ABSTRACTS

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SECTION 2

Lectures

Field and Environment

LECTURES

OCCURRENCE OF *ASPERGILLUS* SECTION *NIGRI* IN GRAPES IN EUROPE AND ISRAEL

Paola Battilani
Istituto di Patologia Vegetale, Università Cattolica del Sacro Cuore, Via E. Parmense 84, 29100 Piacenza (Italy)
e-mail: paola.battilani@unicatt.it

The interest in *Aspergillus* section *Nigri* is mainly related to the role this section, particularly *A. carbonarius*, plays in causing grapes contamination with ochratoxin A (OTA). The presence of black aspergilli is common in vineyards, but the incidence of berries infected by these fungi is notable from veraison, with the highest incidence normally observed at harvesting.

Incidence of berries infected by black aspergilli is highly variable in different countries; the mean trend of a 3-year survey in Southern Europe and Israel showed around 25% of colonised berries at early veraison in most countries, with higher presence in Italy and especially in Israel; at harvesting, the percentage of colonised berries was higher, particularly in Greece, France and Israel, where fungi can be isolated from more than 50% of berries. Variability is also remarkable among years, and 2003 showed to be a conducive year, with more than 85% of berries colonised by black aspergilli both in Israel and France at harvesting.

Among the black aspergilli isolated from berries, uniseriate was the least represented group, always being absent in Greece, sporadically found in Spain and identified in Portugal only in 2003. *A. niger* aggregate was the principal group at ripening; in all countries and years they were dominant in respect to uniseriate and *A. carbonarius* isolates. *A. carbonarius* was detected in all the countries and the years considered, but its incidence was lower than 10% in Italy, Portugal and Spain, around 15% in Israel and around 30% in France and Greece, with high variability among vineyards.

During ripening, spatial variability of black aspergilli is significantly related to latitude and longitude, showing a positive West – East and North – South gradient. Predictive maps of black aspergilli incidence, drawn applying geostatistical analysis, show the same trend in the 3 years, with the highest incidence in 2003, followed by 2001 and 2002. The highest number of berries colonised by black aspergilli was always highlighted in Israel, Greece and Southern France, associated with the highest incidence of *A. carbonarius*. Southern Spain and Southern Italy also have relevant incidence of black aspergilli.

The thermo-wetness maps for the 3 years showed a trend related to the incidence of black aspergilli. The coldest and more rainy year was 2002, while 2003 was the hottest and driest, particularly during August, with Israel being the hottest and driest country, followed by Greece and Southern Italy. In certain locations relative humidity in the vineyards could be affected by the proximity to the sea which can increase the conducive conditions in the microclimate of the grape vine canopy. This could explain the high contamination reported by South Eastern France in the 3-years period.

This indicates that meteorological conditions can contribute to explaining spatial distribution of black aspergilli within the Mediterranean basin.

CHEMICAL AND BIOLOGICAL CONTROL OF SOUR ROT CAUSED BY BLACK ASPERGILLI IN DIFFERENT FARMING SYSTEMS AND GRAPEVINE VARIETIES IN GREECE

Sotiris E. Tjamos, Myrto Dimakopoulou, Polymnia P. Antoniou, Eris C. Tjamos
Agricultural University of Athens, Department of Plant Pathology, Iera odos 75, 11855 Votanikos, Athens, Greece
Tel. 0030 210 529450 Fax 0030 210 294513 ect@aua.gr

The mycotoxin Ochratoxin A (OTA) mainly produced by *Aspergillus niger* and *A. carbonarius* creates serious problems in the winemaking industry of several Mediterranean countries. Although control measures against sour rot agents were not specifically suggested so far, the OTA risk necessitates research on developing control measures against the involved fungi. So, in parallel with chemical evaluation against *A. carbonarius*, the biological approach with epiphytic yeasts was also tested in our laboratory in Greece.

Vineyard tests conducted in Rhodes Island included one or two applications of the fungicide Switch (cyprodinil + fludioxonil). The rate of 0.1% was used either at veraison and/or 20 days before harvesting in vineyards of grapevine variety Cabernet Sauvignon, linear planting in Kameiros region, and Grenache Rouge, cup formation in Kalavarda region during summer 2003 and 2004. The obtained data demonstrate that even one application of Switch was effective in reducing the percentage of infected bunches both in Cabernet Sauvignon and Grenache Rouge. It was also indicated that two Switch applications significantly reduce the OTA level.

The potential of several epiphytic grapevine yeast isolates in controlling sour rot causing fungi was investigated. Two out of 150 tested yeasts initially proven to be highly effective against Aspergilli in laboratory tests were further evaluated in the vineyard. One of them designated as GY-18, was identified as *Cryptococcus laurentii*, while the second one, designated as K-4, has been identified as *Aureobasidium pullulans.*

Vineyard tests carried out in Nemea region on the variety Agiorgitico during summer 2003 and 2004 included beyond Switch the copper fungicide cupravit and separate sprays of the two laboratory evaluated epiphytic yeasts. The obtained data demonstrate that either Switch or yeast K-4 were the most effective applications during the two years of the experimentation period.

Although the obtained data demonstrate that either Switch or yeast K-4 applications significantly reduce incidence of sour rot of berries in Nemea region, a third year experiment was also carried out this summer both in Rhodes Island and in Nemea region to verify our two year results.

ABIOTIC FACTORS DETERMINE GERMINATION, GROWTH AND OTA PRODUCTION BY *ASPERGILLUS CARBONARIUS* STRAINS FROM THE MEDITERRANEAN BASIN

David Mitchell, David Aldred and Naresh Magan
Applied Mycology Group, Institute of BioScience and Technology, Cranfield University, Silsoe, Bedford MK45 4DT, U.K. (n.magan@cranfield.ac.uk).

Studies were conducted to determine the impact of water availability, temperature, pH and gas composition on germination, growth and OTA production by strains of *A.carbonarius*. Generally, germination was rapid at >0.95 water activity (aw), especially at >25^{o}C and usually within 25 hrs. However, at <0.93 a_w 25^{o}C, germination and germ tube extension were significantly slower. Interestingly, comparisons of germination and germ tube extension on grape skins and grape tissue demonstrated that at 0.98-0.995 aw germination was most rapid in grape tissue, and slowest on grape skins. Generally, elevated C02 levels (25-50%) had little impact on germination of strains. However, germ tube extension was decreased by elevated C02 at 0.95 and 0.995 aw, especially at 50% C02.

Growth occurred over a wide range of aw x temperature regimes. Generally, there were some intra-strain significant differences in growth rate between those from different countries. Optimum growth was at 30-35^{o}C and 0.98 a_w for most strains. In contrast OTA production was optimum at 15-20^{o}C and closer to 0.93-0.95 a_w. Contour maps were produced of individual and all strains to obtain a perspective on optimum and marginal conditions for growth and OTA production. These are useful indicators of low and high risk abiotic factors which will influence the development of *A.carbonarius* on grapes. Elevated C02 (up to 50%) decreased growth slightly at different aw levels. However, effect on OTA production was not significant indicating a requirement of greater concentrations for effective control.

Some studies were conducted of the partitioning of OTA into spores, mycelium and medium. These suggested that for many *A.carbonarius* strains >50-60% of the OTA was present in the spores. This is in contrast to *A.ochraceus* where more OTA is present in the mycelium and medium/matrix. This could have implications for the level of OTA contamination found on grapes.

ECOPHYSIOLOGICAL STUDIES ON OTA-PRODUCING SPECIES OF BLACK ASPERGILLI

A.J. Ramos, N. Bellí, S. Marín, V. Sanchis
Food Technology Department. CeRTA-UTPV. Lleida University. Av. Rovira Roure 191. 25198 Lleida (Spain).
Tel. 34 973 702811; Fax. 34 973 702596; e-mail address: ajramos@tecal.udl.es

Ecophysiological studies with *Aspergillus* section *Nigri* have been developed in order to determine the influence of water activity (a_w), temperature and incubation time on growth and OTA production by isolates of black Aspergilli obtained from grapes. These studies have been carried out in a medium with composition similar to that of grapes (Synthetic Nutrient Medium, SNM).

With regard to growth, *A*. section *Nigri* isolates tested (4 *A. carbonarius*, 3 *A. niger* aggregate and 3 uniseriates) grow on SNM at all the temperatures assayed ranging from 10 to 37 ºC, with an optimum between 30 and 37 ºC. However, at 10 ºC the growth of all the isolates was negligible when a_w levels were low (0.90 and 0.93 a_w). The optimum a_w for black Aspergilli to grow, seems to be at 0.98, similar to the a_w of grapes in the field. Thus field conditions are likely to be conducive to optimum growth of these species. Furthermore, there were statistical differences between the growth of the different isolates tested, mainly due to the group to which the isolate belonged (*A. carbonarius*, *A. niger* aggregate and uniseriate), with the *A. niger* aggregate presenting the fastest growth rates in the three trials and *A. carbonarius* the lowest.

Two *A. carbonarius* and two *A. niger* aggregate strains isolated from grapes were tested *in vitro* for OTA accumulation at 25ºC on SNM, over periods of 20 days at different a_w levels, and results have shown the significant influence of a_w and incubation time on OTA accumulation. High a_w (above 0.95) seem to favour OTA production of these isolates and maximum amounts of OTA were found at the earlier growth states of *A*. section *Nigri* isolates tested (5 days for *A. carbonarius* and 7-13 days for *A. niger* aggregate).

As *A. carbonarius* is widely accepted as the main OTA-producing species in grapes in the Mediterranean area, more specific ecophysiological studies were carried out with isolates of this species to determine the influence of different environmental factors, photoperiod, day-night temperature simulating field conditions, and skin damage of grapes on growth and OTA production.

Thus, the effects of a_w (0.90-0.99), temperature (15-37 ºC) and their interaction on growth and OTA production by 8 isolates of *A. carbonarius* were investigated on SNM. Optimum a_w level for growth (0.95-0.99 a_w) seems to correspond with optimum for OTA production, meanwhile the most propitious temperature for the toxin production (20 ºC) was below the best one for growth (35-37 ºC).

The effect of light and temperature regimes simulating day and night in the field in the months preceding grape harvest on *A. carbonarius* growth and OTA production were investigated. Twelve-hour photoperiod affected positively *A. carbonarius* growth with no differences between incubating the mould at day temperature (28 ºC) or alternating day/night temperatures (28 ºC/20 ºC). Slower growth, however, was observed at constant incubation at 20 ºC. Under 12h-alternation periods of day and night temperatures, growth was faster at continuous darkness than under continuous light conditions. No significant differences on OTA production were detected due to either fluctuating temperature or photoperiod. However, as photoperiod enhanced the growth of colonies, it would also enhance OTA accumulation.

To investigate the impact of skin damage on visible *A. carbonarius* colonization and OTA production directly on grapes at different temperatures and relative humidities, 4 ochratoxigenic *A. carbonarius* strains were used. Damaged and undamaged table grapes were surface-disinfected, inoculated, and stored at 3 levels of relative humidity (RH: 80, 90 and 100 %) and at 2 levels of temperature (20 and 30 ºC). After 7 days, the infection percentage of *A. carbonarius* was recorded and OTA accumulation in berries was analysed. Results showed that damaged grapes were more commonly infected and colonies were more developed than in undamaged ones; consequently more OTA was detected in the first ones. Temperature and RH had significant influence on both infection and toxin content. The amount of OTA detected at 30 ºC was higher than at 20 ºC in most of the treatments. The highest RH (100 %) led to maximum amounts of OTA while no significant differences were found between 90 % and 80 % in the OTA content.

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CHARACTERISATION AND ECOLOGY OF OCHRATOXIGENIC BLACKASPERGILLUS SPECIES IN AUSTRALIAN VINEYARDS

Su-lin L. Leong[a,b,c], Ailsa D. Hocking[a,b], Eileen S. Scott[b,c]
[a] *Food Science Australia, PO Box 52, North Ryde, NSW 1670, Australia*
[b] *Cooperative Research Centre for Viticulture, PO Box 154, Glen Osmond, SA 5064, Australia*
[c] *School of Agriculture and Wine, University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia*
Corresponding author: Dr Ailsa Hocking, tel. (+612) 9490 8520, fax (+612) 9490 8581, Ailsa.Hocking@csiro.au

Aspergillus niger and *A. carbonarius* were isolated from vineyard soils in 17 of 17, and four of 17 Australian viticultural regions, respectively. *A. aculeatus* was isolated infrequently. All thirty-two isolates of *A. carbonarius* and three of 100 isolates of *A. niger* produced ochratoxin A (OTA). Of Australian *A. niger* isolates analysed for restriction fragment length polymorphisms within the internal transcribed spacer region of 5.8S ribosomal DNA, 61 of 113 isolates, including the three toxigenic isolates, were of type N pattern, and 52 were type T. Analysis of amplified fragment length polymorphisms for a selection of these *A. carbonarius* and *A. niger* aggregate isolates, as well as imported isolates, clearly differentiated *A. carbonarius* from *A. niger*, and further divided *A. niger* into types N and T. Six polymorphic microsatellite markers, developed specifically for *A. niger*, also differentiated strains into N and T types. There was no clear relationship between genotypic distribution and ochratoxigenicity, substrate or geographic origin.

Growth and toxin production were examined for five Australian isolates of *A. carbonarius* and two of *A. niger* on synthetic grapejuice medium, within the range 0.92-0.98 a_w, and at 15 °C, 25 °C, 30 °C and 35 °C. Maximum growth occurred at *ca* 0.965 a_w / 30 °C for *A. carbonarius* and *ca* 0.98 a_w / 35 °C for *A. niger*. The optimum temperature for OTA production was 15 °C and little was produced above 25 °C. The optimum a_w for toxin production was 0.95 for *A. niger* and 0.95-0.98 for *A. carbonarius*. Toxin was produced in young colonies, however, it decreased as colonies aged.

Populations of *A. carbonarius* inoculated onto bunches of Chardonnay and Shiraz decreased from pre-bunch closure to early veraison. Populations from veraison to harvest were variable, and increased in bunches with tight clustering and splitting. Inoculation of bunches with *A. carbonarius* spore suspension did not necessarily result in Aspergillus bunch rot. For Semillon bunches inoculated with *A. carbonarius* spores with and without berry puncture, increased susceptibility to rot and OTA formation was associated with berry damage, in particular at greater than 12.3 °Brix (20 d before harvest). OTA contamination of bunches was related to the number of mouldy berries per bunch, with shrivelled, severely mouldy berries the primary source of OTA.

THE MYCOBIOTA OF WINE GRAPES IN THE CZECH REPUBLIC IN THE YEAR 2004

Vladimir Ostry [1], Jarmila Skarkova [1], Jiri Ruprich [1], Ivana Prochazkova [1], Alena Kubatova [2]
[1]National Institute of Public Health in Prague, Centre for the Hygiene of Food Chains,
NRC for Microfungi and Mycotoxins in Food Chains, Brno, CZ-612 42, The Czech Republic
[2]CCF - Culture Collection of Fungi, Department of Botany, Faculty of Science,
Charles University, Prague, The Czech Republic

As a response to project "WINE-OCHRA RISK" a pilot study in The Czech Republic was prepared. The aim of study was a monitoring of the mycobiota of wine grapes and a monitoring of toxigenic microfungi producers of ochratoxin A and selected *Alternaria* mycotoxins in wine grapes from domestic crop in the year 2004.

Five vineyards with twenty two grape varieties were selected for the study in the south corner of Moravia in the ZNOJMO WINE REGION. One sample of wine grapes varieties was represented by three subsamples of wine grapes, which were sampled in left, middle and right part of vineyard.

Five berries per bunch of each subsample were randomly selected, plated onto Dichloran Rose Bengal Chloramphenicol (DRBC) agar, and incubated for 5-7 days at 25°C. *Penicillium* spp. and *Aspergillus* spp. were picked off onto Czapek Dox (CZ) agar. *Alternaria, Cladosporium* and other species were picked off onto Potato dextrose agar (PDA) to obtain pure cultures, and for further identification of the individual species.

A sensitive, validated, accreditated HPTLC method for quantification of ochratoxin A in grape fresh juice, must and wine has been developed. Samples of grape fresh juice were centrifuged, purified on commercial immunoaffinity columns (Ochratest VICAM). The detection limit (LoD) was 4 ng/l, the limit of quantification (LoQ) was 8 ng/l of grape fresh juice, must and wine.

Alternaria alternata, Cladosporium (C. herbarum, C. cladosporioides), Penicillium (P. expansum, P. aurantiogriseum, P. spinulosum, P. citrinum), Aspergillus clavatus, Epicoccum nigrum and *Rhizopus nigricans* were isolated from grapes. Strains of *Alternaria alternata* biotype 1 and biotype 2 were determined in 16 (73%) grape samples, *Cladosporium herbarum* in 9 (41%) grape samples, *Rhizopus nigricans* in 6 (27%) grape samples, *C. cladosporioides*, *Penicilium aurantiogriseum* a *P. griseofulvum* in 3 (14%) grape samples, *P. expansum* in 2 (10%) grape samples, *P. citrinum*, *P. spinulosum, Aspergillus clavatus* a *Epicoccum nigrum* in 1 (5%) grape sample. The *Alternaria alternata* strains were divided to two biotypes (biotype 1 and biotype 2) according to a description of morphological criteria (growth on Potato dextrose agar /PDA/ and dichloran malt extract agar /DCMA/) and microscopy of conidia (a conidial length).

Ochratoxigenic microfungi (***Aspergillus carbonarius**,* other ***Aspergillus*** **Section** ***Nigri***, ***Aspergillus ochraceus**, Penicillium verrucosum* and *Penicillium nordicum*) **were not determined in grapes.** The occurrence of **ochratoxin A** in **grape fresh juice, mus**t and **wine was not determined**. The results of pilot study indicate and approve that ochratoxin A incidence and concentration decrease probably in wine products /white, rosé and red/ from Northern regions in Europe and increase in wine products from more Southern (Mediterranean) regions.

PREVALENCE FUNGI FROM GROUP A. NIGER IN VINEYARD REGION OF ARMENIA

K.M. Grigoryan, Graf, A. Kirakosyan, M. Sargsyan
Yerevan State University, Armenia
foodlab@mail.ru

Having extensive vine growing and winemaking history and traditions, the Armenian wine sector has a number of strengths and characteristics that suggest viability as a wine producer and exporter.

The wine industry is very important for Armenian economy and research, which is focused on determining of mycotoxin-ochratoxin A (OTA) and patulin producing fungi that are present in the grape producing system.

OTA puts human health at risk because it is nephrotoxic, teratogenic, immunotoxic and possibly neurotoxic. Now OTA is classified as a carcinogen. It is a one number mycotoxin produced by fungal contaminants ("mould") of food, particularly *P. verrucosum, A. ochraceus, A. carbonarius*. It is found widely in agricultural products, notably cereals, coffee and pulses.

Wine and grape juice have been recently identified as the second most important source of OTA one of the major mycotoxins. *A.carbonarius* is the main species thought to be responsible for OTA in grapes (Horie, 1995; Téren et al., 1996; Wicklow et al., 1996). It has been isolated from grapes in France, Spain, Italy, South America, Greece, Israel, Portugal and Australia (risk due to the high stability of OTA under a variety of environmental factors).

In Armenia fungal contaminants identified in grapes include *Botrytis cinereae*, *Aspergillus niger, Penicillium crustaceum, P. jantinellum* and *Trichothecium roseum.* The raw materials used for the production of Armenian wine may contain 37 species of micromycetes that belong to the genera *Botrytis, Aspergillus* and *Penicillium.* The dominant species in Armenian wine are Penicillium and Aspergillus genus, particularly coremial and sclerotial fungi that are potential producers of patulin and OTA. Patulin was detected in 30% of the grape juice produced in Armenia, and 20% of the juices had concentrations of 55-61 µg/l, exceeding recommended FDA safety limits of 50 µg/l.

The basic purpose of the work is to study a degree of contamination of grape raw material by fungi from 2 winemaking regions of Armenia (Ararat and Vayk regions). In total, more than 100 samples are analyzed.

The lead researches have shown high degree of contamination of raw grapes from Ararat region, with hotter and dry climate, by fungi from genus *Aspergillus*, section *Nigri- A. niger ,A. japonicus, A. carbonarius, A. phoenicus, A.aculeatus.* These species frequently were allocated from the damaged and overripe berries. Primary pollution of grapes occurs during vegetation since the soil is the basic reservoir of these species. In particular, the period from 15 days pre-harvest (12.5^0 brix) until harvest was the critical time for rapid development of *Aspergillus* rots. Also, bad hygienic conditions at winemaking plants promote the further growth of a degree contamination of fungi from section *Nigri*.

These species have been detected in 65 % of the investigated samples from Ararat and 43 % of raw grapes from Vayk region. Among the revealed species from this group, percentage *A. carbonarius* makes 27%.

Studying the basic morphological and some physiological characteristics allowing to differentiate species *A. carbonarius* from relatives of its species, in particular species *A. niger.*

Toxin-producing fungi can be deposited on crops by birds and insects or be introduced to crops from the soil. The fungi present in grape juice and wine are typically of great structural diversity. Fungal growth on grapes and grapes must in storage, and its processing is another major source of contamination.

Studying of toxigenic potential isolated strains has shown, that about 3% strains *A.carbonarius* are producing ochratoxin A. Accordingly (Ueno et al., 1991; Abarca et al., 1994; Heenan et al., 1998; Taniwaki et al., 1999), other related species *A. niger* have also been reported reliably as producers. However, the results of our research have shown that all the toxigenic strains belong to black *Aspergillus,* not produced of OTA, exclude *A.carbonarius*

Thus, the results received show the presence of risk factor of contamination by mycotoxins during storage and realization of wine in Armenia.

OTA AT VINE AND WINE-STAGE: SOLUTIONS TO REDUCE THE CONTAMINATION

C. Prêtet -Lataste[1], L. Guérin[1], J. Béguin[1], D. Solanet[2], P. Cottereau[2], B. Molot[2], J.M. Jarlot[3], D. Caboulet[3], A. Poulard[4]

[1] ITV France Tours BP39537 37095 TOURS cedex 2 (France) Tel : +33247 88 24 20, Fax : +33247 88 24 21, caroline.lataste@itvfrance.com;
[2] ITV France Nîmes : Domaine de Donadille 30230 RODILHAN (France)
[3] ITV France Pech Rouge, Domaine de Pech Rouge, 11430 GRUISSAN (France)
[4] ITV France Nantes, Château de la Frémoire 44 120 VERTOU (France)

ITV France is a French Research Institute for Vine and Wine. It has led 3 years of experimentations in matters of Ochratoxin A (OTA) at vine- and wine-stage. OTA is a mycotoxin which may contaminate Mediterranean wines, namely those located in Languedoc, France. Experiments were carried out on three fields:

- **Microbiology** in order to understand the biology of *Aspergillus carbonarius* which is the main producer of OTA;

  Fungicides screening lead us to precise *in vitro* efficiency of several active materials.: which may limit the growth of *Aspergillus* at vine stage. We have also shown that *Aspergillus carbonarius* is able to grow under acid conditions: at must- or wine-stage, even under the low pH-conditions of musts and wines, *A. carbonarius* is able to germinate and to grow. Furthermore *A. carbonarius* can neither germinate nor grow when treated by high temperature such as a hot prefermentary maceration (60°C during 16h, 60°during 30 min or 85°C during 5 min).

- **Viticulture** to find fungicides solutions and to prevent musts contamination;

  In viticulture, we have shown that at least the three following fungicides have a high efficiency to reduce the OTA-contamination in musts: mepanipyrim; the association of cyprodinil and fludioxonil; pyrimethanil. Using these fungicides, precisely when grapes colour is changing or 15 days later according to the year, may reduce *Aspergillus*- and thus OTA-contamination by 80%. In addition to these chemical solutions, any organic solutions have been studied . We have concluded that *Trichoderma atroviride* may be an antagonist of *A. carbonarius*, but this interesting efficiency is highly dependant on the strain and still needs confirmation

- **Oenology** to establish decontamination solutions during the fermentation process.

In oenology we have compared the efficiency of various yeasts on OTA-decontamination in wine. We have found that strains such as Levuline Ribera can adsorb more than 55% of OTA-contamination during the fermentation for red wines. We have also found that enzymes from *Aspergillus niger* can reduce OTA-contamination from about 60% on musts.

A CROP PROTECTION APPROACH TO ASPERGILLUS AND OTA MANAGEMENT IN SOUTHERN EUROPEAN VINEYARDS

Ewald M Kappes[1], Luca Serrati[2], Jean-Baptiste Drouillard[3], Juan-Miguel Cantus[4], Anastasia Kazantzidou[5]

[1]Syngenta Crop Protection, Schwarzwaldallee 215, CH-4002 Basel, phone +41 61 323 7910, fax +41 61 323 5954, email: ewald.kappesyngenta.com
[2]Syngenta Crop Protection S.p.A., 139 Via Gallarate, I-20151 Milano, [3]Syngenta Agro S.A.S., 20 rue Marat, F-78210 St-Cyr-l'Ecole, [4]Syngenta Agro S.A., Ribera del Loira 8-10, 3a planta, E-28042 Madrid, [5]Syngenta Hellas S.A., Anthoussas Avenue, Anthoussa, GR-153 49 Athens

Ochratoxin A (OTA) is the key mycotoxin found in grapes, wine, grape juice and raisins. In European vineyards OTA can be found predominantly in grape products from the Mediterranean basin. It is produced mainly by *Aspergillus carbonarius* and by some strains of *A. niger*. The epidemiology of these ochratoxygenic fungi is favoured by fruit damage, e.g. damage by grape berry moth (*Lobesia botrana*). Since 2001 Syngenta has been conducting *in vitro* studies to evaluate fungicidal effects on *A. carbonarius* and vineyard studies integrating *Aspergillus* control with grape berry moth control. The aim of these studies is to develop recommendations for growing a healthier grape crop, which is essential for the production of good wine, juice and raisins and the minimization of OTA contamination.

In vitro, cyprodinil, fludioxonil and fluazinam were highly active against spore germination and mycelium growth of *A. carbonarius* (Table 1). Azoxystrobin was far less active, while copper, folpet, mancozeb and sulphur were not active at the tested rates.

Table 1 - *In vitro* activity of different fungicides against *Aspergillus carbonarius* (mean of 10 strains)

IC_{50} (mg/l)	Cyprodinil	Fludioxonil	Fluazinam	Azoxystrobin	$CuSO_4$	Folpet	Mancozeb	Sulphur
Spore germination	0.004	0.1	0.47	34.58	>1000	>1000	>1000	>1000
Mycelium growth	0.01	0.02	0.06	7.02	>1000	>1000	>1000	>1000

The influence of grape berry moth on OTA in the must could be demonstrated in a vineyard trial in Southern France (Table 2). The control of the 2nd and 3rd generation grape berry moth using effective products with ovicidal activity, in order to avoid berry damage, lead to a strong reduction of the OTA in the must.

Table 2 - Effect of grape berry moth control on Ochratoxin A (OTA) in must (Béziers, France 2002)

Insecticide		No. of larvae per bunch	OTA in the must (µg/l)
Untreated		2.99	4.67
Lufenuron + fenoxycarb*	30 g/ha+75 g/ha	0.33	1.07
Lufenuron*	50 g/ha	0.25	0.50

**2 applications at the beginning of the 2nd and 3rd generation*

As a next step, cyprodinil and fludioxonil were included in vineyard trials in Italy, France, Spain and Greece. In France also fluazinam was tested.

Table 3 - Effect of crop protection on *Lobesia*, *Aspergillus* and Ochratoxin A (OTA) in must (Vendrell, Spain 2003)

Botryticide	Insecticide	% att. Lobesia	% att. ASPESP	OTA in must (µg/l)
Fenhexamid (C+D)	No insecticide	14.87	23.41	2.92
Cyprodinil+fludioxonil (C+D)	No insecticide	10.30	6.36	0.76
Fenhexamid (C+D)	Lufenuron + fenoxycarb	0.99	3.29	1.05
Fenhexamid (C) Cyprodinil +fludioxonil (D)	Lufenuron + fenoxycarb	2.27	2.70	0.47
Cyprodinil+fludioxonil (C+D)	Lufenuron + fenoxycarb	0.99	0.84	0.47

Vineyard trials clearly demonstrated that cyprodinil, cyprodinil + fludioxonil and fluazinam are effective fungicides for the control of *Aspergillus* and the reduction of OTA (an example from Spain is shown in Table 3). The most effective timing was 21 days before harvest (Stage D). An additional earlier application at veraison (Stage C) is advisable under high risk conditions. Crop protection using insecticides for an effective grape berry moth control and fungicides with good activity against *Aspergillus* can contribute significantly in the reduction of OTA levels in wine, grape juice and raisins.

EVALUATION METHODOLOGIES OF GRAPEVINE EPIPHYTIC YEASTS AGAINST SOUR ROT OF GRAPES CAUSED BY *ASPERGILLUS CARBONARIUS*

Myrto Dimakopoulou, Nikos Avramidis, Polymnia P. Antoniou, Sotiris E. Tjamos and Eris C. Tjamos
Agricultural University of Athens, Department of Plant Pathology, Iera odos 75, 11855 Votanikos, Athens, Greece
Tel. 0030 210 529450 Fax 0030 210 294513 ect@aua.gr

Aspergillus niger and *A. carbonarius* are among the main sour rot agents of grapes in Greece and other Mediterranean countries. Both fungal species are also very dangerous for raisin and wine production due to the formation of the mycotoxin ochratoxin A.

Control measures against sour rot agents are not suggested so far. However, OTA risk reexamines the control of the involved fungi. So, in parallel with chemical evaluation against *A. carbonarius*, the biological approach with epiphytic yeasts was also tested in our laboratory.

The potential efficacy of epiphytic grapevine yeasts in controlling sour rot, caused by *A. carbonarius* was investigated. Several yeast isolates were selected from a large collection of epiphytic grapevine yeasts collected from vineyards of various regions of Greece.

In the first laboratory tests, individual berries kept in plastic boxes used as sealed chambers (Rh 100%, temp. 22 C, photoperiod 12h) were dipped in yeast suspensions (10^7 cfu /ml) after being wounded by piercing a hole of 2 mm in diameter. After 24 hrs the berries were inoculated with *A. carbonarius* spore suspension (10^4 conidia/ml). Following five days of incubation it was demonstrated that the rot formation was inhibited by 60%-90% compared with the untreated control.

A total of 25 out of 150 yeasts were initially tested. It was indicated that a high percentage ranging from 70-80% were highly effective against the fungus. Two most effective isolates were selected for further experimentation. One of them designated as GY-18, originated from Xinomauro variety from central Macedonia of Greece was identified as *Cryptococcus laurentii*. The second one, designated as K-4, originated from Robola variety Cephalonia Island has been identified as *Aureobasidium pullulans.*

To further test the indication that a high percentage of initially evaluated yeasts applied at a concentration of 10^7 cfu/ml, was able to inhibit rot formation and even sporulation of the fungus on the pierced berries, several isolates were also obtained from various grapevine varieties and regions of Greece. The data obtained from another 18 isolates out of 500, strongly suggested that a high percentage of epiphytic yeasts is able to act antagonistically against *A. carbonarius.* Actually, most of the tested isolates were able to inhibit fungal growth for at least 4-5 days after application while few of them were able to exercise inhibitory activity even 7 days after berry infection.

INFLUENCE OF MYCOFLORA ON OCHRATOXIN A PRODUCTION BY *ASPERGILLUS CARBONARIUS* AT DIFFERENT ENVIRONMENTAL CONDITIONS

V. Sanchis, A. Valero, A.J. Ramos, S. Marín
Food Technology Department, University of Lleida, CeRTA-UTPV, Rovira Roure 191, 25198 Lleida, Spain
Tel 34 973 702535; Fax 34 973 702596; e.mail address: vsanchis@tecal.udl.es

Ochratoxin A (OTA) was first reported in wines in 1996 by Zimmerli and Dick. This mycotoxin has been shown to be nephrotoxic, immunosuppressive, teratogenic and classified as a possible human carcinogen (2B) by the International Agency for Research on Cancer (IARC).

Recent studies have shown that black aspergilli, *Aspergillus carbonarius*, *A. niger* aggregate, and *A.* section *Nigri* uniseriates, are all very common in grapes at harvest, being *A. carbonarius* the main OTA producer in this product. Other species belonging to *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Fusarium* and *Rhizopus* can be found on grapes at the same time competing with black aspergilli in order to colonize the grapes.

Environmental factors such as water activity, temperature, pH, nutritional factors, etc. affect mycelial growth and mycotoxin production by moulds being limiting factors of the fungal colonization. Temperature and water activity are the main factors influencing germination, growth and mycotoxin production by OTA producing isolates.

In this study we aimed to understand the effect of fungal interactions which take place at different environmental conditions in OTA production by *Aspergillus carbonarius*.

Microorganisms used in this study were *Alternaria alternata*, *Cladosporium herbarum*, *Eurotium amstelodami*, *Penicillium janthinellum*, *P. decumbens*, *Trichoderma harzianum*, *Candida* sp. and *A. carbonarius* OTA-positive. They were isolated from grapes and vine dried fruits collected from the South of Spain. Growth in pure cultures of *A. carbonarius* and in paired cultures with the other species at different water activities (0.92 and 0.97) and temperatures (20 and 30ºC) was 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, OTA production was tested after 4, 7, 10, 14 and 18 days of incubation, at four distances along inter-colony axis (1, 2, 3 and 4 cm from *A. carbonarius* inoculation point).

At 0.92 a_w and 20ºC fungal interaction had no significant effect on OTA production. By contrast, at 0.92 a_w and 30ºC OTA was only produced when *A. carbonarius* was paired with *E. amstelodami* and with both penicilli, although the levels were not significant. At 0.97 a_w and 30ºC the interaction affected negatively the OTA production from the 7th day of incubation. Only *E. amstelodami* and *Candida* sp. seem to stimulate the OTA production at 0.97 a_w and 20ºC from the 14th day of incubation.

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BIOLOGICAL ACTIVITIES OF BLACK ASPERGILLI CONIDIA AND OCHRATOXIN A EVOLUTION IN GRAPES AND WINE

Ali Atoui[a], Hend Bejaoui[a], Florence Mathieu[a], Patricia Taillandier[b] and Ahmed Lebrihi[a*]
Laboratoire de Génie Chimique UMR5503 (CNRS-INPT-UPS)
Departement: Bioprocédés et Systèmes Microbiens
[a]*ENSAT/INPT: 1, Av. de l'Agrobiopôle BP32607, Auzeville-Tolozane, Castanet-Tolozan, 31326, France*
[b]*ENSIACET/INPT: 5, rue Paulin Talabot, BP 1301, 31106 Toulouse Cedex 1, France*
[*]Corresponding author. Telephone: 05 62 19 39 44. Fax: 05 62 19 39 01. E-mail: lebrihi@ensat.fr

Ochratoxin A (OTA) is a secondary metabolites produced by fungi belonging to *Aspergillus* and *Penicillium* genera. It has been shown to be nephrotoxic, teratogenic, immunosuppressive and carcinogenic agent. The presence of OTA has been detected in different kinds of foods and beverages such as grapes, grape juices and wines.

The objective of the present work was to investigate the possible presence of OTA in conidia of *A. carbonarius* and to study the OTA excretion ability of conidia of *A. carbonarius* during germination and to follow up the biological removal of OTA by black aspergilli conidia.

The results have shown that conidia *A. carbonarius* collected from the different media tested contained 0.011-0.1pg OTA per conidium. Moreover the attached OTA is excreted into the medium during the first hours of conidial germination contributing to the increase of OTA in must during maceration. OTA has been shown to decrease after 8 hours of germination. Another study has been conducted in order to investigate the biological removal of OTA by black aspergilli conidia. For that living and dead conidia of *Aspergillus niger*, *A. carbonarius* and *A. japonicus* were inoculated in synthetic and natural grape juices and conidia physiological states were followed. Dormant, swollen and germed conidia were able to adsorb OTA and no OTA degradation products were detected. This removal occurred as rapidly after conidia inoculation in synthetic and natural grape juices. Even non-viable conidia (heat-treated) were capable of removing OTA. After 30 hours for *A. niger* and *A. carbonarius* and 14 hours for *A. japonicus*, a degradation process, the second stage of OTA removal, was settled and Otα, an OTA degradation product, was detected and increased while OTA disappeared progressively from the grape juice. Comparisons between the three black aspergilli species tested showed that *A. japonicus* and *A. niger* were the best species, and could be interesting for further OTA detoxification processes in grape juices.

EFFECT OF OTA-PRODUCING *ASPERGILLI* ON STILBENIC PHYTOALEXIN SYNTHESIS IN GRAPES

Luigi Bavaresco [*1)], Silvia Vezzulli [1)], Paola Battilani [2)], Paola Giorni [2)], Amedeo Pietri [3)], Terenzio Bertuzzi [3)]

[1)] *Istituto di Frutti-Viticoltura (IFV), Università Cattolica del Sacro Cuore, 29100 Piacenza, Italy*
[2)] *Istituto di Entomologia e Patologia Vegetale (IEPV), Università Cattolica del Sacro Cuore, 29100 Piacenza, Italy*
[3)] *Istituto di Scienze degli Alimenti e della Nutrizione (ISAN), Università Cattolica del Sacro Cuore, 29100 Piacenza, Italy*
[*] *Corresponding author: Tel: ++39 0523 599267, E-mail: luigi.bavaresco@unicatt.it, Fax: ++39 0523 599268*

Since 1996, occurrence of ochratoxin A (OTA) contamination has been detected in wine and grape juice (*1*, *2*). Mycoflora potentially responsible for OTA in grapes are present in the field; *Aspergilli* are dominant with respect to *Penicillia*, and among these *Aspergilli* section *Nigri,* even though *A. ochraceus* OTA-producers are occasionally isolated. *A. carbonarius* probably plays the main role because of the high percentage of positive strains and the amount of OTA produced. OTA is a carcinogenic toxin in rodents and it possesses teratogenic, immunotoxic and possibly neurotoxic and genotoxic properties. In 1993, the International Agency for Research on Cancer (IARC) has classified OTA as a possible carcinogenic product for humans. In 1998, it has been pointed out that concurrent administration of berry and leaf juice of common grape to mice, along with OTA, significantly reduces the hepatic and renal damage caused by ingestion of this mycotoxin; this action is possibly related to the synthesis of *trans*-resveratrol, compound belonging to the stilbene group (*3*). Stilbenes are low molecular weight phenolics occurring in a number of plant species, including *Vitis* spp. Claimed to have beneficial effects on human health, these compounds include resveratrol, piceatannol, and piceid, and are produced by plants in response to abiotic and biotic stresses, especially after fungal attacks (*4*).

The aim of this work was to investigate the possibility for some OTA producing fungi to elicit stilbenic phytoalexin synthesis in grape berries.

Berries of *Vitis vinifera* L. cv. Barbera were infected, at veraison and during ripening, by a conidial suspension of *A. japonicus, A. ochraceus, A. fumigatus* and two isolates of *A. carbonarius* in order to control OTA production and stilbene induced synthesis. The experimental design provided also for intact and punctured berries and incubation temperature of 25 °C and 30 °C. All the tested fungi, except *A. fumigatus*, significantly increased *trans*-resveratrol synthesis over the control, while *trans*-piceid was not affected; only *A. ochraceus* significantly elicited the berries to synthesize piceatannol. The two isolates of *A. carbonarius* produced higher amounts of OTA than did the other fungi. A positive correlation between OTA and *trans*-resveratrol synthesis occurred. *trans*-Resveratrol and piceatannol showed fungicidal activity against *A. carbonarius*, being able to completely inhibit fungal growth at a concentration of 300 µg/g and 20 µg/g respectively (*5*).

During the following vintage, a further *in vitro* trial has been carried out in order to study the stilbene-synthase (StSy) gene expression levels in berry (skin) after inoculation with *Aspergillus carbonarius*. Barbera (susceptible variety, low stilbene production) and Castor (resistant hybrid, high stilbene production) genotypes were used for this evaluation. Based on chemical and genetic analysis (RT-PCR approach), *A. carbonarius* has been confirmed to act as a stilbene synthesis elicitor in berry tissues.

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