

## COLONIZATION OF GRAPES BERRIES BY *ALTERNARIA* sp. AND THEIR ABILITY TO PRODUCE MYCOTOXINS

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### ABSTRACT

Our research focused on identify the *Alternaria* species from grapes (surface sterilized berries and non-surface sterilized berries) of Slovak origin and characterize their toxinogenic potential in *in vitro* conditions. We analyzed 47 samples of grapes, harvested in years 2011, 2012 and 2013 from various wine-growing regions. For the isolation of species, the method of direct plating berries and surface-sterilized berries (using 1 % freshly pre-prepared chlorine) on DRBC (Dichloran Rose Bengal Chloramphenicol agar) was used. For each analysis was used 50 berries. Only undamaged berries have been used for analysis. The cultivation was carried at  $25 \pm 1^\circ\text{C}$ , for 5 to 7 days in dark. After incubation, the colonies of *Alternaria* were transferred on PCA - potato-carrot agar and CYA - Czapek-yeast extract agar and cultured for 7 days at room temperature and natural light. A total 4 species-groups of the genus *Alternaria* were isolated from grapes berries: *Alternaria alternata* (1369 isolates), *Alternaria arborescens* (734 isolates), *Alternaria infectoria* (143 isolates), and *Alternaria tenuissima* (3579 isolates). According to European Union legislation mycotoxins produced by species genus *Alternaria* are not monitored in foods and food commodities. Mycotoxins such as alternariol and alternariol monomethylether are mutagenic and genotoxic in various *in vitro* systems. Selected strains were tested for production of altenuene, alternariol monomethylether and alternariol. In neither case of *A. infectoria* species-group isolates was confirmed the production of tested mycotoxins in *in vitro* conditions by TLC method. The ability to produce altenuene, alternariol monomethylether and alternariol in *in vitro* conditions was detected in isolates of *Alternaria alternata*, *Alternaria arborescens* and *Alternaria tenuissima* species-groups. Isolates of *Alternaria alternata* species-group (44 tested isolates) were able to produce altenuene (24 isolates), alternariol monomethylether (42 isolates) and alternariol (43 isolates). Only one isolate did not produce any mycotoxins. Isolates of *Alternaria arborescens* species-group (38 tested isolates) were able to produce altenuene (24 isolates), alternariol monomethylether (33 isolates) and alternariol (36 isolates). Only two isolates did not produce any mycotoxins. Isolates of *Alternaria tenuissima* species-group (87 tested isolates) were able to produce altenuene (42 isolates), alternariol monomethylether (41 isolates) and alternariol (73 isolates). Thirteen isolates did not produce any mycotoxins.

**Keywords:** *Alternaria*; altenuene; alternariol; alternariol monomethylether; grape

### INTRODUCTION

Grapes have a complex microbial ecology including filamentous fungi, yeasts and bacteria with different physiological characteristics and effects upon wine production (Barata et al., 2012). The black mould genus *Alternaria* Ness is ubiquitously distributed and includes various saprophytic, endophytic and pathogenic species. Many of the genus *Alternaria* Ness commonly cause spoilage of various food crops in the field or post-harvest decay (Ostrý, 2008; Logrieco et al., 2009). *Alternaria* species are pathogenic and saprophytic fungi widely distributed in soil. They are widespread in both humid and semiarid regions and can infect growing plants in the field. They are the principal contaminating fungi in wheat, sorghum and barley. In addition to cereal crops, *Alternaria* species have been reported to occur in oilseeds such as sunflower and rapeseed, tomato, apples, citrus fruits, olives and several other fruits and vegetables. *Alternaria* species grow at low temperature; hence they are generally associated with extensive spoilage during refrigerated transport and storage (Ostrý, 2008). *Alternaria* genus is the main component of the wine grape mycobiota at harvest

time (Serra et al., 2005; Prendes et al., 2015; Tančinová et al., 2015). The most common fungi spoiling grapes were *Alternaria*, *Botrytis cinerea* and *Cladosporium* (Tournas, et al., 2005). Moreover, several *Alternaria* species are known to produce toxic secondary metabolites, *Alternaria* mycotoxins (Rotem, 1994; Prendes et al., 2015). Mycotoxins are secondary metabolites produced by filamentous fungi that have been detected in food commodities, including grapes and wine (Serra et al., 2005). *Alternaria* species have the ability to produce a variety of secondary metabolites, which plays important roles in food safety (Andresen, et al., 2015). The major *Alternaria* mycotoxins belong to three structural classes: tetramic acid derivate, tenuazonic acid; the dibenzopyrone derivatives, alternariol, alternariol methylether and altenuene; and the perylene derivatives, the altertoxins (Andersen et al., 2002). Food relevant *Alternaria* species are able to produce many more metabolites (Ostrý, 2008; Logrieco et al., 2009). *Alternaria* toxins occurred regularly in cereals, tomato sauces, figs, wine and sunflower seeds. Only incidental occurrence of the *Alternaria* toxins was

observed in fresh apples, fresh citrus, fresh tomatoes and olives (López et al., 2016).

Our research focused on the identify the *Alternaria* species from grapes of Slovak origin and characterize their toxinogenic potential in *in vitro*.

## MATERIAL AND METHODOLOGY

### Samples

Forty-seven samples of grapes, harvested in years 2011, 2012 and 2013 from various wine-growing regions of Slovakia, from small and medium-sized vineyards were analyzed. White and red grape variety were analyzed. *White grape*: Müller Thurgau (1), Velsch Riesling (4), Grüner Veltliner (5), Pálava (1), Pinot blanc (2), Pinot gris (2), Sauvignon (2), Tramin (1), Zala gyöngye (1). *Red grape*: Alibernet (1 sample), André (2 samples), Blaufrankise (8), Cabernet Sauvignon (2), Pinot noir (2), Saint Laurent (1). Samples (3 kg) were collected at the time of technological ripeness. Picked grapes were stored at  $4 \pm 1$  °C and analyzed within 24 h after harvest.

### Mycological analysis

For the isolation of *Alternaria* sp. was used the method of direct plating berries: surface-sterilized berries and non-sterilized berries on DRBC (Dichloran Rose Bengal Chloramphenicol agar) Samson et al., (2002).

The endogenous mycobiota was determined by the method of direct placing of superficially sterilized berries on agar plates (Samson et al., 2002). More than 50 pieces of undamaged berries from each sample were superficially sterilized (using 1% freshly pre-prepared chlorine). Sterilization was carried out for 2 minutes. Berries 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 (agar with dichloran, rose bengal and chloramphenicol) (Samson et al., 2002). Cultivation lasted from 5 to 7 days in darkness at  $25 \pm 1$  °C. For each analysis was used 50 berries. Only undamaged berries have been used for analysis. After incubation, the colonies of *Alternaria* were transferred onto appropriate identification media.

**Identification of *Alternaria* species-groups.** Grown micromycetes were classified into the genera and then isolated by re-inoculation on the identification nutrient media and identified by accepted mycological keys and publications. Isolates of the genus *Alternaria* were re-inoculated on PCA - potato-carrot agar and CYA - Czapek-yeast extract agar (Samson et al., 2002) and cultured for 7 days at room temperature and natural light.

In order to improve study of sporulation pattern we proceeded as follows. The colonized agar (piece of approx. size 0.5 x 1.0 cm) was cut and transferred to the agar surface, outside the colony. The growth was observed as early as one to two days of cultivation on the edge of the removed part. Main used identification keys were Andersen et al., (2001); Andersen et al., (2002); Simmons, (1994); Simmons, (2007) and Simmons and Roberts (1993).

Obtained results were evaluated and expressed in isolation frequency (Fr) at the species level. The isolation frequency (%) is defined as the percentage of samples within which the species occurred at least once (Gautam et al., 2009).

These values were calculated according to González et al., (1996) as follows:

$$Fr (\%) = (ns / N) \times 100$$

where ns = number of samples with a species; N = total number of samples.

### Toxinogenicity analysis

Toxinogenicity of selected isolates was analysed by means of thin layer chromatography (TLC) by Samson et al., (2002). This method was performed with modifications according to Labuda and Tančinová (2006). Testing was focused on determination of the ability to produce mycotoxins altenuene (ALT), alternariol (AOH) and alternariol monomethylether (AME).

The colonies grown on yeast extract sucrose agar (YES) (7, respectively 14 days, in darkness at  $25 \pm 1$  °C) were cut into squares of approximate size 2 cm x 2 cm and placed in an Eppendorf tube with 0.5 mL of extraction solution (chloroform: methanol - 2:1; Reachem, SR). The content of the tubes was stirred for 5 minutes by Vortex Genie® 2 (MO BIO Laboratories, Inc. - Carlsbad, CA). The obtained extracts were applied to silica gel chromatography plate (Alugram® SIL G, Macherey - Nagel, Germany) and plates were put into the TEF solvent (toluene: ethyl acetate: formic acid - 5 :4 :1; toluene - Mikrochem, SR; ethyl acetate and formic acid - Slavus, SR). After elution and drying, the mycotoxins identity was confirmed by visual comparison with the standards of mycotoxins (AME, ALT and AOH - Merck, Germany) under UV light with a wavelength of 254 nm and 366 nm.

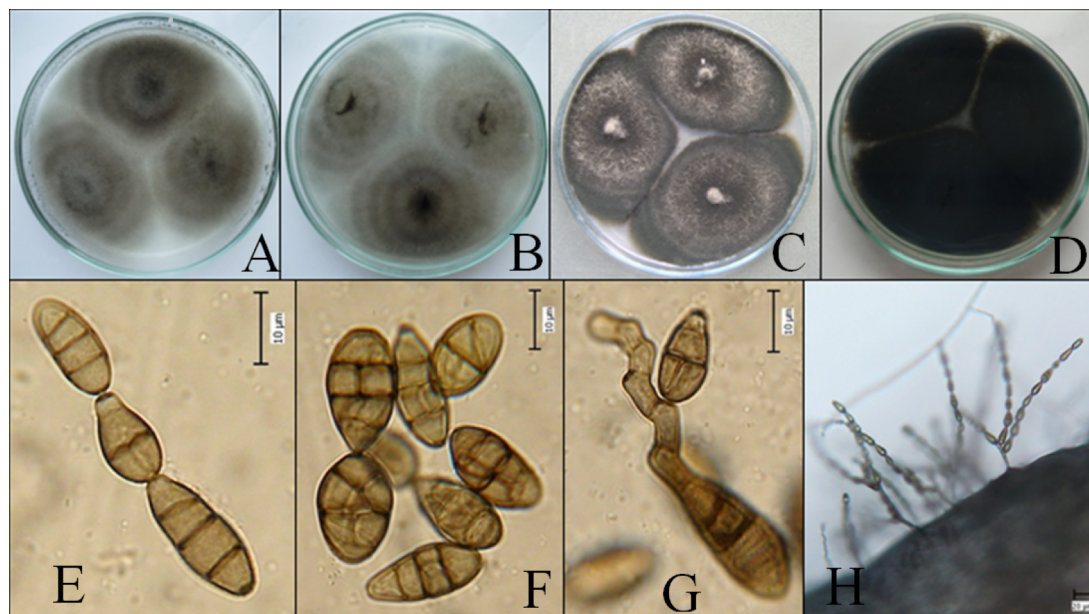
## RESULTS AND DISCUSSION

In the current study from all samples were isolated *Alternaria* species (from superficially sterilized berries and berries without sterilization, too). The cosmopolitan fungal genus *Alternaria* consists of multiple saprophytic and pathogenic species. Based on phylogenetic and morphological studies, the genus is currently divided into 26 sections. *Alternaria* section *Alternaria* contains most of the small-spored species with concatenated conidia, including important plant, human and postharvest pathogens (Woundenberg et al., 2015). A total of 4 species-groups (Table 1) of the genus *Alternaria* (*Alternaria* section *Alternaria*) were isolated from grapes berries, namely *Alternaria alternata* group, *Alternaria arborescens* group, *Alternaria infectoria* group, and *Alternaria tenuissima* group. Isolates, which could not be closer specified or contaminated another species were specified as *Alternaria* sp.. Sporulation patterns of *Alternaria* species-group are listed according to Simmons, (2007). The typical sporulation pattern of *Alternaria alternata* group (Figure 1) comprises a single suberect conidiophore and an apical cluster of branching chains of small conidia separated by short secondary donidiophores. Long, well-defined primary conidiophores of *Alternaria arborescens* group (Figure 2) characteristically bear a few terminal and subterminal branches. Each conidiophore branch bears a branching chain of conidia, giving a relatively tall, three-dimensionally arborescent appearance to the suberect system.

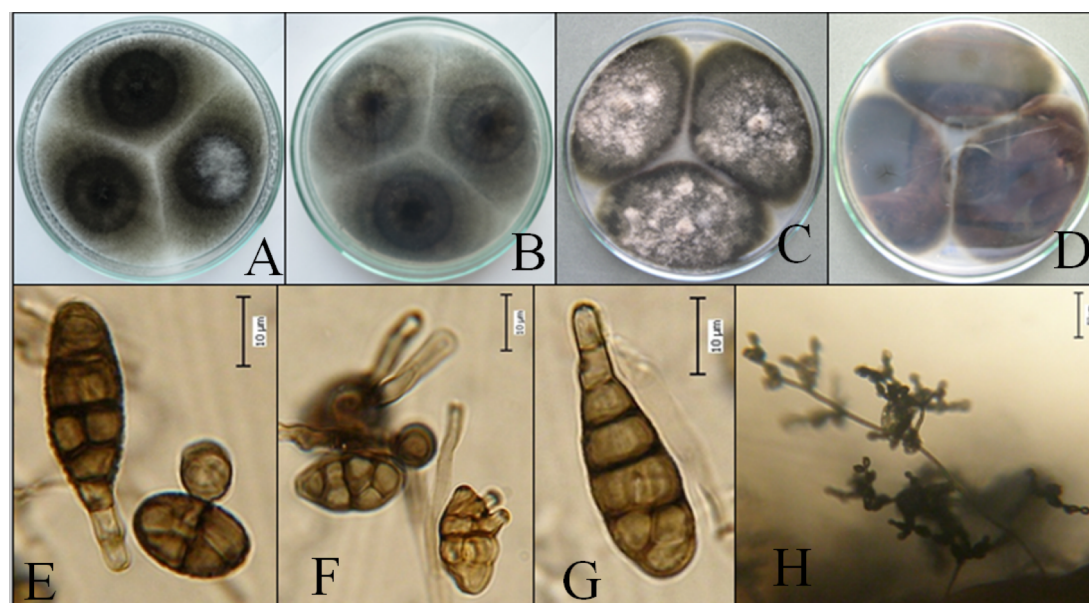
**Table 1** Species-groups of *Alternaria* isolated from berries of Slovak origin determined by using plate direct method on DRBC agar from 47 samples.

<i>Alternaria</i> groups	Superficially sterilized berries		Berries without sterilization	
	Number of isolates	Isolation frequency (%)	Number of isolates	Isolation frequency (%)
<i>Alternaria alternata</i>	662	78.7	707	78.7
<i>Alternaria arborescens</i>	405	34.0	329	57.4
<i>Alternaria infectoria</i>	109	31.9	34	29.78
<i>Alternaria tenuissima</i>	1644	93.6	1935	89.36
<i>Alternaria</i> sp.	144	53.2	94	46.81

Note: DRBC - Dichloran Rose Bengal Chloramphenicol agar.



**Figure 1** *Alternaria alternata* group 7 days of incubation. A-B colonies on PDA (A – top, B – reverse), C-D colonies on CYA (C – top, D – reverse), E-F conidia (scale bar = 10 µm), H – conidium sporulation pattern (scale bar = 20 µm) Photo: Mašková.

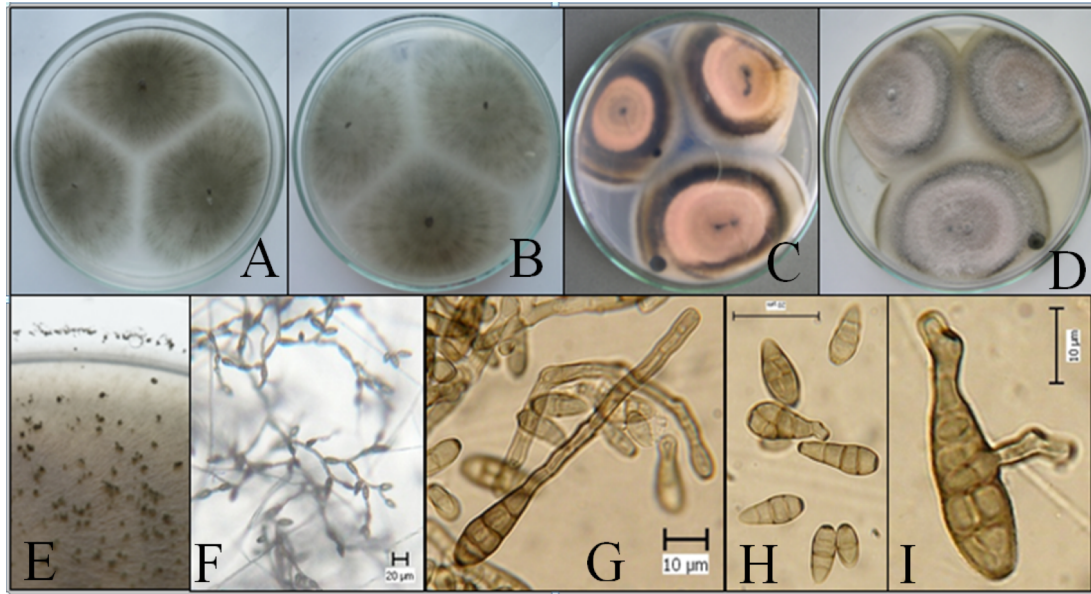


**Figure 2** *Alternaria arborescens* group 7 days of incubation. A-B colonies on PDA (A – top, B – reverse), C-D colonies on CYA (C – top, D – reverse), E-F conidia (scale bar = 10 µm), H – conidium sporulation pattern (scale bar = 50 µm), Photo: Mašková.

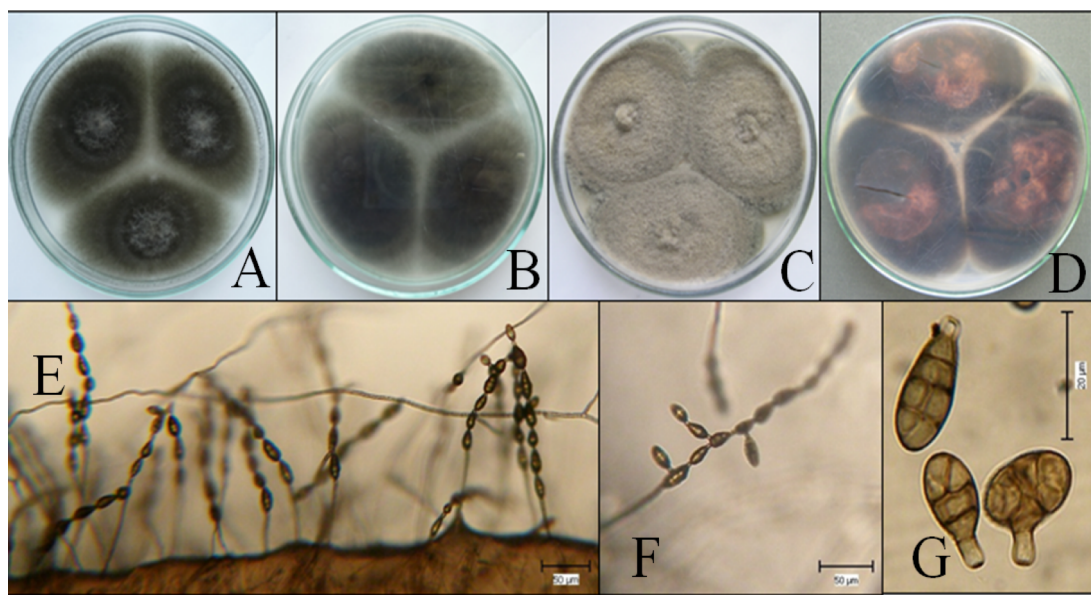
**Table 2** Potential ability of *Alternaria* species groups isolates to produce mycotoxins in *in vitro* conditions, tested by TLC method.

Species groups of <i>Alternaria</i>	Number of tested isolates	Number of isolates without the production of mycotoxins	Mycotoxins		
			Altenuene	Alternariol monomethylether	Alternariol
<i>Al. alternata</i>	44	1	24	42	43
<i>Al. arborescens</i>	38	2	24	33	36
<i>Al. infectoria</i>	15	15	0	0	0
<i>Al. tenuissima</i>	87	13	42	41	73

Note: TLC - thin layer chromatography, *Al.* – *Alternaria*.



**Figure 3** *Alternaria infectoria* group 7 days of incubation. A-B colonies on PDA (A – top, B – reverse), C-D colonies on CYA (C – top, D – reverse), E – granular look of colonies, F – conidium sporulation pattern (scale bar = 20 μm), G-I conidia (scale bar = 10, 20, 10 μm), Photo: Mašková.



**Figure 4** *Alternaria tenuissima* group 7 days of incubation. A-B colonies on PDA (A – top, B – reverse), C-D colonies on CYA (C – top, D – reverse), E-F – conidium sporulation pattern (scale bar = 50 μm), G – conidia (scale bar = 20 μm), Photo: Mašková.

Conidiophores of *Alternaria infectoria* group (Figure 3) that sporulate in the surface mass commonly are unbranched but have 1 – 3 geniculate extensions and conidiogenous loci incorporated in a total length of 50 – 100 µm. *Alternaria tenuissima* group (Figure 4) – produce uncrowded chains of up to 12 conidia on branching hyphae. The initial 1 – 2 and sometimes even 4 – 5 lowest conidia of a chain usually have only transverse septa; only one or two mature conidia in a chain have the helpfully diagnostic median, subconstricting transverse septum that is such a striking feature of field conidia. The occurrence of the genus *Alternaria* in grape berries reported: Serra et al., (2005, 2006); Tournas et al., (2005); Ostrý et al., (2007); Polizzotto et al., (2012); Chunmei et al., (2013); Mašková et al., (2013); Lorenzini and Zapparoli (2014); Roseusseaux et al., (2014); Prendes et al., (2015); Tančinová et al., (2015) and other authors. According Bau et al., (2005) predominant mycobiota of grape berries belonged to *Alternaria* spp., *Cladosporium* spp. and *Aspergillus* spp. These three genera were isolated from 75.6%, 22.5% and 17.3% of plated berries, respectively. Magnoli et al., (2003) reported that *Alternaria* genus was the most frequent (80% positive samples) from the surface-disinfected berries from Argentina. *Alternaria alternata* was the only species identified from this genus. Ostrý et al., (2007); Diguta et al., (2011); Prendes et al., (2015) recorded incidence of *Alternaria alternata*, also. Other authors mentioned the occurrence of *Alternaria* on the grape berries as follows: *Alternaria alternata* and *Alternaria tenuissima* (Rousseaux et al., 2014); *Alternaria alternata* and *Alternaria arborescens* (Lorenzini et al., 2014); *Alternaria arborescens* species-group and *Alternaria tenuissima* species-group (Polizzotto et al., 2012). Isolation of *A. infectoria* species-group mentioned Mašková et al., (2013), from Slovakian samples of grapes, too. In our sample was dominant *Alternaria tenuissima* group (1644 isolates – berries superficially sterilized and 1935 isolates – berries without sterilization), follow by *Alternaria alternata* group (662, respectively 707 isolates), *Alternaria arborescens* group (405 and 329 isolates) and *Alternaria infectoria* (109 and 34 isolates).

Mycotoxins are abiotic hazards produced by certain fungi that can grow on a variety of crops (Marin et al., 20013). According to European Union legislation mycotoxins produced by species genus *Alternaria* are not monitored in foods and food commodities. Mycotoxins such as alternariol and alternariol monomethylether are mutagenic and genotoxic in various *in vitro* systems. In addition, it has been suggested that in certain areas in China *Alternaria* toxins in grains might be responsible for oesophageal cancer. Hence, due to their possible harmful effects, *Alternaria* toxins are of concern for public health (EFSA, 2011). According to Prendes et al., (2015), *Alternaria*, one of the most mycotoxigenic genus commonly found in wine grapes, could represent a high risk for the wine consumer's health. Representative isolates were selected for analysis to produce mycotoxins in *in vitro* conditions randomly from all obtained isolates. The results are presented in Table 2. A total of 184 isolates were tested. Production of selected secondary metabolites demonstrated the toxinogenicity of isolates and on the other hand, it also served as an auxiliary indicator for identification (chemotaxonomy), mainly to

distinguish the *Alternaria infectoria* species-group from the others (Mašková et al., 2012). Production of mycotoxins by any of *Alternaria infectoria* strains still has not been demonstrated (Andersen et al., 2002; Labuda et al., 2008; Piovarčiová et al., 2007). Conversely, *Alternaria alternata* and *Alternaria tenuissima* are known to produce several types of mycotoxins (Andersen et al., 2002; Piovarčiová et al., 2007), which were confirmed in our study (Table 2). In neither case of the 15 tested isolates of *Alternaria infectoria* species-group we confirmed the production of mycotoxins ALT, AOH and AME. Although, the reputation of "nontoxigenic" strains of the *Alternaria infectoria* species-group has been undermined in recent years by isolation unknown metabolites (Mašková et al., 2012). Conversely, isolates of other tested species-groups proved to be highly toxigenic (Table 2). Only one isolates of *Alternaria alternata* species-group and two isolates of *Alternaria arborescens* species-group did not produce tested mycotoxins in *in vitro* conditions detectable by TLC method. Robiglio and Lopez were tested eleven *Alternaria alternata* strains, isolated from Red Delicious apples in cold storage in Argentina, for alternariol and alternariol methyl ether production in laboratory media and in whole fresh fruits. Most of them were able to produce both toxins in all media. They were detected also in mycelium free filtrates from liquid cultures and in asymptomatic tissues from inoculated fruit. Thus, in the evaluation of mouldy core incidence in apples, the presence of *Alternaria alternata* toxins in tissues should be considered even in the absence of mycelia (Robiglio and Lopez, 1995).

Small-spored *Alternaria*, such as *Alternaria alternata* group, *Alternaria arborescens* group, *Alternaria infectoria* group and *Alternaria tenuissima* group are important producers of mycotoxins, or other unknown metabolites but they were dominant fungal consortium in grapes berries in our samples. Considering that literature reported about the effectiveness of *Alternaria* endophytes against important grapevine pathogens, it should be interesting to elucidate the chemical structure of *Alternaria* unknown metabolites and to evaluate them as new biological method in the control of grapevine diseases (Polizzotto et al., 2012).

## CONCLUSION

From the 2350 surface-sterilized (47 samples) grape berries have been isolated 2964 strains of genus *Alternaria* and from the same number of non-sterilized berries 3099 isolates of this genus. Isolates were identified according to sporulation patterns to four species groups: namely *Alternaria alternata* (1369 isolates), *Alternaria arborescens* (734), *Alternaria infectoria* (143), and *Alternaria tenuissima* (3579) and 238 isolates were not identified to species group. There were found out the ability to produce following mycotoxins: altenuene, alternariol and alternariol monomethylether in *in vitro* conditions by TLC method of chosen strains of genus *Alternaria*. In another research would be advisable to follow occurrence of these mycotoxins in grapes, must, wine and another grape products.

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