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Biodiversity of *Aspergillus* section *Nigri* populations in Argentinian vineyards and ochratoxin A contamination

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ABSTRACT

Aspergillus section *Nigri* are described as the main source of ochratoxin A (OTA) contamination in grapes and wine worldwide. The knowledge of the factors affecting grape contamination by species included in this section and OTA production is essential to be able to reduce their presence, not only to improve wine quality, but also to maintain their safety. Therefore, the aims of this study were to determine the incidence of *Aspergillus* section *Nigri* species harvested in different grape-growing regions from Argentina, their ability to produce OTA, to correlate with meteorological conditions and geographical coordinates with their prevalence and to evaluate the OTA natural occurrence in grapes and wines. The morphological identification showed that *Aspergillus niger* aggregate species were the most prevalent ones, followed by *Aspergillus carbonarius* and *Aspergillus* uniseriate. These populations were confirmed through using AFLP markers and sequencing and, *Aspergillus tubingensis* was separated from *A. niger* aggregate. Climatic factors, altitude, longitude and latitude have influenced on the distribution of species included in the section. *A. carbonarius* and *A. niger* were OTA producers but differed in their OTA producing ability. Temperature was the factor which influenced the most over the highest incidence of *A. carbonarius* in La Rioja and San Juan regions. The trellis system in vineyards and drip irrigation also influenced the species isolation. The OTA levels detected in grapes and wines were low, but grape variety was more important in susceptibility to fungal infection and OTA levels.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by species belonging to *Aspergillus* and *Penicillium* genera. Its occurrence has been reported in a wide variety of food and beverages. Grapes and their derived products as grape juice, dry vine fruits and wine, are frequently contaminated with this toxin (Majerus and Otteneder, 1996; Zimmerly and Dick, 1996). OTA has been shown to have nephrotoxic, immunotoxic, genotoxic, neurotoxic and teratogenic properties. The IARC has classified OTA as a possible human carcinogen, group 2B (IARC, 1993). Based on the available scientific toxicological and exposure data, the European Union has established 2 μg/kg as OTA level maximum permitted in wines (European Commission, 2006).

Several studies have demonstrated that OTA contamination severity depends on agro-climatic conditions, vintage, vineyard location and cropping system (Bellí et al., 2005a; Battilani et al., 2006a; Leong et al., 2006a). The grape-growing area in Argentina has a wide latitudinal extension which combined with the topography of its valleys determine ecological variations that allow the classification of well-demarcated regions. In general, the regions have cold winters, hot summers and good sunshine. Low precipitation requires artificial irrigation from rivers or groundwater, forming oases perfectly defined and separated. The altitude varies between 450 m and 1800 m above sea level (Catania and Avagnina, 2010).

Aspergillus section *Nigri* species are frequently isolated in Argentinian vineyards showing different potential of OTA production (Magnoli et al., 2004; Chulze et al., 2006; Ponsone et al., 2007). In regions with higher mean temperatures such as La Rioja and San

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Juan, the highest biodiversity of species and *Aspergillus carbonarius* incidence have been observed (Chiotta et al., 2009). However, the incidence of these fungi during several vintages has not been examined. Thus, the risk of OTA contamination could not be predicted.

The aims of this study were: i) to evaluate the biodiversity of *Aspergillus* section *Nigri* in vineyards through different vintages, ii) to determine the toxigenic ability of the isolated strains, iii) to evaluate OTA contamination in grapes and wines, iv) to determine the effect of agro-meteorological conditions and cropping system on both the prevalence of the species and OTA contamination.

2. Materials and methods

2.1. Vineyards

Vineyards from grape-growing regions located in Argentina were evaluated during 2006/07, 2007/08, 2008/09, 2009/10 and 2010/11 vintages. The total geographical area selected for sampling was located between 67° and 69° west longitude and 29°–39° south latitude and was divided in seven areas: La Rioja – Chilecito, San Juan – Tulum Valley, Mendoza – Uco Valley, Mendoza North-East, Mendoza ZARM (high area near Mendoza River), Mendoza South and Neuquén – Río Negro.

Each vintage, meteorological data (air temperature, relative humidity, and rainfall) were collected from automatic weather stations located as close as possible to each experimental vineyard. Cropping system and soil type data were also obtained from each vineyard.

2.2. Grape sampling and fungal isolation and identification

To determine the incidence of *Aspergillus* section *Nigri*, from each area five vineyards were evaluated. From each vineyard, 10 plants were sampled during the ripening stage (late February–March) along two diagonal transects of vineyard and 3 bunches of grapes were taken from each plant. Therefore, from each vineyard 30 bunches were considered as a sample. From each bunch, ten berries were randomly selected (300 berries per sample), surface-disinfected for 1 min in sodium hypochlorite solution (1%), rinsed in sterile distilled water (three times), and 100 berries placed on the surface of dichloran-rose bengal-chloramphenicol (DRBC) medium. The plates were incubated at 25 °C for 7 days. After the incubation period, the percentage of infected grapes was determined. From each sample a representative number of *Aspergillus* section *Nigri* strains (the square root of the total number) isolated from each vineyard were evaluated. The colonies were sub-cultured on malt extract agar (MEA) medium at 28 °C and the strains were identified following the methodology described by Klich (2002). The black aspergilli species were classified into three main groups: *Aspergillus niger* aggregate, *A. carbonarius* and *Aspergillus* uniseriate. Molecular characterization of the strains was performed using AFLP markers and sequencing according to Chiotta et al. (2011a, 2011b).

2.3. OTA production by Aspergillus section Nigri strains

Ochratoxin A production by *A. carbonarius*, aggregate *A. niger* (*A. niger* and *Aspergillus tubingensis*) and *A.* uniseriate strains was evaluated according to Bragulat et al. (2001). Strains were inoculated in Czapek yeast agar (CYA) medium (containing 1 g of K_2HPO_4, 10 mL of Czapek concentrate, 1 mL of trace metal solution, 5 g of yeast extract, 30 g of sucrose, 15 g of agar and water up to 1 L) and incubated at 25 °C for 7 days. Three agar plugs (3 mm) of the fungal colony grown in the medium were taken along the radius from the inoculum point and placed in a vial with 1 mL of methanol. After 60 min, the extract was filtered through a membrane filter (Syringe filters, 17 mm, 0.45 μm, nylon membranes, TITAN) and submitted to a high performance liquid chromatography (HPLC) analysis as it is described in 2.6.

The strains identified as *A. carbonarius* which did not produce detectable OTA levels in CYA medium were further analyzed using different liquid culture media: yeast extract-sucrose (YES) broth containing 2% yeast extract, 15% sucrose, and potato-dextrose (PDB) broth (Oxoid, Basingstoke, UK). After the incubation period, the cultures were filtered and the extraction of OTA was performed by mixing 100 μL of culture with 900 μL of mobile phase. The extract was filtered again through a nylon membrane filter (0.45 μm) prior to analysis by HPLC.

Culture extracts of *A. tubingensis* strains grown on CYA and YES media incubated 7 days at 20 °C and 28 °C were analyzed by Q-TOF LC/MS to confirm OTA production.

2.4. Ochratoxin A occurrence in grapes

For toxin analysis, three bunches per plant were considered as a sample. Berries were randomly selected, homogenized, mechanically crushed, and OTA content was determined following the methodology proposed by Visconti et al. (2001) with some modifications. Fifty grams of grapes were mixed with 150 mL of polyethylene glycol and sodium hydrogen carbonate solution (5% $NaHCO_3$, 1% PEG 8000). The mixture was homogenized in an oscillating shaker for 30 min and filtered through a filter paper (Whatman N° 4). The extract was centrifuged at 6400 rpm for 20 min, at 4 °C and immediately filtered through a microfibre filter (Whatman, 0.45 μm). The pH of each sample was adjusted to 7.4 with HCl (0.1 M). A ten-milliliter portion was taken and added to an inmunoaffinity column (OchraTestTM; Vicam, Digen Ltd, Oxford, UK). The column was washed with 5 mL of sodium chloride and polyethylene glycol solution (2.5% NaCl, 0.5% $NaHCO_3$), followed by 5 mL of distilled water. Ochratoxin A was eluted from the column with 1.5 mL methanol and the eluate blown to dryness under nitrogen. The residue was immediately re-dissolved in mobile phase and submitted to HPLC analysis.

2.5. Ochratoxin A occurrence in wine

OTA content in wine was determined following the methodology described by Visconti et al. (2001). Ten mL of wine were mixed with 10 mL of water solution containing PEG 8000 (1%) and $NaHCO_3$ (5%). The extract was filtered and a fraction of 10 mL was passed through an immunoaffinity column. The column was washed with 5 mL of an aqueous solution of 2.5% NaCl and 0.5% $NaHCO_3$ and, then with 5 mL double distilled water. Ochratoxin A was finally eluted with 2 mL of methanol (HPLC grade), at a flow rate of 1–2 drops per second. The extract was evaporated to dryness at 50 °C under N_2 stream, and the residues were re-dissolved in 250 μL of the HPLC mobile phase.

2.6. OTA detection and quantification

The HPLC apparatus used for determination of OTA was a Hewlett–Packard (Hewlett–Packard company, Palo Alto, CA, USA) chromatograph with a loop of 50 μL, equipped with a fluorescence detector (λexc = 330 nm and λem = 460) and a C18 column (150 × 4.6 mm, 5 μm particle size; Luna-Phenomenex, Torrance, CA), connected to a pre-column (20 × 4.6 mm, 5 μm particle size; Supelguard LC-ABZ, Supelco). The mobile phase was pumped at 1.0 mL/min, and consisted of an isocratic system composed by acetonitrile, water, acetic acid (99:99:2). OTA was quantified on the

basis of HPLC fluorometric response compared with the OTA standard.

The *A. tubingensis* extracts were analyzed using an Agilent 1290 Infinity LC coupled to an Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Q-TOF LC/MS System. The separation was performed in an Agilent Zorbax Eclipse Plus C_{18} Rapid Resolution HD column (2.1 × 100 mm, 1.8 μm). The dual AJS ESI ionization source operated in positive mode was performed using the following parameters: gas temperature, 140 °C; nebulizer, 40 psig; sheath gas temperature, 350 °C; sheath gas flow, 11 L/min; capillary voltage, 4000 V; nozzle voltage, 500 V. Analysis was done in gradient mode, being solvents: (A) water with 1% formic acid and (B) acetonitrile with 1% of formic acid. The gradient was performed as follows: startup, 30% B; linear increase to 100% B in 7.5 min; 100% B was held during 1.5 min for cleaning; the composition was returned to 30% in 1.5 min and held during 2 min for equilibration. The analysis was performed with a flow rate of 0.35 mL/min. The retention time of OTA was 4.39 min.

2.7. Statistic analysis

Data on fungal and OTA contamination in grapes was analyzed by ANOVA test, followed by Tukey mean separation test ($p < 0.001$, $p < 0.05$). Pearson correlation coefficient was used to evaluate the relationship between the frequency of colonized grapes with *Aspergillus* section *Nigri* species and the meteorological condition (maximum, mean and minimum temperatures, rain and relative humidity), soil type and management practices during grape-growing stages. All statistical analyses were carried out using the Software SigmaStat for windows version 3.5 (SPSS, Chicago, USA).

Multivariate analysis (principal components) was done to determine the association among the evaluated variables using the InfoStat program version 1.0 (Argentina). Compositional data was transformed using the index Atkinson: I = log (strains proportion of a species + 0.01)/(strains proportion of other species determined + 0.01). The confirmation of the results was carried out through a canonical correlation analysis, which provides a measure of correlation between a linear combination of variables in a set (variables: index *A. niger* aggregate/*A. carbonarius*, index *A. carbonarius*/*A. niger* aggregate, index *A.* uniseriate/*A. niger* aggregate, OTA levels produced by *A. niger* aggregate and *A. carbonarius*) with a combination of variables in another set (climate variables: temperature, relative humidity and rainfall) (Hotelling, 1936).

3. Results

3.1. Aspergillus section Nigri incidence

Aspergillus section *Nigri* isolation frequency varied according to the area and the vintage evaluated. The highest incidence in La Rioja, San Juan and Mendoza North-East regions was observed during 2007/08 vintage, while in Mendoza ZARM, Mendoza – Uco Valley regions during 2008/09 vintage and in Mendoza South and Rio Negro – Neuquén regions during 2009/10 vintage. Significant differences have been observed among regions ($p < 0.05$). In general and regardless of the vintage, in those regions located to the north from grape growing areas (La Rioja, San Juan and Mendoza North-East) and Río Negro – Neuquén region the higher infection levels were observed compared to the other found in area's Mendoza (ZARM, Uco Valley and South).

The species biodiversity within *Aspergillus* section *Nigri* was similar during all vintages. Out of 834 strains isolated from grapes, most of them were morphologically identified as species belonging to *A. niger* aggregate, representing 81.5%, 88.2%, 84.7%, 84.9% and 94.4% of the strains during 2006/07, 2007/08, 2008/09, 2009/10 and 2010/11 vintages, respectively. Molecular analysis showed that within *A. niger* aggregate, A. *tubingensis* and *A. niger* were identified. From *A. niger* aggregate strains separated by sequencing analysis ($n = 83$), 82% were identified as *A. tubingensis* and 18% as *A. niger*. In AFLP analysis, of total strain analyzed ($n = 119$), 74% were identified as *A. tubingensis* and 26% remaining as *A. niger* aggregate (Table 1). *A. carbonarius* isolation frequency was lower than *A. niger* aggregate and represented 9.8%, 6.9%, 8.5%, 15% and 1.9% of the strains isolated during 2006/07, 2007/08, 2008/09, 2009/10 and 2010/11 vintages, respectively. *Aspergillus* uniseriate percentages were the lowest during all vintages, except in the last one where *A. carbonarius* presence was lower.

Aspergillus niger aggregate species were dominant in all grape-growing regions while *A. carbonarius* was relevant in La Rioja and San Juan regions ($p < 0.05$) (Fig. 1). *Aspergillus* uniseriate were isolated in higher percentages in La Rioja during the first three vintages evaluated. They were not isolated during 2009/10 vintage

Table 1
Aspergillus section *Nigri* species identified by molecular analysis.

Analysis	Species	Regions[a]	Vintage
AFLP	*A. niger* aggregate	La Rioja – Chilecito (3%)	2009/10
		San Juan – Tulum Valley (3%)	2006/07
		Mendoza – Uco Valley (7%)	2007/08
		Mendoza North-East (39%)	2006/07 - 2009/10
		Mendoza ZARM (29%)	2006/07 - 2007/08 - 2009/10
		Mendoza South (16%)	2009/10
		Neuquén – Río Negro (3%)	2007/08
	A. tubingensis	La Rioja – Chilecito (15%)	2006/07 - 2007/08
		San Juan – Tulum Valley (17%)	2006/07 - 2007/08
		Mendoza – Uco Valley (8%)	2006/07 - 2007/08 - 2009/10
		Mendoza North-East (14%)	2006/07 - 2007/08 - 2009/10
		Mendoza ZARM (10%)	2007/08 - 2009/10
		Mendoza South (9%)	2006/07 - 2007/08 - 2009/10
		Neuquén – Río Negro (27%)	2006/07 - 2007/08 - 2009/10
	A. carbonarius	La Rioja – Chilecito (60%)	2006/07 - 2007/08 - 2009/10
		San Juan – Tulum Valley (40%)	2006/07 - 2007/08 - 2009/10
	A. uniseriate	La Rioja – Chilecito (57%)	2006/07 - 2007/08
		Mendoza ZARM (29%)	2006/07
		Mendoza South (14%)	2006/07
Sequencing	*A. niger*	La Rioja – Chilecito (7%)	2008/09
		San Juan – Tulum Valley (15%)	2006/07 - 2080/09
		Mendoza – Uco Valley (21%)	2008/09
		Mendoza North-East (21%)	2006/07 - 2007/08
		Mendoza ZARM (21%)	2006/07 - 2080/09
		Mendoza South (15%)	2008/09
	A. tubingensis	La Rioja – Chilecito (9%)	2006/07 - 2007/08 - 2008/09
		San Juan – Tulum Valley (14%)	2006/07 - 2007/08 - 2008/09
		Mendoza – Uco Valley (20%)	2007/08 - 2008/09
		Mendoza North-East (20%)	2006/07 - 2007/08 - 2008/09
		Mendoza ZARM (6%)	2007/08 - 2008/09
		Mendoza South (9%)	2008/09
		Neuquén – Río Negro (22%)	2006/07 - 2007/08 - 2008/09
	A. carbonarius	La Rioja – Chilecito (29%)	2008/09
		San Juan – Tulum Valley (57%)	2008/09
		Mendoza – Uco Valley (14%)	2008/09
	A. japonicus	La Rioja – Chilecito (20%)	2008/09
		Mendoza – Uco Valley (20%)	2008/09
		Mendoza South (40%)	2008/09
		Neuquén – Río Negro (20%)	2008/09

[a] The percentage of strains isolated by region is showed in parentheses.

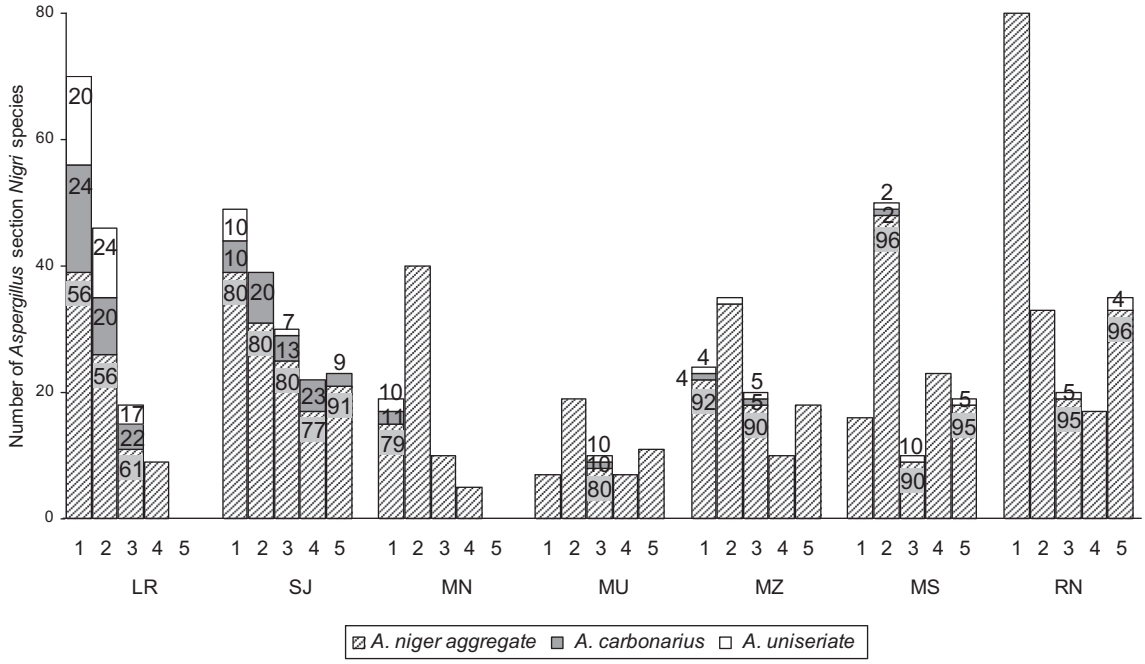

Fig. 1. *Aspergillus* section *Nigri* species isolated from different grape-growing regions during 2006/07 (1), 2007/08 (2), 2008/09 (3), 2009/10 (4) and 2010/11 (5) vintages. The values given on the bars refer to the mean isolation frequency (%) of the different species in each region. **LR:** La Rioja – Chilecito, **SJ:** San Juan – Tulum Valley, **MN**: Mendoza North-East, **MU:** Mendoza – Uco Valley, **MZ:** Mendoza ZARM, **MS**: Mendoza South, **RN:** Neuquén-Río Negro.

but were isolated in regions located to the South in low percentages during 2010/11 vintage (Mendoza South and Rio Negro – Neuquén).

3.2. Toxigenic profile by Aspergillus strains

Aspergillus niger aggregate and *A. carbonarius* showed some potential to produce ochratoxin A. All the *A. carbonarius* strains ($n = 70$) were OTA producers and the levels produced varied according to the evaluated vintage. The strains isolated during 2007/08 vintage showed the highest levels of toxin production (mean: 1442 ng/g) whereas the strains isolated during the other vintages produced OTA in lower levels (mean ranging from 45 to 252 ng/g). Within *A. niger* aggregate species ($n = 718$), a lower number of toxigenic strains were isolated during 2008/09 ($n = 12$), 2009/10 ($n = 7$), and 2010/11 ($n = 13$) vintage in comparison with 2006/07 ($n = 54$) and 2007/08 ($n = 25$) vintages, but the production levels detected were higher, especially during 008/09 vintage (mean: 118 ng/gr). Using AFLP and sequencing analysis, 202 strains were analyzed within aggregate *A. niger*. From this group, only 36 stains were identified as *A. niger sensu stricto* which 12 were ochratoxigenics. Previously, OTA peaks detected in *A. tubingensis* extracts by LC with fluorescence detection were not confirmed using Q-TOF LC/MS. The main ion $[M+H]^+$ with m/z 404 produced a peak in the mass spectra of the OTA standard at 4.38 min, but in the extract cultures was not observed. During the OTA standard injections, the ion with m/z 239.01 consistent with the lost of the phenylalanine residue was also observed in a very low proportion. Among *Aspergillus* uniseriate strains ($n = 46$) none of the isolated were OTA producers.

3.3. Influence of geographic coordinates and climatic condition on the Aspergillus section Nigri populations

The incidence of infected berries by *Aspergillus* section *Nigri* species was significantly correlated with geographic coordinates (Fig. 2). A negative correlation was obtained between black aspergilli and longitude, indicating that its incidence increases from west to east. In particular, the percentage of *A. niger* aggregate species increases to greater longitude. The altitude also showed influence on the species isolation rates. A higher *A. carbonarius* incidence in areas with higher altitude was observed, while *A. niger* aggregate incidence decreased with altitude. Latitude showed a positive correlation with *A. niger* aggregate and negative with *A. carbonarius* and *A.* uniseriate. This correlation shows that isolation frequency of *A. niger* aggregate species increases toward the south of Argentina, while the isolation frequency of *A. carbonarius* and *A.* uniseriate were higher in northern regions of Argentina.

Among all climatic conditions evaluated, temperature was the factor which influenced the most on the isolation frequency of *Aspergillus* section *Nigri*, since the same behavior was observed in all vintages evaluated (Table 2). The incidence of *A. carbonarius* and *A.* uniseriate positively correlated with maximum, mean and minimum temperature, while *A. niger* aggregate species was negatively correlated. Such correlation showed that the isolation frequency of *A. carbonarius* and *A.* uniseriate increased as temperature increased, whereas the isolation frequency of *A. niger* aggregate species increased as temperature decreased.

Rainfall and *A. niger* aggregate populations showed a negative correlation during 2006/07, 2007/08 and 2008/09 vintages. In contrast, rainfall recorded and *A. carbonarius* and *A.* uniseriate isolation positively correlated. Significant differences were observed during 2007/08 vintage for *A. carbonarius* and *A.* uniseriate ($p < 0.05$). The same behavior was obtained with relative humidity for *A. niger* aggregate and *A. carbonarius* but only during 2007/08 vintage. However, no significant differences were observed. The results indicate that *A. carbonarius* and *A.* uniseriate populations predominated in regions with higher rainfall, in contrast to the data observed for *A. niger* aggregate species.

Biplot graph from multivariate analysis shows the interaction among climatic variables evaluated (maximum, mean and minimum temperature, relative humidity and rainfall), Atkinson index

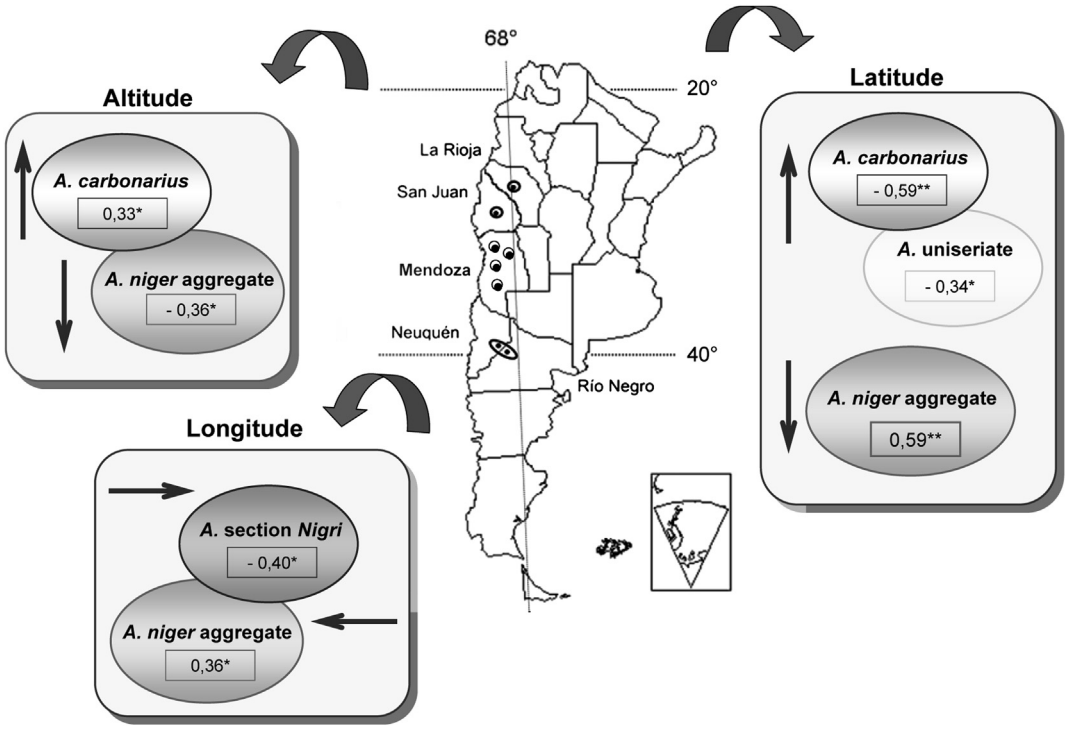

Fig. 2. Pearson correlation coefficients between geographical coordinates, altitude and isolation frequency of *Aspergillus* section *Nigri* species. Significant correlation $p < 0.05$ (*) and $p < 0.001$ (**).

of *Aspergillus* section *Nigri* species and OTA levels produced (Fig. 3). The regional variability was greater than inter-vintage variability. However, some regions depending on the vintage evaluated were more closely related to any other particular climatic variable, which influenced on the variability of species isolation percentages. In general, higher *A. carbonarius* and *A.* uniseriate percentages were observed in La Rioja and San Juan regions, where the highest temperatures were recorded. On the other hand, *A. niger* aggregate species were isolated in higher levels in the remaining regions evaluated. The canonical correlation analysis clearly shows that higher mean temperatures influenced the higher *A. carbonarius* isolation percentages ($r = 0.87$, $p < 0.001$).

Table 2
Correlation between meteorological parameters and *Aspergillus* section *Nigri* species isolated during all vintages evaluated.

Meteorological data	*A. niger* aggregate	*A. carbonarius*	*A.* uniseriate
T maximum (°C)	−0.58 (06/07)	0.59 (06/07)	0.56 (06/07)
	−0.73 (07/08)	0.77 (07/08)*	0.53 (07/08)
	−0.59 (08/09)	NO	0.52 (08/09)
	−0.82 (09/10)*	0.82 (09/10)*	NO
	−0.53 (10/11)	0.61 (10/11)	NO
T mean (°C)	−0.84 (06/07)*	0.84 (06/07)*	0.83 (06/07)*
	−0.92 (07/08)*	0.94 (07/08)**	0.61 (07/08)
	−0.59 (08/09)	0.68 (08/09)	NO
	−0.91 (09/10)*	0.91 (09/10)*	NO
	NO	0.48 (10/11)	NO
T minimum (°C)	−0.86 (06/07)*	0.86 (06/07)*	0.85 (06/07)*
	−0.92 (07/08)*	0.88 (07/08)**	0.64 (07/08)
	−0.54 (08/09)	0.76 (08/09)*	NO
	−0.80 (09/10)*	0.80 (09/10)*	NO
	NO	NO	NO
RH (%)	NO	NO	NO
	−0.58 (07/08)	NO	0.58 (07/08)
	NO	NO	NO
	NO	NO	NO
	NO	NO	NO
R (mm)	−0.54 (06/07)	0.54 (06/07)	NO
	−0.82 (07/08)*	0.63 (07/08)	0.94 (07/08)**
	−0.57 (08/09)	0.54 (08/09)	0.47 (08/09)
	NO	NO	NO
	NO	NO	NO

T: temperature, HR: relative humidity, R: Rainfall. NO: no correlation. Significative correlation $p < 0.05$ (*) and $p < 0.001$ (**).

3.4. Influence of viticulture practices on Aspergillus section Nigri populations

3.4.1. Vine training systems

Aspergillus carbonarius was isolated in higher percentages in vineyards with parral and low vertical shoot positioned trellis (VSP) (19.5%, 16.5%, respectively), while in vineyards under high VSP lower percentages were observed (2%). Uniseriate species showed a similar isolation frequency, whereas *A. niger* aggregate species were isolated in higher percentages in the vineyards conducted under high VSP (96%). Although the incidence of *A. niger* aggregate strains was high in this system, the percentage of OTA producer strains was higher in parral system (Fig. 4).

3.4.2. Soil and irrigation systems

There were no significant differences between the soil texture (sandy, clay and silty) and *Aspergillus* section *Nigri* species isolation from grapes. However, in vineyards where irrigation was performed using the drip system higher percentages of *A. carbonarius* and *A.* uniseriate were isolated (13.5% and 11.5% of total strains, respectively) in comparison with furrow irrigation (5.3% and 0.9%, respectively). The incidence of *A. niger* aggregate was high under both irrigation systems evaluated, but the percentage of ochratoxigenic strains was higher in vineyards under drip system (Fig. 4).

3.5. Aspergillus and ochratoxin A incidence in grapes and wines

Bonarda, Syrah and Cabernet Sauvignon grape varieties showed the highest *A. carbonarius* percentage, representing 29%, 12% and

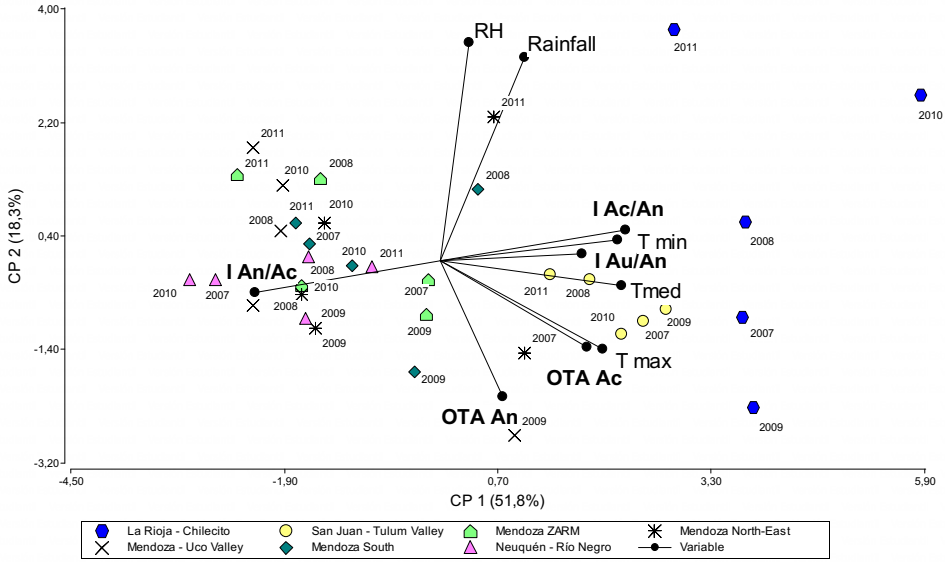

Fig. 3. Principal component analysis of recorded meteorological conditions vs. *Aspergillus* section *Nigri* species percentages and OTA levels produced. An: *A. niger* aggregate, Ac: *A. carbonarius*, Au: *A.* uniseriate, I: Atkinson index.

10% of the strains isolated respectively. *A. niger* aggregate ochratoxigenic strains were isolated in high percentages from Pinot Noir, Syrah and Cabernet Sauvignon varieties, representing 36%, 26% and 21% of the isolated strains, respectively. Uniseriate species remained in Bonarda variety (19%) although they were not OTA-producers.

Although ochratoxigenic species were isolated in all regions, the OTA levels detected in grape and wine samples were low, ranging from 0.1 to 5.4 ppb (ng/g) and from 0.01 to 4.82 ppb (ng/mL), respectively (Table 3). As regards grapes, Cabernet Sauvignon and Syrah varieties have shown the highest levels of OTA contamination, whereas in wine it has only been detected in Syrah variety. In La Rioja and San Juan regions, the highest number of positive samples for OTA contamination was detected.

4. Discussion

Biodiversity of *Aspergillus* section *Nigri* was represented by *A. niger* aggregate species, *A. carbonarius* and *Aspergillus* uniseriate in vineyards Argentina during 5 vintages. These populations were confirmed by using AFLP markers and sequencing and, *A. tubingensis* was clearly separated from *A. niger* aggregate. Altitude, longitude and latitude have shown some influence on the species distribution. Isolation frequency of *A. niger* aggregate species increases toward the south and west of grape-growing area, while the isolation frequency of *A. carbonarius* and *A.* uniseriate was higher in the northern regions. These results agree with studies done in southern Europe and Israel, where a geostatistical analysis was used to determine regions with high risk of *Aspergillus* section *Nigri* species contamination and OTA production (Battilani et al., 2006b).

The geographic region was relevant in the isolation frequency of a particular species within *Aspergillus* section *Nigri*, but was intrinsically related to temperature and humidity conditions recorded in each area. The role of temperature and water activity (a_W) on the survival of *Aspergillus* section *Nigri* species is complex. Through the present study it has been observed a positive correlation between temperature and *A. carbonarius* isolation and a

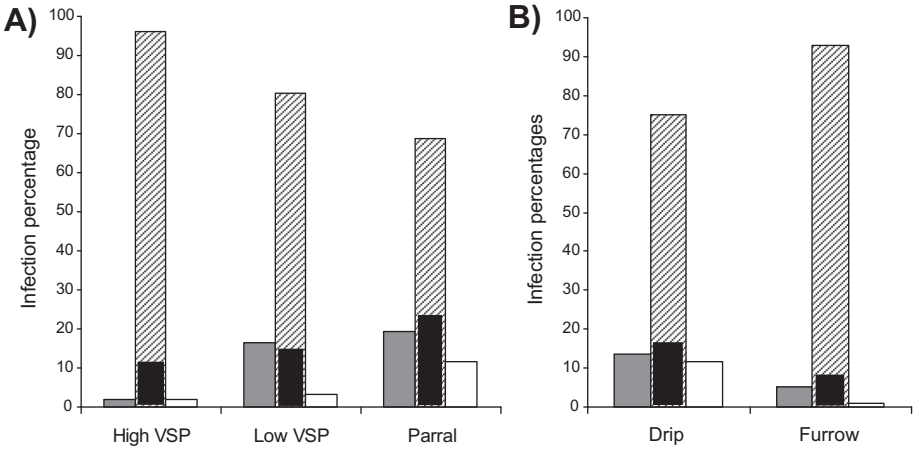

Fig. 4. Biodiversity of *Aspergillus* section *Nigri* species in different conduction (A) and irrigation (B) systems. ▒ *A. carbonarius*, ▨ *A. niger* aggregate, ■ ochratoxigenic *A. niger* aggregate strains, □ *A.* uniseriate.

Table 3
Natural occurrence of ochratoxin A in grapes and wines.

Samples	Grape-growing regions	Ochratoxin A		
		Positive samples/total[b]	Range[c] (ppb)[a]	Mean[d] (ppb)[a]
Grapes	La Rioja – Chilecito	10/20	0.1–1.6	0.7
	San Juan – Tulum Valley	8/19	0.2–5.4	2.6
	Mendoza North-East	2/13	0.9–2.9	1.9
	Mendoza – Uco Valley	6/13	0.3–1.0	0.6
	Mendoza ZARM	3/14	0.1–0.3	0.1
	Mendoza South	3/15	0.2–0.3	0.1
	Río Negro – Neuquén	4/30	0.1–0.3	0.1
Wines	La Rioja – Chilecito	7/10	0.1–2.0	0.4
	San Juan – Tulum Valley	4/9	DL	–
	Mendoza North-East	3/7	DL	–
	Mendoza – Uco Valley	4/17	DL	–
	Mendoza ZARM	1/14	DL	–
	Mendoza South	8/12	0.1–4.8	0.7
	Río Negro – Neuquén	1/6	DL	–

DL: Detection limit: 0.01 μg/kg.
[a] ppb: ng/gr and ng/mL of OTA detected in grapes and wine samples, respectively.
[b] Samples contaminated with OTA of total.
[c] OTA level ranges obtained.
[d] Mean levels of OTA detected in grapes and wines.

negative correlation between the percentages of *A. niger* aggregate isolation and temperature. It is important to highlight that such negative correlation was not due to the lower number of *A. niger* aggregate strains isolated in warmer regions but to the increase of isolation percentages of *A. carbonarius* and *A.* uniseriate strains. Previous studies have shown that *A. niger* aggregate species had higher growth rates than *A. carbonarius* at temperatures above 20 °C (Mitchell et al., 2003; Bellí et al., 2004; Leong et al., 2007; Astoreca et al., 2010). Optimal temperatures for *A. niger* aggregate species growth varied between 35 and 37 °C, while *A. carbonarius* grew more rapidly around 30 °C. However, in the range from 0.90 to 0.99 a_W, *A. carbonarius* has shown optimal growth from 0.98 to 0.99, while *A. niger* aggregate has not shown any differences in growth within the range from 0.90 to 0.995 (Mitchell et al., 2003; Bellí et al., 2004, 2005b). The higher growth rate and tolerance to a wide temperature and a_W range, could explain the increased isolation frequency of *A. niger* aggregate species in all the grape-growing regions evaluated; while *A. carbonarius* showed a higher incidence in northern regions, where higher temperature and humidity levels were found.

The highest *Aspergillus* section *Nigri* species incidence does not necessarily imply OTA contamination on grapes. Ecological conditions that influence the ochratoxigenic species growth are different from those that allow optimum OTA production. Most OTA production by *A. carbonarius* isolated from grapes from different geographical regions occurred in the range from 0.95 to 0.99 a_W, and a temperature of 20 °C, followed by 15 °C, and decreased remarkably in the range of 30–37 °C (Mitchell et al., 2004; Bellí et al., 2004, 2005b). *A. carbonarius* and *A. niger* have been OTA-producers in this study. These species differed in their OTA producing ability, as the percentage of *A. carbonarius* OTA-producing strains and the levels produced were higher than those observed by *A. niger* strains. Potential producers of OTA belonging to *A. carbonarius* were isolated from regions with higher temperatures (La Rioja and San Juan) suggesting an OTA contamination potential risk as a wide temperature range has been observed in these regions. Higher temperatures during the day could favor fungal growth and lower temperatures during the night could favor OTA production.

In previous studies, we described OTA production by *A. tubingensis* strains based in LC with fluorescence detection analysis (Chiotta et al., 2011a,b). In the present work, we have used Q-TOF LC/MS analysis to confirm this aspect and data showed that the *A. tubingensis* strains were not able to produce OTA. This result agrees with previous studies using mass spectrometry (Nielsen et al., 2009; Storari et al., 2012). Conflicting results obtained in relation to the OTA production by *A. tubingensis* could be associated to conditions of culture media and strain storage which could be modifying the ability of the strains to produce OTA. The methodology of analysis could also produce incorrect results, as it was confirmed in the present work. Further studies need to be done with this species to clarify this.

Trellis systems and irrigation method affected species isolation. Microclimatic conditions generated by each trellis system around clusters may explain the differences in the presence of fungal species (Vail and Marois, 1991; Pieri et al., 2001; Ferrer et al., 2008). Parral systems have an outer surface higher than other systems which are constantly exposed to sun activity. The foliage has rarely good extension and develops an appropriate microclimate bellow the vegetation plane to favor fungal diseases due to the lack of ventilation (Hidalgo, 2003). In the vineyard conducted with VSP, on the other hand, the highest number of bunches exposed, which allows more light and ventilation, resulted in a decrease in temperature and relative humidity differences between the canopy and the environment and could benefit a greater penetration of phytosanitary products (Smart and Robinson, 1991; Egger, 1994; Ferrer et al., 2001, 2008). These conditions could explain the highest *A. carbonarius* and A. uniseriate incidence in Parral and *A. niger* aggregate species in high VSP. The highest *A. niger* isolation in VSP could probably be due to its faster growth rate and increased tolerance to high temperatures and low water activity as it was observed by Battilani et al. (2003), Leong et al. (2004), Serra et al. (2003, 2005), Bau et al. (2005), and Bellí et al. (2005a). Great species biodiversity isolated, also under low VSP could be attributed to bunch position which is closer to the soil. Previous studies have shown that air movement deposited soil spores on berries surface and the incidence of *A. carbonarius* spores increased in air samples collected in areas close to the soil (Battilani et al., 2004a; Kazí et al., 2004; Leong et al., 2006a).

In vineyards, soil is the primary inoculum source for *Aspergillus* section *Nigri*. Soil physical features and water could influence the biodiversity of isolated species. The highest percentages of *A. carbonarius*, *A.* uniseriate and *A. niger* ochratoxigenic strains have been observed in the present study under the drip irrigation system. Other studies have shown that *A. carbonarius* incidence is higher in soil surface than depth, and increased more in soil under the vines than in the area among the vines (rows) (Kazí et al., 2004, 2006; Leong et al., 2006b). Data has shown that the incidence increase of these populations could be due to the location of the drip irrigation on vine soil, which could favor the dissemination of fungal spore. Instead, soil populations could be sweeping during the flood under the furrow irrigation.

The OTA levels detected in grapes and wines were low. Therefore, the low OTA levels in relation to ochratoxigenic species incidence could be explained on the basis of grape's good health at harvest stage over the different vintages evaluated, considering that the sampling has been performed randomly and there has not been a selection of damaged and undamaged grapes. However, the presence of a high percentage of OTA-producing species is relevant as berries damage could favor the risk of OTA contamination. A previous study has shown that the skin-damaged berries by *Planococcus ficus* affected the incidence of ochratoxigenic species and OTA contamination in grapes (Chiotta et al., 2010).

Furthermore, it has been shown that grape variety has been important in relation to the susceptibility of fungal infection and OTA contamination. More compact varieties (Cabernet Sauvignon, Syrah, Bonarda and Pinot Noir) were more susceptible to infection

by *Aspergillus* section *Nigri* species, since they have greater contact surface among berries. Therefore, this results in a low deposition of cuticle and epicuticular waxes being more sensitive to attack by fungal species (Vail and Marois, 1991). Moreover, these contact surfaces have higher humidity which could favor the spread of rot areas. Some training systems, such as lira, keep the area ventilated and show decreases up to 50% of damage by rot (Ferrer et al., 2001, 2008; Pieri et al., 2001; Pertot et al., 2007). Other authors also showed through "in vitro" studies that grape variety regardless of bunch structure, affected the levels of contamination with *Aspergillus* section *Nigri* and OTA contamination level (Battilani et al., 2004b).

The results obtained in this study provide relevant information to establish the potential risk areas for OTA contamination in Argentinian vineyards. Moreover, the data will be useful for the application of appropriate management strategies to reduce or prevent the development of ochratoxigenic species.

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