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Mycobiota and toxicogenic *Alternaria* spp. strains in Malbec wine grapes from DOC San Rafael, Mendoza, Argentina

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ABSTRACT

Alternaria, one of the most mycotoxigenic genus commonly found in wine grapes, could represent a high risk for wine consumer's health. The aims of this work were to identify the mycobiota of Malbec wine grapes under the influence of routine control viticulture practices, to identify *Alternaria* spp. strains by morphological and molecular methods and characterize their toxicogenic ability and pathogenicity. *Alternaria* was the main component of the wine grape mycobiota of the DOC San Rafael at harvest time (81%) followed by *Cladosporium* (7%) and only in minor percentage by *Penicillium* (4%) and *Aspergillus* (3%) among others. The application of an organic or non-organic treatment in the vineyard did not affect significantly the incidence of the present genera. According to morphological and molecular identification based on the genetic marker *Alt a 1*, all *Alternaria* isolates were included into *Alternaria alternata* species. Of 34 analyzed *Alternaria* strains, 97% were able to produce at least one of the three mycotoxins analyzed: alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TA) and 53% co-produced the three mycotoxins. TA was the toxin produced at highest frequency (97%) and at highest levels in a range from 11.2 to 1941.0 ppm. It was followed by AOH produced by 71% of the strains, in a range from 1.8 to 437.0 ppm and AME produced by 59% of the strains, in a range from 0.6 ppm to 663.4 ppm. The 55% of the *Alternaria* strains were very pathogenic, 31% moderately pathogenic and only 14% were slightly pathogenic. In the present work, a high incidence and prevalence of *Alternaria* genus was reported despite the use of routine control viticulture practices, as well as a high percentage of toxicogenic and pathogenic *Alternaria* strains.

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1. Introduction

Argentina ranks fifth among wine-producing countries, and exports its wines to important markets such as United Kingdom, Denmark, the Netherlands, Russia, United States, Canada and Brazil. The area of DOC “San Rafael” is a well-recognized winemaking region located in the south of Mendoza province, in Western Argentina. This region has distinctive ecological features (*terroir*) that allows the production of high quality wines. In addition, Malbec is one of the most typical and used red wine grape varieties in Argentina. Several studies were already carried out in this region with the purpose of improving winemaking through the use of indigenous microorganisms (Martín & Morata de Ambrosini, 2014; Merín, Mendoza, & Morata de Ambrosini, 2014).

Filamentous fungi impact negatively in the production, sensory quality and safety characteristics of wine in several ways. Their development in wine grapes brings significant yield losses for winemaking, alters the chemical composition of wine grapes and produces secondary fungal metabolites and enzymes that together adversely affect wine flavor and color as well as yeast and lactic acid bacteria growth during vinification (Fleet, 1999). Among them, it is of great concern the presence of toxicogenic fungi in wine grapes

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capable of producing mycotoxins that could persist during the winemaking process up to wine, being a high risk for consumer's health.

The application of copper and sulphur (organic treatment) and/or ditiocarbamate related fungicides (non-organic treatment) in the field during the vineyard development are routine control viticulture practices which achieve an effective prevention against causal fungal agents of yield losses like *Uncinula necator*, *Plasmopara viticola*, *Botrytis cinerea* and *Stereum necator*, among others. Despite that, their mode of action might be nonspecific and might act on the mycobiota other than the targets fungus (Cadez, Zupan, & Raspor, 2010).

The genus *Alternaria* is ubiquitously distributed and includes both saprophytic and opportunistic plant-pathogenic species, which may affect crops in the field or cause harvest and postharvest decay of plant products. Moreover, several *Alternaria* species are known to produce toxic secondary metabolites, *Alternaria* mycotoxins. The major *Alternaria* mycotoxins are: the tetramic acid derivative, tenuazonic acid (TA) and the dibenzopyrone derivatives, alternariol (AOH) and alternariol monomethyl ether (AME). 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 hematological 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 (Logrieco, Moretti, & Solfrizzo, 2009; Ostry, 2008). Despite the toxic effects of the *Alternaria* toxins and their documented occurrence, they have not yet received the same attention as others mycotoxins and up to now there is no regulation about them (EFSA, 2011).

Alternaria is part of the main wine grape mycobiota from different winemaking regions worldwide (Rousseaux, Diguta, Radoï-Matei, Alexandre, & Guilloux-Bénatier, 2014). As an opportunistic pathogen, it has the potential to cause a grape berry rot in the field under high disease pressure situations. Additionally, strains of *Alternaria alternata* are reported to produce AOH and AME mycotoxins on grapes (Tournas & Stack, 2001), and natural occurrence of AOH and AME in grape juices and wine has also been reported (Pizzutti et al., 2014; Scott, Lawrence, & Lau, 2006) even in Argentina (Broggi et al., 2013). Strikingly, *Alternaria* has not been extensively studied in wine grapes as a hazardous genus.

The aims of this study were: i) to identify the mycobiota of Malbec wine grapes at harvest, under the influence of routine control viticulture practices in the vineyards, and ii) to identify the *Alternaria* strains isolated from Malbec wine grapes and characterize their toxicogenic potential and pathogenicity.

2. Materials and methods

2.1. Vineyard treatments and sampling procedures

To evaluate the mycobiota and the influence of routine control viticulture practices, an experiment was performed in a vineyard of Malbec variety (*Vitis vinifera* L.) from INTA Experimental Station – Rama Caída (lat. 34.7° S, long. 68.4° W, altitude 750 m) belonging to DOC San Rafael wine grape-growing region (Mendoza, Argentina) during 2010/2011 (2011) and 2011/2012 (2012) vintages. The experimental field comprised three rows of vines within which a randomized trial of six blocks with four plants per block was used. In three out of the six blocks an organic treatment consisting of 3 spraying (in January, February and March) with 3 g/L copper oxychloride and 3 g/L sulphur was applied (organic treatment) and the rest of them were untreated. An independent sample was taken at harvest time from each block containing grape bunches collected at 1.5 m from the ground from three out of the four plants (a bunch per plant). Additionally, during 2012/2013 (2013) vintage, six representative vineyards of Malbec variety from DOC San Rafael under organic and non-organic treatments were sampled. The total geographical area selected for sampling was located between 34.3° and 34.8° S latitude, 67.4° and 68.5° W longitude, and 500 and 800 m altitude. The six vineyards were treated with copper and sulphur as previously described above, but the non-organic vineyards (two of them) were first sprayed with Mancozeb 80% WP (2.1 g/L), a dithiocarbamate related fungicide. An independent sample was taken at harvest time from each vineyard containing grape bunches collected at 1.5 m from the ground from nine plants located in three different parts of the vine (a bunch per plant).

All samples were kept in plastic bags and transferred to the laboratory as soon as possible.

2.2. Fungal isolation and morphological identification

Asymptomatic grape berries (15 in 2011, 30 in 2012 and 90 in 2013 vintages) were randomly selected from each sample, surface-disinfected with 1% (w/v) sodium hypochlorite solution for 1 min and rinsed in sterile distilled water three times and placed directly onto Dichloran-Rose-Bengal-Chloramphenicol Agar (DRBC) medium (Pitt & Hocking, 2009) plates to be incubated at 25 °C for 7 days.

The resulting fungal colonies in DRBC medium were enumerated and grouped according to their morphology to calculate the relative abundance of each distinguishable genus, and a representative number of colonies from each genus (in relation to its relative abundance) were randomly selected. They were sub-cultured in Czapek-Yeast extract-Agar (CYA) medium (Pitt & Hocking, 2009) at 25 °C and single conidial colonies were obtained and identified according to Pitt and Hocking (2009). Out of 53 identified isolates from 2011 to 2012 vintages, 38 belonged to *Alternaria* genus, which were placed on Potato-Carrot-Agar (PCA) medium (Pitt & Hocking, 2009) and incubated at 25 °C under cool-white fluorescent lamps with an alternating 8/16 light/dark cycle to be identified at species level according to sporulation patterns and conidial morphology (Simmons, 2007).

2.3. DNA extraction and molecular identification

Each of the 38 *Alternaria* isolates was grown on CYA and inoculated in 100 mL of Wikerman medium (glucose 4%, malt extract 0.3%, yeasts extract 0.3% and peptone 0.5%). The shaken cultures (120 rpm) were incubated for at least three days at 25 °C. Mycelia were harvested by filtration and dried mycelia were stored frozen at −20 °C until ground. Fungal DNA was extracted with the cetyltrimethylammonium bromide (CTAB) method (Leslie & Summerell, 2006). Quality and quantity of DNA were checked by comparison with standard DNA markers (DNA Molecular Weight Marker II, λ DNA *Hind*III digested, 120 bp-23,100 bp; Roche, Germany) on 0.8% agarose gel.

To identify *Alternaria* species-group, the protocol described by Pavón, González, Pegels, Martín, and García (2010) was used. Briefly, it consisted of amplification by PCR of different regions of *Alt a 1* gen with the specific primer set for *Alternaria* genus: Dir5cAlta1 (GAGAAC AGCTTCATGGACTTCTCTTT) and Inv4Alta1 (CGCGGCAGTAGTTGGG AA); and each primer set for the 4 defined species-groups, *A. alternata*: AaltDAlta1 (CGCATCCTGCCCTGTCA) and AinflAlta1 (GTTGGTAGCCTT GATGTTGAAGC), *Alternaria infectoria*: AinfDAlta1(CGCATCCTGCCC AGTTG) and AinflAlta1 (GTTGGTAGCCTTGATGTTGAAGC), *Alternaria radicina*: AraDAlta1 (CCCGCCAGGACAACGCT) and AsolIAlta1 (GTTGGTGGCCTTGATGTTGAAG), and *Alternaria porri*: AsolDAlta1 (CGCATCCTGCCCCGTCT) and AsolIAlta1 (GTTGGTGGCCTTGATGTTGAAG). The gels were stained with ethidium bromide (0.5 μg/mL).

Fragment sizes were estimated comparing with a 100-bp ladder (Biodynamics, Madison, USA) and visualized by UV transilumination.

2.4. Mycotoxin production

The ability to produce alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TA) toxins were evaluated in 34 *Alternaria* strains out of 38 identified isolates. Briefly, Petri plates containing ground rice-corn steep liquor medium (GRCS; ground rice 50 g, corn steep liquor 5 g, agar 15 g, 1000 mL distilled water) were inoculated centrally with a 4 mm diameter agar disc taken from the margin of a 7-day-old colony of each isolate grown on CYA at 25 °C and transferred facedown to the centre of each plate. Inoculated plates were incubated at 25 °C in darkness (Chulze, Torres, Dalcero, & Combina, 1994).

The extraction method was based on a micro-scale extraction (Smedsgaard, 1997) modified into a three-step extraction procedure suited for *Alternaria* metobolites by Andersen, Krøger, and Roberts (2001). At 14 days of incubation, three agar plugs (4 mm diameter) were cut from the edge of a colony from each Petri plate and placed in a 4-mL amber screw-cap vial. The plugs were extracted in 1.5 mL of chloroform-methanol (2:1, v/v) for 60 min in an ultrasonic bath. The extract was transferred to clean 4-mL amber vials and evaporated to dryness (N_2, 50 °C). The same plugs were then extracted ultrasonically for 60 min in 1.3 mL of ethyl acetate containing 1% (v/v) formic acid. The second extract was transferred to the amber vial containing the first dried extract and evaporated. The plugs were then extracted ultrasonically for 60 min with 1.5 mL of 2-propanol and the extract was transferred to the amber vial with the two previous extracts and evaporated. The pooled, dried extract was re-dissolved ultrasonically in 1 mL of methanol and 1 mL of acetonitrile/water (25:75, v/v), filtered through a 0.45-μm-pore-size filter, and transferred to a clean 1.5-mL amber vial to use in HPLC analysis.

The HPLC system consisted of a Hewlett Packard model 1100 pump (Palo Alto, CA, USA) 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. For AOH and AME the mobile phase consisted of two consecutive isocratic mobile phase mixtures containing acetonitrile/water at 25:75 (v/v, solvent A) and acetonitrile/water at 50:50 (v/v, solvent B). Solvent A was pumped for 3.5 min at 1 mL/min, and then solvent B was pumped for 16.5 min at 1 mL/min. The detector was set at 256 nm for AOH and AME and the retention times of AOH and AME 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 in acetonitrile/water (25:75, v/v). For TA, the mobile phase consisted of two consecutive isocratic mobile phase mixture containing acetonitrile/0.027 mol/L sodium dihydrogen phosphate solution (25:75, v/v, Solvent C) and acetonitrile/0.027 mol/L sodium dihydrogen phosphate solution (50:50, v/v, Solvent D). Solvent C was pumped for 3.5 min at 1 mL/min followed by solvent D for 16.5 min at 1 mL/min. The detector was set at 279 nm and the retention time of TA was 7.0 min. Quantification was relative to external standards of 0.5, 1.0, 2.0, and 3.0 mg/mL in acetonitrile/0.027 mol/L sodium dihydrogen phosphate solution (25:75, v/v).

Recovery experiment was performed on GRCS medium at levels of 0.1–10 ppm with AOH, AME and TA, respectively. Mean recovery and repeatability (relative standard deviation) ranged from 85 to 98% (0.2–1.4 %), from 88 to 97% (0.1–2%), from 86% to 92% (0.5–2.5 %) for AOH, AME and TA, respectively. Limit of detection (LOD, signal-to-noise ratio 3) was 0.01 ppm for the three toxins and the quantification limit (LOQ) was established as three times the detection limit.

2.5. Pathogenicity

The pathogenicity of 29 identified *Alternaria* strains was evaluated on Malbec grapes following the toothpick-inoculation technique suitable for grapes (Greco, Patriarca, Terminiello, Fernández Pinto, & Pose, 2012) with some modifications. Briefly, halved toothpicks were autoclaved 3 times in distilled water and then placed on 90 mm Petri dishes containing Potato dextrose Agar (PDA) medium (Pitt & Hocking, 2009). The toothpicks were inoculated with the *Alternaria* strains and were incubated at 25 °C under cool-white fluorescent lamps with an alternating 8/16 light/dark cycle for 2 weeks to allow complete colonization. Wine grape berries were surface disinfected with sodium hypochlorite solution (1%, v/v) for 1 min and rinsed in sterile distilled water three times. Seven berries were inoculated with one toothpick each, placed in a glass Petri dish (90 mm diameter), and incubated 7 days at 25 °C with an alternating 8/16 light/dark cycle for each fungal strain and for the negative control (toothpicks free of fungal growth). To archive 100% relative humidity (RH) a glass of water were placed in the incubator. After the incubation period, external and internal lesions were evaluated. The degree of pathogenicity was assessed by calculating the lesion extension and was confirmed according to Koch's postulates. In the pathogenicity test, three different levels were established according to the lesion size: isolates slightly pathogenic (1, lesion includes a quarter or less of the grape's surface), isolates moderately pathogenic (2, lesion includes more than a quarter and up to half of the grape's surface), and isolates very pathogenic (3, lesion includes more than half to the whole surface of the grape berry). For each strain, three repetitions were done.

2.6. Statistical analysis

Data on relative abundance of filamentous fungi and mycotoxin production were analyzed by ANOVA, and transformation were applied when necessary, followed by Fisher LSD test ($p < 0.05$ or $p < 0.01$). Tables of contingencies were also applied in some cases. Pearson correlation coefficients were used to evaluate the relationship between different mycotoxins. Statistical analysis on pathogenicity data was performed by the non parametric Kruskal–Wallis test ($p < 0.05$).

Statistical analyses were done using the Software Infostat (InfoStat versión 2013, FCA, Universidad Nacional de Córdoba, Argentina) and STATGRAPHICS Plus 5.1 (Manugistics, Rockville, MD, USA).

3. Results

3.1. Characterization of mycobiota

Mycological survey of the experiments carried out in INTA Rama Caída from DOC San Rafael during 2011 and 2012 vintages and in all the DOC San Rafael during 2013 vintage, revealed the presence of four relevant genera of filamentous fungi at harvest time in Malbec grapes. Among them, *Alternaria* spp. was predominant in terms of relative abundance (58% in 2011, 81% in 2012 and 2013) followed by *Cladosporium* (22% 2011, 19% 2012 and 7% 2013) and alternatively by *Aspergillus* (11% 2011 and 3% 2013) or *Penicillium* (3% 2011 and 4% 2013) (Fig. 1). Besides, a minor portion (<5%) of *Drechslera* and genera belong to Zygomycetes among others, was found through the different vintages analyzed (data not shown).

No significant differences were observed ($p < 0.05$) in the relative abundance of *Alternaria*, *Cladosporium*, *Aspergillus* nor

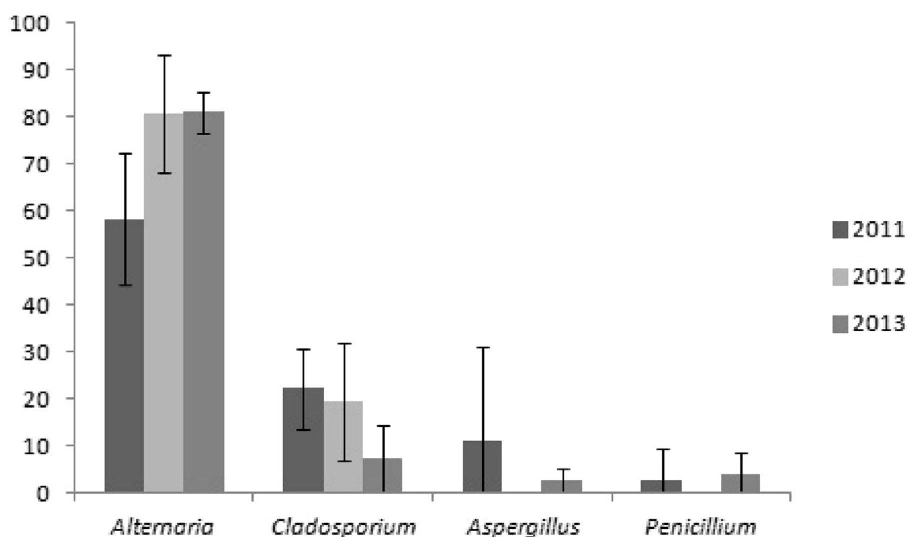

Fig. 1. Relative abundance (%) of the four relevant filamentous fungi genera found in Malbec grapes at harvest time in INTA Rama Caída from DOC San Rafael during 2011 and 2012 vintages and in all DOC San Rafael during 2013 vintage.

Penicillium between the application or not of the organic treatment (copper oxychloride and sulphur) during 2011 and 2012 vintages or between vineyards under organic and non-organic treatments during 2013 vintage.

3.2. Isolation and identification of Alternaria strains

According to their sporulation patterns, all of the 38 *Alternaria* isolates were identified as *A. alternata*.

In addition, the molecular identification of the *Alternaria* strains based on the *Alt a 1* marker showed positive amplification of 195 bp corresponding to the primer set Dir5cAlta1-Inv4Alta1, confirming that all of them belonged to *Alternaria* genus (data not shown). They were also included in *A. alternata* species-group since all of them amplified for the primer set AaltDAlta1 – AinflAlta1 (data not shown). There was no amplification for the primer sets corresponding to *A. infectoria*, *A. radicina* or *A. porri* species-groups of *Alternaria* genus, neither for negative controls (data not shown).

3.3. Toxicogenic characterization of Alternaria strains

The 97% of the 34 analyzed *Alternaria* strains were able to produce at least one of the three mycotoxins tested (AOH, AME and TA) (Table 1). TA was the toxin produced by the majority of the strains (97%) and at highest levels (a maximum of 1941.0 ppm, and a minimum of 11.2 ppm). It was followed by AOH produced by 71% of the strains, with a maximum of 437.0 ppm and a minimum of 1.8 ppm and AME produced by 59% of the strains, with a maximum of 663.4 ppm and a minimum of 0.6 ppm. Among the toxin-producer strains ($n = 33$), different profiles were found: 53% of the isolates co-produced TA, AOH and AME, 18% co-produced TA and AOH and 3% co-produced TA and AME. In addition, 21% of the isolates were able to produced exclusively TA.

No significant difference ($p < 0.05$) was observed in the production of each mycotoxin (TA, AOH and AME) between the strains isolated from blocks with the application or not of the organic treatment (copper oxychloride and sulphur) neither significant difference was found ($p < 0.05$) in the toxicogenic capacity between strains isolated in different vintages (2011 and 2012) for each mycotoxin.

A significant positive correlation between AOH and AME was observed ($r = 0.46$, $p = 0.006$). No correlation was found between AOH and TA or AME and TA.

3.4. Pathogenic characterization of Alternaria strains

From the 29 *Alternaria* strains evaluated, 55% were very pathogenic, 31% moderately pathogenic and only a minor portion (14%) were slightly pathogenic (Table 1).

No significant difference ($p < 0.05$) was observed in pathogenic capacity between the strains isolated from blocks with the application or not of the organic treatment (copper oxychloride and sulphur) or from different vintages (2011 and 2012).

4. Discussion

Alternaria genus was the main component of the wine grape mycobiota of the DOC San Rafael at harvest time, which is in agreement with previous studies carried out in several winemaking regions worldwide (Bau, Bragulat, Abarca, Minguez, & Cabañes, 2005; Belli et al., 2006; Da Rocha Rosa et al., 2002; Magnoli, Violante, Combina, Palacio, & Dalcero, 2003; Medina, Mateo, Laura Lopez-Ocana, Valle-Algarra, & Jimenez, 2005; Sage, Krivobok, Delbos, Seigle-Murandi, & Creppy, 2002). It was followed by *Cladosporium* and only in minor percentage by *Penicillium* and *Aspergillus*, among others, which may or may not be present. Magnoli et al. (2003) have reported that *Alternaria* (9.2% of infected berries) was followed by *Aspergillus* (7.6%), *Fusarium* (5.5%), *Ulocadium* (3.3%), *Eurotium* (3.0%), *Penicillium* (2.4%) and *Cladosporium* (2.0%) in wine grapes from Mendoza, Argentina, during 2001 vintage. The differences in the location and years studied, among the most important factors, could explain the possible discrepancies.

To our knowledge, this is the first report analyzing the effect of application of an organic treatment (copper oxychloride and sulphur) and/or non-organic treatment (dithiocarbamate related fungicides) in the field during the vineyard development on the occurrence of the mycobiota in wine grapes, and so far it seems that the current routine control viticulture practices have an ineffective role against them. These findings would be particularly important when considering high frequency mycotoxigenic genus, such as *Alternaria*.

Table 1
Characterization of *Alternaria* strains isolated during 2011 and 2012 vintages.

Isolation vintage	Organic treatment[a]	Species[b]	Species-group[c]	Isolates tested	Toxin production (ppm)			Pathogenicity[d]
					TA	AME	AOH	
2011	Treated	*Alternaria alternata*	*Alternaria alternata*	20	162.1	153.4	nd	1
				25.1	1070.8	nd	12.2	2
				27	44.2	nd	nd	3
	Untreated			2	265.3	149.5	29.6	3
				3	1056.8	63.7	27.2	–
				4	583.8	16.0	35.1	1
				7	1176.8	0.6	8.6	–
				8	225.1	nd	nd	3
				15	1080.0	236.5	146.8	1
				18	136.0	nd	18.2	2
2012	Treated			5.1	73.5	nd	nd	3
				5.2	55.5	123.9	1.75	2
				5.3	798.4	nd	nd	2
				5.4	459.4	nd	nd	3
				5.5	73.6	663.4	142.8	2
				7.1	148.2	5.7	19.3	3
				7.2	282.1	9.9	107.5	3
				7.3	441.3	22.6	2.2	3
				7.4	46.0	nd	nd	1
				7.5	1941.1	10.8	60.4	3
				8.1	11.2	nd	nd	3
				8.2	–	–	–	–
				8.3	119.9	289.5	180.6	2
				8.4	726.5	6.7	17.1	–
	Untreated			1.1	655.3	139.5	437.0	–
				1.2	387.1	117.7	19.9	3
				1.3	–	–	–	–
				1.4	45.1	nd	12.3	3
				2.1	152.9	nd	nd	3
				2.2	594.1	nd	101.7	3
				2.3	101.9	34.5	71.3	3
				2.4	433.7	0.9	7.4	2
				2.5	1412.0	nd	26.9	2
				3.1	nd	nd	nd	–
				3.2	–	–	–	
				3.3	594.4	nd	21.2	3
				3.4	–	–	–	–
				3.5	539.1	24.9	2.9	2
Average 2011–2012					481.6	108.9	62.9	

TA: Tenuazonic acid; AOH: Alternariol; AME: Alternariol monomethyl ether.
nd: not detected below the LOQ.
–: not determined.
[a] Organic treatment consisting of 3 spraying (in January, February and March) with 3 g/L copper oxychloride and 3 g/L sulphur.
[b] Morphological species.
[c] Molecular species-group: *A. alternata*, *A. infectoria*, *A. porri* and *A. radicina*.
[d] Pathogenicity grade: 1. slightly pathogenic; 2. moderately pathogenic; 3. very pathogenic.

Due to the high intra-species variations among *Alternaria* species and the significant influence of environmental conditions on sporulation patterns there is a need of others tools besides the traditional morphological methods for *Alternaria* identification (Thomma, 2003). In this context, we have successfully applied the molecular identification method developed by Pavón et al. (2010), which resulted in concordance with the morphological identification, since *A. alternata* is included, among others, in the *A. alternata* species-group. Similarly, Mikušová, Sulyok, and Šrobárová (2014) identified two species, *A. alternata* and *Alternaria tenuissima*, in Slovakian grape berries based on microscopic and macroscopic characteristics.

Mikušová et al. (2014) have analyzed the toxicogenic ability of 11 *Alternaria* strains isolated from grape berries in three Slovak winemaking regions in CYA (Czapek Yeast extract Agar) and YES (Yeast Extract Sucrose) agar media using HPLC-MS/MS, but the values obtained were very low (0.0058–0.0337 ppm of AOH and 0.0044–0.0494 ppm of AME) and even unquantifiable (altenuene and TA) in comparison with our results. In contrast, the GRCS medium, used during the present work, has been described as the most suitable for *Alternaria* mycotoxins screening according to Chulze et al. (1994) supporting a good production of *Alternaria* toxins and easy extraction. Nevertheless, Mikušová et al. (2014) have detected *Alternaria* toxins from dried wine berries, supporting the possibility to find them also in wine grapes.

We have found a high percentage of the analyzed *Alternaria* strains capable of producing at least one of the three mycotoxins analyzed (AOH, AME and TA) in agreement with several previous reports on toxin production on autoclaved polished rice or GRCS medium by *Alternaria* strains isolated from different substrates (Li, Toyazaki, & Yoshizawa, 2001; Oviedo, Sturm, Reynoso, Chulze, & Ramirez, 2013; Patriarca, Azcarate, Terminiello, & Fernández Pinto, 2007; Somma et al., 2011). In addition, the majority of the analyzed strains (53%) were able to co-produce the three mycotoxins, as previously reported (Greco et al., 2012; Patriarca et al., 2007; Somma et al., 2011) showing a potential risk of synergistic effects.

Moreover the application of organic treatment did not produce a difference in the toxicogenic potential of *Alternaria* strains isolated from grapes. This fact reflected once more the inefficiency of such a

routine control viticulture practice to prevent the presence of toxicogenic *Alternaria* strains. Additionally, the toxicogenic potential was maintained from one year to the next one.

The frequency of the analyzed strains producing TA (97%) was higher than *Alternaria* strains isolated from other substrates (65%, Greco et al., 2012; 32%, Li et al., 2001; 10%, Oviedo et al., 2013; 72%, Patriarca et al., 2007; 70%, Somma et al., 2011), although a more frequent production of AOH and AME was reported in some cases. Therefore, the toxicogenic profile of the *Alternaria* strains isolated from wine grapes seems to be distinct from that of other substrates.

Likewise, TA was produced at higher amounts than AOH and AME. This was in agreement with Greco et al. (2012) and Somma et al. (2011) among others, as well as the maximum TA concentration achieved (1941 ppm) was in agreement with the 2778 ppm obtained for *Alternaria* strains from blueberries (Greco et al., 2012), the 4200 ppm for *A. alternata* from tomatoes (Bottalico & Logrieco, 1998), the 3563 ppm for *A. alternata* from Chinese wheat (Li et al., 2001) and the 6800 ppm for *A. alternata* from mandarin fruits (Logrieco, Bottalico, Mule, Moretti, & Perrone, 2003).

Similar maximum concentrations of AOH (437 ppm) and AME (663 ppm) were already reported in the literature, such as 119 and 902 ppm, respectively, for *A. alternata* from blueberries (Greco et al., 2012), 600 and 100 ppm for *A. alternata* from tomatoes (Bottalico & Logrieco, 1998), 178 and 98 ppm for *A. alternata* strains from wheat (Li et al., 2001), and 20 and 20 ppm for *A. alternata* from mandarins (Logrieco et al., 2003).

The positive correlation between the production of AOH and AME by *Alternaria* strains isolated from other substrates was already observed (Li et al., 2001; Oviedo et al., 2013; Patriarca et al., 2007), possibly because they share the same chemical precursors (Hiltunen & Söderhäll, 1992). Additionally, no correlation was found between production of AOH, AME or TA in GRCS medium and pathogenicity of the same isolates in grape berries.

The percentage of pathogenic *Alternaria* strains was high (55% very pathogenic and 31% moderately pathogenic isolates) taking into account that they were isolated from grape berries without damage or visible symptoms of disease. Besides, it is similar to that obtained in *Alternaria* strains isolated from substrates where *Alternaria* rot is well-characterized like blueberries and tomatoes (Greco et al., 2012; Somma et al., 2011). Despite this, there are few reports of *Alternaria* rot in grape berries (Kakalíková, Jankura, & Šrobárová, 2009; Nair, 1985; Swart & Holz, 1994; Tournas & Katsoudas, 2005). The explanation for this apparent contradiction could reside in the opportunistic-pathogen nature of *Alternaria* genus, being dependent on favorable conditions to cause infections (Thomma, 2003).

Therefore, if *Alternaria* is capable of infecting wine grapes in any of its developmental stages in the field, it is also likely to produce its mycotoxins *in situ*, given that the production of AOH and AME by toxicogenic *Alternaria* strains in grape berries was already proven (Tournas & Stack, 2001). These toxins could remain in the grape berry, without visible disease symptoms (Barkai-Golan & Paster, 2008), and persist during winemaking up to wine, as has been already reported for AOH and AME (Broggi et al., 2013; Pizzutti et al., 2014; Scott et al., 2006), being a potential toxicological risk for consumers.

In conclusion, this study is the first polyphasic approach to characterize *Alternaria* spp. strains from wine grapes by morphological and molecular identification, toxicogenic ability and pathogenicity. Our results indicate a high incidence and prevalence of *Alternaria* genus under different years as well as despite the use of routine control viticulture practices in vineyards. Also, they showed a high percentage of toxicogenic and pathogenic *Alternaria* strains and a high production of toxins, especially TA, suggesting that *Alternaria* could be a hazardous genus in wine grapes. Nevertheless, more studies should be done in order to assess the extent of *Alternaria* mycotoxins contamination of wine grapes and wine, and to establish standardized methods developed for this purpose with the aim of contributing to a guideline limits set for these toxins.

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