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Relationship between environmental factors, dry matter loss and mycotoxin levels in stored wheat and maize infected with Fusarium species

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Methods/Techniques:	Mycology, LC/MS
Additives/Contaminants:	Mycotoxins, Mycotoxins - fumonisins, Mycotoxins - fusarium, Mycotoxins - trichothecenes
Food Types:	Cereals and grain, Cereals
Abstract:	<p>This study examined the relationship between storage environmental factors (water activity (aw) (0.89-0.97) and temperature (15-30°C)), colonisation of wheat and maize by <i>Fusarium graminearum</i> and <i>F. verticillioides</i> respectively and the dry matter losses (DMLs) caused, and quantified the contamination with deoxynivalenol (DON), zearalenone (ZEA) and fumonisins (FUMs) during storage. Fungal growth was assessed by the amount of CO₂ produced under different interacting conditions of aw and temperature. DMLs were quantified using the cumulative CO₂ data and these were shown to increase as temperature and aw increased. The amount of DON, ZEA (wheat for human consumption) and FUMs (feed maize) produced was significantly affected by the storage conditions. The three toxins however showed different patterns of production. Optimum for DON was at the wettest conditions (0.97 aw) and the highest temperature assessed (30°C), while for ZEA this shifted to 25°C. FUMs were produced in higher amounts in maize at 30°C and 0.97 aw, however, at intermediate aw levels (0.955 aw) the highest production occurred at 25°C followed by 20°C. Polynomial models were developed for the effect of the storage factors on DMLs and toxin production. DMLs under different environmental conditions were significantly correlated with DON and FUMs. DON contamination was above the EU limits in at least 80% of the wheat</p>

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	samples with DMLs>1%, while at least 70% of the same samples contained ZEA above the respective EU legislative limits. Similarly, at least 75% of the maize samples with DMLs≥0.9% exceeded the EU limits for the sum of fumonisins in feed. These results show that it may be possible to use temporal CO2 production during storage of grains as an indicator of the level of contamination of the grain with mycotoxins.

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Relationship between environmental factors, dry matter loss and mycotoxin levels in stored wheat and maize infected with *Fusarium* species

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Running title: Dry matter losses and mycotoxins in wheat and maize

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15 This study examined the relationship between storage environmental factors (water
16 activity (a_w) (0.89-0.97) and temperature (15-30°C)), colonisation of wheat and maize
17 by *Fusarium graminearum* and *F. verticillioides* respectively and the dry matter
18 losses (DMLs) caused, and quantified the contamination with deoxynivalenol (DON),
19 zearalenone (ZEA) and fumonisins (FUMs) during storage. Fungal growth was
20 assessed by the amount of CO₂ produced under different interacting conditions of a_w
21 and temperature. DMLs were quantified using the cumulative CO₂ data and these
22 were shown to increase as temperature and a_w increased. The amount of DON, ZEA
23 (wheat for human consumption) and FUMs (feed maize) produced was significantly
24 affected by the storage conditions. The three toxins however showed different
25 patterns of production. Optimum for DON was at the wettest conditions (0.97 a_w) and
26 the highest temperature assessed (30°C), while for ZEA this shifted to 25°C. FUMs
27 were produced in higher amounts in maize at 30°C and 0.97 a_w , however, at
28 intermediate a_w levels (0.955 a_w) the highest production occurred at 25°C followed by
29 20°C. Polynomial models were developed for the effect of the storage factors on
30 DMLs and toxin production. DMLs under different environmental conditions were
31 significantly correlated with DON and FUMs. DON contamination was above the EU
32 limits in at least 80% of the wheat samples with DMLs>1%, while at least 70% of the
33 same samples contained ZEA above the respective EU legislative limits. Similarly, at
34 least 75% of the maize samples with DMLs≥0.9% exceeded the EU limits for the
35 sum of fumonisins in feed. These results show that it may be possible to use
36 temporal CO₂ production during storage of grains as an indicator of the level of
37 contamination of the grain with mycotoxins.

Keywords: Respiration, dry matter loss, *Fusarium graminearum*, *F. verticillioides*, water activity, temperature, grain storage, deoxynivalenol, zearalenone, fumonisins, mycotoxins, EU legislative limits

Introduction

Wheat, maize and rice are the most important staple foods world-wide (FAO, 2009). They represent the key food chains and are the major nutritional components for both food and feed. Thus any deterioration in the quality of these commodities due to fungal spoilage or contamination with mycotoxins results in a significant impact on consumer and animal health.

Wheat and maize are commonly colonised by *Fusarium* species from the field. Poor drying during the harvesting phase or storage of damp grain can facilitate rapid colonisation by spoilage and mycotoxigenic moulds at the post-harvest stage (Magan and Aldred, 2007; Magan et al., 2010). This results in quality, dry matter and nutritional losses with significant economic consequences. Colonisation by *Fusarium* species can also result in contamination with mycotoxins. Thus, *F. graminearum* can contaminate wheat with deoxynivalenol(DON), nivalenol and zearalenone (ZEA), while *F. verticillioides* can contaminate maize with fumonisins (FUMs). In recent years type A trichothecenes (T-2 and HT-2 toxins) produced by *F. langsethiae* and *F. sporotrichioides* have also become of concern especially in oats and barley, but also in wheat. Contamination of these grains with fusarium toxins has generally been considered as more significant at the pre-harvest stage (Cleveland et al, 2003; Miller, 2008). However, several studies have demonstrated that fusarium toxins may also proliferate during storage when damp conditions occur (Wagacha and Muthomi,

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62 2008), during harvesting and due to poor management of the drying phase.
63 Therefore effective management of storage conditions is essential for conservation
64 of both grain quality and safety post-harvest.

65 Due to the toxic effects of fusarium toxins on animals and humans, many
66 countries world-wide have established legislative limits for these compounds in food
67 and feed. In Europe these limits are harmonised (European Commission (EC),
68 2006a) and methods for the sampling and analysis of these toxins in different
69 products are also established (EC, 2006b). Finally, inspection and control measures
70 enforce these limits in products produced within Europe and check the compliance of
71 imported products from third countries.

72 However, despite the stringent control measures in place, contaminated
73 products are still identified in Europe. Since 2005 between 650 and 1000
74 notifications for mycotoxins in food and feed are received each year by the Rapid
75 Alert System for Food and Feed (RASFF), the vast majority of which concerns
76 aflatoxins. Notifications concerning fusarium toxins have usually been <20per year
77 mostly for FUMs, but also DON and ZEA in cereals, cereal products or feed (EC,
78 2005-2009). Fusarium toxins are very stable molecules and once formed, traditional
79 food and feed processing techniques are inefficient in removing them from the
80 products. It is therefore important to investigate further the conditions conducive to
81 contamination by *Fusarium* species in order to be able to efficiently predict and
82 prevent toxin accumulation in grain.

83 There have been many attempts to develop “storability risk indexes” for quality
84 losses during storage. These were reviewed by Fleurat-Lessard (2002) and include
85 the time to the appearance of microscopic or visible mould, ergosterol measurement,

the analysis of volatiles associated with fungal growth and quantitative enzyme production. Recently, a similar approach, based on time to visible moulding, has been used to develop safe storage guidelines for durum wheat although the accuracy of this approach needs to be debated (Nithya et al., 2011). Dry matter losses (DMLs) have also been considered as a quality indicator and values as low as 0.04% DML has been reported in relation to seed germination and visible moulding in wheat (White et al., 1982a, b; Lacey et al., 1994). Seitz et al. (1982) indicated that 0.5% DML would be enough to downgrade maize from food to feed quality with an increased risk of aflatoxin contamination depending on the initial spore load at the time of storage. However, not much information is available on the risk of fumonisin contamination of maize.

In terms of predicting toxin contamination in stored grain, Lund and Frisvad (2003) and Lindblad et al. (2004) related colony forming units (CFUs) of *Penicillium verrucosum* to the storage conditions and the probability of exceeding the EU legislative limits for ochratoxin A. Yong and Cousin (2001) obtained some good correlations comparing *Aspergillus parasiticus* colony counts to enzyme-linked immunosorbent assay (ELISA) quantification and ergosterol measurements in order to detect aflatoxin contamination in maize and peanut samples. More recently Pasquali et al. (2010) related the counts of a nivalenol chemotype *Fusarium culmorum* strain to the mycotoxin content of grain, while Casado et al. (2010) found no correlation between the number of infected kernels and fumonisin contamination of a maize bulk. However, no studies have attempted to quantitatively relate DMLs to toxin contamination during storage and use these losses as a safety risk index. Such information would be very valuable in assessing the potential for exceeding the legislative limits for DON and ZEA in wheat or for FUMs in maize.

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The objectives of this study were to (a) quantify the DMLs caused during *F. graminearum* and *F. verticillioides* colonisation of wheat for human consumption and feed maize respectively based on the amounts of CO₂ produced under different combinations of a_w x temperature interactions, (b) quantify the production of DON and ZEA in wheat and FUMs in maize under the same conditions and (c) develop models to relate DMLs to mycotoxin contamination taking into account a_w x temperature effects and identify the threshold DMLs which would result in exceeding the EU legislative limits.

Materials and methods

Fungal cultures

The *F. graminearum* strain (isolate L1-2/2D) was supplied by the University of Lleida, Catalonia, Spain; it was isolated from wheat and is a known high producer of deoxynivalenol (DON) and zearalenone (ZEA) (Marín et al., 2010). The *F. verticillioides* strain (MPVP 294) originated from maize in Italy and is a known high producer of fumonisins B₁ and B₂ (Etcheverry et al., 2009). This strain was supplied by Università Cattolica del Sacro Cuore (UCSC), Piacenza, Italy. The cultures were maintained on Malt Extract Agar (MEA) media (OXOID, malt extract, 30; mycological peptone, 5; agar, 15 g/l).

Grain treatment and water activity adjustment of wheat and maize

Wheat used in this experiment was obtained from a local Bedfordshire farm while feed maize was supplied by UCSC, Piacenza, Italy. Both cereals (2008/2009

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3 134 harvest year) were treated with 12 kGy gamma irradiation (Isotron, Plc, Swindon,
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5 135 UK) and stored at 4°C. At this irradiation dose the cereals retained germinative
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7 136 capacity but were free of fungal contamination (Hope et al., 2005).
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10 137 Water adsorption curves were prepared for both grains as previously described
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12 138 (Mylona and Magan, 2011). Although the water adsorption curves were relatively
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14 139 stable over the first three days of measurement, the data of the 3rd day were used for
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16 140 the determination of the amount of water required to achieve the target a_w levels.
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18 141 Irradiated grain was modified to the treatment a_w levels by the addition of sterile
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20 142 distilled water and the treatments used for wheat were: 0.89, 0.94 and 0.97 a_w and
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22 143 for maize: 0.91, 0.955 and 0.97 a_w which equated to 20.5, 26 and 32% and 19, 25 and
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24 144 27.5% moisture content (wet weight basis) respectively. These a_w levels were
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26 145 selected to simulate ambient drying of moist grain or poor storage management
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28 146 practices that may occur when moist grain is mixed with drier grain. These a_w levels
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30 147 are representative of marginal and optimum conditions for the growth of the relevant
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32 148 fungal species in these cereal types (Sanchis and Magan, 2004).
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43 150 ***Grain inoculation and storage***

44 151 Wheat and maize treatments were inoculated with 1ml of spore suspension and
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46 152 thoroughly mixed. Spore suspensions were obtained from 10 day old cultures of *F.*
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48 153 *graminearum* and *F. verticillioides* flooded with Tween 80 solution (one drop of
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50 154 Tween 80 (ACROS organics) in 100ml of sterile water) and gently scraping the
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52 155 culture surface with a sterile spatula. The concentration of the spores was
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54 156 determined using a haemocytometer (Olympus BX40 microscope, Microoptical Co.;
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56 157 slide Marienfeld superior, Germany) and adjusted by dilution to 10^4 spores ml^{-1} .
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Three replicates of 10g (wet weight) were placed in 40ml vials (Chromacol Ltd, UK) and incubated in 10L closed containers in which the target equilibrium relative humidity was maintained using 0.5l of an appropriate glycerol/water solution. The samples were stored at four temperatures (15, 20, 25 and 30 °C) for 10 days.

Respiration measurement by Gas Chromatography (GC)

Treatment samples were sealed daily for one hour with injection caps to allow for CO₂ accumulation in the headspace. 5 ml of the headspace were withdrawn by a syringe and directly inserted in the Gas Chromatograph (GC) for CO₂ analysis. The GC used was a GC 8000 Series, Carlo Erba Instruments with a Hot Wire Detector (HWD) and Helium as a carrier gas. For the CO₂ analysis a column packed with Porapak Q was used. Chromatograms were obtained by a DP 800 Data Processor, Carlo Erba (Mylona and Magan, 2011).

The respiration rate and the total DMLs were calculated for each sample from the percentage CO₂ concentration data obtained, as previously described (Mylona and Magan, 2011). DMLs were adjusted as per initial dry weight of treatments. At the end of the 10-day incubation period the samples were frozen at -40°C for subsequent toxin analysis.

Toxin extraction/analysis

The samples were oven-dried at 60°C for 24 hours and then milled in a small laboratory blender (Waring Commercial, Christison, UK). Samples were analysed for fusarium toxins by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) (Sulyok et al., 2006; Vishwanath et al., 2009). Quantitation was based on external

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3 181 calibration using certified liquid standards, no isotopically-labelled internal standards
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5 182 were used. Results were corrected for apparent recovery that was 82% in case of
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7 183 zearalenone as determined by analysis of the untreated wheat sample after spiking.
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9 184 The same approach was not applicable for DON and the fumonisins, as the
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11 185 untreated samples were already contaminated. Therefore, the apparent recovery
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13 186 obtained during method validation (57% for FB₁, 70% for FB₂, 95% for DON; Sulyok
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15 187 et al. 2006) was used to correct the results. The accuracy of the method was
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17 188 externally checked by participation in proficiency testing organized by BIPEA
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19 189 (Bureau Inter Professionel d'Etudes Analytiques; Gennevilliers, France). Z-scores
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21 190 were 0.4 and 0.62 for DON in two wheat samples, -0.8 and -1.09 for ZON in two
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23 191 wheat samples and 1.36 and 1.55 for FB₁ and FB₂, respectively in a sample of
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33 194 ***Statistical analysis and modelling of the results***

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36 195 Data were analysed with Microsoft Office Excel 2007 and with the package
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38 196 STATISTICA 9 (StatSoft®, Inc. 2010. STATISTICA (data analysis software system,
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40 197 version 9.1. www.statsoft.com)).
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47 199 ***Statistical analysis of DML and toxin analysis data***

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50 200 Total DML data and toxin analysis data were assessed by the Shapiro-Wilk, W
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52 201 test for normality. Data not normally distributed were logarithmically transformed in
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54 202 order to stabilise the variance and their normality reassessed by the Shapiro-Wilk W
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56 203 test. Normally distributed log-transformed data were analysed by one and two-way
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ANOVA for determination of the significance of the effect of each factor and their interaction on the variable. Log-transformed data still not normally distributed were analysed by the Kruskal-Wallis test by ranks.

Model development

Forward stepwise regression (STATISTICA 9) and Regression (Microsoft Office Excel 2007) were used to obtain polynomial equations for DML and toxin production with regard to the storage conditions (a_w and temperature). The assumptions of linearity and normally distributed residuals were assessed, producing normal plots of the residuals. Where residuals were not normally distributed the variable was logarithmically transformed and the normality reassessed. Contour maps were also constructed (Microsoft Office Excel 2007) to indicate the effects of storage conditions on DMLs and toxin production for both wheat and maize.

Correlations between DMLs and toxin production data

DMLs were plotted against toxin production for DON, ZEA (wheat) and FUMs (maize). The Spearman Rank Order Correlations test was used to determine the significance of the correlation between the two variables in each case.

Results

Respiration rates and CO₂ production by *F. graminearum* in wheat and *F. verticillioides* in maize as affected by the storage conditions

Overall, respiration in wheat at 0.89 a_w and 15°C was constantly very low throughout the 10 day storage period (data not shown). This can be attributed to the grain, since no fungal growth was observed at these conditions. Respiration rates generally started increasing after 2-4 days incubation and this was due to the initiation of fungal growth (data not shown). Respiration data obtained until that point in time were attributed to the respiration of the grain and they were generally stable under a set combination of storage conditions, but higher with increasing a_w and temperature. Once fungal growth was initiated, respiration rates started increasing with time and the increase was generally higher under a set combination of storage conditions. Thus the fungal contribution to the total respiration was increasingly more significant representing >75% of the total respiration in the wheat experiment by the 10th day of incubation under the most favourable storage conditions. Similar observations were also made in the maize experiment, however in this case respiration rates started increasing from the second day in the most favourable combinations of storage conditions ($T \geq 20^\circ\text{C}$, $a_w \geq 0.955$). In maize under the most favourable conditions tested >85% of the respiration measured on the 10th day of storage was due to fungal colonisation. The respiration of the grain under each combination of storage conditions has not been subtracted from the total respiration measured, as this is considered unrealistic, since in practice these two could not be dissociated, i.e. during CO₂ monitoring in silos.

Figure 1 shows the effects of a_w and temperature on the cumulative CO₂ production during wheat colonisation by *F. graminearum*. This was very low at 0.89 a_w , but generally increased with increasing a_w and temperature over time and the maximum was observed under the wettest conditions and 30°C where the total accumulated CO₂ production was also the highest.

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A similar trend in the respiration rates and total CO₂ production with increasing a_w and temperature over time was observed during *F. verticillioides* colonisation of maize. Overall, the cumulative CO₂ production during the respiration of *F. verticillioides* on maize was higher than that of *F. graminearum* on wheat.

Effect of water activity and temperature on dry matter losses in wheat and maize during storage

Dry matter losses (DMLs) in wheat and maize during colonisation by *F. graminearum* and *F. verticillioides* respectively increased with increasing a_w and temperature. The maximum was observed under the wettest conditions and highest temperature assessed. Overall, DMLs were higher in maize than in wheat, which paralleled the higher respiration rates observed in these studies. Figure 2 shows the DMLs caused in (a) wheat and (b) maize after 10 days colonisation by *F. graminearum* and *F. verticillioides* respectively, under different combinations of storage conditions. The maximum standard errors (SE_{max}) in the determination of DMLs in each experiment have been calculated and are included in the legend of Figure 2. As temperature and a_w increased the relative DMLs also increased. At 30°C DMLs were highest and were about 7 and 15% respectively.

Statistically, the effect of a_w on logDMLs in wheat (normally distributed: W, p=0.090) was highly significant (p=0,000), while the effect of temperature was significant (p=0.043). The interaction of the two factors (a_w×T) on logDMLs was also highly significant (p=0,000).For maize, logDML data were also normally distributed (W, p=0.498) and in this case the effects of both a_w (p=0.000) and temperature (p=0.000) were highly significant, as well as their interaction (a_w×T) (p=0.006).

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275 Mycotoxin production in wheat and maize under different storage conditions

276 Figure 3 shows the pattern of (a) DON and (b) ZEA production in wheat by *F.*
277 *graminearum* and (c) fumonisins production by *F. verticillioides* in maize under
278 different combinations of a_w and temperature. The maximum standard errors (SE_{max})
279 in the determination of each toxin have also been calculated and are included in the
280 legend of Figure 3. DON and ZEA levels were low at all temperatures and 0.89 a_w ,
281 and at all a_w levels and 15°C. However, some DON may be produced at 0.97 a_w and
282 15°C. The optimum for DON production was observed at 30°C while for ZEA at 25°C
283 at the wettest conditions. The total combined FUMs (sum of fumonisins B₁ and B₂)
284 production was higher at 0.97 a_w and 30°C while at reduced a_w levels the optimum
285 appeared to be at 25°C. No FUMs were produced at 15°C in any of the samples over
286 the time scale of this experiment, while under the driest conditions tested (0.91 a_w)
287 some FUMs were produced at 25°C. Data were also collected on the individual
288 FUMs. Interestingly, the ratio of fumonisin B₁/fumonisin B₂ was found to be between
289 4 and 8 and to increase with increasing temperature and a_w (data not shown).

290 For DON and ZEA, the Kruskal-Wallis test by ranks was used for the analysis of
291 the raw data. For DON the effect of a_w was highly significant ($p=0.000$), while the
292 effect of temperature was not significant ($p=0.071$). For ZEA the effect of a_w was also
293 highly significant ($p=0.001$), while the effect of temperature was significant
294 ($p=0.043$). The Kruskal-Wallis test was also highly significant for the effect of a_w on
295 the total FUMs ($p=0.000$), while the effect of temperature was found significant
296 ($p=0.042$).

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Development of models of DML and toxin production with regard to storage conditions

The following equation was fitted for the effect of a_w and temperature as well as their interaction ($a_w \times T$) on the DMLs and DON production in wheat colonised by *F. graminearum*:

$$DML_{wheat} = b_0 + b_1 a_w + b_2 T + b_3 a_w^2 + b_5 a_w T \quad (eq. 1)$$

The quadratic effect of temperature (T^2) was not significant in either DMLs or DON production in wheat. The values for the coefficients b_0 - b_5 as well as the statistical significance of the factors in each case are presented in Table 1.

For maize, the residuals for DMLs were not normally distributed and therefore the data were logarithmically transformed before regression. Only the effects of a_w^2 , T^2 and $a_w \times T$ were significant on logDMLs (eq. 2).

$$\log DML_{maize} = b_0 + b_3 a_w^2 + b_4 T^2 + b_5 a_w T \quad (eq. 2)$$

The values for the coefficients as well as the statistical significance of the factors in each case are presented in Table 1.

For the sum of fumonisins only the effects of T , T^2 and $a_w \times T$ were found to be significant on logFUMs (eq. 3) and the coefficients and the statistical significance of each of the factors are presented in Table 1.

$$\log FUMs = b_2 T + b_4 T^2 + b_5 a_w T \quad (eq. 3)$$

Due to the pattern of ZEA production which increased up to 25°C and sharply decreased at 30°C, it has been considered more relevant to only include the data up to 25°C for input in the regression. A similar approach has been previously used by

Marín et al. (2008) in order to model toxin production by *Aspergillus carbonarius* in pistachio nuts. Using this approach, all factors were significant on ZEA except of T^2 and an equation similar to eq.1 could be used for the effect of storage factors on ZEA production in stored wheat, for temperatures up to 25°C. The coefficients for each factor and the statistical significance are provided in Table 1.

Contour maps were constructed for the DMLs and DON in wheat and log DMLs and logFUMs in maize with regard to the storage conditions, based on the above equations (Figure 4). From these maps the a_w ×temperature regions where DMLs and toxin production are optimum and those where these are marginal can be clearly identified.

Correlation between dry matter losses and toxin production

Figure 5 shows a scatter plot of the DML and DON production data obtained in wheat under different combinations of storage conditions. There was a highly significant positive correlation between DMLs and DON (Spearman Rank Order Correlations test: $R=0.9572$, $p=0.0000$). The solid straight line denotes the EU limit established for DON in wheat for human consumption ($1750\mu\text{g kg}^{-1}$). More than 80% of the samples with >1% DMLs had DON levels exceeding the EU limits for this toxin in wheat. A significant positive correlation ($R=0.8371$, $p=0.0000$) was also obtained between DMLs caused in maize and FUMs (total) (Figure 6) and in this case the solid straight line denotes the EU limits for the sum of fumonisins in feed maize ($4000\mu\text{g kg}^{-1}$). In this case more than 75% of the maize samples with >0.9% DMLs had total fumonisin levels above the EU limits, however as opposed to the wheat samples, one sample contained total fumonisin levels above the EU limits with as little as 0.66% DMLs.

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Plotting the data for ZEA against DMLs in a similar way also gave a statistically highly significant positive correlation ($R=0.5536$, $p=0.0005$) (data not shown). A much higher significant correlation ($R=0.9160$, $p=0.0000$) between the two variables could be obtained if only the data between 15 and 25°C were considered (Figure 7). Due to the pattern of ZEA production under different combinations of storage conditions, just above 70% of the samples with DMLs >1% exceeded the legislative limits for this toxin in the wheat samples.

Discussion

In this study, growth of *F. graminearum* and *F. verticillioides* on wheat for human consumption and feed maize respectively was determined by the amount of CO₂ produced during storage. Using this technique we have previously determined the growth boundaries of *F. langsethiae* on oat grain for the first time (Mylona and Magan, 2011).

Growth optima for *F. graminearum* were found in this study to be similar to those reported in the literature, determined either visually by semi-quantitative methods or by fungal colony diameter measurements (Cuero et al., 1987; Hope et al., 2005). As opposed to previous studies where 0.92 a_w was found marginal for the growth of *F. verticillioides* by either colony measurements or scanning electron microscopy (SEM) (Marín et al., 1995; Torres et al., 2003; Samapundo et al., 2005), we observed growth even at 0.91 a_w at >20°C. Optima were at 30°C and 0.97 a_w similar to those reported in previous studies. Overall, the results presented in this work are in good agreement with published data, suggesting that fungal growth boundaries on grains may be accurately estimated by the amount of CO₂ produced during storage. It is

believed that *in situ* this method may be more accurate for monitoring potential for spoilage in cereals and improve the management of the post-harvest storage phase.

Using the CO₂ data we calculated the dry matter losses (DMLs) caused in wheat and maize during fungal colonisation by *F. graminearum* and *F. verticillioides* respectively and developed model relationships and contour maps to visualise the effect of storage conditions on the losses. Similar maps were also constructed for the effect of storage conditions on mycotoxin production. These maps may be very useful in identifying high- and low-risk conditions for mycotoxin contamination in stored wheat and maize, since a_w or temperature stress applied during control strategies could stimulate the production of other toxins instead. For example, storing grain at <15°C could reduce the risk of DON or FUMs contamination, but would not be very efficient against ochratoxin A formation by *Penicillium* species, which has been shown to occur in as low as 10°C (Lindblad et al. 2004). On the other hand, an increase in storage temperature of wheat to 30°C would dramatically increase the chances of DON contamination, while causing a reduced presence of ZEA. This kind of switches in the major mycotoxin contamination in food/feed may be important when considering and developing predictions of the effects of an increase in the average global temperature due to climate change (Magan et al, 2011). However, similar information should also be obtained for other metabolites produced by the same species which may be optimally produced under different combinations of storage conditions.

We have obtained some highly significant correlations between DMLs and toxin production in stored wheat and maize. From the correlations obtained in this study between DMLs and DON in wheat and DMLs and total FUMs in feed maize, it is suggested that in both cases EU limits are likely to be exceeded when very small

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392 DMLs are reached (0.9 – 1%). This means that the tolerances are very low and this
393 type of information could be used practically for improving management of grain in
394 the post-harvest component of the food chain. Thus continuous monitoring of CO₂
395 concentration in silos and the determination of DMLs as a possible indicator of the
396 level of contamination of stored grain with mycotoxins could be beneficial. This could
397 minimise the chances of grain lots exceeding the legal limits for the toxins of
398 concern. DMLs had previously been considered only as an indicator of quality
399 deterioration of grain (White et al., 1982a, b; Lacey et al., 1994) while methods
400 suggested for the estimation of toxin contamination in stored grains involved colony
401 counts of mycotoxigenic fungi or ergosterol measurements (Lund and Frisvad, 2003;
402 Lindblad et al., 2004;Yong and Cousin, 2001). Previous studies in wheat which
403 examined the effect of *Penicillium verrucosum* colonisation, ochratoxin A (OTA)
404 contamination and environmental conditions suggested that for this species the
405 presence of much lower populations (Log 3 CFUs g⁻¹) would result in OTA
406 contamination above the EU legislative limits (Lund and Frisvad, 2003; Lindblad et
407 al., 2004). The serial dilution method used is more appropriate for highly sporulating
408 fungi such as *Penicillium* and *Aspergillus* species while this technique is less
409 appropriate for *Fusarium* species which may be underestimated (Thrane, 1996).
410 Despite this, *F. verticillioides* populations isolated from the maize samples in this
411 study after 10 days storage under conducive conditions were higher than log 7 CFUs
412 per gram grain in all the samples where fumonisin levels were above the EU limits
413 (data not shown). We also observed a significant correlation between the CFU
414 populations of *F. verticillioides* and logDML (R=0.69, p<0.001), while the correlation
415 between CFUs and the sum of FUMs (R=0.45) was significant at p<0.05 level. This
416 suggests that where microconidia are produced, perhaps CFUs could be used as a

contamination risk index in stored maize for long term monitoring of a stored batch. However, CO₂ monitoring as a non-invasive technique can be observed in real time which may be more appropriate than traditional mycological methods.

Ecological studies often focus on the determination of single toxins produced by the fungal species of concern, usually the ones controlled by legislation, while several metabolites are usually produced on naturally contaminated grain. Consequently very little is known about the effect of storage conditions on “secondary” toxins or the full range of metabolites that may be produced by single species. In this study we determined fumonisin B₁ and B₂ production in maize and observed that at intermediate a_w levels the optimum production shifted at lower temperatures. This has also been observed previously with *F. verticillioides* and *F. proliferatum* (Marín et al., 1999). In our study fumonisins B₃ and B₄ were also detected in all the maize samples. While fumonisin B₄ was present in smaller amounts, fumonisin B₃ was present at levels similar to that of fumonisin B₂ and occasionally even higher – especially at 30°C and 0.955 and 0.97 a_w (data not shown). This may be of particular importance under temperature and water stress shifts which are important in predicting the impacts of climate change scenarios on mycotoxins in staple food commodities. Thus shifts to fumonisin B₃ instead of B₁ and B₂ which is the focus of control strategies at present could become important.

In conclusion, this study has shown that CO₂ data under different interacting environmental conditions can be used to quantify the DMLs which can be caused by mycotoxigenic fungi in both wheat and maize. It is also possible to relate the DMLs to the EU legislative limits for DON and FUMs in wheat and maize respectively. This suggests that for these toxins the relative threshold tolerances for minimising the chances of exceeding the EU legislative limits are small. We have also shown that it

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may be possible to use CO₂ production in stored cereals to improve management of grain post-harvest as part of a food chain prevention strategy.

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Table 1.Values of coefficients b_0 - b_5 and statistical significance of the relevant factors in the model equation for DMLs and for toxin production as determined by forward stepwise regression. Highly significant p-values are shown in bold.

	DMLs wheat		logDMLs maize		DON		ZEA (up to 25°C)		logFUMs	
	b	p-value	b	p-value	b	p-value	b	p-value	b	p-value
Intercept (b_0)	768.54	0.000	-6.8	0.000	16964772.53	0.000	2056528.45	0.000	-2.67	0.042
a_w (b_1)	-1610.15	0.000	-	-	35575748.93	0.000	-4233344.85	0.000	-	-
T (b_2)	-3.94	0.000	-	-	66142.57	0.000	-12371.46	0.000	-0.61	0.000
a_w^2 (b_3)	839.39	0.000	4.87	0.000	18579066.67	0.000	2164948.37	0.000	-	-
T ² (b_4)	-	-	-0.003	0.000	-	-	-	-	-0.01	0.000
T * a_w (b_5)	4.42	0.000	0.2	0.000	73379.66	0.000	13692.58	0.000	1.25	0.000

Figure legends

Figure 1: Effect of a_w and temperature on the cumulative CO₂ production during wheat colonisation by *F. graminearum* over time. Bars indicate the standard errors in the determination of total CO₂ production under each combination of storage conditions.

Figure 2: Effect of *F. graminearum* and *F. verticillioides* colonisation of wheat and maize respectively expressed as mean DMLs (%) after 10 days of storage under different a_w and temperature conditions (wheat: SE_{max} = 0.33, maize: SE_{max} = 1.38).

Figure 3: Production of different mycotoxins (in µg kg⁻¹) by *F. graminearum* in wheat (DON: SE_{max}=0.59(E+04) and ZEA: SE_{max}=0.82(E+03)) and *F. verticillioides* (Sum of fumonisins: SE_{max}=2.33(E+05)) in maize with respect to the storage conditions.

Figure 4: Contour maps describing the DMLs (a) and DON accumulation (b) caused in irradiated wheat during the growth of *F. graminearum* and the logDMLs (c) and log Fumonisin production (d) in maize during the growth of *F. verticillioides* on irradiated maize under different combinations of environmental conditions.

Figure 5: Scatter plot of the relationship between DMLs and DON production during colonisation of stored wheat by *F. graminearum*. The thick black line indicates the EU legislative limits for DON in wheat for human consumption.

Figure 6: Scatter plot of the relationship between DMLs and Sum of fumonisins production during colonisation of stored maize by *F. verticillioides*. The thick black line indicates the EU legislative limits for the Sum of fumonisins in feed maize.

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Figure 7: Scatter plot of the relationship between DMLs and ZEA production between 15-25°C, during colonisation of stored wheat by *F. graminearum*. The thick black line indicates the EU legislative limits for ZEA in wheat for human consumption.

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Figure 1: Mylona et al.

a) wheat

b) maize

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