Research Article

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Factors affecting distribution and abundance of *Aspergillus* section *Nigri* in vineyard soils from grapevine growing regions of Argentina†

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Abstract

BACKGROUND: *Aspergillus* species belonging to section *Nigri* are the main fungi responsible for ochratoxin (OTA) contamination in grapes and wine. These species live as saprophytes in the superficial layer of the vineyard soil. We evaluated the biodiversity of potentially ochratoxigenic strains of *Aspergillus* section *Nigri* isolated from vineyard soils from different grapevine growing regions of Argentina. The isolates were characterized by classical and molecular methods. A multiple correspondence analysis was performed to identify the overall correlation of the *Aspergillus* group distribution with environmental conditions, geographical characteristics and vineyard practices.

RESULTS; *Aspergillus niger* aggregate was the prevalent group (71%) and *A. carbonarius* made up only 2%. Species discrimination by species-specific primers showed that in *A. niger* aggregate 89% were *A. tubingensis*; 97% of the uniseriate were *A. japonicus*/*A. aculeatus*. Isolates belonging to these groups were unable to produce OTA. Our results clearly demonstrate a strong association between presence of *A. carbonarius*, high average temperatures and drip irrigation. Precipitation levels appear as a secondary factor, and altitude, vineyard age, predominant species, grape variety or total fungal count showed no association with *A. carbonarius*.

CONCLUSION: We demonstrated a low prevalence of ochratoxigenic species in vineyard soil from the grape-growing regions of Argentina.

Keywords: ochratoxin; vineyard soil; *Aspergillus* section *Nigri* distribution; multiple correspondence analysis

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin, produced by species belonging to *Aspergillus* and *Penicillium* genera, reported as one of the three most important and harmful mycotoxins in the world.[1] The toxin is a very strong nephrotoxin, with carcinogenic, teratogenic and immunosuppressive properties, classified as Group 2B by the International Agency for Research on Cancer.[2] OTA has been detected worldwide in different foods and beverages, including cereals, coffee beans, wine, cocoa beans, beer and dried fruit, and also in feed sources.[3–6] OTA was detected in wine for the first time in 1996.[7] Today, in Europe, wine – and especially red wine – has been determined as the second major source of human exposure to OTA after cereals, followed by beer and coffee.[8] Thus the European Commission has fixed the maximum limit for OTA in wine and grape juice at 2.0 μg kg^{-1}.[9]

Aspergillus species belonging to section *Nigri*, commonly known as black aspergilli – in particular, *Aspergillus carbonarius* and species belonging to the *Aspergillus niger* aggregate – have been identified as the main fungi responsible for OTA contamination in grapes and wine. They are opportunistic fungi that may occur and form OTA on grapes from veraison to harvest.[10]

In vineyards the best way to reduce OTA production is to control the presence of ochratoxigenic fungi.[11] For this purpose, knowledge of the ecological factors that affect occurrence of black aspergilli in the vineyard plays an important role. The different OTA-producing species live as saprophytes in the superficial layer of the vineyard soil, which constitutes the major inoculum reservoir of *Aspergillus* section *Nigri* species. Australian authors have demonstrated that vineyard soil at a depth of 0–5 cm beneath the vines is the primary reservoir of black aspergilli.[12] Concentrations were also higher in the soil directly beneath the vines compared to the inter-row area. It has been postulated that air movement deposits the spores from the soil on to the grape berry surfaces;

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thus the risk of contamination with OTA in wines might be related to the presence of toxigenic strains in the soil.[13]

Argentina is the fifth largest wine-producing country in the world. In 2009, approximately 564 million liters were exported, mainly to North America and Europe. The grape-growing area is located along the country's western border in the Andes region with latitudinal amplitude from north to south (22–40° latitude). They extend for over 2000 km, where different microclimates give to the different areas some ecological particularity. These kinds of differences have been demonstrated to be important for OTA contamination; for example, wines from southern and warmer regions of Europe showed incidence and levels of contamination higher than those from northern European areas. As was reviewed by Covarelli *et al.*,[14] different climatic conditions may affect the distribution of OTA-producing fungi and final OTA concentration; the origin of the wine seems to be a determining factor of its final OTA content.

According to the review by Somma *et al.*,[11] the occurrence of black *Aspergillus* species in Australia is similar to that in Mediterranean countries, with a prevalence of *A. niger* and *A. carbonarius*, while *A. niger* is the most common species in South America on grapes. The distribution and factors influencing this distribution of *Aspergillus* section *Nigri* have never been evaluated in vineyard soil in Argentina; this knowledge and ochratoxigenic-producing potential of strains could be critical in evaluating the risk of ochratoxin contamination in Argentinean wine.

The aim of this study was to evaluate the biodiversity of potentially ochratoxigenic strains of *Aspergillus* section *Nigri* group isolated from vineyard soils from different grapevine growing regions of Argentina considering different parameters such as harvest year, environmental conditions, geographical characteristics and vineyard practices.

MATERIALS AND METHODS

Study area

Seven grapevine growing regions of Argentina were chosen for this study based on their climatic differences and national economic importance, in particular: La Rioja, San Juan, Mendoza Uco Valley, Mendoza North, Mendoza ZARM (high area near the Mendoza river), Mendoza South and Neuquén–Rio Negro (Patagonia).

Surveys were conducted in 2008 and 2009 growing seasons to evaluate the distribution of *Aspergillus* section *Nigri* in vineyard soils immediately prior to harvest. Geographic coordinates, altitude, average annual precipitation and average annual temperatures for each of these regions are shown in Table 1. In each region, four vineyards planted with red grapes were selected. The average size of the vineyards was about 5000 m^2 (1.24 acres).

Soil sampling

In order to ensure representativeness in the soil sample from each vineyard, two soil subsamples were randomly collected under vine lines and in the middle of rows in five consecutive rows located in the central portion of the stand. In this way, 10 soil subsamples (approximately 100 g) were obtained from each vineyard with a trowel from the top 5 cm of soil, and subsequently mixed into a single composite sample. Samples were collected in paper bags and stored at 4 °C until laboratory analysis. Data on cropping systems, soil characteristics, vineyard age and irrigation system were obtained from each vineyard manager. Meteorological conditions (temperature and precipitation levels) were obtained from weather stations close to the vineyards.

Mycological analysis

Each soil sample (10 g) was mixed with 90 mL peptone water (1 g L^{-1}) and shaken on a horizontal shaker for 20 min. This mixture was decimally diluted. A 0.1 mL aliquot of each dilution was spread on the surface of Dichloran Rose Bengal Chloramphenicol agar (DRBC) to enumerate total cultivable fungi. Plates were incubated at 25 °C for 5–7 days. For counting, plates containing 10–100 colonies were used and the results were expressed as colony-forming units per gram of soil (CFU g^{-1}).[15]

Filamentous fungi were identified at genus level according to macroscopic and microscopic criteria in accordance with Samson *et al.*[16]

Representative colonies suspected to belong to *Aspergillus* section *Nigri* were transferred for subculturing and classified according to microscopic criteria into three groups: uniseriates, *A. niger* aggregate and *A. carbonarius*.[17]

Molecular identification

To confirm the morphological identification, selected strains were grown on MEA and inoculated in 250 mL Erlenmeyer flasks containing 50 mL Wickerman's medium (40 g glucose, 5 g peptone, 3 g yeast extract, 3 g malt extract L^{-1}). The shaken cultures (150 rpm), incubated at 25 °C, were filtered after 3 days and frozen with liquid nitrogen, ground to a fine powder and kept at −80 °C. Fungal DNA was extracted with a cetyltrimethylammonium bromide (CTAB) method described by Leslie and Summerell.[18]

Polymerase chain reaction (PCR) amplification

PCR conditions were optimized for each pair of primers that would allow the identification of uniseriate *Aspergillus* section *Nigri* (*A. aculeatus*/*A. japonicus*), *A. niger* aggregate (*A. niger* and *A. tubingensis*) and *A. carbonarius*. Primer reaction mix was done as described by Perrone *et al.*[19] and Susca *et al.*[20] PCR reactions were carried out on a PTC-2000 Thermal Cycler (MJ Research Inc., Watertown, MA, USA) and the reactions were performed using the following PCR conditions: denaturation at 94 °C for 5 min; 30 cycles (primer *TUB1/2* and *NIG1/2*) and 35 (primer *JAPO1/2*; *CARBO1/2*) of denaturation at 94 °C for 50 s, annealing at 60 °C (primer *TUB1/2* and *NIG1/2*) and 58 °C (primer *JAPO1/2*; *CARBO1/2*) for 50 s, extension at 72 °C for 1 min; final extension at 72 °C for 7 min, followed by cooling at 4 °C for 10 min. Amplification products where examined using agarose gel electrophoresis and ethidium bromide staining. The molecular masses of the amplified DNA were estimated by comparison with the 100 bp DNA ladder (New England Biolabs, Ipswich, MA, USA).

OTA production by potential producers

Ochratoxin-producing ability was quantified for *Aspergillus* section *nigri* isolated strains randomly selected from each of the collected soil samples. Vials (4 mL containing 2 mL yeast extract sucrose (YES)) were inoculated with approximately 2×10^4 conidia suspended in 5 µL of 10 mL L^{-1} Tween 80 in water. The vials were incubated at 25 °C in the dark, over 7 days. After incubation, the cultures were filtered through Whatman No. 1 filter paper. Filtrate (100 µL) diluted with $CHCL_3$ (900 µL) was centrifuged (8603 × *g*, 5 min), then the aqueous phase transferred to a fresh Eppendorf tube, evaporated with N_2 and stored at −20 °C. The dry extract was dissolved in 250 µL mobile phase and an aliquot of 50 µL was injected into a C18 column (150 × 4.6 mm, 5 µm particle size; Supelcosil LC-ABZ, Supelco, Bellefonte, PA, USA), connected to a pre-column (20 × 4.6 mm, 5 µm particle

Table 1. Geographic coordinates and climatic condition of the different regions included in this study

Region	Geographic coordinates	Altitude (m above sea level)	Average annual precipitation (mm)	Average annual temperature (°C)
La Rioja	29° 10′ 49″ S, 67° 28′ 44″ W	1080	200	19.3
San Juan	31° 39′ 03″ S, 68° 23′ 49″ W	580	180	18.1
Mendoza North	32° 43′ 17″ S, 68° 35′ 45″ W	610	200	14.3
Mendoza ZARM (high area near the Mendoza river)	33° 04′ 53″ S, 69° 02′ 45″ W	1160	200	14.2
Mendoza Uco Valley	33° 21′ 54″S, 69° 08′ 41″W	1050	200	14.0
Mendoza South	34° 40′ 14″ S, 68° 21′ 56″ W	680	340	15.6
Neuquén–Rio Negro	39° 02′ 11″ S, 67° 33′ 45″ W	240	200	11.5

size; Supelguard LC-ABZ, Supelco) (25 × 4.6 mm, 5 µm; Brownlee, PerkinElmer, Waltham, MA, USA) on a high-performance liquid chromatography system equipped with fluorescence detection (HPLC-FLD; λ_{exc} 330 nm; λ_{em} 460 nm, Hewlett Packard 1046-A programmable fluorescence detector). The mobile phase consisted of an isocratic system composed of acetonitrile–water–acetic acid (99:99:2) pumped at 1 mL min^{-1} flow (pump, Hewlett Packard Serise 1100). OTA was quantified on the basis of HPLC fluorometric response compared with an OTA standard solution using data module Hewlett Packard Kayak XA (HP ChemStation Rev. A.06.01).

The calibration curve was prepared with 4.5, 7.5 and 12 µg L^{-1} OTA standard (Sigma) solutions. The retention time of OTA was at 5.7 min. Detection and quantification limits (LOD and LOQ) were 0.02 µg OTA L^{-1} and 0.06 µg OTA L^{-1}, respectively. The recovery for OTA on YES media was 94 ± 1.5% (mean ± SD, $n = 5$).

Strains that showed a small or odd shaped peak on the same retention time that OTA were analyzed using the method described by Storari *et al.*[21] The isocratic mobile phase consisted of acetonitrile–acetic acid solution (46 g L^{-1}) (57:43, v/v) pumped at 1.0 mL min^{-1}. The retention time of OTA was 4.2 min.

Statistical analysis

Data on fungal contamination and species distribution were analyzed by ANOVA test, followed by LSD Fisher test ($P < 0.05$). The microbial diversity was determined based on the diversity index of Shannon–Wiener.[22]

Pearson correlation coefficient was used to evaluate the relationship between *Aspergillus* section *Nigri* count and altitude, precipitation levels and temperature registered during the previous three months to harvest.

Multiple correspondence analysis (MCA) is an exploratory multivariate statistical technique used to produce a simplified representation of the information for a large dataset. MCA allows the investigation of several qualitative parameters and permits a geometrical representation of all the information.[23] An MCA was performed to identify the overall correlation of the *Aspergillus* group distribution in the different vine-growing regions with environmental conditions, geographical characteristics and vineyard practices. All quantitative variables were categorized into classes, generating ordinal data, the variables, classes and numbers of samples per class are shown in Table 2. The numeral variables investigated were categorized as shown in Table 2. All statistical analyses were was performed using InfoStat® software (Version 2011e).

RESULTS

Evaluation of the total mycobiota

Analysis of the mycobiota of soil samples collected from vineyards in different regions of Argentina revealed the presence of a variety of fungal genera. The prevalent genera, in decreasing order, were *Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium*, *Ulocladium*, *Fusarium* and *Trichoderma*. The mean CFU of fungal colonies per gram of soil was variable among the regions. CFU counts were not significantly different between years. The soil samples from Neuquén–Rio Negro region showed the highest total fungal counts in both sampling years (mean = 6.72 × 10^4 CFU g^{-1}), while samples collected in La Rioja showed the lowest (mean = 2.55 × 10^4 CFU g^{-1}). The level of fungal inoculum in samples from Neuquén–Rio Negro was significantly higher than in the other regions ($P < 0.05$) (Fig. 1).

Distribution of *Aspergillus* section *Nigri* species

Aspergillus section *Nigri* species were detected in all soil samples evaluated. The species belonging to this section accounted for 11% of the total fungal species, and 94% of the total *Aspergillus* species isolated from soil samples collected in the different regions. Soil samples from Neuquén–Rio Negro were significantly the most contaminated ($P < 0.05$) with black aspergilli each year. In this region, levels ranged from 1.5 × 10^4 to 1.8 × 10^4 CFU g^{-1} of soil (mean = 1.69 × 10^4 CFU g^{-1}) (Fig. 2). There was no significant difference between both sampling years in relation to total count and distribution of black aspergilla on each region, so data were pooled for posteriori analyses.

The numbers of black aspergilli isolated from soil were correlated to the climatic conditions. The Pearson's correlation coefficient was calculated for values from each region. The incidence of black aspergilli was negatively correlated with the minimum temperature registered in the previous 3 months to harvest ($r_p = -0.73$, $P < 0.05$). Also altitude showed a negative correlation with black aspergilli count ($R = -0.80$). No correlation was found between precipitation levels, average temperature and maximum temperature ($P < 0.05$).

Black aspergilli species distribution in the wine regions is showed in Fig. 3. *Aspergillus niger* aggregate was the most common group in all regions (71%), especially in the region of Uco Valley, where they represent 96% of the black aspergilli isolated. In contrast, uniseriate isolates were recovered with an average incidence of 27% considering all regions, with variations between 4% and 40%, in Uco Valley and La Rioja, respectively. *Aspergillus carbonarius* made up only 2% of section *Nigri* isolates collected and only occurred in two regions La Rioja and San Juan (Fig. 3).

Table 2. Variables and numerical data categorization used in MCA, and number of samples per class

	Class 1		Class 2		Class 3	
Variable	Name	No.	Name	No.	Name	No.
Black aspergilli count	Low count <2000 CFU g^{-1}	12	Intermediate count 2000–5000 CFU g^{-1}	19	High count >5000 CFU g^{-1}	5
Average temperature	Low T < 25 °C	25	High T > 25 °C	11	–	–
Average precipitation	Low ppt <25 mm	9	Moderate ppt 25–50 mm	9	High ppt >50 mm	18
Altitude[a]	Low alt. <400 m	8	Medium alt. 400–800 m	8	High alt. >800 m	20
Irrigation	Dripping	12	Flood	24	–	–
A. carbonarius	Absence	30	Presence	6	–	–
Predominant species	Uniseriate	6	*A. niger* aggregate	30	–	–
Zone (regions)	La Rioja, San Juan, Mendoza Uco Valley, Mendoza North, Mendoza ZARM, Mendoza South and Neuquén–Rio Negro					

[a] Altitude: meters above mean sea level.

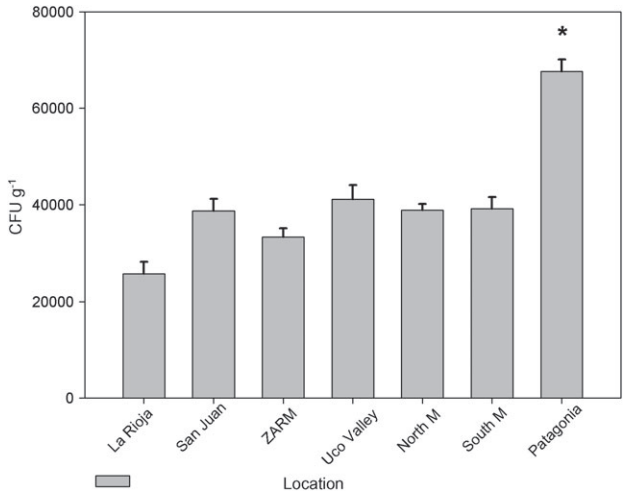

Figure 1. Total fungal count (CFU g^{-1}) in vineyard soil samples in the 2008/2009 growing season in the main Argentina grapevine growing regions. Asterisk indicates significant significant differences (*P < 0.05) from others regions.

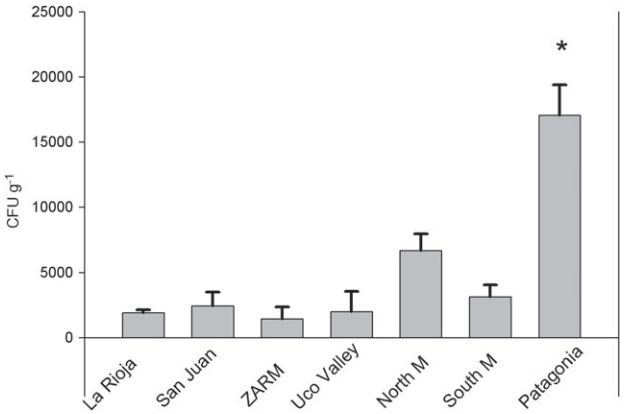

Figure 2. Black aspergilli count (CFU g^{-1}) in vineyard soil samples in the 2008/2009 growing season in the main Argentina grapevine growing regions. Asterisk indicates significant significant differences (*P < 0.05) with others regions.

Shannon–Wiener index was calculated for every region. In the 2008 growing season, San Juan showed the highest index (H1= 0.877), while the lowest was found in Mendoza Uco Valley (H1= 0.278). In the 2009 growing season, the highest index was found in La Rioja region (H1= 0.698), while the lowest was again observed in Mendoza Uco Valley (H1= 0.098).

Molecular identification of *Aspergillus* section *Nigri* species

From the 423 *Aspergillus* section *Nigri* strains isolated from vineyard soil from different regions of Argentina, 133 *A. niger* aggregate strains, 36 uniseriate strains, and 6 *A. carbonarius* strains were selected and identified using a PCR-based method. 119 isolates (89%) of *A. niger* aggregate were molecularly identified as *A. tubingensis* and 14 isolates (11%) as *A. niger*. The species *A. tubingensis* was more frequently isolated from wine-growing areas of the central west area (Mendoza and San Juan), while strains of *A. niger* were isolated more frequently in Mendoza ZARM. Thirty-five out of the 36 uniseriate strains (97%) tested with primers JAPO1/JAPO2, amplified a 583 bp fragment specific for *A. japonicus*/*A. aculeatus*, whereas the remaining strain did not amplify that product. The species *A. japonicus*/*A. aculeatus* occurred more frequently in the regions of La Rioja and Mendoza. All the strains morphologically identified as *A. carbonarius* amplified a 371 bp fragment specific for this species.

Toxigenic capacity of strains

A total of 423 black aspergilli, with confirmed identification by molecular methods, was tested for OTA production. No OTA production by the *A. niger* aggregate and uniseriate isolates was detected. Data on OTA production by the *A. carbonarius* isolates showed that 100% of the isolates produced levels between 10.4 and 521.2 µg kg^{-1} YES. The average OTA production was 205.5 µg kg^{-1} YES.

Multiple correspondence analysis

Interpretation of correspondence analysis graphs is based on the proximity of two variables (the closer the stronger, e.g. *A. carbonarius* presence and high average temperature), and second on the distance between such proximate variable to the origin of axes (the larger, the more significant). Figure 4 shows the graphic display of the variables on the plane defined by the two first axes of the analysis. This shows a strong association between the presence of *A. carbonarius*, high average temperature and drip irrigation. As a counterpart, another association is between absence of *A. carbonarius*, low average temperature and flood irrigation.

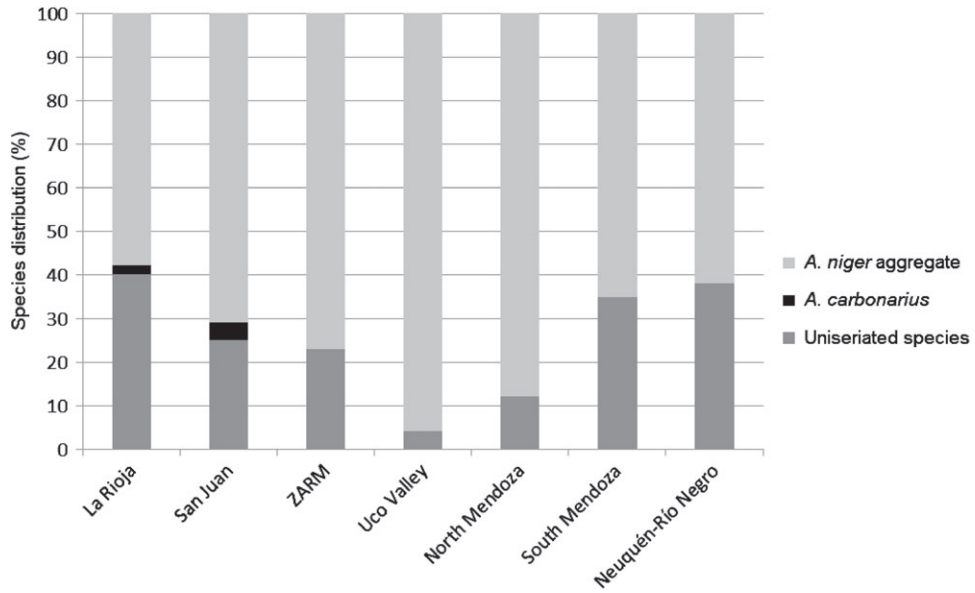

Figure 3. Distribution of *Aspergillus* group in vineyard soils sampled in 2008/2009 growing season in the main Argentina grapevine growing regions.

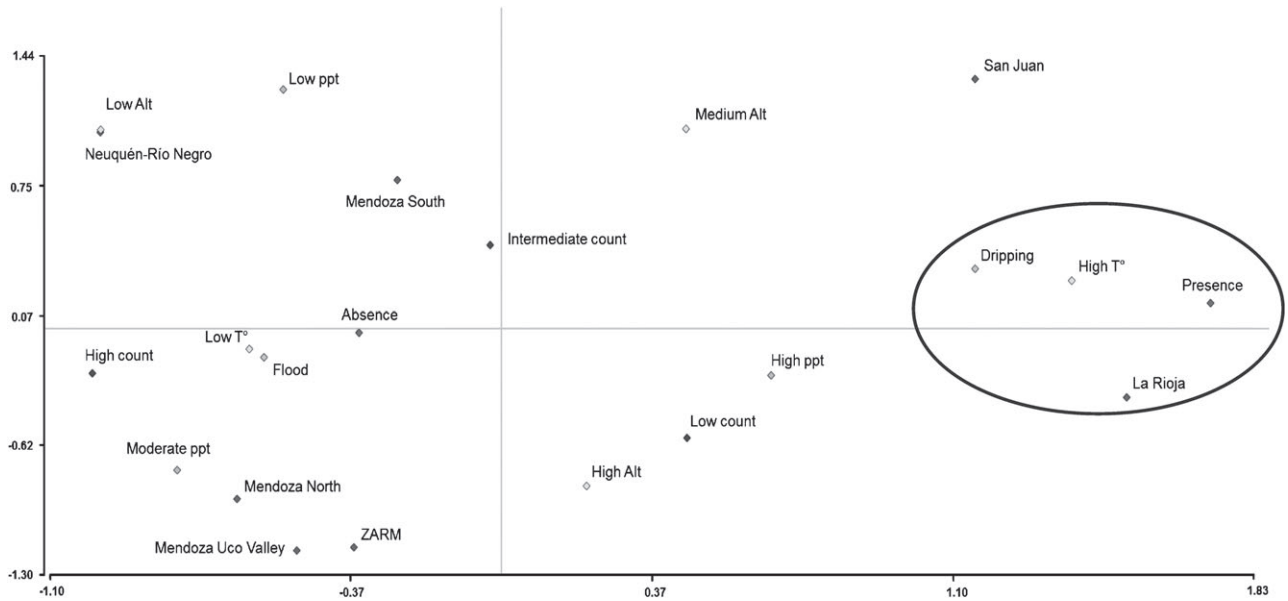

Figure 4. Multiple correspondence analysis of the diverse factors assumed to affect *A. carbonarius* presence in the 2008/2009 growing seasons in the main Argentina grapevine growing regions. Alt, altitude; ppt, precipitation.

Precipitation levels appear as a secondary factor, but this can also influence *A. carbonarius* incidence. Altitude, sampled region and *Aspergillus* section *Nigri* count showed no association with the presence of *A. carbonarius* (Fig. 4).

Neither vineyard age, predominant species, grape varieties or total fungal count showed an association with *A. carbonarius* presence (data not shown).

DISCUSSION

Information on fungal contamination of vineyards soils is scarce, since most studies are focused in grapes. In the present study, seven fungal genera were identified from vineyard soil samples, revealing a mycobiota that can be compared to the genera encountered naturally on grapes. Studies carried out in grape demonstrated that the isolated genera are the same that those found in this work.[24] Gómez *et al.*[25] studied fungal populations present in grapes from Spain and found *Aspergillus* species as the most abundant, followed by species of *Alternaria*, *Penicillium* and *Cladosporium*. Similarly, Magnoli *et al.*[26] also encountered seven fungal genera in grapes collected in Mendoza (Argentina) during the 2001 harvest season, being *Alternaria alternata* the predominant species, followed by *Aspergillus* spp. Furthermore, *Penicillium*, *Cladosporium* and *Botrytis* species were prevalent in grapes from Portugal.[27] Also, *A. alternata*, *Cladosporium* spp., *Penicillium* spp. and *Epiccoccum* spp. were frequently isolated in grapes from Uruguay (Bettucci, personal communication).

The control of the presence of ochratoxigenic fungi in vineyards is the best way to reduce OTA contamination.[11] It was demonstrated that the main fungi involved in ochratoxin A contamination

of grapes and derived products are black aspergilli and that these fungi live as saprophytes in the superficial layer of the vineyard soil, constituting the major inoculum reservoir. However, there are only a few studies of *Aspergillus* section *Nigri* in vineyard soil.[13,28] Most of the studies were done to evaluate the distribution of aspergilli in grapes.

In the present study, we found that *Aspergillus* section *Nigri* species showed a similar pattern of distribution in both years evaluated, *A. niger* aggregate being the dominant group, followed by uniseriate *Aspergillus* species and as a minor percentage *A. carbonarius*. The Shannon–Wiener index varied from region to region and between the different growing seasons. A higher index is an indication that either there is relatively high species evenness or that there are a relatively high number of unique species. Thus La Rioja had the highest index in the second growing season, because *A. carbonarius* was isolated only from this region and Mendoza Uco Valley the lowest index due to the prevalence of *A. niger* aggregate over uniseriate *Aspergillus* species (higher than 95%).

Our results are similar to those obtained in France by Bejaoui *et al.*, who found that *A. niger* aggregate species were the most frequently isolated (54% in average) from grapes, followed by *A. carbonarius* (36% on average).[29] Previous to the present study, in our laboratory we evaluated the incidence of *Aspergillus* section *Nigri* species in grapes harvested in the same wine-producing regions of Argentina, but during the 2006/2007 growing season. We found that the species belonging to *A. niger* aggregate were the most frequently isolated (81%), followed by *A. carbonarius* (11%) and uniseriate *Aspergillus* species (8%).[24] Similarly, other authors reported that *A. niger* aggregate species were the most frequently isolated, followed by *A. carbonarius* and finally uniseriate *Aspergillus* in Argentinean, Tunisian and Australian grapes.[26,28,30]

In the present study *A carbonarius*, with identity confirmed by molecular methods, was isolated in low frequencies and only in the regions of La Rioja and San Juan, showing that the prevalence of *A. carbonarius* in soil is very low. *Aspergillus carbonarius* was recognized as the dominant contributor to OTA contamination of grape in Mediterranean countries and Australia. However, some authors considered that *A. niger* aggregate may slightly contribute to OTA contamination of grape as this group is the most common species of *Aspergillus* present in vineyards.[11] In general, the percentage of OTA-producing strains belonging to *A. niger* aggregate is quite variable, ranging from 0.6% to 50%.[31,32] This variability could be due to incorrect delimitation of species, since *A. niger* and *A. tubingensis* can only be differentiated by molecular methods, as well as to the different number of strains tested and *in vitro* OTA production conditions (synthetic medium, incubation time and temperature). In the present study *A. niger* aggregate isolates were not able to produce OTA; the molecular characterization of these isolates showed that 89% of the total were confirmed as *A. tubingensis* and only 11% as *A. niger*.

Although some authors have confirmed the ability of *A. tubingensis* to produce OTA,[33,34] recently Storari *et al.*[21] concluded that *A. tubingensis* cannot be counted among the ochratoxigenic species. According to the authors, these false-positives were due to the misinterpretation of the chromatograms obtained by HPLC-FLD. For this reason, in this study the *A. tubingensis* isolated were checked in their ability to produce OTA using two HPLC mobile phases. The results showed that all isolates were unable to produce the toxin.

Three closely related species are recognized within the uniseriate group: *A. japonicus*, *A. aculeatus* and *A. uvarum*.[35] We confirmed by molecular method the identity of 35 out of 36 isolates. The strain not identified using specific primers could be *A. uvarum* or other species not yet identified. Morphologically, *A. uvarum* is closely related to *A. japonicus*. The ability of the uniseriate *A. aculeatus* and *A. japonicus* to produce OTA has been reported, but it is possible the isolates were misidentified, because several subsequent studies demonstrated that uniseriate species are not OTA producers.[17,24,32]

Our results showed that *A. carbonarius* (100% of strains) were able to produce OTA, with an average level of 205.5 μg kg^{-1} culture medium (YES). This species is the largest producer of this mycotoxin in grapes in France, Spain, Portugal and Italy.[29,36–38] Previous studies have demonstrated its invasive nature to colonize and penetrate the clusters during veraison.[39]

In the present study, we found a strong association between presence of *A. carbonarius*, high average temperatures and drip irrigation. Most *A. carbonarius* strains have an optimum *in vitro* growth temperature of 30–35 °C and no development below 15 °C. The presence of *A. carbonarius* in the Northwest Region (La Rioja) could be attributed to warm to hot weather. As a counterpart, the absence of this species at higher latitudes would be influenced by the cool climate of these regions (average annual temperature of 14.5 °C) like in the southern regions (Patagonia).

There are no available data of relationship between drip irrigation and *A. carbonarius* presence.

The effects of a_w on *A. carbonarius* spore germination in culture media are well known,[40] but the role of temperature and a_W in the survival of *A. carbonarius* in soil is complex. Kazi *et al.*[13] reported a maximum survival level of *A. carbonarius* in soil at 25 °C, and increased soil moisture content decreased the survival of *A. carbonarius*. Besides, when the vineyards were irrigated the counts of *A. carbonarius* collapsed, but returned to previous levels when the soil dried.

In the present study no relation between grape variety and vineyard age with *A. carbonarius* presence was found (data not shown). In contrast to our results, Battilani *et al.*[41] found a correlation between *A. carbonarius* colonization level and vineyard age, in which the older the vineyard, the more probable it would be colonized by *A. carbonarius.* As was mentioned, all previous studies were done in grape and generally the researchers turn to field plots or small-scale studies to evaluate the influence of specific variables on the overall crop.

A limited survey, carried out in the same locations evaluated in the present study, showed a low incidence (8.5%) of OTA contamination in wine samples. The OTA levels detected ranged from 0.02 to 4.82 μg L^{-1} (mean 0.37 μg L^{-1}) and only two out of 47 samples showed levels higher than 2 μg L^{-1}.[42] The OTA contamination levels are similar to those obtained in Chile, where the climatic conditions are very similar to Argentina. The results obtained in Chilean wine samples showed that only 2.9% from 1188 samples were contaminated with OTA, with the highest concentration of 0.35 ± 0.09 μg L^{-1} in one sample.[43]

Results of the current study demonstrated a low prevalence of ochratoxigenic species in vineyard soil from the main grape-growing regions of Argentina; these results and the low contamination level in wine samples obtained from this area suggest that OTA contamination does not represent a serious threat to Argentinean wine production. Besides, this is the first study where the main ecological factors and cultural management were considered together, evaluating their influence in the distribution of *Aspergillus* section *Nigri* in vineyard soil in Argentina.

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REFERENCES

1 Palencia ER, Hinton DM and Bacon CW, The black *Aspergillus* species of maize and peanuts and their potential for mycotoxins production. *Toxins* **2**:399–416 (2010).
2 International Agency for Research on Cancer (IARC), *Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occurring Substances; Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins*, Vol. 56. IARC, Lyon, France, pp. 489–521 (1993).
3 Gumus T, Arici M and Demirci M, A survey of barley, malt and beer contamination with ochratoxin A in Turkey. *J Inst Brewing* **110**:146–149 (2004).
4 Clouvel P, Bonvarlet L, Martinez A, Lagouarde P, Dieng I and Martin P, Wine contamination by ochratoxin A in relation to vine environment. *Int J Food Microbiol* **123**:74–80 (2008).
5 Batista LR, Chalfoun SM, Silva CF, Cirillo M, Varga EA and Schwan RF, Ochratoxin A in coffee beans (*Coffea arabica* L.) processed by dry and wet methods. *Food Control* **20**:784–790 (2009).
6 Magalhães J, Sodré G, Viscogliosi H and Grenier-Loustalot F, Occurrence of ochratoxin A in Brazilian cocoa beans. *Food Control* **22**:744–748 (2011).
7 Zimmerli B and Dick R, Ochratoxin A in table wine and grape juice: occurrence and risk assessment. *Food Addit Contam* **13**:655–668 (1996).
8 European Commission, Reports on tasks for scientific cooperation. Reports of experts participating in Task 3.2.7. Assessment of dietary intake of ochratoxin A by the population of EU Member States, SCOOP Task 3.2.7 (2002).
9 European Commission, Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Offic J EU* **364**:5–24 (2006).
10 Visconti A, Perrone G, Cozzi G and Solfrizzo M, Managing ochratoxin A risk in the grape-wine food chain. *Food Addit Contam* **25**:193–202 (2008).
11 Somma S, Perrone G and Logrieco AF, Diversity of black Aspergilli and mycotoxin risks in grape, wine and dried vine fruits. *Phytopathol Mediterr* **51**:131–147 (2012).
12 Leong SL, Hocking AD and Scott ES, *Aspergillus* species producing ochratoxin A: isolation from vineyard soils and infection of Semillon bunches in Australia. *J Appl Microbiol* **102**:124–133 (2007).
13 Kazi BA, Emmett RW, Nancarrow N and Clarke K, Effects of temperature, moisture and/or irrigation on the survival *of Aspergillus carbonarius* in soil, in *Proceedings of 8th International Congress of Plant Pathology*, Vol. 2, p. 140, Christchurch, New Zealand. (2003).
14 Covarelli L, Beccari G, Marini A and Tosi L, A review on the occurrence and control of ochratoxigenic fungal species and ochratoxin A in dehydrated grapes, non fortified dessert wines and dried vine fruit in the Mediterranean area. *Food Control* **26**:347–356 (2012).
15 Pitt JI and Hocking AD, *Fungi and Food Spoilage*. Blackie, London, 1997.
16 Samson RA, Hockstra ES, Frisvad JC and Filtenborg O, *Introduction to Food and Airborne Fungi*. Wageningen Press, Wageningen, Netherlands (2002).
17 Bellí N, Mitchell D, Marín S, Alegre I, Ramos AJ, Magan N *et al.*, Ochratoxin A-producing fungi in Spanish wine grapes and their relationship with meteorological conditions. *Eur J Plant Pathol* **113**:233–239 (2005).
18 Leslie J and Summerell B, *The Fusarium Laboratory Manual*. Blackwell, Ames IA (2006).
19 Perrone G, Susca A, Stea G and Mule G, PCR assay for identification of *Aspergillus carbonarius* and *Aspergillus japonicus*. *Eur J Plant Pathol* **110**:641–649 (2004).
20 Susca A, Stea G, Mule G and Perrone G, Polymerase chain reaction (PCR) identification of *Aspergillus niger* and *Aspergillus carbonarius* based on the calmodulin gene. *Food Addit Contam* **24**:1154–1160 (2007).
21 Storari M, Bigler L, Gessler C and Broggini GAL, Assessment of the ochratoxin A production ability of *Aspergillus tubingensis*. *Food Addit Contam* **29**:1450–1454 (2012).
22 Krebs C, *Ecological Methodology*. Harper & Row, New York, pp. 361–367 (1989).
23 Greenacre M and Hastie T. The geometric interpretation of correspondence analysis. *J Am Stat Assoc* **82**:437–447 (1987).
24 Chiotta ML, Ponsone ML, Combina M, Torres AM and Chulze SN, *Aspergillus* section *Nigri* species isolated from different wine grape growing regions in Argentina. *Int J Food Microbiol* **136**:137–141 (2009).
25 Gómez C, Bragulat MR, Abarca ML, Mínguez S and Cabañes FJ, Ochratoxin A-producing fungi from grapes intended for liqueur wine production. *Food Microbiol* **23**:541–545 (2006).
26 Magnoli C, Violante M, Combina M, Palacio G and Dalcero A, Mycoflora and ochratoxin-A producing strains of *Aspergillus* Section *Nigri* in wine grapes in Argentina. *Lett Appl Microbiol* **37**:179–184 (2003).
27 Abrunhosa L, Paterson RRM, Kozakiewicz Z, Lima N and Venancio A, Mycotoxin production from fungi isolated from grapes. *Lett Appl Microbiol* **32**:240–242 (2001).
28 Leong SL, Hocking AD, Pitt JI, Kazi BA, Emmett RW and Scott ES, Black *Aspergillus* species in Australian vineyards: from soil to ochratoxin A in wine, in *Advances in Food Mycology*, ed. by Hocking AD, Pitt JI, Samson RA and Thrane U. Springer, *New York*, pp. 153–171 (2006).
29 Bejaoui H, Mathieu F, Taillandier P and Lebrihi A, Black aspergilli and ochratoxin A production in French vineyards. *Int J Food Microbiol* S46–S52 (2006).
30 Lasram S, Belli N, Chebil S, Nahla Z, Ahmed M, Sanchis V and Ghorbel A, Occurrence of ochratoxigenic fungi and ochratoxin A in grapes from Tunisian vineyard. *Int J Food Microbiol* **114**:376–379 (2007).
31 Abarca ML, Accensi F, Cano J and Cabañes FJ, Taxonomy and significance of black aspergilli. *A Van Leeuw J Microb* **86**:33–49 (2004).
32 Perrone G, Susca A, Cozzi G, Ehrlich K, Varga J, Frisvad JC *et al.*, Biodiversity of *Aspergillus* species in some important agricultural products. *Stud Mycol* **59**:53–66 (2007).
33 Medina A, Mateo R, Valle-Algarra F, Mateo E and Jiménez M, An overview of ochratoxin A in beer and wine. *Int J Food Microbiol* **119**:79–83 (2007).
34 Perrone G, Mule G, Susca A, Battilani P, Pietri A and Logrieco A, Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. *Appl Environ Microbiol* **72**:680–685 (2006).
35 Perrone G, Varga J, Susca A, Frisvad JC, Stea G, Kocsubé S *et al.*, *Aspergillus uvarum* sp. nov., an uniseriate black *Aspergillus* species isolated from grapes in Europe. *Int J Syst Evol Microbiol* **8**:1032–1039 (2008).
36 Cabañes FJ, Accensi F, Bragulat MR, Abarca ML, Castella G, Minguez S *et al.*, What is the source of ochratoxin A in wine? *Int J Food Microbiol* **79**:213–215 (2002).
37 Serra R, Abrunhosa L, Kozakiewicz Z and Venancio A, Black *Aspergillus* species as ochratoxin A producers in Portuguese wine grapes. *Int J Food Microbiol* **88**:63–68 (2003).
38 Battilani P, Giorni P, Bertuzzi T, Formenti S and Pietri A, Black aspergilli and ochratoxin A in grapes in Italy. *Int J Food Microbiol* **111** (Suppl 1): 53–60 (2006).
39 Leong S, Hocking AD and Pitt JI, Occurrence of fruit rot fungi (*Aspergillus* Section *Nigri*) on some drying varieties of irrigated grapes. *Aust J Grape Wine Res* **10**:83–88 (2004).
40 Leong SL, Hocking AD and Scott ES, Effects of water activity and temperature on the survival of *Aspergillus carbonarius* spores in vitro. *Lett Appl Microbiol* **42**:326–330 (2006).
41 Battilani P, Barbano C, Marin S, Sanchis V, Kozakiewicz Z and Magan N, Mapping of *Aspergillus* section *Nigri* in Southern Europe and Israel based on geostatistical analysis. *Int J Food Microbiol* **111**:72–82 (2006)
42 Ponsone ML, Chiotta ML, Combina M, Torres AM, Knass P, Dalcero A and Chulze S, Natural occurrence of ochratoxin A in musts, wines and grape vine fruits from grapes harvested in Argentina. *Toxins* **2**(8):1984–1996 (2010).
43 Vega M, Ríos G, von Baer D, Mardones C, Tessini C, Herlitz E *et al.*, Ochratoxin A occurrence in wines produced in Chile. *Food Control* **20**(1):147–150 (2012).

