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Short communication

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

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ABSTRACT

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 0.995 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.

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 (Harteveld 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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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 5.5×10^5 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 × 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 in 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 0.995 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

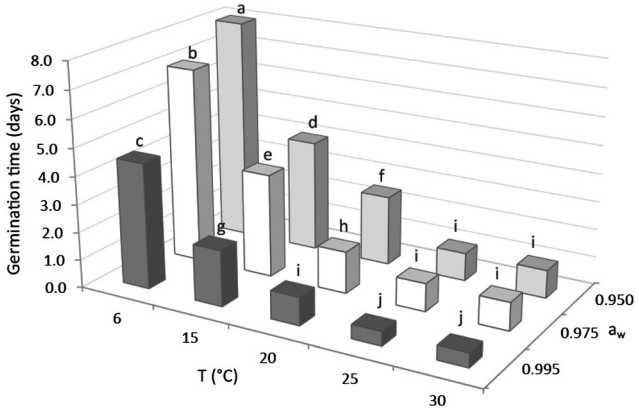

Fig. 1. Effect of water activity (a_w) and temperature (T) on the germination of *Alternaria arborescens* on tomato pulp agar. Bars with the same letter are not significantly different ($p < 0.05$).

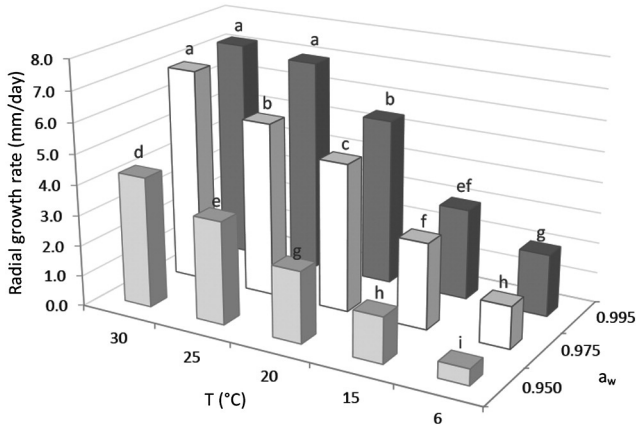

Fig. 2. Effect of water activity (a_w) and temperature (T) on radial growth rate (mm/day) of *A. arborescens* on tomato pulp agar. Bars with the same letter are not significantly different ($p < 0.05$).

(0.52 mm/day) was observed at 0.950 a_w and 6 °C. At the same temperature and the highest a_w level, 0.995, the growth rate was four times higher (2.03 mm/day). At 0.95 a_w the growth rates increased linearly with temperature, whereas at the higher a_w 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; Polizzotto et al., 2012).

Previous studies (Somma 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 during the storage period which may extend for several weeks. Tomatoes 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 a_w 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 a_w, 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 a_w. The same was observed at 0.95 a_w, 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 a_w 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 a_w and 21 and 15 °C respectively, but *A. arborescens* growth rates were 4.8 and 2.8 mm/day at 0.975 a_w at the same temperatures. At 0.95 a_w, *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 a_w 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 a_w levels favor the development of both closely related species. Data reported in the literature on *A. alternata* also agree on these results. The optimum a_w 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 a_w 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 a_w (0.98) and moderate temperature (21 °C), the maximum AOH accumulation occurred at the same temperature but at lower a_w (0.95). For AME biosynthesis, the optimum conditions were 35 °C and 0.95 a_w.

Low storage temperatures (6 °C) resulted in a great reduction in the amount of toxin produced, even at its corresponding optimum a_w 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 a_w) or concentrated tomato paste (0.93–0.95 a_w) 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 a_w. 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 a_w 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.

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