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# Impact of interacting climate change factors on growth and ochratoxin A production by *Aspergillus* section *Circumdati* and *Nigri* species on coffee

Asya Akbar, Angel Medina and Naresh Magan

Applied Mycology Group, Environment and AgriFood Theme, Cranfield University, Cranfield, Beds. MK43 AL5, U.K.

Corresponding author: Prof. N. Magan, Applied Mycology Group, AgriFood Theme, Cranfield University, Cranfield, Beds. MK43 0AL, U.K. E.mail: n.magan@cranfield.ac.uk

Running title: Climate change factors, coffee and ochratoxin contamination

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**Abstract**

The objectives of this study were to evaluate the effect of interacting climate change (CC) factors (water stress [water activity, $a_w$; 0.99-0.90]); temperature [30, 35$^{o}$C]; and elevated $CO_2$ [400 and 1000 ppm] on (a) lag phases prior to growth, (b) growth and (c) ochratoxin A (OTA) production by species of *Aspergillus* sections *Circumdati* and *Nigri* on coffee-based media and stored coffee beans. The lag phases, prior to growth, of all strains/species were

slightly increased as $a_w$, temperature and $CO_2$ were modified. The interacting CC factors showed that most strains/species examined grew well at 30°C and slightly less so at 35°C except for *A. niger* (A 1911) which could tolerate the higher temperature. In addition, the interaction of elevated $CO_2$ (1000 ppm) + temperature (35°C) increased OTA production when compared with 30°C but only for strains of *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 stored coffee beans there was only a significant stimulation of OTA production by *A. westerdijkiae* strains in elevated $CO_2$ (1000) at 0.90 $a_w$. These results suggest differential effects of CC factors on OTA production by species in the Sections *Circumdati* and *Nigri* in stored coffee and that for most species there is a reduction in toxin production.

**1. Introduction**

It has been suggested that climate change (CC) 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 +2¬4°C, linked to an increase in atmospheric $CO_2$ from approx. 400 ppm to 700-800 or 1000¬1200 ppm (x2 or x3 increase) accompanied by episodes of heavy rainfall or periods of extreme drought (Magan et al. 2011; Medina et al. 2014; Medina et al., 2015a; Paterson & Lima, 2010; Wu et al. 2011; Botana and Sainz, 2015).

It has been suggested that CC impacts on pests and fungal diseases in terms of diversity and migration may cause significant increases in damage to crops allowing increased mycotoxigenic fungal colonisation and perhaps increased mycotoxin production (Bebber et al., 2013; 2014; Medina et al., 2015b). Recent studies by Vaughn et al. (2014) demonstrated that in elevated $CO_2$ *Fusarium verticillioides* infection of maize will increase although fumonisins would remain relatively unchanged. Vary et al. (2015) demonstrated that cultures of *Fusarium graminearum*, when repeatedly cultured under elevated $CO_2$ conditions, become tolerant of CC factors and when inoculated onto ripening wheat ears results in increased *Fusarium* head blight. This may potentially lead to higher deoxynivalenol (DON) contamination of harvested grain, although this was not tested. Studies also suggest

that the risks from DON contamination will be influenced by CC factors (Skelsey and Newton, 2015; Van Der Fels-Klerx et al., 2012; Botana and Sainz, 2015).

A recent study by Medina et al. (2015a) examined the effect of CC interacting factors on growth of *Aspergillus flavus* and aflatoxin $B_1$ ($AFB_1$) production. This suggested that, while growth was not significantly affected, $AFB_1$ production was significantly stimulated when exposed to these combined CC factors. No other detailed studies have been carried out with mycotoxigenic fungi to identify the impact that CC factors may have on colonisation and mycotoxin contamination of different commodities. Coffee is an economically important export crop from many tropical/sub-tropical regions of the world. There have been concerns of the possible impact on CC factors on coffee production and concomitant OTA contamination because of the legislative limits which exist in many importing countries. Thus, there was a need to examine the key species involved in contamination of coffee with OTA and to evaluate how CC factors may impact on the growth and OTA production by members of the *Aspergillus* sections *Circumdati* and *Nigri* which are responsible for toxin contamination.

The objective of this study was to determine the impact of interacting CC factors of $CO_2$ (400ppm vs. 1000ppm), water stress (0.99, 0.98, 0.95 water activity, $a_w$) and temperature (30, 35°C) on (a) *in vitro* effects on the lag phases (λ, days) prior to growth, growth and OTA production on a coffee-based medium for 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) and (b) determine the effect of CC interacting factors on OTA production *in situ* by *A. westerdijkiae* (B 2, CBS 121986), *A. niger* (A 1911), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14).

## 2. Material and methods

### Fungal strains

*Aspergillus* section *Circumdati* strains: Two strains of *A. westerdijkiae* (B2 isolated from green coffee; and a type strain CBS 121986), one strain of *A. steynii* (CBS 112814) and a strain of *A. ochraceus* (ITAL 14) isolated from Arabica coffee beans (kindly provided by Dr. B. Patino, Complutense University, Madrid, Spain and Dr M. Taniwaki, ITAL, Campinas, Brazil).

These strains are all kept in their respective Culture Collections and the identification was confirmed by comparison with type strains.

*Aspergillus* section *Nigri* strains: One strain of *Aspergillus niger* (A 1911), kindly provided by Prof. Paola Battilani (Università Cattolica del Sacro Cuore, Italy), and one strain of *A. carbonarius* (ITAL 204) both isolated from Arabica coffee were used. The latter was also supplied by Dr M. Taniwaki.

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

#### *Media preparation, inoculation and growth measurements*

The experiments were conducted on a Robusta Coffee Meal Extract Agar (CMEA). This was prepared by boiling 300g of ground green coffee beans in 1L of distilled water for 30min (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%), 20% concentrated coffee extract and glycerol were added (23, 9.2, 2.7 g) to adjust the media to 0.95, 0.98, and 0.99 water activity ($a_w$) respectively. The media were autoclaved at 121ºC for 10 min and poured into 9 cm sterile Petri plates and kept at 4ºC 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 colonies on unmodified CMEA at 25°C for 7 days. Spore suspensions were prepared by rubbing 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 7-10 µL of the spore suspension. The replicates of the same treatment were enclosed together in plastic environmental chambers.

The cultures with the same $a_w$ were enclosed together in the environmental chambers containing valves at each end: one for $CO_2$ intake and the other for exit. Two 500 ml beakers of glycerol/water solution of the same $a_w$ as the treatment were included in the chamber to maintain the equilibrium relative humidity (ERH).

The chambers were flushed with 5 L of 1000 ppm $CO_2$ from a gas cylinder (British Oxygen Company, 1000 ppm $CO_2$ cylinder) for about 10 minutes and then the valves sealed as detailed previously (Medina et al., 2015a). The same procedure was carried out for the control treatment (400 ppm) with air from a BOC cylinder. The environmental chambers were incubated for 10 days at 30 and 35°C and fungal growth rates measured every two days. At the end of this period the cultures were removed for OTA analysis. Growth of the colonies was measured in two directions at right angles to each other every two days. Immediately after measurement the environmental chambers were flushed with the treatment $CO_2$ for 10 mins and then the valves at each end sealed and incubated at the treatment temperature.

The change in the colony radius (mm) vs time (days) for each replicate of each strain under the different treatment 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 square of the linear correlation coefficients was ≥0.98.

**In vitro *effect on OTA production***

Five plugs (4mm diameter) from three replicates of each strain and treatment were transferred using a sterile cork borer to 2ml Eppendorf tubes and weighed. 750µl 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 15000xg. The supernatant was filtered directly into HPLC vials.

The conditions for OTA quantification using HPLC and a fluorescence detector were by using a Waters system (Waters Corp., Milford, MA, USA; λexc 333nm, λem 460) and a C18 Column (Poroshell 120, length 100ml, diameter 4.6 mm, particle size 2.7 micron; 600 Bar). The mobile phase was acetonitrile (57%):water (41%):acetic acid (2%) at a flow rate of 1ml $min^{-1}$, with a run time of 12 mins. The LOD and LOQ were 0.09 ng/g and 0.32 ng/g, respectively. The methodology used is detailed in Sultan et al. (2014).

## *In situ* effect of interacting climate change factors on OTA production by the Aspergillus species in stored coffee

### *Coffee moisture adsorption curves*

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

A moisture adsorption curve 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 the addition of known amounts of water to 10 g green coffee bean sub-samples and stored at 4°C for 24 hr to allow water adsorption. The samples were then removed and after equilibration at 25$^{o}$C the $a_w$ of the hydrated green coffee beans measured using an Aqualab 3TE (Decagon Devices Inc., Pullman, Washington, U.S.A). The coffee bean samples were then dried at 110°C for 24 hr and kept in a desiccator at room temperature for 1 hr and then weighed to determine the moisture content. Subsequently 325 g of irradiated raw coffee was weighed and water added using the adsorption curve to obtain the required target aw levels and kept at 4°C for 24 hr for equilibration. The coffee beans were then divided into six sub-samples (50 g) in solid substrate culture vessels (Magenta, Sigma Ltd, UK) which had permeable microporous membrane lids.

### *Inoculation of coffee treatments*

Five OTA producing species including strains of *A. westerdijkiae* (B 2, CBS 121986), *A. niger* (A 1911), *A. carbonarius* (ITAL 204) and *A. ochraceus* (ITAL 14) previously isolated from coffee beans were cultured on CMEA at 25°C for 7 days, and spore suspensions prepared as detailed previously. The concentration was adjusted by dilution to ~$10^4$ spores/ml and confirmed using a haemocytometer. Using the methodology of Palacios-Cabrera et al. (2004), 0.5 ml of spore suspension ($10^4$ CFUs/ml) of each strain 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 placed in the environmental chambers. The methodology was the same as that used for the *in vitro* studies for $CO_2$ flushing of either air (400 ppm) or 1000 ppm $CO_2$. The inoculated coffee beans 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 quantification. In these *in situ* studies *A. steynii* was not included as *A. westerdijkiae* was considered to be the most important species in coffee, followed by *A. ochraceus*.

***Ochratoxin A extraction and quantification***

Clean up, extraction and quantification of OTA from the *in situ* CC experiments. Initial clean-up was done using Neogen immunoaffinity columns (Neogen, Neocolumn method). Ten grams of milled coffee beans were extracted with 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). 1.5 ml was dried and 0.5 ml of acetonitrile:water (50:50) added. The final extracts were analysed by HPLC as detailed previously. The retention time of OTA under the conditions described was approximately 2.5 min. The mobile phases used were acetonitrile (57%): acetic acid (2%): water (41%) (Sultan et al., 2014).

**2.4 Statistical analysis**

A full factorial design with three factors: water activity, temperature and $CO_2$ was applied. Each treatment, $a_w$ x temperature x $CO_2$ combination was carried out in triplicate, both for growth rate assessment and OTA production and repeated once. Normality was checked using the Kolmogorov-Simonov test. Analysis of data, the effects of $a_w$, temperature, $CO_2$ 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 the 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. Results**

***In vitro* effect of interacting Climate Change factors on lag times before growth, growth and ochratoxin A (OTA) production by species/strains of *Aspergillus* sections *Circumdati* and *Nigri*.**

***Effects on lag phases prior to growth and relative growth rates of strains/species***

Figures 1 and 2 compares the lag phases (λ, days) prior to growth and relative growth rates of all the six strains examined. Overall, there was an increase in the lag phases for all species/strain when water stress was imposed, especially at 30$^{O}$C. There were some differences in the lag phases specially for *A. westerdijkiae* (CBS 121986) and *A. carbonarius* (ITAL 204), with an increase when exposed to 0.95 $a_w$ and 1000 ppm $CO_2$ compared to the control (see Figures 2b). Overall, water stress impacted most significantly on the increases in lag phases prior to growth (Table 1). The $CO_2$ alone was not statistically significant for lag phases, but $CO_2$ x temperature was significant for *A. carbonarius*, *A. ochraceus* and *A. steynii.* Temperature alone also significantly impacted on lag phases prior to growth (see Table 1).

Figure 1 and 2 also compare the growth rates for the different strains when exposed to air and elevated $CO_2$ (x 2.5 existing levels), at the different $a_w$ x temperature treatments. The optimum growth for most of the strains examined was at about 0.98 $a_w$ and then 0.99 $a_w$, with the slowest at 0.95 $a_w$. The only exception was *A. steynii* (CBS 112814) where optimum was at 0.95 $a_w$, then 0.98 $a_w$ and the lowest growth 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. When the temperature was elevated to 35°C, growth was generally similar to what was observed at 30$^{O}$C. Maximum growth rates and tolerance of 35°C were observed for *A. niger* (A 1911), while the slowest growth was observed in *A. westerdijkiae* (B 2, CBS 121986) strains at all $a_w$ levels when compared to the same conditions at 30°C.

The statistical analyses for impacts on growth of the strains/species are shown in Table 1. Growth was significantly ($P<0.05$) affected by elevated $CO_2$ at 30ºC and 0.95 and 0.99 $a_w$ for strains of *A. westerdijkiae* (B 2; CBS 121986) at all $a_w$ levels. At 35°C, the effect of $a_w$ was statistically significant for most strains. Indeed, growth of *A. niger* (A 1911) and *A. steynii* (ITAL 14) was significantly stimulated by $CO_2$ (1000 ppm). The effect of the various factors and their interactions were more significant at 35°C than at 30°C.

**In vitro** ***effects of interacting Climate Change factors on ochratoxin A (OTA) production***

Figure 3 and 4 show the comparison between existing and future interacting CC factors on OTA production in the *in vitro* assays. For most strains examined, there was no effect of elevated $CO_2$ on OTA production at 30°C. For both *A. westerdijkiae* strains (B 2, CBS 121986) and *A. steynii* (CBS 112814 strain), there was a significant increase in OTA production at $35^{o}$C/1000 ppm $CO_2$ at 0.95-0.99 $a_w$. For other strains/species there was often a reduction in OTA production. The highest OTA production was by *A. westerdijkiae* (B 2) and the lowest by *A. niger* (A 1911 at 0.95 $a_w$). Statistically, the effect of these interacting CC factors, are shown in Table 2.

***In situ* effect of interacting Climate Change conditions on ochratoxin A (OTA) production in stored coffee**

The treatment effects on OTA production by five of the different strains/species colonising stored coffee when exposed to 400 or 1000 ppm $CO_2$ at different $a_w$ levels (90, 0.95 and 0.97) at 30 and 35°C is shown in Figures 5 and 6. The highest OTA production was achieved by *A. westerdijkiae* (B 2, CBS 121986) when compared to other strains at 0.90 and 0.95 $a_w$ in elevated $CO_2$ (1000 ppm) at 30°C (Figure 5a). The exposure to elevated $CO_2$ concentrations (1000 ppm) at 30°C resulted in some stimulation of OTA production by these strains/species after 12 days storage. In some cases stimulation occurred under water stress (0.90 $a_w$) when combined with elevated $CO_2$. For example, *A. westerdijkiae* strains (B 2; CBS 121986) produced significantly higher OTA amounts in elevated $CO_2$ in contrast to that at 400 ppm. For most of the species/strains tested, when exposed to 35°C compared to 30°C, OTA production was reduced at different $a_w$ levels and elevated $CO_2$ (1000 ppm). However, for *A. westerdijkiae* (B 2), OTA production was increased significantly (x 60 times) by the combination of CC factors. Similar increases were found for the CBS 121986 strain of the same species at 0.90 and 0.95 $a_w$.

Table 3 shows the statistical analyses of the effect of single and interacting factors on OTA production. For the two *A. westerdijkiae* (B 2, CBS 121986) strains there was a significant increase ($P=0.05$) in OTA contamination in stored coffee in elevated $CO_2$ at 0.90 and 0.97 $a_w$, and for *A. niger* (A 1911) at 30°C. $A_w$ and $CO_2$ 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 $CO_2$ on OTA production by *A. carbonarius* (ITAL 204). There was a significant effect of temperature, at 30°C and 35°C, on OTA production for all strains except *A. carbonarius* (ITAL 204).

**4. Discussion and Conclusions**

The present study has shown that interacting CC factors can influence lag phases prior to growth, growth rates and for some species, OTA production. This may be particularly important in the Arabian Gulf climates since coffee beans have to be imported from producer countries. However, the coffee beans may be stored under humidity conditions that may enhance coffee spoilage. There have been practically no studies on the three-way interactions of these important CC environmental factors on mycotoxigenic fungi, and much less so on mycotoxigenic species and contamination of coffee (Medina et al., 2015b; Paterson et al., 2014). The present studies suggest that approx. 2.5 times existing $CO_2$ levels together with drought stress and increased temperature may enhance OTA production by 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 coffee-based media. Overall, the *in vitro* studies showed that there was less effect on lag phases prior to growth, and growth while there was a significant effect on OTA production ($p=0.05$) by some species. However, the effect does vary between species and strains.

The strains tested in this study grew well at 30°C, and slightly less so at 35°C except for *A. niger* (A1911). This strain appeared to be more tolerant of these higher temperatures. Also, the interaction of elevated $CO_2$ (1000 ppm) + 35°C only increased OTA production when compared with the controls at 30°C + existing $CO_2$ concentrations for strains of *A. westerdijkiae* (B 2), *A. ochraceus* (ITAL 14) and *A. steynii* (CBS 112814). Most strains had optimum growth at 0.95 $a_w$ and 35°C, while at 30°C, the optimum was at 0.98 $a_w$. Most studies to control species from these two *Aspergillus* Sections have been in relation to modified atmosphere storage where very high concentrations of $CO_2$ have been used (15-

75% CO2; Paster et al., 1983; Paterkai et al., 2007; Cairns-Fuller et al., 2008; Valero et al., 2008).

Experimental studies on the impact of CC factors on mycotoxin production are scarce. The study by Medina et al. (2015a) examined *A. flavus* growth and aflatoxin $B_1$ ($AFB_1$) production under CC conditions including 650 and 1000 ppm $CO_2$, drought stress and +4°C temperature above optimum conditions. While growth was unaffected at 37°C regardless of $CO_2$ concentration and water availability, there was a significant stimulation of $AFB_1$ production (e.g. 0.92 and 0.95 $a_w$ x 37°C and 650 or 1000 ppm $CO_2$). Molecular analyses of biosynthetic genes involved in $AFB_1$ production confirmed the mycotoxin results. This suggests that the three-way interacting factors involved in CC may represent physiological stress on the fungus which results in a stimulation of secondary metabolite production as a defence response (Magan & Aldred, 2007).

The impact of these 3-way interacting CC factors on OTA production by strains of Sections *Circumdati* and *Nigri* on stored coffee beans have not been examined previously. CC factors had a significant impact on OTA production by *A. westerdijkiae* strains. This stimulation was similar to that observed with *A. flavus* (Medina et al., 2015a). Although growth was not significantly affected, studies with *Fusarium graminearum* and *F. verticillioides* suggest that CC factors may modify the patterns of growth (Medina et al., 2015b). However, for other species, especially *A. carbonarius* and *A. niger,* there was a reduction in OTA production under CC conditions. This suggests that the impacts of CC factors will differ between species, especially in terms of mycotoxin production. Paterson et al. (2014) suggested that Robusta coffee, grown mainly at lower altitudes, may be able to tolerate CC factors better than Arabica which is grown at higher altitudes and may be more sensitive to such interacting stresses. They emphasised the importance of obtaining knowledge on the impact of CC factors on both pests and diseases of coffee because of the impacts, especially in Ethiopia, where Arabica in grown. Increases in specific pests may lead to an increase in infection with mycotoxigenic species because of the increased level of damage of the ripening coffee beans. Indeed they postulated as to whether CC scenarios may lead to other more competitive toxigenic species such as *A. flavus* or *F. verticillioides* becoming more important

at elevated temperatures, resulting in aflatoxins or fumonisin contamination becoming more important (Paterson et al., 2014).

It is interesting to note recent studies on the impact of CC factors on infection of wheat and maize by *F. graminearum* and *F. verticillioides*, respectively (Vaughan et al., 2014; Vary et al., 2015). In the former case, acclimatisation of cultures for 10-20 generations in CC conditions prior to infection of wheat exposed to CC conditions resulted in a significant increase in Fusarium Head Blight, although mycotoxin production was not determined. For maize grown in CC conditions there was an increase in fungal biomass of the pathogen but no increase in fumonisin contamination. This suggests that different mycotoxigenic fungi may respond differently to CC conditions and thus extrapolation needs to be done with care.

In conclusion, both in *in vitro* and *in situ* exposure of species of *Aspergillus* Sections *Circumdati* and *Nigri* to CC factors resulted in changes in either lag phases prior to growth, some effects on growth and on OTA production. There were differential effects on mycotoxigenic species and on OTA production. In this study, OTA production was increased in vitro for strains of *A. westerdijkiae* and *A. steynii* on coffee-based media. In stored coffee, *A. westerdijkiae* (35$^{o}$C) and *A. niger* (30$^{o}$C) produced higher amounts of OTA under CC conditions. For other strains/species there was no change or a decrease in OTA production. The present studies were carried out on stored harvested coffee beans. However, CC conditions will alter the physiology of growth and production of coffee beans which may alter the interaction with mycotoxigenic fungi (Paterson et al., 2014; Medina et al., 2015b). Other aspects such as exposure to fluxes of UV radiation may also need to be taken into account (Garcia-Cela et al., 2015). In addition, the possible impact of acclimatisation of these strains/species of toxigenic fungi to CC conditions needs to be also examined in terms of whether this will influence colonisation rates of important commodities and whether this may lead to enhanced or reduced mycotoxin contamination.

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**Table** 1**:** Summary statistical results for lag phase (days) and growth rate of six ochratoxigenic *Aspergillus* strains in relation to $CO_2$, water activity ($a_w$) and $CO_2$ x $a_w$ at 30 and 35$^{o}$C using the Kruskal-Wallis Test (non-normality data).

| **Temperature 30°C Factors** | | | | |
|---|---|---|---|---|
| **Strains** | **$CO_2$ (1000ppm)** | **$a_w$** | **$CO_2$ X $a_w$** | **Response** |
| ***A. westerdijkiae* (B 2)** | NS | NS | NS | Lag time (λ, days) |
| | NS | S | NS | Growth rate (mm day$^{-1}$) |
| ***A. westerdijkiae* (CBS 121986)** | NS | S | NS | Lag time (λ, days) |
| | NS | NS | S | Growth rate (mm day$^{-1}$) |
| ***A. niger* (A 1911)** | NS | NS | NS | Lag time (λ, days) |
| | NS | S | S | Growth rate (mm day$^{-1}$) |
| ***A. carbonarius* (ITAL 204)** | NS | S | S | Lag time (λ, days) |
| | NS | S | S | Growth rate (mm day$^{-1}$) |
| ***A. ochraceus* (ITAL 14)** | NS | NS | NS | Lag time (λ, days) |
| | NS | NS | NS | Growth rate (mm day$^{-1}$) |
| ***A. steynii* (CBS 112814)** | NS | NS | NS | Lag time (λ, days) |
| | NS | S | NS | Growth rate (mm day$^{-1}$) |
| **Temperature 35 °C** | | | | |
| **Strains** | **$CO_2$ (1000ppm)** | **$a_w$** | **$CO_2$ X $a_w$** | **Response** |
| ***A. westerdijkiae* (B 2)** | NS | NS | NS | Lag time (λ, days) |
| | NS | S | NS | Growth rate (mm day$^{-1}$) |
| ***A. westerdijkiae* (CBS 121986)** | NS | S | NS | Lag time (λ, days) |
| | NS | S | NS | Growth rate (mm day$^{-1}$) |
| ***A. niger* (A 1911)** | S | NS | S | Lag time (λ, days) |
| | S | S | NS | Growth rate (mm day$^{-1}$) |
| ***A. carbonarius* (ITAL 204)** | S | S | S | Lag time (λ, days) |
| | NS[a] | S | S | Growth rate (mm day$^{-1}$) |
| ***A. ochraceus* (ITAL 14)** | S | S | S | Lag time (λ, days) |
| | NS | S | S | Growth rate (mm day$^{-1}$) |
| ***A. steynii* (CBS 112814)** | S | S | S | Lag time (λ, days) |
| | S | S | NS | Growth rate (mm day$^{-1}$) |
| **Temp 30 and 35°C** | | | | |
| | **$CO_2$ (1000ppm)** | **$a_w$** | **Temp 30+35°C** | |
| ***A. westerdijkiae* (B 2)** | NS | NS | S | Lag time (λ, days) |
| | NS | NS | S | Growth rate (mm day$^{-1}$) |
| ***A. westerdijkiae* (CBS 121986)** | NS | S | NS | Lag time (λ, days) |
| | NS | S | S | Growth rate (mm day$^{-1}$) |
| ***A. niger* (A 1911)** | NS | NS | S | Lag time (λ, days) |
| | NS | S | S | Growth rate (mm day$^{-1}$) |
| ***A. carbonarius* (ITAL 204)** | NS | NS | S | Lag time (λ, days) |
| | NS | S | S | Growth rate (mm day$^{-1}$) |
| ***A. ochraceus* (ITAL 14)** | NS | NS | S | Lag time (λ, days) |
| | NS | NS | S | Growth rate (mm day$^{-1}$) |
| ***A. steynii* (CBS 112814)** | S | S | NS | Lag time (λ, days) |
| | NS | NS | S | 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.
NS not significant
S significant

Table 2. Summary of statistical results for ochratoxin A production by six ochratoxigenic *Aspergillus* strains at different water activity ($a_w$) x temperature x $CO_2$ data, using the Kruskal-Wallis Test (non-normality analyses) in the in vitro assays on a coffee-based medium.

| | **Temperature 30°C** | | |
|---|---|---|---|
| | **Factors** | | |
| **Strains** | **$CO_2$ (1000ppm)** | **$a_w$** | |
| ***A. westerdijkiae* (B 2)** | **S** | **S** | |
| ***A. westerdijkiae* (CBS 121986)** | NS | **S** | |
| ***A. niger* (A 1911)** | NS | **S** | |
| ***A. carbonarius* (ITAL 204)** | NS | **S** | |
| ***A. ochraceus* (ITAL 14)** | NS | NS | |
| ***A. steynii* (CBS 112814)** | **S** | **S** | |
| | **Temperature 35°C** | | |
| ***A. westerdijkiae* (B 2)** | **S** | **S** | |
| ***A. westerdijkiae* (CBS 121986)** | **S** | **S** | |
| ***A. niger* (A 1911)** | **S** | NS | |
| ***A. carbonarius* (ITAL 204)** | **NS** | **S** | |
| ***A. ochraceus* (ITAL 14)** | S | NS | |
| ***A. steynii* (CBS 112814)** | **S** | NS | |
| | **Temperature 30 and 35°C** | | |
| | **$CO_2$ (1000ppm)** | **$a_w$** | **Temp 30+35** |
| ***A. westerdijkiae* (B 2)** | **S** | **S** | S |
| ***A. westerdijkiae* (CBS 121986)** | **S** | **S** | S |
| ***A. niger* (A 1911)** | NS | NS | S |
| ***A. carbonarius* (ITAL 204)** | **S** | **S** | S |
| ***A. ochraceus* (ITAL 14)** | NS | NS | S |
| ***A. steynii* (CBS 112814)** | **S** | **S** | S |

*S,*significant (*P*<0.05)

NS, not significant (*P*>0.05)

**Table 3:** Summary statistical effects of climate change treatments on ochratoxin A production by *Aspergillus* section Circumdati and Nigri species/strains at different water activity ($a_w$) and temperatures in stored coffee beans using the Kruskal-Wallis Test (non-normality data).

| | **Temperature 30°C** | |
|---|---|---|
| | Factors | |
| **Strains** | **$CO_2$** | **$a_w$** |
| ***A. westerdijkiae* (B 2)** | **NS** | **NS** |
| ***A. westerdijkiae* (CBS 121986)** | **S** | NS |
| ***A. niger* (A 1911)** | **NS** | **S** |
| ***A. carbonarius* (ITAL 204)** | NS | **S** |
| ***A. ochraceus* (ITAL 14)** | NS | **S** |
| | **Temperature 35°C** | |
| ***A. westerdijkiae* (B 2)** | **S** | **S** |
| ***A. westerdijkiae* (CBS 121986)** | **S** | NS |
| ***A. niger* (A 1911)** | NS | **S** |
| ***A. carbonarius* (ITAL 204)** | NS | **S** |
| ***A. ochraceus* (ITAL 14)** | NS | **S** |

| | **Temperature 30+35°C** | | |
|---|---|---|---|
| | **$CO_2$ (1000ppm)** | **$a_w$** | **Temp 30+35°C** |
| ***A. westerdijkiae* (B 2)** | NS | NS | S |
| ***A. westerdijkiae* (CBS 121986)** | **S** | NS | S |
| ***A. niger* (A 1911)** | NS | NS | S |
| ***A. carbonarius* (ITAL 204)** | NS | **S** | NS |
| ***A. ochraceus* (ITAL 14)** | NS | NS | S |

*S*,significant ($P$<0.05)
NS, not significant ($P$>0.05)

**Figure legends**

Figure 1. Comparison of the effect of water activity ($a_w$) x $CO_2$ x temperature on (i) the mean lag time (λ, in days) prior to growth, and (ii) growth rate (mm/day) of (a-d) *A. westerdijkiae* (strains B 2, CBS 121986), (e-f) of *A. niger* (strain A1911) on a coffee-based medium at 30 and 35°C. Bars indicate standard error of the mean.

Figure 2. Comparison of the effect of water activity ($a_w$) x $CO_2$ x temperature on (i) the mean lag time (λ, in days) prior to growth, and (ii) growth rate (mm/day) of (a-b) *A. carbonarius* (strain ITAL 204), (c-d) *A. ochraceus* (strain ITAL 14) and (e-f) *A. steynii* (strain CBS 112814) on a coffee-based medium at 30 and 35°C. Bars indicate standard error of the mean.

Figure 3. *In vitro* effect of water activity ($a_w$) x $CO_2$ on ochratoxin A (OTA) production by (a-d) strains of *A. westerdijkiae* (B 2, CBS 121986) and (e-f) *A. niger* (strain A1911) on a coffee-based medium at 30 and 35°C. Bars indicate standard error of the mean. Note: scale ranges are different for OTA production.

Figure 4. *In vitro* effect of water activity ($a_w$) x $CO_2$ on ochratoxin A (OTA) production by (a-b) *A. carbonarius* (strain ITAL 204), (c-d) *A. ochraceus* (strain ITAL 14) and (e-f) *A. steynii* (strain CBS 112814) grown on a coffee-based medium at 30 and 35°C. Bars indicate standard error of the mean. Note: scale ranges are different for OTA production.

Figure 5. *In situ* effect of water activity ($a_w$) x $CO_2$ x temperature effects on ochratoxin A (OTA) production by strains of *A. westerdijkiae* (a, b: B 2; c, d: CBS 121986) grown on stored coffee beans. Bars indicate standard error of the mean. Note: scale ranges are different for OTA product.

Figure 6. Effect of water activity ($a_w$) x $CO_2$ x temperature on ochratoxin A (OTA) production by *A. niger* (a, b: strain A1911), *A. carbonarius* (c, d: strain ITAL 204) and *A. ochraceus* (e, f: strain ITAL 14) grown on stored coffee beans. Bars indicate standard error of the mean. Note: scale ranges are different for OTA product

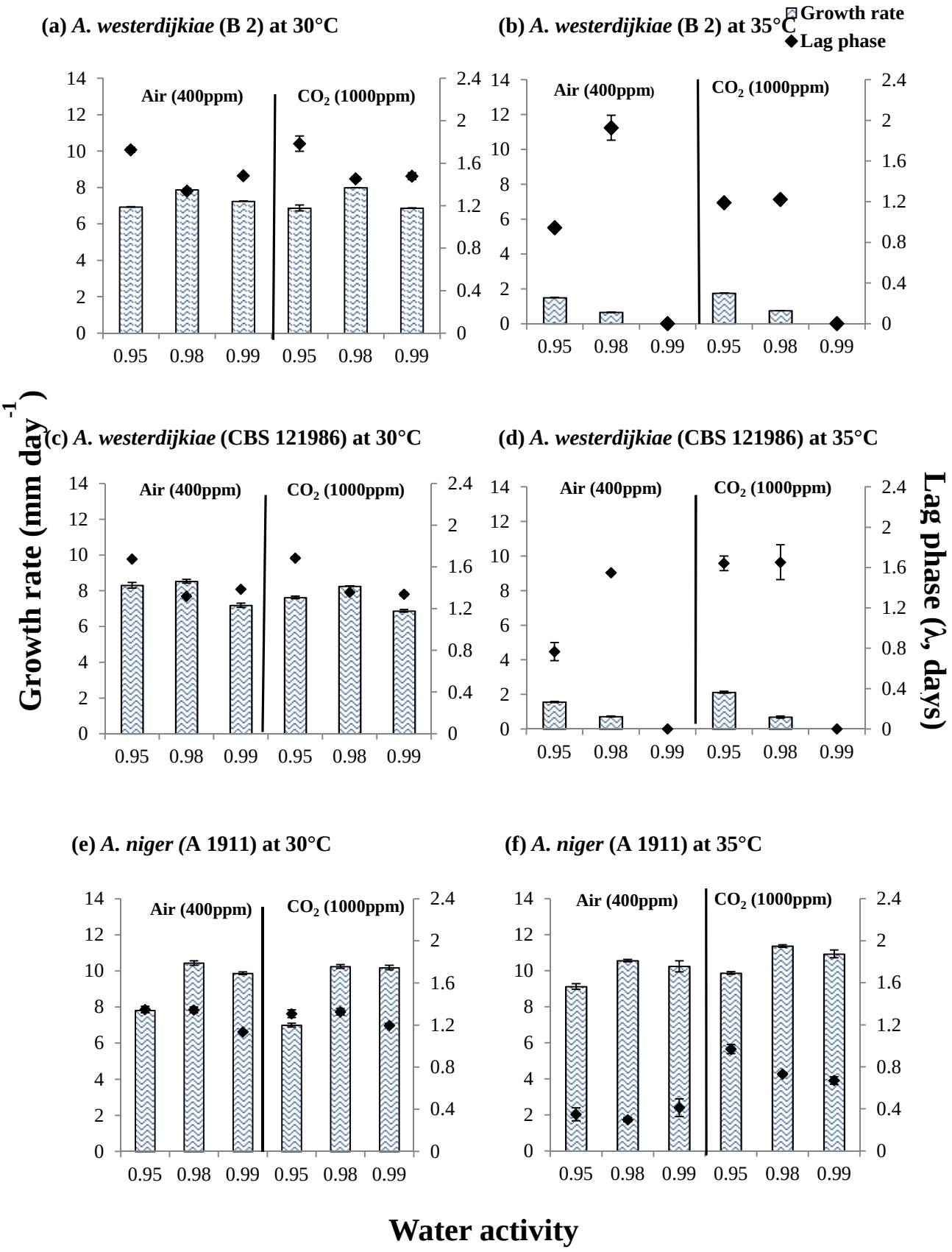


Figure 1. Akbar, Medina and Magan

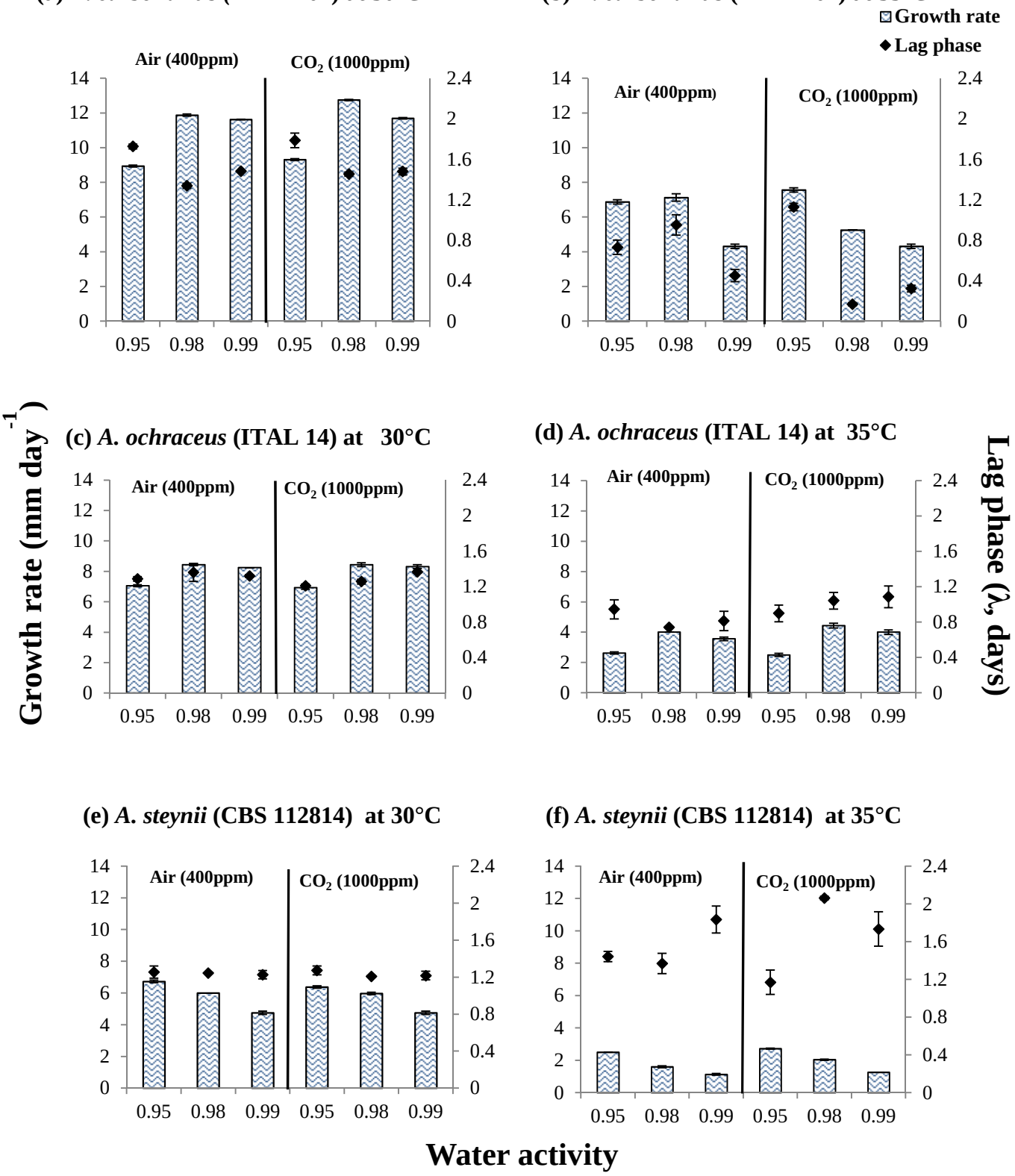


Figure 2. Akbar, Medina and Magan

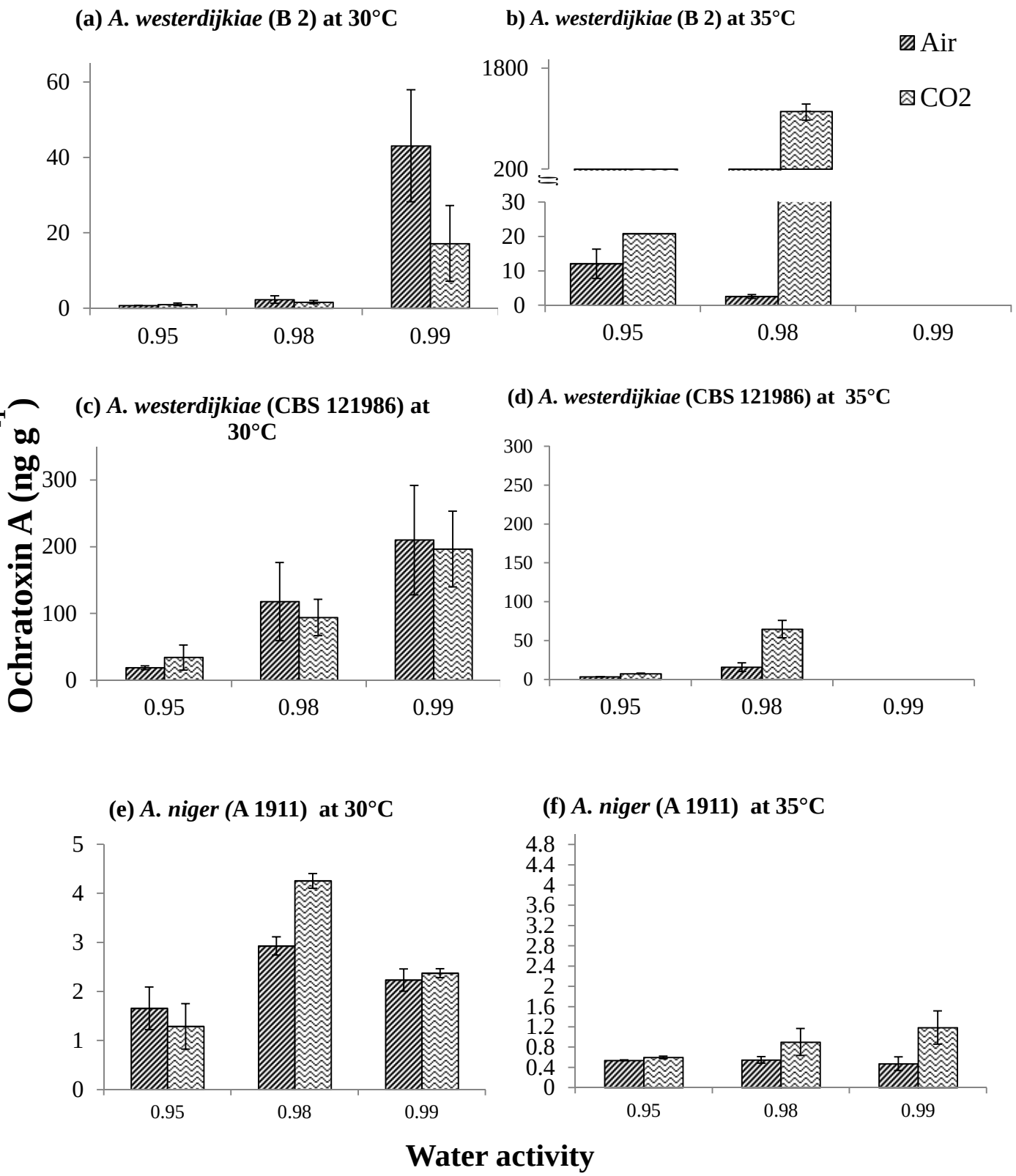


Figure 3. Akbar, Medina and Magan

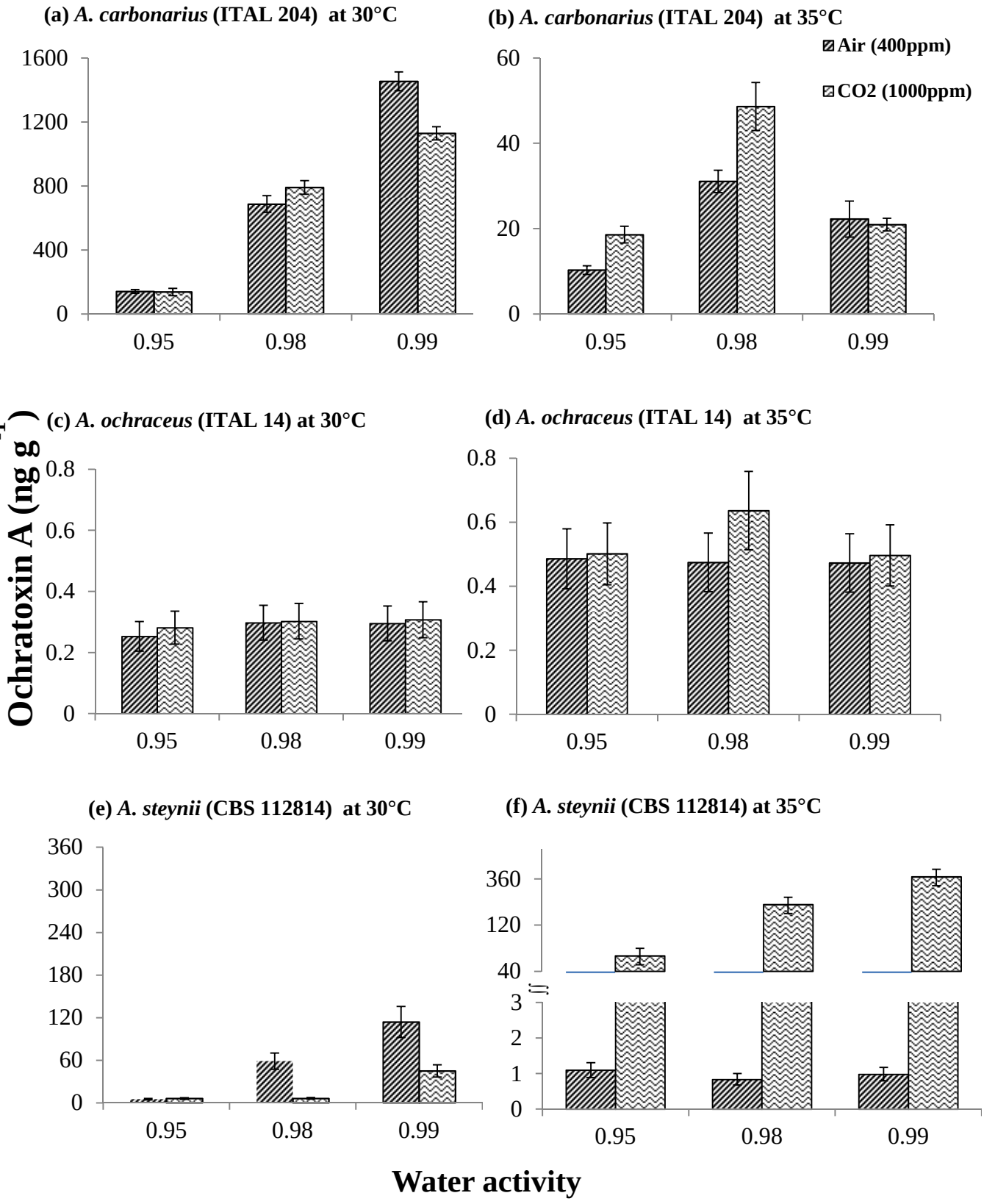


Figure 4. Akbar, Medina and Magan

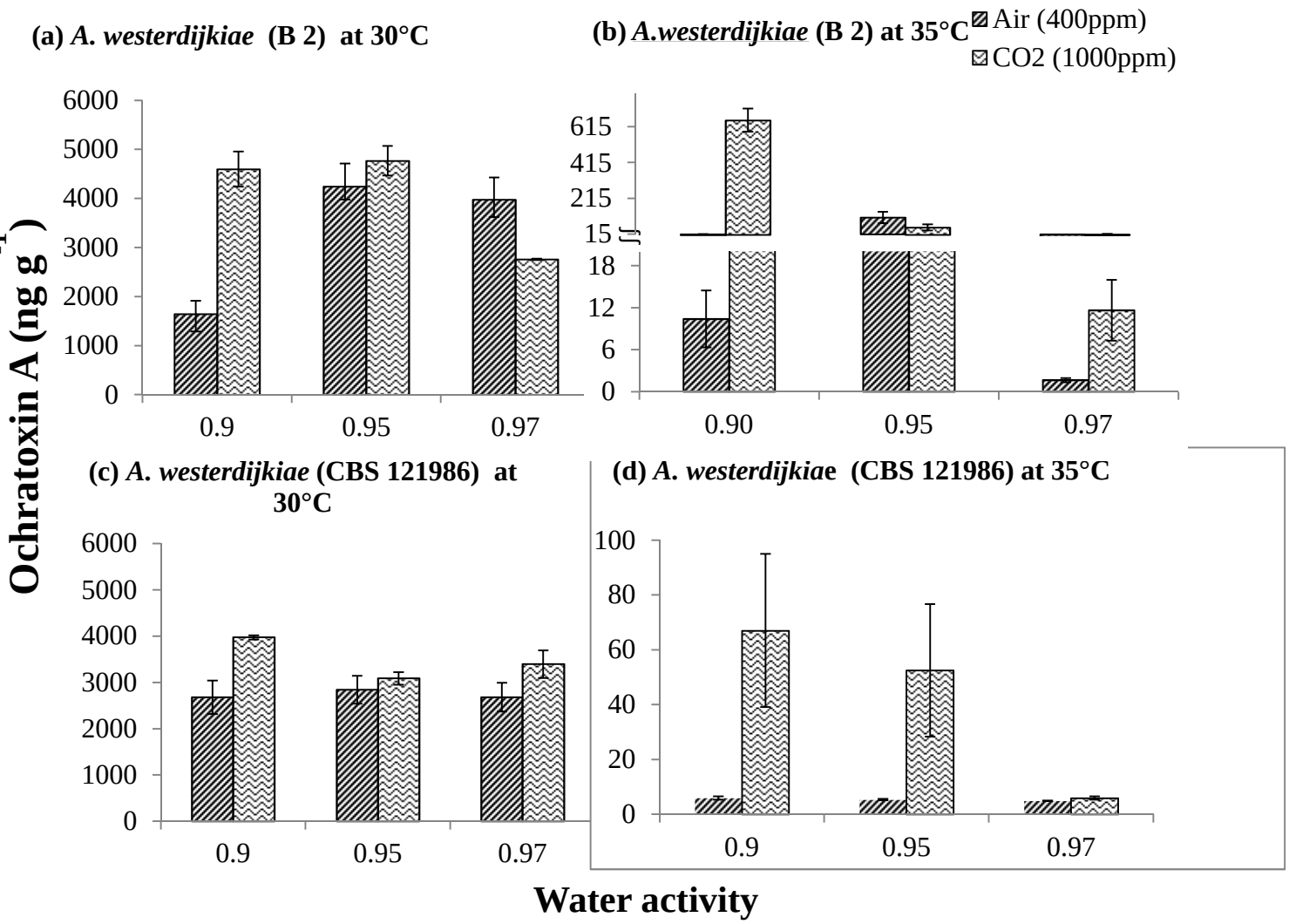


Figure 5. Akbar, Medina and Magan

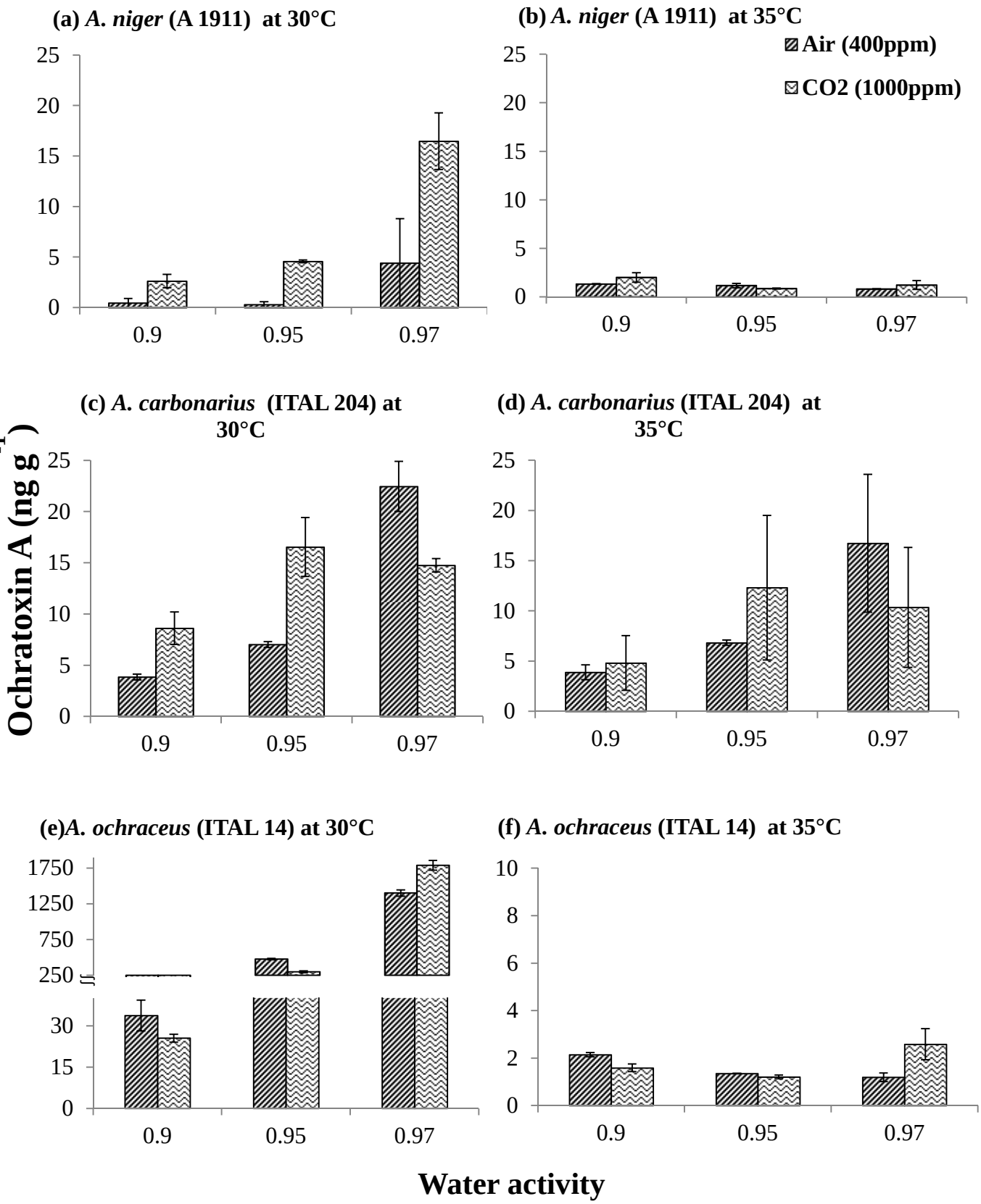


Figure 6. Akbar, Medina and Magan

# Impact of interacting climate change factors on growth and ochratoxin A production by Aspergillus section Circumdati and Nigri species on coffee

Akbar, Asya

2016-06-06



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