CRANFIELD UNIVERSITY

KALLIOPI MYLONA

FUSARIUM SPECIES IN GRAINS: DRY MATTER LOSSES, MYCOTOXIN CONTAMINATION AND CONTROL STRATEGIES USING OZONE AND CHEMICAL COMPOUNDS

CRANFIELD HEALTH

PhD THESIS
Academic Year: 2011 - 2012

Supervisor: PROFESSOR NARESH MAGAN
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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

This Project identified the relationships between storage conditions, dry matter losses (DMLs) caused by *Fusarium* species in cereal grains and mycotoxin contamination and assessed novel control strategies for post-harvest grain management including chemical control and ozone.

F. graminearum, *F. verticillioides* and *F. langsethiae* were inoculated on wheat, maize and oats and stored under environmental conditions where marginal to optimum spoilage and mycotoxin contamination can occur. DMLs were calculated from the CO_2 produced and were significantly correlated with deoxynivalenol (DON), zearalenone (ZEA), fumonisins (FUMs) and T-2 and HT-2 toxins respectively. Mycotoxin levels in wheat and maize exceeded the EU legislative limits with 0.9-1% DMLs. Therefore, CO_2 monitoring during storage can indicate the level of contamination in a stored batch.

Using CO_2 production data at different water activity (a_w) and temperature conditions, the environmental regimes at which *F. langsethiae* can grow and contaminate oats with T-2 and HT-2 toxins were identified for the first time.

Five acids were examined *in vitro* and little effect was observed on *Fusarium* growth, in the aqueous form, while the effect on mycotoxin production varied. Dissolved in ethanol, adipic, fumaric and ferulic acids inhibited fungal growth and controlled DON and FUMs, but T-2 toxin was stimulated by the ethanol.

Two garlic essential oils, propyl-propylthiosulfinate (PTS) and propyl propylthiosulfonate (PTSO) were studied for the first time. *In vitro*, 200 ppm reduced fungal growth (50-100%) and mycotoxin production by >90%. The efficacy was species-dependent. In naturally contaminated oats of 0.93 a_w stored for 20 days, 16 ppm PTSO reduced T-2 and HT-2 toxins by 66% and ochratoxin A (OTA) by 88%, while 200 ppm PTS reduced OTA by 95%. In wheat, 100 ppm PTS reduced DON and ZEA and 300 ppm PTS reduced fumonisins by 40-80%. PTSO:PTS (1:1) at 400 and 600 ppm was very effective against DON and ZEA in wheat of 0.92 a_w.

Ozone (O_3) exposure at 200 ppm for 30 min delayed *Fusarium* spore germination on media of 0.98 a_w and inhibited germination at 0.94 a_w. O_3 was more effective against fungal spores than mycelium and little effect was observed on growing cultures. *In vitro,* mycotoxin production after exposure depended on the stage of life of the fungi. O_3 reduced fungal populations in grains. Mycotoxin production in wet grains treated with 100-200 ppm O_3 for 60 min and stored for up to 30 days was reduced or completely inhibited, depending on the species and the exposure system. Simultaneous drying of the grain due to the O_3 passage was observed.

Keywords:

Wheat, maize, oats, cereals, *F. graminearum*, *F. verticillioides*, *F. langsethiae*, post-harvest, storage, adipic, tartaric, fumaric, trans-cinnamic, ferulic, PTSO, PTS, thiosulfinates, thiosulfonates, garlic, essential oils, fumigation, food, feed, atmosphere, headspace, deoxynivalenol, zearalenone, nivalenol, fumonisins, T-2, HT-2, trichothecenes, preservation, silo, quality, safety, legislative limits

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LIST OF ABBREVIATIONS

AcN	Acetonitrile
ANOVA	Analysis of Variance
Apx	Appendix
a_w	water activity
bw	body weight
CFU	colony forming unit
DNA	deoxyribonucleic acid
DON	deoxynivalenol
DML	dry matter loss
EC	European Commission
ED_{XX}	Effective Dose
EU	European Union
e.g.	*exempli gratia*
ELEM	equine leukoencephalomalacia
ELISA	enzyme-linked immunosorbent assay
ERH	equilibrium relative humidity
et al.	*et alii*
FAO	Food and Agricultural Organisation
FAOSTAT	Food and Agricultural Organisation Statistics database
FB_x	Fumonisin B_x
FDA	Food and Drug Administration
FP7	7^{th} Framework Programme
FUMs	Fumonisins
g	Grams
GC	Gas Chromatography
GMP	Good Manufacturing Practice
GRAS	generally recognised as safe
h	Hour
HACCP	Hazard Analysis Critical Control Point
HPLC	High Pressure Liquid Chromatography
H_2O	water
IARC	International Agency for Research on Cancer
i.e.	*id est*
JECFA	Joint Expert Committee on Food Additives
kg	kilogram
kGy	kiloGrey
l/L	litre
LOD	limit of detection
LOQ	limit of quantification
log	logarithm

M	Molarity
m	metre
m^2	square metre
m^3	cubic metre
MEA	Malt Extract Agar
MeOH	methanol
mg	milligram
MIC	minimum inhibitory concentration
min	minute
ml	millilitre
MS	Mass Spectroscopy
MT	Metric Tonnes
ng	nanogram
NIV	nivalenol
No	number
OAc	OAcetyl
OTA	ochratoxin A
p / p_0	vapour pressure / vapour pressure of pure water
PDA	Potato Dextrose Agar
Plc	public limited company
PPE	porcine pulmonary edema
ppm	parts per million
psi	pounds per square inch
RASFF	Rapid Alert System for Food and Feed
RNA	ribonucleic acid
SCF	Scientific Committee for Food
TDI	Tolerable Daily Intake
UK	United Kingdom
UCSC	Università Cattolica del Sacro Cuore
US / USA	United States / United States of America
USDA	United States Department of Agriculture
UV	Ultraviolet
YERBA	yeast extract-rose bengal agar
ZEA	zearalenone
µg	microgram

1 LITERATURE REVIEW

1.1 General Introduction

The Earth's population is constantly rising and so does the demand for food. Healthy and nutritious food is a necessity for humans but also animals, since they constitute a major source of food for humans. Grains such as wheat, maize, rice, barley and oats have a very high nutritive value and they are produced and consumed in hundreds of millions of tonnes annually as food and feed (FAO, 1988). From the United Nations Food and Agricultural Organisation Statistics database FAOSTAT which provides data on the food and agriculture of 200 countries (FAO, 2009), in 2007, considering only the 20 highest producing countries, the world's wheat production was around 526 million metric tonnes (MT) while the maize production was around 682 million MT. However, grains are produced at certain periods in the year while the demand remains the same throughout the year and therefore production is usually followed by storage for a few days, months or even longer times.

Grains are usually dried before storage; however, drying may some times be inefficient due to limited resources, wet weather at harvest or due to poor post-harvest management practices. In combination with mild temperature, conditions may become favourable for the colonisation of stored grain by microorganisms such as bacteria, yeasts and filamentous fungi. This can result in visible spoilage, changes in the physicochemical and organoleptic properties of the crops and losses in dry matter and nutritional quality. Sometimes this can also result in contamination with mycotoxins, substances of varying toxicity to humans and animals, that can cause acute or chronic illness and in some cases death. It is estimated that annually worldwide at least 25% of crops are contaminated with mycotoxins, while a FAO estimate was for 50% (FAO, 1991). Even though growth of mycotoxigenic fungi and toxin production in stored commodities are only favoured at certain conducive environmental conditions, due to the international trade this becomes of global concern.

The associated economic losses are also huge, since these products cannot be traded or consumed. Estimates in the United States of America (USA) vary and refer to losses of between $0.5-5 billion per year; however, the actual worldwide losses cannot be accurately estimated. According to Dohlman (2003) the economic losses from mycotoxin contamination of wheat, maize and peanuts alone represented almost $1 billion; however, this did not include the cost of quality control measures and sample analysis or health-related costs.

In order to protect public health and the consumer, many countries have enforced legislative limits for the amounts of toxins that may be present in different products, as well as methods for sampling and analysis of these toxins (European Commission (EC), 2006a; 2006b, 2010). This legislation has been harmonised in the different EU Member States in order to prevent restriction of trade. However, different limits for certain mycotoxins are established in other parts of the world, while still certain countries have not yet established such legislation (FAO, 2004).

In Europe the Rapid Alert System for Food and Feed (RASFF) was put in place by the European Commission (EC) in order to allow Member States to notify one another in the case of an identified health threat, either through border rejections, market official control inspections or other means. Between the years 2005 and 2010, 650-1000 notifications for mycotoxins in food and feed were received each year by RASFF, the vast majority of which concerned aflatoxins. Notifications concerning fusarium toxins have usually been <20 per year, mostly for fumonisins, but also deoxynivalenol and zearalenone in cereals, cereal products or feed (EC, 2005-2010). Figure 1.1 shows the relevant proportions of the mycotoxins of interest in this Project in cereals and feed over the total mycotoxin notifications received by RASFF in the period 2005-2010. The data presented in this graph are the average of the values contained in the RASFF reports of that period (EC, 2005-2010).

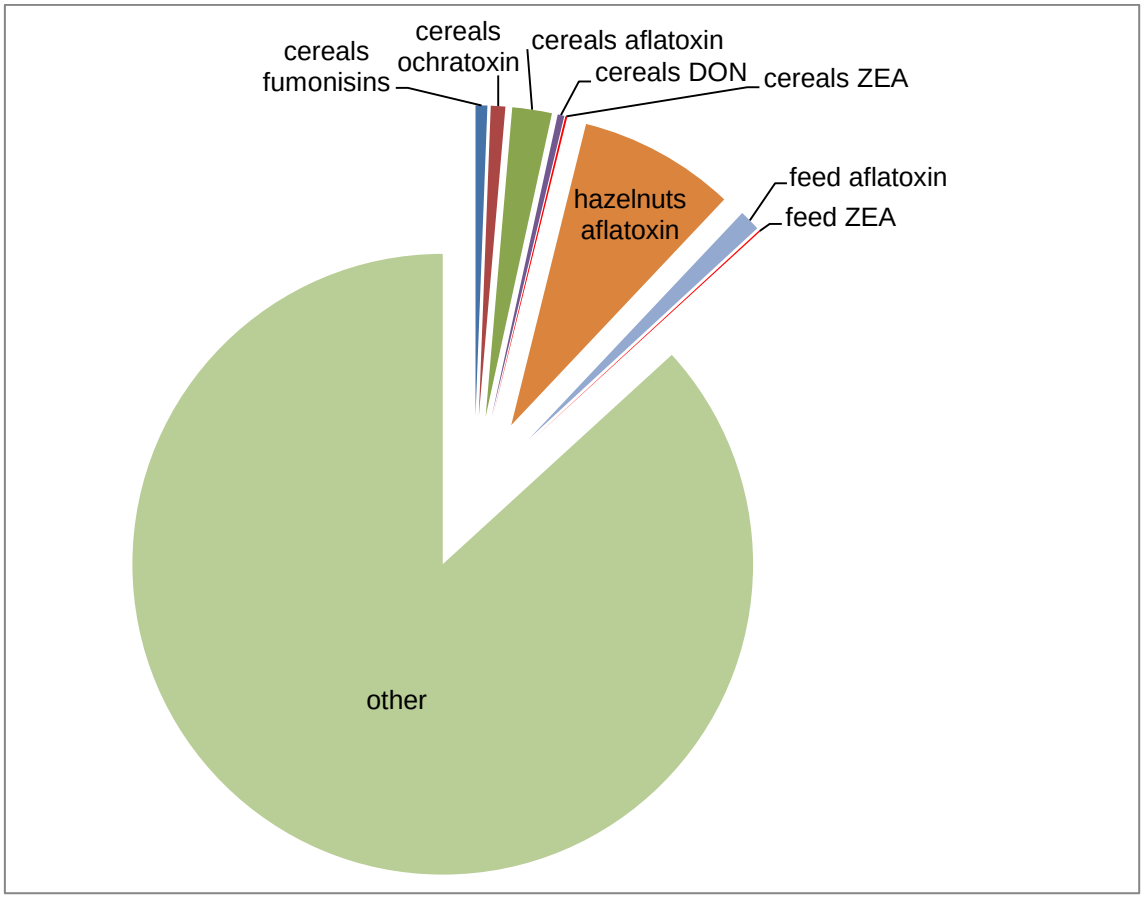


Figure 1.1: Average relevant proportion of the mycotoxins of interest in this Project in cereals and feed in relation to the average total mycotoxin notifications received by RASFF in the period 2005-2010.

Prevention seems to be the best way to avoid contamination of cereal grains with mycotoxins. However this is not always easy, since contamination may start pre-harvest in the field, but may also be introduced post-harvest due to poor management of the food chain. Therefore, many techniques have been developed for both pre-harvest and post-harvest control of mycotoxins in food and feed. These can generally be divided into physical, chemical and biological or combinations of those techniques. In any case the final aim is to ensure the safety of the final product for the consumer.

The aim of this project was to identify the conditions under which certain mycotoxins may be formed in wheat, maize and oats during storage and

examine the possibility of predicting when stored grain may be contaminated beyond the relevant European legislative limits. Subsequently, novel techniques were assessed for efficacy in the post-harvest prevention and control of these toxins in the food and feed chain, so as to ensure safe food for the consumers.

1.2 Post-harvest storage of grains

In many parts of the world grain is harvested when still slightly moist and it is subsequently dried either in the field or at the post-harvest stage using ambient or heated air, before being transported or stored for a few weeks, months or even longer until further processing and use.

After harvest, grain is still alive and respiring and it is a very rich source of nutrients. Harvested grain is often accompanied by extraneous matter such as soil, organic matter, water, but also by microorganisms such as bacteria, yeasts and filamentous fungi and also insect pests. Extraneous and metabolic water combined with heat can facilitate the growth of spoilage fungi which may lead to mycotoxin contamination of grain during storage.

Sinha (1973) was the first to consider grain stored in bags or in bulk as a simply structured man-made ecosystem and include all the above interactions using a holistic approach. This provided a very useful tool for the effective post-harvest management of stored grain. Sinha (1995) also adopted the definition of Odum (1989) for the term ecosystem for general use: *"the biotic community and its nonliving environment treated together as a functional system of complimentary relationships including transfer and circulation of energy and material"*, as well as his grouping of the worlds' ecosystems and he classified the stored-grain ecosystem as a "human-subsidised, solar-powered ecosystem".

Depending on the storage conditions, many interactions take place in stored grain. These cause changes in terms of quality and nutritional value, losses in dry matter, organoleptic properties (flavour, colour, odour) and germination and they can also result in the contamination of the stored grain with mycotoxins.

Tipples (1995) described the stored grain ecosystem as a complex ecological system and identified four categories of factors that account for changes in stored grain: the condition of the grain, the environmental conditions, the invasive forces and the grain treatment. He also provided a schematic diagram (Figure 1.2) which describes in more detail those four factors.

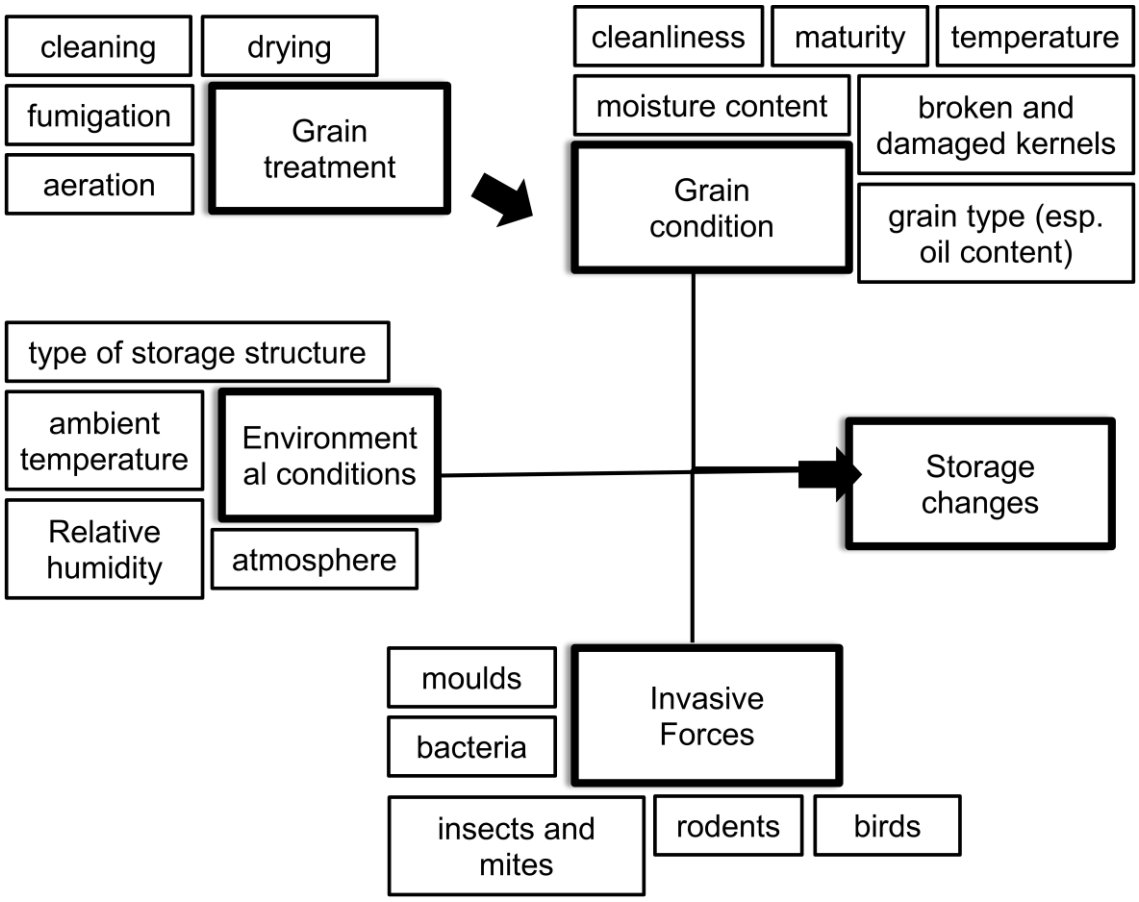


Figure 1.2: The total storage effect: a complex ecology (adopted from Tipples, 1995).

A similar diagram of the factors affecting the various interactions in a stored grain ecosystem was also provided by Sinha (1995). More recently, Magan et al. (2004) considered the interactions between intrinsic (grain characteristics, i.e. water activity, nature, nutrient composition), implicit (micro-ecosystem, spore load, insects/mites), extrinsic (environmental conditions) and processing

factors (agronomic practices, drying, mechanical damage, preservatives, atmospheres) through the different stages of the food chain (pre-harvest, at harvest and post-harvest) and suggested another model for the fungal colonisation of stored grains and the likely mycotoxin contamination.

All the factors previously described are very important parameters that should be thoroughly considered when trying to design an effective management scheme for the safe post-harvest storage of such crops.

1.2.1 Factors affecting storage

(a) Water content / drying efficiency

Water can be bound to a substrate by different forces and therefore the following three states of water can be identified: the water of constitution, which is part of the substrate and is firmly bound to it and cannot be removed without disrupting the molecule, the absorbed water which is linked to the substrate by physical forces and therefore is less firmly bound than the water of constitution and finally the “free” water which is more loosely linked to the substrate and thus more available for different kinds of interactions (Pixton, 1967). Magan et al. (2004) suggested that this more freely available water is the fraction which is used by spoilage microorganisms when they colonise a substrate, rather than the total water content of the substrate.

Water activity (a_w) is one way of expressing the water availability. This is defined as the ratio of the vapour pressure (p) of water in the substrate to that of pure water (p_0) at the same temperature (Scott 1957) and is also related to the equilibrium relative humidity (ERH) by the following relationship:

$$a_w = \frac{p}{p_0} = \frac{(ERH)(\%)}{100} \qquad \textbf{Equation 1-1}$$

The water activity is affected by the temperature. The relationship between a_w and water content for a specific substrate can be expressed by the moisture sorption isotherms under a constant temperature. Depending on whether water is added to or removed from the substrate, a hysteresis effect is observed in the moisture sorption isotherms.

Depending on the water content of grain during storage, different microorganisms are able to grow. Thus, fungi can be classified into the following categories according to the relative humidity range over which they can grow (Moreau, 1979):

- Xerophilic fungi can grow at relative humidity as low as 80% although their optimum is around 95% relative humidity
- Mesophilic fungi can grow at relative humidities in the range 80-90% while their optimum lies in the range 95-100% relative humidity
- Hydrophilic fungi can grow at relative humidity above 90% while their optimum is close to 100%.

Drying of grain prior to storage is the main measure employed over the centuries in order to reduce the water content of grain and thus restrict the growth of microorganisms causing spoilage. Several different drying techniques have been developed and can be generally categorised as natural or artificial. Natural drying employs environmental factors such as wind and temperature for the drying of grain; in many areas of the world where environmental conditions allow, grain is sun-dried in the field after harvest to the required moisture content. Alternatively grain can be stored in ventilated storage areas where it can be dried by warm air. However, in several parts of the world natural drying cannot be achieved due to high moisture air or reduced sunshine and in these cases artificial drying is essential. Hundreds of different techniques have been developed for artificial drying and in most cases an air flow of elevated temperature is required which is produced by heating.

However, water may still be produced during storage as a result of the metabolic activity of the grain and of any accompanying organisms, but also due to accidental water leakage. Therefore, drying of grain before storage

should be efficient enough, decreasing the water content below the levels known to allow microbial growth, but also allowing for a safety threshold in case water is reintroduced into the system during storage.

(b) Temperature

Temperature affects the growth of filamentous fungi on different substrates and thus plays a very important role during grain storage. Fungi can be categorised depending on the temperature range at which they can grow, however in general terms the majority of them can grow in the range 10-40°C with an optimum at 25-35°C (Magan et al., 2004). Storage at low temperature is well known to restrict the growth of many microorganisms including fungi; however, due to the high cost of this technique its application is very limited.

During storage the temperature might rise, not only due to the environmental conditions, but also from within the grain, due to the metabolic activity of the microorganisms present. Depending on the storage conditions different fungi may grow and produce water and energy. When this energy cannot escape the system, the temperature may rise followed by the growth of a succession of mesophilic, thermotolerant and thermophilic microorganisms resulting in even higher temperatures. This has been described as “spontaneous heating” or development of “hot spots” and temperatures as high as 75°C have been reported leading to complete loss of the stored grain (Lacey, 1980; Sauer, 1992; Magan et al., 2010).

(c) Substrate

The nutritional characteristics of the substrate are very important since they determine the moisture content/water activity relationship. Carbohydrate rich grains (i.e. wheat: 71% carbohydrate, maize: 74%, oats: 66% (USDA, 2009)) are less hygroscopic than lipid rich seeds (i.e. hazelnuts: 60.75% lipid content) which can absorb water from the atmosphere, rendering them more susceptible to invasion by fungi or affecting their storage properties.

The crop cultivar may also be considered, since different varieties have been reported to have different resistance against fungal invasion. Most grains are structurally similar. They comprise:

- the endosperm which is the major part of the grain, it is rich in starch and provides the food for the embryo (germ),
- the aleurone layer which is the outer layer of the endosperm,
- the pericarp which surrounds the aleurone and together they comprise the bran and
- the husk which is the outer layer of the grain. Oats differ from the rest of the cereals of interest in this project in that they are accompanied by the husk at harvest while other cereals are only pre-harvest (Hoseney and Faubion, 1992).

The composition of each of the parts differs between different grain types and this determines the differences in their characteristics and properties.

Many studies have also noted the importance of the condition of the grain and the degree of mechanical damage of the grain kernels on the susceptibility to fungal colonisation (Tuite et al., 1985; Ng et al., 1998). Tuite et al. (1985) concluded that the closer the damage is to the maize germ (embryo), the more susceptible the seed is to mould deterioration. However, they suggested that this is still subject to genotypic effects with certain more resistant genotypes maintaining their resistance even when damaged. Ng et al. (1998) tried to relate the dry matter losses calculated by measuring CO_2 production during storage of corn to the percentage of mechanical damage. They observed that as the percentage of mechanically damaged kernels increased up to 40%, the allowable storage time for corn decreased, while it remained relatively stable between 40 and 50% damaged kernels. They also suggested that the permissible dry matter loss (DML) for corn with 25-35% mechanically damaged kernels is 0.35% before corn is downgraded from Grade 2 (according to the United States corn grading system) which is even lower than 0.5% DML that had previously been suggested by Saul and Steele (1966).

(d) Fungal contamination of the grain

Grain at harvest is contaminated with a wide tange of bacteria, yeasts and filamentous fungi. Depending on the storage conditions, different microorganisms may grow due to their different ecological requirements and depending on the presence of other competing species. Under favourable conditions fungal spores start to germinate and fungal colonisation of stored grain proceeds rapidly causing spoilage and quality deterioration. Colonisation of grain by some fungi can be visible within a few days however other species colonise with microscopic mycelium strands, making it more difficult to identify infection at an early stage.

(e) Presence of insects / pests

Grain at harvest may also be contaminated with insects and their larvae, which can develop under bad storage conditions initiating spoilage. Storage silos should be free of pests however poor post-harvest hygiene management can result in their introduction also facilitating the initiation of spoilage. Insects and pests can modify the intergranular storage atmosphere, they can increase the grain moisture content by the products of their metabolism, they can cause damage to grain kernels rendering them more susceptible to fungal invasion and finally they carry spores on their bodies which they can spread throughout the grain bulk.

(f) Composition of the storage atmosphere

The storage atmosphere also plays an important role during grain storage. When storage is air-tight, CO_2 accumulates while O_2 concentration decreases due to the respiration of both the grain and its accompanying microorganisms. This has been considered as a benefit and was initially employed as a method for controlling insect infestation and later as a control measure against fungal spoilage of grain for use as animal feed. However, Magan and Aldred (2007) suggested that many fungi are micro-aerophilic rather than obligate aerobes and can therefore survive very low O_2 concentrations, even lower than 0.14%

and cause spoilage and even mycotoxin production in stored grain (Magan et al., 2010).

Often it is necessary to aerate grain in order to prevent temperature rise due to metabolic activity or environmental conditions. In this case aerobic conditions are maintained and CO_2 cannot accumulate in the system. Grain aeration is a very extensive subject and it is not within the scope of this thesis; here it is only referred as a technique for the adjustment of the composition of the storage atmosphere.

Several approaches are available on the use of modified atmosphere storage or fumigation of stored grain for the prevention of fungal colonisation and toxin production and these will be reviewed in Section 1.5 on Control Strategies. The effectiveness of modified atmosphere storage depends upon many factors, including the concentration used, the temperature and moisture content of the grain, the time and the length of application as well as the microorganisms against which it is used and the stage of their life-cycle. Certain controlled atmospheres and their effectiveness against fungal colonisation and mycotoxin contamination of stored grains are also reviewed in Section 1.5.

(g) Presence of preservatives / antifungal agents

The application of different chemical substances to grain at the time of storage has long been used for the inhibition of fungal growth and toxin production, especially on grain for use as animal feed. This is another factor that needs to be considered in the stored grain ecosystem. Some of the substances previously used have now proven ineffective or leave chemical residues in the product which are not acceptable under current legislation requirements. In combination with the consumer demand for chemical-free products, new and known substances are now screened for antifungal properties and evaluated for use as natural preservatives for stored products. These may be phytochemicals or antioxidants that naturally occur in plants or even plant extracts and essential oils and these will be further reviewed in Section 1.5.3 on Chemical Control Strategies.

(h) Storage time

Storage time has also been discussed by many authors as a factor that should be considered during grain storage. Under well-managed storage conditions grain may remain unaffected for long periods (6-12 months). Some studies have even suggested that storage of grain under such conditions may improve the quality of the grain due to some ageing (Tipples, 1995). However, when storage conditions become favourable for the growth of spoilage fungi, it can be presumed that longer storage times will allow for further proliferation of the spoilage microorganisms, resulting in more significant losses of grain quality. In this study only short-term storage studies have been considered (up to 30 days).

1.2.2 Respiration / Dry matter losses during grain storage

(a) Respiration

Grain at harvest is contaminated with a range of microorganisms including bacteria, yeasts, filamentous fungi and insects or pests, which altogether form an ecosystem that is alive and unless controlled, continues to respire while in storage.

Respiration has previously been used to measure metabolic activity in stored grain (Baily and Gurjar, 1918; Milner et al., 1947a, b; White et al., 1982a, b). Respiration can be approximately considered as the aerobic oxidation of carbohydrates, represented by the following equation (Saul and Lind, 1958):

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 677.2\,cal\ (or\ 2835\,kJ)$$ **Equation 1-2**

Respiration in stored grain includes the respiration of the grain as well as that of the accompanying microorganisms. The relevant contribution of the microorganisms and the grain to the total respiration has been debated for many years and some studies have been conducted to address this issue. Seitz et al. (1982a) used surface-sterilised maize and showed that the grain

respiration was the major contributor to the dry matter loss since even though surface-sterilised, the maize showed 0.5% dry matter loss without significant fungal growth during storage. However, in a subsequent study (Seitz et al., 1982b) they suggested that respiration of both the grain and fungi contributes to dry matter loss. The contribution of the fungi is usually small at the beginning of storage, but increases as fungi grow, paralleled by an increase in dry matter loss depending on moisture content, temperature, amount of kernel damage and amount and type of fungal inoculum on the grain (Seitz et al., 1982b). Willcock and Magan (2001) used wheat straw (a senescent material) to directly quantify the respiratory activity of the microorganisms present. Fleurat-Lessard (2002) reviewed several studies to conclude that the major contributor in CO_2 production and dry matter loss in cereals is indeed fungal activity.

Respiration rates are influenced by all the factors that affect grain storage (Lacey et al., 1997; Willcock and Magan, 2001). Respiration, as described by Equation 1-2, can be determined by the amount of CO_2 produced by the grain or by the amount of O_2 consumed. Some methods used to measure respiration are destructive, thus not allowing continuous monitoring. Seitz et al. (1982a, b) determined the respiration rate of high moisture corn as mg CO_2 per kilogram of dry matter per hour by the following formula:

$$R = \frac{(C - A) \cdot F \cdot D}{M} \qquad \textbf{Equation 1-3}$$

where

C: is the concentration (by volume) of CO_2 in the effluent air (i.e. ml CO_2/ml air),

A: is the concentration of CO_2 in the ambient air (by volume),

F: is the airflow in ml/hour,

D: is the density of CO_2 (mg/ml) which was considered as 1.75 for the conditions of the experiment and

M: is the mass of dry matter at the beginning of the storage.

Pomeranz (1992) used sealed units to measure respiration however O_2 was not reintroduced in the system while CO_2 accumulated, which might have inhibited the growth of microorganisms. Hamer et al. (1991) used an automatic electrolytic respirometer to study the respiration of cereals by continuous monitoring of oxygen uptake and measurement of the total CO_2 produced. CO_2 did not accumulate in this system and therefore microbial respiration was not inhibited, more samples could be analysed simultaneously and some of the errors of earlier systems could be avoided.

(b) Dry matter loss (DML)

From the equation of the aerobic oxidation of carbohydrates (Equation 1-2), 14.7 g of CO_2 per kilogram of grain are equivalent to 1% DML (Steele et al., 1969). The amount of DML that is allowable before grain is deemed unfit for use in food or downgraded for use as feed varies between grains.

From the studies of Seitz et al. (1982a; b), curves of the %DML against time were determined by integrating the respiration rate data against time. The storage time was divided into intervals and the amount of CO_2 during each interval was calculated by the respiration rate at the end of the interval. The sum of the CO_2 amounts of those intervals was the total CO_2 produced during the storage period and from that the DMLs were determined.

Willcock and Magan (2001) showed that DML caused by single species on sterile straw is different than that of mixed populations on naturally contaminated straw. The percentage DML caused in the first case is much higher than that caused by mixed populations, showing that there may be antagonistic interactions between different species that affect respiration in naturally contaminated grain.

(c) Respiration / Dry matter losses in the management of grain storage

From Equation 1-2 the main products of respiration are CO_2, water and heat. These are very important parameters in the sense that they are responsible for a chain of other reactions and activities that overall affect the stability of the stored grain ecosystem and are the ones that have to be monitored closely. At

the same time these are the parameters that have to be controlled for prolonged safe storage of grains.

Studies with maize showed that a loss of 0.5% in dry matter was unacceptable, even without visible moulding (Seitz et al., 1982a). It was therefore suggested that the visual examination sometimes may not be adequate in order to establish the quality of grain. This has also been discussed by other studies (Hamer et al., 1991; Lacey et al., 1997). Magan (1993) suggested that microscopic growth rather than visible moulding should be used as a more effective indication of contamination.

With regard to wheat, allowable DMLs have been shown to vary between 0.1 and 2% in different studies (Kreyger, 1972; White et al., 1982a). White et al. (1982a) using as a criterion 0.1% DML, suggested that wheat with 18.4% water content could be safely stored for 55 days, however after just 23 days visible mould appeared suggesting that the allowable DML is only 0.04%.

Considering Bailey's suggestion (Bailey, 1940) that the respiration rate of grain is proportional to the kernel size, Brook (1987) suggested that DML of 0.5% in maize is equivalent to 0.085% DML in wheat, results that were also supported by the studies of Lacey et al. (1994; 1997).

Equations have been developed that relate the permissible storage time before grain has reached certain DML level, depending on temperature, moisture content and mechanical damage of the grain. The following equation can be used to determine the storage time before maize has lost 0.5% dry matter at 15.5°C for maize grain with 25% water content and 30% mechanically damaged kernels:

$$T = T_R \cdot M_T \cdot M_M \cdot M_D \qquad \textbf{Equation 1-4}$$

where T: is the estimated allowable time for 0.5% dry matter loss,

T_R: is the time for 0.5% dry matter loss under the above conditions and

M_T, M_M and M_D: are the multipliers used in the above formula to correct for the actual temperature, water content and mechanical damage (Steele et al., 1969).

Thompson (1972) used Equation 1-4 to determine the relationship between allowable storage time and CO_2 production during storage. The following equation was suggested:

$$y = 1.3\,(exp(0.006t) - 1) + 0.015t \qquad \textbf{Equation 1-5}$$

where y: is the grams CO_2 produced per kilogram dry matter and

t: is the time in hours.

It is also possible to predict the DMLs caused by dividing Equation 1-5 by 14.7.

Fleurat-Lessard (2002) concluded that the rate of CO_2 production can be used as a "storability risk index" as has been suggested by other studies in the past.

1.3 Fungal species / Mycotoxins of interest

Mycotoxins are secondary metabolites produced by filamentous fungi when they colonise various substrates and are toxic to humans and animals. Many different mycotoxins have been identified. Some mycotoxins are produced in the field, others are produced during storage when the conditions are favourable for the growth of the fungus and some can be produced both in the field and during storage.

Mycotoxins are very stable molecules and once grain has been contaminated with mycotoxins its decontamination is very difficult. Indeed, contaminated foodstuffs must not be deliberately detoxified using chemical treatments (EC, 2006a)

Mycotoxins of interest in this project are:

- trichothecenes: deoxynivalenol (DON) and nivalenol (NIV) produced by *Fusarium graminearum* in wheat,
- zearalenone (ZEA) produced by *Fusarium graminearum* in wheat,
- fumonisins produced by *Fusarium verticillioides* in maize,
- T-2 and HT-2 toxins produced by *Fusarium langsethiae* in oats,

- other toxins (ochratoxin A, aflatoxins) produced in the above grains by naturally co-occuring fungal species (*Penicillium, Aspergillus*).

Generally, the above toxins are produced when grain with moisture content above 15% (equivalent to ~0.70 a_w) is stored under temperature conditions favourable for the growth of some of the above fungal species (generally T>20°C).

1.3.1 Mycotoxigenic *Fusarium* species

Species of the genus *Fusarium* are very common plant pathogens of cereal crops. They are of particular importance because they affect key cereal commodities for human and animal nutrition (wheat, maize, barley, oats) reducing their yield, quality and nutritional value. In addition they contaminate these crops with mycotoxins (trichothecenes: Deoxynivalenol, nivalenol, T-2, HT-2; zearalenone; fumonisins).

(a) *Fusarium graminearum*

F. graminearum is a plant pathogen causing *Fusarium* head blight in wheat, which is associated with significant economic losses as well as with the contamination of wheat grain with type B trichothecenes. It also affects barley and maize to a lesser extent. *F. graminearum* infects wheat at the time of anthesis and thus it is generally considered that trichothecene contamination of wheat occurs at the pre-harvest stage (Cleveland et al., 2003; Miller, 2008). However, evidence exists that contamination may also proliferate during harvesting and at the post-harvest stage, due to inefficient drying or under damp post-harvest storage conditions that are more common in certain parts of the world (Wagacha and Muthomi, 2008).

(b) *Fusarium verticillioides*

F. verticillioides is a fungal species most commonly infecting maize and it is responsible for significant losses in quality and nutritional value as well as for the contamination of this crop with fumonisins, toxins associated with significant

health effects in animals and possibly related to oesophageal cancer in humans. Contamination of maize with fumonisins can occur at the field and at the post-harvest stage.

(c) *Fusarium langsethiae*

Fusarium langsethiae was only described as a new species in 2004 as previously it was confused with *Fusarium sporotrichioides* and *Fusarium poae* two closely related species. It was first identified as a producer of T-2 and HT-2 toxins in cereals in Norway however the environmental conditions under which these toxins are optimally produced in grains have not been previously identified.

1.3.2 Trichothecenes

Trichothecenes are tetracyclic sesquiterpenoid compounds with a 12, 13-epoxy group and can be classified in two groups depending on whether they have a carbonyl group at C-8 (group A; e.g. T-2 and HT-2 toxins) or a group other than carbonyl at C-8 (group B; e.g. Deoxynivalenol and nivalenol). Trichothecenes are very stable at 120°C, moderately stable at temperatures as high as 180°C and may decompose at 210°C (JECFA, 2001a).

(a) Group A trichothecenes: T-2 toxin, HT-2 toxin

T-2 toxin is a generally toxic, heamatotoxic and immunotoxic substance and can inhibit protein synthesis and even synthesis of DNA and RNA. There is limited evidence of tumour formation in experimental animals. Usually T-2 toxin and HT-2 toxin occur together and T-2 toxin is metabolised to HT-2 toxin which may produce further metabolites. A combined Tolerable Daily Intake (TDI) of 0.06 µg/kg bw was initially suggested for the sum of T-2 and HT-2 toxins (SCF, 2002; JECFA, 2001a). However an updated scientific opinion published recently by JECFA (2011) established a new group TDI of 100 ng/kg bw for T-2 and HT-2 toxins after a re-evaluation of new data for these toxins during 2005-2010. The new evaluation refers to possible genotoxic and cytotoxic effects and limited

data on tumourigenicity in animals, although no new studies presented evidence for carcinogenicity in animals or humans. The animals more seriously affected by the presence of these toxins are pigs and cats. Again no maximum limits were established for T-2 and HT-2 toxins in Europe. However, a review of maximum limits established in other countries of the world was presented and these limits are most commonly established at ~100 µg/kg (in food grains) in some central and eastern-European countries (JECFA, 2011). Even more countries have established maximum limits for these toxins in feed and these seem to be different for different animal species.

The chemical structure of T-2 and HT-2 toxins is presented in Figure 1.3.

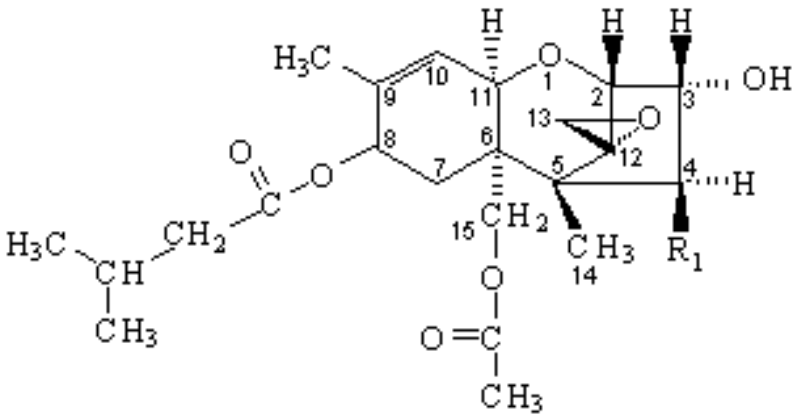


Figure 1.3: Chemical structure of T-2 and HT-2 toxins. R_1 is (OAc) for T-2 toxin and (OH) for HT-2 toxin

(b) Group B trichothecenes: Deoxynivalenol, nivalenol

Deoxynivalenol (DON) is commonly produced by *F. graminearum* and *Fusarium culmorum* which grow in the field and contaminate many different grains such as wheat, maize, oats and barley. DON once formed is very stable in storage and also during grain processing and cooking and does not degrade in high temperatures (SCF, 1999). DON is also known as vomitoxin because an acute dose causes vomiting in pigs. DON is generally toxic and immunotoxic however

there is no evidence of carcinogenicity or mutagenicity. Finally a tolerable daily intake (TDI) of 1 µg/kg bw was established (SCF, 1999).

Nivalenol (NIV) is a toxin produced most commonly by *Fusarium cerealis* and *Fusarium poae*, but also by *F. culmurum* and *F. graminearum*. NIV is evaluated as a generally toxic substance, immunotoxic and heamatotoxic, while information on its genotoxicity is only limited (SCF, 2000a). For NIV a TDI of 0.7 µg/kg bw was established (SCF, 2000a). Figure 1.4 shows the chemical structures of DON and NIV:

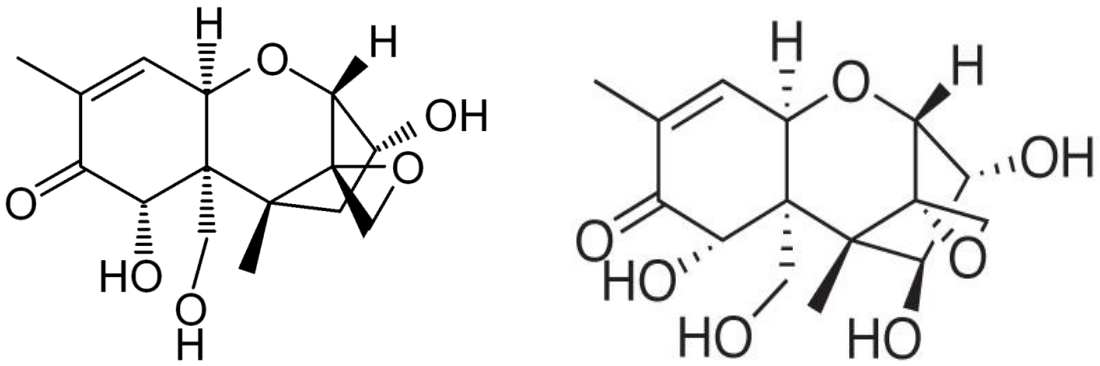


Figure 1.4: Chemical structures of deoxynivalenol (DON) (left) and nivalenol (NIV) (right)

1.3.3 Fumonisins

Fumonisins were first identified in 1988 and they are a group of structurally related compounds FB_1, FB_2, FB_3 and FB_4 however more studies have focused on FB_1 and FB_2 and their toxicity. Fumonisins are produced by the fungal species *Fusarium verticillioides* (*Fusarium moniliforme*) and *Fusarium proliferatum* when they colonise maize. Recent studies have also identified the ability of *Aspergillus niger* to produce fumonisin B_2 (Frisvad et al., 2007).

FB_1 is a water-soluble molecule and therefore its concentration can be reduced during wet milling for the manufacture of corn-starch. Contrarily, FB_1 is stable during dry milling or other processing techniques, while other treatments such as ammoniation or nixtamalisation reduce the FB_1 content but increase the

concentration of hydrolysed fumonisin, the toxicity thus remaining. Figure 1.5 shows the chemical structure of fumonisin B_1.

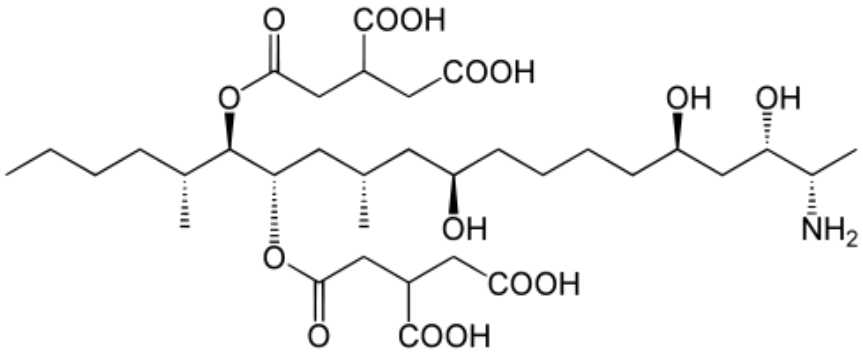


Figure 1.5: Chemical structure of fumonisin B_1

Many studies have suggested that FB_1 and its metabolites are retained in the liver and kidney and in animal studies it has demonstrated increased apoptosis leading to tumour formation. FB_1 has also been associated with porcine pulmonary edema (PPE) in pigs and equine leukoencephalomalacia (ELEM) in horses. Finally increased FB_1 intake has been associated with increased occurrence of oesophageal cancer in certain regions in South Africa and China and possibly in Italy and recently there have been indications that the cardiovascular effects of FB_1 could also induce toxic effects.

The International Agency for Research on Cancer (IARC, 1993; 2002) considering the toxicity of FB_1 concluded that there is inadequate evidence for fumonisin carcinogenicity in humans, but that there is sufficient evidence for the carcinogenicity in experimental animals and therefore it was classified in Group 2B, as possibly carcinogenic to humans. The Scientific Committee on Food expressed a final opinion that there is no adequate evidence on the genotoxicity of FB_1 (SCF, 2000c) and considering the ELEM in horses allocated a TDI of 2 µg/kg bw. This was later expanded as a group TDI to fumonisins B_1, B_2 and B_3 alone or in combination (JECFA, 2001b; SCF, 2003).

1.3.4 Zearalenone

Zearalenone is an estrogenic mycotoxin produced by *Fusarium* species (*F. graminearum*, *Fusarium culmorum*) mainly in maize, wheat, barley and oats. It is a very stable compound during processing and storage and unaffected by high temperatures. During wet milling there is evidence that ZEA remains in the gluten fraction to high amounts.

The mode of toxic action of ZEA and is metabolites is believed to be through binding to estrogenic receptors. Pigs are the animals more sensitive to ZEA, as well as sheep. ZEA has been evaluated by IARC as not carcinogenic however immunotoxic effects and effects on the reproductive system of experimental animals were observed (SCF, 2000b).

Some more recent studies reviewed by Zinedine et al. (2006) have indicated that there might be some relationship between ZEA and breast cancer and that ZEA is genotoxic.

Figure 1.6 shows the chemical structure of zearalenone.

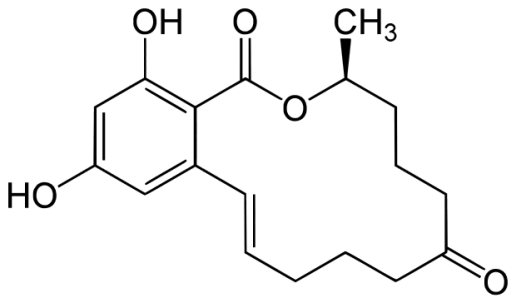


Figure 1.6: Chemical structure of zearalenone

1.3.5 Other toxins of interest

(a) Aflatoxins

Aflatoxins were first identified in 1961. They are generally produced by three species: *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*. They occur in areas of the world where the climate is hot and humid, but due to

the international trade they are of worldwide importance. Four major aflatoxins have been identified, aflatoxins B_1, B_2, G_1 and G_2, named after the colour (blue or green) that they show under ultraviolet (UV) light. Aflatoxin M_1 is a metabolic product of aflatoxin B_1. The chemical structure of aflatoxin B_1 is presented in Figure 1.7.

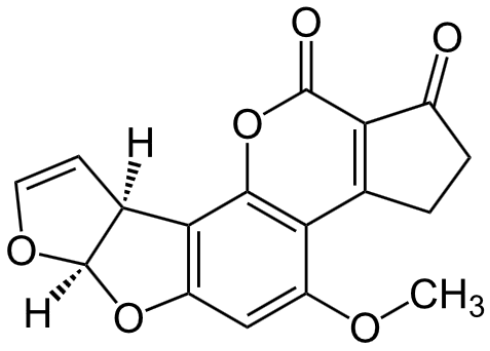


Figure 1.7: Chemical structure of aflatoxin B_1

In 1993 the International Agency for Research on Cancer concluded that aflatoxin B_1 and naturally occurring mixtures of aflatoxins are carcinogenic to humans (IARC, 1993). Also there was sufficient evidence that aflatoxins B_1, G_1 and M_1 and naturally occurring mixtures of aflatoxins are carcinogenic to experimental animals. Finally IARC (2002) suggested that aflatoxin M_1 is possibly carcinogenic to humans; however, there was insufficient evidence for the carcinogenicity of aflatoxins B_2 and G_2.

In 1996 the Scientific Committee for Food of the European Commission after reviewing several evaluations concluded on the carcinogenicity of the different aflatoxins and that aflatoxins, especially aflatoxin B_1, are genotoxic carcinogens producing adducts in humans and animals *in vivo* and chromosomal anomalies in animals (SCF, 1996). Finally, it was concluded that levels of exposure of as low as 1 ng/kg body weight (bw)/day can still contribute to a risk of liver cancer. For aflatoxin M_1 it was suggested that it can occur in the absence of other aflatoxins and that it is a genotoxic carcinogen, its potency being around 10 times lower than the one of aflatoxin B_1.

(b) Ochratoxin A

Ochratoxin A (OTA) is produced by species of *Penicillium* and *Aspergillus* and is found in a range of foodstuffs including cereals. It is categorised as a nephrotoxic substance and it has carcinogenic, teratogenic, immunotoxic, genotoxic and possibly neurotoxic properties (SCF, 1996; 1998). It was initially categorised as “possibly carcinogenic to humans” (IARC, 1993) while it is carcinogenic in animals. OTA is associated with Balkan Endemic Nephropathy and maybe with urinary tract tumours in humans.

It is a very stable compound and only temperatures above 250°C have been reported to have some significant effect on OTA. It has been suggested that exposure to OTA should be below 5 ng/kg bw per day (EFSA, 2006). The chemical structure of OTA is presented in Figure 1.8.

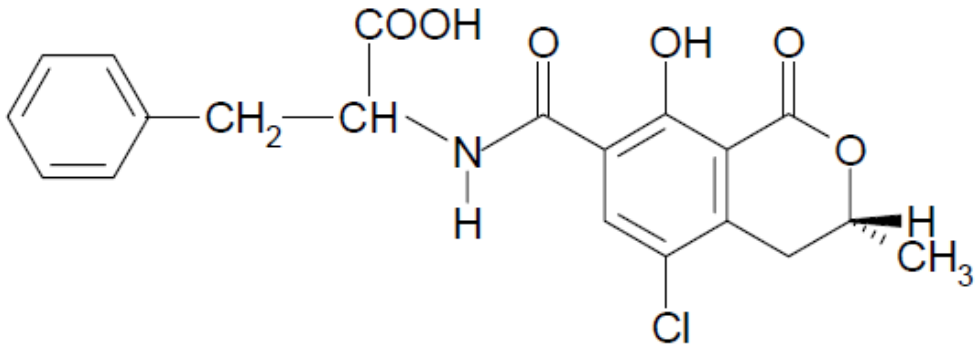


Figure 1.8: Chemical structure of ochratoxin A

1.4 Legislative limits for mycotoxins in grains

One of the objectives of this project is to develop control systems and strategies that will prevent mycotoxin contamination of cereal grains of interest in this project at the post harvest stage, in order to be compliant with the limits laid down by the European legislation. Maximum levels have been established for mycotoxins in food and/or feed by Commission Regulation (EC) No 1881/2006

which is reviewed when new information becomes available, while Regulation (EC) No 401/2006 provides official methods for the analysis of mycotoxins in these products.

Table 1.1 shows the maximum levels established by the European Commission for the mycotoxins of interest in the project in the commodities of interest. Foodstuffs not complying with these levels must not be used as food ingredients and must not be mixed with foodstuffs that comply with these levels (EC, 2006a; 2010). Also foodstuffs that contain mycotoxins must not be detoxified by chemical treatments while foodstuffs treated by physical means or sorting in order to reduce contamination levels must not be mixed with food for direct human consumption or with food ingredients.

When there is concern that certain foodstuffs imported in the European Union from third countries are contaminated, provisions may be enforced specifically for the products of concern. For example, Commission Decision 2006/504/EC lays down special conditions for hazelnuts imported from Turkey due to aflatoxin contamination risks (EC, 2006c).

Table 1.1: Maximum levels for the mycotoxins of interest in the project in the cereals of interest

Deoxynivalenol	(µg/kg)	
Unprocessed durum wheat and oats	1750	
Wheat for direct human consumption	750	
Zearalenone	(µg/kg)	
Unprocessed cereals other than maize	100	
Wheat for direct human consumption	75	
Fumonisins	Sum of B_1 and B_2 (µg/kg)	
Unprocessed maize except of unprocessed maize for wet milling	4000	
Maize for direct human consumption	1000	
Aflatoxins	B_1 (µg/kg)	Sum (B_1, B_2, G_1, G_2) (µg/kg)
Maize and nuts to be subjected to sorting, or other physical treatment, before human consumption or use in foodstuffs	5.0	10.0
Nuts and processed products thereof, intended for direct human consumption or use in foodstuffs	2.0	4.0
Cereals and their products (except of maize)	2.0	4.0
Ochratoxin A	(µg/kg)	
Unprocessed cereals	5.0	
Processed cereal products and cereals for direct human consumption	3.0	

1.5 Control strategies for the prevention of mycotoxin formation during storage

Due to the stability of mycotoxins, their adverse effects on human and animal health and due to the legislation in force, contaminated grains cannot be traded resulting in significant economic losses. Since to date it is not possible to completely eliminate the contamination of grains with mycotoxins (Codex, 2003), it is of great importance to develop strategies aimed at preventing such contamination.

For the control of fungal growth and mycotoxin contamination in stored grains effective pre-harvest as well as post-harvest management is required and these steps were examined under the concept of Hazard Analysis and Critical Control Point (HACCP) by Aldred and Magan (2004).

Pre-harvest management techniques include the use of grain varieties that have proven to be more resistant to fungal invasion, crop rotation, planting, sowing and harvesting at the right times and use of the appropriate fertilisers and insecticides at the appropriate concentrations (Codex, 2003; Magan and Aldred, 2007). The use of genetically engineered hybrids resistant to fungal infection has also been suggested (Munkvold and Desjardins, 1997; Jouany, 2007).

Post-harvest strategies can be divided into those preventing the formation of mycotoxins by inhibiting fungal growth and those involving mycotoxin binders or compounds that can degrade mycotoxins for use in the feed chain. The most important preventive measure for the safe storage of grains is drying to moisture content below 15% (Codex, 2003) or the equivalent to 0.65-0.70 a_w (Aldred and Magan, 2004; Magan and Aldred, 2007). Efficient cleaning, hygiene management of storage areas and effective pest control are also important (Codex, 2003; Magan and Aldred, 2007).

Other techniques aimed at the preventing the mycotoxin contamination of stored grains generally involve physical methods, such as moving of stored grain and aeration or the use of chemical compounds such as preservatives, some of which have even been reported to be beneficial for feed grains (Codex,

2003). Several techniques are based on the manipulation or use of gases. Hermetic storage relies on the accumulation of CO_2 for the control of aerophilic microorganisms, but modified atmosphere storage and fumigation have also been examined. Finally in recent years the use of biological control agents which can out-compete toxigenic strains *in situ* has received much attention. The use of chemical compounds for the preservation of grains and the use of gases were the techniques of prior interest in this project.

1.5.1 Modified atmosphere storage / Fumigation

The use of gases with the scope of modification of the storage atmosphere or for short-term treatment has been widely employed over the years for the control of fungal growth and mycotoxin contamination during post-harvest grain storage. Generally techniques involving gases can be categorised into those where the storage atmosphere is modified (usually by altering the ratios of CO_2 and O_2 or introducing additional gases) or those where a gas is flushed through the stored grain for a certain length of time or repeatedly (fumigation). Due to safety concerns related to the most widely used fumigants and their effect on the environmental (Montreal Protocol) or because of resistance developed by certain species after repeated exposure to such fumigants, the need for alternatives is a high priority at present (Bell, 2000; Tiwari et al., 2010).

(a) CO_2 / O_2 modified atmospheres

CO_2 rich or O_2 poor atmospheres have been the focus of most studies for the post-harvest storage of grains and such studies have been extensively reviewed (Banks, 1981; Jayas and Jeyamkondan, 2002). Overall it is suggested that for the successful long-term storage of wet grains atmospheres of <1% oxygen or >60% CO_2 are required (Banks, 1981).

Cairns-Fuller et al. (2005) suggested that growth of *Penicillium verrucosum* on wheat and OTA production was reduced as CO_2 concentration in the atmosphere increased up to 50% while reducing the water activity also enhanced the inhibitory effect. Paster et al. (1983) found that the mycelial

growth of *Aspergillus ochraceous* Wilhelm inoculated on solid synthetic media was completely inhibited with 80 or 100% CO_2 for 14 days storage. However, subsequent storage in air resulted in growth and toxin production similar to that of the controls, suggesting that the effect of CO_2 at the concentrations applied was not lethal to the fungi but fungistatic. Overall total inhibition of OTA production occurred at >30% CO_2 regardless of the O_2 level and at O_2:CO_2 (1%:20%). Other O_2:CO_2 combinations resulted in both reduced and enhanced levels of OTA. They also suggested that the CO_2 concentration necessary for inhibition of fungal growth and toxin production depends on the species studied and that in general it is higher than that required for insect control.

More recently Samapundo et al. (2007a) concluded that both the initial headspace CO_2 concentration and the a_w had a significant effect on the growth of *F. verticillioides* and *F. proliferatum*. 10% CO_2 completely inhibited FB_1 production by *F. verticillioides* at all a_w levels examined, while higher levels (30-40%) were required for complete inhibition of toxin production by *F. proliferatum*. In contrast, 2-20% O_2 had no effect on the growth rates of the fungi, while as a_w decreased, the optimum for toxin production moved to lower O_2 concentrations (Samapundo et al., 2007b). The maximum inhibition of toxin production was 70% for *F. verticillioides* and 20% for *F. proliferatum* and the authors suggested that O_2 modified atmospheres should not be regarded as a means for the control of fungal growth and mycotoxin production. Complete inhibition of the growth of both fungi was only achieved in the absence of O_2 and vacuum packaging. In a similar study Giorni et al. (2008) found that CO_2:O_2:N_2 mixtures containing 50:1:49 and 75:1:24 v/v could reduce aflatoxin production in maize stored for 3 weeks by 46 and 58% respectively. They attributed this effect to the CO_2 concentration, without however considering any likely additional effect of the N_2 concentration.

(b) Fumigation

Fumigation is a technique primarily used for the elimination of storage insects and pests in storage facilities. Substances that have been used as fumigants are methyl bromide, phosphine, sulphuryl fluoride, carbonyl sulphide, ethyl

formate and hydrogen cyanide. From these, methyl bromide cannot be used after 2015 worldwide under the Montreal Protocol. Phosphine-resistant strains of certain insects have already started appearing while for the rest of the above substances certain advantages and disadvantages have been discussed by Bell (2000).

Fumigation has also extended to the scope of prevention of fungal growth in stored grains. SO_2 is used as food additive and preservative and for the prevention of mould growth on raisins, however its use as fumigant is limited due to the corrosion it causes to the pipes used for its delivery (Magan and Aldred, 2007). Nitrogen has also been employed in some studies however it has proven ineffective in the presence of even 1% oxygen (Bell and Armitage, 1992). Finally the effectiveness of helium, ethylene and ozone (McKenzie et al., 1998; Kim et al., 1999) has been studied for controlling fungal growth.

1.5.2 Ozone (O_3) in grain storage

(a) O_3 gas

O_3 is a triatomic molecule and is one of the allotropic forms of oxygen; it is naturally occurring in the atmosphere but it is very unstable and within 20-50 minutes it decomposes to oxygen without leaving any residues. It is a powerful oxidising agent. The US-FDA has approved the use of O_3 in the gaseous and aqueous phases during the treatment, processing and storage of food as an antimicrobial agent under the conditions prescribed in the Federal Register (FDA, 2001) and under Good Manufacturing Practice (GMP). In addition it is also approved for use as an antimicrobial agent in bottled water and in 2004 the US-FDA published a guidance document for the processors of apple juice and cider on the use of O_3 for the reduction of pathogens (US-FDA, 2004).

In the past there were certain limitations for its use such as the absence of efficient systems for high O_3 generation and the associated economic costs. Today different methods for O_3 production are available including the corona

discharge method, electrochemical production and more recently O_3 production from air. O_3 can thus be produced at the site which offers a significant advantage when compared to alternative treatments for which production, storage and transportation costs are involved. Also ozone can be employed either in its gaseous form or dissolved in water and applied as an aqueous solution, offering the possibility for even more applications.

O_3 has been studied for the decontamination of fresh fruits and vegetables or for the control of fungi causing disease in fruit (Tiwari et al., 2010; Minas et al., 2010). Also its efficacy against storage insects has been demonstrated (Kells et al., 2001; Tiwari et al., 2010). Ozone has also been studied for application in other areas including the use in building remediation against the “sick building syndrome” a condition where fungal or bacterial contamination of building materials adversely affects the human health, or for decontamination in health care units (Hudson and Sharma, 2009). The efficacy of ozone against wood pest infestation was not very successful (Taylor and Morrell, 2009).

(b) O_3 efficacy *in vitro*

Very few studies have examined the *in vitro* effect of O_3 on fungal spore germination and mycelia growth (Freitas-Silva and Venâncio, 2010). In one of the first studies reported, Hibben and Stotzky (1969) examined the effect of different O_3 concentrations (10-100 parts per hundred million) and exposure times (1-6 hours) on the *in vitro* spore germination of several fungal species (including *Aspergillus, Penicillium, Botrytis, Fusarium, Alternaria, Verticillium, Rhizopus*) on solid and liquid yeast extract-rose bengal agar (YERBA) and also on dried spores. Spore germination decreased with increasing O_3 concentration and time of exposure while generally high O_3 concentration at short exposure time had similar effects with low O_3 concentration for longer time. They even observed stimulation of germination after short exposures at low O_3 concentrations and reduced efficacy when spores had formed clumps. They suggested that the effect of O_3 on the spores was fungicidal and that it was not associated with changes in the media. Delayed or slower germination and differences in the appearance of the cultures formed from treated spores and

their sporulation was observed after certain treatments but they were not due to O_3 effects to the media. Results obtained in liquid media were worse than those in solid media, while the effect of O_3 exposure on dried spores was limited. Finally they suggested different mechanisms in order to explain the results obtained including effects on the membrane integrity or on enzymes involved in cell development. They also suggested that the presence of water could have a synergistic effect, but they could not explain the different results obtained on solid and liquid media.

Antony-Babu and Singleton (2009) studied the *in vitro* effect of O_3 on *Aspergillus nidulans* and *Aspergillus ochraceus* (200 and 300 μmol/mol for 10 min and 0.2 μmol/mol for 12 days, at 18°C). Spore germination at higher O_3 concentrations was reduced by 50% compared to the controls, while only 15% in the low dose extended exposure. Contrarily spore production was reduced in the low dose continuous exposure while unaffected in the short time-high O_3 treatment. Different behaviour between the two species was also noticed, especially with regard to biomass production which was reduced in *A. ochraceus* while stimulated in *A. nidulans*. In a more recent study the same authors showed that the substrate is an important factor determining the effect of O_3 on fungal spores, since the sugar content of the media affected the efficacy of the O_3 dose applied against *Eurotium* spores (Antony-Babu and Singleton, 2011). For 100% elimination of the spores, 300 μmol O_3 for 2 hours were required. The authors finally noted differences in the appearance of the cultures formed from the treated spores. However these studies did not examine any possible effect of the water activity to the efficacy of the treatment or during subsequent storage nor did they examine the effect of O_3 treatment on mycotoxin production by surviving fungal spores.

Minas et al. (2010) obtained up to 50% inhibition in the growth of *Botrytis cinerea in vitro* on Potato Dextrose Agar (PDA) (inoculated with agar plugs) with a combination of O_3 (0.3 μl/l) at 0°C and 95% RH after 6 days exposure and showed that these results were not due to effects of O_3 to the agar. However, this inhibition was not maintained once the cultures were stored at 22°C for 24

and 48 h suggesting a fungistatic effect. They also studied the effect of the above O_3 exposure conditions on the germination of *Botrytis cinerea in vitro* and found that complete inhibition of germination even after 3 days incubation at 22°C was obtained after 8 h O_3 exposure.

Finally, Zotti et al. (2010) studied the effect of O_3 on *Aspergillus* cultures of different ages and suggested that colonies at an earlier stage of growth were more susceptible, though certain aspects of this study could be challenged.

(c) O_3 efficacy *in situ*

Recent large scale *in situ* studies have attempted to practically apply O_3 concentrations for the treatment of stored grains. Kells et al. (2001) filled a 12.7 tonne steel bin with grain and continuously applied O_3 (50 ppm for 3 days or 25 ppm for 5 days) from the top of the bin, since this is the area that is considered as most susceptible to insect accumulation and fungal growth, due to the higher moisture content caused by condensation or leaks in the storage bin. Up to 90% of insects were eliminated, while only 63% or insignificant reduction of *A. parasiticus* conidia was observed for each treatment respectively.

Wu et al. (2006) studied the effect of gaseous O_3 produced from pure oxygen in combination with the duration of the treatment, the water activity and the temperature on the inactivation of stored wheat fungi (assessed by enumeration of fungi by the spread plate method). They achieved 96.9% spore inactivation with a 0.33 mg/g/min dose for 5 min and 100% inactivation with the same dose for 15 min at 0.90 a_w and 20°C. The efficiency of the O_3 treatment generally increased with increasing a_w (0.80-0.90) and increasing temperature (10-40°C). They also suggested that a stable O_3 concentration at the exit of the system was paralleled with maximum spore inactivation and could be used as a control parameter for the ozonation process. Similar results were obtained by Allen et al. (2003); 5 min at 0.1 mg/g/min on barley of 0.98 a_w and 20°C was sufficient to inactivate 96% of spores and mycelium and they suggested that mycelium was more vulnerable to O_3 than the fungal spores. They also suggested that O_3 is more efficient at higher water availability since it reacts more easily with water and forms free radicals that are more efficient.

White et al. (2010) attempted to relate the effect of O_3 treatment on high moisture maize to dry matter losses (used as a quality indicator) after 9 days storage, however with not much success. Even though the O_3 concentrations applied were high (1000-9000 ppm) and the exposure times long (up to 24 hours), small reduction of DMLs was obtained in the treated samples compared to the controls and no clear relationship was observed with regard to the experimental parameters. Also no attempt has been made to determine the effect of the treatments on possible mycotoxin production by the fungi in the samples.

Tiwari et al. (2010) reviewed studies suggesting that O_3 treatment can cause up to 3 log reduction of populations in different crops depending on other exposure parameters.

(d) O_3 dispersion through the grain

The mechanism of O_3 dispersion through grains has been the focus of some studies aiming to optimise the exposure systems. Strait (1998) and Kim et al. (1999) suggested that although it was possible for O_3 to disperse through the whole grain mass in small scale storage bins, in large-scale bins two phases could be identified in the dispersion of O_3. In the first phase several interactions between O_3 and the grain consume an amount of the applied dose described as “the ozone demand of the medium” (Kim et al., 1999). In the second phase these interactions are eliminated, O_3 disperses easier and the desired concentration can be established. Considering this previous work, Kells et al. (2001) studied the characteristics of O_3 penetration through the grain in a 12.7 tonne capacity steel bin and defined the “apparent velocity of ozone” through the grain mass as the ratio of the total airflow rate (m^3/s) to the cross-sectional area of the storage bin (m^2). They suggested that there is a linear relationship between the O_3 concentration in the storage bin and the depth in the grain mass which they defined as the “stable degradation rate”. They concluded that an apparent velocity of 0.03 m/s is required for the O_3 front to disperse through the grain mass in a reasonable time. Mendez et al. (2003) debated later that this might differ for different kinds of grains. Overall, both studies suggested that

much smaller velocities and less time are required for the same level of O_3 penetration through the grain during the second phase of O_3 dispersion or in grain that has previously been treated with O_3 possibly due to the previous elimination of the active sites of the grain. Tiwari et al. (2010) in their review suggested that O_3 movement and adsorption in a grain mass depends on several factors, including the characteristics of the grain, the presence of extraneous matter, the contamination of the grain and the moisture content.

(e) Quality of grain treated with O_3

Another aspect of O_3 treatment is the possible effect on grain nutritional quality, organoleptic and technological properties. Relevant studies have been reviewed by Tiwari et al. (2010). Akbas and Ozdemir (2006) studied the effect of ozonation on certain physicochemical and organoleptic properties of pistachio nuts and concluded that no significant changes occurred in the kernels, but that some organoleptic properties were affected in ground samples. Wu et al. (2006) showed that 98 mg/g/min O_3 treatment of wheat for 15 min had no adverse effects on the seed germination of wheat which is much higher than the dose necessary for inactivation of the fungal spores. Mendez et al. (2003) also showed that treatment with 50 ppm O_3 for 30 days had no detrimental effect on certain quality characteristic of several grains. However they noted that formation of nitric acid when using ambient air to generate O_3 especially by corona discharge might affect the organoleptic properties of the grains (Mendez et al. 2003). The economics of O_3 treatment for long periods also needs to be examined for feasibility.

(f) O_3 in the degradation of mycotoxins

Several studies have also focused on whether O_3 can degrade mycotoxins, on the toxicity of the treated mixtures and on the possibility that more toxic by-products may be formed. Aqueous solutions of aflatoxins and trichothecenes have been successfully degraded with low O_3 doses and with relatively short treatments and the toxicity of the treated samples investigated (McKenzie et al., 1997; Young et al., 2006). In contaminated grains O_3 degradation has also proven very efficient. Approximately 70% aflatoxin degradation was achieved in

contaminated cornmeal slurry (20% O_3 for 5 min) (McKenzie et al., 1997) and more than 95% aflatoxin B_1 degradation in 30 kg naturally contaminated maize in stainless steel columns (McKenzie et al., 1998). No adverse effects were observed on the turkey poults fed this O_3-decontaminated maize. Combination of gaseous O_3 (4.2% per weight) and mild heating (25, 50 and 75°C) has been assessed for the degradation of aflatoxins in peanuts and peanut flour and the efficacy generally increased with increasing time (5-15 min) and temperature, though the effect of temperature was not so significant when longer times were applied (Proctor et al., 2004). A higher degradation was obtained in peanut kernels (77±2%) than in peanut flour (56±3%). Similarly higher degradation of aflatoxins in pistachio kernels than in ground pistachios has been observed under the same conditions possibly due to reduced penetration of O_3 in the ground nuts (Akbas and Ozdemir, 2006). The authors also observed higher effect of O_3 on aflatoxin B_1 than on the other aflatoxins in accordance with previous studies (Akbas and Ozdemir, 2006).

Mycotoxin degradation with O_3 is not within the scope of this project but it has been considered here as grains subject to O_3 treatment prior to storage may already be contaminated with mycotoxins to some extent from the field (especially fusarium toxins: DON, NIV, ZEA, T-2, HT-2, fuminosins) and it is therefore very important to know whether the O_3 treatment could have any undesirable effects, such as the formation of more toxic secondary products from the degradation of certain mycotoxins.

1.5.3 Chemical methods for the post-harvest control of mycotoxins

Several different chemical methods have been examined for the control of mycotoxin contamination in stored grains. Hydrolysis and nixtamalisation have not proved very successful, ammoniation has found more use as feed treatment (Park, 1993) while a combination of H_2O_2 and $NaHCO_3$ has been reported to reduce fumonisin concentration by up to 100% in contaminated maize even

though further research was required (Munkvold and Desjardins, 1997; Park et al., 1996).

Consumers require chemical-free products and research has now focused on natural substances and compounds naturally present in plants and grains, essential oils, plant extracts and natural antioxidants for use as preservatives and antifungal agents.

(a) Chemicals / antioxidants with antifungal properties

Several studies have examined different organic acids for efficacy in the preservation of stored grain (Jones et al., 1974, Singh et al., 1987, Petterson et al., 1989; Higgins and Brinkhaus, 1999) although they did not always include the effect of the treatment on mycotoxin contamination (Vandegraft et al., 1975). The use of formic, acetic and propionic acids and their mixtures for preservation of wet grain and prevention of mould growth has been patented since 1971 (BP Chemicals, 1971). Propionic acid has been extensively studied and several commercial products based on this or its mixtures with other compounds are available on the market for application in grain preservation (Sauer and Burroughs, 1974; Bothast et al., 1978; White and Coates, 1998; Higgins and Brinkhaus, 1999). It has also been suggested that the above acids are more efficient than their salts (Sauer and Burroughs, 1974; Marín et al., 2000).

Sorbate applied at 1% was shown to control fungal growth and aflatoxin production in high-moisture maize (Lee et al., 1986) although the authors suggested that application at such concentration would not be cost-efficient. Application at 0.5% however had no effect on aflatoxin production. Other more recent studies have also shown that treatment of moist grain with acids at sub-inhibitory concentrations or inefficient coverage of grains could even result in stimulated fungal growth and mycotoxin production (Marín et al., 1999; Marín et al., 2000). This is due to the fact that the compounds do not eliminate the fungi but instead act as fungistats (Marín et al., 2000; Magan and Aldred, 2007; Magan et al., 2010). Marín et al. (2000) observed that treatment with propionates had different effects on different fungal species. This has previously been suggested to lead to favoured growth of the species more tolerant to the

treatment. Also they reported that in maize treated with propionates fumonisin B_1 production by *F. verticillioides* was unaffected while increasing propionate concentrations increasingly controlled fumonisin B_1 production by *F. proliferatum* (Marín et al., 2000). Thus, research for other compounds that may have a wider inhibitory effect on several fungal species encountered in naturally contaminated grains is required.

Chipley and Uraih (1980) studied the effect of several derivatives of benzoic acid on the growth of *A. flavus* and *A. parasiticus* and aflatoxin production with varying results. Among those, trans-cinnamic acid at 2.5 mg per 25 ml of the chemically defined basal medium significantly reduced mycelial growth and toxin production while it was completely inhibited by 5 mg per 25 ml. Ferulic acid also showed significant inhibition of growth (39%) and toxin production (75%) at these two concentrations. They noted that, interestingly, inhibition of mycelial growth did not always result in a similar inhibition in toxin production.

Beekrum et al. (2003) suggested that naturally occurring phenolic compounds that have been used as antimicrobials since 1867 could also have antifungal activity. They found that chlorophorin, iroko, maakianin, vanillic acid and caffeic acid inhibited the growth of *F. verticillioides* and reduced Fumonisin B_1 production by 88-94%. Interestingly ferulic acid had no effect on fungal growth but it reduced toxin production by 90%. Benzoic acid did not demonstrate any inhibitory effect in this study.

Nesci and Etcheverry (2006) achieved various levels of inhibition of the growth of *A. flavus* and *A. parasiticus* with ferulic acid, trans-cinnamic acid and mixtures of the two *in vitro*, while aflatoxin B_1 decreased with decreasing water activity. In a subsequent *in situ* study Nesci et al. (2007) showed that 30 mM ferulic acid and 20 and 25 mM trans-cinnamic acid could be used for the control of aflatoxin B_1 production in maize of 0.93 a_w stored for 10 and up to 35 days though at some combinations of water activity and storage times stimulation was occasionally observed. Varying results were also obtained with mixtures of the two compounds at different proportions but the mixture 25 mM:30 mM trans-

cinnamic acid: ferulic acid caused complete inhibition of aflatoxin B_1 production in maize of all water activities stored for up to 35 days.

Recently Boutigny et al. (2009) studied the effect of ferulic acid on the growth of *Fusarium* species and trichothecene production in liquid cultures, additionally considering the time of application of the compound. Fungal biomass was inhibited by 39% and 85% with 2.5 and 5 mM ferulic acid respectively, while trichothecene production was completely inhibited with both treatments. Concentrations 0.1 to 1 mM ferulic acid had no significant effect on the fungal biomass produced but toxin production was inhibited between 68% and 99%, an observation that led to the suggestion that ferulic acid may be affecting the secondary metabolism of the *Fusarium* species. They also observed that the effect of ferulic acid on trichothecene production was the same, irrespective of whether it was added at the spore germination step or during the mycelia development when trichothecenes start to accumulate. However addition of ferulic acid at both steps had an accumulating significant effect achieving 96% inhibition of toxin production (Boutigny et al., 2009). These studies however were performed mostly in liquid cultures and did not consider the possible additional effects of water availability to the effect of the compounds.

(b) Plant extracts / essential oils with antifungal properties

Several essential oils and plant extracts have been screened for their efficacy against fungal growth and toxin production.

Bluma et al. (2008) studied the efficiency of 96 plant extracts as either ethanolic extracts, essential oils or aqueous extracts and they found that essential oils were more effective and some even affected the growth of *A. flavus* strains. Clove and poleo essential oils completely inhibited aflatoxin B_1 production at 0.982 a_w, while thyme and eucalyptus achieved a maximum of 85-90% inhibition. With slight water stress however (0.955 a_w) a maximum of 80% inhibition in toxin production was observed with the essential oils. In a subsequent study Bluma and Etcheverry (2008) studied the effect of the same essential oils in maize grain. 100% inhibition of the growth of *A. flavus* and *A. parasiticus* was observed when 2000 and 3000 µg/g of boldus, poleo and

mountain thyme essential oils were applied at 0.955 and 0.90 a_w and 25°C after 11 days, while anise and clove achieved 100% growth inhibition only at 3000 µg/g. However, after 35 days the inhibition did not remain the same in all cases. Aflatoxin synthesis was also completely inhibited by 2000 and 3000 µg/g of boldus, poleo and mountain thyme essential oils at 0.982 a_w and at all concentrations at 0.955 and 0.90 a_w after 11 days, but this effect again did not last for more than 35 days.

Aldred et al. (2008) examined the efficacy of the essential oils of bay, clove and cinnamon on the growth of *Penicillium verrucosum* and *Aspergillus westerdijkiae* and ochratoxin A production in wheat grain. More than 90% inhibition of *in vitro* growth and toxin production was achieved with 500 µg/g of the essential oils, but the treatments were not so effective in natural grain. In the same study the antioxidant resveratrol was found to be more efficient achieving 60% inhibition of growth and toxin production even on naturally contaminated grain.

In both studies it was observed that in some cases even though growth was inhibited, toxin production was stimulated, something that has been previously suggested by Magan et al. (2002), possibly due to the combination between the water stress and the stress caused by the antifungal agent.

Onion and garlic both members of the *Allium* family have always been considered as having significant antimicrobial properties and several studies have focused on their antifungal efficiency. They contain organosulfur compounds and it has been suggested that their antifungal activity is due to the compounds allicin and ajoene (Yoshida et al., 1987). Yin and Tsao (1999) examined the antifungal activity of seven plants of the *Allium* family against three *Aspergillus* species and garlic was shown to be more effective though generally the concentrations required were species dependent. Also the presence of acetic acid increased the antifungal efficiency at 25°C however higher temperatures decreased the efficiency.

Some very early studies even supported such properties in thiosulfinates (Small et al., 1947) though they have not found application in grain preservation

possibly due to their reduced stability and strong odour. Garlic extract has been approved for use as a pesticide (although currently under re-evaluation) and commercial products are available for such applications.

Benkeblia (2004) studied the effect of essential oils of three types of onions and garlic on the growth of three fungi *in vitro*. All concentrations of red onion and garlic essential oils showed strong inhibitory effect on the growth of *A. niger* as well as higher concentrations (200, 300 and 500 ml/l) of green and yellow onion essential oils; similar results were obtained for *Penicillium cyclopium*. *Fusarium oxysporum* was mostly affected by the essential oils of garlic and only the higher concentrations of the onion oils (300, 500 ml/l). In another study Singh and Singh (2005) studied the effect of *Allium sativum* extracts and other plant extracts on the growth of *A. flavus* and aflatoxin production in liquid cultures and two application times. Addition of the *Allium sativum* extract at the beginning of the incubation showed 84.59% inhibition in the mycelia growth and 100% inhibition in aflatoxin production, but when added on the fourth day of incubation toxin inhibition was only marginal. However these studies were performed in liquid cultures and therefore no information is available on the efficacy of these compounds on naturally contaminated grains. Also the authors did not consider any likely additional effects of the water availability of the substrate to the efficacy of the treatments.

1.5.4 Physical methods for the post-harvest control of mycotoxins

Mechanical separation is one physical method that has been employed for the control of mycotoxin contamination in grains. This method however is considered impractical since, if contaminated material remains in storage, contamination can easily spread to the rest of the stored product and it is also time consuming (Park, 1993).

The efficacy of irradiation in mycotoxin control has also been studied, however food irradiation is considered a sensitive issue and it is likely that consumers are concerned about the safety of irradiated foods. European Commission has

taken into consideration these facts and irradiation has only been authorised in order to control pathogenic or spoilage organisms in certain products and only if it is necessary and safe for the consumer and provided that this treatment is declared on the label of the product (EC, 1999).

1.5.5 Biological methods for the post-harvest control of mycotoxins

Several yeasts and lactic acid bacteria have been screened for their efficacy as biological control agents against mycotoxins. Their efficiency may rely on their ability to dominate against mycotoxigenic fungi, on the production of certain compounds inhibitory to the growth of the mycotoxigenic fungi, or on their ability to bind to different mycotoxin molecules and degrade them.

(a) Yeasts for biological control

The efficiency of *Pichia anomala* as a biocontrol agent has been studied in feed grain stored in silos under different conditions of air permeability (ranging from airtight to non-airtight) and it was shown that it inhibited the growth of *Penicillium roqueforti*. Different possible mechanisms of action were suggested, such as oxygen depletion caused by oxygen consumption by the yeast but also competition for nutrient between the two microorganisms and production of toxins or other compounds that would cause inhibition of *P. roqueforti* growth (Druvefors et al. 2002; Petersson et al. 1999).

(b) Lactic acid bacteria for biological control

Numerous studies in the last 10-15 years have focused on the use of lactic acid bacteria for the control of food spoilage and mycotoxin production by fungi. The lactic acid bacteria that have been screened and the mechanisms of their action have been reviewed by Dalié et al. (2010). The bacterial genera that have been screened are mainly *Lactococcus, Lactobacillus, Leuconostoc and Pediococcus* and their antifungal activity has been studied against certain fungal species, mainly *Aspergilli* but also *Fusaria* and *Penicillia*. The suggested mechanisms of action include the production of certain antifungal compounds, binding of the

mycotoxins (El- Nezami et al. 2002a; 2002b) or inhibition of their biosynthesis (Gourama and Bullerman, 1995). In most studies it was also demonstrated that the antifungal activity of lactic acid bacteria would be affected by certain factors such as the temperature, the time of application as well as the incubation period, the nutrient availability and the pH.

Biological control by competitive exclusion of mycotoxigenic-producing strains by non-mycotoxin producing ones has been attempted successfully for *A. flavus* and could be studied for other mycotoxigenic fungi as well (Munkvold and Desjardins, 1997; Cotty, 1994).

1.6 Aim and objectives of the project and thesis structure

The overall aim of this project was to identify possible mechanisms for the prediction of when a stored grain batch is likely to reach high levels of mycotoxin contamination and be rejected for further use and identify the most effective control strategies that could be used as part of a stored grain management system. The specific objectives were:

- To determine the respiratory activity of *F. graminearum* and *F. verticillioides* during storage at different environmental conditions, the dry matter losses caused in wheat and maize respectively and the effects on mycotoxin production
- To determine the environmental conditions under which *F. langsethiae* can grow and produce T-2 and HT-2 toxins in oats during storage and calculate the dry matter losses caused to the oats at the same conditions
- To identify any relationship between dry matter losses and toxin contamination that could be used as a predictive factor during the management of stored grains
- To examine novel compounds *in vitro* for efficacy on the control of fungal growth and mycotoxin production and determine the amounts of the compounds required for 50% control (ED_{50} values)

- To study the most promising compounds from the *in vitro* trials *in situ* on artificially inoculated grains for the efficient preservation and control of mycotoxin contamination during storage at different environmental conditions
- To examine different O_3 concentrations (generated from ambient air) and exposure durations *in vitro* for efficacy in the control of fungal spore germination, fungal growth and mycotoxin production
- To examine different O_3 concentrations, exposure durations and exposure systems *in situ* for the control of fungal populations in naturally contaminated grains
- To examine the efficacy of the most efficient O_3 treatments *in situ* for the safe storage and control of mycotoxin production in artificially inoculated rewetted grain

The thesis is thus divided in two different sections. In the first part the studies focus on the effect of different environmental conditions on the growth of different *Fusarium* fungal species on wheat, maize and oats during storage, on the dry matter losses caused to the grain and on the production of different mycotoxins at the same conditions (Chapters 2 and 3). On the second part of the thesis, different control strategies are examined initially *in vitro* and subsequently *in situ* for efficacy in controlling mycotoxin production in treated grains stored for up to 30 days at different environmental conditions (Chapters 4-6).

Some additional studies on control strategies have been carried out with *A. flavus* in order to also examine their effect on this fungal species and facilitate comparisons with published studies which most commonly focus on the control of *A. flavus* and aflatoxin production. These were not immediately within the scope of this Thesis which was focused on *Fusarium* species and thus have been included in Appendix H for reference.

The different components of the work that was carried out during this project are presented in the following schematic flow diagram (Figure 1.9).

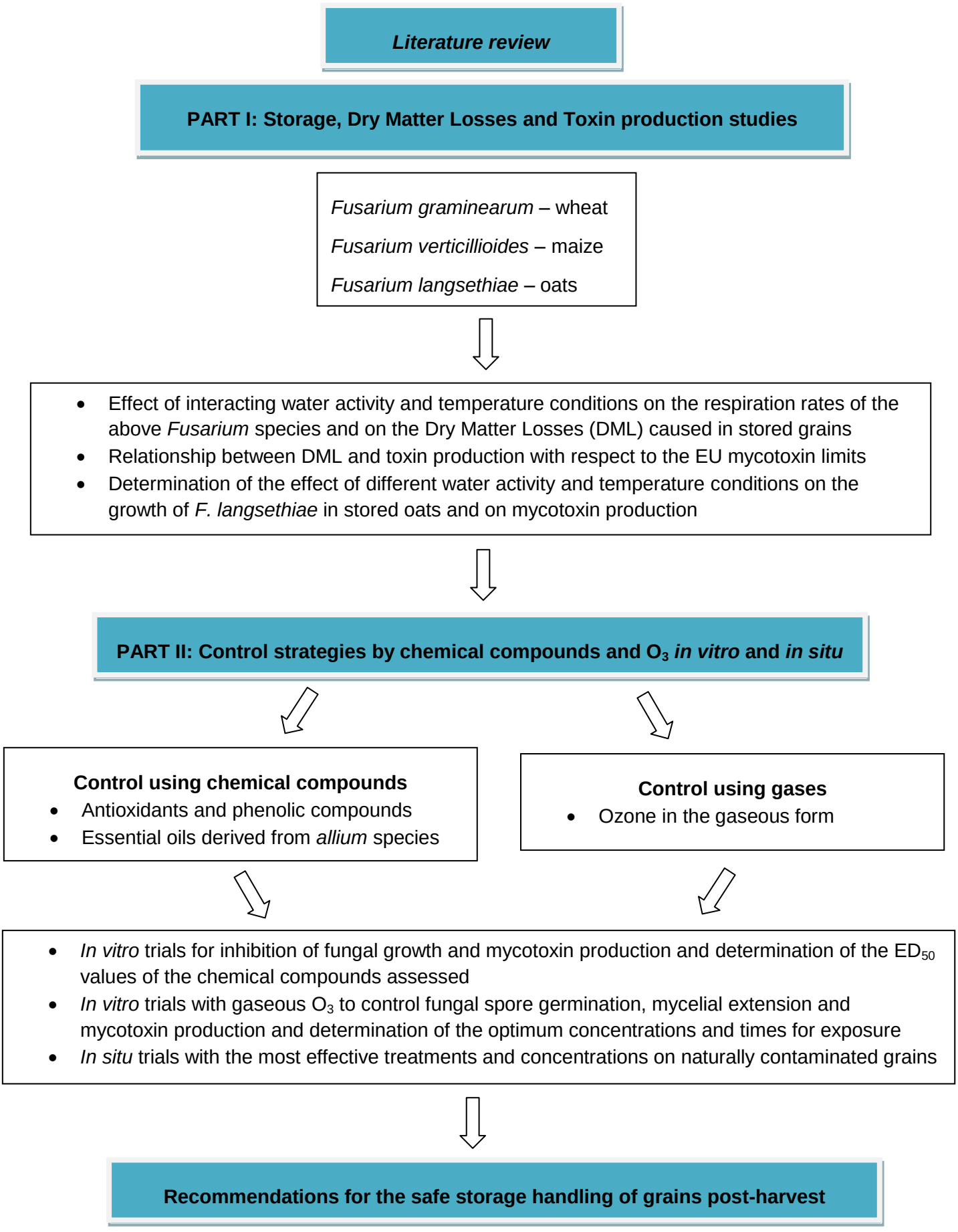


Figure 1.9: Schematic flow diagram of the work carried out in this project.

2 WHEAT AND MAIZE: DRY MATTER LOSSES AND MYCOTOXIN PRODUCTION BY *FUSARIUM* SPECIES

2.1 Introduction

Wheat and maize are major staple foods world-wide (FAO, 2009) and quality losses due to fungal spoilage or contamination with mycotoxins could have a significant impact on human and animal health and also significant economic consequences. *F. graminearum* can contaminate wheat with deoxynivalenol (DON), nivalenol and zearalenone (ZEA), while *F. verticillioides* can contaminate maize with fumonisins (FUMs). The environmental conditions under which these species grow and produce mycotoxins are generally understood (Hope et al., 2005; Marín et al., 1995; Samapundo et al., 2005). Grain contamination with fusarium toxins has generally been considered as more common at the pre-harvest stage (Cleveland et al., 2003; Miller, 2008) however, they may also proliferate during harvesting due to inefficient drying or in damp post-harvest storage conditions (Wagacha and Muthomi, 2008).

Therefore it is very important to monitor the condition of stored grain in order to be able to predict when a stored batch is likely to have reached unacceptable contamination levels. Different “storability risk indexes” have been suggested including the appearance of microscopic or visible fungal growth, ergosterol measurement, volatile analysis for identification of compounds associated with fungal infection and quantitative enzyme production (Fleurat-Lessard, 2002; Magan and Aldred, 2007).

Dry matter losses (DMLs) have been considered as a quality indicator and values as low as 0.04% DML have been reported as a threshold for seed germination and visible moulding in wheat (White et al., 1982a, b; Lacey et al., 1994). Seitz et al. (1982) suggested that 0.5% DML would be enough to downgrade maize from food to feed quality while at the same time the risk of aflatoxin contamination could be increased. However, little information is available on the risk of fumonisin contamination of maize.

In terms of predicting toxin contamination in stored grain, colony forming units (CFUs) of *Penicillium verrucosum* have been related to the storage conditions and to the probability of exceeding the EU legislative limits for ochratoxin A in wheat (Lund and Frisvad, 2003; Lindblad et al., 2004). Yong and Cousin (2001) obtained some good correlations comparing *Aspergillus parasiticus* colony counts to enzyme-linked immunosorbent assay (ELISA) quantification and ergosterol measurements for the detection of aflatoxin contamination in maize and peanuts. More recently Pasquali et al. (2010) related the counts of a nivalenol chemotype of a *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 in maize. However, no studies have attempted to quantitatively relate DMLs to toxin contamination during storage.

The objectives of this study were to (a) quantify the DMLs caused during *F. graminearum* colonisation of wheat and *F. verticillioides* colonisation of maize from the amounts of CO_2 produced under different combinations of storage a_w and temperature, (b) quantify the production of DON and ZEA in wheat and FUMs in maize under the same conditions and (c) develop models to relate the DMLs and mycotoxin contamination to the environmental conditions and (d) identify the threshold DMLs at which a stored batch would be contaminated by the above mycotoxins beyond the relevant EU legislative limits.

2.2 Materials and methods

2.2.1 Grain matrices

The following food matrices were used to perform the respiration and dry matter loss experiments:

a) Wheat supplied by local UK farms, 2008-2010 harvest,
b) Maize supplied by MYCORED partners in Università Cattolica del Sacro Cuore (UCSC), Piacenza, Italy, 2008 and 2009 harvest.

The grains were irradiated at 12 kGy (Isotron, Plc, Swindon, UK) in order to retain germinative capacity but be free of fungal contamination (Hope et al., 2005) and they were stored at 4°C until further use.

2.2.2 Water activity adjustment of grain matrices

Known volumes of sterile water were added to 10 g sub-samples of wheat and maize in sterile Universal vials. The samples were sealed, thoroughly mixed and incubated (at 25°C) in order to allow for the water to be absorbed. The a_w of the grain was determined daily until stabilised, using an AQUALAB, Model Series 3TE, Decagon device (LabCell.). In order to check the accuracy of the a_w readings, standard solutions of known water activities (a_w) were prepared by adding specified amounts of glycerol (glycerol for analysis, ACROS organics, Fisher Scientific) to sterile water according to the Table of Appendix A, modified from Dallyn and Fox (1980). The a_w of these solutions was determined at the beginning and the end of each a_w measurement series.

The a_w of the samples was plotted against the amount of added water. From these curves, the amount of sterile water needed for 10 g sub-samples of the dried grain to obtain the target a_w for the respiration experiments could be determined (dry weight). The time required for each grain type to equilibrate accurately to the target a_w level was also determined.

2.2.3 Fungal strains and preparation of the inoculum

F. graminearum isolate L1-2/2D was supplied by the University of Lleida, Catalonia, Spain. This strain 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 B_1 and B_2 (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).

Cultures of the above fungal species were prepared on MEA and incubated at 25°C for 10 days. A Tween 80 solution was prepared by addition of one drop of Tween 80 (ACROS organics) in 100 ml sterile water. Spore suspensions were prepared by gently scraping the culture surface with a sterile spatula and transferring the spores into sterile Universal vials containing Tween 80 solution. The spore suspensions were filtered through glass wool in order to remove any mycelia fragments. The spore concentration was determined using a haemocytometer (Olympus BX40 microscope, Microoptical Co.; slide Marienfeld superior, Germany; microscope glass cover slips, No 3, 18×18mm, Chance proper LTD, UK) and adjusted by dilution to 10^7 spores ml^{-1}.

2.2.4 Inoculation of the grains and incubation conditions

200 g of irradiated grain were modified to three different a_w levels by addition of sterile water according to the water adsorption curves previously developed and allowed to equilibrate for 72 h. 1 ml of fungal spore suspension was used for inoculation of the grain. Once equilibrated, the grain was inoculated and thoroughly mixed in order for the spores to disperse throughout the whole amount. Three replicates of 10 g (wet weight) were placed in 40 ml vials (Chromacol Ltd, UK) and incubated at 15, 20, 25 and 30°C in sandwich boxes containing glycerol/water solutions of the same a_w (prepared according to the Table of Appendix A) to help maintain the relevant a_w level. The samples were incubated for 10 days and respiration was measured daily.

2.2.5 Respiration measurements by Gas Chromatography

On a daily basis the samples were sealed with injection caps for one hour to allow for CO_2 accumulation in the headspace. After this period, 5 ml of ambient air were inserted in the sealed vials by use of a syringe and subsequently 5 ml

of the headspace were withdrawn and directly inserted in the Gas Chromatograph (GC) for CO_2 analysis. The GC was a GC 8000 Series, Carlo Erba Instruments.

(a) Calibration of the GC

For the calibration of the GC the following standard was used: 10.06% CO_2, 2% O_2 in nitrogen (BOC, UK). Gas samples were withdrawn from the gas cylinder and inserted in the GC. The average of three calibration samples was used to determine the retention factor (RF) and once this was determined, another sample was injected in the GC to confirm the accuracy of the calibration.

(b) CO_2 analysis

A molecular sieve 5A was used and a Hot Wire Detector at 120°C. For the CO_2 analysis a column packed with Porapak Q was used. The file containing the parameters needed for the CO_2 analysis was selected on the integrator on the MFC800 control panel, as well as the correct polarity and the correct position of the selector detector. Chromatograms were obtained by using a DP 800 Data Processor, Carlo Erba.

(c) Calculation of respiration rates and dry matter losses

From the percentage CO_2 concentration data obtained, the respiration rate at certain times during the incubation period and the total DMLs at the end of the incubation period were calculated for each sample. DMLs were adjusted as per dry weight.

Respiration rates per hour, R (mg/kg/h) were determined with the following relationship:

$$R = \frac{C \cdot V \cdot d}{m \cdot t} \qquad \textbf{Equation 2-1}$$

where,

C is the GC readings of CO_2 (%vol),

V is the volume of the headspace and that of the added air (45 ml in this experiment),

d is the density of CO_2 (1.977 mg/ml),

m is the weight of the grain sample and

t is the time break (h).

Dry matter losses were determined with the following relationship:

$$DML = \frac{R \cdot T}{14.7 \cdot 1000 \cdot W} \qquad \textbf{Equation 2-2}$$

where,

T (h) is the total storage time and

W is the factor that accounts for the adjustment of the grain weight per dry weight at each a_w level.

At the end of the 10 day incubation period the samples were frozen at –40°C for subsequent toxin analysis.

2.2.6 Enumeration of fungal populations

In order to confirm the growth of the fungi during the incubation period, the populations under different combinations of storage conditions were analysed using the serial dilution technique. Thus, 1 g of the samples used for the respiration experiments and frozen at –40°C was placed in 9 ml of sterile Tween 80 solution and vigorously shaken (10^{-1} dilution). 1 ml of this solution was then transferred to another 9 ml of Tween 80 solution (10^{-2} dilution) and so on and in this way serial dilutions were prepared. 0.1 ml from each of the dilutions was spread on MEA media of the same water activity as that of the grain treatment conditions (3 replicates for each sample) and the colonies were counted after 5-7 days incubation at 25°C, as appropriate.

2.2.7 Toxin extraction / analysis

The grain samples were oven-dried at 60°C for 24-48 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) by the MYCORED Project partners at the Centre for Analytical Chemistry, Department of Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna, Austria. The analysis was performed according to the methods described by Sulyok et al. (2006) and Vishwanath et al. (2009). The recoveries of these methods were 57% for FB_1, 70% for FB_2, 95% for DON and 82% for ZEA and the limits of detection (LOD) 25, 25, 16 and 1 µg/kg for each of the toxins respectively.

2.2.8 Statistical analysis

All experiments were performed in triplicate. Data were analysed with Microsoft Office Excel 2007 and with the package STATISTICA 9 (StatSoft®, Inc. 2010, STATISTICA (data analysis software system), version 9.1. www.statsoft.com). The standard error of the mean was calculated in all trials and is denoted with vertical bars in the Figures or included in the Figure legend in 3-D graphs.

(a) Statistical analysis of DML and toxin analysis data

The Shapiro-Wilk, W test for normality was used to assess the normality of the total DML and toxin data while the homogeneity of the variance within the data was assessed by the Levene's test. Data not normally distributed were logarithmically transformed in order to stabilise the variance and their normality reassessed by the Shapiro-Wilk W test. Normally distributed data were analysed by one and two-way Analysis of Variance (ANOVA) for determination of the significance of the effect of each factor (a_w and temperature) and their interaction ($a_w \times T$) on the variable. Log-transformed data still not normally distributed were analysed by the Kruskal-Wallis test by ranks.

The statistical significance was provided using the p-values rounded to 3 decimal points. Where this resulted in p=0.000, p<0.001 was used instead. Highly significant results are highlighted in bold in the ANOVA Tables which can be found in Appendix B.

(b) Development of models

Regression (Microsoft Office Excel 2007) and forward stepwise regression (STATISTICA 9) were used in order to build polynomial equations for the DML and toxin data in wheat and maize with regard to the storage conditions. In forward stepwise regression the tolerance used for inclusion of the factors in the model equation was set to 0.0001. Normal plots of the residuals were produced in order to assess their linearity and normal distribution. Where residuals were not normally distributed, the variable was logarithmically transformed and the normality reassessed. Contour maps were constructed (Microsoft Office Excel 2007), including only those factors that were found to be significant, in order to indicate the effects of the storage conditions on the DMLs and toxin production.

(c) Correlations between DML and toxin production data

Scatter plots of the DML data against the toxin data were produced 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.

2.3 Results

2.3.1 Water adsorption curves for irradiated wheat and maize

Figure 2.1 shows the relationship between added water and a_w levels obtained after 24, 48 and 72 h of equilibration at 25°C, after addition of known volumes of water to 10 g samples of irradiated wheat and maize.

It can be observed that the curves for both wheat and maize remained relatively stable over the 72 h period. However, the data of the third day were used to

calculate the amount of water needed for the grains to reach the target a_w levels in this experiment.

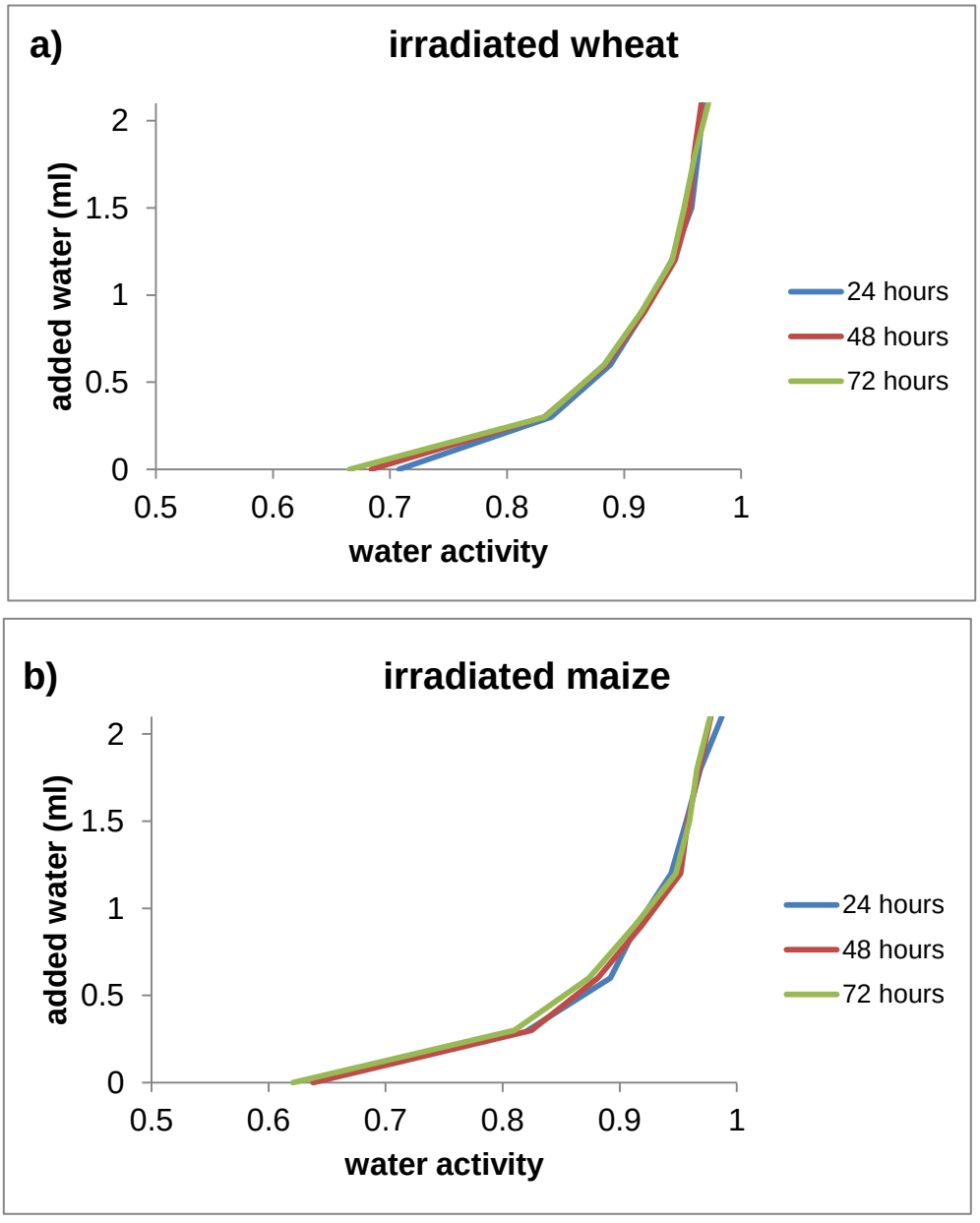


Figure 2.1: Relationship between added water (ml) and water activity (a_w) of irradiated wheat (a) and maize (b) (10 g samples) at 25°C after 24-72 hours of equilibration.

2.3.2 Respiration rates and CO_2 production in wheat and maize as affected by the storage conditions

Figure 2.2 shows the instantaneous (daily) respiration rates in irradiated wheat adjusted to different a_w levels, inoculated with *F. graminearum* and stored at different temperatures for 10 days.

The respiration at 0.89 a_w was constantly very low at all temperatures and decreased with time. At 0.94 and 0.97 a_w respiration rates generally started increasing after 2-4 days. It can also be observed that respiration rates on the first day of the experiment were higher at higher a_w and temperatures.

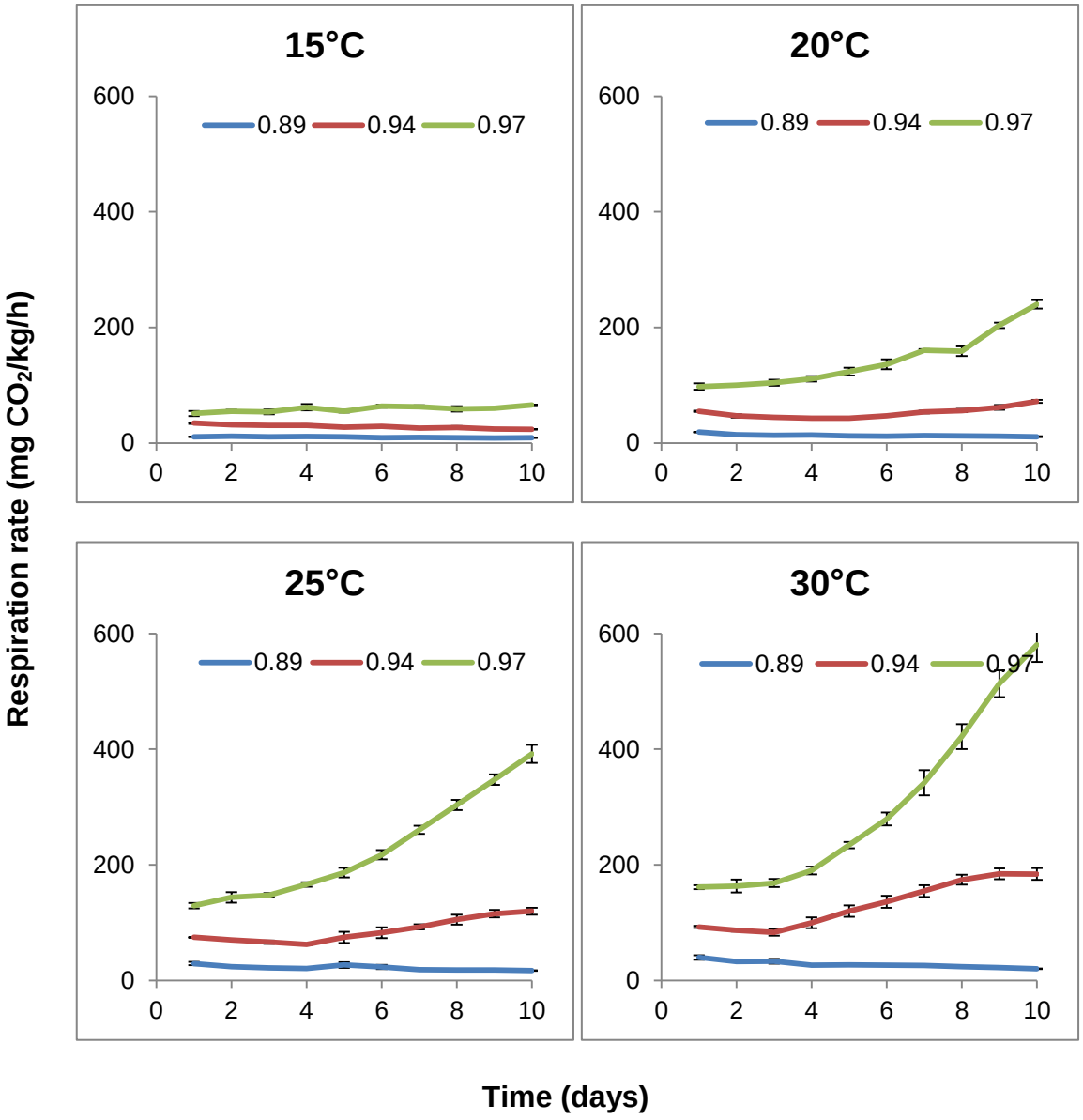


Figure 2.2: Respiration rates (mg CO_2/kg/h) of irradiated wheat inoculated with *F. graminearum* and stored at different combinations of water activity and temperature over 10 days. Vertical bars indicate the standard error of the means.

Similarly, the respiration rates of irradiated maize adjusted to different a_w levels, inoculated with *F. verticillioides* and stored under different temperatures for a 10 day period can be observed in Figure 2.3.

In this storage experiment respiration at 0.91 a_w was again very low especially at the lower temperatures. The respiration measured on the first day was higher under wetter and warmer conditions. Respiration generally started increasing from the 2nd day at the most favourable storage conditions (T≥20°C, a_w≥0.955).

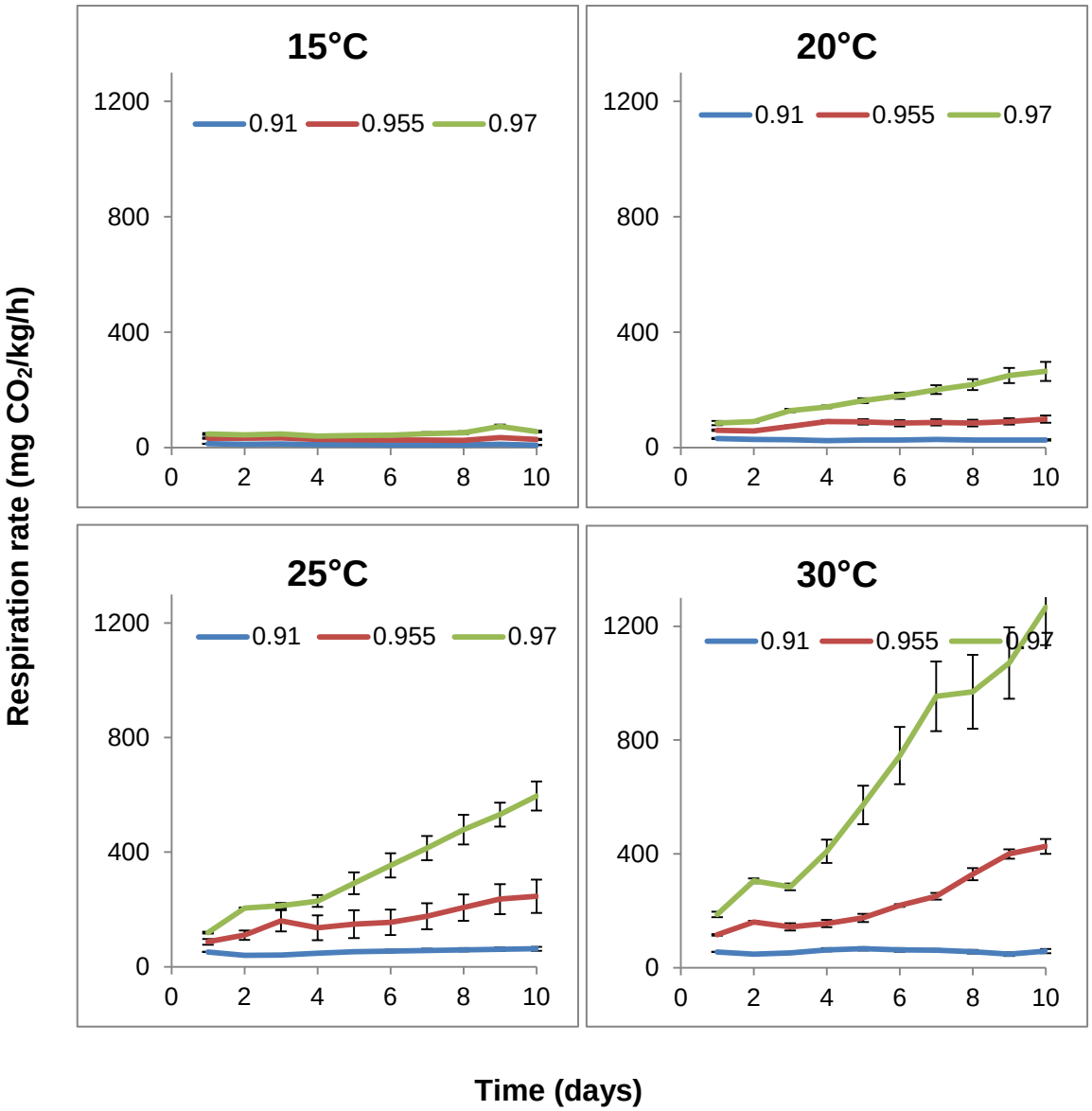


Figure 2.3: Respiration rates (mg CO_2/kg/h) of irradiated maize inoculated with *F. verticillioides* and stored at different combinations of water activity and temperature over 10 days. Vertical bars indicate the standard error of the means.

From the respiration rates the total accumulating CO_2 production was calculated under each combination of storage conditions. Figure 2.4 shows the total accumulating CO_2 production per kg irradiated wheat, inoculated with *F. graminearum* and stored at different a_w and temperature conditions for 10 days.

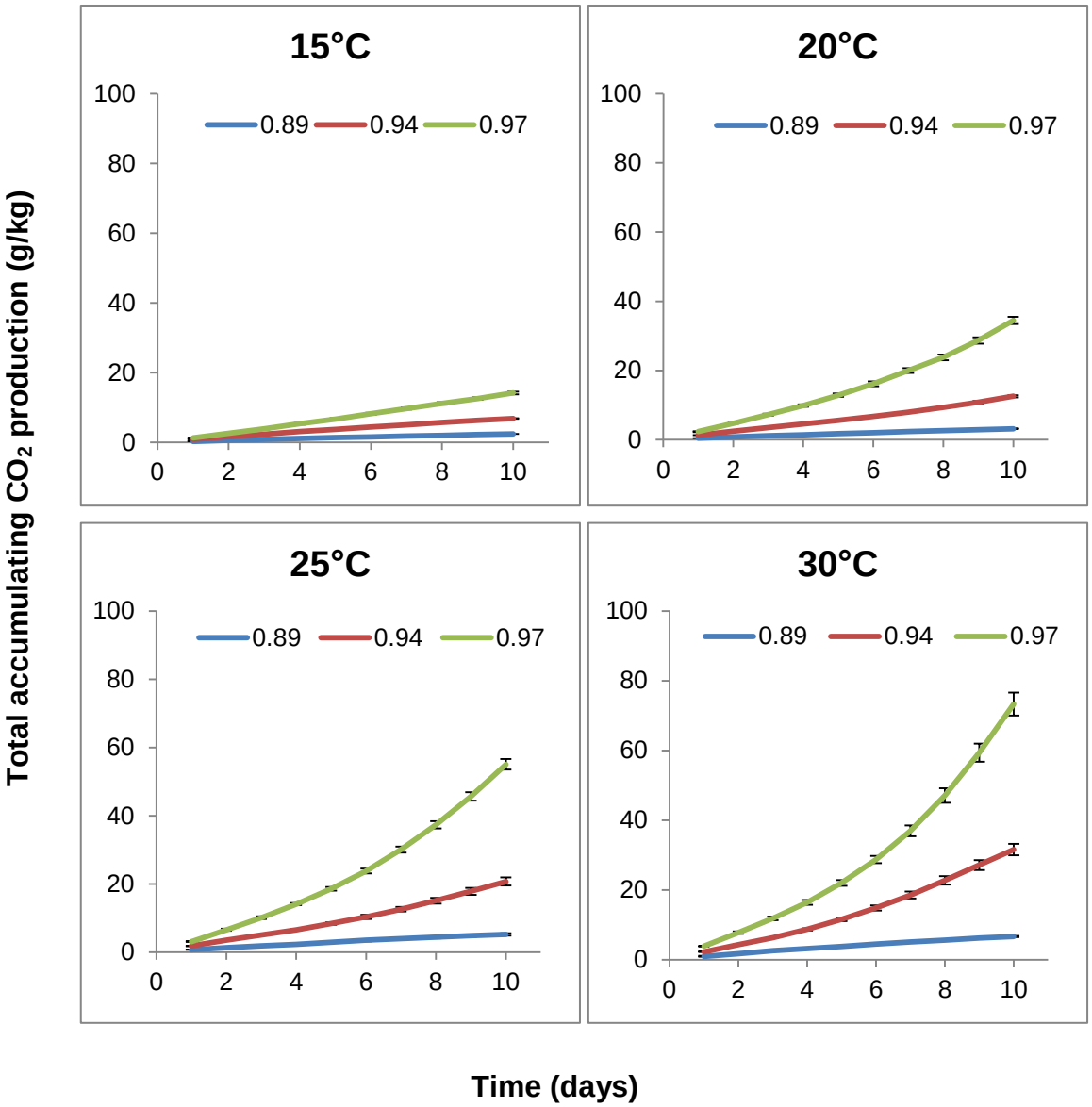


Figure 2.4: Total accumulating CO_2 production (g/kg) in irradiated wheat inoculated with *F. graminearum* and stored at different combinations of water activity and temperature over 10 days. Vertical bars indicate the standard error of the means.

It can be observed that total CO_2 production was very low in the driest conditions (0.89 a_w), but generally increased with increasing a_w and T over time and the maximum was observed at the wettest conditions and 30°C. A similar trend for the total CO_2 production with increasing a_w and T over time was observed during *F. verticillioides* colonisation of maize (Figure 2.5).

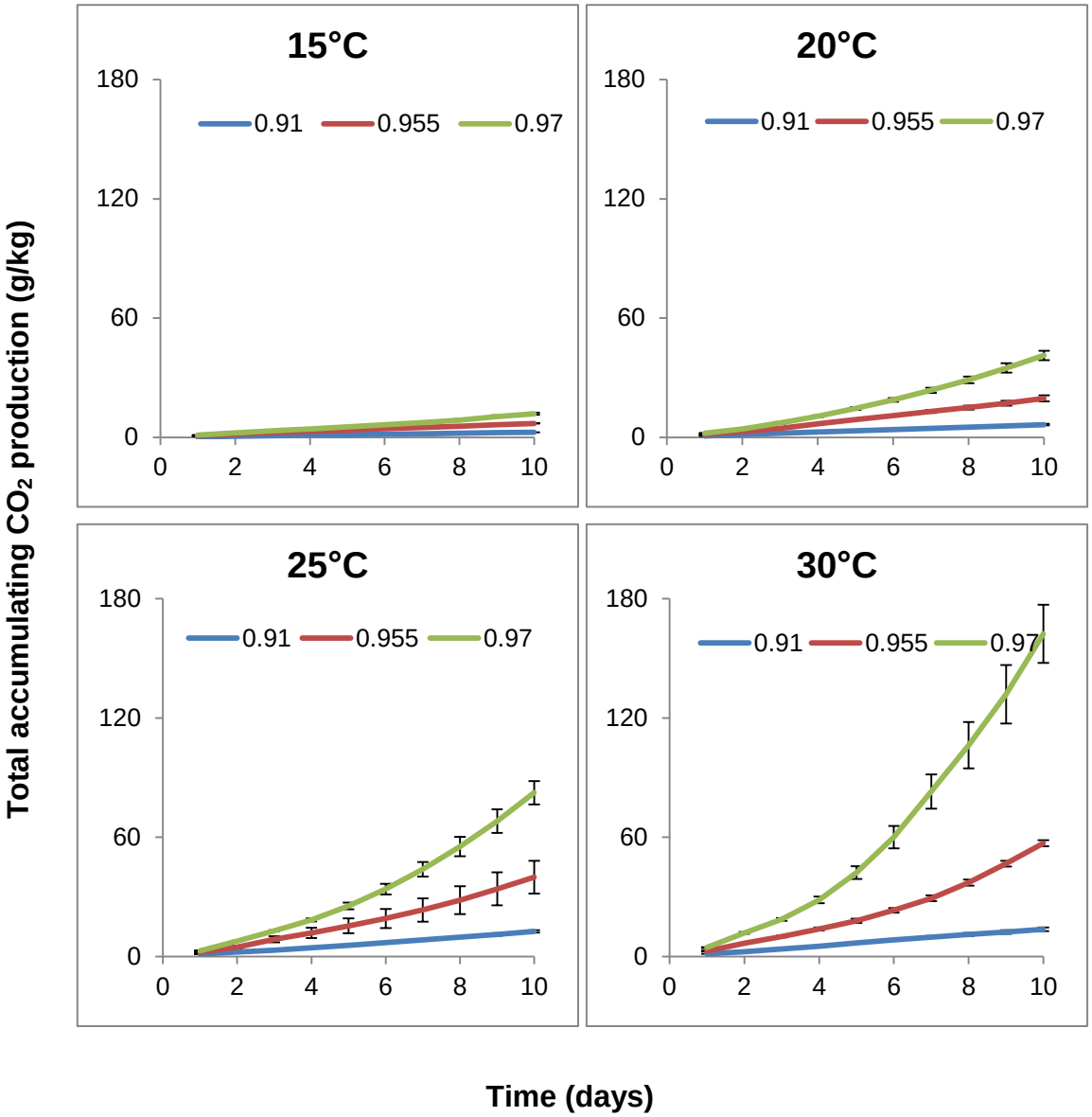


Figure 2.5: Total accumulating CO_2 production (g/kg) in irradiated maize inoculated with *F. verticillioides* and stored at different combinations of water activity and temperature over 10 days. Vertical bars indicate the standard error of the means.

Overall, the total accumulating CO_2 production in the maize experiment was higher than that in the wheat experiment, in line with the higher respiration rates observed in the maize experiment (Figure 2.2 and Figure 2.3).

2.3.3 Dry matter losses in wheat and maize as affected by the storage conditions

Figure 2.6 shows the DMLs caused in wheat inoculated with *F. graminearum* (a) and maize inoculated with *F. verticillioides* (b) after 10 days storage at different combinations of water activity and temperature. The maximum standard errors (SE_{max}) in the determination of DMLs are included in the legend of Figure 2.6.

Dry matter losses (DMLs) in wheat and maize caused by *F. graminearum* and *F. verticillioides* respectively increased with increasing a_w and temperature. The maximum in both cases was observed at the wettest conditions and highest temperature. DMLs were higher in maize (~15%) than in wheat (~7%).

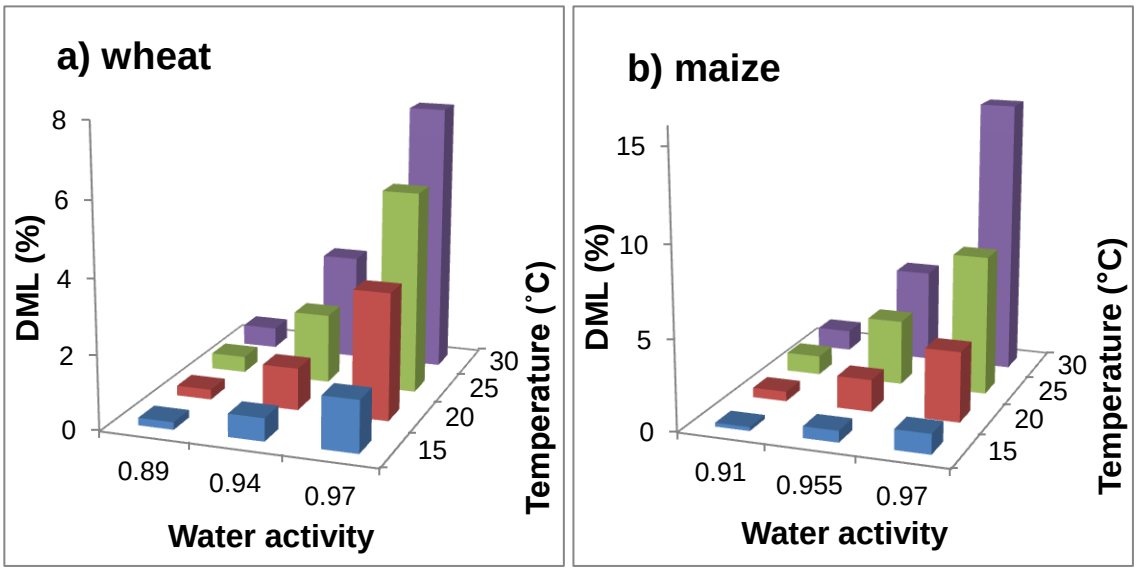


Figure 2.6: Mean DMLs (%) in (a) wheat inoculated with *F. graminearum* and (b) maize inoculated with *F. verticillioides* after 10 days storage at interacting a_w and temperature conditions (wheat: SE_{max} = 0.33, maize: SE_{max} = 1.38).

Statistically, the effect of temperature, a_w and their interaction ($T \times a_w$) on log_{DMLs} in wheat was highly significant (Apx. Table B.1). For maize the effects of temperature and a_w were highly significant on DMLs while the effect of their interaction ($T \times a_w$) was significant (Apx. Table B.2).

2.3.4 Enumeration of fungi isolated from the grain samples after 10 days storage

Table 2.1 shows the logarithm of the *F. verticillioides* colony forming units (log_{CFUs}) isolated from the maize samples after 10 days storage at different combinations of storage conditions.

Table 2.1: Logarithm of *F. verticillioides* colony forming units (CFUs) isolated from the maize samples after 10 days storage at different combinations of water activity and temperature conditions

a_w \ T	15°C	20°C	25°C	30°C
0.91	-	6.16	7.30	7.57
0.955	6.74	>7	>7	>7
0.97	7.00	>7	>7	>7

The serial dilution technique and the spread plate method were also performed for the wheat samples inoculated with *F. graminearum* however it was not possible to accurately count the CFUs in this case.

Plate 2.1 shows the (a) wheat and (b) maize samples of this experiment inoculated with *F. graminearum* and *F. verticillioides* respectively, after 10 days of storage at the wettest and warmer conditions (0.98 a_w, 25 and 30°C).

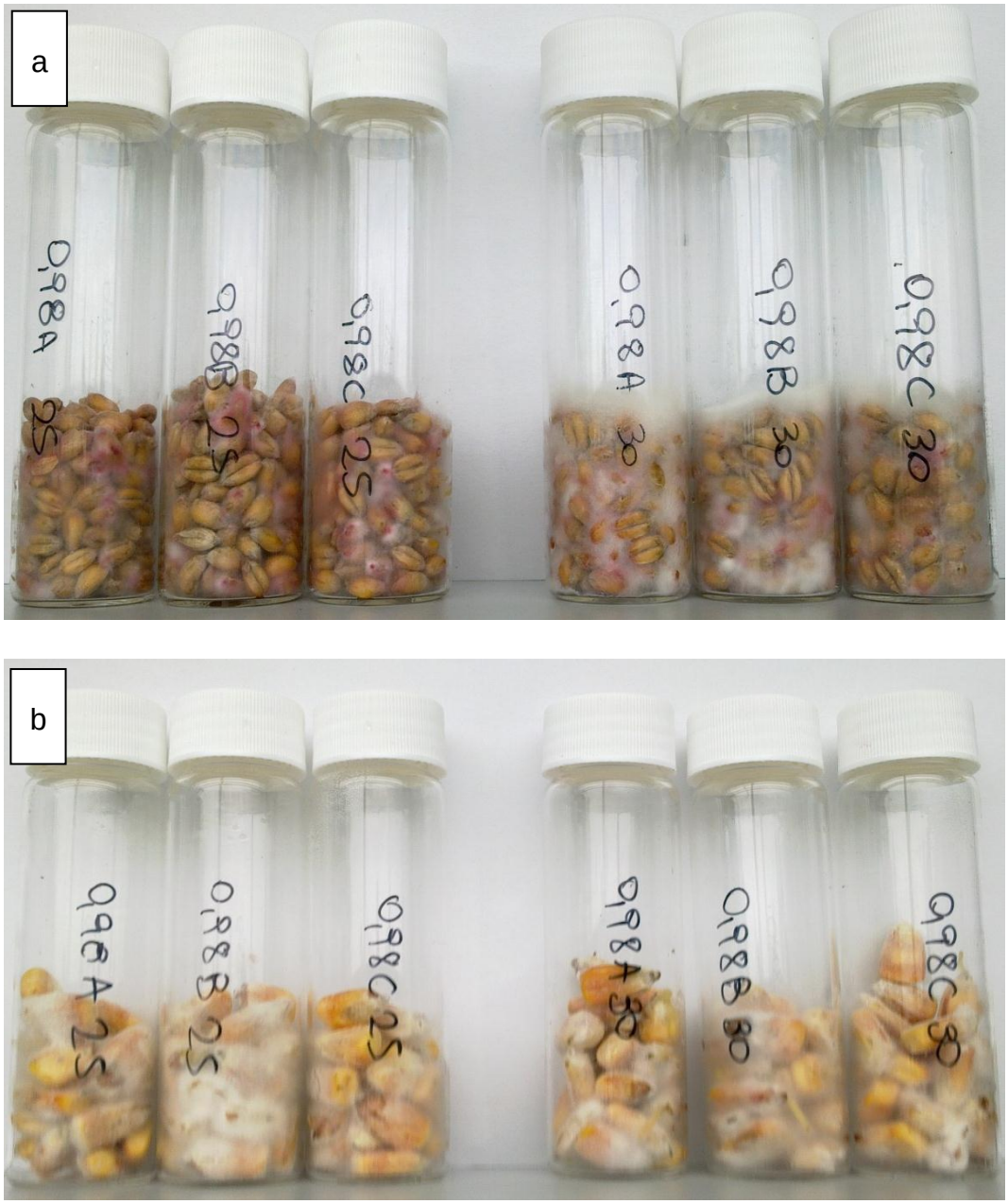


Plate 2.1: Irradiated (a) wheat and (b) maize samples of 0.98 a_w inoculated with *F. graminearum* and *F. verticillioides* respectively after 10 days storage at 25 (left) and 30°C (right).

2.3.5 Mycotoxin production in wheat and maize as affected by the storage conditions

Figure 2.7 shows the production of (a) DON and (b) ZEA by *F. graminearum* in wheat at different combinations of a_w and temperature. The maximum standard error (SE_{max}) in the determination of each toxin is also included in the legend.

DON and ZEA levels were low at all temperatures at 0.89 a_w and at all a_w levels at 15ºC, although some DON was produced at 0.97 a_w and 15°C. Optimum DON production was observed at 30°C while optimum ZEA production at 25°C at the wettest conditions.

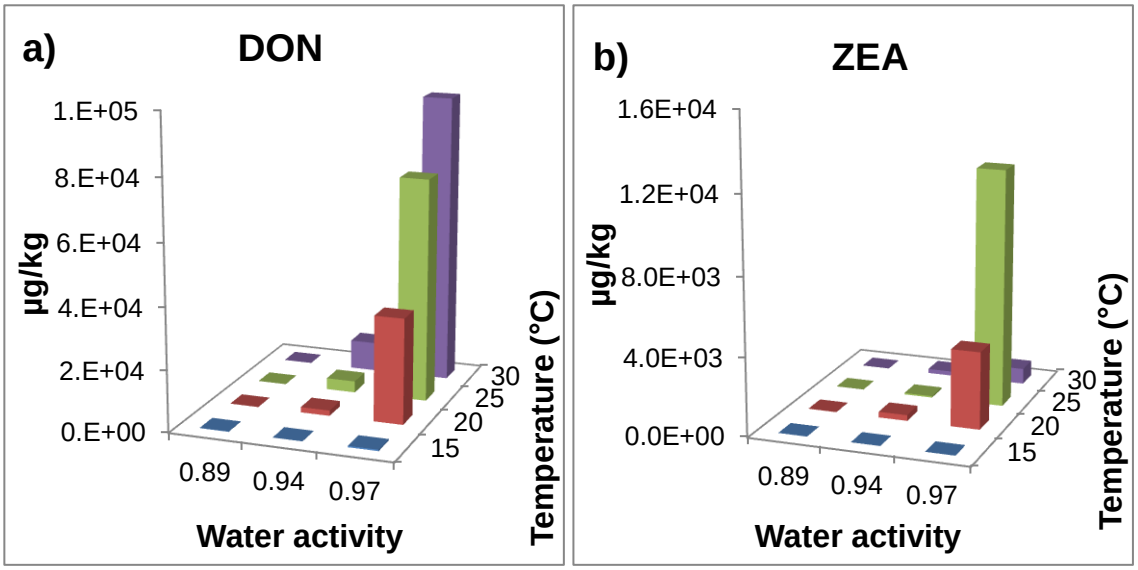


Figure 2.7: Production of (a) DON and (b) ZEA in wheat (DON: $SE_{max}=5.9\times10^3$ and ZEA: $SE_{max}=8.2\times10^2$) by *F. graminearum* at different storage conditions.

Figure 2.8 shows fumonisins production in maize by *F. verticillioides* at different combinations of a_w and temperature. The maximum standard error (SE_{max}) in the determination is included in the legend of the Figure.

Fumonisins (FB_1+FB_2) production was higher at 0.97 a_w and 30°C while at lower a_w levels the optimum was at 25°C. No FUMs were produced at 15°C in

any of the samples over the time scale of this experiment, while under the driest conditions tested (0.91 a_w) some FUMs were produced at 25°C.

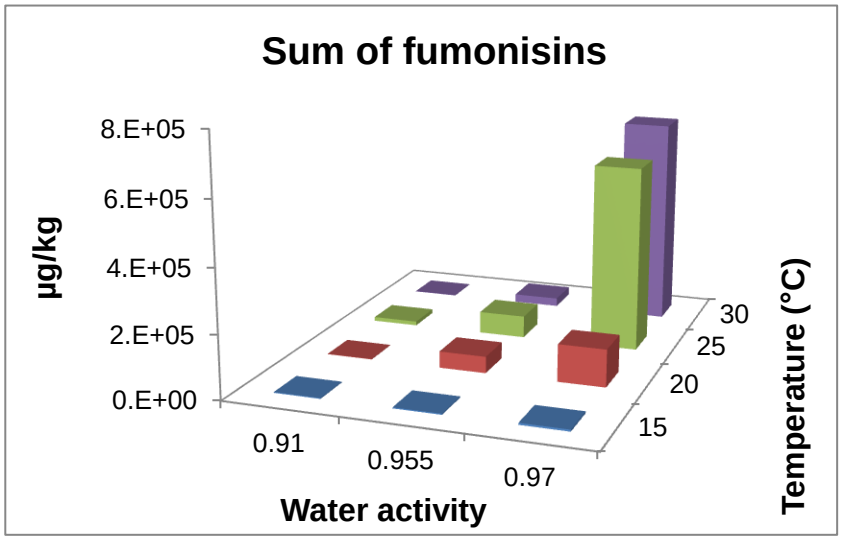


Figure 2.8: Production of fumonisins (B_1+B_2) in maize (FUMs: SE_{max}=1.4×10^5) by *F. verticillioides* at different storage conditions.

Data were also collected on the production of each individual fumonisin (B_1, B_2, B_3 and B_4) produced by *F. verticillioides* in the maize samples at the different storage conditions and they are presented in Figure 2.9.

The optimum for FB_1 and FB_3 production in the maize samples was at 0.97 a_w and 30°C, while at 0.955 a_w optimum production shifted at 25°C. Higher amounts of FB_2 and FB_4 were produced at 25°C at all water activities.

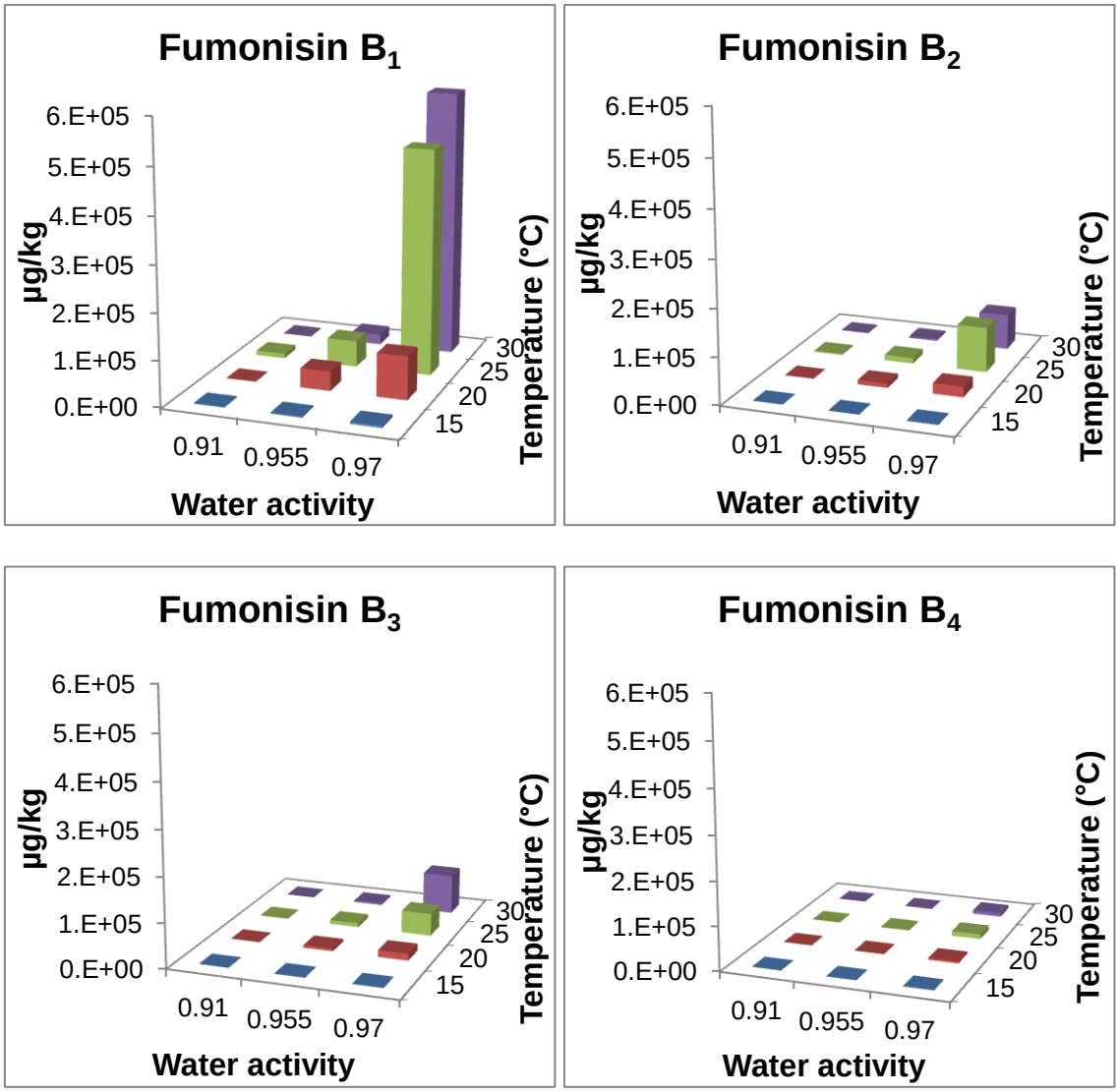


Figure 2.9: Production of fumonisins B_1, B_2, B_3 and B_4 by *F. verticillioides* in stored maize under different environmental water activity and temperature conditions (FB_1: $SE_{max}=2\times10^5$, FB_2: $SE_{max}=0.15\times10^5$, FB_3: $SE_{max}=0.16\times10^5$, FB_4: $SE_{max}=0.03\times10^5$).

The ratios between the different fumonisins produced in the maize samples at different combinations of storage conditions were also calculated and are presented in Tables 2.2-2.5. Storage conditions at which toxin contamination was above the control (T≥20°C, a_w≥0.955) are shown in bold in the Tables.

Table 2.2: Ratio between fumonisin B_1 and fumonisin B_2 (FB_1/FB_2) in maize inoculated with *F. verticillioides* at different storage conditions

a_w \ T	15°C	20°C	25°C	30°C
0.91	5.79	7.67	5.07	6.67
0.955	6.80	**4.69**	**5.33**	**5.81**
0.97	5.86	**4.28**	**4.95**	**7.61**

At the conditions where toxin contamination was above the control (T≥20, a_w≥0.955), the ratio FB_1/FB_2 was found to be between 4 and 8 and to increase with increasing temperature and a_w.

The following Tables show the ratios between fumonisins B_2/B_3, B_1/B_3 and B_2/B_4 in the maize samples of this experiment under different combinations of storage conditions. Where toxin contamination was above the control, the amount of FB_2 produced was 1-2 times that of FB_3, except at 0.97 a_w and 30°C where more FB_3 was produced.

Table 2.3: Ratio between fumonisin B_2 and fumonisin B_3 (FB_2/FB_3) in maize inoculated with *F. verticillioides* at different storage conditions

a_w \ T	15°C	20°C	25°C	30°C
0.91	2.14	1.83	2.51	1.97
0.955	1.62	**1.65**	**1.24**	**1.07**
0.97	2.78	**1.48**	**1.82**	**0.86**

From Table 2.4 it can be observed that for T≥20 and a_w≥0.955, between 6 to 9 times more FB_1 was produced than FB_3 and the pattern of production was irregular at different combinations of storage conditions.

Table 2.4: Ratio between fumonisin B_1 and fumonisin B_3 (FB_1/FB_3) in maize inoculated with *F. verticillioides* at different storage conditions

a_w \ T	15°C	20°C	25°C	30°C
0.91	10.31	14.33	11.14	12.11
0.955	11.00	**7.61**	**6.41**	**6.22**
0.97	15.16	**6.30**	**9.07**	**6.51**

At T≥20 and a_w≥0.955, 5-10 times more FB_2 was produced than FB_4 while the rate of FB_2 production increased with increasing temperature for a given a_w.

Table 2.5: Ratio between fumonisin B_2 and fumonisin B_4 (FB_2/FB_4) in maize inoculated with *F. verticillioides* at different storage conditions

a_w \ T	15°C	20°C	25°C	30°C
0.91	10.05	13.29	13.55	14.17
0.955	16.16	**6.31**	**7.24**	**7.67**
0.97	11.53	**5.22**	**8.46**	**9.06**

The Kruskal-Wallis test by ranks showed that the effect of a_w was highly significant on DON production, while the effect of temperature was not significant. For ZEA and FUMs the effect of a_w was highly significant, while the effect of temperature was significant (Apx. Tables B.3-B.5).

2.3.6 Development of models for the effect of storage conditions on dry matter losses and toxin production in wheat and maize

The general Equation 2-3 containing linear and quadratic effects of a_w and temperature and their interaction (a_w×T) was used to describe the effect of the

environmental factors on the DMLs as well as on toxin production by *F. graminearum* and *F. verticillioides* in stored wheat and maize respectively.

$$Y = b_0 + b_1 a_w + b_2 T + b_3 a_w^2 + b_4 T^2 + b_5 a_w T \qquad \textbf{Equation 2-3}$$

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.

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 2.6.

The quadratic effect of temperature (T^2) was not significant in either DMLs or DON production in wheat. Using only the data between 15-25°C for input in the regression, all factors except T^2 were found significant in ZEA production in wheat.

For maize, the residuals for DMLs were not normally distributed and therefore the data were logarithmically transformed before regression. All the effects were significant on $\log_{DMLs}$ except of the effect of T, while only the effects of T, T^2 and a_w×T were significant on $\log_{FUMs}$.

Table 2.6: Values of coefficients b_0-b_5 and statistical significance of the relevant factors in the model equation for DMLs and for toxin production (Equation 2-3) as determined by forward stepwise regression. Highly significant p-values are shown in bold.

	DMLs wheat		**log_{DMLs} maize**		**DON**		**ZEA (up to 25ºC)**		**log_{FUMs}**	
	b	**p-value**	**b**	**p-value**	**b**	**p-value**	**b**	**p-value**	**b**	**p-value**
Intercept (b_0)	**769.04**	**<0.001**	**137.81**	**<0.001**	**16946335**	**<0.001**	**2065876**	**<0.001**	5.84	0.254
a_w (b_1)	**-1610.15**	**<0.001**	**-305.32**	**<0.001**	**-35565498**	**<0.001**	**-4233345**	**<0.001**	-	-
T (b_2)	**-3.99**	**<0.001**	-0.13	0.119	**-64863**	**<0.001**	**-13347**	**<0.001**	**-1.31**	**0.005**
a_w^2 (b_3)	**839.39**	**<0.001**	**166**	**<0.001**	**18574400**	**<0.001**	**2164948**	**0.013**	-9.52	0.090
T^2 (b_4)	0.00	0.668	**-0.003**	**<0.001**	-27	0.704	24	0.424	**-0.01**	**<0.001**
T * a_w (b_5)	4.42	**<0.001**	**0.33**	**<0.001**	73320	**<0.001**	13693	**<0.001**	**2**	**<0.001**

Contour maps were constructed for the DMLs and DON in wheat and log_{DMLs} and log_{FUMs} in maize with regard to the storage conditions with Microsoft Office Excel 2007, including only those factors that were found to be significant (Table 2.6). These maps are presented in Figure 2.10. From these maps the

combination of storage conditions where DMLs and toxin production are optimum and those where these are marginal can be clearly identified.

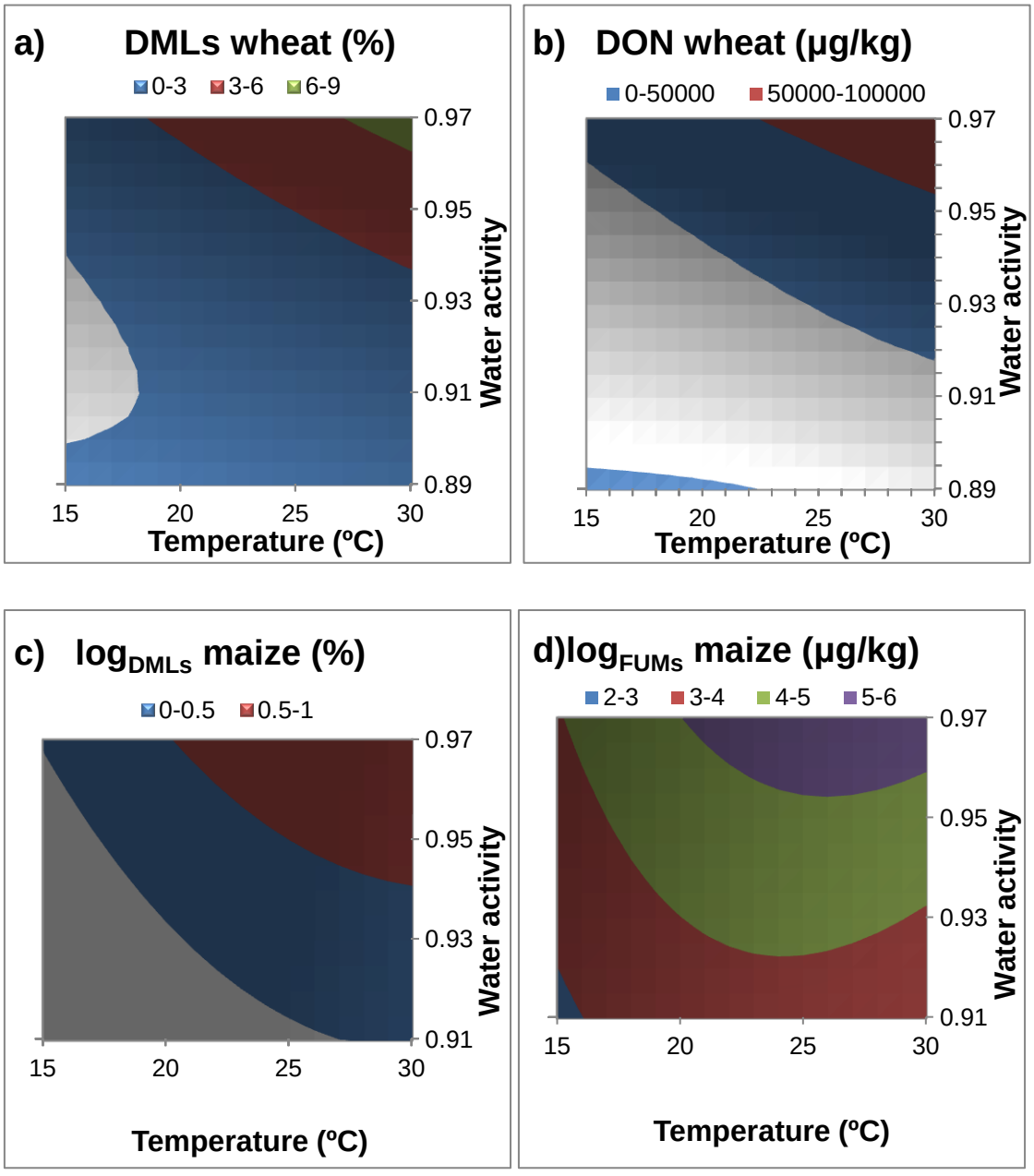


Figure 2.10: Contour maps describing (a) the DMLs and (b) DON accumulation in irradiated wheat during colonisation by *F. graminearum* and (c) the $\log_{DMLs}$ and (d) $\log_{FUMs}$ production in maize during colonisation by *F. verticillioides* under different combinations of environmental conditions.

2.3.7 Correlation between dry matter losses and toxin production in wheat and maize

DML data in wheat were plotted against DON data (Figure 2.11). A highly significant positive correlation was obtained between the two variables (Spearman Rank Order Correlations test: R=0.957, p<0.001). The green line denotes the EU limit for DON in wheat (1750 µg/kg). More than 80% of the samples with >1% DMLs exceeded the EU limits for DON in wheat.

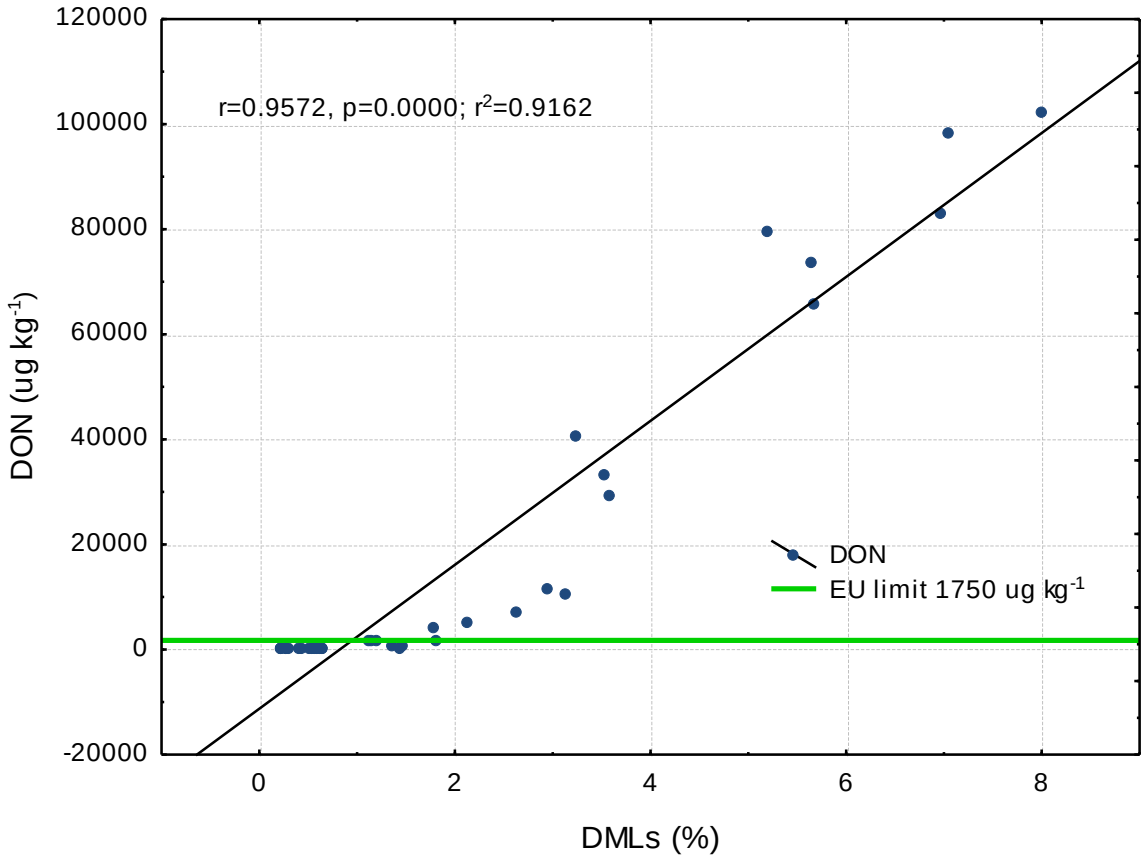


Figure 2.11: Scatter plot of DMLs and DON production during colonisation of stored wheat by *F. graminearum*. The green line indicates the EU legislative limit for DON in wheat.

A highly significant positive correlation (R=0.8371, p<0.001) was also obtained between DMLs and the sum of fumonisins (B_1 and B_2) in maize (Figure 2.12). The green line denotes the EU limits for total fumonisins in maize (4000 µg/kg). More than 75% of the maize samples with >0.9% DMLs exceeded the EU limits for fumonisins. Notably, one maize sample contained total fumonisin levels above the EU limits with as little as 0.66% DMLs.

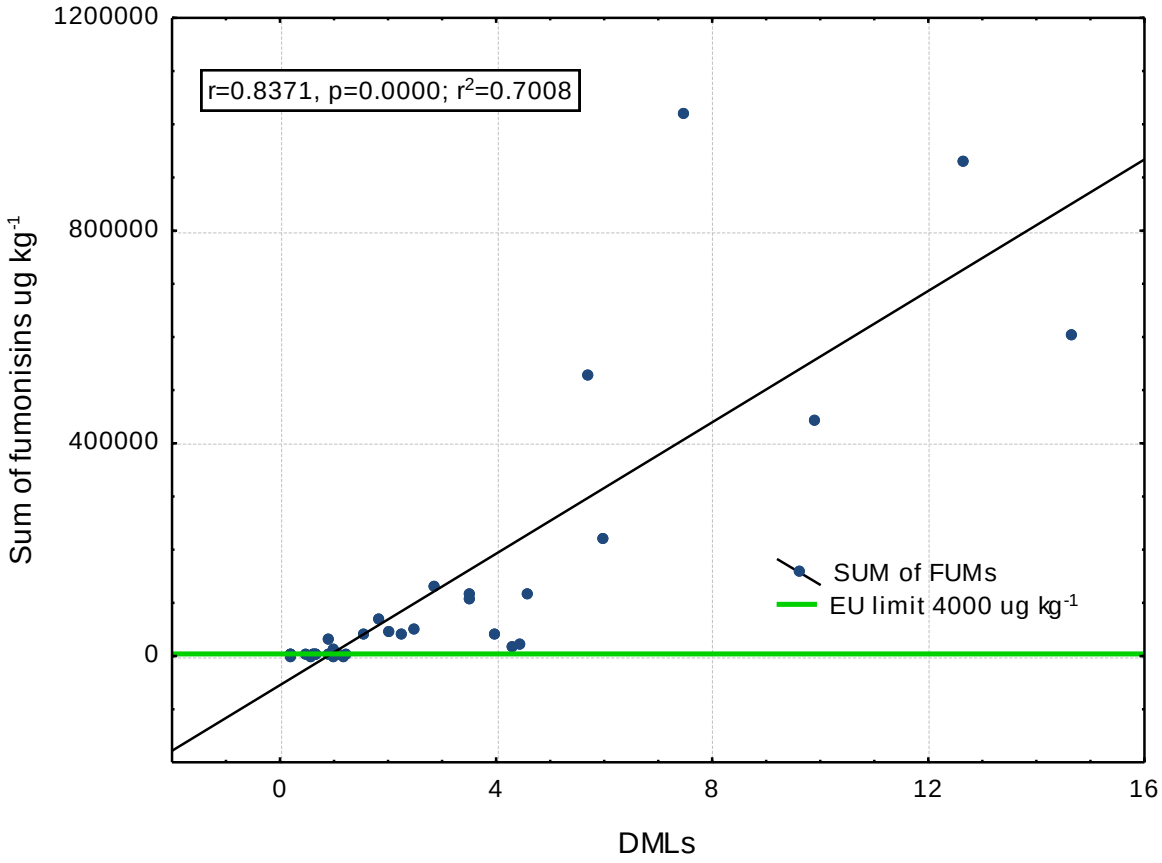


Figure 2.12: Scatter plot of DMLs and sum of fumonisins production during colonisation of stored maize by *F. verticillioides.* The green line indicates the EU limit for the sum of fumonisins in unprocessed maize.

Plotting the data for ZEA against DMLs in a similar way also gave a statistically highly significant positive correlation (R=0.5536, p<0.001). However, a much

higher significant correlation (R=0.916, p<0.001) between ZEA and DMLs in wheat was obtained if only the data between 15 and 25°C were considered (Figure 2.13). Just above 70% of the samples with DMLs >1% exceeded the EU legislative limits for ZEA in the wheat samples.

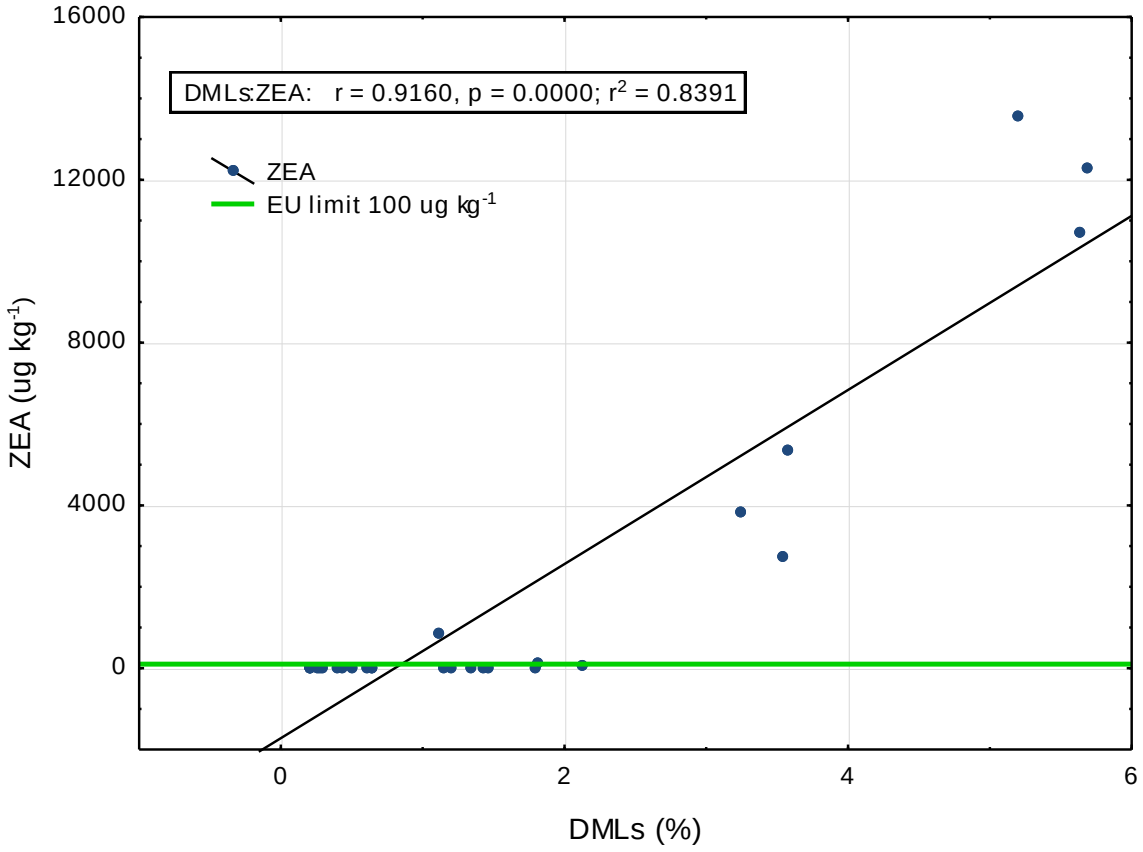


Figure 2.13: Scatter plot of DMLs and ZEA production during colonisation of stored wheat by *F. graminearum* for the temperature range 15-25°C. The green line indicates the EU limits for ZEA in wheat.

2.4 Discussion

2.4.1 Respiration and dry matter losses in stored wheat and maize at different combinations of storage conditions

In the wettest conditions of this experiment respiration rates generally started increasing after 2-4 days incubation, probably coinciding with the initiation of fungal growth. In contrast, in the driest treatments (0.89 a_w) the respiration was constantly very low. This respiration can be attributed to the grain, since no other signs of fungal growth were observed at these conditions. The slight reduction in the respiration rate over time may be attributed to loss of some water during storage. If the respiration observed on the first day of the experiment (time zero) is considered as mainly that of the grain, this increased with increasing a_w and temperature suggesting that grain respiration is higher under wetter and warmer conditions (Figure 2.2). The contribution of the growing fungi to the total respiration increased more significantly with time. It was calculated that on the 10^{th} day of storage and under the most favourable storage conditions >85% of the total respiration in wheat could be attributed to the fungi. Similarly in maize, under the most favourable conditions tested >90% of the respiration measured on the 10^{th} day of storage was due to *F. verticillioides* colonisation. The respiration of the grain under each combination of storage conditions has not been subtracted from the total respiration measured as this was considered unrealistic, since in practice these two could not be dissociated, i.e. during CO_2 monitoring in silos.

Respiration during storage could thus be paralleled to the growth of *F. graminearum* on wheat and *F. verticillioides* on maize. The higher respiration in wetter and warmer conditions parallels the reported growth optima for *F. graminearum* (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), the CO_2 data in this study indicate that this species may grow even at 0.91 a_w at T>20°C. Highest respiration in maize was observed at 30°C and 0.97 a_w, conditions that have

been reported as optima for *F. verticillioides* 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 CO_2 produced during storage. It is believed that *in situ* this method may be more accurate for monitoring the potential for spoilage in cereals and improve the management of the post-harvest storage phase.

In line with the increasing respiration rates and CO_2 production, dry matter losses in both the wheat and maize experiments were higher at higher water activity and higher storage temperature. Also a highly significant correlation was observed between the $\log_{CFU}$ populations of *F. verticillioides* and $\log_{DML}$ data ($R=0.69$, $p<0.001$) which may be explained by the fact that increasing fungal populations could cause higher uptake of nutrients from the grain and therefore higher losses to the grain dry matter. DMLs observed in the maize experiment were higher (~15%) than in the wheat (~7%). One reason for this could be higher *F. verticillioides* populations in maize than *F. graminearum* in wheat. However this cannot be confirmed since it was not possible to obtain accurate CFU counts in the wheat experiment. Another reason could be the higher number of broken kernels in the maize used in this experiment which was of lower quality than the wheat. This has been suggested to affect susceptibility to fungal infection (Tuite et al., 1985; Ng et al. 1998) as it provides a more easily accessible substrate and higher nutrient availability to the fungi.

2.4.2 Models for the effect of storage conditions

The contour maps developed for the effect of interacting a_w×temperature conditions on DMLs and on mycotoxin production may be very useful tools in identifying high- and low-risk environmental conditions for the safe storage of grains. The data obtained in this study can be used as input to models previously developed (Lindblad et al., 2004; Magan et al., 2010) and be integrated in storage management systems. However it is of particular importance to develop similar maps for the whole range of metabolites that can

be produced under different combinations of storage conditions by a single species or by mixed populations which are more often found in naturally contaminated grain. By combining such maps, it could be possible to identify the conditions under which contamination of the grain is less likely since different fungal species produce mycotoxins optimally at different environmental conditions. Thus, temperature or a_w stress applied during control strategies may reduce the risk of contamination with one toxin but could in fact stimulate the production of other toxins. 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 production by *Penicillium* species, which has been shown to occur at temperatures 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 is also of particular importance due to the observed climate change and the increasing global temperatures.

2.4.3 Correlations between dry matter losses and mycotoxin contamination

Highly significant correlations have been obtained between DMLs and toxin production in stored wheat and maize. These suggest than the EU limits for DON and ZEA contamination of wheat or fumonisin contamination of maize are likely to be exceeded when very small DMLs have been reached (0.9-1%). This information could be valuable when designing management strategies for the safe storage of grains post-harvest. Continuous monitoring of CO_2 in silos and determination of DMLs as a possible indicator of mycotoxin contamination of the stored grain could minimise the chances of grain lots exceeding the legal limits for the toxins of concern.

DMLs have previously been considered only as an indicator of quality deterioration in grain (White et al., 1982a, b; Lacey et al., 1994). In terms of ensuring the safety of stored grains, suggested methods for the estimation of

toxin contamination involved colony counts of mycotoxigenic fungi or ergosterol measurements (Lund and Frisvad, 2003; Lindblad et al., 2004; Yong and Cousin, 2001). Lund and Frisvad (2003) and Lindblad et al. (2004) suggested that for *Penicillium verrucosum* the presence of around 3 $\log_{CFUs}$/g of wheat could indicate OTA contamination above the EU legislative limits. Generally the serial dilution and spread plate technique is considered more appropriate for highly sporulating fungi (*Penicillium*, *Aspergillus*) while less appropriate for *Fusarium* species (Thrane, 1996). Despite this, in this study the *F. verticillioides* populations isolated from the maize samples that contained total fumonisin levels above the EU limits after 10 days storage were >7 $\log_{CFUs}$/g grain (Table 2.1). The correlation between CFUs and the sum of FUMs (R=0.45) was significant although such relationships have to be considered with caution since fungi do not necessarily produce higher amounts of toxin at the conditions of optimum growth. As was shown in the maize experiment at intermediate a_w fumonisin production was optimum at 25°C while *F. verticillioides* growth was optimum at 30°C. Several studies have suggested that fungi produce high levels of toxins under conditions of temperature or water stress.

The results obtained in this study suggest that *F. verticillioides* CFUs could be used as a contamination risk index in stored maize for long term monitoring of a stored batch. However, there are certain limitations to this technique, mostly related to representative sampling, lengthy incubation time for CFU counts to be obtained and thus the likelihood of reduced accuracy. Contrarily CO_2 production in a stored grain batch can be observed in real time and it is more accurate and therefore may be more appropriate than traditional mycological methods for the monitoring of the condition of grain during in silo storage.

2.4.4 Relationships between other toxins produced

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, fumonisin B_1 and B_2 production was determined in maize and was observed that at intermediate a_w levels the optimum production shifted at lower temperatures, something that has also been observed previously with *F. verticillioides* and *F. proliferatum* (Marín et al., 1999).

In addition in this study fumonisins B_3 and B_4 were also detected in all the maize samples. While fumonisin B_4 was present in smaller amounts, fumonisin B_3 was present at levels similar to that of fumonisin B_2 and occasionally even higher – especially at 30°C and 0.955 and 0.97 a_w (Table 2.3). This is of particular importance since the maximum legislative limits established for fumonisins in maize (EC, 2006a) only take into account fumonisins B_1 and B_2. Therefore the total fumonisin content of a sample may be significantly underestimated if FB_3 is also present in higher amount than FB_2. Also under climate change scenarios a global temperature rise may shift mycotoxin contamination in maize to fumonisin B_3 instead of FB_1 and FB_2.

It has also been observed that FB_3 seems to be following the same pattern of production as FB_1 (optimum at 0.97 a_w and 30°C and shift of optimum production at 25°C at lower temperatures) while FB_4 seems to be produced optimally under similar conditions as FB_2 (optimum production at 25°C at all water activities). This may indicate that fumonisins B_1 and B_3 are produced by *F. verticillioides* via the same biosynthetic pathway in maize, while fumonisins B_2 and B_4 by a different common mechanism. While significant progress has been made in the recent years in elucidating the mechanism of fumonisin biosynthesis, still the relationship between the individual fumonisins is unknown (Alexander et al., 2009).

3 *FUSARIUM LANGSETHIAE:* GROWTH, T-2 AND HT-2 TOXIN PRODUCTION AND DRY MATTER LOSSES IN OATS

3.1 Introduction

Fusarium langsethiae is a relatively newly identified species in the *Fusarium* genus. It was first identified as a T-2 and HT-2 producer in cereals in Norway and was originally described as a "powdery *Fusarium poae*" (Torp and Langseth, 1999; Langseth and Rundberget, 1999) due to its similar metabolite profile to that of *Fusarium sporotrichioides* and its similar morphological resemblance to *Fusarium poae*. It was finally described as a new species in 2004 by Torp and Niremberg (2004).

T-2 and HT-2 toxins belong to the trichothecenes (Group A) and they are very similar in structure, differing only at the C-4 functional group. T-2 toxin is very easily metabolised to HT-2 toxin, often resulting in the co-occurrence of the two toxins in contaminated cereals (JECFA, 2001). These toxins are very similar in structure to Group B trichothecenes (deoxynivalenol, nivalenol) produced by several *Fusarium* species. T-2 and HT-2 toxin have been evaluated as generally toxic, immunotoxic and heamatotoxic, they can inhibit protein synthesis as well as DNA /RNA synthesis *in vitro* and *in vivo,* while more recent evidence suggests genotoxic and cytotoxic effects and even tumorigenicity in animals (JECFA, 2011). However, since no sufficient evidence exists for carcinogenicity in animals or humans (JECFA, 2001; SCF, 2001; JECFA, 2011), maximum levels have not yet been set for these toxins in food or feed in Europe. Limits established in other parts of the world were however reviewed during the recent re-evaluation of these trichothecenes (JECFA, 2011) and these are most commonly established around 100 µg/kg.

To date most studies on *F. langsethiae* have focused on molecular techniques (Wilson et al., 2004; Yli-Mattila et al. 2008; Nicolaisen et al., 2009; Fredlund et al., 2010) and metabolite profiles (Thrane et al., 2004) in an attempt to clearly

differentiate *F. langsethiae* from its closely related species *F. poae* and *F. sporotrichioides*, with different levels of success. *F. langsethiae* has recently received more attention due to its association with T-2 and HT-2 toxin production.

Some preliminary information on the effect of environmental factors on the growth of *F. langsethiae* and T-2 and HT-2 production was made available by *in vitro* studies (Medina and Magan, 2010; 2011). Also Kokkonen et al. (2010) studied the growth of different *Fusarium* species including *F. langsethiae* in oats, however the storage conditions examined were limited and the study was aimed at comparing toxin production to that by *F. sporotrichioides*. Thus, no information is available on the conditions under which these toxins are formed in cereal grains during storage and data obtained from *in vitro* studies may only be extrapolated to the *in situ* behaviour with caution.

Growth boundaries of other fungal species on different grains have been determined either by semi-quantitative methods or by fungal colony diameter measurements (Cuero et al., 1987; Marín et al., 1995; Hope et al., 2005). It has been shown (Chapter 2) that CO_2 production during storage may be used as an accurate measurement of fungal growth at different interacting environmental conditions. Also, the impact of *F. langsethiae* infection on the dry matter losses (DMLs) in oats has not been examined before. Previously it was shown that DMLs can be associated with the amount of toxin produced in stored wheat and maize under different combinations of storage conditions (Chapter 2). However, such information is not available for oats and T-2 and HT-2 contamination.

The aims of this study were (a) to use CO_2 production data during storage in order to determine the environmental conditions (water activity and temperature) over which *F. langsethiae* may grow on oats (b) to analyse the effect on T-2 and HT-2 toxin production in the oat samples under the same storage conditions and (c) to calculate the DMLs caused in oats and identify any relationship to T-2 and HT-2 toxin production. These data could be very useful tools for determining the safe storage boundaries for oats as well as for predicting the risk conditions for toxin contamination in stored oats.

3.2 Materials and methods

3.2.1 Oat grain

Oats supplied by local UK farms (Bedfordshire, 2009 harvest season) were irradiated at 12 kGy (Isotron, Plc, Swindon, UK) in order to kill any fungal spores (Hope et al., 2005) and were stored at 4°C.

3.2.2 Water activity adjustment of irradiated oats

A water adsorption curve was prepared for irradiated oats as described in Paragraph 2.2.2. The time required for the oats to equilibrate accurately to the target a_w level was also determined.

3.2.3 *Fusarium langsethiae* strain and preparation of the inoculum

Fusarium langsethiae strain 2004/59, kindly supplied by Professor Simon Edwards, Harper Adams University College, UK was used in these experiments. The strain was isolated from oats in the UK and produces T-2 and HT-2 toxins. The culture was maintained on Malt Extract Agar (MEA) media (OXOID, malt extract, 30; mycological peptone, 5; agar, 15 g/l).

A spore suspension was prepared as described in Section 2.2.3 and the concentration of the spores was adjusted by dilution to 10^7 spores/ml using a haemocytometer.

3.2.4 Inoculation of the oats and incubation conditions

Irradiated oats were modified to 0.89, 0.945 and 0.97 a_w by addition of sterile water according to the water adsorption curve previously developed and allowed to equilibrate for 72 hours. The a_w levels chosen were representative of marginal, intermediate and very wet conditions and the temperatures relevant to

both northern and central European climates where *F. langsethiae* has been most commonly isolated from oats. The oats were inoculated with 1 ml spore suspension and thoroughly mixed. 3 replicates of 10 g (wet weight) were placed in 40 ml vials (Chromacol Ltd, UK) and incubated at 15, 20, 25 and 30°C in sandwich boxes containing glycerol/water solutions of the same a_w (prepared according to the Table of Appendix A).

3.2.5 Respiration measurements by Gas Chromatography

On a daily basis the samples were sealed for 1 hour to allow CO_2 accumulation in the headspace and 5 ml were analysed by Gas Chromatography (GC) as described in Section 2.2.5. The calibration of the GC, CO_2 analysis and calculation of the respiration rates and DMLs was performed as in Section 2.2.5.

3.2.6 *F. langsethiae* CFU counts

Initially, 1 h after inoculation of the oats with *F. langsethiae* and at the end of the 10-day storage, the samples were thoroughly mixed and 1 g used for serial dilutions and determination of the CFU counts by the spread plate technique as described in Section 2.2.6.

3.2.7 Toxin extraction / analysis

The oat samples were oven-dried at 60°C for 24-48 h, milled in a small laboratory blender and analysed by LC-MS/MS as in Section 2.2.7.

3.2.8 Statistical analysis

All experiments were performed in triplicate. Data were analysed as in Section 2.2.8. The standard error of the mean was calculated in all trials and is shown with vertical bars in the Figures or included in the legends of 3-D graphs.

The statistical significance has been determined and the ANOVA Tables can be found in Appendix C.

Polynomial equations were obtained and contour maps constructed as in Section 2.2.8. Correlations were assessed using the Spearman Rank Order test as described in Section 2.2.8.

3.3 Results

3.3.1 Water adsorption curves for irradiated oats

Figure 3.1 shows the water adsorption curves obtained for 10 g oat samples after addition of known volumes of water and equilibration for 24-72 h at 25°C.

The curve remained relatively stable over the equilibration period however the data of the 3rd day were used for calculation of the amount of water to be added to the oats in order to reach the target a_w levels in these experiments.

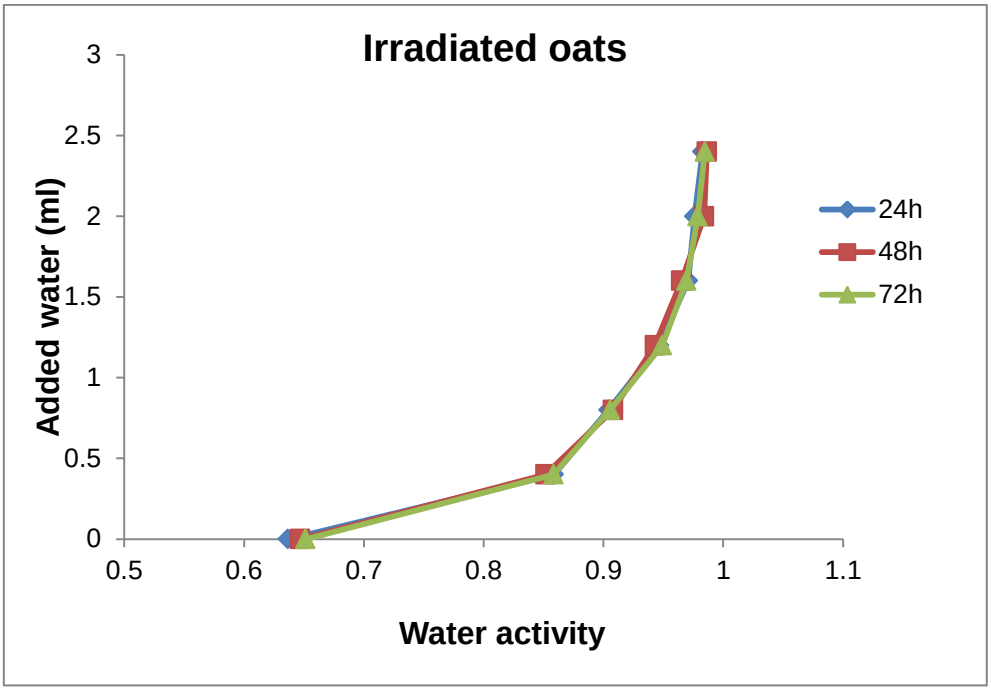


Figure 3.1: Relationship between added water (ml) and water activity of irradiated oats (10 g samples) at 25°C after 24, 48 and 72 hours of equilibration

3.3.2 Respiration rates and CO_2 production in oats as affected by the storage conditions

Figure 3.2 shows the daily respiration rates of oats adjusted to different a_w levels, inoculated with *F. langsethiae* and stored at 15-30°C for 10 days.

Respiration was constantly very low at 0.89 a_w at all temperatures over the 10 day storage period and decreased with time. At 0.945 and 0.97 a_w respiration followed an irregular pattern over time with a peak being observed at each combination of storage conditions at some point during the 10 days. Overall, higher respiration rates were observed in the wettest grain at 25 and 30°C.

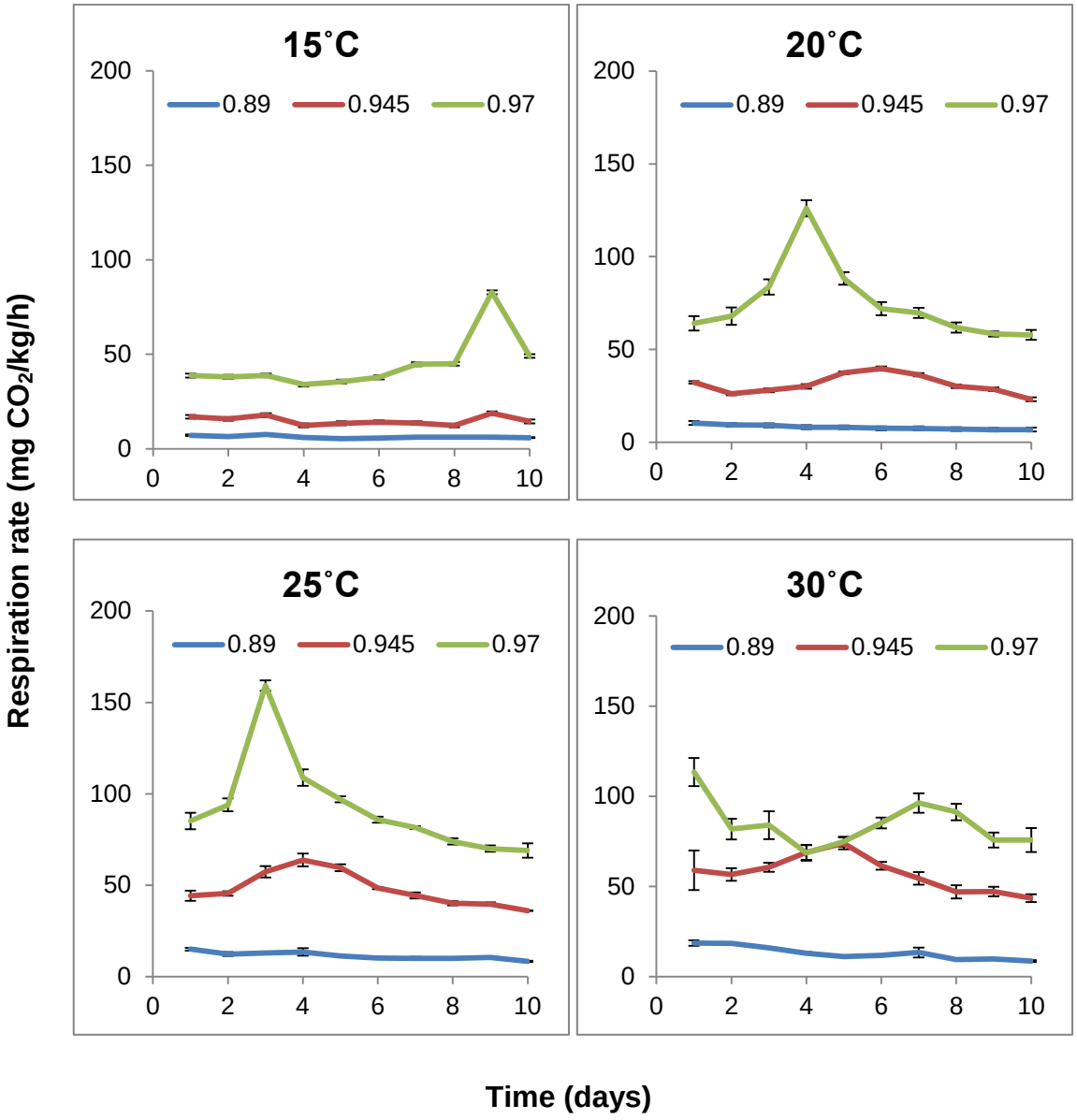


Figure 3.2: Respiration rates (mg CO_2/kg/h) of irradiated oats inoculated with *F. langsethiae* at different a_w×T conditions over 10 days. Vertical bars indicate the standard error of the means.

From the respiration rates the total accumulated CO_2 production during colonisation of oats by *F. langsethiae* over 10 days was calculated under each set of storage conditions and it is shown in Figure 3.3.

The accumulated CO_2 production increased with time at all combinations of storage conditions and the maximum was observed at 0.97 a_w and 25°C.

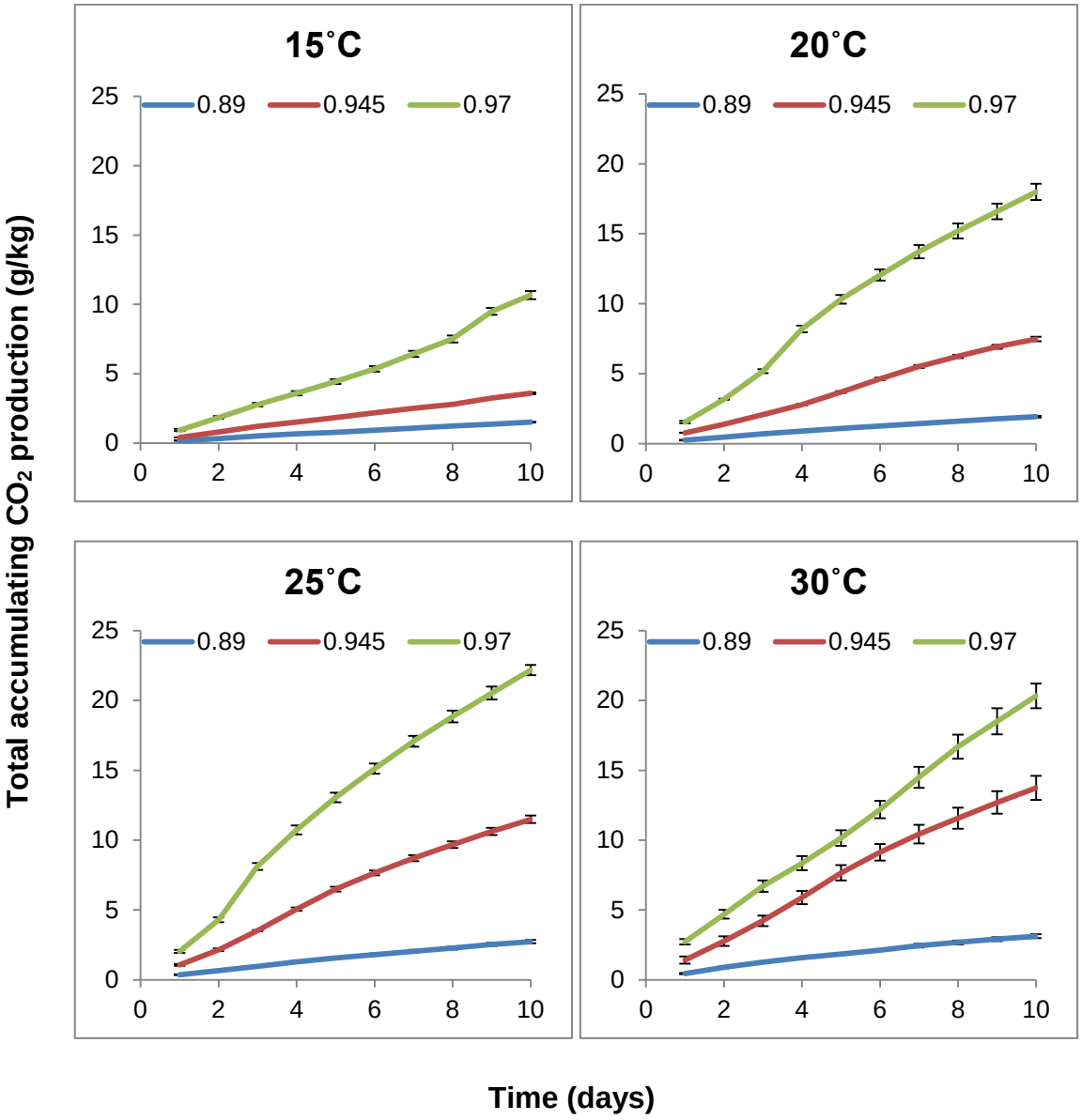


Figure 3.3: Total accumulating CO_2 production (g/kg) in irradiated oats inoculated with *F. langsethiae* at different a_w×T conditions over 10 days. Vertical bars indicate the standard error of the means.

3.3.3 Dry matter losses in oats as affected by the storage conditions

Figure 3.4 shows the DMLs caused in oats colonised by *F. langsethiae* after 10 days storage at different combinations of storage conditions.

DMLs increased with temperature at 0.89 and 0.945 a_w, however at the wettest treatment (0.97 a_w) maximum DMLs were observed at 25°C.

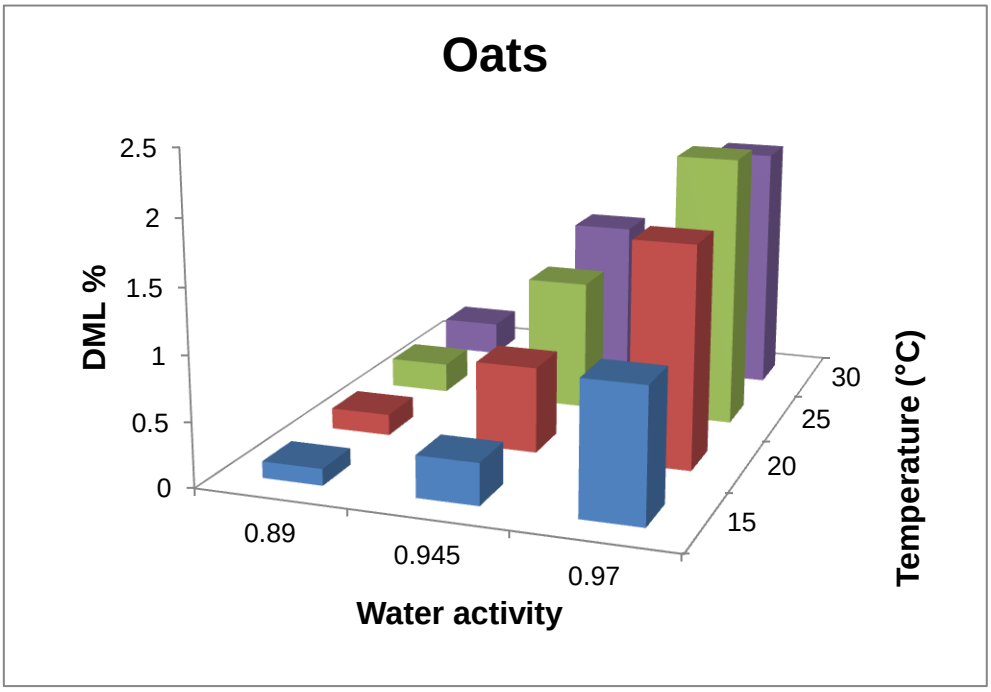


Figure 3.4: Mean DMLs (%) after 10 days of storage under interacting a_w and temperature conditions in oats inoculated with *F. langsethiae* (SE_{max} = 0.09).

The Kruskal-Wallis test by ranks showed that the effect of a_w was highly significant on DMLs but the effect of temperature was not significant (Apx. Table C.1).

A model equation containing linear and quadratic effects of a_w and temperature and their interaction (a_w×T) was used to describe the effect of the environmental factors on the DMLs in oats. All factors were significant and the complete equation developed for DMLs in oats is as follows:

$$DMLs\ oats = 260.42 - 567.2\ a_w - 0.47\ T + 306.37\ a_w^2 - 0.003\ T^2 + 0.72\ a_w\ T$$ **Equation 3-1**

A contour plot showing the DMLs in oats with regard to the storage a_w and temperature is shown in Figure 3.5.

The areas where DMLs are likely to be higher and where DMLs are small can be clearly seen.

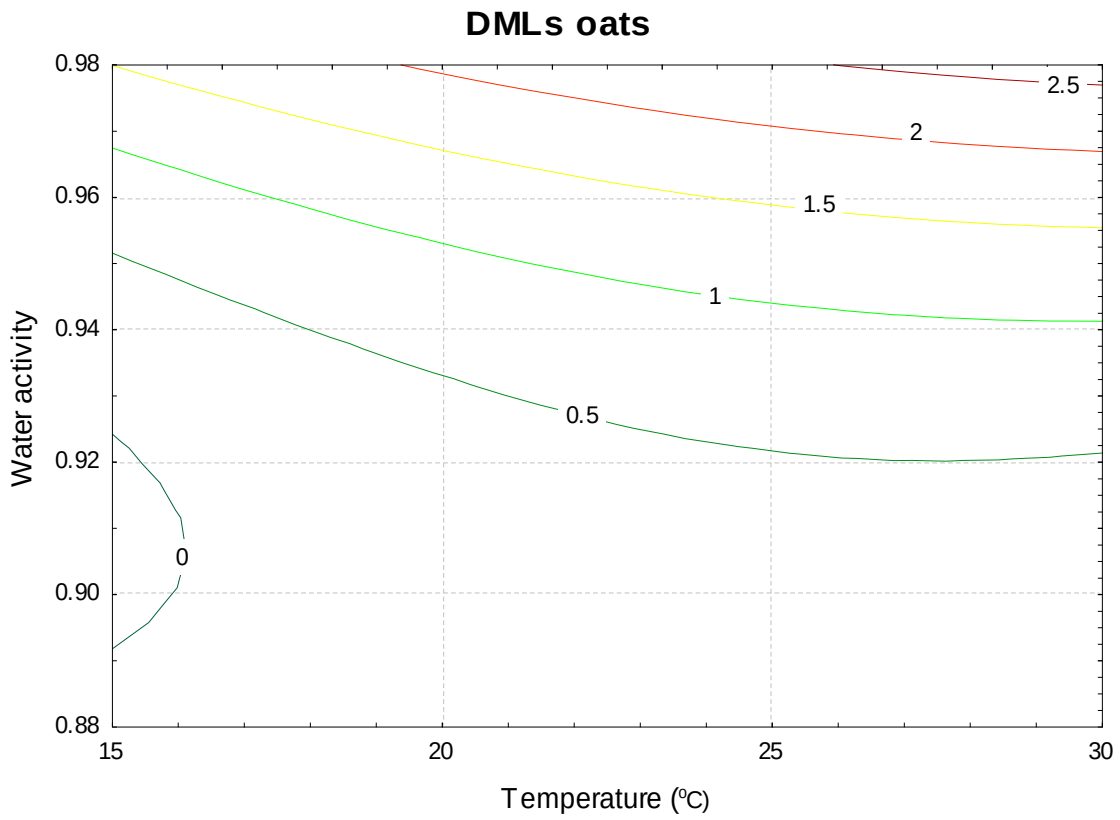


Figure 3.5: DMLs caused in irradiated oats inoculated with *F. langsethiae* under different combinations of environmental conditions.

3.3.4 Enumeration of *F. langsethiae* populations isolated from the oats after 10 days storage

The number of *F. langsethiae* colony forming units (CFUs) per gram isolated from the oats 1 h after inoculation at the beginning of the experiment was ~log4.

Table 3.1 shows the logarithm of *F. langsethiae* CFUs isolated from the oat samples after 10 days incubation at different a_w×T storage conditions.

Table 3.1: Populations of *F. langsethiae* $\log_{CFUs}$/g isolated from the oat samples after 10 days storage at different a_w and temperature conditions

a_w \ T	15°C	20°C	25°C	30°C
0.89	3.22	4.07	4.26	0
0.945	4.50	6.83	6.22	6.01
0.97	6.16	>7	>7	>7

Overall higher *F. langsethiae* populations were isolated from the samples of 0.97 a_w but no clear distinction could be made at temperature ≥20°C. At 0.89 and 0.945 a_w *F. langsethiae* populations were higher at 20 and 25°C.

3.3.5 T-2 and HT-2 toxin production in oats as affected by the storage conditions

Figure 3.6 shows the mean of the sum of T-2 and HT-2 toxins produced in the oat samples at each combination of storage conditions. The mean relative amount of the two individual toxins can also be identified; the shaded part of the bars corresponds to T-2 toxin, while the transparent part to HT-2 toxin production at each combination of storage conditions. The error bars indicate the standard error in the determination of the sum of the two toxins at each combination of storage conditions.

In the wettest treatments of the experiment (0.945 and 0.97 a_w) the sum of T-2 and HT-2 toxins increased with increasing a_w and temperature up to 25°C, while at 30°C it was even lower than at 15°C and 0.97 a_w. At 0.945 a_w toxin production was optimum at 15-25°C. Similar observations were also made for the two individual toxins at these storage conditions. Overall T-2 toxin was produced in higher amounts than HT-2.

In the driest samples of the experiment (0.89 a_w) the production pattern of the two toxins was different. T-2 production was generally very low with only some

of the replicates containing more T-2 toxin than the blank control samples. T-2 toxin increased with increasing temperature up to 25°C, while at 30°C it was the lowest. HT-2 toxin at 0.89 a_w was generally below the limit of detection, while one of the replicate samples at 15 and 25°C contained relatively high amounts of HT-2 toxin, even higher than the amount of T-2 toxin in the relevant samples. This is also the reason for the relatively big error bars at these two treatments.

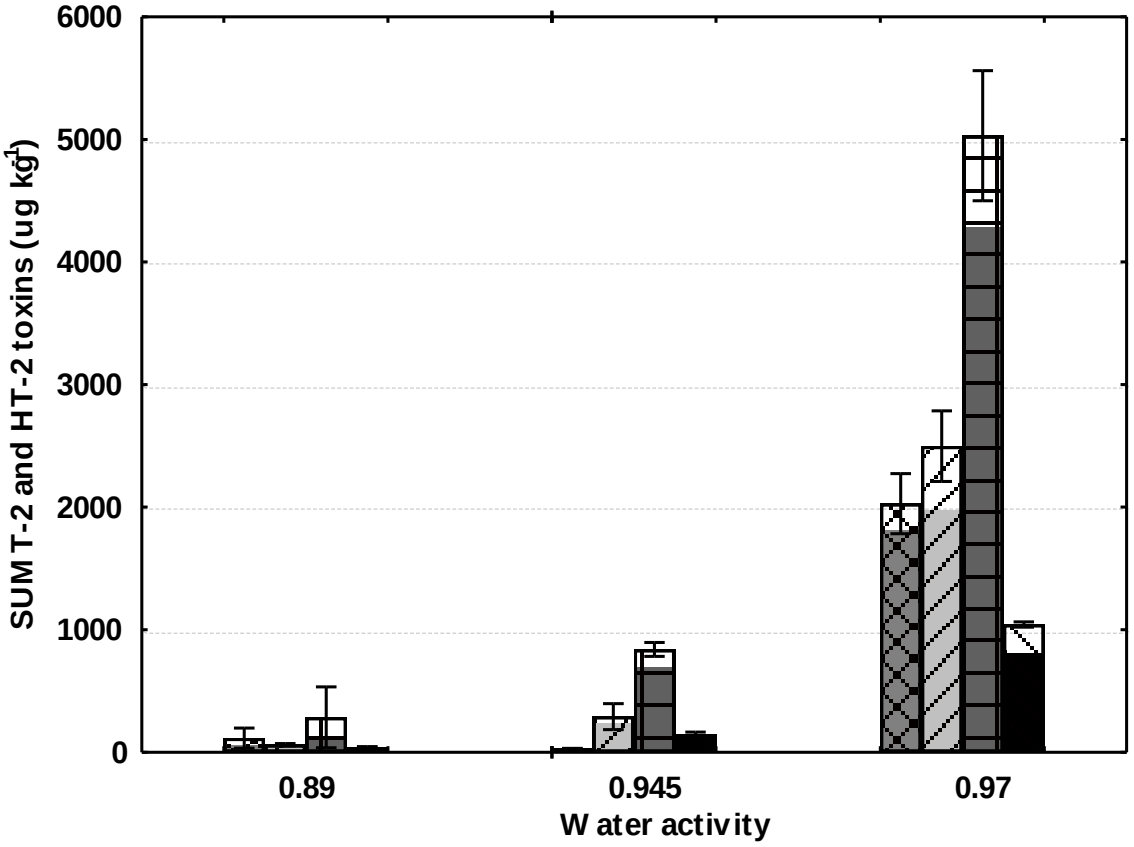


Figure 3.6: Combined effect of a_w and temperature on the sum of T-2 and HT-2 toxins and on the two individual toxins produced by *F. langsethiae* in irradiated oats after 10 days storage. The four bars for each a_w level represent 15, 20, 25 and 30ºC from left to right. The shaded part of the bars corresponds to T-2 toxin production while the transparent part to HT-2 toxin production. The error bars indicate the standard error in the determination of the sum of the two toxins.

Using the Kruskal-Wallis test by ranks, the effect of a_w was found highly significant on the sum of T-2 and HT-2 toxins in oats, while the effect of temperature was not significant (Apx. Table C.2).

A model equation containing linear and quadratic effects of a_w and temperature and their interaction (a_w×T) was used to describe the effect of the environmental factors on the sum of T-2 and HT-2 toxins in oats. Due to the pattern of total toxin production which increased up to 25ºC and sharply decreased at 30ºC, it was considered more relevant to only include the data up to 25ºC 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. All factors were significant except of the effect of T^2 and the complete equation developed for the sum of T-2 and HT-2 toxins in oats is as follows:

$$Sum\ T2\ and\ HT\text{-}2\ toxins = 1184677 - 2528659\ a_w - 2811.96\ T + 1345999\ a_w^2 + 3149.32\ a_w\ T$$ **Equation 3-2**

A contour map showing the sum of T-2 and HT-2 toxins in oats at different storage conditions is shown in Figure 3.7.

The areas where toxin production is likely to be higher and those where it is small can be clearly identified.

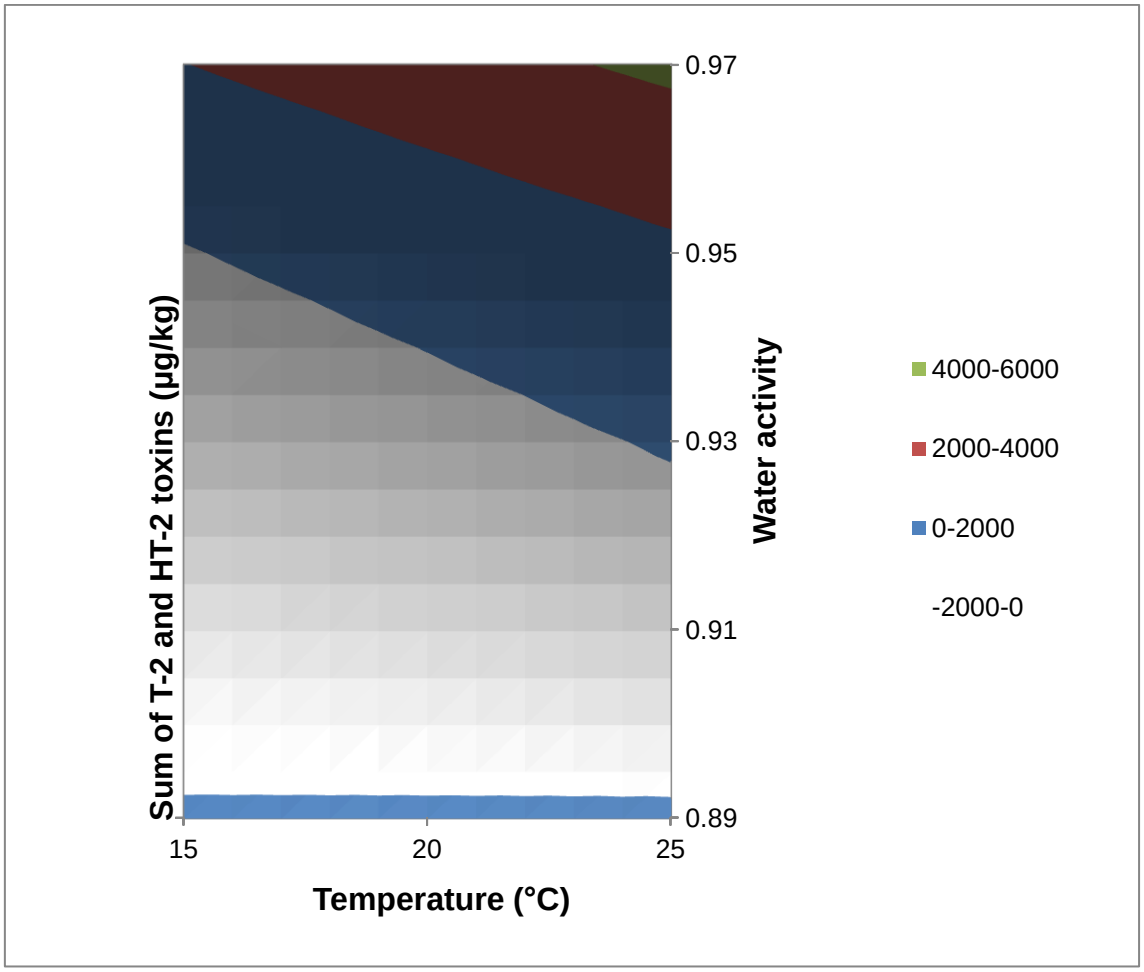


Figure 3.7: Contour map of the sum of T-2 and HT-2 toxin production in irradiated oats by *F. langsethiae* under different combinations of environmental conditions.

3.3.6 Correlation between dry matter losses and toxin production in oats

Figure 3.8 shows a scatter plot of the DML and the total toxin production in oats. A highly significant positive correlation was obtained between the two variables (Spearman Rank Order Correlations test: R=0.7503, p<0.001). The broken green line indicates the legislative limit most commonly established in different parts of the world for the sum of the two toxins in oats (100 µg/kg). >95% of the samples with >0.6% DMLs exceeded this limit. Notably, two oat samples

exceeded this limit with as little as 0.13 and 0.23% DMLs (280 and 780 µg/kg respectively).

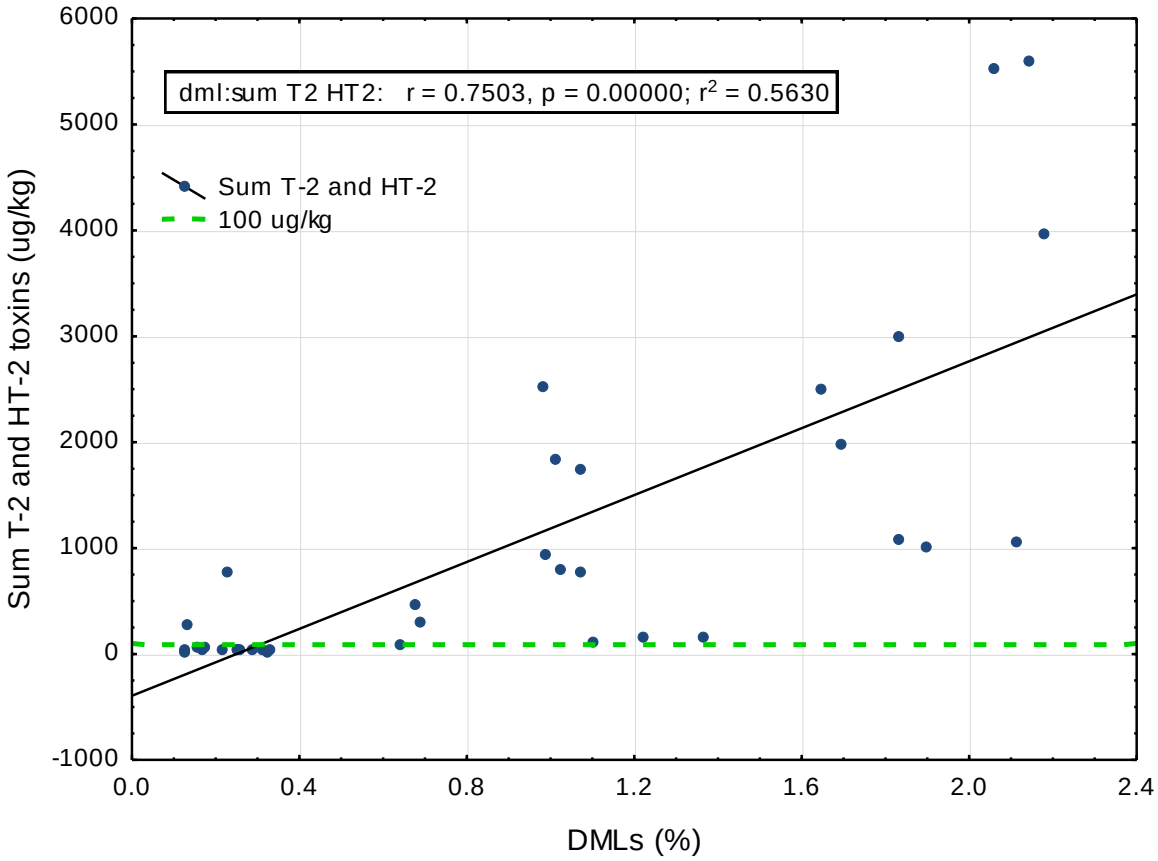


Figure 3.8: Scatter plot of DMLs and the sum of T-2 and HT-2 toxin production during colonisation of stored oats by *F. langsethiae*. The broken green line indicates the legislative limit most commonly established in different parts of the world for the sum of the two toxins in oats.

A much higher significant correlation was obtained (Spearman Rank Order Correlations test: R=0.909, p<0.001) if only the data between 15 and 25°C were considered (Figure 3.9) due to the irregular production of T-2 and HT-2 toxins which increased up to 25°C and then sharply decreased at 30°C.

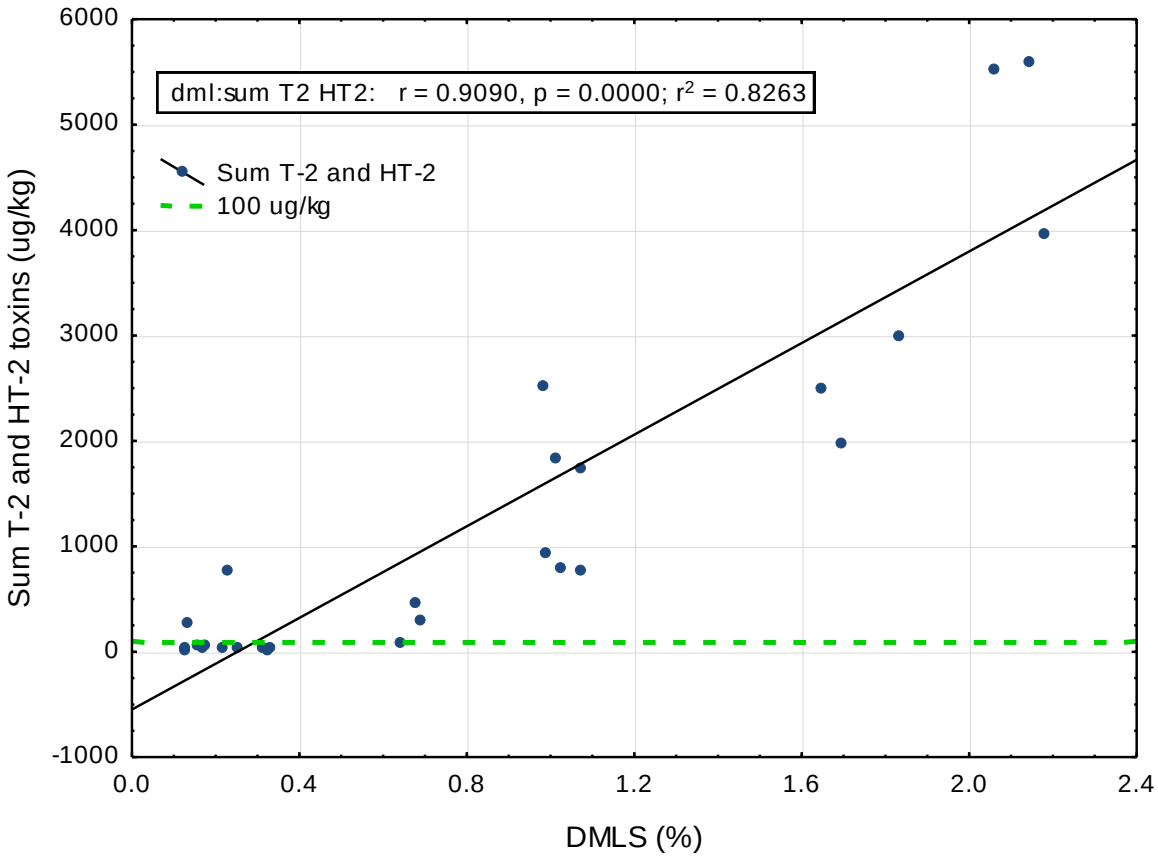


Figure 3.9: Scatter plot of DMLs and the sum of T-2 and HT-2 toxin production during colonisation of stored oats by *F. langsethiae* for the temperature range 15-25°C. The broken green line indicates the legislative limit most commonly established in different parts of the world for the sum of the two toxins in oats.

3.4 Discussion

3.4.1 Growth boundaries for *F. langsethiae* in oats

The respiration measured initially on the first day of the oat storage experiment may be predominantly attributed to the grain since very little fungal growth, if any, could have occurred within the first 24 hours of storage. This initial respiration was higher under wetter conditions and higher temperature (Figure 3.2). This suggests that the grain respiration is higher under wetter and warmer conditions as has been discussed previously (Section 2.4.1).

At 0.89 a_w respiration was constantly very low at all temperatures and although some fluctuations were observed, overall it was lower on the 10th day of storage than on the first day possibly due to loss of some water over time.

Combining the respiration data with the CFU population data at 0.89 a_w and 20 and 25°C, it can be suggested that *F. langsethiae* may grow marginally at these conditions. This contradicts the *in vitro* findings of Medina and Magan (2010) on oat-based media, where no growth was observed after 10 days for any of the *F. langsethiae* strains studied. However, the different nutrient availability between a 2% oat-based medium and oat grains could be the main reason for this. Growth of *Fusarium* species at a_w levels as low as 0.89 has been reported before for *F. verticillioides* (*in vitro*) at 25-30˚C (Marín et al., 1995) and for *F. culmorum* (Magan and Lacey, 1984).

In an attempt to determine the optimum conditions for the growth of *F. langsethiae* on oats, the respiration data and the total CO_2 data suggest that this lies at the wettest conditions (0.97 a_w) and 25°C. At intermediate a_w (0.945) the total CO_2 seems to be similar at 25 and 30°C, but if the higher respiration of the grain at warmer conditions is also taken into account, it can be suggested that optimum temperature for the growth of *F. langsethiae* is probably at 25°C. Overall, these results suggest that the growth of *F. langsethiae* is optimum at 25ºC under intermediate to very wet conditions which is in good agreement with *in vitro* findings for several *F. langsethiae* strains (Medina and Magan, 2010).

A peak observed at some point during the 10 day storage period at 0.945 and 0.97 a_w and all temperatures may correspond to the time when the growth of *F. langsethiae* was optimum at each combination of storage conditions. Examining the respiration rates of Figure 3.2 more carefully while also considering the above suggestions with regard to the growth boundaries of *F. langsethiae*, it can be further suggested that as the environmental conditions for the growth of *F. langsethiae* become less favourable, the respiration peak moves later into time. For example, considering 25°C and the highest water activity level as the optimum conditions for the growth of *F. langsethiae*, the peak in the respiration rate is observed earlier, on the 3rd day, while at lower a_w the peak has shifted to

the 4th day. Similarly, at 20°C which is considered as less favourable temperature than 25°C, the peak is observed on the 4th day at the wettest conditions while has shifted to the 6th day at lower a_w. At even less favourable conditions (15°C), a peak can only be observed on the 9th day while at 30°C safe conclusions cannot be drawn, as this is not considered a favourable temperature for the growth of *F. langsethiae*, but also due to the higher respiration of the grain at this temperature. These observations however cannot explain the difference in the respiration rate pattern observed for *F. langsethiae* in oats compared to the constantly increasing pattern previously observed for *F. graminearum* and *F. verticillioides* in wheat and maize respectively (Chapter 2). Also from these data it is not possible to calculate the relative contributions of the grain and of the fungi to the total respiration as previously done in stored wheat and maize.

3.4.2 Dry matter losses in stored oats colonised by *F. langsethiae* at different combinations of storage conditions

Dry matter losses in oats were highest at 0.97 a_w and 25°C further contributing to the assumption that these were the optimum conditions for the growth of *F. langsethiae*. This can be explained by the fact that higher fungal populations could cause higher losses in grain dry matter.

Total DMLs observed in oats in this experiment (~2.5%) were much lower than those observed previously in the wheat (~7%) and maize experiments (~15%). In contrast, the populations isolated from the oat samples were comparable to those isolated from maize samples in previous studies (Chapter 2). This may be due to the specific growth pattern of *F. langsethiae* or due to the characteristic nutritional components of oats. For example as previously discussed the carbohydrate composition of maize is ~74%, while that of oats ~66% (USDA, 2009) and since respiration has been considered as the aerobic break down of carbohydrates (Equation 1-2) a lower CO_2 production in oats can be expected. Also oats are accompanied by the husk at harvest as opposed to other cereals

(Hoseney and Faubion, 1992) which may also make it more difficult to fungi to access the nutrients. The cultivars must also be considered, since different varieties have been reported to have different resistance against fungal invasion. Also the amount of broken kernels was quite high in the maize used while the oats were of better quality.

3.4.3 Toxin production boundaries for *F. langsethiae* in oats

In this study T-2 and HT-2 toxins were produced optimally at the wettest storage conditions 0.97 a_w tested and 25°C, followed by 20 and 15°C where toxin production was more or less similar and finally 30°C. Also at all a_w levels more T-2 and HT-2 toxins were produced at 25 followed by 20°C, which seem to be the most favourable temperatures for toxin production by *F. langsethiae*. These data are in very good agreement with those of Doohan et al. (2003) where optimum toxin production by *F. poae* and *F. sporotrichioides,* the species most closely related to *F. langsethiae,* was observed at very wet conditions (0.99 a_w) and intermediate temperatures (20-25˚C). Also these results are generally in good agreement with the *in vitro* toxin production data of Medina and Magan (2011) by different *F. langsethiae* strains, although in that study optimum toxin production was observed at 20-30ºC.

The minima for toxin production by *F. langsethiae* were determined by this study at as low as 0.89 a_w at 25°C. *In vitro,* however, none of the strains studied produced toxins at 0.90 a_w (Medina and Magan, 2011). In a previous study on autoclaved oats T-2 toxin levels were found above the control samples at all temperatures at 0.90 a_w but this may be due to the higher availability of nutrients in the autoclaved grain (data not shown).

Interestingly even though the respiration and the CFU count data indicated that growth of *F. langsethiae* did occur at 0.945 a_w and 15˚C, toxin production was below that of the control in all of the replicate samples at these conditions.

In this study, *F. langsethiae* predominantly produced T-2 toxin in oats, while the amounts of HT-2 toxin were generally much lower, in agreement with the *in vitro* results of Medina and Magan (2011) and the study of Kokkonen et al. (2010) on mixed grain. However, at 0.89 a_w and 15 and 25˚C the level of HT-2 toxin in one of the replicate samples in each case was almost twice as high as the level of T-2 toxin, contrarily to all the other experimental samples. Langseth and Rundberget (1999) found higher HT-2 levels in oats and other cereals studied but in their study grains were either dried directly or stored in conditions more likely to have been unfavourable for the growth of *Fusarium* species, imposing stress on the fungus. Edwards (2009) also detected higher amounts of HT-2 toxin than T-2 when analysing more than 450 oat samples in the period 2002-2005. Also he found higher amounts of toxin in the samples produced conventionally than in organic grains. It would be of interest to know the actual distribution of T-2 and HT-2 toxins between the samples of the two different agricultural practices. It is possible that stress imposed by chemical control treatments shifted toxin production from T-2 to HT-2. Also the fungal species responsible for the production of these two toxins at that time was unknown (Edwards, 2009), but the results of this study suggest that this may have been *F. langsethiae*. Medina and Magan (2011) also observed a change in the ratio of T-2 and HT-2 toxins *in vitro* under environmental stress. It has been suggested that T-2 and HT-2 toxins usually co-occur and that T-2 toxin is metabolised to HT-2 toxin (SCF, 2002). The results presented in this Chapter suggest that higher amounts of HT-2 toxin are found under stress conditions. However, it cannot be concluded whether HT-2 toxin is preferably produced at such conditions or whether T-2 toxin is produced and metabolised to HT-2 due to the water stress.

The models developed for the sum of T-2 and HT-2 toxin production with respect to the storage conditions for *F. langsethiae* can be used to predict the environmental storage boundaries for oats that are considered safe and those that represent a higher risk for toxin accumulation in the grain. These models may be very useful in the oat handling milling and processing industry.

3.4.4 Estimation of fungal infection and toxin contamination of stored oats

Several studies have discussed whether the deterioration of stored cereals due to fungal infection could be assessed by visible or even microscopic growth and it has been suggested that microscopic growth could be more accurate (Hamer et al., 1991; Magan, 1993; Lacey et al. 1994). *F. langsethiae* colonisation of oats in this study consisted of very fine mycelium which was difficult to see with the naked eye. Even under the most favourable conditions for the growth of this species, only microscopic growth was observed, as opposed to the extensive mycelial growth observed in wheat and maize infected by other *Fusarium* species (Chapter 2). This could be due to the nutritional components of oats or due to the specific growth pattern of *F. langsethiae*. At the same time the sum of T-2 and HT-2 toxins was more than 5 mg/kg of oats in the above samples. This suggests that visible or even microscopic growth may occasionally prove inefficient as indicators of fungal spoilage in grains, since certain fungal species such as *F. langsethiae* may produce high levels of toxins even with little visible growth. This could be of concern as the stored grain may look healthy while being contaminated with very high levels of T-2 and HT-2 toxins.

Other studies have suggested that DMLs can be used as an indicator of quality deterioration in grain (White et al., 1982a, b; Lacey et al., 1994). The highly significant correlations obtained between DMLs and toxin contamination in the wheat and maize experiments (Chapter 2) suggest that DMLs could be used as an accurate indicator of the level of contamination of a stored batch. Similarly a highly significant correlation has also been obtained in this experiment between DMLs and the sum of T-2 and HT-2 toxin production in stored oats. This suggests that continuous monitoring of CO_2 and DMLs in silos can be used as a safety management tool for stored grains.

Some studies have suggested that the estimation of toxin contamination in stored grains could involve colony counts of mycotoxigenic fungi (Lund and Frisvad, 2003; Lindblad et al., 2004). Despite the fact that the serial dilution and spread plate technique is considered less appropriate for *Fusarium* species

(Thrane, 1996), in this study all the samples that contained >6log *F. langsethiae* populations contained >100 µg/kg of T-2 and HT-2 toxins. Legal limits have not yet been established in Europe for the sum of T-2 and HT-2 toxins in oats. However in some countries of the world maximum limits are set at ~100 µg/kg in oats. If these limits are considered, 95% of the oat samples of this experiment with DMLs as little as 0.6%, would be exceeding these limits. Notably, one of the samples in this experiment contained 279 µg/kg toxin with as little as 0.13% DML due to the very high presence of HT-2 toxin. Another sample contained 782 µg/kg toxin with as little as 0.23% DML. These thresholds are much lower than those previously described for wheat and maize (Chapter 2) rendering oat contamination with T-2 and HT-2 toxins an emerging significant risk.

4 CHEMICAL CONTROL OF FUNGAL GROWTH AND MYCOTOXIN PRODUCTION USING ACIDS

4.1 Introduction

Chemical compounds find applications at various steps in the grain chain: seed preservation, weed control, insect and pest control, fumigation, making use of their chemical properties that render them toxic to different groups of organisms. The efficacy of chemical methods for the control of fungal growth and possibly mycotoxin production in stored grain has received significant attention. Also, due to the increased environmental awareness and consumer demand for chemical-free products, research has now focused on compounds naturally present in plants and grains, essential oils, plant extracts and natural antioxidants for use as preservatives and antifungal agents.

Propionic, sorbic or benzoic acids and their mixtures are often used for the treatment of moist grain and several products containing these acids, their salts and other substances are available on the market. Preliminary studies suggested they have significant efficacy against fungal growth (Sauer and Burroughs, 1974; Bothast et al., 1978; Higgins and Brinkhaus, 1999), but the effect on mycotoxin formation in the treated grain was scarcely examined (Vandegraft et al., 1975). More recently, it was shown that sub-inhibitory concentrations of propionates and sorbates or inefficient coverage of the grains could result in stimulated growth and mycotoxin production (Lee et al., 1986; Marín et al., 1999; Marín et al., 2000). This has been attributed to the fact that the compounds do not kill the fungi but instead act as fungistats (Marín et al., 2000; Magan and Aldred, 2007; Magan et al., 2010). Marín et al. (2000) also observed that the efficacy of propionates was species-dependent which can lead to enhanced growth of the species more tolerant to the treatment. Beekrum et al. (2003) suggested that benzoic acid did not demonstrate any inhibitory effect against *F. verticillioides* growth and fumonisin production *in vitro*. Several derivatives of benzoic acid have been tested against the growth of *A. flavus* and *A. parasiticus* and aflatoxin production with varying results (Chipley and Uraih,

1980). Among these, trans-cinnamic acid significantly reduced mycelia growth and toxin production at 2.5 mg per 25 ml, and caused complete inhibition at 5 mg per 25 ml.

Ferulic acid has also been the focus of many studies. Chipley and Uraih (1980) observed 50% reduction in aflatoxin production with 5 mg/25 ml and noted that inhibition of mycelial growth did not always result in a similar inhibition of toxin production. Beekrum et al. (2003) found no effect on fungal growth but observed 90% reduction of fumonisin production. Nesci and Etcheverry (2006) using ferulic acid, trans-cinnamic acid and mixtures of the two, achieved various levels of inhibition of growth and aflatoxin B_1 production and noticed increased efficacy with decreasing water activity, although occasionally they also observed stimulation. Complete control of aflatoxin B_1 production in maize of all water activities for storage up to 35 days was observed with a mixture 25 mM:30 mM trans-cinnamic acid: ferulic acid (Nesci et a., 2007). Boutigny et al. (2009) showed that 2.5 and 5mM ferulic acid caused 39 and 85% inhibition of fungal biomass of *Fusarium culmorum* respectively in liquid cultures, while trichothecene production was completely inhibited by both concentrations. Lower amounts (0.1 to 1 mM) of ferulic acid had no significant effect on the fungal biomass produced while toxin production was inhibited 68-99%. However these studies are unrealistic, as they were carried out in liquid media and without any simulation of relevant environmental parameters. Boutigny et al. (2009) suggested that ferulic acid may be affecting the secondary metabolism of the *Fusarium* species. They also observed that the effect of ferulic acid on trichothecene production was the same, irrespective of whether it was added at the spore germination stage or during mycelial development when trichothecenes start to accumulate. Addition of ferulic acid at both stages caused a total of 96% inhibition of trichothecenes. Other naturally occurring phenolic compounds have also proven very efficient against *F. verticillioides* and fumonisin B_1 production (88-94% reduction) (Beekrum et al., 2003).

Some of the chemical compounds studied have shown promising results however the effective concentrations, time and means of application and the

efficacy against a range of fungal species that may be encountered in naturally contaminated grains are far from being established. Also, very few studies have examined the presence of residues in treated grains and the likely toxicological issues that may arise. Some of the acids studied are corrosive and thus require especially designed equipment for their application and may also generate odours when applied on moist grain which could limit their suitability for application in feed only. Also the acceptability of treated grain by different animal species and the likely side-effects on characteristic properties and nutrients of the grains have rarely been examined (Bothast et al., 1978).

The aim of this study was to examine different chemical compounds for efficacy in the control of the growth of different fungal species and mycotoxin production. The compounds studied were selected either because they have been reported as effective for the above purpose by previous studies but still more data are required, because they are structurally similar to such compounds or because they are permitted for use as food additives but have never been assessed for antifungal efficacy.

4.2 Materials and methods

4.2.1 Preparation of stock solutions of chemical compounds

Stock solutions of the following compounds were prepared in sterile distilled water. The solutions were then filter-sterilised through a sterile 0.2 µm Millipore filter (Minisart, Sartorius) into sterile containers. When higher concentrations of the compounds were required or when the solubility of the compounds did not allow the preparation of water solutions, stock solutions were prepared in absolute ethanol (HPLC Grade).

(a) Adipic acid

A stock solution of 10000 ppm was prepared by dissolving 1 g of adipic acid (99%, Sigma Aldrich) in 100 ml of sterile distilled water. A second stock solution

(1 M) was prepared by dissolving 14.614 g adipic acid in 100 ml absolute ethanol.

(b) Tartaric acid

A stock solution of 10000 ppm was prepared by dissolving 1 g of tartaric acid (L(+)tartaric acid, Sigma Chemical Co.) in 100 ml of sterile distilled water.

(c) Fumaric acid

A stock solution of 5000 ppm was prepared by dissolving 1 g of fumaric acid (laboratory reagent grade, Fisher Scientific) in 200 ml sterile distilled water. A second stock solution (1 M) was prepared by dissolving 11.6 g fumaric acid in 100 ml absolute ethanol (HPLC Grade).

(d) Trans-cinnamic acid

A stock solution of 400 ppm was prepared by dissolving 0.4 g of trans-cinnamic acid (Aldrich) in 1 L of sterile water into a 1 L volumetric flask, by use of mild heating and stirring.

(e) Ferulic acid

A stock solution (1 M) was prepared by dissolving 14.614 g ferulic acid in 100 ml absolute ethanol (HPLC Grade).

4.2.2 Grains, culture media and water activity adjustment

Naturally contaminated wheat, maize (Section 2.2.1) and oats (Section 3.2.1) were milled and stored at 4°C. Synthetic agar media were prepared using 2% milled grain, 2% technical agar (Agar No 3, OXOID) and glycerol/water solutions (prepared as described in Section 2.2.2) for modification of the water activity. The mixtures were autoclaved and either directly poured into sterile 9 cm Petri dishes in the case of control samples, or poured after the addition of appropriate volumes of the above stock solutions of the compounds to be tested and vigorous mixing to ensure homogeneous concentration. For the initial

screening, concentrations in the range 10-200 ppm were used on wheat agar media of unmodified water activity (0.995 a_w). Where no effect was observed, higher concentrations were tested, using appropriate amounts of the compounds dissolved in ethanol. Ethanolic solutions were also used for the compounds that were not water soluble. In this case controls containing the respective percentage of ethanol were also prepared in addition to the plain controls.

4.2.3 Fungal strains and preparation of the inoculum

F. verticillioides isolate MPVP 294, *F. graminearum* isolate L1-2/2D (Section 2.2.3) and *Fusarium langsethiae* strain 2004/59 (Section 3.2.3) were used in this study. The strains were maintained on Malt Extract Agar (MEA) media (OXOID, malt extract, 30; mycological peptone, 5; agar, 15 g/l).

Agar plugs (4 mm diameter) cut from 10-day-old cultures with a sterile cork borer, were used as inoculum for the *in vitro* trials. For the *in situ* trials, spore suspensions were prepared as previously described (Section 2.2.3) and were filtered through glass wool in order to remove any mycelia fragments. Inoculation with agar plugs did not prove appropriate for the highly sporulating *F. verticillioides* strain during preliminary trials, as spores caused the formation of secondary colonies making the measurement of colony diameters difficult. For this reason a *F. verticillioides* spore suspension was used as inoculum in subsequent experiments.

4.2.4 *In vitro* effect of chemical compounds on mycelia growth rate

Grain media were centrally inoculated with agar plugs/spores. Modified a_w media were stored in polyethylene bags containing replicates of the same a_w treatment in order to maintain the conditions.

The cultures were incubated at 25°C for 10 days, or until the Petri plates were completely colonised by the fungi. Single fungal treatments were kept in each polyethylene bag to prevent cross-contamination.

Two diameters of the colonies formed (at right angles of each other) were measured daily and compared against the diameters of the controls. From these data the % inhibition of the growth rate of the fungi was determined at different chemical compound concentrations and different water activities.

4.2.5 *In vitro* effect of chemical compounds on mycotoxin production

On the 10th day of incubation agar plugs were cut out from each of the replicate plates by use of a 5 mm diameter cork borer, according to the patterns shown in Appendix D. The agar plugs were placed in 2 ml safe-lock Eppendorf® tubes, their weight was recorded and they were frozen at -40°C for subsequent toxin analysis. The extraction and analysis of the relevant toxins for each fungal species were performed according to the following methods:

(a) Fumonisin extraction and analysis

For the extraction of fumonisins 1 ml of extraction solvent AcN:H_2O:Acetic acid (79:20:1) was added in each Eppendorf® tube and the samples were extracted for 1 hour in the dark at room temperature in an orbital shaker at 250 rpm. The extracts were filtered though a 0.2 μm Millipore filter (Minisart, Sartorius) into new tubes and dried in an oven at 60°C for 24 hours. The dried extracts were redissolved in a mixture of AcN:H_2O (1:1) containing 1% Acetic acid and they were analysed for the individual fumonisins by the MYCORED Project partners in IFA-Tulln, Austria using LC-MS/MS according to the method of Vishwanath et al. (2009).

(b) Deoxynivalenol analysis

The analysis of type B trichothecenes in grain is most commonly performed by Gas Chromatography – Electron Capture Detection (GC-ECD) or Liquid Chromatography (LC) coupled with Mass Spectrometry (MS) (Krska et al.,

2007) although LC-DAD detection has also been suggested (Mateo et al., 2001). No specific method for *in vitro* application exists although most commonly the method of Cooney et al. (2001) is used with modifications. This method involves a cleaning step using an in-house prepared column containing charcoal and alumina (Romer, 1986) for the removal of interferences that can hinder the determination and several modifications have been applied over the years (Lauren and Agnew, 1991; Cooney et al., 2001). These methods however are laborious and require increased use of solvents. Also the cost associated with alternative cleaning columns, i.e. MycoSep columns or immunoaffinity columns (Krska et al. 2007), makes their use for routine analysis non cost-effective.

Several modifications of the above methods were assessed in order to obtain a simpler and faster method that would be suitable for the simultaneous analysis of DON, NIV, 3AcDON and 15AcDON from wheat agar media using LC-DAD detection. Amongst those a method involving a cleaning step using silica was selected as more suitable and this method will be published in more detail elsewhere. Briefly, extraction was performed using 1 ml acetonitrile:water (AcN:H_2O) (84:16), the mixture more commonly used for trichothecenes extraction (Krska et al., 2007) and the tubes were shaken in an orbital shaker at 200 rpm in the dark for 60 min at 25°C. The extract was transferred in a new tube and oven-dried overnight at 60°C. Subsequently, it was redissolved in 1 ml 90:10 (H_2O: AcN) and vortexed for a few seconds. The cleaning step involved the addition of 150 mg/ml Alumina directly into the redissolved extract followed by vortexing the mixture for 15 sec, instead of passing the extract through the charcoal and alumina column used in previous methods. The treated extract was then filtered through a 0.2 µm Millipore filter (Minisart, Sartorius) into an amber silanised LC vial and inserted into the LC-DAD for analysis.

The LC equipment used consisted of an Agilent 1200 Series system equipped with a UV diode array detector (DAD) set at 220.4 nm (Agilent Technologies, Palo Alto, CA, USA). The column used for the chromatographic separation was a Phenomenex® Gemini C_{18}, 150 mm×4.6 mm, 3 µm (Phenomenex,

Macclesfield, UK) preceded by a Phenomenex® Gemini 3 mm guard cartridge and the column temperature was set at 25°C.

The chromatographic analysis was performed in the gradient mode, using water (solvent A) and acetonitrile (solvent B). The starting composition of the mobile phase was 5% B, at a flow rate of 0.5 ml/min held for 2 min. The composition was then gradually changed to 25% B over 15 min and maintained for further 3 min. Then it increased gradually to 30% B over 3 min at the same flow rate. The composition was then changed to 99% B during 1min with a flow rate of 1 ml/min in this case, in order to achieve a fast cleaning step and maintained at 99% B for 4 more minutes. Afterwards the composition of the mobile phase was changed linearly to 5% B in 1 min at a flow rate of 1 ml/min and held for 4 min for further cleaning. In a last step the composition was maintained at 5% B but the flow rate changed to 0.5 ml/min for 1 min, in order to be the same as the starting composition of the mobile phase for the following chromatographic run. The injection volume was 50 µl. The total time for the analysis of each sample was 35 min, but this was due to the fact that 4 trichothecenes were analysed simultaneously. DON was eluted from the column at 16.2 min, while NIV at 12.6 min, 15Ac-DON at 24.38 min and 3 Ac-DON at 24.61 min.

The software used was the Agilent ChemStation Software (Ver. B Rev. 03.01, Agilent Technologies, Palo Alto, CA, USA) and the calculations were performed by comparing each peak area to the relevant peak area of trichothecene standards prepared in 90:10 (H_2O:AcN). The mean recovery for DON using this method was 63.2±2.8%. The limit of detection (LOD) for DON was 6.5 ng/g and the limit of quantification (LOQ) was 19.5 ng/g.

(c) Trichothecenes A (T-2 and HT-2 toxin) analysis

T-2 and HT-2 toxins were extracted from the oat-based media and analysed according to the method of Medina et al. (2010) with slight modifications. Briefly 1 ml of AcN:H_2O (84:16, v/v) was added to the thawed agar plugs in the 2 ml safe-lock Eppendorf® tubes and the tubes were shaken for 1 hour at 150 rpm at 25°C in the dark in an orbital shaker. The samples were then centrifuged at 1150xg for 15 min. The extract was filtered through a 0.2 µm Millipore filter

(Minisart, Sartorius) directly into an HPLC silanised amber vial and injected in the chromatograph. The apparatus used for the analysis was an Agilent 1100 Series HPLC system equipped with a UV diode array detector (LC-DAD) set at 200 nm (Agilent Technologies, Palo Alto, CA, USA). The column used was a Phenomenex® Gemini C_{18}, 150 mm×4.6 mm, 3 µm (Phenomenex, Macclesfield, UK) preceded by a Phenomenex® Gemini 3 mm guard cartridge. The analysis was performed in the gradient mode with a mobile phase of AcN:H_2O at a flow rate of 1 ml/min and the conditions were 3 min 30% AcN, changed linearly to 55% AcN over 18 min, changed to 99% AcN in 1 min and held to 99% AcN for 5 min. The mean recoveries for this method were 99±1.53% for T-2 toxin and 101.28±3.11% for HT-2 toxin.

4.2.6 Statistical analysis

All experiments have been performed in triplicate. Data were analysed with Microsoft Office Excel 2007 and with the package STATISTICA 9 (StatSoft®, Inc. 2010. STATISTICA (data analysis software system), version 9.1. www.statsoft.com) as described in Section 2.2.8. The standard error of the mean was calculated in all trials and it is denoted with vertical bars in the Figures. ANOVA Tables from this Chapter can be found in Appendix E.

4.3 Results

4.3.1 *In vitro* efficacy of aqueous acids against fungal growth

Figure 4.1 shows the effect of 0-200 ppm of aqueous adipic, tartaric, fumaric and trans-cinnamic acids on the radial growth rates of *F. graminearum* and *F. langsethiae* on wheat agar media at 25°C.

The growth of *F. graminearum* was slightly stimulated by 10-50 ppm of all the acids studied, while the growth of *F. langsethiae* was only stimulated by tartaric acid. The maximum growth inhibition observed for *F. graminearum* was 13.4%

with 200 ppm trans-cinnamic acid, while for *F. langsethiae* it was ~27% with tartaric acid.

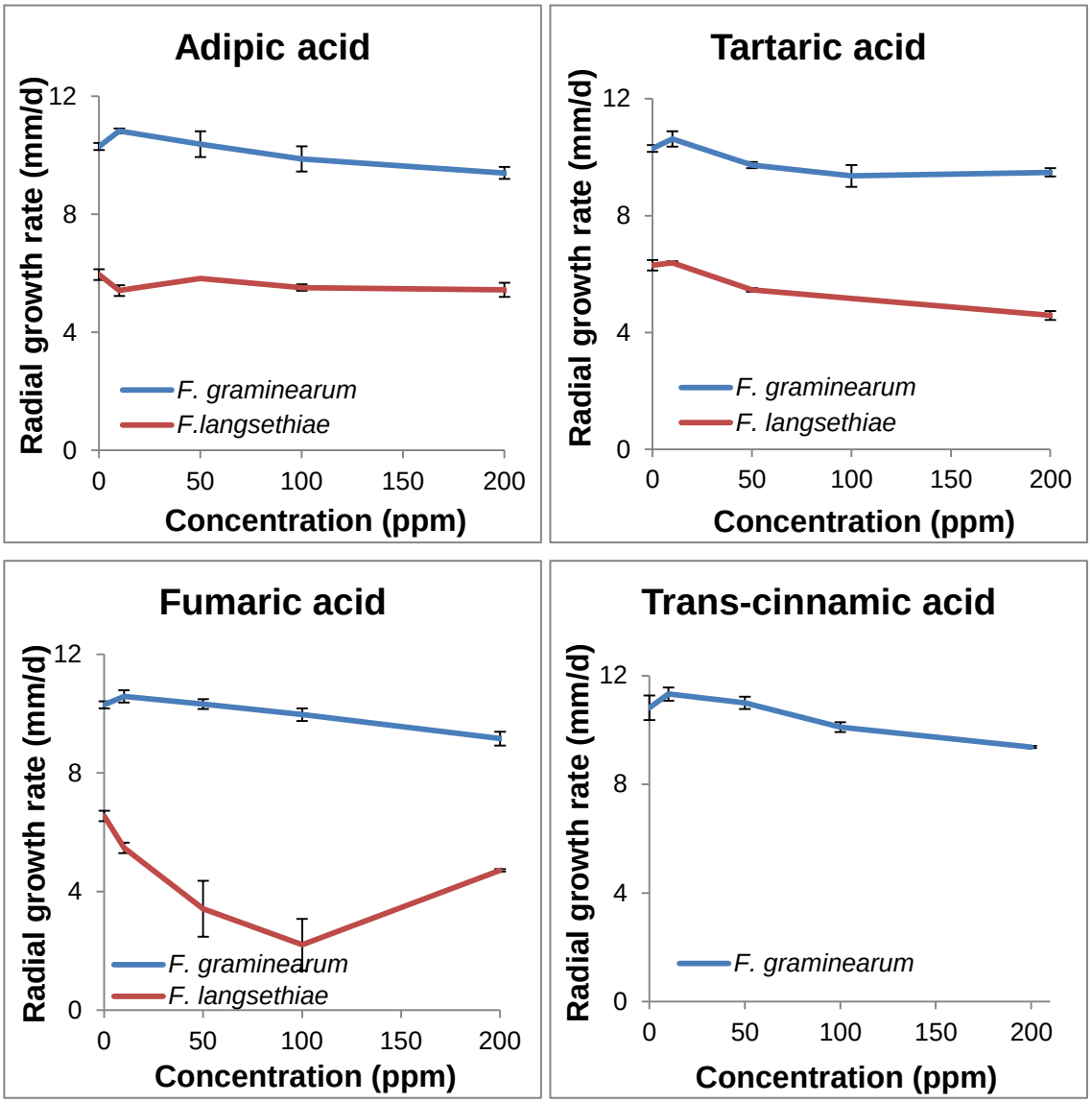


Figure 4.1: Effect of 0-200 ppm aqueous adipic, tartaric, fumaric and trans-cinnamic acids on the *in vitro* radial growth rates of *F. graminearum* and *F. langsethiae.* Vertical bars indicate the standard error of the means.

ANOVA showed that the effect of the concentration of fumaric and trans-cinnamic acids was highly significant on the growth of *F. graminearum* while the

concentration of adipic and tartaric acids was significant. For the growth of *F. langsethiae* the concentration of tartaric and fumaric acids was highly significant, while that of adipic acid was not significant (Apx Tables E.1, E.2).

4.3.2 *In vitro* efficacy of acids dissolved in ethanol against fungal growth

Figure 4.2 shows the effect of different concentrations of fumaric, adipic (0-10 mM) and ferulic (0-20 mM) acids (dissolved in 100% ethanol) on the radial growth rate of *F. graminearum* on wheat agar media at 25°C.

The growth rate of *F. graminearum* was reduced by 35% in the control samples containing ethanol compared to the plain controls.

Increasing concentrations of fumaric acid up to 10 mM (or 1160 ppm) inhibited the growth of *F. graminearum* by up to 82.4% compared to the non-ethanol control (or 73% compared to the control with ethanol).

Similarly, growth inhibition was observed with increasing concentration of adipic acid up to 7.5 mM (1095 ppm) and of ferulic acid up to 15 mM (2913 ppm) to a maximum of 77.5% and 88.5% respectively, compared to the plain control.

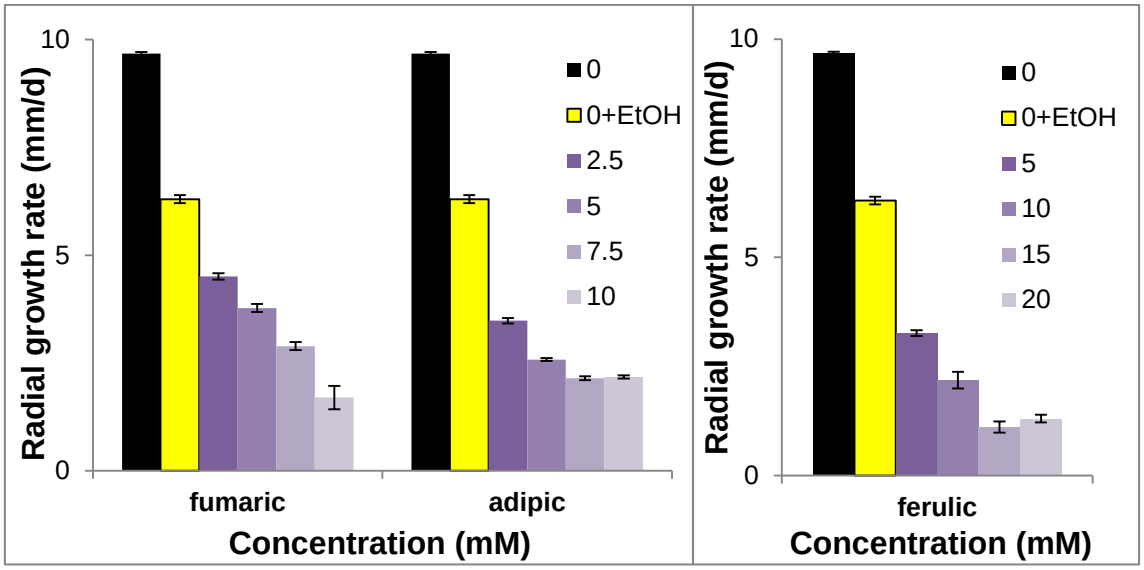


Figure 4.2: Effect of different concentrations of fumaric, adipic and ferulic acids (dissolved in ethanol) on the radial growth rate of *F. graminearum* on wheat agar media at 25°C. Vertical bars indicate the standard error of the means.

The Kruskal-Wallis ANOVA by ranks showed that the effect of acid concentration was highly significant on the growth rate of *F. graminearum in vitro* for all three acids when applied as ethanolic solutions (Apx Table E.3).

Plate 4.1 shows *F. graminearum* cultures on media treated with 0-20 mM ferulic acid (from left to right), after 10 days at 25°C.

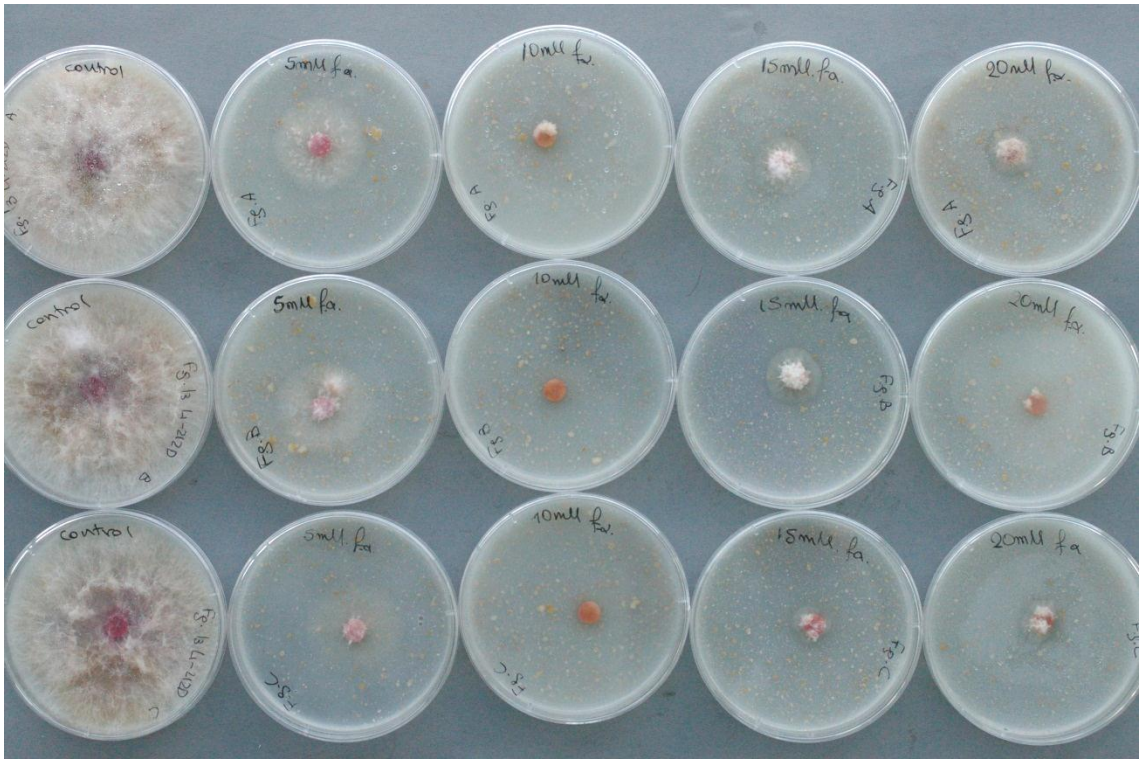


Plate 4.1: Effect of ferulic acid on *F. graminearum* cultures after 10 days at 25°C, from left to right: 0, 5, 10, 15 and 20 mM (3 replicates for each treatment).

Figure 4.3 shows the effect of different concentrations of fumaric, adipic and ferulic acids (dissolved in ethanol) on the *in vitro* growth rate of *F. langsethiae* at 25°C.

Increasing concentration of all three acids caused better inhibition of growth of *F. langsethiae*. The maximum reductions observed were 97.5% for fumaric acid and 94% for adipic acid compared to the non-ethanol control (or 95.6% and 90% respectively compared to the control with ethanol) at 10 mM (1160 and 1460 ppm respectively). For ferulic acid, maximum *F. langsethiae* growth inhibition was observed with 20 mM (3884 ppm) and this was 94.3% compared to the plain control (or 90% compared to the control with ethanol).

The addition of ethanol in the control samples caused a 42.8% reduction in the growth rate of *F. langsethiae* compared to the plain control.

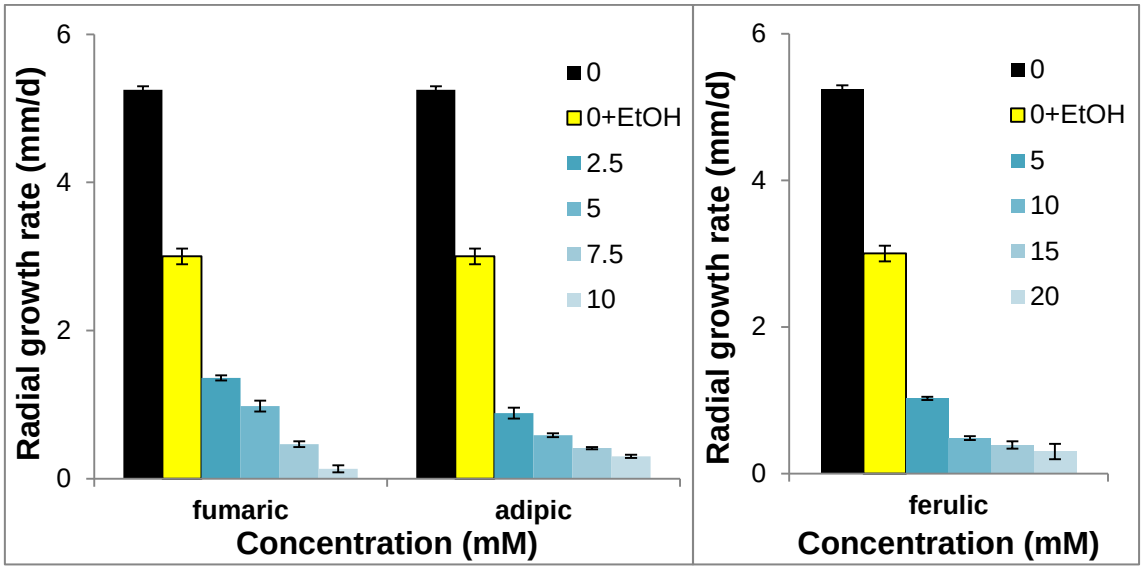


Figure 4.3: Effect of different concentrations of fumaric, adipic and ferulic acids (dissolved in ethanol) on the *in vitro* radial growth rate of *F. langsethiae* at 25°C. Vertical bars indicate the standard error of the means.

The Kruskal-Wallis ANOVA by ranks showed that the effect of acid concentration was highly significant on the *in vitro* growth rate of *F. langsethiae* for all three acids when applied as ethanolic solutions (Apx Table E.4).

Figure 4.4 shows the effect of different concentrations of ethanolic fumaric, adipic and ferulic acids on the *in vitro* growth rate of *F. verticillioides*.

The addition of ethanol alone caused a 16.5% growth inhibition in the control samples compared to the untreated controls.

Increasing concentrations of fumaric and adipic acid caused better control of the growth rate of *F. verticillioides* to a maximum of 71 and 74% respectively, compared to the plain control samples (or 65 and 69% respectively compared to the control with added ethanol).

Ferulic acid at 10 and 15 mM (1940 and 2913 ppm) caused complete inhibition of the growth of *F. verticillioides*, however some growth was observed at 20 mM (3884 ppm).

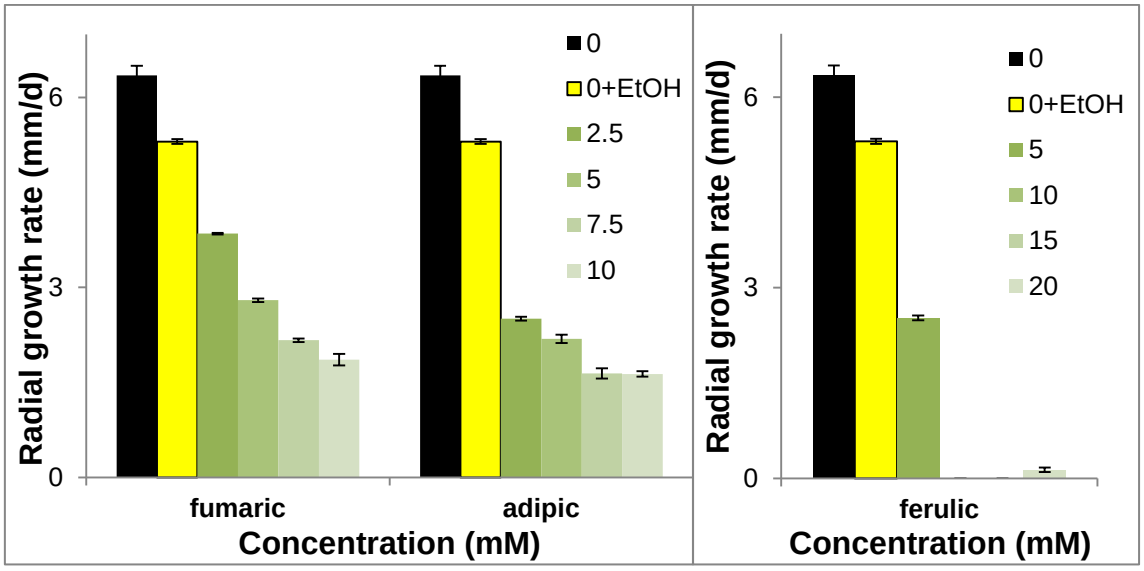


Figure 4.4: Effect of different concentrations of fumaric, adipic and ferulic acids (dissolved in ethanol) on the *in vitro* radial growth rate of *F. verticillioides*. Vertical bars indicate the standard error of the means.

The Kruskal-Wallis ANOVA by ranks showed that the effect of acid concentration was highly significant on the growth rate of *F. verticillioides* for all three acids when applied as ethanolic solutions (Apx Table E.5).

Plate 4.2 shows *F. verticillioides* cultures on media treated with ethanolic fumaric acid (from left to right: 0-10 mM) after 10 days storage at 25°C.

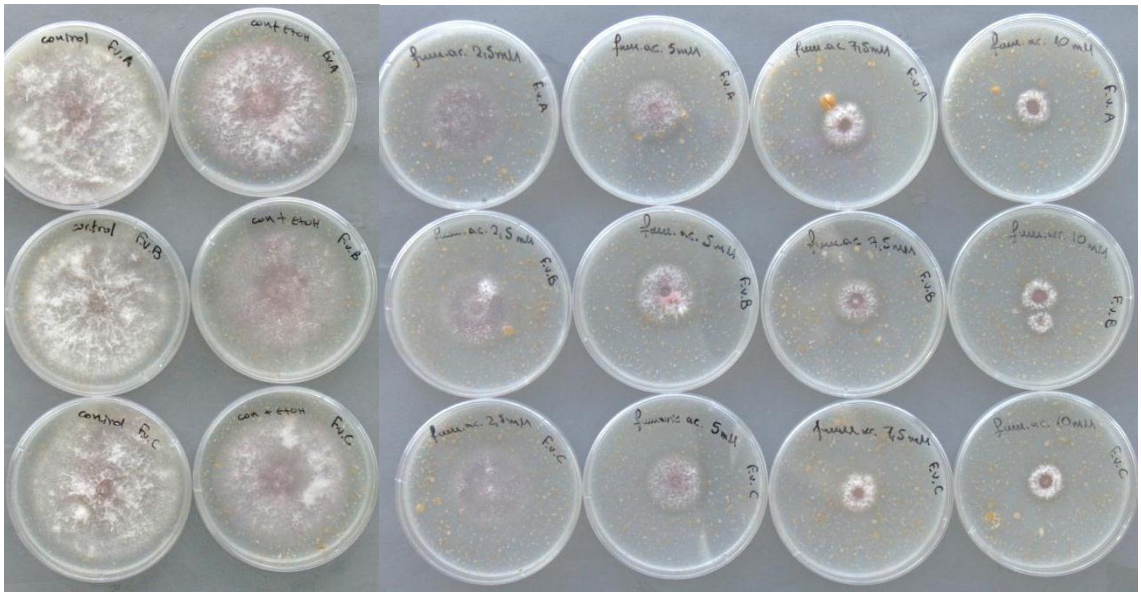


Plate 4.2: Effect of fumaric acid on *F. verticillioides* cultures after 10 days at 25°C, from left to right: control media, control with ethanol, 2.5-10 mM.

4.3.3 *In vitro* fusarium mycotoxin control using aqueous acids

Figure 4.5 shows the effect of different concentrations of adipic, tartaric and fumaric acid on the production of T-2 and HT-2 toxins as well as on the sum of the two toxins by *F. langsethiae* in wheat media at 25°C.

Adipic acid slightly stimulated T-2 and HT-2 toxin production at 10 ppm. The production of both toxins decreased thereafter, but was similar in media treated with 100 and 200 ppm. HT-2 toxin was completely inhibited with 200 ppm.

In the presence of ≤50 ppm tartaric acid T-2 and HT-2 toxin production was stimulated while with increasing acid concentration it decreased. At 200 ppm, T-2 production by *F. langsethiae* was 80% less than in the control, while HT-2 toxin production was completely inhibited.

The addition of fumaric acid in the media stimulated HT-2 toxin production by *F. langsethiae* for the range of concentrations used. The pattern of T-2 toxin production in the same samples was irregular and only marginal inhibition occurred at 200 ppm.

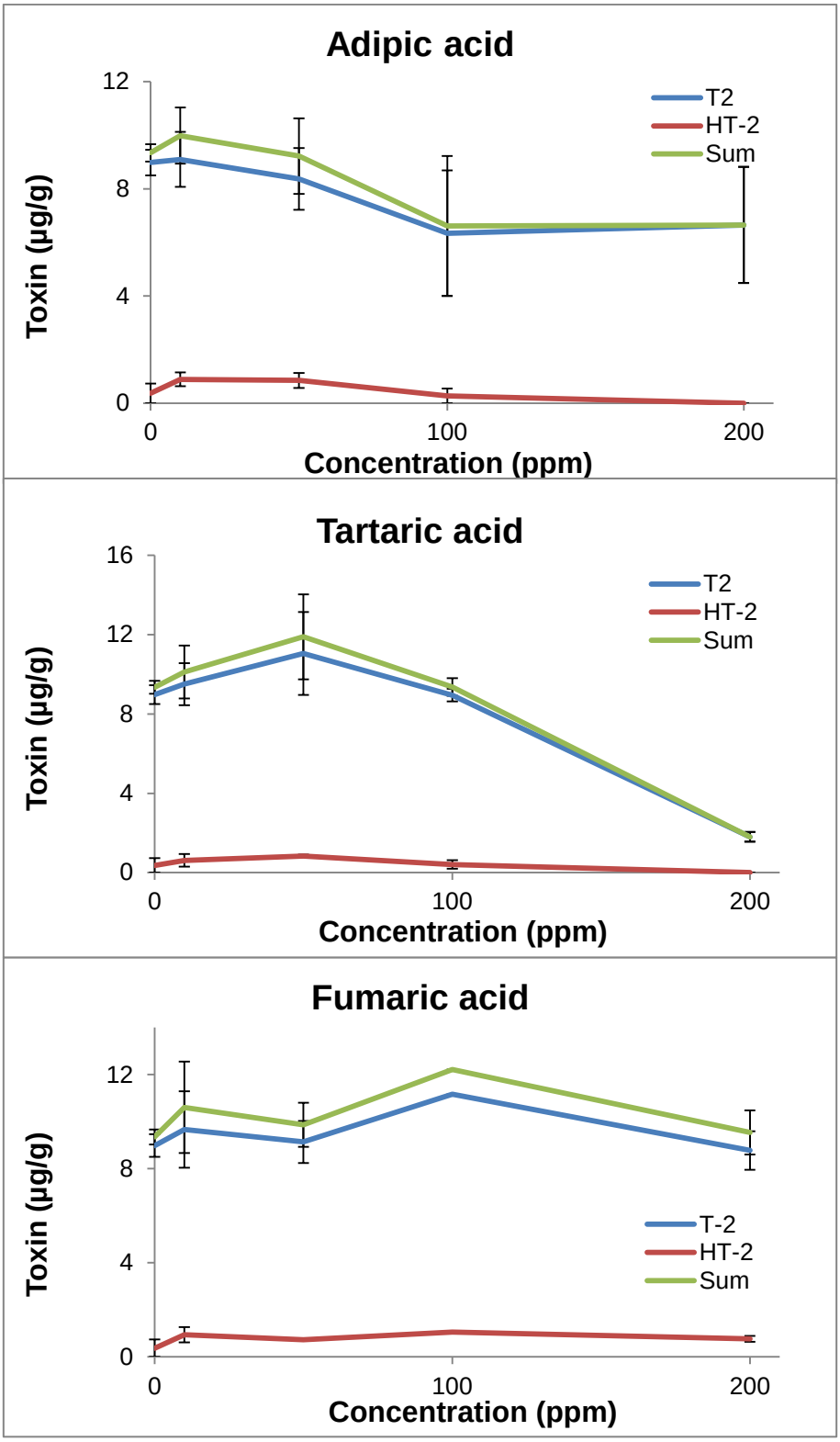


Figure 4.5: Effect of 0-200 ppm adipic, tartaric and fumaric acid on *in vitro* T-2 and HT-2 toxins production and their sum by *F. langsethiae* at 25°C. Vertical bars indicate the standard error of the means.

ANOVA showed that the effect of acid concentration on T-2 toxin production was significant only for tartaric acid, while on HT-2 toxin production it was not significant for any of the acids (Apx Table E.6, E.7).

Figure 4.6 shows the effect of different concentrations of adipic, tartaric and fumaric acid on deoxynivalenol production by *F. graminearum* in wheat agar media at 25°C.

While small amounts (10-50 ppm) of adipic and tartaric acids reduced DON production compared to the control, higher concentrations increasingly stimulated DON production. Similar behaviour was observed for trans-cinnamic acid which caused the maximum inhibition of DON production at 10 ppm (93%).

Fumaric acid inhibited DON production compared to the control to a maximum of 93% at 200 ppm.

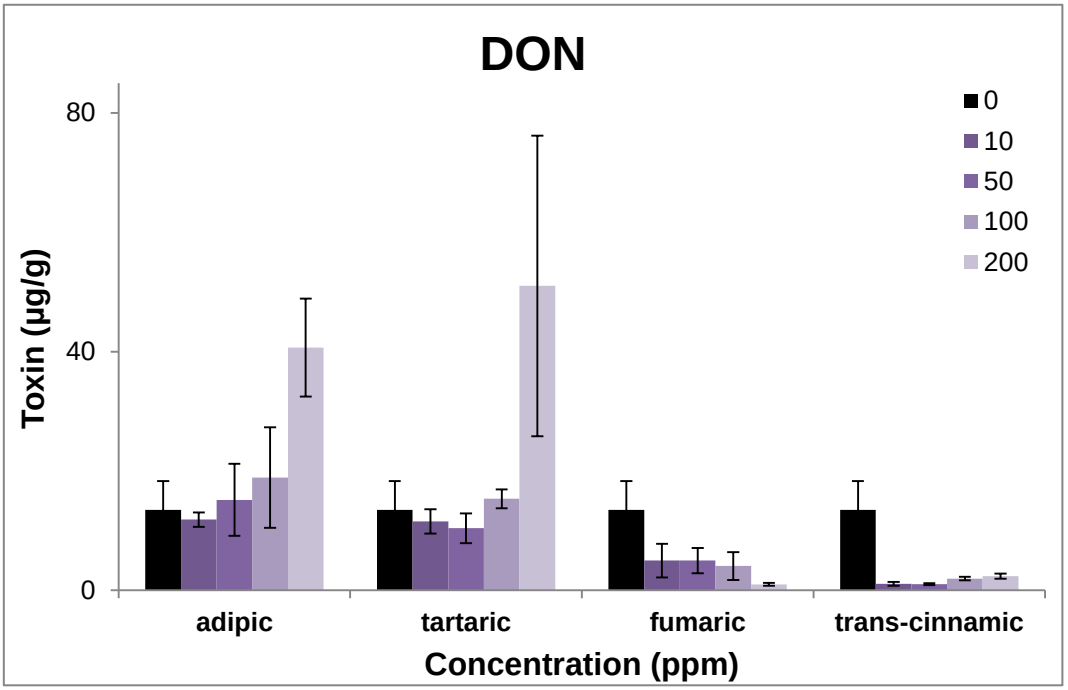


Figure 4.6: Effect of 0-200 ppm of adipic, tartaric, fumaric and trans-cinnamic acids on deoxynivalenol production by *F. graminearum in vitro* in wheat agar media at 25°C. Vertical bars indicate the standard error of the means.

ANOVA showed that the effect of acid concentration was not significant on DON production by *F. graminearum* for any of the four acids assessed (Apx Table E.8).

4.3.4 *In vitro* fusarium mycotoxin control using acids dissolved in ethanol

Figure 4.7 shows the effect of different concentrations of adipic, fumaric and ferulic acids (dissolved in ethanol) on T-2 toxin production by *F. langsethiae in vitro* at 25°C compared to controls with and without ethanol.

The presence of ethanol in the media caused the production of four times more T-2 toxin by *F. langsethiae* than that produced in media without ethanol.

Fumaric acid stimulated T-2 toxin production compared to the non-ethanol control at up to 7.5 mM. At 10 mM (1160 ppm) *F. langsethiae* did not grow and thus toxin production was completely inhibited.

Increasing concentrations of adipic acid stimulated T-2 toxin production compared to the non-ethanol control, while T-2 toxin was generally inhibited if compared to the control with ethanol.

In the samples treated with ferulic acid T-2 toxin production was stimulated 5-10 times compared to the non ethanol control and 2-4 times compared to the control with added ethanol.

HT-2 toxin production was below the limit of detection with all three acids at all concentrations, except of 5 mM ferulic acid where stimulation was observed compared to the plain control (data not shown).

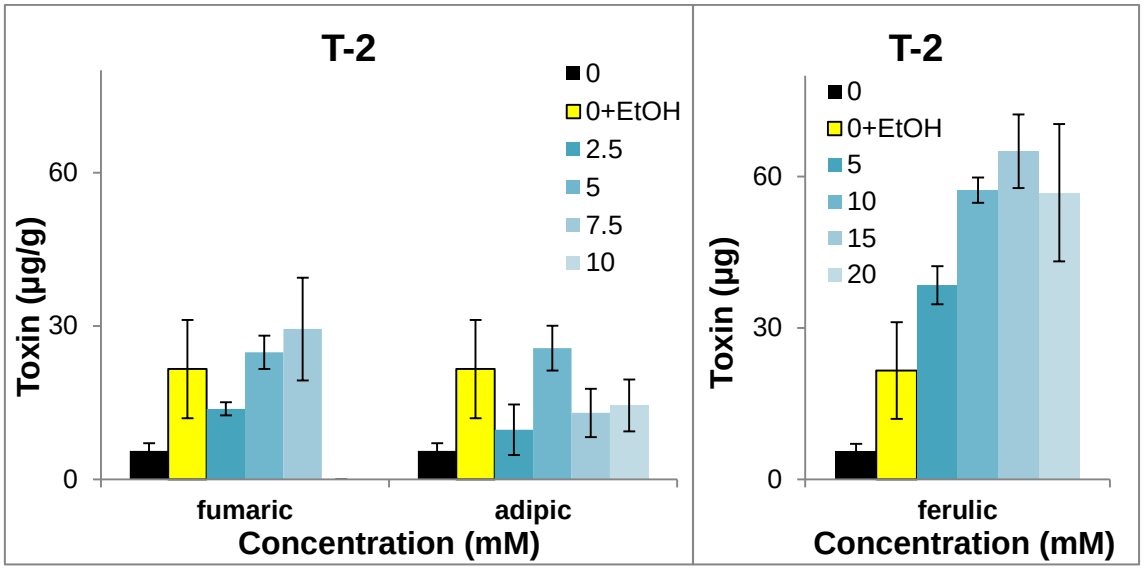


Figure 4.7: Effect of different concentrations of fumaric, adipic and ferulic acids (dissolved in ethanol) on T-2 toxin production by *F. langsethiae* in wheat media at 25°C, compared to non-ethanol and ethanol controls. Vertical bars indicate the standard error of the means.

ANOVA showed that the effect of acid concentration was significant on T-2 toxin production for fumaric and ferulic, while it was not significant for adipic acid when compared to the non-ethanol control. In contrast, when compared to the control with ethanol, the effect of acid concentration was significant on *in vitro* T-2 toxin production only for ferulic acid (Apx Table E.9).

Figure 4.8 shows the effect of different concentrations of adipic, fumaric and ferulic acids (dissolved in ethanol) on the total fumonisins (B_1+B_2) production by *F. verticillioides in vitro* compared to control samples containing ethanol and to plain control samples.

Fumonisin production was inhibited by ~79% due to the addition of ethanol. *In vitro* fumonisins production by *F. verticillioides* was completely inhibited in media treated with ferulic acid at all concentrations.

In media treated with fumaric acid total fumonisins decreased with increasing concentration to a maximum of 99 and 99.8% at 7.5 mM (870 ppm) compared

to the control with and without ethanol respectively, while 10 mM fumaric acid did not cause any further inhibition.

Adipic acid caused 88% control of fumonisin production at 2.5 mM (365 ppm) compared to the control with ethanol (or 97.5% compared to the plain control), while 5-10 mM were less efficient.

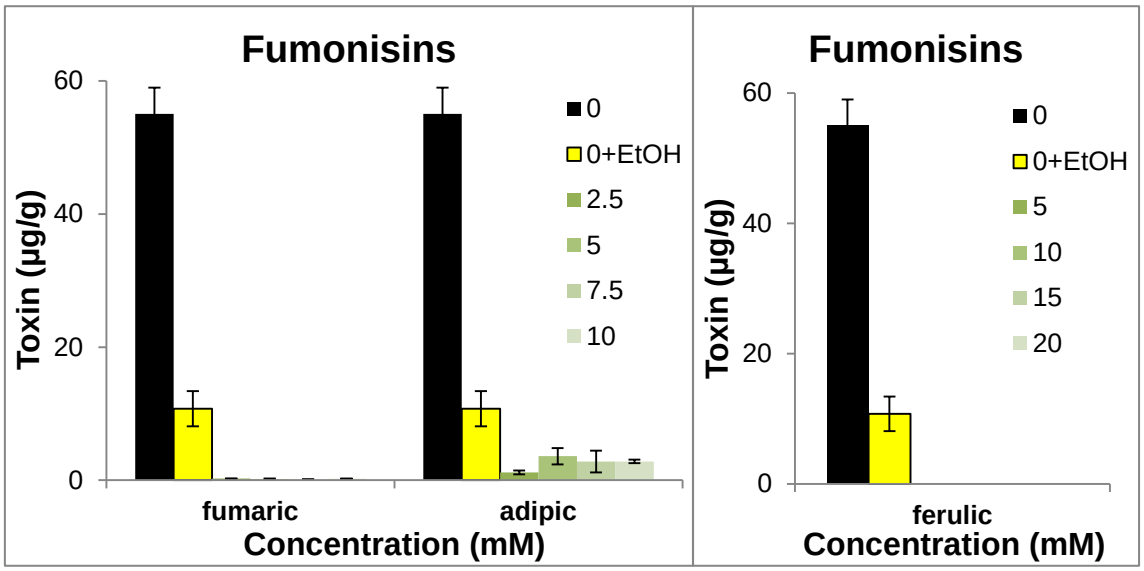


Figure 4.8: Effect of different concentrations of fumaric, adipic and ferulic acids (dissolved in ethanol) on fumonisins (B_1+B_2) production by *F. verticillioides in vitro* at 25°C, compared to non-ethanol and ethanol controls. Vertical bars indicate the standard error of the means.

The Kruskal-Wallis ANOVA by ranks showed that the effect of concentration was significant on *in vitro* fumonisin production by *F. verticillioides* for adipic and ferulic acids but not significant for fumaric acid irrespective of whether comparison was against ethanol or non-ethanol controls (Apx Table E.10).

Figure 4.9 shows the effect of different concentrations of adipic, fumaric and ferulic acid (dissolved in ethanol) on DON production by *F. graminearum* in wheat media at 25°C compared to control samples containing ethanol and to plain control samples.

The presence of ethanol in the media inhibited DON production by *F. graminearum* by 98.8% compared to non-ethanol controls.

DON production increased with increasing concentration of fumaric and adipic acids compared to the control containing ethanol, though it was always reduced compared to the plain control.

In contrast, DON production was reduced in samples containing ≥5 mM ferulic acid compared to both controls and the maximum inhibition was obtained at 20 mM and was 99.6% compared to the plain control (or 63.7% compared to the control with ethanol).

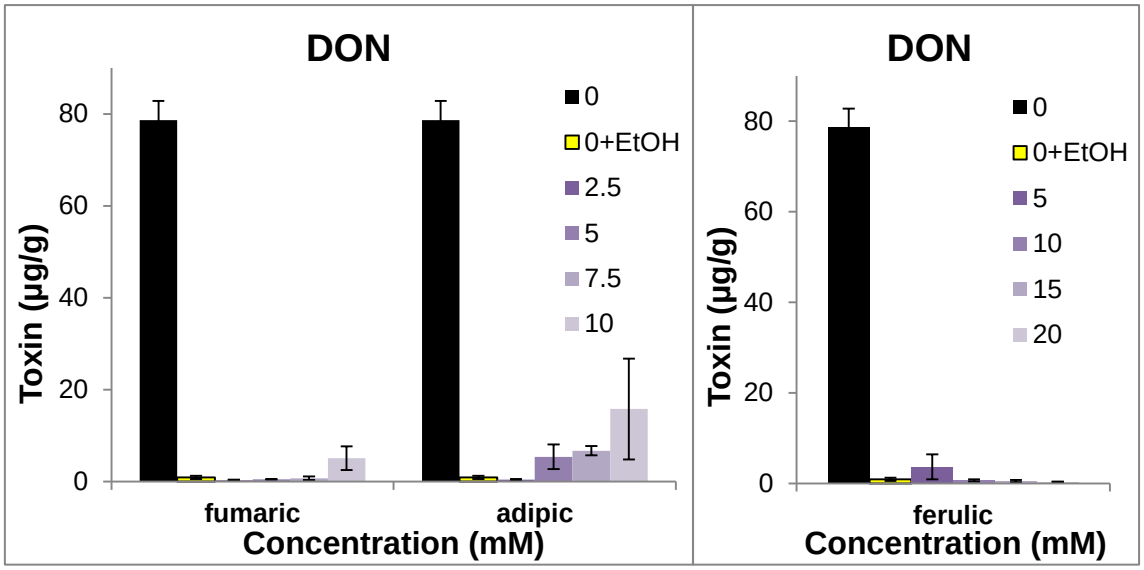


Figure 4.9: Effect of different concentrations of fumaric, adipic and ferulic acids (dissolved in ethanol) on DON production by *F. graminearum* in wheat media at 25°C, compared to non-ethanol and ethanol controls. Vertical bars indicate the standard error of the means.

ANOVA showed that the effect of acid concentration was highly significant on DON production by *F. graminearum in vitro* for fumaric acid, irrespective of whether the controls contained ethanol or not. In contrast, the effect of acid concentration was significant for adipic and ferulic acids when compared to the

plain controls, but not significant when compared to the controls with added ethanol (Apx Table E.11).

Table 4.1 summarises the results obtained in this Chapter on the effects of different acids on the growth of different fungal species and mycotoxin production *in vitro*.

Table 4.1: Summary of the effects of different acids in the aqueous or ethanolic form on the growth and mycotoxin production by different fungal species. Green: effect was observed, Red: no effect observed/stimulation, Blue: further work required, White: not tested. Where numbers are presented, these indicate specific conditions for such effect.

	Acid	Growth				Mycotoxin			
		F. g	***F. v.***	***F. l.***	***A. f.***	***F. g***	***F. v.***	***F. l.***	***A. f.***
Aqueous (0-200 ppm)	**Trans-cinnamic**								
	Ferulic								
	Adipic								
	Tartaric								
	Fumaric								
Ethanolic (5-20 mM, equivalent 0-3884 ppm)	**Trans-cinnamic**								
	Ferulic								
	Adipic					2.5 mM	2.5 mM		
	Tartaric								
	Fumaric					<5 mM		>10 mM	

4.4 Discussion

4.4.1 *In vitro* control of fungal growth and mycotoxin production using acids

Aqueous adipic, tartaric, fumaric and trans-cinnamic acids at the range of concentrations used in this study (0-200 ppm) had little inhibitory effect on the *in vitro* growth rates of *F. graminearum* and *F. langsethiae.* When applied as ethanolic solutions much higher concentrations could be reached (2000-4000 ppm) which generally caused higher growth inhibition. It was observed that the addition of ethanol (alone) in the media caused a 16.5-43% inhibition of the *Fusarium* species growth compared to the plain controls. The synergistic effect of ethanol and the acids caused maximum growth rate inhibitions of 70-100%. Generally the growth of *F. langsethiae* was consistently more affected than that of *F. graminearum* and *F. verticillioides*, irrespective of whether the acids were used as aqueous or ethanolic solutions.

Toxin production was not necessarily reduced at the conditions where growth was inhibited and occasionally stimulation was observed. Also the three *Fusarium* species studied behaved differently to the addition of different acids and this will be discussed in more detail for each individual compound.

The effects observed on secondary metabolite production were different depending on whether the acids were applied as aqueous or ethanolic solutions. Specifically, the production of T-2 toxin by *F. langsethiae* was stimulated by the addition of ethanol compared to the plain control, while DON production by *F. graminearum* and fumonisin production by *F. verticillioides* were inhibited. Dao and Dantigny (2011) reviewed several studies on the inhibition of the germination of different fungal spores and of fungal growth with ethanol, applied either in the liquid form or as vapours and showed that the minimum inhibitory concentration (MIC) for most of the fungi studied is around 4% per weight. However, they did not include in their review any study on the effect of ethanol on toxin production by different fungal species. Despite this, they concluded that ethanol vapour can be used for the control of mycotoxin

contamination in grain storage without any data in support of that assertion (Dao and Dantigny, 2011). The results obtained in the present study suggest that the presence of ethanol can in fact stimulate secondary metabolite production and in particular T-2 toxin production by *F. langsethiae* and notably aflatoxin production by *A. flavus*. This information is of particular importance when designing strategies for the control of fungal spoilage and mycotoxin contamination at the post-harvest stage.

(a) Trans-cinnamic acid

Trans-cinnamic acid at just 10 ppm stimulated the growth of *F. graminearum* while causing 92% inhibition of DON production. Also while the growth of *F. graminearum* was inhibited by just 13.4% at the range of concentrations studied, DON production was significantly reduced (82-92%). It appears that concentrations higher than those used here are required (≥200 ppm) for any significant inhibitory effect on the growth of different fungal species, i.e. *A. flavus* and *Fusarium oxysporum* f. sp. *niveum* (Chipley and Uraih, 1980; Nesci and Etcheverry, 2006; Wu et al., 2008) though at such concentrations different results have been observed on secondary metabolite production.

Trans-cinnamic acid has only been tested for the control of aflatoxin B_1 production. Nesci and Etcheverry (2006) observed complete inhibition with 1 mM trans-cinnamic acid (148 ppm) in maize agar media of 0.99 a_w but stimulation at reduced water activity or with 5 mM (740 ppm). The same authors also observed complete control of aflatoxin B_1 production in maize treated with 25 mM trans-cinnamic acid after 35 days storage (Nesci et al., 2007). In a study carried out to examine the effect of aqueous trans-cinnamic acid on *A. flavus* (Appendix H) it was observed that 10 ppm stimulated growth but reduced aflatoxin B_1 by 43%. Higher concentrations did not affect the growth of *A. flavus* (±5%) but aflatoxin B_1 was stimulated even beyond 100%. Chipley and Uraih (1980) using similar concentrations obtained ~50-100% inhibition of aflatoxin B_1 production in liquid media however this technique may not be as accurate as solid grain media for such studies.

What is of particular importance is that in both previous studies (Chipley and Uraih, 1980; Nesci and Etcheverry, 2006) the treatments were compared to controls with added ethanol while non-ethanol controls were not taken into account. From our data, the addition of small amounts of ethanol (<4%) stimulated aflatoxin B_1 production >4 times compared to the non-ethanol control which may partly explain the different results. This denotes the importance of including both controls with and without ethanol when testing compounds dissolved in this solvent.

(b) Ferulic acid

Ethanolic ferulic acid (10 and 15 mM) completely inhibited the growth of *F. verticillioides* though very little growth was observed at 20 mM. Fumonisins were below the limit of detection in all the treated samples. Beekrum et al. (2003) obtained 90% fumonisin reduction without any significant effect on fungal growth, but the ferulic acid concentrations used in that study were much lower.

DON production was stimulated by 5 mM ferulic acid, but higher concentrations (10-20 mM) caused reduction to a maximum of 99.6% compared to the non-ethanol control. These results are comparable to those of Boutigny et al. (2009) who obtained complete inhibition of trichothecenes production by *F. culmorum* with up to 5 mM ferulic acid in liquid cultures. The same authors obtained 27% reduction of trichothecenes production by *F. graminearum* with 0.5 mM, much lower concentration than those used here. It appears that using liquid cultures control of growth and mycotoxin production is easier and thus the required concentrations of different compounds for any inhibitory effect are underestimated compared to solid grain media.

Contrarily to all the above studies, ferulic acid was very efficient against the growth rate of *F. langsethiae* (max. 94% inhibition) while in the same samples T-2 toxin production was stimulated up to 10 times compared to the control at all concentrations assessed. This may be due to the presence of ethanol which, as was shown previously, can stimulate toxin production by *F. langsethiae*. The

production of HT-2 toxin on the other hand was below the limit of detection at ≥5 mM. To our knowledge no other studies have examined the effect of ferulic acid on this fungal species.

Boutigny et al. (2009) suggested that ferulic acid may be affecting the biosynthetic pathways of secondary metabolites by *Fusarium* species. If this is the case, the results obtained in this study suggest that ferulic acid plays a different role in the mechanism of secondary metabolism in *F. langsethiae* than in the other *Fusarium* species examined. Also ferulic acid seems to affect the mechanism of HT-2 toxin production but not that of T-2 toxin.

Boutigny et al. (2009) also observed that the effect of ferulic acid on trichothecene production was the same, irrespective of whether it was added at the spore germination stage or during the mycelial development when trichothecenes start to accumulate, with addition at both stages causing a 96% inhibition of toxin production. This is of particular practical importance because if ferulic is equally efficient in inhibiting growth and mycotoxin production *in situ* on grains, it could be successfully applied even after the onset of fungal growth, where most control strategies fail to have a significant effect. Control strategies have previously been shown as more efficient against fungal spores than against mycelium colonisation (Schmidt-Heydt et al., 2011). In any case it has to be noted that as discussed earlier, effects observed in liquid cultures may be deviating significantly from those on solid media or on grains and therefore the results of Boutigny et al. (2009) should be confirmed by *in situ* studies.

Ferulic acid has also been studied for the control of *A. flavus* and aflatoxin production. Nesci and Etcheverry (2006) achieved >95% growth inhibition *in vitro* investigating the additional effect of reduced water activity, while aflatoxin B_1 production was below the limit of detection at ≥1 mM (194 ppm). In a study carried out with *A. flavus*, (Appendix H) ferulic acid was slightly less efficient against the growth rate of this species compared to the *Fusarium* species used (max. inhibition 68%), while aflatoxin B_1 production was reduced by 97.3-98.1%. These results are higher than those of Chipley and Uraih (1980) possibly due to the higher ferulic acid concentrations used here.

Ferulic acid is a natural component found in different amounts in grains, fruits, vegetables and also coffee (Zhao and Moghadasian, 2008), in either its free or conjugated form and can be metabolised by humans and animals. Despite this, it has not been approved for use as an additive/preservative in food or feed Europe, nor has it been given a GRAS status by the US FDA.

(c) Tartaric acid

Increasing concentrations of tartaric acid (10-200 ppm) inhibited the growth rate of *F. langsethiae* to a maximum of 27%, while T-2 toxin production was significantly reduced with >100 ppm (~80%). HT-2 toxin production followed a similar pattern and at 200 ppm it was below the limit of detection. In contrast, tartaric acid had a very small inhibitory effect on the growth of *F. graminearum* while DON production was stimulated at ≥100 ppm. Similar results were also obtained in a study carried out with *A. flavus* (Appendix H). Atoui et al. (2007), in one of the very few studies on the use of tartaric acid for the control of fungal growth and mycotoxin production, observed reduced ochratoxin production *in vitro* with 8 g/L, while concentrations up to 16 g/L did not cause any further inhibition. These concentrations however are much higher than those used in the present study. Tartaric acid is permitted for use in food (E 334) at a level no higher than that necessary to achieve a specific effect (Good Manufacturing Practice (GMP)) (EC, 1995). Therefore, much higher concentrations than those used in the present study could be tested for efficacy, since some effect was observed on toxin production by *F. langsethiae* which has been identified as the most vulnerable *Fusarium* species in previous trials.

(d) Fumaric acid

Aqueous fumaric acid (10-200 ppm) had almost no effect on the growth rate of *F. graminearum* while DON production in the same samples was inhibited to a maximum of 93%. At the same concentration range, *F. langsethiae* growth was

inhibited by up to 18% (although at some concentrations irregular values of up to 78% were observed) but T-2 toxin production was stimulated (up to 25%).

Increasing concentrations of ethanolic fumaric acid inhibited the growth rates of all three *Fusarium* species. Fumonisins were almost completely inhibited at all concentrations (max. 99.8%). DON production was controlled by all ethanolic fumaric acid concentrations compared to the control, but maximum inhibition was observed at 2.5 mM (290 ppm). In contrast, T-2 toxin production was stimulated >5 times the control with fumaric acid at up to 870 ppm (7.5 mM) but was below the limit of detection at 10 mM (1160 ppm). This is probably due to the effect of ethanol which, as has been shown can stimulate toxin production by *F. langsethiae*.

In only one study identified on the use of fumaric acid against aflatoxin biosynthesis, some inhibitory effect was observed in the presence of acetic acid (Shantha and Murthy, 1981). In a study that was carried out with *A. flavus* (Appendix H), aqueous fumaric acid had almost no effect on its growth, while aflatoxin B_1 production in the same samples was inhibited to a maximum of 36%. Ethanolic fumaric acid on the other hand, almost halved the growth rate of *A. flavus* and caused up to 96.5% inhibition of aflatoxin B_1 production at 7.5 mM (870 ppm).

Fumaric acid is also permitted for use in food under European legislation to a maximum level of 4 g/kg (EC, 1995), concentration much higher than the ones used in the above studies. Thus, this compound seems promising for the control of mycotoxin production and the preservation of stored grains.

(e) Adipic acid

Aqueous adipic acid was not efficient in inhibiting the growth rate of any of the fungi studied at the range 0-200 ppm (max. <10%). While aqueous adipic acid inhibited T-2 toxin production at ≥50 ppm, DON production was stimulated. Ethanolic adipic acid stimulated T-2 toxin production compared to the plain control, while DON and fumonisins were controlled effectively. However,

optimum inhibition was observed at the lower adipic acid concentration assessed (365 ppm). In a similar study carried out with *A. flavus* (Appendix H) aqueous adipic acid stimulated aflatoxin B_1 production while ethanolic adipic acid inhibited aflatoxin B_1 production at all concentrations, to a maximum of 89.5% with 1460 ppm compared to the plain control.

No studies were identified on the use of adipic acid for the control of growth and toxin production by mycotoxigenic fungi. Flors et al. (2004) observed significant inhibition of the *in vitro* growth of *Alternaria solani* and *Botrytis cinerea* with 10-500 ppm adipic acid monoethyl ester and complete inhibition of spore germination at the same concentrations in liquid media, noting however that the effect was fungistatic. They suggested that this compound may be used as an alternative to fungicides. Adipic acid is also permitted for use in foods (desserts, cakes) to a maximum level of 10 g/kg (EC, 1995), which is much higher than the levels used in this study, thus allowing for the assessment of higher concentrations for efficacy in grain preservation.

4.4.2 *In situ* control of mycotoxin production using ferulic acid

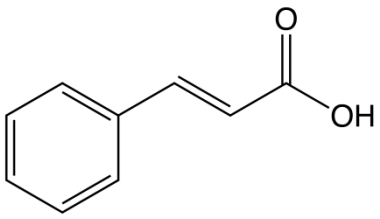
Ferulic, among the acids examined, showed very promising results against fungal growth and mycotoxin production *in vitro*. However one disadvantage was that while it was particularly efficient against DON production by *F. graminearum*, it stimulated T-2 toxin production by *F. langsethiae*. And while this may primarily be due to the presence of ethanol, which was shown to stimulate T-2 toxin production, other compounds have also been shown to inhibit one toxin while stimulate the other when applied at specific concentrations *in vitro* (Table 4.1). Marín et al. (2000) also observed species-dependent efficacy of compounds which can lead to enhanced growth of the more tolerant species. This is of particular importance since *F. graminearum* and *F. langsethiae* are both known to contaminate wheat, barley and oats especially in Northern Europe and their likely co-occurrence in such grains cannot be ignored. Thus, application of a compound that controls DON

production may have the exact opposite results on other toxins. This may also explain the findings of Edwards (2009) who observed higher amounts of toxin in the samples produced conventionally than in organic grains. Other authors have also suggested that sub-inhibitory concentrations of compounds or inefficient coverage can result in stimulated mycotoxin production (Lee et al., 1986; Marín et al., 1999; Marín et al., 2000). For this reason, this compound was not assessed on naturally contaminated wheat or oats.

Ferulic acid was particularly efficient *in vitro* against the production of aflatoxin B_1 by *A. flavus* and for this reason it was tested *in situ* on hazelnuts for control of artificially inoculated *A. flavus* (Appendix H). 25 mM ethanolic ferulic acid reduced aflatoxin B_1 production in hazelnuts of 0.90 a_w stored for 10 days to a maximum of 97.6% and up to 49.5% in hazelnuts stored for 20 days. These results are promising, since such conditions may be encountered immediately after harvest and prior to drying or accidentally during storage, due to water leakage or metabolic activity and treatment with ferulic acid can significantly control aflatoxin contamination. In hazelnuts of 0.95 a_w the treatment was not effective and aflatoxin B_1 production was stimulated particularly in the samples treated with 12.5 mM ferulic acid. Significantly less aflatoxin B_1 was produced in the samples treated with 25 mM and stored for up to 20 days (though still above the control), suggesting that higher concentrations may be more effective. These conditions however are far beyond safe storage and have been used here for comparison only. The results obtained are comparable to those of Nesci et al. (2007) who observed stimulation of aflatoxin B_1 production by *A. flavus* in maize of 0.95 a_w treated with 30 mM ferulic acid and stored for 35 days, despite observing control at other water activity levels (0.93-0.99 a_w). Several other studies have referred to stimulation of fungal growth and toxin production under different environmental conditions, in the presence of fungicides, or in moist grain after treatment with acids at sub-inhibitory concentrations or as a result of inefficient coverage (White and Coates, 1998; Marín et al., 1999; Marín et al., 2000; Magan et al., 2002; Ramirez et al. 2004).

The above results show that it may be possible to use ferulic acid for the control of *A. flavus* and aflatoxin contamination during storage. Ferulic acid was also found particularly effective *in vitro* in controlling fumonisins production by *F. verticillioides* at the same range of concentrations. *A. flavus* and *F. verticillioides* are common pathogens of maize in several parts of the world and often they co-occur. Therefore, ferulic acid could be further examined for application on naturally contaminated maize for the control of the mycotoxins produced by these two species.

Trans-cinnamic and ferulic acids have quite similar structures as can be observed in Figure 4.10. For this reason several studies have examined their similarity in efficacy against fungal growth and mycotoxin production (Nesci and Etcheverry, 2006; Nesci et al., 2007). Also mixtures of ferulic and trans-cinnamic acid at different proportions have been examined and complete control of aflatoxin B_1 production in maize treated with a mixture of 25 mM trans-cinnamic acid and 30 mM ferulic acid after 35 days storage was observed by Nesci et al. (2007).

(a) 

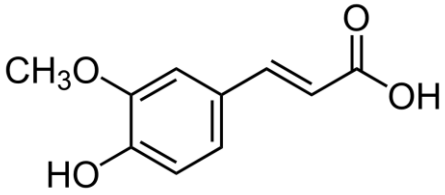
(b) 

Figure 4.10: Chemical structures of (a) trans-cinnamic acid and (b) ferulic acid.

None the less, some concerns related to the practical application of ferulic acid for grain treatment have to be expressed. First of all, due to the low water solubility of this compound, it can only be applied as an ethanolic solution. The ethanol evaporates immediately after addition to the media/grain and the compound remains. Thus, uniform application of ferulic acid was not easily achieved since ferulic acid crystals became attached to the surface of the

hazelnuts and very efficient mixing was required. This caused the peal of the hazelnuts to be removed as well as breaking of some kernels, especially at the highest water activity treatment. Therefore, different methods for the application of this compound on grains should be examined. Also, the applied ferulic acid formed a layer on the surface of the hazelnuts that would require effective washing to be removed. However, Nesci et al. (2007) do not make any reference to such issues during the application of ferulic acid on maize. The concentrations assessed in their study and the mixtures with similar concentrations of trans-cinnamic allow for concerns on the feasibility of practical application of such treatments as well as on the associated cost. However, it may be worth assessing the efficacy of this treatment for application on hazelnuts in shell, as after removal of the shell the seed may be intact and thus unaffected by the treatment. There would also be no need to remove the ferulic acid added for preservation in this case, reducing the associated costs.

Even though ferulic acid is a natural compound, its use in food/feed has not been accepted to date and therefore further studies should be conducted in order to determine that there are no toxicological risks associated with its consumption. Finally, ferulic acid should be tested against other mycotoxigenic fungal species that may be commonly found in small grains in order to examine the effect on secondary metabolite production by those species.

4.4.3 Adipic, tartaric and fumaric acids

The *in vitro* results obtained for fumaric acid indicate that applying this compound at 2.5 mM would significantly decrease DON, while stimulate T-2 toxin production, while 10 mM would completely eliminate T-2 toxin but the efficiency against DON would be compromised. These results are of particular importance since certain grains (wheat, oats, barley) can be contaminated by both *F. graminearum* and *F. langsethiae* in certain parts of the world. Detailed studies should therefore be performed on different grains with mixtures of the

two fungi, in order to determine the optimum concentration of application of this compound for optimum control of both toxins.

Also, since fumaric acid was particularly efficient against both *A. flavus* and *F. verticillioides*, fungal species that are commonly found to contaminate maize in certain parts of the world, further experiments could be carried out on maize with mixed inoculum to examine the efficacy on the simultaneous control of aflatoxins and fumonisins.

Figure 4.11 shows the chemical structures of adipic, tartaric and fumaric acids, as well as propionic acid, a compound widely used for the control of fungal growth on moist grain.

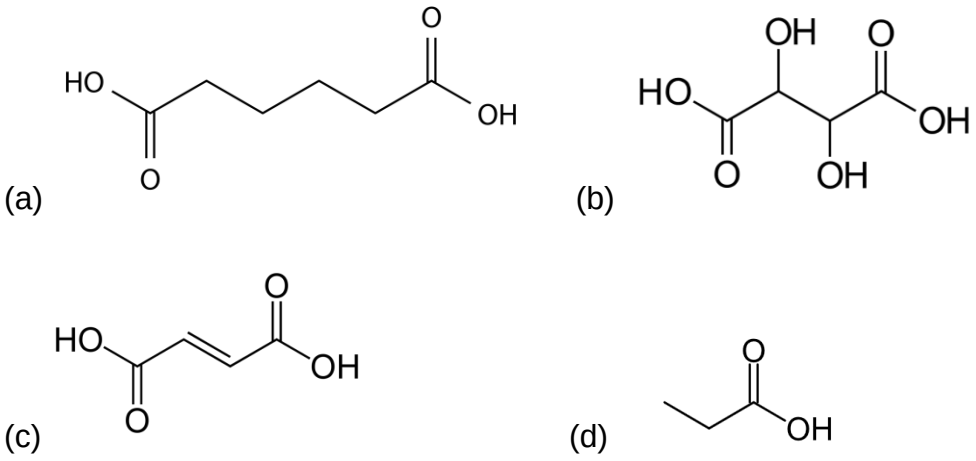


Figure 4.11: Chemical structures of (a) adipic acid, (b) tartaric acid, (c) fumaric acid and (d) propionic acid.

Similarities can be observed in the structure of these compounds. Despite this, they caused different effects on secondary metabolite production by different *Fusarium* species *in vitro* (Table 4.1). This has also been suggested by Marín et al. (2000) who observed that propionates had different effects on different fungal species on naturally contaminated maize. The importance of this has been discussed previously as *F. graminearum* and *F. langsethiae* may co-occur in different grains and control of one species may enhance the growth and toxin production by the other. Therefore, very detailed studies must be conducted

over a wide range of concentrations and using different solvents than ethanol for water-insoluble compounds, before these compounds can have any practical application. Also it may be worth examining mixtures of these acids, for efficacy against mixed fungal populations. Finally, comparisons with commercially available similarly structured compounds may also be of particular significance.

5 CHEMICAL CONTROL OF FUNGAL GROWTH AND MYCOTOXIN PRODUCTION USING ESSENTIAL OILS

5.1 Introduction

Essential oils and extracts derived from plants or grains also offer an attractive alternative for the control of fungal growth and mycotoxin production or for use as preservatives. These are also more widely acceptable by the consumers as they are regarded as natural products.

Several essential oils and plant extracts have been screened for efficacy against fungal growth and toxin production. Bluma et al. (2008) screened 96 plant extracts and suggested that essential oils were more effective against mycotoxin production than aqueous or ethanolic extracts. Some compounds inhibited aflatoxin B_1 production while in some cases the efficacy depended on the water activity. Also the efficacy has been shown to decline with extended storage time (Bluma and Etcheverry, 2008). Differences have also been observed between results *in vitro* and on grain (Aldred et al., 2008) and in some studies even though growth was inhibited, toxin production was stimulated, possibly due to the combination of water stress and physiological stress caused by the antifungal agent itself (Magan et al., 2002).

Onion and garlic, both members of the *Allium* family, have always been considered as having significant antimicrobial properties. Their antifungal efficiency has been studied despite the relative instability of some of their compounds or their strong odour. Yin and Tsao (1999) found garlic out of seven *Allium* plants to be the most effective against three *Aspergillus* species. Species-related efficacy was also observed as higher concentrations of all treatments were required against *A. flavus* and *Aspergillus fumigatus* than against *Aspergillus niger*. At 25°C the presence of acetic acid increased the antifungal efficacy, however higher temperatures decreased the effect. Yoshida et al. (1987) suggested that the antifungal activity of garlic was due to the compounds allicin and ajoene. Benkeblia (2004) observed growth inhibition of *A. niger* and *Penicillium cyclopium* by red onion and garlic essential oils as well

as by higher concentrations (200-500 ml/L) of green and yellow onion essential oils. *Fusarium oxysporum* was mostly affected by the essential oils of garlic and by 300 and 500 ml/L of the onion oils. Singh and Singh (2005) studied the effect of *Allium sativum* extracts and other plant extracts on the growth of *A. flavus* and aflatoxin production in liquid cultures. Addition of the extract at the beginning of the incubation showed 85% inhibition in the mycelia growth and 100% inhibition in aflatoxin production, but when added on the fourth day of incubation toxin was only marginally inhibited.

Some very early studies supported such properties in thiosulfinates (Small et al., 1947) though they have not found application in grain preservation possibly due to their reported reduced stability and their strong odour. Garlic extract has been approved for use as a pesticide (although currently under re-evaluation) and commercial products are available for such applications.

Some of the essential oils studied have shown promising results however studies have mostly focused on *Aspergillus* and *Penicillium* species and thus very limited information is available on the efficacy of essential oils on *Fusarium* species. Also, such compounds have rarely been examined on naturally contaminated grains where a range of different species may be encountered. Several of the compounds used in the above studies also have strong odours which may hinder their suitability for practical applications especially in food grains.

The aim of this study was to examine two novel essential oils, PTS and PTSO, derived from garlic (Mousala, S. L., 2006) for efficacy in the control of fungal growth and mycotoxin production for the first time. Experiments were conducted *in vitro* for a preliminary assessment of these compounds and determination of the effective concentrations for control of growth and mycotoxin production. Subsequently these concentrations were applied *in situ* on naturally contaminated grains, rewetted and stored at 25°C for 10 to 20 days.

5.2 Materials and methods

5.2.1 Preparation of stock solutions of the garlic essential oils

Stock solutions of the following compounds were prepared in sterile distilled water as in Section 4.2.1. Stock solutions were also prepared in 80% absolute ethanol (HPLC Grade).

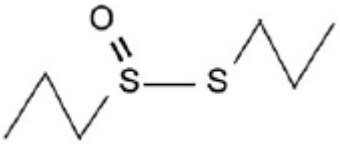
(a) PTS (Propyl propylthiosulfinate)

A stock solution of 5000 ppm was prepared by dissolving 1.1 g PTS (90% PTS, Domca, S.A., Granada, Spain) (Mousala, S. L., 2006) into a 200 ml container containing 200 ml of sterile distilled water and vigorously shaking. Due to the oily nature of PTS the stock solution had the appearance of a stable water emulsion. A second stock solution of 20000 ppm was prepared by dissolving 2.2 g PTS into 100 ml of a mixture of ethanol:H_2O (80:20). This solution was clear.

(b) PTSO (Propyl propyl thiosulfonate)

A stock solution of 10000 ppm was prepared by dissolving 1.1 g PTSO (90% PTSO, Domca, S.A., Granada, Spain) (Mousala, S. L., 2006) into a 100 ml container containing 100 ml sterile distilled water and vigorously shaking. Due to the oily nature of PTSO the stock solution had the appearance of a water emulsion however less stable than the PTS one. A second stock solution of 20000 ppm was prepared as for PTS.

Figure 5.1 shows the chemical structures of PTS and PTSO.

a) PTS: 

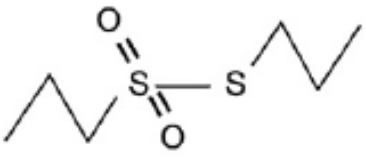
b) PTSO: 

Figure 5.1: Chemical structures of a) PTS and b) PTSO

5.2.2 Grains, culture media and water activity adjustment

Naturally contaminated wheat, maize (Section 2.2.1) and oats (Section 3.2.1) were used for the *in situ* experiments. Water adsorption curves were prepared for these grains as described in Section 2.2.2 and they can be found in Appendix F. The samples were allowed to equilibrate at 4°C prior to use, in order to prevent fungal growth. For the *in vitro* trials synthetic agar media were prepared and modified to different concentrations as described in Section 4.2.2. For the initial screening concentration in the range 10-200 ppm were used on wheat agar media of unmodified water activity (0.995 a_w). Some trials were also performed in media of modified water activities (0.90-0.96 a_w).

5.2.3 Fungal strains and preparation of the inoculum

As described in Section 4.2.3.

5.2.4 *In vitro* effect of essential oils on mycelia growth rate

As described in Section 4.2.4.

5.2.5 *In vitro* effect of essential oils on mycotoxin production

As described in Section 4.2.5.

5.2.6 *In situ* fungal growth and mycotoxin production in grains treated with essential oils

Grains were rewetted according to water adsorption curves of Appendix F, substituting part of the water by the amount of the essential oil stock solutions required to obtain the target compound concentration and allowed to equilibrate for 2-3 days at 4°C. Grain (100 g) was inoculated with 1 ml spore suspension

containing ~10^5 spores/ml of the fungi to be studied and thoroughly mixed using a roler mixer in order for the spores to disperse throughout the mass. Sub-samples (~15 g) were weighed in plastic bottles and stored in sandwich boxes for 10-20 days. The ERH was maintained using glycerol-water solutions of the same a_w.

At the end of each storage period 3 replicates were removed from storage and frozen at -20°C for subsequent toxin analysis. Grain samples were oven-dried overnight at 60°C and milled and they were extracted and analysed by LC-MS/MS as previously described (Section 2.2.7).

5.2.7 Statistical analysis

All experiments have been performed in triplicate. Data were analysed as described in Section 2.2.8. The standard error of the mean was calculated in all trials and it is denoted with vertical bars in the Figures. ANOVA Tables from this Chapter can be found in Appendix G.

5.3 Results

5.3.1 *In vitro* efficacy of garlic essential oils against fungal growth

Figure 5.2 shows the effect of 0-200 ppm PTS and PTSO on the *in vitro* radial growth rates of *F. graminearum, F. verticillioides* and *F. langsethiae.*

Both PTS and PTSO showed very good inhibitory effect on the growth of all three *Fusarium* species examined and this increased with increasing concentration. Complete inhibition of the growth of *F. langsethiae* was observed with 100 ppm PTS and of *F. verticillioides* with 200 ppm. Growth of *F. graminearum* was also significantly inhibited, although complete inhibition was not achieved in the range of concentrations examined. 200 ppm PTSO completely inhibited the growth rate of *F. langsethiae* and were very effective

against *F. graminearum* and *F. verticillioides* although complete inhibition was not achieved.

PTS was always more efficient than PTSO when applied at the same concentration against the same fungal species.

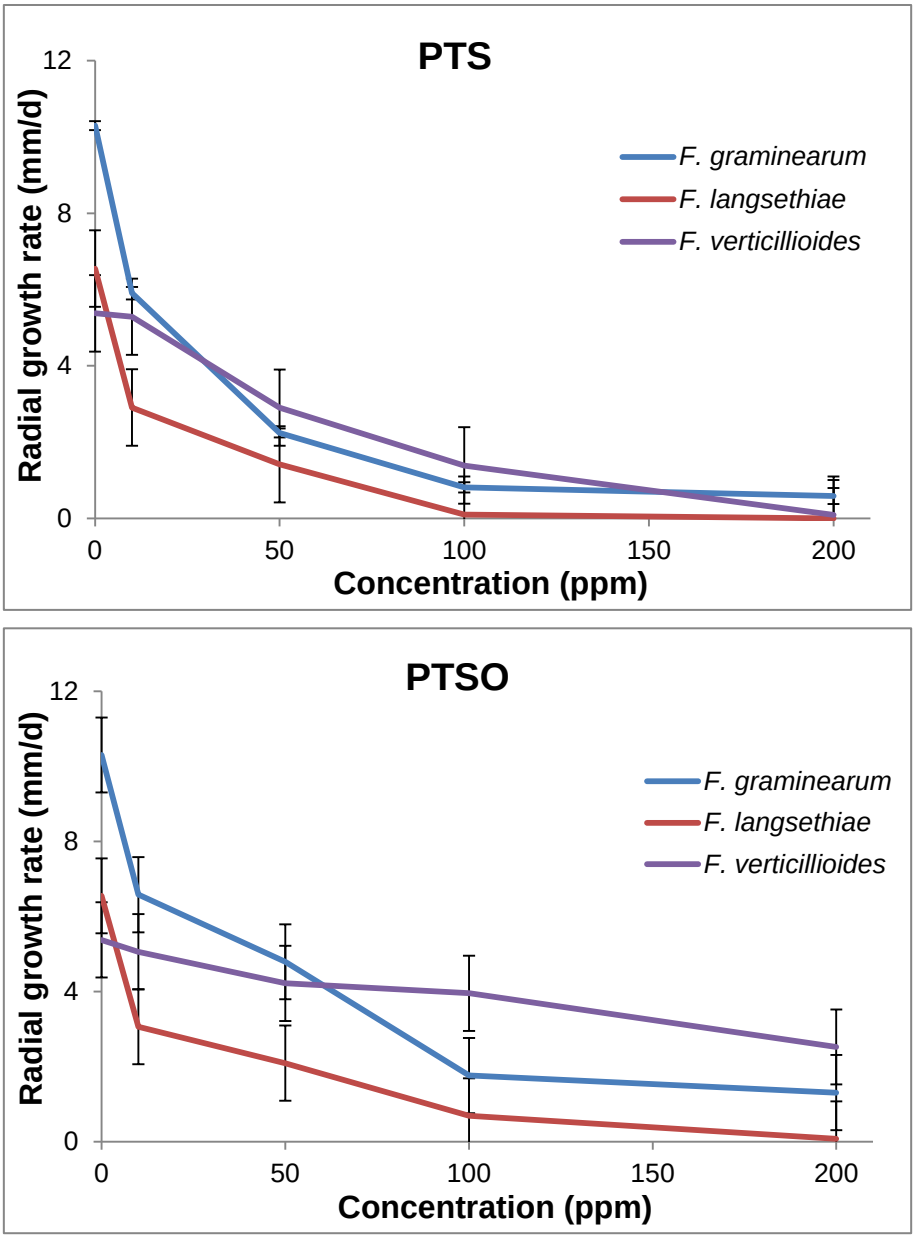


Figure 5.2: Effect of 0-200 ppm PTS and PTSO on the *in vitro* radial growth rate of *F. graminearum*, *F. langsethiae* and *F. verticillioides.* Vertical bars indicate the standard error of the means.

Plate 5.1 shows *F. graminearum* cultures on wheat agar media treated with (a) PTSO and (b) PTS (0-200 ppm from left to right) after 10 days storage at 25°C.

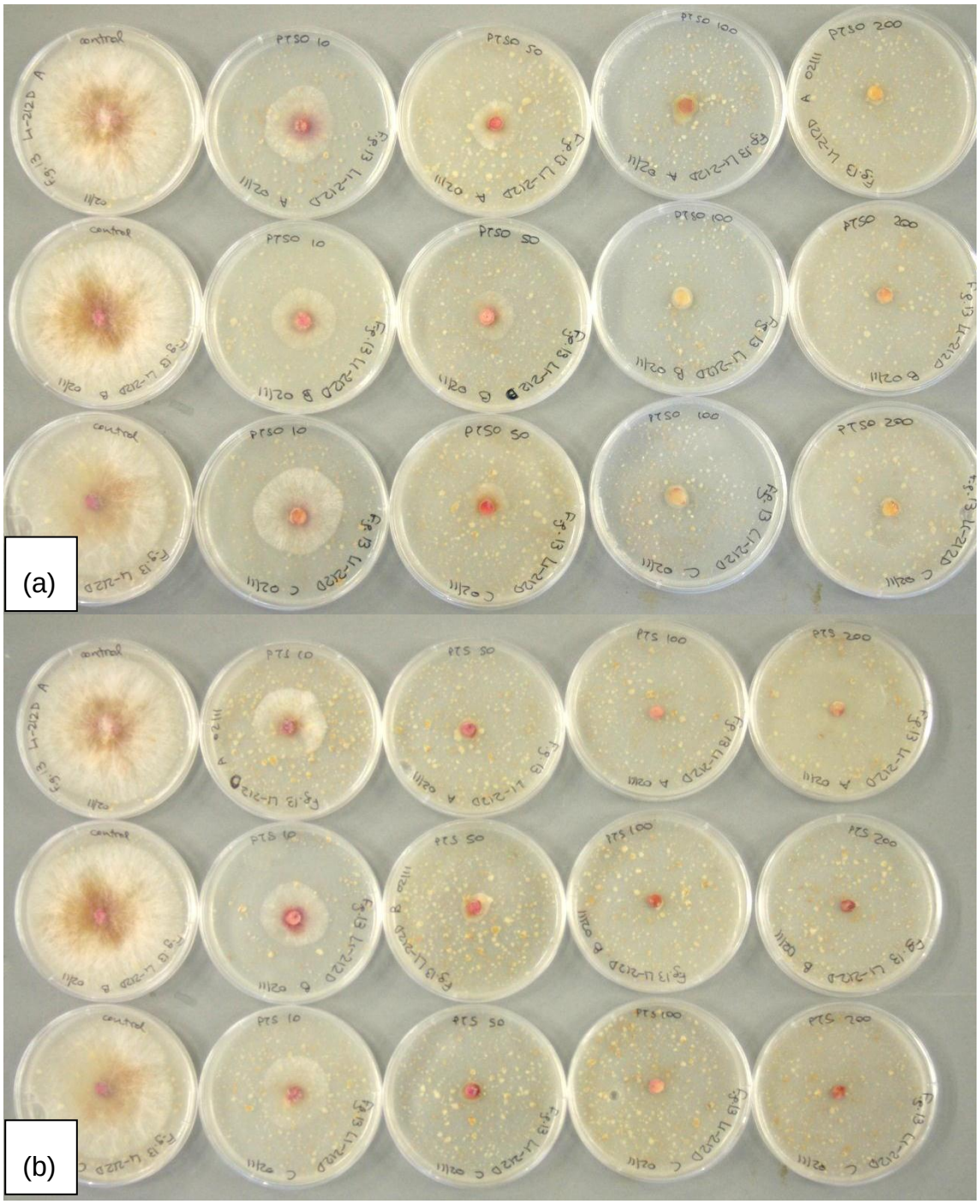


Plate 5.1: *F. graminearum* cultures on wheat agar media treated with (a) PTSO and (b) PTS (0-200 ppm from left to right) after 10 days storage at 25°C.

The Kruskal-Wallis ANOVA by ranks showed that the effect of PTS and PTSO concentration was highly significant on the growth rate of all three *Fusarium* species, while the effect of fungal species was also significant on the growth rate (Apx Tables G.1 and G.2).

5.3.2 *In vitro* efficacy of PTSO against fungal growth in media of reduced water activity

Figure 5.3 shows the effect of 0-100 ppm PTSO on the growth rate of *F. graminearum* and *F. langsethiae* on wheat agar media of different a_w levels.

The radial growth rates of both fungi were significantly inhibited with increasing PTSO concentration at 0.94 and 0.92 a_w compared to the control. Maximum % inhibition was observed at the highest PTSO concentration and 0.995 a_w (~83% for *F. graminearum* and ~90% for *F. langsethiae*) and this was followed by 0.94 a_w for *F. graminearum*, while by 0.92 a_w for *F. langsethiae.*

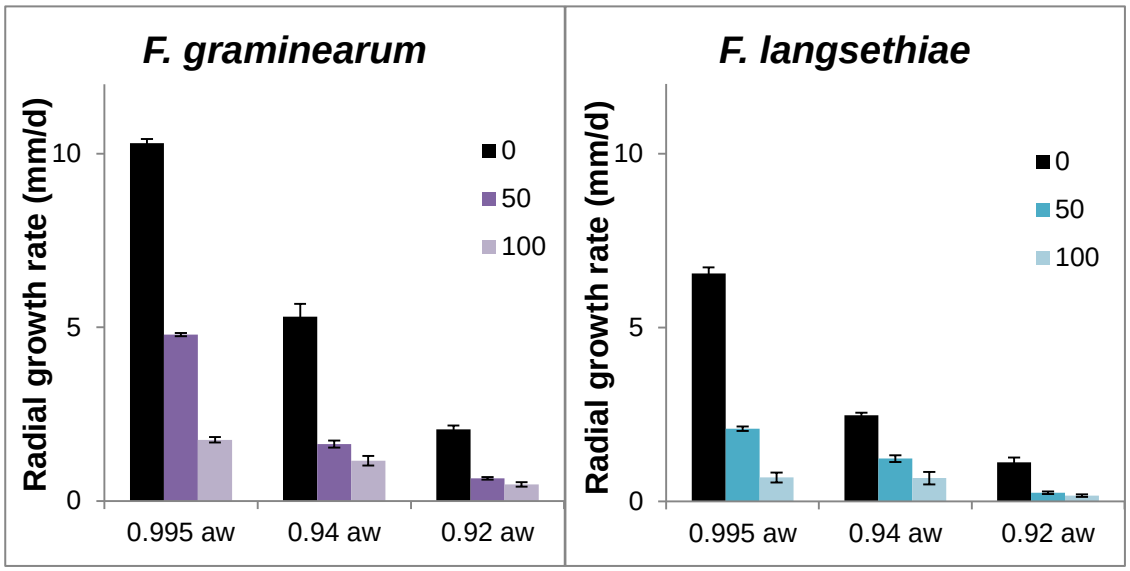


Figure 5.3: Effect of 0-100 ppm PTSO and substrate a_w on the radial growth rates of *F. graminearum* and *F. langsethiae in vitro* at 25°C. Vertical bars indicate the standard error of the means.

ANOVA showed that the effects of PTSO concentration, water activity and fungal species were highly significant on the log-transformed radial growth rate data, while the effects of the interactions between these factors were not significant (Apx Table G.3).

Table 5.1 summarises the ED_{50} values (effective dose for 50% *in vitro* growth inhibition) of PTS and PTSO for *F. graminearum*, *F. langsethiae* and *F. verticillioides* on wheat agar media modified to certain a_w levels and 25°C.

Generally lower levels of PTS than PTSO were required for 50% inhibition of the growth rate of the above three *Fusarium* species. However, reduction of the substrate a_w did not necessarily parallel the need for lower amounts of PTSO for 50% growth inhibition. Also, overall, lower concentrations of both compounds were required for 50% inhibition of the growth of *F. langsethiae* than for the other two *Fusarium* species.

Table 5.1: ED_{50} values (ppm) of PTS and PTSO for 50% growth inhibition of *F. graminearum, F. verticillioides* and *F. langsethiae* on wheat agar media of different water activities at 25°C.

	F. graminearum	***F. verticillioides***	***F. langsethiae***
PTS (0.98 a_w)	15.5	55	9
PTSO (0.98 a_w)	42	189	9.5
PTSO (0.94 a_w)	36	>100	50
PTSO (0.92 a_w)	37	>100	32

5.3.3 *In vitro* fusarium mycotoxin control using garlic essential oils

Figure 5.4 shows the effect of different concentrations of PTSO and PTS dissolved in (a) water and (b) aqueous ethanol (80%) on DON production by *F. graminearum* in wheat media at 25°C.

DON production in media treated with aqueous solutions of both compounds was generally reduced compared to the control. Inhibition by aqueous PTSO was irregular with compound concentration. DON production was below the limit of detection with 200 ppm aqueous PTS.

Irregular DON levels were observed in media treated with ethanolic PTSO and the inhibition caused by the addition of ethanol alone was higher than that achieved by any PTSO concentration. DON production at 250 ppm PTSO was ~4 times more than in the control. In contrast, DON production in media treated with ethanolic PTS decreased with increasing concentration and was below the limit of detection at ≥150 ppm PTS.

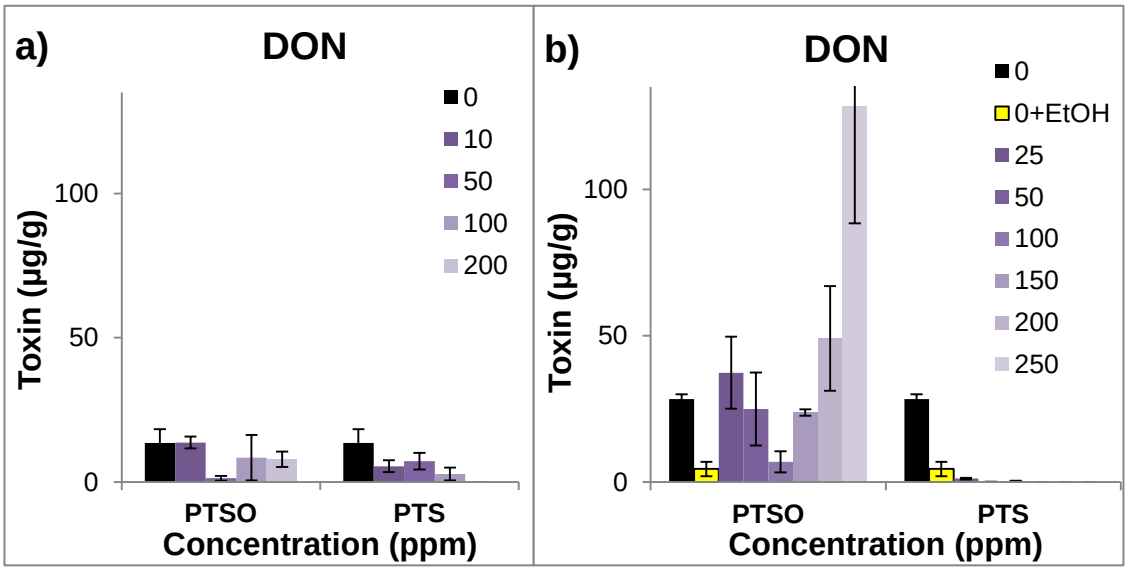


Figure 5.4: Effect of different concentrations of PTSO and PTS dissolved in (a) water and (b) aqueous ethanol (80%) on *in vitro* DON production by *F. graminearum* in wheat agar media at 25°C. Vertical bars indicate the standard error of the means.

ANOVA showed that the concentration of aqueous PTSO was not significant for *in vitro* DON production by *F. graminearum*, while the concentration of aqueous PTS was significant. Similar results were obtained for the effect of PTS and PTSO dissolved in 80% ethanol on DON production when compared to the plain control, but when compared to controls with ethanol the concentration of both essential oils was significant (Apx Table G.4, G.5).

Figure 5.5 shows the effect of different concentrations of PTSO and PTS dissolved in either water (a) or 80% ethanol (b) on the production of T-2 and HT-2 toxins by *F. langsethiae* in wheat media at 25°C.

In media treated with aqueous PTSO, T-2 toxin production by *F. langsethiae* was generally less than in the control, although no specific pattern was observed with concentration for the range examined. HT-2 toxin production was below the limit of detection at all PTSO concentrations.

T-2 production decreased with increasing PTS concentration and was below the limit of detection with 200 ppm. HT-2 toxin in the same samples was not detected at ≥50 ppm PTS.

The production of T-2 and HT-2 toxins by *F. langsethiae* was 4 times higher in the control samples containing 80% ethanol than in the non-ethanol controls.

T-2 and HT-2 toxin production was stimulated in media treated with PTSO dissolved in 80% ethanol at all concentrations compared to the non-ethanol control, but was generally reduced compared to the control with ethanol.

In media treated with PTS dissolved in 80% ethanol, at concentrations ≤100 ppm T-2 toxin was stimulated but HT-2 was inhibited, while at ≥150 ppm both toxins were completely inhibited.

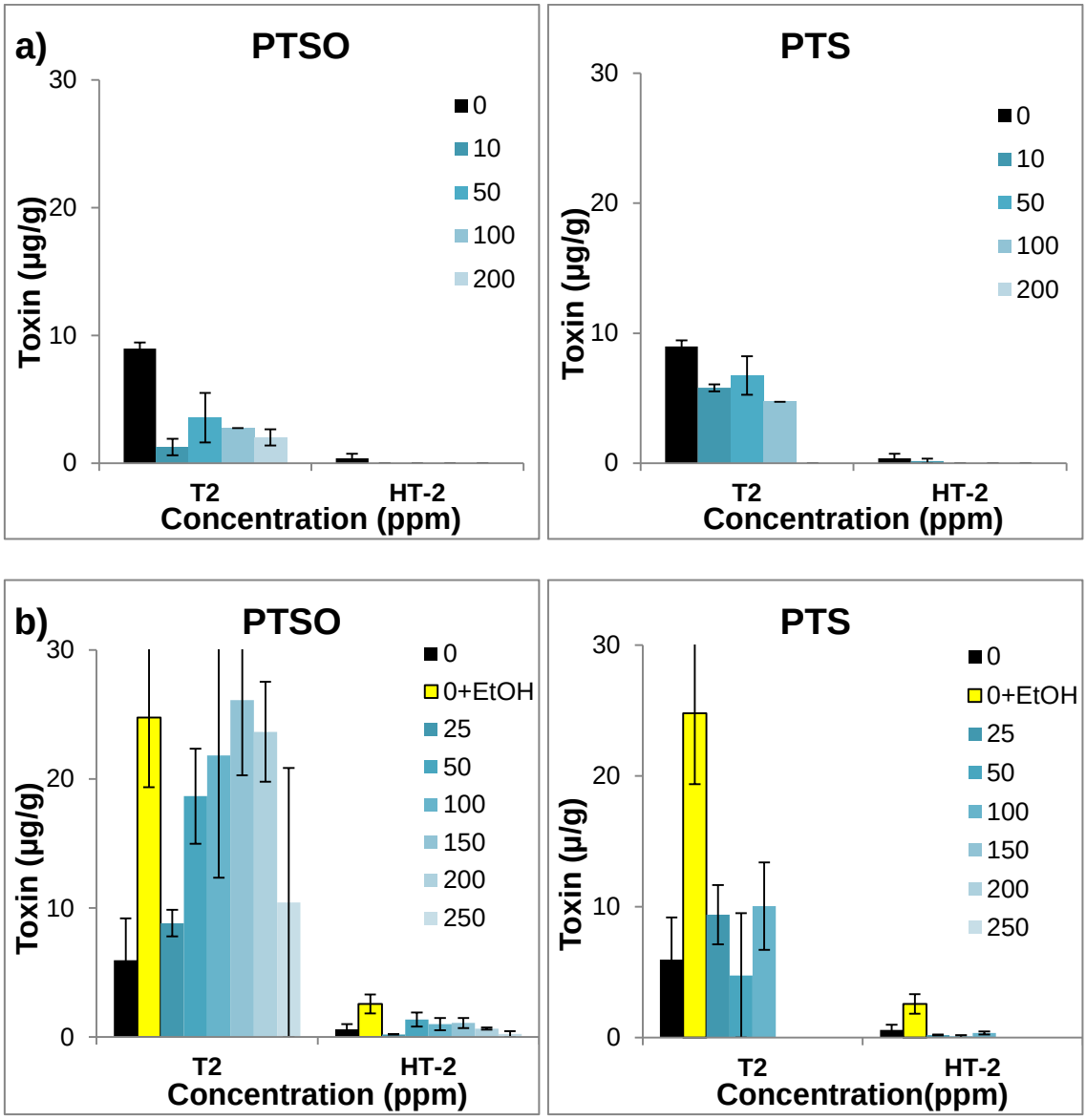


Figure 5.5: Effect of different concentrations of PTSO and PTS dissolved in (a) water and (b) 80% ethanol on T-2 and HT-2 toxins production by *F. langsethiae in vitro* at 25°C. Vertical bars indicate the standard error of the means.

Statistically the effect of aqueous PTS concentration was significant on T-2 toxin production but not significant on HT-2 while the concentration of aqueous PTSO was not significant on any of the two toxins (Apx Table G.6).

When the essential oils were used dissolved in 80% ethanol, the effect of their concentration was not significant on either T-2 or HT-2 toxin production

compared to the plain control (Apx Table G.7). Comparing the treated samples to the control with added ethanol, PTSO concentration was not significant on any of the toxins while PTS concentration was significant on both toxins (Apx Table G.8).

Figure 5.6 shows the effect of different concentrations of aqueous PTSO and PTS on the *in vitro* production of fumonisins B_1 and B_2 by *F. verticillioides*.

The production of both toxins was stimulated with up to 100 ppm PTSO, but it was inhibited with 200 ppm. In contrast, PTS inhibited FB_1 and FB_2 toxins at concentrations >10 ppm to a maximum of ~90% inhibition with 200 ppm PTS.

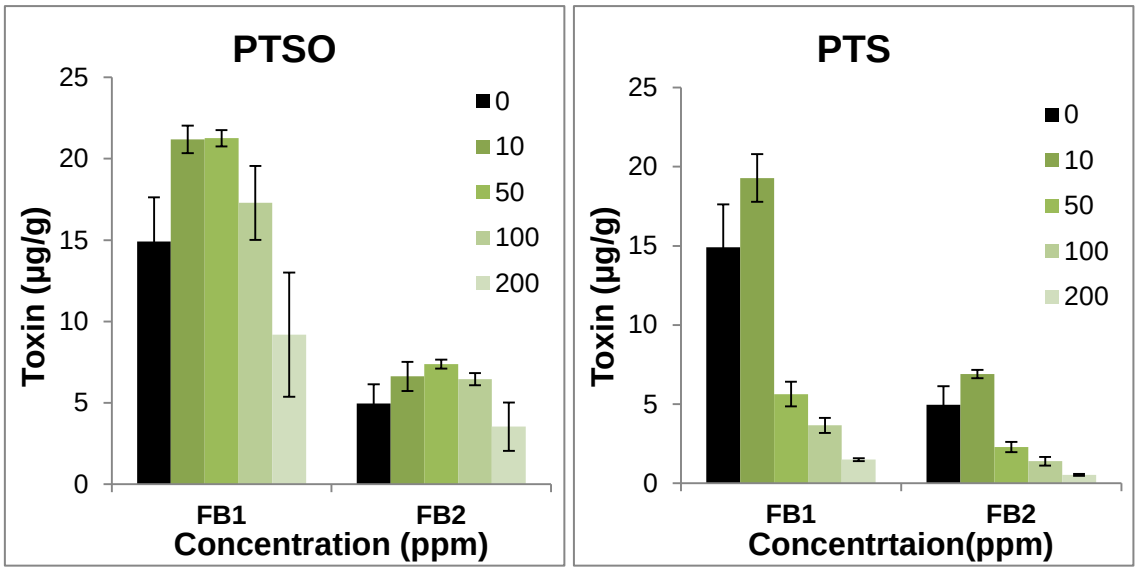


Figure 5.6: Effect of 0-200 ppm aqueous PTSO and PTS on the production of fumonisins B_1 and B_2 by *F. verticillioides in vitro* at 25°C. Vertical bars indicate the standard error of the means.

ANOVA showed that the effect of PTSO concentration was significant on FB_1 production by *F. verticillioides in vitro* but it was not significant on the production of FB_2. The effect of PTS concentration was highly significant on the log-transformed data of both FB_1 and FB_2 (Apx Table G.9).

5.3.4 *In vitro* fusarium mycotoxin control using garlic essential oils in media of reduced water activity

Figure 5.7 shows the effect of 0-100 ppm aqueous PTSO on DON production by *F. graminearum* in wheat media of two different water activity levels at 25°C.

DON production decreased with increasing PTSO concentration in media of 0.96 a_w (max. 62% reduction) while at 0.94 a_w there was no significant difference between 50 and 100 ppm PTSO (78 and 77% reduction respectively). Strangely more DON was produced in the control samples at 0.94 a_w than at 0.96 a_w.

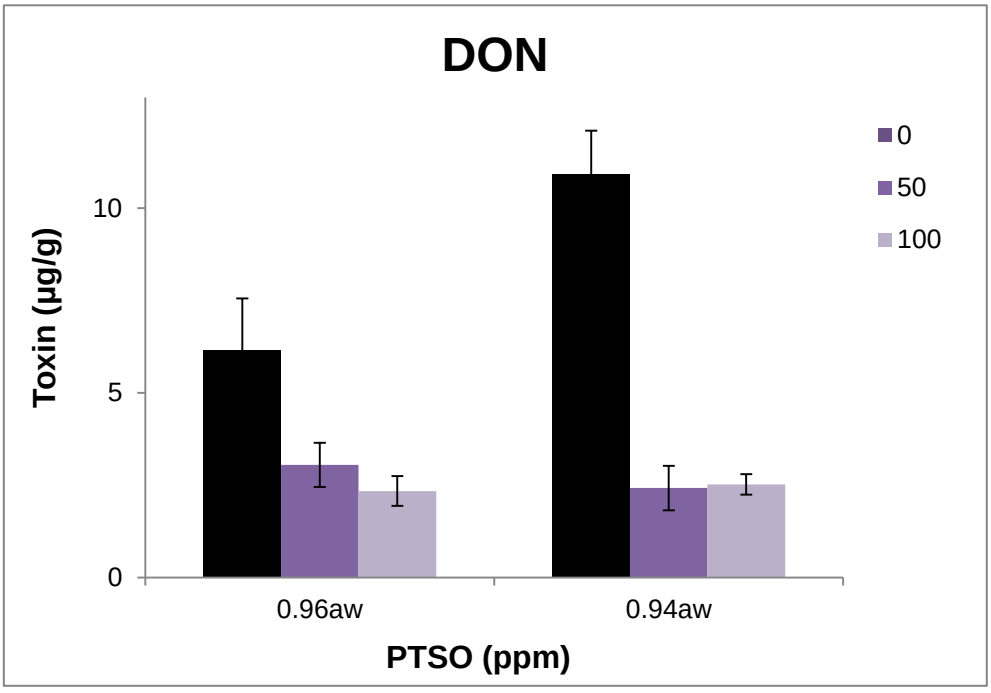


Figure 5.7: Effect of 0-100 ppm aqueous PTSO on *in vitro* deoxynivalenol production by *F. graminearum* in wheat agar media adjusted to 0.96 and 0.94 a_w, at 25°C. Vertical bars indicate the standard error of the means.

ANOVA showed that the effect of aqueous PTSO concentration was highly significant on DON production in media of reduced a_w, but the effects of water

activity and of the interaction of the two factors were not significant (Apx Table G.10).

Figure 5.8 shows the effect of different concentrations of aqueous PTSO on T-2 and HT-2 toxin production by *F. langsethiae* in wheat media adjusted to two different water activity levels at 25°C.

Production of both trichothecenes was generally reduced in the samples treated with PTSO compared to the control, except for the combination of 0.94 a_w and 100 ppm where HT-2 toxin was stimulated (25%). For both toxins better inhibition was consistently obtained with 50 ppm PTSO and the maximum was observed at 0.96 a_w (T-2 toxin: 81%; HT-2 toxin: 67%) which appears to be the more effective treatment.

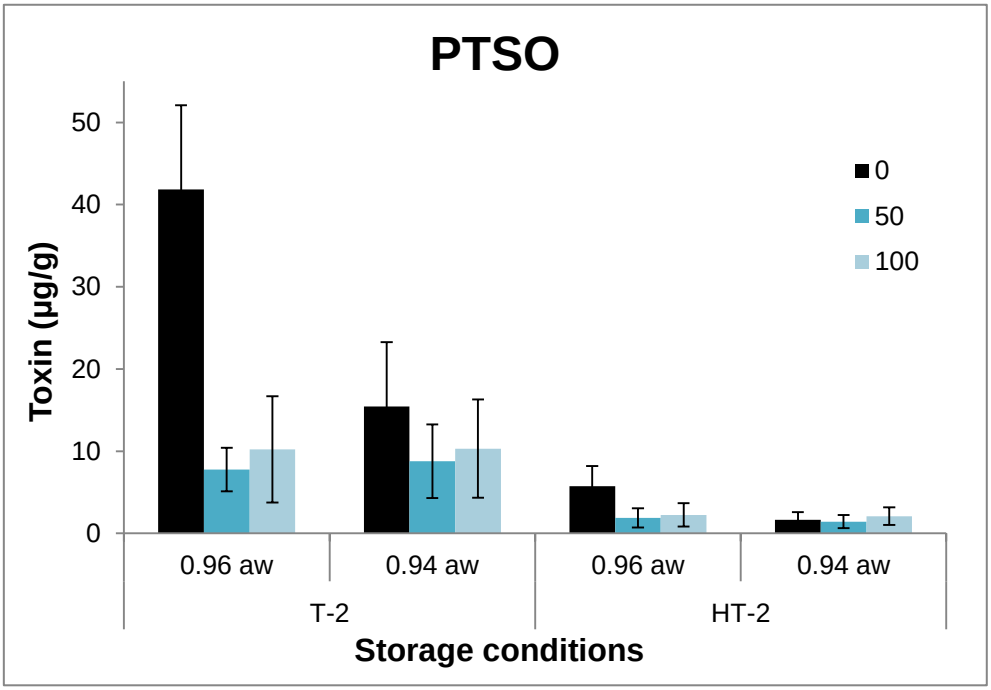


Figure 5.8: Effect of 0-100 ppm aqueous PTSO on *in vitro* T-2 and HT-2 toxin production by *F. langsethiae* in media adjusted to 0.96 and 0.94 a_w, at 25°C. Vertical bars indicate the standard error of the means.

The Kruskal-Wallis ANOVA by ranks showed that the effects of water activity and PTSO concentration were not significant on T-2 and HT-2 toxin production (Apx Table G.11).

5.3.5 *In situ* mycotoxin control using PTSO

Figure 5.9 shows the effect of PTSO on deoxynivalenol and zearalenone production in naturally contaminated wheat rewetted to two different a_w levels, inoculated with *F. graminearum* and stored for 10 and 20 days at 25°C.

A small reduction in DON production (~33%) compared to the control was observed with 80 ppm PTSO in the samples stored for 10 days at both a_w levels, while stimulation was observed with 40 ppm. Treatment with PTSO was inefficient in the samples stored for 20 days and toxin production was higher than in the controls at both a_w levels.

ZEA production was more efficiently reduced with 80 ppm PTSO in wheat of 0.93 a_w, to a maximum of 53.5% and 30% compared to the control after 10 and 20 days storage respectively, while at 0.95 a_w 40 ppm PTSO were more efficient (61% and 48% respectively).

ANOVA showed that the effect of storage time was highly significant on $\log_{DON}$ production in stored wheat, while the effects of water activity and PTSO concentration as well as the interactions of the factors were not significant (Apx Table G.12). The Kruskal-Wallis ANOVA showed that PTSO concentration and wheat a_w were not significant on ZEA production by *F. graminearum*, but the effect of storage time was highly significant (Apx Table G.13).

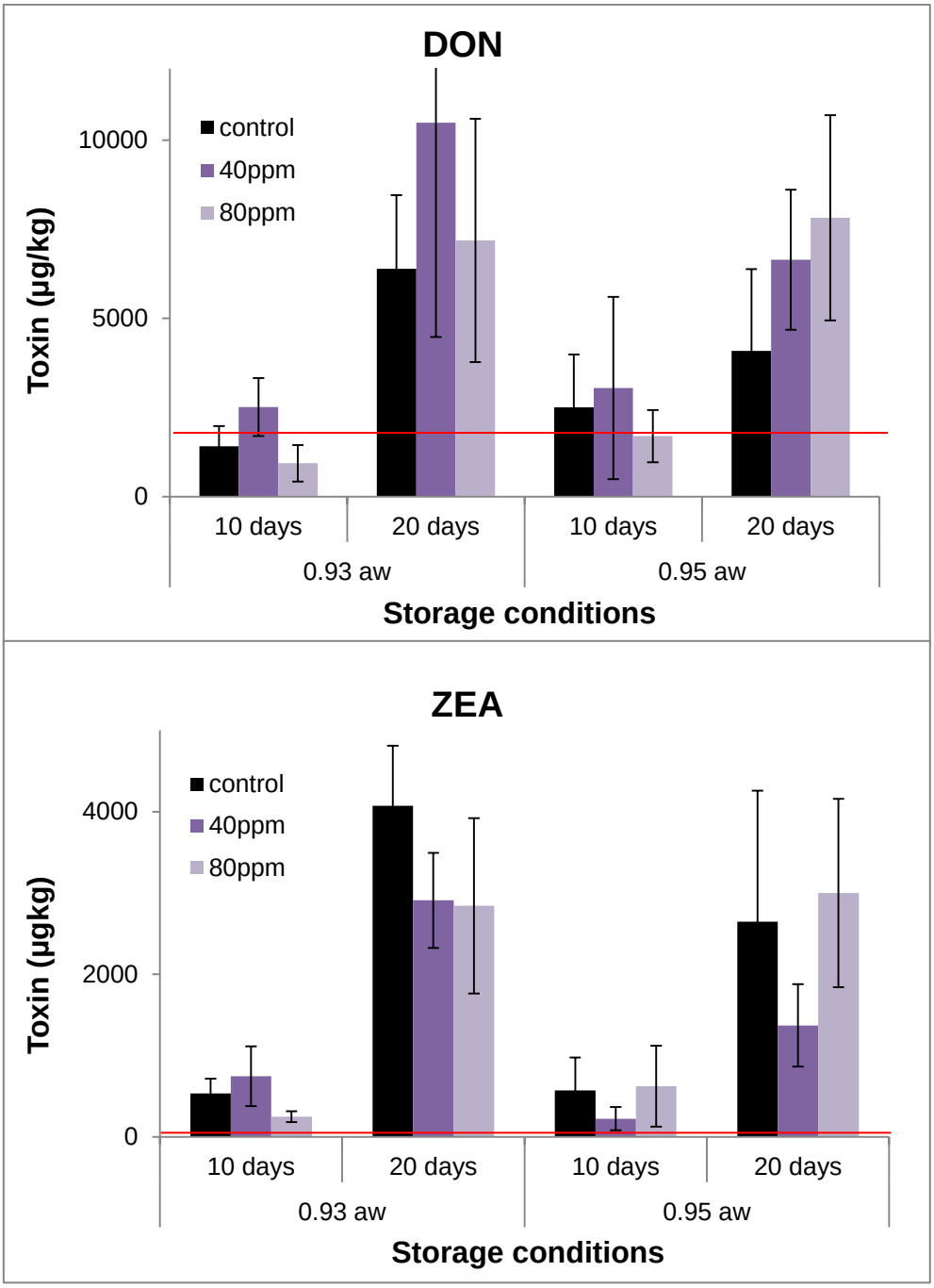


Figure 5.9: Effect of 0-80 ppm aqueous PTSO on deoxynivalenol and zearalenone production by *F. graminearum* in wheat rewetted to 0.93 and 0.95 a_w and stored at 25°C for 10 and 20 days. Vertical bars indicate the standard error of the means. The red lines show the EU legislative limits for each of the toxins in wheat.

Figure 5.10 shows the production of T-2 and HT-2 toxins in naturally contaminated oat grain rewetted to two water activity levels, adjusted to 0-16 ppm PTSO, inoculated with *F. langsethiae* spores and stored for 10 and 20 days at 25°C. The broken green line shows the legislative limits more commonly established around the world for the sum of T-2 and HT-2 toxins.

16 ppm PTSO caused reduced production of T-2 and HT-2 toxins compared to the control at all conditions for storage up to 20 days, while 8 ppm PTSO generally stimulated toxin production. The maximum inhibition observed was 66-67% compared to the control at 0.93 a_w/20 days and at 0.95 a_w/10 days.

With regard to the individual toxins, HT-2 toxin was produced in higher amounts than T-2, but was also more effectively inhibited by PTSO (data not shown).

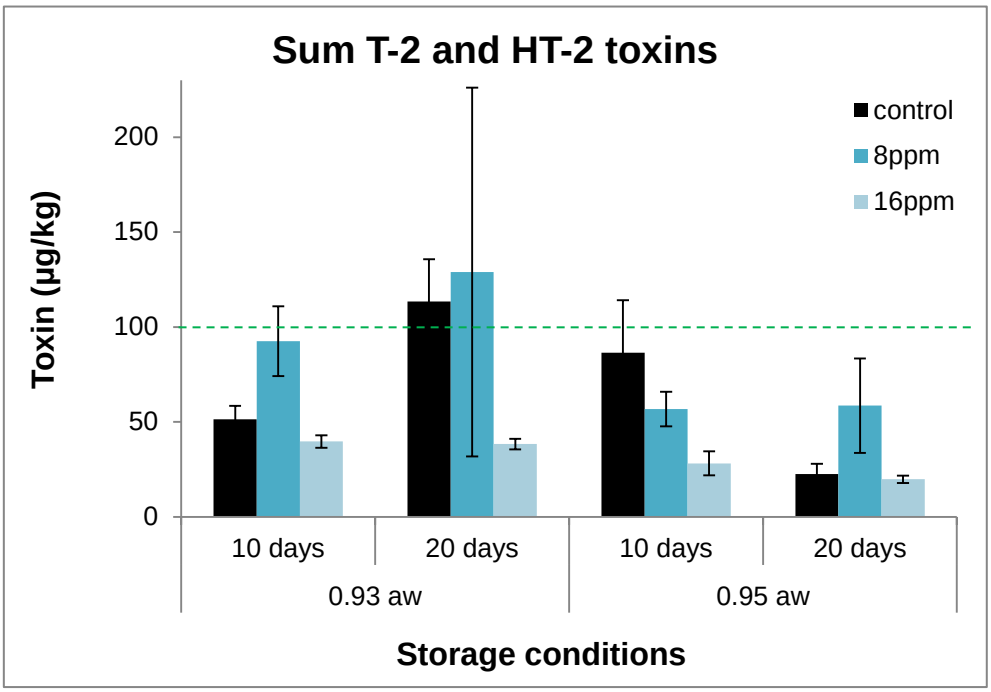


Figure 5.10: Effect of 0-16 ppm PTSO on the sum of T-2 and HT-2 toxin production by *F. langsethiae* in naturally contaminated oats rewetted to 0.93 and 0.95 a_w and stored for 10 and 20 days at 25°C. Vertical bars indicate the standard error of the means. The green broken line shows the legislative limits more commonly established around the world for T-2 and HT-2 toxins.

ANOVA showed that both PTSO concentration and grain water activity were significant on T-2 and HT-2 toxin production in oats, while the effect of storage time and the interactions of the factors were not significant (Apx Table G.14).

Figure 5.11 shows the production of ochratoxin A due to the naturally present *Penicillium* populations in the above oat samples treated with PTSO.

A maximum of 82-83% inhibition of OTA production was observed with 8 ppm PTSO at 0.93 a_w after 10 and 20 days storage at 25°C compared to the control. At 0.95 a_w, samples treated with PTSO and stored for 10 days had OTA levels similar to the control, while for longer storage some effect was observed with 16 ppm PTSO. The OTA levels observed in the oat samples due to the naturally present *Penicillium* populations were very high and above the relevant EU legislative limits in all the samples.

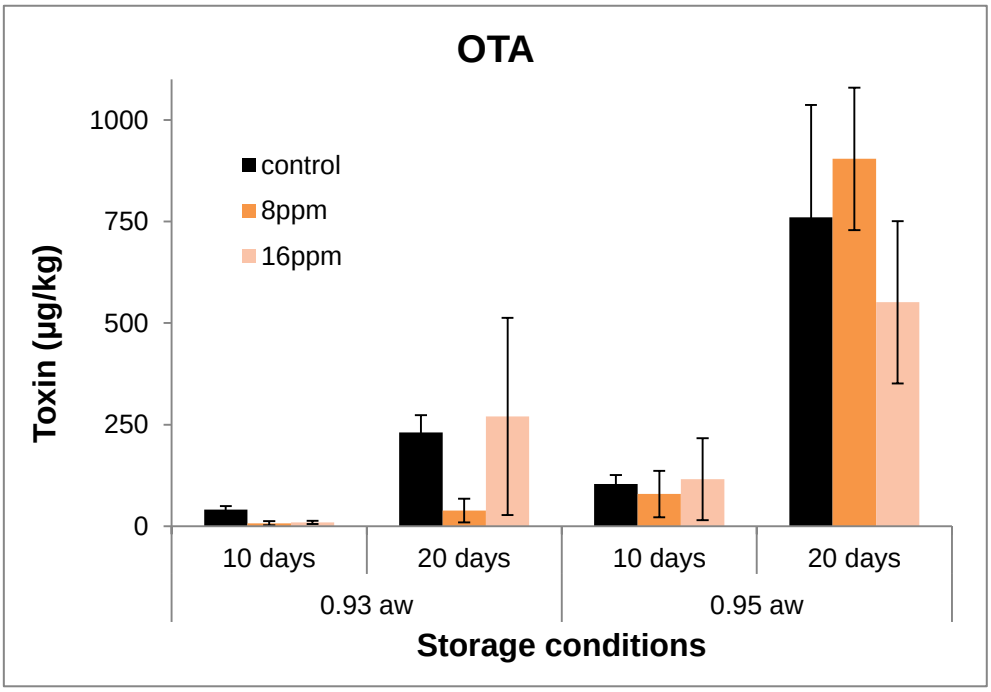


Figure 5.11: Effect of 0-16 ppm PTSO on ochratoxin A production in naturally contaminated oats rewetted to 0.93 and 0.95 a_w and stored for 10 and 20 days at 25°C. Vertical bars indicate the standard error of the means. EU limit for OTA: 5 µg/kg.

The Kruskal-Wallis ANOVA showed that the effect of PTSO concentration was not significant on OTA production in stored oats, but the effect of grain a_w was significant and the effect of storage time was highly significant (Apx Table G.15).

5.3.6 *In situ* mycotoxin control using PTS

Figure 5.12 shows DON and ZEA production in naturally contaminated wheat rewetted to two a_w levels, treated with 0-300 ppm aqueous PTS, inoculated with *F. graminearum* and stored for 10 and 20 days at 25°C. The red lines show the EU limits for each of the toxins in wheat.

DON production was reduced in all the PTS-treated samples of 0.93 a_w stored for up to 20 days at 25°C compared to the control. However, the inhibitory effect of PTS was generally higher at lower concentrations. Maximum inhibition compared to the control was obtained with 100 and 200 ppm PTS after 10 days and 20 days storage (76% and 95%) respectively. In the wheat samples of 0.95 a_w stored for 10 days DON production increased with increasing PTS concentration, while after 20 days irregular results were obtained ranging from 90% inhibition of DON production with 200 ppm PTS to >100% stimulation at 300 ppm.

Zearalenone production, as opposed to DON, was occasionally stimulated in the samples of 0.93 a_w stored for 10 days. The most efficient PTS concentration against ZEA production at 0.93 a_w was 100 ppm, similarly to DON, achieving a maximum of 13 and 89% inhibition after 10 and 20 days storage respectively. In the 0.95 a_w samples stored for 10 days the maximum inhibition compared to the control was obtained with 100 ppm PTS (~64%). In the samples stored for 20 days twice as much ZEA was produced compared to the control, while 200 ppm PTS inhibited ZEA production by ~96%.

Overall, the most efficient PTS concentrations for the control of both toxins were 100 ppm for the wheat of 0.93 a_w and 200 ppm for the samples of 0.95 a_w.

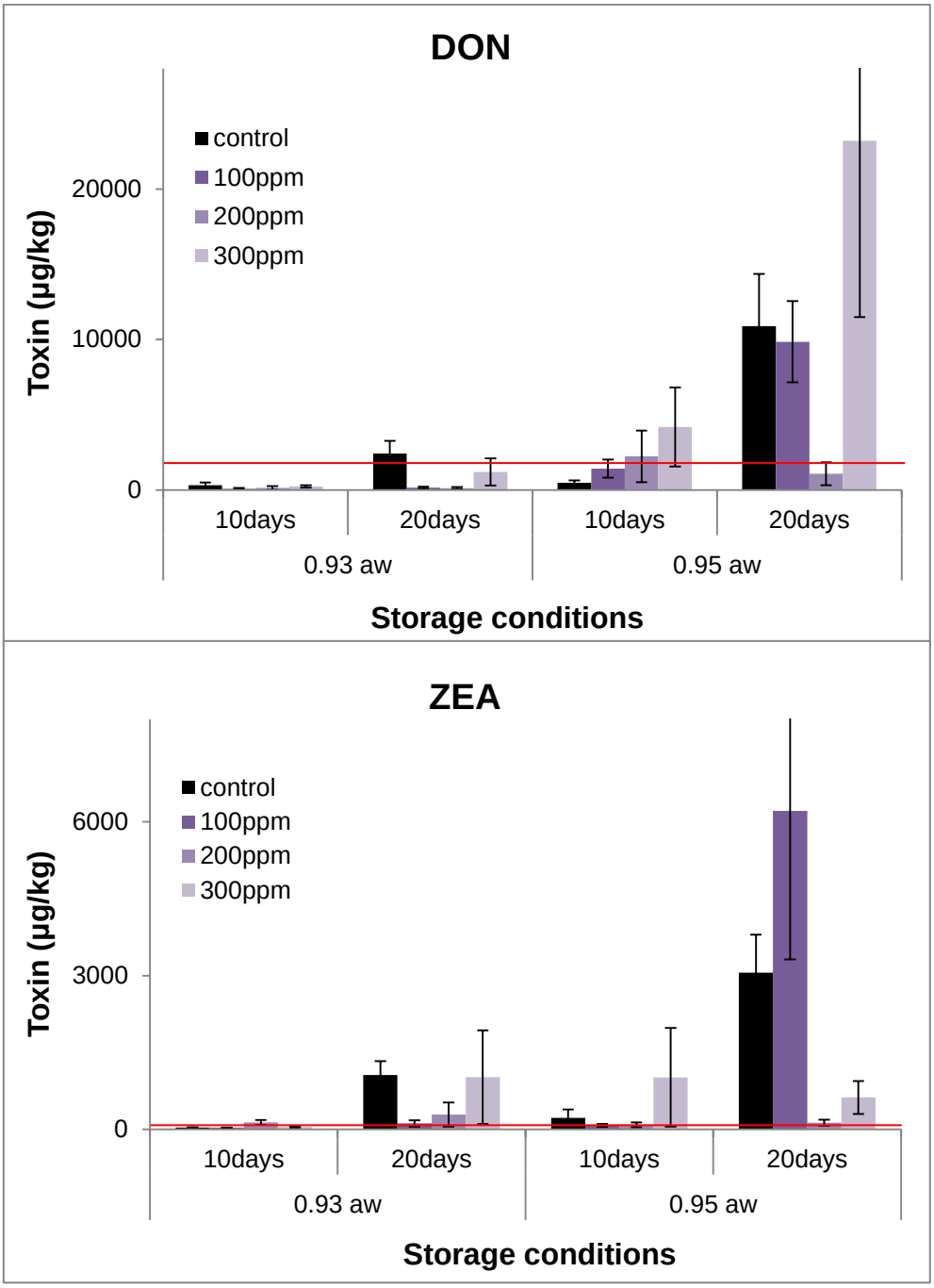


Figure 5.12: Effect of 0-300 ppm PTS on deoxynivalenol and zearalenone production by *F. graminearum* in wheat of 0.93 and 0.95 a_w and stored at 25°C for 10 and 20 days. Vertical bars indicate the standard error of the means. The red lines show the EU legislative limits for each of the toxins in wheat.

ANOVA showed that the effects of PTS concentration and storage time were significant on $\log_{DON}$ production by *F. graminearum* in stored wheat, while the effect of grain a_w was highly significant. The effects of the interactions between the factors were not significant (Apx Table G.16). For zearalenone the Kruskal-Wallis ANOVA by ranks showed that the effects of PTS concentration and grain water activity were not significant on the amount of toxin produced in the stored samples while the effect of storage time was highly significant (Apx Table G.17).

Figure 5.13 shows T-2 and HT-2 toxin production in naturally contaminated oats rewetted to two water activity levels, adjusted to 0-300 ppm aqueous PTS, inoculated with *F. langsethiae* spores and stored for 10 and 20 days at 25°C.

Aqueous PTS overall stimulated the production of the sum of T-2 and HT-2 toxins in oats of 0.93 a_w and particularly in the samples stored for longer time. In contrast, in the wetter oats (0.95 a_w), PTS inhibited toxin production compared to the controls. The most effective concentration was 100 ppm PTS and toxin production in the oats was reduced by 63 and 42% after 10 and 20 days storage respectively.

With regard to the two individual toxins, HT-2 toxin was produced in 1-4 times the amount of T-2 toxin, although the levels of the two trichothecenes were generally very low and overall below the limit most commonly established for these toxins in some parts of the world. HT-2 toxin production was slightly more affected by the treatment than T-2 toxin production (data not shown).

ANOVA showed that the effects of PTS concentration, grain a_w and storage time were not significant on $\log_{(T\text{-}2+HT\text{-}2)}$ toxin production by *F. langsethiae* in stored oats, while the interaction between a_w and storage time was significant (Apx Table G.18).

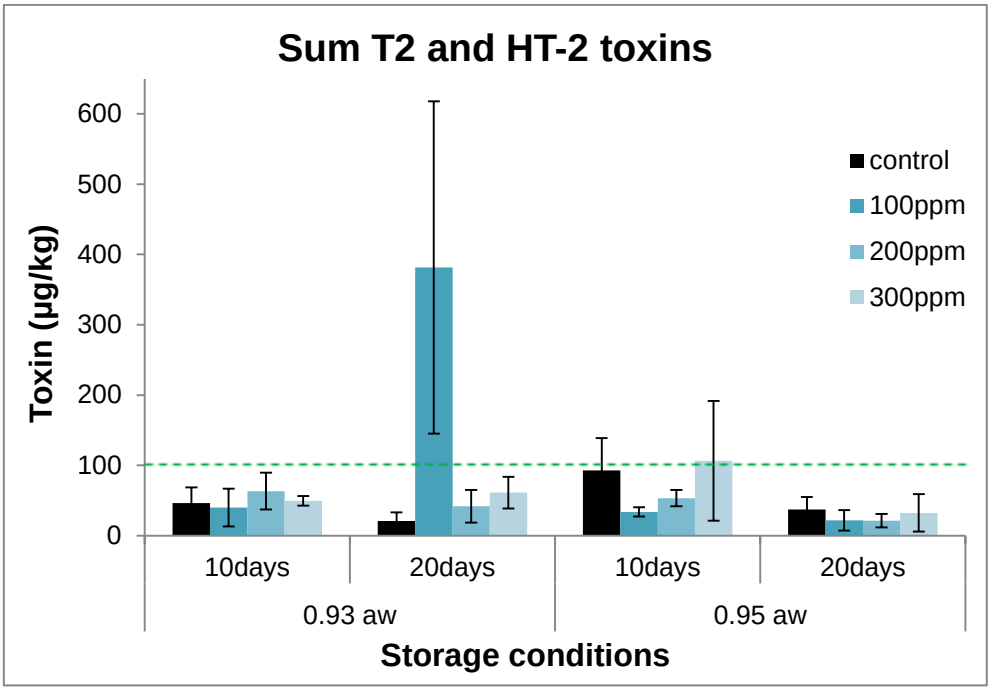


Figure 5.13: Effect of 0-300 ppm aqueous PTS on T-2 and HT-2 toxin production by *F. langsethiae* in oats rewetted to 0.93 and 0.95 a_w and stored for 10 and 20 days at 25° C. Vertical bars indicate the standard error of the means. The green broken line shows the legislative limits more commonly established around the world for T-2 and HT-2 toxins in oats.

Figure 5.14 shows the production of ochratoxin A in the above oat samples treated with aqueous PTS due to the naturally present *Penicillium* populations.

PTS was particularly effective against OTA production by naturally occuring species in oats of 0.93 a_w. The most effective treatment was 200 ppm causing >95 and >99% reduction of OTA production after storage for 10 and 20 days respectively. At 0.95 a_w a maximum of 70% reduction was observed in the samples treated with 200 ppm PTS after 10 days storage, while 300 ppm PTS were required for efficient control of OTA (>95%) in the samples stored for 20 days. Generally the OTA levels observed in the oats due to the naturally present *Penicillium* populations were very high, but were reduced below the relevant EU legislative limits by certain treatments even after 20 days storage at wet and warm storage conditions.

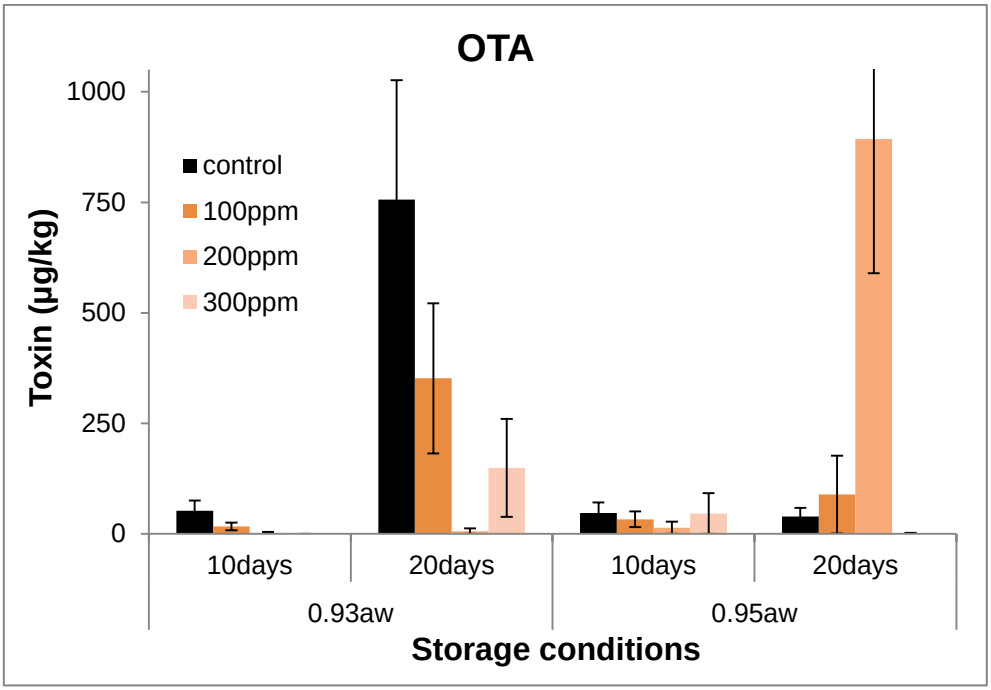


Figure 5.14: Effect of 0-300 ppm aqueous PTS on ochratoxin A production in naturally contaminated oats rewetted to 0.93 and 0.95 a_w and stored for 10 and 20 days at 25°C. Vertical bars indicate the standard error of the means.

The Kruskal-Wallis ANOVA showed that the effects of PTS concentration and grain a_w were not significant on OTA production in stored oats, but the effect of storage time was significant (Apx Table G.19).

Plate 5.2 shows the oat samples of the above trial rewetted at 0.95 a_w treated with 0-300 ppm PTS and inoculated with *F. langsethiae* after 20 days storage at 25 °C.

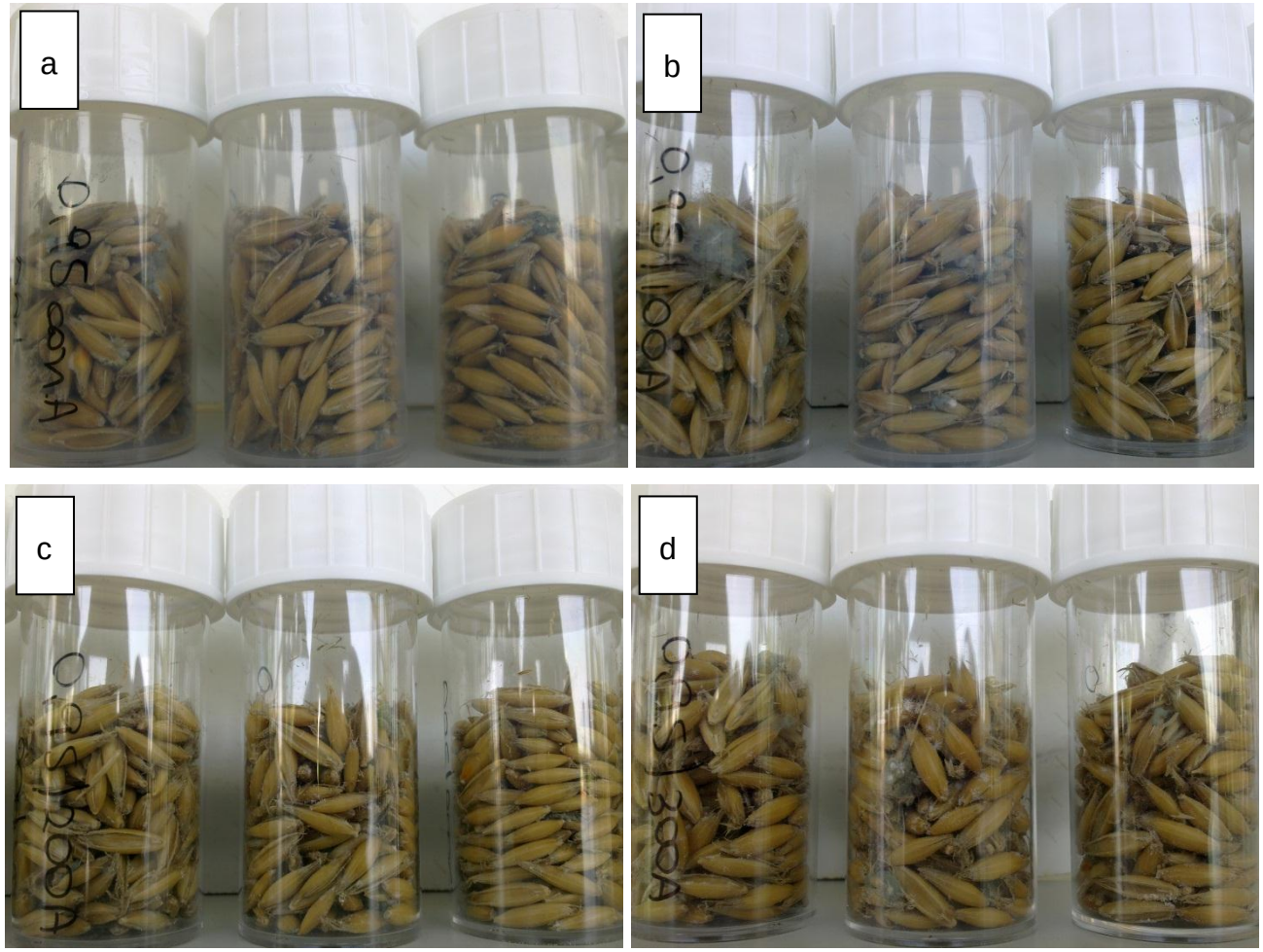


Plate 5.2: Naturally contaminated oat samples rewetted at 0.95 a_w, treated with 0, 100, 200 and 300 ppm PTS (a to d) and inoculated with *F. langsethiae* after 20 days storage at 25 °C.

Figure 5.15 shows the production of fumonisins (B_1+B_2) in naturally contaminated maize rewetted to 0.91 and 0.94 a_w, adjusted to 0-300 ppm aqueous PTS, inoculated with *F. verticillioides* spores and stored for 10 and 20 days at 25°C. The red line shows the EU legislative limits established for the sum of fumonisins in maize.

At 0.91 a_w, 100 ppm PTS slightly stimulated fumonisin production while higher concentrations reduced fumonisin production to a maximum of 39% that of the control after 10 days storage and 80% after 20 days storage with 300 ppm PTS. In the latter samples the fumonisin content was below the relevant EU limit. At 0.94 a_w reduction of fumonisins was observed only at 300 ppm PTS and this was 40 and 57% in the samples stored for 10 and 20 days respectively.

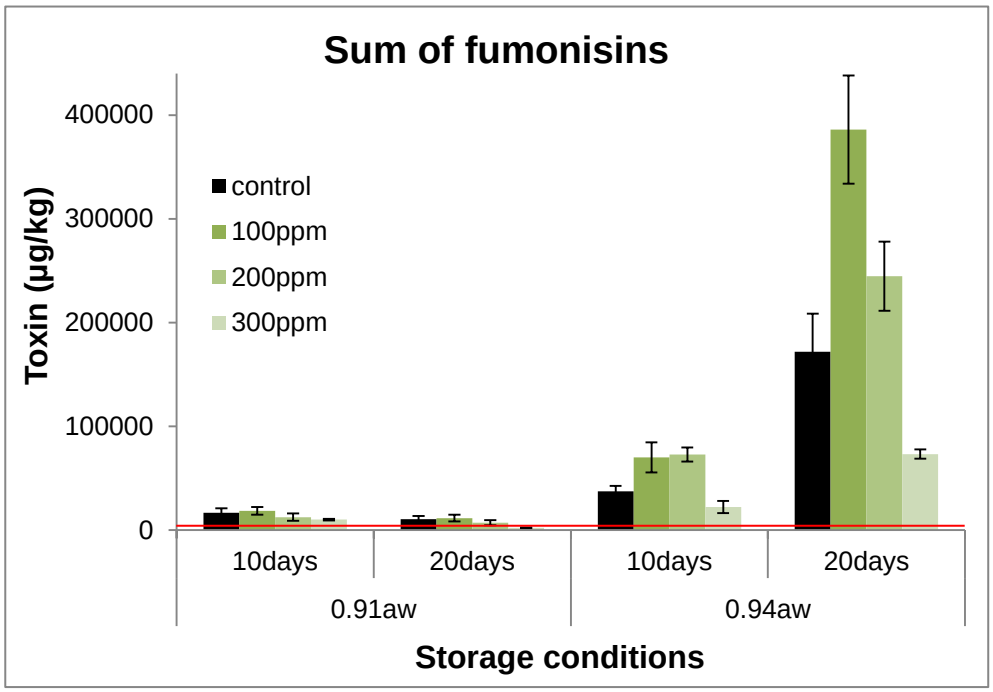


Figure 5.15: Effect of 0-300 ppm aqueous PTS on fumonisins (B_1+B_2) production by *F. verticillioides* in maize rewetted to 0.91 and 0.94 a_w and stored at 25°C for 10 and 20 days. Vertical bars indicate the standard error of the means. The red line shows the EU legislative limits established for the sum of fumonisins (B_1+B_2) in maize.

The effect of PTS on the two individual fumonisins FB_1 and FB_2 was generally similar, the only difference being that in the samples of 0.91 a_w FB_2 was inhibited by all PTS concentrations as opposed to FB_1.

ANOVA showed that the effects of PTS concentration, maize water activity and storage time were highly significant on the logarithm of total fumonisin production (FB_1+FB_2). The interaction of a_w×storage time was also highly significant, the interaction of a_w×PTS concentration was significant while the interactions of PTS×storage time and of all three factors PTS×a_w×storage time were not significant (Apx Table G.20).

Plate 5.3 shows the maize samples of the above trial rewetted to 0.91 and 0.94 a_w, treated with 100-300 ppm PTS, inoculated with *F. verticillioides* and stored for 10 days at 25 °C.

Plate 5.3: Naturally contaminated maize samples rewetted to (a) 0.91 and (b) 0.94 a_w, treated with 100, 200 and 300 ppm PTS (left to right) and inoculated with *F. verticillioides*, after 10 days storage at 25 °C.

5.3.7 *In situ* mycotoxin control using a mixture of PTS:PTSO (1:1)

Figure 5.16 shows DON production in wheat rewetted to 0.92 and 0.94 a_w, adjusted to 200-600 ppm aqueous mixture of PTS:PTSO (1:1), inoculated with *F. graminearum* and stored for 10 and 20 days at 25°C.

In wheat of 0.92 a_w DON production was stimulated with 200 ppm of the mixture but 400 ppm reduced DON production to a maximum of 83% and 90% after 10 and 20 days storage respectively, compared to the control. DON production was marginal in all the samples of 0.94 a_w stored for 10 days, while in the 20-day samples DON was inhibited compared to the control only in the samples treated with 200 ppm of the mixture (~37%). Higher concentrations of the mixture caused the production of very high amounts of DON, beyond the EU limits, in one of the replicate samples in each case (~100 times more than the maximum observed in the other two replicates).

Similar observations were made for ZEA production in the above wheat samples. ZEA was inhibited >96% in the samples of 0.92 a_w with 400 and 600 ppm of the mixture of the essential oils, while in wetter wheat (0.94 a_w) ZEA was controlled only by 200 ppm of the mixture and stimulated with higher concentrations.

Interestingly, more ZEA than DON was produced in the control wheat samples of this experiment and higher concentrations of both toxins were produced in the control samples of 0.92 a_w than at 0.94 a_w. Also DON was generally below the EU legislative limit established for this toxin in wheat (shown with a red line in the Figure 5.16), while ZEA was above the relevant EU limit, but controlled by the treatment below this level.

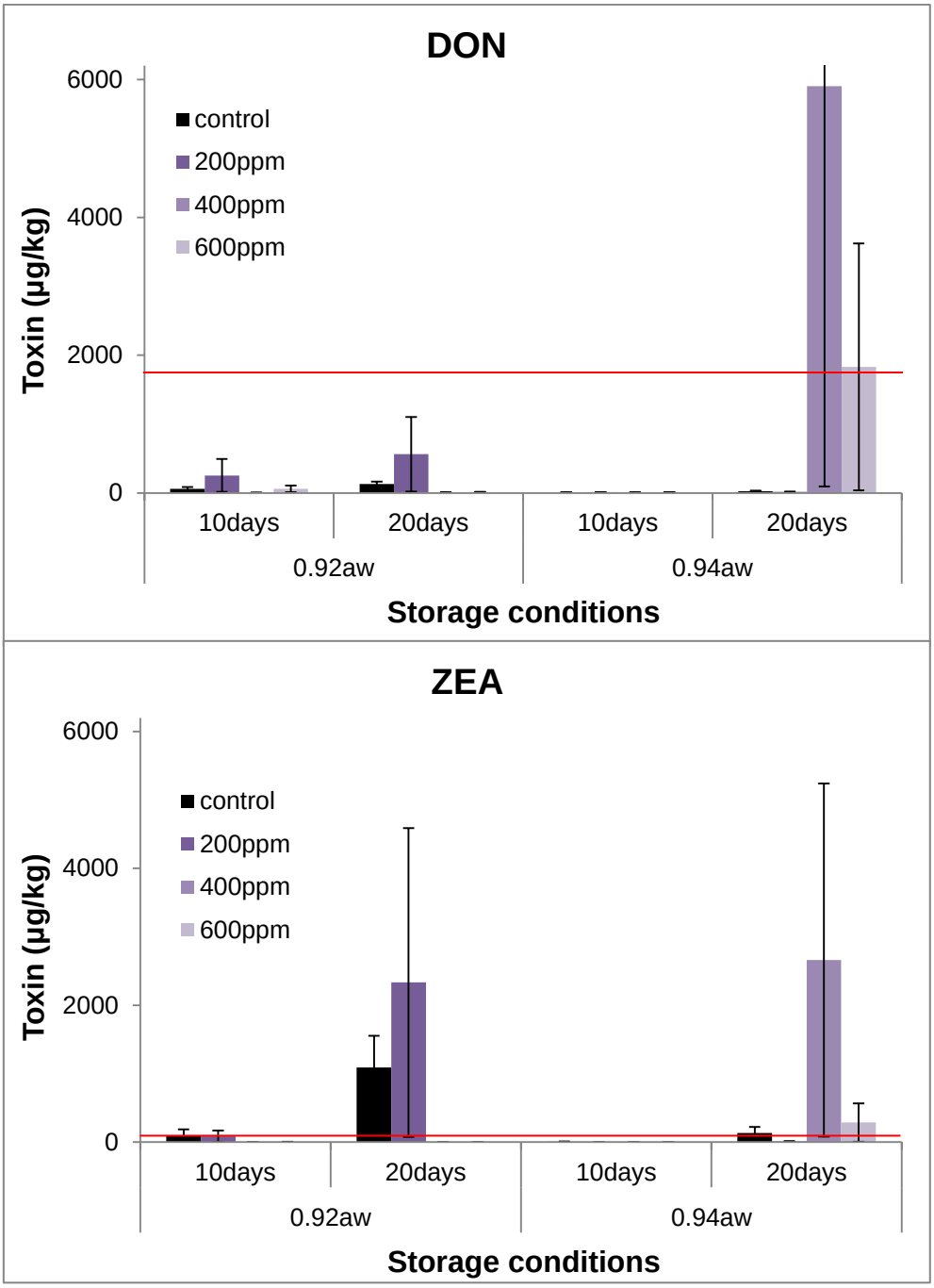


Figure 5.16: Effect of 0-600 ppm aqueous mixture of PTS:PTSO (1:1) on deoxynivalenol and zearalenone production by *F. graminearum* in wheat rewetted to 0.92 and 0.94 a_w and stored at 25°C for 10 and 20 days. Vertical bars indicate the standard error of the means. The red lines show the EU legislative limits for each of the toxins in wheat.

The Kruskal-Wallis ANOVA showed that the concentration of the PTS:PTSO mixture and wheat water activity were not significant on DON or ZEA production during storage, but the effect of storage time was highly significant for DON and significant for ZEA (Apx Table G.21, G.22).

5.4 Discussion

5.4.1 *In vitro* control of fungal growth and mycotoxin production using garlic essential oils

Aqueous PTS and PTSO were very efficient at inhibiting the *in vitro* growth rate of the different fungal species against which they were tested. The inhibitory effect increased with increasing concentration and generally the level of inhibition was species-dependent. *F. langsethiae* was the *Fusarium* species mostly affected by both compounds and complete inhibition of its growth was achieved with 100 ppm PTS and with 200 ppm PTSO. In a similar experiment the growth of *A. flavus* (Appendix H) was completely inhibited with 50 ppm of both compounds, much less than that needed for the *Fusarium* species. However, this was probably due to the fact that inoculation of the media in this case was performed with fungal spores as opposed to the use of agar plugs in the case of *Fusarium langsethiae*. This suggests that fungal spores may be more sensitive to the effect of PTS and PTSO than mycelium, something that has also been suggested by other studies (Schmidt-Heydt et al., 2011). PTS was more efficient in inhibiting the growth rate of the different fungi than PTSO when applied at the same concentration.

In terms of toxin production different results were obtained *in vitro* depending on whether the garlic essential oils were dissolved in water or in ethanol. As has been discussed previously, the presence of ethanol alone in the media caused reduced DON production by *F. graminearum* compared to the plain control, while stimulated T-2 toxin production by *F. langsethiae*.

Both compounds used as aqueous solutions caused reduced DON, T-2 and HT-2 toxins and aflatoxin B_1 production compared to the control, while surprisingly PTSO only started showing some inhibitory effect on fumonisins B_1 and B_2 at 200 ppm. Thus >4 times the amount of PTS was required compared to PTSO for similar effect to be observed. Generally PTS was more efficient against toxin production than PTSO except in the case of T-2 toxin where aqueous PTSO seems to have performed more effectively at <200 ppm. Similarly, HT-2 toxin was completely inhibited at all PTSO concentrations and at PTS concentrations ≥50 ppm. Also it was observed that generally higher PTS concentrations (200 ppm) completely inhibited the production of all toxins, except of fumonisins. Thus higher concentrations of this compound should be examined against *F. verticillioides*.

When the compounds were applied as ethanolic solutions the efficacy of PTS against toxin production was enhanced compared to when applied as aqueous solution, except in the case of T-2 toxin where stimulation was observed at PTS ≤100 ppm, possibly due to the presence of ethanol. In contrast, PTSO applied as an ethanolic solution caused stimulation of the production of all of the toxins compared to the plain control in the samples where fungal growth was observed.

This is the first study to examine the efficacy of PTS and PTSO garlic essential oils against fungal growth and mycotoxin production by the relevant species *in vitro*, despite some very early studies that supported such properties in thiosulfinates (Small et al., 1947).

5.4.2 *In situ* efficacy of garlic essential oils against mycotoxin production in grains

Overall PTS and PTSO showed very good efficacy for the treatment of relatively wet grain and subsequent storage for up to 20 days. The efficacy of the treatments however was dependent on the specific “fungal species – toxin” system and differences were observed with regard to the production of different

toxins by a single fungal species as well as by mixed populations. Similar observations have been made before in grains treated with different fungicides and stored at different environmental conditions (Marín et al., 2000; Magan et al., 2002; Ramirez et al. 2004).

An additional advantage of those compounds, particularly when compared to the acids tested in Chapter 4, is that they are liquid and relatively water soluble/miscible and as such they can be applied to the grains and reach the target concentrations easier. Also the range of concentrations required for the control of mycotoxin production during prolonged storage is much smaller.

Their strong odour, however, might limit their practical applications to feed or to certain food categories. Also toxicological studies must be performed in order to examine the likely effects of any residues or by-products present in the treated grains. Garlic extract has been approved for use as pesticide (though currently under re-evaluation) and commercial products are available for such applications.

(a) PTSO

Treatment of wet grain with PTSO was generally capable of reducing toxin contamination after storage at 0.93-0.95 a_w for 10-20 days compared to the control untreated samples, even though these conditions are far from those recommended for safe storage. These conditions were used in order to imitate accidental rise of the grain water activity to levels where the fungal species of interest in this project can grow, for example in the case of hot spots. DON production in wheat treated with 80 ppm PTSO and stored for 10 days was ~33% less than the control and in fact it was reduced below the relevant EU limits. This treatment however was not efficient for extended storage up to 20 days. ZEA production in the same samples was more effectively reduced even after 20 days storage and while 80 ppm PTSO were more efficient in the samples of 0.93 a_w (30-53% reduction), at 0.95 a_w better control was observed with 40 ppm PTSO (48-60%). Notably all the samples contained ZEA levels above the relevant EU limits. While not of primary interest in this project, it is worth mentioning that in the same experiment nivalenol production was

controlled successfully only in the samples of 0.93 a_w stored for 10 days (36%). In the rest of the samples stimulation was observed, which notably increased with increasing PTSO concentration in the samples stored for 20 days (data not shown).

PTSO was overall more efficient in controlling the production of T-2 and HT-2 toxins by *F. langsethiae* in oats with as little as 16 ppm. This treatment was generally more effective against HT-2 toxin (8-78%) than T-2 toxin (18-42%) and this is of particular importance since HT-2 toxin was generally produced in higher amounts than T-2 in this trial. In the same oat samples high levels of ochratoxin A were detected due to the natural presence of *Penicillium* species. PTSO at only 8 ppm was particularly efficient in controlling ochratoxin A production in the samples of 0.93 a_w (82-83.4%) irrespective of the length of storage time (10 or 20 days) while at 0.95 a_w reduction was ~25% with 8 ppm PTSO for 10 days storage while with 16 ppm for 20 days.

As it has been shown previously by the *in vitro* studies, *F. langsethiae* is the *Fusarium* species less tolerant to these treatments. Therefore, higher PTSO concentrations could also be examined for efficacy in mycotoxin reduction in wheat.

(b) PTS

Wheat of 0.93 a_w was successfully stored for up to 20 days and DON production in all the treatments was reduced compared to the untreated grain and below the relevant EU limits. The most efficient treatment was 100 ppm PTS causing 76-94% control of DON production. This was also the most effective treatment for the control of ZEA production which was reduced to below or near the relevant EU limits after 10 and 20 days storage respectively, as well as for the control of nivalenol (data not shown) in the same samples. It has to be noted however that at certain combinations of storage conditions and PTS concentration toxin production was stimulated. In particular, in wheat of 0.95 a_w, inconsistent results were obtained and while a certain concentration was more effective against one or more of the toxins, the same concentration stimulated the production of other toxins.

For the control of total fumonisins in stored maize of 0.91 a_w and 0.94 a_w 300 ppm of PTS were required, achieving 39-80% inhibition compared to the control samples. These results suggest that higher concentrations of PTS could also be assessed for even more efficient control of fumonisins in stored maize.

In the same maize samples treated with 300 ppm PTS, aflatoxin B_1 produced by the naturally present fungal species was below the limit of detection as opposed to the control samples where 50-150µg/kg were detected. Similarly ochratoxin A production was either reduced or below the limit of detection in the above samples (data not shown).

In naturally contaminated oats of 0.93 a_w PTS was not efficient and overall the production of T-2 and HT-2 toxins was stimulated, particularly after longer storage. In the same samples, ochratoxin A production was inhibited 95-99% with 200 ppm PTS, irrespective of the storage time. In contrast, in the oats of 0.95 a_w stored for 10 days, 100 ppm PTS caused 45% and 72% inhibition of T-2 and HT-2 toxins respectively, while OTA was reduced by 30%. For longer storage higher concentrations were more efficient; 200 ppm against HT-2 toxin, 100 ppm for T-2 toxin and 300 ppm for OTA. It is likely that the effect of PTS is species-dependent and that control of one toxin (i.e. OTA) caused enhanced production of another toxin (i.e. trichothecenes). Similar observations have also been made by Marín et al. (2000).

(c) PTSO:PTS mixture (1:1)

The mixture of the two garlic essential oils (1:1) when applied at 400 ppm was very effective at inhibiting DON production in wheat of 0.92 a_w for up to 20 days, reducing DON production 82.5-90% compared to the untreated grain. At 0.94 a_w some irregular toxin levels obtained do not allow for safe conclusions to be reached. 400 and 600 ppm of the mixture were particularly effective against ZEA production in wheat of 0.92 a_w, causing >96% inhibition, while in wheat of 0.94 a_w the most effective treatment was 200 ppm causing up to 94% inhibition of ZEA for storage up to 20 days. Overall, higher mixture concentrations were required for more efficient control at drier conditions, while in wetter grain less amount of the mixture was required against both DON and ZEA.

It was observed however that in the same wheat samples of lower a_w, nivalenol was generally inhibited at all concentrations of the mixture for storage up to 20 days, while in the wetter samples nivalenol was stimulated at all concentrations of the mixture.

In vitro, PTSO was shown to be ~4 times less effective than PTS against certain fungal species. Thus, mixtures with higher proportion of PTSO than PTS could also be examined for efficacy in controlling trichothecenes and ZEA production in wheat.

6 OZONE FOR THE CONTROL OF FUNGAL GROWTH AND MYCOTOXIN PRODUCTION

6.1 Introduction

Ozone (O_3) is a powerful oxidising agent, very unstable and decomposes to oxygen without leaving residues. Nowadays, efficient techniques for its generation and its reduced costs compared to alternatives have rendered it an attractive option for the treatment of grains post-harvest prior to storage with the scope of reducing microbial contamination and insect populations.

Few studies are available on the *in vitro* effect of O_3 on the germination of fungal spores, mycelial growth and mycotoxin production. The concentrations assessed, the experimental techniques, the appliances for the generation of O_3, the fungal species studied, the environmental factors and the culture media used vary significantly and make comparisons difficult.

Generally studies that used low O_3 concentrations (~1 ppm) employed longer exposure times (6 hours-6 days) (Hibben and Stotzky, 1969; Minas et al., 2010; Zotti et al., 2010) and observed reduced spore germination compared to the controls with increasing O_3 concentration and time of exposure. Occasionally stimulation of germination was observed after short exposures at low O_3 concentrations. 100% elimination of fungal spores has been claimed by using 300μmol O_3 for 2h (Antony-Babu and Singleton, 2011) and by 0.3μl/l after 8h continuous exposure at 0°C and 95% ERH (Minas et al., 2010). Overall, differences in the appearance of the cultures formed from treated spores as well as in their sporulation were observed after longer exposures at low doses (Hibben and Stotzky, 1969; Antony-Babu and Singleton, 2009). Different species behaved differently to O_3 exposure (Antony-Babu and Singleton, 2009) while contradictory opinions have been expressed on whether spores or mycelia are more sensitive (Allen et al., 2003; Freitas-Silva and Venâncio, 2010; Minas et al., 2010; Zotti et al., 2010) and on whether the effect of O_3 is fungicidal or fungistatic (Hibben and Stotzky, 1969; Minas et al., 2010). The

experimental conditions of the different studies have to be carefully examined to draw safe conclusions.

Mechanisms suggested for the effect of O_3 on fungal spores include effects on the membrane integrity or on enzymes involved in cell development. The presence of water could have a synergistic effect possibly by formation of free radicals (Hibben and Stotzky, 1969; Allen et al., 2003) while the role of the substrate is another point for consideration (Antony-Babu and Singleton, 2011).

To our knowledge no studies have examined the effect on mycotoxin production *in vitro* by fungal spores or fungal colonies after O_3 exposure. Most studies have focused on the use of O_3 for decontamination purposes in aqueous solutions of mycotoxins (McKenzie et al., 1997; Young et al., 2006) or on naturally contaminated grains and nuts (McKenzie et al., 1997, 1998; Akbas and Ozdemir, 2006; Proctor et al., 2004) and in some of these studies the toxicity of the treated samples was also assessed.

Of particular interest is whether O_3 can be successfully applied for the treatment of grains at the time of storage reducing/eliminating fungal spores and other microorganisms and thus reducing the risk of mycotoxin contamination of stored grains and extending the safe post-harvest storage time.

Kells et al. (2001) obtained 63% reduction of *A. parasiticus* conidia with 50 ppm O_3 for 3 days but insignificant reduction with 25 ppm for 5 days. Wu et al. (2006) achieved 96.9% and 100% spore inactivation with 0.33 mg/g/min O_3 for 5 min and 15 min respectively at 0.90 a_w and 20°C, while Allen et al. (2003) inactivated 96% of spores and mycelium after 5 min at 0.1 mg/g/min on barley of 0.98 a_w at 20°C, suggesting that mycelium is more vulnerable to O_3 than fungal spores. Recently, White et al. (2010) attempted to relate the effect of O_3 treatment of high moisture maize to dry matter losses after 9 days of storage. Despite using high O_3 concentrations and long exposure times, small reduction of DMLs was observed in the treated samples compared to the controls and no clear pattern with regard to the O_3 dose applied or the exposure time. No attempt was made to determine the effect of the treatments on possible mycotoxin production by the fungi in the samples.

Ozone has also proven effective against insects (Kells et al., 2001; Tiwari et al., 2010) and this is an additional advantage for an integrated approach in the post-harvest management of grain.

Ozone dispersion in a grain mass must be optimised for increased efficacy during application in large scale storage bins which differs from small scale trials. Two distinct phases have been identified (Strait, 1998; Kim et al. 1999) (a) "the O_3 demand of the medium" (Kim et al., 1999) and (b) the phase where interactions have been eliminated and O_3 disperses easier and so the desired concentration can be established. Kells et al. (2001) suggested that an "apparent velocity" of 0.03m/s is required for O_3 to disperse through the grain mass in a reasonable time. Smaller velocities and less time are required during the second phase of O_3 dispersion or in repeated O_3 exposures (Kells et al., 2001; Mendez et al., 2003; Hardin et al., 2010). The importance of the grain condition and the presence of foreign matter on the rate of O_3 decay have also been noted (Hardin et al., 2010; Tiwari et al., 2010).

Any likely effects on quality characteristics of the grains after O_3 treatment are also very important. 50 ppm O_3 for 30 days had no detrimental effect on certain quality characteristic of several grain types (Mendez et al., 2003). Akbas and Ozdemir (2006) noted some differences between the organoleptic properties of pistachios depending on whether O_3 exposure took place on kernels or in ground nuts. Wheat germination was unaffected by exposure at 0.98 mg/g/min O_3 for 15 min (Wu et al., 2006), while in barley this dose was safe for up to 10 min and lower O_3 doses were safe for up to 45 min (Allen et al., 2003). Muthukumarappan et al. (2000) pointed to the possible effects on oxidative stability of foods due to the presence of singlet oxygen and other reactive species related to O_3 exposure.

Overall, the O_3 concentrations required for the effective control of fungal growth and mycotoxin production are not yet established, nor are the optimum exposure times for the highest efficiency. However, the effects of other factors such as water availability, substrate, temperature, foreign matter and degree of spoilage during O_3 treatment of grains are not fully understood.

The aim of this study was to examine the efficacy of gaseous O_3 at inhibiting spore germination, mycelia growth rate and toxin production by different *Fusarium* fungal species *in vitro.* Experiments were also conducted on dried naturally contaminated grains, in order to examine whether gaseous O_3 was effective in reducing the total populations. Grains were also rewetted to target water activity levels, artificially inoculated with known numbers of spores of the above mycotoxigenic fungal species, exposed to gaseous O_3 and stored at 25°C for 10-30 days. At the end of the storage time the total fungal populations were enumerated and identified to species level where possible and the samples were analysed for mycotoxin production.

6.2 Materials and methods

6.2.1 O_3 gas

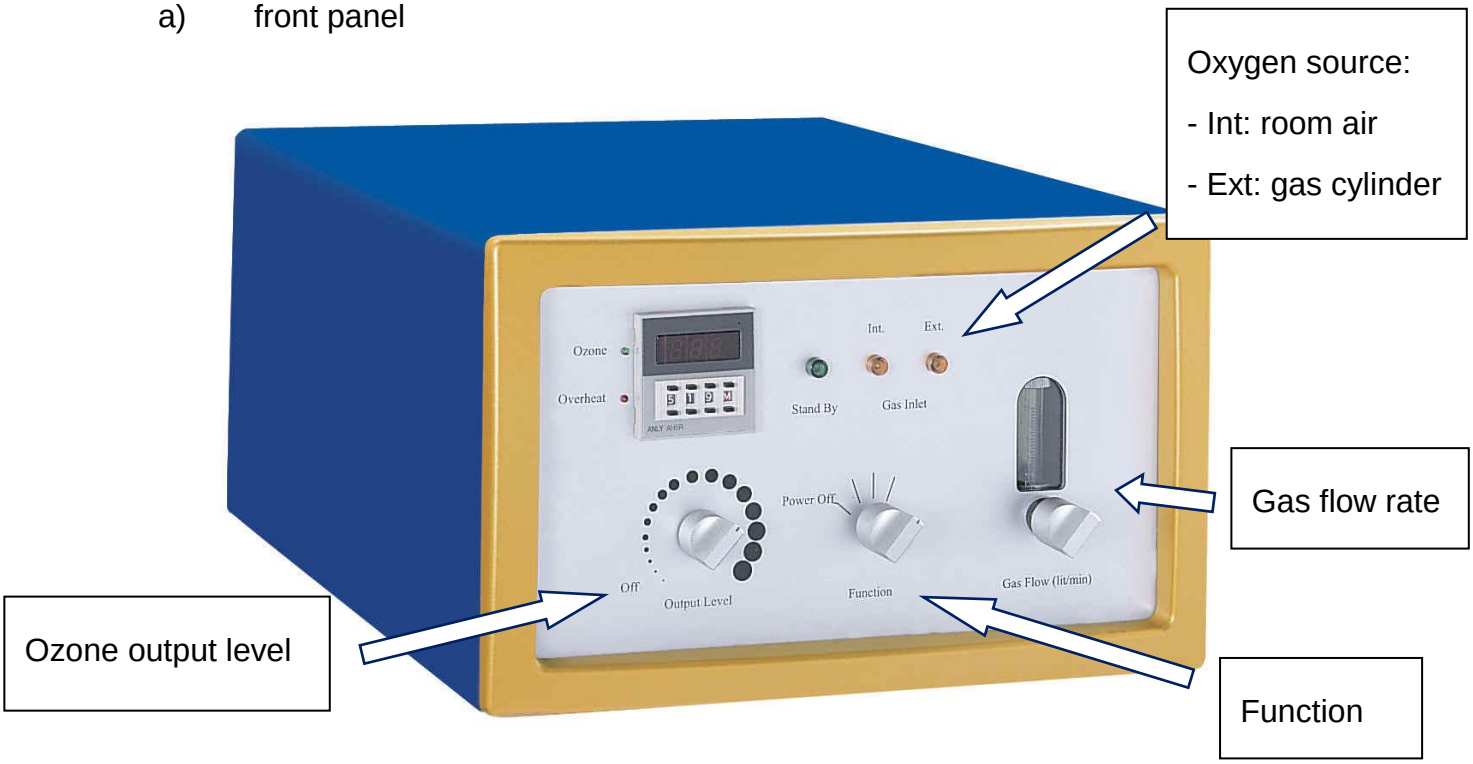
Ozone (O_3) is a molecule consisting of three oxygen atoms. It is an allotropic form of oxygen and therefore less stable. It is a gas with a light blue colour and a characteristic smell detectable by humans in extremely low concentrations. It occurs naturally in the atmosphere but it is very unstable and decomposes to oxygen. Ozone is a very powerful oxidising agent and it is harmful to humans as it can cause irritation to the eyes and the respiratory system at very low concentrations. The maximum concentration of exposure of workers for 8 h not likely to cause any health effects is established at 0.1 ppm of O_3, while exposure to 50 ppm for more than 30 min may be potentially lethal. For this reason certain safety precautions must be in place. A Risk Assessment was performed for the use of the O_3 generator in the laboratory.

6.2.2 Apparatus for O_3 generation and exposure chamber

The apparatus used in this experiment for the generation of O_3 was a C-Lasky series Ozone generator, Model CL010DS purchased from AirTree Ozone

Technology Co., Ltd., (Sijhih City, Taipei County, Taiwan). Figure 6.1 shows the front and back panels of the apparatus:

a) front panel



b) back panel

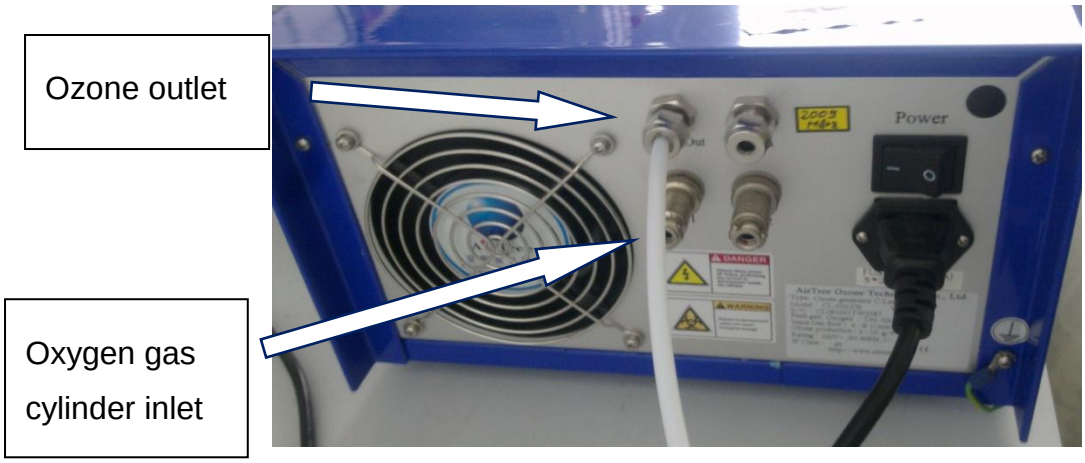


Figure 6.1: Ozone generator, C-Lasky series, Model CL010DS (AirTree Ozone Technology Co., Ltd., Sijhih City, Taipei County, Taiwan); front (a) and back (b) panel

O_3 was generated by this equipment by corona discharge between the surfaces of two quartz tubes without the presence of metals for improved efficiency, stable O_3 production and low energy consumption. This generator can produce 2 g/h O_3 from dry ambient air or up to 10 g/h O_3 if fed with oxygen from an external oxygen supply at a flow rate of 6 L/min. O_3 produced by the Ozone generator was transferred into the exposure chamber by a Teflon tube connected to the generator with stainless steel joints. The exposure chamber was placed into a fume cupboard in order to ensure that O_3 could not escape into the laboratory area.

The air around the O_3 generator and the fume cupboard was monitored using Ozone test stripes (Macherey-Nagel, Epak Electronics Ltd., Chard, Somerset, UK) which change colour depending on the O_3 concentration in the air.

O_3 exposures were performed in two different exposure chambers (set-ups) which can be seen in Figure 6.2:

(a) an airtight 5 L glass jar system

An airtight glass jar (5 L) was used as the exposure chamber for the *in vitro* experiments but also for some grain exposures. A Teflon tube inserted from the lid of the jar and reaching the bottom of the jar was used as the O_3 inlet in the system, while the O_3 outlet was at the lid of the jar. O_3 exposure in this system was therefore based on contact due to settling of O_3 on the samples and diffusion through the grains.

(b) a 100 ml volume glass tube

For some of the *in situ* experiments a 100 ml glass tube was used as the exposure chamber in order to imitate storage in a column set-up. The O_3 inlet was at the bottom of the column and the outlet was at the top of the system. The column was filled with grain and the O_3 generated was forced upwards through the grain mass at a specified flow rate.

An O_3 analyser Model UV-100, Eco Sensors (Santa Fe, New Mexico 87505 USA) was used in order to measure the O_3 concentration at the exit of the

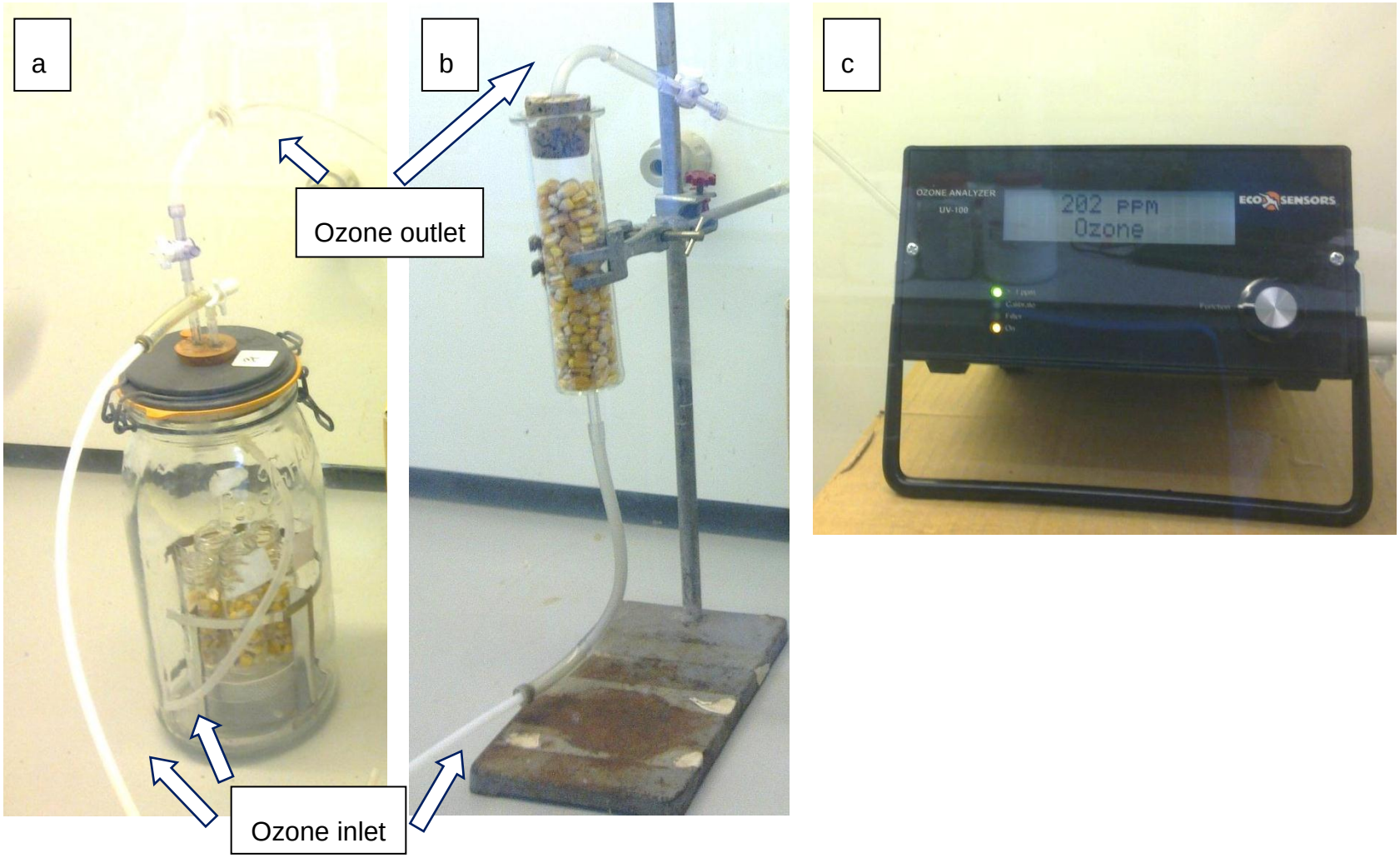


Figure 6.2: Ozone exposure chambers: a 5 L air-tight glass jar (a) and a 100 ml glass tube (b) and ozone analyser (c)

exposure chamber (Figure 6.2). The reading obtained was used as the actual exposure concentration inside the exposure chamber.

The O_3 concentrations reached the target level inside both chambers within 1-2 minutes from the beginning of the exposure and once the exposure was stopped the O_3 concentration decreased to <3 ppm in less than one minute thus preventing hold time at intermediate O_3 concentrations.

6.2.3 Grains, culture media and water activity adjustment

For the *in vitro* trials agar media were prepared using 2% milled grain, 2% technical agar (Agar No 3, OXOID) and appropriate amounts of glycerol/water solutions for modification of the water activity (as described in Section 4.2.2). Naturally contaminated wheat, maize and oats (Section 4.2.2) were used for the *in situ* O_3 experiments.

6.2.4 Fungal strains and preparation of spore suspensions

F. verticillioides isolate MPVP 294 (2.2.3), *Fusarium langsethiae* strain 2004/59 (Section 3.2.3) and *F. graminearum* isolate WP/08/031 supplied by Professor Simon Edwards (Harper Adams University College, UK) isolated from wheat in the UK and a known producer of deoxynivalenol were used in this study. The strains were maintained on Malt Extract Agar (MEA) media (OXOID, malt extract, 30; mycological peptone, 5; agar, 15 g/l). Spore suspensions were prepared as previously described (Section 2.2.3) and filtered through glass wool in order to remove any mycelial fragments.

6.2.5 *In vitro* effect of O_3 on spore germination

100 µl of spore suspension were spread on the surface of the grain media. The plates were allowed to dry for a couple of hours and were then placed inside the

glass jar without the lids and exposed to O_3. Two plates were used at each exposure experiment to serve as replicates one placed above the other, the distance between them being around 5 cm. Plates exposed to air were used as controls. Preliminary experiments were performed using 50-200 ppm O_3, at a flow rate of 6 L/min for 30 min.

The replicate plates were then stored at 25°C for 10 days. On a daily basis, one agar plug (~1 cm diameter) was removed from each plate, placed on a glass microscope slide (Fisherbrand, UK), stained with Lactophenol Cotton Blue (ProLab Diagnostics, UK), covered with glass slips and examined under the microscope in order to assess the germination of the spores. Spores were considered as germinated when the germ tube length was equal to or longer than the size of the spore. Two sets of 100 spores were assessed for germination on each plug and the average germination determined for each plate. The average of the two replicates was used as the % germination at each combination of water activity and O_3 concentration. Subsequent experiments were performed at the above O_3 concentrations for different lengths of time (15, 60min).

6.2.6 *In vitro* effect of O_3 on mycelia growth rate

Grain media were centrally inoculated with 10µl spore suspension and incubated at 25°C in polyethylene bags containing replicates of the same a_w treatment. The cultures were allowed to grow, the 0.98 a_w treatments for a couple of days and the 0.94 a_w treatments for 5-6 days. The colony diameters were recorded and preliminary exposures to 0-200 ppm O_3 were performed for 30 min. The cultures were then stored at 25°C and two diameters of the colonies at right angles to each other were measured on a daily basis and compared against the diameters of the control cultures that had been exposed to air. From these data the % inhibition of the growth rates of the fungi was determined at different combinations of O_3 concentration and substrate a_w. Subsequent experiments were performed for (15 and 60 min).

6.2.7 *In vitro* effect of O_3 on mycotoxin production by *Fusarium* species

On the 10th day of storage after O_3 exposure, agar plugs were taken from each replicate plate used for the germination and growth studies by use of a 5 mm diameter cork borer according to the patterns shown in Appendix C. The agar plugs were placed in 2 ml safe-lock Eppendorf® tubes, their weight was recorded and they were frozen at -40°C for subsequent toxin analysis. The extraction and analysis of the relevant toxins for each fungal species were performed as in Section 4.2.5.

6.2.8 *In situ* effect of O_3 on the total fungal populations on naturally contaminated grain

Naturally contaminated wheat was weighed into Universals (13 g) and exposed for 30 min to 100 and 200 ppm O_3 in the glass jar set-up at a flow rate of 6 L/min. 75 g of naturally contaminated maize were exposed to 100 and 200 ppm O_3 for 60 min in the column set-up at a flow rate of 6 L/min.

Immediately after the exposure 1 g grain from each sample exposed in the jar set up and 1 g grain from the bottom, middle and top of the column were used for assessment of the fungal populations using the serial dilution technique. 100 µl of each dilution were spread on Malt extract agar (MEA) in order to compare the populations isolated from the treated samples to those of the control untreated grain.

6.2.9 *In situ* fungal growth and mycotoxin production in grains inoculated with *Fusarium* species and exposed to O_3

Grains were rewetted according to water adsorption curves previously developed (Appendix F) and allowed to equilibrate for 2-3 days at 4°C. They were then inoculated with 1 ml spore suspension containing ~10^5/ml spores of

the fungi to be studied per 100 g of grain and thoroughly mixed using a roller mixer in order for the spores to be dispersed throughout the mass of the grain. Preliminary exposures were performed by exposing six replicates (10-15 g inoculated grain in Universals) inside the glass jar (~7 cm from the base of the jar) to 100 and 200 ppm O_3 for 1 hour at a flow rate of 6 L/min. The effect of O_3 on the total populations of the exposed samples was determined as described above (Section 6.2.8). The samples were then stored in sandwich boxes, the ERH maintained using glycerol-water solutions of the same a_w, for 10-30 days. At the end of each storage period 1 g from each sample was used to determine the fungal populations by the serial dilution technique.

Subsequent experiments were performed by filling the glass column with 60-75 g of grain and flushing with different O_3 concentrations through the grain mass at a rate of 6 L/min. Nine subsamples were taken from the grain lot (three from each: bottom, middle and top of the column) and stored for 15 and 30 days in sandwich boxes of controlled ERH. In a repeated experiment autoclaved grain was exposed to O_3 at a flow rate 4 L/min for 1 hour (in the column set-up) while control samples were exposed to air at the same flow rate, in order to study the effect of O_3 dissociated from any likely drying effect. The effect on fungal populations was also examined.

The rest of the grain was frozen at -20°C for subsequent mycotoxin analysis as described in Section 2.2.7.

6.2.10 Statistical analysis

Data on *in vitro* germination, radial growth rate and mycotoxin analysis as well as *in situ* data on fungal populations and mycotoxin analysis were analysed as in Section 2.2.8. ANOVA Tables can be found in Appendix I.

6.3 Results

6.3.1 Effect of O_3 on spore germination *in vitro*

Figure 6.3 shows the effect of 0-200 ppm O_3 on the mean germination of *F. graminearum* spores on 2% wheat agar adjusted to 0.98 and 0.94 a_w and stored at 25°C after exposure for 30 min at a flow rate of 6 L/min.

Spore germination was lower in the treated samples compared to the control and in media of 0.98 a_w this was dependent on the O_3 dose applied. However, with time spores recovered and after 6 days of incubation at 25 ºC germination was 100% in all treatments. On media of 0.94 a_w none of the spores germinated after 30 min exposure irrespective of the O_3 dose used and this was maintained for the 10 days that the study lasted. Indeed, even after 15 days when the plates were re-examined, spore germination had not resumed in any of the treatments (data not shown).

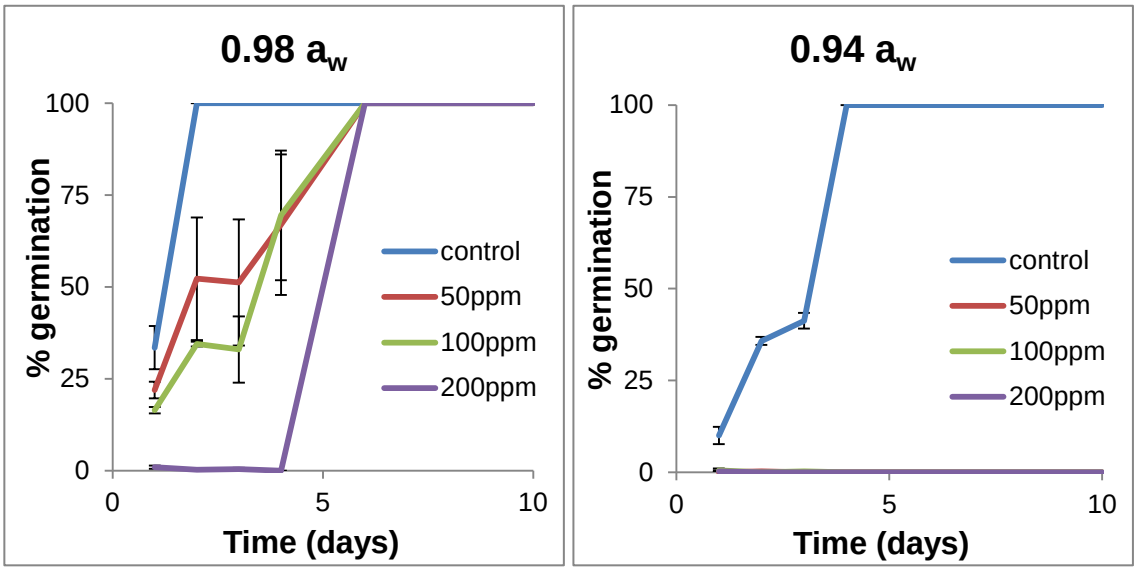


Figure 6.3: Germination (%) of *Fusarium graminearum* WC/08/031 spores over time on 2% wheat agar media adjusted to 0.98 and 0.94 a_w and stored at 25°C after exposure to 0-200 ppm O_3 for 30 min at 6 L/min flow rate. Vertical bars indicate the standard error of the means.

The Kruskal-Wallis ANOVA showed that the effect of O_3 concentration was not significant on the germination of exposed *F. graminearum* spores, while the effect of substrate water activity was highly significant (Apx Table I.1).

Plate 6.1 shows spores of *F. graminearum* WC/08/031 on wheat agar media of 0.94 a_w after 48 hours of storage at 25°C (a) control, germinated and (b) after exposure at 200 ppm O_3 for 30 min, un-germinated.

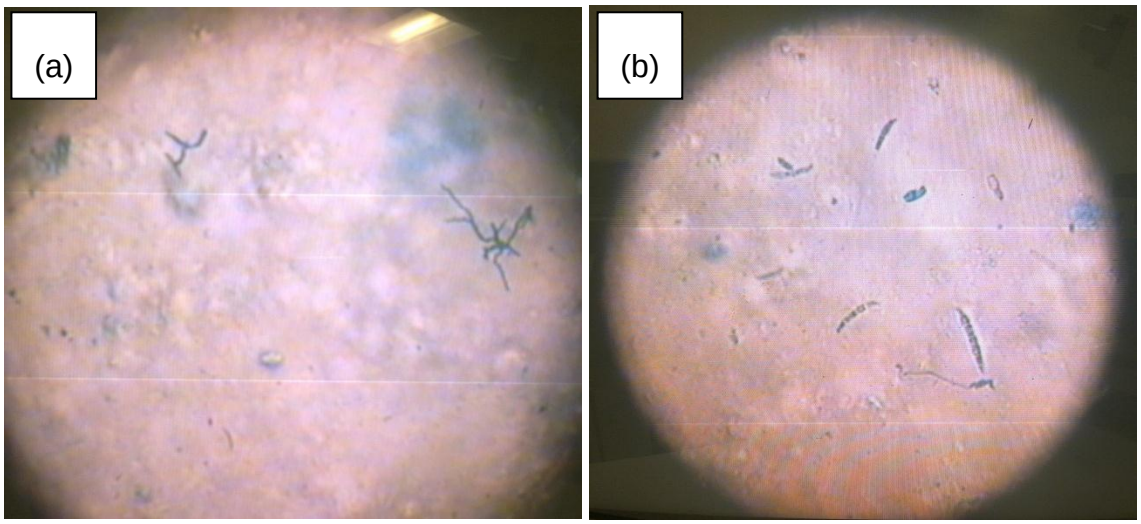


Plate 6.1: Spores of *F. graminearum* WC/08/031 on wheat agar media of 0.94 a_w after 30 min exposure at 200 ppm O_3 and storage at 25°C for 72 hours (a) germinated (control) and (b) un-germinated.

Figure 6.4 shows the mean germination of *F. verticillioides* spores on 2% maize agar adjusted to 0.98 and 0.94 a_w and stored at 25°C after exposure to 0-200 ppm O_3 for (a) 15 min and (b) 30 min at a flow rate of 6 L/min.

All spores treated with O_3 for 15 min germinated irrespective of the O_3 dose applied or of the substrate a_w, though in media of 0.94 a_w 100% germination was reached 1-2 days later than in the media of 0.98 a_w treated with similar O_3 doses. After 30 min exposure, some inhibitory effect was initially observed with increasing O_3 concentration for *F. verticillioides* spores treated with 50-200 ppm O_3 on media of 0.98 a_w, but at the end of the experiment 100% germination had

been reached. In media of 0.94 a_w germination was completely inhibited only for spores treated with 200 ppm O_3.

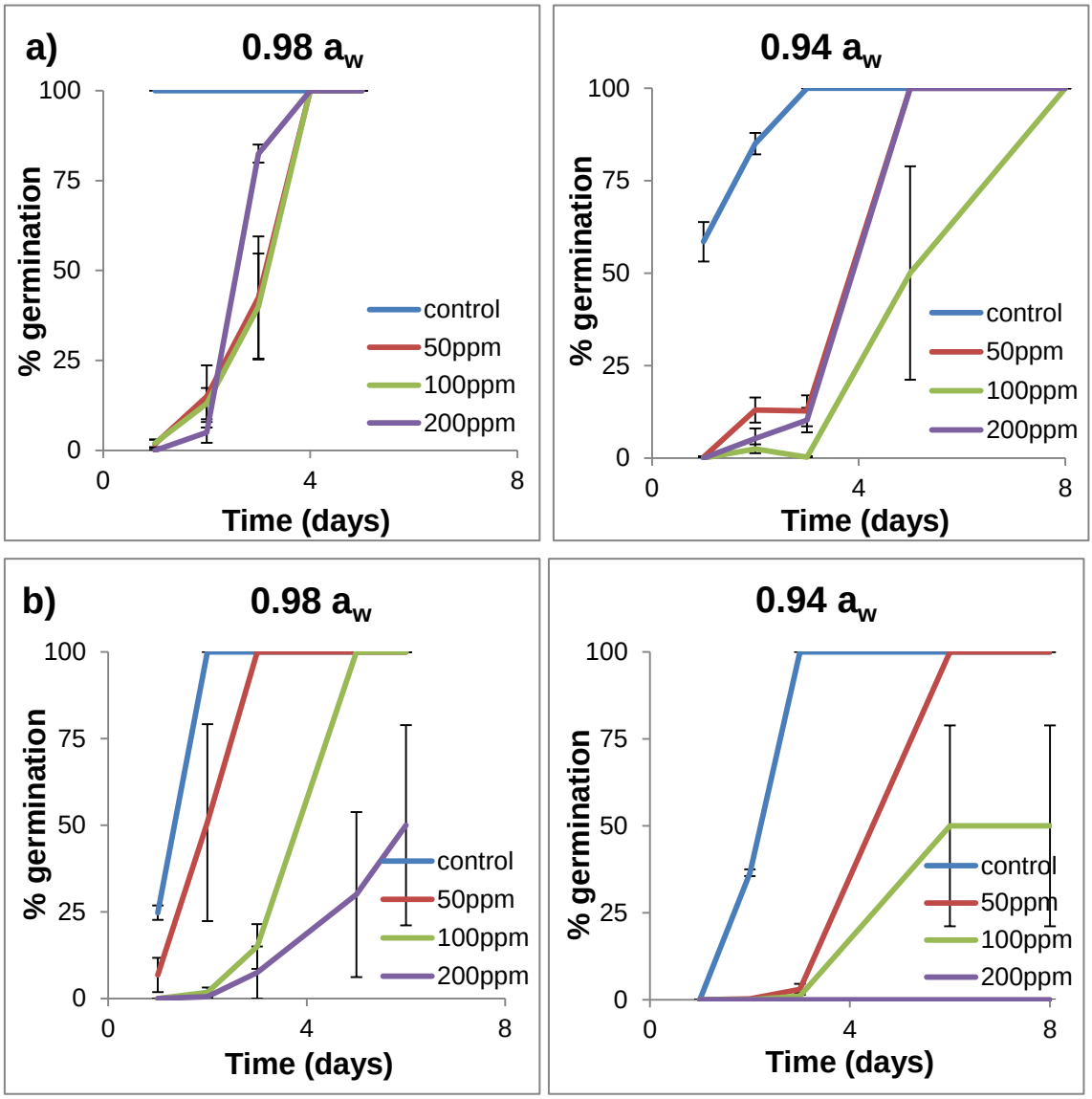


Figure 6.4: Germination (%) of *Fusarium verticillioides* MPVP 294 spores over time on 2% maize agar media adjusted to 0.98 and 0.94 a_w and stored at 25°C after exposure to 0-200 ppm O_3 for (a) 15 min and (b) 30 min at 6 L/min flow rate. Vertical bars indicate the standard error of the means.

The Kruskal-Wallis test showed that the effects of O_3 concentration and exposure time were significant on the germination of *F. verticillioides* spores while the effect of substrate a_w was not significant (Apx Table I.2).

Plate 6.2 shows maize agar media of 0.98 a_w spread with *F. verticillioides* spores and exposed to air, 50, 100 and 200 ppm O_3 for 15 min after 72 hours storage at 25°C. Spore germination at this point was 100% in the controls and 25-75% in the treated samples. A difference can be observed in the colour of the O_3–treated culture plates compared to the control plates treated with air of the same flow rate.

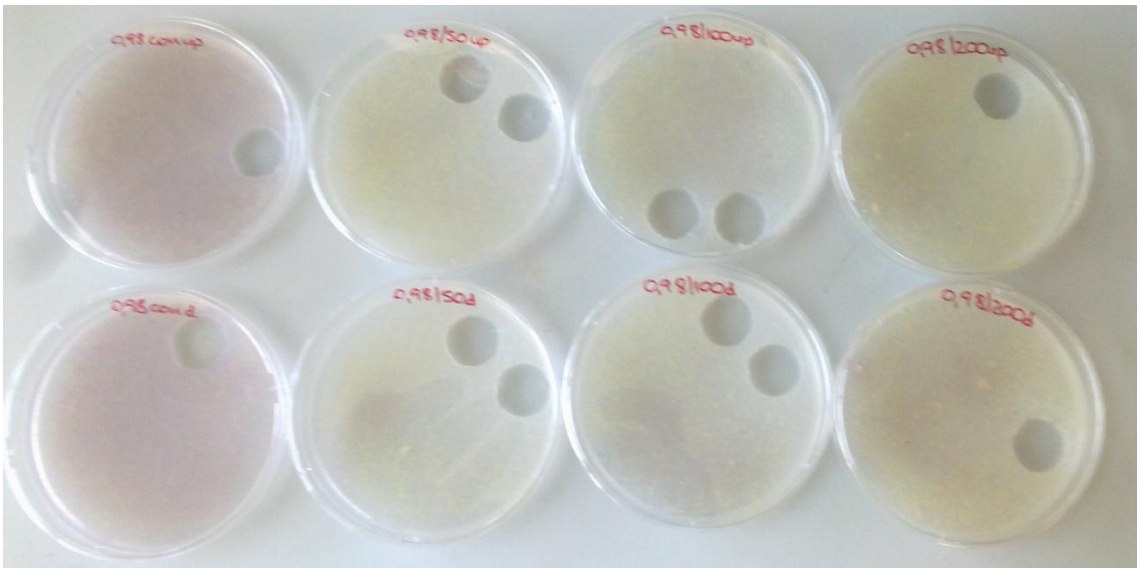


Plate 6.2: Maize agar media of 0.98 a_w spread with spores of *F. verticillioides* MPVP 294, exposed to air, 50, 100 and 200 ppm O_3 (from left to right) for 15 min and stored at 25°C for 72 hours.

6.3.2 Effect of O_3 on mycelia extension *in vitro*

Figure 6.5 shows the effect of 0-200 ppm O_3 on the mycelia extension of *F. graminearum* on 2% wheat agar of 0.98 and 0.94 a_w after 30 min exposure at a flow rate of 6 L/min.

Some inhibitory effect was observed in the radial growth rate of *F. graminearum* at 0.98 a_w, with the maximum inhibition observed at 200 ppm O_3 (~27%) when compared to the control. In the drier samples (0.94 a_w) ~45% inhibition was observed in the treated samples, irrespective of the O_3 dose applied.

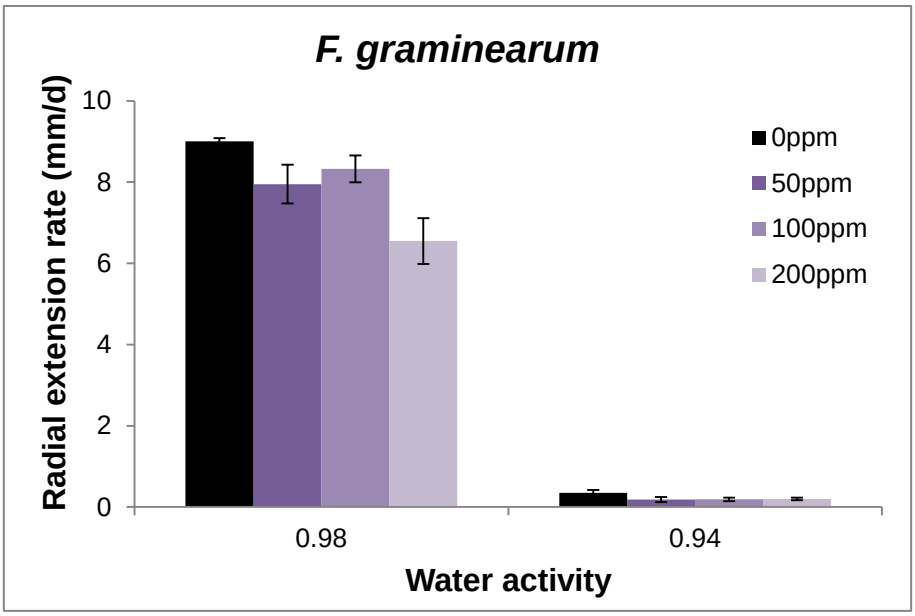


Figure 6.5: Effect of 0-200 ppm O_3 exposure for 30 min at 6 L/min flow rate on the radial growth rate of *Fusarium graminearum* WC/08/031 on 2% wheat agar media adjusted to 0.98 and 0.94 a_w. Vertical bars indicate the standard error of the means.

The Kruskal-Wallis test showed that the effect of O_3 concentration was not significant on the radial growth rate of *F. graminearum*, while the effect of substrate a_w was highly significant (Apx Table I.3).

Figure 6.6 shows the effect of 0-200 ppm O_3 exposure for (a) 30 and (b) 60 min at 6 L/min flow rate on the radial growth rate of *F. verticillioides* actively growing cultures on 2% maize media adjusted to 0.98 and 0.94 a_w.

A maximum inhibition of 12% was observed in the growth rate of *F. verticillioides* at 0.98 a_w after 30 min exposure irrespective of the O_3 dose

applied. In the drier conditions exposure at 100 ppm O_3 for 30 min stimulated (27%) the mycelia extension rate, while a borderline inhibition was observed at 200 ppm (~6%). Exposure of actively growing *F. verticillioides* cultures to the same O_3 dose for 60 min caused a maximum inhibition of the radial growth rate of 14% at 200 ppm O_3 in the wettest samples. In contrast, in media of 0.94 a_w only a slight inhibition (5%) was observed at the same conditions.

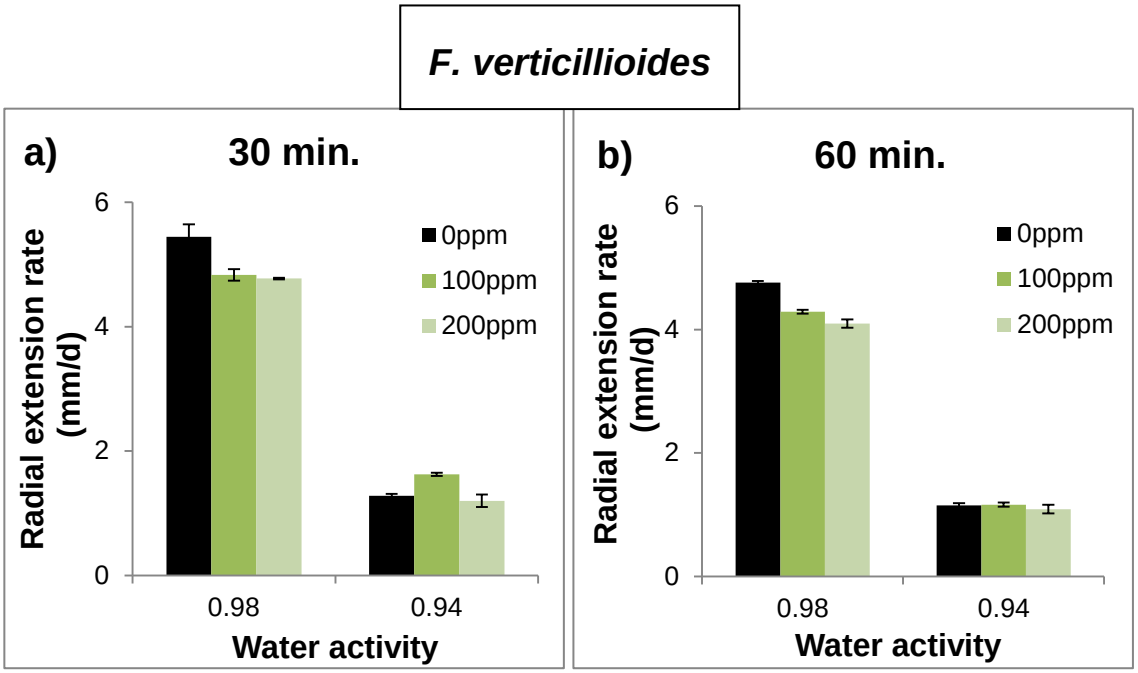


Figure 6.6: Effect of 0-200 ppm O_3 exposure for (a) 30 and (b) 60 min at 6 L/min flow rate on the radial growth rate of *F. verticillioides* actively growing cultures on 2% maize media adjusted to 0.98 and 0.94 a_w. Vertical bars indicate the standard error of the means.

The Kruskal-Wallis test showed that the effect of O_3 concentration was not significant on the radial extension rate of actively growing *F. verticillioides* cultures on maize agar media after O_3 exposure, while the effect of substrate a_w was highly significant and the effect of the duration of the O_3 exposure was significant (Apx Table I.4).

6.3.3 Effect of O_3 on *in vitro* mycotoxin production by *Fusarium* species

Figure 6.7 shows the production of different fumonisins in maize media of 0.98 and 0.94 a_w spread with *F. verticillioides* spores, exposed to 0-200 ppm O_3 for 15 min and stored for 10 days at 25°C.

The production of all four fumonisins was stimulated when *F. verticillioides* spores were exposed to O_3 (before the onset of fungal growth) compared to the control samples treated with air.

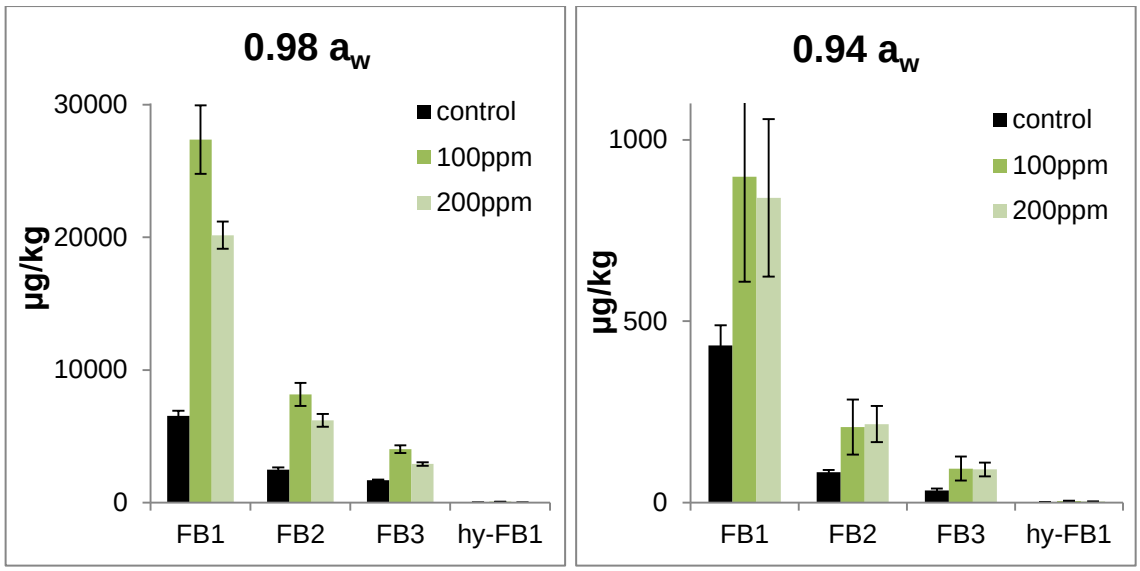


Figure 6.7: Combined effect of O_3 dose and substrate a_w (0.98 and 0.94 a_w) on fumonisins B_1, B_2, B_3 and hydrolysed-FB_1 production in maize media spread with *F. verticillioides* spores, exposed to O_3 (0-200 ppm for 15 min at 6L/min flow rate) and stored at 25°C for 10 days. Vertical bars indicate the standard error of the means.

The Kruskal-Wallis test showed that the effect of O_3 concentration was not significant for fumonisins B_1, B_2 and B_3 production on media spread with *F. verticillioides* spores, exposure for 15 min and incubated for 10 days, while the effect of substrate a_w was highly significant. In contrast, the effects of O_3 dose

and substrate a_w were highly significant on hydrolysed-FB_1 while the effect of their interaction ($O_3 \times a_w$) was not significant (Apx Tables I.5, I.6).

Figure 6.8 shows the production of different fumonisins by actively growing *F. verticillioides* cultures exposed to 0-200 ppm O_3 for 60 min and stored at 25°C for 10 days.

When actively growing cultures were treated with O_3, the production of all four toxins was reduced compared to the control samples treated with air in media of 0.98 a_w, but no significant difference was observed between the two O_3 doses. At 0.94 a_w exposure at 100 ppm O_3 slightly stimulated FB_1 and FB_2 production, while 200 ppm O_3 reduced the production of all four toxins compared to the control (30-50% reduction). Statistically, the effect of O_3 dose was not significant on fumonisins production by growing *F. verticillioides* cultures, while the effect of substrate a_w was highly significant (Apx Tables I.7, I.8).

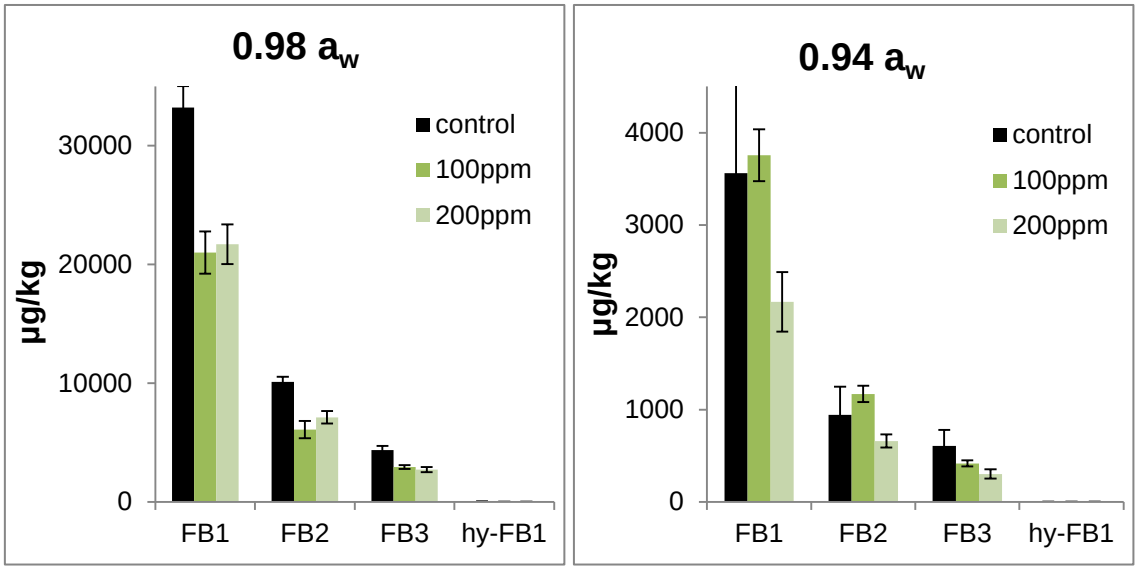


Figure 6.8: Combined effect of O_3 dose and substrate a_w (0.98 and 0.94) on the production of fumonisins B_1, B_2, B_3 and hydrolysed-FB_1 by growing *F. verticillioides* cultures exposed to 0-200 ppm for 60 min at 6 L/min flow rate and stored at 25°C for 10 days. Vertical bars indicate the standard error of the means.

6.3.4 *In situ* efficacy of O_3 on fungal populations isolated from naturally contaminated and artificially inoculated grain after exposure

Figure 6.9 shows the effect of 30 min O_3 exposure in the jar set-up on the total fungal populations (CFUs) isolated from naturally contaminated wheat immediately after the exposure.

The fungal populations isolated from the wheat samples exposed to 100 ppm O_3 for 30 min were ~0.5 log less than those from the control, while no effect was observed in the samples exposed to 200 ppm O_3.

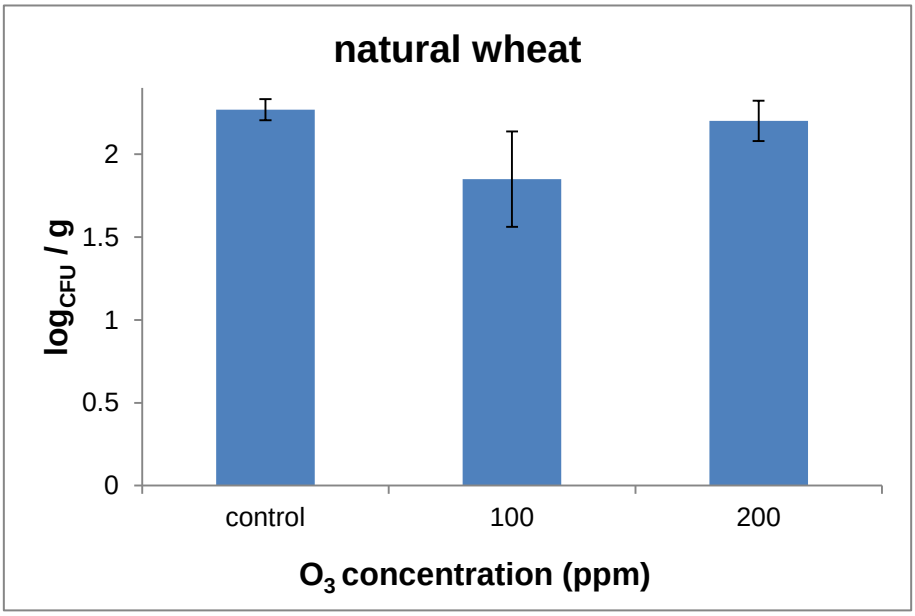


Figure 6.9: Effect of 100 and 200 ppm O_3 exposure for 30 min (in the jar system) at a flow rate of 6 L/min on the logarithm of the populations (log_{CFUs}) isolated from naturally contaminated wheat, compared to the counts of the control untreated wheat. Vertical bars indicate the standard error of the means.

ANOVA showed that the effect of O_3 concentration was not significant on the log_{CFU} counts isolated from naturally contaminated wheat after exposure in the jar system (Apx Table I.9).

Figure 6.10 shows the log_{CFU} counts isolated from naturally contaminated maize after 60 min exposure to 100 and 200 ppm O_3 at a flow rate of 6 L/min in the column set-up compared to the control.

Exposure at 100 ppm O_3 caused 1.25 logs reduction in the total fungal populations isolated from the maize samples, while exposure at 200 ppm caused almost 2 logs reduction compared to the control samples that were treated with air at the same flow rate.

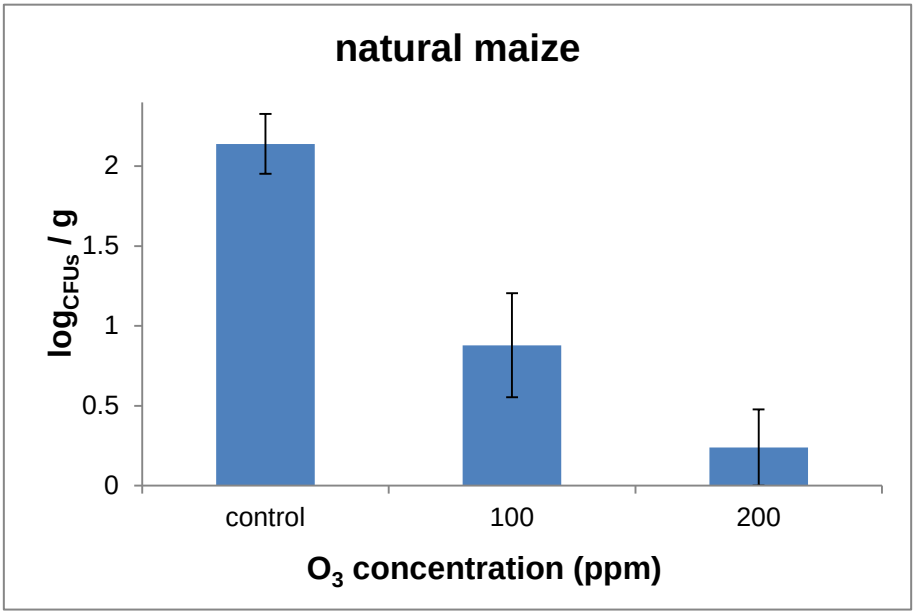


Figure 6.10: Effect of 100 and 200 ppm O_3 exposure for 60 min (in the column system) at a flow rate of 6 L/min on the logarithm of the total fungal populations (CFUs) isolated from naturally contaminated maize, compared to the control maize treated with air. Vertical bars indicate the standard error of the means.

ANOVA showed that the effect of O_3 concentration was significant on the log_{CFU} counts isolated from naturally contaminated maize after 60 min exposure in the column system (Apx Table I.10).

Figure 6.11 shows the effect of 100 and 200 ppm O_3 on the log_{CFU} counts isolated from (a) autoclaved maize rewetted to 0.90 and 0.93 a_w and (b)

naturally contaminated maize rewetted to 0.92 and 0.88 a_w, both inoculated with *F. verticillioides* spores (~10^4/ml), after exposure to 0-200 ppm O_3 for 60 min in the column system.

Some effect was observed in the total fungal populations isolated from the O_3-treated samples compared to those isolated from the control samples treated with air. This effect however was not proportional to the O_3 dose applied and was more pronounced in the naturally contaminated maize samples than in the samples that had been autoclaved prior to additional inoculation.

The Kruskal-Wallis test showed that the effect of grain a_w was not significant on the $\log_{CFU}$ counts isolated from the maize samples after exposure in any of the two experiments. In contrast, the effect of O_3 concentration was significant on the $\log_{CFU}$ counts isolated from natural maize, but not significant on the $\log_{CFU}$ counts isolated from autoclaved maize immediately after exposure (Apx Tables I.11, I.12).

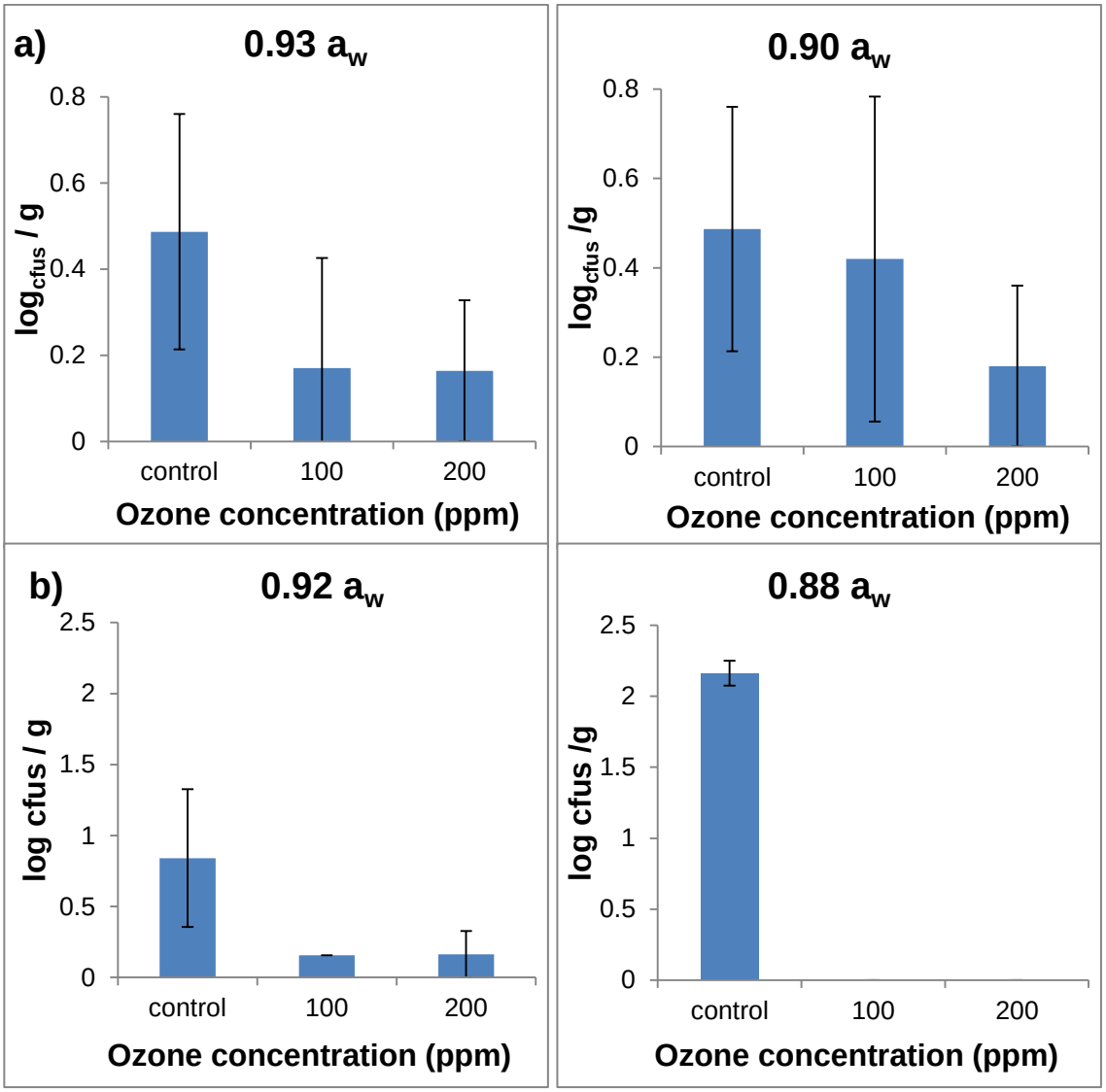


Figure 6.11: Logarithm of total fungal populations isolated from (a) autoclaved maize rewetted to 0.93 and 0.90 a_w and (b) naturally contaminated maize rewetted to 0.92 and 0.88 a_w, both additionally inoculated with ~10^4 *F. verticillioides* spores/ml, after exposure at 100 and 200 ppm O_3 for 60 min in the column system, at a flow rate of 4 L/min and 6 L/min respectively, compared to the control maize treated with air. Vertical bars indicate the standard error of the means.

6.3.5 *In situ* mycotoxin production in stored grains exposed to O_3 in the glass jar system

Figure 6.12 shows the amount of DON produced in wheat samples rewetted to 0.98 and 0.94 a_w, inoculated with *F. graminearum* WP/08/031, exposed at 100 and 200 ppm O_3 for 30 min at a flow rate of 6 L/min in the glass jar set-up and subsequently stored at 25°C under controlled ERH for 20 days. The red line shows the EU legislative limits for DON in wheat.

It can be observed that after 20 days storage at 25°C DON production was significantly reduced (~70%) in the wheat of 0.98 a_w exposed to 100 ppm O_3, while in the samples exposed to 200 ppm O_3 it was higher than in the control. In contrast, in the treated wheat of 0.94 a_w DON production increased compared to the control after 20 days storage, by 40% in the samples exposed to 100 ppm O_3 while by 100% in the samples exposed to 200 ppm O_3.

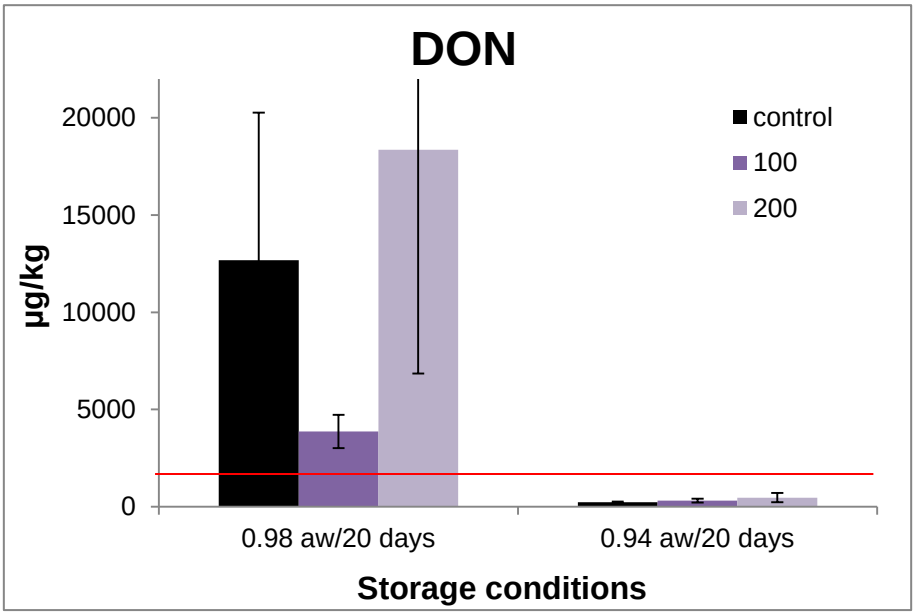


Figure 6.12: Effect of O_3 exposure to 100 and 200 ppm for 30 min in the jar system on DON production in wheat of 0.98 and 0.94 a_w, inoculated with *F. graminearum* WP/08/031 and stored at 25°C for 20 days. Vertical bars indicate the standard error of the means. The red line shows the EU legislative limits for DON in wheat.

ANOVA showed that the effect of O_3 concentration was not significant on DON production after 20 days storage, the effect of a_w was highly significant and the effect of the interaction of the two factors was not significant (Apx Table I.13).

Figure 6.13 shows the sum of fumonisins (B_1+B_2) production in naturally contaminated maize samples rewetted to 0.94 and 0.91 a_w, inoculated with *F. verticillioides,* exposed to 100 and 200 ppm O_3 for 1 h (in the jar set-up) and stored at 25°C for 10 days. The red line shows the EU legislative limit for the sum of fumonisins in maize.

In the wetter samples (0.94 a_w) fumonisins production decreased with increasing O_3 concentration to a maximum of 40% in the samples exposed to 200 ppm O_3 for 1 h. In the samples of 0.91 a_w fumonisins production after 10 days storage was reduced compared to the control in the samples exposed to 100 ppm O_3, but was unaffected in the samples exposed to 200 ppm O_3.

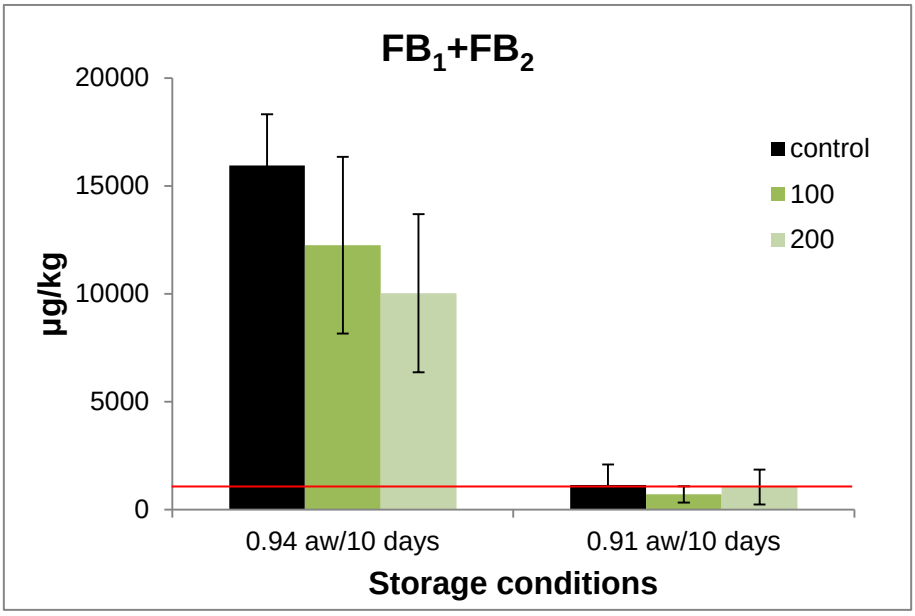


Figure 6.13: Effect of O_3 exposure at 100 and 200 ppm for 1 hour (in the jar set-up) on the sum of fumonisins (B_1+B_2) production in maize of 0.94 and 0.91 a_w, inoculated with *F. verticillioides* and stored at 25°C for 10 days. Vertical bars indicate the standard error of the means. The red line shows the EU legislative limit for the sum of fumonisins in maize.

ANOVA showed that the effect of O_3 concentration was not significant on the total fumonisin production after 10 days storage, the effect of a_w was highly significant and the effect of the interaction of the two factors was not significant (Apx Table I.14).

6.3.6 *In situ* mycotoxin production in stored grains exposed to O_3 in the glass column system

Figure 6.14(a) shows the total fumonisins (B_1+B_2) produced in maize samples rewetted to 0.88 and 0.92 a_w, inoculated with *F. verticillioides*, exposed at 100 and 200 ppm O_3 in the glass column at a flow rate of 6 L/min for 1 hour and stored at 25°C for 15 and 30 days.

Fumonisins were not produced in any of the maize samples at 0.88 a_w, while at 0.92 a_w fumonisin production was completely inhibited in the O_3-treated samples even after 30 days storage.

Figure 6.14(b) shows the production of ochratoxin A in the above maize samples due to the natural contamination by *Penicillium* species.

OTA production was generally inhibited at 0.88 a_w and even after 30 days storage the amount of OTA was below the EU legislative limits. The irregular values observed in the 15-day samples are due to one replicate containing high levels of OTA in each a_w. Otherwise, in the rest of the maize samples of 0.92 a_w, 85-90% inhibition of OTA production was observed in the treated samples compared to the controls.

The Kruskal-Wallis test showed that the effects of O_3 concentration and grain water activity were significant on fumonisins production but the effect of storage time was not significant. Contrarily in the same samples the effects of O_3 concentration and storage time were not significant on ochratoxin A production but the effect of water activity was highly significant (Apx Table I.15).

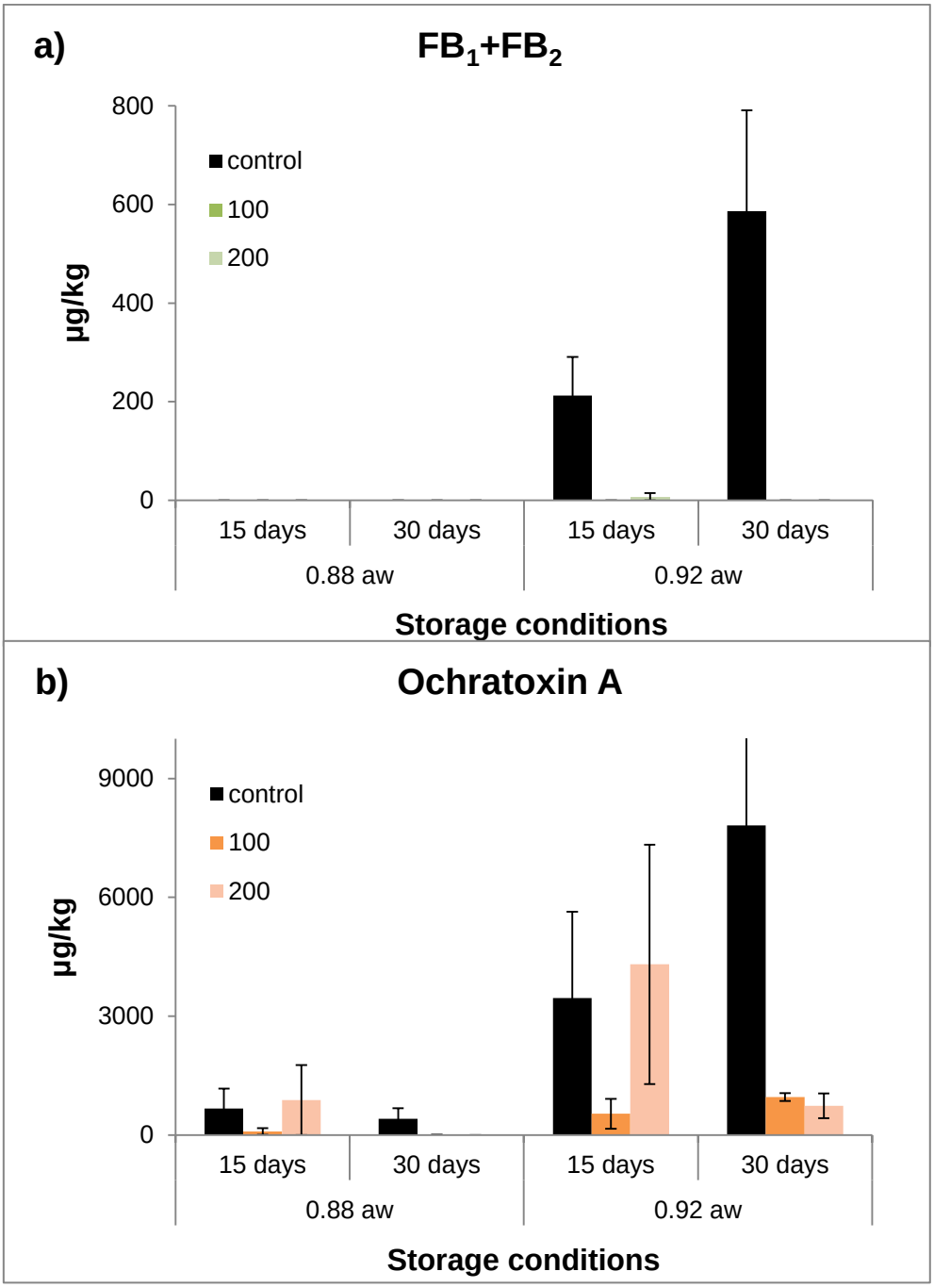


Figure 6.14: Effect of O_3 exposure at 100 and 200 ppm for 1 hour in the column system on (a) the sum of fumonisins (B_1+B_2) and (b) ochratoxin A production in naturally contaminated maize of 0.88 and 0.92 a_w, inoculated with *F. verticillioides* and stored at 25°C for 15 and 30 days. Vertical bars indicate the standard error of the means.

Figure 6.15(a) shows the sum of T-2 and HT-2 toxins produced in oat samples rewetted to 0.88 and 0.91 a_w, inoculated with *F. langsethiae*, exposed to 0-200 ppm O_3 in the glass column system at a flow rate of 6 L/min for 1 hour and stored at 25°C for 15 and 30 days.

The sum of T-2 and HT-2 toxins was generally reduced in the treated oat samples compared to the controls (38-99%), except on two occasions where toxin production in the samples treated with 100 ppm O_3 was higher than in the controls (0.88 a_w/30 d, 0.91 a_w/15 d).

With regard to the two individual toxins, HT-2 was the toxin produced in higher amounts in the oat samples and the pattern of production under the different combinations of treatment×storage time was the same as for the sum of the two toxins. The pattern of T-2 toxin was different from that of the sum in the samples of 0.88 a_w treated with 200 ppm O_3 and stored for 15 days, where toxin production was higher than in the control (data not shown).

Figure 6.15 (b) shows the production of ochratoxin A in the above oat samples due to the natural presence of *Penicillium* species.

OTA production in the oats of 0.88 a_w treated with O_3 and stored for up to 30 days was reduced by 74-99.9% when compared to the untreated grain. In the oats of 0.91 a_w OTA production in the samples treated with O_3 was 83-95% less than in the control.

ANOVA showed that the effects of O_3 concentration, oat a_w, storage time and their interactions were not significant on the sum of T-2+HT-2 toxins produced in the oat samples (Apx Table I.16). In contrast, for OTA production in the oats, the Kruskal-Wallis ANOVA by ranks showed that the effect of grain a_w was highly significant while the effects of O_3 concentration and storage time were significant (Apx Table I.17).

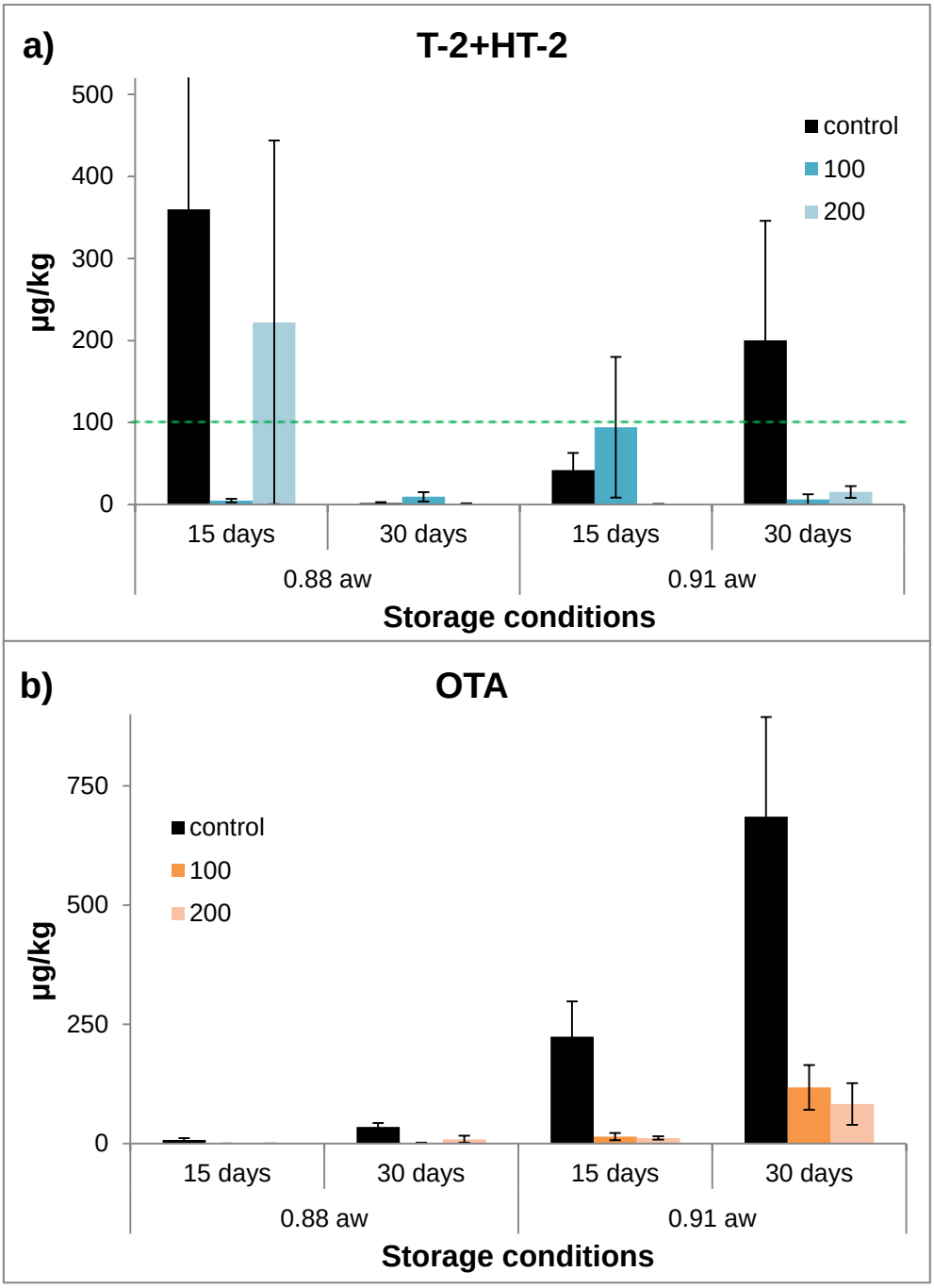


Figure 6.15: Effect of O_3 exposure to 0-200 ppm for 1 hour on (a) the sum of T-2 and HT-2 toxins and (b) ochratoxin A production in oats of 0.88 and 0.91 a_w, inoculated with *F. langsethiae,* exposed in the column system and stored at 25°C for 15 and 30 days. Vertical bars indicate the standard error of the means. The green broken line shows the legislative limits more commonly established around the world for T-2 and HT-2 toxins in oats.

Figure 6.16 shows the fumonisins (B_1+B_2) production in autoclaved maize of 0.90 and 0.93 a_w, inoculated with *F. verticillioides,* exposed to 0-200 ppm O_3 in the column system at a flow rate of 4 L/min for 1 hour and stored for 15 and 30 days at 25°C.

In the samples of 0.90 a_w exposure at 100 ppm O_3 was more efficient in inhibiting total fumonisin production (~20%) than 200 ppm, irrespective of the storage time. In the samples of 0.93 a_w exposed at 100 and 200 ppm O_3 and stored for 15 days, up to 40 and 60% inhibition of fumonisins was observed respectively. In contrast, fumonisin production in the samples stored for 30 days was higher than the control after treatment with 100 ppm O_3 although similar to the control in the samples treated with 200 ppm O_3.

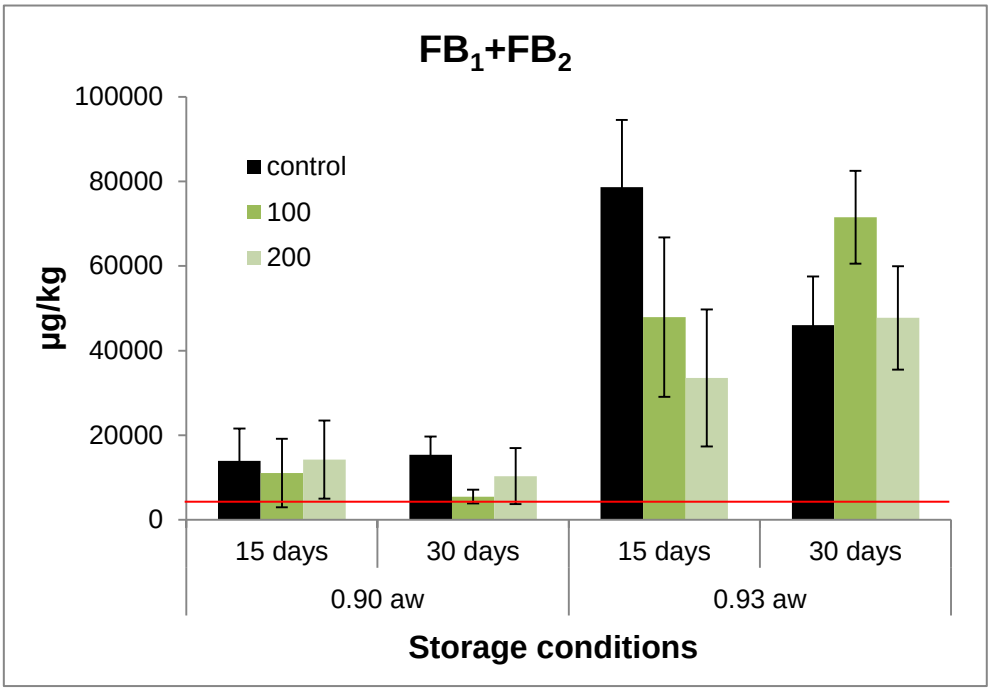


Figure 6.16: Effect of O_3 exposure to 0-200 ppm for 1 hour in the column system on fumonisins (B_1+B_2) production in autoclaved maize of 0.90 and 0.93 a_w, inoculated with *F. verticillioides* and stored at 25°C for 15 and 30 days. Vertical bars indicate the standard error of the means. The red line shows the EU legislative limits for the sum of fumonisins in maize.

The Kruskal-Wallis test showed that the effects of O_3 concentration and storage time were not significant on fumonisins (B_1+B_2) production in autoclaved maize samples, but the effect of grain a_w was highly significant and the effect of the position of the sample in the column was significant (Apx Table I.18).

Figure 6.17 shows the actual fumonisins (B_1+B_2) production in each of the maize samples of the above experiment, in relation to their location (top, middle and bottom) in the glass column system.

Fumonisin production was generally higher in the samples taken from the top of the column, less in the middle and lowest in the bottom of the column.

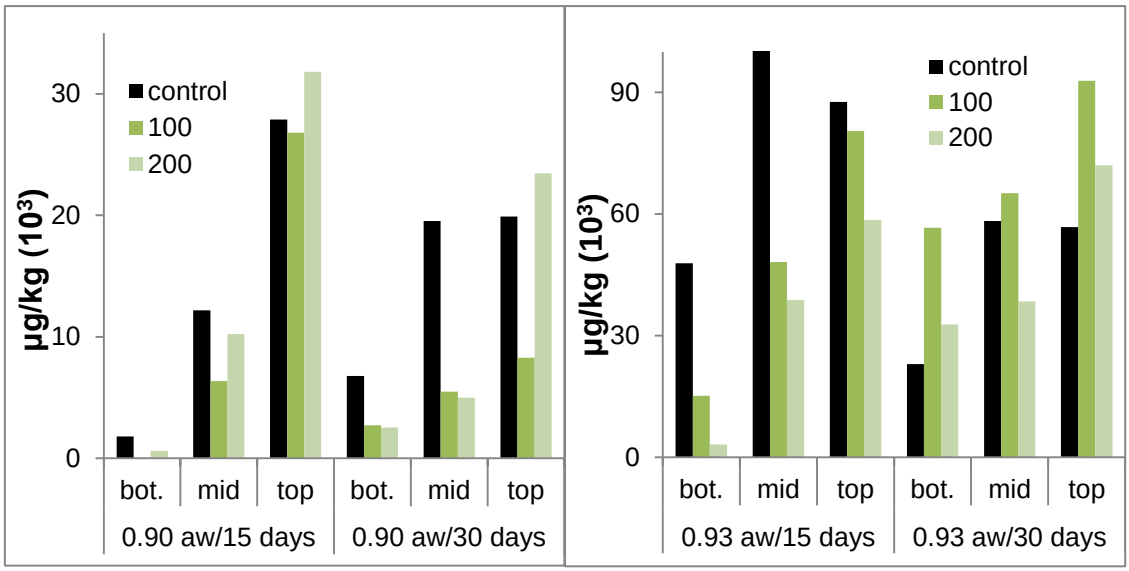


Figure 6.17: Fumonisins (B_1+B_2) production in autoclaved maize of 0.90 and 0.93 a_w inoculated with *F. verticillioides,* exposed at 0-200 ppm O_3 for 1 hour in the column system and stored at 25°C for 15 and 30 days, in relation to the location of the grain in the column during exposure (bottom, middle and top).

Figure 6.18 shows the autoclaved maize samples of the above trial inoculated with *F. verticillioides,* exposed to 0-200 ppm O_3 for 1 hour and stored at 25°C for 15 days.

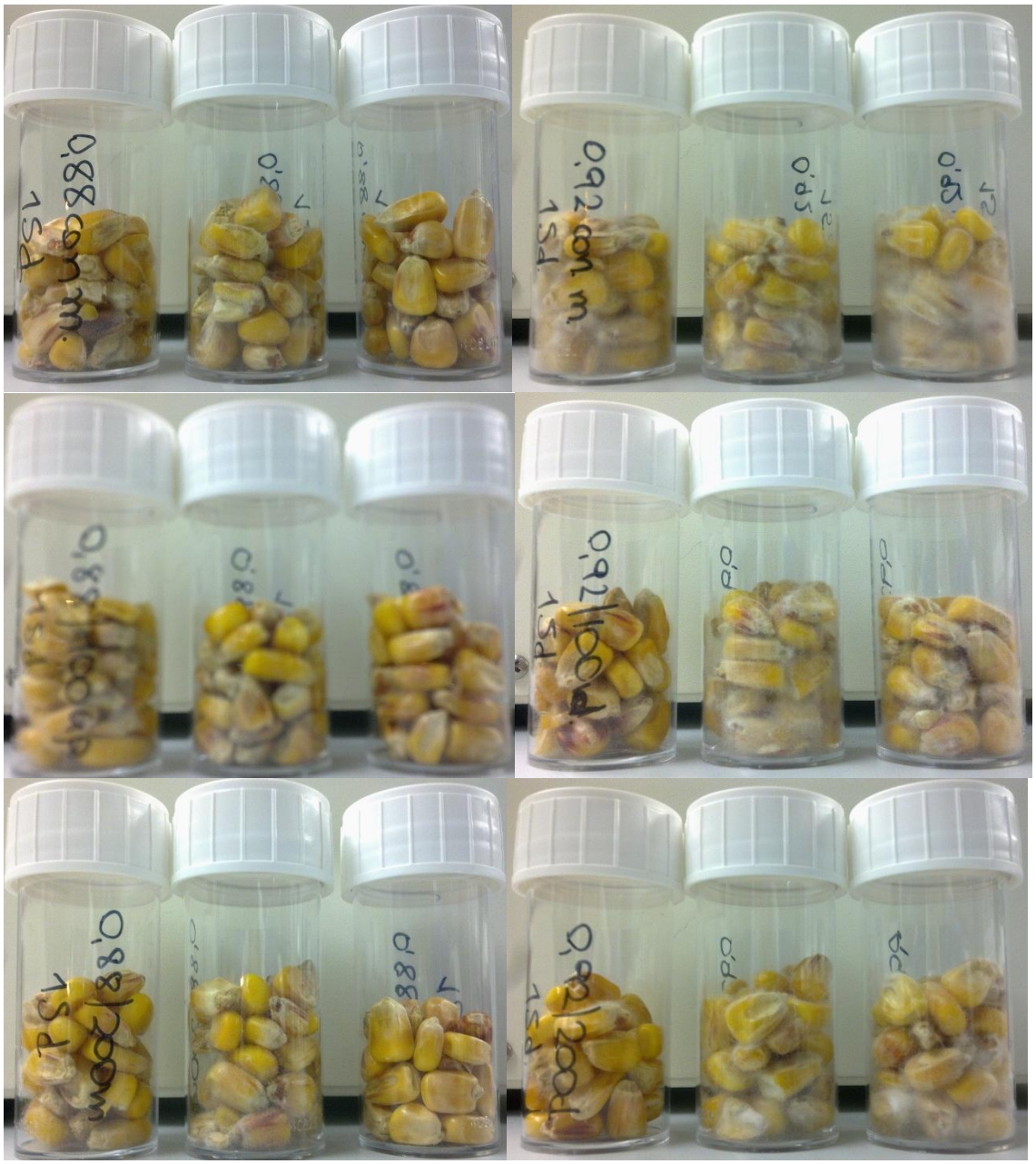


Figure 6.18: Autoclaved maize samples of 0.88 (left) and 0.92 a_w (right), inoculated with *F. verticillioides* spores and exposed to 0-200 ppm O_3 (top to bottom) for 1 hour in the column system, after 15 days storage at 25°C.

6.3.7 Fungal populations isolated from grain inoculated, exposed to O_3 and stored

Figure 6.19 shows the $\log_{CFU}$ *Penicillium* populations isolated from naturally contaminated maize rewetted to 0.88 and 0.92 a_w, inoculated with *F. verticillioides*, exposed at 100 and 200 ppm O_3 at a flow rate of 6 L/min for 1 hour in the column system and stored at 25°C for 15 and 30 days.

The *Penicillium* counts isolated from the O_3-treated samples were lower than those isolated from the control samples at all storage conditions tested.

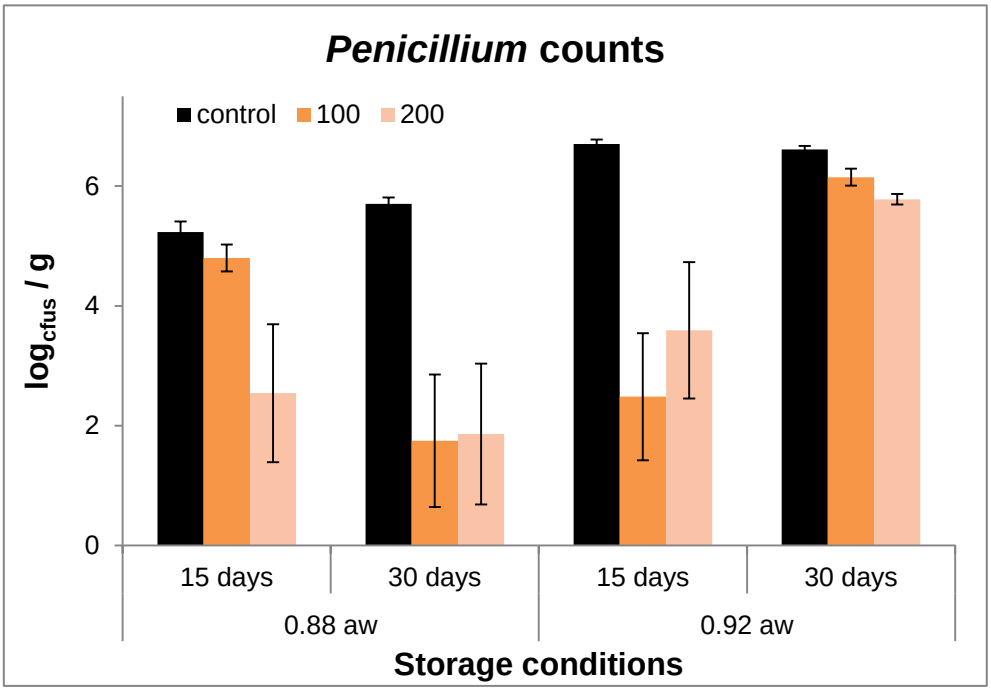


Figure 6.19: Effect of O_3 exposure at 0-200 ppm for 1 hour in the column system, at a flow rate of 6 L/min, on the $\log_{CFUs}$ of *Penicillium* species isolated from maize compared to the control maize treated with air. Vertical bars indicate the standard error of the means.

The Kruskal-Wallis test showed that the effects of O_3 concentration and maize a_w were significant on the lof_{CFU} counts isolated after 15 and 30 days storage

from the maize samples but the effect of storage time was not significant (Apx Table I.19).

Figure 6.20 shows the *F. verticillioides* populations (log_{CFU}/g) isolated from inoculated maize of 0.90 and 0.93 a_w, exposed at 0-200 ppm O_3 at a flow rate of 4 L/min for 1 hour in the column system and stored at 25°C for 15 and 30 days.

Generally, the *F. verticillioides* populations isolated from the O_3-treated samples were lower than in the controls, except of those exposed to 100 ppm O_3 and stored for 30 days where counts were slightly higher than in the controls.

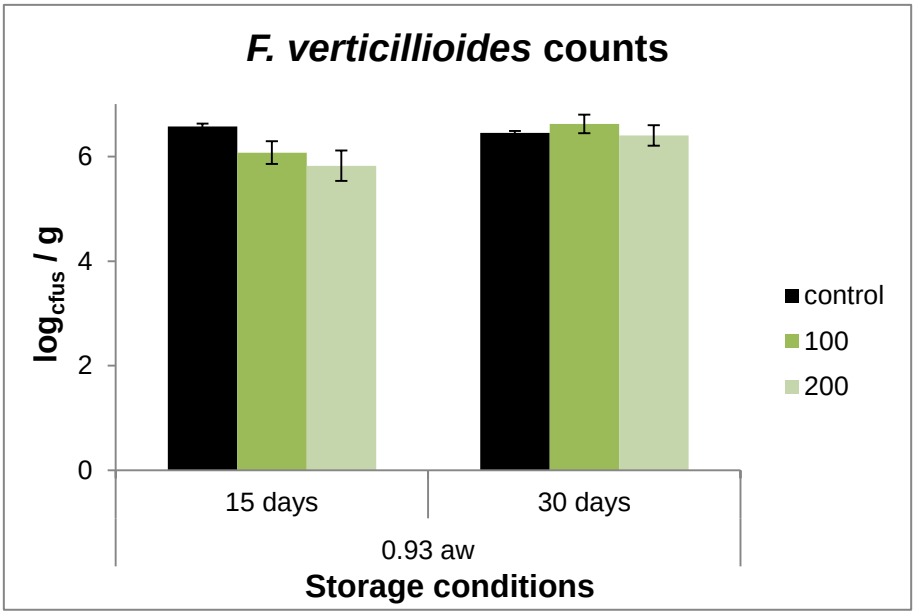


Figure 6.20: Effect of O_3 exposure to 100 and 200 ppm for 1 hour, in the column system, at a flow rate of 4 L/min, on the populations of *F. verticillioides* (log_{CFUs}/g) isolated from maize compared to the control treated with air. Vertical bars indicate the standard error of the means.

ANOVA showed that the effects of O_3 concentration, storage time and their interactions were not significant on the *F. verticillioi*des populations isolated from the maize samples exposed to O_3 in the column system and stored at 25°C for 15 and 30 days storage (Apx Table I.20).

6.4 Discussion

6.4.1 Effect of O_3 on spore germination *in vitro*

In vitro studies with two different *Fusarium* species showed that O_3 exposure at up to 200 ppm for 30 min can delay rather than inhibit spore germination on media of very high a_w (0.98) and this effect was concentration dependent. Contrarily, in drier media (0.94 a_w), spore germination was completely inhibited at the above exposure conditions. These results are comparable to those of Antony-Babu and Singleton (2011) who obtained 100% elimination of *Eurotium* spores after 2 hours exposure at 300 µmol O_3. In our study the additional effect of water stress was examined, to which Antony-Babu and Singleton (2011) surprisingly make little reference. It is likely that the a_w of their media was affected by the addition of sucrose (Dallyn and Fox, 1980), although this was not measured.

Whether the effect of O_3 is fungicidal or fungistatic has been debated by several previous studies (Hibben and Stotzky, 1969; Minas et al., 2010). The results obtained in the present studies suggest that this depends on the actual experimental conditions. Therefore, some combinations of O_3 concentration and exposure time under favourable environmental conditions (i.e. substrate a_w) may render the effect of O_3 fungicidal while it may otherwise be fungistatic.

Other factors may also influence the efficacy of the O_3 treatment. It was observed that individual spores were more sensitive to O_3, while clumps of spores could survive certain O_3 treatments, something that has also been observed previously (Hibben and Stotzky, 1969).

In one of the studies with *F. verticillioides* it was observed that mycelial fragments (accidentally contained in the inoculum) survived the O_3 treatment and grew, allowing the formation of micro-colonies which subsequently grew normally, while spore germination was inhibited. This suggests that mycelial fragments are less sensitive to the range of O_3 doses used in this study. This was also noticed by Antony-Babu and Singleton (2009) while it contradicts the conclusions of Allen et al. (2003). It appears however that in their study (Allen et

al., 2003) mycelial growth was only allowed for 16 hours at 0.98 a_w and 25°C, conditions at which limited mycelial extension would have occurred for most of the fungi commonly found in stored grain. Studies with insects have also shown that populations of different ages have different resistance to O_3 (Bell, 2000) which could be paralleled to the different resistance of spores and mycelia of fungi to O_3.

Secondary effects of O_3 on the nutrients contained in the media, rendering them toxic to the spores rather than the O_3 dose itself, has been rejected by previous studies (Hibben and Stotzky, 1969). Also drying of the media due to the O_3 flow should be excluded as spores germinated unaffected on control media exposed to air of the same flow rate.

Differences were observed in the colour of the cultures derived from O_3–exposed spores when compared to the controls treated with air (Plate 5.2). Similar observations have also been discussed by previous studies (Hibben and Stotzky, 1969; Antony-Babu and Singleton, 2009).

Overall, where complete inhibition of spore germination was not achieved, the combined effect of water stress and O_3 concentration could only extend the time to reach specific % germination by 1-2 days. The exposure time was another important factor. Reducing the exposure time could be more cost effective and with less likely side-effects on nutrients for practical applications however it was shown that the efficacy against fungal spores may be compromised.

Similar results have also been obtained for the *in vitro* germination of spores of *A. flavus* and *P. verrucosum*. Germination of these spores on media of 0.95 a_w was delayed compared to the controls with 100 and 200 ppm O_3 for 30 min while the same concentrations completely inhibited spore germination on media of 0.90 a_w (data not shown).

6.4.2 Effect of O_3 on mycelia extension *in vitro*

Exposure of actively growing cultures at 100-200 ppm O_3 for 30 min gave varying and slightly contradictory results with regard to the mycelial growth rate of two *Fusarium* species. Doubling the exposure time did not significantly improve the efficacy of the O_3 treatment, as opposed to the increased efficacy previously observed on spore germination. The maximum inhibition observed was 45% in cultures on media of 0.94 a_w exposed at 200 ppm O_3, but the results were not consistent between the two *Fusarium* strains at similar conditions. Also stimulation of fungal growth was occasionally observed in the treated samples. Similar results were also obtained in additional studies performed with actively growing cultures of *A. flavus* and *P. verrucosum* (data not shown).

Previously, Zotti et al. (2010) studied the effect of O_3 on actively growing cultures of different ages (3, 6 and 9 days) and suggested that O_3 treatment was more effective in younger cultures and that the efficacy decreased with increasing culture age. This effect was not examined in the studies presented in this Chapter. However, it has been discussed previously that mycelial fragments were not affected by certain O_3 treatments, although spore germination was completely inhibited. This suggests that earlier stages of fungal growth were more susceptible to O_3. The O_3 dose used by Zotti et al. (2010) was only 1 ppm, much lower than in our studies. Also *Aspergillus* colonies after 6 or 9 days of growth on MEA of unmodified a_w would have colonised most of the surface of a 9 cm Petri dish. Thus, these results may need to be treated with caution especially since the statistical significance of the results was not examined.

6.4.3 Effect of O_3 exposure on fungal populations isolated from grains

Two different exposure systems were used in order to compare the efficacy of O_3 on the fungal populations of naturally contaminated dry grain immediately after exposure. O_3 treatment was more efficient in reducing fungal populations when O_3 was forced through a grain column from the bottom to the top at a flow

rate of 4-6 L/min (53-87% reduction) than when diffused in an exposure chamber at the same flow rate, but allowed to settle and disperse through the grains (18% reduction). This may be due to more efficient contact with a higher number of grain kernels. Some studies have fed O_3 from the top of the storage container because this area is considered more susceptible to fungal growth due to its higher moisture content (Kells et al., 2001) while in other studies O_3 gas has been inserted from the bottom (White et al. 2010). The results obtained in the present study suggest that the exposure system may influence significantly the efficacy of the O_3 treatment.

When rewetted grain was exposed to 100 and 200 ppm O_3 for 1 h up to 100% reduction in the populations isolated was obtained, although no clear difference was observed between the two O_3 doses applied. The experimental conditions used in this study are not directly comparable with other published studies (Kells et al., 2001; Wu et al., 2006; Allen et al., 2003) but overall it appears that longer exposure times may have a more pronounced effect. Allen et al. (2003) suggested that the effect of O_3 on the fungal populations is more significant under wetter conditions although from the present study no clear difference was observed between grains adjusted to different a_w levels.

6.4.4 Effect of O_3 on the *in vitro* control of mycotoxin production

In the present study O_3 exposure at different stages of fungal growth (a) spores and (b) mycelial colonies, had a different impact on fumonisin production by *F. verticillioides*.

When culture media spread with spores were exposed to O_3 (100 and 200 ppm for 15 min), production of all four fumonisins analysed (fumonisin B_1, B_2, B_3 and hydrolysed-FB_1) was stimulated compared to the control samples treated with air. As this treatment was inefficient in completely eliminating the fungal spores, it may have imposed stress and thus caused increased toxin production by the surviving spores. Another possible explanation is that O_3 treatment may have caused the release of more nutrients from the culture media allowing the spores

to grow and produce toxins more easily, but this needs to be further examined. Exposure at 100 and 200 ppm O_3 for 30 min in media of 0.94 a_w completely inhibited spore germination and thus no colonies were formed and no toxin production occurred.

When actively growing cultures were treated with O_3 (100 and 200 ppm for 1 h) the *in vitro* production of all four toxins was generally reduced (maximum 50%) compared to the control samples treated with air. Differences were observed in the behaviour of the four fumonisins; at 0.98 a_w FB_1 and FB_2 were more inhibited by 100 ppm O_3, FB_3 was more inhibited by 200 ppm, while for hy-FB_1 no difference was observed between the two treatments. Notably, slight stimulation of FB_1 and FB_2 production was observed at 0.94 a_w after exposure to 100 ppm O_3 although 200 ppm were efficient against all four toxins (30-50% reduction).

An important observation is that irrespective of whether the O_3 exposure took place before the onset of fungal growth or in actively growing cultures, the amount of all four fumonisins produced was similar in the treated samples at 0.98 a_w. In contrast, at 0.94 a_w, toxin production by O_3-treated cultures, even though reduced compared to the control, was 3-5 times higher than that produced by spores that survived the O_3 treatments, except of hydrolysed FB_1. Further studies need to be conducted in order to explain these results.

This is the first study on *in vitro* mycotoxin production by fungal spores or fungal cultures after treatment with O_3 and subsequent incubation for up to 10 days. Studies were also carried out with spores of *A. flavus* and *P. verrucosum* spread on the surface of culture media, exposed to 0-200 ppm O_3 and stored for 10 days at 25°C (data not shown). Aflatoxin B_1 production was generally stimulated in the wettest media examined (0.95 a_w) although stimulation was lower in the samples that were treated with higher O_3 doses. In media of 0.90 a_w aflatoxin B_1 production was reduced in all the samples treated with O_3 to a maximum of 99%. Similar results were also obtained for OTA production in media of 0.95 a_w; OTA was stimulated at all O_3 doses, although stimulation was again lower at 200 ppm O_3. At 0.90 a_w OTA production was below the limit of

detection in the samples treated with ≥50 ppm O_3 (data not shown). These results are slightly different from those observed with regard to fumonisin production by *F. verticillioides* spores exposed to O_3 detailed in this Chapter. One reason may be the wider range of a_w for germination and growth of *Aspergillus* and *Penicillium* species compared to the *Fusarium* species.

6.4.5 Effect of O_3 on mycotoxin production in grains treated and stored for several days

Exposure of grain to O_3 and subsequent storage resulted in different levels of mycotoxin contamination depending on the exposure system used as well as on the fungal species present.

DON was significantly reduced (~70%) compared to the controls in wheat of 0.98 a_w after exposure to 100 ppm O_3 for 30 min in the glass jar chamber, but stimulated by treatment with 200 ppm O_3, as well as in wheat of 0.94 a_w (40-100%). In contrast, fumonisin production in maize was reduced in treated samples stored for 10 days (max ~40%) but the reduction was not directly related to the water activity of the grain or the O_3 dose applied. Thus no clear conclusions can be drawn with regard to the effects of a_w and O_3 dose on toxin production in wet grain samples treated in this set-up and stored for several days. However, it is likely that fungal populations that survived the O_3 exposure may have subsequently been able to colonise rapidly, perhaps in the absence of competing species resulting in higher mycotoxin production under favourable environmental conditions.

When O_3 exposure took place in the column system, fumonisin production was completely inhibited in maize of 0.92 a_w even after 30 days storage. OTA production in the same samples was generally inhibited and even after 30 days storage contamination was below the relevant EU legislative limits. One reason could be partial drying of the grain due to the O_3 flow which could have limited the potential for fumonisins and OTA to occur during storage. However, the sum of T-2 and HT-2 toxins in treated oat samples was inhibited 38-99% compared

to the control grain treated with air while OTA production in the same samples was 74-99.9% less than in the control.

In a trial with artificially inoculated autoclaved maize, 100 ppm O_3 were more efficient than 200 ppm in inhibiting fumonisin production in the drier samples even after 30 days. In the wetter samples treatment with increasing O_3 doses was very efficient for storage up to 15 days, while irregular results were obtained in grain stored for longer periods. It is possible that surviving *F. verticillioides* spores were able to colonise grain rapidly, producing more fumonisins when stored for longer time under relatively favourable environmental conditions. Also autoclaving may have released more nutrients that may have favoured fungal growth from the surviving spores. In this experiment a clear pattern in fumonisin production was observed depending on the location of the sample in the column. Total fumonisins were higher in the samples taken from the top of the column, less in those taken from the middle and even lower in those taken from the bottom of the column, nearer to the O_3 inlet. An explanation could be local drying of the grain nearer to the O_3 inlet due to the air flow and formation of gradient of moisture contents in the grain column, with the wettest samples observed at the top of the column. This pattern however was less obvious in the control samples, where toxin production was always lower at the bottom of the column though no significantly different between the middle and top of the column, especially in the samples stored for longer time. Surprisingly this effect was not observed to such extent in the other experiments on naturally contaminated grains. One reason for this may be the non-uniform distribution of fungal populations in naturally contaminated grains as opposed to the autoclaved maize that had been artificially inoculated and therefore fungal spores should be contained to a similar amount through the grain. Another reason may be the alteration of the inner structure of the maize kernels due to autoclaving, allowing easier movement of moisture.

Overall better control of toxin production after several days of storage was obtained in the grain samples that were exposed to O_3 in the column set-up. As

the effect of drying should be eliminated by similar treatment of the control with air, this exposure system may allow for better penetration of O_3 through the grains and thus for more efficient exposure of a larger number of grain kernels.

This is the first study to have examined mycotoxin production in wet grains treated with O_3 and stored for up to one month.

White et al. (2010) exposed maize to O_3 and stored it for 9 days and observed fungal growth first at the top of the columns. They suggested that more spores may have been eliminated by O_3 near the inlet (bottom of column). However, this cannot be explained, as the concentration of O_3 should be the same throughout the whole length of the column. What they have not considered is the possibility of drying of the grain caused due to the O_3 flow which would be most evident at the lower part of the column since this is nearer to the inlet and the development of a gradient of moisture contents causing higher water availability at the top of the column. Also if more fungal growth is associated with higher mycotoxin contamination (as we have previously shown in Chapter 2 and 3), it is likely that mycotoxin contamination at the top of their grain columns may have also been higher. However, no attempt was made in that study (White et al., 2010) to determine mycotoxin production in the grain samples. This is particularly important since localised rise of the water content of stored grain may give rise to the formation of hot spots with all the associated consequences (Lacey, 1980; Sauer, 1992; Magan et al., 2010).

7 OVERALL DISCUSSION

7.1 *Fusarium* species growth, dry matter losses and mycotoxin contamination during grain storage

The studies on the storage of wheat, maize and oats at interacting water activity and temperature conditions have shown that losses in dry matter (DMLs) caused by mycotoxigenic fungi and calculated by the CO_2 produced are significantly correlated with the mycotoxin contamination of the grains. This relationship can be used as a tool for the real time monitoring of a stored grain batch through the introduction of CO_2 sensors at various positions in grain silos. Under normal circumstances, a more or less stable CO_2 signal with time should be received, fluctuating around a baseline. This would depend on the grain moisture content and temperature. In contrast, a continuously increasing CO_2 signal with time could possibly be attributed to the onset of fungal growth. At that time management intervention should take place in order to avoid the formation of hot spots and grain contamination with mycotoxins.

The initiation of fungal colonisation of a stored grain batch has been shown to be localised due to the formation of hot spots in the grain. Thus, the use of several CO_2 sensors in a big capacity grain silo could possibly even indicate the location of the problem. This will however depend on the engineering aspects of the storage system. Nonetheless, the costs associated with designing such a highly efficient system could be balanced by the reduced costs of chemical intervention or the resulting costs of chemical analysis and even possibly of the rejection of a whole batch.

It has also been shown that the relative threshold DMLs for minimising the chances of a grain batch exceeding the EU legislative limits for mycotoxins are very small. While in wheat and maize 70-80% of the samples with 0.9-1% DMLs were above the EU limits for DON, ZEA and fumonisins, in oats 95% of the samples with 0.6% DMLs contained >100 µg/kg T-2 and HT-2 toxins which is the limit set in some parts of the world for these toxins.

This threshold level also indicates the importance of contamination of oats with T-2 and HT-2 toxins by *F. langsethiae*. This is a relatively newly identified species and information on the environmental conditions for its growth and mycotoxin production in oats has become available for the first time from the present study. It has been shown that toxin production occurs optimally at 25°C and very wet conditions while it may take place in conditions as marginal as 0.89-0.90 a_w and 20-25°C. This is of particular importance since such conditions are characteristic of the climate of Northern European countries. The results obtained in the present study also suggest that *F. langsethiae* may be the main species largely responsible for T-2 and HT-2 toxin contamination of oats in UK.

It is known that different fungal species can produce more than one mycotoxin and that naturally contaminated grains can be infected with a range of fungal species which can result in contamination with a variety of secondary metabolites. The results obtained reinforce the importance of examining grains for a range of toxic substances. It has been shown for example that fumonisin B_3 may be produced in maize in higher amounts than FB_2, depending on the environmental conditions. However, FB_3 content is not examined by most commonly used analytical methods, as the regulatory limits only consider fumonisins B_1 and B_2. Thus, high levels of FB_3 may lead to significant underestimation of the mycotoxin content of maize.

7.2 Chemical control of fungal growth and mycotoxin production in stored grains

Some of the compounds examined in Chapters 4 and 5 have similar structure to substances known for their antifungal activity. This, however, did not necessarily seem to account for similar antifungal efficacy, not even between fungi of the same species. As different mycotoxins are produced by different fungal species through different metabolic pathways which are not well understood to date, it should be expected that control strategies could not be

universal. Different compounds may affect the secondary metabolite biosynthetic pathways of *Fusarium* species in different ways.

Some promising results were obtained for some of the compounds examined, in particular for the garlic essential oils, which notably demonstrated significant effect against mycotoxin production in wet grains stored for up to 20 days even though used in very small amounts (8-200 ppm). Also aqueous fumaric and trans-cinnamic acid showed some effect against mycotoxin production *in vitro* which however was not accompanied by significant effect on fungal growth and was also species-dependent. Fumaric acid, a food additive, dissolved in ethanol, was particularly efficient in inhibiting mycotoxin production by three *Fusarium* species and *A. flavus in vitro.* Thus, further studies should be conducted to examine its efficacy as a preservative for stored grains

One very significant observation from the studies on chemical control was that ethanol, a compound widely used as a carrier for water-insoluble chemicals due to its GRAS status, was shown to stimulate the production of T-2 toxin and aflatoxins, two very potent mycotoxins. Although the stimulating effect of ethanol was usually maintained until a certain concentration of the different compounds beyond which control was achieved, inefficient coverage by the treatment may result in stimulation of mycotoxin production with significant consequences. Therefore, the carrier solvent for the application of chemical control strategies must be very carefully selected and extensively studied against a range of microorganisms likely to be present in naturally contaminated grains.

Similarities were observed between the results obtained for the acids and the garlic essential oils. Some compounds were found to affect mycotoxin production by different fungi at different concentration ranges. This is very important as on naturally contaminated grains mixed populations are encountered. In such cases, those concentrations that offer best efficacy against the different mycotoxins and minimise the chance of stimulation, in particular of the most potent ones, should be determined and used for practical applications. Also some treatments showed toxin-specific or species-specific

efficacy which may lead to uninhibited growth of the most resistant species leading thus to higher levels of the relevant toxins. Occasionally, control of some toxins was compromised or even induced, especially at higher grain a_w or after longer storage times. However, it has to be taken into consideration that some of the conditions used in these studies are far beyond safe storage and were selected in order to allow for comparisons to be made; therefore, the results obtained should not be underestimated.

The occasional stimulation of toxin production at lower concentrations denotes the importance of the uniform application of the treatment and so the method of application must be considered with caution. The need for examining alternative methods for the application of compounds intended for the preservation of grains was demonstrated; for example acids could be applied in their solid (crystalline) form rather than dissolved, as far as efficient mixing can be achieved. This may be more feasible in such grains where the empty space between different grains is smaller. Also it may be worth examining whether contact is required for control by certain compounds or whether their vapours could also be effective, in particular for the essential oils.

Nonetheless, consideration must be given to the treatments required for the removal of the applied acids or garlic essential oils prior to consumption of the treated grain, especially when high concentrations have been applied, either due to their irritating effects or due to their strong odour. For the garlic essential oils it was observed that the odour of PTS was stronger than PTSO and that it was generally stronger at higher concentrations while it appeared to fade with time. The relationship between odour and efficacy needs to be examined since they may be related and an attempt to reduce the first could also reduce the efficacy of the treatments. The odour of these compounds may not be of primary importance if they are applied to feed grains, since it may actually attract certain animal species, but it is believed that it could reduce the acceptability of grain for human consumption except than for certain applications, i.e. garlic bread.

Also before these compounds can be commercially applied, their likely side effects on certain properties of the grains must be examined. European legislation requires that feed additives have favourable effects on the treated grains as well as on the animals that consume those and on the environment (EC, 2003). Very limited studies have dealt with such aspects of chemical control and more studies in these areas are required, especially for compounds that show promising results and thus could be filed for approval. Also extensive toxicological evaluations need to be performed with regard to the acids not currently included in the lists of permitted food/feed additives for example ferulic acid and for PTSO, PTS and their mixtures as well as any likely by-products that these may decompose into after application on the grain, in order to ensure safety for humans and animals. Apart from preserving grain being non-toxic, having no effects to their nutritional value and leaving no residues to the animal tissues, Jones et al. (1974) also suggested that the compounds used as grain preservatives should follow known metabolic pathways.

Therefore extensive research must be undertaken for the most promising compounds identified before these can be accepted for general use. This should also include studies on a range of environmental conditions, against mixed populations and even on different substrates, in order to establish the most efficient concentration for each “grain-fungi-environmental conditions” system. The cost associated with the treatment of grains with the most promising chemical compounds is another factor that must be examined. It would be very interesting to compare this cost to that of alternative techniques or to that of drying the grain to a water activity level considered safe for long term storage.

7.3 Ozone gas for the control of fungal growth and mycotoxin production in stored grains

Overall considering both the results of the *in vitro* studies and of the studies conducted on grains it can be concluded that O_3 can indeed affect fungi and

thus it can be considered as a control strategy for the safe storage of grain. This effect appears to be dependent on several factors, including the characteristics of the grain (type, water activity, temperature), the parameters of the treatment (O_3 dose applied, length of exposure, exposure system) and the characteristics of the microorganisms present (species, populations, age). Also the environmental factors (ERH, temperature) under which grain is stored after O_3 exposure determines the length of safe storage as was shown in Chapters 2 and 3. However, the extent to which each of these factors affects the results obtained as well as their interactions still need to be determined.

Although several studies have examined the efficacy of O_3 in reducing fungal populations on grains, none of them has examined the effect on mycotoxin production after storage of the treated grain. The highest treatment used in the present studies was up to 200 ppm O_3 for up to 1 hour. At these conditions the production of all the mycotoxins of prior interest in this project after storage of the treated grain for up to 30 days has been significantly reduced compared to the untreated grain (up to 99%). The range of O_3 concentrations used here has been shown not to affect certain grain quality parameters (Allen et al., 2003; Wu et al., 2006).

Treatment with 200 ppm O_3 did not consistently give better efficacy than 100 ppm. Therefore, either higher O_3 concentrations or longer exposure durations need to be assessed, or even a combination of the two, in order to determine the optimum treatments, while at the same time examining the effects on quality parameters. Another option that has not been examined is repeated O_3 treatments. The results of the present studies suggest that surviving spores may grow normally after the O_3 treatment, leading to spoilage during extended storage. This, in fact, can even lead to smaller inhibition of the production of some mycotoxins, as competing fungi are eliminated by the treatment. Thus, repeated O_3 exposures could inhibit those spores or could eliminate new generations of spores produced from the surviving ones.

Overall much better control of toxin production was obtained after storage for several days following O_3 exposure in the column system than in the jar system.

This is of particular importance as use of the most effective exposure system may allow reduction of other parameters as exposure time or exposure dose, having thus less side-effects and reduced cost.

It was also observed that O_3 flow through the grain can additionally cause partial drying of the grain. This could be considered as an additional advantage since grains after harvest need to be dried for safe long-term storage.

Although several studies have focused on the kinetics of O_3 diffusion through grain (Strait, 1998; Kim et al. 1999; Kells et al., 2001; Mendez et al., 2003; Hardin et al., 2010) none of them has considered any of these points: drying effects, formation of moisture gradients, sites of reduced efficacy. Maybe different exposure techniques should be considered than *in silo*, such as mixing of the grain during exposure (White et al., 2010), exposure through more than one channels placed at different levels, or repeated exposures after rotation of the grain.

It was also observed that fungal mycelium demonstrates an increased tolerance to the effects of O_3 compared to fungal spores. This means that grains should be treated with O_3 as soon as possible after harvest, in order to avoid onset of fungal growth before exposure, especially at high moisture content levels. Also it has been suggested that O_3 treatment is more efficient when grains are wetter (Allen et al., 2003). However, this did not appear to be the case in the studies presented in this Thesis. O_3 treatment at an early stage could dry the grain and also eliminate fungal spores, insects (Kells et al., 2001; Tiwari et al., 2010) and other contamination before long term storage.

Another advantage of gaseous O_3 treatment of particular importance compared to other techniques traditionally used for the treatment of grains is that the storage silo does not need to be airtight, as opposed to modified atmosphere storage (CO_2/O_2/other gases) or fumigation. In fact, treatment could take place in one silo specifically designed for this purpose, possibly with additional structural/engineering characteristics for increased efficacy and then transferred to ordinary storage silos for long-term storage. Also O_3 treatment of grains can

offer higher control of mycotoxin contamination during subsequent storage than that reported by the above techniques, at much lower cost.

In summary, it can be suggested that treatment of relatively wet grains with O_3 has many advantages compared with other techniques traditionally used in the grain trade industry. One point of consideration that was identified is the possible formation a moisture content gradient in the grain column after O_3 exposure. However, this was observed to a bigger extent in an experiment performed with autoclaved grain, the inner structure of which may have been somehow altered due to autoclaving, affecting thus water movement during exposure. This could also be prevented by mixing the grain during exposure or by other structural characteristics in the exposure column, introduced as appropriate.

7.4 Other mycotoxins detected in the naturally contaminated grain samples

A wealth of metabolite production data has been obtained from the LC-MS/MS analysis of the grain samples from all the experiment carried out on cereal grains. These data sets will be used for the publication of wider secondary metabolite production changes under different interacting water activity and temperature conditions. The UK oats used contained, apart from T-2 and HT-2 toxins, high levels of OTA but also a total of more than 50 other metabolites. Some of the toxins detected in the oat samples included: T-2 tetraol, DON, NIV, enniatins, beauvericin, 3 nitro-propionic acid, aurofusarin, apicidin, *Alternaria* toxins, citrinin, viridicatin. The maize samples contained, apart from fumonisins, aflatoxins (B_1, B_2 and even G_1, G_2, M_1), OTA, 3 nitro-propionic acid, DON, NIV, ZEA, mycophenolic acid and viridicatin. In the wheat samples the following were detected, in addition to DON, NIV and ZEA: moniliformin, apicidin, aurofusarin, enniatins, beauvericin, culmorin and viridicatin.

The toxicological effects of some of these metabolites on humans and animals are not well known and in particular effects of mixtures containing several of

these metabolites simultaneously. There is thus a need to better understand the combined effects of such compounds and mixtures in terms of their additive or synergistic toxicological impact on humans and animals.

8 CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

8.1 Conclusions

- CO_2 production and dry matter loss data are correlated to mycotoxin contamination of grains. Thus, CO_2 measurements can be used to predict the level of mycotoxin contamination in a stored grain batch.
- EU limits for DON and ZEA in wheat and fumonisins (B_1+B_2) in maize are likely to be exceeded when 0.9-1% DMLs have been reached. In oats, if limits similar to those established in some countries of the world were considered, these could be exceeded at as little as 0.6% DMLs.
- The growth boundaries of fungal species on grains can be accurately determined using respiration data at different environmental water activity and temperature conditions. This approach, considered here for the first time, is more accurate than colony diameter measurements.
- T-2 and HT-2 toxin contamination of oats by *F. langsethiae* can occur at 0.9 a_w and 20-25°C with only microscopic growth. Therefore visible or even microscopic growth may occasionally prove inefficient as indicators of fungal spoilage in grains.
- Aqueous trans-cinnamic and fumaric acid at <200 ppm showed particular efficacy against DON production by *F. graminearum* while aqueous adipic and tartaric acids were particularly efficient against T-2 and HT-2 toxin production by *F. langsethiae*, without any significant effect on fungal growth. These results however were species-specific.
- Ethanol addition in the culture media (<4%) caused 16.5-43% inhibition of the *Fusarium* species growth compared to the non-ethanol controls but stimulated secondary metabolite production, in particular T-2 and HT-2 toxins by *F. langsethiae* and aflatoxin production by *A. flavus*.
- Ferulic, fumaric and adipic acid (dissolved in ethanol) showed some promising results in inhibiting the growth and mycotoxin production of three different *Fusarium* species *in vitro* and similar results were also observed against *A. flavus* and aflatoxin contamination.

- PTS and PTSO, two essential oils derived from garlic, showed very high efficacy in inhibiting fungal growth and mycotoxin production both *in vitro* and on grains, against introduced fungal species and against naturally occurring species.
- O_3 exposure at 200 ppm for 30 min can delay spore germination on media of 0.98 a_w but completely inhibit germination on media of 0.94 a_w. Cultures derived from O_3–treated spores had different colour than controls. Clumps of spores were more resistant to O_3 than individual spores.
- O_3 exposure of actively growing cultures at 100-200 ppm O_3 for up to 60 min did not significantly affect the growth rate of the fungi.
- O_3 exposure (100-200 ppm for 1 h) reduced fungal populations in naturally contaminated dry grain up to 87%. The exposure system may influence the efficacy of the O_3 treatment.
- Secondary metabolite production after O_3 treatment may be affected by the stage of life of the fungi (spores, mycelial colony) at the time of exposure and this is of particular importance in the treatment of naturally contaminated grains. This effect may also differ between different toxins produced by a single species.
- Mycotoxin contamination in grains of >0.9 a_w stored for up to 30 days at 25°C after O_3 exposure (≤200 ppm for 60 min) in a column system was reduced by 60-64% for fumonisins in maize, 99% for T-2 and HT-2 toxins and 95% for OTA in oats. This effect depended on the exposure system, the condition of the grain and was species-dependent.
- O_3 exposure in a column system, with the gas being flushed vertically upwards through the grain, was more efficient in controlling mycotoxin production in the grains during subsequent storage.
- O_3 exposure in a column at a flow rate of 4-6 L/min may also partially dry the grain.
- O_3 treatment of grains offers a very attractive alternative to modified atmosphere storage or fumigation, as grain can be transferred after exposure to a normal storage silo which does not have the strict

airtightness requirements of the above techniques, while at the same time fungal spores, insects and mycotoxin contamination are significantly reduced with non-significant side-effects on grain quality.

- Fungal mycelium is more resistant to O_3 and more resistant to chemical treatment compared to fungal spores. Thus grains should be treated with the preferred control strategy as soon as possible after harvest.

8.2 Suggestions for future work

Based on the findings of this study further work could include:

- CO_2 monitoring *in situ* in small grain silos containing grain of different water activity levels, for assessment of the feasibility of this technique for practical application.
- Investigation of the effect of longer O_3 treatments at relatively low concentrations (~100 ppm) and of repeated O_3 exposures (i.e. daily) on mycotoxin production in rewetted grain during post-treatment storage and simultaneous examination of secondary effects on different grain nutrients, as few such studies are available at the moment.
- Assessment of the efficacy of fumaric acid in maize for the simultaneous control of aflatoxins and fumonisins and on wheat/oats for the simultaneous control of DON and T-2 and HT-2 toxins. Examination of mixtures of fumaric, adipic and ferulic acids on different grains as such mixtures could exceed the adverse effect of ethanol observed against certain fungal species. Assessment of mixtures of aqueous fumaric and trans-cinnamic acids on wheat.
- Optimisation of the required garlic essential oils concentration for the simultaneous control of a range of mycotoxins produced by different fungal species in naturally contaminated grains. Mixtures with different proportions than (1:1) of the two oils can also be examined.
- Assessment of the antifungal and anti-mycotoxigenic efficacy of PTS and PTSO vapours that could possibly be used as fumigants, instead of

applied directly on the grain, thus leaving less residues and reducing the odour of the treated grain.

- Detailed studies on the toxicity of mixtures of mycotoxins as grains are seldom contaminated by just one toxin. Investigation of the effect of O_3 exposure on such mixtures.
- Molecular work on the expression of different genes related to mycotoxin production in order to understand the mechanism involved in mycotoxin production, control or stimulation during different control strategies.

8.3 Publications

8.3.1 Peer reviewed publications

Mylona, K., Sulyok, M. and Magan, N., 2012. Relationship between environmental factors, dry matter loss and mycotoxin levels in stored wheat and maize infected with *Fusarium* species. Food Additives and Contaminants, Part A, 29:7, 1118-1128.

Mylona, K. and Magan, N., 2011. *Fusarium langsethiae*: storage environment influences dry matter losses and T-2 and HT-2 toxin contamination of oats. Journal of Stored Products Research 47, 321-327.

Magan, N., Aldred, D., Mylona, K. and Lambert, R.J.W., 2010. Review: Limiting mycotoxins in stored wheat. Food Additives and Contaminants, Part A, 27:5, 644-650.

8.3.2 Manuscripts in preparation

Mylona, K., Medina, A. and Magan, N. An HPLC-DAD method for the simultaneous analysis of Deoxynivalenol, Nivalenol, 3-AcDON and 15-AcDON produced by *Fusarium graminearum* in synthetic wheat-based culture media. MANUSCRIPT IN PREPARATION

Ozone in the control of spore germination, fungal growth and mycotoxin production by different mycotoxigenic fungal species *in vitro*. MANUSCRIPT IN PREPARATION

Mycotoxin control in stored grains treated with ozone gas. MANUSCRIPT IN PREPARATION

PTS and PTSO garlic essential oils against fungal growth and mycotoxin production *in vitro* and *in situ* in stored grain. MANUSCRIPT IN PREPARATION

Aflatoxin contamination of hazelnuts: predicting the safe storage time and examining different control strategies. MANUSCRIPT IN PREPARATION

8.3.3 Oral presentations

Mycotoxins and quality losses in stored grain and potential control using chemicals and ozone. Postgraduate Conference, Cranfield Health, Cranfield University, UK, 23 September 2011.

Fusarium fungal species: effect of post-harvest storage conditions on contamination of grains with *Fusarium* toxins. In International Congress of Post-harvest Pathology, Lleida, Spain, 11-14 April 2011.

Quantifying losses by mycotoxigenic fungi in relation to the risk of exceeding EU mycotoxin legislative limits. In BerlinFOOD2010 European PhD Conference in Food Science and Technology, Berlin, Germany, 8-10 September 2010.

Quantifying dry matter losses post harvest: relationship between mycotoxigenic fungi, mycotoxin contamination and the risks of exceeding EU mycotoxin legislative limits. In International Mycotoxin Conference, MycoRed, 2010, Global Mycotoxin Reduction Strategies: Asia and Pacific Rim, Penang, Malaysia, 1-4 December 2010.

8.3.4 Poster presentations

Can we predict mycotoxin contamination in stored grains? Kalliopi Mylona and Naresh Magan, MYCORED North America Conference, Ottawa, Canada, 24-28 June 2012.

Ozone effects on fungal growth and mycotoxin production in stored grain. Kalliopi Mylona and Naresh Magan, MYCORED North America Conference, Ottawa, Canada, 24-28 June 2012.

Efficacy of plant extracts (PTS, PTSO) against growth and toxin production of mycotoxigenic *Aspergillus, Fusarium* and *Penicillium* species, *in vitro* and *in situ*. Kalliopi Mylona and Naresh Magan. MYCORED Africa Conference, Cape Town, South Africa, 4-6 April 2011.

Effect of environmental factors on colonisation rates, T-2 and HT-2 toxin production and dry matter losses in oats. Kalliopi Mylona and Naresh Magan. MYCORED Africa Conference, Cape Town, South Africa, 4-6 April 2011.

Quantifying losses by mycotoxigenic fungi in relation to the risk of exceeding EU mycotoxin legislative limits. Kalliopi Mylona and Naresh Magan. 6th Conference of the World Mycotoxin Forum, Noordwijkerhout, the Netherlands, 8-10 November 2010.

Relating losses by *F. graminearum* in wheat with mycotoxin contamination. Kalliopi Mylona and Naresh Magan. Cranfield Health Postgraduate Student Conference, Cranfield University, UK, September 2010.

Effect of environmental factors on mycotoxigenic fungal respiration and dry matter losses in cereal and nut matrices. Kalliopi Mylona, David Aldred and Naresh Magan. International Society of Mycotoxicology, ISM Conference 2009, Worldwide Mycotoxin Reduction in Food and Feed Chains, Tulln, Austria.

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APPENDICES

Appendix A

The relationship of Molality to a_w of aqueous glycerol solutions and to the amount of glycerol (g) per 100 ml of water for the preparation of aqueous glycerol solutions (modified from Dallyn and Fox, 1980)

Molality	a_w	g glycerol to add to 100 ml water	Molality	a_w	g glycerol to add to 100 ml water
0.1	0.998	0.921	5.5	0.901	50.652
0.2	0.996	1.842	6.0	0.892	55.256
0.3	0.995	2.763	6.5	0.884	59.861
0.4	0.993	3.684	7.0	0.875	64.466
0.5	0.991	4.605	7.5	0.867	69.07
0.6	0.989	5.526	8.0	0.858	73.675
0.7	0.987	6.447	8.5	0.850	78.28
0.8	0.986	7.368	9.0	0.841	82.884
0.9	0.983	8.289	9.5	0.833	87.489
1.0	0.982	9.209	10	0.825	92.094
1.2	0.978	11.051	11	0.809	101.303
1.4	0.975	12.893	12	0.793	110.513
1.6	0.971	14.735	13	0.777	119.722
1.8	0.967	16.577	14	0.762	128.931
2.0	0.964	18.419	15	0.747	138.141
2.5	0.955	23.023	16	0.733	147.35
3.0	0.946	27.628	17	0.719	156.56
3.5	0.937	32.233	18	0.705	165.769
4.0	0.928	36.838	19	0.691	174.978
4.5	0.919	41.442	20	0.678	184.188
5.0	0.910	46.047	22	0.650	202.606

Appendix B

Table B.1: Analysis of Variance for the effect of T, a_w and their interaction ($T \times a_w$) on the logDMLs caused in stored wheat inoculated with *F. graminearum*

Effect	SS	DF	MS	F	p
Intercept	0.270	1	0.270	334.301	**<0.001**
T	1.888	3	0.629	778.919	**<0.001**
a_w	6.509	2	3.254	4029.017	**<0.001**
$T \times a_w$	0.088	6	0.015	18.177	**<0.001**
Error	0.019	24	0.001	-	-

Table B.2: Analysis of Variance for the effect of T, a_w and their interaction $T \times a_w$ on the logDMLs caused in stored maize inoculated with *F. verticillioides*

Effect	SS	DF	MS	F	p
Intercept	1.722	1	1.722	363.458	**<0.001**
T	3.711	3	1.237	261.112	**<0.001**
a_w	4.436	2	2.218	468.189	**<0.001**
$T \times a_w$	0.115	6	0.019	4.042	0.006
Error	0.114	24	0.005	-	-

Table B.3: Kruskal-Wallis ANOVA by ranks for the effect of T and a_w on DON production in stored wheat by *F. graminearum*

Effect	Code	Valid N	Sum of Ranks	Mean Rank
T	1	9	101.000	11.222
	2	9	167.500	18.611
	3	9	180.500	20.056
	4	9	217.000	24.111
a_w	1	12	99.000	8.250
	2	12	229.000	19.083
	3	12	338.000	28.167

* T: H (3, N=36) =7.05; a_w: H (2, N=36) =21.5

Table B.4: Kruskal-Wallis ANOVA by ranks for the effect of T and a_w on ZEA production in stored wheat by *F. graminearum*

Effect	Code	Valid N	Sum of Ranks	Mean Rank
T	1	9	96.000	10.667
	2	9	193.500	21.500
	3	9	203.500	22.611
	4	9	173.000	19.222
a_w	1	12	136.000	11.333
	2	12	215.500	17.958
	3	12	314.500	26.208

* T: H (3, N=36) =8.13; a_w: H (2, N=36) =13.72

Table B.5: Kruskal-Wallis ANOVA by ranks for the effect of T and a_w on the sum of fumonisins production in stored maize by *F. verticillioides*

Effect	Code	Valid N	Sum of Ranks	Mean Rank
T	1	9	98.000	10.889
	2	9	169.000	18.778
	3	9	225.000	25.000
	4	9	174.000	19.333
a_w	1	12	101.000	8.417
	2	12	240.000	20.000
	3	12	325.000	27.083

* T: H (3, N=36) =8.19; a_w: H (2, N=36) =19.2

Appendix C

Table C.1: Kruskal-Wallis ANOVA by ranks for the effect of T and a_w on DMLs caused in stored oats by *F. langsethiae*

Effect	Code	Valid N	Sum of Ranks	Mean Rank
T	1	9	112.000	12.444
	2	9	154.000	17.111
	3	9	194.000	21.556
	4	9	206.000	22.889
a_w	1	12	78.000	6.500
	2	12	236.000	19.667
	3	12	352.000	29.333

T: H (3, N=36) =5.45; a_w: H (2, N=36) =28.4

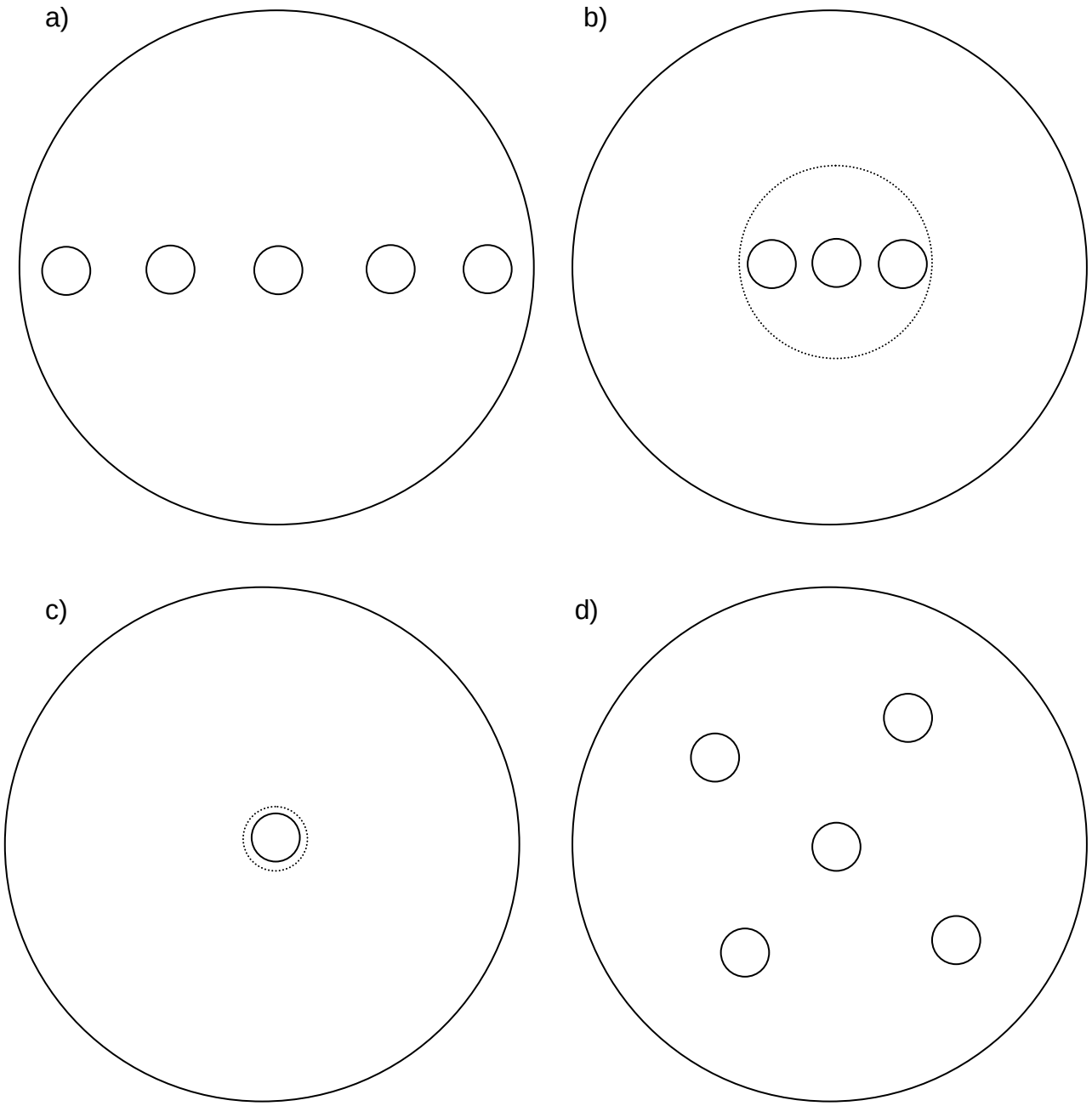
Table C.2: Kruskal-Wallis ANOVA by ranks for the effect of T and a_w on the sum of T-2 and HT-2 toxins produced in stored oats by *F. langsethiae*

Effect	Code	Valid N	Sum of Ranks	Mean Rank
T	1	9	126.000	14.000
	2	9	181.000	20.111
	3	9	209.000	23.222
	4	9	150.000	16.667
a_w	1	12	120.000	10.000
	2	12	180.000	15.000
	3	12	366.000	30.500

T: H (3, N=36)= 3.93; a_w: H (2, N=36)= 24.7

Appendix D

Patterns for cutting agar plugs from the culture media for toxin analysis a) when the size of the colony was filling the Petri dish, b) for average colony sizes, c) when the colony size was very small, d) for spread plate



Appendix E

Table E.1: ANOVA for the effect of acid concentration on the radial growth rate of *F. graminearum* in wheat agar media

	Code	Valid N	Sum of Ranks	Mean Rank
Adipic acid*	1	6	89.500	14.917
	2	6	148.000	24.667
	3	6	115.000	19.167
	4	6	76.000	12.667
	5	6	36.500	6.083

*H (4, N= 30)=15.09, p=0.005

	Effect	SS	DF	MS	F	p
Tartaric acid	**Intercept**	2940.300	1	2940.300	7992.117	**<0.001**
	ppm	7.083	4	1.771	4.813	0.005
	Error	9.198	25	0.368		
Fumaric acid	**Intercept**	3040.133	1	3040.133	13825.07	**<0.001**
	ppm	7.339	4	1.835	8.34	**<0.001**
	Error	5.497	25	0.220		
Trans-cinnamic acid	**Intercept**	3324.32	1	3324.32	7886.26	**<0.001**
	ppm	14.71	4	3.68	8.724	**<0.001**
	Error	10.54	25	0.42		

Table E.2: One way ANOVA for the effect of acid concentration on the radial growth rate of *F. langsethiae* in wheat agar media

	Effect	SS	DF	MS	F	p
***Adipic acid**	**Intercept**	16.849	1	16.849	17194.05	**<0.001**
	ppm	0.009	4	0.002	2.33	0.084
	Error	0.025	25	0.001		
Tartaric acid	**Intercept**	933.534	1	933.534	5802.553	**<0.001**
	ppm	14.056	4	3.514	21.842	**<0.001**
	Error	4.022	25	0.161		

* Calculations based on log data

	Code	Valid N	Sum of Ranks	Mean Rank
Fumaric acid*	1	6	154.000	25.667
	2	6	124.000	20.667
	3	6	52.000	8.667
	4	6	30.000	5.000
	5	6	105.000	17.500

***H** (4, N=30)=22.55, **p<0.001**

Table E.3: Kruskal-Wallis ANOVA by ranks for the effect of different concentrations of fumaric, adipic and ferulic acids dissolved in ethanol on the radial growth rate of *F. graminearum*

	Code	Valid N	Sum of Ranks	Mean Rank
Fumaric acid	1	6	165.000	27.500
	2	6	129.000	21.500
	3	6	93.000	15.500
	4	6	56.000	9.333
	5	6	22.000	3.667
Adipic acid	1	6	165.000	27.500
	2	6	129.000	21.500
	3	6	93.000	15.500
	4	6	35.000	5.833
	5	6	43.000	7.167
Ferulic acid	1	6	141.000	23.500
	2	6	105.000	17.500
	3	4	50.000	12.500
	4	4	16.000	4.000
	5	6	39.000	6.500

* fumaric: H (4, N=30)=27.73, **p<0.001**; adipic: H (4, N=30)=26.55, **p<0.001**; ferulic: H (4, N=26)=23.18, **p<0.001**

Table E.4: Kruskal-Wallis ANOVA by ranks for the effect of different concentrations of fumaric, adipic and ferulic acids dissolved in ethanol on the radial growth rate of *F. langsethiae*

	Code	Valid N	Sum of Ranks	Mean Rank
Fumaric acid	1	6	165.000	27.500
	2	6	129.000	21.500
	3	6	93.000	15.500
	4	6	57.000	9.500
	5	6	21.000	3.500
Adipic acid	1	6	165.000	27.500
	2	6	129.000	21.500
	3	6	93.000	15.500
	4	6	55.500	9.250
	5	6	22.500	3.750
Ferulic acid	1	6	165.000	27.500
	2	6	129.000	21.500
	3	6	71.500	11.917
	4	6	53.000	8.833
	5	6	46.500	7.750

* fumaric: H (4, N=30)=27.89, **p<0.001**; adipic: H (4, N=30)=27.66, **p<0.001**; ferulic: H (4, N=30)=23.35, **p<0.001**

Table E.5: Kruskal-Wallis ANOVA by ranks for the effect of different concentrations of fumaric, adipic and ferulic acids dissolved in ethanol on the radial growth rate of *F. verticillioides*

	Code	Valid N	Sum of Ranks	Mean Rank
Fumaric acid	1	6	165.000	27.500
	2	6	129.000	21.500
	3	6	93.000	15.500
	4	6	51.000	8.500
	5	6	27.000	4.500
Adipic acid	1	6	165.000	27.500
	2	6	129.000	21.500
	3	6	42.000	7.000
	4	6	42.000	7.000
	5	6	87.000	14.500
Ferulic acid	1	6	165.000	27.500
	2	6	129.000	21.500
	3	6	92.500	15.417
	4	6	37.500	6.250
	5	6	41.000	6.833

* fumaric: H (4, N=30)=27.12, **p<0.001**; adipic: H (4, N=30)=27.43, **p<0.001**; ferulic: H (4, N=30)=26.41, **p<0.001**

Table E.6: One-way ANOVA for the *in vitro* effect of acid concentration on T-2 toxin production by *F. langsethiae*

Toxin	Acid	Effect	SS	DF	MS	F	p
T-2 toxin	Adipic acid	**Intercept**	932.90	1	932.9	118.96	**<0.001**
		ppm	20.41	4	5.1	0.65	0.639
		Error	78.42	10	7.84		
	Tartaric acid	**Intercept**	973.66	1	973.66	274.95	**<0.001**
		ppm	155.51	4	38.88	10.98	0.001
		Error	35.41	10	3.54		
	Fumaric acid	**Intercept**	910.35	1	910.35	279.57	**<0.001**
		ppm	5.08	4	1.27	0.39	0.810
		Error	22.79	7	3.26		

Table E.7: Kruskal-Wallis ANOVA by ranks for the effect of adipic and tartaric acid concentration and one-way ANOVA for the effect of fumaric acid concentration on *in vitro* HT-2 toxin production by *F. langsethiae*

		Code	Valid N	Sum of Ranks	Mean Rank
HT-2 toxin	Adipic acid*	1	3	20.000	6.667
		2	3	35.000	11.667
		3	3	34.000	11.333
		4	3	19.000	6.333
		5	3	12.000	4.000
	Tartaric acid*	1	3	23.000	7.667
		2	3	30.000	10.000
		3	3	34.000	11.333
		4	3	21.000	7.000
		5	3	12.000	4.000

*Adipic acid: H (4, N=15)=7.52, p=0.111; tartaric acid: H (4, N=15)=5.37, p=0.251

		Effect	SS	DF	MS	F	p
HT-2 toxin	Fumaric acid	Intercept	5.358	1	5.358	21.884	0.001
		ppm	0.715	4	0.179	0.73	0.592
		Error	2.448	10	0.245		

Table E.8: Kruskal-Wallis ANOVA by ranks for the effect of adipic and tartaric acids concentration and one-way ANOVA for the effect of fumaric and trans-cinnamic acids concentration on *in vitro* DON production by *F. graminearum*

	Effect	Code	Valid N	Sum of Ranks	Mean Rank
DON	Adipic acid*	1	3	23.000	7.667
		2	3	18.000	6.000
		3	3	24.000	8.000
		4	3	26.000	8.667
		5	3	29.000	9.667
	Tartaric acid*	1	3	26.000	8.667
		2	3	12.000	4.000
		3	3	19.000	6.333
		4	3	29.000	9.667
		5	3	34.000	11.333

*Adipic acid: H (4, N=15)=1.1, p=0.894; tartaric acid: H (4, N=15)=4.97, p=0.291

		Effect	SS	DF	MS	F	p
DON	Fumaric acid*	Intercept	1.243	1	1.243	2.939	0.117
		ppm	2.974	4	0.743	1.759	0.214
		Error	4.227	10	0.423		
	Trans-cinnamic acid	Intercept	861.495	1	861.495	31.502	**<0.001**
		ppm	137.175	4	34.294	1.254	0.350
		Error	273.474	10	27.347		

*Calculations based on log DON data

Table E.9: One-way ANOVA for the effect of acid concentration (dissolved in ethanol) on T-2 toxin production by *F. langsethiae in vitro* when compared to the plain control and when compared to the control with added ethanol

	Acid	Effect	SS	DF	MS	F	p
Compared to plain control	**Fumaric acid**	**Intercept**	3251.217	1	3251.217	46.881	**<0.001**
		ppm	1856.788	4	464.197	6.694	0.007
		Error	693.499	10	69.350		
	Adipic acid	**Intercept**	2461.384	1	2461.384	43.892	**<0.001**
		ppm	704.999	4	176.250	3.143	0.065
		Error	560.776	10	56.078		
	Ferulic acid	**Intercept**	26358.48	1	26358.48	136.677	**<0.001**
		ppm	6313.30	4	1578.32	8.184	0.003
		Error	1928.52	10	192.85		
Compared to control with ethanol	**Fumaric acid**	**Intercept**	4819.367	1	4819.367	39.087	**<0.001**
		ppm	1592.439	4	398.110	3.229	0.060
		Error	1232.980	10	123.298		
	Adipic acid	**Intercept**	3845.804	1	3845.804	34.954	**<0.001**
		ppm	624.379	4	156.095	1.419	0.297
		Error	1100.257	10	110.026		
	Ferulic acid	**Intercept**	30539.37	1	30539.37	123.741	**<0.001**
		ppm	3436.21	4	859.05	3.481	0.05
		Error	2468.00	10	246.80		

Table E.10: ANOVA for the effect of acid concentration (dissolved in ethanol) on *in vitro* total fumonisins production by *F. verticillioides* when compared to the plain control and to the control with added ethanol

	Effect	Code	Valid N	Sum of Ranks	Mean Rank
Compared to plain control	Fumaric acid	1	3	42.000	14.000
		2	3	25.000	8.333
		3	3	17.500	5.833
		4	3	12.500	4.167
		5	3	23.000	7.667
	Adipic acid	1	3	42.000	14.000
		2	3	8.000	2.667
		3	3	25.000	8.333
		4	3	19.000	6.333
		5	3	26.000	8.667
	Ferulic acid	1	3	42.000	14.000
		2	3	19.500	6.500
		3	3	19.500	6.500
		4	3	19.500	6.500
		5	3	19.500	6.500

* adipic: H(4, N=15)=10.17, p=0.038; ferulic: H(4, N=15)=13.8, p=0.008; fumaric acid (H(4, N=15)=8.36, p=0.079)

Compared to control with ethanol	**Fumaric acid**	1	3	42.000	14.000
		2	3	25.000	8.333
		3	3	17.500	5.833
		4	3	12.500	4.167
		5	3	23.000	7.667
	Ferulic acid	1	3	42.000	14.000
		2	3	19.500	6.500
		3	3	19.500	6.500
		4	3	19.500	6.500
		5	3	19.500	6.500

* ferulic acid H (4, N=15)=13.8, p=0.008; fumaric acid: H (4, N=15)=8.36, p=0.079)

Compared to control with ethanol	**Adipic acid***	**Effect**	**SS**	**DF**	**MS**	**F**	**p**
		Intercept	3.240	1	3.240	52.794	**<0.001**
		ppm	1.435	4	0.359	5.844	0.011
		Error	0.614	10	0.061		

*Calculations based on log fumonisins data

Table E.11: ANOVA for the effects of fumaric, adipic and ferulic acids concentration (ethanolic and aqueous) on DON production by *F. graminearum in vitro* compared to plain control and to control with added ethanol

	Acid	Effect	SS	DF	MS	F	p
Compared to plain control	**Fumaric acid**	**Intercept**	2.31	1	2.31	44.269	**<0.001**
		ppm	11.611	4	2.903	55.628	0.001
		Error	0.522	10	0.052		
	Adipic acid	**Intercept**	11.527	1	11.527	35.108	**<0.001**
		ppm	6.287	4	1.572	4.787	0.02
		Error	3.283	10	0.328		
	Ferulic acid	**Intercept**	0.112	1	0.112	0.169	0.69
		ppm	13.974	4	3.494	5.277	0.015
		Error	6.621	10	0.662		
Compared to control with ethanol	**Fumaric acid**	**Intercept**	0.001	1	0.001	0.01	0.926
		ppm	3.181	4	0.795	11.911	**<0.001**
		Error	0.668	10	0.067		
	Adipic acid	**Intercept**	3.424	1	3.424	9.986	0.010
		ppm	3.650	4	0.913	2.661	0.095
		Error	3.429	10	0.343		

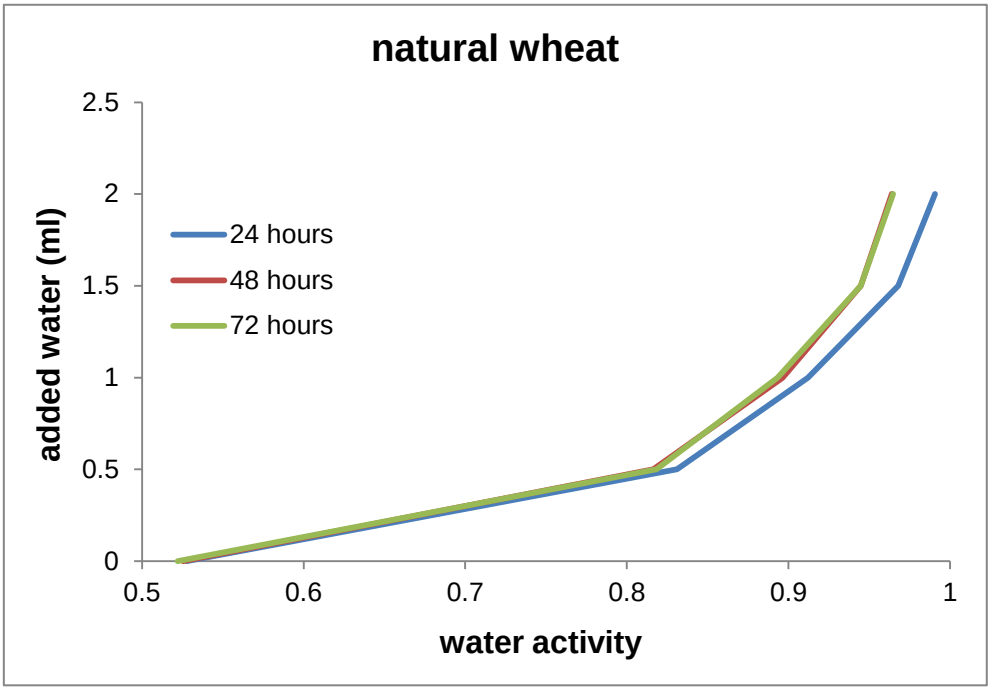
*Calculations based on log DON data

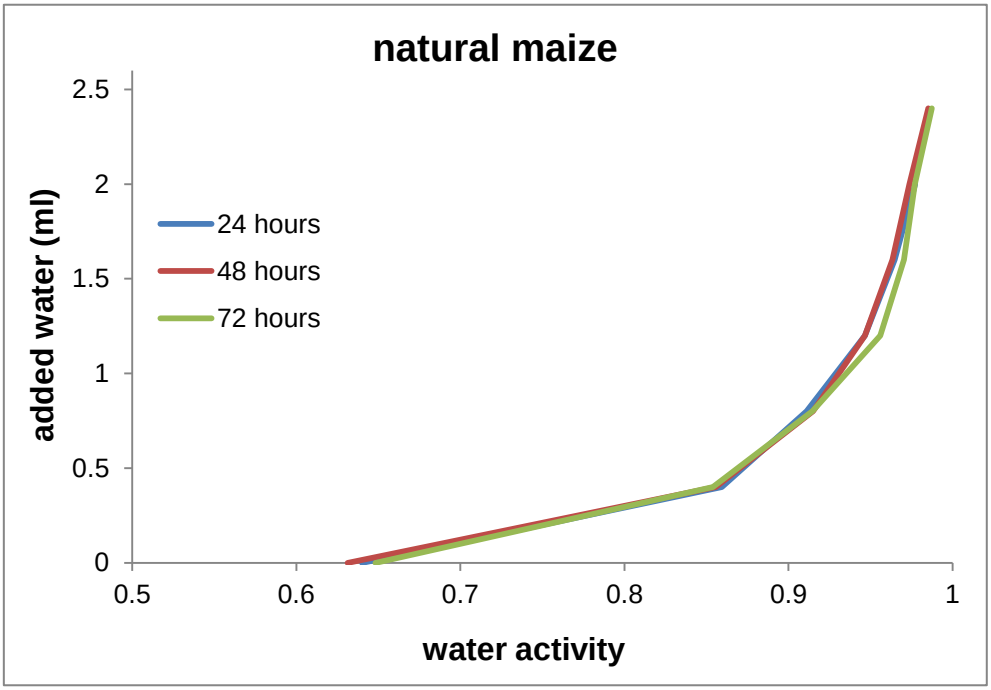
		Code	Valid N	Sum of Ranks	Mean Rank
DON	**Ferulic acid**	1	3	26.000	8.667
		2	3	31.000	10.333
		3	3	16.500	5.500
		4	3	24.500	8.167
		5	3	22.000	7.333

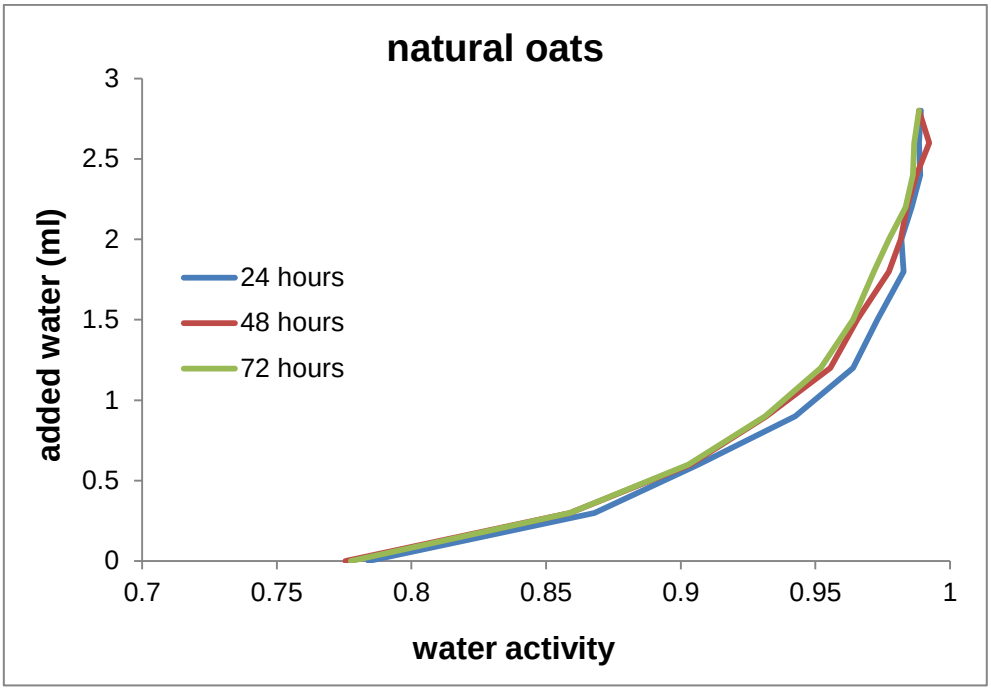
***** H (4, N=15)=1.9, p=0.755 ***** compared to control with added ethanol

Appendix F

Water adsorption curves for wheat, maize and oats in their natural state over 24, 48 and 72 hours of equilibration:






natural oats
added water (ml)
3
2.5
2
1.5
1
0.5
0
24 hours
48 hours
72 hours
0.7
0.75
0.8
0.85
0.9
0.95
1
water activity

Appendix G

Table G.1: Kruskal-Wallis ANOVA by ranks for the effects of different PTS concentrations and of fungal species on the radial growth rates of *F. graminearum, F. verticillioides* and *F. langsethiae*

	Effect	Code	Valid N	Sum of Ranks	Mean Rank
PTS	*F. graminearum*	1	6	165.000	27.500
		2	6	129.000	21.500
		3	6	93.000	15.500
		4	6	43.500	7.250
		5	6	34.500	5.750
	F. langsethiae	1	6	165.000	27.500
		2	6	129.000	21.500
		3	6	93.000	15.500
		4	6	45.000	7.500
		5	6	33.000	5.500
	F. verticillioides	1	6	150.000	25.000
		2	6	144.000	24.000
		3	6	93.000	15.500
		4	6	57.000	9.500
		5	6	21.000	3.500
	Fungal species	1	30	1427.000	47.567
		2	30	1506.000	50.200
		3	30	1162.000	38.733

* *F. graminearum*: H (4, N=30)=26.612, **p<0.001**; *F. langsethiae*: H (4, N=30)=27.647, **p<0.001**; *F. verticillioides*: H (4, N=30)=26.522, **p<0.001**; fungal species: H (2, N=90)=3.176, p=0.204

Table G.2: Kruskal-Wallis ANOVA by ranks for the effects of different PTSO concentrations and of fungal species on the radial growth rates of *F. graminearum, F. verticillioides* and *F. langsethiae*

	Effect	Code	Valid N	Sum of Ranks	Mean Rank
PTSO	***F. graminearum***	1	6	165.000	27.500
		2	6	129.000	21.500
		3	6	93.000	15.500
		4	6	54.000	9.000
		5	6	24.000	4.000
	F. langsethiae	1	6	165.000	27.500
		2	6	129.000	21.500
		3	6	93.000	15.500
		4	6	57.000	9.500
		5	6	21.000	3.500
	Fungal species	1	30	1570.000	52.333
		2	30	1580.000	52.667
		3	30	945.000	31.500

* *F. graminearum*: H (4, N=30)=27.451, **p<0.001**; *F. langsethiae*: H (4, N=30)=27.877, **p<0.001**; fungal species: H (2, N=90)=12.926, p=0.002

		Effect	SS	DF	MS	F	p
PTSO	***F. verticillioides***	**Intercept**	535.499	1	535.499	3036.918	**<0.001**
		ppm	29.963	4	7.491	42.481	**<0.001**
		Error	4.408	25	0.176		

Table G.3: ANOVA for the effect of PTSO concentration, substrate a_w, fungal species and their interactions on *in vitro* radial growth rate of *F. graminearum* and *F. langsethiae*

Effect	**SS**	**DF**	**MS**	**F**	**p**
Intercept	0.038	1	0.038	1.109	0.297
ppm	5.771	2	2.885	84.198	**<0.001**
a_w	3.232	1	3.232	94.305	**<0.001**
species	1.593	1	1.593	46.478	**<0.001**
ppm×a_w	0.167	2	0.084	2.44	0.096
ppm×species	0.002	2	0.001	0.028	0.972
a_w×species	0.010	1	0.01	0.298	0.587
ppm× a_w×species	0.166	2	0.083	2.417	0.098
Error	2.056	60	0.034		

Table G.4: ANOVA for the effect of different concentrations of aqueous PTS and PTSO on *in vitro* DON production by *F. graminearum*

		Effect	SS	DF	MS	F	p
DON	PTSO	Intercept	1192.322	1	1192.322	20.486	0.001
		ppm	309.315	4	77.329	1.329	0.325
		Error	582.029	10	58.203		

	Effect	Code	Valid N	Sum of Ranks	Mean Rank
DON	PTS	1	3	37.000	12.333
		2	3	27.000	9.000
		3	3	31.000	10.333
		4	3	19.000	6.333
		5	3	6.000	2.000

* (H (4, N=15)=9.67, p =0.046)

Table G.5: ANOVA for the effect of different concentrations of PTS and PTSO (80% ethanol solutions) on *in vitro* DON production by *F. graminearum* compared to plain control and to control with added ethanol

	Effect	Code	Valid N	Sum of Ranks	Mean Rank
Compared to plain control	PTS	1	3	60.000	20.000
		2	3	51.000	17.000
		3	3	33.000	11.000
		4	3	42.000	14.000
		5	3	15.000	5.000
		6	3	15.000	5.000
		7	3	15.000	5.000

	PTSO	1	3	34.000	11.333
		2	3	34.000	11.333
		3	3	31.000	10.333
		4	3	8.000	2.667
		5	3	25.000	8.333
		6	3	42.000	14.000
		7	3	57.000	19.000

* PTS: H (6, N=21)=19.78, p =0.003; PTSO: H (6, N=21)=11.71, p =0.069

Compared to control with ethanol	PTS	1	3	60.000	20.000
		2	3	51.000	17.000
		3	3	33.000	11.000
		4	3	42.000	14.000
		5	3	15.000	5.000
		6	3	15.000	5.000
		7	3	15.000	5.000
	PTSO	1	3	13.000	4.333
		2	3	39.000	13.000
		3	3	31.000	10.333
		4	3	13.000	4.333
		5	3	33.000	11.000
		6	3	45.000	15.000
		7	3	57.000	19.000

* PTS: H (6, N=21)=19.78, p =0.003; PTSO: H (6, N=21)=13.51, p =0.036

Table G.6: Kruskal-Wallis ANOVA by ranks for the effect of different concentrations of aqueous PTS and PTSO on *in vitro* T-2 and HT-2 toxin production by *F. langsethiae*

		Code	Valid N	Sum of Ranks	Mean Rank
PTS	T-2 toxin	1	3	40.000	13.333
		2	3	27.000	9.000
		3	3	32.000	10.667
		4	3	12.000	4.000
		5	3	9.000	3.000
	HT-2 toxin	1	3	29.000	9.667
		2	3	28.000	9.333
		3	3	21.000	7.000
		4	3	21.000	7.000
		5	3	21.000	7.000
PTSO	T-2 toxin	1	3	42.000	14.000
		2	3	15.000	5.000
		3	3	24.000	8.000
		4	3	23.000	7.667
		5	3	16.000	5.333
	HT-2 toxin	1	3	30.000	10.000
		2	3	22.500	7.500
		3	3	22.500	7.500
		4	3	22.500	7.500
		5	3	22.500	7.500

* PTS: T-2 toxin: H (4, N=15)=12.06, p=0.017; HT-2: H (4, N=15)=3.24, p=0.519
PTSO: T-2 toxin: H (4, N=15)=7.89, p=0.096; HT-2: H (4, N=15)=4.00, p=0.406

Table G.7: ANOVA for the effect of different concentrations of PTS and PTSO (80% ethanol solutions) on *in vitro* production of T-2 and HT-2 toxins by *F. langsethiae* compared to the plain control

		Code	Valid N	Sum of Ranks	Mean Rank
PTS	T-2 toxin	1	3	48.000	16.000
		2	3	53.000	17.667
		3	3	35.000	11.667
		4	3	32.000	10.667
		5	3	21.000	7.000
		6	3	21.000	7.000
		7	3	21.000	7.000
	HT-2 toxin	1	3	53.000	17.667
		2	3	48.000	16.000
		3	3	33.000	11.000
		4	3	34.000	11.333
		5	3	21.000	7.000
		6	3	21.000	7.000
		7	3	21.000	7.000
PTSO	HT-2 toxin	1	3	26.000	8.667
		2	3	16.000	5.333
		3	3	47.000	15.667
		4	3	42.000	14.000
		5	3	44.000	14.667
		6	3	39.000	13.000
		7	3	17.000	5.667

* PTS: T-2: H (6, N=21)=12.04, p=0.061; HT-2: H (6, N=21)=12, p=0.062 PTSO: HT-2: H (6, N=21)=8.91, p=0.179

		Effect	SS	DF	MS	F	p
PTSO	T-2 toxin	Intercept	5717.034	1	5717.034	48.941	**<0.001**
		ppm	1151.722	6	191.954	1.643	0.208
		Error	1635.409	14	116.815		

Table G.8: ANOVA for the effect of different concentrations of PTS and PTSO (80% ethanol solutions) on *in vitro* production of T-2 and HT-2 toxins by *F. langsethiae* compared to control with added ethanol

		Code	Valid N	Sum of Ranks	Mean Rank
PTS	T-2 toxin	1	3	60.000	20.000
		2	3	46.000	15.333
		3	3	32.000	10.667
		4	3	30.000	10.000
		5	3	21.000	7.000
		6	3	21.000	7.000
		7	3	21.000	7.000
	HT-2 toxin	1	3	60.000	20.000
		2	3	45.000	15.000
		3	3	31.000	10.333
		4	3	32.000	10.667
		5	3	21.000	7.000
		6	3	21.000	7.000
		7	3	21.000	7.000
PTSO	HT-2 toxin	1	3	56.000	18.667
		2	3	12.000	4.000
		3	3	41.000	13.667
		4	3	36.000	12.000

		5	3	38.000	12.667
		6	3	33.000	11.000
		7	3	15.000	5.000

* PTS: T-2: H (6, N=21)=15.19, p=0.019; HT-2: H (6, N=21)=14.85, p=0.021
PTSO: HT-2: H (6, N=21)=12.06, p=0.061

		Effect	**SS**	**DF**	**MS**	**F**	**p**
PTSO	**T-2 toxin**	**Intercept**	7732.691	1	7732.691	61.896	**<0.001**
		ppm	871.970	6	145.328	1.163	0.379
		Error	1749.030	14	124.931		

Table G.9: One-way ANOVA for the effect of different PTS and PTSO concentrations on *in vitro* fumonisins (B_1 and B_2) production by *F. verticillioides*

		Effect	**SS**	**DF**	**MS**	**F**	**p**
PTS	**logFB$_1$**	**Intercept**	9.169	1	9.169	933.238	**<0.001**
		ppm	2.450	4	0.613	62.346	**<0.001**
		Error	0.098	10	0.01		
	logFB$_2$	**Intercept**	1.737	1	1.737	109.022	**<0.001**
		ppm	2.365	4	0.591	37.106	**<0.001**
		Error	0.159	10	0.016		
PTSO	**FB$_1$**	**Intercept**	4216.142	1	4216.142	204.658	**<0.001**
		ppm	302.680	4	75.670	3.673	0.043
		Error	206.009	10	20.601		
	FB$_2$	**Intercept**	502.71	1	502.71	154.425	**<0.001**
		ppm	28.237	4	7.059	2.169	0.146
		Error	32.554	10	3.255		

Table G.10: ANOVA for the effect of PTSO concentration and substrate water activity on DON production by *F. graminearum*

PTSO	DON	Effect	SS	DF	MS	F	p
		Intercept	5.682	1	5.682	265.751	**<0.001**
		ppm	1.026	2	0.513	23.998	**<0.001**
		a_w	0.020	1	0.020	0.948	0.350
		ppm×a_w	0.107	2	0.053	2.492	0.124
		Error	0.257	12	0.021		

Table G.11: Kruskal-Wallis ANOVA by ranks for the effect of PTSO concentration and substrate water activity on the production of T-2 and HT-2 toxins by *F. langsethiae*

	Effect	Code	Valid N	Sum of Ranks	Mean Rank
T-2 toxin	**ppm PTSO**	1	6	81.000	13.500
		2	6	43.000	7.167
		3	6	47.000	7.833
	a_w	1	9	77.000	8.556
		2	9	94.000	10.444
HT-2 toxin	**ppm PTSO**	1	6	69.000	11.500
		2	6	45.000	7.500
		3	6	57.000	9.500
	a_w	1	9	75.000	8.333
		2	9	96.000	10.667

* T-2: a_w: H (1, N=18)=0.57, p=0.452; ppm PTSO: H (2, N=18)=5.12, p=0.077; HT-2: a_w: H (1, N=18)=0.88, p=0.349; ppm PTSO: H (2, N=18)=1.72, p=0.423.

Table G.12: ANOVA for the effects of PTSO concentration, wheat a_w, storage time and their interactions on DON production by *F. graminearum*

Effect	SS	DF	MS	F	p
Intercept	421.51	1	421.51	2273.621	**<0.001**
PTSO ppm	0.129	2	0.065	0.349	0.709
a_w	0.002	1	0.002	0.013	0.912
Storage time	3.349	1	3.349	18.064	**<0.001**
ppm×a_w	0.126	2	0.063	0.339	0.716
ppm×st.time	0.200	2	0.100	0.540	0.590
a_w×st.time	0.048	1	0.048	0.260	0.615
ppm×a_w×st.time	0.153	2	0.076	0.412	0.667
Error	4.449	24	0.185		

Table G.13: Kruskal-Wallis ANOVA by ranks for the effects of PTSO concentration, wheat a_w and storage time on zearalenone production by *F. graminearum*

	Effect	Code	Valid N	Sum of Ranks	Mean Rank
PTSO	**Concentration**	1	12	240.000	20.000
		2	12	204.500	17.042
		3	12	221.500	18.458
	Water activity	1	18	371.500	20.639
		2	18	294.500	16.361
	Storage time	1	18	187.000	10.389
		2	18	479.000	26.611

* PTSO conc.: H (2, N=36)=0.47, p=0.789, a_w: H (1, N=36)=1.48, p=0.223, storage time: H (1, N=36)=21.34, **p<0.001**

Table G.14: ANOVA for the effects of PTSO concentration, oat a_w, storage time and their interactions on logsum(T-2+HT-2) toxin production by *F. langsethiae*

Effect	SS	DF	MS	F	p
Intercept	100.503	1	100.503	1707.967	**<0.001**
PTSO ppm	0.720	2	0.360	6.119	0.007
a_w	0.459	1	0.459	7.799	0.01
Storage time	0.067	1	0.067	1.143	0.296
ppm×a_w	0.012	2	0.006	0.105	0.901
ppm×st.time	0.001	2	0.001	0.011	0.989
a_w×st.time	0.222	1	0.222	3.768	0.064
ppm×a_w×st.time	0.361	2	0.181	3.070	0.065
Error	1.412	24	0.059		

Table G.15: Kruskal-Wallis ANOVA by ranks for the effects of PTSO concentration, oat a_w and storage time on ochratoxin A production in naturally contaminated oats

	Effect	Code	Valid N	Sum of Ranks	Mean Rank
PTSO	**Concentration**	1	12	272.000	22.667
		2	12	187.000	15.583
		3	12	207.000	17.250
	Water activity	1	18	243.000	13.500
		2	18	423.000	23.500
	Storage time	1	18	224.500	12.472
		2	18	441.500	24.528

* PTSO concentration: H (2, N=36)=2.97, p=0.227, a_w: H (1, N=36)=8.11, p=0.004, storage time: H (1, N=36)=11.79, **p<0.001**

Table G.16: ANOVA for the effects of aqueous PTS concentration, wheat a_w, storage time and their interactions on logDON production by *F. graminearum*

Effect	SS	DF	MS	F	p
Intercept	368.66	1	368.66	887.676	**<0.001**
PTS ppm	3.761	3	1.254	3.019	0.044
a_w	13.558	1	13.558	32.644	**<0.001**
Storage time	3.032	1	3.032	7.300	0.011
ppm×a_w	2.012	3	0.671	1.615	0.205
ppm×st.time	2.748	3	0.916	2.205	0.107
a_w×st.time	0.001	1	0.001	0.002	0.963
ppm×a_w×st.time	0.455	3	0.152	0.365	0.779
Error	13.289	32	0.415		

Table G.17: Kruskal-Wallis ANOVA for the effects of PTS concentration, wheat a_w and storage time on zearalenone production by *F. graminearum*

	Effect	Code	Valid N	Sum of Ranks	Mean Rank
PTSO	**Concentration**	1	12	343.000	28.583
		2	12	283.000	23.583
		3	12	279.000	23.250
		4	12	271.000	22.583
	Water activity	1	24	498.000	20.750
		2	24	678.000	28.250
	Storage time	1	24	420.000	17.500
		2	24	756.000	31.500

* PTS concentration: H (3, N=48)=1.39, p=0.707, grain a_w: H (1, N=48)=3.44, p=0.064, storage time: H (1, N=48)=12, **p<0.001**

Table G.18: ANOVA for the effects of aqueous PTS concentration, oat a_w, storage time and their interactions on log(T-2+HT-2) toxin production by *F. langsethiae*

Effect	**SS**	**DF**	**MS**	**F**	**p**
Intercept	113.097	1	113.097	502.321	**p<0.001**
PTS ppm	0.106	3	0.035	0.157	0.924
a_w	0.451	1	0.451	2.002	0.167
Storage time	0.464	1	0.464	2.062	0.161
ppm×a_w	1.011	3	0.337	1.497	0.234
ppm×st.time	0.993	3	0.331	1.47	0.241
a_w×st.time	0.997	1	0.997	4.427	0.043
ppm×a_w×st.time	0.716	3	0.238	1.060	0.38
Error	7.205	32	0.225		

Table G.19: Kruskal-Wallis ANOVA by ranks for the effects of aqueous PTS concentration, oat a_w and storage time on ochratoxin A production in naturally contaminated oats

	Effect	Code	Valid N	Sum of Ranks	Mean Rank
PTS	**Concentration**	1	12	379.000	31.583
		2	12	313.000	26.083
		3	12	257.000	21.417
		4	12	227.000	18.917
	Water activity	1	24	616.500	25.688
		2	24	559.500	23.313
	Storage time	1	24	455.000	18.958
		2	24	721.000	30.042

* Concentration: H (3, N= 48)=5.895, p=0.117, a_w: H (1, N= 48)=0.359, p=0.549, storage time: H (1, N= 48)=7.757, p=0.005

Table G.20: ANOVA for the effect of aqueous PTS concentration, maize a_w, storage time and their interactions on the logarithm of total fumonisins (B_1+B_2) production by *F. verticillioides*

Effect	SS	DF	MS	F	p
Intercept	919.250	1	919.250	19109.64	**<0.001**
PTS ppm	2.538	3	0.846	17.59	**<0.001**
a_w	15.785	1	15.785	328.15	**<0.001**
Storage time	1.011	1	1.011	21.01	**<0.001**
ppm×a_w	0.637	3	0.212	4.41	0.011
ppm×st.time	0.102	3	0.034	0.71	0.555
a_w×st.time	1.280	1	1.280	26.61	**<0.001**
ppm×a_w×st.time	0.137	3	0.046	0.95	0.428

Error	1.539	32	0.048		

Table G.21: Kruskal-Wallis ANOVA by ranks for the effects of aqueous PTS:PTSO mixture concentration, wheat a_w and storage time on deoxynivalenol production by *F. graminearum*

	Effect	Code	Valid N	Sum of Ranks	Mean Rank
PTS:PTSO mixture	**Concentration**	1	12	344.000	28.667
		2	12	324.000	27.000
		3	12	229.000	19.083
		4	12	279.000	23.250
	Water activity	1	24	611.000	25.458
		2	24	565.000	23.542
	Storage time	1	24	421.000	17.542
		2	24	755.000	31.458

* Mixture concentration: H (3, N=48)=3.34, p=0.342; a_w: H (1, N=48)=0.225, p=0.635, storage time: H (1, N=48)=11.86, **p<0.001**

Table G.22: Kruskal-Wallis ANOVA by ranks for the effects of aqueous PTS:PTSO mixture concentration, wheat a_w and storage time on zearalenone production by *F. graminearum*

	Effect	Code	Valid N	Sum of Ranks	Mean Rank
PTS:PTSO mixture	**Concentration**	1	12	379.0000	31.58333
		2	12	299.5000	24.95833
		3	12	238.5000	19.87500
		4	12	259.0000	21.58333
	Water activity	1	24	624.0000	26.00000

		2	24	552.0000	23.00000
	Storage time	1	24	444.0000	18.50000
		2	24	732.0000	30.50000

* Mixture concentration: H (3, N=48)=4.92, p=0.178; a_w: H (1, N=48)=0.551, p=0.458, storage time: H (1, N=48)=8.817, p=0.003

Appendix H

H.1 Trials on chemical control of *A. flavus* growth and aflatoxin production

Some trials on chemical control of fungal growth and toxin production were performed with an aflatoxigenic strain of *Aspergillus flavus* in order to examine whether results comparable to those obtained with *Fusarium* species could be obtained. Also this would allow for comparisons with published literature since most studies on chemical control focus on aflatoxins due to the fact that these mycotoxins are carcinogenic to humans.

H.1.1 Methods and materials

Hazelnuts without the shell were supplied by MYCORED partners in TUBITAK MAM, Marmara Research Centre, Turkey. Water adsorption curves were prepared for the nuts in the natural state as described in Section 2.2.2 and they were used for the *in situ* experiments. The preliminary *in vitro* screening for the efficacy of the chemical compounds of Section 4.2.1 was performed on wheat agar media, while further trials were performed on 2% hazelnut agar media prepared as described in Section 4.2.2.

Aspergillus flavus isolate 1217 supplied by TUBITAK MAM, Turkey was used in these studies. Spore suspensions of *A. flavus* were used as inoculum for both *in vitro* and *in situ* trials. These were prepared in Tween 80 solution from 10 day old cultures as described in Section 2.2.3 and the concentration was adjusted by dilution to 5×10^7 spores/ml using a haemocytometer. 10 µl spore suspension were used to centrally inoculated media. The radial growth rates of the colonies formed were determined as in Section 4.2.4. On the 10^{th} day of incubation agar plugs were cut out from each of the replicate plates, they were placed in 2 ml safe-lock Eppendorf® tubes, their weight was recorded and they were frozen at -40°C for subsequent toxin analysis.

The extraction and analysis of aflatoxins was performed as follows: 1 ml chloroform was added to the thawed agar plugs in the Eppendorf® tubes and they were shaken for 1 hour in an orbital shaker. The upper layer was discarded and the chloroform was evaporated to dryness. The residue was derivatised according to the method of the Association of Analytical Chemists (AOAC, 2005). 200 µl HPLC grade hexane were added to the residue followed by 50 µl Trifluoroacetic acid (TFA) and the mixture was vortexed for 30 sec. The samples were left to stand for 5 min and subsequently 950 µl H_2O:AcN (9:1) were added and the mixture was vortexed for 30 sec. After 10 min the upper layer was discarded and the extracts were filtered through 0.2 µm Millipore filter (Minisart, Sartorius) directly into amber HPLC vials. The samples were analysed by an Agilent 1100 HPLC equipped with a fluorescence detector (Millipore Waters, Corp., Milford, MA, USA) (excitation: 333 nm, emition: 460 nm). The column used was a C_{18} Luna Spherisorb ODS2 150 x 4.6 mm, 5 µm (Water Corp.) The analysis was performed at the isocratic mode with a mobile phase of Methanol:H_2O:AcN (30:60:10) and 1 ml/min flow rate.

For the *in situ* trials hazelnuts were rewetted according to water adsorption curves prepared and modified to the required concentrations as in Section 2.2.4.

H.1.2 Results

Figure H.1 shows the effect of 0-200 ppm of adipic, tartaric, fumaric and trans-cinnamic acids and PTS and PTSO on the *in vitro* radial growth rate of *A. flavus.*

It can be observed that all four acids used had almost no effect on the growth rate of *A. flavus,* while in fact low concentrations (10-50 ppm) caused slight stimulation to the growth rate. Contrarily, concentrations as low as 50 ppm PTS and PTSO were able to completely inhibit the growth of *A. flavus in vitro*.

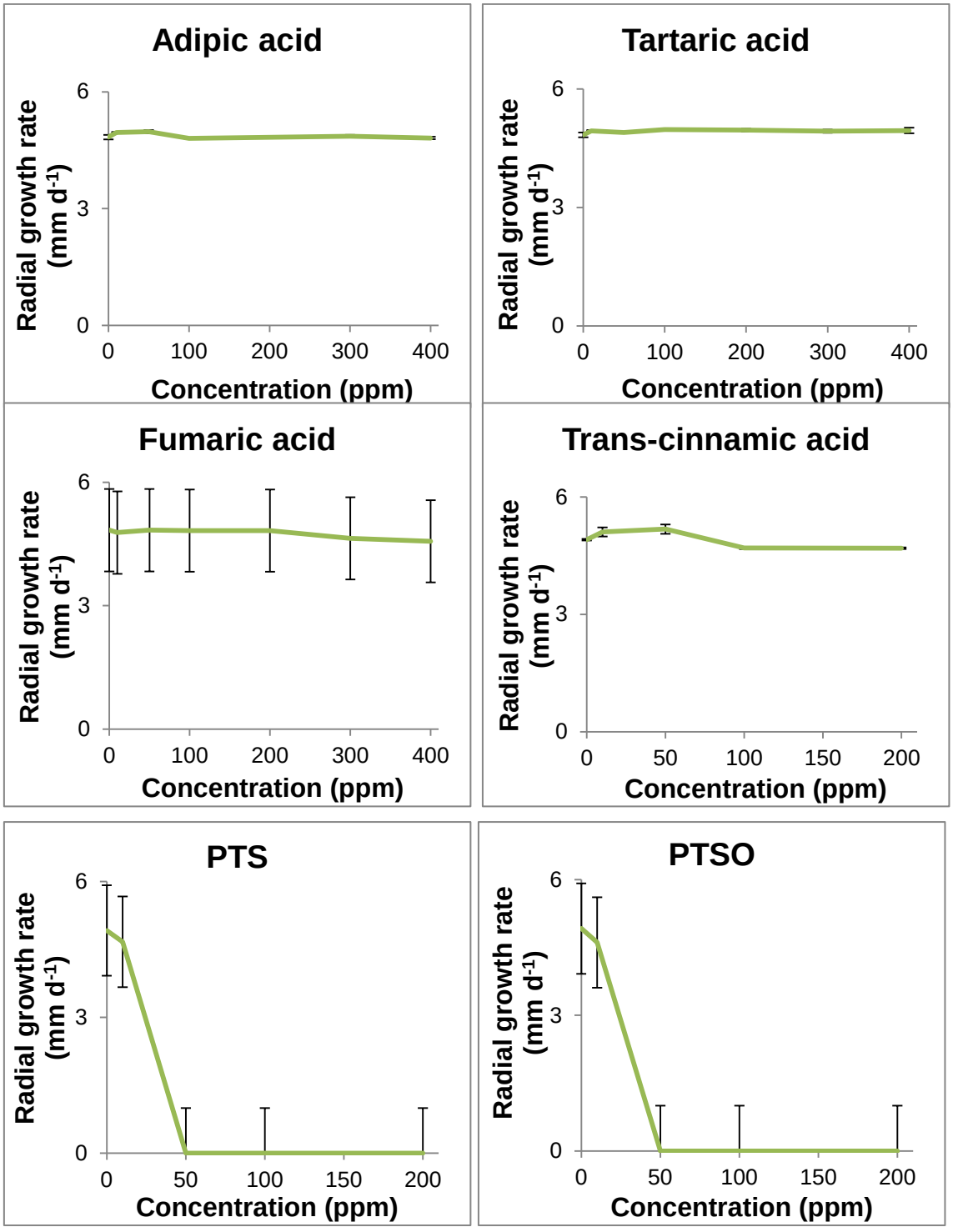


Figure H.1: *In vitro* effect of 0-200 ppm of adipic, tartaric, fumaric and trans-cinnamic acids, PTS and PTSO on the radial growth rate of *A. flavus.* Vertical bars indicate the standard error of the means.

Figure H.2 shows the effect of different concentrations of fumaric, adipic and ferulic acids on the *in vitro* radial growth rate of *A. flavus.*

Increasing concentrations of all three acids caused increasing inhibition to the radial growth rate of *A. flavus* to a maximum of 49, 46 and 68% compared to the plain control from left to right (or 39, 36 and 62% respectively, compared to the control with added ethanol).

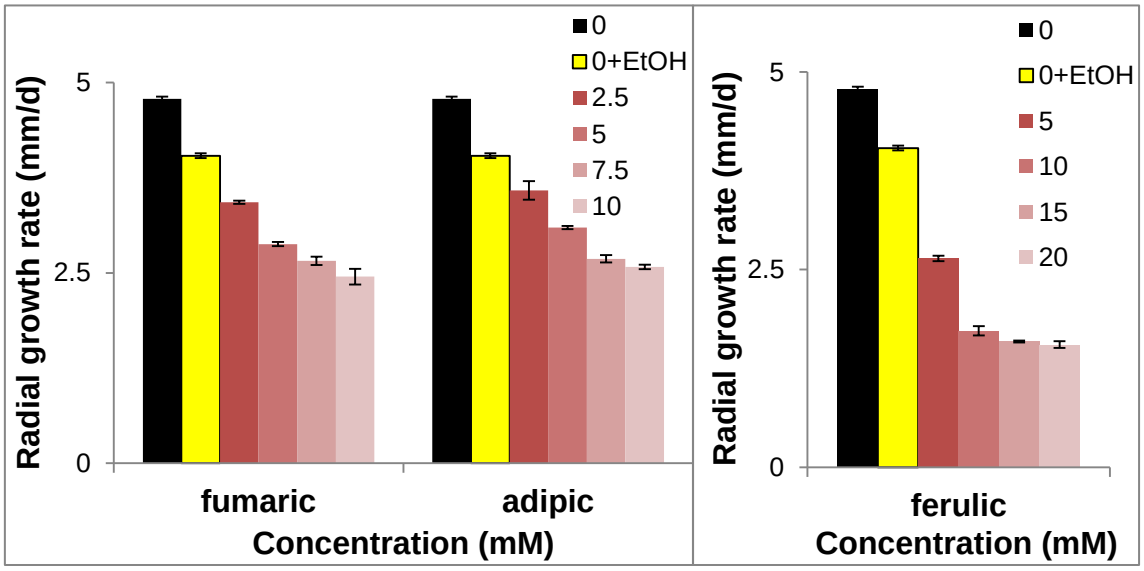


Figure H.2: *In vitro* effect of different concentrations of fumaric, adipic and ferulic acids on the radial growth rate of *A. flavus.* Vertical bars indicate the standard error of the means.

Figure H.3 shows the effect of 0-100 ppm PTSO on the *in vitro* radial growth rate of *A. flavus* in media of different a_w levels.

Growth of *A. flavus* was completely inhibited by 10 ppm PTSO at 0.87 a_w, while at 0.93 a_w 25 ppm PTSO were required for complete inhibition of *A. flavus* growth.

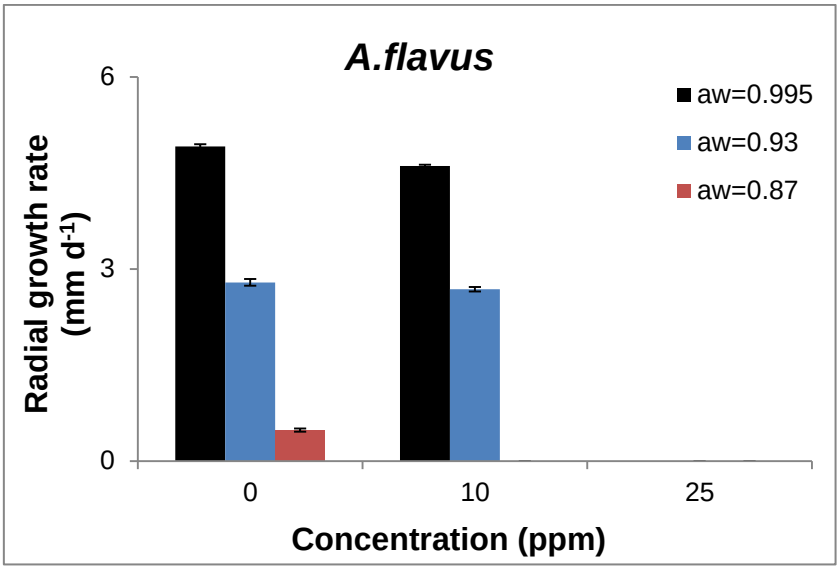


Figure H.3: Effect of different concentrations of PTSO on the *in vitro* radial growth rate of *A. flavus* in wheat agar media of different a_w levels. Vertical bars indicate the standard error of the means.

Table H.1 summarises the ED_{50} values (effective dose for 50% growth inhibition) of PTS and PTSO for *A. flavus in vitro* in wheat agar media of certain a_w levels at 25°C.

Table H.1: ED_{50} values of PTS and PTSO for *A. flavus in vitro* in wheat agar media of certain a_w levels at 25°C

A. flavus	**PTS (0.995 a_w)**	**PTSO (0.995 a_w)**	**PTSO (0.93 a_w)**	**PTSO (0.87 a_w)**
ED_{50} (ppm)	29	29	17	4.9

Figure H.4 shows the effect of different concentrations of adipic, fumaric and tartaric acids on *in vitro* aflatoxin B_1 production by *A. flavus*.

Adipic acid stimulated aflatoxin B_1 production with increasing concentration, tartaric acid stimulated aflatoxin B_1 production at 300 ppm but to a lesser extent at 400 ppm, fumaric acid inhibited aflatoxin B_1 production to a maximum of 36% compared to the control at 400 ppm while trans-cinnamic acid at 10 ppm caused 43% inhibition of aflatoxin B_1 though stimulation was observed with higher concentrations.

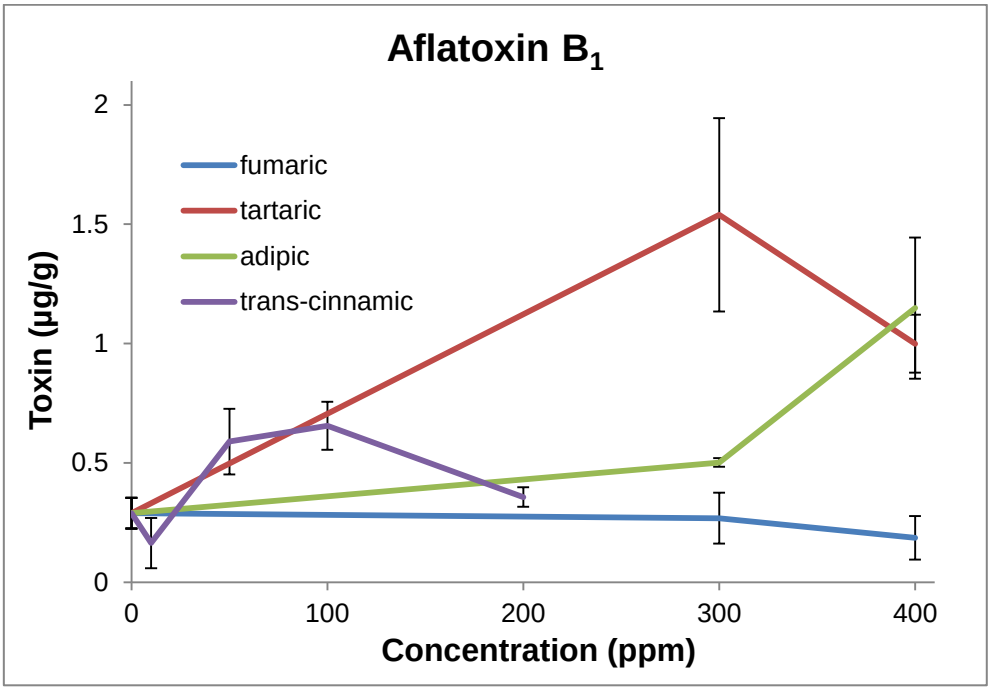


Figure H.4: Effect of different concentrations of adipic, fumaric, tartaric and trans-cinnamic acids on *in vitro* aflatoxin B_1 production by *A. flavus*. Vertical bars indicate the standard error of the means.

Figure H.5 shows the effect of different concentrations of ethanolic solutions of fumaric, adipic and ferulic acids on *in vitro* aflatoxin B_1 production by *A. flavus*.

2.5 mM of fumaric and adipic acid had no significant effect on aflatoxin B_1 production compared to the plain control but higher amounts caused inhibition to a maximum of 96.5% for fumaric acid (at 7.5 mM – 870 ppm), 89.5% for

adipic acid (at 10 mM – 1461 ppm) and 97-98% for ferulic acid at all concentrations compared to the plain control.

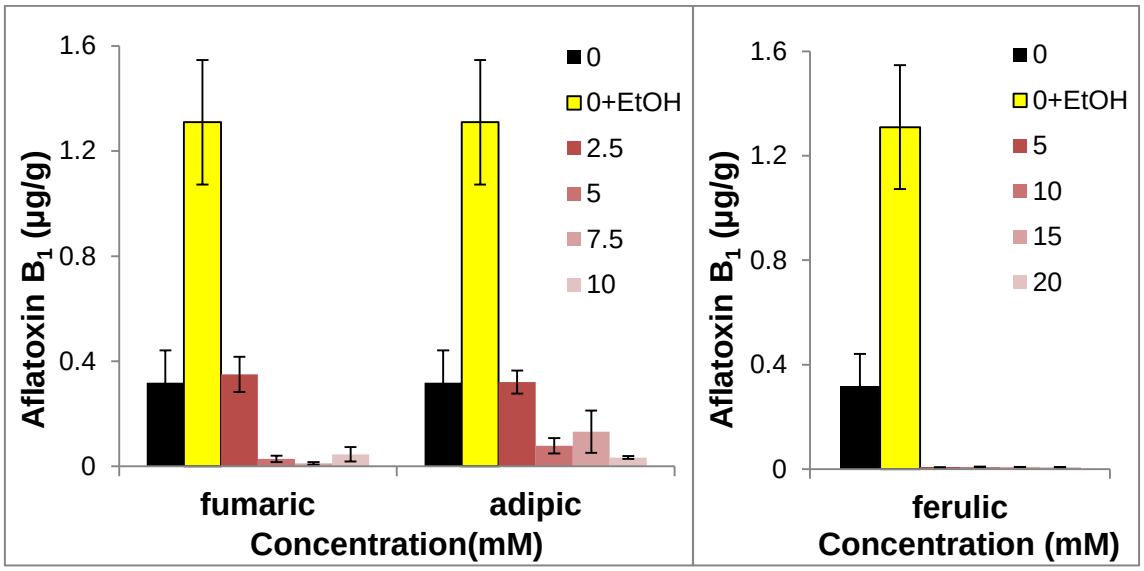


Figure H.5: Effect of different concentrations of fumaric, adipic and ferulic acids dissolved in ethanol on *in vitro* aflatoxin B_1 production by *A. flavus*. Vertical bars indicate the standard error of the means.

The addition of ethanol in the media stimulated aflatoxin B_1 production by *A. flavus* in the control samples (>300%).

Figure H.6 shows the effect of different concentrations of PTSO and PTS on *in vitro* aflatoxin B_1 production by *A. flavus*.

Aflatoxin B_1 production was reduced compared to the control with 10 ppm aqueous PTS and PTSO while it was completely inhibited with higher concentrations of the two compounds.

The addition of ethanol to the media stimulated aflatoxin B_1 production compared to the plain control. PTSO at 25 ppm (dissolved in ethanol) stimulated aflatoxin B_1 production compared to the control (>4 times), while

higher concentrations completely inhibited toxin production. Aflatoxin B_1 was not produced at any concentration of PTS dissolved in ethanol.

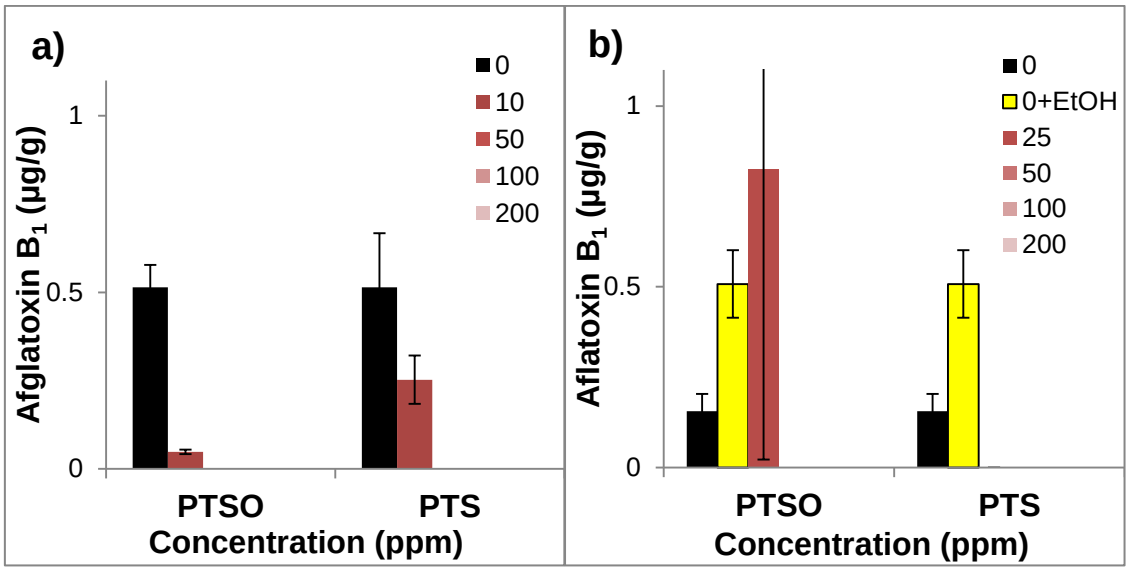


Figure H.6: Effect of different concentrations of PTSO and PTS dissolved in (a) water and (b) ethanol (100%) on *in vitro* aflatoxin B_1 production by *A. flavus* at 25°C. Vertical bars indicate the standard error of the means.

Figure H.7 shows the effect of different concentrations of ethanolic solutions of ferulic acid on aflatoxin B_1 production by *A. flavus* on hazelnuts rewetted to two water activity levels and stored for 10 and 20 days at 25°C.

Aflatoxin B_1 production was reduced in all the hazelnut samples of 0.90 a_w treated with ferulic acid compared to the controls and in fact was below the EU limits in the samples stored for 10 days for both treatments. The maximum inhibition of aflatoxin B_1 was observed in the samples of 0.90 a_w stored for 10 days and this was ~64% for the hazelnuts treated with 12.5 mM ferulic acid and ~98% for those treated with 25 mM ferulic acid. In the 0.90 a_w samples stored for 20 days the inhibitions were 20 and 49.5% respectively, compared to the control. In the hazelnuts of 0.95 a_w aflatoxin B_1 production was stimulated in all

the treated samples compared to the control, though 25 mM ferulic acid seemed to cause less stimulation.

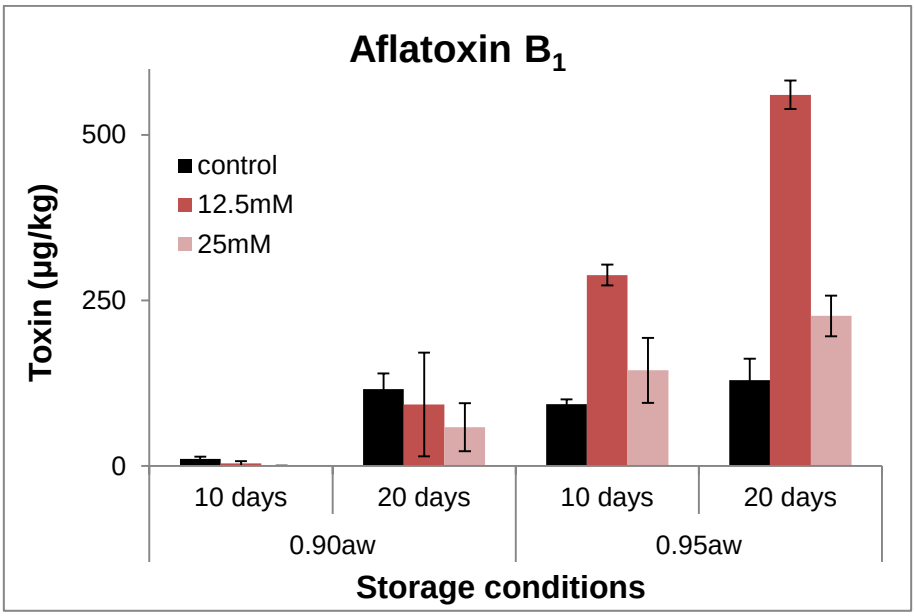


Figure H.7: Effect of different concentrations of ferulic acid dissolved in ethanol on aflatoxin B_1 production by *A. flavus* on hazelnuts rewetted to 0.90 and 0.95 a_w and stored for 10 and 20 days at 25°C. Vertical bars indicate the standard error of the means.

The Kruskal-Wallis ANOVA by ranks showed that the effect of ferulic acid concentration was not significant on aflatoxin B_1 production by *A. flavus* in the hazelnuts but the effect of the water activity of the nuts was highly significant and the effect of the storage time was significant (Table H.2).

Table H.2: Kruskal-Wallis ANOVA by ranks for the effects of ferulic acid concentration, hazelnut a_w and storage time on aflatoxin B_1 production by *A. flavus*

	Effect	Code	Valid N	Sum of Ranks	Mean Rank
Ferulic acid	**Concentration**	1	12	202.000	16.833
		2	12	266.000	22.167
		3	12	198.000	16.500
	Water activity	1	18	202.000	11.222
		2	18	464.000	25.778
	Storage time	1	18	265.000	14.722
		2	18	401.000	22.278

* concentration: H (2, N=36)=2.19, p=0.335; hazelnuts a_w: H (1, N=36)=17.18, **p<0.001**; storage time: H (1, N=36)=4.63, p=0.031

Appendix I

Table I.1: Kruskal-Wallis ANOVA by ranks for the effect of O_3 concentration and substrate a_w on *F. graminearum* spore germination *in vitro*

Effect	Code	Valid N	Sum of Ranks	Mean Rank
O_3 (ppm)	1	8	180.000	22.500
	2	8	116.000	14.500
	3	8	116.000	14.500
	4	8	116.000	14.500
a_w	1	16	360.000	22.500
	2	16	168.000	10.500

* O_3: H (3, N=32) =6.2, p= 0.102; a_w: H (1, N=32) =18.26, **p<0.001**

Table I.2: Kruskal-Wallis ANOVA by ranks for the effect of O_3 concentration, substrate a_w and exposure duration on the *in vitro* germination of *F. verticillioides* spores

Effect	Code	Valid N	Sum of Ranks	Mean Rank
O_3 (ppm)	1	16	584.000	36.500
	2	16	584.000	36.500
	3	16	520.000	32.500
	4	16	392.000	24.500
a_w	1	32	1104.000	34.500
	2	32	976.000	30.500
Exposure duration	1	32	912.000	28.500
	2	32	1168.000	36.500

***** O_3: H (3, N=64)=13.5, p=0.004; a_w: H (1, N=64)=2.25, p=0.134; storage time: H (1, N=64)=9, p=0.003

Table I.3: Kruskal-Wallis ANOVA by ranks for the effect of O_3 concentration and substrate a_w on the *in vitro* radial growth rate of *F. graminearum* actively growing cultures after exposure to 0-200 ppm O_3

Effect	Code	Valid N	Sum of Ranks	Mean Rank
O_3 (ppm)	1	8	165.000	20.625
	2	8	122.500	15.313
	3	8	133.500	16.688
	4	8	107.000	13.375
a_w	1	16	392.000	24.500
	2	16	136.000	8.500

* O_3: H (3, N=32)=2.57, p=0.463; a_w: H (1, N=32)=23.31, **p<0.001**

Table I.4: Kruskal-Wallis ANOVA by ranks for the effect of O_3 concentration, substrate a_w and exposure duration on the *in vitro* radial growth rate of *F. verticillioides* actively growing cultures after exposure to 0-200 ppm O_3

Effect	Code	Valid N	Sum of Ranks	Mean Rank
O_3 (ppm)	1	16	433.500	27.094
	2	16	410.000	25.625
	3	16	332.500	20.781
a_w	1	24	876.000	36.500
	2	24	300.000	12.500
Exposure duration	1	24	703.000	29.292
	2	24	473.000	19.708

* O_3: H (2, N=48)=1.782, p=0.41; a_w: H (1, N=48)=35.283, **p<0.001**; exposure duration: H (1, N=48)=5.626, p=0.018

Table I.5: Kruskal-Wallis ANOVA by ranks for the effect of O_3 dose and substrate a_w on the *in vitro* production of fumonisins B_1, B_2 and B_3 after 15min exposure of *F. verticillioides* spores to 0-200 ppm O_3

Toxin	Effect	Code	Valid N	Sum of Ranks	Mean Rank
Fumonisin B_1	**O_3 (ppm)**	1	8	76.000	9.500
		2	8	118.000	14.750
		3	8	106.000	13.250
	a_w	1	12	222.000	18.500
		2	12	78.000	6.500
Fumonisin B_2	**O_3 (ppm)**	1	8	73.000	9.125
		2	8	118.000	14.750
		3	8	109.000	13.625
	a_w	1	12	222.000	18.500
		2	12	78.000	6.500
Fumonisin B_3	**O_3 (ppm)**	1	8	73.000	9.125
		2	8	120.000	15.000
		3	8	107.000	13.375
	a_w	1	12	222.000	18.500
		2	12	78.000	6.500

* FB_1: O_3: H (2, N=24)=2.34, p=0.31; a_w: H (1, N=24)=17.28, **p<0.001**

FB_2: O_3: H (2, N=24)=2.84, p=0.242; a_w: H (1, N=24)=17.28, **p<0.001**

FB_3: O_3: H (2, N=24)=2.95, p=0.229; a_w: H (1, N=24)=17.28, **p<0.001**

Table I.6: ANOVA for the effect of O_3 dose, substrate a_w and their interaction ($O_3 \times a_w$) on *in vitro* hydrolysed-FB_1 production after 15min exposure of spores to 0-200 ppm O_3

Effect	SS	DF	MS	F	p
Intercept	11.921	1	11.921	136.849	**p<0.001**
O_3	4.848	2	2.424	27.826	**p<0.001**
a_w	3.643	1	3.643	41.82	**p<0.001**
$O_3 \times a_w$	0.502	2	0.251	2.882	0.082
Error	1.568	18	0.087		

Table I.7: Kruskal-Wallis ANOVA by ranks for the effect of O_3 dose and substrate a_w on *in vitro* fumonisin B_1 production after exposure of actively growing cultures to 0-200 ppm O_3 for 60min at 6 L/min flow rate

Toxin	Effect	Code	Valid N	Sum of Ranks	Mean Rank
Fumonisin B_1	**O_3 (ppm)**	1	8	118.000	14.750
		2	8	99.000	12.375
		3	8	83.000	10.375
	a_w	1	12	222.000	18.500
		2	12	78.000	6.500
Fumonisin B_2	**O_3 (ppm)**	1	8	116.000	14.500
		2	8	98.000	12.250
		3	8	86.000	10.750
	a_w	1	12	222.000	18.500
		2	12	78.000	6.500
Fumonisin B_3	**O_3 (ppm)**	1	8	120.000	15.000
		2	8	98.000	12.250
		3	8	82.000	10.250

	a_w	1	12	222.000	18.500
		2	12	78.000	6.500

* FB_1: O_3: H (2, N=24)=1.54, p=0.464; a_w: H (1, N=24)=17.28, **p<0.001**

FB_2: O_3: H (2, N=24)=1.14, p=0.566; a_w: H (1, N=24)=17.28, **p<0.001**

FB_3: O_3: H (2, N=24)=1.82, p=0.403; a_w: H (1, N=24)=17.28, **p<0.001**

Table I.8: Kruskal-Wallis ANOVA by ranks for the effect of O_3 concentration and substrate a_w on *in vitro* hydrolysed-FB_1 production after exposure of actively growing cultures to 0-200 ppm O_3 for 60 min at 6 L/min flow rate

Effect	Code	Valid N	Sum of Ranks	Mean Rank
O_3 (ppm)	1	8	119.000	14.875
	2	8	95.000	11.875
	3	8	86.000	10.750
a_w	1	12	222.000	18.500
	2	12	78.000	6.500

* O_3: H (2, N=24)=1.46, p=0.483; a_w: H (1, N=24)=17.28, **p<0.001**

Table I.9: Analysis of Variance for the effect of O_3 dose on log_{CFU} counts isolated from natural wheat after 30 min exposure at 100 and 200 ppm O_3

Effect	SS	DF	MS	F	p
Intercept	26.637	1	26.637	479.276	**<0.001**
O_3	0.203	2	0.102	1.826	0.303
Error	0.167	3	0.056		

Table I.10: ANOVA for the effect of O_3 dose on log_{CFU} counts isolated from natural maize after exposure to 100 and 200 ppm for 60 min

Effect	SS	DF	MS	F	p
Intercept	10.602	1	10.602	54.819	**<0.001**
O_3	5.616	2	2.808	14.521	0.005
Error	1.160	6	0.193		

Table I.11: Kruskal-Wallis ANOVA by ranks for the effect of O_3 concentration and grain a_w on the log_{CFU} counts isolated from autoclaved maize inoculated with *F. verticillioides* immediately after exposure

Effect	Code	Valid N	Sum of Ranks	Mean Rank
O_3 (ppm)	1	6	58.000	9.667
	2	6	58.000	9.667
	3	6	55.000	9.167
a_w	1	9	91.500	10.167
	2	9	79.500	8.833

* O_3: H (2, N=18)=0.05, p=0.975; grain a_w: H (1, N=18)=0.4, p=0.528

Table I.12: Kruskal-Wallis ANOVA by ranks for the effect of O_3 concentration and grain a_w on the log_{CFU} counts isolated from naturally contaminated maize inoculated with *F. verticillioides* immediately after exposure

Effect	Code	Valid N	Sum of Ranks	Mean Rank
O_3 (ppm)	1	6	93.000	15.500
	2	6	38.500	6.417
	3	6	39.500	6.583
a_w	1	9	87.000	9.667
	2	9	84.000	9.333

* O_3: H (2, N=18)=13.71, p=0.001; grain a_w: H (1, N=18)=0.02, p=0.884

Table I.13: ANOVA for the effect of O_3 concentration and grain a_w on logDON production in naturally contaminated wheat inoculated with *F. graminearum* exposed to 100 and 200 ppm O_3 (jar set-up) and stored for 20 days at 25°C

Effect	**SS**	**DF**	**MS**	**F**	**p**
Intercept	178.399	1	178.399	1188.930	**<0.001**
O_3	0.21	2	0.105	0.698	0.517
a_w	8.549	1	8.549	56.972	**<0.001**
$O_3 \times a_w$	0.186	2	0.093	0.619	0.555
Error	1.801	12	0.150		

Table I.14: ANOVA for the effect of O_3 concentration and grain a_w on logfumonisins production in natural maize inoculated with *F. verticillioides* exposed to 100 and 200 ppm O_3 (jar set-up) and stored for 10 days at 25°C

Effect	**SS**	**DF**	**MS**	**F**	**p**
Intercept	198.777	1	198.777	535.433	**<0.001**
O_3	0.213	2	0.107	0.287	0.756
a_w	9.269	1	9.269	24.967	**<0.001**
$O_3 \times a_w$	0.015	2	0.007	0.02	0.981
Error	4.455	12	0.371		

Table I.15: Kruskal-Wallis ANOVA by ranks for the effect of O_3 concentration, grain a_w and storage time on fumonisins (B_1+B_2) production in maize rewetted to two a_w levels, inoculated with *F. verticillioides*, exposed to 100 and 200 ppm O_3 for 1 hour in the column set-up and stored for 15 and 30 days at 25°C

Toxin	Effect	Code	Valid N	Sum of Ranks	Mean Rank
Sum of fumonisins (B_1+B_2)	**O_3 (ppm)**	1	12	291.000	24.250
		2	12	180.000	15.000
		3	12	195.000	16.250
	a_w	1	18	270.000	15.000
		2	18	396.000	22.000
	Storage time	1	18	337.000	18.722
		2	18	329.000	18.278
Ochratoxin A	**O_3 (ppm)**	1	12	287.000	23.917
		2	12	176.000	14.667
		3	12	203.000	16.917
	a_w	1	18	211.000	11.722
		2	18	455.000	25.278
	Storage time	1	18	356.000	19.778
		2	18	310.000	17.222

* fumonisins: O_3: H (2, N=36)=11.41, p=0.003; grain a_w: H (1, N=36)=8.32, p=0.004; storage time: H (1, N=36)=0.03, p=0.855

OTA: O_3: H (2, N=36)=5.11, p=0.077; grain a_w: H (1, N=36)=15.13, **p<0.001**; storage time: H (1, N=36)=0.54, p=0.463

Table I.16: ANOVA for the effect of O_3 concentration, grain a_w, storage time and their interactions on the sum of T-2 and HT-2 toxins produced in oats rewetted to two a_w levels, inoculated with *F. langsethiae*, exposed to 0-200 ppm O_3 for 1 hour in the column set-up and stored at 25°C for 15 and 30 days

Effect	SS	DF	MS	F	p
Intercept	8.935	1	8.935	4.957	0.036
O_3	6.303	2	3.152	1.748	0.196
a_w	0.0001	1	0.0001	0.00003	0.996
Stor. time	2.845	1	2.845	1.578	0.221
O_3×a_w	0.42	2	0.21	0.117	0.891
O_3×stor. Time	4.054	2	2.027	1.124	0.341
a_w×stor. Time	1.124	1	1.124	0.624	0.437
O_3×a_w×stor. Time	10.573	2	5.287	2.933	0.073
Error	43.264	24	1.803		

Table I.17: Kruskal-Wallis ANOVA by ranks for the effect of O_3 concentration, grain a_w and storage time on OTA produced in oats of to two a_w levels, exposed to 0-200 ppm O_3 for 1 hour in the column set-up and stored at 25°C for 15 and 30 days

Toxin	Effect	Code	Valid N	Sum of Ranks	Mean Rank
Ochratoxin A	**O_3 (ppm)**	1	12	314.000	26.167
		2	12	174.000	14.500
		3	12	178.000	14.833
	a_w	1	18	215.000	11.944
		2	18	451.000	25.056
	Storage	1	18	269.000	14.944

	time	2	18	397.000	22.056

* O_3: H (2, N=36)=9.687, p=0.008; grain a_w: H (1, N=36)=14.157, **p<0.001**; storage time: H (1, N=36)=4.164, p=0.041

Table I.18: Kruskal-Wallis ANOVA by ranks for the effect of O_3 concentration, grain a_w, storage time and position in the column on fumonisins (B_1+B_2) production in autoclaved maize rewetted to two a_w levels, inoculated with *F. verticillioides*, exposed to O_3 in the column set-up and stored for 15 and 30 days at 25°C

Effect	Code	Valid N	Sum of Ranks	Mean Rank
O_3 (ppm)	1	12	246.000	20.500
	2	12	219.000	18.250
	3	12	201.000	16.750
a_w	1	18	194.000	10.778
	2	18	472.000	26.222
Storage time	1	18	328.000	18.222
	2	18	338.000	18.778
Position in the column	1	12	297.000	24.750
	2	12	233.000	19.417
	3	12	136.000	11.333

* O_3: H (2, N=36)=0.77, p=0.68; grain a_w: H (1, N=36)=19.34, **p<0.001**, storage time: H (1, N=36)=0.03, p=0.874; position: H (2, N=36)=9.87, p=0.007

Table I.19: Kruskal-Wallis ANOVA by ranks for the effect of O_3 concentration, a_w and storage time on the *Penicillium* log_{CFU} counts isolated after 15 and 30 days from naturally contaminated maize rewetted to two a_w levels, exposed to 0-200 ppm O_3 in the column set-up and stored at 25°C

Effect	Code	Valid N	Sum of Ranks	Mean Rank
O_3 (ppm)	1	12	320.000	26.667
	2	12	179.000	14.917
	3	12	167.000	13.917
a_w	1	18	422.000	23.444
	2	18	244.000	13.556
Storage time	1	18	281.000	15.611
	2	18	385.000	21.389

* O_3: H (2, N=36)=10.9, p=0.004; grain a_w: H (1, N=36)=7.95, p=0.005; storage time: H (1, N=36)=2.71, p=0.1

Table I.20: ANOVA for the effects of O_3 concentration, storage time and their interaction on *F. verticillioi*des log_{CFU} counts isolated from autoclaved maize exposed to O_3 in the column set-up after 15 and 30 days storage at 25°C

Effect	SS	DF	MS	F	p
Intercept	720.279	1	720.279	2489.314	**<0.001**
O_3	0.489	2	0.245	0.845	0.454
Storage time	0.503	1	0.503	1.739	0.212
O_3×stor.time	0.484	2	0.242	0.837	0.457
Error	3.472	12	0.289		