Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Water activity and temperature effects on growth and mycotoxin production by *Alternaria alternata* strains isolated from Malbec wine grapes

L.P. Prendes[1,2], V.G.L. Zachetti[2,3], A. Pereyra[3], V.I. Morata de Ambrosini[1,2] and M.L. Ramirez[2,3]

1 Facultad de Ciencias Aplicadas a la Industria, Universidad Nacional de Cuyo, San Rafael, Mendoza, Argentina
2 Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Sede Central, Buenos Aires, Argentina
3 Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina

Keywords
Alternaria alternata, growth, mycotoxins, temperature, water activity, wine grapes.

Correspondence
Luciana Prendes, Facultad de Ciencias Aplicadas a la Industria, Universidad Nacional de Cuyo, Bernardo de Irigoyen 375, 5600 San Rafael, Mendoza, Argentina.
E-mails: luciana_prendes@hotmail.com; lpprendes@fcai.uncu.edu.ar

2017/1862: received 23 August 2016, revised 27 October 2016 and accepted 11 November 2016

doi:10.1111/jam.13351

Abstract

Aims: To study the effects of water activity (a_W; 0·99, 0·98, 0·97, 0·96 and 0·95), temperature (15, 25 and 30°C), incubation time (7–28 days), and their interactions on mycelial growth and alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TA) mycotoxin production on a synthetic nutrient (SN) media similar to grape composition by three strains of *Alternaria alternata* isolated from wine grapes from Argentina.
Methods and Results: Interacting conditions of a_W, temperature and time of incubation were used to examine growth and mycotoxin production. All strains were able to grow at 0·95 a_W, but maximum growth rates were obtained at 0·99 a_W and 25°C. Maximum levels of AOH, AME and TA were obtained at 0·99 a_W and 25°C, but high amounts of TA were also obtained at 0·96 a_W and 15 or 30°C. Production of AOH and AME was favoured over TA at 25°C. TA levels were more sustained than AOH and AME.
Conclusion: The optimum and marginal conditions for growth and mycotoxin production by *A. alternata* on a SN media similar to grape composition were in agreement, but certain stressful conditions for growth evaluated also promote mycotoxin production.
Significance and Impact of the Study: Temperature and a_W conditions that allows growth and mycotoxin production are those normally found during wine grape ripeness in the field. Therefore, efforts should be made to prevent *Alternaria* presence and mycotoxin production in wine grapes.

Introduction

Nowadays, wine consumption has become a synonym of culture and lifestyle worldwide, and it has also been associated with beneficial effects for human health (Artero *et al.* 2015). In 2015, worldwide production of wine has been significant reaching 274·4 mhL. Argentina, with a production of 13·4 mhL during 2015, ranks fifth among wine-producing countries and exports its wines to important markets such as United Kingdom, Denmark, the Netherlands, Russia, the United States, Canada and Brazil (OIV 2016).

Alternaria is the main component of wine grape mycobiota from different winemaking regions in Argentina and worldwide (Magnoli *et al.* 2003; Rousseaux *et al.* 2014; Prendes *et al.* 2015; Tančinová *et al.* 2015), and it has been associated to grape berry rot in the field under conducive environmental conditions (Pearson and Gohheen 1996; Cucchi and Becerra 2009; Kakalikova *et al.* 2009; Steel *et al.* 2013). Fungal contamination of grapes could occur before or during harvest and processing. In addition, *Alternaria alternata* strains isolated from Malbec wine grape have demonstrated the *in vitro* ability to produce alternariol (AOH), alternariol monomethyl ether

(AME) and tenuazonic acid (TA) in synthetic media (Prendes *et al.* 2015; Trinidad *et al.* 2015). Also, the natural occurrence of some of these mycotoxins in wine grapes, grape juices and wine has already been reported (Scott *et al.* 2006; Broggi *et al.* 2013; Pizzutti *et al.* 2014; Fan *et al.* 2016; Fontana *et al.* 2016; López *et al.* 2016).

The exposure to *Alternaria* toxins has been linked to a great variety of adverse effects to both human and animal health (Dall'Asta *et al.* 2014). 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.* 1991, 1992). The toxicity of TA has been described in plants and numerous animal species, including chicken, guinea pigs, mice, rabbits, dogs and rhesus monkeys, and it has been associated with human haematological disorders such as onyalai, a form of thrombocytopenia. AOH and AME are mutagenic and cytotoxic to bacterial and mammalian cells and are suspected to be carcinogenic (Ostry 2008; Logrieco *et al.* 2009; Dall'Asta *et al.* 2014). Even though, there is not neither international nor Argentinian regulations about the presence of *Alternaria* toxins in food and feed. The European Food Safety Authority (EFSA) suggested in 2011 that *Alternaria* toxins are of high concern for public health (EFSA 2011). Altogether, these results indicate that the presence of *Alternaria* genera in wine grapes could represent a risk in the health of wine consumers.

Fungal growth and mycotoxin production are the result of complex interactions between diverse biotic and abiotic factors, and knowledge of the effect of each involved factor is crucial for prediction, understanding and prevention of mycotoxins contamination in food and by-products. Among these factors, water activity (a_W) and temperature have been described as the most important pre- and postharvest environmental factors (Sanchis and Magan 2004).

The influence of temperature and a_W in the growth and mycotoxin production (AOH, AME, TA and ALTX-II) of *Alternaria* strains isolated from different substrates (wheat, sorghum, sunflower, soya beans and tomatoes) in synthetic media with similar composition has been recently revised (Lee *et al.* 2015). However, at present, no studies have compared the effect of a_W and temperature on growth of *A. alternata* strains isolated from wine grapes and identified the conditions conducive to growth and mycotoxin production. Such information will be important in developing realistic forecasting systems for predicting risk of grapes colonization and mycotoxin production by *A. alternata*.

Since the *Alternaria* genus has been found in all developmental stages of wine grapes and in different winemaking regions, the knowledge of optimal and suboptimal a_W and temperature for growth and mycotoxin production of these species can be an important aid to predict and prevent the presence of such mycotoxins in wine grapes and wine. Therefore, the aim of this work was to study the effects of a_W, temperature and incubation time on growth and AOH, AME and TA production on a synthetic grape media which simulates grape composition by three strains of *A. alternata*.

Materials and methods

Fungal strains

Three *A. alternata* strains (5·5, 7·5 and 25·1) previously isolated from asymptomatic Malbec wine grapes from DOC or DO (Denomination of Origin) San Rafael (Mendoza, Argentina) during 2011 and 2012 vintage were used. This wine grape-growing region is located between 34·3° and 34·8°S latitude, 67·4° and 68·5°W longitude, and 500 and 800 m altitude. These isolates have been previously identified by morphological and molecular methods and were selected as representative because of its high but different production profiles of AOH, AME or TA in ground rice-corn steep liquor medium (GRCS). Also, its moderate to high pathogenicity grade in a detached berry test (grade 2 or 3), similar to the majority of the *A. alternata* strains isolated from Malbec grapes, was analysed during a previous study (Prendes *et al.* 2015). Cultures were maintained in 15% glycerol (v/v) at −80°C.

Medium and water activity adjustment

Studies were carried out *in vitro* using a synthetic nutrient (SN) media with composition similar to grapes at mid-veraison (Bellí *et al.* 2005; Leong *et al.* 2006). The medium had the following composition: D (+) glucose, 70 g; D (−) fructose, 30 g; L (−) tartaric acid, 7 g; L (−) malic acid, 10 g; $(NH_4)_2SO_4$, 0·67 g; $MgSO_4$ (7 H_2O), 1·5 g; NaCl, 0·15 g; $CuCl_2$, 0·0015 g; $FeSO_4$ (7H_2O), 0·021 g; $ZnSO_4$ (7H_2O), 0·0075 g; (+) catechin, 0·05 g; agar, 25 g; distilled water, 1000 ml. The pH of the medium was adjusted to 4·2 with KOH (6 mol l^{-1}), and this pH correspond to those present in Malbec grapes from DOC San Rafael at harvest (Martin and Morata de Ambrosini 2014). The a_W of this basal medium was 0·99. The a_W of SN media was modified to 0·98, 0·97, 0·96 and 0·95 by adding different amounts of glycerol (M.L. Ramirez, personal communication). The media were autoclaved at 120°C for 20 min. Flasks of molten media were thoroughly shaken, prior to pouring into sterile 9-cm Petri dishes. The a_W of representative samples of media was checked with a water activity metre (Aqualab

Series 3; Decagon Devices, Inc., Pullman, WA). Additional control plates were prepared and measured at the end of the experiment to detect any significant deviation of the a_W.

Inoculation, incubation and growth assessment

SN media agar plates were inoculated centrally with a 3-mm agar disk taken from the margin of a 7-day-old colony of each isolate grown on synthetic nutrient agar (SNA) at 25°C. Inoculated plates of the same a_W were sealed in polyethylene bags and incubated at 15, 25 and 30°C for 28 days. A full factorial design was used, where the factors were a_W, temperature and strains, and the response was growth (total numbers of plates: 5 a_W × 3 temperatures × 3 strains × 3 replicates). The entire experiment was repeated twice.

Assessment of growth was made every day during the incubation period, and two diameters of the growing colonies were measured at right angles to each other until the colony reached the edge of the plate. Colonies radii were plotted against time, and linear regression was applied in order to obtain the growth rate (mm day^{-1}) as the slope of the line.

AOH, AME and TA extraction, detection and quantification

The extraction method used was based on a microscale extraction (Smedsgaard 1997) modified by Andersen *et al.* (2001) into a three-step extraction procedure suited for *Alternaria* metabolites. At 7, 14, 21 and 28 days of incubation, three plugs (4-mm diameter) were removed from the inner, middle and outer area of each colony. Plug were weighed and introduced into 4-ml amber screw-cap vial. The plugs were extracted, re-dissolved and filtered to use in HPLC analysis, as previously described by Prendes *et al.* (2015).

The HPLC system consisted of a Hewlett Packard model 1100 pump (Palo Alto, CA) connected to a Hewlett Packard 1100 Series variable wavelength detector and a data module Hewlett Packard Kayak XA (HPChemStation Rev. A.06·01). Chromatographic separations were performed on a Symmetry C18 (100 × 4·6 mm i.d., 5-μm particle size) connected to a guard column SecurityGuard (20 × 4·6 mm i.d.) filled with the same phase. The AOH, AME and TA mobile phase and running conditions were those described by Prendes *et al.* (2015). For AOH and AME, the detector was set at 256 nm and the retention times were 11·8 and 17·5 min, respectively. Quantification was relative to external standards of 0·5, 1·0, 2·0 and 3·0 mg ml^{-1} in acetonitrile/water (25 : 75, v/v). For TA, the detector was set at 279 nm and the retention time was 7·0 min. Quantification was relative to external standards of 0·5, 1·0, 2·0 and 3·0 mg ml^{-1} in acetonitrile/0·027 mol l^{-1} sodium dihydrogen phosphate solution (25 : 75, v/v).

The number of toxin analysis was as follows: 5 a_W × 3 temperatures × 3 strains × 4 times of incubations × 3 replicates.

Recovery experiment was performed on SN media at levels of 10–1000 ng g^{-1} with AOH, AME and TA, respectively. Mean recovery ranged from 87% to 94%, from 89% to 92% and from 85% to 98% for AOH, AME and TA, respectively. Limit of detection (LOD, signal-to-noise ratio 3) was 0·001 μg g^{-1} for the three toxins, and the quantification limit (LOQ) was established as three times the detection limit.

Statistical analysis

The growth rates and mycotoxin concentration were evaluated by analysis of variance (ANOVA) to determine the effect of a_W, temperature and *A. alternata* strains and two- and three-way interactions. When the analysis was statistically significant, the *post hoc* Tukey's multiple comparison procedure was used for separation of the means. Statistical significance was judged at the level $P \leq 0{\cdot}001$. Statistical analysis was done using SigmaStat for Windows Version 2·03 (SPSS Inc., Chicago, IL). Surface response and contour map graph were produced using Sigma Plot Version 10·0 (Systat Software Inc., Hounslow, London, UK).

Results

Effects of a_W and temperature on growth

Figure 1 gives a diagrammatic representation of the interaction of a_W and temperature on growth rate of all *A. alternata* strains studied in SN media. Optimal a_W levels for growth rate ranged from 0·97 to 0·99 at 15 to 30°C, with maximum growth at the highest a_W used (0·99) and 25°C. We defined as optimal those growth rates that were above 50% of the maximum growth rates obtained. The three strains were able to growth at the lowest a_W tested (0·95) at the three temperatures evaluated (15, 25 and 30°C).

The strains showed differences in the growth rates at the highest temperature evaluated (30°C). The strains *A. alternata* 7·5 and 25·1 showed a similar behaviour, decreasing its growing rates at 30°C with respect to 25°C at all a_W evaluated, with an exception at 0·95 a_W for 7·5 strain. Meanwhile, the growth rates for *A. alternata* 5·5 at 30°C and 0·98, 0·97, 0·96 and 0·95 a_W were the same as at 25°C, with an exception at 0·99 a_W, where a decrease was observed.

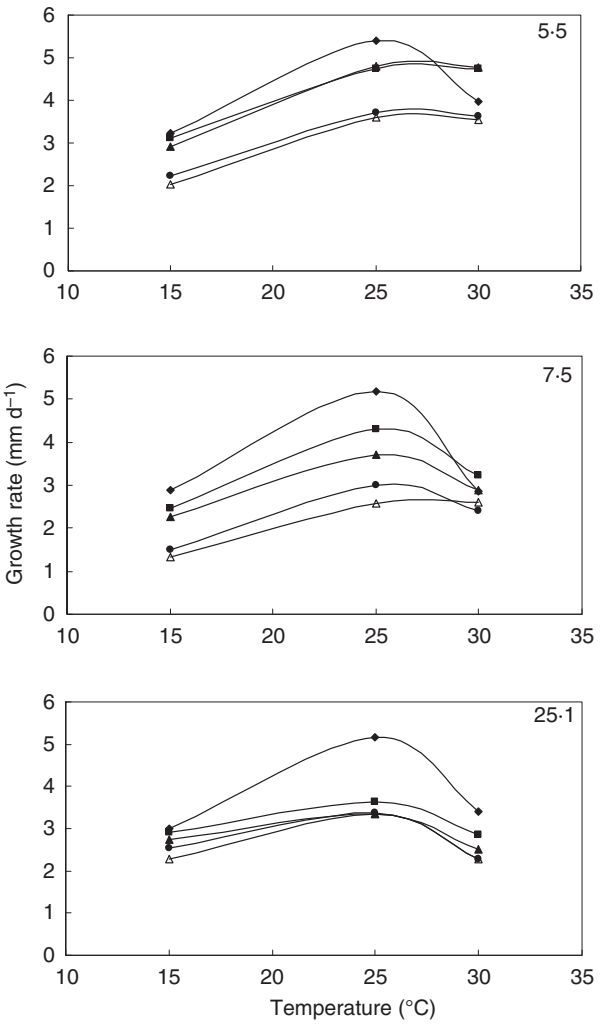

Figure 1 Effect of water activity, 0·95 (△), 0·96 (●), 0·97 (▲), 0·98 (■) y 0·99 (♦) and temperature on growth rates of *Alternaria alternata* strains 5·5, 7·5 and 25·1, on SN media.

In general, growth of all strains decreased as a_W of the media was reduced. However, strain 5·5 showed the same growth rate at 0·98 and 0·97 a_W and at 0·96 and 0·95 a_W in all temperatures evaluated, and particularly, growth rate at 30°C and 0·97 and 0·98 was higher than at 0·99 a_W. Strain 7·5 showed a higher growth rate at 0·98 a_W, followed by 0·99, 0·97, 0·95 and 0·96 a_W at 30°C. Meanwhile, strain 25·1 showed a higher growing rate at 25°C and 0·99 a_W followed by 0·98 but the rest of the a_W showed the same growing rate.

The ANOVA of the effect of single variables (isolate, a_W and temperature) and two- and three-way interactions revealed that all variables alone and all interactions had a significant effect on growth rates ($P < 0·001$; Table 1), being the temperature the most important factor (F-Snedecor: 1959·753; Table 1).

Table 1 Analysis of variance on the effects of different strains (S), water activity (a_W) and temperature (*T*), and their interactions on growth of *Alternaria alternata* on synthetic nutrient media

Source of variation	Degrees of freedom	Mean square	F-Snedecor
S	2	9·831	813·197*
a_W	4	7·017	580·458*
T	2	23·692	1959·753*
S × a_W	8	0·510	42·154*
S × *T*	4	2·031	168·006*
a_W × *T*	8	0·884	73·109*
S × a_W × *T*	16	0·156	12·944*

*$P < 0·001$.

The conditions under which equivalent growth rates occurred under different environmental conditions were joined to produce contour lines to map the relative optimum and marginal conditions for growth of the *A. alternata* strains examined (Fig. 2). The fastest growth for the three strains tested occurred around 0·99 a_W and 25°C, but a rapid growth was also observed in a broader range of 0·97–0·99 a_W and 25–30°C for strain 5·5.

Effect of a_W, temperature and incubation time on AOH, AME and TA production

The surface response curves of AOH, AME and TA at 15, 25 and 30°C over 28 days of incubation are shown in Figs 3–5. All metabolites were produced in whole range of temperature and a_W evaluated (15–30°C and 0·95–0·99, respectively). In general, the maximum toxin levels were produced at the highest a_W evaluated (0·99) and at 25°C and the production declined in the following order 25, 30 and 15°C and as a_W was reduced. However, TA levels were shown to be more sustained than AOH and AME, and high levels of AOH, AME and TA production were also detected at 0·96 a_W and 15 or 30°C, depending on the strain and the toxin considered.

The highest level of AOH was produced at 25°C and 0·99 a_W by the strain 25·1 after 14 days of incubation (more than 25 000 $\mu g\ g^{-1}$ followed by strain 7·5 after 21 days of incubation (more than 1600 $\mu g\ g^{-1}$) and the strain 5·5 after 21 days (more than 250 $\mu g\ g^{-1}$). Increments in AOH production were also detected at 0·96 a_W and 15°C after 28 days of incubation for the strains 7·5 and 5·5.

The highest level of AME was produced at 25°C and 0·99 a_W by the strain 25·1 after 14 days of incubation (more than 16 000 $\mu g\ g^{-1}$) followed by the strain 7·5 after 14 or 21 days of incubation (more than 3000 $\mu g\ g^{-1}$) and the strain 5·5 after 21 days (more than 900 $\mu g\ g^{-1}$). A slight increase was also detected at 0·96 a_W and 15°C after 21 days of incubation for the strain 7·5 and

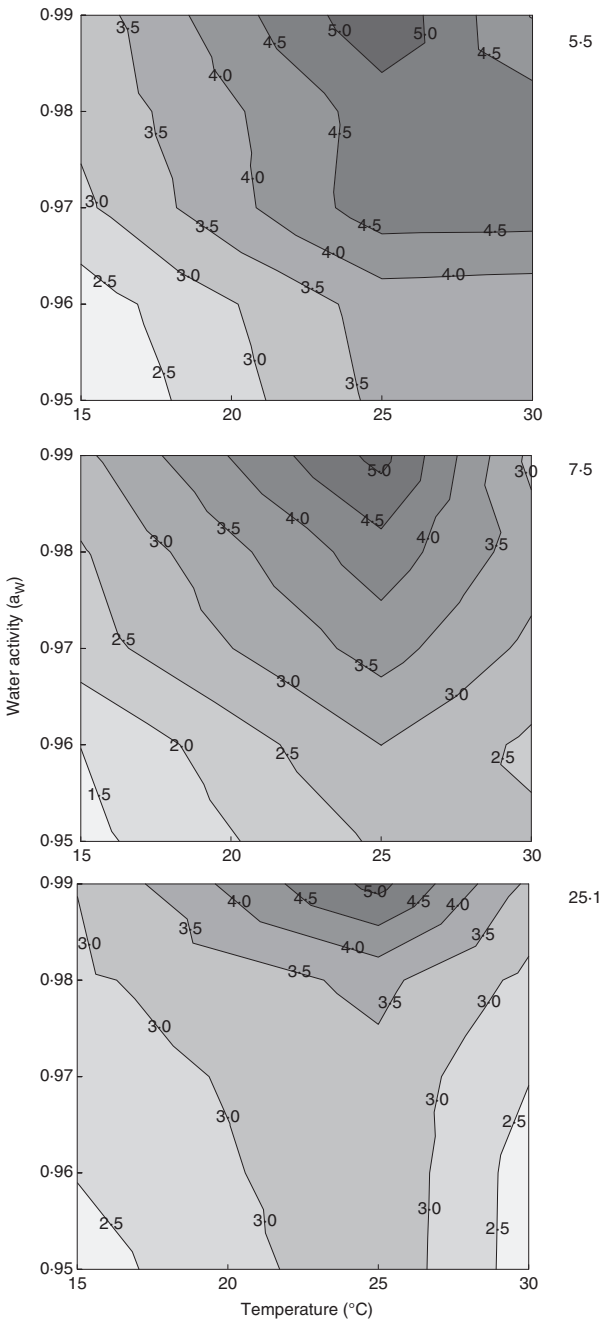

Figure 2 Two-dimensional contour map of growth profile of *Alternaria alternata* strains 5·5, 7·5 and 25·1, in relation to temperature and water activity. The numbers on the isopleths refer to similar growth rates (mm day^{-1}).

at 0·96 and 15 or 25°C after 21–28 days of incubation for the strain 5·5.

The highest level of TA was observed at 25°C and 0·99 a_W by the strain 25·1 after 14 days of incubation (more than 10 000 μg g^{-1}) followed by the strain 7·5 after 21 days of incubation (more than 1200 μg g^{-1}) and the strain 5·5 after 21 days (200 μg g^{-1}). As a_W was reduced, less TA was produced but the levels were sustained. Besides, there were also peaks of high production at 0·96 a_W at 15 and 30°C after 7, 14 or 28 days of incubation for the strain 7·5. Also, similar behaviour was observed at 0·96 a_W and 30°C at all incubation times for the strain 25·1 and at 0·96 a_W and 15 and 25°C after 21 and 28 days of incubation for the strain 5·5.

Besides, studied strains had different AOH, AME and TA production profiles related to incubation temperatures (Figs 3–5). At 25°C, *A. alternata* 5·5 and 7·5 produced higher levels of AME, followed by AOH and TA. Meanwhile, strain 25·1 produce higher levels of AOH, followed by AME and TA. At 15°C like as 30°C strains 7·5 and 25·1 had similar patterns of production, the most produced toxin was TA followed by AOH and AME indistinctly (at 15°C) or first by AOH and then by AME (at 30°C). However, strain 5·5 was a better producer of AME, followed indistinctly by AOH or TA at 15°C or equally produces TA, AOH and AME at 30°C.

In order to determinate the effect of variables evaluated on the production of AOH, AME and TA for the three strains, an ANOVA on the effect of single variables (a_W, temperature and days of incubation) and the two- and three-way interactions was done (Tables 2–4). Only the a_W factor alone was statistically significant ($P < 0{\cdot}001$; Table 2) in the AOH production by the three strains evaluated. Meanwhile, the effects of the other factors and its interaction on the production of AME and TA varied among the strains considered.

Data obtained were used to develop contour maps to identify the optimum conditions of a_W and temperature and the range of conditions for production of different quantities of AOH, AME and TA (Fig. 6). As could be observed, the three toxins tested (AOH, AME and TA) showed a similar range of temperature and the production of TA showed the widest spectrum of a_W.

Discussion

This study compared for the first time the impact of both a_W and temperature on growth and AOH, AME and TA production on a SN media with composition similar to grapes by three strains of *A. alternata* representatives of *A. alternata* populations of Malbec grapes.

Fruits are particularly prone to fungal development, because of its high a_W, sugar content and the presence of organics acids that give a low pH to the pulp (Tournas and Katsoudas 2005). The DOC San Rafael area present a hot and dry weather, with cold nights and moderate drought, with risk of late frosts and hail, annual rainfall ranging around 250 mm and moderate incidence of fungal diseases (Catania *et al.* 2012). However, in the latest

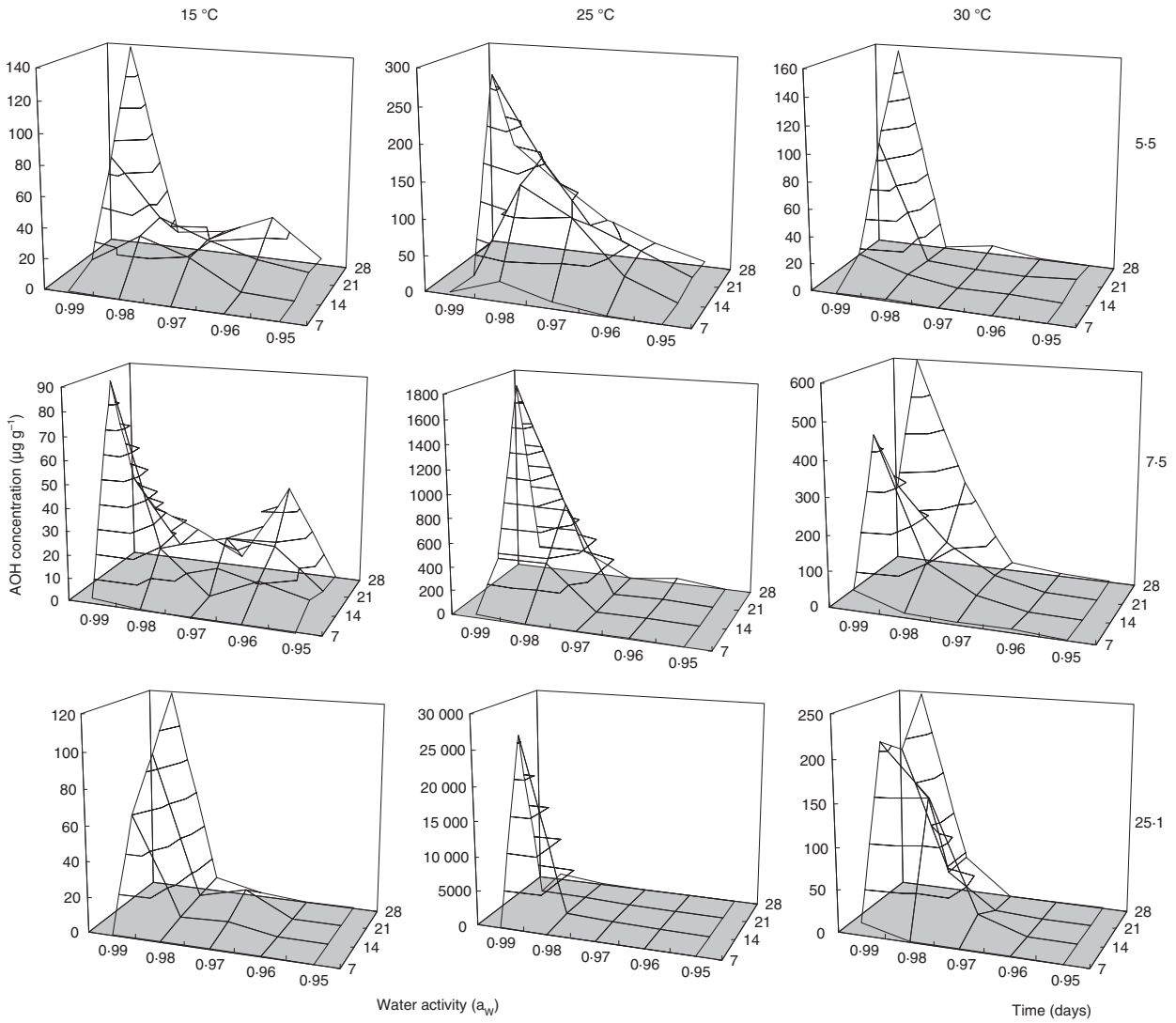

Figure 3 Alternariol levels (μg g^{-1}) produced by strains of *Alternaria alternata*, 5·5, 7·5 and 25·1, in a SN media adjust to different levels of water activity and temperature during 28 days.

years, it has also been affected by global climate change (warmer climate, heat weaves, an increase in rainfall and drought) which may influence the wine grapes mycobiota and mycotoxin production as has been reported for others crops (Paterson and Lima 2010). Previous studies have report that a_W of wine grapes change from veraison to harvest (0·98 to 0·96 or 0·95) as a consequence of sugar accumulation (Leong *et al.* 2006; Tassou *et al.* 2009) and that rainfall increment the a_W in the grape surface (Rousseau and Donèche 2001). Therefore, this study results valuable to predict the growth and mycotoxin production by *A. alternata* on wine grapes under field conditions, due to the use of the SN media similar to grape composition, the a_W (0·95, 0·96, 0·97, 0·98 and 0·99) and temperatures (15, 25 and 30°C) that simulate those conditions present on grapes between veraison to ripeness in DOC San Rafael.

During this study, a_W and temperature have influenced the growth of the three *A. alternata* strains evaluated, being temperature the most important factor. Meanwhile, the a_W, the temperature and the incubation time influenced the production of AOH, AME and TA depending on the strain evaluated.

Recently, the optimum conditions of temperature and a_W for the growth of *A. alternata* strains isolated from other substrates have been revised (Lee *et al.* 2015), and some studies have found similar optimum conditions as observed in the present work. Magan and Aldred (2007) found that growth of *A. alternata* isolated from wheat was optimum at about 25°C and growth

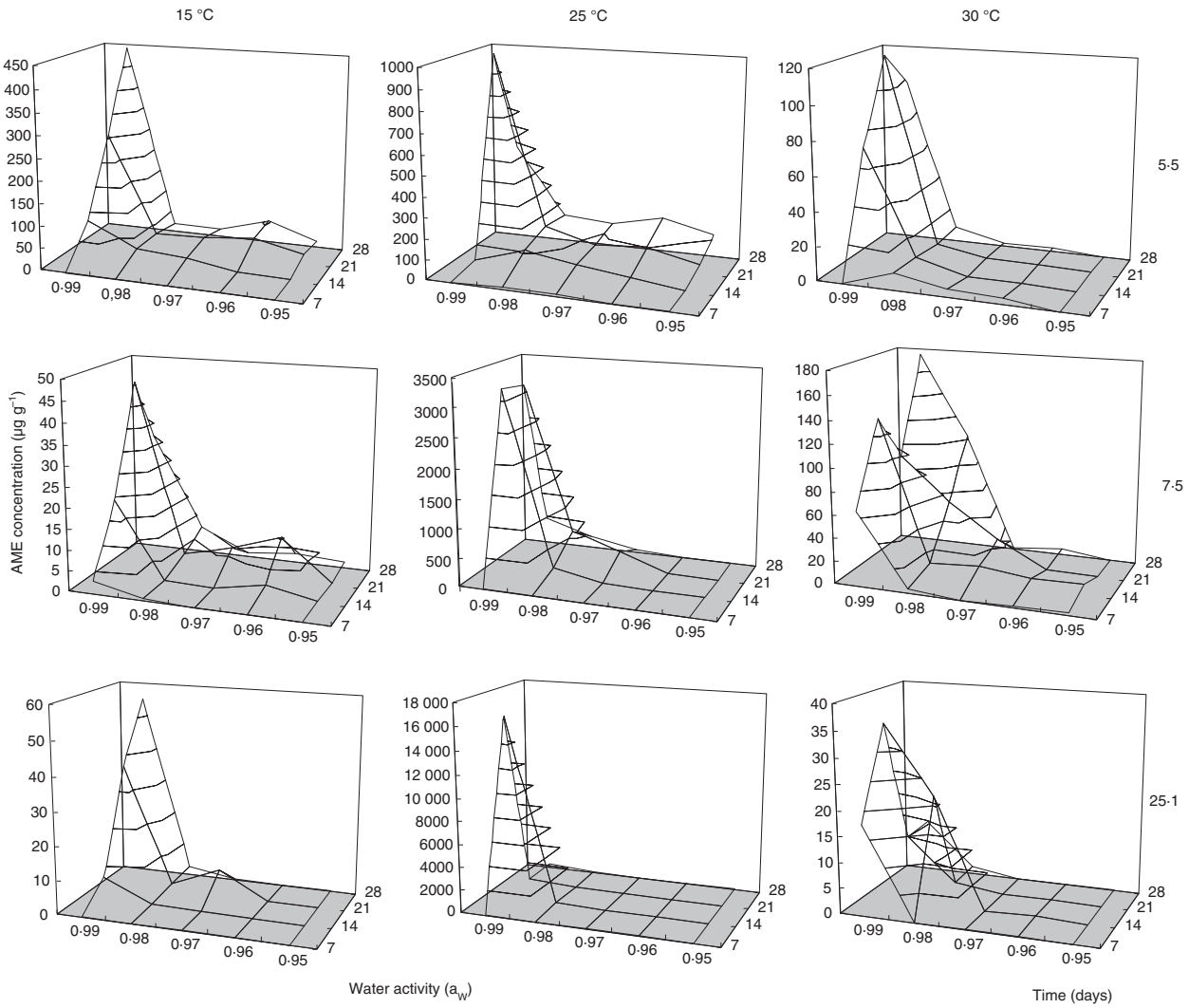

Figure 4 Alternariol monomethyl ether levels (μg g^{-1}) produced by strains of *Alternaria alternata*, 5·5, 7·5 and 25·1, in a SN media adjust to different levels of water activity and temperature during 28 days.

occurred over the a_W range 0·88–0·89 to 0·995 on wheat-based medium. Oviedo *et al.* (2009) reported that *A. alternata* strains isolated from soya beans have optimum growth at a_W ranging from 0·92 to 0·995 and 25°C, although they grew well until 35°C. Meanwhile, Pose *et al.* (2009) reported an optimum growth at 0·982 a_W and 21°C, for a cocktail of five strains of *A. alternata* isolated from tomato fruits, although they grew well in the range from 6 to 35°C and 0·922 to 0·982 a_W in a synthetic medium based on tomato. In general, all these studies suggest that growth was similar for different strains from the same niche, for isolates from cereals, tomatoes, sorghum, sunflower and soya beans (Lee *et al.* 2015). However, in our work, we found one of the *A. alternata* strains evaluated (5·5) with a similar growth at 25 and at 30°C, which would mean a different growth behaviour among the *A. alternata* populations of Malbec grapes.

Besides several studies have analysed the effects of environmental conditions on the mycotoxin production by *A. alternata* strains from different substrates in synthetic media, this is the first work on *A. alternata* strains isolated from wine grapes in synthetic grape media.

In this study, the suitable ranges of temperature and a_W for AOH and AME productions were slightly narrower than for growth, in agreement with several previous studies (Lee *et al.* 2015). Particularly, they were similar with the optimum growth range (0·97–0·99 a_W at 15, 25 and 30°C), except for the strain 25·1 at 25°C, in which the production of both mycotoxins was almost exclusively at 0·99 a_W. However, TA production was detected in almost all the range of growth

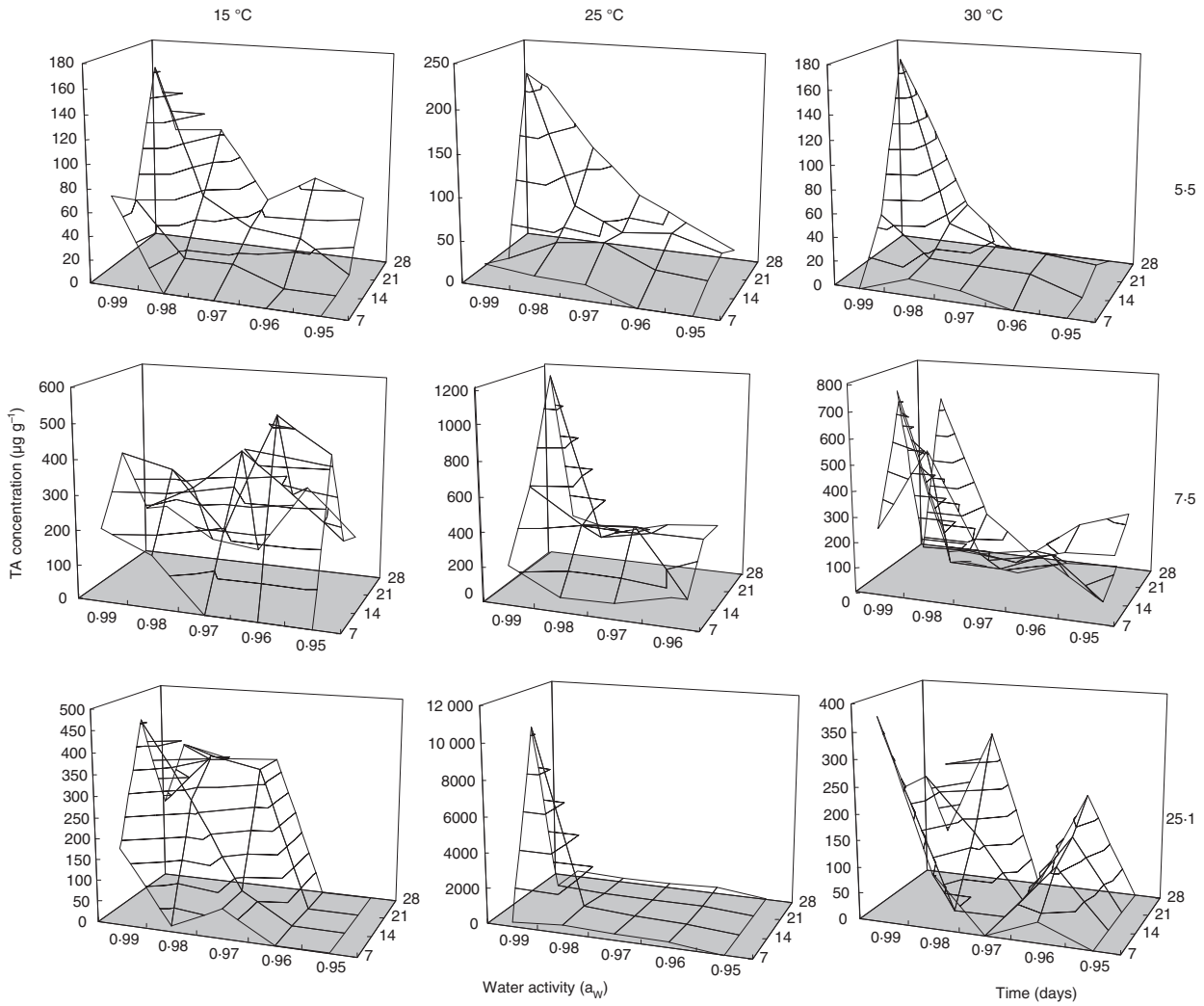

Figure 5 Tenuazonic acid levels (μg g^{-1}) produced by strains of *Alternaria alternata*, 5·5, 7·5 and 25·1, in a SN media adjust to different levels of water activity and temperature during 28 days.

Table 2 Analysis of variance on the effects of water activity (a_W), temperature (*T*) and incubation time (i) on alternariol production by three *Alternaria alternata* strains on a synthetic nutrient media with composition similar to grape

Source of variation	Degrees of freedom	5·5		7·5		25·1	
		Mean square	F-Snedecor	Mean square	F-Snedecor	Mean square	F-Snedecor
T	2	33 210·638	19·636*	2 705 749·554	5·820	40 051 380·246	5·009
i	3	23 191·552	13·712*	711 537·178	1·530	33 198 664·594	4·152
a_W	4	30 831·884	18·229*	2 532 521·135	5·447*	39 742 293·860	4·971*
T × i	6	5098·976	3·015	685 967·980	1·475	32 457 949·017	4·060*
T × a_W	8	5078·383	3·003	2 026 650·001	4·359*	36 182 047·872	4·525*
i × a_W	12	11 142·123	6·588*	566 780·098	1·219	32 191 126·571	4·026*
T × i × a_W	4	1909·279	1·129	55 022·216	1·194	32 077 646·736	4·012*

*$P < 0·001$.

evaluated, showing a more sustained production, increasing the chances of natural occurrence of TA in wine grapes.

However, the existence of high levels of AOH, AME and TA production in suboptimum growth conditions (0·96 a_W and 15 or 30°C) evidence that fungal conditions

Table 3 Analysis of variance on the effects of water activity (a_W), temperature (*T*) and incubation time (i) on alternariol monomethyl ether production by three *Alternaria alternata* strains on a synthetic nutrient media with composition similar to grape

Source of variation	Degrees of freedom	5·5		7·5		25·1	
		Mean square	F-Snedecor	Mean square	F-Snedecor	Mean square	F-Snedecor
T	2	200 276·270	9·367*	24 130·360	1·010	15 125 947·617	4·778
i	3	156 630·289	7·326*	179 298·142	7·502*	13 040 188·593	4·119
a_W	4	248 504·137	11·623*	486 462·504	20·353*	14 691 251·076	4·641
T × i	6	56 753·952	2·654	259 976·259	10·877*	12 999 595·904	4·107*
T × a_W	8	35 662·930	1·668	131 607·752	5·506*	14 275 763·990	4·510*
i × a_W	12	73 362·160	3·431*	41 933·753	1·754	12 835 179·237	4·055*
T × i × a_W	4	32 141·577	1·503	105 161·640	4·400*	12 845 952·108	4·058*

*$P < 0{\cdot}001$.

Table 4 Analysis of variance on the effects of water activity (a_W), temperature (*T*) and incubation time (i) on tenuazonic acid production by three *Alternaria alternata* strains on a synthetic nutrient media with composition similar to grape

Source of variation	Degrees of freedom	5·5		7·5		25·1	
		Mean square	F-Snedecor	Mean square	F-Snedecor	Mean square	F-Snedecor
T	2	1672·299	0·199	418 959·984	9·266*	7 309 418·936	4·551
i	3	54 471·158	6·483*	271 013·529	5·994*	5 046 266·575	3·142
a_W	4	82 781·853	9·852*	669 830·144	14·815*	7 491 387·017	4·665
T × i	6	8237·097	0·980	241 260·390	5·336*	5 332 375·728	3·320
T × a_W	8	5961·227	0·709	178 767·203	3·954*	4 939 698·218	3·076
i × a_W	12	23 834·374	2·837	121 388·287	2·685	5 402 994·817	3·364*
T × i × a_W	4	6967·039	0·829	129 730·055	2·869*	4 942 599·593	3·078*

*$P < 0{\cdot}001$.

that favour growth not necessarily agree with those that promote mycotoxin production, so we also could found them on intact substrates and derivatives (Barkai-Golan and Paster 2008). Also, stress situations, which might be derived from climate changes, have already been reported as effective elicitors of mycotoxin production but in other genera different from *Alternaria* (Magan *et al.* 2011).

As reported in previous works (Lee *et al.* 2015), mycotoxin production by *Alternaria* strains was more variable than growth in this study. Different AOH, AME and TA production profiles were found between the analysed strains and those profiles for each particular strain were related to temperature. It seems that the temperature of 25°C favours the production AOH and AME above TA in all strains tested. In addition, a decrease of *Alternaria* mycotoxins after reaching maximum levels under different a_W and temperature conditions was shown in all strains tested. This phenomenon could be explained with possible toxin degradation by *A. alternata* as has been suggested by other authors (Etcheverry *et al.* 1994).

Also, at similar conditions of temperature, a_W and time of incubation (25°C; 0·99; 14 days), there were differences between the production profile and quantities of AOH, AME and TA for each particular strain evaluated in SN media and the previously evaluated in GRCS media (Prendes *et al.* 2015). *Alternaria alternata* 7·5 was a better producer of AME (3000 $\mu g\ g^{-1}$) followed by TA (600 $\mu g\ g^{-1}$) and AOH (400 $\mu g\ g^{-1}$) in SN media meanwhile was a better producer of TA (1941 $\mu g\ g^{-1}$) followed by marginal production of AOH and AME (11 and 61 $\mu g\ g^{-1}$, respectively) in GRCS media. *Alternaria alternata* 25·1 was an excellent producer of AOH (25 000 $\mu g\ g^{-1}$) followed by AME (16 000 $\mu g\ g^{-1}$) and TA (10 000 $\mu g\ g^{-1}$) in SN media meanwhile was a better producer of TA (1071 $\mu g\ g^{-1}$) followed by marginal production of AME (12 $\mu g\ g^{-1}$) in GRCS media. In the case of *A. alternata* 5·5, it was a bad producer of AOH, AME and TA in SN media (0, 0 and 50 $\mu g\ g^{-1}$, respectively) and a good producer of AME (663 $\mu g\ g^{-1}$), followed by AOH (143 $\mu g\ g^{-1}$) and TA (74 $\mu g\ g^{-1}$) in GRCS media. This shows the importance of the nutritional food status in mycotoxin production (Lee *et al.* 2015). Toxicogenic species can grow and produce toxins on a wide range of substrates, although some of them were more adequate for mycotoxin synthesis than others, determining the production or not of the toxins.

Several previous works have been developed to determinate the influence of environmental factors on growth and mycotoxin production by *Alternaria* strains, being a_W and temperature the most important (Sanchis and

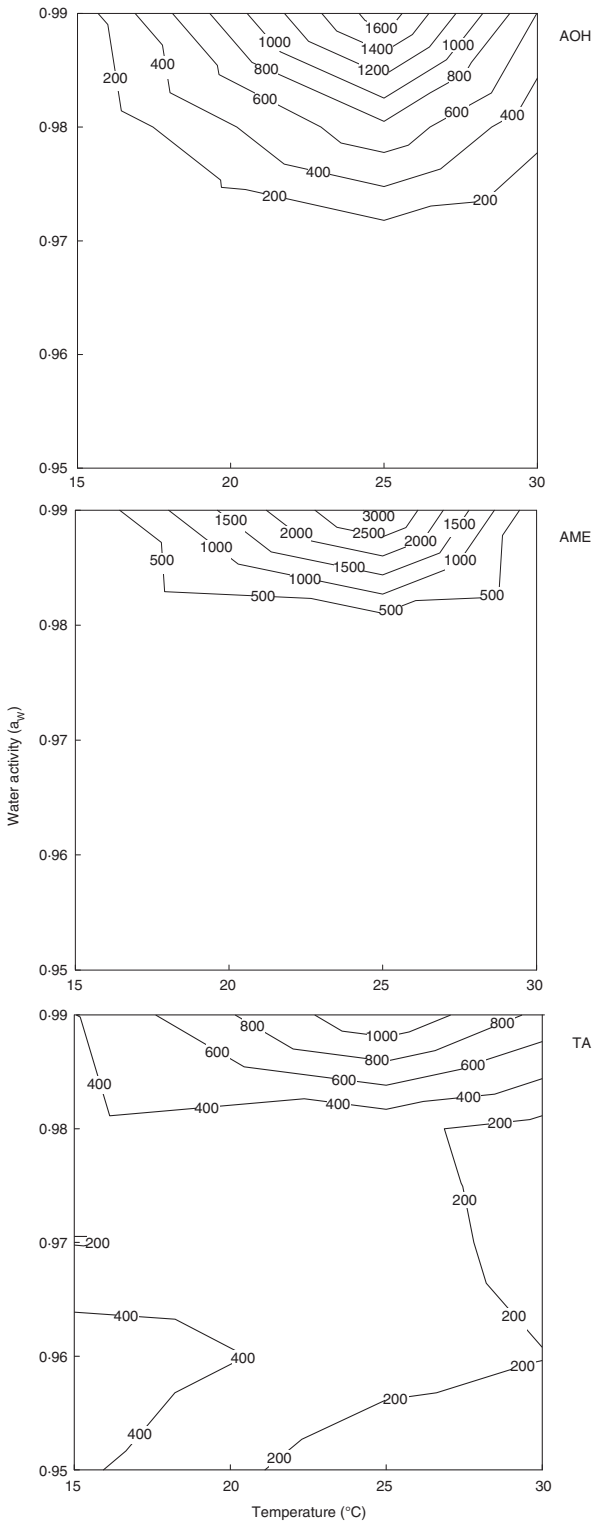

Figure 6 Two-dimensional contour maps of alternariol, alternariol monomethyl ether and tenuazonic acid production profiles of a representative *Alternaria alternata* strain in relation to temperature and water activity. The numbers of the isopleths refer to similar toxin concentrations ($\mu g\ g^{-1}$).

Magan 2004; Lee *et al.* 2015). But apart from these environmental factors, chemical (type of substrate, antifungal agents) and biological factors (strain variability, inoculum size, competing microflora) would clearly play a role in natural environments as has been showed for ochratoxigenic moulds (Astoreca *et al.* 2010).

In conclusion, the knowledge of the interaction among the marginal and suboptimum conditions of a_W and temperature provided in this work results in useful information for predicting the possible risk factors for AOH, AME and TA contamination of wine grape from veraison in the field and until start of vinification. Moreover, the wide range of conditions of a_W (0·95–0·99) and temperature (15–30°C) for AOH, AME and TA production in a SN media similar to grape composition by *A. alternata* strains demonstrated in the present work shows a high risk to be found in these mycotoxins in wine grapes. Supporting this hypothesis, the natural occurrence of TA has been recently detected in wine grapes collected in Argentina (Fontana *et al.* 2016), and numerous previous studies have found AOH, AME and TA in derivatives as grape juice and wine (Lau *et al.* 2003; Scott *et al.* 2006; Broggi *et al.* 2013; Pizzutti *et al.* 2014; Fan *et al.* 2016; López *et al.* 2016).

Considering the sustained production of AOH, AME and TA mycotoxins in some cases not corresponding to higher growth, the mere presence of *Alternaria* genus in wine grapes should be considered a toxicological risk for wine consumers and therefore steps must be taken to control it as well as the development and production of its toxins in wine grapes.

Acknowledgements

This research was financially supported by CONICET (PIP No. 11220110100823 project) and SECTyP-UNCuyo (No. 06/L131 project). L.P.P. is a fellow of CONICET and V.I.M. and M.L.R. and V.G.L.Z. are members of the Research Career of CONICET.

Conflict of Interest

Authors declare no conflict of interest.

References

Andersen, B., Krøger, E. and Roberts, R.G. (2001) Chemical and morphological segregation of *Alternaria alternata*, *A. gaisen* and *A. longipes*. *Mycol Res* **105**, 291–299.

Artero, A., Tarín, J.J. and Cano, A. (2015) The impact of moderate wine consumption on health. *Maturitas* **80**, 3–13.

Astoreca, A.L., Magnoli, C.E. and Dalcero, A.M. (2010) Ecophysiology of *Aspergillus* section *Nigri* species potential ochratoxin a producers. *Toxins* **2**, 2593–2605.

Barkai-Golan, R. and Paster, N. (2008) Mouldy fruits and vegetables as a source of mycotoxins: part 1. *World Mycotoxin J* **1**, 147–159.

Bellí, N., Ramos, A.J., Coronas, I., Sanchis, V. and Marín, S. (2005) *Aspergillus carbonarius* growth and ochratoxin A production on a synthetic grape medium in relation to environmental factors. *J Appl Microbiol* **98**, 839–844.

Broggi, L., Reynoso, C., Resnik, S., Martinez, F., Drunday, V. and Romero Bernal, A. (2013) Occurrence of alternariol and alternariol monomethyl ether in beverages from the Entre Rios Province market, Argentina. *Mycotoxin Res* **29**, 17–22.

Catania, C.D., Avagnina de del Monte, S., Uliarte, M.E., del Monte, R.F. and Tonietto, J. (2012) El clima vitícola de las regiones productoras de uvas para vinos de Argentina. In *Clima, Zonificación y Tipicidad del Vino en Regiones Vitivinícolas Iberoamericanas* ed. Tonietto, J., Sotés Ruiz, V. and Gómez-Miguel, V. pp. 51–96. Madrid: CYTED.

Cucchi, N. and Becerra, V. (2009) Manual de tratamiento fitosanitario para cultivos de clima templado bajo riego. In *Vid* ed. Cucchi, N. and Becerra, V. Tomo I, Sección III pp. 176–178. Argentina: EDICIONES INTA.

Dall'Asta, C., Cirlini, M. and Falavigna, C. (2014) Mycotoxins from *Alternaria*: toxicological implications. In *Advances in Molecular Toxicology* ed. Fishbein, C. and Heilman, J.M. pp. 107–121. Amsterdam: Elsevier B.V.

EFSA on Contaminants in the Food Chain (CONTAM). (2011) Scientific opinion on the risk for animal and public health related to the presence of *Alternaria* toxins in feed and food. *EFSA J* **9**, 2407. http://dx.doi.org/10.2903/j.efsa.2011.2407.

Etcheverry, M., Chulze, S., Dalcero, A., Varsavsky, E. and Magnoli, C. (1994) Effect of water activity and temperature on tenuazonic acid production by *Alternaria alternata* on sunflowers seeds. *Mycopathologia* **126**, 179–182.

Fan, C., Cao, X., Liu, M. and Wang, W. (2016) Determination of *Alternaria* mycotoxins in wine and juice using ionic liquid modified countercurrent chromatography as a pretreatment method followed by high-performance liquid chromatography. *J Chromatogr A* **1436**, 133–140.

Fontana, A.R., Prendes, L.P., Morata, V.I. and Bottini, R. (2016) High-throughput modified QuEChERS method for the determination of the mycotoxin tenuazonic acid in wine grapes. *RSC Advances* **6**, 95670–95679.

Kakalikova, L., Jankura, E. and Srobarova, A. (2009) First report of *Alternaria* bunch rot of grapevines in Slovakia. *Australas Plant Dis Notes* **4**, 68–69.

Lau, B.P.Y., Scott, P.M., Lewis, D.A., Kanhere, S.R., Cléroux, C. and Roscoe, V.A. (2003) Liquid chromatography–mass spectrometry and liquid chromatography–tandem mass spectrometry of the Alternaria mycotoxins alternariol and alternariol monomethyl ether in fruit juices and beverages. *J Chromatogr A* **998**, 119–131.

Lee, H.B., Patriarca, A. and Magan, N. (2015) *Alternaria* in food: ecophysiology, mycotoxin production and toxicology. *Mycobiology* **43**, 93–106.

Leong, S.L., Hocking, A.D., Pitt, J.I., Kazi, B.A., Emmett, R.W. and Scott, E.S. (2006) Australian research on ochratoxigenic fungi and ochratoxin A. *Int J Food Microbiol* **110**, S10–S17.

Liu, G.T., Qian, Y.Z., Zhang, P., Dong, Z.M., Shi, Z.Y., Zhen, Y.Z., Miao, J. and Xu, Y.M. (1991) Relationships between *Alternaria alternata* and oesophageal cancer. In *Relevance to Human Cancer of N-Nitroso Compounds, Tobacco Smoke and Mycotoxins* ed. O'Neill, I.K., Chen, J. and Bartsch, H. pp. 258–262. Lyon, France: International Agency for Research on Cancer.

Liu, G., Qian, Y., Zhang, P., Dong, W., Qi, Y. and Guo, H. (1992) Etiological role of *Alternaria alternata* in human esophageal cancer. *Chin Med J* **105**, 394–400.

Logrieco, A., Moretti, A. and Solfrizzo, M. (2009) *Alternaria* toxins and plant diseases: an overview of origin, occurrence and risks. *World Mycotoxin J* **2**, 129–140.

López, P., Venema, D., de Rijk, T., de Kok, A., Scholten, J.M., Mol, H.G. and de Nijs, M. (2016) Occurrence of *Alternaria* toxins in food products in The Netherlands. *Food Control* **60**, 196–204.

Magan, N. and Aldred, D. (2007) Why do fungi produce mycotoxins? In *Food Mycology: A Multifaceted Approach to Fungi and Food* ed. Dijksterhuis, J. and Samson, R.A. pp 121–133. Boca Raton: Taylor and Francis.

Magan, N., Medina, A. and Aldred, D. (2011) Possible climate-change effects on mycotoxin contamination of food crops pre- and postharvest. *Plant Pathol* **60**, 150–163.

Magnoli, C., Violante, M., Combina, M., Palacio, G. and Dalcero, A. (2003) Mycoflora and ochratoxin-producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina. *Lett Appl Microbiol* **37**, 179–184.

Martin, M.C. and Morata de Ambrosini, V.I. (2014) Effect of a cold-active pectinolytic system on colour development of Malbec red wines elaborated at low temperature. *Int J Food Science Technol* **49**, 1893–1901.

Organización Internacional de la Viña y el Vino (OIV) (2016) *Aspectos de la coyuntura mundial*. Available at: http://www.oiv.int/public/medias/2936/oiv-noteconjmars2016-es.pdf.

Ostry, V. (2008) *Alternaria* mycotoxins: an overview of chemical characterization, producers, toxicity and occurrence in foodstuffs. *World Mycotoxin J* **1**, 175–188.

Oviedo, M.S., Ramirez, M.L., Barros, G.G. and Chulze, S.N. (2009) Effect of environmental factors on tenuazonic acid production by *Alternaria alternata* on soybean-based media. *J Appl Microbiol* **107**, 1186–1192.

Paterson, R.R.M. and Lima, N. (2010) How will climate change affect mycotoxins in food? *Food Res Int* **43**, 1902–1914.

Pearson, R.C. and Goheen, A.C. (1996) Enfermedades producidas por factores bióticos. In *Plagas y enfermedades de la vid* ed. Pearson, R.C. and Goheen, A.C. pp. 26–27. Madrid: Mundi-Prensa.

Pizzutti, I., Kok, A., Scholten, J., Righi, L., Cardoso, C., Rohers, G. and Da Silva, R.C. (2014) Development, optimization and validation of a multimethod for the determination of 36 mycotoxins in wines by liquid chromatography tandem mass spectrometry. *Talanta* **129**, 352–363.

Pose, G., Patriarca, A., Kyanko, V., Pardo, A. and Fernández Pinto, V. (2009) Effect of water activity and temperature on growth of *Alternaria alternata* on a synthetic tomato medium. *Int J Food Microbiol* **135**, 60–63.

Prendes, L.P., Merín, M.G., Andreoni, M.A., Ramirez, M.L. and Morata de Ambrosini, V.I. (2015) Mycobiota and toxicogenic *Alternaria* spp. strains in Malbec wine grapes from DOC San Rafael, Mendoza, Argentina. *Food Control* **57**, 122–128.

Rousseau, S. and Donèche, B. (2001) Effects of water activity (a_W) on the growth of some epiphytic micro-organisms isolated from grape berry. *Vitis* **40**, 75–78.

Rousseaux, S., Diguta, C.F., Radoï-Matei, F., Alexandre, H. and Guilloux-Benatier, M. (2014) Non-*Botrytis* grape-rotting fungi responsible for earthy and moldy off flavors and mycotoxins. *Food Microbiol* **38**, 104–121.

Sanchis, V. and Magan, N. (2004) Environmental conditions affecting mycotoxins. In *Mycotoxins in Food: Detection and Control* ed. Magan, N. and Olsen, M., pp 174–189. Oxford: Woodhead Publishing Ltd..

Scott, P., Lawrence, B. and Lau, B. (2006) Analysis of wines, grape juices and cranberry juices for *Alternaria* toxins. *Mycotoxin Res* **22**, 142–147.

Smedsgaard, J. (1997) Micro-scale extraction procedure for standardized screening of fungal metabolites production in cultures. *J Chromatogr A* **760**, 264–270.

Steel, C.C., Blackman, J.W. and Schmidtke, M. (2013) Grapevine bunch rots: Impacts on wine composition, quality and potential procedures for the removal of wine faults. *J Agric Food Chem* **61**, 5189–5206.

Tančinová, D., Rybárik, L., Masková, Z., Felsöciová, S. and Císarová, M. (2015) Endogenal colonization of grapes berries. *J Microbiol Biotech Food Sci* **4**, 69–73.

Tassou, C.C., Natskoulis, P.I., Magan, N. and Panagou, E.Z. (2009) Effect of temperature and water activity on growth and ochratoxin A production boundaries of two *Aspergillus carbonarius* isolates on a simulated grape juice medium. *J Appl Microbiol* **107**, 257–268.

Tournas, V.H. and Katsoudas, E. (2005) Mould and yeast flora in fresh berries, grapes and citrus fruits. *Int J Food Microbiol* **105**, 11–17.

Trinidad, A.V., Ganoza, F.Q., Pinto, V.F. and Patriarca, A. (2015) Determination of mycotoxin profiles characteristic of *Alternaria* strains isolated from Malbec grapes. In *BIO Web of Conferences*. Vol. 5, p. 02004. EDP Sciences. Available at http://dx.doi.org/10.1051/bioconf/20150502004.