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Mycotoxin production of selected *Fusarium* species at different culture conditions

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ABSTRACT

The toxin producing capacity of seven *Fusarium* species (*F. langsethiae*, *F. sporotrichioides*, *F. poae*, *F. avenaceum*, *F. tricinctum*, *F. graminearum* and *F. culmorum*) and the effect of culture conditions on the toxin production were studied. The strains were isolated from Finnish grains and cultivated on a grain mixture at three different water activity/temperature combinations (i.e. 0.994/15 °C; 0.994/25 °C; 0.960/25 °C). The mycotoxins produced were analyzed with a multi-toxin method based on liquid chromatography–tandem mass spectrometry enabling the simultaneous determination of 18 different *Fusarium* toxins. The general toxin profiles revealed *F. langsethiae* and *F. sporotrichioides* as producers of diacetoxyscirpenol, neosolaniol, HT-2 and T-2-toxins. *F. sporotrichioides* produced additionally beauvericin. In the *F. poae* cultures, only beauvericin was detected. *F. avenaceum* and *F. tricinctum* were capable of producing enniatins, moniliformin and antibiotic Y, and *F. graminearum* and *F. culmorum* produced zearalenone, deoxynivalenol and 3-acetyl deoxynivalenol. Differences existed in the quantitative toxin production between the individual strains representing the same species. Additionally, the culture conditions affected the range and amounts of toxins produced. In general, a_w 0.994 and temperature of 15 °C favoured the type-A trichothecene production of *F. langsethiae* and *F. sporotrichioides*. The beauvericin production of *F. sporotrichioides* occurred more favourably at a_w 0.960 and 25 °C. *F. poae* produced the highest concentrations of beauvericin under two different conditions, namely at a_w 0.994/15 °C and a_w 0.960/25 °C. None of the combinations particularly favoured toxin production of *F. avenaceum*, with all three toxins being produced extensively at all culture conditions. *F. tricinctum* produced enniatins most efficiently at a_w 0.994/25 °C. The moniliformin production of both these two species occurred readily at a_w 0.960/25 °C. *F. culmorum* and *F. graminearum* produced the highest concentrations and variety of mycotoxins at a_w 0.960/25 °C. The results give valuable information on the toxigenicity of some important *Fusarium* species. Additionally, this is the first in-depth study to investigate the influence of environmental conditions on the toxin production by *F. langsethiae*, *F. poae*, *F. avenaceum* and *F. tricinctum*.

1. Introduction

Fungi of the genus *Fusarium* are important pathogens of small-grain cereals, especially in the temperate regions of the world. Infection with *Fusarium* commonly results in reduction of the quality and yield of the crop. Additionally, many of these fungi are capable of producing toxic secondary metabolites (mycotoxins) which exert a broad spectrum of toxic properties and may evoke adverse effects on human and animal health if they gain access to the food and feed chain. Fusaria are also frequent contaminants of grains grown in Finland. Among the most common species are *F. avenaceum*, *F. tricinctum*, *F. culmorum*, *F. graminearum* as well as *F. langsethiae*, *F. sporotrichioides* and *F. poae* (Yli-Mattila et al., 2002, 2004; Ylimäki et al., 1979). All of these species have been associated with several mycotoxins such as trichothecenes, beauvericin (BEA), enniatins (ENNs) and moniliformin (MON) (Jestoi et al., 2008; Langseth et al., 1998, 2001; Logrieco et al., 2002; Morrison et al., 2002; Thrane et al., 2004; Yli-Mattila et al., 2006). Many of these mycotoxins are frequently detected as natural contaminants also in grains harvested in Finland (Eskola et al., 2000, 2001; Jestoi et al., 2004a, 2004b; Yli-Mattila et al., 2008). Generally, the concentration levels have been low. However, they vary in different years and can be high occasionally.

Mycotoxin production of fungi is a complex process currently not fully understood. It is known that both the growth of fungi and their toxigenic potential are affected by environmental factors, such as temperature, water activity, pH and both nutrient composition and availability (Doohan et al., 2003; Shwab and Keller, 2008). Under field conditions there are many factors which influence the mycotoxin production: climate, agricultural practises, host plant and the presence of other microorganisms (Xu et al., 2007). Secondary metabolism of fungi is regulated by genetic mechanisms which respond to various stimuli from the environment (Shwab and Keller,

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2008). However, it is not known exactly how all these factors act and are regulated at the molecular level during the toxin synthesis. The same fungus can produce a very different range of mycotoxins under different culture conditions (Kostecki et al., 1999; Velluti et al., 2000; 2001; Vogelgsang et al., 2008a). Additionally, fungal species and strains can vary substantially in their ability to produce mycotoxins, and not all fungi possess the genes for the production of certain metabolites. On the other hand, even if a species or a strain has the necessary mechanisms for the biosynthesis, the toxins are not necessarily formed under particular conditions, in either laboratory or field.

A better understanding of the role of individual factors in mycotoxin production would help in estimating the related risks and planning preventative strategies. This knowledge may become even more crucial with climate change which is believed to affect the occurrence of fungi and their production of mycotoxins (Miraglia et al., 2009). Thus *in vitro* studies can be useful in predicting the mycotoxin profiles that might be expected in the field or under storage conditions. The toxin production of different *Fusarium* species has been examined earlier both *in vitro* (Jestoi et al., 2008; Langseth et al., 1998; Thrane et al., 2004) as well as *in planta* (Jestoi et al., 2008; Vogelgsang et al., 2008b). Many of the *in vitro* studies have been focused on toxin production profiles of a fungus in rather optimal conditions. The studies examining the role of the culture conditions have mainly concentrated on *F. graminearum* and *F. culmorum*, these species being the most important *Fusarium* head blight (FHB) pathogens and deoxynivalenol (DON) producers around the world (Bottalico and Perrone, 2002; Goswami and Kistler, 2004). Some of the *Fusarium* species prevalent in the northern European climates, such as *F. avenaceum*, *F. tricinctum*, *F. sporotrichioides*, *F. langsethiae*, and *F. poae*, have attracted much less attention. Mateo et al. (2002) studied the effect of temperature and water activity on toxin profiles of *F. sporotrichioides* on different cereal substrates. Marasas et al. (1987) and Park and Chu (1993) applied different temperatures when studying the toxin production of this species. Vogelgsang et al. (2008a) carried out experiments with *F. avenaceum* and *F. poae* focusing on the influence of the growth substrate. The prevalent *Fusarium* species in Finland have been studied previously in field trials and on rice cultures (Jestoi et al., 2008). However, the effect of environmental factors was not investigated. Furthermore, *F. langsethiae* was not included in that study. Recent reports indicate that this species is becoming more prevalent in the northern European countries including Finland (Edwards et al., 2009; Parikka et al., 2008). It has also been proposed that *F. langsethiae* is the primary T-2/HT-2 toxin producer in this region (Edwards et al., 2009). Therefore, more information is needed on the toxin producing characteristics of this fairly recently identified (Torp and Nirenberg, 2004) fungal species.

In this study, strains representing seven prevalent *Fusarium* species in Finland were cultured on a grain mixture under different environmental conditions. The mycotoxins produced were analyzed with a multi-toxin method based on liquid chromatography–tandem mass spectrometry (LC–MS/MS). The objectives of the study were (1) to investigate the toxin production capacity of the selected strains on a cereal substrate and (2) to examine the effect of different a_w and temperature combinations on the type and amount of toxins produced.

2. Materials and methods

Fusarium strains representing *F. langsethiae*, *F. sporotrichioides*, *F. poae*, *F. avenaceum*, *F. tricinctum*, *F. graminearum* and *F. culmorum* were isolated from Finnish grains in the harvest year 2005 (Table 1). The species were identified morphologically according to Gerlach and Nirenberg (1982) except *F. langsethiae* which was identified morphologically according to Torp and Nirenberg (2004). Hyphal tip cultures of the strains were inoculated onto a mixture of wheat, oats and barley grains (1:1:1, w:w:w). Each of the three isolates was cultivated in triplicate at three a_w/temperature combinations (0.994/15 °C; 0.994/25 °C; 0.960/25 °C) for four weeks. These combinations were selected to approximate the conditions encountered in the field during the growing season. Here, 100 g of the grain mixture was weighed into a one litre glass bottle, and distilled water (either 120 or 40 ml depending on the desired a_w) was added onto the mixture. The bottles were autoclaved twice for 1 h at 120 °C. After cooling, the *Fusarium* isolates were inoculated onto the substrate. After the incubation period, the grain cultures were air dried at 45 °C and ground into a homogenous flour. The control samples had the same treatment as described above, but contained no fungal culture.

Table 1
Fusarium strains examined.

Species	Strain code	Host plant
F. langsethiae	Fl 05010	Oat
F. langsethiae	Fl 05013	Wheat
F. langsethiae	Fl 05014	Barley
F. sporotrichioides	Fs 05002	Barley
F. sporotrichioides	Fs 05003	Wheat
F. sporotrichioides	Fs 05004	Oat
F. poae	Fp 05009	Oat
F. poae	Fp 05016	Barley
F. poae	Fp 05019	Wheat
F. avenaceum	Fa 05001	Barley
F. avenaceum	Fa 05020	Wheat
F. avenaceum	Fa 05021	Oat
F. tricinctum	Ft 05005	Oat
F. tricinctum	Ft 05006	Barley
F. tricinctum	Ft 05007	Wheat
F. graminearum	Fg 05011	Barley
F. graminearum	Fg 05012	Wheat
F. graminearum	Fg 05017	Oat
F. culmorum	Fc 05008	Wheat
F. culmorum	Fc 05015	Barley
F. culmorum	Fc 05018	Oat

The grain mixture was chosen as a substrate to simulate the natural habitat of the strains to obtain a better estimate of the toxin production *in planta*. As the strains were isolated from different host plants, a grain mixture was chosen instead of a single cereal variety. The amount of water added on the grain mixture to achieve the desired a_w was calculated on the basis of moisture adsorption curves. These were constructed experimentally. Volumes of water in the range from 20 to 140 ml were added onto 100 g of grain mixture, mixed carefully and autoclaved twice at 120 °C. After letting the grains cool down and the moisture equilibrate, the water activity was measured with AquaLab CX-2 device (Decagon Devices Inc., Pullman, Washington, USA). The curves were constructed by plotting the a_w against the amount of water added.

A total of 18 *Fusarium* mycotoxins was analyzed from the ground cultures by using LC–MS/MS multi-toxin method as described by Kokkonen and Jestoi (2009). The mycotoxins analyzed are listed in Table 2 together with method validation parameters: recovery and limit of detection and quantification (LOD and LOQ). Enniatins (ENN) were reported as a sum of ENN A, A1, B and B1. All the standards for mycotoxins were purchased from Sigma-Aldrich (Saint Louis, MO, USA), except for antibiotic Y which was provided by Dr. Kristian Fog Nielsen (DTU, Denmark). In brief, the analytes were extracted from a 10 g grain sample with accelerated solvent extraction (ASE, Dionex, Sunnyvale, USA) with 90% acetonitrile (ACN). A five ml portion of the filtered extract was concentrated by nitrogen evaporation and thereafter reconstituted in 0.2 ml of 50% ACN. A volume of 10 µl was injected into the LC–MS/MS instrument (Waters Alliance 2965 and MicroMass Quattro Micro, Waters corp., Manchester, UK) where the analytes were separated in an Inertsil ODS-EP (GL Sciences, Torrance, CA, USA) column and detected utilizing both positive and negative