

## ORIGINAL ARTICLE

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

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## Keywords

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

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## 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 Gohéen 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 onyala, 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<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.67 g; MgSO<sub>4</sub> (7 H<sub>2</sub>O), 1.5 g; NaCl, 0.15 g; CuCl<sub>2</sub>, 0.0015 g; FeSO<sub>4</sub> (7H<sub>2</sub>O), 0.021 g; ZnSO<sub>4</sub> (7H<sub>2</sub>O), 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<sup>-1</sup>), 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<sup>-1</sup>) 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- $\mu$ m 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<sup>-1</sup> 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<sup>-1</sup> in acetonitrile/0.027 mol l<sup>-1</sup> 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<sup>-1</sup> 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  $\mu$ g g<sup>-1</sup> for the three toxins, and the quantification limit (LOQ) was established as three times the detection limit.

#### Statistical analysis

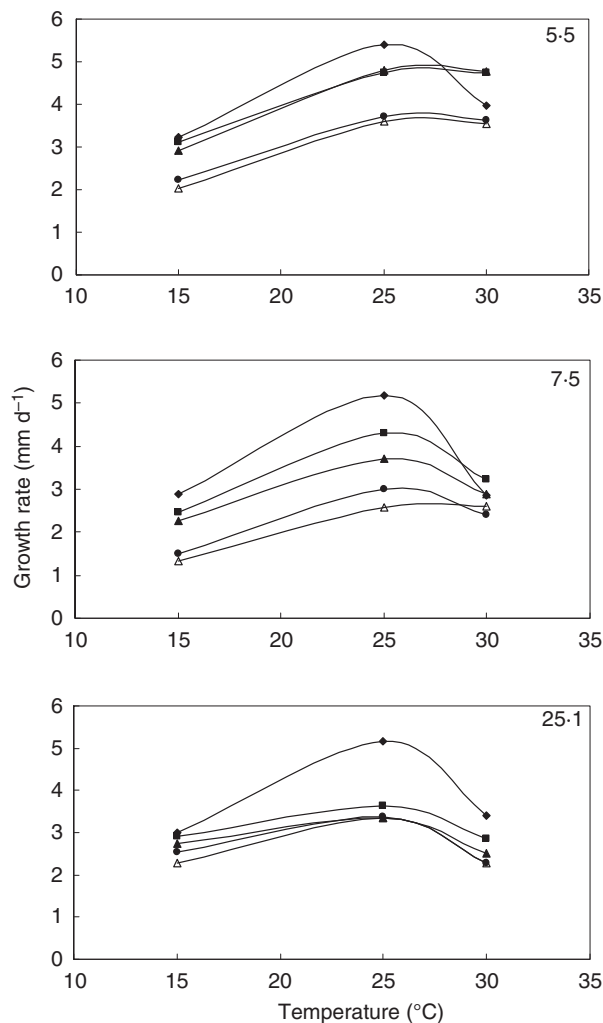
The growth rates and mycotoxin concentration were evaluated by analysis of variance (ANOVA) to determine the effect of  $a_w$ , temperature and *A. alternata* strains and two- and three-way interactions. When the analysis was statistically significant, the *post hoc* Tukey's multiple comparison procedure was used for separation of the means. Statistical significance was judged at the level  $P \leq 0.001$ . 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 ( $\Delta$ ), 0.96 ( $\bullet$ ), 0.97 ( $\blacktriangle$ ), 0.98 ( $\blacksquare$ ) y 0.99 ( $\blacklozenge$ ) 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*
$S \times a_w$	8	0.510	42.154*
$S \times T$	4	2.031	168.006*
$a_w \times T$	8	0.884	73.109*
$S \times a_w \times T$	16	0.156	12.944*

\* $P < 0.001$ .

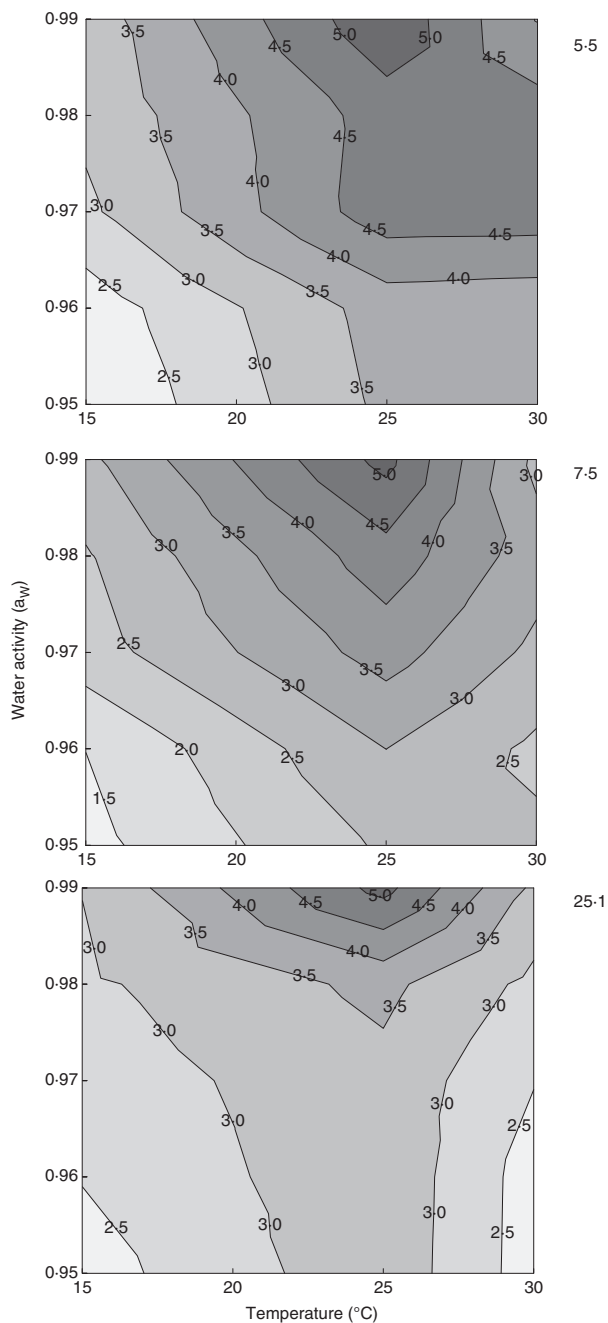
The conditions under which equivalent growth rates occurred under different environmental conditions were joined to produce contour lines to map the relative optimum and marginal conditions for growth of the *A. alternata* strains examined (Fig. 2). The fastest growth for the three strains tested occurred 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  $\mu\text{g g}^{-1}$ ) followed by strain 7.5 after 21 days of incubation (more than 1600  $\mu\text{g g}^{-1}$ ) and the strain 5.5 after 21 days (more than 250  $\mu\text{g g}^{-1}$ ). 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  $\mu\text{g g}^{-1}$ ) followed by the strain 7.5 after 14 or 21 days of incubation (more than 3000  $\mu\text{g g}^{-1}$ ) and the strain 5.5 after 21 days (more than 900  $\mu\text{g g}^{-1}$ ). 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 ( $\text{mm day}^{-1}$ ).

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  $10\,000\ \mu\text{g g}^{-1}$ ) followed by the strain 7.5 after 21 days of incubation (more than  $1200\ \mu\text{g g}^{-1}$ ) and the

strain 5.5 after 21 days ( $200\ \mu\text{g g}^{-1}$ ). 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.

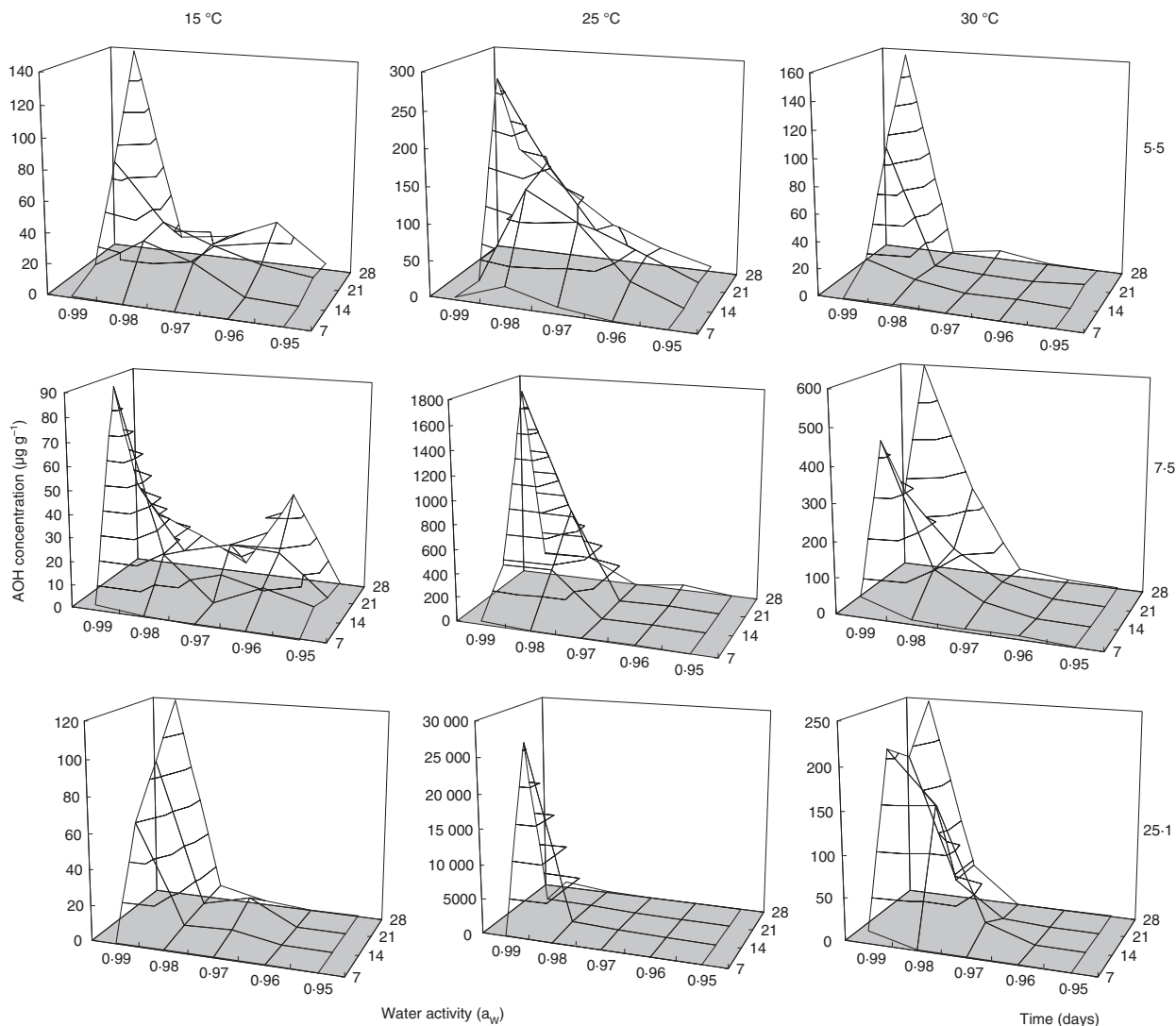
In order to determinate the effect of variables evaluated on the production of AOH, AME and TA for the three strains, an ANOVA on the effect of single variables ( $a_w$ , temperature and days of incubation) and the two- and three-way interactions was done (Tables 2–4). Only the  $a_w$  factor alone was statistically significant ( $P < 0.001$ ; 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



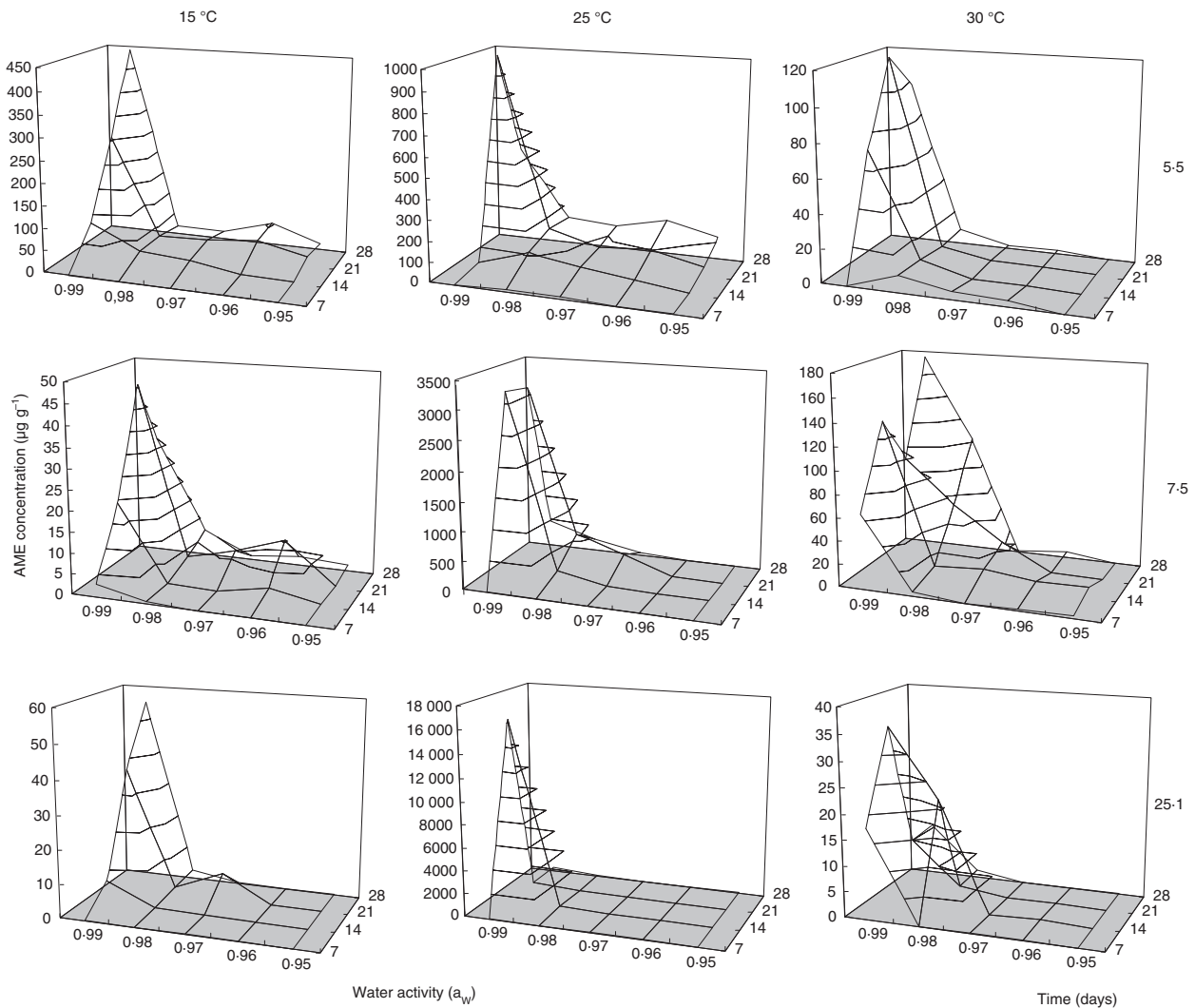
**Figure 3** Alternariol levels ( $\mu\text{g g}^{-1}$ ) produced by strains of *Alternaria alternata*, 5-5, 7-5 and 25-1, in a SN media adjust to different levels of water activity and temperature during 28 days.

years, it has also been affected by global climate change (warmer climate, heat waves, 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



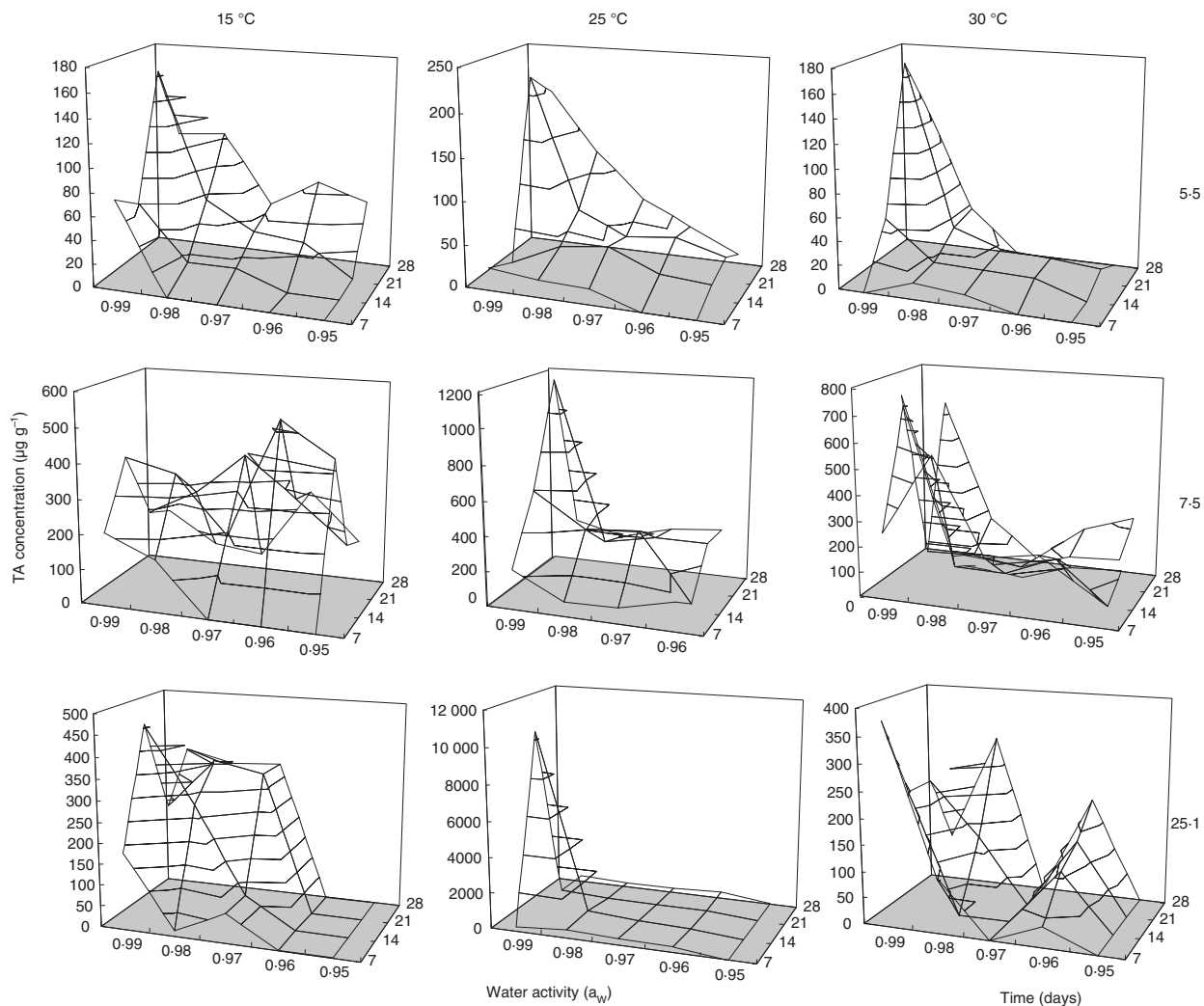
**Figure 4** Alternariol monomethyl ether levels ( $\mu\text{g g}^{-1}$ ) produced by strains of *Alternaria alternata*, 5-5, 7-5 and 25-1, in a SN media adjust to different levels of water activity and temperature during 28 days.

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 ( $\mu\text{g g}^{-1}$ ) 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*
$T \times i$	6	5098.976	3.015	685 967.980	1.475	32 457 949.017	4.060*
$T \times a_w$	8	5078.383	3.003	2 026 650.001	4.359*	36 182 047.872	4.525*
$i \times a_w$	12	11 142.123	6.588*	566 780.098	1.219	32 191 126.571	4.026*
$T \times i \times a_w$	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
$T \times i$	6	56 753.952	2.654	259 976.259	10.877*	12 999 595.904	4.107*
$T \times a_w$	8	35 662.930	1.668	131 607.752	5.506*	14 275 763.990	4.510*
$i \times a_w$	12	73 362.160	3.431*	41 933.753	1.754	12 835 179.237	4.055*
$T \times i \times a_w$	4	32 141.577	1.503	105 161.640	4.400*	12 845 952.108	4.058*

\* $P < 0.001$ .**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
$T \times i$	6	8237.097	0.980	241 260.390	5.336*	5 332 375.728	3.320
$T \times a_w$	8	5961.227	0.709	178 767.203	3.954*	4 939 698.218	3.076
$i \times a_w$	12	23 834.374	2.837	121 388.287	2.685	5 402 994.817	3.364*
$T \times i \times a_w$	4	6967.039	0.829	129 730.055	2.869*	4 942 599.593	3.078*

\* $P < 0.001$ .

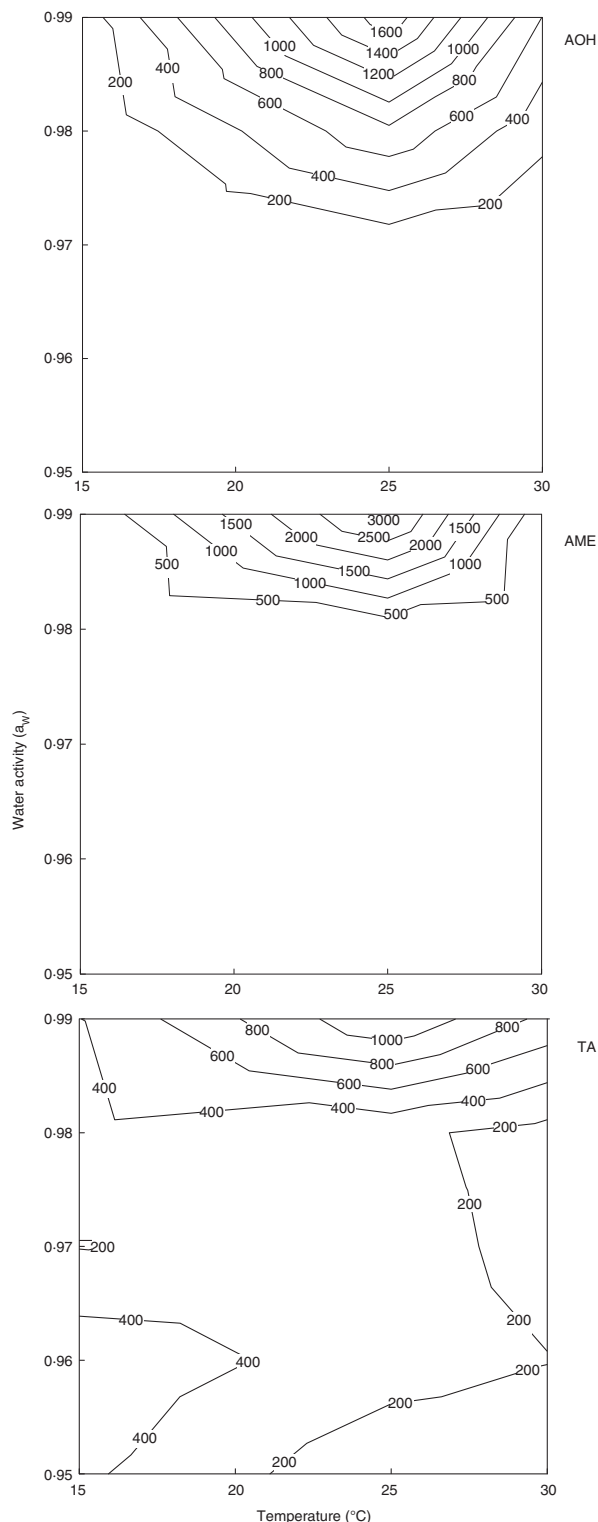
that favour growth not necessarily agree with those that promote mycotoxin production, so we also could found them on intact substrates and derivatives (Barkai-Golan and Paster 2008). Also, stress situations, which might be derived from climate changes, have already been reported as effective elicitors 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  $\mu\text{g g}^{-1}$ ) followed by TA (600  $\mu\text{g g}^{-1}$ ) and AOH (400  $\mu\text{g g}^{-1}$ ) in SN media meanwhile was a better producer of TA (1941  $\mu\text{g g}^{-1}$ ) followed by marginal production of AOH and AME (11 and 61  $\mu\text{g g}^{-1}$ , respectively) in GRCS media. *Alternaria alternata* 25.1 was an excellent producer of AOH (25 000  $\mu\text{g g}^{-1}$ ) followed by AME (16 000  $\mu\text{g g}^{-1}$ ) and TA (10 000  $\mu\text{g g}^{-1}$ ) in SN media meanwhile was a better producer of TA (1071  $\mu\text{g g}^{-1}$ ) followed by marginal production of AME (12  $\mu\text{g g}^{-1}$ ) 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  $\mu\text{g g}^{-1}$ , respectively) and a good producer of AME (663  $\mu\text{g g}^{-1}$ ), followed by AOH (143  $\mu\text{g g}^{-1}$ ) and TA (74  $\mu\text{g g}^{-1}$ ) 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 determine 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 ( $\mu\text{g g}^{-1}$ ).

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.

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### Conflict of Interest

Authors declare no conflict of interest.

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