International Journal of Food Microbiology 273 (2018) 33–42

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Fungal community, *Fusarium* head blight complex and secondary metabolites associated with malting barley grains harvested in Umbria, central Italy

Giovanni Beccari[a], Maria Teresa Senatore[a,1], Francesco Tini[a], Michael Sulyok[b], Lorenzo Covarelli[a,*]

[a] Department of Agricultural, Food and Environmental Sciences, University of Perugia, Borgo XX Giugno 74, 06121 Perugia, Italy
[b] Centre for Analytical Chemistry, Department of Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences (BOKU), Vienna, Konrad Lorenz Strasse 20, A-3430 Tulln, Austria

ARTICLE INFO

Keywords:
Fusarium
Barley
Mycotoxins
LC-MS/MS
Enniatins
Beer

ABSTRACT

In recent years, due to the negative impact of toxigenic mycobiota and of the accumulation of their secondary metabolites in malting barley grains, monitoring the evolution of fungal communities in a certain cultivation area as well as detecting the different mycotoxins present in the raw material prior to malting and brewing processes have become increasingly important. In this study, a survey was carried out on malting barley samples collected after their harvest in the Umbria region (central Italy). Samples were analyzed to determine the composition of the fungal community, to identify the isolated *Fusarium* species, to quantify fungal secondary metabolites in the grains and to characterize the *in vitro* mycotoxigenic profile of a subset of the isolated *Fusarium* strains. The fungal community of barley grains was mainly composed of microorganisms belonging to the genus *Alternaria* (77%), followed by those belonging to the genus *Fusarium* (27%). The *Fusarium* head blight (FHB) complex was represented by nine species with the predominance of *Fusarium poae* (37%), followed by *Fusarium avenaceum* (23%), *Fusarium graminearum* (22%) and *Fusarium tricinctum* (7%). Secondary metabolites biosynthesized by *Alternaria* and *Fusarium* species were present in the analyzed grains. Among those biosynthesized by *Fusarium* species, nivalenol and enniatins were the most prevalent ones. Type A trichothecenes (T-2 and HT-2 toxins) as well as beauvericin were also present with a high incidence. Conversely, the number of samples contaminated with deoxynivalenol was low. Conjugated forms, such as deoxynivalenol-3-glucoside and HT-2-glucoside, were detected for the first time in malting barley grains cultivated in the surveyed area. In addition, strains of *F. avenaceum* and *F. tricinctum* showed the ability to biosynthesize *in vitro* high concentrations of enniatins. The analysis of fungal secondary metabolites, both in the grains and *in vitro*, revealed also the presence of other compounds, for which further investigations will be required. The combination of microbiological analyses, of molecular biology assays and of multi-mycotoxin screening shed light on the complexity of the fungal community and its secondary metabolites released in malting barley.

1. Introduction

Barley (*Hordeum vulgare*) is one of the most important cereal crops worldwide. With a production of 988,285 tons in 2016 it is also one of the most cultivated cereals in Italy (FAO, 2018). A significant amount (10–15%) of the Italian barley production is used for malt obtainment, mostly destined to the national beer industry. In Italy, beer obtained from malting barley was estimated in 1 296,800 tons in 2014 (FAO, 2018) and this beverage is becoming increasingly important within the national food-processing economy. In fact, over the last three decades

Abbreviations: EU, European Union; FHB, *Fusarium* head blight; FIESC, *Fusarium incarnatum-equiseti* species complex; DON, deoxynivalenol; 3AcDON, 3-acetyldeoxynivalenol; 15AcDON, 15-acetyldeoxynivalenol; NIV, nivalenol; MON, moniliformin; ENs, enniatins; BEA, beauvericin; DON-3G, deoxynivalenol-3-glucoside; T-2G, T-2-glucoside; HT-2G, HT-2-glucoside; PDA, potato dextrose agar; SE, standard error; EDTA, ethylenediamine-tetraacetic acid disodium salt dehydrate; TAE, trizma base-glacial acid acetic-ethylenediamine tetra-acetic acid disodium salt dehydrate; *tef1α*, *translation elongation factor 1α*; LC-MS/MS, liquid chromatography-tandem mass spectrometry; ESI, electrospray ionization; MRM, multiple reaction monitoring; ENB, enniatin B; ENB1, enniatin B1; ENA1, enniatin A1; ENA, enniatin A; ZEN, zearalenone; TeA, tenuazonic acid; Ten, tentoxin; AOH, alternariol; AME, alternariol methyl ether; Macro, macrosporin; EAs, ergot alkaloids; ENB2, enniatin B2; ENB3, enniatin B3; HIV-1, human immunodeficiency virus type 1

* Corresponding author.
E-mail address: lorenzo.covarelli@unipg.it (L. Covarelli).
[1] Present address: Department of Agricultural Sciences, Alma Mater Studiorum University of Bologna, Viale G. Fanin 44, 40127, Bologna, Italy.

https://doi.org/10.1016/j.ijfoodmicro.2018.03.005
Received 17 November 2017; Received in revised form 15 February 2018; Accepted 10 March 2018
Available online 12 March 2018
0168-1605/

Italian beer consumption *pro capite* increased, while, in the last ten years the number of microbreweries had a 5-fold increase (AssoBirra, 2016; Colen and Swinnen, 2016).

Unfortunately, the quality of malting barley as well as malt production and the brewing process could be damaged by several fungal microorganisms (Laitila et al., 2007; Noots et al., 1998). Furthermore, the fungal community colonizing malting barley grains mainly consists of toxigenic genera (*i.e. Alternaria*, *Fusarium*, *Aspergillus*, *Penicillium*) (Beccari et al., 2016, 2017; Medina et al., 2006). In fact, the fungal species belonging to these genera are able to biosynthesize mycotoxins, secondary fungal metabolites causing a wide range of toxic effects in humans (Ferrigo et al., 2016; Ostry, 2008). The presence of a toxigenic mycobiota, along with a negative repercussion on the quality of malting and brewing (Oliveira et al., 2012, 2013; Sarlin et al., 2005; Schwarz et al., 2002; Wolf-Hall and Schwarz, 2002; Wolf-Hall, 2007), also represents a non-negligible risk factor for consumer's health (Inoue et al., 2013; Rodríguez-Carrasco et al., 2015). With this regard, maximum and recommended levels have been established for certain mycotoxins in foodstuffs in the European Union (EU) (Commission Recommendation, 2013; Commission Regulation, 2006, 2007), including traditional raw materials for beer production such as barley and wheat. In addition, other mycotoxins have not been legislated yet as well as many other fungal secondary metabolites with a still unclear impact on final consumer's health.

Among the typical fungal genera commonly colonizing malting barley grains, the *Fusarium* genus is considered the main threat to the obtainment of qualitatively good malt and beer, being able to infect the heads during the cultivation phase and to biosynthesize a wide range of mycotoxins. In fact, several *Fusarium* species are the causal agents of *Fusarium* head blight (FHB) and, at the same time, the most important mycotoxin source in malting barley. FHB is a complex disease caused by at least 17 *Fusarium* spp. (Parry et al., 1995), even if, only a few of them, such as *Fusarium graminearum*, *Fusarium poae*, *Fusarium avenaceum* and *Fusarium culmorum* are considered to be the main FHB agents (Xu et al., 2008). Other species such as *Fusarium sporotrichioides*, *Fusarium langsethiae*, *Fusarium tricinctum*, *Fusarium acuminatum* and members of the *Fusarium incarnatum-equiseti* species complex (FIESC) could be present with a low incidence. However, FHB complex composition is considered to vary, (Beccari et al., 2017; Ferrigo et al., 2016) as a consequence of climatic factors such as temperature and humidity during anthesis (Xu et al., 2008) as well as by previous crop (Schöneberg et al., 2016). Furthermore, a "single-field" cohabitation of more than one *Fusarium* species is common (Xu and Nicholson, 2009).

For these reasons, mycotoxin contamination of grains cultivated during a specific season and in a particular geographic area is the consequence of the *Fusarium* community composition (Vogelgsang et al., 2017). In fact, each *Fusarium* species possesses a specific mycotoxigenic profile, even if differences in the ability of biosynthesizing secondary metabolites were detected also among different strains of the same species (Ferrigo et al., 2016). *F. graminearum* and *F. culmorum* are the typical producers of type B trichothecenes such as deoxynivalenol (DON), with its acetylated forms (3-acetyldeoxynivalenol, 3AcDON and 15-acetyldeoxynivalenol, 15AcDON), and nivalenol (NIV) (Geraldo et al., 2006). Conversely, *F. avenaceum* is able to produce moniliformin (MON) and depsipeptides, a chemical family which includes enniatin analogues (ENs), and beauvericin (BEA) (Jestoi et al., 2008; Luz et al., 2017). ENs and BEA are also produced by *F. tricinctum* and by *F. poae* (Covarelli et al., 2015a; Cuomo et al., 2013; Jestoi, 2008; Logrieco et al., 2002). This last species is also able to biosynthesize NIV and, even if with low frequencies and quantities, type A trichothecenes such as T-2 toxin and HT-2 toxin (Jestoi et al., 2008; Stenglein, 2009; Vogelgsang et al., 2008). Finally, these last two mycotoxins are typically biosynthesized at high levels by *F. sporotrichioides* and *F. langsethiae* (Imathiu et al., 2013; Jestoi et al., 2008).

Some of the above-mentioned substances could be metabolized by plants, fungi, mammals or during food processing, originating conjugated (masked) forms (Berthiller et al., 2013). Due to their implication on malting barley, the most investigated "masked" mycotoxins are the conjugated forms of trichothecenes such as deoxynivalenol-3-glucoside (DON-3G), T-2-glucoside (T-2G) and HT-2-glucoside (HT-2G) (Lancova et al., 2008; Lattanzio et al., 2013). Glucoside conjugates of trichothecenes may represent a potential threat to consumer's safety because they can be hydrolyzed to their toxic native forms during digestion (Berthiller et al., 2013).

In addition, in the last years, the development of multi-mycotoxin methods showed the occurrence of a high number of secondary metabolites, many of them without a clear role on fungal physiological functions and having an unknown impact on barley, malt and beer quality and on final consumer's health (Streit et al., 2013).

During the last years, due to the negative repercussions of the toxigenic mycobiota, particularly of *Fusarium* infections, and of the accumulation of their secondary metabolites in malting barley grains, monitoring and characterizing the evolution of the FHB complex in a certain cultivation area as well as assessing the different types of mycotoxins present in the raw material prior to the malting and brewing processes have become increasingly important (Schwarz, 2017).

For this reason the aim of this study was to: 1) determine the mycobiota composition of malting barley grains with particular attention to the microorganisms belonging to the *Fusarium* genus; 2) molecularly identify the *Fusarium* species isolated from the grains; 3) analyse a wide range of fungal secondary metabolites in the grains, including the EU legislated, conjugated and less known compounds; 4) characterize the ability of a subset of *F. avenaceum* and *F. tricinctum* strains isolated from the grains to *in vitro* biosynthesize a wide range of secondary metabolites.

2. Materials and methods

2.1. Sampling and determination of malting barley mycobiota

The present study was conducted on 52 malting barley samples. All samples were cultivated in different areas of the Umbria region (central Italy) during the season 2013–2014. The sampling strategy aimed at covering the most important cultivation areas to obtain a representative overview of the investigated region (Supplementary Fig. S1). Immediately after the harvest, the 52 grain samples (500 g) were stored at 4 °C until analysis. Sampling locations for each sample are reported in Supplementary Table S1. All samples belonged to the variety "Quench". Each grain sample was divided into two sub-samples: one used for mycological analyses (250 g) and the other one for fungal secondary metabolites analyses (250 g).

Mycological analyses were performed as described by Beccari et al. (2016). In detail, about 30 g of the sampled kernels were externally disinfected for 2 min using a water-ethanol (95%, Sigma Aldrich, Saint Louis, MO, USA)-sodium hypochlorite (7%, Carlo Erba Reagents, Milan, Italy) solution (82:10:8% vol.) and rinsed with deionized sterile water for 1 min. One-hundred kernels were placed onto potato dextrose agar (PDA, Biolife Italiana, Milan, Italy) supplemented with streptomycin sulphate (0.16 g/L, Sigma Aldrich) and 2,6 dichloro-4-nitroaniline (0.006 g/L, Sigma Aldrich) into 10 Petri dishes (100 mm diameter), containing 10 kernels each. The dishes were kept at 22 °C in the dark and after 5 days of incubation a combination of visual and stereomicroscope (SZX9, Olympus, Tokyo, Japan) observations were carried out on each single kernel (10 kernels per replicate, 10 replicates per sample) to determine fungal development.

The incidence (%) of developed fungal genera recovered during the whole survey was indicated as the average (± standard error, SE) of the 52 malting barley analyzed samples. The incidence (%) of each fungal genus recovered from each sample was calculated as the average (± SE) of 10 biological replicates (Petri dishes).

Visual observation data were subject to one-way analysis of variance by considering the "genus" as a factor (incidence of developed