreasing by 24.25% (from 303.50 g/m^2 to 229.90 g/m^2) followed by León with a decrease of 13.56% (from 293.50 g/m^2 to 253.70 g/m^2). In the same way, for TKW the genotype most affected was León decreasing by 6.44% (from 50.96 g to 47.68 g) followed by Proteo with a decrease of 2.04% (from 42.21 g to 41.35 g).

Protein composition. The HMW-GS (h*igh molecular weight gluten subunits*) allelic composition of each wheat genotypes was the following for *Glu-A1*, *Glu-B1* and *Glu-D1*, respectively: AGP Fast (not identified; 13+16, 5+10); León (2*, 7+9, 2+12); Nutria (2*, 7+9, 5+10); Pleno (2*, 7+8, 19 5+10) and Proteo (1, 7+9, 5+10). The analysis of protein fractions was significant only in 2014, while during 2015 and 2016 no significant differences were observed. In 2014, significant differences ($p<0.05$, n=60) were detected for *Fusarium* treatments and genotypes (Supp.Info 2). Protein degradation varied between *Fusarium* treatments, with *F. graminearum* showing more preference to degrade glutenins, while *F. poae* showed more preference to degrade

gliadins. Interestingly, when both *Fusarium* species were inoculated together, both protein fractions were affected (Fig. 3).

The values of relative abundance (pixel intensity) indicate that glutenins were affected by FP_0FG_1 decreasing in 70.82% compared to the control (Fig. 4), followed by FP_1FG_0 (61.97%) and FP_1FG_1 (60.91%). On the other hand, FP_1FG_0 was the treatment that most affected the gliadin fraction reducing in 29.42 %, followed by FP_0FG_1 (25.14%) and FP_1FG_1 (16.51%). Regarding the GLI/GLU ratio, there were no statistical differences, but a slight trend was observed indicating a higher ratio compared to the control for FP_1FG_1 (3.54 fold) followed by FP_0FG_1 (2.91 fold) and FP_1FG_0 (2.25 fold).

Regarding glutenin fractions, HMW-GS was more affected by FP_0FG_1 decreasing in 60.00%, followed by FP_1FG_0 (57.80%) and FP_1FG_1 (38.85%), while LMW-GS fraction was also affected by FP_0FG_1 reducing by 74.93 % and then followed by FP_1FG_1 (69.23%) and FP_1FG_0 (63.49%). However, no significant differences were found in the HMW-GS/LMW-GS ratio. For ω-gli fraction, it was observed that FP_1FG_0 was the treatment with the highest degradation of this protein fraction (37.85%) followed by FP_0FG_1 (37.52%) and FP_1FG_1 (26.82%). In addition, FP_1FG_0 was the treatment that most affected α-β-γ-gli decreasing by 26.34 %, followed by FP_0FG_1 (20.30%) and FP_1FG_1 (12.60%). Regarding ω-gli/α-β-γ-gli ratio, no significant differences were found.

Mycotoxins contamination. Due to the natural existence of *Fusarium* spp. present in the environment, the analysis of the control plots indicated slight contamination with mycotoxins. In 2014, the highest concentrations of DON (5.06 ± 2.47 µg/g) and 15-ADON (0.13 ± 0.04 µg/g) were observed in FP_0FG_1 grains after the treatment (hereafter *Fusarium* treatment), while the major values for 3-ADON (55.76 ± 31.37 µg/g) were in FP_1FG_1 (Table 1). For DON, significant differences (p=0.0377,

n=60) were observed only for genotype, showing that the genotype with the highest DON accumulation was Proteo (12.01 ± 3.67 µg/g), while the least contaminated was Pleno (0.84 ± 0.19 µg/g) (Fig. 5). For 3-ADON and 15-ADON no significant differences were found. Regarding NIV concentrations, only two genotypes showed contamination above the limit of quantification during 2014(Proteo: 0.36 ± 0.14 µg/g; and León: 0.18 ± 0.03 µg/g).

Similarly, in 2015 the highest concentrations of DON (4.54 ± 1.62 µg/g) and 15-ADON (2.98 ± 1.49 µg/g) were observed in FP_0FG_1, while the major values for 3-ADON (11.10 ± 2.21 µg/g) were in FP_1FG_1. Significant differences were observed only for genotypes in DON ($p<0.0001$, n=80) (Fig. 5). In the first case, Proteo was the genotype with the highest amount of DON (8.74 ± 2.01 µg/g), while León showed the lowest values (1.66 ± 0.68 µg/g). For acetylated derivatives, no significant differences were found in this growing season. Regarding NIV, during 2015 and 2016 concentrations were below the limits of quantification.During 2016, the highest values of mycotoxins were observed in FP_0FG_1 for DON (1.01 ± 0.29), 3-ADON (30.96 ± 7.21) and 15-ADON (1.03 ± 0.19). . For all the mycotoxins analyzed in this growing season, no significant differences were found between *Fusarium* treatments or genotypes.

Discussion

The three growing seasons evaluated were different from each other in terms of environmental conditions such as relative humidity, temperature, and accumulated rainfall during the period from anthesis to harvest (Fig. 1). For disease parameters significant differences between the *Fusarium* treatments were found only in 2014, but no statistical differences that could demonstrate antagonism or synergism were

observed when *F. graminearum* and *F. poae* were inoculated together (Fig. 2). In 2014, we registered the highest disease values, due to warm temperatures and wet conditions at the flowering stage that were favorable for the development of the disease In contrast, during2015 and 2016 the lowest temperatures and the driest conditions decrease FHB development causing minor visual symptoms. In agreement with Lori *et al.*[25], we observed that FHB symptoms are associated with the total rainfall accumulated and also with the pattern of distribution during the flowering stage. The difference observed in visual symptoms between *F. poae* and *F. graminearum* treatments could be explained because *F. poae* colonize floral tissues to a greater extent than they do grain, while *F. graminearum* may colonize both tissue types to a similar extent[13].

Tillage practices were similar among the three years, therefore our results indicated that the variations in climatic factors played an important role in disease symptoms. It is known that environmental conditions play a fundamental role in *Fusarium*–host interactions, depending on the different climatic factors of each growing season, mainly during the flowering stage[12]. Different thermo-hygrometric conditions in this stage can explain the variations in the presence of different *Fusarium* species, and when the conditions were not favorable for the main causal agents of FHB such as *F. graminearum*, other species as *F. poae* could increase significantly their presence.[4] Wet conditions and warm temperatures (about 28°C) are favorable for *F. graminearum* infection, while dry conditions and temperatures around 25°C are for *F. poae*[16]. Furthermore, it could be observed that the lowest values of symptoms in 2015 and 2016 were due to daily minimum temperatures below 9°C and maximum temperatures greater than 26°C registered around anthesis that may inhibit or decrease fungal growth[26].

Fusarium species cause yield loss and deterioration on wheat grain quality[27]. In the current study, we did not observe statistical differences between the *Fusarium*

treatments in terms of parameters such as PC, SDSS and WG, showing only differences among genotypes (Supp.Info 1). In the same way, other studies agree with our results showing that no differences were in *Fusarium* infected samples for these parameters. Eggert *et al.*[28] and Wang *et al.*[29] found that protein concentration did not change significantly with respect to *F. graminearum* or *F. culmorum* infection, while in naturally infected samples Hysing & Wiik[30] found that there were no significant differences for wet gluten and protein concentration at all levels of infection tested. Regarding GY and TKW, we observed a decrease of up to 24.25% and 6.44% (respectively) depending on the genotype, coinciding with Hysing & Wiik[30] that reported a decrease of 4.00% in TKW and a correlation with the level of infection with *Fusarium* spp.($r=0.76$, $p=0.020$). These differences between genotypes for GY and TKW could suggest that the existing genetic differences are likely to be more important than other agronomic factors (such as soil tillage and the use of fertilizer) against *Fusarium* infection[25].

Gluten proteins play an important role in determining the wheat processing quality, being glutenins and gliadins responsible for the viscoelastic properties of the dough[2] *Fusarium* infection can affect the baking properties in relation to changes in the protein fractions [29,31]. In the current work, we observed statistical differences for *Fusarium* treatments (Supp.Info 2), decreasing the glutenin fractions such as HMW-GS (52. 22%) and LMW-GS (69.22%). Furthermore, we observed a reduction in the gliadin fractions, for ώ-gliadins (34.06%) and α-β-γ-gliadins (19.75%). Therefore, the ratio GLI/GLU was increased because we observed a higher degradation of the glutenins (64.57%) than the gliadins (23.69%). These results could be explained since *Fusarium* infection produces different hydrolytic enzymes such as cellulases, cutinases, proteinases, and xylanases. The fungal proteases activity such as trypsin protease or

serine protease (which are known to be protein-degrading enzymes) is part of the exoproteome of *Fusarium* and they could be responsible for the changes observed in the gliadins/glutenins ratio[32]. This change in the GLI/GLU in favor of gliadins fraction, was reported by Eggert et al.[11] in wheat and emmer (*Triticum dicoccum*), showing a preferential degradation of glutenins with respect to gliadins. In addition, a high amount of degraded glutenins was found within the gliadin fraction after digestion and extraction. These results can explain the increase in the GLI/GLU ratio after infection with *Fusarium* and may mask the degradation of gliadins and. Other causes that may explain the increase in GLI/GLU ratio: 1) the reduced protein synthesis produced by *Fusarium* infection; 2) the subsequent inhibition of protein synthesis by the accumulation of DON; 3) the formation of polymeric glutenins in the later maturation stages[29].

As regards to the potential impact on baking quality, several authors have reported that the presence of proteolytic enzymes that can affect glutenin/gliadin fractions are consistent with: 1) the significant reduction in the resistance to extension; 2) an inverse relationship between loaf volume and the level of FDK contamination; 3) a decrease in dough functionality and loaf volume accentuated in baking processes that involve long fermentation periods[31]. In our work, we observed that *Fusarium graminearum* degraded in more proportion glutenin fractions, while *F. poae* affected gliadins. In the same way, Brzozowski *et al.*[33] demonstrated in wheat that proteolytic intra and extracellular enzymes synthesized by *F. poae* were capable of gliadin hydrolysis in a greater proportion than other species such as *F. graminearum*. In our work, when both *Fusarium* species were inoculated together, gliadins (ω and α-β-γ gliadins) and glutenins (HMW and LMW) were degraded in a remarkable proportion,

although there was no evidence to support synergism regarding the impact on protein fractions and baking properties.

We found mycotoxins in all the *Fusarium* treatments evaluated. Between *Fusarium* treatments no statistical differences were observed for mycotoxins concentration (Table 1), assuming that synergism was not found under these experimental conditions. However, we reported genotype effects in 2014 and 2015 (Fig. 5), showing different mycotoxin contamination in each genotype. Because the flowering patterns between genotypes were similar in the years analyzed, these differences in mycotoxin contamination could be due to differences in FHB resistance (polygenic resistance)[34].In 2014 we expected a higher mycotoxin contamination compared to 2015 and 2016, related to the major values of incidence/FHB index observed in the field. In 2014 we observed a slight predominance of *F. graminearum* respect to *F. poae*, therefore we expected to find a major proportion of DON and its acetylated derivatives compared to NIV. In this way, we observed that mycotoxin concentration varied greatly between *Fusarium* treatments, genotypes and years, observing the highest level of DON in 2014 (5.06 ± 2.47 µg/g) and for 15-ADON in 2015 (2.98 ± 1.49 µg/g) in both cases for *F. graminearum* treatment, while for 3-ADON the contamination was the highest (55.76 ± 31.37 µg/g) in 2014 for *F. graminearum* x *F. poae* treatment (Table 1). Regarding NIV, in several previous studies it was the most frequent mycotoxin found, being more toxic than DON or its acetylated derivatives[9,35,36,37]. In the present work, we observed low values of NIV (0.19 ± 0.05 µg/g) in 2014 for *F. poae* treatment. Stenglein *et al.*[38] found higher levels of NIV in wheat genotypes inoculated under field conditions (ranging between 0.30 and 16.10 µg/g). These differences in the NIV concentration may be due to differences in the *Fusarium* isolates used, differences in the genotypes and/or differences in the environmental conditions during the flowering stage. Recently, Nazari

et al.[39] found in vitro conditions that the optimum temperature for *F. poae* NIV production is 27.5 °C (ranging between 20 °C and 35°C), while in our work the average temperatures during the flowering stage were below in 2014, 2015, 2016 (18.22 °C, 17.65 °C and 17.62 °C, respectively). In general terms, the most common trichothecene found in cereal grains is DON, which often occurs along with relatively small amounts of acetylated derivatives[14]. In the last years, several authors have reported the simultaneous production of 3-ADON and 15-ADON in comparable amounts by *F. graminearum*, finding a tendency to increase the simultaneous production of both DON-acetylated derivatives[15,18,22].In the current work, we found a major concentration of 3-ADON along the different years (Table 1), despite the fact that the *F. graminearum* isolates used were DON, 3-ADON and 15-ADON producers. A possible explanation for the prevalence of 3-ADON is that the secondary metabolite biosynthesis could be affected mainly by temperature and other environmental conditions[40]. Recently, Ramírez Albuquerque *et al.*[41] found under *in vitro* conditions that the temperature plays an important role in the mycotoxin production, being the production of 3-ADON maximum at 25-30°C, while the production of 15-ADON is maximum at 10°C. In addition, these results could also depend on the interaction between the *Fusarium* isolates chosen for the mixture and the chemotype of each of them[42].

Conclusions

This research presents novel information about the interaction between *F. graminearum* and *F. poae* on wheat genotypes under field conditions.However, many questions remain to be addressed and further work is clearly needed to determine how plant-pathogen interaction can impact on the disease development, grain quality and mycotoxin contamination. We conclude that: 1) at least in years with favorable conditions for FHB development (*F. graminearum*) a trend was observed suggesting

that synergism between *F. graminearum* and *F. poae* could exist in disease parameters; 2) degradation of different protein fractions depends on each *Fusarium* species (glutenins were degraded preferably by *F. graminearum*, gliadins were degraded preferably by *F. poae*, both protein fractions were degraded when both *Fusarium* species were present); 3) different concentrations of mycotoxins (mainly DON) were observed among the genotypes evaluated, that could be useful in the future for genetic improvement. Nevertheless, it is known that exist strong effects of the genotype (variations in plant-pathogen interaction) and the year (variations in climatic conditions) on the *Fusarium*-wheat pathosystem, therefore more studies are deemed necessary even under controlled conditions to confirm these results.

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Conflict of interest

The authors declare no have conflict of interest.

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Table 1. Grains contamination with Deoxynivalenol (DON), 3-Acetyl Deoxynivalenol (3-ADON), 15-Acetyl Deoxynivalenol (15-ADON) and Nivalenol (NIV) in five wheat genotypes during 2014, 2015 and 2016 growing seasons.

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.	0.19 ± 0.05
	F. graminearum	5.06 ± 2.47	44.45 ± 35.18	0.13 ± 0.04	n.d.
	F. poae / *F. graminearum*	3.00 ± 1.22	55.76 ± 31.37	0.12 ± 0.04	0.12 ± 0.04
	Control	1.03 ± 0.23	8.02 ± 1.25	0.09 ± 0.04	0.08 ± 0.03
2015	*F. poae*	n.d.	n.d.	n.d.	0.11 ± 0.02
	F. graminearum	4.54 ± 1.62	10.75 ±3.16	2.98 ± 1.49	n.d.
	F. poae / *F. graminearum*	3.53 ± 0.80	11.10 ±2.21	1.59 ± 1.12	0.11 ± 0.02
	Control	1.95 ± 0.95	9.11 ± 1.67	0.49 ± 0.08	0.09 ± 0.02
2016	*F. poae*	n.d.	n.d.	n.d.	0.02 ± 7.3e-4
	F. graminearum	1.01 ± 0.29	30.96 ± 7.21	1.03 ± 0.19	n.d.
	F. poae / *F. graminearum*	0.99 ± 0.31	22.28 ± 5.43	0.71 ± 0.23	0.02 ± 1.5e-3
	Control	0.79 ± 0.11	22.57 ± 2.83	0.50 ± 0.07	0.02 ± 1.5e-3

n.d.: non detected.

Figure legends

Fig.1. Relative humidity (RH %), precipitation (mm), maximum temperature (TMax), medium temperature (TMed) and minimum temperature (TMin), from inoculation to harvest. **A**: 2014, **B**: 2015, **C**: 2016.

Fig.2. Incidence **(A)**, severity **(B)** and FHB Index **(C)** values for different treatments in 2014, 2015 and 2016. 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. Different letters are statistical differences according to Tukey´s test at $p \leq 0.05$.

Fig.3. Degradation of different protein subunits in *Fusarium* damaged kernels (FDK). **A)** Relative abundance and fractions of polyacrylamide gel (SDS-PAGE; T% = 13.5%) showing the pattern of bands in the high molecular weight glutenins (HMW) and low molecular weight (LMW). **B)** Relative abundance and fractions of polyacrylamide gel (SDS-PAGE; T% = 13.5%) showing the pattern of bands in the ω-gliadins and α,β, γ-gliadins. Treatments: *F. poae* (FP_1FG_0), *F. graminearum* (FP_0FG_1), both pathogens (FP_1FG_1) and control without *Fusarium* (FP_0FG_0).

Fig.4. Relative abundance of each protein subunit for different treatments in *Fusarium* damaged kernel (FDK) in 2014 growing season. Treatments: *F. poae* (FP_1FG_0), *F. graminearum* (FP_0FG_1), both pathogens (FP_1FG_1) and control without *Fusarium* (FP_0FG_0). GLU: glutenins; GLI: gliadins; HMW: high molecular weight glutenins; LMW: low molecular weight glutenins; ω-GLI: ω-gliadins; α-β-γ-GLI: α, β, γ-gliadins.

Mean ± SEM. Columns with different letters are statistically different according to Tukey´s test at $p \leq 0.05$.

Fig.5. Differences between genotypes for the concentration of deoxynivalenol. Mean ± SEM. Columns with different letters are statistically different according to Tukey´s test at $p \leq 0.05$.