BIO Web of Conferences **5**, 02004 (2015)
DOI: 10.1051/bioconf/20150502004

Determination of mycotoxin profiles characteristic of *Alternaria* strains isolated from Malbec grapes

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Abstract. The world grape production has increased, reaching 751 million quintals (Mql) in 2013. Many *Alternaria* species have been studied for their ability to produce secondary metabolites in foods, some of which have toxic properties with tenuazonic acid (TA), alternariol (AOH), alternariol methyl ether (AME) being the most important ones. The aim was to determine the characteristic mycotoxin production profiles of *Alternaria* strains isolated from Malbec grapes in the Patagonian region of Argentina. Fifty *Alternaria* isolates (5 *A. alternata*, 5 *A. arborescens* and 40 *A. tenuissima*) were analyzed for the production of mycotoxins (TA, AOH and AME) in autoclaved rice media by High Performance Liquid Chromatography (HPLC). All isolates were found to be producers of mycotoxins; the 100% was producer of TA (0.016–21.031 mg/kg), 98% produced AOH (0.003–0.057 mg/kg) and 36% produced AME (0.001–0.133 mg/kg). Thirty-three isolates co-produced the three mycotoxins. In this study, it was demonstrated a high toxigenic potential of *Alternaria* isolates. Although *Alternaria* growth on grapes has been amply demonstrated, there are few studies about the incidence their more characteristic mycotoxin sand their toxicogenic capacity determination in grapes, wines and derivatives. In addition, mycotoxins studied in this work are not regulated in oenology. Therefore, further studies should be conducted to assess the health risk due to the presence of *Alternaria* toxins in grapes, wine, grape juice and raisins.

1. Introduction

World production of grapes, until 2013, was 751 Mql and according to preliminary estimates of 2014 there were 271 million hl (Mhl) wine worldwide. Argentina reached a production of 15.2 Mhl, in the same period; the country is still considered the fifth largest producer of wines worldwide [1].

The genus *Alternaria* includes pathogenic species found in a variety of agronomically important plants such as cereals, oilseeds, vegetables and fruits [2]. A wide variety of *Alternaria* strains was isolated from different hosts including grapes [3–6]. Species such as *Alternaria alternata*, *A. arborescens* and *A. tenuissima* have frequently been isolated from grapes during development in the vineyard or postharvest storage [4,7–9].

Alternaria cause rot bunch damaged berries, scrape and pedicels [10,11]. From table grapes infected with *A. alternata* and cold storage, it was demonstrated that the pathogen is able to penetrate into the host tissue through the stomata and lenticels microcracks of the scarfskin [10].

The mycobiota frequently isolated from grapes includes the genera *Aspergillus*, *Penicillium*, *Mucor*, *Rhizopus*, *Alternaria*, *Cladosporium*, *Botrytis* and *Fusarium* [7,8, 12–25].

The main *Alternaria* mycotoxins belong to three structural classes: the tetramic acid derivative (TA), dibenzopyrone derivatives (AOH, AME and altenuene) and perylenquinone derivatives (Altertoxins: ALT) [26]. The most common toxins produced by this genus in foods are AOH, AME and TA. TA toxicity has been reported in plants, in chicken embryos and several other animal species, including guinea pigs, mice, rabbits, dogs and rhesus monkeys. AOH and AME are mutagenic and cytotoxic for bacterial and mammalian cells. TA presents higher acute toxicity than AOH, AME and ALT [27–32].

In foodstuffs, *Alternaria* mycotoxins can occur with other toxins produced by different genera. Theirco-occurrence can contribute to an increase in the ingestion of mycotoxins in the human diet. Human exposure to these mycotoxins is difficult to assess since few data of their natural occurrence is available [32].

Globally, *Alternaria* has been isolated from grapes, wines, musts and raisins; however, the presence of their most common mycotoxins has rarely been studied in these substrates. There are not specific international regulations of *Alternaria* toxins in food.

The aim of this study was to determine the characteristic mycotoxin production profiles of *Alternaria* strains (TA, AOH and AME) isolated from Malbec grapes for wine production in the Patagonian region of Argentina.

2. Materials and methods

2.1. Grape samples, isolation and identification of fungi

A total of 10 samples of Malbec grapes (bunches) with and without apparent fungal contamination was analyzed. The samples were collected during the 2013 harvest

from Maique and Azul valley in the Rio Negro province in Patagonian region of Argentina. Samples were sent to Food Microbiology Laboratory, FCEN, Buenos Aires University, where they were stored at 4°C until their analysis. A total of 95 *Alternaria* spp. isolates were obtained and identified from all grape samples [33]. Fifty *Alternaria* isolates (5 *A. alternata*, 5 *A. arborescens* and 40 *A. tenuissima*) were selected for the evaluation of mycotoxin production.

2.2. Mycotoxin production

The strains were cultured on autoclaved rice. Flasks containing 12.5 g of autoclaved polished rice were conditioned at 40% moisture. The rice was inoculated with a 5 mm disk cut from a colony margin (using a sterile cork borer) of 1-week-old Potato Carrot Agar (PCA) single spore culture and incubated at 25°C for 3 weeks in the dark [34].

2.3. Extraction *Alternaria* toxins

The culture material (5 g) was homogenized with 10 mL of methanol and 8 mL of 20% ammonium sulfate and filtered. The filtrate was extracted with 6 mL of hexane and the aqueous phase was divided into two parts. One part was extracted twice with 1 mL of chloroform. The organic phases were combined, evaporated to dryness and dissolved in 1 mL of methanol for AOH and AME analysis by HPLC. The other part was adjusted to pH 2 with 6N HCl and extracted twice for TA with 5 mL of chloroform. The chloroformic phase was then partitioned into 6 mL of 5% sodium bicarbonate, acidified to pH 2 again, and extracted twice with 3 mL of chloroform. The final chloroform extracts were combined, washed with 5 mL water, and evaporated to dryness. The residue was dissolved in 1 mL methanol and analyzed for TA by HPLC [34].

2.4. HPLC detection

The extracts obtained were analyzed by HPLC. The HPLC system consisted of a Shimadzu LC-142 CA liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Rheodyne sample valve fitted with a 20 µl loop and a Shimadzu SPD M10Avp UV photodiode array detector. The analytical column was Jupiter 4.6 × 250 mm 5 µ C18 (Phenomenex, USA). Standards of TA (as a copper salt), AME and AOH were purchased from SIGMA Chemical Company (St. Louis, MO, USA). The mobile phase was methanol/water (80:20) containing 300 mg $ZnSO_4 \cdot H_2O$/L, for AME and AOH, and methanol/water (90:10) containing 300 mg $ZnSO_4 \cdot H_2O$/Lf or TA. The wavelength for recording chromatograms was 258 nm for AOH and AME, and 280 nm for TA. The calibration curve was constructed using the toxins standard and correlating peak-area versus the mass of analyte injected. Reference spectra were acquired during the elution of the associated standards and used for peak identification by comparison after spectra normalization. The detection limits were 11 µg/kg for TA, 2 µg/kg for AME to 5 µg/kg for AOH.

3. Results

3.1. Production profiles of secondary metabolites

The toxigenic profile of the 50 *Alternaria* isolates from Argentinean Malbec grapes is shown in Table 1. TA, AOH and AME production was tested. All the isolates were able to produce mycotoxins. TA was produced by 100% (50/50) in a range from 0.016 to 21.031 mg/kg. AOH was produced by 98% (49/50) in a range from 0.003 to 0.057 mg/kg. AME production was observed in 36% (18/50) in a range from 0.001 to 0.133 mg/kg. TA was the toxin produced at higher levels.

3.2. Co-production of secondary metabolites

The co-production of mycotoxins is shown in Fig. 1. A 36% of the isolates (18/50) co-produced the three mycotoxins, 62% (31/50) simultaneously produced TA and AOH. One isolate produced only one mycotoxin (TA).

Table 1. Production of *Alternaria* mycotoxins.

Species	No. of isolates	Mycotoxin production			
		Mycotoxin	No. of positive	Average (mg/kg)	Range (mg/kg)
A. tenuissima	40 (78.4%)	TA	40	3.195	0.016–21.031
		AOH	39	0.013	0.003–0.057
		AME	14	0.052	0.001–0.133
A. alternata	5 (9.8%)	TA	5	3.227	0.019–10.093
		AOH	5	0.007	0.003–0.016
		AME	2	0.021	0.020–0.022
A. arborescens	5 (9.8%)	TA	5	1.551	0.016–3.587
		AOH	5	0.009	0.003–0.018
		AME	2	0.017	0.017

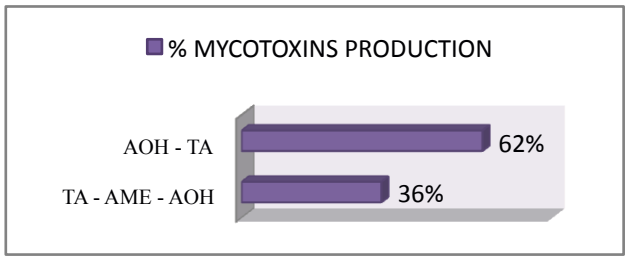

Figure 1. Co-production of *Alternaria* toxins.

4. Discussion

Alternaria growth on grapes has been reported in several countries such as Argentina, Brazil, Spain, Italy, Portugal, USA, Slovakia, Hungary and Czech Republic. The incidence of *Alternaria* in grapes has been reported in different percentages with respect to total mycobiota: 5–23% in USA, 25% in Italy, 80% in Argentina; 3–18%, 24%, 17% in different studies in Portugal, 75%, 3–58%, 13% in Spain, and *A. alternata* and *A. tenuissima* were reported in 16–19% in Slovakia [7,8,13,15,24,25,35–42].

A decrease in the percentage of grapes contaminated with *Alternaria* spp. was observed with the maturation of the berries [36,37,39,41]; however in Slovenia, *A. alternata* increased its incidence in 2008 and decreased in 2009; while the incidence of *A. tenuissima* was maintained in 2008 and declined in 2009 [13].

Even though the occurrence of AOH, AME and TA has been reported in wine [46–48], the presence of *Alternaria* isolates in the vineyard, harvest or post-harvest could not be correlated with the presence of mycotoxins in wine due to the lack of studies.

Few studies are available in the literature on the toxigenic profile of *Alternaria* species isolated from grapes. In this study, the production profiles of the most common *Alternaria* mycotoxins (TA, AOH and AME) isolated from Malbec grapes were determined. TA was the toxin produced at the highest concentration. This result demonstrates a lower production of *Alternaria* toxins compared to strains isolated from different substrates as Argentine wheat, blueberries and tomatoes [34,43–45].

Autoclaved rice was used as a substrate for mycotoxins testing of *Alternaria*, following the method described by Li et al. [34]. This substrate is suitable for the mycotoxins biosynthesis because mechanical barriers and grains defenses, which are thermolabile, have been suppressed, thus allowing *Alternaria* isolates to express their toxicogenic potential in the substrate analyzed [43]. It is necessary to evaluate the toxicogenic capacity in grapes or a media that representing this substrate to know the real potential risk of these mycotoxins.

5. Conclusion

The present work shows the toxigenic potential of the *Alternaria* species isolated from Malbec grapes, which indicates the potential risk of mycotoxin accumulation in the fruits. More work should be done to assess the health risk implied by the presence of *Alternaria* toxins in grapes, musts, wines, spirits, and raisins, since there is little information in Argentina and worldwide. The only mycotoxin regulated in wines is ochratoxin A, while there are not regulations for AOH, AME and TA. Grape producers should be advised to prevent fungal growth and mycotoxin contamination.

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