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Effect of fenpropimorph, prochloraz and tebuconazole on growth and production of T-2 and HT-2 toxins by *Fusarium langsethiae* in oat-based medium

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Abstract

Fusarium langsethiae has been isolated from infected cereals in central and northern Europe where it has been identified in the last decade as the main species involved in the occurrence of high levels of T-2 and HT-2 toxins, mainly in oats. The efficacy of three fungicides (prochloraz, tebuconazole, fenpropimorph) for controlling growth of two strains of *F. langsethiae* isolated from oats was examined at 0.96 and 0.98 a_w at 15, 20 and 25 ºC on oat-based media. The concentrations necessary for 50 and 90% growth inhibition (ED_{50} and ED_{90} values) were determined. The effect on the trichothecene type A mycotoxins T-2 and HT-2 were also determined. Without fungicides both strains grew faster at 0.98 than at 0.96 a_w and the influence of temperature on growth rates was 25>20>15 ºC. Prochloraz and tebuconazole were more effective than fenpropimorph against *F. langsethiae*. Strain, temperature and type of fungicide significantly influence the ED_{50} and ED_{90} values for growth. The concentration ranges under different environmental conditions were: prochloraz (0.03-0.1 and 0.3-1.5), tebuconazole (0.06-0.9 and 1.3-8.2), and fenpropimorph (22-59 and 125-215 mg l^{-1}). Production of T-2 and HT-2 toxins was influenced by temperature, a_w, type of fungicide and dose. Levels of T-2 were usually higher than those of HT-2 under

the same conditions. The biosynthesis of T-2 toxin increased after 10 days incubation, but was reduced with decreasing temperature and increasing fungicide dose. At 0.98 a_w T-2 levels increased in cultures containing fenpropimorph while at 0.96 a_w the toxin concentrations increased in response to the other two fungicides. Low doses of prochloraz or tebuconazole enhanced toxin production when compared with untreated cultures for strain 2004-59 at 0.96 a_w and 20-25 ºC. HT-2 was hardly detectable in the treatments with prochloraz or tebuconazole at 0.98 a_w. This is the first study on the effect of these anti-fungal compounds on control of growth of *F. langsethiae* and effects on T-2 and HT-2 toxins in oat-based media.

1. Introduction

Fusarium head blight (FHB) is a wide-spread destructive disease of small-grain cereal crops caused by a wide number of *Fusarium* spp. and some *Microdochium* spp. (Xu et al., 2005). *Fusarium* spp. in general reduces grain yield and/or contaminates the grain with a range of toxic metabolites detrimental to human and animal health.

Fusarium langsethiae has been isolated from infected oats, wheat and barley in central and northern Europe (Torp and Adler, 2004; Torp and Nirenberg, 2004). This species, with the morphological characteristics of *Fusarium poae* and metabolic profile of *Fusarium sporotrichioides* (Torp and Nirenberg, 2004; Schmidt et al., 2004; Thrane et al., 2004; Wilson et al., 2004, Yli-Mattila et al., 2004, 2008), was identified a decade ago (Torp and Langseth, 1999). However, it does not produce any visible symptoms, which makes colonisation assessment and effect of fungicides difficult to test in small grains. Although a small percentage of wheat ears with infected 'glume spots' thought to have been caused by *F. langsethiae* in the field have been observed in Austria (Adler and Torp, 2004), *F. langsethiae* can readily be isolated from symptomless oats, wheat and barley grains. The epidemiology of this specie is not understood and host plant preference is unknown, though recent results suggest that *F. langsethiae* may have developed some host preference for oats (Imathiu et al., 2009).

F. langsethiae has been involved in the production of high levels of T-2 and HT-2 mycotoxins in cereals in Norway (Langseth and Rundberget, 1999; Torp and Langseth, 1999) and in oats in the UK (Edwards, 2007), where recent studies have shown a high incidence of both toxins in wheat, barley and especially in oats (Edwards, 2009a, 2009b, 2009c; Scudamore et al., 2007, 2009). The highly toxic type A trichothecenes T-2 toxin and its deacetylated form HT-2 toxin are of special interest because T-2 toxin has been shown to inhibit DNA, RNA and protein synthesis and to induce DNA fragmentation characteristic of apoptosis (Beasley, 1989; Canady et al.,

2001; Prelusky et al., 1994; Schuhmacher-Wolz et al., 2010). T-2 and HT-2 mycotoxins, in unprocessed cereals and by products are currently being considered for legislation in the EU (European Commission, 2006).

The main factors that influence fungal growth and mycotoxin production are temperature, water activity (a_w) and presence of anti-fungal substances (Aldred and Magan, 2004; Edwards, 2004, Llorens et al., 2004a, 2004b; Logrieco and Visconti, 2004; Magan and Aldred, 2007; Magan et al., 2002; Medina et al., 2007a, 2007b; Ramírez et al., 2004, 2006). A significant focus has been on the development and use of fungicides to prevent and control infection of pathogenic *Fusarium* spp. during ripening of small grain cereal crops. Less attention has been paid, in practice, to the effect that such fungicide applications may have on mycotoxin production. This is important as it has been observed that sub-lethal doses of some fungicides may lead to a stimulation of trichothecene production by *Fusarium* species (D'Mello et al., 1997, 1998; Hope et al., 2002; Mathies et al., 1999; Moss and Frank, 1985; Placinta et al., 1996; Ramírez et al., 2004). It is surprising that these previous reports, with the exception of Hope et al. (2002) and Ramírez et al. (2004), took no account of the interactions between the efficacy of the fungicides and key environmental factors, such as a_W or temperature.

Fungicide applications pre-harvest are a reality whether in intensive or sustainable cereal production systems. Fusarium species colonising ripening cereals will thus be exposed to azole fungicides, such as prochloraz (imidazole), or tebuconazole (triazole) and morpholines, such as fenpropimorph, in agricultural environments (Serfling et al., 2007). In describing the response of an isolate to a fungicide, an EC_{50} (50% effective concentration) or ED_{50} (50% effective dose) value is usually given. This is the concentration or dose that reduces growth rate to 50% compared with that observed in the absence of fungicide. An EC_{90} or ED_{90} value is sometimes also given. It provides additional information about growth over a wider range of fungicide concentrations (Serfling et al., 2007; Tzatzarakis et al., 2001). Thus, these parameters deal with the efficacy to control fungal growth but their impact or relationship to the level of mycotoxin accumulation in the substrate has not been studied in detail (Pateraki et al., 2007). Prochloraz, tebuconazole and fenpropimorph are extensively applied in agriculture to control fungal growth in cereals and other crops in many European countries. So far, no study has been carried out to examine the effect of these anti-fungal agents on the growth of *F. langsethiae* strains or their ability to produce T-2 and HT-2 toxins.

Therefore, the objectives of this study were to assess the efficacy of prochloraz, tebuconazole, and fenpropimorph in an oat-based medium, under different a_w/temperature regimes on the control of (i) growth of two strains of *F. langsethiae* isolated from oats from different countries and (ii) T-2 and HT-2 production by these strains. The ED_{50} and ED_{90} values for fungicides in relation to growth and toxin production under different $a_w \times$ temperature conditions were determined.

2. Materials and Methods

2.1. Fungal strains and growth conditions

Two strains of *F. langsethiae*, 2004-59 and M562, isolated from oats in 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. Cultures were preserved in 15% glycerol at –20 ºC. Before carrying out the study of ecological factors on growth and T-2 and HT-2 accumulation, the strains were grown on 3% oat agar. The culture medium was prepared by boiling 30 g of oats kernels in pure water for 1 h. After filtration, the liquid was brought to 1 l and 2% w/v of agar was added. The mixture was autoclaved at 115 °C for 30 min and poured into Petri dishes. The strains were inoculated on the centre of 9 cm Petri plates and incubated at 25 ºC for 7 days. These fresh cultures were used to prepare inocula for further experiments on efficacy of fungicides on fungal growth and toxin production.

2.2. *Modification of media with fungicides at different a_w conditions*

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); 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 deionised water and used immediately after preparation.

A solid medium containing oat extract was prepared as indicated in Section 2.1. The water activity of the media were adjusted to 0.98 and 0.96 with glycerol and, after autoclaving (115 ºC, 20 min), was let to cool to about 50 ºC (Medina and Magan, 2010b). The fungicides were then added to obtain the target concentrations. Flasks of molten media were thoroughly shaken, prior to pouring into 9 cm sterile Petri dishes, to ensure that an even dispersion of the fungicide treatment was obtained. 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: prochloraz (0.01, 0.1, 0.3, 0.7, 0.9, 1.5 mg/l), tebuconazole (0.1, 0.3, 0.5, 0.7, 0.9, 1.5, and 2 mg/l; additionally, 4, 8 and 12 mg/l were tested for 0.96 a_w) and fenpropimorph (10, 20, 25, 30, 50, 90, 150 and 180 mg/l; additionally, 40 and 70 were assayed at 0.98 a_w, and 200 and 230 mg/l were tested for strain M562 at 0.98 a_w).

All media, with and without fungicides, 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 the same a_w were enclosed in sealed plastic containers together with beakers of a glycerol-water solution matching the same a_w as the treatment to maintain a constant equilibrium relative humidity (ERH) inside the boxes. The experiments were carried out in triplicate. Treatments were incubated 15 ºC, 20 ºC and 25 ºC for 10 days.

2.2.1 Growth evaluation

Assessment of growth was made every day during the incubation period by measurement of two diameters of the growing colonies at right angles to each other until the colony reached the edge of the plate. The radii of the colonies were plotted against time, and linear regression applied in order to obtain the growth rate (mm/day) as the slope of the line. The growth rates were plotted and the ED_{50} and ED_{90} concentrations calculated by comparison with the controls at each temperature and a_w level.

2.2.2 Chemical analysis

Reagents and standards

Standards of T-2 and HT-2 toxins were supplied by Sigma (Sigma–Aldrich, Alcobendas, Spain). Acetonitrile and methanol were supplied from J.T. Baker (Deventer, The Netherlands). Pure water was obtained from a Milli-Q system (Millipore Co., Billerica, MA, USA). All solvents were HPLC grade.

Extraction of T-2 and HT-2 toxins from the F. langsethiae *cultures*

Approx. 5-6 agar discs were taken from the replicates and treatment agar plates using a cork borer and placed in previously weighed 2 ml Eppendorf safe-lock tubes and then reweighed. This provided about 0.75 g agar/culture for extraction. A total of 3 replicates per treatment and controls, respectively, were collected and analysed for T-2 and HT-2 toxins as described by Medina et al. (2010a).

Chromatographic analysis

The LC system consisted of a Waters 600E system controller, a Millipore Waters 717 Plus autosampler and a Waters 996 Photodiode array detector (DAD) (Waters, Milford, MA, USA).

A volume of 50 µL of the extract was injected into the LC system. T-2 and HT-2 toxins were separated using a C18 Zorbax Eclipse Plus® (150 x 4.6 mm, 3.5µm) (Agilent Technologies, Waldbronn, Germany), with a guard column of the same material. Analysis was performed in the gradient mode using two solvents (Medina et al., 2010a).

Statistical Analysis

Statistical analysis was performed using Statgraphics Centurion XV.2.11 (Statpoint Inc., VA, USA). Multifactor ANOVA and *post hoc* analysis of factors with more than two levels (Duncan's test of multiple comparisons) were applied to data. A 95% confidence level was used to assess influence of individual and interacting treatments.

3. **Results**

3.1. Effect of fungicides on growth of the two strains of F. langsethiae *used in this study.*

The relative growth of the two strains was initially examined in relation to the treatment conditions of $a_w \times$ temperature (Fig. 1). This shows that growth was favoured by temperature (growth rates increase in the order 25 > 20 > 15 ºC) and was faster at 0.98 than 0.96 a_w, regardless of temperature. The statistical analyses using ANOVA showed that single factors of a_w and temperature were significant ($P < 0.05$) while strain differences and interacting factors were not. Duncan's test showed three homogeneous non-overlapping groups with regard to temperature.

Figs. 2 and 3 show the effects of fenpropimorph and the two azoles (prochloraz and tebuconazole), respectively, on the growth rate (GR, mm/day) of the two strains of *F. langsethiae* on oat-based media under the all the treatment conditions assayed. In general, there was a decrease of the RGR as the fungicide dose was increased regardless of a_w, temperature and strain. These data were used to calculate the ED_{50} and ED_{90} of each fungicide, which are shown in Table 1

For fenpropimorph, the ED_{50} and ED_{90} values were in the ranges 22-59 and 125-215 mg/l, respectively (Table 1). Both indices increased with temperature so that this fungicide was more effective at 15 than 25 ºC. ANOVA revealed that the ED_{50} was

significantly affected ($P < 0.05$) by temperature but not by a_w or the strain. However, the ED_{90} was significantly affected by all single factors as well as by the interaction $a_w \times$ strain and the Duncan's test placed each temperature in a separate homogeneous group.

For prochloraz, Table 1 shows the ranges of ED_{50} (0.03-0.1 mg/l) and ED_{90} (0.3-1.5 mg/l). There were significant differences ($P < 0.05$) among the ED_{50} related to a_w, temperature and the interaction temperature $\times$ a_w. Temperature originates three homogeneous, non-overlapping groups (Duncan's test). ED_{90} was influenced by temperature, a_w and strain but there were interactions between a_w and strain or temperature. Efficacy was higher at 15 than 20-25 ºC.

For tebuconazole, the ED_{50} and ED_{90} values were in the ranges 0.06-0.9 and 1.3-8.2 mg/l, respectively (Table 1). The ED_{50} was significantly affected only by a_w ($P < 0.05$). The fungicide was more effective at 0.98 a_w. However, the ED_{90} was significantly influenced by temperature, a_w and the interaction $a_w \times$ temperature. The Duncan's test applied to ED_{90} shows that control of growth was better at 15 than 20-25 ºC.

ANOVA of all the data including class of fungicide, a_w, strain and temperature revealed that these factors (except for a_w) significantly influenced ($P < 0.05$) the ED_{50} values. The interactions between type of fungicide and temperature or type of fungicide and strain were also significant. The average ED_{50} of fenpropimorph was higher than that for the azole fungicides (Table 1), which clustered together in a single homogeneous group (Duncan's test). With regard to temperature effects on the ED_{50}, the Duncan's test showed two homogeneous groups (15 ºC and 20-25 ºC). With regard to the ED_{90}, the four single factors and their interactions were also statistically significant ($P < 0.05$). There were differences between efficacy of the fungicides ($P < 0.001$). The Duncan's test placed each fungicide and each temperature in a separate non-overlapping group.

To summarise, overall prochloraz was the most active antifungal agent to control growth of *F. langsethiae*. Tebuconazole was about 4-6 times less effective than prochloraz, but differences are significant only regarding the ED_{90}. Fenpropimorph was by far the least active fungicide (100-500 times less effective than prochloraz). Influence of a_w and strain was not conclusive as it was generally affected by interactions. However, efficacy of fungicides was always higher at 15 ºC than 25 ºC.

3.2. Effect of fungicides on T-2 and HT-2 mycotoxin production by F. langsethiae *strains*

Fig. 4 shows the effect of a_w and temperature on T-2 and HT-2 production by both strains of *F. langsethiae* at different a_w/temperature regimes in control cultures. This shows that T-2 toxin was predominantly produced by the strains with significantly less HT-2 toxin produced regardless of environmental conditions. Overall, strain M562 produced more T-2 than strain 2004-59 under the same conditions. The maximum T-2 level (11.6 μg/g) was found in control cultures of strain M562 incubated at 0.98 a_w and 25ºC (see Fig. 4). Overall, T-2 concentration was always lower a 15 ºC than at 20 or 25 ºC. Levels of HT-2 were < 1.5 μg/g and this toxin was sometimes undetectable in cultures of strain M562.

The hypothesis of a normal distribution of mycotoxin data cannot be rejected with 95% confidence level according to the Shapiro-Wilks test. However, transformation of data using the expression log (x + 1*)*, where *x* is toxin concentration was carried out to improve normality. Multifactor ANOVA (without interaction, as interaction was not significant) revealed that T-2 concentration in control cultures is significantly affected ($P \leq 0.05$) by a_w (less concentration at 0.96 a_w) and temperature (less concentration at 15 ºC than at 25 ºC) but not by the strain. Duncan's test showed two homogeneous overlapping groups with regard to the influence of temperature (15-20 ºC and 20-25 ºC). HT-2 production was not significantly affected ($P > 0.05$) by any of the factors.

Fenpropimorph: Fig. 5 shows the levels of T-2 and HT-2 in cultures of both strains in oat-based medium after 10 incubation days in presence of fenpropimorph. The highest level of T-2 (6.6 μg/g) was produced by strain M562 at 10 mg fenpropimorph/l, 0.98 a_w and 25 ºC. Overall, production of both toxins decreased when fungicide dose increased. T-2 production was reduced regarding untreated controls carried out under the same conditions at all doses. However, under some conditions (0.96 a_w) a slight increase of T-2 toxin level when fungicide dose increased (from 10 to 25 mg/l) was found.

Prochloraz: Fig. 6 shows toxin accumulation data in presence of prochloraz. The maximum T-2 toxin level (3.7 μg/g) was found in cultures of strain M562 supplemented with 0.01 mg/l at 0.96 a_w and 25 ºC. In general, toxin levels were higher at 0.96 a_w than at 0.98 a_w, and higher in cultures of strain M562 than strain 2004-59. These levels decreased with decreasing temperature and increasing dose. No toxin was detected a doses > 0.7 mg/l. At 0.98 a_w detectable levels were found only at the two lowest doses. Interestingly, the 0.01 mg/l dose at 0.96 a_w and 20-25 ºC seemed to promote T-2 production with regard to untreated cultures of strain 2004-59.

Tebuconazole: The effect of tebuconazole on accumulation of these mycotoxins in oat-based medium is shown in Fig. 7. The maximum concentration of T-2 (4.4 μg/g) was recorded in cultures of strain 2004-59 at 0.96 a_w, 25 ºC, and 0.1 mg tebuconazole/l. Toxin levels were higher at 0.96 a_w than at 0.98 a_w (as happened with prochloraz) and both toxins were undetected in cultures supplemented with > 2.0 mg/l. HT-2 was not usually detected at 0.98 a_w. As in the case of prochloraz and unlike fenpropimorph, at low doses of tebuconazole T-2 production increased with regard to untreated cultures of strain 2004-59 at 0.96 a_w and 20-25 ºC.

ANOVA of T-2 production data in presence of fenpropimorph indicated that strain, temperature, and fungicide dose significantly influenced T-2 production ($P < 0.05$) although the interactions $a_w \times$ dose, $a_w \times$ temperature and strain $\times$ temperature were also significant. Strain M562 significantly produced more T-2 toxin than strain 2004-59. Overall, increasing temperature favoured toxin production and increasing fungicide dose caused a decrease in toxin accumulation. Duncan's test showed three homogeneous non-overlapping clusters related to temperature. HT-2 toxin production was significantly influenced by dose and a_w. The interactions strain $\times$ temperature and $a_w \times$ strain $\times$ temperature were also significant.

ANOVA of T-2 production data in presence of prochloraz showed that fungal strain did not significantly influence T-2 level but the remaining single factors did. The interactions $a_w \times$ dose, $a_w \times$ temperature, and temperature $\times$ dose were also significant. Duncan's test classified the tested temperatures and doses into three different non-overlapping clusters (for dose, 0.01, 0.1 and the remaining ones). HT-2 toxin production was influenced by a_w, dose and the interactions $a_w \times$ dose and $a_w \times$ dose $\times$ strain.

ANOVA and Duncan's test of T-2 production data in presence of tebuconazole gave similar results to those obtained in media amended with prochloraz. However, in the case of HT-2 toxin all factors significantly influenced HT-2 production: its level increased with temperature and strain M562 produced less toxin than strain 2004-59.

To summarise, prochloraz and tebuconazole were more effective than fenpropimorph with regard to the reduction of T-2 toxin production. Effectiveness increased with the concentration of each antifungal agent and with decreasing temperature in the order 15 > 20 > 25 ºC. Prochloraz and tebuconazole were more effective at 0.98 a_w than at 0.96 a_w while fenpropimorph was on average equally effective at both values. Fungal strain influenced T-2 level only in the case of fenpropimorph. Concerning reduction of HT-2 toxin production, effectiveness also increased with the concentration of each fungicide and was higher at 0.98 a_w but did

not change with temperature or strain, except for tebuconazole. The existence of interactions between the variables revealed by ANOVA enables to assume that there are complex relationships that govern the production of these mycotoxins in oat-based medium amended with any of the three fungicides tested.

4. Discussion

Some anti-fungals agents used in small grain cereals have been examined in detail for efficacy against strains of *F. langsethiae* in relation to both growth and production of T-2 and HT-2 toxins under different environmental conditions. This study has shown that there are complex interactions between abiotic factors fungicides and toxin production. There are differential effects on both growth and toxin which were clearly shown by the ED_{50} and ED_{90} values for the three fungicides tested. This study has provided new data that suggest that relevant changes in the response of the fungi to a_w and temperature take place in the presence of sub-inhibitory concentrations of the assayed fungicides. Consequently, treatments with these anti-fungal substances might affect the natural presence and distribution of *F. langsethiae* and production of T-2 and HT-2 in cereals.

In the absence of fungicides (controls) and under the experimental conditions, both strains grow faster at 0.98 a_w than at 0.96 a_w and the order of growth rate is 25 > 20 > 15 ºC. These results agree with the recent data on the influence of these parameters on growth profiles of 8 strains of *F. langsethiae* from northern Europe (Magan et al., 2011; Medina and Magan, 2010b). Production of T-2 and HT-2 in the absence of fungicides was also higher in the media where the values of a_w and temperature were higher. These results also agree with previous reports (Kokkonen et al., 2010; Medina et al., 2011) and might explain differences in T-2/HT-2 contamination of cereals depending on the country of origin. Scudamore et al. (2009) have found higher contamination in samples from the UK and Ireland than in samples from Scandinavia.

Even when the studied fungicides were effective to control growth and production of T-2 and HT-2, overall, fenpropimorph was the least effective of the three fungicides examined with higher concentrations required to inhibit growth (high ED_{50}, ED_{90} values). This chemical was more effective against other

species, such as *Microdochium nivale* (ED_{50} = 0.13 mg/l) (Debieu et al., 2000), although the assay conditions were different. This anti-fungal agent belongs to the morpholine group of sterol biosynthesis inhibitors (Campagnac et al., 2009; Debieu et al., 1992; Marcireau et al., 1990) and is widely used to control pathogens, such as powdery mildew, rusts and leaf blotch diseases of cereals (Leroux, 2003). The results of the present study show that the ED_{50} of fenpropimorph against *F. langsethiae* is not significantly affected ($P > 0.05$) by the factors a_w, strain and temperature or their mutual interactions. However, the ED_{90} is significantly affected by the three single factors and the interaction $a_w \times$ strain. These results provide interesting information that can be very useful to control *F. langsethiae* and other toxigenic fungi in cereals.

Like fenpropimorph, in general the azole-based compounds, prochloraz and tebuconazole, were more effective at 15 than 20-25 ºC. This may be important as this *Fusarium* species is known to cause problems in cooler climatic regions such as the UK, Ireland or Scandinavia, where ripening and slow drying can result in conducive conditions for this species to colonise oats. The ED_{50} concentrations required to control growth were very similar for these two azoles. However, the ED_{90} value for prochloraz was lower than for tebuconazole suggesting better efficacy overall in controlling growth of this *F. langsethiae*. Previous studies with the efficacy of these fungicides *against F. graminearum* also suggested that prochloraz was more effective *in vitro* than *in situ* (Ramírez et al., 2004). However, this was on wheat. The ED_{50} and ED_{90} of prochloraz and tebuconazole were determined against the maize pathogen *Colletotrichum graminícola* (Serfling et al., 2007) and also showed that this pathogen was more sensitive to prochloraz than to tebuconazole. Moreover, this behaviour was similar in non-adapted and adapted strains to these fungicides. Thus, ED_{50} and ED_{90} of prochloraz against a non-adapted strain of *C. graminicola* was 0.004 mg/l and 0.087 mg/l, respectively, while in the adapted strain these concentrations were 0.011 and 0.720 mg/l, respectively. Against other *Fusarium* species on PDA the range was 0.24 – 6.5 mg/l (the less sensitive was *F. crookwellense* and the most sensitive was *F. sporotrichioides*) (Mullenborn et al., 2008). All these reports and the results obtained in the present study indicate that the efficacy of these fungicides is dependent on the

fungal species, strain, ecological conditions and the interactions amongst all these factors. Azoles differing in structure but exhibiting the same mode of action are used to treat both fungal diseases of plants and medical mycoses. These anti-fungal agents interfere with the metabolism of fungal pathogens, mainly by inhibition of ergosterol biosynthesis (Hewitt, 2004). Studies on the efficacy of azoles on the growth of phytopathogenic and mycotoxigenic fungi on cereals are needed to find the optimal doses for a suitable control of these fungi and to minimise the risk of resistant strain build-up.

It is concluded that, in the presence of fenpropimorph, the highest levels of T-2 and HT-2 were obtained at 0.98 a_w, which was also the most favourable value for fungal growth, both in controls and in the presence of the fungicide. Similar results were obtained in previous reports (Kokkonen et al., 2010; Medina et al., 2011) with *F. langsethiae* in absence of fungicides. The results obtained in the present study show that fenpropimorph added in the range 10-70 mg/l decreases production of T-2 and HT-2 with regard to controls but does not prevent their biosynthesis under the assayed conditions.

It is worth to emphasise that T-2 and HT-2 production by *F. langsethiae* in media amended with the two examined azoles was higher at 0.96 a_w than at 0.98 a_w. These results suggest that fungal sensitivity to these fungicides increases and toxin production is reduced in media with 0.98 a_w, which is more favourable to growth. In cereals, even during pre-harvest, a_w rarely reaches values higher than 0.96. Hence, special attention should be paid to the effect that sub-inhibitory concentrations of these fungicides have on the production of T-2 and HT-2 toxins by *F. langsethiae* in crops. In the assays performed in the media amended with low doses of the two azoles the levels of T-2 and HT-2 increased under some conditions. Studied carried out with strains of different fungal species, from diverse geographical origin and isolated from different host also aim that mycotoxin production in presence of sub-inhibitory concentrations of some fungicides can be boosted under water or thermal stress conditions (Medina et al., 2007a, 2007b; Ramírez et al., 2004). However, ED_{50} or ED_{90} was not determined or the species *F. langsethiae* was not included in these studies.

Unfortunately, as far as we know, there are not previous studies on EDs 50 and 90 of the assayed fungicides on mycotoxigenic fungi, which makes the discussion of the obtained results difficult.

Production of T-2 and HT-2 toxins was dependent on the type of fungicide and its dose, temperature, a_w and strain. The presence of any of the three fungicides produced a general decrease in the accumulation of the two toxins in cultures even at the lower doses taking the controls as references. In fungicide-containing cultures, toxin concentration generally decreased with temperature at a given dose but some exceptions were found; for example, with tebuconazole and prochloraz, HT-2 level was higher at 20 ºC than at 25 ºC if a_w was 0.96. At 0.96 a_w, especially at 15 ºC, comparable levels of T-2 and HT-2 toxin were produced by strain 2004-59. Some authors have suggested that a biotransformation of T-2 into HT-2 might occur (Lattanzio et al., 2009) and this could be boosted under conditions of physicochemical stress. This may explain, at least in part, the fact that natural oats samples contaminated with levels of HT-2 higher than T-2 have been found (Edwards, 2009; Langseth and Rundberget, 1999).

Studies on the efficacy of fenpropimorph and azoles against phytopathogenic and mycotoxigenic fungi are very necessary not only for control of these fungi and mycotoxins in crops but also because, especially, the azoles are extensively applied in agriculture and medicine. A relationship between the development of azole resistance in agriculture and the development of azole resistance in clinical practice may exist. It has been reported that treatment of the maize pathogen *C. graminicola* with an agricultural azole causes cross-resistance to medical azoles and boosts caspofungin, amphotericin B, and nystatin efficacy (Serfling et al., 2007). Moreover, it has been described that tebuconazole increased T-2 toxin levels in rye and winter triticale infested with *Fusarium* spp. with respect to untreated cereals (Mankeviciene et al., 2008). Deoxynivalenol (DON) production was also increased in barley crops treated with this fungicide (Malachova et al., 2010). Prothioconazole, another triazole fungicide, was found to trigger DON biosynthesis when added at sub lethal doses in cultures of *F. graminearum* (Audenaert et al., 2010). Therefore, it is very necessary to perform additional

studies on the influence that ecological factors and sub-inhibitory doses of the most commonly used agricultural antifungal agents have not only on the development of fungal resistance but also on mycotoxin production. So far, the last aspect has been very scarcely investigated and no study has been published regarding *F. langsethiae* and production of T-2 and HT-2.

Acknowledgements

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Figure captions

Fig. 1. Growth rates (GR) of two strains of *F. langsethiae* cultured on oat-based medium at different water activities and temperatures. Error bars mean standard errors. A) strain 2004-59; B) strain M562.

Fig. 2. Growth rates (GR, mm/day) of two strains of *F. langsethiae* on oat-based medium supplemented with different doses of fenpropimorph.

Fig. 3. Growth rates (GR, mm/day) of two strains of *F. langsethiae* on oat-based medium supplemented with different doses of prochloraz and tebuconazole.

Fig. 4. Accumulation of T-2 and HT-2 toxins in (control) cultures of two strains of *F. langsethiae* in oat-based medium after 10 days incubation at 0.96 and 0.98 a_w and at 15, 20 and 25ºC.

Fig. 5. Change of levels of T-2 and HT-2 toxins in oat-based medium cultures of two strains of *F. langsethiae* with different doses of fenpropimorph at 0.98 and 0.96 a_w and 15, 20 and 25ºC. Incubation time was 10 days. Please refer to Figure 4 for control data.

Fig. 6. Change of levels of T-2 and HT-2 toxins in oat-based medium cultures of two strains of *F. langsethiae* with different doses of prochloraz at 0.98 and 0.96 a_w and 15, 20 and 25 ºC. Incubation time was 10 days. Please refer to Figure 4 for control data.

Fig. 7. Change of levels of T-2 and HT-2 toxins in oat-based medium cultures of two strains of *F. langsethiae* with different doses of tebuconazole at 0.98 and 0.96 a_w and

15, 20 and 25 ºC. Incubation time was 10 days. Please refer to Figure 4 for control data.

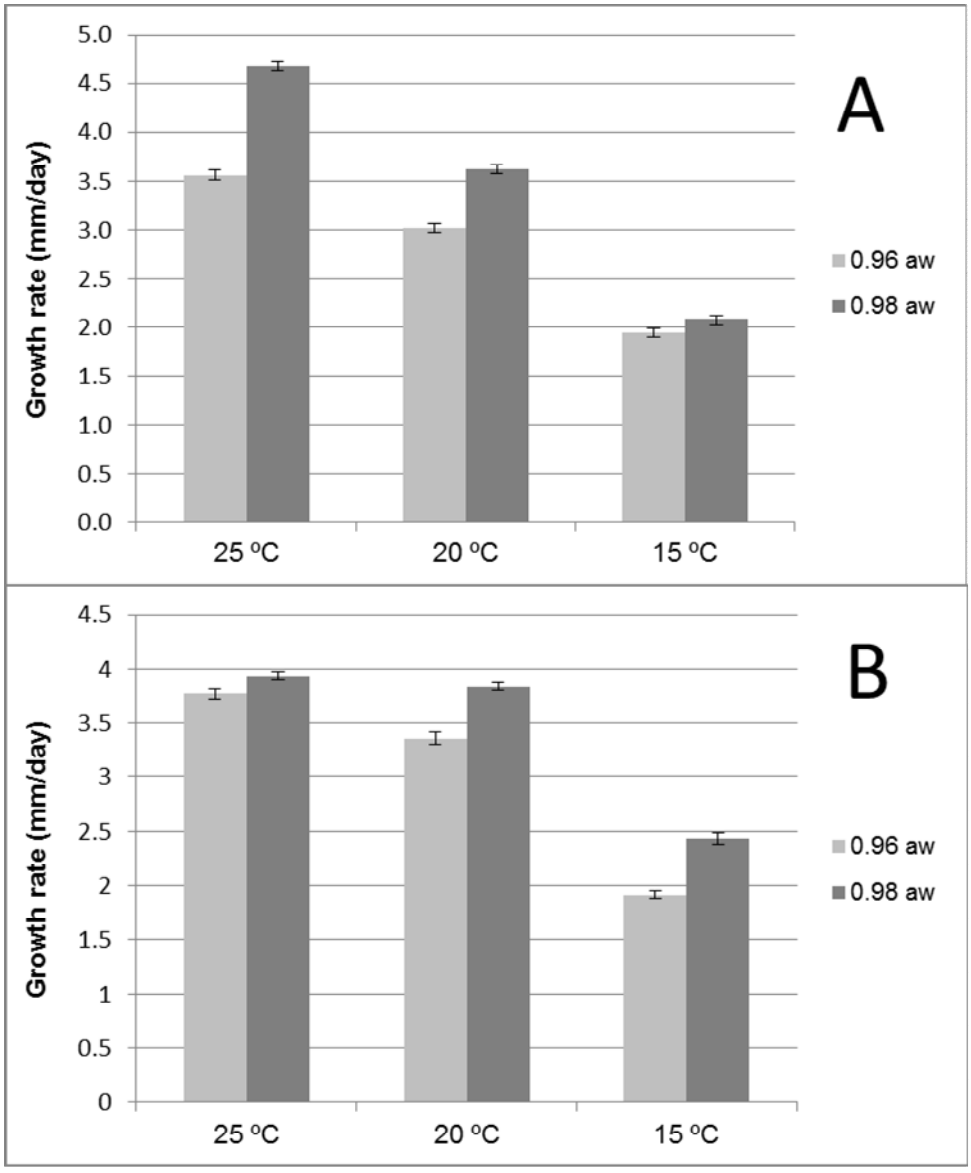

Figure 1

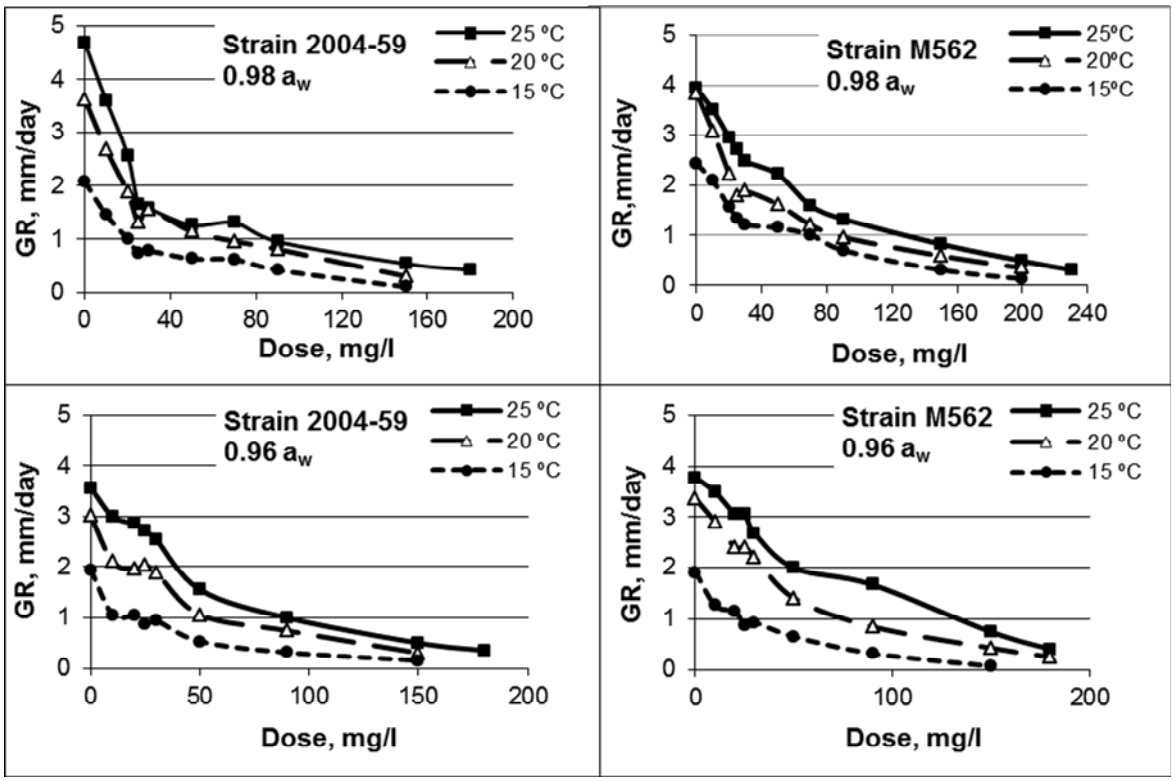
Strain 2004-59
0.98 a_w
25 °C
20 °C
15 °C
GR, mm/day
Dose, mg/l
Strain M562
0.98 a_w
25°C
20°C
15°C
GR,mm/day
Dose, mg/l
Strain 2004-59
0.96 a_w
25 °C
20 °C
15 °C
GR, mm/day
Dose, mg/l
Strain M562
0.96 a_w
25 °C
20 °C
15 °C
GR, mm/day
Dose, mg/l

Figure 2.

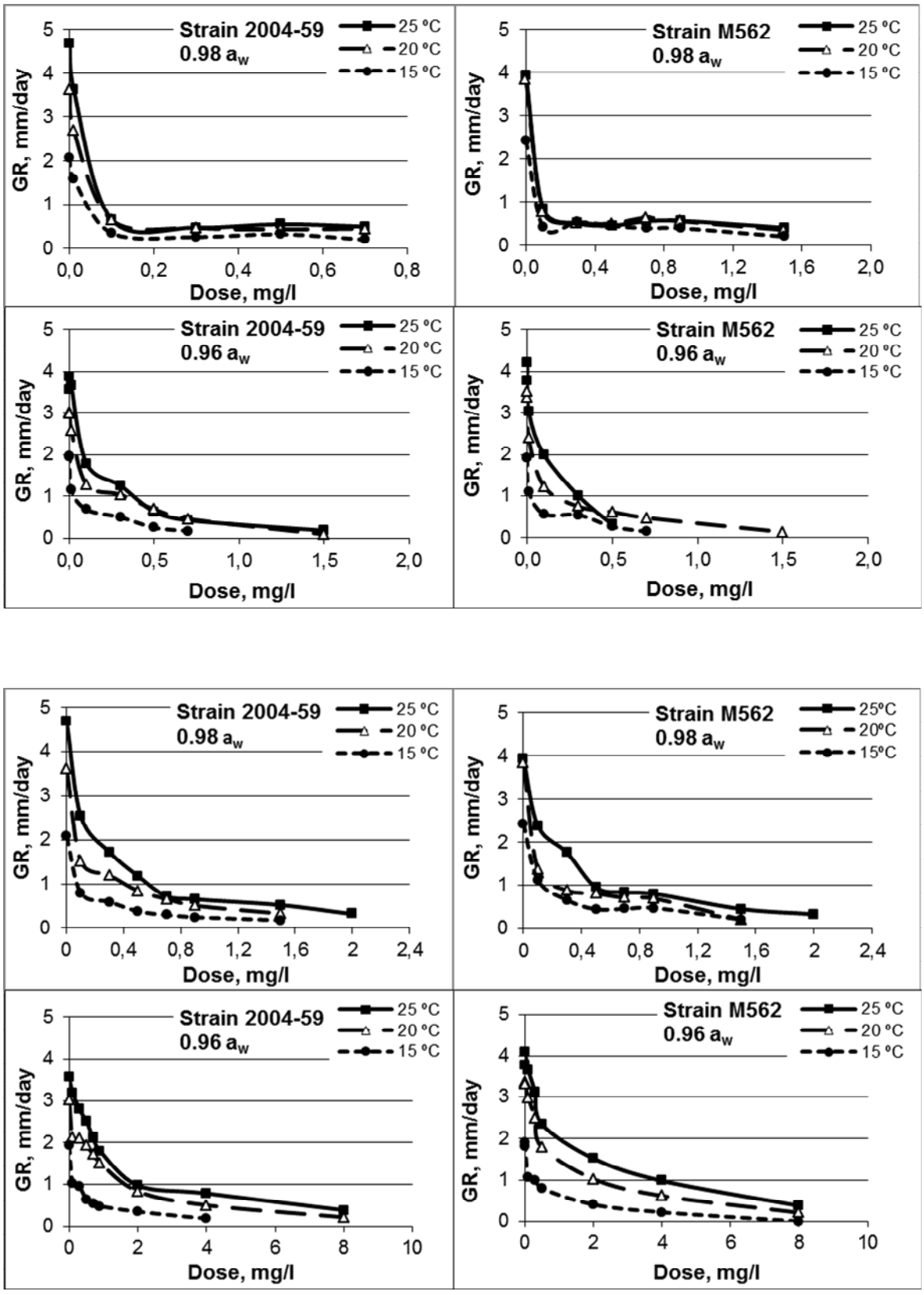
Strain 2004-59
0.98 a_w
25 °C
20 °C
15 °C
GR, mm/day
Dose, mg/l
Strain M562
0.98 a_w
25 °C
20 °C
15 °C
GR, mm/day
Dose, mg/l
Strain 2004-59
0.96 a_w
25 °C
20 °C
15 °C
GR, mm/day
Dose, mg/l
Strain M562
0.96 a_w
25 °C
20 °C
15 °C
GR, mm/day
Dose, mg/l
Strain 2004-59
0.98 a_w
25 °C
20 °C
15 °C
GR, mm/day
Dose, mg/l
Strain M562
0.98 a_w
25°C
20°C
15°C
GR, mm/day
Dose, mg/l
Strain 2004-59
0.96 a_w
25 °C
20 °C
15 °C
GR, mm/day
Dose, mg/l
Strain M562
0.96 a_w
25 °C
20 °C
15 °C
GR, mm/day
Dose, mg/l

Figure 3.

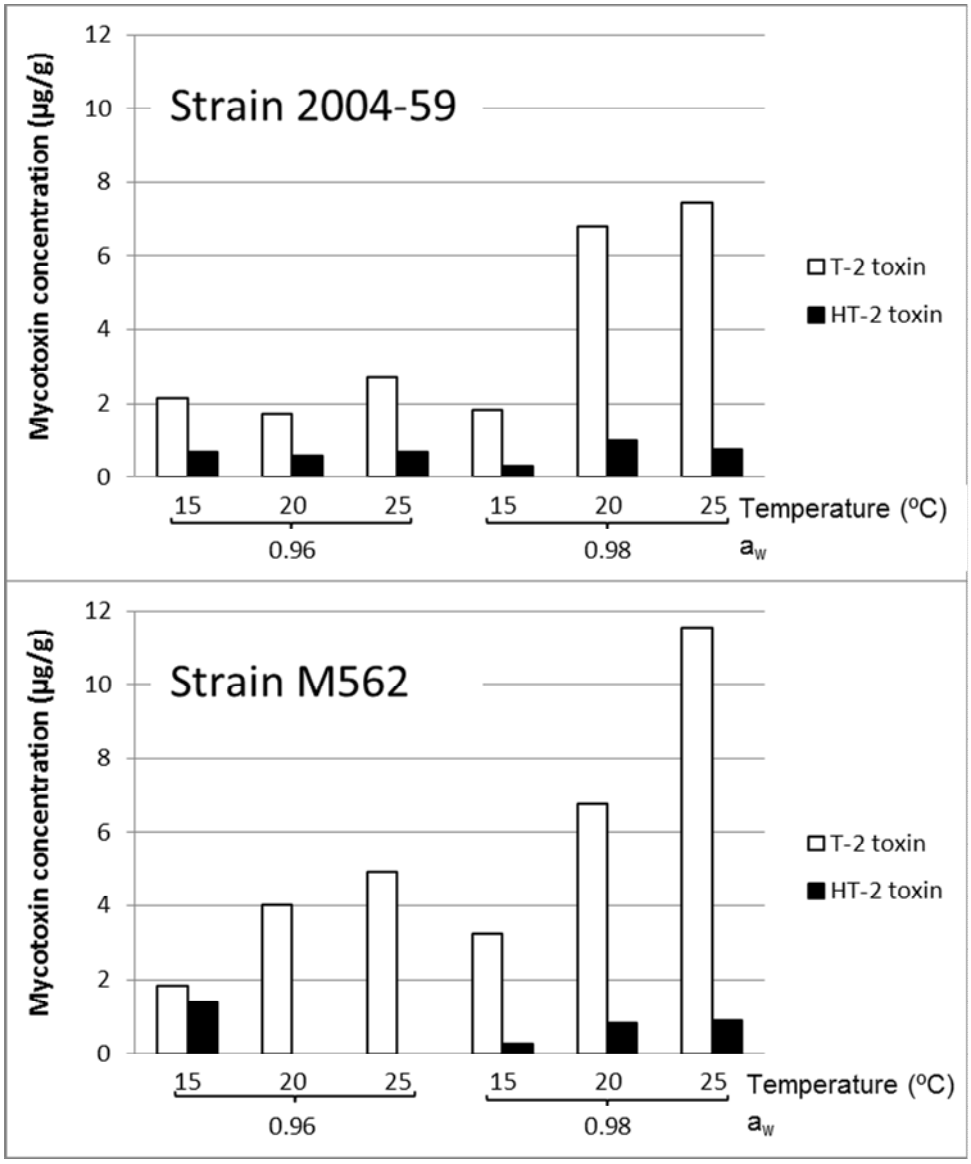
Strain 2004-59
Mycotoxin concentration (µg/g)
12
10
8
6
4
2
0
T-2 toxin
HT-2 toxin
15
20
25
15
20
25
Temperature (ºC)
0.96
0.98
a_w
Strain M562
Mycotoxin concentration (µg/g)
12
10
8
6
4
2
0
T-2 toxin
HT-2 toxin
15
20
25
15
20
25
Temperature (ºC)
0.96
0.98
a_w

Figure 4.

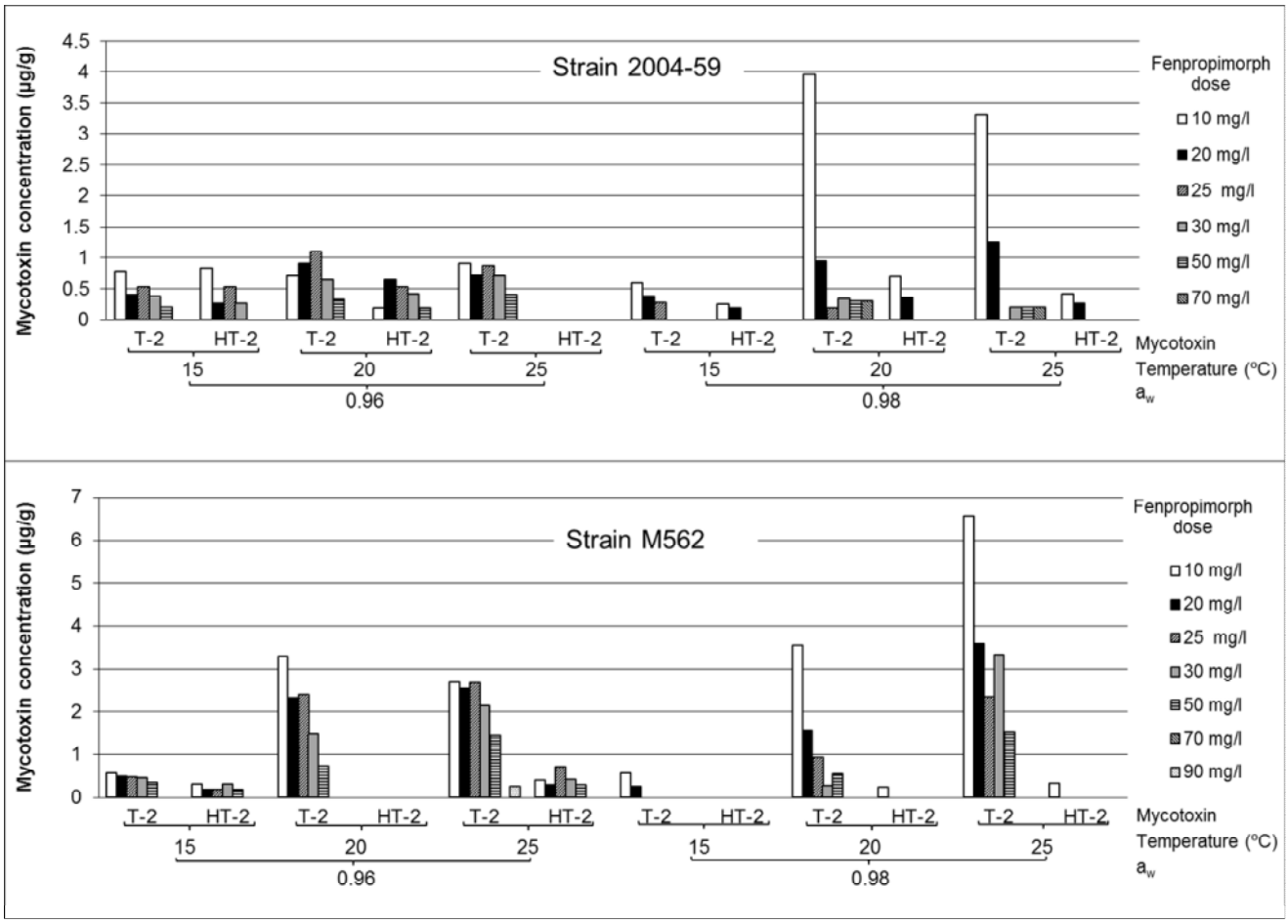
Strain 2004-59
Mycotoxin concentration (µg/g)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
T-2
HT-2
15
20
25
0.96
0.98
Fenpropimorph dose
10 mg/l
20 mg/l
25 mg/l
30 mg/l
50 mg/l
70 mg/l
Mycotoxin
Temperature (°C)
a_w
Strain M562
Mycotoxin concentration (µg/g)
7
6
5
4
3
2
1
0
Fenpropimorph dose
10 mg/l
20 mg/l
25 mg/l
30 mg/l
50 mg/l
70 mg/l
90 mg/l
Mycotoxin
Temperature (°C)
a_w

Figure 5.

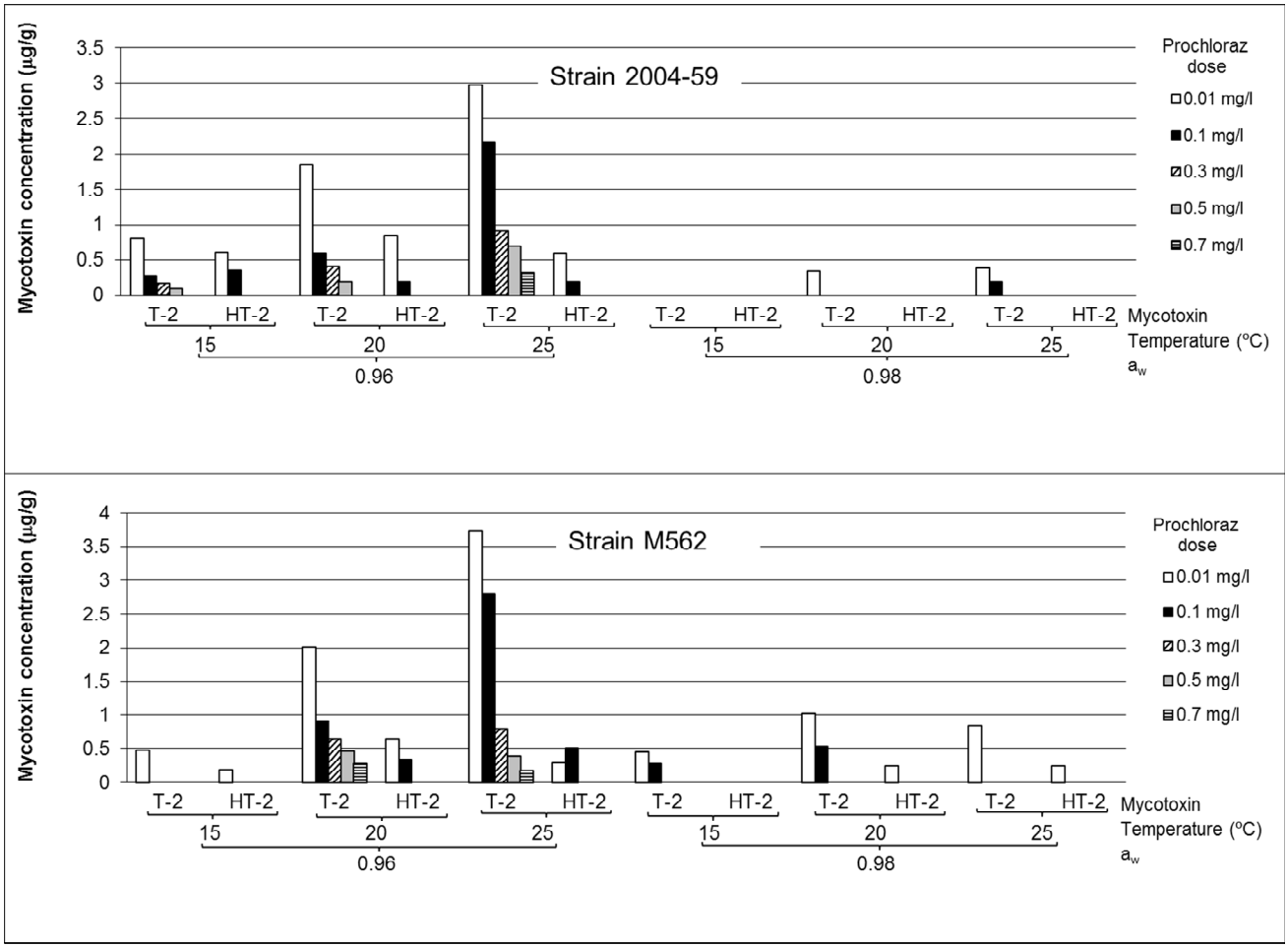
Strain 2004-59
Mycotoxin concentration (µg/g)
3.5
3
2.5
2
1.5
1
0.5
0
Prochloraz dose
0.01 mg/l
0.1 mg/l
0.3 mg/l
0.5 mg/l
0.7 mg/l
T-2
HT-2
15
20
25
0.96
0.98
Mycotoxin
Temperature (°C)
a_w
Strain M562
Mycotoxin concentration (µg/g)
4
3.5
3
2.5
2
1.5
1
0.5
0
Prochloraz dose
0.01 mg/l
0.1 mg/l
0.3 mg/l
0.5 mg/l
0.7 mg/l
T-2
HT-2
15
20
25
0.96
0.98
Mycotoxin
Temperature (°C)
a_w

Figure 6.

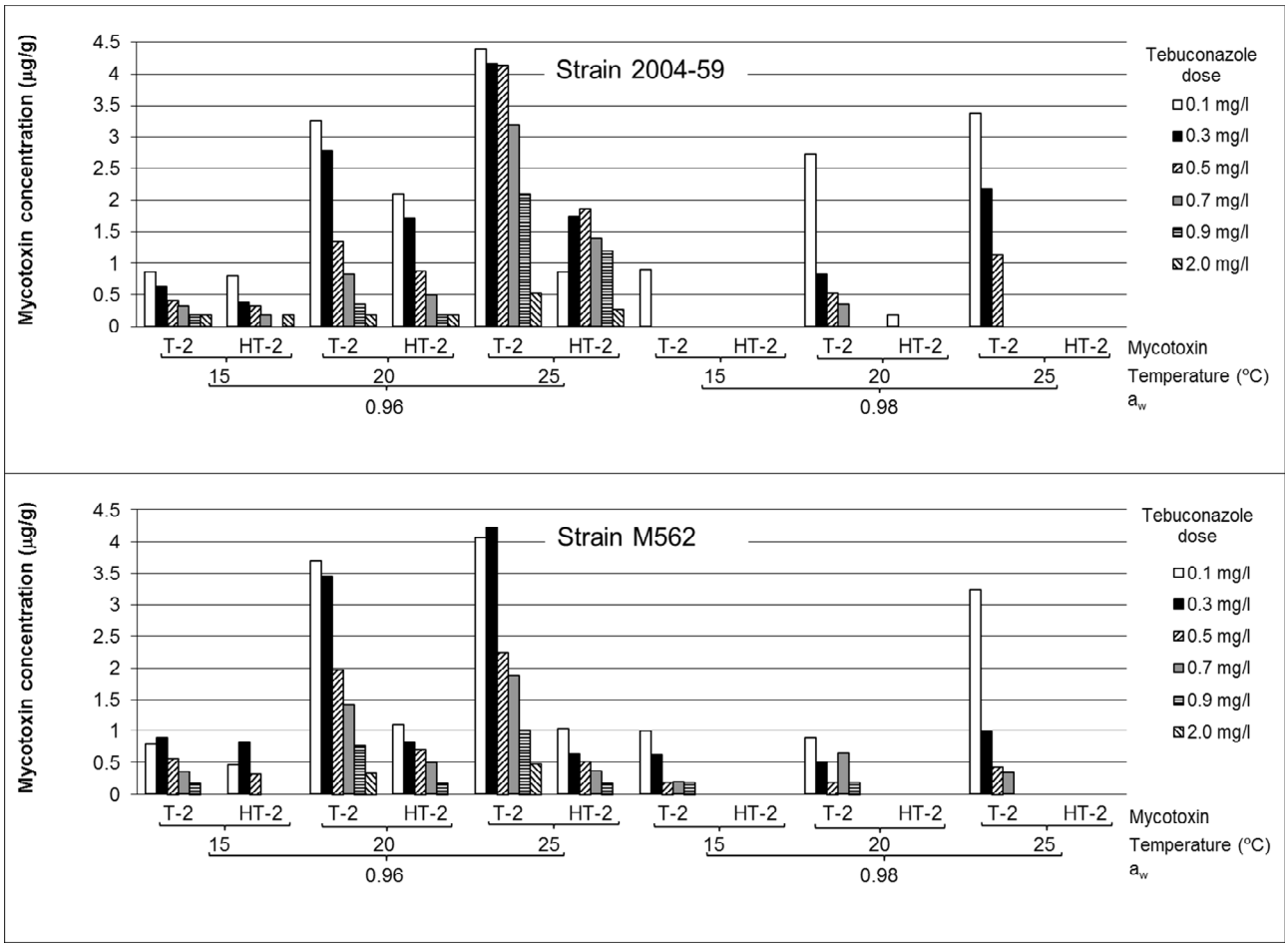
Strain 2004-59
Mycotoxin concentration (µg/g)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Tebuconazole dose
0.1 mg/l
0.3 mg/l
0.5 mg/l
0.7 mg/l
0.9 mg/l
2.0 mg/l
T-2
HT-2
15
20
25
0.96
0.98
Mycotoxin
Temperature (°C)
a_w
Strain M562
Mycotoxin concentration (µg/g)

Figure 7.