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Temperature and water stress impacts on growth and production of altertoxin-II by strains of *Alternaria tenuissima* from Argentinean wheat

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RESEARCH ARTICLE

Abstract

Alternaria tenuissima is commonly isolated from wheat in Argentina. The objective of this study was to examine the effects of temperature (15-34 °C) and water activity (0.98, 0.95 a_w) on growth and temporal altertoxin II (ALTX-II) production by two strains over 14-21 days on a milled wheat agar. It was shown that growth occurred over the whole temperature range tested and was optimum at 25-30 °C and 0.98 a_w, and 30 °C at 0.95 a_w. The incubation time did not show any significant effect on ALTX-II accumulation. The optimum conditions for ALTX-II production were 0.98 a_w and 30 °C for both strains. The strains also accumulated significant amounts of this toxin at 34 °C. This is the first study to evaluate the ecology of growth and production of ALTX-II by strains of *A. tenuissima*.

Keywords: temperature, water stress, growth, *Alternaria*, altertoxin II

1. Introduction

Alternaria is a ubiquitous fungal genus, with species commonly contaminating ripening cereals and harvested grain (Kosiak *et al.*, 2004; Li *et al.*, 2001; Logrieco *et al.*, 2009; Mercado Vergnes *et al.*, 2006). In Argentina, several studies have demonstrated that *Alternaria* species are important colonisers of ripening wheat, and it was found to be the major component of the wheat mycota (Broggi *et al.*, 2007; González *et al.*, 1996; 1999, Patriarca *et al.*, 2007). *Alternaria tenuissima* has been shown to be the major species isolated from Argentinean wheat (Patriarca *et al.*, 2007). It has been isolated more frequently than *Alternaria alternata* and *Alternaria infectoria*, which have been reported as the predominant species in cereals in several studies worldwide (Andersen *et al.*, 1996; Kosiak *et al.*, 2004; Li *et al.*, 2001; Logrieco *et al.*, 2009; Medina *et al.*, 2006; Webley *et al.*, 1997). Previous studies have demonstrated that the most common *Alternaria* toxins present in Argentinean wheat were tenuazonic acid (TeA), alternariol (AOH), and alternariol monomethyl ether (AME) (Azcarate *et al.*, 2008). The toxicological aspects of these mycotoxins have been described. Of particular health concern is the association found between *A. alternata* contamination in cereal grains and the high levels of human oesophageal cancer in China (Liu *et al.*, 1992). TeA is toxic to several animal species, e.g. mice, chicken and dogs. In dogs, it caused haemorrhages in several organs; in chicken it reduced feed efficiency, suppressed weight gain and increased internal haemorrhaging. AOH and AME might cause cell mutagenicity, could combine with the DNA isolated from human foetal oesophageal epithelium, and AOH could induce squamous cell carcinoma of the foetal oesophagus. AOH has been reported to possess cytotoxic, genotoxic and mutagenic properties *in vitro* (Ostry, 2008).

Recently, it was demonstrated that altertoxin II (ALTX-II) is more mutagenic than alternariols in terms of DNA strand breaking in mammalian cells (Fleck *et al.*, 2012). It induced mutations at the hypoxanthine guanine phosphoribosyltransferase gene locus at concentrations similar to that of the established mutagen 4-quinoline-*N*-oxide, thus proving to be at least 50 times more potent mutagen than the alternariols. Several *Alternaria* species isolated from wheat grown in Argentina have shown the capability of producing ALTX-II. For example, of 17

A. tenuissima strains isolated from wheat, 13 (76%) were found to produce ALTX-II (A. Patriarca, unpublished data). However, detailed studies of the kinetics of ALTX-II by *A. tenuissima* have not been examined.

Fungal growth and mycotoxin production has been shown to be markedly affected by environmental factors, especially water availability (water activity, a_w) and temperature (Sanchis and Magan, 2004). The production of TeA, AME, alternuene (ALT) and AOH by *Alternaria* species in relation to these factors have been described in different substrates including cereals and soyabeans (Magan *et al.*, 1984; Oviedo *et al.*, 2010, 2011; Pose *et al.*, 2010). However, no data are available on the biosynthesis of ALTX-II under different individual or interacting environmental conditions. Because of the potential toxicity of ALTX-II there is a need to better understand the relationship between these ecological factors and the production of this mycotoxin. The objective of this study was to examine the effect of temperature (15-34 °C) and a_w (0.98, 0.95) interactions on (1) growth and (2) ALTX-II production by two strains of *A. tenuissima* on a wheat-based matrix.

2. Materials and methods

Fungal strains

Two *A. tenuissima* strains (W11.1.2 and W43.3.1) isolated from wheat cultivated in Argentina were used for this experiment. For identification, single germinating conidia were transferred to potato carrot agar (PCA; Simmons, 2007) and incubated under standardised conditions according to Simmons (2007). The 9 cm Petri plate cultures were incubated in a single layer under light 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. The colony and sporulation characteristics of the strains were compared to those of representative cultures of *A. tenuissima* EGS 34.015 (Dr. Emory Simmons collection, Mycological Services, Crawfordsville, IN, USA) in standard culture conditions. Based on sporulation patterns and conidial morphology, the isolates were identified as *A. tenuissima*. The strains are maintained in the IBT collection at the Department of Systems Biology, DTU (Kgs. Lyngby, Denmark).

Medium, inoculation, incubation conditions and growth measurements

A 2% milled wheat agar medium was used in this study. Milled wheat was prepared by homogenisation for 5 min in a laboratory homogeniser. Mixtures of 2% (w/v) wheat flour in water were made and 2% (w/v) agar was added. Water used to prepare the medium was modified with glycerol to achieve a final a_w level of 0.95 and 0.98, respectively. The medium was autoclaved at 121 °C and poured into 9 cm diameter Petri dishes (15 ml per plate).

Agar discs (4 mm diameter) from 7-day-old cultures of each of the two strains grown on malt extract agar (MEA; Pitt and Hocking, 2009) were cut using a sterile cork-borer and all the treatment and replicate plates centrally inoculated. A total of five replicates per treatment were incubated at 15, 20, 25, 30 and 34 °C for 14 and 21 days at each 0.95 and 0.98 a_w level. The experiment was carried out twice. To minimise water transfer from or to the medium, additional blank Petri plates corresponding to the same a_w level were placed in closed polyethylene bags. Growth was measured for periods of 14-21 days every two days or as required in two directions at right angles to each other. The temporal extension rates were used to determine the growth rates by using the linear regression of the diametric extension rates.

Altertoxin-II extraction and analysis

At the end of the 14 and 21 days (three) separate replicates of the colonised wheat agar plates were weighed and extracted by mixing with 15 ml methanol and shaking for 30 min at 250 rpm at 25 °C. To this methanol extract was added 5 ml 10% ammonium sulphate solution and filtered. The filtrate was extracted twice with 5 ml chloroform and the chloroform phase was separated and evaporated to dryness. Dry extracts were re-dissolved in 500 µl of HPLC grade methanol and kept at -20 °C until analysis.

The analyses were carried out on a system consisting of an Agilent 1100 Series HPLC equipped with a UV diode-array detector set at 258 nm (Agilent Technologies, Palo Alto, CA, USA). The column used was an Agilent Eclipse Plus C18 (4.6×150 mm, 3.5 µm), preceded by a Phenomenex Gemini C18 (3 mm, 3 µm) guard cartridge. The mobile phase consisted of methanol:water (90:10, v/v) containing 300 mg $ZnSO_4 \cdot H_2O$/l. A flow rate of 0.4 ml/min was used, and the column temperature was 25 °C. Signals were processed by Agilent ChemStation software (Rev. B.03.01). ALTX-II standard was provided by the Institute of Applied Biosciences (Karlsruhe Institute of Technology, Karlsruhe, Germany). The standard was dissolved in HPLC grade methanol at a concentration of 1.0 mg/ml and stored at -20 °C. Working standard solutions (100, 50, 10, 5, and 1 µg/ml) were prepared by appropriate dilution of the stock standard with methanol and used to obtain calibration curves for HPLC-diode array detector analysis. Quantification was achieved through comparison of peak areas of the chromatograms of the samples with those of the standard solutions.

Experimental design and data analyses

A full factorial design with two variables (a_w and T) was used for growth analysis. Five independent replicates for each a_w-temperature combination were made for each of the *A. tenuissima* strains and the experiment was carried out twice (for growth studies). For toxin analysis, the

full factorial design was made with three variables (a_w, T, and time), with 3 independent replicates of each a_w-temperature-time combination for each strain. Statistical analysis was performed using the Statistica 8 software (StatSoft, Inc., Tulsa, OK, USA). The effect of temperature and water activity on growth rate was examined by ANOVA. Normality of mycotoxin production data was tested by Shapiro-Wilk test. Due to non-normality of the data, Kruskal-Wallis analysis of ranks was used for examining differences between groups, considering a_w, temperature, and time as variables for all data sets and for each strain individually. The two *A. tenuissima* strains were compared using two-sample t tests.

3. Results

Effect of temperature × water activity on growth of *Alternaria tenuissima* strains

Figure 1 shows the effect of the interacting conditions examined on the diametric growth rates of the two strains. This shows that both strains had a very broad temperature range for growth with an optimum at 25-30 °C at 0.98 a_w for strain W11.1.2, while for the other strain (W43.3.1) this was 30 °C and 0.98 a_w. At 0.95 a_w, growth was reduced by >50-60% regardless of temperature. For both strains, the optimum temperature at 0.95 a_w was 30 °C. The strains were still able to grow relatively similar at 0.95 a_w at 34 °C as at 30 °C. Statistically, all factors, a_w, temperature, strain and their interactions, significantly affected growth rate ($P<0.0001$).

Effect of temperature × water activity on temporal altertoxin II production

Figure 2 shows the relative changes in production of ALTX-II by the two strains of *A. tenuissima* in relation to the temperature range and two a_w levels examined. ALTX-II was produced by both *A. tenuissima* strains under all the conditions tested. The higher a_w level (0.98) favoured the accumulation of this toxin at all temperatures and incubation times. The general trend in the behaviour of both strains as a function of temperature was similar at 0.95 a_w, with low production at 15 °C, the optimum at 20 °C, with a relative decrease at 25-30 °C, and a second increment at 34 °C. The incubation time did not show any significant effect on ALTX-II accumulation, even though a higher amount of toxin was detected after 21 days incubation at most of the treatment conditions examined. At 0.98 a_w, the optimum production was observed at 30 °C for both strains. For *A. tenuissima* W11.1.2, the maximum amount of toxin was detected at 0.98 and 30 °C after 21 days incubation (1,403 ng/g), while for *A. tenuissima* W43.3.1 it was produced at the same temperature and a_w after 14 days (2,021 ng/g). Strain W11.1.2 also produced high amounts of toxin at 20 and 34 °C at 0.98 a_w. For *A. tenuissima* strain W43.3.1, the production was much lower at temperatures different from the optimum, except at 34 °C, where the amount of toxin produced was 47% of the optimum production. Statistically, there was an overall significant effect of temperature on ALTX-II production ($P<0.01$). However, examining each strain individually showed that temperature was a significant factor only for strain W11.1.2 ($P<0.05$). Furthermore, there was a significant difference in toxin production between the two strains ($P=0.05$). The effect of a_w was significant for both strains ($P<0.0001$).

4. Discussion and conclusions

There has been much discussion about the taxonomy of *Alternaria* species. This has been revised regularly in recent years. The traditional identification techniques, based solely on morphological characteristics of conidia, have led to the general belief that *A. alternata* was the most abundant species in nature. After a series of revisions, Simmons (2007) finally organised the genus into 276 species and developed the concept of 'species-group', a group sharing the same three-dimensional sporulation apparatus, characterised by a representative species. With the new taxonomic tools, species other than *A. alternata* were reported as

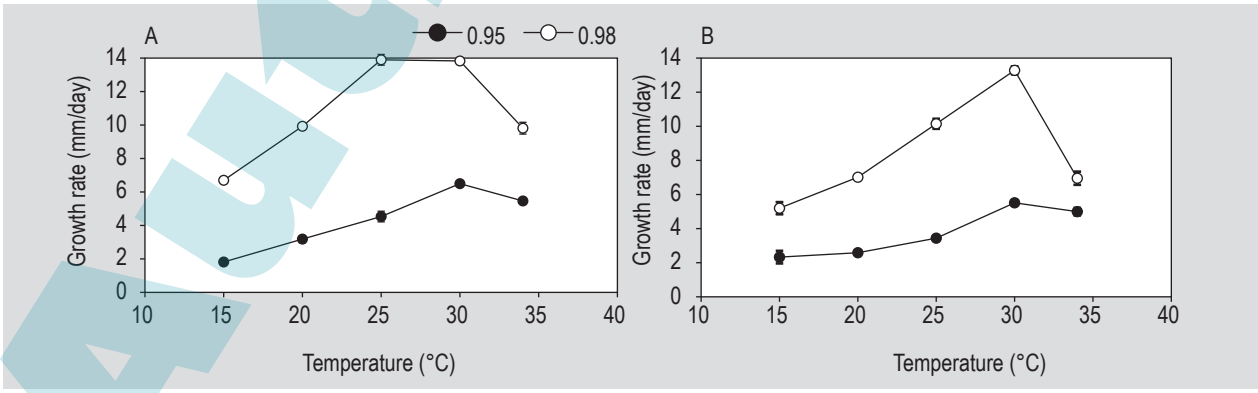

Figure 1. Effect of temperature × water activity on diametric growth rates (mm/day) of strains W11.1.2 (A) and W43.3.1 (B) of *Alternaria tenuissima* on a 2% milled wheat agar medium. Bars indicate standard errors of the mean.

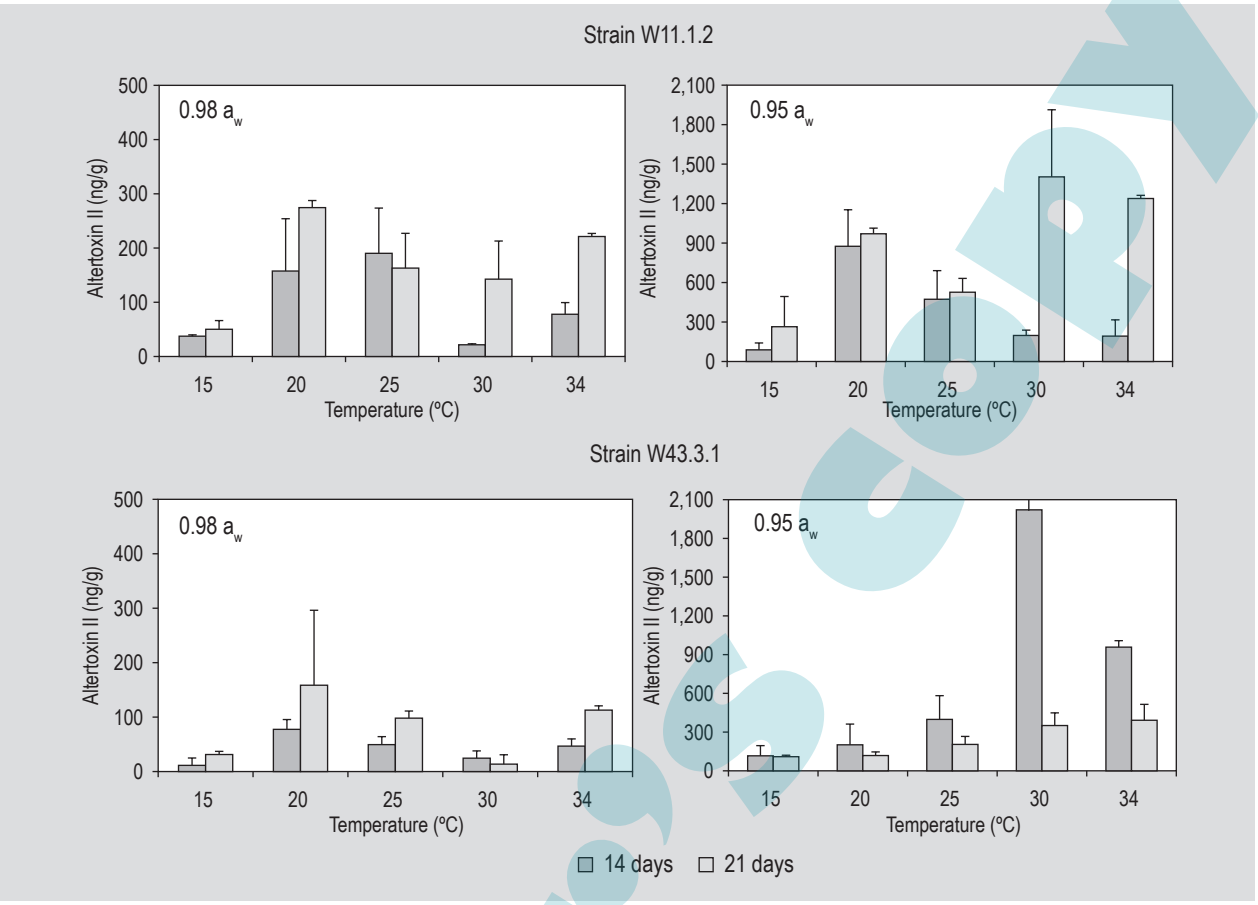

Figure 2. Kinetics of altertoxin-II production by two strains of *Alternaria tenuissima* over periods of 14 and 21 days on 2% milled wheat agar medium. Bars indicate standard errors. Please note different Y-axis range for 0.98 and 0.95 water activities.

predominant in several crops (Andersen and Frisvad, 2004; Greco *et al.*, 2012; Polizzotto *et al.*, 2012). *A. tenuissima* has been found to be the predominant species in Argentinean wheat in previous studies (Patriarca *et al.*, 2007).

This is the first study to evaluate the ecology of growth and production of ALTX-II by strains of *A. tenuissima* isolated from wheat. The relationship between interacting environmental conditions (a_w × temperature) and growth of the *A. tenuissima* strains was similar to that reported for *A. alternata.* Based on the water availability range known for *A. alternata*, the water activity levels chosen for the present study were selected as conditions representing intermediate water stress (0.95) and higher water availability (0.98), under which both growth and mycotoxin production can occur. This also represents the conditions that occur during wheat ripening at the milky ripe to mid-dough stage when mycobiota, including *Alternaria* and *Fusarium,* are able to colonise the ripening ears of cereals. Previous studies showed that the optimum a_w for *A. alternata* was between 0.98-1.00, and the optimum temperature in the range of 25-30 °C, while the maximum and minimum ranges for growth were 32-35 °C and 5-6.5 °C, respectively (Magan and Lacey, 1984; Magan *et al.*, 1984; Oviedo *et al.*, 2010, 2011; Pose *et al.*, 2009; Sanchis and Magan, 2004; Sautour *et al.*, 2002). Pose *et al.* (2009) found that *A. alternata* strains isolated from Argentinean tomato fruits showed relatively high growth rates at 35 °C and a_w levels of 0.954 (3.96 mm/day) and 0.982 (4.62 mm/day). The *A. tenuissima* strains used in the present study had even higher growth rates at 34 °C and both a_w levels. Strain W11.1.2 grew 5.46 mm/day at 0.95 a_w and 9.81 mm/day at 0.98 a_w, while the strain W43.3.1 grew 5.0 and 6.96 mm/day at the same a_w levels, respectively. This is in contrast with the available data on *A. alternata* isolated from wheat in the UK. Magan and Lacey (1985) showed that growth of these strains occurred at 30 °C but with a sharp decline to no growth at 35 °C. They showed 8 mm/day diametric growth at 25 °C with 0.2 mm/day diametric growth at 30 °C and no growth at 35 °C. In Argentina, growth rates of 5-6 mm/day were found at 35 °C, which suggests a better adaptation to elevated temperatures than in the UK.

The maximum production of ALTX-II was observed at 0.98 a_w and 30 °C for both strains, which were also the optimum conditions for growth. No data are available on the effect of a_w × temperature effects on production of AOH, AME or TeA by *A. tenuissima* on wheat matrices. An early study

by Young *et al.* (1980) examined TeA production by *A. tenuissima* on cottonseed. Maximum production was at 20 °C and 37.5% moisture content (=1.00 a_w). Production was reduced by 50% when a_w was changed to 0.95. They suggested that probably 20 °C and >0.90 a_w was required for TeA production on cottonseed. However, the temperature range examined in the present study was not included. Magan *et al.* (1984) reported that AOH, AME and ALT were produced in highest amounts by *A. alternata* at 0.98 a_w and 25 °C both on wheat extract agar and wheat grain. Other studies reported optimum production of TeA between 21-30 °C at 0.98 a_w (Oviedo *et al.*, 2009; Pose *et al.*, 2010), for AOH between 15-25 °C and 0.95-0.98 a_w (Oviedo *et al.*, 2010, 2011; Pose *et al.*, 2010), and for AME between 30-35 °C and 0.92-0.98 a_w (Oviedo *et al.*, 2010, 2011; Pose *et al.*, 2010) in different substrates. Magan and Baxter (1994) isolated a range of *Alternaria* strains from sorghum, which were screened for TeA production on Fries medium and then on sorghum-based medium or sorghum grain. All six strains were able to produce TeA *in vitro* on a modified Fries medium with most produced after 28 days. Studies with one isolate (Alt5) showed that TeA was produced over the range 10-30 °C and a_w levels of 0.998-0.93. On sorghum-based media, the maximum production was observed at 0.95 a_w and 25 °C after 28 days. On sorghum grain, the production was best at 0.995 a_w after 14-21 days storage at 25 °C. However, these strains were not definitively identified as *A. tenuissima*.

Overall, there is a great diversity in the optimum conditions reported for toxin production by *Alternaria* spp. depending on the substrate and due to intraspecific differences. However, the general trend suggests that high temperatures and a_w levels would lead to a significant accumulation of toxins, especially ALTX-II, TeA and AME. The existence of synergistic effects between these mycotoxins is still unknown but further toxicological studies are necessary to evaluate the implication of their co-occurrence in food products.

The ability of *A. tenuissima* strains isolated from Argentinean wheat to tolerate elevated temperatures (34 °C) and synthesise a considerable amount of ALTX-II in a range of 30-34 °C indicates that this species could play an important role under climate change conditions. The Argentinean wheat production area is divided into five regions (I to V), further divided into subregions (North or South), according to agrometeorological conditions. Regions IIS, IV, VS and IIN are the main production areas, with maximum temperatures and humidity progressively increasing from VS to IV, IIS and IIN. The sowing of long cycle wheat starts between the months of May and June, and the flowering occurs in spring during September-November, with average temperatures in the field of 15 to 25 °C, and maximum temperatures occasionally reaching 35 °C in the IIN region. Actual climate conditions are favourable for *A. tenuissima* growth and production of ALTX-II, especially in the warmer crop regions. In a climate change scenario, this pathogen could become a potential hazard in most of the cultivated areas, and the presence of this mycotoxin in wheat grains could become important.

In conclusion, these data suggests that, while growth of strains of *A. tenuissima* under different interacting conditions may vary only slightly, the amounts of ALTX-II that can be produced vary with temperature and a_w. There appear to be different temperatures at each a_w level at which production is optimum, i.e. 30 °C at 0.98 a_w and 20 °C at 0.95. It is also notable that both strains were able to produce high amounts of ALTX-II, even at 34 °C, over the a_w range tested. These are useful additional data of the potential of such mycotoxigenic fungi and their mycotoxins becoming important under changing climatic conditions.

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