Effect of water activity and temperature on growth of *Alternaria alternata* on a synthetic tomato medium

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ABSTRACT

Alternaria alternata is a toxigenic fungus, predominantly responsible for Blackmould of ripe tomato fruits, a disease frequently causing substantial losses of tomatoes, especially those used for canning. The objective of this study was to determine the effect of water activity (a_w, 0.904, 0.922, 0.954, 0.982) and temperature (6, 15, 21 and 35 °C) on germination and radial growth rate on a synthetic tomato medium of a cocktail inoculum of five strains of *A. alternata* isolated from tomato fruits affected by Blackmould. The shortest germination time (1.5 days) was observed at 0.982 a_w, both at 21 °C and 35 °C. The germination time increased with a reduction on a_w. The fastest growth rate was registered at 0.982 a_w and 21 °C (8.31 mm/day). Growth rates were higher when a_w increased. No growth or germination was observed at the lowest a_w level evaluated (0.904) after 100 days of incubation at 6 °C and 15 °C. A temperature of 6 °C caused a significant reduction in growth rates, even at the optimum a_w level. The knowledge on the ecophysiology of the fungus in this substrate is necessary to elaborate future strategies to prevent its development and evaluate the consumer health risk.

1. Introduction

Tomato (*Lycopersicum esculentum*) and tomato products are widely consumed in Argentina. It constitutes one of the vegetables with the highest processing volume in this country and in the rest of the world. In Argentina, the production is destined for fresh consumption and for processing into industrialized products such as tomato puree, pulp, juice, sauces, etc. The per capita consumption of tomato products is estimated in 10 kg/year (Franco, 2006).

The genus *Alternaria* contains numerous species that are both saprophytic and pathogenic on many plants, including several plants used for food (Bottalico and Logrieco, 1998). Because of their thin skin, tomatoes are very susceptible to fungal decay, and *Alternaria* is the most common fungus on moldy tomatoes (Andersen and Frisvad, 2004). In tomatoes, *A. alternata* requires injured or weakened tissue for penetration and growth (Barkai-Golan, 2001). *A. alternata* is the main causal agent of Blackmould of ripe tomato fruits, a disease frequently causing substantial losses of tomatoes, especially those used for canning (Bottalico and Logrieco, 1992). This species was found to be the most prevalent fungus causing ripe fruit rot in California processing tomatoes (Morris et al., 2000). In southern Italy, *A. alternata* is the dominant fungal species although *A. tenuissima* has

also been recovered (Logrieco et al., 2003). In Argentina *A. alternata* and *A. tenuissima* have been found as the main species associated to tomato fruits spoilage (Pose, 2007).

During the colonization of the host, the pathogen can produce toxic metabolites in the infected plants and when they accumulate in plant parts used as food, they can be a hazard to human health (Bottalico and Logrieco, 1998). The major *Alternaria* mycotoxins belong to three structural classes: the tetramic acid derivative, tenuazonic acid (TA); the dibenzopyrone derivatives, alternariol (AOH), alternariol monomethyl ether (AME) and altenuene; and the perylene derivatives, the altertoxins (Logrieco et al., 2003). AME and AOH are not very acutely toxic, but they have mutagenic effects. There is also some evidence of carcinogenic properties. Tenuazonic acid is not mutagenic in bacterial systems. However, it is toxic to several animal species; in dogs, it caused hemorrhages in several organs, and in chickens sub-acute toxicity was observed (Scott, 2004). *A. alternata* toxins were associated with human esophageal cancer in Linxian, China (Liu et al., 1991).

Previous studies have demonstrated the presence of *Alternaria* toxins in tomato products processed and sold in Argentina. From 80 tomato puree samples analyzed, 39 (49%) were contaminated with *Alternaria* toxins. TA was found in 23 samples at levels ranging from 39 to 4021 µg/kg; AOH in 5 samples at levels from 187 to 8756 µg/kg, and AME in 21 samples ranging from 84 to 1734 µg/kg (Terminiello et al., 2006).

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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 and its relation with these factors have been described in different substrates (Lacey, 1992). However, no studies have been carried out on isolates from tomatoes or on tomato as growing substrate. Such information is important in developing realistic forecasting systems for predicting risk of colonization and mycotoxin production.

Due to the high incidence of *Alternaria* and its mycotoxins in commodities and food products 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. alternata* isolates causing Blackmould on tomato fruits.

2. Materials and methods

2.1. Fungal strains

Five strains of *A. alternata* isolated from ripe tomato fruits affected by Blackmould were used in this study. The isolates were identified according to Simmons (2007). The colony and sporulation characteristics of representative cultures of *A. tenuissima* EGS 34.015, *A. alternata* EGS 34.016, *A. arborescens* EGS 39.128 and *A. infectoria* EGS 27.193 (Mycological Services, Crawfordsville, IN) were determined and compared with those of the tomatoes isolates in standard condition culture. Single germinating conidia were transferred to Petri dishes containing Potato–Carrot-Agar (PCA) and incubated for 7 days at 25 °C. Based on sporulation patterns and conidial morphology, the isolates used in this work were grouped as *A. alternata*. The strains toxigenic capability was evaluated in autoclaved polished rice after incubation in the dark at 25 °C for 21 days. The five strains selected were capable of producing AOH, AME and TA. Analysis of *Alternaria* toxins in synthetic tomato medium will be performed in a future work. All of them are maintained in the culture collection of Universidad Nacional de Quilmes, Buenos Aires, Argentina and in the culture collection of the Istituto di Scienze delle Produzioni Alimentare (ITEM fungal culture collection) of the Consiglio Nazionale delle Ricerche, Bari, Italy.

2.2. Medium

Growth rate and spore germination were determined on tomato pulp agar (TPA) designed for this purpose. This medium contained 800 ml/l of pulp of fresh tomatoes, 200 ml of distilled water and 15 g of agar (pH 4.39). The a_w of the medium was adjusted with glycerol 87% analytical grade (Merck 4094) to 0.982; 0.954; 0.922 and 0.904 ± 0.003 (Dallyn and Fox, 1980). Water activity was measured with a water activity meter (Aqualab CX-2, Decagon Devices Inc., USA).

2.3. Inoculation and incubation

The isolates were grown on water agar (agar 2%) for 15 days at 25 °C to obtain heavily sporulated cultures (Larone, 2002). A cocktail inoculum was prepared with the five strains according to Hocking and Miscamble (1995). Spores of each strain 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 3×10^5 spores/ml. TPA plates were inoculated centrally with a 1 µl calibrated loop of spore suspension. The plates were incubated at 35, 21, 15 and 6 °C for a maximum period of 100 days. To minimize water transfer from or to the medium, plates corresponding to 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 by 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 stereo-microscope. The criterion for germination was the production of a germination tube of longitude similar to the diameter of the conidia in at least 50% of the inoculum (approximately 100–150 spores) (Hocking and Miscamble, 1995). The first measurement was done after 12 h after inoculation and thereafter twice a day. The radial mycelial growth was determined by 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 designed variables (a_w and T) at four levels was applied. Four replicates were used per each a_w– temperature combination both for germination and growth rate assessment. The responses recorded were germination time and colony diameter along time. The effects of a_w, temperature, and their interaction were examined by ANOVA using Statistica software v6.0 (StatSoft, Inc., 1984–2001, Tulsa, OK, USA).

3. Results and discussion

3.1. Water activity and temperature effect 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. alternata* on tomato pulp agar (TPA).

The shortest germination time (1.5 days) was observed at 0.982 a_w, both at 21 °C and 35 °C (Fig. 1). The germination time increased with a reduction on a_w. At the lowest a_w level evaluated (0.904) no germination was registered after 100 days of incubation at 6 °C and 15 °C. The highest temperature levels studied (21 °C and 35 °C) were the most favorable for germination. At those temperatures the germination times were very similar at each a_w level, except at 0.904, where the germination occurred slightly sooner at 21 °C (5 days) than at 35 °C (5.75 days). At a_w 0.922, a decrease in temperature from 15 °C to 6 °C
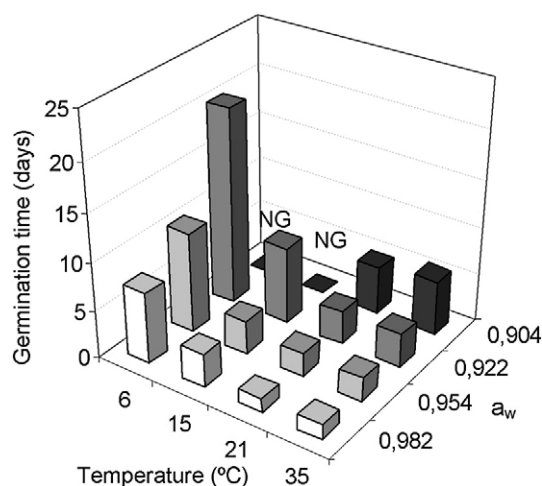

Fig. 1. Effect of water activity (a_w) and temperature on germination time of *Alternaria alternata* on tomato pulp agar (NG: no growth).

increased the germination time by 12.5 days, the latter being the a_w–temperature combination with the longest germination time in this study (20.5 days).

The results obtained in the present work were in agreement with data from other authors. The optimum temperature reported for germination of *A. alternata* varied between 25 and 30 °C, while the minimum and maximum were 5 °C and 35 °C respectively. The minimum a_w reported for germination was 0.84 (Chandrashekar and Ball, 1980; Dickinson and Bottomley, 1980; Magan and Lacey, 1984).

3.2. Water activity and temperature effect on radial growth rate

Statistical analysis of variance (ANOVA) showed that a_w, temperature, and their interaction significantly affected radial growth rate of *A. alternata* ($p < 0.0001$).

Fig. 2 shows the radial growth rate of *A. alternata* on a tomato pulp medium at four different levels of a_w and temperature. The optimum condition for growth was a_w 0.982 and 21 °C (8.31 mm/day). At this a_w level, the growth rates were higher at all temperatures evaluated, which indicates that high a_w is favorable for this mould. Growth was slow at a_w 0.922 at all temperatures and no growth or germination was observed at 6 °C and 15 °C at 0.904 a_w. The effect of temperature was also observed. The fastest growth was registered at 21 °C at all a_w levels. At 35 °C the growth rates were higher than at 15 °C, except at the maximum a_w level 0.982. A temperature of 6 °C caused a significant reduction in growth rates, even at the optimum a_w level.

According to the literature, the minimum a_w for growth of *A. alternata* in different culture media was determined between 0.84 and 0.88 (Magan and Lacey, 1984; Rowan et al., 1999; Sautour et al., 2001), while the optimum was described in a range of 0.98–1.00 a_w. These data agree with the results of the present work. The optimum temperature reported for growth was in the range of 25–30 °C, and the maximum and minimum ranges were 32–35 °C and 5–6.5 °C respectively (Chandrashekar and Ball, 1980; Dickinson and Bottomley, 1980; Magan and Lacey, 1984; Sautour et al., 2002). The temperature range allowing growth of *A. alternata* on tomato medium was similar to those reported in other media; however, relatively high growth rates were observed at 35 °C at a_w levels 0.954 and 0.982 (3.96 and 4.62 mm/day respectively). This might suggest that *A. alternata* would be able to grow at slightly higher temperatures on tomato medium. Comparisons between our results and literature data are difficult because no reports have been found on the growth or germination of *A. alternata* on tomato and tomato products.
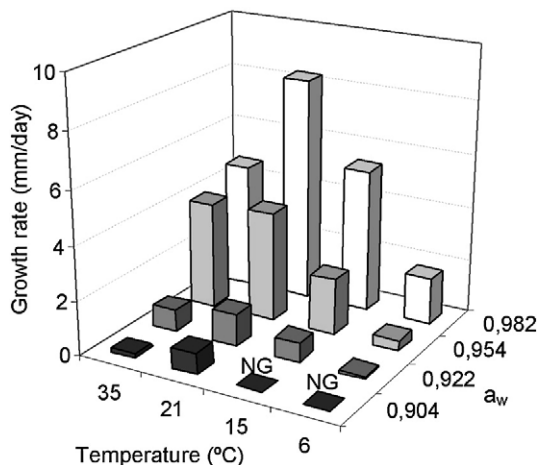
The concept of using cocktail inocula was introduced for physiological studies on foodborne bacterial pathogens, particularly in acquisition of data for predictive modelling studies, as a way of determining the extremes of growth limits for particular species. The use of bulked spore suspensions was applied for the first time to studies on the a_w tolerances of fungi by Hocking and Miscamble (1995). Although this approach can be criticized because of loss of information about the responses of individual strains of a species, it is accepted as a legitimate method for establishing the most extreme conditions under which a particular species is capable of growth. The use of a cocktail inoculum in the present study provided data closer to the real conditions at which tomato and tomato products are exposed.

A synthetic medium (TPA) similar to tomato fruits composition was developed. Its use provides a practical advantage because it simplifies the determination of germination time and the measurement of colony diameter, which would be difficult to perform on tomato fruits.

The range of temperatures selected for the present study is representative of ambient temperatures at which tomato fruits are stored in warm temperate regions such as our country in the different seasons (35 °C, 21 °C, and 15 °C, in summer, autumn and winter respectively); and 6 °C was selected as a refrigeration temperature. The extremes of the range were chosen close to the values registered in the literature as limitant for the growth of *A. alternata* on other substrates (Lacey, 1992). However, our results showed that tomato medium allowed higher growth rates in the upper limits of temperature. This could indicate that warm storage temperatures increase the risk of contamination with *Alternaria*.

The a_w range studied was selected considering the optimum and minimum a_w values reported for growth (0.98 and 0.88 respectively) (Lacey, 1992; Sautour et al., 2002). Intermediate values were chosen according to a_w values of tomato products (tomato sauce: 0.986; tomato chutney: 0.955; concentrated tomato paste: 0.93–0.85). Although *Alternaria* is probably inactivated during thermal processes, the raw material (tomato paste) is prone to contamination if it is not stored at adequate temperatures before processing. According to our results, concentrated tomato pastes, which are often used in the production of tomato sauces and purees, are also susceptible to *Alternaria* contamination in spite of their low a_w. Refrigeration of these products could be advised in order to prevent *Alternaria* growth and toxin production. Growth of *Alternaria* during the early stages of sun-drying of tomatoes is also of concern, especially in high temperature climate.

The germination and growth of *A. alternata* on tomato medium at high water activities, even at low temperatures, evidence the potential of this species for spoiling tomato fruits during storage, including refrigerated products. Considering the results obtained and the high a_w of tomato fruits (0.99) the recommended refrigeration temperatures should be below 6 °C. Temperature is one of the major environmental factors that affect the life of tomato fruits and their rate of deterioration by fungi.

4. Conclusions

The present study is the first report on the effect of a_w and temperature on the ecophysiology of *Alternaria alternata* on tomato medium. The results obtained could be extrapolated to evaluate the risk of spoilage in tomato fruits and tomato products caused by this pathogen, the main causal agent of Blackmould of ripe tomato fruits.

The substantial post-harvest losses registered justify studies to control the pathogen. The knowledge on the ecophysiology of the fungus in this substrate is necessary to elaborate strategies combining different methodologies and controlled environmental factors to prevent its development. The data presented here provide a matrix of growth and germination responses to water activity and temperature that may be used in constructing a mathematical model for the

Fig. 2. Effect of water activity (a_w) and temperature on growth rate of *Alternaria alternata* on tomato pulp agar (NG: no growth).

prediction of the shelf life of tomato products. Such a model will be of benefit to manufacturers of these and related products in development of new formulations and processes.

Knowledge of the environmental factors influencing fungal growth is important so that storage environments can be made unfavorable for mycotoxin formation. Furthermore, considering the toxigenic potential of *Alternaria* strains, understanding the effects of environmental factors on mycotoxins production by this fungus on tomato is necessary to evaluate the consumer health risk.

Acknowledgements

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