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ORIGINAL ARTICLE

Influence of *Planococcus ficus* on *Aspergillus* section *Nigri* and ochratoxin A incidence in vineyards from Argentina

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Keywords

Argentinean vineyards, *Aspergillus* section *Nigri,* grapes, ochratoxin A, *Planococcus ficus*.

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Abstract

Aim: The aim of this work was to evaluate the effect of *Planococcus ficus* infection in red wine grapes on *Aspergillus* section *Nigri* and ochratoxin A (OTA) contamination.

Methods and Results: During 2006/2007 and 2008/2009 vintages, Merlot, Malbec and Cabernet Sauvignon varieties divided into two categories of grape samples (undamaged and damaged by *P. ficus*) were evaluated. Regardless of the grape variety and the harvest season evaluated, *Aspergillus* section *Nigri* incidence and the mean OTA concentration in damaged berries were significantly higher than that in the undamaged ones ($P < 0{\cdot}05$; $P < 0{\cdot}001$). The Merlot variety showed the highest level of black aspergilli contamination in damaged grapes during the 2006/2007 vintage (53·5% of infection), whereas Malbec presented the highest incidence during the 2008/2009 vintage (57·6% of infection). The Cabernet Sauvignon variety showed the highest OTA levels, ranging from 0·1 to 140 μg kg^{-1}.

Conclusions: The presence of *P. ficus* in vineyards increased the risk of OTA occurrence in grapes, suggesting the need to implement insect control at preharvest stage to reduce the entry of OTA in the wine production chain.

Significance and Impact of the Study: This study is the first report on the influence of *P. ficus* on the potential risk of OTA contamination in grapes.

Introduction

Aspergillus bunch rot is a preharvest mouldy disease caused by species belonging to *Aspergillus* section *Nigri* (Emmett *et al.* 1992). Some species of black aspergilli are able to produce ochratoxin A (OTA) on several substrates, mainly *Aspergillus carbonarius* and *Aspergillus niger* aggregate, that are known to produce this toxin in grapes and raisins (Varga and Kozakiewicz 2005; Battilani *et al.* 2006a; Leong *et al.* 2006). Berry damage may occur because of birds, insects or infection by fungal species, being these the primary factors affecting the *Aspergillus* bunch rots and the subsequent production of OTA in grapes.

Ochratoxin A has been demonstrated to be nephrotoxic and has been associated with a fatal human kidney disease, referred to as Balkan Endemic Nephropathy (BEN) and with an increase in tumour incidence of the upper urinary tract (Marquardt and Frohlich 1992). It has also showed carcinogenic, immunotoxic, genotoxic, teratogenic and possibly neurotoxic properties. The presence of OTA has been reported in a wide range of foods and beverages, in body fluids and in kidneys of animals and humans (EFSA 2006). Natural occurrence of OTA has been observed in grapes and derived products such as dried vine fruit, grape juices and wine (Zimmerli and Dick 1996; Serra *et al.* 2003; Magan and Olsen 2004). Specific regulations for OTA have established 2 μg kg^{-1} as the maximum level of OTA allowed for wines, musts and grapes in the European Union (European Commission 2006).

High infestation levels by *Planococcus ficus* Signoret (Order *Hemiptera*, Family *Pseudococcidae*) have been

found in most grape-production areas throughout the world. It is of particular economic importance on grapevines in the Mediterranean region, South Africa, Pakistan and Argentina (Ben-Dov 1994; Ben-Dov and Matile-Ferrero 1995). Recently, high infestation levels have been reported in the vineyards of Mendoza province, one of the most important wine production regions from Argentina. This zone accounts for 70% of the vineyards and 158 833 ha of the total area, which is cultivated with varieties suitable for making high-quality wines (Fanzone *et al.* 2010). Grapes are susceptible to insect attack and fungal diseases that can reduce the quality of the harvested grapes and affect the organoleptic features of wines. During the life cycle of *P. ficus,* six generations are completed in 1 year. In winter, the insect remains primarily as cottony egg masses grouped under the bark of the plants. As soon as temperature rises, it moves out onto aerial portions of the plants, setting on buds and leaves. As from the third generation (veraison season), the insect remains all over the plant through the rest of the season. In the fall, the downward migration of the vine mealybug takes place once again (Becerra *et al.* 2006). On bunches, *P. ficus* dehydrates grapes by perforating the grape skin, produces sugary substances that allow spoilage fungi development and, consequently, decreases the wine quality. Previous studies showed the presence of potential OTA producer species in wine grapes from Argentina (Magnoli *et al.* 2003; Chulze *et al.* 2006; Ponsone *et al.* 2007; Chiotta *et al.* 2009). The presence of the insect in grapevines could be responsible for the increase in *Aspergillus* section *Nigri* species and OTA occurrence in damaged berries. Therefore, the aim of this study was to evaluate the interaction between berries damaged by *P. ficus*, level of infection with *Aspergillus* section *Nigri* and OTA occurrence in wine grapes.

Materials and methods

Grape sampling

Vineyards from a grape-growing region located in Mendoza, Argentina, were sampled during 2006/2007 and 2008/2009 vintages. All the vineyards were settled at 31° 1′ South Latitude 68° 52′ West Longitude. The sampling was conducted during the ripening stage (late February–March) in vineyards that had a history of *P. ficus* infestation. Malbec, Merlot and Cabernet Sauvignon grape varieties were analysed. Areas of infection by *P. ficus* were observed in all the vineyards. Bunches of grapes were collected from each plant with visual infection symptoms and categorized as damaged berries. The samples collected from neighbouring healthy plants were categorized as undamaged ones. Three bunches of plants were considered as a sample, and twenty samples were taken from each vineyard, ten of each category: damaged and undamaged berries.

Fungal isolation and identification

To determine the incidence of fungal genera 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, all fungal colonies were identified according to Samson *et al.* (2000), and the percentage of grapes infected by each genus was determined. *Aspergillus* section *Nigri* colonies were picked and subcultured on malt extract agar at 28°C. Identification at species level was performed according to the methodology described by Klich (2002). The species were classified into three main proposed groups: *A. niger* aggregate, *A. carbonarius* and *Aspergillus uniseriate*.

Ochratoxin A occurrence

From each vineyard, 20 samples (10 from damaged grapes and 10 from undamaged grapes) were collected by variety. For toxin analysis, three bunches/plant were considered 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 was 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 2290 ***g*** 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 mol l^{-1}). A ten-millilitre 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 resuspended in mobile phase and submitted to HPLC analysis.

The HPLC system 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 precolumn (20 × 4·6 mm i.d., 5-μm particle size; Security Guard, Phenomenex). The mobile phase was pumped at 1·0 ml min^{-1} and consisted of an isocratic system composed by acetonitrile/water/acetic acid (99 : 99 : 2, by vol.). OTA was quantified based on HPLC fluorometric response compared with the OTA standard. The limit of detection was 0·01 μg kg^{-1}.

Statistical analysis

To determine whether there were significant differences between undamaged and damaged grapes by *P. ficus* in relation to *Aspergillus* section *Nigri* species infection and OTA occurrence, data were analysed by ANOVA test, followed by Tukey mean separation test ($P < 0{\cdot}001$; $P < 0{\cdot}05$).

Results

Mycoflora in wine grape samples

Over the two vintages of study, different levels of fungal contamination were observed that were related to the vintage year, grape variety and category (damaged and undamaged grape). During the 2006/2007 vintage, *Alternaria* species showed the highest incidence in all varieties independent of grape damage. *Alternaria* species infection ranged from 45·8 to 91·6% (mean 69·8%) in damaged grapes and ranged from 21·4 to 79·6% (mean 53·7%) in undamaged grapes. *Aspergillus, Penicillium* species and yeasts were the other most frequent mycoflora isolated, and their infection levels were higher in damaged grapes than that in undamaged ones. On the other hand, *Cladosporium*, *Fusarium* and *Trichoderma* species were isolated at low percentages, <10%. During the 2008/2009 vintage, the grape samples showed high percentages of yeast contamination, reaching 99% in the Cabernet Sauvignon variety. The grape infection with *Alternaria* and *Aspergillus* species was relevant only in damaged grapes from the Malbec variety, with a percentage of 57 and 57·6%, respectively. Statistical significant differences in the fungal infection levels between undamaged and damaged berries were observed, and the higher levels were detected in damaged berries ($P < 0{\cdot}05$; $P < 0{\cdot}001$) (Figs 1 and 2).

Aspergillus section *Nigri* incidence

Ninety-nine per cent of *Aspergillus* species isolated from all grape samples analysed were identified as *Aspergillus* section *Nigri.* The frequency of grape samples contaminated ranged from 70 to 100% during the 2006/2007 vintage, whereas during the 2008/2009 vintage only from

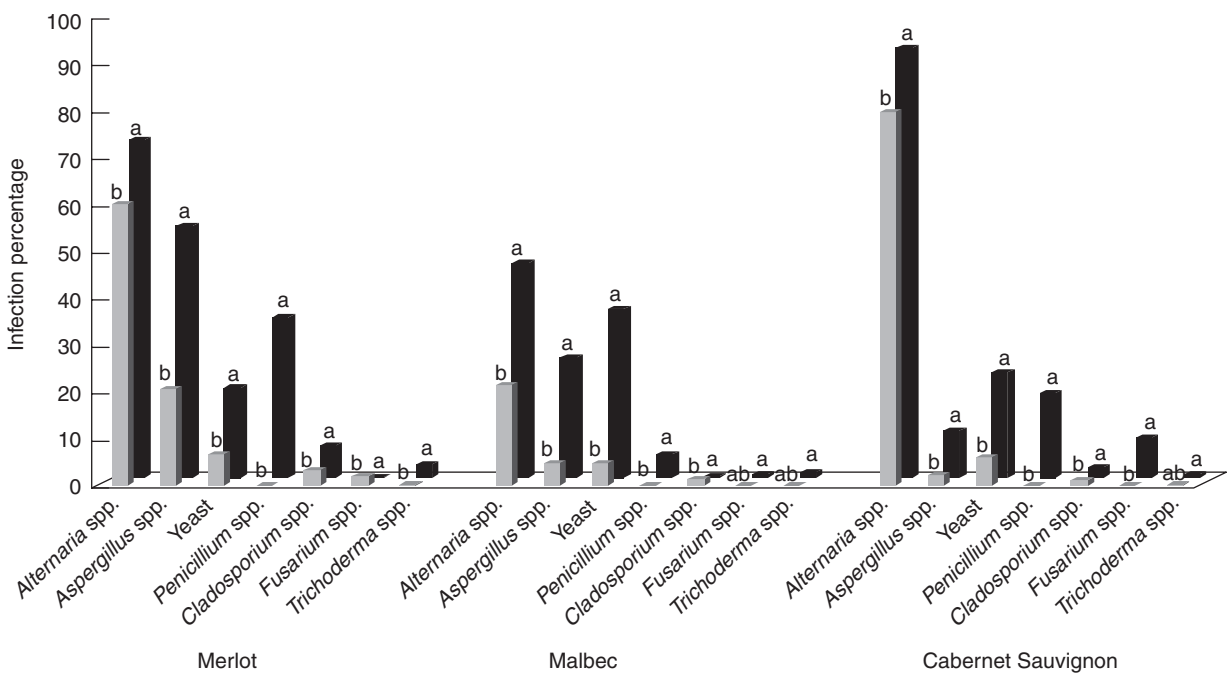

Figure 1 Mycoflora isolated from undamaged and damaged grapes by *Planococcus ficus* during 2006/2007 vintage. Different letters over bars indicate significant differences in the infection percentage of the mycoflora between damaged and undamaged grapes ($P < 0.05$; $P < 0.001$). (▩) Damaged and (■) Undamaged.

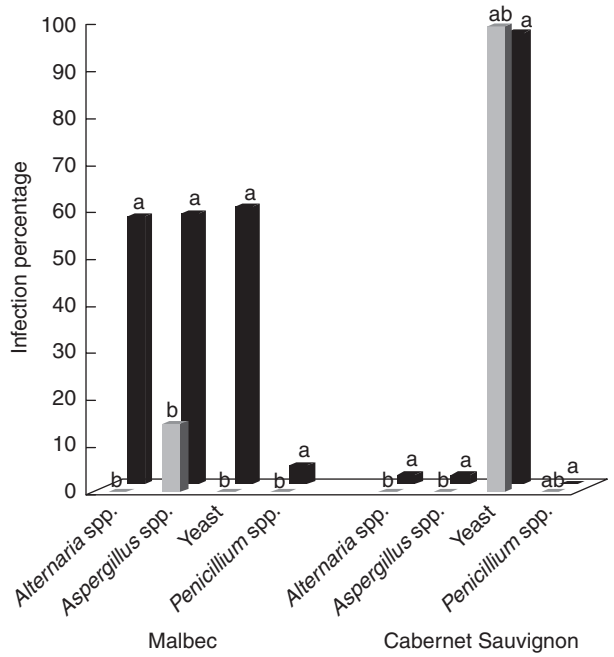

Figure 2 Mycoflora isolated from undamaged and damaged grapes by *Planococcus ficus* during 2008/2009 vintage. Different letters over bars indicate significant differences in the infection percentage of the mycoflora between damaged and undamaged grapes ($P < 0.05$; $P < 0.001$). (■) Damaged and (▒) Undamaged.

0 to 50% of the samples were infected (Table 1). Regardless of the grape variety and the vintage evaluated, damaged berries showed the highest levels of contamination with *Aspergillus* section *Nigri* ($P < 0{\cdot}05$). The stains isolated were identified as species belonging to *A. niger* aggregate (98%). *Aspergillus uniseriate* species were occasionally isolated (2%), and no *A. carbonarius* was found in any of the vintages or varieties sampled.

The Merlot variety showed the highest levels of *Aspergillus* section *Nigri* contamination, 53·5% and 20% in damaged and undamaged berries, respectively during the 2006/2007 vintage. In contrast, the Malbec variety showed the highest incidence, 57·6 and 14% in damaged and undamaged berries, respectively, during 2008/2009 vintage (Fig. 3).

Ochratoxin A occurrence in wine grapes

Of 100 samples evaluated, 27% showed ochratoxin A contamination in levels ranging from 0·1 to 140·2 μg kg^{-1} (Table 1). The mean ochratoxin A concentration was significantly higher in damaged berry samples than that in undamaged ones ($P < 0{\cdot}001$). Merlot and Cabernet Sauvignon varieties showed OTA levels ranging from 0·1 to 140·2 μg kg^{-1}, in the 2006/2007 vintage. However, lower OTA levels were detected in the 2008/2009 vintage, ranging from 0·1 to 11·8 μg kg^{-1}. The Cabernet Sauvignon variety showed the highest levels of toxin detected in damaged grapes during both vintages evaluated (mean levels: 51·2 and 8·6 μg kg^{-1} in 2006/2007 and 2008/2009, respectively).

Discussion

This study provides new data on the infection of *P. ficus* on wine grapes, and the subsequent effect on *Aspergillus* section *Nigri* inoculum distribution and OTA occurrence.

Table 1 *Aspergillus* section *Nigri* and ochratoxin A levels in undamaged and damaged grapes by *Planococcus ficus*

			Aspergillus section *Nigri* incidence*		Ochratoxin A ocurrence		
Vintage	Grape varieties	Grape categories	Range (%)†	Frequency (%)‡	Samples positive/total	Range (μg kg^{-1})	Mean (μg kg^{-1})
2006/2007	Merlot	Undamaged	6–70	100	5/10	0·5–15·6	7·2a
		Damaged	2–78	100	7/10	1·3–50·2	15·3b
	Malbec	Undamaged	2–14	80	0/10	ND	–
		Damaged	2–88	90	0/10	ND	–
	Cabernet sauvignon	Undamaged	4–70	90	1/10	0·1	0·1a
		Damaged	2–80	70	9/10	2·7–140·2	51·2b
2008/2009	Malbec	Undamaged	0–25	50	0/10	ND	–
		Damaged	4–70	20	3/10	0·1–1·0	0·6
	Cabernet sauvignon	Undamaged	ND	0	1/10	5·4	5·4a
		Damaged	0–20	10	7/10	0·1–11·8	8·6b

Detection limit: 0·01 μg kg^{-1}. Significant differences: $P < 0{\cdot}001$.
ND, not detected.
*The main species isolated from damaged and undamaged grapes belonged to *Aspergillus niger* aggregate.
†*Aspergillus* section *Nigri* infection ranges obtained among the ten samples analysed by category.
‡Isolation frequency: number of infected samples of total samples analysed by vineyard.

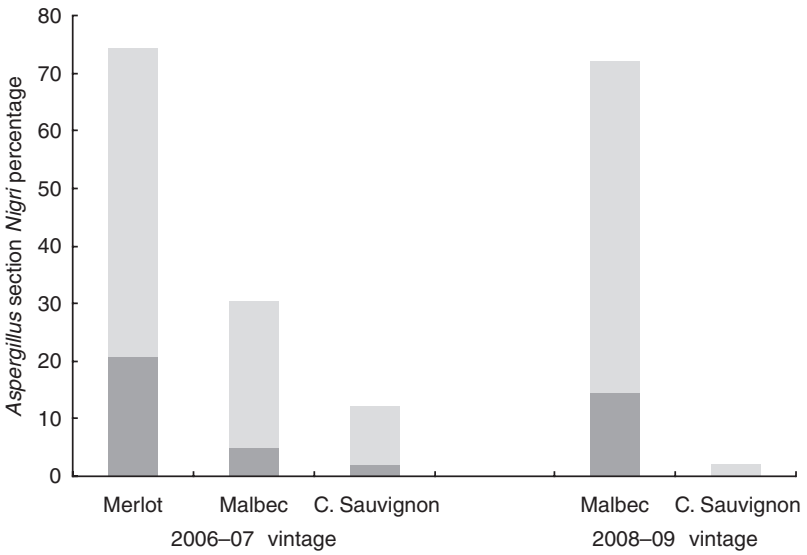

Figure 3 Percentage of berries colonized by *Aspergillus* section *Nigri* strains in damaged and undamaged grapes. (▒) Damaged grapes and (■) Undamaged grapes.

Alternaria species were detected in high percentage in the three grape varieties evaluated during the 2006/2007 vintage and were relevant only in the Malbec variety during the 2008/2009 vintage. The presence of *Alternaria* species in grape needs to be considered because these species also have been described as potential mycotoxins alternariol (AOH) and alternariol monomethyl ether producers in red wines (Scott *et al.* 2006). *Aspergillus* was the other relevant fungal genus isolated in both vintages, the infection frequency varied with the grape variety and insect damage. *Penicillium, Cladosporium, Fusarium*, and *Trichoderma* species were occasionally isolated from the grapes. These results agree with a previous study on the wine grapes mycoflora from Argentina, where the authors found that *Alternaria* and *Aspergillus* were the most frequent genera in the same wine grape-growing region (Magnoli *et al.* 2003).

During the 2008/2009 vintage, a high percentage of yeasts were isolated from the damaged and undamaged grape samples in the two grape varieties evaluated. These findings could explain the lower incidence of filamentous species during this vintage. It is important to highlight the presence of yeasts, as studies on mechanisms of antagonism for space and nutrients have demonstrated that yeasts can be used as potential biocontrol agents against ochratoxigenic *Aspergillus* on grapes. Bleve *et al.* (2006) showed that *Cryptococcus laurentii* and *Aureobasidium pullulans* were the most promising species. Preliminary results obtained in our laboratory have also shown the ability of *Kluyveromyces thermotolerants* strains to inhibit *Aspergillus* section *Nigri* growth and OTA accumulation (Ponsone *et al.* 2008).

Grapes infested by *P. ficus* showed the highest *Aspergillus* section *Nigri* incidence, mainly species belonging to *A. niger* aggregate. This insect could contribute to grape damage while it is actively feeding, affecting the integrity of the berries and favouring the colonization of ochratoxigenic species. Besides, *P. ficus* secretes sugary substances covering leaves and fruits, being important for the survival of several species of ants. The sugary substances could also be a good substrate for fungal growth. In our study, ants were collected from infected plants and plated onto the surface of the DRBC medium. Black aspergilli growth was observed after 7 days of incubation and showed that ants can also disseminate black *Aspergillus* on grapes (data not shown).

Ochratoxin A accumulation was dependent of the grape category, grape variety and the vintage year. The highest OTA contamination levels were detected in grapes damaged by *P. ficus* being relevant during 2006/2007 vintage. Different OTA levels observed between vintages could be attributed to the variations in climatic conditions since during the 2006/2007 vintage higher humidity levels in comparison with the 2008/2009 vintage were observed (51 and 39% of relative humidity, respectively) (INTA 2010). These data agree with previous findings, where we showed a positive correlation between OTA levels and rain during harvest time (Chiotta *et al.* 2009). Similar results were obtained by Visconti *et al.* (2008) in Italy, who found the highest OTA levels in more humid regions. In addition, other studies have demonstrated that meteorological conditions contributed to the variation in *Aspergillus* section *Nigri* incidence and OTA occurrence on grapes (Sage *et al.* 2004; Battilani *et al.* 2006a,b; Bellí *et al.* 2006; Serra *et al.* 2006). With regard to the variety, although Cabernet Sauvignon showed low *Aspergillus* section *Nigri* incidence, high OTA levels were found in damaged berries during both vintages. These

findings can be explained by the different susceptibility of some varieties to berry splitting, and the consequent greater risk for black aspergilli infection and subsequent OTA contamination. Studies carried out in Europe also showed that the grape variety affected the incidence of *Aspergillus* section *Nigri* and OTA levels, being the Cabernet Sauvignon variety the most susceptible (Battilani *et al.* 2004).

The effect of insect pest damage on ochratoxigenic *Aspergillus* infection and OTA content in grape berries was observed by *Lobesia botrana*. This insect is the principal grape berry moth in the vineyards of Southern Europe, and its larvae can either contribute to spore dispersal or act as spore vectors, by trapping conidia in the cuticle ornamentation, then facilitating a rapid fungal penetration by tunnelling into berries, as demonstrated for *Botrytis cinerea* (Fermaud and Le Menn 1989, 1992; Cozzi *et al.* 2006). The results obtained in this study showed the important role of the insect in grape skin damage in wine grapes in relation to fungal infection. *P. ficus* can be considered as a source of grape skin damage because *Aspergillus* section *Nigri* and ochratoxin A contamination increased in vineyards infested by this insect. Therefore, the implementation of preventive measures to minimize berry damage in the field, such as controlling pathogenic fungi and insects during grape growing and the remotion of visibly damaged grapes at harvest, could significantly reduce OTA contamination in grapes.

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