Food Microbiology 46 (2015) 618–626

Contents lists available at ScienceDirect

Food Microbiology

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

Biosynthesis of beauvericin and enniatins *in vitro* by wheat *Fusarium* species and natural grain contamination in an area of central Italy

L. Covarelli [a, 1, *], G. Beccari [a, 1], A. Prodi [b], S. Generotti [a, c], F. Etruschi [a, c], G. Meca [c], C. Juan [c], J. Mañes [c]

[a] Department of Agricultural, Food and Environmental Sciences, University of Perugia, Borgo XX Giugno 74, 06121, Perugia, Italy
[b] Department of Agricultural Sciences, Alma Mater Studiorum Bologna University, Viale Fanin 40, 40127, Bologna, Italy
[c] Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrès Estellès s/n, 46100, Burjassot, València, Spain

ARTICLE INFO

Article history:
Received 5 April 2014
Received in revised form
15 September 2014
Accepted 18 September 2014
Available online 26 September 2014

Keywords:
Beauvericin
Enniatins
Fusarium avenaceum
Fusarium poae
LC-MS/MS
LC-DAD

ABSTRACT

Contamination of wheat grain by beauvericin (BEA) and enniatins (ENs) is a global emerging mycotoxicological food problem. In this study, strains of *Fusarium avenaceum* (FA), *Fusarium poae* (FP), *Fusarium equiseti* and *Fusarium sporotrichioides*, all potential BEA and EN producers, isolated from 162 grain samples of durum and soft wheat harvested in 2009 and 2010 collected in an area of central Italy, were preliminarily screened for the presence of the *esyn1* gene, encoding the multifunctional enzyme enniatin-synthetase for the detection of potential hexadepsipeptide-producing isolates. All positive isolates were tested for their ability to biosynthesize BEA and ENs *in vitro*. In addition, all wheat samples were investigated for the natural presence of BEA and ENs (ENA, ENA1, ENB, ENB1). All FA and FP strains resulted to be positive for the presence of the *esyn1* gene. All FA strains showed the ability to biosynthesize ENs *in vitro* but not BEA. Conversely, all FP strains resulted to be BEA producers and some of them co-biosynthesized ENs. A remarkable presence of "emerging" mycotoxins was found in the grains, particularly ENs. Co-contamination by BEA and ENs also occurred. This study gives an important contribution to assess the risk posed by mycotoxigenic fungi and their mycotoxins in food.

1. Introduction

Durum wheat (*Triticum durum* Desf.) and soft wheat (*Triticum aestivum* L.) can be attacked by several *Fusarium* species able to cause *Fusarium* Head Blight (FHB), a disease which provokes yield losses and quality reduction due to mycotoxin contamination. Mycotoxins are toxic secondary metabolites produced by different fungal species, including those belonging to the *Fusarium* genus. They represent one the most prevalent sources of food contamination in the world, with strong acute or chronic negative impacts on human health. *Fusarium* species are able to produce a wide range of mycotoxins that, considering the globally available data about their toxicity, occurrence and contamination levels, are usually classified into two distinct groups of the so called "traditional" and "emerging" mycotoxins. "Traditional" *Fusarium* mycotoxins of wheat are in particular represented by trichothecenes, while, "emerging" *Fusarium* mycotoxins of this crop are mainly represented by hexadepsipeptides like beauvericin (BEA) and enniatins (ENs). Some methods have been described in the scientific literature for the detection of BEA and ENs in cereals and derived products (Jestoi et al., 2005; Santini et al., 2009; Serrano et al., 2012) and BEA and EN analogues (A, A1, A2, B, B1, B2 and B4) have been detected in different food commodities during the last ten years. These mycotoxins have been found to be common contaminants of cereals and derived products in many countries such as Spain (Meca et al., 2010; Serrano et al., 2013, 2012), Morocco (Mahnine et al., 2011; Sifou et al., 2011; Zinedine et al., 2011), Tunisia (Oueslati et al., 2011), Italy (Jestoi et al., 2004b; Juan et al., 2013a, 2014), Portugal (Blesa et al., 2012), Poland (Stepień et al., 2013), Finland (Jestoi et al., 2004a, 2004b; Logrieco et al., 2002), Norway (Uhlig et al., 2006), Sweden (Lindblad et al., 2013) and Canada (Tittlemier et al., 2013).

FHB is associated with up to 17 *Fusarium* species (Parry et al., 1995) but just a few of them are important worldwide in terms of diffusion, qualitative and quantitative economic impact. *Fusarium graminearum* Schwabe (syn. *Gibberella zeae* (Schwein.) Petch) is often the prevalent species in many FHB affected world areas while other three species, *Fusarium culmorum* (W.G. Smith), *Fusarium avenaceum* (Fr.) Sacc. and *Fusarium poae* (Peck) Wollenw. are frequently found to cause FHB. Their geographical distribution is

* Corresponding author. Tel.: +39 075 585 6464; fax: +39 075 585 6482.
E-mail address: lorenzo.covarelli@unipg.it (L. Covarelli).
[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.fm.2014.09.009
0740-0020/

related to temperature and humidity conditions (Osborne and Stein, 2007). In fact, *F. graminearum*, even if globally present, is more common in warmer and temperate regions including central and southern Europe. Among the mentioned species, *F. avenaceum* and *F. poae*, even if generally classified as secondary FHB casual agents, are considered two important species under a mycotoxicological point of view (Bottalico and Perrone, 2002; Uhlig et al., 2007) and their presence has increased during past years. *F. avenaceum* has been isolated from infected grain over a range of climatic zones (Uhlig et al., 2007) and, even if this species is predominant in the colder areas of northern Europe (Jestoi et al., 2004a; Yli-Mattila et al., 2004) and Canada (Turkington et al., 2002), it can be also present at considerable levels in central Europe (Bottalico and Perrone, 2002; Wiśniewska et al., 2014). Furthermore, *F. poae* has been detected in different countries, along with the most frequently isolated FHB pathogens, such as Argentina (González et al., 2008), Canada (Bourdages et al., 2006) and also in some northern European areas (Birzele et al., 2002). In Italy, these two species have also been found with a high frequency in the last years both in wheat (Bottalico and Perrone, 2002; Covarelli et al., 2012, 2008, 2010; Infantino et al., 2012; Pancaldi et al., 2010; Shah et al., 2005; Xu et al., 2005) and in barley (Giannini et al., 2013). In particular, Infantino et al. (2012), investigating the community structure of the *Fusarium* complex in the wheat seed in Italy, found *F. poae* to be the most abundant species and a considerable presence of *F. avenaceum*, particularly in the northern and central areas of the country, was detected. Pancaldi et al. (2010), during several surveys conducted from 1995 to 2007 in an area of northern Italy, reported *F. poae* as the third FHB causal agent. In an area of central Italy (Umbria), Covarelli et al. (2008) showed the prevalent occurrence of *F. poae* in the years 2004–2006, while *F. avenaceum* was detected among the main FHB agents in 2008 in the same region (Covarelli et al., 2010). Investigations conducted by the same authors in the years 2009–2010 showed that *F. poae* and *F. avenaceum* increased their presence when climatic conditions were not favorable for the development of the main FHB causal agents, such as *F. graminearum*, which decreased its incidence (Covarelli et al., 2014).

BEA and ENs are biosynthesized by more than 20 *Fusarium* species (Jestoi, 2008) and *F. poae* is reported to be one of the main BEA and EN producers (Jestoi, 2008; Stenglein, 2009). In addition, this species is able to produce trichothecenes (nivalenol, T-2, HT-2 toxin and diacetoxiscirpenol) (Thrane et al., 2004). On the contrary, *F. avenaceum* is unable to biosynthesize trichothecenes (Kulik et al., 2011) but it is able to biosynthesize BEA and ENs (Jestoi, 2008). Also other important FHB associated agents such as *Fusarium equiseti* (Jestoi, 2008; Logrieco et al., 1998) and *Fusarium sporotrichioides* (Thrane et al., 2004) showed the ability to produce BEA and ENs, cyclic hexadepsipeptides with a similar biosynthetic pathway. The multifunctional enzymes that catalyze the biosynthesis of these mycotoxins are BEA or EN synthetases (Sy-Cordero et al., 2012; Xu et al., 2008). The corresponding gene is called *esyn1* (Haese et al., 1993) and a PCR approach based on this gene for the molecular detection of potential enniatin-producing *Fusarium* species has been developed by Kulik et al. (2007). This is a useful tool to perform a preliminary screening in order to assess the incidence of *Fusarium* spp. isolated from grains which are able to biosynthesize both the hexadepsipeptides ENs and BEA, considering that the *esyn1* gene, coding for a multifunctional enzyme, is also responsible for BEA synthesis and not just ENs (Koncz et al., 2009; Kulik et al., 2007). BEA is composed of an alternated D-α-hydroxyisovaleryl-(2-hydroxy-3-methylbutanoic acid) and N-methyl-L-phenylalanyl residues, whereas ENs of three D-α-hydroxyisovaleryl and three N-methyl-L-amino acid residues (Jestoi, 2008). Showing similar structure, BEA and ENs can be assumed to present the same toxic actions (Serrano et al., 2012). The toxicity of BEA and ENs is related to their ability to promote the transport of cations through the membranes with strong repercussions on their normal physiological levels in the cell, affecting ionic homeostasis (Juan-García et al., 2013; Kamyar et al., 2004; Kouri et al., 2003). An increase of intracellular cations, such as calcium, activates the calcium-dependent endonucleases and causes subsequent DNA fragmentation that is a typical step of apoptosis (Elmore, 2007). It has been demonstrated that BEA and ENs have citotoxic effects in different cell lines (Ferrer et al., 2009; Kamyar et al., 2004). Recent studies showed, in human colon adenocarcinoma (Caco-2) cells, a high increase of reactive oxygen species (ROS) level in the presence of BEA and ENs as well as cell-death induction by a mitochondria-dependent apoptotic process with loss of mitochondrial membrane potential (Prosperini et al., 2013a, b).

The toxic effect of BEA and ENs causes a strong negative impact on food commodities including cereals like wheat.

During the last years, the approach based on the screening of fungal isolates for the evaluation of their *in vitro* ability to biosynthesize mycotoxins has been developed to assess the potential mycotoxin contamination in a certain area and permitted to assess the mycotoxigenic profiles and the different levels of mycotoxin production of fungal strains as well as to determine the potential co-production of several mycotoxins by a single isolate (Fotso et al., 2002; Jestoi et al., 2008; Munkvold et al., 1998; Shephard et al., 1999). A mycotoxigenic characterization based on the combination of molecular and *in vitro* assays could represent a useful tool to map a *Fusarium* population and to identify differences between populations in different areas.

Therefore, considering the increasing importance of BEA and ENs, especially for their potential, but not well established, toxicological properties on humans and animals, a study was carried out in order to determine the potential genetic ability to produce hexadepsipeptides and the *in vitro* production of BEA and ENs by fungal strains of *F. avenaceum*, *F. poae*, *F. equiseti* and *F. sporotrichioides* isolated from durum and soft wheat kernels harvested in an area of central Italy (Umbria region) and to detect BEA and EN contaminations of the same grains from which fungal strains were isolated.

2. Materials and methods

2.1. Grain sampling and obtainment of Fusarium strains

The present survey was conducted on 162 durum and soft wheat grain samples harvested in the seasons 2009 and 2010 at several farms located in the Umbria region in central Italy. Samples (one sample of 500 g per each location) were collected just after their harvest, immediately stored at 4 °C until use and then divided into two bulks of 250 g each, one for seed mycological analysis and one, finely ground by a laboratory blender, for mycotoxin analysis. Mycological analysis and *Fusarium* species molecular identification have been described in a previous study (Covarelli et al., 2014). After species identification, a sub-sample of about 25% of each group of strains, identified as *F. avenaceum* ($n = 22$), *F. poae* ($n = 12$), *F. equiseti* ($n = 3$) and *F. sporotrichioides* ($n = 1$), was selected and used for subsequent analyses. Strain selection was conducted based on their place of origin in order to analyze strains evenly sampled across the surveyed area.

2.2. Detection of the esyn1 gene to assess the fungal potential to biosynthesize hexadepsipeptides

The above mentioned *Fusarium* strains were grown in Petri dishes containing potato dextrose agar (PDA). After 10 days of