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Impact of three sterol-biosynthesis inhibitors on growth of *Fusarium langsethiae* and on T-2 and HT-2 toxin production in oat grain under different ecological conditions

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ABSTRACT

The health-beneficial properties of oats have led to an increase in the consumption of oats and oat-based food products in recent years. *Fusarium langsethiae* grows on small grain cereals, especially oats and can result in contamination with type A trichothecenes (T-2 toxin (T-2) and HT-2 toxin (HT-2)) in crops pre- and post-harvest. The aim of this work was to assess the efficacy of three fungicides (fenpropimorph, prochloraz and tebuconazole) on temporal growth of two *F. langsethiae* strains and the accumulation of T-2 and HT-2 in oat grains, under different water activities (a_w; 0.95 and 0.98) and temperatures (15 and 25 °C). All the antifungal agents reduced growth rates when compared to controls, and this increased with increasing fungicide dose. The ranges of ED_{50} values (mg/kg) were 65–270 for fenpropimorph, 0.25–4.2 for prochloraz, and 0.3–14 for tebuconazole. The ED_{90} values (mg/kg) ranged from 170 to > 800, 0.5 to > 10 and 0.5 to > 15 for these three fungicides respectively. The ED_{50} values were not statistically significantly affected by the factors a_w, temperature or strain. However, there were significant differences among the fungicides. Fenpropimorph proved less efficient that the two azoles, which do not differ significantly. In general, levels of HT-2 were higher than T-2 in all cultures regardless of environmental conditions. Overall, HT-2 concentration was always higher at 25 than 15 °C and increased from day 14 to day 21. Levels of both toxins generally decreased with increasing fungicide dose regardless of fungicide type, strain and incubation time. No toxins were detected in cultures at 0.95 a_w in the presence of any of the three fungicides. Also under wetter conditions at 0.98 a_w neither mycotoxin was found in cultures treated with prochloraz at doses >1 mg/kg (15 °C) or >3 mg/kg (25 °C) or tebuconazole at doses >6 mg/kg (15 or 25 °C). ANOVA showed that in treatments with each fungicide the factors dose, time and temperature significantly affected toxin production while there was no difference between the strains.

1. Introduction

There has been increased consumer awareness of the health-promoting properties of oats and its by-products characterized by good taste, dietetic properties, high beta-glucan content and anti-carcinogenic effects (Gallaher, 2000; Salminen et al., 1998). Beta-glucan is known as a prebiotic, stimulating the growth of some beneficial residential colon microorganisms such as bifidobacteria (Connolly Lovegrove, & Tuohy, 2010; Jaskari et al., 1998; Wood & Beer, 1998). Because of these benefits, oats are becoming popular as part of a healthy diet for both humans and animals (Biel, Bobko, & Maciorowski 2009; Flander, Sanmenkallio-Marttila, Suortti, & Autio, 2007; Peltonen-Sainio, Kontturi, & Rajala, 2004; Ryan, Thondre, & Henry, 2011) and new oat-based functional food products have been developed (Angelov, Gotcheva, Kuncheva, & Hristozova, 2006; Gupta, Cox, & Abu-Ghannam, 2010).

Fusarium langsethiae has been isolated from infected oats, wheat and barley in central and northern Europe (Torp & Adler, 2004; Torp & Langseth, 1999; Torp & Nirenberg, 2004). This species has been implicated in the production of high levels of T-2 and HT-2 toxins in cereals, especially in oats (Edwards, 2007, 2009a, 2009b, 2009c; Langseth & Rundberget, 1999; Scudamore, Baillie, Patel, & Edwards, 2007; Scudamore, Patel, & Edwards, 2009; Torp & Langseth, 1999). This mycotoxigenic species is difficult to detect because it does not produce any visible symptoms on the oat grains. *F. langsethiae* is closely related to *Fusarium sporotrichioides and Fusarium sibiricum,* a novel type A trichothecene producing *Fusarium* species recently identified by Yli-Mattila et al. (2011) and isolated from cereals

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grains in Siberia and the Russian Far East (Burkin, Soboleva, & Kononenko, 2008).

T-2 toxin (T-2) is a potent inhibitor of DNA, RNA, protein synthesis and mitochondrial function. It shows immunosuppressive and cytotoxic effects both *in vivo* and *in vitro* and induces DNA fragmentation characteristic of apoptosis (Bouaziz et al., 2008; Caloni Ranzenigo, Cremonesi, & Spicer, 2009; Schuhmacher-Wolz, Heine, & Schneider, 2010). T-2 is readily metabolized to HT-2 toxin (HT-2) *in vivo* and *in vitro* (Dohnal, Jezkova, Jun & Kuca, 2008; Königs, Mulac, Schwerdt, Gekle, & Humpf, 2009). The few comparative data available on T-2 and HT-2 indicate that they induce adverse effects with similar potency (WHO, 2001; Schuhmacher-Wolz et al., 2010).

The interest in the area of food safety is increasing. Risk managers may face the problem that reducing the risk of one compound may increase the risk of another compound (Muri, Van der Voet, Boon, Van Klaveren, & Brüschweiler, 2009). An example is the potential increase in mycotoxin levels due to a reduced use of fungicides in crop production (Finamore et al., 2004; Juan, Moltó, Lino, & Mañes, 2008). Other studies have found a higher extent of contamination in conventional than in organic food (Birzele, Meier, Hindorf, Jramer, & Dehne, 2002; Edwards, 2009b, 2009c). Overall, from a food safety point of view, the health risks associated with fungicide residues in foods are considered a lower risk than the potential impacts of mycotoxins on public health (Muri et al., 2009).

However, studies are needed to determinate the sensitivity of the most relevant mycotoxigenic species to fungicides commonly used in cereal production systems and the influence these treatments have on mycotoxin production (Magan, Hope, Colleate, & Baxter, 2002). These studies should concurrently cover the influence and interactions of ecological variables linked to climate, such as humidity and temperature, on mycotoxigenic fungal growth and on biosynthesis of mycotoxins. This is important, for example, in predictive studies on the occurrence of different mycotoxins in various agro-climatic regions and under the pressure of regional and global climate change (Magan, Medina, & Aldred 2011). The suitable selection of fungicides and their doses avoiding the risks related to their usage (poor or excessive) constitutes a priority.

Fenpropimorph, prochloraz and tebuconazole are antifungal agents extensively applied in agriculture to control fungal growth in cereals and other crops in many European countries. However, little is known about the impact of sub-lethal doses of these fungicides on the accumulation of mycotoxins in cereal grain (Ramírez, Chulze, & Magan, 2004). To our knowledge, the only previous study which compared the *in vitro* efficacy of these fungicides against *F. langsethiae* was reported by Mateo, Valle-Algarra, Mateo-Castro, Jiménez, & Magan (2011). Fenpropimorph belongs to the morpholine group of sterol biosynthesis inhibitors (Campagnac et al., 2009) and is widely used to control pathogens, such as powdery mildew, rusts and leaf blotch diseases of cereals (Leroux, 2003). Prochloraz and tebuconazole, two azoles that differ in structure but exhibit the same mode of action, are used to treat both fungal diseases of plants and medical mycoses. These antifungal agents interfere with the metabolism of fungal pathogens, mainly by inhibition of ergosterol biosynthesis (Hewitt, 1998). No studies have been carried out to examine the effect of these antifungal agents on growth of *F. langsethiae* strains or their ability to produce T-2 and HT-2 in oat grain.

The aims of the present study were: (i) to assess the efficacy (ED_{50}, 50% effective dose and ED_{90}, 90% effective dose) of fenpropimorph, prochloraz, and tebuconazole under different ecological conditions (temperature, a_w and their possible interactions), on growth of two strains of *F. langsethiae* in oat grain; (ii) to determine the effect of each variable and its interactions on T-2 and HT-2 production in oats.

2. Materials and methods

2.1. Fungal strains and growth conditions

Two strains of *F. langsethiae*, 2004/59 and M562, isolated from oats in the UK and Sweden, respectively, were used. These strains are held in the Applied Mycology Group Culture Collection (Cranfield University, UK). They were kindly provided by Prof. S. Edwards, Harper Adams University College, U.K. and Dr. M. Olsen, Swedish Food Authority, Sweden. Strains were preserved in 15% glycerol at −20 °C. Before carrying out the study about efficacy of fungicides and influence of ecological factors on growth and mycotoxin accumulation, the strains were grown on 3% oat agar. Milled oat was prepared by homogenization for 5 min in a Waring laboratory science homogenizer model 7009G (Waring Laboratory Science, CT, USA). A mixture of 3% (w/v) oat flour in water was prepared and 2% (w/v) agar was added. The culture medium was autoclaved for 20 min at 121 °C. The medium was poured into 9-cm diameter Petri dishes. The two strains of *F. langsethiae* were inoculated and incubated for 7 days at 25 °C. These fresh cultures were used to prepare inocula for further experiments on efficacy of fungicides on fungal growth and toxin production in oat grains.

2.2. Effect of environmental conditions and fungicides on growth. Growth evaluation in oat grain cultures

The active ingredient, product name, concentration and company of the fungicides used in this study were the following: fenpropimorph (Funbas®, EC 750 g a.i./l, BASF Crop Protection, Spain), prochloraz (Dogma® 400 g a.i/l, Industrias Afrasa S.A., Paterna, Valencia, Spain) and tebuconazole (Folicur® 250 g a.i./l, Bayer CropScience, Paterna, Valencia, Spain). Diluted solutions of the fungicides were prepared by mixing appropriate amounts of each fungicide (based on concentration of the a.i.) in sterile deionized water and used immediately after preparation.

Oat grains (15 g), previously analysed to ensure they had undetectable levels of T-2 and HT-2, were placed in Erlenmeyer flasks and autoclaved for 20 min at 121 °C. Then, the water activity was adjusted to 0.95 and 0.98 by addition of sterile distilled water using a moisture adsorption curve. An appropriate aliquot of water was replaced by solution of a suitable fungicide treatment (prochloraz, tebuconazole or fenpropimorph) to obtain the final target concentrations of each fungicide. Preliminary experiments were performed to choose the range of concentrations for each fungicide to be added to obtain dose–response curves. Based on these assays the doses used were: fenpropimorph (100, 200, 300 and 800 mg/kg), prochloraz (0.1, 1.0, 3.0 and 10.0 mg/kg) and tebuconazole (0.5, 2.0, 6.0 and 15.0 mg/kg). Controls containing only water were also prepared.

Flasks with oats, with and without fungicides, were refrigerated at 4 °C for 48 h with periodic shaking to allow adsorption and equilibration and a good distribution of the fungicides. At the end of this period, a_w-values were checked with an Aqualab Series 3 (Labcell Ltd., Basingstoke, Hants, UK). The hydrated oat grains were placed in sterile 9-cm Petri dishes to form a layer of grains (15 g). All treatments were inoculated centrally with a 3-mm diameter agar disk taken from the margin of a 5–7-day-old growing colonies. Inoculated Petri plates of same a_w were enclosed in sealed plastic containers together with beakers of a glycerol-water solution matching the same a_w as the treatments to maintain a constant equilibrium relative humidity inside the boxes. The experiments were carried out in triplicate and repeated once. Treatments were incubated at 15 and 25 °C and two sampling-time periods used (14 and 21 days).