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A 2018-2019 survey on Deoxynivalenol (DON) in Romanian barley (*Hordeum vulgare* L.) samples

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Abstract

Objective: Mycotoxin contamination represents a clear public health concern. In this context, a barley survey was conducted in Romania, to monitor the occurrence of deoxynivalenol (DON) in barley samples collected during the 2018 and 2019 growing seasons.

Methods: A total number of 122 barley samples was collected along with information regarding the specific location of fields, the applied agronomic practices and cropping systems. Enzyme linked immunosorbent assay (ELISA) was used for the quantification of DON.

Results: The results showed 89 contaminated samples (72.95%). Out of these samples, 19 samples (15.57%) registered DON levels higher than the limit of 1250.00 µg kg^{-1}, settled by the Commission Regulation (EC) No 1881/2006 for unprocessed cereals other than durum wheat, oats and maize. The highest DON level was 1592.51 µg kg^{-1}, noted by a barley sample from Brăila County (Macroregion 2, the South-East development region).

Conclusion: When referring to the analysed samples, DON contamination was independent of the type of barley cultivar, but strongly influenced by the pedo-climatic differences between counties. These results highlight the importance of an effective and sustainable mycotoxin management along the food and feed chain, as well as the need of mapping the mycotoxin hotspot areas.

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Introduction

Barley (*Hordeum vulgare* L.) is one of the most important cereals in the world with an estimated global production of approximately 138 million tons produced in 48 million hectares in 2018-2019 [28]. At European Union (EU) level, barley represented the second most spread cereal crop after wheat in 2018 and registered a percent of 22.1% of the total area cultivated with cereals [17].

The industrial use of barley grain has experienced continuous growth, mainly due to its economic importance for malt production [23]. Romania ranks on the 4^{th} place within the EU member states for cereal production, after France, Poland, Germany and Spain. In 2019, there was registered a cultivated area of 5426.34 thousand hectares, with 165.06 thousand hectares more than in 2018 [11]. Both in 2018 and 2019, Romania registered the 7^{th} largest barley cultivated area and production, after Spain, France, Germany, United Kingdom, Poland and Denmark [12]. In 2018, there was registered a barley cultivated area of 423.50 thousand hectares, which noted a total production of 1870.7 thousand tones and an average production of 44417 kg ha^{-1} [17].

Cereals are very susceptible to fungal attacks, both in the field and during storage. The fungi genus that should be taken into account for barley and other small grains is *Fusarium*, which is of great concern to both producers and consumer [20]. *Fusarium* spp. can be associated with a disease called Fusarium Head Blight (FHB), which is widespread in moderate climatic zones around the world, including European cereal-growing areas [21]. The major species frequently associated with FHB of small-grain cereals in Europe are *Fusarium graminearum*, *F. avenaceum* and *F. culmorum*, and, to a lesser extent *F. poae*, *F. cerealis*, Fus *F. arium equiseti*, *F. sporotrichioides* and *F. tricinctum* [23]. One of the key problems associated with FHB in barley is the formation of mycotoxins, toxic secondary metabolites that pose a health risk to both humans and animals, since they can be transferred across the beer production chain from barley to malt and thus to the final product [21].

Deoxynivalenol (DON) is the mycotoxin most often detected in small grains [14,20]. It is a secondary metabolite of toxinogenic *Fusarium* species, which is found in a range of cereal grains [21]. DON is predominantly produced by *Fusarium graminearum* and *F. culmorum*, which are frequently observed in temperate regions of Europe [9,14]. Following exposure with a high acute dose, DON can induce gastrointestinal symptoms such as vomiting both in humans and animals [14]. The accumulation of DON in human and animal bodies can lead to weight gain suppression and anorexia in animals and can induce acute and chronic effects such as immunosuppression, neurotoxicity, embryotoxicity and teratogenicity [14,23]. The provisional maximum tolerable daily intake (PMTDI) of 1 µg kg^{-1} body weight (b.w.) and acute reference dose (ARfD) of 8 µg kg^{-1} b.w. day^{-1} for DON and its metabolites have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [10]. The carcinogenic potential of mycotoxins has been evaluated by International Agency for Research on Cancer (IARC), and DON is placed in Group 3 (not classifiable as to their carcinogenicity to humans) [16]. Given their highly toxigenic nature, at European level the presence of aflatoxins is strictly regulated, being imposed maximum levels in various commodities [6].

The incidence of DON in wheat and other cereals has been reported in many European countries, including Austria [3], Croatia [24], Czech Republic [25], Finland [22], Hungary [27], Italy [1], Serbia [19], Spain [29] and Romania [2,18,13,26]. However, there is little data about DON content of barley in Romania.

In this context, the current study was undertaken to monitor the occurrence of DON in barley samples collected during the 2018-2019 harvests from fields located in various barley-producing Romanian counties. The information on DON levels in the Romanian counties will be essential to identify hotspot regions in Romania and will aid to design appropriate and cost-effective DON management strategies to prevent mycotoxin contamination right at the source.

Materials and methods

Barley samples

One hundred twenty-two (*N* = 122; 1 kg/sample) samples of barley were randomly collected in Romania in 2018 (61 samples) and 2019 (61 samples) from private cereal farmers, immediately after harvest. The sampling was done by inspectors of the County Agriculture Directorates of the Romanian Ministry of Agriculture and Rural Development, according to the European guidelines [7]. Upon arrival, all samples were transferred into paper bags and stored in the dark until their assessment. All samples were received along with information regarding the specific location of fields and the applied agronomic practices (hybrid type, previous crops, incorporation of crop residues, sowing date, fertilisation and fungicide information etc.), which were filled in by farmers into a structured questionnaire dedicated to this study.

Geographic coordinates

In order to reference the origin of samples, the European nomenclature of territorial units for statistics (NUTS) was used, based on the European regulation [8] (Figure 1). The Northern latitude and Eastern longitude of the location of the field of each sample were determined using Google Maps [15], based on the information given by farmers in the questionnaires that accompanied the barley samples.

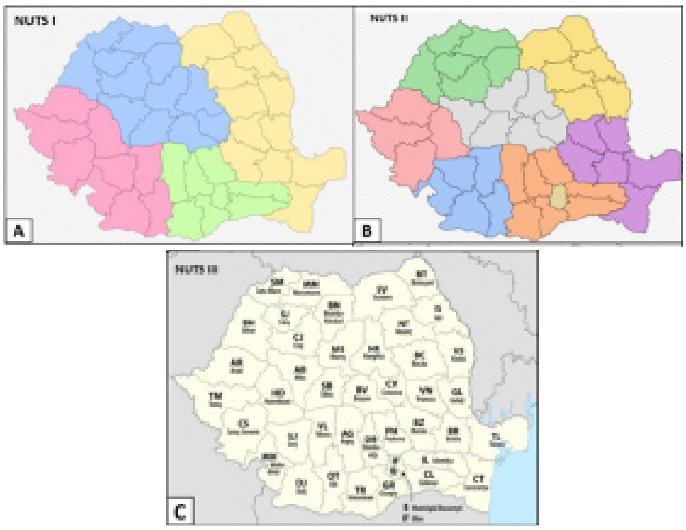


Figure 1: The NUTS (Nomenclature of Territorial Units for Statistics) regions of Romania: **(A)** NUTS I – Macroregions; **(B)** NUTS II – Regions; **(C)** NUTS III – Counties.

Mycotoxin analysis

A competitive enzyme linked immunosorbent assay (ELISA) was selected for the quantitative analysis of DON. The assessment was performed with commercially available test kits, according to the manufacturer's instructions (Ridascreen® DON, R-Biopharm AG, Germany). Thus, all samples were first finely ground using a laboratory mill (MRC Ltd., Israel) and mixed thoroughly to achieve complete homogenization. Furthermore, 5 grams of grinded sample were homogenized in 25 mL distilled water and mixed vigorously for 3 minutes at room temperature using an orbital shaker (GFL Gesellschaft für Labortechnik mbH, Germany). All extracts were then filtered using grade 1 filter paper (Whatman™, UK). There were employed 50 µL standard solutions and prepared samples to separate duplicate wells. A volume of 50 µL of the enzyme conjugate was added to each well, followed by 50 µL of the antibody solution. The plate was gently mixed by hand and incubated for 30 minutes at room temperature in the dark. After the incubation period, the liquid was poured out of the wells and the plate was vigorously taped upside down against absorbent paper to ensure complete removal of liquid from the wells. This was followed by the washing procedure (250 µL washing buffer, repeated three times). There were added 100 µL of substrate/chromogen solution to each well. The plate was again very well mixed by hand and incubated for 15 minutes at room temperature in the dark. After incubation, 100 µL of the stop solution were added to each well. The absorbance was measured at 450 nm using a Sunrise™ plate reader (Tecan Group Ltd., Switzerland). The RIDA®SOFT Win software was used for the evaluation of the immunoassays. A mycotoxin quality control material (Trilogy Reference Material, Naturally Contaminated DON Wheat, Trilogy Analytical Laboratory, Inc., USA) was used for each measurement, to ensure the quality of the analyses.

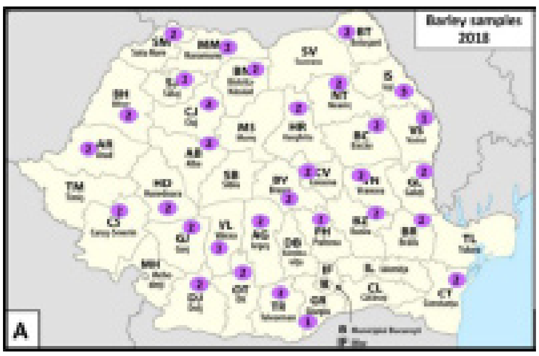
Data analysis

ELISA tests were run in duplicate for each sample. Descriptive statistics (average and standard deviation) of these results have been employed in data analysis. Reported results include the recovery of the used quality control material. The uncertainty of the method was 59.52 µg kg^{-1}. Statistical analysis was performed using IBM® SPSS® Statistics 20 (IBM Corp., USA). Significance was defined at $P < 0.05$.

Results and discussions

Origin of the collected samples

The aim of this study was to monitor the occurrence of DON in 122 barley samples collected during the 2018-2019 growing seasons from fields located in different regions of Romania (Figure 2).



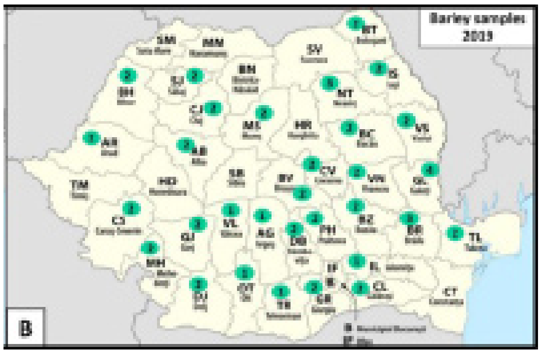


Figure 2: Distribution of the collected barley samples (NUTS III level): **(A)** 2018 harvest; **(B)** 2019 harvest.

In 2018, there were received for analysis 61 barley samples, of which 20 samples (32.79%) from Macroregion 1 (North-West and Central development regions), 19 samples (31.15%) from Macroregion 2 (North-East and South-East development regions), 7 samples (11.48%) from Macroregion 3 (South-Muntenia and Bucharest-Ilfov development regions) and 15 barley samples (24.59%) from Macroregion 4 (South-West Oltenia and West development regions). Thus, the majority of the samples were collected from the Macroregion 1 and Macroregion 2, respectively, where the North-West development region registered the highest number of barley samples (12 samples). While there were received 2 barley samples from each County, Vaslui (Macroregion 2, North-East development region) and Giurgiu (Macroregion 3, South-Muntenia development region) counties sent only one barley sample, while from Vâlcea (Macroregion 4, South-West Oltenia development region) and Iași (Macroregion 2, North-East development region) counties there was received a number of 3 samples. No barley samples were received from counties such as Mureș and Sibiu (Macroregion 1), Suceava and Tulcea (Macroregion 2), Dâmbovița, Ialomița, Călărați and Ilfov (Macroregion 3), Timiș and Mehedinți (Macroregion 4).

A number of 61 barley samples was received for analysis in 2019, also. There were registered 14 samples (22.95%) from Macroregion 1 (North-West and Central development regions), 24 samples (39.34%) from Macroregion 2 (North-East and South-East development regions), 11 samples (18.03%) from Macroregion 3 (South-Muntenia and Bucharest-Ilfov development regions) and 12 barley samples (19.67%) from Macroregion 4 (South-West Oltenia and West development regions). While there was received an average number of 2 barley samples from each County, Ialomița, Argeș, Teleorman (Macroregion 3), Vâlcea and Olt (Macroregion 4) counties sent only one barley sample for analysis. Galați County noted the highest number of collected samples (4 samples), followed by Brăila County, with 3 samples. There were received no barley samples from counties such as Bistrița-Năsăud, Maramureș, Satu-Mare, Harghita and Sibiu (Macroregion 1), Suceava and Constanța (Macroregion 2), Ilfov (Macroregion 3), Hunedoara and Timiș (Macroregion 4).

Prevalence of DON contamination in barley samples in Romania

The current study documents DON levels in 2018-2019 barley samples across Romania. When using ELISA method, an accurate quantification is only possible within the range of the calibrators - values of the given standards provided by the kit multiplied by the corresponding dilution factor (e.g. 5 for cereals and feed), which results in a range of 3.70 – 100.00 µg kg^{-1} DON. The calculation of the results was done using the cubic spline function for RIDA®SOFT Win software. The analysis of 122 barley samples revealed that the 2019 harvest noted an important (31.15%) DON contamination, higher than the threshold set by the European regulations, which stipulates 1250.00 µg kg^{-1} as the maximum level of DON for unprocessed cereals other than durum wheat, oats and maize [6]. Meanwhile, no barley sample showed a DON contamination beyond this level in 2018.

For all samples outside the calibrator range (18.50 – 500.00 µg kg^{-1} DON), the mathematical function had to be extrapolated, although this leaded to an increased uncertainty for these samples [30]. Thus, for 29 samples of the 2018 harvest and 4 samples of the 2019 barley harvest, the concentrations of DON were lower than the limit of detection of the ELISA kit (18.50 µg kg^{-1}). As samples with negative test results still could involve a DON contamination below the limit of detection of the assay, the 'out of range' function of the RIDA®SOFT Win software was applied for these samples, in order to receive a rough estimation of the concentrations of total aflatoxins for the assessed samples.

A number of 3 samples of the 2018 harvest (4.92%) and 34 samples of the 2019 harvest (55.74%) noted DON concentrations higher than the maximum detection limit of the kit (500.00 µg kg^{-1}). Because samples with higher concentrations can be diluted to lie within the range of the calibrators, this step was applied to all samples which were found having DON concentrations higher than the given range. Thus, after applying the 'out of range' function of the RIDA®SOFT Win software, none of the analysed samples was identified as having no detectable DON concentrations (Figure 3). Out of the total number of assessed samples (122 barley samples), 33 samples noted DON concentrations lower than 18.50 µg kg^{-1}. No sample of the 2018 harvest exceeded the deoxynivalenol limit imposed by the European regulations. However, for the 2019 harvest, 19 samples noted DON levels higher than 1250.00 µg kg^{-1} (Table 1). The highest concentration of DON was 1592.51 µg kg^{-1}.

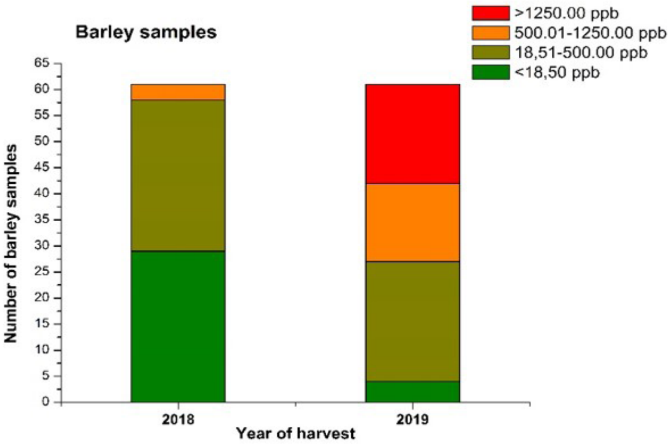


Figure 3: Barley samples, harvest 2018-2019

Table 1: Number (and percent) of 2018-2019 barley samples from Romania with various levels of deoxynivalenol concentrations

Sample category (based on the relevance of the DON concentrations)	Number (and percent) of 2018 barley samples	Number (and percent) of 2019 barley samples	Total number (and percent) of barley samples
Analysed samples	61 (100%)	61 (100%)	122 (100%)
< 18.50 µg/kg*	29 (47.54%)	4 (6.56%)	33 (27.05%)
18.51 – 500.00 µg/kg	29 (47.54%)	23 (37.70%)	52 (42.62%)
500.01 – 1250.00 µg/kg	3 (4.92%)	15 (24.59%)	18 (14.75%)
> 1250.00 µg/kg	0 (0%)	19 (31.15%)	19 (15.57%)

*Results for which the 'Out of range' function of the RIDA®SOFT Win software was applied;

We argue that we detected a wide range of DON levels. However, the concentrations found in most of the examined samples were relatively under the maximum level of 1250.00 µg kg^{-1} (84.43%). Similar results were noted in Czech Republic, where DON was detected in 83% of barley samples at a mean level of 28 µg kg^{-1} within a four-year study, which critically assessed the impact of factors such as weather conditions, growing locality, type of grain of barley cultivar and fungicide treatment on contamination levels of the most abundant Fusarium toxins in various cultivars of spring barley grown in two localities [21]. A study reported that a number of 15 samples of barley from Turkey was assessed. DON was found in 20% of barley samples (lower bound = 85 µg kg^{-1}; upper bound = 102.4 µg kg^{-1}), but levels were below the EU maximum level, with a maximum value of 973 µg kg^{-1} [14]. Another survey assessed a total of 50 malting barley grain samples (considering different varieties) during the 2013 harvest in Brazil and high levels of DON in malting barley cultivated in southern Brazil were found [23]. DON was detected in approximately 18% (9 of 50 samples) of the samples, with levels ranging from 200 µg kg^{-1} to 15100 µg kg^{-1} (mean 3400 µg kg^{-1}), while 10% of the analysed samples were far above the Brazilian and EU international regulations. However, results of mycotoxin analyses reported from different countries are difficult to compare, due to various parameters such as climatic and pedological conditions, type of barley cultivar or applied agronomic practices [20].

Distribution of DON across Romania

This study reports for the first time the distribution and levels of DON in barley samples harvested across Romania during two consecutive years. Results show a significant difference in terms of DON frequency in between 2018 and 2019, respectively. The descriptive statistics are shown in Table 2.

Table 2: Number (and percent) of 2018-2019 barley samples from Romania with various levels of deoxynivalenol concentrations

Origin of samples		Parameter	Year	
NUTS I	NUTS II		2018	2019
Macroregion 1 (*n* = 34)	**North-West** development region (*n* = 18)	No. of samples	12	6
		Frequency (%)	58.33	100.00
		Mean (± SD) (µg kg^{-1})*	196.65 (± 302.23)	1139.31 (± 369.08)
		Range (µg kg^{-1})*	4.40 – 912.11	446.18 – 1487.39
		No. (%) of samples > ML	0	3 (50.00)
		Maximum level (µg kg^{-1})	912.11	1487.39
	Central development region (*n* = 16)	No. of samples	8	8
		Frequency (%)	75.00	100.00
		Mean (± SD) (µg kg^{-1})*	82.60 (± 173.43)	1015.88 (± 603.34)
		Range (µg kg^{-1})*	3.24 – 510.67	136.61 – 1579.96
		No. (%) of samples > ML	0	4 (50.00)
		Maximum level (µg kg^{-1})	510.67	1579.96
Macroregion 2 (*n* = 43)	**North-East** development region (*n* = 21)	No. of samples	10	11
		Frequency (%)	10.00	81.82
		Mean (± SD) (µg kg^{-1})*	15.21 (± 6.16)	334.14 (± 352.50)
		Range (µg kg^{-1})*	4.59 – 25.06	11.93 – 1033.92
		No. (%) of samples > ML	0	0
		Maximum level (µg kg^{-1})	25.06	1033.92
	South-East development region (*n* = 22)	No. of samples	9	13
		Frequency (%)	44.44	84.62
		Mean (± SD) (µg kg^{-1})*	29.47 (± 42.87)	438.76 (± 647.86)
		Range (µg kg^{-1})*	5.15 – 142.69	16.66 – 1592.51
		No. (%) of samples > ML	0	3 (23.08)
		Maximum level (µg kg^{-1})	142.59	1592.51
Macroregion 3 (*n* = 18)	**South-Muntenia** development region (*n* = 18)	No. of samples	7	11
		Frequency (%)	57.14	100.00
		Mean (± SD) (µg kg^{-1})*	28.56 (± 23.18)	866.02 (± 644.65)
		Range (µg kg^{-1})*	13.00 – 79.31	55.19 – 1581.08
		No. (%) of samples > ML	0	5 (45.45)
		Maximum level (µg kg^{-1})	79.31	1581.08
	Bucharest-Ilfov development region (*n* = 0)	No. of samples	0	0
		Frequency (%)	n.a.	n.a.
		Mean (± SD) (µg kg^{-1})*	n.a.	n.a.
		Range (µg kg^{-1})*	n.a.	n.a.
		No. (%) of samples > ML	n.a.	n.a.
		Maximum level (µg kg^{-1})	n.a.	n.a.

Macroregion 4 (*n* = 27)	**South-West Oltenia** development region (*n* = 17)	No. of samples	9	8
		Frequency (%)	66.67	100.00
		Mean (± SD) (µg kg^{-1})*	76.57 (± 77.36)	797.64 (± 274.24)
		Range (µg kg^{-1})*	2.94 – 183.27	414.36 – 1284.79
		No. (%) of samples > ML	0	1 (12.50)
		Maximum level (µg kg^{-1})	183.27	1284.79
	West development region (*n* = 10)	No. of samples	6	4
		Frequency (%)	66.67	100.00
		Mean (± SD) (µg kg^{-1})*	20.89 (± 10.75)	1307.88 (± 241.56)
		Range (µg kg^{-1})*	11.45 – 41.16	948.54 – 1471.48
		No. (%) of samples > ML	0	3 (75.00)
		Maximum level (µg kg^{-1})	41.16	1471.48
Overall (*n* = 122)		No. of samples	61	61
		Frequency (%)	52.46	93.44
		Mean (± SD) (µg kg^{-1})*	72.99 (± 158.28)	745.59 (± 583.27)
		Range (µg kg^{-1})*	2.94 – 912.11	11.93 – 1592.51
		No. (%) of samples > ML	0	19 (31.15)
		Maximum level (µg kg^{-1})	912.11	1592.51
		ML (µg kg^{-1})	1250.00	1250.00

N: number of analysed samples; Frequency: the percent of samples ≥ 18.50 µg kg^{-1}/total number of samples from that region; Range: minimum and maximum values; Mean: average of the positive results; SD: standard deviation; ML: maximum permitted level set by EC Commission Regulation No. 1881/2006 for unprocessed cereals other than durum wheat, oats and maize; *Results for which the 'Out of range' function of the RIDA®SOFT Win software was applied; n.a.: not applicable.

When referring to the 2018 barley samples, results show that Macroregion 1 (*n* = 20) noted 7 barley samples with DON concentrations below the limit of detection of the assay (< 18.50 µg kg^{-1}), 10 samples with DON levels in the range of 18.51 – 500.00 µg kg^{-1} and 3 samples with DON concentrations in the range of 500.01 – 1250.00 µg kg^{-1}. No sample from this geographical area exceeded the maximum level of 1250.00 µg kg^{-1} in 2018. The maximum level of DON (912.11 µg kg^{-1}) noted in Macroregion 1 in 2018 was noted by a barley sample from Bistrița Năsăud County (North-West development region). This was also, the highest level of DON noted for the 2018 barley crop in Romania. In 2019, Macroregion 1 (*n* = 14) registered no barley sample with DON levels under the limit of detection of the ELISA kit. There were 3 samples with DON concentrations in the range of 18.51 – 500.00 µg kg^{-1} and 4 samples with DON concentrations in the range of 500.01 – 1250.00 µg kg^{-1}. The maximum admitted level of 1250.00 µg kg^{-1} was exceeded by 7 barley samples in 2019. The maximum level of DON (1579.96 µg kg^{-1}) noted in Macroregion 1 in 2019 was noted by a barley sample from Covasna County (Central development region).

There were assessed 19 barley samples from Macroregion 2 in 2018 and 14 samples recorded DON concentrations below the limit of detection of the assay, while 5 samples noted concentrations within the range of 18.51 – 500.00 µg kg^{-1}. No sample from this geographical area exceeded the maximum admitted level of 1250.00 µg kg^{-1}. In 2018, the maximum concentration of DON (142.69 µg kg^{-1}) in Macroregion 2 was noted by a barley sample from Constanța County (South-East development region). In 2019, Macroregion 2 (*n* = 24) registered 4 barley sample with DON levels under the limit of detection of the ELISA kit, 14 samples with DON concentrations in the range of 18.51 – 500.00 µg kg^{-1} and 3 samples with DON concentrations in the range of 500.01 – 1250.00 µg kg^{-1}. There were noted 3 samples which exceeded 1250.00 µg kg^{-1} DON in 2019. In 2019, the maximum level of DON (1592.51 µg kg^{-1}) noted in Macroregion 2 was noted by a barley sample from Brăila County (South-East development region). This was also, the highest level of DON noted for the 2019 barley crop in Romania.

In 2018, there were assessed only 7 barley samples from Macroregion 3, where 3 samples noted DON concentrations below the limit of detection of the assay (< 18,50 µg kg^{-1}), while 4 samples recorded levels in the range of 0 – 1.75 µg kg^{-1}. No sample from this geographical area exceeded the maximum admitted level of 1250.00 µg kg^{-1}. The highest concentration (79.31 µg kg^{-1}) of DON was noted in Teleorman County (South-Muntenia development region). In 2019, there were assessed 11 barley samples from Macroregion 3. There were noted 5 samples with DON levels in the range of 18.51 – 500.00 µg kg^{-1} and 1 sample with DON concentrations in the range of 500.01 – 1250.00 µg kg^{-1}. Also, 5 samples from this region exceeded the maximum admitted level. In 2019, the maximum level of DON (1581.08 µg kg^{-1}) noted in Macroregion 3 was noted by a barley sample from Dâmbovița County (South-Muntenia development region).

For the 2018 barley harvest, there were assessed 15 samples from Macroregion 4. A number of 5 samples noted DON levels below the detection limit of the ELISA kit (< 18.50 µg kg^{-1}), while

10 samples noted DON concentrations in the range of 18.51 – 500.00 µg kg^{-1}. The highest concentration (183.27 µg kg^{-1}) of DON was noted in Vâlcea County (South-West Oltenia development region). In 2019, there were assessed 12 barley samples from this geographical area. There was noted one sample with DON levels in the range of 18.51 – 500.00 µg kg^{-1} and 7 samples with DON concentrations in the range of 500.01 – 1250.00 µg kg^{-1}. Also, 4 samples from this region exceeded the maximum admitted level. In 2019, the maximum level of DON (1471.48 µg kg^{-1}) noted in Macroregion 4 was noted by a barley sample from Caraș Severin County (West development region).

In the current study across Romania, the results might indicate prevailing climatic conditions for 2019 which favoured high infection rates with *Fusarium* spp. and increased the incidence of DON in barley samples. Similar climatic conditions were also observed in the summer months of 1997 in the western part of Romania, which resulted in a wide spread contamination with DON and its derivatives in wheat and maize for animal consumption [5]. To the best of the author's knowledge, there is only sporadic published data investigating the occurrence of *Fusarium* mycotoxins in barley from Romania during the last decade. However, the changed mycotoxin pattern between 2018 and 2019 may be also attributed for instance to competition between the various toxinogenic *Fusarium* species, as a significantly higher mean level of DON might be assumed to a large variability in toxinogenic fungi in the growing localities [21].

In 2019, Dâmbovița County (Macroregion 3, South-Muntenia development region) was identified as the region with the highest average of contaminated samples, 1578.24 µg kg^{-1} (± 4.02 µg kg^{-1}). In South-Muntenia development region there were noted other 2 Counties which scored average results which exceeded the DON limit imposed by the European regulations: Giurgiu County, with an average of 1395.30 µg kg^{-1} (± 130.23 µg kg^{-1}) and Teleorman County, with only one barley sample which noted 1355.30 µg kg^{-1}. Macroregion 1 also noted 3 Counties with average levels of DON which exceeded the imposed EU regulations: Alba County (Central development region), which noted an average of 1535.01 µg kg^{-1} (± 46.27 µg kg^{-1}), Cluj county (North-West development region) with an average of 1335.83 µg kg^{-1} (± 3.62 µg kg^{-1}) and Bihor County (North-West development region) that registered an average of 1289.54 µg kg^{-1} (± 279.80 µg kg^{-1}). Thus, the southern, central and north-western areas of Romania acted as hotspot regions for DON, requiring implementation of DON management strategies to reduce mycotoxin contamination in the field, in order to result safe barley crops that will enhance trade and increase income and welfare of farmers and consumers.

DON contamination in correlation with the applied agronomic practices and cropping systems

Growing locality, susceptibility (mycotoxin content taken as criterion) of a particular barley cultivar to *Fusarium* infection and fungicide treatment were employed in our study for the identification of essential pre-harvest factors impacting levels of the target mycotoxin.

For the 2018 barley harvest, the monitoring questionnaires indicated the use of Romanian barley cultivars for 32.79% of the assessed samples. Other types of cultivars belonged to producers such as Saaten Union (16.39%), followed by Caussade Semences (8.20%), Biocrop (6.56%), Syngenta Agro SRL and Limagrain, both of them registering a percent of 4.92%. A number of 7 barley cultivars belonging to other producers were also noted, while for 14.75% of the samples this information was either missing or not very clear in the monitoring questionnaires.

When referring to the 2019 barley samples, the monitoring questionnaires indicated the use of Romanian barley cultivars again for 32.79% of the samples. Other types of cultivars belonged to producers such as Saaten Union (16.39%), Caussade Semences (13.11%), Biocrop (8.20%), Syngenta Agro SRL (4.92%) and Limagrain (4.92%). Barley cultivars belonging to other producers were also noted, but only in a limited number (4 barley samples), while for 13.11% of the samples this information was missing from the monitoring questionnaires.

Different grain species and varieties differ in their susceptibility to *Fusarium* [20]. However, when referring to the analysed samples of this survey, DON contamination was independent of the type of cultivar. Our results are in agreement with another study, which showed that the barley cultivar seemed to have not a significant effect on DON concentrations [21]. However, physiological features of the barley plant such as height of vegetation, resistance to lodging and days to maturity of the barley cultivar (calculated from sowing date to harvest) are important factors in this context [21]. Tall plants are usually more resistant to FHB than short plants and thus their seeds contain less DON. Also, spike characteristic play a major role in FHB infection and DON contamination, as the two-row spike was associated with tall stature and resistance to lodging than the six-row type [4].

Significant differences were noted only in between DON concentrations of the same barley cultivar assessed during the two consecutive years. Thus, variation in the susceptibility to different *Fusarium* species in interaction with climatic factors and agricultural practices during critical phases of plant growth may explain some of the observed differences [20; 23]. A study which assessed about 3000 samples of barley, oats and wheat grown in Norway, showed a significantly higher content of DON in oats than both barley and wheat in grain produced by ordinary grain producers in Norway [20]. Thus, the differences in contamination level between the grain species seemed to be due partly to edaphic and agro-technical factors and partly to variation in the susceptibility to different *Fusarium* species in interaction with climatic factors.

Also, fungicide treatment was selected as a factor with high importance. However, no barley cultivar showed statistically significant ($P > 0.05$) differences in DON content between the different levels of used nitrogen fertilisation in the seasons of 2018 and 2019 in Romania. Generally, the results of studies assessing the impact of fungicides on mycotoxin levels in treated crops are rather contradictory. In any case, besides the type of active ingredient and the method and time of its application, both weather conditions at the time of fungicide use and the extent and aggressiveness of *Fusarium* species invasion play an important role [21]. This was also noticed in our study, as the outcome of similar fungicidal treatments was highly variable in between the two studied years. Thus, none of the tested fungicides, although they represented various classes, guaranteed an effective reduction of DON levels in treated barley in 2019.

Our results showed that southern, western and also central counties proved to represent hotspot regions for DON contamination in 2019 and taking into consideration the information provided by the monitoring questionnaires, some of these counties noted poor agricultural practices, also. Thus, in order to prevent the occurrence of DON, farmers from hotspot regions for mycotoxin contamination should avoid rotating barley directly with corn, as corn residue is a preferred host for *Fusarium*, which can cause DON accumulation on the seed. However, barley does well following legumes like potatoes, as it pulse crops because of the availability of residual nitrogen.

Conclusions

This 2018-2019 survey has been aimed at extending knowledge on the impact of several factors on DON mycotoxin in various barley cultivars across Romania. Our results indicated that barley is a potential source of DON exposure in certain regions of Romania. Hotspot regions for DON were identified in various areas but only when environmental conditions were favourable for the occurrence of toxigenic fungi. For this reason, the results showed that a significant fraction of the analysed samples belonging to the 2019 harvest (31.15%) contained unsafe DON levels, which exceeded the threshold set by the European regulations (1250.00 μg kg^{-1}). However, high concentrations of DON were independent of the type of barley cultivar as well as the different levels of nitrogen fertilisers. Even so, the results should be interpreted with high caution, as mycotoxin presence is never justified by only one single factor, such as climatic conditions, geographic position, seasonal variances, agricultural practices, type of cultivar or physiological features of the barley plant.

However, DON remains a major *Fusarium* toxin in barley. Therefore, measures to reduce mycotoxin contamination are needed in barley. For this reason, the occurrence of this mycotoxin is to be further studied, as the observed changes in between various agricultural years represent factors influencing the pattern of these pathogens in barley crops. Also, a better understanding of grain morphological features on DON levels for barley cultivars in Romania and the interrelationship between plant traits and *Fusarium* resistance are needed in the development of effective and efficient breeding strategies for *Fusarium*-resistant cultivars. Even more, a correct selection of proper agronomic practices which favour reduced occurrence of DON in regard to the specific environmental and climatic conditions of the hotspot areas are desired.

Our results highlight the impact of climate conditions in the occurrence of mycotoxins and furthermore, the obtained knowledge from our current study will aid in the development of risk maps and DON management strategies for the barley crop in Romania.

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