ABSTRACTS

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OCHRATOXIN A IN GRAPES AND WINE: PREVENTION AND CONTROL

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SECTION 3

Post Harvest

LECTURES

SURVIVAL OF OTA-PRODUCING FUNGI DURING STORAGE OF TABLE GRAPES

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Table grapes of cv. 'Superior' were sampled from four vineyards through three seasons, for the occurrence of black *Aspergillus* species that can produce ochratoxin A after storage. The results show that storage of table grapes for 7 d at 20 °C did not have a significant effect on the number or type of isolates, but occasionally their number was much higher than that at harvest. In contrast, storage for one month at 0 °C, with SO_2 generator pads reduced the number of isolates substantially. The ochratoxin A-producing fungus, *A. carbonarius*, could be isolated from all the samples at harvest and it survived all storage procedures that did not include a sufficient level of SO_2. Dipping the bunches in ethanol prior to cold storage did not reduce the number of isolates of the *A. niger* aggregate. In contrast, bunches that were exposed to a final level of 5 ppm of SO_2 after 12 d of cold storage were free of contamination by black *Aspergillus* species. Likewise, *A. carbonarius* inoculated onto Petri dishes that were exposed to the same storage conditions, failed to develop after storage. Exposure to a final level of 0.4 ppm of SO_2 resulted fewer fungal colonies than in the control, and the surviving spores developed into fungal colonies that failed to sporulate. These results demonstrate that the current commercial methods can protect stored grapes from contamination by black *Aspergillus* species.

OCHRATOXIN A: FROM GRAPES TO WINE

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Prevention of ochratoxin A (OTA) accumulation through pre-harvest management is the best method to control mycotoxin contamination; however, the contamination should occur, so the hazards associated with the toxin must be managed through post-harvest procedures. The assignment of CCPs is important to make HACCP effective and is essential to prevent or to eliminate a food safety hazard or to reduce it to an acceptable level. Grapes selection is a preventive measure and the good manufacture practices in winemaking can work as corrective action for reducing contamination.

According to all the experiments that were conducted under “Wine-Ochra Risk” the results show that no OTA is produced during winemaking, but each operation during winemaking can modify OTA content. OTA passes from grapes into the juice during crushing, the maceration increases OTA content, the alcoholic and malo-lactic fermentation cause an OTA reduction. During all clarification steps (either natural sedimentation or by the use of adjuvant), the level of ochratoxin A decreases because of its adsorption into the sediment. The ochratoxin A reduction due to the natural sedimentation is small, while the decrease due to the use of adjuvant depends on the type and amount of adjuvant.

In order to manage the hazard of OTA in winemaking and to verify if OTA content in wine is lower than the legal limit of 2 μg/L, it would be enough to analyze OTA in must and in wine at the end of alcoholic fermentation, since the following steps cause the OTA reduction.
According to the results, it is expected that a significant decrease in ochratoxin A concentration occur from grape at harvest to wine if the following good manufacture practices are achieved:

- Minimize the time interval between harvest and crushing; refrigerate the grapes when crushing will not take place in a short time
- Discard bunches with visible mould grown (specially black moulds)
- Add sulphur dioxide to grapes
- Control OTA level in must after crushing
- In case of high OTA risk, reduce the maceration time, use yeast, lactic acid bacteria, and chemical adjuvants proven to be effective against OTA.

OCHRATOXIN A FATE IN SOME VINIFICATION STEPS

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A few years ago, the presence of ochratoxin A was reported for the first time in wine. Since then several studies have been conducted, being worldwide accepted that grape juice is usually more contaminated than wines, and red grape products are more contaminated than white ones. This knowledge has led researchers to conclude that grape processing could contribute to a reduction in the content of this mycotoxin in grape products, such as the case of wines.
The fate of ochratoxin A during the more common vinification steps was evaluated, for Vinho Verde production. Vinho Verde is a typical wine produced in a wine region from northwest of Portugal. Ripe berries were collected and innoculated with an ochratoxigenic strain of *Aspergillus carbonarius*, in order to obtain grapes with different levels of contamination. Grapes with a content of OTA ranging from 0.43 to 7.48 µg/Kg were obtained and used for vinification. It was found that after crushing this amount of OTA is splitted between must and pomace, being found that after alcoholic fermentation just about 31.8% of OTA was still present in wine. The remaining amount of OTA is mainly found in lees and pomace. After racking this amount decreased to 10.9 %, and after malolactic fermentation to 8.1%. Also, it was found that OTA was present in higher amounts in spent fractions from wine making, such as lees after fermentation or sediment after racking. Based on this data, this reduction is associated with the mycotoxin removal by adsorption into solid wastes or fining agents, and may do not be due to the degradation of ochratoxin A in other compounds.

STRATEGIES FOR OTA REMOVAL

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Ochratoxine A (OTA), produced by *Aspergillus* and *Penicillium,* is known to have nephrotoxic, teratogenic, hepatotoxic or carcinogenic effects. Since 1996, the presence of this toxin was shown in grape juice and wines. From the end of 2005, a maximum value of 2 ppb is fixed by the European Community. In the way to reduce OTA present in the wine and in grape juice different processes can be developed; biological adsorption, biotransformation and biodegradation for examples.
OTA can be eliminated by enzymatic degradation. In this case, black aspergilli can be used as source of enzymatic activities. *A. niger*, *A. carbonarius* and *A. japonicus* are able to transform OTA to Otα in grape juice and in different synthetic media. The percentage of degradation varies according to the isolate tested and the medium selected but it can reach 99%. The degradation of the OTA is checked by the follow-up of the appearance of the Otα in the medium.
Oenological yeasts in growth are able to reduce the level of OTA initially present of 45 % in a synthetic medium. If dead yeasts are used (treatment with heat or the acid), this percentage can reach 75% according to the medium used. In this case, there is no appearance of the Otα, the elimination of the OTA is thus done by adsorption.
Spores of *Aspergillus* of the *Nigri* section (*A. carbonarius*, *A. niger* and *A. japonicus*) can be used to reduce the OTA present in a grape juice. This phenomenon is adsorption which occurs immediately as soon as the spores are added in the medium contaminated by the OTA. Process can be developed with by the use of *A. japonicus* as adsorption material for OTA elimination. All these three biological material are discussed for OTA elimination.
Key words: OTA, grape, grape juice, Aspergillus, yeast, detoxification

ASPERGILLUS SECTION *NIGRI* DOMINANCE DURING THE SUN-DRIED GRAPE PROCESSING

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Previous publications reported the occurrence of black aspergilli, and ochratoxin A (OTA) in sun-dried grapes. Even the European Union has established a specific legal limit in this product. Contamination of grapes and their derivatives can occur during preharvest, harvest and grape processing. *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Fusarium* and *Rhizopus* are regarded as the main natural contaminants in this product. However at the end of the process, isolates of *Aspergillus* section *Nigri* are the predominant fungi and they are the main responsible of the OTA presence in this product.

In this study we aimed to understand the mycobiota succession in grapes and sun-drying through parameters such as temperature, water activity and fungal interactions focusing on niche adaptation and antagonistic capability of black aspergilli.

Microorganisms used in this study were *Alternaria alternata*, *Cladosporium herbarum*, *Eurotium amstelodami*, *Penicillium janthinellum*, *P. decumbens*, *Trichoderma harzianum*, *Candida* sp., *Aspergillus carbonarius* OTA-negative, *A. carbonarius* OTA-positive, *A. niger* aggregate sp. and *A.* section *Nigri* sp. classified as uniseriate. They were isolated from grapes and vine dried fruits collected from the South of Spain. Growth studies in pure cultures and in paired cultures at different water activities (0.82, 0.87, 0.92 and 0.97) and temperatures (20, 30 and 40ºC) were carried out in a medium with composition similar to that of grapes (Synthetic Nutrient Medium, SNM). Growth radii in the line between both inoculation points were recorded daily for 18 days. In addition, each fungus was given a numerical score using the Magan and Lacey criteria (1984).

Effects on black aspergilli growth of temperature, water activity, paired species and all their interactions, proved to be significant ($p< 0.001$). At high temperatures and low water activities, *Penicillium* isolates, *E. amstelodami* and *A. niger* aggregate showed higher growth rates, while *T. harzianum* only grew well at the highest water activity. Among the black aspergilli, *A. niger* aggregate was in general the more inhibited by the interacting species, while both *A. carbonarius* isolates growth was less affected and sometimes stimulated. In addition, *A.* section *Nigri* was dominant in most paired assays, being only surpassed by *T. harzianum* at 0.97 water activity and 20ºC. However this isolate did not grow at low water activities.

During the drying period that can last from 5 days to two weeks, only few microorganisms such as black aspergilli, are capable to resist both germicidal UV light and the strong sunlight heating.

To sum up, prevalence of *Aspergillus* section *Nigri* can be easily explained by its adaptation to environmental conditions of sun-drying, and by its capability to dominate over some other fungal species involved when coming into contact with them

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POTENTIAL FOR CONTROL OF *A.CARBONARIUS* STRAINS FROM VINE FRUITS USING SULPHUR DIOXIDE OR CONTROLLED ATMOSPHERE STORAGE

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In vitro studies were conducted on a red grape juice medium with strains from currants/sultanas (5 strains) and one strain from grapes for wine production to examine potential for control of germination, growth and OTA production using sodium metabisulphite (NaBMS) or controlled atmospheres at different water activity levels (0.985, 0.965, 0.93). Generally, germination and germ tube extension were inhibited by >500 ppm of NaBMS. However, mycelial growth was stimulated by low NaBMS concentrations (100, 250 ppm). A concentration of up to 1000 ppm was required for complete inhibition of growth. The production of OTA was inhibited by up to 750 ppm NaBMS. However, at lowered a_w (0.93) OTA production was inhibited by 500 ppm.

The efficacy of controlled atmospheres x a_w showed that there was very little inhibitory effect on spore germination and germ tube extension, even by 50% CO_2. However, 50% CO_2 inhibited growth after 5 days exposure completely. However, after 10 days growth was not as effectively controlled. OTA production by *A .carbonarius* strains was influenced predominantly by a_w levels and less so by up to 50% CO_2. Overall, there was little difference between the strains examined with that from grapes for wine production.

DISTRIBUTION OF OCHRATOXIN A IN WINE AND WINERY BYPRODUCTS DURING WINE-MAKING OF NEGROAMARO AND PRIMITIVO

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The distribution of ochratoxin A (OTA) in wine and winery byproducts during wine-making of Negroamaro and Primitivo was monitored in two wineries in the vintage 2004/2005. Samples of must, grape pomace, wine and lees were collected and analysed for OTA by using appropriate IMA/LC/FLD analytical methods. The sampling was performed in order to evaluate both the effect of maceration/pressing/sediment separation on OTA concentrations in must and wine and the percentage of OTA distribution in wine and winery byproducts. All tested samples contained measurable concentrations of OTA. Mean concentrations of OTA in must and wine remained roughly the same before and after maceration, pressing and sediment separation for both Negroamaro and Primitivo. Most OTA was found in grape pomace (77-89%) followed by lees (10-23%) and wine (3-6%). These data will be verified in a pilot experiment performed at laboratory scale under controlled conditions.
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FATE OF OCHRATOXIN A DURING WHITE AND RED VINIFICATION IN AUSTRALIA

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White and red grapes were inoculated on the vine by puncturing berries with a syringe containing a suspension of *Aspergillus carbonarius* spores. Inoculated grapes contained ochratoxin A (OTA), and displayed greater total soluble solids due to berry shrivelling. Titratable acidity also increased in inoculated grapes due to production of citric acid by the fungus. Vinification of these grapes simulated standard Australian practice, and samples were collected for OTA analysis. Pressing resulted in the greatest reduction in OTA (68-85% decrease in concentration, compared with that of crushed grapes). Additional reductions occurred at racking from grape and gross lees, and after storage. OTA did not appear to be degraded during vinification, rather, it was removed by binding to marc, grape lees and gross lees. Pectolytic enzyme treatment of white must, bentonite juice fining, recovery of juice or wine from lees, and static or rotary style fermentation of red must containing pomace had negligible effect on OTA contamination. In a Semillon wine, which contained 56 mg/L grape-derived proteins, bentonite added as a fining agent achieved a greater reduction in OTA than proteinaceous agents, such as caseinate and gelatin. However, in a Shiraz wine, which did not contain detectable grape-derived proteins, yeast hulls, and, to a lesser extent, gelatin, were effective for removing OTA.

OCHRATOXIN A REDUCTION BY LACTIC ACID BACTERIA

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Ochratoxin A (OTA) reduction in wine after malo-lactic fermentation (MLF) has been highlighted in many studies. Usually malo-lactic fermentation starts up after alcoholic fermentation because of the lactic acid bacteria naturally present in wine. Selected bacteria are already employed in winemaking to improve the overall wine quality. We report here the OTA reduction in wines by *Lactobacillus plantarum* and *Oenococcus oeni* strains. In particular we have investigated on the OTA removal in wines with different OTA and ethanol contents and the kinetic of OTA reduction.
During the 2004 vintage we have verified the OTA reduction by lactic acid bacteria during the malo-lactic fermentation of Negroamaro naturally contaminated. We have carried out trials using freeze-dried *Lactobacillus plantarum* and *Oenococcus oeni* strains..
We have used masses naturally contaminated and spiked with OTA and with different level of SO_2. The temperature during malo-lactic fermentation (MLF) was 20°C. OTA analyses have been done at different times after the malo-lactic fermentation. OTA levels were lower than the OTA content before MLF.The data confirm the ability of tested strains to wine decontamination.

POSTERS

OCHRATOXIN A REDUCTION BY CHEMICAL ADJUVANTS

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After 1996, when ochratoxin A (OTA) was detected in wine, several studies started with the aim to define the true occurrence of this toxin throughout Europe and to identify the key elements for OTA presence in grape and wine in order to provide tools for preventive and corrective actions. The goal of our work is to obtain a corrective action in must and wine through the use of adjuvants which act as adsorbents. Chemical adjuvants were screened for their ability to degrade OTA in must and wines naturally contaminated or spiked with OTA. Before and after treatments, ochratoxin A was determined. The treatments with chemical adjuvants reduce ochratoxin A in wine when charcoal adsorbent is present but this treatment is now admitted only in must during white wine-making. The treatment efficiency depends both on the type and on the amount of the adsorbent; the employment of high amounts of charcoal irreversibly changes the red wine characteristics because this product has an high affinity to polyphenolic compounds, too. This negative effect on the colour is not very significant with amounts of charcoal lower than 10 g/hL.

MYCOFLORA POPULATION DYNAMICS AND OTA CONTAMINATION DURING DRYING OF VINE FRUITS

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A. carbonarius and *A. niger* aggregate are the key fungi responsible for contamination of vine fruits with the mycotoxin ochratoxin A (OTA). This study examined temporal populations of these two groups and other component mycoflora in grapes prior to harvest and during the drying phase up to storage from fields at three different altitude levels in 2004. The populations of *A. carbonarius* and *A. niger* aggregate increased during drying to reach Log 3-4 CFUs g^{-1} currants prior to storage. Other predominant genera isolated were yeasts and *Penicillium* species. *A. carbonarius* and *A. niger* aggregate were present on grapes before harvest and their population and frequency was increased during currant drying to achieve the highest populations and contamination levels just before storage. OTA was detected in 40% of the samples with positive samples mainly originating from dried currants prior to storage. The frequency of isolation of *A.carbonarius* and *A.niger* aggregate increased earlier when grapes/drying currants were direct plated. About 75% of *A. carbonarius* strains were found to produce OTA. Altitude significantly influenced when OTA was first found in samples and more samples were contaminated as the drying phase proceeded.

INTERACTIONS BETWEEN TWO YEAST SPECIES AND OCHRATOXIN A

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The present study has investigated the effects of different concentrations of Ochratoxin A (OTA) on the fermentative ability and the biofilm formation capacity of two yeast strains. The OTA levels in the fermented samples were significantly lower than those in the control samples for both the yeast strains. Moreover, OTA was not detected in any of the yeast samples, showing that the yeasts are not able to absorb this mycotoxin. No negative effects on the fermentative process and the biofilm formation were seen in the presence of OTA in the must.

EFFECT OF YEASTS AND THEIR BY-PRODUCTS ON THE OCHRATOXIN A LEVEL OF WINE

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The potential for yeast cells to adsorb mycotoxins has been reported. Different study for biological reduction of ochratoxin A on laboratory trials and on commodities have been proposed, but few study for OTA removal has been carried out in must and wine. Our objective in this study is to verify the adsorption capacity of yeasts and their by-products towards the ochratoxin A. Must and wine naturally contaminated or spiked with OTA were treated with different amount of active dry yeast and of yeast cell wall. Samples were taken after different times of contact for the analysis of OTA.
In order to evaluate the adsorption of OTA performed by the yeast walls we have carried out a test where 1 g/L of yeast walls have been added to 5 aliquots, each of 100 ml of red wine naturally contaminated with OTA..
After 3-8-10-15-20 days the OTA reduction in the samples has been tested, following the centrifugation. Each sample were shaken for 24 hours and stored in the laboratory at room temperature (~ 20°C). The kinetics of the OTA trend during the contact of wine and yeast walls shows that the maximum effectiveness is reached 20 days later with a 44% decrease in OTA.
We are also carrying out lab-scale trials in order to evaluate the OTA reduction obtained by Mycosorb, an adjuvant not used in enology, that is extracted from Saccharomyces cerevisiae cell walls and now is used as feed additive to adsorb the mycotoxins.
Each trial has been carried out with naturally contaminated wine, on lab-scale (200 mL of wine), at room temperature and in duplicate. In these trials, Mycosorb concentrations of 0.25 g/L and 1.0 g/L have been used, obtaining a 77% and 88% reduction respectively, after 60 hours of contact.

THE ITALIAN PROJECT OF MIPAF FOR THE STUDY OF OCHRATOXIN A IN GRAPE AND WINE

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After the discovery of the presence of ochratoxin A (OTA) in wine (Zimmerli and Dick, 1996), the Italian Ministry for Agriculture and Forestry (Ministero delle Politiche Agricole e Forestali, MiPAF) started a national research project. The aims were to find out the major factors that could lead to OTA production and to set down the possible agronomic and oenological strategies focused at the reduction of OTA presence.

Different topics were considered:

- Grapevine pathology: composition of berry mycoflora in relation to viticultural environments, agronomical factors and chemical controls
- Oenology: winemaking treatments aimed to reduce and eliminate OTA from wine, methods for the determination and quantification of OTA in must and wine
- Potential risks for the consumer health
- Establishment of guidelines for prevention and reduction of OTA

Viticultural studies were carried out on selected vineyards of Northern Italy (Lombardy, Venetia and Piedmont), Central Italy (Tuscany and Latium), Southern Italy (Apulia and Basilicata), Sicily and Sardinia. In 1999-2004 more than 300 vineyards were inspected and more than 900 grape samples were collected. Analyses were carried out on pressed grapes.

Results showed the presence of a complex mycoflora, which composition was very different according to the geographic area. The role of the cultivar and of the viticultural factors showed to be very important, in particular the presence of wound inflicted by grape moth attacks. OTA determination analyses pointed out a big presence of the toxin on samples collected in Apulia viticultural areas.

Oenological studies showed that the winemaking technique did not influence the OTA amount in wine, saved the duration of maceration, which was individuated as the cause of the difference in OTA concentration between red and white wines. As far as is concerning the treatments aimed to reduce OTA amount in wine, the best results were obtained with the use of decolorizing charcoal. The treatment with 15 g/hL of decolorizing charcoal was sufficient to remove at least 85% of the initial OTA amount. An alternative to decolorizing charcoal was the use of fermentation lees.

Zimmerli B., Dick R. 1996. Ochratoxin A in table wine and grape-juice: occurrence and risk assessment. Food Addit. Contam. 13(6): 655-68.