Influence of environmental parameters on mycotoxin production by *Alternaria arborescens*

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ABSTRACT

Alternaria arborescens has been reported as a common fungal species invading tomatoes and is capable of producing several mycotoxins in infected plants, fruits and in agricultural commodities. Alternariol (AOH), alternariol monomethyl ether (AME), and tenuazonic acid (TeA) are some of the main *Alternaria* mycotoxins that can be found as contaminants of food. This species can produce these toxic metabolites together with AAL toxins (*Alternaria alternata* f. sp. *lycopersicum* toxins), which can act as inhibitors of sphingolipid biosynthesis. The objective of this study was to determine the effect of water activity (a_w, 0.995, 0.975, 0.950) and temperature (6, 15, 20, 25 and 30 °C) on mycotoxin production by *A. arborescens* on a synthetic tomato medium. The optimum production of AOH and AME occurred at 0.975 a_w after 40 days of incubation at 30 °C. The maximum TeA accumulation was observed at 0.975 a_w and 25 °C and at 0.950 a_w and 30 °C. AAL TA was produced in higher quantities at 0.995 a_w and 30 °C. At 6 °C no quantifiable levels of AOH or AME were detected, but significant amounts of TeA were produced at 0.975 a_w. In general, high a_w levels and high temperatures were favorable for mycotoxin production. The greatest accumulation of all four toxins occurred at 0.975 a_w and 30 °C. The results obtained here could be extrapolated to evaluate the risk of tomato fruits and tomato products contamination caused by these toxins.

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1. Introduction

The *Alternaria* genus is widely distributed in soil and decaying organic matter. Many species are plant pathogens and saprophytes that may affect crop plants in the field or cause pre- and postharvest spoilage. As it is common for many soft-skinned vegetables and fruits, tomatoes are especially susceptible to fungal invasion and *Alternaria* spp. have been reported to be the most frequent fungal species invading tomatoes (Barkai-Golan and Paster, 2008).

This fungal genus is known to produce a wide range of mycotoxins which present different degrees of toxicity. The importance of these toxic metabolites is markedly increasing because many plant products incorporated in a large proportion in human and animal diets are frequently infected by species of *Alternaria* capable of mycotoxin production (Moressi et al., 1999). Due to their growth even at low temperature, *Alternaria* species are also responsible for spoilage of these commodities during refrigerated transport and storage (EFSA, 2011).

The chemical and toxicological aspects of the *Alternaria* toxins have been recently reviewed (Dall'Asta et al., 2014; Pavón Moreno et al., 2012). Among the *Alternaria* mycotoxins, tenuazonic acid (TeA), alternariol (AOH), and alternariol monomethyl ether (AME) are the

most frequently detected on plants. TeA is a metabolite characterized by toxicity towards animals (Ostry, 2008); it is acutely toxic for several animal species such as mice, chicken and dogs (Dall'Asta et al., 2014), and it is considered as a possible causal factor of Onyalai, a human hematological disorder (Steyn and Rabie, 1976). TeA has been also shown to inhibit protein production and cell proliferation in three mammalian cell lines (Zhou and Qiang, 2008). AOH and AME, two toxins frequently found in combination as they share a common biosynthesis pathway, were reported to be mutagenic (Schrader et al., 2001), and their DNA-damaging properties have been demonstrated in several mammalian cell lines (Fehr et al., 2009; Fernández-Blanco et al., 2015).

Particularly, *A. arborescens*, the causal agent of tomato stem canker, can produce AAL-toxins (*Alternaria alternata* f. sp. *lycopersicum* toxins). These toxins were found to reproduce the symptoms of the disease when they were tested on susceptible genotypes of tomato leaf (Oikawa et al., 1999). AAL toxins are sphinganine analog mycotoxins (SAMs), structurally related to fumonisins, which can act as inhibitors of sphingolipid biosynthesis. The cellular effects of AAL-toxin exposure have been studied and it was found to be toxic to rat hepatoma cell lines and dog kidney cells (Shier et al., 1991); however, the toxicological impact on humans is still unclear.

Natural occurrence of AOH and AME has been reported in various fruits, including tomatoes, olives, mandarins, melons, peppers, apples and raspberries, and also in grains, sunflower seeds, oilseed rape and

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pecans. The occurrence of low levels of AOH in processed fruit products, such as apple juice, processed tomato products, grape juice, red wine, cranberry nectar, prune nectar and raspberry juice, is of human health interest. AME has also been detected in apple juice, prune nectar and tomato products (Scott, 2004; Terminiello et al., 2006). TeA has been shown to occur in several *Alternaria* infected fruits and vegetables, and in other foodstuffs, such as grains and seeds (Azcarate et al., 2008; Scott, 2001). It has also been found in tomato products and spoiled tomatoes in Canada and the US (Scott and Kanhere, 1980; Stack et al., 1985), in Brazilian tomato products (da Motta and Valente Soares, 2001) and in Argentinean tomato puree (Terminiello et al., 2006). To our knowledge, the natural occurrence of AAL toxins has only been reported in maize silage (Mansfield et al., 2007).

Mycotoxin production depends on the fungal strain, the substrate on which it grows and the environmental conditions, the two most important of which are water activity (a_w) and temperature. Furthermore, the a_w and temperature limits for growth and mycotoxin production are sometimes markedly different (Magan et al., 1984). For this reason, knowledge of the influence of environmental factors on growth and mycotoxin biosynthesis can be an important tool in predicting mycotoxin contamination of food. The production of TeA, AME, ALT and AOH by *Alternaria* species in relation to these factors have been described in different substrates including tomatoes (Magan et al., 1984; Oviedo et al., 2010; Pose et al., 2010), but all of these studies were focused on *A. alternata*.

There are currently no statutory or guideline limits set for *Alternaria* mycotoxins, although their relevance in food and feeds is currently under discussion. The European Food Safety Authority published a report on the risks of *Alternaria* toxins for animal and public health (EFSA, 2011), concluding that between the main vegetables and vegetable products contributing to dietary exposure to *Alternaria* mycotoxins, tomato and tomato products are of particular concern. They also stated that there are not enough relevant data on toxicity of these mycotoxins, and more information is needed on their toxicokinetics, occurrence, and influence of food and feed processing to enable their risk assessment.

The objective of this work was to study the effects of a_w and temperature on the production of AOH, AME, TeA and AAL TA by *A. arborescens* on a synthetic tomato medium with the aim of providing more data on the influence of environmental factors on contamination of tomato products by *Alternaria* toxins.

2. Materials and methods

2.1. Fungal strains

A representative strain of *A. arborescens* (EGS 39128) from the culture collection of Emory G. Simmons (Mycological Services, Crawfordsville, IN, USA), which was isolated from tomato stem lesion, was used in this study. It was inoculated on Potato Carrot Agar (PCA) (Simmons, 1992) and grown under an alternating light/dark cycle consisting of 8 h of cool-white daylight followed by 16 h darkness for 7 days at 25 °C in order to promote sporulation.

2.2. Medium

Toxin production was determined on tomato pulp agar (TPA) designed for this purpose in a previous work (Pose et al., 2009). This medium contained 800 ml/l of pulp of fresh tomatoes, 200 ml distilled water and 15 g agar. The a_w of the medium was adjusted with glycerol 87% analytical grade (Merck 4094) to 0.950; 0.975 and 0.995 ± 0.003. Water activity was measured with a water activity meter (Aqualab).

2.3. Inoculation and incubation

Spores of 7-day-old cultures grown in PCA were placed in an aqueous solution of a_w adjusted with glycerol to avoid affecting the a_w of the culture medium. After dispersal of mycelium and conidial chains, the suspension was counted using a Neubauer chamber. TPA plates were inoculated centrally with a 1 μl calibrated loop of a suspension consisting of 5.5×10^5 spores/ml. The plates were incubated at 6, 15, 20, 25 and 30 °C for a maximum period of 40 days. To minimize water transfer from or to the medium, plates with the same a_w level were placed in closed bags containing a vessel with adjusted glycerol–water solution (Romero et al., 2007). Control plates were prepared and measured at the end of the experiment in order to detect any significant deviation of the a_w, and no change in any tested plate was detected. Each set of conditions ($a_w \times$ temperature) was run in triplicate.

2.4. Mycotoxin extraction

AOH, AME and TeA were determined by a modification of the method described by da Motta and Valente Soares (2000a, 2000b). Briefly, a 10 g portion of tomato pulp agar cultures was weighed and transferred to blender cup with the aid of 25 ml methanol. It was blended at low speed for 3 min, then combined with 5 ml of a 10% ammonium sulfate solution and filtered. The filtrate was transferred to a separating funnel, and 16 ml hexane was added. The mixture was gently shaken for 1 min and the hexane phase was discarded after separation. Twenty ml of water at 8 °C were added to the methanolic phase in order to avoid forming an emulsion. Two extractions with 16 ml chloroform were conducted. The chloroform extract was evaporated in a rotary evaporator at 35 °C. The residue was dissolved in 1 ml HPLC grade methanol and was analyzed for AOH and AME. The methanolic phase was acidified to pH 2 with HCl 6 N. Two extractions with 16 ml chloroform were made. All the chloroform was collected in a separating funnel and washed with 12 ml water. The chloroform extracts were collected and evaporated in a rotary evaporator at 35 °C. The residue was dissolved in 1 ml HPLC grade methanol and was analyzed for TeA.

AAL toxins TA1 and TA2 were determined by the method described by Solfrizzo et al. (2005). A 10 g portion of tomato pulp agar cultures was weighed and extracted with 30 ml acetonitrile–water (50:50) acidified with 0.5 M HCl at pH 3. After shaking for 30 min in an orbital shaker at 300 rpm, the samples were filtered. A 2.4 ml aliquot of the filtrate was mixed with 7.6 ml 1% potassium chloride solution and the pH of the solution was adjusted to 3, where necessary, with HCl 6 N. An Oasis® HLB column (Waters) was conditioned by washing successively with 2 ml acetonitrile and then with 2 ml 1% potassium chloride solution. An aliquot of 10 ml diluted sample extract was applied to the SPE column and the eluate was discarded. The SPE column was washed successively with 2 ml 1% potassium chloride solution and then with 2 ml acetonitrile–water (15:85) and the washings were discarded. TA toxins were eluted with 2 ml acetonitrile–water (70:30) and the eluate was collected in a glass vial. An aliquot of the purified sample extract was derivatized with OPA reagent and analyzed by C18 reversed phase HPLC with fluorometric detection.

2.5. HPLC determination

AOH, AME and TeA were detected by HPLC as described by Pose et al. (2010). Standards and extracts were injected into an HPLC system consisting of a Shimadzu LC-CA liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Rheodyne sample valve fitted with a 20 μl loop and a Shimadzu UV detector Model SPD-6A. The analytical column was Jupiter 4.6×250 mm 5 μ C18 (Phenomenex, USA). The mobile phase was methanol/water (80:20) containing 300 mg $ZnSO_4.H_2O$/l, for AOH and AME, and methanol/water (90:10) containing 300 mg $ZnSO_4.H_2O$/l, for TeA. A flow rate of 1 ml/min was used. The wavelengths for recording chromatograms were 258 nm for AOH and AME, and 280 nm for TeA. Standards of TA (as a copper salt), AME and AOH were purchased from Sigma-Aldrich (St. Louis, MO, USA). From all solid standards, individual stock solutions of 0.5 mg/ml were prepared in methanol and stored at −18 °C. The copper salt was reconverted to
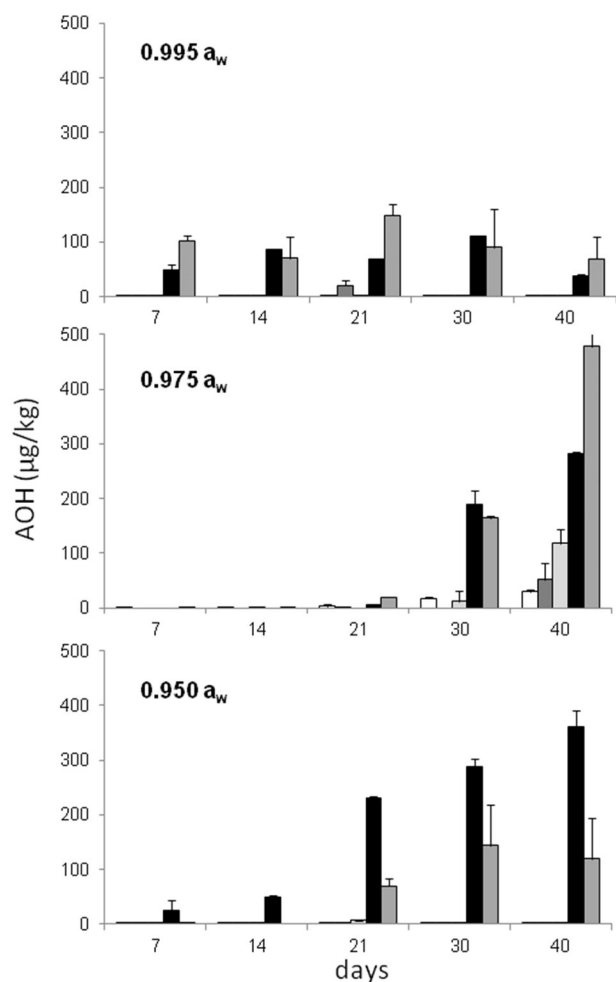
Fig. 1. Alternariol (AOH) accumulation (μg/kg) by *A. arborescens* under three water activity levels (0.995, 0.975, 0.950) at different temperatures (□ 6 °C, ▪ 15 °C, □ 20 °C, ■ 25 °C, ▪ 30 °C) and incubation times. Bars indicate standard errors of the mean.
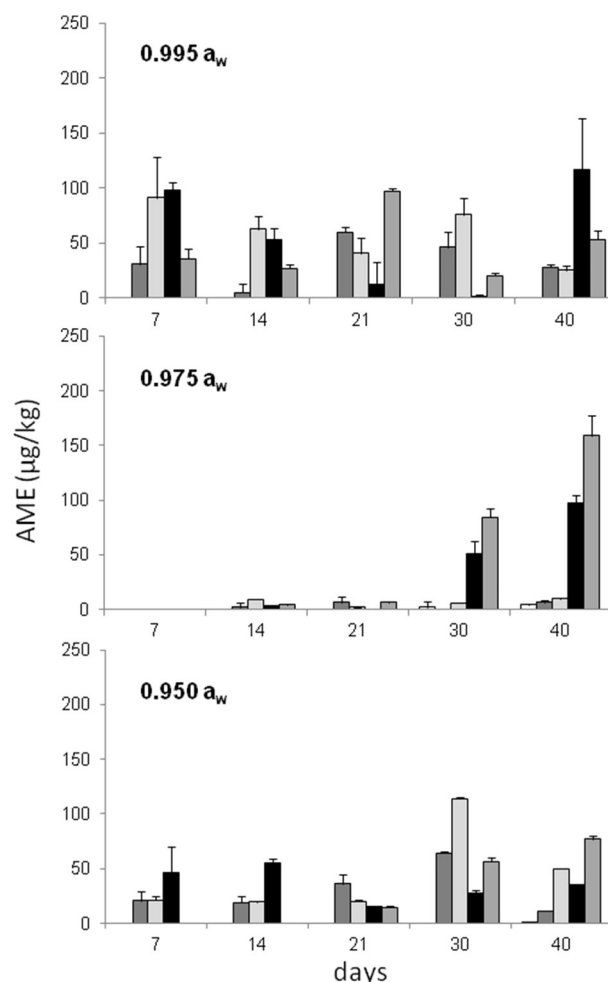
Fig. 2. Alternariol monomethyl ether (AME) accumulation (μg/kg) by *A. arborescens* under three water activity levels (0.995, 0.975, 0.950) at different temperatures (□ 6 °C, ▪ 15 °C, □ 20 °C, ■ 25 °C, ▪ 30 °C) and incubation times. Bars indicate standard errors of the mean.

tenuazonic acid as described by Scott and Kanhere (1980). Working standard solutions of 5 μg/ml of each toxin were then prepared. A calibration curve was constructed for quantification purposes using the toxin standards and correlating peak area versus concentration (levels 100 to 1000 μg/kg). Confirmation of all toxins was achieved by co-injection with the corresponding standard and by using a Shimadzu SPD-M10Avp photodiode array detector and comparing the UV spectra with the corresponding standards. Quantification limits (LOQs) were determined as five times the noise, which was calculated as six times the standard deviation of the lineal regression of the drift at the selected time range. LOQs were 5 μg/kg for AOH, 3 μg/kg for AME, and 8 μg/kg for TA.

Detection and quantification of AAL TA toxins was carried out in the same HPLC system using a Shimadzu fluorescence detector RF-10AXL set at 335 nm (excitation) and 440 nm (emission). The mobile phase consisted of a mixture of acetonitrile–50 mM sodium dihydrogen phosphate solution (43:57) adjusted to pH 3.35 with o-phosphoric acid and eluted at 1 ml/min. AAL toxins TA (TA1 and TA2) were purchased from the Medical Research Council, South Africa. A stock solution was made by dissolving 1 mg of solid standard in 1 ml acetonitrile–water (50:50). Working standard solutions (range 0.03–1 μg/ml) were prepared by appropriate dilution of the stock standard and used to obtain calibration curves for quantification by HPLC-FD. The two isomers of AAL TA eluted as two separate peaks with retention times of 6.6 min (AAL TA$_1$) and 7.2 min (AAL TA$_2$). For the quantification of AAL TA, the sum of the two peak areas was considered (Solfrizzo et al., 2005). LOQ

for AAL TA, calculated as six times the standard deviation of the lineal regression of the drift at the selected time range, was 0.05 μg/g.

2.6. Statistical analysis

The effects of a_w, temperature and incubation time on AOH, AME, TA and AAL TA accumulation by *A. arborescens* were analyzed statistically by ANOVA using Statistica software v6.0 (StatSoft, Inc., 1984–2001, Tulsa, OK, USA). Means were compared by Tukey test to determine significant differences between the treatments assayed.

3. Results

The effects of a_w, temperature and incubation time on mycotoxin production by *A. arborescens* on tomato medium are shown in Figs. 1 to 4. Due to the high difference in concentration between the four toxins, different units were used in the figures to adjust the scale.

The ANOVA of the effect of a_w, temperature, incubation time, and their interactions showed that all factors alone and all the interactions were statistically significant in relation to AOH production ($p < 0.01$), and to AME, TeA and AAL TA production ($p < 0.0001$).

Growth at 0.950 a_w and 6 °C was very slow, and no only trace levels of toxin were detected at these conditions, except for AAL TA, which was produced in a concentration of 7.2 μg/g after 40 days of incubation.
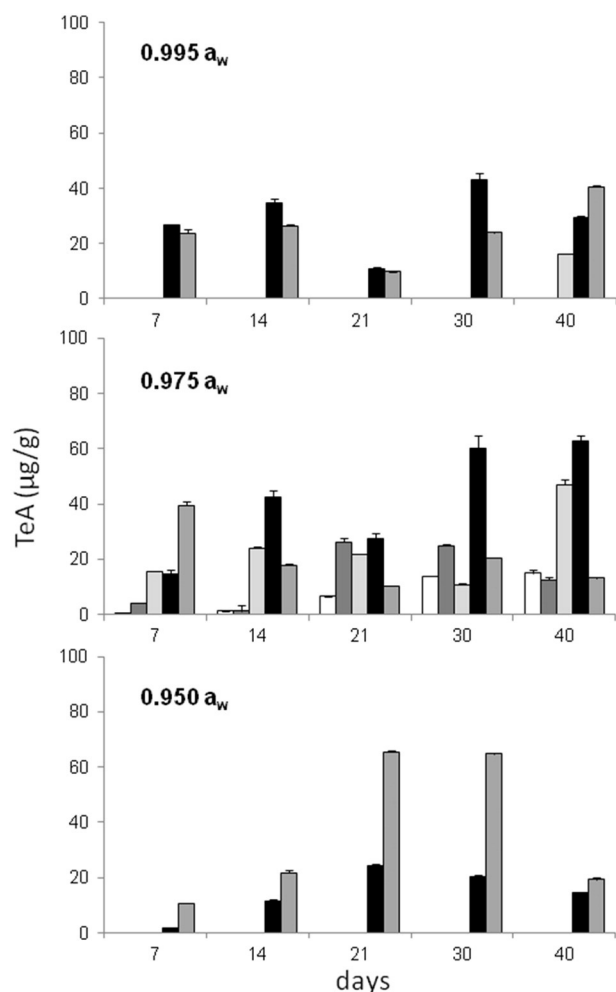
Fig. 3. Tenuazonic acid (TeA) accumulation (μg/g) by *A. arborescens* under three water activity levels (0.995, 0.975, 0.950) at different temperatures (□ 6 °C, ▣ 15 °C, ☐ 20 °C, ■ 25 °C, ▦ 30 °C) and incubation times. Bars indicate standard errors of the mean.
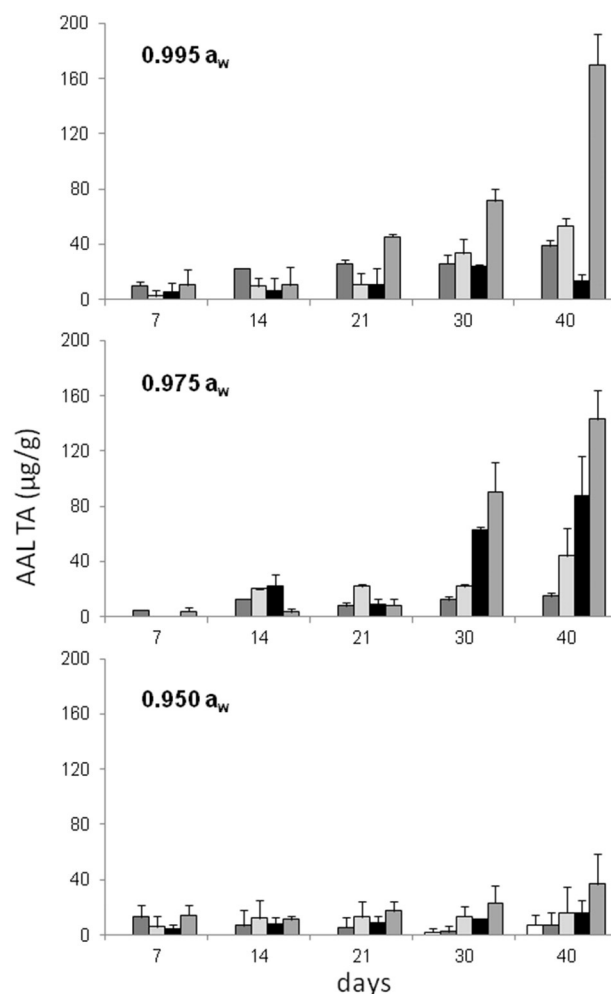
Fig. 4. AAL TA accumulation (μg/g) by *A. arborescens* under three water activity levels (0.995, 0.975, 0.950) at different temperatures (□ 6 °C, ▣ 15 °C, ☐ 20 °C, ■ 25 °C, ▦ 30 °C) and incubation times. Bars indicate standard errors of the mean.

The optimum conditions for production of the four toxins are summarized in Table 1. For AOH and AME production they were 0.975 a_w and 30 °C, with a total accumulation of 477 μg/kg and 159 μg/kg respectively after 40 days of incubation. TeA was optimally produced at 0.975 a_w and 25 °C (62.8 μg/g, 40 days) and at 0.950 a_w and 30 °C (65.4 μg/g, 21 days), with no significant differences between toxin concentration at both set of conditions. Unlike the other toxins, the optimum a_w level for AAL TA biosynthesis was the highest evaluated (0.995 a_w), and the maximum accumulation was observed after 40 days of incubation at 30 °C (170 μg/g).

In order to determine the risk of simultaneous co-occurrence of the four toxins at different temperature and a_w levels, the total mycotoxin accumulation after 40 days of incubation on tomato medium was compared (Fig. 5). A high accumulation of all four toxins occurred at 0.975 a_w and 30 °C, although high levels of toxins could also be found at 25 °C at this a_w level. At 0.995 a_w, 30 °C seems to be the most critical temperature for co-occurrence of the four mentioned toxins. Temperatures below 15 °C would not allow a high accumulation of these metabolites; however, AAL TA can still be found in high concentration at the optimum a_w for its production (38.6 μg/g, 0.995 a_w). At 6 °C only TeA was accumulated in significant amounts after 40 days of incubation at 0.975 a_w (14.9 μg/g); the rest of the toxins were produced at lower levels at the same conditions (AOH 30 μg/kg, AME 4 μg/kg).

4. Discussion

To our knowledge this is the first study to evaluate the combined effect of a_w and temperature on mycotoxin production by *A. arborescens*.

The taxonomy of *Alternaria* has been regularly revised in the past decade, with the small-spored species constituting a particularly controversial group. When previous traditional methods, based on morphological characteristics of conidia (shape, size, color, ornamentation, etc.) were applied, most of the isolates were classified as *A. alternata*, thus leading to a general belief that this is the most common species in food products. In consequence, the few ecophysiology studies on *Alternaria* available in the literature have been developed for *A. alternata* (or cultures identified as *A. alternata*).

Table 1
Optimum conditions for alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA) and AAL TA biosynthesis by *Alternaria arborescens*.

Toxin	Temperature (°C)	Water activity (a_w)	Time (days)
AOH	30	0.975	40
AME	30	0.975	40
TeA	30*	0.950*	21–30*
	25*	0.975*	30–40*
AAL TA	30	0.995	40

(*) No significant differences were observed between toxin concentration produced at both set of conditions.
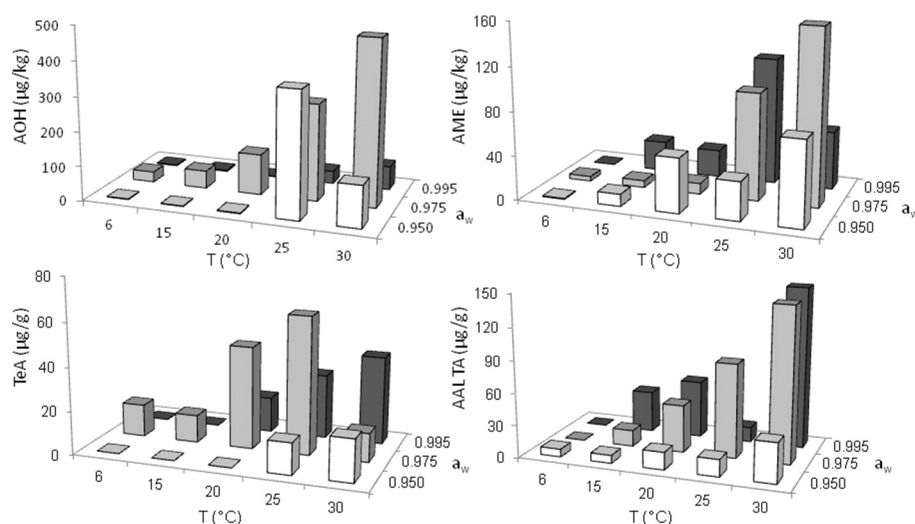
Fig. 5. Total mycotoxin accumulation produced by *A. arborescens* after 40 days of incubation on tomato medium. Please note different concentration units for the four toxins.

Since new taxonomic tools were implemented, other species have been reported as predominant in cereals, fruits and vegetables. In particular, *A. alternata* has been reported as the main pathogen in tomatoes for many years, and the "black mold" disease was attributed to this species as the primary causal agent (Hasan, 1995; Logrieco et al., 2003; Morris et al., 2000). More recent works indicated that other species could also infect tomatoes, such as isolates belonging to *Alternaria tenuissima* species-group and *A. arborescens* sp.-grp. (Andersen and Thrane, 2006; Benavidez Rozo et al., 2014; Somma et al., 2011). *A. arborescens* (Simmons, 2007), previously classified as the *A. alternata* tomato pathotype (*A. alternata* f. sp. *lycopersici*), was only referred to as the causal agent of tomato stem canker. However, in previous studies from our group, *Alternaria* strains isolated from the black fruit lesion were identified as *A. arborescens*, demonstrating its pathogenicity on the fruit as well (Benavidez Rozo et al., 2014; Somma et al., 2011).

Even though *A. alternata* and *A. arborescens* are closely related, their ecophysiology might differ. A previous work (Vaquera et al., 2014) showed that *A. arborescens* had an optimum growth temperature of 30 °C which is higher than that reported for *A. alternata* (21 °C). Regarding toxin production, Pose et al. (2010) analyzed the effect of temperature and a_w on mycotoxin production by *A. alternata* isolated from tomatoes (See Table 2). AOH biosynthesis by *A. alternata* was optimum at 0.954 a_w and 21 °C, while for *A. arborescens* the optimum a_w level detected in this work was 0.975, with 477 μg/kg of AOH detected at 30 °C, although a high concentration of AOH was also produced at 0.95 and 25 °C (361 μg/kg).The biosynthesis of AME by *A. alternata* was optimum at 0.954 a_w and 35 °C; for *A. arborescens*, high temperatures (30 °C) also proved to be more favorable for its accumulation, although a significantly higher concentration of this toxin was detected at 0.975 a_w than at 0.95 a_w. TeA was optimally produced by *A. alternata* at 0.982 a_w and

21 °C, meanwhile for *A. arborescens* lower water activities (0.975, 0.95) and higher temperatures (25, 30 °C) resulted in higher toxin accumulation. Hasan (1995) studied the influence of temperature on mycotoxin production by *A. alternata* when cultured on liquid and tomato homogenate. The optimum for AOH and AME was 28 °C in both cultures, which are in concordance with our results for *A. arborescens*, but TeA was produced in large quantities at 21 °C by *A. alternata*, while *A. arborescens* accumulated more TeA at higher temperatures (25–30 °C). *A. alternata* was also capable of growing at 7 °C and of producing all toxins in reasonable amounts; meanwhile *A. arborescens* could grow at 6 °C at all a_w levels studied but only TeA was detected in considerable amounts at this temperature and 0.975 a_w after incubating for 21–40 days (range 6.5–14.9 μg/g).

Other works on *A. alternata* isolated from substrates other than tomato reported an optimum production of AOH in the ranges 15–25 °C and 0.95–0.98 a_w; 30–35 °C and 0.92–0.98 a_w for AME, and 21–30 °C and 0.98 a_w for TeA (Magan et al., 1984; Oviedo et al., 2010, 2011). Comparisons are difficult due to the high diversity observed, which could be explained by the source, geographical and intraspecific differences of strains. However, in general, higher temperatures were determined as optimum for toxin production by *A. arborescens*, especially for AOH biosynthesis. Further studies with strains isolated from different substrates are necessary to evaluate if this particular behavior is characteristic of the whole species, or only related to tomato pathogens. These differences between both species in relation to environmental parameters are important to design prevention and control strategies. It is also relevant information to establish regulatory limits for *Alternaria* toxins in tomato and industrial tomato products.

Currently, no data are available in the literature regarding water activity and temperature effect on production of AAL toxins in vitro or in vivo. These fungal metabolites were first described by Gilchrist and Grogan (1976), and were found to produce foliar interveinal necrosis, being toxic to certain genotypes of tomato. According to Gilchrist et al. (1992) the toxins involved in stem canker disease of tomato were not produced in culture by non-pathogenic isolates of *A. alternata*, thus AAL toxin production was attributed to the tomato pathotype (*A. alternata* f. sp. *lycopersici*, syn. *A. arborescens*). Our results showed that a combination of high a_w and temperature (0.995, 30 °C) led to a considerable accumulation of AAL TA in tomato medium, and even at a lower a_w level (0.975), high amounts of toxin were detected after 40 days incubation at 30 °C (143 μg/g). As *A. arborescens* is an important tomato pathogen which can cause fruit decay, these toxic metabolites can accumulate in the fruits, and represent a potential risk when moldy fruits are destined to industrial by-products. Their toxic effects

Table 2
Comparison of optimum conditions for toxin biosynthesis by *Alternaria alternata* and *A. arborescens*.

	A. alternata[**]		A. arborescens	
	a_w	T (°C)	a_w	T (°C)
AOH	0.954	21	0.975	30
AME	0.954	35	0.975	30
TeA	0.982	21	0.975*	25*
			0.950*	30*

(*) No significant differences were observed between toxin concentration produced at both set of conditions.
(**) Data from Pose et al. (2010).

on plants have been studied, but little is known of their toxicological potential on animals and humans.

The range of temperatures studied was selected representing ambient temperatures at which tomato fruits are stored in warm temperate regions, in the different seasons (30 °C, 25 °C, 20 °C, and 15 °C, in summer, autumn and winter respectively), and 6 °C was selected as a refrigeration temperature. Our results showed that warm storage temperatures increase the risk of contamination with *Alternaria* mycotoxins, especially in fruits or high a_w products. However, derived products of intermediate a_w, such as tomato sauces or tomato puree (0.98–0.97 a_w), are also susceptible to accumulate high amounts of toxins. Although the fungus is inactivated during thermal processes, the raw material used for elaboration of tomato products (tomato paste) is prone to contamination if it is not stored at adequate temperatures before processing. At this stage mycotoxin accumulation could increase. Even though storage at refrigeration temperatures significantly reduces toxin production, some *Alternaria* toxins, such as TeA can accumulate if the storage period exceeds 14 days. These results are in agreement with Hasan (1995), who recommended a storage temperature below 7 °C for no longer than 10 days.

Ecophysiological data on simultaneous mycotoxin production by different *Alternaria* species constitute a useful tool for producers to determine safe handling conditions to prevent contamination, and by regulatory authorities to establish monitoring programs on susceptible food products.

Acknowledgments

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