Incidence of *Fusarium graminearum* and DON in malting barley grains (*Hordeum vulgare* L.)

Incidencia de *Fusarium graminearum* y DON en granos de cebada cervecera (*Hordeum vulgare* L.)

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Abstract

Fusarium graminearum is a fungal species affecting the quality and safety of malting barley grains, one of the most important cereals worldwide. Fungal growth and mycotoxin production vary among growing seasons and sowing locations, mainly due to weather conditions. This work aimed to assess the incidence of *F. graminearum* and the contamination with Deoxynivalenol (DON) in 40 barley grain samples from different Buenos Aires, Argentina localities during the 2017 and 2018 growing seasons. *F. graminearum* was identified in 80% of the samples. It was isolated in eight of eleven localities in the first and ten in the second growing seasons, with a similar maximum incidence (20% and 17%, respectively). On the other hand, all samples were contaminated with DON, and 75% exceeded the maximum limits established by The European Union (EC 1126/2007). The level of DON contamination was significantly higher in the second growing season, which was rainier and had a higher mean temperature (an average of 2.5 ppm in 2017 and 3.75 ppm in 2018). The results obtained in the present study show the need to establish regulations in Argentina on maximum limits of *Fusarium* mycotoxins in barley.

Keywords: Barley • *Fusarium graminearum* • incidence • Deoxynivalenol • food safety

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Resumen

Fusarium graminearum es una especie fúngica que afecta la calidad e inocuidad de la cebada cervecera, uno de los cereales más importantes a nivel mundial. Tanto el crecimiento fúngico como la producción de micotoxinas varían año a año y por la región de siembra, principalmente por las condiciones climáticas. El objetivo de este trabajo fue evaluar la incidencia de *F. graminearum* y la contaminación con Deoxinivalenol (DON) en 40 muestras de cebada cervecera de distintas localidades de Buenos Aires, Argentina, durante las temporadas de cultivo 2017 y 2018. *F. graminearum* fue identificado en el 80% de las muestras. Se aisló en ocho de once localidades en la primera temporada de cultivo y en diez localidades de la segunda temporada de cultivo, con una incidencia máxima similar (20% y 17% respectivamente). Por otro lado, todas las muestras estuvieron contaminadas con DON y el 75% excedieron los límites máximos establecidos por la Unión Europea (EC 1126/2007). El nivel de contaminación con DON fue significativamente mayor en la segunda temporada de cultivo, la cual fue más lluviosa y tuvo una temperatura media mayor (un promedio de 2,5ppm en 2017 y 3,75ppm en 2018). Los resultados obtenidos en este estudio muestran la necesidad de establecer regulaciones en Argentina sobre límites máximos para micotoxinas de *Fusarium* en cebada.

Palabras clave: Cebada • *Fusarium graminearum* • incidencia • Deoxinivalenol • inocuidad

Introduction

Barley (*Hordeum vulgare* L.) is the fourth most important cereal worldwide after wheat, corn, and rice and is used for both human consumption and animal feed. The world production of barley in the 2022 growing season was 152 million tons, whereas, in Argentina, it was about 4.5 million tons, of which 3 million tons were exported. The main importing countries were China (2,335,983 tons) and Brazil (612,295 tons) (6). The use of barley has increased fundamentally in the brewing industry. In Argentina, in the 2022 growing season, malting barley occupied 98.61% of the sown area, whereas fodder barley occupied only 1.39%. Buenos Aires was the province that presented the largest sown area (26).

Malting barley is often associated with *Fusarium graminearum sensu stricto* (hereafter *F. graminearum*) infection, resulting in yield reduction, lower germination capacity, lower thousand kernel weight, and protein degradation (14, 25). Besides the several economic losses, *F. graminearum* can produce mycotoxins with the potential to cause adverse effects on human and animal health. Many studies demonstrated the ability of *F. graminearum* to produce deoxynivalenol (DON) (4, 7, 18, 22). Remnants of this mycotoxin have been observed after malting and in commercial beer (3, 11, 19, 20). DON is a trichothecene B-type which can cause vomiting, diarrhea, intestinal inflammation, reduced feed intake, and decreased absorption of amino acids and carbohydrates. Chronic diseases include anorexia and immunotoxicity (21). Regulations on maximum limits for DON vary among countries. The European Union (EU) has established a maximum DON limit of 1.25 ppm in unprocessed cereals other than durum wheat, oats, and maize (EC N° 1126/2007) (28). Instead, Brazil, one of the main importing countries in 2022, has established a maximum DON limit of 1 ppm in barley grain, malted barley, and cereal-based products (2). Argentina has established maximum DON limits for wheat and maize (1 ppm) and cereal-based foods for infants and young children (0.2 ppm). However, maximum DON limits for barley in Argentina have not yet been established (8).

Barley is the main raw material in the brewing industry. Since both fungal growth and mycotoxin production vary among growing seasons and sowing locations, this work aimed to assess the incidence of *F. graminearum* as well as the levels of contamination with DON in 40 malting barley samples from different Buenos Aires localities, Argentina, during two consecutive growing seasons (2017 and 2018).

Material and methods

Barley samples were obtained from different Buenos Aires localities, Argentina, during the 2017 and 2018 growing seasons. In the first growing season, 20 barley samples were obtained from Tandil (3 samples), Balcarce (3), Lobería (2), Necochea (1), Tres Arroyos (1), Coronel Pringles (1), Olavarría (1), Guaminí (5), Bolivar (1), San Cayetano (1), and General La Madrid (1). In the second growing season, 20 barley samples were obtained from Tandil (5), Balcarce (1), Lobería (2), Necochea (2), Tres Arroyos (1), Coronel Pringles (1), Olavarría (1), Guaminí (2), Bolivar (2), Coronel Suarez (1), and Saavedra (2) (figure 1).

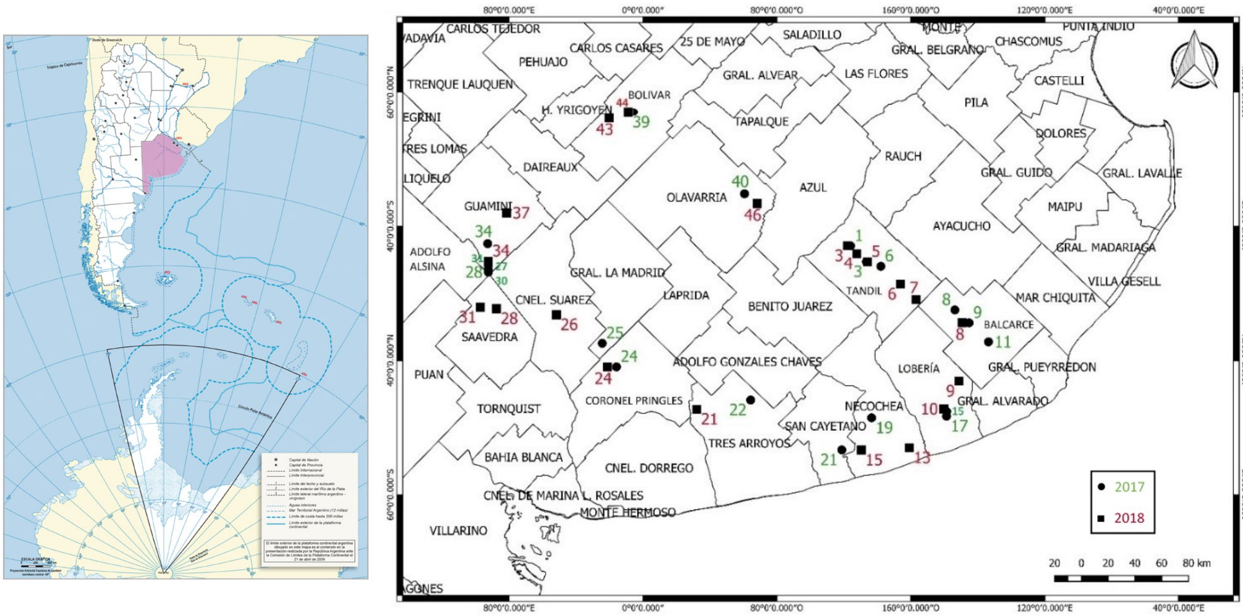

Figure 1. Geographical sampling points from different Buenos Aires localities, Argentina in two consecutive growing seasons. The numbers indicate the sample number.

Figura 1. Puntos geográficos de muestreo de diferentes localidades de Buenos Aires, Argentina, en dos temporadas de cultivo consecutivas. Los números indican el número de muestra.

Meteorological data from each sampled locality was collected during the 2017 and 2018 growing season. In particular, accumulated precipitation and mean temperature were analyzed to evaluate the eventual influence of climatic conditions on the incidence of *F. graminearum* and the levels of contamination with DON in barley grains. Meteorological data was obtained and analyzed from the Giovanni online data system, developed and maintained by the NASA GES DISC and the QGis software (v.3.16) (1). Data was collected during the period of flowering (November) in which barley is susceptible to *Fusarium* infection (figure 2, page XXX).

Grain samples (500 g) were reduced with a grain divider, surfaced disinfected by washing with sodium hypochlorite 5% and ethanol 70%, subsequently, for 2 min, and washed twice with sterile distilled water for 2 min. One hundred grains were randomly plated (10 grains/plate) on potato dextrose agar 2% (PDA) with 0.25 g chloramphenicol/L and incubated at 25 °C for 4-7 days, under a 12 h light/dark cycle. Potential *F. graminearum* colonies (mycelia from white to pale orange to yellow and with red pigments in the agar) were subcultured onto PDA and carnation leaves agar (CLA) and incubated at 25 °C for 15 days, under a 12 h light/dark cycle for identification (12). To confirm morphological results, a *F. graminearum*-specific PCR was performed for a representative subgroup using primers Fg16F and Fg16R (16). Monosporic cultures of previously identified *F. graminearum* isolates were cultured for 6 days on PDA plates at 25 °C. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (27). Polymerase chain reaction (PCR) mixture (25 µL) contained 10-20 ng of genomic DNA, 10X reaction buffer, 2.5 mM $MgCl_2$, 1 µM of each primer, 30 µM of dNTPs (Genbiotech S.R.L., Argentina), 1 U of *Taq* DNA Polymerase (Inbio-Highway, Argentina), 0.014% of Cresol Red solution (Sigma-Aldrich Co. St Louis, MO), 0.0005% Tween 20®, 0.0005% Nonidet P40®.

The amplification of DNA was performed in an XP Thermal Cycler (Bioer Technology Co., China) (16). After electrophoresis separation in 5X TBE buffer at 80 V on 1.5% agarose gel containing 3-4 µL of GelRedTM, the PCR products (≈ 400 base pairs) were visualized under UV light (Biotium, Hayward, USA). The isolate 1-1 was used as positive control (7).

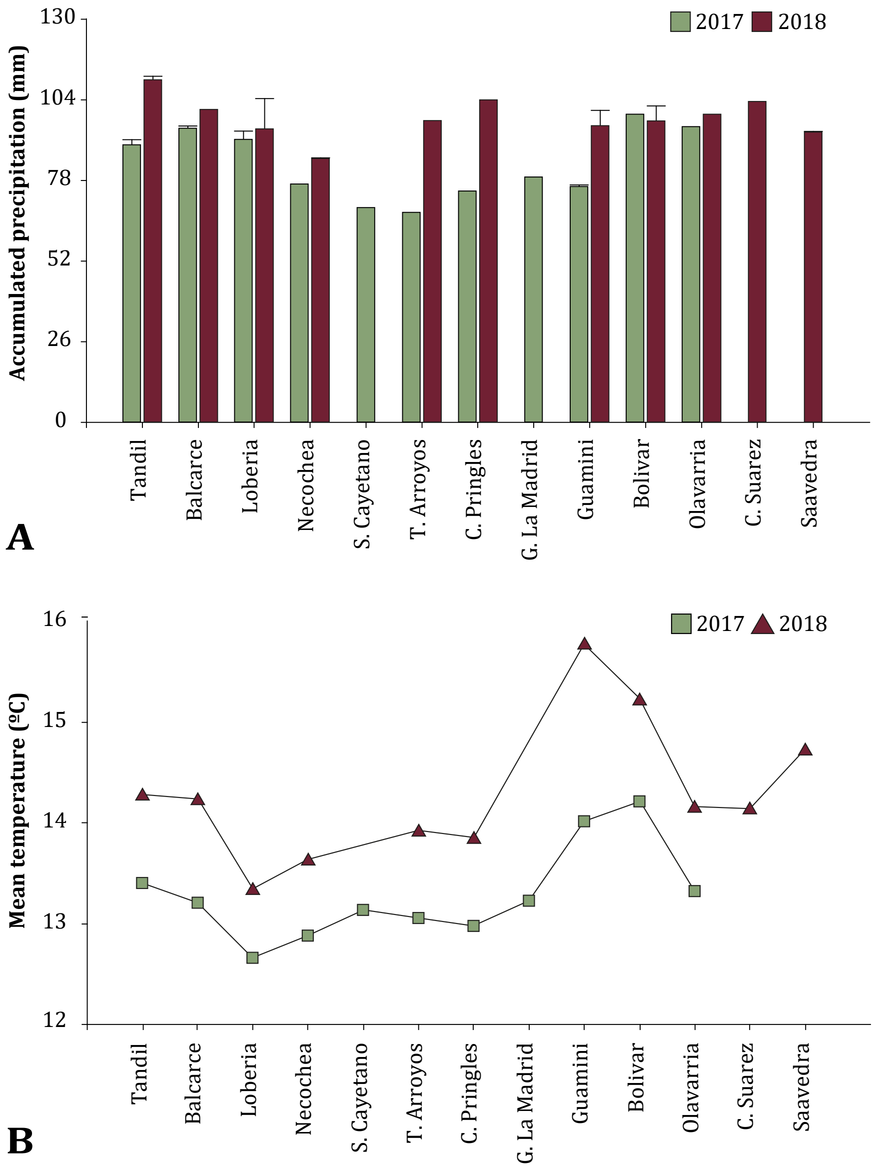

Figure 2. Accumulated precipitation (A) and mean temperature (B) from sampling localities during November in the two growing seasons. The lack of standard deviation in some localities is because only one data was obtained.

Figura 2. Precipitaciones acumuladas (A) y temperatura media (B) de las localidades muestreadas durante noviembre en las dos temporadas de cultivo. La falta de desviaciones estándar en algunas localidades se debe a que solo se obtuvo un dato.

The protein content of grain samples was measured with a NIT analyzer with a double-face monochromator (Agricheck, Bruins Instruments, Germany) to evaluate their relationship with the incidence of *F. graminearum*.

DON contamination was analyzed in a representative subsample (20 g) by enzyme-linked immunosorbent assay (ELISA) following the manufacturers' specifications (Agra-Quant DON, RomerLabs) with a detection limit of 0.2 ppm. The plate was scanned using an automatic plate reader Rayto RT-6000.

Statistical analyses were done using RStudio version 2022 (24). Normal distribution of the data was evaluated using the Shapiro–Wilks test. Analysis of variance (ANOVA) was performed to evaluate differences in *F. graminearum* incidence and DON contamination levels between growing seasons and localities. Pearson's Correlation analysis was performed between the incidence of *F. graminearum* and protein content, the incidence of *F. graminearum* and DON contamination levels, and the DON contamination levels and climatic conditions.

Results and discussion

Our results showed that 32 out of the 40 samples (80%) were contaminated with *F. graminearum*. A total of 236 isolates were morphologically identified, and the selected isolates amplified fragments of ≈ 400 base pairs, which confirmed the morphological observations. The number of samples contaminated by *F. graminearum* was greater than in other studies carried out in Buenos Aires (18), central Italy (4), and Southern Brazil (22, 23). A recent study has demonstrated that warm nights increase the incidence of *F. graminearum* in barley, so it is important to consider that the incidence observed in this study may increase in the coming years (15). The incidence (percentage of infected grains per sample) varied from 0 to 20% in the first growing season, with an average incidence of 5.3%, and varied from 0 to 17% in the second growing season, with an average incidence of 6.5% (figure 3). A similar incidence was observed in wheat samples from different Buenos Aires localities (13). Regarding distribution by locality, *F. graminearum* was isolated from eight localities in 2017 and ten localities in 2018 (figure 3). The greatest number of isolates was obtained in Necochea and Tandil in the first and the second growing seasons respectively, whereas the lowest number of isolates was obtained in Balcarce and Saavedra in the first and the second growing seasons respectively (figure 3). *F. graminearum* was not isolated from Bolivar, San Cayetano, and Tres Arroyos in the first growing season, and from Coronel Suarez in the second. Correspondent analyses showed non-significant differences in the incidence of *F. graminearum* in barley between different seasons and different localities. However, we did not have the same number of samples per locality and the same localities per growing season. Besides, an association with the favorable climatic conditions in each locality was not found.

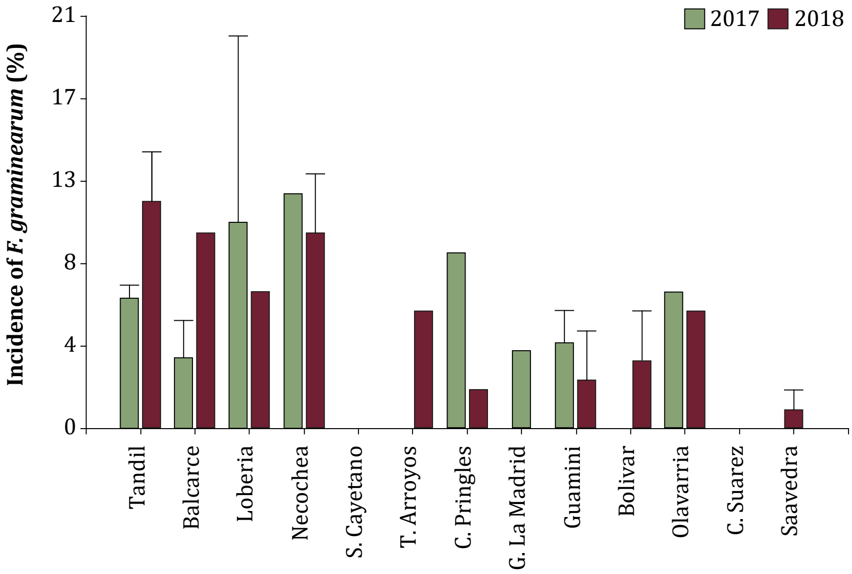

Figure 3. Incidence of *F. graminearum* (average of grains infected per sample) in malting barley samples grown in different Buenos Aires localities, in two consecutive growing seasons. The lack of standard deviation in some localities is because only one data was obtained.

Figura 3. Incidencia de *F. graminearum* (porcentaje de granos infectados por muestra) en muestras de cebada cervecera en diferentes localidades de Buenos Aires, en dos temporadas de cultivo consecutivas. La falta de desviaciones estándar en algunas localidades se debe a que solo se obtuvo un dato.

Protein content analysis showed that 67.5% of the samples did not meet the regulation (5), two samples exceeded the maximum limit of protein content (13%) and 25 samples did not reach the minimum limit of protein content (9.5%). Although the incidence of *F. graminearum* was slightly higher in the second growing season, 90% of the samples of the first growing season and 45% of the second did not meet the regulation. Pearson's Correlation analysis was non-significant and showed a low correlation between protein content and the incidence of *F. graminearum* ($r = 0.226$, $p > 0.05$). In other studies, protein content was evaluated after inoculation with *Fusarium* species, and no effects were observed, attributing the fluctuation in protein content to environmental conditions during crop development (9), and genotype evaluated (14).

All the samples analyzed were contaminated with DON in different concentrations. In barley studied in Buenos Aires (18), central Italy (4), and Southern Brazil (23), the number of contaminated samples was lower than in this study. In contrast, in wheat samples studied in Buenos Aires, the number of contaminated samples was similar to the number observed (13). Significant differences ($p < 0.05$) were found only between growing seasons. The 2018 growing season samples showed higher DON concentrations than the 2017. The second growing season was rainier and showed higher mean temperature than the first; however, only a moderate correlation was observed between DON content and accumulated precipitations ($r = 0.365$, $p < 0.05$). A similar association was observed by Gonzalez *et al*. (2008) and Piacentini *et al*. (2015). In the present study, 30 out of 40 samples (75%) exceeded the maximum limit established by the EU (1.25 ppm) and 32 out of 40 (80%) exceeded the maximum limit established by Brazil (1 ppm). These values warn of the need to establish control measures to guarantee the production of safe grains, suitable for commercialization and industrialization. As observed in figure 4, DON contamination levels greater than 5 ppm were observed in 35% of the samples in the first growing season and 55% in the second growing season, whereas DON contamination levels lower than 1.25 ppm were observed in 45% of the samples in the first growing season and 5% in the second growing season. In other studies carried out in Buenos Aires (18) and Brazil (23) DON contamination levels were similar to those observed in this study. However, some authors obtained DON contamination levels lower than our results, and even, none samples (4) and only one sample (17), exceeded the maximum limit established by the EU.

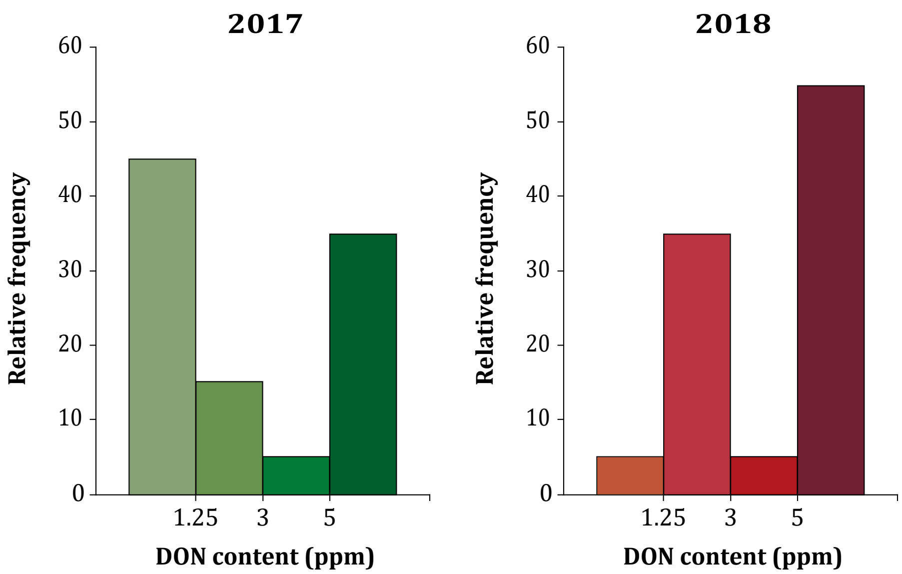

Figure 4. Relative frequency of DON content in malting barley samples grown in two consecutive growing seasons.

Figura 4. Frecuencia relativa del contenido de DON en muestras de cebada cervecera de dos temporadas de cultivo consecutivas.

Pearson's Correlation analysis was performed between the incidence of *F. graminearum* and DON contamination levels, showing a statistically significant correlation between variables (r = 0.467, p <0.05). In the first growing season, Tandil, one of the localities with the highest DON contamination level, had an incidence of *F. graminearum* higher than average (7%). In contrast, in Bolivar, the locality with the lowest DON contamination level, *F. graminearum* was not isolated. These results may indicate the presence of other DON-producing *Fusarium* species (13, 17, 18) or that *F. graminearum* was not obtained with the isolation method used (13). In the second growing season, Tandil, one of the localities with the highest DON contamination level, had the highest *F. graminearum* incidence. In contrast, Guaminí, the locality with the lowest DON contamination level, had an incidence of *F. graminearum* lower than average (3%). The correlation between the incidence of *F. graminearum* and DON contamination levels was also demonstrated in other studies (4, 13, 22).

Conclusion

In conclusion, our results demonstrate that 80% of malting barley samples from Buenos Aires, Argentina were contaminated with *F. graminearum* in the 2017 and 2018 growing seasons. The incidence of *F. graminearum* observed was greater than in other studies in Buenos Aires in barley. The incidence of *F. graminearum* and precipitations influenced DON contamination levels. All the samples analyzed were contaminated with this mycotoxin. The results obtained in the present study show the need to establish regulations in Argentina on maximum limits of *Fusarium* mycotoxins in barley and to carry out continuous monitoring to prevent the negative impact on consumers' health.

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