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Temperature and water activity influence on simultaneous production of AAL toxins by *Alternaria arborescens* on tomato medium

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Abstract *Alternaria spp.* have been reported to be the most frequent phytopathogenic fungi invading tomatoes. Certain species are capable of producing several mycotoxins in infected plants and in agricultural commodities. AAL toxins are produced by *Alternaria arborescens,* which is the causal agent of tomato stem canker, and one of the species involved in the tomato black mold. AAL toxins include five congeners, TA, TB, TC, TD and TE, structurally similar. The objective of this study was to determine the effect of water activity (a_w, 0.995, and 0.950) and temperature (6, 15, 20, 25 and 30 °C) on simultaneous AAL toxins production by *Alternaria arborescens.* The maximum accumulation of AAL toxins occurred at 0.995 a_w after 40 days of incubation at 30 °C. None of the toxins was detected at temperatures below 30 °C at any a_w during the whole incubation period. The results obtained here could be extrapolated to evaluate the risk of toxin accumulation in tomato fruit and tomato products.

Keywords *Alternaria arborescens* · AAL toxins · Water activity · Temperature · Tomato

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The genus *Alternaria* contains numerous species that are both saprophytic and pathogenic on many plant species. Certain *Alternaria* species are capable of producing several mycotoxins in infected plants and in agricultural commodities (Logrieco et al. 2009). In particular, *A. arborescens* (syn. *Alternaria alternata* f. sp. *lycopersci*) is a ubiquitous fungus that can be isolated from many plants and other substrata.

Alternaria has been reported to be the most frequent fungal genus invading tomatoes (Andersen and Frisvad 2004). Tomato fruit are easily infected because of their soft skin and weak tissues that allow a rapid penetration and fungal growth (Pitt and Hocking 1997). *A. arborescens* is known to be the causal agent of tomato stem canker and is also one of the species responsible of the tomato black mold, being capable of developing primary infection of leaves, stems and fruit in susceptible cultivars (Caldas et al. 1994, Somma et al. 2011).

AAL toxins, synthesized by *A. arborescens,* together with fumonisins, produced by *Fusarium* spp., are called sphinganine analog mycotoxins (SAMs) due to their similarity to sphinganine, which is the backbone precursor of sphingolipids. AAL toxins are five compounds structurally alike, TA, TB, TC, TD and TE (Fig. 1). Fumonisins, the other SAMs produced by *Fusarium* spp., whose toxicity is widely studied, are highly toxic; they can cause leukoencephalomalacia and pulmonary edema syndrome in animals and are associated to human esophageal cancer and neural tube defects. AAL toxins and fumonisins have shown similar toxicity to plants and mammalian cells and both exhibited

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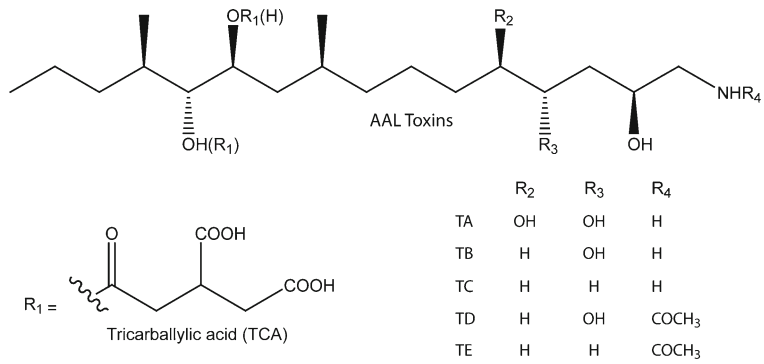

Fig. 1 Chemical structure of AAL toxins

inhibitory activity to ceramide synthase, which is involved in sphingolipid biosynthesis (Tsuge et al. 2012). A survey on the responses of 88 plant species to AAL-toxins revealed that some plants are highly sensitive to them (Dayan and Duke 2014). Because of their high incidence and toxicity, fumonisins are regulated worldwide, but there are no regulations on *Alternaria* toxins at present. More investigations on the toxic potential of these fungal metabolites and their hazard for human consumption are needed.

Mycotoxin production depends on the fungal strain, the substrate on which it grows and the environmental growth conditions. Water activity (a_w) and temperature are the two most important environmental factors on the development of fungi and mycotoxin production (Pose et al. 2010). Knowledge on the influence of environmental factors on growth and mycotoxin formation can be an important aid in predicting food spoilage and mycotoxin contamination. Furthermore, the a_w and temperature limits for growth and mycotoxin production are sometimes markedly different (Pose et al. 2010). Growth and toxin production of *Alternaria* species and its relation with these factors have been described in different substrates (Magan et al. 1984, Oviedo et al. 2010, Pose et al. 2010, Vaquera et al. 2014). In a recent study (Vaquera et al. 2016), factors influencing alternariols, tenuazonic acid and AAL TA production by *Alternaria arborescens* isolated from tomatoes were investigated. However, the incidence of these environmental parameters on the simultaneous production of all AAL toxins (TA, TB, TC, TD and TE) was not determined. Such information is important to evaluate biological activity, assess potential health risks, and address possible economic consequences of food contamination by this family of mycotoxins.

The objective of this study was to evaluate the effects of a_w and temperature on the simultaneous production of AAL toxins by *A. arborescens* on a synthetic tomato medium.

The reference strain *A. arborescens* (EGS 39–128) from the collection of Dr. Emmory G. Simmons, Indiana, USA, isolated from tomato stem lesion, was used in this study. The strain is available in the collection of the CBS Fungal Biodiversity Centre, Utrecht, the Netherlands. It was selected because it is able to synthesize all AAL toxins: TA, TB, TC, TD and TE (Benavidez Rozo et al. 2014, Andersen et al. 2015).

Stock solution of AAL toxin TA of concentration 1 mg/ml in acetonitrile (Merck, Darmstadt, Germany, HPLC grade) was prepared from commercial standard purchased from the Medical Research Council, PROMEC Unit, South Africa.

AAL toxins TB, TC, TD and TE were generated from a culture of *A. arborescens* (EGS 39–128) with the method described as follows: conidia from PCA cultures of the strain were inoculated onto seven media, dichloran rose bengal yeast extract sucrose agar (DRYES), malt extract agar (MEA), nitrite sucrose agar (NS), oat meal agar (OAT), potato carrot agar (PCA), potato sucrose agar (PSA) (Andersen et al. 2002) and Agar Tomato (Pose et al. 2010), in order to ensure all AAL toxins were expressed. All plates were incubated at 25 °C for 14 days. The extraction technique was based on a microscale method (Andersen et al. 2002). The final, dried extract was analyzed by HPLC-MS as it is described below. All AAL toxins were detected (Fig. 2). All molecular ion m/z ratios were obtained and MS/MS spectra (Table 1) were used to confirm identity of mycotoxins.

In order to investigate the influence of environmental parameters on the simultaneous production of AAL toxins, tomato pulp agar (TPA), designed for this purpose by Pose et al. (2010), was used. This medium contained 800 ml of pulp of fresh tomatoes, 200 ml of

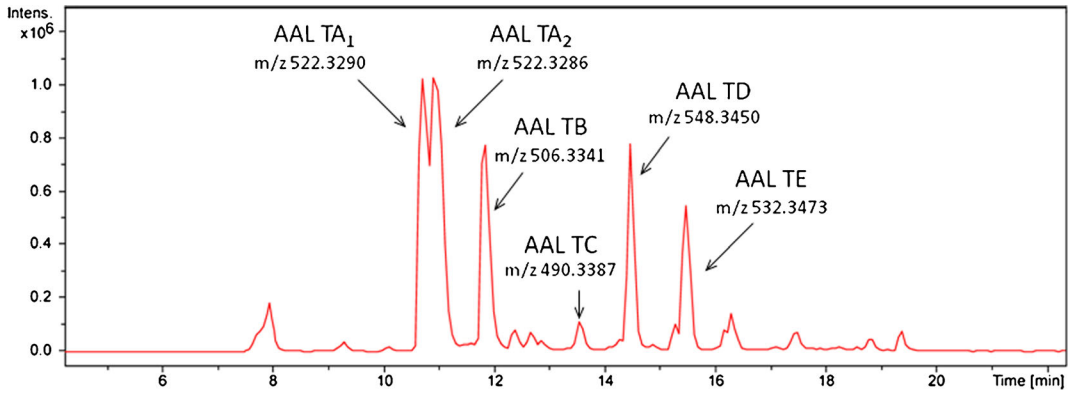

Fig. 2 HPLC-MS chromatogram of AAL toxins production in seven media at 25 °C by *Alternaria arborescens* EGS 39–128

distilled water and 15 g of agar per liter (pH 4.39). The a_w of the medium was adjusted with glycerol 87% p.a. (Merck 4094) to 0.995 and 0.950. Water activity was measured with a water activity meter (Aqualab CX-2, Decagon Devices Inc., USA).

The *A. arborescens* strain was grown on Potato Carrot Agar (PCA) (Simmons 2007) 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 obtain heavily sporulated cultures. Spores were placed in an aqueous solution of 0.05% Tween 80 (Biopack) of a_w adjusted with glycerol, to avoid affecting the a_w of the culture medium. After homogenizing, the suspension was counted using a Neubauer chamber. Under these conditions the inoculum concentrations varied between 1.5 and 3.0×10^5 spores/ml. TPA plates were inoculated centrally with a 1 μl calibrated loop of spore suspension. Inoculated plates of the same a_w were placed in closed sterile polyethylene bags containing a vessel with adjusted glycerol/water solution to minimize water transfer from or to the medium. 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. Sets of each treatment were incubated at 6, 15, 20, 25 and 30 °C. AAL toxins produced in each plate after incubating for 7, 14, 21, 30 and 40 days were determined. Each set of conditions (aw × temperature × time) was run by triplicate.

The analytical method for extraction of AAL toxins was described by Solfrizzo et al. (2005). The extracts were analyzed by HPLC-MS. Analysis by HPLC-MS was performed on a Bruker MicrOTOF-Q II® mass spectrometer (Bruker Daltonics, Billerica, MA, USA) coupled to an Agilent series 1200®HPLC with autosampler and diode array detector. The MS and MS/MS were performed simultaneously. MS conditions: ESI positive capillary mode. Potencial: 4.5 kV; Nebulizer gas: 3 bar; Drying gas: 6 l/min; Temperature: 200 °C. MS/MS conditions: automatic mode selection in the quadrupole ion was used, and collision energies (CID) of between 15 and 25 eV were applied according to the mass range in

Table 1 Fragmentation patterns of AAL toxins in positive ESI mode

AAL	RT (min)	Observed m/z ($[M + H]^+$)	Molecular formula $[M + H]^+$	Mass accuracy (ppm)	Fragment ions (m/z)
TA	10.8	522.3285	$C_{25}H_{47}NO_{10}$	-2.4	504.3157, 486.3048, 346.2958, 310.2762, 292.2629
TB	11.8	506.3338	$C_{25}H_{47}NO_9$	-2.9	488.3213,470.3110, 330.3021, 294.2795, 276.2664
TC	13.5	490.3381	$C_{25}H_{47}NO_8$	-1.3	472.3232, 454.3121, 314.3052, 278.2842
TD	14.5	548.3437	$C_{27}H_{49}NO_{10}$	-1.5	530.3317, 512.3213, 372.3097, 336.2899, 318.2806
TE	15.5	532.3469	$C_{27}H_{49}NO_9$	2.2	514.3381, 496.3262, 356.3153, 320.2946

the collision cell. Argon was used as collision gas. Multipoint mass calibration was carried out using a mixture of sodium formate from *m/z* 50 to 900 HPLC conditions: solvent A, 0.1% HCOOH; solvent B, MeOH 0.3 ml/min, 30 °C temperature. Linear gradient elution was performed as follows: 20% B (0–1 min), 20–100% B (1–15 min), 100% B (15–25 min). The data acquisition and processing of the spectra was performed using the Bruker software Compass®DataAnalysis 4.0. Each experiment was repeated at least three times in order to ensure reproducibility. All the relations m/z were obtained with high accuracy, enabling to obtain the molecular formulas of metabolites subject to analysis. Spectra MS/MS were used to confirm identity of mycotoxins, in addition to the comparison of their retention times with the standards.

A fully randomized factorial design run in triplicate was used to compare the toxin concentrations at different times and a_w levels for each AAL toxin separately. The effect of a_w, time, and their interaction was examined by ANOVA using Statistica software v6.0 (StatSoft, Inc., 1984–2001, Tulsa, OK, USA). Temperature was excluded as factor, since toxin production was detected at only one of the tested temperatures.

AAL toxins production in arbitrary units (AU) on TPA at a_w 0.995 and 0.950, 30 °C, and 21, 30 and 40 days is shown in Fig. 3. Statistical analysis of variance (ANOVA) showed that the effect of aw, time, and their interaction significantly influenced the biosynthesis of AAL TA, TD and TE ($p < 0.001$).

Although growth was observed at all conditions assayed, no production of AAL toxins was detected at both a_w during the whole incubation period at any temperature below 30 °C. No AAL TC production was observed in any of the tested conditions, which suggests that TPA is not a good substrate for the production of this toxin. The only temperature level that supported the rest of AAL toxins production was 30 °C, and they were detected after 21, 30 and 40 days of incubation. The maximum accumulation of all AAL toxins was observed at 0.995 a_w after 40 days of incubation, and the minimum for TA, TD and TE at a_w 0.950 and 21 days with no production of AAL TB in these conditions (Fig. 3). The a_w of the medium had a strong influence on the production of AAL TA, TD and TE; much higher quantities were detected at 0.995 and 40 days, than at 0.950 in the same conditions. On the contrary, in the case of AAL TB, quantities produced at 0.995 a_w (1500 AU) and at 0.950 a_w (1300 AU) were no significantly different. Regarding the incubation time, its effect was significant on all AAL toxins. The highest increment in the amount of toxin accumulated was observed between 30 and 40 days, for AAL TA, TD and TE at the optimum a_w level (0.995). After 40 days of incubation these toxins concentration increased from 8 to 15 times with respect to 30 days. Even though the effect of incubation time was significant on the synthesis of AAL TB, a much lower increment on toxin concentration was observed for this toxin along the whole incubation period. However, at 0.995 a_w, TB was only detected after 40 days of incubation.

Figure 4 shows AAL toxins production in TPA at the optimum conditions (0.995 a_w, 30 °C and 40 days). The fragmentation patterns of AAL toxins observed in the ESI MS/MS experiments of ions $[M + H]^+$ m/z reproduced those obtained with the standards (Table 1) and were similar to those described in ESI MS

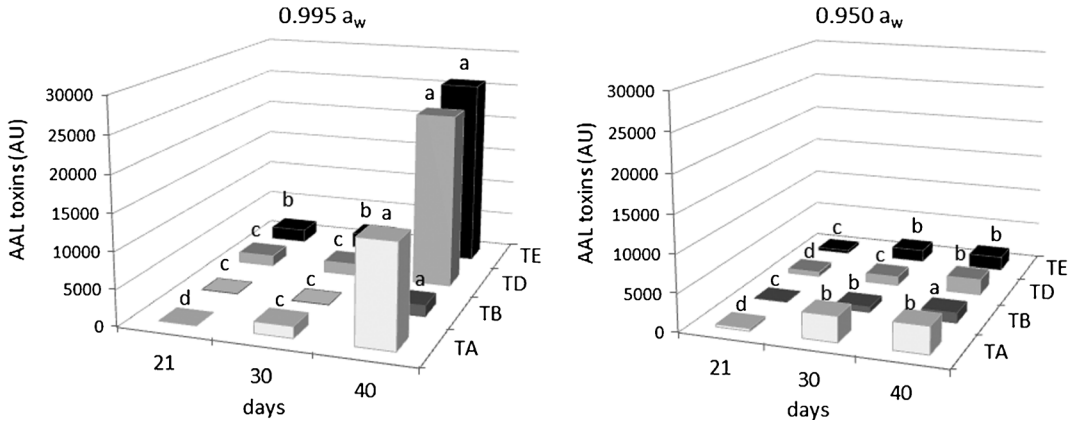

Fig. 3 AAL toxins production by *Alternaria arborescens* on TPA at 0.995 and 0.950 a_w, 30 °C, and 21, 30 and 40 days. Bars with the same letter within each AAL toxin are not significantly different ($p < 0.001$)

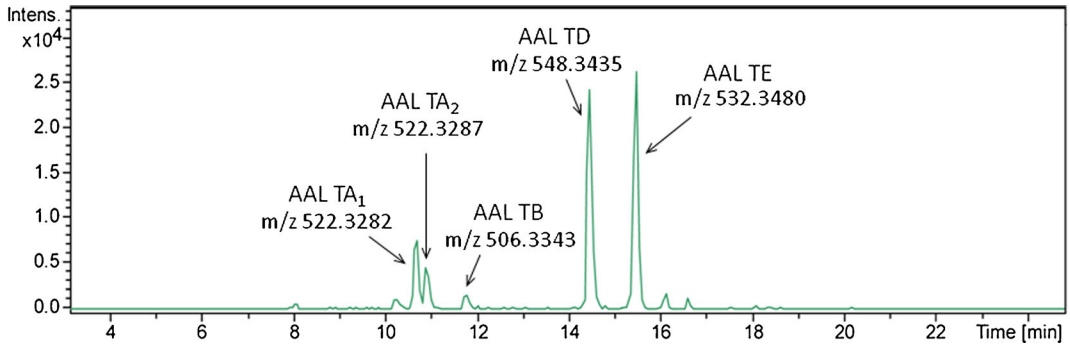

Fig. 4 HPLC-MS chromatogram of AAL toxins production in TPA after 40 days of incubation at 0.995 a_w and 30 °C

experiments by Caldas et al. (1995) and Benavidez Rozo et al. (2014).

To our knowledge, there is no data on the influence of a_w and temperature on the simultaneous production of AAL toxins. Mansfield et al. (2007) reported the incidence of AAL TA and TB in maize silage and correlated its presence with weather data (temperature and relative humidity) during the growing season. They found that temperature during maize development was negatively correlated with AAL TA, while moisture events positively correlated with it. No correlation with environmental conditions was possible for AAL TB due to the scarce number of samples contaminated with this toxin. In a previous work, Vaquera et al. (2016) investigated how environmental parameters affect mycotoxin production by *Alternaria arborescens*. Alternariol (AOH), alternariol methyl ether (AME), tenuazonic acid (TeA) and only AAL TA were investigated. In this study AAL TA was determined by HPLC with fluorescence detection. Although the method had a good performance and a high sensitivity for AAL TA determination, the simultaneous detection of all five AAL toxins, involving each isomer well resolved from one another, and the unequivocal identification of peaks could only be achieved by HPLC-MS. This methodology was also preferred by Mansfield et al. (2007) to detect both AAL TA and TB from maize silage.

The lack of information about AAL toxins incidence and their in vitro production by *Alternaria* spp. isolates could be attributed to several factors. According to our results, AAL toxins only accumulate in high levels at a temperature not lower than 30 °C, and generally, the studies on fungal toxigenic potential are standardized at 25 °C. In the study by Vaquera et al. (2016), AAL TA was detected at high a_w (0.995) at temperatures below 30 °C, such as 25 °C or 20 °C, but in much lower amounts than at 30 °C. These quantities can only be detected if a high sensitive method as HPLC with fluorescence detection is applied for its determination. It is also worth noticing that high amounts of toxins were only accumulated after a period of 40 days, which exceeds the standard incubation periods involved in most in vitro studies. Thus, the lower amounts produced in a shorter period could be unnoticed if no high sensitive method are applied for its detection or if the substrate is not conducive to their accumulation.

On the other hand, the taxonomy of the *Alternaria* genus is still under revision. As a consequence, it is quite common that closely related species among the small-spore *Alternaria* are identified as *A. alternata* when traditional taxonomic systems, based on morphological characteristics are applied. AAL toxins have been classified as host-specific toxins (HSTs), as they are only toxic to a specific host plant of the fungus. Most HSTs are pathogenicity factors, required by the fungi to invade tissue and induce disease (Tsuge et al. 2012). When several plant diseases caused by *Alternaria* were discovered, the responsible pathogens, which were HST-producers, were classified as *A. alternata* pathotypes (Nishimura 1980). In particular, the AAL toxins producer, which is the causal agent of tomato stem canker, was established as the *A. alternata* tomato pathotype (syn. *A. alternata* f. sp. *lycopersici*). However, this classification is in disagreement with that of Simmons (2007), who sorted a number of small-spored *Alternaria* into separate and morphologically distinguishable species, based on three-dimensional conidiation patterns and conidia morphology. According to Simmons (2007) the tomato pathotype is *A. arborescens,* a species morphologically different from *A. alternata*. This species was associated with tomato stem canker, although in several studies it was

also isolated from the lesions of tomato fruit affected by black mold (Benavidez Rozo et al. 2014; Somma et al. 2011). Somma et al. (2011) have also shown that strains of *A. arborescens* isolated from black mold tomato fruit have a high toxigenic capacity, with most of the isolates being able to produce other common *Alternaria* toxins, such as AOH, AME and TeA. AOH is a key colonization factor during *Alternaria* infection of tomatoes (Graf et al. 2012). The representative *A. arborescens* strain used in the present work is known to produce these mycotoxins together with AAL toxins in high levels (Vaquera et al. 2016; Benavidez Rozo et al. 2014; Somma et al. 2011).

The tomato pulp agar (TPA) has previously proved its use for ecophysiology studies on the genus *Alternaria* (Pose et al. 2010, Vaquera et al. 2014, 2016), since its composition resembles that of tomato fruit. The results obtained here could be extrapolated to the natural contamination of the fruit.

The knowledge on how environmental factors impact on toxin production by the pathogen is relevant to develop prevention strategies for the control of the disease. *A. arborescens* is able to grow in the fruit in the ripening stages and during the storage period. Tomatoes are cultivated during spring and summer in Argentina, with average temperatures between 25 and 30 °C during the harvest season. Moreover, storage is usually performed at room temperature. The combination of high temperatures and the high water activity of tomato (0.995) result in a fast growth of the pathogen (Vaquera et al. 2014) and an increasing risk of AAL toxins accumulation.

The optimum a_w/temperature combination for AAL toxins biosynthesis (0.995/30 °C) could also lead to the production of high levels of TeA, AME and AOH (Vaquera et al. 2016), increasing the risks of synergistic effects between the toxins. When these compounds accumulate in edible parts of the plant, such as tomato fruit, they can exert toxic effects on humans and animals. Mycotoxins are considered to be heat-stable molecules and are not easily destroyed by conventional food processing. This issue has a particular importance in tomato products, given that, in developing countries, fruit with high fungal contamination are frequently used for industrialized derived products (tomato pasta, puree, etc), with a consequent higher mycotoxin accumulation.

The present study is the first report on the effect of a_w and temperature on AAL toxins simultaneous production by *Alternaria arborescens*. AAL toxins only accumulate in high levels at a temperature not lower than 30 °C. The combination of different pre- and post-harvest technologies and controlled environmental factors during ripening and storage stages could prevent the pathogen development and the biosynthesis of its multiple toxic metabolites, which could persist and concentrate in the by-products.

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