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Fungi and the natural occurrence of deoxynivalenol and fumonisins in malting barley (*Hordeum vulgare* L.)

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ABSTRACT

The industrial use of barley grain has experienced continuous growth, mainly due to its economic importance for malt production. From a technological perspective, fungal persistence can reduce product marketability and cause economic losses. In this sense, the aim of the present study was to determine the presence and identification of mycoflora and the occurrence of deoxynivalenol (DON) and fumonisins (FBs) in malting barley. The samples presented a low count of fungal colonies, with values ranging from10.5 to 0.5×10^1 CFU g^{-1} and the species most found were *Fusarium graminearum* and *Fusarium verticillioides* with 26% and 12% of incidence, respectively. In the samples analyzed for mycotoxins occurrence, DON and FBs were present in 18% and 12%, respectively. The high concentrations of toxins found in the malting samples may be strongly influenced by agricultural practices and the weather conditions during critical phases of plant growth.

1. Introduction

Barley (*Hordeum vulgare* L.) is one of the most important cereals in the world with an estimated global production of approximately 141 million tons produced in 50 million hectares in 2013 (USDA, 2011). The industrial use of barley grain has experienced continuous growth mainly due to its economic importance for malt production (Oliveira, Mauch, Jacob, Waters, & Arendt, 2012).

Several works have demonstrated that the barley can be contaminated with fungi and, from a technological perspective, fungal persistence can reduce product marketability causing economic losses. This lowers the quality of the grain and, consequently, the final product is affected (Noots, Delcour, & Michiels, 1999; Schwarz, Schwarz, Zhou, Prom, & Steffenson, 2001).

The fungi genus that should be taken into account for barley and other small grains is *Fusarium*, and this can be associated with a disease called *Fusarium* head blight (FHB) (Ibáñez-Vea, Lizzaraga, González-Peñaz, & López de Cerain, 2012 and Neuhof, Koch, Rasenko, & Nehls, 2008). The major species associated with FHB in Europe are *Fusarium graminearum*, *Fusarium avenaceum*, and *Fusarium culmorum*, and, to a lesser extent *Fusarium poae*, *Fusarium cerealis*, *Fusarium equiseti*, *Fusarium sporotrichioides* and *Fusarium tricinctum* (Bottalico & Perrone, 2002; Ibáñez-Vea et al., 2012).

Contamination of barley by these pathogenic fungi negatively affects vegetation and leads to reduced germination capacity and grain malting quality, as well as losses in yield (Oliveira et al., 2012). Although micromycetes of *Fusarium* spp. are known as "field fungi", they can also grow during storage (Hashmi & Ghaffar, 2006; Oliveira et al., 2012).

Among the mycotoxins associated with FHB, such as trichothecenes, deoxynivalenol (DON), nivalenol, T-2 and HT-2 toxins, DON is the most common found in barley grains. 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 (Pestka, 2007; Wijnands & Van Leusden, 2000).

Furthermore, an important problem found in the industry concerning DON presence in barley and consequently in beer, is "gushing", i.e. excessive foaming and overflowing upon opening a bottle. This has been reported frequently in the last few years and can seriously damage the beer quality and the reputation of the brewery.

Another relevant *Fusarium* species that can be found in barley is *Fusarium verticillioides* and this is a producer of fumonisins (FBs). FBs are a family of toxic and carcinogenic mycotoxins that cause serious diseases affecting humans and animals (Marasas et al., 2004). More than ten types of FBs have been isolated and characterized. Of these, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and

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fumonisin B_3 (FB_3) are the major FBs produced in nature, with FB_1 being the most prevalent and toxic (Musser & Plattner, 1997). Additionally, these toxins are catalogued as group 2B carcinogens by the International Agency for Research on Cancer (International Agency for Research on Cancer, 1993).

With respect to the mycotoxins produced by *Fusarium*, it can be said that they are produced mainly in the field, although some toxin synthesis may occur during storage. These may also increase during germination of the barley during the malting and brewing process of the beer (Pietri, Bertuzzi, Agosti, & Donaldini, 2010; Wolf-Hall, 2007). Basically, the temperature and moisture conditions are crucial factors and thereby affect fungal infection and toxin synthesis (Doyle, 1997).

Due to the health risks associated with the consumption of contaminated commodities, their occurrence is currently under regulation in many countries. In 2012, the Brazilian regulation proposed a maximum tolerable level (MTL) of 1.75 μg/g for DON in malted barley grains. The limit will be decreased over time to allow grain producers and the industry to adapt to the legislation without causing a shortage of barley. As of January 2017, DON limits for malted barley will be set at 0.75 μg/g (Brasil, 2011, 2013).

Currently, the limit fixed for DON by the Commission of the European Communities is equal to 1.75 μg/g for cereals and sub products (Commission of the European Communities, 2006). It is important to emphasize that there is no specific regulation for FBs in barley and, therefore, the raw material should be carefully monitored.

For the reasons stated above, the aim of the present study was to determine the presence and identification of mycoflora and also the occurrence of DON and FBs in malting barley from Brazil by means of High Performance Liquid Chromatography. It is important to mention that this research has not been developed in Brazil previously, and can therefore be a benchmark for the Brazilian beer industry from here onwards and cultivation/storage of this widespread cereal.

2. Materials and methods

2.1. Materials

2.1.1. Samples

A total of 50 malting barley grain samples were collected during the 2013 harvests, with a minimum weight of 1 kg (considering different varieties) recommended for cultivation in Rio Grande do Sul-RS, Parana-PR and Santa Catarina-SC states, in Southern Brazil. Samples were received after the cleaning and drying stages (up to a maximum of 60 °C) in the storage unit, packed in polyethylene bags and stored at 4 °C for immediate mycoflora, DON and FBs analysis.

2.2. Reagents and materials

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). Mycotoxin standards (DON and FBs) and OPA reagent were obtained from Sigma Aldrich Chemicals (St. Louis, MO, USA). All other chemicals and reagents were of analytical or HPLC grades.

2.3. Mycotoxins standards and preparation

A stock solution of DON was prepared by dissolving 1 mg of DON in 1 ml of acetonitrile. The standard curve solutions were prepared from appropriate dilutions of the stock solutions (200 μg/ml) with Milli-Q water (0.15–15 μg/ml). A stock solution of FB_1 and FB_2 was prepared by dissolving 1 mg of FB_s in 1 ml of methanol. The standard curve solutions were prepared from appropriate dilutions of the stock solutions (50 μg/ml) with methanol (0.005–2.5 μg/ml). For the clean- up stage, immunoaffinity columns were used from DON-test Vicam (Milford, MA, USA) and solid phase extraction (SPE) columns (Quarternary amino N+, 500 mg/6 ml) from Phenomenex (Torrance, CA, USA).

2.4. Moisture content and water activity

To determine water activity (aw) the barley grains (2 g) were submitted to the Aqua-Lab 4TE Decagon Devices (Sao Jose dos Campos, SP, Brazil). In addition, for moisture content determination, the barley grains (2 g) were submitted to a drying process in an Olidef-cz oven (Ribeirao Preto, SP, Brazil) (105 ± 5 °C) up to a constant weight using a gravimetric method. All analyses were performed in triplicate and in accordance with the Association of Official Analytical Chemists – Association of Official Analytical Chemists (2005).

2.5. Mycological analysis

Total fungal load counts were determined according to Silva et al., 2010, as an enumeration technique. Twenty-five grams of each sample were added to 225 ml of 0.1% peptone dissolved in water under sterile conditions. The mixture was stirred on a rotary shaker Marconi (Piracicaba, SP, Brazil) for 2 min and dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were obtained. 0.1 ml aliquots of each dilution were plated on the surface of the PDA medium containing chloramphenicol (100 mg l^{-1}) and incubated in a microbiological incubator, Quimis (Diadema, SP, Brazil) for 7 days, at 28 °C in the dark. The analyses were carried out in duplicate. The results were presented taking into account the colony forming units per gram (CFU g^{-1}) in the 10^{-1} dilution.

2.6. Incidence of fungal species

To accomplish the identification of fungal genera and species, the isolated strains were morphologically selected and then subcultured on PDA, MEA, GN25 and CYA media. Species identification was performed through microcultivation in Czapek-dox for all fungi genera (Weber and Pit, 2000; Samson, Hong, & Frisvad, 2006; Savi, Piacentini, Tibola, & Scussel, 2014). The isolates were examined under a light microscope (LM), CH-Bl45-2, Olympus (Shinjuku, Tokyo, Japan) (100× and 400× magnifications) and species identification was carried out according to available taxonomic keys and guides (Nelson, Toussoun, & Marassas, 1983; Pitt & Hocking, 1997; Raper & Fennel, 1965).

2.7. ON and FBs analysis

The malting barley samples were analysed for DON using immunoaffinity columns for the cleaning step and LC/UV for detection, according to the Vicam protocol DON test, N_o G1005 USA (Vicam, 2013), with some modifications. In short, 25 g of each sample were mixed into an industrial blender with 100 ml of ultrapure water, and blended for 30 s. Then, the mixture was filtered twice.

For sample clean-up and concentration, a 1 ml aliquot of the extract was applied to an immunoaffinity column (DON Test HPLC) at a flow rate of one drop per second. This column was previously conditioned with 1 ml of HPLC grade water. The sample was followed by 2.5 ml of HPLC grade water to wash the column, and the toxin was slowly eluted with 2 ml of 100% HPLC grade