Short communication

Water activity and temperature effects on growth of *Alternaria arborescens* on tomato medium

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**A R T I C L E  I N F O**

Article history:
Received 14 February 2014
Received in revised form 14 May 2014
Accepted 9 June 2014
Available online 13 June 2014

Keywords:
* Alternaria arborescens
  Growth
  Germination
  Temperature
  Water activity
  Tomato medium

**A B S T R A C T**

*Alternaria arborescens* is the causal agent of tomato stem canker, a disease frequently responsible of substantial economic losses. *A. arborescens* can produce several mycotoxins, such as alternariol, alternariol monomethyl ether and tenuazonic acid and phytotoxins such as the AAL toxins. The objective of this study was to determine the effect of water activity (a$_w$, 0.950, 0.975, 0.995) and temperature (6, 15, 20, 25 and 30 °C) on the germination and radial growth rate of *A. arborescens* on a synthetic tomato medium. Germination followed by growth was observed at all temperatures and a$_w$ levels analyzed. The shortest germination time (0.5 days) was observed at 0.950 a$_w$, both at 25 °C and at 30 °C. The germination time increased with a reduction of a$_w$ and temperature. The highest growth rate was registered at 0.995 a$_w$ and 30 °C (7.21 mm/day) while the lowest occurred at 0.950 a$_w$ and 6 °C (0.52 mm/day), conditions at which the longest lag phase was observed (8 days). Growth rates increased with a$_w$ and temperature. Knowledge of the ecophysiology of the fungus in this substrate is necessary to formulate future strategies to prevent its development and evaluate the consumer health risk posed by potential exposure to the toxins.

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1. Introduction

In 2012 in Argentina, 7000 hectares from different regions were under cultivation of tomatoes. This fruit is destined mainly for industry, especially for the production of tomato puree. Domestic consumption was reported to be 530,000 tons during 2012, mostly covered from domestic production with some imports from Chile, China and USA (Informe frutihortícola, 2012).

Many *Alternaria* species are saprophytic fungi. However, some species have acquired pathogenic capacities, causing disease over a broad host range. In general, *Alternaria* species cause a relatively slow destruction of host tissues through the reduction of photosynthetic potential. Typically, tissues weakened by stress, senescence or wounding are more susceptible to *Alternaria* sp. infection (Thomma, 2003).

*Alternaria arborescens* is a ubiquitous fungus that can be found in many kinds of plants and other substrata. It has been isolated from fruits such as tomato (Somma et al., 2011), blueberries (Greco et al., 2012), grapevines (Polizzotto et al., 2012), apples (Hartveld et al., 2013), cherries (Andersen and Thrane, 2006), nuts, including pistachio (Ma and Michailides, 2004), walnuts and hazelnuts (Belisario and Santori, 2009; Hong et al., 2006), and less frequently from cereals, such as wheat (Patriarca et al., 2007) and barley (Andersen et al., 2002).

Tomato fruits are easily infected because their thin skin and soft tissue allow rapid penetration and growth of the different *Alternaria* infecting species (Pitt and Hocking, 1997). Tomato is commonly infected by *A. arborescens* after harvest and extended storage. This fungus is capable of developing primary infection of leaves, stems and fruit of susceptible tomato cultivars. It is known to be the causal agent of tomato stem canker, one of the most devastating tomato diseases worldwide, responsible for significant economic losses sustained by tomato producers each year (Esmailzadeh et al., 2008).

Natural occurrence of *Alternaria* species on tomato has been often detected and the isolates showed a high capability for production of mycotoxins such as tenuazonic acid (TA), alternariol (AOH) and alternariol monomethyl ether (AME) (Andersen and Frisvad, 2004; Logrieco et al., 2003; Pose et al., 2004; Somma et al., 2011). AAL toxins were first isolated as host-specific toxins from *A. arborescens* (synonym *A. alternata f. sp. lycopersici*) (Caldas et al., 1994). These compounds cause apoptosis in susceptible tomato cells and mammalian cells by inhibiting ceramide biosynthesis (Yamagishi et al., 2006). Previous studies have demonstrated the presence of *Alternaria* toxins in tomato products processed and sold in Argentina (Terminiello et al., 2006).

There are currently no statutory or guideline limits set for *Alternaria* mycotoxins. The European Food Safety Authority published a report on the risks of *Alternaria* toxins for animal and public health (EFSA, 2011), concluding that there are not enough relevant data on toxicity of these mycotoxins, and more information is needed on their toxicokinetics,

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http://dx.doi.org/10.1016/j.ijfoodmicro.2014.06.007
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occurrence, and influence of food and feed processing to enable their risk assessment. They also concluded that, between the main vegetables and vegetable products contributing to dietary exposure to AOH, AME, TA and tentoxin, tomato and tomato products are of particular concern.

Fungal growth is markedly affected by different environmental factors, the two most important being water activity ($a_w$) and temperature (Magan and Lacey, 1984). Growth of mycotoxigenic Alternaria species in relation to these factors has been described in different substrates (Lacey, 1992; Oviedo et al., 2010; Pose et al., 2009). However, no studies have been carried out on isolates from the A. arborescens species group. Such information is important in developing realistic forecasting systems for predicting risk of colonization and mycotoxin production.

Due to the high incidence of A. arborescens and its mycotoxins in tomato fruits and byproducts in Argentina, the objective of this study was to determine the effects of $a_w$, temperature and their interaction on the growth and conidial germination of A. arborescens causing tomato stem canker on tomato fruits.

2. Materials and methods

2.1. Fungal strain

A representative strain of A. arborescens (EGS 39-128) from the culture collection of Emory G. Simmons (Mycological Services, Crawfordsville, IN, USA) isolated from tomato was used in this study. It was inoculated on Potato Carrot Agar (PCA) (Simmons, 1992) and grown under standardized conditions in order to promote sporulation. The unsealed plates were incubated in a single layer under lights with an alternating light/dark cycle consisting of 8 h of cool-white daylight followed by 16 h darkness for 7 days at 25 °C (Simmons, 2007).

2.2. Medium

Growth rate and spore germination were 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 CX-2, Decagon Devices Inc., USA).

2.3. Inoculation and incubation

Spores of 7-day-old cultures grown in PCA were placed in an aqueous solution the $a_w$ of which was 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 $5.5 \times 10^5$ 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 quadruplicate.

2.4. Examination of the germination and growth measurement

For determination of the germination time, the plates were observed at 40× magnification under a stereomicroscope. The criterion for germination was the production of a germination tube of length similar to the diameter of the conidia at least 50% of the inoculum (Hocking and Miscamble, 1995). The first measurement was done 12 h after inoculation and thereafter twice a day. The radial mycelial growth was determined by a periodical measurement of two right-angled diameters of the colonies. Radial growth vs time was plotted and radial growth rates (mm/day) were calculated from the slope by linear regression (Patriarca et al., 2001).

2.5. Experimental design and data treatment

A full factorial design with two variables ($a_w$ and T) was used. Four independent replicates per $a_w$–temperature combination were made both for germination and growth rate assessment. The responses recorded were germination time and radial growth rate. The effects of $a_w$, temperature, and their interaction were examined by ANOVA using Statistica software v8.0 (StatSoft Inc., 1984–2007, Tulsa, OK, USA).

3. Results

3.1. Effect of water activity and temperature on germination time

Statistical analysis of variance (ANOVA) showed that all effects ($a_w$, temperature, and their interaction) were significant ($p < 0.0001$) on the germination time of A. arborescens on tomato pulp agar (TPA).

Germination followed by growth occurred at all the $a_w$–temperature combinations evaluated. The shortest germination time (0.5 days) was observed at 0.995 $a_w$ at both 25 °C and 30 °C (Fig. 1). The germination time increased with a reduction of $a_w$ and temperature; at the lowest $a_w$ and temperature levels evaluated (0.950, 6 °C), it reached its highest value (8 days). The two highest temperature levels, 25 °C and 30 °C, were equally favorable for germination, as the germination times observed at these temperatures were not significantly different at any $a_w$ level. The optimum temperature for germination was between 25 and 30 °C. At refrigeration temperatures, the influence of $a_w$ was more pronounced than at high temperatures; at 0.995 $a_w$ and 6 °C, it took 4.5 days for A. arborescens to germinate on a synthetic tomato medium, whereas at the same temperature and 0.975 $a_w$ the germination time increased to 7 days.

3.2. Effect of water activity and temperature on radial growth rate

Statistical analysis of variance (ANOVA) showed that all effects ($a_w$, temperature, and their interaction) were significant ($p < 0.0001$) on the radial growth rate of A. arborescens on TPA.

The optimum conditions for A. arborescens growth were the highest $a_w$ and temperature levels (0.995 $a_w$ and 30 °C; 7.21 mm/day) (Fig. 2). However, radial growth rates were not significantly different at 0.975 $a_w$ and 30 °C (6.99 mm/day) and at 0.955 $a_w$ and 25 °C (6.97 mm/day). The growth rate increased with $a_w$ and temperature, the latter being the most significant of both environmental factors. The lowest growth rate
(0.52 mm/day) was observed at 0.950 aw and 6 °C. At the same temperature and the highest aw level, 0.995, the growth rate was four times higher (2.03 mm/day). At 0.95 aw, the growth rates increased linearly with temperature, whereas at the higher aw levels, 0.975 and 0.995 the ratio of increment in growth rates changed abruptly between 15 and 20 °C, reaching its maximum value between 25 and 30 °C.

4. Discussion

To our knowledge there are no data on the ecophysiology of A. arborescens in the literature. This could be because the taxonomy of the genus Alternaria is still under discussion. The traditional methods for identification, primarily based on morphological characteristics of the reproductive structures have led to a great number of isolates classified as A. alternata and a general belief that this species was the most abundant in nature. The criteria classifying Alternaria taxa according to host-specificity or forma specialis also resulted in different pathotypes of A. alternata, and the causal agent of tomato stem canker was recurrently referred to as A. alternata f. sp. lycopersici in the literature. Several revisions of taxonomy made by Emory Simmons (1992, 1993, 1994, 1995, 1999), finally organized the genus into 276 species and developed the “species-group” concept by referring to certain groups using a representative species, for instance, the A. arborescens species-group (Simmons, 2007). Since then, species other than A. alternata have been reported as predominant in several food substrates (Andersen and Frisvad, 2004; Greco et al., 2012; Patriarca et al., 2007; Polizotto et al., 2012).

Previous studies (Somnia et al., 2011) have shown that A. arborescens is present in tomato fruits affected by black mold in Argentina, and its toxigenic capacity is high, with most of the isolates being able to produce AOH, AME and TA, and frequently at high levels. Understanding how environmental factors affect pathogen growth is relevant to development of prevention strategies. This fungus is able to grow in the fruits especially if stored at room temperature in warm climate regions. Refrigerated chambers are usually stored at room temperature in Argentina, and eventually in refrigerated chambers. At room temperature (25 to 30 °C in spring and summer respectively) and at the high water activity of tomato fruits (0.995) the fungus is able to germinate in less than 1 day, and its growth at these conditions is extremely fast (6.97–7.21 mm/day), which confirms that warm storage temperatures increase the risk of contamination with A. arborescens. During autumn, average storage temperatures are between 15 and 20 °C, conditions that reduce germination times to 1 or 2 days, and growth rates to 2.98 and 5.46 mm/day respectively. Under refrigeration temperatures (5 °C), germination took 4 days at high aw and growth was slower (2 mm/day). Even though refrigeration temperatures represent the best strategy to control the pathogen, extremely low temperatures are not recommended for storage of fresh fruits as there may be a significant reduction in sensorial quality and color development.

The results obtained in the present work were in agreement with data reported for A. alternata in other substrates. The optimum temperature for germination varied between 25 and 30 °C, with a minimum at 5 °C and maximum at 35 °C (Magan and Lacey, 1984). The germination times for A. arborescens were also similar to those reported for A. alternata isolates from tomato fruits affected by black mold (Pose et al., 2009). At 0.98 aw, germination times for A. alternata (1.5, 3.5, and 7.5 days at 21, 15 and 6 °C, respectively) were in accordance to those observed for A. arborescens at 0.975 aw. The same was observed at 0.95 aw, and 21 and 15 °C, with germination times of 2.5 and 3.5 days, respectively, for A. alternata. However, A. arborescens germinated after 8 days of incubation at 6 °C, whereas it took more than 10 days for A. alternata to germinate at this temperature.

At similar aw levels and temperatures, Pose et al. (2009) reported higher radial growth rates for A. alternata from tomatoes than those observed for A. arborescens. A. alternata grew at a rate of 8.3 and 5.3 mm/day at 0.98 aw and 21 and 15 °C respectively, but A. arborescens growth rates were 4.8 and 2.8 mm/day at 0.975 aw at the same temperatures. At 0.95 aw, A. alternata growth rates were 4.1 and 2.1 respectively and A. arborescens grew at 2.3 and 1.5 mm/day respectively at these temperatures. At 6 °C growth rates for both species were similar; 1.4 and 0.5 mm/day at 0.98 and 0.95 aw respectively for A. arborescens and 1.7 and 0.4 mm/day for A. alternata.

The present results indicate that A. arborescens has an optimum growth temperature (25–30 °C) higher than A. alternata (21 °C) in TPA (Pose et al., 2009), although high aw levels favor the development of both closely related species. Data reported in the literature on A. alternata also agree on these results. The optimum aw for A. alternata growth in different culture media was in a range of 0.98–1.0; the optimum, maximum and minimum temperature ranges were 21–30 °C, 32–35 °C and 5–6 °C, respectively (Pose et al., 2009; Magan and Lacey, 1984; Sautour et al., 2001).

There are no data on the effect of aw and temperature on mycotoxin production by A. arborescens. A previous work on A. alternata strains isolated from tomatoes in Argentina demonstrated that the production of each toxin on tomato medium was affected differently by environmental factors (Pose et al., 2010). While TA production was optimal at high aw (0.98) and moderate temperature (21 °C), the maximum AOH accumulation occurred at the same temperature but at lower aw (0.95). For AME biosynthesis, the optimum conditions were 35 °C and 0.95 aw.

Low storage temperatures (6 °C) resulted in a great reduction in the amount of toxin produced, even at its corresponding optimum aw level. Currently, studies are being carried out to determine the effect of both environmental factors on toxin production by A. arborescens in order to evaluate if there are interspecific differences in their behavior.

Tomato products such as tomato purees, sauces and chutney (0.97–0.98 aw) or concentrated tomato paste (0.93–0.95 aw) are also susceptible to contamination with Alternaria sp. and their mycotoxins. Although the fungal spores are probably inactivated during thermal processes, the tomato paste used as raw material is prone to contamination if it is not stored at adequate temperatures before processing. In the present study low germination times and relatively high growth rates were observed at both 0.975 and 0.950 aw. According to our results, concentrated tomato pastes used as raw material for different tomato products of reduced water activity are susceptible to A. arborescens contamination, especially if stored at room temperature in warm climate regions. Refrigeration of these products at 6 °C or below is advised in order to prevent A. arborescens growth and toxin production.

5. Conclusions

The present study is the first report on the effect of aw and temperature on the germination and growth of A. arborescens. The results...
obtained in a synthetic tomato medium could be extrapolated to evaluate the risk of spoilage in tomato fruits and tomato byproducts caused by this pathogen, the main causal agent of stem canker of tomato, with high toxigenic potential. The substantial losses due to this pathogen make it necessary to widen the knowledge on its ecophysiology, to elaborate adequate strategies of control. The combination of different postharvest technologies and controlled environmental factors during storage could prevent its development. In addition, considering the toxigenic potential of A. arborescens strains, the prevention of the growth would result in reducing associated consumer health risks.

Acknowledgments

Financial support of Universidad de Buenos Aires (UBACYT 2013-2016 No 20020120100016) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 2012-2014 No 112 20110100383) is acknowledged.

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