ABSTRACTS

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OCHRATOXIN A-PRODUCING SPECIES FROM SPANISH WINE GRAPES: A 3 SEASON STUDY

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The objective of the study was to identify the ochratoxin A (OTA) producing mycobiota of grapes at different developmental stages of the berries. During 2001, 2002 and 2003 seasons, a total of 7 vineyards located mainly along the Mediterranean coast of Spain were studied. Besides, OTA-producing ability of the isolates of potentially ochratoxigenic species was analyzed. Predominant mycobiota of grapes belonged to *Alternaria* spp., *Cladosporium* spp. and *Aspergillus* spp. *Penicillium* spp. represented less than 3% of the isolates. With the maturation of berries, the incidence of *Alternaria* spp. and *Cladosporium* spp decreased while there was a clear increase of *Aspergillus* spp. In all cases, statistically significant differences were found in the occurrence of those genera at each sampling time. During the 3 years of the study, a total of 1812 isolates of *Aspergillus* spp. were recovered from grapes, of these, 1423 isolates belonged to *A. niger* aggregate and 226 were *A. carbonarius* isolates. Only 13 isolates of *A. ochraceus* were identified. A total of 214 isolates belonging to *Penicillium* spp. were recovered from berries. The sole isolate of *P. verrucosum* identified was able to produce citrinin but did not produce OTA. Isolates belonging to *Aspergillus* section *Nigri* constituted about 90% of the total *Aspergillus* isolates. Although they were recovered in all the stages sampled, there was a statistically significant increase at harvesting. Even though the number of isolates recovered in DRBC medium were higher than on MEA, these differences were not statistically significant.

OTA-PRODUCING SPECIES FROM SPANISH WINE GRAPES

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OTA has been reported in wine since 1996. Wine is estimated to be the second source of OTA in the diet after cereals, as it can represent up to 15 % of the total OTA intake. OTA possesses teratogenic, nephrotoxic and immunotoxic properties and has been classified as a possible human carcinogen (Group 2B).

There is few data on mycoflora and potential OTA-producing fungi in Spanish wine grapes. This study focus on the identification of the common mycoflora in wine grapes from four important grape growing regions of Spain, studying their evolution along grape formation in 2001, 2002 and 2003. Four wine producing regions representing a cross section of five important *Designations of Origin* of Spain (La Rioja, Costers del Segre, Utiel-Requena, Penedés and Conca de Barberà) were chosen for the study. Samples were taken at three growth stages (one month after setting, veraison and harvest time).

Mycoflora was determined in berries and all potential OTA producers were grown onto Czapek Dox agar (CZ) for classification, and onto Czapek Yeast Extract agar (CYA) for OTA production assessment. As morphological identification of black *Aspergilli* was notoriously difficult, in this study they have been classified according to the morphology of their spores and conidial heads into three groups: uniseriates, *A. niger* aggregate (biseriates excluding *A. carbonarius*) and *A. carbonarius*.

The highest number of *A.* section *Nigri* were detected at harvest in the four regions all years; this suggests that late ripening marks a profound change in the ecological factors affecting dissemination of spores as well as microbial growth. Moreover, grapes are more susceptible to fungal infection when approaching harvest as sugar content increased and the berry texture becomes less harder. All of that, together with the increasing temperatures in the month preceding harvest, sometimes above 30 ºC, could influence on black *Aspergilli* development.

The general pattern in the apparition of the species in grapes, was not significantly different among 2001, 2002 and 2003, thus results can be considered representative of the situation in the sampled areas. However, more black *Aspergilli* were isolated in 2003 than in the two previous years, probably because 2003

was an extremely hot year in Spain. Moreover, a positive correlation between the number of black *Aspergilli* found in grapes and the temperature in the field was found. No significant correlation between their presence and other meteorological factors such as relative humidity, rainfall, etc. could be established.

Mean percentages of uniseriates, *A. niger* aggregate and *A. carbonarius* were 18 %, 64 % and 18 %, respectively. Among the total mycoflora, 464 and 648 *Aspergillus* section *Nigri* were isolated in 2002 and 2003, respectively. *A. carbonarius* presented the highest percentage of OTA-positive strains (82 % in 2002 and 76 % in 2003) and produced the highest levels of toxin (2.5-25 μg g^{-1}). Authors agree that the high number of *A. carbonarius* encountered in grapes, together with their huge ability to produce OTA, make them relevant for the toxin presence in the field and therefore, in grape products.

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DETECTION OF OCHRATOXIN MOLDS IN GRAPES OF SIX ORIGIN DENOMINATION IN SPAIN

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Ochratoxin A (OTA) is one of the most important mycotoxins because of its high toxicity to humans. OTA is found in a variety of agro-food products such as cereals, coffee and wine and it is mainly produced by species of the genus *Aspergillus* (*A. ochraceus, A. niger and A. carbonarius)* and *Penicillium* (*P. verrucosum* and *P. nordicum*). The occurrence of these species is affected by climatic and geographical factors. Therefore, in order to predict the risk of contamination of commodities by OTA, and prevent OTA entering the food chain, it is necessary to perform an accurate detection of those species. We have developed PCR-based diagnostic protocols in our laboratory to detect the main OTA-producing species *Aspergillus* species (*A. ochraceus, A. niger and A. carbonarius*), including *A. japonicus,* recently described as OTA producer. We have used these tools to analyse the occurrence of those species in a wide sample of vineyards located in two important Spanish regions of quality wines. These regions, Castilla and León and La Rioja, produce 268.9×10^3 and 345.0×10^3 Tm of wine grapes, respectively. We have analysed 14 grapes varieties (6 of white grapes and 7 of red grapes). Additionally, we have analysed by conventional methods the presence of *Penicillium* species. The results we present indicate the absence of OTA-producing *Penicillium* species and *A. carbonarius*, and a extremely low occurrence of *A. ochraceus* (absent in the region Castilla and León). The most relevant OTA-producing species was *A. niger.* The influence of the grape variety is also discussed.

OCHRATOXIN A PRODUCTION BY *ASPERGILLUS SPP* ISOLATED FROM GRAPES GROWN IN SPAIN

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In an attempt to study the mycobiota responsible for the production of ochratoxin A (OTA) in Spanish wines, four red varieties of grapes (Bobal, Tempranillo, Garnacha and Monastrell) and one white variety (Moscatel) grown in Spain were screened. The number of samples was 52. They were taken in different vineyards in the provinces of Albacete, Alicante, Cuenca, Málaga, Murcia, Rioja and Valencia. The main fungal genera isolated were *Alternaria*, *Cladosporium*, and *Aspergillus*. The isolation frequency of *Aspergillus* spp. section *Nigri* in contaminated samples was 82%. Production of OTA was tested using yeast extract-sucrose broth supplemented with 5% bee pollen. An overall 205 isolates of *Aspergillus* section *Nigri* (120 of *Aspergillus carbonarius*, 21 of *Aspergillus tubingensis* and 64 of *Aspergillus niger*) were cultured. Then, after extraction and

clean-up, OTA analyses were performed by liquid chromatography with fluorescence detection. Eventual confirmation of OTA was accomplished by liquid chromatography–ion trap-mass spectrometry.

It was found that 74.2% of *A. carbonarius* isolates produced OTA at levels ranging from 1.2 to 3,530 ng/ml of culture medium, whereas 14.3% of *A. tubingensis* isolates produced OTA at levels ranging from 46.4 to 115.5 ng/ml of culture medium. However, no *A. niger* isolate had the ability to produce this toxin under the assayed conditions. Identification of the *A. niger* aggregate isolates was based on PCR amplification of 5.8S rRNA genes and its two internal transcribed spacers ITS1 and ITS2, followed by digestion of the PCR products with restriction endonuclease RsaI. The restriction patterns were compared with those from type strains of *A. niger* (CECT 2807) and *A. tubingensis* (CECT 20393). DNA sequencing of OTA-producing isolates of *A. tubingensis* was performed on the ITS1-5.8S rRNA gene-ITS2 region. A very high degree of agreement (99-100%) was found with the DNA sequence of *A. tubingensis* type strain CBS 643.92. The results showed that there are significant differences concerning the isolation frequency of OTA-producing fungi in the different grape varieties but these differences have no correlation to berry colour.

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FUNGAL FLORA AND OCHRATOXIN A PRODUCTION DURING WINE GRAPE MATURATION IN FRENCH VINEYARDS DURING THREE YEARS

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A survey was conducted in France over three years (2001-2003) in 10 vineyards from four winemaking regions with different geographical locations and climatic conditions. *Aspergillus* and *Penicillium* were isolated from the grapes. During 2001 and 2002, from setting to harvest, the total numbers of fungal isolates were respectively 721 and 711 increasing in 2003 to reach 1035. The *Aspergillus* genus was essentially represented by *Nigri* section (99%) and was predominant (80% ±4.6) when compared to *Penicillium* (20% ±4.6). Regardless of sampling year, 32.5% (±σ = 1.26) of the fungal isolates were OTA producers and 93% (±σ = 2.65) belonging to black aspergilli. The ochratoxigenic potential of the isolates and their occurrence on grapes revealed that *A. carbonarius* was the main OTA producer on French grapes. At harvest time, the fungal population was maximal and this was the most critical period influencing OTA contamination. Grapes from Languedoc-Roussillon region were most infested with ochratoxigenic fungi and had the highest concentrations of OTA. All these results could be of prime importance for further preventive action against ochratoxigenic species in vineyards to limit the contamination of grapes and grape products in France.

OCHRATOXIGENIC FUNGI IN GRAPES OF GREECE

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Different fungi of the grapes mycoflora were isolated and their ability to produce ochratoxin A production was determined. The grapes were provided by ARKAS SA and ACHAIA CLAUSS SA both companies established in Peloponnese region in South Greece, during the harvest of 2004. There were grapes from cultivars Mavrodafni, Roditis, Moschoflero, Agiorgitiko, Cabernet-Sauvignon, Sauvignon Blanc, Sauvignon Blanc-Chardonnay, Cabernet-Merlot. The majority of the isolates were black aspergilli while the rest belonged to the genus Penicillium, Alternaria, Botrytis and some of the isolates were not identified. Eight of the ninety isolates of black aspergilli were able to produce ochratoxin A, all of them being isolated from a certain cultivar Roditis. Ochratoxin was not detected in any sample of grapes (limit of detection 0.005 μg kg^{-1}), while it was detected at

the beginning of fermentation (0.5-1.4 µg kg^{-1}) and reached high levels during the fermentation (6.9-7.2 µg kg^{-1}) of the cultivar Roditis. These preliminary results showed a strong correlation between the presence of ochratoxin-producing fungi on grapes and presence of OTA in musts.

REGIONAL DIFFERENCES IN THE MYCOFLORA OF PORTUGUESE WINE GRAPES – A 3 YEAR-STUDY

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The mycoflora of healthy ripe berries from Portuguese winemaking regions was studied between the years 2001 and 2003. Four regions were selected: Alentejo, Douro, Ribatejo and Vinhos Verdes, located in the southeast, northeast, southwest and northwest of the Portuguese mainland, respectively. All the regions have Mediterranean climates apart from Vinhos Verdes, which is Submediterranean, more humid than the other regions due to Atlantic influences. Eleven vineyards were analysed (2 to 3 vineyards *per* region), and a total of 32 grape samples were taken, of 50 berries each. The mycoflora of grapes was evaluated by plating methods. During this study a total of 3623 strains were isolated and identified to genus level. The *Aspergillus* and *Penicillium* strains were identified to species level. The differences in the mycoflora of grapes between the 4 regions were analyzed using the non-parametric test Kruskal-Wallis H. Ostensibly, the classification of the grapes into their geographical origin based on its mycoflora was attempted using a decision tree algorithm (C4.5) based on the Shannon Information Theory. The success of the models to classify and predict the region of origin of the samples was compared. Furthermore, due to the increasing interest on the presence of *A. carbonarius* in grapes due to ochratoxin A (OTA) production, the relationship between the presence of this species in grapes and the remaining mycoflora was studied trough the Spearman correlation coefficient (r_s).

Twenty-seven (27) genera of fungi were identified. The most frequent genera in grapes by descending order were *Cladosporium*, *Botrytis*, *Alternaria*, *Aspergillus*, *Penicillium*, *Aureobasidium*, *Rhizopus, Epicoccum* and *Trichothecium*, with a mean frequency in the samples of 54%, 36%, 36%, 34%, 32%, 10%, 8%, 6% and 2%, respectively. The mean frequency of the remaining 18 genera in the samples was below 2%. Three genera varied its incidence significantly according to the region of origin of the samples: *Aspergillus*, *Botrytis* and *Ulocladium*. The mean incidence of *Aspergillus* and *Botrytis* in Vinhos Verdes samples was significantly lowest and highest than in the other regions, respectively. *Ulocladium* was significantly higher in Alentejo than in the other regions. The 524 *Aspergillus* strains identified belonged to 14 species. The most frequent were by far black aspergilli, namely *A. niger* aggregate (79% of the isolated strains) and *A. carbonarius* (13%). The only species that varied significantly its frequency between regions was *A. niger* aggregate, in the same way as the genus *Aspergillus*. The 446 *Penicillium* strains identified belonged to 25 species. The most frequent were *P. brevicompactum* (32%), *P. thomii* (29%) and *P. glabrum/spinulosum* (14%). Six *Penicillium* species differed significantly between regions. Using decision trees it was possible to classify successfully 91% of the samples according to 3 sample classes: Vinhos Verdes, Douro and South samples (Alentejo and Ribatejo). In the model it was used as decision criteria the low incidence (≤ 8%) of *A. niger* aggregate in grapes to classify the Vinhos Verdes samples and the highest incidence of *P. thomii* (> 4%) in Douro grape samples to separate this region from the South, but it could not discriminate accurately between the two south regions, Alentejo and Ribatejo. The estimated predictive ability of the model in the 3 classes was 82%.

Regarding the OTA producing species *A. carbonarius*, its presence varied between regions, but not significantly with the statistics tests used. The strongest significant correlation found between *A. carbonarius* and the remaining species was with the *A. niger* aggregate (r_s = 0.539, P<0.001).

The data presented here indicate that grapes are consistently exposed to a particular mycoflora that varies according its geographical origin. The positive significant correlation of *A. carbonarius* with the low OTA producing *A. niger* aggregate species suggests that the *A. niger* aggregate presence in grapes may indicate the presence of the rarer species *A. carbonarius*.

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OCCURRENCE OF OCHRATOXIN A PRODUCING FUNGI IN WINE AND TABLE GRAPES IN ISRAEL

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A 3-year survey was conducted to assay the number and *in vitro* ochratoxin A (OTA) production capacity of *Aspergillus* section Nigri isolates in 10 vineyards in Israel. The survey included field sampling of two wine cultivars, 'Sauvignon Blanc' and 'Cabernet Sauvignon' as well as the table grape cultivar 'Superior'. The major finding, which confirms previous reports in other countries, is that *A. carbonarius* is the most consistent producer of OTA, with approximately 35% of the isolates identified as positive *in vitro*. In comparison, approximately 5% of other isolates from the *A. niger* aggregate produced OTA *in vitro*. In contrast, none of the isolates with a uniseriate head morphology could be proven to produce OTA. The number of isolates, collected early in the season was less than 10-fold compared to veraison, and this number doubled at harvest. In general, the composition of isolates in the samples did not differ during berry development. The total number of positive isolates fluctuated from year to year, but a few vineyards were generally more contaminated than others with positive isolates. On the average, more OTA producing isolates were retrieved from the surface of the table grapes cv. 'Superior' compared to 'Sauvignon Blanc', but this difference could also be attributed to the larger size of the berry that was in contact with the sampling medium. None of the samples collected at harvest contained traces of OTA in the juice. The results of this study prove that, as in all the countries around of the Mediterranean basin, Israel suffers from contamination of the grape berry surface by fungal producers of OTA. The findings of this study could facilitate better management of the risks posed by OTA to grape products.

PRESENCE OF BLACK ASPERGILLI AND OCHRATOXIN A IN VINEYARDS IN ITALY

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The presence of black aspergilli in grapes grown in Italy was investigated and the effect of environmental and cultural factors able to influence fungal incidence and OTA presence studied. In the period 2001-2004, black aspergilli were present in bunches starting from setting but their colonisation of berries increased at early veraison. *A. carbonarius* was never dominant at the different growth stages, or in different geographic areas and years. Nevertheless, it was confirmed as the key fungus because of the relevant percentage of high OTA producer isolates in the population. Statistical correlation was never found between the number of strains able to produce OTA, collected from each vineyard at different growth stages, and OTA content in bunches.

Role of environmental and cultural factors able to influence fungal incidence and OTA presence were considered. The effect of geographic area on fungal flora was confirmed by statistical analysis that showed a significant effect of location and year, even if a major role was played by meteorological conditions, both on fungal colonisation and OTA content in bunches. The effect of cropping system has not yet been clarified, for example, time of harvesting seems to be more relevant than grape variety, but grape variety, perhaps in relation to time of ripening, and use of fungicides, may also be important. In particular, white grape varieties are at a lower risk of contamination, probably because of earlier harvesting; besides, the mixture of Cyprodinil and Fludioxonil, sprayed at veraison, seems to control OTA synthesis.

This research demonstrated the possibility of defining geographic areas where the risk of OTA presence is high, low or absent; this information can be obtained from annual meteorological data, collected from veraison, onward.

DYNAMICS OF *ASPERGILLUS* AND *PENICILLIUM* SPECIES IN DIFFERENT ITALIAN VINEYARDS

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Samples of grape clusters were collected throughout the Italian territory from Piedmont to Sicily in order to investigate their fungal community with particular reference to toxigenic species. Despite the wide variations observed in the contamination levels, the average number of CFU/g of fresh weight was higher in samples collected in Southern and Central Italy, while fungal communities detected in Northern regions and in Sardinia were generally more reduced. Black *Aspergilli* and in particular *A. carbonarius*, were isolated in all the sampling areas, but were more frequent and abundant in Southern Italy. *A. ochraceus*, even if not frequently isolated, was present all over the Italian country as well as *A. flavus*. Several *Aspergillus* species were seldom detected in different areas. The most commonly isolated *Penicillium* species, particularly frequent in Northern Italy, were *P. brevicompactum*, *P. citrinum* and *P. expansum*. Few strains of *A. carbonarius* isolated from all the sampled regions, except from Sardinia, produced OTA *in vitro*. Some isolates of other species belonging to the *Nigri* group accumulated OTA *in vitro.* OTA was produced by almost all the *A. ochraceus* isolates and synthetized also by some *Eurotium* strains, *A. terreus, P. aurantiogriseum, P. expansum* and *P. variabile*. OTA, not found in samples collected in Northern Italy, contaminated very few samples, coming from Central Italy, Sicily and Sardinia. More than 70 % of samples collected in Southern Italy contained variable quantities of OTA.

FUNGAL SPECIES ASSOCIATED WITH GRAPE CLUSTERS IN LOMBARDY

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In 2003 and 2004, 13 varieties, cultivated in vineyards located in six different viticultural areas in Lombardy, were sampled at beginning of veraison, late veraison and harvest, in order to evaluate the presence of toxigenic fungi. The contamination level was higher in 2004 in comparison with 2003. In both years, the number of UFC/g of fresh weight and of the fungal species progressively increased from veraison to harvest. Black *Aspergilli* were more frequently isolated at late veraison and harvest in 2003, while in 2004 their presence decreased on mature clusters. *A. carbonarius* was isolated only at late veraison and harvest in 2003. Few UFC/g of *Eurotium* species were constantly found on berries. *Penicillium* species were either more numerous and more frequently isolated during the late samplings. Some strain of *A. aculeatus*, *A. awamori*, *A. carbonarius* and *A. foetidus*, isolated in 2003, produced ochratoxin A, while none of the black *Aspergilli*, isolated in 2004, was ochratoxigenic. The other species, isolated in both years, were no able to produce OTA *in vitro*. Very low levels of OTA were detected only in four samples collected at the beginning of veraison in 2003. Other potentially toxigenic species, such as *A. flavus*, *P. citrinum* and *P. expansum* were found on the collected samples.

OCCURRENCE AND DISTRIBUTION OF OCHRATOXIN-PRODUCING FUNGI IN VINEYARDS IN SOUTH ITALY

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Ochratoxin A (OTA), a mycotoxin produced prevalently by fungi belonging to the genera *Aspergillus* and *Penicillium*, is a common contaminant of numerous foods and feeds. The Reg. (CE) n. 466/2001 establishes the maximal tolerable level of OTA in numerous agricultural commodities. Recently, the limit of 2.0 μg kg^{-1} (ppb) has been fixed also for wine (red, white and rosé) and other wines and/or grape-juice based drinks [Reg. (CE) N. 123/2005 of 26.1.2005]. The limit will be applied after the vintage of 2005.

In last years numerous researches have been carried out in order to evaluate the risk of OTA contamination in wine and to understand its origin. The results of a five-year (1999-2004) monitoring on fungi associated to bunches and on OTA contamination in musts and wines, carried out in more than 150 vineyards of 24 cultivars located in main grape-growing areas of Puglia, Calabria, Campania, Molise and Basilicata, are reported herein.

Samples of bunches (25-50 kg) were collected in each vineyard at the vintage time established by the grower, singularly examined for bunch rot (grey mould, sour rot and secondary fungal rots), and subjected to micro-vinification for red and/or white wine. At different times, musts or wines were sampled for mycological analysis and assessment of OTA contamination.

Sour rot was the most common bunch rot, while grey mould was particularly severe on late-ripening cultivars with thin-skin berries.

Secondary rots due to *Aspergillus* sp. were observed in around 80% vineyards, mainly located in Calabria, Puglia and Molise, with an incidence generally lower than 10%. 'Chardonnay', 'Gaglioppo', 'Lambrusco', 'Montepulciano', 'Negroamaro', 'Primitivo' and 'Sangiovese' were the most susceptible cultivars, whereas 'Cabernet Sauvignon' showed the lowest incidence. Secondary rots by *Penicillium* sp. were detected in less than 50% of surveyed vineyards with an incidence generally lower than 5%. 'Cabernet Sauvignon', 'Gaglioppo' and 'Primitivo' were the cultivars more affected.

Mycological analysis revealed a high fungal contamination of musts (10^4-10^8 Colony-Forming Units, CFU, per ml) prevalently caused by *Aspergillus* sp. (84%) and *Penicillium* sp. (16%). *Aspergillus niger* Van Tieghem was largely prevalent (82%), followed by *Aspergillus carbonarius* (Bainier) Thom (14%), *Aspergillus wentii* Wehemer (1.5%), *Aspergillus aculeatus* Lizuka (0.5%) and other species. The most common species of *Penicillium* were *Penicillium variabile* Sopp (43%), *Penicillium paxilli* Bainier (27%), *Penicillium janthinellum* Biorurge (14%) *Penicillium implicatum* Biorurge (12%).

Selected isolates representative of different species were evaluated for their ability to produce OTA through HPLC analysis of cultural filtrates. *A. carbonarius* appeared the most important, if not exclusive, OTA producer. The fungus was found in all monitored areas, and was more abundant on 'Lambrusco', 'Negroamaro', 'Primitivo', 'Sangiovese' and 'Trebbiano'.

OTA was detected in about 75% of musts, prevalently those obtained by grapes with dark berries, at concentrations ranging from 0.1 to 23.8 ng ml^{-1} depending on different years, cultivars and vineyards. About 15% of must samples exceeded the maximum tolerable limit. Generally, high OTA contamination was found in musts from 'Gaglioppo', 'Negroamaro', 'Primitivo' and 'Sangiovese'.

EPIDEMIOLOGY OF OCHRATOXIN A PRODUCING FUNGI IN ORGANIC APULIAN VINEYARDS.

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The presence of pesticide may influence the epiphytic mycoflora occurring on grapes significantly. In order to evaluate this, during the 2004 grape growing season, we studied the occurrence and toxigenicity of black aspergilli on grape bunches in eight organic vineyards of Primitivo and Negroamaro variety located in the Salentum area. 320 samples of bunches were processed and the CFU/g of fresh berries were calculated for the three predominant black aspergilli found: *A. niger* aggregate, *A. carbonarius* and *A. japonicus*. *A. niger* aggregate was predominant from early veraison to ripening representing 80-85% of contamination, *A. carbonarius* increased from veraison to ripening to *A. japonicus*'s detritment and represent 15-20% of

contamination in the late growing stage. The OTA contamination of processed berries was assessed and the results were correlated with the incidence of black aspergilli population, and, in particular, with the increasing CFU values of *A. carbonarius*. 242 black aspergilli representative of the sampling were isolated, identified and characterized for OTA production. Only two of 164 *A. niger* aggregate strains resulted OTA producers, while all the *A. carbonarius* (53 strains) and none of the *A. japonicus* were positive to OTA production. This evidence confirmed that overall the mycoflora responsible of OTA contamination in grapes and wine is mainly associated to *A. carbonarius*.

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ASPERGILLIA AND *PENICILLIA* ON GRAPES IN SOME SICILIAN VINEYARDS.

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Observations on species of *Aspergillus* and *Penicillium* associated with berries were carried out in Western Sicily, from 1999 to 2003. In particular, the investigated vineyards were located in several areas of Palermo, Trapani and Agrigento, and the tested wine cultivars, red and white, were the following: Cabernet, Catarratto, Grecanico, Grillo, Insolia, Malvasia, Merlot, Nero d'Avola, Sirah and Trebbiano.
In the first two years (1999 – 2000), the observation were carried out at harvest and post-harvest, while, from 2001 at middle veraison and pre-harvest also.
The same percentages of species of *Penicillia* an *Aspergillia* were detected during 1999 and 2000, while, despite the wide variations in the contamination levels, the population of genus *Aspergillus* was always prevailed over genus *Penicillium*, from 2001 to 2003.
In all the sampled areas *A. niger* is the most frequent, while *A. carbonarius*, *A. ochraceus* and *A. flavus* were not frequently isolated. During our observations, population of *Penicillia* shown an high biodiversity, and only in the last year *P. verrucosum* was isolated.
The Istituto per la Viticoltura di Conegliano Veneto is still testing the presence of OTA in different samples of berries and wines.

SURVEY OF BLACK ASPERGILLI IN SICILY

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Black *Aspergilli* are common fungi responsible for post-harvest decay of fresh fruits and they are found on the surface of healthy grapes at all stages. To evaluate the incidence of fungi responsible for ocratoxin A (OTA) production in Sicily, a systematic survey has been scheduled in vineyards representative of sicilian grape-growing areas. Monitoring of air and soil was performed with the aim of studying the dynamics of *Aspergilli* populations in fields and results were expressed as colony forming units per gram of dry soil. Isolates of *Aspergillus* spp. were purified, characterised by morphological and molecular methods. Fungi responsible for OTA production in grapes are quite present in all vineyards: *Aspergilli* section *Nigri* are dominant to *Penicillia*. *Penicillium verrucosum* was not found. Most of black *Aspergilli* strains were identified as belonging to the *A.niger* aggregate. *A.carbonarius*, the main OTA producers in grapes, was also found and was readily recognized. The capacity of the isolates to produce OTA is under evaluation.

OCHRATOXIN A AND FILAMENTOUS FUNGI EVALUATION IN GRAPES (*VITIS VINIFERA)* FROM SANTA CATARINA STATE, SOUTHERN BRAZIL

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The quality of wines has been evaluated traditionally according to its organoleptic properties. Recently, safety issues have been raised, such as pesticide residues and mycotoxins (*e.g.*, ochratoxin A), with the introduction of new agricultural practices and the development of analytical methods with higher sensitivity. Ochratoxin A (OTA) is produced by some *Aspergillus* and *Penicillium* species and its contamination of wine is now a major safety issue.

The mycobiota and OTA in Southern Brazilian grapes were assessed. 30 samples of grapes, collected in 16 vineyards from the two most important wine sub-regions of the State of Santa Catarina, Brazil, were analysed for their micobiota and 9 were also analysed for OTA. The mycobiota was evaluated by plating 10 grapes from each sample in Dichloran Rose Bengal Chloranfenicol Agar and Sabouraud Dextrose Agar, supplemented with chloranfenicol. Czapeck Yeast Agar was used for the assessment of OTA production by black *Aspergillus* strains. OTA determination from grapes was by chromatography with immunoaffinity clean-up.

Cladosporium (found in 86.7% of plated berries), *Alternaria* (80.0%), *Botrytis* (70.0%), *Aspergillus* (66.7%), *Penicillium* (63.3%), *Aureobasidium* (30.0%), *Rhyzopus* (16.7%), *Fusarium* (13.3%), *Epicoccum* (10.0%), and *Nigrospora* (6.7%) were the main fungi isolated from the grapes. 62 *Aspergillus* strains were isolated, of which 16 were black. All these black *Aspergillus* were from the *A. niger* aggregate and from which OTA was not detected. OTA was analysed in 9 samples, from which 6 were positive, at levels from 0.16 µg /Kg to 0.77 µg/Kg.

In conclusion, black *Aspergillus* strains were found in grapes, but, despite of not being able to produce detectable amounts of OTA, OTA was also found in some grape samples.

ASPERGILLUS SECTION *NIGRI* AND OCHRATOXIN A IN ARGENTINIAN WINE GRAPES

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The evolution of contamination with *Aspergillus* section Nigri and ochratoxin A occurrence was evaluated in four vineyards located at Mendoza province, Argentina during 2003-2004. The survey included two grape varieties, one of late maduration (*Bonarda*) and other of early maduration (*Tempranillo*). The vineyards were set under conventional and organic cropping systems. Bunches of grapes at different growth stages were collected, and berry samples were plated on Petri dishes containing Dichloran 18% glycerol agar (DG18) and Dichloran Rose Bengal Chloramphenicol agar (DRBC) media (Pitt and Hocking, 1997). After a incubation period of 7 days at 25 ºC ± 1 ºC, the mycoflora belonging to *Aspergillus* section *Nigri* was identified. The ability to produce ochratoxin A (OTA) by the potential ochratoxigenic species was evaluated on YES medium (2% yeast extract, 15% sucrose). The cultures were incubated at 30 ºC ± 1ºC for 10 days in darkness. The OTA content of the grapes was determined by HPLC. Through the different growth stages, from setting to harvest, the grapes increased the contamination with Aspergillus section Nigri. The main species isolated belonged to *A. niger* aggregate. From 246 strains evaluated 24% were ochratoxigenic. The levels of toxin produced ranged from 1.3 to 50 ng/mL of culture medium. OTA was not detected in grapes during the survey, considering a detection limit of 1 ng/g. The climatic conditions were recorded and were quite different from those recorded in a survey carried out in Italy. It appears that the climatic differences among different grape-growing areas may be responsible for differences in OTA contamination in grapes.

MANAGEMENT OF *ASPERGILLUS CARBONARIUS* IN AUSTRALIAN VINEYARDS

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On grapes in Australia, three species of *Aspergillus* section *Nigri*, viz. *A. aculeatus*, *A. carbonarius* and *A. niger*, can cause Aspergillus bunch moulds. All Australian isolates of *A. carbonarius* produce the mycotoxin, ochratoxin A (OTA), an undesirable fungal by-product in grapes. To ensure the integrity of Australian dried grapes, research was conducted to increase knowledge of the biology and management of *A. carbonarius* in vineyards.

Surveys of vineyards in north west Victoria showed that soil under vines was the primary source of *A. carbonarius* and spores of *A. carbonarius* were dispersed by wind from the soil to grape berries on vines. Vineyards with a high incidence of *A. carbonarius* in soil and air during the growing season had a high incidence of spores of *A. carbonarius* on grapes at harvest. Incidence of *A. carbonarius* was highest in surface soil under vines (to a depth of 0-1 cm) and in air near the soil surface (at a height of 10 cm). Differences in the incidence of *A. carbonarius* in soil and on grapes occurred between seasons in the same vineyard and between vineyards in some seasons.

Soil type, temperature and moisture influenced the survival of *A. carbonarius* in vineyard soils. Temperatures of 15-35^{o}C were most favourable for the survival of *A. carbonarius* in dry soil. Highest reductions in survival were observed when initial soil saturation was over 50% by weight at optimum temperatures for *A. carbonarius*. High temperatures and moisture, after soil was initially dry and in an aerobic condition, completely eliminated *A. carbonarius*. Reductions in the survival of *A. carbonarius* were higher in soil with higher water-holding capacities.

Floor management also influenced the incidence of *A. carbonarius* in vineyards. Incidence of *A. carbonarius* in soil was highest in vineyards where alternate vine rows were cultivated every fortnight throughout the vine-growing season and green manure crops were sown into the cultivated rows. Incidence of *A. carbonarius* was lowest in the soil of vineyards where there was no cultivation for three years and the vineyard floor between vine rows was covered with weeds and grasses. Incidence of *A. carbonarius* in soil decreased after irrigation but later increased when the soil dried. Amendment of soils with selected cover crop materials (eg. lucerne, oat or rye hay) also reduced populations of *A. carbonarius.*

This and previous research showed that selected vine and vineyard floor management practices, along with the use of grape varieties with resistance to rain damage, can minimise the incidence of *A. carbonarius* and Aspergillus rot in bunches on vines and OTA in dried grapes. In Australia, these management practices are being adopted and used with dried grape testing and processing strategies to optimise the quality of dried grape products. In years when the risk of mould damage is significant, consignments of dried grapes from producers are tested before they are processed to ensure that they are not contaminated with OTA. During processing, laser scanners are used to identify and remove dark berries that may have moulds. Further testing after processing is also used to ensure product integrity.

INFLUENCE OF FARMING SYSTEM AND CLIMATE ON OCCURRENCE OF *ASPERGILLUS* SECTION *NIGRI* IN VINEYARDS OF THE MEDITERRANEAN AREA

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Grapes contamination with ochratoxin A (OTA) is related to *Aspergillus* section *Nigri* infection. These fungi are present on bunches at setting, but their incidence increases at veraison and till ripening with relevant differences in incidence of infected berries among geographic areas and years. The aim of this study was to evaluate the influence of cultural and environmental factors on black aspergilli incidence in vineyards of the Mediterranean area.

Data on incidence of contaminated berries at harvesting, collected yearly in more than 100 vineyards in France, Greece, Italy, Israel, Portugal and Spain during the 3-year period 2001-2003, were included in a database. Questionnaires were prepared including queries on general aspects of vineyards and on the cropping system. Data obtained from questionnaires were organised to be included in a database. In particular, for each variable considered, the answers were attributed to a group, and between 3 and 5 groups were defined for each variable. I.e., soil type was divided in 1) sandy, 2) loamy and 3) clayey and all answers were included in these 3 groups. Meteorological data (mean daily temperature and relative humidity and summation of rain) were collected for all vineyards. Summation of daily mean air temperature and rain during August were also added to the database.
Data on the incidence of infected berries at harvesting were standardised on yearly basis. Then, mean incidence of infected berries, per vineyard for all years, was computed. Two groups of vineyards were separated: 1) *low incidence*, with those vineyards having 3-year mean lower than zero; 2) *high incidence*, with those vineyards having 3-year mean higher than 0. This variable was considered as a group variable for further data analysis.
Correspondence analysis (CA) was applied to data on training system and grape varieties to group them, using the software package SPSS ver. 11.5.1. The aim of this analysis is to account for as much variation in the data as possible; plots show the relationships between categories of the variables. Five and four groups were defined for grape variety and training system respectively.
Discriminant analysis was run using the software package SPSS ver. 11.5.1. This statistical model looks for the best linear combination of variables for distinguishing among several groups. Variables included in the discriminant analysis were: soil type, number of plants per hectare, altitude/exposure, age of the vineyard, grape colour, grape variety, summation of mean daily temperature and daily rain in August. Data on cropping system, like manuring, irrigation or pest and disease control, were not included because they were frequently not complete or not reliable.
A discriminant model was first run including only general vineyard features; it classified correctly 77% of cases. The addition of meteorological data increased the discriminant capacity of the model and the number of cases classified correctly increased to 84%. In 8% of cases the model operated an overestimate, classifying vineyards with low incidence to the wrong group (high incidence), and in 8% of cases an underestimate. The most relevant variables to discriminate low and high incidence of black aspergilli in vineyards were soil type and grape variety; each of these variables were able to classify correctly more than 60% of cases when considered alone. Rain during August increased the discriminant capacity of the model, in particular abundant rain was associated to low incidence. The most favourable soil was clay and local varieties grown in France, Greece, Italy, Portugal and Spain were the more susceptible. These results could contribute to the development of a Decision Support System (DSS) planned to manage the grape-wine chain to maintain OTA as low as possible.

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SAMPLING OF BUNCHES IN VINEYARD TO ASSESS OCHRATOXIN A CONTENT IN MUST

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Quantification of mycotoxins in foods and feeds is a relevant issue, because of the risk of harmful effects on human and animal health and the importance to make correct decisions when a lot must be classified as acceptable or unacceptable with respect to a legal limit. Specific studies were managed to design sampling plans for the determination of mycotoxins, but all these studies examine post-harvest mycotoxin quantification procedures, even if most mycotoxin problems originate in the field.
The aim of this work was to study the spatial variability of OTA in grape vineyards and to define a reliable sampling protocol for grape bunches, in order to assess OTA content before harvesting; a correct quantification of OTA in vineyard would enable a better management of the wine production chain to reduce the risk for consumers.
In 2002, two vineyards placed in San Pancrazio Salentino (Brindisi), in Southern Italy, were chosen. Grape varieties 'Negroamaro' and 'Sangiovese' were cultivated respectively in vineyard 1 and 2 with 'Alberello' (head-trained spur pruned) and 'Tendone' (overhead trellis, cane pruned). The same sampling design was applied to both vineyards. Ten plants were chosen following the X-shaped path of the whole vineyard and 10 plants along the 2 central cross perpendicular lines; all bunches on the East-exposed branch were collected from the 20 plants in each vineyard. Bunches harvested from the X-shaped path, in both vineyards, were numbered progressively, weighted and crushed separately; juices obtained were analysed for OTA content. Bunches obtained from each plant on the cross perpendicular lines were crushed together and juices obtained were

analysed for OTA content; HPLC was used for all samples. Basic statistic and analysis of spatial correlation was applied to data.
A simulated approach for sampling design was used to assess the influence on measured OTA content. Three different designs were followed: a) completely randomised sampling; b) sampling in a pre-defined position on each plant (systematic); c) taking a fixed number of samples from 1-2 plants.
High variability was observed in OTA content both among bunches and among plants in the two vineyards, despite different contamination levels. Very high amounts of OTA were detected in 1 or 2 strongly contaminated bunches in both vineyards. This variability is totally random; in fact, no relation was found between OTA content and the position of a bunch on the branch or the location of the plant in the vineyard.
Sampling design markedly influenced the assessment of must contamination. The best results were obtained when sampling was planned taking into account plants and when bunches were collected in a pre-defined position, especially if central in the branch. The difference from mean OTA contamination of the vineyard decreased when the number of sampled plants increased. A reduction of variability was also obtained, as expected, increasing the number of sampled bunches.
Because of the large variability among OTA levels detected in bunches collected from the same plant and in the same vineyard, estimated means differed from calculated mean OTA content in both vineyards. If the true contamination in a vineyard is 2 µg Kg^{-1}, i.e. the limit fixed in Europe for OTA content in must and wine, the range of OTA content assessed by sampling 1 bunch in the central position of 10 plants should lie between 2.9 and 1.4 µg Kg^{-1}. The accuracy could be considered acceptable and good indications can be obtained for the management of the must.

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EFFECTS OF FUNGICIDES ON OCHRATOXIGENIC BLACK ASPERGILLI

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Ochratoxins are mycotoxins which exhibit nephrotoxic, immunosuppressive, teratogenic and carcinogenic properties The ability to produce ochratoxin A (OTA) by members of *Aspergillus* section *Nigri* (black aspergilli) has been demonstrated and *Aspergillus carbonarius* has been reported as the main responsible of the OTA levels found in grapes and wine.

A study to evaluate the impact of the application of several fungicide treatments to grapes on *A. carbonarius* growth and OTA production was developed. The experimental design was divided in 3 trials in order to: i) screen 26 fungicides used in Spanish vines, assayed at doses recommended by manufacturers, to test their efficiency against *A. carbonarius* growth and OTA production on a grape-like synthetic nutrient medium (SNM) at 20 and 30 ºC; ii) find out the minimum dose of each fungicide that allows the growth of *A. carbonarius* on SNM; iii) investigate the effect of several fungicides on growth and OTA production of *A. carbonarius* inoculated on grapes.

i) Results showed that 10 fungicides reduced the growth rate of *A. carbonarius*, meanwhile 13 completely inhibited its growth. OTA production was in general higher at 20 ºC than at 30 ºC. Fungicides that stopped fungal growth also inhibit OTA production. Fungicides that reduce *A. carbonarius* growth, also reduced OTA production, except in 4 treatments which in general favoured OTA production.

ii) For this study the 13 fungicides selected were those that prevented *A. carbonarius* growth in the previous experiment. Temperatures assayed were 20 ºC and 30 ºC. The doses used for this trial were: D (dose recommended by the manufacturer), d1 (3/4 D), d2 (1/2 D), d3 (1/4 D), d4 (1/10 D), d5 (1/100 D) and d6 (1/200 D). Results showed that each fungicide had a different effect on *A. carbonarius* growth, but most of them allowed growth at d4. At the optimum temperature for *A. carbonarius* growth, 30 ºC, higher amounts of fungicide were needed than at 20 ºC to prevent fungal growth.

iii) Disinfected table grapes, were dipped in a fungicide solution for 30 seconds. 3 fungicides (F3: azoxystrobin; F14: fenhexamid 50%; F 26: copper hydroxide 80%+copper 50%) were used at the dose recommended by the manufacturer and 3 more (F1: penconazole 10%; F9: cyprodinil 37,5% + fludioxonil 25%; F23: mancozeb 80%) were used at d4 (1/10 D). A spore suspension of 2 *A. carbonarius* isolates (W38 and W120) were sprayed separately onto the grapes. Regarding fungal growth, best results were achieved with F9, as percentages of infection were greatly reduced, with maximum reduction of 100 % for W120 strain at 20 ºC. F1, F3 and F9 fungicides showed a reduction of the OTA production, that was higher at 20 ºC than at 30 ºC. With the other fungicides, and in some cases, an increase on OTA production was observed.

Finally, a field trial on the effect of two fungicides on ochratoxigenic fungi of grapes was developed. The fungicides used were Switch (cyprodinil 37,5% + fludioxonil 25%) and/or Chorus (cyprodinil 50%), always combined with a treatment with the insecticide Lufox. Switch seems to be active against black aspergilli as in

most cases vineyards treated with a double application of this fungicide and a high application of Lufox showed the minimum percentage of black aspergilli on grapes. Two other different treatments with Chorus also gave good results but in both of them one application of Switch at veraison was carried out. It would be interesting to find out how this fungicide affects the different species in the section Nigri.

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INFLUENCE OF INTEGRATED PROTECTION OF VINEYARDS ON OCHRATOXIN PRODUCING FUNGI IN SOUTH ITALY

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Ochratoxin A (OTA) contamination in wine is a phenomenon arising in vineyards but causing severe problems to wine industry. The Reg. (CE) N. 123/2005 of 26.1.2005 establishes in 2.0 μg kg^{-1} the maximum tolerable level of OTA in wine (red, white and rosé) and in others wine and/or drink based on grape-juice. Healthy grape is essential to produce wine of high quality and with OTA concentrations lower than the new limit. In this respect, integrated crop protection strategies in vineyards play a key role to avoid or reduce OTA contamination in grapes and wine.

During the last five years (1999-2004), field trials were carried out in order to evaluate the effectiveness of different spray schedules against ochratoxigenic fungi and other predisposing factors (i.e., powdery mildew, grape moth) on OTA contamination in wine. This reports deals with results of comparison of spray schedules based on fungicides selected among those with the highest activity *in vitro* against *Aspergillus niger* and *Aspergillus carbonarius*.

Filed trials were carried out in vineyards cv Aglianico, Gaglioppo, Montepulciano, Negroamaro and Primitivo, grown with different training systems (small tree "alberello" or wall-trained tree). The vineyards were divided in 1,500-5,000 m^2 plots that were submitted to different spray schedules based on cyprodinil+fludioxonil (37.5+25%; Switch, Syngenta Crop Protection; 800 g ha^{-1}); pyrimethanil (37.4%; Scala, Basf Italia; 2,000 g ha^{-1}) and iprodion (50%; Rovral, Basf Italia, 1,500 g ha^{-1}). Fungicides were applied using farmer engines and distribution volumes of 600-1,000 l ha^{-1}. The sprays were carried out according to the normal spray schedule used against grey mould. Treatments were carried out at the following phenological stages: just before berry touch (B), véraison (C) and at the end of August (D). The last spray was not done on 'Primitivo' (early ripening cultivar). The fungicides were tested either alone or in alternation with others.

At the vintage time, one sample of bunches (25-50 kg) was collected in each plot, observed for assessing incidence of grey mould, sour rot and secondary fungal rots and then submitted to micro-vinification. Samples of must or wine were submitted to mycological and chemical analyses.

Grey mould was particularly severe in raining and wet seasons. All tested spray schedules controlled effectively the disease as compared to the untreated check. The spray at the stage of pre-berry touch (B) proved to be more effective than that carried out at véraison (C), but less than the spray done at the end of August (D). As expected, none of tested schedules was able to control sour rot. Berry rot caused by *Penicillium* sp. was only erratic in vineyards, whereas berries with obvious *Aspergillus* sp. sporulation were found in all experimental fields. Several combinations of spray with cyprodinil+fludioxonil, pyrimethanil and iprodion limited effectively rot severity showing, on the average, a protection level of about 50% as compared to the untreated check. The control was particularly appreciable in vineyards were *Aspergillus* sp. was more abundant on bunches.

As to the fungal contamination in musts is concerned, the most effective schedules decreased the number of cfu (colony forming units) per ml of must of *Aspergillus* sp., but less those of *Penicillium* sp.. On the average, cfu of *A. niger* and *A. carbonarius* were reduced, respectively, of 30% and 50%.

In agreement with above reported data, OTA contamination in musts and wines was decreased of around 50% as a result of the most effective spray schedules, especially in vineyards where fungicide distribution was accurate.

LABORATORY STUDIES OF FUNGICIDES USED ON GRAPEVINE TOWARDS MYCOTOXIN-PRODUCING FUNGI

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Among the various microorganisms detected on grape-wine berries after veraison, several species of *Aspergillus* and *Penicillium* produce mycotoxins which can contaminate grape-juice and wine. Laboratory trials were conducted to study the effect, on the growth and germination, of fungicides (twenty three) used in vineyards, against several *Aspergillus* and *Penicillium* (four species isolated on berries and four others of collections).
The most toxic active ingredients were fluazinam, fludioxonil, cyprodinil, pyriméthanil and carbendazime.
Seven fungicides (among the twenty three tested before), choosen for their potential interest in practice were tested in-vitro against two strains of *Aspergillus carbonarius* and one strain of *Aspergillus alliaceus*, producing ochratoxin A (OTA). The impact of the fungicides on the production of ochratoxin A of these strains was tested in this case. Inhibitors of respiration like fluazinam, folpel and azoxystrobin as well as fosetyl-Al, and pyrimethanil decreased the production of the mycotoxin. On the other hand, difenoconazole and carbendazime induced an increase of the production of this mycotoxin.
These results are compared those obtained in field trials.

INHIBITION OF OCHRATOXIN A SYNTHESIS BY NEW SYNTHETIC COMPOUNDS

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Ochratoxin A is a mycotoxin produced by some *Aspergillus* and *Penicillium* species which is usually detected in beer, cereals, coffee, feeds, figs, sultans or wine. Some fungicides have been found to be effective in preventing fungal growth, but, in same cases an increase in the synthesis of mycotoxins was reported. Also, the pressure to use less aggressive compounds to the environment stimulates the exploration of new and better compounds.

This purpose directed the choice of synthetic compounds that were prepared and tested on the growth and on ochratoxin A production of an *Aspergillus alliaceus*, an *A. ochraceus*, an *A. carbonarius* and an *A. niger* strain. Most of these new synthetic compounds have a linear structure incorporating a urea and/or a phenolic unit. Fungi were grown in YES media supplemented with 50 μM of each one of the tested compounds in triplicate, for 6 days. Growth was recorded by measuring the diameter of colonies each 24 hours, and ochratoxin A was quantified after 5 days of growth using HPLC and fluorescence detection.

Growth of the *A. ochraceus* and the *A. carbonarius* strain was not inhibited by most of these compounds. However, some lead to a decrease in ochratoxin A synthesis. Compounds without the phenolic unit were found to be less effective, while those compounds with both urea and phenolic units were the most effective, inhibiting ochratoxin A synthesis up to 90%. The *A. alliaceus* and *A. niger* strains growth was inhibited by one of these compounds in 22 and 27%, respectively and in less extent by others (6 to 13%).

This approach will lead to the selection of functional groups able to inhibit the synthesis of ochratoxin A which could be inserted into more powerful antifungal compounds. These data, as well as the relation between the chemical structure of the synthetic antifungal compound and the inhibition of growth and of ochratoxin A production, will be discussed.

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NATAMYCIN EFFICACY FOR CONTROL OF GROWTH AND OCHRATOXIN PRODUCTION BY *ASPERGILLUS CARBONARIUS* ISOLATES UNDER DIFFERENT ENVIRONMENTAL REGIMES

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Natamycin is a fungicide which is produced by *Streptomyces natalensis* and commonly used in food products for controlling spoilage, especially by moulds. It has a broad spectrum of activity against spoilage moulds and is considered to be a very stable product with efficacy against some mycotoxigenic species. However, no studies have been previously carried out to examine effectiveness against *A. carbonarius* growth and OTA production. We tested the effect of natamycin in the range 0-10 ppb for control of growth of two isolates of *A. carbonarius* at 0.98-0.94 a_w and 15-25^oC on a red grape extract medium. Inhibition of growth was dependent on temperature and a_w level. At 15^oC, 5-10 ppb of natamycin was effective at reducing growth almost completely. However, at 20-25^oC and all three a_w levels growth was only slightly inhibited by 5-10 ppb natamycin.

There were some isolate differences with regard to inhibition of OTA. OTA was stimulated by 1 ppb natamycin at 15^oC with 10 ppb required to inhibit OTA production by >90%. However at 0.96 and 0.94 a_w almost complete inhibition occurred. At 20^oC, OTA production was only significantly inhibited by 10 ppb natamycin at 0.94 a_w. At 0.96 and 0.98 a_w some inhibition occurred by 5-10 ppb but greater concentrations would be required for effective inhibition. At 25^oC 5 ppb was effective at all a_w levels. This suggests that under optimum temperature x a_w conditions for *A. carbonarius* growth the fungicide was least effective with > 10 ppb required. However at 15 and 25^oC and a wide range of aw levels natamycin can effectively control OTA production. Natamycin is considered to be effective at 5 ppb against a range of other mycotoxigenic moulds. However, for food applications where *A. carbonarius* needs to be controlled probably at least 10 ppb is required.

IN VITRO EVALUATION OF EPIPHYTIC VINE YEASTS ACTIVITY FOR BIOCONTROL OF OCHRATOXIGENIC GRAPES' MOLDS

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One hundred and fourty-four strains of epiphytic micro-organisms, isolated from berries of "Negroamaro ", an apulian grape wine variety, were screened for antagonistic activity against *Aspergillus carbonarius* and *A. niger*. These black Aspergilli are considered the main ochratoxigenic species responsible for the accumulation of ochratoxin A in grape. Twenty eight yeast isolates were selected for their inhibitory effects on the above fungal species and assayed by an *in vitro* nutritional competition test for their antagonistic capacity towards three selected ochratogenic strains. Six yeast isolates belonging to five species, namely 2 isolates of *Issatchenkia orientalis* and one each of *Metschnikowia pulcherrima, Kluyveromyces thermotolerans, Issatchenkia terricola and Candida incommunis* species, were finally selected and screened on wounded grape berries for their ability to inhibit the contemporary infection by ochratoxigenic molds and reduce the formation of ochratoxin A. With the exception of the *K. thermotolerans* isolate, when inoculated at 10^9 CFU/wound, the other five challenger yeasts reduced the *A. carbonarius* and *A. niger* colonization on grape berry ($P < 0.05$). In particular, the best antagonistic activity was shown by the two *I. orientalis* isolates. Results suggest that antagonist yeasts with the potential to control of *A. carbonarius* and *A. niger* on grape can be found among the microflora associated with the berries.

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OCHRATOXIN A DETOXIFICATION AND ANTAGONISTIC ACTIVITY BY FOUR *AUREOBASIDIUM PULLULANS* BIOCONTROL STRAINS

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Ochratoxin A (OTA) is a possibly carcinogenic mycotoxin contaminating wine because of the attack to wine-grape by *Aspergillus carbonarius*. In this study, we assessed the ability of 4 *Aureobasidium pullulans* strains (LS30, AU14-3-1, AU34-2, AU18-3B) of a) achieving biological detoxification of OTA in *in vitro* experiments, b) exerting biocontrol activity against *Aspergillus carbonarius* on wine grape berries (cv Montepulciano) and c) their influence on OTA contamination of berries in lab-scale experiments. HPLC analyses of liquid media incubated for 6 days showed decreases in OTA concentration of 91.2%, 89.5%, 83% and 77% for strains AU34-2, AU14-3-1, LS30 and AU18-3B, respectively. Liquid chromatography-mass spectrometry analyses showed that, for all the strains, the main products of OTA degradation were the less toxic ochratoxin α (OT-α) and the aminoacid phenylalanine, putatively derived from carboxy-peptidase activity of these biocontrol agents. In biocontrol activity assays, the 4 strains lowered the levels of infections by *A. carbonarius*, especially in the case of strain AU18-3B that yielded 61.4% of disease incidence reduction. HPLC analyses of extracted berries treated with the biocontrol strains and inoculated with *A. carbonarius* showed striking decreases of OTA contamination as compared to untreated control: 88.4%, 83.1%, 72.4% and 64.0 % in the presence of AU18-3B, LS30, AU34-2 and AU14-3-1, respectively. No OT-α was detected insofar, but further experiments are in progress to confirm this finding.

CHEMICAL AND BIOLOGICAL CONTROL OF SOUR ROT CAUSED BY BLACK ASPERGILLI IN THE GRAPEVINE VARIETY AGIORGITICO OF KORINTH REGION

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Black aspergilli such as *Aspergillus niger* and *A. carbonarius* are among the main sour rot agents of wine grapes in Greece and other Mediterranean countries. Development of control measures against sour rot agents is urgently needed. This work reports data related to the evaluation of chemicals or epiphytic yeasts in minimizing the negative effect of these fungi in the field

Consecutive field tests were carried out in vineyards of Nemea region during summer 2003, 2004 and currently 2005 with the variety agiorgitico

Two applications of the chemicals Switch (cyprodinil + fludioxonil) at a rate of 0.1% and cupravit 0.5% were used (1st spray at veriason and 2nd one 20 days before harvesting).

Similarly vineyard sprays of two laboratory evaluated epiphytic yeasts were used. One designated as GY-18, originated from Xinomauro variety (*Cryptococcus laurentii)* and the second designated as K-4, originated from Robola variety (*Aureobasidium pullulans).*

The obtained data demonstrate that switch and yeast K-4 were the most effective applications during the two years of the experimentation period. Indeed for 2003 the corresponding figures for the percentage of infected bunches was 14.9% for switch, 37.4% for Cupravit, 16% for yeast GY-18, *12% for K-4*, while 33.7% for untreated control. Figures for the 2004 experiment were 10.94% for switch, 11.62% for Cupravit, 22.8% for GY-18, 10.6% for K-4 *while* 28.4% for untreated control.

As for the composition of *Aspergillus* populations the corresponding percentages for *Aspergillus niger* (A.n.) and *Aspergillus carbonarius* (A.c.) in 2003 were: A.n. 60% and A.c. 1% for switch, A.n. 63% and A.c. 15% for Cupravit, A.n. 35% and A.c. 57% for GY-18, A.n. 32% and A.c. 20% for K-4, and A.n. 58% A.c.25% for the untreated control.

Figures for the 2004 experiment were A.n. 20.5% and A.c. 9% for switch, A.n. 13.5% and A.c. 31.5% for Cupravit, A.n. 7% and A.c. 41.5 for yeast GY-18, 9% and A.c. 22.5% for yeast K-4, A.n. 9.5% and A.c. 65% for untreated control

Although the obtained data demonstrate that either switch or yeast K-4 applications significantly reduce incidence of sour rot of berries a third year experimentation is currently planned to verify our results.

CHEMICAL CONTROL OF BLACK ASPERGILLI IN DIFFERENT FARMING SYSTEMS AND VARIETIES IN RHODES ISLAND OF GREECE

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Vineyard surveys of wine producing grape cultivars Cabernet Sauvignon and Grenache Rouge on the island of Rhodes, Greece, during 2003 and 2004, demonstrated the occurrence of various *Aspergillus* spp. on berries of bunches at harvest. *Aspergillus niger*

This study provides the first evidence that chemical applications with the fungicide Switch, especially under low to intermediate *Aspergillus* infection of vineyards, could both significantly reduce the occurrence of OTA producing *Aspergillus* spp. and restrict sour rot severity in different farming systems.

Field tests were carried out in vineyards of Kameiros region (variety Cabernet Sauvignon, linear planting) and Fanes village (Grenache Rouge, cup formation) of the island during summer 2003 and 2004.

Two applications of the chemicals Switch (cyprodinil + fludioxonil) at a rate of 0.1% were used (1st spray at veriason and 2nd one 20 days before harvesting).

The obtained data demonstrate that two applications of switch were the most effective during the two years of the experimentation period. Indeed for 2003 the corresponding figures for the percentage of infected bunches in Cabernet Sauvignon was 5.65% for one application switch, 8.07% for two while 20.26% for untreated control. Figures for the 2004 experiment were 3.52% for one application switch, 3.77% for two while 23.63% for untreated control.

As for Grenache Rouge for 2003 was 11.72% for one application switch, 11.75% for two while 33.25% for untreated control. Figures for the 2004 experiment were 14.07% for one application switch, 5.78% for two, while 54.56% for untreated control.

As for the composition of *Aspergillus* populations in vineyard of Cabernet Sauvignon the corresponding percentages for *Aspergillus niger* (A.n.) and *Aspergillus carbonarius* (A.c.) in 2004 were: A.n. 22.5% and A.c. 45.5% for one switch application, A.n. 18% and A.c. 22% for two switch applications, and A.n. 4% A.c. 96% for the untreated control.

As for the composition of the populations in the experimental field of the Grenache Rouge the corresponding percentages for A.n. and A.c. in 2004 were: A.n. 21.5% and A.c. 62.5% for one switch application, A.n. 28% and A.c. 52% for two switch applications, and A.n. 10.5% A.c. 89.5% for the untreated control.

Finally OTA level in ppb quantified in must of the Grenache Rouge experiment for 2004 showed that for one switch application we had 3.45 ppb, for two switch applications 0.037 and 15,95 for the untreated control.

The obtained data demonstrate that the varieties and their formation scheme affect disease incidence and the composition of Aspergillus populations. Furthermore, indicate that two switch applications significantly reduce the OTA level. Before suggesting chemical application to the farmers a third year experimentation is currently planned to verify our previous results.

LOBESIA BOTRANA - AN OCHRATOXIN A RISK FACTOR IN GRAPEVINE MANAGEMENT

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The presence of ochratoxin A (OTA) in wine represent a recent mycotoxicological risk caused by berry contamination in field by black aspergilli, and primarily by strains of *Aspergillus carbonarius* in southern Europe. Available evidence indicates that these black aspergilli are mainly saprophytes and responsible for secondary rot. A large diversity of associations between insects and toxigenic fungi has been demonstrated. Damaged berries by wounds caused by insect may provide an entry to berry tissue for these fungi, *Lobesia botrana* is the major grape berry moth in vineyards of southern Europe. In this respect, two year study was carried out to evaluate the influence of *L. botrana* attacks on ochratoxin A accumulation in the field. In both years surveys, higher levels of OTA were found in both intact and rotten berries originating from bunches damaged by *L. botrana* larvae as compared to bunches without *L. botrana* attacks. The levels of toxin, measured in the two year harvests, ranged from 0.02 to 681 ng/g of fresh berries. This is the first evidence of an interaction between *L. botrana* damaged berries and OTA contamination, in the field. Preliminary results of a field trial showed that an accurate control of the third generation of *L. botrana* reduces the inoculum of *Aspergilli* and the formation of OTA in grapes.

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INCIDENCE OF HARVEST CONDITIONS OF GRAPES ON OCHRATOXIN A IN WINE

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An experimentation was led on Ganson (red metis Jurançon x Grenache) in Herault. A block randomized with 4 repetitions was organized. Different protection programs were compared. The control had only a basic mildiou and oïdium protection. The protected part was issued of 3 different lots. A complete program against Lobesia botrana was applied (on 1st, 2nd and 3 rd generation); cyprodinil + fludioxonil (SWITCH), 1 kg/ha, was added either at stage C or D.

On the 24/8 there wer 0,07 perforations per bunch on the protected, 4,49 on the control. The attacks of Botrytis and Aspergillus are reported in the following table.

		Control	Protected
Botrytis	Frequency	55.6 a	23.4 b
	Intensity	7.0 a	1.5 b
Aspergillus	Frequency	99.4 a	10.5 b
	Intensity	51.9 a	0.5 b

Each part was harvested and split into 2 lots: one kept intact, the other one gently pressed in order to extract 2 % of juice. There was a delay of 5 h before crushing at the experimental wiery. Analysis of ochratoxin A were realized on must, fermenting must after 3 days and 5 days, wines after alcoholic and malolactic fermentations, and wine in bottle.

The levels of ochratoxin A in bottle were the following:

	ochratoxin A (µg/l)
Protected intact	0,21
Protected maceration	0,3
Control intact	6,23
Control maceration	11,62

The protected grapes have very low levels of ochratoxin A (0,21 and 0,3 µg/l). There is no significant incidence of maceration during harvest and transportation of grapes.
The control grapes have very high level of ochratoxin A (6,23 and 11,62 µg/l, 3 to 6 times higher than the UE regulation at 2 µg/l). This confirms the importance of pest control, and particularly grape moth control, on the prevention of ochratoxin A in wines.
On control grapes, maceration increases significantly the level of ochratoxin A: + 87 %. During transportation, a light grape maceration was enough to induce ochratoxin A synthesis by *Aspergillus* mycelium present on grapes.

INCUBATION TIME AND TEMPERATURE EFFECTS IN OCHRATOXIN A PRODUCTION BY *ASPERGILLUS CARBONARIUS*

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Wine is considered, after cereals, the second major source of OTA intake. There is a great concern on this metabolite in the Mediterranean countries because many results show that wines from the Mediterranean area contain higher levels of OTA than wines from Northern areas in Europe. Many surveys carried out in different countries from Southern Europe, with Mediterranean climate, have reported that *Aspergillus* section *Nigri* (black aspergilli), in particular *A. carbonarius*, is the main responsible of OTA production in grapes. It is therefore very important to know the kinetics of accumulation of OTA by this species, and the impact of field temperature in it.

The effects of incubation time (up to 10 days) and temperature (7, 15, 20, 25, 30, 35 and 42 ºC) on OTA-producing capacity and OTA accumulation by *A. carbonarius* isolated from grapes, were studied on grape-like medium. The limits of growth and OTA production have been identified. OTA production was significantly higher at 20 ºC, followed by 25, 15, 30, 35 ºC. No growth was observed at 7 ºC and 42 ºC after 10 days of incubation and consequently, no OTA was detected at these temperature levels. In general, maximum OTA-producing capacity was found earlier with increasing incubation temperatures. However, at 35 ºC, OTA was rarely detected although growth was maximum at this temperature. OTA accumulation was maximum after 10 days of incubation for all the temperatures except at 30 ºC, where the maximum was detected at earlier incubation time (6-8 days) and then remained stable.

As a conclusion OTA production and accumulation may occur in the temperature range 15-30 ºC and in time periods as short as 2-4 days. OTA accumulation which has been reported in grapes in the field near harvest (average of minimum, mean and maximum temperatures in Spain at harvest = 15.5, 22.0, 31.5 ºC, respectively) might optimally occur during the night when temperature decreases to optimum levels for OTA production.

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OBSERVATIONS ON OCHRATOXIN A PRODUCTION IN *ASPERGILLUS CARBONARIUS* AND *ASPERGILLUS NIGER*

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Ochratoxin A (OTA) is a mycotoxin frequently found as contaminant of a great variety of agricultural commodities all over the world. Originally isolated as secondary metabolite of *Aspergillus ochraceus* Wilhem, OTA is produced by several species of *Aspergillus* and *Penicillium*. Fungal and OTA contamination were determined in grapes and musts from vineyards located in South Italy. More than 80% of the samples analysed by direct plating showed contamination by fungal propagules recognized as *Aspergillus niger* Van Tieghem or *Aspergillus carbonarius* (Bainier) Thom.

The OTA-producing ability of isolates of *Aspergillus* and *Penicillium* representative of fungal populations located in the monitored area was evaluated using different techniques.

The green-blue fluorescence in Coconut Agar, detectable on the reverse of Petri dishes under NUV light (365 nm), was evaluated on more than 20,000 isolates including different species of *Aspergillus* and *Penicillium*. None of 14,700 *A. niger* isolates, 216 *Aspergillus aculeatus* Lizuka and 50 for each of 7 species of *Penicillium* (*Penicillum brevicompactum* Dierckx, *Penicillum implicatum* Biourge, *Penicillum janthinellum* Biourge, *Penicillum paxilli* Bainier, *Penicillum purpurogenum* Stoll and *Penicillum variable* Sopp. yielded fluorescence in the medium. The green-blue fluorescence typical for OTA production was observed for 331 of 2,207 isolates of *A. carbonarius* (15%) and 10 of 2,000 isolates of *Aspergillus wentii* Wehmer (0.5%).

The ability to produce OTA was determined by Thin Layer Chromatography (TLC) in 508 isolates of *A. niger*, 265 of *A. carbonarius*, 51 of *A. aculeatus*, 5 of *A. wentii*, and 35 isolates of *Penicillium* (5 for each of the 7

species reported above). Positive results, a green-blue fluorescent spot under NUV light showing the same migration of OTA (Rf = 0.5), were obtained by 95% of *A. carbonarius* isolates and 20% *A. wentii* isolates. HPLC analysis of cultural filtrates of representative isolates confirmed the results obtained by TLC for most fungal species. However, *A. wentii* never showed OTA production, and only 2 of 28 *A. niger* isolates released OTA after at least one-week growing at concentrations (0.02-0.03 ng ml^{-1}) very close to the detection limit (0.01 ng ml^{-1}).

A broad variability in OTA production was observed among *A. carbonarius* isolates. These could be discriminated in 3 groups on the ground of the level and temporal evolution of OTA concentrations in cultural filtrates sampled at 24-h intervals during 7-day growing: i) less than 10 ng ml^{-1} of OTA remaining constant; ii) high OTA concentrations increasing over the time (13 to 572 ng ml^{-1}); iii) high OTA concentrations decreasing with time (398 to 35 ng ml^{-1}).

An assay on the influence of temperatures ranging from 15 to 40°C on OTA production in *A. carbonarius* showed that optimal temperature is 20-25°C (506 to 1,578 ng ml^{-1}). OTA production and mycelial growth seemed negatively correlated.

Single berries of different grape cultivars were artificially inoculated with *A. carbonarius* or *A. niger*: OTA determination by HPLC confirmed that *A. carbonarius* is the principal if not exclusive responsible of OTA contamination in musts. Single berries inoculated with *A. carbonarius* showing rot on 50% of surface (4-5 days after inoculation) contained OTA in amounts variable from 532 to 1,028 µg. OTA was detected even in quiescent conidia of *A. carbonarius* (41 ng from 10^3 conidia).

ROLE OF TEMPERATURE ON GROWTH AND OCHRATOXIN A PRODUCTION BY *ASPERGILLUS CARBONARIUS*

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Sixty strains of *A. carbonarius* isolated from grape in Italy were tested on synthetic grape medium (SGM), representative of grape composition at early veraison, to verify the effect of strain and temperature on fungal growth and Ochratoxin A (OTA) production. Strains were inoculated centrally on Petri dishes and incubated at 15°C and 25°C for 14 days. Measure of diameter to determine fungal growth was conducted as well as analyses for the determination of OTA production (Bragulat *et al.*, 2001).
All the isolates resulted able to grow and to produce OTA at both considered temperatures but with significant difference between 15°C and 25°C. The mean daily growth was 1.3 mm at 15°C and 2.3 mm at 25°C while the mean daily OTA production was 126 ppb and 81 ppb respectively.
In particular, the fungal growth resulted mainly dependant on temperature and OTA production on strain.
The mean daily growth resulted to have opposite behaviour respect to OTA production: in fact, the highest daily growth rate was observed at 25°C while the optimum for toxin production resulted, for almost all strains, at 15°C.
A cluster analysis was applied to data collected in order to classify strains in homogenous groups. This analysis resulted in 5 clusters: two of them, cluster 1 (15% of considered strains) and cluster 5 (18% of considered strains), consider opposite situation taking into account only isolates able to produce high amount of toxin at only one temperature, respectively at 25°C and at 15°C. All the other clusters consider intermediate situations, in particular: cluster 2 (37% of considered strains) group strains able to produce OTA at both temperature but with higher amounts at 15°C; cluster 3 (12% of considered strains) includes isolates with low OTA production at both temperatures; cluster 4 (18% of considered strains) collects strains with low toxin production with slightly higher production at 15°C. No correlation with geographic provenience of strains was noted.
Optimum for *A. carbonarius* growth resulted to be different from its optimum for OTA production. This have to be taken into account considering OTA contamination in grapes; in fact, limited presence of fungi on grapes can result in great amounts of the toxin.

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INFECTION OF GRAPE BERRIES BY A. CARBONARIUS IN VITRO

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Ten bunches of the grape varieties Barbera and Trebbiano were collected, the first one at early veraison and ripening and the other one only at ripening, in an experimental vineyard placed in Piacenza. Samples of berries with a peduncle of 1 cm were prepared from bunches choosing only those without damages or fungal growth. Berries were surface disinfected, rinsed twice and then dried. Berries were dipped in a spore suspension of *A. carbonarius* with concentration of 10^7 conidia/mL taking them by the peduncle and paying attention to avoid the wetting with the inoculum of the insertion point of the peduncle, where micro lesions are frequently present.
At ripening also Cabernet Sauvignon bunches were collected from the same experimental vineyard and berries were inoculated with further dilution of the initial inoculum going from 10^7 to 10^1 conidia/mL to verify the role of spore load in fungi contamination of grape.
Berries were then put in a humid chamber, well separated, without the possibility to touch each other, and incubated at 25°C for 7 days. After incubation, all berries with visible symptoms of *A. carbonarius* growth were counted, and healthy berries were incubated on DRBC at 25°C for 7 days directly or after disinfection. After incubation, the number of berries showing fungal growth was determined as well as OTA content.
At early veraison only 6% of berries of Barbera showed visible symptoms of fungal growth while at ripening the same grape variety showed an higher incidence of fungal growth (80%) underlining that grape at this growing stage is less resistant to fungi attack. After incubation on DRBC, all berries resulted infected. Different was the behaviour of Trebbiano that showed only 15% of berries with fungal growth at ripening that increased up to 80% of berries after incubation on DRBC showing that this grape variety is more resistant to fungi penetration. Significant difference were found between 'untreated' and 'disinfected' berries.
Spore load resulted relevant in grape infection of Cabernet Sauvignon. Only berries inoculated with a concentration of at least 10^4 conidia/mL showed fungal growth and the number of berries with visible symptoms of fungal colonization increased along with inoculum concentration. The same seems to happen to OTA production that is present only in berries with symptoms and progressively increased along with inoculum up to 10^6 conidia/mL. Anyway, *A. carbonarius* resulted able to penetrate berries also without visible symptoms; after disinfection and incubation on DRBC, berries without symptoms resulted free from fungal growth only with inoculum concentration of 10^2 or lower.

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