Oct 28th, 4:30 PM - 6:00 PM

Microbiological analysis of the Aspergillus carbonarius and Penicilliumverrucosum in Kosovo vineyards

Valon Durgati
University for Business and Technology

Aneliya Georgieva
University of Food Technologies

Galena Angelova
University of Food Technologies

Angel Angelova
University of Food Technologies

Zyri Bajrami
University of Tirana

Microbiological analysis of the Aspergillus carbonarius and Penicillium verrucosum in Kosovo vineyards

Valon Durguti [1]*, Aneliya Georgieva [2], Galena Angelova[2], Angel Angelov [2], Zyri Bajrami [3]

[1]*UBT - Higher Education Institution, Lagjja KALABRIA p.n., Prishtinë, Kosovë
10000 Prishtina, Kosovo
[2] University of Food Technologies – Departament of Biotechnology, Food Safety and Microbiology, Plovdiv – Bulgaria
[3] University of Tirana, Departament of Biology, Tirana –Albania
valon.durguti@ubt-uni.net

Abstract: Through the use of the microbiological semi-selective media (MEA-B and DYSG) for the species Aspergillus carbonarius and Penicillium verrucosum we have analyzed different varieties of the table and wine grapes in the region of Suhareka, south part of Kosovo. The purpose of this scientific work it was to determine through the microbiological analysis the main species of the fungi that produce the micotoxin known as Ochratoxin A. Analysis of the samples was done in the Department of Microbiology, University of Food and Technology, Plovdiv – Bulgaria.

Keywords: grape, fungi, microbiology, ochratoxin A, mycotoxin

INTRODUCTION

Fungi in their cellular organization are clearly eukaryotes and for that are now recognized as one of the five eukaryotic kingdoms.

Different kinds of large fungi or macrofungi have been recognized for thousands of years but in the early nineteenth it was established that many serious plant diseases were caused by infection of the plant by minute living organisms, recognized as microscopic fungi or microfungi. Microfungi were also found attracking dead organic materials and they were termed moulds, spelt molds in USA [6].

Molds are filamentous fungi that are classified based on the morphology of asexual or vegetative mycelia elements and their spore structures. Molds are ubiquitous with various genera commonly found on grapes, examples include *Aspegillus, Botrytis and Penicillium*, and to a lesser extent *Phytophtora, Moniliella, Alternaria and Cladosporium* [5].

The growth of fungi in a particular food is governed largely by a series of physical and chemical parameters (water activity, temperature, nutrient status, pH, preservatives, etc), and these factors do not act independently, but synergistically [11].

The greatest risk from the growth of fungi on fruit (grapes) is their ability to synthesize in the optimal conditions the substances (secondary metabolites) known as micotoxins, which are very dangerous to human health, animals and plants [3].

Ochratoxin A, N-[(3R)-(5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl) carbonyl]-L-phenylalanin is a mycotoxin produced by certain species of *Aspergillus* and *Penicillium* filamentous fungi. OTA contaminates cereals and cereal products, coffee, beans, pork meat and meat products, milk and milk products, eggs, wine and beer all over the world [2].

The *Penicillium* species that is associated with ochratoxin A production is *Penicillium verrucosum*, which is considered to be the largest producer of OTA in cereals, such as wheat, oats, barley and rye, in areas where the cold climate prevails [7]. It grows only at temperatures below 30°C and at a lower water activity. *Penicillium* species may produce ochratoxin A at temperatures as low as 5°C [1].

Aspergillus ochraceus is the best known species of ochratoxin A – producing *Aspergillus*. It grows at moderate temperatures and at a high water activity and is a significant source of ochratoxin A in cereals. It infects coffee beans usually during sun-drying causing contamination in green coffee [1].

Aspergillus carbonarius is highly resistant to sunlight and survives sun-drying because of its black spores and therefore grows at high temperatures. It is associated with maturing fruits and is the source of ochratoxin A in grapes, dried vine fruits, and wine and is also another source of ochratoxin A in coffee [1].

Aspergillus niger is another minor source of ochratoxin A production in infected coffee beans and dried vine fruits.

Mycotoxins can cause serious health problems in animals and humans known as mycotoxicosis [4] . The mycotoxin has been detected in various food stuffs such as dried fruits, coffee, maize, sorghum, wheat, pulses and wine [10]. The toxic effects of mycotoxins (e.g. ochratoxins, fumonisins, zearalenone, etc.) are mostly known from veterinary practice. Mycotoxicoses, which can occur in both industrialized and developing countries, arise when environmental, social and economic conditions combine with meteorological conditions (humidity, temperature) which favour the growth of moulds [8].

The OTA levels in grape and wine depend on different factors such as the climate, the date of harvesting and different wine-making procedures. Climatic conditions (high humidity and temperature) and geographical location are important factors favouring OTA accumulation in grape berries [13].

OTA exposure has been associated with increased levels of oxidative DNA, lipid, and protein damage. Second, various biological processes known to be mobilized under oxidative stress were shown to be altered by OTA. These effects have been observed in both in vitro and in vivo test systems. In vivo, active doses were often within doses documented to induce renal tumors in rats [9].

OTA is arguably risk factor for Balkan Endemic Nephropathy (BEN). BEN is a chronic tubulointerstitial kidney disease that occurs in some areas of Bosnia and Herzegovina, Bulgaria, Croatia, Romania, Serbia, and Monte Negro [14].

International Agency for Cancer Research classifies OTA as potential carcinogenic substance for man (group 2B). Zimmerli and Dick (1995) were the first ones to report the existence of OTA in grape and wine [15].

MATERIALS AND METHODS

For isolation and microbiological analysis of these two types of the fungi responsible for synthesizing of ochratoxin A (*Aspergilus carbonarius* and *Penicillium verrucosum*) we have used the recommendations given by ICFM - International Comission of Food Mycology [11].

We have applied two kinds of methods for cultivation and isolation of the fungi: a) direct plating and b) dilution plating.

In the beginning of the work we have prepared the semi – selective media, MEA – B (*Malt Extract Agar – Boscalid*) for cultivation and potential isolation of the fungi known as *Aspergillus carbonarius* and DYSB (*Yeast Extract Sucrose Glycerol Agar*) for cultivation and potential isolation of the fungi known as *Penicillium verrucosum*.

a. The composition of the microbiological semi-selective media MEA-B (Malt Extract Agar – Boscalid)

- Malt Extract 20 g/l, LOT: BCBQ0197V – Fluka Company (Sigma Aldrich) – France
- Agar 20 g/l, Fillab - EOOD
- Chloramphenicol 0.005 g/l, Fillab - EOOD
- Dichloran 1.0 ml (0.2%), LOT: STBF2742V, Sigma Aldrich
- Boscalid 10 mg/l, LOT: SZBF099XV – Fluka Company (Sigma Aldrich) – Germany
- Pepton 0.1%, LOT: 63927JD, Difcolaboratories
- Ethanol 70%
- H20 1000 ml

b. The composition of the microbiological semi-selective media DYSG (Yeast Extract Sucrose Glycerol Agar)

- Yeast extract 20 g/l, LOT: BCBQO966V, Fluka Company (Sigma Aldrich) -France
- Sucrose 150 g, Fillab - EOOD
- K2HPO4 1 g, Fillab - EOOD
- MgSO4 + 2 H20 0.5g, Fillab - EOOD
- Chloramphenicol 2 x 0.05 g, Fillab - EOOD
- Dichloran 1.0 ml, LOT: STBF2742V, Sigma Aldrich
- ZnSO4 + 2H20 0.01 g, Fillab - EOOD
- CuSO4 + 5 H20 0.005g, Fillab - EOOD
- Agar 20 g, Fillab - EOOD
- Glycerol 220 ml, LOT: SZBFO62DV, Sigma Aldrich
- Pepton 0.1%, LOT: 63927JD, Difcolaboratories
- Ethanol 70%
- H20 1000 ml

DIRECT PLATING

.Sample preparation

After semi – selective media preparation and their sterilization in autoclave with steam pressure at 121 ºC for 15 minutes, the grapes of different varieties are disinfected first with alcohol (ethanol 70%) for 2 minutes and then are washed (cleared) with water for 1 minute.
The analyzed varieties were: Sauvignon Blanc, Gamay, Franconia, Pinot Blanc and Muscat Hamburg.
The grape plating is done in sterile box, part of the microbiological laboratory in the University of Food Technologies, Plovdiv – Bulgaria.

PLATING

After sample preparation, we have taken 4 grape berries from each variety and set them into the Petri dishes, three replicates for each one. After deciding the grape berries into the Petri dishes, all of the samples are placed in an incubator, in the temperature 25 ºC for 5 day.

Examination

After incubation phase, initially we have made a visual examination of the Petri dishes through the appropriate lenses, counted the infected grape berries and the results are presented as a percentage. After that, through the use of the microscope we have made microscopic analysis, for the purpose of greater security during identification and analysis of certain characteristics of the analyzed species. Microscopic examination is done by taking a handful of developed fungi colonies and their placement by microbiological needle in the microscope slide. Then we have thrown a drop of alcohol (ethanol 70%) in the microscopic sample, left the alcohol to evaporate and finally we have thrown a drop of lactic acid, covered the sample with cover slide and thus the sample was ready for microscopic examination.

Dilution plating

Sample preparation

ICFM - International Comission of Food Micology (I.Pitt.J and D.Hocking.A, 2006) recommends two usual methods for sample preparation (homogenization) in the initial stage of the dilution plating: a) through the homogenizer known as stomaching machine and b) through the homogenizer known as blending machine.

We have used a blending machine (BEER), but in advance grape berries as well as other methods are treated with alcohol (ethanol 70%) for 2 minutes and then washed (cleared) by water for 1 minute.

Diluents and dilution

In this study work we have used aqueous solution of peptone 0.1%. We have prepared a series of dilutions recommended by ICFM, while for inoculation we have used dilutions of 10^{-3} - 10^{-5}, three dilutions for each variety and each semi - selective media. After the preparation a series of

dilutions, we have taken a drop (0.1 ml) from a series of test tubes (10^3 - 10^{-5}) and have thrown it in the Petri dishes, in both semi – selective media. Then through microbiological shoulder we have distributed the material (drop) uniformly through the semi – selective media. Shoulder in each case is treated with alcohol (70% ethanol) and then sterilized with flame.
At the end the Petri dishes are placed in an incubator, in temperature 25 ºC for 5 day. The results of this method (method of dilution) are presented as well as for bacteria, as colonies per gram of analyzed sample (viable counts / gram of the sample). Even to this method as well as to direct plating, after completion of the incubation the samples are examined under a microscope, previously treated with alcohol (ethanol 70%) and lactic acid in microscope slide.

Results and discussion

Table 1. The percentage (%) of moulds infection in the five grape varieties (Direct Plating in MEA-B)

MEA – B	The Petri Dish nr. 1	The Petri Dish nr. 2	The Petri Dish nr. 3	Average %
Sauvignon Blanc	25	25	25	25%
Gamay	43,75	25	25	31,25%
Franconia	25	31,25	25	27,08%
Pinot Blanc	37,5	25	25	29,17%
Muscat Hamburg	6,25	6,25	43,75	18,75%

From the obtained results from the direct and dilution plating in the semi – selective media MEA - B we have seen the pronounced presence of the genera *Alternaria* and other kinds of the moulds, as well as lack of the genera *Aspergillus* (*Aspergillus carbonarius*) and *Penicillium* (*Penicillium verrucosum*).

Based on the results (% of the infection) presented in the tab.1. we can see that in any of the analyzed grape varieties by direct plating, the percentage of infection does not exceed 50% and this probably as a result of applying the appropriate process of splashing the grapes with fungicides in vineyards where sampling is done.

Table 2. The total number of colonies (Dilution Plating in MEA-B)

The total number of colonies in each dilution						
MEA – B	10^{-3}		10^{-4}		10^{-5}	
	The Petri dish nr. 1	The Petri dish nr. 2	The Petri dish nr. 1	The Petri dish nr. 2	The Petri dish nr. 1	The Petri dish nr.2
Sauvignon Blanc	19	15	3	2	-	-
Gamay	24	28	6	2	-	2 (2 fungi colonies)
Franconia	3	1	-	-	-	-
Pinot Blanc	6	11	1	-	-	3
Muscat Hamburg	41	25	2 (1fungi colonies)	7	-	3

From the results of the tab.2. we can see that the dilution to the extent of 10-3 is suitable for the cultivation of moulds of different genres compared to the level of other dilutions, in terms of total number of colonies expressed after the incubation phase.

Table 3. Morphological charachteristics of the moulds isolated in MEA-B (Direct Plating)

The cultures characteristics		The morphological characteristics		Gender	Variety
The colony colour	The pigmentation presence	Hyphae	Spores		
Gray-white colony	Dark brown to black pigmentation	Hyphae - septated	Brown, septated with pyriform shape	Alternaria	Gamay Franconia M. Hamburg Sauvignon Blanc
Olive green colony	Black pigmentation	Hyphae - septated	Green, lemon-shaped	Cladosporium	Franconia Sauvignon Blanc
Light to dark brown	Brown pigmentation	Septated, walls are thick	Not determined	Not determined	Gamay
White colony	No pigmentation	Hyphae - septated	Not determined	Not determined	Gamay Franconia
Dark brown to black colony	Yellow pigmentation	Hyphae - septated	Conidia – brown, oval	Aspergillus carbonarius	M. Hamburg

Of particular importance is the fact that we have isolated at the grape variety *Muscat Hamburg* a colony which according to the morphological characteristics belongs to the genera *Aspergillus*, concretely the *Aspergillus carbonarius* (fig.1.). During the work we have used in the MEA-B the fungicide called boscalide, which inhibits the growth and development of other species of the *Aspergillus* and allows only the growth of the *Aspergillus carbonarius.*

Table 4. The percentage (%) of moulds infection in the five grape varieties (Direct Plating in DYSG)

DYSG	The Petri dish nr. 1	The Petri dish nr. 2	The Petri dish nr. 3	Average %
Sauvignon Blanc	6,25	0	0	2,08%
Gamay	25	6,25	6,25	12,5%
Franconia	6,25	25	12,5	14,58%
Pinot Blanc	0	18,75	0	6,25%
Muscat Hamburg	12,5	0	0	4,17%

Based on the results (% of the infection) presented in the tab.4 we can see that also here like in the tab.1 in any of the analyzed grape varieties by direct plating in the semi – selective media DYSG the percentage of infection does not exceed 50% and the infection rate here is lower compared with the infection in semi – selective media known as MEA – B.

Table 5. The total number of colonies (Dilution Plating in DYSG)

The total number of colonies for each dilution						
DYSG	10^{-3}		10^{-4}		10^{-5}	
	The Petri dish nr. 1	The Petri dish nr. 2	The Petri dish nr. 1	The Petri dish nr. 2	The Petri dish nr. 1	The Petri dish nr.2
Sauvignon Blanc	9	14	2	-	-	-
Gamay	5	5	-	-	-	-
Franconia	1	-	-	2	-	-
Pinot Blanc	7	6	-	-	1	-
Muscat Hamburg	27	26	3	2	-	4

As well as in the tab.2 we can see also here that the dilution to the extent of 10^{-3} is suitable for the cultivation of moulds of different genres compared to the level of other dilutions.

Table 6. Morphological charachteristics of the moulds isolated in DYSG (Direct Plating)

The culture characteristics		The morphological characteristics		Gender	Variety
The colony colour	The presence of pigmentation	Hypha	Spore		
Gray-white	Dark brown to black pigmentation	Hyphae - septated	Brown, septated with pyriform shape	Alternaria	Gamay Francon ia
Brown		Hyphae – septated and thick walls	Oval shape	Not determined	Francon ia

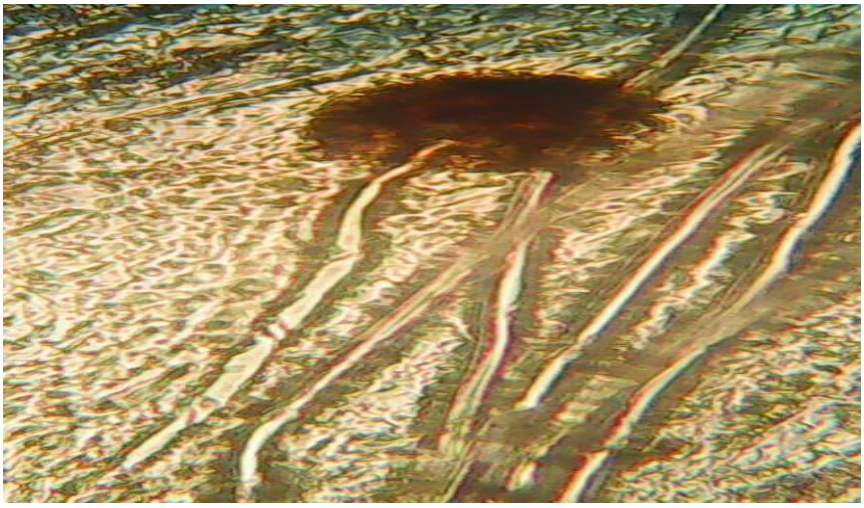

Fig.1. Aspergillus carbonarius isolated in the variety Muscat Hamburg (MEA-B), Suhareka-Kosovo

CONCLUSIONS

After completing of this study work we can conclude that the main species of the fungi responsible for OTA synthesis (*Aspergillus carbonarius* and *Penicillium verrucosum*) appear in very small extent in the vineyards which we have analyzed, or specifically in only one case and that to the type of the table grape known as Muscat Hamburg.
This probably is due to climatic conditions not so favorable for the growth and development of the fungi responsible for the OTA synthesis in Kosovo, or due to adequate application of the splashing process in the analyzed vineyards with substances known as fungicides.
From the obtained results we can see the slightly greater presence of the genera *Alternaria* and that in both semi – selective media (MEA-B and DYSG).Then we can see the presence of the

genera *Cladosporium* only in the MEA-B, this typical for grape together with genera *Alternaria*, as well as other types of microorganisms.
In the future it would be of particular interest to study the effect of the fungicides in the species responsible for synthesizing of OTA in grapes (*Aspergillus carbonarius* and *Penicillium verrucosum*). Concretely, to analyse in microbiological or genetic way these two species in the grape samples, previously treated with fungicide and then untreated with fungicide, in order to confirm the potential effect of the fungicides in these main species of the fungi responsible for the OTA synthesizing.
We can finally conclude that the citizens of Kosovo are not endangered by the consumption of the table and industrial grapes, also the consumption of the wines produced from these grape varieties, in the sense of intoxication with micotoxin known as ochratoxin A (OTA).

REFERENCES

1. Centre for Food Safety, Food and Environmental Hygiene Department, The Government of the Hong Kong Special Administrative Regiondried vine fruits: Ochratoxin A in Food.(2006) Report nr.23; available at: http://www.cfs.gov.hk/
2. D.Flajs, A-M.Domijan, D.Ivić, B.Cvjetković and M.Peraica: ELISA and HPLC analysis of ochratoxin A in red wines of Croatia, (2009) Food Control; 590 – 592.
3. E.Zain., M: Impact of mycotoxins on humans and animals. Journal of Saudi Chemical Society, Volume 15, Issue 2, (2011) Pg: 129–144.
4. El Khoury, A., and Atoui, A: Ochratoxin A: General Overview and Actual Molecular Status. Toxins 2(4): (2010) 461–493.
5. Fugelsang.K and Edwards.Ch. : Wine Microbiology, (2007),USA.
6. J Carlile M., Dr, C Watkinson S., and W Gooday G.: The Fungi (Second Edition), Copyright © 2001 Elsevier Ltd.
7. Javier Cabañes F., Rosa Bragulat M., and Castellá G: Ochratoxin A Producing Species in the Genus Penicillium. Toxins (Basel); (2010) 2(5): 1111–1120.
8. M. Peraica., B. Radic., A. Lucic., and M. Pavlovic: Toxic effects of mycotoxins in humans, Bulletin of the World Health Organization, (1999) 77 (9).
9. Marin-Kuan M., Ehrlich V., Delatour T., Cavin C., and Schilter B: Evidence for a Role of Oxidative Stress in the Carcinogenity of Ochratoxin A. Journal of Toxicology (2011) 645361.
10. Marquardt R.R., Frohlich A.A. (1992): A review of recent advances in understanding ochratoxicosis. PubMed J Anim.Sci. (1992) 70 (12), 3968-88.
11. Pitt J.I. and Hocking A.D: Fungi and Food Spoilage, (2009) Springer, USA.
12. Ribereau-Gayon.P., Dubourdie D., Doneche B., and Lonvaud A: Handbook of Enology, Volume 1, The Microbiology of Vine and Vinifications 2-nd Edition, (2006), France.
13. Visconti. A., Perrone. G., Cozzi. G. and Solfrizzo., M: Managing ochratoxin A risk in the grape-wine food chain, Food Additives & Contaminants; (2008) 193-202.
14. Yordanova, P., Wilfried, K., and Dimitrov, P. (2010): Ochratoxin A and β2 Microglobulin in BEN Patients and Controls. Journal List Toxins (Basel), (2010) V2 (4).
15. Zimmerli B. and Dick R: Ochratoxin A in table wine and grape-juice: Occurrence and risk assessment. Food Additives and Contaminants (1996) 13, 655-668