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GROWTH OF *ASPERGILLUS OCHRACEUS, A. CARBONARIUS* AND *A. NIGER* ON CULTURE MEDIA AT DIFFERENT WATER ACTIVITIES AND TEMPERATURES

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ABSTRACT

The objective of this paper was to determine the influence of three culture media with different water activities, times of incubation and temperatures on the growth of *A. ochraceus, A. carbonarius* and *A. niger*. Spores of *A. ochraceus, A. carbonarius* and *A. niger* were inoculated onto three culture media: Czapeck Yeast extract Agar (CYA), Dichloran 18% Glycerol Agar (DG18) and Malt Yeast extract 40% Glucose Agar (MY40G). The plates were incubated at five different temperatures (8, 25, 30, 35 and 41ºC). The growth of fungi was evaluated every 24h, measuring the colony diameter (mm). None of the species grew at 8°C in any culture media. For *A. carbonarius*, 30ºC was the best temperature for growth while for *A. niger* temperatures above 30ºC were better in all culture media. *A. ochraceus* presented good growth at 25 and 30ºC in all culture media, while at 35ºC, growth was slower, especially on CYA. At 41ºC, *A. ochraceus* did not grow in any culture media and *A. carbonarius* was significantly inhibited. *A. niger* grew at 41ºC and was shown to be the most xerophilic fungi when compared to *A. carbonarius* and *A. ochraceus*.

Key words: *Aspergillus ochraceus, Aspergillus carbonarius, Aspergillus niger,* growth measurement, water activity, temperature

INTRODUCTION

Fungi are significant environmental microrganisms especially in foods where they are responsible for spoilage, production of mycotoxins and, in some cases desirable bioconversions. Consequently, it is important to know their requirements for water, temperature, nutrients, oxygen and other factors for their growth. The most common genera of fungi in food are *Aspergillus, Penicillium* and *Fusarium*. Several species of these genera are able to produce mycotoxins, which are of concern to public health. Among them, ochratoxin A is nefrotoxic and carcinogenic to some animals (20,25) and has been detected in different types of foods such as cocoa and cocoa products (14), coffee (11,22,23), dried fruits (12,13), cereals (9), wines (15,26,27), beer (5) and others (2). Ochratoxin A (OA) is believed to be produced in nature by three main species of fungi, *Aspergillus ochraceus, A. carbonarius* and *Penicillium verrucosum*, with a minor contribution by *A. niger* (8). *P. verrucosum* is believed to occur only in cool temperate climates, and is mainly associated with cereals (18,19). *A. carbonarius* and *A. niger* were described as sources of OA (1,6,24). *A. ochraceus* has been isolated from several green coffee samples originating from coffee producing countries (4,23).

Several data of growth conditions for *A. niger* and *A. ochraceus* have been recorded. However, little has been published about *A. carbonarius*. Most information about its physiology is assumption based on *A. niger*, because of its similarity. It may be closely related, but it differs from *A. niger* most notably in the production of larger spores (10). The importance of studying *A. carbonarius* is because of its ability to produce ochratoxin A, which is much greater than that of *A. niger*. *A. niger* has been reported to grow optimally at relatively high temperatures, with a maximum of 45 to 47ºC and optimal conditions from 35 to 37ºC. This species has been reported as a

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xerophile with germination reported at 0.77 a_w at 35ºC (19). *A. ochraceus* has been described as a mesophilic xerophile. Growth occurs between 8 and 37ºC, with the optimum from 24 to 31ºC (16,19,21). Previous studies suggest that the a_w minima for *A. ochraceus* varies from 0.76 to 0.88 depending on the substrate (3) and the optimum from 0.98 to 0.96 (21). The objective of this work was to study the effect of temperature, culture media and water activity (a_w) on the growth of three OA producing fungi: *A. niger, A. carbonarius* and *A. ochraceus.*

MATERIALS AND METHODS

Fungal species

Three species of fungi producers of ochratoxin A isolated from green coffee from São Paulo State, Brazil by Taniwaki *et al*. (23) were studied: *A. niger* ITAL 704, *A.carbonarius* ITAL 170 and *A. ochraceus* ITAL 118.

Culture media

The following media were used: Czapeck Yeast Extract Agar (CYA), a_w 0.997 (17); Dichloran 18% Glycerol Agar (DG18), a_w 0.955 (7) and Malt Yeast 40% Glucose Agar (MY40G), a_w 0.897 (19).

Cultivation

Cultures were grown on each medium in standard size (85mm) plastic Petri dishes. Inocula were prepared from 5 day old cultures grown on Malt Extract Agar (MEA) (17). A suspension of spores was prepared in a phosphate buffer (pH 7.2) with 0.1% of Tween 80. The spores were counted in a haemacytometer giving 10^4 spores/ml. A drop of suspension of each fungus was inoculated, separately, on the centre of each culture medium. Plates were incubated upright at temperatures of: 8, 25, 30, 35 and 41ºC. All experiments were performed in 6 replicates.

Growth Measurements

Fungal growth was measured at intervals of 24h after the third day of incubation. The reverse side of the colonies was measured in millimetres with a ruler.

Statistical analysis

Co-variance was used to analyse colony diameter so that the effects of one or more factors (a_w, temperature, species and time) could be assessed separately, for statistically significant differences. The SAS System (version 6.12, SAS Institute, Cary, NC 27513, USA) statistical package was used.

RESULTS

The growth of *A. ochraceus, A. carbonarius* and *A. niger* on different culture media, time and temperatures is shown in Figs. 1 to 3, respectively.

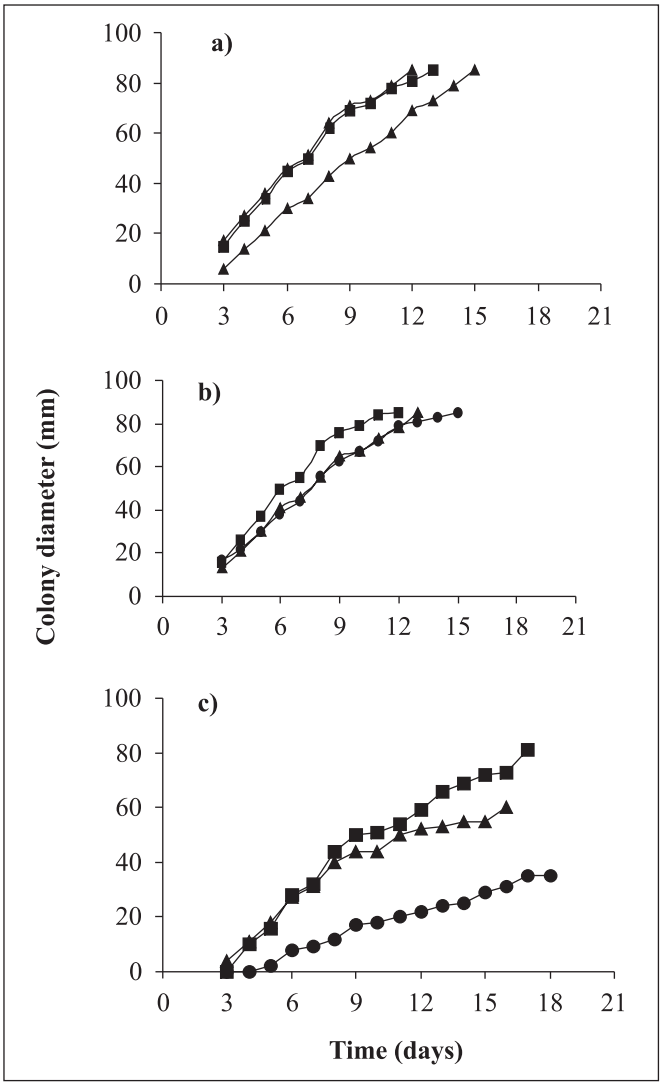

Figure 1. Growth of *A. ochraceus* on three different culture media (● CYA; ■ DG18; ▲ MY40G) at the temperatures of a) 25ºC; b) 30ºC; c) 35ºC.

The results represent an average of 6 replicates. At 8ºC there was no growth of any of these three species on the three culture media. As shown in Fig. 1, the temperatures of 25 and 30ºC favoured fast colony diameter growth for *A. ochraceus*. On the other hand, slower growth can be observed at 35ºC and total inhibition at 41ºC on all of the culture media. At 35ºC, *A. ochraceus* grew better on DG18 and MY40G than on CYA. A more xerophilic character may be attributed to this fungus at 35ºC. For *A. carbonarius*, 30ºC was shown to be the best temperature for its growth on CYA, DG18 and MY40G. *A. carbonarius* grew poorly at 41ºC reaching 12, 33 and 40 mm on CYA, DG18 and MY40G, respectively, and with no more growth after 6 to 8 days (Fig. 2). In general, growth of *A. niger* was

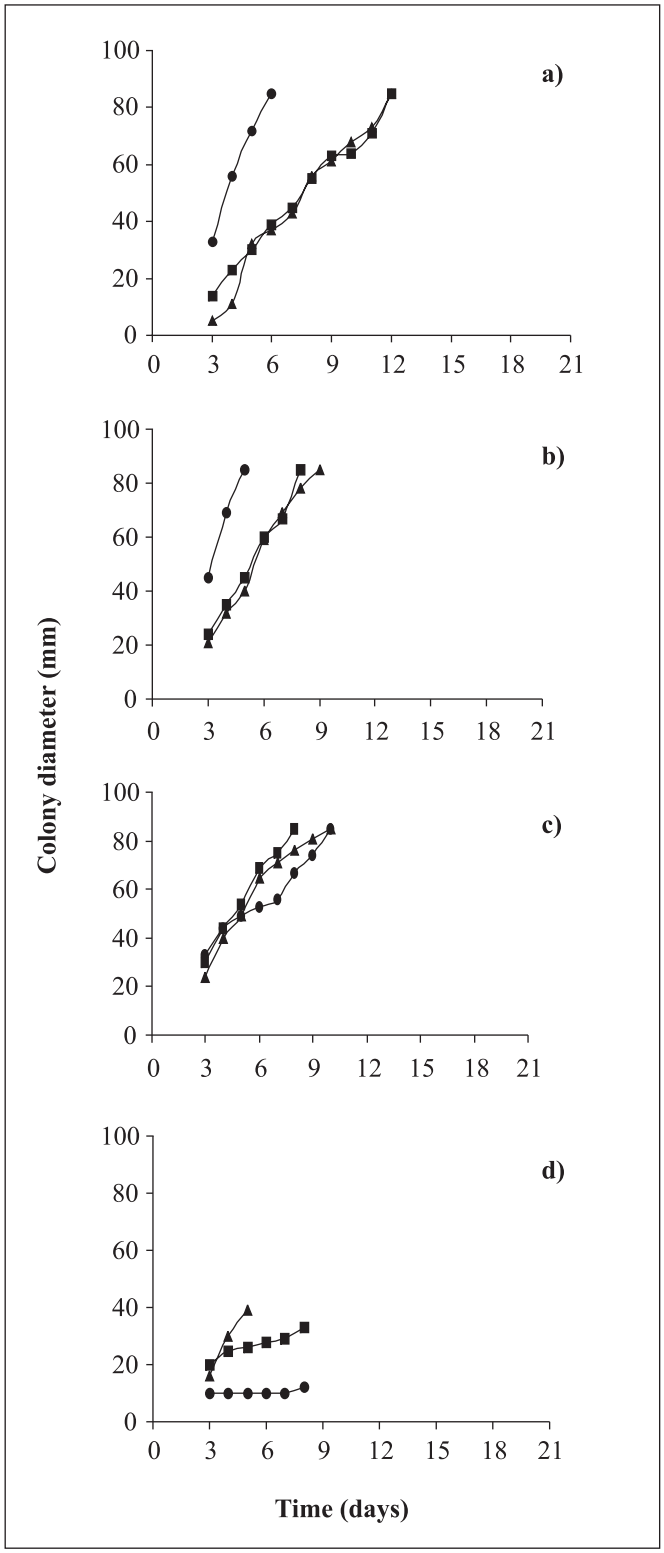

Figure 2. Growth of *A. carbonarius* on three different culture media (● CYA; ■ DG18; ▲ MY40G) at temperatures of a) 25ºC; b) 30ºC; c) 35ºC; d) 41ºC.

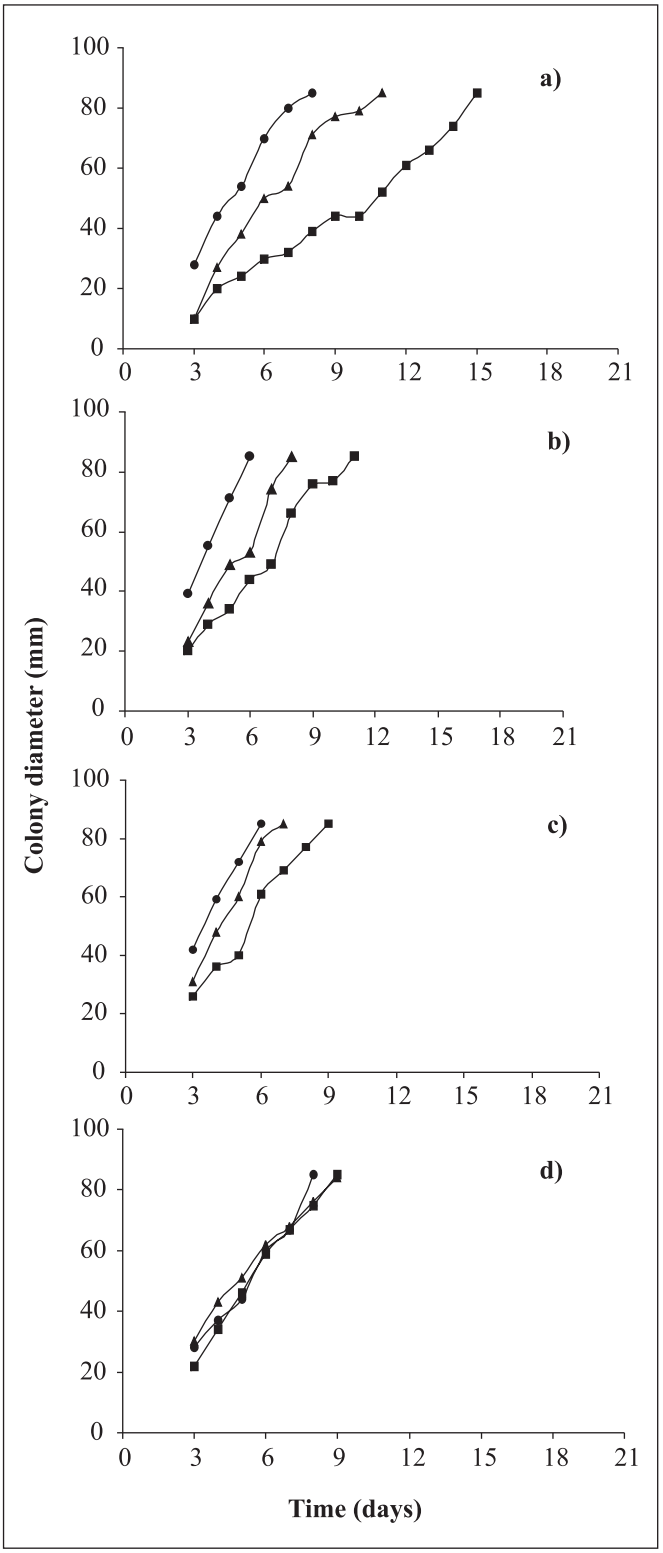

Figure 3. Growth of *A. niger* on three different culture media; (● CYA; ■ DG18; ▲ MY40G) at the temperatures of a) 25ºC; b) 30ºC; c) 35ºC; d) 41ºC.

faster at 35ºC on all culture media. This shows that *A. niger* prefers to grow at temperatures higher than 30ºC. At 41ºC, this species spread over the whole plate (85 mm) on CYA, DG18 and MY50G after 8, 9 and 10 days, respectively (Fig. 3). At the temperature of 41ºC, *A. carbonarius* was significantly inhibited in all culture media. The range of temperatures between 25 to 41ºC was not a limiting factor for growth of *A. niger*. *A. carbonarius* grew faster than *A. ochraceus* at temperatures above 30ºC, especially at 35ºC. Significant differences in a_w and temperature were observed for the growth of all the species tested.

Statistical analyses showed significant differences between species ($P < 0.001$) due to a_w, temperature, species, and two-and three-way interactions (Table 1).

Analysing the data for species as it is shown in Table 2 a similar tendency can be observed from the data of Table 1, in relation to the single effect. The differences appeared when the interactions of the effects of a_w X temperature were compared. However, they were only significant for *A. ochraceus.* Another fact which should be emphasized is the interaction of time X a_w, which was only statistically significant for *A. niger* (Table 2).

Table 1. Analysis of co-variance of the effect of water activity of the culture media (a_w), temperature (T), time (t) and species (S) and their interactions, on the colony diameter of *Aspergillus ochraceus*, *Aspergillus niger* and *Aspergillus carbonarius*.

Factor	df	MS	F
S	2	2763.90	285.01*
a_w	2	1759.57	181.44*
T	2	620.09	63.94*
t	2	6125.14	315.81*
S X a_w	4	710.49	73.27*
S X T	4	320.57	33.06*
S X t	4	72.72	7.50
a_w X T	4	323.68	33.38*
a_w X t	4	31.38	3.24
T X t	4	13.01	1.34
S X T X t	8	7.27	0.75
S X a_w X t	8	28.84	2.97
S X a_w X T	8	80.38	8.29*
a_w X T X t	8	12.05	1.24

* Significant $P < 0.001$.

DISCUSSION

The temperature of 8ºC was inhibitory for *A. ochraceus, A. carbonarius* and *A. niger*. Pitt and Hocking (19), have recorded minimum growth of *A. niger* and *A. ochraceus* at 6 and 8ºC, respectively, which differs from the data obtained in the present experiment. This may be due to the differences among the strains isolated from different origins around the world. *A. carbonarius* although similar to *A. niger*, has its own distinct growth character, especially at temperatures higher than 35ºC. While *A. niger* grew very well at 41ºC on all media, *A. carbonarius* did so poorly. *A. ochraceus* did not grow at all at 41ºC in all culture media. The media with reduced a_w such as DG18 and MY40G, were not a limiting factor for these three species. On MY40G (a_w 0.897), the three species were able to grow at temperatures from 25 to 35ºC. *A. carbonarius* grew better at MY40G than CYA at 41ºC. These observations may suggest that the substrate may influence the thermotrophic behaviour of the fungi. MY40G has a higher sugar concentration than CYA. Although DG18 is not a suitable medium to study the kinetic of fungal growth, it was also included because it is commonly used to isolate fungi from food and it was important to evaluate the behaviour of these three species on this medium. Studies on culture media with different compositions and water activities at the laboratorial level are useful in order to follow the kinetic of fungal growth under different conditions.

Table 2. Analysis of co-variance of the effect of water activity of the culture media (a_w), temperature (T), time (t) on the colony diameter of *Aspergillus ochraceus*, *Aspergillus niger* and *Aspergillus carbonarius* on CYA, DG18 and MY40G.

Species	Factor	df	MS	F
A. ochraceus	a_w	2	900.60	53.41*
	T	2	2167.31	128.52*
	time	10	2478.75	146.99*
	a_w X T	4	796.53	47.24*
	a_w X time	20	20.62	1.22
	T X time	20	61.78	3.66*
A. niger	a_w	2	1438.48	1117.67*
	T	2	719.37	558.94*
	time	2	1272.93	989.04*
	a_w X T	4	13.81	10.73 (p= 0.0027)
	a_w X time	4	53.37	41.47*
	T X time	4	2.09	1.63
A. carbonarius	a_w	2	1681.81	61.20*
	T	2	435.59	15.85 (p= 0.0016)
	time	2	1436.26	52.26*
	a_w X T	4	346.48	12.61 (p= 0.0016)
	a_w X time	4	30.15	1.10
	T X time	4	11.26	0.41

*Significant $P < 0.001$.

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RESUMO

Crescimento de *Aspergillus ochraceus, A. carbonarius* e *A. niger* em meios de cultura com diferentes atividades de água e temperaturas

Esporos de *A. ochraceus, A. carbonarius* e *A. niger* foram inoculados em três meios de cultura: Agar extrato de levedura Czapeck (CYA), Agar Glicerol 18% com dicloran (DG18), Agar Extrato de levedura e malte com 40% de glicose (MY40G). As placas foram incubadas em 5 temperaturas diferentes: 8, 25, 30, 35 e 41ºC. O crescimento dos fungos foi avaliado medindo o diâmetro da colônia a cada 24h. O objetivo deste trabalho foi determinar a influência de três meios de cultura com atividade de água diferente tempo de incubação e temperaturas, sobre o crescimento de *A. ochraceus, A. carbonarius* and *A. niger*. Nenhuma das espécies cresceu à 8ºC em nenhum dos meios de cultura. Para *A. carbonarius*, 30ºC foi a melhor temperatura para o seu crescimento enquanto para *A. niger* temperaturas acima de 30ºC foram melhores em todos os meios de cultura. *A. ochraceus* apresentou bom crescimento a 25 e 30ºC em todos os meios de cultura, enquanto seu crescimento à 35ºC foi mais lento, especialmente no meio CYA. A 41ºC, *A. ochraceus* não cresceu em nenhum dos meios de cultura estudados e *A. carbonarius* foi significantemente inibido. *A. niger* cresceu à temperatura de 41ºC e apresentou-se como o fungo mais xerofílico, comparado com *A. carbonarius* e *A. ochraceus*.

Palavras-chave: *Aspergillus ochraceus, Aspergillus carbonarius, Aspergillus niger*, medida de crescimento, atividade de água, temperatura

REFERENCES

1. Abarca, M.L.; Bragulat, G.; Castellá, G.; Cabanes, F.J. Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. *Appl. Environ. Microbiol.*, 60, 2650-2652, 1994.
2. Bucheli, P.; Taniwaki, M.H. Research on the origin, and on the impact of post-harvest handling and manufacturing on the presence of ochratoxin A in coffee. *Food Addit. Contamin.*, 19, 655-665, 2002.
3. Christensen, C.H. Invasion of stored wheat by *Aspergillus ochraceus. Cereal Chem.* 39, 100-106, 1962.
4. Frank, J.M. On the activity of fungi in coffee in relation to ochratoxin A production. *19th ASIC Coffee Conference*, Trieste, Italy, 2001. CD room.
5. Guldborg, M. Ochratoxin A in Danish beer. *Brygmesteren.*, 54, 16-17, 1997.
6. Heenan, C.N.; Shaw, K.J.; Pitt, J.I. Ochratoxin A production by *Aspergillus carb*onarius and A. niger isolates and detecting using coconut cream agar. *J. Food Mycol.*, 1, 67-72, 1998.
7. Hocking, A.D.; Pitt, J.I. Dichloran-glycerol medium for enumeration of xerophilic fungi from low moisture foods. *Appl. Environ. Microbiol.*, 39, 488-492, 1980.
8. JECFA (Joint FAO/WHO Expert Commitee on Food Additives) Safety evaluation of certain mycotoxins in foods. WHO Food Additives Series 47, World Health Organization, Geneva, 2001.
9. Jorgensen, K.; Bilde, B. Occurrence and estimated dietary intakes of ochratoxin A in European countries – results from SCOOP Project. *Food Addit. Contamin. Supplem.*, 13S, 15-16, 1996.
10. Klich, M.A.; Pitt, J.I. *A Laboratory Guide to Common Aspergillus species and their Teleomorphs.* CSIRO Division of Food Science and Technology, Sydney, Australia, 1988.
11. Levi, P.C.; Trenk, H.L.; Mohr, H.K. Study of the occurrence of ochratoxin A in green coffee beans. *J. Assoc. Offic. Analyt. Chem.*, 57, 866-870, 1974.
12. MacDonald, S.; Wilson, P.; Barnes, K.; Damant, A.; Massey, R.; Mortby, E.; Shepherd, M.J. Ochratoxin A in dried vine fruit: method development and survey. *Food Addit. Contamin.*, 16, 253-260, 1999.
13. MAFF (Ministry of Agriculture, Fisheries and Food). Survey of aflatoxins and ochratoxin A in cereals and retail products. MAFF Food Surveillance Information Sheet 130, London, 1997.
14. Matissek, R.; Raters, M. Ochratoxin A in cocoa and human health aspects. *13th International Cocoa Research Conference*, Kota Kinabalu, Malaysia, 9-14 October, 2000.
15. Otteneder, H.; Majerus, P. Occurrence of ochratoxin A (OTA) in wines: influence of the type of wine and its geographical origin. *Food Addit. Contamin.*, 17, 793-798, 2000.
16. Paster, N.; Chet, I. Effects of environmental factors on growth and sclerotium formation in *Aspergillus ochraceus. Can. J. Bot.*, 58, 1844-1850, 1980.
17. Pitt, J.I. *The Genus Penicillium and its Teleomorphic States Eupenicillium and Talaromyces.* London: Academic Press, 1979.
18. Pitt, J.I. *Penicillium viridicatum, Penicillium verrucosum* and production of ochratoxin A. *Appl. Environ. Microbiol.*, 53, 266-269, 1987.
19. Pitt, J.I.; Hocking, A.D. Fungi and Food Spoilage Blackie, London, 1997.
20. Pohland, A.E.; Nesheim, S.; Friedman, L. Ochratoxin A: a review. *Pure Appl. Chem.*, 64, 1029-1046, 1992.
21. Ramos, A.J.; Labernia, N.; Marín, S.; Sanchis, V.; Magan, N. Effect of water activity and temperature on growth and ochratoxin production by three of *Aspergillus ochraceus* on a barley extract medium and on barley grains. *Intern. J. Food Microbiool.*, 44, 133-140, 1998.
22. Stegen, G.V.D.; Jorissen, U.; Pittet, A.; Saccon, M.; Steiner, W.; Vincenzi, M.; WIinkler, M.; Zapp, J.; Schaltter, C. Screening of European coffee final products for occurrence of ochratoxin A (OTA). *Food Addit. Contamin.*, 14, 211-216, 1997.
23. Taniwaki, M.H.; Pitt, J.I.; Teixeira, A.A.; Iamanaka, B.T. The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. *Intern. J. Food Microbiol.*, 82, 173-179, 2003.
24. Téren, J.; Varga, J.; Hamari, Z.; Rinyu, E.; Kevei, É. Immunochemical detection of ochratoxin A in black *Aspergillus* strains. *Mycopathol.*, 134, 171-176, 1996.
25. WHO *Report of the 56th Meeting of the Joint FAO/WHO Expert Committee on Food Additives*, Geneva, Switzerland, 6-15 February, 2001.
26. Zimmerli, B.; Dick, R. Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid chromatography with enhanced fluorescence detection and immunoaffinity column cleanup methodology and Swiss data. *J. Chromat.*, 666, 85-99, 1995.
27. Zimmerli, B.; Dick, ROchratoxin A in table wine and grape-juice: occurrence and risk assessment. *Food Addit. Contamin.*, 13, 655-668, 1996.