

CRANFIELD UNIVERSITY

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**GROWTH AND OCHRATOXIN A PRODUCTION BY
ASPERGILLUS SPECIES IN COFFEE BEANS: IMPACT OF
CLIMATE CHANGE AND CONTROL USING O₃**

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ABSTRACT

Coffee is an important beverage product in many parts of the world. During the production and processing of coffee the prevailing environmental factors and rate of drying can have a profound influence on colonisation by mycotoxigenic fungi and contamination with ochratoxin A (OTA). In Kuwait, coffee beans are imported from various parts of the world. The objectives of this project were to (a) to examine the diversity of mycotoxigenic fungi found in green and roasted coffee beans bought in the Kuwaiti market from different source countries and identify the dominant fungal populations, (b) to examine the ecology and ochratoxin A production by the ochratoxigenic strains and species isolated, (c) determine the effect of caffeine concentrations *in vitro* on growth and OTA production by strains from the *Aspergillus* section *Circumdati* and Section *Niger* groups, (d) evaluate the impact of interacting climate change factors (water activity (a_w) x temperature x elevated CO₂) on growth and OTA production *in vitro* and *in situ*, (e) determine the effect of a_w and temperature interactions on ecology of strains of two new species, *A. aculeatinus* and *A. sclerotiicarbonarius*, isolated from coffee beans and (f) evaluate the efficacy of gaseous ozone (O₃) for controlling OTA producing fungi and control of contamination in coffee beans after treatment and after storage.

The predominant genera isolated from the coffee samples were *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus* and some yeasts. The highest fungal populations in coffee beans imported were Ethiopia. The ecology of the most common toxigenic species in coffee types examined were in the *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati*. Strains of *A. westerdijkiae* (B 2, CBS 121986), *A. niger* (A 1911), *A. carbonarius* (ITAL 204), *A. ochraceus* (ITAL 14) and *A. steynii* (CBS 112814) grew optimally and produced most OTA at 0.95-0.98 a_w and 25-30°C. At reduced a_w levels and 35°C, growth was slower and less OTA produced on coffee-based matrices. Changing the type of coffee-based medium had little effect on the ecology of these strains and species. Use of different coffee concentration (10-80%) *in vitro* had little effect on relative lag phase (λ , days), growth and OTA production by the strains/species tested. Growth was high in 10% coffee concentration for *A. niger* and *A. westerdijkiae* (B2, CBS 121986) while optimum for *A. carbonarius* (ITAL 204), *A. ochraceus* (ITAL

14) and *A. steynii* (CBS 112814) was in the range of 20-70%. The production of OTA was lowest in 10% coffee extract and highest in 70-80% coffee base-media. The caffeine concentration in the medium significantly affected both growth and OTA production in the tested strains/species. Generally, for strains of *A. niger* (A 1911) and *A. carbonarius* (ITAL 204), there was complete inhibition of both growth and OTA production by >1% caffeine concentration.

Interacting climate change factors showed that most strains examined grew well at 30°C and slightly less at 35°C except for *A. niger* (A 1911) which can tolerate higher temperature. In addition, the interaction of elevated CO₂ (1000ppm) plus high temperature (35°C) increased OTA production when compared with 30°C for strains *A. westerdijkiae* (B 2), *A. ochraceus* (ITAL 14) and *A. steynii* (CBS 112814). Most of the strains had optimum growth at 0.95 a_w, at 35°C while, at 30°C the optimum was at 0.98 a_w. On coffee beans there was a significant impact of elevated CO₂ (1000), at 0.90 a_w on OTA production by *A. westerdijkiae* spp. Thus, OTA production was stimulated when combined stresses of elevated temperature (35°C), water stress and increased CO₂ (2.5x) conditions were applied.

The ecology of two new species, *A. aculeatinus*, *A. sclerotiicarbonarius*, isolated from coffee, showed that overall growth of three strains of each was similar over the 20-37°C and 0.85-0.99 a_w ranges. The lag phases prior to growth was <1 day at 0.95-0.98 a_w and 25-37°C and increased to 2-3 days at marginal temperatures and a_w levels. The growth of strains of *A. aculeatinus* was optimum at 0.98 a_w and 30-35°C. For *A. sclerotiicarbonarius* this was 0.99 a_w and 30°C. This species was not able to grow at 37°C. None of the strains of the two species grew at 0.85 a_w, regardless of temperature. Integrated profiles based on the data from three strains of each species were developed to show optimum, maximum and marginal conditions of interacting a_w x temperature conditions for growth. None of the strains produced OTA. This information may be important as these species are part of the mycobiota of coffee and may influence OTA contamination during coffee processing.

Use of gaseous O₃ (600ppm) had little effect on natural populations on coffee beans immediately after treatment, regardless of initial a_w level. However, after storage,

there was some reduction (26%) observed in coffee at 0.95 a_w . In addition, no fungal populations or OTA contamination was found at 0.75 and 0.90 a_w when coffee treated with 600 ppm gaseous O_3 for 60 min was stored. It appears that under wetter conditions (≥ 0.95 a_w) it is more difficult to control fungal populations and OTA contamination. Also, 400 and 600 ppm O_3 applied to coffee beans inoculated with toxigenic strains showed there was less effect on fungal populations at lowered a_w (0.75). While, fungal populations significantly increased directly after exposure and storage at 0.90 and 0.95 a_w . Most of species produced high amounts of OTA in both O_3 treatments at 0.90 and 0.95 a_w . Under drier conditions (0.75 a_w) no fungal growth or OTA was produced.

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ABBREVIATIONS

A.	<i>Aspergillus</i>
AcN	Acetonitrile
ANOVA	Analysis of Variance
Apx	Appendix
a_w	water activity
a_w max	maximum water activity for growth
a_w min	minimum water activity for growth
a_w opt	optimal water activity
CCA	Coconut cream agar
CD	Electrochemical
CFU	colony forming unit
CO₂	Carbon dioxide
CR	Commission Regulation
DG18	Dicloran -18- glycerol agar
EC	European Commission
EOG	Electrolytic ozone generator
ERH	equilibrium relative humidity
et al.	<i>et alii</i>
EU	European Union
F.	<i>Fusarium</i>
FAO	Food and Agricultural Organisation
FDA	Food and Drug Administration
g	Grams
H₂O	water
HPLC	High Pressure Liquid Chromatography
IARC	International Agency for Research on Cancer
kg	kilogram
L L⁻¹	litre
log	logarithm
LOQ	limit of quantification

LOD	Limit of Detection
M	Molarity
m	metre
m²	square metre
MC	Moisture content
MEA	Malt Extract Agar
MeOH	methanol
mg	milligram
min	minute
ml	millilitre
mm	millimeter
μg	Microgram
μmax	maximum growth rate (mm d ⁻¹)
μmol	Micromole
ng	nanogram
No	number
O₂	Oxygen
O₃	Triatomic oxygen (ozone)
OSHA	Federal Occupational Safety and Health
OT	Ochratoxin
OTA	Ochratoxin A
OTA max	Maximum ochratoxin
OTA min	Minimum ochratoxin
P.	<i>Penicillium</i>
PDA	Potato Dextrose agar
ppb	Parts per billion
PPM	Part per Million
RH	Relative Humidity
S	significant
SNM	Synthetic grape juice medium
Tmax	maximum temperature for growth (°C)

$T_{\min}$	minimum temperature for growth (°C)
T_{opt}	T at which $\mu_{\max}$ is optimal (°C)
UK	United Kingdom
US / USA	United States / United States of America
UV	Ultraviolet
wt. %	% of weight
x g	Times gravity
YES	Yeast extract agar
λ_{em}	Emission wavelengths
λ_{ex}	Fluorescence excitation wavelengths

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Coffee, of various types, including Arabian, Turkish, French and Greek, is considered one of the most popular soft drinks in many parts of the world. This includes the Middle East, in countries such as Kuwait. During its production and processing of coffee beans, it becomes contaminated with a wide range of microorganisms, including mycotoxigenic fungi. In Kuwait the biodiversity generally, as well as food quality, are under severe stress due to global warming phenomena. Climate change may exacerbate food safety problems and increase the potential for mycotoxin contamination. Kuwait is a country that imports coffee from India, Africa and Yemen. During different phases of coffee production including the harvesting, fermentation, handling, processing, storage and transport, mycotoxins can be produced, influencing the safety and quality of the end product. Fluctuations in environmental conditions, especially climate change parameters, may enhance coffee spoilage.

The awareness of mycotoxin contamination of coffee has risen throughout the world. Dashti (2005) collected 25 coffee samples from supermarkets in Kuwait and they tested positive for the mycotoxin ochratoxin A (OTA). There is thus increasing consumer concern about food and drink safety and quality (Magan & Olsen, 2004). Silva (2000) studied the most frequent fungal spoilage genera of coffee and cherries and reported *Aspergillus* and *Penicillium* species as being dominant. These genera produce ochratoxins which have nephrotoxic, immunosuppressive, carcinogenic and genotoxic effects in humans and animals (Sedmíková et al., 2001). The incidence of OTA in raw coffee was reported by Levi et al. (1974). The European Commission (Commission Regulation (EC) No. 123/2005) have established regulatory limits for OTA in roasted coffee beans and ground roasted coffee at 5 $\mu\text{g Kg}^{-1}$, and soluble coffee (instant) at 10 $\mu\text{g Kg}^{-1}$ (El Khoury and Atoui, 2010). In Kuwait, there is no specific legislation for these mycotoxins in coffee at present.

1.2 Mycotoxins

The term mycotoxin is derived from the Greek word “Mykes” meaning mould and the Latin word “Toxicum” meaning toxic or poison (Goldblatt, 1972). Several genera of microfungi produce toxic compounds affecting most grain crops, some fruits, vegetables, herbs, nuts, legumes, cocoa, spices and coffee. Mycotoxins are low molecular weight compounds. They are a relatively diverse, large group of naturally occurring toxins, many of which have been identified as chemical agents of toxic diseases in animals and humans. Research on mycotoxins was initiated about 35 years ago, when the first group of mycotoxins (aflatoxins) was isolated and described in 1969 after several acute animal diseases in 1960 (Goldblatt, 1969). The second important mycotoxin to be discovered after aflatoxin, was ochratoxin A (Van Der Merwe et al., 1965a; 1965b).

1.2.1 Ochratoxins

Ochratoxins are toxins produced as secondary metabolites by several fungal species of the *Aspergillus* and *Penicillium* genera. Van der Merwe et al. (1965a) isolated OTA from a culture of *Aspergillus ochraceus* (section *Circumdati*). This section has recently been taxonomically reclassified and this species has been reclassified as *A. westerdijkiae* (the predominant producer of OTA), with other OTA producers being *A. steynii* and *A. ochraceus* (low OTA producer). Samson et al. (2004) isolated two new OTA producing species from coffee beans. These two species, *A. lacticoffeatus* and *A. sclerotioniger*, produce large yellow to red-brown sclerotia and abundant OTA. Other ochratoxigenic *Aspergillus* species found in coffee samples include *A. niger* and *A. carbonarius*. In the genus *Penicillium*, the species producing OTA are *P. verrucosum* in cereals, *P. nordicum* in cured meats and *P. viridicatum* (Pitt, 1987; Larsen et al., 2001). Table 1.1 lists the mycotoxigenic fungi, which produce OTA. After aflatoxins, Ochratoxin A is the most important mycotoxin of concern for human health, whereas ochratoxins B and C are sometimes produced but are of less importance.

Table 1.1: Summary of fungal species, which produce ochratoxin A (Abrunhosa et al., 2010).

Fungal species	References
<i>Aspergillus</i> section <i>Circumdati</i>	
<i>A. cretensis</i>	Frisvad et al.(2004)
<i>A. flocculosus</i>	Frisvad et al.(2004)
<i>A. melleus</i>	Hesseltine et al. (1972)
<i>A. ochraceus</i>	Van der Merwe et al. (1965a); Frisvad et al. (2004)
<i>A. ostianus</i>	Hesseltine et al. (1972)
<i>A. persii</i>	Ciegler, (1972)
<i>A. petrakii</i>	Hesseltine et al. (1972)
<i>A. pseudoelegans</i>	Frisvad et al.(2004)
<i>A. roseoglobulosus</i>	Frisvad et al. (2004)
<i>A. sclerotiorum</i>	Frisvad et al. (2004); Hesseltine et al. (1972); Varga et al. (1996)
<i>A. steynii</i>	Frisvad et al. (2004)
<i>A. sulphureus</i>	Frisvad et al. (2004); Hesseltine et al. (1972); Varga et al. (1996)
<i>A. westerdijkiae</i>	Frisvad et al. (2004)
<i>Aspergillus</i> section <i>Flavi</i>	
<i>A. alliaceus</i> (<i>Petromyces alliaceus</i>)	Varga et al.(1996)
<i>Petromyces albertensis</i>	Frisvad et al. (2006)
<i>Aspergillus</i> section <i>Nigri</i>	
<i>A. carbonarius</i>	Horie, (1995); Samson et al. (2004)
<i>A. lacticoffeatus</i>	Samson et al.(2004)
<i>A. niger</i>	Samson et al.(2004); Abarca et al.(1994)
<i>A. sclerotioniger</i>	Samson et al. (2004)
<i>Penicillium</i>	
<i>P. nordicum</i>	Larsen et al.(2001)
<i>P. verrucosum</i>	Pitt, (1987); Van Walbeek et al. (1969); Ciegler et al. (1973)
<i>P. viridicatum</i>	Ciegler et al. (1973)

These mycotoxigenic species are able to grow in different climatic conditions and contaminate plant products worldwide with OTA (Aish et al., 2004). These include coffee, cocoa, dried fruit, beer, wine, cereals and spices. The ochratoxins are composed of a polyketide-derived di-hydroisocoumarin moiety linked via the 7-carboxy group to L- β -phenylalanine by an amide bond, except for ochratoxin α (Figure 1.1).

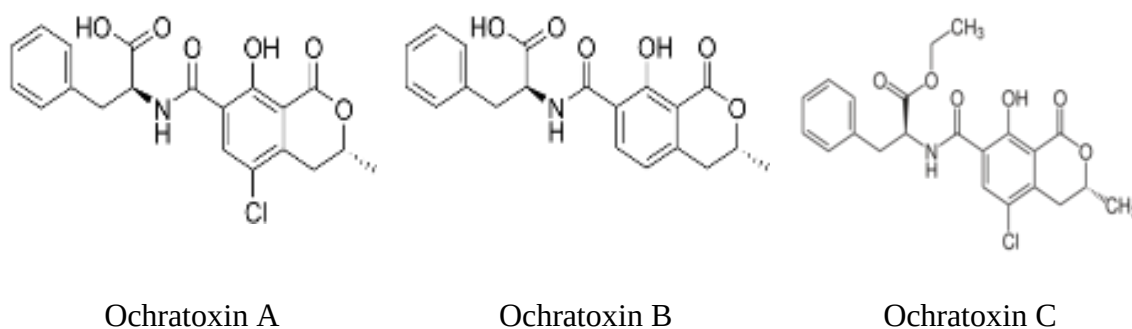


Figure 1.1: Chemical structures of Ochratoxin A, Ochratoxin B, Ochratoxin C.

OTA produces an intense fluorescence under UV light, green in acidic media and blue in alkaline conditions (El Khoury and Atoui, 2010). It is difficult to destroy because of its high temperature resistance and tolerance to low acidity. Boudra et al. (1995) showed that the OTA had thermostability at 250°C in wheat during cooking.

1.2.2 Coffee production

The potential for the formation of OTA during coffee production can be determined by examining the stages of this process. Firstly, pre-harvest: there is no data or studies showing contamination with mycotoxigenic fungi at this stage, and only slight, if any, OTA in immature coffee cherries (Pitt et al., 2013). Secondly, post-harvest, including storage and transport of raw coffee: contamination happens due to variation in land regions and possibly the coffee type determines which fungal species are dominant. During the sun-drying stage, coffee cherries can be contaminated with various fungal species (Taniwaki et al, 2001 and 2003). OTA can be decreased by a mechanical drying process and increased by poor drying processes. Well managed and controlled warehouse storage accounts for less OTA contamination. Finally, processing of raw coffee cherries either manually or using laser sorters to remove broken coffee cherries results in low contamination and OTA production (Taniwaki et al., 2003).

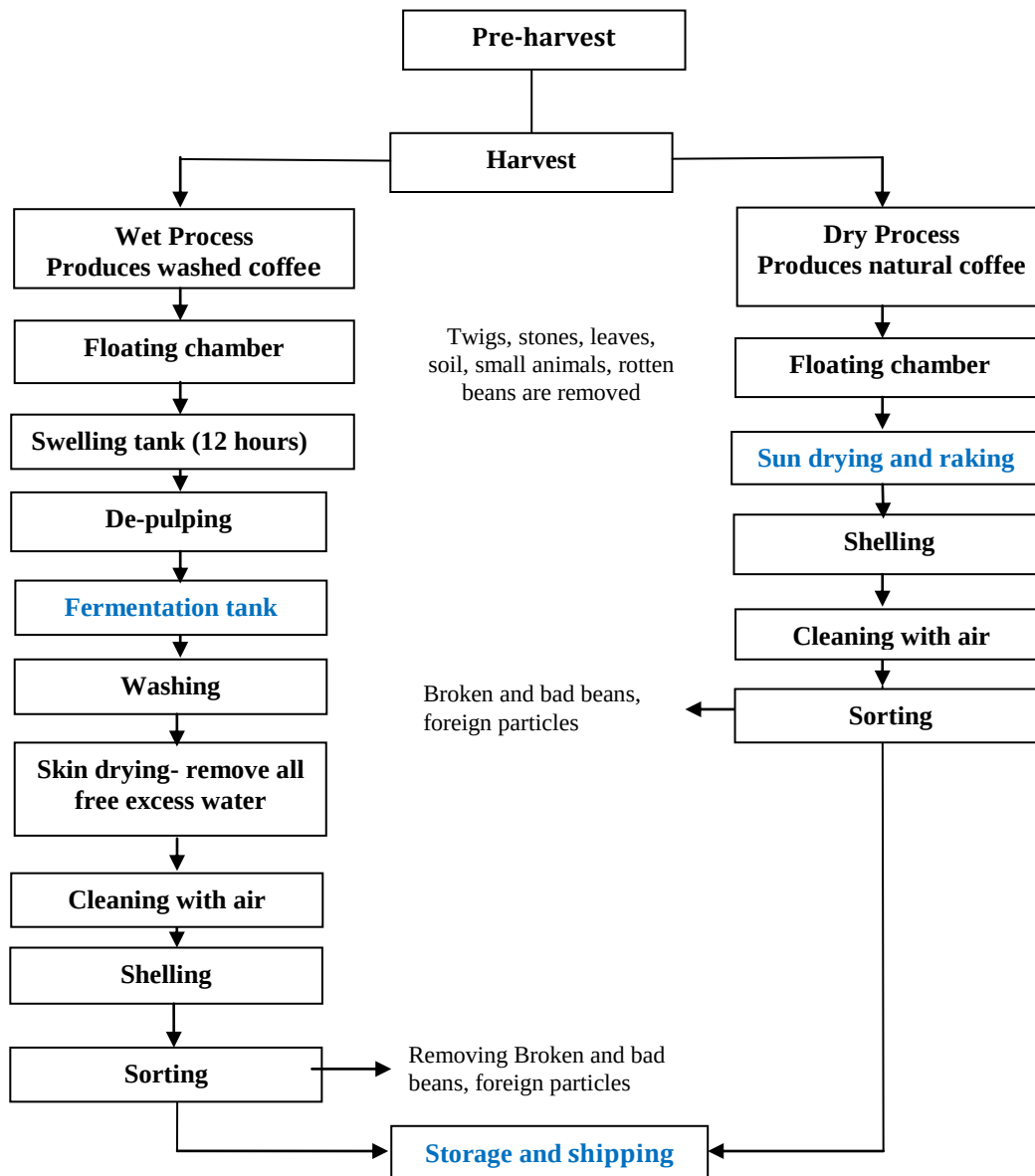


Figure 1.2: General flow chart for coffee production (Augstburger et al., 2000). The stages highlighted in blue are important critical control points (CCPs) where OTA can be formed in the process.

1.3 Impact of environmental factors on mycotoxigenic fungi

Coffee is the most common enjoyable drink in the vast numbers of cafes and homes around the world. In addition, by 2019, the coffee consumption worldwide had almost doubled over 40 years and is predict to be > 9 million tonnes (Fairtrade Foundation, 2012). Coffee cultivation has been threatened by different pests, diseases and also

contamination by mycotoxigenic fungi for many decades (Levi et al., 1974; Paterson et al., 2001). Furthermore, the significance of climate alteration (IPCC, 2013) to coffee production (Davis et al., 2012) and the impact that climate change may have on coffee quality and mycotoxin contamination requires urgent consideration. Global warming expected to have effect on food safety worldwide (Magan et al., 2011; Paterson & Lima, 2011, 2012). The environmental stress factors such as high temperature, humidity and elevated CO₂ influences coffee contamination with mycotoxigenic fungi and therefore may increase the chance of mycotoxin contamination (FAO, 2008; Magan et al., 1984b). There are few studies and little information regarding the impact of climate change on fungal colonisation and mycotoxin production in coffee.

It have been suggested that CO₂ levels will double from 350 to 700 or triple to 900 to 1000 ppm in the next 10-25 years which will effect crops grown worldwide (Medina et al., 2014). Also, predicted increases in temperature of 4–6°C due to climate change in the next 50 years is predicted in different regions of the world (Magan et al., 2011; Paterson & Lima, 2010, 2011, 2012). Thus, although climate change impacts an of wide concern its potential impacts on crop cultivation, colonisation by mycotoxigenic fungi, and mycotoxin contamination have received little attention (Magan et al., 2011; Wu et al., 2011). .

Water activity (a_w) is a measure of the amount of water available in a substrate for microbial growth (Magan and Aldred, 2007). Magan et al. (2011) also suggested that mesophilic fungi may be less competitive than xerophilic fungi such as *Wallemia sebi*, *Xeromyces bisporus* and *Chrysosporium* species which may become more dominant in food crops due to interacting climate change factors (a_w x temperature x CO₂) as they are able to grow in water stress conditions of 0.65 to 0.75 a_w (Magan, 2006; Magan and Aldred, 2007).

Several studies have suggested that modified climate change is already affecting mycotoxigenic fungi. For example, in 2003/2004 and in 2012 in Northern Italy drought and elevated temperatures resulted in colonization and contamination of maize by *Aspergillus* section *Flavi* and aflatoxins (AFs) and subsequent entry of aflatoxin M1

into the dairy chain via the milk (Giorni et al., 2007; Battilani et al., 2012). More recently studies done on maize in Serbian maize samples in 2009 to 2011 showed no aflatoxin contamination. However, prolonged drought during the spring and summer of 2012 resulted in 69% of maize contaminated with aflatoxins (Kos et al., 2013). Similarly, in Hungary, it was also been reported that elevated temperatures has led to an increase in aflatoxin contamination in maize (Dobolyi et al., 2013).

1.4 The ecology of fungi involved in coffee spoilage

Roasting, grading and green coffee beans can all become contaminated by different microorganisms during the production stages at harvest, post-harvest, processing, and during storage and transport. The drying stage is a critical one as uneven drying can lead to colonisation by mycotoxigenic fungi and result in OTA contamination. Understanding the ecological factors that enhance OTA production could help in controlling and reducing the incidence of contamination. There are some key factors that play a major role in growth of these fungi and OTA contamination such as competing mycobiota as well as abiotic factors like water activity (a_w), temperature, pH and moisture content (MC) and intergranular gaseous atmosphere. The moisture content during harvest can vary from 16 to 30% in cherries, 35 to 50% in coffee raisins and 50 to 70% in ripe cherries (Kamau, 1980). At the end of the drying stage the MC must be <12% (approx. =0.65-0.70 a_w); at <9% the coffee loses flavour, while at >13% the risk of OTA contamination increases. Coffee is very hygroscopic and during storage and transport, it can absorb moisture from the environment (Magan and Aldred, 2005). The effect of moisture and temperature on coffee contamination has been studied. Palacios-Cabrera et al. (2003) reported that fluctuations between relative humidity (RH) and temperatures of 14 and 25°C resulted in little or no OTA production in raw coffee at 80%. The optimal conditions for OTA production was found to occur at 87 and 95% RH in a humidity-cycling environment.

1.4.1 Temperature

Temperature is one of the factors that influences the rate of spore germination, mycelial colonisation and mycotoxin production (Magan and Aldred, 2007). Suarez-Quiroz et al. (2004a) reported that temperature, a_w and their interactions had a significant effect on growth and OTA production by strains of *A. ochraceus* and *A. carbonarius*. Taniwaki et al. (2001) and Suarez-Quiroz et al. (2004b) both found that in coffee beans inoculated with two strains of *A. ochraceus* and *A. carbonarius* the minimum a_w for growth was 0.80 and the maximum 0.95 for OTA at 25°C. Furthermore, production was higher in cycling temperatures than at a constant 25°C after 25 days storage. OTA production by *Aspergillus ochraceus* in alternating temperatures (25 and 14°C) was higher than constant temperatures due to condensation and an increase in MC (= a_w) of the coffee beans (Palacios-Cabrera et al., 2004).

Abdel-Hadi & Magan (2009) examined the effect of interaction of a_w and temperature on growth and OTA production of strains of the three new taxonomic species, *A. ochraceus*, *A. steynii*, and *A. westerdijkiae*, in the Section *Circumdati*. They reported that maximum growth occurred for all three species at 0.99-0.95 a_w and 30°C. Furthermore, a previous study on barley-based media showed optimal a_w for three isolates of *A. ochraceus* (= *A. westerdijkiae*) was at 0.98-0.96 a_w and 30°C for two isolates, and 25-30°C for the third. The highest growth rate was at 0.95 a_w (Ramos et al., 1998). Alborch et al. (2011) showed that, in *Aspergillus sclerotioniger* isolated from coffee beans, OTA production was detected from 10-35°C and the T_{max} was 15°C after 10 days. OTA production in *Aspergillus laticoffeatus* was detected from 15-45°C and the T_{max} was after 5 days at 25°C. Astoreca et al. (2010a) reported that *A. niger* strains produced maximum OTA production at 0.973 and 0.995 a_w at 30°C. Kouadio et al. (2007) showed that the optimal growth rate for *A. niger*, *A. carbonarius* and *A. ochraceus* was the same, at 30°C and 0.99 a_w , and OTA production was inhibited at 42°C and 0.75 a_w on a coffee-based medium. In addition, the effect of temperature x a_w interactions on growth and OTA production on green coffee-based media was studied for strains of *A. ochraceus* (Pardo et al., 2005a). Optimal conditions for germination and growth were observed at 0.95-0.99 a_w and 20-30°C. Maximum growth was found at

30°C and 0.95-0.99 a_w while maximum OTA production was at 20°C and 0.99 a_w (Pardo et al., 2005b). Six *A. carbonarius* strains were studied for OTA production in two media: Czapek yeast autolysate (CYA) agar and yeast extract sucrose (YES) agar. CYA was found to be a better culture medium than YES, with maximum OTA being produced at 15°C rather than at 30°C (Esteban et al., 2006). Table 1.2 summarises some of the ecological data on fungal growth and OTA production by different mycotoxigenic fungi in the literature.

1.4.2 Water availability

Water availability is a critical parameter, as it not only affects flavour and aroma of the coffee product, but also influences the contamination of coffee beans by mycotoxigenic fungi. Water activity is related to the water content of the substrate and is referenced to pure water and equilibrium relative humidity (ERH) at a standard temperature and pressure. An ERH of 98% would be equivalent to a a_w of 0.98. It is related to the concept of water potential, which is mainly used in soil microbiology. This allows the components, solute, matric potential and turgor forces to be differentiated. However, in food matrices such as coffee, the water content changes are predominantly due to solute potential and the a_w concept does not allow this differentiation to be made. However, a_w will have a significant influence on germination, growth and mycotoxin production. For coffee, the water content also influences quality and flavour. It is thus important to balance the requirements of a good quality product with minimising conditions conducive to OTA contamination. A number of studies have examined OTA production by different OTA producing fungi. For example, Astoreca et al. (2007) studied the effect of a_w and temperature on the growth of two strains of *Aspergillus* section *Nigri* isolated from coffee beans, where the maximum growth was at 0.97 a_w and 30°C. The a_w for growth depends on the temperature. In studies by Pardo et al. (2005c; 2005b) on *A. ochraceus* strains they found that optimum OTA production was between 0.95-0.99 a_w at 30°C, and the maximum OTA was produced at 0.99 a_w and 20°C. While at 10°C, no OTA was produced. The a_w optimum for growth of two *Aspergillus* section *Nigri* strains grown on irradiated green coffee beans was at 0.995 a_w and 25°C. The maximum OTA production was reported to be between 0.973 and 0.995 a_w at 30°C (Astoreca et al.

2010b). On the other hand, Kouadio et al. (2007) reported $T_{\max}$ of 42°C and 0.75 a_w for OTA production in *Aspergillus niger*, *Aspergillus carbonarius* and *Aspergillus ochraceus* was inhibited. Six *A. carbonarius* have been studied for the effect of a_w and temperature on growth and OTA production. Growth and OTA production were detected only over the range of 0.94-0.99 a_w (Esteban. et al., 2006). Table 1.2 summarises some of the ecological data on fungal growth and OTA production by different mycotoxigenic fungi in the literature.

Table 1.2: Summary of ochratoxigenic species and effect of temperature/a_w on optima and minima for growth and toxin production found in the literature.

Species	Substrate used in Experiment	Tmax	Tmin	a _w min	Optimal a _w growth	Optimal T growth	Optimal a _w OTA production	Optimal T OTA production	Reference
<i>A. ochraceus</i>	Irradiated green coffee bean (media)		10°C				0.99	20°C	Pardo et al. (2005c)
<i>A. ochraceus</i>	Green coffee extract agar medium			0.80 0.85	0.95 0.99	20-30°C			Pardo et al. (2004)
<i>A. ochraceus</i>	CMEA (coffee meal extract agar)			0.80	0.95	30-35°C	0.90	30°C	Suárez-Quiroz et al. (2004b)
<i>A. niger</i>				0.85	0.99	20-30°C			
<i>A. niger</i>	Irradiated coffee bean de-hydrated				0.995	25°C	0.973-0.995	30°C	Astoreca et al. (2010)
<i>A. niger</i> <i>A. carbonarius</i> <i>A. ochraceus</i>	Coffee based media	42°C Inhibit		0.75 Inhibit	0.99	30°C			Kouadio et al. (2007)
<i>A. ochraceus</i>	Irradiated coffee media (In vitro) More OTA produced in coffee media than in grains, grapes, YES media			Lowest OTA production 0.90			0.98	25°C	Pardo et al. (2005b)
<i>A. ochraceus</i>	Coffee meal extract agar (CMEA)+ tested with caffeine (reduce) and chlorogenic acid (little effect)		OTA Can occur at 10°C		0.95-0.99- (0.80 germination)- (0.85 mycelial growth)	20-30°C			Suárez-Quiroz et al. (2004a)
<i>A. carbonarius</i> <i>A. ochraceus</i>	Coffee bean, cycling temperatures of 25°C for 12 hr and 14°C for 12 hr. OA production was higher at cycling temperatures than at 25°C constant temperature.			0.80				14-25°C	Taniwaki et al. (2001)
<i>A. westerdijkiae</i> <i>A. ochraceus</i>					0.95	30°C	0.99	30°C	
	MEA +YES				0.99 Spore production 0.95	30°C Spore production 35°C			Abdel-Hadi, (2009)
<i>A. steinii</i>									

1.4.3 Modified atmospheres for control of ochratoxin producing fungi

Packing food under modified atmospheres is one way to inhibit microbial growth and extend the shelf life of food. This can have the advantage of minimizing the need for chemical preservatives. Modified atmosphere requires packing food by decreasing oxygen levels and replacing them with nitrogen and CO₂ (Zardetto, 2005). Exposing fungi to elevated carbon dioxide (CO₂) is known to inhibit or slow down their growth (Hoogerwerf et al., 2002; Taniwaki et al., 2009). Different species have different level of sensitivity/tolerance to CO₂ concentration. Between 4-20%, CO₂ can stimulate growth of fungi (Wells and Uota, 1970). Although CO₂ concentrations >40% were shown to inhibit or slow down the growth of mycotoxigenic fungi (Taniwaki et al., 2009).

a) *In vitro* effects of modified atmospheres on mycotoxigenic fungi.

Giorni et al. (2008) reported inhibition of aflatoxin production on synthetic media and on stored maize grains with 75% CO₂ on 0.95 and 0.92 a_w. Cairns-Fuller et al. (2005) studied the effect of temperature, a_w and CO₂ on production of OTA by *P. verrucosum* on a milled wheat medium and on stored wheat. The study was performed on gamma irradiated wheat grain over the range 0.75-0.99 a_w, 10-25°C and up to 50% CO₂. This showed that the minimum growth was about 0.80 a_w and inhibition of OTA production and growth occurred with 50% CO₂. Studies with *A. ochraceus* with up to 30% CO₂ on agar-based media inhibited production of OTA after 14 days (Paster et al., 1983). Pateraki et al. (2005) also showed that up to 50% CO₂ affected germination of spores of *A. carbonarius* and mycelial growth but was not very effective in controlling OTA production. Interactions between temperature, a_w and CO₂ suggested that a_w was more important than CO₂. Surprisingly, Valero et al. (2008) found that 1% O₂ combined with only an increased CO₂ level to 15% reduced fungal growth and OTA synthesis by *A. carbonarius* and *A. niger* on synthetic grape juice medium (SNM).

Previous studies by Han & Nout (2000) on interaction effects of temperature, a_w and CO₂ on strains of *Rhizopus* spp. suggested no relationship between these factors on growth. They were more sensitive to a_w than CO₂ and the effect was more pronounced at 40°C at 0.995 a_w.

Magan & Lacey (1984a, 1984b), showed that *Alternaria alternata* growth was inhibited by >5% CO₂ at 0.98 and 0.95 a_w. In addition, they reported a stimulation of growth of some species of *Aspergillus* and *Penicillium* spp. by 5–10% CO₂ concentration on wheat extract agar at 0.98 a_w incubated at 23 or 14 °C. Taniwaki et al. (2009) reported reduction of aflatoxin, patulin, and roquefortine C produced by *Mucor plumbeus*, *Fusarium oxysporum*, *Byssoschlamys fulva*, *Byssoschlamys nivea*, *Penicillium commune*, *Penicillium roqueforti*, *Aspergillus flavus*, *Eurotium chevalieri* and *Xeromyces bisporus* after exposure to 20, 40 and 60% CO₂ plus <0.5% O₂ on Czapek YES and PDA. Zardetto (2005) observed inhibition in growth of *Penicillium aurantiogriseum* on Czapek yeast agar and in atmospheres containing 70% CO₂ for 550 hr. and incubated at 15°C.

Wilson & Jay (1975) reported low production of aflatoxin produced by *A. flavus* when exposed to 61.7% CO₂ in moist maize. Guynot et al. (2003) showed that spoilage was prevented when bakery products were exposed to 70% CO₂ at 0.80 a_w, and delayed at 0.85 and 0.90 a_w. Similarly, Suhr & Nielson (2005) reported *A. flavus* could grow on wheat and rye bread when exposed to 75% CO₂. Other studies showed that 30% CO₂ at 0.98 a_w had an inhibitory effect on production of fumonisin B₁ produced by *Fusarium* section *Liseola* species on yellow dent corn (Samapundo et al., 2007). Diener and Davis (1977) showed that modified atmosphere 20% CO₂ reduced the growth of *A. flavus* in peanuts, and to some extent inhibit growth and sporulation at 25% CO₂ while aflatoxin increased. Giorni et al. (2008) reported inhibition of aflatoxin production on maize grains with >50% CO₂, while 25% CO₂ had little effect. Previous studies showed that growth was observed after 4 weeks on moist maize when it was exposed to 61.7% CO₂ combined with O₂ and N₂ and aflatoxin was increased in air (Wilson and Jay, 1975). Table 1.3 shows some examples of efficacy of CO₂ on mycelial growth or OTA production by mycotoxigenic fungi reported in the literature.

Table 1.3: Summary of the effect of CO₂ on fungal growth and on mycotoxin production by different fungal species from the literature.

Species	CO ₂ %	Effect	Other factors	Media	Author
<i>Botrytis cinerea</i> <i>C. herbarum</i> <i>Rhizopus stolonifer</i>	16%	Growth inhibition 90%	21% O ₂	liquid glucose-salt medium	Wells and Uoto, (1970)
<i>Fusarium roseum</i>	>32%	Growth inhibition 50%			
<i>Alternaria tenuis</i>	45%	Growth inhibition			
<i>P. commune</i> <i>Eurotium chevalieri</i> <i>Xeromyces bisporus</i>	20%	No growth	(< 0.5%) O ₂	Czapek Yeast Extract agar and Potato Dextrose agar	Taniwaki et al. (2009)
<i>P. roqueforti</i> <i>A. flavus</i>	40%	No growth			
<i>Mucor plumbeus</i> <i>Fusarium oxysporum</i> <i>Byssoschlamys fulva</i> <i>Byssoschlamys nivea</i>	20,40,60%	Growth			
	75%	Growth inhibited			
<i>A. flavus</i>	50%	Significant reduction in Aflatoxin			
			0.95-0.92 25°C	synthetic medium and maize grain	Giorni et al. (2008)
<i>P. verrucosum</i>	50%	Growth inhibition	0.75-.99 a _w 10-25°C	Milled wheat medium	Cairns-fuller et al. (2005)
<i>A. ochraceus</i>	>30%	Inhibition of OTA		Agar based media	Paster et al. (1983)
<i>A. carbonarius</i>	50%	Effect on germination but not OTA	0.965 and 0.93 a _w at 25°C	White Grape Juice Agar medium	Pateraki et al. (2005)
<i>A. carbonarius</i> <i>A. niger.</i>	15%	Reduce OTA and growth	1% O ₂	synthetic grape juice medium (SNM)	Valero et al. (2008)
<i>Aspergillus spp.</i> <i>Penicillium</i>	0.03-15%	5-10% stimulate growth, mostly effect lag phase	(14-21%) O ₂ 0.98-0.80 a _w 23-14°C	Wheat extract agar	Magan et al. (1984)
<i>Mucor plumbeus</i> , <i>Fusarium</i> <i>oxysporum</i> , <i>Byssoschlamys fulva</i> , <i>Byssoschlamys nivea</i> , <i>Penicillium</i> <i>commune</i> , <i>Penicillium</i> <i>roqueforti</i> , <i>Aspergillus flavus</i> , <i>Eurotium chevalieri</i> and <i>Xeromyces</i> <i>bisporus</i>	80% CO ₂	Low hyphal lengths were and reduction in Ergosterol production	20% O ₂	Czapek Yeast Extract agar, Potato Dextrose agar , Czapek Yeast Extract 20% Sucrose agar and Malt Yeast 50% Glucose agar	Taniwaki et al. (2010)

1.4.4 Effect of roasting on OTA levels and mycotoxigenic fungi

Roasting coffee by using high temperatures (180 or 250°C) for periods of 5 to 15 min increases the flavour of coffee and at the same time can reduce mould contamination and OTA content. There are three different types of roasting: light (Arabic), medium, and dark. Roasting of coffee beans decreases the water content, which helps to protect beans from infection and OTA production. Since 1988, it has been thought that OTA was broken down during roasting. Blanc et al. (1998) reported full destruction of OTA after roasting Thai Robusta green coffee. Another study showed that between 69 and 96% was destroyed (Van Der Stegen et al., 2001). However, other studies reported little reduction in OTA in green coffee. For example, Tsubouchi et al. (1987) reported the levels of OTA decreased by only 0-12% at 200°C for 10 or 20 min in the dried whole beans.

1.4.5 Effect of caffeine on growth and OTA production by fungi

Caffeine (1, 3, 7- trimethylxanthine) is a white crystalline xanthine alkaloid which exists naturally in different seeds and leaves. It has been studied for many years and has been reported to have an effect on different biological systems. It has been suggested that pure caffeine acts as an antifungal agent. For example, studies by Arora and Ohlan (1997) reported the effect of growth of ten different wood-rotting fungi in eight samples of tea and two samples of coffee, Most of the fungi were inhibit with 0.3% caffeine. Caffeine can act as a natural fungicide and replace other chemical fungicides which, with time, can lead to a build-up of resistance and the problems associated with pesticide residues in the environment.

Some studies show that caffeine can inhibit toxin production by *Aspergillus* and *Penicillium* species (Buchanan and Fletcher, 1978; Buchanan et al., 1982; 1983). For example, aflatoxin production by *Aspergillus parasiticus* was inhibited by caffeine in liquid culture studies. Some studies have also suggested that decaffeinated coffee beans contaminated with *A. parasiticus* can contain high levels of aflatoxin compared with green and roasted coffee beans (Nartowicz et al., 1979). Lenovich (1981) reported that low levels of aflatoxin was produced by *Aspergillus parasiticus* on cocoa beans containing $>1.8 \text{ mg g}^{-1}$ caffeine.

Other work suggested that some microbial species can degrade caffeine as carbon source (Nayak et al. 2012). Microbial species such as *Pseudomonas putida*, *Serratia*, *Klebsiella*, *Rhodococcus*, *Alcalignes*, *Aspergillus tamari*, *Penicillium roqueforti*, *Penicillium verrucosum*, *Fusarium*, and *Stemphylium* have the ability to degrade caffeine (Schwimmer et al., 1971; Woolfolk, 1975; Asano et al., 1994; Mazzafera et al., 1994; Roussos et al., 1995; Yamaoka-Yano & Mazzafera, 1999; Madyastha et al., 1999; Hakil et al., 1999; Roussos et al., 1994; Mohapatra et al., 2006). The initial studies on caffeine degradation as the sole source of carbon and nitrogen done by Kurtzmann and Schwimmer (1971) used strains of *P. roqueforti* and *Stemphylium* sp. Dash and Gummadi (2006) reported caffeine degradation of 5 g L⁻¹ within 48 hr by a *Pseudomonas* species isolated from soil of a coffee plantation area. Furthermore, 90% of caffeine was degraded in solid-state fermentation by *A. niger* isolated from coffee husk (Brand et al., 2000).

Recent studies by Pai et al. (2013) showed that caffeine degradation is enhanced by nitrogen supplementation in the medium. The strains used in these studies were *Gliocladium roseum*, *Fusarium solani*, and *Aspergillus restrict*. In the absence of nitrogen, only *Chrysosporium keratinophilum*. *Fusarium solani*, *Gliocladium roseum* *Chrysosporium keratinophilum*, and *Aspergillus restrictus* were able to resist high caffeine concentrations when grown in solid minimal medium containing caffeine as the sole carbon source (Nayak et al., 2013). Roussos et al. (1994) showed that *P. verrucosum* could not degrade caffeine in media containing NH₄Cl and urea. However, in the absence of these compounds complete degradation occurred. *Penicillium crustosum*, when grown on roasted coffee containing 0.45-0.59 mg mL⁻¹ caffeine, was able to completely degrade the caffeine. However, few detailed ecological studies have been done to examine the efficacy of caffeine concentrations on the growth and OTA production by the key ochratoxigenic fungi colonising coffee. This could be important as Arabica and Robusta coffee have different natural concentrations of caffeine present and this could influence the colonisation by ochratoxigenic fungi and OTA contamination.

1.5 Strategies for reducing OTA production in coffee

1.5.1 Ozonation

Ozone (O_3), triatomic oxygen O_3 , is relatively unstable and decomposes in 20- 25 min to oxygen without any residue and is found naturally in the atmosphere. It is a powerful oxidizing agent and effective as a sanitizer and disinfectant. Processing, storage and food treatment in gaseous and liquid phases had been approved by the FDA, USA for use as an antimicrobial agent (US-FDA, 2001). In addition, it has been used as sanitizer and treatment for bottled water and has been recommended for reducing contamination by pathogens in apple juice and cider (US-FDA, 2004). Exposure to O_3 can cause some harmful health effects. The limit for O_3 exposure is 0.1 ppm for 8 hr continuously and 0.3 ppm for 15 min, regulated by the Federal Occupational Safety and Health Administration (OSHA) (Suslow, 2004).

O_3 gas can be produced by using different methods including Corona discharge method (CD) electrochemical and Electrolytic ozone generation (EOG). The most common and widely used O_3 generator is the Corona discharge; there are two electrodes, one with a high-tension electrode and the other with a low tension electrode separated by a narrow discharge gap (Figure 1. 3). Dissociation of oxygen atom occurs when electrons have sufficient energy, O_3 formed from individually oxygen atom when a certain fraction of these collisions happen. (Guzel-Seydim et al., 2004; Ozone Solutions, 2007). The output ozone from the CD generator when using dry air contains from 1% to 3% O_3 , and 3% to 6% O_3 with pure oxygen (McKenzie et al., 1997; Kim et al., 1999; Mahapatra et al., 2005)

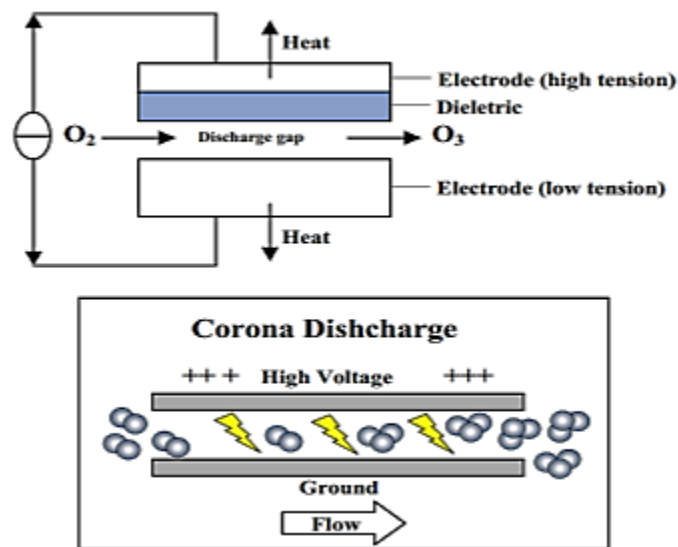


Figure 1.3: Scheme of Corona discharge method: Oxygen is passing in to high voltage plates to simulate corona discharge which broken apart and recombines into ozone (Goncalves, 2009).

Many studies have evaluated the effectiveness of O_3 in reducing and controlling fungi and mycotoxins, and also for decontamination of fruit and vegetables (Minas et al., 2010). However no, studies have been done on controlling mycotoxigenic fungi in coffee beans and OTA production.

a) O_3 action as antimicrobial *in vitro*

No studies have been examined the effect of O_3 on fungi isolated from contaminated coffee beans, fungal populations and control of OTA contamination in coffee. However, some studies have been conducted *in vitro* for efficacy of O_3 against spore germination and mycelia growth (Krause and Weidensaul, 1977; Freitas-Silva and Venancio, 2010; El-Desouky et al., 2012).

Early studies by Hibben and Stotzky (1969) examined the effect on spore germination *in vitro* for spore of 14 fungal species (including *Aspergillus*, *Penicillium*, *Botrytis*, *Fusarium*, *Alternaria*, *Verticillium*, *Rhizopus*) at concentrations of 10-100 pphm (parts per hundred

million) O_3 for 1-6 hr. *in vitro* on solid and liquid yeast extract-rose bengal agar. Comparing the effect of different O_3 concentration, similar effects were shown on spore germination when exposed to high O_3 concentration for a short time, or low concentrations for a longer time. They observed that spore germination was stimulated at lower doses of O_3 . Also, abnormal growth features was shown in species exposed to O_3 . Little inhibitory effect of O_3 exposure on spores was observed in liquid culture although some efficacy was found against dry spores. Heagle (1973) reported the effects of O_3 on germination, fungal growth and sporulation in media but surprisingly suggested that O_3 can inhibited mycelial growth, but rarely cause death of the colony.

A study by Antony-Babu and Singleton (2009) reported the effects of O_3 (200, 300 $\mu\text{mol mol}^{-1}$ and 0.2 $\mu\text{mol mol}^{-1}$ for 10 min and 12 days respectively) at 18°C on *Aspergillus nidulans* and *Aspergillus ochraceus*. At higher O_3 concentration, spore germination was reduced by 50%, while a 15% decrease was found for both the species. The biomass production for *A. nidulans* was stimulated, while that of *A. ochraceus* was reduced. More recent studies by Antony-Babu and Singleton (2011) showed that efficacy against fungal spores was affected by substrate, with the sugar content interacting with O_3 dose when exposing *Eurotium* conidia. However, $a_w \times O_3$ effects were not considered although *Eurotium* species are xerophiles. Minas et al. (2010) reported complete elimination of *Botrytis cinerea* on kiwi fruit when treated continuously with gaseous O_3 (0.3 $\mu\text{l L}^{-1}$) for 8 hr. at 0°C, RH 95%. Some studies done with *A. flavus* showed good efficacy against spore germination, but little effect of O_3 on mycelial extension at any a_w and temperature (Sultan, 2012).

Zotti et al. (2010) found that exposure for 3, 6, and 9 days to O_3 was more effective on fresher cultures of *Aspergillus* and less effective in older cultures. Recent studies showed inhibition of hypha growth of *F. graminearum* and *P. citrinum* after treatment with 60 $\mu\text{mol mol}^{-1}$ O_3 for 120 min at flow rate 1 L min^{-1} (Savi and Scussel, 2014).

Recent studies by El-Desouky et al. (2012) reported inhibition of *A. flavus* growth by 40 ppm O_3 gas for 20 min on PDA media. Sultan (2012) suggested that O_3 had little effect on mycelial extension of *A. flavus* on YES media, regardless of a_w and temperature conditions or O_3 concentration used.

b) O₃ action as an antimicrobial *in situ*

Gaseous O₃ treatment to control fungal populations *in situ* in stored grains has been used, but none has examined the efficacy on coffee beans. Zhao and Cranston (1995) reported that microbial growth can be inactivated by using O₃ on black pepper; the efficiency of O₃ to decrease fungal spore germination was strongly affected by the moisture content of the ground black pepper. According to Kells et al. (2001) a 63% reduction of *A. parasiticus* populations in maize kernels occurred when exposed to 50 mg L⁻¹ of gaseous O₃ for 3 days. Wu et al. (2006) exposed wheat to O₃ and reported inactivation of 96.9 and 100 % of fungal spores with 0.33 mg g⁻¹ for 5 and 15 min, respectively, at 0.90 a_w and 20°C. Allen et al. (2003) showed that inactivation of 96% of fungal spores occurred when treating barley with 0.1 mg min⁻¹ O₃ for 5 min at 0.98 a_w and 20°C. They suggest that O₃ is more effective in higher moisture contents grain where it can react with water and form free radicals. White et al. (2013) reported that O₃ concentrations of 0, 50, 500, 1000 and 15.000 ppm could penetrate high moisture maize (18, 22 and 26% MC) and reduce the presence of *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium*, *Mucor*, *Penicillium* and *Rhizopus* when exposed for 1 hr at a flow rate of 0.5 L min⁻¹. Other studies with *Eurotium* species isolated from naan bread showed that exposure to 300 µmol mol⁻¹ for 5 to 120 min caused complete inhibition of growth (Antony-Babu and Singleton, 2011).

Some studies on the mechanism of action of O₃ as an antimicrobial agent have been done. Hoigne & Bader (1983) showed that the presence of water in organic substances can increase O₃ reactions because the radical form is a powerful oxidant, as O₃ decomposes more readily in water than in the atmosphere. Others suggested that the mechanism of action of O₃ is through lysing of cellular walls by oxidizing organic materials in the microbial membrane, which leads to increasing permeability of the cell walls (Pryor and Rice, 1998). Table 1.4 lists the mycotoxigenic fungi, mycotoxin of concern for human health in some of food products and the efficacy of O₃ from the literature.

Table 1.4: Effect of O₃ on fungi in different food source.

Fungi	Treatment condition	Food type	O ₃ effect	Reference
Total mycoflora	Gaseous O ₃	Strawberry	Reduction of fungal counts	Perez et al. (1999)
<i>Aspergillus parasiticus</i>	0.1 g m ⁻³ of gaseous O ₃ (occasional fumigation)	Maize	Reduction of 63% fungal counts after 3 days	Kells et al. (2001)
<i>Penicillium</i> spp.	Gaseous O ₃ at different levels	Cheese	Reduction of fungal counts in the ripening room and on cheese surface	Serra et al. (2003); Lanita and Silva, (2008)
Total mycoflora	Gaseous O ₃	Barley	Reduction mycelia grow and spores germinate	Allen et al. (2003)
Total mycoflora	0.01–0.02 g m ⁻³ of gaseous O ₃ for 3 and 4 hours	Dried Figs	Reduction of fungal counts	Oztekin, et al. (2006)
Micromycetes (<i>Fusarium</i> , <i>Geotrichum</i> , <i>Myrothecium</i> , <i>Mucor</i> , <i>Alternaria</i> , <i>Verticillium</i> , <i>Penicillium</i> , and <i>Aspergillus</i>)	1.4 g m ⁻³ of gaseous O ₃	Wheat	Reduction of fungal counts	Raila et al.(2006)
<i>A. flavus</i> and <i>A. parasiticus</i>	21 mg L ⁻¹ after 96 hr	Peanut	Reduction in colony-forming and control	De Alencar et al. (2012)
<i>P. nordicum</i>	1.5 mg L ⁻¹ of O ₃	sausage	Prevented growth and production of OTA	Comi et al. (2013)
<i>Fusarium avenaceum</i> , <i>F. graminearum</i> , <i>F. poae</i> , <i>F. solani</i> , <i>F. tricinctum</i> <i>F. sporotrichioides</i>	O ₃ -air mixture. (1250 ppb) for 1 hr	grain	50% decreased of fungi	Steponavičius et al. (2012)
<i>Fusarium verticillioides</i>	100-200 ppm for 60 min, flow rate 6 L min ⁻¹ ,	Maize	Conidial germination inhibition	Mylona et al. (2014)

c) Efficacy of O₃ for mycotoxin control

Some investigation have been done on the action of high concentrations O₃ for degradation and detoxification of common mycotoxins (McKenzie et al., 1997). Aflatoxin in cottonseed meal and peanut was reduced when treated with 29 ppm O₃ (Dwarakanath et al., 1968; Dollear, 1968). Prudente Jr. (2002) reported 10 to 12 % (by weight) of O₃ degraded aflatoxins

in maize. Wang et al. (2010) showed that the wet method of O₃ fumigation of grains is a more effective method to degrade OTA than a dry method. However, moistening grain prior to treatment would require subsequent drying which could cause additional mycotoxin problems to arise where this step is not managed properly. *A. ochraceus* growth and OTA production was inhibited by ~ 1ppm gaseous O₃ (Iacumin et al., 2012). Jie et al. (2011) reported degradation of OTA after exposure to O₃ 30 g m⁻³ for 120 min or at 60 g m⁻³ for 90 min. According to Allen et al. (2003) gaseous O₃ at a concentration of 0.16 mg of O₃ g⁻¹ (barley) 5 min, was effective to inactivate 36% mixtures of fungal spores. Mason et al. (1997) reported that growth, sporulation and mycotoxin production of *A. flavus* and *Fusarium verticillioides* were inhibited by exposure to 5 mg L⁻¹ O₃. Some studies reported that moisture enhanced the reaction between ozone and mycotoxin. For example, Young et al. (2006) showed that trichothecenes contamination was reduced by 90% when moist maize was exposed to 1000 ppm O₃ with a 70% reduction in dry maize. Recent studies investigated the effect of O₃ on fumonisins (FUMs) produced by *Fusarium verticillioides* on maize *in situ* and *in vitro*. Mylona et al. (2014) reported inhibition of conidial germination when treated with 100 and 200 ppm O₃ for 30 and 60 min at a flow rate of 6 L min⁻¹ for 24 hr. However, the germination of spores recovered after 8-10 days storage. On the other hand, FUMs productions were reduced after 10 days with little effect on mycelium extension. *In situ*, there was a reduction in the total fungal populations directly after treatment with O₃ for 60 min, and little FUM B 1 in maize compared to control after 15 and 30 days. Table 1.5 summarises the main studies available on O₃ efficacy on mycotoxigenic fungi and mycotoxins in food.

Table 1.5: Effect of ozone treatment on mycotoxin.

Food grain or product	Effect on	Treatment conditions	Degradation	Reference
Peanut meals	Aflatoxins	25 mg min ⁻¹ O ₃	AFB ₁ and G1 (100% destruction) AFB ₂ (78%)	Dwarakanath et al. (1968)
Corn	Aflatoxins	O ₃ (10-12 wt. %)	Reduced aflatoxin levels by 92%	Prudente Jr, (2002)
Corn	Aflatoxin	92 hr with O ₃ at 200 mg min ⁻¹	>95% reduction in AFB ₁	McKenzie et al. (1998)
Barley	Fungal spores and mycelia	0.16 -0.1 mg of O ₃	96% fungal spore inactivation	Allen et al. (2003)
Maize	<i>A. parasiticus</i>	50 ppm for 3 days	63% reduction	Kells et al. (2001)
Wheat	Fungal spores and mycelia	0.33 mg of O ₃	96.9% fungal spore inactivation	Wu et al. (2006)
Barley	<i>Fusarium</i>	Gaseous O ₃ treatment 11 and 26 mg g ⁻¹ for 15 min	24–36% decrease in <i>Fusarium</i> survival	Kottapalli et al. (2005)
Peanut	Aflatoxin	Gaseous O ₃ (4.2 wt. %) Temperature 25, 50, 75°C	AFB ₁ (77%): 10 min at 75°C AFB ₁ (80%): 5–10 min at 75°C	Proctor et al. (2004)
Dried figs	Total mycoflora	13.8 g m ⁻³ of gaseous O ₃ and 1.7 g m ⁻³ aqueous O ₃	Inactivation of fungi after 15 min	Zorlugenç et al., (2008)
Date fruits	Total mycoflora	0.002, 0.006, and 0.01 g m ⁻³ of O ₃ for 1 hr	Reduction of fungal counts	Najafi and Khodaparast, (2009)
Date fruit	Mycotoxins and <i>Fusarium oxysporum</i> , <i>P. citrinum</i> and <i>A. flavus</i>	8 ppm O ₃ gas for 4 hr	Reduction of fungal count and mycotoxin	Al Ahmadi et al. (2009)
Barley	<i>Fusarium</i> and Deoxynivalenol (DON)	26 mg of O ₃ for 120 min after 2 and 6 hr of steeping	Reduced infection and no effect on DON	Dodd et al. (2011)
Wheat grains	Aflatoxin and citrinin (CTR)	60 µmol mol ⁻¹ of O ₃ for 180 min	AFB ₁ and AFB ₂ levels reduced	Savi et al. (2014)
Maize	<i>F. verticillioides</i>	100-200 ppm for 60 min	Fumonisin	Mylona et a. (2014)

1.6 AIMS AND OBJECTIVES

The overall hypotheses was to test whether different imported coffees represented a risk of OTA intake by Kuwaitis, to determine whether climate change factors may enhance exposure to mycotoxins such as OTA and the potential for controlling such contamination using gaseous O₃.

Phase I: What is the biodiversity of mycotoxigenic fungi in green and roasted coffee from the Kuwaiti market and do they represent a risk?

- i. Isolating mycotoxigenic fungi from coffee (raw green and roasted) from Kuwait coffee.
- ii. Qualitative and quantitative determination of ochratoxigenic fungi and their contribution to the total populations.
- iii. Determination of potential for production of ochratoxin A.

➤ Can *in vitro* studies of impact of water activity, temperature, coffee concentration and caffeine on lag phases, growth and production of ochratoxin A (OTA) by strains of *Aspergillus* section *Circumdati* and Section *Nigri* isolated from coffee be used to predict risks in stored coffee?

- i. Determination of the effect of various concentrations (%), 10, 20, 30, 40, 50, 70 and 80% of coffee extract on the lag phase (λ , days), growth of strains and/or ochratoxin A production in CMEA medium.
- ii. The effect of water activity and temperature on lag phases (λ , days), growth and OTA production
- iii. Evaluation of different caffeine concentration (0.5-4%) on the lag phase (λ , days), growth of strains and/or ochratoxin A production in a conducive YES medium.

Phase II: Will climate change interacting factors (a_w x temperature x CO₂) increase or decrease the capacity for colonisation and OTA production by key toxigenic species (*A. westerdijkiae*, *A. steynii*, *A. ochraceus* and *A. carbonarius*) and increase the relative risk to consumers?

- i. To examine *in vitro* and *in situ* effects of CO₂ (400 ppm vs 1000 ppm) on germination, growth and OTA production by these species. Studies were carried out on different a_w levels and temperature to simulate climate change type fluctuations of drought stress and elevated temperatures.

Phase III: Is there a similarity between new species of *Aspergillus* section *Nigri* isolated from Thai coffee beans under different interacting environmental conditions with other toxigenic species

- i. The effect of water activity and temperature on the lag phases (λ , days), growth and OTA production

Phase IV: Can gaseous O₃ treatment be used to control toxigenic populations on coffee beans and OTA after treatment and after storage?

- i. *In situ* efficacy of O₃ on fungal populations and OTA production isolated from naturally contaminated and artificially inoculated coffee beans
- ii. Effect of O₃ on the fungal populations and OTA production by *A. westerdijkiae*, *A. carbonarius* and *A. ochraceus* after inoculation on irradiated coffee beans
- iii. Effect of O₃ on the *in situ* control of growth and ochratoxin A production produced by *A. carbonarius*, *A. westerdijkiae* and *A. ochraceus* in irradiated coffee beans

Figure 1.4: summarises the different phases of the work carried out in this research project. The research work is presented as distinct Chapters with individual short Introductions and Materials and Methods Sections, Results and Discussion sections. This is followed by the overall Conclusions and possible areas for future work.

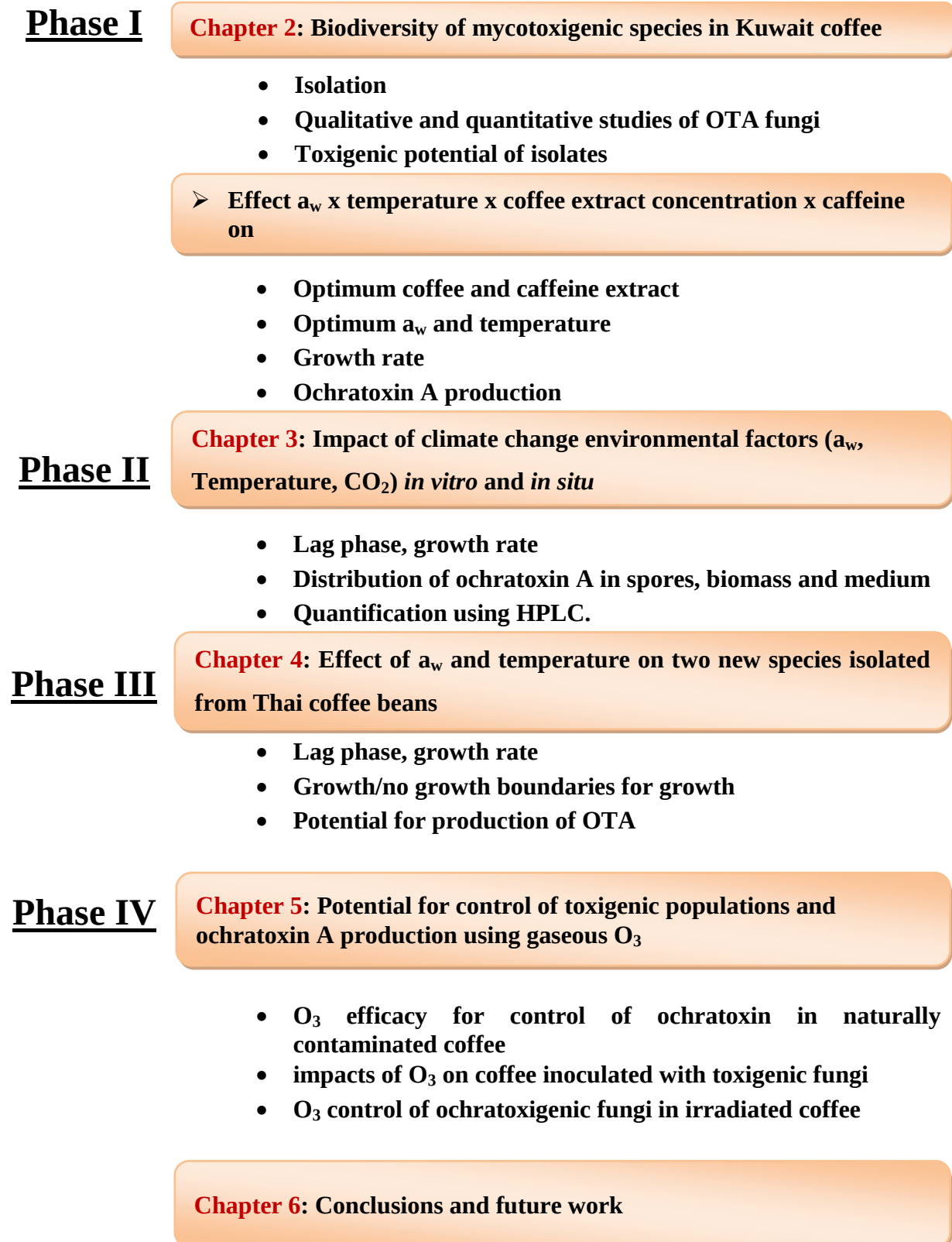


Figure 1.4: Flow diagram of the experimental work carried out in this thesis.

CHAPTER 2

BIODIVERSITY OF MYCOTOXINS IN KUWAITI COFFEE AND EFFECT OF a_w , TEMPERATURE, COFFEE CONCENTRATION AND CAFFEINE ON LAG PHASE, GROWTH AND OTA PRODUCTION

2.1 Biodiversity of mycotoxigenic fungi from Kuwaiti coffee, green and roasted.

2.1.1 Introduction

Mycotoxins are secondary metabolites of filamentous fungi, which are toxic, and predominantly produced by the genera of *Aspergillus*, *Penicillium* and *Fusarium*. Their occurrence in food and feed products can have a negative impact on human and animal health. The FAO (2001) has estimated that up to quarter of the world's basic food and feed crops are contaminated with mycotoxigenic fungi, causing significant economic losses.

Ochratoxin A (OTA) is an important mycotoxin which is produced by species of *Aspergillus* and *Penicillium*. OTA is a potent nephrotoxic and nephrocarcinogenic toxin, which affects humans and animals. For example, EFSA (2006) and Pfohl-Leszkowicz et al. (2002) found OTA had teratogenic and immunosuppressive properties in animals. In addition, it has been suggested that it is responsible for Danubian endemic familial nephropathy (DEFN) in humans (EFSA, 2006). Moreover, it has been classified by the International Agency for Research on Cancer (IARC, 1993) as a possible human carcinogen (Class 2B) (Walker, 2002; Benford et al., 2001).

The major fungal species known to produce OTA are *P. verrucosum*, *P. nordicum*, *A. carbonarius*, *A. niger* and *A. ochraceus* (Cabanes et al., 2010). Recently, two new species, *A. westerdijkiae* and *A. steynii*, that can produce OTA, have been taxonomically separated from *A. ochraceus*, which only produces moderate OTA levels (Frisvad et al., 2004). Thus, the main species causing OTA contamination of many products including coffee are considered to be from the *Aspergillus* section *Circumdati*, especially, *A. westerdijkiae* (Abdel-Hadi and Magan, 2011). These mycotoxigenic species were found to contaminate mainly raw food products during post-harvest storage and transport (Magan and Aldred, 2007).

Several abiotic factors including temperature, a_w , pH and nutritional content of the commodity affect fungal growth and OTA production (Esteban et al., 2004; Magan and Aldred, 2005; Astoreca et al., 2007; Schmidt-Heydt et al., 2010, 2011). Frisvad et al. (2006) reported that *P. verrucosum* was the main mycotoxigenic species found in wheat and barley. However, coffee is grown in warmer climatic regions where *Aspergillus* species are predominant. Coffee berries have been reported to be contaminated with *Aspergillus* species. *A. ochraceus* was isolated mainly in warm humid conditions in countries producing coffee (Suárez-Quiroz et al., 2004b). Morello et al. (2007) found *A. westerdijkiae* in Brazilian coffee. In addition, *A. steynii* and *A. carbonarius* have been found in dried Thai coffee beans (Noonim et al., 2008).

The objectives of this Chapter were: (a) to examine the diversity of mycotoxigenic fungi found in green and roasted coffee bought in the Kuwaiti market from different source countries and the relative proportions of the total fungal populations present (Section 2.1); (b) to examine the effect of a_w x temperature effects on the OTA producing fungi in relation to lag phased prior to growth, growth and OTA production on coffee-based media (Section 2.2); (c) to examine the effect of different coffee concentrations on five OTA producing strains (Section 2.2); and (d) to assess the effect of different caffeine concentrations on the growth and OTA production by the five OTA producing strains isolated from imported Kuwaiti coffee (Section 2. 3).

2.1.2 Materials and methods

1. Collection of coffee bean samples

A total of 20 different coffee samples (Al Ameen coffee; fresh green coffee beans; full roasted coffee, both beans and ground; half-roasted coffee, beans and ground; light roasted coffee, beans and ground; ground French coffee; and ground roasted Greek coffee) were all analysed for fungal populations and OTA contamination. Additional samples were purchased from 6 Kuwaiti supermarkets in Kuwait. All samples were stored at 4°C prior to analysis.

a) Determination of water activity and moisture content of coffee beans

The water activity (a_w) of the coffee bean samples was measured using an Aqualab 3TE (Decagon Devices, Inc., Pullman, Washington, USA). The moisture content was determined by weighting 10 g of coffee beans and oven-drying at 110°C for 25 hr. The samples were cooled in desiccators and re-weighed. The percentage moisture content (MC) was calculated as $MC\% = ((W_w - W_d)/W_w) * 100$.

2. Isolation media

The following media were used in these studies.

Malt extract agar (MEA): (Oxoid, Basingstoke, Hampshire, UK) is a broad spectrum medium for isolating, culturing and enumerating fungal species. A small amount of anti-bacterial agent, chloramphenicol (250 $\mu\text{g ml}^{-1}$) was added to inhibit bacterial growth. Once the components were mixed, the medium was autoclaved at 121°C and 1 atm for 15 min and poured into 9 cm sterile Petri plates, cooled and stored at 4°C in polyethylene bags for a maximum period of 21 days until used.

DG18 (Dichloran-18-Glycerol Agar) (Hocking and Pitt, 1980; Oxoid, Basingstoke, Hampshire, UK): This was used for isolation and enumeration of xerotolerant and xerophilic fungi at $<0.90 a_w$. Additional selectivity against bacterial growth was achieved by the incorporation of the heat-stable antibiotic chloramphenicol (250 $\mu\text{g ml}^{-1}$). The sterilised agar was allowed to cool to approximately 50°C before being

aseptically poured into 9 cm Petri dishes and kept at 4°C in sealed polyethylene bags for a maximum period of 21 days.

Czapek Dox Agar: this medium was used for morphological identification of the mycotoxigenic strains. It is a semi-synthetic medium used for the cultivation of fungi, containing sodium nitrate as the sole source of nitrogen and sucrose as the source of carbon. The autoclaved agar was poured into 9 cm Petri dishes and kept at 4°C in sealed polyethylene bags for a maximum period of 21 days.

Yeast extract sucrose agar (YES): this is a conducive medium for the production of extracellular secondary metabolites for analyses by HPLC. It contains 20 g yeast extract (Oxoid, Basingstoke, Hampshire, UK) and agar, Sucrose 150 g and 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 885 ml distilled water. Once the components were homogenized, the medium was autoclaved, poured into 9 cm Petri plates and kept at 4°C for a maximum period of 21 days.

Coconut cream agar medium (CCA): this medium was prepared with 50% coconut homogenate in distilled water and solidified by the addition of 2% agar. The medium was autoclaved at 121°C for 10 min, mixed well and poured into 9 cm Petri plates and kept at 4°C until used (Dyer and Mc Cammon, 1994).

3. Mycotoxigenic fungal isolation from coffee

Direct plating: Coffee beans (fresh green coffee beans, full roasted coffee (beans and ground), half roasted coffee (beans and ground), light roasted coffee (beans and ground), ground French coffee, and ground Greek coffee, were directly plated (5X2) on MEA agar media and incubated for 7 days at 25°C. The frequency of fungi was counted and recovered and maintained on MEA at 4°C for later checking for toxin production potential.

Serial dilution: The fungal populations on the coffee samples were enumerated on solid MEA and DG18 media, using the surface spread method. Whole beans and seeds were homogenised by blending 10 g of each sample in 90 ml of sterile water plus tween

80 solutions in a stomacher (Lab-Blender 400, Seward Medical, London, UK) for 2 min. For ground coffee beans and whole coffee beans, 1 g was added to 9 ml sterile water. Serial dilutions were done (10^{-1} to 10^{-4}) and 200 μ l from each dilution spread plated onto MEA and DG18 agar plates in duplicate. The Petri dishes were incubated at 25°C for 7 days before fungal enumeration.

4. Extraction, detection and quantification of OTA in coffee

Two methods were used to determine and confirm the presence of OTA in coffee: (a) using coconut cream agar (CCA) medium and (b) HPLC analyses for quantification of OTA production.

Screening for OTA production using CCA media: A preliminary screening for OTA production by the strains was performed by growing them on the CCA medium and the fluorescence under long-wave UV light (365 nm) examined. All strains were centrally inoculated onto CCA plates using a spore suspension and incubated at 25°C for 7 days. Those colonies that showed a blue fluorescence on the reverse side of the colony under UV light were considered positive for OTA.

Quantitative method: Confirmation of the CCA media results was performed by HPLC analyses as detailed below. Five plugs from strains isolated from coffee were extracted from each colony after 10 days incubation at 25°C on YES media, transferred to 2 ml Eppendorf tubes and weighed. 1000 μ l methanol was added to each Eppendorf tube. The samples were then shaken using a KS 501 digital orbital shaker for 30 min and centrifuged for 10 min at 15000 x g. The supernatant was filtered and analysed with the HPLC system (Agilent, Berks., UK). 20 μ l of the extracted toxin from the YES medium were injected into the HPLC system. 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 100 mm, diameter 4.6 mm, particle size 2.7 micron; 600 Bar)
Temperature of column	25°C

Excitation	330 nm
Emission	460 nm
Flux	1 ml min ⁻¹
Volume of sample injected	20 µl
Retention time	Approximately 2.49 min
Run time	17 min
Limit of detection	0.01 ng g ⁻¹
Limit of Quantification	0.039 ng g ⁻¹

b) Effect of water activity (a_w), temperature (°C), media and their interactions on growth rate and OTA production

1. Fungal strains used in these studies

Thirteen strains were isolated from six imported coffee bean samples from different Kuwaiti supermarkets. The strains, including *Penicillium*, *Aspergillus* section *Circumdati* (strains 2A3, 22A, 25, 1C2, 26A2, A1, 23A3, 23A4, 26A4, 22, 25A) and an unknown yellow colony, were examined in these experiments. Other strains included in these studies were: strains of *Aspergillus* section *Circumdati* (B 2/2, B 2, B 1/1, C 1/1, A1, C 2/2) isolated from green coffee beans from Saudi Arabia, as well as some type strains such as *A. steynii* (3.53, CBC 112814) and *A. westerdijkiae* (CCT 6795, CBS 121986), kindly provided by Dr. B. Patino (Complutense University, Madrid, Spain). Other strains such as *A. niger* (A 1911) and *A. carbonarius* (A 877) were kindly supplied by Prof. P. Battilani (Università Cattolica del Sacro Cuore, Italy).

2. Media

Two media were used to determine the effect of nutrients on the growth and OTA production by ochratoxigenic fungi:

CMEA (Green coffee meal extract agar): The medium was made by boiling 30 g of green coffee beans in 1 L of distilled water for 30 min. A double layer of muslin was used for filtering the resulting mixture, the volume made up to 1 L Technical agar No. 3 (2%), and glycerol was added to adjust the media to 0.99, 0.98, 0.95, and 0.90 a_w . The

media were autoclaved at 121°C for 10 min and poured into 9 cm Petri plates, cooled and kept at 4 °C until used (Pardo et al., 2005). The final a_w levels were checked with a water activity meter, the Aqualab 3TE (Decagon Devices, Inc., Pullman, Washington, USA). They were found to be accurate to 0.003 a_w .

RMEA (High roasted coffee meal extracted agar): The same method as described above was used, but using high roasted coffee beans.

3. Calculations of lag phases prior to growth and growth rates

The diameter of the colonies was measured in two directions at right angles to each other every day, until the colonies reached the Petri plate (9.cm) rim. The change in the colony radius (mm) vs time (days) for each strain under the different a_w , temperature and substrate conditions was plotted in Microsoft Excel®. After data plotting, a linear model was used to calculate the relative growth rates (mm day^{-1}) and the lag phases. The growth rates (mm day^{-1}) were obtained as the slope of the line. By using the same equation, lag time (in days) were calculated by the regression line formula. The squares of the linear correlation coefficients was ≥ 0.98 .

Ochratoxin A:

As described in section 2.1.2 (4)

c) Experimental design and statistical analysis

A full factorial design was applied, with three (4x3x2) factors: water activity, temperature and media at three levels. Each treatment combination was carried out in triplicate, both for growth rate assessment and OTA production. Normality was checked using the Kolmogorov-Simonov test. The effects of water activity, temperature, media and their interactions were examined by the Kruskal-Wallis (nonparametric analyses), used when the data are not normally distributed. For normally distributed data, the data sets were analysed using Minitab® 16 package (Minitab Inc., 2010. State College, PA, USA). The statistical significant level was set at $P < 0.05$ for all single and interacting factors (Table 2.2).

Table 2.1: Summary of the single and interacting factors used in the experimental design for this experiment. The key to media: CMEA, green coffee meal extract agar medium; RMEA, roasted coffee meal extract agar medium.

Factor	Level			
Water activity	0.90	0.95	0.98	0.99
Temperature (°C)	25°C	30°C	35°C	
Media	CMEA	RMEA		

2.1.3 Results

a) Biodiversity of mycotoxigenic fungal species in imported coffee beans

During this study, the first set of samples of imported coffee beans from one source in Kuwait did not yield any fungal populations. Subsequent samples did contain fungal surface contamination (Plate 2.1). Subsequently, six samples from different sources in Kuwait showed contamination with up to 13 fungal genera/species. Figure 2.1 shows the frequency of isolation of the mycotoxigenic fungal species from imported green coffee samples from Kuwait based on direct plating results.

A. carbonarius, *Penicillium* and *Fusarium* spp. were observed in coffee from India, but none from Ethiopia or Yemen. *A. niger* and *A. westerdijkiae* were found in all the coffee samples. *A. carbonarius* represented 7.3, 12.8 and 30%, while *Penicillium* spp. 21.3 and 4.3% and *Fusarium* 2.4 and 4.3% of the total isolations in the samples from India. The species that was dominant in all coffee samples were representatives of the *A. niger* group, some of which have the ability to produce OTA (46, 91, 21, 15, 87 and 90% respectively). The second mycotoxigenic species found in coffee was *A. westerdijkiae* (5, 21.3, 10, 9 and 5%). The frequency of isolation of most of the species was higher in coffee beans imported from India, when compared with the other countries of origin.

Table 2.2 shows the total fungal populations isolated from the coffee samples from the samples as $\log_{10}$ CFUs g^{-1} dry weight of *Aspergillus* and *Penicillium* species. The fungal populations in samples of coffee from the Yemen (sample 1) and Ethiopia (1) were significantly higher when compared to Yemen (sample 2). Species from the *Aspergillus* section *Nigri* were the highest populations contaminating coffee imported from the Yemen (sample 1; $4.45 \log_{10}$ CFUs g^{-1} dry weight).



Plate 2.1: Example of the fungal species present on directly plated green coffee beans on MEA and incubated at 25°C for 7 days.

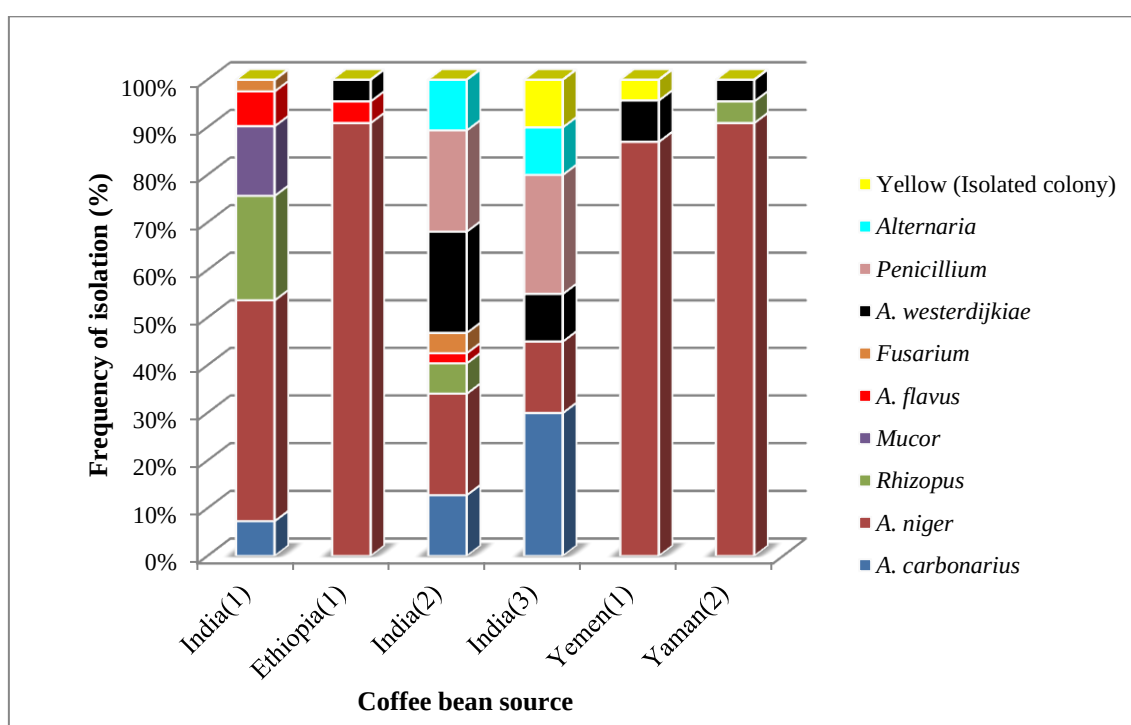


Figure 2.1: Frequency of isolation of the dominant fungal species from imported coffee beans from different countries based on direct plating on MEA at 25°C after 7 days incubation. Mean of 10 coffee beans.

Table 2.2: Dominant fungal populations (Log_{10} CFUs g^{-1} dry weight $\pm$ standard error) isolated from green coffee beans from different sources based on serial dilution on DG18 at 25°C for 7 days.

Sample No.	<i>Aspergillus</i> section <i>Nigri</i>	<i>Penicillium</i>	Total fungal population
Yemen (sample 1)	4.45 $\pm$ 0.7	3.9 $\pm$ 0.8	4.55 $\pm$ 0.9
Yemen (sample 2)	2.4 $\pm$ 0.003	ND	2.4 $\pm$ 0.001
Ethiopia (sample 1)	2.7 $\pm$ 0.05	3.4 $\pm$ 0.60	4.45 $\pm$ 0.5

ND: non detectable, below the detectable level

b) Water activity and moisture content of the coffee beans.

The moisture content and a_w of the samples were measured (Figure 2.2). This showed that the a_w and the MC of the coffee samples was low but relatively similar. In all cases this represented a_w levels <0.45 at which no fungal growth would occur. However, at these MC levels, flavour would most probably be affected.

Table 2.3: Water activity and water content of imported coffee bean samples from different sources collected in Kuwait.

Coffee bean Sample	Water activity (a_w)	MC % (wet wt. basis)
India(1)	0.46	8.55
Ethiopia(1)	0.46	7.77
India(2)	0.426	8.28
India(3)	0.46	8.52
Yemen(1)	0.45	7.47
Yemen(2)	0.42	7.49

c) Screening for OTA production using coconut cream agar medium (CCA) and HPLC.

Twenty three strains of *Aspergillus* section *Flavi*, *Aspergillus* section *Circumdati*, *Aspergillus* section *Nigri* and *Penicillium* species were evaluated for the ability to produce OTA. This

was assayed using the fluorescence on CCA and by quantification using HPLC (Table 2.3) Blue fluorescence was observed under UV in *Aspergillus* section *Circumdati* strains isolated from green coffee beans imported from Saudi Arabia (B 2/2, B 2, B 1/1, C 1/1, A 1, C 2/2) and *A. westerdijkiae* strains (23A3; 2A3; A1; and in the unidentified Yellow colony. However, no blue fluorescent was detected in *Aspergillus* section *Flavi*, *Aspergillus* section *Nigri* or the *Penicillium* strains examined. The strains with negative results in CCA media were excluded for quantification of OTA using HPLC.

Twenty-seven strains and four type strains tested (*A. niger* A 1911, *A. carbonarius* A 877, *A. steynii* CBS 112814 and *A. westerdijkiae* CBS 212986), produced OTA. The *Penicillium* strains and *A. carbonarius* (A877) gave negative results in terms of OTA production in YES medium. The OTA produced by positive strains was in the range 232.4-82250.1 ng g⁻¹ medium; one strain was a very high producer (82250.1 ng g⁻¹), eight strains were high producers (62322.2-5595.6 ng g⁻¹) and two strains were low OTA producers (232.4-570.5 ng g⁻¹). The results obtained using CCA were confirmed using HPLC analyses.

Table 2.4: Ability of *Aspergillus* section *Circumdati*, *Aspergillus* section *Nigri*, *Penicillium* species and other isolated strains to produce ochratoxin A (OTA) on a conducive YES medium and based on HPLC analyses (ng g⁻¹ medium ± standard error).

Isolated strains	No. Strains	Medium(CCM)	Mean OTA (ng g ⁻¹ medium)
<i>Aspergillus</i> section <i>Circumdati</i>	14 ^b	+++	12342.4± 3416.4
<i>A. westerdijkiae</i> (B2/2)	1	+++	82250.06± 2509.8
<i>A. westerdijkiae</i> (B 2)	1	+++	59681.9± 9493.6
<i>A. westerdijkiae</i> (B 1/1)	1	+++	62322.2± 315.4
<i>A. westerdijkiae</i> (C 1/1)	1	+++	32712.1± 1171.8
<i>A. westerdijkiae</i> (A 1)	1	+++	5595.6± 3486.7
<i>A. westerdijkiae</i> (C 2/2)	1	+++	20422.6± 19175.7
Unknown yellow	3	+	232.4± 90.5
<i>A. niger</i> (A 1911)	1	+++	59681.9± 667.7
<i>A. carbonarius</i> (A 877)	1	-	ND
<i>A. steynii</i> (CBS 112814)	1	++	570.5± 138.1
<i>A. westerdijkiae</i> (CBS 121986)	1	+++	7927.1± 329.4

^a *Aspergillus flavus* strains

^b *Aspergillus westerdijkiae* strains(ex: 2A3)

^c *Aspergillus niger* strains

ND: non detectable, below the detectable level

d) Effect of water activity (a_w), temperature ($^{\circ}\text{C}$), media and their interactions on growth and OTA production by the strains isolated from coffee.

Figures 2.3-2.8 show the relative growth rate of *A. westerdijkiae* (2 A3, C 1/1, B 2, CBS 121986), *A. steynii* (CBS 112814) and the yellow (unidentified colony) strains at different a_w , temperature and media conditions over periods of 12 days. The highest growth rate was observed at 25°C when compared with that at 30 and 35°C , at 0.95 and 0.98 a_w on CMEA. The exception was the yellow (unidentified colony) at 0.95 a_w and 30°C .

Furthermore, OTA quantification shows that optimum, on the green coffee extract medium, was at 0.95 a_w at 35°C for *A. westerdijkiae* (2A3) (Table 2.4). While, for the other strains of *A. westerdijkiae* (C 1/1, CBS 121986) optimum a_w for OTA production was 0.98 and 30°C . The highest production was observed at 0.98 a_w and 35°C ($5206.97 \text{ ng g}^{-1}$) for *A. westerdijkiae* (strain B 2). The lowest production was observed by the yellow unidentified colony at 0.98 a_w at 25°C . The best a_w and temperature for OTA production by *A. steynii* (CBS 112814) was at 0.99 a_w and 30°C .

Table 2.5 summarises the statistical analyses for mycelial growth and OTA production for six strains at different a_w , media and temperature conditions. The growth rate was significantly ($P < 0.05$) affected by a_w (0.90, 0.95, 0.98 and 0.99) and temperature (25 , 30 and 35°C) for all strains except the unidentified yellow colony, which was unaffected by temperature ($P > 0.05$). There was a significant effect of the two different coffee media for some strains, except those of *A. westerdijkiae* (2A3, B 2, CBS 121986).

For OTA production, a_w was a significant factor for all strains except the yellow unidentified one ($P < 0.05$) and *A. steynii* (CBS 112814). There were similar amounts of OTA produced by *A. westerdijkiae* (C1/1) in the two media, although for the other *A. westerdijkiae* strains (2A3, B 2, CBS 121 986) there was a significant effect of medium type. Overall, the strains grew best at 25°C in CMEA and RMEA, with significant OTA production at 30°C (See Appendix I).

The individual factors assayed (water activity, temperature, media) as well as the interaction of $a_w \times \text{temperature} \times \text{media}$ (CMEA; RMEA) showed a significant influence on growth and OTA production. Most of the strains produced OTA in both media at 0.95 and 0.98 a_w but at different temperatures.

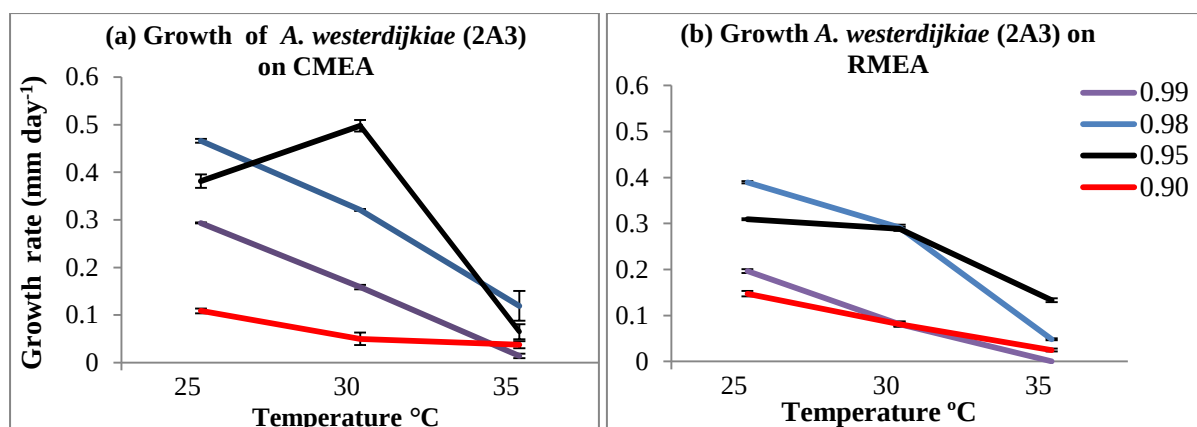


Figure 2.2: Effect of different temperatures (25, 30, 35°C) and media type (a) CMEA and (b) RMEA on growth of *A. westerdijkiae* (2A3) at different a_w levels (0.99, 0.98, 0.95, 0.90) after 12 days. Bars indicate standard error of the mean. Key: CMEA, green coffee meal extract agar; RMEA, high roasted coffee meal extract agar.

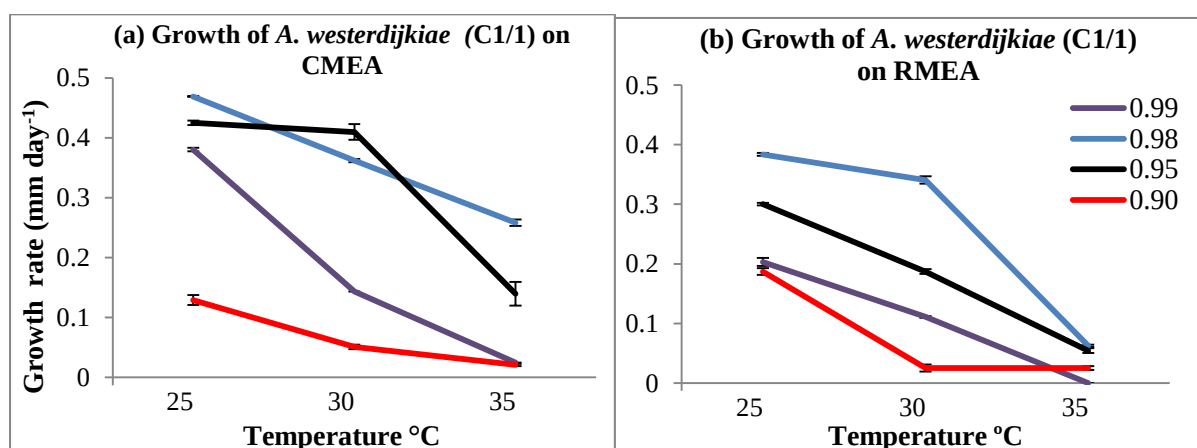


Figure 2.3: Effect of different temperatures (25, 30, 35°C) and media type, (a) CMEA and (b) RMEA, on growth of *A. westerdijkiae* (C1/1) at different a_w levels (0.99, 0.98, 0.95, 0.90) after 12 days. Bars indicate standard error of the mean. Key: CMEA, green coffee meal extract agar; RMEA, high roasted coffee meal extract agar.

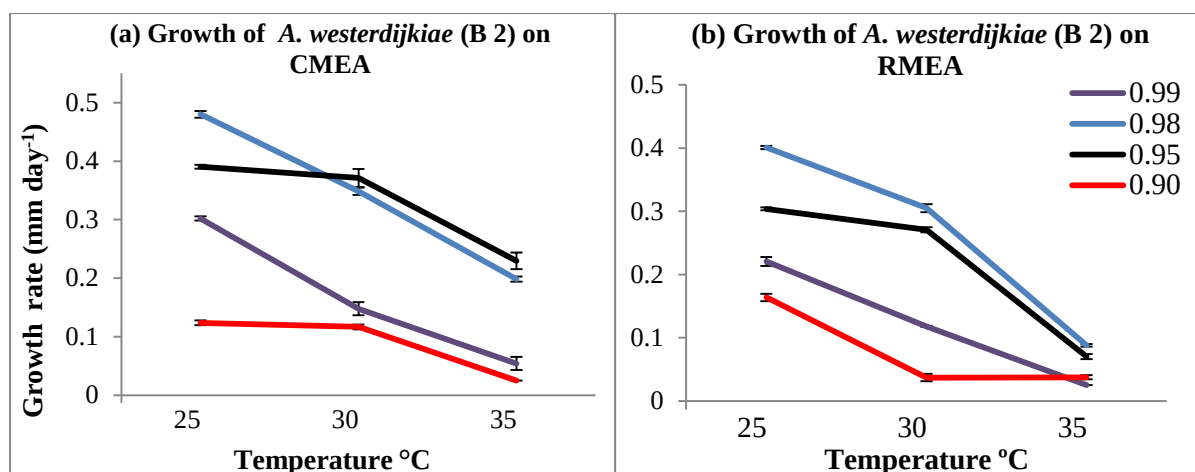


Figure 2.4: Effect of different temperatures (25, 30, 35°C) and media type (a) CMEA and (b) RMEA on growth of *A. westerdijkiae* (B 2) at different a_w levels (0.99, 0.98, 0.95, 0.90) after 12 days. Bars indicate standard error of the mean. Key: CMEA, green coffee meal extract agar; RMEA, high roasted coffee meal extract agar.

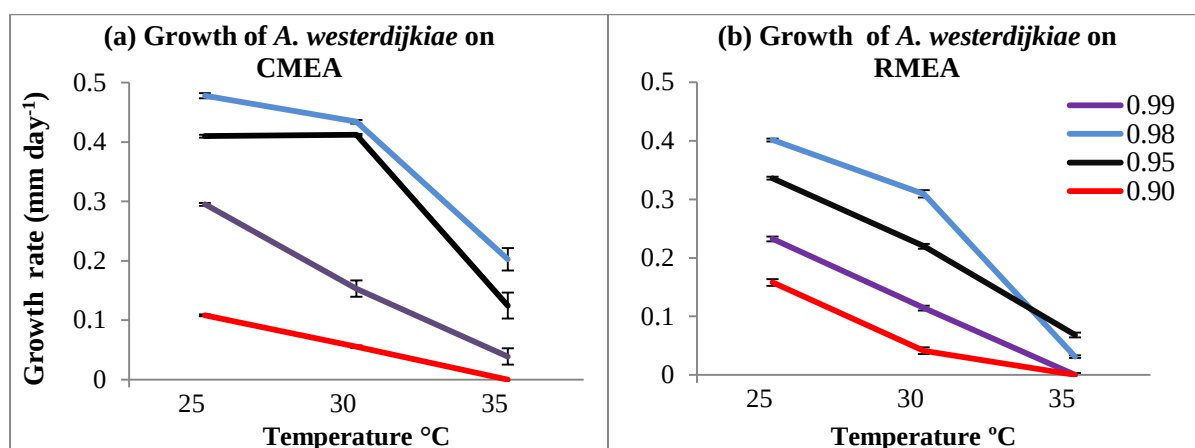


Figure 2.5: Effect of different temperatures (25, 30, 35°C) and media type (a) CMEA and (b) RMEA on growth of *A. westerdijkiae* (CBS 212986) at different a_w levels (0.99, 0.98, 0.95, 0.90) after 12 days. Bars indicate standard error of the mean. Key: CMEA, green coffee meal extract agar; RMEA, high roasted coffee meal extract agar.

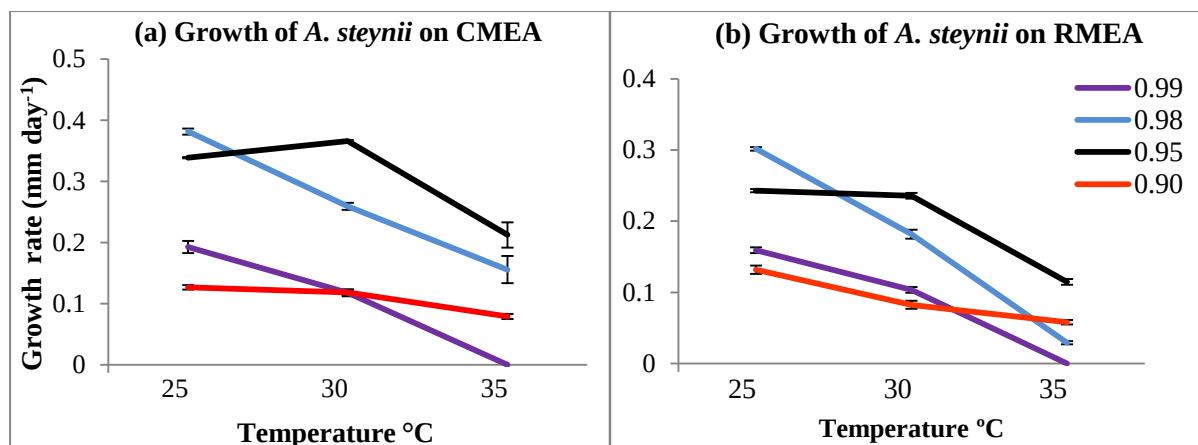


Figure 2.6: Effect of different temperatures (25, 30, 35°C) and media type (a) CMEA and (b) RMEA on growth of *A. steynii* (CBS 112814) at different a_w levels (0.99, 0.98, 0.95, 0.90) after 12 days. Bars indicate standard error of the mean. Key: CMEA, green coffee meal extract agar; RMEA, high roasted coffee meal extract agar.

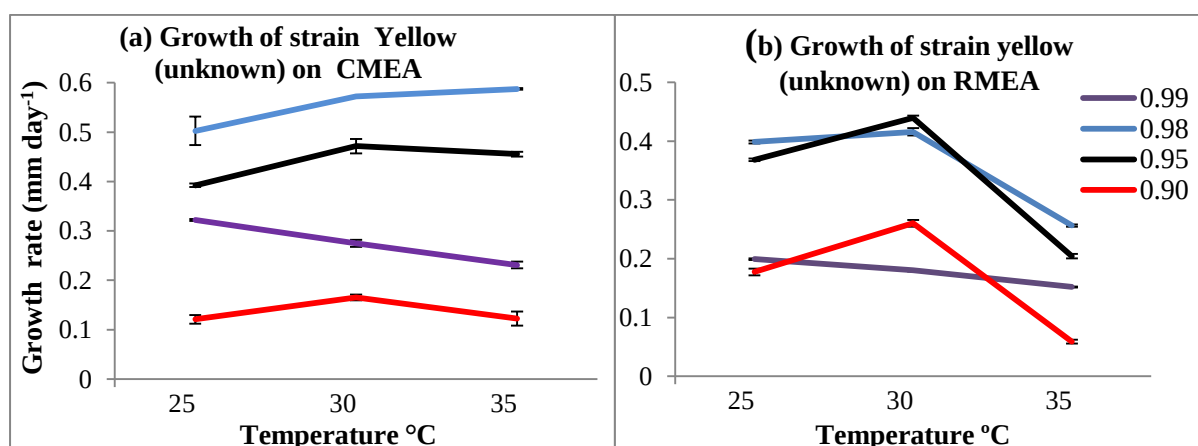


Figure 2.7: Effect of different temperatures (25, 30, 35°C) and media type, (a) CMEA and (b) RMEA, on growth of the yellow (unidentified strain) at different a_w levels (0.99, 0.98, 0.95, 0.90) after 12 days. Bars indicate standard error of the mean. Key: CMEA, green coffee meal extract agar; RMEA, high roasted coffee meal extract agar.

Table 2.5: Effect of a_w , temperature and media on OTA production (ng g^{-1}). Key to media: CMEA, green coffee meal extract agar; RMEA, high roasted coffee meal extract agar, section 2.1.2 (b-2).

Temperature		25°C		30°C		35°C	
Strains		CMEA ^a	RMEA	CMEA	RMEA	CMEA	RMEA
<i>A. westerdijkiae</i> (2A3)	0.99	ND	ND	ND	ND	ND	ND
	0.98	4.08±1.12	<LOQ	8.29± ^b 3.88	<LOQ	10.29 ^a	ND
	0.95	8.69±0.87	2.64±0.17	<LOQ	<LOQ	46.41^a	ND
	0.9	ND	<LOQ	ND	ND	<LOQ	ND
<i>A. westerdijkiae</i> (C1/1)	0.99	<LOQ	ND	ND	ND	ND	ND
	0.98	<LOQ	<LOQ	48.3±13.75	ND	9.65 ^a	ND
	0.95	3.24±0.37	<LOQ	ND	ND	ND	ND
	0.9	ND	<LOQ	ND	ND	ND	ND
<i>A. westerdijkiae</i> (B 2)	0.99	ND	ND	40.29±5.05	ND	ND	ND
	0.98	9.42±5.17	<LOQ	1802.8±416.36	ND	5206.97±433.13	ND
	0.95	3.65±0.15	<LOQ	3.24±0.37	<LOQ	28.13±1.12	<LOQ
	0.9	ND	ND	<LOQ	ND	<LOQ	ND
Yellow (unidentified colony)	0.99	<LOQ	ND	ND	ND	ND	ND
	0.98	<LOQ	ND	ND	ND	ND	ND
	0.95	<LOQ	ND	ND	ND	ND	ND
	0.9	ND	<LOQ	ND	ND	ND	ND
<i>A. westerdijkiae</i> (CBS 121986)	0.99	<LOQ	ND	<LOQ	<LOQ	ND	ND
	0.98	<LOQ	<LOQ	280.35±17.18	ND	64.3±15.77	ND
	0.95	30.55±6.21	<LOQ	5.38±2.91	ND	52.81	ND
	0.9	ND	<LOQ	<LOQ	ND	ND	ND
<i>A. steynii</i> (CBS 112814)	0.99	ND	ND	6.33±0.75	2.6±0.1	ND	ND
	0.98	3.41	<LOQ	ND	ND	ND	ND
	0.95	<LOQ	ND	ND	ND	ND	ND
	0.9	ND	ND	ND	ND	ND	ND

<LOQ: Low level of quantification.

ND: None detected, below detectable level.

a: media

Table 2.6: Summary of the statistical analyses of the effect of the factors on growth rate and OTA production by six strains in relation to a_w , temperature, medium and their interactions using the Kruskal-Wallis Test (non-normality data).

Factors				
Strains	Temperature	Media (CMEA ,RMEA)	Water activity (a_w)	Response
<i>A. westerdijkiae</i> (2A3)	S	NS	S	Growth rate (mm day ⁻¹)
	S	S	S	OTA (ng g ⁻¹)
<i>A. westerdijkiae</i> (C1/1)	S	S	S	Growth rate (mm day ⁻¹)
	S	NS	S	OTA (ng g ⁻¹)
<i>A. westerdijkiae</i> (B 2)	S	NS	S	Growth rate (mm day ⁻¹)
	S	S	S	OTA (ng g ⁻¹)
Yellow (Unidentified colony)	NS	S	S	Growth rate (mm day ⁻¹)
	NS	NS	NS	OTA (ng g ⁻¹)
<i>A. westerdijkiae</i> (CBS 121986)	S	NS	S	Growth rate (mm day ⁻¹)
	S	S	S	OTA (ng g ⁻¹)
<i>A. steynii</i> (CBS 112814)	S	S	S	Growth rate (mm day ⁻¹)
	NS	NS	NS	OTA (ng g ⁻¹)

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

NS not significant

S significant

2.1.4 Discussion

Green coffee beans are one of the largest trade commodities worldwide with an annual production of 7 billion Kg. It is thus the second most valuable product after oil (Sibanda, 2006). Like other agricultural commodities, it is subject to contamination by mycotoxigenic fungi resulting in potential risks to human health (Napolitano et al., 2007). Six Coffee bean types were collected from different supermarkets and the diversity of fungi was analysed by direct and serial dilution plating. This showed that the predominant genera were *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus* and some yeasts. Mycoflora and OTA contamination of coffee has been previously studied (Levi et al., 1974; Micco et al., 1989). Mislivec et al. 1983, suggested that *Aspergillus* species are the most dominant fungal flora in 944 coffee bean samples. High levels of contamination by yeasts and moulds in green coffee samples has been found in coffee at different stages of processing (Mislivec et al., 1983; Urbano et al., 2001). In the present study a high frequency of isolation of *A. niger* was observed (15 to 91%) for coffee imported from India. For *A. westerdijkiae*, the populations represented about 4.5-10%. Overall, Indian coffee beans were more contaminated than other coffees imported into Kuwait. Isolates of *Aspergillus flavus* were only isolated in low frequencies from coffee beans, while isolates of *Aspergillus* section *Nigri* represented 30 % of the infected beans, and about 21% of the samples were contaminated with *Penicillium*.

The MC of coffee is an important quality criterion and influences the colonisation of mycotoxigenic fungi. Thus, 10-12% MC (=50-70% RH) is considered a safe level to prevent any spoilage of green coffee and reduce the potential for mycotoxin contamination without losing quality (Taniwaki et al., 2001). Similarly, Betancourt and Frank (1983) showed the limits for green coffee spoilage by fungi if stored at >14 % moisture content (approximately 0.80 a_w) and a relative humidity of 75%. Viani (2002) concluded that at >0.80 a_w , the length of time prior to roasting can represent a high risk of mould growth and OTA contamination of coffee beans. In this study, the moisture content of Indian coffee beans ranged from 8.2-8.5%, while for coffee from Ethiopia 7.7% and Yemen 7.4%. The equivalent range was 0.423-0.455. This is very dry and should, in reality, affect coffee flavour. Overall, there were higher fungal populations in coffee beans imported from India and Ethiopia (4.55-4.45 $\log_{10}$ CFUs g^{-1}) than from the other countries.

Strains of *Aspergillus* section *Flavi*, *Aspergillus* section *Nigri*, *Aspergillus* section *Circumdati* and *Penicillium* were isolated from the green bean samples collected from different Kuwaiti markets, Saudi Arabia and other sources. OTA production was measured using two methods: a qualitative coconut agar method and a quantitative HPLC method. By using these two methods, there was 85% compatibility of the results. Frank (1999), Taniwaki et al. (2003) and Urbano et al. (2001) reported high percentage of OTA production by isolates of *A. ochraceus* from green coffee. However, this was before the taxonomic reclassification of the *Aspergillus* section *Circumdati* grouping and it is possible that these strains would now be reclassified as *A. westerdijkiae* (Abdel-Hadi and Magan, 2011).

It is important to note the relative mixture of non-toxigenic and toxigenic contaminating species of coffee and the prevailing environmental conditions will influence the actual OTA contamination levels in the coffee, especially during processing and storage. Magan and Aldred (2007) showed that the interactions between different toxigenic and non-toxigenic species was in a state of flux and that the dominance may change both spatially and temporally. In Brazil, Taniwaki et al. (1999) found that *A. ochraceus* (= *A. westerdijkiae*) isolated from green coffee produced about 90% of the OTA. However, Urbano et al. (2001) found that 88.1 % of *A. niger* and *A. ochraceus* from Brazilian green coffee beans produced OTA. Surprisingly, Ilic et al. (2007) examined coffee beans from Vietnam and isolated only one species, *A. niger*, and found that eight of 92 strains (8.7%) produced OTA.

The present study showed high OTA production by *A. westerdijkiae* strains, in the range of (82250-5595 ng g⁻¹ medium). *A. niger* produced 59682 ng g⁻¹ and *A. steynii* 571 ng g⁻¹ medium. Thus, *A. westerdijkiae*, occasionally *A. niger*, and less commonly *A. steynii* are probably responsible for OTA production in green coffee beans.

a) Interactions of a_w x temperature x media on growth rates and OTA production.

In this study some *A. westerdijkiae* (2A3, C1/1, B 2 and CBS 121986), the unknown Yellow isolate and *A. steynii* (CBC 112814) were tested for their relative growth rates and OTA production ability in both green and roasted coffee-based media in relation to a_w x temperature conditions. These experiments showed the significant influence of the two key abiotic parameters, a_w and temperature on both growth and OTA production. The OTA

production and growth rate pattern was higher at 0.95-0.98 a_w and 25-30°C with much slower growth and less OTA production at 0.90 a_w and 35°C on the green coffee meal extract medium. Much higher OTA production by a strain of *A. westerdijkiae* (B2; 5207-1803 ng g⁻¹) at 0.98-0.95 a_w at 35-30°C was found on the green coffee-based extract medium. The strain of *A. steynii* (CBC 112814) and the Yellow isolate produced very low or no OTA. The type of nutritional medium had no overall influence on growth of the mycotoxigenic fungi and OTA production. It is possible that the roasting process affected the nutrients and thus would have less influence than in the green unroasted coffee medium. .

Several species in the *Aspergillus ochraceus* group (= *A. westerdijkiae*) (Ciegler, 1972), *A. niger*, *A. carbonarius* (in section *Nigri*) have been reported to produce OTA (Abarca et al., 1994; Heenan et al., 1998). Urbano et al. (2001), Taniwaki et al. (2003) reported that *A. ochraceus* was the major cause of OTA contamination in green coffee. The minimal a_w for growth was 0.85 and overall growth was reduced by 50% at 0.93 a_w (Astoreca et al., 2007). Pardo et al. (2005a) reported optimal conditions for growth on green coffee-based media for *A. ochraceus* species at 0.95–0.99 a_w and 20–30°C. The minimum a_w level for germination was 0.80, and 0.85 for mycelial growth. The optimum and minimum a_w for *A. ochraceus* were 0.80 and 0.95 respectively, 0.85 and 0.99 for *A. niger*. While the OTA production for *A. ochraceus* was limited at 0.90 and optimum at 0.95 a_w , for *A. niger* this was at 0.99 a_w at 35°C (Suarez-Quiroz et al., 2004a). The maximum growth for *A. westerdijkiae* on YES media occurred at 0.95 a_w and 30°C, while for *A. steynii* this was 0.99 a_w and 30°C. Previous studies by Abdel-Hadi and Magan (2009) found that optimum OTA production was at 0.99 a_w for *A. steynii* at 30°C, and 25°C for *A. westerdijkiae*. This type of information may be important for a better understanding of the boundary environmental conditions which may allow growth and those which may allow OTA production. This is important in developing strategies for the minimisation of OTA in coffee during processing and storage.

2.2 Effect of a_w , temperature and coffee concentration on lag phases, growth and production of ochratoxin A (OTA) by five strains of *Aspergillus* section *Circumdati* and Section *Nigri* isolated from coffee.

2.2.1 Introduction

Various studies have used coffee as a substrate to evaluate its effect on growth and OTA production by species from the *Aspergillus* section *Circumdati* and *Aspergillus* section *Nigri* groups. For example, Arora and Ohlan (1997) tested two coffee media, Bru instant and Nestle coffee powder in boiling distilled water to study the growth of various fungi. They showed inhibition of approximately 50% of the spoilage fungi examined. Other studies have compared cocoa powder with coffee powder agar media. Overall, for *A. ochraceus*, *A. niger* and *P. nordicum* OTA production was higher in coffee powder than cocoa powder media (Ban-koffi et al., 2009). However, a_w was not modified in these studies. Masoud et al. (2005) used grounded green coffee bean agar and reported that two yeasts, *Pichia anomala* and *Pichia kluyveri*, inhibited OTA production by *A. ochraceus*. In addition, Pardo et al. (2005) studied the effect of various a_w and temperature levels on germination and growth of *A. ochraceus* on 3% (w/v) green coffee meal extract agar (CMEA). None of these studies examined the effect that different concentrations of coffee may have on growth and OTA production by different OTA producing strains. This is important to determine whether the in vitro system will simulate what may occur in the natural substrate so that it can be utilised effectively in modelling contamination in natural coffee.

The objectives of the present work was to determine the effect of various concentrations of coffee extracts (10, 20, 30, 40, 50, 70, and 80%) on the lag phase (λ , days), growth and OTA production by strains of *A. westerdijkiae* (B 2, CBS 121986), *A. niger* (A 1911), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14). Subsequently 6% coffee extract medium was used to evaluate the effect of a_w and temperature on the lag phases (λ , days), growth and OTA production by these strains.

2.2.2 Materials and methods

a) Fungal strains

One strain from the *Aspergillus* section *Circumdati* (B 2) was isolated from green coffee beans imported into Saudi Arabia. In addition, *Aspergillus westerdijkiae* (CBS 121986) and *A. steynii* (CBC 112814) isolated from coffee beans, were kindly provided by Dr. B. Patino (Complutense University, Madrid, Spain) and also used in this study. A strain of *Aspergillus niger* (A 1911), kindly provided by Prof. Paola Battilani (Università Cattolica del Sacro Cuore, Italy), and one strain each of *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14), isolated from Arabica coffee were used (kindly provided by Dr. Marta Taniwaki, ITAL, Campinas, Brazil).

b) Media used to evaluate the effect of coffee concentration

The following media were used to determine the effect of coffee concentration (%) on growth and OTA production by these strains:

CMEA (Coffee meal extract agar): The medium was made by boiling 300g of ground green coffee beans in 1L of distilled water for 30 min (concentrated coffee extract). A double layer of muslin was used for filtering the resulting mixture and the volume was made up to 1L. Technical agar No 3 (2%), 10, 20, 30, 40, 50, 70 and 80% concentrated coffee extract and glycerol were added (9.2 g) to adjust to 0.98 water activity (a_w). The media were autoclaved at 121°C for 10 min and poured into 9 cm Petri plates and kept at 4°C until used. The final a_w levels were checked with a water activity meter, Aqualab 3TE (Decagon Devices, Inc., Pullman, Washington, USA). Media were used to evaluate the effect a_w x temperature interactions on growth and OTA production.

CMEA (Coffee meal extract agar): The media was made by boiling 300 g of ground green coffee beans in 1 L of distilled water for 30 min (concentrated coffee extract). A double layer of muslin was used for filtering the resulting mixture and the volume was made up to 1 L. Technical agar No 3 (2%), 6% concentrated coffee extract and glycerol were added (50.6 , 23, 9.2, 2.7 g) to adjust to 0.90, 0.95, 0.98, and 0.99 water activity (a_w). The media were autoclaved at 121°C for 10 min and poured into 9 cm Petri plates and kept at 4 °C until used.

The final a_w levels were checked with a water activity meter, Aqualab 3TE (Decagon Devices, Inc., Pullman, Washington, USA).

c) Inoculum preparation

The inoculum was prepared by growing each strain on MEA at 25°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. The CMEA media treatments and replicates were centrally point inoculated with 7 μ L of the spore suspension and incubated at 30°C for the coffee percentage experiment and 20, 25, 30, 35 and 37°C for 9 days. Three replicates per treatment were used for each strain.

d) Calculation of lag phase prior to growth and growth rate

As described in section 2.1.2 (b-3).

e) Extraction, detection and quantification of OTA

The same procedure as detailed in section 2.1.2 (4).

f) Data analysis

A factorial design was applied for growth rate assessment and OTA production. A full factorial design with two (4 x 5) factors: water activity and temperature was applied. Each treatment, water activity x temperature combination was carried out in triplicate, both for growth rate assessment and OTA production (Table 2.7)

Normality was checked using the Kolmogorov-Simonov test. Analysis of data, the factors and response and their interaction were examined by the Kruskal-Wallis (nonparametric) if the data was not normally distributed. For normally distributed data, the data sets were analysed using Minitab® 16 package (Minitab Inc., 2010. State College, PA, USA). The statistical significant level was set at $P < 0.05$ for all single and interacting treatments.

Table 2.7: Summary of the different treatments and levels used in the experiment.

Factor		Level			
Water activity		0.90	0.95	0.98	0.99
Temperature (°C)		20°C	25°C	30°C	35°C
					37°C

2.2.3 Results

a) Effect of Coffee concentration on Lag phase, growth and OTA production

Overall, the lag phases prior to growth were all <24 hrs. However, there were statistical differences during the 0-24 hr for some of strains (Figures 2.9-2.14). These figures also show the information on growth and OTA production in the different treatment conditions for each strain examined. The growth rate of strains of *A. westerdijkiae* (B 2, CBS 121986), *A. niger* (A 1911), *A. carbonarius* (ITAL 204), *A. ochraceus* (ITAL 14) and *A. steynii* (CBS 112814) on 0.98 a_w CMEA medium containing various concentrations of coffee shows that there was relatively little difference in the relative growth rate. The fastest growth among all strains was observed for *A. carbonarius* (ITAL 204) in 70% coffee extract where it reach 12.25 mm day⁻¹; see Figure 2.12; Plate 2.2a. For other strains such as the *A. niger* (A 1911) and *A. westerdijkiae* (B 2, CBS 121986) growth was best with 10% coffee extract and slowest in 80%, see Figure 2.9, 2.10, 2.11; Plate 2.2b. For *A. carbonarius*, isolated from Brazilian coffee, the optimum was over a range of coffee concentrations of 20-70%. Similar results were obtained for *A. ochraceus* (ITAL 14; 8mm day⁻¹), *A. steynii* (CBS 112814; 6mm day⁻¹) and *A. westerdijkiae* (CBS 121986; 8.08 mm day⁻¹), respectively. However, the lowest growth was observed in 10% coffee extract for one of the *A. carbonarius* strains (ITAL 204), *A. ochraceus* (ITAL 14); and 80% coffee extract for *A. westerdijkiae* (B 2) and the *A. steynii* (CBS 112814) strain at 30°C.

Overall, there was a higher amount of OTA produced as the coffee concentration increased for most of the strains examined with the maximum in the 70-80% coffee-based media. Often, in the 10-20% concentration coffee-based media, the OTA amounts were the lowest. The strains, which produced the highest OTA amounts, were *A. carbonarius* (ITAL 204) and *A. steynii* (CBS 112814) with approx. 1607 and 1383 ng g⁻¹ respectively. The lowest amount was by *A. ochraceus* (ITAL 14) in 10% coffee medium at about 0.67 ng g⁻¹.

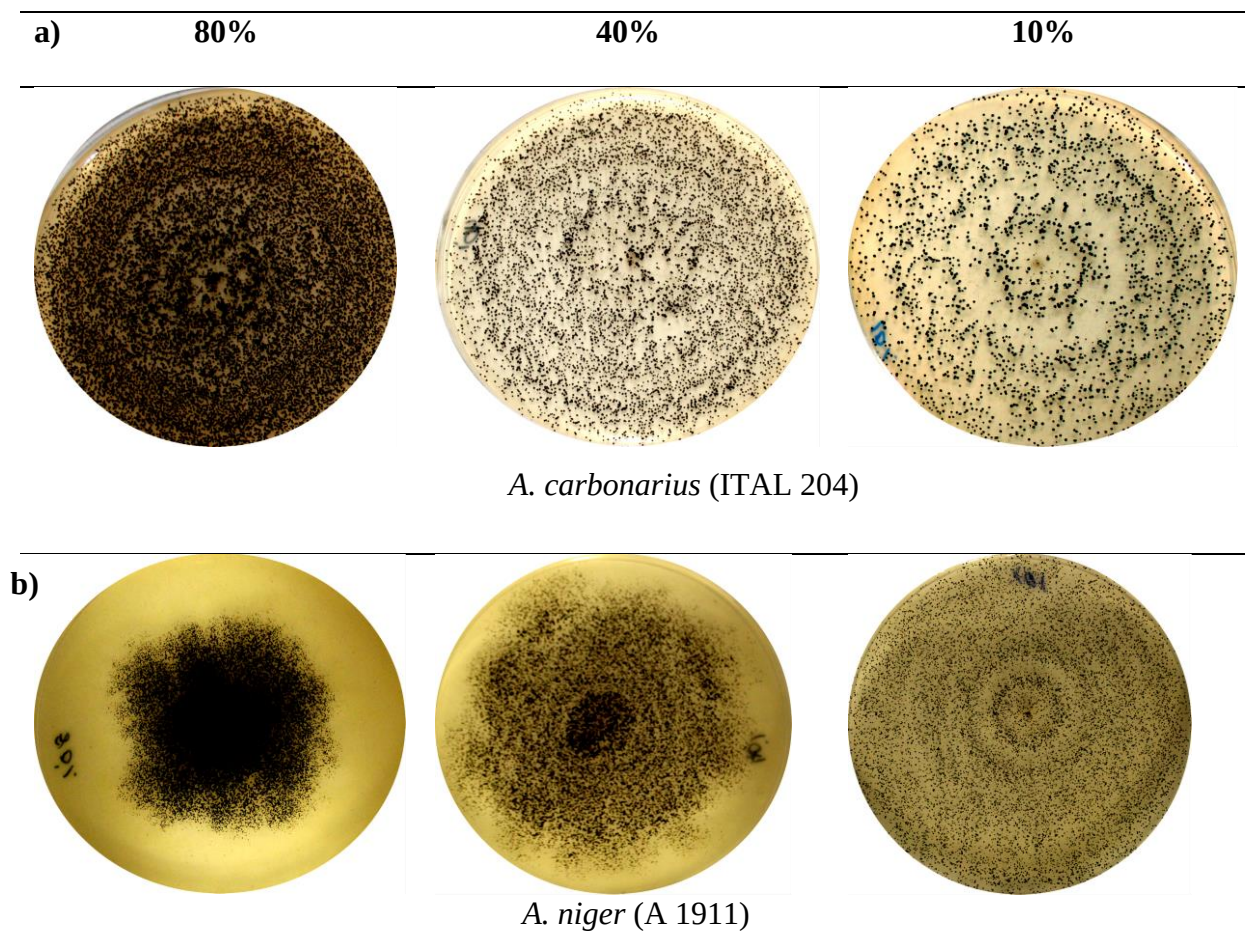


Plate 2.2: shows coffee medium concentration (10, 40 and 80%) on a) *A. carbonarius* (ITAL 204) and b) *A. niger* (A 1911) effects growth on 0.98 a_w CMEA at 30°C for 9 days.

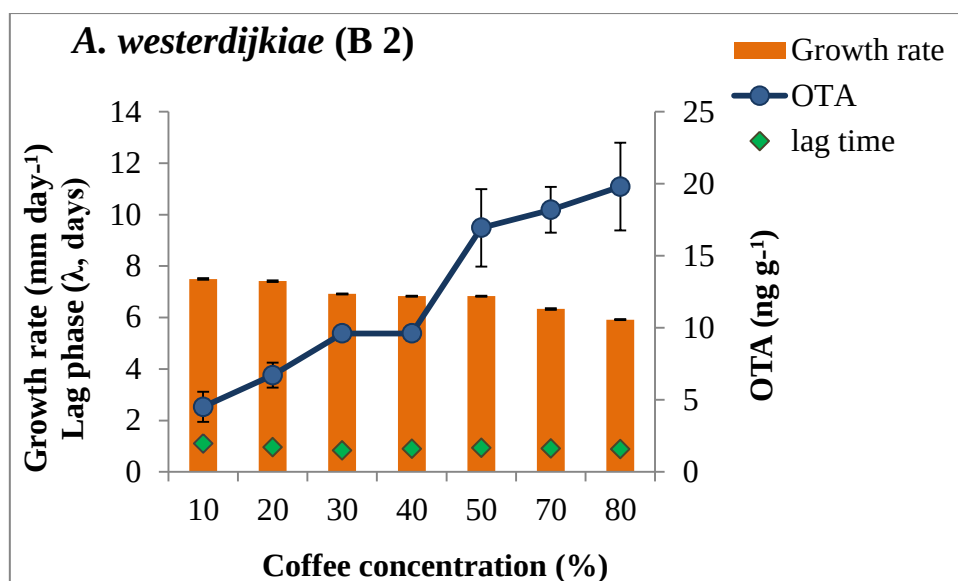


Figure 2.8: Comparison of coffee medium concentration (10, 20, 30, 40, 50, 70 and 80%) effects on lag phase (λ , days), growth and OTA production of *A. westerdijkiae* (B 2) on 0.98 a_w CMEA at 30°C for 9 days. Bars indicate standard error of the mean.

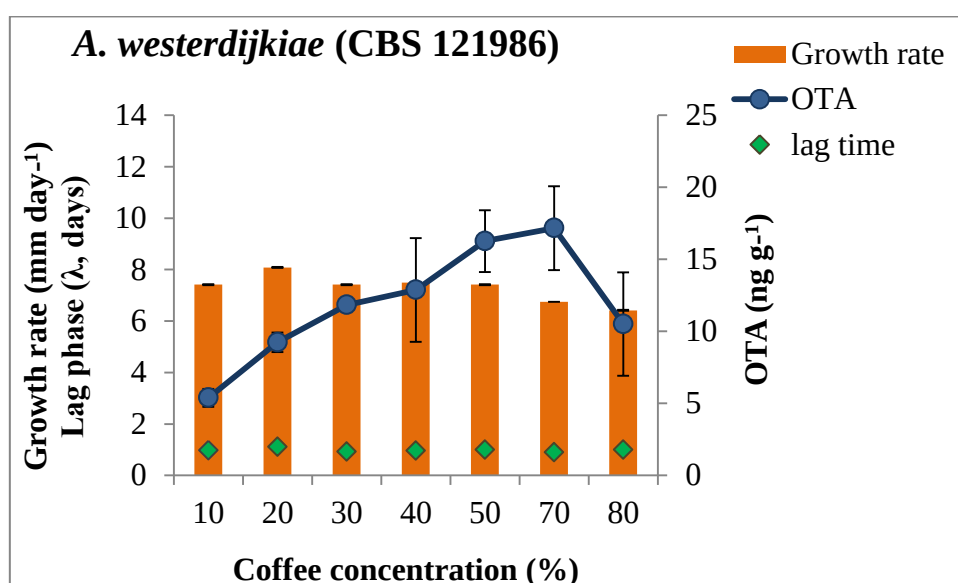


Figure 2.9: Comparison of the coffee concentration (10, 20, 30, 40, 50, 70 and 80%) effects on lag phase (λ , days), growth and OTA production of *A. westerdijkiae* (CBS 121986) on 0.98 a_w CMEA at 30°C for 9 days. Bars indicate standard error of the mean.

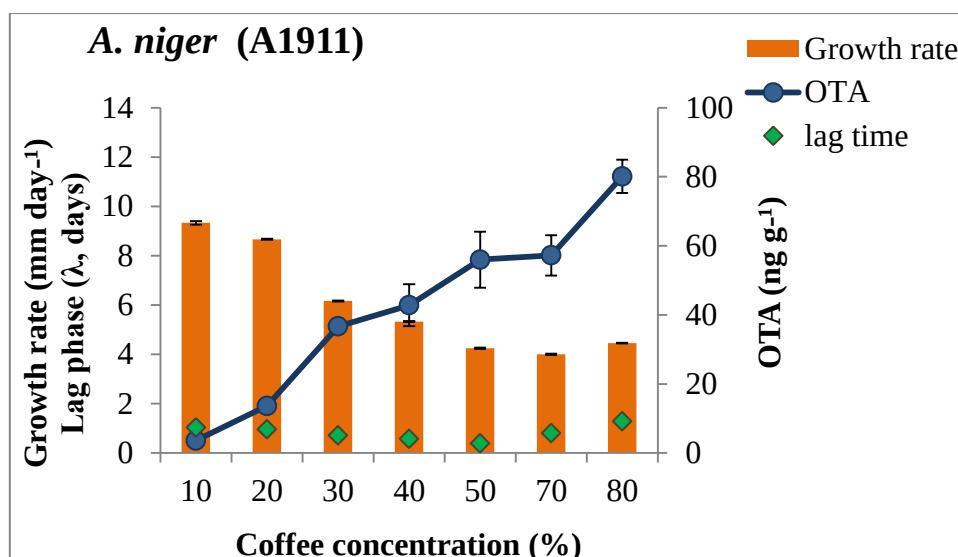


Figure 2.10: Comparison of the coffee concentration (10, 20, 30, 40, 50, 70 and 80%) effects on lag phase (λ , days), growth and OTA production of *A. niger* (A 1911) on 0.98 a_w CMEA at 30°C for 9 days. Bars indicate standard error of the mean. Note: the OTA range is different from the other figures and cannot be directly compared.

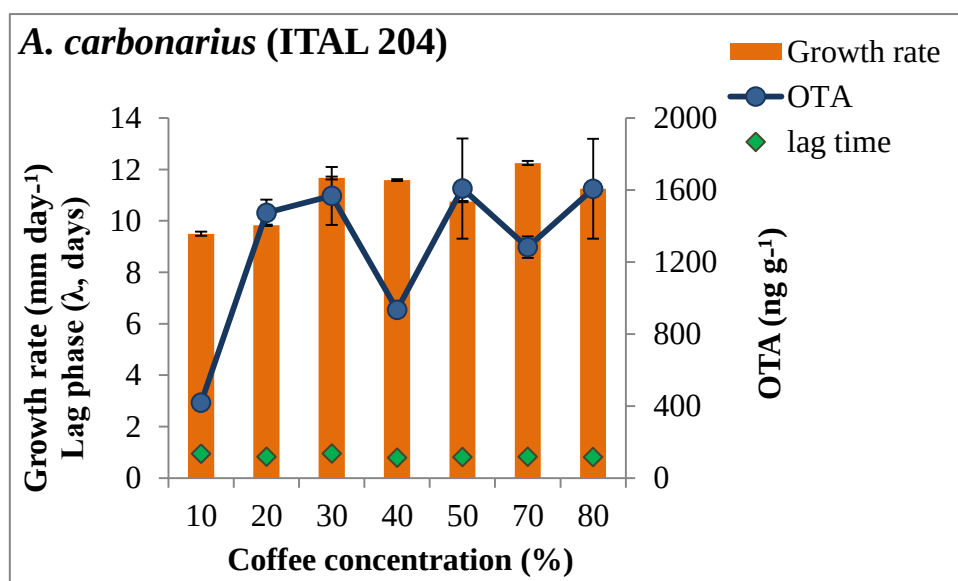


Figure 2.11: Comparison of the coffee concentration (10, 20, 30, 40, 50, 70 and 80%) effects on lag phase (λ , days), growth and OTA production of *A. carbonarius* (ITAL 204) on 0.98 a_w CMEA at 30°C for 9 days. Bars indicate standard error of the mean. Note: the OTA range is different from the other figures and cannot be directly compared.

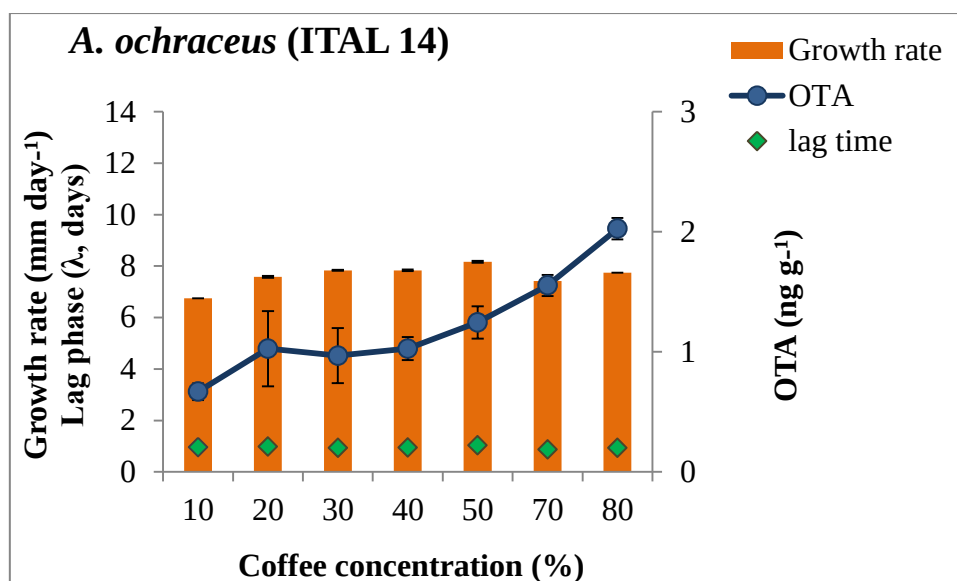


Figure 2.12: Comparison of the coffee concentration (10, 20, 30, 40, 50, 70 and 80%) effects on lag phase (λ , days), growth and OTA production of *A. ochraceus* (ITAL 14) on 0.98 a_w CMEA at 30°C for 9 days. Bars indicate standard error of the mean. Note: the OTA range is different from the other figures and cannot be directly compared.

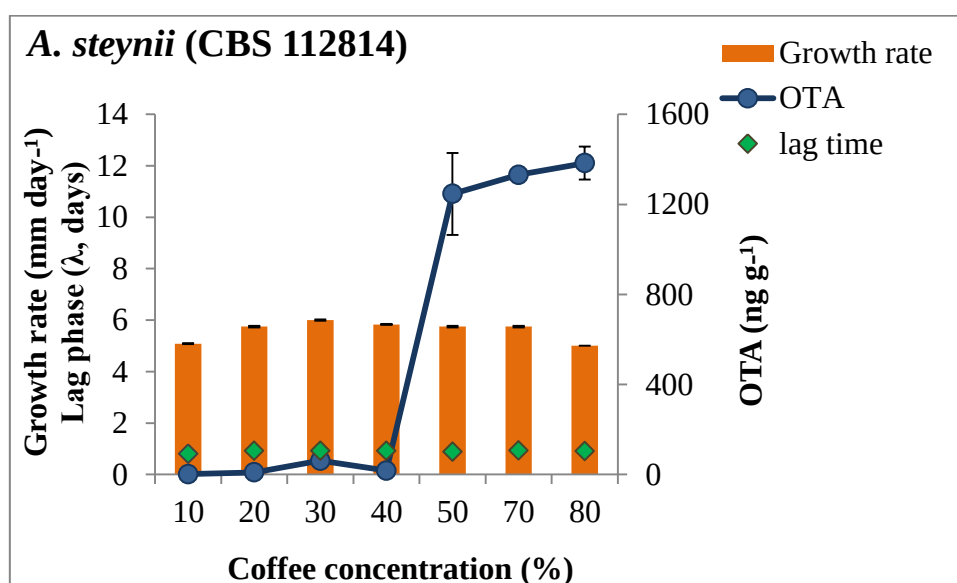


Figure 2.13: Comparison of the coffee concentration (10, 20, 30, 40, 50, 70 and 80%) effects on lag phase (λ , days), growth and OTA production of *A. steynii* (CBS 112814) on 0.98 a_w CMEA at 30°C for 9 days. Bars indicate standard error of the mean. Note: the OTA range is different from the other figures and cannot be directly compared.

Statistically, the coffee concentration had significant effects ($p < 0.05$) on the lag phases prior to growth, growth and OTA production except for *A. niger* (A 1911) (Table 2.7, also see Appendix I).

The growth rates for all strains was highly significantly ($P < 0.05$) affected by coffee concentration, with the highest growth observed on 10% coffee concentration for the strains *A. westerdijkiae* (B 2) and *A. niger* (A 1911) (See Appendix I). Except for other strains, the highest growth of *A. westerdijkiae* (CBS 121986), *A. steynii* (CBS 112814) *A. ochraceus* (ITAL 14) and *A. carbonarius* (ITAL 204) respectively was on 20, 30, 50 and 70% coffee extract (Table 2.7, also see Appendix I)

Table 2.7 also shows the statistical analyses of OTA production for each individual strain. This show that overall, the OTA produced by *A. carbonarius* (ITAL 204) *A. steynii* (CBS 112814) and *A. niger* (A 1911) were highest, respectively, at 80% coffee concentration. Interestingly, for the type strain from the culture collection, *A. steynii* (CBS 112814), grew very slowly and produced little OTA production in 10-40% coffee concentration, with higher production in 50, 60 and 70% coffee extract.

Table 2.8: Summary statistical table for the lag phase, growth rate and OTA production of the five strains on media containing different coffee concentrations by using the Kruskal-Wallis Test (non normality data) and ANOVA (normality data).

Strains	Factors (Coffee concentration (%))	Response
<i>A. westerdijkiae</i> (B 2)	NS ^a	Lag time (λ , days)
	S ^b	Growth rate (mm day ⁻¹)
	S ^b	OTA (ng g ⁻¹)
<i>A. westerdijkiae</i> (CBS 121986)	NS ^b	Lag time (λ , days)
	S ^a	Growth rate (mm day ⁻¹)
	S ^b	OTA (ng g ⁻¹)
<i>A. niger</i> (A 1911)	S ^b	Lag time (λ , days)
	S ^a	Growth rate (mm day ⁻¹)
	S ^b	OTA (ng g ⁻¹)
<i>A. carbonarius</i> (ITAL 204)	NS ^b	Lag time (λ , days)
	S ^b	Growth rate (mm day ⁻¹)
	S ^b	OTA (ng g ⁻¹)
<i>A. ochraceus</i> (ITAL 14)	NS ^b	Lag time (λ , days)
	S ^a	Growth rate (mm day ⁻¹)
	S ^b	OTA (ng g ⁻¹)
<i>A. steynii</i> (CBS 112814)	NS ^b	Lag time (λ , days)
	S ^a	Growth rate (mm day ⁻¹)
	S ^a	OTA (ng g ⁻¹)

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

^a Kruskal-Wallis test.

^b ANOVA.

NS not significant

S significant

b) Effect of a_w and temperature on lag phases, growth and production of ochratoxin A (OTA) by five strains of *Aspergillus* section *Circumdati* and *Aspergillus* Section *Nigri* isolated from coffee.

Figure 2.15 shows the effect of different temperature x a_w levels on the lag times prior to growth initiation for all strains tested. Very short lag phases occurred at 35–37°C and 0.98 to 0.99 a_w . At lower temperatures and a_w levels the lag times increased to >1.5 days, especially at 0.90 a_w and 20°C. Similar trends were found for *A. carbonarius* (ITAL 204), *A. ochraceus* (ITAL 14) and *A. niger* (A 1911). While for *A. westerdijkiae* (B 2; CBS 121986) the shortest lag times and fastest growth occurred at 0.99 a_w and 37°C.

Figure 2.16 compares the relative growth rate of the strains of *A. westerdijkiae* (B 2; CBS 121986), *A. niger* (A 1911), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) at

different a_w x temperature conditions. The highest growth rate was observed at 25 and 30°C when compared to the other temperatures tested (20, 37°C). In addition, the limit for growth of *A. westerdijkiae* (B 2; CBS 121986), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) was 0.90 a_w at all temperatures over the time periods of the experiments. For all strains examined, the growth was optimum at 0.98 and 0.99 a_w and this decreased under water stress imposition. Furthermore, the lowest growth rate for most species was observed at 0.90 a_w at 37°C. The only exception was *A. niger* (A 1911) which had the highest growth rate at 37°C at 0.99 a_w and the lowest at 20°C at 0.90 a_w .

Comparison of OTA production by all strains in relation to temperature x a_w conditions is shown in Table 2.8. In almost all cases, for the strains examined optimum OTA production was at 0.99-0.98 a_w at 20, 25 and 30°C. At 37°C there was significantly less OTA produced even when water was relatively freely available. Overall, for all the strains and species examined at $< 0.95 a_w$ on the coffee medium there was a significant decrease in OTA production. The highest OTA production was at 0.99 a_w at 25°C ($321.8 \pm 17.3 \text{ ng g}^{-1}$) for *A. carbonarius* (ITAL 204; Figure 2.17).

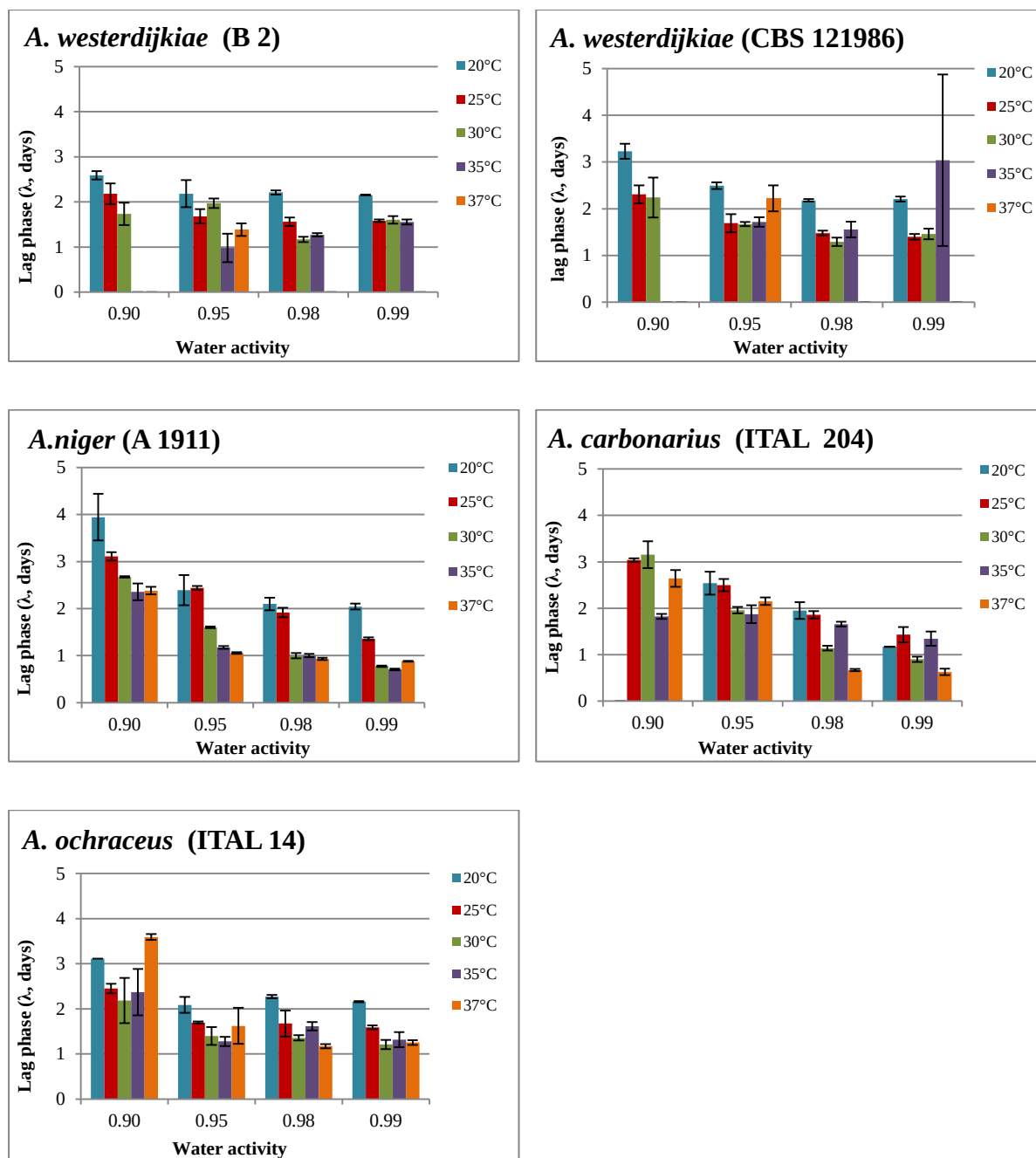


Figure 2.14: Comparison of the effect of water activity x temperature on lag phase (λ , in days) of *A. westerdijkiae* (B 2), *A. westerdijkiae* (CBS 121986), *A. niger* (A 1911), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) on CMEA for 9 days. Bars indicate standard error of the mean.

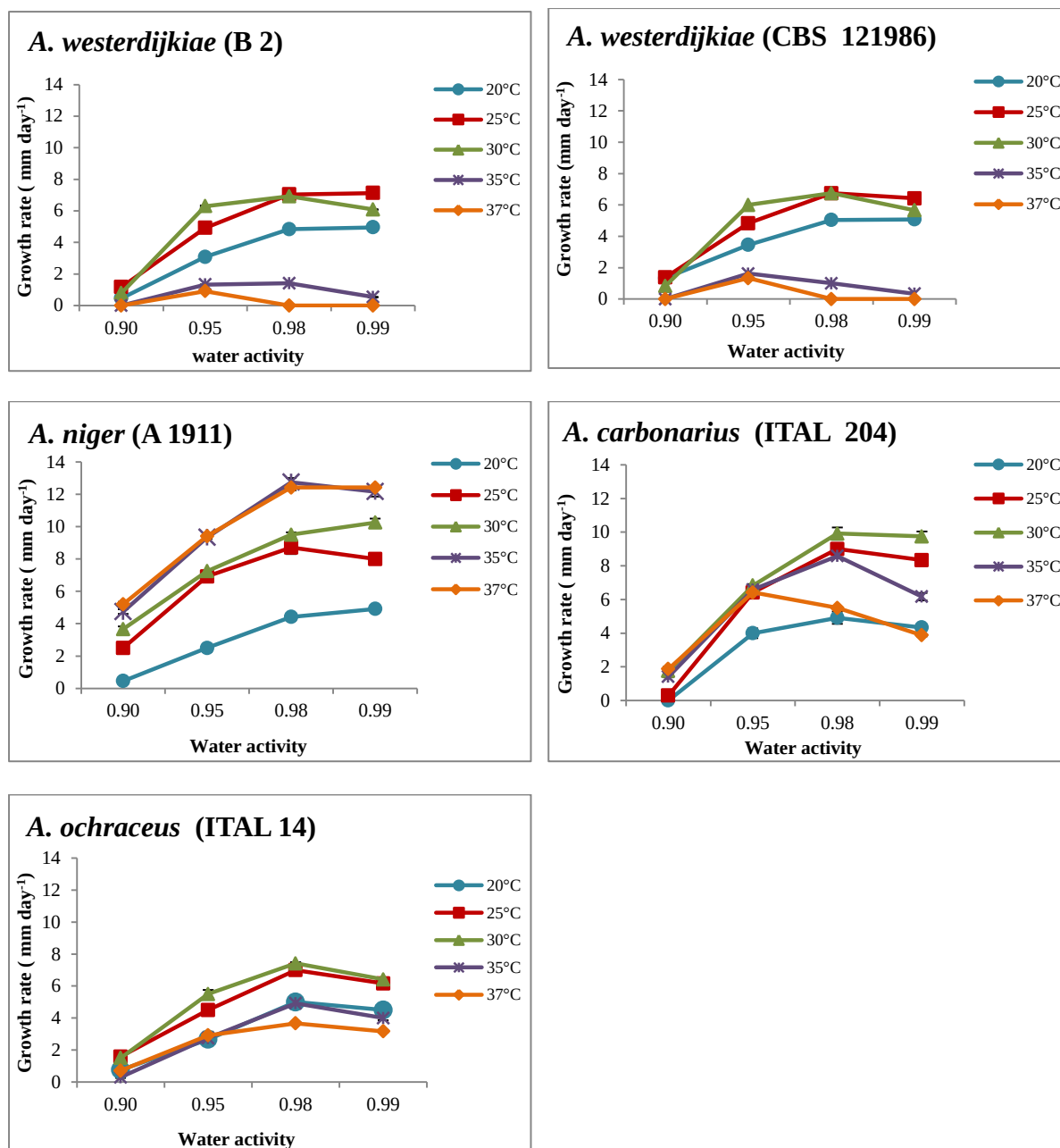


Figure 2.15: Comparison of the effect of water activity x temperature on growth of *A. westerdijkiae* (B 2), *A. westerdijkiae* (CBS 121986), *A. niger* (A 1911), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) CMEA for 9 days. Bars indicate standard error of the mean.

Table 2.9: Ochratoxin A production (ng g^{-1}) by strains of four species in relation to a_w x temperature conditions when grown on a green coffee-based extract medium (CMEA).

Temperature		20°C	25°C	30°C	35°C	37°C
Strains		OTA $\text{ng g}^{-1} \pm \text{SE}^b$	OTA $\text{ng g}^{-1} \pm \text{SE}^b$	OTA $\text{ng g}^{-1} \pm \text{SE}^b$	OTA $\text{ng g}^{-1} \pm \text{SE}^b$	OTA $\text{ng g}^{-1} \pm \text{SE}^b$
<i>A. westerdijikiae</i> (B 2)	0.90	0.6±0.1(3)	LOQ(3)	LOQ(3)	0.3±0.2(3)	0
	0.95	24.6±2.3(3)	1.05±0.6(3)	1.6±0.5(3)	8.6±0.9(3)	4.7±1.4(3)
	0.98	40.5±2.5(3)	0.4±0.09(3)	39.8±18.0 (3)	34.8±11.5(3)	0
	0.99	116±22.5(3)	1.8±1.4(2)	153.7±50.2(3)	76.4±5.7(3)	0
<i>A. westerdijikiae</i> (CBS 121986)	0.90	LOQ(2)	LOQ(3)	2±2.2(3)	2.3±1.9(3)	0
	0.95	22.8±7.8(3)	LOQ(3)	3.5±2.2(3)	7.5±5.7(3)	13.6±4.9(3)
	0.98	1±0.6(3)	0	16.4±9.3(3)	17.4±5.2(3)	0
	0.99	11.6±10.7(3)	0.3(1)	310.5±69.3(3)	8.1±1.9(3)	0
<i>A. carbonarius</i> (ITAL 204)	0.90	0	0.4±0.4 (3)	0.5±0.3 (3)	1.6±1.2 (3)	1.1±0.2 (3)
	0.95	69.4±4.5(3)	57.3±9.6(3)	34.6±2.6 (3)	16.5±0.5 (3)	28.3±1.3(3)
	0.98	168.3±6.4(3)	208.1±1 (3)	26.8±1.3(3)	35.5±1.8 (3)	17.6±0.6 (3)
	0.99	286.6±21.1 (3)	321.8±17.3(3)	301.8±12.1 (3)	22.3±7.9(3)	11.8±2.8(3)
<i>A. niger</i> (A 1911)	0.90	2.8(1)	0.4 (1)	0	0	0
	0.95	4.7±1(3)	0.8±0.3 (3)	3.5±0.8 (3)	0	0.5±0.1 (2)
	0.98	50.3±4.8(3)	7.9±1.4(3)	0	0	0
	0.99	81.8±6.4 (3)	14±2.6(3)	0.4±0.2(3)	0	0
<i>A. ochraceus</i> (ITAL 14)	0.90	LOQ(2)	LOQ(3)	0.7±0.5 (3)	0.6±0.2 (3)	0.4 ±0.2(2)
	0.95	0.4 (3)	0.7 (3)	0.8± (3)	0.4 (3)	LOQ(3)
	0.98	0.5±0.1 (3)	0.55 (3)	0	0.4 (3)	LOQ(3)
	0.99	0.6±0.1 (3)	0.59 (3)	0.5±0.02(3)	0.5 ±0.3(3)	0.4±0.1 (3)

<LOQ: Low level of quantification

() Number of positive samples used for calculation.

b: standard error.

Table 2.9 summarises the statistical analyses for the different individual and interacting factors for the strains/species examined. Mycelial growth was significantly affected by temperature and a_w for all strains, and two-way interactions of a_w x temperature was significant for *A. ochraceus* (ITAL 14) and *A. carbonarius* (ITAL 204). In addition, there was a significant difference in OTA production by different strains with the exception of *A. ochraceus* (ITAL 14). Furthermore, a significant difference between lag phases (λ day⁻¹) by individual strains was observed. The box plots and details of the single and two-way interacting factors on OTA production, and the ANOVA results are detailed in Appendix I.

A. niger (A 1911) examined on CMEA showed optimum growth rate 0.99 a_w and 35 and 37°C respectively (Figure 2.17). The growth rates for *A. westerdijkiae* (B 2), *A. westerdijkiae* (CBS 121986), *A. ochraceus* (ITAL 14) and *A. carbonarius* (ITAL 204) were optimum was at 0.98 a_w and 25-30°C (see Appendix I). Most of the stains had minimum growth at 0.90 a_w at 37°C except for *A. niger* (A 1911) at 20°C (these results are detailed in Appendix I)

The OTA production was similar for *A. westerdijkiae* (B 2), *A. westerdijkiae* (CBS 121986), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) at 0.95 a_w , optimal temperature range of 20-30°C. The optimal OTA production for *A. niger* (A 1911) was at 0.99 a_w . Lowest OTA production was found at temperature 37°C for all strains and 0.90 a_w except *A. ochraceus* (ITAL 14) at 0.98 a_w (as detailed in Appendix I).

Statistically there was significant effect of temperature on lag phase (λ , days) at 20-35°C for all strains. For a few strains: *A. niger* (A 1911), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) there was a significant effect of a_w . *A. carbonarius* (ITAL 204) was the only strain that showed a significant effect of the interaction of a_w x temperature on lag phase.

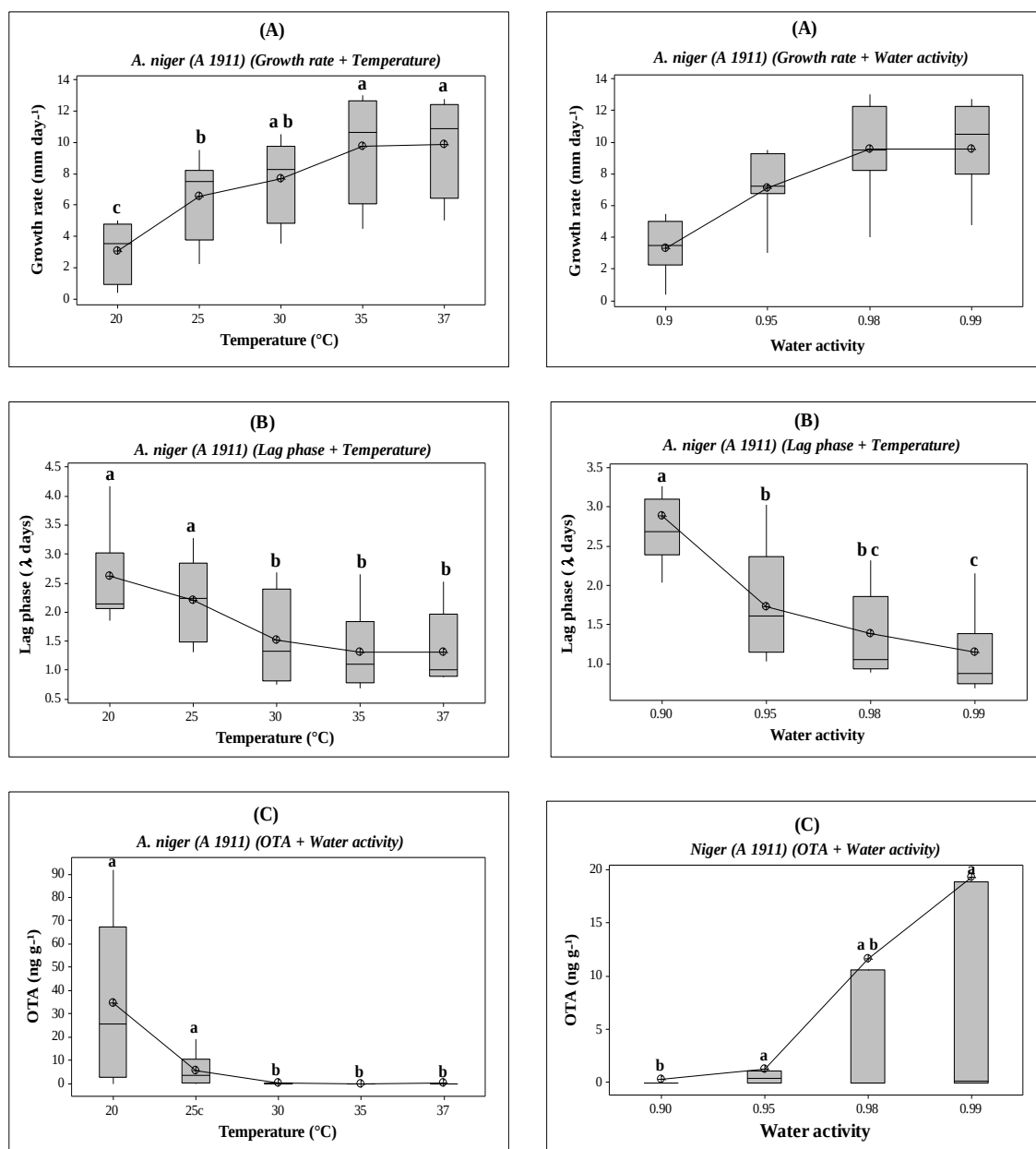


Figure 2.16: Box-plot analysis for fungal growth (A), lag phase (λ , days) (B) and OTA production (C) of *A. niger* (A 1911) inoculated on CMEA and incubated at 5 different temperature (20, 25, 30, 35 and 37°C) for 9 days. The box-plot analysis shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

Table 2.10: Summary statistical table for the growth rate and OTA production of five strains on different a_w and temperature by using Kruskal-Wallis Test (non normality data) and ANOVA (normality data).

Factors				
Strains	a_w	Temp (°C)	$a_w \times \text{Temp}(\text{°C})$	Response
<i>A. westerdijkiae</i> (B 2)	$P<0.05^{*a}$	$P<0.05^{*a}$	N/A	Growth rate (mm day ⁻¹)
	$P>0.05^a$	$P<0.05^{*a}$	N/A	Lag phase (λ , days)
	$P<0.05^{*a}$	$P<0.05^{*a}$	N/A	OTA (ng g ⁻¹)
<i>A. westerdijkiae</i> (CBS 121986)	$P<0.05^{*a}$	$P<0.05^{*a}$	N/A	Growth rate (mm day ⁻¹)
	$P>0.05^a$	$P<0.05^{*a}$	N/A	Lag phase (λ , days)
	$P<0.05^{*a}$	$P<0.05^{*a}$	N/A	OTA (ng g ⁻¹)
<i>A. niger</i> (A 1911)	$P<0.05^{*a}$	$P<0.05^{*a}$	N/A	Growth rate (mm day ⁻¹)
	$P<0.05^{*a}$	$P<0.05^{*a}$	N/A	Lag phase (λ days)
	$P<0.05^{*a}$	$P<0.05^{*a}$	N/A	OTA (ng g ⁻¹)
<i>A. carbonarius</i> (ITAL 204)	$P<0.05^{*b}$	$P<0.05^{*b}$	$P<0.05^{*b}$	Growth rate (mm day ⁻¹)
	$P<0.05^{*b}$	$P<0.05^{*b}$	$P<0.05^{*b}$	Lag phase (λ , days)
	$P<0.05^{*a}$	$P<0.05^{*a}$	N/A	OTA (ng g ⁻¹)
<i>A. ochraceus</i> (ITAL 14)	$P<0.05^{*b}$	$P<0.05^{*b}$	$P<0.05^{*b}$	Growth rate (mm day ⁻¹)
	$P<0.05^{*a}$	$P<0.05^{*a}$	N/A	Lag phase (λ , days)
	$P<0.05^b$	$P<0.05^b$	$P<0.05^{*b}$	OTA (ng g ⁻¹)

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

^a Kruskal-Wallis test.

^b ANOVA.

* Significant

N/A Not Applicable

2.2.4 Discussion

This is the first study that has focused on the effect of different coffee concentrations (10-80%) on the lag phase (λ , days), growth and OTA production by strains of these species. This is important for utilising the information to simulate what might occur in naturally contaminated coffee beans. The use of a model system is valuable if it accurately parallels what may happen naturally, especially when developing predictive models.

For most of the strains, the relative lag phase was not affected by the concentration of coffee extract used in the media. Overall, in the low concentration (10%) coffee extract medium, production of OTA was reduced for strains of *A. westerdijkiae* (B 2, CBS 121986) and *A. niger* (A 1911) when compared with the other extract concentrations tested. At 80% coffee extract, the highest OTA production was found, although growth was significantly reduced. Except for the strains of *A. carbonarius* (ITAL 204), *A. ochraceus* (ITAL 14) and *A. steynii* (CBS 112814), which had low growth rates, the OTA production was low at concentrations in the range 10-30% coffee media, but increased with higher coffee concentrations in the media. This study certainly suggests that coffee concentration will have an impact on OTA production and thus possibly OTA contamination. It may be that the nutritional differences represent a stress effect, which thus influences the secondary metabolite production, in this case, OTA.

In studies with a standard coffee concentration, it was found that a_w , temperature and $a_w \times$ temperature had a profound influence on growth and OTA production by these strains/species on a green coffee-based medium. This study has shown that both these factors and their interactions will have an effect on the dominance of these fungi in stored coffee and that the optimum for these species for (a) growth and (b) OTA production may be different. The growth rate pattern for most of strains was higher at 0.98-0.99 and 25-30°C except for *A. niger* (A 1911) at 37°C. For OTA production, *A. carbonarius* (ITAL 204) produced the highest amounts at 0.99 a_w and 25°C. In contrast, low OTA production was produced by *A. ochraceus* (ITAL 14). Temperatures of 25-35°C had a more significant effect than 20 and 37°C. The lowest OTA production for most of strains was at 37°C and 0.90 a_w except for *A. ochraceus* (ITAL 14) at 0.98 a_w .

As the conditions became more stressful, the lag time increased and the growth rate decreased. This also shows that there is a differential effect of these two climate-related factors on both growth and OTA production *in vitro* and needs to be tested *in situ* on stored coffee.

Taniwaki et al. (2003) reported that *A. ochraceus* (= *A. westerdijkiae*) was the major species responsible for OTA in green coffee. The minimal a_w for growth was 0.85. Overall, growth has been shown to be reduced by 50% at 0.93 a_w (Astoreca et al., 2007). Pardo et al. (2005a) reported the optimal conditions for growth on CMEA of *A. ochraceus* were at 0.95–0.99 a_w and 20–30°C. The minimum a_w level for germination was 0.80, and for mycelial growth 0.85 a_w . While, the maximum and no OTA production for *A. ochraceus* were 0.99 and 0.80 a_w respectively. These were 0.99 and 0.85 for *A. niger*, respectively. Previous studies suggest that optimum OTA production by *A. ochraceus* was at 0.95 a_w with no production at 0.90 a_w . For *A. niger* the optimum was at 0.99 a_w and 35°C (Suarez-Quiroz et al., 2004a). The maximum growth for type strains of *A. westerdijkiae* on YES media occurred at 0.95 a_w and 30°C. Optimum OTA production was at 25°C (Abdel Hadi et al., 2009). With the exception of the work of Pardo et al. (2005a) most of the other studies did not use a coffee-based medium. For coffee-based media, *in vitro* systems may not accurately simulate what may occur *in situ*. This is important as it suggests that studies in natural coffee beans may be more important. However, the type of data in this study can be utilised to understand whether the boundary conditions for growth and OTA production may change with nutrition. The optimum conditions for OTA production may be different in naturally contaminated coffee beans.

2.3 Effect of caffeine concentration on ochratoxigenic fungal strains

2.3.1 Introduction

Caffeine (1,3,7-trimethylxanthine) has been shown to have an inhibitory effect on a number of mycotoxigenic *Aspergillus* and *Penicillium* species by reducing both growth and mycotoxin production such as aflatoxins, ochratoxin A, sterigmatocystin, patulin and citrinin (Buchanan et al., 1978; 1982; 1983; Nartowicz et al., 1979). The caffeine concentration endogenously present in coffee varies with type of bean. Thus, Arabica coffee contains approx. 0.6% caffeine, while Robusta coffee about 4% (Oestreich-Janzen, 2010). Therefore, the question arises as to whether caffeine concentration may influence the ability of ochratoxigenic fungi to colonise and produce OTA in different concentrations of caffeine.

Some studies have shown that caffeine plays a role in aflatoxin inhibition in coffee (Nartowicz et al., 1979). Tsubouchi et al. (1985) reported inhibition of growth and OTA production by *A. ochraceus* IFM 0458 in YES medium containing 1% caffeine. In contrast, the production of OTA was found to be higher in the presence of 0.5 and 1.0% caffeine in another *A. ochraceus* IFM S-235 strain. There are few studies regarding caffeine degradation in fungi; nine strains of *A. ochraceus*, three of *A. elegans* and one of *A. sclerotiorum* isolated from green coffee beans and grown on ground coffee-based media were shown to significantly degrade caffeine (Tsubouchi et al., 1985).

Kurtzmann & Schwimmer (1971) studied caffeine degradation by *Stemphylium* and *Penicillium roqueforti* in roasted coffee infusion media with 0.45-0.59 mg ml⁻¹ caffeine. *Pseudomonas putida*, which was isolated from coffee plantation soil, was able to degrade caffeine at up to 5 g L⁻¹ (Dash and Gummadi, 2006). In addition, a strain of *A. niger* isolated from coffee husk was able to degrade caffeine by up to 90% in solid-state fermentation (Brand et al., 2000). Penaloza et al. (1985) showed suppression of caffeine degradation when inorganic nitrogen was added to moist coffee pulp media during solid-state fermentation by *A. niger*. In contrast, Roussos et al. (1994) found no degradation of caffeine by *Penicillium verrucosum* when NH₄Cl and a nitrogen source

(urea) were added during solid-state fermentation of coffee pulp and complete degradation in the absence of both.

Recently, Nayak et al. (2013) showed an increase in caffeine degradation by *Gliocladium roseum*, *Fusarium solani*, and *Aspergillus restrictus* when a nitrogen source was used in media. However, for *Chrysosporium keratinophilum* degradation was better in the absence of nitrogen. In addition, some species appear to have a resistance to high concentrations of caffeine such as *Fusarium solani*, *Gliocladium roseum*, *Chrysosporium keratinophilum*, and *Aspergillus restrictus* when grown in solid minimal medium with caffeine as the sole carbon source.

The objective of the present study was to examine the effect of different concentrations of caffeine on the lag phase, growth and OTA production by *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* strains on conducive YES-based media at 0.98 a_w and 30°C.

2.3.2 Materials and methods

a) Fungal strains

The same five fungal strains were used as previously detailed in Section 2.2.2 (a).

b) Media

The YES media were prepared as described in Section 2.1.2. To this medium caffeine concentrations (%) (Sigma-Aldrich) were added in the range 0.5-4%. The pH (Hanna Instrument HI 8519N pH meter) of the coffee stock and caffeine were measured and found to be 5.8 and 4.5 respectively. The YES medium pH was adjusted to pH 6 by using a Universal buffer mixture by combining 63.15 g of 0.2 M Na_2HPO_4 ml^{-1} and 36.85 g of 0.1 M Citrate acid ml^{-1} (McIlvaine's buffer solutions). After caffeine addition, glycerol was added (9.2 g) to adjust to final a_w to 0.98. The media were autoclaved at 121°C for 10 min and poured into 9 cm Petri plates, cooled and kept in closed polyethylene bags at 4°C until used.

c) Inoculum preparation

This was made as described previously in Section 2.2.2 (d).

d) Extraction, detection and quantification of OTA

As described in section 2.3.2 (e).

e) Calculation of lag phase prior to growth and growth rate

As described in section 2.1.2 (b-3)

f) Data analysis

As described in section 2.3.2 (f).

2.3.3 Results

The effect of caffeine (0.5-4%) on the lag phase (λ , days), growth (mm day^{-1}) and OTA production (ng g^{-1}) of *A. westerdijkiae* (B2, CBS 121986), *A. niger* (A 1911), *A. carbonarius* (ITAL 204), *A. steynii* (CBS 112814) and *A. ochraceus* (ITAL 14) on YES media pH 6 at 0.98 a_w and 30°C is shown in Figures 2.18 to 2.23.

This clearly shows that for all the strains examined, there was an increase in the lag phase as the caffeine concentration was increased. In terms of growth rate *A. westerdijkiae* (B2, 121986), were reduced by almost 60% with 0.5% caffeine and by >90% in 4% caffeine concentration in the strains when compared to the controls (Figure 2.18, 2.19). However, for *A. niger* (A 1911) and *A. carbonarius* (ITAL 204) there was almost complete inhibition with >0.5% caffeine concentration (Figures 2.20, 2.21). For *A. ochraceus* (ITAL 14) there was a decrease in growth by about 50% and 94% in 0.5 and 4% caffeine respectively. In the case of *A. steynii* (CBS 112814), growth was inhibited by almost 70% in 0.5% caffeine and reduced by >90% in 4% caffeine compared to control (Figure 2.23).

Similar results were observed for OTA production. For almost all the strains, with the exception of *A. steynii* (CBS 112814) and *A. ochraceus* (ITAL 14), at 0.5% caffeine concentration, OTA was inhibited. *A. steynii* (CBS 112814) was relatively tolerant to 0.5-1.0% caffeine with only a 24% reduction in OTA production. The only exception was *A. ochraceus* (ITAL 14) where OTA production was significantly increased in the presence of 2-4% caffeine. The highest OTA production of *A. ochraceus* (ITAL 14) reached was 8.7 ng g^{-1} in 3% caffeine concentration.

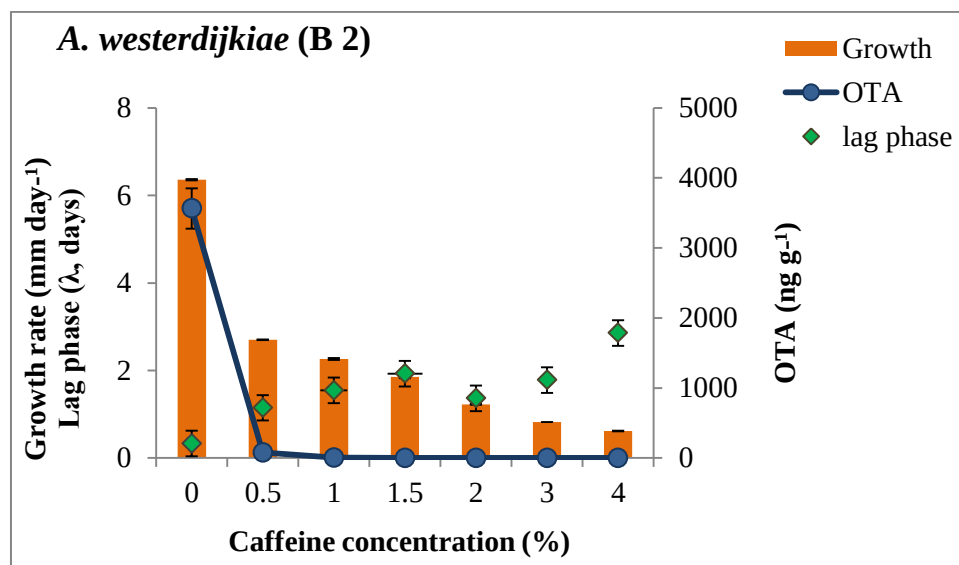


Figure 2.17: Effect of caffeine concentration (0.5-4%) on lag phase (λ , days), growth and OTA production of *A. westerdijkiae* (B 2) on 0.98 a_w YES medium at 30°C for 9 days. Bars indicate standard error of the mean.

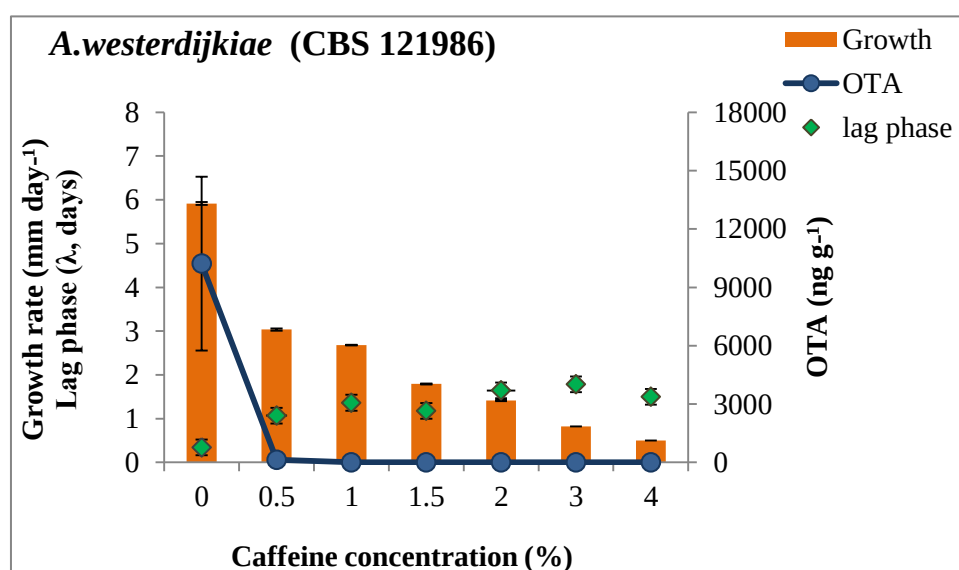


Figure 2.18: Effect of the caffeine concentration (0.5-4%) on lag phase (λ , days), growth and OTA production of *A. westerdijkiae* (CBS 121986) on 0.98 a_w YES medium at 30°C for 9 days. Bars indicate standard error of the mean.

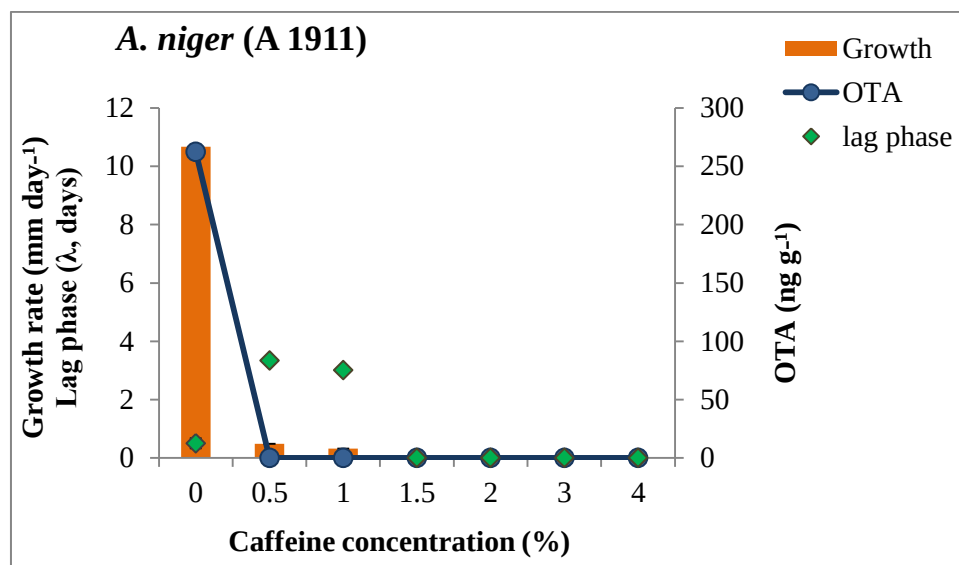


Figure 2.19: Effect of caffeine concentration (0.5-4%) on lag phase (λ , days), growth and OTA production of *A. niger* (A 1911) on 0.98 a_w YES medium at 30°C for 9 days. Bars indicate standard error of the mean. Note: in 1.5-4% caffeine no growth occurred and thus no OTA analyses were done.

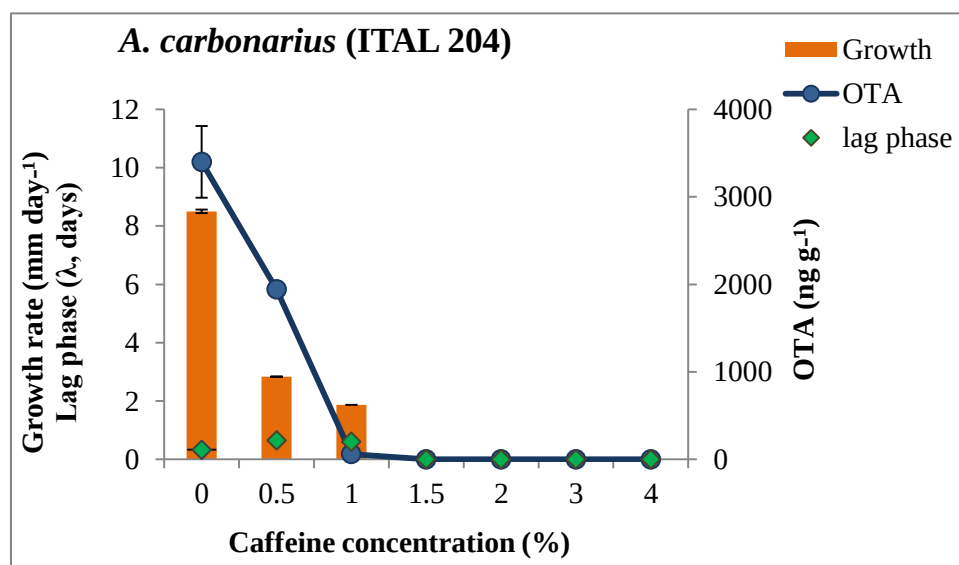


Figure 2.20: Effect of caffeine concentration (0.5-4%) on lag phase (λ , days), growth and OTA production of *A. carbonarius* (ITAL 204) on 0.98 a_w YES medium at 30°C for 9 days. Bars indicate standard error of the mean. Note: in 1.5-4% caffeine, no growth occurred and thus no OTA analyses were done.

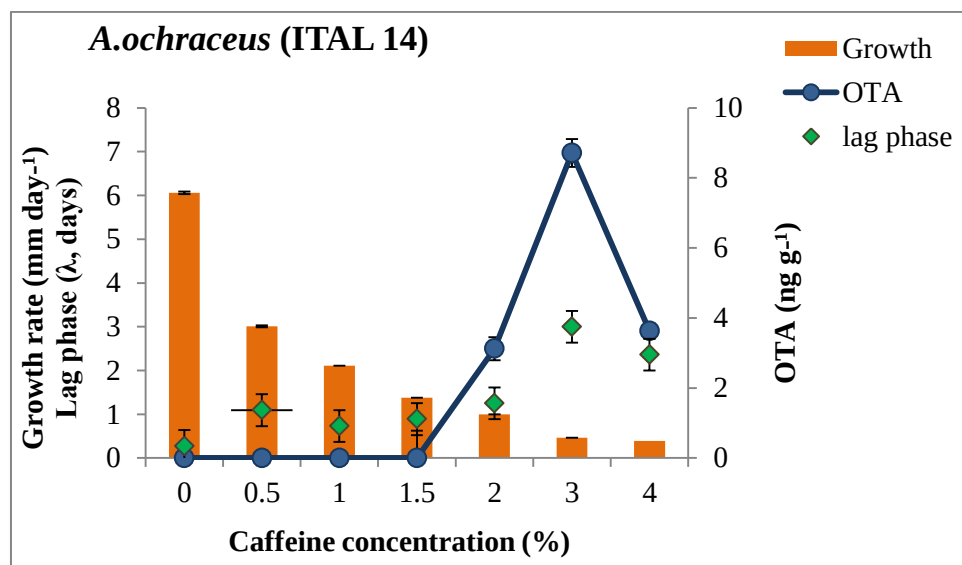


Figure 2.21: Effect of caffeine concentration (0.5-4%) on lag phase (λ , days), growth and OTA production of *A. ochraceus* (ITAL 14) on 0.98 a_w YES medium at 30°C for 9 days. Bars indicate standard error of the mean.

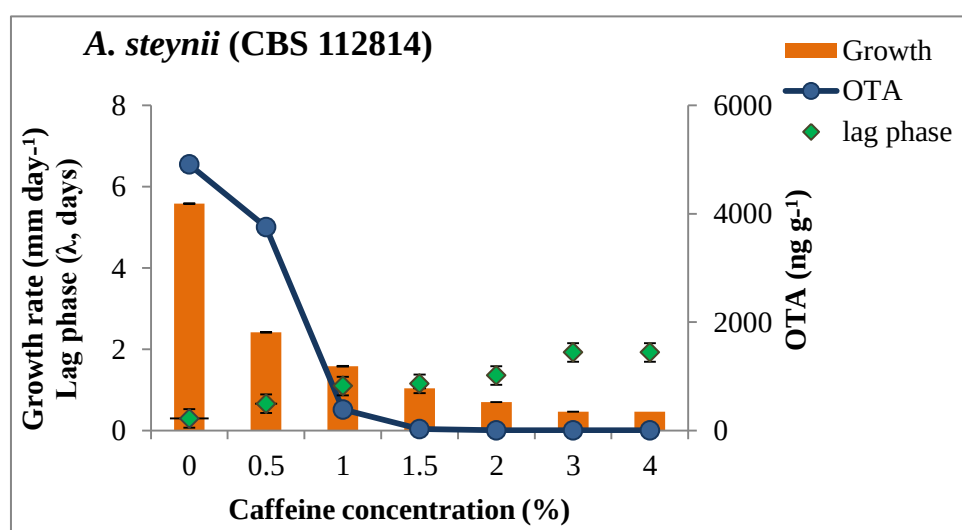


Figure 2.22: Effect of caffeine concentration (0.5-4%) on lag phase (λ , days), growth and OTA production of *A. steynii* (CBS 112814) on 0.98 a_w YES medium at 30°C for 9 days. Bars indicate standard error of the mean.

Statistically, the caffeine concentration had a significant effect ($p < 0.05$) on the lag phases prior to growth, growth and OTA production (Table 2.10). The detailed statistical analyses are presented in Appendix I.

Appendix I shows the effect of the lag phase (λ , days) on the different strains subject to different levels of caffeine concentration (0, 0.5, 1, 1.5, 2, 3 and 4%) and 0.98 a_w at 30°C for 9 days. This shows that an increase in caffeine results in an increase of the lag in all strains.

The growth of all strains was significantly ($P < 0.05$) affected by caffeine concentration, with the highest growth at the lowest concentration 0.5% for all strains and this was reduced with increasing caffeine concentration (see Appendix I).

Appendix I shows significant differences between amounts of OTA produced by an individual strain. Statistical analysis showed that, overall, the mean OTA production were variable among all strains. A similar effect on OTA production was found in the two strains at *A. westerdijkiae* (B 2, CBS 121986), where a large reduction in OTA production occurred in all the caffeine concentrations. The highest OTA production was found in *A. carbonarius* (ITAL 204) and *steynii* (CBS 112814) at 0.5% caffeine concentration. This was reduced as caffeine concentration was increased. In contrast, little production of OTA was found in *A. ochraceus* (ITAL 14).

Table 2.11: Summary statistical table for the Lag phase (λ , days), growth rate and OTA production of 6 strains on caffeine concentration by using Kruskal-Wallis Test (non normality data) and ANOVA (normality data).

Factors		
Strains	Caffeine concentration (%)	Response
<i>A. westerdijkiae</i> (B 2)	S ^b	Lag time (λ , days)
	S ^a	Growth rate (mm day ⁻¹)
	S ^a	OTA (ng g ⁻¹)
<i>A. westerdijkiae</i> (CBS 121986)	S ^b	Growth rate (mm day ⁻¹)
	S ^a	Lag phase (λ , days)
	S ^a	OTA (ng g ⁻¹)
<i>A. niger</i> (A 1911)	S ^a	Growth rate (mm day ⁻¹)
	S ^a	Lag phase (λ days)
	S ^a	OTA (ng g ⁻¹)
<i>A. carbonarius</i> (ITAL 204)	S ^a	Growth rate (mm day ⁻¹)
	S ^a	Lag phase (λ , days)
	S ^a	OTA (ng g ⁻¹)
<i>A. ochraceus</i> (ITAL 14)	S ^a	Growth rate (mm day ⁻¹)
	S ^a	Lag phase (λ , days)
	S ^a	OTA (ng g ⁻¹)
<i>A. steynii</i> (CBS 112814)	S ^a	Growth rate (mm day ⁻¹)
	S ^a	Lag phase (λ , days)
	S ^a	OTA (ng g ⁻¹)

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

S Significant

^a Kruskal-Wallis test.

^b ANOVA.

2.3.4 Discussion

No previous studies have examined the effect of different concentrations of caffeine on growth and OTA production by strains of the *Aspergillus* sections *Circumdati* and *Nigri*. This study has shown that increasing concentrations of caffeine have a significant impact on both growth and OTA production by the examined strains/species. Overall, for strains of *A. niger* (A 1911) and *A. carbonarius* (ITAL 204) there was inhibition of both growth and OTA production by >1% caffeine concentration.

Overall, more *Arabica* (*Coffea arabica*) than *Robusta* (*Coffea canephora*) is consumed which accounts for 75% and 25% of the total world-wide consumption. Robusta coffee beans have a more concentrated amount of caffeine reaching 4–5% and is characterised by its bitterness, and is less aromatic. Arabica coffee has a much more flavour and aroma and has less than half the caffeine of Robusta beans (Kreicbergs et al., 2011). This could be important in terms of colonisation by ochratoxigenic fungi and OTA contamination in these two types of coffee beans. However, few studies have considered this aspect.

Previously, Buchanan et al. (1981) reported an inhibitory effect of caffeine on OTA biosynthesis and growth of *A. ochraceus*. This might be due to caffeine interfering with lipid accumulation. Some fungal species accumulate lipids, including *A. flavus* and *A. ochraceus* as a result of secondary metabolism (Gupta et al., 1970; Shin and Marth, 1974). Previously, growth and OTA production by a strain of *A. ochraceus* was completely inhibited by caffeine at 1.0 and 2.0% concentrations on YES medium after 3–21 days (Nehad et al., 2005). It may be possible that caffeine content affects the colonisation and production of OTA. This will be investigated in the next experiment

Interestingly, *A. steynii* was more tolerant while *A. ochraceus* (ITAL 14) was stimulated to produce high amounts of OTA at >2% caffeine concentrations. Previously, caffeine concentrations of 1-2% were found to completely inhibit the growth and OTA production by *A. ochraceus* on a conducive YES medium over 3-21 days incubation (Nehad et al., 2005). In addition, Buchanan et al. (1981) reported an inhibitory effect of caffeine on growth and OTA biosynthesis of *A. ochraceus*. It was suggested that this

may be due to the caffeine interfering with lipid accumulation. Some fungal species accumulate lipids as a result of secondary metabolism, including *A. flavus* and *A. ochraceus* (Gupta et al., 1970; Shin and Marth, 1974). It appears that a higher caffeine concentration actually inhibits growth and mycotoxin production by some mycotoxigenic species, although there may be some exceptions. This area needs more investigation to better understand the relationship between caffeine content of coffee, the contamination with mycotoxigenic fungi and the interactions at a molecular and physiological level.

It has been reported that decaffeinated green coffee beans, when compared with the normal green coffee beans, have a higher content of aflatoxins produced by *A. flavus* (Soliman, 2002). When 1-2% caffeine was added, the growth of *A. flavus* decreased by 50%, and aflatoxin was inhibited. However, these studies were carried out in a liquid medium only.

CHAPTER 3

IMPACT OF CLIMATE CHANGE FACTORS (a_w x TEMP x CO₂) ON GROWTH AND OTA PRODUCTION *IN* *VITRO* AND *IN SITU*

3.1 Introduction

It has been suggested that climate change factors will have a profound impact on sustainable food production systems. Based on existing information on climate change, it is estimated that there will be regions in the world where the temperature will increase by between 2 and 4°C, linked to an increase in atmospheric CO₂ from approx. 350-400 ppm to 650-700 or 1000 ppm (x2 or x3 increase) and episodes of heavy rainfall or periods of drought (Paterson & Lima, 2010; Wu et al. 2011; Magan et al. 2011; Medina et al. 2014; Medina et al. 2015). The only detailed studies so far examining these three factors is the work on *A. flavus* and aflatoxin production by Medina et al. (2015). This suggests that, while growth would not be significantly affected, aflatoxin B₁ (AFB₁) production would be stimulated when exposed to these combined factors together. No such studies have been carried out with fungi which contaminate coffee with OTA from the *Aspergillus* sections *Circumdati* and *Nigri*.

Most previous studies with CO₂ have considered the utilisation of modified atmospheres at much higher levels to control mycotoxin production, especially during storage. Wilson and Jay (1975) showed that *A. flavus* could grow after 4 weeks when treated with modified atmospheres but AFB₁ was reduced when treated with 61.7% CO₂ balanced with O₂ and N₂ in moist maize at 27°C. In addition, *A. flavus* can grow with up to 75% CO₂ when balanced with different O₂ and N₂ concentrations in both wheat and rye bread. Pateraki et al. (2007) suggested that elevated CO₂ at up to 50% , regardless of a_w level, does not effectively control germination of *A. carbonarius* on grape-based media. However, other studies with *A. carbonarius* and *A. niger* in 15% CO₂ combined with 1% O₂ resulted in a reduction in fungal growth and OTA production (Valero et al. 2008). Giorni et al. (2007) found that at least 50% CO₂ was needed to reduce AFB₁ production by *A. flavus* on synthetic maize-based and in stored maize grain at 0.95 and 0.92 a_w for 21 days at 25°C. Up to 75% CO₂ was required to inhibit AFB₁ production. Other studies with *A. ochraceus* suggest that 80% CO₂ was required for effective inhibition of OTA on agar-based media (Paster et al., 1983). For other mycotoxigenic fungi such as *Penicillium verrucosum*, at least 50% CO₂ in the range of 0.90-0.95 a_w was required for significant inhibition of OTA production (Cairns-Fuller et al., 2005).

The hypothesis was whether combinations of a_w x temperature increase (30 or 35°C) and elevated (400 or 1000 ppm CO₂) will increase or reduce lag phases prior to growth, growth rates and ochratoxin A production *in vitro* and *in situ* in stored coffee. These studies were carried out with strains of *A. westerdijkiae* (B 2, CBS 121986), *A. niger* (A 1911), *A. carbonarius* (ITAL 204), *A. ochraceus* (ITAL 14) and *A. steynii* (CBS 112814).

3.2 Materials and methods

3.2.1 *In vitro* effects of interacting climate change environmental factors on growth and OTA production

a) Fungal strains

As described in Section 2.2.2. (c)

b) Media used in these studies

In this study, a Coffee meal extract agar (CMEA) was used as described in Section 2.2.2. (b). The media were modified to 0.95, 0.98, and 0.99 a_w using glycerol. The media were autoclaved at 121°C for 10 min and poured into 9 cm Petri plates and kept at 4°C until used. The final a_w levels were checked with a water activity meter (Aqualab 3TE, Decagon Devices, Inc., Pullman, Washington, USA).

c) Inoculum preparation, inoculation and measurement

The inoculum was prepared by growing each strain on MEA at 25°C for 7 days. The spore suspensions were prepared by agitating the colony surface with a sterile spatula in 9ml of sterile distilled water containing 0.05% Tween 80. The CMEA media were centrally point inoculated with 7 μ l of the spore suspension. Three replicates were prepared for each strain. The replicates of the same treatment were enclosed together in a plastic chamber. Cultures with the same a_w were enclosed together in a plastic chamber with two valves: one for the intake of CO₂ and the other for flushing out. A 500 ml beaker of glycerol/water solution of the same a_w 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 5 L of 1000 ppm CO₂ from a gas cylinder (British Oxygen Company, 1000 ppm CO₂ cylinder) for about 8 minutes and then sealed (Medina et al., 2015). The same procedure was carried out for the control treatment (400 ppm). The chambers were incubated for 9 days at 30 and 35°C and fungal growth measured daily.

d) Calculation of lag phase prior to growth and growth rate

As described previously in Section 2.1.2 (b-3)

e) Extraction and quantification of OTA from *in vitro* studies using HPLC

Five plugs from each replicate treatment and from each strain were extracted from each colony after 9 days incubation at 30 and 35°C on CMEA media, transferred to 2 ml Eppendorf tubes and weighed. 750 µl methanol were added to each Eppendorf tube. The samples were then shaken using a KS 501 digital orbital shaker for 30 min and centrifuged for 10 min at 15000 xg. The supernatant was filtered and analysed with a HPLC system (Agilent, Berks., UK). 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 100 mm, diameter 4.6 mm, particle size 2.7 micron; 600 Bar)
Temperature of column	25°C
Excitation	333 nm
Emission	460 nm
Flux	1 ml min ⁻¹
Volume of sample injected	50 µL
Retention time	Approximately 3.842 min
Run time	12 min
Limit of detection	0.097 ng g ⁻¹
Limit of Quantification	0.323 ng g ⁻¹

f) Data analysis

A full factorial design with three factors: water activity, temperature and CO₂ was applied. Each treatment, a_w x temperature x CO₂ combination was carried out in triplicate, both for growth rate assessment and OTA production. Normality was checked using the Kolmogorov-Simonov test. Analysis of data, the effects of a_w , temperature, CO₂ and their interaction were examined by the Kruskal-Wallis (nonparametric) if the data was not normally distributed. For normally distributed data, the data sets were analysed using Minitab® 16 package (Minitab Inc., 2010. State College, PA, USA). The statistically significant level was set at $P < 0.05$ for all single and interacting treatments.

3.2.2 *In situ* effect of interacting climate change factors on growth and OTA production on coffee

Raw Arabica coffee beans from the Al Ameen coffee company (Kuwait) were used in these studies. 12-15 Kg of coffee beans were irradiated and kept at 4°C in sealed bags until used (12-15 K Grays, Isotron Ltd, Swindon, Berks.).

a) Preparation of spore suspensions from the strains

Five OTA producing species including *A. westerdijkiae* (B 2, CBS 121986), *A. niger* (A 1911), *A. carbonarius* (ITAL 204) *A. steynii* (CBS 112814) and *A. ochraceus* (ITAL 14) previously isolated from coffee beans were cultured on MEA at 25°C for 7 days, and spore suspensions prepared by agitating the colony surface with a sterile spatula in 9 ml of sterile distilled water containing 0.05% Tween 80. The concentration was adjusted by dilution to $\sim 10^4$ spores/ml and confirmed using a haemocytometer.

b) Determination of the moisture adsorption curve of green coffee beans

A moisture adsorption curve (MAC) was constructed to determine the amounts of water to be added to the irradiated green coffee beans to obtain the target a_w levels of 0.90, 0.95 and 0.97. The curve was prepared by adding different known amounts of water to 10 g green coffee bean samples and equilibrating at 4°C for 24 hr, then measuring the a_w of the hydrated green coffee beans. The coffee was then dried at 110°C for 24 hr and then kept in a desiccator at room temperature for 1 hr and the coffee beans weighed to determine the moisture content. The a_w of the green coffee beans was measured using an Aqualab 3TE (Decagon Devices, Inc., Pullman, Washington, USA). Figure 3.1 shows the moisture adsorption curve and the relationship between added water and a_w values.

Subsequently 325 g of irradiated raw coffee was weighed and water added using the MAC to obtain the required a_w levels and kept at 4°C for 24 hr. The moist raw coffee beans were then divided into six sub-samples (50 g) in solid substrate culture vessels (Magenta, Sigma Ltd, UK) which could be closed using lids with a permeable microporous membrane lid.

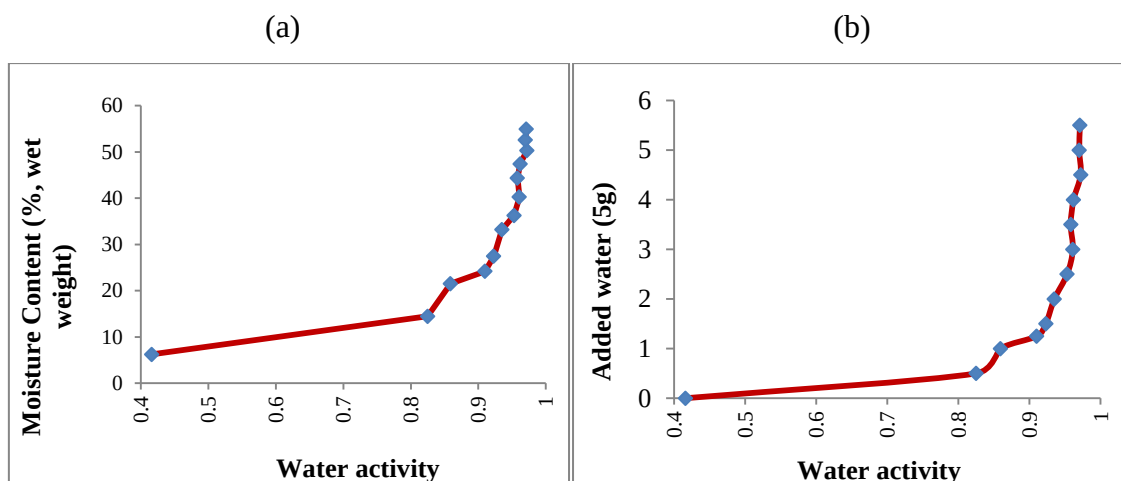


Figure 3.1: (a) Adsorption moisture curve of raw coffee beans and (b) amounts of added water required to obtain target water activity levels to 5 g of green coffee.

c) Inoculation of coffee beans with different OTA producing strains

Using the methodology of Palacios-Cabrera et al. (2004), 0.5 ml of spore suspension (10^4 CFU ml^{-1}) [*A. westerdijkiae* (B 2), *A. westerdijkiae* (CBS 121986); *A. niger* (A 1911), *A. carbonarius* (ITAL 204), *A. ochraceus* (ITAL 14)] of each were added to 50 g of raw green coffee beans and shaken well. Twenty five grams of coffee beans were used as a control at each a_w level. The replicates of the same treatment were enclosed together in a plastic chamber. Culture vessels with the same a_w were enclosed together in a plastic chamber with two openings: one for intake of CO_2 and other for flushing out. The methodology was the same as that used for the *in vitro* studies for CO_2 , flushing either air (400 ppm) or 1000 ppm CO_2 . The inoculated vessels were incubated for 12 days at 30 and 35°C. They were then destructively sampled by grinding 25 g of coffee beans at the end of the storage period. These were stored at -20°C until OTA extraction and analysis was carried out by HPLC.

d) Extraction and quantification of OTA from the coffee experiments

OTA was extracted using an immunoaffinity column clean up procedure based on the Neogen Neocolumn method. Ten grams of milled coffee beans were extracted with a 50 ml solution of methanol: water (70:30) in 1% sodium bicarbonate. The extracts were filtered and 5 ml diluted with 45 ml phosphate buffered saline (PBS/Tween (0.01% v/v)

and applied to an immunoaffinity column (Neogen Europe Ltd, UK) as shown in Figure 3.2. 1.5 ml was dried and 0.5 ml of acetonitrile: water (50:50) added. The final extracts were injected (50 μ l) into the HPLC system (flow rate: 1 ml min⁻¹) equipped with a fluorescence detector (λ_{ex} 333 nm; λ_{em} 460 nm) and C18 column (Poroshell 120, length 100 mm, diameter 4.6 mm, particle size 2.7 μ m). The retention time of OTA under the conditions described was approximately 2.5 min. The mobile phases used were acetonitrile (57%): acetic acid (2%) and water (41%) (Medina and Magan, 2014).

e) Data analysis

As described in section 3.2.1 (e).

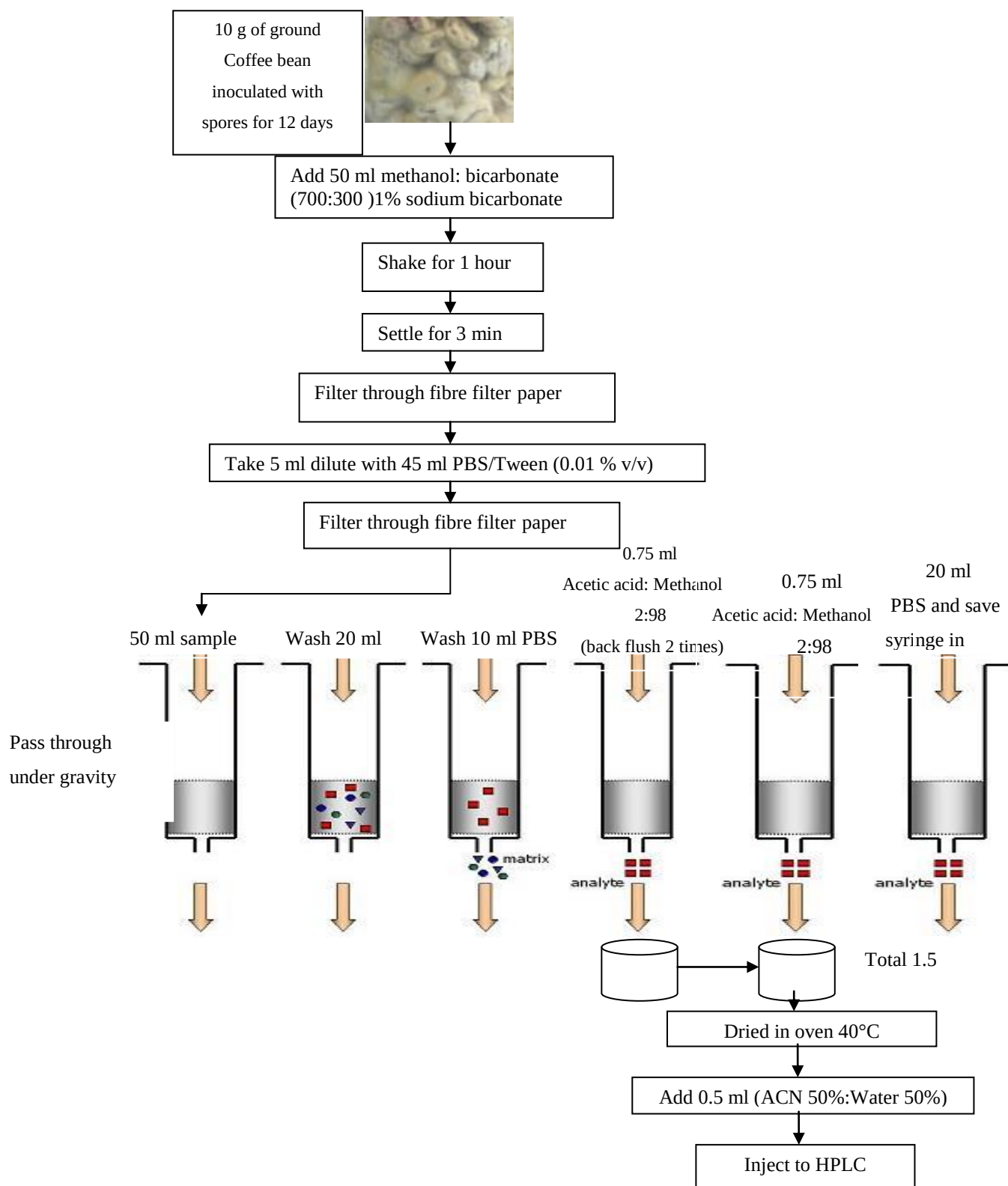


Figure 3.2: Pathway for the extraction of OTA in raw coffee beans inoculated with spores for 12 days used in this study.

3.3 Results

3.3.1 *In vitro* effect of water activity x elevated CO₂ x temperature on lag times prior to growth, growth and OTA production

The data for both lag phases prior to growth and relative growth rates are presented on the same Figures for each strain and species examined.

a) Effects of climate change on lag phase

Figures 3.3 to 3.8 compare the lag phase (λ , days) for all the six strains examined. Overall, there was an increase in the lag phases prior to growth for all species as water stress was imposed at 30°C. When the temperature was increased to 35°C, there was a change in the lag periods for some strains, especially when exposed to 1000 ppm CO₂ in the different a_w treatments. There were some differences in the lag phases prior to growth among the groups: *A. westerdijkiae* (CBS 121986) and *A. carbonarius* (ITAL 204) showed an increase in lag phase periods when exposed to 0.95 a_w and 1000 ppm CO₂ as compared to the control [see Figures 3.4 (b), 3.4 (b)]. In contrast, *A. westerdijkiae* (B 2) and *A. carbonarius* (ITAL 204) showed a decrease in lag phase periods when exposed to 0.98 a_w in air (400 ppm) when compared to the treatment with CO₂ (1000 ppm) [see Figures 3.3 (b), 3.6 (b)]. *A. niger* (A 1911) showed a longer delay in lag phases in all a_w levels [Figure 3.5 (b)].

b) Effects of climate change on relative growth of strains

The same Figures 3.3 to 3.8 compare the growth rate for different strains when exposed to air and elevated CO₂ (x 2.5 existing levels), different a_w levels and temperatures. The optimum growth for most of the strains examined was at about 0.98 a_w then 0.99 a_w , with a minimum at 0.95 a_w . The only exception was *A. steynii* (CBS 112814) where the optimum was 0.95 then 0.98 a_w and lowest growth observed was at 0.99 a_w and 30°C. In contrast, the *A. niger* (A 1911) and *A. carbonarius* (ITAL 204) isolates grew similarly at all a_w levels at 30°C. The minimum growth was observed for the *A. ochraceus* (ITAL 14) strain.

When the temperature was elevated to 35°C, growth was noticeably influenced by both CO₂ (1000 ppm) and a_w and their interaction. Generally, there was increase in growth

for all species in response to water stress at 35°C. Maximum growth rates and toleration of 35°C was observed in *A. niger* (A 1911), while the slowest growth was observed in *A. westerdijkiae* (B 2, CBS 121986) at all a_w levels when compared to the same conditions at 30°C [see Figures 3.3 (b), 3.4 (b), 3.5 (b)]. Compared to the controls (400ppm), mycelial growth increased in both *A. niger* (A 1911) and *A. steynii* (CBS 112814) all a_w levels (0.95, 0.98 and 0.99), respectively at 30°C [see Figure 3.5 (b), 3.8 (b)].

c) Statistical analyses of results

Statistically, Table 3.1 shows that the factors assayed (water activity and CO₂), as well as their interactions, had a significant effect on lag phase (λ , days) and mycelial growth for the six strains at 30°C and 35°C by using the Kruskal-Wallis and ANOVA Test. had a significant effect for most of the strains at 30°C. Moreover, the lag phases were significantly ($p < 0.05$) affected by elevated CO₂ and a_w and the interaction of $a_w \times$ modified atmosphere (CO₂) at all a_w levels tested, for *A. carbonarius* (ITAL 204), and *A. ochraceus* (ITAL 14), and at 0.95 and 0.98 a_w for *A. steynii* (CBS 112814) at 35°C. In addition, there was a significant effect of elevated in CO₂ (1000 ppm) and the interaction of $a_w \times$ CO₂ for *A. niger* (A 1911). A_w had an effect on *A. westerdijkiae* (B 2, CBS 121976) (see Appendix II).

Growth was significantly ($P < 0.05$) affected by elevated CO₂ at 30°C and 0.95 and 0.99 a_w for *A. westerdijkiae* (B 2) and *westerdijkiae* (CBS 121986) at all a_w levels. Furthermore, the growth rate of all strains, except *A. westerdijkiae* (CBS 121986), was influenced by a_w on CMEA. At 35°C, there was a statistically significant effect for most strains by a_w . Growth of *A. niger* (A 1911) and *A. steynii* (ITAL 14) was significant stimulated by CO₂ (1000 ppm). The effect of the various factors and their interactions were more significant at 35°C than at 30°C (see Appendix II).

Temperatures of 30°C and 35°C had a significant effect on lag phases and growth for all strains except on the lag phases for *A. westerdijkiae* (CBS 121986) and *A. steynii* (CBS 112814) (Table 3.1)

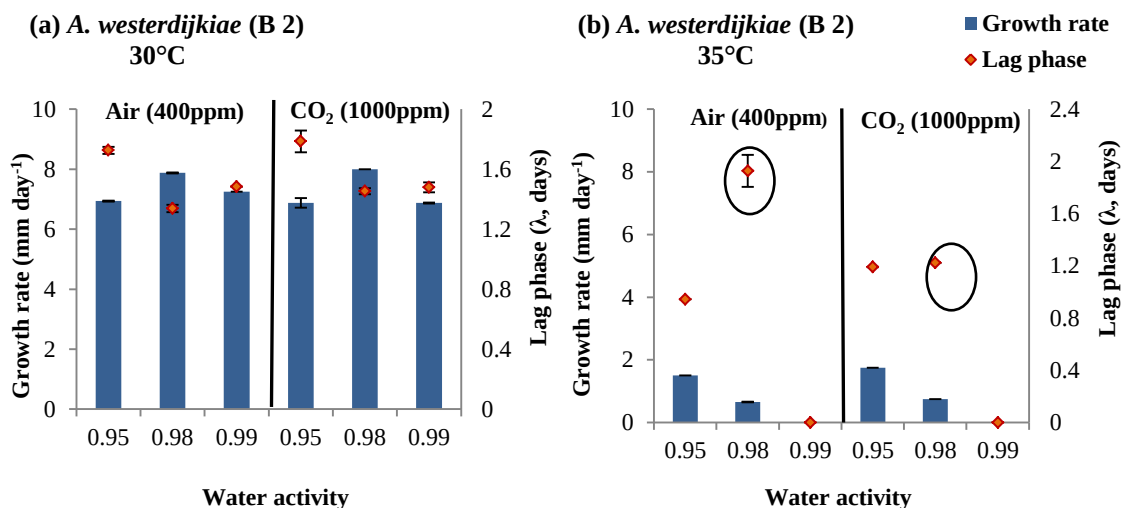


Figure 3.3: Effect of $a_w \times \text{CO}_2 \times$ temperature effects on growth (mm day⁻¹), the mean lag time (λ , in days) of *A. westerdijkiae* (B 2) grown on CMEA for 9 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean. Circle indicates significant difference.

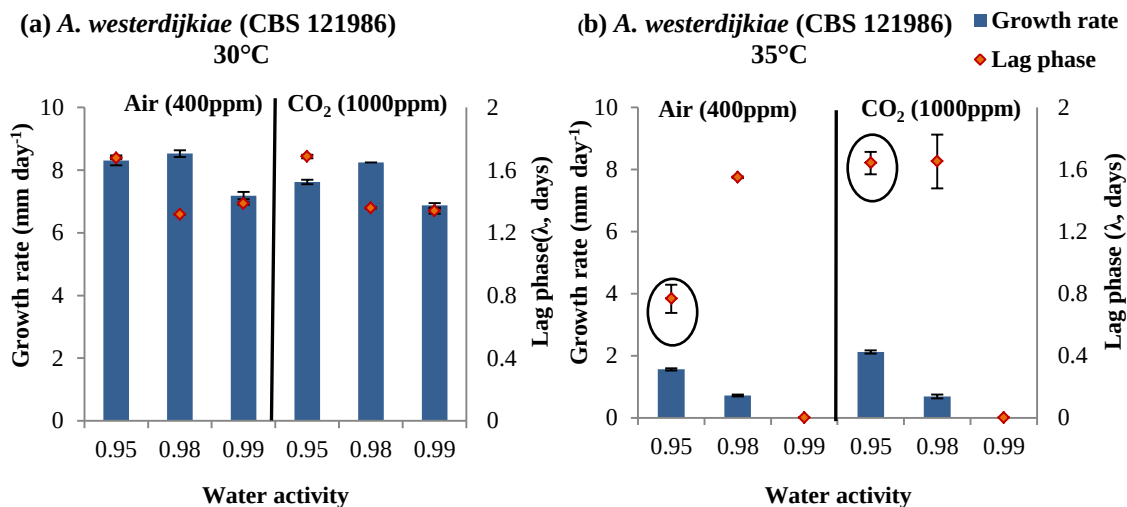


Figure 3.4: Effect of $a_w \times \text{CO}_2 \times$ temperature on growth (mm day⁻¹), the mean lag time (λ , in days) of *A. westerdijkiae* (CBS 121986) grown on CMEA for 9 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean. Circles indicate significant difference.

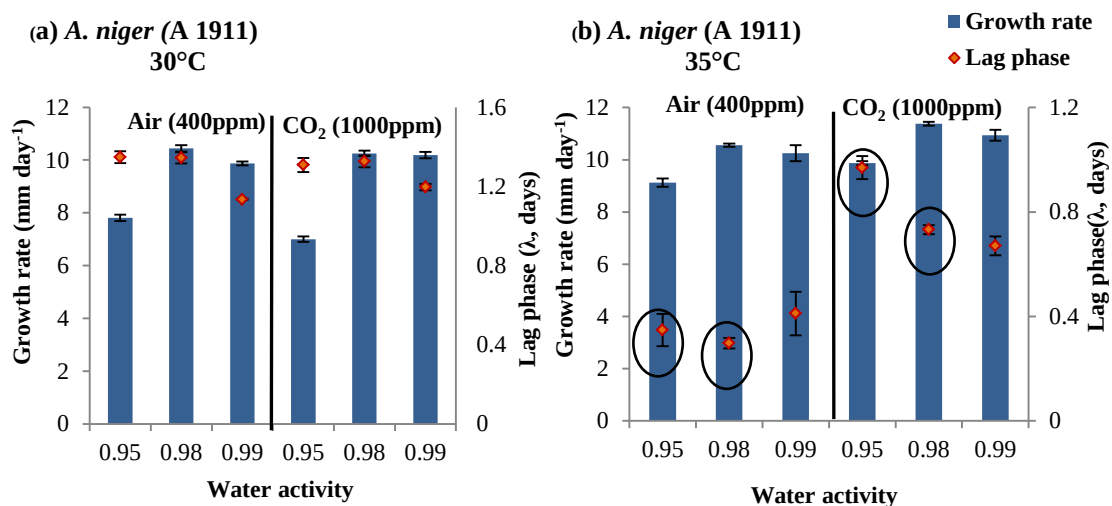


Figure 3.5: Effect of $a_w \times \text{CO}_2 \times$ temperature on growth (mm day⁻¹), the mean lag time (λ , in days) of *A. niger* (A 1911) grown on CMEA for 9 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean. Circles indicate significant difference.

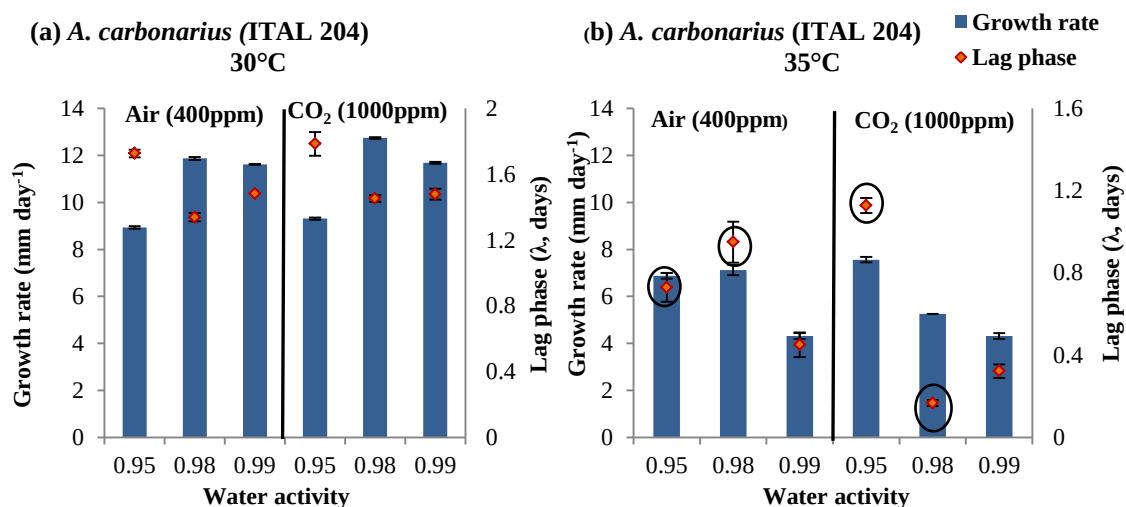


Figure 3.6: Effect of $a_w \times \text{CO}_2 \times$ temperature on growth (mm day⁻¹), the mean lag time (λ , in days) of *A. carbonarius* (ITAL 204) grown on CMEA for 9 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean. Circles indicate significant difference.

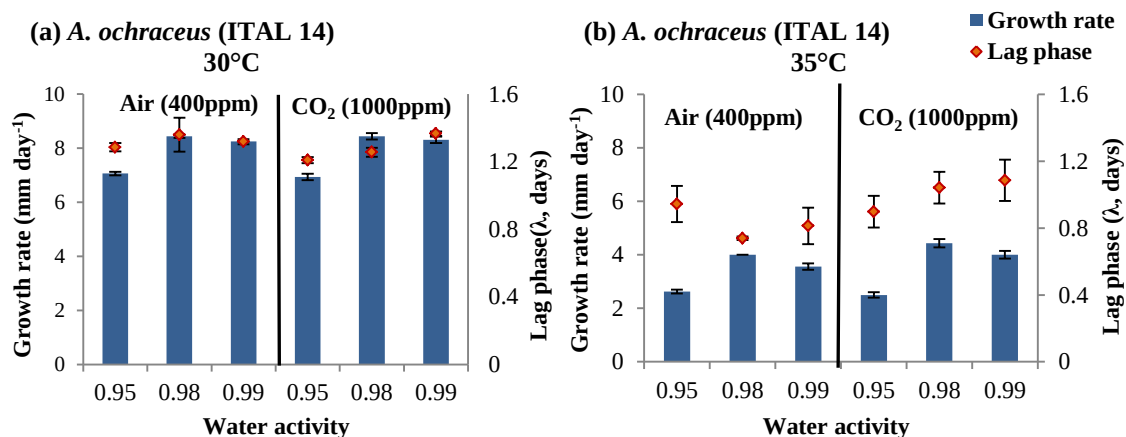


Figure 3.7: Effect of $a_w \times \text{CO}_2 \times$ temperature on growth (mm day⁻¹), the mean lag time (λ , in days) of *A. ochraceus* (ITAL 14) grown on CMEA for 9 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean.

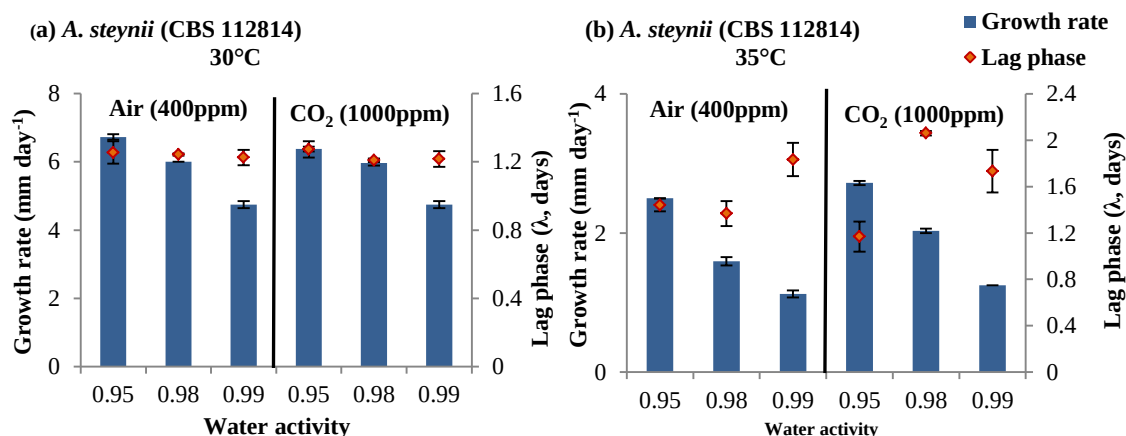


Figure 3.8: Effect of $a_w \times \text{CO}_2 \times$ temperature on growth (mm day⁻¹), the mean lag time (λ , in days) of *A. steynii* (CBS 112814) grown on CMEA for 9 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean.

Table 3.1: Summary statistical results for lag phase and growth rate of six strains in relation to CO₂, a_w and CO₂ x a_w at 30 and 35°C using the Kruskal-Wallis Test (non normality data) and ANOVA (normality data).

Temperature 30°C				
Factors				
Strains	CO ₂ (1000ppm)	a _w	CO ₂ X a _w	Response
<i>A. westerdijkiae</i> (B 2)	NS ^a	S ^a	-	Lag time (λ, days)
	NS ^a	S ^b	NS ^b	Growth rate (mm day ⁻¹)
<i>A. westerdijkiae</i> (CBS 121986)	NS ^b	S ^b	NS ^b	Lag time (λ, days)
	S ^a	NS ^a	-	Growth rate (mm day ⁻¹)
<i>A. niger</i> (A 1911)	NS ^b	NS ^b	NS ^b	Lag time (λ, days)
	NS ^a	S ^a	-	Growth rate (mm day ⁻¹)
<i>A. carbonarius</i> (ITAL 204)	NS ^a	S ^a	-	Lag time (λ, days)
	NS ^a	S ^a	-	Growth rate (mm day ⁻¹)
<i>A. ochraceus</i> (ITAL 14)	NS ^b	NS ^b	NS ^b	Lag time (λ, days)
	NS ^a	S ^a	-	Growth rate (mm day ⁻¹)
<i>A. steynii</i> (CBS 112814)	NS ^b	NS ^b	NS ^b	Lag time (λ, days)
	NS ^a	S ^a	-	Growth rate (mm day ⁻¹)
Temperature 35 °C				
Strains	CO ₂ (1000ppm)	a _w	CO ₂ X a _w	Response
<i>A. westerdijkiae</i> (B 2)	NS ^a	S ^a	-	Lag time (λ, days)
	NS ^b	S ^b	NS ^b	Growth rate (mm day ⁻¹)
<i>A. westerdijkiae</i> (CBS 121986)	NS ^a	S ^a	-	Lag time (λ, days)
	NS ^a	S ^a	-	Growth rate (mm day ⁻¹)
<i>A. niger</i> (A 1911)	S ^b	NS ^b	S ^b	Lag time (λ, days)
	S ^b	S ^b	NS ^b	Growth rate (mm day ⁻¹)
<i>A. carbonarius</i> (ITAL 204)	S ^b	S ^b	S ^b	Lag time (λ, days)
	NS ^a	S ^a	-	Growth rate (mm day ⁻¹)
<i>A. ochraceus</i> (ITAL 14)	S ^b	S ^b	S ^b	Lag time (λ, days)
	NS ^a	S ^a	-	Growth rate (mm day ⁻¹)
<i>A. steynii</i> (CBS 112814)	S ^b	S ^b	S ^b	Lag time (λ, days)
	S ^b	S ^b	NS ^b	Growth rate (mm day ⁻¹)
Temp 30 and 35°C				
	CO ₂ (1000ppm)	a _w	Temp 30+35°C	
<i>A. westerdijkiae</i> (B 2)	NS ^a	NS ^a	S ^a	Lag time (λ, days)
	NS ^a	NS ^a	S ^a	Growth rate (mm day ⁻¹)
<i>A. westerdijkiae</i> (CBS 121986)	NS ^a	S ^a	NS ^a	Lag time (λ, days)
	NS ^a	S ^a	S ^a	Growth rate (mm day ⁻¹)
<i>A. niger</i> (A 1911)	NS ^a	NS ^a	S ^a	Lag time (λ, days)
	NS ^a	S ^a	S ^a	Growth rate (mm day ⁻¹)
<i>A. carbonarius</i> (ITAL 204)	NS ^a	NS ^a	S ^a	Lag time (λ, days)
	NS ^a	S ^a	S ^a	Growth rate (mm day ⁻¹)
<i>A. ochraceus</i> (ITAL 14)	NS ^a	NS ^a	S ^a	Lag time (λ, days)
	NS ^a	NS ^a	S ^a	Growth rate (mm day ⁻¹)
<i>A. steynii</i> (CBS 112814)	S ^a	S ^a	NS ^a	Lag time (λ, days)
	NS ^a	NS ^a	S ^a	Growth rate (mm day ⁻¹)

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

^a Kruskal-Wallis test.

^b ANOVA.

NS not significant

S significant

d) *In vitro* effects of climate change factors on OTA production

The effect of elevated CO₂ in different a_w and temperature treatments on OTA production are compared in Figures 3.9-3.14. For most strains examined, there was no effect of elevated CO₂ on OTA production at 30°C. However, OTA production was reduced from approx. 43.1 to 17.2 ng g⁻¹, 1454.1 to 1128.6 ng g⁻¹ and 113.9 to 45.0 ng g⁻¹ for *A. westerdijkiae* (B 2), *A. carbonarius* (ITAL 204) and *A. steynii* (CBS 112814) respectively, when exposed to 1000 ppm CO₂ at 0.99 a_w [see Figures 3.9, 3.12, 3.14 (a)]. There was an increase in OTA production by about 45% by *A. niger* (A 1911) in elevated CO₂ at 0.98 a_w at 30°C [see circle in Figures 3.11(a)].

When the temperature was increased to 35°C in the presence of CO₂ (1000ppm), there was a stimulation effect on OTA production: 702.9, 64.74 and 48.63 at 0.98 a_w for *A. westerdijkiae* (B 2, CBS 121986) and *A. carbonarius* (ITAL 204) respectively compared to the controls of 2.6, 15.75 and 31.06ng g⁻¹ [see Figures 3.9,3.10,3.12 (b)]. The highest OTA production was by *A. westerdijkiae* (B 2) and the lowest was by *A. steynii* (CBS 112814) at 0.95 a_w [as shown in Figures 3.9 and 3.14 (b)].

Statistically, The OTA production was significantly affected by elevated CO₂, a_w and the interaction between a_w and modified atmosphere (2.5 x CO₂) at 30 and 35°C (see Table 3.2 and Appendix II).

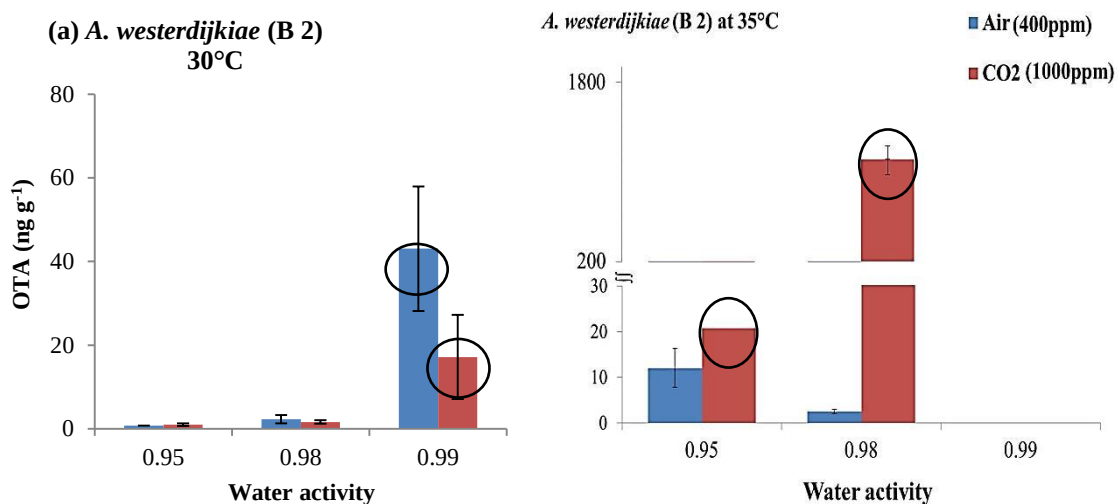


Figure 3.9: Effect of $a_w \times \text{CO}_2$ on OTA production by *A. westerdijkiae* (B 2) grown on CMEA for 9 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean. Circles indicate significant difference. Note: scale ranges are different for OTA production.

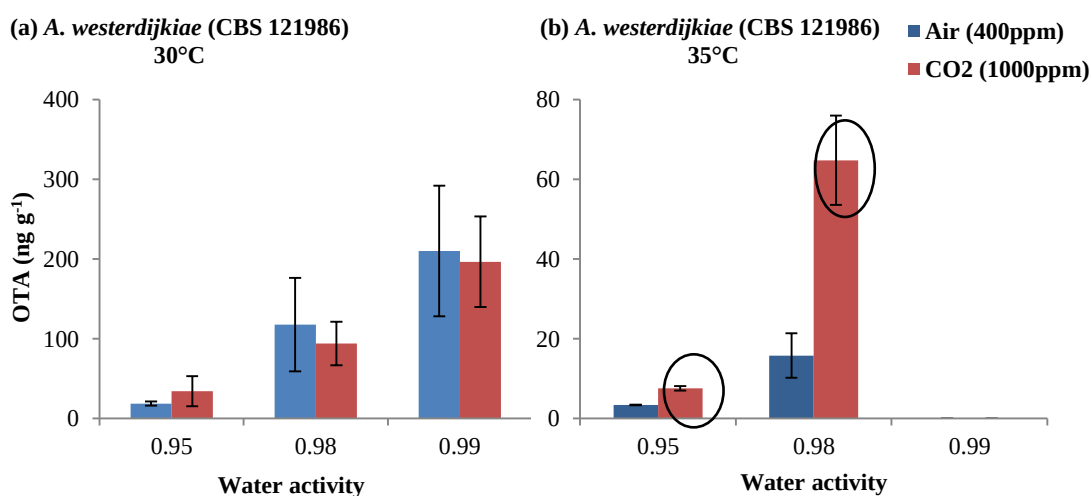


Figure 3.10: Effect of $a_w \times \text{CO}_2$ on OTA production by *A. westerdijkiae* (CBS 121986) grown on CMEA for 9 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean. Circles indicate a significant difference. Note: scale ranges are different for OTA production.

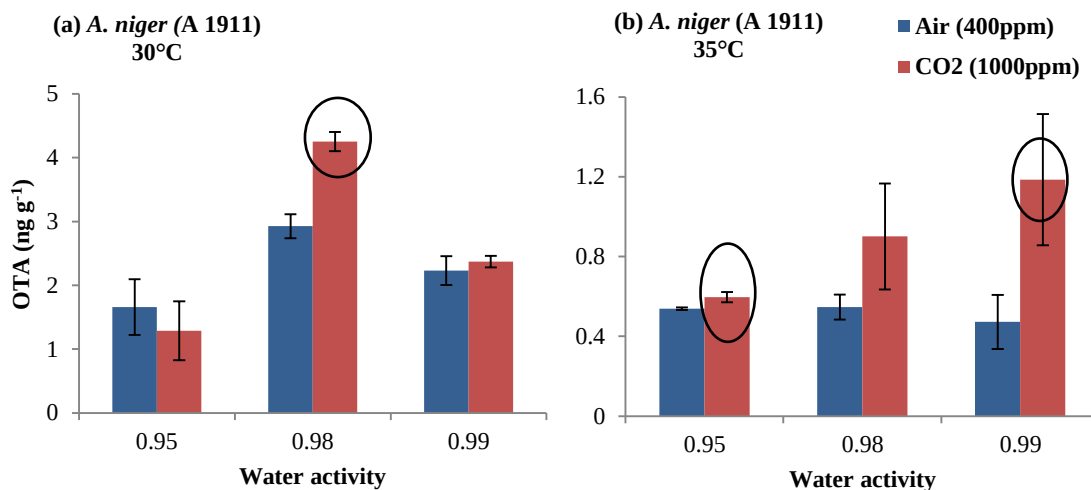


Figure 3.11: Effect of $a_w \times \text{CO}_2$ on OTA production by *A. niger* (A 1911) grown on CMEA for 9 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean. Circles indicate significant difference. Note: scale ranges are different for OTA production.

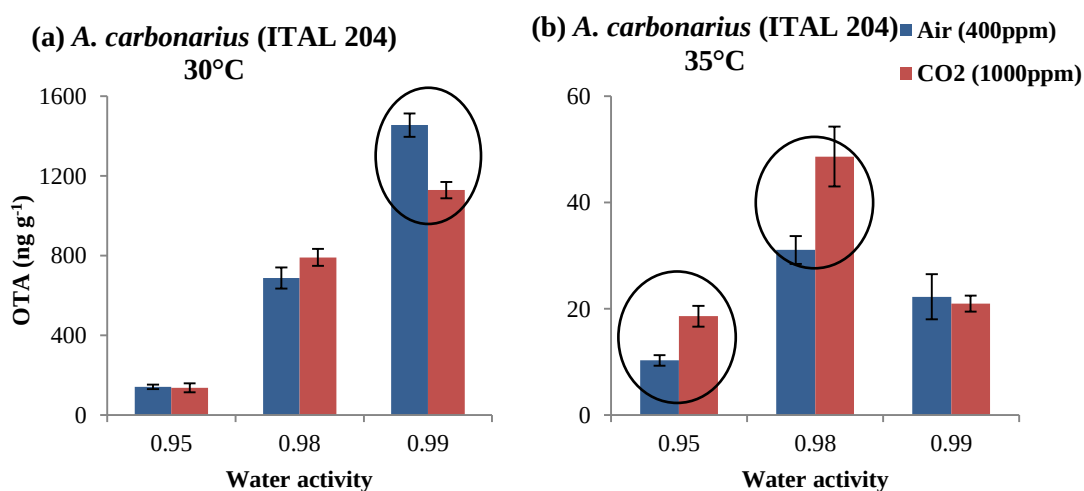


Figure 3.12: Effect of $a_w \times \text{CO}_2$ on OTA production by *A. carbonarius* (ITAL 204) grown on CMEA for 9 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean. Circles indicate significant difference. Note: scale ranges are different for OTA production.

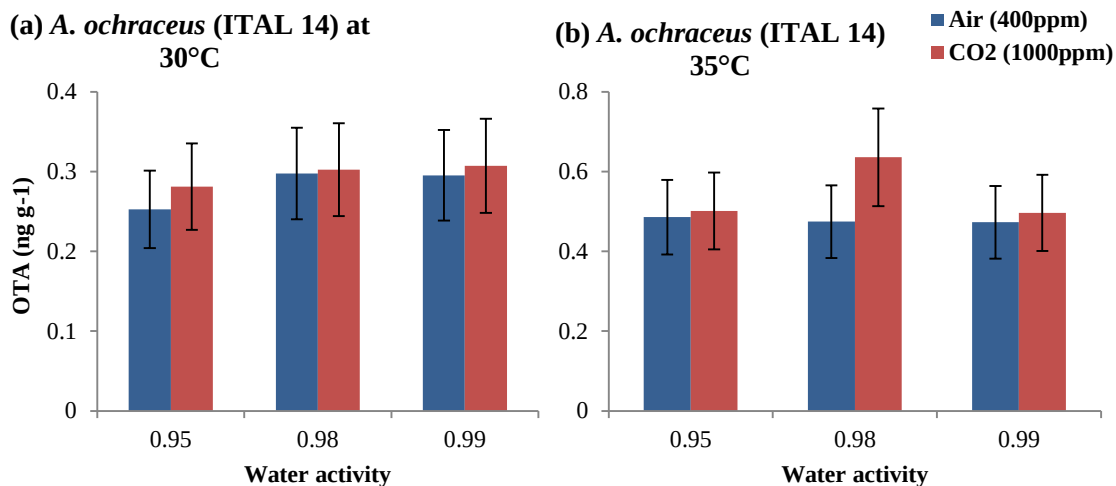


Figure 3.13: Effect of $a_w \times \text{CO}_2$ on OTA production by *A. ochraceus* (ITAL 14) grown on CMEA for 9 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean.

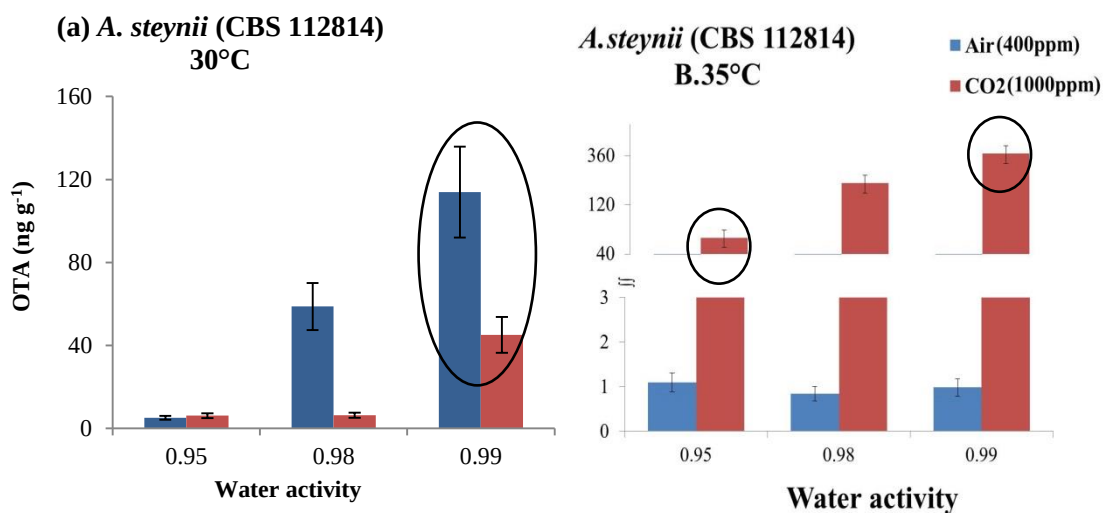


Figure 3.14: Effect of $a_w \times \text{CO}_2$ on OTA production by *A. steynii* (CBS 112814) grown on CMEA for 9 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean. Circles indicate significant difference. Note: scale ranges are different for OTA production.

Table 3.2: Summary of statistical results for the OTA production of six strains on different a_w , using Kruskal-Wallis Test (non-normality data) and ANOVA (normality data).

Temperature 30°C Factors			
Strains	CO ₂ (1000ppm)	a_w	CO ₂ x a_w
<i>A. westerdijkiae</i> (B 2)	S ^a	S ^a	-
<i>A. westerdijkiae</i> (CBS 121986)	NS ^b	S ^b	NS ^b
<i>A. niger</i> (A 1911)	S ^b	S ^b	S ^b
<i>A. carbonarius</i> (ITAL 204)	S ^b	S ^b	S ^b
<i>A. ochraceus</i> (ITAL 14)	NS ^a	NS ^a	-
<i>A. steynii</i> (CBS 112814)	S ^a	S ^a	-
Temperature 35°C			
<i>A. westerdijkiae</i> (B 2)	S ^a	S ^a	-
<i>A. westerdijkiae</i> (CBS 121986)	S ^a	S ^a	-
<i>A. niger</i> (A 1911)	S ^a	NS ^a	-
<i>A. carbonarius</i> (ITAL 204)	S ^b	S ^b	S ^b
<i>A. ochraceus</i> (ITAL 14)	NS ^b	NS ^b	NS ^b
<i>A. steynii</i> (CBS 112814)	S ^a	NS ^a	-
Temperature 30 and 35°C			
	CO ₂ (1000ppm)	a_w	Temp 30+35
<i>A. westerdijkiae</i> (B 2)	S ^a	S ^a	S ^a
<i>A. westerdijkiae</i> (CBS 121986)	S ^a	S ^a	S ^b
<i>A. niger</i> (A 1911)	NS ^a	NS ^a	S ^b
<i>A. carbonarius</i> (ITAL 204)	S ^a	S ^a	S ^a
<i>A. ochraceus</i> (ITAL 14)	NS ^a	NS ^a	S ^b
<i>A. steynii</i> (CBS 112814)	S ^a	S ^a	S ^b

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

^a Kruskal-Wallis test.

^b ANOVA.

NS not significant

S significant

3.3.2 *In situ* effect of water activity x elevated CO₂ x temperature on OTA production at 30°C and 35°C in irradiated coffee beans

Figures 3.15-3.20 shows the effect of the treatments on OTA production by the different strains and species when exposed to 400 or 1000 ppm CO₂ at different a_w levels (0.90, 0.95 and 0.97) on irradiated coffee beans at 30°C and 35°C for 12 days.

The highest OTA production was by *A. westerdijkiae* (B 2, CBS 121986) when compared to other strains at 0.90, 0.95 a_w in elevated CO₂ (1000ppm) at 30°C (see Figure 3.16, 3.17 (a)).

The exposure to elevated CO₂ concentrations (1000ppm) at 30°C resulted in some stimulation of OTA production by these strains/species after 12 days incubation. In some cases stimulation occurred under water stress (0.90 a_w) when combined with elevated CO₂. For example, *A. westerdijkiae* (B 2 CBS 121986) produced 4598.8 and 3974.2 ng g⁻¹ at elevated CO₂ in contrast to 400ppm respectively [see Figure 3.16, 3.17 (a)] (Plate 3.2b) . In addition, strain *A. niger* (ITAL 14) was affected at 0.97 a_w where OTA was increased to 33.38ng g⁻¹ when compared to air where the OTA mean was 6.54 ng g⁻¹ [Figure 3.18 (a)] (Plate 3.2a).

When the temperature was increase to 35°C, as compared to 30°C, OTA production for most strains was reduced at different a_w levels and CO₂ (1000ppm). However, for *A. westerdijkiae* (B 2), OTA production was increased when exposed to CO₂ (1000 ppm) at 35°C, to about 651.54 ng g⁻¹ when compared to air, about 10.38 ng g⁻¹, at 0.90 a_w [see Figure 3.16 (b)]. Similarly, for *A. westerdijkiae* (CBS 121986), at 0.90 and 0.95 a_w [(see Figure 3.17 (b))]. The highest production for OTA in *A. niger* (A 1911) was found to be at 0.90 a_w [see Figure 3.18 (b)] (Plate 3.3.a). For *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) the maximum OTA production was at 0.97 a_w [Figure 3.19, 3.20 (b)].

Statistically, Table 3.3 shows that the effect of a_w x elevated CO₂ (1000 ppm) x temperature varied between species. For *A. westerdijkiae* (B 2, CBS 121986) there was a significant increase ($P=0.05$) in OTA production in the presence of elevated CO₂ at

0.90 and 0.97 a_w for *A. niger* (A 1911) at 30°C (details in Appendix II). A_w and CO₂ and their interaction significantly ($P<0.05$) affected OTA production by *A. westerdijkiae* (B 2), *A. niger* (A 1911) and *A. carbonarius* (ITAL 204) species on stored coffee beans at 30°C. There were no effects of a_w on *A. westerdijkiae* (CBS 121986) and no effect of CO₂ on OTA production by *A. carbonarius* (ITAL 204) (see Appendix II)

Appendix II also shows there was a significant stimulation by increased CO₂ (1000 ppm) and a_w on OTA production by *A. westerdijkiae* (B 2) and by CO₂ (1000 ppm) for the strain of *A. westerdijkiae* (CBS 121986) at 35°C. Furthermore, there were significant effects of a_w on OTA production by *A. niger* (A 1911), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) (details in Appendix II).

There was a significant effect of temperature, at 30°C and 35°C, on OTA production for all strains except *A. carbonarius* (ITAL 204).

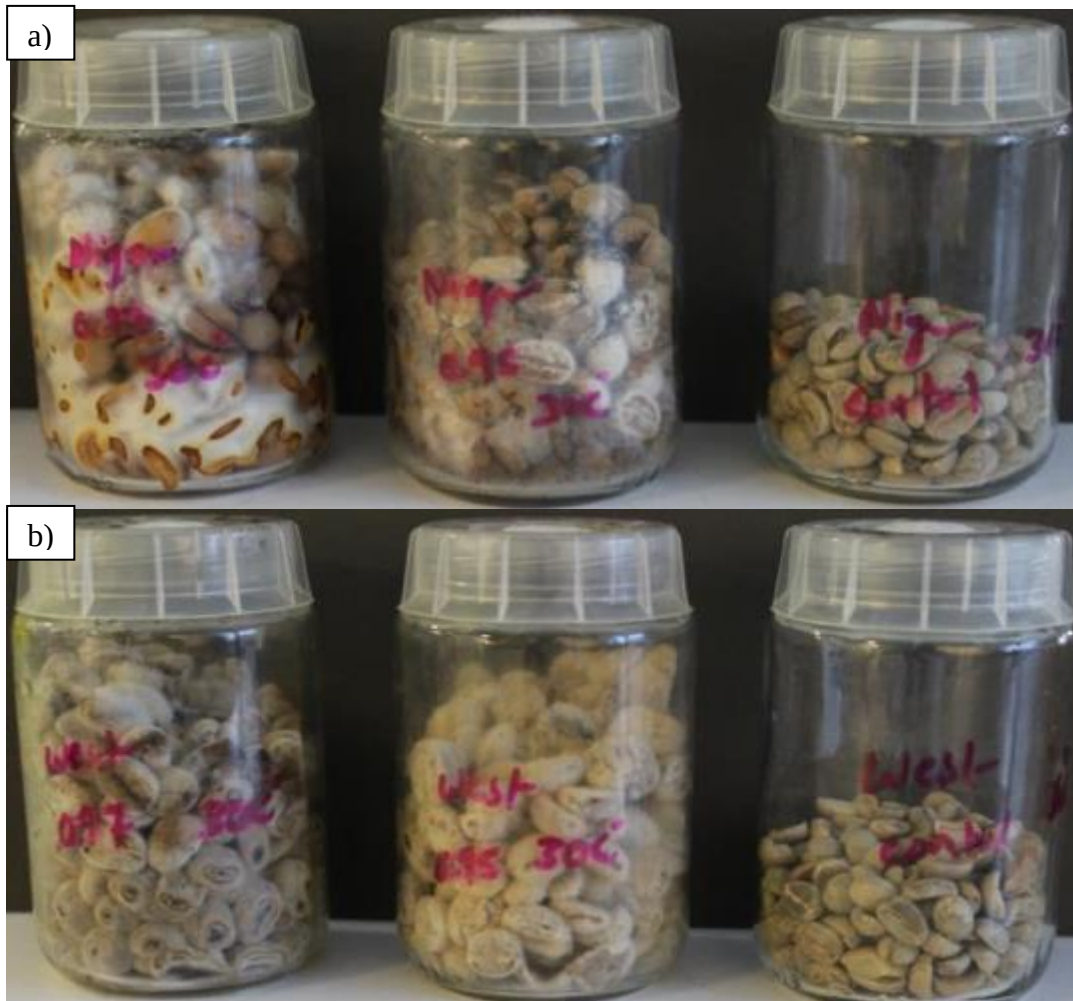


Plate 3.1: Contaminated irradiated coffee bean samples with a) *A. niger* (A 1911) and b) *A. westerdijkiae* (CBS 121986) rewetted at 0.90, 0.95 and 0.97 a_w at elevated CO_2 (1000 ppm) after 12 days storage at 30 °C.



Plate 3.2: Contaminated irradiated coffee bean samples with a) *A. niger* (A 1911) and b) *A. westerdijkiae* (CBS 121986) rewetted at 0.90, 0.95 and 0.97 a_w at elevated CO_2 (1000 ppm) after 12 days storage at 35 °C.

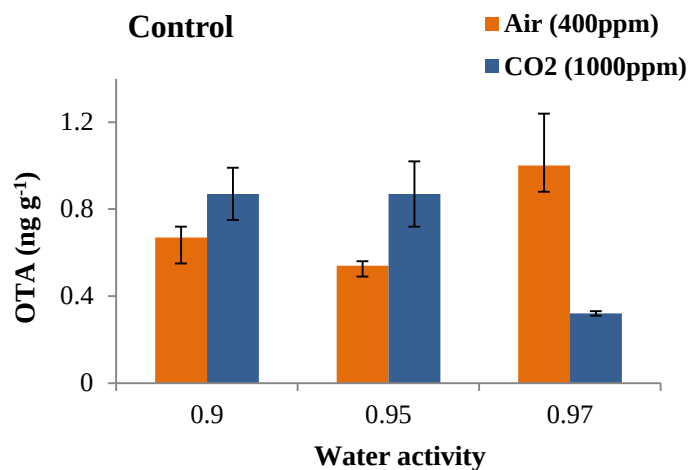


Figure 3.15: Effect of $a_w \times \text{CO}_2$ on OTA production of irradiated coffee beans (control) for 12 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean.

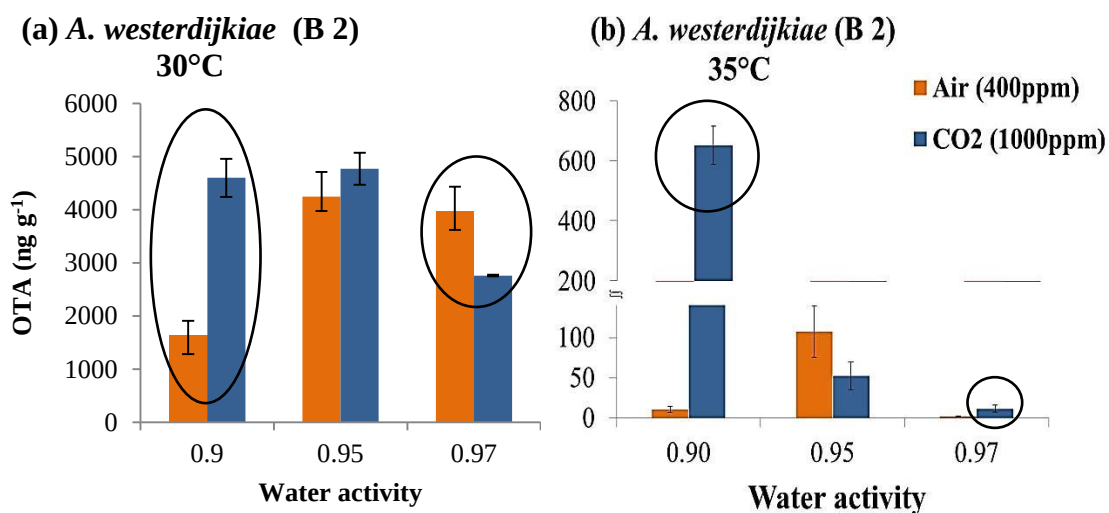


Figure 3.16: Effect of $a_w \times \text{CO}_2 \times \text{temperature}$ on OTA production by *A. westerdijkiae* (B 2) grown on irradiated stored coffee beans for 12 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean. Circles indicate significant differences. Note: scale ranges are different for OTA production.

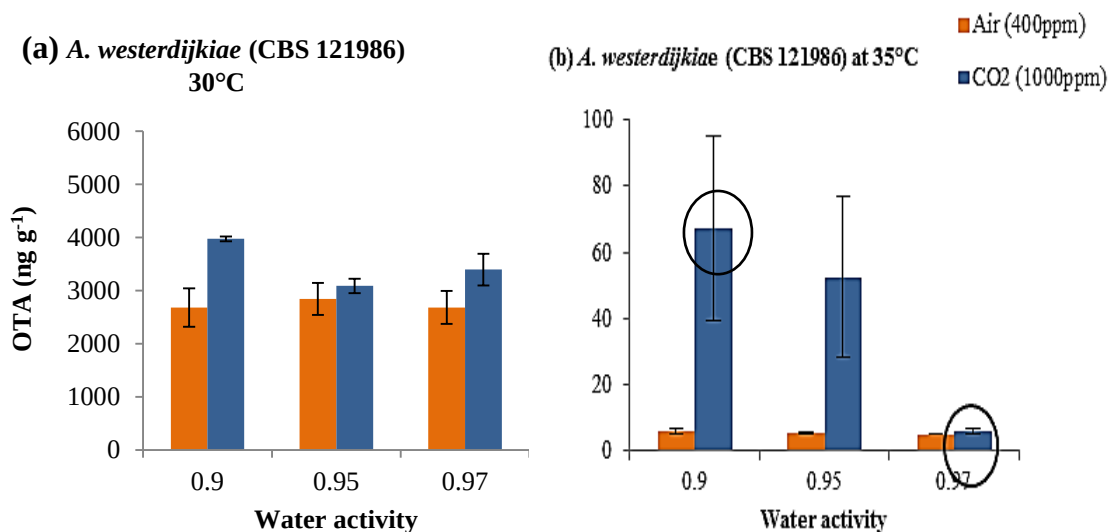


Figure 3.17: Effect of $a_w \times \text{CO}_2 \times \text{temperature}$ on OTA production by *A. westerdijkiae* (CBS 121986) grown on stored irradiated coffee beans for 12 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean. Circles indicate significant differences. Note: scale ranges are different for OTA production.

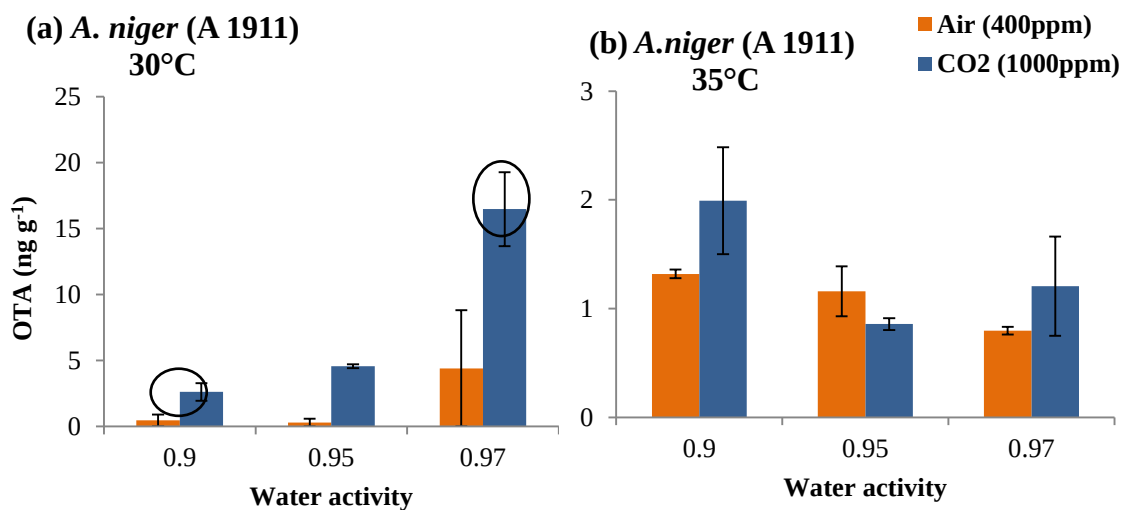


Figure 3.18: Effect of $a_w \times \text{CO}_2 \times \text{temperature}$ on OTA production by *A. niger* (A 1911) grown on stored irradiated coffee beans for 12 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean. Circles indicate significant differences.

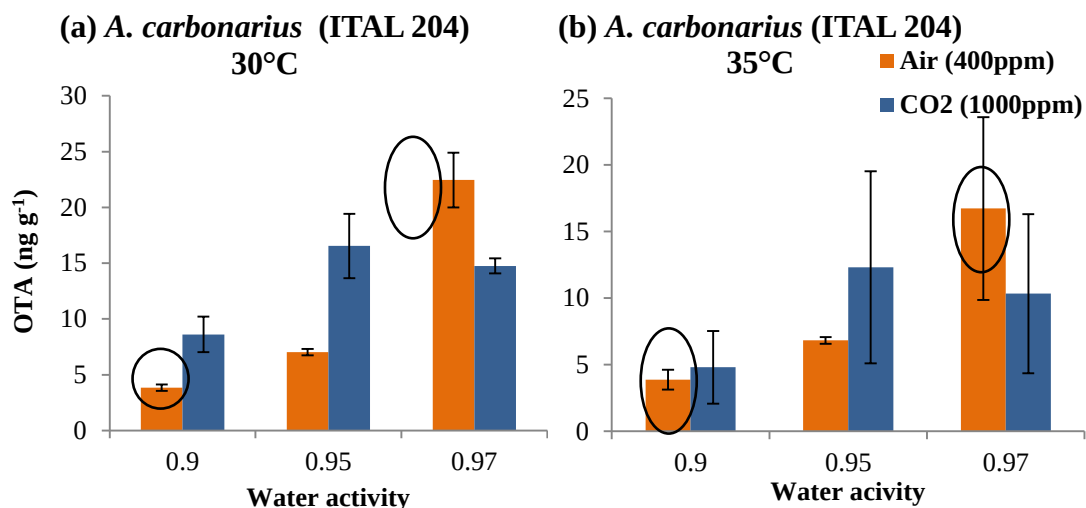


Figure 3.19: Effect of $a_w \times \text{CO}_2 \times$ temperature on OTA production by *A. carbonarius* (ITAL 204) grown on stored irradiated coffee beans for 12 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean. Circles indicate significant differences.

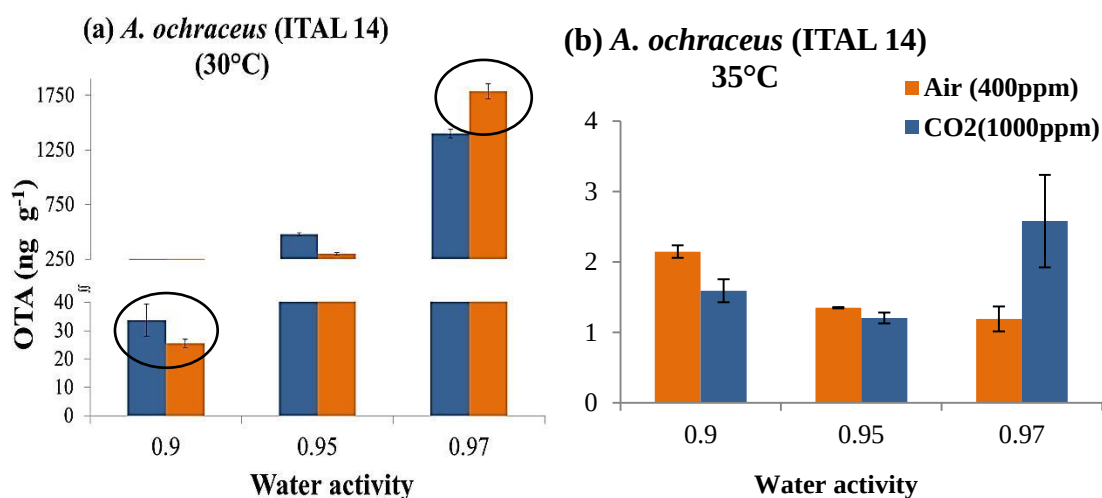


Figure 3.20: Effect of $a_w \times \text{CO}_2 \times$ temperature on OTA production by *A. ochraceus* (ITAL 14) grown on stored irradiated coffee beans for 12 days at (a) 30°C and (b) 35°C. Bars indicate standard error of the mean. Circles indicate significant differences. Note: scale ranges are different for OTA production.

Table 3.3: Summary statistical table for growth rate and OTA production of 5 strains on different a_w and temperatures by using Kruskal-Wallis Test (non normality data) and ANOVA (normality data).

Temperature 30°C			
Strains	Factors		
	CO ₂	a_w	$a_w \times \text{CO}_2$
<i>A. westerdijkiae</i> (B 2)	S ^b	S ^b	S ^b
<i>A. westerdijkiae</i> (CBS 121986)	S ^{*b}	NS ^b	NS ^b
<i>A. niger</i> (A 1911)	S ^{*b}	S ^b	S ^b
<i>A. carbonarius</i> (ITAL 204)	NS ^b	S ^b	S ^b
<i>A. ochraceus</i> (ITAL 14)	NS ^a	S ^a	N/A
Temperature 35°C			
<i>A. westerdijkiae</i> (B 2)	S ^a	S ^a	N/A
<i>A. westerdijkiae</i> (CBS 121986)	S ^a	NS ^a	N/A
<i>A. niger</i> (A 1911)	NS ^b	S ^b	NS ^b
<i>A. carbonarius</i> (ITAL 204)	NS ^a	S ^a	N/A
<i>A. ochraceus</i> (ITAL 14)	NS ^a	S ^a	N/A
Temperature 35°C			
	CO ₂ (1000ppm)	a_w	Temp 30+35°C
<i>A. westerdijkiae</i> (B 2)	NS ^a	NS ^a	S ^a
<i>A. westerdijkiae</i> (CBS 121986)	S ^a	NS ^a	S ^a
<i>A. niger</i> (A 1911)	NS ^a	NS ^a	S ^a
<i>A. carbonarius</i> (ITAL 204)	NS ^a	S ^a	NS ^a
<i>A. ochraceus</i> (ITAL 14)	NS ^a	NS ^a	S ^a

S significant ($P < 0.05$)

NS not significant ($P > 0.05$)

^a Kruskal-Wallis test

^b ANOVA

N/A Not Applicable

3.4 Discussion

3.4.1 *In vitro* effect on lag times prior to growth, growth and OTA production at 30°C and 35°C

The present study shows the crucial role that climate change factors such as elevated CO₂ and water stress can have on lag phases prior to growth, growth rates and indeed OTA production. This may be particularly important in the Arabian Gulf climates. Coffee beans are not produced in Kuwait but imported from different countries. The coffee beans may however be stored under humidity conditions that may enhance coffee spoilage. There have been practically no studies on the three-way interactions of these important climate change environmental factors on mycotoxigenic fungi. The present studies suggest that slightly elevated CO₂ levels of existing concentrations combined with drought stress and increased temperature may enhance OTA production of some mycotoxigenic species on coffee-based media. These are the first studies to examine these effects on strains of *A. westerdijkiae*, *A. niger*, *A. carbonarius*, *A. ochraceus* and *A. steynii* on green coffee-based media.

Interestingly, these studies have shown that the effect of increased CO₂ (1000 ppm; $p < 0.05$) had less impact on lag phases prior to growth and on growth, but more effects on OTA production. However, the effect does vary between species. In this study, most of the strains grow well at 30°C and slightly less so at 35°C except for *A. niger* (A1911). This strain appears to be more tolerant at high temperatures of 35°C. Also, the interaction of increased CO₂ (1000 ppm) plus elevated temperature (35°C) increased OTA production when compared with 30°C for strains of *A. westerdijkiae* (B 2), *A. ochraceus* (ITAL 14) and *A. steynii* (CBS 112814). Most strains had an optimum growth at 0.95 a_w, at 35°C, while at 30°C, the optimum was at 0.98 a_w.

There have been some other studies on the effect of CO₂ on OTA production by strains of these and other species. However, most previous studies used very high levels of CO₂ as part of an approach to develop modified atmosphere storage. For example, Cairns-Fuller (2005) suggested that 50% CO₂ was required to inhibit growth and OTA production by *P. verrucosum* in moist grain by >75% at 0.90-0.995 a_w. Paster et al.

(1983) reported that no growth of *A. ochraceus* occurred at 80% or 100% CO₂ and that growth was partially inhibited by 60% CO₂. Similarly, Pateraki et al. (2005) found that 50% CO₂ inhibited *A. carbonarius* growth after 5 days.

Some studies have reported a reduction in growth rate at 5-10% of CO₂ of some species at low a_w levels and an increase in the lag phases prior to growth. Although at $\geq 15\%$ CO₂ most strains showed inhibition of growth, especially under water stress (Magan and Lacey (1984a). Valero et al. (2008) found that there was significant reduction in growth observed by *A. carbonarius* and *A. niger* at 1% O₂ and combination of 1% O₂ and 15% CO₂. Also, inhibition of *B. cinerea* by exposure to 15% CO₂ plus 5% O₂ has been reported (Berry and Aked, 1997). Valero et al. (2008) reported reductions of growth and OTA production by combination of 1% O₂ and 15% CO₂ of *A. carbonarius* and *A. niger*.

Recently Medina et al. (2015), carried out studies on *A. flavus* and aflatoxin B₁ (AFB₁) production under slightly elevated CO₂ (650 and 1000 ppm), a_w and temperature conditions. They found a significant stimulation of AFB₁ production, especially under drought stress at 37°C. In addition, there was a significant increase in AFB₁ at 0.92 and 0.95 a_w x 37°C and 650 or 1000 ppm CO₂. They suggested that the three-way interaction of these factors together influenced mycotoxin production than slightly elevated CO₂ alone. It is possible that the interacting factors provide stress conditions, which affect the physiology of the fungus and stimulates secondary metabolite production as a defence response (Magan & Aldred, 2007).

3.4.2 *In situ* effect of water activity x elevated CO₂ x temperature on OTA production at 30 and 35°C

This study demonstrated a significant impact of a_w x CO₂ interactions on OTA production by the strains examined when grown on stored green coffee beans. The results showed the highest OTA production was at 0.97 a_w on stored green coffee beans (=44% MC) and the minimum was at 0.90 a_w (=22% MC). This indicates the differences between optimum conditions for OTA production for these strains *in situ*. The best

strains with the highest capacity for OTA production at 0.90, 0.95 and 0.97 a_w were *A. westerdijkiae* (B 2) then *A. westerdijkiae* (CBS 121986) and *A. ochraceus* (ITAL 14) at 0.97 a_w . OTA production was highest in 0.95 a_w by *A. westerdijkiae* (B2) at 30°C.

The present work shows that in coffee stored at 0.90 a_w and elevated CO₂ (1000 ppm), OTA production by *A. westerdijkiae* spp. can be significantly impacted. Thus, stimulation of OTA production was observed when combined stresses of elevated temperature (35°C), water stress and increased CO₂ conditions were imposed. Moisture content equivalents for 0.90, 0.95 and 0.97 are approx. 22, 34 and 45.37% respectively. Palacios-Cabrera et al. (2004) reported high amounts of OTA produced by *A. carbonarius* in irradiated raw coffee beans with equilibrium relative humidity of 100% (0.99 a_w). Similarly, maximum growth rate of *A. ochraceus* on irradiated green coffee beans was found at 30°C and 0.95-0.99 a_w and OTA production ranged from 40-17000 ng g⁻¹ (Pardo et al. 2005b). In addition, they noticed a marked decrease of growth was observed when temperature (10°C) and a_w (0.80) levels were reduced. Limits of moisture for safe storage of green coffee beans have been suggested at 10-12% (approximately 0.65-0.70 a_w ; Kulaba, 1981).

Taniwaki et al. (1999) reported lowest OTA accumulation for *A. ochraceus* at 0.80 a_w at 25 °C with 0.2 ng g⁻¹ in green coffee, and high an OTA levels at 0.95 a_w with production of 7178 ng g⁻¹ after 21 days incubation. Bucheli et al. (1998), showed lowest a_w levels for *A. ochraceus* to produce OTA in green coffee as 0.85 and 20 °C and 30°C. They suggested that to prevent OTA production in green coffee, the a_w should be < 0.72.

Joosten et al. (2001) showed that the maximum fungal growth in green coffee beans occurs between 25 and 30°C. Lowest growth rates were obtained at 10°C and the maximum at 30°C. Kulaba (1981) and Sterling (1980) found that growth rates declined in green coffee when the temperature was below 26°C. However, these studies did not examine increased temperatures of >30°C.

Other studies of OTA production on different substrates by Damoglou et al. (1984) and Haggblom (1982) showed maximum OTA levels were detected at 20°C and 0.85 a_w after 19 days, and no OTA production at 10°C in barley grains. The optimum OTA

production was at around 28°C in corn, rice and wheat (Trenk et al. 1971). Ramos et al. (1998) reported maximum OTA production by *A. ochraceus* on barley grains at 25-30°C and 0.98 a_w.

This study suggests that even under conditions optimum for growth, OTA production may be stimulated under water stress and elevated CO₂ conditions representing 2.5x the current value for climate change conditions. More studies are required to evaluate in more detail the potential impact that interacting climate change factors may have a naturally contaminated coffee for processing.

CHAPTER 4

THE IMPACT OF WATER AND TEMPERATURE INTERACTION ON LAG PHASE, GROWTH AND POTENTIAL OCHRATOXIN A PRODUCTION BY TWO NEW SPECIES, *A. ACULEATINUS* AND *A. SCLEROTIICARBONARIUS*, ON A GREEN COFFEE-BEASED MEDIUM

4.1 Introduction

Coffee beans (*Coffea arabica*, *Coffea canephora* var. *robusta*) are colonised by a range of mycobiota during ripening (Ilic et al., 2007; Leong et al., 2007; Noomin et al., 2008; Taniwaki et al., 2003). It is known that species of the *Aspergillus* section *Circumdati* and *Aspergillus* section *Nigri* are the predominant components of the mycobiota on coffee (Astoreca et al., 2010a; Batista et al., 2003; Taniwaki et al., 2003; Perrone et al., 2007). The focus has been on those species from these two Sections that produce ochratoxins (OTA) for which legislative limits exist (soluble coffee, 10 µg g⁻¹; roasted/ground coffee, 5.0 µg g⁻¹; EU Commission Regulation (EC) No 1811/2006). Indeed the water and temperature relations for growth and OTA production by strains of species of both these Sections have been studied in detail (Abdel-Hadi & Magan, 2009; Belli et al., 2004; Mitchell et al., 2004; Pardo et al., 2005a).

In a survey of the mycobiota of Arabica coffee (Northern Thailand) and Robusta coffee (Southern Thailand) in Thailand two new species were discovered and classified in the *Aspergillus* section *Nigri* group (Noonim et al., 2008a). The two new species were identified and given the names *Aspergillus aculeatinus* and *A. sclerotiicarbonarius*. These were classified as uniseriate and biseriate species respectively, within the *Aspergillus* section *Nigri*. The two species were only isolated from Robusta coffee in Southern Thailand (Noomin et al., 2008b). There is practically no knowledge of the effect of environmental factors on the growth of strains of these species on coffee-based media. Samson et al. (2007) grouped a range of black *Aspergillus* species into four groups based on growth at 12 different temperature levels (6-40°C). *A. sclerotiicarbonarius* was in their Group 2 with an optimum of 33°C and *A. aculeatus* was in their Group 3 had an optimum of 36°C. They both grew over the range 12-40°C on defined media. These two species are considered to be non-toxigenic. However, as they occupy the same niche in coffee as other mycotoxigenic species, they could compete with these and affect OTA contamination. It has been shown that an understanding of the temperature x water activity relations of such species is important as these combined factors influence interactions between different mycotoxigenic and non-mycotoxigenic fungi (Magan and Aldred, 2007; Magan et al., 2010).

The hypothesis was to determine whether these new species isolated from coffee behaved in a similar manner to toxigenic species in relation to interacting environmental conditions. Thus, the objectives of this study were to examine the effect of water activity (a_w) and temperature interactions on (a) lag phases prior to growth, (b) relative growth rates and ecological profiles on a green coffee-based medium by three strains each of *A. aculeatinus* *A. sclerotiicarbonarius* previously isolated from Thai Robusta coffee beans. We also tested whether these strains previously identified as non-OTA producers may be stimulated to produce OTA on a green coffee-based medium.

4.2 Material and methods

4.2.1 Fungal strains

The fungal strains of each species used in this study were *Aspergillus aculeatinus* (CBS 115570, CBS 121875, and CBS 121872) and *Aspergillus sclerotiicarbonarius* (CBS 121056, CBS 121851, and CBS 121853). These were kindly supplied by Dr Robert Samson (Head, Applied and Industrial Mycology Group, CBS, Baarn, Holland).

4.2.2 Media, inoculation and growth measurements

The experiments were conducted on a Robusta Coffee Meal Extract Agar (CMEA). This was prepared by boiling 300 g of ground green coffee beans in 1 L of distilled water for 30 min (concentrated coffee extract). A double layer of muslin was used for filtering the resulting mixture and the volume was made up to 1 Technical agar No 3 (2%), 6% concentrated coffee extract and glycerol were added (50.6, 23, 9.2, 2.7 g) to adjust the media to 0.90, 0.95, 0.98, and 0.99 water activity (a_w) respectively. The media were autoclaved at 121°C for 10 min and poured into 9 cm Petri plates and kept at 4 °C until used. The final a_w levels were checked with a water activity meter (Aqualab 3TE; Decagon Devices, Inc., Pullman, Washington, USA).

Inoculum of each strain was prepared by growing each strain on unmodified CMEA at 25°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. The resulting spore suspension was used to point inoculate the treatment and replicate 9 cm Petri plates with 7 μ l of the spore suspension. Three replicates of each a_w treatment was incubated at 20, 25, 30, 35 and 37°C for 9 days. The treatments and replicates were kept in closed polyethylene bags and the colony diameter was measured in two directions at right angles to each other every day.

4.2.3 Examination of potential production of OTA

To confirm that these were non-mycotoxigenic strains each was inoculated on the CMEA modified to 0.98 a_w and incubated for a 9 days at 30°C. Five plugs (4 mm diameter) from three replicates was transferred to 2 ml Eppendorf tubes and weighed. 750 μ l methanol was added to each Eppendorf tube. The samples were then shaken using a KS 501 digital orbital shaker for 30 min and centrifuged for 10min at 15000 x g. The supernatant was filtered directly into HPLC vials.

The conditions for HPLC analysis of OTA quantification was by using a fluorescence detector (Waters Corp., Milford, MA, USA)(λ_{exc} 333nm, λ_{em} 460) and a C18 Column (Poroshell 120, length 100 ml, diameter 4.6 mm, particle size 2.7 micron; 600 Bar). The mobile phase were Acetonitrile (57%): Water (41%): Acetic acid (2%) at flow rate 1 ml min^{-1} , run time of 12 min (Hunt et al., 1980). The LOD and LOQ were 0.09 ng g^{-1} and 0.32 ng g^{-1} respectively.

4.2.4 Statistical analysis

A full factorial design with two (4 x 5) factors: water activity, temperature was applied. Each treatment, water activity x temperature combination, was carried out in triplicate, for calculating the lag phase (prior to growth) and the growth rate (diametric growth rate, mm/day) based on the temporal increase in colony diameter over the 9 days of the experiment. Only the linear portions of the growth curves were used for obtaining the linear regression lines. Normality was checked using the Kolmogorov-smirnov test. Analysis of data, the effects of a_w , temperature and their interaction were examined by

the Kruskal-Wallis (non-parametric) where data was not normally distributed. For normally distributed data the data sets were analysed using Minitab® 16 package (Minitab Inc., 2010. State College, PA, USA). The statistical significant level was set at $P < 0.05$ for all single and interacting treatments.

4.3 Results

4.3.1 Effect of a_w x temperature on lag phases prior to growth, growth rates and growth profiles

Figure 4.1 shows the effect of different temperatures on the lag times prior to growth initiation at different a_w levels for three strains of each species examined. This shows that as water stress (drier conditions) and temperature stress was imposed the time prior to growth increased. This was particularly evident for the strains of both *A. aculeatinus* and *A. sclerotiicarbonarius*. At marginal temperatures for growth (20°C) and all a_w levels there was an increase in lag times when compared to other conditions. The shortest lag times were at 0.99 a_w x 20-35°C and the longest were at 20°C and 0.90 a_w .

Table 4.1 shows that overall, statistically, there were significant ($P<0.05$) differences between the three strains of *A. aculeatinus* in relation to lag phases prior to growth. However, there was no difference in growth rates between strains, although there were effects in relation to a_w and temperature. For *A. sclerotiicarbonarius* lag and growth rates were similar for all three strains. However, a_w and temperature had a significant effect on strains as shown in Appendix III.

Figure 4.2 shows effect of a_w x temperature interactions on the relative growth rates of each of the strains of *A. aculeatinus* and *A. sclerotiicarbonarius* examined. Optimum growth was at 0.98-0.99 a_w at 30-35°C. The lowest growth rate for all strains of the two species was at 0.90 a_w at 20 and 37°C. None of the strains grew at 0.85 a_w , regardless of temperature. The strains of *A. aculeatinus* appear to be more tolerant of elevated temperatures than *A. sclerotiicarbonarius* (plate 4.1). Thus, at 37°C, the strains of the latter species were unable to grow over the whole a_w range examined.

Table 4.2 shows the detailed statistical analyses for each strain in relation to lag phases prior to growth and growth rates in relation to the factors examined. This shows that for each individual strain there was less effect of a_w and temperature on lag phases prior to

growth than on actual growth rate. Overall, both a_w and temperature had a significant effect on growth as shown in Appendix III.

Figure 4.3 shows an integration of the data from three strains of each species to develop a a_w x temperature profile of the maximum, optimum and minimum conditions for the growth of these two species on a coffee based medium.

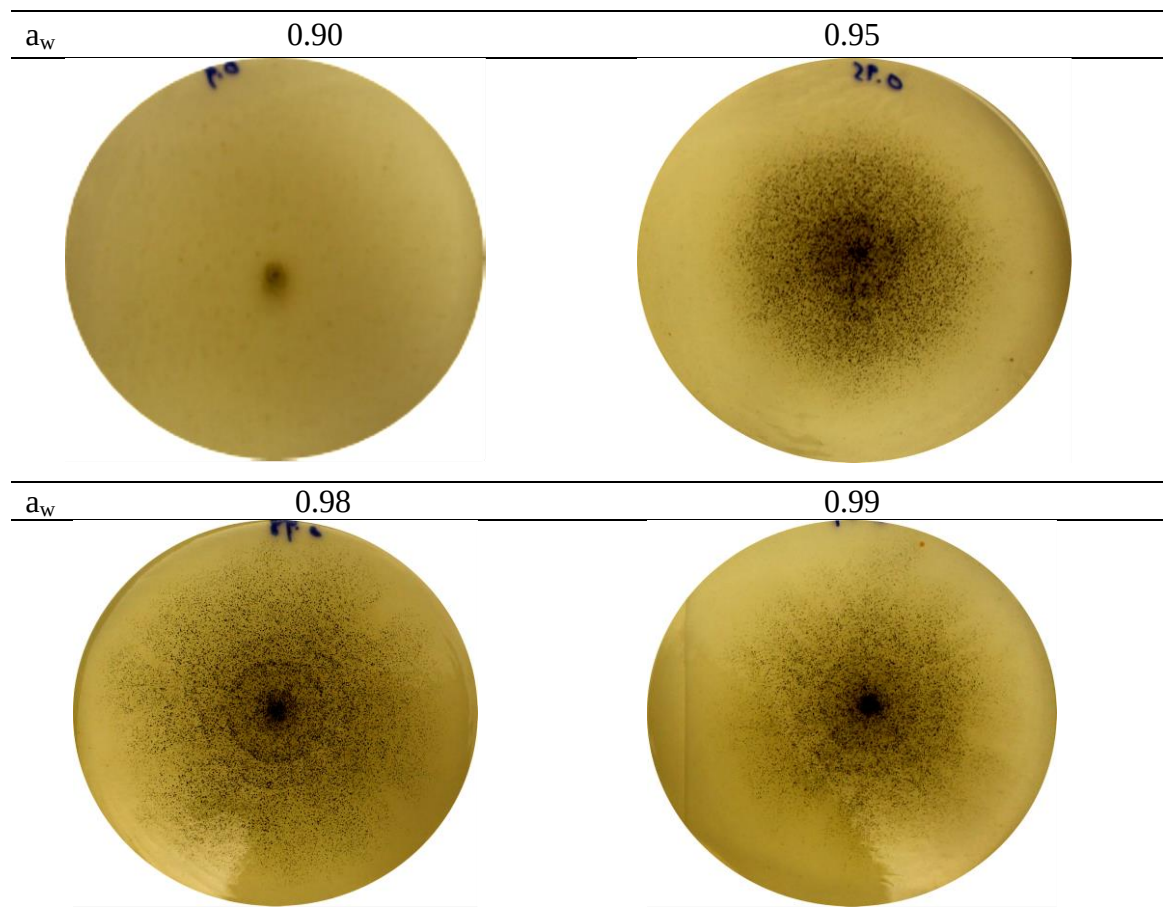


Plate 4.1: shows *A. aculeatinus* (CBS 115570) at different a_w × temperature levels on a coffee based medium at 37°C

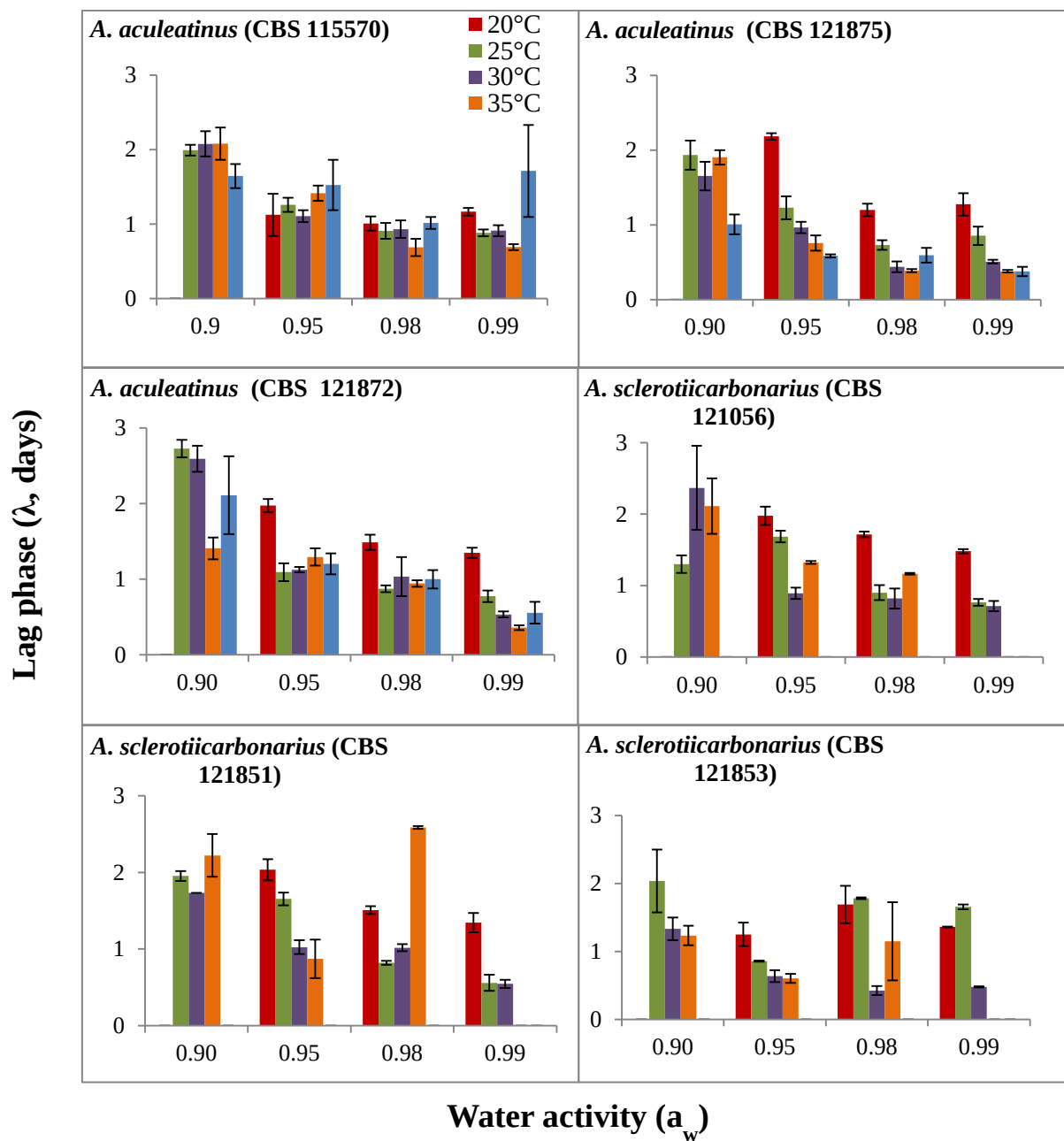


Figure 4.1: Comparison of the lag phases prior to growth (in days) of three strains each of *A. aculeatinus* and *A. sclerotiicarbonarius* at different $a_w \times$ temperature levels on a coffee based medium. Bars indicate standard error of the mean.

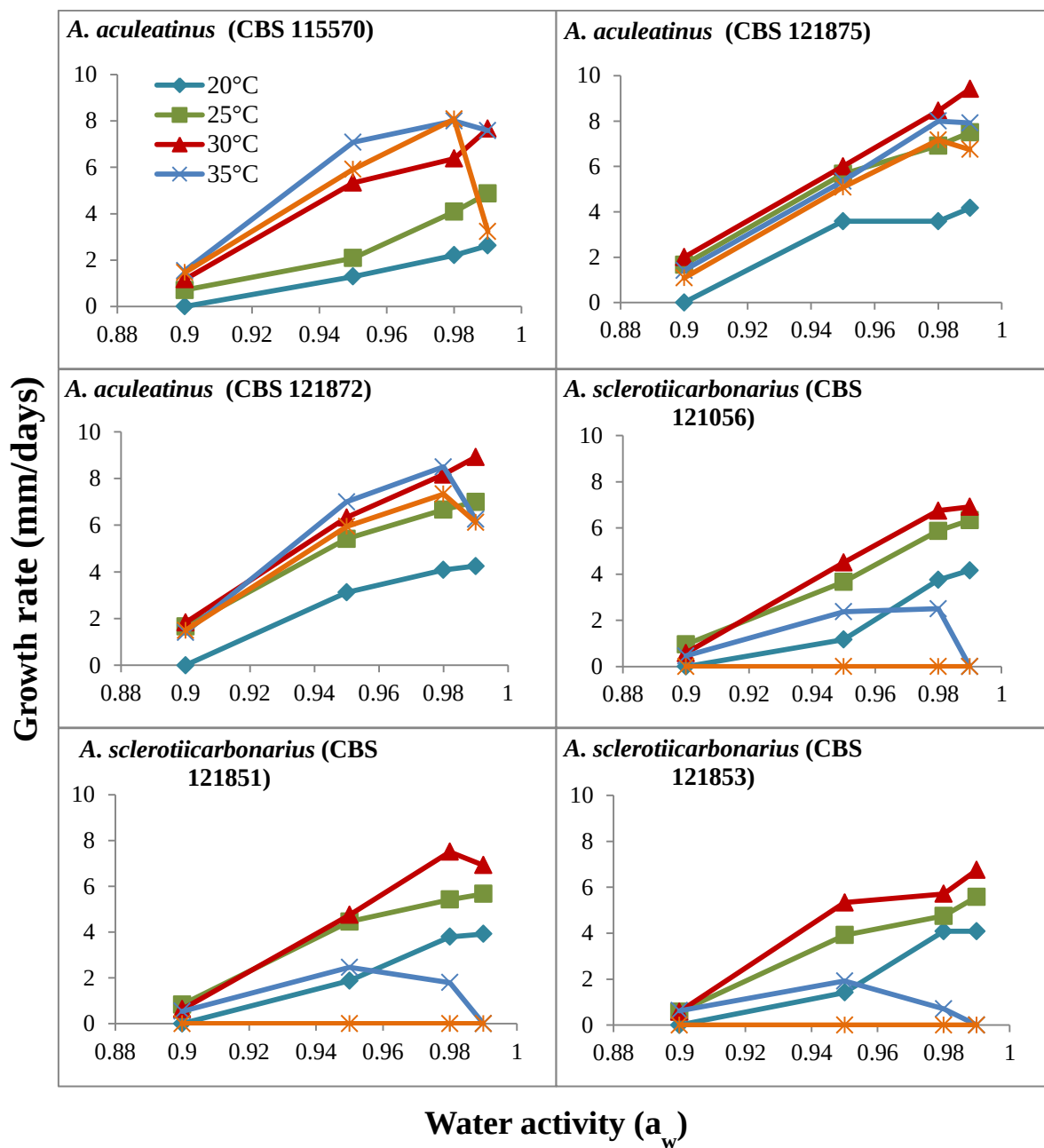


Figure 4.2: Effect of different temperature $\times a_w$ on relative growth rates of three strains of *A. aculeatinus* and *A. sclerotii carbonarius* on a coffee-based medium.

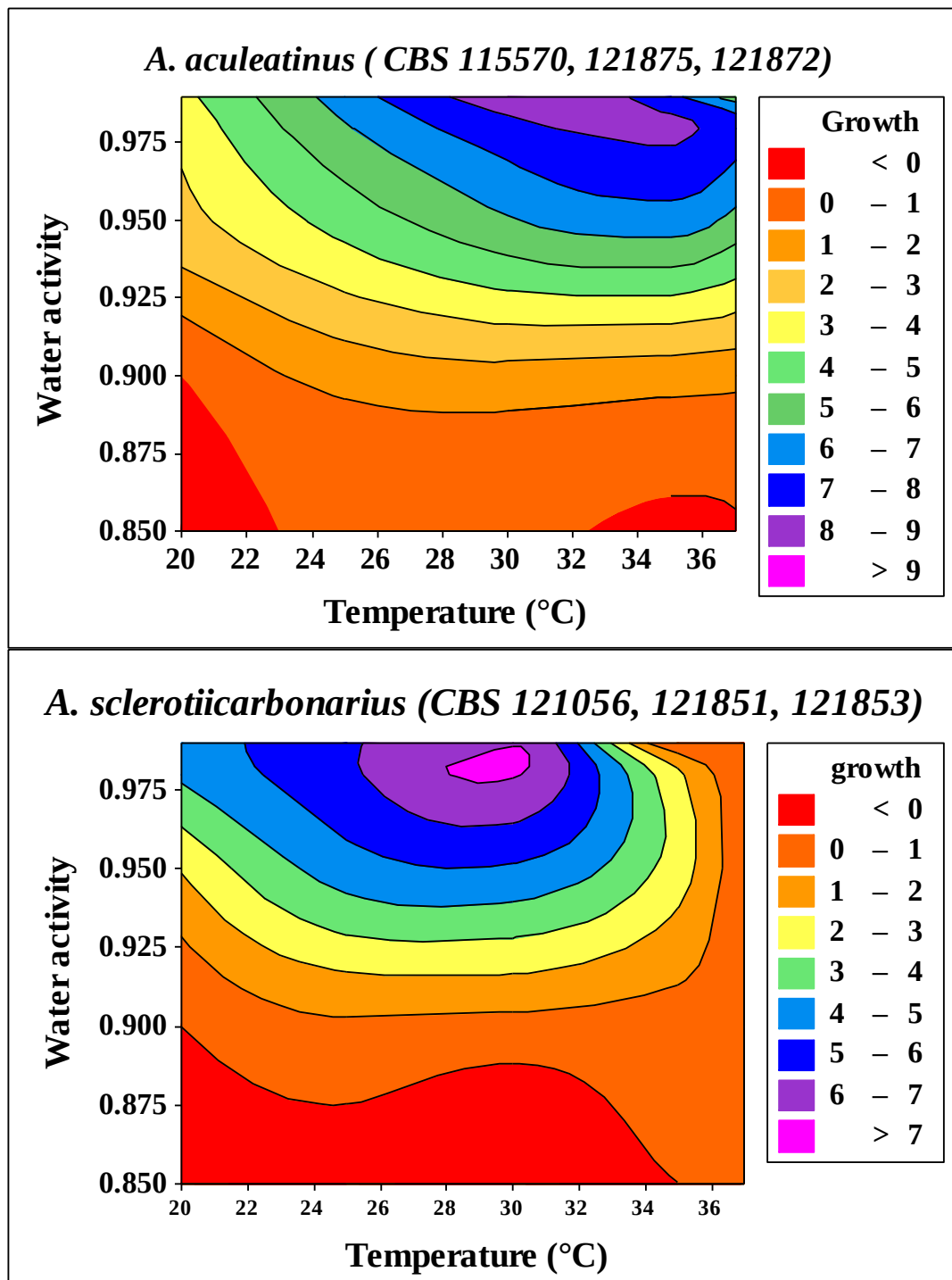


Figure 4.3: Mean contour plots of the relative growth rate profiles of the combined data from three strains of each species of *A. aculeatinus* and *A. sclerotiicarbonarius* in relation to $a_w \times$ temperature on a coffee-based medium.

Table 4.1: The statistical analyses of the three strains of each species examined in relation to both lag phases (λ , days) and the growth rate in relation to strain $\times$ a_w and temperature using the Kruskal–Wallis Test (non-normality data) and ANOVA (normality data). The significant factors are in bold.

Factors				
Strains	Strains	a_w		Temp (°C)
<i>A. aculeatinus</i> (CBS 115579, CBS 121875, CBS 121872)	$P < 0.05^{a*}$	$P < 0.05^{a*}$	$P < 0.05^{a*}$	Lag phase (λ , days)
	$P > 0.05^a$	$P < 0.05^{a*}$	$P < 0.05^{a*}$	Growth rate (mm day ⁻¹)
<i>A. sclerotiicarbonarius</i> (CBS 121056, CBS 121851, CBS 121853)	$P > 0.05^a$	$P < 0.05^{a*}$	$P < 0.05^{a*}$	Lag phase (λ , days)
	$P > 0.05^a$	$P < 0.05^{a*}$	$P < 0.05^{a*}$	Growth rate (mm day ⁻¹)

Table 4.2: Summary of statistical analysis effects on lag phases prior to growth (λ , days) and growth of 6 strains at different a_w and temperature levels using the Kruskal-Wallis Test (non-normality data) and ANOVA (normality data).

Factors				
Strains	a_w	$a_w \times \text{Temp}$	Temp (°C)	
<i>A. aculeatinus</i> (CBS 115579)	$P > 0.05^a$	N/A	$P > 0.05^a$	Lag phase (λ , days)
	$P < 0.05^{*a}$	N/A	$P < 0.05^{*a}$	Growth rate (mm day ⁻¹)
<i>A. aculeatinus</i> (CBS 121875)	$P < 0.05^{*a}$	N/A	$P > 0.05^a$	Lag phase (λ , days)
	$P < 0.05^{*b}$	$P < 0.05^{*b}$	$P < 0.05^{*b}$	Growth rate (mm day ⁻¹)
<i>A. aculeatinus</i> (CBS 121872)	$P > 0.05^a$	N/A	$P > 0.05^a$	Lag phase (λ , days)
	$P < 0.05^{*a}$	N/A	$P < 0.05^{*a}$	Growth rate (mm day ⁻¹)
<i>A. sclerotiicarbonarius</i> (CBS 121056)	$P > 0.05^a$	N/A	$P < 0.05^{*a}$	Lag phase (λ , days)
	$P < 0.05^{*a}$	N/A	$P < 0.05^{*b}$	Growth rate (mm day ⁻¹)
<i>A. sclerotiicarbonarius</i> (CBS 121851)	$P > 0.05^a$	N/A	$P < 0.05^{*a}$	Lag phase (λ , days)
	$P < 0.05^{*a}$	N/A	$P < 0.05^{*a}$	Growth rate (mm day ⁻¹)
<i>A. sclerotiicarbonarius</i> (CBS 121853)	$P > 0.05^a$	N/A	$P < 0.05^{*a}$	Lag phase (λ , days)
	$P < 0.05^{*a}$	N/A	$P < 0.05^{*a}$	Growth rate (mm day ⁻¹)

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

^a Kruskal-Wallis test.

^b ANOVA.

* Significant

N/A Not applicable

4.3.2 Ochratoxin A production

The three strains of each species were examined for the production of OTA. The analyses showed that at 0.98 a_w on coffee-based media these strains did not produce any OTA based on analyses using HPLC and the limits of detection and quantification for the method employed.

4.4 Discussion

Overall, the data suggests that they have similar ecological behaviour in relation to the abiotic factors of a_w x temperature with optimum conditions for growth of approx. 0.98-0.99 a_w and 30-35°C. The main difference between the uniseriate *A. aculeatinus* and the biseriate *A. sclerotiicarbonarius* strains of these species was the inability of the biseriate strains to grow at 37°C, regardless of the a_w level. In all cases no growth was observed at 0.85 a_w suggesting limits for growth of around 0.88 a_w based on extrapolation from the study.

Previously, studies by Samson *et al.* (2007) had reported maximum temperature for growth of these strains as 33°C for *A. sclerotiicarbonarius* and about 36°C for *A. aculeatinus*. However, these temperature optima were included mixed groups of uniseriate and biseriate species. For example, There group 3 (36°C) included *A. aculeatinus* as well as *A. carbonarius* (biseriate) and the uniseriate species *A. japonicus* and *A. uvarum*. They also reported no growth at 5 and 9°C, respectively, but did not examine interactions with a_w . The data should be compared with that available for other species in the *Aspergillus* section *Nigri* groups (Sanchis & Magan, 2004; Samson *et al.*, 2007). Studies by Leong *et al.* (2004) found that the uniseriate *A. aculiatus* had a lower optimum temperature than the biseriate *A. carbonarius* or *A. niger*. Indeed, *A. niger* had the highest thermal tolerance of the species isolated from irrigated grapes in Australia.

There have been a number of detailed studies which have examined the optimum, minimum and maximum a_w x temperature conditions for growth of biseriate members of the *Aspergillus* section *Nigri* group (Belli *et al.*, 2004; Mitchell *et al.*, 2004; Natskoulis *et al.*, 2009; Tassou *et al.*, 2007a,b). These studies have compared a wide range of strains of *Aspergillus carbonarius* and *Aspergillus niger* isolated from grapes and vine fruits from different regions of Europe. For *A. carbonarius*, Tassou *et al.* (2009) found the lowest a_w for growth in about 0.85-0.88 a_w depending on the time of incubation. These are lower than that found by Belli *et al.* (2004) and Mitchell *et al.* (2004) but it was suggested that this could be explained by regional climatic conditions and adaptation, perhaps making those from Greece more xerotolerant (Tassou *et al.*,

2009). No growth was observed for the strains of *A. carbonarius* or *A. niger* isolated from grapes at 10 and 40°C although the one exception was at 0.98 a_w /40°C where slight growth was observed on a grape-based nutrient medium. Optimum conditions for growth for strains of both these species (*A. carbonarius*, *A. niger*) was 30-35°C at 0.98-0.99 a_w . Interestingly, Mitchell et al. (2004) found no correlation between strains of *A. carbonarius*, growth and country of origin. In contrast, OTA production was found to be optimum at 15-25°C and 0.95-0.98 a_w regardless of origin of species (Belli et al., 2004; Mitchell et al., 2004; Tassou et al., 2007).

The other important group of Aspergilli which produces OTA is the species in the section *Circumdati* group. The effect of a_w x temperature on growth of *A. ochraceus* was examined on green coffee extract agar (Pardo et al., 2005b). However, only 20, 25 and 30°C was examined. Optimal conditions for growth were 0.95-0.98 a_w and 20-30°C. Minimum a_w for growth was 0.85 a_w although they found germination did occur at 0.80 a_w . This study did not consider higher temperatures which were shown in this study to favour the growth of the strains of these two species from the section *Nigri*.

The integration of the data for three strains of each species provides an overall picture of the impact of a_w x temperature on both optimum, maximum conditions for growth and the boundary conditions based on the data ranges used for the first time. This suggests that the uniseriate *A. aculeatinus* species has a wider optimum temperature and a_w range (28-36°C; 0.99-0.97 a_w) and grows faster over a wider temperature range than the biseriate *A. sclerotiicarbonarius* (28-30°C; 0.99-0.975 a_w).

Marginal a_w conditions for a growth were similar. There is no other information on uniseriate section *Nigri* species or strains. However, comparisons can be made with that for a biseriate species such as *A. carbonarius* (predominantly OTA producers) and *A. niger* (predominantly non-OTA producers; Belli et al., 2004; Mitchell et al., 2004; Tassou et al., 2009). These suggest marginal conditions for both *A. carbonarius* and *A. niger* at around 0.88-0.90 a_w with a temperature range of 15-35/37°C.

Although these strains and species have been confirmed to be non-OTA producers on defined nutritional media (Samson et al., 2007; Nielsen et al., 2009) we were interested to examine whether growth on coffee-based media might result in biosynthesis of OTA. The present study showed that at 0.98 a_w on coffee-based media these strains did not produce any OTA, based on analyses using HPLC and the limits of detection and quantification for the method employed. Previous studies have found that the uniseriate species in section *Nigri* such as *A. aculeatinus*, *A. aculeatus*, *A. japonicus* and *A. uvarum* do not produce OTA, although they may produce other extralites. In contrast, biseriate section *Nigri* species such as *A. carbonarius* (most strains) and *A. niger* (some strains) produce OTA. Other biseriate species including *A. sclerotiicarbonarius*, produce a range of other extralites (Samson et al., 2007).

In summary, this study has provided ecological data on the growth profiles of strains of *A. aculeatinus* (uniseriate) and *A. sclerotiicarbonarius* (biseriate) section *Niger* species in relation to a_w x temperature for the first time. It has also identified the optimum and marginal conditions for growth which will be useful in understanding the complex interactions which can occur between mycotoxigenic and non-mycotoxigenic *Aspergillus* species in the coffee ecosystem during cultivation and processing which may influence the level of OTA found.

Since these species do not produce any OTA it may be possible that they have an influence on ochratoxigenic species. Thus, they could be examined as potential antagonistic competitors of toxigenic species and be utilised as biocontrol candidates for control of OTA contamination. Thus it may be of interest to examine the use of non-ochratoxigenic strains of *A. aculeatinus* and *A. sclerotiicarbonarius* to competitively exclude ochratoxin A producing strains and minimise coffee contamination with OTA.

CHAPTER 5

CONTROL STRATEGIES FOR FUNGAL GROWTH AND OCHRATOXIN A PRODUCTION BY USING *IN SITU* GASEOUS OZONE TREATMENT

5.1 Introduction

Ozone (O₃) is a highly unstable triatomic molecule and powerful oxidation agent that breaks down in the lower atmosphere to normal O₂, without leaving any residue. In recent years, different techniques to oxidize and sterilize food using O₃ have been applied in the agri-food sector. There have been few, if any, studies on the efficacy of gaseous O₃ to control the germination of spores, colonisation and OTA production by *Aspergillus* section *Circumdati* and section *Niger* species in coffee-matrices.

Fungal species may have a variable sensitivity/tolerance to gaseous O₃ exposure depending on time x concentration used (Hibben and Stotzky, 1969; Antony-Babu and Singleton, 2009). Sensitivity/tolerance is also influenced by moisture content, substrate, and spore morphology (Hibben and Stotzky, 1969). Inactivation of spores and inhibition of mycelial growth, when exposed to gaseous O₃, have shown variable results. For example, Vijayanandraj et al. (2006) reported that *A. niger* mycelial growth was reduced but that spore germination was unaffected by O₃ treatment. Interestingly, mycelial growth and sporulation were inhibited by continuous O₃ exposure on peaches when inoculated with *Monilinia fructicola*, *Botrytis cinerea*, *Mucor piriformis* or *Penicillium expansum* and kept in store (Palou et al., 2002). There is debate as to whether gaseous O₃ treatment is fungistatic or fungicidal (Nicoué et al., 2004; Minas et al., 2010). Minas et al. (2010) and Hibben and Stotzky (1969) reported that germination rates for *Alternaria* and *Stemphylium* spp. with thick walled multicellular spores was the same when exposed to air or 1 ppm O₃. However, germination of thin-walled spores of *Rhizopus stolonifer*, *Trichoderma viride*, *A. niger*, *P. egyptiacum* at the same O₃ concentration was reduced. However, these studies did not examine whether recovery of germination might occur subsequently. Mylona et al. (2014) showed that exposure of *Fusarium verticillioides* conidia to 200-300 ppm O₃ exposure for 1 hr was initially effective but that over a subsequent 10 day period the spore viability could recover and indeed resulted in fumonisin production under different a_w conditions.

Although the inhibition of spore viability by gaseous O₃ has been examined, the ability for physiological repair has not been studied in detail. O₃ may oxidize cell components

including polyunsaturated fatty acids, glycoprotein and glycolipids and enzymes, and weaken the cell wall that can lead to cell lysis (Roushdy et al., 2011; Pryor and Rice, 1998).

To my knowledge, no previous studies have examined the effect of gaseous O₃ on growth of ochratoxigenic fungal colonisation of coffee and indeed OTA production *in vitro* or *in situ*. Some studies have examined the effect of O₃ on decontamination of fungal populations and degradation of mycotoxins in cereals and grains in aqueous solutions (Wang et al., 2010; Young et al., 2006) or naturally contaminated grains and nuts (Proctor et al., 2004; Akbas and Ozdemir, 2006) to control mycotoxin contamination. Allen et al. (2003) reported that mycelia were less resistant to O₃ than fungal spores and both were reduced in barley grains by 96% after exposure to O₃ gas at 0.1 mg g⁻¹min⁻¹ for 5 min at 0.98 a_w and 20°C. Kell et al. (2001) achieved 63% inactivation of *A. parasiticus* populations in maize with 50 ppm O₃ after treatment for 3 days. Less inactivation occurred with 25 ppm exposure for 5 days. Wu et al. (2006) obtained 100% reduction in fungal spores with 0.33 mg g⁻¹ min⁻¹ O₃ for 5 min and a reduction of 96.9% for 15 min at 0.90 a_w and 20°C.

The effect of O₃ treatment on physiology of grain has been studied. Wu et al. (2006) suggested that wheat germination after exposure to O₃ at 0.98 mg g⁻¹ min⁻¹ for 15 min was unaffected. Mendez et al. (2003) reported no effect on physiological quality of maize grains when exposed to 50 ppm O₃ for 3 days. Rozado et al. (2008) confirmed this, where 50 mg Kg⁻¹ at a flow rate of 0.8 L min⁻¹ O₃ was used to treat maize effectively, and by Santos et al. (2007) using 100 ppm injected at a flow rate of 4.6 L min⁻¹. However, the germination of treated barley grain depended on O₃ concentration. The germination was decreased by 28%, if the dose was higher than 0.98 mg g⁻¹ and for more than 45 min (Allen et al., 2003).

The control of fungal growth and OTA in coffee beans by using different concentrations of gaseous O₃ has not been studied previously. Thus, the aim of this study was to examine the efficacy of different gaseous O₃ concentrations to reduce or inhibit fungal spore germination, OTA production by three types of species dominant in coffee beans (*A. westerdijkiae*, *A. ochraceus* and *A. carbonarius*) *in situ*. Studies were carried out

using two systems: (a) irradiated coffee which was inoculated with spores of the ochratoxigenic species, and (b) naturally contaminated stored coffee beans at different a_w conditions and exposed to gaseous O_3 (600 ppm) and subsequently stored for 10-12 days. Subsequently, the effect on fungal populations and OTA production was determined.

5.2 Materials and methods

5.2.1 Ozone gas

Ozone gas has a strong odour and is detectable at very low concentrations of 10 ppm. It is found naturally in small amounts in the atmosphere and it is made up of three oxygen atoms. It is very unstable and therefore breaks down in the lower atmosphere to O_2 . O_3 is an oxidizing agent and can thus cause damage to the mucus layer and respiratory system in concentrations >100 ppm. There are no health effects on workers who are exposed to 0.1 ppm O_3 for up to 8 hr. However, it can be lethal if people are exposed to ≥ 50 ppm O_3 for more than 30 min. Safety precautions and thus a detailed risk assessment was completed and all O_3 treatments were done in a fume cupboard with the necessary safety controls.

5.2.2 Ozone generator and exposure chamber

The O_3 gas used in the experiments was generated from a corona discharge ozone generator (C-Lasky series, Model CL010DS, AirTree Ozone Technology Co., Ltd., Sijhih City, Taipei County 22150 Taiwan). Figure 5.1 shows the O_3 generator, analyser and treatment glass column used in these studies.

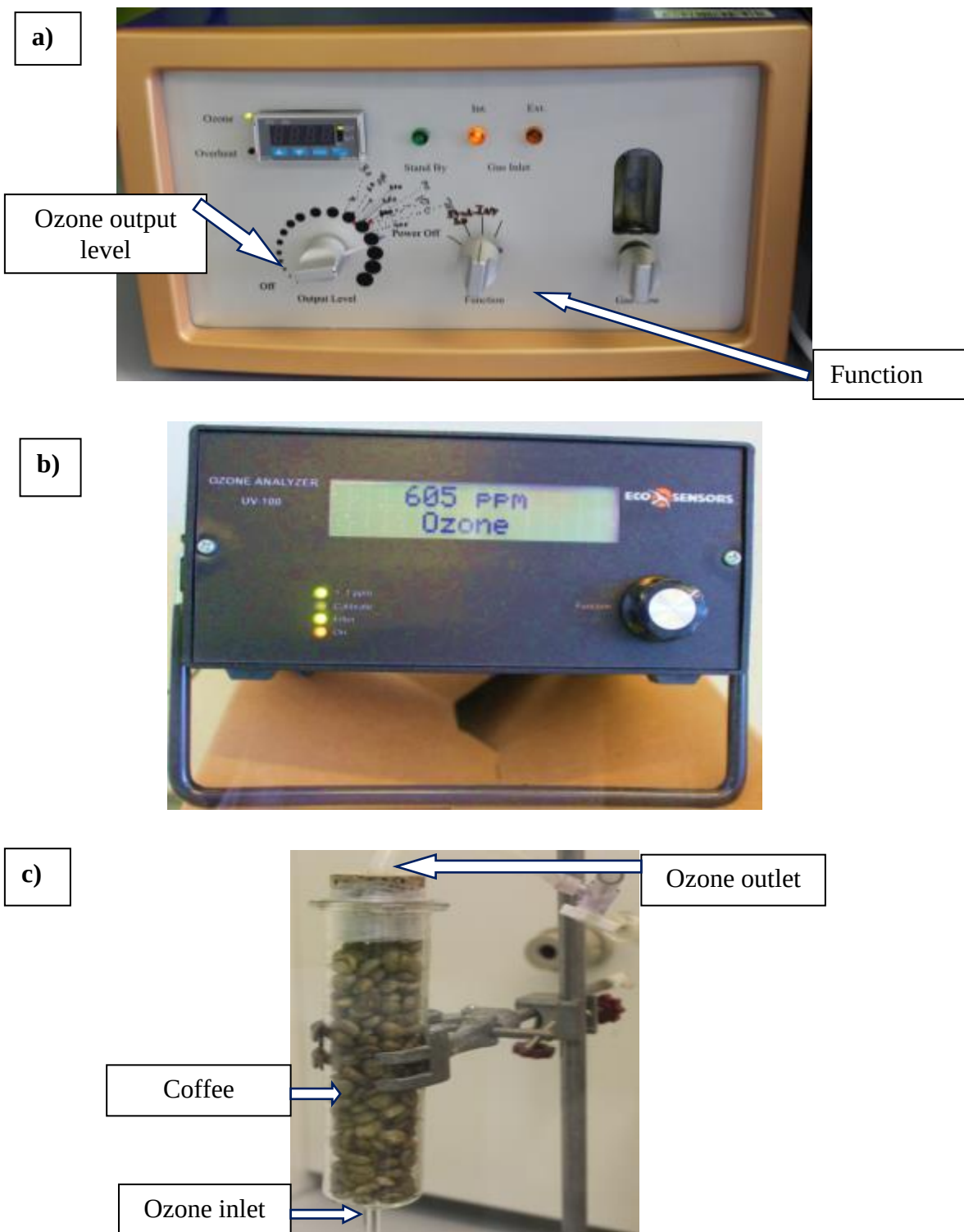


Figure 5.1: (a) Ozone generator, C-Lasky series, Model CL010DS (AirTree Ozone Technology Co., Ltd., Sijhih City, Taipei County, Taiwan), (b) Ozone analyser, and (c) 100ml glass column (c).

The O₃ generator can produce 2 g hr⁻¹ O₃ from dry air at a flow rate of 6 L min⁻¹. O₃ formed by the O₃ generator was pumped into the base of the glass column using a Teflon tube connected to the generator with stainless steel joints. The exposure chamber was filled with coffee beans and O₃ passed through the column at a specific flow rate. At the top of the glass column, the outlet tube was also connected to a O₃ analyser (Model UV-100, Eco Sensors Inc., Santa Fe, New Mexico 87505 USA). This was to ensure that the exposure of the coffee bean samples was kept at the target O₃ treatment level during the treatment period accurately. The rest of the O₃ was exited via the fume hood to the exterior.

5.2.3 Selection and preparation of spore suspensions the strains

Three strains of OTA producing species of *A. westerdijkiae* (CBS 121986), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) previously isolated from coffee were used. The preparation for these strains was described in Section 3.2.2 (a)

5.2.4 Preparation of coffee beans samples

a) Determination of the moisture adsorption curve of natural and irradiated green coffee beans

A moisture adsorption curve (MAC) was constructed to determine the amounts of water to be added to both irradiated and naturally contaminated green coffee beans to obtain the target a_w levels of 0.75, 0.90 and 0.95. The curves were prepared by adding different known amounts of water to 5 g green coffee bean subsamples and equilibrating at 4°C for 24 hr, then measuring the a_w of the hydrated green coffee beans. The coffee was then dried at 110°C for 24 hr, and kept in a desiccator at room temperature for 1hr and the coffee beans weighed to determine the moisture content. The a_w of the green coffee beans was measured using an Aqualab 3TE (Decagon Devices, Inc., Pullman, Washington, USA). Figure 5.1 shows the moisture adsorption curve and the relationship between added water and a_w values for normal contaminated coffee beans. For irradiated coffee beans the moisture adsorption curve was described in Section 3.1.1 (b). Table 5.1 shows the amounts of H₂O required to obtain the target a_w levels in both natural and irradiated coffee beans.

Table 5.1: The amounts of water required for 5 g subsamples of the two types of coffee beans to get an exact a_w .

a_w	Naturally contaminated coffee beans	Irradiated coffee beans
0.75	0.25 ml	0.75 ml
0.90	0.9 ml	1.5 ml
0.95	2.1 ml	2.5 ml

Subsequently 45 g of raw coffee was weighed and water added using the MAC to obtain the required a_w level and kept at 4°C for 24 hr. The moist raw coffee beans were inoculated and exposed to gaseous O_3 and then stored in solid substrate culture vessels (Magnet, Sigma Ltd, UK) which could be closed using lids which contained a permeable microporous membrane. These were stored in environmental chambers where the a_w level was maintained using glycerol/water solutions to obtain the target ERH values for the a_w levels of the coffee treatments.

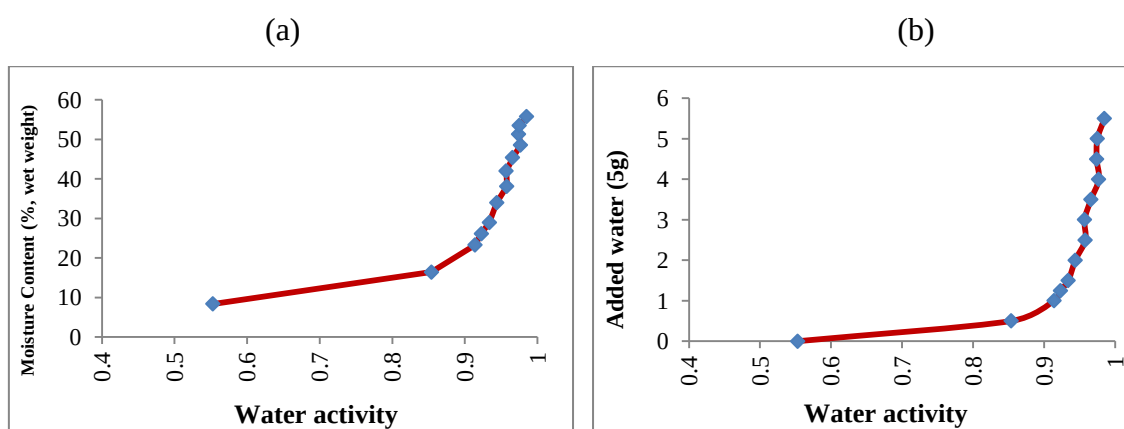


Figure 5.1: (a) Adsorption curve of raw coffee beans and (b) amount of added water required to obtain target water activity levels in raw coffee beans.

5.2.5 Efficacy of O₃ on natural and inoculated coffee beans:

a) Effect of O₃ on the total fungal populations in naturally contaminated coffee beans.

Naturally contaminated coffee beans were rewetted according to the water absorption curve as described previously to obtain the target a_w levels of 0.75, 0.90 and 0.95. Three replicates of each treatment (45 g) were exposed for 60 min to 600 ppm O₃ in the glass column system at a flow rate of 6 L min⁻¹.

Directly after the treatment with O₃, ~2.2 g sub-samples were collected from the top, middle and bottom of the glass column, combined, and used for the determination of the total fungal populations. To compare the fungal populations from O₃ and control treatments serial dilutions were carried out, and 200 µl of each dilution spread plated onto malt extract agar (MEA) and incubated at 30°C for 4-5 days before enumeration. The coffee beans were stored for 12 days in vessels with microporous lids, and placed together in plastic chambers. To maintain the EHR, a beaker with a solution of glycerol-water at the appropriate a_w was placed in each chamber. Fungal populations were determined at the end of the storage time, using serial dilution as described above. The OTA production levels in naturally contaminated coffee beans were measured after storage as describe in Chapter 3, Section 3.2.2 (d).

b) Effect of O₃ on the fungal populations of *A. westerdijkiae*, *A. carbonarius* and *A. ochraceus* when inoculated onto naturally contaminated coffee beans.

A moisture adsorption curve (MAC) as described in Section 5.2.4 (a) was prepared. 5 ml spore suspensions at a concentration of 10⁴ each, of *A. westerdijkiae* (CBS 121968), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) were prepared and mixed together to give a total of 15 ml. The three replicates of rewetted contaminated coffee beans were inoculated with 1.5 ml of the spore suspension mixture and mixed well for 2 min. The coffee beans were then placed in the glass column system and exposed to 600 ppm O₃ at a flow rate of 6 L min⁻¹ for 60 min.

The fungal populations were determined immediately after treatment and after storage for 12 days as described previously using serial dilution. The OTA contamination was determined after removal from storage.

c) Effect of O₃ on the fungal populations of *A. westerdijkiae*, *A. carbonarius* and *A. ochraceus* on irradiated coffee bean.

The irradiated coffee beans were modified to the target a_w levels of 0.75, 0.90 and 0.95 using the previously described MAC for irradiated coffee beans (see Table 5.1). A spore suspension at a concentration of 10^4 spores $m\ L^{-1}$ of *A. westerdijkiae* (CBS 121976), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14), each, was prepared separately as described in Section 5.2.3. The coffee bean treatments were inoculated as described previously, the beans well shaken and then introduced into the glass column (45 g). The three replicates of each treatment were exposed to 400 and 600 ppm O₃ for 60 min at a flow rate of 6 L min^{-1} .

The fungal populations immediately after treatment and after 12 days storage and OTA analysis were performed as detailed previously.

5.2.6 Data analysis

As described in Chapter 3, Section 3.2.1 (e).

5.3 Results

5.3.1 *In situ* efficacy of O₃ on fungal populations isolated from naturally contaminated and artificially inoculated coffee beans after exposure and storage

Figures 5.2 and 5.3 show the effect of O₃ exposure (0 or 600 ppm; flow rate of 6 L min⁻¹), for 60 minutes, on the total fungal populations (log₁₀+1 CFUs g⁻¹ dry weight) isolated from naturally contaminated coffee beans and naturally contaminated coffee beans inoculated with a mixture of strains of *A. westerdijkiae* (CBS 12986), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) rewetted to 0.75, 0.90 and 0.95 a_w. The populations were measured both in the glass column directly after exposure and after storage for 12 days at 30°C.

Exposure of naturally contaminated coffee to O₃ had little effect on the fungal populations at 0.90 and 0.95 a_w isolated immediately after exposure when compared to the control (Figure 5.2). After storage, there was a reduction of 24% at 0.95 a_w in the 600 ppm O₃ treatment when compared to the controls. In addition, no fungal populations were isolated from the 0.75 and 0.90 a_w treatments when compared to the untreated controls in the dilutions which were plated.

Figure 5.3 shows that when the coffee was inoculated with a mixture of the three ochratoxigenic strains there was only a slight reduction in the populations isolated immediately after treatment with gaseous O₃. In contrast, when compared to the controls, no fungi were isolated at 0.75 and 0.90 a_w after 12 days storage at 30°C.

Table 5.1 shows the statistical results using the Kruskal-Wallis test. This shows that treatment of the coffee with 600 ppm O₃ had a significant effect and reduced the populations of fungi in stored coffee beans after treatment at both 0.90 and 0.95 a_w. In the coffee beans inoculated with ochratoxigenic fungi there was a statistically significant effect of the O₃ treatment at all three a_w levels.

Appendix IV shows the summary statistics for fungal populations for both contaminated coffee beans and those inoculated with the three ochratoxigenic strains. A significant reduction of populations at 0.95 and 0.90 was found after storage.

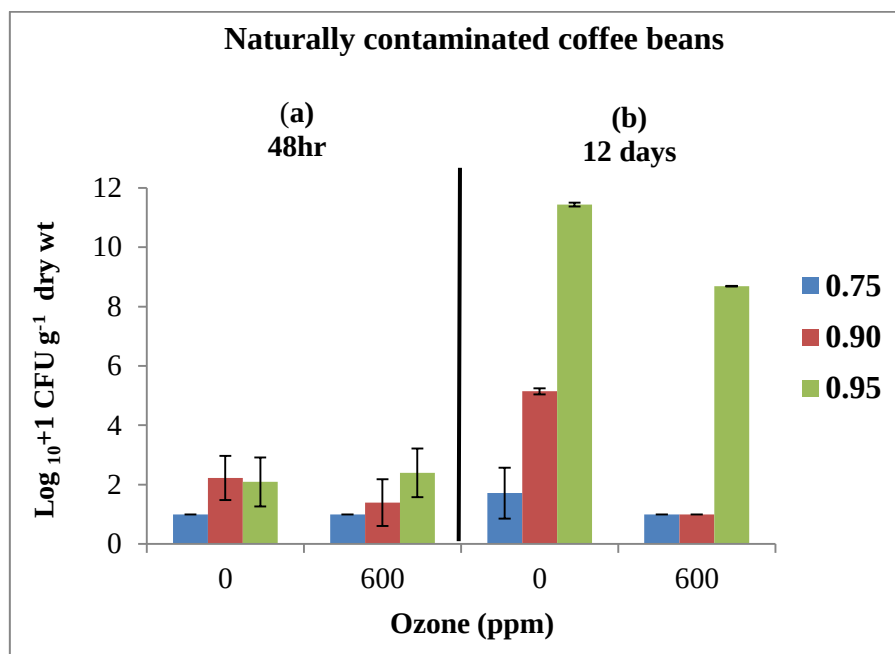


Figure 5.2: Effect of air or 600 ppm O₃ exposure for 60 min (in 100 ml glass columns) at a flow rate of 6 L min⁻¹ on the log₁₀+1 of the populations isolated from naturally contaminated coffee beans, adjusted to 0.75, 0.90 and 0.95 a_w, compared to the counts of the control (untreated 0 ppm) untreated (a) directly after exposure and (b) after 12 days storage at 30°C. Vertical bars indicate the standard error of the mean.

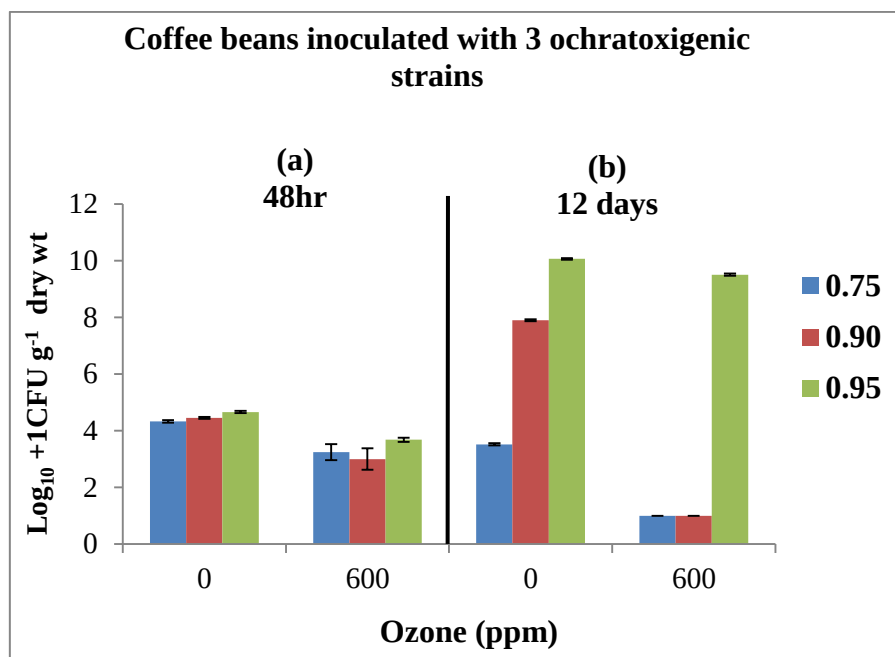


Figure 5.3: Effect of air or 600 ppm O₃ exposure for 60 min (in 100 ml glass column) at a flow rate of 6 L min⁻¹ on the logarithm of the populations (log₁₀+1 CFUs g⁻¹ dry weight) isolated from naturally contaminated coffee beans, plus inoculated with *A. westerdijkiae* (CBS 12986), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) adjusted to 0.75, 0.90 and 0.95 a_w, compared to the controls (untreated 0 ppm)untreated (a) directly after exposure and (b) after 12 days storage at 30°C. Vertical bars indicate the standard error of the means.

Table 5.2: The statistical results for control of fungal populations ($\log_{10} +1$ CFUs g^{-1} dry weight) on (a) naturally contaminated coffee beans and that inoculated with the three ochratoxigenic strains, and (b) compares naturally contaminated coffee beans and contaminated coffee beans inoculated with 3 strains by using Kruskal-Wallis Test (non-normality data).

Factors						
(a)	a_w	Ozone	Days	Ozone (0, 600ppm)	a_w (0.75,0.90,0.95)	Days (48hr, 12days)
Naturally contaminated coffee beans (1)	0.75	NS	NS	S	S	S
	0.90	S	NS			
	0.95	S	S			
Coffee beans inoculated with 3 ochratoxigenic strains (2)	0.75	S	S	S	S	S
	0.90	S	S			
	0.95	S	S			
(b)	a_w	Ozone	Days	Ozone (0, 600ppm)	a_w (0.75,0.90,0.95)	Days (48hr, 12days)
(1) versus (2)	0.75	S	NS	S	S	S
	0.90	S	NS			
	0.95	S	S			

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

S Significant

NS Not significant

5.3.2 Ochratoxin A contamination in naturally contaminated and artificially inoculated coffee beans after exposed to O₃ in the glass column system

Figure 5.4 and 5.5 show the amount of OTA produced (ng g⁻¹) in naturally contaminated coffee beans and those inoculated with the ochratoxigenic fungi at 0.75, 0.90 and 0.95 a_w and exposed to 600 ppm O₃ for 60 min at a flow rate of 6 L min⁻¹ in the glass column system, and subsequently stored at 30°C under controlled ERH for 12 days.

Figure 5.4 shows that after 12 days storage OTA production was inhibited in the naturally contaminated coffee beans exposed to O₃ treatment at 0.75 and 0.90 a_w when compared with the controls. However, there was no effect at 0.95 a_w.

Where the coffee was inoculated with a mixed inoculum of the three ochratoxigenic fungal species of *A. westerdijkiae* (CBS 121986), *A. ochraceus* (ITAL 14) and *A. carbonarius* (ITAL 204) and then exposed to O₃, there was a significant reduction in OTA contamination after storage for 12 days at 0.75 and 0.90 a_w treatments when compared to the untreated controls (Figure 5.5). In addition, there was an 88% decrease in OTA production at 0.95 a_w (21.4ng g⁻¹ to 2.6 ng g⁻¹). The highest OTA production was observed in the 0.95 a_w treatment.

The statistical effect of the O₃ treatments are shown in Table 5.2.

Appendix IV shows the detailed statistical results of the Kruskal-Wallis test which shows the significant effect of 600 ppm O₃ in 0.75, 0.90 a_w in naturally contaminated, and in all the a_w treatments inoculated with the ochratoxigenic strains.

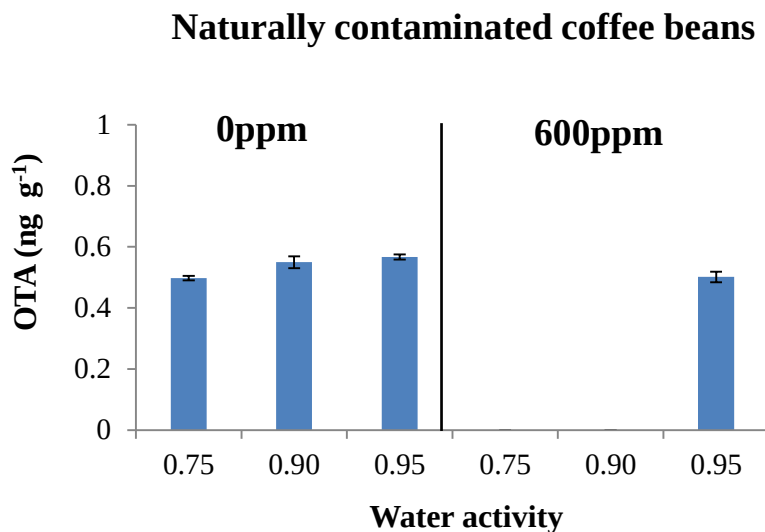


Figure 5.4: Combined effect of O₃ dose and a_w (0.98 and 0.94) on OTA production in contaminated coffee beans, exposed to O₃ (0-600 ppm for 60 min at 6 L min⁻¹ flow rate) and stored at 30°C for 12 days. Vertical bars indicate the standard error of the means.

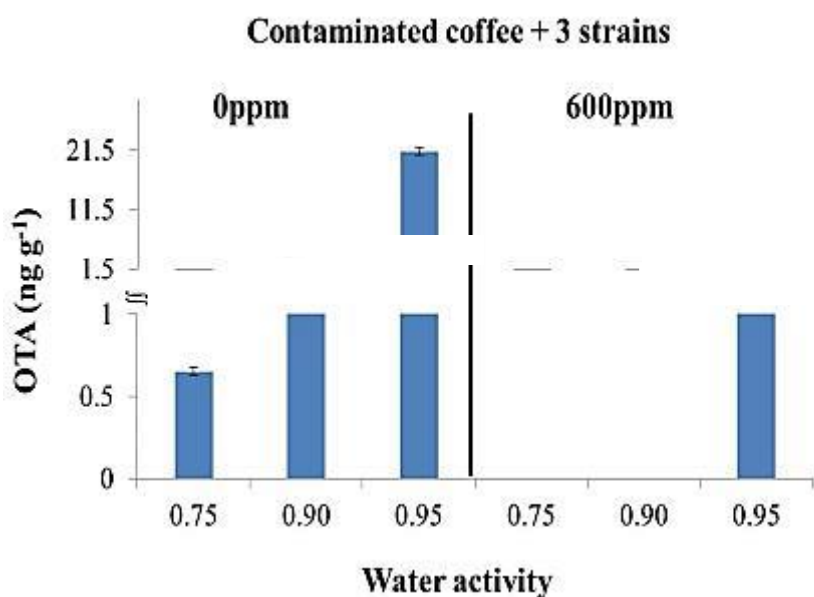


Figure 5.5: Combined effect of O₃ dose and a_w (0.98 and 0.94 a_w) on OTA production in contaminated coffee beans inoculated with *A. westerdijkiae* (CBS 121986), *A. ochraceus* (ITAL 14) and *A. carbonarius* (ITAL 204), exposed to O₃ (0-600 ppm for 60 min at 6 L min⁻¹ flow rate) and stored at 30°C for 12 days. Vertical bars indicate the standard error of the means.

Table 5.3: The statistical analysis of the effect of O₃ treatment on OTA (ng g⁻¹) production in (a) naturally contaminated coffee beans and (b) that inoculated with 3 toxigenic strains compared to the control.

Factors				
(a)	a _w	Ozone	Ozone (0, 600ppm)	a _w (0.75,0.90,0.95)
Naturally contaminated coffee beans (1)	0.75	S	S	NS
	0.90	S		
	0.95	NS		
Coffee beans inoculated with 3 ochratoxigenic strains (2)	0.75	S	S	S
	0.90	S		
	0.95	S		
(b)	a _w	Ozone	Ozone (0, 600ppm)	a _w (0.75,0.90,0.95)
(1) versus (2)	0.75	S	S	S
	0.90	S		
	0.95	S		

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

S Significant

NS Not significant

5.3.3 Effect of O₃ on populations of *A. westerdijkiae* (CBS 121986), *A. ochraceus* (ITAL 14) and *A. carbonarius* (ITAL 204) after inoculation on irradiated coffee beans.

Figure 5.6 shows the effect of gaseous O₃ immediately after treatment with 400 and 600 ppm (60 min, 6 L min⁻¹) on populations of *A. carbonarius* (strain ITAL 204). There was little difference between the controls and O₃ treatments immediately after exposure. However, after 12 days storage the fungal populations in the 0.75 a_w treatment remained

low, while in the 0.90 and 0.95 a_w treatments, regardless of O_3 exposure level, there was a significant increase in the populations of these species isolated from the coffee beans, suggesting there was no inhibitory effect at these two a_w levels with either of the O_3 treatments.

Figures 5.7 and 5.8 show that for the coffee beans inoculated with *A. westerdijkiae* and *A. ochraceus* at 0.75 a_w and exposed to 600 ppm O_3 , there was some decrease in populations after treatments, and also after storage. In contrast, there was an increase in fungal populations of these two fungi at both 0.90 and 0.95 a_w at 400 and 600 ppm O_3 when compared to the controls.

Tables 5.3 and Table 5.4 show the statistical results using the Kruskal-Wallis test on the populations of *A. westerdijkiae* (CBS 121986), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14), the effect of O_3 (400, 600 ppm) and time of incubation and the interaction of $a_w \times O_3$ treatments. There was a significant reduction effect of O_3 treatment, at 0.75 a_w , while an increase at 0.90 and 0.95 a_w (for details see Appendix IV).

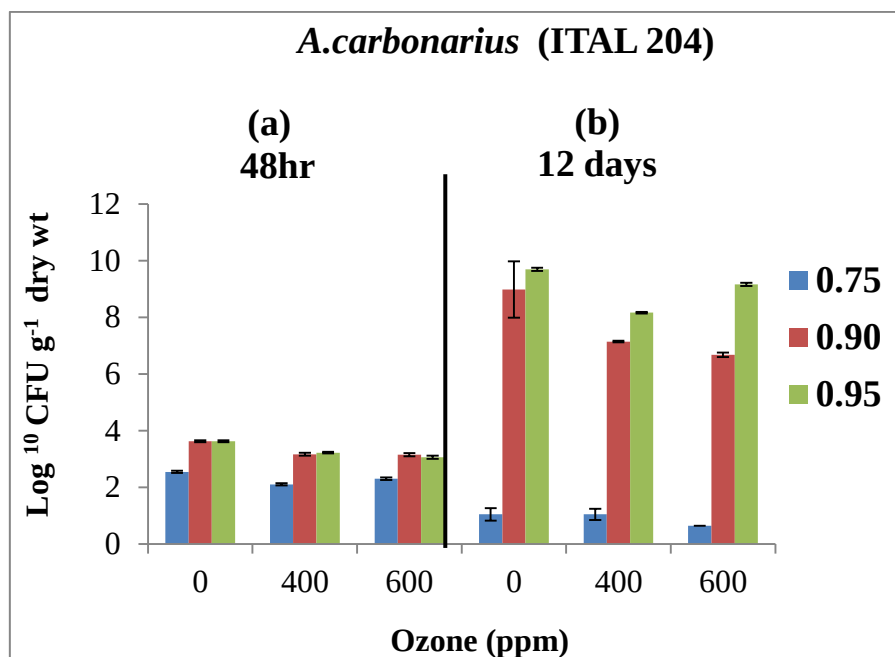


Figure 5.6: Effect of 0, 400 and 600 ppm O_3 exposure for 60 min at a flow rate of 6 L min^{-1} on the log_{10} populations of *A. carbonarius* (ITAL 204) pre-inoculated on irradiated coffee beans adjusted to 0.75, 0.90 and 0.95 a_w , compared to the control (untreated, 0 ppm); (a) directly after exposure and (b) after 12 days storage at 30°C. Vertical bars indicate the standard error of the means.

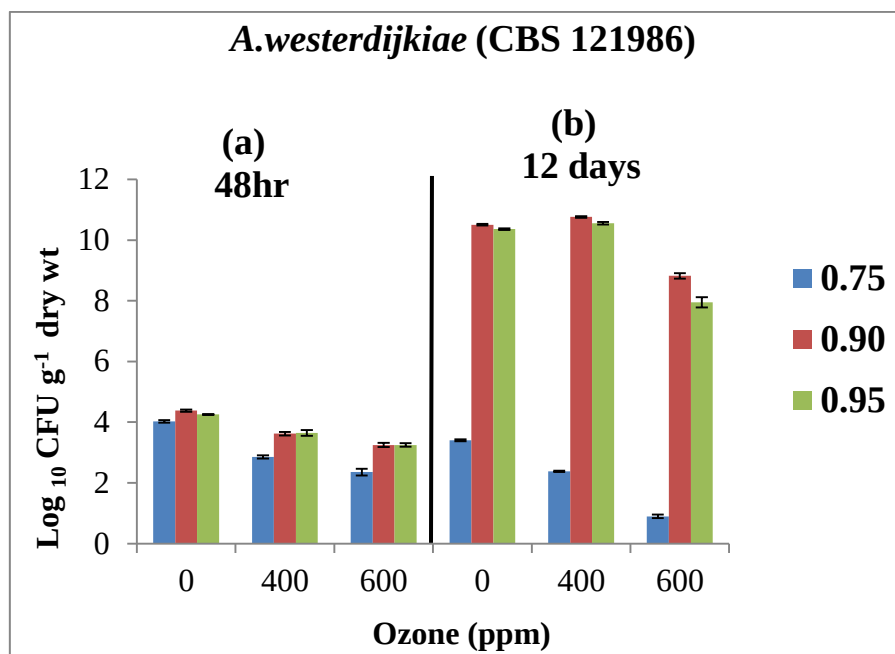


Figure 5.7: Effect of 0, 400 and 600 ppm O₃ exposure for 60 min at a flow rate of 6 L min⁻¹ on the log₁₀ populations of *A. westerdijkiae* (CBS 121986) inoculated on irradiated coffee beans adjusted to 0.75, 0.90 and 0.95 a_w, compared to the control (untreated 0 ppm); (a) directly after exposure and (b) after 12 days storage at 30°C. Vertical bars indicate the standard error of the means.

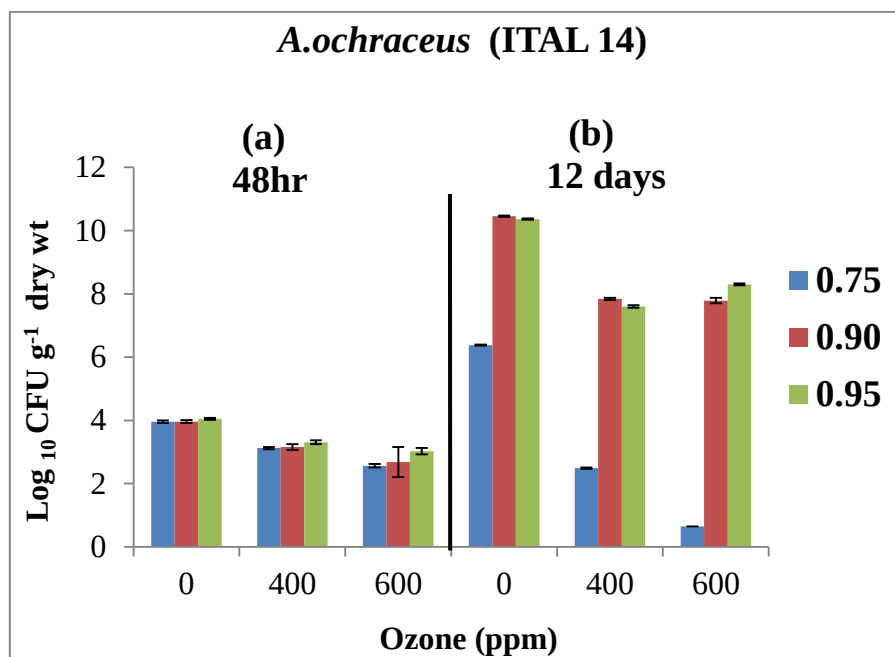


Figure 5.8: Effect of 0, 400 and 600 ppm O₃ exposure for 60 min at a flow rate of 6 L min⁻¹ on the log₁₀ populations of *A. ochraceus* (ITAL 14) inoculated on irradiated coffee beans adjusted to 0.75, 0.90 and 0.95 a_w, compared to the control (untreated, 0 ppm); (a) directly after exposure and (b) after 12 days storage at 30°C. Vertical bars indicate the standard error of the means.

Table 5.4: Summary of the statistical effect of O₃ treatments on fungal populations ($\log_{10}$ CFU g⁻¹ dry weight) of *A. westerdijkiae* (CBS 121986), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) in relation to a_w (0.75, 0.90, 0.95) and time (48 hr, 12 days) by using the Kruskal-Wallis Test (non-normality data).

Strains	Factors		
	a_w	Ozone (0, 400, 600ppm)	Time (48hr, 12days)
<i>A. carbonarius</i> (ITAL 204)	0.75	S	S
	0.90	S	S
	0.95	S	S
<i>A. westerdijkiae</i> (CBS 121986)	0.75	S	S
	0.90	S	S
	0.95	S	S
<i>A. ochraceus</i> (ITAL 14)	0.75	S	S
	0.90	S	S
	0.95	S	S

Table 5.5: The statistical analyses of the effect of O₃ on the fungal populations ($\log_{10}$ CFU g⁻¹ dry weight) of *A. westerdijkiae* (CBS 121986), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) at different concentrations (0, 400, 600 ppm), a_w and time (48 hr, 12 days) using ANOVA (normality data).

	Ozone (ppm)	a_w	$a_w \times$ Ozone	Time
<i>A. carbonarius</i> (ITAL 204)	S	S	S	S
<i>A. westerdijkiae</i> (CBS 121986)	S	S	S	S
<i>A. ochraceus</i> (ITAL 14)	S	S	S	S

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

5.3.4 Effect of O₃ on the *in situ* control of ochratoxin A production by *A. carbonarius* (ITAL 204), *A. westerdijkiae* (CBS 121986) and *A. ochraceus* (ITAL 14) in irradiated and stored coffee beans

Figure 5.9 shows the OTA contamination of irradiated coffee beans inoculated with *A. carbonarius* (ITAL 204) at 0.90 and 0.95 a_w, exposed to 400 or 600 ppm O₃. OTA was reduced by about ~55 and 80% respectively.

When *A. westerdijkiae* (CBS 121986) was examined in coffee beans exposed to O₃ at 0.90 a_w, a decrease in OTA production of about ~ 70, 32% was observed at 400 and 600 ppm respectively (Figure 5.10). On the other hand, at 0.95 a_w, OTA production was higher in the 400 ppm than 600 ppm O₃ treatments.

Figure 5.11 shows that OTA production by *A. ochraceus* (ITAL 14) was only slightly reduced when compared with the controls at 0.90 a_w, with about 10% at 400 ppm O₃. There was no effect of O₃ treatment at 0.95 a_w regardless of O₃ concentration when compared to the controls.

Tables 5.5 and 5.6 show the statistical analyses of the effects of O₃ and a_w and their interaction on OTA production by the three ochratoxigenic species on coffee beans. The a_w and O₃ (600 ppm) significantly affected OTA production by *A. carbonarius* (ITAL 204) and *A. westerdijkiae* (CBS 121986) at all a_w levels tested. The only exception was *A. ochraceus* (ITAL 14) where OTA production was controlled at 0.75 a_w only (see Appendix IV for details).

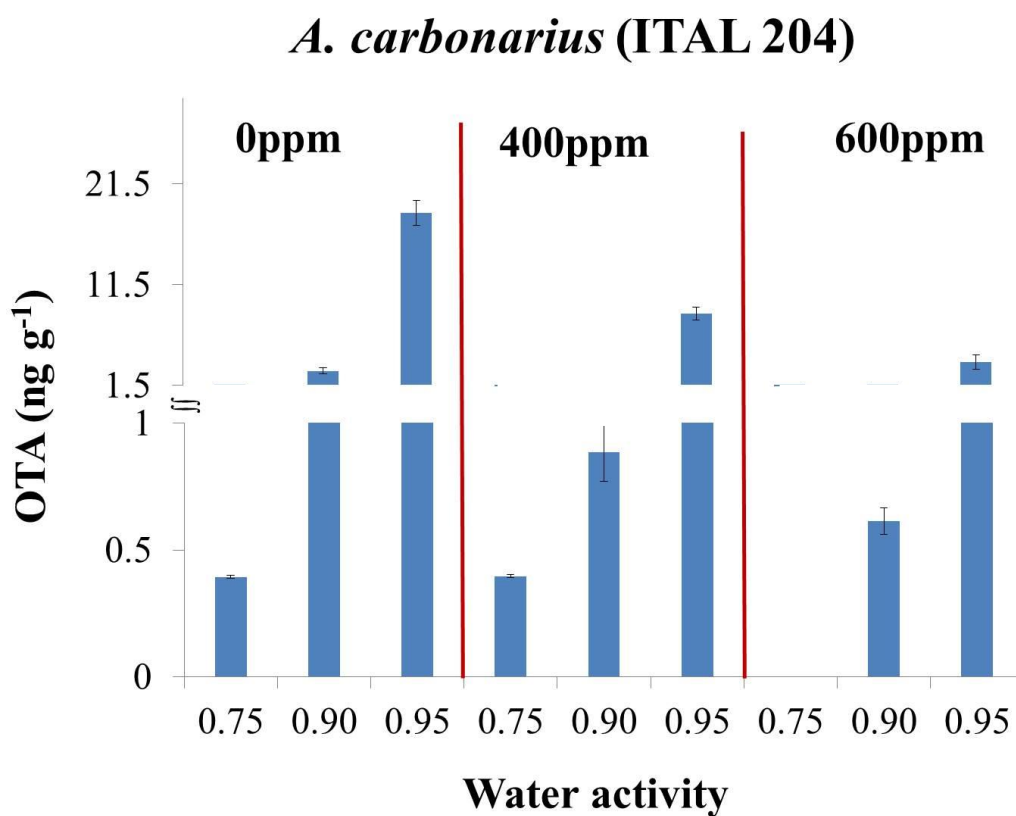


Figure 5.9: Effect of O₃ dose (0, 400, 600 ppm) and a_w (0.75, 0.90, 0.95 a_w) on OTA production by *A. carbonarius* (ITAL 204) in irradiated coffee beans exposed for 60 min at 6 L min⁻¹ flow rate and subsequently stored at 30°C for 12 days. Vertical bars indicate the standard error of the means.

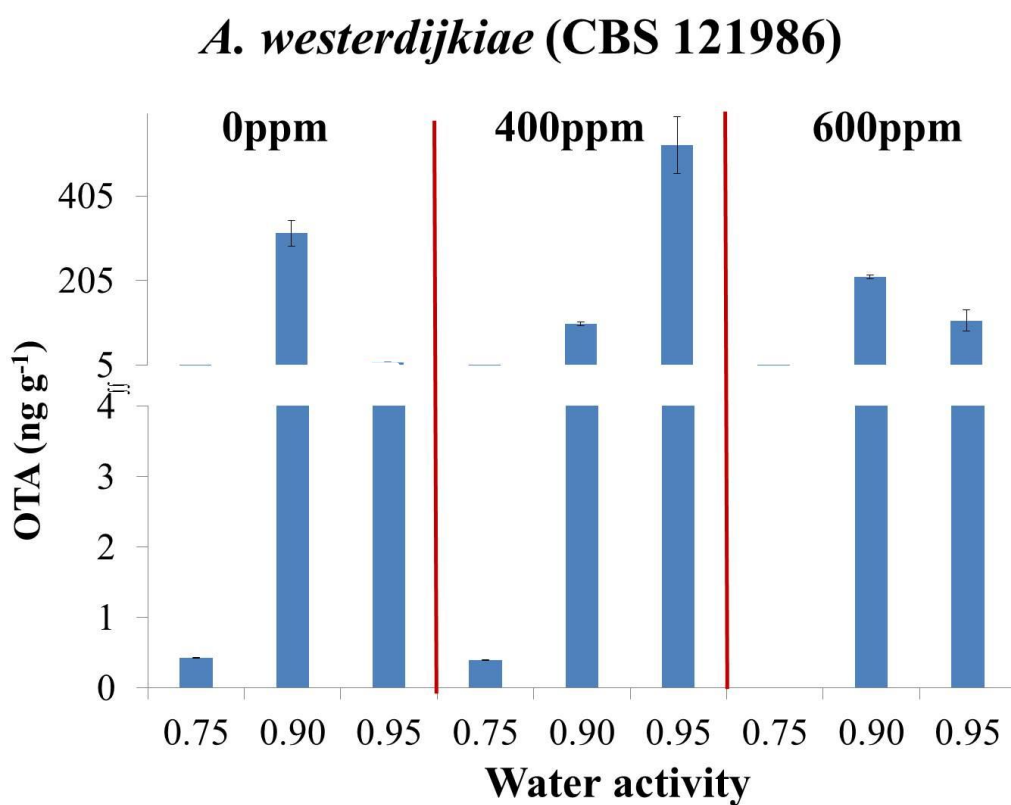


Figure 5.10: Effect of O₃ (0, 400, 600 ppm) and a_w (0.75, 0.90, 0.95 a_w) on OTA production by *A. westerdijkiae* (CBS 121986) on irradiated coffee beans, exposed for 60 min at 6 L min⁻¹ and stored at 30°C for 12 days. Vertical bars indicate the standard error of the means.

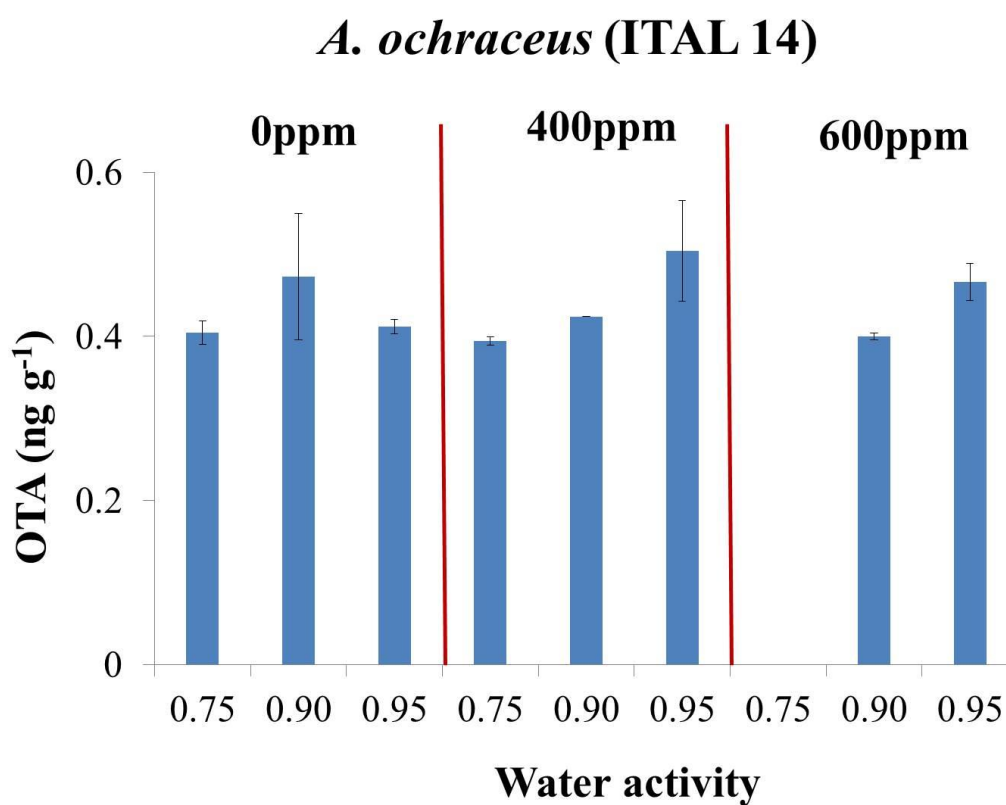


Figure 5.11: Effect of O₃ (0, 400, 600 ppm) and a_w (0.75, 0.90, 0.95 a_w) on OTA production *A. ochraceus* (ITAL 14) on irradiated coffee beans, exposed for 60 min at 6 L min⁻¹ and stored at 30°C for 12 days. Vertical bars indicate the standard error of the means.

Table 5.6: Summary of the statistical results of O₃ treatment on OTA (ng g⁻¹) production by *A. westerdijkiae* (CBS 121986), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) in relation to a_w (0.75, 0.90, 0.95) after 12 days storage using the Kruskal-Wallis Test (non-normality data).

Strains	Factors	
	a _w	Ozone (ppm)
<i>A. carbonarius</i> (ITAL 204)	0.75	S
	0.90	S
	0.95	S
<i>A. westerdijkiae</i> (CBS 121986)	0.75	S
	0.90	S
	0.95	S
<i>A. ochraceus</i> (ITAL 14)	0.75	S
	0.90	NS
	0.95	NS

Table 5.7: Statistical comparison of OTA production by the species in relative to O₃ and a_w x O₃ using ANOVA.

	Ozone (ppm)	a _w	a _w x Ozone
<i>A. carbonarius</i> (ITAL 204)	S	S	-
<i>A. westerdijkiae</i> (CBS 121986)	S	S	-
<i>A. ochraceus</i> (ITAL 14)	NS	S	-

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

S Significant

NS not significant

5.4 Discussion

5.4.1 *In situ* efficacy of O₃ on fungal populations and OTA production in naturally contaminated and artificially inoculated coffee beans.

A relatively high exposure concentration (600 ppm) of gaseous O₃ was used in order to compare the efficacy to control fungal populations and OTA production of naturally contaminated coffee beans and that inoculated with a mixture of three ochratoxigenic strains immediately after exposure (48 hr) and after storage at different a_w levels. There have been no previous studies done on the effects of O₃ on controlling fungal or OTA contamination. Directly after exposure, there appeared to be no impact on the fungal populations, regardless of initial a_w level treatment. However, after storage, there was some reduction (26%) observed in coffee at 0.95 a_w and treated with 600 ppm O₃. In addition, no fungal populations and OTA contamination was found at 0.75 and 0.90 a_w when the coffee was treated with 600 ppm gaseous O₃ for 60 min. It appears that under wetter conditions ($\geq 0.95 a_w$) it is more difficult to control fungal populations and OTA contamination.

Some studies have shown that in environmental stressed conditions O₃ treatment may be more effective in controlling fungal contamination and perhaps mycotoxin production. For example, Mylona et al. (2014) exposed maize grains *in situ* to gaseous O₃ (100, 200 ppm) for 60 min and subsequently stored the grain initially inoculated with *Fusarium verticillioides* for 15 and 30 days at (0.88, 0.92 a_w). They observed that fungal populations, directly after exposure, was significantly reduced and fumonisins, especially at 0.88 a_w was minimal. After storage, very low amounts of fumonisin FB1 occurred at 0.92 a_w . Similarly Mylona (2013) studied effect of O₃ on *F. langsethiae* which produces T-2 and HT-2 toxin production in oats and found that toxin production was reduced by 38-99% at different a_w levels and with 200-400 ppm O₃. Also, a 74-99.9% reduction in OTA production by *Penicillium verrucosum* was found in wheat grain treated with O₃ and stored for 30 days (Mylona, 2013).

In the present study, similar effects were found in coffee beans inoculated artificially with a mixture of three ochratoxigenic species. When compared to the controls after

storage there was a significant reduction in fungal population after treatment with 600 ppm O₃. Complete inhibition of both fungal populations and OTA occurred at both 0.75 and 0.90 a_w when treated with 600 ppm O₃. Some studies have used higher doses of O₃ (75 mg L⁻¹) and longer exposure times (60min) to decrease fungal growth in grains (Luo et al., 2014). For instance, Prudente and King (2002) used 10 to 12 % (by weight) of O₃ gas to degrade 92% of aflatoxins in corn at a flow rate of 2 L min⁻¹ for 96 hr. White et al. (2013) suggested that up to 1000-15,000 ppm of gaseous O₃ can reduce fungal growth in high moisture maize at 0.5 L min⁻¹ treatment for 1 hr. It may be that by optimizing the three key factors: O₃ concentration level x time of exposure and water availability control efficiency can be optimized.

5.4.2 Effect of O₃ on the fungal populations and OTA production by *A. carbonarius* (ITAL 204), *A. westerdijkiae* (CBS 121986) and *A. ochraceus* (ITAL 14) when inoculated on irradiated coffee beans

Two O₃ concentrations 400 and 600 ppm, were used in order to compare efficacy for controlling fungal populations of the three ochratoxigenic species in coffee beans. Our study showed that O₃ has little effect on fungal populations at 0.75 a_w, while at 0.90 and 0.95 a_w, which are much more conducive conditions for growth, the populations increased significantly, even after exposure to O₃. All three species produced high amounts of OTA in the 400 and 600 ppm O₃ treatments at both 0.90 and 0.95 a_w. Under conditions which probably do not allow these fungal species to grow (0.75 a_w) little if any OTA was produced. Some fungal spores are less sensitive to ozone than bacteria (Restaino, 1995; Jindal et al, 1995). It has been reported that <0.003 mg L⁻¹ has no effect on fungal spores. The growth and sporulation is repressed by concentrations higher than 0.03 mg L⁻¹. It has been suggested that >20 mg L⁻¹ of O₃ destroys conidia, other asexual spores and fungal populations (Steponavicius et al, 2012). Aflatoxin B₁ (AFB₁), produced by *Aspergillus flavus* has been reported to be degraded by O₃ (25 mg min⁻¹) on cottonseed (Dwarakanath et al, 1968). Similarly, a reduction in AFB₁ has been reported with treatment of 25 mg min⁻¹ O₃ of peanuts (Dollear et al., 1968). Also, Maeba et al. (1988) reported inactivation of pure AFB₁ by 1.1 mg L⁻¹ for 5 min. However. Allen et al. (2003) suggested that fungal mycelium was less resistant to

gaseous O₃ than conidia. This is surprising, as both Sultan (2012) and Mylona et al. (2014) found that spores were significantly more sensitive to O₃ than mycelial extension which was relatively unaffected.

Mixture of gaseous O₃ (1250 ppb) and air on wet grains, were found to suppress *Fusarium avenaceum*, *F. graminearum*, *F. poae*, *F. solani*, *F. tricinctum*, *F. sporotrichioides* and *Alternaria alternata* top surface layer in grain for 2 hr a days, this study did not consider the mycotoxins production (Steponavicius et al. 2012).

O₃ possesses mutagenic effects which reduces AFB₁ and fumonisins in corn (Prudente and King, 2002). The present study shows that a high dose of gaseous O₃ (600ppm), regardless of a_w level, does not appear to degrade OTA, especially when contamination is by *A. carbonarius*. The tolerance of *Aspergillus* spp. may be due to their dark pigmentation and relatively thick walled conidia which can provide protection against UV-light, sunlight and perhaps gases such as O₃. Hibben & Stotzky (1969) indicated that small hyaline spores are more sensitive to O₃, while, large and pigmented spores, like *A. niger*, were more resistant (Restaino et al., 1995). Spores of *A. fumigatus* have been found to be particularly resistant to O₃ (Whistler & Sheldon, 1988). Of course, the relationship between O₃ concentration x time of exposure is particularly important as has been shown by Mylona (2014), Raila et al. (2006) and Steponavicius et al. (2012).

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

This study has shown that coffee imported into Kuwait from different countries is very dry but still contains fungal contamination. However, the low moisture content means that flavour may be affected. The coffee was also contaminated with ochratoxigenic fungi, which could produce ochratoxin A. This study from samples in 2011 suggests that there is some risk of exposure to OTA in Kuwaiti population due to coffee intake in various forms. There now needs to be another survey to see how consistent this is and whether chronic exposure to OTA may be occurring in the Kuwaiti population. Unfortunately, there are no present legislative limits for imported commodities like coffee in Kuwait at the present time.

Robusta and Arabica coffees contain very different amounts of caffeine. There have, surprisingly been no studies to examine whether the caffeine content may influence growth of ochratoxigenic fungal colonisation and OTA contamination. This study has demonstrated that indeed, species from the *Aspergillus* section *Circumdati* and Section *Nigri* are very sensitive to caffeine concentrations >0.5% and higher concentrations often inhibited growth and OTA production. This suggests that caffeine may in fact be a good inhibitor of mycotoxin production by some mycotoxigenic species. It was hoped that *in vitro* studies on different concentrations of coffee extract would simulate what occurred in stored coffee beans. However, while I was able to get a significant amount of information on lag phases prior to growth, growth rates and OTA production in relation to a_w x temperature conditions these were different from that in coffee. Thus, studies on coffee may give more accurate information on the ways to try to minimise growth and OTA contamination of coffee beans.

Studies on strains of two new species suggested that these had a similar range of activity to toxigenic species (Akbar & Magan, 2014). This provided new information on species, which colonise coffee beans. Since they were demonstrated to not produce OTA, they could be very good candidates for utilisation as antagonistic microorganisms of ochratoxigenic species. Thus, it would be interesting to utilise these in different ratios with toxigenic species and examine whether they may reduce OTA contamination as has been shown for aflatoxin control (Mohale et al., 2013).

Climate change scenarios are considered to have significant impacts on staple commodities including beans used for coffee and cocoa production (Magan et al., 2011). There has been only one other detailed study to examine whether interacting conditions of elevated temperature and CO₂ with drought stress may impact on *A.flavus* and aflatoxin production in maize (Medina et al., 2014; 2015). The studies carried out in this project provide new data, which suggests that there may be differences between species in their response to these combined factors. While growth may not be affected significantly by the three interacting factors, for some species there was a statistically significant increase in OTA production at 35°C and 1000 ppm CO₂, especially under drought stress. This would suggest that the prevailing future climate changes may result in more rather than less OTA contamination in hygroscopic commodities such as coffee and cocoa. More studies are probably needed at both a molecular and physiological level to better understand the relationship between the impact of these changes and secondary metabolite production.

It was hoped that gaseous O₃ might provide a method for minimisation of OTA contamination of coffee. However, very high concentrations were required when compared to other studies (Mylona et al., 2014). This may be because the species of *Aspergillus* involved have a high pigmentation levels, especially the black *Aspergilli*. This may limit the ability of O₃ to be lethal against spores or indeed any mycelial colonisation or OTA contamination. Under marginal conditions for growth of species from the *Aspergillus* section *Circumdati* and *Nigri*, and under drier conditions, O₃ appeared to be effective at inhibiting OTA contamination. It was interesting to note that in naturally contaminated coffee which was inoculated with toxigenic species the results were better than those obtained in irradiated “clean” coffee beans. It may be that the process of irradiation changes the components of the coffee beans such that infection and establishment is better and the reaction of O₃ with the surface of the coffee beans is different from that in naturally contaminated coffee beans. However, overall O₃ does not appear to be an effective alternative approach to minimising OTA in coffee beans during storage.

Below are lists of potential future lines of research.

Chapter 2

a) Biodiversity of mycotoxigenic fungi from Kuwaiti coffee, green and roasted.

- The predominant genera were *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus* and some yeasts. *Aspergillus* species were dominant on coffee beans.
- Screening of the strains isolated from the different coffee samples showed, both on a coconut-based medium, and in coffee-based media using HPLC, that OTA production occurred in strains of *A. westerdijkiae*, *A. niger* and *A. steynii*.
- Faster growth and higher OTA production was at 0.95-0.98 a_w and 25-30°C for much slower growth and less OTA was produced at 0.90 a_w and 35°C on CMEA.
- The two types of nutritional media, CMEA and RMEA, had no overall influence on relative growth of the mycotoxigenic fungi or indeed OTA production.

a) Effect of a_w x temperature x coffee extract concentration by five OTA producing strains of *Aspergillus*.

- Overall, as environmental stress (a_w x temperature) was imposed, the lag phases prior to growth increased under the conditions examined *in vitro*.
- A higher production of OTA occurred in 80% coffee extract concentration, although growth rates were reduced. This study suggests that coffee extract concentration has an impact on OTA production and thus possibly OTA contamination of coffee beans.
- Examination of the interaction of a_w x temperature on growth and OTA production *in vitro* showed that growth rate pattern for most of strains was higher at 0.98-0.99 and 25-30°C except for *A. niger* (A 1911) at 37°C.
- The highest OTA production occurred in *A. carbonarius* (ITAL 204) at 0.99 a_w and 25°C and the lowest OTA production was by a strain of *A. ochraceus* (ITAL 14) at 0.90 a_w and 37°C.

b) Effect of caffeine concentration on growth and OTA production

- Both growth and OTA production was inhibited by >0.5% caffeine concentration for strains of *A. westerdijkiae*, *A. niger* and *A. carbonarius*.
- *A. steynii* was tolerant of increased caffeine concentration, while *A. ochraceus* (ITAL 14) was stimulated to produce high amounts of OTA at >2% caffeine concentrations.

Chapter 3

a) Impact of interacting climate change environmental factors on growth and OTA production *in vitro* and *in situ*.

- Regardless of temperature or a_w level, increasing the CO₂ concentration from 400 to 1000 ppm had little impact on the lag phases prior to growth, and on growth rates but significant effects on OTA production.
- Most of the OTA producing strains grew well at 30°C and slightly slower at 35°C except for *A. niger* (A1911). Also, optimum growth was at 0.95 a_w and 35°C, while at 30°C the optimum was at 0.98 a_w *in vitro*.
- Relative interacting stress conditions such as a_w , temperature and elevated CO₂ and stimulated growth and OTA production.
- The highest OTA production was at 0.97 a_w and the minimum at 0.90 a_w in irradiated coffee beans. This indicated some differences between optimum conditions for OTA production for these strains *in situ*.
- There was a statistically significant increase in OTA production by interacting climate change conditions when cultures were exposed to a_w x temperature x 1000 ppm CO₂ when compared to the controls (400 ppm CO₂).

Chapter 4

a) **The impact and comparison of a_w x temp interactions on lag phase prior to growth, growth and potential OTA production by strains of two new species, *A. aculeatinus* and *A. sclerotiicarbonarius* isolated from coffee beans.**

- The study showed that different strains of the two species have similar a_w x temperature interaction.
- The optimum conditions for growth were at approx. 0.98-0.99 a_w and 30-35°C, with the main difference the inability of those of *A. sclerotiicarbonarius* to grow at 37°C, regardless of the a_w level.
- No growth was observed at 0.85 a_w suggesting limits for growth of around 0.88 a_w .
- The strains of these two species did not produce OTA. However, they may interact with toxigenic strains in coffee and influence OTA contamination levels depending on the prevailing conditions during drying.

Chapter 5

a) **Control of fungal growth and OTA from naturally contaminated and artificially inoculated coffee beans after exposure to O_3 *in situ*.**

- Treatment of coffee with 600 ppm of gaseous O_3 impacted on the fungal populations; regardless of the initial a_w level treatment directly after exposure.
- There was significant reduction in fungal populations in the O_3 (600 ppm) treated coffee at 0.95 a_w .
- Exposure of the naturally contaminated coffee to 600 ppm gaseous O_3 for 60 min resulted in no fungal populations or OTA production isolated from the 0.75 and 0.90 a_w treatments.
- In wetter coffee, ≥ 0.95 a_w it was difficult to control fungal populations and OTA contamination.

b) Control of fungal growth and OTA by three ochratoxigenic strains after inoculation of irradiated coffee beans and exposure to gaseous O₃

- 400 and 600 ppm O₃ has little effect on fungal populations at water stressed 0.75 a_w, while at 0.90 and 0.95 a_w, the populations increased significantly.
- All three species produced high amounts of OTA in the 400 and 600 ppm O₃ treatments at both 0.90 and 0.95 a_w, and little if any at 0.75 a_w.

6.2 Suggestions for future work

- A further more comprehensive study on the ochratoxigenic strains in imported into the Kuwaiti marketplace is required to understand the potential exposure risk, especially when consuming poor quality coffee.
- More detailed studies of the role of the *A. niger* group when exposed to longer period of CO₂ (1000 ppm), low a_w (0.6-0.7) at 37°C.
- Potential for using modified atmosphere storage with 50%+ CO₂ and low O₂ concentrations (<1.0%) as a method of controlling ochratoxigenic fungi in post-harvest processing and packaging.
- Would higher concentrations of O₃ be effective in controlling growth and OTA production by strains of *A. westerdijkiae*, which appear to be particularly tolerant to such treatment, especially under conducive environmental conditions.
- Could alternative compounds, which may also impart flavour, be used to control OTA contamination post-harvest. Examples could be different essential oils and antioxidants including cardamom and clove extracts which are popular in the middle east.

- Molecular work on gene expression of key biosynthetic genes involved in OTA production, e.g. PKS genes.
- Potential for using interference RNA (RNAi) to inhibit steps in the biosynthetic pathway of OTA production, either via the coffee plant or by direct treatment of coffee beans during fermentation or the drying process.

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APPENDICES

APPENDIX I

Appendix I. 1: Effect of water activity (a_w), temperature ($^{\circ}\text{C}$), media and their interactions on growth and OTA production by the strains isolated from coffee.

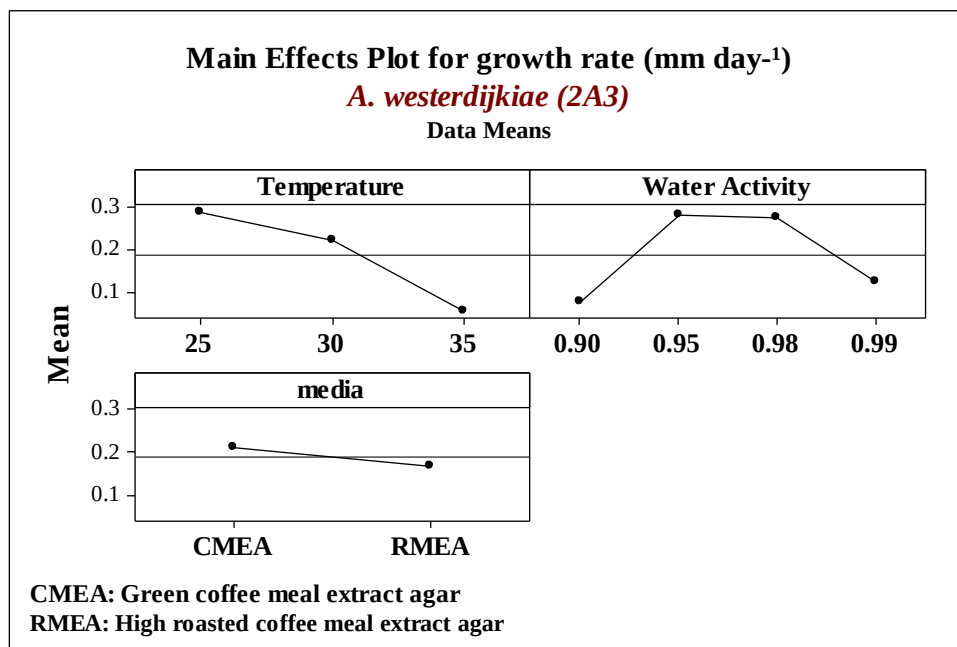
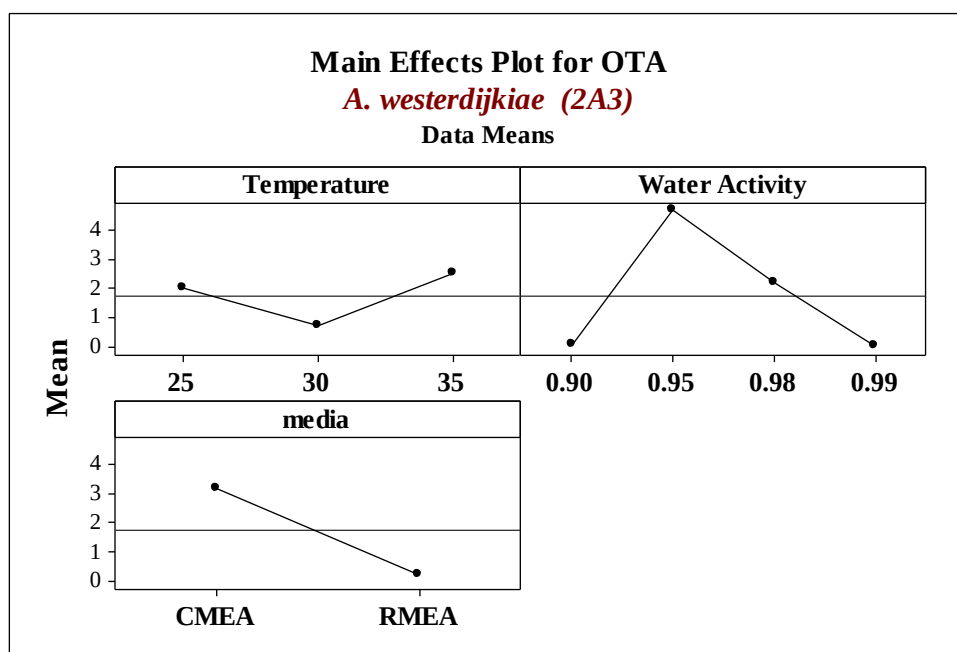


Figure I 1.a: Main effect plot of the mean of growth rate and OTA production versus temperature, a_w and media for strains *A. westerdijkiae* (2A3).

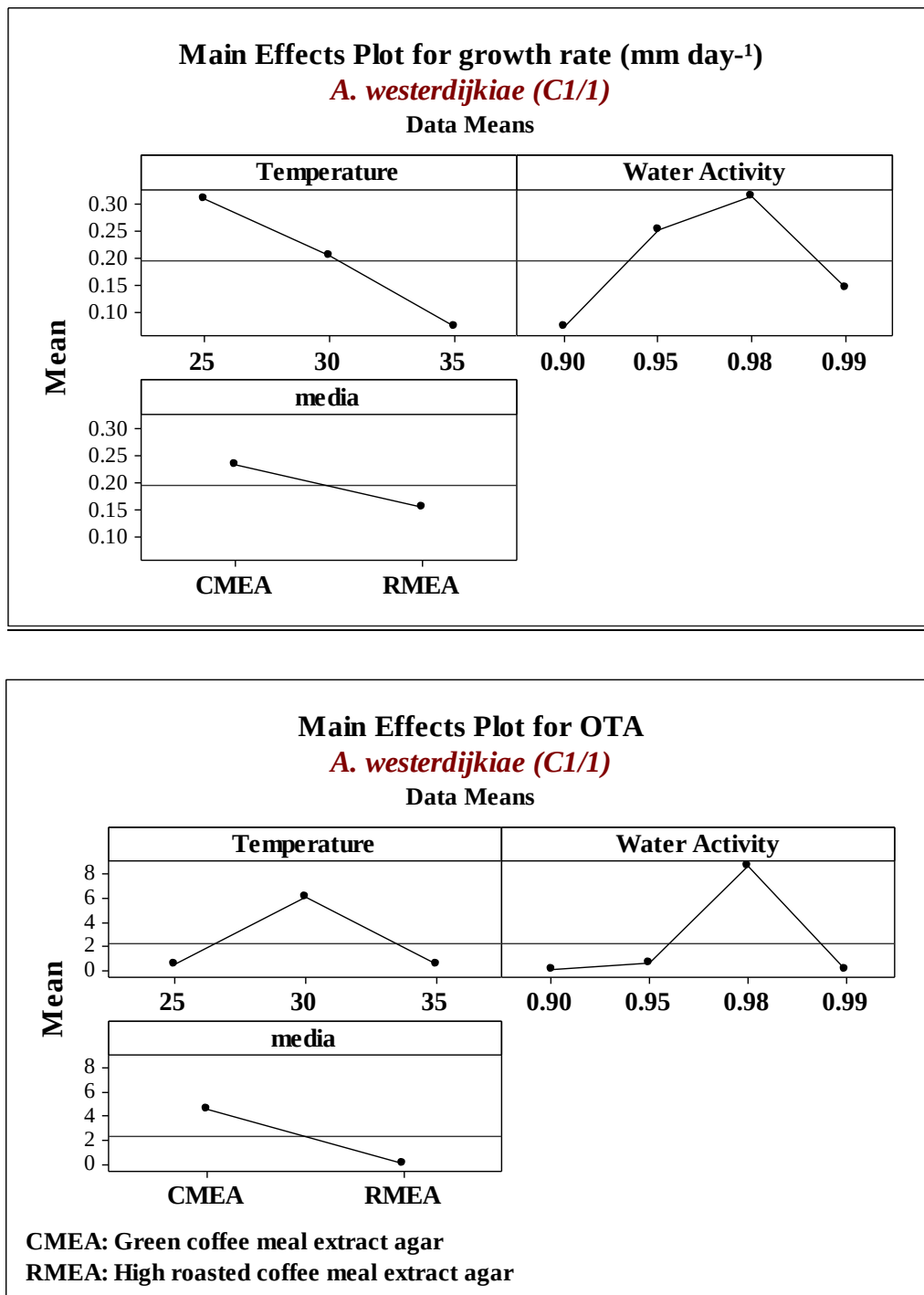


Figure I 2.b: Main effect plot of the mean of growth rate and OTA production versus temperature, a_w and media for strains *A. westerdijkiae* (C1/1).

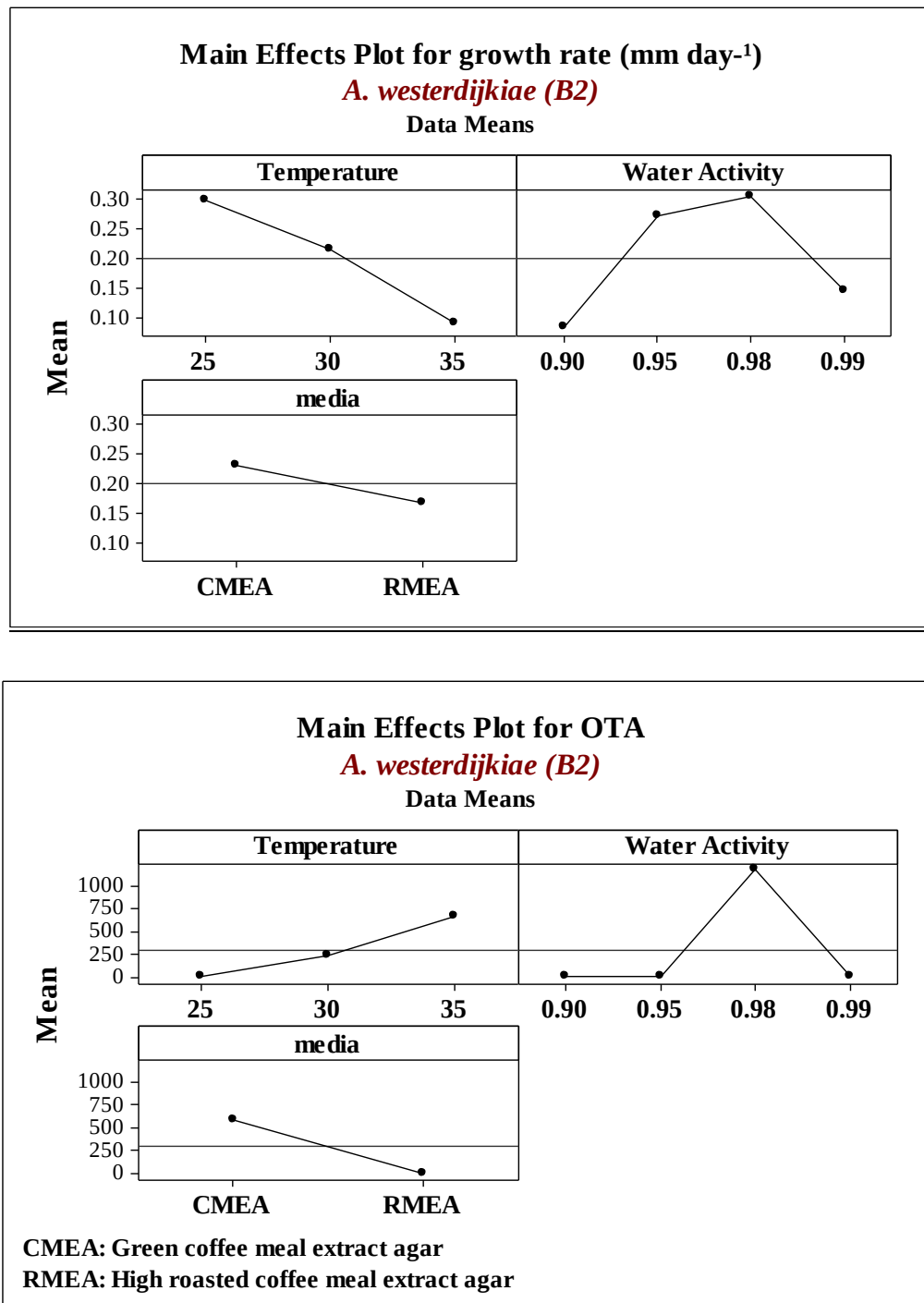


Figure I 3.c: Main effect plot of the mean of growth rate and OTA production versus temperature, a_w and media for strains *A. westerdijkiae* (B2).

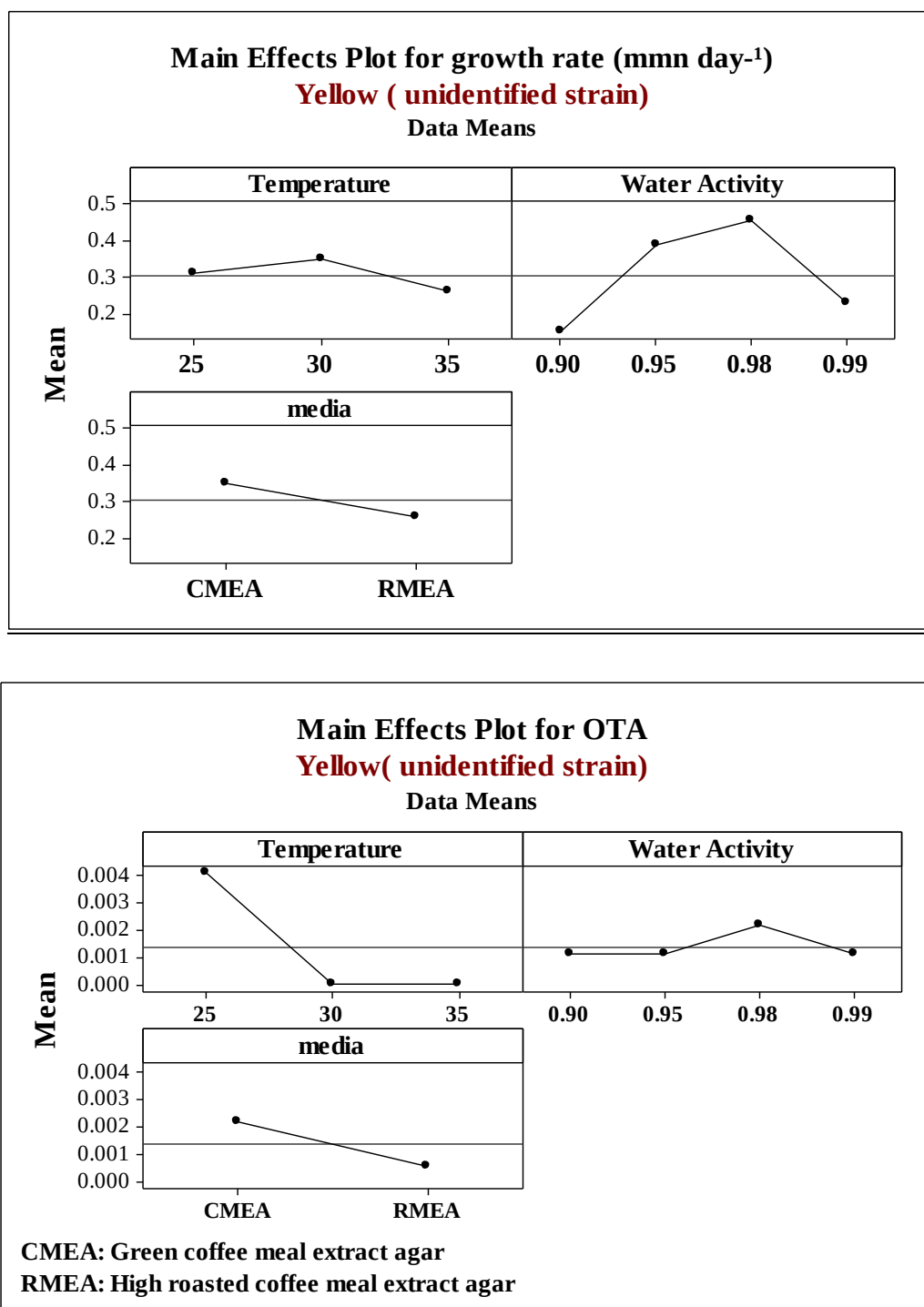


Figure I 4.d: Main effect plot of the mean of growth rate and OTA production versus temperature, a_w and media for strains Yellow (unidentified strain).

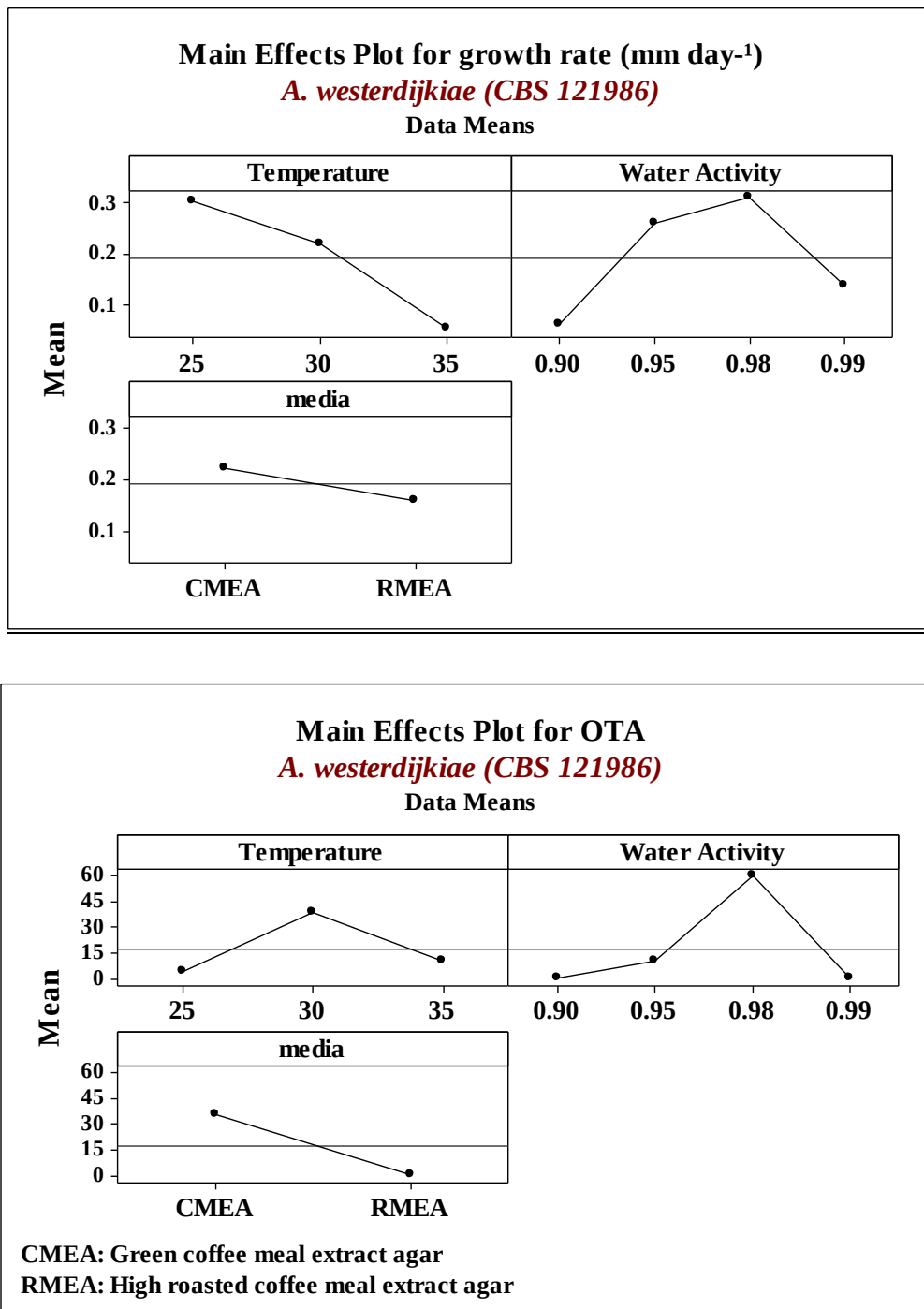


Figure I 5.e: Main effect plot of the mean of growth rate and OTA production versus temperature, a_w and media for strains *A. westerdijkiae* (CBS 121986).

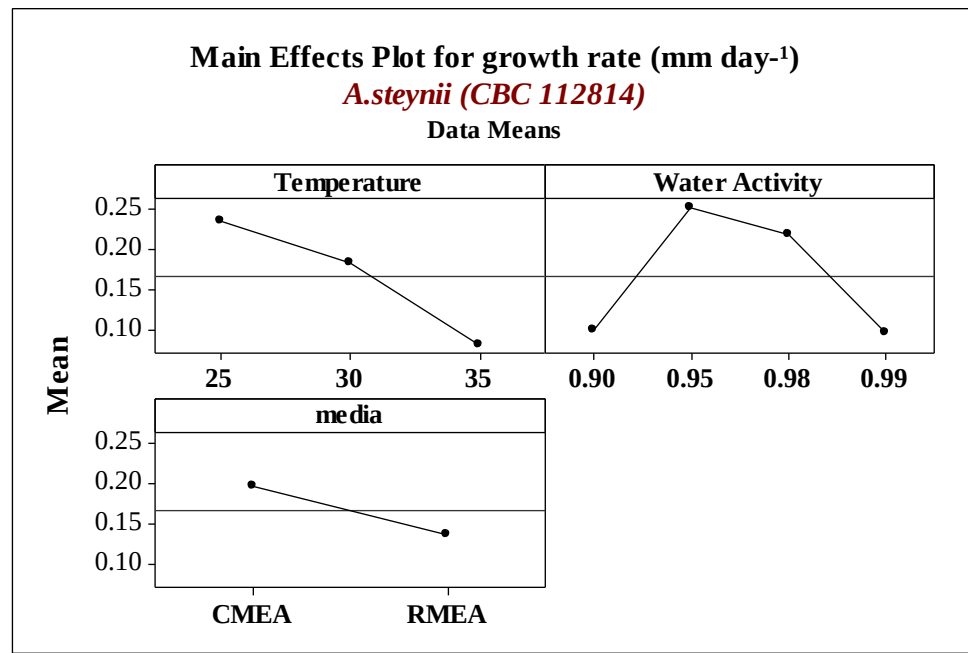
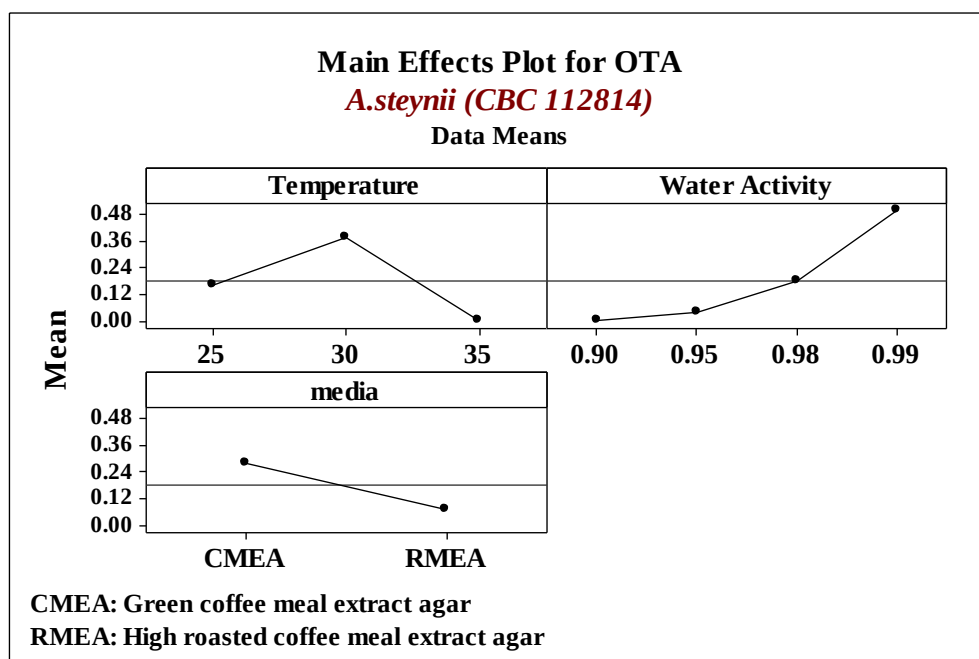


Figure I 6.f: Main effect plot of the mean of growth rate and OTA production versus temperature, a_w and media for strains *A. steynii* (CBS 112814).

Appendix I. 2: Effect coffee concentration on lag phases, growth and production of ochratoxin A (OTA) by five strains of *Aspergillus* section *Circumdati* and Section *Nigri* isolated from coffee.

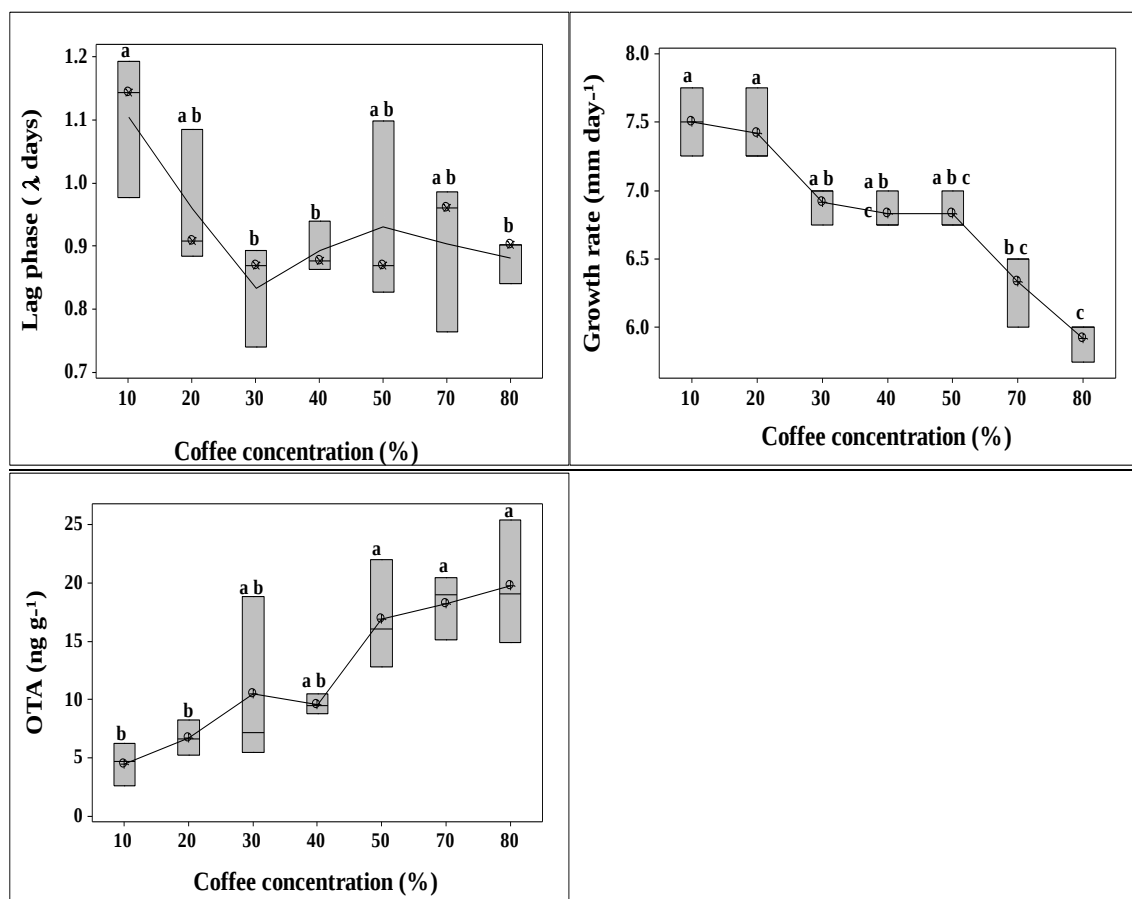


Figure I.2 a: Box-plot analysis for fungal growth (a), lag phase (λ , days) (b) and OTA production (c) of *A. westerdijkiae* (B 2) inoculated on CMEA and incubated in different coffee extract concentration (10, 20, 30, 40, 50, 70 and 80%) and 0.98 a_w at 30°C for 9 days. The box-plot shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

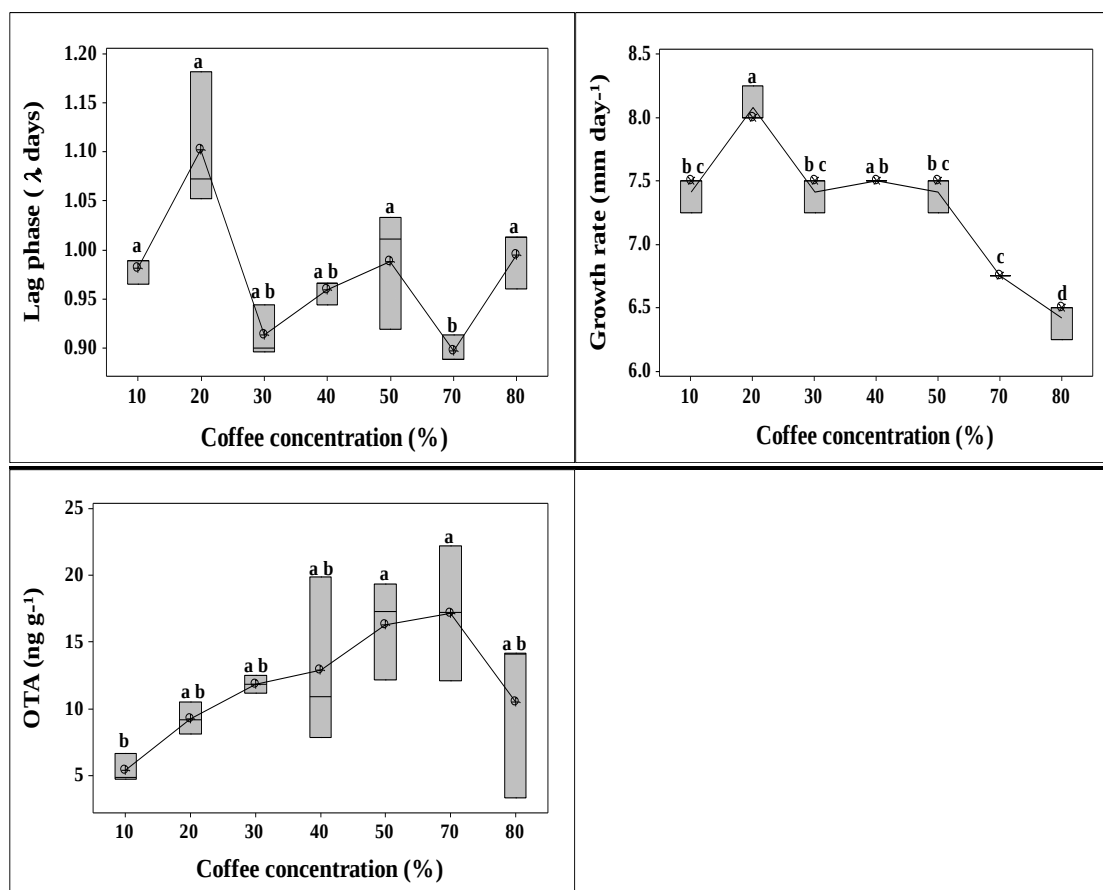


Figure I.2.b: Box-plot analysis for fungal growth (a), lag phase (λ , days) (b) and OTA production (c) of *A. westerdijkiae* (CBS 121986) inoculated on CMEA and incubated in different coffee extract concentration (10, 20, 30, 40, 50, 70 and 80%) and 0.98 a_w at 30°C for 9 days. The box-plot shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

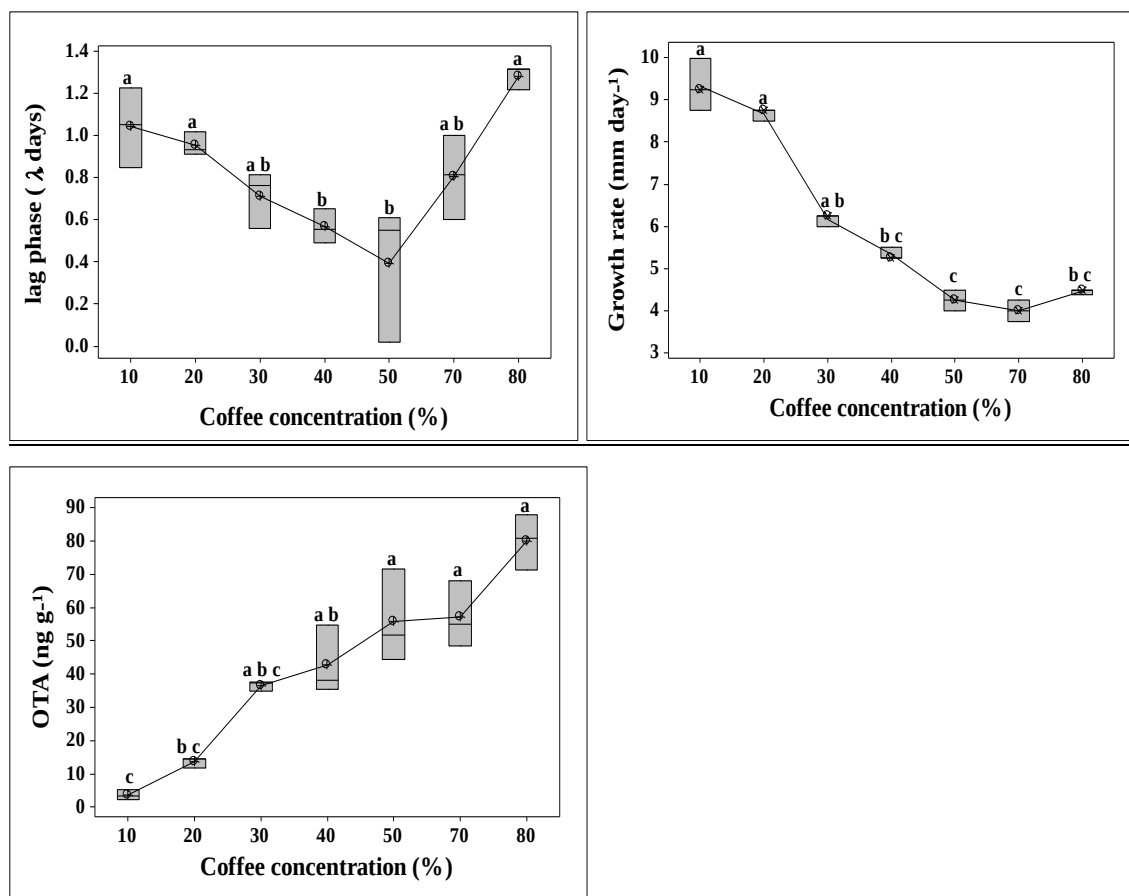


Figure I.2.c: Box-plot analysis for fungal growth (a), lag phase (λ , days) (b) and OTA production (c) of *A. niger* (A 1911) inoculated on CMEA and incubated in different coffee extract concentration (10, 20, 30, 40, 50, 70 and 80%) and 0.98 a_w at 30°C for 9 days. The box-plot shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

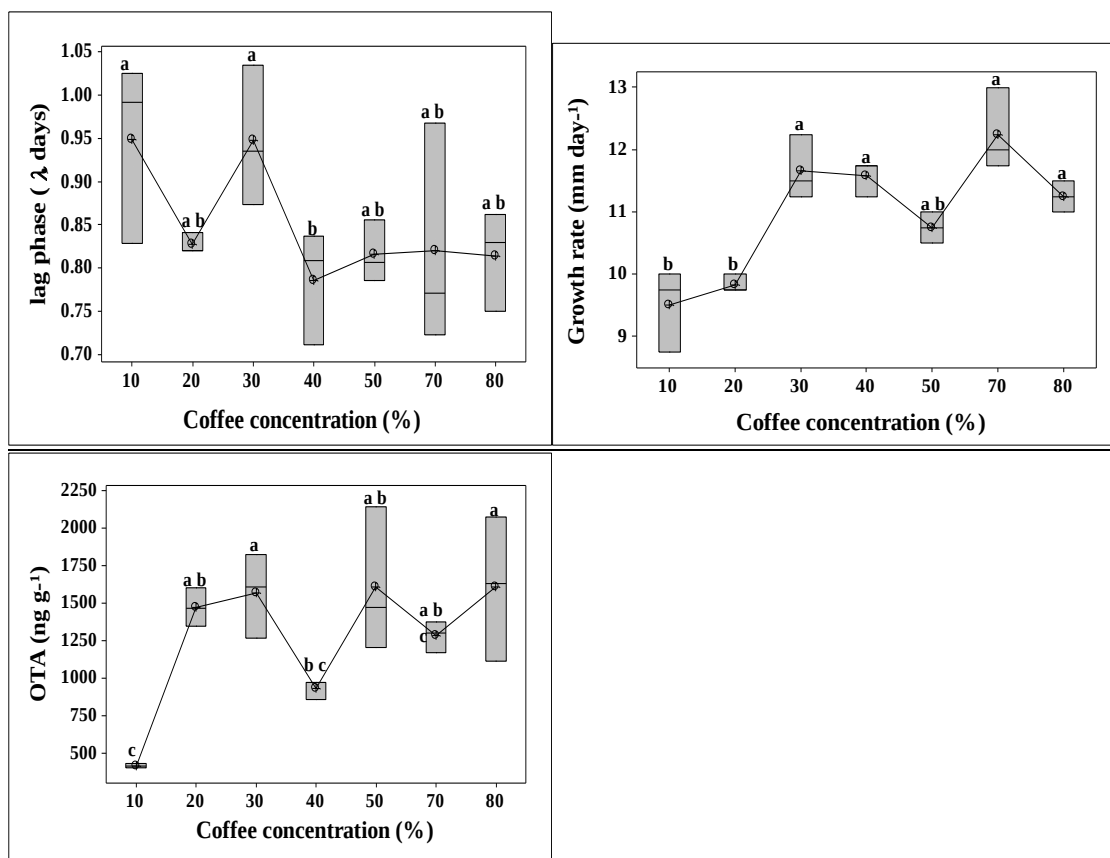


Figure I.2.d: Box-plot analysis for fungal growth (a), lag phase (λ , days) (b) and OTA production (c) of *A. carbonarius* (ITAL 204) inoculated on CMEA and incubated in different coffee extract concentration (10, 20, 30, 40, 50, 70 and 80%) and 0.98 a_w at 30°C for 9 days. The box-plot shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

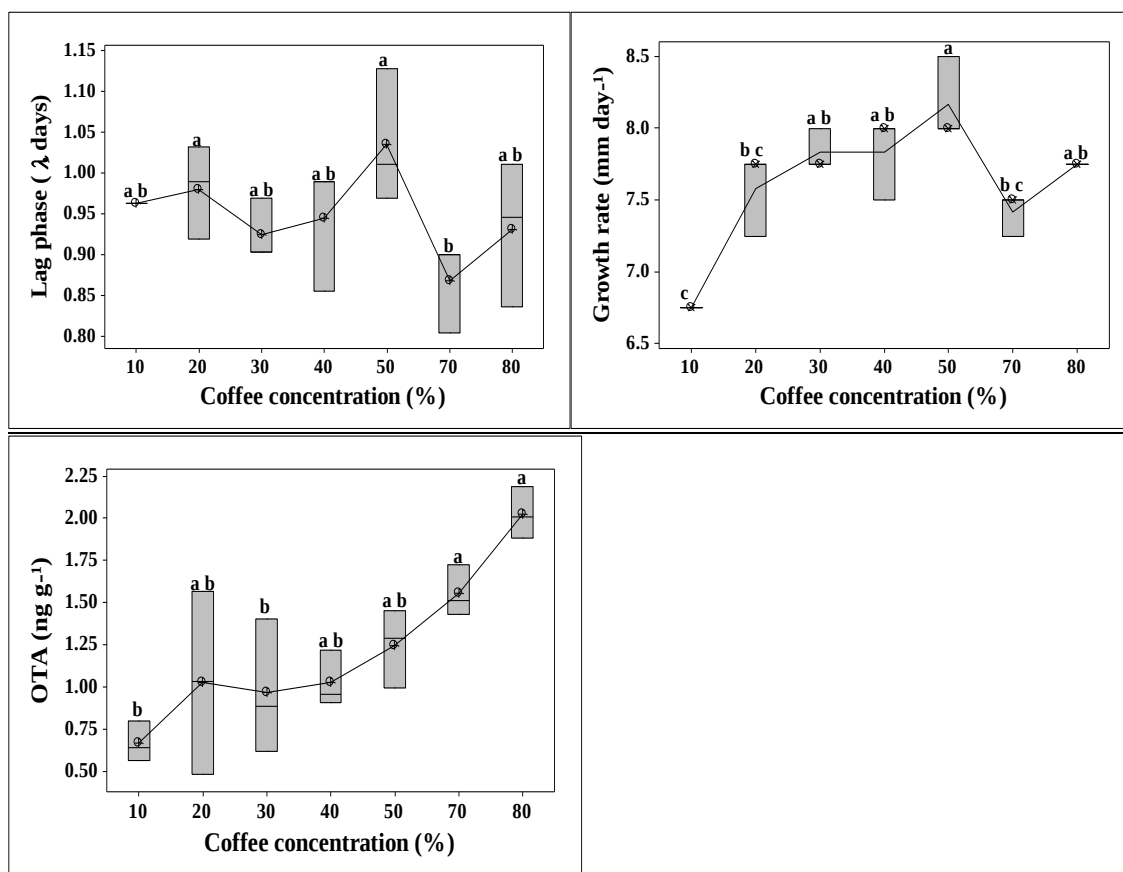


Figure I.2.e: Box-plot analysis for fungal growth (a), lag phase (λ , days) (b) and OTA production (c) of *A. ochraceus* (ITAL 14) inoculated on CMEA and incubated in different coffee extract concentration (10, 20, 30, 40, 50, 70 and 80%) and 0.98 a_w at 30°C for 9 days. The box-plot shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

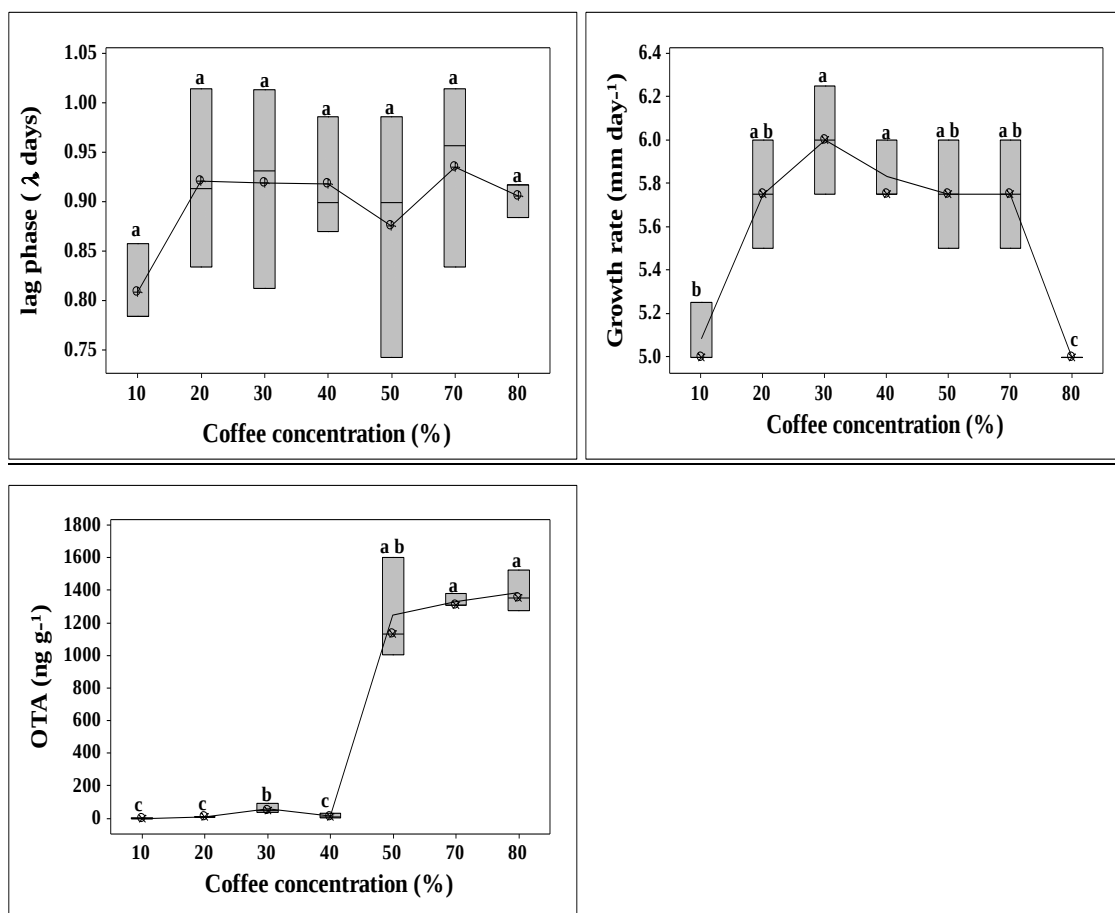


Figure I.2.f: Box-plot analysis for fungal growth (a), lag phase (λ , days) (b) and OTA production (c) of *A. steynii* (CBS 112814) inoculated on CMEA and incubated in different coffee extract concentration (10, 20, 30, 40, 50, 70 and 80%) and 0.98 a_w at 30°C for 9 days. The box-plot shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

Appendix I.3: Effect of a_w , temperature and coffee concentration on lag phases, growth and production of ochratoxin A (OTA) by five strains of *Aspergillus* section *Circumdati* and Section *Nigri* isolated from coffee.

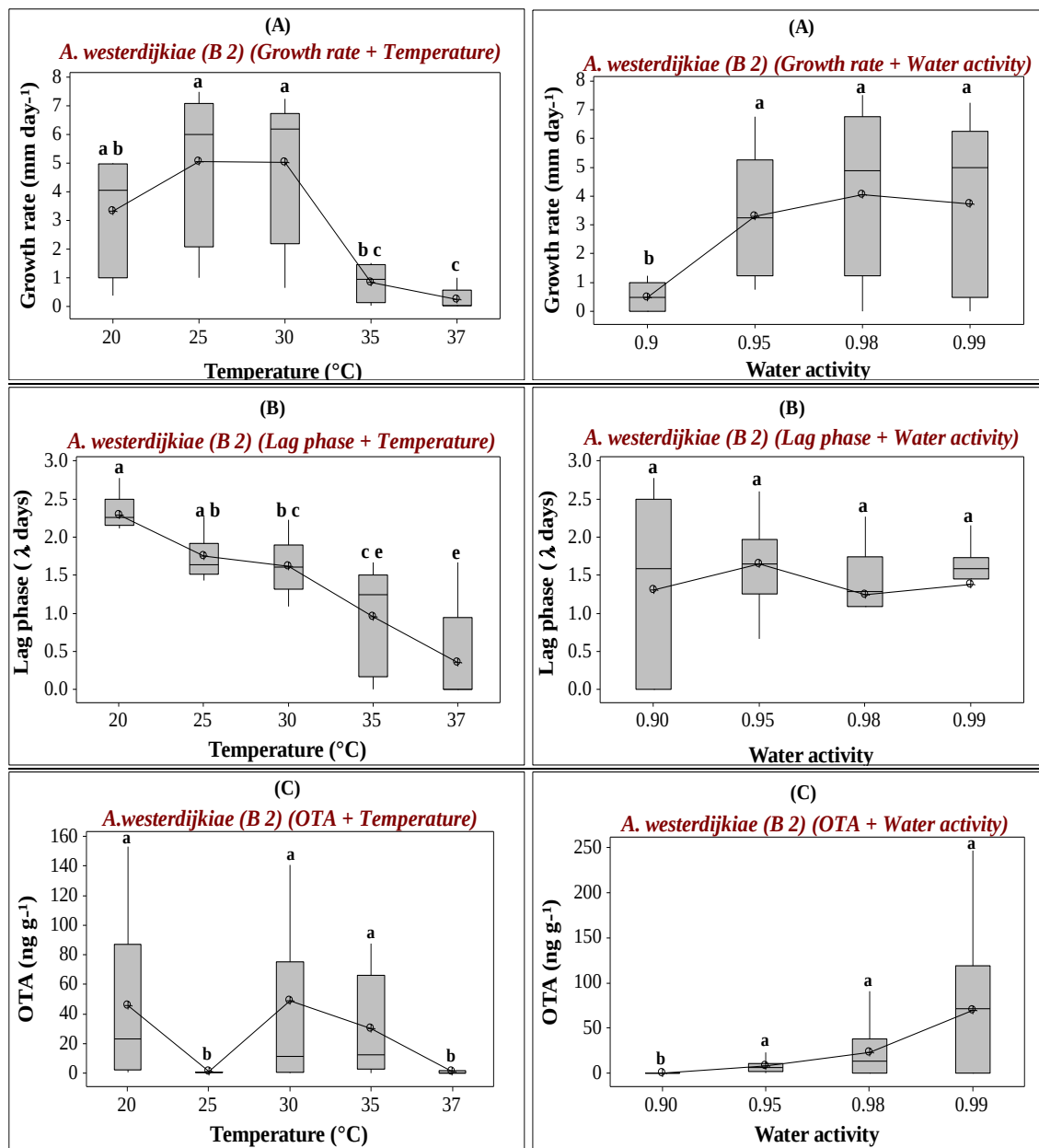


Figure I.3.a: Box-plot analysis for fungal growth (A) Growth rate (B) Lag phase and (C) OTA production of *A. westerdijkiae* (B 2) inoculated on CMEA and incubated at 5 different temperature (20, 25, 30, 35 and 37°C) for 9 days. The box-plot analysis shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

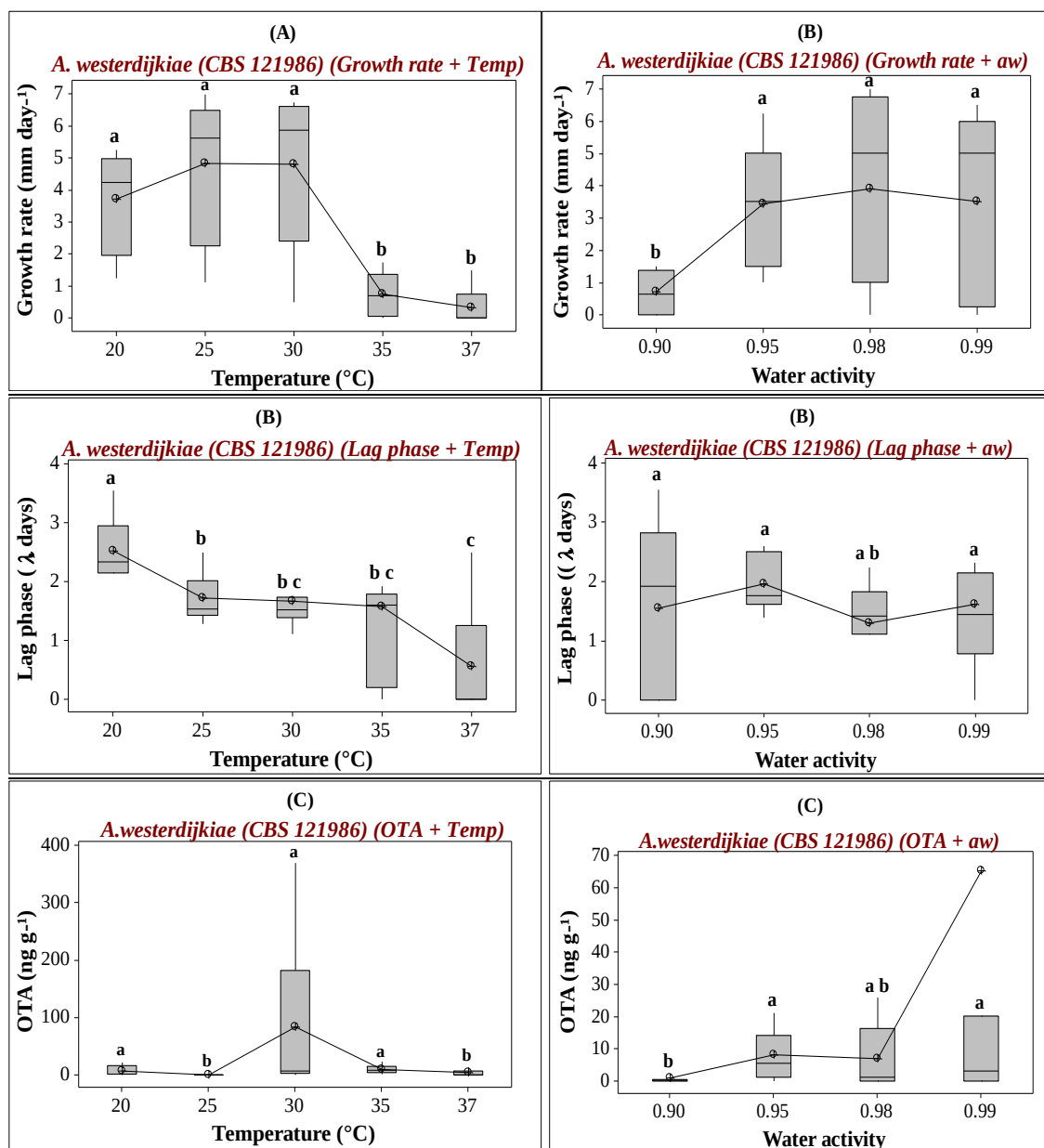


Figure I.3.b: Box-plot analysis for fungal growth ((A) Growth rate (B) Lag phase and (C) OTA production of *A. westerdijkiae* (CBS 121986) inoculated on CMEA and incubated at 5 different temperature (20, 25, 30, 35 and 37°C) for 9 days. The box-plot analysis shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

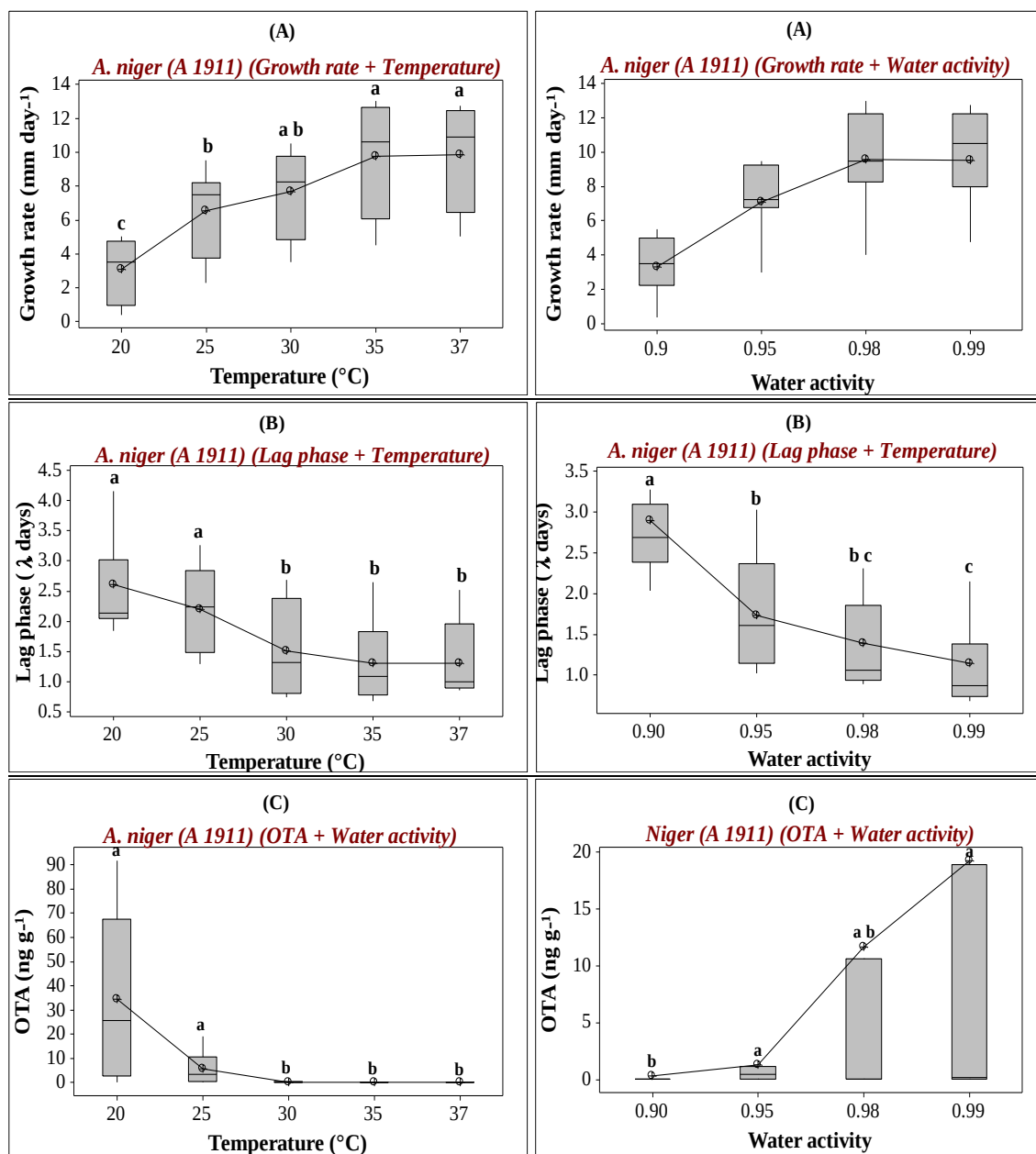


Figure I.3.c: Box-plot analysis for fungal growth (A), lag phase (λ , days) (B) and OTA production (C) of *A. niger* (A 1911) inoculated on CMEA and incubated at 5 different temperature (20, 25, 30, 35 and 37°C) for 9 days. The box-plot analysis shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

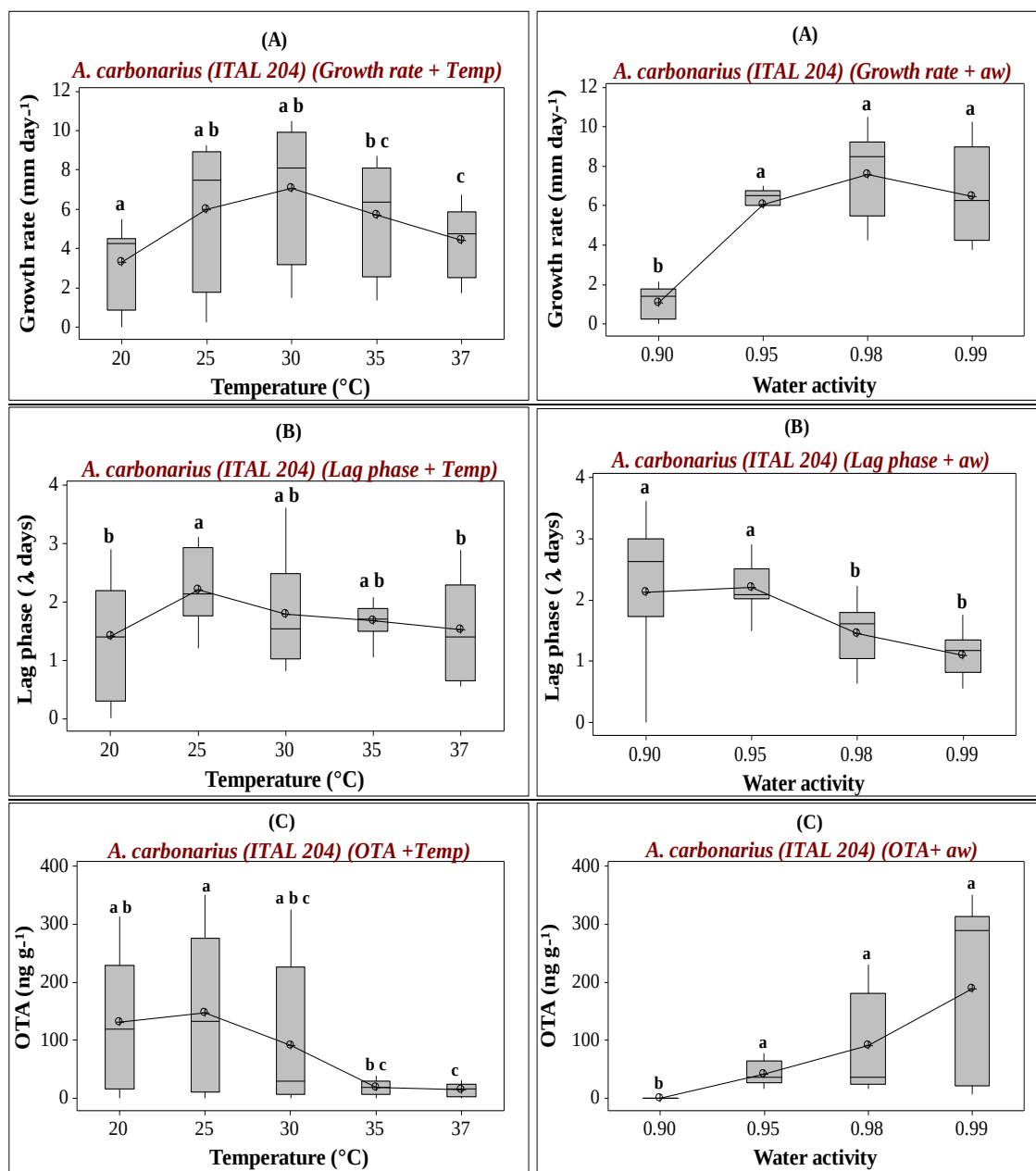


Figure I.3.d: Box-plot analysis for fungal growth (A), lag phase (λ , days) (B) and OTA production (C) of *A. carbonarius* (ITAL 204) inoculated on CMEA and incubated at 5 different temperature (20, 25, 30, 35 and 37°C) for 9 days. The box-plot analysis shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

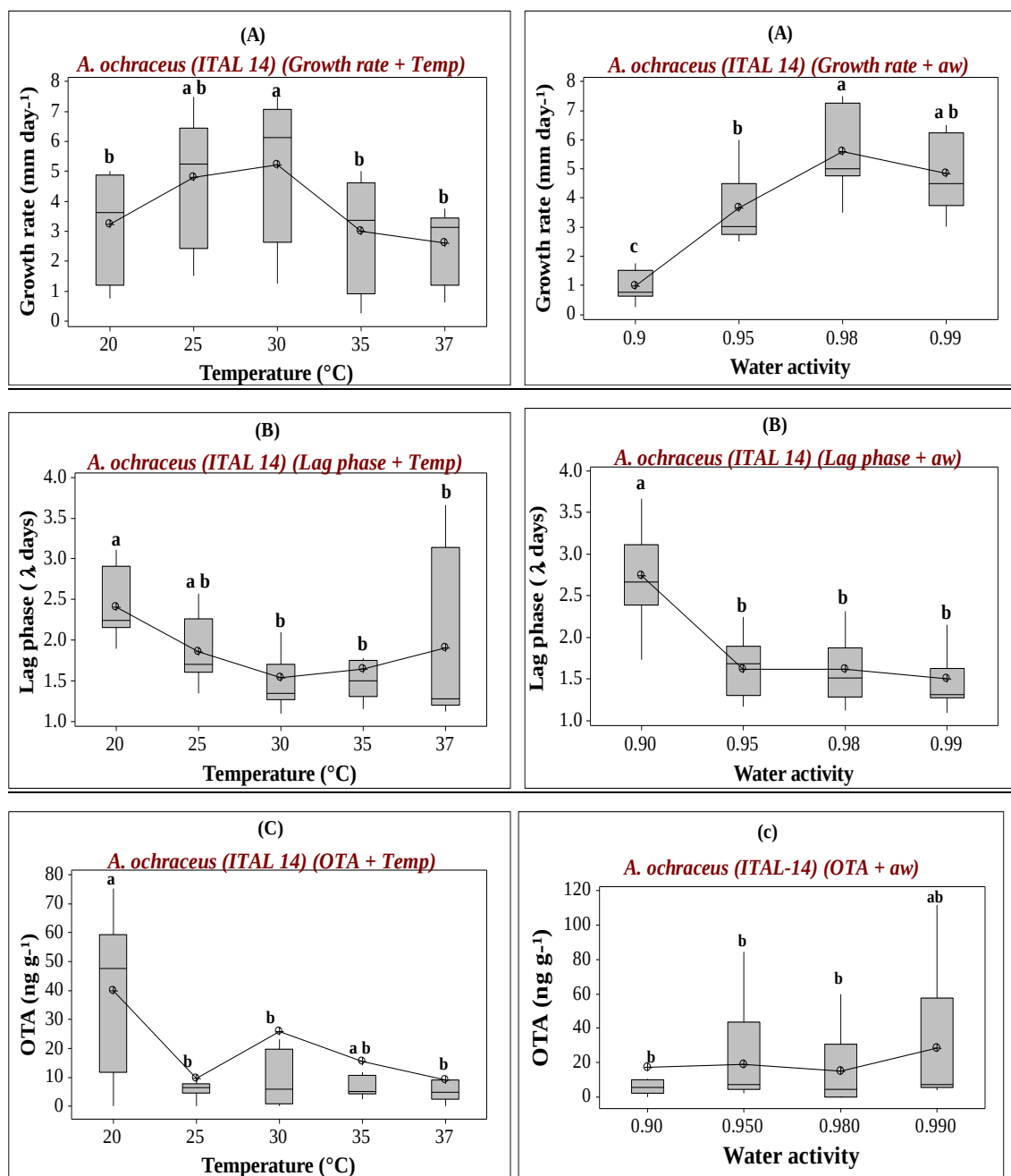


Figure I.3.e: Box-plot analysis for fungal growth (A) Growth rate (B) Lag phase and (C) OTA production of *A. ochraceus* (ITAL 14) inoculated on CMEA and incubated at 5 different temperature (20, 25, 30, 35 and 37°C) for 9 days. The box-plot analysis shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

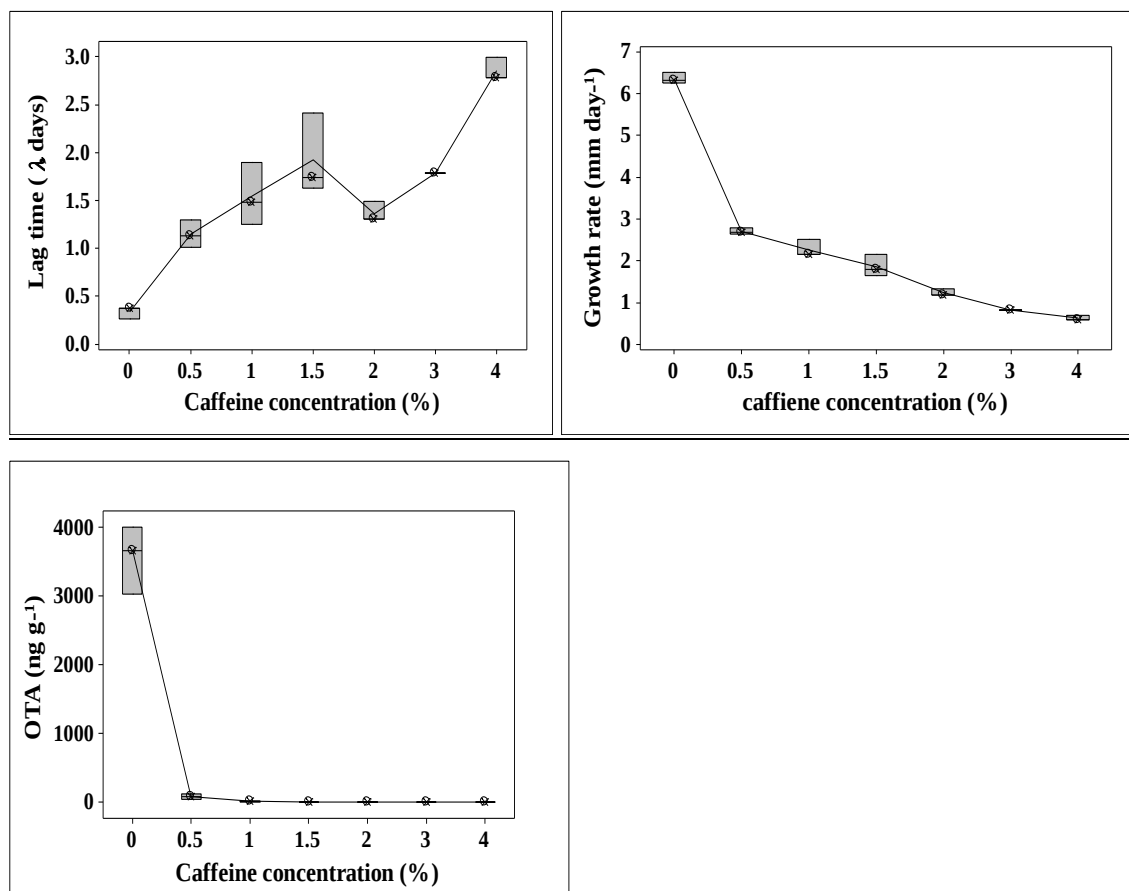
Appendix I.4: Effect of caffeine concentration on ochratoxigenic fungal strains

Figure I.4.a: Box-plot analysis for fungal growth, lag phase (λ , days) and OTA production of *A. westerdijkiae* (B2) inoculated on YES and incubated in different caffeine concentration (0, 0.5, 1, 1.5, 2, 3 and 4%) and 0.98 a_w at 30°C for 9 days. The box-plot shows the median (line inside the box).

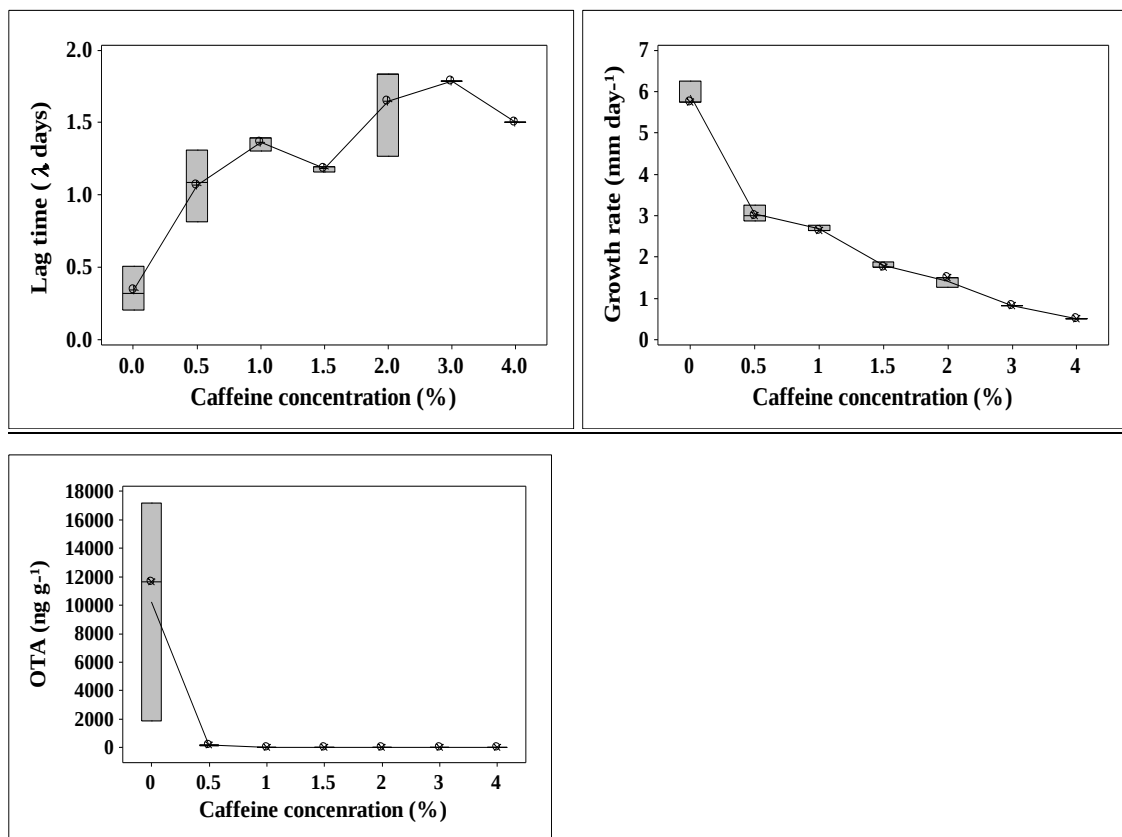


Figure I.4.b: Box-plot analysis for fungal growth, lag phase (λ , days) and OTA production of *A. westerdijkiae* (CBS 121986) inoculated on YES and incubated in different caffeine concentration (0, 0.5, 1, 1.5, 2, 3 and 4%) and 0.98 a_w at 30°C for 9 days. The box-plot shows the median (line inside the box).

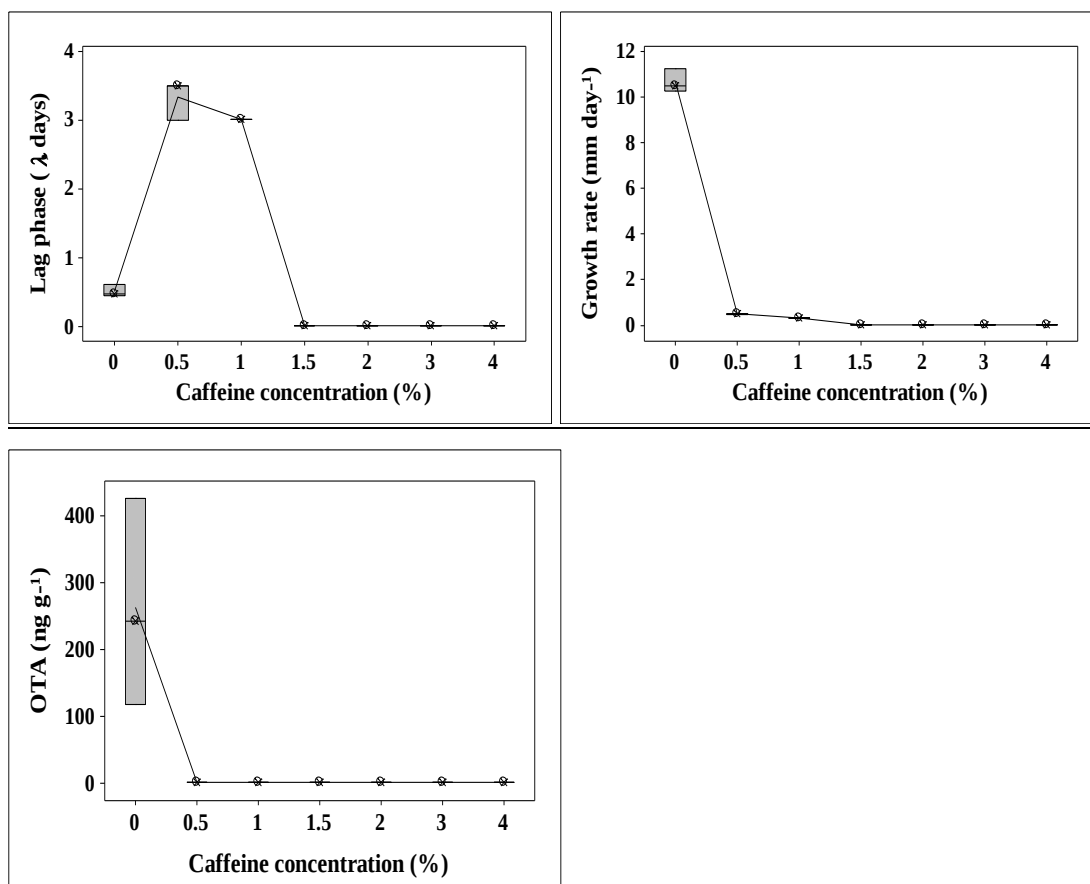


Figure I.4.c: Box-plot analysis for fungal growth, lag phase (λ , days) and OTA production of *A. niger* (A 1911) inoculated on YES and incubated in different caffeine concentration (0, 0.5, 1, 1.5, 2, 3 and 4%) and 0.98 a_w at 30°C for 9 days. The box-plot shows the median (line inside the box).

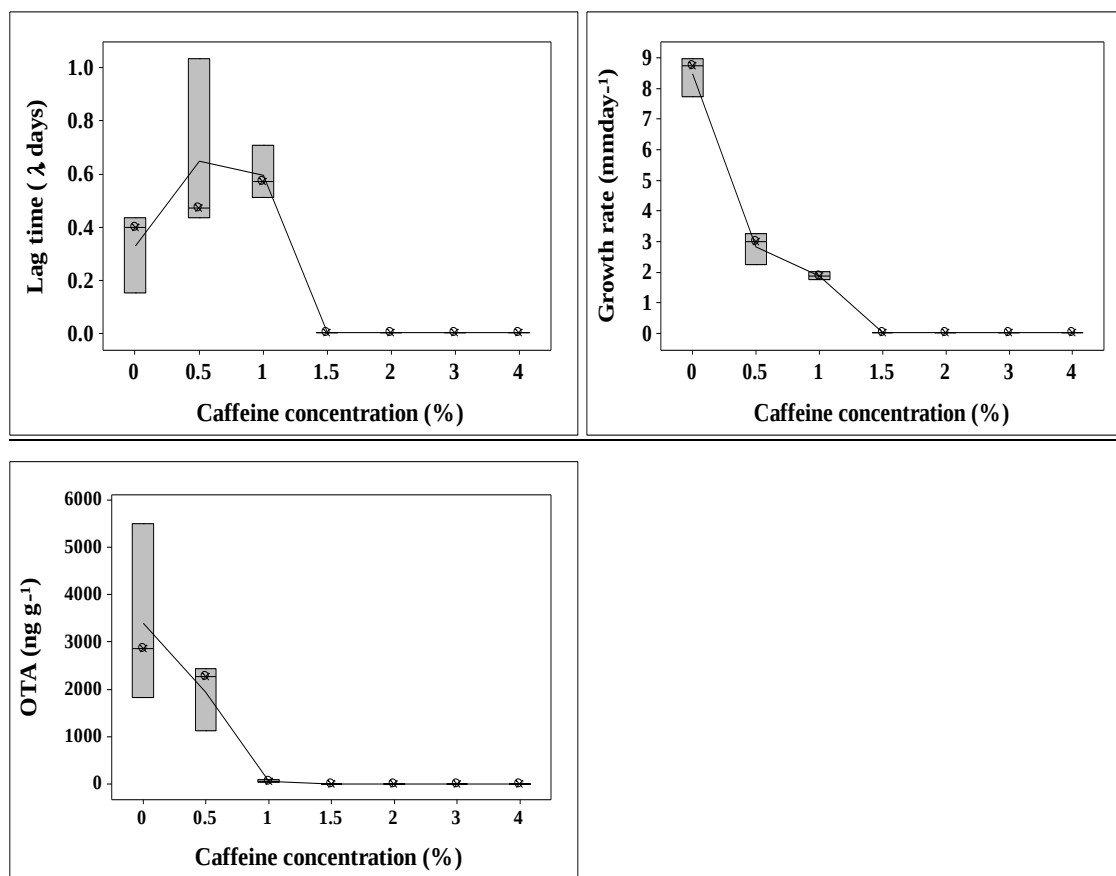


Figure I.4.d: Box-plot analysis for fungal growth, lag phase (λ , days) and OTA production of *A. carbonarius* (ITAL 204) inoculated on YES and incubated in different caffeine concentration (0, 0.5, 1, 1.5, 2, 3 and 4%) and 0.98 a_w at 30°C for 9 days. The box-plot shows the median (line inside the box).

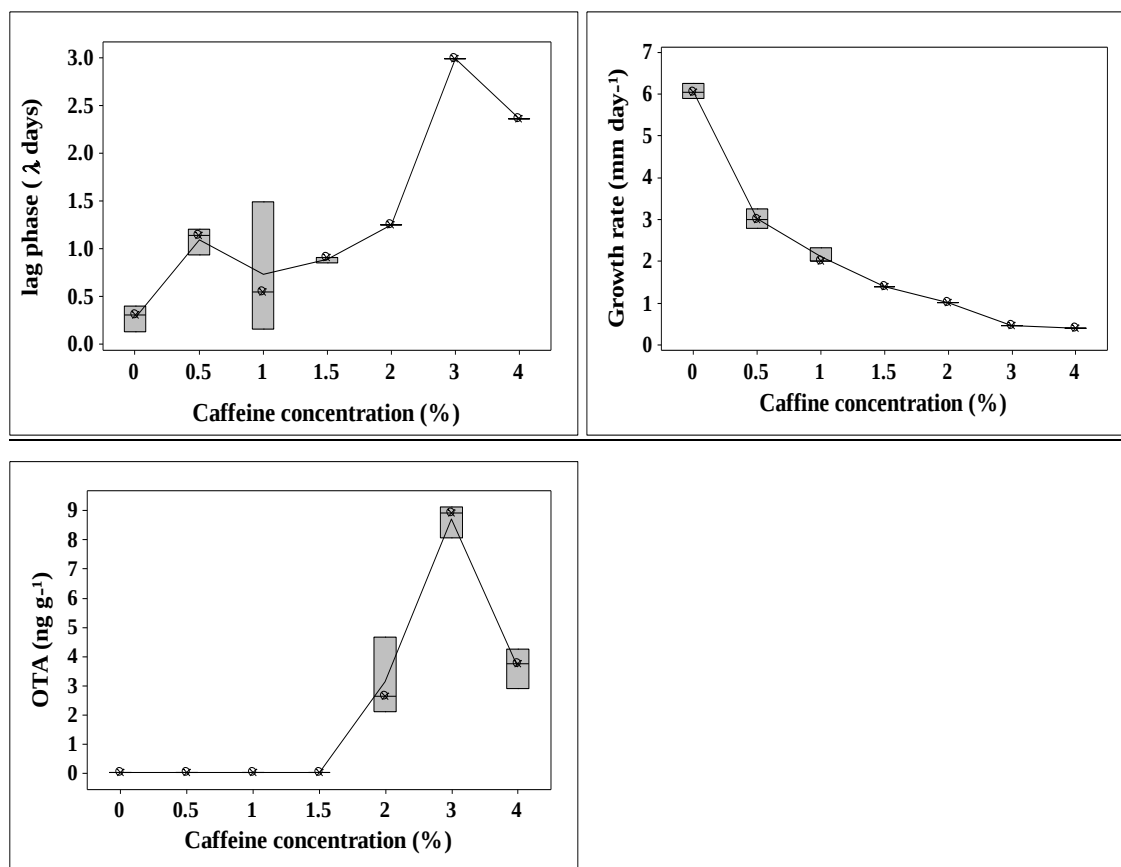


Figure I.4.e: Box-plot analysis for fungal growth (a), lag phase (λ , days) (b) and OTA production (c) of *A. ochraceus* (ITAL 14) inoculated on YES and incubated in different caffeine concentration (0, 0.5, 1, 1.5, 2, 3 and 4%) and 0.98 a_w at 30°C for 9 days. The box-plot shows the median (line inside the box).

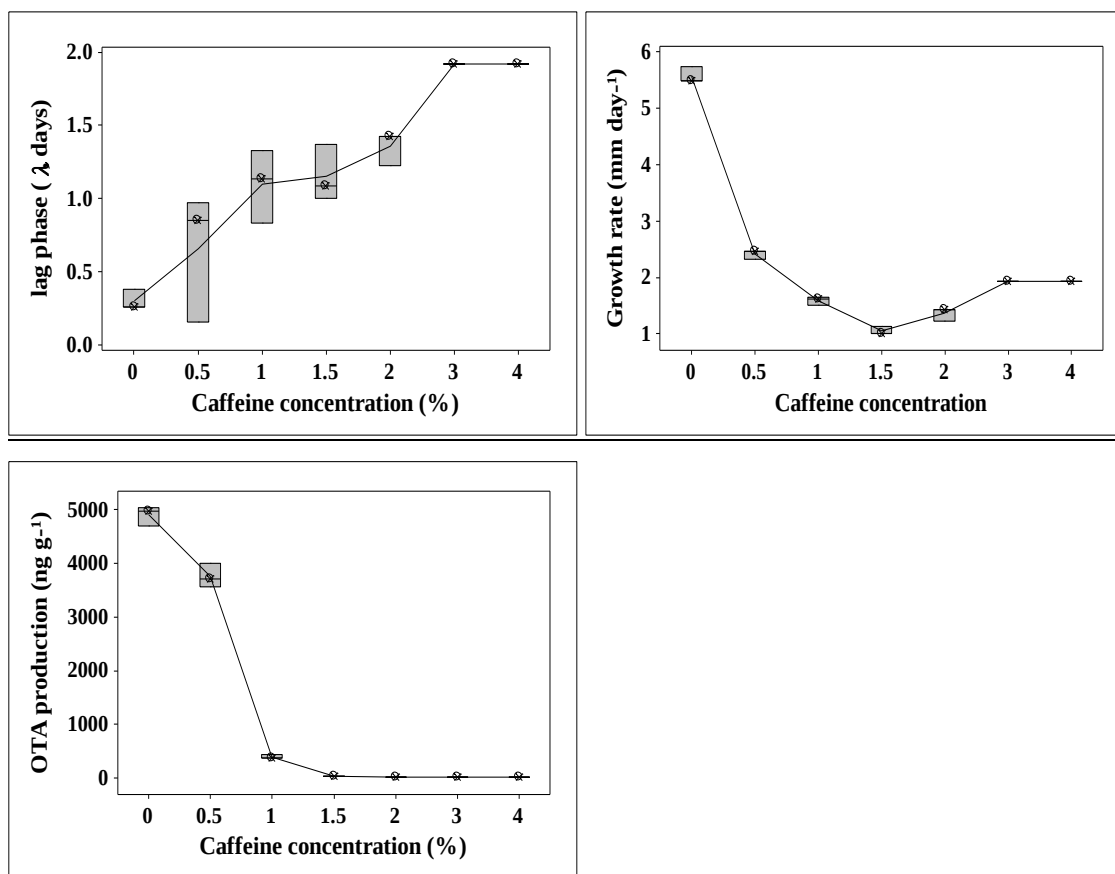


Figure I.4.f: Box-plot analysis for fungal growth, lag phase (λ , days) and OTA production of *A. steynii* (CBS 112814) inoculated on YES and incubated in different caffeine concentration (0, 0.5, 1, 1.5, 2, 3 and 4%) and 0.98 a_w at 30°C for 9 days. The box-plot shows the median (line inside the box).

APPENDIX II

Appendix II. 1: *In vitro* effect of water activity x elevated CO₂ on lag times prior to growth, growth and OTA production at 30°C

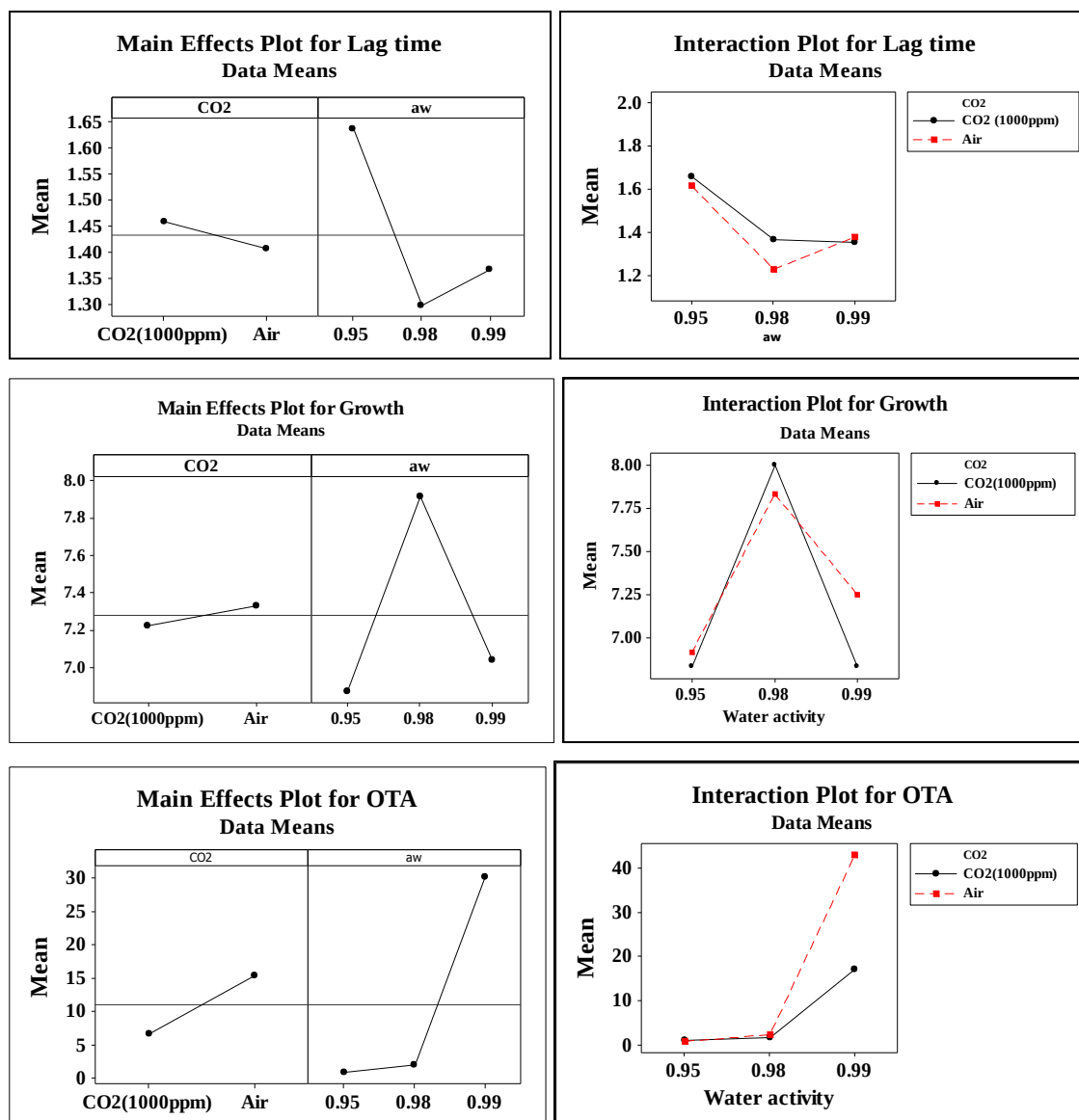


Figure II.1.a: Main effects plot of the mean of lag phase (λ , days) and OTA production (ng g^{-1}) growth rate (mm day^{-1}) versus CO₂, a_w for normal distribution of strains *A. westerdijkiae* (B 2) inoculated on CMEA (20%) and incubated at temperature 30°C and a_w (0.95, 0.98 0.99) for 9 days *in vitro*.

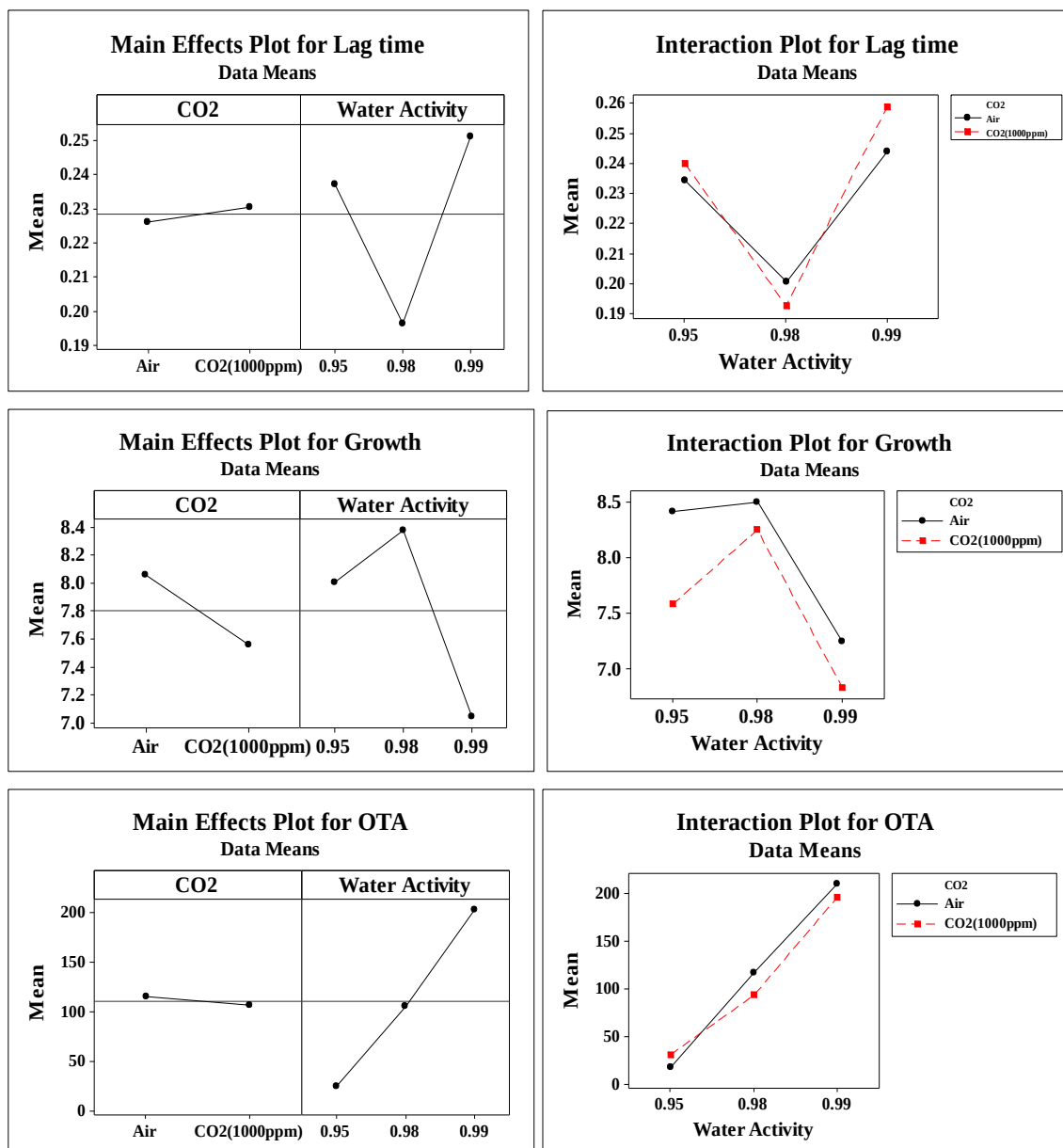


Figure II.1.b: Main effects plot of the mean of lag phase (λ , days), growth rate (mm day^{-1}) and OTA production (ng g^{-1}) versus CO_2 , a_w for normal distribution of strains *A. westerdijkiae* (CBS 121986) inoculated on CMEA (20%) and incubated at temperature 30°C and a_w (0.95, 0.98 0.99) for 9 days *in vitro*.

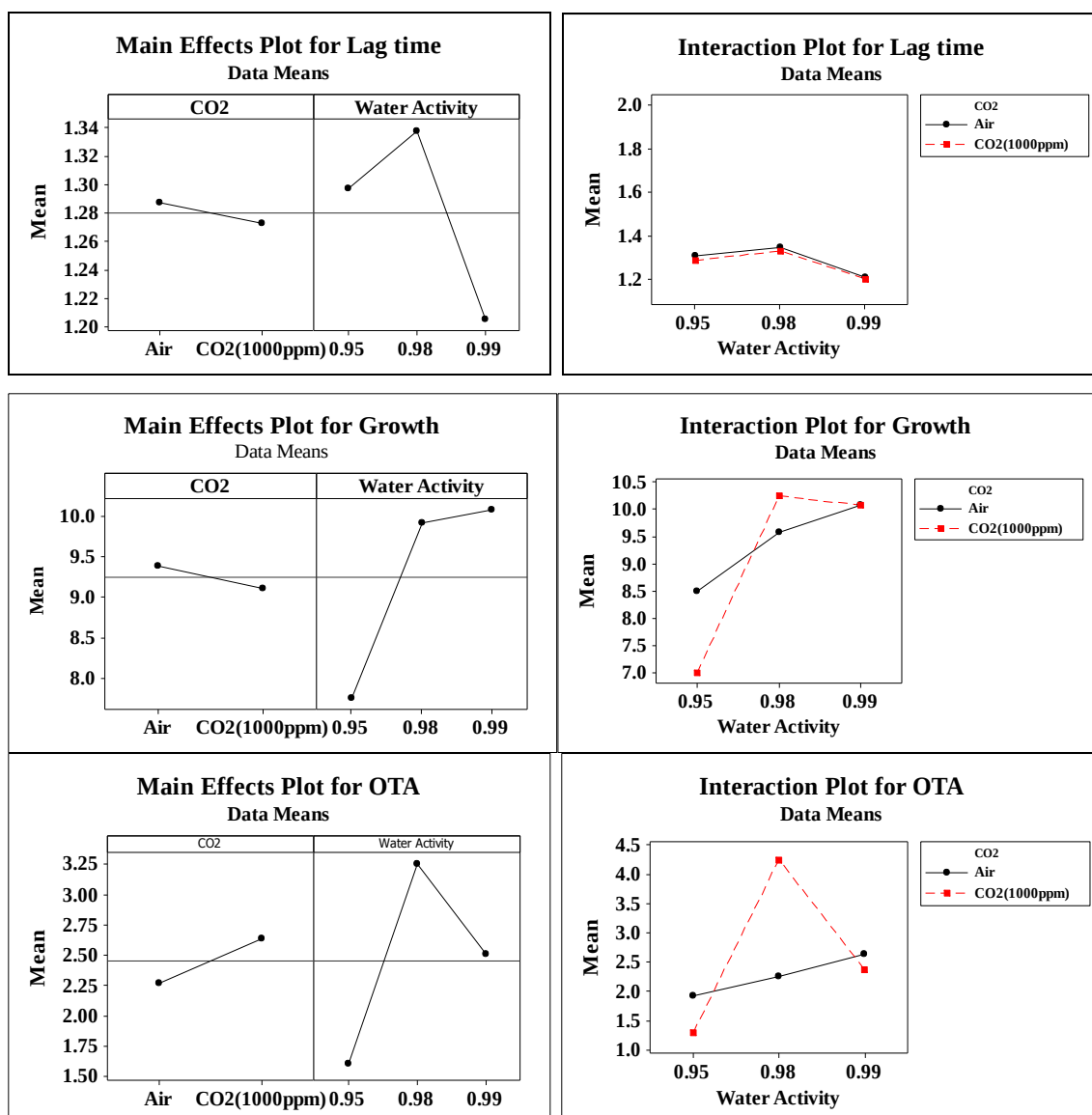


Figure II.1.c: Main effects plot of the mean of lag phase (λ , days), growth rate (mm day^{-1}) and OTA production (ng g^{-1}) versus CO_2 , a_w for normal distribution of strains *A. niger* (A 1911) inoculated on CMEA (20%) and incubated at temperature 30°C and a_w (0.95, 0.98 0.99) for 9 days *in vitro*.

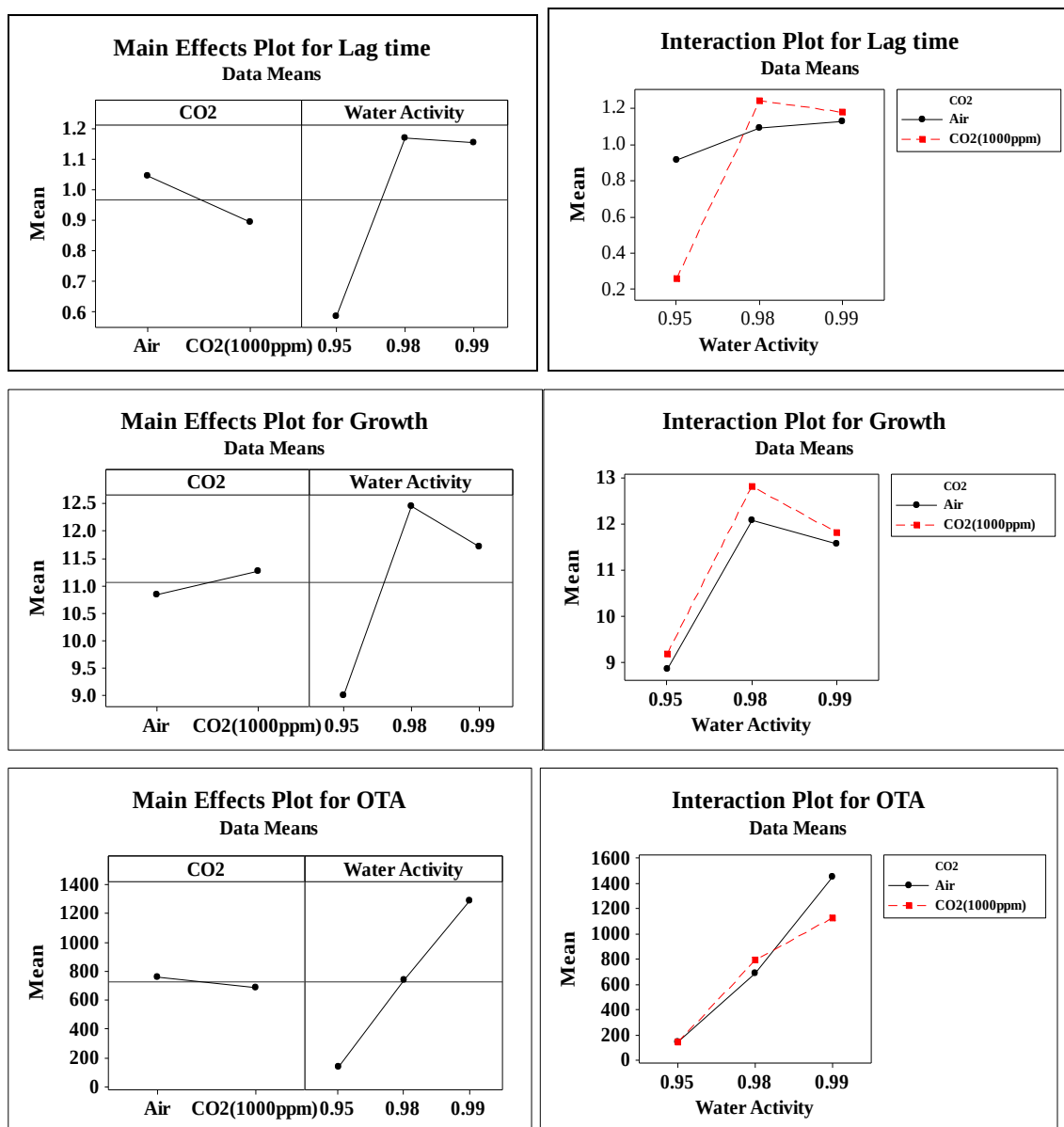


Figure II.1.d: Main effects plot of the mean of lag phase (λ , days) growth rate (mm day⁻¹) and OTA production (ng g⁻¹) versus CO₂, a_w for normal distribution of strains *A. carbonarius* (ITAL 204) inoculated on CMEA (20%) and incubated at temperature 30°C and a_w (0.95, 0.98 0.99) for 9 days *in vitro*.

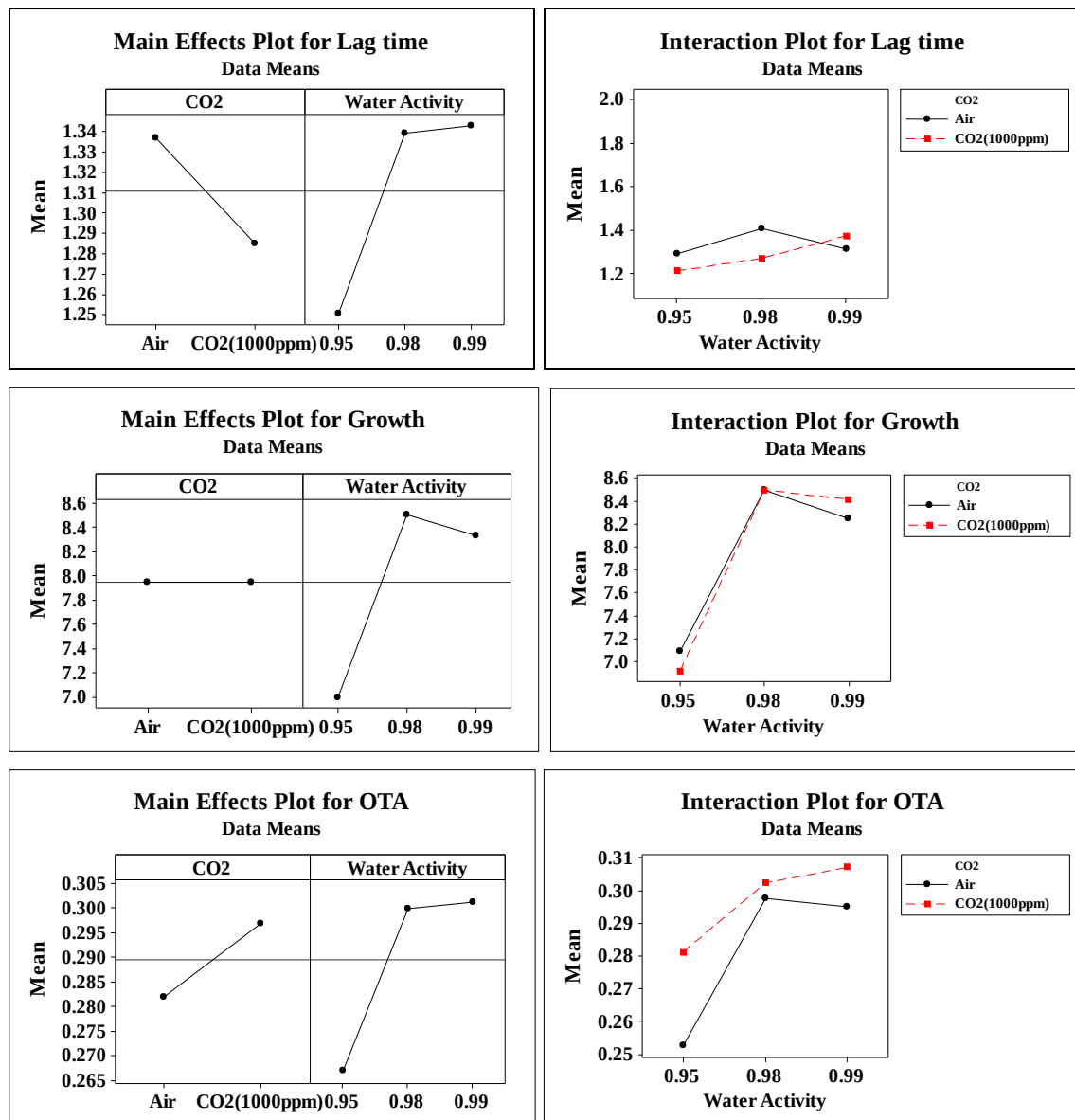


Figure II.1.e: Main effects plot of the mean of lag phase (λ , days) growth rate (mm day⁻¹) and OTA production (ng g⁻¹) versus CO₂, a_w for normal distribution of strains *A. ochraceus* (ITAL 14) inoculated on CMEA (20%) and incubated at temperature 30°C and a_w (0.95, 0.98 0.99) for 9 days *in vitro*.

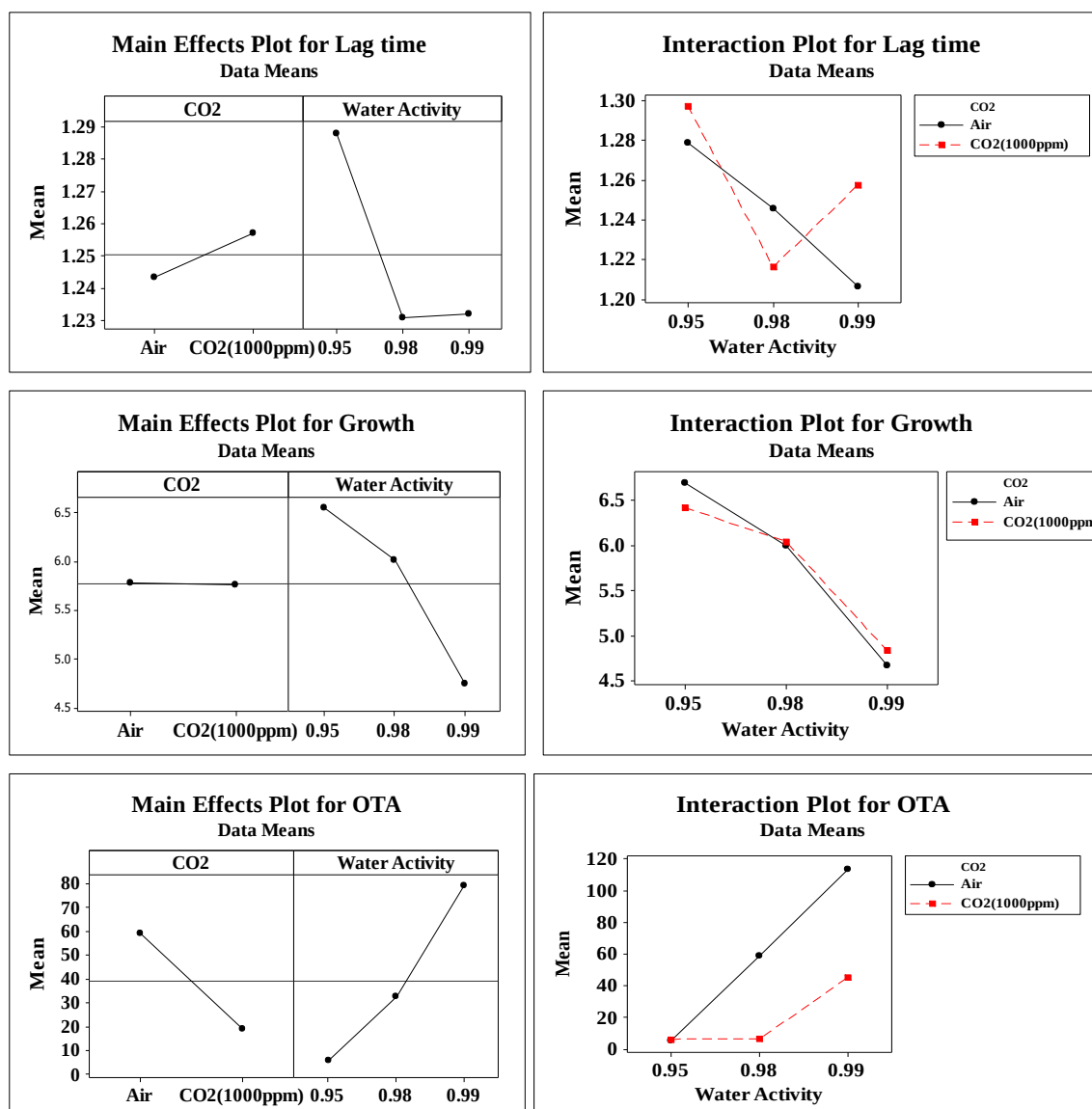


Figure II.1.f: Main effects plot of the mean of lag phase (λ , days) growth rate (mm day^{-1}) and OTA production (ng g^{-1}) versus CO_2 , a_w for normal distribution of strains *A. steynii* (CBS 112814) inoculated on CMEA (20%) and incubated at temperature 30°C and a_w (0.95, 0.98 0.99) for 9 days *in vitro*.

Appendix II 2: *In vitro* effect of water activity x elevated CO₂ on lag times prior to growth, growth and OTA production at 35°C

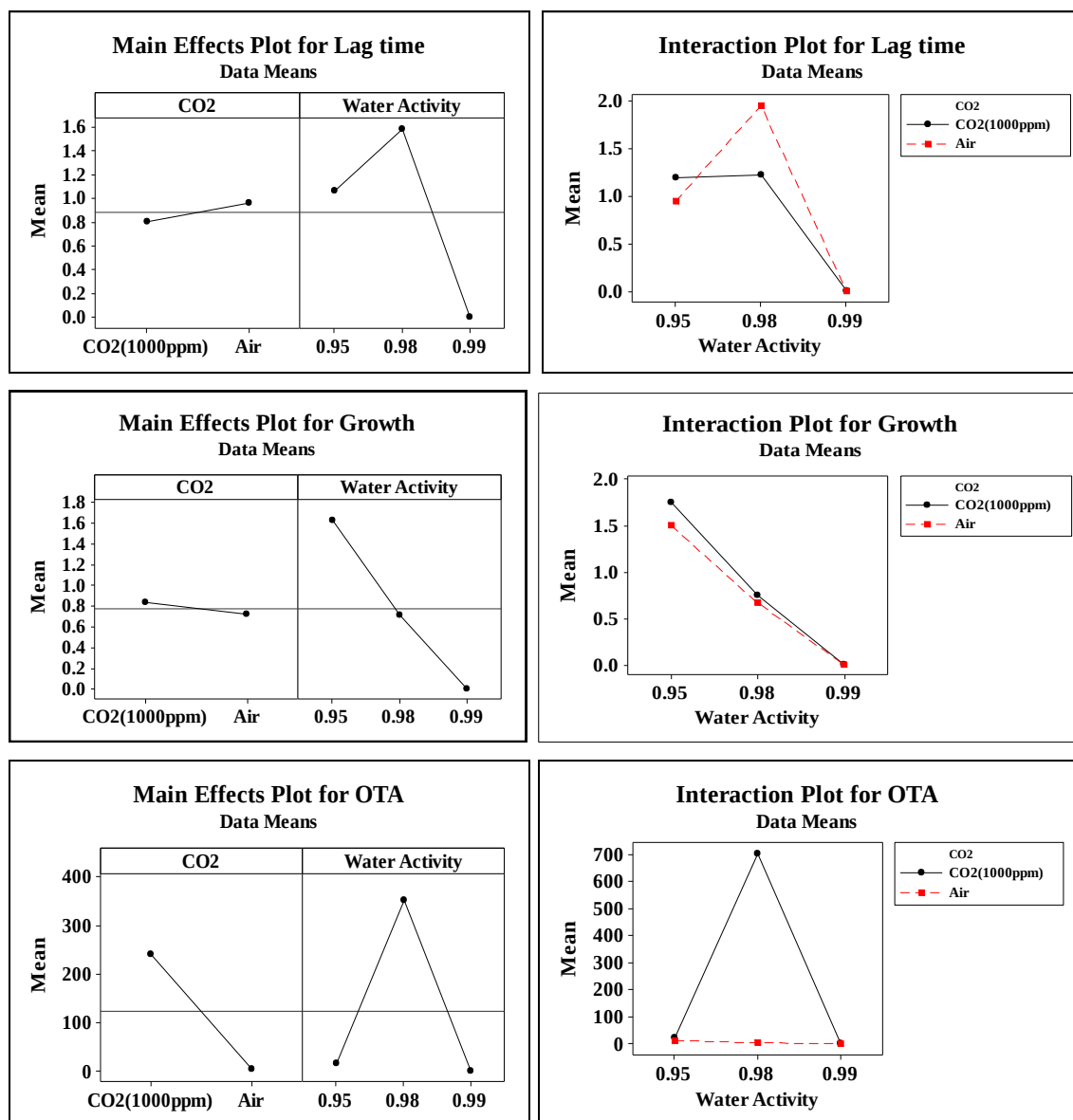


Figure II.2.a: Main effects plot of the mean of lag phase (λ , days) growth rate (mm day^{-1}) and OTA production (ng g^{-1}) versus CO₂, a_w for normal distribution of strains *A. westerdijikiae* (B 2) inoculated on CMEA (20%) and incubated at temperature 35°C and a_w (0.95, 0.98 0.99) for 9 days *in vitro*.

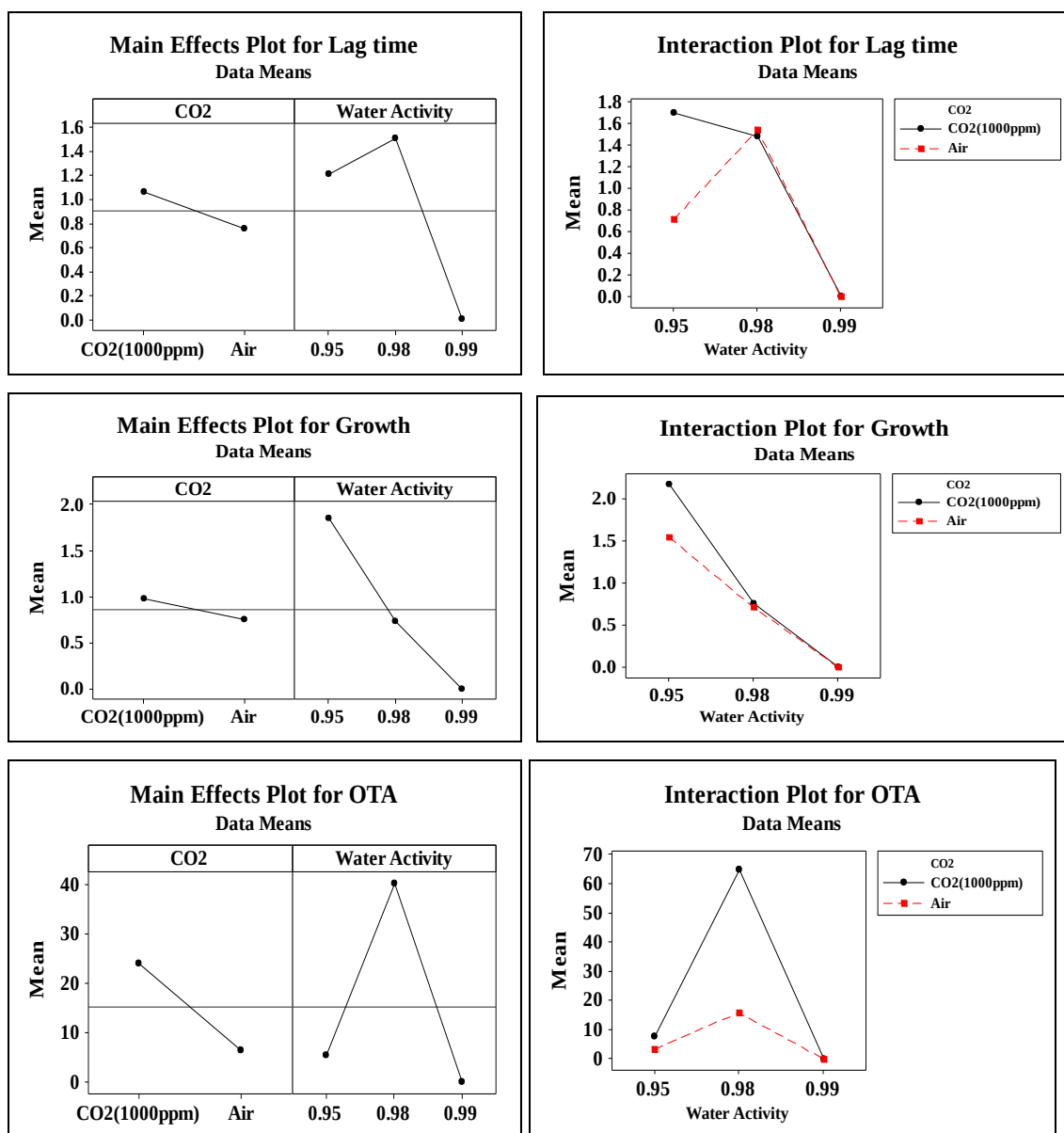


Figure II.2.b: Main effects plot of the mean of lag phase (λ , days) growth rate (mm day^{-1}) and OTA production (ng g^{-1}) versus CO₂, a_w for normal distribution of strains *A. westerdijkae* (CBS 121986) inoculated on CMEA (20%) and incubated at temperature 35°C and a_w (0.95, 0.98 0.99) for 9 days *in vitro*.

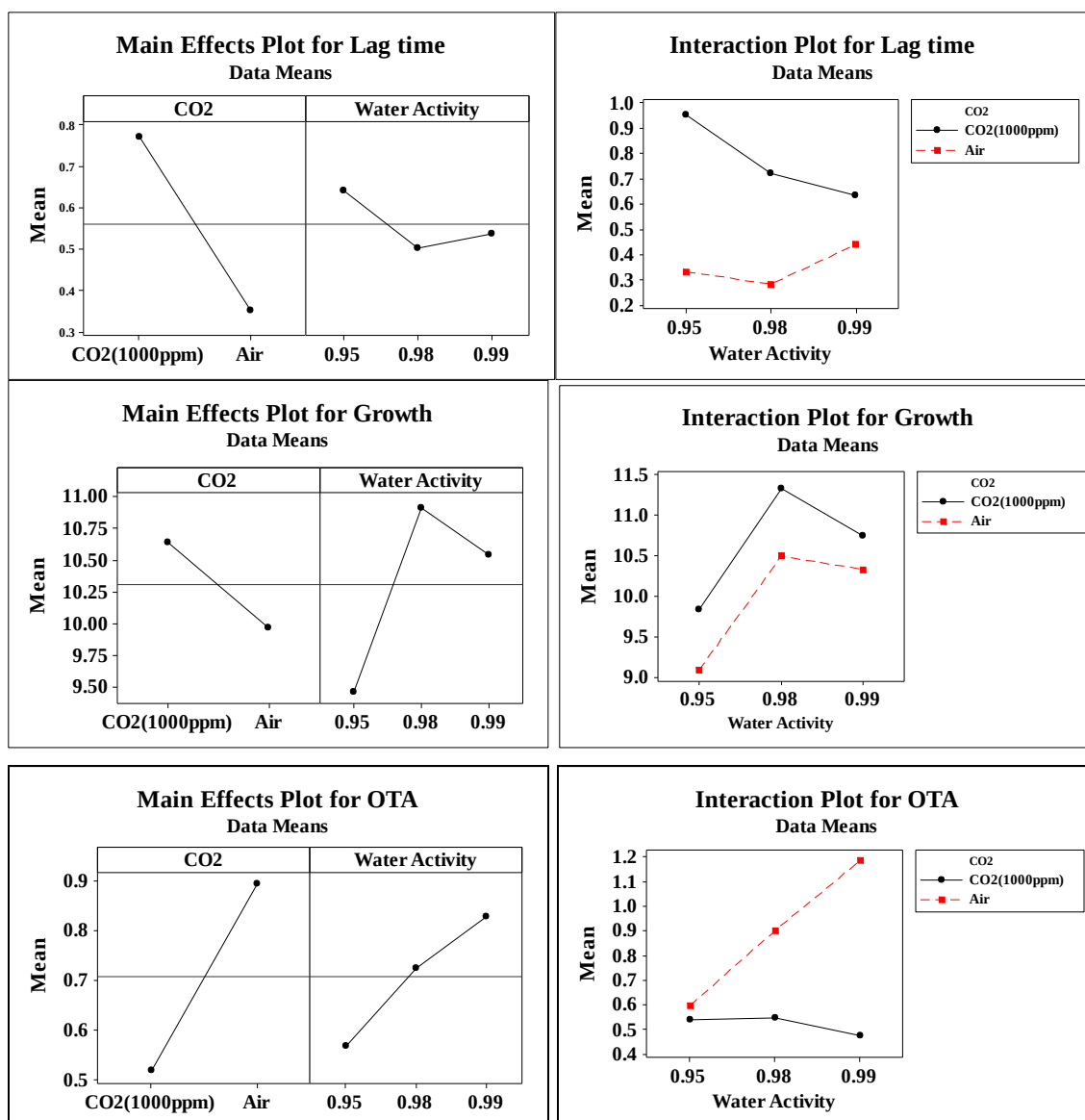


Figure II.2.c: Main effects plot of the mean of lag phase (λ , days) growth rate (mm day^{-1}) and OTA production (ng g^{-1}) versus CO_2 , a_w for normal distribution of strains *A. niger* (A 1911) inoculated on CMEA (20%) and incubated at temperature 35°C and a_w (0.95, 0.98 0.99) for 9 days *in vitro*.

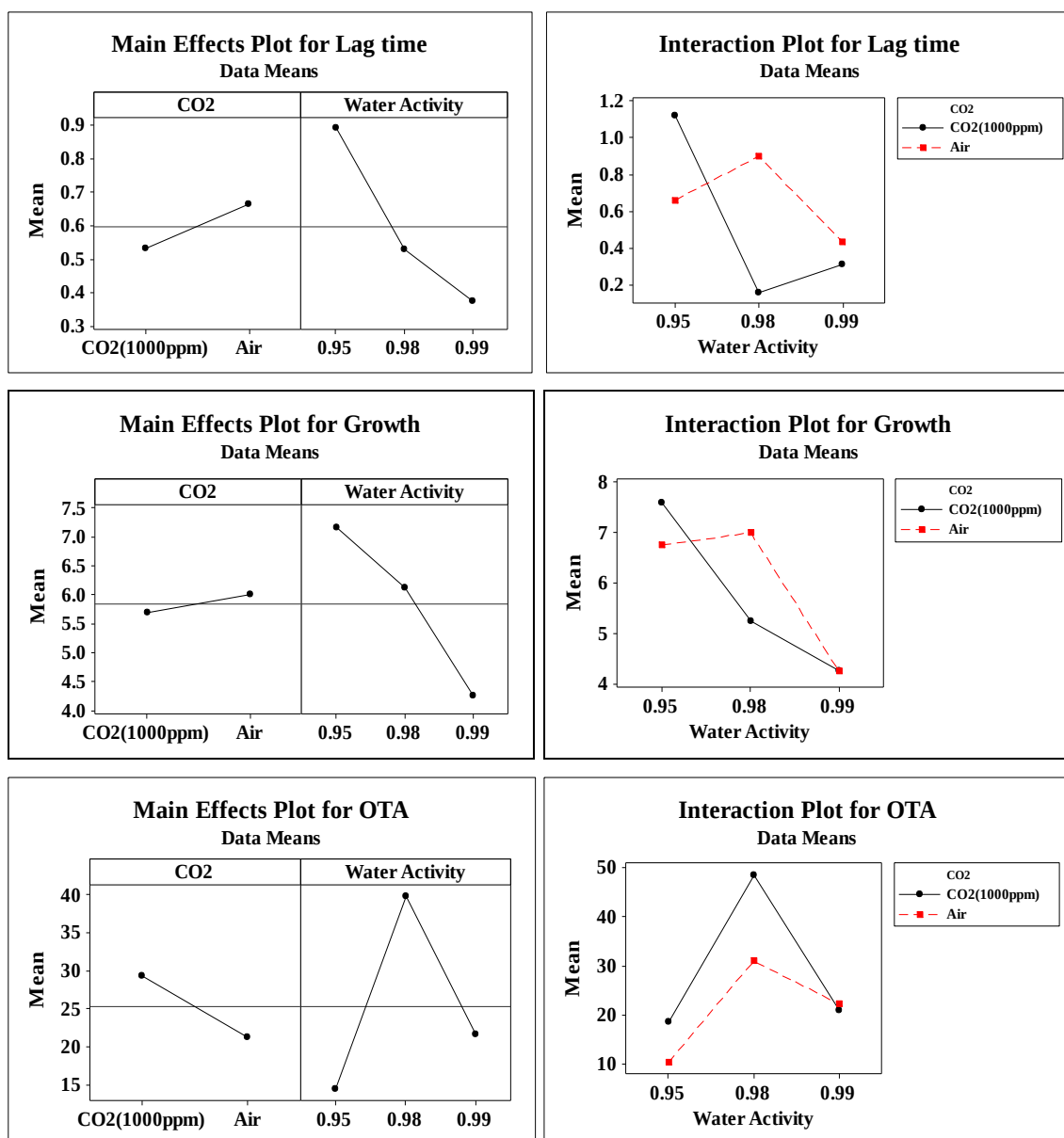


Figure II.2.d: Main effects plot of the mean of lag phase (λ , days) growth rate (mm day^{-1}) and OTA production (ng g^{-1}) versus CO₂, a_w for normal distribution of strains *A. carbonarius* (ITAL 204) inoculated on CMEA (20%) and incubated at temperature 35°C and a_w (0.95, 0.98 0.99) for 9 days *in vitro*.

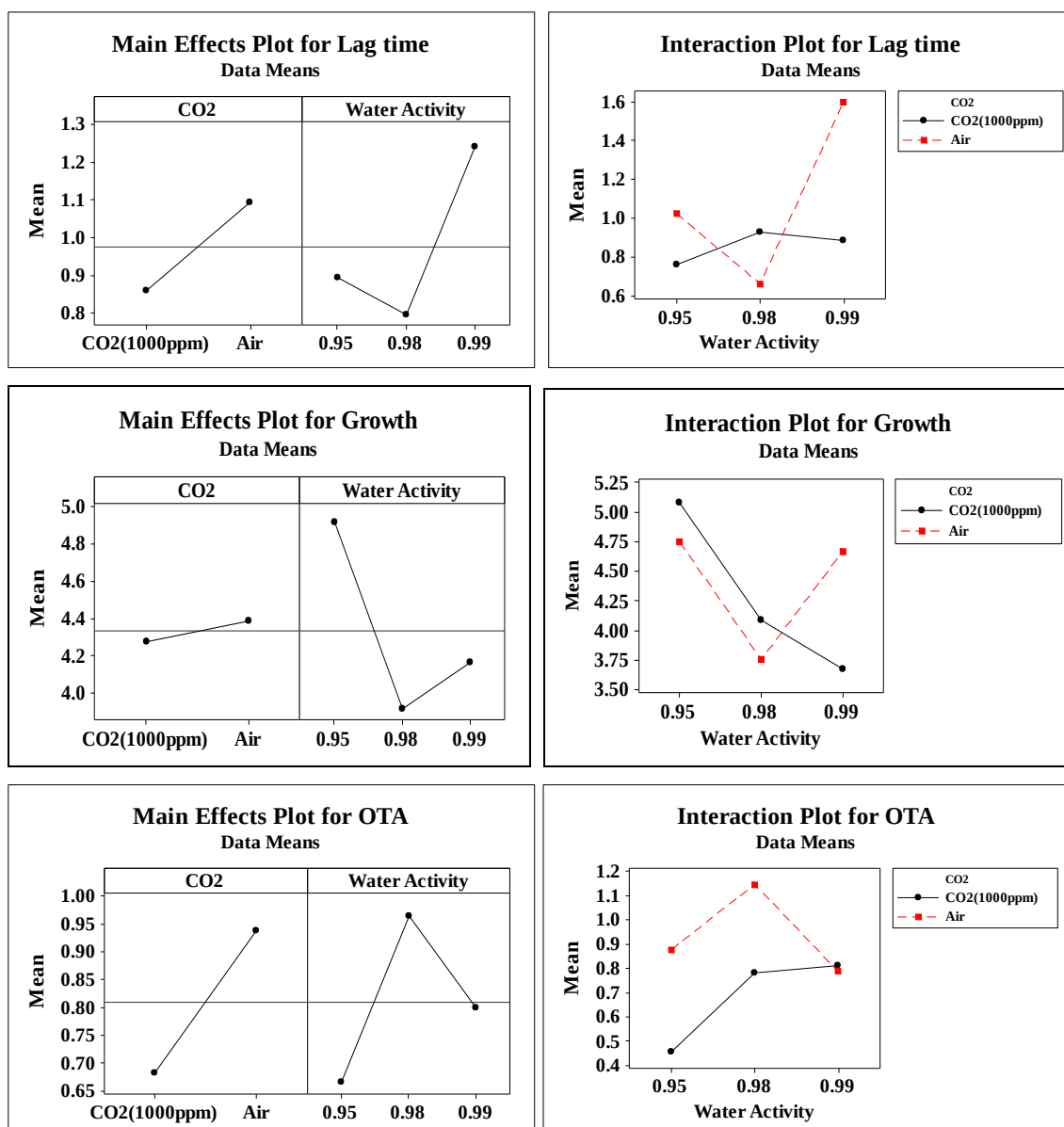


Figure II.2.e: Main effects plot of the mean of lag phase (λ , days) growth rate (mm day^{-1}) and OTA production (ng g^{-1}) versus CO_2 , a_w for normal distribution of strains *A. ochraceus* (ITAL 14) inoculated on CMEA (20%) and incubated at temperature 35°C and a_w (0.95, 0.98 0.99) for 9 days *in vitro*

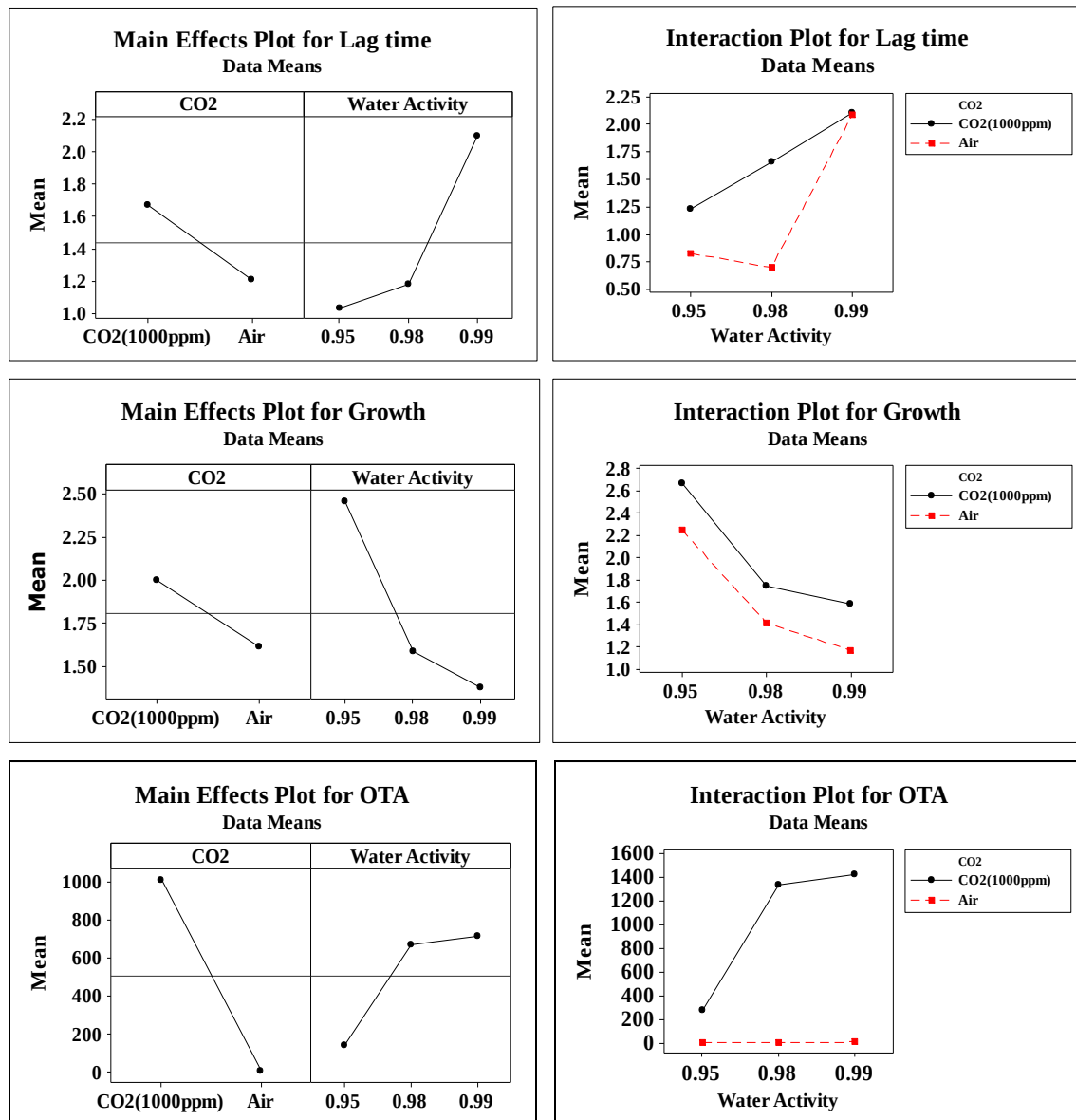


Figure II.2.f: Main effects plot of the mean of lag phase (λ , days) growth rate (mm day⁻¹) and OTA production (ng g⁻¹) versus CO₂, a_w for normal distribution of *A. steynii* (CBS 112814) inoculated on CMEA (20%) and incubated at temperature 35°C and a_w (0.95, 0.98, 0.99) for 9 days *in vitro*.

Appendix II 3: *In vitro* comparison of the effect of water activity x elevated CO₂ x temperature on lag times prior to growth, growth and OTA production.

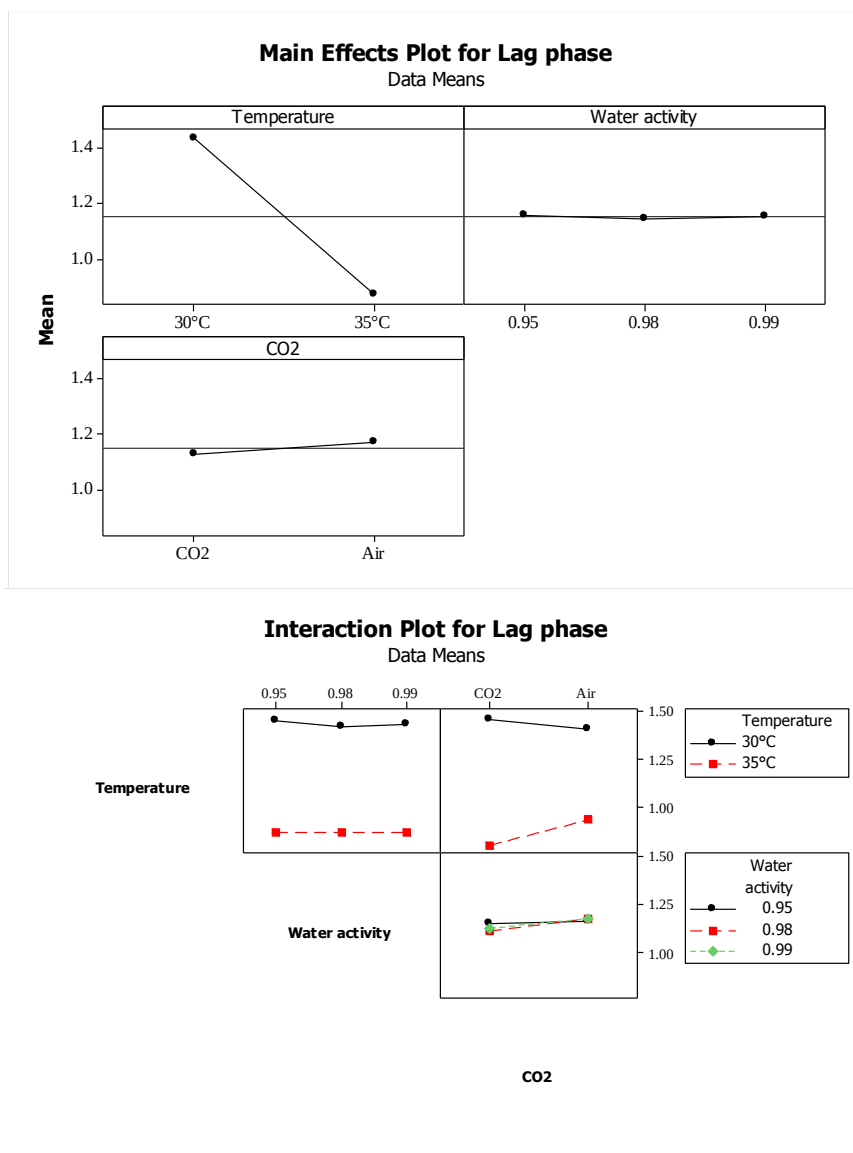


Figure II.3.a: Comparison of main effects plot of the mean of fungal lag phase (λ , days) versus CO₂, a_w and temperature for normal distribution of strains *A. westerdijkiae* (B 2) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98 0.99) for 12 days *in vitro*.

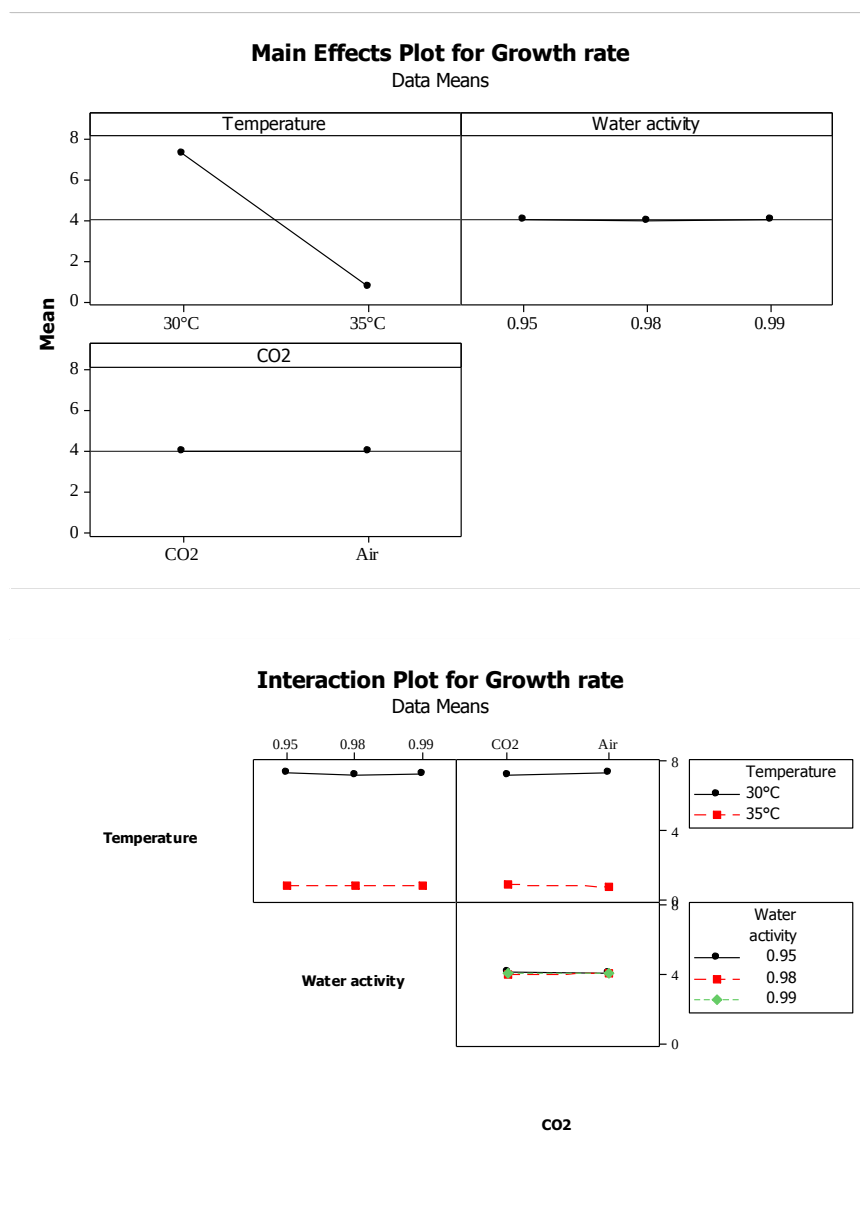


Figure II.3.b: Comparison of main effects plot of the mean of fungal growth rate (mm day⁻¹) versus CO₂, a_w and temperature for normal distribution of strains *A. westerdijkiae* (B 2) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98 0.99) for 12 days *in vitro*.

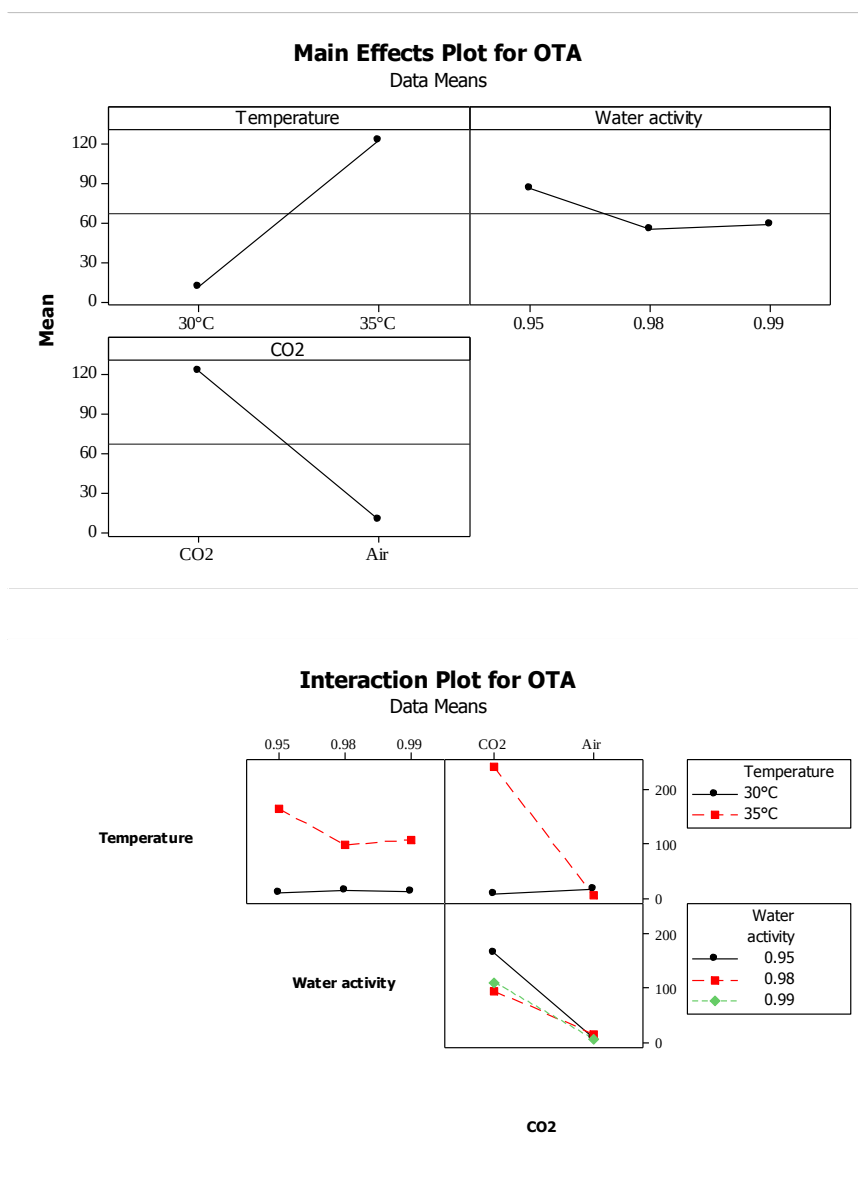


Figure II.3.c: Comparison of main effects plot of the mean of fungal OTA production (ng g^{-1}) versus CO_2 , a_w and temperature for normal distribution of strains *A. westerdijikiae* (B 2) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98 0.99) for 12 days *in vitro*.

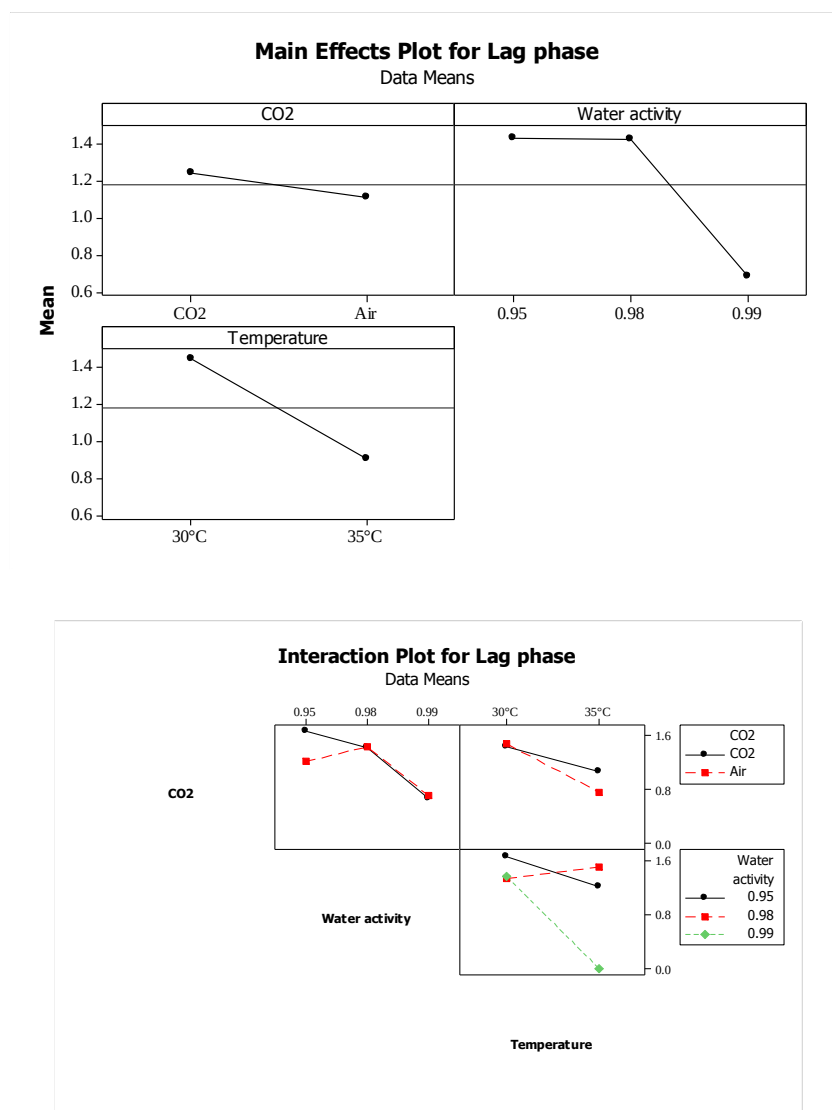


Figure II.3.d: Comparison of main effects plot of the mean of fungal lag phase (λ , days) versus CO₂, a_w and temperature for normal distribution of strains *A. westerdijkiae* (CBS 121986) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98 0.99) for 12 days *in vitro*.

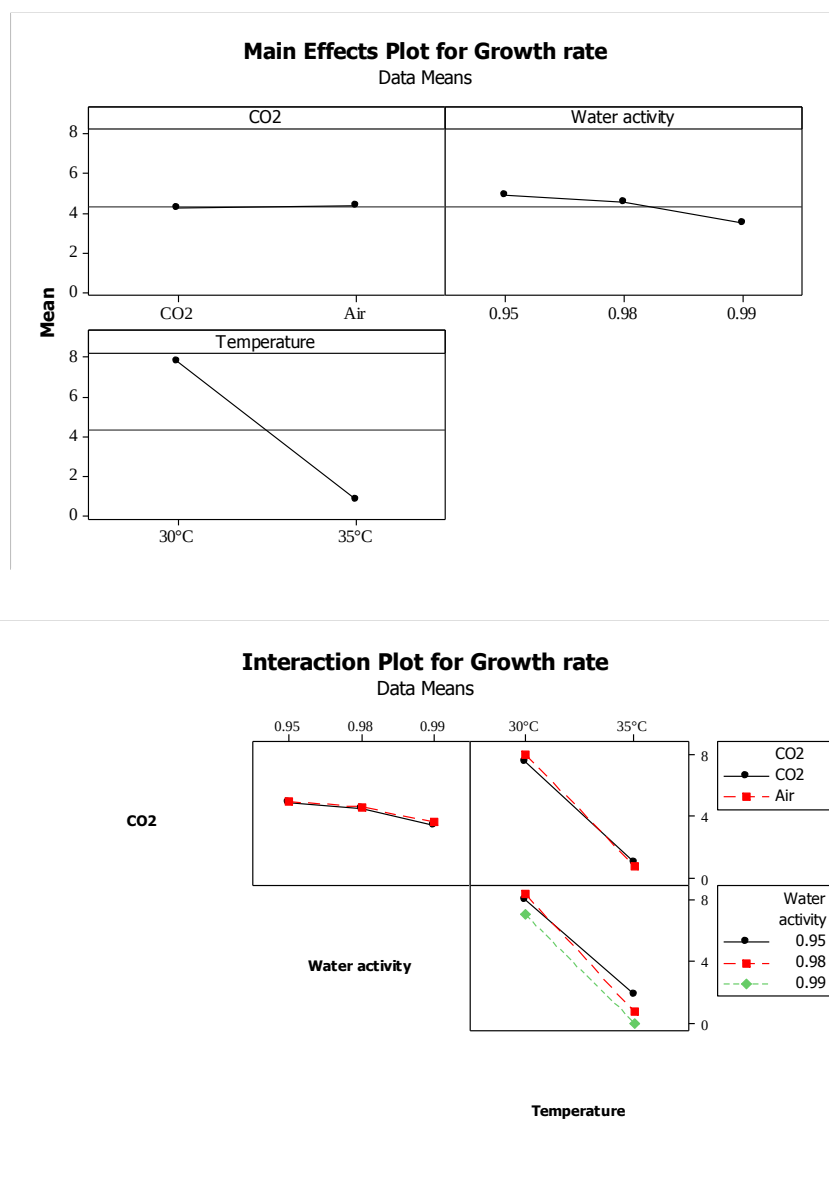


Figure II.3.e: Comparison of main effects plot of the mean of fungal growth rate (mm day⁻¹) versus CO₂, a_w and temperature for normal distribution of strains *A. westerdijikiae* (CBS 121986) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98 0.99) for 12 days *in vitro*.

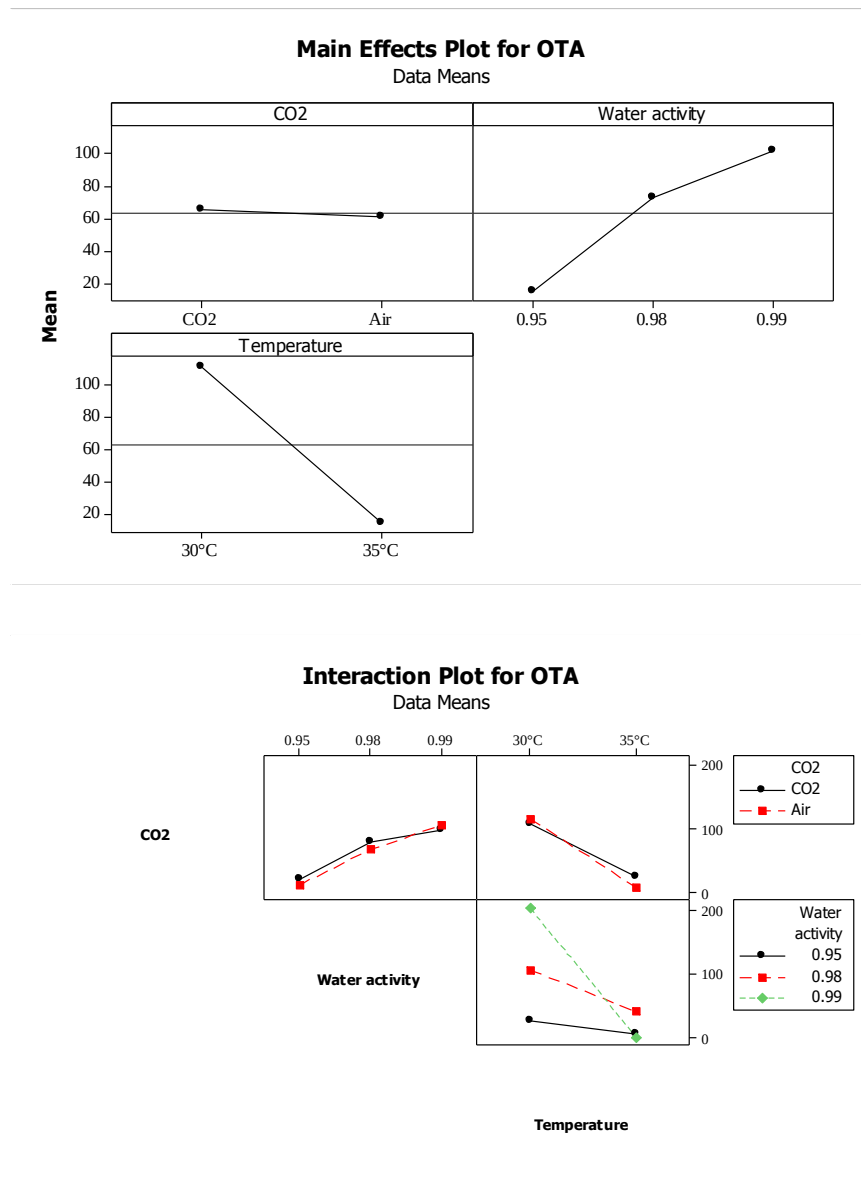


Figure II.3.f: Comparison of main effects plot of the mean of fungal OTA production (ng g^{-1}) versus CO_2 , a_w and temperature for normal distribution of strains *A. westerdijikiae* (CBS 121986) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98 0.99) for 12 days *in vitro*.

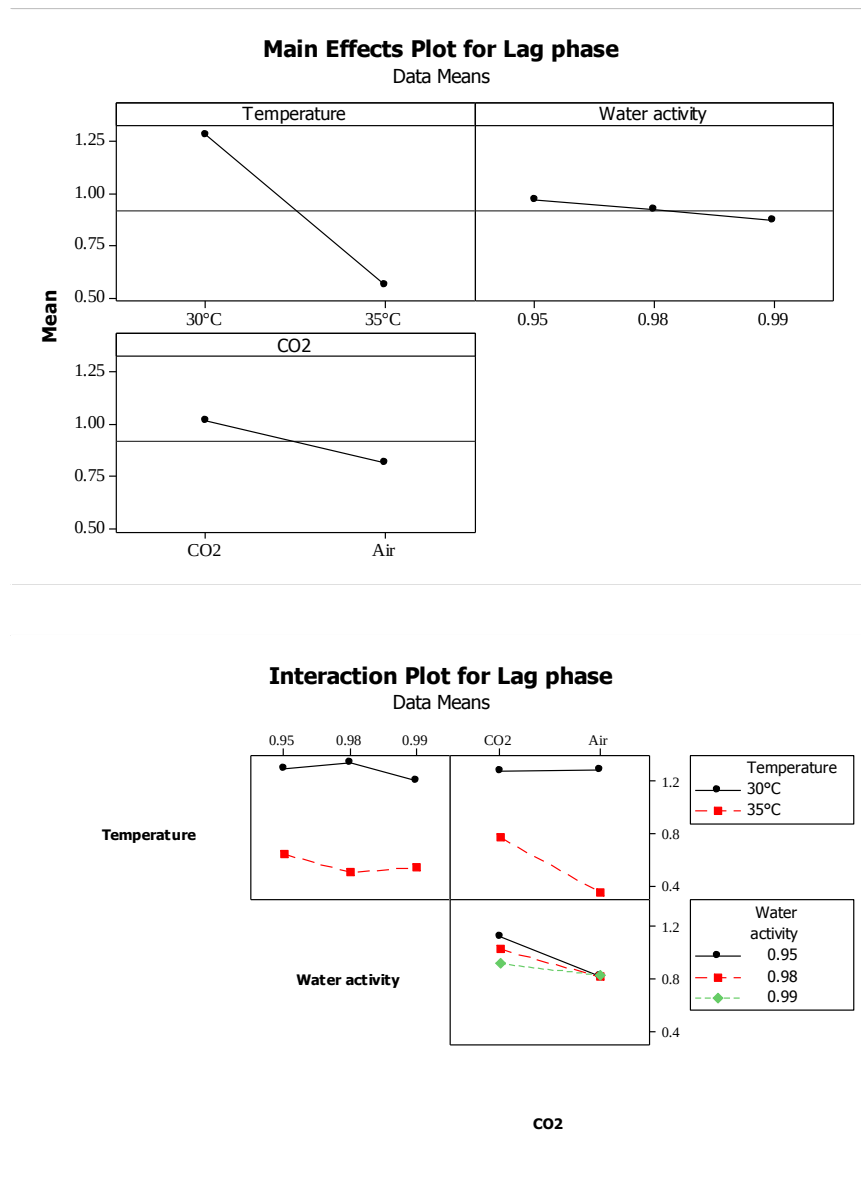


Figure II.3.g: Comparison of main effect plot of the mean of fungal lag phase (λ , days) versus CO_2 , a_w and temperature for normal distribution of strains *A. niger* (A 1911) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98 0.99) for 12 days *in vitro*.

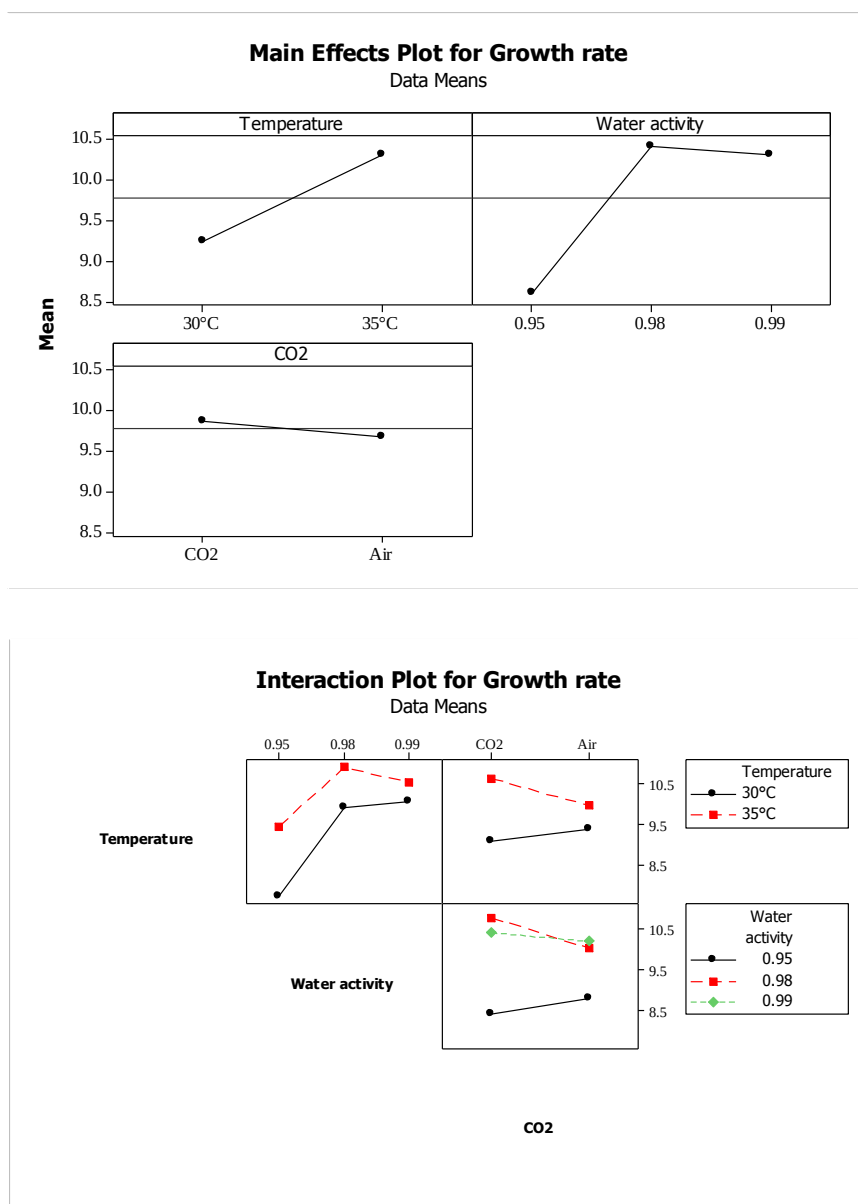


Figure II.3.h: Comparison of main effects plot of the mean of fungal growth rate (mm day⁻¹) versus CO₂, a_w and temperature for normal distribution of strains *A. niger* (A 1911) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98 0.99) for 12 days *in vitro*.

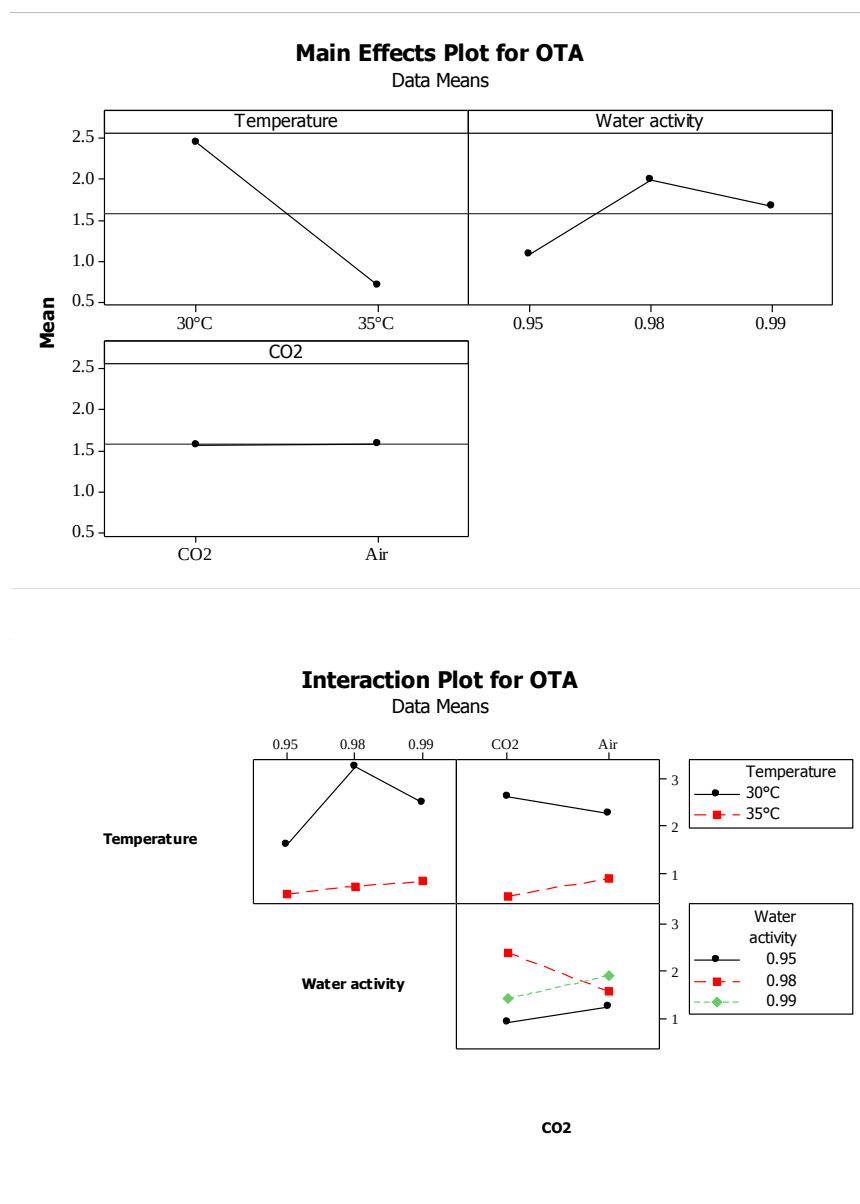


Figure II.3.i: Comparison of main effects plot of the mean of fungal OTA production (ng g⁻¹) versus CO₂, a_w and temperature for normal distribution of strains *A. niger* (A 1911) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98 0.99) for 12 days *in vitro*.

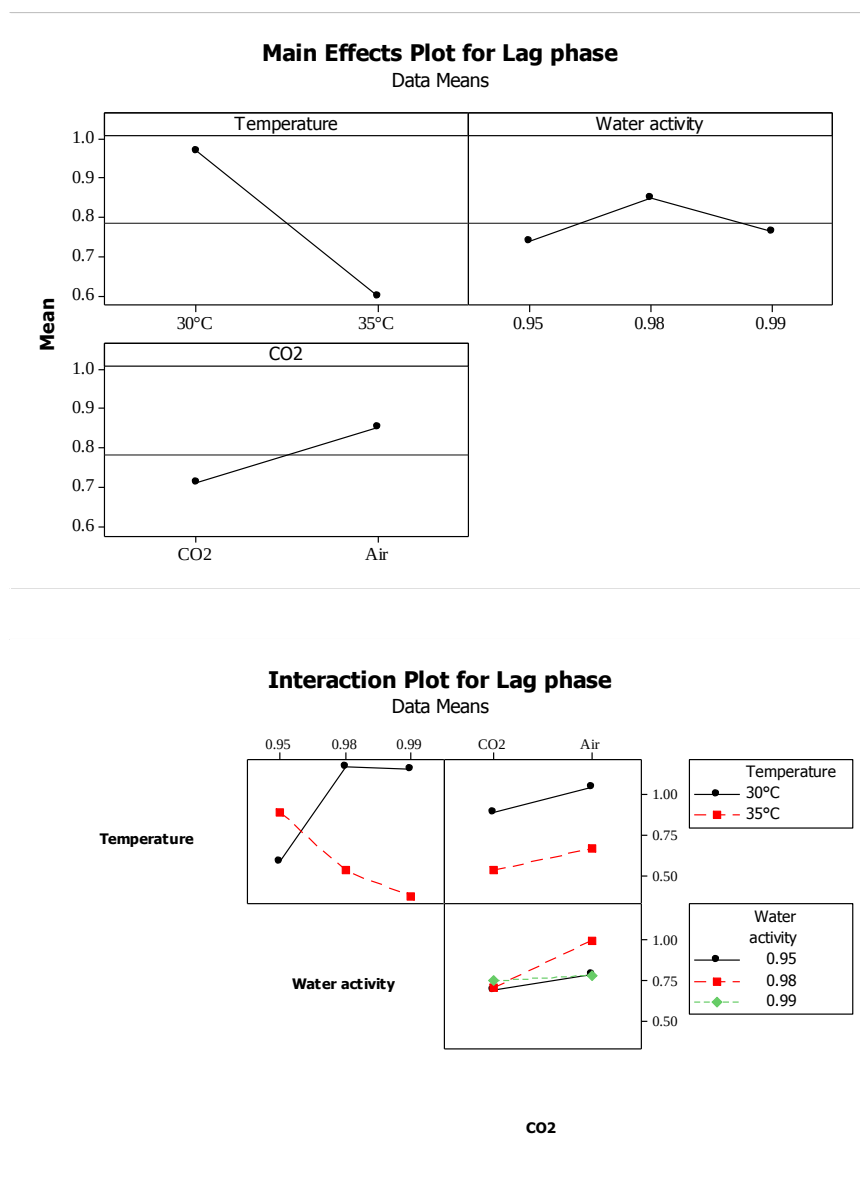


Figure II.3.j: Comparison of main effects plot of the mean of fungal lag phase (λ , days) versus CO₂, a_w and temperature for normal distribution of strains *A. carbonarius* (ITAL 204) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98 0.99) for 12 days *in vitro*.

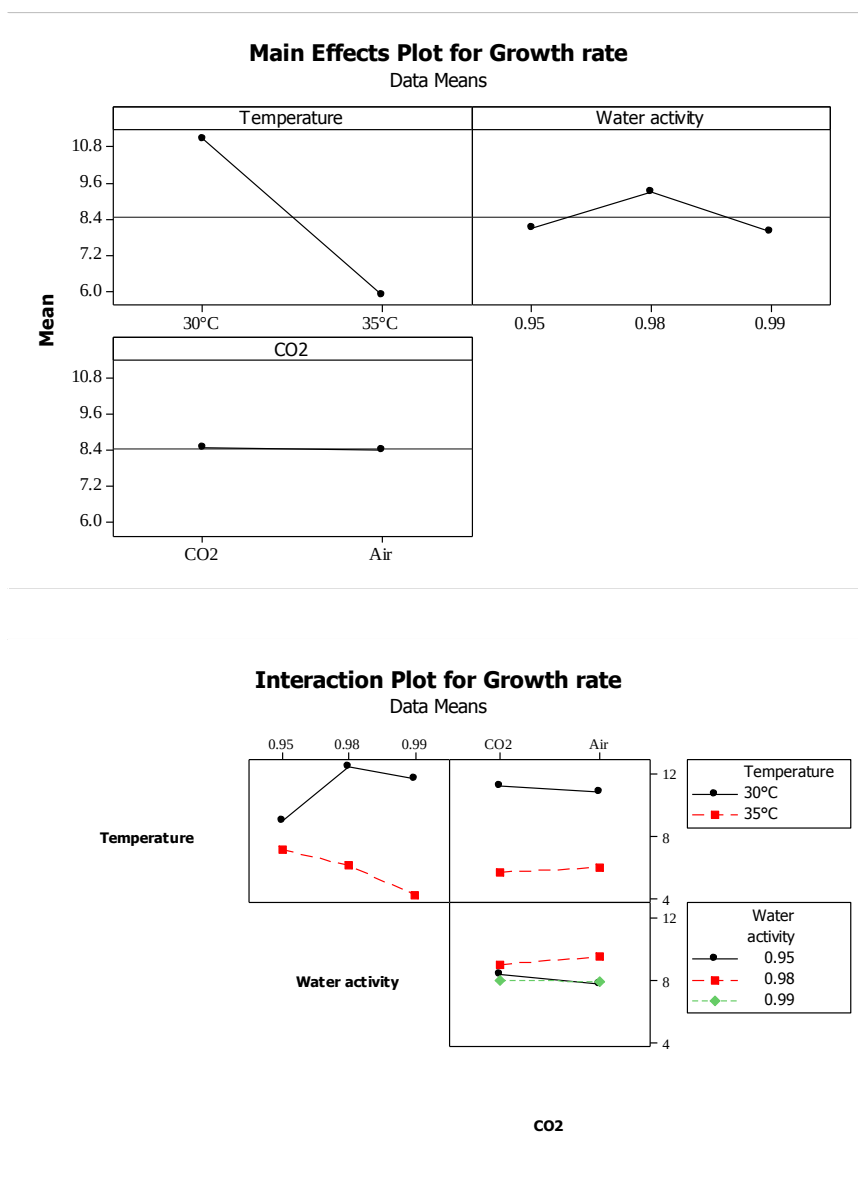


Figure II.3.k: Comparison of main effects plot of the mean of fungal growth rate (mm day⁻¹) versus CO₂, a_w and temperature for normal distribution of strains *A. carbonarius* (ITAL 204) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98, 0.99) for 12 days *in vitro*.

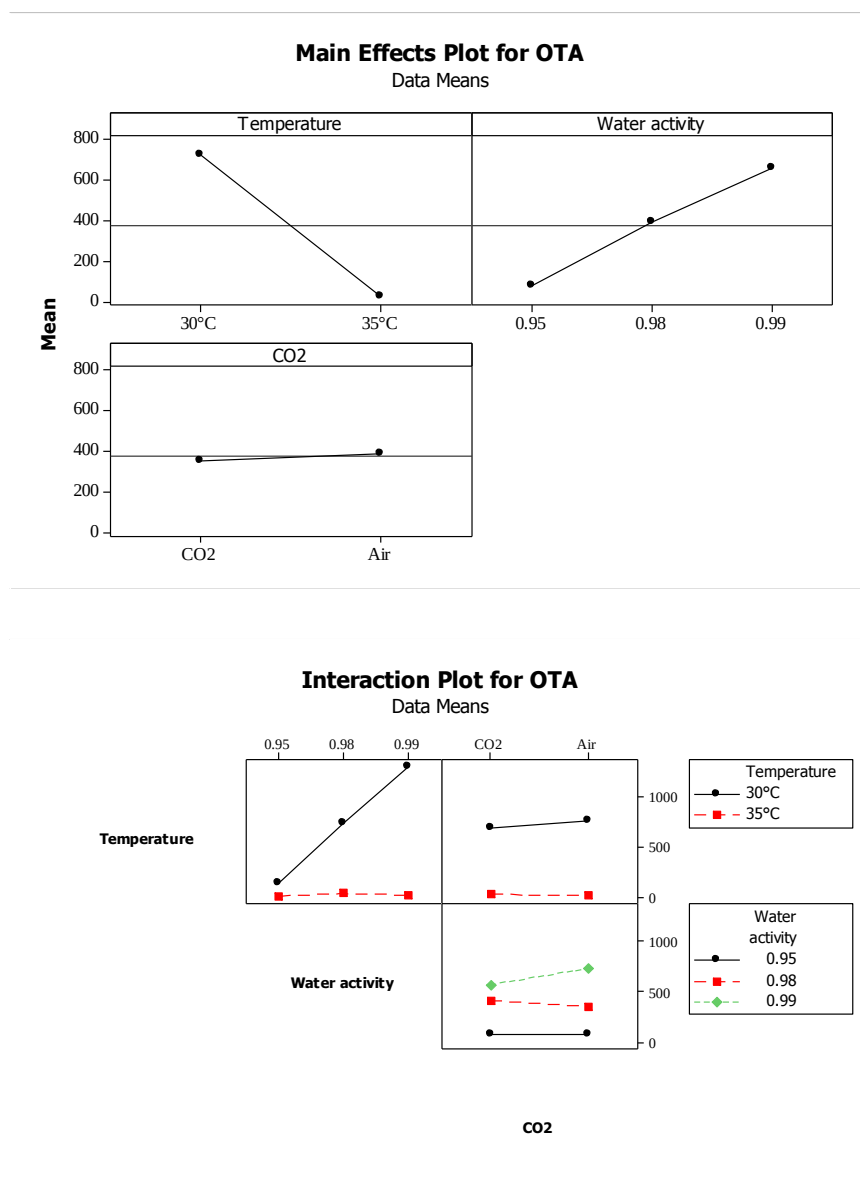


Figure II.3.I: Comparison of main effects plot of the mean of fungal OTA production (ng g⁻¹) versus CO₂, a_w and temperature for normal distribution of strains *A. carbonarius* (ITAL 204) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98, 0.99) for 12 days *in vitro*.

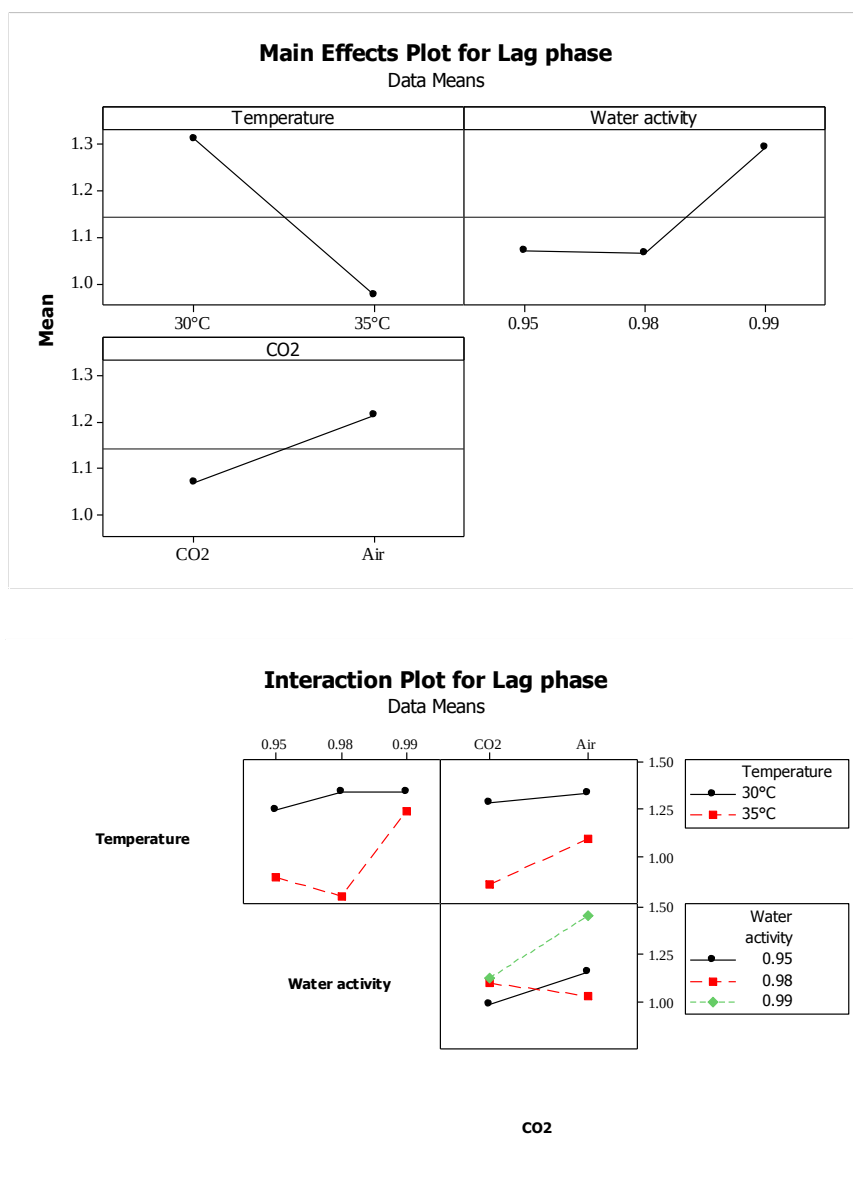


Figure II.3.m: Comparison of main effects plot of the mean of fungal lag phase (λ , days) versus CO₂, a_w and temperature for normal distribution of strains *A. ochraceus* (ITAL 14) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98, 0.99) for 12 days *in vitro*.

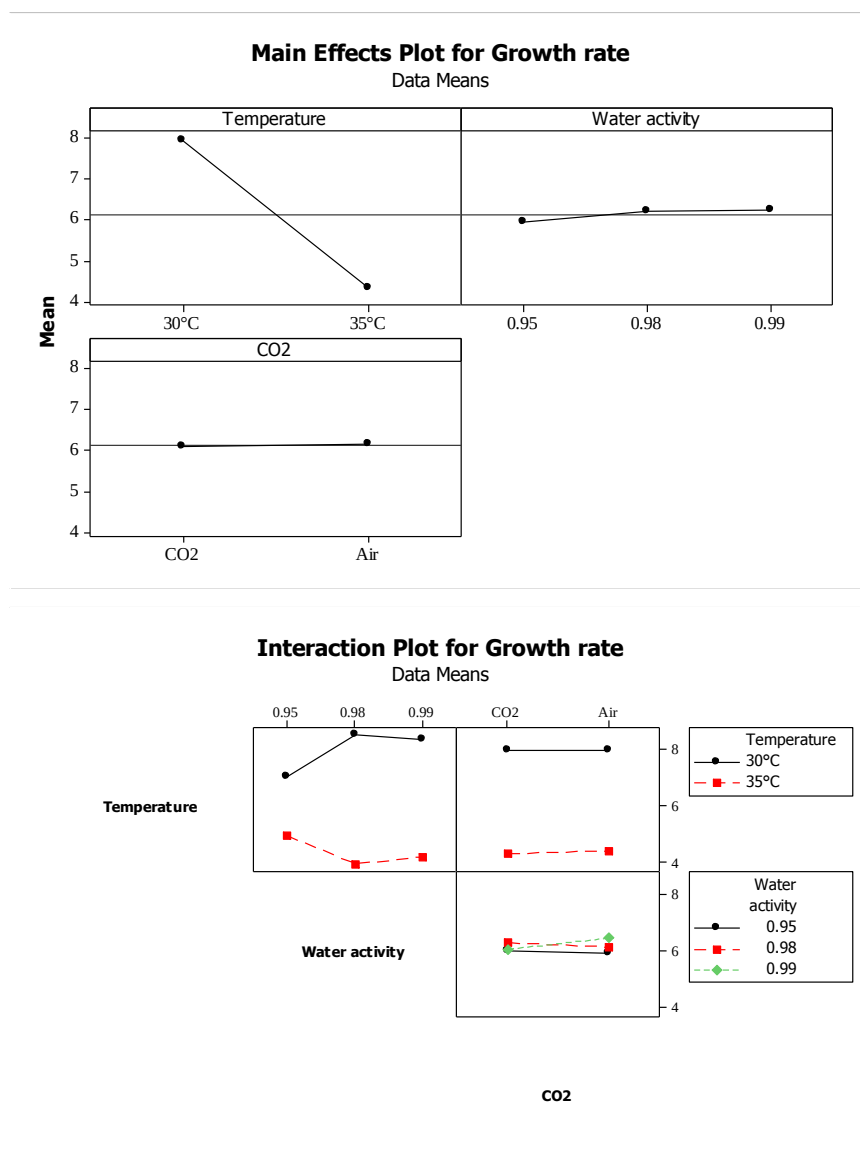


Figure II.3.n: Comparison of main effects plot of the mean of fungal growth rate (mm day⁻¹) versus CO₂, a_w and temperature for normal distribution of strains *A. ochraceus* (ITAL 14) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98, 0.99) for 12 days *in vitro* .

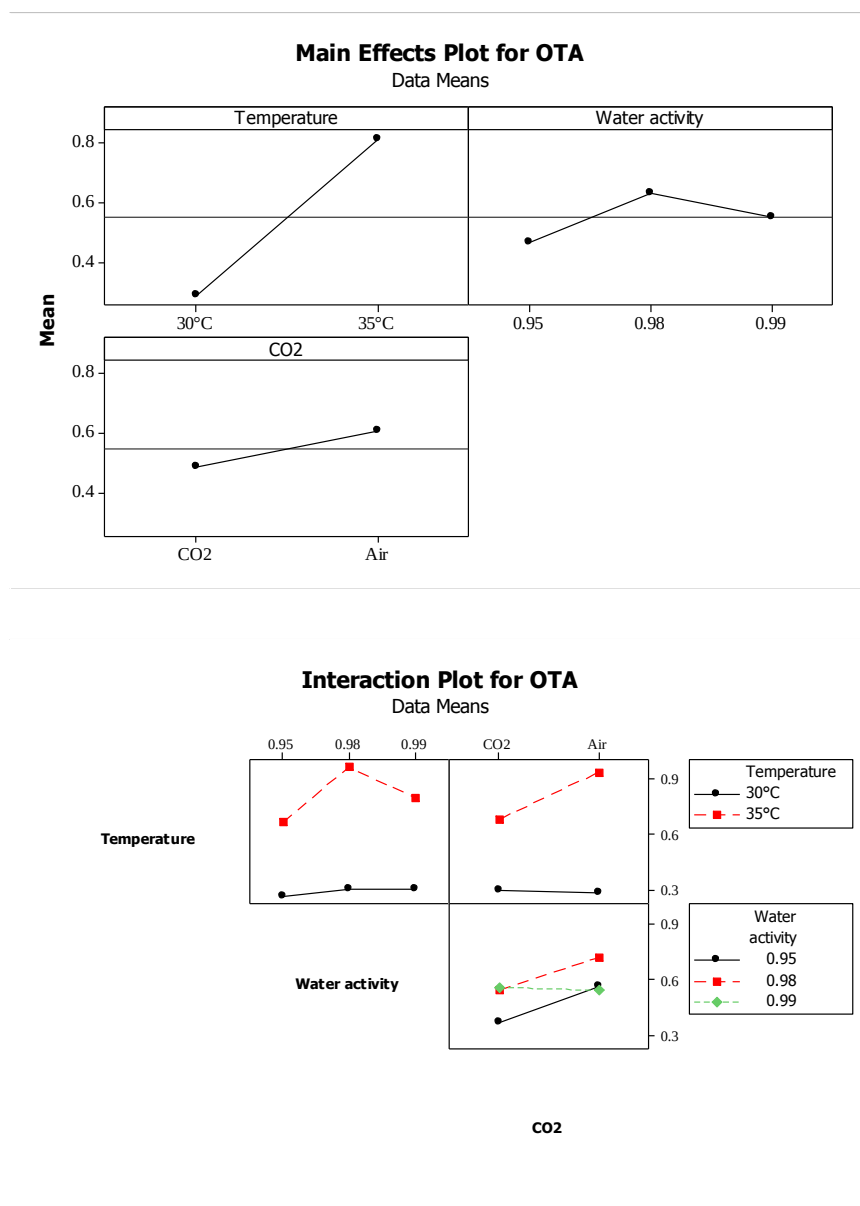


Figure II.3.o: Comparison of main effects plot of the mean of fungal OTA production (ng g⁻¹) versus CO₂, a_w and temperature for normal distribution of strains *A. ochraceus* (ITAL 14) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98, 0.99) for 12 days *in vitro*.

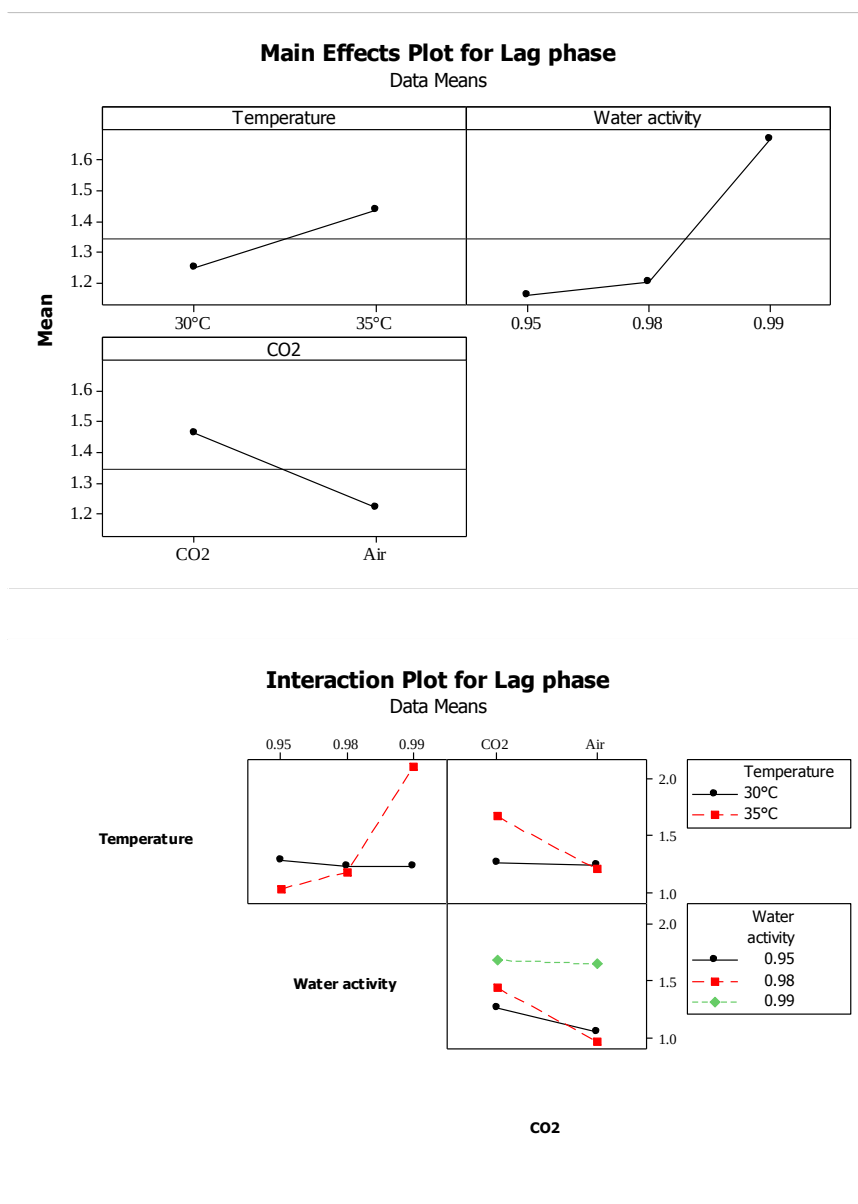


Figure II.3.p: Comparison of main effect plot of the mean of fungal lag phase (λ , days) versus CO_2 , a_w and temperature for normal distribution of strains *A. steynii* (CBS 112814) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98, 0.97) for 12 days *in vitro*.

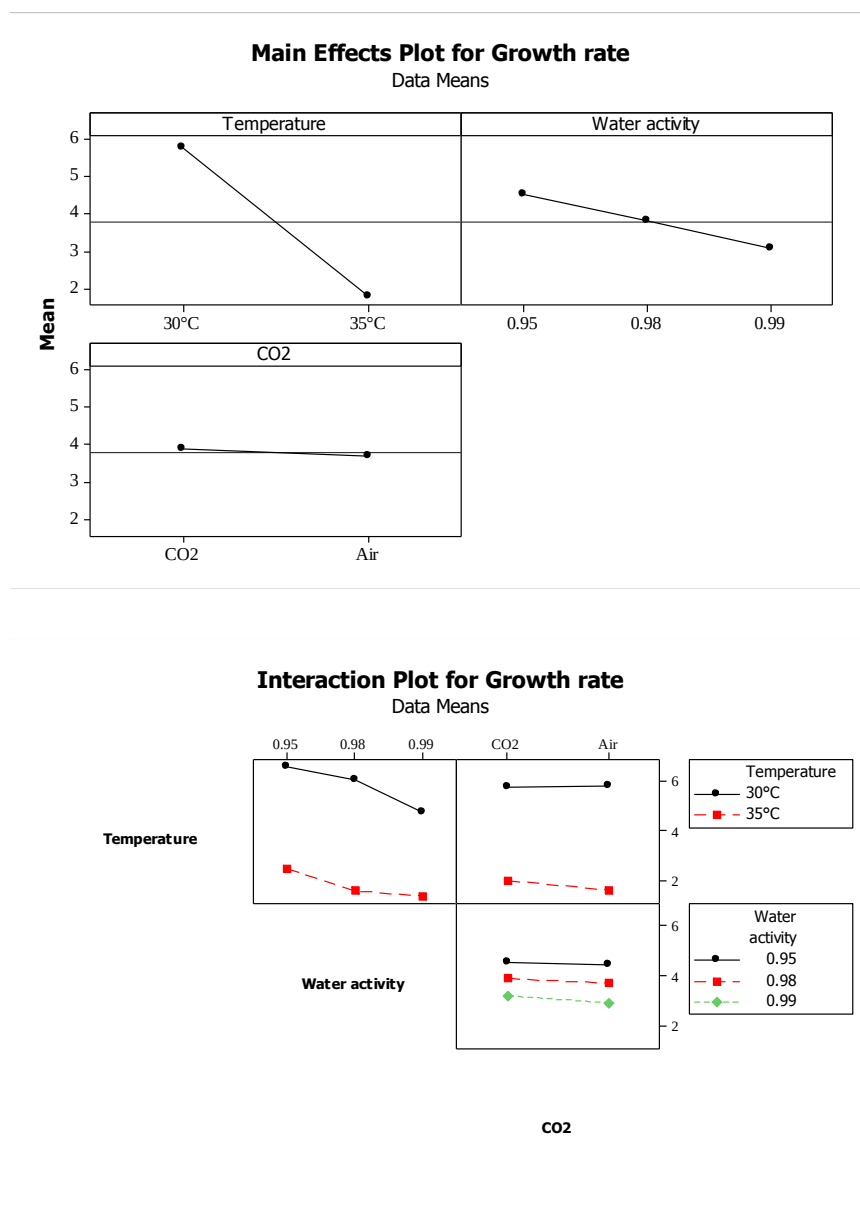


Figure II.3.q: Comparison of main effects plot of the mean of fungal growth rate (mm day⁻¹) versus CO₂, a_w and temperature for normal distribution of strains *A. steynii* (CBS 112814) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98, 0.99) for 12 days *in vitro*.

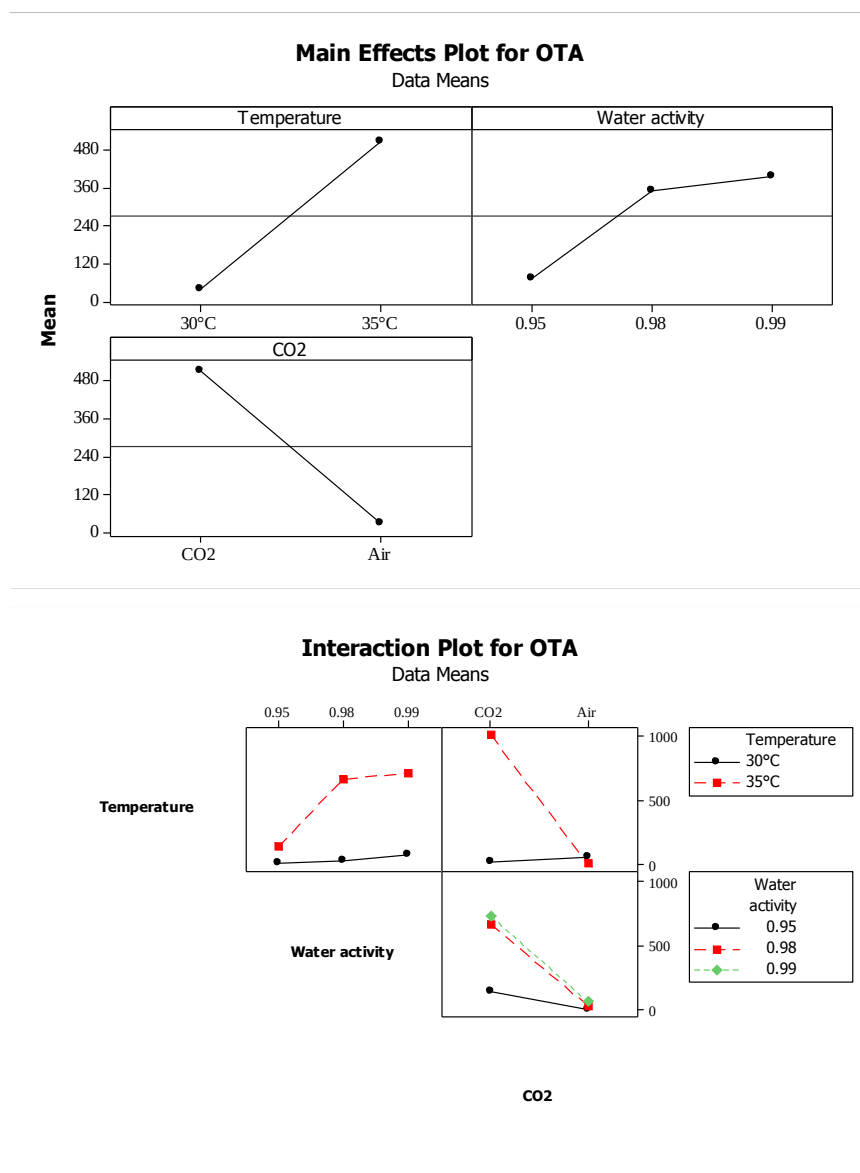


Figure II.3.r: Comparison of main effects plot of the mean of fungal OTA production (ng g⁻¹) versus CO₂, a_w and temperature for normal distribution of strains *A. steynii* (CBS 112814) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.95, 0.98, 0.99) for 12 days *in vitro*.

Appendix II 4: *In situ* effect water activity x elevated CO₂ x temperature on OTA production at 30°C in irradiated coffee beans

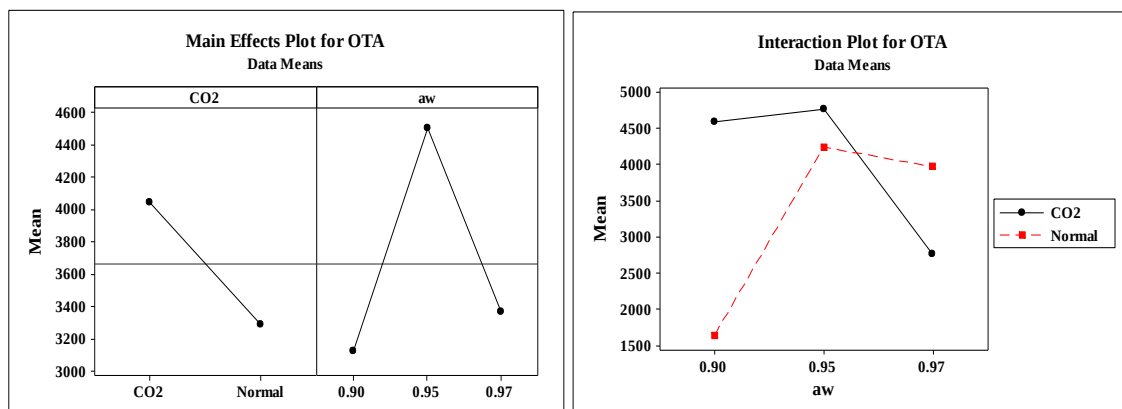


Figure II.4.a: Main effects plot of the mean of fungal OTA production versus CO₂, a_w for normal distribution of strains *A. westerdijkiae* (B 2) inoculated on irradiated coffee beans and incubated at temperatures 30°C and a_w (0.90, 0.95 0.97) for 12 days *in situ*.

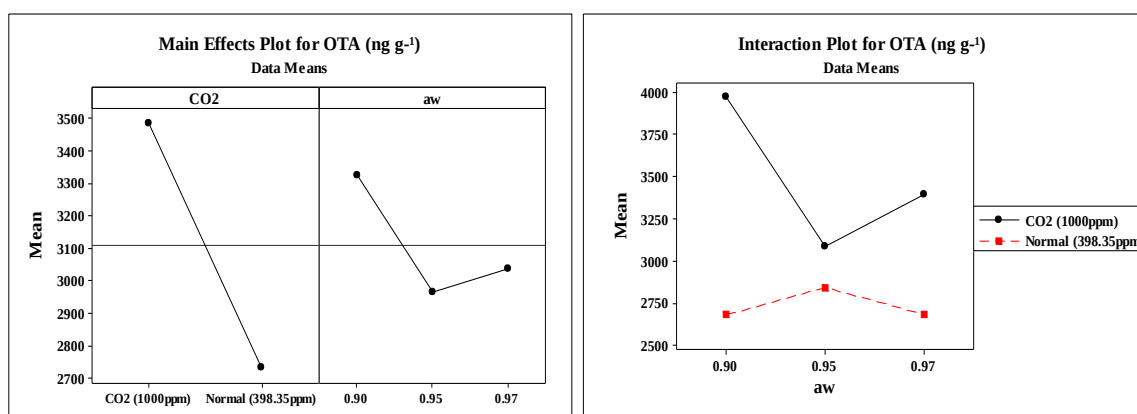


Figure II.4.b: Main effects plot of the mean of fungal OTA production versus CO₂, a_w for normal distribution of strains *A. westerdijkiae* (CBS 121986) inoculated on irradiated coffee beans and incubated at temperatures 30°C and a_w (0.90, 0.95 0.97) for 12 days *in situ*.

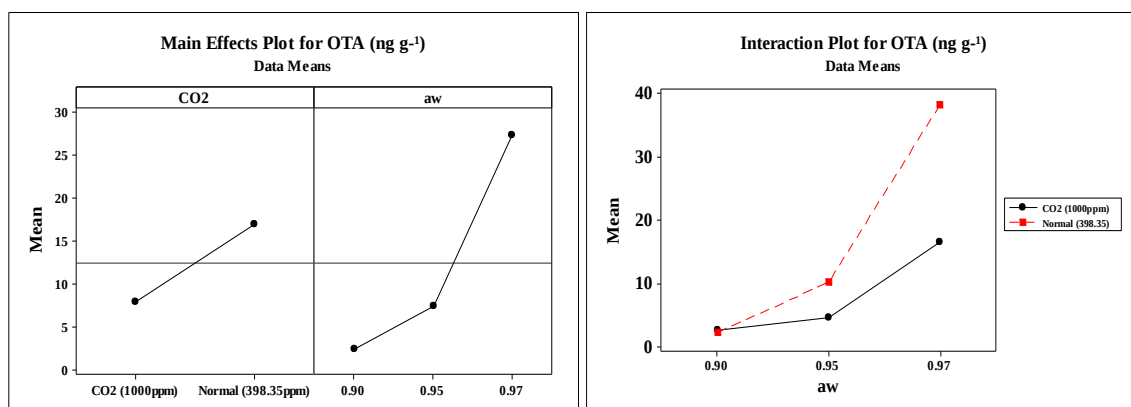


Figure II.4.c: Main effects plot of the mean of fungal OTA production versus CO₂, a_w for normal distribution of strain *A. niger* (A 1911) inoculated on irradiated coffee beans and incubated at temperatures 30°C and a_w (0.90, 0.95 0.97) for 12 days *in situ*.

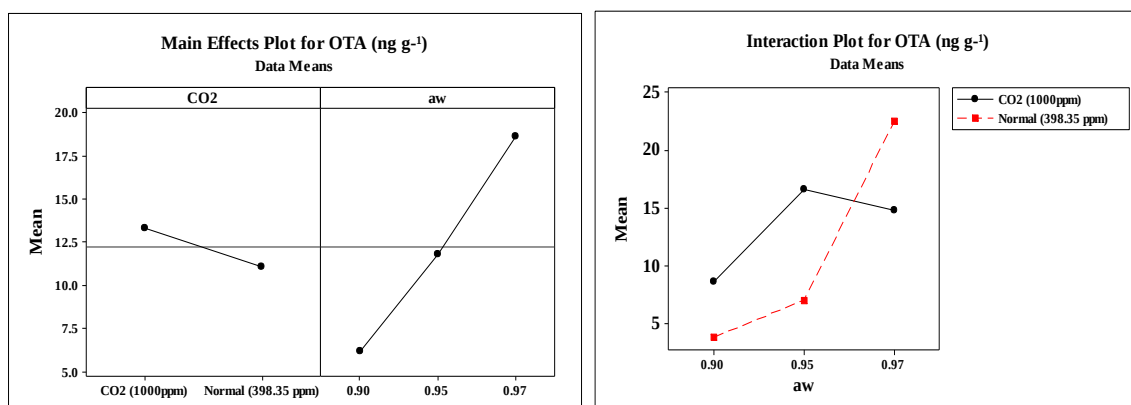


Figure II.4.d: Main effects plot of the mean of fungal OTA production versus CO₂, a_w for normal distribution of strain *A. carbonarius* (ITAL 204) inoculated on irradiated coffee beans and incubated at temperatures 30°C and a_w (0.90, 0.95 0.97) for 12 days *in situ*.

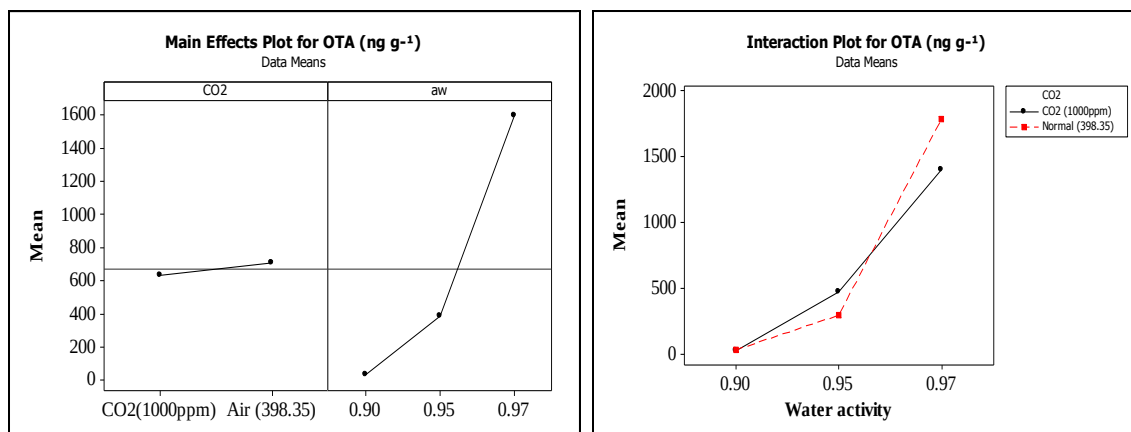


Figure II.4.e: Main effects plot of the mean of fungal OTA production versus CO₂, a_w for normal distribution of strain *A. ochraceus* (ITAL 14) inoculated on irradiated coffee beans and incubated at temperatures 30°C and a_w (0.90, 0.95 0.97) for 12 days *in situ*.

Appendix II 5: *In situ* effect water activity x elevated CO₂ x temperature on OTA production at 35°C in irradiated coffee beans

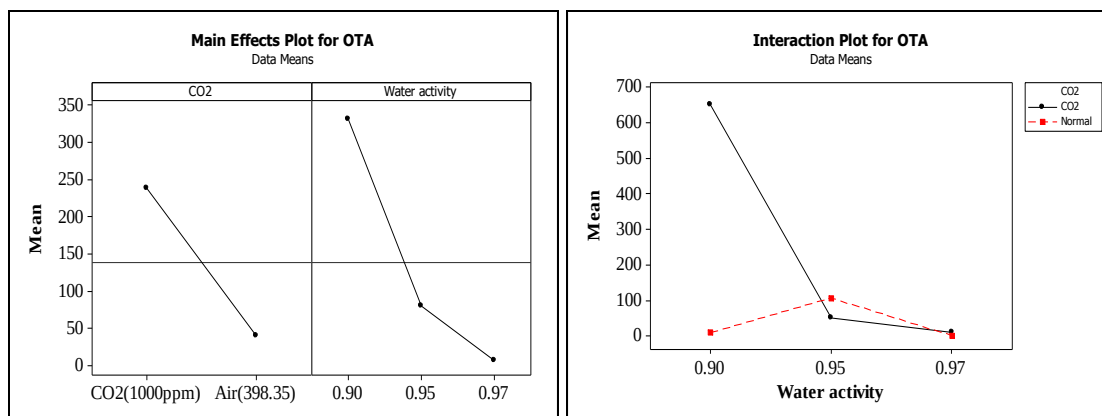


Figure II.5.a: Main effects plot of the mean of fungal OTA production versus CO₂, a_w for normal distribution of strain *A. westerdijkiae* (B 2) inoculated on irradiated coffee beans and incubated at temperatures 35°C and a_w (0.90, 0.95 0.97) for 12 days *in situ*..

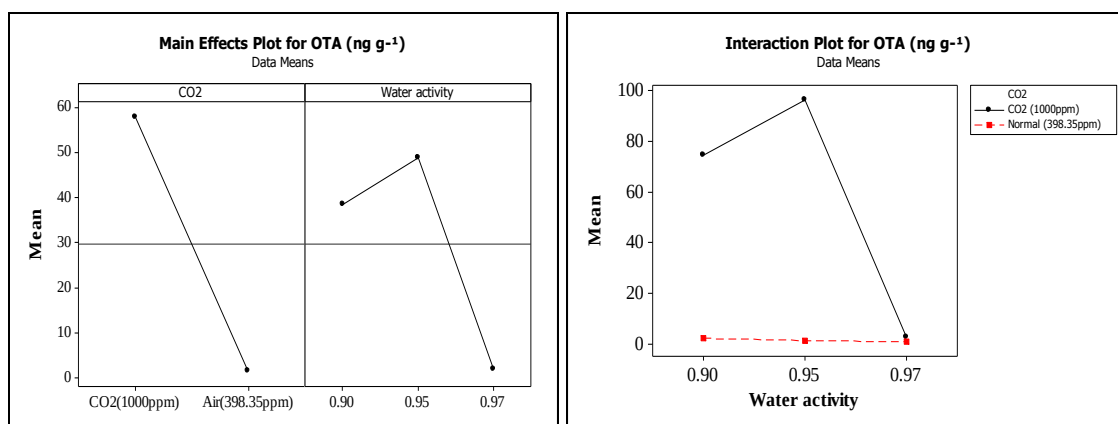


Figure II.5.b: Main effects plot of the mean of fungal OTA production versus CO₂, a_w for normal distribution of strain *A. westerdijkiae* (CBS 121986) inoculated on irradiated coffee beans and incubated at temperatures 35°C and a_w (0.90, 0.95 0.97) for 12 days *in situ*.

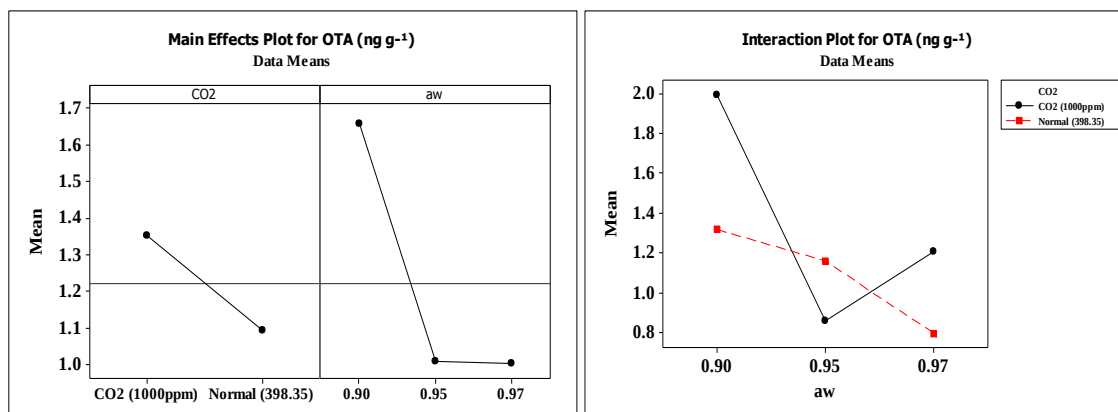


Figure II.5.c: Main effects plot of the mean of fungal OTA production versus CO₂, a_w for normal distribution of strains *A. niger* (A 1911) inoculated on irradiated coffee beans and incubated at temperatures 35°C and a_w (0.90, 0.95 0.97) for 12 days *in situ*..

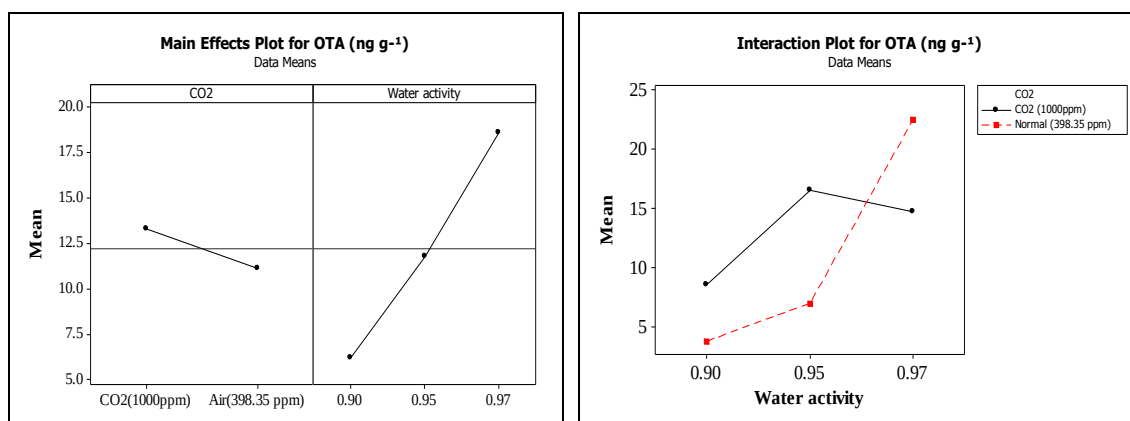


Figure II.5.c: Main effects plot of the mean of fungal OTA production versus CO₂, a_w for normal distribution of strains *A. carbonarius* (ITAL 204) inoculated on irradiated coffee beans and incubated at temperatures 35°C and a_w (0.90, 0.95 0.97) for 12 days *in situ*.

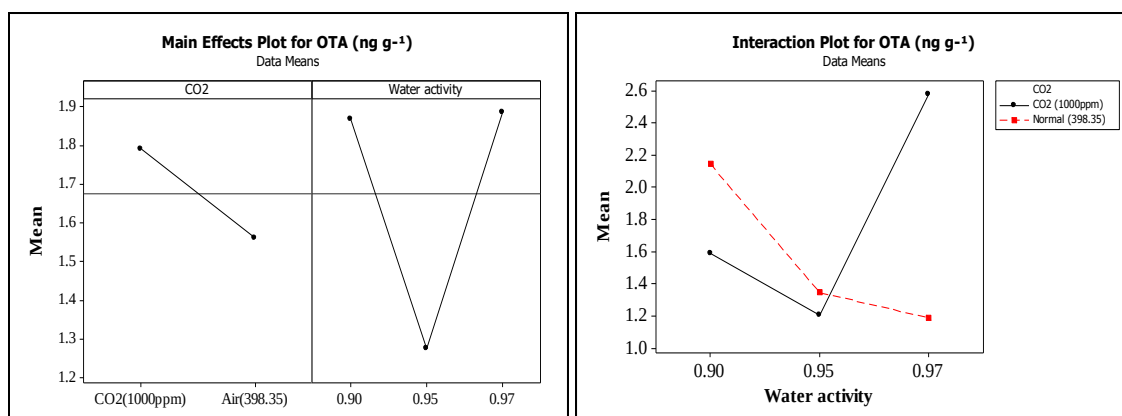


Figure II.5.d: Main effects plot of the mean of fungal OTA production versus CO₂, a_w for normal distribution of strains *A. ochraceus* (ITAL 14) inoculated on irradiated coffee beans and incubated at temperatures 35°C and a_w (0.90, 0.95 0.97) for 12 days.

Appendix II 6: *In situ* effect water activity x elevated CO₂ x temperature on OTA production at 30°C and 35°C in irradiated coffee beans

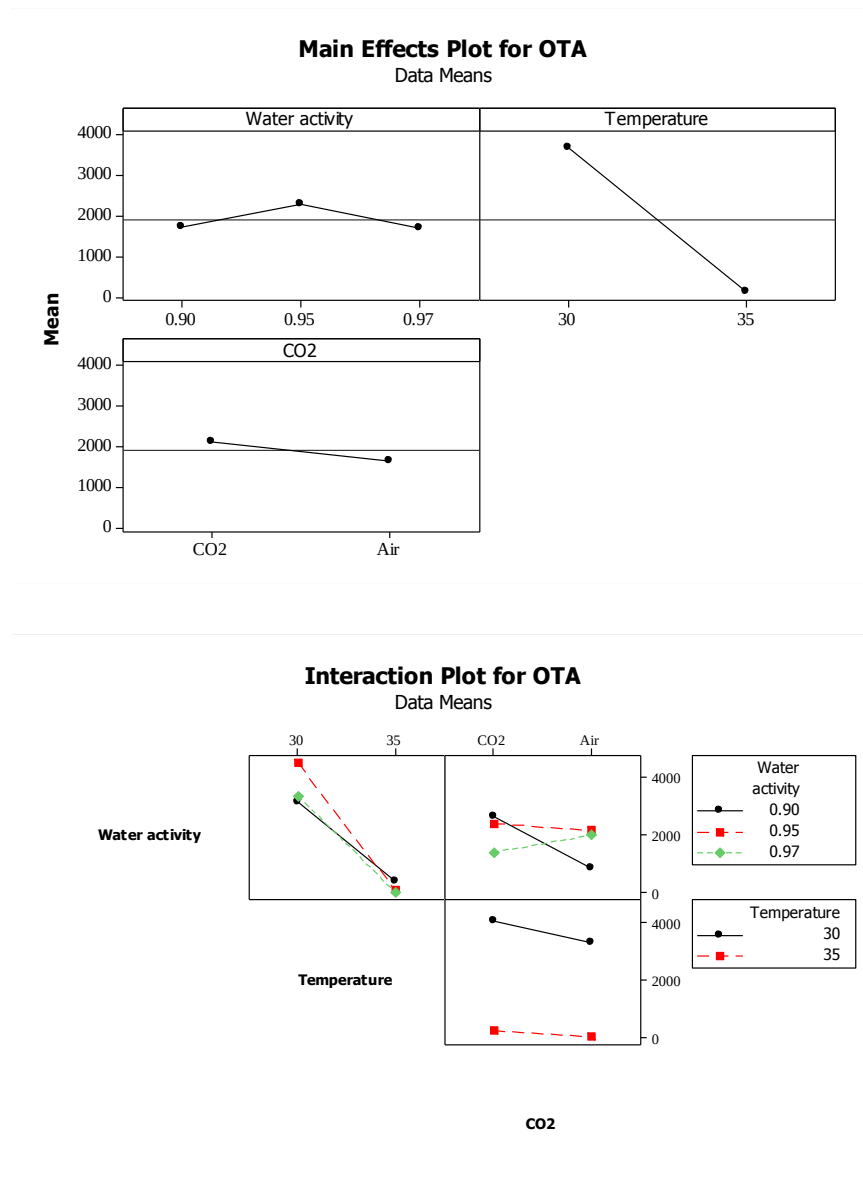


Figure II.6.a: Comparison of main effects plot of the mean of fungal OTA production versus CO₂, a_w and temperature for normal distribution of strain *A. westerdijkiae* (B 2) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.90, 0.95, 0.97) for 12 days *in situ*.

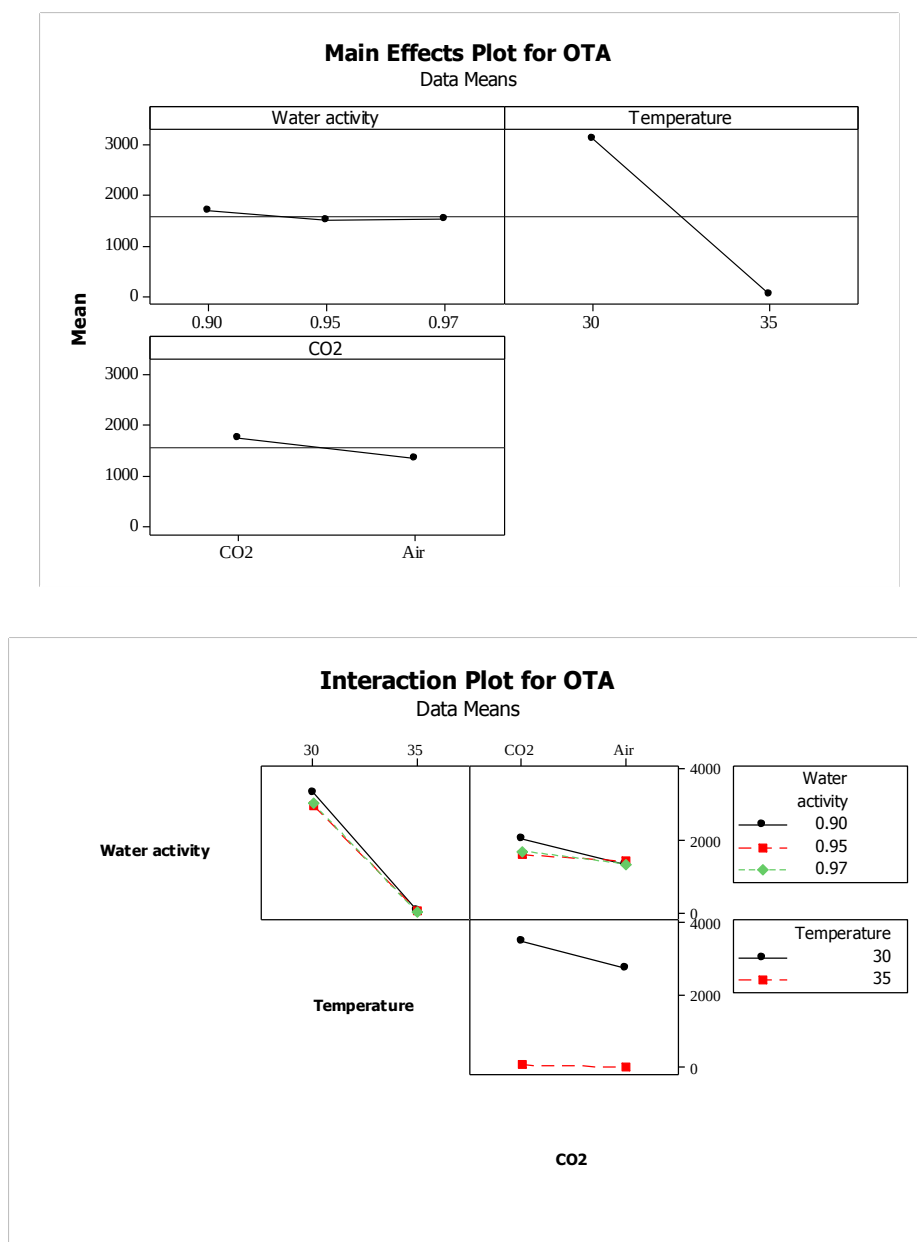


Figure II.6.b: Comparison of main effects plot of the mean of fungal OTA production versus CO₂, a_w and temperature for normal distribution of strain *A. westerdijkiae* (CBS 121986) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.90, 0.95, 0.97) for 12 days *in situ*.

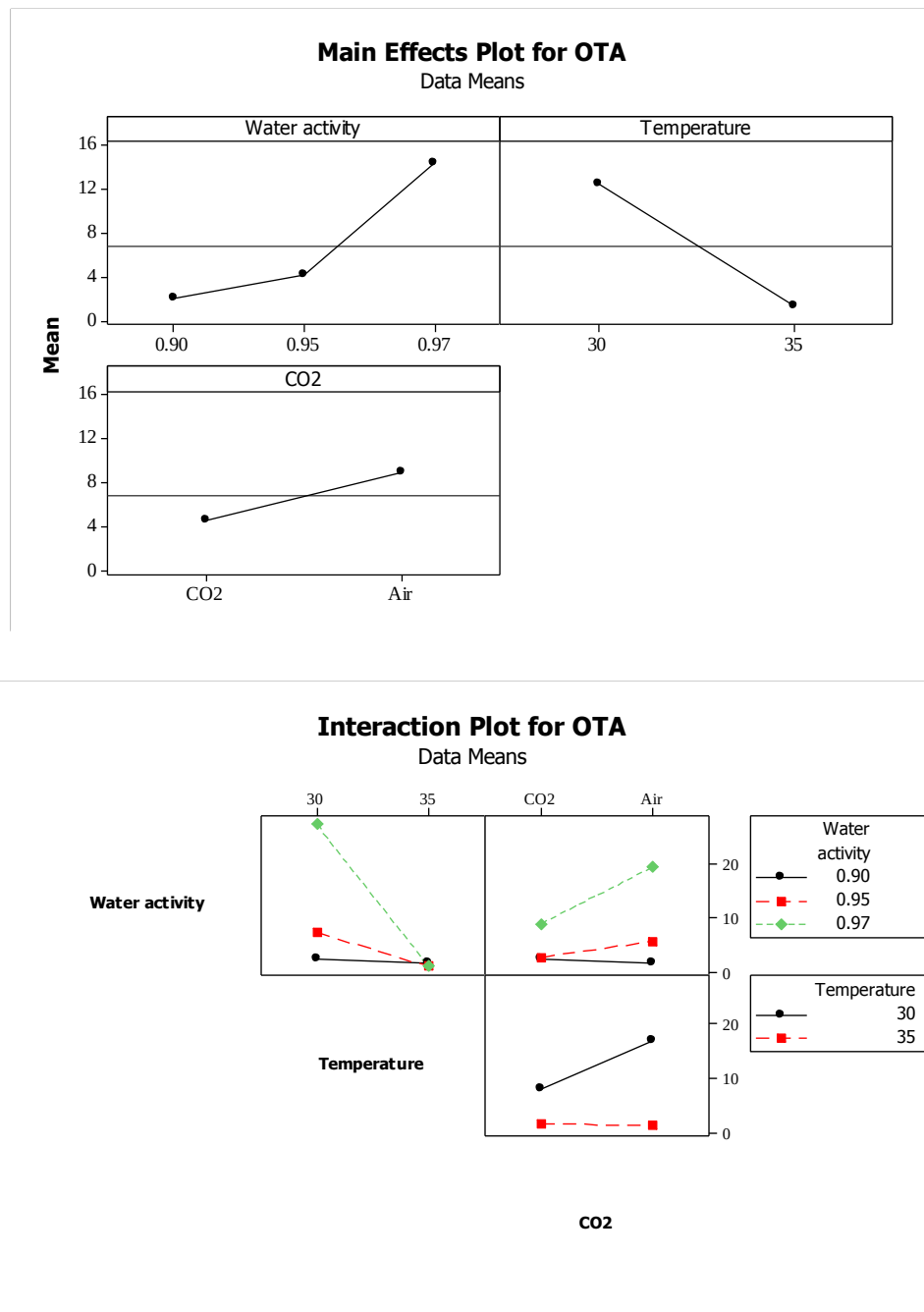


Figure II.6.c: Comparison of main effects plot of the mean of fungal OTA production versus CO₂, a_w and temperature for normal distribution of strain *A. niger* (A 1911) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.90, 0.95, 0.97) for 12 days *in situ*.

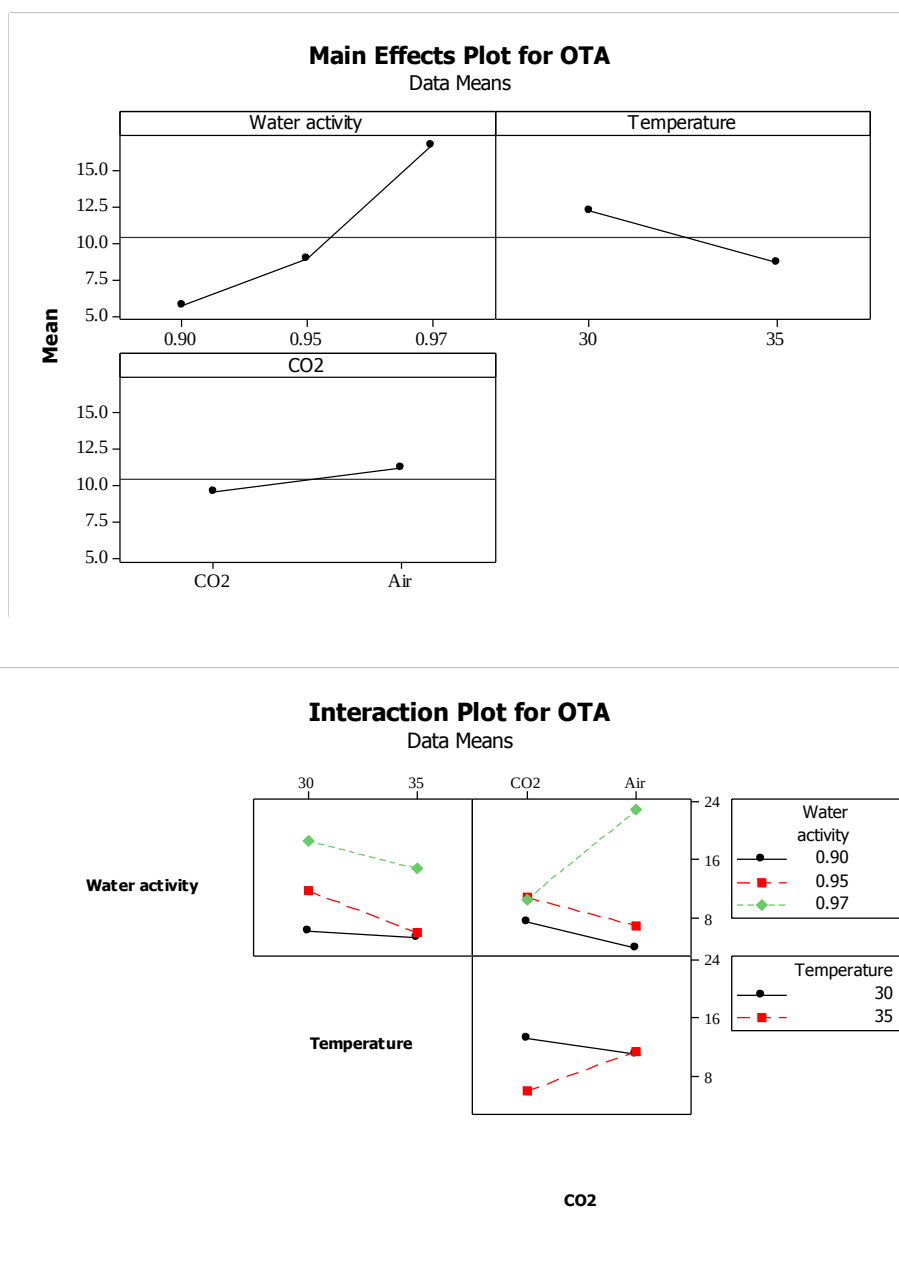


Figure II.6.d: Comparison of main effects plot of the mean of fungal OTA production versus CO₂, a_w and temperature for normal distribution of strain *A. carbonarius* (ITAL 204) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.90, 0.95, 0.97) for 12 days *in situ*.

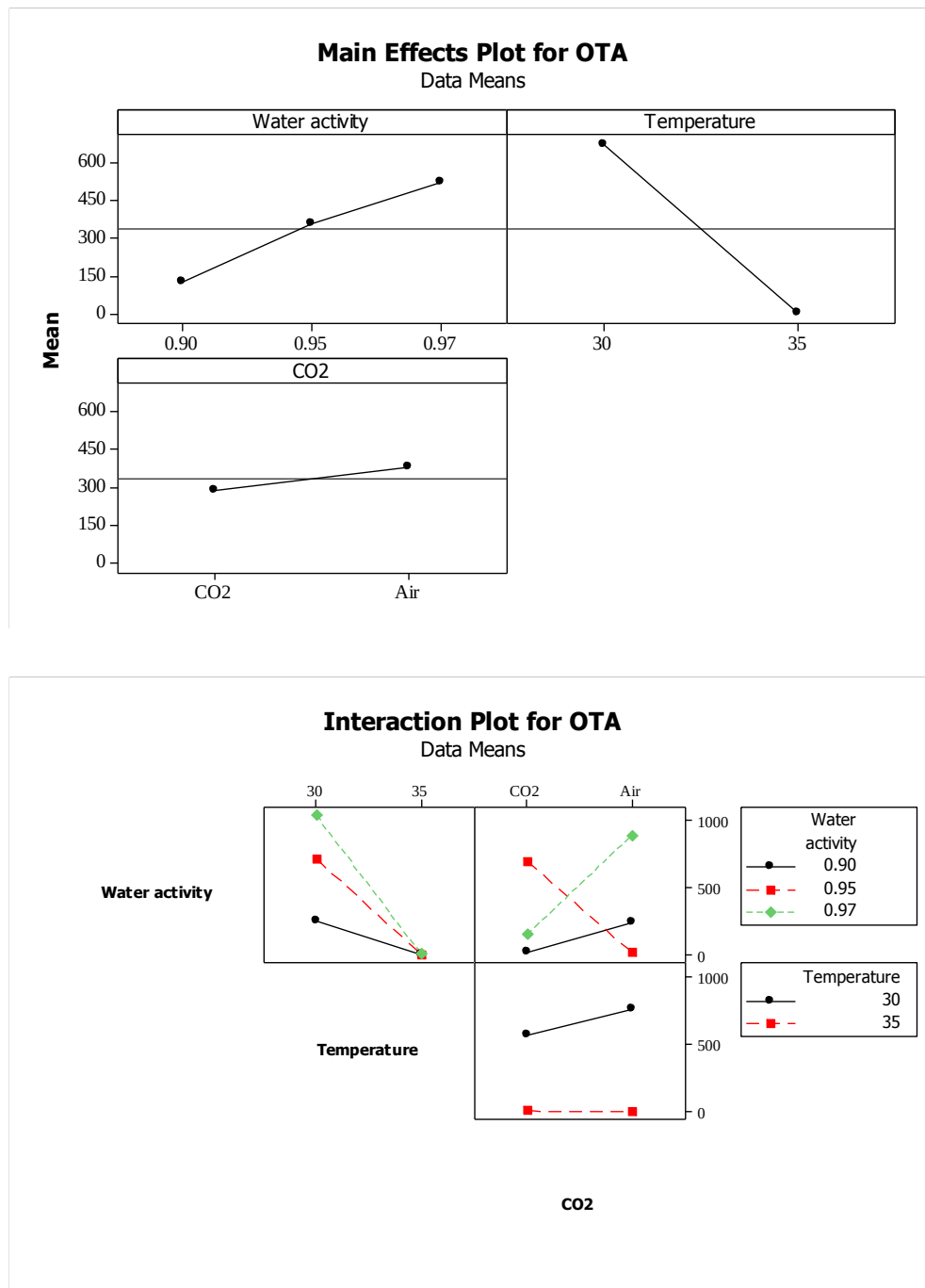


Figure II.6.e: Comparison of main effects plot of the mean of fungal OTA production versus CO₂, a_w and temperature for normal distribution of strain *A. ochraceus* (ITAL 14) inoculated on irradiated coffee beans and incubated at temperatures 30 and 35°C and a_w (0.90, 0.95, 0.97) for 12 days *in situ*.

APPENDIX III

Appendix III 1: Effect of a_w x temperature on fungal growth of *A. aculeatinus* (CBS 115570) and *A. sclerotii carbonarius* (CBS 121056) inoculated on CMEA

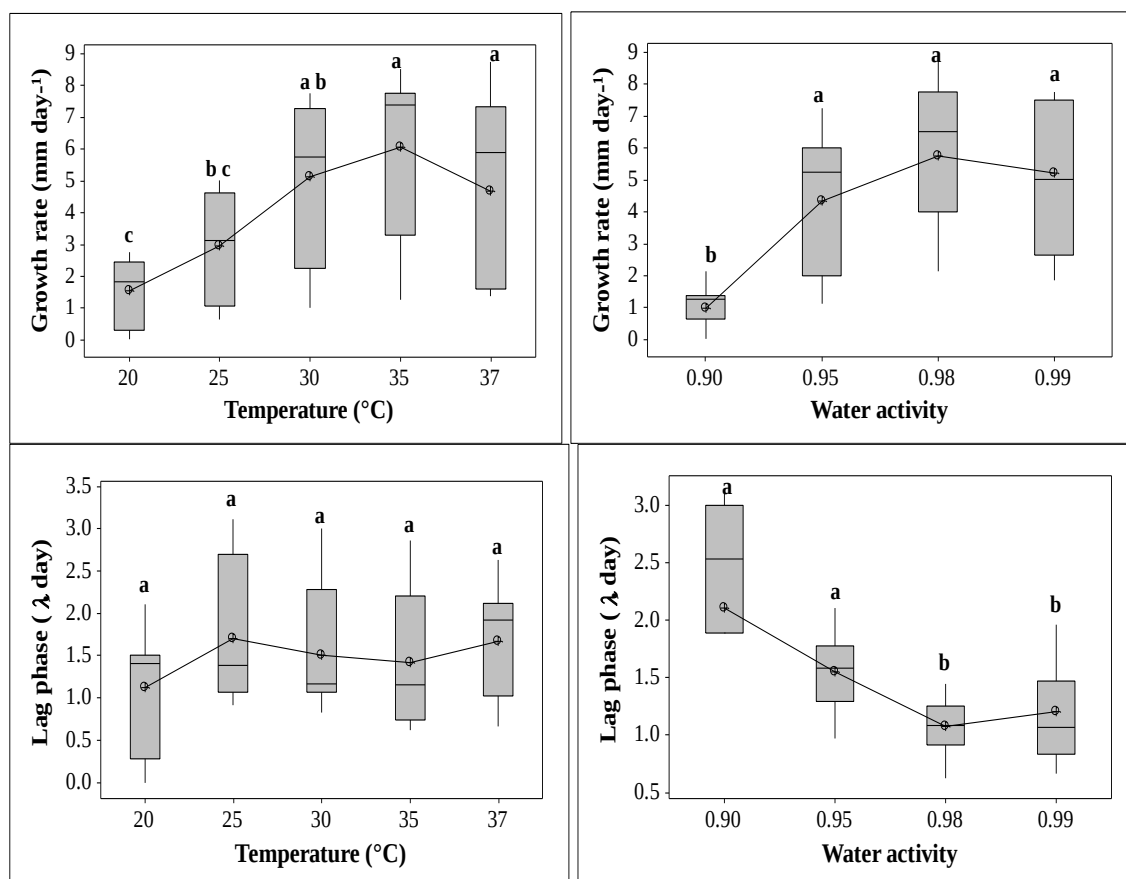


Figure III. 1: Box-plot analysis for fungal growth and lag time (λ , day) of *A. aculeatinus* (CBS 115570) inoculated on CMEA and incubated at 5 different temperature (20, 25, 30, 35 and 37°C) and 4 a_w (0.90, 0.95, 0.98, 0.99) for 9 days. The box-plot shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

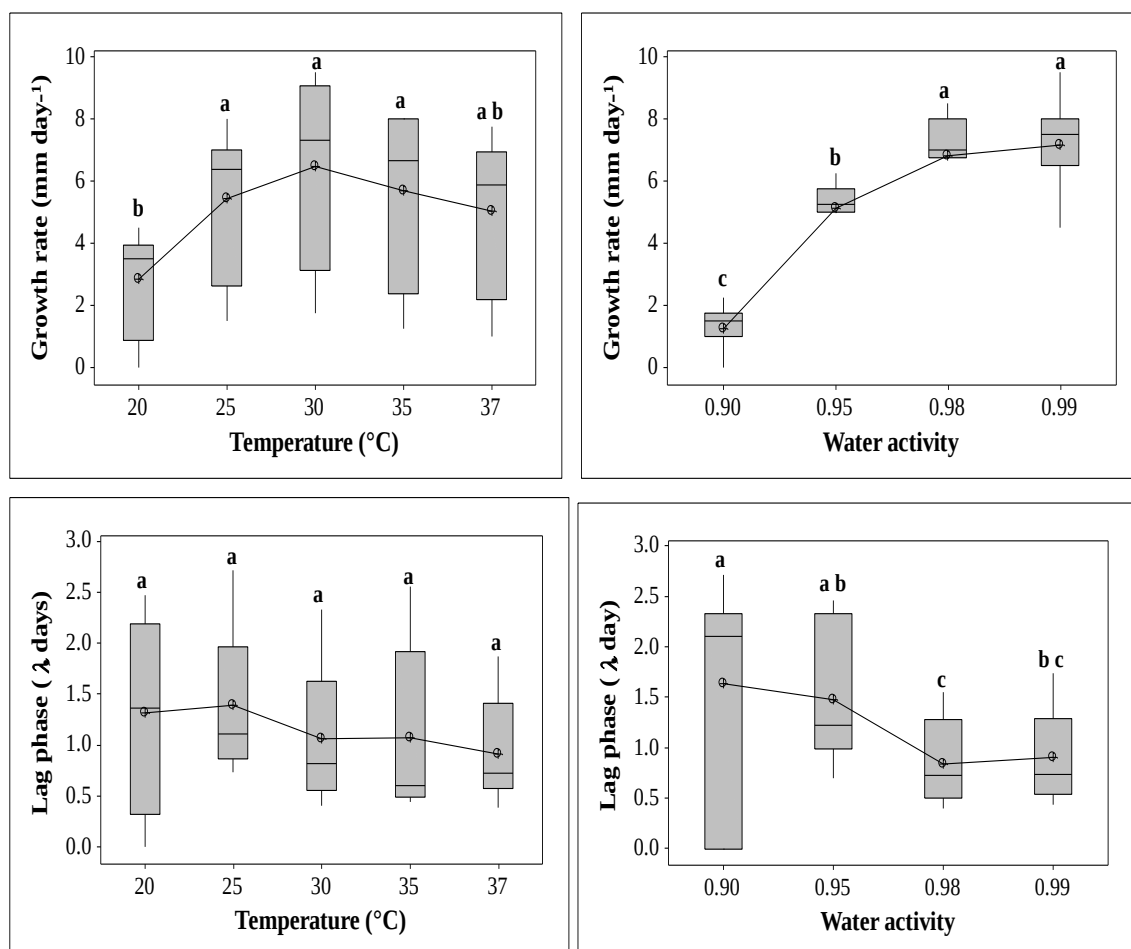


Figure III 2: Box-plot analysis for fungal growth and lag time (λ , days) of *A. aculeatinus* (CBS 121875) inoculated on CMEA and incubated at 5 different temperature (20, 25, 30, 35 and 37°C) and 4 a_w (0.90, 0.95, 0.98, 0.99) for 9 days. The box-plot shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

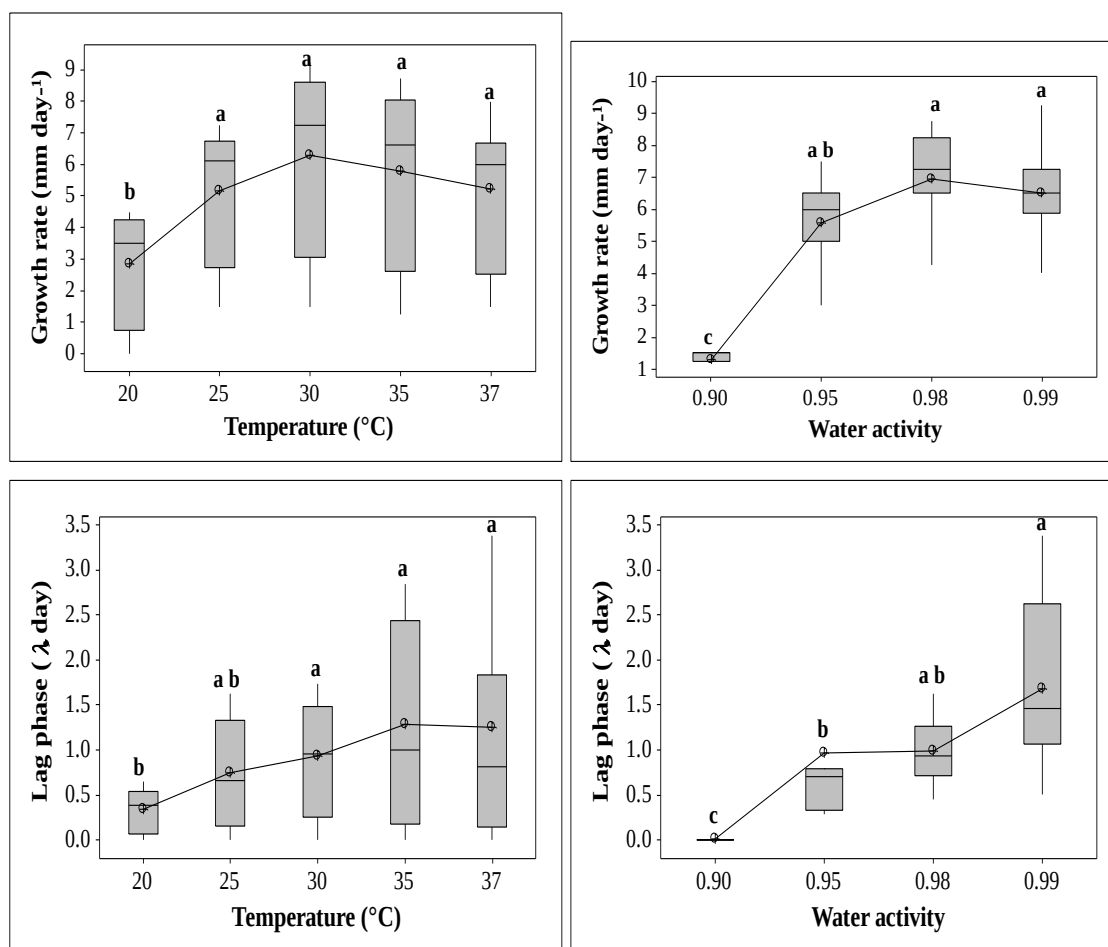


Figure III 3: Box-plot analysis for fungal growth and lag phase (λ , days) of *A. aculeatinus* (CBS 121872) inoculated on CMEA and incubated at 5 different temperature (20, 25, 30, 35 and 37°C) and 4 a_w (0.90, 0.95, 0.98, 0.99) for 9 days. The box-plot shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

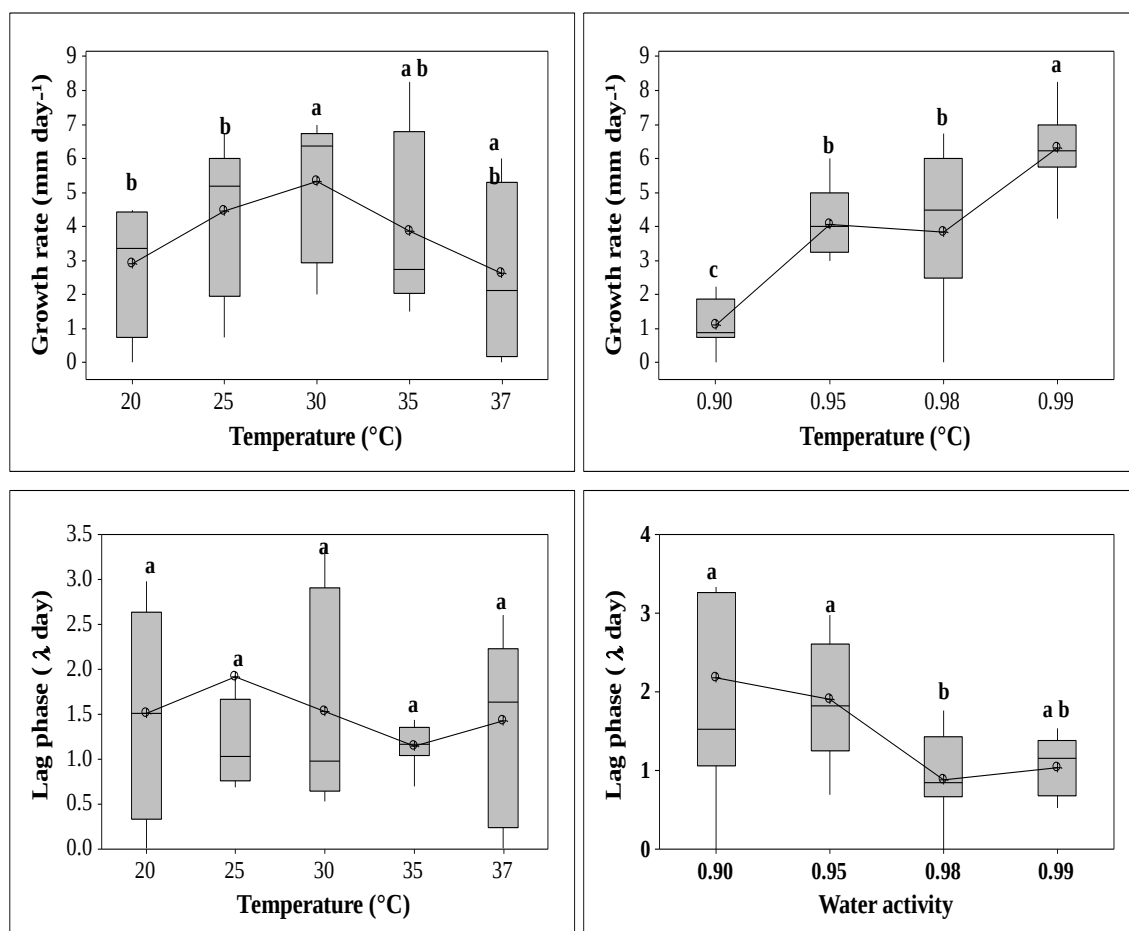


Figure III 4: Box-plot analysis for fungal growth and lag phase (λ , days) of *A. sclerotiicarbonarius* (CBS 121056) inoculated on CMEA and incubated at 5 different temperature (20, 25, 30, 35 and 37°C) and 4 a_w (0.90, 0.95, 0.98, 0.99) for 9 days. The box-plot shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

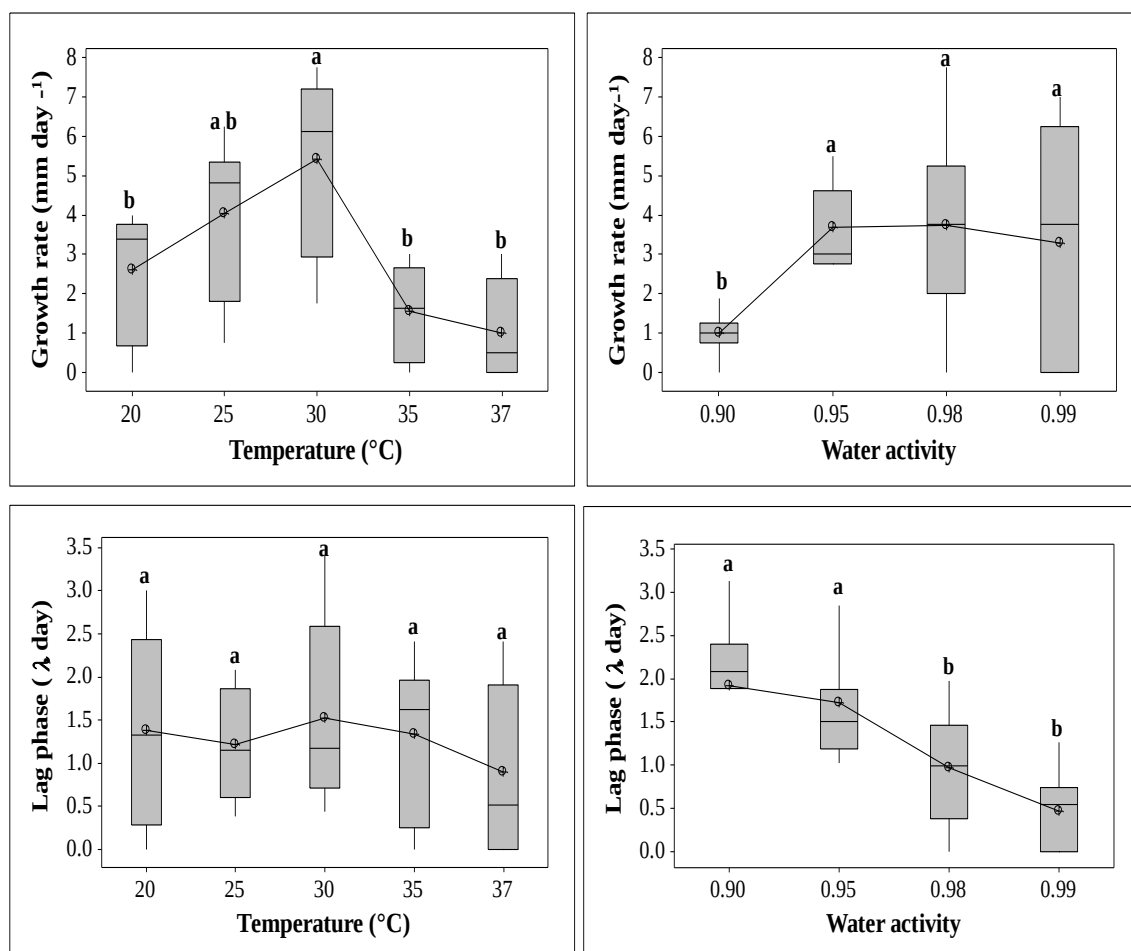


Figure III 5: Box-plot analysis for fungal growth and lag time (λ , day) of *A. sclerotiicarbonarius* (CBS 121851) inoculated on CMEA and incubated at 5 different temperature (20, 25, 30, 35 and 37°C) and 4 a_w (0.90, 0.95, 0.98, 0.99) for 9 days. The box-plot shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

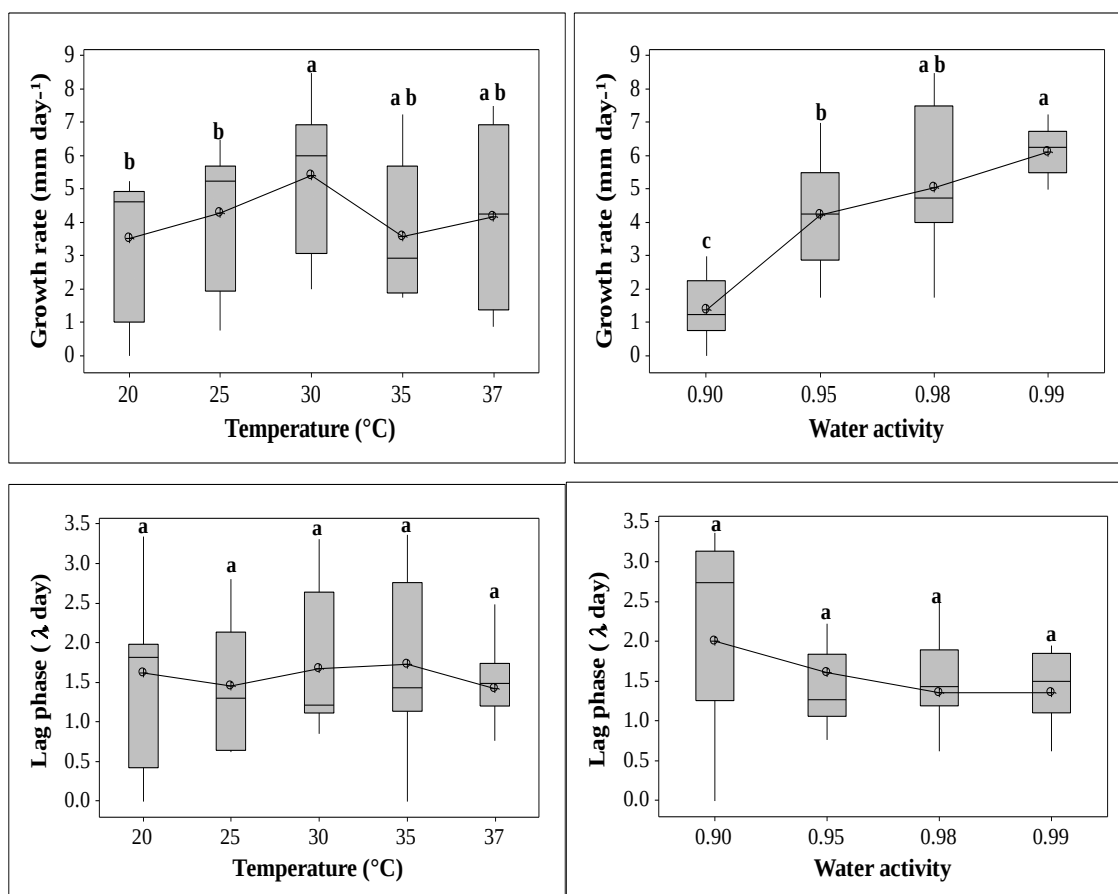


Figure III 6: Box-plot analysis for fungal growth and lag phase (λ , days) of *A. sclerotiicarbonarius* (CBS 121853) inoculated on CMEA and incubated at 5 different temperature (20, 25, 30, 35 and 37°C) and 4 a_w (0.90, 0.95, 0.98 0.99) for 9 days. The box-plot shows the median (line inside the box). Symbol shows the mean and boxes labelled with same letter indicate sharing the group.

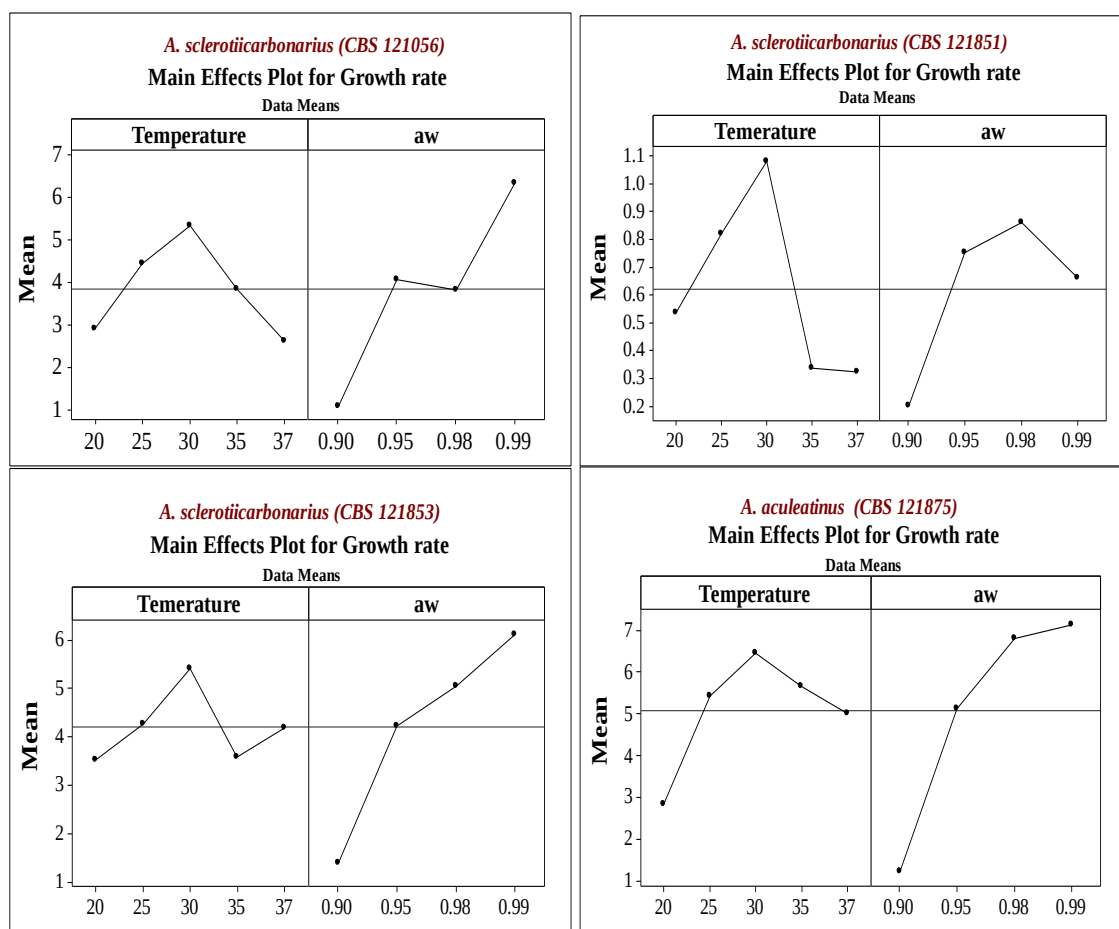


Figure III 7: Main effect plot of the mean of growth rate versus Temperature, a_w for normal distribution of strains *A. sclerotii carbonarius* (CBS 121056), *A. sclerotii carbonarius* (CBS 121851), *A. sclerotii carbonarius* (CBS 121853) and *A. aculeatinus* (CBS 121875).

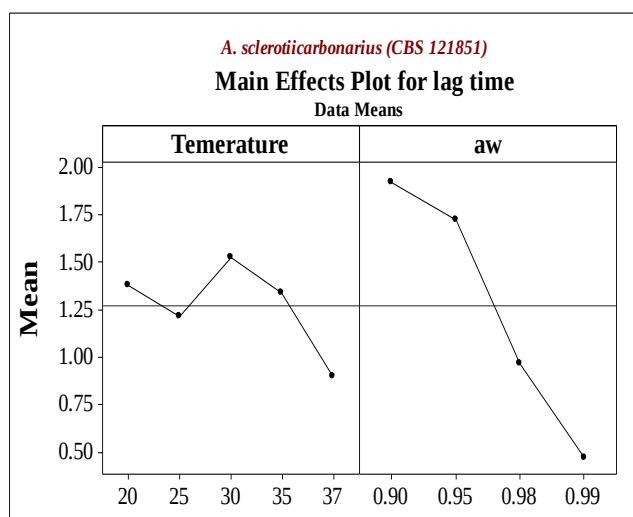


Figure III 8: Main effect plot of the mean of lag phases (λ , days) versus Temperature, a_w for normal distribution of strains *A. Scleroticarbonarius* (CBS 121851).

APPENDIX IV

Appendix IV 1: Effect of O₃ on the total fungal populations on naturally coffee bean

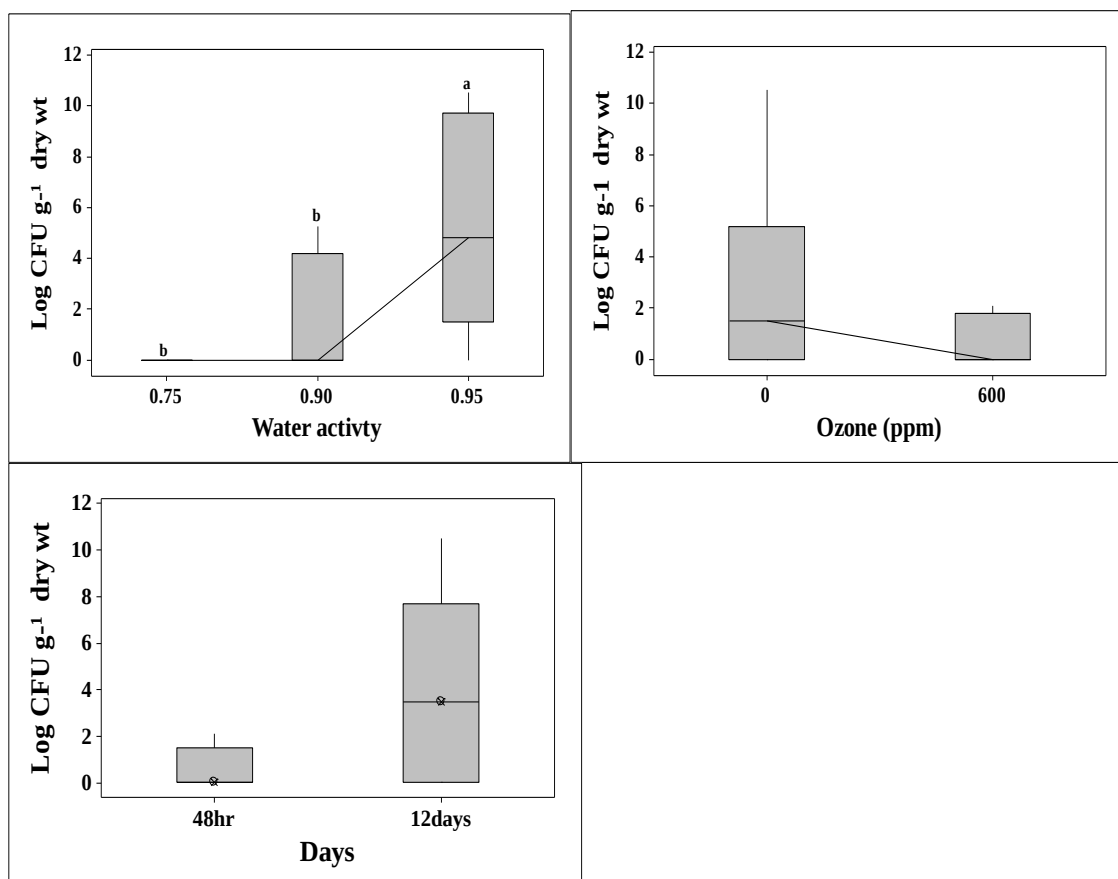


Figure IV.1.a: Box-plot analysis of fungal populations, effect by ozone (0, 600ppm) and isolated from naturally contaminated coffee bean adjusted to 0.75, 0.90 and 0.95 a_w , compared to the counts of the control (untreated 0ppm)untreated directly after exposure (48) and 12 days of incubation. Vertical bars indicate the standard error of the means.

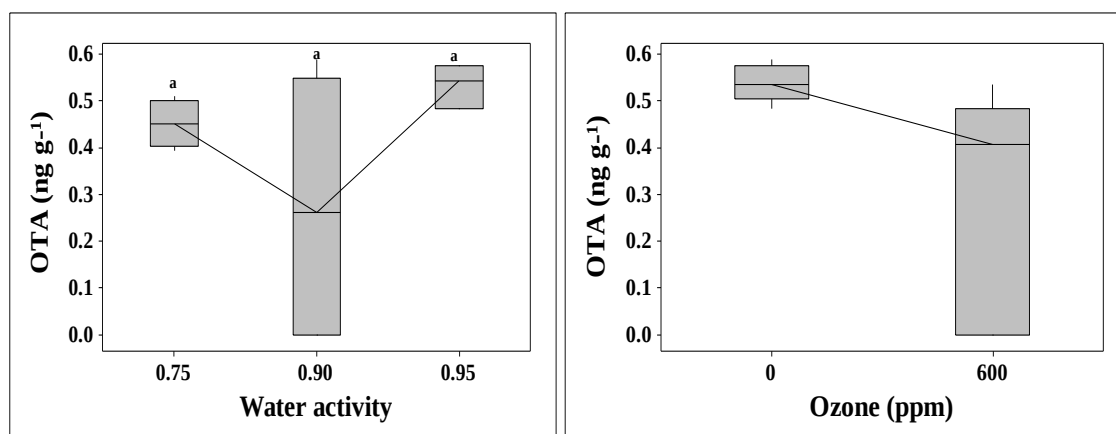


Figure IV.1.b: Box-plot analysis OTA production (ng g⁻¹) by ozone (0, 600ppm) and isolated from naturally contaminated coffee bean adjusted to 0.75, 0.90 and 0.95 a_w , compared to the counts of the control after 12 days of incubation. Vertical bars indicate the standard error of the means.

Appendix IV 2: Effect of O₃ on the fungal populations of *A. westerdijkiae*, *A. carbonarius* and *A. ochraceus* on naturally coffee bean

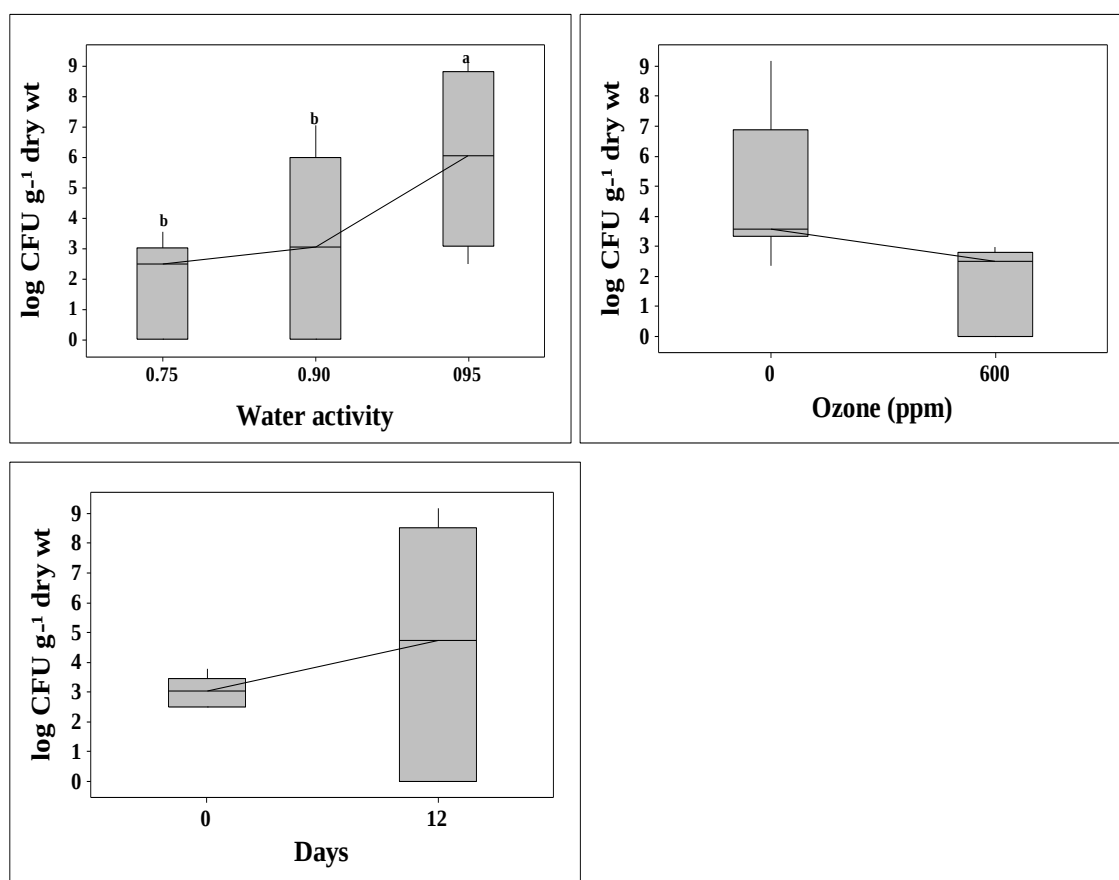


Figure IV.2.b: Box-plot analysis of fungal populations, effect by ozone (0, 600ppm) and isolated from naturally contaminated coffee bean plus inoculated with *A. westerdijkiae* (CBS 12986), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) adjusted to 0.75, 0.90 and 0.95 *a_w*, compared to the counts of the control (untreated 0ppm)untreated directly after exposure (48) and 12 days of incubation. Vertical bars indicate the standard error of the means.

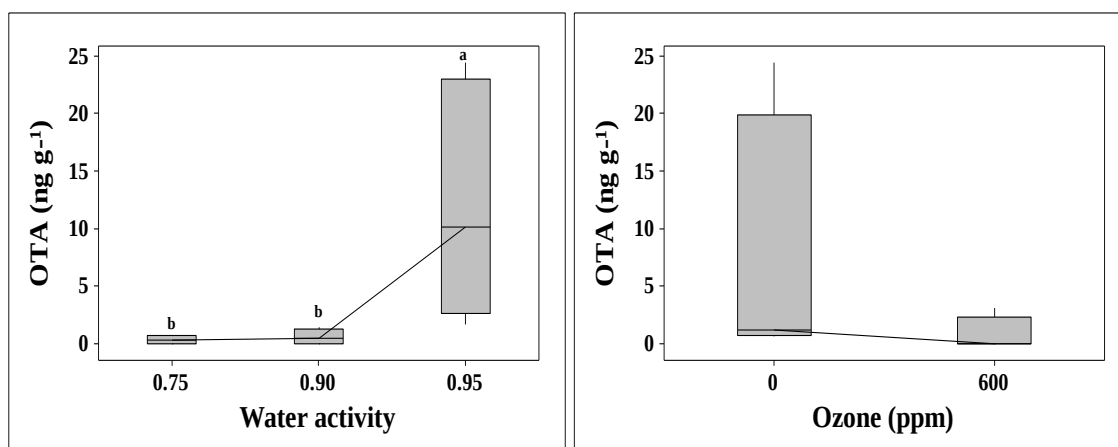


Figure IV.2.b: Box-plot analysis OTA production (ng g⁻¹) by ozone (0, 600ppm) and isolated from naturally contaminated coffee bean plus inoculated with *A. westerdijkiae* (CBS 12986), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) adjusted to 0.75, 0.90 and 0.95 *a_w*, compared to the counts of the control after 12 days of incubation. Vertical bars indicate the standard error of the means.

Appendix IV 3: Effect of O₃ on the fungal populations of *A. westerdijkiae*, *A. carbonarius* and *A. ochraceus* on irradiated coffee bean.

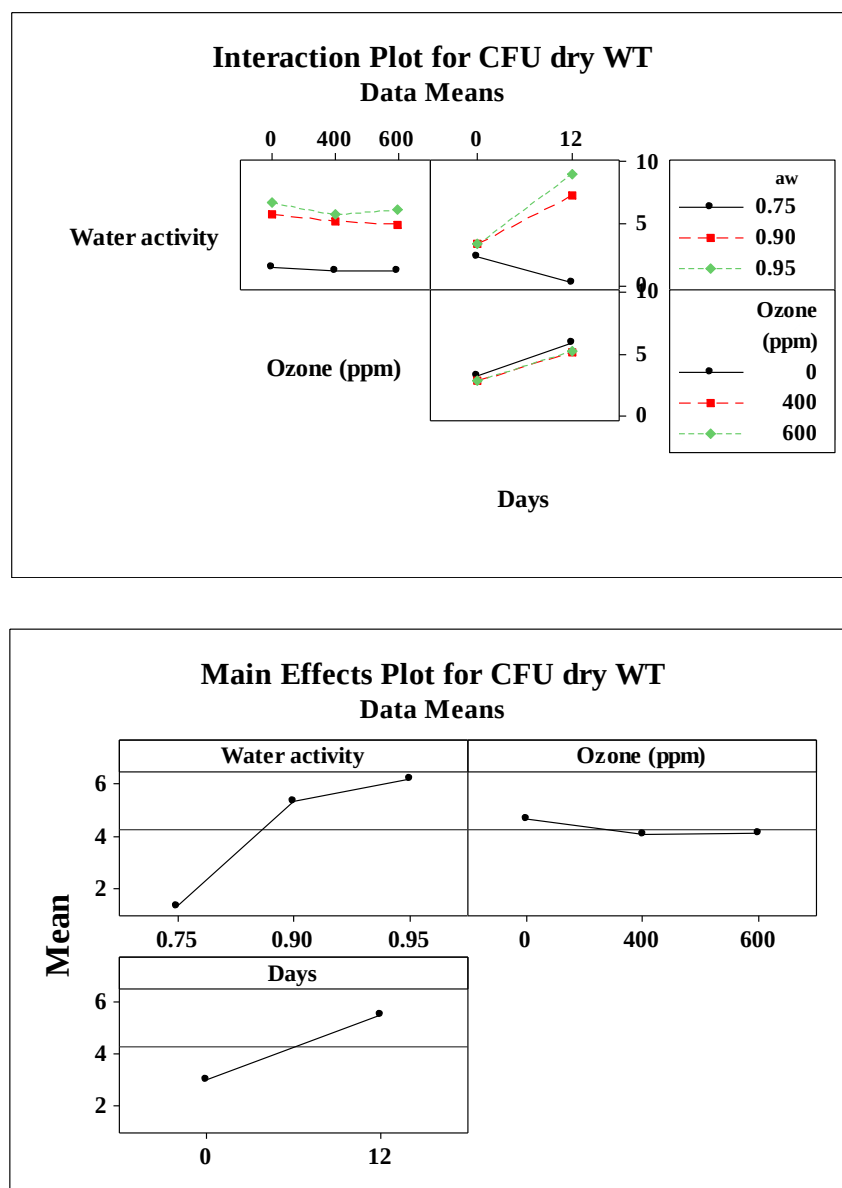


Figure IV.3.a: Main effect plot of the mean of fungal population effect by ozone (0, 400, 600 ppm) and isolated from irradiated coffee bean plus inoculated with *A. carbonarius* (ITAL 204) adjusted to 0.75, 0.90 and 0.95 *a_w*, compared to the counts of the control (untreated 0ppm)untreated directly after exposure (48) and 12 days of incubation.

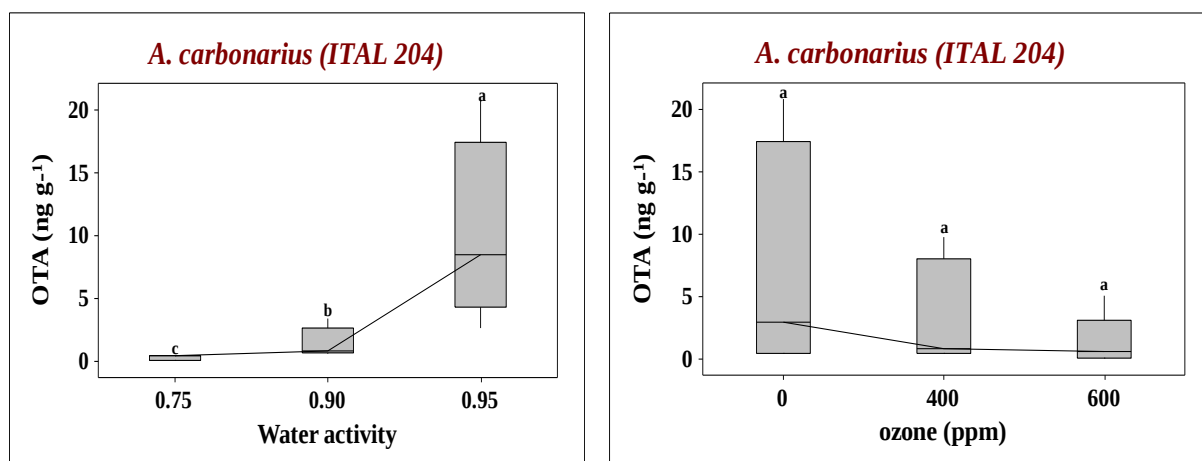


Figure IV.3.b: Box-plot analysis OTA production (ng g⁻¹) by ozone (0, 400, 600ppm) and isolated from irradiated coffee bean plus inoculated with *A. carbonarius* (ITAL 204) and adjusted to 0.75, 0.90 and 0.95 *a_w*, compared to the counts of the control after 12 days of incubation. Vertical bars indicate the standard error of the means.

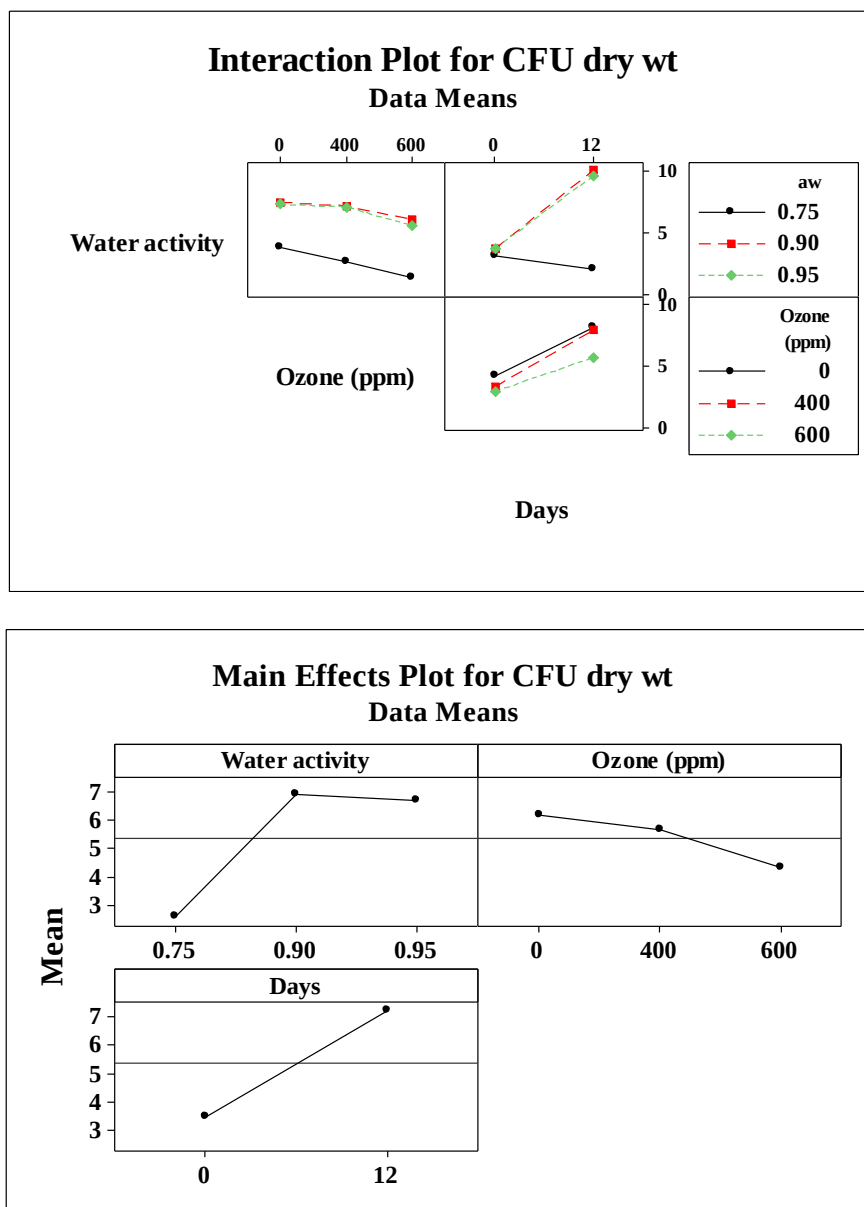


Figure IV.3.c: Main effect plot of the mean of fungal population effect by ozone (0, 400, 600ppm) and isolated from irradiated coffee bean plus inoculated with *A. westerdijkiae* (CBS121986) adjusted to 0.75, 0.90 and 0.95 a_w , compared to the counts of the control (untreated 0ppm)untreated directly after exposure (48) and 12 days of incubation.

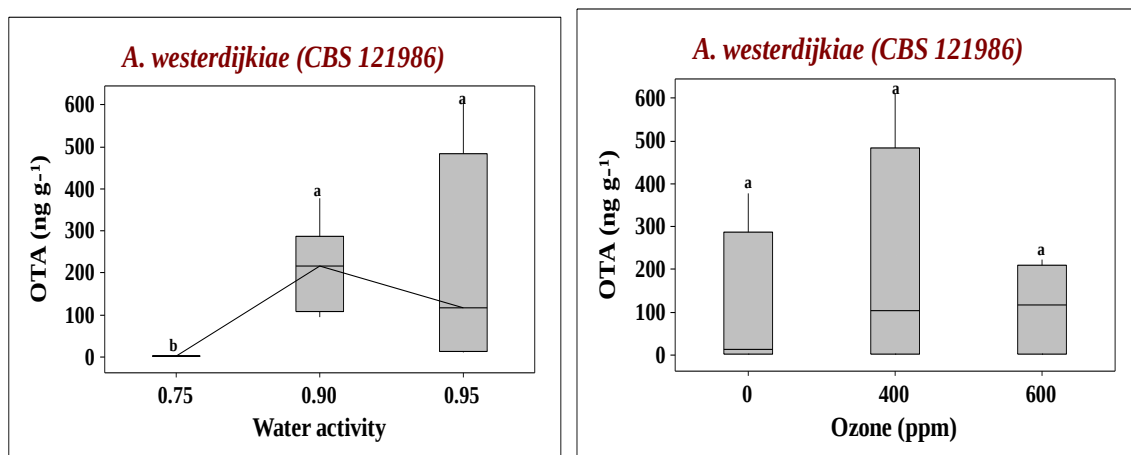


Figure IV.3.d: Box-plot analysis OTA production (ng g^{-1}) by ozone (0, 400, 600ppm) and isolated from irradiated coffee bean plus inoculated with *A. westerdijkiae* (CBS 121986) adjusted to 0.75, 0.90 and 0.95 a_w , compared to the counts of the control after 12 days of incubation. Vertical bars indicate the standard error of the means.

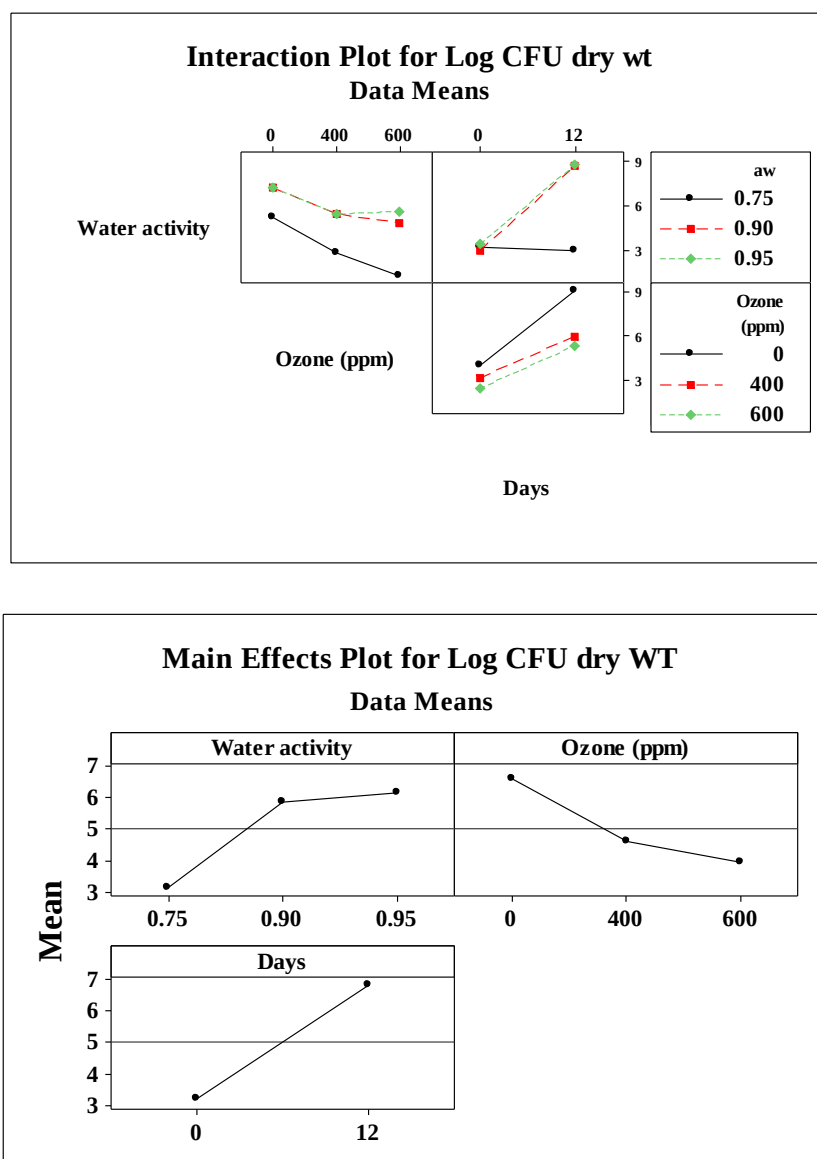


Figure IV.3.e: Main effect plot of the mean of fungal population effect by ozone (0, 400, 600ppm) and isolated from irradiated coffee bean plus inoculated with *A. ochraceus* (ITAL 14) adjusted to 0.75, 0.90 and 0.95 a_w , compared to the counts of the control (untreated 0ppm)untreated directly after exposure (48) and 12 days of incubation.

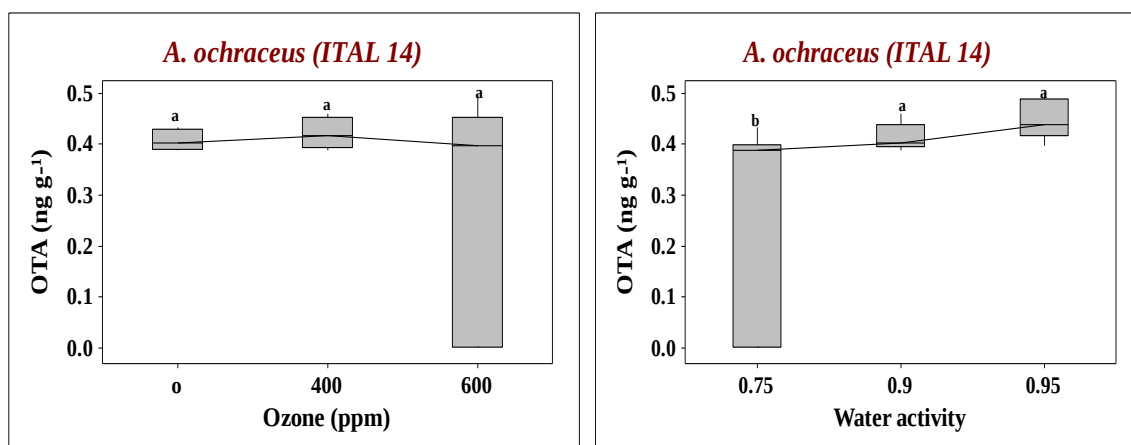


Figure IV.3.f: Box-plot analysis OTA production (ng g⁻¹) by ozone (0, 400, 600ppm) and isolated from irradiated coffee bean plus inoculated with *A. ochraceus* (ITAL 14) and *A. ochraceus* (ITAL 14) adjusted to 0.75, 0.90 and 0.95 *a_w*, compared to the counts of the control after 12 days of incubation. Vertical bars indicate the standard error of the means.

PUBLICATION

1. Publications

Akbar, A., & Magan, N. (2014). The impact of water and temperature interactions on lag phase, growth and potential ochratoxin A production by two new species, *Aspergillus aculeatinus* and *A sclerotiicarbonarius*, on a green coffee-based medium. *International Journal of Food Microbiology*, 188, 116-121.

2. Poster presentations.

Conference 1

Fungal Interactions [British Mycological Society Annual Meeting]
3–6 September 2012
Universidad de Alicante, Alicante University, Spain



Poster:

“Effect of climate change factors on growth and ochratoxin A production by *Aspergillus* sections *Nigri* and *Circumdati* strains found in coffee”

(Asya Akbar, Angel Medina and Naresh Magan)

Conference 2:

Poster:

Meetings & Forays



ANNUAL SCIENTIFIC MEETING 2013

FUNGI AND ENVIRONMENTAL CHANGE

School of Biosciences

Cardiff University

Tuesday 10th September, 2013 – Friday 13th September, 2013

“Water activity and elevated CO₂ interaction effects on the lag phase, growth and ochratoxin A production by isolates of *Aspergillus* section *Circumdati* and Section *Nigri* isolated from coffee beans”.

(Asya Akbar, Angel Medina and Naresh Magan)

It has been awarded with the second best poster award at BMS meeting, Cardiff.



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Short communication

The impact of water and temperature interactions on lag phase, growth and potential ochratoxin A production by two new species, *Aspergillus aculeatinus* and *A. sclerotii carbonarius*, on a green coffee-based medium

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ABSTRACT

Two new species of *Aspergillus* (*A. aculeatinus*, *A. sclerotii carbonarius*) were previously isolated from coffee in Thailand. The objective of this study was to examine the effect of interacting environmental factors of water availability (water activity, a_w) and temperature on lag phases prior to growth, growth and potential for ochratoxin A (OTA) production by three strains of each species on a green coffee-based medium for the first time. This showed that overall the growth of the three strains of each species was similar over the 20–37 °C and 0.85–0.99 a_w ranges. The lag phase prior to growth was <1 day at 0.95–0.98 a_w and 25–37 °C and increased to 2–3 days at marginal temperatures and a_w levels. The growth of strains of the uniseriate species *A. aculeatinus* was optimum at 0.98 a_w and 30–35 °C. For the biseriate *A. sclerotii carbonarius* strains this was 0.99 a_w and 30 °C. This species was not able to grow at 37 °C. None of the strains of the two species grew at 0.85 a_w , regardless of temperature. Integrated profiles based on the data from three strains of each species have been developed to show the optimum, maximum and marginal conditions of interacting a_w and temperature conditions for growth. None of the strains produced OTA on a green coffee-based medium. This information is important as these species are part of the mycobiota of coffee and may influence OTA contamination by other ochratoxigenic species during coffee processing.

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1. Introduction

Coffee beans (*Coffea arabica*, *Coffea canephora* var. *robusta*) are colonised by a range of mycobiota during ripening (Ilic et al., 2007; Leong et al., 2007; Noonim et al., 2008a,b; Taniwaki et al., 2003). It is known that species of the *Aspergillus* section *Circumdati* and *Aspergillus* section *Nigri* are the predominant components of the mycobiota on coffee (Astoreca et al., 2010; Batista et al., 2003; Taniwaki et al., 2003; Perrone et al., 2007). The focus has been on those species from these two sections that produce ochratoxins (OTA) for which legislative limits exist (soluble coffee, 10 µg/g; roasted/ground coffee, 5.0 µg/g; EU Commission Regulation (EC) No. 1831/2003). Indeed the water and temperature relations for growth and OTA production by strains of species of both these sections have been studied in detail (Abdel-Hadi and Magan, 2009; Belli et al., 2004; Mitchell et al., 2004; Pardo et al., 2005a).

In a survey of the mycobiota of Arabica coffee (Northern Thailand) and Robusta coffee (Southern Thailand) in Thailand two new species were discovered and classified in the *Aspergillus* section *Nigri* group (Noonim et al., 2008a). The two new species were identified and

given the names *Aspergillus aculeatinus* and *A. sclerotii carbonarius*. These were classified as uniseriate and biseriate species respectively, within the *Aspergillus* section *Nigri*. The two species were only isolated from Robusta coffee in Southern Thailand (Noonim et al., 2008b). There is practically no knowledge of the effect of environmental factors on the growth of strains of these species on coffee-based media. Samson et al. (2007) grouped a range of black *Aspergillus* species into four groups based on growth at 12 different temperature levels (6–40 °C). *A. sclerotii carbonarius* was in their Group 2 with an optimum of 33 °C and *A. aculeatinus* was in their Group 3 with an optimum of 36 °C. They both grew over the range 12–40 °C on defined media. These two species are considered to be non-toxicogenic. However, as they occupy the same niche in coffee as other mycotoxigenic species, they could compete with these and affect OTA contamination. It has been shown that an understanding of the temperature × water activity relations of such species is important as these combined factors influence interactions between different mycotoxigenic and non-mycotoxigenic fungi (Magan and Aldred, 2007; Magan et al., 2010).

The objectives of this study were to examine the effect of water activity (a_w) and temperature interactions on (a) lag phases prior to growth, (b) relative growth rates and ecological profiles on a green coffee-based medium by three strains each of *A. aculeatinus*

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and *A. sclerotiiicarbonarius* previously isolated from Thai Robusta coffee beans. We also tested whether these strains previously identified as non-OTA producers may be stimulated to produce OTA on a green coffee-based medium.

2. Material and methods

2.1. Fungal strains

The fungal strains of each species used in this study were *A. aculeatinus* (CBS 115570, CBS 121875, CBS 121872) and *A. sclerotiiicarbonarius* (CBS 121056, CBS 121851, CBS 121853). These were kindly supplied by Dr Robert A. Samson (Head, Applied and Industrial Mycology Group, CBS, Baarn, Holland).

2.2. Media, inoculation and growth measurements

The experiments were conducted on a Robusta Coffee Meal Extract Agar (CMEA). This was prepared by boiling 300 g of ground green coffee beans in 1 l of distilled water for 30 min (concentrated coffee extract). A double layer of muslin was used for filtering the resulting mixture and the volume was made up to 1 l. Technical agar No. 3 (2%), 6% concentrated coffee extract and glycerol were added (50.6, 23, 9.2, 2.7 g) to adjust the media to 0.85, 0.90, 0.95, 0.98, and 0.99 water activity (a_w) respectively. The media were autoclaved at 121 °C for 10 min and poured into 9 cm Petri plates and kept at 4 °C until used. The final a_w levels were checked with a water activity meter (Aqualab 3TE; Decagon Devices, Inc., Pullman, Washington, USA).

Inoculum of each strain was prepared by growing each strain on unmodified CMEA at 25 °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. The resulting spore suspension was used to point inoculate the treatment and replicate 9 cm Petri plates with 7 μ l of the spore suspension. Three replicates of each a_w treatment were incubated at 20, 25, 30, 35 and 37 °C for 9 days. The treatments and replicates were kept in closed polyethylene bags and the colony diameter was measured in two directions at right angles to each other every day.

2.3. Examination of potential production of OTA

To confirm that these were non-mycotoxigenic strains each was inoculated on the CMEA modified to 0.98 a_w and incubated for 9 days at 30 °C. Five plugs (4 mm diameter) from three replicates were transferred to 2 ml Eppendorf tubes and weighed. Methanol (750 μ l) was added to each Eppendorf tube. The samples were then shaken using a KS 501 digital orbital shaker for 30 min and centrifuged for 10 min at 15,000 $\times$ g. The supernatant was filtered directly into HPLC vials.

The conditions for HPLC analysis of OTA quantification were determined by using a fluorescence detector (Waters Corp., Milford, MA, USA) (λ_{exc} 333 nm, λ_{em} 460) and a C18 Column (Poroshell 120, length 100 ml, diameter 4.6 mm, particle size 2.7 μ m; 600 bar). The mobile phase was acetonitrile (57%):water (41%):acetic acid (2%) at a flow rate of 1 ml/min, run time of 12 min. The LOD and LOQ were 0.09 μ g/g and 0.32 μ g/g respectively.

2.4. Statistical analysis

A full factorial design with two (4×5) factors: water activity and temperature, was applied. Each treatment, water activity $\times$ temperature combination, was carried out in triplicate, to calculate the lag phase (prior to growth) and the growth rate (diametric growth rate, mm/day) based on the temporal increase in colony diameter over the 9 days of the experiment. Only the linear portions of the growth curves were used for obtaining the linear regression lines. Normality was checked using the Kolmogorov–Smirnov test. Analysis of data, the effects

of a_w , temperature and their interaction were examined by the Kruskal–Wallis (non-parametric) where data was not normally distributed. For normally distributed data the data sets were analysed using Minitab® 16 package (Minitab Inc., 2010, State College, PA, USA). The statistical significant level was set at $P < 0.05$ for all single and interacting treatments.

3. Results and discussion

Fig. 1 shows the effect of different temperatures on the lag times prior to growth initiation at different a_w levels for three strains of each species examined. This shows that as water stress (drier conditions) and temperature changes were imposed, the time prior to growth increased. This was particularly evident for the strains of both *A. aculeatinus* and *A. sclerotiiicarbonarius*. At marginal temperatures for growth (20 °C) and all a_w levels there was an increase in the lag times when compared to other conditions. The shortest lag times were at 0.99 $a_w \times 20$ –35 °C and the longest were at 20 °C and 0.90 a_w .

Table 1 shows that overall, statistically, there were significant ($P < 0.05$) differences between the three strains of *A. aculeatinus* in relation to lag phases prior to growth. However, there was no difference in growth rates between strains, although there were effects in relation to a_w and temperature. For *A. sclerotiiicarbonarius* lag and growth rates were similar for all three strains. However, a_w and temperature had a significant effect on strains.

Fig. 2 shows the effect of $a_w \times$ temperature interactions on the relative growth rates of each of the strains of *A. aculeatinus* and *A. sclerotiiicarbonarius* examined. Optimum growth was at 0.98–0.99 a_w and 30–35 °C. The lowest growth rate for all strains of the two species was at 0.90 a_w at 20 and 37 °C. None of the strains grew at 0.85 a_w , regardless of temperature. The strains of *A. aculeatinus* appear to be more tolerant of elevated temperatures than *A. sclerotiiicarbonarius*. Thus, at 37 °C, the strains of the latter species were unable to grow over the whole a_w range examined.

Table 2 shows the detailed statistical analyses for each strain in relation to lag phases prior to growth and growth rates in relation to the factors examined. This shows that for each individual strain there was less effect of a_w and temperature on lag phases prior to growth than on actual growth rates. The data suggest that they have similar ecological behaviour in relation to the abiotic factors of $a_w \times$ temperature with optimum conditions for growth of approximately 0.98–0.99 a_w and 30–35 °C. The main difference between the strains of uniseriate *A. aculeatinus* and the biseriate *A. sclerotiiicarbonarius* was the inability of the biseriate strains to grow at 37 °C, regardless of the a_w level. In all cases no growth was observed at 0.85 a_w suggesting limits for growth of around 0.88 a_w based on extrapolation from the study.

Previously, studies by Samson et al. (2007) had reported maximum temperature at which these strains were unable to grow as 33 °C for *A. sclerotiiicarbonarius* and about 36 °C for *A. aculeatinus*. However, these temperature maxima included mixed groups of uniseriate and biseriate species. For example, their group 3 (36 °C) included *A. aculeatinus* as well as *A. carbonarius* (biseriate) and the uniseriate species *A. japonicus* and *A. uvarum*. They also reported no growth at 5 and 9 °C, respectively, but did not examine interactions with a_w . The data should be compared with that available for other species in the *Aspergillus* section *Nigri* groups (Sanchis and Magan, 2004; Samson et al., 2007). Studies by Leong et al. (2004) found that the uniseriate *A. aculeatus* had a lower optimum temperature than the biseriate *A. carbonarius* or *A. niger*. Indeed, *A. niger* had the highest thermal tolerance of the species isolated from irrigated grapes in Australia.

There have been a number of detailed studies which have examined the optimum, minimum and maximum $a_w \times$ temperature conditions for growth of biseriate members of the *Aspergillus* section *Nigri* group (Belli et al., 2004; Mitchell et al., 2004; Natskoulis et al., 2009; Tassou et al., 2007a,b). These studies have compared a wide range of strains

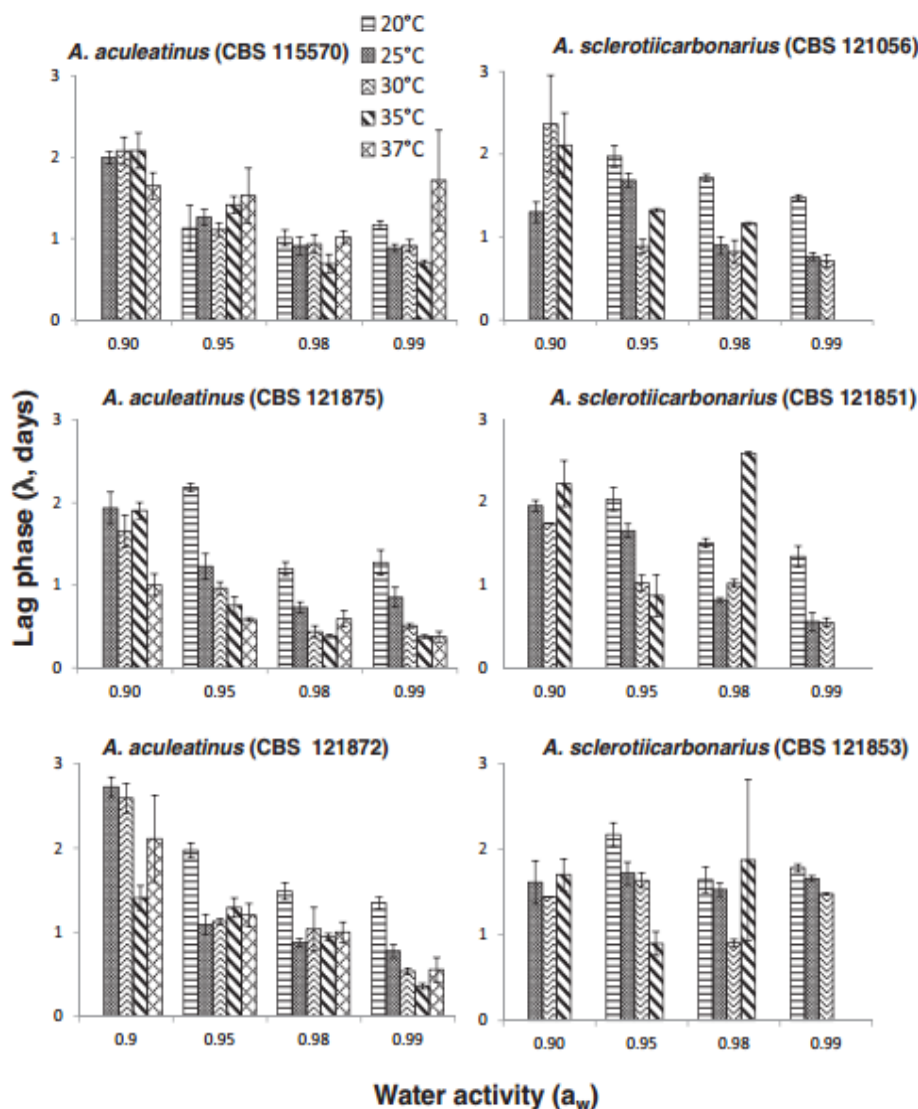


Fig. 1. Comparison of the lag phases prior to growth (in days) of three strains each of *A. aculeatinus* and *A. sclerotii carbonarius* at different $a_w \times$ temperature levels on a coffee-based medium. Bars indicate standard error of the mean.

of *A. carbonarius* and *A. niger* isolated from grapes and vine fruits from different regions of Europe. For *A. carbonarius*, Natskoulis et al. (2009) found the lowest a_w for growth at about 0.85–0.88 a_w depending on

the time of incubation. These are lower than that found by Belli et al. (2004) and Mitchell et al. (2004) but it was suggested that this could be explained by regional climatic conditions and adaptation, perhaps

Table 1

The statistical analyses of the three strains of each species examined in relation to both lag phases (λ , days) and the growth rate in relation to strain $\times$ water activity and temperature using the Kruskal–Wallis Test (non-normality data) and ANOVA (normality data). The significant factors are in bold.

Factors				
Strains	Strains	a_w	Temperature ($^{\circ}\text{C}$)	
<i>A. aculeatinus</i> (CBS 115579, CBS 121875, CBS 121872)	$P < 0.05^{*,a}$	$P < 0.05^{*,a}$	$P < 0.05^{*,a}$	Lag phase (λ , days)
	$P > 0.05^b$	$P < 0.05^{*,a}$	$P < 0.05^{*,b}$	Growth rate (mm day^{-1})
<i>A. sclerotii carbonarius</i> (CBS 121056, CBS 121851, CBS 121853)	$P > 0.05^a$	$P < 0.05^{*,a}$	$P < 0.05^{*,a}$	Lag phase (λ , days)
	$P > 0.05^a$	$P < 0.05^{*,a}$	$P < 0.05^{*,a}$	Growth rate (mm day^{-1})

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

^a Kruskal–Wallis test.

^b ANOVA.

* Significant.

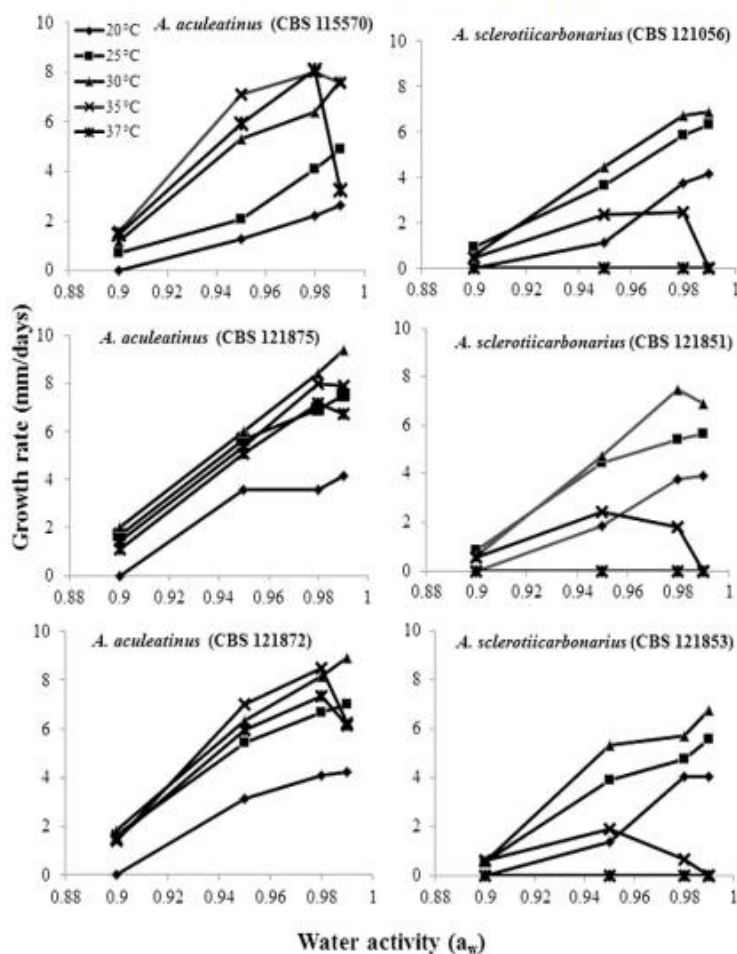


Fig. 2. Effect of different temperature $\times$ water activity on relative growth rates of three strains of *A. aculeatinus* and *A. sclerotiumcarbonarius* on a coffee-based medium.

making those from Greece more xerotolerant (Natskoulis et al., 2009). No growth was observed at 10 and 40 °C for the strains of *A. carbonarius* or *A. niger* isolated from grapes although the one exception was at 0.98

a_w /40 °C where slight growth was observed on a grape-based nutrient medium. Optimum condition for growth for strains of both these species (*A. carbonarius*, *A. niger*) was 30–35 °C at 0.98–0.99 a_w .

Table 2

P values for the growth rate and lag phases (λ , days) of 6 strains on different water activities and temperatures by using Kruskal–Wallis test (non-normality data) and ANOVA (normality data).

Factors				
Strains	a_w	$a_w \times$ Temperature	Temperature (°C)	
<i>A. aculeatinus</i> (CBS 115579)	$P > 0.05^a$	N/A	$P > 0.05^a$	Lag phase (λ , days)
	$P < 0.05^{*,a}$	N/A	$P < 0.05^{*,a}$	Growth rate (mm day $^{-1}$)
<i>A. aculeatinus</i> (CBS 121875)	$P < 0.05^{*,a}$	N/A	$P > 0.05^a$	Lag phase (λ , days)
	$P < 0.05^{*,b}$	$P < 0.05^{*,b}$	$P < 0.05^{*,b}$	Growth rate (mm day $^{-1}$)
<i>A. aculeatinus</i> (CBS 121872)	$P > 0.05^a$	N/A	$P > 0.05^a$	Lag phase (λ , days)
	$P < 0.05^{*,a}$	N/A	$P < 0.05^{*,a}$	Growth rate (mm day $^{-1}$)
<i>A. sclerotiumcarbonarius</i> (CBS 121056)	$P > 0.05^a$	N/A	$P < 0.05^{*,a}$	Lag phase (λ , days)
	$P < 0.05^{*,a}$	N/A	$P < 0.05^{*,b}$	Growth rate (mm day $^{-1}$)
<i>A. sclerotiumcarbonarius</i> (CBS 121851)	$P > 0.05^a$	N/A	$P < 0.05^{*,a}$	Lag phase (λ , days)
	$P < 0.05^{*,a}$	N/A	$P < 0.05^{*,a}$	Growth rate (mm day $^{-1}$)
<i>A. sclerotiumcarbonarius</i> (CBS 121853)	$P > 0.05^a$	N/A	$P < 0.05^{*,a}$	Lag phase (λ , days)
	$P < 0.05^{*,a}$	N/A	$P < 0.05^{*,a}$	Growth rate (mm day $^{-1}$)

P value of 0.05 or less is evidence that there is at least one significant effect in the model. N/A, not applicable.

^a Kruskal–Wallis test.

^b ANOVA.

^{*} Significant.

Interestingly, Mitchell et al. (2004) found no correlation between strains of *A. carbonarius*, growth and country of origin. In contrast, OTA production was found to be optimum at 15–25 °C and 0.95–0.98 a_w regardless of origin of species (Belli et al., 2004; Mitchell et al., 2004; Tassou et al., 2007a,b).

The other important group of *Aspergilli* which produces OTA are species in the section *Circumdati* group. The effect of $a_w \times$ temperature on growth of *A. ochraceus* was examined on green coffee extract agar (Pardo et al., 2005b). However, only 20, 25 and 30 °C were examined. Optimal conditions for growth were 0.95–0.98 a_w and 20–30 °C. Minimum a_w for growth was 0.85 a_w although they found that germination did occur at 0.80 a_w . This study did not consider higher temperatures which were shown in this study to favour the growth of the strains of these two species from the section *Nigri*.

In Fig. 3 we have integrated the data from the three strains of each species to develop $a_w \times$ temperature profiles of the maximum, optimum and minimum conditions for the growth of these two species on a green coffee-based medium. The integration of the data for three strains of each species provides an overall picture of the impact of $a_w \times$ temperature on both optimum, maximum conditions for growth and the boundary conditions based on the data ranges used for the first time. This suggests that the uniseriate species *A. aculeatinus* has a wider optimum temperature and a_w range (28–36 °C; 0.99–0.97 a_w) and grows faster over a wider temperature range than the biseriate *A. sclerotici carbonarius* (28–30 °C; 0.99–0.975 a_w).

Marginal a_w conditions for growth were similar. There is no other information on uniseriate section *Nigri* species or strains. However, comparisons can be made with that for a biseriate species such as *A. carbonarius* (predominantly OTA producers) and *A. niger* (predominantly non-OTA producers; Belli et al., 2004; Mitchell et al., 2004; Natskoulis et al., 2009). These suggest marginal conditions for

both *A. carbonarius* and *A. niger* at around 0.88–0.90 a_w with a temperature range of 15–35/37 °C.

Although these strains and species have been confirmed to be non-OTA producers on defined nutritional media (Samson et al., 2007; Nielsen et al., 2009) we were interested to examine whether growth on coffee-based media might result in biosynthesis of OTA. The present study showed that at 0.98 a_w on coffee-based media these strains did not produce any OTA, based on analyses using HPLC and the limits of detection and quantification for the method employed. Previous studies have found that the uniseriate species in section *Nigri* such as *A. aculeatinus*, *A. aculeatus*, *A. japonicus* and *A. uvarum* do not produce OTA, although they may produce other extralites. In contrast, biseriate section *Nigri* species such as *A. carbonarius* (most strains) and *A. niger* (some strains) produce OTA. Other biseriate species including *A. sclerotici carbonarius*, produce a range of other extralites (Samson et al., 2007).

In summary, this study has provided ecological data on the growth profiles of strains of *A. aculeatinus* (uniseriate) and *A. sclerotici carbonarius* (biseriate) section *Nigri* species in relation to $a_w \times$ temperature for the first time. It has also identified the optimum and marginal conditions for growth which will be useful in understanding the complex interactions which can occur between mycotoxigenic and non-mycotoxigenic *Aspergillus* species in the coffee ecosystem during cultivation and processing which may influence the level of OTA found.

Acknowledgements

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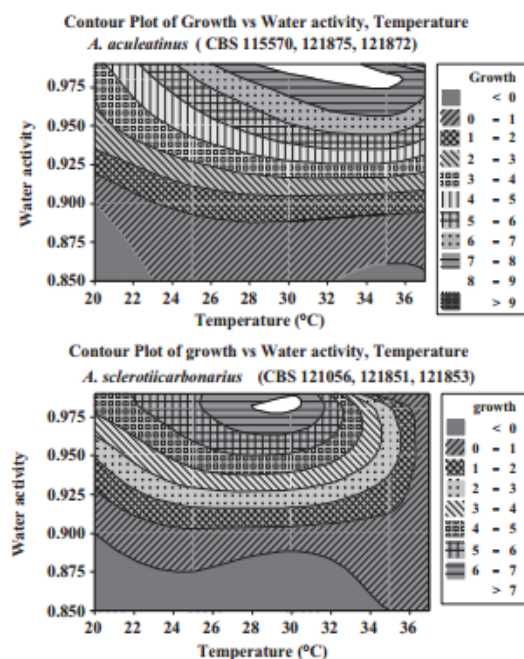


Fig. 3. Mean contour plots of the relative growth rate profiles of the combined data from three strains of each species of *A. aculeatinus* and *A. sclerotici carbonarius* in relation to water activity $\times$ temperature on a coffee-based medium.

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Cranfield Health

Effect of climate change factors on growth and ochratoxin A production by *Aspergillus* sections *Nigri* and *Circumdati* strains found in coffee

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Introduction

- There has been interest in the potential effect of climate change factors on the growth and mycotoxin production by spoilage fungi.
- This requires examination of the efficacy of combinations of the key environmental factors such as elevated CO₂ (750-1000 ppm) x elevated temperature (+3-5°C) and drought stress on growth and mycotoxin production.
- Very few studies have focused on such combination of factors to examine the relative risk of changes in mycotoxin production under such climate changes scenarios, in vitro and on food-grade commodities.
- The objective of this study was to examine the effects of CO₂ (350, 1000 ppm) and interactions with a_w and temperature on growth and OTA production by strains from the *Aspergillus* section *Nigri* and section *Circumdati* isolated in vitro and in situ on coffee-based matrices

Materials and Methods

Media: Green Coffee extract meal agar (GCMEA) was modified to 0.90, 0.95 and 0.98 water activity with glycerol.

Strains: Strains of *Aspergillus* section *Nigri* (2A3, C1/1, B2) type strains of *A. westerdijkiae* and *A. niger* were used.

Treatments, inoculation and CO₂ flushing: The treatment plates were inoculated centrally with spores and treatments/replicates of the same a_w were enclosed in a plastic chamber with a intake and exit tube for flushing with CO₂. A beaker of glycerol/water solution of the same a_w was placed in the chamber to maintain the equilibrium relative humidity (ERH). The plastic chambers were flushed every day with 5L of 1000 ppm CO₂ from a gas cylinder (British Oxygen Company, 1000 ppm CO₂ cylinder) for about 8mins and then sealed. The chambers containing treatments at each a_w level and CO₂ level were stored at 30 and 35°C.

Measurements: Growth rates were measured after 3, 6, 9, 12 days. At each measurement time the chambers were flushed with CO₂ as described earlier. The relative growth rates were compared.

Ochratoxin A (OTA) analyses: After 12 days incubation, 5 x 4mm agar plugs from the colonies were transferred to Eppendorf tubes, weighed and frozen at -20°C for analysis by HPLC.

Results

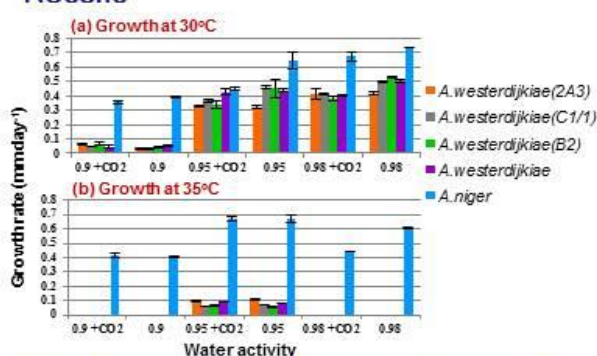


Figure 1. Growth of mycotoxigenic fungi in modified atmospheres and different water activity levels (a) 30°C and (b) 35°C.

Table 1. OTA production (ng g⁻¹) by five isolates at different water activity levels at 30 and 35°C grown for 12 days on GCMEA in air or in modified CO₂ conditions.

CO ₂ Concentration (ppm)	30° C					
	Normal CO ₂ (350 ppm)			Modified CO ₂ (1000 ppm)		
	0.9	0.95	0.98	0.9	0.95	0.98
	Mean ± S.E.			Mean ± S.E.		
<i>A. westerdijkiae</i> (2A3)	ND	ND	<LOQ(2)	ND	ND	ND
<i>A. westerdijkiae</i> (C1/1)	ND	ND	5.9±2.65(3)	ND	ND	<LOQ(2)
<i>A. westerdijkiae</i> (B2)	ND	ND	<LOQ(2)	ND	ND	7.78(1)
<i>A. westerdijkiae</i> (control)	ND	23.34(1)	<LOQ(1)	ND	ND	ND
<i>A. niger</i>	ND	ND	ND	ND	ND	ND
	35° C					
	Normal CO ₂ (350 ppm)			Modified CO ₂ (1000 ppm)		
<i>A. westerdijkiae</i> (2A3)	ND	12.5±11.7(2)	ND	ND	<LOQ(2)	ND
<i>A. westerdijkiae</i> (C1/1)	ND	<LOQ(3)	ND	ND	ND	ND
<i>A. westerdijkiae</i> (B2)	ND	<LOQ(3)	ND	ND	ND	ND
<i>A. westerdijkiae</i> (Control)	ND	<LOQ(3)	ND	ND	ND	ND
<i>A. niger</i>	ND	ND	ND	ND	ND	ND

(1) indicate number of positive samples used for calculation. Samples under <LOQ with clear peak half amount were considered (0.107ng g⁻¹). a: Standard error. ND: Nondetectable, below detectable level.

Factors	Growth rate (mm day ⁻¹)						Mean lag time (h, in days)					
	a _w × Temperature						a _w × Temperature					
	0.90+30°C	0.95+30°C	0.98+30°C	0.90+35°C	0.95+35°C	0.98+35°C	0.90+30°C	0.95+30°C	0.98+30°C	0.90+35°C	0.95+35°C	0.98+35°C
<i>A. westerdijkiae</i> (2A3)	0.43	0.03*	0.91	-	0.11	-	0.001*	0.7	0.25	-	0.13	-
<i>A. westerdijkiae</i> (C1/1)	0.58	0.8	0.0002*	-	0.006*	-	0.0027*	0.25	0.0082*	-	0.89	-
<i>A. westerdijkiae</i> (B2)	0.17	0.0503	0.0019*	-	0.0057*	-	0.0038*	0.06	0.25	-	0.0019*	-
<i>A. westerdijkiae</i> (Control)	0.58	0.8	0.0002*	-	0.0061*	-	0.0027*	0.25	0.0082*	-	0.89	-
<i>A. niger</i>	0.43	0.03*	0.09	0.201	0.11	0.0001*	0.0001*	0.7	0.54	0.1846	0.13	0.4226

Table 2. Comparison of growth rate and lag time of the five strains subject to modified CO₂ and normal conditions on GCMEA with different water activity at 30 and 35°C by using The t test and Pooled t (depend on equal and unequal variance)

Conclusions

- The present study showed a crucial role of the environmental factors such as carbon dioxide, water activity and temperature on growth rates and OTA production by the isolates examined, especially in Arabian gulf climates.
- Generally, combination of CO₂, temperature and water activity had an enhanced inhibitory effect on growth and OTA production by *A. westerdijkiae* and *A. niger* strains on green coffee media.
- Statistically exposure to 1000ppm reduced growth of *A. westerdijkiae* at 0.98 a_w at 30°C and 0.95 a_w at 35°C. The only exception was *A. niger* 0.95 a_w and 30°C.
- The lag time were extended significantly at 0.90 a_w and 30°C in the presence of elevated CO₂.
- In terms of OTA production, the amounts produced was inhibited except for the strain of *A. westerdijkiae* which produced more OTA than the other strains tested, despite growth effects being similar to other strains.
- More studies are in progress to examine the efficacy on these strains and some new OTA producing species which have been isolated from coffee.

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Water activity and elevated CO₂ interaction effects on the lag phase, growth and ochratoxin A production by isolates of *Aspergillus* section *Circumdati* and Section *Nigri* isolated from coffee beans

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Introduction

- There has been interest in the potential effect of climate change factors on the growth and mycotoxin production by spoilage fungi.
- This requires examination of the efficacy of combinations of the key environmental factors such as elevated CO₂ (3x, 1000 ppm) x water stress on growth and mycotoxin production.
- Very few studies have focused on such combination of factors to examine the relative risk of changes in mycotoxin production under such climate changes scenarios, *in vitro* and on food commodities.
- The objective of this study was to examine the effects of CO₂ (air; 1000 ppm) and interactions with a_w on lag phase prior to growth, growth and ochratoxin (OTA) production by strains from the *Aspergillus* section *Nigri* and section *Circumdati* isolated *in vitro* and *in situ* on coffee-based matrices

Materials and Methods

Media: Green Coffee extract meal agar (CMEA) was modified to 0.95, 0.98 and 0.99 water activity with glycerol.

Strains: *A. westerdijkiae* (B 2, CBS 121986), *A. niger* (A 1911), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) predominantly isolated from coffee beans were used.Treatments, inoculation and CO₂ flushing: The treatment plates were inoculated centrally with spores and treatments/replicates of the same a_w were enclosed in a plastic chamber with an intake and exit tube for flushing with CO₂. A beaker of glycerol/water solution of the same a_w was placed in the chamber to maintain the equilibrium relative humidity (ERH). The plastic chambers were flushed every day with 5L of 1000 ppm CO₂ from a gas cylinder (British Oxygen Company, 1000 ppm CO₂ cylinder) for about 8mins and then sealed. The chambers containing treatments at each a_w level and CO₂ level were stored at 30°C.Measurements: Growth rates were measured everyday. At each measurement time the chambers were flushed with CO₂ as described earlier. The relative growth rates compared.

OTA: After 9 days incubation, 5 x 4mm agar plugs from the colonies were transferred to Eppendorf tubes, weighed and frozen at -20°C for analysis by HPLC (fluorescent detector).

Results

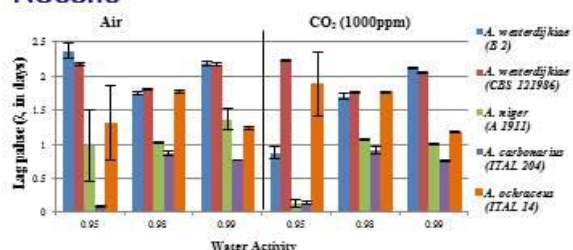


Figure 1. The mean lag time (λ , in days) for the tested strains in relation to a_w x CO₂ treatments at 30°C. Bars indicate standard error of the mean.

Table 1. Statistical analysis of effect of a_w , CO₂ and interaction by using Kruskal-Wallis Test (non normality data) and ANOVA (normality data).

Strains	Factors			Response
	a_w	CO ₂	a_w x CO ₂	
<i>A. westerdijkiae</i> (B 2)	\$	\$	NS	Lag time (λ , days)
	\$	NS	\$	Growth rate (mm day ⁻¹)
	\$	NS	N/A	OTA (ng g ⁻¹)
<i>A. westerdijkiae</i> (CBS 121986)	\$	NS	\$	Lag time (λ , days)
	\$	\$	N/A	Growth rate (mm day ⁻¹)
	\$	NS	NS	OTA (ng g ⁻¹)
<i>A. niger</i> (A 1911)	\$	NS	NS	Lag time (λ , days)
	\$	\$	N/A	Growth rate (mm day ⁻¹)
	\$	NS	NS	OTA (ng g ⁻¹)
<i>A. carbonarius</i> (ITAL 204)	\$	NS	NS	Lag time (λ , days)
	\$	\$	\$	Growth rate (mm day ⁻¹)
	\$	\$	N/A	OTA (ng g ⁻¹)
<i>A. ochraceus</i> (ITAL 14)	NS	NS	NS	Lag time (λ , days)
	NS	NS	NS	Growth rate (mm day ⁻¹)
	\$	NS	N/A	OTA (ng g ⁻¹)

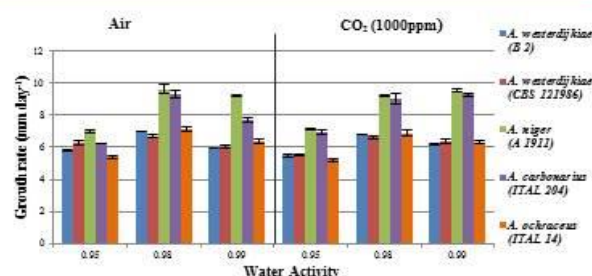


Figure 2. Comparison of the effect of a_w x CO₂ effects on growth (mm day⁻¹) of tested strains grown on CMEA for 9 days. Bars indicate standard error of the mean.

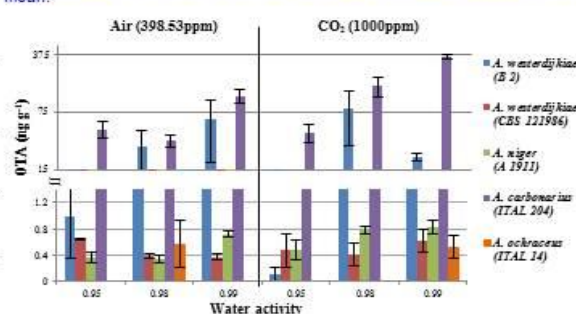


Figure 3. Comparison of the effect of a_w x CO₂ effects on OTA (ng g⁻¹) production by five strains grown on CMEA for 9 days.

Conclusions

- The present study showed the critical role of the environmental factors such as CO₂ and a_w on growth rates and OTA production by the isolates examined, especially in Arabian gulf climates.
- There was increase in the lag phase for the strains *A. westerdijkiae* (B 2), *A. niger* (A 1911) and *A. carbonarius* (ITAL 204) at 0.95 and 0.99 a_w .
- The growth rate was not affected by a_w and elevated atmosphere (CO₂) at 30°C except *A. carbonarius* (ITAL 204) in 0.99 a_w with this treatment.
- Generally, combinations of CO₂ x a_w stimulated OTA production by *A. westerdijkiae* and *A. niger* strains on CMEA.
- This study suggested that the effect of increased CO₂ (1000ppm) on lag phases, growth and OTA production varies between species.
- Studies are in progress to examine the effect of these interacting abiotic factors on OTA contamination of stored coffee beans.

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