Effect of *Aspergillus carbonarius* amounts on winemaking and ochratoxin A contamination

Chunmei Jiang a, Junling Shi a,b,c,*, Yongle Cheng a, Yanlin Liu d

aCollege of Food Science and Engineering, Northwest A&F University, 28 Xinong Road, Yangling, Shaanxi Province 712100, China

bSchool of Life Sciences, Northwestern Polytechnical University, 127 Youyi West Road, Xi’an, Shaanxi Province 710072, China
cKey Laboratory for Space Bioscience and Biotechnology, Northwestern Polytechnical University, 127 Youyi West Road, Xi’an, Shaanxi Province 710072, China
dCollege of Enology, Northwest A&F University, 23 Xinong Road, Yangling, Shaanxi Province 712100, China

**Abstract**

*Aspergillus carbonarius* is the major ochratoxin A (OTA)-producing fungus that contaminates wine grapes. To investigate the effect of the initial amount of *A. carbonarius* on winemaking and ochratoxin A contamination, different conidial concentrations of *A. carbonarius* were manually added to the grape musts before fermentation. Sampling was carried out at different stages in alcoholic fermentation, including crushing, maceration, pressing and alcoholic fermentation. The levels of alcohols, soluble solids, and reducing sugars in the musts were analyzed before and during the whole procedure of alcoholic fermentation. *Aspergillus* spp. and other fungal contaminants increased rapidly after crushing, however most died at 48 h. OTA levels in the musts increased during the first 8–48 h and then decreased sharply in *A. carbonarius* inoculants (1 × 10⁴ to 1 × 10⁶ spores/g) in a spore concentration-dependent manner. Most OTA was retained in the pomaces fraction after the pressing operation. High amounts of *A. carbonarius* did not significantly affect yeast growth, sugar use, and alcoholic production during fermentation. In conclusion, high levels of contamination with *A. carbonarius* in the grape musts did not inhibit alcoholic fermentation, but caused high OTA residues in the wine produced.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Ochratoxin A (OTA) is an isocoumarin derivative linked through the carboxy-group to an L-b-phenylalanine, and is a mycotoxin with nephrotoxic, carcinogenic, immunotoxic, genotoxic, and teratogenic effects, associated with fatal human kidney disease (Abouzied et al., 2002). OTA is classified as a possible (category 2B) human carcinogen by the International Agency for Research on Cancer (Horvath, Upham, Ganev, & Trosko, 2002). OTA is receiving increasing attention for its toxic effects and high incidence in a wide range of food commodities, including cereal-based products, coffee, spices, nuts, olives, grape-derived products, beans, figs, and cocoa (Battilani, Mangan, & Logrieco, 2006; Perrone et al., 2007). Apart from cereals, grapes and wines are the commodities with the highest incidence of OTA contamination (Bau, Bragulat, Abarca, Mínguez, & Cabañas, 2005; Belli et al., 2004). Contamination of OTA in wines has been widely reported around the world (Belli et al., 2006; Solfrizzo, Panzarini, & Visconti, 2008; Visconti, Pascale, & Centonze, 1999; Zimmerli & Dick, 1995). The European Union limit of OTA in wine is 2.0 µg/L (European Commission, 2006). It is urgent and essential to inhibit OTA accumulation in wine products.

The presence of OTA in musts and wine is due to fungal contamination of the grapes that may develop pre- or post- harvest, or during the phases before wine-making. *Aspergillus* species, particularly *Aspergillus carbonarius*, have been identified as the major fungi responsible for OTA contamination in grapes and wine (Battilani et al., 2006; Fabiani, Corzani, & Arfelli, 2010; Péteri, Téren, Vágóvölgyi, & Varga, 2007). Temperature, moisture, aeration, infection period, and interaction between different fungi are factors influencing mycotoxin development (Scudamore, Patel, & Breeze, 1999).

Red wine has been found to have higher amounts of OTA than white or rosé wines, and processing has been thought to play an important role in diminishing the potential risks of OTA contamination (Grazioli, Fumi, & Silva, 2006). Much research has been focused on the change of OTA concentration in grape musts during fermentation (Esti, Benucci, Liburdi, & Acciaro, 2012; Grazioli et al., 2006; Meca, Blaiotta, & Ritieni, 2010; Ratola, Abade, Simoes, 2011; Ratola, Abade, Simoes, 2012).
Attempts have been made to reduce OTA during winemaking through the use of yeasts and other microorganisms (Meca et al., 2010; Scott, Kanhere, Lawrence, Daley, & Farber, 1995). Fungal contaminants such as *Botrytis cinerea*, *Aspergillus* spp., and *Penicillium* spp., have been reported to produce metabolites with the ability to retard yeast growth during fermentation (Donèche, 1993; Lafon-Lafourcade & Ribereau-Gayon, 1984; Reed & Nagodawithana, 1988). However, information on the development and OTA production of *A. carbonarius* and its influence on winemaking have not been available.

The current study was therefore aimed at investigating the influence of *A. carbonarius* amounts on winemaking process, including yeast growth and ethanol production, and OTA accumulation in wine. The study was hoped to provide useful information on predicting the influence of *A. carbonarius* contamination in grape berries on wine quality and OTA contamination in winemaking process.

### 2. Materials and methods

#### 2.1. Grapes

A wine grape Cabernet Sauvignon cultivar (sugars: 17 °Brix; pH: 3.60; titratable acidity: 9.35 g/L) was collected from local vineyards in Shaanxi Province, China, and used to ferment dry red wine (v/v, 12%).

#### 2.2. *Aspergillus carbonarius*

*A. carbonarius* CCTCC AF 2011004, a strain previously isolated from grape berries and showed high OTA producing ability in vitro was used. It was maintained at the China Center for Type Culture Collection (Wuhan, China). *A. carbonarius* was prepared as a spore suspension by flushing the surface of 7-day-old cultures grown at 25 °C on potato dextrose agar (PDA) with sterile water, then filtering through sixteen layers of sterile medical gauze. The suspensions were finally adjusted to $1 \times 10^7$ spores/mL with sterile water using a hemocytometer.

#### 2.3. Winemaking

For each treatment, winemaking trials were carried out using the technology currently use in wineries, similarly to the methods outlined by Grazioli et al. (2006) and Leong, Varelis, Giannikopoulos, and Scott (2006). The detail information was shown as a flow chart in Fig. 1. Vinification trials started by destemming and crushing the grapes, yielding a must with pomaces (skins and seeds) included. In each treatment, about 7 kg grapes were used and all of the musts with pomaces were put in to a 10-L glass tank. Then, 50 mg/L of SO$_2$ and 40 mg/L of pectinase was added and 200 mg/L of *Saccharomyces cerevisiae* powder (Excellence SP, Lamothe-Abiet, France) was inoculated to this must. After 24 h, about 42 g of sucrose was added to each kilogram of must with pomaces to get enough high sugar content for producing 12% (alcohol content, v/v) dry wine. Each of the above inoculated must samples was placed into a tank and kept at 20–25 °C during maceration and alcoholic fermentation. Ice bags were placed into the tank center if the temperature was higher than 25 °C. After 3–6 days, the liquid phase was separated from the pomaces by pressing. After completion of alcoholic fermentation, a first racking was carried out to remove the lees from the wine.

---

**Fig. 1.** Flow chart of winemaking, showing the treatments and sampling times.
To investigate the effects of *A. carbonarius* before fermentation, the fungus was added to the grape musts together with yeast, pectinase, and SO₂, using different inocula sizes of 1 × 10⁶, 1 × 10⁷, 1 × 10⁸, 1 × 10⁹ spores/g; coded A, B, C, and D, respectively. Another batch of must with pomaces, inoculated with yeast and 1 × 10³ spores of *A. carbonarius* per gram of must, without SO₂ addition, was prepared to study the effects of SO₂, and was coded E. The musts with only SO₂ and yeasts added, without *A. carbonarius* inoculation, were considered as controls and coded CK.

### 2.4. Sampling

For each treatment, sampling was carried out starting with the whole grape (0 h) until to the end of the alcoholic fermentation (216 h) as shown in Fig. 1. Sampling was carried out in the musts at times listed in Fig. 1, in triplicate. All the obtained samples were subjected to analysis of *A. carbonarius* and yeasts amounts, OTA concentration, ethanol content, soluble solids, and reducing sugar content.

### 2.5. Mycological analysis

Mycological analysis was performed as previously described for wine grapes (Abrunhosa, Paterson, Kozakiewicz, Lima, & Venâncio, 2001; Pitt & Hocking, 2009) with some modifications. A 25 mL quantity of must was added to 225 mL of sterile solution of 0.1% (w/v) peptone, and then shaken vigorously in a vortex (Vortex Genie 2; Scientific Industries, Bohemia, NY, USA) for 1 min. The above mixture (0.1 mL) was inoculated onto dichloran rose bengal chloramphenicol agar (DRBC) (Becton–Dickenson, Franklin Lakes, NJ, USA) using the spread plate method (Pitt & Hocking, 2009). To separately analyze the fungal contamination in the liquid phase and pomaces of the musts, the musts obtained after the pressing operation were also separated by four layers of medical gauze before inoculation by spreading. In this operation, about 1.0 g of the pomaces and 0.1 mL of the liquid phase were used. When the number of fungi was too high to count, a serial 10-fold dilution was made using 0.1% peptone solution, and plated onto DRBC medium. The inoculated plates were incubated at 25 °C for 2–3 days and the number of colonies was recorded. After incubation, the fungal and yeast colonies were counted and the results were expressed as cfu/mL sample (Brugalat, Martínez, Castellà, & Cabañes, 2008). *Aspergillus* and *Penicillium* strains were counted separately based on their colony morphology and morphological characteristics as previously reported (Klich, 2002; Pitt, 1979, 1988). Other species of fungi were also identified and counted based on their morphological characters as previously described (Pitt & Hocking, 2009; Von Arx, 1970). Every sample was tested in triplicate, and the mean results were reported with standard deviations.

### 2.6. OTA analysis

The OTA concentration in each sample of the obtained must was analyzed following the method reported by Visconti et al. (1999) with slight modification. In addition, the OTA levels in the liquid phase and pomaces of the musts obtained after the pressing operation were separately analyzed after separation using four layers of medical gauze. The samples for OTA measurements were pretreated using the method reported by Visconti et al. (1999). In brief, the musts and liquid phases of the musts obtained after the pressing operation were directly diluted with a water solution containing polyethylene glycol (PEG, 10 g/L) and NaHCO₃ (50 g/L), mixed, filtered, and cleaned up using Ochraprep immunoaffinity columns (IAC) (R-Biopharm, Darmstadt, Germany). The pomaces samples were homogenized in a blender (Moulinex) for 10 min; with a 1 min break every 3 min. Then, 25 g of each pomaces sample was weighed and mixed with water containing PEG (10 g/L) and NaHCO₃ (50 g/L). The solution was mixed for 30 min in magnetic mixer (IKA, Guangzhou, China), and then centrifuged for 20 min at 8500 rpm and 4 °C. The supernatant was collected and subjected to IAC treatment.

All of the IAC-treated samples were subjected to the following treatments for OTA measurement. Methanol was used as the elution solution to collect the adhered OTA in the columns. The OTA methanol extracts were analyzed for OTA concentration using a reversed phase high performance liquid chromatography (HPLC) (LC-20A, Shimadzu, Kyoto, Japan) with fluorescence detector. The HPLC system included a LC-20AB binary pump equipped with a RF 20A fluorescence detector (330 nm excitation wavelength; 470 nm emission wavelength). Chromatographic separations were performed using a Shimadu Wondasil C18-column (5 μm particles, 4.6 × 250 mm). The mobile phase consisted of an acetonitrile–water–acetic acid mixture (99:99:2 v/v/v) eluted at a flow rate of 1.0 mL/min. The sample injection volume was 20 μL. Quantification of OTA was performed by measuring peak area at OTA retention time (approximately 11 min) and comparing it with the calibration curve. The limit of detection (LOD) and limit of quantification (LOQ) obtained on the basis of a signal-to-noise ratio of 3:1 and 10:1 were 0.02 μg and 0.08 μg OTA/L must, respectively. The results of OTA concentration are reported as μg/L of musts.

### 2.7. Measurements of alcohols, reducing sugars, and soluble solids

Levels of alcohols and reducing sugars were determined following the official methods of the Office international de la Vigne et du Vin (OIV) (OIV, 1990). Soluble solids were detected using a WYTO-90 hand refractometer (Huruiming, China).

### 2.8. Statistical analysis

All statistical analyses were performed using the Statistic Package for Social Sciences (SPSS version 13.0; IBM, Armonk, NY, USA) for Windows.

### 3. Results and discussion

#### 3.1. Fungal contaminants in musts

Fig. 2 shows the changes in quantities of *Aspergillus* spp., *Penicillium* spp., and other fungi at different stages during alcoholic fermentation. It indicates the fate of the contaminated fungi during alcoholic fermentation.

As shown in Fig. 2–CK, the main species of natural fungal contaminants in the musts were *Aspergillus* and *Penicillium*. The total quantities of other fungi, including *Alternaria* sp., *Cladosporium* sp., and *Botrytis* sp. (identification data are not shown), were below 1 × 10⁵ cfu/mL throughout the whole fermentation process; this is also true for all treatments with *A. carbonarius* inoculation (see Fig. 2 A–D). The occurrence of these fungal genera was consistent with their occurrences on grape surfaces (Sage, Kri沃bok, Delbos, Seigle-Murandi, & Creppy, 2002). In addition, the total amount of *Aspergillus* spp. in musts increased after the destemming and crushing treatment (3 h), indicating that large quantities of these fungi were introduced to the winemaking process at this stage. Nevertheless, the amount of *Penicillium* spp. increased sharply after surce addition (24 h), and then decreased rapidly after 48 h.

For the treatments with differing quantities of added *A. carbonarius* (Fig. 2A, B, C, and D), the maximum quantity of *Aspergillus* spp. was lower than the initial inoculated *A. carbonarius*. 4
conidial concentration, indicating that the growth or spore germination of Aspergillus spp. was inhibited during fermentation. In addition, the highest quantity of Aspergillus spp. appeared immediately after inoculation (8 h) and then decreased sharply; except that treatment B (A. carbonarius inoculation of 1 × 10^6 spores/g) yielded the highest value at 24 h. In addition, the highest quantity of Aspergillus spp. at 8 h in the musts increased with the inoculation size of A. carbonarius, implying that the increased development of Aspergillus spp. would be mainly caused by the inoculated A. carbonarius. It is interesting that the development of Penicillium spp. in the musts was greatly inhibited by the addition of A. carbonarius in a concentration-dependant manner. When 1 × 10^6 spores/g of A. carbonarius was added, Penicillium spp. was almost completely inhibited (Fig. 2A).

By comparing Fig. 2D and E, SO2 treatment significantly inhibited the development of all the tested fungal contaminants, although more significantly for Penicillium spp. than for Aspergillus spp. After SO2 treatment, the amount of Penicillium spp. (Fig. 2D) was reduced by nearly four times, whereas, the amount of Aspergillus spp. was reduced by 55%, compared with that of the control without SO2 treatment (Fig. 2E). This is consistent with the results reported by Franck, Latorre, Torres, and Zoffoli (2005).

3.2. Quantities of yeast in musts

As shown in Fig. 3, the total quantities of yeasts increased after inoculation (8 h) and reached maximum value at 48 h for all treatments except that the treatments inoculated with 1 × 10^3 spores/g of A. carbonarius and without SO2; this showed the highest yeast amount at 72 h, and then decreased sharply until 144 h. After 144 h, the amount of yeast increased again, up to its second peak at fermentation (168 h) and the pressing operation (192 h). After the pressing operation, the amount of yeast decreased rapidly until to the lowest level (216 h). No significant difference was found between yeast amounts among treatments with differing A. carbonarius inoculation sizes, indicating that A. carbonarius did not affect the growth of yeasts in grapes and fermenting musts.

The highest quantity of yeasts in the sample without SO2 treatment (Fig. 2E). This is consistent with the results reported by Franck, Latorre, Torres, and Zoffoli (2005).
treatment, indicating that the addition of SO2 may inhibited yeast growth during alcoholic fermentation. Furthermore, Constanti et al. (1998) reported that the addition of SO2 inhibited the growth of non-Saccharomyces yeasts originating from grape surfaces. It was also found that non-Saccharomyces yeasts isolated at the early stages (0–48 h) were not detected in the last stage of winemaking process in the current study.

By comparing the data shown in Figs. 2 and 3, it was found that the increase in yeast amount was consistent with the decrease in the amount of Aspergillus spp., including the trend and peak time/lowest time. This indicated that the increase in yeast quantity had an inhibiting effect on the development of Aspergillus spp., whereas the inhibition effect decreased when the initial A. carbonarius inoculation increased. The mechanism is that S. cerevisiae has activity on inhibiting the growth and OTA production by A. carbonarius and Aspergillus ochraceus in vitro and in vivo (Cubaiu, Abbas, Dobson, Budroni, & Migheli, 2012). Additionally, ethanol accumulation produced by S. cerevisiae is another intrinsic factor that inhibits the development of fungal contamination during alcoholic fermentation since a 20% ethanol could inactivate significantly fungal spores at 25 °C (0.7 kPa) (Dao & Dantigny, 2011; Legan, 1993). This can also be seen from our results (Fig. 5): decrease in the quantity of Aspergillus spp. was consistent with the high ethanol accumulation at this stage (6–8%).

3.3. Distribution of fungal contamination and OTA in different parts of musts in the pressing operation

As shown in Table 1, after the pressing operation, Aspergillus spp. was not detected in the liquid phase of musts, but was detected (2–3 colonies per gram of sample) in pomaces samples. This illustrates that most fungal contamination, especially by Aspergillus spp., is removed from the liquid phase of fermenting musts and retained in the pomaces phase. There is, therefore, little fungal contamination in wine products.

It can also be seen that high levels of A. carbonarius contamination in the musts resulted in high OTA residues in the liquid phase and the pomaces (Table 1); this is consistent with the trend in the amount of Aspergillus spp. When 1 × 10⁶ spores/g of A. carbonarius were added to the musts, the OTA level increased by almost 128 times in the must, 79 times in the liquid phase, and 132 times in the pomaces. It should be mentioned that when SO2 treatment was not used, the OTA level in the liquid phase of the must with 1 × 10³ spores/g of A. carbonarius inoculation was much lower than that in the control (without extra A. carbonarius inoculation), although it was higher in the whole must and the pomaces phase. This would be caused by the non-Saccharomyces that were not killed with the SO2 and could degrade the ochratoxin as they produce more extracellular enzymes than Saccharomyces (Strauss, Jolly, Lambrechts, & Van Rensburg, 2001). Another reason is that the yeasts may adsorb (Caridi, Sidari, Pulvirenti, Meca, & Ritieni, 2012; Perrone et al., 2007) and take the OTA together to the pomaces phase in the pressing operation. In addition, the high OTA content and quantities of Aspergillus spp. in the pomaces could be attributed to the adsorption effect by grape skins, seeds, and yeasts (Esti et al., 2012; Leong et al., 2006; Visconti, Perrone, Cozzi, & Solfrizzo, 2008).

3.4. Accumulation of OTA in the musts during alcoholic fermentation

Fig. 4 shows the accumulation of OTA in the musts during the alcoholic fermentation process. It can be seen that the OTA levels in all must samples increased in the first few hours and then decreased sharply during fermentation. For all samples with SO2 treatment, the residue of OTA in the musts increased with the inoculation size of A. carbonarius, although it was higher in the whole must and the pomaces phase. This extra addition of A. carbonarius shortened the time when OTA accumulation reached its peak value from 72 h (control) to 8–48 h. Furthermore, when 1 × 10³ spores/g of A. carbonarius was added and SO2 treatment was used, OTA accumulation in the musts remained at a stable high level after sucrose addition (24 h) until the latter stage of fermentation (168 h). However, in the musts with 1 × 10³ spores/g of A. carbonarius inoculation but without SO2 treatment, stable high OTA accumulation did not appear. Meanwhile OTA accumulation decreased sharply after 48 h, which was consistent with the fast increase in yeasts during this period (from 48 to 72 h) (see Fig. 3).

The fast increase in OTA accumulation from 8 to 48 h (Fig. 4) was also consistent with the growth of Aspergillus spp. (Fig. 2) and the increase in alcohol concentration (Fig. 5) in the musts during this period. This result is also consistent with that previously reported by Grazzioli et al. (2006). OTA reduction in red musts during alcoholic fermentation has been reported to be caused by two ways: biodetoxification (Abrunhosa, Paterson, & Venâncio, 2010) (microbial enzymatic hydrolysis of toxin molecules) and adsorption (Meca et al., 2010). The latter way is attributable to binding interactions with suspended solids, such as whole or fragmented grape skins, and/or yeast cell walls, which act like a sponge coated with negative charges that interact with the acidic features of toxins (Huwig, Freimund, Käppeli, & Dutler, 2001; Ponsone, Chiotta, Combina, Dalcero, & Chulze, 2009). Polysaccharides, proteins, and
lipids of viable yeast cell walls in particular, exhibit numerous and easily accessible adsorption sites, by hydrogen bonding, ionic or hydrophobic interaction, that increase OTA adsorption (Cecchini, Morassut, Garcia Moruno, & Di Stefano, 2006).

3.5. Levels of soluble solids, reducing sugars, and alcohols during fermentation

As shown in Fig. 5, in comparison with the control, addition of A. carbonarius reduced the levels of soluble solids in the middle stage of fermentation, especially from 24 to 144 h (soluble solids) and 96 h (reducing sugars), but did not have significant influence on alcohol production during the fermentation. No significant difference was found among the samples with different A. carbonarius inoculation sizes. The extra reduction in soluble solids and reducing sugars in the must could be caused by the growth of the extra A. carbonarius introduced into the musts. In addition, the insignificant differences among different A. carbonarius inoculation sizes indicated that this fungus did not disturb the major properties of the normal alcoholic fermentation process.

4. Conclusions

It was found that the quantity of Aspergillus spp. and other fungal contaminants in musts increased rapidly after crushing, but decreased after 48 h when yeasts grew quickly. After the pressing operation, most OTA accumulated in fermenting musts was removed from the liquid phase and retained in the pomaces; this was true for all samples. High quantities of A. carbonarius resulted in high OTA production and high OTA residue in wine. Extra A. carbonarius addition had no significant influence on alcohol production or the composition of the wine. SO2 treatment significantly inhibited the development of fungal contaminants and yeast growth during the alcoholic fermentation stage of winemaking.

Fig. 4. OTA content in musts during alcoholic fermentation. Grapes were inoculated with A. carbonarius at inoculum sizes of $1 \times 10^6$ (A), $1 \times 10^5$ (B), $1 \times 10^4$ (C), and $1 \times 10^3$ spores/g (D and E). The control (CK) is without inoculation. Group E was not treated with SO2. Error bars represent the standard deviations of three individual replicates.
Acknowledgments

This research was supported by grants from the Agriculture Department of China (CARS-30).

References


