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REGULAR ARTICLE

COLONIZATION OF GRAPE BERRIES BY REPRESENTATIVES OF THE GENUS *ALTERNARIA* AND THEIR OCCURRENCE IN THE STUM

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ABSTRACT

The aim of the study was to analyze the mycobiota occurring on the grapes originating from Slovak wine-growing regions. The main focus was to discover the presence of the representatives of the genus *Alternaria* on/in grape berries and in the stum. Using the direct method of placing grape berries on the culture medium was analyzed their total and endogenous (after superficial sterilization) mycobiota. Plate dilution method was used for stum mycobiota determination. *Alternaria* spp. colonized grapes on the surface and inside with an isolation frequency (Fr) of 100%. Moreover, 100% of the stum samples were positive for the presence of this genus. Their relative density (RD) was 44.9% (unsterilized grapes), 57.9% (sterilized grapes) and 6.35% (stum). In all the areas analysed we recorded 4 species-groups: *A. alternata*, *A. arborescens*, *A. infectoria* and *A. tenuissima*. With the highest Fr and RD occurred *A. tenuissima* species-group, followed by *A. alternata* and *A. arborescens* species-groups. All of them are potential producers of mycotoxins as alternariol, alternariol monomethylether, tenuazonic acid, altertoxin I, II and III.

Keywords: *Alternaria* sp., grape berries, mycobiota, stum

INTRODUCTION

Grapes have a complex microbial ecology including filamentous fungi, yeasts and bacteria with different physiological characteristics and effects upon wine production. Some species are only found in grapes, such as parasitic fungi and environmental bacteria, while others have the ability to survive and grow in wines, constituting the wine microbial consortium. The proportion of these microorganisms depends on the grape ripening stage and on the availability of nutrients **(Barata *et al.,* 2012)**. Contamination by different moulds occurs during preharvesting, harversting and grape processing. During these periods, temperature and humidity are important factors in mycelium growth and conidia germination **(Lozada, 1995)**. Rotting and spoilage of grape berries before harvest can be caused by a variety of fungal species. *Botrytis cinerea*, which causes bunch rot, is the main species. Other species include *Penicillium*, *Aspergillus*, *Alternaria* and *Cladosporium*. Grapes that are heavily infected with moulds alter in chemical composition and mould enzymes adversely affect wine flavour and colour and the growth of yeasts during alcoholic fermentation **(Fleet, 1999, 2001)**. *Botrytis cinerea* damages the berries and has a detrimental effect on the organoleptic properties. Moulds like *Aspergillus* spp. or *Penicillium* spp., possibly producing mycotoxins, are active mostly in the vineyard, although their metabolites may affect wine quality during grape processing **(Barata *et al*., 2012; Serra *et al*., 2006; Pitt et Hocking, 1997, 2009)**. The main concern from the viewpoint of mycotoxin contamination is the black Aspergilli, *Aspergillus carbonarius* and *A. niger*. These fungi are capable of producing ochratoxin A (OTA) which may contaminate grapes and grape products such as wine, grape juice and dried vine fruit **(Hocking *et al.,* 2007)**.

Surveys of the fungi to which grapes are exposed in the vineyard were conducted in the main wine-producing countries in Europe such as France **(Sage *et al*., 2004)**, Greece **(Tjamos *et al.,* 2004)**, Italy **(Battilani *et al.,* 2003)**, Portugal **(Abrunhosa *et al.,* 2001; Serra *et al.,* 2003)** and Spain **(Bau et al. 2005)**, and also in countries such as Australia **(Leong *et al.,* 2004)**, Argentina and Brazil **(Rocha Rosa *et al.,* 2002)**.

In view of the fact that there are a number of fungi that can produce toxins in vineyards, it is necessary to examine their distribution and potential to endanger the health security of the grape products. The fact, that on the detection of *Alternaria* mycotoxins in wine there are very few publications, there is no guarantee that in the wine and other products from grapes they are not present and they do not constitute any risk.

The aim of this work was to evaluate the mycobiota of healthy Slovak grapes destined for commercial winemaking at harvest time and to identify the species-groups of *Alternaria* with the potential to produce mycotoxins.

MATERIAL AND METHODS

The study was focused on the mycological analysis of grapes and stum (grape juice) with a focus on potentially toxigenic representatives of genera *Alternaria*.

For the analysis we used 20 samples collected from various Slovak localities in 2011 (Table 1). The collection of grape samples took place in the time of their technological ripeness. The grapes were picked at random by the diagonal of the land and each sample was made up of around 3 kg of grapes. Samples were collected in sterile plastic containers, stored in a cool place and transported to the mycological laboratory for analysis up to 24 hours from the collection. Totally 16 samples came from vineyards with conventional way of management, 3 samples (No 14, 15, 16) from the vineyards with the bio-production and sample No 10 was collected from a variety produced without chemical protection, with a natural resistance to economically important vine disease such as powdery mildew and diseases caused by *Peronospora* and *Botrytis*.

The **total mycobiota** was determined by the method of direct placing of grape berries on agar plates **(Samson *et al.*, 2002b)**. Exactly 50 berries from each sample were placed on DRBC plates (agar with dichloran, rose bengal and chloramphenicol) **(Samson *et al.*, 2002a)**. Cultivation lasted from 5 to 7 days in darkness at 25 ± 1 °C.

The **endogenous mycobiota** was determined by the method of direct placing of superficially sterilized berries on agar plates **(Samson *et al.*, 2002b)**. More than 50 pieces of undamaged berries from each sample were superficially sterilized with chloramine solution, prepared from 10 ml of distilled water and 5 g of chloramine. Sterilization was carried out 2 minutes. Grains were rinsed 3 times with sterile distilled water and dried on sterile filter paper. Exactly 50 berries from each sample were placed on DRBC plates **(Samson *et al.*, 2002a)**. Cultivation lasted from 5 to 7 days in darkness at 25 ± 1 °C.

The **analyses of stum** were determined by using the plate dilution method. From each sample were squeezed more than 250 g of randomly selected berries and 20 ml of the stum has been added to 180 ml of sterile peptone water containing 0.02% Tween 80. Prepared suspensions were shaken on a Stomacher easyMix®. Dilutions 10^{-1}, 10^{-2} and 10^{-3} were in the

double repetition surface-inoculated in amount of 0.1 ml on DRBC agar plates. Cultivation lasted from 5 to 7 days in darkness at 25 ± 1 °C.

Table 1 Sampling points of mycologically analyzed Slovak grape berries and their varieties

No	Town or village	Wine-growing rayon	Wine-growing region	Variety
1.	Báb	Šintavský	Nitrianska	Chardonnay
2.	Nitra	Nitriansky	Nitrianska	mixture
3.	Oponice	Radošinský	Nitrianska	Chardonnay
4.	Beladice	Zlatomoravecký	Nitrianska	Riesling Italico
5.	Vinodol	Vrábeľský	Nitrianska	Chardonnay
6.	Komjatice	Žitavský	Nitrianska	Riesling
7.	Čaka	Želiezovský	Nitrianska	Riesling Italico
8.	Nová Dedina	Tekovský	Nitrianska	Green veltliner
9.	Brhlovce	Pukanecký	Nitrianska	Pinot blanc
10.	H. Moravce	Hontiansky	Stredoslovenská	Konkordia
11.	Gbelce	Strekovský	Južnoslovenská	Riesling Italico
12.	Mužla	Štúrovský	Južnoslovenská	Riesling Italico
13.	Pribeta	Hurbanovský	Južnoslovenská	Riesling
14.	V. Krtíš	Modrokamenský	Stredoslovenská	Pinot gris
15.	V. Krtíš	Modrokamenský	Stredoslovenská	Pinot noir
16.	V. Krtíš	Modrokamenský	Stredoslovenská	Sauvignon
17.	Modra	Modranský	Malokarpatská	Pinot blanc
18.	Zeleneč	Trnavský	Malokarpatská	Cabernet Sauvignon
19.	Báb	Šintavský	Nitrianska	Traminer
20.	Báb	Šintavský	Nitrianska	Blaufränkisch

Grown micromycetes were classified into the genera and then isolated by re-inoculation on the identification nutrient media and identified through macroscopic and microscopic observation in accordance with accepted mycological keys and publications. Isolates of the genus *Alternaria* were re-inoculated on PCA - potato-carrot agar **(Samson et al., 2002a)** and cultured for 7 days at room temperature and natural light. The colonies were examined according to the classification schemes proposed by **Simmons et Roberts (1993)**.

Another used identification keys were **Andersen *et al.* (2001)**, Andersen et al. (2002), **Dugan and Peever (2002)**, **Simmons (1994)** and **Simmons (2007)**.

The obtained results were evaluated and expressed in isolation frequency (Fr) and relative density (RD) at the genus and species level. The isolation frequency (%) is defined as the percentage of samples within which the species or genus occurred at least once. The relative density (%) is defined as the percentage of isolates of the species or genus, occurring in the analyzed sample **(Guatam *et al.*, 2009)**. These values were calculated according to **González *et al.* (1996)** as follows:

Fr (%) = (ns / N) x 100 RD (%) = (ni / Ni) x 100

where ns = number of samples with a species or genus; N = total number of samples; ni = number of isolates of a species or genus; Ni = total number of isolated fungi.

RESULTS AND DISCUSSION

Submitted study monitored the occurrence of micromycetes from the genus *Alternaria*, which are able to produce mycotoxins and thus aggravate the impact of wine products on human health. Results confirmed a claim of **Scott (2001)**, that *Alternaria* spp. are important fungal contaminants of fruits and fruit products, including *A. alternata*, a contaminant of various fruits. Mycological analyses (Figure 1) have shown that the representatives of this genus occur on grape berries with a 100% isolation frequency (Fr) (within the total as well as the endogenous mycobiota). In addition, their averaged relative density (RD) was also quite high - 44.9% within the total and 57.9% within the endogenous mycobiota. Some other authors have similar findings. According to **Bau *et al.* (2005)**, predominant mycobiota of the Spanish wine grapes belonged to *Alternaria* spp., *Cladosporium* spp. and *Aspergillus* spp.. **Serra *et al.* (2006)** reported, that *Alternaria* was one of the most frequent genera on grapes without surface disinfection in all the regions of Portugal. Most of the fungi found are ubiquitously distributed, such as the field fungi *Alternaria*, *Cladosporium* and *Epicoccum*, which occur commonly in the air, plant surfaces, debris and soil. **Magnoli *et al.* (2003)** noted that *Alternaria* genus was the most frequent (80% of the samples) genus found on surface-disinfected wine grapes from Argentina. *Alternaria alternata* was the only species identified from this genus. In samples from Slovakia we recorded the presence of 4 groups of the genus *Alternaria* – *A. alternata* species-group, *A. arborescens* species-group, *A. infectoria* species-group and *A. tenuissima* species-group. Referred species-groups were identified according to the classification schemes proposed by

Simmons et Roberts (1993) and within each group is still possible to observe some differences. Isolates, which could not be closer specified were marked as *Alternaria* sp.. Isolation frequency and relative density of each group (within the genus) from grape berries are listed in the Table 2. Sample No 10 (variety produced without chemical protection, with a natural resistance to economically important vine diseases) was actually the least inhabited by representatives of this genus (unsterilized grapes – RD 16.8%, sterilized grapes – RD 28.2%). On the contrary, in the samples from bio-production (No 14, 15, 16), the values of RD were relatively high and ranged between 31.1 – 50.4% (unsterilized grapes) and 60.0 – 67.5% (sterilized grapes).

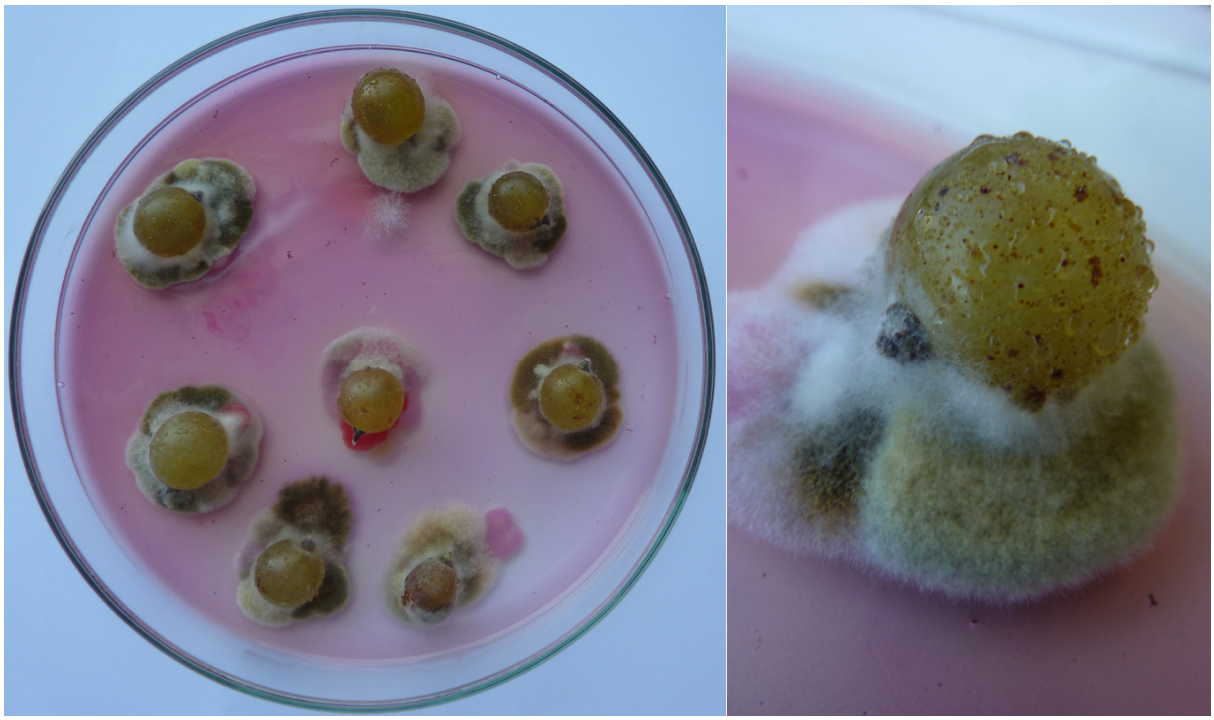

Figure 1 Grape berries of Slovak origin, colonized mostly by *Alternaria* species (agar with dichloran, rose bengal and chloramphenicol)

The study has shown that representatives of the genus *Alternaria* come into stum in lower numbers. Isolation frequency of the genus was still 100%, but relative density was only 6.4%. The dominant genus of the stum was *Cladosporium*. Isolation frequency and relative density of each group (within the genus) isolated from the stum are listed in the Table 3.

Mycological analyses of all 3 articles (unsterilized, sterilized grapes and stum) have found that the most dominant species-group is *A. tenuissima*, followed by *A. alternata* and *A. arborescens* species-groups (Figure 2). The toxins produced by this species (alternariol,

alternariol monomethylether, tenuazonic acid, altertoxin I, II and III) have been reported in various fruits **(Tournas and Stack, 2001)** and authors **Sage *et al.* (2004)** assessed that among the 90 species identified from the French grape samples, almost half were listed as mycotoxin producers. On the basis of the study, which we discussed in the past **(Mašková *et al.*, 2012)**, it is highly probable that the *Alternaria* strains present at the grapes berries in Slovak conditions are able to produce mentioned mycotoxins.

Table 2 Isolation frequency (Fr) and relative density (RD, within the genus) of *Alternaria* species-groups isolated from grape berries, harvested from various localities of Slovakia in 2011

Species-group	Unsterilized grapes		Sterilized grapes	
	Fr [%]	RD (min – max) [%]	Fr [%]	RD (min – max) [%]
A. alternata	80.0	0.0 – 100.0	75.0	0.0 – 98.3
A. arborescens	50.0	0.0 – 90.9	70.0	0.0 – 97.9
A. infectoria	40.0	0.0 – 7.6	50.0	0.0 – 34.8
A. tenuissima	80.0	0.0 – 100.0	90.0	0.0 – 92.2
Alternaria spp.	15.0	0.0 – 3.0	25.0	0.0 – 16.7

Table 3 Isolation frequency (Fr) and relative density (RD, within the genus) of *Alternaria* species-groups isolated from the stum obtained from grapes harvested from various localities of Slovakia in 2011

Species-group	Fr [%]	RD (min – max) [%]
A. alternata	50.0	0.0 – 87.5
A. arborescens	40.0	0.0 – 100.0
A. infectoria	20.0	0.0 – 100.0
A. tenuissima	70.0	0.0 – 100.0
Alternaria spp.	20.0	0.0 – 100.0

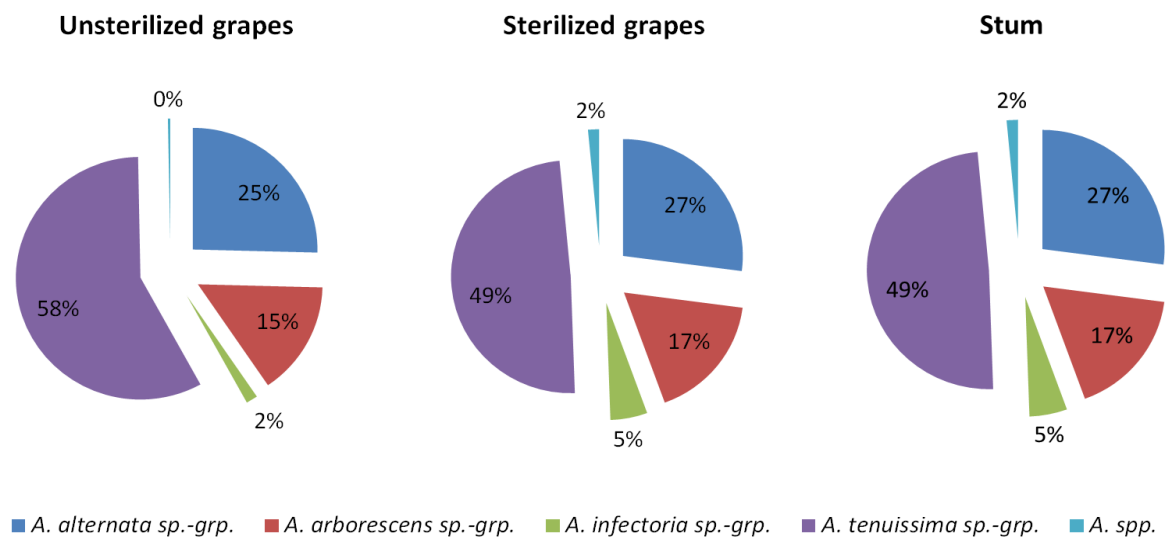

Figure 2 The average relative density of *Alternaria* species-groups isolated from grape berries of Slovak origin (the percentage of particular representatives of the genus *Alternaria*)

CONCLUSION

In the field of grapes mycobiota monitoring are carried out many studies, particularly in the southern wine regions of the Europe (Spain, Italy, France, Greece, Portugal), where are the wine products contaminated by mycotoxins in larger quantities. In these areas, attention focuses mainly on ochratoxin A. However, producers of this mycotoxin can overshadow other potential toxigenic micromycetes, such as *Alternaria* spp. and their metabolites.

Our study has shown that representatives of the genus *Alternaria* occur in all monitored regions of Slovakia and even they represent for approximately half of all isolates. For that reason, they constitute a significant risk of mycotoxin contamination. Next studies should be directed to the testing of the ability of *Alternaria* isolates to produce mycotoxins and to the monitoring of the mycotoxins presence in grape products.

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REFERENCES

ABRUNHOSA, L. – PATERSON, R. R. – KOZAKIEWICZ, Z. – LIMA, N. – VENÂNCIO, A. 2001. Mycotoxin production from fungi isolated from grapes. In *Letters in Applied Microbiology*, 32, p. 240–242.

ANDERSEN, B. – KROGER, E. – ROBERTS, R. G. 2001. Chemical and morphological segregation of *Alternaria alternata*, *A. gaisen* and *A. longipes*. In *Mycological Research*, no. 105, p. 291-299.

ANDERSEN, B. – KROGER, E. – ROBERTS, R. G. 2002. Chemical and morphological segregation of *Alternaria arborescens*, *A. infectoria* and *A. tenuissima* species-groups. In *Mycological Research*, no. 106, p. 170-182.

BARATA, A. – MALFEITO-FERREIRA, M. - LOUREIRO, V. 2012. The microbial ecology of wine grape berries. In International Journal of Food Microbiology, 153, p. 243-259.

BATTILANI, P. – PIETRI, A. – BERTUZZI, T. – LANGUASCO, L. – GIORNI, P. – KOZAKIEWICZ, Z. 2003. Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. In *Journal of Food Protection*, 66, p. 633–636.

BAU, M. - BRAGULAT, M. R. – ABARCA, M. L. – MINGUEZ, S. – CABAÑES F. J. 2005. Ochratoxigenic species from Spanish wine grapes. In *International Journal of Food Microbiology*, 98, p. 125–130.

DUGAN, F. M. – PEEVER, T. L. 2002. Morphological and cultural differentiation of described species of *Alternaria* from Poaceae. In *Mycotaxon*, vol. 83, p. 229 – 264.

FLEET, G. H. 1999. Microorganisms in food ecosystems. In *International Journal Food Microbiology*, 50, p. 101–117.

FLEET, G. H. 2001. Wine. In *Doyle, M. P., Beuchat, L. R. and Montville, T. J. 2001. Food Microbiology Fundamentals and Frontiers*, 2nd ed., Washington, DC: ASM Press. p. 747–772.

GONZÁLES, H. H. L. – PACIN, A. – RESNIK, S. L. – MARTINEZ, E. J. 1996. Deoxynivalenol and contaminant mycoflofa in freshly harvested Argentinean wheat in 1993. In *Mycopathologia*, vol. 135, no. 2, p. 129-134.

GUATAM, A. – SHARMA, S. – BHADAURIA, R. 2009. Detection of toxigenic fungi and mycotoxins in medicinally important powdered herbal drugs. In *Internet Journal of Microbiology*, vol. 7, no. 2.

HOCKING, A. D. – LEONG, S. L. – KAZI, B. A. – EMMETT, R. W. – SCOTT, E. S. 2007. Fungi and mycotoxins in vineyards and grape products. In *International Journal of Food Microbiology*, 119, p. 84-88.

LEONG, S. – HOCKING, A. – PITT, J. I. 2004. Occurrence of fruit rot fungi (*Aspergillus* section Nigri) on some drying varieties of irrigated grapes. In *Australian Journal of Grape and Wine Research*, 10, p. 83–88.

LOZADA, A. F. 1995. Isolation and identification of mycotoxigenic fungi in selected foods and feeds. In *Food Additives and Contaminants*, 3, p. 509–514.

MAGNOLI, C. – VIOLANTE, M. – COMBINA, M. – PALACIO, G. – DALCERO, A. 2003. Mycoflora and ochratoxin-producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina. In *Letters in Applied Microbiology*, 37, p. 179–184.

MAŠKOVÁ, Z. – TANČINOVÁ, D. – BARBORÁKOVÁ, Z. – FELŠÖCIOVÁ, S. – CÍSAROVÁ, M. 2012. Comparison of occurrence and toxinogenity of *Alternaria* spp. isolated from samples of conventional and new crossbred wheat of Slovak origin. In *Journal of Microbiology, Biotechnology and Food Science*, 1 (February special issue), p. 552-562. ISSN 1338-5178.

PITT, J. I. - HOCKING, A. D. 2009. Fungi and Food spoilage. 3. vyd. New York : Springer. 519 p. e-ISBN 978-0-378-92207-2.

PITT, J. I. – HOCKING, A. D. 1997. Fungi and food Spoilage 2nd ed. London : Blackie Academic & Professional. 593 p. ISBN 0-8342-1306-0.

ROCHA ROSA, C. A. – PALÁCIOS, V. – COMBINA, M. – FRAGA, M. E. – OLIVEIRA REKSON, A. - MAGNOLI, C. E. – DALCERO, A.M. 2002. Potential ochratoxin A producers from wine grapes in Argentina and Brazil. In *Food Additives and Contaminants*, 19, p. 408–414

SAGE, L. – GARON, D. –SEIGLE-MURANDI, F. 2004. Fungal microflora and ochratoxin A risk in french vineyards. In *Journal of Agricultural and Food Chemistry*, 52, p. 5764–5768.

SAMSON, R. A. – HOEKSTRA, E. S. – FRISVAD, J.C. – FILTENBORG, O. 2002a. Introduction to food- and airborne fungi. Utrecht : Centraalbureau voor Schimmelcultures. 389 p. ISBN 90-70351-42-0.

SAMSON, R. A. – HOEKSTRA, E. S. – LUND, F. – FILTENBORG, O. – FRISVAD, J.C. 2002b. Method for the detection, isolation and characterisation of food-borne fungi. In *Samson, R. A., Hoekstra, E. S., Frisvad, J. C. & Filtenborg, O. Introduction to food- and airborne fungi*. Utrecht : Centraalbureau voor Schimmecultures. p. 283-297. ISBN 90-70351-42-0.

SCOTT P. M. 2001. Analysis of agricultural commodities and food for *Alternaria* mycotoxins. In *Journal of AOAC International*, 84, p. 1809–1817.

SERRA, R. – ABRUNHOSA, L. – KOZAKIEWICZ, Z. – VENÂNCIO, A. 2003. Black *Aspergillus* species as ochratoxin A producers in Portuguese wine grapes. In *International Journal of Food Microbiology*, 88, p. 63–68.

SERRA, R. – LOURENÇO, A. – ALÍPIO, P. – VENÂCIO, A. 2006. Influence of the region of origin on the mycobiota of grapes with emphasis on *Aspergillus* and *Penicillium* species. In *Mycological Research*, 110, p. 971-978.

SIMMONS, E. G. 1994. *Alternaria* themes and variations (106 – 111). In *Mycotaxon*, no. 50, p. 409-427.

SIMMONS, E. G. 2007. *Alternaria*, An Identification Manual. Utrecht : CBS Fungal Biodiversity Centre. 775 p. ISBN 978-90-70351-68-7.

SIMMONS, E. G. – ROBERTS R. G. 1993. *Alternaria* themes and variations (73). In *Mycotaxon*, no. 40, p. 109-140.

TJAMOS, S. E. – ANTONIOU, P. P. – KAZANTZIDOU, A. – ANTONOPOULOS, D. F. – PAPAGEORGIOU, I. – TJAMOS, E.C. 2004. *Aspergillus niger* and *Aspergillus carbonarius* in Corinth raisin and wine-producing vineyards in greece: population composition, ochratoxin a production and chemical control. In *Journal of Phytopathology*, 152, p. 250–255.

TOURNAS, V. H. – STACK, M. E. 2001. Production of alternariol and alternariol monomethylether by *Alternaria* grown on fruits at various temperatures. In *Journal of Food Protection*, 64, p. 528–532.