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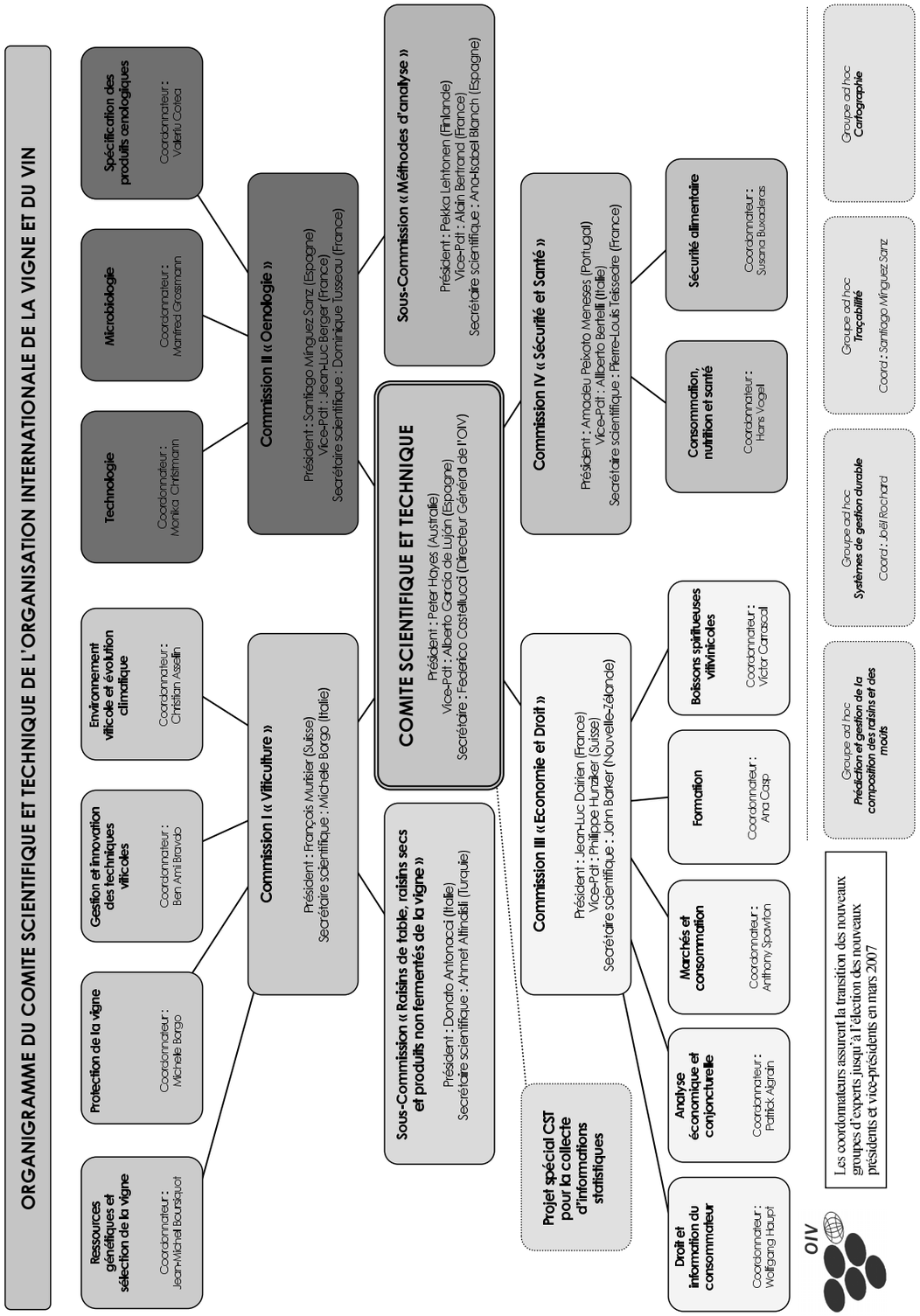

Rapid characterization of potential ochratoxin-producing fungi isolated from grapes and study of natamycin efficacy for its control[1]

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KEY WORDS: oenology, grape, fungy characterisation, *A. carbonarius, A. tubingensis, A. niger*, natamycin, OTA production.

ABSTRACT

Fungal flora on ripe grape and fungi characterization are very critical for assessing the risk of OTA presence in wine, a mycotoxin classified as possible carcinogen to humans.

In the present work, the PCR-RFLP technique has been applied to the ITS1-5.8S-ITS2 region of the rDNA to carry out the characterization of potential OTA producing species of *Aspergillus* section *Nigri* from different grape varieties grown in Spain. The toxin production levels of the different isolates have also been analysed and the ability of natamycin, a fungicide, to control fungal development and OTA production *in vitro* has been studied, as well as the interaction with environmental factors (water activity, temperature).

The results obtained from *in vitro* cultures of 205 isolates of species in *Aspergillus* section *Nigri* showed that 74.2 % of *A. carbonarius* isolates and 14.3 % of *A. tubingensis* isolates were capable of producing OTA at different levels. No isolate of *A. niger* showed OTA producing capacity under the experimental conditions. *A. carbonarius* was the most frequently isolated ochratoxigenic species.

The effect of natamycin against isolates of *A. carbonarius* has been studied according to different level, water activity and temperature. It can be seen that the efficacy of very low concentrations of this fungicide on fungal growth depends on water activity and temperature. (Bulletin de l'OIV, 2006, vol. 79, n° 909-910, pp. 649-661)

1 A paper from the XXIXth World Congress of Vine and Wine. OIV, Logroño (Spain), 25-30 June 2006.

1. INTRODUCTION

Ochratoxin A (OTA) was discovered in 1965 as a secondary metabolite of a strain of *Aspergillus ochraceus* (Van der Merwe et al., 1965). OTA exhibits intestinal fragility, nephrotoxicity, immunosuppresion, theratogenicity, carcinogenicity (JECFA, 1996; Bondy and Armstrong, 1998; Dirheimer, 1998), cytotoxicity in hepatic cell lines (Bondy and Armstrong, 1998), and also induces iron deficiency anemia (Huff et al., 1979). OTA could be responsible for Balkan endemic nephropathy (BEN), observed in Balkanic countries. The International Agency for Research on Cancer (IARC) classifies OTA in group 2B (possible carcinogen to humans) (IARC, 1993).

Fungi from two other genera are known to produce ochratoxins. In genus Penicillium, OTA is produced by P. verrucosum (Pitt, 1987) and in genus Aspergillus, by A. ochraceus, A. melleus, A. auricomus, A. ostianus, A. petrakii, A. sclerotiorum, and A. sulfureus, all in section Circumdati (formerly the A. ochraceus group) (Hesseltine et al., 1972; Abarca et al., 2001; Bayman et al., 2002). A. alliaceus and A. albertensis, formerly placed in section Circumdati, but recently shown to be more closely related to section Flavi, have also been described as producers of OTA (Peterson, 2000). In recent years, some members of Aspergillus section Nigri (formerly A. niger group) such as A. niger var. niger and A. carbonarius have been reported as ochratoxigenic fungi (Abarca et al., 1994; Heenan et al., 1998) and more recently, the ability of the uniseriate species of black aspergilli A. japonicus to produce OTA has been mentioned (Battilani et al., 2003). In A. niger aggregate it has always been difficult to distinguish one taxa from another by morphological means because the differences are very subtle. The division of this A. niger aggregate into two species, namely A. niger and A. tubingensis, according to RFLP analysis of total DNA was proposed by Kuster van Someren et al. (1991). Studies involving a molecular approach followed and substantially confirmed these results (Logrieco et al., 2002). Although the ability of A. niger to produce OTA has been described (Abarca et al., 1994), the species A. tubingensis has not been reported as OTA producer (Abarca et al., 2004).

Ochratoxin A has been detected in human blood (Burdaspal and Legarda, 1998) and food and drinks such as cereals (mainly wheat, barley, corn and oats), seeds, beans, pulses, peanuts, dried fruits, coffee, milk, beer and, in recent years, in wine (Otteneder and Majerus, 2000; López de Cerain et al., 2002). Due to the presence of OTA in food and drinks typical in the human diet, the study of OTA has become increasingly important. The Joint FAO/WHO Expert Committee on Food Additives has discussed the imposition of a maximum tolerable weekly intake of 100 ng of toxin/kg body weight, and a maximum level of 5-20 µg OTA/kg in cereals, both processed and non-processed. The OIV fixed 3 ng/kg as a maximum level of OTA in wine (OIV, 2002).

Wine is a product of great economic relevance around the world, especially in producing countries. Recently, it has been shown that OTA is stable in wine for at least one year (López de Cerain et al., 2002).

Fungal flora on ripe grape is very critical for assessing the risk of OTA presence in wine. Therefore, some researchers have recently studied grape mycobiota in different countries (Abrunhosa et al., 2001; Cabañes et al., 2002), but no attention has been paid to the study of ochratoxigenic fungi co-occurrence in different grape varieties.

Natamycin (pimaricin) is a fungicide produced by *Streptomyces natalensis*, which is commonly used in dairy-based food products for controlling spoilage by moulds, especially in cheese (De Ruig and Von der Berg, 1985). Studies by Basilico et al. (2001) have shown that natamycin at 2% (v/v) and the parabens (0.5% w/v) effectively controlled thread mould growth in vacuum-packed hard cheeses. It has a broad spectrum of activity against spoilage moulds and is considered to be a very stable product with efficacy against *A. flavus* and aflatoxin production (Rusul and Marth, 1988), although the interaction with different environmental factors has not been studied in detail. However, detailed studies of interaction with environmental factors and efficacy on mycotoxigenic species have not been examined.

In the present work, we have applied the PCR-RFLP technique to the ITS1-5.8S-ITS2 region of the rDNA to carry out the characterization of potential OTA producing species of *Aspergillus* section *Nigri* from different grape varieties grown in Spain. We have also analysed the toxin production levels of the different isolates and studied the ability of natamycin to control fungal development and OTA production *in vitro*.

2. MATERIALS AND METHODS

2.1. Fungal isolation and identification

In this study, a total of 52 grape (44 red grapes and 8 white grapes) samples (*Vitis vinifera*) were analyzed. Table 1 shows the studied grape varieties, the geographical origin of the vineyards and the number of samples taken for the different grape varieties.

Table 1. Characteristics of the grape samples used in the study.

Color of berries	*Grape variety*	*Vineyard location* Town (Province)	*Number of samples*
Red	Bobal	Requena (Valencia)	3
		Iniesta (Albacete)	6
		Villamalea (Cuenca)	5
	Garnacha	Iniesta (Albacete)	7
	Tempranillo	Iniesta (Albacete)	7
		Haro (Rioja)	11
	Monastrell	Jumilla (Murcia)	3
		El Pinoso (Alicante)	5
		Sax (Alicante)	5
White	Moscatel	Málaga (Malaga)	4
		Titaguas (Valencia)	2
		Villar del Arzobispo (Valencia)	2

Fifty berries were picked from all the parts of the bunches and homogenized in a stomacher (IUL Instruments, Barcelona, Spain). From the homogenate, decimal seriate dilutions were made under sterile conditions. These solutions were used to inoculate Petri dishes containing Malt Extract Agar (Cultimed, Panreac Química S.A., Barcelona, Spain). Petri dishes were then incubated at 28ºC for 5-7 days in the dark. After incubation, the number of colony forming units of filamentous fungi per millilitre of berry homogenate (CFU/ml) was evaluated. Taxonomic identification of all isolates was achieved through macroscopic and microscopic observation with the aid of guidelines published for each genus or general guidelines.

A. carbonarius was identified through microscopic observation, and *Aspergillus niger* aggregate *(A. niger* and *A. carbonarius)* was identified on the basis of the determination of restriction patterns of PCR-amplified rDNA products. Fungal DNA was isolated according to the method described by Lee and Taylor (33). An ITS1-5.8S-ITS2 rDNA gene was amplified by PCR. Two oligonucleotide fungal primers described by White et al. (1990) were used for amplification (ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS2: 5'-TCCTCCGCTTATTGATATGC-3'). Amplified random products were digested overnight at 37ºC with restriction endonuclease Rsa I (Boehringer Mannheim). PCR products and restriction fragments were separated by electrophoresis in 1% and 2% agarose gels, respectively, with 0.5 x TBE buffer. After electrophoresis, gels were stained with ethidium bromide (0.5 µg/ml), and the DNA bands were visualized with UV transilluminator. DNA sizes were estimated by comparison with a DNA length standard (100 bp molecular marker, Gibco BRL Life Technologies Inc., Rockville, Md., USA). The restriction patterns obtained for the different isolates from grape samples were compared with those obtained in the same conditions from two strains (*A. niger* No 2807 and *A. tubingensis* No 20393) held at the Spanish Collection of Type Cultures (CECT, Valencia University, Burjassot, Valencia, Spain).

LC-ion trap-mass spectrometry was used to unambiguously confirm the presence of OTA in cultures. The analysis was carried out on an Agilent 1100 liquid chromatograph (Agilent Technologies, Waldbronn, Germany), equipped with a Zorbax SB-C18 column (150 x 4.6 mm, 5 µm particle size) (Agilent Technologies) and coupled to a Bruker Esquire 3000 Plus ion trap mass spectrometer (Bruker Instruments, Billerica, Ma., USA). The mobile phase was programmed following a linear gradient at a flow-rate of 0.5 ml/min. Solvent A was water with 0.05% trifluoroacetic acid, solvent B was methanol with 0.05% tifluoroacetic acid. The gradient program was as follows: 0 min, 40% B; 1.5 min, 40% B; 15 min, 100% B. The ionization method was electrospray ionization (ESI) in positive mode by using the following ionization source parameters: N_2 nebulizer gas at 60 psi, dry gas at 10 l/min, dry temperature at 220ºC, and capillary voltage at 3 kV. Confirmation by MS was based on the protonated molecule $[M+H]^+$ and the most abundant product ion $[(M+H)^+ - HCOOH]$ whose m/z ratios are 404 and 358, respectively.

2.2. Study of natamycin efect

Once the first study was concluded, four isolates of *A. carbonarius* isolated from wine grapes were selected to study the capacity of natamycin to control both fungal growth and OTA production *in vitro*. Only two of them were OTA-producers. The formulation used in these studies was Delvocid (50% a.i.).

A freshly prepared red grape extract from organic table grapes, which was modified with glycerol to the required a_w levels (0.98, 0.96, 0.94), was used. The temperatures of incubation were 15, 20 and 25ºC based on the information available indicating that optimum temperature conditions for OTA production are 15-20ºC while those for growth are about 30-35ºC (Mitchel et al., 2004). Mycelial extension rates were measured over periods of 12 days and the linear regression of the linear parts of the radial extension rates was used to determine the growth rates (mm/day).

2.3. OTA extraction and analysis

Twenty grams of each fungal culture (agar + fungal biomass) was cut into small pieces and extracted with 50 ml methanol (Sigma, UK) by shaking at 110 rpm for 1 hr at 25ºC in the dark. The extracts were cleaned up by filtration through filter paper (Whatman No. 4) containing 5-10 g of Celite 545 (Aldrich Chemical Co., UK). One ml of each filtrated extract was removed and centrifuged at 1100 rpm for 15 min for final purification. The supernatant of each sample was removed and placed in LC amber vial for analyse.

The LC system used consisted of a Millipore Waters 600E system controller, a Millipore 712 WISP autosampler and a Millipore Waters 470 scanning fluorescence detector (Millipore Corporation Ma., USA)(excitation and emission wavelengths were 330 and 460 nm, respectively). The samples were separated using a C_{18} Luna Spherisorb ODS2 column (150 x 4.6mm, 5µm) (Phenomenex, Macclesfield, UK), with a guard column of the same material. Run time for samples was 12 min with OTA being detected at about 5.75 min. The flow rate of the mobile phase (acetonitrile-water-acetic acid; 57:41:2 v/v/v) was 1 ml/min. Standards used were in the 50-1200 ng/ml range. The recovery rate was 88% from the agar-based medium with a limit of detection of <0.01 µg OTA/g medium, based on a signal-to-noise ratio of 3:1. Analysis of the results was carried out on a computer with Kroma systems 2000 software (Bio-Tek Instruments, Milan, Italy).

3. RESULTS

Eight fungal genera were mainly isolated from the grape samples (*Aspergillus*, *Alternaria*, *Acremonium*, *Penicillium*, *Cladosporium*, *Fusarium*, *Rhizopus*, and *Phoma*).

When considering the *A. niger* aggregate (*A. niger* and *A. tubingensis*) and *A. carbonarius*, the ANOVA shows that there are significant differences (*p*-value = 0.000) in contamination levels among the different grape varieties. The most contaminated grape varieties by *Aspergillus* section *Nigri* were Monastrell, Moscatel and Bobal, where *A. carbonarius* average contamination levels were 400, 227, and 165 CFU/ml, respectively. The CFUs of *A. carbonarius*/ml in Garnacha and Tempranillo varieties were significantly lower (71 and 32, respectively). Species of the *A. niger* aggregate were isolated less often. Due to morphological similarity within isolates in *A. niger* aggregate, identification of isolates in this group was based on PCR amplification of 5.8S rDNA and its two intergenic spacers ITS1 and ITS2, followed by the subsequent digestion of PCR products with restriction endonuclease Rsa I. The ITS1-5.8S-ITS2 region was amplified from all 85 isolates of the *Aspergillus niger* aggregate. Product size was about 600 bp (596-600 bp). The restriction patterns obtained from this amplified region with enzyme Rsa I were compared with those from the strains *A. tubingensis* CECT 20393 and *A. niger* CECT 2807 supplied by the CECT. Figure 1 shows the results obtained for four isolates of *A. tubingensis* and one isolate of *A. niger*. OTA was detected in cultures of *A. tubingensis*.

As can be seen, enzyme Rsa I does not cut the PCR product. This result was the same for all *A. tubingensis* isolates (T-pattern) regardless of their capacity for producing OTA. Two fragments of 523 and 75 bp (N-pattern) were obtained from all *A. niger* isolates in the same conditions. The PCR-RFLPs for *A. niger* isolate Bo62 from the Bobal grape variety can be seen in Figure 1. No isolate displaying the N-pattern was OTA producer. As can be observed, the T- and N-patterns of isolates of *Aspergillus niger* aggregate from grapes were identical to the patterns obtained from strains *A. tubingensis* CECT 20393 and *A. niger* CECT 2807, respectively.

Figure 1. PCR products digested by Rse I and separated on a 2% agarose gel.

Lanes 1 and 9 are the 100-bp DNAladder (Gibco BRL) used as size markers. Lane 2 Tpattern: strain of *A. tubingensis* CECT 20393. Lane 3 Npattern: strain of *A. niger* CECT 2807. Lanes 4, 5, 7 and 8 shows different isolates of *A. tubingensis*. Lane 6 shows an *A. niger* isolate.

Of an overall 205 *Aspergillus* section *Nigri* isolates that were tested for OTA production using yeast extract-sucrose broth (YES; 2% yeast extract, 15% sucrose) supplemented with 5% bee pollen to increase OTA production, 92 of them were able to produce this toxin. Eighty-nine of these isolates were classified as *A. carbonarius* while the remaining 3 were classified as *A. tubingensis*. OTA was not detected in *A. niger* cultures (Table 2).

Table 2. OTA production capacity of *Aspergillus* section *Nigri* isolates from grapes grown in Spain when cultured in yeast extract sucrose (YES) broth supplemented with 5% bee pollen. Culture conditions: 28 days at 25ºC in the dark.

Fungi	Number of isolates		% positive isolates	OTA (ng/ml)	
	Assayed	Positive		Average*	Range
A. carbonarius	120	89	74.2	155.0	1.2 - 3531.4
A. niger	64	—	—	—	—
A. tubingensis	21	3	14.3	70.7	46.4 - 111.5
Total	205	92	44.9		

* Average OTA level in cultures of positive isolates

Analysis by LC-ion trap-MS-MS of the extracts confirmed the identity of OTA in cultures. The peaks produced by the [M+H]- ion (m/z 404) and the [(M+H)- - HCOOH] ion (m/z 358) were observed in the mass spectra of the OTA standard and the cultures where OTA was previously detected by LC-fluorescence detection.

Evaluation of natamycin for controlling fungal growth and OTA production

Initial studies were carried out on malt extract agar and grape juice-base medium at 25ºC and approx. 0.99 a_w. On the former medium no growth occurred at >0.5 µg natamycin/ml while on grape-juice based medium no growth occurred at >0.25 µg natamycin/ml. Thus, detailed studies were subsequently carried out over a narrow range of concentrations of natamycin to examine efficacy on growth and OTA production.

Figure 2 shows an example of the linear regression of the temporal mycelial extension in relation to different natamycin and water availability conditions for a strain. At 20 ng/ml, the mycelial growth of a strain of *A. carbonarius* was inhibited.

The efficacy was influenced by a_w and temperature. There were some differences between the efficacy with regard to the strains. Growth was sometimes faster at low a_w and very low concentrations of natamycin (1-5 ng/ml), especially at 20-25ºC. Growth was inhibited most effectively at 15ºC over the whole a_w range tested.

Figure 2. Effect of natamycin (10 ng/ml) on mycelial radial extension of *A. carbonarius* on a red grape juice extract medium at three a_w levels and 25ºC. Bars indicate standard errors of means.

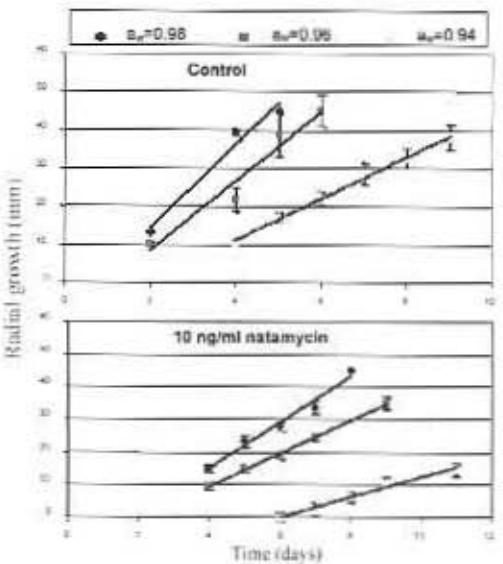
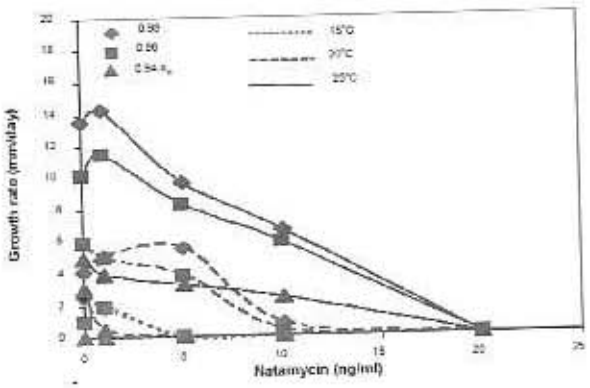


Figure 3 shows the effect of natamycin level, a_w and temperature on the growth rate of *A. carbonarius*. It can be seen that the efficacy of very low concentrations of this fungicide on fungal growth depends on a_w and temperature.

The results obtained with the OTA-producing strains showed that natamycin at 5-10 ng/ml inhibited growth of *A. carbonarius* in cultures at 0.98-0.94 a_w and 25ºC. However, at 20ºC, OTA production was only significantly inhibited using 10 ng/ml. At least this concentration should be added to the medium to ensure the absence of OTA at 0.96 and 0.94 a_w (Data not shown).

Figure 3. Effect of natamycin on growth rate of *A. carbonarius* on a red grape juice extract medium at 15-25ºC and 0.98-0.96 a_w.



4. CONCLUSIONS

Before this report, *A. tubingensis* had not been found to be able to produce OTA. However, in this work, three OTA-producing isolates (two from the Bobal grape variety, and one from the Monastrell) were found.

The results obtained from *in vitro* cultures of 205 isolates of species in *Aspergillus* section *Nigri* showed that 74.2 % of *A. carbonarius* isolates and 14.3 % of *A. tubingensis* isolates were capable of producing OTA at levels ranging from 1.2 to 3531 ng/ml, and from 46.4 to 111.5 ng/ml, respectively. No isolate of *A. niger* showed OTA producing capacity under the experimental conditions. *A. carbonarius* was the most frequently isolated ochratoxigenic species.

Natamycin is very effective against isolates of *A. carbonarius*. Generally, 20-25 ng/ml completely inhibited the growth of the isolates examined, regardless of environmental conditions. However, the efficacy of very low concentrations on growth and OTA control depended on both a_w and temperature. At 25ºC growth inhibition was effective at 5-10 ng/ml and 0.98-0.94 a_w. At 20ºC, OTA production was only significantly inhibited by 10 ng/ml at 0.96 and 0.94 a_w.

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BIBLIOGRAPHY

ABARCA M. L., M. R. BRAGULAT, G. CASTELLA AND F. J. CABAÑES. **Ochratoxin A production by strains of Aspergillus niger var. niger.** In Appl. Environ. Microbiol., 1994, n°60, p. 2650- 2652.

ABARCA, M. L., F. ACCENSI, M. R. BRAGULAT AND F. J. CABAÑES. **Current importance of ochratoxin A-producing Aspergillus sp.** In J. Food Protec., 2001, n°64, p. 903-906.

ABARCA, M. L., F. ACCENSI, J. CANO AND F. J. CABAÑES. **Taxonomy and significance of black Aspergilli**. In Ant. van Leeuwen, 2004, n°86, p. 33-49.

ABRUNHOSA, L., R. R. M. PATERSON Z. KOZAKIEWICZ, N. LIMA, AND A. VENÂNCIO. **Mycotoxin production from fungi isolated from grapes.** In Lett. Appl. Microbiol., 2001, n°32, p. 240-242.

BASILICO, J.C, DEBASILICO, M.Z., CHIERICATTI, C., VINDEROLA, C.G. **Characterization and control of thread mould in cheese.** In Lett. Appl. Microbiol., 2001, n°32, p. 419-423.

BATTILANI P., A. PIETRO, T. BERTUZZI, L. LANGUASCO, P. GIORNI AND Z. KOZAKIEWICZ. **Ocurrence of ochratoxin A-producing fungi in grapes grown in Italy.** In J. Food Protec., 2003, n°66, p. 633- 636.

BAYMAN, P., J. L. BAKER, M. A. DOSTER, T. J. MICHAILIDES AND N. E. MAHONEY. **Ochratoxin production by Aspergillus ochraceus group and Aspergillus alliaceus.** In Appl. Environ. Microbiol., 2002, n°68, p. 2326-2329.

BONDY G. S. AND C. L. ARMSTRONG. **Cytotoxicity of nephrotoxic fungal toxins to kidney-derived LLC-PK1 and OK cell lines.** In Cell Biol. Toxicol., 1998, n°14, p. 323-332.

BURDASPAL, P. A. AND T. M. LEGARDA. **Datos sobre la presencia de ocratoxina A en plasma humano en España.** In Alimentaria , 1998, n°292, p. 103-109.

CABAÑES, F. J., F. ACCENSI, M. R. BRAGULAT, M. L. ABARCA, G. CASTELLÁ, S. MINGUEZ AND A. PONS. **What is the source of ochratoxin A in wine?** In Int. J. Food Microbiol., 2002, n°79, p. 213- 215.

DE RUIG W.G., VAN DEN BERG, G. **Influence of the fungicides sorbate and natamycin in cheese coatings on the quality of cheese.** In Neth. Milk Dairy J., 1985, n°39, p. 165-169.

DIRHEIMER, G. **Recent advances in the genotoxicity of mycotoxins.** In Rev. Méd. Vét. Toulouse, 1998, n°149, p. 605-616.

HUFF, W. E., C. F. CHANG, M. F. WARREN AND P. B. HAMILTON. **Ochratoxin A-induced iron deficiency anemia**. In Appl. Microbiol., 1979, n°37, p. 601-604.

IARC. **Ochratoxin A. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins,** vol. 56, p. 489-521. In IARC monographs on the evaluation of carcinogenic risks to humans. International Agency for Research of Cancer, Lyon, France, 1993.

JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES (JECFA). **Ochratoxin A: A safety evaluation of certain food additives and contaminants.** WHO Food Additives Series, 1996, n°35, p. 363-376.

KUSTERS VAN SOMEREN, M. A., R. A. SAMSON AND J. VISSER. **The use of RFLP analysis in classification of the black Aspergilli: Reinterpretation of Aspergillus niger aggregate**. In Curr. Genet., 1991, n°19, p. 21-26.

LOGRIECO, A., G. STEA, P. BATTILANI AND G. MULÈ. **AFLP and sequence analyses of different ochratoxin producing strains of Aspergillus section Nigri from grape in Europe.** In G. Vannacci and S. Sarrocco. Proceedings, 6th European Conference of Fungal Genetics, Pisa, Italy, 2002, p. 426.

LÓPEZ DE CERAIN, A., E. GONZÁLEZ-PEÑAS, A. M. JIMÉNEZ AND J. BELLO. **Contribution to the study of ochratoxin A in Spanish wines.** In Food Addit. Contam., 2002, n°19, p. 1058-1064.

MITCHEL, D., PARRA, R., ALDRED, D. AND MAGAN, N. **Water and temperature relations of growth and ochratoxin A production by Aspergillus carbonarius strains from grapes in Europe and Israel .** In J. Appl. Microbiol., 2004, n°97, p. 439-445.

ORGANISATION INTERNATIONALE DE LA VIGNE ET DU VIN. **Reduction of ochratoxin in wines.** Resolution CST 1/2002.

PETERSON, S. W. **Phylogenetic relationships in Aspergillus based on rDNA sequence analysis.** In R. A. Samson and J. I. Pitt. Classification of Penicillium and Aspergillus: Integration of modern taxonomic methods. Reading (UK): Harwood Publishers, 2000, p. 323-356.

PITT, J.I. **Penicillium viridicatum, Penicillium verrucosum and production of ochratoxin A.** In Appl. Environ. Microbiol., 1987, n°53, p. 266-269.

RUSUL, G. AND MARTH E. H. **Growth and aflatoxin production by Aspergillus parasiticus in a medium at different pH values and with or without pimaricin.** In Eur. Food Res. Technol., 1998, n°187, p. 436-439.

VAN DER MERWE, K.J., P.S. STEYN AND L. FOURIE. **Ochratoxin A, a toxic metabolite produced by Aspergillus ochraceus.** In Wilh. Nature (London), 1965, n°205, p. 1112-1113.

WHITE, T. J., T. BRUNS, S. LEE AND J. TAYLOR. **Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics**. In M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White. PCR protocols: A guide to methods and applications. San Diego (California): Academic Press Inc., 1990, p. 315-322.