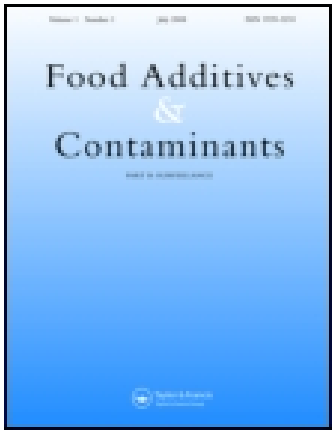

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Mycoflora and deoxynivalenol in whole wheat grains (*Triticum aestivum* L.) from Southern Brazil

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The fungal species *Fusarium graminearum* is related to deoxynivalenol (DON) formation. The aim of this study was to evaluate mycoflora and DON occurrence in 53 whole wheat grain samples collected in Southern Brazil during the 2012 crop. Wheat grains showed adequate values of water activity ranging from 0.48 to 0.72, within the required limits of moisture content, ranging from 9.1% to 13.9%. In addition, low counts of fungal colonies, ranging from 10 to 8.2×10^2, were found. For *Fusarium* genera, there was predominance of *Fusarium verticillioides* (34%) and *F graminearum* (30.2%). For *Aspergillus* species, 37.7% of *Aspergillus flavus* was determined. Regarding the *Penicillium* species, *Penicillium digitatum* (49%) was the most found species. DON was detected in 47.2% (25 out of 53) of the samples analysed, with levels ranging from 243.7 to 2281.3 μg kg^{-1} (mean: 641.9 μg kg^{-1}).

Keywords: *Fusarium graminearum*; wheat grains; deoxynivalenol; mycoflora

Introduction

Cereals may be contaminated by toxigenic fungal strains under favourable conditions during all stages of plant development in the field and storage steps (Scussel et al. 2011). The main fungal genera reported in harvest or stored grains are *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium* and *Penicillium* (Bräse et al. 2009). However, *Fusarium, Aspergillus* and *Penicillium* sp. are more often found in wheat grain worldwide (González et al. 1996, 1998; Berghofer et al. 2003; Muthomi et al. 2008; Riba et al. 2008; Roigé et al. 2009; Riba et al. 2010). In the literature, *Fusarium* species, especially *Fusarium graminearum*, is frequently associated with *Fusarium* head blight (FHB) in wheat (Del Pont et al. 2012). FHB is a fungal disease that occurs primarily by inoculum of *F. graminearum* in the wheat plant. *F. graminearum* appear to be capable of producing ascopores (sexual spores) in the natural state, which are produced in perithecia on wheat crop residues. These spores are carried by wind over long distances and deposited in anthers, cause infection in the plant and, consequently, promote depigmentation of affected spikelets. Depigmentation occurs as green to white/brown/yellow. If the environment is moist enough, pathogens produce masses of conidia, which appear pink in colour. Conidia are also a major source of inoculums (Panisson et al. 2002; Lima 2004).

Among the mycotoxins associated with FHB, such as deoxynivalenol (DON), nivalenol, T-2 and HT-2 toxins, DON is the most common in wheat grains (Muthomi et al. 2008; Bensassi et al. 2010; Soleimany et al. 2012; Stanković et al. 2012; Santos et al. 2013). The accumulation of DON in human and animal bodies after ingestion of contaminated food can induce acute and chronic effects, such as immunosuppression, neurotoxicity, embryotoxicity and teratogenicity (Rotter et al. 1996; Wijnands & Van Leusden 2000; Pestka 2007).

In the last two years in Brazil, around 5.09 million tons of wheat were harvested, mainly in the Southern Brazil region (94%). Nevertheless, the annual national demand for wheat grain is about 10 million tons. For this reason, Brazil imports approximately 5.7 million tons of wheat grain, mainly from Argentina (CONAB 2012). The subtropical climate in this region may cause exposure of grains and seeds to contamination by fungi and mycotoxins, due to environmental factors, mainly temperature and humidity (Astolfi et al. 2012; CONAB 2012).

Since 2012, the Brazilian regulation has proposed maximum levels (MLs) of 2000 μg kg^{-1} for DON in whole wheat grains for human consumption. This limit will decrease over time to allow grain producers and the industry to meet the legislation requirements, without causing a shortage of wheat. From January 2014, DON limits for whole wheat grain will be set at 1500 μg kg^{-1} and in January 2016, at 1000 μg kg^{-1} (Brasil 2011). Nowadays, the lower limit of DON is equal to 1250 μg kg^{-1}, as fixed by the European Commission for unprocessed cereals and 1750 μg kg^{-1} for unprocessed durum wheat (EC 2006a).

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In order to evaluate whether the quality of wheat produced in Brazil is in accordance with the Brazilian regulation, this paper aims to assess the occurrence of DON contamination in whole wheat grains, especially in the most important productive regions in Southern Brazil. In addition, the detection and identification of mycoflora in wheat grains was performed. A method was validated for determination of DON content in whole wheat grains, involving mycotoxin extraction from the sample by a clean-up step using an immunoaffinity column and quantitative analysis by high-performance liquid chromatography (HPLC)/UV.

Materials and methods

Reagents and materials

Culture media and reagents used were Pro-analysis grade. Potato dextrose agar (PDA), malt extract agar (MEA) and peptone bacteriology media were purchased from Himedia (Curitiba, Parana, Brazil). Czapek-dox, 25% glycerol nitrate (GN25), czapek yeast extract (CYA) media and chloramphenicol were obtained from Vetec (Duque de Caxias, RJ, Brazil).

DON standard was supplied by Sigma Aldrich Chemicals (St. Louis, MO, USA), reconstituted in a concentration of 1 mg ml^{-1} acetonitrile and stored at –20°C before use. Working standard solutions, ranging from 0.15 to 15 μg ml^{-1}, were prepared from suitable dilutions of the stock solution in the mobile phase (acetonitrile:water, 10:90, v/v) and stored at 4°C. The solvents acetonitrile and methanol were obtained from Vetec (Duque de Caxias, RJ, Brazil) at LC grade. High-purity milli-Q water (18.2 MΩ cm^{-1}) was obtained from a Millipore Synergy system (Millipore, Bedford, MA, USA). For sample clean-up step, an immunoaffinity column from DON-Test (Vicam, Milford, MA, USA) was used according to the manufacturer procedures.

Instruments

Moisture content (mc) and water activity (a_w) were determined using a drying oven, Olidef-cz (Ribeirao Preto, SP, Brazil) and Aqua-Lab 4TE Decagon Devices (Sao Jose dos Campos, SP, Brazil), respectively.

Regarding the mycological tests, the following equipment were required: light microscopes (LM), CH-Bl45-2, Olympus (Shinjuku, Tokyo, Japan); autoclave, Phoenix (Araraquara, SP, Brazil); microwave oven, Philco (Sao Paulo, SP, Brazil); laminar flow cabinet, Veco (Campinas, SP, Brazil); fume cabinet, Quimis (Diadema, SP, Brazil); rotary shaker, Marconi (Piracicaba, SP, Brazil) and microbiological incubator, Quimis (Diadema, SP, Brazil).

Whole wheat samples were ground in a laboratory mill Romer 1301 (Union, MO, USA). The determination of DON was carried out by HPLC equipment model 321 of Gilson (Middleton, WI, USA) equipped with an isocratic pump model 805, a manual injector (20 μL loop), an ultraviolet–visible (UV) detector model 118 set at 218 nm and a chromatographic column C18 250 × 4.60 mm reversed-phase, with 4 μ particle size Fusion-RP 80, Phenomenex (Madrid Avenue, Torrance, CA, USA).

Sampling

A total of 53 whole wheat grain samples were collected during the 2012 crop, of different varieties recommended for cultivation in Rio Grande do Sul-RS, Parana-PR and Santa Catarina-SC states, in Southern Brazil. The samples were collected from bulk batches, after dirt removal and drying (up to a maximum of 60°C) in the storage units. Collection was performed using a grain auger from different points of the bulk batches, with a minimum final weight of ca. 10 kg. Each sample was homogenised and reduced in portions varying around 2.0 kg. Samples were packed in polyethylene bags and stored at 4°C for immediate analysis of mycoflora, in the Laboratory of Mycology, and DON analysis, in the Laboratory of Mycotoxicology and Food Contaminants, Food Science and Technology Department, Center of Agricultural Sciences at the Federal University of Santa Catarina, Florianopolis, Santa Catarina.

Moisture content, water activity and mycological analysis

To determine mc, wheat grains (2 g) were submitted to a drying process in an oven (105 ± 5°C) up to a constant weight using gravimetric method. In addition, for a_w determination, the wheat grains (2 g) were submitted to Aqua-Lab 4TE equipment. All analyses were performed in triplicate and in accordance to Association of Official Analytical Chemists – AOAC (2005).

Mycological analysis was applied as enumeration technique to evaluate fungal total load (Silva et al. 2010). Twenty-five grams of each sample was added to 225 ml of 0.1% peptone dissolved in water in sterile conditions. The mixture was stirred on a rotary shaker for 2 min., dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were obtained, 0.1 ml aliquots of each dilution were spread (in duplicate) on the surface of the PDA medium containing chloramphenicol (100 mg l^{-1}) and incubated for a maximum of 7 days, at 28°C in the dark.

The results were presented taking into account the colony forming units per gram (CFU g^{-1}) in the dilution 10^{-1}. For identification of fungal genera and species, the isolated strains were sub-cultured on PDA, MEA, GN25 and CYA media. Species identification was performed through microcultivation in carnation leaf agar for *Fusarium* and Czapek-dox for *Aspergillus* and *Penicillium*

species, as described by Weber and Pitt (2000) and Samson et al. (2006). The isolates were examined under a light microscope (100× and 400× magnifications) and species identification was carried out according to available taxonomic keys and guides (Raper & Fennel 1965; Pitt 1979; Nelson et al. 1983; Pitt & Hocking 1997).

DON quantification

Whole wheat samples (2.0 kg) were ground in a Romer mill (with automatic quartering) and portions of 25 g were taken for DON analysis, using clean-up with immunoaffinity columns according to the Vicam protocol DON Test, No. G1005 USA (Vicam 2013) with slight modifications. Briefly, 25 g of each sample were mixed into an industrial blender and set into an industrial blender jar with 100 ml of ultra pure water. The mixture was blended for 30 seconds, filtered twice and cleaned by an immunoaffinity column (DONTest HPLC), which was first conditioned with 1 ml ultra pure water, at a flow rate of one drop per second. After washing the column with 2.5 ml of ultra pure water, the mycotoxin was slowly eluted with 2 ml of 100% LC grade methanol. The eluate was evaporated using a heating block set at 40°C with a gentle nitrogen stream and the dry residue was then re-dissolved in 100 µL of mobile phase (acetonitrile:water, 10:90, v/v). The extract (20 µL) was injected in the LC/UV system set at 218 nm and a flow rate of 0.6 ml min^{-1}. The DON quantification was performed by measurement of peak area at DON retention time compared with the standard solutions used for the calibration curve (0.15, 0.20, 0.25, 0.50, 1, 2, 3, 4, 5, 7.5, 10 and 15 µg ml^{-1}) with correlation coefficient (r) = 0.996. Recoveries were obtained by spiking DON-free wheat samples at DON concentrations of 250, 1000 and 1500 µg kg^{-1} at the same day and under same HPLC conditions. This work was performed in the LABMICO laboratory, which is accredited by Ministry of Agriculture and Food Supplies (MAPA), following ISO/IEC 17025 (2005) procedures. For quality control, samples were analysed on five different days. Proficiency test was performed in an interlaboratory study with matrix reference materials of Romer Labs, following ISO/IEC 17043:2010 (Conformity Assessment General Requirements for Proficiency Testing) with z-scores between –1 and 1.7 for DON. Measurement uncertainty (data not shown in the table) was performed according to European Commission Regulation No. 401/2006 (EC 2006b).

Results and discussion

Moisture content, water activity and mycological analysis

Under favourable conditions, such as high temperature (25–35°C), mc (13–16%) and a_w (0.70–0.90), toxigenic fungi can produce mycotoxins (Scussel 2002). Therefore, maximum mc required in wheat, according to official Brazilian classification, is 13%, as defined in Normative Instruction No. 7 (Brasil 2010). In this study, the whole wheat grains showed values from 0.48 to 0.72 (mean 0.58 ± 0.01) a_w, which meet the required mc limits ranging from 9.14% to 13.94% (mean: 11.38 ± 1.06%). In addition, it presented low count fungal colonies, ranging from 10 to 8.2×10^2 (mean: $1.9 \times 10^2 \pm 1.4 \times 10^2$ CFU g^{-1}). According to the Brazilian regulation, there is no maximum limit for fungal amount in unprocessed wheat grains; however, the presence of toxigenic fungal species in food can be indicative of potential mycotoxin accumulation.

With respect to the whole wheat grain samples, it is possible to highlight the presence of different isolated fungal genera, especially the *Fusarium* (34% of *Fusarium verticillioides* and 30.2% of *F. graminearum*), *Aspergillus* and *Penicillium* spp., as shown in Table 1. *Fusarium graminearum* can be responsible for DON production, as found in this study. DON levels in wheat grains are determined by conditions in the field before harvest. In Southern Brazil, rain and high humidity periods in the flowering periods can increase contamination by *F. graminearum*, which can cause FHB and DON

Table 1. Fungal species isolated from whole wheat grain samples from regions in South Brazil.

Wheat grains		Total samples contaminated	Isolated fungi (%)[a]
Genera	Species		
Fusarium			
Fusarium	*F. verticillioides*	18	34.0
	F. graminearum	16	30.2
	F. oxysporum	1	1.9
Other fungi			
Aspergillus	*A. flavus*	20	37.7
	A. parasiticus	7	13.2
	A. nivea	6	11.3
	A. penicillioides	2	3.8
	A. oryzae	2	3.8
	A. terreus	2	3.8
	A. niger	1	1.9
	A. ochraceus	1	1.9
Penicillium	*P. digitatum*	26	49.0
	P. olsonii	5	9.4
	P. expansum	4	7.5
	P. thomii	4	7.5
	P. citrinum	2	3.8
Mucor	*M. plumbeus*	9	17
Cladosporium	*C. herbarum*	4	7.5
Trichoderma	*T. harzianum Rifae*	3	5.7
Cumminghamella	*C. bertholettia*	3	5.7
Rhizopus	*R. oryzae*	3	5.7
Alternaria	*A. alternate*	2	3.8
Byssochlamys	*B. fulva*	1	1.9
Monascus	*M. ruber*	1	1.9

Note: [a]Percentage of isolated fungal species found in the investigated wheat samples (n = 53).

accumulation. It is important to mention that the level of resistance of wheat cultivar also has effects on DON contamination (Astolfi et al. 2012; Del Pont et al. 2012). Previous studies in the country showed DON to be the main mycotoxin in wheat grains (Calori-Domingues et al. 2007; Santos et al. 2013). Similarly, in wheat grains collected in Argentina, *F. graminearum* also was the most frequently isolated species (González et al. 1996, 1998), like it was in Kenya (Muthomi et al. 2008). Co-occurrence of DON with other mycotoxins in naturally contaminated wheat, such as fumonisins (FBs), ochratoxin and zearalenone, was also reported (Birzele et al. 2000; Marques et al. 2008; Stanković et al. 2012). The major FB-producing fungus, *F. verticillioides*, was often found in this study.

Among the *Aspergillus* species, the most found was *Aspergillus flavus* (around 37.7%), followed by *Aspergillus parasiticus* (13.2%) and *Aspergillus nivea* (11.3%), which is in accordance with the Riba et al. (2008, 2010) studies. *Penicillium* genera were often found in this study, with predominance of *Penicillium digitatum* (49%) and *Penicillium olsonii* (9.4%). These genera are also mentioned in studies in Argentina (Roigé et al. 2009) and Australia (Berghofer et al. 2003). Other fungal species were found in lower predominance (Table 1).

Method validation of DON

The HPLC/UV method for DON separation and the validation parameter linearity, limits of detection and quantification (LOD and LOQ), reproducibility, repeatability and recovery obtained have shown to be quite adequate. Under the chromatographic conditions used, the retention time (Rt) of DON was ca. 17 ± 0.5 min. Linearity was determined from the calibration curve, which was linear in the range 0.15–15 μg ml^{-1}, with a correlation coefficient (r) equal to 0.996. The LOD (signal-to-noise ratio = 3) and LOQ (signal-to-noise ratio = 10) were 66.7 and 119.1 μg kg^{-1}, respectively. The recovery experiments were determined by blank wheat grains spiked with DON at concentrations of 250, 1000 and 1500 μg kg^{-1} and analysed in triplicate. They showed yields equal to 87 ± 9%, 96 ± 6% and 93 ± 3%, respectively, with a mean of 92 ± 4%.

DON levels in wheat grains

DON was detected in approximately 47.2% (25 out of 53) of the samples analysed, at levels of 243.7 to 2281.3 μg kg^{-1} (mean: 641.9 μg kg^{-1}), as presented in Table 2. Considering European Comission rules (2006a) regarding to the ML for DON in unprocessed cereals (set

Table 2. DON levels in whole wheat grain samples from regions in South Brazil.

Number of wheat samples	Distribution levels of DON (μg kg^{-1})	Frequency (%)	Positive samples (μg kg^{-1})	Mean contamination of positive samples (μg kg^{-1})
28	<119.1[a]	52.8	0	0
13	200–500	24.5	243.7	320.9
			257.8	
			258.6	
			287.2	
			287.8	
			291.3	
			298.5	
			318.3	
			358.9	
			362.3	
			374.1	
			405.9	
			427.6	
8	500–1000	15.1	508.8	677.0
			527.3	
			547.3	
			726.2	
			731.5	
			746.5	
			805.2	
			823.65	
3	1000–2000	5.7	1370.1	1392.5
			1382.9	
			1424.6	
1	2000–3000	1.9	2281.3	2281.3
Total: 53		100	243.7–2281.3	641.9

Note: [a]Method LOQ = 119.1 μg kg^{-1}.

Table 3. DON levels in wheat grain samples found in other studies.

Country		Frequency (%)	Range (μg kg^{-1})	Mean (μg kg^{-1})	References
Brazil		94	90–4573	332	Calori-Domingues et al. (2007)
Brazil		66.4	206.3–4732.3	1894.9	Santos et al. (2013)
Serbia	2005[a]	85.7	52–3306	605.5	Stanković et al. (2012)
	2007[a]	93.3	50–1090	282.8	
Kenya	Nakuru	75	105–303	132.7	Muthomi et al. (2008)
	Nyandarua	59	105–289	113	
Tunisia		83	12800–30500 μg kg^{-1}	21520	Bensassi et al. (2010)
Malaysian		25	5.5–18.6	–	Soleimany et al. (2012)
India		40	70–4730	910	Mishra et al. (2013)
Morocco		11.1	321–1310	502.1	Ennouari et al. (2013)

Note: [a]year of the study.

at 1250 μg kg^{-1}) and the Brazilian regulations (set at 2000 μg kg^{-1}), only 4 (7.5%) and 1 (1.9%) samples disagreed in both regulations, respectively. However, considering future Brazilian regulations for DON levels in whole wheat grains from 2016, foreseen at 1000 μg kg^{-1}, four (7.5%) samples would be in regulation disagreement (Brasil 2011). Similar to these results, Santos et al. (2013) found in northern and central/southwestern region of Parana state, Brazil, 66.4% contaminated samples with DON levels from 206.3 to 4732.3 μg kg^{-1} (mean 1894.9 μg kg^{-1}). In 2007, Calori-Domingues et al. reported for Southern states of Brazil in Sao Paulo, Parana and Rio Grande, 94% DON-contaminated wheat grains with levels from 90 to 4573 μg kg^{-1} (mean 332 μg kg^{-1}). Other studies reported DON occurrence in wheat grains worldwide, as presented in Table 3.

Conclusion

Whole wheat grain samples produced in Rio Grande do Sul-RS, Parana-PR and Santa Catarina-SC states in Southern Brazil, showed low fungal load and DON levels below the ML, as set by the EU and Brazil regulations in almost all samples. High contamination levels occurred in 4 (7.5%) samples above the EU ML, which in only 1 (1.9%) sample exceeded the Brazilian ML (2000 μg kg^{-1}). However, the heterogeneous and wide occurrence of DON, even at low concentrations in some cases, must be a matter of concern and show the importance the study. The knowledge of mycoflora and DON levels may contribute to the adoption of corrective measures, such as prevention and control by means of grain-monitoring programmes throughout the production and storage periods. Thus, it will be possible to verify quickly any irregularity and consequently adopt corrective measures to reduce risks of mycotoxin contamination.

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