Making wine safer: the case of ochratoxin A

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Abstract

This study aims to assess the risk of ochratoxin A (OTA) in European wine with the objective of reducing toxin levels through an integrated management of production and processing. All European countries of the Mediterranean basin are involved. Preliminary results indicate that OTA producing fungi are already present on grapes in the vineyard, prior to harvest. Vineyard location has more influence on OTA levels than grape variety. Weather patterns also seem to influence OTA levels. Results obtained from applications of

various adjuvants aimed at reducing and/or eliminating OTA in wine are discussed.

Keywords: Wine; ochratoxin A; *Aspergillus*

1. Introduction

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppressive properties, which has received interest both from the scientific community and from various food committees (Battaglia *et al.*, 1996; Walker, 1999). It has been detected in various foods, including grape juice and wine, where it was reported for the first time by Zimmerli and Dick (1995). Since then numerous surveys have been conducted to monitor OTA levels in wine and grape juice (Burdaspal and Legarda, 1999; Pietri *et al.*, 2001; Sage *et al.*, 2002). Results indicate that OTA incidence and concentration increases in products from more southern regions, with red wine having higher levels of OTA than white (Zimmerli and Dick, 1996; Otteneder and Majerus, 2000). This latter point has been interpreted as due to differences in red wine processing compared to that of white wine, indicating that this is a postharvest technology problem. Whilst the higher levels of OTA producing fungi in grapes grown in southern (warmer) regions was considered the probable cause for higher OTA incidence in these regions, presuming that this may be a pre-harvest problem.

Fungal genera reported to be able to produce OTA are *Penicillium* and *Aspergillus.* They are common saprophytes and may be present in vineyards. Among the aspergilli Section *Circumdati*, the yellow aspergilli (Krogh, 1987) and Section *Nigri*, the black aspergilli, are the main OTA producers (Abarca *et al.*, 1994; Teren *et al.*, 1996). Indeed, black aspergilli are commonly present in vineyards and have the ability to cause berry rot, known as *Aspergillus* rot or black mold (Snowdon, 1990). Among these black aspergilli *Aspergillus carbonarius* has been reported to have the highest ochratoxigenic potential, with most isolates being able to produce OTA in synthetic media (Heenan *et al.*, 1998). Various authors have suggested that *A.carbonarius* may be the fungus responsible for OTA production in grapes (Pitt, 2000; Cabañes *et al.*, 2002; Abarca *et al.*, 2003).

Most of the studies to date, have been concerned with quantifying levels of OTA in wine, with little information available on the origins of the contamination. In May 2001 a multidisciplinary project (Risk assessment and integrated ochratoxin A management in grape and wine. Acronym: WINE-OCHRA RISK. Contract n. QLK1-CT-2001-01761) was initiated within the V Framework Programme of the European Union. The project aimed at assessing the risk of OTA in wine in Europe with the purpose of reducing toxin levels through an integrated management of production and processing. All wine producing countries of the Mediterranean basin are involved (Italy, France, Spain, Portugal, Greece and Israel). The project is designed to answer the following questions, with the aim of providing preventive and corrective actions. Which are the fungi responsible for OTA production in grapes? When and where do the fungi first produce OTA? What are the conditions which trigger OTA production? Is pre-harvest the origin of the problem? Does post-harvest wine processing technology contribute to the problem?

The present paper describes the qualitative findings collected during the first two years of the project.

2. Materials and methods

2.1. Fungal samples

One-hundred-and-seven vineyards were selected for the study. The vineyards were distributed across each of the participating countries in order to be as representative of the whole terrain as possible. Grape varieties chosen were those of relevance to the country, except one variety, Cabernet Sauvignon, that was common to all. Sampling had to include two neighbouring vineyards, one of Cabernet Sauvignon and one of the local variety. There were four sampling dates: fruit setting, one month later, early veraison, and harvesting. Ten plants were chosen along the diagonals of each vineyard, and at each sampling date the next plant in the same row was sampled. A bunch was picked from each plant in a central position that was defined *a priori*. Bunches were collected with care, placed in paper bags and stored in cooled boxes, with as a short transfer time back to the laboratories as possible.

Five berries per bunch were randomly selected, plated onto Dichloran Rose Bengal Chloramphenicol (DRBC) agar, and incubated for 5-7 days at 25 °C.

Wherever possible whole berries were used, otherwise they were aseptically cut in half. Only penicillia and aspergilli were picked off onto Czapek Dox (CZ) agar to obtain pure cultures, and for further identification of the individual species.

2.2. OTA Fungal screening for OTA production

All strains, or at least those known to be ochratoxin producers, were tested for OTA production using a method adapted from Bragulat, Abarca and Cabañes (2001).

2.3. Influence of grape variety, farming methods and climate

Data on air temperature, relative humidity and rainfall were collected on a monthly basis by each partner.

Two questionnaires were produced in order to study the factors which influence the prevalence of OTA producing fungi and the levels of OTA, at every stage of grape production from the field to wine making. One questionnaire was sent to the vineyards, and dealt with information on locality, farming practices etc. The other was sent to the wineries, and dealt with wine technologies, uses of adjuvants etc.

2.4. Artificial inoculation of berries using OTA producing fungal strains

Berries obtained from grape bunches were inoculated with several species of *Aspergillus* strains and incubated at two different temperatures. Intact and artificially damaged berries were considered. Un-inoculated berries served as controls. Incidence of visible fungal colonization and OTA content were quantified (Bavaresco *et al.*, 2003).

2.5. Effect of grape processing on OTA content

2.5.1. OTA levels in musts

Ten musts and wines from two red grapes, and five musts from white grapes were analysed. The influence of different wine making phases on OTA content were also evaluated during full scale wine production. Samples were taken

in duplicate during crushing, end of maceration and alcoholic fermentation, and after malolactic fermentation.

2.5.2. Effect of chemical and biological adjuvants on OTA levels of wine

Four materials, all containing charcoal, two with calcium caseinate and two with silica, were tested for their ability to reduce OTA content.

Lactic acid bacteria, namely *Lactobacillus plantarum* and *Oenococcus oeni* were used in the trials. *L.plantarum* strains were grown on MRS agar (Difco) for 48h at 28°C. Cells were centrifuged and washed, and suspended in physiological solution to yield an initial population in must of 10^8cfu/ml (must 1).

O. oeni in dry commercial form was rehydrated (1:20) in sterile water at 40°C for 15 min, and then washed and suspended in must to yield an initial population of 10^8 cfu/ml (must 2). Both musts were incubated at 25°C for 5 to 12 days. Laboratory trials with musts and wines were conducted in duplicate. OTA levels were measured before and after treatments (Silva *et al.*, 2002).

3. Results

3.1. Fungal samples

Both aspergilli and penicillia were isolated from all the countries involved in the project, with aspergilli dominating. Three *Aspergillus* sections were identified, namely *Nigri, Circumdati* and *Fumigati.* Section *Nigri* formed more than 90 % of the total isolations*; it* was present at setting, and increased in frequency through veraison and ripening. Furthermore, fungi of this section were all isolated from asymptomatic berries.

Identification of the species is notoriously difficult in this section (*Nigri*), and therefore the isolates were grouped into three categories, namely uniseriate black aspergilli (consisting of *Aspergillus japonicus* and *Aspergillus aculeatus*), *Aspergillus niger* aggregate (consisting of *A.niger* and *Aspergillus tubingensis*) and *Aspergillus carbonarius*, which is relatively easy to identify because of its large conidia. In all cases isolates were sent to an expert for

further authentication and verification. Over half of the isolates were categorized as *A.niger* aggregate, 30% were identified as *A.carbonarius* and 20% as uniseriates.

3.2. Fungal screening for OTA production

All *A.niger* aggregate, *A.carbonarius* and uniseriate isolates were screened for their OTA producing ability. Most of the *A.carbonarius* strains were OTA positive, and produced the toxin at high levels. Only about 5% of the *A.niger* aggregate proved positive, with much lower OTA production. OTA production in uniseriate species is still under study. Furthermore, all isolates are being further screened using molecular techniques in order to unequivocally identify the OTA producing strains, particularly within the *A.niger* aggregate.

3.3. Influence of grape variety, farming methods and climate

These studies are still on-going, and so only preliminary results can be given. It appears that temperature (north - south differences), rainfall and relative humidity all influence OTA levels in grape in the field (Serra, Kozakiewicz, Lima and Venâncio, 2001; Battilani, Pietri, Bertuzzi, Languasco, Giorni and Kozakiewicz, 2002). There are differences in OTA content between vineyards, even though the cultivars and training techniques may be the same. As yet different farming techniques have not shown an influence on OTA levels.

3.4. Artificial inoculation of berries using OTA producing fungal strains

Both symptoms and OTA content were higher in damaged compared with undamaged berries. OTA content was not significantly influenced by temperature. Fungi appeared to be more invasive at harvesting and symptoms were high in both damaged and undamaged berries. However, OTA content appeared to be higher in younger and damaged berries. *A.carbonarius* infected berries produced the highest OTA levels.

3.5. OTA levels in musts

None of the white musts was contaminated with OTA. Three of the red musts and three red wines contained OTA. The wine phase which removed most OTA was the malolactic fermentation.

3.6. Effect of chemical and biological adjuvants on OTA levels of wine

Products containing charcoal were efficient in decreasing levels of OTA in wine; maximal reduction was about 90%. When OTA levels in wine were high, all the adjuvants/absorbents strongly modified the wine colour.

All *L.plantarum* and *O.oeni* strains exhibited the ability to reduce OTA levels in must. However, *L.plantarum* isolates were more effective.

Some strains of *L.plantarum* were considered and differences between strains were noticed in their ability to reduce OTA content (from 30% to 40%) (Silva *et al.*, 2002).

4. Discussion and conclusions

Studies carried out on trans-European populations of fungi on grapes, indicate that *Aspergillus* species in general and Section *Nigri* in particular, are the dominant OTA producers. These results concur with those of Sage *et al.* (2002) for France, and Da Roche *et al.* (2000) for South America. Furthermore, *A.carbonarius* appears to be the strongest OTA producer, followed by the *A.niger* aggregate. Over 80% of *A.carbonarius* isolates produced the toxin, results which fit well with those of previous reports. Heenan *et al.* (1998) stated that 90.9% of their isolates produced the toxin, whilst (Teren *et al.*, 1996) identified 41.7% producers in another survey.

Fungi of the Section *Nigri* were isolated from grapes commencing at fruit setting and increasing in frequency through veraison, ripening and harvesting. Contamination by black aspergilli has always been considered to be a post-harvest problem. But, the fact that these strains were isolated from asymptomatic grapes, whilst still in the field, suggests that the pre-harvest period may play a significant role in determining OTA content of grapes. The incidence of black aspergilli was shown to be significant with more isolations from southern Europe (Serra *et al.*, 2003; Battilani *et al.*, 2002). Black aspergilli are very resistant to sunlight and to hot dry environments (Pitt and Hocking, 1985). They are perfectly adapted to the conditions observed in the vineyards of the Mediterranean basin. Other factors which may influence OTA production are rainfall and relative humidity (Battilani *et al.*, 2002).

Studies carried out during wine making have shown the presence of OTA in red must. Levels of OTA were variable during wine making/processing, but the amount present in the final product was lower than that in the original must. Malolactic fermentation particularly appears to play a significant role in reducing levels of OTA.

The use of adjuvants, particularly charcoal-based products, appears to be efficient in reducing OTA levels in wine. However, their use is limited because charcoal can and does alter the colour of red wine. Low amounts of charcoal can reduce OTA level by about 50% with no effect on colour but high amounts substantially modify the colour. The removal of OTA in wine using lactic acid bacteria appears to be a more suitable approach. *Lactobacillus plantarum* reduced OTA content by 50% in must and approximately 45% in wine. However, further research is required before this treatment can be recommended.

Results to date, support the hypothesis that black aspergilli, in particular *A.carbonarius* and the *A.niger* aggregate, are the fungal species responsible for OTA contamination in grapes and wine from wine-growing areas with warm and dry conditions. However, more studies are required on the effects of agronomic practices, climate and rainfall on OTA levels in the field. Trials to reduce OTA content in musts and wine using various chemical and biological adjuvants appear promising, but further work is required. Finally, this project conducted throughout the Mediterranean basin, using the same protocols and approaches, should contribute significantly to understand the role which the various factors play in causing OTA contamination of grapes and wine.

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