International Journal of Food Microbiology 149 (2011) 127–132

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Influence of water activity and temperature on growth and mycotoxin production by *Alternaria alternata* on irradiated soya beans

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ARTICLE INFO

Article history:
Received 28 March 2011
Received in revised form 6 June 2011
Accepted 16 June 2011
Available online 24 June 2011

Keywords:
Alternaria alternata
Growth
Two dimensional profiles
Soybean
Environmental factors
Mycoroxins

ABSTRACT

The aim of this study was to determine the effects of water activity (a_w) (0.99–0.90), temperature (15, 25 and 30 °C) and their interactions on growth and alternariol (AOH) and alternariol monomethyl ether (AME) production by *Alternaria alternata* on irradiated soya beans. Maximum growth rates were obtained at 0.980 a_w and 25 °C. Minimum a_w level for growth was dependent on temperature. Both strains were able to grow at the lowest a_w assayed (0.90). Maximum amount of AOH was produced at 0.98 a_w but at different temperatures, 15 and 25 °C, for the strains RC 21 and RC 39 respectively. Maximum AME production was obtained at 0.98 a_w and 30 °C for both strains. The concentration range of both toxins varied considerably depending on a_w and temperature interactions. The two metabolites were produced over the temperature range 15 to 30 °C and a_w range 0.99 to 0.96. The limiting a_w for detectable mycotoxin production is slightly greater than that for growth. Two-dimensional profiles of $a_w \times$ temperature were developed from these data to identify areas where conditions indicate a significant risk from AOH and AME accumulation on soya bean. Knowledge of AOH and AME production under marginal or sub-optimal temperature and a_w conditions for growth can be important since improper storage conditions accompanied by elevated temperature and moisture content in the grain can favour further mycotoxin production and lead to reduction in grain quality. This could present a hazard if the grain is used for human consumption or animal feedstuff.

1. Introduction

Soya bean (*Glycine max* L. Merr.) is an Asiatic leguminous plant cultivated in many parts of the world for its oil and proteins, which are extensively used in the manufacture of animal and human foodstuffs (FAO, 2004; Hepperly 1985). Production in Argentina reached 46 million tons during the 2007/2008 harvest season ranking in which Argentina was the third largest soya bean producer in the world. In Argentina, during the last quarter of the century, soya bean production has increased at an unprecedented rate from a cultivated area of 38,000 ha in 1970 to 16 million ha today. Around 70% of the soybean harvested is processed, providing 81% and 36% of the world's exported soya bean oil and meal, respectively (MAGyP, 2010). Saprophytic and parasitic fungi may be present in or on soybean seeds, pods, and flowers. The occurrence of fungi in seeds has received far more attention than the occurrence of fungi in pods and flowers. Infected seeds and infected seedlings developing from them represent greater economic risk in soya bean production and contamination with mycotoxins represents a health risk to humans and animals. *Alternaria* and *Fusarium* species are the most commonly isolated fungi from soya bean in Argentina and in other regions of the world (Barros et al., 2011; Boca et al., 2003; Broggi et al., 2007; Gally et al., 2006; Roy et al., 2000; Villarroel et al., 2004).The most common *Alternaria* species found on soya beans is *Alternaria alternata* (Barros et al., 2011; Broggi et al., 2007).

Alternaria species are well known for the production of toxic secondary metabolites, such as alternariol (AOH) and alternariol monomethyl ether (AME). During the last 5 years numerous studies dealing with AOH and AME toxicity have been published. Both mycotoxins have been reported to have genotoxic, mutagenic and carcinogenic effects (Logrieco et al., 2009; Ostry 2008). Also, the mutagenicity of AOH may have bearing on the carcinogenicity of this mycotoxin. Recently Tiemann et al. (2009) have demonstrated that AOH and AME negatively affected progesterone synthesis in porcine granulosa cells in vitro. Since granulosa cells directly influence the metabolic and structural growth of the oocyte (Albertini et al., 2001), exposure to AOH or AME may eventually affect reproductive performance by interfering with follicular development in swine and possibly other mammalian species. Therefore *Alternaria* toxins in feedstuffs should be carefully controlled.

Recently we have found natural contamination with AOH and AME in soya bean samples collected in Cordoba province, Argentina

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doi:10.1016/j.ijfoodmicro.2011.06.007

(Barros et al., 2011). Although only a limited number of samples were evaluated, the high frequency of natural occurrence of both *Alternaria* metabolites indicated that further studies on natural occurrence, effect of food processing on toxin concentration, and prevention strategies are needed. Considering the extensive use of soya bean in the manufacture of animal and human foodstuffs, the risk to both populations arising from continuing low-level exposure to AOH and AME should be taken into account.

There is an increasing world consumer demand for high quality and healthy food and drink products with the lowest possible level of contaminants such as mycotoxins. As a result, the food industry in the developed world demands raw ingredients of the best quality and that conform to statutory limits for mycotoxins where these have been set. Because mycotoxins are unavoidable, it is important to know how the concentrations of mycotoxins present in raw materials change through the food and feed chains. The development of prevention strategies today has been predominantly based on using the HACCP approach and to identify the critical control points in the pre- and post-harvest food chain. This approach enables strategies to be developed for minimising consumer exposure through appropriate management of products (Sanchis and Magan, 2004; Scudamore 2004). The key environmental determinants of pre and post harvest are water availability and temperature (Magan and Aldred, 2007). Biosynthesis of mycotoxins is significantly influenced by environmental conditions such as pH, water activity (a_w) and temperature (Astoreca et al., 2007; Ramirez et al., 2006).

Studies in relation to ecophysiological parameters (a_w, temperature and incubation time) on in vitro growth and AOH, AME and TA by two strains of *A. alternata* from soya beans have already been carried out (Oviedo et al., 2009, 2010), but there are no data about this subject on soya beans as a natural substrate.

The objective of this study was to determine the impact of a_w and temperature on growth and mycotoxin production (AOH and AME) on irradiated soya beans by two strains of *A. alternata* isolated from soya bean in Argentina.

2. Materials and methods

2.1. Fungal strains

Two *A. alternata* strains (RC 21 and RC 39) isolated from soya bean in Argentina were used. These isolates have been morphologically characterised according to Simmons (2007), noting, in particular, three-dimensional sporulation patterns. Both isolates were found to produce AOH, AME and TA by HPLC detection when tested in ground rice-corn steep liquor medium and soya bean extract agar (Oviedo et al., 2009, 2010). These strains are deposited at the Department of Microbiology and Immunology, Universidad Nacional de Rio Cuarto culture collection (RC). Cultures are maintained in 15% glycerol at −80 °C.

2.2. Grain

Soya beans (14.5% moisture content, 1 kg batches) were gamma irradiated (7 kGy) using a Cobalt radiation source and stored aseptically at 4 °C. The irradiated soya beans contained no microbial infection or mycotoxin contamination and had retained germinative capacity of about 75%, although respiration was reduced by about 30% and shoot length by about 65–70% (Hamer, 1994). The initial a_w of the grain was 0.711. Four hundred grams of irradiated soya beans were weighed into sterile beakers and rehydrated to the required a_w (0.99, 0.98, 0.96 and 0.90) by addition of sterile distilled water using a moisture absorption curve. Flasks were subsequently refrigerated at 4 °C for 48 h with periodic shaking to allow absorption and equilibration. Finally, the a_w levels were confirmed by using an Aqualab Series 3 water activity metre (Labcell Ltd., Pullman, USA).

2.3. Inoculation, incubation and growth assessment

Rehydrated soya bean was placed in sterile 9-cm Petri dishes to form a monolayer of grains (20 g). Then a 3-mm-diameter agar disc was taken from the margin of a 7-day-old colony of each isolate growing on synthetic nutrient agar (Gerlach and Nirenberg, 1982) at 25 °C and transferred face down onto the centre of each plate. To maintain the correct equilibrium of relative humidity inside the boxes, Petri plates containing grains of the same a_w were enclosed in plastic containers together with two beakers of glycerol–water solution of the same a_w as the treatments. Containers were incubated at 15, 25 and 30 °C and the experiment consisted of a fully replicated set of treatments with three replicates per treatment.

Assessment of growth was made daily during the incubation period, with soya bean cultures being examined using a binocular magnifier (×10). Two diameters of the growing colonies were measured at right angles to each other until the colony reached the edge of the plate. The radii of the colonies were plotted against time, and a linear regression was applied to obtain the growth rate as the slope of the line. Three complete Petri plate cultures per treatment were destructively sampled after 28 days of incubation, dried at 50 °C for 24 h and stored at −20 °C until toxin analysis was carried out.

2.4. Mycotoxin analysis

From each replicate, ~20 g of soya beans was extracted with 2 g NaCl, 30 mL of hexane and 50 mL of acetonitrile: methanol: water at 45:10:45 (vol/vol, pH 3) by shaking in an orbital shaker for 60 min. The extract was filtered through filter paper Whatman No. 4, and 10 mL of filtrate was collected. The filtered extract (2.5 mL) was applied to a C_{18} column (10 mL capacity, containing 500 mg of C_{18} sorbent, Waters, Milford, MA, USA) fitted to a Supelco solid-phase extraction (SPE) manifold (Supelco, Bellefonte, PA), previously conditioned by the passage of 6 mL acetonitrile until air comes through the column. Toxins were eluted with 2 mL of acetonitrile: glacial acetic acid at 99:1 (vol/vol). The eluted extract was collected in a 4-mL screw-cap amber vial and evaporated under a moderate stream of nitrogen at 50 °C in a heating block (Thermo Scientific, Rockford, IL, USA). The residue after cleanup was redissolved in 200 μL of methanol by vortexing for 1 min and diluted with 200 μL of acetonitrile:0.027 M NaH_2PO_4 at 25:75 (vol/vol) and stored at 4 °C until HPLC analysis (Barros et al., 2011).

The HPLC system consisted of an HP 1100 pump (Hewlett Packard, Palo Alto, CA) connected to an HP 1100 series variable wavelength detector and a data module Hewlett Packard Kayak XA (HP ChemStation rev. A.06.01). Chromatographic separations were performed on a Symmetry C_{18} column (100 by 4.6 mm inside diameter and 5 μm particle size) connected to a SecurityGuard™ guard column (20 by 4.6 mm inside diameter) filled with the same phase. The mobile phase consisted of two consecutive isocratic mobile phase mixtures containing acetonitrile:water at 25:75 (vol/vol, solvent A) and acetonitrile:water at 50:50 (vol/vol, solvent B). Solvent A was pumped for 3.5 min at 1.0 mL/min, and then solvent B was pumped for 16.5 min at 1.0 mL/min. The detector was set at 256 nm for AOH and AME. Injection volume was 50 μL and the retention time of AOH and AME were 11.9 and 18.3 min respectively. Quantification was relative to external standards of 0.5, 1.0, 2.0 and 3 μg/mL in acetonitrile: water (25:75, vol/vol).

Recovery experiments were performed in triplicate by spiking 20 g of ground blank soya bean samples with AOH and AME toxins at levels of 50, 100, 500, 1000, 2000 and 3000 ng/g. Spiked samples were left overnight at room temperature to allow solvent evaporation prior to proceed with the extraction step. Mean recoveries for AOH and AME from soya beans seed spiked at levels between 50 and 3000 ng/g were 94.90% and 98.98%, respectively, with a within-laboratory relative standard deviation (RSD) 20% and 5.9% for AOH and AME respectively.

Detection limits were 8 and 16 ng/g for AOH and AME, respectively, based on the signal to noise ratio (3:1) and the limit of quantification was established as 3 times the limit of detection (24 and 48 ng/g).

2.5. Statistical treatment of results

The linear regression of increase in radius against time (in days) was used to obtain the growth rates (mm/day) under each set of treatment conditions. The growth rates and mycotoxin concentration were then evaluated by analysis of variance (ANOVA) using SigmaStat for Windows Version 2.03 (SPSS Inc.). Statistical significance was judged at the level $P<0.001$. Contour plots for growth rate and maximum AOH and AME yield over the range of water activities and temperatures examined were prepared in Sigmaplot (v. 9.1, Systat Sofware, Inc., Point Richmond, CA, USA).

3. Results and discussion

This study compared the impact of $a_w \times$ temperature regimens on growth and AOH and AME production on irradiated soya bean by two strains of *A. alternata* isolated from soya bean in Argentina, for the first time. There is no information about the effect of environmental factors on growth and mycotoxin production by *A. alternata* in irradiated soya beans in the literature, and few reports on other substrates. This makes direct comparison difficult.

3.1. Effect of a_w and temperature on growth

Fig. 1 gives a diagrammatic representation of the interaction of a_w and temperature on growth rate (mm/day) of both *A. alternata* strains studied on irradiated soya bean. Maximum growth rates were obtained at 25 °C and 0.98 a_w for both strains. Minimum a_w level for growth was 0.90 at 25 °C and 0.96 a_w at 15 and 30 °C. Optimum a_w level for growth was dependent on temperature and also dependent on strain. At 30 and 25 °C both strains showed the same behaviour with the optimum a_w for growth being 0.98.

The conditions under which the same growth rates occurred were joined to produce contour lines which produce a map of the relative optimum and marginal rates of growth of the *A. alternata* strains (Fig. 2).

Abiotic factors (a_w and temperature) and their interaction had a significant influence on mycelial growth of *A. alternata* strains on irradiated soya beans (Table 1).

Maximum growth rate was obtained at 25 °C in both in vitro (on soybean based media; Oviedo et al., 2010) and in situ studies (on irradiated soya beans, present work) but at different a_w, 0.99 and 0.98 respectively. It is important to emphasise that the strains showed lower growth rates on irradiated soya beans than on soybean based medium (in vitro). The present results agree with previous studies on the water and temperature requirements for *A. alternata* growth demonstrating that its minimum a_w is 0.84 to 0.88 with optimal growth at 0.98 to 1.0 a_w at 25 °C (Magan and Baxter, 1994; Magan and Lacey, 1984).

It has been suggested that nutrient source may also influence the minimum a_w for growth (Griffin, 1972) and, consequently, studies on artificial substrates may not accurately reflect capabilities for growth on natural substrates (Magan and Lacey 1984). For this reason this study, to gain information specifically on the relationship between colonisation patterns and mycotoxin production, was carried out on gamma irradiated soya beans which had retained germinative

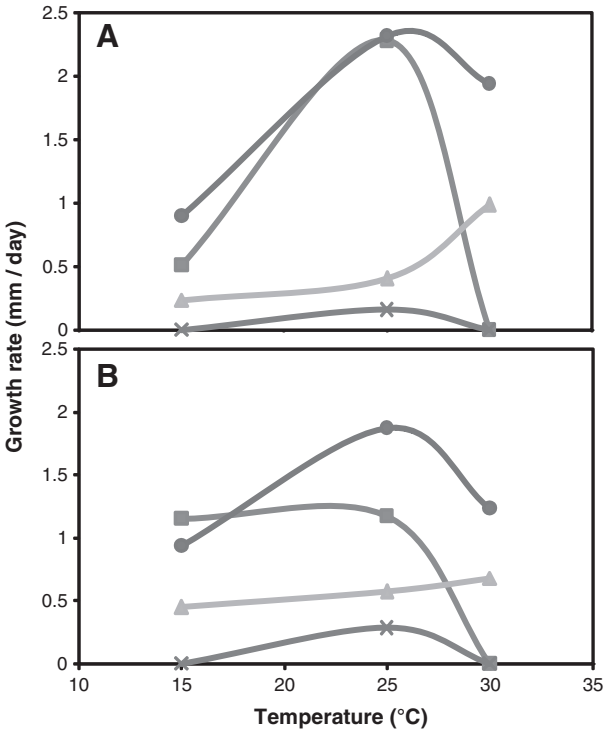

Fig. 1. Effects of a_w, 0.90 (×) 0.96 (▲), 0.98 (●) and 0.99 (■), and temperature on the growth rate of two *Alternaria alternata* strains on irradiated soya beans. (A) Strain RC 21. (B) Strain RC 39.

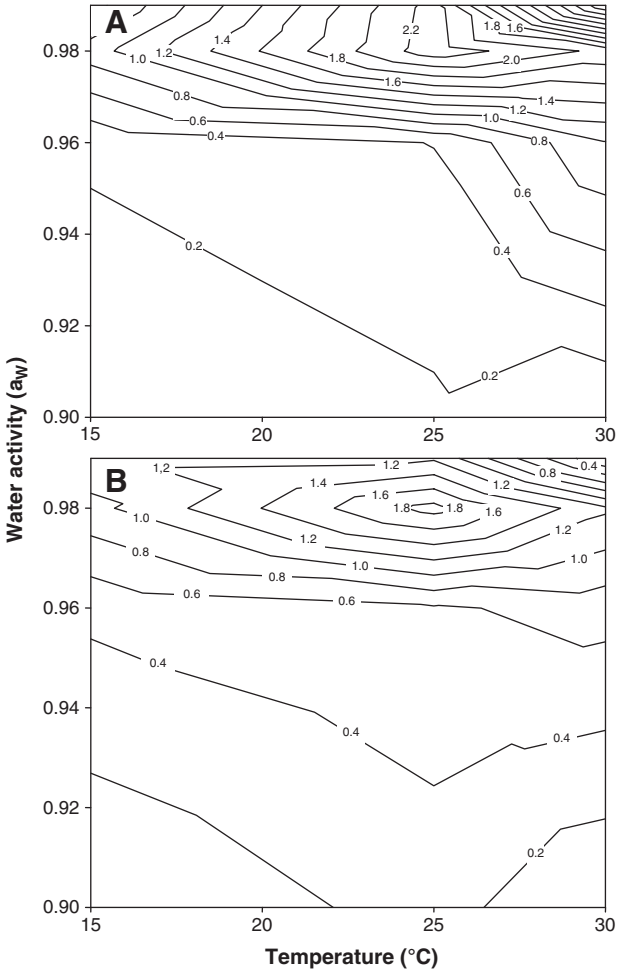

Fig. 2. Contour maps for growth rates of two strains of *Alternaria alternata* showing the relationship between water activity and temperature. The numbers on the contour lines refer to growth rates (mm/day). (A) Strain RC 21. (B) Strain RC 39.

Table 1
Analysis of variance on the effects of water activity (a_w), temperature (T), and different isolates (i) and their interactions on growth of *Alternaria alternata* on irradiated soya beans.

Source of variation	df[a]	MS[b]	*F*[c]
I	1	0.142	1.147
T	2	1.407	11.328*
a_w	3	5.753	46.322*
T×i	2	0.511	4.111
a_w×i	3	0.175	1.408
a_w×T	6	1.052	8.472*
a_w×T×i	6	0.188	1.516

[a] Degrees of freedom.
[b] Mean square.
[c] Snedecor F.
* $P<0.001$.

capacity without the natural mycoflora being present (Lacey and Magan, 1991).

3.2. Effect of a_w, temperature and incubation time on alternariol and alternariol monomethyl ether production

The surface response curves of AOH and AME production at 15, 25 and 30 °C after 21 days of incubation are shown in Fig. 3. No significant production of either toxin was observed at 0.90 a_w for either strain at all temperature levels assayed. The maximum amounts of AOH were obtained 0.98 a_w but at different temperatures: 15 and 25 °C for the strains RC 21 and RC 39, respectively. Maximum amounts of AME were obtained at 25 °C and 0.98 a_w after 21 days of incubation, for both strains.

At the highest temperature assayed (30 °C) maximum AOH production was obtained at 0.98 a_w for both strains. However, the strains differed in the a_w for maximum AME production, being 0.98 and 0.96 for the strain RC 21 and RC 39, respectively.

At 25 °C, maximum AOH and AME production was obtained at 0.98 a_w after 21 days incubation for both strains. Both strains were able to produce AOH at 0.99 and 0.96 a_w.

Reducing the temperature to 15 °C resulted in similar trends for AOH and AME production for both strains. AME production was only found at 0.98 a_w. Maximum amounts of AOH were obtained at 0.98 a_w for both strains and very low amounts of this toxin was obtained at 0.99 and 0.96 a_w.

Abiotic factors (a_w and temperature) and their interaction had a significant influence on AOH and AME production by *A. alternata* strains on irradiated soya beans (data not shown).

Data obtained for the two strains were used to develop contour maps in order to identify the optimum conditions of a_w and temperature and the range of conditions for production of different quantities of AOH and AME (Figs. 4 and 5).

The range of both toxin concentrations varied considerably depending on a_w and temperature interactions. Further both metabolites were produced over the temperature range from 15 to 30 °C and a_w range from 0.995 to 0.96 a_w. The limiting a_w for detectable mycotoxin production was slightly higher than that for growth. From the results obtained, it is evident that the AOH and AME production by *A. alternata* is favoured by different temperatures.

In a previous study, working with the same *A. alternata* strains, we were able to demonstrate the effect of interacting environmental conditions on growth and AOH and AME production on soya bean-based media (in vitro) (Oviedo et al., 2010). Maximal AOH production occurred at 0.98 a_w and 25 °C for both strains. Maximum AME production was obtained for both strains at 30 °C, but at different a_w: 0.92 and 0.94 for the strains RC 21 and RC 39 respectively. Based on previous and the present results, it appears that maximum AOH concentration occurs under the same conditions (a_w and temperature) in both in vitro and in situ studies. Different combinations of a_w and temperature are necessary for optimal production of these two toxins by *A. alternata*.

A few studies have examined the effect of different environmental conditions on AOH and AME production by *A. alternata* on sunflower seeds and wheat (Magan and Lacey, 1984; Torres et al., 1992), but no studies have been done on irradiated soya beans seeds. Magan and Lacey (1984) showed the effect of temperature (5, 15, 25 and 30 °C) and water relations (0.98, 0.95 and 0.90 a_w) on AOH, AME and altenuene production by *A. alternata* on autoclaved wheat grains. All

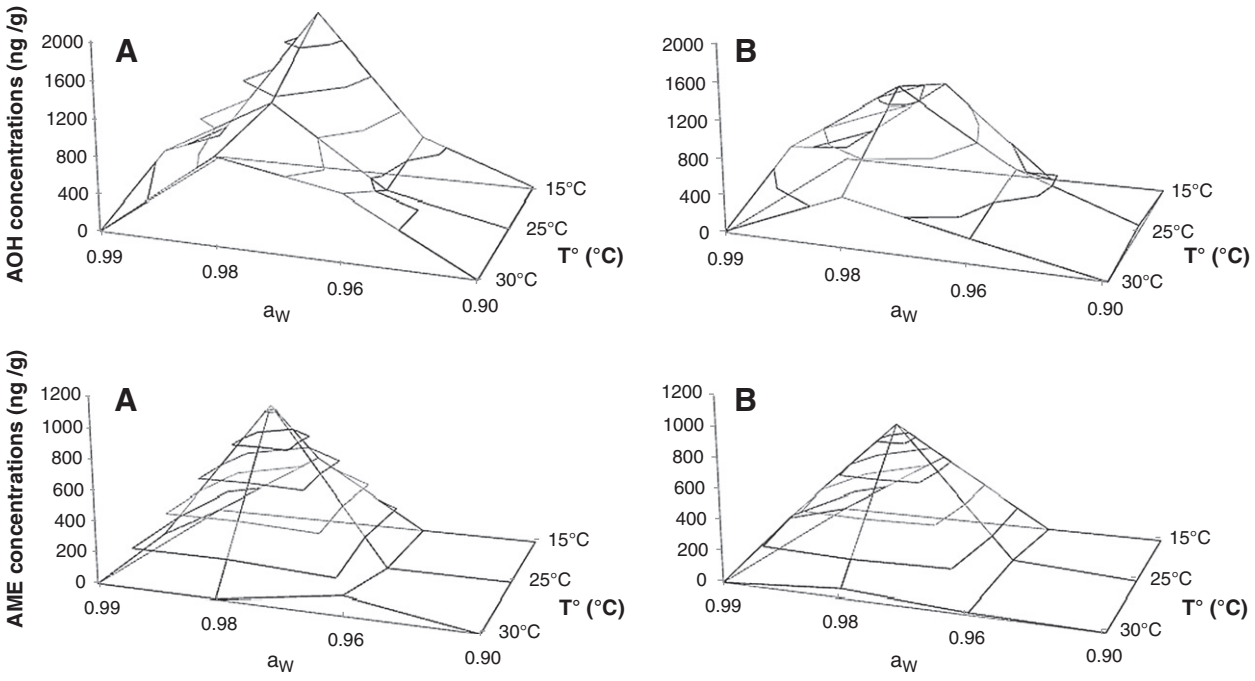

Fig. 3. Alternariol and alternariol monomethyl ether concentrations (ng/g) produced by two *Alternaria alternata* strains inoculated on irradiated soya beans adjusted to different water activity levels and incubated at 15, 25, and 30 °C for 21 days. (A) RC 21; (B) RC 39.

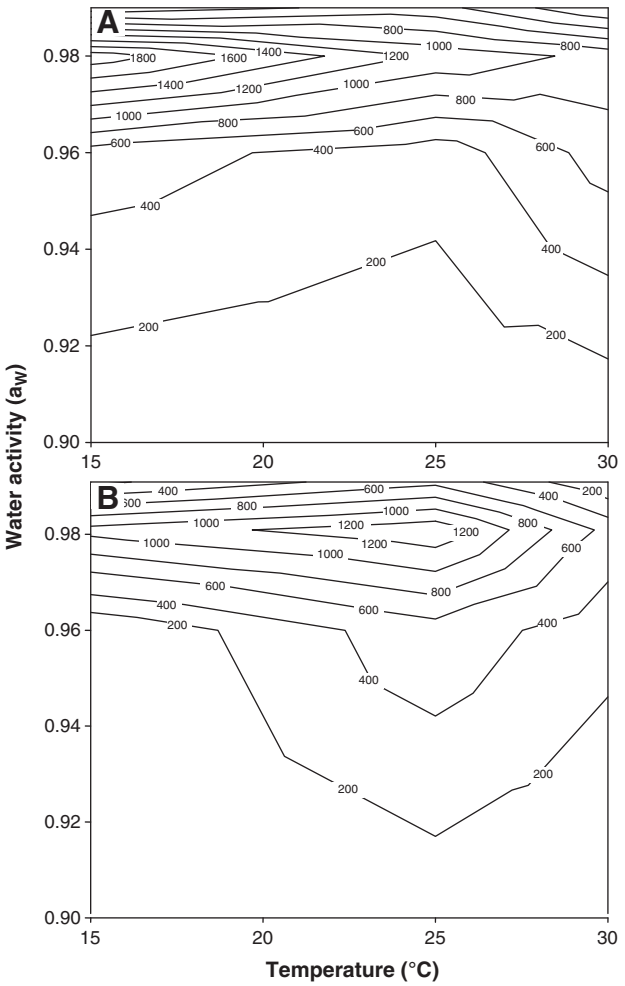

Fig. 4. Contour maps for alternariol production by two strains of *Alternaria alternata* showing the relationship between water activity and temperature. The numbers on the contour lines refer to mean concentrations (ng/g) of alternariol. (A) RC 21; (B) RC 39.

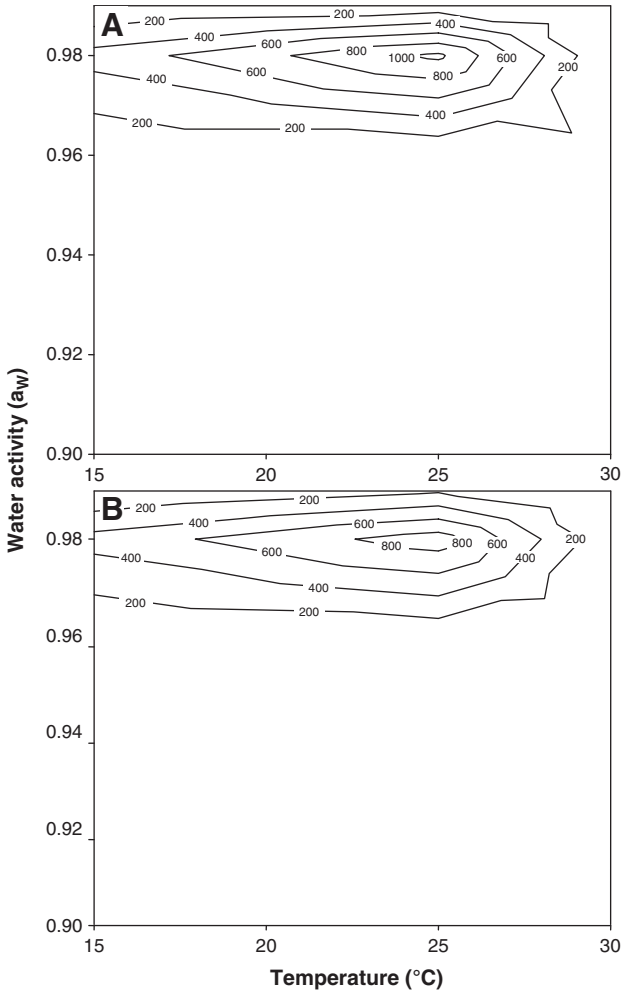

Fig. 5. Contour maps for alternariol monomethyl ether production by two strains of *Alternaria alternata* showing the relationship between water activity and temperature. The numbers on the contour lines refer to mean concentrations (ng/g) of alternariol monomethyl ether. (A) RC 21; (B) RC 39.

three toxins were produced optimally at 25 °C and 0.98 a_w. At all temperatures, much more toxin was produced at 0.98 than at lower a_w.

In another study, Torres et al. (1992) showed the effect of temperature (20, 25 and 32 °C) and a_w (0.98, 0.90, 0.87 and 0.80) on AOH and AME production by two strains of *A. alternata* on autoclaved sunflower seeds. They found that the optimal temperature for production of both toxins was 25 °C and 0.90 a_w. These previous studies are difficult to compare with our results because the substrates and the range of a_w and temperature used were different from those used during the present work.

The trends for growth and AOH and AME production on soybean have several implications for contamination of soya beans seeds and soy by-products with these mycotoxins. The a_w during soya bean development in the field changes from approximately 0.992 to 0.70 during a period of 45 days between pod formation and grain development until maturity is reached (R3 to R8). It has also been demonstrated that *A. alternata* is the most common species isolated from flowers and pods during stage R3, immature seeds (stage R6) and also during stage R8 (full maturity) where the water content of the seeds dropped dramatically (Barros et al., 2011). Thus the range of a_w from pod formation until harvest supports optimal and sub-optimal growth and AOH and AME formation, respectively. Permanent storage capacity has not increased at the same pace as the soya bean production in our country, so a substantial portion of the 17 million - tonne harvest is stored in temporary hermetic storage systems, called silo-bags, that remain in the field for long periods (over 5 months). Normally the bags are filled with soya bean at 12.5 to 15.5% MC (wet basis) (0.7 to 0.8 a_w). During the storage period, between Autumn and Summer, the MC remains unchanged, and is below the critical moisture threshold for fungal growth and development. Temperatures in the upper sections of the bags normally start to increase in August to a maximum of about 21 °C in October, in line with changes in environment. A major risk of mould related to the quality loss of soya bean in silo-bags occurs at localised points of moisture aggregation due to condensation and seepage of water into the grain through punctures. The moisture content of relatively small parcels of grain can get very high thus the likelihood of quality loss due to mould growth is also high. Furthermore, as condensation and leaks are likely to occur at the boundaries of a bag, oxygen ingress is more immediate, resulting in the loss of the hermetic atmosphere with subsequent mould growth (Rodriguez et al., 2004). In this scenario the conditions of a_w and temperature are appropriate for *A. alternata* growth and mycotoxin production according to our results.

In the present study, the knowledge of interacting environmental conditions provides very useful information for predicting the possible risk factors for AOH and AME contamination of soya bean. The a_w and temperature range used in this study simulate those occurring during grain ripening. The data demonstrated for the second time of the contrasting impact of a_w and temperature on growth and AOH and

AME production by the two strains were examined. The knowledge of AOH and AME production under marginal or sub-optimal temperature and a_w conditions for growth can be important since improper storage conditions accompanied by elevated temperature and moisture content in the grain can favour further mycotoxin production and lead to reduction in grain quality. This could present a hazard if the grain is used for human consumption or animal feedstuff.

Since *A. alternata* is isolated at a very high frequency from soya bean around the world the contour maps generated by the present study on growth and AOH and AME production may provide very useful guidelines for facilitating effective management of predicting risk for growth and mycotoxin production during ripening, harvesting and storage of soya bean.

Acknowledgements

This work was supported by the Secretaría de Ciencia y Técnica, Universidad Nacional de Río Cuarto (SECyT-UNRC). Oviedo, M.S. is a fellow of CONICET and Ramirez, M.L., Barros, G.G. and Chulze, S., are members of the Research Career of CONICET.

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