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Ecophysiology and water relations of growth and ochratoxin A production by *Penicillium verrucosum* and *Aspergillus westerdijkiae*, impacts of climate change and control using preservatives

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ABSTRACT

Penicillium verrucosum and *Aspergillus westerdijkiae* contaminate cereal grains and coffee beans with ochratoxin A (OTA) for which legislative limits exist. These fungi reside in soil and contaminate grain during harvesting and post-harvest storage, and post-fermentation during drying of coffee beans. There is a lack of information on the impacts of environmental conditions which influence the inoculum potential in soil and on cereal grain and coffee beans and potential control of OTA during post-harvest storage phases. The objectives of this project were to: (a) examine the water and temperature relations of strains of *P. verrucosum* (two strains) and *A. westerdijkiae* (three strains) in relation to growth and OTA production, (b) compare the sensitivity and tolerance of one strain of each species to solute (ionic and non-ionic) and matric stress on growth kinetics, expression of key genes in the OTA cluster (*otapks*, *otanrps*) and phenotypic OTA production for the first time, (c) examine the impact of three way interacting climate change related abiotic environmental factors (water activity, temperature, and CO_2 levels) on growth, gene expression and OTA production by strains of the two species, and (d) screen a range of potential preservatives *in vitro* control of growth and OTA production for *in situ* control of contamination of grain and coffee under different a_w and temperature conditions.

The ecological studies revealed that both strains of *P. verrucosum* (OTA11; straw21) could grow over a wide range of a_w levels and temperatures. However, under drier conditions (0.90 a_w) growth was much slower. For OTA production, optimum production was at 25°C and 0.98 a_w for strain OTA11 when compared to the other strain on a conducive YES medium. On wheat-based media, both strains were able to grow efficiently over a range of a_w levels (0.98, 0.95 and 0.90 a_w) at 15, 20 and 25°C. On wheat-based matrices, the OTA production was much lower than on the conducive YES medium, regardless of a_w and temperature for both strains after 10 days incubation. However, after 20 days, the strain Straw21, produced higher amounts of OTA under water stress condition (0.90 a_w) at 25°C. For *A. westerdijkiae* strains growth occurred over a wide range of a_w levels and different temperatures on the conducive YES media. However, the strain *A. westerdijkiae* CECT produced the highest amount of OTA at 0.98 a_w and 25-30°C. Comparison of growth under solute and matric stress showed that the growth of *P. verrucosum* strain OTA11 is more tolerant of matric potential stress than Straw21 over a wide range of water potentials at 25°C. The strain OTA11 grew well under all non-ionic solute potential stress and under matric stress. However, under ionic solute stress no growth occurred under extreme water stress of -19.6 MPa (=0.86 a_w). Upon examination

of the expression pattern of the *otapks* gene under such stress conditions, there was an increase in the gene expression in the non-ionic (glycerol) solute stress-imposed treatments when compared with ionic solute (NaCl) and matric stress treatments. This suggests that this is a key gene involved in OTA biosynthesis. There were some parallels with the phenotypic OTA production. For *A. westerdijkiae* species, both tested strains (CECT and CCT) grew well under matric stress at all stress levels imposed (-1.4—19.6 MPa = 0.99-0.86 a_w). As for *P. verrcuosum*, this strain was more sensitive to ionic solute stress imposed with NaCl with growth inhibited at -19.6 MPa (=0.86 a_w). For this species, the gene expression of *otapks* was significantly increased under moderate stress conditions -9.8 MPa (=0.95 a_w) modified with the non-ionic solute (glycerol). However, under matric stress this expression was significantly reduced when compared to solute stress. For OTA production, this was increased at -9.8 MPa (=0.95 a_w) under non-ionic solute stress when compared to the other treatments.

Overall, the impact of climate change related abiotic factors, especially elevated CO_2 levels (400 vs 1000 ppm CO_2) had no significant effect on the growth of *P. verrucosum* when compared with existing conditions under matric stress. However, under non-ionic stress (glycerol imposed), no growth was reported at -2.8 MPa (=0.95 a_w) at 1000 ppm and 30°C. Overall, the growth pattern in non-ionic solute stress was lower under elevated levels of CO_2 than in matric stress conditions when compared with existing conditions. For the *otapks* gene, expression was increased under elevated CO_2 levels in matric stress treatments when compared to existing conditions. This pattern was paralleled with production of OTA under these conditions. With regard to *A. westerdijkiae*, surprisingly no growth occurred at 37°C in all the conditions tested. However, at 30°C, the elevated levels of CO_2 had no significant impact on growth under matric and non-ionic stress when compared with existing CO_2 levels. For the *otapks* gene expression, this increased in matric imposed water stress in all conditions examined. However, there was no gene expression in non-ionic stress conditions, and this paralleled the OTA production pattern.

Initial screening of six potential preservatives showed that for both growth and OTA control by one strain of each species SM, TCA and PP were the most effective compounds. They inhibited growth of *P. verrucosum* at 250 ppm on wheat-based matrices. While, for FE, it was the least effective treatment as ED_{50} and ED_{90} values show that 1000 mg/l and 2700 mg/l are required for controlling the growth rate at both water stress levels (0.95 and 0.95 a_w) respectively and for the MIC for toxin production. However, on stored wheat grains, some growth and OTA was produced in the SM and TCA treatments at 250 ppm treatment.

For *A. westerdijkiae*, the most effective compounds inhibiting growth were at 500 and 1000 ppm of SM, TCA and PP at 0.95 and 0.98 a_w on coffee-based media. With PP, no toxin was produced at 100 ppm at both water stress levels although some growth occurred. Also, FE was the least effective treatment with ED_{50} and ED_{90} values of 1000 and 1530 mg/l respectively at both water stress levels. In stored coffee beans, the results were different with some growth found at 1000 ppm in treatments of TCA and SM at both a_w levels. In addition, high amounts of OTA were produced in the 1000 ppm treated and stored coffee beans at both a_w levels. Overall, *in vitro* efficacy was not an accurate guide to *in situ* efficacy, especially in relation to toxin control in both stored wheat and coffee beans under different a_w levels.

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First of all, I would like to dedicate my project to the soul of my mother, passed away for three years now, who always believed in the value of education and learning. I could not complete this project without her valuable advice that inspired me throughout my project. Sincerely, I am so grateful for everything she did for me and for my brothers, promising, I will achieve my best for my career and for my life to be proud of me.

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ABBREVIATIONS

A	*Aspergillus*
AcN	Acetonitrile
Afs	Aflatoxins
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of Variance
Apx	Appendix
asp	alkaline serine protease
a_w	water activity
ß-tubulin	Beta tubulin
CC	Climate change
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CP	Calcium propionate
DON	Deoxynivalenol
EC	European Commission
λ_{em}	Emission wavelengths
ERH	Equilibrium Relative humidity
F	*Fusarium*
FE	Ferulic acid
λ_{ex}	Fluorescence excitation wavelenghts
FUMs	*Fumonisins*
g	Grams
HOG	High Osmolarity glycerol
HPLC	High pressure Liquid Chromatography
LOD	Limit of detection
LOQ	Limit of quantification
L	Litre
Ψ_m	Matric potential
max	maximum
m.c	moisture content

MEA	Malt Extract agar
MPa	Mega Pascals
µmol	micromole
mg	milligram
µg	microgram
min	minimum
min	minute
ml	millilitre
NaCl	Sodium Chloride
ng	nanogram
N_2	Nitrogen
NS	non-significant
nrps	non-ribosomal peptide synthetase
O_2	Oxygen
OTA	Ochratoxin A
P	*Penicillium*
PEG	Polyethylene glycol
pks	polyketide synthetase
PS	Potassium sorbate
PP	Propyl parapen
ppb	parts per billion
ppm	parts per million
qPCR	Quantitative Polymerase chain reaction
RAPD	Random-amplified-polymorphism DNA
RT	Reverse transcriptase
RNA	Ribonucleic acid
S	Significant
SM	Sodium metabisulphite
SD	Standard deviation
Ψ_s	Solute potential
X g	times gravity
TCA	Trans-cinnamic acid
UK	United Kingdom

USA	United states of America
wt %	% of weight
YES	Yeast extract agar
ZEN	Zearalenone

CHAPTER ONE

LITERATURE REVIEW

1.1.General Introduction

Mycotoxins are natural toxic chemical compounds produced by certain genera of fungi. There are many different mycotoxins, with the most important ones found in different raw food and feed commodities such as cereals, nuts, coffee, cocoa, spices and fruit juices (Lund and Frisvad, 2003). They are heat stable and very difficult to destroy once they enter the food/feed processing chains.

The effects of some food-borne mycotoxins are acute, symptoms of severe illness appearing very quickly. Other mycotoxins in food have longer-term chronic or cumulative effects on health, including the induction of cancers and immune deficiency. There are some key mycotoxins, or groups of mycotoxins, that often occur in food and for which legislative limits exist: these are the type A and B trichothecenes (T-2/HT-2 toxins and deoxynivalenol (DON) respectively); zearalenone (ZEN); ochratoxins (OTA); fumonisins (FUMs); and aflatoxins (AFs)**.**

Raw food/feed commodities can be contaminated with mycotoxigenic fungi and mycotoxins pre-harvest, during the harvesting process, drying and storage. Inefficient drying of cereals, or pockets of damp commodities in poorly managed storage facilities can result in hot spots which can result in growth of spoilage moulds and pests which can increase the temperature to >60^{o}C and cause significant quality loss as well as mycotoxin contamination (Magan *et al.*, 2003 a). Poor post-harvest management can also cause undesirable effects such as off-odours, discolouration, and loss of nutritional quality.

Spoilage fungi which colonise cereals have been classified into two groups, i.e., the so-called "field fungi" which colonise the phyllosphere of cereals prior to harvest. These include yeasts, *Cladosporium, Epicoccum* and *Aureobasdium* species that do not produce mycotoxins and *Fusarium* species, such as *Fusarium graminearum, F. culmorum, F. langsethiae* and *F. sporotrichioides* and *Alternaria* species which do (Magan & Lacey, 1986). The 2nd group of so-called "storage fungi" include those which can cause spoilage and produce mycotoxins post-harvest during storage. A good example is *P. verrucosum,* which only contaminates grain with OTA post-harvest during poorly managed storage. However, this may apply in temperate regions, but in tropical and sub-tropical regions this does not apply as many spoilage mycotoxigenic fungi occur both pre- and post-harvest. For example, *Aspergillus flavus* (cereals/nuts), *A. westerdijkiae* (maize, coffee, cocoa) and *A. carbonarius* (grapes, vine fruits).

It has been shown that mycotoxigenic fungi such as *F. verticilliodes* (FUMs contamination of maize), and *F. graminearum* (DON, wheat) can cause significant dry matter losses under marginal conditions for growth (Mylona *et al*., 2013). They also suggested that <0.5% in dry matter losses due to the activity of these mycotoxigenic fungi may result in mycotoxin levels above the EU legislative limits (Garcia-Cela *et al*., 2018). There is thus interest in understanding the relationship between environmental factors, growth of key spoilage mycotoxigenic fungi and the biosynthesis of the toxic secondary metabolites.

1.2. Important mycotoxins and their effects

Mycotoxins are classified as toxic secondary metabolites produced by a wide variety of fungi that contaminate agricultural crops prior to harvest or during storage, post-harvest (Zychowski *et al.*, 2013). Table 1 shows the most important mycotoxins worldwide and their relative toxic effects**.**

Table 1.1. Summary of the main mycotoxins for which legislation exists, and their relative toxicity. Key to mycotoxins: AFs, aflatoxins; OTA, ochratoxin A; DON, deoxynivlenol; ZEN, zearalenone; FB_1, fumonisin B_1; total FUMs, total fumonisins.

Toxin	Relative Toxicity	Reference
Total AFs	• Hepatocellular carcinoma • Reduced reproductivity • Immunosuppression	(Wu and Santella, 2012) (Marin *et al*., 2013) (Jiang *et al.*, 2008)
OTA	• Nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxin effects • Cause human Balken endemic nephropathy (BEN)	(El Khoury and Atoui, 2010) (Leszkowicz *et al*., 2002)
DON	• Abdominal pains, dizziness, headaches, throat irritation, nausea, vomiting, bloody stools, diarrhea • Immune disorders such as (immunosuppression or immunostimulation), weight loss, anorexia, and decreased nutritional efficiency	(Rotter *et al*., 1996) (Lori and Rizzo, 2007)
ZEN	• Has role in human breast cancer	(Yu *et al.*, 2005)

	• Cause reproductive problems	(Gromadzka *et al*., 2009; Wood, 1992).
FB_1; total FUMS	• Have carcinogenic and cardiovascular toxic effects • Hepatocarcinogenic • Oesophageal cancer • Associated with neural tube defects (NTDs)	(Gelderblom *et al.*, 1988) (Gelderblom *et al*., 1991) (Yoshizawa, *et al.*, 1994) (Sydenham *et al.*, 1990). (Missmer *et al.*, 2006).

1.2.1 Ochratoxins

Van der Merwe *et al*. (1965) isolated OTA from a culture of *Aspergillus ochraceus.* This species was subsequently reclassified as *A. westerdijkiae* (the predominant producer of OTA), with other OTA producers in the section Circumdati being *A. steynii* and *A. ochraceus* (Samson *et al.*, 2004). OTA is also produced by *Aspergillus* section *Nigri* species (especially *A. carbonarious*), and a few *Penicillium* species (*P. verrucosum, P. nordicum*). The ochratoxins are composed of a polyketide-derived di-hydroisocoumarin moiety linked via the 7-carboxy group to L-ß-phenylalanine by an amide bond, except for ochratoxin α (Figure 1.1). OTA has particular characteristics which includes it heat stability as it is resistance to acidic conditions (El khoury and Atoui, 2010). Thus, its elimination is very difficult. In addition, OTA is considered the most important ochratoxin of concern for human health, whereas ochratoxins B and C are sometimes produced but are of less importance. Table 1.2 lists the mycotoxigenic fungi which produce OTA.

Table 1.2. Summary of fungal species, which produce ochratoxin A *(*Abrunhosa *et al*., 2010). Species in bold are the most important ochratoxin producers in food commodities.

Aspergillus* section *Circumdati	***A.cretensis***	(Samson *et al*., 2004)**.**
	A.flocculosus	(Samson *et al*., 2004),
	A.melleus	(Hasseltine *et al*., 1972)
	A.ochracheus	(Van der Merwe *et al*., 1965; Samson *et al*., 2004).
	A.ostianus	(Hasseltine *et al*., 1972)
	A.persii	(Ciegler, 1972)
	A.petrakii	(Hasseltine *et al*., 1972)
	A.pseudoelegans	(Samson *et al*., 2004)
	A.roseoglobulosus	(Samson *et al*., 2004)
	A.sclerotiorum	(Samson *et al*., 2004; Hasseltine *et al*.,1972; Varga *et al*., 1996)
	A.steynii	(Samson *et al*., 2004).

	A.sulphureus	(Samson *et al*., 2004; Hasseltine *et al*., 1972; Varga *et al*., 1996)
	A.westerdijkiae	(Samson *et al*., 2004).
Aspergillus* section *Nigri	***A.carbonarius***	(Horie, 1995; Samson *et al*., 2004)
	A.lacticoffeatus	(Samson *et al*., 2004)
	A.niger	(Samson *et al*., 2004; Abarca *et al*., 1994)
	A.sclerotioniger	(Samson *et al*., 2004)
Penicillium species	***P.nordicum***	(Larsen *et al*., 2001)
	P.verrucosum	(Pitt, 1987; Van Walbeek *et al*., 1969; Ciegler *et al*., 1973).
	P.viridicatum	(Ciegler *et al*., 1973).

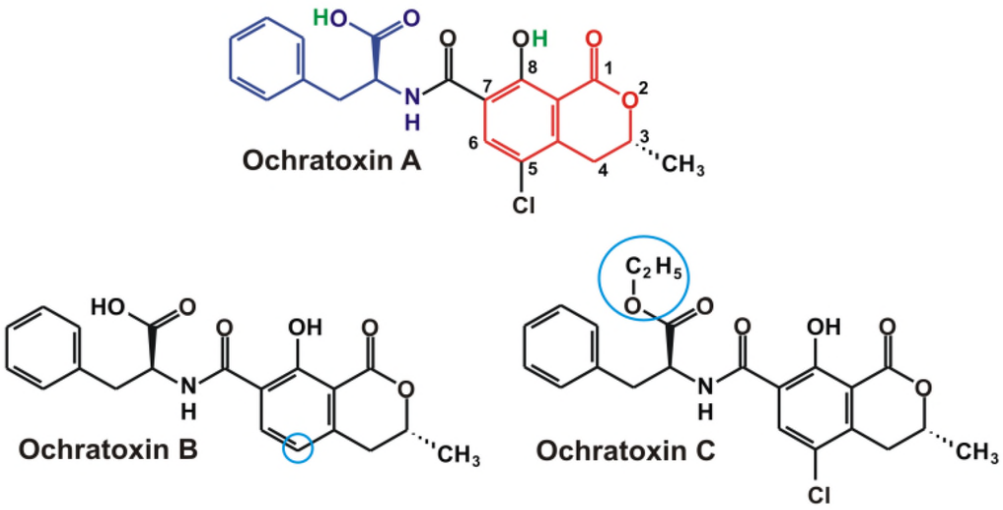

Figure 1.1. Chemical structures of Ochratoxin A (dark blue: phenylalanine part, red: dihydro-isocoumarin ring, green: acidic hydrogens), B, and C. The highlighted structures are characteristic to the three different ochratoxin molecules (light blue). (From Koszegi and Poor, (2016).

1.2.2. Regulations and Legislation for Ochratoxin A in different commodities

Because of the potential effects of mycotoxins on the health of humans and animals, legislative authorities have set up measures in many countries to control and minimize exposure to mycotoxins. The European Commission Regulation sets maximum levels for certain contaminants in food stuffs including OTA contamination. These have been set for unprocessed cereals, including rice and buckwheat at 5µg/kg. For cereal-derived products, maximum OTA contamination is fixed at 3 µg/kg. For contamination of vine fruits, the limit is 10 µg/kg (European Commission, 2002). The European Commission (2005) highlighted the contribution of many food products such as wine, grape juice and coffee to human OTA exposure. Thus, limits also exist for OTA contamination of grape juices, wines (red, white and rosé) at 2 µg/L. In coffee, the regulation

allows a maximum level for OTA of 5 µg/kg in coffee beans and 10 µg/kg in instant coffee. Overall, different strategies are being developed to try and minimize contamination of food products by mycotoxins. Table 1.3 summarizes the maximum legislative limits for OTA in different products.

Table 1.3. Maximum Levels for Ochratoxin A regulated by Commission Regulation (EC) No 1881/2006.

Product	OTA (µg/kg) Maximum level
Un processed cereals	5
Cereal derived products	3
Dry grapes	10
Grape juices, wines	2
Coffee beans	5
Instant coffee	10

1.3. Commodities contaminated with ochratoxigenic fungi and OTA

The predominant fungal species responsible for contamination of commodities with OTA is influenced by the prevailing climatic conditions. Thus, in sub-tropical and tropical regions which are warmer and more humid *Aspergillus* section *Circumdati* species, especially *A. westerdijkiae,* and sometimes *Aspergillus* section *Nigri* species, such as *A. carbonarius,* are important. In temperate regions *P. verrucosum* is important as it grows and contaminates grain with OTA in damp cool conditions (Belli *et al*., 2004).

1.3.1. Cereals and OTA contamination

Many surveys have shown that *P. verrucosum* predominantly infects temperate cereals, especially wheat and barley. Lund & Frisvad (2003) showed that *P. verrucosum* contaminated cereal grain after the harvesting process and during drying and storage. Apparently, the drying phase prior to storage is a key step in preventing OTA formation. After harvesting, if the drying process is slow under less than ideal conditions of temperature especially in the presence of rain, it permits the growth of *P. verrucosum* in the grain (Magan and Olsen, 2004). Thus, cereals should be dried quickly after harvesting and kept at <14.5-15.0% moisture content (m.c.) during storage to avoid OTA contamination (Tittlemeir *et al*., 2012) . Figure 1.2 shows key points where OTA contamination could be minimised after harvesting in cereals.

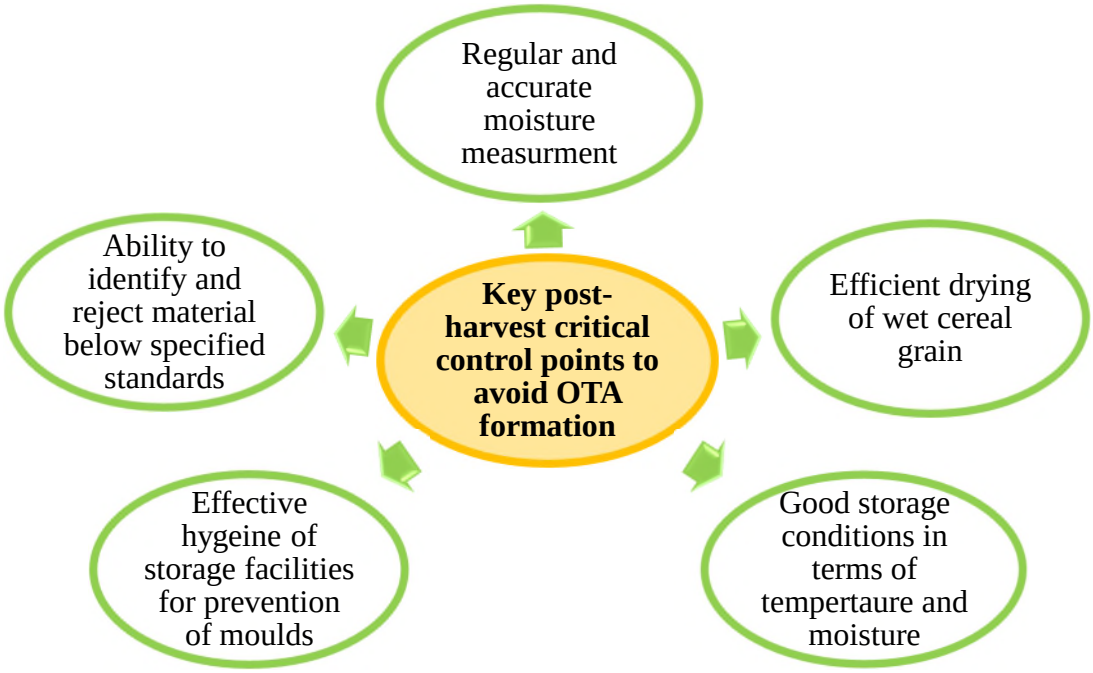

Figure 1.2 Key post-harvest critical control points for OTA formation (adapted from Pitt *et al*., 2013).

1.3.2. Ochratoxin A in coffee

Coffee berries develop best at near 30°C, while temperatures below 19°C are required for flowering. *A. westerdijkiae* and *A. carbonarius* are reported to infect coffee cherries in elevated tropical regions. To understand when OTA is formed in coffee, it is important to highlight the different stages of coffee production. Firstly, it has been shown that OTA is not present in cherries at harvest, with *A. westerdijkiae* and *A. carbonarius* not infecting immature coffee cherries (Pitt *et al.*, 2013). Secondly, after harvest, it has been found that these species can infect coffee when the drying conditions are less than ideal in conjunction with rain as well as when the coffee cherries are sundried (Pitt *et al*., 2013; Taniwaki *et al.*, 2018). Thus, good mechanical drying is effective in eliminating OTA from coffee (Pitt *et al*., 2013; Taniwaki *et al.*, 2018**).** Thirdly, during the farm storage phase, it has been reported that poor management of coffee crops are the major source of OTA. During the final stage, coffee processing reduces OTA levels by rejection of defective cherries using modern laser sorters. In addition, the roasting process reduces OTA levels in coffee from 8 to 98% depending on the time and temperature of roasting (Ferraz *et al.*, 2010). Figure 1.3 illustrates the critical control phases for OTA formation in coffee.

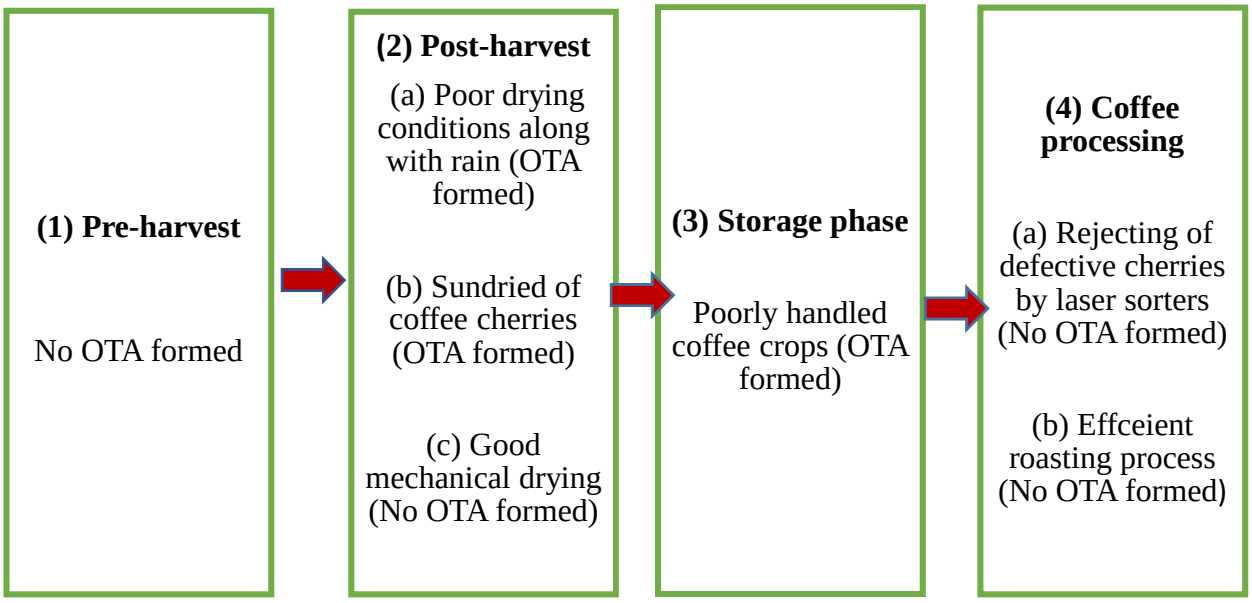

Figure 1.3 Time course of OTA formation in coffee processing (adapted from Pitt *et al*., 2013)

1.4. Key species producing OTA: effects of environmental factors on growth and ochratoxin A production

Generally, mycotoxigenic fungi, like all microorganisms, are influenced by environmental factors such as water availability, temperature, pH and gas composition and their interactions (Magan, 2007). Often the range of conditions which are optimum for growth are different from those for mycotoxin production (Sanchis and Magan, 2004). Figure 1.4 shows the relationship between key abiotic and biotic factors which interact and influence the life cycle of spoilage and mycotoxigenic fungi.

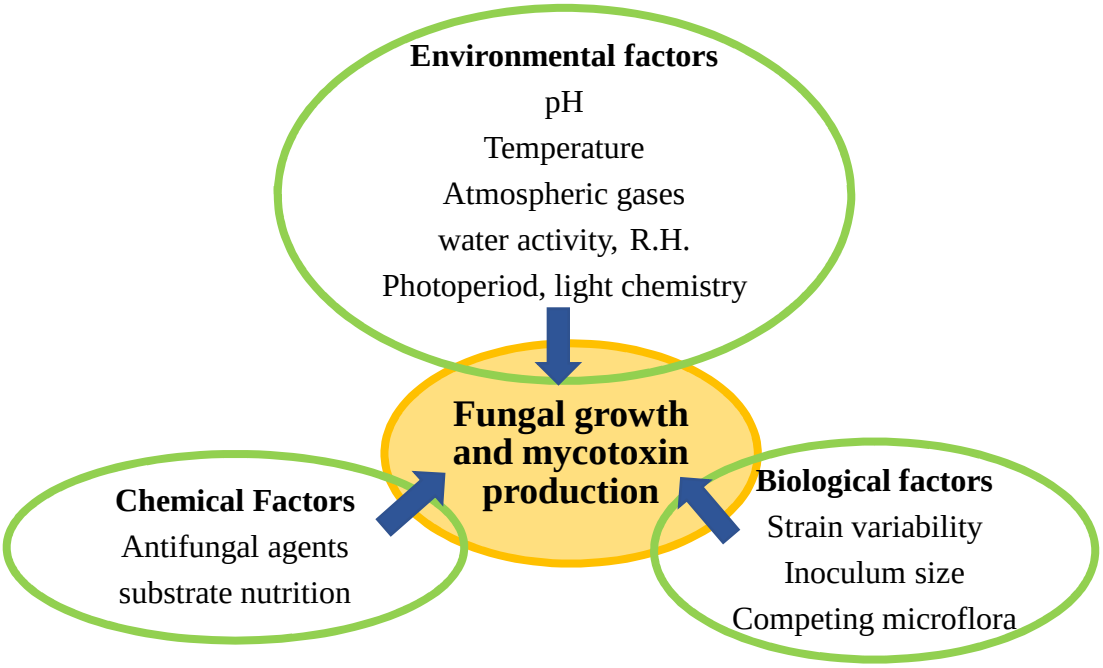

Figure 1.4 Principal factors influencing fungal growth and mycotoxin production (EC, 1994).

1.4. 1. Effect of environmental factors on the growth of ochratoxigenic fungi.

I. Effect of temperature

Temperature is an important environmental factor affecting growth and mycotoxin production by moulds. Fungi can be divided according to their tolerance to temperature into psychrotolerant, psychrophilic, mesophilic, thermotolerant and thermophilic fungi. Ochratoxigenic fungi are all mesophilic although they have different optima and marginal temperatures for growth.

With respect to *Aspergillus* section *Circumdati*, it has been shown that the optimum growth is close to 30°C for *A. ochraceus*, *A. steynii* and *A. westerdijkiae* respectively (Abdel-Hadi and Magan, 2009). For species from the *Aspergillus* section *Nigri group*, Palumbo *et al*. (2015) reported that the optimum temperature range for *A. carbonarius* growth was at 30-35°C. Moreover, Marín *et al*. (2008) confirmed that the optimum temperature required for growth of *A. carbonarius* on pistachio nuts was 30-35°C. For *A. niger*, Leong *et al*. (2006) reported that optimum growth occurred at 35°C.

With regard to *Penicillium* species, including *P. verrucosum* and *P. nordicum*, it was found that the former species has optimum growth at 25°C, whereas the latter showed best growth at 20°C

on dry-cured sausages (Rodríguez *et al*., 2015). Pardo *et al*. (2006) found that optimum growth of *P. verrucosum* was at 20°C on barely-based media. Previous studies by Carins-Fuller *et al*. (2005) conducted on wheat grain suggested that optimum growth by *P. verrucosum* was at between 15-25°C.

II. Effect of Water activity

Water activity (a_w) is defined as the amount of water in a substrate which is available for microbial growth. This is related to the water that is freely available in a substrate and related to the moisture content of each specific commodity. A_w is the ratio of the water vapour pressure of the water in a food to the water vapour pressure of pure water under the same temperature and pressure conditions. Thus:

$a_w = P/P_0$

a_w is related to other measures such as the Equilibrium Relative Humidity (ERH) and to the total water potential (sum of: osmotic, matric and turgor potentials) which is predominantly used in soil microbiology and soil science (Magan, 2007).

It has been previously shown that a_w is an important criterion for understanding the ecology of mycotoxigenic species and is related to interactions with temperature to obtain profiles for growth and mycotroxin production (Sanchis and Magan, 2004). Studies by Abdel-Hadi and Magan (2009) revealed that optimum growth of *A. ochraceus, A. steynii* and *A. westerdijkiae* in the section *Circumdati* was at 0.99-0.95 a_w.

In *Aspergillus* section *Nigri*, it was reported that growth by *A. carbonarius* was strongly reduced under moderately increasing ionic stress conditions using NaCl as the osmotic solute. Studies by Tassou *et al*. (2007) reported that no growth was observed at 0.85 a_w by *A. carbonarius* isolates from Greece. However, the optima values for growth were at 0.96-0.97 a_w. On the other hand, studies by Astoreca *et al*. (2009) indicated that optimum growth by *A. niger* aggregate strains on corn grains occurred at 0.95-0.91 a_w.

Studies have also identified the effect of a_w on growth of ochratoxigenic *Penicillium* species. Studies by Cairns-Fuller *et al*. (2005) showed that 17–18% moisture content (0.80-0.83 a_w) could

limit any potential growth by *P. verrucosum* in wheat grain. This is also in agreement with studies of Lindblat *et al.* (2004) who suggested that growth can occur under some conditions at 0.80 a_w. *P. nordicum* is an ecologically specialised species which grows in a high salt environment and is thus a xerophilic species. This species colonises dry-cured meats during the ripening process. Rodríguez *et al.* (2014) showed that at 0.87 a_w, strains of this species are able to grow better than in conditions where water is more freely available. Interestingly, *P. nordicum* can effectively colonise NaCl rich foods with less competition from other mycobiota (Schmidt-Heydt *et al.*, 2011). However, it was revealed that toxigenic and non-toxigenic strains of *P. nordicum* showed limited growth at 0.80-0.85 a_w on ham-based media where the optimum colony size was at 0.95 a_w after 14 days incubation (Leggieri *et al.*, 2011). Table 1.4 summarizes the ranges of temperature and a_w conditions for growth of different ochratoxigenic fungi.

Both *A. westerdijkiae* and *P. verrucosum* inoculum for contamination of coffee and cereal grain comes from soil. Thus, it is important to understand the relative tolerance of these two species to water stress in soil and perhaps survival on crop residue. This is predominantly determined by tolerance of matric stress (in soil) and solute stress (in crop debris). There have been very few studies to examine the relative sensitivity/tolerance of these two species and mycotoxigenic species in general to these important parameters of water stress. For example, Ramos *et al*. (1999) showed that *A. ochraceus* strains were relatively tolerant of both matric and solute stress in terms of growth. They also showed that the strains of this species were able to produce mixtures of compatible solutes (sugar alcohols) which enabled the growth and colonisation under matric stress effectively.

Studies by Jurado *et al.* (2008) with *Fusarium verticillioides* showed that both growth and expression of key *FUM* biosynthetic genes were more sensitive to matric than solute stress. There has been little information on *P. verrucosum* and *A. westerdijkiae* in relation to matric vs solute stress to understand its life cycle and ability to colonise cereals and coffee post-harvest.

Table 1.4. Summary of the optimum temperature and water activity range for growth of ochratoxigenic fungi as reported in the literature.

Fungal species	Optimum Temperature(°C)	Optimum Water activity (a_w)
A. westedijkiae ***A. steynii*** ***A. ochraceus***	**30** (Abdel-Hadi and Magan, 2009)	**0.95-0.99** (Abdel-Hadi and Magan, 2009)
A. carbonarius	**30-35** (Mitchell *et al*., 2004)	**0.96-0.97** (Tassou *et al*., 2007)
A. niger	**35** (Leong *et al*., 2006)	**0.91-0.95** (Astoreca *et al*., 2009)
P. verrucosum	**25** (Rodríguez *et al*., 2015)	**0.80-0.83** (Frisvad and Samson, 1991)
P. nordicum	**20** (Rodríguez *et al*., 2015)	**0.87** (Rodríguez *et al.*, 2014)

1.4.2. Effect of environmental factors on OTA production by ochratoxigenic fungi.

I. Effects of temperature

It should be kept in mind that the optimum conditions for growth are not always the same as those for secondary metabolite production. Abdel-Hadi and Magan (2009) demonstrated that *A. steynii* and *A. westerdijkiae* produced maximum OTA amounts at higher temperatures of 30-35°C, and *A. ochraceus* at 25-30°C. Ramos *et al*. (1998) revealed that maximum amounts of OTA produced by *A. ochraceus* were at 25-30°C on barely-based media and in barely grains. Pardo *et al*. (2005) found that maximum OTA was produced by *A. ochraceus* at 20°C on green coffee beans while at 10°C no OTA was produced regardless of a_w.

Mitchell *et al*. (2004) showed that 20-25°C is optimum for OTA production by *A. carbonarius* strains from different parts of Europe, on grape-based media. Belli *et al*. (2007) artificially inoculated damaged and undamaged table grapes with four ochratoxigenic *A. carbonarius* strains

which were stored at different RH values (80, 90, and 100%) at 20 and 30ºC. This showed that OTA production was significantly induced at 30°C rather than 20°C in most of the treatments. A model was developed by Marín *et al.* (2008) to predict OTA production boundaries by an *A. carbonarius* strain in relation to moisture content and storage temperature of pistachios. They demonstrated that OTA accumulation sharply increased at 15-20°C. In addition, studies by Astoreca *et al*. (2009) reported that *A. niger* aggregate yielded high amounts of OTA at 25°C on corn grain media. However, it was suggested that optimum OTA production was at 15°C by *A. niger* aggregate strains on grape juice media (Leong *et al*., 2006). Table 1.5 lists the different temperature conditions for OTA production by ochratoxigenic fungi.

For *Penicillium* species, in particular *P. verrucosum* and *P. nordicum*, they exhibit different patterns in terms of toxin production. Battilani *et al*. (2010) found that the temporal OTA production increased with time (7, 14, 21 days) with optimum production at 20°C by *P. nordicum* in a dry-cured meat. Moreover, Rodríguez *et al.* (2015) showed that *P. nordicum* produced optimum OTA at 10-20°C. Furthermore, they showed that *P. nordicum* could produce high amount at 25°C. In contrast, *P. verrucosum* produced OTA over the temperature range of 15-30°C (Rodríguez *et al.,* 2015). Similarly, Cairns Fuller *et al*. (2005) reported that optimum conditions for OTA production by this species was at 25°C.

II. Effects of Water activity

Sanchis and Magan (2004) have shown earlier that a_w is an important criterion for understanding the ecology of mycotoxigenic species in terms of toxin production by ochratoxigenic species. It was found that *A. steynii* and *A. westerdijkiae* generated maximum OTA amounts at intermediate water stress (0.95-0.90 a_w) (Abdel-hadi and Magan, 2009). Ramos *et al*. (1998) revealed that maximum amounts of OTA were produced by *A. ochraceus* at the highest a_w tested (0.98 a_w) on barely extract malt agar and in barely grains. In contrast, optimum a_w levels for OTA production by *A. ochraceus* on barely grain was found at 0.99 a_w (Pardo *et al*., 2004).

It was found that the ionic stress conditions have a significant influence on OTA production by *A. carbonarius.* Stoll *et al*. (2013) demonstrated that OTA production was strongly reduced in *A. carbonarius* under moderately increasing ionic stress conditions when increasing NaCl concentrations were used. In contrast, at increasing glucose concentrations, OTA biosynthesis was moderately reduced. Previously, studies on four ochratoxigenic *A. carbonarius* strains on grapes showed that maximum OTA amounts were at the highest RH (100%), while no significant differences were found between 90% and 80% RJ in terms of OTA content (Belli *et al*., 2007).

Table 1.5 illustrates the different a_w ranges for OTA production by *Aspergillus* section *Nigri* species.

Lindblat *et al*. (2004) and Cairns-Fuller *et al*. (2005) reported that OTA production may be limited to about 0.83 a_w. In contrast, it was determined that optimum production of OTA by *P. verrucosum* was at 0.97 a_w with a minimum at 0.89 a_w (Rodríguez *et al.*, 2015). However, these strains may not have been isolated from cereal grain. In addition, they elucidated that *P. nordicum* possessed a different pattern where they produced optimum OTA at 0.90 a_w and very little at 0.85 a_w.

Table 1.5 Summary of optimum temperature and optimum water activity for OTA production by ochratoxigenic fungi as reported in literature.

Fungal species	Optimum Temperature(ºC)	Optimum Water activity (a_w)
A. westedijkiae	**30-35°C** (Abdel-Hadi and Magan, 2009)	**0.90-0.95** (Abdel-Hadi and Magan, 2009)
A. steynii		
A. ochraceus	**25-30°C** (Abdel-Hadi and Magan, 2009)	**0.99** (Pardo *et al*., 2004).
A. carbonarius	**20-25** (Mitchell *et al*., 2004)	**0.95-0.98** (Mitchell *et al*., 2004)
	30 (Belli *et al*., 2007)	**0.99** (Belli *et al*., 2007)
A. niger	**25** (Astoreca *et al*., 2009)	**0.97** (Astoreca *et al*., 2009)
P. verrucosum	**15-30** (Rodríguez *et al.*, 2015)	**max 0.97, min 0.89** (Rodríguez *et al.*, 2015)
P. nordicum	**20** (Battilani *et al*., 2010)	**Optima 0.90, min0.85** (Rodríguez *et al.*, 2015)
	10-20 (Rodríguez *et al*., 2015)	

III. Effect of modified atmosphere on growth and OTA production by ochratoxigenic fungi.

Most mycotoxigenic fungi are able to tolerate low O_2 conditions and are thus microaerophilic. For the control of spoilage in different commodities modified atmospheres by reducing O_2 to <1% and increasing CO_2 to >50-75% have been examined (Pateraki *et al*., 2007; Giorni *et al*., 2008).

Magan *et al*. (2004) demonstrated that the three interacting abiotic factors temperature, water activity and gas compositions can influence the growth and OTA production by these spoilage fungi. These studies showed that lag phases prior to growth and growth of a range of field and spoilage fungi is reduced by decreasing O_2 to <0.14%. Alternatively, increasing CO_2 to >50% is often necessary for inhibition of mycelial growth. However, some species, e.g., spoilage fungi such as *P. roqueforti,* are able to grow and infect grain at > 80% CO_2 and 4% O_2 is present (Magan and Aldred, 2007). The use of modified O_2 and CO_2 post-harvest systems can prevent deterioration as well as (O_2 free) N_2 systems. Both growth and mycotoxin production by mycotoxigenic fungi show different trends when exposed to CO_2. This was detected in *P. verrucosum* where 25% CO_2 reduced growth but resulted in only a limited reduction in OTA accumulation **(**Magan and Alderd, 2007). Generally, growth and OTA production were highest in air, followed by 25 and 50% CO_2 regardless of the a_w level tested. Meanwhile, CO_2 and a_w together caused an enhanced inhibitory effect, although this was not synergistic (Magan and Aldred, 2007). It was shown that growth and OTA production by *P. verrucosum* are slightly affected by up to 50% CO_2 regardless of water activity *whether* in vitro and in stored wheat grain (Cairns-Fuller *et al*., 2005).

Studies have examined the interaction effects of CO_2 × temperature× a_w on growth and mycotoxin production by black *Aspergilli*. Pateraki *et al*. (2007) confirmed that up to 50% CO_2 had only a slight impact on OTA production by *A. carbonarius* over a range of a_w conditions, with a_w the more important factor than CO_2. Interestingly, Valero *et al*. (2008) found that 1% O_2 combined with only an increased level of CO_2 to 15% reduced fungal growth and OTA synthesis by *A. carbonarius* and *A. niger* on synthetic grape juice medium (SNM).

A new perspective of using controlled atmospheres along with a_w and temperature storage regime has been examined for OTA control in cereal grain. It was reported that 25% CO_2 reduced growth of *P. verrucosum* as well as OTA accumulation (Magan and Alderd, 2007). Moreover, studies by Cairns-Fuller (2004) suggested that conidial germ tube length is significantly inhibited by 50% CO_2 at 0.90-0.995 a_w for *P. verrucosum.* Table 1.6 listed the effects of CO_2 on growth and OTA production by ochratoxigenic fungi.

Table 1.6. Summary of the effect of CO_2 on fungal growth and mycotoxin production by ochratoxigenic fungi from the literature.

Species	CO_2%	Effect	Author
P. verrucosum	50%	Growth inhibition	Carins-fuller *et al*., (2005)
A. ochraceus	>30%	Inhibition of OTA	Paster *et al*., (1983)
A. carbonarius	50%	Effect on germination but not OTA	Pateraki *et al*., (2007)
A. carbonarius ***A. niger***	15%	Reduce OTA and growth	Valero *et al*., (2008)
Aspergillus spp. ***Penicillium***	0.03-15%	5-10% stimulate growth, mostly effect lag phase	Magan *et al*., (1984).

IV. Potential role of future climate change factors on growth and mycotoxin production

Climate change (CC) is expected to have a marked effect on our landscape worldwide. Ray *et al*. (2015) suggested that CC may account for yield variability in a third of the key staple commodities on a global basis. It is assumed that atmospheric concentrations of CO_2 are expected to double or triple (from 400 to 800-1200 ppb) in the next 25-50 years. This will result, consequently, in a global temperature rise of between 2-4°C depending on levels of man-made industrial activity. It is important to consider the effect of CC on the infection of staple food commodities and the possible contamination with mycotoxins. In studies performed on wheat, Vary *et al*. (2015) suggests that physiology of wheat is modified when comparing 390 ppm CO_2 with elevated levels of 780 ppm CO_2. They also examined the effect of acclimatisation of *Septoria tritici* blotch (STB) disease and *Fusarium* Head Blight (FHB) disease when cultivated for up to 10 generations before inoculating the wheat plants. This resulted in an increase in pathogenicity and disease. Unfortunately, no data on mycotoxin contamination was collected from this study. However, it has been reported that fungi are more tolerant to elevated CO_2 levels as an individual factor. However, they are less tolerant to such increased CO_2 concentrations when combined with other environmental factors (Magan *et al*., 2011; Medina *et al*., 2015a). This suggests that colonisation of staple food crops including cereals and mycotoxin contamination may be affected by interacting conditions of elevated temperature × water stress × elevated CO_2 levels. However, few studies

have examined the influence of these three-way interactions on growth and mycotoxin production by *Aspergillus, Penicillium* and *Fusarium* species.

A recent study by Medina *et al.* (2015) examined the effects of these three-way interacting factors on growth and aflatoxin B_1 (AFB_1) production by *A. flavus*. Overall, growth was unaffected by CC conditions. In contrast, they had a significant effect on AFB_1 production with increases of up to 79 times the control values at 30^{o}C and existing climate conditions. This was confirmed by relative increases in the expression of both a regulatory and structural gene involved in the biosynthesis of AFB_1. Recent studies on *Aspergillus* section *Circumdati* and *Nigri* species suggests that CC effects on growth and OTA production are variable with some effects on lag phases prior to growth and some stimulation of OTA production (Akbar *et al*., 2017). At present, no information is available on effects on spore germination, growth, sporulation or OTA production by species such as *P. verrucosum.*

1.5. Biosynthetic gene clusters and phenotypic ochratoxin A production by ochratoxigenic fungi

1.5.1. OTA gene cluster in *Aspergillus* species

Harris and Mantle (2001) proposed a biosynthetic pathway based on a mechanistic model according to the structure of OTA. They stated that OTA biosynthesis occurs in three steps. The first step is polyketide synthesis of ochratoxin α via mellein involving a polyketide synthase. The second includes acyl activation: mellein is methylated and oxidized to 7-Carboxy-Mellein (=OTβ) followed by chlorination leads to OTα. The second precursor, phenylalanine, is synthesized *via* the shikimic acid pathway, followed by ethyl ester activation so that it can participate in the subsequent acyl displacement reaction. The third step includes the linkage of the produced activated precursors via a synthetase generating OTC, an ethyl ester of OTA that is de-esterified by an esterase resulting in OTA molecule. Figure 1.5 shows the suggested hypothetical pathway for OTA biosynthesis by *Aspergillus* species.

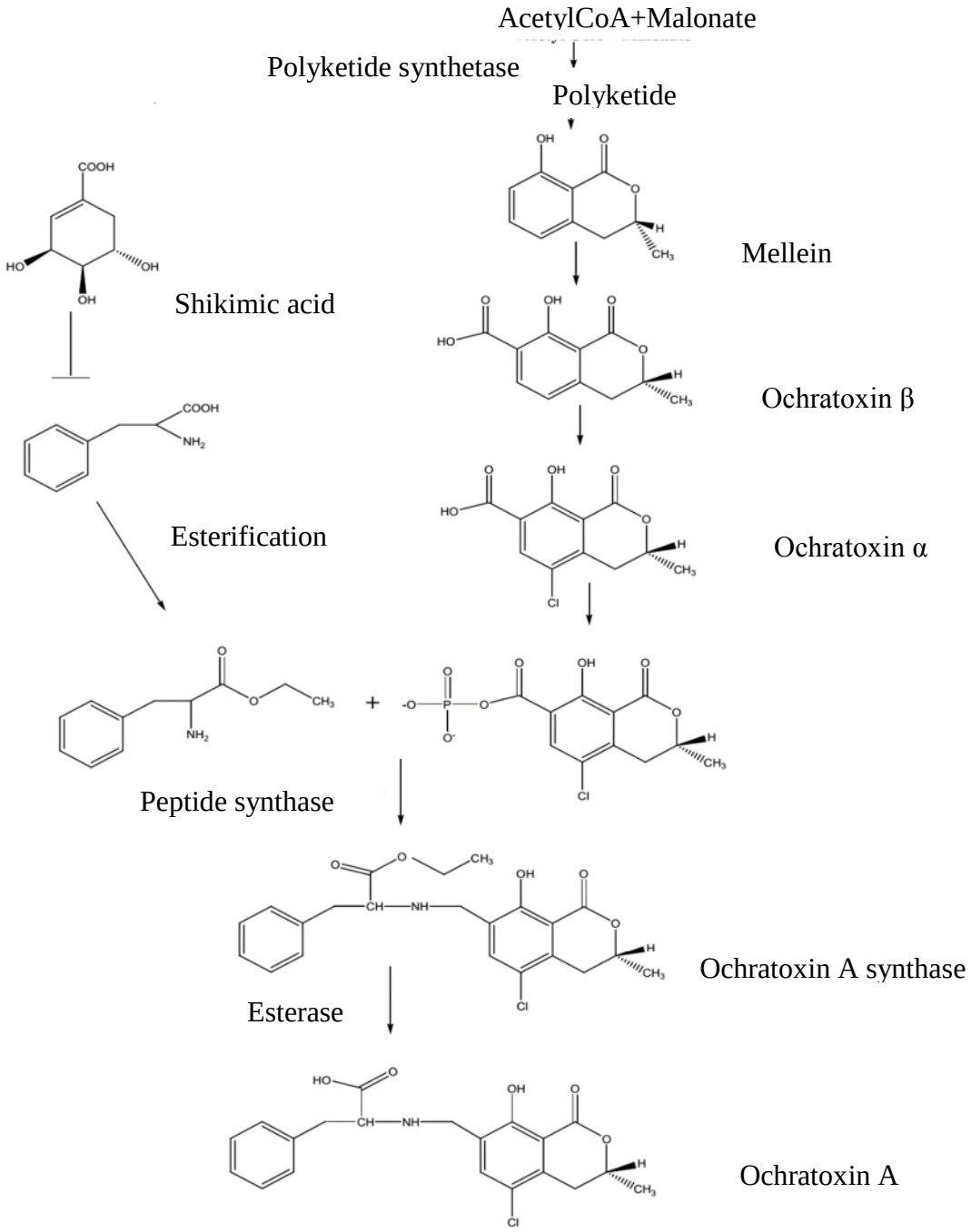

Figure 1.5. Schematic representation of the hypothetical OTA biosynthetic pathway as proposed by Harris and Mantle (2001).

1.5.2. OTA gene cluster in *P. verrucosum*

The two *Pencillium* species that are mainly responsible for OTA production are *P. verrucosum* occurring in cereals and *P. nordicum* found in NaCl and protein rich foods. The main characteristic of these food environments is the high content of proteins, peptides and amino acids. Schmidt-Heydt *et al*. (2011) have developed a schematic representation of the cluster of the biosynthetic genes of OTA, which showed that the expression of the *otapksPN* gene, the gene encoding for the OTA polyketide synthase, can be regarded as a key gene in the first step of OTA biosynthesis (Figure 1.6 a). Ringot *et al*., (2006) have illustrated the key biosynthetic genes including in OTA biosynthetic pathway (Figure 1.6 b).

Figure 1.6 (a). Schematic representation of the cluster of the biosynthetic genes of OTA (adapted from Schmid-heydt *et al.*, 2011).

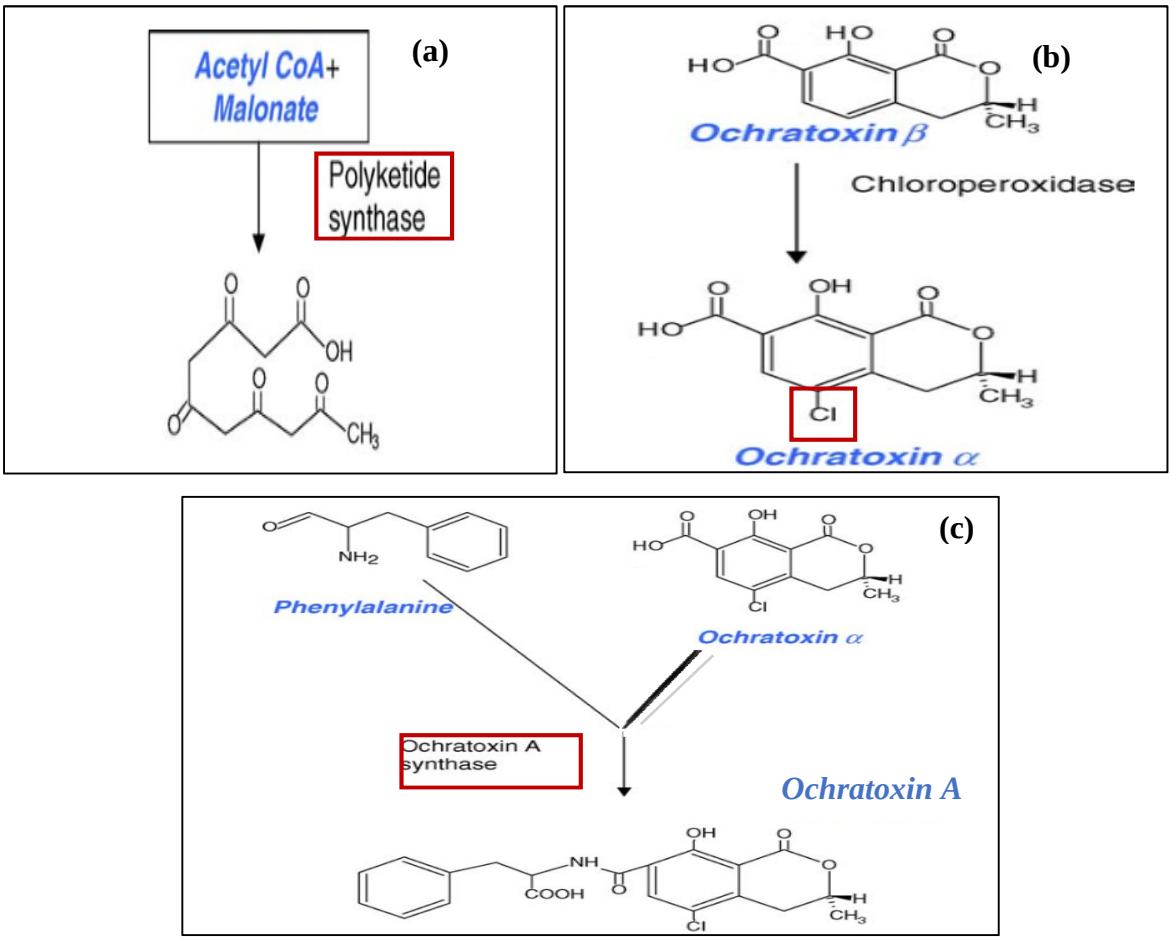

Figure 1.6 (b). OTA biosynthetic pathway showing the key biosynthetic genes that control each step. (a) Polyketide formation by the product of the *otapks* gene. (b) Chlorination by the activity of chlorinating enzyme (otachI). (c) Peptide formation by the product of the *otanrps* gene (adapted from Ringot *et al*., 2006).

Interestingly, it was suggested that several signalling cascade pathways responded to external parameters and play a role in the activation of the mycotoxin biosynthesis genes (Garcia-Rico *et al*., 2008; Shwab and Keller, 2008). For *P. verrucosum*, it was shown that a HOG-like cascades (high osmolarity glycerol) is involved in ochratoxin regulation under NaCl stress conditions where the signal is transmitted at the gene level by MAP kinases (such as HOG) or heterometric G-proteins to either activate or repress gene function (Schmidt-Heydt *et al*., 2011). In addition, Adam *et al*. (2008) estimated that the HOG MAP kinase pathways can be induced by other abiotic factors. It has to be taken into account that the *otapksPN* gene is highly activated within 24 h before OTA is detected. This could thus be a very good early indication of OTA contamination (Schmidt-Heydt *et al*., 2011). Figure 1.7 illustrates the sequential steps in the activation of mycotoxin biosynthesis genes via a signal transduction pathway. Generally global regulatory genes are influenced which firstly transmit the signal to more specialized transcription factors, which by itself activates the mycotoxin biosynthetic structural genes.

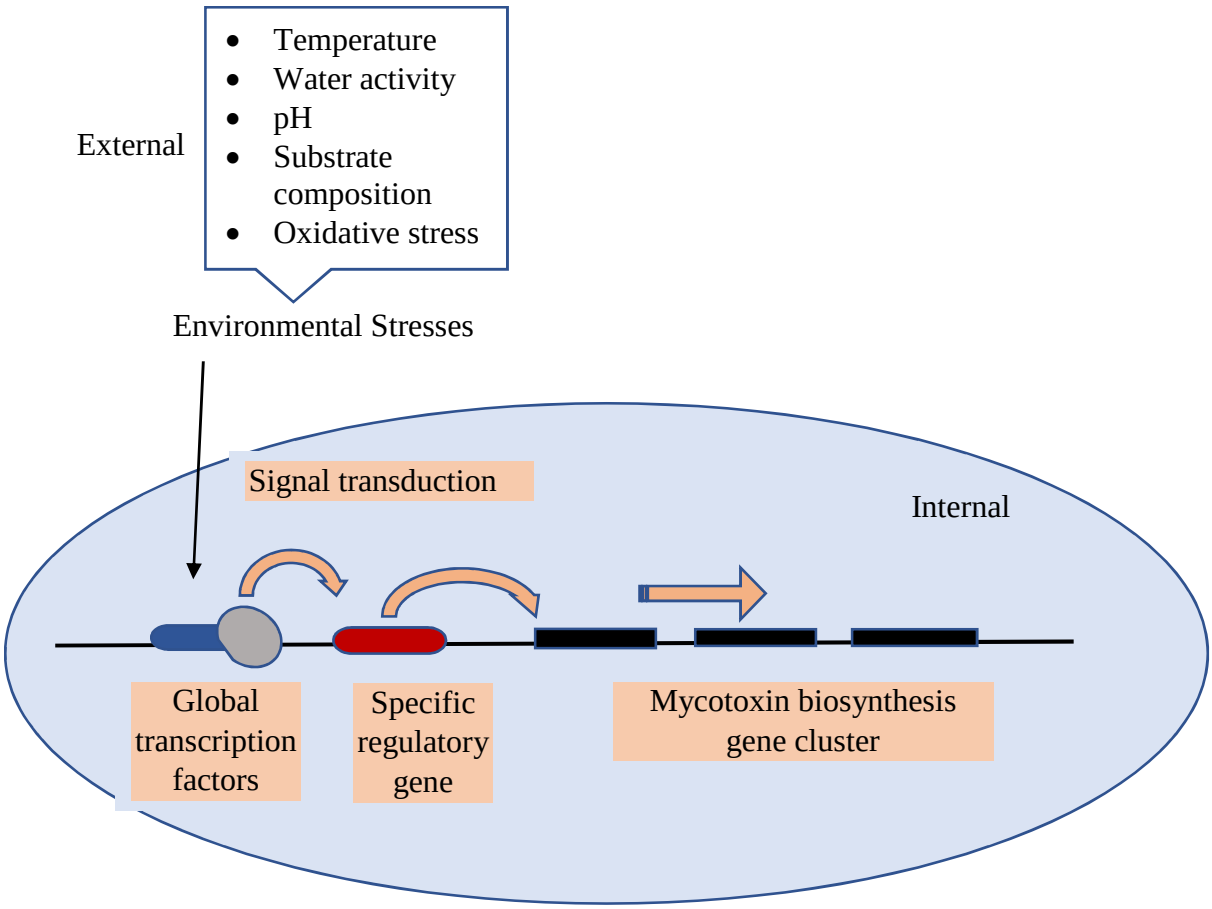

Figure 1.7 Diagram of the influence of external stress parameters on the activation of mycotoxin biosynthesis genes via a signal transduction pathway (modified from Schmidt-Heydt *et al*., 2011)

In addition to other studies, Geisen *et al*. (2006) suggested that the optima expression profile of the different genes occurs consecutively in the order of the biochemical reactions: the polyketide synthase gene, the non-ribosomal peptide synthase, the putative chlorinating enzyme and the putative transport protein. Figure 1.8 illustrates the consecutive profile of OTA biosynthesis in these two *Penicillium* species.

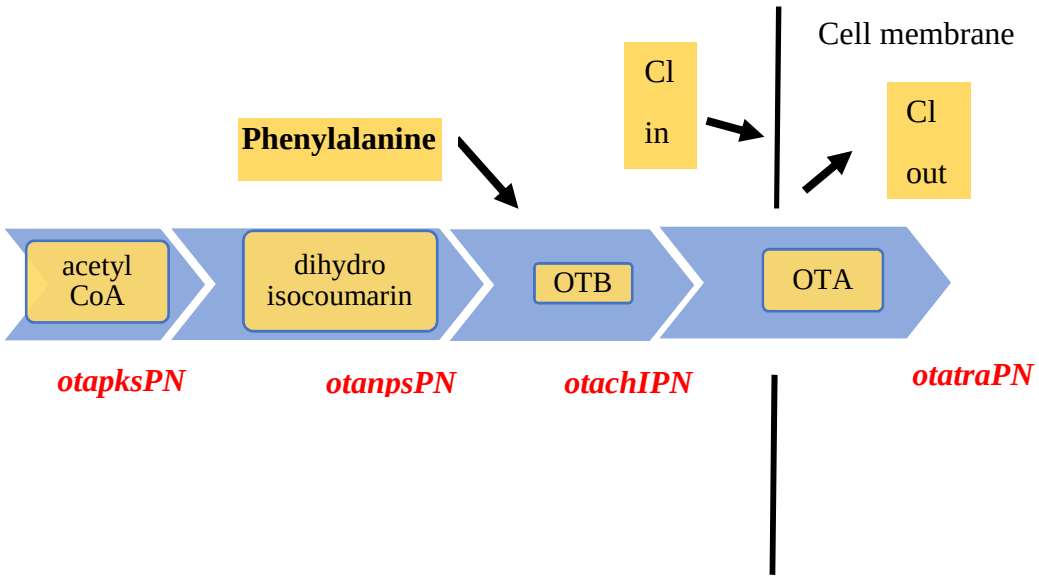

Figure 1.8 Suggested pathway of OTA biosynthesis in *Penicillium verrucosum* and *P. nordicum* (modified from Geisen *et al*., 2006).

1.5.3. Effect of environmental factors on gene expression and OTA biosynthesis of ochartoxigenic fungi

Abiotic factors such as temperature, a_w, and pH have a strong influence on the expression of mycotoxin biosynthesis genes (Magan *et al*., 2013). This is in agreement with the studies by Schmidt-Heydt *et al*. (2008) who evaluated the general expression profile of the *otapks*PV gene of *P. verrucosum* in relation to abiotic factors. They reported that the major gene expression peak was at 0.98 a_w, whereas optimal growth was at 0.99 a_w. The same trend was with the temperature as 25°C was optima for *otapks*PV expression whereas 30°C for growth. Also, optimum pH for *otapks*PV expression was at 8 whereas for growth this was at pH 6. Furthermore, they reported that the major and minor peaks of OTA production were found at the same parameter values as

the gene expression peaks. Hence, a_w and temperature had a greater impact on *otapks*PV gene expression compared with pH.

It has been shown that OTA biosynthesis in *A. ochraceus* is affected by the pH of the growth medium, as higher levels were produced in the lower pH range (O'Callaghan *et al*., 2006). Hence, the transport of substrates in *A. ochraceus* is affected by pH where the rate of uptake of glutamate was dependent on culture pH and production of OTA was reduced where uptake of glutamate was low (Geisen, 2004). Thus, at higher pH values the uptake of substrates essential for OTA biosynthesis may be reduced or that primary metabolism in the fungus may be affected so that the availability of acetate for OTA biosynthesis is reduced. O'Callaghan *et al*. (2006) suggested that the effect of pH is mediated at the level of gene transcription. Moreover, they found that the reduced production of OTA at higher pH values is accompanied by a reduction in *pks* gene transcript accumulation.

Thus, mycotoxin biosynthetic genes are often co-ordinately regulated (Kato *et al*., 2003) and arranged in clusters (Yu *et al*., 2004). Studies by O'Callaghan *et al*., (2006) showed that both of the putative p450 genes were co-regulated with the *pks* gene, exhibiting increased levels of expression in a restrictive medium supplemented with yeast extract and decreased levels of expression at higher pH values.

Not only environmental parameters like temperature and a_w are important, but also the nutritional composition of the substrate plays a role. Also, whether ionic or non-ionic solutes are added will also affect OTA biosynthesis by the toxigenic species. In an attempt to examine the effect of NaCl rich environments on OTA biosynthesis and gene activation, studies performed by Schmidt-Heydt *et al*. (2011) on *P. nordicum* grown on YES medium with increasing concentrations of NaCl showed that the highest production of the toxin was in the range between 10 and 30 g/L NaCl. They showed that the concentration of NaCl and thus a_w had a strong impact on OTA biosynthesis and gene activation.

The ability of *P. verrucosum* to adapt to such high ionic stress conditions contributes to the presence of the putative chlorinating and transport activity at the very end of the pathway of gene activation. This mechanism probably accounts for the continuous production and excretion of OTA that leads to the continuous flow of chloride out of the cell that ensures chloride homeostasis within the cell, especially at higher chloride concentrations. This is considered to be the mechanism responsible for production of OTA and the ability of these species to grow in high osmotic concentrations (Schmidt-Heydt *et al*., 2011).

As mentioned before, fungi have different signal cascades that are activated by different signals. The cascade which is activated by high osmolarity in the medium is the HOG pathway (high osmolarity glycerol). It was found that there is a paralleled activation of HOG phosphorylation and of OTA biosynthesis. This was confirmed in *P. nordicum* which is adapted to high salt and other extreme conditions where there is a steady phosphorylation of HOG in NaCl rich environments, and this is paralleled with high production of OTA (Sonjak *et al*., 2011). Similarly, *P. verrucosum* is also able to adapt to dry cured or salted food environments due to this mechanism where phosphorylation occurs only at higher concentrations of NaCl that activates OTA biosynthesis (Stoll *et al*., 2013). However, it has to be kept in mind that OTA can be synthesised by other species when HOG phosphorylation is low or is not activated, especially under glucose conditions, than in high NaCl conditions. This is clearly so in *A. carbonarius* where OTA biosynthesis occurs at lower concentrations of NaCl. This indicates that perhaps other factors may also play a role in the regulation of OTA biosynthesis in some OTA producing species (Stoll *et al*., 2013).

1.6. Molecular detection of ochratoxigenic fungi and genes involved in ochratoxin A biosynthesis.

A real time PCR (RT-PCR) method has now become an established DNA-based technique which provides a tool for fungal detection and quantification by monitoring and quantifying their DNA. The chemical markers used in RT-PCR are SYBR Green 1 dye (Gonzalez *et al*., 2009) and TaqMan (Livak *et al*., 1995).

One of the major advantages of the development of PCR based systems allows the early indication of the switching on of the biosynthetic genes involved in phenotypic OTA contamination of key food commodities. Farber and Geisen (2004) demonstrated a positive correlation between OTA content and DNA quantity by using real time PCR for both *P. nordicum* and *A. ochraceous*. This correlation was established for the mycotoxin biosynthesis genes with the quantitative real-time PCR (Geisen *et al*., 2004) and using primers targeting sequences of the housekeeping genes for comparison as a marker (Mule *et al*., 2006).

As mentioned before, the product of the *otapks* gene, a polyketide synthase, is the key enzyme responsible for the biosynthesis of OTA. Thus, other studies have reported the uses of *otapks PN* sequence to monitor the growth and OTA production of *P. nordicum* by RT-PCR. (Geisen *et al*., (2004; Farber and Geisen, 2004). Furthermore, they demonstrated a strong correlation between the copy number of *otapksPN* gene and the colony forming units (CFUs) of the toxigenic species.

Studies conducted by O'Callaghan *et al*. (2013) demonstrated that the *pks* gene is the key one responsible for OTA biosynthesis. They used a suppression subtractive hybridization PCR-based approach and revealed that the *pks* gene was expressed only under OTA permissive conditions and during the early stages of the mycotoxin synthesis. Moreover, they showed that a mutant which had the *pks* gene blocked was unable to synthesize OTA.

There are many systems of PCR-based detection and quantification of OTA including AFLP (Amplified fragment length polymorphism), RAPD (Random-amplified-polymorphism DNA) marker primers based or using mycotoxin biosynthetic gene for identifying ochratoxigenic fungi. The choice depends on the goal of the study (El Khoury and Atoui, 2010).

For example, AFLP and RAPD could be the best choice when the purpose of the study is (1) to discriminate between relevant OTA producer species or (2) to show the polymorphism of some isolated strains belonging to the same species. However, when dealing with the detection of the fungus, the best way is to conduct conventional PCR using primers designed from housekeeping genes or mycotoxin biosynthetic genes as described in the previous section (see El khoury and Atoui, 2010).

Regarding the molecular techniques used in food systems, Real time PCR technology provides an advantage over AFLP and RAPD in terms of determining the mycotoxigenic status of food sample. In addition, it has the power to estimate the mycotoxin content. However, for AFLP and RAPD techniques the isolation of the DNA and the quality of the extracts are critical.

1.7. Strategies for reducing OTA production

1.7.1. Food preservatives

Preservatives such as Calcium propionate (CP) and potassium sorbate (PS) are used to control the growth of mycotoxigenic spoilage fungi, although consumer pressure is to reduce the use of these preservatives and replace them with other more natural ones. Since these preservatives are fungi stats, they need to be effectively mixed to ensure that there are no untreated pockets which may allow growth of spoilage fungi (Magan and Aldred, 2006). Moreover, it has been shown that sub-optimal levels stimulate growth and mycotoxin production by *P. verrucosum* strains (Arroyo *et al*., 2005). It has also been shown that both CP and PS over a range of low concentrations (150 ppm in the case of CP and 150-300ppm in the case of PS) can cause an upregulation of the *otapks*PV gene and subsequently an increase in OTA production. Thus, reduction of the use of

such fungistats would lead to problems with mycotoxin contamination, especially in bakery products (Schmidt-Heydt *et al*., 2007).

Aldred *et al*. (2008) showed that essential oils were not effective in controlling growth of *P. verrucosum* on wheat grains. In contrast, the antioxidant resveratrol was more effective in controlling growth and OTA contamination of stored wheat. In addition, some essential oils at low concentrations were able to stimulate mycotoxin production by this species. There is thus interest in examining alternative compounds which may be potentially useful to control growth of ochratoxigenic fungi and more importantly OTA contamination in cereals and coffee.

1.8. Objectives of the project

This project examined the water relations and ecology of two important OTA producing fungi (*P. verrucosum, A. westerdijkiae*) responsible for contamination of stored wheat and coffee. The work examined, for the first time, the impact of solute (ionic and non-ionic) and matric potential (PEG 8000) stress on growth, expression of key OTA cluster genes and toxin production by these two species and the relationship with phenotypic OTA production. In addition, the effects of interacting abiotic climate change related factors (a_w, temperature and elevated levels of CO_2) were examined to evaluate the impact on growth and toxin production under both solute and matric potential stress. Finally, a number of potential preservatives were screened for potential control of these ochratoxigenic species in terms of growth and toxin production *in vitro* and *in situ* to evaluate whether they could be used practically to minimise contamination of these commodities.

The studies that were carried out included:

Phase I

Chapter 2

Effect of interactions between water stress and temperature on lag phases prior to growth, growth and OTA production by *P. verruocosum* and *A. westerdijkiae* strains *in vitro*.

(i) Effect of temperature and a_w on lag phases prior to growth, growth and OTA production by *P. verrucosum* strains on a conducive YES medium.

(ii) Effect of interacting a_w x temperature conditions on growth and OTA production on wheat-based media

(iii) Relationship between these interacting abiotic factors and growth and OTA production by strains of *A. westerdikiae* on a conducive YES medium

(iv) Effect of a_w x temperature effects on colonisation and OTA production by *A. westerdijkiae* strains on a coffee-based medium.

Phase II

Chapter 3

Comparison of effect of solute and matric stress on growth, key biosynthetic genes and phenotypic OTA production by *P. verrucosum* and *A. westerdijkiae*

i) Comparison of solute and matric potential stress on growth and key OTA gene expression (*otapks*, *otanrps*) of *P. verrucosum* (strain OTA11).

ii) Solute and matric potential stress on phenotypic OTA production by *P. verrucosum*.

iii) Comparison of solute and matric potential water stress on growth and gene expression by *A. westerdijkiae* (strain CECT).

iv) Relationship between solute and matric potential water stress and phenotypic OTA production by *A. westerdijkiae* (strain CECT).

Phase III

Chapter 4

Effect of interacting climate change abiotic factors on growth and biosynthetic genes involved in OTA production by *P.verrucosum* and *A. westedijkiae*

i) Effect of water potential x elevated CO_2 x temperature on growth, gene expression and OTA production by *P. verrucosum* on milled wheat-based media.

ii) Impact of three-way climate change abiotic factors on growth, gene expression and OTA production by *A. westerdijkiae* on milled coffee-based media.

Phase IV

Chapter 5

Effect of preservatives to control growth and OTA production of *P. verrucosum* and *A. westerdijkiae in vitro* and *in situ*.

i. Screening of 6 different preservatives for controlling growth of *P. verrucosum* on wheat-based matrices.

ii. Determination of the ED_{50} and ED_{90} concentrations of preservatives for control of growth and OTA production by *P. verrucosum in vitro* under different a_w conditions

iii. Effect of the best preservatives on colonisation and OTA production by *P. verrucosum* in stored wheat at different a_w levels.

iv. Screening of 6 preservatives for control of growth of *A. westerdijkiae in vitro* on milled coffee-based media.

v. Determination of the ED_{50} and ED_{90} concentrations of the preservatives on the growth and OTA production by *A. westerdijkiae.*

vi. Effect of the best preservatives on the colonisation and OTA contamination by *A. westerdijkiae* in stored coffee beans.

Chapter 6

Conclusions and future work

CHAPTER 2

Effect of water stress and temperature on growth and OTA production by *P. verrucosum* and *A. westerdijkiae in vitro*.

2.1 Introduction

Ochratoxin A (OTA) was originally discovered as a toxic metabolite of *Aspergillus ochraceous* (= *A.westerdijkiae*) (Van der *et al*., 1965). It was subsequently found to be produced by both *Aspergillus* and *Penicillium* species. It is present in a wide variety of food commodities and is thus of economic importance. For example, *P. verrucoum* is responsible for OTA contamination of raw cereal commodities and cereal-based products, *P. nordicum* contaminates some dry-cured meats, *A. carbonarius, A. westerdijkiae* (*= A. ochraceous),* and *A. steynii* contaminate coffee, cocoa, spices, and dried fruits (Frisvad *et al*., 2011; Lund and Frisvad, 2003; Magnoli *et al*., 2007; Perrone *et al*., 2007; Sanchez-Hervas *et al*., 2008; Taniwaki *et al*., 2003). OTA is a potent nephrotoxin that has hepatotoxic, teratogenic, immunosuppressive, and carcinogenic effects (Abouzied *et al*., 2002; EFSA, 2006; EFSA, 2010; FAO/WHO, 2001; Petzinger and Ziegler, 2000; Pfohl-Leszkowicz and Manderville, 2007). Thus, the International Agency for Research on Cancer (IARC) has classified this toxin as a class 2B human carcinogen (IARC, 1993).

Magan and Lacey (1986) showed that fungal growth and mycotoxin production were markedly influenced by different environmental factors, the two most important being water availability (water activity, a_w) and temperature. The interaction of these two abiotic factors influence germination, growth, sporulation and mycotoxin production (Sanchis and Magan, 2004). Changes in a_w and temperature have been shown to affect the growth of *A. ochraceus* (*=A. westerdijkie*) and *A. niger* isolates (Ayerst, 1969; Marin *et al*., 1998) and the ability to compete with other spoilage fungi and influence OTA production (Ramakrishna *et al*., 1996; Magan *et al*., 2003). It has been shown that a_w and temperature are important criteria for understanding the ecology of mycotoxigenic species. The interaction between these variables determines whether mould growth will occur and, if so, the relative development of the fungal community. It is very important to determine the optimum and marginal conditions for growth and OTA production by species such as *P. verrucosum* as it can be used to provide guidelines of the level of risk of contamination of stored cereal commodities.

The objectives of this study were to compare the effect of interactions between water availability and temperature on: (a) growth of different isolates of *P. verrucosum* on a conducive YES and milled wheat agar media; (b) growth of isolates of *A. westerdijkiae* on a conducive YES medium and (c) a comparison of the effect of these interacting abiotic conditions on OTA production on the conducive YES medium.

2.2 Materials and methods

2.2.1. Fungal strains

Two strains of *P. verrucosum* (P.V OTA11, P.V STRAW 21) and three strains of *A. westerdijkiae* (A.W VMSO, A.W CCT 6795 and A.W CECT 2948) were used in this study. The former strains were isolated from wheat grain and wheat straw respectively, and the latter ones all from coffee. We are grateful to Dr Monica Olsen (National Food Authority, Sweden) and Dr. M.H. Taniwaki (ITAL, Campinas, Brazil) for the supply of the strains.

2.2.2. Inoculum preparation and inoculation

Fungal strains were sub-cultured on malt extract agar (30.0 g malt extract, 5.0g peptone and 15g agar) at 25°C in the dark for up to 10 days. The spores were gently dislodged from the colony surface by using a surface sterilised loop and placing them into suspensions of 9 ml sterile distilled water containing 0.05 % Tween-80 in 25ml Universal bottles. The suspensions were shaken and then the spore concentration determined using a haemoEffect cytometer and adjusted to 10^6 spores/ml. This was used for inoculation.

2.2.3. Effect of water activity x temperature conditions on the growth of the ochratoxigenic fungal strains and species

All strains were inoculated on a yeast extract sucrose (YES) medium (20g/l yeast extract, 150g/l sucrose, 20g/l agar) after adjusting a_w levels using glycerol/water solutions to obtain target treatments of 0.98, 0.95 and 0.90 a_w. Media were sterilised by autoclaving at 121°C, for 1h. Molten cooled media were poured into 9 cm Petri dishes. The a_w of the treatments were checked using an Aqua lab (TE4) (Decagon Devices. Pullman, WA99163 USA) instrument for confirmation of the actual a_w levels.

The treatments and replicates were inoculated with 5 µl of the spore suspension and incubated at 15, 20, 25, 30 and 35°C. The experiments were carried out with at least four replicates per treatment.

2.2.4. Effect of water activity x temperature conditions on growth and OTA production by *Penicillium verrucosum* strains on wheat-based media

For this study 2% (w/v) milled wheat agar (1.5% w/v technical agar No. 3, Oxoid LTD, Hampshire, UK) was used as the basic medium (0.995 a_w). The a_w of the basic medium was modified to 0.85, 0.90, 0.95, 0.98 a_w using glycerol/water solutions (Magan *et al*., 2003). Media were sterilized at 121ºC and then cooled to 45-50 ºC before pouring into 9 cm sterile Petri plates.

2.2.5. Growth assessment

Colony diameters of four replicate plates were measured in two directions at right angles to each other. Measurements were recorded daily during growth for up to ten days and the growth rate was calculated by plotting the radial mycelial growth against time and the slope of the linear growth phase was used to obtain the radial growth rates (mm/day; Patriarca *et al*., 2001).

2.2.6 Quantification of OTA production

The treatments and replicates were harvested after 11 days. Plugs were taken across the diameter of the colony using a surface-sterlised cork borer (6 mm diameter). The five plugs were placed into 2 ml Eppendorf tubes and weighed. OTA was extracted by adding 1 ml HPLC grade methanol and shaken for 1 hour. The plugs were discarded after centrifugation. The extracts were filtered directly into amber (0.22 µm) HPLC vials. The conditions for OTA detection and quantification were as follows:

Mobile Phase	Acetonitrile (57%): Water (41%): Acetic acid (2%)
Column	120CC-C18 column (Poroshell 120, length 100ml, diameter 4.6mm, particle size 2.7 micron;600 Bar).
Temperature of column	25ºC
Excitation	330nm
Emission	460nm
Flux	1ml min^{-1}
Retention time	Approximately 2.6 min
Run time	10 min

2.2.7 Statistical analyses of data

For all experiments, four replicates were used for both growth studies and OTA production (Table 2.1). The normality of the data was checked using the Shapiro test and homogenecity was checked using the Levene test. The factors and response and their interaction were examined by the Kruskal-Wallis (non-parametric) if the data were not normally distributed. For normally distributed data, the data sets were analysed using ANOVA (JMP software). The statistical significant level was set at $p<0.05$ for all single and interacting factors.

Table 2.1. Summary of the single and interacting factors used in the experimental design for this experiment.

Factor	Level				
Water activity (a_w)	0.98	0.95	0.90		
Temperature (°C)	15	20	25	30	35
Species and strains: *P. verrucosum*	PV OTA11	PV STRAW21			
A. westerdijkiae	AW CCT	AW CECT	AW VMSO		

2.3. Results

2.3.1 Effect of temperature and water activity on growth of *P. verrucosum* strains on YES medium.

Figure 2.1. compares the growth rates in relation to water availability at three different temperatures using media modified with glycerol for strain OTA11. This showed that the optimum temperature for growth was 25ºC regardless of the level of water stress imposed. The optimum a_w for growth was 0.98, followed by 0.95 and 0.90 a_w. Although OTA11 grows over a wide range of temperatures and water stress conditions, statistically the imposed water stress had a significant effect on the growth rate. However, there was no significant difference between the different temperatures examined (Table 2.2).

Similar patterns in relation to a_w and temperatures were observed for the Straw21 strain, where the temperatures applied had no significant effect on growth rate, while there was a significant effect of all a_w treatments (Figure 2.2).

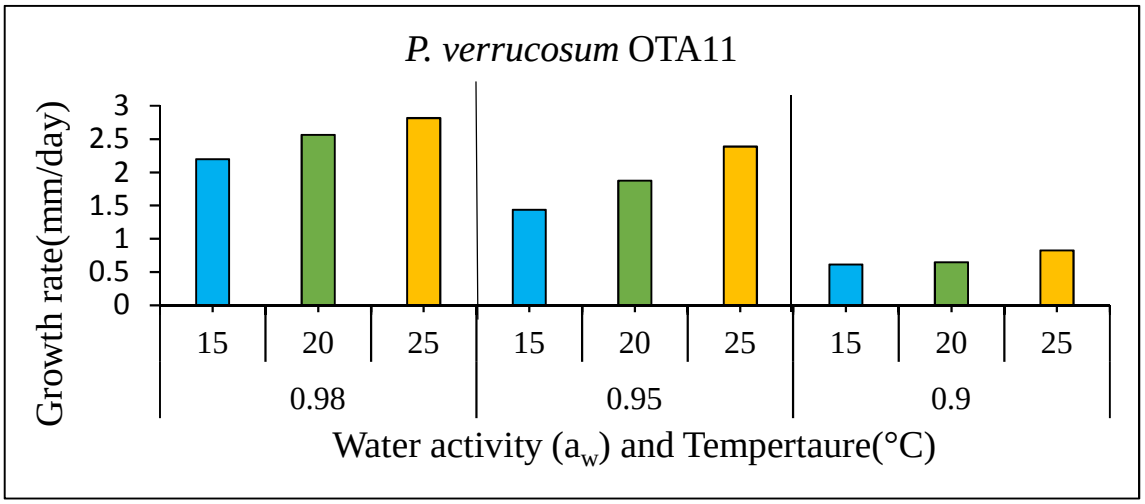

Figure 2.1. Effect of temperature and water activity on radial growth rate (mm/day) of *Penicillium verrucosum* (isolate OTA11) on YES medium modified with glycerol to obtain target a_w levels.

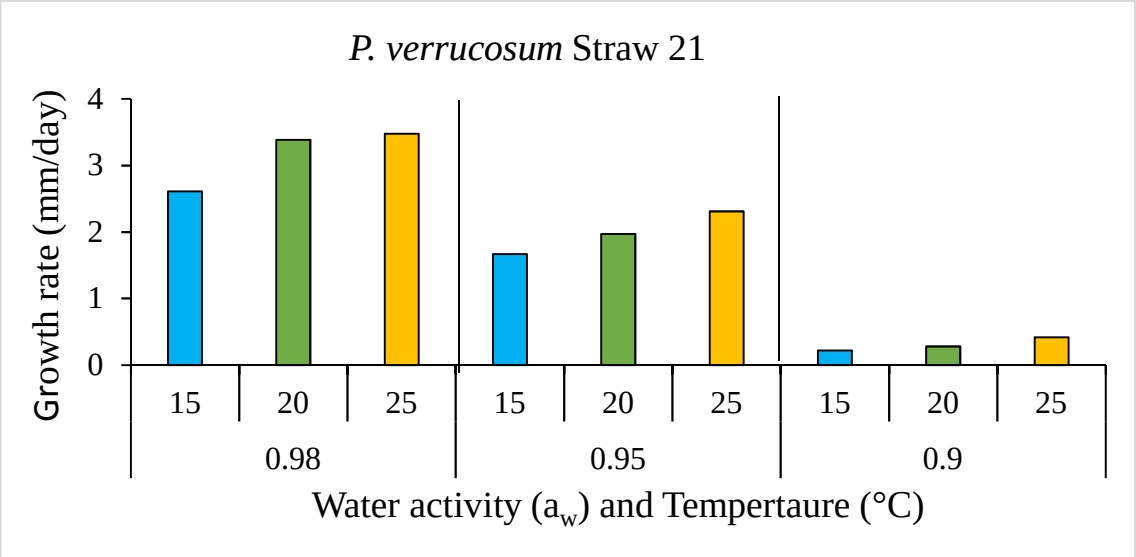

Figure 2.2. Effect of temperature and water activity on radial growth rate (mm/day) of the *Penicillium verrucosum* strain (straw 21) on YES medium modified with glycerol to obtain the target a_w levels.

Table 2.2. The lists the *p*-values for the growth rate two strains of *P.verrucosum* in relation to different a_w and temperature conditions using the Kruskal-Wallis Test (non- normality data).

Strain	a_w	Temp (°C)	a_w×Temp (°C)	Straw21×OTA11	Response
OTA11	$p<0.05^{*}$	$p>0.05^{**}$	N/A	$p>0.05^{**}$	Growth rate
Straw21	$p<0.05^{*}$	$p>0.05^{**}$	N/A		Growth rate

p values of 0.05 or less are often considered evidence that there is at least one significant effect in the model.

*Significant; **Non-significant; N/A Not Applicable.

2.3.2 Effect of water activity x temperature conditions on OTA production by *Penicillium verrucosum* strains on YES medium.

Comparison of OTA production by the two strains of *P. verrucosum* in relation to three a_w levels at different temperatures after 11 days incubation is shown in Table 2.5. Significant difference between the amounts of OTA produced by strain OTA11 under different a_w levels was found. However, temperatures had a no significant effect on toxin production. Overall, *P. verrucosum* (strain OTA11) produced higher amounts of OTA than the other strain isolated from crop residue at all temperatures and a_w levels examined. The strain Straw21 produced no OTA at 0.90 a_w regardless of the temperature, and at 0.98a_w and 15°C, and this treatment was excluded from the statistical analyse.

Table. 2.3. Ochratoxin A production (µg/g) by strains of *P.verrucosum* in relation to a_w × temperature conditions when grown on yeast extract sucrose medium.

Temperature	15°C	20°C	25°C
Strain	**OTAµg/g ±SD**	**OTAµg/g ±SD**	**OTAµg/g ±SD**
P.verrucosum			
(OTA11) 0.98	43.95±11.12(3)	33.67±4.46 (3)	100.87±15.8(3)
0.95	4.87±3.9 (3)	0.43±0.06 (3)	36.9±8.8 (3)
0.90	1.34±1.4 (3)	0.0772±0.075(3)	0.90±0.73(3)
Straw21			
0.98	No toxin	0.54±0.123 (2)	1.4±0.1 (3)
0.95	0.025±0.003(2)	0.1±0.02 (3)	0.09±0.1 (3)
0.90	No toxin	No toxin	No toxin

The numbers of replicates used for calculation are in parentheses; SD, standard deviation.

Table 2.4. The lists the *p*-value for the OTA production by two strains of *P.verrucosum* in relation to different a_w and temperature conditions using the Kruskal-Wallis Test (non- normality data) and ANOVA (parametric data).

Strain	a_w	Temp (°C)	a_w×Temp (°C)	Straw21×OTA11	Response
OTA11	$p<0.05^{*k}$	$p>0.05^{a**}$	N/A		OTA
Straw21	$P<0.05^{*a}$	$p>0.05^{**K}$	N/A	$p<0.05^{*k}$	OTA

p values of 0.05 or less are often considered evidence that there is at least one significant effect in the model.

*Significant; **Non-significant; N/A Not Applicable, [a] ANOVA, [k] Kruskal-Wallis.

2.3.3. Effect of water activity x temperature conditions on lag phases prior to growth and growth of *P. verrucosum* strains on wheat-based media.

Figure 2.3 and 2.4 show the relative growth rates of the two strains of *P. verrucoum* together with the lag phases on a 2% milled wheat medium at 15, 20, 25°C. They all grew faster at 25°C with optimum growth at about 0.98 a_w while no growth occurred at 0.85 a_w at all temperatures. Statistical analyses showed that a_w significantly affected relative growth rates by ANOVA (parametric test) and Kruskal-Wallis test for OTA11 and straw21 strains respectively. In contrast, temperature had no significant impact on the growth rate of both strains.

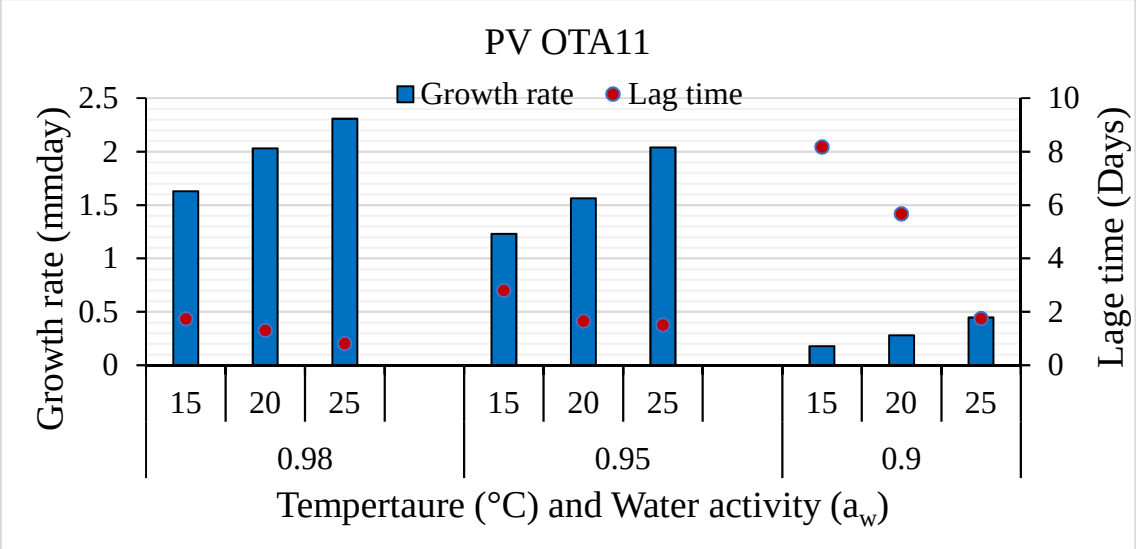

Figure 2.3. Comparison of the water activity and temperature effects on the lag phase (ƛ, days) and growth rate of *P. verrucosum* (OTA11).

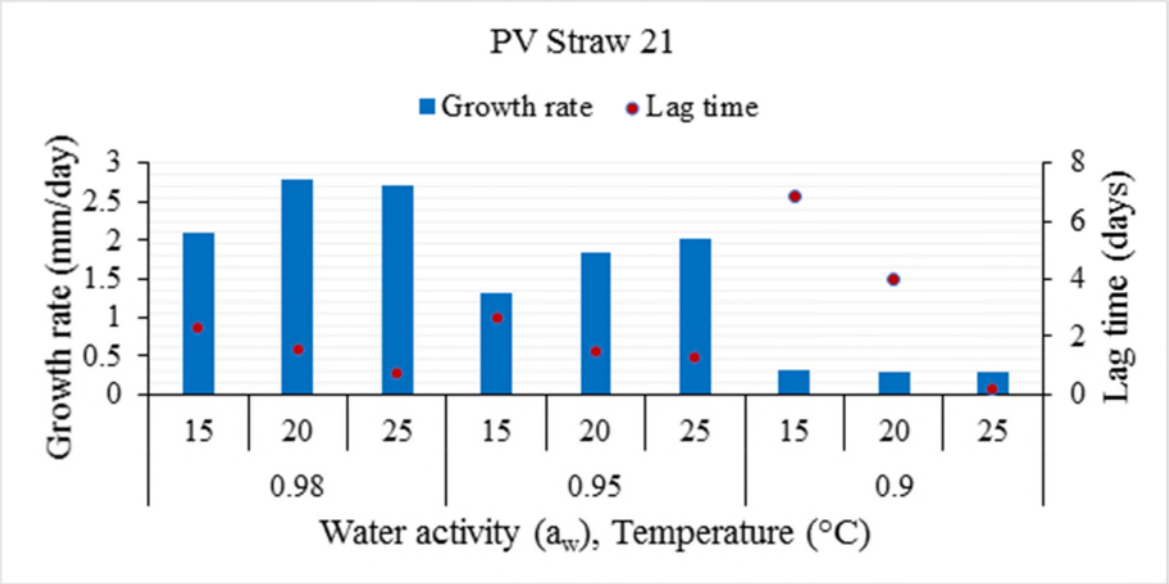

Figure 2.4. Comparison of the water activities and temperatures effects on lag phases (ƛ, days) and growth rate of *P. verrucosum* (Straw 21).

2.3.4 Effect of water activity x temperature conditions on OTA production by *P. verrucosum* strains on wheat-based media.

Tables 2.5 and 2.6 compares the OTA production by the two strains after 11- and 20-days incubation respectively. This showed that most OTA was produced by strain OTA11 after 11 days incubation. Meanwhile, significant amounts of toxin were produced at 0.95 a_w and 25°C after 20 days by strain OTA11. At 0.90 a_w and all temperatures for both strains, no toxin was detected and was thus excluded from the statistical analyses.

Table 2.5. Ochratoxin A production (µg/g) by strains of *P. verrucosum* in relation to a_w × temperature when grown on a 2% wheat medium after 11 days.

Temperature	15°C	20°C	25°C
Strain	**OTAµgg$^{-1\pm SD}$**	**OTAµgg$^{-1\pm SD}$**	**OTAµgg$^{-1\pm SD}$**
P.verrucosum			
(OTA11)	LOQ	0.73±0.26 (2)	0.82±0.28(3)
0.98	LOQ	0.044±0.02 (3)	0.18±8.8 (3)
0.95	No growth after 11	so no toxin	
0.90	days	detected	
Straw21			
0.98	0.014±0.006 (3)	0.069±0.01 (4)	0.19±0.002 (3)
0.95	LOQ	0.14±0.005(4)	0.027±0.011(3)
0.90	No growth after	So, no toxin	
	11 D	detected	

The numbers of positives used for calculation are in the parentheses; SD, standard deviation; <LOQ: below the level of quantification.

Table 2.6. Ochratoxin A production (µg/g) by strains of *P. verrucosum* in relation to a_w × temperature when grown on a 2% wheat medium after 20 days.

Temperature		15°C	20°C	25°C
Strain		**OTAµgg$^{-1\pm SD}$**	**OTAµgg$^{-1\pm SD}$**	**OTAµgg$^{-1\pm SD}$**
P.verrucosum				
(OTA11)		LOQ	0.65±0.006 (2)	0.63±0.13(3)
0.98		0.22±0.1(3)	0.63±0.085 (3)	6.81±2.6 (4)
0.95		0.03±0.022(4)	0.01±0.002(3)	0.59±0.26(3)
0.90				
Straw21	**0.98**	0.032±0.01(4)	0.19±0.03(4)	0.25±0.035(4)
	0.95	0.01±0.002(4)	0.01±0.003(4)	0.03±0.011(3)
	0.90	0.028±0.002(3)	0.25±0.005(2)	23.4±2(2)

The numbers of positives used for calculation are in parentheses; SD, standard deviation; <LOQ: below the level of quantification.

Table 2.7. The summary *p*-values for the growth rate and lag phases and OTA production by two strains of *P. verrucosum* on wheat media using the Kruskal-Wallis Test (non-normality data) and ANOVA (normality data).

Strain	a_w	Temp (°C)	a_w×Temp (°C)	Straw21×OTA11	Response
OTA11	$p<0.05^{*}$	$p>0.05^{**k}$		$p>0.05^{**k}$	Growth rate
	$p<0.05^{*k}$	$p<0.05^{*k}$		$p>0.05^{**k}$	Lag phase
	$p>0.05^{**k}$	$p<0.05^{*k}$	N/A	$p<0.05^{*k}$	OTA (11D)
	$p<0.05^{*k}$	$p<0.05^{*k}$		$p<0.05^{*k}$	OTA (20D)
Straw21	$p<0.05^{*}$	$p>0.05^{**a}$			Growth rate
	$p>0.05^{**k}$	$p<0.05^{*k}$			Lag phase
	$p<0.05^{*a}$	$p>0.05^{**k}$	N/A		OTA (11D)
	$p<0.05^{*k}$	$p>0.05^{**k}$			OTA (20 D)

p values of 0.05 or less are often considered evidence that there is at least one significant effect in the model.

*Significant; **Non-significant; N/A Not Applicable, ^{k} Kruskal -Wallis, ^{a} ANOVA.

2.3.5 Effect of water activity x temperature conditions on growth of *Aspergillus westerdijkiae* strains on YES medium.

The maximum growth rates were observed at temperature levels of 25-30°C at 0.98 a_w for *A.westerdijkiae* (strain VMSO; Figure 2.5). Statistically, a_w had a significant effect on the growth rate (Table 2.8). The optimum temperature for growth was dependent on the a_w level. Thus at 30-35°C, growth rates were higher at 0.95 a_w, while at 0.90 a_w the growth rates were slower at 30 and 35°C.

Figure 2.5 compares the growth rates in relation to water availability at three different temperatures using media modified with glycerol for *A. westerdijkiae* strain CCT 6795. Maximum growth occurred at 30°C and 0.98 a_w with a decrease under slightly dryer conditions (0.95 a_w) at 30°C. Growth was slowest at 0.90 a_w regardless of the temperature tested. Statistically, the imposed water stress had a significant impact on growth rate, whereas the temperature change showed no significant effect on the growth (see Table 2.8).

For strain CECT 2948 of this species growth was best at 30°C followed by 25°C at the same a_w 0.98; see Figure 2.5). There was no significant difference at 0.98 and 0.95 a_w on the relative growth rate.

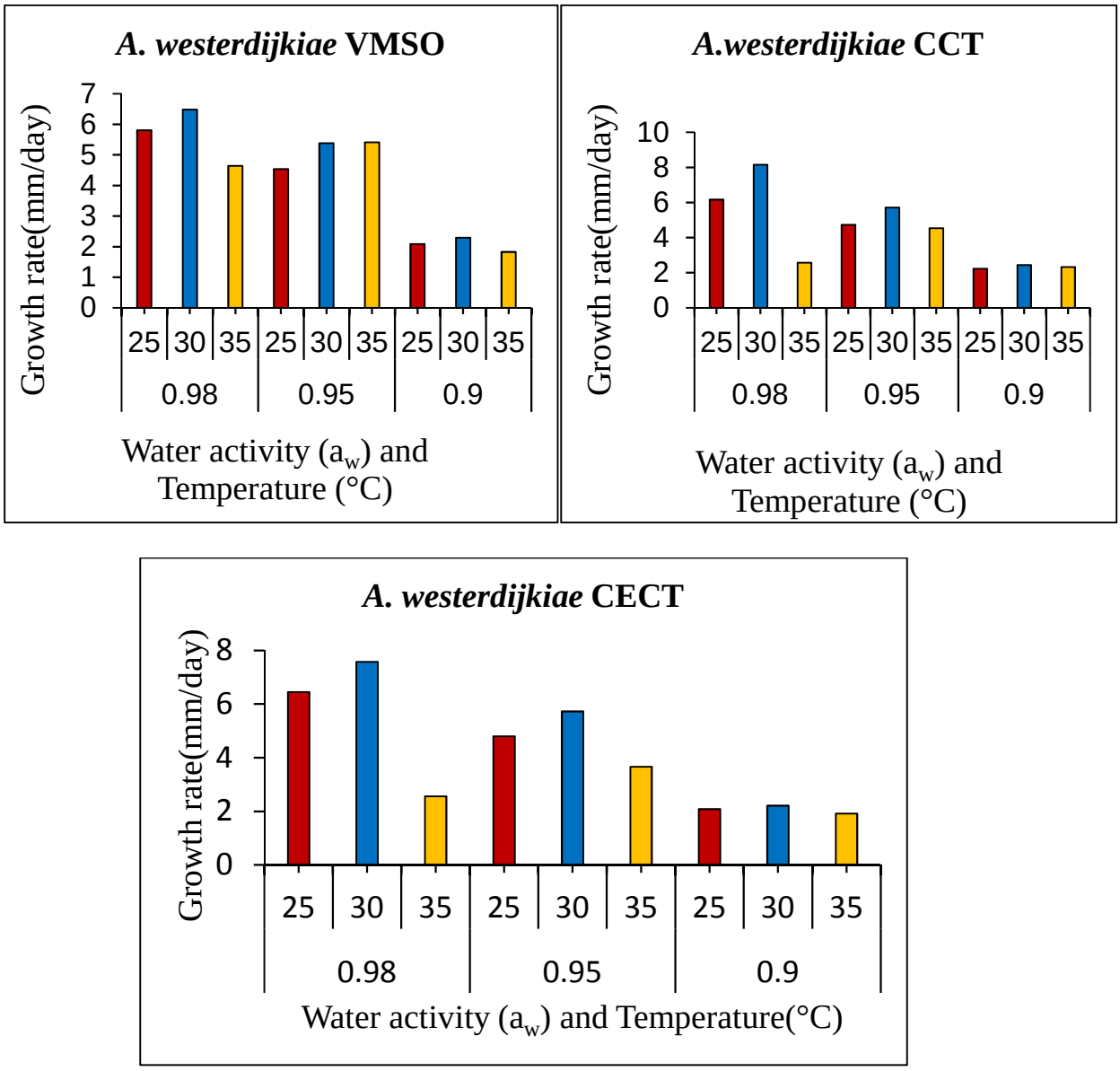

Figure 2.5. Comparison of the effect of the water activity x temperature on growth rate of the three strains of *A. westerdijkiae* (VMSO; CCT6795; CECT2948) on YES modified medium with glycerol.

Table. 2.8. The lists the *p*-value for the growth rate of the three strains of *A.westerdijkiae* in relation to different a_w and temperature conditions using Kruskal-Wallis Test (non- normality data).

Strain	a_w	Temp (°C)	a_w x Temp	VMSO x CCT x CECT	Response
VMSO	$p<0.05^{*}$	$p>0.05^{**}$	N/A	$p>0.05^{**}$	Growth rate (mm/day)
CCT	$p<0.05^{*}$	$p>0.05^{**}$	N/A		
CECT	$p<0.05^{*}$	$p>0.05^{**}$	N/A		

p values of 0.05 or less are often considered evidence that there is at least one significant effect in the model.

*Significant; **Non-significant; N/A Not Applicable.

2.3.6 Effect of water activity x temperature conditions on OTA production by *Aspergillus westerdijkiae* strains on YES medium.

The strain CECT produced higher amounts of OTA at 0.98 a_w and 25°C (Table 2.9). Statistically, a significant difference in OTA production was found between 0.98 and 0.90 a_w at 30 and 35°C respectively. For the strain CCT, temperature had a significant impact on OTA production regardless of the imposed water stress. In contrast, for strain VMSO there was a significant effect of a_w regardless of temperature. Statistically, the OTA toxin production was excluded in the analyses at 0.98 and 0.95 a_w at 25°C and for the CCT strain at 0.98 a_w and 25ºC as they were below the level of quantification, respectively. Interestingly, there was a significant difference in the intraspecific toxin production between strains CECT and CCT and VMSO and CECT (see Table 2.10).

Table 2.9. Ochratoxin A production (µg/g) by strains of *A. westerdijkiae* in relation to a_w × temperature when grown on yeast extract sucrose medium.

Temperature		25°C	30°C	35°C
Strain		OTAµgg$^{-1\pm SD}$	OTAµgg$^{-1\pm SD}$	OTAµgg$^{-1\pm SD}$
A.westerdijkiae				
(VMSO)	**0.98**	LOQ	0.51±0.022(3)	0.42±0.13(3)
	0.95	LOQ	1.9e-5±3e-(2)	0.177±0.02(3)
	0.90	0.096±0.002(2)	0.093±0.018(3)	0.16±0.031(3)
CCT	**0.98**	LOQ	2.3±0.75(3)	0.29±0.011(3)
	0.95	1.5e-5±1.9e-6 (2)	1.49±0.28(2)	0.21±0.057(3)
	0.90	1.3e-5±2e-6 (2)	2.44±1.3(2)	1.8±0.27 (2)
CECT	**0.98**	35.23±0.75(3)	31.7±1.9(3)	2.1±0.29(2)
	0.95	3.5±0.75(3)	5.4±0.94(2)	8.1±2.6(2)
	0.90	2±0.56(3)	17.08 ±2.5(2)	0.70±0.02(2)

The numbers of positives used for calculation are in the parentheses; SD, standard deviation; <LOQ: below the level of quantification.

Table 2.10. The lists the *p*-value for the OTA production by the three strains of *A. westerdijkiae* in relation to different a_w and temperature conditions using the Kruskal-Wallis Test (non-normality data) and ANOVA test.

Strain	a_w	Temp (°C)	a_w x Temp	VMSO x CCT x CECT	Response
VMSO	$p<0.05^{*}$	$p>0.05^{**}$	N/A	$P<0.05^{*}$	OTA production (µg/g)
CCT	$p>0.05^{**}$	$P<0.05^{*}$	N/A		
CECT	$p<0.05^{*}$	$P<0.05^{*a}$	N/A		

p values of 0.05 or less are often considered evidence that there is at least one significant effect in the model.

*Significant; **Non-significant; N/A Not Applicable, [a]ANOVA.

2.4 Discussion

2.4.1 Effect of environmental parameters on growth and OTA production by *P. verrucosum* strains on YES and wheat agar medium respectively

The present study showed that environmental factors such as a_w and temperature play a crucial role in determining the growth of strains of *P. verrucosum.* The two strains showed the same trend in terms of optimum conditions for growth on YES and wheat agar media which was 25°C and 0.98 a_w. Pitt and Hocking (1977) classified *P. verrucosum* as a xerophilic spoilage fungus capable of growing at 0.80 a_w. Although the minimum a_w level was obtained for growth on YES in the current study, at 0.90 a_w, it was higher than that detected by previous studies using Czapek agar medium and BMEA (Barley Malt Extract Agar), as 0.88 and 0.85 a_w (Pardo *et al*., 2006). In the present study, the minimum a_w where growth was lower at all temperature conditions was 0.90 a_w. This is consistent with Marin *et al*. (1998) who reported that the minimum conditions for growth by some spoilage *Penicillium* species was also at 0.90 a_w on maize-based medium. Others have suggested that the nutritional status may influence the marginal conditions for growth under water stress conditions (Gqaleni *et al*., 1996) as well as influence the secondary metabolite production (Madhyastha *et al*., 1990). More recently, Rodriguez *et al*. (2015) compared the ability of both *P. nordicum* and *P. verrucosum* for growth and OTA production on cured-meat based media. In this study the ecological boundaries for growth of *P. verrucosum* were quite wide, while the conditions of OTA production were very narrow with a higher sensitivity to NaCl-modified media than *P. nordicum*. The relative differences ecologically have between these two OTA producing species was also been reviewed recently (Perrone *et al*., 2019).

The International Commission on Microbiological specifications for foods (ICMSF) (1996) classified *P. verrucosum* as a fungus which can grow from 0 to 31°C with an optimum at 20°C. Hill and Lacey (1984) reported fungal growth occurred at 2-20°C and 0.90-0.98 a_w levels. The current study demonstrated that the *P. verrucosum* strains can grow over a wide range of temperatures and water activities on YES and wheat agar media respectively. In this sense, Cairns-Fuller *et al*. (2005) have reported that optimum conditions for growth and OTA production were at about 25°C and 0.95 a_w.

This study has highlighted a consistent relationship between the relative growth rate and mycotoxin production on both YES and wheat agar media. The optimum conditions for OTA production in both media were at about 0.98 a_w and 25°C for both strains. However, the amount of OTA produced in wheat agar media was lower than that in YES medium. It is known that YES

is a conducive medium for secondary metabolite production. In addition, it is clear that the interacting abiotic conditions as well as nutritional status will determine the colonisation and mycotoxin production by these strains and most mycotoxigenic fungi (Payne, 1999; Cole, 1989). Several studies have reported YES medium as the best for OTA production (Skrinjar and Dimic, 1992; Bragulat *et al*., 2001). This is probably because yeast extract is an excellent source of vitamins, amino acids, small peptides, nucleotides, minerals and other nutrients (Zang *et al*., 2003). The present study also found higher amounts of OTA were produced by Straw21 strain under water stress conditions on wheat-based media after 20 days incubation. It is possible that there are two different regulatory impacts of such imposed stress on secondary metabolite production, under optimal and under stress conditions (Geisen *et al*., 2006).

2.4.2. Effect of environmental parameters on growth and OTA production by *A. westerdijkiae* strains on YES medium

The current study has demonstrated that maximum OTA production was observed at 0.98 a_w at 25-30°C for two of the strains examined (CCT; CECT) which paralleled the optimum conditions for growth. These results are similar to those previously found by Ramos *et al*. (1998) who found that *A. ochraceus* (= *A.westerdijkiae*) strains produced a maximum amounts of OTA at 0.98 a_w and 25-30°C on barley extract agar and on stored barley grains. However, a previous study which compared species within the *Aspergillus* section *Circumdati* group showed that OTA production was maximum at 0.95-0.90 a_w and high temperatures of 30-35°C for *A. steynii,* 25-30^{o}C for *A. westerdijkiae* and the same temperatures for *A. ochraceous* (Abdel-Hadi and Magan, 2009).

Some studies have been carried out on other OTA producers such as *A. ochraceus* and *Aspergillus carbonarius* before the reclassification of the former species. For example, Pardo *et al*. (2005) demonstrated that maximum conditions for *A. ochraceus* colonization on green coffee beans were at 30°C and 0.95-0.99 a_w. Optimum conditions for growth of *A. carbonarius* were found to be 30°C and 0.98 a_w. In contrast, maximum OTA accumulation on synthetic grape juice medium by two strains of this species isolated from grapes was 0.96 a_w and 25°C. (Belli *et al*., 2004a; Mitchell *et al*., 2004).

This study is an initial characterisation of abiotic conditions that influence the development of ochratoxigenic *P. verrucosum* and *A. westerdijkiae* strains to be used in later studies. This study has provided data on the relative effects of these interacting abiotic factors which can be utilised

for the development of predictive models of the lag phases prior to growth, mycelial growth, and OTA production by these two species.

CHAPTER 3

Effect of solute and matric potential stress on growth, gene expression and Ochratoxin A production by *Penicillium verrucosum* and *Aspergillus westerdijkiae*

3: Effect of solute and matric stress on growth, gene expression and OTA production by *P. verrucosum* and *A. westerdijkiae*.

3.1. Introduction

Contamination of cereals with OTA occurs during harvesting, drying and storage of cereal grain. This is mainly due to the contamination with *P. verrucosum* that can subsequently contaminate the grain with OTA. There has thus been interest in the source of *P. verrucosum* contamination and the effect of the prevailing environmental factors on its survival and subsequent contamination of cereal grain. Thus, *P. verrucosum* needs to be able to tolerate fluxes in water stress both in soil and on crop debris (Magan, 2007).

In soil, the effect of matric water stress is an important stress factor, while in crop debris solute stress is an important component of water stress (Magan, 1988). Thus, the relative tolerance to both solute and matric stress by this fungus is important to understand its life cycle and ability to colonise and survive in soil and on crop residues. To clarify the difference between the solute and matric potential stress, it is important to know that a_w can be expressed in term of the total water potential (Ψ), a measure of the total water content available for microbial growth which is measured in Megapascals (MPa) (Magan, 2007). This is the sum of three factors: (i) the osmotic or solute potential due to presence of ions or other solutes, (ii) the matric potential due directly to forces required to remove water bound to the matrix (e.g., soil) and (iii) the turgor potential of microbial cells balancing their internal status with the external environment. However, in soil, matric stress is the most important component.

In contrast, in crop residues there are about 10% soluble sugars, cellulose and hemicelluloses and some lignin and this matrix represents solute potential stress. Thus, the behaviour of *P. verrucosum* in response to these types of water stress may determine the capacity to contaminate cereal grain during the harvesting process. Previously, Ramos *et al*. (1999) showed that the growth rate of a strain of *A. ochraceus* (*=A. westerdijkiae)* isolated from cereals was slightly slower under matric stress when compared to solute stress, either ionic or non-ionic. For other non-xerotolerant fungi such a *Fusarium* species it has been shown that both spore germination and growth was significantly more sensitive to matric than solute (ionic/non-ionic) water stress (Magan, 1988, Magan *et al*., 1995; Ramirez *et al*., 2004; Jurado *et al*., 2008). However, few of these studies examined the effects on mycotoxin production. It is known that for both *P. verrucosum* and *P. nordicum* the biosynthetic genes involve in OTA production has been partially elucidated (Wang *et al*., 2016). In *P. nordicum*, a putative OTA biosynthetic cluster has been identified containing

biosynthetic genes encoding for (i) a PKS (*otapks*PN), (ii) a non-ribosomal peptide synthetase (NRPS) (*otanrps*PN) responsible for the formation of the peptide bond between the polyketide and the phenylalanine, other putative genes such as *otachI*PN potentially encoding a chlorinating enzyme, and *otatra*PN which potentially encodes a transporter protein involved in OTA export (Geisen *et al*., 2006; Karolewiez and Geisen, 2005). Geisen *et al*. (2004) correlated the expression of *otapks*PN from *P. nordicum* with the biosynthesis of OTA. In addition, it was shown that the OTA biosynthetic pathways in *P. nordicum and P. verrucosum* are highly homologous, except for the polyketide synthase gene (*otapks*) (Geisen *et al*., 2006; Wang *et al*., 2016).

The majority of OTA-producing species are included in the genus *Aspergillus*. Several species of *Aspergillus* section *Circumdati* are capable of producing OTA. Although, *A. ochraceus* was considered the most important OTA producer especially in warmer climates, new species in the *Aspergillus* section *Circumdati* have been described that are able to produce OTA, in particular *A. steynii* and *A. westerdijkiae*. (Frisvad *et al*., 2004).

In the present study two strains of *A. westerdijkiae* were examined for their tolerance to matric potential stress in comparison with solute stress for growth and OTA production. The species is important as it colonises drying coffee beans after fermentation and has also been implicated in contamination of both coffee and cocoa with OTA.

Thus, the objectives of this study were to examine the impact of (a) solute stress (either ionic or non-ionic), (b) matric potential stress on growth, expression of the *otapks*PV and *otanrps*PV gene and *otapks*AW and *otanrps*AW gene involved in OTA production and (c) phenotypic OTA production by *P. verrucosum* and *A. westerdijkiae* on a conducive YES medium for the first time.

3.2. Materials and methods

3.2.1. Fungal strains

Two strains of *P. verrucosum* (OTA11, Straw 21) were used in this study. They were isolated from wheat grain and wheat straw respectively. These were kindly supplied by Dr Monica Olsen, National Swedish Food Authority, Uppsala, Sweden. Two strains of *A. westerdijkiae* (A.W CCT 6795; A.W CECT 2948) were used in this study. These strains were both isolated from coffee.

3.2.2. Inoculum preparation and inoculation

The fungal strain was sub-cultured on malt extract agar (MEA) at 25°C in the dark for 7 days. The spores were gently dislodged from the colony surface into 9 ml sterile distilled water containing 0.05 % Tween-80 in 25 ml Universal bottles. The fungal spore concentration was determined using a haemocytometer and adjusted to 10^6 spores/ml. This was used for inoculation by taking 5µL of an inoculum and spreading onto a MEA plate which was incubated over night at 25°C. The germlings were then used as the inoculum. 4 mm agar discs were taken with a surface sterilised cork borer and centrally used to inoculate the treatment plates which were overlayed with a sterile 85 mm cellophane layer. Subsequently, the inoculated treatments and replicates were incubated at 25°C for 10 days.

3.2.3. Effect of the solute and matric stress on growth and OTA production by *P. verrucosum* strains and *A. westerdijkiae*.

The YES agar media were modified osmotically by the addition of the ionic solute NaCl (Lang, 1967) and the non-ionic solute glycerol (Dallyn and Fox, 1980) to obtain target levels of -1.4 MPa (=0.99 a_w), -7.0 MPa (0.95 a_w), -9,8 MPa (0.93 a_w), -14.0 MPa (0.90 a_w), and -19.6 MPa (0.86 a_w) to the YES agar medium.

For modification of the matric potential, the agar was omitted and the yeast extract with the sucrose were added together with known amounts of PEG 8000 to obtain the same matric potentials detailed above. These matric potentials were checked using the Aqua Lab 4 TE (Decagon Devices. Pullman, WA, 99163, USA). It has been shown that the water potential generated by PEG 8000 is predominantly (99%) due to matric forces (Steuter *et al.*, 1981). The media were prepared in 9 cm Petri plates which contained a 8.5 cm base layer of sterile circular discs of capillary matting. After decanting 15 ml of the sterile cooled medium into the Petri plates they were then overlayed with sterile polyester fibre layer and then a 85 mm sterile cellophane layer. This method has been

detailed previously (Jurado *et al*., 2008). The different treatments were kept in different polyethylene bags and kept closed to avoid moisture loss and changes in water potential/matric potential regimes.

3.2.4. Growth assessment

Colony diameters of 4-5 replicate plates were measured in two directions at right angles to each other. Measurements were recorded daily or as required for up to ten days. The growth rate was calculated by plotting the radial mycelial growth against time and the linear regression of the slope of the linear growth phase was used to obtain the radial growth rates (mm/day, Patriarca *et al*., 2001).

3.2.5. Isolation of total RNA

The fungal biomass was harvested after 10 days of incubation in the presence of Liquid nitrogen to keep the integrity of the RNA and stored at -80°C for molecular work, and -20°C for OTA analysis.

The fungal cell walls were disrupted using the bead-beating method recommended by Leite *et al*. (2013). The RNA was extracted using the Total RNA Spectrum Plant Kit (Sigma, UK) following the manufacturers protocol. To remove genomic DNA contamination, samples were treated with an on-column DNase digestion using the RNase-Free DNase Set Kit (Qiagen, UK). The RNA concentration and purity (A_{260}/A_{280} ratio) & (A_{260}/A_{230} ratio) were determined spectrophotometrically using a 2.5µL aliquot on the Picodrop (Spectra Services Inc., USA). For checking the RNA integrity, the Experion™ Automated Electrophoresis System using the Experion RNA StdSens analysis kits (Bio-Rad Laboratories Ltd., Hertfordshire, UK) was used, where the RQI that the minimum quality control was set at RQI >7.

3.2.6. RT-qPCR assays and relative quantification

RT-qPCR assays were used to amplify the *otapks*PV and *otanrps*PV genes, with the ß-tubulin gene used as the reference. For *A. westerdijkiae*, RT-qPCR assays were used to amplify the *otapks*AW and *otanrps*AW genes as target genes with the Actin-1 gene used as the reference gene.

(a) Primers

For *P. verrucosum*, the primer pairs PV-bentaqfor/rev, previously designed from the *otanrps*PV gene involved in the OTA biosynthetic pathway (Rodríguez *et al*., 2011) and the β-tubulin gene (Leite, 2013) were used. Nucleotide sequences of primers used in the RT-qPCR assays are shown in Table 3.1. For *A. westerdijkiae*, the primer pairs A_w-Actin-1 F/Rev were used as the reference gene. *pks wes* for/rev and *nrps wes* for/rev were designed from Gil-Serna *et al*. (2018) involved in the OTA biosynthetic pathway. Nucleotide sequences of primers used in the RT-qPCR assays are shown in Table 3.2.

(b) Assessment of PV primers efficiency.

A different set of amplicons were also amplified, targeting one reference gene in order to normalise the expression levels between the different experiments and samples. For the generation of the standard curves a pool of total RNA from different samples was reversibly transcripted into cDNA. The cDNA pool was 10-fold diluted starting from the equivalent transcript amount of 2µg total RNA quantified by spectrometry.

The analysis of the standard curves shows the gene efficiency correction used with the Pfaffi method of gene quantification (Figure 3.1a). This method takes into account differences of amplification efficiencies from each reaction to normalize cycle threshold (CT) differences in relative quantification. Relative quantification allows the normalization of gene expression with differences of mRNA material input. The initial differences of mRNA amounts can be normalized due to stable expressed gene ß-tubulin which was used as a reference gene. This gene is stable over a wide range of conditions tested. Differences in the quantification of this amplicon relates to the initial differences of mRNA quantities and the levels of *otapks*PV were normalized against this gene. Standard curves relating the Ct values and the amount of amplicon were generated. A good linear relationship between the increasing Ct values and the target template was observed (Figure 3.1 b).

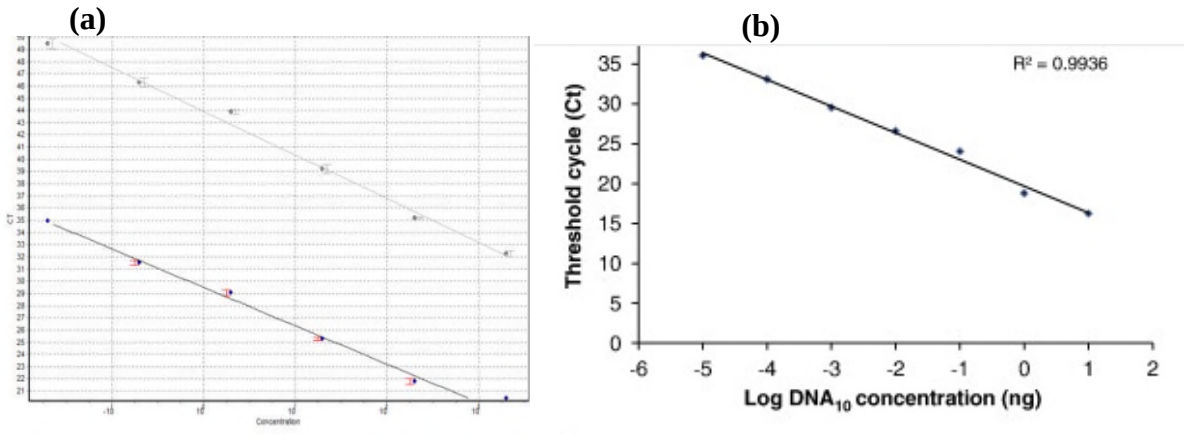

Figure 3.1. (a) Standard curves used to calculate amplification efficiency for each gene (*otapks*PV and β-tubulin as the reference gene) for relative quantification using the Pfaffi method. The light grey line represents the *otapks*PV standard curve while darker line represents the β-tubulin standard curve. The vertical line represents the mean standard error. The efficiency of *otapks*PV was of 1.08 with an R value of 0.99 while the efficiency of β-tubulin was 0.90 with an R value of 0.99. **(b)** Standard curves showing the $\log_{10}$ DNA amount vs. threshold cycle (Ct) values.

Table 3.1. Nucleotide sequences of primers for RT-qPCR assays**.**

Primer pairs	Gene	Nucleotide sequences (5'-3')	Product size (pb)	Publication
PV-bentaq-for	ß-tubulin	CTAGGCCAGCGCTGACAAGT	63	Leite, (2013)
PV-bentaq-rev	ß-tubulin	CTAGGTACCGGGCTCCAA		
*otapks*PV-for	*otapks*PV	TTGCGAATCAGGGTCCAAGTA	51	Schmidt-Heydt *et al*., (2007 b)
*otapks*PV-rev	*otapks*PV	CGAGCATCGAAAGCAAAAACA		
*otanprs*PV-for	*otanrps*PV	GCCATCTCCAAACTCAAGCGTG	117	Rodriguez *et al*., (2011)
*otanprs*PV-rev	*otanrps*PV	GCCGCCCTCTGTCATTCCAAG		

Table 3.2. Nucleotide sequences of primers for RT-qPCR assays of *A.westerdijkiae.*

Primer pairs	Gene	nucleotide sequences (5'-3')	Product size(pb)	Reference
actin-for	actin-1*wes*	CCTATCTACGAGGGTTTCGCC	73	
actin-rev		AGTCGGTAAGATCACGACCAG		Verheecke *et al*., (2015)
pks_wes for	*pkswest*	GCAGATATCAGACAAGGCATCT	177	Gil-Serna
*pks*_wes-rev		GCACGTAAGATGCGCTAGAGT		*et al*., (2018)
NRPS_wes for	*nrpswest*	GCTTGCTGACAAGCCGATGAC	124	Gil-Serna
NRPS_wes rev		GGTCGTCAGTCGCTCATCCA		*et al*., (2018)

(c) cDNA synthesis and qPCR.

cDNA was synthesized according to the Omniscript RT kit protocol (Qiagen, UK). The qPCR was prepared with three replicates of RNA control samples together with a template-free negative control which was included in the runs. The reaction mixture consisted of 5 µL of SYBR (Sso Advanced ^{Tm} Universal Syber $^{®}$ Green Supermix), 300nM of each primer, and 1 µL of cDNA template in a final volume of 10 µL. After an activation step of 10 min at 95°C, all subsequent 40 cycles were performed according to the following temperature regime: 95°C for 15s and 60°C for 30s. After the final qPCR cycle, a melting curve analysis of the qPCR products was performed. The Ct determinations was perform using Bio-Rad CFX software.

(d) Relative quantification Gene Expression.

Relative quantification of the expression of *otapks*PV and *otanrps*PV genes was performed using the reference ß-tubulin gene as an endogenous control to normalise the quantification of the target in the relative quantification assays and used for all treatments. Quantification cycle (Cq) determinations were automatically performed by the instrument using the default parameters, and the expression ratio was calculated using the $2^{-\Delta\Delta Ct}$ method proposed by Livak and Schmittgen (2001). The non-modified medium was used as the control.

3.2.7. Quantification of OTA production

See Chapter 2 section 2.2.6.

3.2.8. Statistical analyses of data

Each treatment was carried out in triplicate, for growth rate assessment, gene expression and OTA production. The normality was checked using the Shapiro test and homoscedasticity was checked using the Leven test. The factors and their interactions were examined by the Kruskall-Wallis (non-parametric) test if the data were not normally distributed. For normally distributed data, the data sets were analysed using ANOVA (JMP software). The statistical significant level was set at $p<0.05$ for all single and interacting treatments.

3.3. Results

3.3.1. Effect of matric stress on growth of *P. verrucosum* strains

Figure 3.2 shows the effect of matric stress (Ψ_m) on relative growth rates of the two strains examined. Both grew over a wide range of Ψ_m stress. Optimum growth was at -7.0 MPa (=0.95 a_w) for both strains. However, *P. verrucosum* straw21 strain was more sensitive to this type of stress than the OTA11 one at 25°C. The growth rate of the Straw21 strain was significantly less than the OTA11 strain (p=0.05).

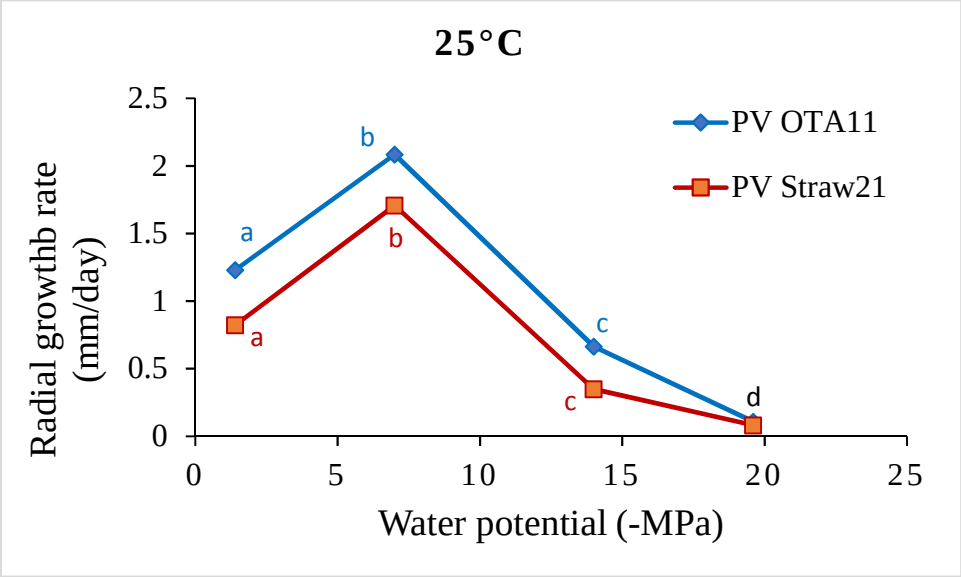

Figure 3.2. Effect of matric potential stress modified with PEG 8000 on the radial growth rate of two strains of *P. verrucosum* (OTA11, Straw21) on YES medium at 25ºC. Different letters indicate a significant difference.

3.3.2. Comparison of solute and matric potential stress on growth and gene expression of *P. verrucosum* (strain OTA11).

Figure 3.3. compares the effect of different solutes (ionic and non-ionic) with Ψ_m stress on growth of the toxigenic strain OTA11. This shows that -7.0 MPa (=0.95 a_w) was optimal for growth, regardless of whether solute or Ψ_m water stress was imposed. Indeed, growth was faster under Ψ_m stress in this treatment. *P. verrucosum* was more sensitive to ionic solute stress with no growth observed at the lowest water potential (Ψ_t) tested (-19.6 MPa= 0.86 a_w). Statistically, the ANOVA test showed that solute and matric stress had a significant effect on the growth rate.

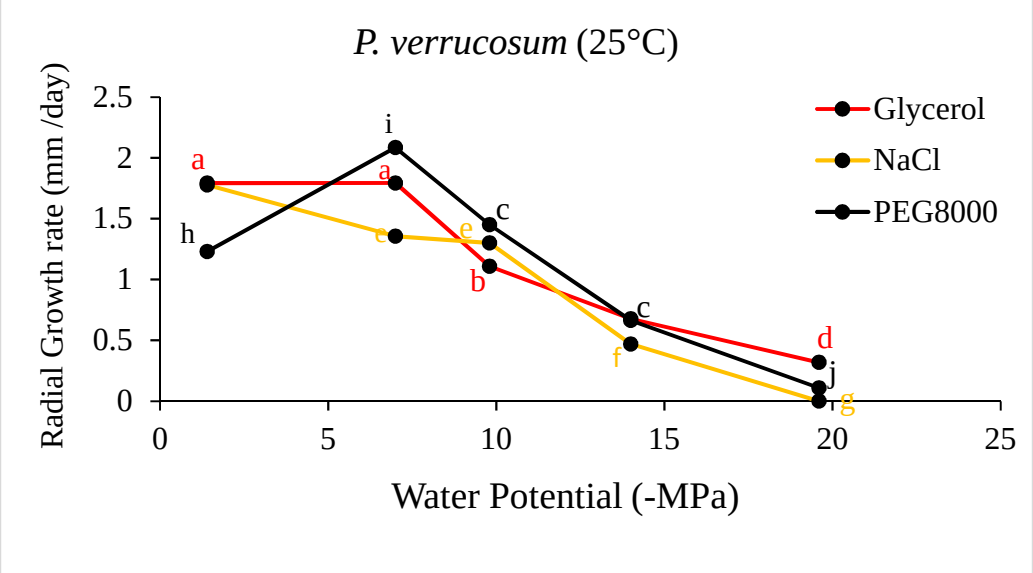

Figure 3.3. Comparison of the effect of solute potential modified with NaCl or glycerol and matric potential stress modified with PEG 8000 on the relative growth rates of *P. verrucosum* strain OTA11 on YES medium at 25ºC over periods of 10 days. Different letters indicate significant differences.

a) Comparison of solute and matric potential stress on *otapks*PV gene expression.

Figure 3.4. shows the impact of the interaction between the solute (Glycerol, NaCl) and matric potential (PEG 8000) stress on the relative *otapks*PV expression in relation to the different treatments. The pattern of production is clear where solute stress was imposed with maximum expression at -7.0 and -1.4 MPa conditions with a decrease at -9.8 and -14.0 MPa. Very low expression was observed where matric stress was imposed. There was thus a significant difference in relative expression of this biosynthetic gene for OTA production in response to imposed solute or matric stress.

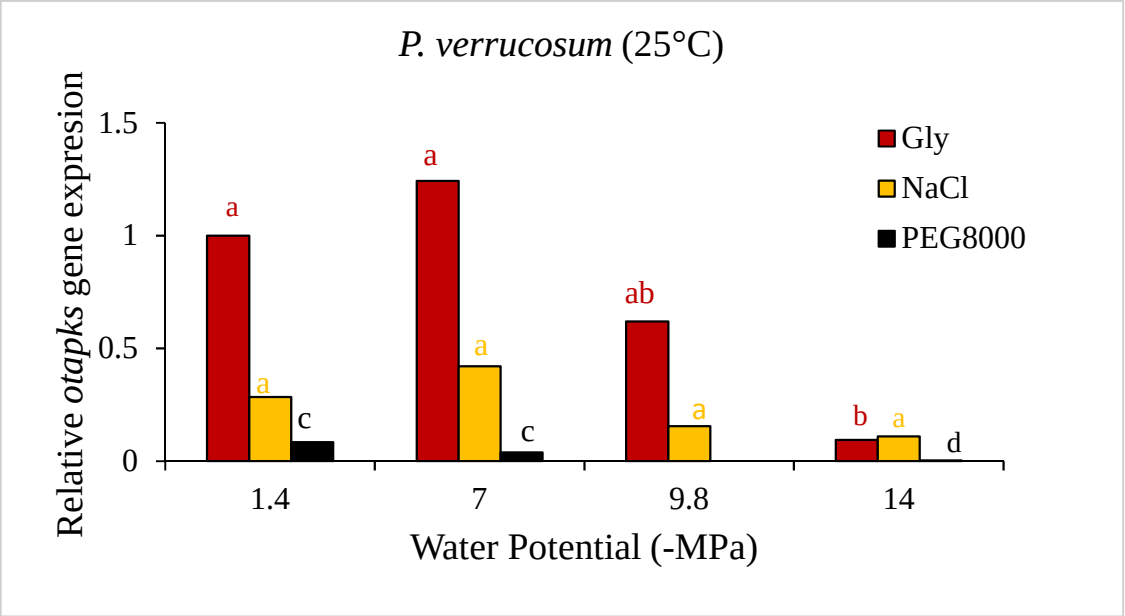

Figure 3.4. Relative gene expression values of *otapks*PN gene expression in *P. verrucosum* strain OTA11 grown on YES medium incubated at 25°C for 10 days when compared with the non-modified YES medium used as a calibrator. Different letters indicate a significant difference between the levels of each treatment.

b) Comparison of solute and matric potential stress on *otanrps*PV gene expression.

Figure 3.5. shows the influence of different solutes (Glycerol, NaCl) and matric potential stress on the second gene examined, *otanrps*PV. The results here were different. There appeared to be a very high expression of this gene under freely available water conditions in the matric stress-imposed treatment. Under additionally imposed matric stress this was significantly decreased. In this case, in the glycerol-imposed solute stress conditions the expression was generally low. However, for ionic-imposed stress there was some increase in expression at -9.8 and -14.0 MPa. There was less of a pattern of expression of this gene in relation to the different imposed water stress treatments.

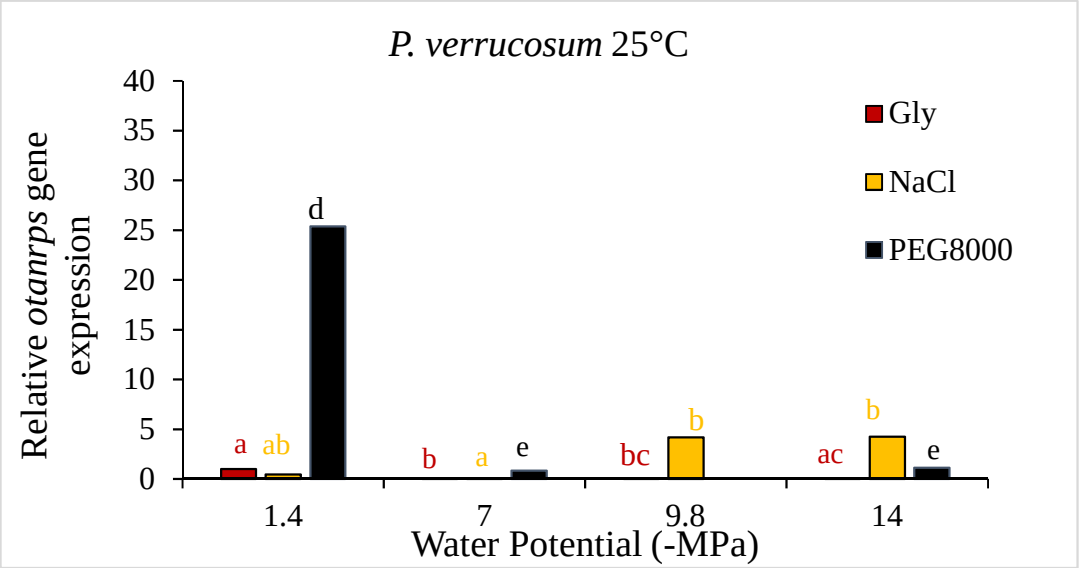

Figure 3.5. Relative gene expression values of *otanrps*PV gene expression in *P. verrucosum* OTA11 grown on YES medium incubated at 25°C for 10 days with regard to non-modified YES medium used as a calibrator. Different letters indicate significant difference between levels of each treatment.

3.3.3. Comparison of solute and matric potential stress on OTA production by *P. verrucosum*.

For OTA production, the *P. verrucosum* strain was more sensitive to Ψm stress under all treatments when compared to the two solute imposed stress conditions (Figure 3.6). Optimum OTA production was produced under solute stress when imposed with glycerol and NaCl, under freely available water conditions (-1.4 MPa=0.99 a_w). Very little OTA was produced under the imposed Ψm stress conditions. Kruskal-Wallis test revealed that imposed matric stress of -14 MPa (=0.90 a_w) had a significant impact on toxin production when compared with the other water stress treatments. However, under non-ionic solute stress, *P. verrucosum* produced significantly more OTA in freely available water conditions (-1.4 MPa = 0.99 a_w). This was threefold more than that at in moderate water stress (-7.0 MPa = 0.95 a_w).

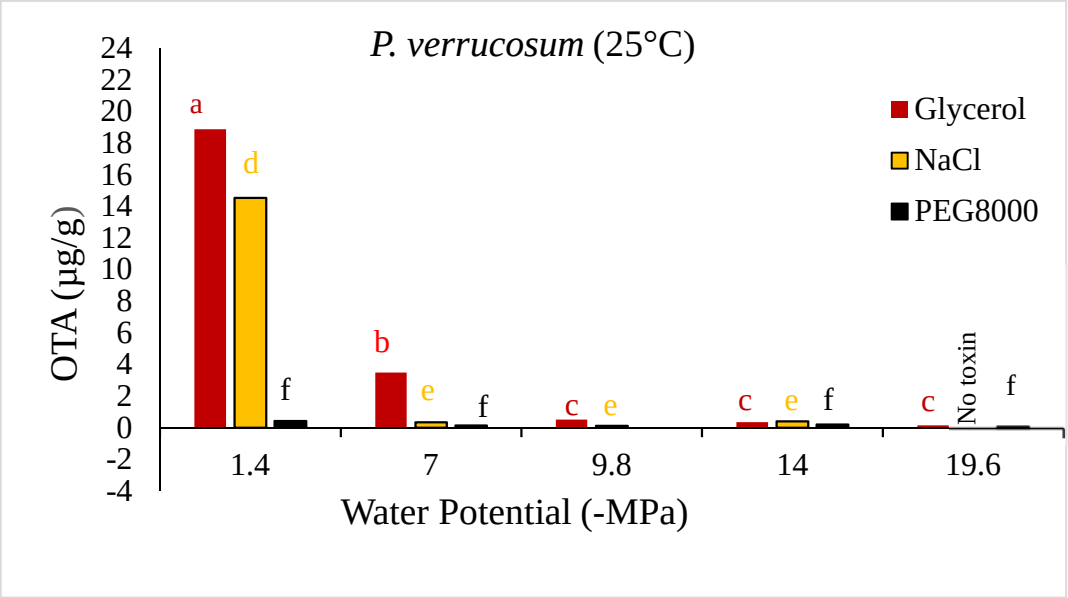

Figure 3.6. Comparison of the effect of solute and matric potential stress on ochratoxin A production by *P. verrucosum* OTA11 on a conducive YES medium after 10 days at 25ºC. Different letters indicate significant difference.

3.3.4. Effect of matric water stress on growth rate and OTA production by two strains of *A. westerdijkiae* (CCT, CECT).

Figure 3.7. shows the effect of matric stress on growth of the two strains was slightly different. The CECT strain was more tolerant to matric stress with a higher growth rate at -7.0 MPa (=0.95 a_w) when compared to the non-modified medium. Interestingly, ANOVA revealed no significant difference between growth at -1.4 and -14.0 MPa (= 0.99 and 0.90 a_w) for the CECT strain. However, overall, there was no significant difference between growth at -14 and -19.6MPa (0.90 and 0.86 a_w) respectively for the CCT strain. Statistically, the intraspecific differences between the two strains was not significant in terms of growth rates.

In contrast, the profile of OTA production was quite different from the growth pattern for the CECT strain. Statistically, this strain produced significantly higher amounts of toxin at 0.86 a_w (-19.6 MPa) when compared to the other treatments. Although the CCT strain grew at all water stress levels and with the different solutes, no toxin was produced and was thus excluded from the statistical analyses (Figure 3.8).

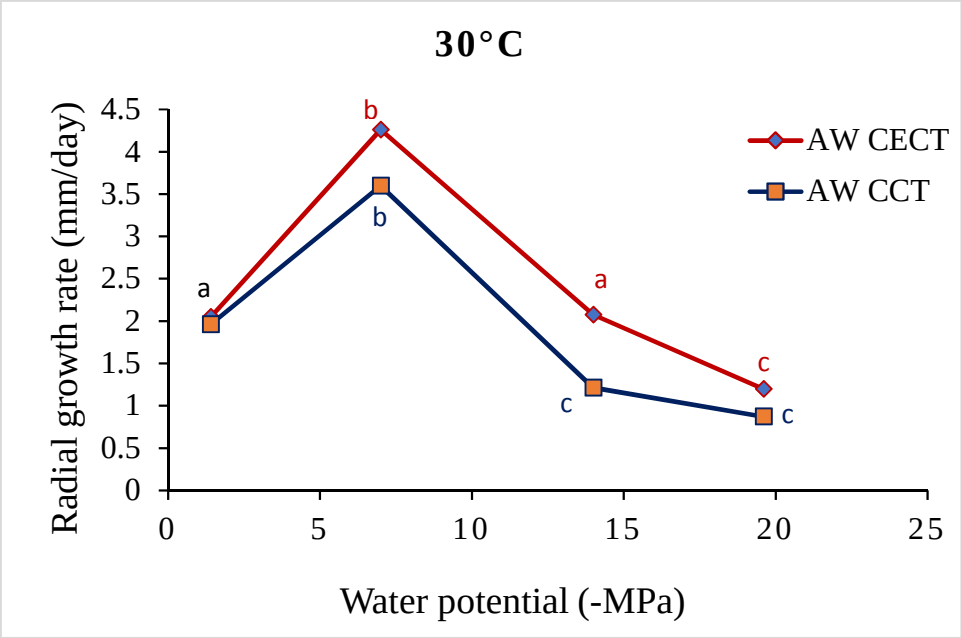

Figure 3.7. Effect of matric potential modified with PEG8000 on the growth of two strains of *A.westerdijkiae* (CECT, CCT) on YES media at 30ºC. Different letters indicate significant differences.

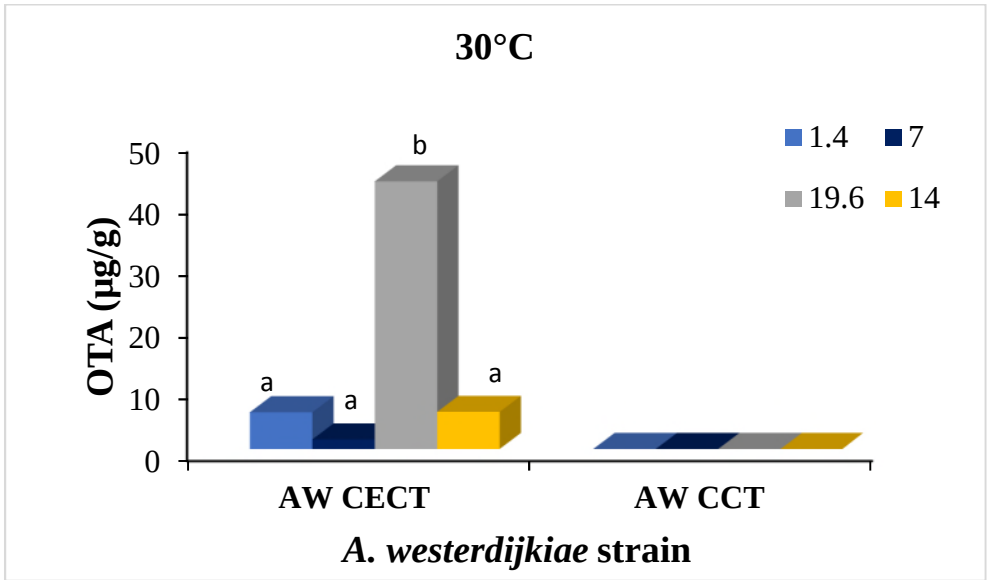

Figure 3.8. Effect of matric potential modified with PEG 8000 on OTA production by two strains of *A. westerdijkiae* (CECT, CCT) on YES media at 30ºC. Different letters indicate significant differences.10.83<SD>0.74.

3.3.5. Comparison of solute and matric potential water stress on growth and gene expression by *A. westerdijkiae* (strain CECT).

Figure 3.9. compares the effect of solute and matric water stress on growth of this *A. westerdijkiae* strain. Optimum growth occurred at intermediate solute/matric water stress and not when water was freely available (-1.4 MPa; 0.99 a_w). The growth patterns show more sensitivity to ionic solute stress than non-ionic or matric stress. Also, while NaCl inhibited growth at -19.6 MPA (0.86 a_w) in the non-ionic stress (glycerol) and matric stress, growth still occurred. Statistically, there was a significant difference between growth rates on glycerol/NaCl modified media at 30°C assessed by one-way ANOVA test. However, there was no significant difference at -9.8 and -14.0 MPa (=0.93 and 0.90 a_w) on growth with the ionic solute NaCl. Also, the growth rate of *A. westerdijkiae* on matrically-modified media was higher than with solute-modified media with an optimum growth in the range -7.0-14.0 MPa matric potential (=0.95 to 0.90 a_w). Interestingly, there was no significant difference in the growth rate under matric stress at -1.4 and -14.0 MPa (=0.99 and 0.90 a_w) respectively. No growth was recorded in relatively dry conditions (-19.6=0.86 a_w) under imposed ionic solute conditions (NaCl) and was thus excluded from the statistical analyses.

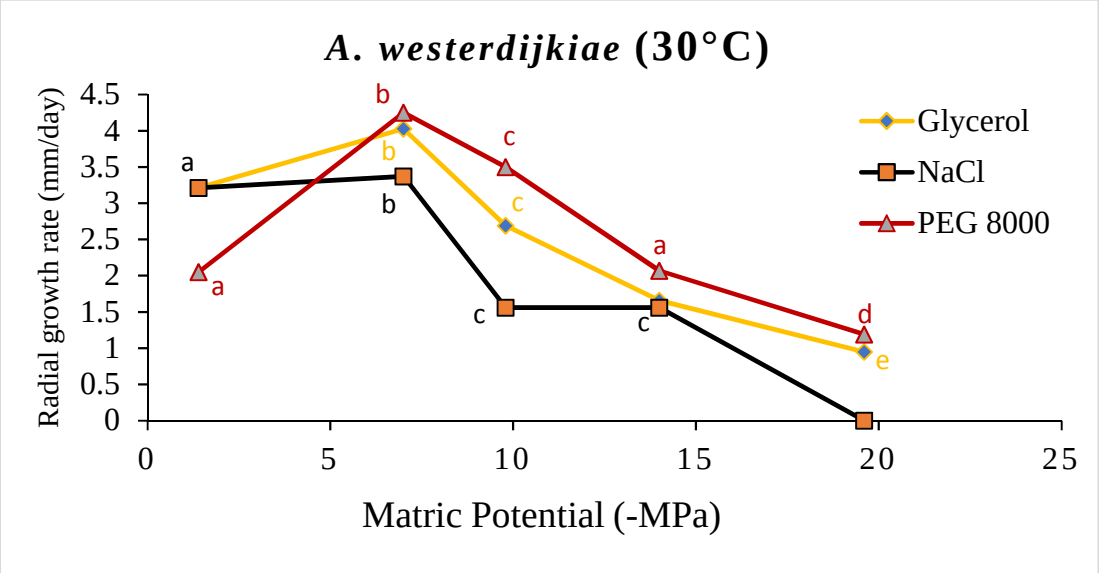

Figure 3.9. Comparison of the effect of solute (ionic, NaCl; non-ionic, glycerol) and matric potential stress (PEG 8000) on growth of *A. westerdijkiae* CECT on YES medium at 30°C. Different letters indicate significant differences**.**

Figure 3.10. compares the effect of solute and matric potential stress on ota*pks*AW expression at two different water stress levels. A significant sharp peak was observed at moderate water stress -9.8 (=0.93 a_w) under non-ionic stress when compared with the other solutes. However, at -9.8 (=0.93 a_w) matric potential stress, the gene expression was more sensitive when compared to other solutes at the same water stress. Moreover, there was a significant impact between the gene expression at -9.8 (=0.93 a_w) under NaCl and matric potential amended media when compared with that at at -1.4 (=0.99 a_w).

Figure 3.11. compares the ota*nrps*AW expression pattern with different solute treatments. A significant peak was detected at -9.8 (=0.93 a_w) under non-ionic solute stress. However, the gene expression profile was apparently constant when the water was freely available (0.99 a_w) under solute and matric stress. Statistically, the matric stress had a significant impact on gene expression when compared with the non-ionic solute stress (glycerol-amended media).

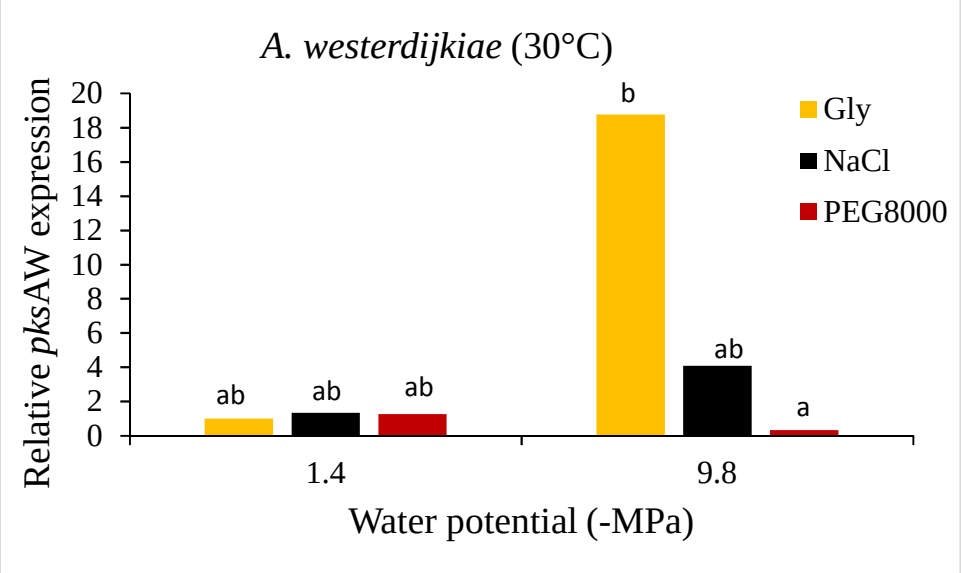

Figure 3.10. Relative gene expression values of ota*pks*AW gene expression in *A. westerdijkiae* grown on YES medium at 30°C for 10 days with regard to non-modified YES medium as a calibrator. Different letters indicate significant differences.

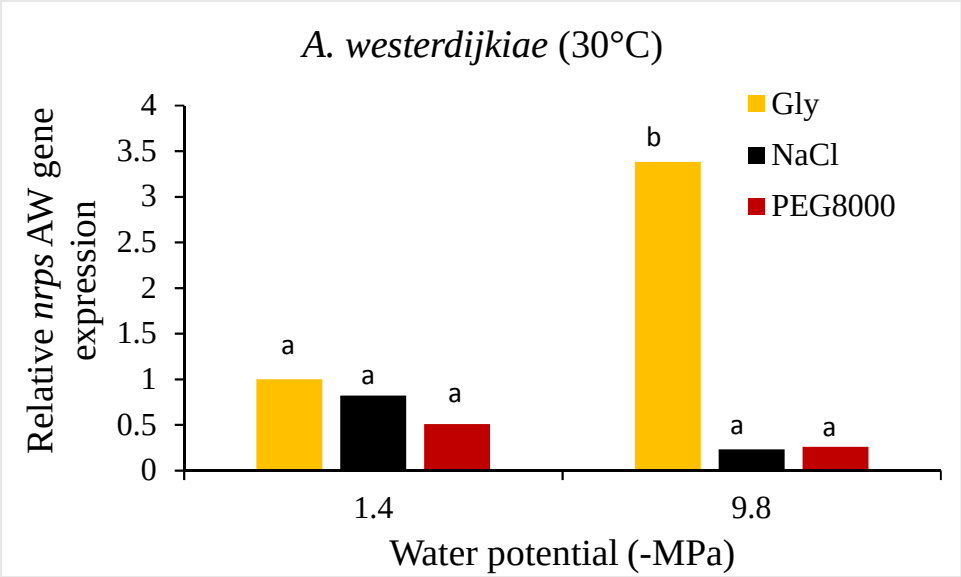

Figure 3.11. Relative gene expression values of ota*nrps*AW gene expression in *A.westerdijkiae* grown on YES medium incubated at 30°C for 10 days when compared with non-modified YES medium used as a calibrator. Different letters indicate significant differences.

3.3.6. Comparison of solute and matric potential water stress on OTA production by *A. westerdijkiae* (strain CECT).

Figure 3.12. compares the OTA production in relation to the different water stress treatments at 30ºC after 10 days incubation. The highest OTA production was actually under water stress with the matric potential treatment (19.6 MPa). There was really little difference between the treatments with solutes or matric stress under other conditions.

Under drier conditions, however, at -14 MPa (0.90 a_w), the strain was more resistant under ionic NaCl modified medium as producing high significant amount of toxin comparing to the other treatments at the same water stress. Overall, the strain was more sensitive towards the non-ionic solute stress as the amount of toxin decreased gradually upon increasing the water stress.

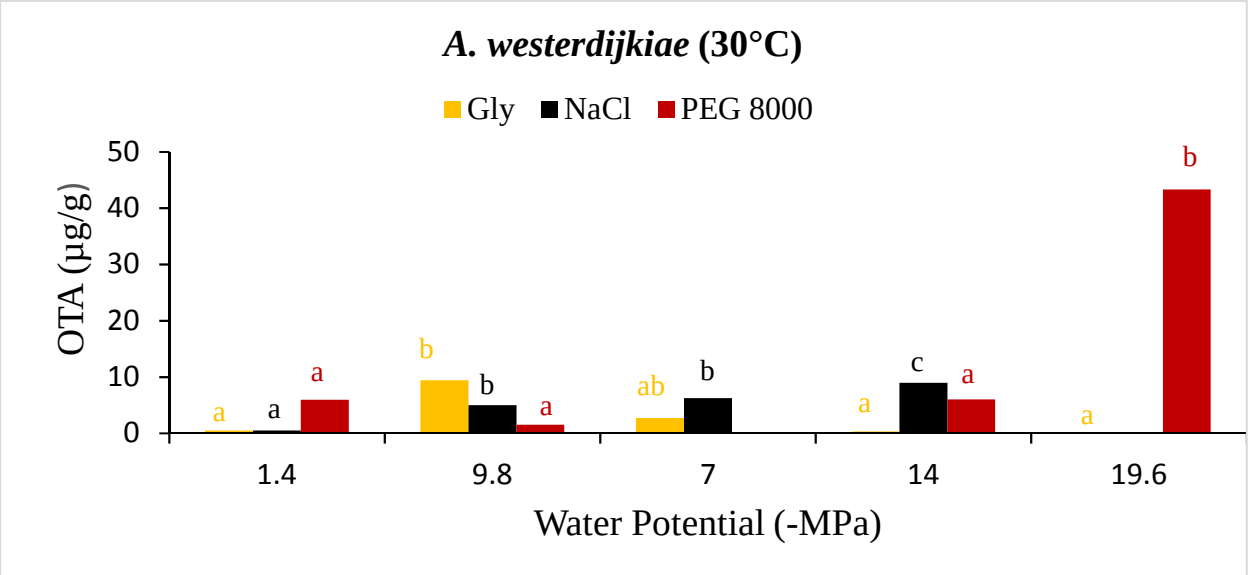

Figure 3.12. Comparison of the effect of solute stress (ionic, NaCl; non-ionic, glycerol) and matric potential stress (PEG 8000) on OTA production by *A. westerdijkiae* on YES medium at 30ºC. Different letters indicate significant difference.

3.4. Discussion

3.4.1. Solute and matric stress effects on growth of *P. verrucosum*

This is the first study to examine the impact of different solute and matric imposed stress on growth, biosynthetic gene expression and phenotypic OTA production by *P. verrucosum*. This certainly suggests that *P. verrucosum* is well suited to survival in soil and also on crop residues based on the colonisation over a range of water stress regimes, including matric stress which is supposedly more difficult to overcome (Magan, 2007). Overall, ionic solute stress was more toxic with high concentrations at -19.6 MPa inhibiting the ability to grow.

P. verrucosum was able to grow optimally at -7.0 MPa (=0.95 a_w) which is relatively dry conditions when considering soil moisture relations as the wilting point of plants is around -1.4 MPa. This tolerance of matric stress would suggest that this species has evolved to successfully not just survive but actively grow in such stressed conditions. These results are very different from those observed for *Fusarium* species such as *F. verticillioides* and *F. graminearum*, which were found to be more sensitive to matric stress than solute stress in terms of growth (Jurado *et al*., 2008; Ramirez *et al*., 2004). These other species are not xerophilic or xerotolerant. In contrast, studies with an *A. ochraceus* strains isolated from cereals had relatively similar tolerances to solute and matric stress (Ramos *et al*., 1999).

3.4.2 Effects on biosynthetic gene expression and phenotypic OTA production

In this study, the expression of *otapks*PV was not parallel with the growth data. This is in good agreement with the results previously obtained by Jurado *et al*., (2008) who showed that osmotic stress has a profound influence on induction of the transcript level of the *FUM1* gene, despite the fact that growth of *F. verticillioides* was reduced.

High OTA production was observed under non-ionic solute stress, which was paralleled by high *otapks*PV expression. However, at lower water potentials, which means more stressful conditions (-9.8; 14.0 MPa=0.93, 0.90 a_w) under ionic stress, a considerable amount of toxin could be observed, higher than in the other conditions. This indicates perhaps two different regulatory principles of OTA production, one acting under optimal and one acting under stress conditions may be operating (Geisen *et al*., 2006).

This study has confirmed a good correlation between the expression data of the *otapks*PV gene and the biosynthesis of OTA under the treatment conditions. This confirms previous work suggesting that the *otapks*PV gene is a key gene of the OTA biosynthetic pathway in *P. verrucosum* and regulated by environmental stimuli (Schmidt-Heydt *et al*., 2011).

The *otanrps*PV expression pattern was different from *otapks*PV expression and not consistent with toxin production. In this sense, Geisen *et al*. (2004) demonstrated that the *otapks*PN gene expression is paralleled by the induction of toxin production during the time course of the experiment. Furthermore, they reported that the first mRNA could be detected after 4 days incubation (growth phase), where first traces of OTA were analysed, then the amount of *otapksPN* mRNA reached its maximum at day 6 and remained at this level until day 8 (stationary phase) where the OTA concentration remained constant. Subsequently, the mRNA disappeared slowly, and was then degraded very rapidly until day 11, when no *otapks*PN mRNA could be observed.

Taken together, the window in which *otanps*PV gene expression was demonstrated to be active in the current study, it can perhaps be deduced that while the *otapks*PV is up regulated under certain conditions, the *otanps*PV is down regulated at the same conditions confirming that NRPS operates in a manner independent of the PKS to enable this species to produce OTA as a self-protection mechanism under stress conditions.

This feature may be due to the fact that NRPSs has a modular structure, with one module being a semi-autonomous unit that recognizes, activates, and modifies a single residue of the final peptide by means of three typical domains: (i) the adenylation domain (A), which is responsible for substrate specificity and activation; (ii) the peptidyl carrier domain (P), which covalently binds the substrate to the enzymes via a thioester linkage; and (iii) the condensation domain (C), which catalyses peptide bond formation. Figure 3.13 (Finking and Marahiel, 2004; Schwarzer *et al*., 2003; Challis and Naismith, 2012). Subsequently, the module would be able to ligate the isocoumarin group to the amino acid phenylalanine through the carboxyl group in the OTA molecule.

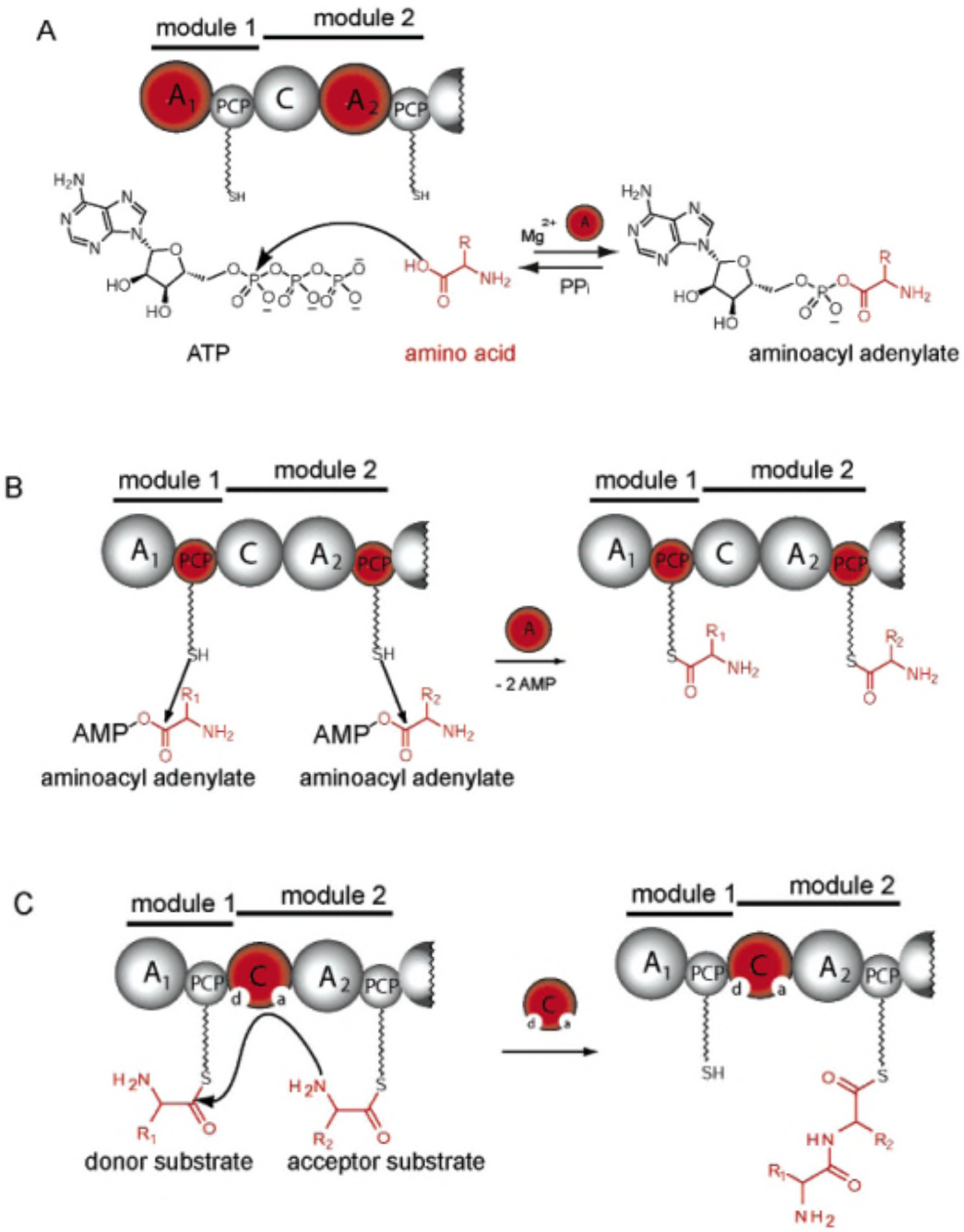

Figure 3.13. Domain catalysed reactions. Domains in action are indicated in red. Activation (A) domain recognizes the amino acid. Peptidyl carrier protein (PCP) domain gets aminoacylated by the amino acid. Condensation (C) domain transfers the amino acid to the next module.(adapted from Challis and Naismith, 2012).

The profile of OTA production under different solute stresses was completely different from those for growth. Optimum OTA production was observed at 0.99 a_w when water was freely available regardless of the solute used. At 0.95 a_w there was a significant decrease for both solute types. Meanwhile, the amount of toxin was significantly reduced, when imposing high water stress in the ionic NaCl-amended medium. This again suggests more sensitivity to ionic solute stress than

non-ionic water stress. In contrast, some other toxigenic *Penicillium* species are ecologically specialised to grow in very high ionic environments (Rodriguez *et al*., 2014). For example, *P. nordicum* which can colonise cured meats is actually adapted to colonise high salt environments more rapidly than in low salt environments. This study did show that *P. verrucosum* can be found occasionally on NaCl rich products and produce OTA which has been shown in some previous studies (Peintner *et al.,* 2000; Andersen, 1995 and Corni *et al*., 2004), where *P. verrucosum* was occasionally found in dry cured ham matrices.

Previously, Schmidt-Heydt *et al*. (2011) showed that *P. verrucosum* can shift the secondary metabolite profile towards OTA on substrates with increased concentrations of NaCl. Also, they demonstrated that the production of OTA under high NaCl conditions is an adaptive reaction. Moreover, OTA carries a chlorine in its molecule and under high chloride ion concentrations, which can be toxic for the cell, the permanent production and excretion of OTA leads to a flow of chlorine out of the cell to ensure maintenance of the chloride homeostasis (Samapundo *et al*., 2010; Ayodele and Ojoghoro, 2007). Under matric stress, however, the amount of toxin was significantly decreased by increasing this type of water availability when compared to the other two solutes. This suggests that matric stress is more inhibitory than changes in ionic and non-ionic solute stress.

3.4.3. Comparison of matric water stress effects on growth and OTA production by two *A. westerdijkiae* strains

The growth pattern was optimum over a wide matric stress range showing no significant difference between -1.4MPa and -14MPa and, -14MPa and -19.6MPa for the two strains examined. However, this contrasted with OTA production where one strain produced significant amounts of OTA while the other strain did not produce any. This suggests that there are marked intra-strain differences, perhaps not in terms of colonisation but in terms of OTA production. Previous studies of different strains of *A. westerdijkiae* found some differences in OTA production and growth in response to solute stress but not as found in the present study (Abdel-Hadi and Magan, 2009).

3.4.4. Comparison between effects of solute and matric water stress on growth and gene expression of *A. westerdijkiae.*

This is the first study that has attempted to compare the influence of different types of water stress on the growth and OTA production by a strain of *A. westerdijkiae*. This is a xerophilic,

mycotoxigenic species which grew best at -7.0 MPa (=0.95 a_w) matric stress than under imposed ionic solute stress. Also, growth was optimum over a wide range of of matric potential stress (-1.4 to -19.6 MPa) and with solute stress modified with the non-ionic solute, glycerol. This is surprising as matric potential stress has always been assumed to represent a much higher level of water stress than solute stress (Griffin, 1981). In addition, the growth was better under drier conditions, down to 19.6 MPa (=0.86 a_w) when glycerol was used as a solute. These differences may be due to glycerol being utilised as a carbon source under extreme water stress to act as a compatible solute to enable growth to occur (Medina *et al.*, 2014). Previously, Ramos *et al.* (1999) showed that growth of a strain of *A. ochraceus* was very similar when cultures were grown on non-ionic vs matric stress. Moreover, they found that with the ionic solute this species was more sensitive at higher concentrations which appeared to be toxic. The results are very similar to those obtained in this study on effects on growth.

In contrast, other studies have found that germination and germ tube extension of a range of soil fungi were more sensitive to changes in matric than solute potential stress including *Penicillium* species, with the exception of *Gliocladium roseum* and *G.virens* (Magan, 1988). In addition, other studies reported that mycelial extension of other fungi such as *Alternaria alternata*, *Microdochium bolleyii* and a range of basidiomycetes was significantly more sensitive to matric than solute stress (Magan *et al*., 1995).

This study has confirmed the consistent relationship between *otapks*AW gene expression and OTA production. A high induction peak of *otapks*AW was observed at moderate water stress (=0.95 a_w) under glycerol amended medium that paralleled toxin production. This induction suggests that the activation of OTA biosynthesis is stress regulated (Schmidt-Heydt *et al.,* 2008). Other studies found that osmotic solute stress has a profound impact on induction of the transcript level of the FUM1 gene, although the growth of *F. verticilliodes* was retarded (Jurado *et al*., 2008).

Interestingly, the current study has shown that the major peak of *otapks*AW gene activation was close to the optimum growth rates observed under mild stress conditions (=0.95 a_w) with non-ionic solute stress imposed with glycerol. At the optimal growth rate, the priority may be for the primary metabolism, resulting in a slightly later effect on switching the biosynthetic gene clusters for synthesis of the secondary metabolites such as OTA (Schmidt-Heydt *et al.,* 2008).

Interestingly, the expression of the *otanrps*AW gene was highest with relatively freely available

water at 0.99 a_w (-1.4 MPa) while under ionic stress the *otapks*AW gene expression was decreased Similar trends were observed previously with *P. nordicum* and *otanps*PN gene was increased, this was parrarled by a decrease in the *otapks*PN gene (Geisen and Karolewiez, 2005)**.**

3.4.5. Comparison between effects of solute and matric water stress on OTA production by *A. westerdijkiae*.

With regard to OTA production, the current study suggests that there was slightly more OTA produced under ionic stress as well as under extreme matric stress (-14 MPa (=0.90 a_w). This may be due to *A. wetserdijkiae* producing more OTA because of ionic stress resulting in increasing amounts of toxin due to a self-protection mechanism. Thus excretion of OTA could lead to a flow of chloride out of the mycelium ensuring chloride homeostasis within under higher chloride concentrations ((Schmidt-Heydt *et al.*. 2011). In contrast, Stoll *et al*., (2013) showed that for *A. carbonarius* there was reduced growth and OTA biosynthesis under moderately increasing ionic stress conditions (NaCl). However, at high glucose concentrations, growth was induced and a moderate reduction of OTA biosynthesis was reported (Geisen *et al*., 2013).

Interestingly, high amounts of toxin was also detected under matric stress when compared to the other solutes. Leong *et al*., (2015) in their study on *Xeromyces bisporus* found that exposure to extreme conditions of 0.68 a_w, that elements of the HogA pathway and glycerol biosynthetic pathways were up-regulated. Moreover, other studies have also suggested that cultures *A. parasiticus*, *A. flavus* and *A. ochraceus* were able to modify the synthesis of sugar alcohols as water stress was imposed to facilitate their tolerance to the interacting environmental stress conditions (Magan, 2007).

Indeed, Ramos *et al*. (1999) and Nesci *et al*. (2004) showed that for *A. ochraceus* there was a switch from the synthesis of high molecular weight sugar alcohols (mannitol, arabitol) to low molecular weight sugar alcohols (erythritol, glycerol) and sometimes an accumulation of the sugar trehalose, which is a desiccation protectant increased (Ramos *et al*., 1999, Nesci *et al*., 2004; Ramirez *et al*., 2004). The increase in glycerol and erythritol is important as they are able to provide a more stable internal cellular water potential than the other high molecular weight sugar alcohols (Magan, 2007).

CHAPTER FOUR

Effect of interacting abiotic climate change factors on growth, gene expression and OTA production by *P. verrucosum* and *A. westerdijkiae*

4.1. Introduction

The major challenge for the next 25-50 years will be to produce and provide enough food on a global basis which is safe for consumers. It has been suggested that extreme weather events and climate change (CC) abiotic factors such as changes in temperature, CO_2 and drought/flooding will have a significant impact on food safety and food security (Paterson and Lima, 2010; Wu *et al*, 2011; Medina *et al*., 2017).

Recent studies with mycotoxigenic species and contamination of staple food grains such as maize have suggested that interacting CC abiotic factors (+2-4^{o}C, 400 vs 1000 ppm CO_2 and drought stress) will have a significant impact on mycotoxin contamination (Medina *et al*., 2017). For example, it has been shown that while growth of *A. flavus* is not affected by CC factors, both biosynthetic genes involved in aflatoxin production and phenotypic toxin production were stimulated, both *in vitro* on maize-based media and on stored maize grain (Medina *et al*., 2015, 2017a; Gilbert *et al*., 2017). It has also been suggested that ochratoxin A production by *A. westerdijkiae* might be affected by CC abiotic factors while *A. carbonarius* is not affected (Akbar *et al*., 2016). Studies with *F. verticillioides* and fumonisin production on maize between silking and maturity showed that the biomass of the pathogen increased but that toxin production was unaffected by elevated CO_2 exposure. Subsequent studies with drought stress x CO_2 exposure showed a stimulation of fumonisins (Vaughan *et al*., 2014, 2016). However, there have been no previous studies on *P. verrucosum* in terms of effects of CC interacting abiotic factors on growth and OTA production.

The objectives of this study were to examine the effect of interacting CC change factors on:

(a) Growth of *P. verrucosum* and *A. westerdijkies* under interacting temperature x a_w stress and existing CO_2 (400 ppm) vs elevated CO_2 (1000 ppm) on wheat and coffee-based media respectively under non-ionic solute and matric stress.

(b) effects of treatments on key biosynthetic genes involved in OTA production.

(c) Effects on phenotypic OTA production

4.2. Materials and Methods

4.2.1 Fungal strains

One strain of *P. verrucosum* (OTA11) and one of *A. westerdijkiae* (CECT 2948) were used in these studies. The former strain was isolated from wheat grain and the latter from coffee beans.

4.2.2. Inoculum preparation and inoculation

These are described in Chapter 3, Section 3.2.2

4.2.3. Media used in these studies and growth measurements

A 2% (w/v) milled wheat and a 2% (w/v) milled coffee agar media were used as the basal medium for studies with *P. verrucosum* and *A. westerdijkiae* respectively. The media were modified to -7.0 (=0.95 a_w) and -2.8 (=0.98 a_w) using glycerol. The media were autoclaved at 121°C and poured into 9 cm Petri plates and kept at 4°C until used. The final a_w levels were checked with an Aqua Lab TE4 (Decagon Devices. Pullman, WA, 99163, USA).

The modification of the matric potential of the media has been described in Chapter 3, Section 3.2.3. The only modifications were that the YES nutrients were replaced with milled wheat grain or milled coffee beans.

The solute modified media were centrally point inoculated with 5 µl of the spore suspension. For matric potential modified media, a 4 mm agar plug of the fungi was used to centrally inoculate the treatments as described in Chapter 3, Section 3.2.3. Four replicates of each treatment were used for each species. Cultures with the same a_w were enclosed together in a plastic chamber with two valves: one for the intake of CO_2 and the other for flushing out. A 500 ml beaker of glycerol/water solution of the same aw was included in the chamber to maintain the equilibrium relative humidity (ERH). The colonies were measured every day and the plastic chambers flushed immediately afterwards with air or 3 L of 1000 ppm CO_2 from a gas cylinder (British Oxygen Company, 1000 ppm CO_2 cylinder) for about 12 minutes and then sealed (Medina *et al*., 2014). The same procedure was carried out for the control treatment (400 ppm). The chambers were incubated for 10 days at 25°C, 30°C for *P. verrucosum* and 30°C and 37°C for *A. westerdijkiae.* The fungal growth was measured daily as described in Chapter 3, Section 3.2.4.

4.2.4. Isolation of total RNA

This was the same as described in Chapter 3, Section 3.2.5

4.2.5. RT-qPCR assays and relative quantification

This was described in Chapter 3, Section 3.2.6 (a, b, c) and Section 3.3.6 (a, b, c).

4.2.6. Quantification of OTA production

See Chapter 3, Section 3.2.7

4.2.7. Statistical analyses of Data

See Chapter 3, Section 3.2.8.

4.3. Results

4.3.1. Effect of water potential x elevated CO_2 x temperature on growth, gene expression and OTA production by *P. verrucosum* on wheat-based media.

(a) Effect of climate change on relative growth rate at 25°C and 30°C on wheat media.

Figure 4.1a, b shows the effect of treatments on the relative growth rates in response to both solute and matric stress when exposed to the 3-way interacting CC environmental conditions. Growth was significantly affected when exposed to 30^{o}C and -2.8 MPa (= 0.98 a_w) and 1000 ppm CO_2 where no growth occurred. However, at -7.0 MPa (0.95 a_w) and 1000 ppm CO_2 increased growth when compared to existing conditions. With matric stress there was no effect on growth, with similar colonisation rates under all the treatments at 30^{o}C (see Figure 4.1b).

Statistically, the impact of treatments showed that there was a significant effect on growth at 25°C. At 30^{o}C, under glycerol solute stress, there was a significant effect with 1000 ppm CO_2 at -2.8 MPa water potential (=0.98 a_w).

(b) Effect of climate change factors on gene expression at 25°C and 30°C on wheat media.

Effect on *otapks* gene expression: At 25°C, the pattern of gene expression at the two water stress levels in the different media was quite different. At -2.8 MPa water potential (= 0.98 a_w) the gene expression was slightly higher at 1000 ppm when compared to the existing conditions in the glycerol modified media. However, under matric stress, the gene expression was significantly low in the 1000 ppm CO_2 and -7.0 MPa water potential media (= 0.95 a_w) when compared to existing conditions.

Statistically, the imposed water stress and type of solute applied had significant effect on the relative *otapks* gene expression at 25°C. Under matric stress of -7.0 MPa (0.95 a_w) and existing CO_2 concentrations (400 ppm), the gene expression was significantly higher when compared to that with elevated CO_2 (Figure 4.2a).

However, at 30°C, the pattern of gene expression was significantly stimulated under the imposed interacting stresses. There was a significant effect on gene expression at -7.0 MPa (=0.95 a_w) imposed matric stress under elevated levels of CO_2 (1000 ppm; Figure 4.2b).

Effects on *otanrps* gene expression: Figure 4.3a shows that the *otanrps* expression pattern was consistent with *pks* gene expression under matric potential stress. However, very low gene expression occurred in the solute modified (glycerol) treatments. Statistically, the gene expression was significantly reduced under elevated CO_2 (1000 ppm) exposure and under imposed water stress (0.95 a_w) at 25°C.

At 30°C, the gene expression was significantly higher under elevated levels of CO_2 at -2.8 MPa matric stress (=0.98 a_w) when compared to the existing interacting abiotic conditions. (Figure 4.3b).

(c) Effect of climate change abiotic factors on OTA production

Table 4.1 shows the concentrations of OTA (ng/g) under elevated CO_2 and imposed water stress. The strain was more tolerant of matric stress with a consistently higher toxin production regardless of water stress and elevated CO_2 levels at 25°C. However, in the solute modified media, no OTA was detected.

At 30°C, the OTA production pattern showed that *P. verrucosum* was tolerant of matric stress and was stimulated to produce increased amounts of OTA in 1000 ppm CO_2 exposure than in air (400 ppm), regardless of the water stress treatment (see Table 4.1).

Statistically, the interaction of three abiotic factors in both media showed no significant effect on toxin production (see Table 4.2).

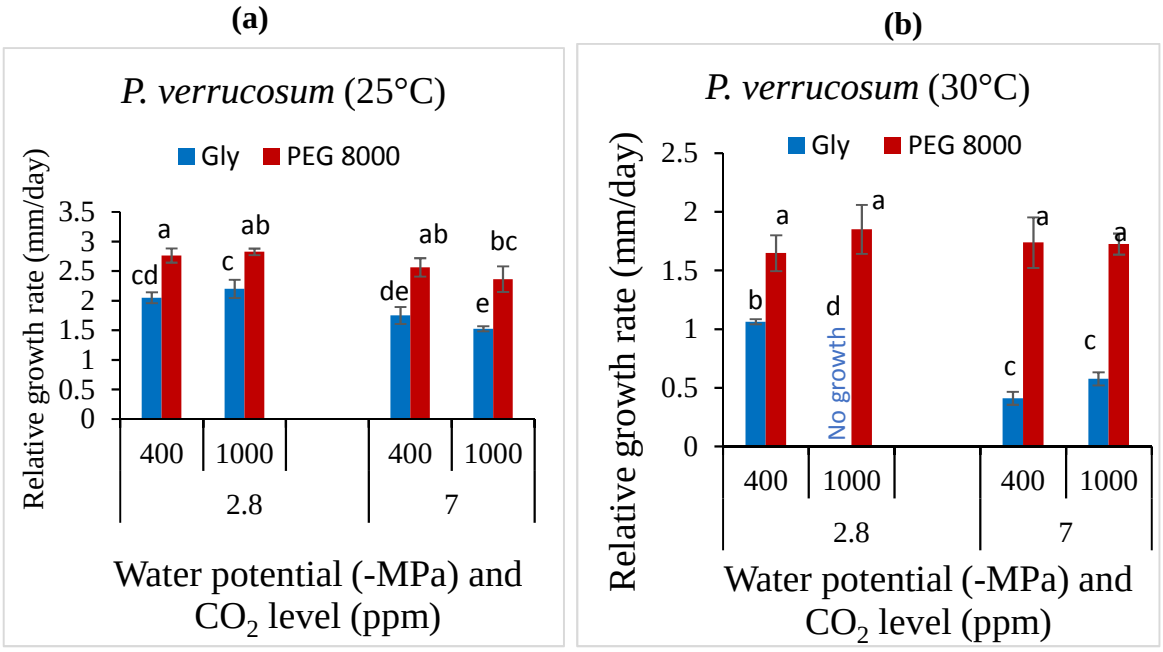

Figure 4.1. Effect of water potential x elevated CO_2 x temperature on relative growth rate of *P. verrucosum* grown on wheat-based media modified with glycerol (solute potential) or PEG 8000 (matric potential) over for 10 days at (a) 25°C and (b) 30°C. Bars indicate standard error of the mean. Different letters indicate significant difference.

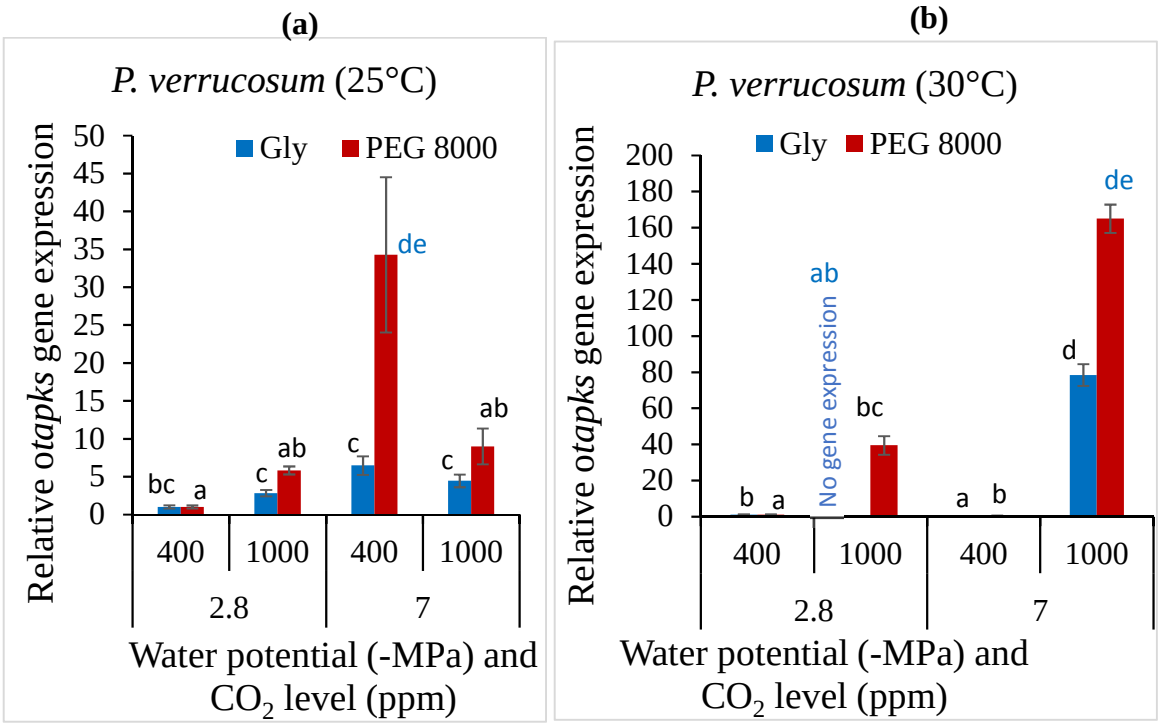

Figure 4.2. Effect of water potential x elevated CO_2 x temperature on relative *otapks* gene expression of *P. verrucosum* grown on wheat-based media under solute (glycerol) and matric (PEG 8000) stress for 10 days at (a) 25°C and (b) 30°C. Comparisons were made with the control media (400 ppm and -2.8 MPa (0.98 a_w) used as the calibrator for each medium separately. Bars indicate standard error of the mean. Different letters indicate significant differences.

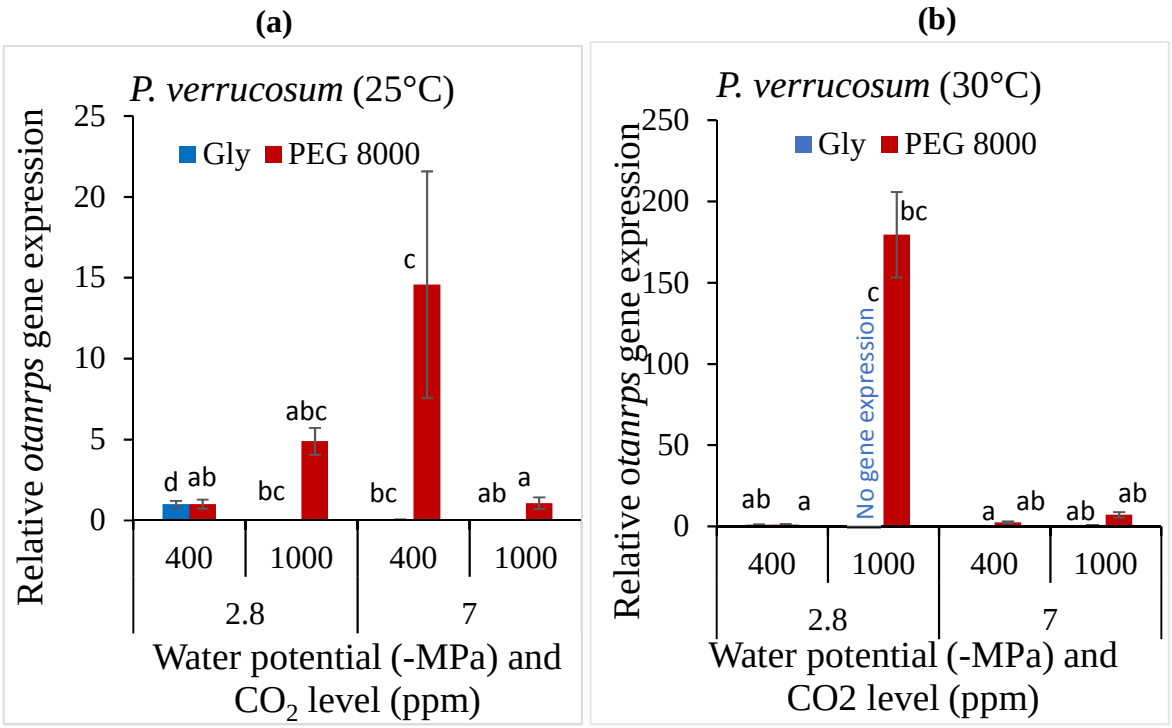

Figure 4.3. Effect of water potential X elevated CO_2 X and temperature on relative *otanrps* gene expression of *P. verrucosum* grown on wheat media modified with glycerol and matric potential to two water activities for 10 days at (a) 25°C and (b) 30°C with regard to control media (400 ppm and 0.98 a_w) used as a calibrator for each media separately. Bars indicate standard error of the mean. Different letters indicate significant difference.

Table 4.1. Effect of water potential x elevated CO_2 x temperature on OTA production (µg/g) on wheat-based media solute (Glycerol) or matric potential modified media at 25°C and 30°C after 10 days incubation.

Temperature			**25°C**		**30°C**	
Strain	**Ψ (-MPa)**	**CO_2 level (ppm)**	**Gly modified media**	**PEG 8000 modified media**	**Gly modified media**	**PEG 8000 modified media**
P. verrucosum	2.8	400	0.75±0.069	55.74±39.75	ND	2.05±1.40
		1000	ND	**83.55±14.16**	No toxin	1.92±0.65
	7.0	400	ND	**156.002±90.90**	ND	0.93±0.46
		1000	ND	102.43±66.95	ND	3.24±4.27

ND: non-detected, below the detectable level.

Table 4.2. Statistical analyses of OTA at 25°C and 30°C by factorial ANOVA test[a].

Source	DF	Sum of Squares	Prob>F
Ψ_t	1	7422785708	**0.0261***
CO_2	1	2153651658	0.2180
Ψ type	1	3.6514e+10	**<.0001***
Temp	1	1.8038e+10	**0.0010***
Ψ_t *CO_2	1	2436136788	0.1908
Ψ_t * Ψ type	1	7662474172	**0.0240***
Ψ_t * Temp	1	3641748743	0.1120
CO_2* Ψ type	1	2153651658	0.2180
CO_2* Temp	1	1171435499	0.3609
Ψ type * Temp	1	1.8e+10	**0.0010***
Ψ_t *CO_2* Ψ type	1	2533413115	0.1824
Ψ_t *CO_2 * Temp	1	1229629827	0.3494
Ψ_t * Ψ type *Temp	1	3760472190	0.1066
CO_2* Ψ type*Temp	1	1171435499	0.3609
Ψ_t *CO_2* Ψ type*Temp	1	1278496019	0.3402

*Significant difference. [a] Shown are the ANOVA results for the effects of Ψ_t (-2.8, -7 MPa), type of water potential (non-ionic solute and matric) and CO_2 level (400 ppm, 1000 ppm) and their interactions on OTA production at 25°C and 30°C. *, significant at $p<0.05$.

4.3.2. Effect of water potential x elevated CO_2 x temperature on growth, gene expression and OTA production by *A. westerdijkiae* on coffee-based media.

(a) Effect of climate change factors on relative growth rate at 30°C and 37°C on coffee-based media.

Figure 4.4 shows the effect of the interacting abiotic CC factors on growth at 30°C. Interestingly, the strain was relatively tolerant of elevated CO_2 with growth generally very similar at 1000 ppm CO_2 as that at 400 ppm, regardless of water stress levels. However, in solute modified media, growth was decreased significantly under imposed water stress (-7.0 (=0.95 a_w, 1000 ppm).

Interestingly, at elevated temperature of 37°C, no growth occurred in both solute and matric modified coffee-based media in any of the treatments over the 10-day incubation period.

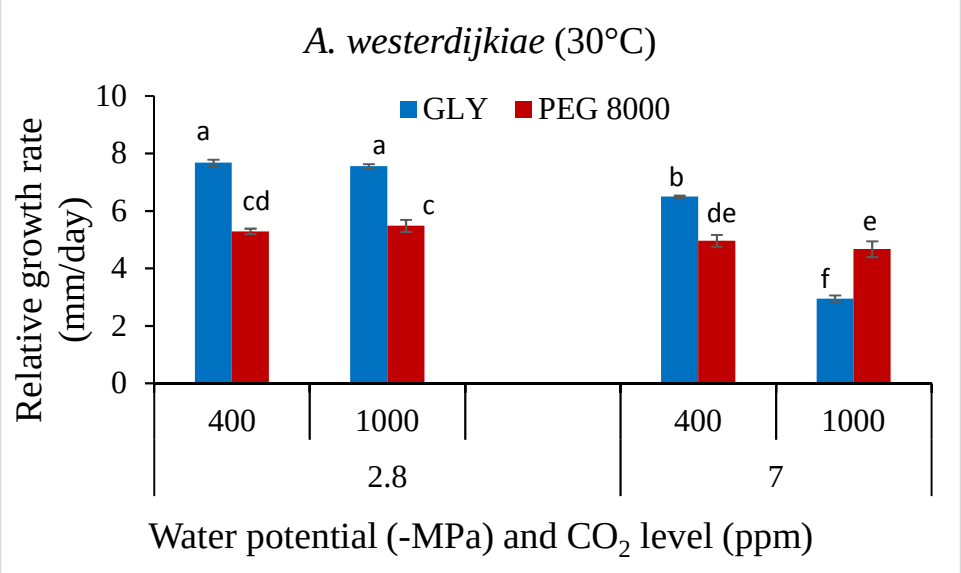

Figure 4.4. Effect of water potential x elevated CO_2 x and temperature on relative growth rate of *A. westerdijkiae* grown on coffee-based solute (glycerol) and matric (PEG 8000) modified media to two water availability treatment levels over 10 days at 30°C. Bars indicate standard error of the mean. Different letters indicate significant differences.

(b) Effect of climate change on gene expression at 30°C on coffee-based media.

Effect on *otapks* gene expression: There was a low gene expression under the imposed elevated levels of CO_2 and high matric potential stress (Figure 4.5). Statistically, the elevated CO_2 and water potential had no significant effect on the gene expression. In 1000 ppm CO_2 exposure and -7.0 MPa (=0.95 a_w), the gene expression was slightly higher compared to the control (400

ppm). Overall, the gene expression was relatively constant at the different CO_2 levels and under imposed water stress. No gene expression occurred in the solute modified media and was thus excluded in the statistical analyses of the data sets.

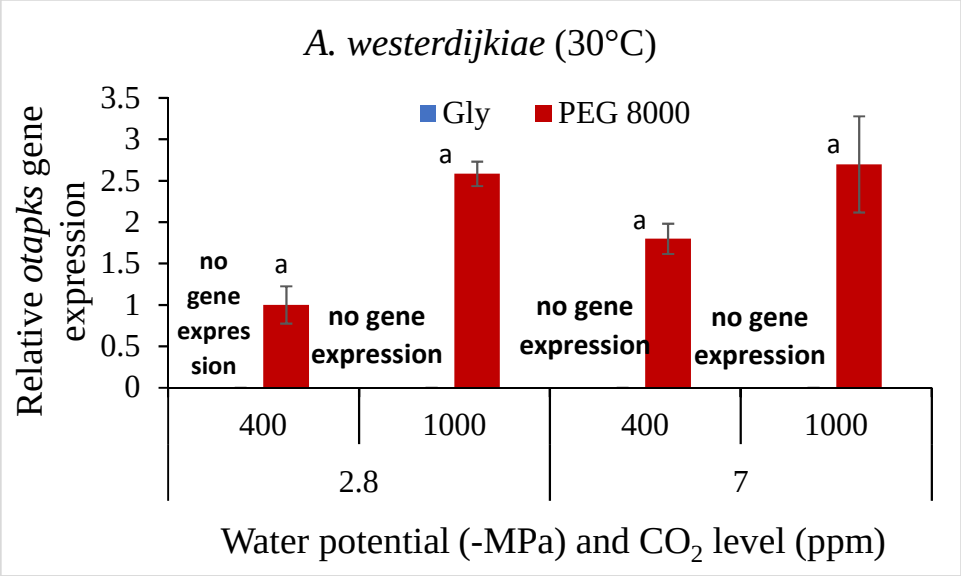

Figure 4.5. Effect of water potential x elevated CO_2 x and temperature on relative *otapks* gene expression of *A. westerdijkiae* grown on coffee-based media modified with a solute (glycerol) or matrically (PEG 8000) at 2 a_w levels for 10 days at 30°C when compared to the control media (400 ppm and 0.98 a_w) used as a calibrator for each media separately. Bars indicate standard error of the mean. Different letters indicate significant differences.

Effect on *otanrps* gene expression: There was no gene expression found in both treatments under the imposed water stress and elevated CO_2 levels.

(c) Effect of climate change abiotic factors on OTA production on coffee-based media.

The strain was more sensitive to the imposed solute stress with glycerol and CO_2 levels as no toxin was detected. However, the strain was more tolerant of matric potential stress as some small amounts of OTA was produced. No statistical differences in the OTA production under matric stress were found. Table 4.3 shows the concentration of OTA found under the impact of CC scenarios.

Table 4.3. Effect of water potential x elevated CO_2 x and temperature on OTA production (ng/g) on coffee-based media with modified solute (Glycerol) and matric stress (PEG 8000) at 30°C for 10 days.

Strain	Ψ_t (-MPa)	CO_2 level (ppm)	Gly modified media	PEG 8000 modified media
A. westerdijkiae	2.8	400	ND	22.08±4.4
		1000	ND	**48.31±32.51**
	7.0	400	ND	16.53±15.67
		1000	No toxin	**21.72±8.21**

ND: non-detected (below the detectable level).

Table 4.4. Statistical analysis of OTA at 30°C by ANOVA[a].

Source	DF	Sum of Squares	Prob>F
Ψ_t	1	26.82	0.75
CO_2	1	20.15	0.78
Ψ type	1	405.08	0.23
Ψ *CO_2	1	159.11	0.44
Ψ_t * Ψ type	1	26.82	0.75
CO_2* Ψ type	1	20.15	0.78
Ψ_t *CO_2* Ψ type	1	159.11	0.44

[a] Shown are the ANOVA results for the effects of Ψ_t (-2.8, -7 MPa), type of water potential (nonionic Ψ_s and Ψ_m) and CO_2 level (400 ppm, 1000 ppm) and their interactions on OTA production at 25°C. *, significant at $p<0.05$.

4.4. Discussion

4.4.1. Effect of interacting abiotic climate change factors on growth, gene expression and OTA production by *P. verrucosum* on wheat-based media.

This is the first study, to my knowledge, which has examined the effect of CC scenarios on the molecular ecology of *P. verrucosum.* This strain was able to grow well over a wide range of solute potentials. There was no significant difference in growth between the non-ionic solute and matric stress conditions at -2.8 (=0.98 a_w) and -7.0 MPa (=0.95 a_w), and different levels of CO_2 at 25°C. The elevated levels of CO_2 also had no impact on growth at both water potential treatments. However, when the temperature was elevated by +5°C, the growth pattern was different in both media. In the solute modified media, growth was decreased significantly in the elevated CO_2 treatments as no growth was observed at 1000 ppm, and -2.8 MPa (=0.98 a_w) treatments. The general pattern was lower than at 25°C. However, under matric potential stress, growth was faster, regardless of the water stress and elevated levels of CO_2.

For the biosynthetic genes involved in OTA production, the *pks and otanrps* gene expression patterns appeared to be only slightly affected by the elevated CO_2 treatment, especially at 25ºC. Interestingly, under solute stress with existing or elevated CO_2 the expression of both *otapks* and *otanrps* genes were very low with no expression recorded at intermediate water stress level of -2.8 MPa and 1000 ppm. However, at -7.0 MPa relative *otapks* expression was significantly increased at 1000 ppm CO_2 exposure. Previously, for other mycotoxigenic fungi such as *A. flavus* it was found that the *aflD* (structural gene) and *aflR* (regulatory gene) were stimulated under elevated temperature in maize–based media and in stored maize (Medina *et al*., 2017).

The relative increase in the *otapks* and *otanrps* expression under elevated temperature and CO_2 conditions thus had a significant influence on the biosynthetic pathways involved in secondary metabolite production by *P. verrucosum*. Overall, this study suggests that perhaps under CC scenarios other secondary metabolites might be produced, especially in areas where coffee and cocoa are grown, fermented, dried and stored. Often the CCP is ensuring effective drying post-fermentation to ensure that there is even drying. Pockets of moist commodity can subsequently lead to OTA contamination (Pitt *et al*., 2013).

4.4.2. Effects of CC interacting abiotic factors on growth, gene expression and OTA production by *A. westerdijkiae* on coffee-based media.

The influence of three way interacting environmental factors on *A. westerdijkiae* was quite different. The strain grew effectively at elevated CO_2 levels in both intermediate and drought conditions when compared to the control (400 ppm) at 30°C. However, when the temperature was increased +7°C, no growth occurred over the 10-day incubation period. Medina *et al*., (2014) previously reported that the growth of *A.flavus* was relatively unaffected by the addition of 2x and 3x existing CO_2 (650, 1000 ppm) levels at 37°C under the different water stress treatments.

The gene expression pattern was not consistent with the growth behaviour since no gene expression of key biosynthetic genes in the OTA cluster, especially *otapks* was found in the solute (glycerol) modified water potential treatment in elevated CO_2 and drought. In the matric modified media, however, the different levels of CO_2 had no significant effect on the gene expression which remained low in all treatments examined. In addition, no expression was detected for the *otanrps* gene. These results are not consistent with those obtained by Medina *et al*., (2014) who demonstrated in their study on *A. flavus* that there is a relative increased expression of both the structural *aflD* and the regulatory *aflR* genes at 37°C and under water stress (0.95, 0.92 a_w) conditions on a conducive medium.

With regard to toxin production, this study showed that OTA production was enhanced under intermediate drought conditions at 1000 ppm in response to matric stress. Overall, OTA production pattern of *A. westerdijkiae* was still low when compared to that of *P. verrucosum*.

The results obtained in the present study are a good prediction for the consequences of CC scenarios, especially in hot spot regions of the world, including Sub-Saharan Africa, parts of America and parts of Asia (Medina *et al*., 2017). Other studies have been carried out on stored coffee beans in relation to OTA production under CC change conditions on *Aspergillus* section *Circumadati* and Section *Nigri* species (*A. westerdijkiae, A. ochraceus, A.steynii; A. carbonarius*) (Akbar, 2015; Akbar *et al*., 2016). They estimated that OTA production was stimulated in *A. westerdijkiae* while in *A. carbonarius*, there was a reduction in OTA production.

Some studies have been carried out in the field to determine the impacts of CC especially on maize. They have estimated, for example, in Serbia, there was no aflatoxin contamination in maize in the 2009-2011 seasons. However, prolonged exposure of maize to hot and dry weather in 2012 resulted in 69% of the samples being contaminated with aflatoxins (Kos *et al.*, 2013).

Overall, some studies have shown that there is intra-strain difference for *A. flavus* in response to growth and OTA production in control conditions and under CC scenarios. They reported that acclimatisation of toxigenic strains for up to 10 generations can influence growth and AFB_1 production. Indeed, this was stimulated in the acclimatised strain when colonising pistachio nuts. However, of the three strains did not respond in this manner. (Medina *et al*., 2017). More detailed studies are necessary on more strains of each species to better understand the impact that the 3-way interacting abiotic factors influence colonisation and toxin production. Previously, Vary *et al*., (2015) showed that 10-20x strain acclimatisation for *F. graminearum* and *Zymospetoria tritici* resulted in higher pathogenicity than unacclimatised strains. Unfortunately, no toxin quantification was done. Overall, fungal pathogens are able to evolve very quickly, especially in response to abiotic stresses including drought, chemical fungicides and temperature fluctuations. Thus, CC scenarios imposes significant stress on the fungus and because of their plasticity they are able to rapidly adapt and colonise crop ecosystems under CC conditions (Vaughan *et al*., 2014; Vary *et al*., 2015; Battilani *et al*., 2016).

CHAPTER 5

Effect of preservatives on growth and OTA production by *P. verrucosum* and *A. westerdijkiae in vitro* and in stored grain and coffee beans

Chapter five

5.1. General Introduction

There is significant interest in the development of minimisation strategies to reduce human and animal exposure to mycotoxins including OTA. A number of studies have examined the use of preservatives to inhibit the growth of toxigenic species. The most commonly used ones have been salts of organic acids as they have GRAS (generally recognised as safe) status by the American Food and Drug Administration (Alcano and Jahn, 2016; Ricke, 2003). Salts of propionic acid and sorbic acids such as calcium propionate (CP) and potassium sorbate (KS) are preservatives that are commonly used in intermediate food products, especially bakery products as well as in animal feeds. However, there is pressure to reduce the concentrations of preservatives used in both raw commodities and processed food products because of consumer concerns about chemical additives. However, it has been demonstrated that the use of sub-optimal concentrations of such preservatives can result in a stimulation of growth of mycotoxigenic fungi and a stimulation of toxin production by some *Penicillium*, *Fusarium*, and *Aspergillus* species (Arroyo *et al*., 2005, Marin *et al*., 2002, Schmidt *et al*., 2007). Thus, alternative but more effective preservatives are required for controlling *P. verrucosum* in wheat and *A. westerdijkiae* in coffee production, to try and minimise OTA contamination of these commodities.

The objectives of this study were to:

(a) Screen a range of different preservatives at different concentrations for efficacy against *P. verrucosum* on wheat-based, and for *A. westerdijkiae,* on coffee-based media under different water activity conditions

(b) Determine the best candidates based on the ED_{50} and ED_{90} concentrations for controlling growth and OTA production

(c) Test the best treatments in stored wheat (*P. verrucosum*) and coffee (*A. westerdijkiae*) for controlling OTA contamination in these two matrices under different a_w conditions.

5.2. Materials and Methods

5.2.1. Fungal strains

One strain of *P. verrucosum* (OTA11) and one of *A. westerdijkiae* (CECT 2948) were used in this study. The former strain was isolated from wheat grain and the latter from coffee beans.

5.2.2. Inoculum preparation and inoculation

Fungal strains were sub-cultured on malt extract agar (30.0 g malt extract, 5.0g peptone and 15g agar at 25°C in the dark for 10 days. The spores were gently dislodged from the colony surface with a surface sterilised loop into 9 ml sterile distilled water containing 0.05 % Tween-80 in 25ml Universal bottles. Fungal spore concentration was determined using a haemocytometer and diluted to 10^6 spores/ml by the addition of sterile water.

5.2.3. *In vitro* screening of preservatives for control of mycelial growth and OTA production.

2% (w/v) milled wheat or 2% (w/v) milled coffee agar media were used as the basal medium for screening efficacy of the preservatives for the two species respectively. The media were modified to the target a_w levels (0.98 and 0.95) with glycerol/water solutions and sterilized at 121ºC, cooled to 50ºC and poured into sterile 90 mm diameter sterile plastic Petri plates. The a_w of the basal media was checked using the Aqualab (TE4) instrument (Decagon Devices. Pullman, WA 99163 USA). The pH of all the treatments was 6.5-7.0. The preservatives tested were Sodium metabisulphite, Potassium sorbate, Calcium propionate, Ferulic acid, Trans-cinnamic acid and Propyl paraben.

Different concentrations of the preservatives were used. In order to obtain these concentrations a stock solution of 50.000 mg L^{-1} was made up and diluted appropriately to obtain treatment levels. These were filter sterilized and added to the cooled molten media, which was then shaken vigorously to facilitate mixing and then poured into the Petri plates (approx. 15 mls per plate).

The treatments and replicates were centrally inoculated with the spore inoculum of each species and these were incubated at 25 or 30°C for *P. verrucosum* and *A. westerdijkiae* respectively. The colonies were measured as described previously (See Chapter 2, Section 2.2.5).

5.2.4. Extraction & Quantification of OTA production from the in-vitro samples.

See Chapter 2, Section 2.2.6.

5.2.5. Efficacy of the best preservatives for control of growth and OTA production in stored wheat grain and green coffee.

Both wheat grain (500 gms) in sealed bags and raw arabica coffee beans (500 gms, Al Ahmed Co., Kuwait) in sealed plastic bags were gamma irradiated at 12-15 kGrys (Synergy Health, Swindon, Berks., U.K.) and stored at 4°C until use.

(a) Determination of the moisture adsorption curve of wheat & coffee grains.

Moisture adsorption curves were constructed for both wheat and coffee. Thus, different known amounts of water were added to 5 gm quantities of wheat or coffee in 25 ml Universal bottles. These were shaken regularly and stored at 4°C for equilibration. After equilibration at 25°C the actual a_w of the samples was measured and the moisture contents also determined by drying at 105°C for 17 hrs. The a_w of the wheat/coffee was measured using an Aqua lab TE4 instrument. Figure 5.1 and 5.2 show the moisture adsorption curve and the relationship between added water and a_w values for irradiated wheat and coffee grains.

Subsequently, 864 g of wheat grains and 420 g of the coffee beans were weighed, and sterile water added using the MAC to obtain the required a_w levels and kept at 4°C for 24 hr. The moist wheat and coffee grains were divided into four replicates for each treatment (12 g for wheat grains) and (5.7 g for coffee beans) which are placed in single layers in the 9 cm Petri plates and inoculated centrally with 0.5 ml of spore suspension (10^6 CFU ml^{-1}). The replicates of the same treatment were enclosed together in a plastic chamber and incubated at 25°C and 30°C for *P. verrucosum* and *A. westerdijkae* respectively. The samples were then ground at the end of the storage period. These were stored at -20°C until OTA extraction and analysis were carried out by HPLC.

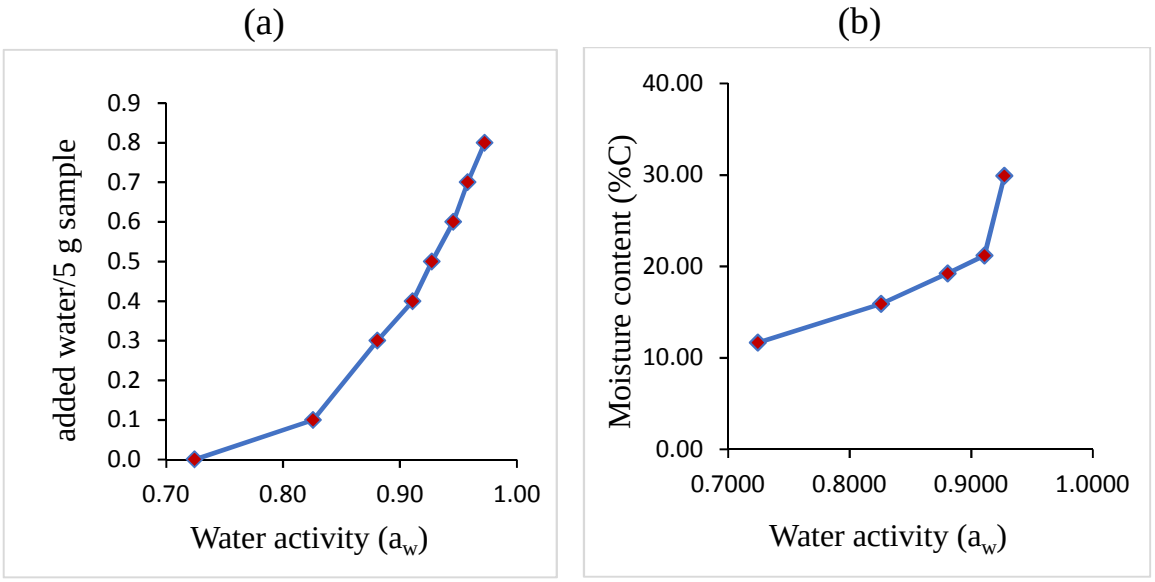

Figure 5.1. (a) Relationship between added water and water activity values and (b) adsorption moisture curve for wheat grains.

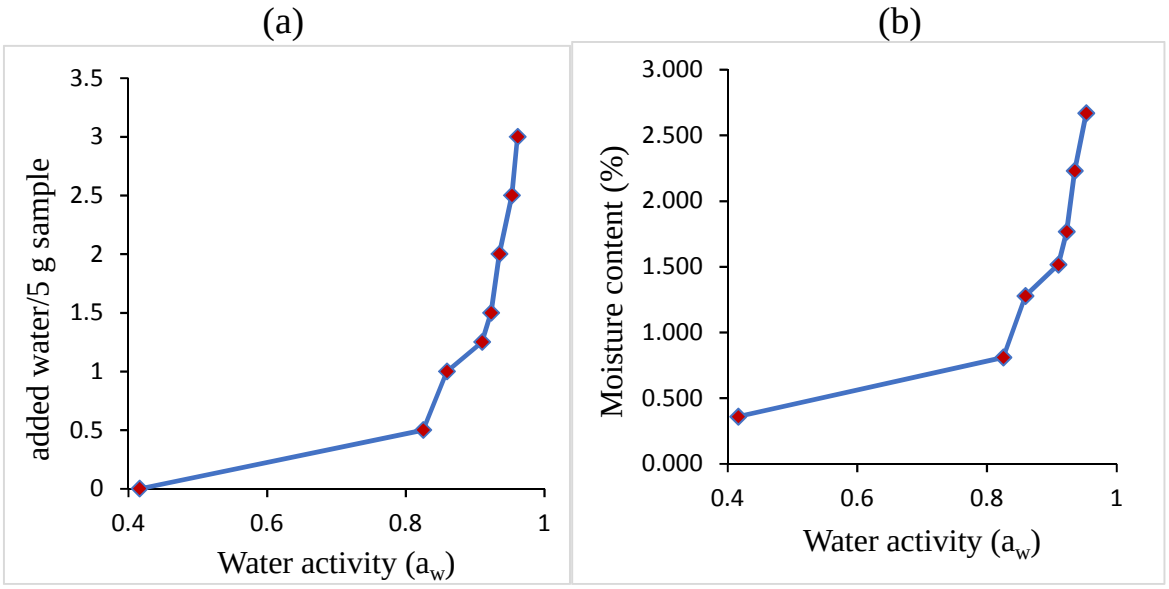

Figure 5.2. (a) Relationship between amounts of added water and water activity values in 5 g sub-samples of coffee beans and (b) moisture adsorption curve for the coffee beans.

(b) Preparation of spore inoculum for stored wheat grain/coffee bean studies

The strains were grown on MEA at 25^{o}C for 7 days. Spore suspensions were prepared by agitating the colony surface with a sterile spatula in 9 ml of sterile distilled water containing 0.05% Tween 80. This was decanted into a sterile 25 ml Universal bottle and the concentration determined. This was adjusted with sterile water to obtain an inoculum of 10^6 spores/ml using a haemocytometer.

(c) Extraction and quantification of OTA from the stored wheat and coffee experiments

OTA was extracted using the multistep 229 Ochra column method of Romer Ltd. (Austria). Twelve grams of milled wheat grains and 5.7 g of milled coffee beans were extracted with 50 ml of 84+16 ACN+H_2O (Acetonitrile+Water) and 24 ml of 84+16 ACN+H_2O (Acetonitrile+Water) for wheat rains and coffee beans respectively and shaken for 30 minutes. The extract was filtered and 6 mls acidified with 60µl acetic acid. This was then passed through the MultiSep column. Then all the liquid was removed, and 4 ml of the eluate was evaporated to dryness and re-dissolved in 400 µl mobile phase as shown in Figure 4.3. The final extract was injected (20 µl) into the HPLC system (flow rate: 1 ml min^{-1}) equipped with a fluorescence detector (λ_{ex} 333nm; λ_{ex} 460nm) and C18 column (Poroshell 120, length 100mm, diameter 4.6 mm, particle size 2.7 µm). The retention time of OTA under the conditions described was approximately 2.5 min. The mobile phase used were Acetonitrile (57%): Water (41%): Acetic acid (2%). (Medina *et al*., 2014).

5.3. Data analysis

See Section 2.2.7

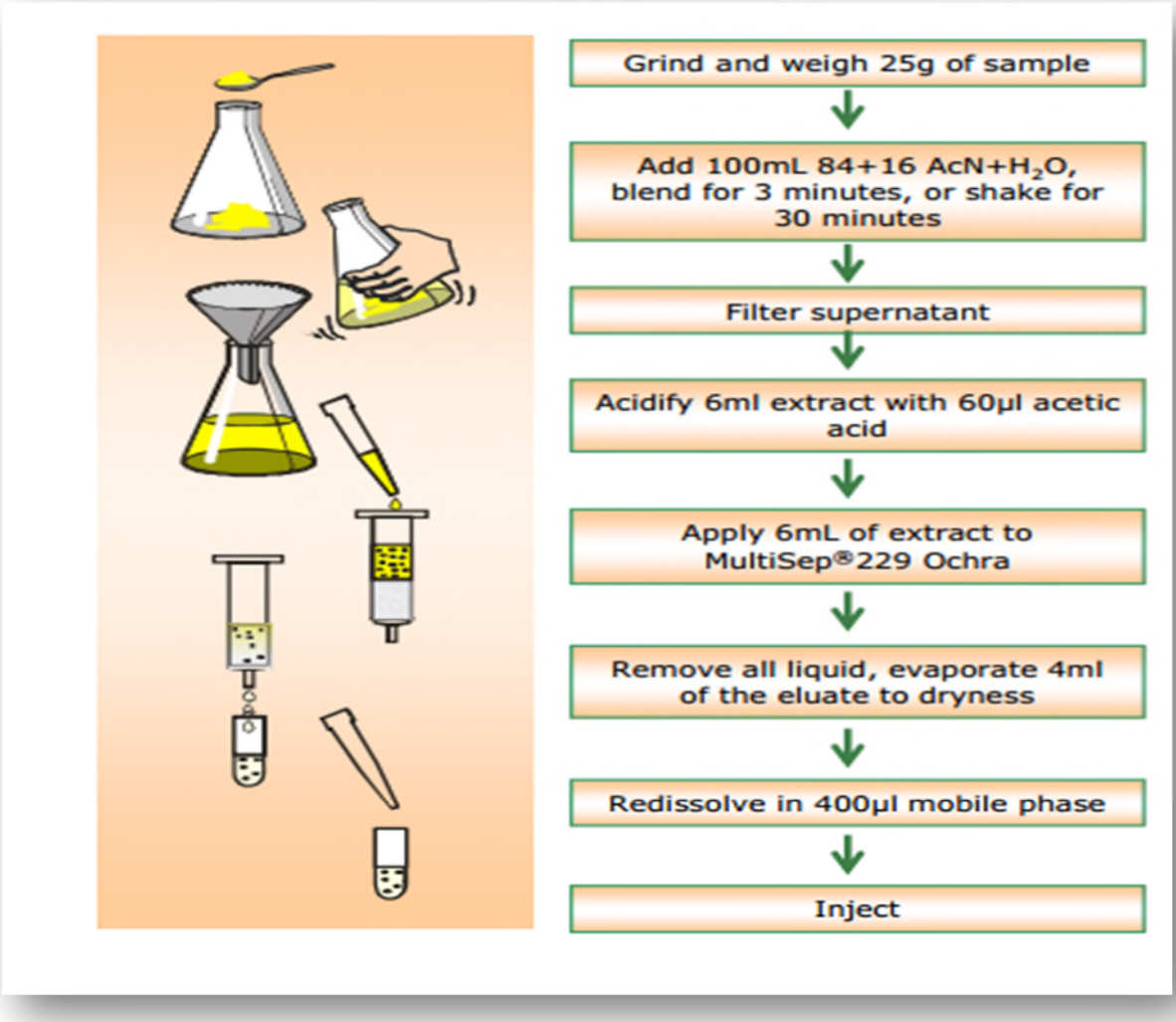

Figure 5.3. Pathway for the extraction of OTA from wheat grains/Coffee beans (adapted from Romer Labs IAC Instructions).

5.4. Results

5.4.1. Screening preservatives for controlling growth of *P. verrucosum* on wheat-based matrices.

Table 5.1 shows the effect of the tested preservatives on relative growth of the *P. verrucosum* strain on wheat-based media at 25ºC at both 0.98 and 0.95 a_w. The use of Sodium metabisulphite inhibited growth completely at higher concentrations (250-1000 mg L^{-1}) as well as trans-cinnamic acid and propyl paraben at both 0.98 and 0.95 a_w. However, the strain showed a strong tolerance to the other preservatives examined even at 1000 mg L^{-1}. Thus, based on these results higher concentrations of these compounds were subsequently tested. More detailed studies were carried out with sodium metabisulphite to examine the effects on both growth and OTA control against *P. verrucosum*.

Table 5.1. Screening the effect of different preservatives on growth of *P. verrucosum* on wheat-based medium modified to two water activities at 25ºC.

			0.95 a_w			
Concentration (mg L^{-1})	**0**	**50**	**100**	**250**	**500**	**1000**
SM (Sodium metabisulphite)	**++++**	**+++**	**+**	-	-	-
PS (Potassium sorbate)	+++	+++	+++	+++	+++	+++
CP (Calcium Propionate)	++++	++++	+++	+++	+++	+++
FE (Ferulic acid)	++++	++++	++++	++++	++++	+
TCA (Trans-cinnamic acid)	**++++**	**++++**	**++++**	-	-	-
PP (Propyl parapen)	**++++**	**++++**	**++**	-	-	-
			0.98 a_w			
SM	**++++**	**++++**	**++++**	-	-	-
PS	++++	++++	++++	++++	++++	++++
CP	++++	++++	++++	++++	++++	++++
FE	++++	++++	++++	++++	++++	+
TCA	**++++**	**++++**	**++++**	-	-	-
PP	**++++**	**++++**	**+++**	-	-	-

Key to effects: ++++, similar to control; +++, 75% of control; ++, 50% of control; +, 25% of control; -, no growth.

(a) Effect of Sodium metabisulphite on growth and OTA production by *P. verrucosum* on wheat-based matrices

Figure 5.4. shows the effect of different concentrations of SM on growth of the *P. verrucosum* strain in wheat media at 25°C at both 0.98 and 0.95 a_w. Growth was inhibited at high concentrations of SM confirming the initial screening studies. Growth was thus significantly inhibited by higher concentrations of this preservatives at both 0.98 and 0.95 a_w.

For toxin production, there is a significant effect of the applied SM on OTA production, with none being produced under moderate water stress at >100mg/l (Figure 5.5).

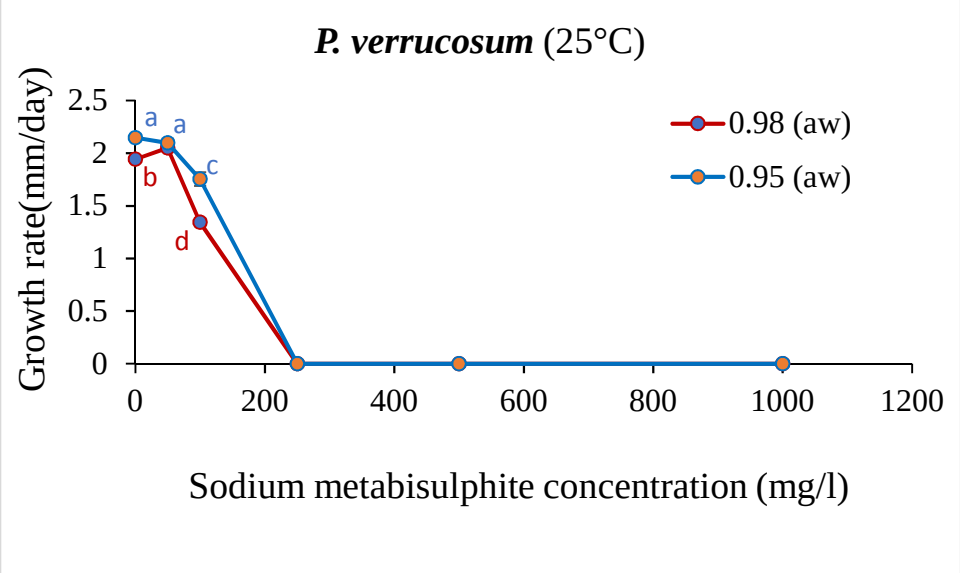

Figure 5.4. Effect of sodium metabisulphite concentration on radial growth rate (mm/day) and by *P. verrucosum* on 2% wheat medium modified to two water activity levels at 25ºC after 10 days. Different letters indicate significant difference.

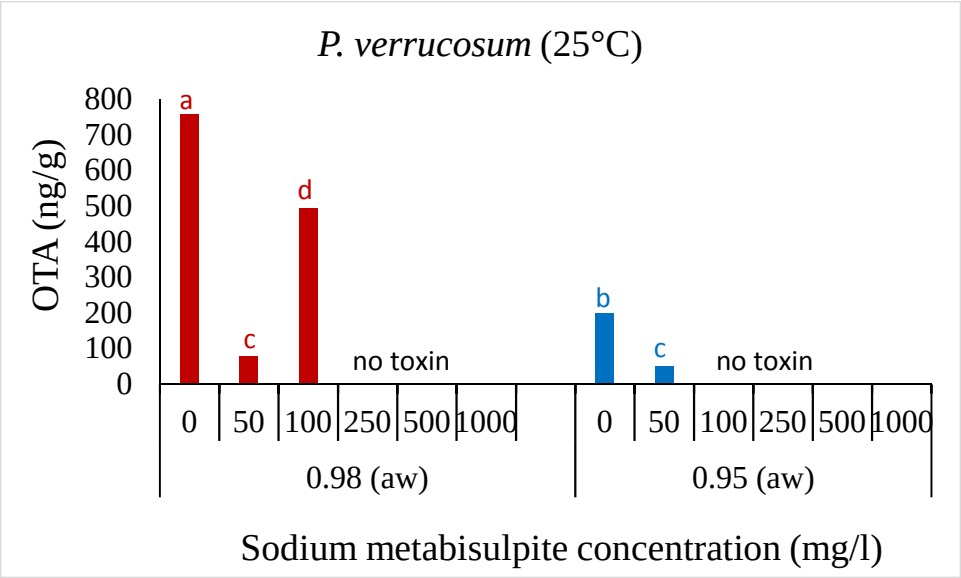

Figure 5.5. Effect of sodium metabisulphite concentration on OTA production by *P. verrucosum* on 2% wheat medium modified to two water activity levels. Different letters indicate significant difference.

(b) Effect of Ferulic acid (FE) on growth and OTA production by *P. verrucosum*.

Figure 5.6 shows the effect of this treatment on both growth and OTA production. As the concentration of FE was increased, the growth rate was significantly reduced with up to 2000 mg/l regardless of the imposed water activities. However, the effect on OTA production was very different. Thus, under wetter conditions there was some inhibition of OTA production. However, at intermediate a_w (0.95) there was a relatively low production with low concentrations of FE. However, as this was increased there was a stimulation of OTA production especially with 2000 mg/l (Figure 5.7).

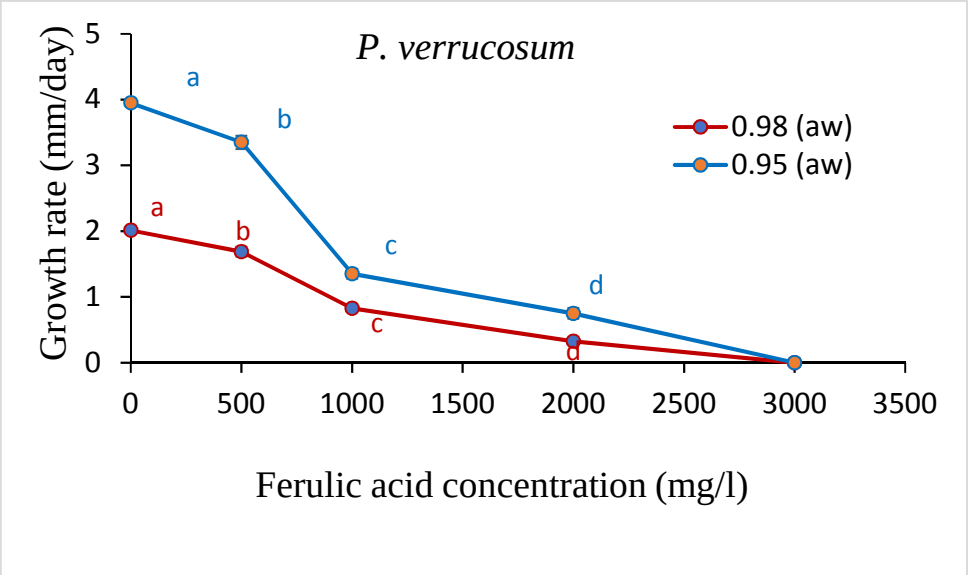

Figure 5.6. Effect of Ferulic acid concentration on radial growth rate (mm/day) of *P. verrucosum* on 2% wheat medium modified to two water activity at 25ºC after 10 days. Different letters indicate significant differences.

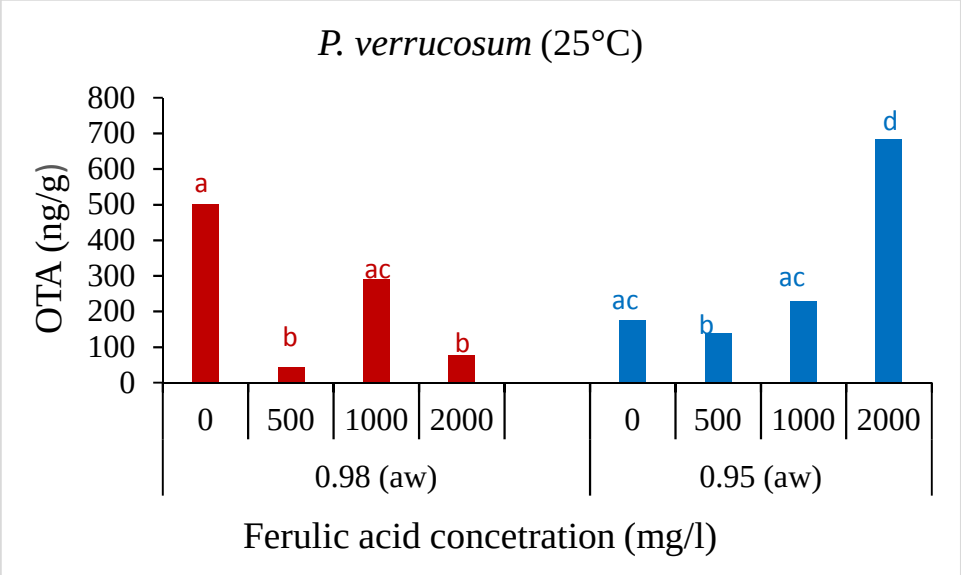

Figure 5.7. Effect of Ferulic acid concentration on OTA production by *P. verrucosum* on 2% wheat medium modified to two water activity at 25ºC after 10 days. Different letters indicate significant differences.

(c) Effect of trans-cinnamic acid on growth and OTA production by *P. verrucosum*

This preservative had a significant effect on growth of *P. verrucosum* at both a_w levels with no growth occurring at 250 mg/l (Figure 5.8). For OTA production, toxin was produced at all concentrations except the 250 mg/l where no growth occurred. At 0.98 a_w there was little difference in toxin production between 0, and 50-100 mg/l. However, at 0.95 a_w 50 and 100 mg/l significantly reduced OTA production. Table 5. 2. shows the statistical effect of the treatments.

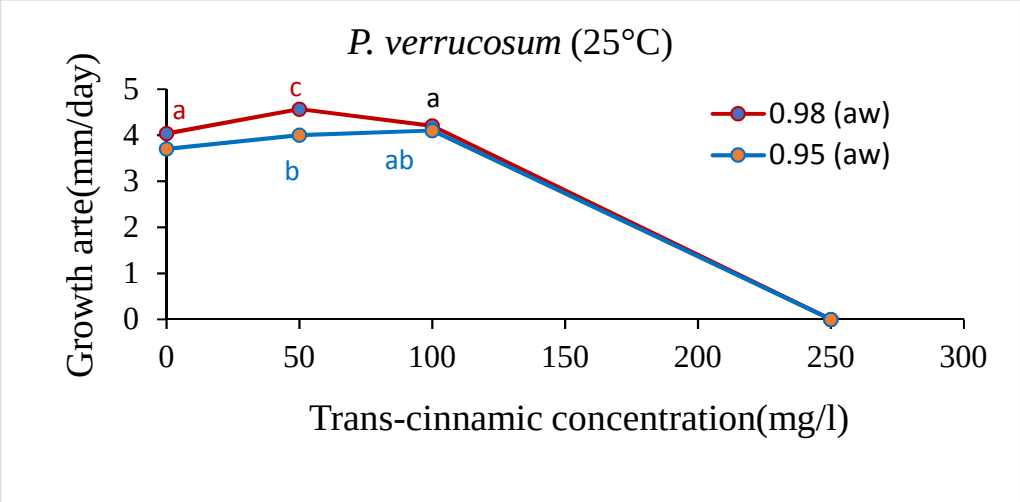

Figure 5.8. Effect of Trans-cinnamic acid concentration on radial growth rate (mm/day) of *P. verrucosum* on 2% wheat medium modified to two water activity at 25ºC after 10 days. Different letters indicate significant differences.

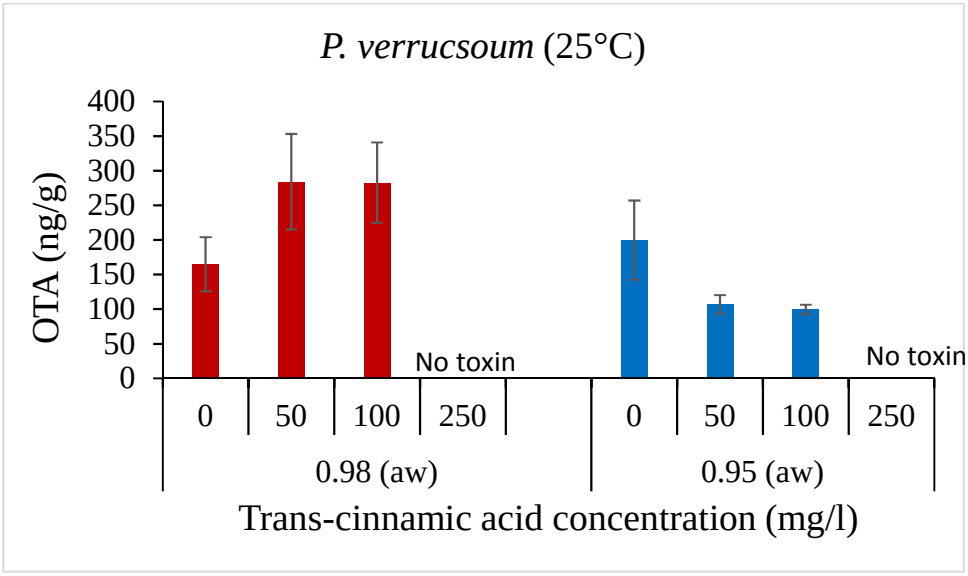

Figure 5.9. Effect of Trans-cinnamic acid concentration on OTA production by *P. verrucosum* on 2% wheat medium modified to two water activity at 25ºC after 10 days. Bars represent SEM.

Table 5.2. List of *p*-values for OTA production using the Kruskal-Wallis test (non-parametric data).

Strain	TCA acid Concentration (mg/l)	a_w	Conc*a_w	Response
P. verrucsoum	NS*	S*	N/A	OTA (ng/g)

* Kruskal-Wallis test; S significant; NS: non-significant; NA: non-applicable. $p<0.05$.

(d) Effect of Propyl Paraben on growth and OTA production by *P. verrucosum*

Figure 5.10 shows the effect of this anti-oxidant treatment on both growth and OTA production at two a_w levels. Again, PP was able to completely inhibit growth and OTA production at 250 mg/l concentration. With more freely available water OTA was only slightly inhibited with 100 mg/l while at 0.95 a_w. Both 50 and 100 mg/l appeared to reduce production significantly. Table 5.3 shows the statistical effect of PP on OTA production.

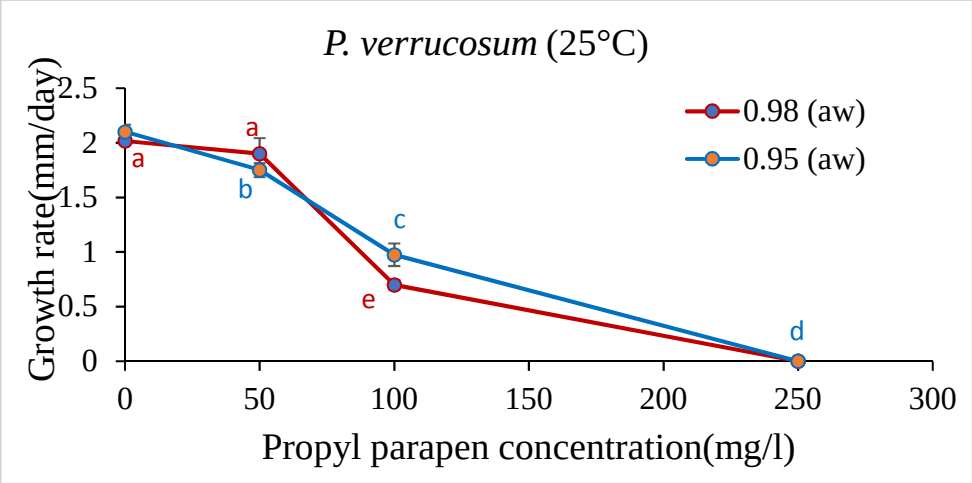

Figure 5.10. Effect of Propyl Paraben concentration on radial growth rate (mm/day) of *P. verrucosum* on 2% wheat medium modified to two water activity at 25ºC after 10 days incubation. Different letters are significant different.

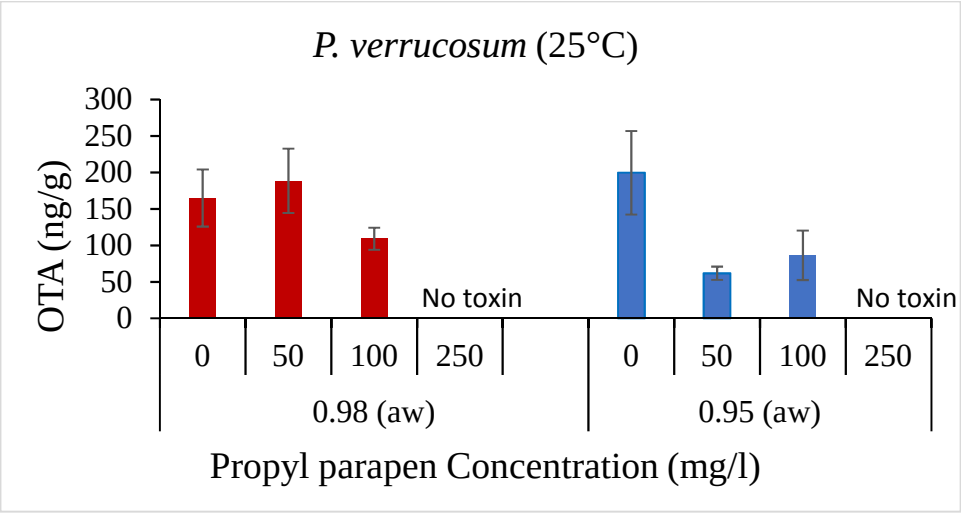

Figure 5.11. Effect of Propyl Paraben concentration on OTA production by *P. verrucosum* on 2% wheat medium modified to two water activity at 25ºC after 10 days incubation. Bars represent SEM.

Table 5.3. List of *p*-values for OTA production using the Kruskal-Wallis test (non-parametric data).

Strain	PP acid Concentration (mg/l)	a_w	Conc*a_w	Response
P. verrucsoum	S*	NS*	N/A	OTA (ng/g)

* Kruskal-Wallis test; S significant; NS: non-significant; NA: non-applicable. $p<0.05$.

(e) Determination the ED_{50} and ED_{90} for the effect of preservatives on the growth and toxin production by *P. verrucosum*

Tables 5.4 and 4.5 show the ED_{50} and ED_{90} values for TCA, FE, and SM in relation to the concentrations required for inhibition of growth and OTA production at both a_w levels examined.

Table 5.4. The calculated ED_{50} and ED_{90} concentrations of the three best treatments on growth and OTA production by *P. verrucosum* at 0.98 a_w.

0.98 a_w		
	ED_{50} (mg/l)	ED_{90} (mg/l)
Ferulic acid		
Growth rate	1000	2000
OTA	300	500
TCA		
Growth rate	150	240
OTA	210	60
SM		
Growth rate	175	250
OTA	40	230

Table 5.5. The calculated ED_{50} and ED_{90} concentrations of the three best treatments on growth and OTA production by *P. verrucosum* at 0.95 a_w. MIC, minimum inhibitory concentrations

0.95 a_w		
	ED_{50} (mg/l)	ED_{90} (mg/l)
Ferulic acid		
Growth rate	900	2700
OTA	MIC	MIC
TCA		
Growth rate	150	240
OTA	100	210
SM		
Growth rate	176	250
OTA	40	70

5.4.2. Effect of preservatives on growth and OTA production by *P. verrucosum* in stored wheat.

(a) Effect of Sodium metabisulphite on control of growth and OTA production in stored wheat

Figure 5.12 shows the effect of treatments with this preservative on both colonisation rates and OTA production by the strain of *P. verrucosum* examined. Results were very different from that observed *in vitro*. At 0.95 a_w there was no effect on growth of any of the preservative concentrations. However, under drier conditions growth was stimulated by 100 mg/l. For OTA control this was effect at 0.95 a_w as the concentration was increased. However, under drier conditions there was again some stimulation in OTA production, even with 250 mg/l although this was not statistically significant. Table 5.6 shows the statistical effect of the treatments.

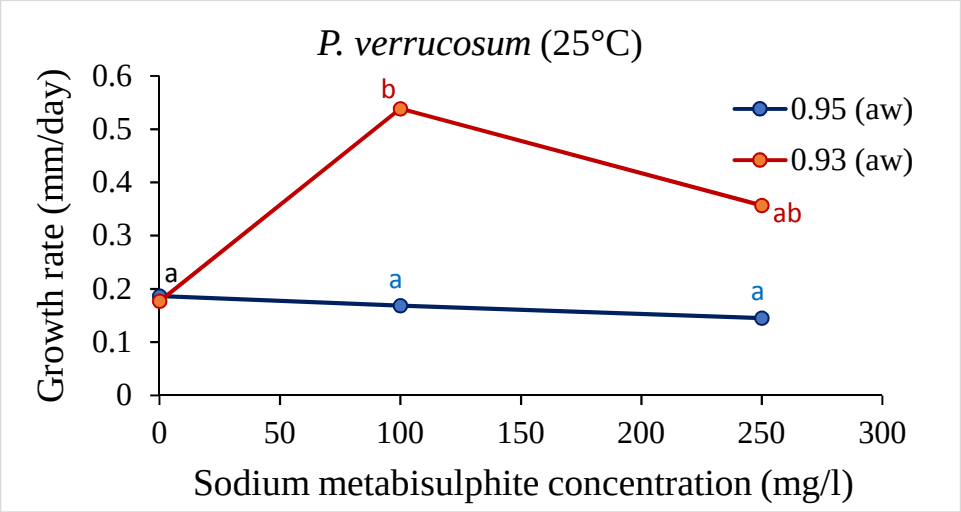

Figure 5.12. Effect of Sodium metabisulphite concentration on radial growth rate (mm/day) of *P. verrucosum* on layers of wheat grain modified to two water activity at 25ºC after 30 days storage. Different letters are significant different.

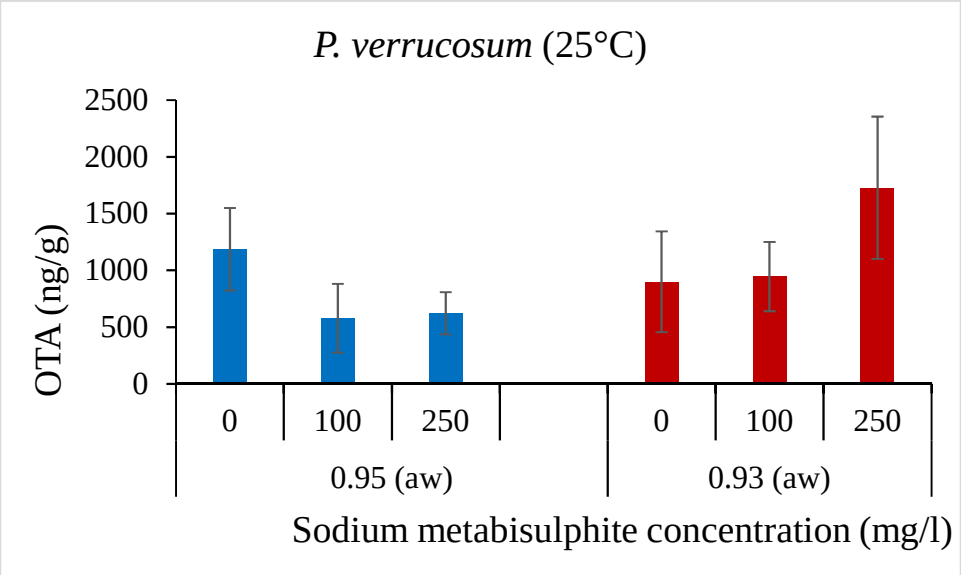

Figure 5.13. Effect of Sodium metabisulphite concentration on OTA production by *P. verrucosum* on layers of wheat grain modified to two water activity at 25ºC after 30 days storage. Bars represent SEM.

Table 5.6. List of *p* values for the growth rate and OTA production by ANOVA test.

Strain	a_w	SM Conc	a_w*Conc	Response
P. verrucosum	NS[a]	NS[a]	NS[a]	OTA (ng/g)

S; significant, NS; non-significant, NA; not applicable.

(b) Effect of Ferulic acid on growth and OTA production in stored wheat

The effect of different concentrations on both growth and OTA at two a_w levels is shown in Figure 5.14. Growth was completely inhibited at the highest concentration examined regardless of a_w level. Control of OTA production was very effective at both 1000-2000 mg/l treatment levels when compared with the untreated control. Table 5.7 shows the statistical effect of the different treatments.

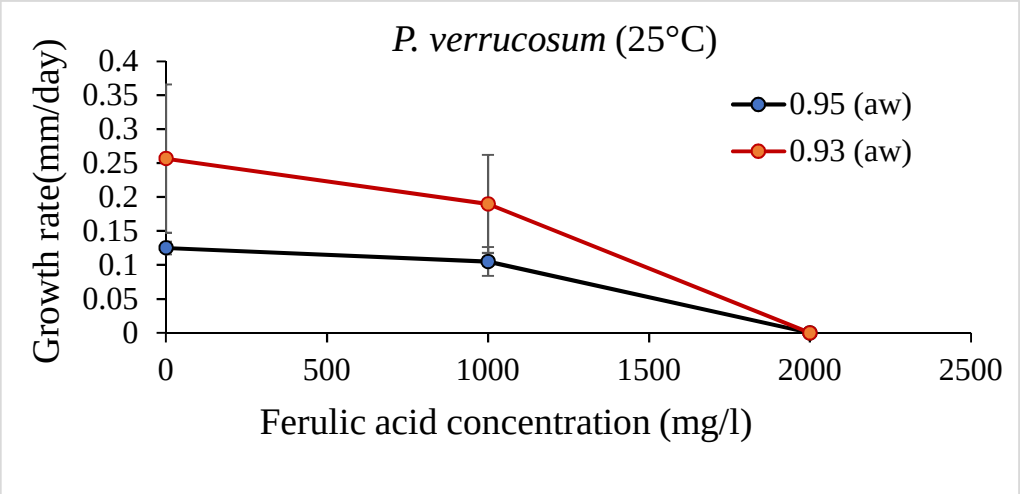

Figure 5.14. Effect of Ferulic acid concentration on radial growth rate (mm/day) of *P. verrucosum* on layers of wheat grains modified to two water activity at 25ºC after 30 days incubation. Bars represent SEM.

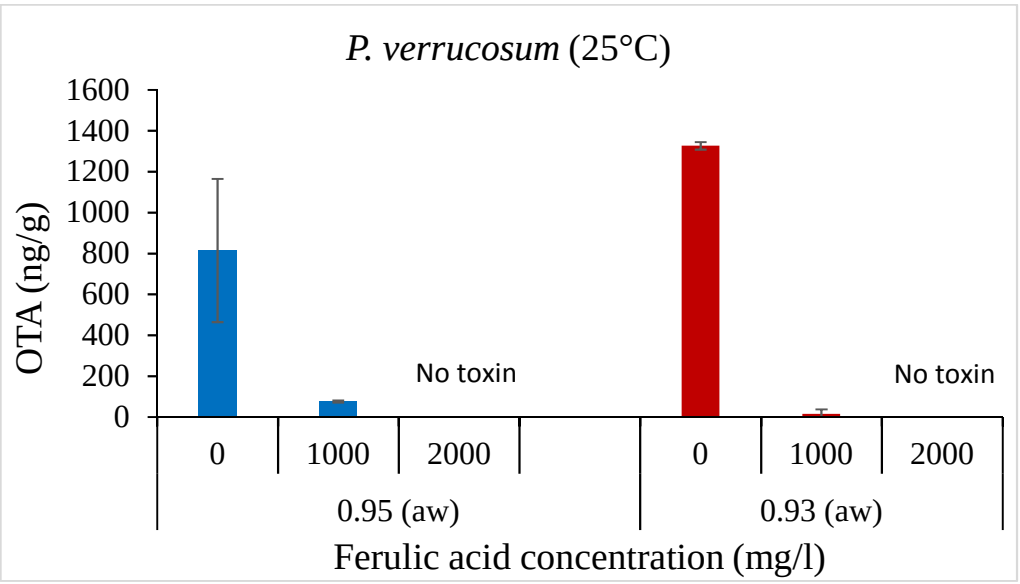

Figure 5.15. Effect of Ferulic acid concentration on OTA production by *P. verrucosum* on layers of wheat grains modified to two water activity at 25ºC after 30 days incubation. Bars represent SEM.

Table 5.7. List of *p* values for the growth rate and OTA production by Kruskal-Wallis test and ANOVA test.

Strain	a_w	FE Conc	a_w*Conc	Response
P. verrucosum	S[a]	NS[a]	NS[a]	Growth rate (mm/day)
	NS[*]	S[*]	NA	OTA (ng/g)

S; significant, NS; non-significant, NA; not applicable.
[*]Kruskal-Wallis test, [a]ANOVA test.

(c) Effect of Trans-cinnamic acid on growth and OTA production by *P. verrucosum* in stored wheat grain

Figure 5.16 shows the effect of different concentrations tested on layers of wheat grain of different a_w levels. Overall, there was little effect of treatments on growth rate at both a_w levels, although colonisation was faster under drier conditions. For OTA control there was less produced at 0.95 a_w regardless of treatments with higher production at 0.93 a_w. However, there was no significant control of OTA observed. There was thus no statististically significant effects of treatment with Trans-cinnamic acid (Table 4.8).

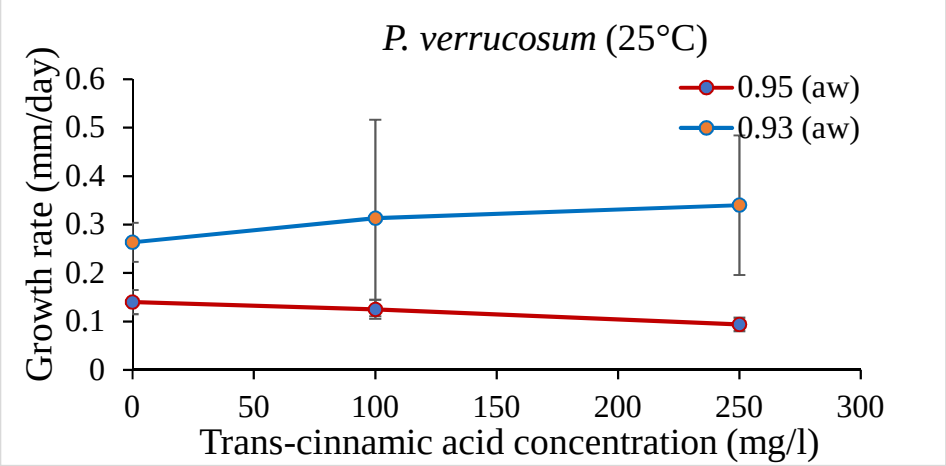

Figure 5.16. Effect of Ferulic acid concentration on radial growth rate (mm/day) of *P. verrucosum* on layers of wheat grains modified to two water activity at 25ºC after 30 days incubation. Bars represent SEM.

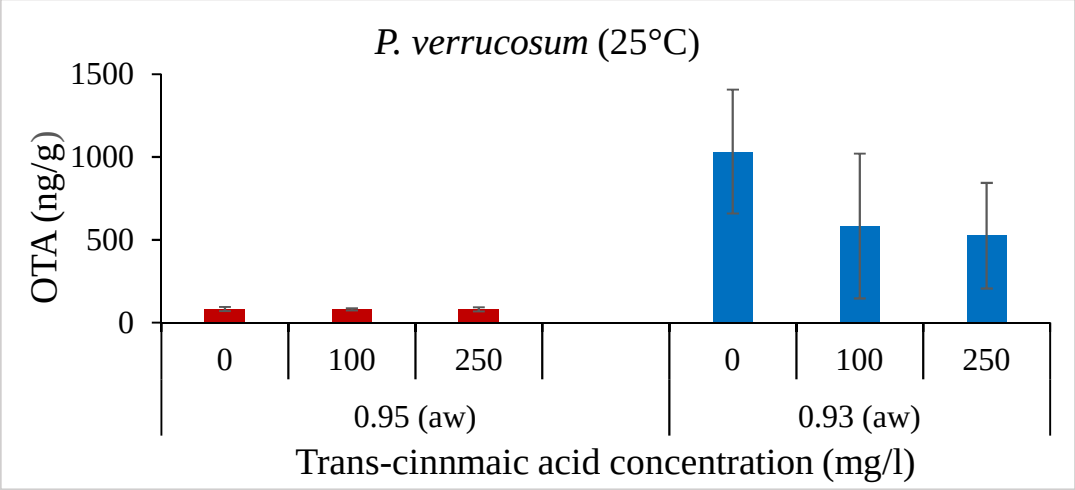

Figure 5.17. Effect of Trans-cinnamic acid concentration on OTA production by *P. verrucosum* on wheat grain modified to two water activity at 25ºC after 30 days incubation. Bars represent SEM.

Table 5.8. List of *p* values for the growth rate and OTA production by Kruskal-Wallis test and ANOVA test.

Strain	a_w	TCA Conc	a_w*Conc	Response
P. verrucosum	NS[a]	NS[a]	NS[a]	Growth rate (mm/day)
	S[*]	NS[*]	NA[*]	OTA (ng/g)

S; significant, NS; non-significant, NA; not applicable.
[*]Kruskal-Wallis test, [a]ANOVA test.

5.4.3. Screening preservatives for control of growth of *A. westerdijkiae in vitro* on coffee-based media.

Table 5.9. shows the effect of the preservatives on growth of the strain of *A. westerdijkiae* on coffee-based medium modified to 0.98 and 0.95a_w at 30ºC with different concentrations of the compounds relative to the control. Overall, SM, TCA and PP were most effective at 500-1000 mg/l concentrations.

Table 5.9. Screening for the effect of different preservatives on growth rate of *A. westerdijkiae* on coffee medium modified to two water activities.

	0.95 a_w					
Concentration (mg/l)	**0**	**50**	**100**	**250**	**500**	**1000**
SM	**++++**	**+++**	**+++++**	**+++**	**++**	-
PS	++++	++++	++++	++++	++++	++++
CP	++++	++++	++++	++++	++++	++++
FE	++++	++++	++++	++++	++++	++++
TCA	**++++**	**++++**	**++++**	**++++**	**++++**	-
PP	**++++**	**++++**	**++++**	**++**	-	-
	0.98a_w					
SM	**++++**	**+++++**	**++++**	**+++**	**++**	**+**
PS	++++	++++	++++	++++	++++	++++
CP	++++	++++	++++	++++	++++	++++
FE	++++	++++	++++	++++	++++	++++
TCA	**++++**	**++++**	**++++**	**++++**	**++++**	**-**
PP	**++++**	**++++**	**++++**	**++++**	-	-

Key to effects: ++++, similar to control; +++, 75% of control; ++, 50% of control; +, 25% of contol; -, no growth.

(a) Effect of Sodium metabisulphite on growth and OTA production by *A. westerdijkiae*

Figure 5.18 shows the effect of this preservative on the relative growth rate and OTA production by this species at two a_w levels. At 1000 mg/l there was complete inhibition of growth of this species at both a_w levels. Overall, both preservative concentration and the a_w level has a significant effect on growth.

For the production of OTA, in the controls on coffee-based media the production was below the level of quantification at both a_w levels tested. However, when 250 or 500 mg/l was used this

appeared to stimulate OTA production. However, at 1000 mg/l there was complete inhibition of OTA production.

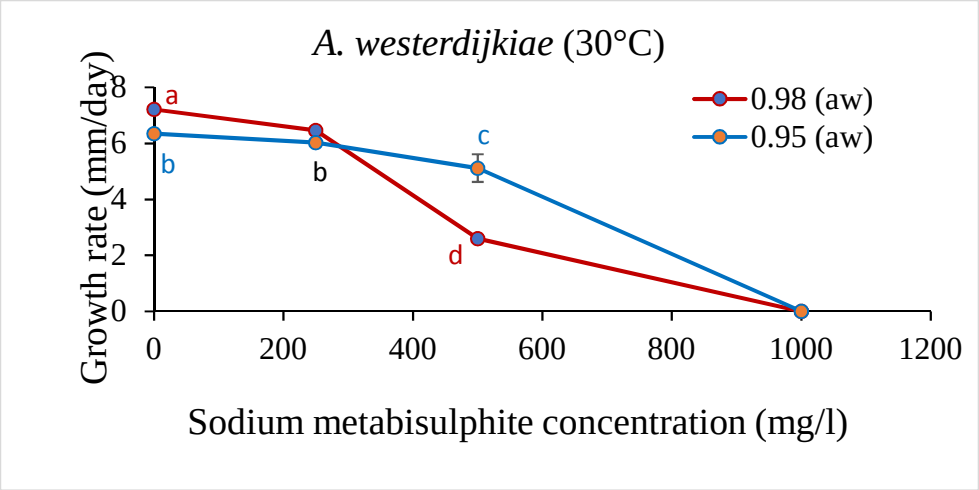

Figure 5.18. Effect of Sodium metabisulphite concentrations on radial growth rate (mm/day) of *A. westerdijkiae* on 2% coffee medium modified to two water activity levels at 30ºC after 10 days incubation. Data for growth with different letters are significant.

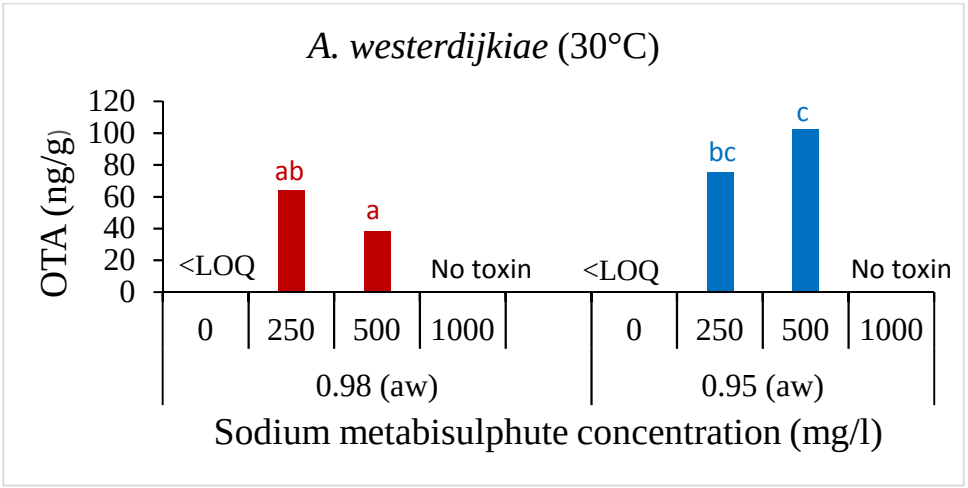

Figure 5.19. Effect of Sodium metabisulphite concentrations on OTA production by *A. westerdijkiae* on 2% coffee medium modified to two water activity levels at 30ºC after 10 days incubation. Data with different letters are significant difference.

b) Effect of Ferulic acid on growth and OTA production by *A. westerdijkiae*

Statistically, both the FE concentrations and water stress imposed had significant effects on the growth rate. Interestingly, the growth was significantly reduced by half at sub-optimal concentration (1000 mg/l) compared to the growth rate at 250 mg/l at both water stress levels. Growth was completely inhibited at 2000 mg/l at both water stress levels, thus it was excluded from the statisitical analyses. (Figure 5.20).

For toxin production, Interestingly, no toxin was detected at 1000 mg/l and 0.98 a_w, in contrast, a significant amount of toxin was reported at 1000 mg/l at 0.95 a_w (Table 5.10).

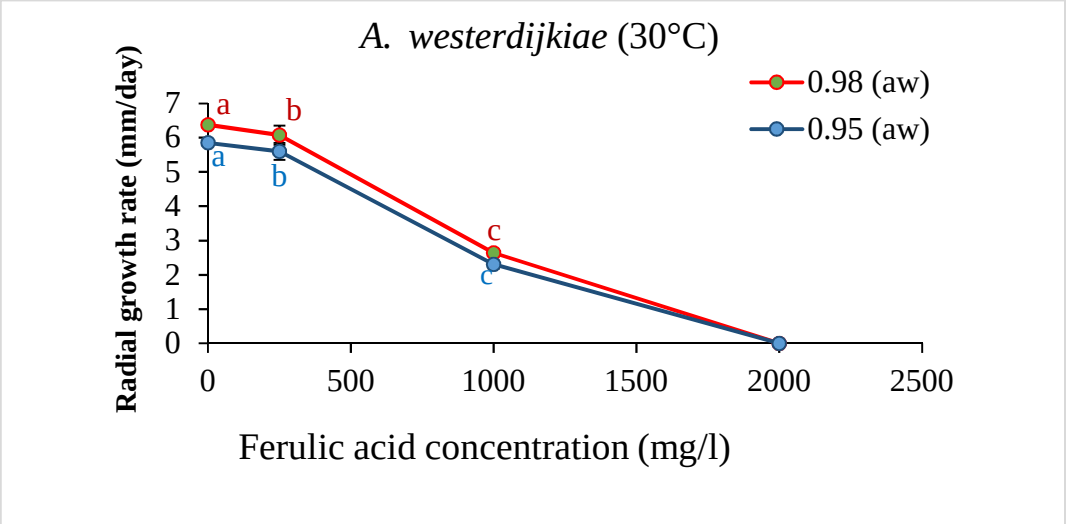

Figure 5.20. Effect of Ferulic acid concentrations on radial growth rate (mm/day) of *A. westerdijkiae* on 2% coffee medium modified to two water activity at 30ºC after 10 days. Bars represent SEM. Levels with different letters are significant different.

Table 5.10. Ochratoxin A production under different concentrations of Ferulic acid at two water activities by *A. westerdijkiae* and statisitical analysis by ANOVA test.

Strain	**a_w**	**0 mg/l**	**250 mg/l**	**1000 mg/l**	**2000 mg/l**
A. westerdijkiae		OTA (ng/g)±SD	OTA (ng/g)±SD	OTA (ng/g)±SD	OTA (ng/g)±SD
	0.98	<LOQ	<LOQ	No toxin	No toxin
	0.95	42.08±7.39*	No toxin	47.78±4.3*	No toxin

*No significant difference.

c) Effect of Propyl parapen on growth and OTA production by *A. westerdijkiae*.

This strain of *A. westerdijkiae* was sensitive when exposed to PP concentrations in terms of growth rate. Since, the growth rate was reduced significantly when high doses of PP was used at both water activities. At 500 mg/l the growth rate was inhibited, thus it was excluded from the statistical analyses (Figure 5.21).

For toxin production, the strain was more resistant under sub-optimal concentrations (250 mg/l) at 0.95 a_w as it produced high amounts of toxin when compared to the control as well as to less water availability. However, no significant differences were found between treatments.

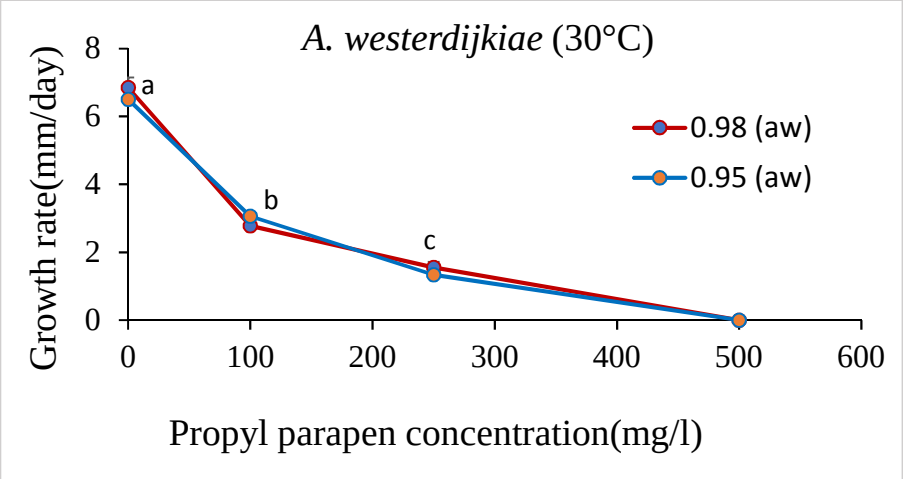

Figure 5.21. Effect of Propyl parapen concentrations on radial growth rate (mm/day) of *A. westerdijkiae* on 2% coffee medium modified to two water activity at 30ºC after 10 days. Bars represent SEM. Different letters indicate significant difference.

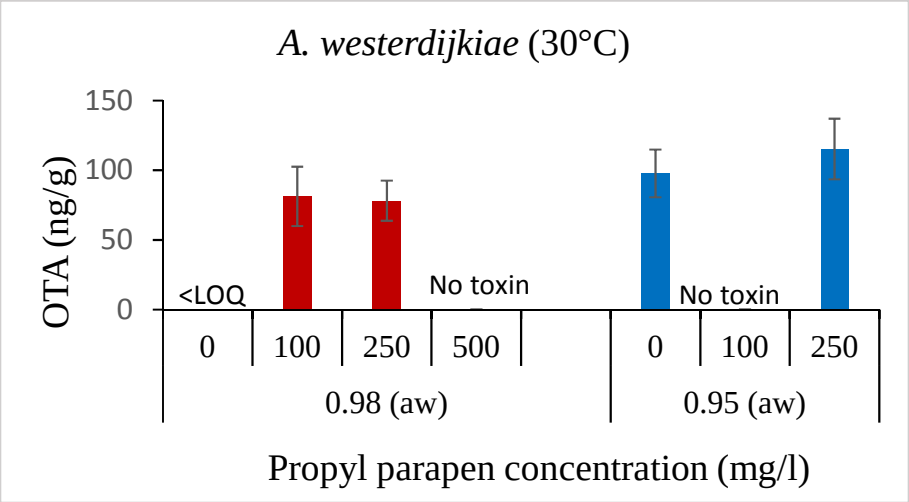

Figure 5.22. Effect of Propyl parapen concentrations on OTA production by *A. westerdijkiae* on 2% coffee medium modified to two water activity at 30ºC after 10 days. Bars represent SEM.

d) Effect of Trans-cinnamic acid on growth and OTA production by *A. westerdijkiae*

Statistically, different doses of TCA have significant effects on the growth rate of the strain regardless of water stress (Figure 5.23). The growth was inhibited at 1000 mg/l at both water activities.

For toxin control, there was more resistance under moderate water stress (0.95 a_w) and the sub-optimal doses of TCA (Table 5.11). Hence, the amount of toxin was below the level of quantification at 0.98 a_w under control conditions and no toxin was detected at 250 mg/l at 0.98 a_w stress, thus these were excluded in the statistical analyses.

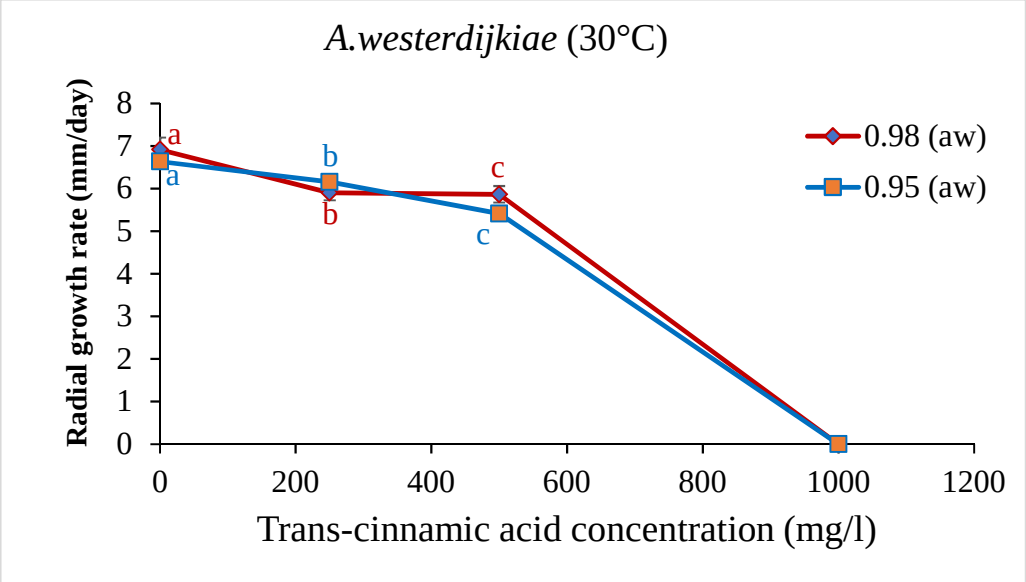

Figure 5.23. Effect of Trans-cinnamic acid concentrations on radial growth rate (mm/day) of *A. westerdijkiae* on 2% coffee medium modified to two water activity at 30ºC after 10 days. Bars represent SEM. Different letters indicate significant difference.

Table 5.11. ANOVA test of Ochratoxin A production under different concentrations of Trans-cinnamic acid at two water activities by *A.westerdijkiae*.

Strain	a_w	**0 mg/l**	**250 mg/l**	**500 mg/l**	**1000 mg/l**
A. westerdijkiae		OTA (ng/g) ±SD	OTA (ng/g) ±SD	OTA (ng/g) ±SD	OTA (ng/g) ±SD
	0.98	<LOQ[a]	<LOQ[a]	124± 16.7[b]	No toxin
	0.95	101.9±10.57[a]	102.9±26.5	96.2±16.6[a]	No toxin

Different letters indicate significant difference.

e) Determination the ED_{50} and ED_{90} for the effect of preservatives on the growth and toxin production by *A. westerdijkiae*

Table 5.12 and 5.13 show the ED_{50} and ED_{90} values for TCA, FE, and SM in respect to the growth rate and OTA control.

Table 5.12. ED_{50} and ED_{90} values of growth and toxin production by *A. westerdijkiae* for FE, TCA and SM at 0.98a_w.

0.98 a_w		
	ED_{50} (mg/l)	ED_{90} (mg/l)
Ferulic acid		
Growth rate	900	1530
OTA	MIC	MIC
TCA		
Growth rate	700	950
OTA	170	250
SM		
Growth rate	450	225
OTA	40	MIC

Table 5.13. ED_{50} and ED_{90} values of growth and toxin production by *A. westerdijkiae* for FE, TCA and SM at 0.95 a_w.

0.95 a_w		
	ED_{50} (mg/l)	ED_{90} (mg/l)
Ferulic acid		
Growth rate	900	1530
OTA	1300	MIC
TCA		
Growth rate	700	650
OTA	90	220
SM		
Growth rate	800	1000
OTA	MIC	50

5.4.4. Effect of preservatives on the growth rate and OTA production by *A. westerdijkiae* on stored coffee beans.

a) Effect of Ferulic acid on growth and OTA production.

The strain was more resistant towards different doses of ferulic acid in terms of growth rate than found in the in vitro media-based studies. However, water stress had a significant effect on the growth rate of the strain. At drier conditions, 0.93 a_w, the growth was reduced compared to when less water was available (Figure 5.24).

In terms of toxin control, the strain produced high amount of toxin regardless of the water stress and FE concentrations used (Figure 5.25. Table 5.15 shows the statistical analysis for the growth rate and OTA production under the impact of FE doses at the two water activities tested.

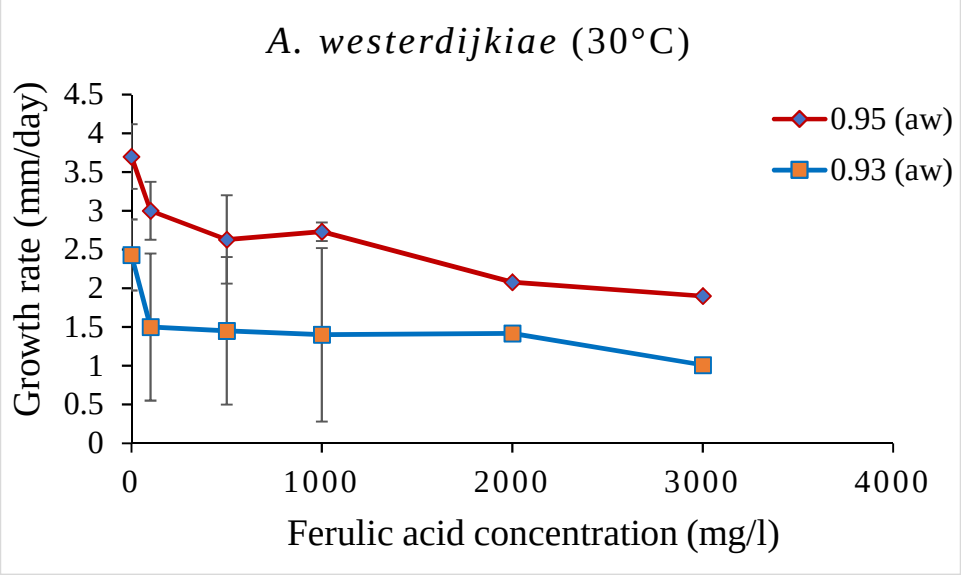

Figure 5.24. Effect of Ferulic acid concentrations on radial growth rate (mm/day) of *A.westerdijkiae* on irradiated coffee grains modified to two water activity at 30ºC after 10 days. Bars represent SEM.

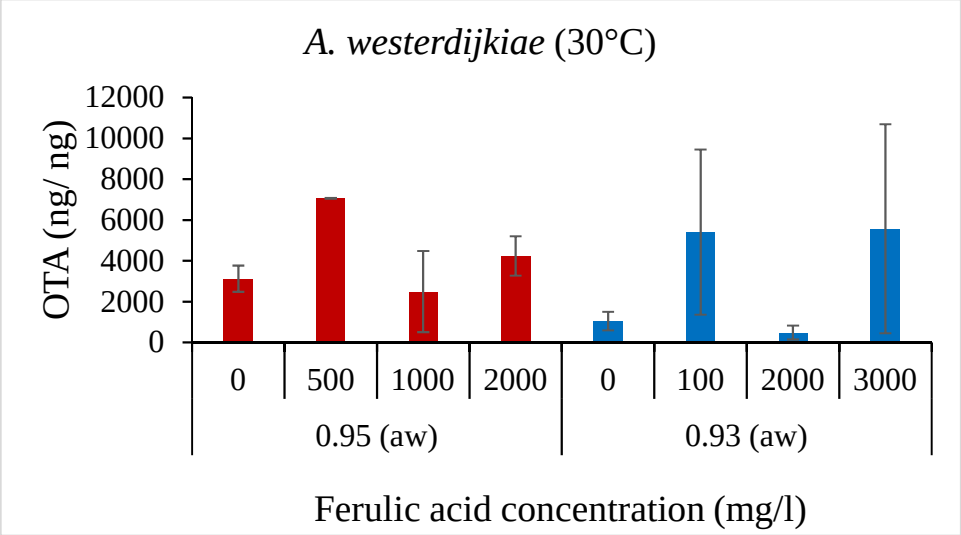

Figure 5.25. Effect of Ferulic acid concentrations on OTA production by *A. westerdijkiae* on 2% coffee medium modified to two water activities at 30C after 10 days. Bars represent SEM.

Table.5.14. List of *p*-values of growth &OTA production by *A.westerdijkiae* by Kruskal-Wallis test and ANOVA test.

Strain	**a_w**	**FE concentrations (mg/l**	**a_w*Conc**	**Response**
A.westerdikiae	S[a]	NS[a]	NS[a]	Growth rate (mm/day)
	NS[*]	NS[*]	N/A	OTA (ng/g)

S, significant; NS, non-significant; N/A, non-applicable; [*]Kruskal Wallis test.

b) Effect of Trans-cinnamic acid concentrations on growth and OTA production.

The effect of TCA concentrations and two aw levels had no significant effect on the growth rate of the strain (Figure 5.25).

For OTA control, observable amounts were produced at both aw levels. However, under high water stress (0.93 a_w and 1000 mg/l), toxin production was significantly higher when compared to less stressful conditions (Table 5.16).

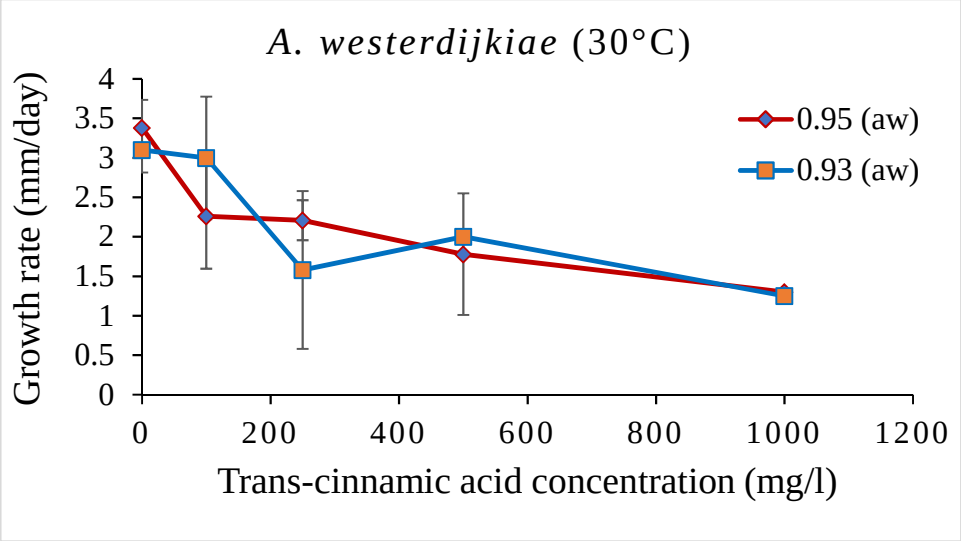

Figure 5.26. Effect of Trans-cinnamic acid concentrations on radial growth rate (mm/day) of *A.westerdijkiae* on irradiated coffee grains modified to two water activity at 30ºC after 10 days. Bars represent SEM.

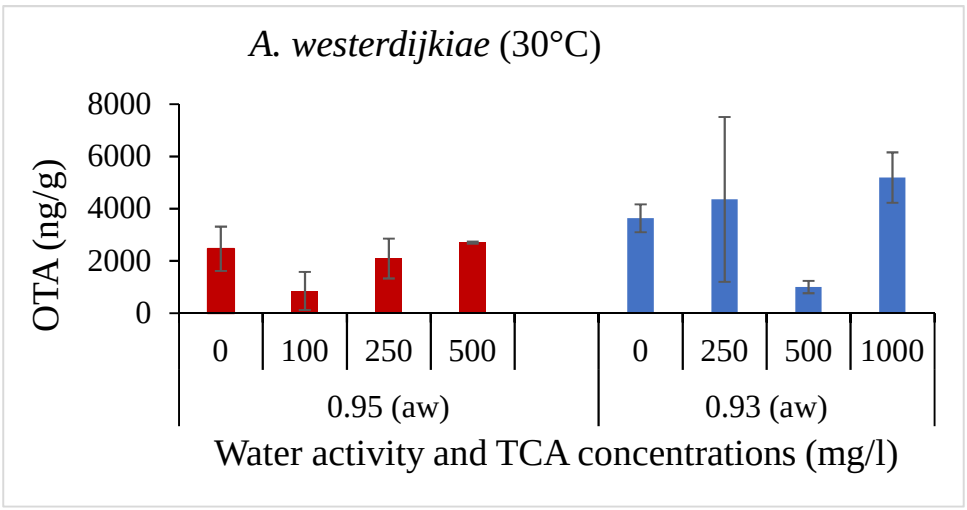

Figure 5.27. Effect of Trans-cinnamic acid concentrations on OTA (ng/g) by *A. westerdijkiae* on irradiated coffee grains modified to two water activity at 30ºC after 10 days. Bars represent SEM.

Table 5.16. List of *p*-values of growth &OTA production by *A. westerdijkiae* by Two Way ANOVA test.

Strain	a_w	Conc	a_w*Conc	Response
A.westerdijkiae	NS	NS	NS	Growth rate (mm/day)
	NS	S	NS	OTA (ng/g)

S;significant, NS; non-significant.

c) Effect of Sodium metabisulphite concentrations on growth and OTA production.

Both water stress and SM concentrations had no significant effect on the growth rate of the *A. westerdijkiae* strain examined (Figure 5.27). The strain was relatively tolerant to the range of concentrations used in contrast to the in vitro coffee-media based studies.

For OTA control, only the highest concentrations inhibited production at both a_w levels (Figure 5.28).

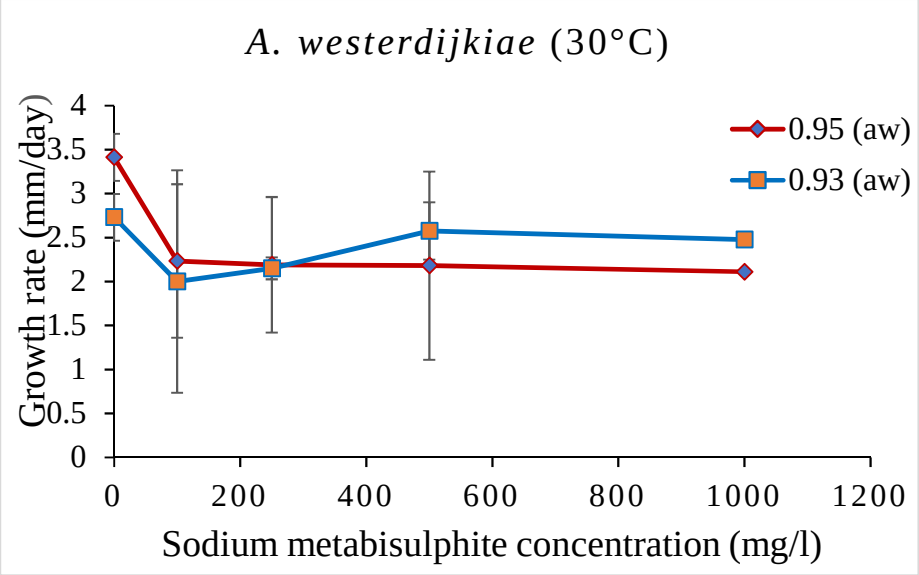

Figure 5.28. Effect of Sodium metabisulphite concentrations on radial growth rate (mm/day) of *A.westerdijkiae* on irradiated coffee grains modified to two water activity at 30ºC after 10 days. Bars represent SEM.

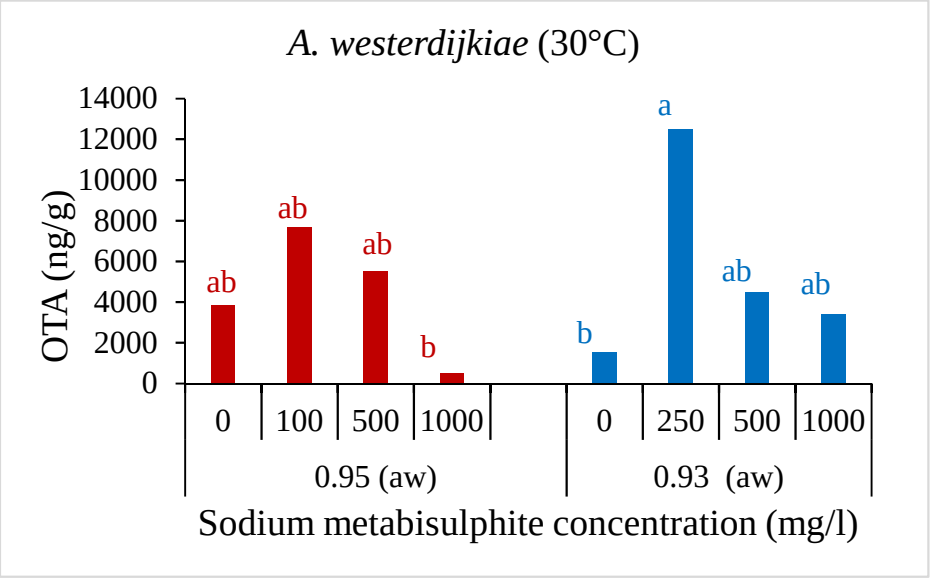

Figure 5.29. Effect of Sodium metabisulphite concentrations on OTA production by *A. westerdijkiae* on irradiated coffee grains modified to two water activity at 30ºC after 10 days. Different letters indicate significant differences from the control.

5.5. Discussion

5.5.1. Effect of preservatives on growth and OTA production by *P.verrucosum in vitro* and on stored wheat

Interestingly, very little information was available on the impact of preservatives on the potential for control of OTA production on wheat grains and coffee beans by spoilage and mycotoxigenic moulds. For this reason, the potential for control of OTA production by *P. verrucosum* and *A. westerdijkiae* on wheat- and coffee-based media was evaluated in this study.

In vitro studies demonstrated that the three compounds SM, TCA and PP were found to be very effective in controlling both growth and OTA production by *P. verrucosum* on milled wheat-based agar media over a range of a_w levels. The growth was initiated up to 100 mg/l at both water activities, afterwards, the growth was inhibited completely.

In contrast, contrary to the effects observed for growth, OTA production by *P. verrucosum* was favoured by 0.98 a_w up to 100 mg/l although high production occurred in the control treatments. However, this strain was more sensitive to the use of SM concentrations at 0.95 a_w as this was gradually decreased until no toxin was observed at 100 mg/l. SO_2 can be highly toxic to microorganisms as it has mutagenic effects and thus inactivates mRNA and reacts with disulphide linkages in proteins, enzyme cofactors, aldehyde and ketone structures of five and six carbon sugars. It is also able to deaminate cytosine derivatives to uracil, and uracil and has deleterious effects on membrane permeability (Babich and Stotzky, 1980). In contrast, small quantities of SO_2 may stimulate growth as the sulphur is an essential element for growth. The addition of Ferulic acid to different food commodities at both high and sub-optimal doses had a significant effect on growth and OTA production. Generally, for *P. verrucosum*, growth occurred over a range of concentrations at 0.95 a_w when compared with that at 0.98 a_w. However, a significant decrease in the growth rate was observed when a high concentration was used (2000 mg/l) regardless of a_w level. Despite this effect on growth, there was a stimulation of OTA production under water stress at the high concentration used (2000 mg/l) at 0.95 a_w.

P. verrucosum was relatively resistant to use of the preservatives CP and PS, for both growth and toxin production. However, TCA and PP doses inhibited growth 250 mg/l at both a_w levels. However, OTA was still produced with these concentrations. These results partially match the classical theory of inhibition by organic acids. Organic acids do not completely dissociate in water (Theron & Lues, 2007) and thus the undissociated acid form is the main agent responsible for

antifungal activity (Lund *et al*., 1987). This results in the undissociated acid molecules being lipophilic and able to pass easily through the plasma membrane by diffusion. In the cytoplasm, with a pH close to 7.0, the acid molecules would be dissociated into charged anions and protons. Because they are deprotonated once inside the cell, they cannot pass across the lipid bilayer and accumulate in the cytoplasm, thus decreasing the pH (Salmond *et al*., 1984). This acidification process slows down metabolism of glycolytic enzymes impacting on cellular functions (Krebs *et al*., 1983).

It is important to examine colonisation of *P. verrcuosum* of wheat grain to simulate what might happen in reality when using these preservatives. Thus, for one of the best *in vitro* preservative's SM was relative ineffective in wheat grain with little control.

In layers of wheat grain Ferulic acid was able to control OTA production at sub-inhibitory doses (1000 mg/l) at both 0.95 and 0.93 a_w when compared to TCA and SM. Previously, studies on *Fusarium* species which cause head blight of wheat were inhibited by essential oils such as cinnamon oil (500 ug/g) at 0.995 a_w and 25^{o}C in terms of both growth and trichothecene production in wheat. However, care is needed in using sub-optimal concentrations. For example, Magan *et al*. (2002) showed that sub-optimal fungicide concentrations could stimulated deoxynivalenol (DON) production by *F. culmorum* in wheat grains. They also showed that efficacy *in vitro* may be very different from what might occur *in situ*. In the present study growth was inhibited by 250 mg/l TCA and SM on wheat-based medium. However, colonisation of wheat grain still occurred at 0.95 a_w/250 mg/l of TCA and SM.

Indeed, Magan and Aldred (2007) suggested that combinations of abiotic stresses combined with chemical stress and the nutritional nature of the matrix may together result in a stimulation of toxin production in the presence of sub-optimal concentrations of biocides. Kang *et al*. (2001) previously showed that DON levels in the cell walls, cytoplasm, mitochondria and vacuoles of the hyphae of *F. culmorum* were significantly higher in presence of fungicides such as metaconazole and tebuconazole at 20 µg/ml than in the controls. Magan *et al*., (2002) also showed that sub-optimal fungicide concentrations stimulated DON production *in vitro* and in wheat grain by *F. graminearum* strains.

5.5.2. Effect of preservatives on growth and OTA production by *A. westerdijkiae* in vitro and in stored coffee

Interestingly, *A. westerdijkiae* grew faster over a wide range of SM concentrations up to 500 mg/l at both a_w levels. For complete inhibition of mycelial growth at least 1000 mg/l was required, regardless of the a_w level used. Moreover, OTA production behaviour was consistent with the growth data as the strain was more tolerant to the preservative at 500 mg/l producing high amounts of toxin at 0.95 a_w.

Previously, Magan (1993) examined the effect of different concentrations of SO_2 in relation to different interacting temperature x a_w conditions *in vitro* and *in situ* on grain for control of *Penicillium* and *Aspergillus* spp. The growth of *Aspergillus spp.* (*Aspergillus terreus, Aspergillus flavus* and *A. ochraceus*) was inhibited by 50 mg/l dissolved SO_2 on a malt extract-based medium at 0.995 and 0.95 a_w. Some *Penicillium spp* and *Aspergillus niger* were tolerant of up to 250 mg/l. In contrast, growth of other *Penicillium spp*. was stimulated by 100 mg/l.

Previously, King *et al*. (1981) showed that SO_2 binding substances enabled yeasts to be tolerant to higher concentrations of SO_2. In the present study it is possible that a percentage of SO_2 was absorbed and bound to the glycerol substance reducing its antifungal activity against *A. westerdijkiae*. For other preservatives examined such as FE, PS, and CP there was much less inhibitory impacts on growth and OTA production. Eklund (1985) suggested that the accumulation of toxic anions plays an important role in determining the toxicity and/or tolerance to acid preservatives by spoilage moulds. In addition, Copetti *et al*. (2012) pointed out that certain acids, such as sorbic acid, use other mechanisms to simultaneously inhibit the introduction of protons into the cell cytoplasm by acting directly on the cell membrane as a specific inhibitor of metabolism (Stratford and Anslow, 1998). This could help to explain the differential sensitivity of spoilage moulds to sorbic acid. Furthermore, the efficacy of the preservatives is normally more effective at acidic pH levels as the antimicrobial effect of the undissociated acid is much stronger than the dissociated acid. Luck and Jager, (1995) showed that optimum pH where efficacy is around 4.8-5.5 for sodium propionate. Above this the preservative is less effective (Liewen and Marth, 1985).

Overall, propionates were found to be relative ineffective, regardless of a_w levels examined. Previous studies have also found that these acids were not very effective in controlling growth and toxin production by *Fusarium* and other toxigenic spoilage moulds (Brachfeld, 1969; Razavi-Rohani and Griffins, 1999; Marin *et al*., 2000). Both TCA and PP had a significant effect on

reducing the growth of *A. westerdijkiae* with complete inhibition at 500 mg/l, However, the toxin production pattern was very different with higher toxin production occurring at 250 mg/l regardless of the a_w level used. Previous studies have reported that propyl paraben is an effective anti-oxidant against *Fusarium* Section Liseola species. Etcheverry *et al*. (2002) found that PP inhibited *Fusarium verticilliodes* and *F.proliferatum* growth and their production of fumonisins.

Passone *et al*. (2007) suggest that binary and tertiary mixtures of analytical and industrial grade parabens were effective against *A. flavus* and aflatoxin production in short term storage of peanuts. Others believe that the effectiveness of the parabens increases with an increase in the chain length of the ester group (Thompson *et al*., 1993; Thompson, 1994). Moreover, it is known that PP works mainly at the cell membrane level eliminating the pH-related component of the promotive force and affecting energy transduction and substrate transport (Khan *et al*., 2001).

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 General discussion

This project has examined the factors which ecologically make *P.verrucosum* and *A. westerdijkiae* such resilient species which are able to survive and grow in the soil ecosystem under a range of water stress conditions. In addition, the tolerance to both matric and solute stress suggests that soil and crop debris are the key reservoirs for these fungi for subsequent contamination of cereals and coffee and perhaps cocoa in temperate and tropical regions respectively. This knowledge is important in the understanding of the ecophysiology and molecular ecology of these species, which was not previously known. In addition, this type of information is important in understanding the source for contamination and the relative risk of toxin contamination in these commodities. Both these species are xerophiles and thus may be able to survive such environmental stresses by the production of appropriate compatible solutes, especially low molecular weight sugar alcohols (glycerol, erythritol) and trehalose which helps in desiccation tolerance (Magan, 2007).

No previous studies have addressed the impact that interacting CC abiotic factors may have on both growth and OTA production. Recent studies of *A. flavus* and *A. westerdijkiae* showed stimulation of mycotoxin contamination of maize and coffee respectively (Akbar et al., 2016; Medina *et al*., 2017). In contrast, *A. carbonarius* growth and OTA production were reduced or unaffected, in coffee-based medium, or in stored coffee beans (Akbar et al., 2016). Previously, Vary et al. (2015) showed that acclimatisation of *F. graminearum* under CC conditions for 10+ generations resulted in increased pathogenicity in ripening wheat. Thus, there may be differential impacts of CC conditions on individual mycotoxigenic species. The present study examined how the interactions of increased temperature, elevated CO_2 and drought stress (solute and matric potential) may influence both growth and OTA production for the first time. There was relatively little effect of CC abiotic factors on ecology of *P. verrucosum* regardless of whether exposed to solute or matric water stress. This was supported by both OTA biosynthetic gene expression and phenotypic toxin production. Previously, Magan *et al*. (2011) showed that CC scenarios had a drastic impact on xerophilic fungi such as *Wallemia sebi* and *Xeromyces bisporus* under very dry conditions (0.65-0.75 a_w) where there is much less competition from other mesophilic fungi and other microorganisms (Magan, 2006; Magan and Aldred, 2007). Moreover, Pieckova and Kunova (2002) reported that *W. sebi* can produce metabolites such as walleminol and walleminone that can be toxic to animals and humans, even under extreme stress conditions. The present study will be beneficial in terms of a better understanding of the resilience of these two species in temperate

cereals and coffee and the reasons why they predominantly contaminate these commodities post-harvest, as a result of their effective survival on crop debris and in soil. Subsequnetly studies were carried out to examine the potential for developing minisation strategies for OTA in these commodities using different existing and natural preservatives. These studies showed that in most cases *in vitro* assays are not a true reflection of *in situ* efficacy. Indeed, sub-optimal concentrations when applied to the actual commodity can lead to a stimulation of mycotoxin production as a stress response (Mutasa and Magan, 1990; Magan et al., 2002; Mylona et al., 2019). This has been shown for *Fusarium* species, *A.flavus*, *A. carbonarius* and *A. westerdijkiae*. Overall, much higher concentrations of the effective preservatives are needed to ensure that mycotoxin production can be either completely inhibited or reduced during storage of temperate cereals or coffee. Future post-harvest control strategies should take into account the above potential problems to ensure that mycotoxin contamination can be minimised to levels below the legislative limits.

6.2 Conclusions

- The Ochratoxigenic fungi grow over a wide range of water activity and temperature conditions. However, OTA production occurred over a narrower range of water activities and temperatures.
- Although both strains of *P. verrucosum* grew on both YES and wheat agar media, growth rates on wheat agar media were slower than on YES.
- Under water stress conditions (0.90 a_w) and high temperature (35°C) *A. westerdijkiae* (CECT strain) both growth and OTA production were significantly lower than under optimum conditions.
- Although both species (*P. verrucosum*, *A. westerdijkiae)* are xerophylic, they were more sensitive to ionic than non-ionic or matric stress. In terms of toxin production, however, *P. verrucosum* was more sensitive to matric stress. This was paralleled by the *otapks* gene expression, suggesting that this gene is the key regulatory gene involved in OTA biosynthesis. In contrast, *otapks* gene expression in *A. westerdijkiae* was low under moderate matric stress -9.8 MPa (=0.95 a_w).
- Interacting climate change factors of elevated levels of CO_2 in combination with high temperature had no significant effect on the growth rate of *P. verrucosum.* However, in terms of the *otapks* gene expression, no gene expression was observed in the 1000 ppm CO_2 and -2.8 MPa (=0.98 a_w) non-ionic glycerol-amended media. In addition, the amount

of toxin was reduced at -7.0 (=0.95 a_w) in the 1000 ppm CO_2 than in the 400 ppm CO_2 in the matric stressed media at 25°C.

- For *A. westerdijkiae*, the growth rate was decreased significantly at 1000 ppm + solute stress at -7.0 MPa (=0.95 a_w) in glycerol-amended media when compared to existing conditions at 30°C. However, no growth was detected at 37°C in all the climate change related treatments.
- Control of growth and OTA production by *P. verrucosum* in stored wheat using preservatives was found to be not as effective as that observed in the *in vitro* studies. For sodium metabisulphite, growth occurred with up to 250 ppm and was faster at 0.93 a_w when compared to 0.95 a_w although there were some inhibitions by this compound. However, the toxin production was lower at 0.95 a_w than at 0.93 a_w at all the examined doses.
- Potential control of growth and OTA production by *A. westerdijkiae* using preservatives *in situ* experiments showed that colonisation of coffee occurred over a wide range of sodium metabisulpite concentrations at both aw levels examined. However, at 1000 ppm dose reduced OTA contamination especially at 0.95 a_w when compared to the other compounds.

6.3 Suggestions for future work

- A comprehensive study is needed with *P. verrucosum* and *A. westerdijkiae* in longer term studies in relation to the effect of water activity x temperature effects on colonisation rates, relative kinetics of biosynthetic gene expression and toxin production *in situ*. This would provide more realistic data on the behaviour of these species and the relative risks of OTA contamination in stored wheat and coffee respectively.

- Further studies should be conducted to examine the whole genome of both species under the influence of solute and matric stress to estimate the behaviour of other gene clusters and other secondary metabolites which may be produced, especially under solute and matric stress. What is the role of these secondary metabolites in the competitiveness of these species in colonising wheat and coffee, respectively, especially post-harvest.

- Further evaluation of the impact of three interacting abiotic climate change scenarios in terms of *in situ* exposure to elevated CO_2 levels (1000 ppm), higher temperatures (+2-4˚C)

and drought conditions especially for *P. verrucosum* species to improve knowledge of toxin contamination of a staple grain and whether the risks may increase under these conditions and influence food safety for consumers.

- More studies are necessary on the relationship between preservative action and the stimulation of toxin production, especially related to biosynthetic gene expression and enzymes involved in grain colonisation to target control systems which will reduce or switch off toxin production.

- Examine the potential for using interference RNA (RNAi) approaches to inhibit the OTA biosynthetic pathway genes to prevent or reduce the potential for OTA biosynthesis and contamination of these commodities.

References

References

Abarca, M.L., Bragulat, M.R., Castellá, G. and Cabañes, F.J. (1994). Ochratoxin A production by strains of *Aspergillus niger var. niger*. *Applied and Environmental Microbiology* 60, 2650–2652.

Abdel-Hadi, A. and Magan, N. (2009). Influence of physiological factors on growth, sporulation and ochratoxin A/B production of the new *Aspergillus ochraceus* grouping. *World Mycotoxin Journal* 2, 429–434.

Abrunhosa, L., Paterson, R.R.M. and Venâncio, A. (2010). Biodegradation of Ochratoxin A for Food and Feed Decontamination. *Toxins* 2, 1078–1099.

Abouzied, M.M., Horvath, A.D., Podlensy, P.M. and Regina, NP. (2002). Ochratoxin A concentrations in food and feed from a region with Balkan endemic nephropathy. *Food Additives & Contaminants* 19, 755-764.

Akbar, A., (2015). Growth and ochratoxin a production by *Aspergillus* species in coffee beans: impact of climate change and control using O_3. PhD thesis, Cranfield University.

Akbar, A., Medina, A and Magan, N. (2017). Impact of interacting climate change factors on growth and ochratoxin A production by *Aspergillus* section *Circumdati* and *Nigri* species on coffee. *World Mycotoxin Journal* 9, 864-874.

Akbar, A., Medina, A. and Magan, N. (2016). Impact of interacting climate change factors on growth and ochratoxin A production by *Aspergillus* section *Circumdati* and *Nigri* species on coffee. *World Mycotoxin Journal* 9, 864-874.

Alcano, M., Jahn, D. and Scherer, C. (2016). Susceptibility of *Aspergillus spp* to acetic and sorbic acids based on pH and effect of sub-inhibitory doses of sorbic acid on ochratoxin A production. *Food Research International* 81, 25-30.

Aldred, D., Cairns-Fuller, V. and Magan, N. (2008). Environmental Factors affect Efficacy of Some Essential Oils and Resveratrol to Control Growth and Ochratoxin A Production by *Penicillium verrucosum* and *Aspergillus westerdijkiae* on Wheat Grain. *Journal of Stored Products Research* 44, 341–346.

Andersen, S.J. (1995). Compositional changes in surface mycoflora during ripening of naturally fermented sausages. *Journal of Food Protection* 58, 426-429.

Arroyo, M., Aldred, D. and Magan, N. (2005). Environmental factors and weak organic acid interactions have differential effects on control of growth and ochratoxin A production by

Penicillium verrucosum isolates in bread. *International Journal of Food Microbiology* 98, 223-231.

Astoreca A., Barberis C., Magnoli C., Combina M. and Dalcero, A. (2009). Eco-physiological factors effect on growth rate, lag phase and ochratoxin A production by *Aspergillus niger* aggregate strains in irradiated peanut seeds. *Food Microbiology* 129, 131–135.

Ayerst, G. (1969). The effects of moisture and temperature on growth and spore germination in some fungi. *Journal of Stored Products Research* 5, 127-141.

Ayodele, S.M. and Ojoghoro, O.J. (2007). Salt stress effects on the vegetative growth of *Pleurotus tuberregium* (FR) sing. J*ournal of Biological Sciences* 7, 1278-1281.

Babich, H. and Stotzky, G. (1980). Gaseous and heavy metal pollutants. In: Burns, R.G., Slater, J.H. (Eds.). *Experimental Microbial Ecology*. Blackwell, Oxford, p. 631.

Battilani, P., Toscano, P., Van der Fels-Klerx, H.J., Moretti, A., Camardo Leggieri, M., Brera, C., Rortais, A., Goumperis, T. and Robinson, T. (2016). Aflatoxin B1 contamination in maize in Europe increases due to climate change. *Scientific Reports* 6, 24328.

Battilani, P., Formenti, S., Toscani, T. and Virgili, R. (2010). Influence of abiotic parameters on ochratoxin A production by a *Penicillium nordicum* strain in dry-cured meat model systems. *Food Control* 21, 1739–1744.

Bellí N., Marín S., Coronas I., Sanchís V. and Ramos A.J. (2007). Skin damage, high temperature and relative humidity as detrimental factors for *Aspergillus carbonarius* infection and ochratoxin A production in grapes. *Food Control* 18, 1343–1349.

Belli, N., Ramos, A.J., Sanchis, V. and Marin, S. (2004a). Incubation time and water activity effects on ochratoxin A production by *Aspergillus* section *Nigri* strains isolated from grapes. *Letters of Applied Microbial* 38, 72-77.

Brachfeld, B.A. (1969). Antimicrobial food additives. Baker's Digest 43, 60–65.

Bragulat, MR., Abarca, ML. and Cabanes, FJ. (2001). An easy screening method for fungi producing Ochratoxin A in pure culture. *International Journal of Food Microbiology* 71, 139-144.

Cairns-Fuller, V., Aldred, D. and Magan, N. (2005). Water, temperature and gas composition in- teractions affect growth and ochratoxin A production by isolates of *Penicillium verrucosum* on wheat grain. *Journal of Applied Microbiology* 99, 1215–1221.

References

Cairns-Fuller, V. (2004). Dynamics and control of ochratoxigenic strains of *Penicillium verrucosum* and *Aspergillus ochraceus* in the stored grain ecosystem. PhD Thesis, Cranfield University, Silsoe, U.K.

Challis, G.L. and Naismith, J.H. (2012). Structural Aspects of Non-Ribosomal Peptide Biosynthesis. *Europe PMC Funders Group* 14, 748–756.

Ciegler, A., Fennell, D.I., Sansing, G.A., Detroy, R.W. and Bennett, G.A. (1973). Mycotoxin-producing strains of *Penicillium viridicatum*: Classification into subgroups. *Applied Microbioogy* 26, 271–278.

Cole, RJ. (1989). Technology of Aflatoxin denonatmination. In: Natori S, Hashimoto k, Uueno Y (eds) Mycotoxins and Phytoxins 88. Elsevier Scientific Publishing Co., Amsterdam, 177-184.

Copetti, M., Iamanaka, B., Mororó, R., Pereira, J., Frisvad, J. and Taniwaki, M. (2012). The effect of cocoa fermentation and weak organic acids on growth and ochratoxin A production by *Aspergillus* species. *International Journal of Food Microbiology* 155, 158 –164.

Corni, G., Orlic, S., Redzepovic, S., Urso, R. and Lacumin, L. (2004). Moulds isolated from Istrian dried ham at the pre-ripening and ripening level. *International Journal of Food Microbiology* 96, 29-34.

European Food Safety Agency (2010). Opinion of the Scientific panel on contaminants in the food chain on a request from the commission related to Ochartoxin A in food. EFSA J. 365. 1-56.

Eklund, T. (1985). Inhibition of microbial growth at different pH levels by benzoic and propionic acids and esters of p-hydroxybenzoic acid. *International Journal of Food Microbiology* 2, 159–167.

El Khoury, A.E. and Atoui, A. (2010). Ochratoxin a: General Overview and Actual Molecular Status. *Toxins* 2, 461–493.

Etcheverry, M., Torres, A., Ramirez, M.L., Chulze, S. and Magan, N. (2002). In vitro control of growth and fumonisin production by *Fusarium verticillioides* and *F. proliferatum* using antioxidants under different water availability and temperature regimes. J*ournal of Applied Microbiology*. 92. 624–632.

European Union (2006). Commission {Regulation} ({EC}) {No} 1881/2006 of 19 {December} 2006 setting maximum levels for certain contaminants in foodstuffs.

European Commission. Commission Regulation (EC) No 123/2005 of 26 January (2005). amending Regulation (EC) No 466/2001 as regards ochratoxin A. *European Communities, 25,* 3–5.

European Commission. Commission Regulation (EC) No 472/2002 of 12 March (2002). *European Communities 75,* 18–20.

European Commission. (1994). Mycotoxins in human nutrition and health. Agroindustrial research division of the European Commission Directorate-General XII for scientific research and development, 36.

Farber, P. and Geisen, R. (2004). Analysis of differentially-expressed ochratoxin A biosynthesis gene of *Penicillium nordicum. European Journal of Plant Pathology* 110, 661-669.

FAO/WHO (2001). Safety evaluation of certain mycotoxins in food. Fifty sixth meeting of the joint FAO/WHO expert Committee on food additives (JECFA)-WHO Food additives series 47. FAO food and nutrition paper 74. Food and Agriculture Organisation, World health Organisation, Geneva, Switzerland.

Ferraz, M.B.M., Farah, A., Iamanaka, B.T., Perrone, D., Copetti, M. V., Marques, V.X., Vitali, A. a. and Taniwaki, M.H. (2010). Kinetics of Ochratoxin A Destruction during Coffee Roasting. *Food Control* 21, 872–877.

Finking, R. and Marabiel, M. A. (2004). Biosynthesis of non-ribosomal peptides. *Annual Review of Microbiology* 58, 453-488.

Frisvad, JC., Thomas, OL., Thrane, UIF., Martin, M. and Robert A, Samson. (2011). Fumonisin and Ochratoxin production in industrial *Aspergillus niger* strains. *Plos One* 6, e23496.

Frisvad, J.C., Frank, J.M., Houbraken, J.A.M.P., Kuijpers, A.F.A., and Samson, R.A. (2004). New Ochratoxin A Producing Species of *Aspergillus* Section *Circumdati*. *Studies in Mycology* 50, 23–43.

Frisvad, J.C. and Samson, R.A. (1991). Filamentous fungi in food and feeds: ecology, spoilage and mycotoxin contamination.' in Handbook of Applied Mycology, Vol. 3, Foods and Feed ed. Arora, D.K. Mukerji, K.G. and Marth, E.H, 61–68. New York: Marcel Dekker.

Garcia-Cela, E., Kiaitsi, E., Sulyok, M., Medina, A., and Magan, N. (2018). *Fusarium graminearum* in Stored Wheat: Use of CO_2 Production to Quantify Dry Matter Losses and Relate

This to Relative Risks of Zearalenone Contamination under Interacting Environmental Conditions. *Toxins* 10, Article No. 86.

Garcia-Rico, R. O., Fierro, F., Mauriz, E., Gomez, A., Fernandez- Bodega, M. A. and Martın, J. F. (2008). The heterotrimeric alpha protein PGA1 regulates biosynthesis of penicillin, chrysogenin and roquefortine in *Penicillum chrysogenum*. *Microbiology* 154, 3567-3578.

Geisen, R., Stoll, D. and Schmidt-Heydt, M. (2013). Differences in the Regulation of Ochratoxin a by the HOG Pathway in *Penicillium* and *Aspergillus* in Response to High Osmolar Environments. *Toxins* 7, 1282–1298.

Geisen, R., Schmidt-Heydt, M. and Karolewiez, A. (2006). A gene cluster of the ochratoxin A biosynthetic genes in *Penicillium*. *Mycotoxin Research* 22, 134- 141.

Geisen, R. and Karolewiez, A. (2005). Cloning a part of the ochratoxin A biosynthetic gene cluster of *Penicillium nordicum* and chartcetrization of the ochratoxin polyketide synthase gene. *Systematic and Applied Microbiology* 28, 588-595.

Geisen, R., Mayer, Z., Karolewiez, A. and Färber, P. (2004). Development of a Real Time PCR System for Detection of *Penicillium nordicum* and for Monitoring Ochratoxin A Production in Foods by Targeting the Ochratoxin Polyketide Synthase Gene. *Systematic and Applied Microbiology* 27, 501–507.

Gelderblom, W.C., Kriek, N.P., Marasas, W.F.O. and Thiel, P.G. (1991). Toxicity and carcinogenicty of the *Fusanum moniliforme* metabolite, fumonisin B1, in rats. *Cracinogenesis* 12, 1247-1251.

Gelderblom, W.C., Marasas, W.F., Jaskiewicz, K., Thiel, P.G., Horak, R.M., Vleggaar, R and Kriek, N.P. (1988). Fumonisins-novel mycotoxins with cancer-promoting activity produced by Fusarium moniliforme. *Applied and Environmenatl Microbiology* 54, 1806-1811.

Gilbert, M., Medina, A., Mack, B., Lebar, A., Rodriguez, D., Bhatnagar, D., Magan, N., Obrian, G and Payne, G. (2017). Carbon dioxie mediates the response to temperature and water activity levels in *Aspergillus flavus* during infection of maize kernels. *Toxins* 10, 5.

Gil-Serna, J., García-Díaz, M., González-Jaén, M.T., Vázquez, C., and Patiño, B. (2018). Description of an Orthologous Cluster of Ochratoxin A Biosynthetic Genes in *Aspergillus* and *Penicillium* Species. A Comparative Analysis. *International Journal of Food Microbiology* 268, 35–43.

Giorni, P., Battilani, P., Pietri, A., and Magan, N. (2008). Effect of a_w and CO_2 Level on *Aspergillus Flavus* Growth and Aflatoxin Production in High Moisture Maize Post-Harvest. *International Journal of Food Microbiology* 122, 109–113.

Gqaleni, N., Smith, JE., Lacey, J. and Gettinby, G. (1996). Production of the mycotoxin cyclopiazonic acid by *Penicillium commune* on solid agar media: effects of water activity, temperature and incubation time. *Journal of Food Protection* 59, 864-868.

Gonzalez-Salgado, A., Patino, B., Gil-Serna, J., Vazquez, C. Gonzalez, M.T. (2009). Specific detection of *Aspergillus carbonarius* by SYBR Green and TaqMan quantitative PCR assays based on the multicopy ITS2 region of the rRNA gene. *FEMS Microbiology Letters* 295, 57-66.

Griffin, D.M. (1981). Water and microbial stress. In Advances in Microbial Ecology 5 (ed. M. Alexander). 91-136. Plenum Publishing Co.

Gromadzka, K., Waśkiewicz, A., Goliński, P. and Świetlik, J. (2009). Occurrence of estrogenic mycotoxin- zearalenone in aqueous environmental samples with various NOM content. *Water Research* 43, 1051-1059.

Harris, J.P. and Mantle, P.G. (2001). Biosynthesis of diaporthin and orthosporin by *Aspergillus ochraceus*. *Phytochemistry* 57, 165-169.

Hesseltine, C.W., Vandegraft, E.E., Fennell, D.I., Smith, M.L. and Shotwell, O.L. (1972). *Aspergilli* as ochratoxin producers. *Mycologia* 64, 539–550.

Hill, RA. and Lacey, J. (1984). *Penicillium* species associated with barley grain in the UK. *Transactions of the British Mycological Society* 82, 297-303.

Horie, Y. (1995). Productivity of ochratoxin A of *Aspergillus carbonarius* in *Aspergillus* section *Nigri*. *Nippon Kingakkai Kaiho* 36, 73–76.

IARC, 1993. Some naturally occurring substances, food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC monographs on the evaluation of Carcinogenic risks to humans. 56. IARC, Lyon, France.

International Commission on Microbiological specifications for foods. (ICMSF), 1996. Toxigenic fungi: *Aspergillus*. In: Roberts, T.A; BairdParker, AC; Tompkin, RB, Microorganisms in Foods. Characteristics of food Pathogens. Blackie Academic & Professional, London. pp. 347-381.

Jiang, Y., Jolly, P.E., Preko, P., Wang, J.-S., Ellis, W.O., Phillips, T.D. and Williams, J.H. (2008). Aflatoxin-Related Immune Dysfunction in Health and in Human Immunodeficiency Virus Disease. *Clinical & Developmental Immunology.* 790309.

Jurado, M., Marın, P, Magan, N. and Teresa Gonzalez-Jaen, M. (2008) Relationship between Solute and Matric Potential Stress, Temperature, Growth, and. *Applied and Environmental Microbiology* 74, 2032–2036.

Kang, Z., Huang,L., Krieg, U., Mauler-Machrik, A. and Buchenaue, H. (2001). Effects of tebuconazole on morphology, structure, cell wall components and trichothecene production of *Fusarium culmorum in vitro*. *Pest Management Science* 57, 491-500.

Karolewiez, A. and Geisen, R. (2005). Cloning a part of the ochratoxin A biosynthetic gene cluster of *Penicillium nordicum* and characterization of the ochratoxin polyketide synthase gene. *Systematic and Applied Microbiology* 28, 588-595.

Koszegi, T., Poor, M. (2016). Ochratoxin A: Molecular Interactions, Mechanisms of Toxicity and Prevention at the molecular level. *Toxins* 8, 111.

Kato, N., Brooks, W. and Calvo, A.M. (2003). The expression of sterigmatocystin and penicillin genes in *Aspergillus nidulans* is controlled by veA, a gene required for sexual development. *Eukaryotic Cell* 2, 1178–1186.

Khan, S.H., Aked, J. and Magan, N. (2001). *In vitro* potential for antioxidant chemicals to control the anthracnose pathogens of bananas, *Colletotrichum musae*. *Plant Pathology* 50, 601–608.

King, A.D., Ponting, J.D., Sanschuck, D.W., Jackson, R. and Mihara, K. (1981). Factors affecting death of yeast by sulphur dioxide. *Journal of Food Protection* 44, 92-97.

Kos, J., Mastilovic, J., Janic Hajnal, E. and Saric, B. (2013). Natural occurrence of aflatoxins in maize harvested in Serbia during 2009-2012. *Food Control* 34, 31-34.

Krebs, H., Wiggins, D., Stubs, M., Sols, A. and Bedoya, F. (1983). Studies on the mechanism of the antifungal action of benzoate. *Biochemical Journal* 214, 657–663.

Larsen, T.O., Svendsen, A. and Smedsgaard, J (2001). Biochemical Characterization of Ochratoxin A-Producing Strains of the Genus *Penicillium*. *Applied and Environmental Microbiology* 67, 3630–3635.

Leggieri, M.C., Pont, N.P., Battilani, P. and Magan, N. (2011). Detection and discrimination between ochratoxin producer and non-producer strains of *Penicillium nordicum* on a ham-based medium using an electronic nose. *Mycotoxin Research* 27, 29-35.

Leite, G. (2013). Potential for control of spoilage and mycotoxigenic species using mixtures of anti-oxidants, aliphatic acids and molecular approaches using RNAi. PhD thesis. Cranfield University.

Leong, S., Pettersson, O.V., Rice, T., Hocking, A.D and Schnurer, J. (2010). The extreme xerophilic mould *Xeromyces bisporus*- growth and competition at various water activities. *International Journal of Food Microbiology* 145, 57-63.

Leong, S.L.; Hocking, A.D. and Scout, E.S. (2006). Effect of temperature and water activity on growth and ochratoxin A production by Australian *Aspergillus carbonarius* and *A. niger* isolates on a simulated grape juice medium. *Food Microbiology* 10, 209–216.

Leong, S.L., Lantz, H., Pettersson, O.V. and Schnurer, J. (2015). Genome and physiology of the ascomycete filamentous fungus *Xeromyces bisporus*, the most xerophilic organism isolated to date. *Environmental Microbiology* 17, 496-513.

Leszkowicz, A., **Petkova-Bocharova, T., Chernozemsky, N and Castegnaro, M.** (2002). Balkan endemic nephropathy and associted urinary tract tumors: a review on aetiological causes and the potential role of mycotoxins. *Food Additives and Contaminants* 19, 282-302.

Liewen, M.B. and Marth, E.H. (1985). Growth and inhibition of microorganisms in the presence of Sorbic acid-a review. *Journal of Food Protection* 48, 364-375.

Lindblat, M., Johnsson, P., Jonsson, N., Lindqvist, R. and Olsen, M. (2004). Predicting noncompliant levels of ochratoxin A in cereal grain from *Penicillium verrusocum* counts. *Journal of Applied Microbiology* 97, 609– 616.

Livak, K.J. and Schmittgen, T.D. (2001) . Analysis of Relative Gene Expression Data Using Real-Time Quantititative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25, 402-408.

Livak, K.J., Flood, S.J., Marmaro, J., Giusti, W. and Deetz, K. (1995). Oligonucleotides with fluorescent dyes at opposite ends provide a quenched system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods and Applications* 4, 357-362.

Lori, G.A. and Rizzo, I. (2007). Deoxinivalenol. In: Soriano, J.M. (ED). *Micotoxinas en Alimentos Díaz de Santos, Madrid,* 269-292.

Luck, E. and Jager, M., (1995). Antimicrobial Food Additives—Characteristics, Uses, Effects, 2nd ed. Springer-Verlag, Berlin, Germany. 260 pp.

Lund, B., George, S. and Franklin, J. (1987). Inhibition of type A and type B (proteolytic) *Clostridium botulinum* by sorbic acid. *Applied and Environmental Microbiology* 53, 935–941.

Lund, F. and Frisvad, J.C. (2003). *Penicillium Verrucosu*m in Wheat and Barley Indicates Presence of Ochratoxin A. *Journal of Applied Microbiology* 95, 1117–1123.

Madhyastha, S.M., Marquardt, R.R. Frohlich, A.A., Platford, G. and Abramson, D. (1990). Effects of different cereal and oil seed substrates on the growth and production of toxins by *Aspergillus alutaceus* and *Penicillium verrucosum*. *Journal of Agricultural and Food Chemistry* 38, 1506–1510.

Magan, N., Hope, R., Cairns, V. and Aldred, D. (2003 a). Post-Harvest Fungal Ecology: Impact of Fungal Growth and Mycotoxin Accumulation in Stored Grain. *European Journal of Plant Pathology* 109, 723–730.

Magan, N., and Lacey, J. (1986). Effects of gas composition and water activity on growth of field and storage fungi. *Transactions of the British Mycological Society* 82, 305-314.

Magan, N. and Olsen, M. (2004). Mycotoxins in Food: Detection and Control. Woodhead Publishing Ltd, Cambridge, UK.

Magan, N. (2007). Fungi in extreme environments. In Kubicek CP, Druzhinina IS (eds) Environmental and Microbial relationships: the Mycota IV, 2nd edn. *Springer, Berlin*, 85–103.

Magan, N., Sanchis, V. and Aldred, D. (2004). Role of spoilage fungi in seed deterioration. Chapter 28, In *Fungal Biotchnology in Agricutural, Food and Environmental Applications* ed. D.k. Aurora. 311-323.

Magan, N and Aldred. D. (2007). Post-harvest control strategies: minimizing mycotoxins in the food chain. *International Journal of Food Microbiology* 119, 131-139.

Magan, N., Cayley, G. and Lacey, J. (1984). The effect of water activity and temperature on mycotoxin production by *Alternaria alternata* in culture and on wheat grain. *Applied and Environmental Microbiology* 47, 1113-1117.

Magan, N., Medina, A. and Aldred, D., (2011). Possible climate change effects on mycotoxin contamination of food crops pre- and post- harvest. *Plant Pathology* 60, 150-163.

References

Magan, N., Geisen, R., Schmidt-Heydt, M. and Parra, R. (2013). Integrated molecular and ecophysiological data for modelling mycotoxin production. In *Predictive Mycology*, 279-301.

Magan, N., Medina, A and Aldred, D. (2011). Possible climate change effects on mycotoxin contamination of food crops pre- and post-harvest. *Plant pathology* 60, 150-163.

Magan, N. and Aldred, D. (2006): Managing microbial spoilage in cereal and bakery products. In: Blackburn, Clive de W. (Ed), *Food Spoilage Microorganism*. Woodhead Publications, Cambridge, U.K., 194-212.

Magan, N. (2006). Mycotoxin contamination of food in Europe: early detection and prevention strategies. *Mycopathologia* 162, 245-253.

Magan, N and Aldred, D. (2007). Post-harvest control strategies minimizing mycotoxins in the food chain. *International Journal of food Microbiology* 119, 131-139.

Magan, N. and Lacey, J. (1986). Effects of gas composition and water activity on growth of field and storage fungi. *Transactions of the British Mycological Society* 82, 305-314.

Marin, S., Sanchis, V., Saenz, R., Ramos, A.J., Vinas, I. and Magan, N. (1998). Ecological determinants for germination and growth of some *Aspergillus* and *Penicillium* spp. From maize grain. *Journal of Applied microbiology* 84, 25-36.

Magan, N., Arroyo, M. and Aldred, D. (2003): Mould prevention in bread, Chap.24. In Bread Making: Improving Quality ed. Cauvain, S.P. Cambridge, UK: Woodhead Publishing.

Magan, N. (1988). Effect of water potential and temperature on spore germination and germ tube growth in vitro and on straw leaf sheaths. *Transactions of the British Mycological Society* 90, 97-107.

Magan, N. (2007). Fungi in extreme environments. in Kubicek CP, Druzhinina IS (eds) *Environmental and Microbial relationships*: the Mycota IV, 2nd edn. *Springer, Berlin*, 85–103.

Magan, N., Hope, R., Colleate, A. and Baxter, E.S. (2002). Relationship between growth and mycotoxin production by *Fusarium* species, biocides and environment. *European Journal of Plant Pathology* 108, 685–690.

Magan, N., Challen, MP. and Elliot, TJ. (1995). Osmotic, matric and temperature effects on in vitro growth of isolates of *Agaricus bisporus* and *A.bitorquis*. In: Elliot, T.J. (ed), *Science and technology of edible mushrooms*. Rotterdam, Balkemaar. 773-780.

Magan, N. (1993). Tolerance of fungi to sulphur dioxide. Chapter 7, In *Stress tolerance of fungi*, edt. D.H.Jennings, pp. 173-187. Marcell Dekker.

Magnoli, C.E., Astroce, A. and Ponsone, ML. (2007). Ochratoxin A and *Aspergillus* section *Nigri* in peanut seeds at different months of storage in Cordoba, Argentina. *International Journal of Microbiology* 119, 213-218.

Marin, S., Ramos, A.J., Cano-Sancho, G. and Sanchis, V. (2013). Mycotoxins: Occurrence, Toxicology, and Exposure Assessment. *Food and Chemical Toxicology* 60, 218–37.

Marín, S., Hodzić, I., Ramos, A. J. and Sanchis, V. (2008). Predicting the growth/no-growth boundary and ochratoxin A production by *Aspergillus carbonarius* in pistachio nuts. *Food Microbiology* 25, 683–689.

Marín, S., Magan, N., Abellana, M., Canela, R., Ramos, A.J. and Sanchis, V. (2000). Selective Effect of Propionates and Water Activity on Maize Mycoflora and Impact on Fumonisin B1 Accumulation. *Journal of Stored Products Research* 36, 203–214.

Marin, S., Sanchis, V., Saenz, R., Ramos, A.J., Vinas, I. and Magan, N. (1998). Ecological determinants for germination and growth of some *Aspergillus* and *Penicillium* spp. From maize grain. *Journal of Applied Microbiology* 84, 25-36.

Marin, S., Guynot, M. and Neira, P. (2002). Risk assessment of the use of suboptimal levels of weak acid preservatives in the control of mould growth on bakery products. *International Journal of Food Microbiology* 79, 203-211.

Marin, S., Sanchis, V and Magan, N. (1995). Water activity, temperature and pH effects on growth of *Fusarium moniliforme* and *F. proliferatum* isolates from maize. *Canadian Journal of Microbiology* 41, 1063-1070.

Medina, A., Rodriguez, A. and Magan, N. (2015a). Changes in environmental factors driven by climate change: effects on the ecophysiology of mycotoxigenic fungi Chapter 4.' in Climate Change and Mycotoxins, Eds. Botana Luis Mi., Sainz Marıa Jric Berlin, Germany: De Gruyter; 71-90. ISBN: 978-3-11-033361-9.

Medina, A., Rodriguez, A., Sultan, Y. and Magan, N. (2015). Climate change factors and *A. flavus*: effects on gene expression, growth and aflatoxin production. *World Mycotoxin Journal* 8, 171-179.

Medina, A., Rodriguez, A. and Magan, N. (2014). Effect of Climate Change on *Aspergillus*

flavus and Aflatoxin B1 Production. *Frontiers in Microbiology* 1–7.

Medina, A., Akbar, A., Baazeem, A., Rodriguez, A. and Magan, N. (2017). Climate Change, Food Security and Mycotoxins: Do We Know Enough?. *Fungal Biology Reviews* 31, 143–154.

Medina, A., Rodriguez, A. and Magan, N. (2014). Effect of Climate Change on *Aspergillus Flavus* and Aflatoxin B1 Production. *Frontiers in Microbiology.* 5 . 1–7.

Missmer, S.A., Suarez, L., Felkner, M., Wang, E., Merrill, A.H., Rothman, K.J. and Hendricks, K.A (2006). Exposure to Fumonisins and the Occurrence of Neural Tube Defects along the Texas-Mexico Border. *Environmental Health Perspectives* 114, 237–41.

Mitchell, D., Parra, R., Aldred, D. and Magan, N. (2004). Water and temperature relations of growth and ochratoxin A production by *Aspergillus carbonarius* strains from grapes in Europe and Israel. *Journal of Applied Microbiology* 97, 439–45.

Mule, G., Susca, A., Logrieco, A., Stea, G. and Visconti, A. (2006). Development of a quantitative real-time PCR assay for the detection of *Aspergillus carbonarius* in grapes. *International Journal of Microbiology* 111, 28-34.

Mutasa, E.S and Magan, N. (1990). Utilization of potassium sorbate by tobacco spoilage fungi. *Mycological Research* 94, 965-970.

Mylona, K., Sulyok, M. and Magan, N. (2012). *Fusarium graminearum* and *Fusarium verticillioides* colonisation of wheat and maize, environmental factors, dry matter losses and mycotoxin production relevant to the EU legislative limits. *Food Additives and Contaminants* 29, 1118-1128.

Mylona, K., Garcia-Cela, E., Sulyok, M., Medina, A. & Magan, N. (2019). Effects of two garlic extracts [Propyl propane thiosulfonate (PTS) and Propyl propane thiosulfinate (PTSO)] on growth and mycotoxin production by *Fusarium* species in vitro and in stored cereals. *Toxins* 11, 495. ***Doi:10.3390/toxins11090495.***

Neschi, A., Etcheverry, M. and Magan N. (2004). Osmotic and matric potential effects on growth and compatible solute accumulation in *Aspergillus* section *flavi* strains from Argentina. *Journal of Applied Microbiology* 96, 965–972.

O'Callaghan, J., Coghlan, A., Abbas, A., García-Estrada, C., Martín, J. and Dobson, A. D.W. (2013). Functional characterization of the polyketide synthase gene required for ochratoxin A biosynthesis in *Penicillium verrucosum*. *Food Microbiology* 161, 172-181.

O'Callaghan, J., Stapleton, P. C. and Dobson, A.D. W. (2006). Ochratoxin A biosynthetic genes in *Aspergillus ochraceus* are differentially regulated by pH and nutritional stimuli. *Fungal Genertics and Biology* 43, 213–21.

Pardo, E., Malet, M., Marín, S., Sanchis, V. and Ramos, A.J. (2006). Effects of water activity and temperature on germination and growth profiles of ochratoxigenic *Penicillium verrucosum* isolates on barley meal extract agar. *Food Microbioogy* 106, 25–31.

Pardo, E., Ramos, A.J, Sanchis, V and Marin S. (2005). Effect of water activity and temperature on mycelial growth and ochratoxin A production by isolates of *Aspergillus ochraceus* on irradiated green coffee beans. *Journal of Food Protection* 68, 133–138.

Pardo, E., Marín, S., Sanchis, V. and Ramos, A. J. (2004). Prediction of fungal growth and ochratoxin A production by *Aspergillus ochraceus* on irradiated barley grain as influenced by temperature and water activity. *Food Microbiology* 95, 79–88.

Pardo, E., Malet, M., Marín, S., Sanchis, V. and Ramos, A.J. (2006). Effects of water activity and temperature on germination and growth profiles of ochratoxigenic *Penicillium verrucosum* isolates on barley meal extract agar. *Food Microbiology* 106, 25–31.

Passone, M.A., Resnik, S. and Etcheverry, M.G. (2007). Antiaflatoxigenic property of food grade antioxidants under different conditions of water activity in peanut grains. *International Journal of Food Microbiology* 118, 8–14.

Paster, N., Lisker, N. and Chet, I. (1983). Ochratoxin production by *Aspergillus flavus* Wilhem grown under controlled atmospheres. *Applied and Environmental Microbiology* 45, 1136-1139.

Patriarca, A., Vaamonde, G., Fernandez, V. and Comerio, R. (2001). Influence of water activity and Temperature on the growth of *Wallemia sebi*: application of a predictive model. *International Journal of Food Microbiology* 68, 61-67.

Pateraki, M., Dekanea, A., Mitchell, D., Lydakis, D. and Magan, N. (2007). Efficacy of sulphur dioxide, controlled atmospheres and water availability on in vitro germination, growth and ochratoxin A production by strains of *Aspergillus carbonarius* from grapes and vine fruits. *Postharvest Biology and Technology* 44, 141–149.

Paterson, R.R.M. and Lima, N. (2010). How Will Climate Change Affect Mycotoxins in Food?' *Food Research International* 43, 1902–1914.

Payne, GA. (1999). Ear and Kernel rots. In: White DG (ed) Compendium of Corn diseases. *The American Phytopathology Society Press*. 44-47.

Peintner, U., Geiger, J. and Pöder, R. (2000). The mycobiota of speck, a traditional tyrolean smoked and cured ham. Journal of *Food Protection* 63,1399‑1403.

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, JC. and Samson, RA. (2007). Biodiversity of *Aspergillus* species in some important agricultural products. *Studies in Mycology* 59, 53–66.

Perrone, G., Rodriguez, A., Magistà, D. and Magan, N. (2019). Insights into existing and future fungal and mycotoxin contamination of cured meats. *Current Opinion in Food Science 29, 1-8.*

Petzinger E, Ziegler K. 2000. Ochratoxin A from a toxicological perspective. *Journal of Veterinary Pharmacology and Therapeutics* 23, 91-98.

Pfohl-Leszkowicz, A. and Manderville, RA. (2007). Ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans. *Molecular Nutrition. Food Research* 51. 61–99.

Pieckova, R and Kunova, Z. (2002). Indoor fungi and their ciliostatic metabolites. *Annals of Agricultural and Environmantal Medicine* 9, 59-63.

Pitt, J.I. (1987). *Penicillium viridicatum, Penicillium verrucosum*, and production of ochratoxin A. *Applied and Environmental Microbiology* 53, 266–269.

Pitt, J., Taniwaki, M.H. and Cole, M. (2013). Mycotoxin production in major crops as influenced by growing, harvesting, storage and processing, with emphasis on the achievement of Food Safety Objectives. *Food Control* 32, 205-215.

Pitt, J.I. and Hocking, A. (1977). Influence of solute and hydrogen ion concentration on the water relations of some xerophilic fungi. *Journal of General Microbiology* 101, 25–40.

Pitt, J.I., Taniwaki, M.H. and Cole, M.B. (2013). Mycotoxin Production in Major Crops as Influenced by Growing, Harvesting, Storage and Processing, with Emphasis on the Achievement of Food Safety Objectives. *Food Control* 32, 205–215.

Ramos, A.J., Magan, N. and Sanchis, V. (1999). Osmotic and matric potential effects on growth, sclerotia and partitioning of polyols and sugars in colonies and spores of *Aspergillus ochraceous*.

Mycological Research 103,141-147.

Ramos, A.J., Laberia, N., Marin, S. and Magan, N. (1998). Effect of water activity and temperature on growth and ochratoxin production by three strains of *Aspergillus ochraceus* on a barley extract medium and on barley grains. *Food Microbiology* 44, 133-140.

Ramirez, M.L., Chulze, S.N. and Magan, N. (2004). Impact of Osmotic and Matric Water Stress on Germination, Growth, Mycelial Water Potentials and Endogenous Accumulation of Sugars and Sugar Alcohols in Fusarium Graminearum. *Mycologia* 96, 470–478.

Ramakrishna, N., Lacey, J. and Smith, J.E. (1996). Colonization of barley grain by *Penicillium verrucosum* and Ochratoxin A formation in the presence of competing fungi. *Journal of Food Protection* 59, 1311-1317.

Ramos, A.J., Laberia, N., Marin, S. and Magan, N. (1998). Effect of water activity and temperature on growth and ochratoxin production by three strains of *Aspergillus ochraceus* on a barley extract medium and on barley grains. *Food Microbiology* 44, 133-140.

Ramos, A.J., Magan, N. and Sanchis, V. (1999). Osmotic and matric potential effects on growth, sclerotia and partitioning of polyols and sugars in colonies and spores of *Aspergillus ochraceous*. *Mycological Research* 103, 141-147.

Ray, D.K., Gerber, J.S., MacDonald, G.K. and West, PC. (2015). Climate variation explains a third of global crop yield variability. *Nature Communications* http://dx.doi.org/10.1038/ncomms6989.

Razavi-Rohani, S.M. and Griffiths, M.W. (1999). Antifungal effects of sorbic acid and propionic acid at different pH and NaCl conditions. *Journal of Food Safety* 19, 109–120.

Rodríguez, A., Borges, D., Medina, A., Córdoba, J.J. and Magan, N. (2015). Relationship between ecophysiological factors, growth and ochratoxin A contamination of dry-cured sausages. *International Journal of Food Microbiology* 194, 71-77.

Rodríguez, A., Medina, Á., Córdoba, J.J., and Magan, N. (2014). The Influence of Salt (NaCl) on Ochratoxin A Biosynthetic Genes, Growth and Ochratoxin A Production by Three Strains of *Penicillium Nordicum* on a Dry-Cured Ham-Based Medium. *International Journal of Food Microbiology* 178, 113-119.

Rodríguez, A., Rodríguez, M., Luque, M.I., Justesen, A.F. and Córdoba, J.J. (2011).

Quantification of Ochratoxin A-Producing Molds in Food Products by SYBR Green and TaqMan Real-Time PCR Methods. *International Journal of Food Microbiology* 149, 226–235.

Rodríguez, A., Medina, Á., Córdoba, J.J. and Magan, N. (2014). The Influence of Salt (NaCl) on Ochratoxin A Biosynthetic Genes, Growth and Ochratoxin A Production by Three Strains of *Penicillium Nordicum* on a Dry-Cured Ham-Based Medium. *International Journal of Food Microbiology* 178, 113–119.

Ricke, S. (2003). Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. *Poultry Science* 82, 632-639.

Ringot, D., Chango, A., Schneider, Y-J and Larondelle, Y. (2006). Toxicokinetics and toxicodynamics of ochratoxin A, an update. *Chemico-Biological Interactions* 159, 18-46.

Rotter, B.A., Prelusky, D.B. and Pestka, J.J. (1996). Toxicology of deoxinivalenol (vomitoxin). *Toxicology and Environmental Health* 48, 1-34.

Salmond, C., Kroll, R. and Booth, I. (1984). The effect of food preservatives on pH homeostasis in *Escherichia coli*. *Journal of General Microbiology* 130, 2845–2850.

Samapundo, S., Deschuyffeleer, N., Van Laere, D., De Leyn, I. and Devlieghere, F. (2010). Effect of NaCl Reduction and Replacement on the Growth of Fungi Important to the Spoilage of Bread. *Food Microbiology* 27, 749–756.

Samson, R. A., Houbraken, J. A M.P., Kuijpers, A.F. A, Frank, J.M. and Frisvad, J.C. (2004). New Ochratoxin A or Sclerotium Producing Species in *Aspergillus* Section *Nigri*. *Studies in Mycology* 50, 45–61.

Samson, R.A., Hoekstra, E.S., Frisvad, J.C and Filtenborg, O. (2002). Introduction to food and airborne fungi. *Centraalbureau voor Schimmelcultures.* Utrecht, Netherlands.

Sanchis, V. and Magan, N. (2004). Environmental profiles for growth and mycotoxin production in Mycotoxins in Food: Detection and Control ed. Magan, N. and Olsen, M. Cambridge: Woodhead Publishing Ltd, 174–189.

Sanchez-Hervas, M., Gil, JV., Bisbal, F., Ramon, D. and Martinez-Culebras, PV. (2008). Mycobiota and Mycotoxin producing fungi from Cocoa beans. *International Jounal of Food Microbiology* 125, 336-340.

Schmidt-Heydt, M., Baxter, E. and Geisen, R. (2007 b). Physiological relationship between food preservatives environmental factors Ochratoxin and OTA*pks* PV gene expression by *Penicillium verrucosum*. *International Journal of Food Microbiology* 119, 277-283.

Schmidt-Heydt, M., Graf, E., Batzler, J. and Geisen, R. (2011). The application of transcriptomics to understand the ecological reasons of ochratoxin a biosynthesis by *Penicillium nordicum* on sodium chloride rich dry cured foods. *Trends in Food Science and Technology,* S39–S48.

Schmidt-Heydt, M., Magan, N. and Geisen, R. (2008). Stress induction of mycotoxin biosynthesis genes by abiotic factors. *FEMS Microbiology Letters* 284, 142–149.

Schmidt-Heydt, M. and Geisen, R. (2007). A Microarray for Monitoring the Production of Mycotoxins in Food. *International Journal of Food Microbiology* 117, 131–140.

Schwarzer, D., Finking, R. and Marabiel, MA. (2003). Nonribosomal peptides from genes to products. *Natural Product Reports* 20, 275-287.

Shwab, E. K. and Keller, N. P. (2008). Regulation of secondary metabolite production in filamentous ascomycetes. *Mycological Research* 112, 225-230.

Skrinjar, M. and Dimic, G. (1992). Ochratoxigenicity of *Aspergillus ochraceus* group and *Penicillium verrucosum* var. *cyclopium* strains on various media. Acta Microbiologica Hungary 39, 257-261.

Sonjak, S., Ličen, M., Frisvad, J. C. and Gunde-Cimerman, N. (2011). Salting on dry-cured meat—A potential cause of contamination with the ochratoxin A-producing species *Penicillium nordicum*. *Food Microbiology* 28, 1111–1116.

Steuter, A. A., Mozafar, A. and Goodin, J.R. (1981). Water potential of aqueous polyethylene glycol. *Plant Physiology* 67, 64-67.

Stoll, D., Schmidt-Heydt, M. and Geisen R. (2013). Differences in the regulation of ochratoxin A by the HOG pathway in *Penicillium* and *Aspergillus* in response to high osmolar environments. *Toxins* 5, 1282–1298.

Stratford, M. and Anslow, P. (1998). Evidence that sorbic acid does not inhibit yeast as a classic "weak acid" preservative. *Letters in Applied Microbiology* 27, 203-206.

Sydenham, E.W., Thiel, P.G., Marasas, W.F., Shephard, G.S., Schalkwyk, D.J. Van. And

Koch, K.R (1990). Natural Occurrence of Some Fusarium Mycotoxins in Corn from Low and High Esophageal Cancer Prevalence Areas of the Transkei, Southern Africa. *Journal of Agricultural and Food Chemistry* 38, 1900–1903.

Taniwaki, M.H., Pitt, J.I., Teixeira, a. a. and Iamanaka, B.T. (2003). The Source of Ochratoxin A in Brazilian Coffee and Its Formation in Relation to Processing Methods. *International Journal of Food Microbiology* 82, 173–179.

Tassou, C. C., Panagou, E. Z., Natskoulis, P. and Magan, N. (2007). Modelling the effect of temperature and water activity on the growth of two ochratoxigenic strains of *Aspergillus carbonarius* from Greek wine grapes. *Journal of Applied Microbiology* 103, 2267–2276.

Theron, M. and Lues, J. (2007). Organic acids and meat preservation: A review. *Food Reviews International* 23, 141–158.

Thompson, D.P. (1994). Minimum inhibitory concentration of esters of p-hydroxybenzoic acid (paraben) combinations against toxigenic fungi. *Journal of Food Protection* 57, 133–135.

Thompson, D.P., Metevia, L. and Vessel, T., (1993). Influence of pH alone and in combination with phenolic antioxidants on growth and germination of mycotoxigenic species of *Fusarium* and *Penicillium*. *Journal of Food Protection* 56, 134-138.

Tittlemeir, S.A., Roscoe, M., Blagden, R., Kobialka, C. and Nowiki, T. (2012). An overview of ochratoxin A in Canadian grains. *Canadian Journal of Plant Pathology* 34, 346.

Van der Merwe, K.J., Steyn, P.s. and Fourie, L. (1965). Mycotoxins. Part II. The constitution of ochratoxins A, B, and C, metabolites of *Aspergillus ochraceus* wilh. *Chemical Society,*7083-7088.

Van Walbeek, W., Scott, P.M., Harwig, J and Lawrence, J.W. (1969). *Penicillium viridicatum* Westling: A new source of ochratoxin A. *Canadian Journal of Microbiology* 15, 1281–1285.

Van der Merwe, KJ., Steyn, PS. and Fourie, L. (1965). Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus*. *Nature* 205, 1112-1113.

Valero, A., Begum, M., Hocking, A. D., Marín, S., Ramos, A. J. and Sanchis, V. (2008). Mycelial growth and ochratoxin A production by *Aspergillus* section *Nigri* on simulated grape medium in modified atmospheres. *Journal of Applied Microbiology* 105, 372-379.

Varga, J., Kevei, E., Rinyu, E., Teren, J. and Kozakiewicz, Z. (1996). Ochratoxin Production

by *Aspergillus* Species. *Applied and Environmental Microbiology* 62, 4461–4464.

Vary, Z., Mullins, E., Mcelwain, J.C., and Doohan, F. (2015). The severity of wheat diseases increases when plants and pathogens are acclimatised to elevated carbon dioxide. *Global Change Biology* 21, 2661-2669.

Vaughan, M.M., Huffaker, A., Schmelz, E.A., Dafoe, N.J., Christensen, S.A., McAuslane, H.J., Alborn, H.T., Allen, L.H. and Teal, P.E.A. (2016). Interactive Effects of Elevated CO_2 and Drought on the Maize Phytochemical Defense Response against Mycotoxigenic *Fusarium verticillioides*. *PLoS ONE* 11, 1–24.

Vaughan, M.M., Huffaker, A., Schmelz, E.A., Dafoe, N.J., Christensen, S., Sims, J., Martins, V.F., Swerbilow, J., Romero, M., Alborn, H.T., Allen, L.H. and Teal, P.E.A. (2014). Effects of Elevated [CO_2] on Maize Defence against Mycotoxigenic *Fusarium verticillioides*. *Plant, Cell and Environment* 37, 2691–2706.

Verheecke, C., Liboz, T., Anson, P., Diaz, R. and Mathieu, F. (2015). Reduction of Aflatoxin Production by *Aspergillus flavus* and *Aspergillus parasiticus* in Interaction with Streptomyces. *Microbiology (United Kingdom)*161, 967–972.

Wang, Y., Wang, L., Liu, F., Wang, Q., Selvaraj, J.N., Xing, F., Zhao, Y., and Liu, Y. (2016). Ochratoxin A Producing Fungi, Biosynthetic Pathway and Regulatory Mechanisms. *Toxins* 8, 1–15.

Wu, F., Bhatnagar, D., Bui-Klimke, T., Carbone, I., Hellmich, R., Munkvold, G., Paul, P., Payne, G., and Takle, E. (2011). Climate Change Impacts on Mycotoxin Risks in US Maize. *World Mycotoxin Journal* 4, 79–93.

Yoshizawa, T., Yamashita, A. and Luo, Y (1994). Fumonisin Occurrence in Corn from High- and Low-Risk Areas for Human Esophageal Cancer in China. *Applied and Environmental Microbiology* 60, 1626–1629.

Yu, J., Chang, P.K., Ehrlich, K.C., Cary, J.W., Bhatnagar, D., Cleveland, T.E., Payne, G.A., Linz, J.E., Woloshuk, C.P. and Bennett, J.W. (2005). Clustered pathway genes in aflatoxin biosynthesis. *Applied and Environmental Microbiology* 70, 1253–1262.

Zychowski, K., Rodrigues, A., Jaclyn, H and Phillips, T. (2013). The effct of Aflatoxin-B1 on Red Drum (Sciaenops ocellatus) and Assessment of Dietary Supplemetation of NovaSil for the

Prevention of Aflatoxicosis. *Toxins* 5, 1555-1573.

APPENDICES

APPENDIX I

Appendix I. 1. Effect of preservatives on growth and OTA production by *P. verrucosum* on 2% wheat based medium modified to two water activity at 25°C after 10 days. Bars represent SEM.

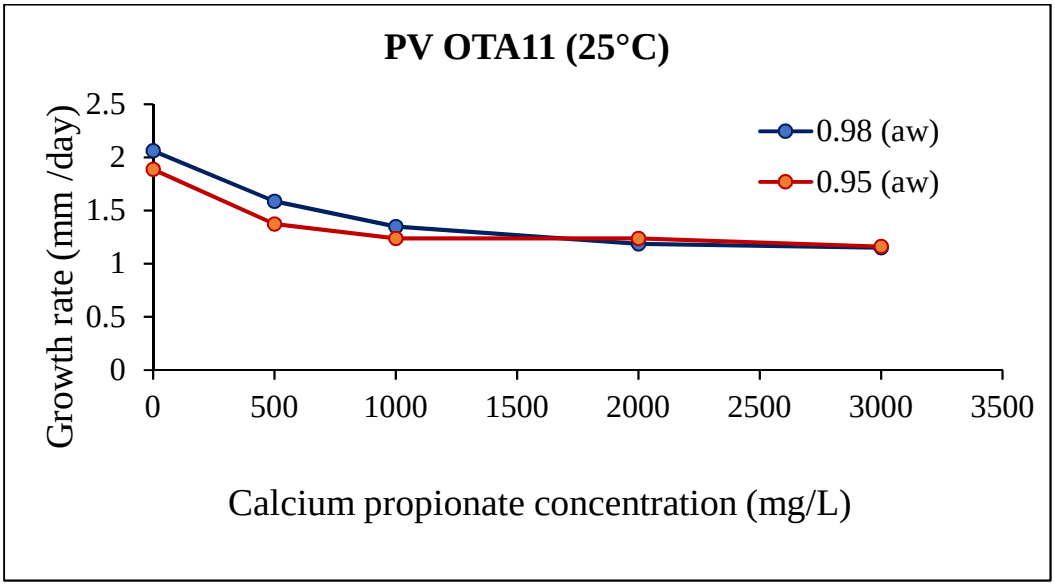

Figure I 1.a: Effect of Calcium propionate on growth rate

Table I 1. a: ANOVA of growth rate of *P. verrucosum* under the impact of CP on 2% milled wheat based medium for 10 days.

Source	DF	Sum of squares	F ratio	Prob> F
conc	4	5.52	76.42	<.0001*
a_w	1	0.24	13.54	0.0009*
conc * a_w	4	0.42	5.84	0.0013*

* Significant.

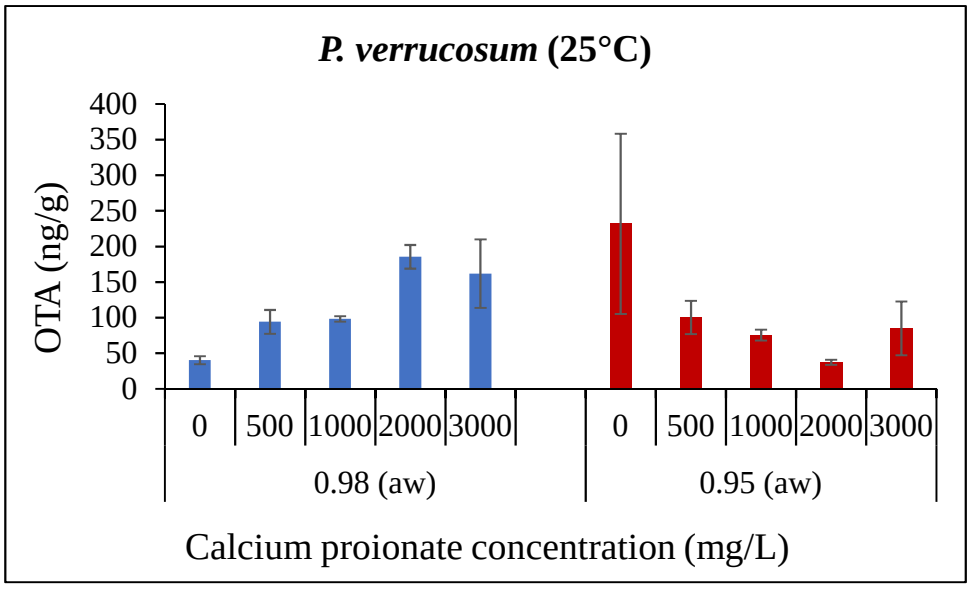

Figure I 2. b. Effect of calcium propionate on OTA production

Table I 2. b: ANOVA of OTA production by *P. verrucosum* under the impact of CP on 2% milled wheat based medium for 10 days.

Source	DF	Sum of squares	F ratio	Prob> F
conc	4	24121.96	8.742	0.0003*
a_w	1	21624.007	31.3491	<.0001*
conc * a_w	4	61539.223	22.3039	<.0001*

* Significant.

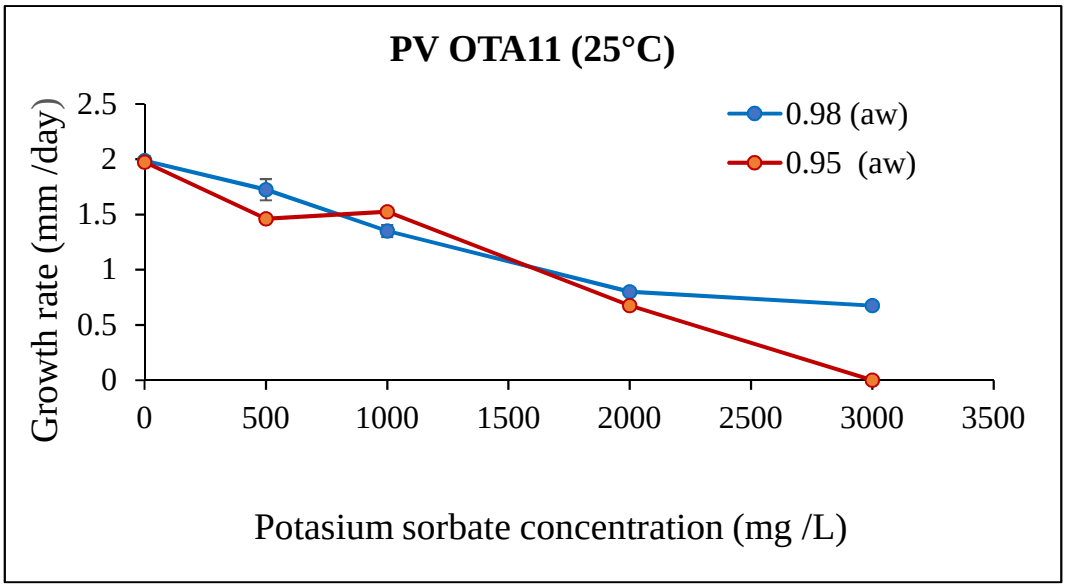

Figure I. 3.c. Effect of Potasium sorbate on growth rate.

Table I. 3. c: ANOVA of growth rate of *P. verrucosum* under the impact of PS on 2% milled wheat based medium for 10 days.

Source	DF	Sum of squares	F ratio	Prob> F
conc	4	37.66	482.82	<.0001*
a_w	1	0.0012	0.0641	0.8019
conc * a_w	4	2.89	37.07	<.0001*

* Significant.

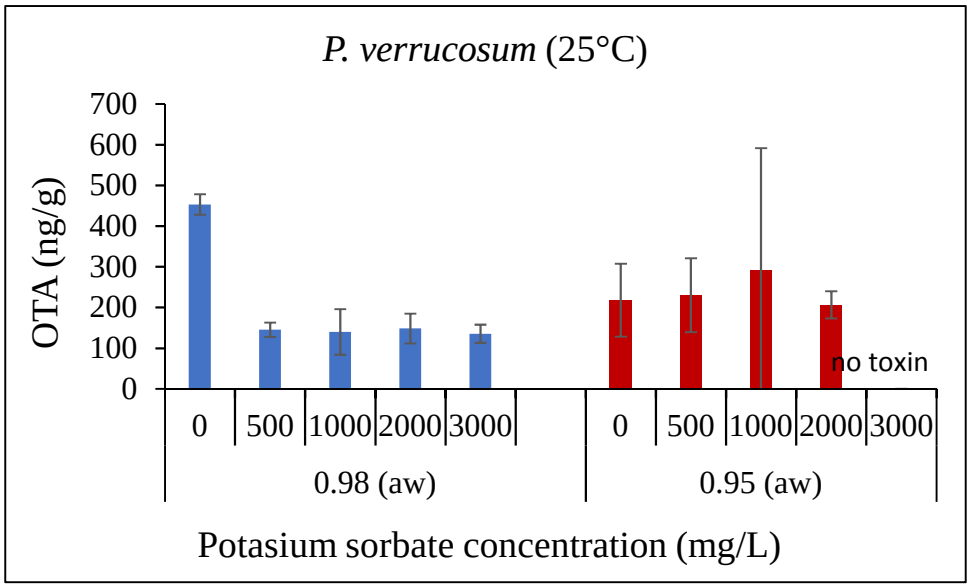

Figure I 3.d. Effect of Potasium sorbate on OTA production.

Table I 4. d: ANOVA of OTA production by *P. verrucosum* under the impact of PS on 2% milled wheat based medium for 10 days.

Source	DF	Sum of squares	F ratio	Prob> F
conc	4	146332.24	2.1435	0.1129
a_w	1	82894.48	4.8570	0.0394
conc * a_w	4	157754.41	2.3108	0.0932

* Significant.

Appendix II

Appendix II. 1. Effect of preservatives on growth and OTA production by *A. westerdijkiae* on 2% coffee based medium modified to two water activity at 30°C after 10 days. Bars represent SEM.

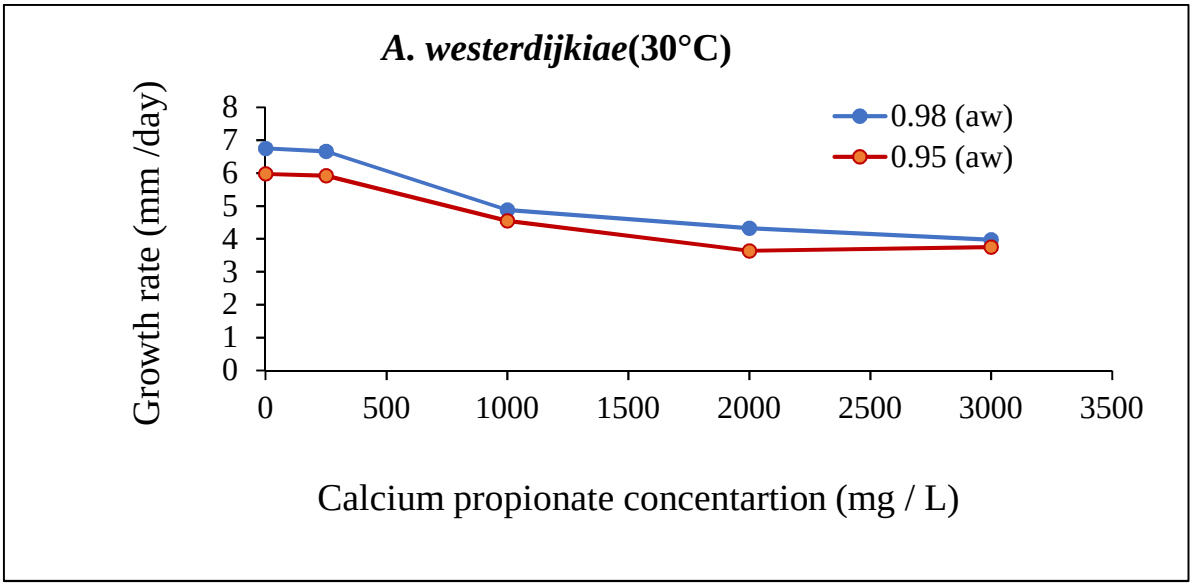

Figure II. 1.a: Effect of calcium propionate on the growth rate.

Table II. 1.a: ANOVA of growth rate of *A. westerdijkiae* under the impact of CP on 2% milled coffee based medium for 10 days.

Source	DF	Sum of squares	F ratio	Prob> F
conc	4	82.51	641.33	<.0001*
a_w	1	4.80	149.37	<.0001*
conc * a_w	4	2.08	16.22	<.0001*

* Significant.

Table II. 1.b: Effect of CP on OTA production (ng/g).

Strain *A. westerdijkiae*	**a_w**	**0 mg/l OTA (ng/g)±SD**	**250 mg/l OTA (ng/g)±SD**	**1000 mg/l OTA (ng/g)±SD**	**2000 mg/l OTA (ng/g)±SD**	**3000 mg/l OTA (ng/g)±SD**
	0.98	<LOQ	<LOQ	No toxin	No toxin	<LOQ
	0.95	97.82±29	<LOQ	105.87±4.36	52.47±13.96	<LOQ

Table 11.1.c: ANOVA of OTA production by *A.westerdijkiae* under the impact of CP on 2% milled coffee based medium for 10 days.

Source	**DF**	**Sum of squares**	**F ratio**	**Prob> F**
conc	4	27671.54	39.53	<.0001*
a_w	1	14167.87	80.96	<.0001*
conc * a_w	4	13835.77	19.76	<.0001*

* Significant.

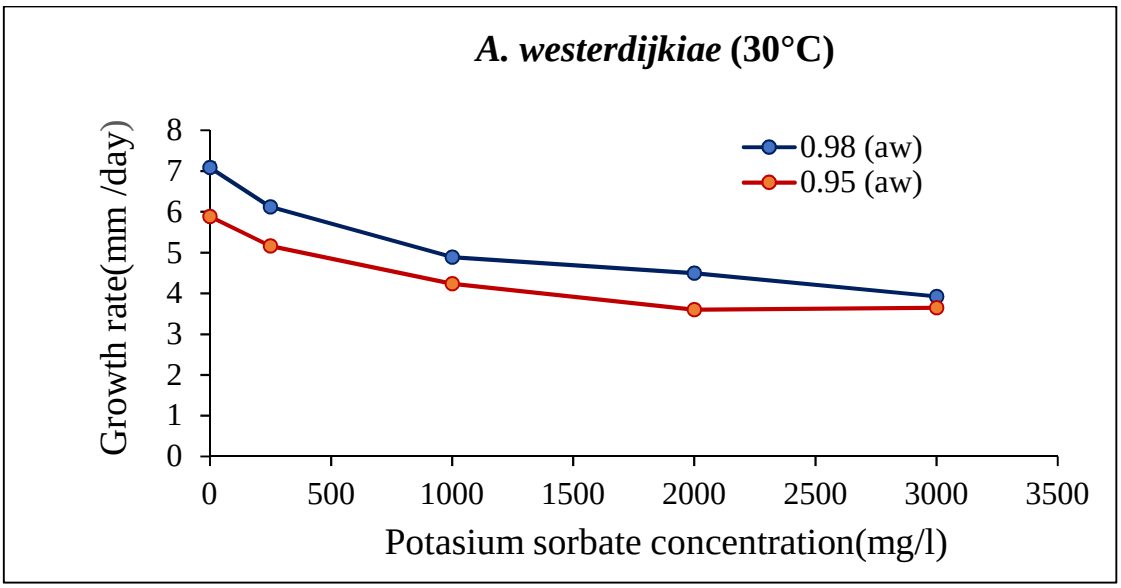

Figure II. 1. b. Effect of PS on growth rate.

Table 11.1. d: ANOVA of growth rate of *A. westerdijkiae* under the impact of PS on 2% milled coffee based medium for 10 days.

Source	DF	Sum of squares	F ratio	Prob> F
conc	4	63.44	518.6	<.0001*
a_w	1	11.52	376.67	<.0001*
conc * a_w	4	3.95	32.33	<.0001*

* Significant.

Table II.1.e. Effect of PS on OTA production (ng/g).

Strain	**a_w**	**0 mg/l OTA (ng/g)±SD**	**250 mg/l OTA (ng/g)±SD**	**1000 mg/l OTA (ng/g)±SD**	**2000 mg/l OTA (ng/g)±SD**	**3000 mg/l OTA (ng/g)±SD**
A. westerdijkiae	0.98	<LOQ	<LOQ	No toxin	9.6±11.1	27.3±1.7
	0.95	12.23±1.41	22.2±4.51	45.4±10.19	66.29±2.65	28.84±4.89

Table II.1. f. ANOVA of OTA production by *A.westerdijkiae* under the impact of PS on 2% milled coffee based medium for 10 days.

Source	DF	Sum of squares	F ratio	Prob> F
conc	4	5421.99	31.85	0.0407
a_w	1	203.62	4.78	<.0001*
conc * a_w	4	3164.23	18.59	<.0001*

* Significant.

Publications

1. Poster presentations.

Conference 1

Poster:

Solute and matric potential effects on growth and ochratoxin A production by *Penicillium verrucosum* and *Aspergillus westerdijkiae* contaminating wheat and coffee

(Shaimaa Abdel Mohsen, Angel Medina and Naresh Magan)

Conference 2

Poster:

Solute and matric potential stress and *Penicillium verrucosum*: impacts on growth, gene expression and ochratoxin A production

(Shaimaa ABDELMOHSEN*, Carol VERHEECKE-VAESSEN, Esther GARCIA-CELA, Angel MEDINA and Naresh MAGAN).

Conference 3

Mycotoxins and Phycotoxins (GRS)
Gordon Research Seminar

Deciphering Biotoxin Production, Cell Mechanisms and Mitigation in a Changing Environment

June 15 - 16, 2019

Chairs
Carol Verheecke-Vaessen and
Zacharias J. Smith

Contact Chairs

Poster:

Solute and matric potential stress and *Penicillium verrucosum*: impacts on growth, gene expression and ochratoxin A production

(Shaimaa ABDELMOHSEN*, Carol VERHEECKE-VAESSEN, Esther GARCIA-CELA, Angel MEDINA and Naresh MAGAN).