Post-harvest strategies for the prevention of fungal growth and mycotoxin production in corn

Simbarashe Samapundo (MSc.)

FACULTEIT BIO-INGENIEURSWETENSCHAPPEN

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Reason has always existed
But not always in a reasonable form

KARL MARX

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Post-harvest strategies for the prevention of fungal growth and mycotoxin production in corn

Thesis submitted in fulfillment of the requirements

for the degree of Doctor (PhD) in Applied Biological Sciences

Titel van het doctoraat in het Nederlands:

Na-oogst bewaringsstrategieën voor het voorkomen van schimmelgroei en mycotoxineproductie in maïs

Illustration: Electron micrograph of a conidial head of *Aspergillus flavus*. Source: www.rhodes.edu/biology/hill/hill/hill.html

To refer to this thesis:

Samapundo, S. 2006. Post-harvest strategies for the prevention of fungal growth and mycotoxin production in corn. Thesis submitted in fulfillment of the requirements for the degree of doctor (Ph.D.) in Applied Biological Sciences. Faculty of Bioscience Engineering, University of Ghent

ISBN number: 90-5989-135-X

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Simbarashe Samapundo was supported by the Belgian Technical Cooperation (BTC).

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ACKNOWLEDGEMENTS

First and foremost I would like to express my profound gratitude to my promoters Prof. dr. ir. Frank Devlieghere and Prof. dr. ir. Bruno De Meulenaer. The work presented in this thesis is a testament of their invaluable advice, foresight and dedication. I would like to wish them unending success in their exemplary professional and family lives. I would also like to thank Dr. Annemie Geeraerd for some of the most productive correspondence I had during the study, her input in the modelling phase of the study was invaluable. I would also like to sincerely thank the Belgian Technical Cooperation for their financial support, which enabled this study to be done. I also extend my sincere gratitude to the members of the Examination Commission for participating in the fruition of this thesis and their efforts to improve the work I had presented to them.

I would like to express my sincere gratitude to Prof. Johan Debevere for initially granting me permission to pursue this study and supporting my applications for financial assistance. To my colleagues from the Laboratory of Food Microbiology and Food Preservation and the Laboratory of Food Chemistry and Human Nutrition, I say thank you for all the support, I really do hope everyone remains successful in their individual endeavors and continue to contribute as a unified group in making these laboratories even more successful than they are today. Special thanks to Prof. Mieke Uyttendaele, Vicente, Maria, Lieve, An De Coen, Andreja, Mirjana, Katleen, Martin, Mie Remaut, Peter, Kjell, Danny, Tineke, Nathalie, Frederic, Leen, Vicky, Ils, Sarah, Karl, Katty, Lisbeth, Ann Opsomer, Nicole, An V., Nancy, Linsey and the ladies from the Accredited laboratory.

I have had the luxury of working with some very dedicated and talented MSc Food Science Technology thesis students on various parts of the study. To them, Abel Atukwase, Yendouban Lamboni and David Osei-Nimoh, I say thank you for the excellent input and output, for helping to improve how the experiments were conducted and for ensuring we worked within the deadlines of the study. I sincerely hope for only the very best for you and your families. I am also indebted to the unconditional friendships of Dr.

Tonderayi Murimwa, Steven Mudawarima, Farai Chauke, Malvern Chirume and Dr. Liberty Sibanda. May we continue to encourage and challenge each other to be the best at what we do and who we are.

To my brothers, sister and nephew - Nyasha, Takudzwa, Tendekai, Tatenda and Munashe – and my parents Mr. Saul Samapundo and Mrs. Mabel Samapundo, words will never be able to fully explain my gratitude for your unconditional love, support and encouragement. To my beloved wife Griet, thank you for your unwavering love, support and patience during the good, bad and the very bad days. Thank you for the warm smile and hug that greeted me at the end of each day, that made going home after a hard day that much more special. I also thank you for continuously encouraging me, inflating my ego when I felt deflated, for proof reading some papers and translating the summary. Thank you for everything.

In fair honesty it is impossible to mention everyone I am immensely indebted to for their help in and outside of the study, but to those I didn't mention thank you and may you be blessed with only the best in your lives.

Thank you – Ndinotenda

Simba
14 September 2006, Gent

NOMENCLATURE

NOMENCLATURE

AccQ.Fluor	6-amino-quinolyl N-hydroxysuccinimidylcarbamate
a_w	water activity
a_{wmin}	minimum water activity for growth
a_{wmax}	maximum water activity for growth
a_{wopt}	optimum water activity for growth
BET	Brunauer-Emmett-Teller
BGY-F	bright greenish-yellow fluorescence
b_w	transformed a_w: $b_w = \sqrt{1-a_w}$
CAST	Council for Agricultural Science and Technology
CFU	colony forming units
CL	confidence limit
CMI	cardinal model with inflexion
CO_2	carbon dioxide
D_{max}	maximum diameter
DNA	deoxyribonucleic acid
ECB	European corn borer
EHC	Environmental Health Criteria
ELISA	enzyme linked immunosorbent assay
FAO	Food and Agricultural Organization
FAO/CIMMYT	Food and Agricultural Organization/International Maize and Wheat Improvement Centre
FAOSTAT	Food and Agricultural Organization Statistical Databases
FDA	Food and Drug Administration
FMOC	9-fluorenylmethyl chloroformate
GAB	Guggenheim-Andersen-de Boer
GC	Gas chromatography
H_2O_2	hydrogen peroxide
HCC	human hepatocellular carcinoma
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin layer chromatography
IARC	International Agency for Research on Cancer

ICMSF	International Commission for Microbiological Specifications for Foods
IH CO_2	initial headspace carbon dioxide
IH O_2	initial headspace oxygen
IPCS/WHO	International Programme on Chemical Safety
IITAB	International Institute of Tropical Agriculture Benin
k	germination rate factor (d^{-1})
KCN	potassium cyanide
kGy	kiloGray
LEM	leucoencephalomalacia
LC	liquid chromatography
MIC	minimal inhibitory concentration
m_0	monolayer moisture content
MA	modified atmosphere
MAP	modified atmosphere packaging
MS	mass spectrometry
MSE	residual mean square error
NBDF	4-fluoro-7-nitrobenzofuran
ND	not detected
NPK	Nitrogen Phosphorus Potassium
NTD	Neural Tube Defects
NTP	National Toxicity Programme
OPA	*o*-pthaldialdehyde
OPLC	over pressure liquid chromatography
PBS	phosphate buffered saline
PDA	potato dextrose agar
PES	pulmonary edema syndrome
P_{max}	maximum percent of germinated spores
Q_{st}	net isosteric heat of water sorption
R	universal gas constant (8.314 J mol^{-1} K)
SAX	strong anion exchange
SPE	solid phase extraction
RIA	radio-immunoassay
RIVM	National Institute of Public Health and the Environment

TLC	thin layer chromatography
T	temperature
T_{max}	maximum growth temperature
T_{min}	minimum growth temperature
T_{opt}	optimum growth temperature
UV	Ultraviolet
WHO	World Health Organization
λ	lag phase duration (d)
γ	gamma iradiation
τ	time (d) for 50% of spores to germinate
ΔH_c	enthalpy diference between monolayer and multilayer (J mol^{-1} K)
ΔH_k	difference between heat of condensation and heat of sorption of the multilayer (J mol^{-1} K)
μ_m	maximum specific germination rate (h^{-1})
°C	degree Celsius
°K	degree Kelvin

INTRODUCTION AND RESEARCH OBJECTIVES

INTRODUCTION AND RESEARCH OBJECTVES

Introduction

The importance of corn as food, feed and raw material for industry and the importance of moulds, after pests such as insects and rodents, as the most important causes of cereal grain yield loss are clearly demonstrated in the previous review. Moulds cause yield losses directly through spoilage and depletion of dry matter (Christensen and Sauer, 1982; Bottalico et al., 1999) and indirectly through poisoning with their mycotoxins (de Campos et al., 1980). The economic consequences of both sources of yield loss are far reaching and are set to become more important as demand on the world's food crops rises sharply with the current population boom taking place especially in developing countries.

Corn provides an excellent substrate for the growth of several mycotoxigenic fungi. This results in corn, when consumed as a staple food, being a major dietary source of mycotoxins of importance to both public and animal health such as aflatoxins and fumonisins. The risk posed by these mycotoxins in developing countries is compounded by the fact that the financial and technological resources to tackle these problems are very limited in these regions. In addition, although the ubiquitous and cosmopolitan nature of most fungi result in these problems occurring worldwide, these become more pronounced in tropical developing countries as a result of their higher humidities, temperatures and generally poor pre- and post-harvest agricultural practices (Fandohan et al., 2003; Hell et al., 2003). It can also be deduced from the previous review that despite the extensive studies in almost half a decade of 'mycotoxicology' and even longer of 'mycology', decisive solutions for these problems have not yet been fully resolved which are applicable to both resource rich and resource poor regions of the world. Predictive mycology, by providing tools allowing for the prediction of fungal growth and mycotoxin production, could play a very important role in improving the quality and safety of food (Dantigny, 2003; Dantigny et al. 2003, 2005a). The review also reveals that although there is an abundance of studies on the effect of environmental factors on the growth of mycotoxigenic fungi of importance to corn, none of these reports have attempted to develop validated models of their growth on corn itself. There is also an apparent over

reliance on observation of growth on artificial growth substrates. In addition, the relationship between fungal growth and mycotoxin production and the influence of environmental factors on this relationship has not yet been adequately described.

Of recent concern has been the emergence of insect and fungal resistance to methods that have been applied worldwide to partially control biological causes of yield loss were resources permit. Resistance has been observed to insecticides and fungicides on one hand (Placinta et al., 1999) and to transgenic crops genetically engineered for resistance to insects such as *Bt* corn, on the other hand (Linacre and Thompson, 2004). This implies that an urgent need exists for alternative methods to be evaluated that can be applied in developed and developing countries to abate this problem. Negative consumer perceptions about chemical preservatives in the food chain now, more than ever, play a very important role in the choice of methods that can be applied. To date, several post-harvest techniques for the prevention of growth and mycotoxin production have also been proposed including the application of compounds with antifungal effects such as synthetic antioxidants, essential oils, bicarbonate salts, weak organic acids and their salts, natural phenolic compounds, sorbates, propionates, benzoates or the use of modified atmospheres. However, most of the studies have been carried out on artificial media and their effects would still need to be validated on corn and other cereals. The influence of a_w, the single most important determinant of fungal growth, on the efficacy of the proposed techniques and the possibility and consequences of its interactions with other ecophysiological growth determinants has also been largely ignored. In addition, the impact of some of the chemicals evaluated on the sensorial properties of the corn has surprisingly (if not disappointly) yet to be reported in literature, given that the treated product should first and foremost be sensorially acceptable.

Major research objectives

The study was set-up and carried out to address some of the issues and paucities highlighted above. Clearly a need exists for the development of validated predictive models that can describe the influence of environmental and preservative factors on the growth of fungi of importance to corn, contributing in this way to the evaluation of solutions that can be used in developed and developing countries for protecting

grain stores. Several models including (i) Arrhenius type models (ii) cardinal models, (ii) Bělehrădrek type and (iv) polynomial models that could be potentially used to describe the trends observed (and sourced from both predictive mycology and predictive microbiology) were to be evaluated. The best fitting model/s would provide the basis for the modelling of the combined effects of a_w and various preservation techniques evaluated in later stages of the studies. As corn exhibits a wide water content gradient from the time it is harvested till its dry and kept in store, the role of its a_w/moisture content on the efficacy of these solutions has to be clarified. The increasing consumer influence on the use of chemical preservatives in the food chain also means that non-chemical techniques or techniques based on the application of natural antifungal chemicals need to be developed. In this context the major objectives of this study were to model the growth of *Fusarium* and *Aspergillus* species of greatest importance to corn and to evaluate the potential of non-chemical and chemical techniques to inhibit both fungal growth and mycotoxin production on corn during the post-harvest period, applying predictive modelling techniques were possible. Due to (potentially large) differences in the structural, compositional and nutrient availabilities between corn and artificial corn simulating media such as corn meal agar, all the experiments conducted in this study were carried out directly on corn. To achieve these goals the work was organized into three workpackages:

Work package 1. Characterization of the water relations of the growth substrate - yellow dent corn.

Work package 2. Modelling of the effects of the most important environmental factors (water activity (a_w) and temperature) on growth and evaluation of the effects of these factors on the relationship between growth and mycotoxin production of the most important fungal contaminators of corn.

Work package 3. Evaluation of non-chemical and chemical techniques to inhibit growth and mycotoxin production of the most important fungal contaminators of corn.

Specific research objectives

Work package 1

(i) To develop and model sorption isotherms for yellow dent corn to fully characterize the water relations of the growth substrate for the experiments. The best fitting sorption model was then to be used as a means of reproducibly rehydrating corn to particular a_w values in the subsequent experiments.

Work package 2

(ii) To evaluate models for their ability to describe the influence of a_w and temperature on the radial growth of *Fusarium* and *Aspergillus* species of greatest importance to corn.

(iii) To develop and validate a protocol to evaluate growth at the individual spore level and to apply this protocol to determine the variation in the colony growth rates (mm d^{-1}) and lag phase durations (d) of individual spores of *F. verticillioides* and *A. flavus*, and the influence of a_w and temperature on the observed variation.

(iv) To determine the relationship between radial growth and fumonisin B_1 production on yellow dent corn by *F. verticillioides* and *F. proliferatum*.

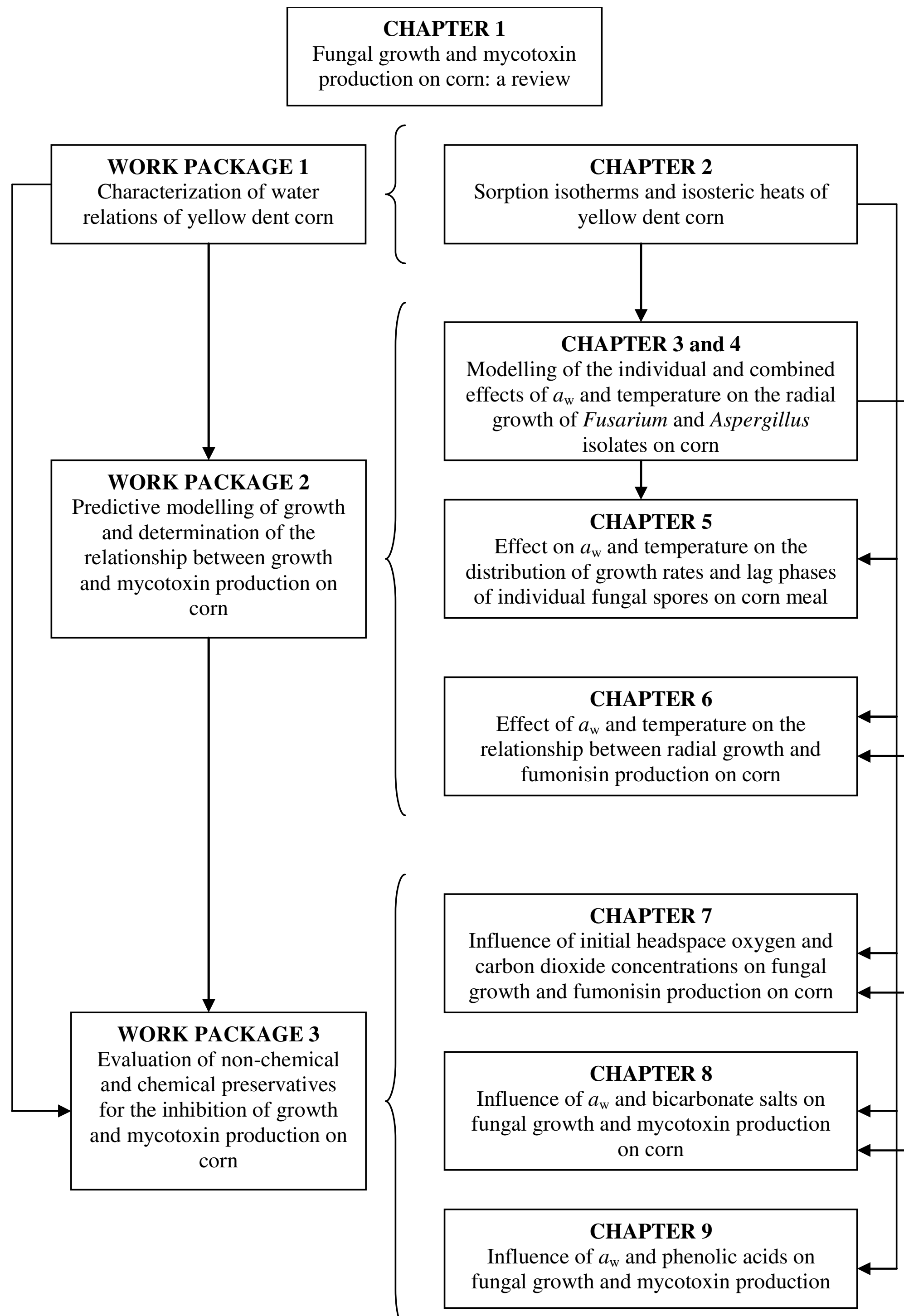
Work package 3

(v) To evaluate non-chemical and chemical (synthetic and natural) post-harvest techniques for inhibiting fungal growth and mycotoxin production, applying where possible the model/s developed in work package 2:

- The influence of modified atmosphere packaging (initial headspace oxygen or carbon dioxide concentration) on growth and fumonisin B_1 production by *F. verticillioides* and *F. proliferatum* on corn at different a_w values.

- The combined effect of bicarbonate salts and a_w on the radial growth and mycotoxin (fumonisin B_1 and aflatoxin B_1) production by *Fusarium* and *Aspergillus* species of greatest importance to corn and the sensorial quality of the treated corn.
- The combined effect of natural phenolic compounds and a_w on the radial growth and mycotoxin (fumonisin B_1 and aflatoxin B_1) production by *Fusarium* and *Aspergillus* species of greatest importance to corn.

OUTLINE OF STUDY



CHAPTER 1

Fungal growth and mycotoxin production in corn: a review

Chapter 1. Fungal growth and mycotoxin production in corn: a review

1.1 Abstract

This chapter presents the amalgamation of several existing literature sources to provide a broad review of the causes and consequences of fungal growth and mycotoxin production on corn and the various methods used to manage or eradicate these problems. In the first segment an overview of the importance of corn, routes for yield loss of corn and the most important eco-physiological determinants and consequences of fungal growth on corn are elaborated. The review thereafter discusses the physico-chemical and toxicological properties of the mycotoxins most important to corn - fumonisins and aflatoxins - and their mechanisms of toxicity. The current pre- and post-harvest strategies used to minimize the production of mycotoxins on corn are then evaluated, paying particular attention to the methods that have been proposed for fumonisins and aflatoxins. This is then followed by an overview of the physical, chemical and biological methods that are used or have been proposed to detoxify mycotoxin contaminated corn. The analytical techniques available for the detection and quantification of fumonisins and aflatoxins are then discussed followed by a description of the legislation currently applied worldwide to limit the amount of fumonisins and aflatoxins in corn and corn products for use as food and feed. To complete the literature study, a brief review of what is now known as 'Predictive Mycology' is given to provide an overview of the process of modelling fungal growth and the difficulties that have hindered the modelling of fungal growth. The models that have been successfully used to describe fungal growth are then discussed in this section.

In describing these different aspects of fungal spoilage, mycotoxin production and methods for their management, the review provides a sound basis for the justification of the research objectives of this study.

1.2 Worldwide importance of corn

Botanically, corn (*Zea mays* Linnaeus) is a tall annual plant that belongs to the grass family (*Graminaeae*) (FAO, 1992). Corn is also commonly referred to as *maize*, an American Indian word which literally means 'that which sustains life' (FAO, 1992). The cultivation of corn is thought to have most probably originated in Central America, particularly in Mexico, from where it spread northward to Canada and southward to Argentina (FAO, 1992). Corn cultivars may be divided into six general types - popcorn (*Z. mays* everta), flint corn (*Z. mays* indurata), dent corn (*Z. mays* indenta), flour corn (*Z. mays* amylacea, also known as soft corn), sweet corn (*Z. mays* saccharata) and pod corn (*Z. mays* tunicata) (Rooney and Serna-Saldivar, 1987). Dent corn has so far been the most important cultivar worldwide (Lynch et al., 1999). Several hundred sub-cultivars now exist mostly due to cross-breeding and to a lesser extent genetic modification aimed at increasing yields or resistance to common plant pests and diseases (Widstrom et al., 1984; D'Mello and Macdonald, 1997; Troyer, 2004).

Corn together with wheat and rice are the most important cereal grains in the world (FAO, 1992; Lynch et al., 1999). Although corn is the most widely cultivated cereal, it ranks third behind rice and wheat in terms of global production (FAO/CIMMYT, 1997). According to the FAO, global production of corn was 721.4 million tonnes in 2004 on 147 million hectares of land (FAOSTAT, 2005). The report identified the United States, China, the European Union, Brazil and Mexico as the world's largest producers of corn. Annual corn production has been in continuous ascendancy and current world production and demand is expected to double by 2020. Significant increases in world maize production have in part been realized as a result of additional land being cultivated, but more significantly as a result of the use of genetically improved cultivars, more efficient field practices and fertilizer application, and the introduction of more highly reproductive corn varieties (FAO, 1992). International trade flows in corn are highly regionalized, with 80% of all shipments from the United States being directed towards Latin America and the Caribbean Islands, whereas about 75% of the exports from South Africa, Zambia and Zimbabwe remain in sub-Saharan Africa (FAO/CIMMYT, 1997).

Corn is utilized in three basic manners – as food, as feed for livestock and as a raw material for industry (FAO, 1992). As food, corn is intrinsically linked to many societies past and present, and today provides a staple food for approximately 400 million people worldwide (FAO/CIMMYT, 1997). It also accounts for approximately as much as 15-20% of the total daily calories in the diets of people in more than 20 developing countries, primarily located in Latin America and Africa (Dowswell, et al., 1996). Corn is also an important cereal in the diets of people with celiac disease (gluten intolerance). The importance of corn as a feed grain in both developed and developing countries stems from its more efficient conversion to animal products such as meat, milk and eggs compared to other grains (Gatch and Munkvold, 1999). As much as 60% of the crop is devoted to the production of animal feed in the United States (Gatch and Munkvold, 1999; Troyer, 2004). Worldwide corn also serves as a basic raw material for the industrial production of corn starch, syrup, dextrose, high fructose corn syrup, oil, protein, alcohol, biodegradable chemicals, plastics, paper, textiles, ready-to-eat snack foods, breakfast cereals, cornmeal and more recently fuel (Rooney and Serna-Saldivar, 1987; FAO/CIMMYT, 1997; Lynch et al., 1999).

1.3 Routes for yield loss of corn

Current global production levels could far exceed demand if the loss of yields which occur during the pre-harvest period, during harvesting and in store could be minimized. Total yield loss in an agricultural season have been reported to be as high as 30% in tropical humid countries and about 10-15% in cooler temperate areas (FAO, 1992). Poor post-harvest practices tend to exacerbate the situation resulting in losses in excess of 50% often being reported in many parts of the world (Harris and Lindblad, 1978; Sode et al., 1995). An example being storage losses of up to 58% that have been reported in Nigeria as a result of insect and mould attack (Okereke and Nwosu, 1987). Whereas physical and mechanical damage (that occur mostly during harvesting) contribute to yield loss, pests in the form of insects and rodents (Udoh et al., 2000) together with moulds (Ominski et al., 1994) share the greatest responsibility for yield losses. Their intertwined roles are discussed below.

1.3.1 Role of insects in the yield loss of corn

Insects and other pests such as rodents are considered as the principal causes of grain losses (Udoh et al., 2000). They are able to cause severe damage at any stage in the pre-harvest or post-harvest periods. In addition, damage from insect feeding provides preferential sites for penetration by fungi, with some insects also acting as vectors of fungi (Dowd, 1995; Munkvold et al., 1997a, 1997b; Sobek and Munkvold, 1999). A prominent example being the European corn borer (ECB) (*Ostrinia nubilalis* Hb), a major pest of corn in central Europe which causes severe physical damage and yield losses as a result of tunnelling into the stalks and ears, in addition to promoting the infection of corn with *Fusarium* spp. and *A. flavus* (Wicklow, 1991; Magg et al., 2002). Moths of corn earworm (*Helicoverpa zea*) and the sap beetle (*Sitophilus zeamais*) have also been determined to be vectors of *A. flavus* (Wicklow, 1991; Rodriguez-del-Bosque et al., 1998).

1.3.2 Role of fungi in the yield loss of corn

The contamination of cereal grains by fungi is often an additive process, which begins in the field and potentially increases during harvest, drying and storage (CAST, 2003). Fungi are generally ranked as the second most important cause of grain yield loss (Ominski et al., 1994). Corn in particular is regarded as being highly vulnerable to degradation by fungi (Munkvold and Carlton, 1997). In addition to grain yield losses, the fungal infection of corn has been determined to decrease the processing and nutritional quality of the grain (Christensen and Sauer, 1982; Bechtel et al., 1985; Bottalico et al., 1989; Marin et al., 1998d; Marin et al., 1999a). The extent of reduction in grain quality is logically related to the degree of fungal development (Vieira, 2003). The losses incurred as a result of fungal growth are not only of economic importance but are also of significant public and animal health concern due to the possible production of mycotoxins by these fungi (de Campos et al., 1980).

An important classification has traditionally been made which broadly classifies the fungal contaminators of corn and other cereals as either field (pathogenic) or storage (saprophytic) fungi (Magan and Lacey, 1984; 1988). Field fungi are those that predominate on the field and are assumed to have insignificant consequences in the

post-harvest period, whereas storage fungi dominate the mycoflora during store and may also be present on the crop during the pre-harvest period (Christensen and Kaufmann, 1969; Magan and Lacey, 1984, 1988). The *Fusarium* spp. are generally considered as field fungi, whereas the *Penicillium* and *Aspergillus* are considered as storage fungi (Northolt and Bullerman, 1982; Magan and Lacey, 1988; Sweeney and Dobson, 1998). This classification however loses its integrity in view of the numerous cases worldwide where poor post-harvest practices enable typical field fungi to become important in the storage period (CAST, 2003). In addition some fungi such as *A. flavus* are considered as both pathogens and saprophytes of corn (Northolt and Bullerman, 1982).

1.3.2.1 Fungal contaminants of corn responsible for pre-harvest losses

A wide variety of fungi colonize corn plants during the vegetative stage, the most important of which are members of the *Aspergillus*, *Fusarium* and *Penicillium* genera (Bankole, 1994; Almeida et al., 2000; Bankole and Mabekoje, 2004). Of these, the Fusaria are regarded as the most frequent fungal contaminators of field corn (Mills, 1989, Bakan et al., 2002), with *F. verticillioides* in particular being the most prevalent species on corn worldwide (Marasas et al., 1981, 1988; Logrieco and Bottalico, 1988; Bottalico et al., 1989; Bacon and Nelson, 1994, Bankole, 1994; Bankole and Mabekoje, 2004). Although these findings are generally accepted worldwide, it is also well known that the prevailing biotic and abiotic factors largely determine which species become dominant (Marin et al., 1998).

On the field, the Fusaria are responsible for a number of corn diseases which ultimately lead to lower yields. Among these diseases are *Fusarium* ear rot, headblight, kernel rot and stalk rot (Dowd, 1995; Munkvold and Desjardins, 1997). *Fusarium* ear rot is the most common ear rot disease of corn and it can be found on nearly every corn field at harvest in the Corn Belt of the US (Smith and White, 1988), whereas *Fusarium* headblight is chronic in cereal-growing areas of Asia, Africa and South America (Bhat and Miller, 1991). *Fusarium* ear rot is caused by *F. verticillioides*, *F. proliferatum*, or *F. subglutinans* (Miller, 2001) and the symptoms are often highly correlated with ear damage by European corn borer and corn earworm larvae (Munkvold et al., 1999). Infection has also been reported to occur

without the development of symptoms (Munkvold et al., 1997a; Nelson et al., 1993). Although quality and yield are negatively impacted, the primary importance of *Fusarium* ear rot is its association with mycotoxins of public health significance, particularly the fumonisins (Smith and White, 1988; Dowd, 1998; Dowd and Munkvold, 1999). Kernel rot caused by *A. flavus* and *A. parasiticus*, is also frequently reported and has been associated with insect damage to ears and the production of the most notorious group of mycotoxins in corn, the aflatoxins (Smith and White, 1988).

1.3.2.2 Fungal contaminants of corn responsible for post-harvest losses of corn

The deterioration and loss of quality in stored grain is usually a result of the action of fungi, insects and mites - acting individually or in different combinations at various times of the storage period (Sinha et al., 1986). The Aspergilli are generally considered as the most important fungal contaminators on corn during store (Magan and Lacey, 1988; Bankole and Mabekoje, 2004). This has been attributed to their ability to grow and produce mycotoxins at more water compromised conditions than the *Fusarium* spp., which dominate the pre-harvest period (Magan and Lacey, 1988). Although the *Fusarium* spp. are predominantly considered as field fungi, a number of reports strongly suggest they may also play an important role in some cases during the post-harvest period. Pelhate (1968) suggested that the Fusaria should be considered as an intermediate group between field and storage fungi as they can develop in stored moist grain whereas Marin et al. (2004) reported that fumonisin production by members of the Fusaria may occur post-harvest when the storage conditions are inadequate. Moreover, Bacon and Williamson (1992) reported that the Fusaria are one of the most prevalent fungal species associated with maize worldwide both pre- and post-harvest. In general, the most important species on post-harvest corn are *A. flavus*, *A. parasiticus*, *F. verticillioides* and *F. proliferatum*, (Bacon and Williamson, 1992; Munimbazi and Bullerman, 1996; Ali et al., 1998; Almeida et al., 2000).

1.4 Eco-physiological determinants of fungal growth in bulk stored corn

Bulk stored grains are in principle man-made ecological systems in which respiring grain interacts with microorganisms, insects and the environment (Sinha, 1973; Magan and Lacey, 1988). In these systems the most important eco-physiological factors influencing fungal growth and survival are the availability of water (water

activity, a_w), temperature (Magan and Lacey, 1984, 1988; Sinha, 1995; Marin et al., 1998a), the range of contaminating fungi and their interactions (Wicklow et al., 1980, 1988; Wicklow, 1988; Magan and Lacey, 1988), interactions between the fungi and granary insects and mites (Barney et al., 1995; Sinha et al., 1986) and active resistance of the seeds to fungal infection (Cantone et al., 1983; Yao and Tuite, 1989; Stroshine and Yang, 1990). Importantly, the extent of field contamination largely determines the size of the contaminating mycoflora entering store and consequently the rate of deterioration of stored grain (Chatterjee et al., 1990; Vieira, 2003).

In contrast to bacterial growth were temperature is the most important determinant of growth, water availability is the single most important environmental factor affecting the ability of moulds to germinate, grow and establish themselves on grain and the rate at which they are able to do so (Magan and Lacey, 1988; Magan, 1988; Holmquist, 1983; Dantigny et al., 2005a). Temperature is also very important, this being highlighted in its well documented interactions with a_w in determining fungal growth (Northolt and Bullerman, 1982; Holmquist et al., 1983; Marin et al., 1999b). As a result, mentioning one factor without the other is often not enough. Fungal growth on both corn and artificial substrates is generally characterized by minimal, optimal and maximal a_w or temperature values for both germination and hyphal/mycelial extension.

1.4.1 Interaction between water activity and temperature on the germination of fungi of importance to stored grains systems

With regard to germination, Pitt and Miscamble (1995) reported that minimal a_w (a_{wmin}) for the germination of *A. flavus* and related species changed with temperature from 0.82, 0.81 to 0.80 at 25, 30 and 37°C, respectively. Marin et al. (1998a) also reported similar a_{wmin} for germination that varied between 0.80 and 0.85 over a temperature range of 5-45°C for *Aspergillus* species isolated from corn, including *A. flavus*. Other isolates from corn, including *A. ochraceus*, *A. niger*, *P. aurantiogriseum* and *P. hordei*, were also investigated in this study and interaction between a_w and temperature on germination and the temporal rates of germination was found. Although no optimal a_w (a_{wopt}) were mentioned, germination was determined to be very rapid at a_w values > 0.90. Marin et al. (1996) determined the germination

kinetics of isolates of *F. verticillioides* from corn and found an interaction between the significant effects of a_w and temperature. Sautour et al. (2001) reported a significant positive interaction between a_w and temperature on the rate of germination of *P. chrysogenum* spores on an artificial growth medium. Interaction between the effects of a_w and temperature on the germination and germination rates of *P. verrucosum* on barley malt agar and *A. ochraceus* on barley medium, green coffee-based and grape juice synthetic medium has also been reported by Pardo et al. (2004, 2005a, 2005b, 2006). In general, the ranges of conditions for germination are wider than those for growth of *Fusarium*, *Aspergillus* and *Penicillium* spp. isolated from corn (Magan and Lacey, 1984; Marin et al., 1996, 1998b).

1.4.2 Interaction between water activity and temperature on the mycelial growth of fungi of importance to stored grains systems

Synergistic interaction between a_w and temperature has been reported for the mycelial growth of *F. verticillioides* and *F. proliferatum* on corn and artificial media (Marin et al., 1995, 1999a, 1999b; Velluti et al., 2000). Marin et al. (1996) determined the a_{wmin} for growth of *F. verticillioides* and *F. proliferatum* isolated from corn to be 0.880. They also determined that no growth occurred at a_w 0.850. These results were in agreement with those of Cahagnier et al. (1995), who suggested that the threshold for growth of *F. verticillioides* was a_w 0.850. Contrasting optimum temperatures (T_{opt}) for growth of *F. verticillioides* and *F. proliferatum* on corn and artificial growth medium have been reported. 30ºC has been the most frequently reported T_{opt} value (Marin et al., 1995, 1999a, 1999b), whereas 20°C and 25ºC have been reported for the growth of *F. verticillioides* on autoclaved corn by Alberts et al. (1990) and Le Bars et al. (1994).

Synergistic interaction between a_w and temperature on the mycelial growth of *Aspergillus* and *Penicillium* species isolated from corn has also been observed (Holmquist et al., 1983; Marin et al., 1998a). The shortest lag phases for these isolates were observed at a_w values between 0.95-0.995 over a wide temperature range. Of these, *A. flavus* and *A. niger* both had an optimum a_w (a_{wopt}) for growth of 0.994. The T_{opt} for growth were 30 and 37°C, respectively. Several reports can be found in literature on the cardinal a_w and temperatures for growth of *A. flavus* and *A.*

parasiticus species on both corn and artificial media. In brief a_{wopt} values have generally been found to occur between 0.970 and 0.995 (Ayerst, 1969; Northolt et al., 1977; Holmquist et al., 1983; Gibson et al., 1994; Sautour et al., 2001a, 2002). The a_{wmin} values have generally been found to occur between 0.78 and 0.82 (Pitt and Miscamble, 1995; Sautour et al., 2001a, 2001b). T_{opt} values have been found to occur between 25 and 35°C (Schindler et al., 1967; Trenk and Hartman, 1970; Niles et al., 1985; Holmquist et al. 1983; Pitt and Hocking, 1997; Sautour et al., 2001a, 2005b). Minimal and maximal temperatures for growth of *A. flavus* were found to be 12°C and 43°C, respectively (Ayerst et al., 1969).

1.4.3 Interactions between fungi of importance to stored grains systems and the influence of environmental factors on this relationship

Given conducive environmental conditions, fungi present on the corn will rapidly grow and compete for the corn substrate, resulting in intra- or inter-specific interactions which result in some species eventually becoming more dominant than others (Magan and Lacey, 1988; Marin et al., 1998c). Although some of these fungal species can individually affect the grain quality, they usually act in different combinations at various times in the storage period (Sinha, 1973, 1986). Importantly, fungal interactions in stored grain ecosystems inevitably impact on the ability of component fungi to produce mycotoxins (Cuero et al., 1987).

In recent years *F. verticillioides* and *F. proliferatum* have attracted a lot of interest on research on corn due in part to their competitiveness and ability to produce a wide range of mycotoxins (Cawood et al., 1991). *F. verticillioides* in particular has been demonstrated to be an inhibitor of the infection of seed and pre-harvest corn by *A. flavus* and other *Fusarium* spp. (Wicklow et al., 1980, 1988; Rheeder et al., 1990; Zummo and Scott, 1992). The presence of *F. verticillioides* and *F. proliferatum* has also been found to reduce the presence of *A. flavus*, *A. ochraceus* and *A. niger* on irradiated maize, particularly at 15°C and high water availabilities of a_w 0.95-0.98 (Marin et al., 1998a). Marin et al. (1998a) also determined that temperature and a_w interactions profoundly affected the potential of fumonisin producing *Fusarium* spp. to exclude *Aspergillus* and *Penicillium* spp. from its niche, as inhibition of *A. niger* and *A. flavus* was either slight or did not occur at 25°C. At 25°C *F. proliferatum* has

been determined to overgrow *F. verticillioides* and become dominant regardless of the a_w value. This may partly explain the diminished competitive abilities of *F. verticillioides* at this temperature (Marin et al., 1998a, 1998c). Negative correlations have also been found between the frequency of isolation of *F. verticillioides* and other important *Fusarium* spp. such as *F. graminearum* from corn ears, which were attributed to competition for substrate, production of antagonistic substances and the prevailing environmental conditions (Blaney et al., 1986; Rheeder et al., 1990). Marin et al. (1995) suggested that the ability of Fusaria of the section *Liseola* to produce fumonisins gives them a competitive advantage over other fungal colonizers of corn. This was confirmed by Keyser et al. (1999), who reported that fumonisin B_1 exhibited antifungal activites towards a wide range of fungi including *A. flavus*, *P. expansum* and *F. graminearum*, but showed little or no inhibitory effects on *F. proliferatum* and *F. verticillioides*.

1.5 Mycotoxin contaminants of corn

On the field, during transport and in store some of the fungi that contaminate corn can produce toxic secondary metabolites that are known to have deleterious effects on humans and animals and may elicit physiological responses from plants (Nelson et al., 1993; Peraica et al., 1999; Hussein and Brasel, 2001; CAST, 2003). Mycotoxins have undoubtedly presented a hazard to human and animal health for centuries, which can now only become more important as the demand on available food supply increases with the phenomenal increase in the worlds population (Nelson et al., 1993). As much as 25-50% of the world's food crops are thought to be contaminated with mycotoxins (Dohlman, 2004).

The presence of mycotoxins on corn also has very large economic consequences accruing from lowered animal production and human toxicoses, lowered commodity market value and secondary effects on agricultural production and communities (Cardwell et al., 2001). Other costs include those incurred in mycotoxin management programmes such as research on crop production practices and methods to prevent or minimize the occurrence of mycotoxins in food products (Robens, 2001). In the United States alone, annual losses are estimated to range between US$0.5 and 1.5

billion as a result of aflatoxins in corn and wheat, fumonisins in corn, and deoxynivalenol in wheat (CAST, 1989).

Exhaustive reviews exist in literature about the worldwide distribution of fungal species on cereal plants and the occurrence of mycotoxins on food products including corn (Marasas, 1995, 1996; D'Mello and Macdonald, 1997; FAO/CIMMYT, 1997; Placinta et al., 1999; Bennett and Klich, 2003; FAO, 2004; Soriano and Dragacci, 2004). The ubiquitous and cosmopolitan nature of the most common fungal contaminants of corn such as *F. verticillioides*, *F. proliferatum*, *F. graminearum*, *A. flavus* and *A. parasiticus*, has resulted in their mycotoxins - fumonisins, aflatoxins, and tricothecenes (primarily zearalenone and deoxynivalenol) - being found on corn worldwide (Robens and Richard, 1992; Moss, 1998; CAST, 2003). These mycotoxins have also been identified as the primary sources of yield loss and costs of crop and grain management (Robens and Richard, 1992). Evidence of the co-occurrence of fumonisins and aflatoxins in corn worldwide has been found, further highlighting the relevance of both mycotoxins and their producers on corn (Almeida et al., 2000; Medina-Martinez et al., 2000; Li et al., 2001; Vargas et al., 2001). As a result these two mycotoxins will be discussed in more detail.

1.5.1 Fumonisins

1.5.1.1 Chemical and physical properties

The fumonisins are a group of several toxic structurally related secondary metabolites (Gelderblom et al., 1988) whose basic structure (Fig. 1.1) is a C-20, diester of propane-1,2,3-tricarboxylic acid and a pentahydroxyeicosane containing a primary amino group (Sweeney and Dobson, 1998; Humpf and Voss, 2004). In comparison to other mycotoxins the fumonisins were more recently isolated in 1988 (Gelderblom et al. 1988). Their identification represented a major breakthrough in nearly a century of investigation into animal and human diseases associated with the consumption of corn contaminated with *F. verticillioides* (Munkvold and Desjardins, 1997).

The most abundant fumonisin found in nature is fumonisin B_1 which is also of greatest primary concern as it is considered to be the most active cancer promoting

component within the fumonisins (Gelderblom et al., 1988; Theil et al., 1992). Besides fumonisin B_1 there are at least 12 other fumonisins: fumonisin A_1, A_2, A_3, B_2, B_3, B_4, C_1, C_2, C_3, P_1, P_2 and P_3 (Gelderblom et al, 1991, 1992, 1993; Musser and Plattner, 1997; Sweeney and Dobson, 1998). *F. verticillioides* and *F. proliferatum* are considered to be the most prolific and relevant producers of fumonisins in nature (Weidenborner, 2001), whereas *F. napiforme*, *F. anthophilum*, *F. dlamini*, *F. nyagamai* (Gelderblom et al., 1988; Nelson et al., 1992; NTP, 1999; Rheeder et al., 2002), *Giberrella fujikori* (Desjardins et al., 1997) and *Alternaria alternata* sp. lycopersci (Rheeder et al., 2002) are known to produce fumonisins, but are not significant contributors in nature (Nelson et al., 1992; Thiel et al., 1991a; EHC, 2000; NTP, 1999).

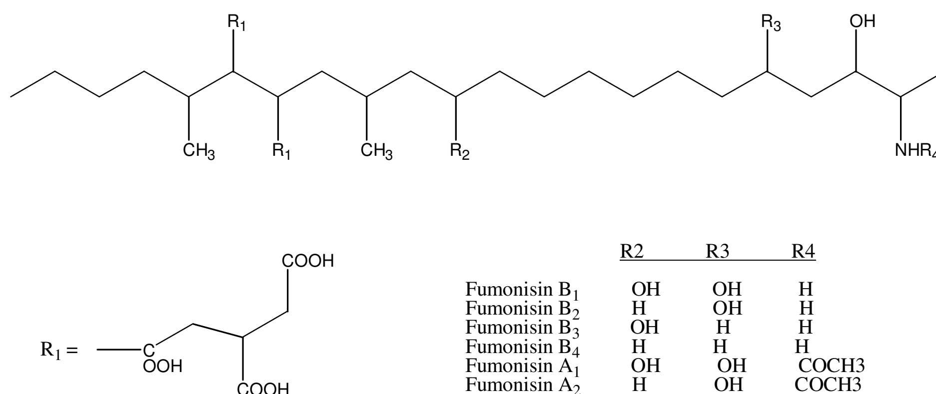


Fig. 1.1. Structural formula of fumonisins. Source: Bezuidenhout et al. (1988).

Fumonisins are rather unique amongst other mycotoxins in that they are water soluble, which contributed to their rather late discovery (Muro-Cacho et al., 2004). Pure fumonisin B_1 is a white, hygroscopic powder, also soluble in acetonitrile-water or methanol (EHC, 2000). It is stable in methanol at -18°C and in acetonitrile-water (1/1, v/v) at 25°C for up to 6 months (Harrison et al., 1991). It however is unstable in methanol at 25°C, in which it breaks down to monomethyl or dimethyl esters (Gelderblom et al., 1992). Fumonisins are relatively heat stable and have been reported to survive many conditions applied during food processing (Humpf and Voss, 2004). Thermal degradation of fumonisins in dried corn culture material was found to follow half-times of 175 minutes, 30-38 minutes, and 10 minutes at 100, 125

and 150°C (Dupuy et al., 1993; Le Bars et al., 1994). Fumonisins have no obvious chromophore in the ultraviolet or visible spectrum and are stable in light (Moss, 1998). Structurally, fumonisins resemble the sphingoid bases sphinganine and sphingosine (Riley et al., 1994a, 1994b; EHC, 2000; Humpf and Voss, 2004), which may help explain their biological activity.

1.5.1.2 Biosynthesis of fumonisins

Fumonisins are thought to be synthesized through the condensation of alanine to an acetate-derived precursor (Branham and Plattner, 1993; Sweeney and Dobson, 1999). Branched chain methyl groups are then added at C-12 and C-16 by an S-adenosyl methionine transferase (Sweeney and Dobson, 1998). The order and number of the subsequent steps involved in the oxygenation and subsequent esterfication of the acetate derived backbone are as yet unknown (Branham and Plattner, 1993; Sweeney and Dobson, 1998). A proposed pathway for fumonisin biosynthesis is shown in Fig. 1.2.

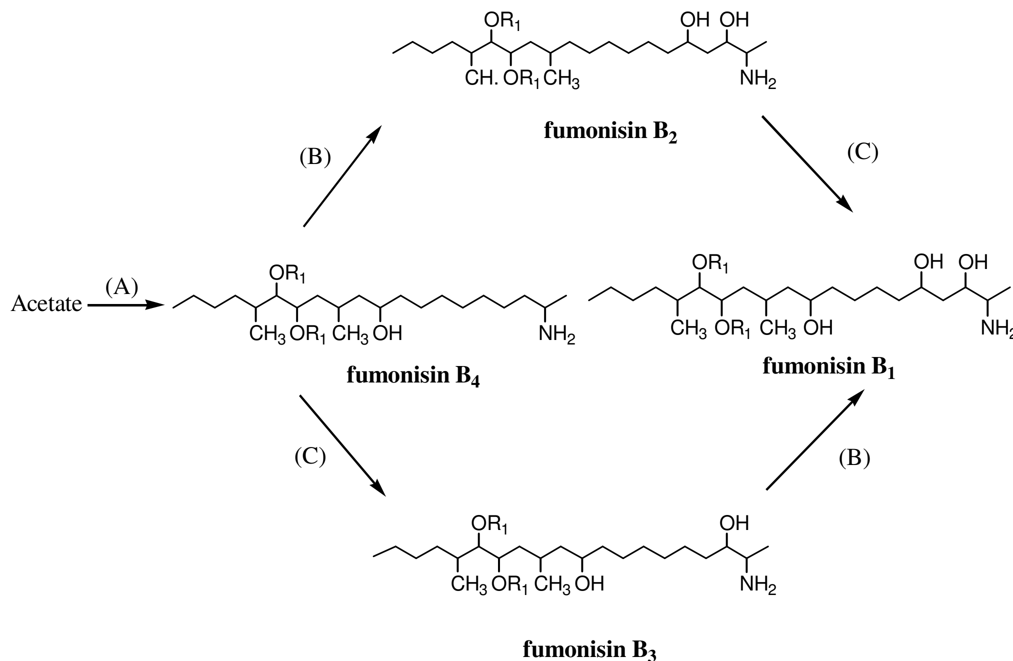


Fig. 1.2. Proposed pathway for fumonisin biosynthesis. R_1 – tricarballyic acid esters, genes involved (A) – *fum* 1, (B) - *fum* 2, and (C) – *fum* 3. Source: Sweeney and Dobson (1998).

1.5.1.3 Toxicological effects of fumonisins on animals and man

Fumonisins have been found to cause a number of toxicological effects in experimental animals, animals of agricultural importance and humans. In horses, leukoencephalomalacia (LEM) has been induced by intravenous injection (Laurent et al., 1989) and by oral dosing of fumonisin B_1 (Kellerman et al., 1990). Wilson et al. (1992) and Ross et al. (1993) elicited the same response by feeding horses and ponies naturally contaminated corn screenings. Field outbreaks of LEM have also been associated with fumonisin contaminated corn, corn screenings and corn based feeds (Sydenham et al., 1990; Ross et al., 1992; Wilson et al., 1992; Ross et al., 1993).

Fumonisins B_1 has been found to cause pulmonary edema syndrome (PES) and hydrothorax in pigs via intravenous injections or through the consumption of contaminated corn screenings (Harrison et al., 1990; Colvin and Harrison, 1992; Haschek et al., 1992; Osweiler et al., 1992). PES outbreaks in the United States during 1989/1990 were associated with corn screenings contaminated with <1-330μg/g fumonisin B_1 and <1-48μg/g of fumonisin B_2 (Harrison et al., 1990; Colvin and Harrison, 1992; Haschek et al., 1992; Ross et al., 1992). Fumonisin B_1 has also been found to be hepatotoxic (Gelderblom et al., 1988, 1991), nephrotoxic (Voss et al., 1993), hepatocarcinogenic (Gelderblom et al., 1991), and cancer promoting and initiating in experimental rats (Gelderblom et al., 1988, 1992, 1993, 1994). Cytotoxicity in a wide range of mammalian cell cultures has also been demonstrated (Norred et al., 1991, 1992a, 1992b; Gelderblom et al., 1993; Cawood et al., 1994).

As a result of ethical considerations, experimental evidence proving or disproving the suspected effects of fumonisins on human subjects cannot be found in literature. Consequently fumonisin B_1 is currently classified by the International Agency for Research on Cancer (IARC) as a Group 2B compound, meaning that it is potentially carcinogenic (IARC, 2002). However, corn intended for human consumption and containing high levels of fumonisins or contaminated by *F. verticillioides* has been associated with high incidences of esophageal cancer in the Transkei region of South Africa (Marasas et al., 1981a, 1981b; Sydenham et al., 1990; Rheeder et al., 1992; Thiel et al., 1992), in Northern Italy (Fransceschi et al., 1990), in the Linxhian region of China (Chu and Li, 1994; Sydenham et al., 1991) and in South-eastern United

States (Gelderblom et al., 1992; Rheeder et al., 1992). Besides the association of human esophageal cancer with the consumption of *F. verticillioides* or fumonisin contaminated corn, the incidence of Neural Tube Defects (NTDs) in Transkei South Africa has been found to be very high, at 61.3 per 10000 births (Venter et al., 1995).

1.5.1.4 Mechanism of toxicity of fumonisins

Wang et al. (1991) reported that the basic backbone of the fumonisin molecule is similar to that of sphinganine, leading to the hypothesis that this mycotoxin may exert its action by competitively inhibiting ceramide synthase. Ceramide synthase plays a central role in the turnover of biologically important complex phospholipids by catalizing the conversion of sphingosine and sphinganine to ceramide (Humpf et al., 1998). Fig. 1.3 illustrates the proposed mechanism by which fumonisins inhibits ceramide synthase. The competitive inhibiton of the conversion of sphinganine and sphingosine to ceramide by the fumonisins results in a elevation of sphinganine levels and sphingosine breakdown products and disruption of overall sphingolipid biosynthesis (Riley et al., 1994a, 1994b). This is believed to initiate a cascade of cellular alterations that may contribute to the toxicity and carcinogenicity of fumonisins (Riley et al., 1994a, 1994b; Merril et al., 1996; Voss et al., 2002).

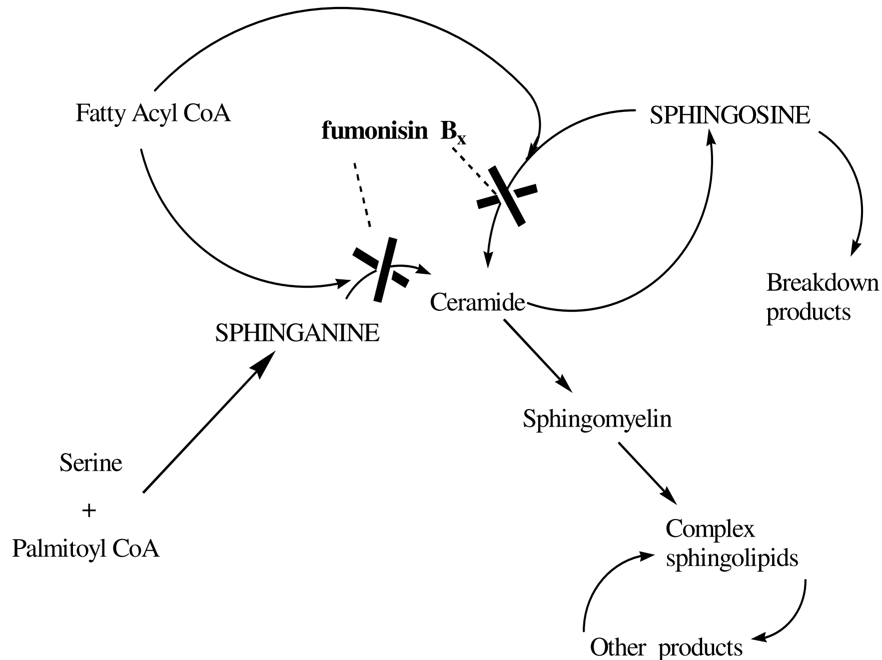


Fig. 1.3 Inhibition of ceramide synthase by fumonisins (fumonisin B_x): ✕ - site of inhibition. Source: Voss et al. (2002).

The toxic effects in horses are usually preceded by elevation in the serum sphinganine to sphingosine ratio (Riley et al., 1994a). The disruption of sphingolipid metabolism has also been reported to stimulate DNA synthesis in cultured Swiss 3T3 fibroblasts and the mitogenic activity of fumonisin B_1 via the accumulation of sphingoid bases may form the molecular mechanism of carcinogenicity (Schroeder et al., 1994). In addition, and with regards to carcinogenicity, the inhibition of hepatocyte proliferation by fumonisin B_1 together with its hepatotoxicity appear to be critical determinants of cancer initiation and promotion (Riley et al., 1994a, 1994b). Other studies have demonstrated that in rat liver, fumonisin B_1 induces changes in phospholipids and fatty acid composition which affect many cell functions that could contribute to its toxicity and carcinogenicity. Such changes in specific polyunsaturated fatty acids were attributed to the disruption of △-6-desaturase and cyclo–oxygenase metabolic pathways which seem to be important in fumonisin B_1 induced toxicity in primary hepatocytes (Gelderblom et al., 2001). With regards to NTDs, it has been suggested that fumonisins block folate uptake (Stevens and Tang, 1997; Hendricks, 1999), which is a critical requirement during organogenesis (Lucock et al., 1998).

1.5.2 Aflatoxins

1.5.2.1 Chemical and physical properties of aflatoxins

Aflatoxins are by far the most studied group of mycotoxins (>5000 publications to date) (Hussein and Brasel, 2001). They were first identified as the probable toxin that killed more than 100,000 turkey poults (Turkey X disease) in England in the early 1960s (Klich et al., 2000; Papp et al., 2002; Kuhn and Ghannoum, 2003). They are difuranocoumarin derivatives (Fig. 1.4), of which more than 20 are known to occur (Hussein and Brasel, 2001; Papp et al., 2002). The four major aflatoxins produced in nature are aflatoxin B_1, B_2, G_1 and G_2 (Muro-Cacho et al., 2004).

The nomenclature, B or G, is based on the fluorescent blue or green colours they produce under UV light on thin layer chromatography plates (Sweeney and Dobson, 1998; Bennett and Klich, 2003). The major producers of aflatoxins on corn and other products are *A. flavus*, *A. parasiticus*, and *A. nominus* (EHC, 1979; Sweeney and

Dobson, 1998; Gourama and Bullerman, 1995; Bennett and Klich, 2003). Other fungi known to produce aflatoxins but encountered less frequently in nature are *A. bombycis*, *A. ochraceoroseus* and *A. pseudotamarii* (Goto et al., 1996; Klich et al., 2000; Peterson et al., 2001). From a physical outlook, pure aflatoxin is a pale white to yellow crystalline, odorless solid (Reddy and Waliyar, 2000). Some of the most important physico-chemical characteristics of the most important aflatoxins are listed in Table 1.1.

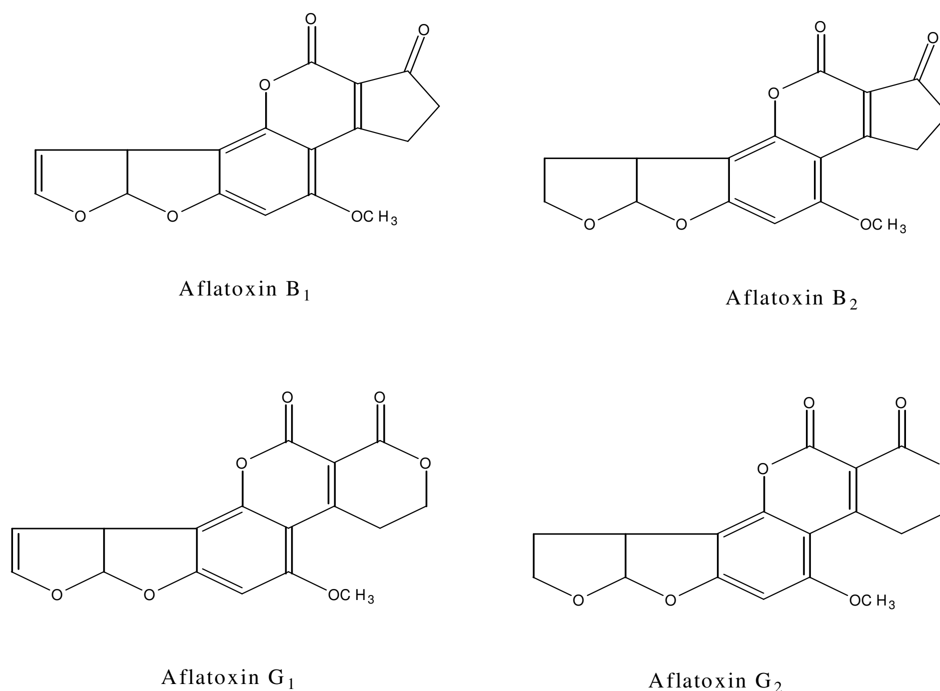


Fig 1.4 Structures of aflatoxin B_1, B_2, G_1 and G_2.

Table 1.1. Selected physico-chemical properties of aflatoxins

Aflatoxin	Molecular formula	Molecular weight (units)	Melting point (ºC)
B_1	$C_{17}H_{12}O_6$	312	268-269
B_2	$C_{17}H_{14}O_6$	314	286-289
G_1	$C_{17}H_{12}O_7$	328	244-246
G_2	$C_{17}H_{14}O_7$	330	237-240
M_1	$C_{17}H_{12}O_7$	328	299

Source: Reddy and Waliyar (2000).

In pure form aflatoxins are very heat stable and their levels are therefore not affected by normal food processing operations such as cooking or pasteurization (EHC, 1979;

Reddy and Waliyar, 2000). Aflatoxins are soluble in methanol, chloroform, acetone and acetonitrile (Reddy and Waliyar, 2000). They are also stable for many years as chloroform or benzene solutions stored in the dark and cold, but are relatively unstable when exposed to light and air, especially when dissolved in highly polar solvents (EHC, 1979). Aflatoxins are also intensely fluorescent when exposed to long wave UV light, which enables their detection at very low levels and also provides the practical basis for most methods used for their quantification (EHC, 1979).

1.5.2.2 Biosynthesis of aflatoxins

The biosynthesis of aflatoxins (Fig. 1.5) is a well understood complex process involving multi-enzymatic reactions that has been discussed in a number of reviews (Trail et al., 1995; Minto and Townsend, 1997; Sweeney and Dobson, 1998). In brief acetate and malonyl CoA are converted to a hexanoyl starter unit by a fatty acid synthase, which is then extended by a polyketide synthase to the decaketide norsolorinic acid, which is considered as the first stable precursor in the aflatoxin biosynthesis pathway (Sweeney and Dobson, 1998). Norsolorinic acid is then transformed via 12 to 17 enzymatic transformations to either aflatoxin B_1 and G_1 in one branch of the pathway or aflatoxin B_2 and G_2 on another branch of the pathway. Sterigmatocystin, a related dihydrofuran toxin, is a late metabolite in the aflatoxin biosynthesis pathway and is also produced as a final product by a number of species such as *A. nidulans* and *A. versicolor* (Bennett and Klich, 2003).

Aflatoxin M_1 and M_2 found in milk, are metabolically biotransformed aflatoxin B_1 and B_2, produced after the consumption of aflatoxin contaminated food or feed (Frobisch et al., 1986; Galvano et al., 1996; Moss, 1998, Moreno and Kang, 1999). The concentration of aflatoxin M_1 is generally 300 times lower than that of aflatoxin B_1 in the feed consumed. This biotransformation strangely provides a route for the excretion of aflatoxins and inadvertently a direct means of their consumption. This route of consumption is very important in light of the fact that milk is typically consumed by vulnerable groups such as the young, old and infirm. Other minor mycotoxins such as P_1, Q_1, B_{2a} and G_{2a} are also thought to be the result of mammalian biotransformation of the major metabolites (Bennett and Klich, 2003).

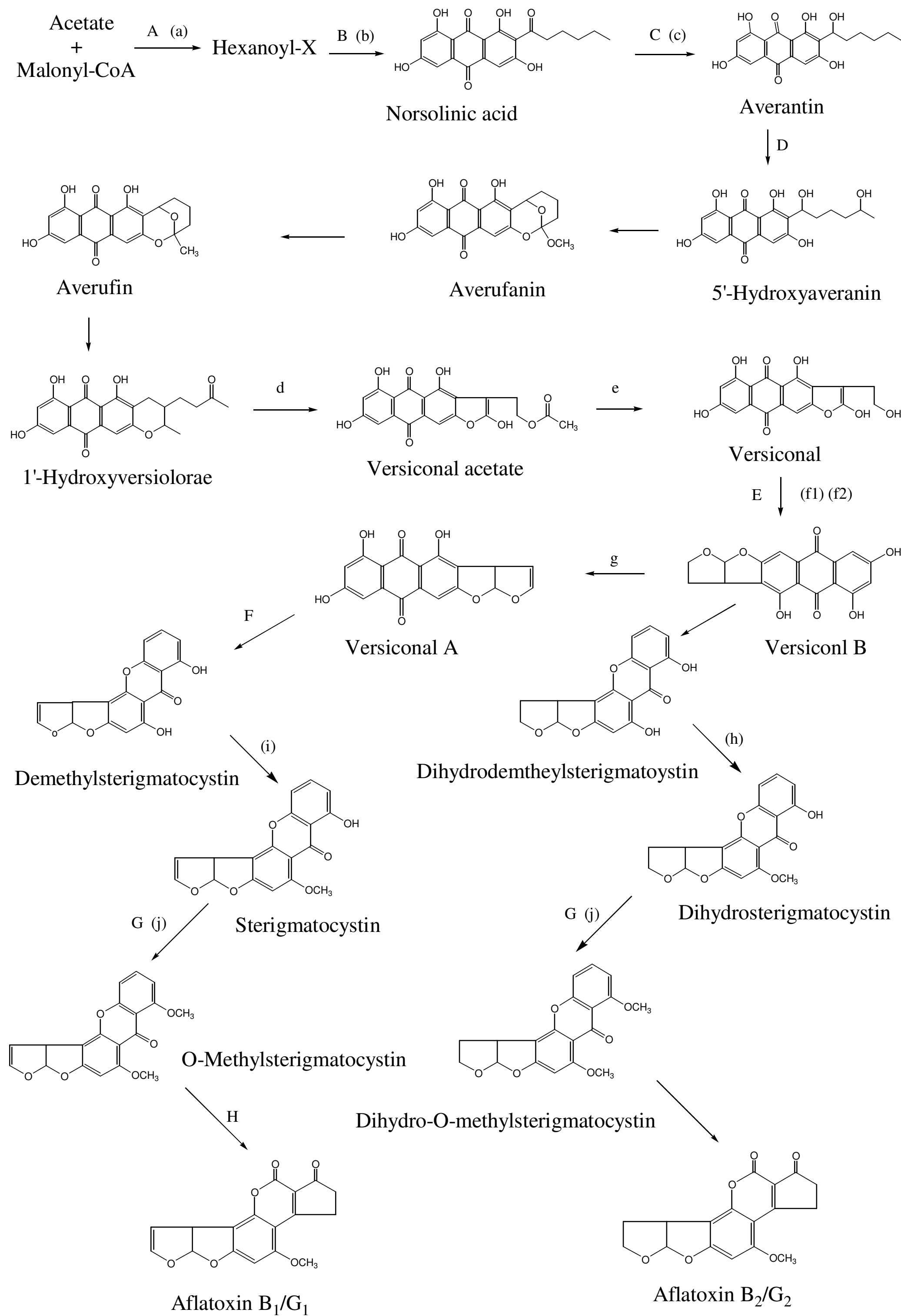


Fig.1.5. Aflatoxin biosynthetic pathway. Enzymes involved: (a) fatty acid synthase, (b) polyketide synthase, (c) norsolorininc acid reductase, (d) versiconal hemiacetal acetate reductase, (e) esterase, (f_1) versicolorin B synthase, (f_2) versiconyl cyclase, (g) desaturase (h) O-methyltransferase (MT-II), (i) O-methyltransferase, (j) O-methyltransferase (MT-I). Genes involved: A *fas*-1A and *fas*-2A, B *pks*A, C *nor*-1, *nor*A, D *avn*A, E *vbs*, F *ver*-1, G *omt*A and H *ord*-1. Source: Sweeney and Dobson (1998).

1.5.2.3 Toxicological effects of aflatoxins in animals and man

The identification of the aflatoxins as the etiological agents responsible for the outbreak of turkey X disease (Klich et al., 2000; Kuhn and Ghannoum, 2003) initiated a new era in scientific investigation now known as mycotoxicology. As a result of their extremely high potency, aflatoxins are currently considered as the mycotoxins of greatest significance in food and feed (Sweeney and Dobson, 1998). Thousands of studies have been carried out with regard to their toxicity and extensive reviews are given by IPCS/WHO (1998) and Eaton and Gallagher (1994). Aflatoxin B_1 in particular is considered as the most potent mycotoxin known to mankind (Squire, 1981) and is classified by the IARC as a Group 1 (carcinogenic) compound (IARC, 2002).

The diseases caused by the ingestion of aflatoxins are loosely termed 'aflatoxicoses' and can be a result of acute, sub-acute or chronic ingestion (Moreno and Kang, 1999; Bennett and Klich, 2003). Aflatoxins are known to be hepatocarcinogenic, mutagenic, teratogenic and immunosuppressive in both animals and man (Moreno and Kang, 1999; Peraica et al., 1999; Pitt, 2000; Hussein and Brasel, 2001; Papp et al., 2002). The liver is generally the primary target organ and liver damage has been demonstrated in poultry, fish, rodents and non-human primates fed aflatoxin B_1 (Bennett and Klich, 2003). Different species have been observed to exhibit different levels of susceptibility to aflatoxins, an example being that in rats and rainbow trout aflatoxin B_1 is amongst the most potent carcinogens known, but is only either a very weak carcinogen or even non-carcinogenic in guinea pigs (Moss, 1998). Aflatoxins have also been determined to be pulmonary carcinogens in experimental animals (Bennett and Klich, 2003).

In humans aflatoxins induce a wide range of diseases. Several studies have linked chronic and acute exposure to dietary aflatoxins with primary liver cancer in humans in many countries worldwide including Uganda, Canada, Germany, Kenya, Mozambique and China (Peers and Linsell, 1973; Van Rensburg et al., 1985; Li et al., 2001; Casado et al., 2001). Exposure to dietary aflatoxins is considered an important risk factor for the development of primary hepatocellular carcinoma in individuals already exposed to hepatitis B (Bennett and Klich, 2003). Synergistic interactions

between aflatoxins and hepatitis B have actually been noted on the etiology of liver cancer (Groopman and Kensler, 1996; Montesano et al., 1997). Evidence has also been found associating aflatoxins with neoplasms in extrahepatic tissues, particularly the lungs (Bennett and Klich, 2003). An example being Hayes et al. (1984), who found a correlation between both respiratory and total cancer in an epidemiological study of Dutch peanut workers exposed to dust contaminated with aflatoxin B_1.

Aflatoxins are thought to be involved in Reye's syndrome, a disease characterized by encephalopathy and fatty degeneration in the viscera of children and adolescents (Hayes, 1980). It has been suggested that kwashiorkor, a severe malnutrition disease, may actually be a form of pediatric aflatoxicoses (Hendrickse, 1997). Aflatoxin exposure in West Africa has also been correlated with stunted growth in children who were exposed right from the neonatal stages (Gong et al., 2002). Maxwell et al. (1998) stated that due to the capacity of aflatoxins to cross the placental barrier, they may cause genetic defects during the foetal stage.

1.5.2.4 Mechanism of toxicity of aflatoxins

From a toxicological point of view, it is clear that the generation of an 8,9 epoxide of aflatoxin is an important prelude to the toxicity observed (Harris et al., 1989; Papp et al., 2002; Kuhn and Ghannoum, 2003). Cytochrome P450 enzymes located in the liver are known to convert aflatoxins to compounds such as the highly reactive 8,9 epoxide, aflatoxicol, aflatoxin Q_1, aflatoxin P_1, and aflatoxin M_1 depending on the genetic predisposition of the species (Eaton and Gallagher, 1994; Smela et al., 2001; Bennett and Klich, 2003; Kuhn and Ghannoum, 2003).

Following its formation, the aflatoxin-8,9-epoxide binds very rapidly to DNA and serum albumin forming aflatoxin-N^7-guanine and lysine adducts, respectively (Eaton and Gallagher, 1994; Smela et al., 2001; Papp et al., 2002). The formation of adducts, shown in Fig. 1.6 for DNA, interrupts the normal metabolic processes of the cell and in the case of DNA adducts, can completely lead to a loss of control over cellular growth and division. With regards to human hepatocellular carcinoma (HCC), the aflatoxin B_1-8,9-epoxide is known to react with guanine at position three in codon 249

of the tumor suppressor p53 gene, the mutational hotspot associated with HCC (Hsu et al., 1991; Moss, 1998; Wang and Groopman, 1999).

Both animals and humans possess enzyme systems which are capable of reducing the damage to DNA and other cellular constituents caused by the aflatoxin-8,9-epoxides. DNA adducts with aflatoxin B_1-8,9-epoxide are usually removed by the glutathione S-transferase mediated nucleotide excision repair pathway (Eaton and Gallagher, 1994; Bennett and Klich, 2003). Failure to repair the DNA may cause GC to TA transversions (point mutations in which a purine is substituted by a pyrimidine or vice versa) and subsequent cellular changes that may lead to cellular transformation (Bennett and Klich, 2003). It has been postulated that humans possess less glutathione S-transferase activity than rats or mice and may therefore be less capable of detoxifying this metabolite (Eaton and Gallagher, 1994).

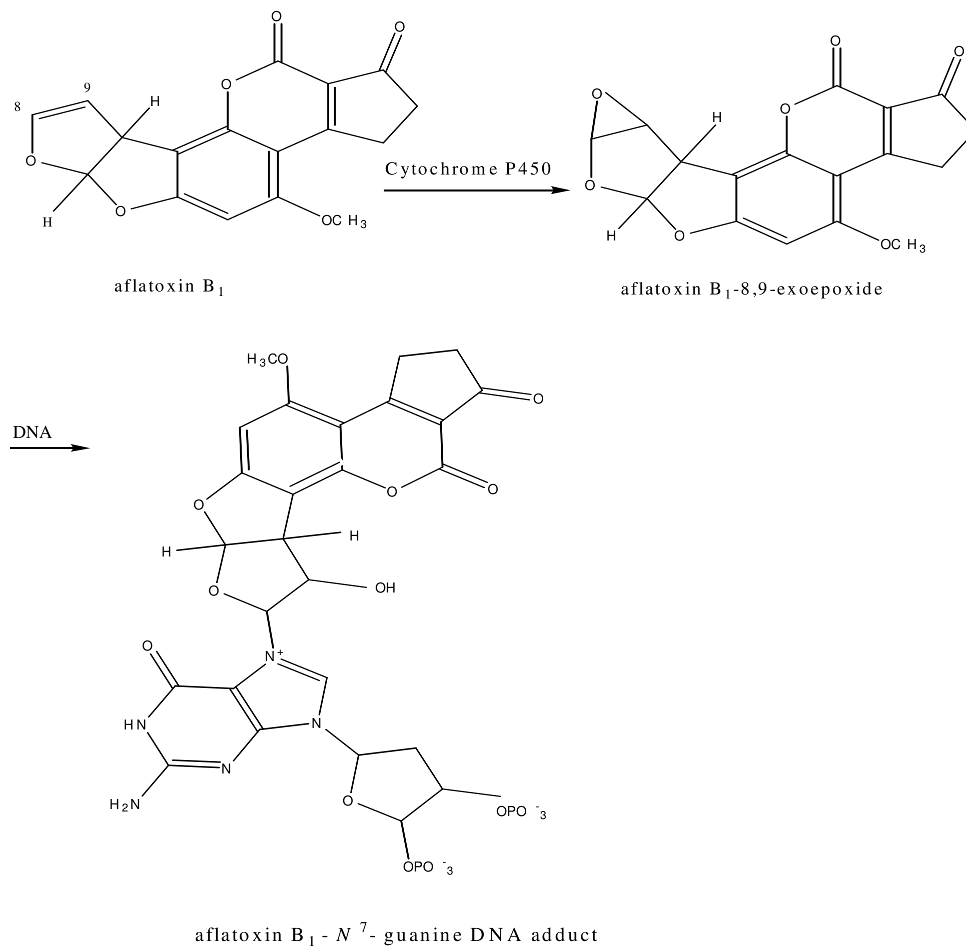


Fig. 1.6. Formation of aflatoxin-DNA adducts. Source: Smela et al. (2001).

1.5.3 Factors affecting the occurrence of aflatoxins and fumonisins on corn

As with fungal growth, the contamination of cereal grains by mycotoxins is often also an additive process, which begins on the field and potentially increases during harvest, drying and storage (CAST, 2003). A multiplicity of factors affects the production or presence of mycotoxins on the field and in stored food and feed such that the isolation of mycotoxigenic fungi from food or feed samples will not necessarily indicate the presence of mycotoxins (Hussein and Brasel, 2001). The occurrence of mycotoxins and magnitude of mycotoxin contamination generally varies with geographical and seasonal factors and with the conditions under which the crops are grown, harvested and eventually stored (EHC, 1979). Moreover, as growth (with or without visual symptoms) is precedent to mycotoxin production, the major factors determining growth which were discussed earlier are equally determinative of mycotoxin production. In an attempt to simplify the description of these factors, D'Mello and Macdonald (1997) categorized the factors as physical, chemical and biological, and they will be discussed as such in this review.

1.5.3.1 Physical determinants of aflatoxin and fumonisin production on corn

Physical factors include the environmental conditions conducive for fungal infection leading to mycotoxin production such as temperature, relative humidity and insect infestation (D'Mello and Macdonald, 1997; Hussein and Brasel, 2001). Although aflatoxin and fumonisin contamination of corn is a global phenomenon, generally crops in tropical and sub-tropical areas are more susceptible to contamination than those in temperate regions, as the high humidity provides conducive conditions for mycotoxin production (EHC, 1979; Fandohan et al., 2003; Hell et al., 2003). Drought stress or moisture stress has also been reported to coincide with higher aflatoxin or fumonisin levels (Payne et al., 1986; Diener et al., 1987; Russell et al., 1991). It however is not yet clear whether the increase in mycotoxin levels is a direct effect of moisture stress on the biosynthetic pathway or an indirect one by predisposition of the plant to infection (Moreno and Kang, 1999).

In experimental studies on both corn and artificial media, fumonisin or aflatoxin production strongly decreases with reduction of a_w regardless of the temperature

(Northolt et al., 1977; Cahagnier et al., 1995; Pitt and Miscamble, 1995; Marin et al., 1999a, 1999c). An exception however was found by Marin et al. (1999a), who reported that *F. proliferatum* produced more fumonisins at a_w 0.95 and 0.92 than at a_w 0.98 at 15°C after two and four weeks. Marin et al. (1999a) postulated that this may indicate the possibility of moisture stress stimulating fumonisin production by *F. proliferatum* at temperatures sub-optimal for growth during the initial growth phases. Minimum a_w values for production of aflatoxins by *A. flavus* and *A. parasiticus* has been reported as 0.82 and 0.87, respectively (Kozakiewicz and Smith, 1994; ICMSF, 1996). Fumonisin production is restricted to higher a_w values of > 0.91 (Sweeney and Dobson, 1998; Marin et al., 1999c). As can be seen, the a_w range for mycotoxin production is generally smaller than that for growth.

Regions with elevated temperatures have generally been associated with increased levels of fumonisins and aflatoxins (Diener et al., 1987; Hirooka et al., 1996; Moreno and Kang, 1999; Velluti et al., 2000; Russell et al., 1991). The effect of temperature on fumonisin production is ambiguous in contrast to that of a_w and appears to be highly dependent on the particular isolate and a_w value of the growth substrate. Unlike the effect of a_w, optimum temperatures for mycotoxin biosynthesis rarely coincide with those for growth. This has clearly been demonstrated for fumonisin (Alberts et al., 1990; Le Bars et al., 1994; Marin et al., 1999a, 1999c) and aflatoxin production (Pitt, 1993) on both corn and artificial growth substrates. In general the optimum temperature for fumonisin or aflatoxin production is between 20 and 25°C depending on the a_w value, whereas that for growth is between 28 and 35°C regardless of the a_w value. As for growth, interactions between a_w and temperature on both aflatoxin (Pitt, 1993) and fumonisin production (Marin et al., 1999c) have also been reported.

As mentioned earlier insects play an important role in the infection of corn by providing preferential sites for fungal penetration or by acting as vectors of mycotoxigenic fungi (Dowd, 1995; Munkvold et al., 1997a, 1997b; Sobek and Munkvold, 1999). The extent of insect damage on corn ears has been consistently associated with higher levels of fumonisins and aflatoxins in corn (Moreno and Kang, 1999; Avantiagatto et al., 2002).

1.5.3.2 Chemical determinants of aflatoxin and fumonisin production on corn

Chemical factors determinative of mycotoxin production include the use of fungicides and/or fertilizers (D'Mello and Macdonald, 1997; Hussein and Brasel, 2001). When used properly fungicides effectively inhibit fungal growth and mycotoxin production (D'Mello and Macdonald, 1997). However, some fungicides such as Fenpropimorph have actually been observed to not only significantly increase aflatoxin B_1 and aflatoxin G_1 production but to also shift production in favour of the more potent aflatoxin B_1 (Badii and Moss, 1988). The use of NPK (Nitrogen, Phosphorus and Potassium) and urea fertilizers has been found to reduce and increase aflatoxin levels on corn, respectively (Hell et al., 2003).

1.5.3.3 Biological determinants of aflatoxin and fumonisin production on corn

Biological factors are based on the interactions between the colonizing mycotoxigenic and non-mycotoxigenic fungal species and the substrate (Hussein and Brasel, 2001). Although contamination by mycotoxins has been observed in asymptomatic grain, in general the level of fungal infection has been correlated to the levels of mycotoxin contamination (Brown et al., 1995; D'Mello and Macdonald, 1997). Interactions between the fungal species on corn are important in determining which fungi dominate and consequently contribute to the mycotoxin contamination of corn. A synergistic relationship was found between *A. flavus* and *F. verticillioides* when they were inoculated on corn ears (Zummo and Scott, 1992). Whereas *F. verticillioides* affected aflatoxin production by the *A. flavus*, *A. flavus* did not affect fumonisin production by *F. verticillioides*. Aflatoxin production was significantly lowered on ears co-inoculated with both isolates than those inoculated with only *A. flavus*. Marin et al. (1998a) and Hirooka et al. (1996) have also observed the *Fusarium* induced inhibition of aflatoxin production of *Aspergillus spp*. Wicklow et al. (1988) suggested that the presence of competing fungi on some kernels and not all, may explain why kernels with high aflatoxin levels may be located next to toxin-free kernels

1.6 Strategies for the prevention and control of fungal growth and mycotoxin contamination of corn

The prevention of contamination of corn by fungi and poisoning by their mycotoxins can be dealt with using strategies targeted at the pre- or post-harvest periods. Detoxification, where possible, provides further reduction of potential risks. Prevention of mycotoxin contamination of cereal grains is indeed better than detoxification as the latter is not always fully effective (Northolt and Bullerman, 1982; Moss, 1998; Placinta et al., 1999). However, prevention is often difficult as many of the conditions on the field cannot be manipulated by man and the mycotoxigenic fungi of importance to corn are ubiquitous in their geographical distribution. Below are some of the methods that have been reported to impact on mycotoxin levels in corn and other cereal grains.

1.6.1 Pre-harvest strategies

As most mycotoxins are actually thought to be produced on the field, most investigators agree that the problem can be managed if contamination is prevented before store (Moreno and Kang, 1999; Placinta et al., 1999). Strategies that have been reported include breeding for resistance to fungal and insect invasion (Widstrom et al., 1984; Brown et al., 1995; Campbell and White, 1995; D'Mello and Macdonald, 1997), genetic engineering to confer resistance to fungal and insect invasion (Munkvold and Desjardins, 1997; Dowd, 2001; Bakan et al., 2002), irrigation to reduce moisture stress (Payne et al., 1986), application of insecticides and fungicides (D'Mello and Macdonald, 1997; Placinta et al., 1999) and the use of competitive atoxigenic strains to out compete toxigenic strains (Brown et al., 1991; Cotty and Bhatnagar, 1994).

The selection of cultivars of cereal crops resistant to *Fusarium* and *Aspergillus* pathogens is currently viewed as a viable and sustainable option for reducing the contamination of grain (Widstrom et al., 1984; D'Mello and Macdonald, 1997). Interestingly it has been observed that the resistance of some cultivars of agriculturally important crops to fungal infection has been correlated with their content of phenolic compounds before or after infection (Assabgui et al., 1993; Carver

et al., 1994; Kofalvi and Nassuth, 1995; McKeehen et al., 1999; El Modafar et al., 2000; Siranidou et al., 2002). As mentioned earlier, fungicides when properly used to control fungal diseases of cereal plants, minimize the possibility of mycotoxin production (D'Mello and Macdonald, 1997; Placinta et al., 1999). A worrying trend of resistance to fungicides by *Fusarium* pathogens has now been observed which may in time drastically reduce their overall impact (Placinta et al., 1999). Insecticides, which indirectly reduce mycotoxin production by reducing insect damage, may also directly inhibit fungal growth and mycotoxin production. An example is Naled which has been found to inhibit growth and aflatoxin production by *A. parasiticus* (Draughton and Ayres, 1981).

Payne et al. (1986) demonstrated in an extensive four year study that the reduction of moisture stress was associated with lower levels of aflatoxin contamination. Despite the important link between moisture stress (drought) and higher mycotoxin levels and the fact that droughts occur commonly, breeding for drought resistance has received little or no interest (Moreno and Kang, 1999). Crop management practices such as weeding, reduce water usage and assist in reducing moisture stress and may therefore contribute to reduced mycotoxin contamination of grain (Moreno and Kang, 1999). Other management practices such mixed cropping with vegetables have been found to reduce aflatoxin contamination of corn, whereas intercropping with cassava, groundnuts or cowpeas and ear damage on the field were found to increase aflatoxin contamination (Hell et al., 2003).

Munkvold and Desjardins (1997) stated that the engineering of plants to produce antifungal proteins or to detoxify mycotoxins *in planta* were feasible approaches to minimizing the risk they pose. Transgenic corn has mostly been genetically manipulated to include the gene from *Bacillus thuringiensis* (*Bt*) responsible for the production of the protein *Bt* cry1Ab, known to be toxic to insects. This corn is now widely known as *Bt* corn and has been demonstrated to have higher yields and lower levels of insect damage and infection by *Fusarium* compared to non-transgenic corn (Pilcher et al., 1997; Bakan et al., 2002; Gatch and Munkvold, 2002). In addition, it has been observed that total fumonisins were reduced in some cases by as much as 30 to 50 times in *Bt* corn hybrids compared to the levels in non-*Bt* hybrids (Dowd, 2000, 2001; Clements et al., 2003; Hammond et al., 2004). The use of genetically modified

corn is still marginal in most parts of the world due to the uncertainity about the long term safety of consuming such products. In addition, the use of *Bt* corn is limited in developing countries where poor communal/rural farmers tend to produce seed from the previous harvest. Moreover, the emergence of insect resistance is now threatening the continued use of *Bt* corn (Linacre and Thompson, 2004).

Brown et al. (1991) and Dorner at al. (1999) reported the use of competitive atoxigenic strains of *A. flavus* to reduce aflatoxin production on corn. The ability of atoxigenic strains of *A. flavus* to prevent the production of aflatoxins and biosynthetic pathway enzymes however varies from strain to strain, which makes the selection of effective isolates critical (Cotty and Bhatnagar, 1994). In reality the use of atoxigenic strains has to be viewed as a 'stop gap' measure as these will normally contribute to loss of the corn through spoilage (Widstrom, 1996). Competitive exclusion of fungi by endophytic bacteria such as *Bacillus mojavensis* and *Bacillus subtilis* has also been proposed as a novel biological control measure for *Fusarium* spp. on the field (Bacon and Hinton, 2000; Bacon et al., 2001)

1.6.2 Post-harvest strategies

Effective pre-harvest inhibition of fungal growth ensures a lower fungal inoculum level enters stores. Thereafter good agricultural/management practices at harvest and during store should effectively ensure the prevention of fungal invasion and related mycotoxin production (Bhat and Miller, 1991). Unlike the pre-harvest period, most of the conditions during the post-harvest period can actually be controlled. Good agricultural practices that have been found to limit mycotoxin production from the point of harvesting and during store include rapid drying to moisture contents of about 13-14% (Munkvold and Desjardins, 1997; Hussein and Brasel, 2001), storage in dry cool rooms with adequate aeration to avoid 'hotspots' from building up and also capable of preventing the entry of pests such as rodents and insects (Jayas and White, 2003), application of effective pesticides and fungicides (Draughton and Ayres, 1981; Badii and Moss, 1988; D'Mello and Macdonald, 1997; Placinta et al., 1999). Prolonged harvesting, and long drying periods on the field should be avoided as they have been associated with higher aflatoxin levels in corn in Benin (Hell et al., 2003).

Several other potential methods to control mycotoxin production on corn in store have also been proposed including the application of compounds with antifungal effects such as synthetic antioxidants (Ahmad and Braunen, 1981; Etcheverry et al., 2002; Farnochi et al., 2005), essential oils (Mishra and Dubey, 1994; Paster et al., 1995; Velluti et al., 2003; Nguefack et al., 2004), bicarbonate salts (Montville and Goldstein, 1987, 1989; El-Nabarawy et al., 1989; Montville and Shih, 1991), weak organic acids and their salts (Przybylski and Bullerman, 1980; Punja and Grogan, 1982; Ray and Bullerman, 1982), natural phenolic compounds (Norton, 1999; Bakan et al., 2003; Beekrum et al., 2003) or the use of modified atmospheres (Wilson et al., 1975; Magan and Lacey, 1984, 1988; Ellis et al., 1993, 1994). Varying degrees of efficacy have been achieved, which have not necessarily resulted in commercial success. Moreover, most of the studies have been carried out on artificial media and their effects would still need to be validated on corn. Of these methods, modified atmosphere storage of grains is beginning to gain the favour of grain farmers in view of the strict regulations being enforced on the use of chemical preservatives and negative perceptions consumers have about chemicals in the food chain (Jayas and Jeyamkondan, 2002).

1.6.3 Decontamination of mycotoxins

Although detoxification is an immediate and effective approach (Moreno and Kang, 1999), it is not always fully effective (Northolt and Bullerman, 1982; Moss, 1998; Placinta et al., 1999). To date numerous physical, chemical and biological techniques have been investigated for the detoxification of corn and corn products, some of which are discussed below.

1.6.3.1 Physical decontamination of corn

Very simple techniques such as stringent hand sorting can reduce the risk posed by mycotoxins in corn (Pitt, 2000). During sorting, potentially aflatoxin contaminated kernels can be identified by their bright greenish-yellow fluorescence (BGY-F) under black light at 365nm (Bothast and Hesseltine, 1975; Hadavi, 2005). The results of this test are only presumptive as the fluorescence is actually emitted by the oxidative action of peroxidases on kojic acid, which is produced at the same time as aflatoxins

(Hadavi, 2005). Automated methods to identify and separate aflatoxin contaminated seed in large lots have also been developed including electronic sorters and mechanical screeners (Goldblatt, 1971). Sydenham et al. (1994) reported that cleaning (the removal of broken kernel fragments, dust and other material < 3mm in size) from bulk grain shipments resulted in a significant reduction by 26 to 69% of the fumonisins present in corn.

Dry milling has been found to result in the redistribution of fumonisins in the resultant fractions (Broggi et al. 2002). It was observed in this study that the bran and germ had fumonisin levels approximately 3, 13 and 29 times higher than those in whole corn, corn flour, corn meal and corn grits, respectively. In a study of the fumonisin B_1, B_2 B_3 contents of corn and corn screenings from Iowa, Wisconsin and Illinois, Murphy et al. (1993) found that the screenings had on average 10 times more fumonisins than the whole corn. It can be seen that in general the larger the size of the milling fraction, the higher the level of fumonisin contamination. The wet milling of contaminated corn was shown to produce starch fractions with either very little or no fumonisins and aflatoxins, while the steep water, gluten fibre, and germ fractions contained most of the two toxins in decreasing order (Bennett and Anderson, 1978; Munkvold et al., 1997). The addition of sodium bisulphite to the initial steep water was reported to further reduce fumonisin levels in these fractions (Pujol et al., 1999). The nature of the degradation products was however not evaluated.

The thermal stability of aflatoxins and fumonisins is already well established. The temperatures applied during normal food processing generally have very little effect on the level of fumonisins and therefore this route of detoxification does not seem to offer much potential as a tool for risk reduction. The effects of thermal processing on fumonisins are comprehensively reviewed in Humpf and Voss (2004). There it is clearly stated that only temperatures above 150-200°C can reduce the fumonisin levels measured in the cooked product. This however does not necessarily translate to reduced toxicity owing to the formation of “hidden” fumonisins which may still be biologically active (Kim et al., 2003).

1.6.3.2 Chemical decontamination of corn

The use of ammonia to detoxify grains has been studied by a number of workers and is considered to be the most acceptable and efficient method on an industrial scale (Moss, 1998). Norred et al. (1991) showed that ammoniation at atmospheric pressure and ambient temperature only slightly reduced the fumonisin levels by hydrolysis to aminopentol. However, when ammoniation is done under high pressure and ambient temperature larger fumonisin and aflatoxin reductions of 79% (Park et al., 1992, 1996) and as much as 93% (Martinez et al., 1994), respectively, were found to occur. Weng et al. (1994) determined that the degradation of aflatoxins by ammoniation was irreversible, helping to ease concern that the degradation products could be converted back to active carcinogens in the stomach. The major limitations of ammoniation are kernel discolouration and a strong ammonia odour, which need to be resolved to boost the chances of industrial success of this form of detoxification (Lillehoj and Wall, 1987).

Nixtamalization, the treatment of corn with lime (calcium hydroxide) and heat to produce massa/tortilla flour, has been shown to reduce fumonisin B_1 levels by hydrolysis to hydrolysed fumonisin B_1 (Sydenham et al., 1995). Contradicting reports have however been published on the safety of nixtamilized corn. Hendrich et al. (1993) found that nixtamilized corn was actually more toxic to rats than fumonisin B_1, whereas Voss et al. (1996) reported that treated corn was less toxic than untreated corn. Hopmans and Murphy (1993) stated that heat treatment under alkaline conditions, as happens during nixtamalization, only removed the tricarballyic residues leaving a molecule which is still toxic.

The Maillard reaction (non-enzymatic browning) between fructose and the amino group of fumonisin B_1 has been shown to result in a significant decrease in the level of detectable fumonisin B_1 (Lu et al., 1997). The reaction results in the removal of the primary amide from the fumonisin, giving rise to a product that has been determined to be non-toxic and non-cancer initiating in rats. Park et al. (1996) also reported the reduction of fumonisin concentrations by up to 100% in contaminated corn by treatment with a combination of hydrogen peroxide (H_2O_2) and sodium bicarbonate ($NaHCO_3$). The reaction products were found to have a much lower toxicity than the

untreated maize. In view of the plethora of proposed chemical detoxification techniques, Munkvold and Desjardins (1997) correctly state that before any of these methods can be used industrially a great deal of work needs to be done to ensure that the products retain their functionality and that their sensorial qualities are not severely compromised. In addition to this, the consequences of the application of these methods on the nutritional and safety aspects of the products need to be determined.

1.6.3.3 Biological decontamination of corn

Biological detoxification can be regarded as any microbial based (whole cell or enzyme) system which results in the biotransformation or degradation of mycotoxins giving rise to metabolites that are either non-toxic or less toxic than the parent molecule (Sweeney and Dobson, 1998). Several microorganisms are known to degrade mycotoxins and therefore only those active on aflatoxins and fumonisins will be discussed.

Flavobacterium aurantiacum has been observed to metabolize aflatoxin B_1 to water soluble products and CO_2 in various foods (Line et al., 1994). Line and Brackett (1995a, 1995b) suggested that the degradation was more likely a mineralization process than a metabolic event as the addition of an extra nutrient source and aflatoxin did not affect the ability of *F. aurantiacum* to degrade aflatoxins. In addition, the age of the bacterial culture had an effect on the ability of *F. aurantiacum* to degrade aflatoxin B_1, with older cultures having a greater ability than younger ones (Line et al., 1994). *A. flavus* itself and *Rhizopus* spp. are fungi known to be capable of degrading aflatoxins (Bol and Smith, 1990). The monooxygenase enzyme system is reportedly involved in the degradation mechanism of *A. flavus*.

Conflicting reports exist about the reduction of mycotoxin levels during alcoholic fermentation. Bennett and Richard (1996) reported that zearalenone, deoxynivalenol and fumonisin levels generally remained constant during ethanol fermentations, whereas Scott et al. (1995) determined that three strains of *Saccharomyces cerevisiae* were able to decrease ochratoxin A, fumonisin B_1 and B_2 levels in wort during alcohol fermentation, resulting in lower mycotoxin levels in the finished beer. Kpodo et al. (1996) reported that aflatoxins were not degraded during the kenkey fermentation

process. Although it is clear that mineralization and microbial metabolism does occur in some cases, much less is known about the degradation products, their toxicity and the enzymatic pathways involved (Sweeney and Dobson, 1998).

1.7 Legislation governing maximum levels of aflatoxins and fumonisins in corn and corn based products for human and animal consumption

Legislation plays an invaluable role in controlling levels of mycotoxins in corn and corn products entering the food chain directly as food or indirectly as feed. Literature reveals two opposite poles with regard to the enforcement of legislature governing mycotoxin levels in corn worldwide. Amongst regulated mycotoxins are on one hand the aflatoxins which are the most widely controlled mycotoxins in the world (FAO, 1997; Van Egmond and Jonker, 2004), whereas on the other hand are the fumonisins which are amongst the least regulated despite their reported importance (FAO, 2004). This is in large due to the current understanding that aflatoxins are the most potent mycotoxins known to mankind (Squire, 1981), whereas there is no conclusive evidence but only epidemiological association implicating fumonisins as human carcinogens (IARC, 2002). Some people are now of the opinion that current mycotoxin regulatory limits have no relationship with food safety, but are unfortunately now more and more resembling bargaining chips for use in trade negotiations (Cardwell et al., 2001). An exhaustive review on worldwide legislature on mycotoxin can be found in FAO (2004), whereas Van Egmond and Jonker (2004) give a comprehensive account of the worldwide regulations on aflatoxins.

Aflatoxins are regulated dually or individually. Currently limits for total aflatoxins in food (shown in Fig. 1.7) range between 0-35 µg/kg, with most countries enforcing a limit of 4 µg/kg (FAO, 2004; Van Egmond and Jonker, 2004). The second most applied limit is 20 µg/kg, which is enforced in 17 countries in mostly Latin America, Africa and the United States. Limits for aflatoxin B_1 in food also follow a similar trend, with 29 countries enforcing a maximum amount of 2 µg/kg, whereas 21 use 5 µg/kg (FAO, 2004; Van Egmond and Jonker, 2004). What is important to note is that countries in which agriculture plays a very important role in the economy apply higher limits (to protect their agricultural sectors) than those who rely on imports. This situation is clearly depicted in that the Food and Drug Administration of the

United States (FDA, 2000) has a limit for total aflatoxins of 20 µg/kg, whereas the European Union enforces a limit of 4 µg/kg (FAO, 2004). With regards to aflatoxin levels in feed, only 39 countries regulated aflatoxin B_1 levels in 2003 (FAO, 2004). The range of limits was between 5 and 50 µg/kg with the majority (27) enforcing a maximum limit of 5 µg/kg. Even fewer countries (21) enforced limits for total aflatoxins in feed, of which most enforced a limit of 20 µg/kg.

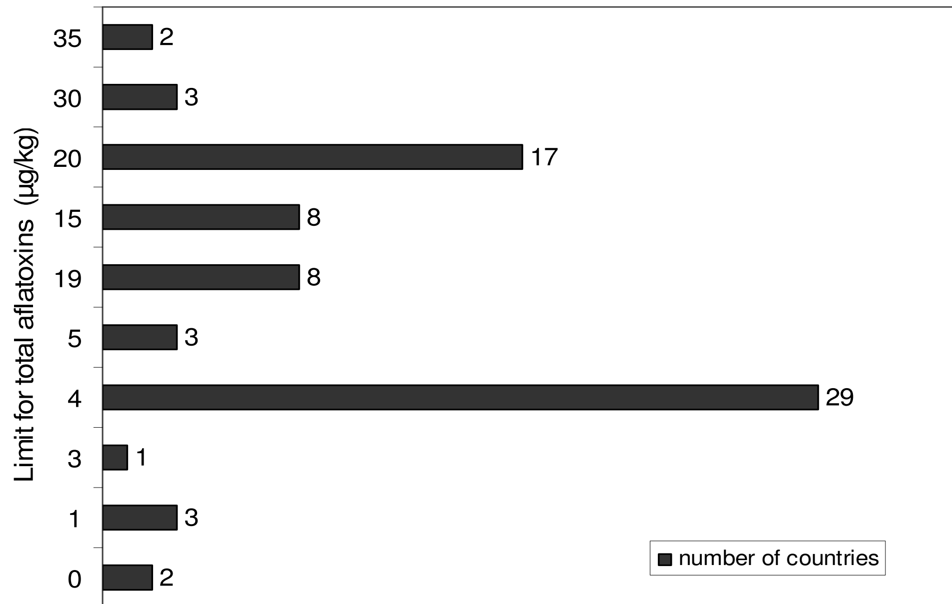


Fig. 1.7. Frequency distribution of worldwide limits for total aflatoxins in food during 2003. Source: FAO (2004).

With regards to fumonisins, very few countries enforce legislation to control their levels in food products. According to the FAO, only six countries had established limits in 2003 (FAO, 2004). Those limits were between 1000 and 3000 µg/kg. In the United States the FDA proposed guidelines for maximum levels of total fumonisins in corn and corn products for human consumption and for use as animal feed shown in Table 1.2 (FDA, 2001). It can be seen that in general the levels permitted in feed tend to be much higher than those allowed in corn and corn products for human consumption. In the Commission Regulation (EC) No. 856/2005, it has been proposed that maximum levels of 2000, 1000, 400 and 200 µg/kg for the sum of fumonisin B_1 and B_2, be applied in the EU as of 1 October 2007 for unprocessed corn, processed corn (i.e. corn flour, grits and semolina), corn-based foods for direct consumption and

processed corn-based for infants and young children, respectively (EU, 2005). Pending the finding of conclusive links to human carcinoma initiation or promotion, fumonisins will likely remain regulated in only a few countries.

Table 1.2. FDA guidelines for maximum levels of fumonisins in corn and corn products.

Human Foods	Total Fumonisins* (µg/g)
Degermed dry milled maize	2
Whole or partially degermed dry milled maize products	4
Dry milled maize bran	4
Cleaned maize intended for masa production	4
Cleaned maize intended for popcorn	3
Animal Feeds	
Feed for equids and rabbits	5
Feed for swine and catfish	20
Feed for breeding ruminants, poultry and mink	30
Feed for ruminants ≥ 3 months old for slaughter	60
Feed poultry for slaughter	100

* Total fumonisins: fumonisin B_1, B_2 and B_3. Source: FDA (2001).

1.8 Analytical tools for the detection and quantification of aflatoxins and fumonisins

The continuous development of improved and new qualitative and quantitative methods for the detection of mycotoxins, including techniques based on gas chromatography (GC), thin layer chromatography (TLC), liquid chromatography (LC), liquid chromatography-mass spectrometry (LC-MS) and immunological assays (i.e. ELISA), has given rise to an increase in the number of cases in which mycotoxins have been implicated in human and animal diseases. Because these methods play an invaluable role in the enforcement of legislation to control the levels of mycotoxins entering the human food and animal feed chain, it is not surprising that a lot of

research has been focused on the improvement of existing or development of new techniques.

1.8.1 Detection and quantification of fumonisins

Although GC (Sydenham et al., 1990; Plattner et al., 1990, 1992; Plattner and Branham, 1994), TLC (Cawood et al., 1991; Pittet et al., 1992; Dupuy et al., 1993), immunological (Azcona-Oliver et al., 1992a, 1992b; Usleber et al., 1994; Pestka et al., 1994; Schneider et al., 1995; Christensen et al., 2000; Paepens et al., 2004) and capillary zone electrophoresis (Hines et al., 1995; Maragos, 1995) techniques have been developed to determine fumonisins, HPLC has supplanted these as the method of choice (Richard et al., 1993; Trucksess, 1998; Shephard, 1998; Arranz et al., 2004). The chromatographic methods developed to date to identify and quantify fumonisins have been reviewed comprehensively by Shephard (1998) and Arranz et al. (2004). The most successful HPLC technique for quantification of fumonisins has been LC with fluorescence detection and precolumn derivatisation, combined with solid-phase extraction (SPE) sample clean-up (Shephard, 1998). Over 90% of laboratories that have reported results have used precolumn derivatisation and LC for quantification (Shephard et al., 1996).

Extraction is generally achieved by using either acetonitrile-water (1:1, v/v) (Wilson et al., 1990; Rice et al., 1995) or methanol-water mixtures containing 70-80% methanol (Shephard et al., 1990; Sydenham et al., 1992; Bennett and Richard, 1994). Other mixtures such as ethyl acetate-acetonitrile or trichloromethane-methanol have also been used (Arranz et al., 2004). The clean-up, to remove matrix impurities and concentrate the fumonisins, can be done by SPE using either reversed-phase (C_{18}) or strong anion-exchange (SAX) cartridges or by immunoaffinity columns (Visconti et al., 1996; Jiménez and Mateo, 1997; Shephard, 1998). The most widely used SPE method is the use of SAX cartridges (Shephard et al., 1996), which have been found to produce better results and are more robust than reversed phase and immunoaffinity cartridges (Bennett and Richard, 1994; Visconti et al., 1996). Although immunoaffinity columns produce a more selective purification of sample extracts, they have a rather limited capacity, which must be strictly adhered to by either dilution or concentration of sample extracts that fall outside of this range (Shephard,

1998). Florisil (an activated magnesium silicate), charcoal-alumina and silica modified with amino groups have also been used to clean samples (Jiménez and Mateo, 1997).

As fumonisins lack any significant chromophores, derivatisation is necessary before HPLC analysis (Arranz et al., 2004) unless detection and quantification is done by mass spectrometry (MS). Various pre-column derivatisation techniques involving reaction of the primary amine group of fumonisins have been reported. The derivatising agents used include maleic anhydride (Alberts et al., 1993), fluorescamine (Ross et al., 1991), *o*-pthaldialdehyde (OPA) (Shephard et al., 1990; Sydenham et al., 1992), naphthalene-2, 3-dicarboxaldehyde with KCN (Bennett and Richard, 1994), 4-fluoro-7-nitrobenzofuran (NBDF) (Scott and Lawrence, 1992), 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride) (Scott and Lawrence, 1992), 9-fluorenylmethyl chloroformate (FMOC) (Holcomb et al., 1993) and 6-amino-quinolyl N-hydroxysuccinimidylcarbamate (AccQ.Flour) (Velázquez et al., 1995, 2000). Despite its relative instability compared to other derivatising agents, OPA has been the most widely used fluorogenic reagent in studies which have reported results (Shephard, 1998; Arranz et al., 2004).

Although detection is mostly done by a fluorescence detector, other types of detectors have been investigated including evaporative light scattering detectors (Wilkes et al., 1995) which enable detection without derivatisation and amperometric electrochemical detection of the OPA/*tert*.-butyl-thiol derivative (Holcomb et al., 1994). Numerous LC-MS methods have also been developed to date which allow for the analysis of food and feed samples for fumonisins without prior derivatisation and enabling strong confirmation of the presence of fumonisins (Doerge et al., 1994; Thakur and Smith, 1996; Paepens et al., 2005). However, the prohibitive cost of a LC-MS system remains a major drawback.

Of recent importance has been the development of portable membrane-based flow-through enzyme immunoassays for the on-site assessment of the contamination of corn by fumonisins (Paepens et al., 2004), based on the patent developed by De Saeger and Van Peteghem (1999). The simplicity of use and analysis of the visual (colour based) results of such assays and their requirement of low-cost

instrumentation undoubetly will result in these assays playing a greater role the management of food safety as they can be easily used anywhere along the food chain (Paepens et al., 2004).

1.8.2 Detection and quantification of aflatoxins

Several techniques have been developed for the detection and/or quantification of aflatoxins in food and feed samples including immunochemical (Chu and Ueno, 1977; Sun and Chu, 1977; Pestka et al., 1980; Flaherty and Payne, 1997; Sibanda et al., 1999; Reddy et al., 2001), TLC (Trucksess et al., 1984; Van Egmond et al., 1991; Stroka et al., 2000), high performance thin layer chromatography (HPTLC) (Coke et al., 1988; Tomlins et al., 1989, Ali et al., 1999), HPLC (Micco et al., 1987; Van Egmond et al., 1991; Trucksess et al., 1994; Scudamore et al., 1998; Akiyama et al., 2001), LC-MS (Delmulle et al., 2006), GC-MS (Flaherty and Payne, 1997) and over-pressure layer chromatography (OPLC) (Otta et al., 2000) methods.

Immunochemical methods for the detection of aflatoxins include solid-phase radio-immunoassays (RIA) (Sun and Chu, 1977), affinity column immunoassay and ELISA (Chu and Ueno, 1977; Pestka et al., 1980; Flaherty and Payne, 1997; Sibanda et al., 1999; Reddy et al., 2001; Delmulle et al., 2005). The ELISA and affinity column techniques are advantageous over the RIA in terms of speed, ease of sample preparation and use, which also makes them more suitable for field use. Portable membrane-based flow-through enzyme immunoassays have also been developed for the field assessment of aflatoxin M_1 in milk (Sibanda et al., 1999) and aflatoxin B_1 in pig feed (Delmulle et al., 2005). Commercially available ELISA kits have been validated against established methods and found to be useful and reproducible, but not down to the maximum levels of aflatoxin B_1 stipulated by many regulatory agencies (Azer and Cooper, 1991; Ward and Morgan, 1991). This has limited their application for quantitative estimations in samples with low levels of contamination.

Despite the advantages of HPLC over TLC, of speed, automation, greater accuracy and precision, TLC has remained very popular for aflatoxin determination especially in resource challenged developing countries (Moss, 1998; Van Egmond et al., 1991; Trucksess et al., 1994). More recently, a lot of effort has been put into developing

reproducible and sensitive methods based on HPTLC (Moss, 1998; Ali et al., 1999). Coke et al. (1988) and Tomlins et al. (1989) achieved limits of detection of less than 1 µg/kg using bi-directional HPTLC.

Both reverse- and normal-phase HPLC methods have been developed for the analysis of aflatoxins (Papp et al., 2002). Extraction is mostly carried out using mixtures of chloroform-water (Van Egmond et al., 1991), methanol-water (Trucksess et al., 1984; Otta et al., 2000), or acetonitrile-water (Trucksess et al., 1994; Otta et al., 2000). Traditionally extract clean-up was achieved using column chromatography and liquid-liquid partitioning. These have now been replaced by the use of silica gel, C_{18} bonded-phase or Florisil based SPE columns (Van Egmond et al., 1991; Papp et al., 2002). Clean-up with immunoaffinity columns prior to analysis is also widely reported (Scudamore et al., 1997; Martins et al., 2001). Due to the diminishing of the fluorescence intensities of aflatoxin B_1 and G_1 in reverse-phase solvent mixtures, derivatisation is often performed pre- or in most cases post-column. Common derivatising agents used include trifluoroacetic acid for pre-column derivatisation (Scott and Lawrence, 1997), and iodine or bromine for post-column derivatisation (Van Egmond et al., 1991; Trucksess et al., 1991). HPLC separation and detection without derivatisation has also been reported (Braga et al., 2005). Moss (1998) stated that HPLC coupled with post-column derivatisation with iodine and fluorescence detection is currently the method of choice owing to its sensitivity and reproducibility. The robustness of this method was demonstrated by Van Egmond et al. (1991) in a European Community collaborative study.

1.8.3 Multi-mycotoxin analysis

The inclusion of regulatory limits for several mycotoxins such as aflatoxins, tricothecenes, ochratoxins and fumonisins that potentially occur together on corn in the legislation of several countries, has lead to an increase in the importance of analytical methods capable of simultaneously analysing these mycotoxins. These methods minimize the labour, costs and time required to acquire results. Frisvad and Thrane (1987) developed a HPLC-UV method for the analysis of 182 structurally different mycotoxins. Their study is regarded as the pioneering work in the area of multi-toxin analysis (Krska et al., 2005). Since then HPLC-MS has shown the greatest

potential for the simultaneous detection of mycotoxins and their degradataion products (Fuchs et al., 2002) and several methods have been developed to date to detect mostly tricothecenes and aflatoxins (Berger et al., 1999; Drifield et al., 2003; Delmulle et al., 2006). Interestingly, the potential use of immunochemical biosensor assays for the rapid (within 25 minutes) detection multiple mycotoxins (aflatoxin B_1, zearalenone, deoxynivalenol and fumonisin B_1) has been reported by van der Gaag et al. (2003).

1.9 Predictive Mycology

1.9.1 Origins of Predictive Mycology

In order to improve the quality and safety of food, a need exists for tools allowing the prediction of fungal growth (Dantigny et al. 2003, 2005a). The inherent differences between fungal and bacterial growth imply that such tools take into account the specificities of mould growth (Gibson and Hocking, 1997; Dantigny et al. 2005a). The term 'predictive mycology' was therefore coined to differentiate the modelling of fungal growth and mycotoxin production from that of bacteria (Dantigny et al., 2005a). Predictive mycology however still borrows a lot of techniques from mainstream predictive microbiology (Gibson et al., 1994; Dantigny et al., 2005a; Cuppers et al., 1997; Pitt, 1993). In light of the agricultural and economic importance of fungi, predictive mycology could be very useful for making predictions on the extent of contamination or growth and toxin production by fungi (Dantigny, 2003).

Due to difficulties in assessment of fungal growth rates and gathering of sufficient, suitable and reproducible data, the modelling of fungal growth has lagged well behind that of bacteria, especially pathogenic bacterial strains (Gibson et al., 1994; Gibson and Hocking, 1997; Marin et al., 2004). Bacteria reproduce by fission, with growth normally taking place only at the surfaces of solid substrates or homogenously through a liquid or slightly viscous or visco-elastic medium. In difference fungi are not unicellular with growth involving germination followed by hyphal extension throughout the physical three-dimensional matrices of foods (Dantigny et al., 2003, 2005a; Gibson and Hocking, 1997). This has resulted in the status quo where no rapid

or simple indirect method exists to estimate fungal growth with respect to time (Gibson and Hocking, 1997; Marin et al., 2004).

Measurement of membrane specific compounds such ergosterol or chitosan cannot be directly related to fungal mass, as they vary with the substrate, growth conditions and age of the mycelium. Colony diameters have been the most frequently used estimate of fungal growth and recently Marin et al. (2004) reported that they are better correlated to fungal growth than CFU counts. Various workers have developed mechanistic/semi-mechanistic and empirical models for a variety of toxigenic and spoilage fungi, describing both germination and hyphal extension. Excellent reviews on the advances in modelling of fungal growth are given by Gibson and Hocking (1997) and Dantigny et al. (2005a, 2006), some of which are described below in the order in which fungal growth occurs. To a lesser extent models describing the influence of environmental factors on mycotoxin have also been developed, and these are also discussed in this section.

1.9.2 Germination models

Marin et al. (1996) modelled for the first time the germination of *F. verticillioides* spores isolated from corn as a function of time using the modified Gompertz equation:

$$\%\ \text{germination} = A\exp\left\{-\exp\left[\left(\frac{\mu_m e}{A}\right)(\delta - t) + 1\right]\right\} \tag{1.1}$$

where A was the value at which % germination becomes constant (100% in most cases), μ_m was the Gompertz rate (h^{-1}) and δ was the lag phase (h). The Gompertz equation has also been used to the model germination kinetics of *Aspergillus* and *Penicillium* spp. on a variety of growth substrates (Marin et al., 1998b; Pardo et al., 2004, 2005a, 2005b, 2006).

Dantigny et al. (2002) used the logistic function shown below to describe the germination kinetics of *Mucor racemosus*.

$$P = \frac{P_{\max}}{(1 + \exp(k(\tau - t)))} \tag{1.2}$$

where P was % germination, P_{max} (%) was maximum percentage of germinated spores (this was substituted with 100 as all spores were capable of germinating), k was the rate factor (h^{-1}) and τ was the time (h) when 50% of the spores had germinated. According to Dantigny et al. (2002), the logistic function seems to perform better than the Gompertz equation. Polynomial equations have been used by Sautour et al. (2001c) to model the interaction of a_w, temperature and pH on the germination of *P. chrysogenum* and El Halouat and Debevere (1997) to model the influence of a_w, headspace $\%CO_2$ and $\%O_2$ on the germination time of moulds isolated from prunes.

1.9.3 Primary growth models

The building of growth models for fungi is in most cases a two-stage process, first being the estimation of the growth parameters under varying conditions by fitting primary models to the raw growth data. Secondary models, describing the effect of the variables investigated, are then fitted to the estimated growth parameters. The primary model developed by Baranyi et al. (1993) describing colony diameter as a function of time has been used to estimate the growth parameters (growth rate and lag phase duration) of *P. roquefortii* (Valik et al., 1999), heat resistant *Byssochlamys fulva*, *Neosatorya fischeri* and *Talaromyces avellanus* (Valík and Piecková, 2001), *Aspergillus* spp. (Gibson at al., 1994; Baranyi et al., 1996) and *P. brevicompactum* (Membré and Kubaczka, 2000).

The growth rates and lag phase durations can also be estimated by a simple regression model:

$$d = \mu(t - \lambda) \tag{1.3}$$

where d is the colony diameter (mm), μ is the growth rate (mm d^{-1}), t is the time (d) and λ is the lag phase duration (d) (Dantigny, 2003; Dantigny et al. 2003, 2005a). For any value of $t < \lambda$, the colony diameter is equal to its initial diameter. Linear regression has been also used to estimate the growth parameters of *Aspergillus*, *Penicillium*, *Cladosporium*, *Mucor*, *Eurotium* and *Alternatia* spp. on a wide variety of growth substrates (Marin et al., 1995, 1998a, 1998b, 1999b; Velluti et al. 2000; Sautour et al., 2001a, 2001b, 2002; Dantigny et al. 2002, 2005b; Lopez-Malo et al., 2005).

The Gompertz function (Eq. 1.1, Marin et al., 1996) described above to model germination kinetics has also been used as a primary growth model by Guynot et al. (2003) to estimate the lag phase before visible growth of *Eurotium*, *Aspergillus* and *Penicillium* spp. under modified atmosphere packaging. Although the lag phase for growth has no biological meaning as it is estimated from macroscopic observations of mycelial colonies (Dantigny et al., 2005a), Dantigny et al. (2002) demonstrated that when the conditions are carefully controlled the lag time coincided with the completion of the germination process for *M. racemosus*. They suggested that this may allow macroscopic examinations to substitute microscopic ones normally employed to determine if germination has occurred or not.

1.9.4 Secondary models

Generally two types of the secondary models – a) mechanistic/semi-mechanistic and b) empirical models – have largely been used to describe the effects of various environmental and preservative agents on fungal growth. Mechanistic models attempt to describe what is actually occurring during growth (Gibson and Hocking, 1997), giving more insight into the behaviour of biological systems than empirical models do (Pitt, 1993). Empirical models simply describe the effect of the conditions under which an experiment is performed on an aspect of growth of the microorganism being investigated (Gibson and Hocking, 1997). Applying the predictions of empirical models outside of the experimental conditions used to develop them is generally considered hazardous.

1.9.4.1 Mechanistic or semi-mechanistic models

Skirdal and Eklund (1993) used a relatively simple mechanistic model to study the effects of sorbic acid and pH on the growth of *P. chrysogenum*, *Cladosporium cladosporiodes* and *Ulocladium atrum*. The Minimum Inhibitory Concentration (MIC) was modelled as a function pH.

$$MIC = \frac{1}{[(1-a)/k_1 + (a/k_2)]} \quad (1.4)$$

where a is the ratio between dissociated and undissociated acid and k_1 and k_2 are the MIC of the dissociated and undissociated acid. Although the model was useful for

explaining the preservative effect of sorbic acid at varying pH levels, it did not take into account the effects of other factors that are present in the foods that may also have an effect on mould growth. Pitt (1993) developed a semi-mechanistic model integrating literature data to describe the effects of temperature, pH, a_w and colony size on mould growth and aflatoxin production. The author stated that the model correctly predicted the decrease in optimum temperature for aflatoxin production with increasing time, but did not accurately represent the effects of spore load on the maximum toxin concentration. This type of semi-mechanistic model may be considered superior to an empirical because it describes rates of growth that are governed by assumptions about mould biology and physical laws of diffusion (Gibson and Hocking, 1997).

1.9.4.2 Empirical models

Empirical models by virtue of their simplicity have been the most widely employed models to describe fungal growth. Gibson at al. (1994) investigated the appropriateness of a model (Eq. 1.5) previously used to predict bacterial growth for the interpretation of the growth data (colony diameter *versus* time) of *A. flavus*, *A. oryzae*, *A. parasiticus* and *A. nomius*:

$$\text{In}(g) = C_0 + C_1 b_w + C_2 b_w^{\ 2} \qquad (1.5)$$

In the model a_w is transformed to b_w, by $b_w = \sqrt{1 - a_w}$ which is more suitable for parabolic fitting (Gibson at al., 1994; Valík and Piecková, 2001). The same model was also successfully used by Valík and Piecková (2001) to describe the effect of a_w on the colony growth rates of heat resistant fungi on Sabouraud agar and has also been used to demonstrate how predictive models could be used to measure the relatedness of some *Aspergillus* species (Baranyi et al., 1997).

Fang et al. (1994) developed a second-order polynomial model (Eq. 1.6) describing the combined effects of chitosan and sugar concentration (°Brix) on the growth of *A. niger* and *A. parasiticus* on low sugar kumquat candies. The 'number of days to visible growth' (y, d) was monitored turbidometrically.

$$y = a + b[chitosan] + c[°Brix] + d[chitosan]^2 + e[chitosan][°Brix] + f[°Brix]^2 \quad (1.6)$$

The coefficients *a*, *b…f* where generated by regression. Pardo et al. (2004, 2005a, 2005b) used polynomial equations to model the influence of a_w and temperature on the lag phase and ochratoxin A production of *A. ochraceus* on irradiated barley and synthetic grape juice medium. Polynomial models have also been used by Marin et al. (1999c) to describe the individual effects of a_w and temperature on fumonisin B_1 production and by Guynot et al. (2003) to describe the influence of a_w and headspace CO_2 concentration on the lag phase before visible growth. Ellis et al. (1993) extended the function used by Guynot et al. (2003) to include the effects of headspace O_2 and pH on the colony diameter (mm). They also found a curvilinear relationship between growth and aflatoxin production: aflatoxin (µg/kg) = 0.017 + 0.060×Growth - 0.048×Growth^2. Although polynomial models tend to find application in many situations and indeed give good fits, they tend to '*overfit'* or give illogical predictions at growth limiting conditions unless some (carefully designed) precautions are taken to avoid this (Geeraerd et al., 2004).

Dantigny et al. (2005b) re-parameterized the Monod-type equation of Houtsma et al. (1994) to develop a model to describe the inhibitory effect of ethanol on the *Aspergillus*, *Cladosporium*, *Eurotium*, *Mucor*, *Penicillium* and *Rhizopus* spp. The model (Eq. 1.7) adequately described curves of growth rate (µ, mm d^{-1}) vs. *E* (ethanol, %), and estimated the concentrations at which growth did not occur (E_{max}, %) to be in the range 3-5%. *K* was a model coefficient related to the shape of the curves.

$$\mu = \mu_{opt} \frac{K(E_{max} - E)}{KE_{max} - 2KE + E_{max}E} \quad (1.7)$$

Cardinal models with inflexion (CMI) have also been used to describe the influence of several environmental factors on the growth of food spoilage moulds (Cuppers et al., 1997, Sautour et al., 2001a). These models enable the estimation of biologically important cardinal parameters such as the minimal, optimum and maximal temperatures or a_w values for growth. In Sautour et al. (2001a) a temperature type CMI model (Eq. 1.8) was developed based on a Rosso-type function to describe the relationship between a_w and the growth rates of *A. flavus*, *P. chrysogenum*, *C.*

cladosprium and *A. alternata* on potato dextrose agar. Excellent correlation was reported between the experimental data and the model predictions, and the estimated cardinal water activities were in accordance with literature data, although they seemed to be slightly underestimated for *A. flavus* and *P. chrysogenum*. Doubt however has been raised with respect to the use of CMI models to describe the effect of temperature (Dantigny et al., 2005a), an example of this being the illogical estimates reported of -12°C for the $T_{\min}$ of *P. roqueforti* by Cuppers et al. (1997).

$$\text{In}\mu = \text{In}\frac{\mu_{opt}(a_w - a_{w\max})(a_w - a_{w\min})^2}{(a_{wopt} - a_{w\min})\left[(a_{wopt} - a_{w\min})(a_w - a_{wopt}) - (a_{wopt} - a_{w\max})(a_{wopt} + a_{w\min} - 2a_w)\right]} \qquad (1.8)$$

CHAPTER 2

Sorption isotherms and isosteric heats of sorption of whole yellow dent corn [2]

[2] Redrafted from: Samapundo, S., Devlieghere, F., De Meulenaer, B., Atukwase, A., Lamboni, Y. and Debevere, J. M. 2006. *Journal of Food Engineering (in press:* doi:10.1016/j.jfoodeng.2006.01.040)

Chapter 2. Sorption isotherms and isosteric heats of sorption of whole yellow dent corn

2.1 Abstract

The adsorption and desorption isotherms of yellow dent corn were determined at 25, 30 and 37°C by the gravimetric method. The GAB, Oswin, Halsey, Henderson, Chung-Pfost and polynomial models were fitted to the experimental sorption data. The isotherms exhibited Type II behaviour and were temperature dependent as the equilibrium moisture content decreased with increase in temperature. Hysteresis was also observed at all temperatures investigated. According to the goodness of fit test applied, the GAB model best described the experimental data followed by the Oswin, Chung-Pfost, polynomial and Henderson models. The Halsey model was the least adequate. The monolayer moisture content was found to decrease with increasing temperature and was higher for desorption than adsorption. The isosteric heat of sorption was determined using the Claussius-Clapeyron equation and it was found to markedly decrease at the average temperatures investigated with increase in moisture content. The GAB model was selected as the model for use in subsequent experiments to adjust the a_w *of the yellow dent corn growth substrate.*

Key words: yellow dent corn, sorption isotherms, isosteric heat of sorption, hysteresis

2.2. Introduction

The development of sorption isotherms for grains and seeds is essential for the establishment of humidity boundaries to inhibit fungal growth and mycotoxin production during storage (Boente et al., 1996). Moisture sorption isotherms represent the relationship between the equilibrium moisture content and water activity (a_w) at constant temperatures and pressures (Kaymak-Ertekin and Gedik, 2004). As food materials have complex compositions and structures, sorption isotherms actually describe the integrated hygroscopic properties of the various constituents or the sorption mechanism and the interaction of food biopolymers with water (Kaymak-Ertekin and Gedik, 2004).

Sorption isotherms are extremely important for modelling drying processes, for the design and optimisation of drying equipment, for prediction of shelf-life stability, for calculation of moisture changes which may occur during storage and for selecting appropriate packaging materials (Gal, 1987). Drying, which results in reduction of a_w, is the most widely used method for preservation of stored grains and seeds (Boente et al., 1996). The most widely used control parameter for drying processes is the moisture content, and therefore an understanding of the relationship between a_w and moisture content is an absolute necessity to achieve optimal storage stability in grains and seeds.

The objectives of this chapter were (*a*) to determine the adsorption and desorption isotherms of yellow dent corn at various temperatures (*b*) to assess several sorption models (including semi-empirical and empirical models) for their ability to describe the experimental sorption data and (*c*) to determine the net isosteric heat of sorption of the corn and the effect of temperature and the sorption process (adsorption or desorption) on its value. The best fitting sorption isotherm was to be used as a tool for reproducibly adjusting the a_w of the yellow corn to desired values in subsequent experiments that determined the combined effects of a_w and other factors such as temperature or preservative factors on the growth and mycotoxin production of fungi of importance to corn. The development of a reliable tool for adjusting the a_w of the target growth substrate was of prime importance to the success of the experiments done in this PhD study as the collection of sufficient and reproducible growth data remains the major bottleneck in predictive mycology.

2.3. Materials and Methods

2.3.1 Materials

Yellow dent corn from Belgian fields was supplied by Aveve NV (Belgium). The corn had an initial moisture content and a_w of 12.79 ± 0.45 kg/100kg dry matter and 0.698 ± 0.015, respectively. The corn was kept in cold storage at 7°C until use. For adsorption isotherms the corn was initially dried for at least 3 weeks over calcium sulphate (Merck, Darmstadt, Germany) until no appreciable weight loss was noted, after which it was used for development of the adsorption isotherms by the static

gravimetric method. For the desorption isotherms the corn was initially placed over pure distilled water until no appreciable weight gain was observed before use. Ten saturated salt solutions of different relative humidity's were selected to give different a_w values in the corn, ranging from 0.110 to 0.972. The relative humidity's/ a_w values of the salt solutions at different temperatures were taken from Labuza (1984), Resnik and Chirife (1988) and McLaughlin and Magee (1998). Approximately 50 g of corn was placed in glass jars containing differing salt solutions and three replications of the same experiment were carried out. A small amount of toluene was placed in each jar to prevent the growth of fungi (Labuza, 1984; Raspusas, 1993). The jars were then placed in incubators at 25 ± 1°C, 30 ± 1°C and 37 ± 1°C. Additional adsorption isotherms were also created at 16 and 22°C (results not shown).

The samples were left to equilibrate until the weight was constant over at least three consecutive days. The total weighing time was maintained at less than 30 s to reduce the sorption of atmospheric moisture. The equilibrium moisture content was determined by drying in a vacuum oven (AOAC, 1980), and the exact value of the corresponding a_w was confirmed by the Novasina Thermoconstanter TH200 (Axair Ltd. Systems, Pfapfikkon, Switzerland).

2.3.2 Modelling of sorption isotherms

Six sorption isotherm models, shown in Table 2.1, were fitted to the experimental data. These sorption models are amongst those most widely used to describe sorption isotherms for various food materials. The parameters of the sorption models were estimated using non-linear regression function of SPSS® Version 11.0 (SPSS Inc., Chicago). The goodness of fit of the models was evaluated by means of the mean relative percentage deviation modulus (P), defined as

$$P(\%) = \frac{100}{N} \sum_{i=1}^{N} \frac{|M_{ei} - M_{ci}|}{M_{ei}} \qquad (2.1)$$

where M_{ei} and M_{ci} are the experimental and predicted moisture content values, respectively, and N is the number of experimental data. A model is considered acceptable if it has a P value less than 10% (Lomauro et al., 1985a).

2.3.3. Determination of the net isosteric heat of sorption

The isosteric heat of sorption is defined as the total heat of sorption of water from the material minus the heat of vaporisation of the water (McLaughlin and Magee, 1998). The Claussius-Clapeyron equation (Eq. 2.2) was used to calculate the net isosteric heat of water sorption (Q_{st}, kJ/mol) as described by Bell and Labuza (2000):

$$Q_{st} = \frac{RT_1T_2}{T_2 - T_1} In \frac{a_{w2}}{a_{w1}} \quad (2.2)$$

where R is the universal gas constant (8.314×10^{-3} kJ/mol K) and a_{w2} and a_{w1} are the water activity values at temperatures T_2 and T_1, respectively. The a_w values at various moisture content levels were calculated using the GAB model.

Table 2.1. Equations used to describe the sorption isotherms

Name	Equation
GAB (Guggenheim-Andersen-de Boer)	$M = \frac{m_0 CKa_w}{[(1-Ka_w)(1-Ka_w+CKa_w)]}$
(Van den Berg, 1985)	
	$C = C_0 \exp(\Delta H_c / RT)$
	$K = K_0 \exp(\Delta H_k / RT)$
Halsey (Halsey, 1948)	$a_w = e^{(-k/M^n)}$
Oswin (Oswin, 1946)	$M = k(a_w / 1 - a_w)^n$
Chung & Pfost (Chung & Pfost, 1967)	$M = a + bIn(-Ina_w)$
Henderson (Henderson, 1952)	$1 - a_w = e^{(-kTM^n)}$
Polynomial	$M = a + ba_w + ca_w^{2}$

a: constant, k: constant, a_w: water activity, b: constant, C: GAB model parameter, K: GAB model parameter, m_0: monolayer moisture content (kg/100kg dry matter), ΔH_c: enthalpy difference between monolayer and multilayer sorption (J mol^{-1}), ΔH_k: difference between heat of condensation and heat of sorption of the multilayer sorption (J mol^{-1}), M: moisture content (kg/100kg dry matter), T: temperature (K), n: constant, c: constant, C_0: constant, K_0: constant, R: universal gas constant (8.314 J mol^{-1} K^{-1}).

2.4. Results and Discussion

2.4.1 Sorption isotherms

The adsorption and desorption isotherms at 25, 30 and 37°C are shown in Fig. 2.1. The isotherms have a sigmoidal shape depicting an increase in the equilibrium moisture content with a_w. This is typical of Type II isotherms (Brunauer et al., 1940), and has been reported for starchy products such as potato and wheat starch (Van den Berg, 1981), potato starch gel (McMinn, 1996) and cookies and corn snacks (Palou et al., 1997).

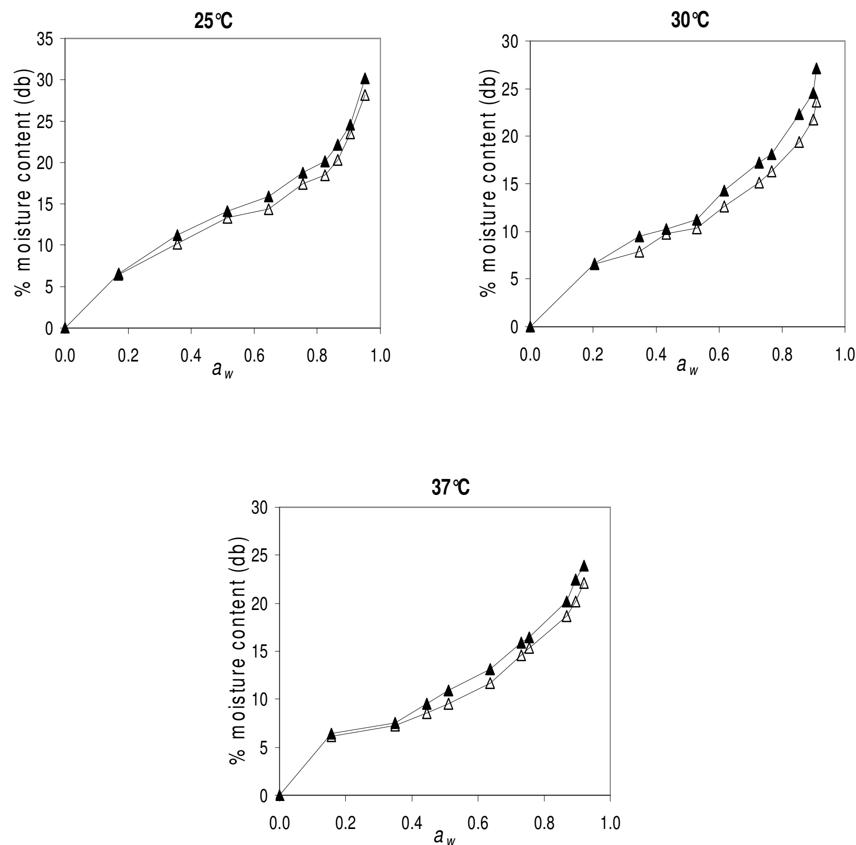


Fig. 2.1. Adsorption (△) and desorption (▲) isotherms of yellow dent corn.

Hysteresis can also be observed over the water range investigated as the equilibrium moisture content for desorption is higher than that for adsorption. Despite the general agreement that some thermodynamically irreversible processes must occur during desorption and/or adsorption for hysteresis to occur, this phenomenon is not fully understood (McLaughlin and Magee, 1998; Al-Muhtaseb et al., 2004). Mohsenin

(1986) proposed that in the wet condition the polar sites available are not entirely satisfied with adsorbed water. The shrinkage that occurs upon drying draws them closer enabling them to satisfy each other. This manifests itself as a reduction of the water binding capacity of the matrix upon subsequent adsorption.

The temperature dependence of the sorption isotherms can be seen in Fig. 2.2 where the equilibrium moisture content is seen to increase with decrease in temperature at the same a_w, or a_w is seen to increase with temperature at the same equilibrium moisture content. This indicates that the corn becomes less hygroscopic when temperature is increased.

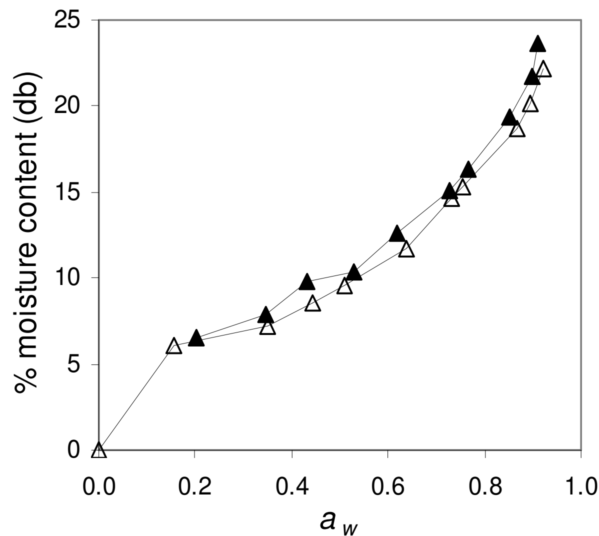


Fig. 2.2. Adsorption isotherms of yellow dent corn at 30 (▲) and 37°C (△).

A number of workers have suggested explanations for this observation. McLaughlin and Magee (1998), Palipane and Driscoll (1992) and Mohsenin (1986) explained this trend as being due to the increased state of excitation of molecules at higher temperatures, leading to an increase in distance and corresponding decrease in attractive forces between them. This gives rise to the observed decrease in degree of water sorption at a given relative humidity as the temperature is increased. Whereas Mazza and LeMaguer (1980) suggested that increase in temperature induces physical and/or chemical changes in the product that can reduce the number of active sites for water binding. The temperature dependence of the equilibrium moisture content has

an important practical bearing on chemical and microbiological reactions associated with spoilage (Al-Muhtaseb et al., 2004). At the same moisture content, higher temperatures entail a higher a_w and consequently faster rates of deterioration (Van den Berg and Bruin, 1981).

Tables 2.2 and 2.3 show the coefficients of the models fitted to the experimental adsorption and desorption data by non-linear regression, respectively; and *P* (%) the mean relative percentage deviation modulus. The determination coefficients (r^2) were in all cases greater than 0.949.

Table 2.2. Estimated parameters and *P* (%) values of the sorption equations fitted to the adsorption isotherm data of yellow dent corn

Model	Temperature (°C)		
	25	30	37
GAB			
m_o	7.444	6.566	6.030
C	34.557	29.169	37.540
K	0.764	0.790	0.792
P (%)	5.56	2.50	3.12
Polynomial			
a	7.684	8.040	7.170
b	-5.835	-11.029	9.663
c	25.324	29.486	27.245
P (%)	8.25	3.79	2.26
Oswin			
k	12.124	10.474	9.851
n	0.287	0.349	0.338
P (%)	4.23	2.75	4.66
Halsey			
k	180.104	79.959	74.560
n	2.266	2.039	2.093
P (%)	12.05	5.79	15.89
Chung-Pfost			
a	9.985	8.449	8.035
b	-5.793	-6.055	5.489
P (%)	3.41	3.87	5.78
Henderson			
k	4.1×10^{-5}	6.21×10^{-5}	5.97×10^{-5}
n	1.664	1.548	1.594
P (%)	7.06	5.28	5.21

With the exception of the Halsey models developed for adsorption data, all models had an average $P \leq 10\%$, and can therefore be considered to be adequate for describing experimental sorption data for yellow dent corn. The Halsey model only had a $P < 10\%$ when fitted to experimental adsorption data at 30°C and desorption

data at 30 and 37°C. Al-Muhtaseb et al. (2004) and Wang and Brennan (1991) have also found the Halsey model to be inadequate for representing the sorption isotherms for starch powders and potatoes, respectively. Tolaba and Saurez (1990) and Boente et al. (1996) however reported that the Halsey model gave a good fit to experimental desorption isotherms for shelled maize and sorption data for flint dent maize, respectively. The GAB model gave the smallest average mean relative percentage deviation followed by the Oswin, Chung-Pfost, polynomial and Henderson models for adsorption.

Table 2.3. Estimated parameters and P (%) values of the sorption equations fitted to the desorption isotherm data of yellow dent corn

Model	Temperature (°C)		
	25	30	37
GAB			
m_o	8.457	7.467	6.838
C	22.975	17.352	24.410
K	0.745	0.794	0.780
P (%)	5.86	3.13	3.13
Polynomial			
a	7.317	8.442	7.097
b	-1.611	-11.719	-7.596
c	23.567	33.538	27.262
P (%)	8.21	5.48	2.95
Oswin			
k	13.112	11.596	10.775
n	0.283	0.363	0.337
P (%)	4.79	3.70	4.10
Halsey			
k	323.559	71.687	70.508
n	2.407	1.926	1.993
P (%)	12.11	7.36	7.96
Chung-Pfost			
a	10.782	9.222	8.757
b	-6.179	-7.098	-6.006
P (%)	3.68	4.12	4.99
Henderson			
k	3.11×10^{-5}	6.19×10^{-5}	4.99×10^{-5}
n	1.713	1.486	1.608
P (%)	5.94	5.58	4.46

The same trend was observed for desorption, the only exception being that the Henderson model had a smaller average mean relative percentage deviation than the polynomial model. However, it must be mentioned that the goodness of fit of a sorption model to experimental data does not describe the nature of the sorption process. Also as water is associated with the food matrix by different mechanisms in

different a_w regions, no single model can be considered accurate over the entire a_w range (Labuza, 1975). The GAB model has also been previously reported to be the best model to predict food isotherms (Van den Berg and Bruin, 1981; Bizot, 1983; Lomauro et al., 1985a, 1985b; McLaughlin and Magee, 1998). The experimental adsorption data and the fitted models are shown in Figs. 2.3-2.8.

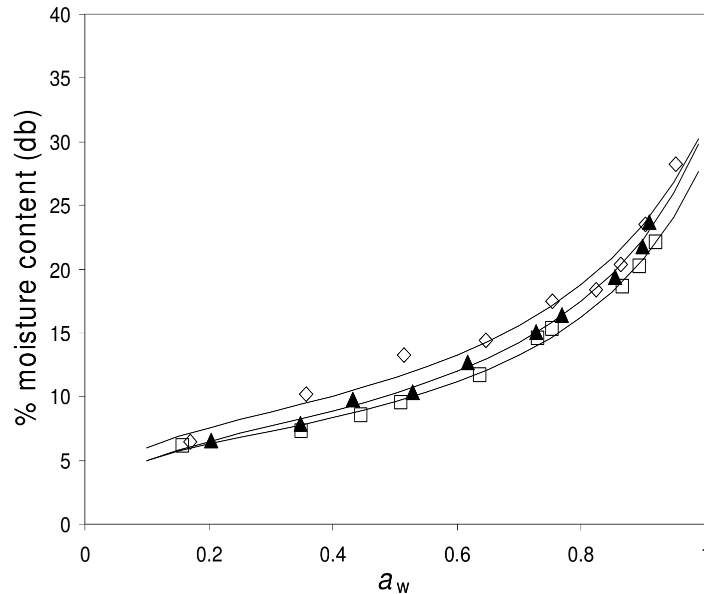


Fig. 2.3. Sorption isotherms of yellow dent corn at 25 (◇), 30 (▲) and 37°C (□) with the fitted GAB model (solid lines).

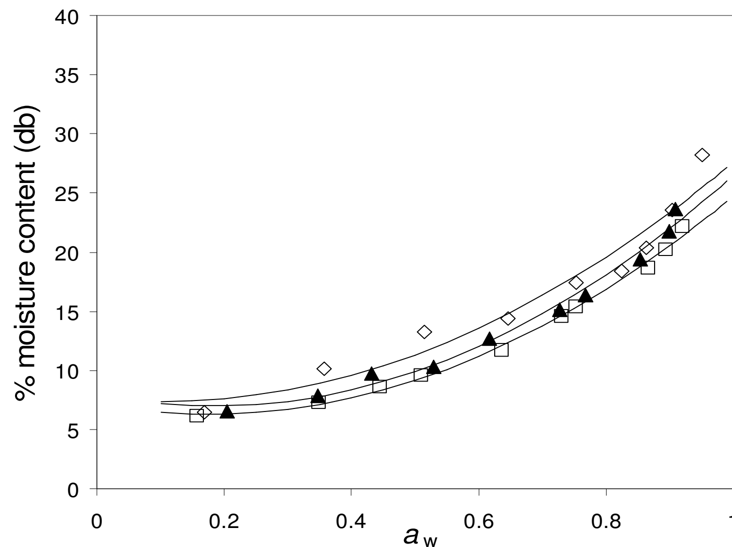


Fig. 2.4. Sorption isotherms of yellow dent corn at 25 (◇), 30 (▲) and 37°C (□) with the fitted polynomial model (solid lines) at different temperatures.

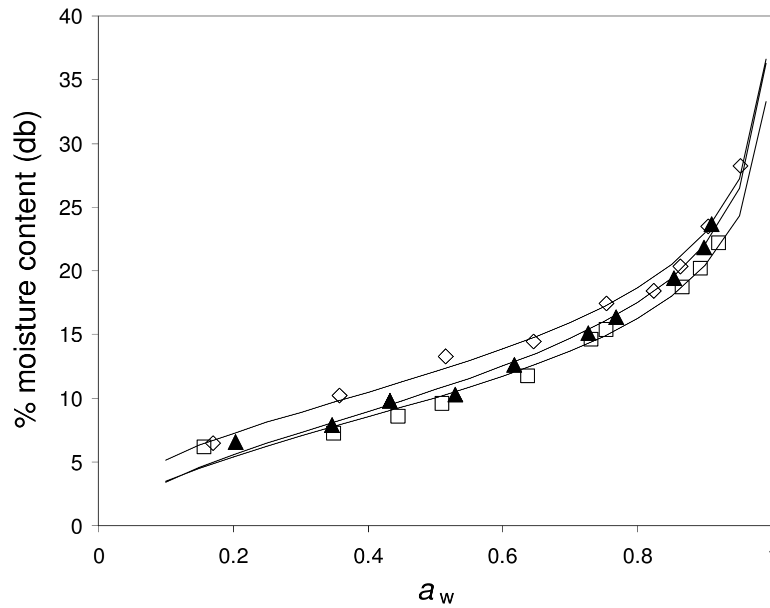


Fig. 2.5. Sorption isotherms of yellow dent corn at 25 (◇), 30 (▲) and 37°C (□) with the fitted Chung-Pfost model (solid lines) at different temperatures.

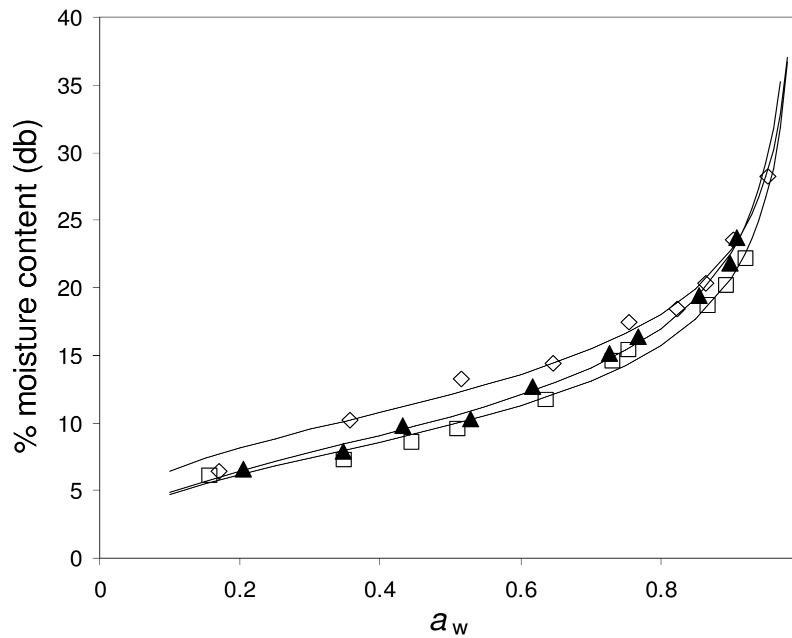


Fig. 2.6. Sorption isotherms of yellow dent corn at 25 (◇), 30 (▲) and 37°C (□) with the fitted Oswin model (solid lines) at different temperatures.

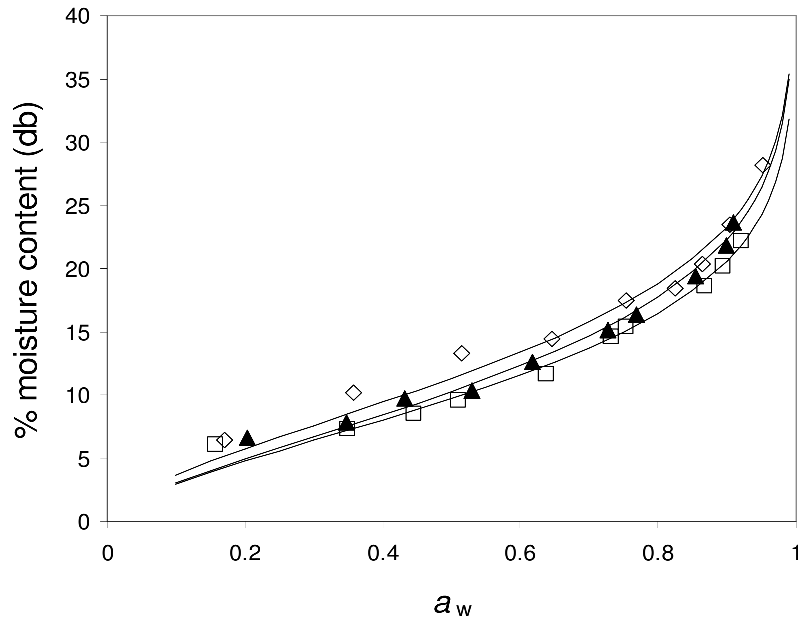


Fig. 2.7. Sorption isotherms of yellow dent corn at 25 (◇), 30 (▲) and 37°C (□) with the fitted Henderson model (solid lines) at different temperatures.

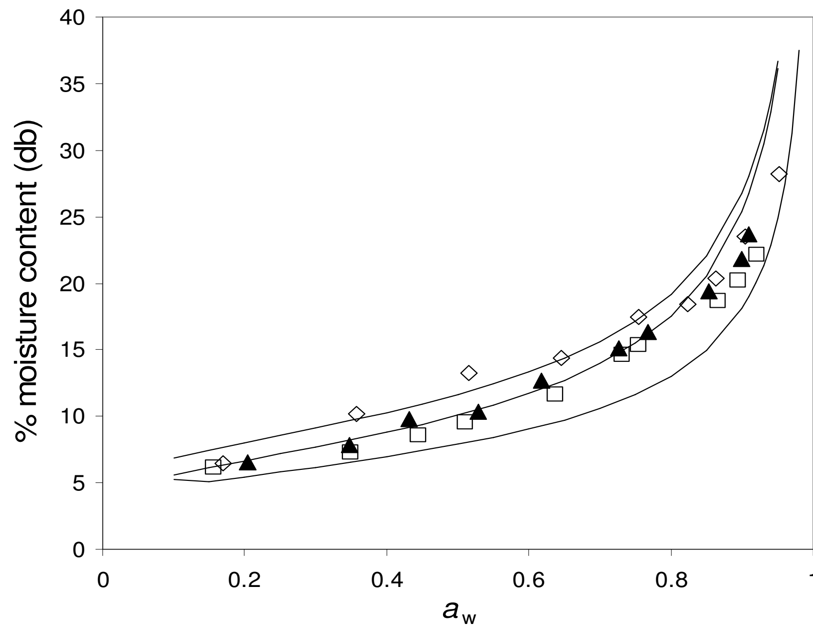


Fig. 2.8. Sorption isotherms of yellow dent corn at 25 (◇), 30 (▲) and 37°C (□) with the fitted Halsey model (solid lines) at different temperatures.

The estimated monolayer moisture contents (m_0) from the adsorption isotherms using the GAB equation were 7.44, 6.57 and 6.03 kg/100kgdm at 25, 30 and 37°C, respectively. Slightly higher monolayer moisture contents of 8.46, 7.47 and 6.84 kg/100kgdm at 25, 30 and 37°C, respectively, were determined from the desorption isotherms. Due to the few data points below a_w 0.4 the BET model could not be applied to the data to estimate m_0 values for comparison with those estimated by the GAB model. The estimated values are comparable to the values reported by Yanniotis (1994) of an average of 7.36 kg/100kgdm for starchy foods in the temperature range of 20-30°C. They are however less than 10.27 kg/100kgdm, reported for degermed corn flour by Kumar (1974) and 9.78 kg/100kgdm for desorption data for corn at 30°C (Hubbard et al., 1957), when the GAB model is used to describe the data. Much more comparable monolayer moisture contents of 7.93 and 7.39 kg/100kgdm for degermed corn flour and corn (desorption data), respectively, were obtained by the same workers using the BET model with moisture contents at a_w values between 0.05 and 0.4.

Values between 3.2 and 16 kg/100kgdm have been reported for starchy foods (Lomauro et al., 1985b). The apparent decrease in the monolayer moisture content with increase in temperature has also been reported by Al-Muhtaseb et al. (2004), McLaughlin and Magee (1998), Westgate et al. (1992) among several workers. This trend can also be explained by the variation of excitation states, distance and attraction between molecules as temperature is varied (McLaughlin and Magee, 1998; Westgate et al., 1992). Palipane and Driscoll (1992) also suggested that at higher temperatures some water molecules can break away from their sorption sites, therefore giving rise to lower monolayer moisture content values as temperature increases. It can also be observed from Tables 2.2 and 2.3 that the values of the values of the monolayer moisture contents are lower, and those of the GAB constant C are higher, for adsorption than for desorption. This can be due to the fact that although less sorption sites are available during adsorption, they have a greater binding energy, resulting in multilayer water molecules deviating more from the free bulk water. The monolayer moisture content (and its temperature and adsorption/desorption dependence) represents a very important storage and process design parameter as it is the optimal moisture content for storage.

2.4.2 Isosteric heats of sorption

Fig. 2.9 shows the isosteric heat of adsorption and desorption of yellow dent corn as a function of moisture content at average temperatures of 27.5 and 33.5°C. It can be clearly seen that the isosteric heats decreased sharply with increase in moisture content at both average temperatures. This has also been observed for sultana raisins (Saravacos et al., 1986), pistachio nuts (Yanniotis and Zarmboutis, 1996), cookies and corn snacks (Palou et al., 1997) and potatoes (McLaughlin and Magee, 1998). This marked decrease can be considered to be due to the fact that sorption initially occurs on the most active primary sites giving rise to higher exothermic interaction energies than those released when these sites become occupied (Iglesias and Chirife, 1976a). The monolayer moisture content also occurs at low moisture contents corresponding to strongly bound water. Iglesias and Chirife (1976b) reported that as net isosteric heat of sorption tends to zero, the influence of the adsorbent on the adsorbed molecules becomes negligible. The maximum isosteric heats calculated are higher than those reported for corn flour (18.6 kJ/mol) by Labuza et al. (1985). This difference could be due to the use of different varieties of corn and different criteria for determination of sorption equilibrium.

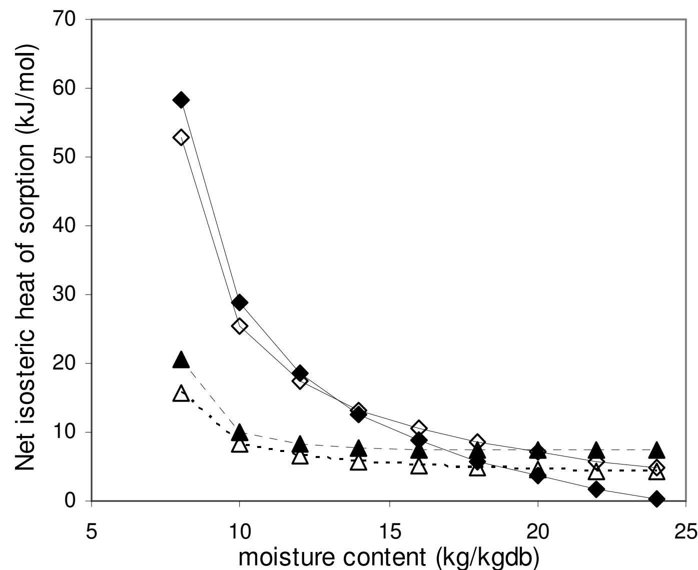


Fig. 2.9. The net isosteric heats of adsorption for yellow dent corn at 27.5 (◇) and 33.5°C (△) and those for desorption at 27.5 (◆) and 33.5°C (▲).

It can also be observed from Fig. 2.9 that the heat of desorption is greater than that of adsorption at low moisture contents for both average temperatures investigated, and remains greater throughout the entire moisture content range at an average temperature of 33.5°C. This indicates that the desorption process requires a greater amount of energy than adsorption. This has also been reported by Wang and Brennan (1991) and McLaughlin and Magee (1998) for potatoes, and Kaymak-Ertekin and Gedik (2004) for grapes, apricots, apples and potatoes. McLaughlin and Magee (1998) attributed this observation as probably being a result of changes in the molecular structure during sorption which could affect the degree of activation of the sorption sites. Knowledge of the isosteric heats of sorption is very important for equipment and process design (Rizvi, 1986), especially that of drying processes as the heats of sorption rise well in excess of the heat of vaporisation as a food is dehydrated to low moisture content (McLaughlin and Magee, 1998). In addition the integrated Claussius-Clapeyron equation also enables the prediction of isotherms at other temperatures (Palou et al., 1997).

2.5. Conclusions

The adsorption and desorption isotherms for yellow dent corn determined at 25, 30 and 37°C exhibit Type II behaviour. Temperature has an effect on the sorption behaviour, higher a_w values being observed at the same moisture content when temperature is increased. Hysteresis is evident as the equilibrium moisture content is higher for desorption than adsorption across the a_w range investigated. Amongst the models evaluated the Halsey model proved the least adequate function to describe the experimental data. The other models fit the data reasonably well, with the best fit being for the GAB model. The net isosteric heat was observed to increase with decrease in moisture content and decreased with increasing temperature. The net isosteric heat is also higher for desorption than adsorption at low moisture contents indicating that the desorption process involves more energy than adsorption.

All these observations play an important role in the determination of suitable preservative process designs, including the optimal drying process and atmospheric conditions for storage. On the basis of the goodness of fit, the GAB model was selected as the best tool for reproducibly adjusting the a_w of the corn. With this in

hand, the study now focused on the experiments to model the effect of a_w and temperature on the growth of the most important fungal contaminators of corn. The following chapter was the first part of this study, in this case modelling growth of *Fusarium verticillioides* and *F. proliferatum*.

CHAPTER 3

Predictive modelling of the individual and combined effect of water activity and temperature on the radial growth of *Fusarium verticillioides* and *F. proliferatum* on corn [3]

[3] Redrafted from: Samapundo, S., Devlieghere, F., De Meulenaer, B., Geeraerd, A.H., Van Impe, J.F., and Debevere, J. M. 2005. *International Journal of Food Microbiology* 105, 35-52.

Chapter 3. Predictive modelling of the individual and combined effect of water activity and temperature on the radial growth of *Fusarium verticillioides* and *F. proliferatum* on corn

3.1 Abstract

The major objective of this study was to develop validated models to describe the effect of a_w and temperature on the radial growth on corn of the two major fumonisin producing Fusaria, namely Fusarium verticillioides and F. proliferatum. The growth of these two isolates on corn was therefore studied at water activities (a_w) between 0.810-0.985 and temperatures between 15-30°C. Minimum a_w for growth was 0.869 and 0.854 for F. verticillioides and F. proliferatum, respectively. No growth took place at a_w values equal to 0.831 and 0.838 for F. verticillioides and F. proliferatum, respectively. The colony growth rates, g (mm d^{-1}) were determined by fitting a flexible growth model describing the change in colony diameter (mm) with respect to time (days). Secondary models, relating the colony growth rate with a_w or a_w and temperature were developed. From preliminary fitting phase a second order polynomial equation and the linear Arrhenius-Davey model provided the best and were further used to describe the combined effect of temperature and a_w on g. The combined modelling approaches, predicting g (mm d^{-1}) at any a_w and/or temperature were validated on independently collected data. All models proved to be good predictors of the growth rates of both isolates on maize within the experimental conditions. The second order polynomial equation had bias factors of 1.042 and 1.054 and accuracy factors of 1.128 and 1.380 for F. verticillioides and F. proliferatum, respectively. The linear Arrhenius-Davey model had bias factors of 0.978 and 1.002 and accuracy factors of 1.098 and 1.122 for F. verticillioides and F. proliferatum, respectively. The developed models can be applied for the prediction of Fusarium growth on corn in store, for the estimation of the environmental boundaries for the growth of these isolates on corn and as a basis for the development of models that incorporate other factors important to mould growth on corn.

Key words: *Fusarium verticillioides, Fusarium proliferatum*, mould growth, predictive modelling

3.2. Introduction

The modelling of fungal growth has lagged well behind that of bacteria, especially pathogenic bacterial strains due to the inherent difficulties in the assessment of fungal growth rates and gathering of sufficient, suitable and reproducible data (Gibson et al., 1994; Gibson and Hocking, 1997). Despite these difficulties a need exists for tools allowing the prediction of fungal growth in order to improve the quality and safety of food (Dantigny et al. 2003, 2005a). The severity of the agricultural problems associated with the spoilage of cereal grains and their poisoning by mycotoxins continues to threaten agricultural based livelihoods in both developed and developing countries. This alone provides sufficient motivation for studies that can contribute to the provision of solutions or mitigation of these problems.

To date literature reveals that the isolation and elucidation of the structure of the fumonisins has stimulated a lot of research on various aspects mostly related to the effect of various environmental factors on growth and toxin production by fumonisin producing Fusaria (Bacon and Williamson, 1992; Le Bars et al., 1994; Cahagnier et al., 1995; Marin et al., 1995,1996, 1998d; Chulze et al., 1999; Marin et al., 1999a,b,c; Velluti et al., 2000; Ono et al., 2002). In these studies a_w has emerged as the single most important determinant of fungal growth (Holmquist et al., 1983), with temperature also having a large but somewhat lesser effect on fungal growth. Despite this interest, till now there has only been one attempt to model the growth of fumonisin producing *Fusarium* species. In this study the percent germination of *F. verticillioides* and *F. proliferatum* with time was modelled by the modified Gompertz equation (Marin et al. 1996). The results presented in this chapter represent the first part of the study done to evaluate and validate predictive models describing the growth of fungi of greatest importance to corn. This is due to the development and modelling of the water relations of the growth substrate (yellow dent corn) in Chapter 2, which allowed for the reliable and reproducible control of the a_w of the corn. The major objective of this chapter was therefore to develop validated models to describe the effect of a_w and temperature on the radial growth on corn of the two major fumonisin producing Fusaria, *F. verticillioides* and *F. proliferatum.*

3.3 Materials and Methods

3.3.1 Fungal Isolates

Fusarium verticillioides Sheldon (25N) and *F. proliferatum* (Matsushima) Nirenberg (73N) were used in the study. The two isolates were obtained courtesy of the Food Technology Department Culture Collection of the University of Lleida, Spain. Both isolates were maintained on potato dextrose agar (PDA) (Oxoid, Basington, UK).

3.3.1.1 Experimental design

A full factorial design was used to investigate the growth of both isolates on corn. Five temperatures fixed at 15, 22, 25, 30 and 37°C and seven a_w values between 0.810 and 0.985 were examined. However, growth did not occur at 37°C. 20 replicates were prepared per condition.

3.3.1.2 Preparation of maize substrate

The maize was sterilized by 25 kGy of γ - irradiation at IBA Mediris (Fleurus, Belgium) to ensure no fungal infection or contamination of the substrate, whilst retaining its ability to germinate. The maize was then stored aseptically at 4-7ºC until further use. The adjustment of a_w was achieved by the direct addition of a certain amount of sterile distilled water as determined by the GAB sorption models developed for the grain in chapter 2. The grain was then allowed to equilibrate in two phases. Initially the grain was placed at 4ºC for 2 days, with periodic mixing. Thereafter, it was placed in perforated aluminium cups for a period of 7 days at the final incubation temperature, over a glycerol-water solution with a_w similar to that desired in the substrate. The final a_w was determined by a Novasina Thermoconstanter, TH200.

3.3.1.3 Preparation of inoculum, inoculation, incubation and growth assessment

An inoculation loop was used to aseptically scrape off sporulating mycelia from the surface of PDA slants on which the isolates were maintained. This was used to inoculate, centrally, the surface of petri plates (90 mm) containing PDA. The

inoculum was then grown first by incubating the petri plates for 6 days at 30°C. The growing colonies were then incubated at 15, 22, 25, or 30°C for one day to enable the isolates to adapt to the incubation conditions they were to be grown. About 24 g of rehydrated maize was placed in each sterile petri plate, to form a single layer of grains. A 5.6 mm diameter agar disk, cut from the margin of the seven day old growing colony on PDA, was transferred to the centre of each plate by means of a sterile cork borer. Petri plates containing grain of the same a_w were then placed over glycerol-water solutions of the same a_w in sealed containers. 20 petri plates were placed in each container. The containers were incubated at 15, 22, 25, and 30ºC.

From each container, four randomly chosen petri plates, containing growing colonies, were assessed periodically for changes in growth. The growth, assessed as the change in diameter of the growing circular colony, was determined by measuring two perpendicular diameters per chosen plate, the average of the four chosen colonies being used to evaluate the change in diameter at that particular time.

3.3.2 Mathematical and Statistical Methods

3.3.2.1 Primary modelling

The average diameters, (y, mm) at each time of measurement (t, days), were used in the growth modelling. The flexible growth function of Baranyi and Roberts (1994) was fitted to the growth data by means of the non-linear function of SPSS® Version 11.0 (SPSS Inc., Chicago). This enabled the determination of the maximum colony growth rate (g, mm d^{-1}) and for some of the curves a short lag phase. Division of g by two gives rise to the radial growth rate (mm d^{-1}). Following the approach of Baranyi and Roberts (1994), the form of the model is:

$$y\,(t) = y_0 + gA(t) - \frac{1}{m}\text{In}\left(1+\frac{e^{mgA(t)}-1}{e^{m(y_{max}-y_o)}}\right) \tag{3.1}$$

where $A(t) = t + \frac{1}{v}\text{In}\left(\frac{e^{-vt}+q_0}{1+q_0}\right)$ (3.2)

and $q_0 = \frac{1}{e^{v\lambda} - 1}$ (3.3)

The parameters are as follows: *m* Richards' curve parameter after the exponential phase (this was fixed as $m = 1$), y_0 diameter of the colony at time $t = 0$, *g* as defined above, y_{max} maximum colony diameter, λ duration of the lag phase, *v* rate of enzymatic reactions. Baranyi and Roberts (1994) made the assumption that as a general principle the growth rate cannot be higher then the rate of the slowest enzymatic reactions causing the bottle-neck in growth, to give a special case of their growth model. Hence *v* should be equal to *g*. This would give final forms of *A(t)* and q_0 used:

$$A(t) = t + \frac{1}{g}\text{In}\left(\frac{e^{-gt} + q_0}{1+q_0}\right) \quad (3.4)$$

and $q_0 = \frac{1}{e^{g\lambda} - 1}$ (3.5)

3.3.2.2 Secondary modelling

Effect of a_w

The maximum colony growth rate was then modelled as a function of a_w as follows. The a_w was initially transformed to b_w (Gibson et al., 1994) to enable better hyperbolic fitting, by: $b_w = \sqrt{1 - a_w}$. The natural logarithms of the colony growth rates were then modelled by the following quadratic function as described by Gibson et al. (1994) (Model 1).

$$\ln g = C_0 + C_1 b_w + C_2 b_w^2 \quad (3.6)$$

The coefficients C_0, C_1, and C_2 and the significance of their associated factors were determined by linear step-wise regression using SPSS Version 11.0.

Combined influence of a_w and temperature

Several potentially suitable functions including (i) Arrhenius type (ii) Bělehrădrek type and (iii) polynomial models, used to describe both fungal and bacterial growth, were fitted to the estimated colony growth rates in a first step during which the two most suitable models were identified on the basis of their residual mean square errors (MSE) and residual plots. In a second step, these two were then further evaluated and validated against independently collected data. Due to the fact there were no suitable estimates for the cardinal values from the experimental data the choice of models used in the first step was limited to mostly polynomial models (with or without parameter or response transformation and with or without constraints to avoid *overfitting*) and the linear Arrhenius-Davey model (Davey, 1989), a model that has been succesfuly used to describe bacterial growth. Of these the combined influence of a_w and temperature was best described and was therefore modelled by the following functions:

model 2 - a second order polynomial equation

$$g = C_0 + C_1 b_w + C_2 b_w^2 + C_3 T + C_4 T^2 + C_5 T b_w \qquad (3.7)$$

where T = degree Celsius (°C)

and model 3 - the linear Arrhenius-Davey equation (Davey, 1989)

$$\ln(g) = C_0 + C_1/T + C_2/T^2 + C_3 a_w + C_4 a_w^2 \qquad (3.8)$$

where T = degree absolute (°K)

The linear Arrhenius-Davey equation is an expansion of the original linear Arrhenius type model introduced and used by Davey (1989) to model the effect of incubation temperatures on bacterial growth: $\ln(k) = C_0 + C_1/T + C_2/T^2$, where k is the growth rate constant. The original activation energy of the Arrhenius equation is replaced by the two coefficients of inverse temperature. However as no physiological interpretation is offered for the significance of the value of the regression coefficients the equation is entirely empirical (Davey, 1989). It is one of the three most studied modifications of the Arrhenius equation used to model the impact of temperature on microbial growth (Buchanan, 1993; Ross and Dalgaard, 2003). Its advantages to similar models are its demonstrated wide application to the growth phase, simplicity,

and ease of use (Davey, 1991). This model, which is widely applied to bacterial growth, was assessed to determine how well it could describe fungal growth.

The coefficients of these two models and the significance of their associated factors were determined by fitting the models onto the estimated colony growth rates using the linear step-wise function of SPSS Version 11.0. It has to be mentioned that due to difficulties in hydrating the maize grain to a_w's greater than 0.985 the cardinal a_w's, optimum and maximum, could not be determined. Consequently other successful approaches for modelling fungal growth that are based on estimated cardinal a_w's as described by Cuppers et al. (1997), Dantigny (1998), Sautour et al. (2001a) and Sautour et al. (2002) could not be applied to the data gathered in these experiments.

3.3.3 Validation

3.3.3.1 Mathematical/Statistical Validation

To validate the models, the experiment was repeated for both isolates using the same four temperatures at which growth took place but at four a_w's different to those examined before, so a total of 16 validation experiments were carried out. The following mathematical and statistical indices were calculated to evaluate the performance of the predictive growth models in describing the observed experimental data adequately: *F*–values and bias and accuracy factors (Ross, 1996; te Giffel and Zwietering, 1999). *F*-values were calculated and compared with the tabulated *F*-values. The bias and accuracy factors provide an objective indication of model performance, by testing the hypothesis that the model being evaluated predicts the true mean or represents it better than another model. The bias and accuracy factors were calculated as follows:

$$\text{Bias factor} = 10^{\left(\Sigma \log(\mu_{\text{observed}} / \mu_{\text{predicted}})/n\right)} \quad (3.9)$$

$$\text{Accuracy factor} = 10^{\left(\Sigma \left|\log\left(\mu_{\text{observed}} / \mu_{\text{predicted}}\right)\right|/n\right)} \quad (3.10)$$

A model is considered to be 'fail safe' when a bias factor of < 1 is obtained (Ross, 1996; te Giffel and Zwietering, 1999). However a bias factor of 0.5 indicates a poor model that is overly conservative as it predicts growth rates that are on average, twice

as large as the observed values. A bias factor larger than 1.1 indicates a 'fail dangerous' model, as the observed growth rates would exceed the predicted growth rates by at least 10% (te Giffel and Zwietering, 1999). The accuracy factor gives an indication of how on average the predictions differ from observations (Ross, 1996).

3.3.3.2 Graphical validation

Graphical validation was also used to assess the generalization properties of the models evaluated. Plots of observed growth rates as a function of predicted growth rates were examined visually to assess the overall reliability of the model. In addition three-dimensional surfaces of model 2 and 3 and the validation data as a function of the investigated variables, were used to obtain a more informative assessment of the performance of the models. These are more useful assessors as they show the interpolating ability of the model by visually depicting the possible *under* or *overfittin*g (Geeraerd et al., 1998, 2004).

3.4 Results

3.4.1 Effect of a_w

The growth curves based on colony diameters were typical of fungal growth for both isolates, characterized by a lag phase, in those cases where growth was hindered by low a_w and/or temperature, and followed by linear growth in all cases. Examples of these growth curves for *F. proliferatum* at 30°C and a_w 0.982 are shown in Fig. 3.1a and Fig. 3.1b, respectively. The upper asymptote was not achieved in most cases owing to the limited growth surface on the petri plates of diameter 90 mm or the maximum period of 6 weeks incubation. At high a_w's and temperatures suitable for growth, the colony rapidly grew reaching the plate diameter within 6 weeks, whereas at less optimal conditions the colony grew at a very slow rate and was unable to reach 90 mm within 6 weeks. Using the modelling approach described earlier the maximum colony growth rate (g), expressed as the increase in diameter per day (mm d^{-1}), was estimated using the non-linear regression function of SPSS Version 11.0 for each condition. These values were then fitted with respect to b_w using the linear regression equation, model 1, for each strain. The coefficients of the models and the significance of the model parameters for both isolates are shown in Table 3.1. Visual

representation of these models relating the colony growth rates (mm d^{-1}), with any a_w at 30, 25, 22, and 15ºC, are shown in Fig. 3.2 for both isolates.

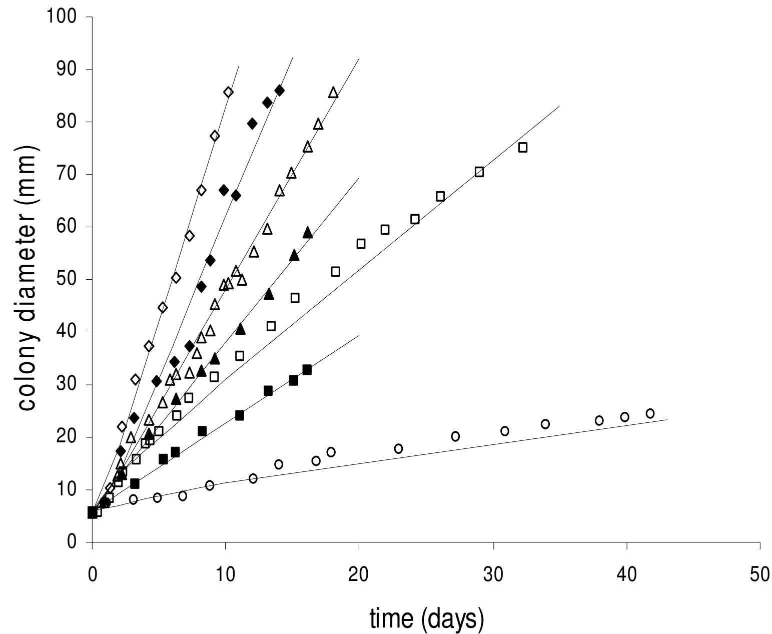


Fig. 3.1a. Plots of colony diameter (mm) versus time (days) for *F. proliferatum* at 30°C. The symbols indicate the experimental growth data at a_w 's 0.982 (◇), 0.972 (◆), 0.948 (△), 0.936 (▲), 0.928 (□), 0.905 (■), and 0.869 (○). The continuous lines indicate the fitted model of Baranyi and Roberts (1994).

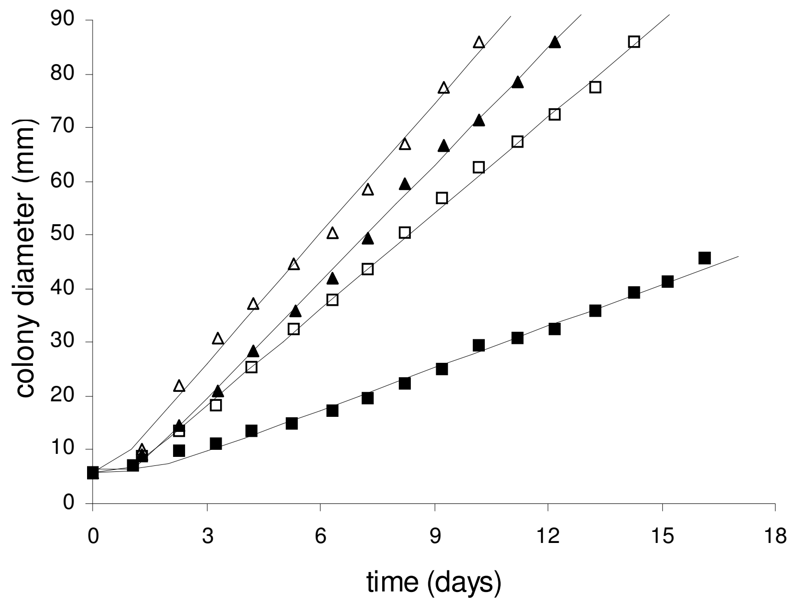


Fig. 3.1b. Plots of colony diameter (mm) versus time (days) for *F. proliferatum* at a_w 0.982. The symbols indicate the experimental growth data at and 30 (△), 25 (▲), 22 (□) and 15ºC (■). The continuous lines indicate the fitted model of Baranyi and Roberts (1994).

Table 3.1 Coefficients for the growth rate model, ln $g = C_0 + C_1b_w + C_2b^2_w$, for *F. proliferatum* and *F. verticillioides* at 15, 22, 25 and 30ºC

	C_0	C_1	C_2	r^2
F. proliferatum				
30ºC	2.575 ± 0.109*	ns	-23.609 ± 1.453*	0.981
25ºC	2.581 ± 0.189*	ns	-26.592 ± 2.522*	0.957
22ºC	2.312 ± 0.214*	ns	-24.267 ± 2.862*	0.935
15ºC	0.524 ± 0.356*	8.523 ± 3.050*	-39.226 ± 6.155*	0.993
F. verticillioides				
30ºC	3.039 ± 0.176*	ns	-19.116 ± 2.145*	0.941
25ºC	4.045 ± 0.196*	-10.037 ± 0.733*	ns	0.974
22ºC	4.063 ± 0.341*	-10.835 ± 1.275*	ns	0.935
15ºC	3.546 ± 0.309*	-10.359 ± 1.152*	ns	0.942

- Significant ($P < 0.05$); ns - Not significant

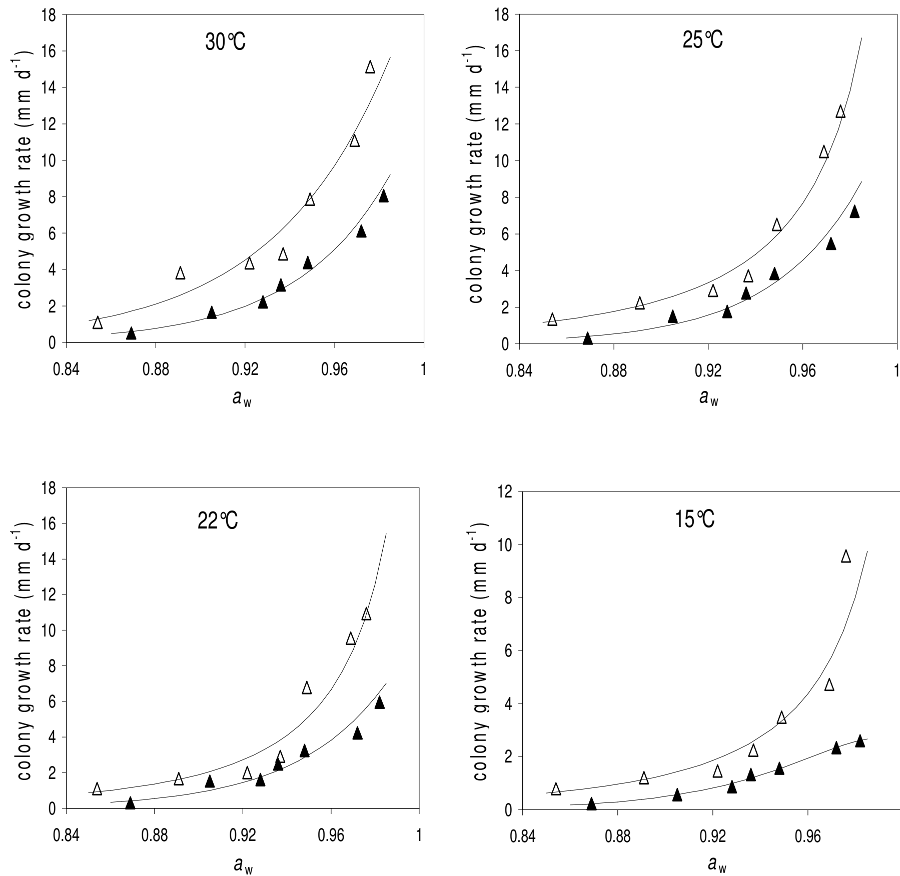


Fig. 3.2. Plots of g (mm d^{-1}) versus a_w. Symbols indicate the colony growth rates estimated from the fitted growth curves at 30, 25, 22, and 15°C for *F. verticillioides* (Δ) and *F. proliferatum* (▲). The continuous lines indicate the fitted g vs. a_w function, where $g = \exp(C_0 + C_1b_w + C_2b_w^2)$.

3.4.3 Combined effect of a_w and temperature

The combined influence of a_w and temperature on the growth rate of *F. proliferatum* and *F. verticillioides* was determined by fitting models 2 and 3 to the estimated colony growth rates using SPSS Version 11.0. The coefficients of the models developed and the significance of their associated factors are shown in Tables 3.2 and 3.3 for models 2 and 3, respectively. Visual representations of model 2 as contour plots are shown in Fig. 3.3a and Fig. 3.3b for *F. proliferatum* and *F. verticillioides*, respectively, whereas those of model 3 are shown in Fig. 3.4a and Fig. 3.4b for *F. proliferatum* and *F. verticillioides*, respectively.

Table 3.2. Coefficients of growth rate model 2, $g = C_0 + C_1b_w + C_2b_w^2 + C_3T + C_4T^2 + C_5Tb_w$, for *F. verticillioides* and *F. proliferatum.*

	F. moniliforme	*F. proliferatum*
Factor	Coefficient	
C_0	18.681 ± 3.162*	-0.193 ± 1.261*
b_w	-144.068 ± 18.642*	-31.539 ± 6.957*
b_w^2	253.270 ± 30.452*	50.656 ± 13.386*
T	0.647 ± 0.104*	0.513 ± 0.043*
T^2	ns	ns
b_wT	-1.600 ± 0.389*	ns
r^2	0.965	0.974

* Significant ($P < 0.05$); ns - Not significant

Table 3.3 Coefficients of the linear Arrhenius-Davey model (Davey, 1989), $\ln g = C_0 + C_1/T + C_2/T^2 + C_3a_w + C_4a_w^2$, for *F. verticillioides* and *F. proliferatum.*

	F. verticillioides	*F. proliferatum*
Factor	Coefficient	
C_0	78.082 ± 21.689*	-87.938 ± 21.592*
$1/T$	ns	ns
$1/T^2$	-683562.8 ± 90382.8*	-853103.3 ± 78965.9*
a_w	-170.359 ± 47.367*	187.940 ± 46.609*
a_w^2	103.311 ± 25.844*	-88.370 ± 25.145*
r^2	0.952	0.971

* Significant ($P < 0.05$); ns - Not significant

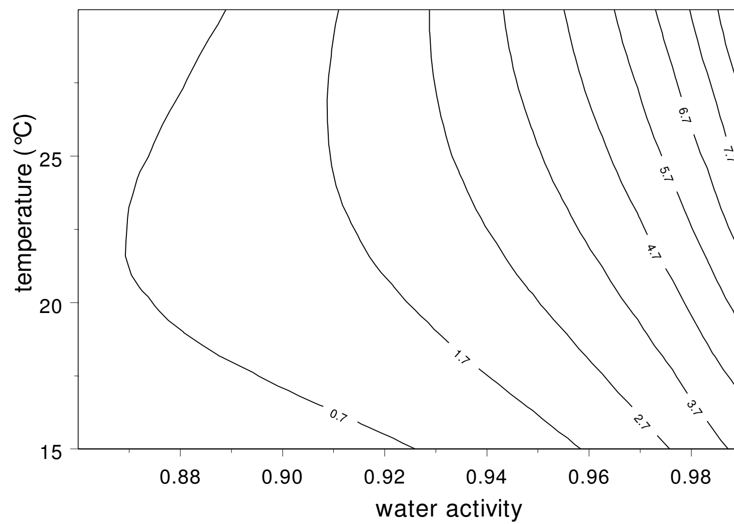


Fig. 3.3a. Contour plot of predicted g (mm d^{-1}) versus a_w and temperature for *F. proliferatum* using model 2, $g = C_0 + C_1b_w + C_2b_w^2 + C_3T + C_4T^2 + C_5Tb_w$.

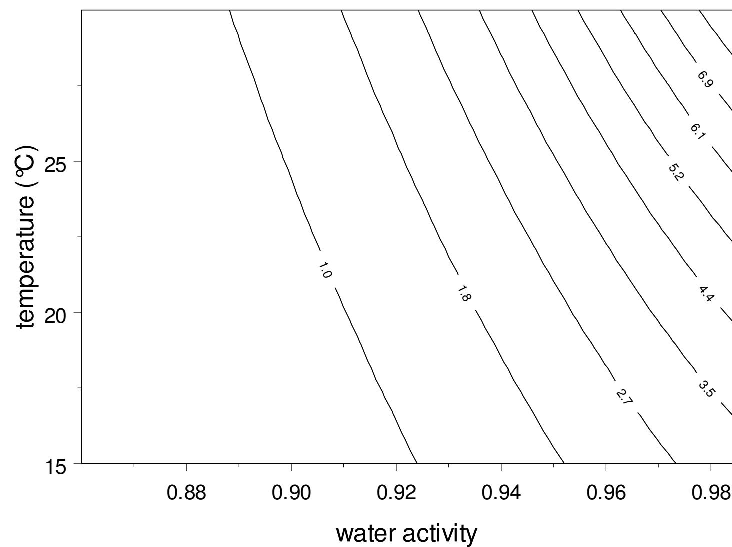


Fig. 3.3b. Contour plot of predicted g (mm d^{-1}) versus a_w and temperature for *F. verticillioides* using model 2, $g = C_0 + C_1b_w + C_2b_w^2 + C_3T + C_4T^2 + C_5Tb_w$.

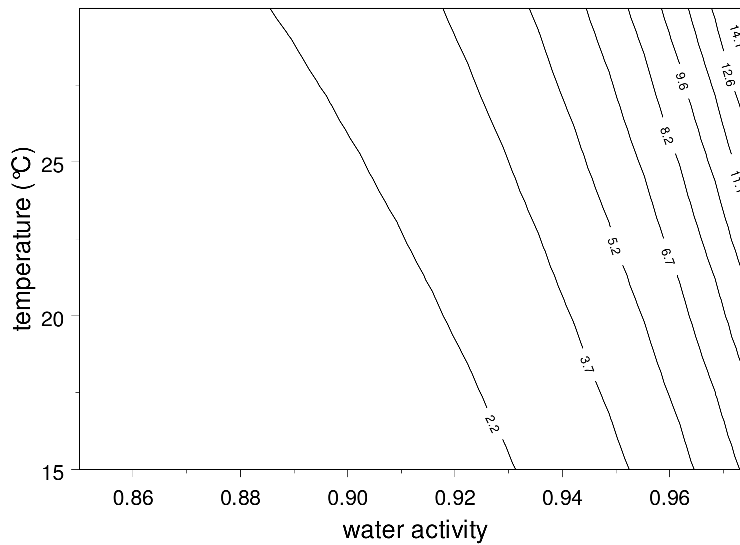


Fig. 3.4a. Contour plot of predicted g (mm d^{-1}) versus a_w and temperature for *F. proliferatum* using model 3, $\ln(g) = C_0 + C_1/T + C_2/T^2 + C_3a_w + C_4a_w^2$.

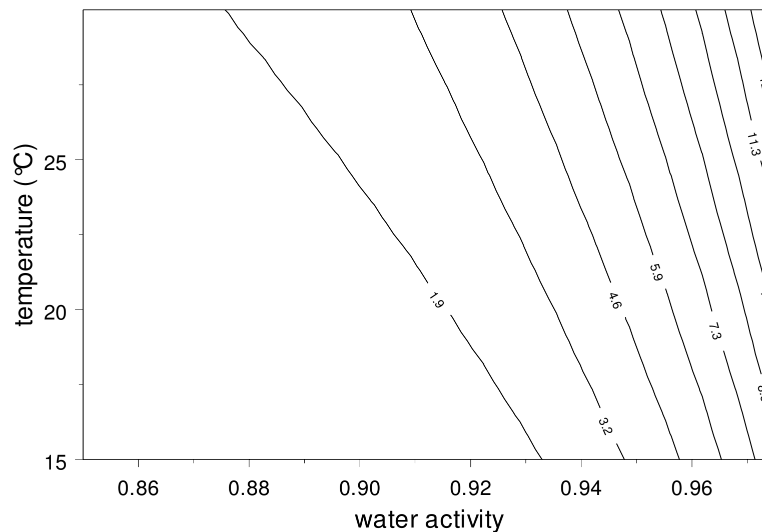


Fig. 3.4b. Contour plot of predicted g (mm d^{-1}) versus a_w and temperature for *F. verticillioides* using model 3, $\ln(g) = C_0 + C_1/T + C_2/T^2 + C_3a_w + C_4a_w^2$.

3.4.4 Validation

The results of the mathematical/statistical analysis used to assess the performance of the models are shown in Tables 3.4, 3.5, and 3.6 for models 1, 2, and 3 respectively. The graphical comparisons of the observed growth rates as a function of the growth rates predicted by model 2 and the three-dimensional surface of this model and the validation data points are shown in Fig. 3.5a and 3.5b for *F. proliferatum* and *F. verticillioides*, respectively. The graphical comparisons of the observed growth rates as a function of the growth rates predicted by model 3 and the three-dimensional surface of this model and the validation data points are shown in Fig. 3.5c and 3.5d for *F. proliferatum* and *F. verticillioides*, respectively.

3.5 Discussion

3.5.1 Effect of a_w

Generally it can be seen from Figs. 3.1a, 3.1b and 3.2 that at any temperature, the higher the a_w value the larger the colony growth rates of both isolates. Le Bars et al. (1994), Cahagnier et al. (1995), and Marin et al. (1995, 1999b, 1999c) have all reported the same trend. Radial growth rates of 4.034 and 7.571 mm d^{-1} were obtained at 30°C and a_w's of 0.982 and 0.976, for *F. proliferatum* and *F. verticillioides*, respectively. These are comparable to the radial growth rates reported by Marin et al. (1999b), of 5.5 and 6.5 mm d^{-1} at 30°C and a_w 0.98 for, *F. proliferatum* and *F. verticillioides*, respectively.

From the same figures it can be noticed that, in the investigated temperature interval, at any a_w the higher the temperature the larger the colony growth rate for both isolates as well. Generally, *F. verticillioides* grew at a faster rate than *F. proliferatum* at all a_w's evaluated as seen in Fig. 3.2. Marin et al. (1999b) also observed this trend. This however contrasts the findings of Marin et al. (1995) where *F. proliferatum* (73N) grew faster than the other isolates tested, including *F. verticillioides* (25N) at high a_w's. The minimum a_w for growth was 0.869 and 0.854 for *F. verticillioides* and *F. proliferatum*, respectively. No growth took place at a_w 0.831 and 0.838 for *F. verticillioides* and *F. proliferatum*, respectively. Similar minimum a_w's for growth

were reported by Marin et al. (1996), where the minimum a_w for growth of both *F. proliferatum* and *F. verticillioides* was 0.880, with no growth taking place at a_w 0.850. Cahagnier et al. (1995) suggested a_w 0.85 was the threshold for growth of *F. verticillioides*.

3.5.3 Validation of the model for the a_w-effect

As shown in Table 3.4 for *F. verticillioides* the *F*-values calculated indicate that growth rates predicted by model 1 and observed values were not significantly different from each other ($P < 0.05$) at all temperatures evaluated. The bias factors are close to 1, and actually < 1 at 15, 25 and 30ºC, indicating that the model was a good predictor of the true mean colony growth rate, and fail safe at 15, 25 and 30ºC. The accuracy factors indicate that on average the predictions differed from the observations by < 13%. For *F. proliferatum* the *F*-values also indicate that at all temperatures the predicted and observed values were not significantly different from each other ($P < 0.05$). The bias factors are also close to 1, and are < 1 at 22 and 25ºC, indicating that the model was also a good predictor of the true mean colony growth rate of *F. proliferatum* and fail safe at 22 and 25°C. The accuracy factors indicate that on average the predictions differed from the observations by < 16%. It can be concluded that model 1 is, within the limits investigated, able to predict the effect of a_w on colony growth rates of both isolates.

These bias and accuracy factors are comparable to those that have been determined for other fungi. Valík and Piecková (2001) modelled the effect of a_w on the growth rate of three heat resistant fungal isolates at 25°C. The bias factors ranged from 1.007 to 1.014 and the accuracy factors from 1.070 to 1.106. These indices have however been more widely used for validating models used to describe bacterial growth. te Giffel and Zwietering (1999) and Ross (1996) have all used these factors to validate models developed to describe the growth of *Listeria monocytogenes*. They obtained accuracy factors that ranged from 1.26 to 4.25. Rasch (2002) obtained bias and accuracy factors of 0.95 and 1.08 respectively for polynomial models used to describe the influence of temperature, salt and pH on the inhibitory effect of reuterin on *Escherichia coli*.

Table 3.4. Validation indices for the performance of the growth rate model 1, ln $g = (C_0 + C_1 b_w + C_2 b^2_w)$, for *Fusarium verticillioides* and *F. proliferatum* at 15, 22, 25 and 30ºC

	15ºC	22ºC	25ºC	30ºC
F. verticillioides				
F-value	0.993	1.092	0.996	0.668
F-table value (95% confidence)	9.277	9.277	9.277	9.277
Bias factor	0.842	1.058	0.949	0.915
Accuracy factor	1.188	1.075	1.082	1.167
F. proliferatum				
F-value	0.863	1.202	1.508	0.724
F-table value (95% confidence)	9.277	9.277	9.277	9.277
Bias factor	1.082	0.903	0.981	1.038
Accuracy factor	1.093	1.218	1.192	1.116

3.5.4 Combined influence of a_w and temperature

It can be observed from the contour plots, Figs. 3.3a, 3.3b, 3.4a and 3.4b, that within the experimental limits the growth rates of both isolates increase with increase of both a_w and temperature. Although both a_w and temperature have a significant effect on the growth rate, the slopes of the contour plots (tending towards the vertical direction) indicate that the influence of a_w is much larger. This confirms previous findings that a_w has a larger effect on fungal growth than temperature (Holmquist et al., 1983; Sautour et al., 2002). Although curvature is evident in almost all the contour plots it is most apparent in the plots for *F. proliferatum* as can be seen in Fig. 3.3a. The significance of the $1/T^2$ term of model 3 for *F. proliferatum* as shown in Table 3.3 also indicates that curvature occurs for the predicted colony growth rates of this isolate. The curvature shows that both models 2 and 3 predict that the relative influence of a_w on the growth rate increases and that of temperature decreases as temperature is increased for *F. proliferatum.*

In Figs. 3.3a and 3.5a it can also be seen that at low a_w's and high temperatures where growth is less optimal, model 2 makes the illogical prediction that an increase in temperature from about 22°C will result in a decrease in the growth rate of *F. proliferatum*. This contrasts the estimated colony growth rates which showed that at all a_w’s the highest growth rates occurred at 30°C, and indicates *overfitting* by model 2. *Overfitting* is typical of polynomial equation predictions at growth limiting

conditions. Model 3 as seen in Figs. 3.4a and 3.5c does not reproduce this *overfitting* for the predicted growth rates of *F. proliferatum*. In contrast, for *F. verticillioides* it can be seen from Figs. 3.3b, 3.5b and 3.5d that both model 2 and 3 predict that the relative effect of a_w and temperature on the growth rate of *F. verticillioides* remains almost constant throughout the a_w and temperature intervals investigated. The slight curvature evident at low a_w's however shows that although the influence of a_w on the colony growth rates remains larger than that of temperature, a marginal decrease in its relative effect occurs at these a_w's. Although the $1/T^2$ term is significant for *F. verticillioides*, this is not apparent in the form of considerable curvature as seen in Fig. 3.5d.

From Table 3.2 it can be seen that significant interaction between a_w and temperature was observed for both *F. verticillioides* and *F. proliferatum*, the negative value of the coefficients C_5 and C_7 for *F. verticillioides* and *F. proliferatum*, respectively, suggests that the interaction is synergistic. The a_w and temperature terms of model 3 are 'additive', and hence there is no 'interaction' predicted by these models theoretically. Davey (1989) suggested that where good agreement between predictions by model 3 and observed growth data occurs, the two factors act independently on fungal growth. However curvature, which is more apparent on the three-dimensional surface of model 3 for both isolates, as seen on Fig. 3.5c and Fig. 3.5d for *F. proliferatum* and *F. verticillioides*, respectively, indicates interaction between the influences of a_w and temperature on the colony growth rates of both isolates. This description of the interaction was incorporated in this theoretically additive model during the parameter estimation procedure. Marin et al. (1998d, 1999a, 1999b) and Velluti et al. (2000) have all reported interaction between the effects of a_w and temperature on the growth rates of *F. proliferatum* and *F. verticillioides*.

3.5.5 Validation of the models for the combined a_w-temperature effect

The *F*-values calculated, shown in Table 3.5 and 3.6, indicate that values predicted by models 2 and 3, for both isolates, did not differ significantly from the observed values. The bias and accuracy factors for both models 2 and 3, and for both isolates, are close to 1 indicating that the models are good predictors of the true mean colony growth rates for both isolates. For *F. verticillioides* the bias factor for model 3 is < 1

indicating that the model was fail safe for this isolate. The observed colony growth rates and the predicted colony growth rates differed by 25.4 and 11% on average, when models 2 and 3 were used to predict the growth rates, respectively. This indicates that on average model 2 predicts colony growth rates approximately twice as deviant from the observed growth rate as those predicted by model 3.

Table 3.5. Validation indices for the performance of the growth rate model 2, $g = C_0 + C_1b_w + C_2b_w^2 + C_3T + C_4T^2 + C_5Tb_w$, for *F. verticillioides* and *F. proliferatum*.

	F. verticillioides	*F. proliferatum*
F-value	0.996	1.118
F-table value (95%confidence)	2.403	2.403
Bias factor	1.042	1.054
Accuracy factor	1.128	1.380

Table 3.6. Validation indices for the performance of the linear Arrhenius-Davey model (Davey, 1989), $\ln g = C_0 + C_1/T + C_2/T^2 + C_3a_w + C_4a_w^2$, for *F. verticillioides* and *F. proliferatum*.

	F. moniliforme	*F. proliferatum*
F-value	1.125	1.023
F-table value (95% confidence)	2.403	2.403
Bias factor	0.978	1.002
Accuracy factor	1.098	1.122

Graphical comparison of plots of the observed and predicted growth rates and the three-dimensional surfaces with the validation data points for the two isolates, when model 2 was used to predict the growth rates, is shown in Fig. 3.5a and Fig. 3.5b for *F. proliferatum* and *F. verticillioides*, respectively. The same graphical comparisons, when model 3 was used to predict the growth rates, are shown in Fig. 3.5c and Fig. 3.5d for *F. proliferatum* and *F. verticillioides*, respectively. It can be seen from these figures that the plots of the observed versus the predicted growth rates are generally more deviant from the line of equivalence for both isolates when model 2 is used to predict the growth rates than when model 3 is used (which can also be seen from the respective accuracy factors). The deviance is larger at low values of a_w and temperature less favourable for growth.

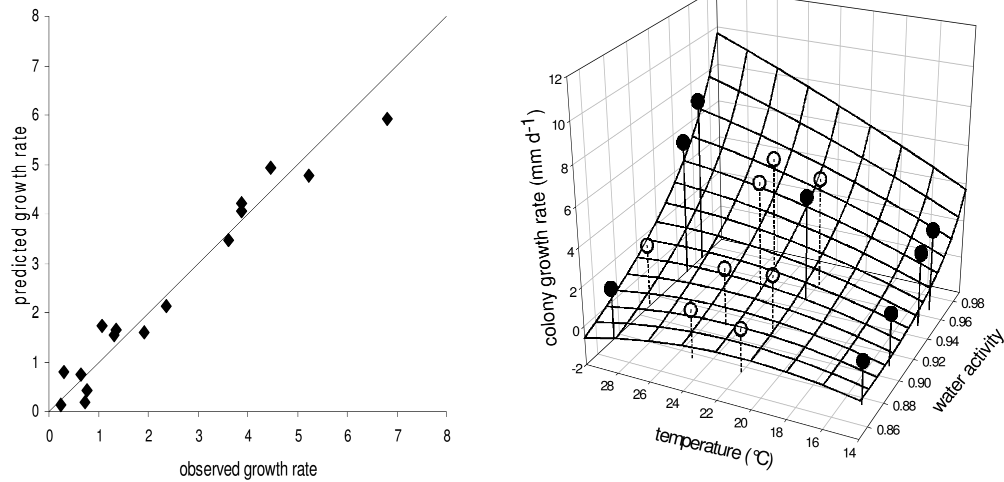


Fig. 3.5a. Left plot: Comparison of predicted colony growth rates by function, where $g = C_0 + C_1b_w + C_2b_w^2 + C_3T + C_4T^2 + C_5Tb_w$, and observed growth rates for *F. proliferatum* (◆). Right plot: Three-dimensional surfaces of the quadratic response surface and the validation data points above (●, with solid drop-line) and below (○, with dashed drop-line) the surface.

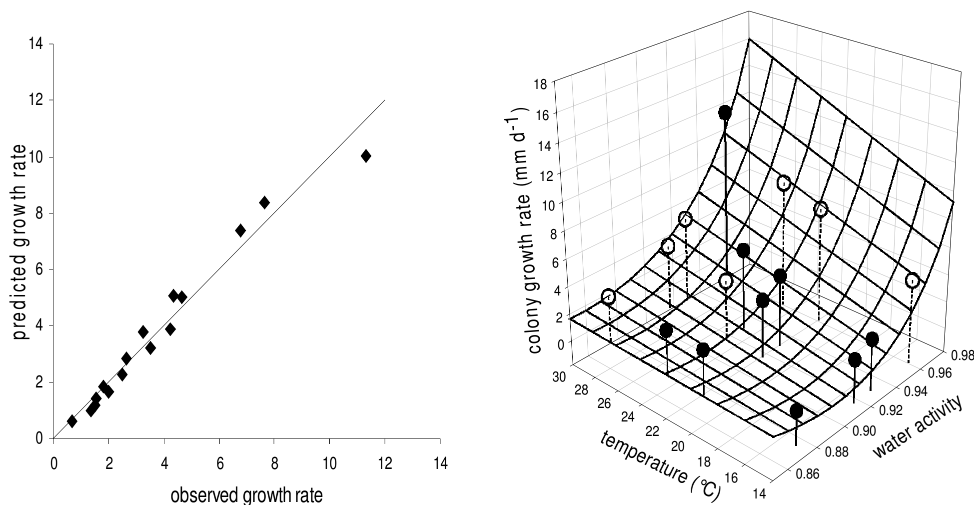


Fig. 3.5b. Left plot: Comparison of predicted colony growth rates by function, where $g = C_0 + C_1b_w + C_2b_w^2 + C_3T + C_4T^2 + C_5Tb_w$, and observed growth rates for *F. verticillioides* (◆). Right plot: Three-dimensional surfaces of the quadratic response surface and the validation data points above (●, with solid drop-line) and below (○, with dashed drop-line) the surface.

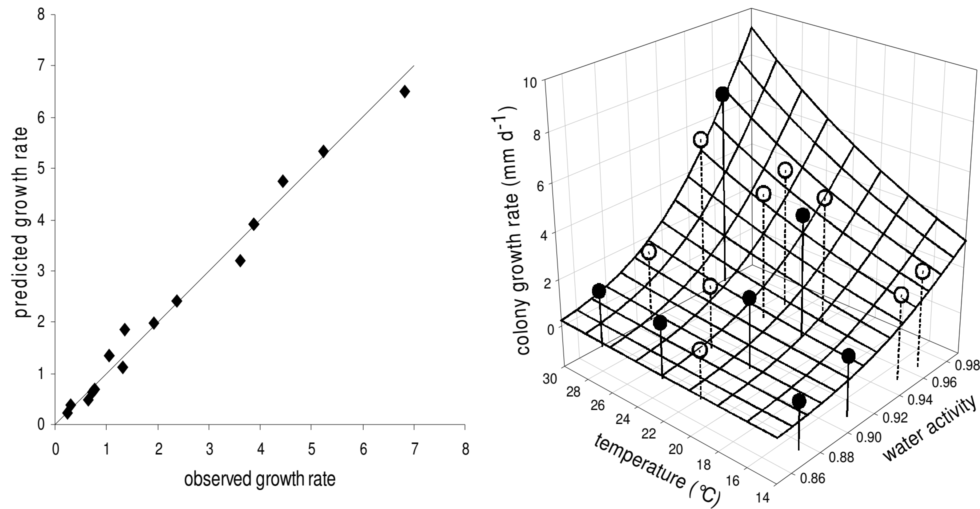


Fig. 3.5c. Left plot: Comparison of predicted colony growth rates by function, where $g = \exp(C_0 + C_1/T + C_2/T^2 + C_3a_w + C_4a_w^2)$, and observed growth rates for *F. proliferatum* (◆). Right plot: Three-dimensional surfaces of the linear Arrhenius-davey model and the validation data points above (●, with solid drop-line) and below (○, with dashed drop-line) the surface.

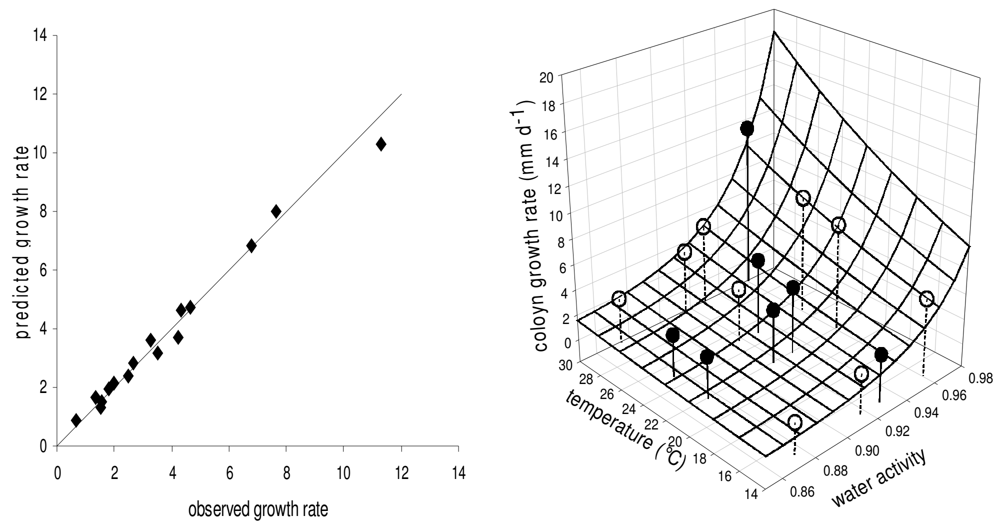


Fig. 3.5d. Left plot: Comparison of predicted colony growth rates by function, where $g = \exp(C_0 + C_1/T + C_2/T^2 + C_3a_w + C_4a_w^2)$, and observed growth rates for *F. verticillioides* (◆). Right plot: Three-dimensional surfaces of the linear Arrhenius-davey model and the validation data points above (●, with solid drop-line) and below (○, with dashed drop-line) the surface.

Overfitting by model 2 is noted at growth limiting conditions for both isolates as seen on the three-dimensional surfaces, shown in Fig. 3.5a and 3.5b for *F. proliferatum* and *F. verticillioides*, respectively. The contour plot, Fig. 3.3a, depicted *overfitting* by model 2 for *F. proliferatum* but this was not observed in Fig. 3.3b for *F. verticillioides*. For *F. proliferatum overfitting* by model 2 is noticed at low a_w's and at low and high temperatures where the predicted colony growth rates are lower than those that were observed. For *F. verticillioides overfitting* is noted at low a_w's and at temperatures around 15°C, where the model illogically predicts that the colony growth rate increases as a_w is decreased from 0.875 to 0.854, the minimum a_w for growth observed for this isolate. This condition, with a very low growth rate, is not included in the contour lines of Fig 3.3b.

It can be concluded that although model 2 is able to predict the effect of a_w and temperature on the colony growth rate of both isolates, the predictions at growth limiting conditions should be treated with caution, as *overfitting* is apparent at these conditions. The observed and predicted growth rates, when model 3 is used to predict the colony growth rates, are close and evenly distributed about the line of equivalence, indicating that model 3 is within the experimental conditions able to predict the effect of a_w and temperature on the colony growth rates of both isolates. The model is also seen to have good interpolating or generalization abilities as the validation data points are evenly distributed about its three-dimensional surface, confirming the deductions made from the plots of the observed versus the predicted growth rates. Model 3, is within the limits investigated, able to predict the effect of a_w and temperature on colony growth rates of both isolates.

Model 3 appears to be a more reliable predictor of the colony growth rates than model 2 when the validation criteria are compared. Importantly the *overfitting* typical of polynomial equations, and as seen on the three-dimensional surfaces, is excluded from the linear Arrhenius-Davey equation making this model a better predictor of the colony growth rate at conditions less optimal for growth where response surface equations become less reliable and tend to over-fit. Therefore the linear Arrhenius-Davey model can be concluded to be the better suited model for describing the colony growth rates of *F. proliferatum* and *F. verticillioides* within the experimental conditions investigated.

3.6 Conclusions

The models that were evaluated are capable of predicting the growth rates of *F. verticillioides* and *F. proliferatum* within the limits of the study. Previous studies of fumonisin producing *Fusarium* species have been limited to the deduction of the effects of a_w and or temperature (Le Bars et al., 1994; Cahagnier et al., 1995; Marin et al., 1995, 1999b,c) without developing any predictive mathematical relationships between growth rates and environmental parameters such as a_w or temperature. However, they also observed the general trend predicted by the models evaluated, namely, an increase in colony growth rate with increase in either a_w and/or temperature. It is noted that effects of temperature on the growth rate would be more accurately described if the study included a wider temperature range, inclusive of the cardinal growth temperatures. Performing the secondary modelling in two steps, first their screening by means of the MSE and residual plots of several potentially suitable models followed by a more detailed evaluation and validation of the two best fitting models, enabled for a wider range of models/model structures to be assessed. This was essential as there is still a limited number of secondary models available in predictive mycology that may adequately describe the trends one might observe. It is important to note however that other parameters that come into play when the maize is still on the field or in storage facilities have to be considered to obtain more meaningful predictions. These other parameters, among others, include the composition of the storage atmosphere, the presence of competitors, cultivar of maize grain, cultural practices and the effect of anti-fungal agents.

After the modelling of the effects of a_w and temperature on the growth of the most important *Fusarium* species on corn, the study now shifted its focus in chapter 4 to the modelling of the individual and combined effects of a_w and temperature on the growth of the most important *Aspergillus* species on corn – *A. flavus* and *A. parasiticus*. The *Aspergillus* species can grow at more water compromised conditions than the *Fusarium* species and produce aflatoxins which are widely considered to be the most potent mycotoxins known to man.

CHAPTER 4

Modelling of the individual and combined effects of water activity and temperature on the radial growth of *Aspergillus flavus* and *A. parasiticus* on corn [4]

[4] Redrafted from: Samapundo, S., Devlieghere, F., Geeraerd, A. H., De Meulenaer, B., Van Impe, J. F. and Debevere, J. M. 2006. *Food Microbiology* (accepted).

Chapter 4. Modelling of the individual and combined effects of water activity and temperature on the radial growth of *Aspergillus flavus* and *A. parasiticus* on corn

4.1 Abstract

A full factorial design of five temperatures (16, 22, 25, 30 and 37°C) and seven a_w values between 0.801 and 0.982 was used to investigate the growth of the two major aflatoxin producing Aspergillus isolates on corn. The colony growth rates (g, mm d^{-1}) and lag phases (λ, d) were estimated by fitting a flexible primary growth model. Subsequently, secondary models relating g or λ to a_w or temperature or a_w and temperature combined, were developed and validated by using independently collected data. The Gibson and linear Arrhenius-Davey model describing the individual effects of a_w or temperature, respectively, on g or λ proved adequate predictors of these growth parameter. Based on the validation criteria, a quadratic polynomial function proved to be more suitable than a Gaussian function or extended Davey model for describing the combined effect of a_w and temperature on g or λ. Both isolates studied had optimum growth temperatures of approximately 30°C. No growth was observed for both isolates at a_w 0.801, growth only occurring at 25 and 30°C at a_w 0.822. Significant interaction between a_w and temperature on g and λ was observed for both isolates. The developed models can be applied for the prediction of Aspergillus growth on corn in store, for the estimation of the environmental boundaries for the growth of these isolates on corn and as a basis for the development of models that incorporate other factors important to mould growth on corn.

Keywords: *Aspergillus*, water activity, temperature, modelling, corn

4.2 Introduction

Members of the *Aspergillus* spp., amongst many other toxigenic fungi, have through time been found to have a strong ecological link with human food supplies (Pitt 2000). They are often associated with food and animal feed during drying and storage, but may also occur as plant pathogens. The contamination of corn is endemic in many growing regions and can be severe, with resultant outbreaks causing large economic

losses and potentially undermining public health (Norton 1999). As a result of their ability to produce aflatoxins and persist pre- and post-harvest as a pathogen and saprophyte in the food supply, *A. flavus* and to a lesser extent *A. parasiticus*, are some of the most widely studied fungal species. To date several studies on the effect of biotic and abiotic factors on growth and aflatoxin production have been published (Schindler, 1967; Trenk and Hartman, 1970; Holmquist et al., 1983; Niles et al., 1985; Lacey, 1989; Gibson et al., 1994; Pitt and Miscamble, 1995; Marin et al., 1998b; Sautour et al. 2001a, 2002).

The modelling of the effect of biotic and abiotic factors on the growth or aflatoxin production of these two species has generally been tackled to lesser extent. This in part being a result of the inherent difficulties in assessment of fungal growth rates and gathering of sufficient, suitable and reproducible data (Gibson et al., 1994; Gibson and Hocking, 1997). With regards to modelling of the growth of these two species, Pitt (1993) developed a mechanistic model integrating literature data on the effect of temperature, a_w, pH, and colony size on *in vitro* mould growth and aflatoxin production. A year later Gibson et al. (1994) described the effect of a_w on the growth rate (g) of *A. flavus* (on artificial media) by means of a linear regression equation (Eq. 3.6). Subsequently Marin et al. (1998b) used a modified Gompertz equation to determine the effect of a_w and temperature on the lag phase and germination rates of various *Aspergillus* species, including *A. flavus*, when grown on corn extract medium. More recently Sautour et al. (2001) developed a temperature type (Rosso type) model to describe the relationship between the growth of several fungi (including *A. flavus*) on potato dextrose agar and water activity, from which cardinal water activities (a_{wmin}, a_{wopt} and a_{wmax}) could be estimated.

Very few modelling studies are available that include *A. parasiticus* growth despite its importance, of which none describe its growth on corn. There are also currently no validated models describing the individual or combined effect of a_w and temperature on the growth of *A. flavus* and *A. parasiticus* on corn despite the plethora of studies to date. The results reported here constitute the second part in the evaluation and validation of different models for their ability to describe the growth of the most important mycotoxigenic moulds on corn. These would provide an empirical mathematical basis for (the planned) modelling of the inhibitory effect of different

preservation chemical and non-chemical factors (where possible) on the growth of these moulds on corn in later studies.

4.3 Materials and Methods

4.3.1 Isolates

Aflatoxigenic *Aspergillus flavus* (IITAB 139) and *A. parasiticus* (IITAB 138) were obtained courtesy of the International Institute of Tropical Agricultural (Cotonou, Benin). They were both isolated from corn and were maintained on potato dextrose agar (PDA) (Oxoid, Basington, UK).

4.3.2 Experimental design

A full factorial design of five temperatures (16, 22, 25, 30 and 37°C) and seven a_w values between 0.801 and 0.982 was used to investigate the growth of the two major aflatoxin producing *Aspergillus* isolates on corn. The *Aspergillus* were grown over a wider temperature range than the *Fusarium* isolates which failed to grow at 37°C, limiting that study to a maximum of 30°C.

4.3.3 Growth substrate

Dried whole yellow corn supplied by Aveve Belgium (NV) was also used as the growth substrate. The corn was treated as described in section 3.3.1.2.

4.3.4 Preparation of inoculum, inoculation, incubation, and growth assessment.

The fungal inoculum was prepared in the same manner as described in 3.3.1.3. The only exceptions being that after the central inoculation of PDA petri plales with sporulating mycelia from the surface of the PDA slants, the inoculum was grown by incubating the petri plates for four rather than six days at 30°C, before further incubation at 16, 22, 25, 30 or 37°C for one more day to enable the isolates to adapt to the incubation conditions they were to be grown. Only five days in total were used instead of seven as the *Aspergillus* isolates grew faster and sporulated earlier than the *Fusarium* isolates. About 24 g of rehydrated corn was placed in each sterile petri

plate, to form a single layer of grains. In addition only ten petri plates instead of 20 were placed in each container, before incubation at 16, 22, 25, 30 or 37°C. Growth was also assessed as described in 3.3.1.3.

4.3.5 Mathematical and Statistical Methods

4.3.5.1 Primary modelling

Primary modelling was done as described in section 3.3.2.1. In brief the flexible growth function of Baranyi and Roberts (1994) was fitted to the growth data, average diameters (*y*, mm) at each time of measurement (*t,* days), by means of the non-linear regression function of SPSS® Version 11.0 (SPSS Inc., Chicago, Ill., USA). This enabled the determination of the colony growth rates (*g*, mm d^{-1}) and the lag phase durations (λ, d) for each experimental condition.

4.3.5.2 Secondary modelling

Due to the ability of the *Aspergillus* isolates to also grow at 37°C, which resulted in maxima (inflexion points) in the temperature profile at 30°C at any a_w value, models such as the linear-Arrenhius-Davey model (Eq. 3.8) which best decribed the growth of the *Fusarium* isolates as function of a_w and temperature were not applicable in this study. Therefore other models had to be evaluated for the combined effects of $\boldsymbol{a}_w$ and temperature which incorporate maxima in their structure. In addition, due to greater differences in colour between the background corn substrate and the *Aspergillus* isolates (green) in comparison to the *Fusarium* isolates (white with a yellow or pinkish hue), better estimates were obtained for the lag phase durations of the *Aspergillus* isolates which were subsequently modelled as well.

4.3.5.2.1 Individual effects of a_w or temperature on the colony growth rates and lag phase durations

The colony growth rate (*g*) and lag phase duration (λ) were modelled as a function of temperature for each a_w level by the modified linear Arrhenius-Davey model (Davey 1989):

$$\ln (g \text{ or } 1/\lambda) = C_0 + C_1/T + C_2/T^2 \quad (4.1)$$

where T = degree Kelvin (K)

The effect of a_w on the colony growth rate and lag phase was modelled for each temperature level by the linear regression equation of Gibson et al. (1994):

$$\ln (g \text{ or } 1/\lambda) = C_0 + C_1 b_w + C_2 b_w^2 \quad (4.2)$$

where a_w is transformed to b_w by $b_w = \sqrt{1 - a_w}$ for better hyperbolic fitting.

The optimum temperatures (T_{opt}, °C), where the growth rate was fastest or the lag phases were shortest, were calculated from the coefficients of Eq. 4.1 as follows:

$$1/T_{opt}(K) = \frac{-C_1}{2C_2} \quad (4.3)$$

therefore $T_{opt}(K) = \frac{-2C_2}{C_1}$

and $$T_{opt}(°C) = \frac{-2C_2}{C_1} - 273 \quad (4.4)$$

The goodness of fit of Eq. 4.1 and 4.2 was determined by the determination coefficient (r^2) and the residual mean square error (MSE).

4.3.5.2.2 The combined influence of a_w and temperature on the colony growth rates and lag phase durations

As performed in chapter 3 several potentially suitable models were fitted to the estimated colony growth rates or lag phases in a first step during which the two most suitable models were identified on the basis of their residual mean square errors (MSE) and residual plots. These two were then further evaluated and validated against independently collected data in second step. According to the MSE and residual plots the two best fitting models were a second order polynomial equation in which the a_w had been transformed to b_w:

$$\ln (g) = C_0+C_1b_w+C_2b_w^2+ C_4T + C_5T^2 + C_6Tb_w \quad (4.5)$$

where T = degree Celsius (°C)

and a Gaussian function:

$$g = A * e^{-0.5\left[\left(\frac{a_w-1}{b}\right)^2+\left(\frac{T-T_{opt}}{c}\right)^2\right]} \quad (4.6)$$

The Gaussian function normally would estimate two optima (T_{opt} and a_{wopt} in this case) for a given data set which would have important biological meaning. However, as mentioned in section 3.3.2.2 difficulties were encountered in reproducibly and evenly hydrating the corn to a_w values > 0.99 during preliminary experiments. This resulted in rather large variations in the colony diameters of replicates. Therefore our experiments were limited to a a_w value of 0.982. As the experimental growth data showed that the a_{wopt} had not been reached at 0.982 (as the growth rates were still in ascendancy), the use of this equation to calculate a_{wopt} by extrapolation was considered to be hazardous and therefore a_{wopt} was replaced by 1. This change marginally affected the estimated model parameters.

Two models were also evaluated for their ability to describe the combined influence of a_w and temperature on the lag phase duration within the experimental conditions investigated. These included a second order polynomial equation as given below:

$$\ln (1/\lambda) = C_0+C_1a_w+C_2a_w^2+C_3T+ C_4T^2 + C_5Ta_w \quad (4.7)$$

where T = degree Celsius (°C)

and the linear Arrhenius-Davey model (Davey 1989), with an added interaction term (a_w/T):

$$\ln (1/\lambda) = C_0+C_1a_w+C_2a_w^2+C_3/T+ C_4/T^2 + C_5a_w/T \quad (4.8)$$

where T = degree Celsius (K)

The coefficients of Eq. 4.5, 4.7 and 4.8 and the significance of their associated parameters were determined by fitting the models to the estimated colony growth rates or lag phases using the linear step-wise regression function of SPSS. The default Levenberg-Marquardt algorithm of the non-linear regression function of SPSS was used to fit the Gaussian function (Eq. 4.6) to the estimated colony growth rates.

4.3.6 Validation

4.3.6.1 Mathematical/Statistical and Graphical validation of models describing the combined effect of water activity and temperature on colony growth rate and lag phase

The models describing the combined effect of a_w and temperature on growth were validated as described in section 3.3.3.1 and 3.3.3.2. In brief, 15 independently collected validation data points were obtained by repeating the experiments at the same five temperatures but at three a_w values different to those examined before. The goodness of fit of the models was determined by means of F–values, bias and accuracy factors and two or three dimensional plots of the models incorporating the validation data.

4.4 Results and Discussion

4.4.1 Growth curves

The growth curves based on colony diameters as a function of time were typical of fungal growth for both isolates. Growth at diameters approximately greater than 60 mm was difficult to accurately assess, especially at high a_w values and temperatures, owing to increased levels of sporulation which spread growth in a non-radial manner over the plate.

4.4.2 Individual effect of a_w or temperature on the growth rate and lag phase duration

As previously described the maximum colony growth rates and the lag phase durations at all conditions were estimated by fitting the flexible function of Baranyi and Roberts (1994) to the experimental growth data. Modelling of the estimated

growth parameters as a function of temperature or a_w was done with Eq. 4.1 and 4.2, respectively. Visual representation of the models developed describing the effect of temperature on the colony growth rate and lag phase are shown in Fig. 4.1 and those describing the effect of a_w are shown in Fig. 4.2. The coefficients of the models, describing g or λ as a function of temperature and their asymptotic standard errors, determination coefficients (r^2) and MSE's are presented in Tables 4.1 and 4.2, respectively. The coefficients of the models describing g or λ as a function of a_w and their asymptotic standard errors, determination coefficients (r^2) and MSE's are presented in Tables 4.3 and 4.4, respectively.

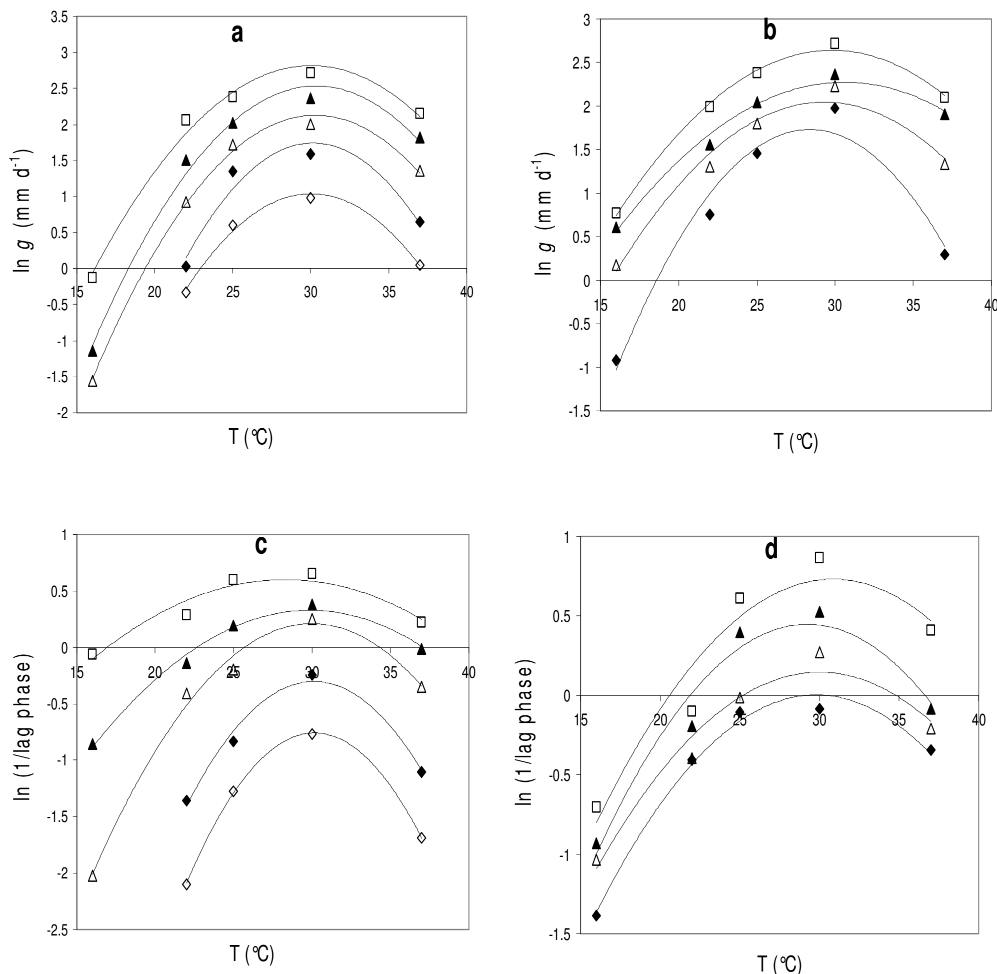


Fig. 4.1. Plots of linear Arrhenius-Davey models (ln g or $1/\lambda = C_0 + C_1/T + C_2/T^2$, solid lines) describing the individual effect of temperature on the growth rate of (a) *A. flavus* and (b) *A. parasiticus*; and the effect of temperature on the lag phase duration of (c) *A. flavus* and (d) *A. parasiticus*. The symbols represent the observed experimental data at a_w values of 0.982 (□), 0.955/0.951 (▲), 0.921 (△), 0.893 (◆) and 0.855 (◇).

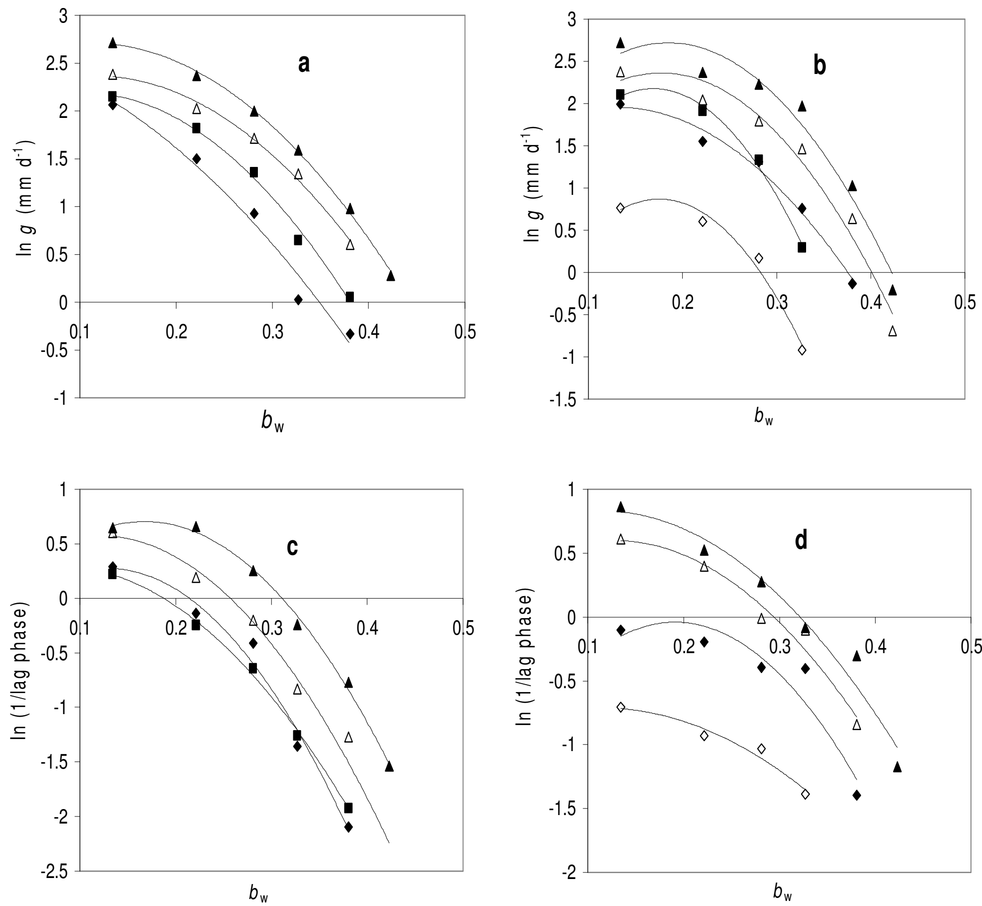


Fig. 4.2. Plots of Gibson models (ln g or $1/\lambda = C_0 + C_1b_w + C_2b_w^2$, solid lines) describing the individual effect of water activity on the growth rate of (a) *A. flavus* and (b) *A. parasiticus*; and the effect of water activity on the lag phase duration of (c) *A. flavus* and (d) *A. parasiticus*. The symbols represent the observed experimental data at incubation temperatures of 37 (■), 30 (▲), 25 (△), 22 (◆) and 16°C (◇).

It can be seen in Fig. 4.1 that for both isolates an increase in incubation temperature from 16 to 30°C resulted in an increase in the colony growth rate and decrease in the lag phase duration. Further increase in temperature from 30 to 37°C resulted in a decrease in the colony growth rate and an increase in the lag phase duration. It can also be observed from Fig. 4.2 that at the temperatures investigated, the higher the a_w value the corn was adjusted to, the faster the colony growth rates and the shorter the lag phase durations for both isolates became, giving rise to the fastest growth rates and shortest lag phases occurring at 30°C and a_w 0.982 for both isolates. From Tables

4.1 and 4.2 it can be noted that for both isolates the estimated optimum temperature (T_{opt}) by Eq. 4.3, at which either the growth rate was fastest or the lag phase duration shortest, was between 28.0 and 30.8°C at the a_w values studied. The estimated T_{opt} are in good agreement to those reported in literature irrespective of the nature of the growth medium. Marin et al. (1998b) reported that the T_{opt} for *A. flavus* grown on maize extract medium was 30°C. Sautour et al. (2001a, 2001b) estimated the T_{opt} of *A. flavus* grown on PDA to be 31°C. Pitt and Hocking (1997) reported that optimum growth occurred in the 32-33°C range. Schindler et al. (1967) and Trenk and Hartman (1970) both reported that the T_{opt} for *A. flavus* was between 29-35°C. Holmquist et al. (1983) found that maximum growth of *A. flavus* and *A. parasiticus* occurred at 33°C, whereas Niles et al. (1985) reported the T_{opt} for growth of *A. flavus* on irradiated corn occurred between 25 and 30°C.

No a_{wopt} were estimated using Eq. 4.2 as the experimental data did not have a maximum and extrapolation outside the experimental limits would be hazardous (Sautour et al., 2001b). The few observed a_{wopt} at some conditions as seen in Fig. 4.2 for *A. parasiticus* are a result of *overfitting* typical for polynomial equations, which occurred as a result of the small difference between colony growth rates and lag phase durations at a_w 0.982 and 0.951 at some temperatures for this isolate. However, some reported values of a_{wopt} include 0.99 for both *A. flavus* and *A. parasiticus* when grown on Sabouraud Dextrose Agar (Holmquist et al., 1983). Ayerst (1969) reported an a_{wopt} of 0.98 for *A. parasiticus*, whereas Marin et al. (1998b) determined the a_{wopt} for mycelial growth of *A. flavus* on maize extract medium to be 0.994. In addition Marin et al. (1998b) found the lag phase to be shortest at a_w 0.994 and 0.95. Values of 0.970 and 0.974 and 0.980-0.995 have been reported by Sautour et al. (2001a, 2002) and Gibson et al. (1994), respectively, for the growth of *A. flavus* on artificial medium. Northolt et al. (1977) reported an a_{wopt} of 0.99 for *A. flavus*. The low MSE's (0.001-0.268) and the high r^2 (0.896-0.999), as seen in Tables 4.1, 4.2, 4.3 and 4.4, indicate that the models evaluated can sufficiently describe the individual effect of temperature or a_w on the colony growth rate or the lag phase duration for both isolates.

Table 4.1. Coefficients, r^2 and MSE's of the linear Arrhenius-Davey models, $\ln(g) = C_0+C_1/T+C_2/T^2$, developed for *A. flavus* and *A. parasiticus*.

A. flavus

a_w	C_0	C_1	C_2	T_{opt} (°C)	r^2	MSE
0.982	$-1.25\times10^3 \pm 1.15\times10^2$	$7.58\times10^5 \pm 6.87\times10^4$	$-1.15\times10^8 \pm 0.10\times10^7$	29.8	0.992	0.020
0.951	$-1.49\times10^3 \pm 1.23\times10^2$	$9.03\times10^5 \pm 7.37\times10^4$	$-1.37\times10^8 \pm 0.11\times10^7$	30.2	0.994	0.023
0.921	$-1.52\times10^3 \pm 6.86\times10^1$	$9.24\times10^5 \pm 4.11\times10^4$	$-1.40\times10^8 \pm 6.15\times10^6$	30.1	0.998	0.007
0.893	$-2.18\times10^3 \pm 5.48\times10^2$	$1.32\times10^6 \pm 3.31\times10^5$	$-2.00\times10^8 \pm 0.50\times10^7$	30.0	0.944	0.083
0.855	$-1.87\times10^3 \pm 1.75\times10^2$	$1.13\times10^6 \pm 1.06\times10^5$	$-1.71\times10^8 \pm 0.16\times10^7$	29.9	0.992	0.009

A. parasiticus

a_w	C_0	C_1	C_2	T_{opt} (°C)	r^2	MSE
0.982	$-8.47\times10^2 \pm 7.69\times10^1$	$5.14\times10^5 \pm 4.60\times10^4$	$-7.78\times10^7 \pm 0.69\times10^7$	29.6	0.992	0.009
0.951	$-6.74\times10^2 \pm 1.12\times10^2$	$4.11\times10^5 \pm 6.71\times10^4$	$-6.23\times10^7 \pm 1.00\times10^7$	30.5	0.980	0.019
0.921	$-9.34\times10^2 \pm 1.75\times10^2$	$5.66\times10^5 \pm 1.05\times10^5$	$-8.55\times10^7 \pm 1.57\times10^7$	29.1	0.962	0.045
0.893	$-1.56\times10^3 \pm 2.73\times10^2$	$9.39\times10^5 \pm 1.63\times10^5$	$-14.1\times10^7 \pm 2.44\times10^7$	28.1	0.956	0.110

Table 4.2. Coefficients, r^2 and MSE's of the linear Arrhenius-Davey models, $\ln(1/\lambda) = C_0+C_1/T+C_2/T^2$, developed for *A. flavus* and *A. parasiticus*.

A. flavus

a_w	C_0	C_1	C_2	T_{opt} (°C)	r^2	MSE
0.982	$-4.02\times10^2 \pm 1.01\times10^2$	$2.42\times10^5 \pm 6.04\times10^4$	$-3.65\times10^7 \pm 0.90\times10^7$	28.0	0.912	0.015
0.951	$-6.38\times10^2 \pm 1.02\times10^2$	$3.85\times10^5 \pm 6.11\times10^4$	$-5.80\times10^7 \pm 0.91\times10^7$	28.5	0.966	0.015
0.921	$-1.09\times10^3 \pm 1.20\times10^2$	$6.61\times10^5 \pm 7.20\times10^4$	$-9.97\times10^7 \pm 1.08\times10^7$	28.9	0.986	0.021
0.893	$-1.63\times10^3 \pm 2.94\times10^2$	$9.89\times10^5 \pm 1.78\times10^5$	$-14.9\times10^7 \pm 2.69\times10^7$	29.6	0.969	0.024
0.855	$-2.00\times10^3 \pm 4.31\times10^1$	$1.21\times10^6 \pm 2.60\times10^4$	$-18.3\times10^7 \pm 0.39\times10^7$	29.6	0.999	0.001

A. parasiticus

a_w	C_0	C_1	C_2	T_{opt} (°C)	r^2	MSE
0.982	$-5.77\times10^2 \pm 2.19\times10^2$	$3.51\times10^5 \pm 1.31\times10^5$	$-5.33\times10^7 \pm 1.96\times10^7$	30.8	0.909	0.071
0.951	$-7.03\times10^2 \pm 1.62\times10^2$	$4.25\times10^5 \pm 9.69\times10^4$	$-6.42\times10^7 \pm 1.45\times10^7$	29.0	0.942	0.039
0.921	$-5.33\times10^2 \pm 1.29\times10^2$	$3.23\times10^5 \pm 7.71\times10^4$	$-4.89\times10^7 \pm 1.15\times10^7$	29.8	0.949	0.025
0.893	$-6.17\times10^2 \pm 5.41\times10^1$	$3.73\times10^5 \pm 3.24\times10^4$	$-5.64\times10^7 \pm 0.48\times10^7$	29.6	0.992	0.004

Table 4.3. Coefficients, r^2 and MSE's of the linear Arrhenius-Davey models, ln (g) = $C_0+C_1b_w+C_2b_w{}^2$, developed for *A. flavus* and *A. parasiticus*.

A. flavus

T (°C)	C_0	C_1	C_2	r^2	MSE
37	1.88 ± 0.43	5.99 ± 3.60	-28.65 ± 6.97	0.993	0.101
30	2.39 ± 0.18	5.59 ± 1.40	-24.77 ± 2.48	0.998	0.052
25	2.02 ± 0.33	5.82 ± 2.80	-24.78 ± 5.40	0.993	0.079
22	2.66 ± 0.92	-2.04 ± 7.72	-16.03 ± 14.92	0.976	0.217

A. parasiticus

T (°C)	C_0	C_1	C_2	r^2	MSE
37	0.07 ± 0.69	24.95 ± 6.46	-73.82 ± 14.03	0.993	0.114
30	1.07 ± 0.94	17.83 ± 7.22	-48.22 ± 12.78	0.964	0.268
25	0.87 ± 0.87	16.80 ± 6.68	-47.31 ± 11.81	0.971	0.247
22	1.39 ± 0.54	8.61 ± 4.53	-32.74 ± 8.75	0.988	0.127
16	-1.44 ± 1.13	26.30 ± 10.66	-75.03 ± 23.16	0.979	0.189

Table 4.4. Coefficients, r^2 and MSE of the linear Arrhenius-Davey models, ln $(1/\lambda)$ = $C_0+C_1b_w+C_2b_w{}^2$, developed for *A. flavus* and *A. parasiticus*.

A. flavus

T (°C)	C_0	C_1	C_2	r^2	MSE
37	0.15 ± 0.27	3.77 ± 2.22	-24.23 ± 4.29	0.997	0.062
30	-0.25 ± 0.25	11.42 ± 1.92	-34.07 ± 3.40	0.996	0.071
25	0.13 ± 0.61	7.45 ± 4.67	-30.83 ± 8.26	0.985	0.173
22	-0.37 ± 0.78	9.90 ± 6.49	-38.14 ± 12.55	0.982	0.183

A. parasiticus

T (°C)	C_0	C_1	C_2	r^2	MSE
30	0.56 ± 0.62	4.56 ± 4.76	-19.64 ± 8.41	0.964	0.176
25	0.25 ± 0.59	5.49 ± 4.91	-21.56 ± 9.50	0.970	0.138
22	-1.29 ± 1.00	13.08 ± 8.38	-34.23 ± 16.20	0.897	0.236
16	-0.89 ± 0.59	3.14 ± 5.55	-13.95 ± 12.06	0.960	0.098

4.4.3 Combined effect of a_w and temperature on colony growth rate

The coefficients of the models developed, a quadratic polynomial and a Gaussian function, describing the combined effect of a_w and temperature on the colony growth rate and their significance and r^2 are shown in Tables 4.5 and 4.6, respectively. The models developed for both isolates are visually depicted as three dimensional surface plots, together with the validation data in Fig. 4.3.

Table 4.5. Coefficients of the model, $\ln(g) = C_0+C_1b_w+C_2b_w^2+C_3T+C_4T^2+C_5Tb_w$, describing the combined effect of a_w and temperature on the growth rates of *A. flavus* and *A. parasiticus*.

Parameter	*A. flavus*	*A. parasiticus*
C_0	-12.01 ± 0.75	8.44 ± 0.96
C_1	ns*	11.732 ± 3.480
C_2	-26.82 ± 3.85	-38.99 ± 6.49
C_3	1.02 ± 0.06	0.70 ± 0.06
C_4	-0.02 ± 0.001	-0.01 ± 0.001
C_5	0.18 ± 0.07	ns
r^2	0.969	0.946

*ns: not significant ($P > 0.05$)

Table 4.6. Coefficients of the model, $g = A * \exp(-0.5((a_w - 1)/b)^2 + ((T - T_{opt})/c)^2)$, describing the combined effect of a_w and temperature on the growth rates of *A. flavus* and *A. parasiticus*.

Parameter	*A. flavus*	*A. parasiticus*
A	15.19 ± 0.51	14.89 ± 0.57
b	0.07 ± 0.002	0.08 ± 0.003
c	6.25 ± 0.22	6.30 ± 0.26
T_{opt}	30.12 ± 0.20	29.91 ± 0.24
r^2	0.976	0.965

*ns: not significant ($P > 0.05$)

In Fig. 4.3 it can be observed that the colony growth rates of both isolates initially increased with increase of both a_w and temperature, maximum growth rates being observed at approximately 29-30°C, after which further increase in temperature resulted in a gradual decrease in the growth rates. Based on an analysis of Davis (1993), Geeraerd et al. (1998) stated that synergistic or antagonistic interaction between factors occurs when the curvature of the dependent variable as a function of one independent variable depends on the value of another independent variable. Based on this, it clearly can be

seen from the curvature of the model surfaces that synergistic interaction occurs between the effects of a_w and temperature on the colony growth rates of both isolates. This visually observed interaction is not necessarily reflected in statistical terms. From Table 4.5, the estimated coefficients of the quadratic polynomial equation show that statistically significant interaction between a_w and temperature was noted only for *A. flavus* and not for *A. parasiticus*. In the later case, the significance of the quadratic terms (b_w^2 and T^2), and the opposite signs of parameters C_3 and C_4 ensure the curvature. In agreement to these findings Marin et al. (1998b) found significant a_w-temperature interactions on the mycelial development of *A. flavus*. Synergism between the effects of a_w and temperature was reported in chapter 3 for *F. verticillioides* and *F. proliferatum* and has also been reported by Velluti et al. (2000). Generally it is well agreed that in contrast to bacterial growth a_w is more important for controlling fungal growth than temperature (Holmquist et al. 1983, Sautour et al. 2001b, Velluti et al. 2000).

It can be seen in Table 4.6 that the T_{opt} estimated by the Gaussian function (Eq. 4.6) are 30.1 and 29.9°C for *A. flavus* and *A. parasiticus*, respectively. These values are in good agreement to those estimated by the linear Arrhenius-Davey models, describing the individual effect of temperature on the colony growth rate and those values mentioned in literature. Both isolates failed to grow at a_w 0.801 and only grew at 25 and 30°C at a_w 0.822. The a_{wmin} reported here are also generally in good agreement with those reported by other workers. Sautour et al. (2001a) have reported that *A. flavus* had an a_{wmin} of 0.822, whereas Ayerst (1969) and Pitt and Miscamble (1995) have reported values of 0.78 and 0.81, respectively. The value of the mathematical/statistical validation parameters used for determining the ability of the models to describe the influence of a_w and temperature on the colony growth rate is shown in Table 4.7. From these it can be observed that although both models provide acceptable fits to the experimental data, the quadratic polynomial equation had smaller MSE's and was more accurate than the Gaussian function. The MSE's were 0.138 and 0.683 on average for the quadratic polynomial and Gaussian functions, respectively. A comparison of the accuracy factors shows that the predicted growth rates by the quadratic polynomial and the Gaussian functions deviated on average from the observed values by 8.05 and 16.3%, respectively,

implying that the predictions made by the Gaussian model were twice as deviant from the validation data as those made by the quadratic polynomial function.

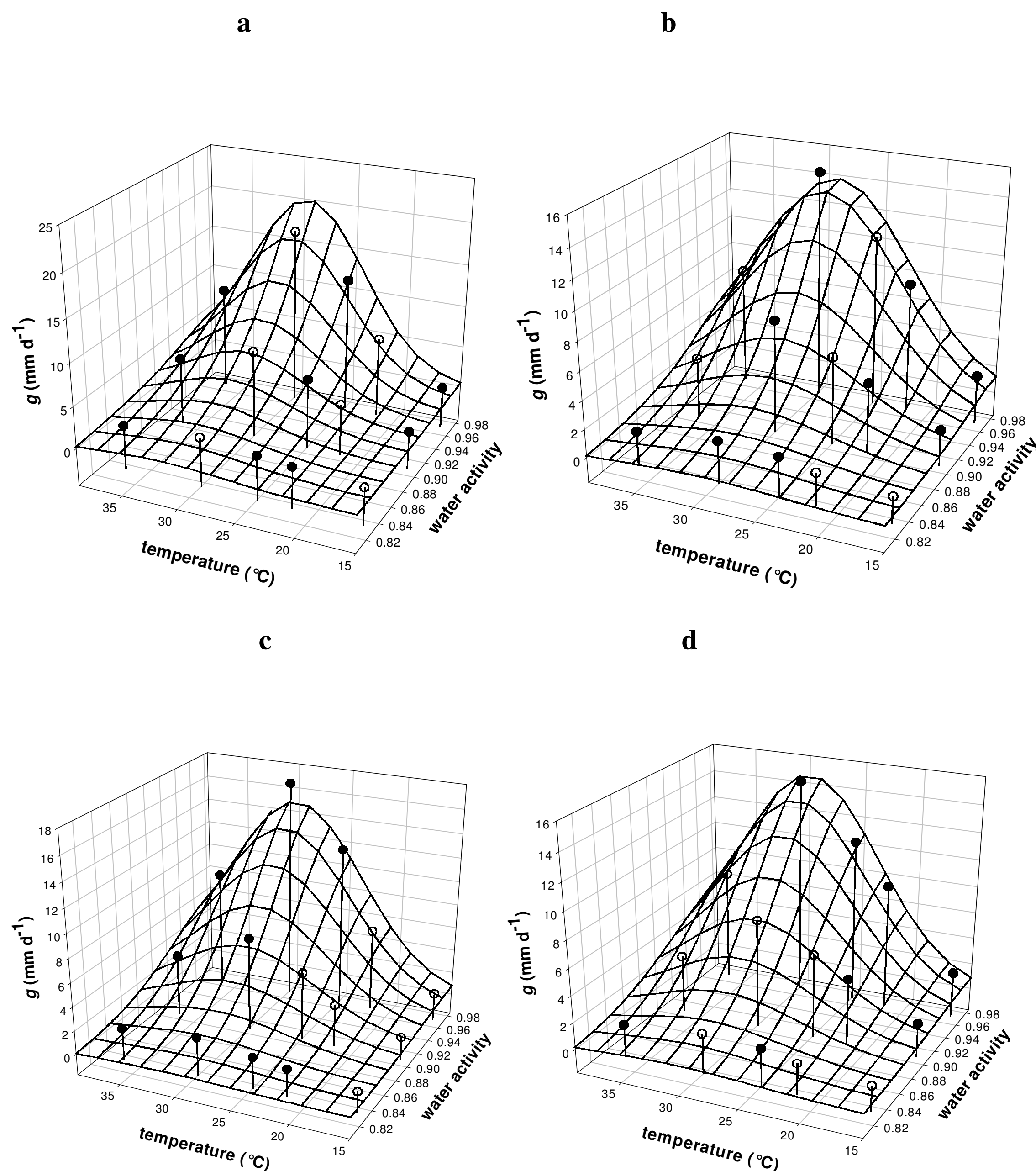


Fig. 4.3. Surface plots of the colony growth rates predicted by ln $(g) = C_0+C_1b_w+C_2b_w^2+C_4T+C_5T^2+C_7Tb_w$ as a function of a_w and temperature for (a) *A. flavus* and (b) *A. parasiticus*; and surface plots of the colony growth rates predicted by $g = A * \exp(-0.5((a_w - 1)/b)^2 + ((T - T_{opt})/c)^2)$ as a function of a_w and temperature for (c) *A. flavus* and (d) *A. parasiticus*. The symbols represent the observed validation data points above (●) and below (○) the model surface.

Table 4.7. Mathematical indices used to validate the growth rate models, describing the combined effect of a_w and temperature on the growth rates of *A. flavus* and *A. parasiticus*.

	A. flavus		*A. parasiticus*	
Validation Indices	Model 1[a]	Model 2[b]	Model 1	Model 2
Bias factor	1.016	0.906	1.036	1.023
Accuracy factor	1.051	1.183	1.110	1.143
F-test	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
MSE	0.154	0.999	0.122	0.367

[a] model 1: $\ln(g) = C_0+C_1b_w+C_2b_w^2+C_3T+C_4T^2+C_5Tb_w$
[b] model 2: $g = A * \exp(-0.5((a_w - 1)/b)^2 + ((T - T_{opt})/c)^2)$,

For *A. flavus* the Gaussian function had a bias factor < 1 indicating that the model was 'fail safe' or rather that it predicted colony growth rates that where larger than those that were observed. Fig. 4.4, showing the plots of the predicted growth rates as a function of the observed growth rates, clearly shows that for both isolates the quadratic polynomial function has better interpolating ability than the Gaussian function owing to the closer and more even distribution of the plot about the line of equivalence. This is confirmed in Fig. 4.3 where it can be seen that the validation data are randomly spread above and below the model surfaces developed from the polynomial models. This is in contrast to the uneven distribution of the validation data about the model surfaces developed from the Gaussian function. This confirms the superiority of the quadratic polynomial equation over the Gaussian function as a predictor of the combined effect of a_w and temperature on the true mean colony growth rates for both isolates.

4.4.4 Combined effect of a_w and temperature on the lag phase

The coefficients of the models developed describing the combined effect of a_w and temperature on the lag phase duration and their significance and r^2 are shown in Table 4.8, whereas the three dimensional surfaces of the models developed for both isolates and the validation data are given in Fig. 4.5.

Both the quadratic polynomial function and the extended Arrhenius-Davey model correctly depicted the experimental data as at any a_w value within the experimental limits,

the T_{opt} (at which the lag phase was shortest) can be clearly seen to occur around 30-33°C for both isolates. Generally it can be observed that at high temperatures and a_w values very short lag phases were encountered, both a_w and temperature having little effect in this zone. Of importance is the observation that in the suboptimal region the effect of a_w on the lag phase becomes much more pronounced the lower the temperature becomes, and vice versa. This gives rise to the significant curvature (steep increase in λ) noted at the growth limiting conditions, indicating that a_w and temperature synergistically influence the lag phase duration.

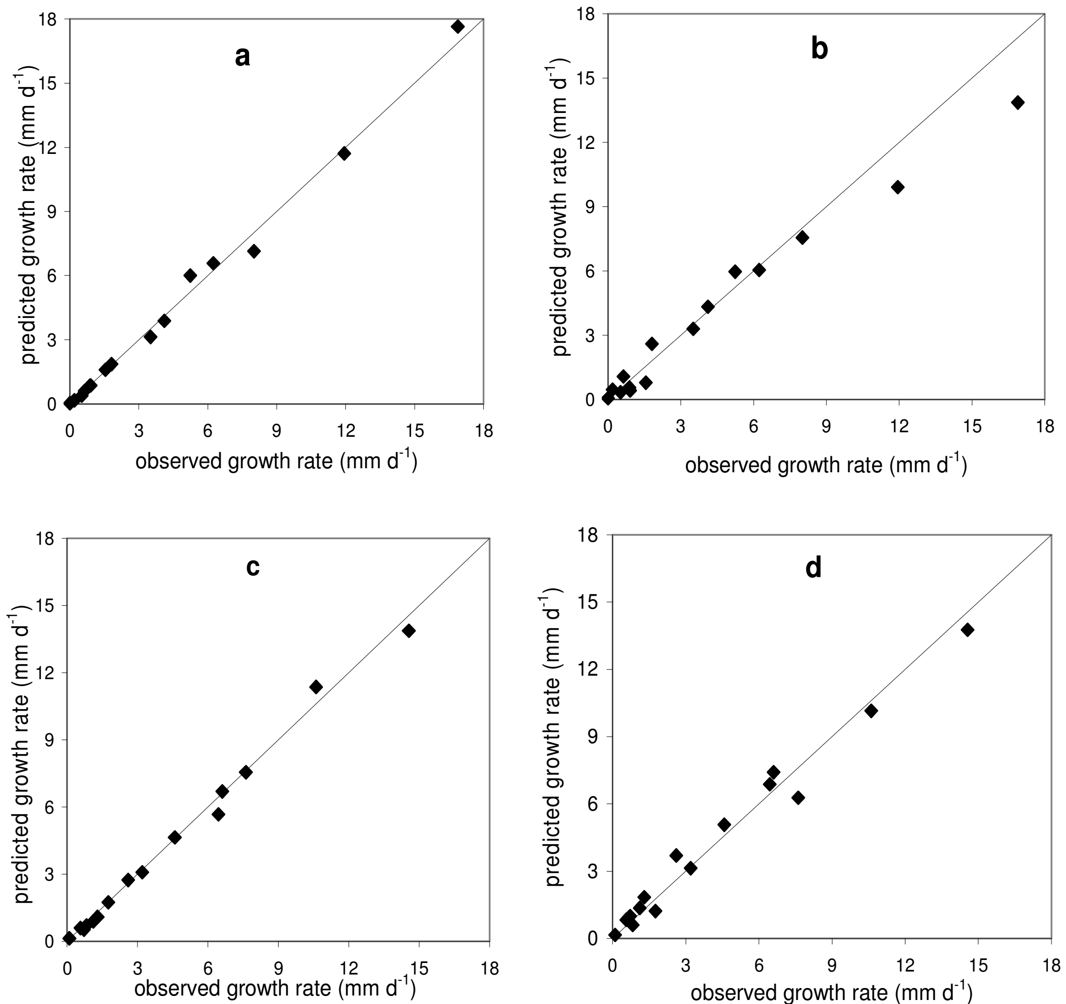


Fig. 4.4. Plots of observed *vs*. colony growth rates predicted by ln $(g) = C_0+C_1b_w+C_2b_w^2+ C_4T + C_5T^2 + C_7Tb_w$ for (a) *A. flavus* and (b) *A. parasiticus*; and plots of observed *vs*. colony growth rates predicted by $g = A * \exp(-0.5((a_w - 1)/b)^2 + ((T - T_{opt})/c)^2)$ for (c) *A. flavus* and (d) *A. parasiticus*.

Table 4.8. Coefficients of the lag phase models describing the combined effect of a_w and temperature on the duration of the lag phase of *A. flavus* and *A. parasiticus*.

A. flavus

Coefficient	Model 1[a]	Model 2[b]
C_0	$-3.74\times10^{1} \pm 0.53\times10^{1}$	$-6.20\times10^{2} \pm 1.04\times10^{2}$
C_1	$3.20\times10^{1} \pm 0.53\times10^{1}$	$-1.54\times10^{2} \pm 5.75\times10^{1}$
C_2	ns*	ns
C_3	$0.10\times10^{1} \pm 2.03\times10^{-1}$	$4.14\times10^{5} \pm 0.61\times10^{5}$
C_4	$-0.90\times10^{-2} \pm 0.10\times10^{-1}$	$-6.97\times10^{7} \pm 0.96\times10^{7}$
C_5	$-5.46\times10^{-1} \pm 1.87\times10^{-1}$	$5.13\times10^{4} \pm 1.73\times10^{4}$
r^2	0.936	0.934

A. parasiticus

Coefficient	Model 1	Model 2
C_0	$-7.24\times10^{1} \pm 1.45\times10^{1}$	$-6.69\times10^{2} \pm 9.22\times10^{1}$
C_1	$1.33\times10^{2} \pm 3.16\times10^{1}$	$1.32\times10^{2} \pm 3.26\times10^{1}$
C_2	$-6.70\times10^{1} \pm 1.74\times10^{1}$	$-6.63\times10^{1} \pm 1.79\times10^{1}$
C_3	$4.32\times10^{-1} \pm 0.51\times10^{-1}$	$3.66\times10^{5} \pm 0.53\times10^{5}$
C_4	$-0.70\times10^{-2} \pm 0.1\times10^{-2}$	$-5.54\times10^{7} \pm 0.79\times10^{7}$
C_5	ns	ns
r^2	0.927	0.922

*ns: not significant ($P > 0.05$)
model 1: $\ln(1/\lambda) = C_0+C_1a_w+C_2a_w^2+C_3T+C_4T^2+C_5Ta_w$
model 2: $\ln(1/\lambda) = C_0+C_1a_w+C_2a_w^2+C_3/T+C_4/T^2+C_5a_w/T$,

From Table 4.8 it can be seen that, as previously observed for the colony growth rate, both the quadratic polynomial and extended linear Arrhenius-Davey functions imply that a statistically significant interaction between a_w and temperature on the lag phase occurs only for *A. flavus* and not for *A. parasiticus*. However, the highly significant curvature of the surface of the models indicates that a synergistic interaction between a_w and temperature on the lag phase occurs for both isolates which is much more pronounced under growth limiting conditions. In agreement with our results Marin et al. (1998b) examined a_w-temperature interactions of various fungi (including *A. flavus*) on maize extract medium and reported that the lag phases were shortest at a_w 0.994 and 0.95 over a wide temperature range as observed for our results, where both a_w and temperature marginally affected the lag phase at conditions optimal for growth. Also in good agreement with these results, was their finding that at marginal temperatures the lag phases increased markedly and that significant interaction between a_w and temperature on the duration of the lag phase occurred.

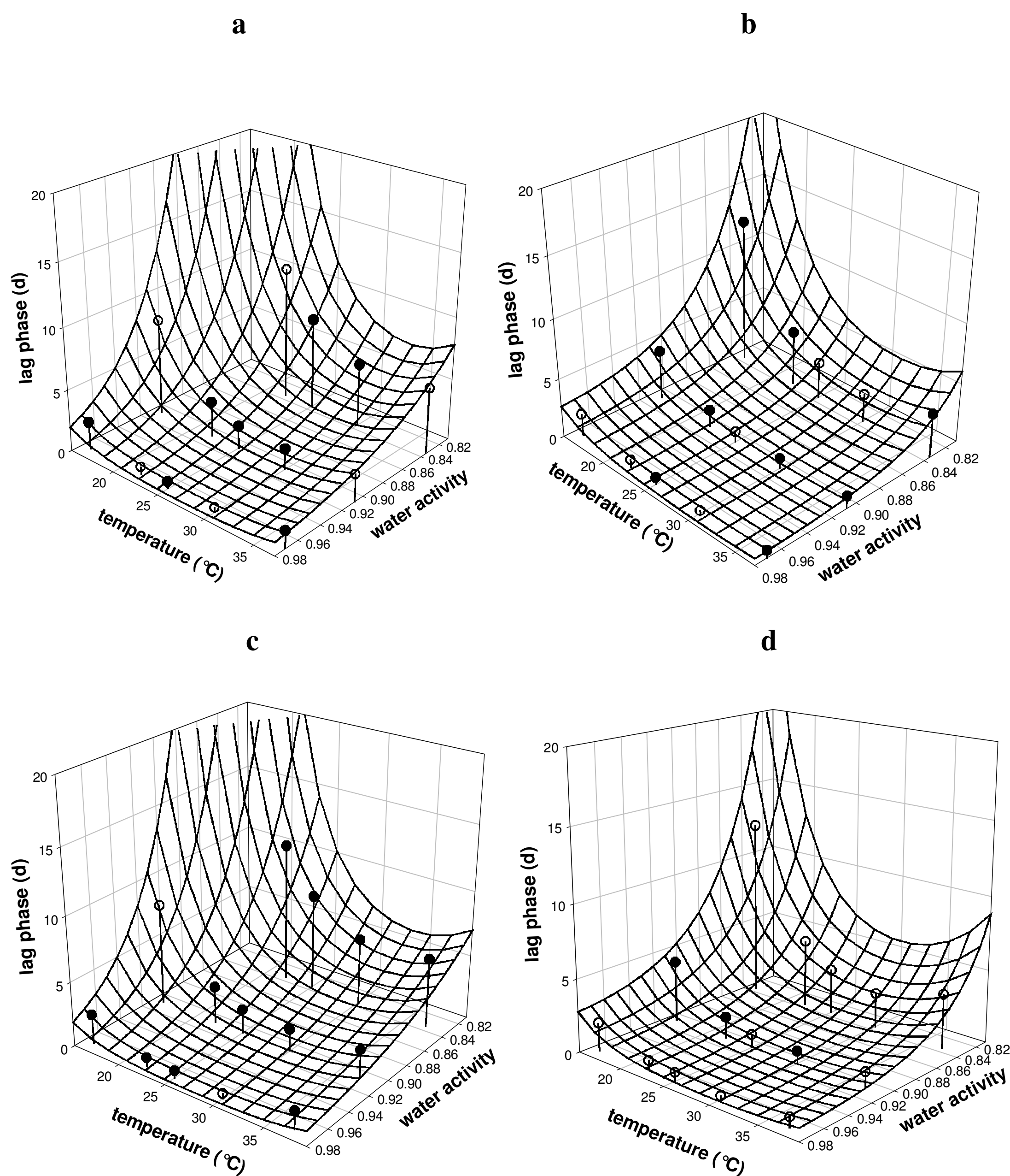


Fig. 4.5. Surface plots of the lag phase predicted by $\ln(1/\lambda) = C_0+C_1a_w+C_2a_w^2+ C_4T + C_5T^2 + C_7Ta_w$ for (a) *A. flavus* and (b) *A. parasiticus*; and when predicted by model $\ln(1/\lambda) = C_0+C_1a_w+C_2a_w^2+ C_4/T + C_5/T^2 + C_7a_w/T$ for (c) *A. flavus* and (d) *A. parasiticus*. The symbols represent the observed validation data points above (●) and below (○) the model surface.

The mathematical/statistical validation criteria calculated to determine the ability of the models developed to describe the influence of a_w and temperature on the lag phase duration are shown in Table 4.9. From these it can be seen that both models generally can be considered to be adequate predictors of the mean lag phase of both isolates. The bias factors are all close to 1, with those of the quadratic polynomial and the linear Arrhenius-Davey actually being 'fail safe' (< 1) for *A. parasiticus* and *A. flavus*, respectively. The lag phases predicted by the quadratic polynomial equation and the linear Arrhenius-Davey differ on average by 10.8% and 12.7% from the observed values, respectively.

Table 4.9. Mathematical indices used to validate the lag phase models, model 1: $\ln (1/\lambda) = C_0+C_1a_w+C_2a_w^2+ C_3T +C_4T^2+C_5Ta_w$ and model 2: $\ln (1/\lambda) = C_0+C_1a_w+C_2a_w^2+C_3/T+C_4/T^2+C_5a_w/T$, describing the combined effect of a_w and temperature on the duration of the lag phase of *A. flavus* and *A. parasiticus*.

	A. flavus		*A. parasiticus*	
Validation Indices	Model 1	Model 2	Model 1	Model 2
Bias factor	1.034	0.912	0.986	1.098
Accuracy factor	1.090	1.121	1.126	1.132
F-test	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
MSE	0.104	0.185	0.060	0.079

The plots of the predicted lag rates as a function of the observed lag phases shown in Fig. 4.6, illustrate that for both isolates the quadratic polynomial function has better interpolating ability than the linear Arrhenius-Davey function owing to the closer and more even distribution of the plot about the line of equivalence. As can be seen in Fig. 4.6b and 4.6d, although the plots of observed *vs*. predicted lag phase are close to the line of equivalence, they are not evenly distributed about it. These observations are confirmed by the distribution of the validation data about the three dimensional surfaces of the models seen in Fig. 4.5. Generally the validation data are more evenly distributed about the model surfaces developed from the quadratic polynomial function than those developed from the linear Arrhenius-Davey model. Consequently the quadratic

polynomial function is a better predictor of the combined effect a_w and temperature on the lag phase duration of both isolates than the linear Arrhenius-Davey model.

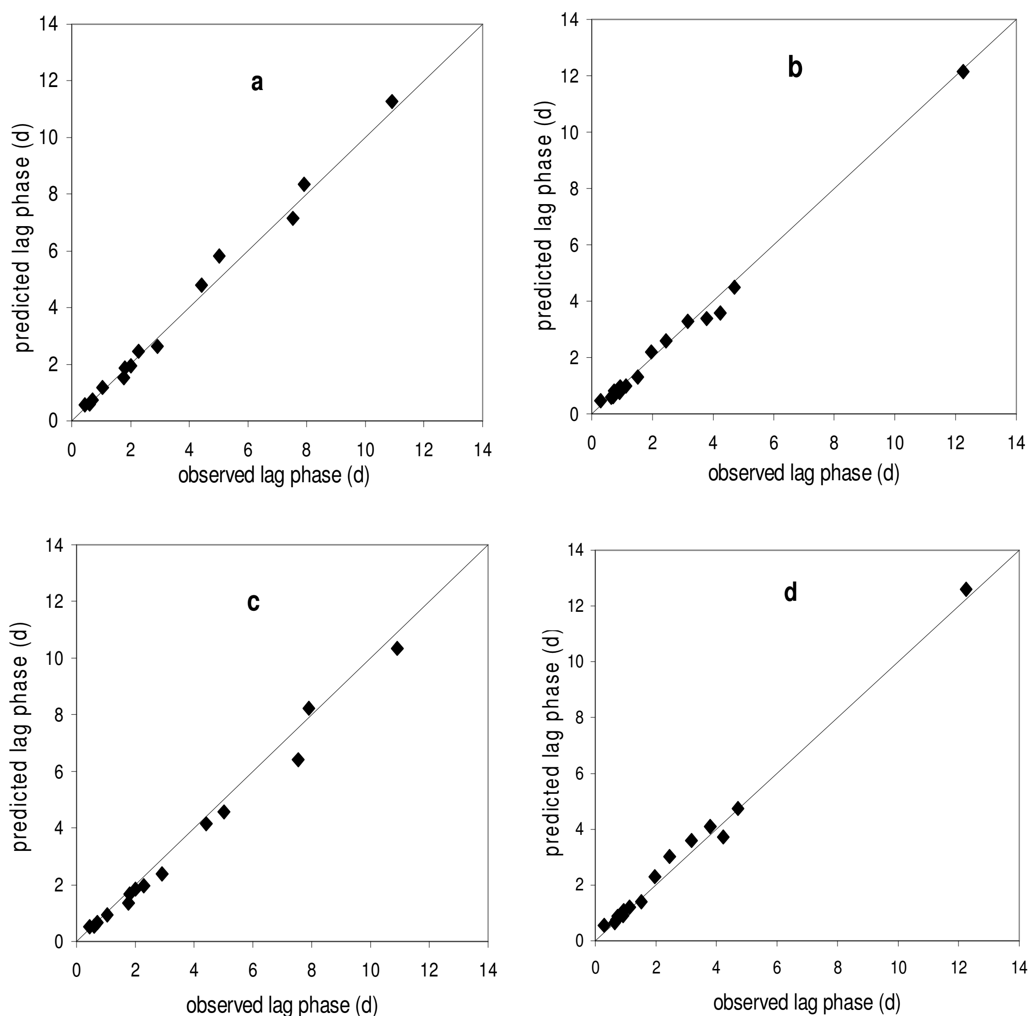


Fig. 4.6. Plots of observed *vs*. lag phase durations predicted by ln $(1/\lambda) = C_0+C_1b_w+C_2b_w^2+ C_4T + C_5T^2 + C_7Tb_w$ for (a) *A. flavus* and (b) *A. parasiticus*; and plots of observed *vs*. lag phase durations predicted by ln $(1/\lambda) = C_0+C_1a_w+C_2a_w^2+ C_4/T + C_5/T^2 + C_7a_w/T$ for (c) *A. flavus* and (d) *A. parasiticus*.

4.5 Conclusions

The models that were evaluated and validated all proved to be suitable predictors of the colony growth rates and lag phase durations of *A. flavus* and *A. parasiticus* on corn within the experimental limits according to the validation criteria applied. However, quadratic polynomial functions proved to be the best predictors of the combined effect of a_w and temperature on either the lag phase and colony growth rates. The estimated T_{opt} and the observed a_{wmin} for growth were in good agreement with those reported by others. Previously developed predictive mathematical relationships between growth rates and environmental parameters such as a_w and/or temperature (Pitt 1993, Marin et al. 1998b, Sautour et al. 2001a) were without validation on the actual growth substrates. However, they also describe the general trend of an increase in colony growth rate with increase in either a_w and/or temperature predicted by other models and reported similar optimal and growth limiting conditions. In light of the demonstrated ability of the polynomial models to adequately describe the growth of both *Aspergillus* and *Fusarium* species (chapter 3), this model was chosen as the basic model for use in the planned modelling of the combined influence of a_w and different non-chemical and chemical preservation factors on the growth of *Fusarium* and *Aspergillus* species on corn.

As observed in chapters 3 and 4, growth from large fungal inoculum exhibits very little variation. This, however, may not be the case when growth emanates from smaller fungal inoculum sizes. The size of fungal inoculum causing natural infection is not known, and it may be that small inoculum sizes are just as important as larger ones. To determine the extent of the variation, a method developed for the isolation of single bacterial cells was modified and validated in chapter 4 for the isolation of single fungal spores and used for determining the extent of variability in the growth of single fungal spores on corn meal as influenced by the most important environmental factors.

CHAPTER 5

Growth kinetics of cultures from single spores of *Aspergillus flavus* and *Fusarium verticillioides* on yellow dent corn meal [5]

[5] Redrafted from: Samapundo, S., Devlieghere, F., De Meulenaer, B. and Debevere, J. M. *Food Microbiology* (accepted).

Chapter 5. Growth kinetics of cultures from single spores of *Aspergillus flavus* and *Fusarium verticillioides* on yellow dent corn meal

5.1 Abstract

A dilution protocol originally developed for the isolation of single bacterial cells was modified to suite the specificities of fungal growth. The modified protocol was used to study the growth kinetics of single spores of A. flavus and F. verticillioides *on yellow dent corn meal. Both* a_w *and temperature significantly influenced the distributions of the colony growth rates and lag phases for growth and the rate at which individual spores of both isolates completed the lag period. An interaction between* a_w *and temperature was noted on the spread of the distributions of these growth parameters. The histograms of the single spore colony growth rates and lag phases generally became wider the more compromising the conditions for growth became, indicating a greater variation in growth ability at these conditions. The rate at which the single spores passed through the lag phases generally decreased with decrease in temperature and/or* a_w, *with an interaction again noted between these two factors on the rate. These results show the potential range and variability in growth of individual fungal spores at the lowest inoculum level possible. In view of these results and the continuing worldwide research on methods for the protection of stored cereal grains from fungal spoilage and the prevention of possible mycotoxin production in those cases, it is important that the influence of these potential techniques is investigated at several inoculum levels (including the individual spore level) to have an idea of the overall variation in the effects of these techniques on both growth and mycotoxin production.*

Key words: *Aspergillus flavus*, *Fusarium verticillioides*, Individual lag phases, individual colony growth rates, water activity, temperature, individual spores

5.2 Introduction

Traditionally the growth and ecophysiological characterization of fungal contaminants of foods has been determined using very high inoculum levels in the form of spore suspensions or circular disks cut from the margins of growing colonies. As observed in chapters 3 and 4 and in numeruous studies by other workers, very little variation is observed in the growth of fungal species evaluated this way. Although sufficient, this approach may overlook the significance of fungal behaviour and variability in growth potential at the individual spore level on a food product. The behaviour and distribution of the growth rates and lag phase duration/germination times of individual spores potentially provides important information with regards to the ability of a single spore to germinate, grow and spoil a food product. It may actually be that contamination at low inoculum levels or by individual spores plays a more important role in fungal infection, spoilage of foods and subsequent production of mycotoxins of public and animal health significance than previously thought. The size of the spore inoculum has been already found to have direct relationship to fumonisin production on corn (Chulze et al. 1999) and has also been found to have a strong effect on the lag duration of *Penicillium chrysogenum* (Sautour et al., 2003). In these studies an increase in inoculum size resulted in an increase in the amount of fumonisins produced and a decrease in the lag durations, respectively.

Fungal growth begins with germination of spores followed by hyphal extension (Dantigny et al., 2005a, 2006), ultimately leading to formation of visible mycelium. Germination is mostly determined microscopically by examination of the extension of the germ tube, whereas mycelial growth is mostly determined macroscopically by evaluating changes in the colony diameter. No single definition of germination has been universally accepted, with a spore generally being considered as having germinated when the length of the germ tube is half, equal to or twice as long as the spore diameter (Dantigny et al., 2005a, 2006). Whilst the microscopic assessment of individual spore germination is possible on translucent artificial media such as solid agar, the major setback to validating these observations on real food products has been that microscopic examination is

severely impeded by the opaqueness of natural food products. This may be overcome by the use of more powerful visualization tools such as Scanning Electron Microscopy (SEM) (Bacon et al., 1992; Torres et al., 2003). Alternatively one could possibly rely on the macroscopic assessment of mycelial extension emanating from an individual spore. An important observation in support of this was made Dantigny et al. (2002), who reported that microscopic observations can be substituted with macroscopic ones, based on their findings that the lag phase coincided with the completion of the germination process. They also stated that the percentage of germinated spores can be considered as the probability a single spore would have germinated. This has important ramifications as in principal the lag phase of a single spore from macroscopic evaluations can be assumed to be a good estimate of its germination time under standardized conditions, thereby allowing for the macroscopic based estimation of germination rates and distribution of individual germination times of fungi on natural food products.

As demonstrated in chapters 3 and 4, growth from large fungal inoculum exhibits very little variation. This may not be the case when growth emanates from smaller fungal inoculum sizes, and indeed the overall extent of variation in growth can be revealed by evaluation of growth at the single spore level. As an extension to the study on the modelling growth of the most important fungal contaminants of corn, an attempt was made to develop in this chapter a working method for the isolation of single spores and for the determination of their ability to grow on corn as influenced by the most important environmental factors. Recently a protocol was developed for the isolation of single bacterial cells of *Lactococcus lactis* by Francois et al. (2003). This method was chosen and modified to isolate single spores on the basis that it was reported to combine a higher chance of having one cell in a microtitre plate with a high yield, and its relative simplicity. In addition to the validation of the protocol for the isolation of single spores of *Aspergillus flavus* and *Fusarium verticillioides*, the method was used to investigate the growth kinetics of these two isolates on yellow dent corn meal and the influence of a_w and temperature on the trends observed. In addition, the influence of a_w and temperature on the rate at which the individual spores completed the lag phase was also determined.

5.3 Materials and Methods

5.3.1 Preparation of fungal inoculum

The isolates used in the study, *F. verticillioides* Sheldon (25N) and *A. flavus* (IITAB 139), were obtained courtesy of the Food Technology Department Culture Collection of the University of Lleida (Spain) and the International Institute of Tropical Agricultural (Cotonou, Benin), respectively. The isolates were sub-cultured on potato dextrose agar (PDA) (Oxoid, Basington, UK). To prepare the inoculum, centrally inoculated PDA plates were incubated at 30°C for seven days to enable sporulation to take place. The plates were then transferred to an incubator at 20 or 30°C for a further day, which enabled the spores to adapt to their final incubation temperature. After incubation 5 ml of wetting agent (3g Tween-20/L distilled H_20) was aseptically added to each plate. A sterile plastic inoculation loop was then used to loosen the colonies (spores and mycelia) from the PDA plates. The suspension created containing the spores and mycelia was then filtered through sterile glass wool into to a sterile 50 ml capacity Falcon tube (Meus, Piove di Sacco, Italy). This process was repeated a further three times.

The spores were then separated from any remaining debris (mostly mycelia) by centrifuging in a Sigma 4K15 centrifuge (Sigma, Göttingen, Germany) at 10000 rpm for 15 min. The wetting agent was then carefully decanted from the pellet of spores and replaced by 20 ml of sterile phosphate buffered saline with Tween-20, pH 7.4 (PBS) (Sigma-Aldrich, Steinheim, Germany). The number of spores per ml of spore suspension was then determined using a Bürker counting chamber (Superior Mareinfeld, Lauda-Königshofen, Germany). A Carl Zeiss Axio Imager A1 microscope (Göttingen, Germany) was used to visualize the spores on 20 cells on the counting chamber. The average number of spores per cell was then calculated and used to estimate the number of spores per ml of spore suspension. In most cases there were approximately 1×10^8 spores/ml of suspension. The spore suspension was then serially diluted to produce a final spore inoculum with a concentration of $\approx1\times10^3$ spores/ml.

5.3.2 Protocol validation

The protocol developed by Francois et al. (2003) for the isolation of single bacterial cells was evaluated for its ability to isolate single fungal spores. The evaluation was performed on PDA. In brief, 200 µl of PBS were placed in all of the wells of a 96 well microtitre plate using a micropipette. 200 µl of the spore suspension, prepared on the same day as described in section 2.1 and adjusted to a concentration of $\approx 10^3$ spores/ml, were then added to the wells in the first column resulting in approximately 200 spores in each of these wells. A ½ dilution series was then prepared sequentially, by thoroughly mixing the contents in these wells before transferring 200 µl to the next well, until the last column of the microtitre plate was reached. This was repeated on 6 microtitre plates, giving rise to 48 dilution series per isolate. The contents of the wells were then transferred and spread aseptically on PDA plates which were incubated at 30°C for 3-5 days before enumeration. The performance of the method was determined by comparison of the mean number of spores per column with the theoretically expected means. A comparison was also made with the results obtained for bacterial growth by Francois et al. (2003). According to Francois et al. (2003), each cell theoretically has 50% probability of staying in the well and an equal probability of going to the next well. This would ideally result in 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125, 0.390625, 0.1953125, 0.09765625 and 0.09765625 cells in 12 consecutive wells.

5.3.3 Growth kinetics of single spores of *A. flavus* and *F. verticillioides* on yellow dent corn meal

5.3.3.1 Experimental design

The growth kinetics and effect of a_w and temperature on the distribution of the colony growth rates and lag phase for growth of single spores of *A. flavus* and *F. verticillioides* on yellow dent corn meal was determined at two a_w and temperature values. For *A. flavus*, a_w values of 0.88 and 0.98 were evaluated, whereas values of 0.92 and 0.98 were evaluated for *F. verticillioides*. As *A. flavus* grows over a wider a_w range than *F. verticillioides*, a larger interval in the experimental a_w limits was used to better reveal any

effect a_w may have on growth at the single spore level. The temperatures evaluated for both isolates were 20 and 30°C. For each condition the colony growth rates and lag phases from about 100 individual spores were determined.

5.3.3.2 Preparation of growth substrate

Yellow dent corn supplied by Aveve NV (Belgium) was used as the growth substrate. Upon reception, the corn was kept in a storage room at 7°C to prevent fungal contamination. The GAB models developed for the corn in chapter 2 were used to determine the amount of sterile distilled water that had to be aseptically added to the corn to reproducibly achieve the desired a_w value. The rehydrated corn was then equilibrated over two days at 4°C before it was aseptically ground to a fine meal using a Waring blender (model 8010E, Torpington, Conn., USA). The ground corn was then incubated for a further day at the final incubation temperature (20 or 30°C) to enable it to attain the incubation temperature. The exact a_w of the treated corn was then determined by the Novasina Thermoconstanter TH200 (Axair Ltd. Systems, Pfapfikkon, Switzerland).

5.3.3.3 Inoculation, assessment and mathematical analysis of the growth

Approximately 22 g of the ground rehydrated corn was aseptically transferred into Petri dishes to form a uniform layer. The spore inoculum was prepared and a ½ dilution series was performed as described in sections 5.3.1 and 5.3.2, respectively. However, only the contents of the last five columns were transferred to the centre of the plates with the ground corn as single spore inoculum. To maintain a constant relative humidity during the incubation period, Petri plates with treated grain of the same a_w were placed in plastic buckets containing glycerol/water solutions of the same a_w. During incubation, growth was assessed regularly by measurement of the perpendicular diameters of each growing colony. Considering the known linear growth of fungi in the growth phase (as observed in chapters 3 and 4), it was decided that at least five or six data points be collected per condition during the linear phase of growth. This was strictly adhered to as Dantigny et al. (2005a) observed that early measurements of diameter of the colony improved the

accuracy of the estimated lag period, when this parameter is obtained by extrapolation. Estimates of the colony growth rates (*g*, mm d^{-1}) and lag phase durations (λ, d) of each spore were determined by linear regression of the growth data collected during the linear phase of growth. The colony growth rate was determined as the slope of the curve whereas the lag phase duration was determined by extrapolating the linear regression equation to the time axis. A very significant advantage of the measurement of the colony growth on the petri plates as opposed to the turbidity measurements employed for bacterial cells on the microtitre plates (Francois et al. 2003), was that it was possible to identify and remove most of the petri plates on which more than one spore grew, allowing for the growth data collected to be largely representative of that of single spores. This is not possible with the turbidity measurements where all the growth data from the wells in the last five columns is taken into consideration, some of which contain more than cell.

The normality of the distributions obtained was determined by means of the Kolmogorov-Smirnov test in SPSS® Version 12.0 (SPSS Inc, Chicago, Ill., USA). All the distributions were determined to be normal (results not shown). Cumulative distributions of the percentage of individual spores that had completed the lag period as a function of time at each condition were also created. A logistic model (Whiting and Call, 1993), also used by Dantigny et al. (2002) to describe the percentage of germinated spores of *Mucor racemosus* as a function of time, was used to describe the percentage of single spores that had completed the lag period as a function of time:

$$P = P_{max} / (1 + e^{k(\tau - t)}) \quad (5.1)$$

where P was percentage (%) of spores which have completed the lag period, P_{max} (%) the maximum percentage which was set to 100 (as all the spores used to generate the data germinated), k (d^{-1}) was the slope term for the rate of increasing single spores that had completed the lag period, τ (d) was the time for the inflexion point when half of the spores have completed the lag period, and t (d) was the time.

The logistic function was fitted to the cumulative distributions using the non-linear regression function of SPSS® Version 12.0. A modified Gompertz function has also been used to model such distributions (Marin et al., 1996; Pardo et al., 2005a, 2006), but according to Dantigny et al. (2005a), the logistic function appears to perform better than the Gompertz equation.

5.4 Results and Discussion

5.4.1 Protocol evaluation

Tables 5.1 and 5.2 show the results of the evaluation of the protocol. As observed for bacterial cells (Francois et al. 2003) the dilution series do not necessarily follow the exact pattern expected theoretically, with some wells actually having no spores followed by wells with one or more spores. Although the mean number of spores per column were not identical to the theoretical mean values, they followed the expected trend and were not significantly different to those of theoretical values according to a t-test ($p < 0.05$). As observed for bacterial cells by Francois et al. (2003), single spores were mostly found in the last five columns, with 78 and 74% of the wells which contained spores having single spores of *A. flavus* and *F. verticillioides*, respectively. This rose significantly to 93 and 89% when only the last four columns were considered for *A. flavus* and *F. verticillioides*, respectively. Similar values of 80 and 87.5% were obtained for *L. lactis* cells in the last five and four columns, respectively by Francois et al. (2003). A high yield of individual spores was obtained, 77 for *A. flavus* and 69 for *F. verticillioides* from 48 ½ dilution series. This yield is much higher than that obtained by Francois et al (2003) for *L. lactis* of 75 from 72 trials. Microscopic images of the fungal spores observed during the enumeration on the counting chamber showed very little clumping of the spores, which may very well play an important role in the higher yields noted in this study. On the basis of these results the protocol could therefore be accepted as a simple high yielding means of isolating single fungal spores and was subsequently applied as such for the study to determine the growth kinetics of single fungal spores and the effect of a_w and temperature on the trends observed.

Table 5.1. ½ dilution series of *A. flavus*, including the 95% confidence limits and the theoretically expected values of the mean numbers of spores in each column. The first 8 of 48 trials are shown.

Column	1	2	3	4	5	6	7	8	9	10	11	12
Trial number												
1	91	44	28	17	10	7	3	2	1	1	1	0
2	102	56	32	11	9	5	1	1	1	0	1	1
3	85	42	29	15	9	4	2	3	0	1	0	0
4	86	43	20	16	12	6	3	0	1	0	1	1
5	92	49	29	12	8	6	3	1	1	1	0	0
6	105	51	22	20	12	5	2	0	2	0	1	0
7	82	45	36	15	7	4	4	1	1	0	0	0
8	84	49	23	10	6	6	2	0	0	0	0	0
...48												
Mean	95.42	48.38	25.46	13.00	8.10	4.79	2.25	1.06	0.67	0.40	0.31	0.19
Upper 95% CL*	97.19	49.94	26.80	13.81	8.76	5.28	2.53	1.33	0.87	0.54	0.45	0.30
Lower 95% CL	93.64	46.81	24.11	12.19	7.45	4.30	1.97	0.79	0.46	0.26	0.18	0.08
Theoretical mean values	100	50	25	12.5	6.25	3.125	1.5625	0.78125	0.39062	0.19531	0.09765	0.09765

* CL: confidence limit

Table 5.2. ½ dilution series for *F. verticillioides*, including the 95% confidence limits and the theoretically expected values of the mean numbers of spores in each column. The first 8 of 48 trials are shown.

Column	1	2	3	4	5	6	7	8	9	10	11	12
Trial number												
1	98	52	26	16	8	4	2	2	1	0	1	0
2	95	43	30	12	7	4	2	1	1	0	1	0
3	105	40	25	12	9	4	1	3	0	1	0	0
4	94	51	27	15	10	5	2	0	1	0	0	1
5	90	50	32	13	7	3	3	1	1	1	0	0
6	93	46	24	13	7	4	3	0	3	1	0	0
7	104	39	21	14	8	3	1	1	1	0	0	0
8	102	48	30	15	6	3	1	0	0	0	0	0
...48												
Mean	97.27	47.52	25.46	12.42	6.48	3.98	2.23	1.10	0.69	0.40	0.17	0.13
Upper 95% CL*	98.66	48.79	26.44	12.97	6.92	4.31	2.48	1.39	0.89	0.54	0.27	0.22
Lower 95% CL	95.89	46.25	24.47	11.86	6.04	3.65	1.98	0.82	0.48	0.26	0.06	0.03
Theoretical mean values	100	50	25	12.5	6.25	3.125	1.5625	0.78125	0.39062	0.19531	0.09765	0.09765

* CL: confidence limit

5.4.2 Effect of water activity and temperature on the growth kinetics of single spores of *A. flavus* and *F. verticillioides* on corn meal

Fig. 5.1 illustrates the typical growth curves obtained and how the colony growth rates and lag phase durations were estimated by linear regression and extrapolation. Table 5.3 shows the mean colony growth rates (*g*, mm d^{-1}) and lag phase durations of the two isolates investigated, also shown are the standard deviations for both growth parameters. Generally high determination coefficients (r^2) were obtained for the linear regression of the growth data of at least 0.92; these are however lower than those normally obtained for fungal growth, where r^2 is usually ≥ 0.99 (Dantigny et al., 2002). This could be possibly be explained by the fact that growth was determined early when greater errors in the estimation of the diameter can occur due to the small size of the colonies.

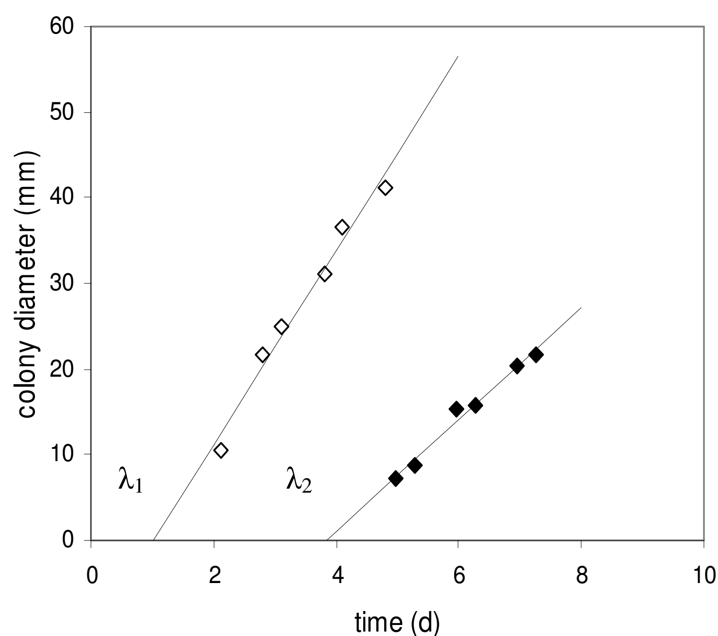


Fig. 5.1. An example of the typical growth curves observed of the individual spores on yellow dent corn meal, indicating the lag durations of spore 1 (λ_1) and spore 2 (λ_2). The colony growth rates of the individual spores were estimated from the slope of the growth curves.

Table 5.3. Mean colony growth rates (g, mm d^{-1}) and lag phase durations (λ, d) of *A. flavus* and *F. verticillioides* on yellow dent corn meal.

	A. flavus							
	a_w 0.98				a_w 0.88			
Temperature	*g* (mm d^{-1})	std. dev.[a]	λ (d)	std. dev.	*g* (mm d^{-1})	std. dev	λ (d)	std. dev.
30°C	11.03	1.55	0.69	0.48	7.89	2.35	1.31	0.58
20°C	9.13	1.62	2.65	0.58	5.43	1.50	2.98	0.73
	F. verticillioides							
	a_w 0.98				a_w 0.92			
Temperature	*g* (mm d^{-1})	std. dev.	λ (d)	std. dev.	*g* (mm d^{-1})	std. dev.	λ (d)	std. dev.
30°C	8.69	1.89	1.45	0.64	6.81	1.84	1.62	0.72
20°C	6.67	1.57	3.44	0.86	3.45	0.79	4.95	1.04

[a] std.dev – standard deviation

As expected the mean colony growth rate decreased whereas the mean lag phase increased with a decrease in a_w and/or temperature. The same trends was also observed in chapters 3 and 4 when high inoculation levels and has also been reported by several other workers (Le Bars et al., 1994; Cahagneir et al., 1995; Marin et al., 1995, 1998b, 1999). In all cases were a high inoculum level was used, a_w was observed to have a greater effect than temperature. Important to note was that the yield of germinating single spores did not differ at any of the conditions within the experimental limits investigated and also did not differ from the yield that had being obtained on PDA during validation of the protocol. This indicated that the ability of the individual spores to germinate on the corn meal was not significantly affected by the a_w and temperature range investigated in this study. This could change if more growth limiting conditions are investigated as Pitt and Miscamble (1995) reported that the a_w minima for germination of *A. flavus* and related species changed with temperature from 0.82, 0.81 to 0.80 at 25, 30 and 37°C, respectively. Marin et al. (1998b) also reported similar minima a_w for germination between 0.80 and 0.85 for *Aspergillus* species including *A. flavus*.

At a_w 0.98, the mean colony growth rates were 11.03 and 8.69 mm d^{-1} at 30°C for *A. flavus* and *F. verticillioides*, respectively. These reduced to 9.13 and 6.67 mm d^{-1} when the temperature was reduced to 20°C. At 30°C a decrease in a_w from 0.98 to 0.88 resulted in a decrease in the mean colony growth rate of *A. flavus* from 11.03 to 7.89 mm d^{-1} at, whereas a decrease from a_w 0.98 to 0.92 at the same temperature resulted in decrease of the mean colony growth rate of *F. verticillioides* from 8.69 to 6.81 mm d^{-1} at. The same trend was observed for both isolates at 20°C. With regards to the lag phase durations of the individual spores, a decrease in temperature at a_w 0.98 from 30 to 20°C resulted in significant increase in the mean lag phase from 0.69 to 2.63 and 1.45 to 3.44 days for *A. flavus* and *F. verticillioides*, respectively.

A similar effect of temperature on the mean lag phases is observed at the lower a_w values investigated, and to a lesser extent for the effect of a_w. The effect of temperature and a_w on the distribution of lag phases and colony growth rates of the individual spores of *A. flavus* is illustrated by the histograms in Fig. 5.2 and 5.3, respectively. Those showing the

effect of temperature and a_w on the individual spores of *F. verticillioides* are shown in Fig. 5.4 and 5.5, respectively.

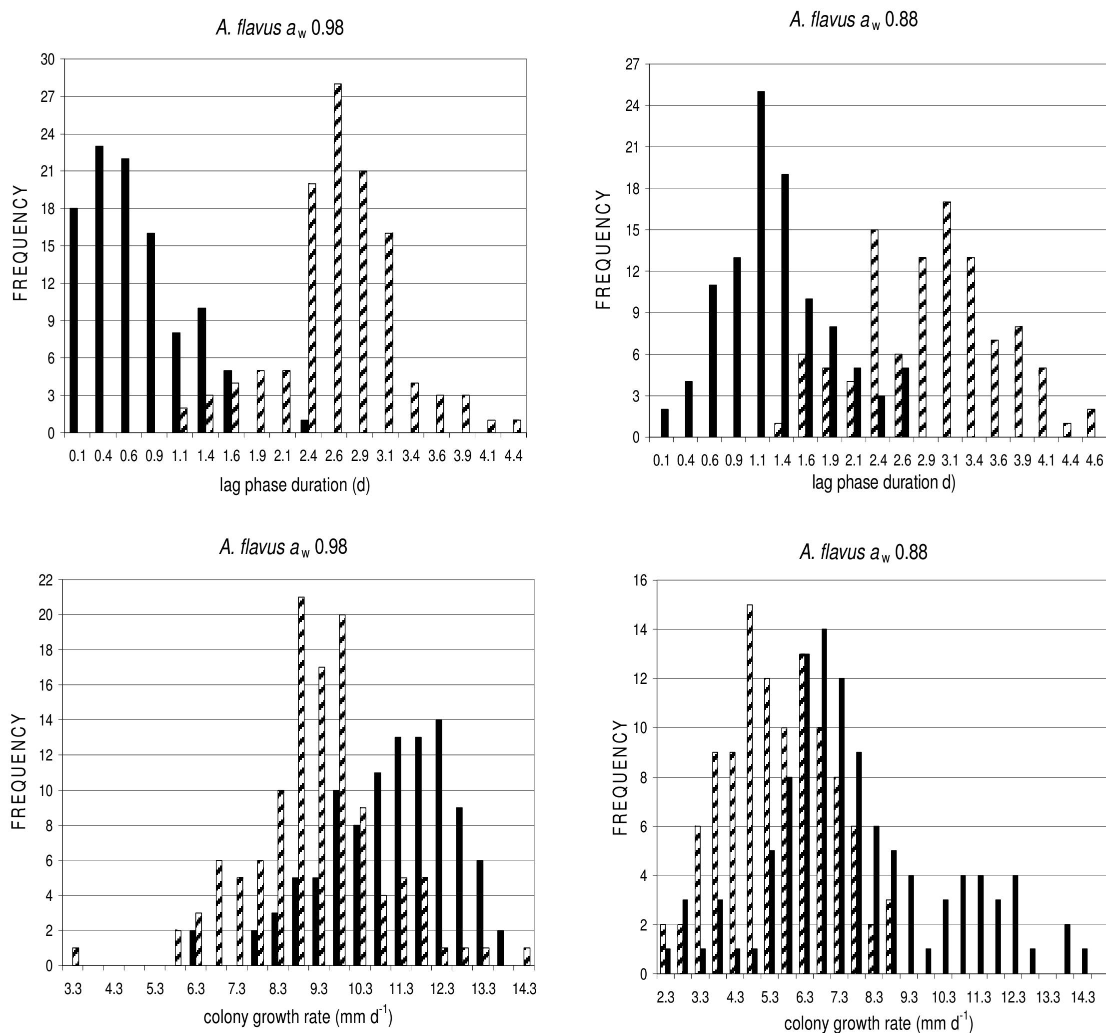


Fig. 5.2. Influence of temperature on the distribution of the lag phases and colony growth rates of individual spores of *A. flavus* at 20 (black and white columns) and 30°C (black columns).

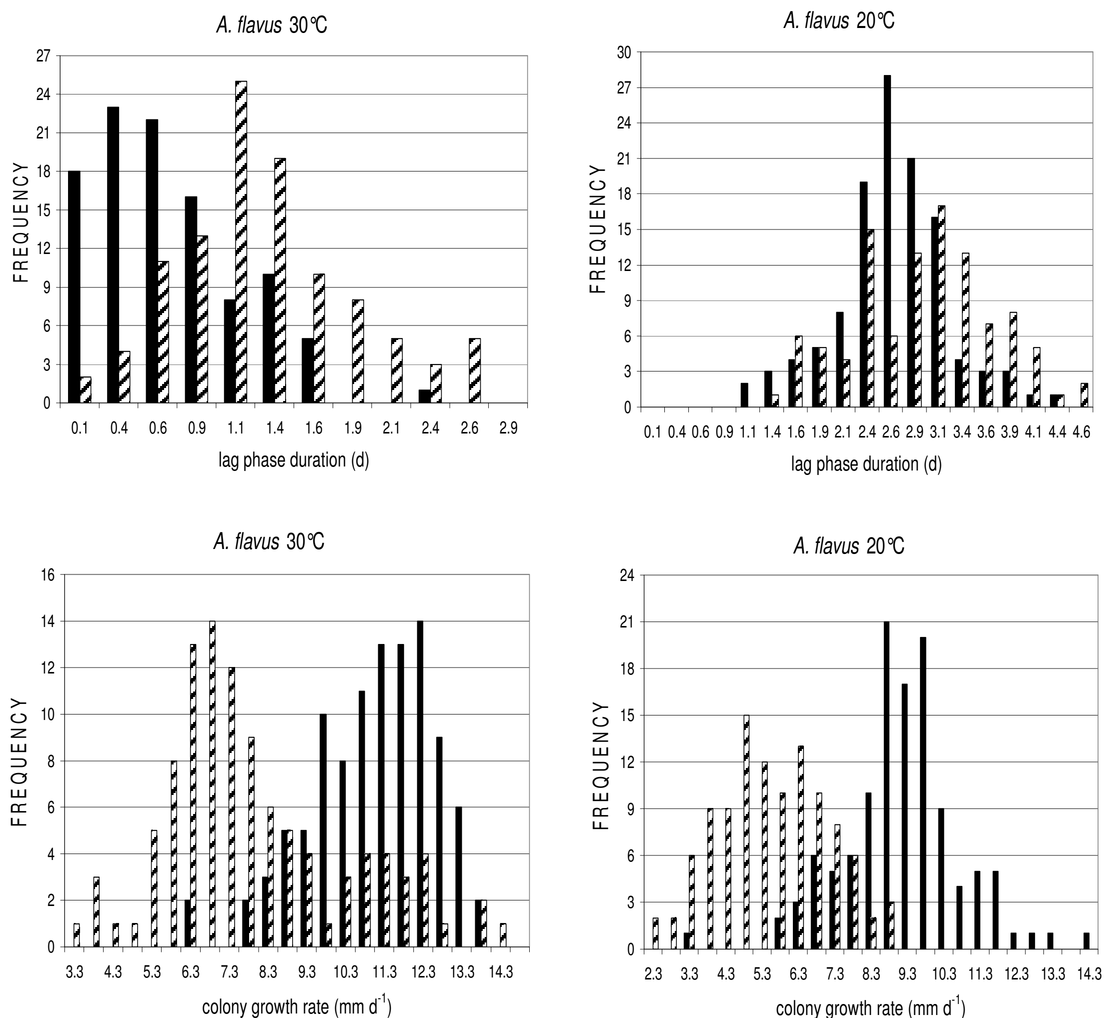


Fig. 5.3. Influence water activity on the distribution of lag phases and colony growth rates of individual spores of *A. flavus* at a_w 0.88 (black and white columns) and 0.98 (black columns).

It can clearly be seen that both temperature and a_w had a very large impact on the distribution of the lag phases and the colony growth rates of the individual spores of both isolates. Most notable is the shift to longer lag phases and smaller colony growth rates when a_w and/or temperature is decreased, alone or in combination. The range of the lag phases and colony growth rates of the individual spores was also observed to be generally larger at more growth compromising conditions than those observed at optimal growth conditions. An example of this being for *A. flavus* at 30°C where at a_w 0.98 the individual spore colony growth rates ranged from 6.16 to 13.76 mm d^{-1} and the lag

phases from 0 to 2.45 days, whereas at a_w 0.88 the individual colony growth rates ranged from 3.45 to 14.36 mm d^{-1} and the lag phases from 0 to 2.69 days. At the most growth limiting condition investigated for *A. flavus* of a_w 0.88 and 20°C the ranges had changed to individual colony growth rates between 2.38 and 8.97 and lag phases between 1.32 and 4.63 days. At 30°C, which had been determined in chapters 3 and 4 to be most optimal growth temperature for the isolates investigated, the distributions of the lag phases and colony growth rates of the individual spores were narrower at both a_w values investigated, for both isolates. At 20°C, which is less optimal for growth, the distributions were spread over a larger range. This indicates that at more optimal conditions for growth, the stress on growth is not sufficient to create large differences in the ability of the spores to germinate and grow.

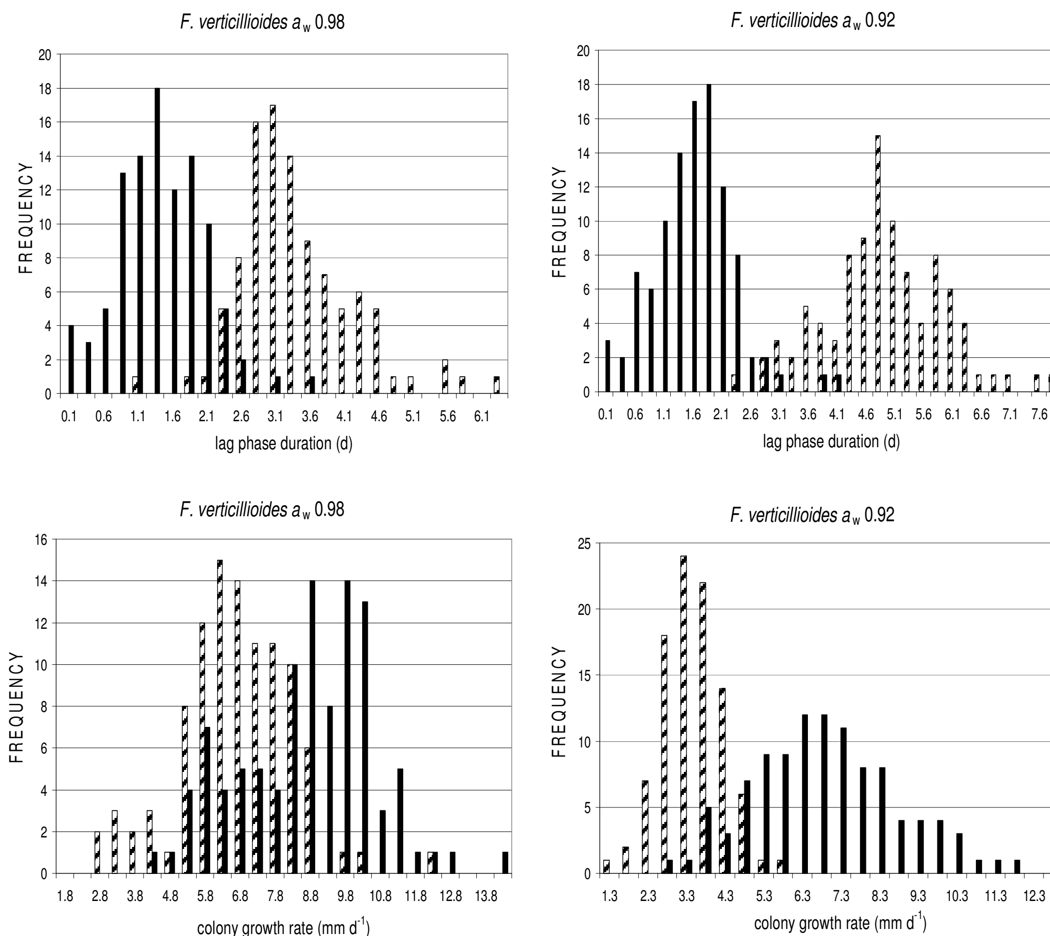


Fig. 5.4. Influence of temperature on the distribution of the lag phases and colony growth rates of individual spores of *F. verticillioides* at 20 (black and white columns) and 30°C (black columns).

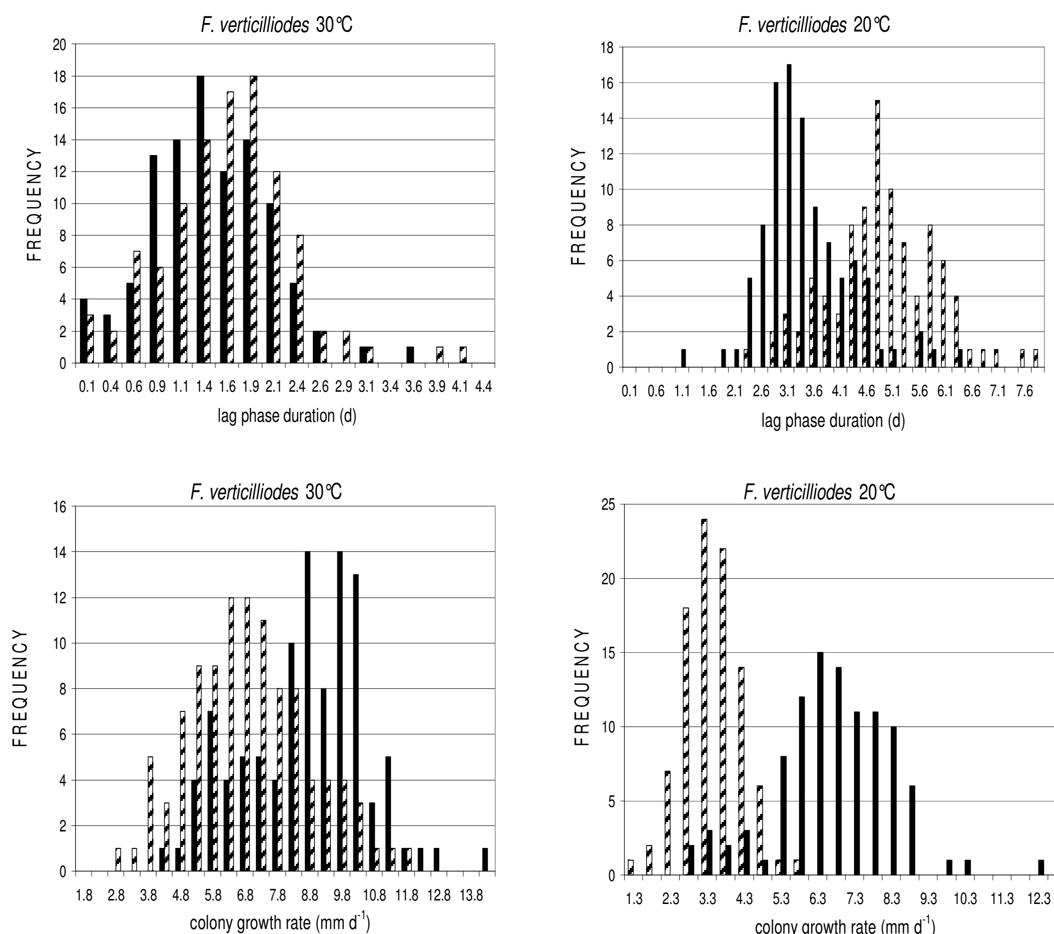


Fig. 5.5. Influence of water activity on the distribution of the lag phases and colony growth rates of individual spores of *F. verticillioides* at a_w 0.88 (black and white columns) and 0.98 (black columns).

At more growth compromising conditions, the stress exerted is clearly depicted in the form of greater variation in the time taken by the spores to start growing and the rate at which they subsequently grow. The effect of temperature appears to be more pronounced on the distribution of the lag phases than it is on the colony growth rates of the individual spores. This can be deduced from the greater degree of overlapping that occurs between the histograms of the colony growth rates compared to those for the lag phases of the individual spores. This may partly be explained by considering that the temperature range investigated may simply be larger in terms of stress imposed on growth compared to the a_w ranges evaluated. Generally at a high a_w value, the distributions of both growth parameters at 20 and 30°C show a greater degree of overlapping than those at a lower less

optimal a_w, indicating that an interaction between a_w and temperature occurs on the distribution of the individual spore lag phases and colony growth rates.

5.4.3 Effect of water activity and temperature on the cumulative distribution of the lag phase durations of single spores of *A. flavus* and *F. verticillioides* on corn meal

The cumulative distributions of the lag phases of the individual spores of both isolates are shown in Fig. 5.6.

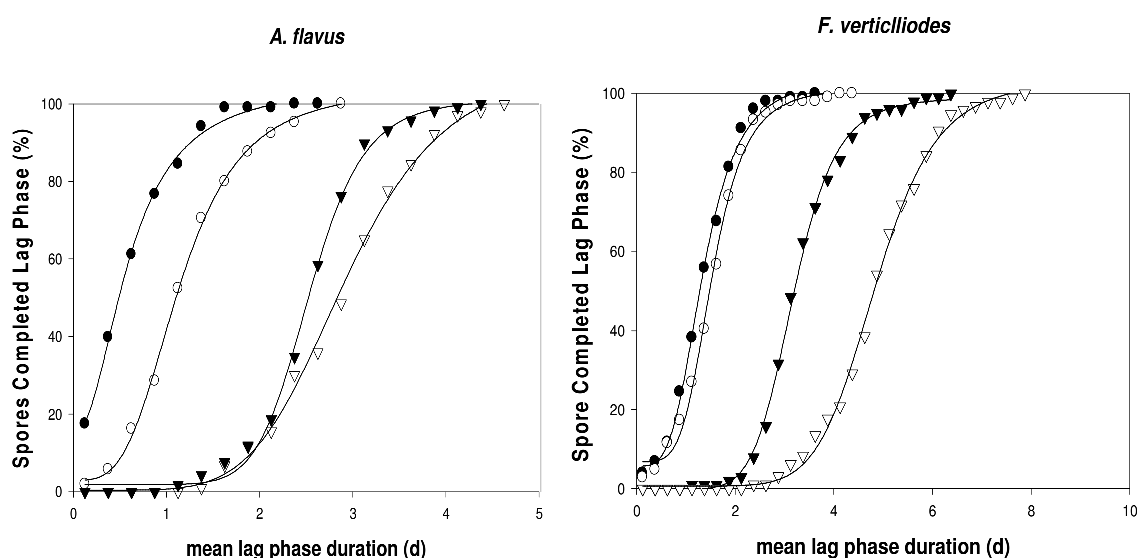


Fig. 5.6. Influence of water activity on the rate of germination of individual lag phases and colony growth rate of *A. flavus* at 30°C a_w 0.98 (●), 30°C a_w 0.88 (○), 20°C a_w 0.98 (▼) and 20°C a_w 0.88 (▽) and *F. verticillioides* at 30°C a_w 0.98 (●), 30°C a_w 0.92 (○), 20°C a_w 0.98 (▼) and 20°C a_w 0.92 (▽).

The parameters of the logistic function fitted to these cumulative distributions are shown in Table 5.4. Very high determination coefficients (r^2) values of ≥ 0.995 were obtained for the fit of the logistic function to the cumulative distributions of both isolates. The effect of both a_w and temperature on the range of single spore lag phases (discussed before in 5.3.2) can also be clearly seen in Fig. 5.6, these generally being wider the more growth limiting the conditions became. For both *A. flavus* and *F. verticillioides*, at the highest a_w value evaluated of 0.98, the slopes of the curves depicting the rate of completion of the lag phase period (k, d^{-1}) at 20 and 30°C are not much different for both isolates. However a comparison of the 95% confidence limits shows that a significant difference occurs for *F. verticillioides*, with a slower rate of completion at 20°C.

Table 5.4. Estimated parameters of the logistic function used to describe the cumulative distribution of the lag phases of *A. flavus* and *F. verticillioides* as a function of time on corn meal.

	A. flavus							
	a_w 0.98				a_w 0.88			
Temperature	k (d^{-1}) [a]		τ (d) [b]		k (d^{-1})		τ (d)	
30°C	3.36	[2.99-3.73]*	0.69	[0.60-0.78]	3.12	[2.77-3.48]	1.14	[1.10-1.18]
20°C	3.33	[3.12-3.54]	2.65	[2.55-2.76]	2.28	[2.13-2.43]	2.85	[2.82-2.88]
	F. verticillioides							
	a_w 0.98				a_w 0.92			
Temperature	k (d^{-1})		τ (d)		k (d^{-1})		τ (d)	
30°C	2.72	[2.59-2.84]	1.31	[1.29-1.33]	2.65	[2.53-2.78]	1.50	[1.48-1.51]
20°C	2.27	[2.05-2.48]	3.24	[3.20-3.29]	1.71	[1.64-1.77]	4.84	[4.81-4.87]

* 95% confidence interval

[a] k - rate of completion of lag phase period

[b] τ – time for half of spores to complete the lag phase

At the lower a_w values evaluated the differences between the slope of the curves (and hence the value of k) at 20 and 30°C becomes more significant for both isolates as seen in both Fig. 5.6 and Table 5.4. This indicates that the rate at which individual spores of both isolates complete the lag period decreases with an increase in the level of stress imposed and further highlights the interaction of the effects of a_w and temperature at the individual spore level. In agreement Sautour et al. (2001c) also found that a significant positive interaction occurred between a_w and temperature on the rate of germination of *Penicillium chrysogenum* spores on an artificial growth medium. Interaction between the effects of a_w and temperature on the germination and germination rates of *Penicillium verrucosum* and *A. ochraceus* on malt extrat agar and green coffee-based medium, respectively, was also reported by Pardo et al. (2005a, 2006).

The influence of both a_w and temperature on τ (d), the time for half of the spores to complete the lag phase, is even more apparent than that on k. This observation has also been reported in studies of *M. racemosus* growth on PDA (Dantigny et al., 2002). Even more striking is the observation that effect of temperature on τ (at the a_w values investigated) is much larger than the effect of a_w at the temperature investigated. An example of this being the increase of τ from 0.69 to 2.65 days for *A. flavus* at a a_w value of 0.98 and from 1.50 to 4.84 days for *F. verticillioides* at a_w value of 0.92 as temperature is lowered from 30 to 20°C. This was already observed in the histograms depicting the distribution of single spore colony growth rates and lag phase durations, and explained by considering that the temperature range investigated may simply be larger in terms of stress imposed compared to the a_w ranges evaluated. It can be deduced from the trend observed for the two parameters that an interaction between a_w and temperature also occurs on the length of time that the individual spore takes to pass through the lag period. As the end of the lag phase is characterized by formation of a visible growing colony which results in a product being considered spoiled, the interaction observed clearly has an important bearing on the shelf-life of food products spoiled by moulds. In assuming that the germination coincides with the lag phase, these same conclusions would be valid for the germination kinetics of the individual spores of both isolates.

5.5 Conclusions

The protocol of Francois et al. (2003), originally developed for the isolation and growth of single bacterial cells, was modified to suit the specificities of fungal growth. The protocol developed fulfilled the criteria required of a simple, reliable and high yielding method. This method was applied to study the effects of a_w and temperature on the distribution of the colony growth rates and lag phase durations of individual spores of *A. flavus* and *F. verticillioides* on corn meal. The effect of the same environmental factors on the rate at which the singles spores pass through the lag period was also discussed.

Both environmental factors significantly influenced the distributions of both growth parameters and the rate at which single spores from both isolates completed the lag period. Interaction was observed between the effects of these two environmental factors on all the growth parameters. In general the range of the colony growth rates and lag phases of the single spores of both isolates generally became wider the more limiting the conditions for growth became. The rate at which the single spores completed the lag phases (k, d^{-1}) generally reduced with a decrease in temperature and/or a_w. These results showed that within the limits investigated yield of the spores was unaffected and that vigorous growth still occurs at the lowest inoculum level possible. In view of these and previously reported results and the continuing worldwide research on methods for the protection of stored cereal grains from fungal spoilage and the prevention of mycotoxin production, it is also important that the influence of these potential techniques be investigated at the individual spore level in addition to the high levels usually studied.

After characterization of the growth of the isolates, the focus of the study shifted in chapter 6 to determining the effect of a_w and temperature on the relationship between radial growth and fumonisin production. In this manner, the growth models developed in chapter 3, together with the relationship established between growth/time and fumonisin production in chapter 6, could be used to predict the size of a colony and estimate the fumonisin production associated with the colony under given temporal combinations of a_w and temperature.

CHAPTER 6

The effect of water activity and temperature on the relationship between fumonisin production and the radial growth of *Fusarium verticillioides* and *F. proliferatum* on corn [6]

[6] Redrafted from: Samapundo, S., Devlieghere, F., De Meulenaer, B., and Debevere, J. 2005. *Journal of Food Protection* 68, 1054-1059

Chapter 6. The effect of water activity and temperature on growth and the relationship between fumonisin production and the radial growth of *Fusarium verticillioides* and *F. proliferatum* on corn

6.1 Abstract

The major objective of this study was to determine the effect of a_w and temperature on the relationship between radial growth and fumonisin production on corn during time, of the two major fumonisin producing Fusaria, Fusarium verticillioides and F. proliferatum. The growth and fumonisin production of these two isolates on corn was studied at water activities between 0.860-0.975 and temperatures between 15-30°C. Both fumonisin production and radial growth (mycelial development) for both isolates increased with a_w at any temperature investigated. The effect of temperature on this relationship was not straightforward. It was however apparent that the effect of temperature on fumonisin production at high a_w values, optimal for growth was only marginal. Whereas at lower a_w values the effect of temperature was more pronounced with more fumonisin production occurring at temperatures less optimal for growth. The optimum temperature for fumonisin production varied between 15 and 25ºC. Notably for F. proliferatum the optimum temperature for growth at any a_w, 30ºC, was the least optimum for fumonisin production. The only other consistent observance for the effect of temperature was that for both isolates the slowest initial rate of fumonisin production was at the 15ºC, where the slowest growth rates were obtained.

Key words: *Fusarium*, fumonisin, corn, growth

6.2 Introduction

Although the *Fusarium* species are best known for the production of the tricothecene mycotoxins (Nelson et al., 1993), species such as *F. verticillioides* and *F. proliferatum* produce a series of other important secondary metabolites including moniliformin, fusarin C, fusaric acid and fumonisins (Bacon and Nelson, 1994). Of current importance are the fumonisins due to their links to both human and animal toxicoses as a result of

consumption of contaminated corn-based food and feeds (Ross et al., 1992; Sydenham et al., 1990).

The effect of environmental factors, competition, and anti-fungal agents on growth and/or fumonisin production by *F. verticillioides* and *F. proliferatum* has been reported by a number of researchers (Bacon and Williamson, 1992; Cahagnier et al., 1995; Marin et al., 1996; Marin et al., 1998d, 1999a, 1999b, 1999c). For those experiments that tried to establish a relationship between growth or time and toxin production, the results have all been based on the effect observed after relatively long fixed periods of time. In most cases single measurements were done after two or more weeks. With regards to the *Aspergillus* species of most importance to corn, the relationship between the aflatoxin production and growth of *A. flavus* has already been well described by Pit (1993). This study was done with the prime objective of generating a better understanding of the relationship between the growth of *Fusarium* species of importance to corn (which was characterized in chapters 3 and 5) and fumonisin production as influenced by a_w and temperature. This would be achieved by evaluating both growth and fumonisin production during time, after short time/growth intervals. The relationship between radial diameter of the colony, time and fumonisin production as influenced by a_w and temperature is therefore reported in detail for the first time.

6.3 Materials and Methods

6.3.1 Experimental design

A full factorial design was used to investigate growth and fumonisin production of both isolates on corn. Four temperatures fixed at 15, 22, 25, and 30°C and four a_w values between 0.860 and 0.975, at which growth took place, were examined with 20 replicates prepared per condition. Samples for fumonisin analysis were collected at six points during the growth to coincide either with time intervals of one week for those colonies that grew slowly or colony diameters of approximately 15, 30, 45, 60, 75 and 85 mm diameter, for those colonies that grew rapidly.

6.3.2 Fungal isolates

Fusarium verticillioides Sheldon (25N) and *F. proliferatum* (Matsushima) Nirenberg (73N), isolated from corn and proven high fumonisin producers, were used in the study. The two isolates were obtained courtesy of the Food Technology Department Culture Collection of the University of Lleida, Spain. Both isolates were maintained on potato dextrose agar (PDA) (Oxoid, Basington, UK).

6.3.3 Preparation of corn substrate

Yellow dent corn supplied by Aveve (NV) Belgium was used as the growth substrate. The a_w of the corn was adjusted to the desired values as previously described in 3.3.1.2.

6.3.4 Preparation of inoculum, inoculation, incubation, and growth assessment

Preparation of the fungal inoculum, inoculation, incubation and growth assessment were done exactly as described in section 3.3.1.3.

6.3.5 Fumonisin analyses

The total fumonisin concentration in the samples was determined in duplicate for each sample by a commercial ELISA kit (enzyme-linked immunosorbent assay), RIDASCREEN Fumonisin Fast (BioPharm, Darmstadt, Germany). The kit is a competitive enzyme immunoassay for the quantitative analysis of fumonisin residues in corn and corn products. The mean lower detection limit of the test is 0.2 µg/g. The upper quantification limit of the ELISA kit for fumonisins in corn and corn products is 9 µg/g. Extraction and preparation of the samples, as well as the test performance, were carried out as suggested and described in the kit. However samples with concentrations greater than 9 µg/g were diluted until their concentration fell within the limits of the kit. As mentioned earlier samples were collected at six points during growth to coincide either with time intervals of one week for those that grew slowly or growth stages of approximately 15, 30, 45, 60, 75 and 85 mm diameter, for those that grew rapidly.

6.4 Results and Discussion

6.4.1 Effect of a_w on the relationship between fumonisin production and radial growth

The effect of a_w on the relationship between colony diameter or time and fumonisin production at different temperatures is shown for *F. proliferatum* and *F. verticillioides* in Figs. 6.1 and 6.2, respectively.

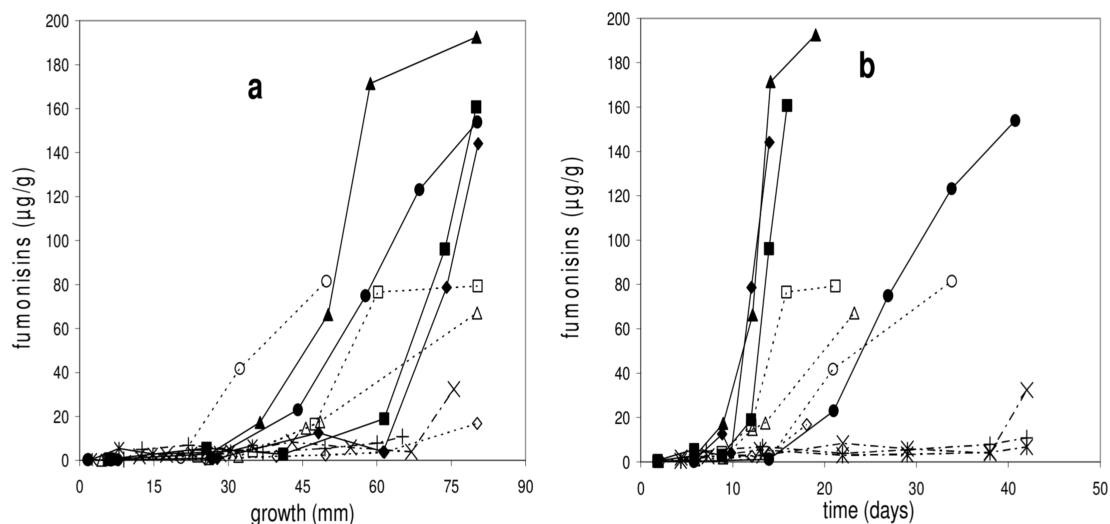


Fig. 6.1. Plots of (a) colony diameter (mm) or (b) time (days) versus fumonisins (µg/g) produced by *F. proliferatum* at at a_w 0.972 (solid symbols) and 30°C (◆), 25°C (■), 22°C (▲), 15°C(●), at a_w 0.948 (empty symbols) and 30°C (◇), 25°C (□), 22°C (△), 15°C (○) and at a_w 0.928 and 25°C (╳), 22°C (✳) and 15°C (+).

It can be seen from Figure 6.1a and 6.2a and 6.2b that for both isolates, at the temperatures investigated, fumonisin production was highly a_w dependent. Generally more fumonisins were produced for the same amount of growth (colony diameter) or time elapsed the higher the a_w. When *F. verticillioides* colonies at 30°C reached 80 mm diameter, 168.1, 3.7, 0.9 and 1.0 µg/g of fumonisins were produced at a_w values of 0.969, 0.949, 0.937, and 0.922 respectively. The greatest differences occurred between a_w 0.969 to 0.949 were the decrease in fumonisin production ranged from 4 to almost 50 fold. The only exception to this trend is noted for *F. proliferatum* at 15ºC and 25°C where the amount of fumonisins produced for the same amount of growth, were initially greater at

a_w 0.948 than at 0.972. However more fumonisins were produced for the same amount of growth at a_w 0.972 than at 0.948 after a colony diameter of approximately 70 mm had been reached at 25°C.

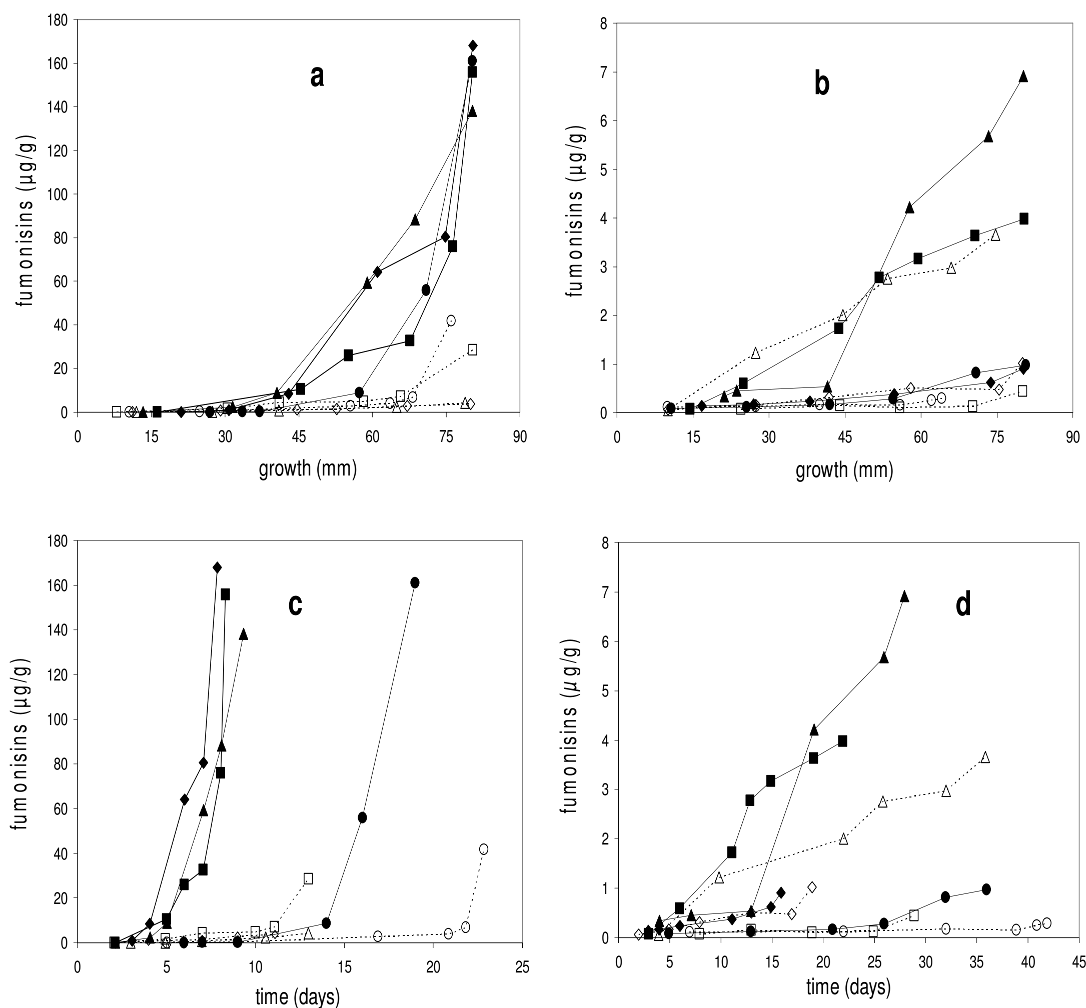


Fig. 6.2. Plots of colony diameter (mm) (a) or (c) time (days) versus fumonisins (µg/g) produced by *F. verticillioides* at a_w 0.969 and 30°C (◆), 25°C (■), 22°C (▲), 15°C (●) and at a_w 0.949 and 30°C (◇), 25°C (□), 22°C (△) and 15°C (○). Plots of colony diameter (mm) (b) or (d) time (days) versus fumonisins (µg/g) produced by *F. verticillioides* at a_w 0.937 and 30°C (◆), 25°C (■), 22°C (▲), 15°C (●) and at a_w 0.922 and 30°C (◇), 25°C (□), 22°C (△) and 15°C (○).

The general trend of a strong magnitude of decrease in the quantities of fumonisins produced and their rate of production with a decrease in a_w at the temperatures investigated, has been reported by other authors but without illustration of fumonisin

production as function of small growth or time intervals. Marin et al. (1999a) observed the same trend as *F. verticillioides* (25N), *F. proliferatum* (75N), and *F. proliferatum* (131N) all generally produced more fumonisins at a_w 0.98 than at 0.95 and 0.92, after four weeks at 15 to 30ºC. Fumonisin B_1 production was also reported by Marin et al. (1999c) to increase with a_w at temperatures between 7 and 30°C for isolates of *F. verticillioides* and *F. proliferatum* grown on corn. Marin et al. (1999a) also observed similar results to ours in that the *F. proliferatum* isolates, at 15°C, produced more fumonisins at a_w at 0.95 and 0.92 than at a_w 0.98 after two and four weeks. This may indicate the possibility of a_w stress stimulating fumonisin production by *F. proliferatum* at temperatures sub-optimal for growth during the initial growth phases. This could also be a species-specific characteristic as it was not observed for *F. verticillioides*.

6.4.2 Effect of temperature on the relationship between growth and fumonisin production

The effect of temperature on fumonisin production by *F. proliferatum* and *F. verticillioides* as a function of growth or time at different a_w values is shown in Figs. 6.1 and 6.2 respectively. Although the effect of temperature on the rate of mycelial development is very clear, its effect on fumonisin production is not clear. For both *F. verticillioides* and *F. proliferatum* the effect of temperature on the rate of fumonisin production, the amount produced for the same amount of growth, and the final amount of fumonisins produced varied largely with a_w.

Fig. 6.1a shows that for *F. proliferatum* at a_w 0.972 the most fumonisins for the same amount of growth were produced at 22ºC, and decreased in the order 15, 25 and 30ºC, respectively. At this a_w 192.6, 160.7, 153.9 and 144.1 µg/g were produced after approximately 80 mm change in colony diameter at 22, 25, 15 and 30°C. At a_w 0.948 the most fumonisins were produced at 15ºC, the amount decreasing in the order 25, 22 and 30ºC for the same amount of growth. At a_w 0.928 much fewer fumonisins were produced in comparison to higher a_w values, 32.4, 10.9, 6.7 and 0.8 µg/g were produced after approximately 80 mm change in colony diameter at 25, 22, 15 and 30ºC respectively. From Fig. 6.1b for *F. proliferatum* at a_w 0.972, the fumonisin production rates are fastest at 22, 25 and 30ºC and slowest at 15ºC. The time for significant fumonisin production to

occur was less, approximately 7-10 days, at the higher temperatures, and longer at 15°C (approximately 14 days). At a_w 0.948, the fumonisin production rate as seen in Fig. 6.1b is greatest at 25ºC and does not differ significantly at 22, 30 and 15ºC. However it generally took a longer time for significant fumonisin production to occur at 30 and 15°C than it took at 22 and 25°C.

An important observation of the results is that although 30ºC was the optimum temperature for growth of *F. proliferatum*, it was the least optimum for fumonisin production at any a_w. The least amount of fumonisins at any colony diameter and after approximately 80 mm change in colony diameter or six weeks of incubation at any a_w were also produced at 30ºC. The effect of temperature on fumonisin production appears to be marginal at higher a_w as fumonisin production by *F. proliferatum* at a_w 0.972 and 30ºC was high. The fumonisin production rate was least at 15°C and also it took a longer time for significant fumonisin production to occur at this temperature. The few reports available for *F. proliferatum* also present findings consistent with ours. Marin et al. (1999c) reported that the optimum temperature for fumonisin B_1 production by *F. proliferatum* was 15ºC followed by 20, 25, 10 and 30ºC at a_w 0.97, which is consistent with the results of this experiment in that the least optimum temperature for fumonisin production by *F. proliferatum* was 30ºC. Marin et al. (1999a) also reported that for *F. proliferatum* (131N) an increase in fumonisin produced after four weeks was noted as temperature was increased from 15 to 30ºC at a_w 0.98. They however noted, in accordance with our results, that more fumonisins were produced after four weeks at 15 and 25°C than at 30°C at lower a_w values of 0.95 and 0.92.

For *F. verticillioides*, Figs. 6.2c and 6.2d, show that temperature, as observed for *F. proliferatum*, had a marked effect on the initial fumonisin production rate as the slowest rate irrespective of a_w was at 15ºC, at which the slowest growth rates were also observed. From Figs. 6.2a and 6.2b it can be seen that the least amount of fumonisins after approximately 80 mm change in colony diameter was produced at 22, 25, 30 and 15ºC at a_w values 0.969, 0.949, 0.937 and 0.922 respectively. The optimum temperature for production by *F. verticillioides* varied with a_w, but was 30ºC at a_w 0.969, 15ºC at a_w

0.949, and 22ºC at a_w values 0.937 and 0.922. Results from other authors are also ambiguous but in most cases consistent with our findings. Le Bars et al. (1994) reported that the optimum fumonisin B_1 production rate by *F. verticillioides* was at 20ºC, and it decreased sharply in the order 25, 15, 30 and 10ºC. Marin et al. (1999a) observed an increase in fumonisin produced after four weeks as temperature was increased from 15 to 30ºC at a_w 0.98 for *F. verticillioides* (25N). They however noted that more fumonisins were produced after four weeks at 15 and 25°C than at 30°C at lower a_w values of 0.95 and 0.92. Marin et al. (1999c) reported that the optimum temperature for fumonisin B_1 production by *F. verticillioides* at a_w 0.97 varied between 20-25-30ºC. Alberts at al. (1990) reported that the maximal production of fumonisin B_1 by *F. verticillioides* on corn cultures was at 20ºC, with very low production at 30°C, they however did not have accurate details of the water content/a_w.

Le Bars et al. (1994) suggested that *F. verticillioides* degraded the fumonisin it produced after a period of time as the toxin was stable in corn. This degradation was reported to start after 13 weeks of incubation at 20-25ºC by Alberts et al. (1990) for *F. verticillioides*, whereas Marin et al (1999a) reported this to occur after approximately five weeks. For this study only a few experiments were actually incubated for the maximum period of six weeks, the incubation time for most of the conditions optimal for growth was less than five weeks, and in some cases a matter of days. The only condition at which fumonisin degradation was noted was for *F. proliferatum* at a_w 0.928 and 30°C, at which a maximum fumonisin concentration of 3.2 µg/g was attained after 14 days. There after the concentration progressively decreased to 0.8 µg/g after 38 days.

6.5 Conclusions

The need for growth stress to stimulate mycotoxin formation has long been postulated. However from these results and reports found in literature, it appears that different stresses have different implications with a_w being positively correlated to the fumonisin production/production rate and temperature having a less direct relationship. Clearly the optimum temperature for growth does not coincide with that for fumonisin production.

The models developed in chapter 3 and the relationships established in chapter 6 between growth and fumonisin production, enable for the prediction of growth at a given a combination of environmental factors (a_w and temperature) and time (duration of growth at particular combinations of these environmental factors), and importantly the consequences of this growth in terms of fumonisin production and therefore safety of the corn.

The following chapters (7, 8 and 9) focused on the evaluation of non-chemical and chemical preservation techniques for the post-harvest preservation of corn. In these chapters the models evaluated in chapters 3 and 4 were evaluated for their ability (where possible) to describe the combined effects of a_w and selected non-chemical and chemical preservation factors.

CHAPTER 7

The influence of initial headspace carbon dioxide and oxygen concentration and their interaction with water activity on the radial growth and fumonisin B_1 production of *Fusarium verticillioides* and *F. proliferatum* on corn[7]

[7] Redrafted from: Samapundo, S., De Meulenaer, B., Atukwase, A., Debevere, J. M. and Devlieghere, F. Part I: The effect of initial headspace carbon dioxide concentration & Part II: The effect of initial headspace oxygen concentration. *International Journal of Food Microbiology* (accepted).

Chapter 7. The influence of initial headspace carbon dioxide and oxygen concentration and their interaction with water activity on the radial growth and fumonisin B_1 production of *Fusarium verticillioides* and *F. proliferatum* on corn

7.1 Abstract

The effect of modified atmospheres on growth and fumonisin B_1 production of Fusarium verticillioides and F. proliferatum on corn was studied. The aspects evaluated included the effects of initial headspace (IH) carbon dioxide and oxygen concentration and their interaction with water activity (a_w) on growth and fumonisin B_1 production. The impact of vacuum packaging and in-cooperation of O_2 scrubbing sachets was also studied. At all a_w values studied, increase in the IH CO_2 concentration generally resulted in a decrease in the colony growth rate (g, mm d^{-1}) and maximum colony diameter (D_{max}, mm), and an increase the lag phase duration (λ, d). Although both a_w and CO_2 had significant synergistic effects on g, a_w had the largest effect. As little as 10% IH CO_2 completely inhibited fumonisin B_1 production by F. verticillioides. F. proliferatum was more resistant and required 40, 30 and 10% CO_2 at a_w 0.984, 0.951 and 0.930, respectively, to completely inhibit fumonisin B_1 production. At all a_w values studied, reduction of IH O_2 concentration from 20 to 2% had no significant effect on g and λ. However, g and λ were positively and negatively correlated to a_w. Although the D_{max} decreased with reduction of the IH O_2 level, the greatest mycelial density occurred at 10% IH O_2 for both isolates. This observation was accompanied by a trend of a decrease in the value of the IH O_2 level at which the most fumonisin B_1 was produced from 15 to 5% when the a_w was decreased from 0.976 to 0.930 for F. verticillioides. For F. proliferatum the optimum conditions for fumonisin B_1 production shifted from 20% at a_w 0.976 to 10% at both 0.951 and 0.930. Vacuum packaging and in-cooperation of O_2 absorbing sachets completely inhibited the growth of both isolates. These results demonstrate that modified atmospheres could potentially be employed in the prevention of fungal growth and mycotoxin production on corn during the post-harvest period.

Key words: *Fusarium verticillioides*, *F. proliferatum*, modified atmospheres, water activity, fumonisin B_1

7.2 Introduction

To date several approaches have been investigated to inhibit fungal growth and mycotoxin production on corn. Novel pre-harvest strategies evaluated to control fungal growth in the field include the use of endophytic bacterium such as *Bacillus mojavensis* and *Bacillus subtilis* as a biological control agents (Bacon and Hinton, 2000; Bacon et al., 2001), the introduction of non-mycotoxigenic strains (Plattner et al., 2000), and the use of cultivars less susceptible to infection (CAST, 2003). Post-harvest strategies evaluated to date have mainly been based on the application of chemical antifungal agents such as sorbates, propionates, benzoates (Punja and Grogan, 1982), butylated hydroxyanisole (Ahmad and Braunen, 1981), and bicarbonate salts (Montville and Shih, 1991).

In the last decade controlled atmospheres (CA) and modified atmosphere packaging (MAP) have successfully been applied as preservation techniques by virtue of their ability to maintain the natural quality of food products in addition to extending the shelf-life and satisfying the ever increasing consumer demand for foods free of chemical residues (Jayas and Jeyamkondan, 2002). As moulds are facultative aerobes and are highly sensitive to CO_2 (Smith et al., 1990; Farber, 1991), potential exists for modified atmospheres (MA) to replace the conventional use of chemical agents to protect bulk stored grain systems from potential fungal growth and mycotoxin production. Despite this potential, to date most studies on stored grains have rather been focused on the ability of MA to control insect pests (Emecki et al., 2002). A useful review of these studies is discussed by Jayas and Jeyamkondan (2002). The success of modified or controlled atmospheres in eradicating insect pests indirectly reduces the possibility of fungal contamination as most insect pests are fungal vectors. The ability of MA to inhibit fungal growth and mycotoxin production on food products normally stored in bulk has been reported by Diener et al. (1972), Wilson et al. (1975, 1977), Magan and Lacey (1984, 1988) and Ellis et al. (1993).

Most of the studies of the effect of MA on fungal growth and mycotoxin production are on *Aspergillus flavus* and aflatoxins (Paster and Bullerman, 1988), resulting in paucity of data on other moulds including fumonisin producing Fusaria. The only reports available on fumonisin producing *Fusarium* species include Wilson et al. (1975) and Gibb and Walsh (1980) who investigated the ability of *F. verticillioides* to survive on corn in high CO_2 atmospheres, whereas Musser and Plattner (1997) investigated fumonisin production under anaerobic conditions in sealed bags. Most of these studies have also been performed at one a_w value/moisture content value and therefore few reports exist on the possible interaction of the effects of modified atmospheres and a_w. The major objective of this chapter was to address this paucity by investigating the effects of initial headspace O_2 or CO_2 concentrations and their interaction with a_w on growth and fumonisin B_1 production of the two major fumonisin producing *Fusarium* spp. on corn. In addition, the effect of vacuum packaging and in-cooperation of O_2 scrubbing sachets in sealed packages on the same was also investigated. This study was the first part of the last phase of this work which now focused on the evaluation of non-chemical and chemical techniques to inhibit growth and mycotoxin production on corn and the application of the polynomial models developed in chapters 3 and 4 to describe the trends observed (where possible).

7.3 Materials and Methods

7.3.1 Fungal isolates

Fusarium verticillioides Sheldon (25) and *F. proliferatum* (Matsushima) Nirenberg (73N) were obtained courtesy of the Food Technology Department Culture Collection of the University of Lleida, Spain. The isolates were maintained at 7°C on potato dextrose agar (PDA) (Oxoid, Basington, UK).

7.3.2 Experimental design

7.3.2.1 Effect of initial headspace CO_2 concentration on the growth and fumonisin B_1 production of F. verticillioides and F. proliferatum on corn

A full factorial combination of six levels of IH CO_2 (0, 10, 20, 30, 40 and 60%) and three a_w values (0.93, 0.95 and 0.98) was used to investigate the effect of headspace CO_2 concentration on growth and fumonisin B_1 production of both isolates at 25°C. All conditions had an IH O_2 concentration of 20 ± 1% O_2 with N_2 making up the balance of the gas.

7.3.2.2 Effect of initial headspace O_2 concentration on the growth and fumonisin B_1 production of F. verticillioides and F. proliferatum on corn

A full factorial combination of six levels of initial headspace (IH) O_2 (0, 2, 5, 10, 15 and 20%) and three a_w values (0.93, 0.95 and 0.98) was used to investigate the effect of headspace O_2 concentration on growth and fumonisin B_1 production of both isolates at 25°C. The balance of gas in the bags was nitrogen (N_2).

7.3.3 Preparation of growth substrate and inoculum

Yellow dent corn was used as the growth substrate and was prepared exactly as described in 3.3.1.3. The fungal inoculum was prepared as stated in section 3.3.1.2.

7.3.4 Inoculation of the corn, packaging and incubation

Approximately 24 g of rehydrated corn was aseptically weighed into each petri plate. Using a sterile cork borer, a 5mm diameter disk was cut from the margin of the seven day old colony on PDA and transferred to the centre of the petri plates containing sterile hydrated corn. Four inoculated plates were placed in high O_2 gas barrier bags (NX90, Euralpack, Wommelgem, Belgium) with an O_2 permeability of 5.2ml/m²/24hr/atm. at 85% R.H. and 23°C. To maintain a constant relative humidity in the package a Petri plate containing approximately 20ml of glycerol/water solution of the same a_w as the

rehydrated grain was also placed in each bag. Two bags were prepared per condition. The desired gas mixtures were introduced into each bag and sealed by means of a Multivac gas packaging machine (Multivac A300/42, Haggenmuller KG, Wolfertschewenden, Germany). All gases used in this study were of the Freshline brand supplied by Air Products (Vilvoorde, Belgium). A gas/product ratio of 8/1 was maintained for all conditions. To achieve an IH O_2 concentration of 0%, AnaeroGen™ (Oxoid Ltd., Basingstoke, UK) O_2 scrubbing sachets, were placed in the bags before sealing. The AnaeroGen sachets were able to reduce the O_2 concentration to 0% within a period of 2 hours. For both isolates two bags with inoculated corn were also vacuum packaged using the Multivac gas packaging machine at all a_w values evaluated. Controls for the experiments were created by hydrating and inoculating the corn in exactly the same manner as described above, after which 10 plates per a_w were incubated under air in sealable plastic containers which contained glycerol/water solutions of the same a_w as the grain.

7.3.6 Growth evaluation and head space gas measurement

Growth was evaluated by periodically measuring two perpendicular diameters from each colony up to the time the colony diameter remained unchanged for at least two consecutive days. As expected this coincided with the time at which O_2 was completely consumed and growth could not possibly occur. The headspace gas composition was analyzed by a SERVOMEX® 1450 Food Package Analyser (Zoetermeer, The Netherlands) at the same time the colony diameters were measured.

7.3.7 Fumonisin B_1 analysis

Two plates were randomly picked from each incubated bag and combined to give one sample after all the O_2 was consumed and growth had stopped. These were stored at -18°C until analysis. This gave rise to two replicates per condition as two bags were prepared per condition. For comparative purposes, two plates from the controls grown in air were collected at the same time as those under MA. The extraction was carried out as follows. The contents of the sampled plates were finely ground using a blender (Braun

Multiquick MR 5000M, Kronberg, Germany). 15g of the ground sample was mixed with 40ml of methanol/water (3/1, v/v) in a plastic flask and blended using an Ultraturrax T25 (Stauffen, Germany) at 9700-9800 rpm for 2 minutes. Pro-analysis methanol was purchased from Chem Lab (Zedelgem, Belgium) and ultra-pure water was produced by a Waters Milli-Q system (Waters, Milford, MA, USA). After blending, the Ultraturrax was rinsed with three 1ml aliquots of methanol/water (3/1) to remove any remaining corn grits. The mixture was then filtered through a 595 ½ filter paper (Schleicher & Schuell Microscience, Dassel, Germany). The plastic flasks were then rinsed with two 3.5ml aliquots of methanol/water (3/1). The pH values of the filtrates were always between 5.8 and 6.5, the normal pH range for corn based samples.

Sample clean-up was then performed by passing a 10ml aliquot of the filtrate through a 10ml capacity Bond-Elut SAX cartridge (Varian, Middelburg, Netherlands) that had been previously conditioned with 5ml methanol followed by 5ml methanol/water (3/1). After passage of the filtrate the cartridges were washed with 8ml of methanol/water (3/1) followed by 3ml of methanol. Fumonisin B_1 was then eluted from the cartridges by 10ml of 1% glacial acetic acid (Chem Lab, Zedelgem, Belgium) in methanol. Throughout the sample clean-up process, the flow rate was maintained at $\leq$ 1ml/min. The eluate was quantitatively transferred to a pear bottomed flask (50ml) and evaporated in a Rotavapor at 30°C until the volume was reduced to $\leq$ 1ml. The residue was then transferred to a test tube and dried under a gentle stream of N_2. The dried samples were kept in closed test tubes at 7°C until HPLC analysis.

Fumonisin B_1 standard was supplied by Sigma (Steinheim, Germany). 5mg of the standard was dissolved in 20ml acetonitrile/water (1/1, v/v) to make the standard stock solution, which was then stored at 7°C until use. Acetonitrile was obtained from Chem Lab (Zedelgem, Belgium). Working standard solutions were prepared by dilution of the standard stock solution with the acetonitrile/water (1/1) solution. Fresh standards were prepared on each day of analysis. The mobile phase was prepared by mixing HPLC grade methanol (Fischer Scientific, Leicestershire, UK) with 0.1*M* sodium dihydrogen phosphate (Janssen Chimica, Geel, Belgium) in the ratio 3:1, respectively. The pH of the

solution was adjusted to 3.35 with *o*-phosphoric acid (UCB, Leuven, Belgium), after which it was filtered through a 0.45 µm filter paper (Pall Corp., Michigan, USA). Fresh mobile phase was always prepared a day before or on the day of analysis. The derivatising reagent was prepared by adding 5ml of 0.1*M* sodium tetraborate and 50µl of 2- mercaptoethanol (Acros Organics, Geel Belgium) to 40mg of *o*–pthaldialdehyde (OPA) (Steinheim, Germany) dissolved in 1ml of HPLC grade methanol. Fresh derivatising reagent was prepared on the day of analysis.

The HPLC system used consisted of a Gilson 307 high pressure pump, Gilson 122 fluorescence detector (Gilson, Wisconsin, USA), 20µl injection loop (Alltech, Deerfield, USA) and a Rheodyne injector (Rheodyne, California, USA). Separations were carried out on a stainless steel Lichrosorb 60-10 RP18 reverse phase column (250 x 4.6mm x 1/4") (Varian, Middelburg, Netherlands) maintained at 22°C. The detector was set at 335nm excitation and 440nm emission. The signals were processed by Varian Star Chromatography Workstation Version 4.5 software (Varian Associates Inc., California, USA). The dried samples were dissolved in 200µl of HPLC grade methanol. 25µl of this solution was then derivatised by adding 200µl of OPA reagent before injection. The standards were treated in exactly the same manner, with 25µl of the freshly prepared standard solutions being derivatised with 200µl of OPA reagent before injection. On the basis of spiked samples within the range 0-1000 µg/kg, the method had an average recovery of 98.3% and a limit of detection of 26.7µg/kg.

During the implemention of the method described above, the effect of mobile phase pH, column temperature, extraction method and derivatisation time on the fluorescence response and recovery of fumonisin B_1 in corn was investigated. The results of these evaluations are described in more detail in Samapundo et al. (2006). In brief, column temperature and mobile phase pH were negatively and positively correlated with the fluorescence response, respectively. Use of an Ultraturrax blender for extraction resulted in higher fumonisin B_1 recoveries compared to a rotary shaker. In contrast to other reports, maximum fluorescence response occurred after a derivatisation time of 8 minutes. The results obtained in this investigation reflected on the absolute requirement

for standardisation of the aforementioned parameters for reliable and reproducible results that can be treated with confidence.

7.3.8 Mathematical and statistical analysis

7.3.8.1 Mycelial growth

The flexible function of Baranyi and Roberts (1994) was fitted to the experimental growth data by means of the non-linear regression function of SPSS Version 11.0 (SPSS Inc, Chicago, IL. USA) as described in section 3.3.2.1. This enabled the objective estimation of important growth parameters such as the maximum colony growth rate (g, mm d^{-1}), lag phase duration (λ, d), and maximum colony diameter (D_{max}, mm). Comparison of the 95% confidence intervals of the growth parameters at different conditions was used to assess whether differences in experimental conditions resulted in a significant effect on one or more growth parameters.

7.3.8.2 Gas evolution

A three parameter sigmoidal function (Eq. 7.1) was fitted to the gas evolution data using SigmaPlot™ (SYSTAT Software Inc., Richmond, CA, USA):

$$\%O_2 or \%CO_2 = a(1+\exp(-(t-t_a)/b)^{-1} \quad (7.1)$$

where a is an estimate of IH O_2 or CO_2 concentration (%), t_a is the time (d) at which % O_2 or CO_2 is $\frac{a}{2}$, t is time (d), and b is a fitting constant.

The first derivative of Eq. 7.1 was used to calculate the O_2 consumption rate (% O_2/d) for each condition examined. Plots of -%O_2/d as a function of time (d) were made to compare the effect of different IH O_2 concentrations on the rate of O_2 consumption of the isolates.

7.4 Results and Discussion

7.4.1 Effect of initial headspace CO_2 concentration and water activity on the growth of *F. verticillioides* and *F. proliferatum* on corn

Although both isolates grew at all IH CO_2 concentrations evaluated, it can be observed in Fig. 7.1 that the colony growth rates and maximum colony diameters of both isolates generally decreased, whereas the lag phases increased, with an increase in IH CO_2 concentration. Differences in mycelial densities were also observed as the higher the IH CO_2 level the more sparsely both isolates grew. The colony growth rates of both isolates also decreased with a_w at all IH CO_2 concentrations evaluated. Comparison of the 95% confidence intervals (results not shown) of these growth parameters confirmed these observations.

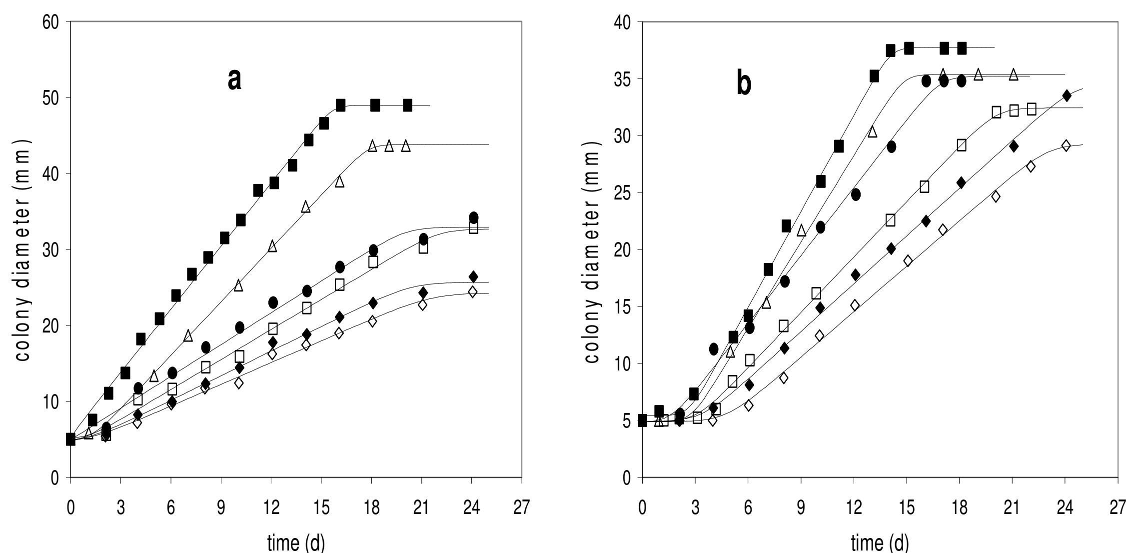


Figure 7.1. Growth curves for *F. verticillioides* at a_w 0.93 (left) and *F. proliferatum* at a_w 0.93 (right) and initial headspace CO_2 concentrations and of 0 (■), 10 (△), 20 (●), 30 (□), 40 (◆) and 60% (◇). The solid lines represent the fitted primary model of Baranyi and Roberts (1994) whereas the symbols represent the experimental growth data.

The combined effects of IH CO_2 concentration and a_w on the growth rates of *F. verticillioides* and *F. proliferatum* was also determined by assessing various forms of the second order polynomial models previously selected in chapters 3 and 4 as the basis for

modelling of the effect of preservation techniques. The models included versions with or without parameter (a_w) or response (growth rate) transformation. Of these, equation 7.2 proved to be the best model according to the MSE and residual plots. In the model a_w was transformed to b_w by $b_w = \sqrt{1-a_w}$.

$$\ln(g) = C_0 + C_1 b_w + C_2 b_w^2 + C_3\%IHCO_2 + C_4\%IHCO_2^2 + C_5 b_w\%IHCO_2 \quad (7.2)$$

The coefficients of the models developed the significance of their associated parameters and their determination coefficients are shown in Table 7.1. The models developed for both isolates are visually depicted as contour plots in Fig. 7.2. As seen in Table 7.1 all model parameters were significant ($P < 0.05$) for both isolates, with the main effects of a_w and IH CO_2 concentration having a positive synergistic effect on the colony growth rates of both isolates. Although both a_w and IH CO_2 concentration have significant individual effects on the colony growth rates of the isolates, the slopes of the contour lines indicate that the most important growth factor is a_w. However, it can also be deduced from these contour plots that the effect of IH CO_2 concentration on the colony growth rate becomes more pronounced as a_w decreases. Also observable in the contour plots is that the models developed make illogical predictions of an increase in colony growth rates with increase in IH CO_2 at low water activity values. This indicates *overfitting* which is typical of polynomial functions at growth limiting conditions.

Table 7.1. Coefficients of the quadratic polynomial equation ($\ln g = C_0+C_1b_w+C_2b_w^2+C_3\%IH\ CO_2 + C_4\%IH\ CO_2^2 + C_5b_wIH\ CO_2$) describing the combined effect of initial headspace CO_2 (IH CO_2) levels and a_w on the colony growth rates of *F. verticillioides* and *F. proliferatum*.

Parameter	*F. verticillioides*	*F. proliferatum*
C_0	37.81±3.22	23.45±1.27
b_w	-253.64±33.31	-149.78±12.77
b_w^2	460.37±82.56	269.92±31.50
%IH CO_2	-0.263±0.034	-0.155±0.018
%IH CO_2^2	0.001±0.000	0.001±0.000
b_w %IH CO_2	0.681±0.127	0.328±0.056
r^2	0.980	0.993

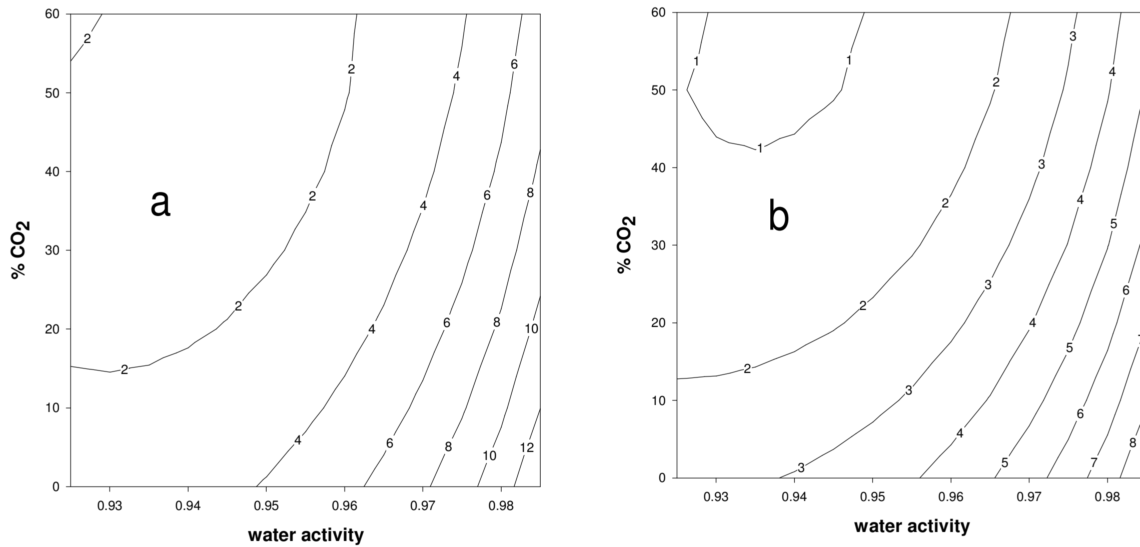


Fig. 7.2. Contour plots describing combined effected of initial headspace CO_2 concentration and a_w on the colony growth rates of a) *F. verticillioides* and b) *F. proliferatum*.

The typical trend in gas evolution is shown in Fig. 7.3, where it can be observed that there was an initial decrease in the concentration of CO_2 in the bags accompanied by an increase in the concentration of O_2. This was surmised to be due to the dissolution of CO_2 into the aqueous phase of the corn. The decrease in CO_2 concentration observed at a particular a_w generally increased with IH CO_2 levels. For example at a_w 0.984 a decrease of approximately 2% occurred in bags with 10% IH CO_2 concentration, whereas at 40 and 60% IH CO_2 the decrease increased to about 5%. Seemingly illogical larger decreases in CO_2 levels (greater CO_2 dissolution) were observed the lower the a_w as seen in Fig. 7.4. This was however due to the fact that at higher a_w values the fungi grew earlier and faster resulting in the CO_2 levels in the bags rising before the full extent of CO_2 dissolution could be observed. At lower a_w values, the longer lag phase and slower growth rates enabled more time for CO_2 dissolution to occur before the metabolically driven increase in CO_2 levels masked this. A decrease in CO_2 concentration of as much as 8% was noted at a_w 0.930 compared to 5% at 0.984 for atmospheres with 60% IH CO_2. This may also partly explain why the effect of IH CO_2 was observed to become more important the lower the a_w value.

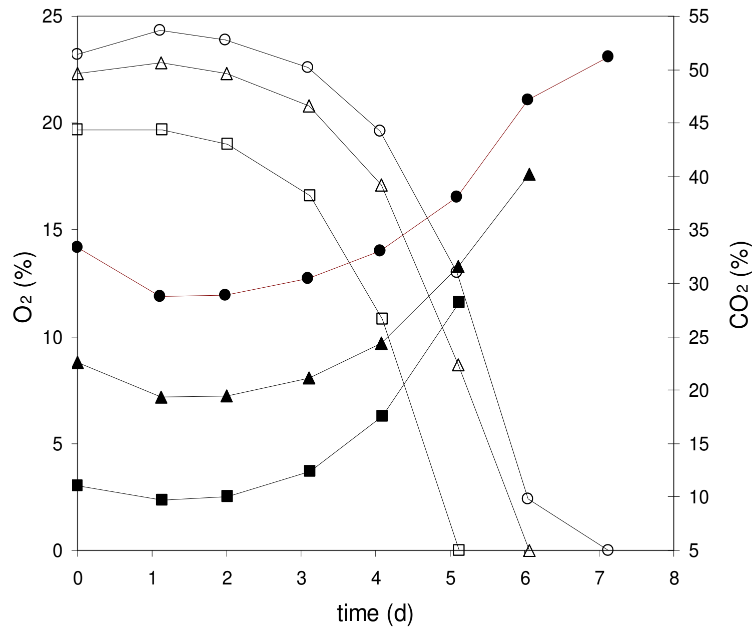


Fig. 7.3. Evolution of gas composition in the bags with different initial headspace CO_2 concentrations for *F. verticillioides* at a_w 0.984. Black symbols indicate evolution of CO_2 concentration in bags with 30 (●), 20 (▲), and 10% (■) initial headspace CO_2 concentrations, whereas the white symbols indicate changes in the O_2 concentration of the bags with 30 (○), 20 (△), and 10% (□) initial headspace CO_2 concentrations.

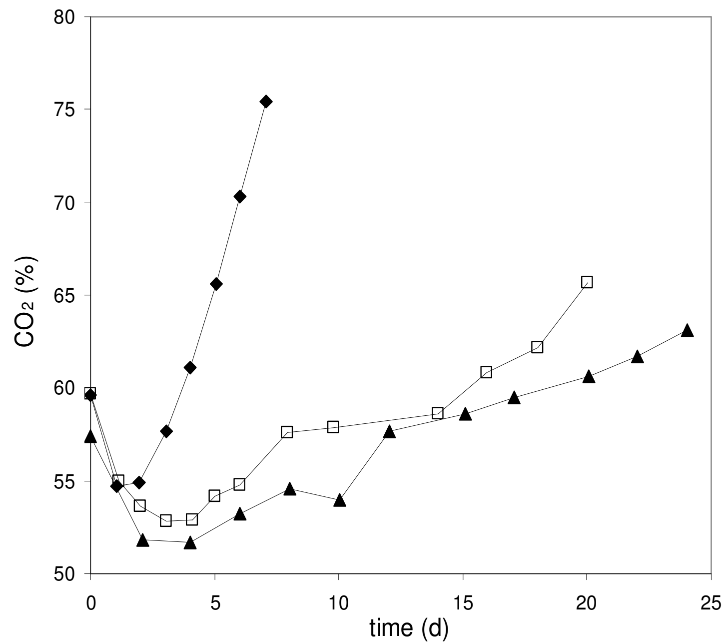


Fig. 7.4. Evolution of the CO_2 concentration in the bags with 60% initial headspace CO_2 for *F. verticillioides* at a_w 0.984 (◆), 0.951 (□) and 0.930 (▲).

The observed effect of CO_2 on growth of moulds and their persistence in CO_2 rich atmospheres has also been reported by a number of authors. Magan and Lacey (1984) reported that significant interaction occurred between CO_2 and a_w on the lag phase of *Aspergillus* and *Penicillium* species, which increased from four days to 16-21 days when CO_2 was increased from 0.03% to 20% in atmospheres with 21% O_2 at a_w 0.90. Paster et al. (1983, 1986) found that inhibition of the growth of *A. ochraceus* and *F. tricinctum* only occurred at CO_2 levels greater than 60% whereas Paster and Bullerman (1988) reported that the growth of *A. flavus*, *A. ochraceus*, *P. patulum* and *F. sporotrichoides* was only inhibited when CO_2 levels exceeded 50%.

Lillehoj et al. (1972) observed that in provided the availability of adequate O_2, only 5% of conidia of *P. aurantiogriseum* (*P. martensii*) were able to germinate under high CO_2 levels. They also determined that the higher the CO_2 level applied, the smaller the range of temperatures that supported germination became. Similarly Ellis et al. (1993) determined that *A. flavus* can grow in CO_2 enriched atmospheres as long as there was some headspace O_2. Smith et al. (1986) observed that although gas mixtures low in O_2 (<0.05-10%) and high in CO_2 were not completely inhibitory, they significantly increased the time for growth to occur from 1-1.5 days to 4-6 days. Resistance to high CO_2 concentration has also been reported for *P. roquefortii* which was found in sealed silos containing barley grain of a_w 0.80-0.87 in which the CO_2 concentration had reached 90% (Clarke and Hill, 1981). Gibb and Walsh (1980), Megan and Lacey (1984) and Ellis et al. (1994) reported the stimulation of fungal growth at CO_2 concentrations of 5 – 10% combined with low O_2 concentrations and high a_w values. This stimulation was probably not observed in this study due to the fact that the effect of CO_2 was only evaluated at a high IH O_2 level of 20% and not at lower values.

7.4.2 Effect of initial headspace CO_2 concentration on fumonisin B_1 production by *F. verticillioides* and *F. proliferatum* on corn

In addition to the observed effect of IH CO_2 concentrations on growth, fumonisin B_1 production by both isolates was also strongly influenced as shown in Table 7.2.

Table 7.2. Effect of initial headspace CO_2 levels and a_w on fumonisin B_1 production on corn by *F. verticillioides* and *F. proliferatum.*

% CO_2	µg/kg	Control (µg/kg)	µg/kg	Control (µg/kg)
F. verticillioides a_w 0.984			*F. proliferatum* a_w 0.984	
0%	381 ± 45	13231 ± 358	7694 ± 1829	76032 ± 1629
10%	ND*	13231 ± 358	4070 ± 243	103141 ± 11231
20%	ND	13231 ± 358	740 ± 25	126170 ± 6850
30%	ND	13231 ± 358	146 ± 24	126170 ± 6850
40%	ND	18301 ± 1037	ND	126170 ± 6850
60%	ND	20142 ± 916	ND	139643 ± 11241
F. verticillioides a_w 0.951			*F. proliferatum* a_w 0.951	
0%	254 ± 52	4716 ± 47	192 ± 13	35216 ± 1922
10%	ND	5926 ± 185	128 ± 18	74206 ± 3349
20%	ND	7230 ± 431	22 ± 6	79292 ± 496
30%	ND	7230 ± 431	ND	79292 ± 496
40%	ND	17632 ± 1629	ND	79292 ± 496
60%	ND	17632 ± 1629	ND	79292 ± 496
F. verticillioides a_w 0.930			*F. proliferatum* a_w 0.930	
0%	52 ± 14	3102 ± 116	100 ± 26	3973 ± 1167
10%	ND	3102 ± 116	ND	4966 ± 409
20%	ND	3632 ± 127	ND	5873 ± 689
30%	ND	3632 ± 127	ND	5873 ± 689
40%	ND	4572 ± 318	ND	6012. ± 1041
60%	ND	4572 ± 318	ND	6012. ± 1041

* ND – fumonisin B_1 not detected

Generally, fumonisin B_1 production markedly decreased with increase in IH CO_2 levels. For both isolates and at all a_w values evaluated complete inhibition of fumonisin B_1 production occurred when there was at least 40% IH CO_2. Fumonisin B_1 production by *F. verticillioides* was inhibited to greater extent by CO_2 than that by *F. proliferatum*, with as little as 10% IH CO_2 completely inhibiting production of fumonisin B_1 at all a_w levels studied. At a_w 0.984, 0.951 and 0.930 fumonisin B_1 production by *F. proliferatum* is completely inhibited at 40, 30, and 10% IH CO_2 concentration, respectively. Importantly this indicates that a synergistic inhibitive effect occurs between a_w and IH CO_2 on fumonisin B_1 production by *F. proliferatum*, as the lower the a_w value, the lower the IH CO_2 concentration required for the complete inhibition of fumonisin B_1 production becomes. As expected fumonisin B_1 production significantly decreased with a_w.

The observed effect of CO_2 on fumonisin B_1 production in this study is consistent with those reported for other mycotoxins. Paster et al. (1983) observed that although inhibition of growth of was *A. ochraceus* was only noted at more than 60% $CO_{2,}$ production of ochratoxin A was completely inhibited by at least 30% CO_2 regardless of the O_2 level. In a study of the effect of atmospheric gases on *Fusarium tricinctum*, Paster et al. (1986) reported that T-2 production decreased at least 5 fold when the mould was grown in an atmosphere of 50% CO_2 or more with 20% O_2 compared to production in air. Also in agreement with our results, studies with *A. flavus* have shown that aflatoxin production on peanuts, corn and synthetic media decreased with increase in headspace CO_2 levels (Landers et al., 1967; Wilson et al., 1977; Diener et al. 1972; Ellis et al., 1993; Ellis et al., 1994). Paster and Lisker (1985) reported that patulin production on synthetic medium by mycotoxigenic *Penicillum* spp. was inhibited at CO_2 levels above 10% in the presence of 20% O_2 whereas Lillehoj et al. (1972) also observed inhibition of penicillic acid production on corn when CO_2 levels were increased at 20% O_2.

Despite numerous reports on the ability of CO_2 to inhibit microbial growth, the mechanism of inhibition remains unclear (Dixon and Kell, 1989; Day, 2000). Four mechanisms have been summarized by Farber (1991) in which the action of CO_2 is explained by a) direct inhibition of enzymes or reduction of the rate of enzymatic

reactions b) cellular penetration leading to intracellular pH changes c) alteration of membrane function including effects on nutrient uptake and absorption and d) direct changes to the physico-chemical properties of proteins. The observation that although growth still occurs (albeit at a reduced rate) in atmospheres with elevated CO_2 levels at which mycotoxin production is strongly inhibited, may be partially explained by the fact that primary metabolism (growth) will be prioritized over secondary metabolic activities such as mycotoxin production when growth stressing conditions are presented.

7.4.3 Effect of initial headspace oxygen concentration on the growth of *F. verticillioides* and *F. proliferatum* on corn

Both isolates required the presence of headspace O_2 for growth. Complete inhibition of growth of both isolates only occurred in the presence of the O_2 scrubbing AnaeroGen bags and under vacuum packaging. It can be seen from Fig. 7.5 that at a particular a_w value the IH O_2 concentration had no effect on the colony growth rates and lag phase durations of both isolates.

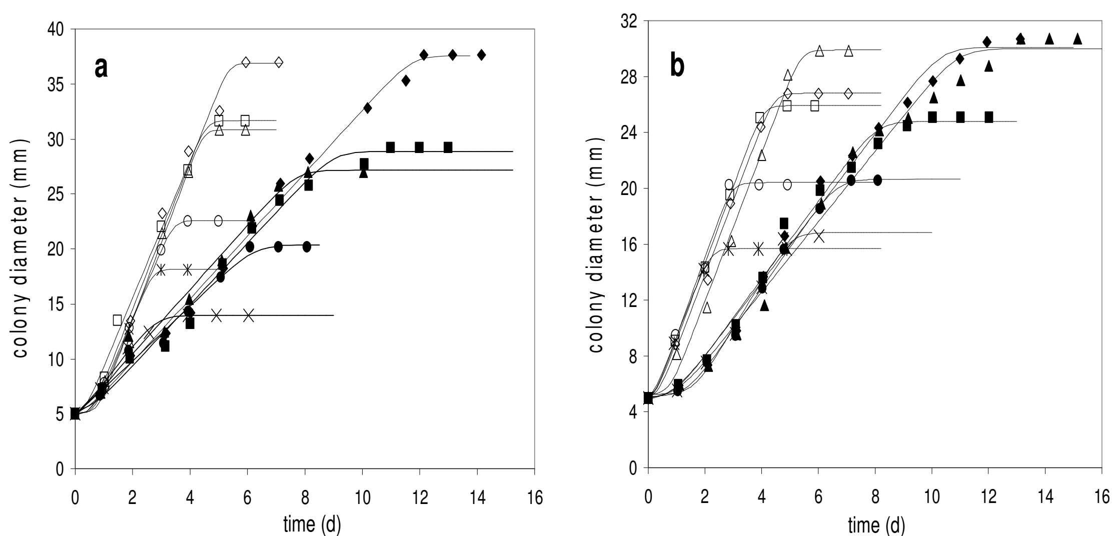


Fig. 7.5. Growth curves for a) *F. verticillioides* and b) *F. proliferatum* illustrating the effect of initial headspace O_2 concentration and a_w. The empty symbols indicate growth data at a_w 0.976 combined with IH O_2 concentrations of 20(◇), 15(△), 10(□), 5(○) and 2% (✳). The solid symbols indicate growth data at a_w 0.951 in (a) and in 0.930 (b) combined with IH O_2 concentrations of 20(◆), 15(▲), 10(■), 5(●) and 2% (✕). The solid lines represent the fitted primary model of Baranyi and Roberts (1994).

It can also be observed that the maximum colony diameter was dependent on the IH O_2 concentration, with higher IH O_2 concentrations resulting in higher D_{max}. Increase in a_w at a particular IH O_2 level was accompanied by an increase in the colony growth rate and decrease in the lag phase duration. With a few exceptions, a_w had no significant effect on D_{max} at a particular IH O_2 concentration. Comparison of the 95% confidence intervals of these growth parameters at a particular a_w value confirmed these deductions. Throughout the study a clear trend was observed with regards to the appearance of the growing colonies of both isolates. The mycelial densities were generally much greater at an IH O_2 concentration of 10% than they were at the other values studied. This implied that although 10% IH O_2 stimulated growth, this stimulation was only in the form of denser mycelia and not by any significant changes in g and λ.

In agreement with our observations Pitt and Hocking (1997) stated that fungi generally appear to be efficient O_2 scavengers, resulting in the fact that the total amount of O_2 available rather than the O_2 tension, determines their ability to grow when other conditions are favourable. Wilson et al. (1975) reported that the growth and consequent spoilage of corn by *F. verticillioides* and *A. flavus* was only delayed and not inhibited by as little as 0.5% O_2 combined with 13.5% CO_2. Bottomley (1950) also noted that mould growth was least affected by variation in O_2 concentration between 21 and 0.1%, but mostly by variation in a_w between 1.00 and 0.70. Magan and Lacey (1984) reported similar results for the growth of some field *Aspergillus* and *Penicillium* spp. on wheat extract agar, where it was observed that lowering of O_2 only slightly increased the lag phase at a_w 0.98 and 23°C. The general ability of moulds to grow unhindered at low O_2 levels has also been reported by Miller and Golding (1949) who observed that the growth of *Aspergillus* spp. was only affected by atmospheres with <5% O_2 at a_w 0.95. Landers et al. (1967) determined that the growth of *A. versicolor* was unaffected by 2% O_2 and that of *A. flavus* was only inhibited by <1% O_2. Smith et al. (1986) reported that a minimal headspace O_2 concentration of 0.4% was required for the growth of *A. niger* and *Penicillium* spores on PDA, where the rest of the atmosphere was a CO_2/N_2 (60/40) mixture. Ellis et al. (1994) and El Goorani (1981) also found that the germination, growth and sporulation of many mould could only be inhibited by O_2 levels of 1% and less.

In agreement to our findings that a reduced IH O_2 level of 10% enhanced growth mainly in form of denser colonies/thicker mycelial masses, Ellis et al. (1994) reported that maximum growth of *A. flavus* occurred at a_w 0.97 and 5-10% headspace O_2 (balance 60/40 CO_2/N_2) after 21 days of storage. Magan and Lacey (1984) and Gibbs and Walsh (1980) reported a stimulatory effect of 5-10% CO_2 at low O_2 concentrations, and attributed the beneficial effect of CO_2 to its fixation as a result of enhanced nutritional requirements at reduced O_2 tensions. Although similarities occur in the headspace O_2 levels at which growth stimulation is observed, in difference the CO_2 levels we employed were initially $< 1\%$ and only reached levels of 5-10% at about the mid-point or towards the end of the incubation period. In addition the stimulation was observed throughout the incubation period in our study, implying that the atmospheric condition at the start of the experiment induced the growth stimulation observed. The complete inhibition of growth by in-cooperation of O_2 scrubbing sachets has also been observed by Smith et al. (1986), who reported that an O_2 absorbing sachet (Ageless) maintained bakery products mould free for more than 60 days. This outcome was anticipated as moulds are facultative aerobic microorganisms, and therefore have an absolute require for O_2 (Pitt and Hocking, 1997).

From Fig. 7.6 the typical evolution in the gas composition of the bags can be seen for *F. verticlliodes* at a_w 0.976 and 0.951. Without exception, a continuous decrease in O_2 concentration occurred due to the metabolic activity of the growing colony. This decrease in O_2 concentration was accompanied by a corresponding increase in CO_2 to values similar to the IH O_2 levels. Plots of the rate of oxygen consumption (-%O_2/d) as a function of time are shown in Fig. 7.7 for both isolates at 20, 5 and 2% IH O_2 concentration. From this figure it can be seen that the IH O_2 concentration had a a_w dependent effect on the O_2 consumption rate of the growing colony. During the first 2-3 days of growth of both isolates, it can be seen that at the highest a_w evaluated (0.976), the rate of O_2 consumption for the same colony area (as the colony growth rates did not differ) was faster the lower the IH O_2 concentration. However, at a_w 0.951 differences in the IH O_2 concentration did not result in any significant difference between the rates of O_2 consumption for both isolates during the same period of growth. However, further

decrease in a_w to 0.930 resulted in a reversal of the trend observed at a_w 0.976, which was more pronounced for *F. verticillioides* than for *F. proliferatum*. In this case the O_2 consumption rate for the same colony area was faster the higher the IH O_2 concentration during the first few days of growth.

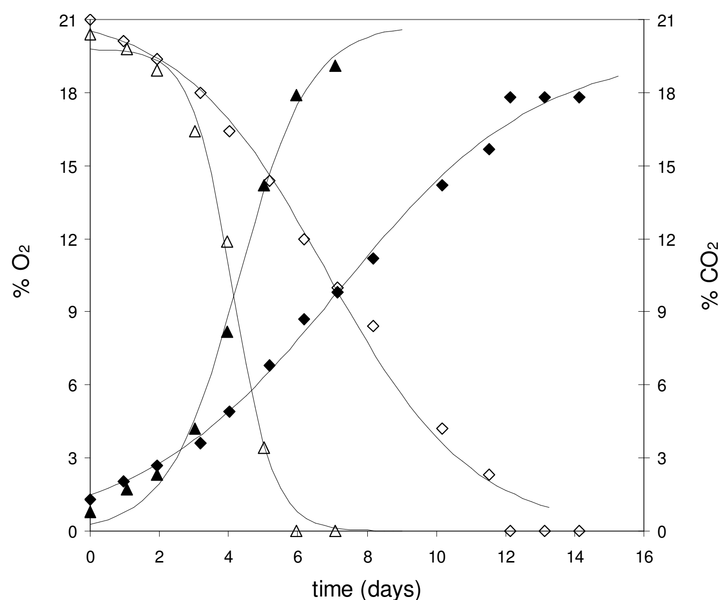


Fig. 7.6. Evolution of O_2 concentration in bags with 20% IH O_2 for *F. verticillioides* at a_w 0.976 (◇) and 0.951 (△) and CO_2 concentration at a_w 0.976 (◆) and 0.951 (▲). The solid lines and the symbols represent the fitted three parameter sigmoidal function (Eq. 7.1) and the experimental data, respectively.

Although it is well known that most fungi are efficient O_2 scavengers (Bottomley et al., 1950; Pitt and Hocking, 1997), their respiratory response to different IH O_2 concentrations is clearly observed to be related to the a_w of the growth substrate. The observed trend in O_2 consumption rate may possibly provide evidence of O_2 stress on the growth which could not be determined through evaluation of the estimated growth parameters. At high a_w values (optimal for growth), theoretically the only source of stress on growth was the reduction of the IH O_2 concentration. This stress appears to be countered by an elevation of the O_2 consumption rate. However, higher stress levels as a result of lowering of a_w combined with a reduction of IH O_2 concentration, combine to lower O_2 consumption rate. It however is also quite clear that despite the differences in the O_2 consumption rate observed at a particular a_w value as a result of the changes in the IH O_2 concentrations; these differences had no effect on the colony growth rate.

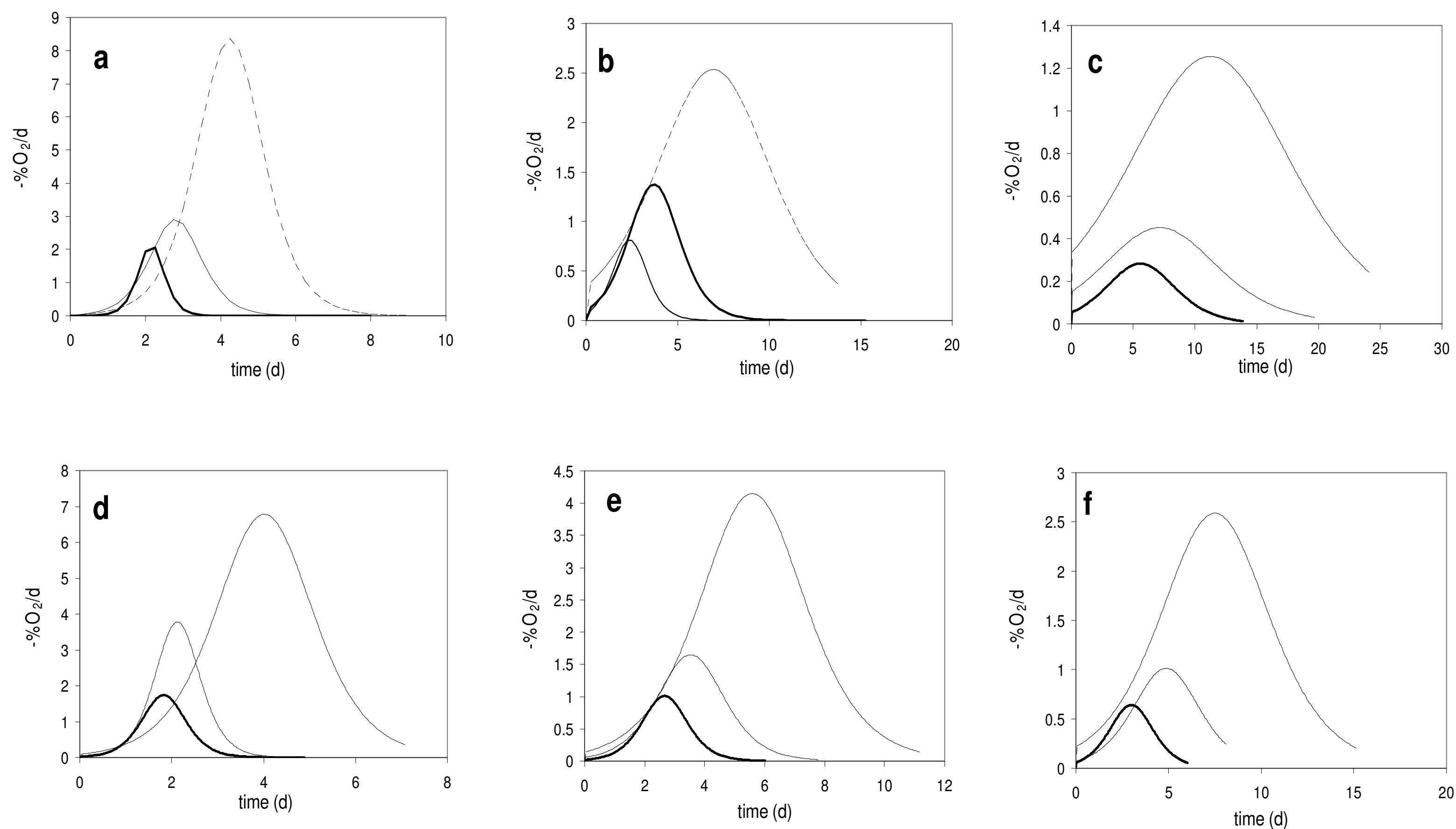


Fig. 7.7. Plots of O_2 consumption rates (-%O_2/d) at 20 (----), 5 (—) and 2 (▬) % initial headspace O_2 concentration as a function time for *F. verticillioides* and *F. proliferatum* at a_w 0.976 a) and d), a_w 0.951 b) and e), and a_w 0.930 c) and f), respectively.

7.4.4 Effect of initial headspace O_2 concentration on the production of fumonisin B_1 by *F. verticillioides* and *F. proliferatum* on corn

The effect of the IH O_2 level on fumonisin B_1 production by the *F. verticillioides* and *F. proliferatum* relative to the controls can be seen in Table 7.3. Although the IH O_2 level did not significantly affect the growth rates and lag phases of both isolates, it can clearly be observed from Table 7.3 that it greatly influenced the amount of fumonisin B_1 produced.

For *F. verticillioides* it can be seen that at the highest a_w value evaluated (0.976), the most fumonisin B_1 was produced at an IH O_2 level of 15%. Peak fumonisin B_1 production subsequently shifted to optimum levels of 10% and 5% IH O_2 at a_w values of 0.951 and 0.930, respectively. In addition it was observed that at all a_w values studied, with the exception of a_w 0.951, more fumonisin B_1 was produced by *F. verticillioides* at 2% IH O_2 than at 20%, despite the fact that maximum colony diameter was at least two times larger at 20 than at 2%. This implies that although very low IH O_2 concentrations have an overall inhibitory effect on fumonisin B_1 production when compared to the controls, in comparison to the other treatments they could have a stimulatory effect on fumonisin B_1 production. Musser and Plattner (1997) reported that fumonisin P_1 production appeared to be enhanced under anaerobic growth conditions, after initial growth under air. They however noted that fumonisin B_1 production did not increase under anaerobic conditions. *F. proliferatum* showed a similar trend of shift in the optimum IH O_2 concentration for fumonisin B_1 production with a_w. However, at the highest a_w evaluated of 0.976 fumonisin B_1 production by *F. proliferatum* was greatest at 20% IH O_2, in contrast to the results for *F. verticillioides*. The amount of fumonisin B_1 produced thereafter decreased with a reduction in the IH O_2 concentration.

However, in agreement with the results for *F. verticillioides*, more fumonisins were produced at a lower IH O_2 concentration of 10% at lower a_w values of 0.951 and 0.930. The influence of a_w on fumonisin B_1 production of both isolates at any IH O_2 level is more clear and logical as the lower a_w the lower the amount of fumonisin B_1 that was

produced. In agreement with the results observed for *F. proliferatum* at high a_w values, Diener et al. (1972), Landers et al. (1967) and Ellis et al. (1994) reported a decrease in aflatoxin production by *A. flavus* on peanuts with a reduction in IH O_2 concentrations. Diener et al. (1972) further determined that in atmospheres without CO_2 the decrease in aflatoxin production became more marked when the IH O_2 concentration was further decreased from 5 to 1%. Orth et al. (1976) also found that although the growth of sterigmatocystin and patulin producing moulds was unaffected by decrease in O_2 to 2%, toxin production was depressed dependent on the strain. They reported that atmospheres with 0.2% O_2 resulted in the complete inhibition of both growth and toxin production. Paster and Lisker (1985) also reported a decrease in patulin and penicillic acid when atmospheric O_2 was reduced to levels $\leq$ 5% in the absence of CO_2. Importantly it was noted in our study that the greatest quantity of fumonisin B_1 was produced in most cases at 10% IH O_2, the condition at which the greatest mycelial densities were also observed. In agreement with these findings, Ellis et al. (1994) reported that both growth and aflatoxin production was also maximal at a headspace O_2 level of 10%. The shift in optimal IH O_2 for fumonisin B_1 production observed for both *F. verticillioides* and *F. proliferatum* is reported for the first time. This shift may also occur for other moulds of importance to stored grain and consequently should be determined and considered as an important input parameter in the design of modified atmospheres for bulk grain systems.

Comparison of fumonisin B_1 production by *F. verticillioides* and *F. proliferatum* under conditions of reduced headspace O_2 levels with production under air (controls) clearly demonstrates the ability of modified atmospheres to reduce potential mycotoxin production. The results show that at the a_w and IH O_2 concentration values studied, a reduction in fumonisin B_1 of at least 70% compared to the controls occurred for *F. verticillioides*. The inhibitory effect at high IH O_2 levels such as 15 and 20% can be partly explained by the fact that CO_2 builds up to levels of at least 10% after approximately four, eight and 12 days at a_w values of 0.976, 0.951 and 0.930, respectively for both isolates. As observed earlier, CO_2 at these levels either completely or strongly inhibited fumonisin B_1 production depending on the a_w value.

Table 7.3. Effect of initial headspace O_2 levels and a_w on aflatoxin B_1 production on corn by *F. verticillioides* and *F. proliferatum*

% O_2	μg/kg	Control (μg/kg)	μg/kg	Control (μg/kg)
	F. verticillioides a_w 0.976		*F. proliferatum* a_w 0.976	
2%	650 ± 24	10559 ± 1691	19 ± 6	1668 ± 43
5%	272 ± 43	17561 ± 896	819 ± 26	1668 ± 43
10%	161 ± 42	25926 ± 185	461 ± 35	1668 ± 43
15%	1623 ± 78	25926 ± 185	2682 ± 44	13231 ± 1985
20%	319 ± 63	32723 ± 431	4008 ± 144	13231 ± 1985
	F. verticillioides a_w 0.951		*F. proliferatum* a_w 0.951	
2%	166 ± 12	1346 ± 100	392 ± 26	1004 ± 134
5%	181 ± 37	2981 ± 366	608 ± 49	1742 ± 25
10%	1410 ± 77	4702 ± 416	2223 ± 267	2511 ± 101
15%	167 ± 14	7162 ± 71	455 ± 55	4716 ± 47
20%	254 ± 32	17817 ± 1002	192 ± 13	4716 ± 47
	F. verticillioides a_w 0.930		*F. proliferatum* a_w 0.930	
2%	165 ± 15	1727 ± 145	98 ± 20	302 ± 51
5%	188 ± 4	3201 ± 189	62 ± 17	1701 ± 91
10%	ND*	3299 ± 289	1920 ± 95	2305 ± 221
15%	100 ± 34	3412 ± 401	151 ± 16	2579 ± 93
20%	106 ± 14	3632 ± 127	100 ± 16	3260 ± 143

* ND – fumonisin B_1 not detected

In chapter 6 where the relationship between growth/time and fumonisin production by the same isolates used in this study was investigated, fumonisin production in air only increased exponentially after approximately 4-5 and 10 days at a_w values of 0.97 and 0.95, respectively. It can be therefore be concluded that the inhibitory effect noted at high IH O_2 levels was a result of the CO_2 levels becoming inhibitory for fumonisin B_1 production before the time at which both isolates have been noted to produce significant quantities of fumonisin B_1 had passed. As the CO_2 levels did not reach the levels reported in this study to be inhibitory to fumonisin B_1 production at lower IH O_2 levels, the effect observed can be attributed to the fact that fumonisin B_1 production is a secondary metabolic activity that maybe sacrificed in favour of primary activities under growth stressing conditions. Stress on the growth as a result of reduction of the IH O_2 levels, observed from analysis of the gas evolution patterns, may be sufficient to produce the inhibitory effect noted.

7.5 Conclusions

The IH CO_2 concentration and a_w were found to have a significant interactive inhibitory effect on the growth of both *F. verticillioides* and *F. proliferatum.* With regards to fumonisin B_1 production, a level of 10% IH CO_2 completely inhibited fumonisin B_1 production by *F. verticillioides* regardless of the a_w. *F. proliferatum* was generally more tolerant to altered atmospheric conditions than *F. verticillioides* as shown by the requirement of at least 40 and 30 % IH CO_2 to completely inhibit fumonisin B_1 production at a_w values of 0.984 and 0.951, respectively. Being a secondary metabolite fumonisin B_1 production is inhibited under conditions that still permit growth. In accordance to the known facultative aerobic nature of most filamentous fungi, both growth and toxin production by both isolates was completely inhibited by vacuum packaging or by placing an O_2 scrubbing sachet in a sealed package. *F. verticillioides* and *F. proliferatum* are tolerant to low O_2 concentrations and exhibit a similar response with regards to their O_2 consumption rates under different a_w and IH O_2 conditions.

The results of the toxin production indicate that the IH O_2 concentration for optimum fumonisin B_1 production decreased from 15 to 5% for *F. verticillioides*, and from 20 to 10% for *F. proliferatum* as a_w was lowered from 0.976 to 0.930. In correspondence to this it was observed that 10% IH O_2 promoted the greatest mycelial density. Reductions in fumonisin B_1 production of at least 70 and 20% compared to the controls were obtained under the modified IH O_2 levels for *F. verticillioides* and *F. proliferatum*, respectively. In addition to prior reports by other workers, these results provide further reason to seriously consider where resources allow the use of MA as a non-chemical preservation technique for the inhibition of fungal growth and mycotoxins during storage of cereal grains. Further research should however be pursued to determine other important aspects such as fungal interaction and competition and their influence on mycotoxin production under modified atmospheres, and the potential for other spoilage processes i.e. alcohol or lactic acid fermentation to occur during long term storage of bulk grain systems under modified atmospheres.

After evaluation of a non-chemical technique, more suitable for application in resource endowed developing countries, the study moved in chapter 8 to the evaluation of chemical techniques based on cheap, easy to apply inorganic chemicals (bicarbonate salts) that can provide post-harvest solutions in resource limited countries.

CHAPTER 8

Interaction of water activity and bicarbonate salts in the inhibition of growth and mycotoxin production by *Fusarium* and *Aspergillus* species of importance to corn [8]

[8] Samapundo, S., Devlieghere, F., De Meulenaer, B., Lamboni, Y., Osei-Nimoh, D. and Debevere, J. M. (submitted).

Chapter 8. Interation of water activity and bicarbonate salts in the inhibition of growth and mycotoxin production by *Fusarium* and *Aspergillus* species of importance of corn

8.1 Abstract

The combined effect of water activity (a_w) and ammonium/sodium bicarbonate on growth and mycotoxin production on corn by Fusarium and Aspergillus species was investigated. The sensorial suitability of corn treated with the bicarbonate salts was also determined. Within the experimental limits, both salts and a_w exerted a synergistic effect on the colony growth rate (mm d^{-1}) and lag phase duration (d). Low levels of both salts enhanced the growth of F. verticillioides and F. proliferatum, with the fastest growth taking place at levels of 0.1-0.2 and 0.5% ammonium and sodium bicarbonate (w/w), respectively. No growth stimulation was observed for the growth of A. flavus and A. parasiticus. Growth of the Fusarium isolates was completely inhibited by 0.8% and 3.5% ammonium and sodium bicarbonate, respectively. At least 1% ammonium bicarbonate completely inhibited the growth of the Aspergillus isolates, whereas 4% sodium bicarbonate failed to completely inhibit their growth at all a_w values evaluated. Increase in concentration of either salt resulted in a decrease of fumonisin B_1 and aflatoxin B_1 production. At least 0.6% ammonium bicarbonate and 2% sodium bicarbonate completely inhibited fumonisin B_1 through 20 days of incubation, whereas 0.8% ammonium bicarbonate achieved the same for aflatoxin B_1 production. However, 4% sodium bicarbonate failed to completely inhibit aflatoxin B_1 production. According to the sensorial analysis, corn treated with up to 1% ammonium bicarbonate was still acceptable for consumption, whereas corn treated with at least 2% sodium bicarbonate was determined to be sensorially unsuitable. It can be concluded from these results that ammonium bicarbonate is more suitable for the protection of corn from fungal invasion and subsequent mycotoxin production, as it was the only one capable of completely inhibiting both growth and mycotoxin production of the Fusarium and Aspergillus isolates of most importance to corn at levels that were still sensorially acceptable. In addition, as it is easy to apply and cheap it could therefore be an appropriate post-harvest solution for use in resource limited developing countries.

Key words: *Fusarium*, *Aspergillus*, corn, ammonium bicarbonate, sodium bicarbonate, aflatoxin B_1, fumonisin B_1.

8.2 Introduction

Evidence of the widespread occurrence (and co-occurrence) of fumonisins and aflatoxins in corn amongst other mycotoxins (Almeida et al., 2000; Medina-Martinez et al., 2000; Li et al., 2001; Vargas et al., 2001) has stimulated the investigation of several methods to either prevent their production or to detoxify foods that may contain them. These methods include nixtamalization, non-enzymatic browning, addition of phenolic compounds and essential oils, ammoniation, detoxification by endophytic bacteria and introduction of non-mycotoxigenic strains (Line and Brackett, 1995a; Murphy et al., 1996; Lu et al., 1997; Bacon and Hinton, 2000; Velluti et al., 2003). Bicarbonate salts are widely available, inexpensive, easy to handle and generally recognized as safe for use in foods (Montville and Goldstein, 1987). They are also commonly used food additives and currently can be used in several foodstuffs at 'quantum satis' levels in European and North American regulations (Lindsay, 1996). They therefore potentially present an attractive alternative to more expensive chemical fungicides, some of which have now been shown to have reduced effects due to the development of resistance by their target fungi (Placinta et al., 1999). Their potential to control fungal growth has been reported by El-Nabarawy et al. (1989), Montville and Goldstein (1987, 1989) and Montville and Shih (1991). They have also been found to have an inhibitory effect on the production of trichothecene mycotoxins (Roinestad et al., 1993, 1994), aflatoxins (El-Nabarawy et al., 1987; Montville and Goldstein, 1987, 1989) and ochratoxin A (Montville and Shih, 1991).

After a successful and favourable evaluation of MA as a non-chemical post-harvest preservation technique the study now focused on the evaluation of inorganic and organic natural chemicals for their potential to protect grain stores from fungal growth and mycotoxin poisoning. In this frame-work bicarbonate salts were selected as the inorganic chemicals to be evaluated for their efficacy on corn. The choice of bicarbonate salts was justified as mentioned above – they are cheap, simple and safe to use and have already been demonstrated to have some antifungal effects. However, despite their potential no reports currently exist on the effect of bicarbonate salts on both growth and fumonisin production to date. Moreover the studies to date have investigated bicarbonate impact at one water activity value or moisture content value, neglecting the possible interaction of the effects of a_w/moisture content and the bicarbonate salts on growth. Their evaluation was completed by determining for the first time the sensorial suitability of bicarbonate

treated corn for human consumption, as ultimately the actual use of such chemical agents will only be possible given that the treated corn remains sensorially suitable for consumption or can be further treated to render it acceptable.

8.3 Materials and Methods

8.3.1 Treatment of corn with bicarbonate salts

Sodium and ammonium bicarbonate obtained from Merck Chemicals (Darmstadt, Germany) were evaluated for their effect on growth, fumonisin B_1 and aflatoxin B_1 production in the study. A full factorial design of three a_w values (between 0.98 and 0.88) and at least six bicarbonate salt concentrations was employed. The concentration range investigated for ammonium bicarbonate was between 0 and 1g/100g of rehydrated corn, whereas that for sodium bicarbonate was between 0 and 4g/100g of rehydrated corn. The GAB sorption model developed for the corn at 25°C in chapter 2 was used to determine the amount of sterile distilled water that had to be aseptically added to the corn to reproducibly achieve the desired a_w value. The rehydrated corn was then equilibrated over two days at 4°C before addition of the bicarbonate salts. Uniform distribution of the salts was achieved by thorough mixing of the corn and salts for at least three minutes in sealed sterile stomacher bags. The treated corn was then incubated for a further day at 25°C to enable the corn to achieve the final incubation temperature. The exact a_w of the treated corn was then determined by the Novasina Thermoconstanter TH200 (Axair Ltd. Systems, Pfapfikkon, Switzerland).

8.3.2 Fungal isolates, preparation of inoculum, inoculation and assessment of growth

Fusarium verticillioides Sheldon (25), *F. proliferatum* (Matsushima) Nirenberg (73N), *Aspergillus flavus* (IITAB 139) and *A. parasiticus* (IITAB 138) were evaluated in the study. *F. verticillioides* and *F. proliferatum* inoculum was prepared and inoculated as as described in section 3.3.1.3 whereas *A. flavus* and *A. parasiticus* inoculum were prepared as described in section 4.3.4, with the exception that ten plates were prepared per condition instead of 20. Growth was then assessed during incubation at 25°C over a maximum period of 42 days as described in section 3.3.1.3.

8.3.3 Mathematical analysis of experimental data

Estimates of the colony growth rate (g, mm d^{-1}) and lag phase duration (λ, d) at each condition were determined by linear regression of the growth data collected during the linear phase of growth as described in section 5.3.3.3. The same modelling procedure as applied in chapter 7 (section 7.4.1). Of the model variations evaluated equation 8.1 provided the best fit to the experimental data on the basis of the MSE and residual plots.

$$\ln(g) = C_0 + C_1 a_w + C_2 a_w^2 + C_3[\text{Salt}] + C_4[\text{Salt}]^2 + C_5 a_w[\text{Salt}] \quad (8.1)$$

where [Salt] was the concentration of bicarbonate salt (% or g/100g of rehydrated corn) and C_0, C_1 …C_5 were estimated model coefficients. The ability of the models developed to describe the influence of a_w and bicarbonate salt on the colony growth rate was determined by their determination coefficients (r^2), MSE, residual plots and by visually assessing the distribution of the experimental growth data about the three dimensional surfaces of the models.

8.3.4 Quantification of effect of bicarbonate salts on fumonisin B_1 production

The effect of the bicarbonate salts on fumonisin B_1 production was determined at a_w 0.960 and at sodium bicarbonate concentrations of 0, 0.5, 1, and 2%, and ammonium bicarbonate concentrations of 0, 0.2, 0.4 and 0.6%. The inoculated plates were incubated in plastic buckets containing glycerol/water solutions of the same a_w, and samples were collected after 10 and 20 days of incubation. For each condition two plates were randomly selected on each day of sampling and stored at -18°C until extraction was performed. The contents of the sampled plates were separately ground using a blender. The method described in section 7.3.7 for extraction, sample clean-up and HPLC analysis was then used to quantify the fumonisin B_1 levels in these samples, with the exception that an Agilent 1100 series HPLC equipped with a fluorescence detector was used to detect fumonisin B_1..

8.3.5 Quantification of effect of bicarbonate salts on aflatoxin B_1 production

The same sampling protocol as described for fumonisin B_1 was used for determination of aflatoxin B_1. However, samples treated with 0, 0.2, 0.4, 0.6 and 0.8% ammonium bicarbonate and 0, 2 and 4% sodium bicarbonate were analysed. After grinding of the

sample, extraction of aflatoxin B_1 was done by mixing 20g of the ground sample with 40ml of methanol/water (3/2, v/v) in a flask. The mixture was then blended for one hour using a gyrating shaker (Stauffen, Germany). The mixture was then filtered through a Whatman No.1 filter paper. The flasks were rinsed with 10ml of methanol/water (3/2) which was also passed through the filter paper. The pH of the filtrate was determined and adjusted where necessary adjusted to 7 by 0.1 *M* NaOH. A 4ml aliquot of the filtrate was then diluted with 15ml of phosphate buffer saline buffer solution pH 7.4 (0.15 *M* NaCL) before sample clean-up. Sample clean-up was achieved by passing the diluted aliquots of the extracts through 3 ml capacity AflaStar^{TM} immunoaffinity columns (Romer Lab Diagnostica GmbH, Herzogenburg, Austria). The columns were then washed with 20ml of water, before slight under-pressure was used to remove any liquid that remained in the columns. Aflatoxin B_1 was then eluted from the cartridges by 2ml of methanol applied as 0.5ml aliquots allowed to rest on the gel bed for about 3-4 minutes. The remaining liquid was removed by applying a slight under-pressure.

Aflatoxin B_1 standard was generously donated by the National Institute of Public Health and the Environment (RIVM, Bilthoven, Netherlands). The standard stock solutions dissolved in methanol were then stored at -18°C until use. Working standard solutions were prepared by dilution of the standard stock solution with methanol/water (1/1). The mobile phase was prepared by mixing HPLC grade methanol (Fischer Scientific, Leicestershire, UK) with water in the ratio 1:1. The Agilent 1100 Series HPLC equipped with a fluorescence detector was also used to detect aflatoxin B_1. Separations were carried out on a stainless steel Discovery® C_{18} reverse phase column (250 x 4.6mm x 5µm) (Supelco, USA) maintained at 25°C. The detector was set at excitation and emission wavelengths of 360nm and 440nm, respectively. On the basis of spiked samples within the range 0-200 µg/kg, the method had an average recovery of 97% and a limit of detection of 1.9 µg/kg.

8.3.6 Sensorial suitability of bicarbonate treated corn

Uncontaminated corn for these tests was first rehydrated to a_w 0.960 by addition of sterile distilled water, after which it was left to equilibrate for two days. The rehydrated corn was then mixed with the bicarbonate salts and left for 1 week at 25°C, after which it was sieved through a 220µm sieve for 2 minutes to reduce the residual levels of bicarbonate

salts on the corn and cooked in a pressure cooker at 1atm (121°C) for 15 minutes. After cooking a triangle test was performed using the untreated corn as the reference by presenting three randomly coded samples at a time to the panelists, two of which were identical.

In the triangle tests 15 panelists of African origin whose staple diet consisted of corn, were asked to note (where perceivable) sensorial differences between cooked untreated corn (reference) and cooked corn that had been treated with 1, 2, 3, and 4% sodium bicarbonate or 0.2, 0.4, 0.6, 0.8 and 1% ammonium bicarbonate. In addition, the panelists were asked to note (when a difference was observed), if the samples were all still sensorially acceptable or not for consumption. In accordance with triangle tests, the treated corn was considered significantly different from the corn ($p < 0.05$) and thus unacceptable for consumption when at least 9 panelists rejected the product.

8.4 Results and Discussion

8.4.1. Effect of bicarbonate salts and their interaction with water activity on the growth of *A. flavus, A. parasiticus, F. verticillioides and F. proliferatum*

Plots of the colony growth rates (mm d^{-1}) and lag phase durations (d) as a function of ammonium bicarbonate or sodium bicarbonate concentration (%) at the a_w values investigated are shown in Fig. 8.1 for *A. flavus* and *A. parasiticus* and Fig. 8.2 for *F. verticillioides* and *F. proliferatum*. The coefficients of the models developed to describe the combined effect of bicarbonate salt concentration and a_w on the colony growth rates of the isolates investigated are shown in Table 8.1.

The three dimensional surfaces of the models developed for the isolates, depicting the colony growth rate as a function of a_w and bicarbonate salt concentration, and also including the experimental growth data are shown in Fig. 8.3 for *F. verticillioides* and *F. proliferatum*, and Fig. 8.4 for *A. flavus* and *A. parasiticus*. The model evaluated (Eq. 8.1) generally gave a good fit as assessed by the determination coefficients (r^2), which were ≥ 0.924, by the MSE (not shown) and by the even and close distribution of the experimental growth data about the three dimensional surfaces of the models developed.

Table 8.1. Coefficients of models developed - $\ln(g) = C_0 + C_1 a_w + C_2 a_w^2 + C_3[\text{Salt}] + C_4[\text{Salt}]^2 + C_5 a_w [\text{Salt}]$ - to describe the combined effect of and bicarbonate salt on the growth rates of *Fusarium* and *Aspergillus* species of importance to corn.

Isolate	Salt	C_0	C_1	C_2	C_3	C_4	C_5	r^2
F. verticillioides	ABC[a]	-12.61 ± 1.76	15.04 ± 1.84	ns[c]	ns	-4.20 ± 0.76	2.00 ± 0.59	0.924
F. verticillioides	SBC[b]	17.45 ± 2.54	19.97 ± 2.67	ns	8.00 ± 1.88	-0.58 ± 0.06	8.91 ± 1.98	0.986
F. proliferatum	ABC	-12.29 ± 1.69	14.37 ± 1.76	ns	3.32 ± 0.61	-8.25 ± 1.01	ns	0.956
F. proliferatum	SBC	-7.87 ± 0.52	ns	10.26 ± 0.58	0.22 ± 0.09	-0.30 ± 0.03	ns	0.983
A. flavus	ABC	-29.86 ± 7.72	59.34 ± 16.80	-29.86 ± 9.13	ns	-0.62 ± 0.12	-0.24 ± 0.10	0.991
A. flavus	SBC	-68.19 ± 22.94	147.38 ± 49.93	-76.82 ± 27.13	-0.25 ± 0.01	ns	ns	0.973
A. parasiticus	ABC	0.41 ± 0.23	ns	2.44 ± 0.27	ns	-1.16 ± 0.09	ns	0.954
A. parasiticus	SBC	-2.23 ± 0.67	4.87 ± 0.73	ns	-0.87 ± 0.27	-0.03 ± 0.01	0.76 ± 0.30	0.991

a: ABC – ammonium bicarbonate

b: SBC – sodium bicarbonate

c: ns – not significant ($p > 0.05$)

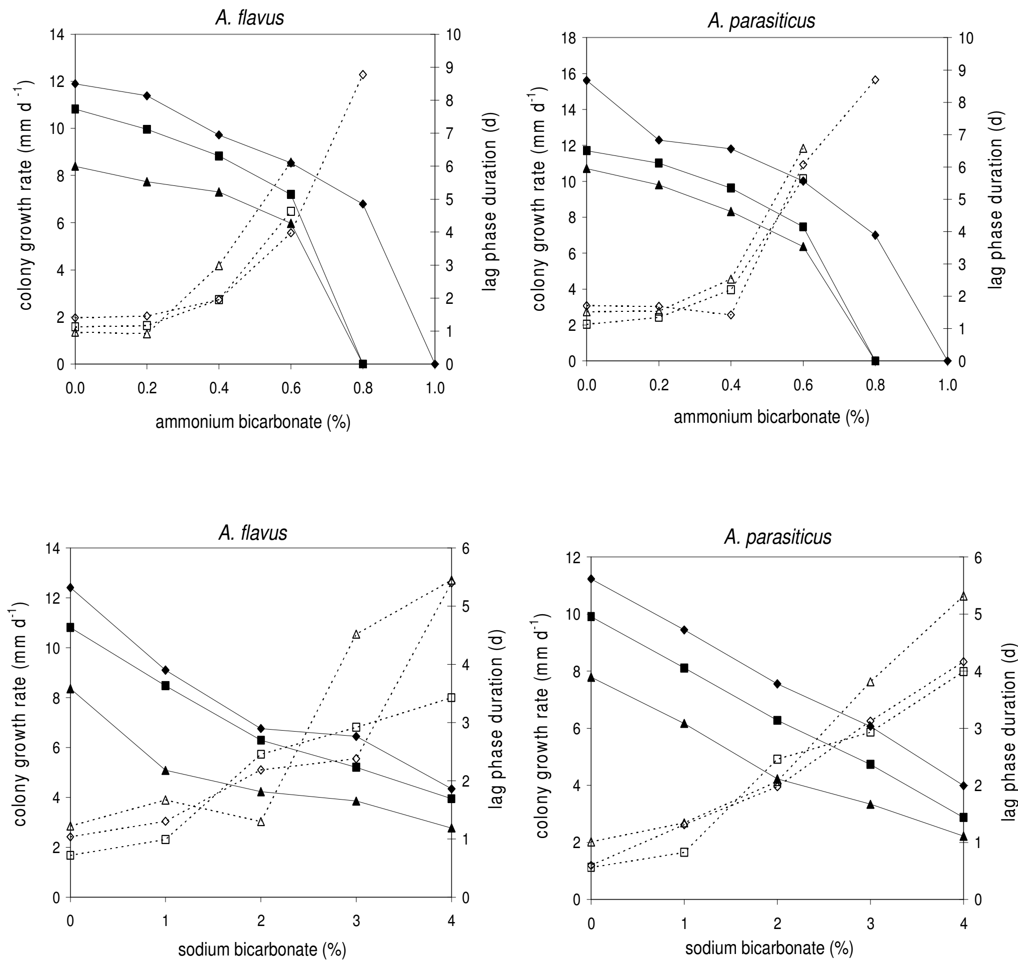


Fig. 8.1. Plots of the colony growth rates (mm d^{-1}, solid black symbols with solid lines) and lag phase durations (d, empty symbols with dotted lines) of *A. flavus* and *A. parasiticus* as a function of ammonium bicarbonate or sodium bicarbonate concentration at a_w values of 0.97 (◆, ◇), 0.92 (■, □) and 0.88 (▲, △).

It can be deduced from Figs. 8.3 and 8.4, and Table 8.1 that the bicarbonate salts and a_w had significant and interactive effects on the colony growth rates of the isolates of the isolates investigated. The trend however differed between the two isolatets. For the *Fusarium* isolates as seen in Figs. 8.2 and 8.3, it was observed that at all the a_w values investigated, an increase in concentration of either salt initially resulted in an increase in the colony growth rate up to a maximum, after which further increment in salt concentration was negatively correlated with the colony growth rate. The fastest growth for *F. verticillioides* and *F. proliferatum* was achieved at ammonium bicarbonate concentrations of 0.1 and 0.2%, respectively, irrespective of the a_w value. On corn treated

with sodium bicarbonate both *Fusarium* isolates grew fastest at a concentration of 0.5% regardless of the a_w value. The ability of some tobacco spoilage fungi to utilize potassium sorbate led Mutasa and Magan (1990) to conclude that some spoilage fungi can metabolize low concentrations of these preservatives. Taking this into consideration, the stimulatory effect observed can be surmised to be a result of the ability of these isolates to utilize the NH_4^+ (from ammonium bicarbonate) and HCO_3^- (from both salts) as growth nitrogen and carbon sources, respectively, at low bicarbonate salt concentration.

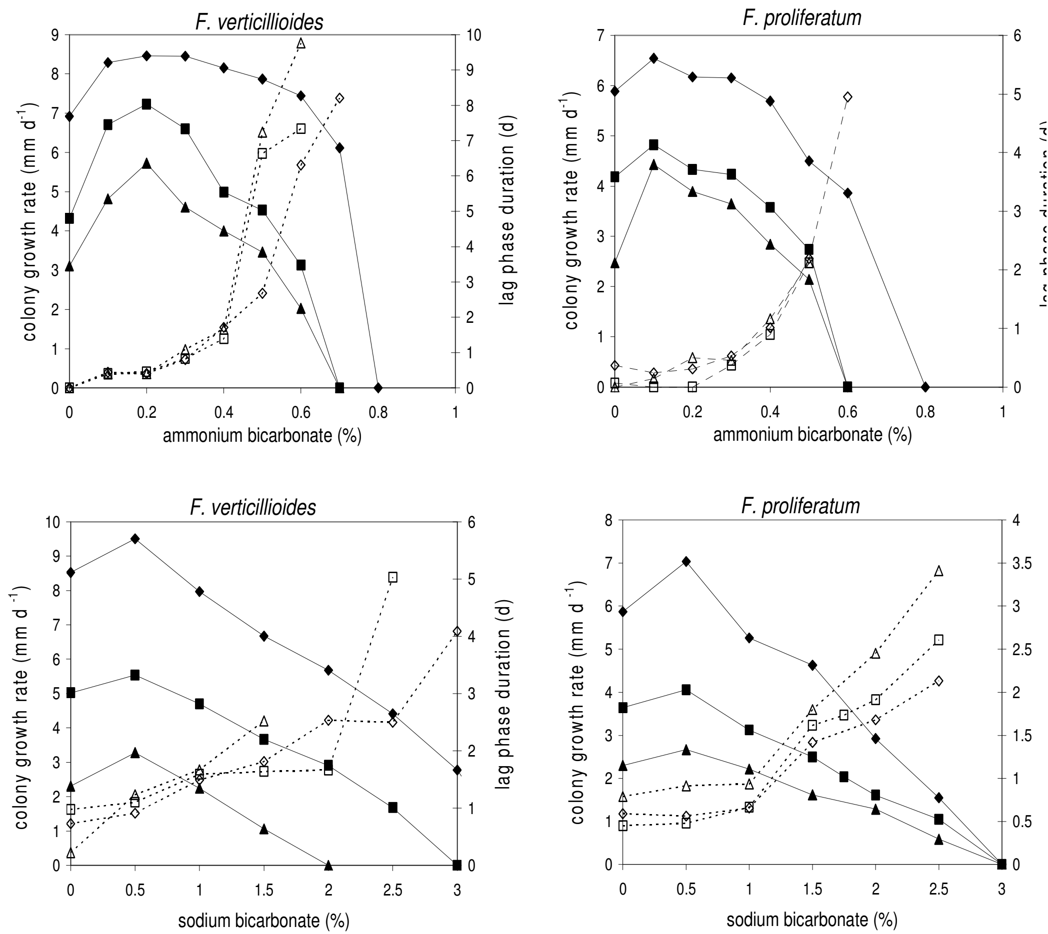


Fig. 8.2. Plots of the colony growth rates (mm d^{-1}, solid black symbols with solid lines) and lag phase durations (d, empty symbols with dotted lines) of *F. verticillioides* and *F. proliferatum* as a function of ammonium bicarbonate or sodium bicarbonate concentration at a_w values of 0.97 (◆, ◇), 0.95 (■, □) and 0.92 (▲, △).

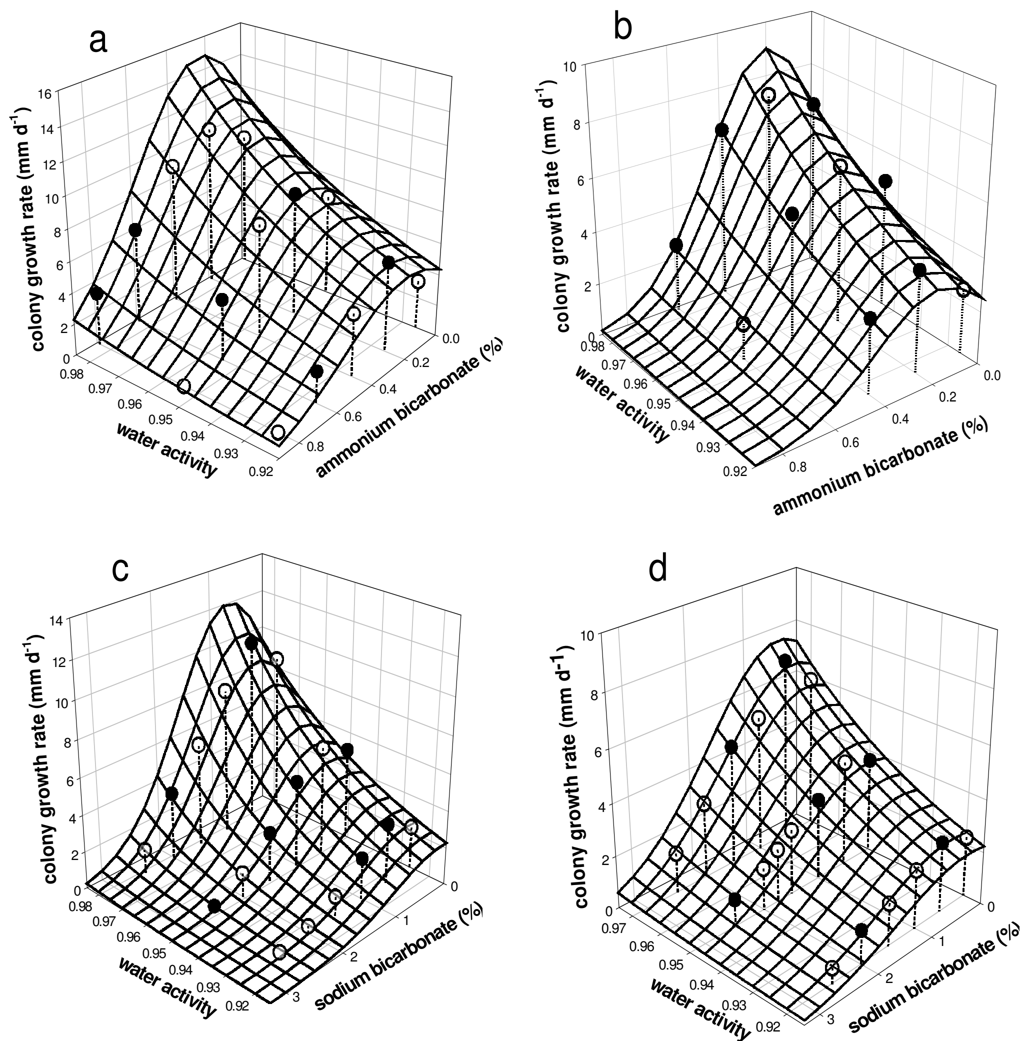


Fig. 8.3. Three dimensional surfaces of the predicted colony growth rates (mm d^{-1}) and the estimated colony growth rates (symbols, ● above and ○ below the model surface) versus a_w and bicarbonate salt concentration (%) for *F. verticillioides* - (a) and (c) and for *F. proliferatum* - (b) and (d).

The significance of the interaction term (shown in Table 8.1) confirms that a syngergistic interaction takes place for *F. verticillioides* between a_w and bicarbonate salt on the colony growth rate, which is also confirmed by the curvature of the model surfaces. Although the interaction term is non-significant for *F. proliferatum*, curvature of the model surface indicates otherwise, highlighting the need to verify mathematical/statistical deductions

visually. The lag phase durations were generally less than two days at ammonium bicarbonate concentrations below 0.4% and 0.6% for *F. verticillioides* and *F. proliferatum*, respectively. These then increased significantly to durations of 5-10 days when higher ammonium bicarbonate concentrations were applied. For the *Fusarium* isolates sodium bicarbonate concentrations below 2.0% generally resulted in lag phases less than three days long, which increased to 4-5 days for *F. verticillioides* when the concentration was raised to concentrations ≥ 2.5% at all a_w values evaluated. At low salt concentrations the a_w value had no significant effect on the lag phase duration, however the influence of a_w on the lag phases is observed in most cases to become larger at higher salt concentrations. This indicates that an interaction occurred between a_w and salt concentration on the lag phase duration.

In contrast to the trend observed for the *Fusarium* isolates, increase in concentration of either salt immediately resulted in a decrease in the colony growth rate and increase in the lag phase duration of both *Aspergillus* isolates at all a_w values investigated. The observation that no growth stimulation occurred at low concentrations of either salt (as observed for both *Fusarium* isolates), is possibly due to differences in metabolic capacities or nutritional requirements for growth at the genus level or this maybe a trait specific to these isolates. As observed for the *Fusarium* isolates, the lag phases of the *Aspergillus* isolates studied increased significantly with an increase in concentration of either salt. An example being *A. flavus* at a_w 0.880 which had a lag phase duration of approximately 1 day at 0 and 0.2% ammonium bicarbonate. This significantly increased to 3 and 6 days at 0.4% and 0.6% ammonium bicarbonate, respectively. As observed for the lag phase durations of the *Fusarium* isolates, an interaction between a_w and bicarbonate is observed as the differences between the lag phases at the three different a_w values are non-significant at low salt concentrations and become larger at higher salt concentrations.

Despite the observation that the salts were initially stimulatory to the growth of the *Fusarium* isolates and not for the *Aspergillus* isolates, the *Fusarium* strains were actually more sensitive to the bicarbonate salts. This can be deduced from the observation that lower concentrations of either bicarbonate salt were required to completely inhibit growth of the *Fusarium* isolates. This was more evident when sodium bicarbonate was applied. 4% sodium bicarbonate was insufficient to prevent *Aspergillus* growth throughout the a_w

range investigated, in contrast to a concentration of 3.5% which completely inhibited *Fusarium* growth at all a_w values studied. 1% ammonium bicarbonate was required at a_w 0.970 to inhibit the growth of the *Aspergillus* isolates whereas a lower level of 0.8% was sufficient to inhibit the growth of the *Fusarium* isolates at all a_w values. For all isolates the concentration of bicarbonate salt required to completely inhibit growth was also observed to decrease with a_w, again reflecting the interactive effect of the salts with a_w on the viability of the isolates. An example of this being that sodium bicarbonate at a concentration of 2.5%, completely inhibited growth of *F. verticillioides* over an incubation period of 42 days at a_w 0.922, whereas 3 and 3.5% was required to achieve the same effect at a_w 0.948 and 0.970, respectively. As observed for the *Fusarium* isolates, the curvature of the model surfaces for *A. flavus* and *A. parasiticus* as seen in Fig. 8.4 also indicates that a_w and bicarbonate salt synergistically influenced the colony growth rate, despite the non-significance of the interaction terms for the models developed for *A. flavus* with sodium bicarbonate and *A. parasiticus* with ammonium bicarbonate.

In agreement with our results the inhibitory effect of ammonium bicarbonate and sodium bicarbonate on the growth of several fungal species has been reported before. DePasquale et al. (1990) reported that ammonium bicarbonate at 0.11*M* (appr. 0.87%) completely inhibited the growth of *F. tricinctum*, *F. graminearum*, *F. sporotrichioides*, *Penicillium griseofulvum*, *P. notatum*, *Aspergillus ochraceus*, *A. flavus* and *A. niger* on PDA. They also found that the sodium bicarbonate applied at about the same concentration (appr. 0.93%) only reduced the viability of *P. griseofulvum*, *A. flavus*, *A. niger* and *P. notatum*. In our study sodium bicarbonate applied at 1% on corn also only slightly reduced the growth rates of the *Fusarium* and *Aspergillus* isolates.

Montville and Shih (1991) observed that ammonium bicarbonate at levels of 1 and 2% completely inhibited monocultures of *F. graminearium*, *A. ochraceus* and *P. griseofulvum* on cracked corn. In addition Montville and Goldstein (1987) reported that both sodium and potassium bicarbonate reduced the viability of *Aspergillus parasiticus* on Czapek's agar. Both DePasquale et al. (1990) and Montville and Shih (1991) observed that ammonium bicarbonate generally has a greater fungicidal activity than sodium bicarbonate, a finding which is confirmed by our results as almost four times more sodium bicarbonate than ammonium bicarbonate was required to completely inhibit the growth of

the *Fusarium* isolates. Moreover, sodium bicarbonate at concentrations as high as 4% only reduced the viability of the *Aspergillus* isolates. Punja and Grogan (1982) and Punja et al. (1986) demonstrated the ability of ammonium bicarbonate to inhibit sclerotial germination and mycelial growth of *Sclerotium rolfsii*. In a study of the effectiveness of sodium bicarbonate in controlling fungal decay on two melon cultivars during prolonged storage periods, Aharoni et al. (1997) found that sodium bicarbonate inhibited the mycelial growth of *Fusarium* spp. Decay incidence of the melon cultivars was reduced by 50% after 14 days of storage at 1.35 and 1% *in vitro* and *in vivo* sodium bicarbonate concentration, respectively. 2% sodium bicarbonate completely eliminated *in vivo* decay. Significant control of blue mold, caused by *P. italicum*, on oranges has been reported to be achieved by sodium bicarbonate solutions of 4% concentration (w/v) at room temperature (Palou et al., 2001).

Several theories on the mechanism of inhibition of growth by bicarbonate salts have been postulated to date. Punja and Grogan (1982) proposed that inhibition by ammonium bicarbonate principally involved unionized ammonia (NH_3) associated with the salt. They also suggested that although the toxicity of the salt to *Sclerotium rolfsii* was not a direct pH effect, it appeared to be pH mediated. The dissociation of NH_4^+ species resulting in the formation of uncharged free NH_3 is pH dependent and governed by the equilibrium equation: $NH_4^+ + H_2O \leftrightarrows NH_3 + H_3O^+$ (pKa = 9.25 at 25°C) (DePasquale and Montville, 1990). Depasquale and Montville (1990) tested the hypothesis that free ammonia caused fungal inhibition by using ammonium sulfate as a model for ammonium bicarbonate. They found that ammonium sulfate was as inhibitory as ammonium bicarbonate at pH values ≥ 8.7, but was completely ineffective at pH values ≤ 7.8. They also found that viability and percent germination of *F. graminearum* and *P. griseofulvum* decreased dramatically as the concentration of free ammonia increased, thereby establishing NH_3 as the toxic agent. They concluded that ammonium bicarbonate inhibits fungal growth because the bicarbonate anion supplies the alkalinity necessary to establish an antifungal concentration of free ammonia. Free ammonia exerts its toxicity via passive intracellular diffusion of the non-ionized NH_3 form, leading to high intracellular concentrations of NH_3 (Tsao and Oster, 1981; Azov and Goldman, 1982; Loffer et al., 1986). High intracellular concentrations of NH_3 have also been found to discharge pH gradients (Gillies and

Deamer, 1979), inhibit cyclic adenosine monophosphate production (Schindler and Sussman, 1979) and inhibit the transportation of amino acids (Roon et al., 1975).

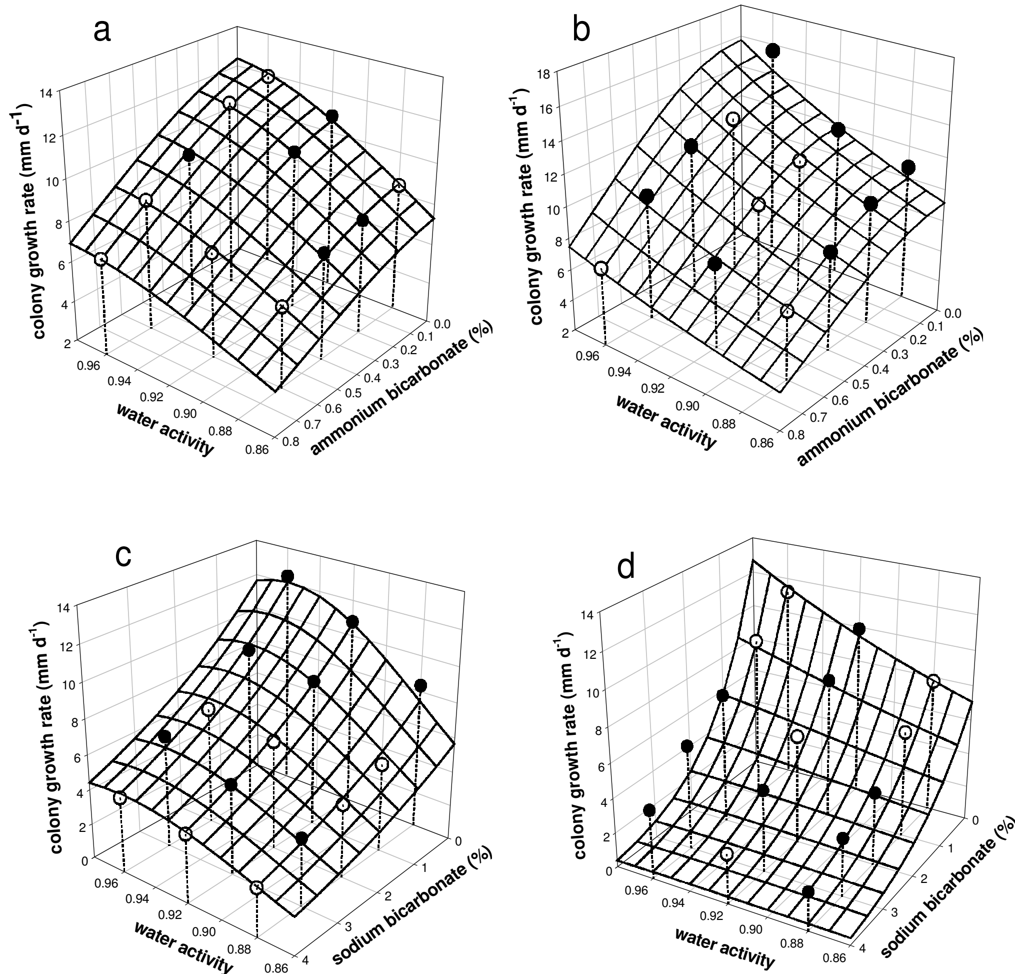


Fig. 8.4. Three dimensional surfaces of the predicted *g* (mm d^{-1}) and the estimated colony growth rates (symbols, ● above and ○ below the model surface) versus a_w and bicarbonate salt concentration (%) for *A. flavus* - (a) and (c) and for *A. parasiticus* - (b) and (d).

The pH values of the extracts for mycotoxin analysis were recorded to have an idea of the possible role of pH in the results observed. In general the extracts from the samples treated

with ammonium bicarbonate for mycotoxin analysis (after 10 days incubation), increased pH from about 5.4 to 8.1 as concentration was increased from 0 to 0.8%. Although these pH values are below the pKa (9.25 at 20°C), the increase in pH with the ammonium bicarbonate concentration certainly results in more free NH_3 being formed, and consequently a larger antimycotic effect as observed. The pH of the extracts was then observed to decrease to values between 5.0 and 6.4 (over the same concentration range) after 20 days incubation. This decrease is possibly a result of the formation of more free NH_3 and/or fungal metabolism. Important to note is that despite this decrease in pH, 0.8% and 1% ammonium bicarbonate completely inhibited *Fusarium* and *Aspergillus* growth, respectively, over a 42 day period.

The bicarbonate ions (HCO_3^-) have also been reported to have an inhibitory effect (Montville and Goldstein, 1987), which is coupled to that of free ammonia in the case of ammoniun bicarbonate. However, this effect may be the sole cause of the inhibition by sodium bicarbonate, as sodium ions have previously been found to be non-inhibitory (Uraih and Chipley, 1976). The principle mode of action of bicarbonate ions is through their buffering capacity, whereby an alkaline environment is sustained. When this happens, organisms will expend more energy on the production of fungal acid to counter the alkaline pH than they use on hyphal extension, giving rise to growth inhibition (Palmer et al., 1997). Fallik et al. (1997) suggested that the inhibitory effect of bicarbonates on *Alternaria alternata* was probably due to reduction in fungal cell turgor pressure which resulted in collapse and shrinkage of hyphae and spores, consequently leading to the inability of the fungi to sporulate. The pH values of the extracts for mycotoxin analysis after 10 days of incubation increased significantly from 5.4 to 9.7 as sodium bicarbonate concentration was raised from 0 to 4%. A smaller decrease in pH was noted after 20 days incubation in comparison to that observed for the extracts from the samples treated with ammonium bicarbonate, therefore hinting that the formation of free NH_3 may be the main reason for the pH decrease observed in that case ammonium bicarbonate. Generally it can be concluded that an increase in sodium bicarbonate concentration results in an increase in both pH and concentration of bicarbonate ions, resulting in inhibition of growth by either or both of the mechanisms mentioned above.

8.4.2. Effect of bicarbonate salts on fumonisin B_1 and aflatoxin B_1 on corn

The effect of the bicarbonate salts on fumonisin B_1 and aflatoxin B_1 production at a_w 0.960 is shown in Table 8.2 and 8.3, respectively. It can be seen that both bicarbonate salts in general had a very large impact on both fumonisin B_1 and aflatoxin B_1 production. Generally the higher the concentration of bicarbonate salt applied the lower the amount of fumonisin B_1 and aflatoxin B_1 produced. In Table 8.2 it can be observed that after 20 days of incubation 3648, 311 and 0 µg/kg fumonisin B_1 were produced at concentrations of 0.2, 0.4 and 0.6%, respectively, representing a reduction of at least 95.3% from the amount produced by the untreated controls (77538 µg/kg) after the same incubation period. The same trend is noted for the effect of ammonium bicarbonate on fumonisin B_1 production after 10 and 20 days by *F. proliferatum*. For *F. verticillioides* sodium bicarbonate completely inhibited fumonisin B_1 production even after 20 days of incubation regardless of concentration applied. However, sodium bicarbonate was less inhibitory on fumonisin B_1 production by *F. proliferatum*. A reduction of at least 76% is observed after 10 days of incubation which subsequently increases to at least 94.3% after 20 days of incubation. Importantly it is noted that although ammonium and sodium bicarbonate concentrations of 0.2 and 0.5%, respectively, enhanced the growth of both isolates relative to their growth on untreated corn of the same a_w, this growth stimulation did not result in an increase in fumonisin B_1 production.

In great contrast to fumonisin B_1 production by the *Fusarium* isolates, was the observation that as much as 4% sodium bicarbonate did not inhibit aflatoxin B_1 production, where only 2% was required to completely inhibit fumonisin B_1 production over the incubation period investigated. At this level *A. flavus* and *A. parasiticus* produced 154 and 186 µg/kg of aflatoxin B_1, respectively, levels that are at least seven times greater than the limit of acceptance of corn set by the United States Food and Drug Administration (FDA) of 20 µg/kg (FDA, 2000) and that of 5 µg/kg set by the European Union (EU) in the Commission Regulation (EC) No. 2174/2003 (EU, 2003). Although not as large as those observed for fumonisin B_1, significant reduction in aflatoxin B_1 production was noted with increase in concentration of the salts. In similarity to fumonisin B_1 production by the *Fusarium* isolates, at least 0.8% ammonium bicarbonate was required to completely inhibit aflatoxin B_1 production by both *Aspergillus* isolates.

Table 8.2. Effect of ammonium and sodium bicarbonate on fumonisin B_1 production by *F. verticillioides* and *F. proliferatum*.

	F. verticillioides fumonisin B_1 (µg/kg)		*F. proliferatum* fumonisin B_1 (µg/kg)	
Ammonium bicarbonate (%)	10 days	20 days	10 days	20 days
0	7162 ± 71	77538 ± 10333	4683 ± 47	79292 ± 3312
0.2	nd[a]	3648 ± 713	nd	517 ± 104
0.4	nd	311 ± 61	nd	461 ± 98
0.6	nd	nd	nd	132 ± 29
Sodium bicarbonate (%)	10 days	20 days	10 days	20 days
0	7162 ± 71	77538 ± 10333	4683 ± 47	79292 ± 3312
0.5	nd	nd	1125 ± 407	4508 ± 314
1.0	nd	nd	137 ± 11	471 ± 141
2.0	nd	nd	nd	nd

a: nd – no fumonisin B_1 detected

Table 8.3. Effect of ammonium and sodium bicarbonate on aflatoxin B_1 production by *A. flavus* and *A. parasiticus*.

	A. flavus aflatoxin B_1 (µg/kg)		*A. parasiticus* aflatoxin B_1 (µg/kg)	
Ammonium bicarbonate (%)	10 days	20 days	10 days	20 days
0	5298 ± 1656	12687 ± 1825	2662 ± 54	4447 ± 190
0.2	1993 ± 68	3910 ± 631	1220 ± 382	3313 ± 200
0.4	890 ± 86	3045 ± 174	610 ± 184	3088 ± 150
0.6	5 ± 1	1503 ± 126	nd	2674 ± 141
0.8	nd[a]	nd	nd	nd
Sodium bicarbonate (%)	10 days	20 days	10 days	20 days
0	5298 ± 1656	12687 ± 1825	2662 ± 54	4447 ± 190
2	501 ± 85	3017 ± 1217	93 ± 35	2700 ± 786
4	nd	154 ± 19	nd	186 ± 12

a: nd – no aflatoxin B_1 detected

The failure of sodium bicarbonate to completely inhibit both growth and aflatoxin B_1 production at the conditions investigated strongly undermines the possibility of its use for protection of corn during storage, particularly when storage starts at a high moisture content. As growth of *Aspergillus* still occurs at a concentration of 0.8% ammonium bicarbonate, at least 1% would be necessary to completely protect the grain from both fungal growth and mycotoxin production. In agreement with our results, sodium bicarbonate has also been found to inhibit the production of trichothecene mycotoxins by *F. tricinctum* (Roinestad et al., 1993, 1994), ochratoxin A by *A. ochraceus* (Montville and Shih, 1991) and aflatoxins by *A. parasiticus* (El-Nabarawy et al., 1989; Montville and Goldstein, 1987, 1989). However, contrary to our findings, Al-Hilli and Smith (1979) found that low concentrations of some food-grade preservatives can stimulate aflatoxin production by *A. flavus*.

Nitrogen metabolism and pH have been reported to have important roles in the regulation of fumonisin biosynthesis (Flaherty et al. 2003; Pirttilä et al. 2004). Keller and Sullivan (1996) and Shim and Woloshuk (1999) reported that fumonisin production begins when ammonium becomes limited in synthetic growth medium containing ammonium salts as the nitrogen source. They also reported the repression of fumonisin B_1 production by 97% when ammonium phosphate was added to cracked corn cultures. With this in mind a different mechanism had to be at work for the inhibitory effect observed for sodium bicarbonate on fumonisin B_1 production. A possible explanation lies in the observation that the optimal pH for fumonisin production has been reported to occur under acidic conditions of pH 3-4 (Keller et al., 1997) and the inhibitory effects of alkaline pH on fumonisin biosynthesis has been demonstrated (Keller et al., 1997; Shim and Woloshuk, 1999; Flaherty et al., 2003; Pirttilä et al. 2004). In addition the role of pH in inhibition of fumonisin B_1 production by determining the expression of genes involved in fumonisin biosynthesis has been demonstrated by a number of workers (Shim and Woloshuk, 1999; Flaherty et al., 2003; Pirttilä et al. 2004). Shim and Woloshuk (2001) reported that *FUM5*, a polyketide synthase gene involved in fumonisin B_1 production was not expressed in a mutant strain of *F. verticillioides* carrying a disrupted gene (*FCC1*) when grown on corn kernels or on a minimal defined medium of pH 6. However, when grown at pH 3 on the defined minimal defined medium the blocks in expression of *FUM5* were suppressed. They concluded that although the *FCC1* gene was not essential for

vegetative growth it played an important role in the regulation of secondary metabolism proceses such as fumonisin biosynthesis and conidiation. In addition Flaherty et al. (2003) reported that although the *PAC1* gene is required for the growth of *F. verticillioides* at alkaline pH, it may also have a role as a repressor of fumonisin biosynthesis under these conditions. In addition they suggested that *FCC1* and *PAC1* may act independently of each other with respect to fumonisin biosynthesis.

The inhibition of mycotoxin production by bicarbonate salts has also been attributed to their ability to impair the activity of enzymes involed in the biosynthetic pathway for mycotoxins (El-Nabarawy et al., 1989; Roinestad et al., 1994). El-Nabarawy et al. (1989) observed the accumulation of the pigments averufin and versicolorin A coincided with the inhibition of aflatoxin when *A. parasiticus* was grown in the presence of sodium bicarbonate. They concluded that the observed repression of aflatoxin production was a result of the inhibition of the enzymes which process versicolorin A and averufin to sterigmatocystin and ultimatelty aflatoxin by bicarbonate. We however did not notice any build-up of pigments on the treated corn during the incubation period. High amounts of aflatoxins have previously been found to be produced by *A. flavus* and *A. parasiticus* only in acidic medium (Cotty, 1988), although divergent or atypical strains have been found as well (Ehrlich et al., 2005). Roinestad et al. (1993, 1994) reported the inhibition of the production of trichothecene mycotoxins by *F. tricinctum* in the presence of sodium bicarbonate. Based on the fact that the trichothecence mycotoxins are biosynthesized from mevalonic acid and that an accumulation of mevalonic acid metabolites was observed in the bicarbonate treated corn, they concluded that a pH related inhibition of mevalonate kinase or a pH related activation of an enzyme responsible in part for the conversion of mevalonic acid to pentoic acid or its precursors resulted in the inhibition of trichothecene production.

8.4.3. Sensorial suitability of corn treated with bicarbonate salts for human consumption

For the samples treated with ammonium bicarbonate, a difference between the treated corn and untreated corn was first noted at a level of 0.4%, by five (33%) of the panelists. They however all considered the corn to be acceptable. At 1% all the

panelists reported a difference between the treated and untreated samples, and one panelist considered the corn to be unacceptable. For corn samples treated with sodium bicarbonate all panelists noted a difference at 1% and 13% of them (2) considered the corn to be sensorially unsuitable for consumption. At 2% the product was considered unsuitable by all the panelists. In view of the fact the treated grain should be sensorially acceptable for consumption, the results of the sensorial evaluation enable for a more informative decision to be made the actual possibility of use of bicarbonate salts as antifungal agents in stored grain systems. The levels at which ammonium bicarbonate is fully inhibitive on fungal growth and mycotoxigenesis favourably coincide with those at which the treated corn is still considered sensorially acceptable. It however is clear that consumption of bicarbonate treated product is not possible without an attempt to reduce residual levels of the salt.

8.5 Conclusions

Ammonium bicarbonate potentially provides an inexpensive, safe, easy to apply and highly effective antimycotic agent that can potentially be used for the protection of bulk grain systems in store, especially in resource limited developing countries. Ammonium bicarbonate completely inhibited both growth and mycotoxin production of both *Fusarium* and *Aspergillus* isolates investigated at levels (1%) that were still sensorially acceptable. Sodium bicarbonate although effective on the growth and fumonisin B_1 production by the *Fusarium* isolates, failed to completely inhibit *Aspergillus* growth and aflatoxin B_1 production at levels (4%) that were sensorially acceptable. A synergistic interaction between the salts and a_w was found to occur on the colony growth rate, lag phase duration and concentration of salt required to completely inhibit growth. The evaluation of several sublethal levels of bicarbonate salt at each a_w value investigated enabled the observation for the first time of *Fusarium* growth stimulation at low salt concentrations, a phenomenon which appears species or genus specific as this was not observed for the *Aspergillus* isolates investigated. The growth stimulation observed however did not translate to an increase in fumonisin B_1.

A wide range of fungi occurs on grain in the immediate post-harvest and storage periods which may have varying tolerances to the bicarbonate salts with regards to both growth and mycotoxin production, as depicted in our results. This alone necessitates further studies to be done to evaluate the effects of these salts on the viability and capacity to produce mycotoxins of these interacting fungi. Methods to minimize the residual levels of the bicarbonate salts before preparation for consumption also have to be further investigated as the levels at which the bicarbonate salts are inhibitory to both growth and mycotoxin production are levels at which they begin to adversely affect sensorial quality of the corn. To complete the study, a chemical technique based on the application of natural phenolic compounds was evaluated in chapter 9. The rise in consumer demands for the reduction of the quantities of synthetic or inorganic chemicals entering the food chain has made the use of such techniques become more and more important.

CHAPTER 9

Can phenolic compounds be used for the protection of corn from fungal invasion and mycotoxin contamination during storage? [9]

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[9] Redrafted from: Samapundo, S., De Meulenaer, B., Osei-Nimoh, D., Lamboni, Y., Debevere, J. and Devlieghere, F. (submitted).

Chapter 9. Can phenolic compounds be used for the protection of corn from fungal invasion and mycotoxin contamination during storage?

9.1 Abstract

The effect of natural phenolic compounds (vanillic and caffeic acid) and water activity (a_w) on the growth and mycotoxin on corn by Fusarium and Aspergillus isolates was investigated. Large differences were observed in the response of the Fusarium and Aspergillus isolates to the phenolic compounds, mostly determined by their genus. Generally for both F. verticillioides and F. proliferatum, an increase in concentration of either caffeic or vanillic acid resulted in a decrease in the colony growth rate and increase in the lag phase duration. Growth of the Fusarium isolates was not completely inhibited at the highest a_w value evaluated of 0.967, with complete inhibition only being observed at combinations of high phenolic acid concentrations ($\geq$ 2000µg/g) and low a_w values ($\leq$ 0.948). Within the experimental limits investigated, growth of the Aspergillus species was not affected by the phenolic compounds. Application of the phenolic compounds significantly reduced fumonisin B_1 and aflatoxin B_1 production. Although the effects noted on artificial media appear to be carried over to corn, rather high concentrations are required to observe similar effects on corn and to completely inhibit growth where possible. This implies the occurrence of interactions of the phenolic compounds with some matrix components which may reduce their overall effectiveness. In addition a strongly bitter taste is imparted at these high levels raising concern about the sensorial acceptability of corn that has been treated by phenolic compounds.

Key words: *Fusarium*, *Aspergillus*, corn, vanillic acid, caffeic acid, aflatoxin B_1, fumonisin B_1.

9.2 Introduction

Interest has recently been inceasing in the possible role of natural phenolic compounds in inhibiting growth and toxin production by fungi. This in part is linked to findings that the resistance of some cultivars of agriculturally important crops to

fungal infection has been correlated with their content of phenolic compounds before or after infection (Assabgui et al., 1993; Carver et al., 1994; Kofalvi and Nassuth, 1995; McKeehen et al., 1999; El Modafar et al., 2000; Siranidou et al., 2002). In addition phenolic compounds have also been found to be inhibitory to the production of several mycotoxins including fumonisins, tricothecenes and aflatoxins (Chipley and Uriah, 1980; Norton, 1999; Bakan et al., 2003; Beekrum et al., 2003). Consumer perception that the use of industrially synthesized food preservatives may be associated with potential toxicological problems has generated interest in use of naturally occurring compounds (Sofos et al., 1998). Being natural plant secondary metabolites found in essentially all plant material (Hammer et al., 1999), phenolic compounds potentially provide an acceptable antifungal agent for application pre- and/or post-harvest. However, to date most studies have been done on artificial media (with a useful inhibitory effect being observed in most cases on both fungal growth and mycotoxin production) resulting in paucity on data for the effect of these compounds when applied on actual food products. The role of possible interactions of these effects with those of a_w (the single most important determinant of fungal growth as shown in chapters 3, 4 and 7) have also largely been ignored. In this context the study marked the final part of the study in which natural chemical compounds of plant origin were to be evaluated for their potential to inhibit fungal growth and mycotoxin production in the post-harvest period as an alternative to the use of non-chemical methods (MA, chapter 7) or inorganic chemicals (bicarbonate salts, chapter 8). The major objectives of this chapter were to ascertain if the inhibitory effects on growth and mycotoxin production observed on artificial media are carried over onto corn, and to determine the influence of a_w on the observed effects and where possible to determine the levels at which growth is completely inhibited.

9.3 Materials and Methods

9.3.1 Treatment of corn with the phenolic compounds

The effect of caffeic acid and vanillic acid (Merck Chemicals, Darmstadt, Germany) on growth of the isolates was evaluated using a full factorial design of three a_w values (0.88-0.97) and at least six phenolic compound concentrations between 0 and 2500 µg/g of corn. The concentration range of phenolic compounds investigated was

determined on the basis of preliminary experiments. These two phenolic acids were selected on the basis of their demonstrated potential to inhibit fungal growth and mycotoxin production on artificial media (Beekrum et al., 2003; Guiraud et al., 1995) and also because they were readily available for use in purified form. Effect of the phenolic compounds on fumonisin B_1 and aflatoxin B_1 production was evaluated at a_w 0.96 and phenolic compound concentrations of 0, 1000 and 2000 µg/g of corn. The a_w of the corn was reproducibly altered to achieve a desired a_w value using the GAB sorption model developed at 25°C in chapter 2. To ensure the even distribution of the phenolic compounds, they were added to the water that was used to rehydrate the corn, after which the water was added to the corn. The final concentration reported (µg/g) takes into account the final weight of the corn (dry corn + distilled water) and assumes no water is lost during the equilibration period. After addition of the water containing the phenolic compounds the treated corn was equilibrated over two days at 4°C. Uniform distribution of the phenolic compounds was further encouraged by periodic mixing of the corn. The treated corn was then incubated for a further day at 25°C to enable it to attain the final incubation temperature. The exact a_w of the treated corn was then determined by the Novasina Thermoconstanter TH200 (Axair Ltd. Systems, Pfapfikkon, Switzerland).

9.3.2 Fungal isolates, inoculation and assessment of growth

Fusarium verticillioides Sheldon (25), *F. proliferatum* (Matsushima) Nirenberg (73N), *Aspergillus flavus* (IITAB 139) and *A. parasiticus* (IITAB 138) were evaluated in the study. *F. verticillioides* and *F. proliferatum* inoculum was prepared and inoculated as as described in section 3.3.1.3 whereas *A. flavus* and *A. parasiticus* inoculum were prepared as described in section 4.3.4, with the exception that ten plates were prepared per condition instead of 20. Growth was then assessed during incubation at 25°C over a maximum period of 42 days as described in section 3.3.1.3.

9.3.3 Mathematical analysis of experimental data

Estimates of the colony growth rate (g, mm d^{-1}) and lag phase duration (λ, d) at each condition were determined by linear regression of the growth data collected during the linear phase of growth as described in section 5.3.3.3.

9.3.4 Quantification of effect of phenolic compounds on fumonisin B_1 production

As mentioned above the effect of the phenolic compounds on fumonisin B_1 production was determined at a_w 0.96 and at phenolic compound concentrations of 0, 1000 and 2000 µg/g of corn. Petri plates containing inoculated grain were incubated in plastic buckets containing glycerol/water solutions of the same a_w, and samples for fumonisin analysis were collected after 10 and 20 days of incubation. For each condition two plates were randomly selected on each day of sampling and stored in a freezer until extraction was performed. The contents of the sampled plates were separately ground using a blender. The method developed in section 7.3.7 for extraction, sample clean-up and HPLC analysis was then used to quantify fumonisin B_1 levels in the samples, with the exception that an Agilent 1100 series HPLC was used to detect fumonisin B_1.

9.3.5 Quantification of effect of phenolic compounds on aflatoxin B_1 production

The same sampling protocol as described for fumonisin B_1 was used for determination of aflatoxin B_1. The extraction, sample clean-up and HPLC analysis was performed as previously described in section 8.3.5.

9.4 Results and Discussion

9.4.1 Effect of phenolic compounds on growth of *Fusarium* and *Aspergillus* isolates on corn

The *Fusarium* and *Aspergillus* isolates responded very differently to the phenolic compounds evaluated, largely determined by their genus. Figs. 9.1 and 9.2, depict the colony growth rates and lag phase durations of *F. verticillioides* and *F. proliferatum* as a function of the phenolic compound concentration at different a_w values, respectively. Fig. 9.3 shows examples of the general trend observed for *A. flavus* and *A. parasiticus*.

It can be seen from Figs. 9.1 and 9.2 that the phenolic compounds had a marked effect on the colony growth rates and lag phase durations of the *Fusarium* isolates, with

increase in caffeic or vanillic acid concentration being positively and negatively correlated to the lag phase durations and the colony growth rates, respectively. In addition to the effect of the phenolic compounds, a_w also had an effect on the colony growth rates and lag phase durations of both *Fusarium* isolates; a slower colony growth rate and longer lag phase being observed the lower the a_w value.

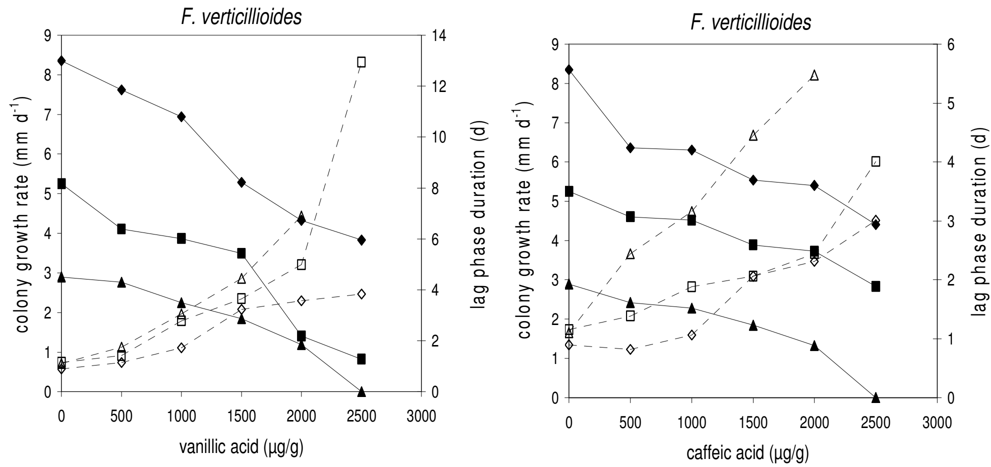

Fig. 9.1. Colony growth rates (mm d^{-1}) (continuous lines and solid symbols) and lag phase durations (d) (broken lines and empty symbols) of *F. verticillioides* as a function of the phenolic compound concentration at a_w values 0.967 (◆, ◇), 0.948 (■, □) and 0.921 (▲, △).

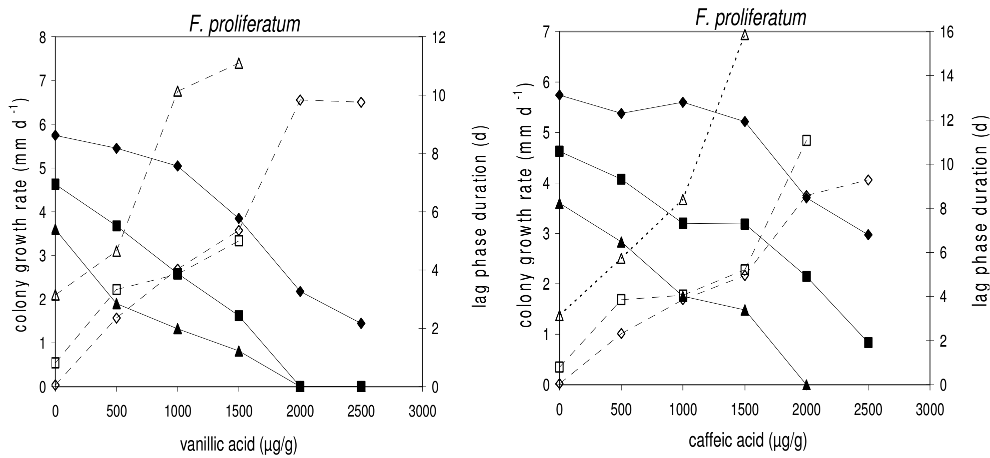

Fig. 9.2. Colony growth rates (mm d^{-1}) (continuous lines and solid symbols) and lag phase durations (d) (broken lines and empty symbols) of *F. proliferatum* as a function of the phenolic compound concentration at a_w values 0.967 (◆, ◇), 0.948 (■, □) and 0.921 (▲, △).

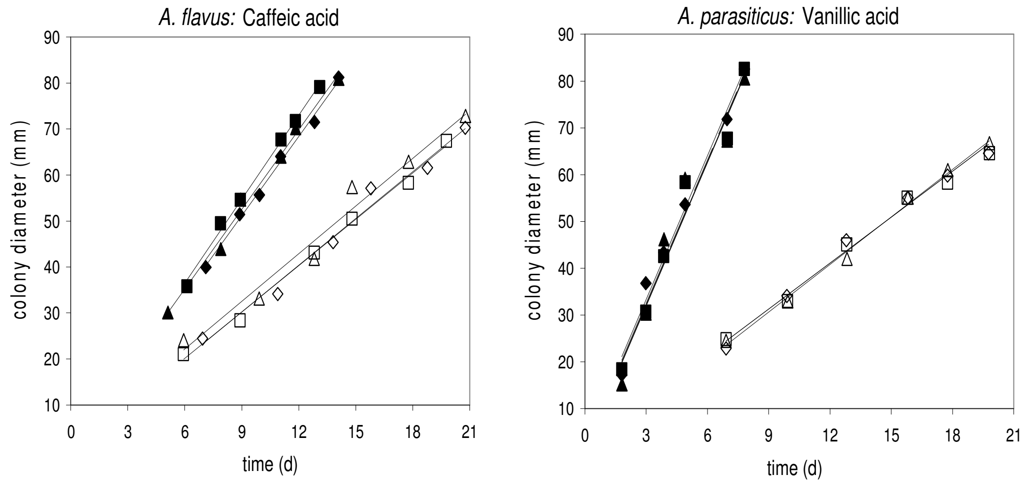

Fig. 9.3. Growth curves, colony diameter (mm) vs time (d), of *A. flavus* and *A. parasiticus* at a_w 0.967 and phenolic acid concentrations of 0 (◆), 500 (■) and 1500 µg/g (▲) and at a_w 0.880 and phenolic acid concentrations of 0 (◇), 500 (□) and 1500 µg/g (△).

At the highest a_w evaluated of 0.967, both phenolic compounds failed to completely inhibit the growth of the *Fusarium* isolates. Complete inhibition of growth *F. verticillioides* was only observed at a_w 0.921 in combination with a caffeic or vanillic acid concentration of 2500 µg/g of corn. *F. proliferatum* was more sensitive than *F. verticillioides* to inhibition by vanillic acid as its growth was completely inhibited at a higher a_w value of 0.948 in combination with a lower vanillic acid concentration of 2000 µg/g. Caffeic acid completely inhibited the growth of *F. proliferatum* at a concentration of at least 2000 µg/g in combination with a a_w value of 0.921. In great contrast to the trend observed for the *Fusarium* isolates, both phenolic compounds did not affect the mycelial growth of the *Aspergillus* species within the concentration and a_w range evaluated. Examples of this are shown in Fig. 9.3 for *A. flavus* and *A. parasiticus*. Also shown in Fig. 9.3 is the expected effect a_w had on the colony growth rates of both isolates, which significantly decreased with a reduction in the a_w value. The colony growth rates of *A. flavus* were on average about 10, 5.7 and 3.5 mm d^{-1} at a_w values of 0.967, 0.921 and 0.880, respectively. Whereas those of *A. parasiticus* were similar to those of *A. flavus* and were on average about 10, 6 and 3 mm d^{-1} at a_w values of 0.967, 0.921 and 0.880, respectively. The lag phases were observed to increase slightly as a_w was decreased.

Phenolic compounds have previously been found to have a potential inhibitory effect on the growth of several food spoiling fungi. In a study on wheat, Siranidou et al. (2002) observed that the cultivar that exhibited the greatest resistance to fungal infection had significantly higher amounts of free phenolic compounds. The susceptible cultivar showed little or no response to fungal infection in terms of its levels of free phenolic compounds. The accumulation of phenylpropanoid compounds after infection by different pathogens has also been reported to be an important resistance factor in cereals to fungal pathogens (Carver et al., 1994; Kofalvi and Nassuth, 1995). Beekrum et al. (2003) reported the inhibitory effect of several plant phenolic compounds, including vanillic acid and caffeic, on the growth of *F. verticillioides* on Sabouraud dextrose agar plates. Both vanillic acid and caffeic acid had minimum inhibitory concentrations of 50 µg/ml. Sinha and Singh (1981) reported that 200µg/g of ferulic acid only reduced the growth of *A. parasiticus* growth by 8.2%. Miller et al. (1996) also reported that 4-acetyl-benzoxazolin-2-one (4-ABOA) had no significant effect on the mycelial weight of *F. culmorum*. Assabgui et al. (1993) reported that levels of 647µg of (E)-ferulic acid per gram of fungal growth retarded the *in vitro* mycelial growth of *F. graminearum* by 50%. Similar levels for inhibition have also been observed in *in vitro* studies on wheat phenolic acids during grain development and their contribution to *Fusarium* resistance (McKeehen et al., 1999). In that study the 50% effective concentration for the inhibition of mycelial growth of *F. graminearum* and *F. culmorum* was found to vary between 329 and 668 µg of ferulic acid and 316 and 793µg of *p*-coumaric acid per gram of growth medium. In addition they noted synergistic activity between ferulic acid and *p*-coumaric in reducing mycelial growth.

Mechanisms for the antimicrobial activity of simple phenolic compounds such as caffeic acid have been proposed to include enzyme inhibition by the oxidized compounds, possibly through reaction with sulphydryl groups or through more non-specific interactions with proteins (Mason and Wasserman, 1987). More complex natural phenolic compounds such as quinones, flavones, flavonoids and flavonols exert their antimicrobial activity by complexing irreversibly with nucleophilic amino acids in proteins leading to inactivation of the protein and loss of function (Cowan, 1999). The most probable targets in microbial cells being surface exposed adhesions, cell wall polypeptides, and membrane-bound enzymes (Cowan, 1999).

Most of the studies mentioned above that have determined the effect of phenolic compounds on fungal growth have mostly been done on artificial media without consideration of the effect of a_w on the trends observed. In addition most of these studies have reported an inhibitory effect without mentioning the levels at which complete inhibition of growth could be achieved; this being an important parameter in determining the suitability of applying these compounds for the prevention of fungal growth on grain in store. This study addressed these issues by determining whether the inhibitory effects observed on artificial media were carried over to a real food product and the influence of a_w on the effects observed in addition to determining whether full inhibition was achieved or not. The inhibitory effects are indeed carried over with regards to growth of the *Fusarium* isolates, although the quantities required to achieve a significant level of inhibition are seemingly larger than those that have been reported or indeed studied on artificial media. This indicates that there may be interactions between the phenolic compounds and some components of the corn matrix that reduce the overall effectiveness of these compounds as antifungal agents. As observed for *A. parasiticus* or *F. culmorum* growth on artificial media (Sinha and Singh, 1981; Miller et al., 1996), the phenolic compounds evaluated in this study did not significantly affect the growth of the *Aspergillus* isolates. This is very critical in the overall assessment of the potential of phenolic compounds as antifungal agents as the *Aspergillus* species are considered to be the predominant fungal species on post-harvest corn.

9.4.2 Effect of phenolic compounds on fumonisin B_1 and aflatoxin B_1 production on corn

The effect of the phenolic compounds on fumonisin B_1 and aflatoxin B_1 production at 25°C and 0.96 a_w is shown in Table 9.1 and 9.2, respectively. Both phenolic compounds were observed to have a significant effect on the ability of all the isolates to produce mycotoxins. With regards to the *Fusarium* isolates the greatest effect appeared to be on *F. verticillioides*, with vanillic acid having a greater effect than caffeic acid. It can be seen that in the presence of vanillic acid (at either 1000 or 2000 μg/g), no fumonisin B_1 was produced after 10 or 20 days of incubation by *F. verticillioides*. Fumonisin B_1 production by *F. proliferatum* was only completely inhibited by vanillic acid at a concentration of 2000 μg/g after 10 days incubation.

Although *F. proliferatum* was able to produce fumonisin B_1 production after 10 days of incubation on corn with 1000 μg/g of vanillic acid, there was a 98% reduction from the amount produced in the control. After 20 days of incubation vanillic acid managed to reduce the production of fumonisin B_1 by *F. proliferatum* by 89% and 95% at levels of 1000 and 2000 μg/g, respectively. The amount of fumonisin B_1 produced was also observed to significantly increase without exception at all conditions when the incubation period was increased from 10 to 20 days irrespective of isolate or phenolic compound evaluated.

Table 9.1. Effect of vanillic acid and caffeic acid on fumonisin B_1 production by *F. verticillioides* and *F. proliferatum*.

	F. verticillioides fumonisin B_1 (μg/kg)		*F. proliferatum* fumonisin B_1 (μg/kg)	
Vanillic acid (μg/g)	10 days	20 days	10 days	20 days
0	7162 ± 71	77538 ± 10333	4683 ± 47	79292 ± 3312
1000	nd[a]	nd	46 ± 13	8879 ± 1165
2000	nd	nd	nd	4341 ± 248
Caffeic acid (μg/g)	10 days	20 days	10 days	20 days
0	7162 ± 71	77538 ± 10333	4683 ± 47	79292 ± 3312
1000	nd	2043 ± 295	151 ± 24	19906 ± 1848
2000	nd	621 ± 72	60 ± 13	18008 ± 1279

a: nd – no fumonisin B_1 detected

Table 9.2. Effect of vanillic acid and caffeic acid on aflatoxin B_1 production by *A. flavus* and *A. parasiticus*.

	A. flavus aflatoxin B_1 (μg/kg)		*A. parasiticus* aflatoxin B_1 (μg/kg)	
Vanillic acid (μg/g)	10 days	20 days	10 days	20 days
0	5298 ± 1656	12687 ± 1825	2662 ± 54	4447 ± 190
1000	611 ± 30	524 ± 21	309 ± 16	430 ± 5
2000	470 ± 41	376 ± 87	218 ± 10	281 ± 21
Caffeic acid (μg/g)	10 days	20 days	10 days	20 days
0	5298 ± 1656	12687 ± 1825	2662 ± 54	4447 ± 190
1000	415 ± 30	451 ± 71	702 ± 34	522 ± 27
2000	408 ± 34	416 ± 84	633 ± 40	496 ± 81

a: nd – no aflatoxin B_1 detected

Caffeic acid was generally less inhibitory than vanillic on fumonisin B_1 production by both *Fusarium* isolates. Complete inhibition by caffeic acid of fumonisin B_1 production by *F. verticillioides* was only observed after 10 days of incubation at both concentrations investigated. After 20 days of incubation, caffeic acid at levels of

1000 and 2000 µg/g managed to inhibit fumonisin production by 97 and 99%, respectively. As mentioned before *F. proliferatum* was generally more resistant to the phenolic acids in terms of fumonisin B_1 production. This can be seen in that the reduction of fumonisin B_1 production by *F. proliferatum* after 20 days of incubation was by a lower level of 75 and 77% at caffeic acid concentrations of 1000 and 2000 µg/g, respectively. Although very large reductions in fumonisin B_1 production were in observed in most cases, the quantities produced were larger than the maximum levels allowed for total fumonisins in corn for human consumption in most cases i.e. the United States Food and Drug Administration (FDA) allows a level of 4000 µg/g total fumonisins (FDA, 2001), whereas only 1000 µg/g total fumonisins is allowed in Switzerland for corn intended for human consumption. These large reductions however could be highly significant in the minimization of the risk posed by fumonisins in corn as fungal contamination and mycotoxin is known to occur in small pockets in bulk stored grains.

Table 9.2 shows the effect of the phenolic compounds on aflatoxin B_1 production. Unlike the trend observed for fumonisin B_1, aflatoxin B_1 was produced at all the conditions evaluated. Despite the observation that the phenolic compounds had no effect of the growth of the *Aspergillus* isolates, a marked effect is noted on aflatoxin B_1 production. Generally the higher the concentration of the phenolic compounds the lower the amount of aflatoxin B_1 that was produced. A reduction of at least 74% was observed from the amount that was produced by the controls for both *Aspergillus* isolates at all the conditions evaluated. In general increase in the incubation period from 10 to 20 days resulted in an increase in the aflatoxin B_1 levels. However some exceptions occurred for *A. flavus* on corn treated with vanillic acid and *A. parasiticus* on corn treated with caffeic acid. In these cases a decrease was observed in the aflatoxin B_1 levels after 20 days of incubation compared to those after 10 days. Time course data for aflatoxin production has also been observed in several studies to rise to a peak concentration followed by a decay to near zero (Pitt, 1993; de Castro et al., 2002; Molina and Giannuzzi, 2002). Although the exact degradation or conjugation mechanisms are yet to be described definitively, Pitt (1993) assumed that aflatoxin decay was initiated by enzymes released during mycelial breakdown. In addition it can be observed in Tables 9.1 and 9.2 that the decrease in fumonisin B_1 and aflatoxin B_1 production was always greatest when the phenolic acid concentration was raised

from 0 to 1000µg/g of corn. After which, an increase to a concentration of 2000µg/g of corn only resulted in a small increase. Although the exact reason for this trend is not known, it may be that the mechanism for this route of inhibition of aflatoxin B_1 production could exhibit a saturation level.

Despite the large reductions in aflatoxin B_1 production as a result of application of the phenolic compounds, the levels produced are at least 20 times those applied as maximum limits in corn for human consumption by the FDA (FDA, 2000) of 20 µg/kg or 5 µg/kg set by the European Union (EU) in the Commission Regulation (EC) No. 2174/2003 (EU, 2003). However, as mentioned earlier the occurrence of fungal contamination and subsequent mycotoxin poisoning occurs in small pockets, in which such large reductions would help to reduce the risk posed by these mycotoxins when the grain is mixed. The inhibitory effect of phenolic compounds on mycotoxin production has been observed before (Chipley and Uraih, 1980; Sinha and Singh, 1981; Desjardins et al., 1988; Norton, 1999; Bakan et al., 2003; Beekrum et al., 2003). Beekrum et al. (2003) reported similar levels of reduction in fumonisin B_1 production by caffeic acid and vanillic acid of 90% at concentrations of only 1µg/g of artificial media. Sinha and Singh (1981) observed that 200 µg/g of ferulic acid inhibited aflatoxin production by a much as 90%. 4-acetyl-benzoxazolin-2-one (4-ABOA) has been reported to inhibit acetyldeoxynivalenol (ADON) production by 50% without a significant effect on mycelium weight of *Fusarium culmorum* (Miller et al., 1996). In contrast to the findings by Miller et el. (1996), Bakan et al. (2003) isolated 4-ABOA from degermed and whole grain fractions, but observed that the inhibition of tricothecene synthesis was not linked to 4-ABOA. They however reported that the soluble phenolic extract from the germ tissues of corn kernels significantly ($p < 0.01$) reduced tricothecene (ADON) production under *in vitro* conditions where growth was only slightly reduced (1.2%) compared to the controls. Although the mechanisms for inhibition of mycotoxin production have not yet been identified it can be assumed that they may be closely related to those described for growth inhibition for the *Fusarium* isolates. Clearly the mechanism of inhibition of aflatoxin B_1 production is divergent from that for growth of the *Aspergillus* isolates.

9.5 Conclusions

Phenolic compounds have in the past shown great promise as potential antifungal agents to protect stored grain from fungal invasion and the associated production of mycotoxins. This has been largely driven by the fact that phenolic compounds are present in essentially all plant material and their association with natural resistance to fungal invasion. In this study the *Fusarium* isolates were found to be generally more susceptible in terms of both growth and mycotoxin production than the *Aspergillus* isolates. Rather high levels of the phenolic compounds combined with low a_w values were required to completely inhibit the growth of *F. verticillioides* and *F. proliferatum*, whereas *A. flavus* and *A. parasiticus* growth was not inhibited within the experimental conditions investigated. Unlike fumonisin B_1 production which was completely inhibited under some conditions, aflatoxin B_1 production occurred at all conditions evaluated. Although it was observed that when mycotoxin production occurred, the quantities of either fumonisin B_1 and aflatoxin B_1 produced exceeded the maximum levels permitted in corn for human consumption in most cases; the large reductions observed would play a very significant role in reduction of risk posed by mycotoxins in corn. Although not reported, it was also noted that the corn became very bitter at the levels investigated which would render the treated corn sensorially unacceptable.

These observations have important ramifications on the potential application of phenolic compounds as antifungal agents to protect stored grain systems. Ideally these agents would have to be able completely inhibit growth and mycotoxin production at levels that do not significantly alter their sensorial or functional properties. In fairness to previous reports, the evaluation of the *in vivo* effects of a larger range of phenolic compounds has to be done to make a definitive conclusion about their usefulness. In view of these results phenolic compounds appear to have a role that may be limited to mycotoxin inhibition in the post-harvest period, given that the corn can be treated before consumption to make it sensorially acceptable.

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

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General discussion

Until the early 1960's when the etiological agent for the Turkey X disease was identified as a secondary metabolite of *A. flavus* (Klich et al., 2000; Papp et al., 2002; Kuhn and Ghannoum, 2003) - ushering in what is now called 'mycotoxicology' - scientists were confounded about the exact cause of the diseases associated with the consumption of mouldy feed or food. To date, several other secondary fungal metabolites with varying deleterious effects in mammals have been identified and collectively termed as mycotoxins. It would be folly to presume that we have identified all the mycotoxins that may be of significant public health as even very important mycotoxins such as the fumonisins were only isolated and identified in 1988 (Gelderblom et al., 1988).

Several studies have been done to date to characterize the eco-physiological determinants of fungal growth and mycotoxin production on both artificial growth substrates and real foods. In addition, an equally large number of reports have been published on potential techniques to inhibit fungal growth and mycotoxin production. These have provided invaluable insight on the conditions capable of supporting or inhibiting fungal growth and/or mycotoxin production. More recently, interest has been increasing in the application of predictive modelling techniques to describe fungal growth and mycotoxin production. This has now given rise to 'predictive mycology', which may play an invaluable role in the development and optimization of methods that are being evalauted for their potential to inhibit fungal growth and mycotoxin production. As predictive mycology continues to grow, it will become even more pertinent that models more specific for fungal growth be developed, as currently most models in use today and used in this study are borrowed from *predictive microbiology*.

Fungal spoilage and mycotoxin contamination of cereal grains continue to be a very important cause of pre- and post-harvest grain yield loss. The cosmopolitan nature of most of the mycotoxigenic fungi, results in as much as 25-50% of all cereal grain

produced in the world being contaminated by mycotoxins (Dohlman, 2004). The current population explosion (especially in poor developing countries), limitations in increasing productivity of cereal crops and excessive pre- and post-harvest losses ensure that demand frequently outstrips production. Worryingly demand is actually expected to double by 2020. Although some or probably most of the formely unknown mycotoxins are now known and well characterized, fungi and their mycotoxins in cereal grains still result in billion dollar losses annually in the United States alone (CAST, 1989). Compounding this problem are the large differences in economic and infrastructural resources between developed and developing countries. These differences mean that those people subsistent on cereals such as corn as a staple food (who are primarily located in developing countries) tend to have the smallest capacities to reduce the risk posed by mycotoxins in these grains. The situation usually worsens during droughts and famines (which occur frequently in these regions) were people are forced to consume visually infested grain they normally would not consider eating during years in which they have good harvests.

The findings that some pests and fungi have developed resistance to traditionally effective insecticides/fungicides (Placinta et al., 1999) and even to transgenic *Bt* corn (Linacre and Thompson, 2004), theoretically puts us back many years in the struggle to address these problems, as these methods have been very successful when properly applied. Decisive steps and decisions have to be made to combat what some agriculturalists have claimed is the greatest threat to agriculture (Cardwell et al., 2001). The provision of sustainable preservation techniques that address the challenges imposed by the enormous difference in economic and infrastructural capacities would ensure the minimization of grain yield losses and risk posed by mycotoxins in cereals. These are the challenges we are faced with and this study attempts to address a small part of what can be done to provide reasonable post-harvest solutions.

Conclusions and recommendations for practical applications

Characterization of the water relations of the growth substrate

Sorption isotherms for cereal grains have traditionally been developed to determine the humidity boundaries to inhibit fungal growth and mycotoxin production during storage. Drying which results in reduction of a_w, is almost universally used to achieve these boundaries in grains, with moisture content being the most widely used control parameter for drying processes. Therefore an understanding of the relationship between a_w and moisture content is an absolute necessity to achieve optimal storage stability in grains and seeds, for calculation of moisture changes which may occur during storage and for selecting appropriate packaging materials.

The desorption sorption isotherm models developed can be applied to determine the moisture content (drying points) associated with the desired stability of the corn at their final storage temperatures. For example if a_w 0.6 is targeted to ensure the microbiological stability of the corn, then using the GAB desorption model it can be estimated that this will correspond to moisture contents of 14.5, 13.4 and 12.3 % (db) if the corn is to be stored at 25, 30 and 37°C, respectively. This means that more energy (and therefore higher costs) will be required to ensure that corn stored at a higher temperature has the same stability as corn stored at lower temperatures. To illustrate the impact of these otherwise small moisture content differences, it can be seen in Fig. 2.9 that the isosteric heats start to increase exponentially with decrease in moisture content in this zone, meaning that in this zone very large quantities of energy are needed to achieve even small reductions in the moisture content. The desorption isotherms developed in this study (through the net isosteric heats of sorption) can then be applied to provide very important details about the actual amount of energy (and therefore the costs) required to achieve a particular level of grain stability in industrialized storage facilities.

By utilizing these two applications together in this way, decisions can be logically made about the drying point/s at which both economic viability and microbiological stability can be ensured. These net isosteric heats have also proven to be invaluable input for the modelling of drying processes and for the design and optimisation of

drying equipment. Reflecting on the situation in the rural areas of developing countries where the drying is done under the sun, it is imperative that even lower moisture contents (a_w values) be attained to provide the same degree of stability as these areas normally have very high average temperatures and the storage facilities are very basic and do not provide meaningful protection to the grain from phenomena such as condensation.

Throughout this study, the relevance of the full characterization of the water relations of a targeted food product (growth substrate) is clearly demonstrated as this provided a reliable basis for the reproducible adjustment of the a_w. In this way, a major bottleneck to the collection of a sufficient and reproducible amount of data to model the growth responses obtained was overcome. In addition, this enabled for one of the objectives of this study and a major paucity in literature – the influence of a_w of the growth substrate on the efficacy of preservation techniques - to be addressed in a repeatable manner. Of the six sorption models fitted to the experimental adsorption data, the GAB model proved to be the best fitting function and was therefore selected and successfully used in the other parts of the study to determine the amount of water that had to be used to reproducibly adjust the a_w of the corn to a desired value. For reliable and reproducible results for use in predictive mycology and other studies, the growth substrate must be fully/adequately characterized to minimize the potential sources of variation in the observed response.

Modelling of the effects of the most important environmental factors on growth of the most important fungal contaminators of corn

This part of the study contributes to predictive mycology by identifying suitable functions (which were validated on independently collected data) to describe the effect of a_w and temperature on the radial growth of the most important fungi on corn. The function that adequately described the growth of both *Aspergillus* and *Fusarium* isolates was succesfully extended in later studies (chapters 7 and 8) that evaluated the combined effects of a_w and non-chemical and chemical preservation techniques on growth. The study also determined the extent of variation in growth (which is not fully revealed when high inoculum sizes are used) and established the relationship

between the radial growth of the *Fusarium* isolates and fumonisin production on corn under various environmental conditions.

With regards to the predictive modelling, two primary models were used in the study, the Baranyi and Roberts (1994) model and simple linear regression. Both alternatives were adequate estimators of the growth parameters. Which primary model is used is largely detemined by the nature of data collected; obviously when the data consists only of points in the linear phase of growth then linear regression should be used. Linear regression is also preferble when estimations of the colony diameter at small diameters are unreliable or are characterized by large variations. The Baranyi and Roberts (1994) model, although more complex, has the distinct advantage that it can model both limited (i.e. as seen in chapter 7 for the influence of IH CO_2) and unlimited growth. Inevitably the simplicity of linear regression (which is easily done in non-specialist software such as Excel) and the fact that the Baranyi and Roberts (1994) does not improve on the estimates of the colony growth rates and lag phases obtained, makes this approach more appealing to most people.

With regards to secondary modelling, the selection of the suitable functions was done in two steps. In the first step several models potentially capable of describing the particular trends observed were fitted to the estimated colony growth rates or lag phase durations (only for the *Aspergillus* isolates). These models included (i) Arrhenius, (ii) cardinal, (ii) Bělehrădrek and (iv) polynomial type models collected from various literature sources. From these, the particular model structure selected for evaluation was largely guided by the trends observed in the colony growth rates or lag phase durations as a function of a_w and temperature or preservative technique. For instance the fact that no cardinal a_w or temperature values could be observed for the estimated colony growth rates of *F. verticillioides* and *F. proliferatum* meant that those model structures incooperating cardinal values (which have been successfully used to model the growth of other fungi) were not applicable for this isolate. Two models were selected from the models evaluated in the first step based on the MSE and residual plots. In the second step these models were further evaluated, validated against independently collected data and reported in the study.

As demonstrated in chapter 3 and 4 it is important to utilize several validation criteria to ensure that the models developed adequately describe the trends observed. These criteria should include mathematical/statistical validation indices such as the MSE, *F*-test, accuracy and bias factors and also graphical analysis in the form of two dimensional plots of the observed *vs*. the predicted growth responses and three dimensional plots of the model showing the distribution of the validation data points about its surface. It is clear that the model selection criteria for fungi still largely resemble those of bacteria as these provide perfectly adequate guidelines. Due to the limited availability of secondary models specific for fungal growth, models typically used to describe bacterial growth can also assessed for their ability to describe fungal growth, as was the case in this study. Other criteria can also be applied to identify appropriate models structure based on (i) aim of the model, (ii) *a priori* knowledge and (iii) the results of historical modelling attempts (Geeraerd et al., 1999).

The second order polynomial function, despite *overfitting* in some cases under growth limiting conditions, was an adequate predictor of both *Aspergillus* and *Fusarium* growth on corn and was therefore selected as the basic model structure for extension in the later studies evaluating various non-chemical and chemical preservation techniques. The models developed to describe the effects of a_w and temperature can be applied with due caution to simulate some real-life situations. Most of this caution is based on the fact that, in for example a real silo or food store, several other factors not accounted for in the model will be omnipresent and will most likely affect the growth of the target organisms, resulting in the model predictions becoming unreliable. These factors include amongst many others the presence of competing mycoflora, insects and fungicides or pesticides.

It contrast it can be argued that as these factors, with the exception of insects, cause the reduced viability of the target fungi, the model predictions would be *fail-safe* as they would predict faster colony growth rates than those observed. Potential applications include the prediction of the amount of spoilage or fungal growth that can be expected to occur under different storage conditions (i.e. with dynamic or static conditions) or the prediction of the a_w and temperature boundaries for growth of these isolates on corn. Furthermore they can also be used a basic model structure for

extension to include the effects of other important storage factors not investigated in this study.

An example of how these models can be applied is given below, where they are applied to predict the expected extent of fungal outgrowth under two different hypothetical post-harvest systems reflecting the conditions in a industrialized farming operation and those in the rural area of a tropical country such as Zimbabwe.

Scenario 1: This case reflects the industrialized farming operation in which corn is harvested and placed in dry silos in which dry air of relative humidity < 10% is circulated. In one silo corn enters at a_w 0.96 whereas in another silo the corn enters at a_w 0.92. The circulation of dry air is done to encourage the fast drying of the corn and it is supposed that the moisture content of the corn decreases as it equilibrates with the air, resulting in the a_w value gradually and uniformly falling 0.96 to a value of 0.80 over a 20 day period. During this time the temperature fluctuates with average night and day temperature of 16 and 28°C, respectively. As the a_w of the corn changes uniformly, we can assign particular a_w values per day i.e. for corn at entering the silo at a_w 0.96 day, it will be 0.952 on day 2; 0.944 on day 3 etc. until it reaches a_w 0.80 on day 20.

Scenario 2: This case reflects an alternate situation in which the corn is harvested towards the end of the wet season (in a tropical humid country) and placed in silos which have no means to allow the circulation of air. This creates a situation where the a_w of the corn hardly changes over the 20 day period. The corn also enters the silos at a_w values of either 0.96 or 0.92.

As an illustration simulations of the outcomes of these preconditions were developed for *A. flavus* and *F. verticllioides* as described below. The simulations consist of two inputs: the duration of the initial lag period (d) and the colony growth thereafter.

For estimation of the duration of the initial lag period of *A. flavus*, Eq. 4.7 was used. As no models were developed for the lag duration of the *Fusarium* isolates, these were estimated from their growth curves at 30°C.

For the estimation of the colony growth some assumptions were made. The first being that once growth has started to occur the isolates will respond immediately to changes in either or both a_w and temperature. Alternatively more complex simulations can also

be made in which the lag phase induced by the changing of the conditions is incooperated in the simulation as these changes normally solicitate an adaptation period. This approach however is less *fail-safe* as it predicts a slower rate of growth than that based on the first assumption. The second assumption that also had to be made is that growth of the fungi occurs at an even rate throughout the three dimensional corn matrix in the silo. After a lag phase had been established and with these assumptions in mind, the models developed to predict the combined effect of a_w and temperature (Eq. 3.7 for *F. verticillioides* and Eq. 4.5 for *A. flavus*) were used to estimate the colony growth rates (mm d^{-1}) at the selected combinations of a_w and temperature. The estimated colony growth rates were then used to estimate the expected change in colony diameters during the cyclic 12 hour periods and therefore the colony diameter or volume/mass of the mycelial infected corn at any time during the storage period (or until the moment the a_{wmin} for growth of a particular isolate is passed). The simulations developed in this manner for the two scenario's is given below in Fig. 10.1 and 10.2 for *A. flavus* and *F. verticillioides*, respectively.

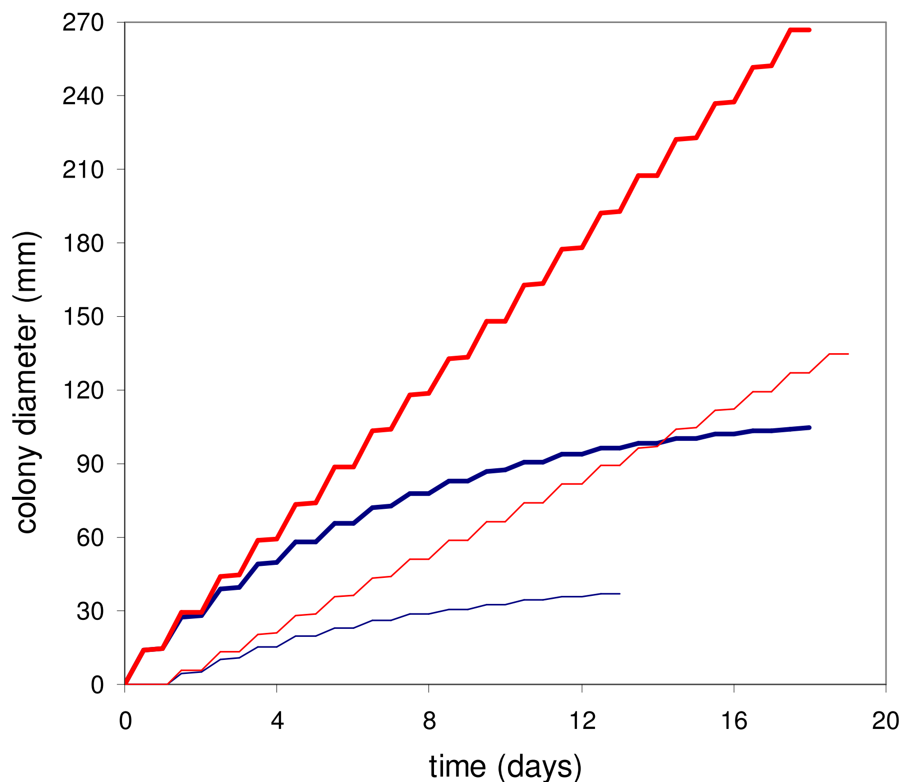

Fig.10.1. Simulation of expected outgrowth of *A. flavus* on corn under scenario 1 at initial corn a_w values of 0.96 (▬) and 0.92 (–) and scenario 2 at a_w values of 0.96 (▬) and 0.92 (–).

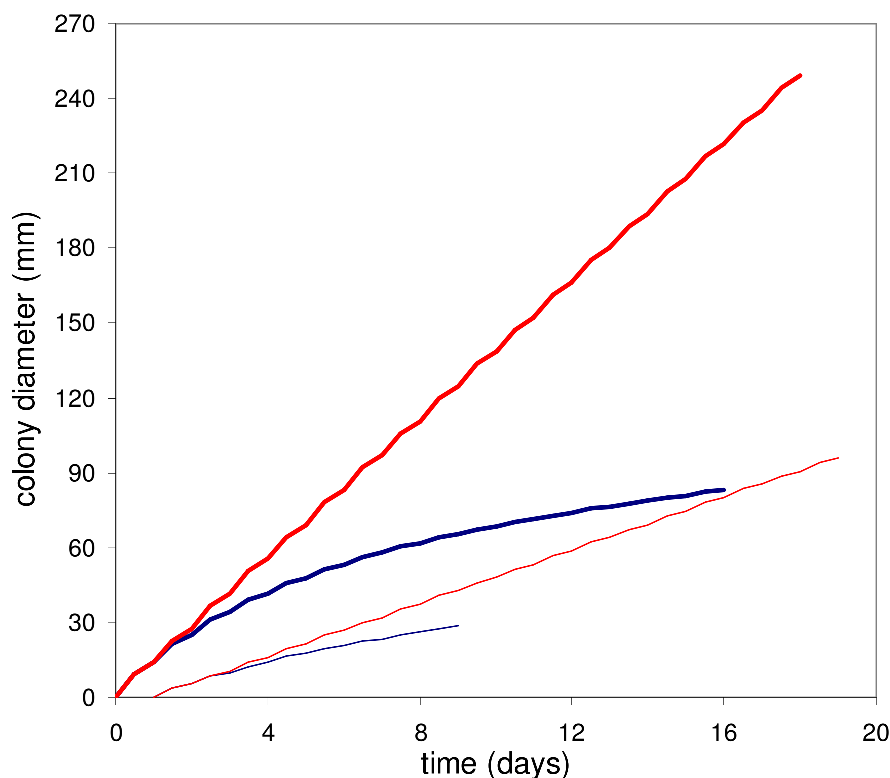

Fig.10.2. Simulation of expected outgrowth of *F. verticillioides* on corn under scenario 1 at initial corn a_w values of 0.96 (▬) and 0.92 (–) and scenario 2 at a_w values of 0.96 (▬) and 0.92 (–).

It can be seen from Fig. 10.1 and 10.2 that the models predict that growth will occur in steps governed by the prevailing temperature in both scenarios and additionally by the gradually reducing a_w of the corn in the first scenario were dry air is circulated in the silo's. The larger steps as seen for *A. flavus* are a result of the observation that larger differences occur between the colony growth rates at 28°C and 16°C in comparison to those for *F. verticillioides*. These differences also importantly mean that although *A. flavus* grows much faster than *F. verticillioides* at high temperatures, the overall growth of the two moulds at a given a_w are almost identical (when cyclic temperatures such as those simulated are encountered) as *F. verticillioides* grows at a faster rate than *A. flavus* at low temperatures.

The model predictions also show for the first scenario, that although growth occurs during the storage period, the overall growth rate continuously decreases as a result of the gradual lowering in the a_w of the corn to 0.80. This is not the case in scenario 2

where the lack of air circulation and the humid surroundings during the wet season ensure that the corn maintains the a_w it had when it entered the silo for a longer time. This therefore results in temperature being the major factor controlling the changes that are observed in the colony growth rate.

Very large differences occur in final diameters between the two conditions simulated in each scenario (i.e. between the two different a_w values for the corn entering the silos). The plots for scenario 1 are constructed up to the time at which the a_{wmin} is reached to visually show how long it takes for the growth to stop. Thereafter the diameters remain constant (not shown). In this way the simulations show that *F. verticillioides* will stop growing earlier than the *A. flavus* isolates as it has a higher a_{wmin} of 0.869 compared to 0.822 for *A. flavus*. Large differences can also be seen between the two scenario's described at a given a_w value. For instance, in Fig. 10.1 it can be seen that at a_w 0.96 at the time growth of *A. flavus* had stopped in the silo in which corn with circulating air (scenario 1), on day 18, the colony in the silo without air circulation had a diameter almost three times larger and moreover growing continues. The same is predicted for *F. verticillioides* at this a_w. At the lower initial a_w value of 0.92 smaller differences are observed in the colony diameters for the two scenario's at the time when the growth in scenario 1 stops, but as time increases these differences obviously become more and more larger.

The models can therefore, by simulating the outcomes of given temporal based changes in the a_w and/or temperature conditions, be used to make an assessment of the importance/consequence of the initial a_w of the grain entering a silo or food store and the consequences of the prevailing conditions on the amount of grain that can be expected to be damaged. Safety assessments may also be incorporated in these assessments based on relationships between mycotoxin production and fungal growth (which can also be assessed experimentally) under specific dynamic/static environmental conditions. The use of the relationships between growth and fumonisin established in chapter 5 to this end was considered harzadous given the fact that cyclic temperatures have been found to significantly influence the production of several mycotoxins, including fumonisin B_1 (Ryu et al., 1999). In a study on the effects of cycling temperatures on fumonisin B_1 production, Ryu et al. (1999) reported that the growth of *F. proliferatum* under 12 hour cycling of temperatures 5 and 20°C or 15

and 30°C significantly increased fumonisin B_1 production in comparison to continuous growth at 25°C after 4 and 6 weeks of incubation. As the relations developed in chapter 5 were established at constant temperatures their use would be viewed to likely produce *fail-dangerous* safety assessments given that cycling temperatures appear to have stimulatory effects on mycotoxin production. The same simulations can also be used for predicting the extent of fungal outgrowth under MA or in the presence of bicarbonate salts, using the models developed in chapter 7 and 8, respectively. These predictions are however are limited to cases at 25°C only. As recommended in the earlier chapters (3, 4, 7 and 8), the effects of competing mycoflora, insects, commonly used fungicides and pesticides etc. should in future studies be incorporated into predictive models to enable them to more closely approximate reality. In addition, systems for the assessment of the prevailing and often dynamic conditions in a food store i.e time-temperature indicators or a_w/relative humidity sensors will have to become part and parcel of grain storage management systems in order to have accurate/reliable data to apply such kinds of models on.

Growth kinetics of individual spores of fungi of importance to corn

So far very little is actually known about the extent of variability in the growth of fungal spores (with the exception of germination kinetics on artificial media) as most studies consider large fungal inoculum levels. This is reflected in the results obtained in chapters 3 and 4 where very little variation occurred in the colony growth rates (mm d^{-1}) and lag phase durations (d) of colonies emanating from large fungal inoculums. It may, as highlighted in chapter 5, be that infection and subsequent mycotoxin production may be very important at the single spore or at low inoculum levels. To determine the extent of variation in growth at the single spore level, a protocol to isolate individual spores was validated and applied. The study revealed rather large variation to occur in both the colony growth rates (mm d^{-1}) and lag phase durations (d) of individual spores of *A. flavus* and *F. verticillioides* and also showed that a_w and temperature have a very strong influence on the variation observed. This may indeed suggest a rethink of the status quo and encourage further studies to be done at several fungal levels which can then be taken into consideration in the modelling process.

Relationship between growth and fumonisin production by the most important Fusarium species to corn

To complete this part of the study the relationship between the well characterized radial growth of *Fusarium* species and fumonisin B_1 production on corn was determined by evaluating both growth and fumonisin production during time (after shorter time/growth intervals). It can be concluded from this study that although a relationship does exist between radial growth and fumonisin production, this relationship is largely determined by the prevailing combination of a_w and temperature. Unlike a_w, temperature has an ambiguous effect on the biosynthesis of fumonisins, with optimum temperatures for growth not coinciding with those for fumonisin production. It was also concluded from these results that temperature stress plays a very important role in stimulating fumonisin production. Evidence of moisture stress stimulating fumonisin biosynthesis by *F. proliferatum* at temperatures sub-optimal for growth was also revealed. On the field, moisture stress has been linked to higher levels of mycotoxin production on cereal crops. The models developed in chapter 3 and the relationship established in chapter 6 between radial growth and fumonisin production, enable for the prediction of growth, given a combination of static environmental factors (a_w and temperature) and time (duration of growth at those particular environmental factors) and furthermore the consequences of this growth in terms of fumonisin production and therefore safety of the corn.

Evaluation of non-chemical and chemical techniques to inhibit fungal growth and mycotoxin production on corn

Considering the staggering losses of up to 50% in grain yields (mostly in developing countries) and the contamination of up to 25% of grain by mycotoxins worldwide, it is not far-fetched to assume that should direct and indirect yield losses be minimized, current world production levels could satisfy demand. The installation of good agricultural practices would undoubtedly go a long way in alleviating yield loss pre- and post-harvest. However, the development of viable and sustainable preservation methods applicable to resource laden developed countries and resource limited developing countries would provide an extra level of food security and safety. The last phase of the study evaluated the potential of various methods to inhibit both

fungal growth and mycotoxin production during the post-harvest period. Special attention was given to the possible influence of a_w (the single most important determinant of fungal growth) on the efficacy of the methods evaluated. This being in light of the fact that corn enters a food store at various a_w levels which may affect the ability of the technique or determine the amount of preservative required to prevent fungal growth and mycotoxin production. In view of negative consumer perceptions towards chemicals in the food chain at least one non-chemical technique was to be evaluated. However, in reality, chemical methods, especially safe, cheap and easy to apply antifungal substances that do not require specialized technology and training or large initial capital investments, appear to be the best solutions for most people in resource limited developing countries. In this part of the study two techniques were chosen, with one based on the use of inorganic chemical compounds (bicarbonate salts) and the other based on the use of organic natural compounds originating from plants (phenolic compounds).

Modified atmospheres (MA) were evaluated as a non-chemical approach, of which the effects of the headspace CO_2 and O_2 levels on both growth and fumonisin B_1 production were investigated. The evaluation of MA was not only based on the fact that fungi are obligate aerobes and therefore have an absolute requirement for O_2, but also that MA has been found to be effective against pests such as insects and rodents which besides causing direct yield loss also act as vectors of fungi. It was demonstrated that the mere presence of O_2 in the sealed bag was sufficient to enable growth, which subsequently stopped when the O_2 was depleted. CO_2 significantly affected the colony growth rates and lag phase durations of both *F. verticillioides* and *F. proliferatum*. The polynomial function was successfully used to model the combined influence of a_w and initial headspace CO_2 on the colony growth rate of both isolates. As described earlier, these models can be used to assess the expected fungal outgrowth under MA, albeit at 25°C. More importantly, were the very large reductions or the complete inhibition of fumonisin B_1 production that were observed when CO_2 was applied which strongly favour the use of MA (where financially and technologically possible) for the protection of cereal grains in bulk store from poisoning by mycotoxins. As the same trends have also been observed by a number of workers on growth and aflatoxin production by *Aspergillus* species on corn and groundnuts (Wilson et al., 1975; Magan and Lacey, 1984, 1988; Ellis et al., 1993,

1994), it can be concluded that MA can provide adequate limitations to both fungal growth and mycotoxin production during grain store. The models developed find similar practical application as stated earlier in the discussion of the modelling of the effects of a_w and temperature on the growth of the fungal isolates investigated in this study. The predictions or simulations that can be made are however limited to a temperature of 25°C.

The potential of bicarbonate salts had been previously reported (Montville and Goldstein, 1987, 1989; El-Nabarawy et al., 1989; Montville and Shih, 1991) but the influence of a_w on the inhibitory effect and the sensorial suitability of corn treated as such had not yet been evaluated. Ammonium bicarbonate was determined to be the most effective bicarbonate salt with regards to the inhibition of both growth and fumonisin B_1 or aflatoxin B_1 production at levels at which the product is still sensorially acceptable. Sodium bicarbonate is only effective on the growth of *Fusarium* species at levels at which the product is no longer sensorially acceptable, and only slightly inhibited the growth of *Aspergillus* within the experimental limits investigated. The polynomial function was also determined to adequately describe the combined effects of a_w and bicarbonate salt concentration on the growth of all isolates investigated. The models developed in this chapter also find similar practical application as stated earlier in the discussion of the modelling of the effects of a_w and temperature on the growth of the fungal isolates investigated in this study. The predictions or simulations that can be made are however (as mentioned for those developed for the influence of MA) limited to a temperature of 25°C. The low costs and simplicity involved in the use of ammonium bicarbonate make it potentially feasible antifungal chemical for use in resource limited developing countries. The growth stimulation observed at low concentrations however highlights the importance of application of such chemicals at the optimal levels.

The natural phenolic compounds investigated were the least successful of the techniques to inhibit growth and mycotoxin production investigated. Although both vanillic and caffeic acid, had an appreciably significant impact on the colony growth rates of the *F. verticillioides* and *F. proliferatum*, they did not affect those of *A. flavus* and *A. parasiticus*. It was also observed that both phenolic compounds produced very large reductions in both fumonisin B_1 and aflatoxin B_1 production, which may be very

important in bulk stored grains where fungal growth and mycotoxin poisoning occurs in small pockets. Drawbacks to their use as antifungal agents for the protection of cereal grains in store include their lack of effect on growth of the *Aspergillus* isolates, the rather high concentrations that are required to achieve significant reductions in mycotoxin production and their current high costs. A method to reduce their levels to sensorially acceptable levels would also need to be developed considering the severe bitterness they impart to treated corn.

Recommendations for further research

i) The development of predictive mycology to the levels that predictive microbiology has reached requires for the development of rapid methods to reliably estimate fungal growth. The current methods of measuring the colony diameters or HPLC analysis of ergosterol are too laborious or time consuming. As an example, methods based on automated image analysis have been used for the determining the morphology filamentous fungi and germination kinetics (Thomas and Paul, 1996; Cox et al., 1998) could be extended to also estimate the growth (hyphal extension) of incubated cultures at regular periods. Such techniques would significantly reduce the work that has to be done to obtain a sufficient quantity of growth data, reduce measurement errors and enable for far more work to be done in the same time.

ii) Current models need to be extended to account for the consequences of fungal competition, the presence of insects, fungicides and pesticides amongst many others. Once such models can be provided then their predictions can be treated with more confidence than those of current models which only account for the effect of what are otherwise considered to be the most important determinants of fungal growth – a_w and temperature.

iii) The importance of inoculum levels on the ability of fungi to grow and contaminate cereal grains with mycotoxins should be thoroughly

investigated. Where possible models incorporating the demonstrated large variation in growth at the individual spore level should be developed to account for the variation that is not revealed when high inoculum levels are used in model development. Alternatively models may also be developed at different inoculum levels.

iv) The efficacy of the techniques investigated in this study and those with potential for application post-harvest should in future studies be assessed in the presence of several interacting fungi capable of producing a wide range of mycotoxins as found on corn. In this manner a better idea would be obtained of the consequences of these techniques on fungal interactions during incubation and the consequences of the observed interactions on mycotoxin production.

v) Although it is easier to work with artificial media, it is recommended that an attempt should also be made to validate the observed results on the target food substrate itself as done in this study. Recently a lot of chemicals have been proposed for application post-harvest to prevent both fungal growth and mycotoxin production after positive evaluation of their effects on artificial media. These effects may not be translated to the same extent on the actual food product owing to interactions that may occur in the more complex food matrix compared to those in artificial media.

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SUMMARY

SUMMARY

In chapter 1 several literature sources are extensively consulted to describe the importance of corn to our societies, routes for yield loss of corn and the most important eco-physiological determinants and consequences of fungal growth on corn. A description is made of the physico-chemical and toxicological properties and mechanisms of toxicity of the fumonisins and aflatoxins, which are considered to be the most important mycotoxins on corn. The pre- and post-harvest strategies evaluated to date to minimize the production of mycotoxins on corn are then discussed in addition to the physical, chemical and biological methods to detoxify mycotoxin contaminated corn. This is followed by a discussion of the analytical techniques available for the detection and quantification of fumonisins and aflatoxins. The legislation currently applied worldwide to limit the amount of fumonisins and aflatoxins in corn and corn products for use as food and feed is then briefly discussed. To complete the literature study, what is now known as 'Predictive Mycology' is reviewed to give an overview of the process of modelling fungal growth and the difficulties that have restricted the modelling of fungal growth. The models that have been successfully used to describe fungal growth are then discussed.

In chapter 2, moisture sorption isotherms fully characterizing the water relations of growth substrate in the study (yellow dent corn) were developed. In addition to characterizing the water relations of the growth substrate, the study was done to determine the sorption model which best described the experimental sorption data. This model was to be used as a tool for reproducibly adjusting the a_w of the growth substrate in the subsequent studies that determined the effects of a_w and other factors such as temperature or preservative agents on the growth and mycotoxin production of the most important fungal contaminators of corn. In this framework six sorption models – the GAB, Oswin, Halsey, Henderson, Chung-Pfost and polynomial models - were evaluated for their ability to describe the experimental isotherms. In general the experimental isotherms exhibited Type II behaviour and were temperature dependent. Hysteresis was also observed at all temperatures investigated. According to the goodness of fit, the GAB

model best described the experimental data followed by the Oswin, Chung-Pfost, polynomial and the Henderson models. The GAB model was therefore selected as the tool to determine the amount of water that had to be added to dried grain to reproducibly achieve a desired a_w value in later studies.

In chapters 3 and 4, the individual and combined effects of a_w and temperature on the growth of *F. verticillioides* and *F. proliferatum* and *A. flavus* and *A. parasiticus*, respectively, were modelled. The best fitting model/s from these studies would be used as the basis for the planned modelling (where possible) of the combined effects of a_w and non-chemical and chemical preservation techniques on the growth of these fungi in subsequent studies. The GAB sorption models developed in chapter 2 were used to ensure the corn had the desired a_w activity. Primary modelling of the growth data obtained was done using the flexible function of Baranyi and Roberts (1994) to estimate the colony growth rates (mm d^{-1}) and lag phase durations (d). For *F. verticillioides* and *F. proliferatum* a second order polynomial function and the linear Arrhenius-Davey model were used to model the combined effect of temperature and a_w on the colony growth rates, whereas Gaussian and second order polynomial functions were used for *A. flavus* and *A. parasiticus*. The different models used (with the exception of the polynomial functions) were necessitated by the observation that the growth data for the *Aspergillus* isolates had inflexion points (temperature maxima) whereas that of the *Fusarium* did not. Due to the greater ease in visually assessing the growth of the *Aspergillus* isolates during the initial phases of growth compared to the *Fusarium* isolates, reliable estimates of the lag phases were obtained for the *Aspergillus* isolates and these were also modelled. A polynomial function and an extended linear Arrhenius-Davey model were used to describe the combined effect of temperature and a_w on the lag phase durations of *A. flavus* and *A. parasiticus*. All the models developed were then validated on independently collected data.

All models proved to be good predictors of the colony growth rates or lag phase durations of both isolates on maize within the experimental limits. Significant interaction was also observed to occur between a_w and temperature on the colony growth rates of both isolates

and lag phase durations of the *Aspergillus* isolates. The linear Arrenhius-Davey model was found to be the best model for describing the colony growth rates of the *Fusarium* isolates, whereas the second order polynomial function proved to be the most suitable predictor of the combined effects of a_w and temperature on both the colony growth rates and the lag phase durations of the *Aspergillus* isolates. Due to the ability of the polynomial functions to adequately describe the growth of both the *Fusarium* and *Aspergillus* isolates, these functions were selected as the basis for the planned modelling of the combined influence of a_w and non-chemical and chemical preservation techniques on the growth of these fungi.

As observed in chapters 3 and 4 and reported in several other studies, growth - expressed as the radial colony growth rates – of colonies emanating from large fungal inoculum sizes is usually characterized by very little variation. This may certainly not be the case when growth arises from smaller inoculum sizes, an event which may actually be very important in the process of natural infection of corn on the field or during storage. In chapter 5 an attempt was made to determine the extent of variation in the colony growth rates and lag phase durations of individual spores of *A. flavus* and *F. verticillioides* on corn meal and the influence of a_w and temperature on the observed variation. To accomplish this, a protocol originally developed for the isolation and growth of single bacterial cells, was modified to suite the specificities of fungal growth. The modified protocol was validated and determined to be a reliable and high yielding method. Both a_w and temperature were found to significantly influence the distributions of the colony growth rates, lag phases and the rates (d^{-1}) at which individual spores of both isolates completed the lag period. In general, the histograms of the single spore colony growth rates and lag phases generally became wider the more compromising the conditions for growth became, indicating a greater variation in growth ability under these conditions. The rate at which the single spores completed the lag phases generally decreased with decrease in temperature and/or a_w. The results show the extent of variation in the growth capacities of individual spores of both isolates on yellow dent corn meal and also demonstrate that even at the lowest inoculum level possible (the individual spore) vigorous growth still occurs.

The relationship between the radial growth of *F. verticillioides* and *F. proliferatum* and fumonisin B_1 production on corn during time and the influence of a_w and temperature on this relationship was determined in chapter 6. In general both fumonisin production and radial growth for both isolates increased with a_w at any temperature investigated. The effect of temperature on the relationship between radial growth and fumonisin production was however not straightforward. It was apparent that the effect of temperature on fumonisin production at high a_w values (optimal for growth) was only marginal, whereas at lower a_w values the effect of temperature became more pronounced with more fumonisin production occurring at temperatures less optimal for growth. The optimum temperature for fumonisin production of both isolates varied between 15 and 25ºC. Notably for *F. proliferatum* it was observed that the optimum temperature for growth at any a_w, 30ºC, was the least optimum for fumonisin production. The only other consistent observation for the effect of temperature was that for both isolates the slowest initial rate of fumonisin production was at 15ºC, where the slowest growth rates were obtained. The models developed in chapter 3 and the relationship established in chapter 6 between radial growth and fumonisin production, enable for the prediction of growth, given a combination of environmental factors (a_w and temperature) and time (duration of growth at particular combinations of these environmental factors), and the consequences of this growth in terms of fumonisin production and therefore safety of the corn.

After modelling of the growth and determination of the relationship between growth and fumonisin production, non-chemical and chemical techniques to inhibit the growth and mycotoxin production by the most important fungal contaminators on corn were investigated. In chapter 7 the influence of initial headspace carbon dioxide (IH CO_2) and oxygen (IH O_2) levels and a_w on the radial growth and fumonisin B_1 production of *F. verticillioides* and *F. proliferatum* was examined. These studies were done to evaluate the potential of modified atmospheres as a non-chemical technique to protect corn during store from fungal contaminators and subsequent mycotoxin poisoning.

Polynomial functions, applied in chapters 3 and 4 to describe the combined influence of a_w and temperature on the colony growth rates of the *Fusarium* and *Aspergillus*, were

modified to describe the combined effect of a_w and IH CO_2 on the colony growth rates of *F. verticillioides* and *F proliferatum*. The models developed adequately described the combined effects of a_w and IH CO_2 on the estimated colony growth rates of both isolates. At all a_w values studied, increase in the IH CO_2 concentration generally resulted in a decrease in the colony growth rate and maximum colony diameter (mm) and an increase the lag phase duration. With regard to fumonisin B_1 production, as little as 10% IH CO_2 completely inhibited fumonisin B_1 production by *F. verticillioides*. *F. proliferatum* was more resistant and required 40, 30 and 10% CO_2 at a_w values of 0.984, 0.951 and 0.930, respectively, to completely inhibit fumonisin B_1 production.

At all a_w values studied, reduction of IH O_2 concentration from 20 to 2% had no significant effect on the colony growth rates and lag phase durations of both isolates. Although the maximum colony diameter decreased with reduction of the IH O_2 level, the greatest mycelial density occurred at 10% IH O_2 for both isolates. This observation was accompanied by a trend of a decrease in the value of the IH O_2 level at which the most fumonisin B_1 was produced from 15 to 5% when a_w was decreased from 0.976 to 0.930 for *F. verticillioides*. For *F. proliferatum* the optimum conditions for fumonisin B_1 production shifted from 20% at a_w 0.976 to 10% at a_w values of 0.951 and 0.930. The results demonstrate the great potential for modified atmospheres to protect cereal grains in store from fungal spoilage and potential poisoning by their mycotoxins during the post-harvest period and the influence of a_w of the corn on the efficacy of this method.

Chapters 8 and 9 evaluated the use of inorganic and organic natural chemical antifungal agents, respectively, to prevent fungal growth and mycotoxin production on corn during the storage period. In chapter 8 the combined effect of a_w and ammonium/sodium bicarbonate on growth and mycotoxin production on corn by *Fusarium* and *Aspergillus* species was investigated. In addition, the sensorial suitability of corn treated with the bicarbonate salts was also determined for the first time as the treated corn should ideally remain sensorially acceptable. Bicarbonate salts were chosen as the inorganic natural chemicals for investigation on the basis that they are cheap, easy to apply and can

currently be used in food products at 'quantum satis' levels in North American and Europe. They have also been previously shown to have strong antifungal effects.

The polynomial models were again modified and applied to describe the combined effects of a_w and bicarbonate salt concentration on the colony growth rates of the *Aspergillus* and *Fusarium* isolates investigated. The models developed were able to adequately describe the effect of the factors mentioned above on the colony growth rates of all the isolates. Within the experimental limits, both bicarbonate salts and a_w exerted a synergistic effect on the colony growth rates and lag phase durations. Low levels of both salts were observed for the first time to enhance the growth of *F. verticillioides* and *F. proliferatum*, with the fastest growth taking place at levels of 0.1-0.2 and 0.5% ammonium and sodium bicarbonate (w/w), respectively. However, no growth stimulation was observed for the growth of *A. flavus* and *A. parasiticus*. Growth of the *Fusarium* isolates was completely inhibited by 0.8% and 3.5% ammonium and sodium bicarbonate, respectively. At least 1% ammonium bicarbonate completely inhibited the growth of the *Aspergillus* isolates, whereas up to 4% sodium bicarbonate failed to completely inhibit their growth at all a_w values evaluated. Increase in concentration of either salt was found to result in a decrease of both fumonisin B_1 and aflatoxin B_1 production. At least 0.6% ammonium bicarbonate and 2% sodium bicarbonate completely inhibited fumonisin B_1 through 20 days of incubation, whereas 0.8% ammonium bicarbonate achieved the same for aflatoxin B_1 production. However, 4% sodium bicarbonate failed to completely inhibit aflatoxin B_1 production. According to the sensorial analysis performed, corn treated with up to 1% ammonium bicarbonate was still acceptable for consumption only after sieving before cooking to reduce the residual levels; whereas corn treated with at least 2% sodium bicarbonate was determined to be sensorially unsuitable. From these results ammonium bicarbonate proved to be more suitable for protection of corn from fungal invasion and subsequent mycotoxin production than sodium bicarbonate as it was the only one capable of completely inhibiting both growth and mycotoxin production of the *Fusarium* and *Aspergillus* isolates of greatest importance to corn at levels that were still sensorially acceptable. As ammonium bicarbonate is easy to apply and cheap it could prove to be an appropriate post-harvest solution in resource limited developing countries.

In chapter 9, natural phenolic compounds (vanillic and caffeic acid) were evaluated for their ability to inhibit growth and mycotoxin on corn by *Fusarium* and *Aspergillus* isolates. Recently interest and research into the potential use of natural antimicrobial compounds has been driven by negative perceptions of consumers about the use of synthetic/inorganic chemical preservatives. In addition, the observations that the natural resistance of some cereal crop cultivars to fungal invasion is correlated to their free phenolic acid content has stimulated investigations into the use of phenolic compounds to inhibit both fungal growth and mycotoxin production. In this study large differences were observed in the response of the *Fusarium* and *Aspergillus* isolates to the phenolic compounds, mostly determined by their genus. Generally for both *F. verticillioides* and *F. proliferatum*, an increase in concentration of either caffeic or vanillic acid resulted in a decrease in the colony growth rates and increase in the lag phase durations. Growth of the *Fusarium* isolates was not completely inhibited at the highest a_w value evaluated of 0.967, with complete inhibition only being observed at combinations of high phenolic acid concentrations ($\geq$ 2000µg/g) and low a_w values ($\leq$ 0.948). Within the experimental limits investigated, growth of the *Aspergillus* species was not affected by the phenolic compounds. The phenolic compounds significantly reduced both fumonisin B_1 and aflatoxin B_1 production. These large reductions may play a very significant role considering that fungal spoilage and mycotoxin poisoning tends to occur in small pockets in bulk stored grains. Several issues however still need to be addressed including the severe bitterness phenolic compounds impart to the treated corn (at these high levels) and their expensiveness before their potentially useful role in reducing mycotoxin levels can be exploited to minimize the contamination of grains in store by mycotoxins.

SAMENVATTING

SAMENVATTING

In hoofdstuk 1 worden verschillende bronnen uit de literatuur uitgebreid geraadpleegd om het belang van maïs in onze samenleving te beschrijven, evenals de manier waarop verlies in opbrengst van maïs optreedt en de meest belangrijke eco-physiologische determinanten en de gevolgen van schimmelgroei op maïs. Vervolgens worden de physico-chemische en toxicologische eigenschappen van fumonisines en aflatoxines en de mechanismen van hun toxiciteit beschreven. Deze toxines worden immers beschouwd als de meest belangrijke mycotoxines in maïs. Ook worden de voor- en na-oogst strategieën besproken die tot nu toe gebruikt worden om de mycotoxineproductie in maïs te minimaliseren samen met de fysische, chemische en biologische methoden om besmet maïs te detoxificeren. Dit wordt gevolgd door een bespreking van de mogelijke analytische technieken voor de detectie en kwantitatieve bepaling van fumonisines en aflatoxines. Nadien wordt kort de wetgeving besproken die momenteel wereldwijd toegepast wordt om de aanwezigheid van fumonisines en aflatoxines in maïs en maïsproducten, die gebruikt worden als voedsel of als veevoeder, te beperken. Om de literatuurstudie te vervolledigen, wordt een overzicht gegeven van het proces om schimmelgroei te modelleren, wat nu bekend is als Predictieve Mycologie, en van de moeilijkheden die hiermee gepaard gaan. Tot slot worden dan de modellen besproken die met succes schimmelgroei kunnen beschrijven.

In hoofdstuk 2, werden de sorptieisothermen ontwikkeld die de relaties tussen het substraat in onderzoek (gele maïs) en water kenmerken. Eveneens werd het sorptiemodel bepaald dat het best de experimentele sorptiegegevens beschrijft. Dit model werd dan gebruikt als een middel om de a_w van het substraat reproduceerbaar aan te passen in de daaropvolgende studies. Deze gaan de effecten van a_w en andere factoren zoals temperatuur en groeiremmende componenten, op de groei en mycotoxineproductie van de meest belangrijke schimmels in maïs na. In dit kader werden zes sorptiemodellen – de GAB, Oswin, Halsey, Henderson, Chung-Pfost en polynomiale modellen – geëvalueerd omtrent hun mogelijkheid om de experimentele isothermen te beschrijven. In het algemeen vertoonden de experimentele isothermen een Type II gedrag en waren ze temperatuurafhankelijk. Hysteresis werd waargenomen bij alle onderzochte temperaturen. Volgens de goodness of fit beschreef

het GAB model de experimentele gegevens het best, gevolgd door het Oswin, Chung-Pfost, polynomiale en het Henderson model. Daarom werd het GAB model geselecteerd als middel om de hoeveelheid water te bepalen dat toegevoegd moest worden aan het gedroogde graan om reproduceerbaar de gewenste a_w te bereiken in latere onderzoeken.

In hoofdstukken 3 en 4 werden de individuele en de gecombineerde effecten van a_w en temperatuur op de groei van *F. verticillioides en F. proliferatum en A. flavus* en *A. parasiticus*, respectievelijk, gemodelleerd. De beste modellen van deze studies werden als basis gebruikt voor de geplande modellering (waar mogelijk) van de gecombineerde effecten van a_w en van niet-chemische en chemische conserveringstechnieken op de groei van deze schimmels in verdere onderzoeken. De GAB sorptiemodellen ontwikkeld in hoofdstuk 2 werden gebruikt om te verzekeren dat de maïs de gewenste a_w activiteit had. Primaire modellen voor de verkregen groeigegevens werden bekomen door gebruik van de flexibele functie van Baranyi en Roberts (1994) om de radiale groeisnelheid van de kolonie (mm d^{-1}) en de duur van de lag fase (d) te schatten. Voor *F. verticillioides* en *F. proliferatum* werden een tweede orde polynomiale functie en het lineaire Arrhenius-Davey model gebruikt om het gecombineerde effect van temperatuur en a_w op de groeisnelheid te modelleren, terwijl Gauss en tweede orde polynomiale functies gebruikt werden voor *A. flavus* en *A. parasiticus*. De verschillende gebruikte modellen (met uitzondering van de polynomiale functies) waren noodzakelijk omdat de groeigegevens voor de *Aspergillus* isolaten buigpunten (temperatuurmaxima) vertoonden terwijl dat voor *Fusarium* niet het geval was. Doordat de groei van de *Aspergillus* isolaten gedurende de initiële fasen van groei gemakkelijker visueel beoordeeld kan worden vergeleken met de *Fusarium* isolaten, werden betrouwbare schattingen van de lagfase bekomen voor de *Aspergillus* isolaten en deze werden ook gemodelleerd. Een polynomiale functie en een uitgebreid lineair Arrhenius-Davey model werden gebruikt om het gecombineerde effect van temperatuur en a_w op de duur van de lagfase van *A. flavus* en *A. parasiticus* te beschrijven. Alle ontwikkelde modellen werden dan gevalideerd met onafhankelijk verzamelde gegevens.

Alle modellen bleken de groeisnelheid en de duur van de lagfase van beide isolaten op maïs binnen de experimentele grenzen goed te voorspellen. Significante interactie

werd eveneens waargenomen tussen de a_w en temperatuur op de groeisnelheid van beide isolaten en op de duur van de lagfase van de *Aspergillus* isolaten. Het lineair Arrenhius-Davey model bleek het beste model te zijn om de groeisnelheid van de *Fusarium* isolaten te beschrijven, terwijl de tweede orde polynomiale functie de meest geschikte voorspeller was van de gecombineerde effecten van a_w en temperatuur op zowel de groeisnelheid als de duur van de lagfase van de *Aspergillus* isolaten. Door de eigenschap van de polynomiale functies om de groei van zowel de *Fusarium* als de *Aspergillus* isolaten goed te beschrijven, werden deze functies geselecteerd als basis voor de geplande modellering van de gecombineerde invloed van a_w en niet-chemische en chemische conserveringstechnieken op de groei van deze schimmels.

Zoals waargenomen in hoofdstukken 3 and 4 en gerapporteerd in verschillende andere studies, wordt de groei van kolonies afkomstig van een groot inoculum – uitgedrukt in radiale groeisnelheid van de kolonie – gewoonlijk gekenmerkt door zeer weinig variatie. Dit is zeker niet het geval wanneer de groei zich voordoet vanuit een kleiner inoculum, een waarneming die zeer belangrijk kan zijn in het proces van natuurlijke besmetting van maïs op het veld of tijdens opslag. In hoofdstuk 5 werd een poging gedaan om de omvang van de variatie in de groeisnelheid en in de duur van de lagfase van individuele sporen van *A. flavus* en *F. verticillioides* op maïsmeel te bepalen, evenals de invloed van a_w en temperatuur op de waargenomen variatie. Om dit te verwezelijken, werd een protocol dat initieel ontwikkeld werd voor de isolatie en groei van individuele bacteriële cellen, aangepast om de specificiteiten van schimmelgroei te volgen. Het aangepaste model werd gevalideerd en gezien als een betrouwbare methode met een hoge opbrengst. Zowel de a_w als temperatuur hadden een significante invloed op de distributies van de groeisnelheid, de lagfase en de snelheid (d^{-1}) waarmee individuele sporen van beide isolaten de lagfase voltooien. In het algemeen, werden de histogrammen van de groeisnelheid van det individuele spore en van de lagfase breder naarmate de condities de groei meer bemoeilijkten, wijzend op een grotere variatie in groeicapaciteit onder deze omstandigheden. De snelheid waarmee de individuele sporen de lagfase voltooiden, daalde over het algemeen met een daling in temperatuur en/of in a_w. De resultaten tonen de grootte van de variatie in de groeicapaciteit van de individuele sporen van beide isolaten in geel maïsmeel en tonen eveneens aan dat zelfs met het kleinst mogelijke inoculum (de individuele spore) snelle groei nog voorkomt.

De relatie tussen de radiale groei van *F. verticillioides* en *F. proliferatum* en fumonisine B_1 productie op maïs in de tijd werd bepaald in hoofdstuk 6 evenals de invloed van a_w en temperatuur op deze relatie. In het algemeen namen zowel de fumonisineproductie als de radiale groei van beide isolaten toe met de a_w bij elke onderzochte temperatuur. Het effect van de temperatuur op de relatie tussen de radiale groei en fumonisineproductie was evenwel niet eenduidig. Het was duidelijk dat het effect van de temperatuur op fumonisineproductie bij hoge a_w waarden (optimaal voor groei) slechts marginaal was, terwijl bij lagere a_w waarden het effect van de temperatuur meer uitgesproken werd met meer fumonisineproductie bij temperaturen suboptimaal voor groei. De optimum temperatuur voor fumonisineproductie van beide isolaten varieerde tussen 15 en 25ºC. Voor *F. proliferatum* in het bijzonder werd waargenomen dat de optimumtemperatuur voor groei bij elke a_w 30ºC was, het minst optimaal voor fumonisineproductie. De enige andere consistente waarneming voor het effect van temperatuur was dat voor beide isolaten de traagste initiële snelheid van fumonisineproductie voorkwam bij 15ºC, waar de laagste groeisnelheden verkregen werden. De modellen ontwikkeld in hoofdstuk 3 en de relatie tussen de radiale groei en fumonisineproductie ontwikkeld in hoofdstuk 6, laten de voorspelling van groei toe wanneer een combinatie van omgevingsfactoren (a_w en temperatuur) en tijd (duur van de groei bij specifieke combinaties van de omgevingsfactoren) gegeven zijn, evenals de gevolgen van deze groei in termen van fumonisineproductie en zodoende van de veiligheid van de maïs.

Na het modelleren van de groei en het bepalen van de relatie tussen groei en fumonisineproductie werden niet-chemische en chemische conserveringstechnieken, gebruikt om de groei en mycotoxineproductie van de meest belangrijke schimmels in maïs te beperken, onderzocht. In hoofdstuk 7 werd de invloed van de initiële concentratie koolstofdioxide (IH CO_2) en zuurstof (IH O_2) in de kopruimte op de radiale groei en fumonisine B_1 productie van *F. verticillioides* en *F. proliferatum* bestudeerd bij verschillende wateractiviteiten. Deze studies werden uitgevoerd om het potentieel te evalueren van gewijzigde atmosferen als een niet-chemische techniek om maïs tijdens opslag te beschermen tegen schimmels en de daaropvolgende mycotoxine besmetting.

De polynomiale functies, gebruikt in de hoofdstukken 3 en 4 om de gecombineerde invloed van a_w en temperatuur op de groeisnelheid van *Fusarium* en *Aspergillus* te beschrijven, werden aangepast om het gecombineerde effect van a_w en IH CO_2 op de groeisnelheid van *F. verticillioides* en *F proliferatum* te bestuderen. De ontwikkelde modellen beschreven de gecombineerde effecten van a_w en IH CO_2 op de geschatte groeisnelheid van beide isolaten op adequate wijze. Bij alle bestudeerde a_w waarden resulteerde een toename in de IH CO_2 concentratie over het algemeen in een daling in de groeisnelheid en in de maximum kolonie diameter (mm), en in een toename van de duur van de lagfase. Met betrekking tot de fumonisine B_1 productie bleek een concentratie van slechts 10% IH CO_2 de productie van fumonisine B_1 door *F. verticillioides* volledig te verhinderen. *F. proliferatum* was meer resistent en vereiste 40, 30 en 10% CO_2 bij a_w waarden van, respectievelijk, 0.984, 0.951 en 0.930, voor een volledig inhibitie van fumonisine B_1 productie.

Bij alle onderzochte a_w waarden, had een reductie van de IH O_2 concentratie van 20 tot 2% geen significant effect op de groeisnelheid en op de duur van de lagfase van beide isolaten. Hoewel de maximum diameter van de kolonies daalde met een reductie van het IH O_2 niveau, kwam de grootste mycelium dichtheid voor bij 10% IH O_2 voor beide isolaten. Deze observatie ging samen met een dalende trend in de waarde van het IH O_2 niveau waarbij het meest fumonisine B_1 werd geproduceerd, van 15 tot 5% wanneer de a_w daalde van 0.976 tot 0.930 voor *F. verticillioides*. Voor *F. proliferatum* verschoven de optimumcondities voor fumonisine B_1 productie van 20% bij een a_w van 0.976 tot 10% bij a_w waarden van 0.951 en 0.930. De resultaten tonen het grote potentieel van gemodificeerde atmosferen aan om graan in opslag te beschermen tegen bederf door schimmels en mogelijke besmetting door hun mycotoxines tijdens de na-oogst periode evenals de invloed van de a_w van de maïs op de efficiëntie van deze methode.

Hoofdstukken 8 en 9 evalueerden het gebruik van anorganische en natuurlijke chemische schimmelwerende componenten, respectievelijk, om schimmelgroei en mycotoxineproductie op maïs tijdens de bewaarperiode te voorkomen. In hoofdstuk 8 werd het gecombineerde effect van a_w en ammonium/natrium bicarbonaat op de groei en de mycotoxineproductie in maïs door *Fusarium* en *Aspergillus* species onderzocht. Daarnaast werd voor de eerste keer ook de sensorische geschiktheid van maïs behandeld met bicarbonaatzouten bepaald aangezien de behandelde maïs sensorisch

aanvaardbaar moet blijven. Bicarbonaatzouten werden gekozen omdat ze goedkoop zijn, gemakkelijk te gebruiken zijn en omdat ze momenteel in voedingsproducten gebruikt kunnen worden in 'quantum satis' hoeveelheden in Noord-Amerika en Europa. Er werd ook reeds aangetoond dat ze schimmelgroei remmen.

De polynomiale modellen werden opnieuw aangepast en gebruikt om het gecombineerde effect van a_w en bicarbonaatzoutconcentratie op de groeisnelheid van de bestudeerde *Aspergillus* en *Fusarium* isolaten te beschrijven. De ontwikkelde modellen konden op een adequate wijze het effect van de hierboven vermelde factoren op de groeisnelheid van alle isolaten beschrijven. Binnen de experimentele grenzen oefenden de bicarbonaatzouten en de a_w een synergistisch effect uit op de groeisnelheid en op de duur van de lagfase. Er werd voor de eerste maal waargenomen dat lage hoeveelheden van beide zouten de groei van *F. verticillioides* en *F. proliferatum* bevorderen. De snelste groei kwam voor bij hoeveelheden van 0.1-0.2 en 0.5% ammonium- en natriumbicarbonaat (w/w), respectievelijk. Nochtans werd er geen groeistimulatie waargenomen voor de groei van *A. flavus* en *A. parasiticus*. De groei van de *Fusarium* isolaten werd volledig verhinderd bij 0.8% en 3.5% ammonium- en natriumbicarbonaat, respectievelijk. Tenminste 1% ammoniumbicarbonaat inhibeerde de groei van de *Aspergillus* isolaten volledig, terwijl tot 4% natriumbicarbonaat onvoldoende was om bij alle onderzochte a_w waarden hun groei volledig te verhinderen. Een toename in concentratie van één van beide zouten bleek te resulteren in een daling van zowel fumonisine B_1 en aflatoxine B_1 productie. Tenminste 0.6% ammoniumbicarbonaat en 2% natriumbicarbonaat verhinderden de productie van fumonisine B_1 gedurende 20 dagen van incubatie volledig, terwijl 0.8% ammoniumbicarbonaat hetzelfde deed voor aflatoxine B_1 productie. Nochtans kon 4% natriumbicarbonaat de productie van aflatoxine B_1 niet volledig verhinderen. Volgens de uitgevoerde sensorische analyse was maïs behandeld met tot 1% ammoniumbicarbonaat nog aanvaardbaar voor consumptie maar enkel na zeven voor koken om de residuen te verminderen; terwijl maïs behandeld met tenminste 2% natriumbicarbonaat sensorisch ongeschikt werd bevonden. Uit deze resultaten bleek dat ammoniumbicarbonaat beter geschikt is voor de bescherming van maïs tegen schimmelinvasie en de daaropvolgende mycotoxineproductie dan natriumbicarbonaat aangezien het de enige is die in staat is

om zowel de groei als de mycotoxineproductie van de *Fusarium* en *Aspergillus* isolaten, van groot belang in maïs, volledig te verhinderen bij concentraties die sensorisch aanvaardbaar zijn. Gezien ammoniumbicarbonaat gemakkelijk te gebruiken is en goedkoop is, zou het een geschikte na-oogst oplossing kunnen zijn voor ontwikkelingslanden met beperkte middelen.

In hoofdstuk 9, werden de natuurlijke fenolische componenten (vanillinezuur en caffeïnezuur) geëvalueerd in verband met hun vermogen om de groei en mycotoxineproductie door *Fusarium* en *Aspergillus* isolaten in maïs te verhinderen. Recent werd de interesse en het onderzoek in het potentieel gebruik van natuurlijke antimicrobiële componenten gedreven door de negatieve houding van consumenten tegenover het gebruik van anorganisch/synthetische chemische bewaarmiddelen. Daarnaast hebben de waarnemingen dat de natuurlijke resistentie van sommige graangewassen tegen schimmelinvasie gecorreleerd is aan hun gehalte aan vrij fenolzuur, de onderzoeken naar het gebruik van fenolische componenten om zowel schimmelgroei als mycotoxineproductie te verhinderen, gestimuleerd. In deze studie werden grote verschillen waargenomen in de reactie van de *Fusarium* en *Aspergillus* isolaten op de fenolische componenten, voornamelijk bepaald door hun soort. In het algemeen resulteerde voor zowel *F. verticillioides* als voor *F. proliferatum* een toename in de concentratie van caffeïnezuur of vanillinzuur in een daling in de groeisnelheid en een toename van de duur van de lagfase. De groei van de *Fusarium* isolaten werd niet volledig verhinderd bij de hoogste onderzochte a_w waarde van 0.967, met enkel een volledige inhibitie bij een combinatie van hoge fenolzuurconcentraties ($\geq$ 2000µg/g) en lage a_w waarden ($\leq$ 0.948). Binnen de experimentele grenzen werd de groei van de *Aspergillus* species niet beïnvloed door de fenolische componenten. De fenolische componenten reduceerden wel significant de productie van zowel fumonisine B_1 en aflatoxine B_1. Deze reducties zouden een belangrijke rol kunnen spelen gezien het feit dat bederf door schimmels en mycotoxinevergiftiging lijken voor te komen op locale plaatsen op in bulk opgeslagen graan. Nochtans belemmeren een aantal zaken de toepassing waaronder de hoge mate van bitterheid die de fenolische componenten (in hoge concentraties) verlenen aan de behandelde maïs en de hoge kosten.

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Lecturer – University of Zimbabwe. Courses - Fruit and Vegetable Technology and Meat Technology (October 2001 – November 2002).

Visiting Lecturer – University of Zambia. Course – Principles of Food Technology (March 2002 – April 2002).

PhD student – Ghent University, Department of Food Safety and Food Quality (December 2002 – September 2006).

EDUCATION

Bachelor of Science degree in Food Science and Technology, 1997, University of Zimbabwe, Zimbabwe). First Class pass, awarded University Book Prizes in 1995-1997.

Masters in Food Science and Technology, 2001, Ghent University, Belgium. Awarded Greatest Distinction (Complimentary Studies) and Great Distinction (Advanced Studies). Thesis title: Effects of residual oxygen on the spoilage and safety of cooked meat products. Promoters: Prof. ir. dr. F. Devlieghere and Prof. ir. dr. J. Debevere.

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