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Fusarium spp. Incidence and DON Contamination in Different Wheat Varieties Correlated with the Environmental Factors

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The genus *Fusarium* consists of multiple diverse species, which, as a result of their frequency in nature and pathogenicity, are significant in agriculture, as well as in human and veterinary medicine. In the course of field trials, by using standard phytopathological methods, and performing analyses of 19 different varieties of wheat and a portion of infected grains gathered from two distinct locations in Slovenia, we have determined the presence of various phytopathogenic species of the genus *Fusarium*. Because of the reliability, the experiment was performed in two consecutive years, 2012 and 2013. A laboratory analysis was conducted with an ELISA test on all grain samples for the determination of deoxynivalenol (DON) concentration. The results show that the main differences in the infection levels (*F. culmorum*+*F. graminearum*; FC+FG) of wheat samples were found in Jable (humid area), at the same time showing higher levels of DON content than Rakičan (dry area). Such a statement is supported by correlation test, where correlation is evident between FC+FG and DON in every variation. The data for both wheat types (awned and awnless) together showed that the grain in Jable is statistically significant more infected by FC+FG when compared to that in Rakičan. Moreover, our descriptive analysis confirms that the infection rate of grain with FC and FG shows a strong correlation with the emergence of DON.

Keywords: DON, environmental factors, field experiment, *Fusarium* spp., wheat varieties

Introduction

Fusarium head blight (FHB; also known as 'scab' or ear blight) is caused primarily by the fungus FG (teleomorph *Gibberella zeae*). It is a devastating disease on wheat capable of infecting the entire wheat host. FHB can lead to several losses in wheat quality, reduced seed germination, but also can reduce nutritional quality through contamination with mycotoxins (Mesterházy 2005). From an economical and environmental aspect, breeding resistant host plant (varieties) is one of the most appropriate methods to control FHB (Ruckenbauer et al. 2001). FG is a facultative parasite, that is, it ordinarily exists as a

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saprophyte but can live as a parasite on plants, causing disease (McMullen et al. 1997; Goswami and Kistler 2004).

The great financial significance is attributed to *Fusarium* diseases of wheat, which are responsible for major crop losses of yield on a global scale. The species of the *Fusarium* genus have also been known to generate a spectrum of toxic secondary metabolites, known as mycotoxins, which can lead to possible health risks in instances of contaminated grain consumption in both human and animal food products (D'Mello et al. 1999; Placinta et al. 1999).

The main *Fusarium* toxic secondary metabolite is deoxynivalenol (DON), produced chiefly by FG and FC, the most important European DON-producing fungi (Bottalico and Perrone 2002). DON incidence is likewise deemed to be an indicator of the probable presence of other, more toxic trichothecenes (Jajić et al. 2008), which might eventually lead to mycotoxicosis by ingestions of cereals and other products derived from *Fusarium*-infected grain (D'Mello and Macdonald 1997).

The purpose of the research was to determine whether there were any differences in ear infection levels between the various wheat varieties, correlated to the weather conditions during the flowering period, the wheat type and the location. Detailed laboratory analyses were conducted to determine whether there were any differences in grain infection and DON contamination in relation to the wheat variety, location, and year.

Materials and Methods

The macro field trials were conducted at two different locations in Slovenia, namely Rakičan and Jable. Both represent official experimental stations for testing new varieties of wheat and their introduction in the general production. The same crop rotation (maize-wheat) was used, for at least a 6-year period, to allow for a greater infection pressure carried by the *Fusarium* spp. In both years (2012 and 2013) and locations of the field trial, the fungicide Prosaro (prothioconazole + tebuconazole), a product of Bayer CS, was applied at a dosage of 1 liter per hectare at the beginning of wheat flowering (BBCH 61).

Rakičan (46°38'N, 16°11'E, altitude: 184 m) is located in the central part of the Prekmurje region, near Murska Sobota. The meteorological station of reference is the Murska Sobota station, also located in Rakičan. In the period 2011–2013, the average air temperature was 11 °C; average annual precipitation was 795.5 mm and typical district brown soil type on holocene gravel debris.

Jable (46°8'N, 14°34'E, altitude: 305 m) is located in the central part of the Ljubljana basin, in pre-alpine Slovenia. The meteorological station of reference is the Brnik station. Average annual temperature (2011–2013) was 9.9 °C; average annual precipitation was 1218.9 mm and moderately clay soil type on a siliceous limestone base.

Monitoring was conducted at both test locations (2012–2013) to determine the length of the flowering of all 19 wheat varieties. Each of the sown samples represented a set of perspective varieties chosen for cultivation in Slovenia, different in origin, type and maturation period. Randomized block design for each wheat variety was 4 × 25 m^2 net value

(100 m^2). At both locations fertilization was done with N:P:K 5:15:30 600 kg ha^{-1} and urea 150 kg ha^{-1}. The number of replications was three. The test locations differ significantly regarding typical weather conditions, but above all regarding precipitation, which strongly favors the development of fusariosis. Each variety was separately catalogued in the database, a procedure of crucial significance in this research because the *Fusarium* spp. are the most infective at that stage of wheat development. In every plot, an integrated manner of wheat production was conducted. The percentile of *Fusarium* ear infection for each wheat variety was determined by the Stack and McMullen (1995) method. The laboratory analyses were carried out on individual grain samples for each wheat variety gaining an accurate representation of all *Fusarium* species.

Statistical analyses

Data collected were analyzed using statistical two-tail Student *t*-test and correlation coefficient. Samples from different populations and variance undergo Student *t*-test Type 3. Statistical analyses were performed using Microsoft Excel Data Analyst add-in.

Identification of Fusarium *spp.*

Over the course of the two-year growing period, 19 samples of different wheat varieties were collected from each of the two distinct locations, continuously. After harvest, all the samples were taken from 30 kg sacks in a total of 2 kg, half of which was used for mycotoxin analysis, and the other half was used for *Fusarium* spp. laboratory identification. In the laboratory, 100 randomly selected wheat grains were surface-disinfected with 1% sodium hypochlorite (NaOCl) in Erlenmeyer flasks for 10 minutes, and further rinsed with sterile distilled water and dried on previously sterilized paper.

Five grains were placed under sterile conditions in each of the 20 Petri dishes, 90 mm in diameter, containing 15 mL of modified potato dextrose agar (PDA) medium: 14 g of potato dextrose agar (Biolife), 10 g technical agar (Biolife), 0.121 g penicillin G (Merck), and 0.542 g streptomycin sulfate (Merck) per liter. They were further incubated under dark conditions at 20 °C, RH 60%, for the period of 7 days, while being observed daily. Following the incubation period, each fungal colony was examined microscopically for identification at genus or species level. The microscopic observations being carried out under 100 and ×400 magnification (Zeiss Axio Scope, Germany). The focus of the observations was on macroconidia morphology, the presence/absence of microconidia and chlamydospores, as well as sporodochia production. In certain cases, due to reliable identification of individual species of fungi, the fragments of colonies developed on seeds and the medium were transferred to carnation leaf agar (CLA) and synthetic nutrition agar (SNA) and incubated at 12 h day/night regime, using combined fluorescent and near ultraviolet light during the daytime period. The CLA and SNA media were prepared according to Burgess et al. (1994) and Nirenberg (1976), respectively. Identification was performed by utilizing the keys of Gerlach and Nirenberg (1982), Burgess et al. (1994) and Leslie and Summerell (2006), noting the considerations of Summerell et al. (2003). The mycelia and spores identified as *Fusarium* spp. were used to establish pure cultures on potato dextrose agar (PDA) and deposited in the refrigerator for the purpose of additional surveys.

ELISA for the determination of DON

The other half of the grain samples were analyzed for DON content by using an ELISA, Ridascreen® Fast DON. The process consisted of the following steps. Firstly, a 20 g sample was taken from the main sample (1 kg) and put into a 250 mL extraction flask and then 100 mL of deionized water was added. After an hour of stirring and filtering the extract, 1 mL was poured into a tube and diluted with 3 mL of deionized water. Using a microtiter plate, we applied 200 µL of the conjugate, then 100 µL of the extract solution in the designated spots. The mixture was moved to a different microtiter plate that contained antibodies and left for 15 minutes. We further washed the plate with a mild detergent solution and added another 100 µL of the substrate, leaving it for 5 minutes, and then adding another 100 µL of the mild detergent solution to stop the reaction. The DON levels were measured with an ELISA reader.

Results

During the flowering period for 2012 in Rakičan, a minimum of 0.1 mm, a maximum of 52.9 mm, and a median value of 15.4 mm of rainfall was observed. The median number of rainy days was 2. The minimal, maximal and median temperatures for the same period were 10.5 °C, 17.9 °C, and 13.7 °C, respectively.

The weather conditions during the flowering period in Rakičan were quite different in 2013 from the same period of the previous year. The minimum amount of rainfall was 0.0 mm, the maximum 16.5 mm and the median data was 5.1 mm. The median for rainy days was 2. The minimum, maximum and median temperatures during the same period were 12.4 °C, 17.3 °C and 15.6 °C, respectively (Table S1*). According to the observations and their descriptive statistics, no correlation was observed at this location for 2012 and 2013 between the weather conditions and the incidence of *Fusarium* head blight. Consequently, there is no direct influence of the total amount of precipitation during flowering and temperature on the average levels of ear infection (Figs S1 and S2).

The acquired data of the wheat flowering period for 2012 in Jable showed a significant difference in precipitation as opposed to Rakičan. The minimum amount of rainfall was 1.5 mm; the maximum was 40.1 mm, and the median data amounted to 34.7 mm. The median for rainy days during flowering was 5. The minimum, maximum and median temperatures for the same period were 13.3 °C, 17.2 °C and 15.3 °C, respectively. In 2013, the minimum amount of rainfall was 44.0 mm, the maximum was 114.9 mm, and the median was 72.1 mm, respectively. The median for rainy days during flowering in 2013 was 8. The minimum, maximum and median temperatures during the same period

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

were 12.6 °C, 20.6 °C and 15.5 °C, respectively (Table S2). For both years in Jable, the descriptive statistics show no correlation between temperature and precipitation on one side and wheat's ear infection on the other, during the flowering period, which was the same as in Rakičan (Figs S1 and S2).

Regarding wheat type, no statistical difference was detected between awned and awnless wheat, based on data on an average ear infection. The same results were obtained when both wheat types were considered over both locations. Due to the unequal ratio (6 awned : 13 awnless) of the wheat types, the results were not generalized.

A part of the grain samples was used for grain analysis. The analysis aimed for the grain infection with FC and FG as well as DON contamination. The results of the analyzed samples are presented in Table S3.

The results obtained from Rakičan in 2012 show an average infestation rate of 0.2% of FC and 2.1% of FG. (Table S3). In 2013, the average presence of FC was 1.1%, while the presence of FG was decreased to 1.6% (Table S3). The data analysis from Rakičan based on FC + FG presence showed that the awned wheat had statistically significant smaller infection levels when compared to the awnless wheat (Table 1). The data for both wheat types revealed that FC + FG significantly infected the grain more in Jable than in Rakičan.

Two positive samples passed the detected limit of 200 μg/kg DON in Rakičan in 2012. The maximum DON content detected in 2012 was 297 μg/kg. One positive sample was detected in 2013, with a maximum DON content of 1033 μg/kg. The total mean value for the DON content in 2012 was 140 μg/kg, and 120 μg/kg in 2013. The limit of 1250 μg/kg imposed by the European Union (EU) for DON content was not exceeded in the analyzed samples. The results indicate that DON presence in Rakičan was at a low level during 2012 and 2013 (Table S3).

The results from Jable in 2012 present an entirely different picture in the occurrence of *Fusarium* spp. and DON contamination. The average presence of FC was 0.5%, and the average of FG was 8.9% (Table S3). In 2013, the presence of FC was higher than 2012 with an average of 1.3%, while the average of FG occurrence had decreased to 3.5% (Table S3). The statistical analysis of the data from Jable showed that there was no statistically significant difference between awned and awnless wheat in relation to FC+FG presence (Table 1).

Table 1. Two-tailed *t*-test of type 3 for both types of wheat based on the data of grain infection with FC + FG (data for both locations and years), with significance level α = 0.05

Location	Rakičan and Jable		Rakičan		Jable		Rakičan	Jable
Wheat types	Awnless wheat	Awned wheat	Awnless wheat	Awned wheat	Awnless wheat	Awned wheat	Both awl+aw	Both awl+aw
Mean	4.81	4.66	2.81	1.66	6.81	7.66	2.45	7.08
Std Dev	5.17	6.71	2.19	0.89	6.44	8.59	1.94	7.08
df	74		36		36		74	
p-value	0.93		0.029*		0.76		0.00015*	

FC + FG – *F. culmorum* + *F. graminearum*; Std Dev – standard deviation; df – degrees of freedom; *statistical significant difference; awl – awnless wheat; aw – awned wheat

In 2012, 16 positive samples passed the detected limit of 200 μg/kg DON in Jable. The maximum DON content detected in 2012 was 3550 μg/kg. In 2013, 16 samples were also detected, with a maximum DON content of 2905 μg/kg. The total mean value for DON in 2012 was 714 μg/kg and 684 μg/kg in 2013. The limit of 1250 μg/kg imposed by the European Union (EU) for DON content was exceeded in two samples in Jable in both 2012 and 2013 (Table S3).

A linear correlation ($r = 0.68$) was observed between FC + FG and DON (Fig. S3) over data from the Rakičan location ($y = 56.742x – 8.8426$, $r^2 = 0.46$). A linear correlation ($r = 0.64$) was also found between FC + FG and DON (Fig. S4) in Jable ($y = 69.507x + 207.1$, $r^2 = 0.41$). By combining the data from both locations (Rakičan and Jable) and both years (2012 and 2013), the highest correlation $r = 0.71$ (Fig. S5) was detected, with the correlation function $y = 77.816x + 43.931$, $r^2 = 0.51$.

Discussion

This research was conducted with the purpose to analyze the *Fusarium* spp. incidence and DON contamination in different wheat varieties correlated with the environmental factors. Through relevant field analyses and laboratory tests, this two-year research work generated valuable information about the *Fusarium* spp. encountered in Slovenia (Rakičan-Jable) on naturally infected wheat and their contamination with DON.

In general, the most often isolated *Fusarium* species from wheat samples were *F. graminearum*, *F. culmorum*, *F. poae* and *F. avenaceum*. These species are pathogenic on wheat ears, causing head blight and mycotoxin contamination of the grain (Parry et al. 1995). With determining the percentile of FHB of each wheat variety individually, by Stack and McMullen's method (1995), it was impossible to predict the mycotoxin contamination by field estimates.

According to the data from the meteorological stations, a far greater intensity of rain was recorded in Jable continuously over the two-year trial period as compared to Rakičan. Regardless of the retrieved data, from the descriptive statistics, no correlation has been determined over both locations for 2012 and 2013 between the weather conditions (temperature/precipitation) and the incidence of *Fusarium* head blight. Consequently, we can also conclude that there is no direct influence of the total amount of precipitation and temperature during flowering on the average levels of ear infection (Figs S1 and S2). These results differ from the results of some other researchers, from the point of view that perhaps there is some other factor besides the environmental conditions responsible for the occurrence of *Fusarium* spp. To be sure in our statement an additional correlation test was conducted between the weather conditions (temperature/precipitation) during flowering and grain contamination with DON. Repeatedly, the same conclusion was confirmed, i.e. a low correlation coefficient was observed ($r = 0.41$ and $r = 0.14$ for temperature and precipitation, respectively) in both locations for 2012 and 2013. A similar conclusion was also confirmed by the correlation test between the weather conditions (temperature/precipitation) during flowering and grain infection with FC + FG ($r = 0.20$ and $r = 0.09$ for temperature and precipitation, respectively) in both locations for 2012 and 2013. Even in both variations, the descriptive statistics have confirmed the impact of other factors responsible for the grain infection with FC + FG and the appearance of DON in grains, besides the weather during the flowering period, even later on in vegetation. Blandino et al. (2012) pointed out that FHB infection and DON contamination of wheat grains can be caused by different factors, while the climatic conditions remain the main factor, but also from agronomic factors such as previous crop residue management, cultivar susceptibility, and fungicide applications. Eiblmeier and von Gleissenthall (2007) gave a similar statement that the climatic conditions during the flowering period, preceding crop, no or minimal tillage, susceptible cultivar, strobilurin as a foliar fungicide and late harvest are considered to be risk factors for increasing levels of DON in wheat grains.

As a result of the specificity of the environmental factors in Jable, the high intensity of the wheat grain infection caused by FG and FC led to a high level of DON contamination. 16 out of 19 samples (2012–2013) showed mycotoxin concentrations above the lower detected limit of 200 μg/kg, but also, two samples in 2012 and 2013 managed to exceed the maximum limit of 1250 μg/kg [Commission Regulation (EC) No 1126/2007 of 28th September 2007], which confirmed the close connection between the presence of FG and FC with the occurrence of DON (Table S3). On the other hand, the Rakičan precipitation was more moderate, resulting in minimal infection caused by FG and FC, and therefore a lower DON contamination. In 2012, there were only two varieties of wheat that exceeded the minimum threshold for DON, while in 2013 only one case was registered. The maximum limit for DON content was not exceeded in the analysed samples in the two-year experimental period (Table S3).

The main differences in the infection levels (FC + FG) of wheat samples were found in Jable, which represents a typical humid area, at the same time showing higher levels of DON content than Rakičan, which is a dry area, the complete opposite of Jable. Such a statement is supported by correlation test, where a correlation is evident between FC + FG and DON for every location separately, as well as for both locations together (Figs S3, S4, and S5). Moreover, our descriptive analysis of discovering the major factors that influence the appearance of DON confirms that the infection rate of grain with FG and FC shows a strong correlation with the emergence of DON.

However, the different levels of mycotoxin contamination between these years were more affected by rainfall than by temperature. It is hard to infer trends or recent developments regarding high DON contamination in grains, because the occurrence of contaminated samples, as well as the environmental factors, is also influenced by many other factors (Koch et al. 2006; Škrbič et al. 2012; Wegulo 2012). Therefore an integrated approach to the disease is appropriate to reduce the risk of high DON contamination in wheat grains. Also, an ear infection cannot be straightforwardly interpreted as a potential insight in grain infection. Contamination levels might also be associated with factors other than climate conditions, such as with mycotoxin formation, crop rotation (maize as a pre-crop for wheat), and growing highly susceptible wheat varieties with no applied fungicide. To obtain further insight into *Fusarium* spp. incidence and DON contamination, additional wheat research is necessary to control food and feed quality. Additionally, to increase the FHB resistance and crop yield, relevant wheat varieties should be preferred for cultivation in Slovenia.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1*. Average amount of rainy days, total rainfall during flowering, ear infection and average temperature during anthesis on 19 different varieties of wheat for Rakičan in years 2012 and 2013

Electronic Supplementary *Table S2*. Average amount of rainy days, total rainfall during flowering, ear infection and average temperature during anthesis on 19 different varieties of wheat for Jable in years 2012 and 2013

Electronic Supplementary *Table S3*. DON content (µg/kg) and percentage representation of *Fusarium culmorum* and *Fusarium graminearum* in 19 different wheat varieties from Rakičan and Jable in 2012 and 2013

Electronic Supplementary *Figure S1*. Visual comparison of the records between AEI (average ear infection) and TRF (total rainfall during flowering) on 19 different wheat varieties for Rakičan and Jable in 2012 and 2013

Electronic Supplementary *Figure S2*. Visual comparison of the records between AEI (average ear infection) and Temp (average temperature during flowering) on 19 different wheat varieties for Rakičan and Jable in 2012 and 2013

Electronic Supplementary *Figure S3*. Correlation between grains infection with FC+FG (*F. culmorum*+*F. graminearum*) and DON content on 19 different wheat varieties for Rakičan in years 2012 and 2013 (y-DON µg/kg, x-grain infested with FC+FG in %)

Electronic Supplementary *Figure S4*. Correlation between grains infection with FC+FG (*F. culmorum* + *F. graminearum*) and DON content on 19 different wheat varieties for Jable in years 2012 and 2013 (y-DON μg/kg, x-grain infested with FC+FG in %)

Electronic Supplementary *Figure S5*. Correlation between grains infection with FC+FG (*F. culmorum* + *F. graminearum*) and DON content on 19 different wheat varieties for both locations Rakičan/Jable in years 2012 and 2013 (y-DON μg/kg, x-grain infested with FC+FG in %)