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ORIGINAL SCIENTIFIC ARTICLE

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TOXIGENIC POTENTIAL OF *Alternaria* SPECIES FROM CEREALS

ABSTRACT: Toxigenic potential of four and one isolate of *A. alternata* and *A. tenuissima*, respectively, on durum wheat cultivar Dušan (*Triticum durum* L.) and common wheat cultivar Barbee (*T. vulgare* L.) were tested. Three different wheat / isolate genotype combinations were used for artificial inoculation of grains under laboratory conditions and toxins production. Alternaria toxins alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), tenuazonic acid (TeA) and altenuen (ALT) concentrations were determined by LC-MS/MS. Cultivar Barbee proved to be a more suitable substrate for toxin production, whereby AOH, AME and TeA were present in highest concentrations. These results underline the possibility of fungal infection and mycotoxin production by *Alternaria* species in field and under storage conditions. Further research is needed for official regulation of acceptable levels of *Alternaria* mycotoxins in food and feed.

KEYWORDS: *Alternaria,* toxin production, wheat

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INTRODUCTION

Wheat is one of the main crops in the Republic of Serbia, with a total production area of about 560.000 ha (Statistical Yearbook, RS, 2021). However, as extreme weather conditions are becoming increasingly prevalent due to climate change and global warming, the risk of mycotoxin contamination has also heightened. As indicated by Vučković et al. (2012), the resulting greater occurrence of phytopathogens on small grains may have negative consequences on the quality and safety of food and feed.

Species of the genus *Alternaria* are significant contaminants of cereals that, in addition to mycotoxins production, cause product degradation during transport and storage. *Alternaria* species produce more than 70 secondary metabolites, some of which e.g., alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), tenuazonic acid (TeA) and altenuen (ALT) are classified as mycotoxins due to their harmful effects on humans and animals (EFSA, 2011, 2016; Escrivá et al., 2017; Gashgari et al., 2019). Moreover, more than one mycotoxin can be found on an infected substrate (Zain, 2011). During the recent years, *Alternaria* species, especially *A. alternata*, have been found as contaminants of wheat and wheat-based products in the Vojvodina Province (Janić Hajnal et al., 2015). For the 2010−2015 period, the EFSA (2016) reported presence of the highest levels of *Alternaria* toxin TeA in tomatoes, nuts, oilseeds, cereals, and fruit. More recent findings reported by Mujahid et al. (2020) further indicate that as emerging mycotoxins, *Alternaria* toxins are candidates for regulation by European authorities. Monitoring and regulation of the permitted levels of *Alternaria* toxins should thus be initiated throughout the EU, according to the SANTE/11356/2019 draft issued by the European Commission.

Mycotoxins produced by *Alternaria* spp. on humans and animals exhibit prolonged and chronic toxic effects, as their intake can be harmful even in low doses. The most common symptoms include nervous system damage, brain bleeds, liver damage and cancer, hyperestrogenism, reproductive system cell degeneration, epithelial cell damage, as well as adverse changes to the mucous membranes, intestinal tract, immune system, and respiratory tract (Liu et al., 1992; European Commission, 2006).

Considering the importance of *Alternaria* as a pathogen and small grain cereals contaminant, the aim of this study was to evaluate the toxigenic potential of *A. alternata* and *A. tenuissima* isolates obtained from wheat and rye by artificially inoculating wheat kernels under laboratory conditions.

MATERIAL AND METHODS

Alternaria isolates and inoculum preparation

Alternaria alternata (isolates A2, A3, A4 originating from wheat) and *A. tenuissima* (isolate A6 originating from rye) isolates were obtained from CRC Genbank of Small Grain Cereals and Microorganisms, Cereal Research Non-

profit Company, Szeged, Hungary. One *A. alternata* isolate (SOR1IIIZA1) obtained from wheat grain grown in the Vojvodina Province was also included in the analyses. To obtain conidia, all isolates were grown on CPA (Carrot = Potato Agar) in Petri dishes of 90 mm diameter for 10 days, under dark conditions at 25 °C. Presence of conidia was monitored on a daily basis. After 10 days, conidia were harvested by washing the culture surface and macerating the medium in sterile distilled water (SDW), after which the concentration was adjusted to 2×10^5 conidia/ml.

Artificial inoculation of wheat kernels

Two wheat genotypes were inoculated under laboratory conditions with different combinations of *Alternaria* isolates: (1) Durum wheat (*Triticum durum* L.) inoculated with isolates A2+SOR1IIIZA1; (2) common wheat cultivar Barbee (*T. vulgare* L.) inoculated with isolates A3+A4+A6; and (3) Durum wheat inoculated with isolates A2+A3+A4+A6+SOR1IIIZA1. Prior to the treatment, 500 g of wheat kernels was weighed and was placed into glass bottles and sterilized at 121 °C for 60 min. After autoclaving and cooling the kernels, 200 ml of the fungal conidia suspension was added and homogenized. Inoculated kernels were incubated at 25 °C in the dark for 20 days. The bottles containing inoculated kernels were shaken on a daily basis for 30 minutes. After the incubation time had lapsed, the glass bottles were exposed to 45 °C for 30 minutes, closed with screw caps and placed into cold storage (at -20 °C) until required for analysis.

Determination of toxin concentrations in artificially inoculated wheat kernels

The analyses were performed using LC-MS/MS methodology adapted from Scott (2001). Briefly, 10 g of the artificially inoculated seed sample was weighed and, after extraction with acetonitrile/water, the resulting aliquot was subjected to solid phase clean-up. The *Alternaria* toxins were then eluted with methanol and quantitatively determined by HPLC-MS/MS after changing the solvent. The *Alternaria* toxin content in the samples was corrected to obtain the required recovery rates. The following toxins were analyzed: (AOH), (AME), (TEN), (TeA) and (ALT) (Table 1).

Table 1. Analyzed toxins produced by *Alternaria* isolates

Toxins	LOQ (µg/kg)	LOD (µg/kg)
Tenuazonic acid (TEA)	10	3
Altenuen (ALT)	10	3
Alternariol (AOH)	2	1
Alternariol monomethyl ether (AME)	2	1
Tentoxin (TEN)	10	3

RESULTS AND DISCUSSION

Alternaria species are common saprophytes or pathogens of a wide range of plants, whereby infection can occur in field as well as during storage. The most frequently analyzed toxins are AOH, AME and TeA, followed by ALT, produced by *A. alternata* or *A. tenuissima*, and TEN toxin produced by *A. alternata*. Due to the occurrence of *Alternaria* species, these mycotoxins can be present as contaminants throughout the entire food and feed chain, especially in cereals, vegetables, fruit and oil seeds (Zwickel et al., 2016).

As a part of the present study, the toxigenic potential of *Alternaria* isolates (*A. alternata* and *A. tenuissima*) obtained from wheat and rye was evaluated, whereby different wheat kernels were subjected to artificial inoculation. As shown in Table 2, all inoculated wheat samples were successfully contaminated with *Alternaria* toxins (AOH, AME, TeA, ALT, and TEN) in high concentrations.

On the durum wheat samples inoculated with the *Alternaria alternata* isolates originating from wheat grown in Hungary (A2) and Serbia (SOR1IIIZA1), the toxin concentration was the lowest. Additionally, analyses related to this combination showed the presence of AOH (>2,000 μg/kg), AME (>1,000 μg/kg), TeA (>9,000 μg/kg), ALT (760±300 μg/kg) and TEN (57±23 μg/kg).

When inoculation was performed on kernel of wheat cultivar Barbee with isolates originating from wheat (A3, A4; *A. alternata*) and rye (A6; *A. tenuissima*), the highest concentrations of the *Alternaria* toxins were recorded, i.e. >30,000 μg/kg, >10,000 μg/kg, >250,000 μg/kg, >5,000 μg/kg and >2,000 μg/kg were maxima attained for AOH, AME, TeA, ALT and TEN, respectively.

Using a mixture of all tested *Alternaria* isolates (A2+A3+A4+A6 and SOR1IIIZA1) in the inoculation of durum wheat kernel resulted in a lower (higher) toxin production (Sample 1) compared to Sample 2. The highest recorded values were as follows: >10,000 μg/kg, >3,000 μg/kg, >50,000 μg/kg, >2,000 μg/kg and 170±68 μg/kg for AOH, AME, TeA, ALT and TEN, respectively. When all results were compared, TeA emerged as the most abundant toxin in all wheat samples, while TEN was present in the lowest concentrations. Finally, the most suitable substrate for *Alternaria* toxin production was wheat Barbee.

Given the high toxicogenic potential of *Alternaria* isolates from small grain cereals and the widespread occurrence of *Alternaria* species, there is a need for the legislation of maximum allowable concentrations of *Alternaria* toxins in food and feed.

Table 2. Determination of toxin concentrations in wheat samples artificially inoculated with *Alternaria* isolates

Wheat samples, mixture of Alternaria isolates	*Toxin*	*Concentration* (μg/kg)
1) Durum, A2 (*Aa*) + SOR1IIIZA1 (*Aa*)	Alternariol (AOH)	>2,000
	Alternariol monomethyl ether (AME)	>1,000
	Tenuazonic acid (TeA)	>9,000
	Altenuen (ALT)	760±300
	Tentoxin (TEN)	57±23
2) Barbee, A3 (*Aa*) + A4 (*Aa*) + A6 (*At*)	Alternariol (AOH)	>30,000
	Alternariol monomethyl ether (AME)	>10,000
	Tenuazonic acid (TeA)	>250,000
	Altenuen (ALT)	>5,000
	Tentoxin (TEN)	>2,000
3) Durum, A2 (*Aa*) + A3 (*Aa*) + A4 (*Aa*) + A6 (*At*) + SOR1IIIZA1 (*Aa*)	Alternariol (AOH)	>10,000
	Alternariol monomethyl ether (AME)	>3,000
	Tenuazonic acid (TeA)	>50,000
	Altenuen (ALT)	>2,000
	Tentoxin (TEN)	170±68

Aa – Alternaria alternata; *At – Alternaria tenuissima*.

CONCLUSION

Fungal species of the genus *Alternaria* represent a potential hazard for food safety owing to their toxin-producing potential. Under laboratory conditions, *A. alternata* and *A. tenuissima* isolates produced a wide range of mycotoxins in high concentrations. As shown in the present study, significant differences exist among wheat types and cultivars in terms of their suitability as a toxin production substrate. Field infections of wheat by these *Alternaria* species can provide conditions conducive to further fungal development during storage and may thus result in significant toxin contamination.

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ТОКСИГЕНИ ПОТЕНЦИЈАЛ ВРСТА ИЗ РОДА *Alternaria* ИЗОЛОВАНИХ СА СТРНИХ ЖИТА

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РЕЗИМЕ: У раду је испитан токсигени потенцијал изолата врста из рода *Alternaria – A. alternata* и *A. tenuissima* у лабораторијским условима на дурум пшеници сорте „Душан" (*Triticum durum* L.), као и на сорти пшенице „Barbee" (*T. vulgare* L.). Током вештачке инокулације у лабораторији коришћене су три различите комбинације генотип пшенице/изолати. Путем LC-MS/MS методе испитан је садржај алтернариа токсина алтернариола (AOH), алтернариол мометилетра (AME), тентоксина (TEN), тенуазоничне киселине (TeA) и алтенуена (ALT). Сорта „Барби" показала се као најпогоднији супстрат за продукцију алтернариа токсина. У испитиваним узорцима утврђена је највиша концентрација токсина AOH, AME и TeA. Резултати ових истраживања указују на могућност остварења инфекције стрних жита од стране *Alternaria* врста током складиштења и на ризик од потенцијалне контаминације од стране микотоксина и уласка отровних једињења у ланац исхране. Даља истраживања и потреба званичне регулације максимално дозвољених количина *Alternaria* токсина у циљу су смањења ризика од тровања секундарним метаболитима које стварају гљиве из рода *Alternaria*.

КЉУЧНЕ РЕЧИ: *Alternaria*, продукција токсина, пшеница