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Impact of Water Activity and Temperature on Growth and Alternariol and Alternariol Monomethyl Ether Production of *Alternaria alternata* Isolated from Soybean

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ABSTRACT

The objective of this study was to determine the effect of water activity (a_w; 0.995, 0.98, 0.96, 0.94, 0.92, and 0.90), temperature (5, 18, 25, and 30°C), incubation time (7 to 35 days), and their interactions on mycelial growth and alternariol (AOH) and alternariol monomethyl ether (AME) production. Two *Alternaria alternata* strains isolated from soybeans in Argentina were grown on 2% soybean extract agar. Maximum growth rates were obtained at the highest a_w (0.995) and 25°C, with growth decreasing as the water availability of the medium was reduced. Maximum amount of AOH was produced at 0.98 a_w and 25°C for both strains. Maximum AME production was obtained for both strains at 30°C but different a_w values, 0.92 and 0.94, for the strains RC 21 and RC 39, respectively. The concentrations of both toxins varied considerably depending on the a_w and temperature interactions assayed. The two metabolites were produced from 5 to 30°C and at a_w values of 0.92 to 0.995. Although at 5 and 18°C little mycotoxin was produced at a_w lower than 0.94. Two-dimensional profiles of a_w by temperature interactions were developed from these data to identify areas where conditions indicate a significant risk from AOH and AME accumulation on soybeans. All the conditions of a_w and temperature that resulted in maximum production of both toxins are those found during soybean development in the field. Thus, field conditions are likely to be conducive to optimum *A. alternata* growth and toxin production.

Soybean (*Glycine max* L.) is a main source of protein and is used worldwide both as food and feed *(11, 13).* Soybean production reached 47.5 million tons during the 2006 to 2007 harvest season, ranking Argentina third in the world among soybean producers. Most of the soybean production is exported to the European Union as oil, seeds, and flour *(24).*

A diverse group of saprophytic and parasitic fungi can colonize and infect soybean pods and seeds before harvesting *(31). Alternaria* and *Fusarium* species are the most commonly isolated fungi from soybeans in Argentina and others regions of the world *(4, 5, 21).*

Most *Alternaria* species are saprophytes that are commonly found in soil or on decaying plant tissues. Some species are opportunistic plant pathogens that, collectively, cause a range of plant diseases that have an economic impact on a large variety of important agronomic host plants, including cereals, ornamentals, oil crops, and vegetables. *Alternaria* species also are well-known postharvest pathogens. Some strains of *Alternaria alternata* are well known for the production of toxic secondary metabolites, some of which are powerful mycotoxins that have been implicated in the development of human esophageal cancer *(29).* Among these metabolites with mammalian toxicity are alternariol (AOH) and alternariol monomethyl ether (AME) *(15, 18).* AOH and AME have cytotoxic, genotoxic, and mutagenic effects in vitro *(6, 10, 14, 32),* and there is some evidence of carcinogenic effects *(33).* These mycotoxins are produced by *Alternaria* species on wheat, tomatoes, sorghum, pecans, sunflower, and cotton *(18, 23).* Recently, we developed a method for AOH and AME extraction, detection, and quantification on soybean seeds and found natural contamination with these toxins in samples collected in Cordoba province, Argentina *(19).* At present, no data on *Alternaria* toxin production on soybeans or on culture medium based on soybeans are available. Few studies have attempted to build two-dimensional profiles for growth of and mycotoxin production by *Alternaria* species *(22).*

Fungal growth and mycotoxin production result from the complex interaction of several factors; therefore, an understanding of each factor involved is essential to understand the overall process and to predict and prevent mycotoxin development *(7).* Temperature and water activity (a_w) are the primary environmental factors that influence growth and mycotoxin production by several *Alternaria* species *(9, 16, 17, 20, 30, 34).*

Alternaria toxins have recently received much attention, both in research programs and in risk assessment studies. No statutory or guideline limits for *Alternaria* mycotoxins have been set by regulatory authorities. Current data on the natural occurrence of *Alternaria* toxins point to low human dietary exposure. Further studies are necessary

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to develop strategies for safe food and feed supplies by developing detection methods, identifying *A. alternata* mycotoxin risk in the production chain, determining the critical points, and developing preventive measures. Accurate information is needed on the impact of key environmental factors such as a_w and temperature and their interactions and on marginal and optimum conditions for fungal growth and toxin production *(22)*. The aim of the present work was to determine the impact of a_w, temperature, and incubation time on growth and AOH and AME production on soybean extract agar by two strains of *A. alternata* isolated from soybeans in Argentina.

MATERIALS AND METHODS

Fungal strains. Two *A. alternata* strains (RC 21 and RC 39) isolated from soybeans in Argentina were used. These isolates have been morphologically characterized according to Simmons *(25, 26)* and Simmons and Roberts *(27),* with most emphasis on three-dimensional sporulation patterns. Both isolates produced AOH, AME, and tenuazonic acid (TA) when tested in ground rice-corn steep liquor medium and by high-performance liquid chromatography (HPLC). 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.

Medium. A 2% (wt/vol) ground soybean agar was used for these studies. The a_w of the basic medium was adjusted to 0.995, 0.98, 0.96, 0.94, 0.92, and 0.90 by addition of different amounts of glycerol *(8)*. The media were autoclaved at 120°C for 20 min, the flasks of molten medium were thoroughly shaken, and the medium was poured into sterile 9-cm petri dishes. The a_w of representative samples of media was checked with a water activity meter (Aqualab Series 3, Decagon Devices, Inc., Pullman, WA). Control plates were prepared and evaluated at the end of the experiment to detect any significant deviation in the a_w.

Inoculation, incubation, and growth assessment. Petri plates were inoculated with a 4-mm-diameter agar disk that was taken from the margin of a 7-day-old colony of each isolate grown on synthetic nutrient agar *(12)* at 25°C and transferred face down to the center of each plate. Inoculated plates of the same a_w were sealed in polyethylene bags and incubated at 5, 18, 25, and 30°C for 35 days. A full factorial design was used where the factors were a_w, temperature, and strain and the response was growth (total number of plates: 6 a_w × 4 temperatures × 2 strains × 3 replicates). All the treatments were repeated three times.

Assessment of growth was made every day during the incubation period, and 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 linear regression was applied to obtain the growth rate (millimeters per day) as the slope of the line.

AOH and AME extraction, detection, and quantification. The extraction method used was based on a microscale extraction *(28)* modified by Andersen et al. *(2)* into a three-step extraction procedure suited for *Alternaria* metabolites. At 7, 14, 21, 28, and 35 days of incubation, three plugs (4-mm diameter) were cut from the edge of a colony from each plate and placed in a 4-ml amber screw-cap vial (three plugs from each replicate). The plugs were extracted in 1.5 ml of chloroform-methanol (2:1, vol/vol) for 60 min in a ultrasonic bath. The extract was transferred to a clean 4-ml amber vial and evaporated to dryness under N_2 at 50°C. The same plugs were then extracted ultrasonically for 60 min in 1.3 ml of ethyl acetate containing 1% formic acid. The second extract was transferred to the amber vial containing the first dried extract and evaporated. The plugs were then extracted ultrasonically for 60 min with 1.5 ml of 2-propanol, and this extract was transferred to the amber vial with the two previous extracts and evaporated. The pooled, dried extract was redissolved ultrasonically in 1 ml of methanol and 1 ml of acetonitrile-water (25:75, vol/vol), filtered through a 0.45-μm-pore-size filter, and transferred to a clean 1.5-ml amber vial for use in HPLC analysis.

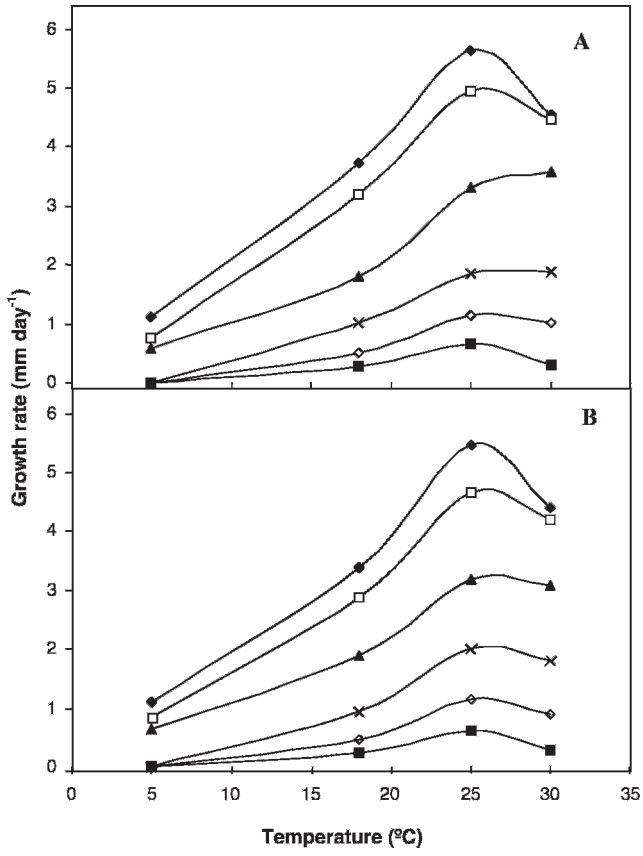

FIGURE 1. *Effect of a_w, 0.90 (■), 0.92 (◇), 0.94 (×), 0.96 (▲), 0.98 (□), and 0.995 (◆), and temperature on growth rate of two* Alternaria alternata *strains on 2% soybean extract agar. (A) Strain RC 21. (B) Strain RC 39.*

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 (inside diameter, 100 by 4.6 mm; 5-μm particle size) connected to a SecurityGuard guard column (inside diameter, 20 by 4.6 mm) 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^{-1}, and then solvent B was pumped for 16.5 min at 1.0 ml min^{-1}. The detector was set at 256 nm for AOH and AME. Injection volume was 50 μl, and the retention times of AOH and AME were 11.8 and 17.5 min, respectively. Quantification was relative to external standards of 0.5, 1.0, 2.0, and 3 μg/ml in acetonitrile-water (25:75).

Recovery experiments were performed on 2% milled soybean agar spiked at concentrations of 0.1 to 10 μg g^{-1} with

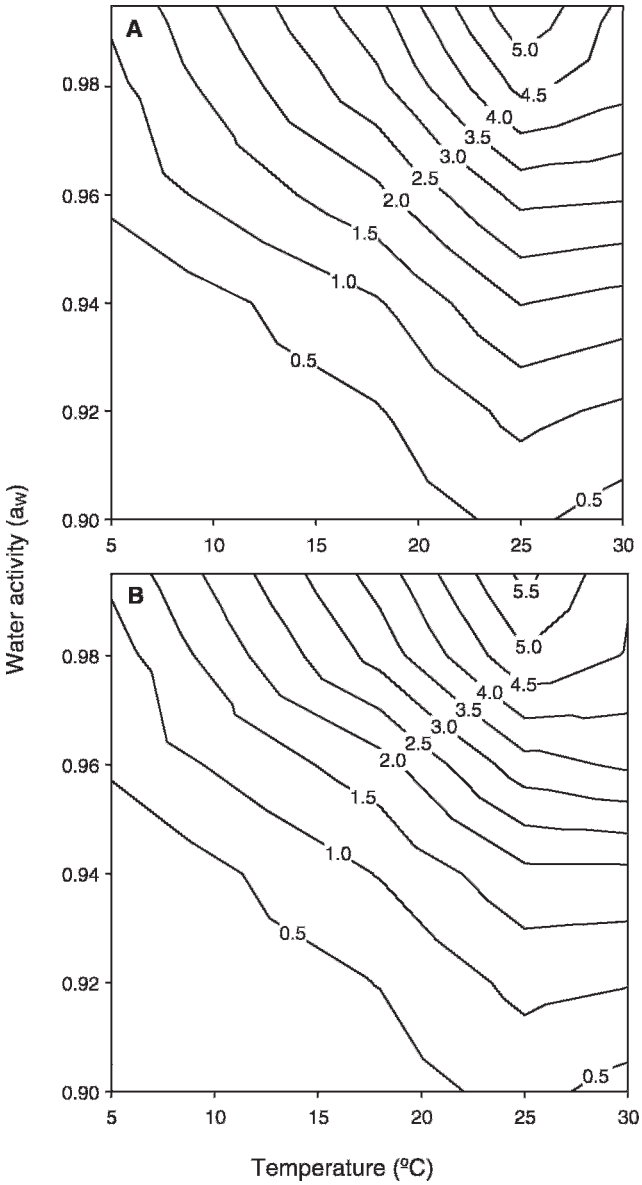

FIGURE 2. *Contour maps for two strains of* Alternaria alternata *showing the relationship between water activity and temperature. The numbers on the contour lines refer to growth rates (millimeters per day). (A) Strain RC 21. (B) Strain RC 39.*

AOH and AME, respectively. Mean recovery and repeatability and relative standard deviation (RSD) ranged from 85 to 98% (RSD, 0.2 to 2%) and 88 to 97% (RSD, 0.2 to 2%) for AOH and AME, respectively. Limit of detection (signal-to-noise ratio 3) was 0.01 μg g^{-1} for both toxins, and the quantification limit was established as three times the detection limit. The number of toxin analyses was 6 a_w values × 4 temperatures × 2 strains × 3 replicates × 5 incubation times.

Statistical analysis. In all cases, the linear regression of increase in colony radius against time (in days) was used to obtain the growth rates (millimeters per 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., Chicago, IL). Statistical significance was determined at $P < 0.05$.

TABLE 1. *Analysis of variance of effect of water activity (a_w), temperature (T), and different isolates (i) and their interactions on growth of* Alternaria alternata *on soybean-based media*

Source of variation	df[a]	MS[b]	F[c]
a_w	5	50.751	2023.21*
T	2	13.626	543.22*
i	1	0.341	13.62*
a_w × T	10	0.793	31.595*
a_w × i	5	0.081	3.24**
T × i	2	0.080	3.21**
a_w × T × i	20	0.413	16.46*

[a] Degrees of freedom.
[b] Mean square.
[c] Snedecor F. * $P < 0.001$; ** $P < 0.05$.

RESULTS

Effect of a_w and temperature on growth. Figure 1 gives a diagrammatic representation of the interaction of a_w and temperature on growth rate of both *A. alternata* strains studied on the soybean-based media. Growth was optimal at 25°C for all a_w levels tested, with maximum growth at the highest a_w (0.995). Both strains were able to growth at the lowest a_w (0.90) at 18, 25, and 30°C. At 0.92 a_w regardless of the temperature, growth was reduced by a factor of 4. Both strains failed to growth at 5°C and an a_w lower than 0.96 during the incubation period. The conditions under which the same growth rates occurred were joined to produce contour lines and a map of the relative optimum and marginal rates of growth of the *A. alternata* strains (Fig. 2).

The ANOVA of the effect of single variables (isolate, a_w, and temperature) and two- and three-way interactions revealed that all variables alone and all interactions had a significant effect on growth rates (Table 1).

Effect of a_w, temperature, and incubation time on AOH and AME production. The surface response curves of AOH and AME production at 5, 18, 25, and 30°C over 35 days of incubation are showed in Figures 3 and 4. No significant production of either toxin was observed at 0.90 a_w for both strains at all temperatures assayed. The maximum amounts of AOH were obtained at 25°C and 0.98 a_w after 35 days of incubation for both strains. The maximum amounts of AME were obtained at 30°C for both strains but at different a_w values of 0.92 and 0.94 for the strains RC 21 and RC 39, respectively, after 28 days of incubation. In general, the toxin concentrations produced by RC 39 were higher than those produced by RC 21 under almost all conditions tested.

AOH production was highest at 25°C and decreased at the following temperatures in order: 30, 18, and 5°C. AME production was higher at 30°C and decreased with decreasing temperature. At the highest temperature assayed (30°C), maximum AOH and AME production by RC 21 were obtained at 0.92 a_w. However, RC 39 had a different behavior, reaching maximum AOH and AME at 0.96 and 0.94 a_w, respectively.

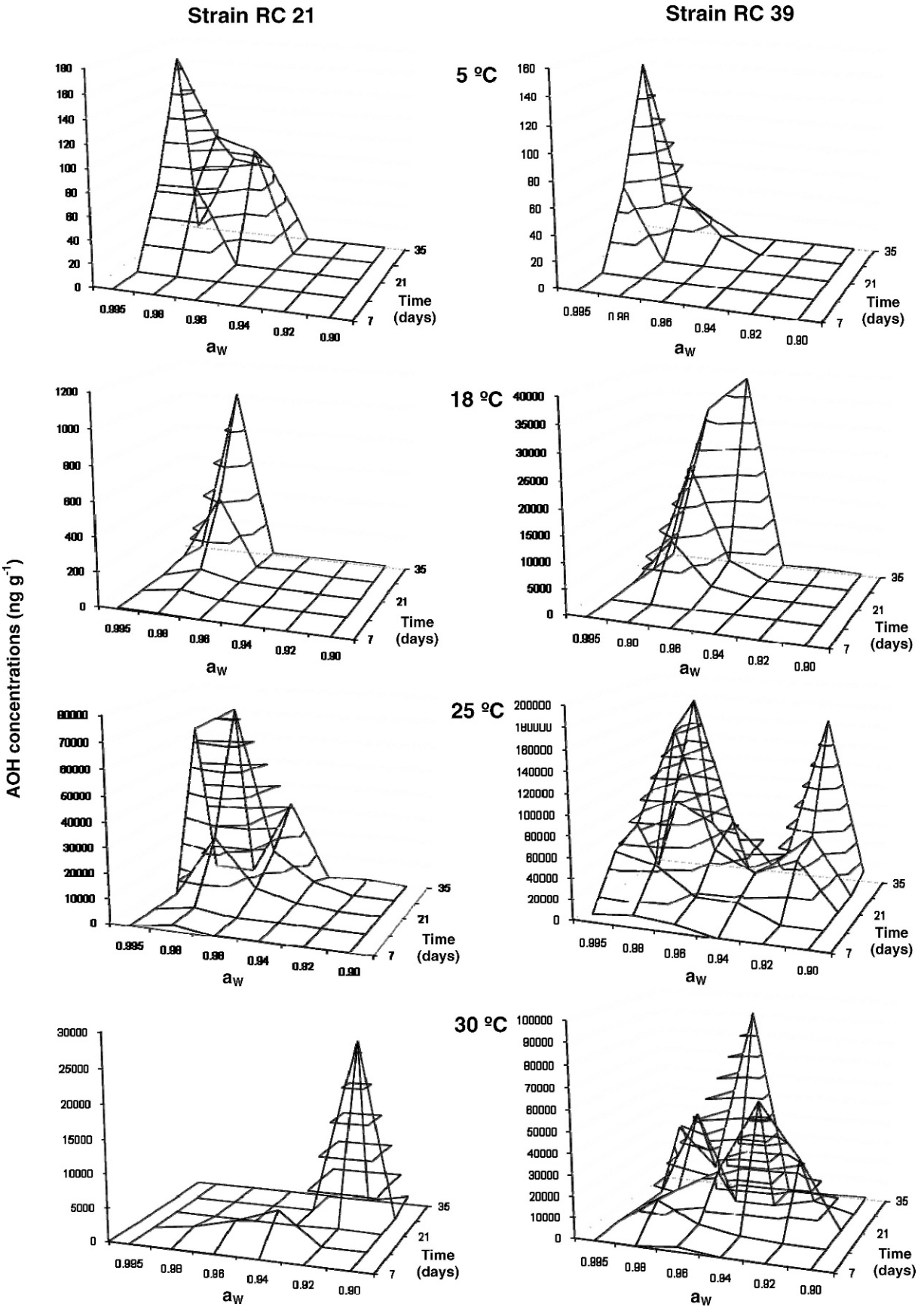

FIGURE 3. *Alternariol (AOH) concentrations (nanograms per gram) produced by two* Alternaria alternata *strains (RC 21 and RC 39) inoculated onto 2% soybean extract agar adjusted to different a_w levels and incubated at 5, 18, 25, and 30°C for 35 days.*

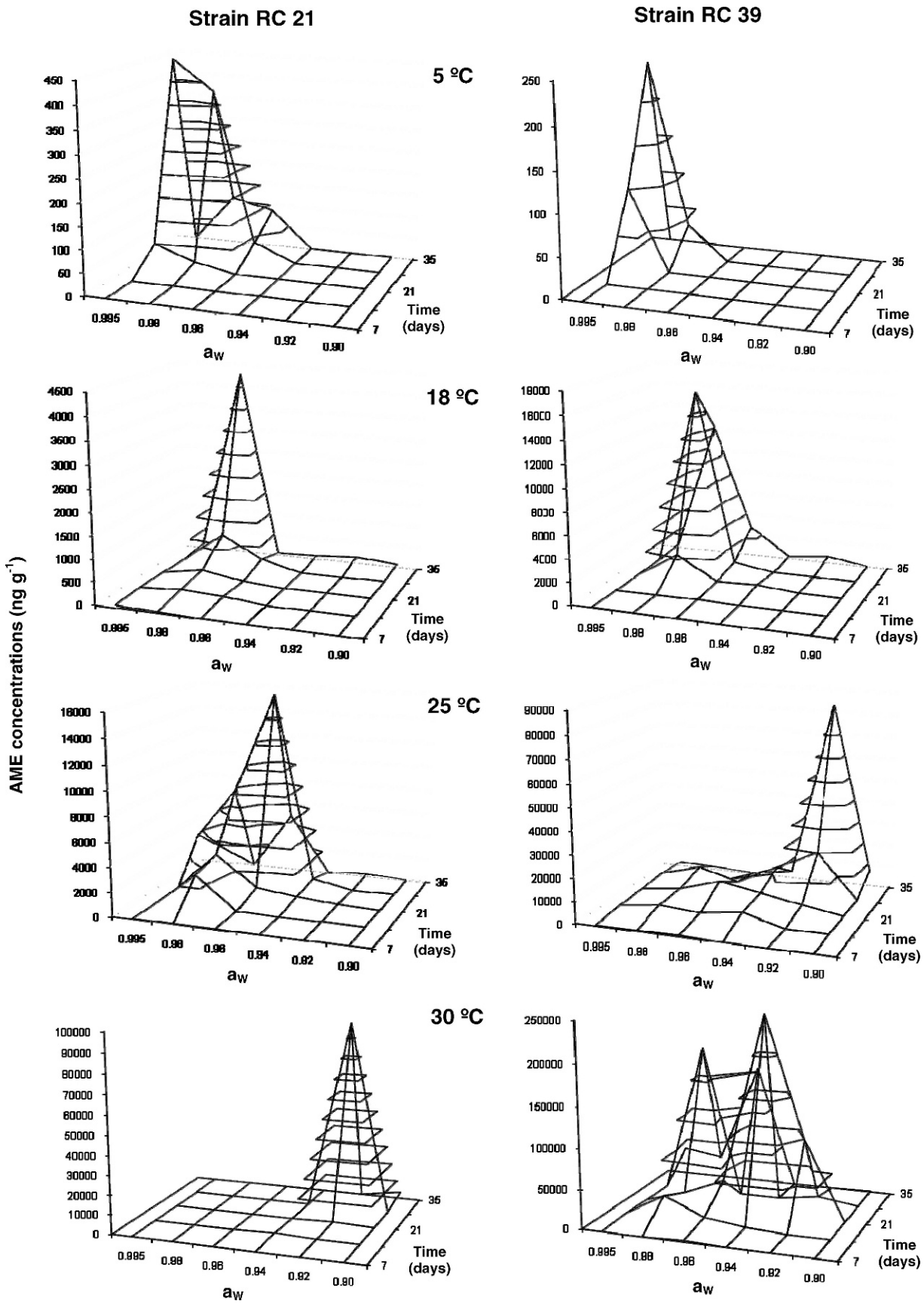

FIGURE 4. *Alternariol monomethyl ether (AME) concentrations (nanograms per gram) produced by two* Alternaria alternata *strains (RC 21 and RC 39) inoculated onto 2% soybean extract agar adjusted to different a_w levels and incubated at 5, 18, 25, and 30°C for 35 days.*

At 25°C, maximum AOH production was obtained at 0.98 a_w after 35 days of incubation for both strains. In general both strains were able to produce AOH at 0.995, 0.96, and 0.94 a_w after 14 days of incubation. The production of high amounts of AOH at 0.92 a_w was observed for RC 39. At the same temperature (25°C), maximum AME production was obtained at 0.96 a_w for RC 21. High AME production also was obtained at 0.98 and 0.995 a_w after 28 days of incubation. For RC 39, AME production increased as the a_w decreased, reaching a maximum at 0.92 a_w after 35 days of incubation.

At 18°C, similar trends for AOH and AME production were observed for both strains. The concentrations of both toxins decreased in comparison to those observed at 25 and 30°C. Maximum amounts of AOH and AME were obtained at 0.98 a_w after 35 days of incubation. Very low amounts of both toxins were obtained at 0.995 and 0.96 a_w.

At the lowest temperature assayed (5°C), similar trends for AOH and AME production were observed for both strains. Maximum concentrations of AOH and AME were reached at 0.995 a_w and decreased as the a_w decreased. Very low concentrations of toxins were produced at 5°C in comparison to the other temperatures tested.

All single variables (a_w, temperature, and days of incubation) and the two- and three-way interactions significantly influenced AOH and AME production for both strains (data not shown).

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

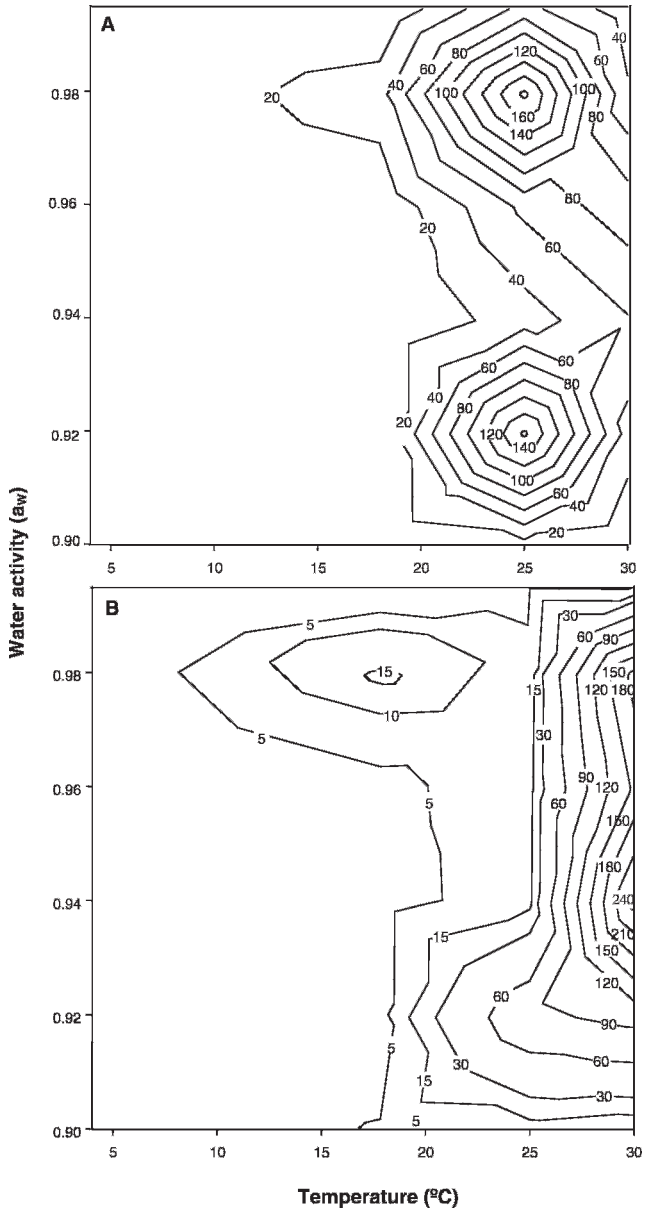

FIGURE 5. *Contour maps for two strains of* Alternaria alternata *showing the relationship between water activity and temperature. The numbers on the contour lines refer to mean concentrations (micrograms per gram) of alternariol (A) and alternariol monomethyl ether (B).*

DISCUSSION

This study compared the impact of a_w and temperature on growth and AOH and AME production on soybean-based media by two strains of *A. alternata* isolated from soybeans in Argentina. Both variables affected growth, and the pattern obtained was independent of the strains evaluated. Optimal a_w levels for growth were 0.995 to 0.92 with an optimal temperature of 25°C. No growth was observed at 5°C at 0.90, 0.92, and 0.94 a_w. Both strains were able to grow slowly at the minimum a_w level assayed (0.90 a_w) at 18, 25, and 30°C. Previous studies on the water and temperature requirements for *A. alternata* growth have revealed that this fungus requires a minimum of 0.84 to 0.88 a_w, and optimal growth was obtained at 0.98 to 1.0 a_w at 25°C *(16, 17).*

Under field conditions, temperature fluctuations, changes in relative humidity, and rainfall all influence the colonization of developing grain by *A. alternata*. The a_w is particularly critical because in the field there is a period of 45 days between pod formation and seed maturity when the water content is appropriate for growth and mycotoxin formation by this species. *Alternaria* spp. have been isolated in very high frequency during different reproductive stages of soybean development in Argentina. During stage R3 (pod formation), 70% of flowers and pods were contaminated with *Alternaria* spp. In stage R6 (full seed), 95% of all immature seed sampled were contaminated; the a_w of the immature seeds was 0.992. Although at stage R8 (full maturity) the water content of the seeds dropped dramatically (0.7 a_w), *Alternaria* spp. continued to be the predominant fungi isolated (60%) *(3).* Because *Alternaria* spp. are present on soybeans for long periods through different reproductive stages in which a_w varies, it is important to know the a_w range for optimal and suboptimal growth of this fungus to predict the risk for AOH and AME production.

In the present study, maximum amounts of AOH were produced at 25°C and 0.98 a_w for both strains evaluated. Maximum AME production was obtained for both strains at 30°C, but different a_w levels (0.92 and 0.94 for the strains RC 21 and RC 39, respectively). In general, all these conditions of a_w and temperature were not optimal for

growth of both isolates. The range of both toxin concentrations differed considerably depending on a_w and temperature interactions. Both metabolites were produced over the temperature range from 5 to 30°C and an a_w range from 0.995 to 0.92. At 5 and 18°C, little mycotoxin was produced at a_w lower than 0.94 a_w. The limiting a_w for detectable mycotoxin production by *A. alternata* was slightly greater than that for growth. The results indicate that AOH and AME production by *A. alternata* is favored at different temperatures.

Few studies have been conducted to examine the effect of different environmental conditions on mycotoxin production by *A. alternata* on natural substrates or on media based on natural substrates *(17, 30),* but no previous studies have been done on soybean-based media. Magan and Lacey *(17)* found an effect of temperature (5, 15, 25, and 30°C) and a_w (0.98, 0.95, and 0.90) on production of AOH, AME, and altenuene by *A. alternata* on both wheat extract agar and wheat grain. All three toxins were produced optimally at 25°C and 0.98 a_w on both substrates. All toxins were produced at 5 to 30°C on agar, and no AME was produced at 30°C and 0.95 or 0.90 a_w. At all temperatures, much more toxin was produced at 0.98 a_w than at lower levels. Changing temperature and a_w altered the relative amounts of the different toxins produced on agar, and peak production occurred at different incubation times: peak AOH after 15 days, peak AME after 30 days, and peak altenuene after 40 days.

Torres et al. *(30)* found an 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 sunflowers seeds. The optimal temperature for production of both toxins was 25°C and 0.90 a_w. Results of previous studies are difficult to compare with our results because the substrates and the range of a_w and temperature were different from those evaluated in the present work.

In a previous study, working with the same *A. alternata* strains we were able to demonstrate the effect of interacting environmental conditions on TA production on soybean-base media *(20)*. Maximum TA production was obtained for both strains at 0.98 a_w but at 30 and 25°C for strains RC 21 and RC 39, respectively. The toxin concentration varied considerably depending on a_w, temperature, incubation time, and strain interactions. TA was produced from 5 to 30°C and at a_w levels of 0.92 to 0.995; however, at 5 and 18°C, little TA was produced at a_w levels below 0.94. Contour maps were developed from these data to identify areas where conditions indicate a significant risk for TA accumulation. Taking into account these previous and the present results, it appears that different combinations of a_w and temperature are necessary for optimal production of these three toxins by *A. alternata* and that the limiting a_w for detectable mycotoxin production is slightly greater than that for growth.

In the present study, an understanding of the interacting environmental conditions provides useful information for predicting the possible risk factors for AOH and AME contamination of soybeans. The a_w and temperature ranges evaluated in this study simulate those occurring during soybean ripening. The data indicate the contrasting impact of a_w, temperature, and incubation time on growth and production of AOH and AME by the two *A. alternata* strains examined. The most important finding is that the temperature and a_w for maximum AOH production are different from those for AME production. An understanding of AOH and AME production under marginal or suboptimal temperature and a_w conditions for growth can be important because improper storage conditions accompanied by elevated temperature and moisture content in soybeans can favor further mycotoxin production and lead to reduction in soybean quality.

Because *A. alternata* has been isolated in very high frequency from soybeans around the world, the contour maps constructed in the present study for growth and production of AOH and AME may provide useful guidelines for facilitating prediction of risk for growth and mycotoxin production during ripening, harvesting, and storage of soybeans.

Results obtained on culture media cannot be readily extrapolated to natural systems because the reactions in the field can be modified by the ecosystem *(1)*. However, these preliminary results can provide an indication of the growth patterns of these fungi as affected by environmental conditions and are a first step for further experimental design under field conditions. Studies working directly with soybean seeds are in progress.

In conclusion, *A. alternata* may grow at all temperatures tested in this study (5 to 30°C); optimal growth occurred between 25 and 30°C, a temperature range that occurs commonly during soybean seed development in the field. However, at 5°C the growth of both *A. alternata* isolates was negligible when a_w levels were low (0.90, 0.92, and 0.94). The optimum a_w for growth was 0.995, similar to the a_w of immature soybean seed in the field. Maximum amounts of AOH and AME were obtained at 25 and 30°C. Both strains were able to produce high amounts of toxins at 18 and 5°C. The optimum a_w for AOH production was 0.98. However, the a_w for maximum AME production was 0.92 or 0.94, depending on the strain. All the conditions of a_w and temperature that resulted in maximum production of both toxins occur frequently during soybean development in the field. Thus, field conditions are likely to be conducive to optimum growth of and toxin production by this species.

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