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Impact of non-selective fungicides on growth and production of ochratoxin A by *Aspergillus ochraceus* and *A. carbonarius* in barley –based medium

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Running head: fungicide effect on *Aspergillus* in barley medium

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Abstract

The aim of this study was to assess the influence of the non-selective fungicides mancozeb, copper oxychloride and sulfur on the growth and capability for producing ochratoxin A (OTA) of ochratoxigenic isolates of *Aspergillus carbonarius* and *A. ochraceus* in barley-based medium. Lag phases and growth rates were determined for each fungicide at different doses, at 15 ºC and 25 ºC and at 0.97 a_w. Mancozeb at 40 mg L^{-1} inhibited fungal growth and provided lag phases > 24 days at 10–20 mg L^{-1} and 15 ºC. OTA was observed only at 25 ºC and doses <10 mg L^{-1}. At 15 ºC, copper oxychloride proved inhibitory at 800 mg L^{-1} while at 25 ºC growth was not delayed and only high doses decreased OTA levels. Sulfur was inhibitory or provided large lag phases at 5–8 g L^{-1} (at 15 ºC) while at 25 ºC growth took place even at 8 g L^{-1} although OTA levels were low or undetectable. The antifungal activity decreased in the order mancozeb > copper oxychloride > sulfur and was lower at 25 ºC than at 15ºC. OTA accumulation was affected by the type of fungicide, dose, temperature and time. The efficacy of these fungicides on the growth of *A. carbonarius* and *A. ochraceus* and OTA production in barley-based medium is assessed for the first time.

Keywords: fungicides, barley, mancozeb, copper oxychloride, sulfur, *Aspergillus,* ochratoxin

Introduction

Barley (*Hordeum vulgare*) constitutes a very nutritious cereal which contains carbohydrates, protein, vitamins and minerals. It represents a good source of fibre and it is known for its significant properties to reduce high blood cholesterol. This cereal is one of the first solid foods given to babies, a common ingredient used in animal feed and a key ingredient in the production of beer, whisky and other drinks. There are various types of barley (whole barley, hulled barley, pearled barley as well as barley flakes). Barley flour is good for making unleavened bread, *chapati,* and *pitta* bread. According to the UN Food and Agriculture Organization, barley is the fourth largest cultivated cereal crop in the world after wheat, rice and corn. In 2007/2008 crop the world total production of barley was 133 million Tm and the top producers of barley crops were Russia, Spain, Canada, Germany, and France (USDA 2009). Barley is the most relevant cereal crop in Spain.

Barley can be contaminated by a wide range of fungi (Ackermann 1998; Andersen et al. 1996; Gareis 1999; Noots et al. 1998). Some of these fungi produce severe grain spoilage but are also capable of producing mycotoxins, which can occur during pre-harvest, harvest/drying and storage, and are determined by intrinsic nutritional, extrinsic, processing and implicit microbial factors (Magan and Aldred 2007; Sinha 1995). In pre-harvest, control of these factors is complicated because it depends on the weather conditions of crop areas. Spain owns a warm climate and barley takes a shorter time to grow and ripen than any other crop, so barley is grown during two seasons, winter and spring, having average temperatures of about 15 to 25 ºC, respectively, and a high relative humidity.

The mycobiota contaminating barley grown in Spain has been reported (Mateo et al. 2004; Medina et al. 2006), and *Alternaria* spp., *Aspergillus* spp., and *Penicillium* spp. were the most important toxigenic fungi isolated.

Ochratoxin A (OTA) is one of the mycotoxins of concern. It is produced by some species of *Penicillium,* such as *P. verrucosum* (Pitt 1987) and *P. nordicum* (Larsen et al. 2001), but also by two *Aspergillus* sections: the section *Circumdati* (the *Aspergillus ochraceus* group)

and the section *Nigri* (*Aspergillus carbonarius* and *Aspergillus niger* aggregate) (Heenan et al. 1998; Medina et al. 2005a; Varga et al. 1996). In Spain, *A. ochraceus* and *A. carbonarius* are the most frequently isolated ochratoxigenic species in barley (Medina et al. 2006). However, the latter has been very scarcely described as a species contaminating cereals. OTA is a potent nephrotoxin also known to be teratogenic, immunosuppresive and carcinogenic. It has been classified by the International Agency for Research on Cancer (IARC 1993) as a possible human carcinogen (group 2B). OTA has been detected in barley, malting barley (Gareis 1999; Trucksess et al. 1999) and by-products such as beer (Legarda and Burdaspal 1998; Medina et al. 2005b; Nakajima et al. 1999; Scott and Kanhere 1995).

Aldred et al. (2004) and Magan and Aldred (2007) examined some important mycotoxins and the post-harvest control strategies that have been developed for effective management to minimise entry of mycotoxins into the food chain. In some cases, pre-harvest decisions can significantly impact the capability for subsequent post-harvest control. Prevention of the growth of mycotoxin-producing fungi is the most effective strategy for controlling the presence of mycotoxins in foods and feed. This could be achieved by knowing the critical limits of different eco-physiological factors affecting fungal infection and mycotoxin synthesis. Moisture, temperature, the type of substrate and some other factors play a major role in the development of OTA-producing fungal isolates and in mycotoxin production (Astoreca et al. 2009a, 2009b; Belli et al. 2004; Kapetanakou et al. 2009; Romero et al. 2007). Culture nutrients affect OTA production by *Aspergillus* spp. (Medina et al. 2008) and OTA biosynthetic genes in *A. ochraceus* are differentially regulated by pH and nutritional stimuli (O'Callaghan et al. 2006). In many cases, usually during pre-harvest, the use of fungicides is the only efficient, cost-effective and often successful way to prevent mould growth (Munimbaz et al. 1997) thus being an attractive strategy to prevent mycotoxin production (FAO/WHO/UNEP 1999). However, fungicides must be applied carefully since some of them, such as carbendazim, can reduce fungal growth but can also stimulate OTA production (Medina et al. 2007b).

Mancozeb [manganese ethylenebis(dithiocarbamate) (polymeric) complex with zinc salt] is a broad range contact non-selective fungicide used to protect many fruit, vegetable, nut and field crops against many fungal diseases. It is also used for seed treatment of cotton, potatoes, peanuts, and, especially, cereal grains (EXTOXNET 1996). It is one of the most used pesticides around the world. Its wide use in agriculture is due to the reported scarce persistence in the environment (Maroni et al. 2000; Wauchope et al. 1992) and low acute toxicity. It is practically non-toxic via the oral route (Edwards et al. 1991) although some adverse effects have been found in mesencefalic cells (Domico et al. 2007). One of its degradation products, ethylenethiourea (ETU), exerts toxic effects in exposed animals (Houeto et al. 1995). The European Commission has set the maximum residue level (MRL) of whole dithiocarbamates in barley to 2 mg/Kg (expressed as CS_2) (European Commission 2009; EC Pesticides Database 2010).

Copper oxychloride and sulfur are classical non-systemic fungicides, widely used, especially in ecological agriculture. The former is used to treat cereals to get better crops in copper-deficient soils (Brennan 1990) and to control a wide range of diseases on many crops. Sulfur is a contact and protectant fungicide used for control of oidium in cereals, brown rot of peaches, powdery mildew of apples, grapes, strawberries, etc. It is toxic to fish but it is non-toxic to birds and bees and has not been proved to be mutagenic (EXTOXNET 1996). These fungicides as well as mancozeb are not considered to be at risk from resistance. According to European regulations the MRL of all copper fungicides in cereals is 10 mg/kg (expressed in copper) while no MRL is required for sulfur (EC Pesticides Database 2010).

Chemical fungicides are widely used to prevent the development of spoilage fungi and mycotoxin production in crops. Thorough studies to know the activity of each fungicide in each crop are necessary, especially for mycotoxin–producing fungi. Studies with some fungicides in grape-like medium (Bellí et al. 2006), dehydrating grapes (Valero et al. 2007), grape berries (Favilla et al. 2008), grape medium (Medina et al. 2007b) or peanut meal extract agar (Barberis et al. 2009) have been performed. Nevertheless, up to date, there are not data on the resistance of *A. ochraceus* and *A. carbonarius* to mancozeb, copper

oxychloride and sulfur in barley or barley-based medium. Moreover, the effect of these fungicides on OTA production by these species on this medium has not been studied.

The aim of this study was to evaluate the effect of mancozeb, copper oxychloride and sulfur on (i) the lag phase to growth, (ii) growth rates and (iii) ochratoxin A production by isolates of *Aspergillus carbonarius* and *A. ochraceus* cultured in a barley-based medium.

Materials and methods

Fungal isolates

An isolate of each *A. carbonarius* (ref. Ac51) and *A. ochraceus* (ref. Ao23) from malting barley grown in Spain and capable of producing OTA (Medina et al. 2006) were chosen for the study. Stock cultures exist in the fungal collection of the 'Mycology and Mycotoxins' group (Department of Microbiology and Ecology, University of Valencia, Spain).

Fungicide formulations

The formulation of mancozeb used was Mancofit [80% active ingredient (a.i), wettable powder (WP)] (Agrofit, Valencia, Spain). It was diluted in water to prepare a stock emulsion containing 1 g mancozeb L^{-1}. The recommended dose is 2.0-3.5 g a.i. L^{-1} (3-4 Kg ha^{-1} on cereal crops).

The formulation of copper oxychloride [$Cu_2Cl(OH)_3$] used was Cuprosan 500 (50% a.i. WP) (Bayer). It was diluted in water to prepare a stock suspension of 10 g L^{-1}. The recommended dose is 2.5 g a.i L^{-1}.

The formulation of sulfur was Quimur [80% a.i. wettable granules (WG)] (Sarabia S.A., Lleida, Spain). The recommended dose is 2.5–7.5 g L^{-1} depending on the crop. A stock suspension of 200 g L^{-1} in water was prepared. Sulfur is insoluble in water so that continuous shaking is needed during aliquot withdrawal from the containing beaker.

Culture medium

The culture medium used, barley-meal extract agar (BMEA), was prepared as follows: 30 g of dry ground barley was boiled in 1 L of deionized water for 30 min. The resulting mixture was filtered through a double layer of muslin and the volume was made up to 1 L. The pH of the mixture was 6.5. A level of 0.97 was chosen for water activity (a_w) to carry out the experiments. This value is between 0.96 and 0.98, which favour growth of *A. ochraceus* (Ramos et al. 1998) and *A. carbonarius* (Medina et al. 2007a, 2007b). Glycerol (120 g) was added to provide 0.97 a_w in the medium (after addition of 20 g of agar to 1 L of suspension). Once the agar was added, the medium was autoclaved (115 ºC, 30 min).

The appropriate volume of each fungicide stock emulsion/suspension was added at around 45 ºC to a series of flasks to obtain the desired concentrations: 1, 3, 5, 10, 20, 30 and 40 mg L^{-1} for mancozeb; 5, 10, 30, 50, 100, 300, 500, 700 and 800 mg L^{-1} for copper oxychloride; and 10, 20, 1000, 3000, 5000 and 8000 mg L^{-1} for sulfur. All concentrations are referred to the a.i. Flasks were vigorously shaken and poured into Petri dishes (20 mL per dish). Controls having no fungicide added were also prepared. The a_w-value was checked after sterilization using non-inoculated Petri dishes and a Novasina RTD 502 equipment (Novasina GmbH, Pfäffikon, Switzerland).

Inoculum preparation

The isolates of *A. carbonarius* and *A. ochraceus* were grown on BMEA for 7 days at 25 ºC. Spores were then suspended in sterile distilled water containing Tween 80 (0.005%). From this suspension of spores, another one containing 1×10^6 spores mL^{-1} was prepared in water modified with glycerol to provide 0.97 a_w for the spore suspension and 3 μL of this one was inoculated at the centre of Petri dishes (previously prepared) under sterile conditions. Incubation was performed at 15 ºC and 25 ºC in closed chambers where there were beakers containing glycerol–water solutions of the same a_w (Llorens et al. 2004). Cultures were checked for a_w at the end of the incubation period. BMEA and incubation conditions were considered suitable for the experiment because of various reasons: the source of the isolates

(barley), the mean temperatures recorded in Spanish crop areas (25 ºC and 15 ºC during spring and winter crops, respectively), and previous studies on the growth of *A. ochraceus* in fungicide-free barley medium.

Growth monitoring

Mycelial extension rates were measured over time. Lag phase was considered as the time (days) to reach a colony five mm diameter (Bellí et al. 2004; Medina et al. 2007a; Astoreca et al. 2009a, 2009b; Samapundo et al. 2007). For each treatment, 15 plates were prepared and two right-angled diameters of the colonies were randomly chosen and measured every day until the colony filled the dish or the cultures were used for OTA determination. The sum of two diameters was divided by four to calculate the mean radius. These measurements were then averaged over the number of measured dishes. The slope of the line obtained by linear regression of colony radius against time (days elapsed from the lag time) was used to determine growth rates (mm day^{-1}).

Ochratoxin A determination

Calibration solutions containing OTA standard (2–1000 ng mL^{-1}) were used to obtain a calibration line by linear regression after injecting 100 µl into the liquid chromatography (LC) system. These solutions were obtained by dissolution of the standard (Sigma-Aldrich, Alcobendas, Spain) in chloroform and further dilution in dimethylsulfoxide (DMSO)/water (60:40, v/v).

Recovery studies were performed by adding variable amounts of OTA standard, dissolved in chloroform, at 45 ºC to Erlenmeyer flasks containing 20 g of autoclaved (115 ºC, 30 min) BMEA. The concentration range for recovery studies was 5–300 ng g^{-1}. After homogenisation, the medium was poured in a Petri dish and let to cool at room temperature. Twenty grams of solid medium was cut into small pieces and homogenised in a stomacher. A fraction of about 1.5 g of the homogenate was taken and extracted with five milliliters of methanol for one h using a rotating plate stirrer (model F-205, FALC Instruments S.R.L., Treviglio, Italy) (Bragulat et al. 2001). Four mL of extract was filtered through filter paper

(Whatman No. 4) containing 5–10 g of Celite 545 (Sigma–Aldrich). The filtrate was dried under a N_2 stream and re-dissolved in 0.50 mL of DMSO/water (60:40, v/v). Finally, 100 μL of solution was injected into the LC system.

For OTA determination, 20 g of culture (substrate plus fungal biomass) was cut into small pieces, homogenised in a stomacher, extracted with methanol (50 mL) and analyzed using the procedure indicated for recovery studies. Once the colony diameter reached 5 mm, the OTA level in the cultures was determined. Samples were taken on days 5, 10, 15 and 20 when incubation temperature was 25 ºC and on days 20, 30, 40 and 60 when incubation temperature was 15 ºC because previous experiments had shown that growth was favoured at 25 ºC. This protocol was performed each time with three dishes from the same treatment. As there were 15 replicates per treatment, the remaining three ones were carried out for safety.

The LC system was a Waters 600E system controller, a Millipore Waters 717 plus autosampler and a Waters 470 scanning fluorescence detector (Waters, Milford, MA, USA). Excitation and emission wavelengths were 330 and 460 nm, respectively. The samples were separated using a C18 Phenomenex Gemini® column (150 × 4.6 mm, 5 μm particle size) (Phenomenex, Macclesfield, UK), with a guard column of the same material. Run time for samples was 20 min with OTA being detected at about 12 min. The flow rate of the mobile phase (acetonitrile/water/acetic acid, 44:55:1, v/v/v) was 1 mL min^{-1}. Measurements were processed using the Millennium® 4.0 software (Waters).

The limit of detection was 0.5 ng OTA g^{-1} culture, based on a signal-to-noise ratio of 3:1. The average recovery rate for OTA from the agar-based medium was 89 %.

Statistical analysis

Multifactor ANOVA and post-hoc analysis were performed using Statgraphics Centurion XV version 15.1.02 (StatPoint, Inc., Herndon, VA, USA). Logarithmic transformation of data according to Barberis et al. (2009) was performed before statistical treatment to increase variance homogeneity. Post-hoc analyses were performed using the Tukey-honestly

significant differences (Tukey-HSD) test, except for variables with two distinct values (temperature) or categories (fungi). For statistical purposes undetectable/unquantifiable levels were considered as zero. Differences were considered significant if $P < 0.05$.

Results

Mancozeb

Lag phases

The lag-times observed in BMEA cultures supplemented with mancozeb are shown in Figure 1A. Lag phases were always longer at 15 ºC than at 25ºC under all the assayed conditions and usually increased with mancozeb dose. A dose of 30 mg L^{-1} prevented fungal growth except in the case of *A. ochraceus* at 25 ºC but growth was always inhibited at 40 mg L^{-1}. At 25 ºC, the lag-times for the cultures of *A. ochraceus* supplemented with mancozeb were about 3 days at 1-10 mg L^{-1}, about 6 days at 20 mg L^{-1} and about 9 days at 30 mg L^{-1}; however, at 15 ºC, lag phases lasted 25 - 40 days at 10 - 20 mg L^{-1} (Figure 1A). The lag phase of *A. carbonarius* also increased with mancozeb dose but they were higher than lag phases observed in *A. ochraceus* cultures under the same conditions.

Multifactor ANOVA showed that lag-times were significantly affected ($P < 0.05$) by the factors fungi, temperature and dose, and their mutual interactions. Lag phases that were significantly different from others with regard to mancozeb dose were clustered in different groups by Tukey-HSD test ($P < 0.05$). In this case, seven groups were found (one for each dose including the control).

Growth rates

Growth rates were higher at 25 ºC than at 15 ºC (Figure 1B), and higher for *A. ochraceus* than for *A. carbonarius* under the same conditions. For both isolates, growth rates at 25 ºC and 15 ºC in cultures containing doses <10 mg L^{-1} were roughly similar to those observed in controls but they decreased at a dose of 20 mg L^{-1}.

Multifactor ANOVA showed that the factors fungi, dose and temperature, and their interactions (except fungal species × temperature) significantly affected growth rate ($P < 0.05$). Tukey-HSD test gave rise to four groups with regard to the influence of the dose on the growth rate (control, 1-10 mg L^{-1}, 20, and 30 mg L^{-1}). Growth rates were set to zero for calculation purposes when no growth was apparent.

OTA levels

OTA levels in cultures carried out at 25 ºC are shown in Table 1. At 25 ºC, OTA was detected on day five in all cultures of *A. ochraceus*, except at 40 mg L^{-1}, and toxin levels usually increased with time. The maximum OTA level (410 ng g^{-1}) was higher than the level found in the control on the same day. However, at 25 ºC, OTA was hardly detected in cultures of *A. carbonarius* on days 5-10 (Table 1). The isolate of *A. carbonarius* produced less OTA in controls than the isolate of *A. ochraceus* under the same conditions. Mean OTA levels in mancozeb-containing cultures of *A. carbonarius* were < 72 ng g^{-1} except at 5 mg L^{-1}.

At 15 ºC, OTA was found in controls but not in mancozeb-containing cultures (Table 2). Temperature, dose and time significantly influenced OTA production in cultures of both fungi ($P < 0.05$).

Regarding the effect of dose at 25 ºC the Tukey-HSD test found three homogeneous but highly overlapped groups for *A. ochraceus* (low, medium and high doses) and four homogeneous groups for *A. carbonarius*. As regards the influence of time only two groups were found by the same test for *A. ochraceus* (day five and the remaining days). However, comparisons are biased because time was cross-related with temperature.

Copper oxychloride

Lag phases

Figure 1C shows that at 25 ºC lag-times for *A. ochraceus* and *A. carbonarius* were practically unaffected (roughly 2.5 days) by copper oxychloride added up to 500 mg L^{-1}. At 15 ºC, however, this fungicide increased lag-times at doses >10 mg L^{-1}. At 15 ºC, lag phases

lasted about 5 days at low doses and increased up to 12-13 days at 700 mg L^{-1} but any isolate was able to grow at 800 mg L^{-1}. The lag phases significantly differed regarding the influence of temperature, fungi, fungicide dose and their interactions. With regard to dose, Tukey-HSD test found eight homogeneous groups with overlapping between the closest means.

Growth rates

Growth rates were higher at 25 ºC than at 15 ºC (Figure 1D). For *A. ochraceus,* growth rates at 25 ºC were roughly 3 mm day^{-1} at doses < 100 mg L^{-1}, but decreased by 50% at higher doses, which seem to hinder mycelial extension.

For *A. carbonarius*, growth rates at 25 ºC decreased by 33% in the 300-800 mg L^{-1} range. At 15 ºC, growth rates also decreased with increasing doses but changes were smoother than at 25 ºC. There were significant differences ($P < 0.05$) among growth rates in cultures containing copper oxychloride with regard to the fungi, temperature, dose and their interactions. Using Tukey-HSD test seven groups appear as regard the dose.

OTA levels

As Table 1 shows, at 25 ºC, the highest OTA level was found in cultures of *A. ochraceus* containing 30 mg fungicide L^{-1}. Low toxin levels were found in the 300–500 mg L^{-1} range and OTA was practically undetectable above 500 mg L^{-1}. At 25 ºC, OTA accumulation was negligible in all *A. carbonarius* cultures containing copper oxychloride. At 15 ºC, OTA maximum level was found in cultures of *A. ochraceus* at a dose of 10 mg L^{-1} while the mycotoxin was undetectable in cultures of this isolate at 100–700 mg L^{-1} (Table 2). At 15 ºC, OTA was undetectable in copper-containing cultures of *A. carbonarius* (Table 2).

Using this fungicide, the factors temperature, dose and fungi significantly influenced accumulation of OTA in cultures of both fungi ($P < 0.05$).

Sulfur

Lag phases

Sulfur also increased the lag phase more at 15 ºC than at 25 ºC (Figure 1E). At 25 ºC, sulfur had no ability to significantly delay fungal development up to a dose of 8 g L^{-1}. At 15 ºC, a dose of 3 g L^{-1} resulted in lag phases of about 21 days for both isolates, which were much longer than lag phases attained at doses ≤ 1 g L^{-1}, while 5 g L^{-1} prevented fungal growth. Temperature, dose and cross interactions, but not the fungi, significantly influenced the lag-times. Tukey-HSD test found six homogeneous groups concerning dose ($P < 0.05$).

Growth rates

Growth rates were higher at 25 ºC than at 15 ºC at the same dose (Figure 1F). At 25 ºC, the growth rate of *A. ochraceus* at 8 g L^{-1} was lower than rates observed at 3 - 5 g L^{-1}. At this temperature, in the case of *A. carbonarius,* there were not significant differences concerning growth rate between controls and cultures at 3 g L^{-1}. At 15 ºC, however, growth rates decreased by 25%. The ANOVA revealed that temperature, fungi and dose and their interactions, except the interaction fungi × temperature, were significant ($P < 0.05$). Tukey-HSD test found six homogeneous groups related to sulfur dose ($P < 0.05$).

OTA levels

At 25 ºC, OTA was not detectable in cultures of *A. ochraceus* at <1 g L^{-1} or at 8 g L^{-1} (Table 1). However, OTA accumulation was high at 3 g L^{-1}. At 15 ºC, OTA levels were significantly higher than those found in controls in cultures containing 0.2 - 3 g sulfur L^{-1} (Table 2).

At 25 ºC OTA level in cultures of *A. carbonarius* was low at intermediate/high sulfur doses. The highest toxin level was found in control and at the dose of 10 mg L^{-1} (Table 1) while at 15 ºC, OTA was not detected (Table 2) and was not assayed when growth was negligible. The influence of temperature, dose and time on OTA accumulation was significant for both isolates. Concerning dose and time Tukey-HSD test found three and two homogeneous groups, respectively, for *A. ochraceus,* and three groups for *A. carbonarius*.

Discussion

In the present study, two ochratoxigenic isolates, one of *A. carbonarius* and another of *A. ochraceus,* were selected to investigate the effect of three antifungal agents on fungal growth and OTA production when the fungi were cultured on barley-based medium at 15 ºC and 25 ºC and at 0.97 a_w.

The doses of the fungicides were chosen on the basis of preliminary experiments performed in our laboratory, which had shown that growth of these isolates in BMEA was inhibited at doses above those used in the present study (data not shown). Sub-inhibitory doses were assayed because mycotoxin production may be stimulated when stressing environmental conditions and low fungicide doses are maintained during growth of mycotoxin-producing fungi (D'Mello et al. 1998; Magan et al. 2002; Medina et al. 2007b; Nesci et al. 2003). This hypothesis is supported by the results obtained in this work.

The lowest assayed temperature (15 ºC) was less favorable than 25ºC to the growth of the studied isolates. The impact of temperature on growth parameters agrees well with studies performed with other strains of *A. carbonarius* (Barberis et al. 2009; Medina et al. 2007b; Romero et al. 2007) and *A. ochraceus* (Pardo et al. 2005; Ramos et al. 1998), even though in all these studies the culture media, a_w-values, presence of fungicides and hosts were different.

OTA accumulation in BMEA was affected by temperature both in fungicide-containing media and controls. Toxin levels were generally higher at 25 ºC than at 15 ºC at a given fungicide dose. These results agree with those of Kapetanakou et al. (2009), who report that OTA production by *A. carbonarius* and *A. ochraceus* in malt extract agar was higher at 25 ºC than at 15, 20 or 30 ºC, although fungicides were not used and a_w conditions were different from those employed in the present study. However, other authors (Barberis et al. 2009) have found that temperature is the factor of minor influence on OTA production by isolates in *Aspergillus* section *Nigri* in media supplemented with butylated hydroxyanisole. In our study,

in addition to temperature, the type of fungicide, their dose and incubation time, clearly influenced OTA accumulation in BMEA.

The isolates used in this study were previously obtained from barley grown in Spain and OTA levels produced in an optimized medium for production of this toxin (yeast extract sucrose supplemented with 5% of bee pollen) (Medina et al. 2006) were lower than those found in BMEA in the present report. Then, BMEA is an excellent substrate for OTA production by the assayed isolates.

Among the three antifungal agents tested, mancozeb was the most active (it inhibited fungal growth at 40 mg L^{-1}) while sulfur was the least active against the tested isolates in BMEA; moreover, large lag phases or even inhibitory effects were observed at low mancozeb doses. It has been reported that mancozeb used up to 10% of the dose recommended by the manufacturer did not prevent the growth of *A. carbonarius* isolated from grapes on synthetic grape-like medium (Bellí et al. 2006). Therefore, mancozeb seems to be more active in BMEA than in synthetic grape medium to control *A. carbonarius*, although the isolates and other conditions were different. This fact may be due to a different degradation rate of the fungicide. It has been reported that in table grapes, mancozeb degrades rapidly to ETU, which suffers from further degradation to relatively non-toxic products (Banerjee et al. 2008). Some of these products are ethyleneurea and 2-imidazoline (Vonk and Sijpesteijn 1970) which further degrades to ethyldiamine and CO_2 (IPCS 1988).

At 25 ºC, mancozeb (up to 30 mg L^{-1}) did not prevent OTA production in cultures of *A. ochraceus* in BMEA but decreased OTA production during the first 15 days of incubation in cultures of *A. carbonarius*. However, at 15 ºC, this chemical effectively arrested OTA production even at the lowest dose. This effect was more evident in the case of *A. ochraceus*. It has been reported that a dose of 3 g mancozeb L^{-1} inhibited growth and OTA production by isolates of *A. carbonarius* from grape (Belli et al. 2006). In the present work inhibitory doses are much lower. Assays aimed at the research of frequent treatment of barley with low doses of mancozeb instead of the employment of high doses should be

carried out. They can be very useful to control and to prevent the growth of *Aspergilli* and production of OTA but also to minimize the toxic effects of ETU in humans and animals.

Any of the assayed doses of copper oxychloride proved inhibitory at 25 ºC. However, at 15 ºC fungal growth was inhibited at 0.8 g L^{-1}. This fungicide produced shorter lag phases and slower growth rates than mancozeb at the same doses, especially in the case of *A. carbonarius*. At 25ºC, OTA levels were undetectable or unquantifiable at doses higher than 500 mg L^{-1} in cultures of *A. ochraceus* and higher than 10 mg L^{-1} in cultures of *A. carbonarius.* At 15ºC OTA production was inhibited at doses higher than 50 mg L^{-1} in cultures of *A. ochraceus* and at all the assayed doses in cultures of *A. carbonarius*. This antifungal agent, though not very effective to control the growth of the assayed species, plays a relevant role in the inhibition of OTA production. Other authors have found that this fungicide was scarcely effective to control *Fusarium oxysporum* f.sp. *cubense* (Nel et al. 2007), that *A. niger* and *Penicillium chrysogenum* were able to grow in a medium containing up to 0.5 g kg^{-1} of this antifungal agent (Gharieb et al. 2004) or that 8 g L^{-1} are needed to completely inhibit the growth of *A. flavus* (Mishra and Dubey 1994). But up to now its effectiveness for controlling the production of OTA in ochratoxigenic species from barley has not been assessed.

Sulfur was unable to produce inhibition of the assayed fungi and its capacity to delay growth was irrelevant at 25 ºC. At 15 ºC, doses equal or higher than 5 g sulfur L^{-1} were needed to inhibit mycelial extension of both isolates. Therefore, sulfur does not appear suitable to control these fungi. In addition, the results shown in the present report indicate that sulfur can increase OTA production by the two species at sub-inhibitory doses, mainly in cultures of *A. ochraceus*. It has been reported that sulfur can inhibit the growth of *A. flavus* at 7 g L^{-1} and 75% inhibition took place at 5 g L^{-1} (Mishra and Dubey 1994) but little is known (Bellí et al 2006) about its effect on mycotoxin production by mycotoxigenic fungi at sub-inhibitory doses. Thus, this antifungal agent should be applied with caution.

The assayed fungi showed some differences concerning lag phase, growth rate and OTA production with regard to the fungicide and its dose. Differences in lag phases are higher at

the lowest temperature in cultures of the isolates of the two assayed species. In cultures containing any of the three fungicides, at 15 ºC, *A. ochraceus* showed shorter lag phases and higher growth rates than *A. carbonarius* with the three antifungal agents; at 25 ºC, the two isolates showed similar lag phases, although growth rates were higher in cultures of *A. ochraceus*, like at 15 ºC. Concerning OTA production, the isolate of *A. ochraceus* produced more toxin that the isolate of *A. carbonarius* in controls, under the same conditions and, generally, this behavior was observed in fungicide-containing cultures at 25 ºC. At 15 ºC, the stress due to temperature significantly affected to OTA production in controls, mainly in those of *A. carbonarius*. However, it is remarkable that at this temperature, less favorable for fungal growth and toxin production, some doses of copper oxychloride and, especially sulfur, have a positive impact on OTA production by *A. ochraceus*. This effect is also observed in cultures at 25 ºC supplemented with certain doses of these two fungicides. Unfortunately, scarcity of previous reports about these fungi and fungicides difficult the discussion of the results, although it is generally known that interspecific and intraspecific differences in fungicide susceptibility are frequent in fungi (Antachopoulos et al. 2007; Pell et al. 2001).

The effects of mancozeb, copper oxychloride and sulfur in isolates of *A. carbonarius* and *A. ochraceus* from barley grown in Spain and cultured on BMEA have been studied for the first time. On the basis of the results obtained in this study it seems that risk of barley contamination with OTA might be higher on spring crops than on winter crops treated with these fungicides at sub-inhibitory levels. Low fungicide doses combined con mild temperatures and relatively high humidity might enhance OTA production in the cereal. Taking into account a) the wide use of these fungicides, b) their toxicity to animals (especially, in the case of mancozeb) and c) the scarcity of studies on these chemicals related to the fungal species here considered, studies on barley in field are needed in order to find the optimal dose of active ingredient against *A. ochraceus* and *A carbonarius* and the way of application to control mycelial development of these fungi in crops and minimize OTA production.

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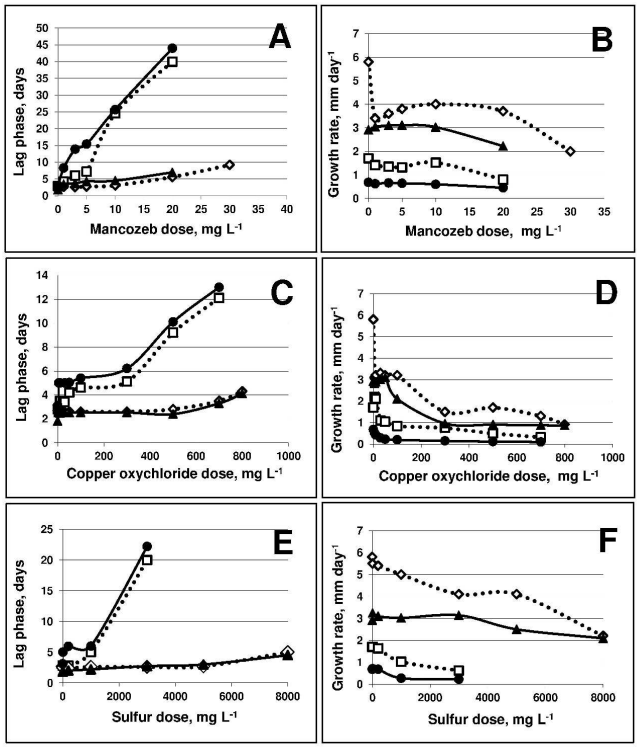
Figure caption

Figure 1. Effect of three fungicides on lag phase and radial growth rate of isolates of *A. ochraceus* and *A. carbonarius* cultured on BMEA at 0.97 a_w. Mancozeb (A and B); Copper oxychloride (C and D); Sulfur (E and F). Line key: *A. ochraceus*: dotted line with white squares (15 ºC) or white rhombi (25 ºC); *A. carbonarius*: solid line with black circles (15 ºC) or black triangles (25 ºC).



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234x303mm (600 x 600 DPI)

Table 1. Mean concentration ± standard deviation of OTA (ng g^{-1}) (N = 3) found in cultures of isolates of *A. ochraceus* and *A. carbonarius* carried out at 25 ºC and 0.97 a_w on BMEA treated with three fungicides.

Fungal species	Fungicide	Dose (mg L^{-1})	Incubation time (days) 5	10	15	20
A. ochraceus	Control*		101 ± 22	270 ± 30	320 ± 30	350 ± 20
	Mancozeb	1	98 ± 25	235 ± 24	295 ± 40	340 ± 30
		3	100 ± 26	260 ± 30	340 ± 30	320 ± 20
		5	130 ± 29	340 ± 40	410 ± 40	340 ± 22
		10	105 ± 24	250 ± 30	312 ± 26	360 ± 30
		20	—	12 ± 6	54 ± 18	153 ± 22
		30	—	ND	15 ± 5	46 ± 13
	Copper oxychloride	5	89 ± 15	241 ± 23	300 ± 30	310 ± 25
		10	99 ± 20	300 ± 22	340 ± 23	345 ± 22
		30	210 ± 29	400 ± 25	550 ± 30	544 ± 25
		50	82 ± 15	195 ± 30	230 ± 30	233 ± 25
		100	122 ± 10	280 ± 30	372 ± 23	368 ± 30
		300	2.2 ± 0.3	5.3 ± 1.9	8.0 ± 1.7	10 ± 4
		500	ND	3.4 ± 0.9	9.1 ± 2.8	12 ± 5
		700	ND	ND	NQ	NQ
		800	ND	ND	ND	ND
	Sulfur	10	ND	ND	ND	ND
		200	ND	ND	ND	ND
		1000	ND	ND	ND	ND
		3000	ND	500 ± 30	1100 ± 40	600 ± 30
		5000	ND	2.0 ± 1.4	11 ± 5	13 ± 6
		8000	ND	ND	ND	ND
A. carbonarius	Control*		20 ± 6	39 ± 15	64 ± 24	56 ± 15
	Mancozeb	1	ND	2.1 ± 1.6	24 ± 5	30 ± 10
		3	ND	ND	NQ	71 ± 12
		5	ND	ND	3.9± 1.0	300 ± 20
		10	ND	ND	10 ± 4	24 ± 8
		20	ND	ND	ND	ND
		30	—	—	—	—
	Copper oxychloride	5	3.1 ± 1.4	6.2 ± 2.1	38 ± 8	42 ± 10
		10	ND	3.2 ± 1.1	15 ± 3	9 ± 3
		30	ND	ND	NQ	NQ
		50	ND	ND	ND	ND
		100	ND	ND	ND	ND
		300	ND	ND	ND	ND
		500	ND	ND	ND	ND
		700	ND	ND	ND	ND
		800	ND	ND	ND	ND
	Sulfur	10	ND	ND	70 ± 17	53 ± 12
		200	ND	1.8 ± 0.9	21 ± 9	30 ± 7
		1000	NQ	1.5 ± 0.5	3.0± 1.6	4.0± 1.9
		3000	ND	ND	ND	ND
		5000	ND	ND	ND	ND
		8000	ND	ND	ND	ND

* No fungicide was added; ND, not detected; NQ, detected but not quantified (< 1.5 ng g^{-1}).

Table 2. Mean concentration ± standard deviation of OTA (ng g^{-1}) (N = 3) found in cultures of isolates of *A. ochraceus* and *A. carbonarius* carried out at 15 ºC and 0.97a_w on BMEA treated with three fungicides.

Fungal species	Fungicide	Dose (mg L^{-1})	Incubation time (days) 20	30	40	60
A. ochraceus	Control*		ND	2.1 ± 1.1	10 ± 5	14 ± 6
	Mancozeb	1	ND	ND	ND	ND
		3	ND	ND	ND	ND
		5	ND	ND	ND	ND
		10	ND	ND	ND	ND
		20	—	—	ND	ND
	Copper oxychloride	5	ND	ND	NQ	2.4 ± 1.9
		10	18 ± 10	42 ± 15	59 ± 21	46 ± 12
		30	ND	ND	10 ± 4	5 ± 3
		50	ND	14 ± 4	18 ± 5	11 ± 3
		100	ND	ND	ND	ND
		300	ND	ND	ND	ND
		500	ND	ND	ND	ND
		700	ND	ND	ND	ND
	Sulfur	10	ND	NQ	7 ± 4	13 ± 5
		200	ND	100 ± 22	122 ± 24	100 ± 25
		1000	ND	NQ	14 ± 3	400 ± 40
		3000	—	ND	2.0 ± 1.1	78 ± 19
A. carbonarius	Control*		ND	ND	NQ	3.2 ± 1.5
	Mancozeb	1	ND	ND	ND	ND
		3	ND	ND	ND	ND
		5	ND	ND	ND	ND
		10	ND	ND	ND	ND
		20	—	—	—	ND
	Copper oxychloride	5	ND	ND	ND	ND
		10	ND	ND	ND	ND
		30	ND	ND	ND	ND
		50	ND	ND	ND	ND
		100	ND	ND	ND	ND
		300	ND	ND	ND	ND
		500	ND	ND	ND	ND
		700	ND	ND	ND	ND
		800	—	—	—	—
	Sulfur	10	ND	ND	ND	ND
		200	ND	ND	ND	ND
		1000	ND	ND	ND	ND
		3000	—	ND	ND	ND
		5000	—	—	—	—
		8000	—	—	—	—

* No fungicide was added; ND, not detected; NQ, detected but not quantified (< 1.5 ng g^{-1}).