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REGULAR ARTICLE

COMPARISON OF OCCURRENCE AND TOXINOGENITY OF *ALTERNARIA* SPP. ISOLATED FROM SAMPLES OF CONVENTIONAL AND NEW CROSSBRED WHEAT OF SLOVAK ORIGIN

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ABSTRACT

The aim of this study was to compare the results of mycological and mycotoxicological analysis of two types of Slovak wheat samples, focusing on *Alternaria* genus. A total of 21 samples of conventional wheat grains and 3 samples of the new crossbred wheat were investigated for exogenous and endogenous mycobiota. The exogenous mycobiota was determined by using plate dilution method and the endogenous mycobiota by the method of direct placing of superficially sterilized grains on agar plates. Toxinogenity of selected isolates was analysed by means of thin layer chromatography. The obtained results of this study show a high isolation frequency of *Alternaria* isolates in samples of conventional as well as new crossbred wheat. A total of 4 species-groups of the genus *Alternaria* were isolated from conventional wheat (*A. alternata*, *A. arborescens*, *A. infectoria*, *A. tenuissima*) and 3 species-groups from new crossbred wheat (*A. arborescens*, *A. infectoria*, *A. tenuissima*). *A. tenuissima* species-group was isolated within the endogenous mycobiota from all samples of conventional and new crossbred wheat. Species-group with the second highest isolation frequency in all tested samples was *A. infectoria*. The highest relative density in all samples belongs to *A. infectoria* and *A. tenuissima* species-groups. Selected strains isolated from both types of wheat 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. The highest toxinogenity (100%) was observed in strains of *A. arborescens* and *A. tenuissima*.

Keywords: *Alternaria*, altenuene, alternariol, alternariol monomethylether, wheat

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important grains crops providing nearly 20 % of the total world food requirement (Uddin *et al.*, 2006). However, the production is affected by numbers of pests and diseases (Mishra *et al.*, 2010). *Alternaria* species are fungi widely distributed in the soil as normal components of its microbiota and are both saprophytes and plant pathogens. They are widespread in both humid and semi-arid regions and can infect growing plants in the field. *Alternaria* species cause plant diseases on many crops, affecting the leaves, stems, flowers and fruits. They are the principal contaminating fungi in wheat, sorghum and barley (Deshpande, 2002). Some *Alternaria* species are able to infect and induce symptoms on plants during their growing stages, while others only cause damage after harvest in storage, trade and processing. Total losses caused by this genus rank among the highest caused by any plant pathogen (Agrios, 2005). During storage, *Alternaria* may also spread from affected plant products to adjacent healthy ones by secondary infections (Rotem, 1994; Barkai-Golan, 2008). *Alternaria* spores are disseminated mainly by air currents, but they can be also rain splashed. Once released, *Alternaria* spores are able to withstand adverse environmental conditions for several days, and conclude infection when weather becomes favourable (Rotem, 1994).

Alternaria species produce more than 70 secondary metabolites which are toxic to plants. A small proportion of these phytotoxins have been chemically characterised and reported to act as mycotoxins to humans and animals (Bottalico and Logrieco, 1992; Barkai-Golan, 2008). Based on their effect on plants, *Alternaria* toxins are divided into non-host-specific toxins and host-specific toxins (Thomma, 2003). Some non-host-specific toxins such as alternariol, alternariol monomethylether, tenuazonic acid and altertoxins have been tested individually and are described to induce harmful effects in animals, including fetotoxic and teratogenic effects (Barkai-Golan, 2008). On the other hand, host-specific toxins such as AAL-toxins have a limited host range and play a critical role in plant pathogenicity, but their animal toxicities have not been fully examined (Thomma, 2003; Barkai-Golan, 2008).

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).

The aim of this study was to compare the results of mycological and mycotoxicological analysis of two types of Slovak wheat samples, focusing on *Alternaria* genus.

MATERIAL AND METHODS

In the study, we analyzed 21 samples of conventional wheat grains and 3 samples of the new crossbred wheat summer form winter grains with purple colour (*Triticum aestivum* L. x *Triticum spelta*), all grown in Slovakia. Samples of conventional wheat were harvested from different regions (Banská Bystrica, Košice, Nitra, Prešov, Trenčín, Trnava and Žilina) and localities of Slovakia in 2008. The new line of wheat with a purple colour was crossbred in Research Center of Plant Production in Piešťany, in Research station of breeding in Vígľaš-Pstruša in 2009.

Exogenous mycobiota of wheat grain analysis

The exogenous mycobiota was determined by using the plate dilution method. Homogenized sample of whole grain or fraction in amount of 20 g was added to 180 mL of peptone water containing 0.02% Tween 80. Prepared suspensions were shaken on a horizontal shaker for 30 minutes. Dilutions 10^{-1}, 10^{-2} and 10^{-3} were in the triple 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.

Endogenous mycobiota of wheat grain analysis

The endogenous mycobiota was determined by the method of direct placing of superficially sterilized grains on agar plates (Samson *et al.*, 2002b). More than 100 pieces of undamaged grains 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 100 grains 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.

Isolation and identification of grown micromycetes

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 (Samson et al., 2002a) and cultured for 7 days at room temperature and natural light. Main used identification keys were Andersen et al. (2001), Andersen et al. (2002), Dugan and Peever (2002), Simmons (1994), Simmons (2007) and Simmons and Roberts (1993).

Results evaluation

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:

$$Fr\ (\%) = (ns / N) \times 100 \qquad RD\ (\%) = (ni / Ni) \times 100$$

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.

Toxinogenity analysis

Toxinogenity of selected isolates was analysed by means of thin layer chromatography (TLC) by Samson et al. (2002b). 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) 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 - Merck, Germany) under UV light with a wavelength of 254 nm and 366 nm. The identity of AOH was established on the device QTrap 4000 LC/MS/MS equipped with TurboIonSpray ESI source and 1100 Series HPLC system. Chromatographic separation was performed at 25 ± 1 °C by Gemini 5 μ C18, 150 mm x 4.6 mm (Phenomenex, USA).

RESULTS AND DISCUSSION

The obtained results of this studies show a high isolation frequency (Fr) of *Alternaria* isolates in samples of conventional as well as new crossbred wheat (Tab 1). Strains of this genus were part of an endogenous mycobiota in all analyzed samples. In general we can say that representatives of *Alternaria* genus were isolated with higher Fr from the interior of the grains than from their surface.

A total of 4 species-groups of the genus *Alternaria* were isolated from conventional wheat, namely *A. alternata*, *A. arborescens*, *A. infectoria* and *A. tenuissima*. Isolates which could not be taxonomically identified because of contamination were recorded as *Alternaria* sp.. A total of 3 species-groups of this genus were isolated from new crossbred wheat, namely *A. arborescens*, *A. infectoria* and *A. tenuissima*. With regard to species-group representation, *A. tenuissima* seems to be the most widespread in wheat of Slovak origin, because it was isolated within the endogenous mycobiota from all samples of conventional and new crossbred wheat (Tab 2). However, from the new wheat samples were also other species-groups isolated with high Fr (100%). In conventional samples was *A. infectoria* species-group also frequently represented, but *A. alternata* and *A. arborescens* were detected in 57.1 and 38.1% of the samples (regardless of the method of isolation).

Table 1 Isolation frequency (Fr) of strains from genus *Alternaria*, isolated from samples of conventional (n = 21) and new crossbred wheat (n = 3), harvested in Slovakia in 2008 and 2009

Mycobiota according to the method of isolation	Isolation frequency [%]	
	conventional wheat	new crossbred wheat
endogenous	100.0	100.0
exogenous	90.5	66.7

Legend: n - number of samples

Table 2 Isolation frequency (Fr) of species-groups from genus *Alternaria*, isolated from samples of conventional (n = 21) and new crossbred wheat (n = 3), harvested in Slovakia in 2008 and 2009

Species-group	Isolation frequency [%]			
	conventional wheat		new crossbred wheat	
	endogenous	exogenous	endogenous	exogenous
A. alternata	57.1	0.0	0.0	0.0
A. arborescens	38.1	9.5	100.0	0.0
A. infectoria	90.5	81.0	100.0	0.0
A. tenuissima	100.0	85.7	100.0	66.7
Alternaria sp.	23.8	0.0	0.0	0.0

Legend: n - number of samples

Average relative density (RD) of *Alternaria* isolates from samples of both types of wheat refers to the fact that representatives of this genus comprise more than half of all isolated micromycetes (Tab 3). From this point of view, the observed genus represents for the future consumer a serious risk in the form of accumulation of toxic secondary metabolites. Within RD of the species-groups from all isolates of this genus we observed again comparable results from both types of wheat. The largest number of isolates belongs to *A. infectoria* and *A. tenuissima* species-groups (Tab 4). On the other hand, *A. arborescens* has been reported in only a small number and *A. alternata* was detected only in samples of conventional wheat. Similar results were obtained even in 2007 (Piovarčiová *et al.*, 2009), when also *A. infectoria* and *A. tenuissima* species-groups occurred with the highest Fr and RD.

Table 3 Relative density (RD) of strains from genus *Alternaria*, isolated from samples of conventional (n = 21) and new crossbred wheat (n = 3), harvested in Slovakia in 2008 and 2009 (regardless of the isolation method)

Relative density [%]	conventional wheat	new crossbred wheat
average	51.3	56.9
minimum - maximum	0.8 – 80.2	49.1 – 69.1

Legend: n - number of samples

Table 4 Average relative density (RD) of *Alternaria* species-groups within the genus, isolated from samples of conventional (n = 21) and new crossbred wheat (n = 3), harvested in Slovakia in 2008 and 2009 (regardless of the isolation method)

Species-group	Relative density [%]	
	conventional wheat	new crossbred wheat
A. alternata	2.0	0.0
A. arborescens	1.3	4.1
A. infectoria	43.4	51.0
A. tenuissima	53.2	44.9
Alternaria sp.	0.1	0.0

Legend: n - number of samples

High numbers of *Alternaria* isolates cause concern mainly because of their potential toxigenic properties. Selected strains isolated from samples of conventional and new crossbred wheat were tested for production of altenuene (ALT), alternariol monomethylether (AME) and alternariol (AOH). According to EFSA (2011), experiments performed in rodents with purified *Alternaria* toxins indicate that the acute toxicity is in the following order: ALT > tenuazonic acid > AME and AOH. Representative strains were selected for analysis randomly from all obtained isolates.

Within the conventional wheat, a total of 79 strains were tested. The largest number of tested strains came from *A. infectoria* and *A. tenuissima* species-groups, because of their high Fr and RD. On the one hand, production of selected secondary metabolites demonstrated the toxinogenity of isolates and on the other hand, it also served as an auxiliary indicator for identification (chemotaxonomy), mainly to distinguish the *A. infectoria* species-group from the others. Production of mycotoxins by any of *A. infectoria* strains still has not been demonstrated (Andersen and Thrane, 1996; Andersen *et al.*, 2002; Labuda *et al.*, 2008; Piovarčiová *et al.*, 2007; Pitt and Hocking, 1997). Conversely, *A. alternata* and *A. tenuissima*

are known to produce several types of mycotoxins (Andersen *et al.*, 2002; Piovarčiová *et al.*, 2007), which was confirmed in our study (Tab 5). In neither case of the 46 tested isolates of *A. infectoria* species-group we confirmed the production of mycotoxins ALT, AOH and AME. Even though, the reputation of "nontoxigenic" strains of the *A. infectoria* species-group was in recent years undermined by force of attention dedicated to the unknown metabolites. Conversely, isolates of other tested species-groups proved to be highly toxigenic (Tab 5). The highest toxinogenity (100%) was observed in strains of *A. arborescens* (AME, AOH) and *A. tenuissima* (AOH).

Within the new crossbred wheat, a total of 26 strains were tested for mycotoxin production. In this case, similar results were found, but toxinogenity of all tested potential toxigenic strains (*A. arborescens* and *A. tenuissima* species-groups) was 100%. Isolates of *A. infectoria* species-group again confirmed their nontoxigenic character (Tab 6).

Table 5 Toxinogenity of selected *Alternaria* strains, isolated from samples of conventional wheat, harvested in Slovakia in 2008

Species-group (number of tests)	positive tests [%]		
	ALT	AME	AOH
A. alternata (4)	75.0	75.0	50.0
A. arborescens (4)	75.0	100.0	100.0
A. infectoria (46)	0.0	0.0	0.0
A. tenuissima (23)	82.6	95.7	100.0
Alternaria sp. (2)	50.0	50.0	50.0

Legend: ALT – altenuene, AME – alternariol monomethylether, AOH - alternariol

Table 6 Toxinogenity of selected *Alternaria* strains, isolated from samples of new crossbred wheat, harvested in Slovakia in 2009

Species-group (number of tests)	positive tests [%]		
	ALT	AME	AOH
A. arborescens (5)	100.0	100.0	100.0
A. infectoria (12)	0.0	0.0	0.0
A. tenuissima (9)	100.0	100.0	100.0

Legend: ALT – altenuene, AME – alternariol monomethylether, AOH - alternariol

CONCLUSION

The outcome of this study is evidence of the fact that *Alternaria* species can significantly affect the quality of Slovak wheat, which can be reflected in the consumer health. The results show a comparable representation of *Alternaria* isolates from conventional and new crossbred wheat. In both cases was demonstrated a high isolation frequency (Fr) of isolates from this genus and high relative density (RD) of *A. infectoria* and *A. tenuissima* species-groups. High numbers of *Alternaria* isolates cause concern mainly because of their potential toxigenic properties. Selected strains isolated from samples of conventional and new crossbred wheat (except isolates of *A. infectoria* species-group) really showed a high ability of mycotoxins production. In addition, *Alternaria* species deserves attention because currently there are no regulations on *Alternaria* toxins in food and feed in Europe or in other regions of the world.

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