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Mycotoxigenic fungi contaminating wheat; toxicity of different *Alternaria compacta* strains

R. Gashgari^a, Fuad Ameen^{b,*}, E. Al-Homaidi^c, Y. Gherbawy^d, S. Al Nadhari^e, V. Vijayan^f^a Department of Biological Science, Faculty of Science, Jeddah University, Jeddah, Saudi Arabia^b Department of Botany & Microbiology, Faculty of Science, King Saud University, Riyadh, Saudi Arabia^c Department of Biological Science, Faculty of Science, Princess Nora University, Riyadh, Saudi Arabia^d Department of Botany, South Valley University, Qena, Egypt^e Department of Plant Protection, Faculty of Agriculture, King Saud University, Riyadh, Saudi Arabia^f JRT Research and Development, Vancouver, Canada

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ABSTRACT

We studied mycotoxigenic fungi contaminating stored wheat grain, measured the toxins they secreted, and assessed their harmfulness. We focused on one common genus *Alternaria*, and chose 19 isolates representing *A. compacta* to study how different strains differed in their mycotoxin secretion and toxicity. Toxicity was assessed in a bioassay with a model bacteria *Bacillus subtilis*. All 19 *A. compacta* strains secreted toxins. Both the mycotoxin pattern and the fungal toxicity differed between the *A. compacta* stains. It seemed that some other toxins than alternariols or altenuene acted as the main virulence factors of *A. compacta* against *B. subtilis*. We suggest that the most commonly studied mycotoxins do not necessarily indicate the toxicity of the fungi. The high variation in the amounts and toxins that different *Alternaria* species and strains secrete pose a challenge to the food supply chain.

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1. Introduction

Food mycotoxins are of great public health concern, especially in many countries in Asia and Africa. One cosmopolitan saprophytic fungal pathogenic species that infects crops is *Alternaria*. Most *Alternaria* species seem to be mycotoxigenic; more than 75% of *Alternaria* species, isolated in Argentina, secreted secondary metabolites that are potential mycotoxins (Andersen et al., 2015). Geographical regions seem to have different fungal species composition and, despite great efforts, the taxa of *Alternaria* has not yet been well organized (Simmons, 2007; Wenderoth et al., 2017). Moreover, the same *Alternaria* species may secrete different toxins depending on the region (Garganese et al., 2016; Lee et al., 2015). Therefore, more monitoring data from different regions are still needed.

The variety of mycotoxins secreted by fungi is great (Fox and Howlett, 2008; Sanzani et al., 2016). More than 70 compounds have been identified to be secreted by *Alternaria* sp.; the most commonly studied are alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tenuazonic acid (TeA) and tentoxin (TEN) (Alexander et al., 2011; Barkai-Golan and Paster, 2011). Recent research has considerably increased knowledge on *Alternaria* secreted toxin concentrations in food, as well as on the actual toxicity of the compounds (Deshidi et al., 2017; Lee et al., 2015; López et al., 2016b, 2016a; Meena et al., 2017; Patriarca et al., 2014). However, the studies have focused on the most common toxins one at the time. The toxicity of the metabolite cocktail, which the fungi secrete, has received less attention and the methods to study the overall harmfulness of different species have not been well developed. The toxicity of the metabolite cocktail needs a multi-mycotoxin approach to be understood.

Our first aim was to increase regional knowledge about fungal species contaminating stored wheat grain; we identified species in Saudi Arabia. The second aim was to assess the harmfulness of wheat grain associated *Alternaria* spp. using a multi-mycotoxin approach. Specifically, we studied how the toxicity varied among different *A. compacta* strains and whether the actual toxicity can be assessed by measuring the most common toxins. We measured three toxins (ALT, AOH and AME) that the different *A. compacta*

* Corresponding author.

E-mail address: Fuadameen@ksu.edu.sa (F. Ameen).

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isolates secreted. We assessed the harmfulness of the fungal strains using a bioassay. In the bioassay, the toxicity was assessed as the capability of the fungi to inhibit a model bacteria *B. subtilis*. We assumed that the more the isolate inhibits the growth of *B. subtilis*, the more it secretes toxins and the higher its toxicity is in general and thus, the higher its potential to be harmful to humans. We collected 100 samples of stored wheat grains from Saudi Arabia, and (a) identified the mycobiota, (b) measured three mycotoxins that the *A. compacta* isolates secreted, and (c) assessed the toxicity of the isolates.

2. Materials and methods

Wheat grains were randomly collected from hundred (100) different grain storages in Riyadh city between May and July 2015. Samples were collected in sterilized polystyrene bottles and stored at 4 °C.

Dilution-plate method was used for isolation the fungal strains from wheat (Youssef et al., 2008). An aliquot of 20 g of grain was weighed (three replicates) into sterile conical flask and sterilized distilled water was added to reach the final volume of 100 ml. After making several dilutions, 1 ml of each dilution were transferred on Dichloran–Rose Bengal Chloramphenicol Agar (DRBC) and Dichloran–chloramphenicol–malt extract agar (DCMA) (HiMedia, India) dishes. The suspension was evenly distributed by moving the dishes and incubated at 25 ± 2 °C for 5–7 days. The fungal endophytic colonies growth was monitored daily. Fungi growing out of the seeds were isolated, purified and identified preliminary according to their morphological characteristics (Abe et al., 2015). The total colony forming units (CFU) were counted. CFU per grain dry weight wheat and the colonization frequency (CF (%); proportion of grain samples colonized by the species) were calculated. Isolates identified as *A. alternata* were chosen for further toxicity studies and their identification was complemented by DNA sequencing.

2.1. DNA extraction, amplification and sequencing

The mycelium was obtained after the spores were first incubated in potato dextrose (PD) broth, thereafter, the DNA was extracted as described by Ameen et al. (2018).

The ITS region of the ribosomal DNA was amplified by PCR with the primers ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gashgari et al., 2016; White et al., 1990). PCR mixture contained 2 ml of DNA with 0.5 mM of each primer, 150 mM of dNTP, 1U of Taq DNA polymerase (Roche company), (Gherbawy, 2005), and PCR reaction buffer at a final volume of 50 µl. The DNA was amplified in a thermal cycler with the cycles: initial denaturation of 10 min at 94 °C, 30 cycles of 30 sec at 94 °C, 30 sec at 58 °C, 30 sec at 72 °C, and a final extension of 10 min at 72 °C.

The more detailed identification of the isolates was based on the separation of RAPD-PCR fragments according to Gherbawy (2005) and Choi et al. (2008). PCR were carried out with five random primers OPA03 (5'-AGT CAG CCA C-3'), OPA04 (5'-AAT CGG GCT G-3'), OPA10 (5'-GTG ATC GCA G-3'), V6 (5'-TGC AGC GTG G-3') and M13 (5'-GAG GGT GGC GGT TCT-3'). The DNA was amplified in a reaction mixture with the final volume of 50 µl by mixing 2 µl of DNA with 0.5 µM of each primer, 150 µM of dNTP, 6 U of Taq DNA polymerase, and PCR reaction buffer. The thermal cycles were: initial denaturation of 3 min at 94 °C, 35 cycles of 1 min. at 94 °C, 1 min. at 50 °C, 1 min. at 72 °C, and a final extension of 10 min. at 72 °C.

Aliquots of PCR products were checked in electrophoresis using 1% agarose gel, ethidium bromide, and visualized by UV trans-illumination. The PCR products were purified by ExoSAPIT (USB Corporation, Amersham Place, UK, under license from GE Healthcare) based on manufacturer's instructions. The purified products were sequenced using an automated DNA sequencer (ABI Prism 3700 automated DNA analyzer) using the BigDye Deoxy Terminator cycle-sequencing kit (Applied Biosystems, Darmstadt, Germany). Sequences were submitted to GenBank on the NCBI website (<http://www.ncbi.nlm.nih.gov>). Sequences were compared with the GenBank database using the BLAST software on the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST/>).

DNA sequences were aligned with Clustal X 1.81 (Thompson et al., 1997). TREECON (de Peer and De Wachter, 1994) was used to construct neighbor-joining tree using Jukes–Cantor model.

RAPD patterns (absence or presence of RAPD-bands) were analyzed as described by Halmschlager et al. (1995). Dendograms were constructed by the Unweighted paired group method of arithmetic average (UPGMA) based on Jaccard's similarity coefficient by using Phoretex 1D Software (version 5.2).

2.2. Bioassay

We selected 19 *A. compacta* strains, which had been identified using RAPD, for the toxicity studies. The potential of the isolates to inhibit the growth of *Bacillus subtilis* was measured as three replicates with a dual culture technique (Oldenburg et al., 1996). The filtrate of the fungal spores was prepared as described in Ameen et al. (2018) Half of the filtrates had been boiled for 15 min and half not boiled.

B. subtilis was provided by the Armed forces hospital, Riyadh city. *B. subtilis* was first grown in Tryptone Glucose Yeast Extract (TGY) broth (HiMedia Laboratories) at 37 °C for 24 h. An aliquot of 1 ml of the bacterial broth was transferred to 100 ml of TGY agar. The inoculated medium was poured on petri dishes (10 ml for each) and left to solidify. At the center of each dish, the agar was punched by a sterilized corks borer to make a hole. A aliquot of 1 ml of sterilized water (control) or filtrate containing fungal spores was added to the holes by a sterilized pipette. Finally, all plates were incubated at 37 °C for 28 h and the radius of the inhibition zone was measured as mm.

2.3. Mycotoxins

The toxins secreted by the isolates were measured in polished rice media (Li et al., 2001). Polished rice (12.5 g) was weighed to flasks (250 ml) that were inoculated by one pure fungus from the plates and incubated at 25 °C for 21 days. Toxins were extracted by adding 30 ml methanol and shaking the mixture until homogeneous. The suspension was filtrated through Whatman filter paper No. 1. The filter was purified by filtering 60 ml of ammonium sulfate (20%). The toxins were isolated with chloroform. High purity chloroform (10 ml) was added, the mixture was shaken and the layer of chloroform was evaporated. The isolation procedure was repeated three times. The dry film was dissolved in 4 ml of methanol and AOH, ALT and AME were analyzed with HPLC combined with UV-detection at 258 nm as previously described (Li et al., 2001). Pure mycotoxins (Wako pure chemicals industries, Ltd) were used as the standards.

The results were expressed as means and standard deviations (SD). Pearson correlation analysis and principal component analysis (PCA) using the correlation matrix were performed for the mycotoxin concentrations and inhibition zones.

3. Results

A total of 50 species and one class belonging to 18 genera were morphologically identified in 100 wheat grain samples (Table 1). The genera were *Alternaria* (9 species), *Cladosporium* (9 species and one class), *Drechslera* (5 species), *Ulocladium* (5 species), *Aspergillus* (4 species), *Stemphylium* (3 species), *Scytalidium* (2 species), *Torula* (2 species) and one species of *Acremonium*, *Embellisia*, *Phoma*, *Penicillium*, *Mycovellosiella*, *Fusarium*; *Staphylotricum*, *Stachybotrys* and *Xylohypha* and an unidentified *Mycelium sterilium*.

A. compacta were the dominant *Alternaria* sp. with a total count of 270 CFU (g dw/grain) and 49% colonization frequency (Table 1). The next dominant *Alternaria* spp. were *A. radicina* and *A. brassicicola* (75 CFU). *A. infectoria*, *A. triticina*, *A. phragmospora*, *A. state of Pleospora infectoria* and *A. longipes* had relatively low CFU.

The phylogenetic tree revealed that all isolates morphologically identified as *A. alternata* belonged to one group of *A. compacta* No. EU1285291, (Fig. 1). The kinship tree for the *A. compacta* isolates divided the strains first into two groups (Fig. 2). In the first group (lowest in Fig. 2), the isolates (Aa2, Aa4 and Aa17) had 65%

Table 1
Total colony forming units (CFU) on DRBC and DCMA media and colonization frequency (CF) of fungi isolated from wheat grain.

Fungi	Total count CFU (g dw/grain)		CF %	
	DRBC	DCMA	DRBC	DCMA
<i>Acremonium strictum</i>	2	–	1	–
<i>Alternaria</i>	325	5.2	56	47
<i>A. alternata</i>	270	25.2	49	43
	265			
	267			
<i>A. brassicicola</i>	75.3	–	1	–
<i>A. dennisii</i>	–	5.4	–	1
<i>A. infectoria</i>	5.13	25.11	4	3
<i>A. longipes</i>	5.2	25.6	1	2
<i>A. phragmospora</i>	5.4	–	2	–
<i>A. radicina</i>	75.21	25.7	4	3
<i>A. infectoria</i>	5.3	–	1	–
<i>A. triticina</i>	5.5	–	2	–
<i>Aspergillus</i>	5.7	75.11	9	13
<i>A. flavus</i>	75.5	75.4	8	8
<i>A. niger</i>	75.1	7	4	8
<i>A. terreus</i>	–	–	–	–
<i>A. ustus</i>	–	–	–	–
<i>Cladosporium</i>	170	75.14	64	60
<i>C. cladosporioides</i>	75.9	5.5	3	2
<i>C. cucumerinum</i>	75.5	32	15	11
<i>C. elatum</i>	25.8	75.4	4	3
<i>C. herbarum</i>	5.81	79	39	39
<i>C. phyllachorae</i>	–	–	–	–
<i>C. psoraleae</i>	25.2	5.2	1	1
<i>C. sphaerospermum</i>	5.5	4	1	1
<i>C. trichoides</i> var. <i>chlamydosporum</i>	5.4	5	2	2
<i>C. uredinicola</i>	75.1	–	1	–
<i>C. spongiosum</i>	75.8	14	3	6
<i>Drechslera</i>	7.14	75.12	57	53
<i>D. austrliensis</i>	25.6	25.6	26	24
<i>D. halodes</i>	75.5	42	21	20
<i>D. indica</i>	5.1	75.4	1	2
<i>D. papendorffii</i>	5.1	–	1	–
<i>D. specifire</i>	25.3	75.24	15	15
<i>Embellisia chlamydospora</i>	–	25.2	–	1
<i>Fusarium oxysporum</i>	50.3	5.2	91	87
<i>Mycelium strelum</i>	89	5.7	30	26
<i>Mycovellosiella perfoliata</i>	5.1	25.7	4	3
<i>Penicillium digitatum</i>	5.6	75.4	10	9
<i>Phoma pomorum</i>	75.5	–	1	–
<i>Scytalidium</i>	12	5.17	6	7
<i>S. lignicola</i>	–	5.1	–	1
<i>S. thermophilum</i>	12	25.16	6	6
<i>Stachybotrys atra</i>	–	–	–	–
<i>Staphylotricum coccusporum</i>	3	–	1	–
<i>Stemphylium</i>	5.75	5.74	27	33
<i>S. herbarum</i>	38	25.4	20	21
<i>S. lycopersici</i>	25.5	25.6	2	1
<i>S. vesicarium</i>	25.14	28	6	11
<i>Torula</i>	–	4	–	2
<i>T. herbarum</i>	–	75.1	–	1
<i>T. herbarum</i> f. <i>quaternella</i>	–	25.2	–	1
<i>Ulocladium</i>	5.2	75.17	75	81
<i>U. atrum</i>	5.97	106	50	55
<i>U. chartarum</i>	5.7	68	30	32
<i>U. chlamydospora</i>	2	–	1	–
<i>U. odemansii</i>	1	75.2	1	1
<i>U. tuberculatum</i>	5.2	–	1	–
<i>Xylohypha pinicola</i>	–	5.6	–	1

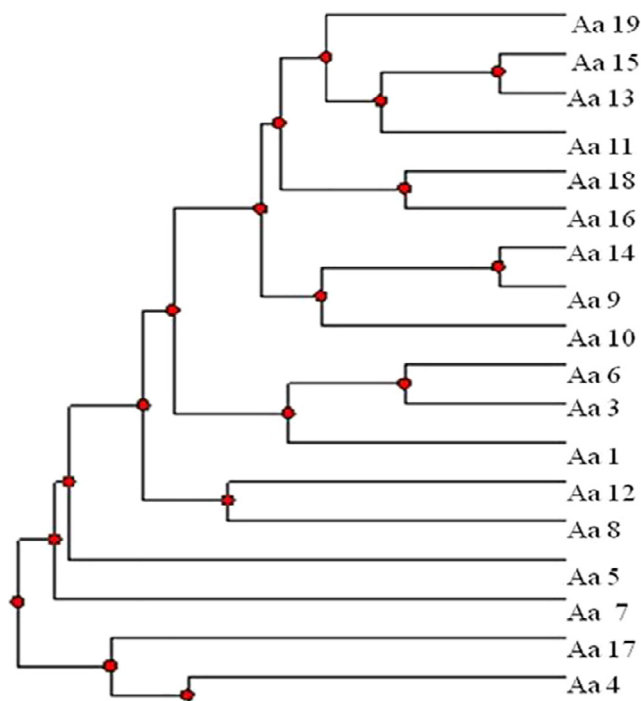


Fig. 2. Kinship tree of the 19 *Alternaria compacta* isolates growing from wheat grains. Sequences were obtained using five random primers.

Table 2

Inhibition (zone, mm, the mean of three replicates, SD = 1.0–1.4 for each) of fungal isolates against *Bacillus subtilis*. Fungal spores derived from wheat grain were studied as such (No Boiling) and after boiling (Boiling).

Isolate	No Boiling	Boiling
Aa1	18.3	19.3
Aa2	18	18.1
Aa3	18.7	19.1
Aa4	19.7	20
Aa5	17.5	18.5
Aa6	18.1	18.2
Aa7	17.7	17.7
Aa8	18	18.1
Aa9	18.3	19.3
Aa10	19	19
Aa11	18.4	19.3
Aa12	20	20.6
Aa13	19.2	20.0
Aa14	16.2	16.9
Aa15	17.8	18
Aa16	18.6	18.9
Aa17	17.5	18.3
Aa18	18.5	19.2
Aa19	16.6	-

et al., 2010; Patriarca et al., 2014). This is supported by our data. Different *A. compacta* strains isolated from wheat had different patterns of toxins secreted; three strains seemed to differ remarkably from the rest of the 16 strains, as indicated by the PCA ordination, where these three species are situated in the left lower corner. The mycotoxin loadings in the PCA indicate that the three strains secreted a combination of relatively high ALT and low AME concentration compared to the other strains. The three strains were not phylogenetically the nearest strains of *A. compacta*, instead they were divided over the kinship tree.

The pathogenicity of fungi depends not only on the mycotoxins secreted but the susceptibility of the organisms to the mycotoxins. Studies on the toxicity to any organisms are limited in general and

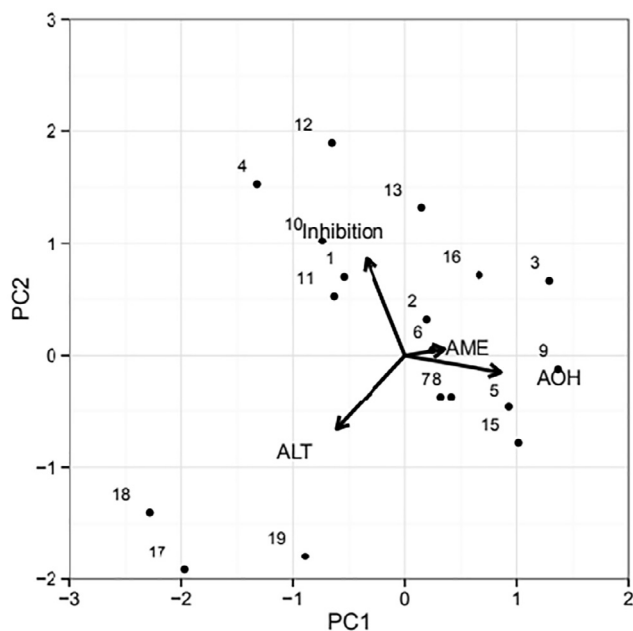


Fig. 3. Sample (19 *A. compacta* isolates) score ordination and the variable loadings (vectors) of principal component analysis using alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT) and inhibition against *B. subtilis* (mean \pm SD, n = 3). PC1 and PC2 explained 38% and 15% of the variation, respectively.

Table 3

Concentrations of altenuene (ALT), alternariol (AOH) and alternariol monomethyl ether (AME) the fungi isolated from wheat grain secreted *in vitro*.

Isolate	Concentration of toxins $\mu\text{g/l}$		
	ALT	AOH	AME
Aa 1	0	0	0
Aa 2	0	0.18 \pm 0.01	0
Aa 3	0.001 \pm 0.0004	0.4 \pm 0.03	0.8 \pm 0.3
Aa 4	0.01 \pm 0.009	0	0
Aa 5	0.002 \pm 0.0002	0.44 \pm 0.03	1.57 \pm 0.04
Aa 6	0.5 \pm 0.04	0.17 \pm 0.01	0.78 \pm 0.026
Aa 7	0.24 \pm 0.02	0.54 \pm 0.03	1.2 \pm 0.18
Aa 8	0.13 \pm 0.03	0.14 \pm 0.04	0.34 \pm 0.04
Aa 9	0.22 \pm 0.03	0.08 \pm 0.01	0.02 \pm 0.01
Aa 10	0.45 \pm 0.04	0.07 \pm 0.014	0.162 \pm 0.03
Aa 11	0.33 \pm 0.02	0.23 \pm 0.032	0.34 \pm 0.03
Aa 12	0.04 \pm 0.03	0.68 \pm 0.036	0.008 \pm 0.0001
Aa 13	0	0.72 \pm 0.03	0.64 \pm 0.035
Aa 14	0	0.03 \pm 0.02	0.88 \pm 0.025
Aa 15	0.80 \pm 0.02	0.59 \pm 0.025	0.57 \pm 0.02
Aa 16	0	0.84 \pm 0.04	0
Aa 17	0.33 \pm 0.04	0	0
Aa 18	0.91 \pm 0.025	0.29 \pm 0.04	0
Aa 19	0.58 \pm 0.03	0	0

in most studies, pure toxins have been tested one at the time. *Alternaria* species are known to have antimicrobial virulence factors (Cota et al., 2008; da Cruz Cabral et al., 2017; Lou et al., 2013; Nemecek et al., 2012). Recently, AOH was found to have antibacterial activity against Gram-positive bacteria *Staphylococcus aureus* (Deshidi et al., 2017). All our *A. compacta* strains inhibited the growth of a common Gram-positive model bacteria *Bacillus subtilis*. We suggest that *A. compacta* has high virulence and therefore, the species may be harmful to humans.

As stated above, wheat originating fungi *A. compacta* secreted toxins that inhibited *B. subtilis*. However, the three toxins, ALT, AOH or AME, seemed not to be especially toxic to *B. subtilis*, because the PCA analysis indicated rather the opposite. Therefore,

it seems that the fungi *A. compacta* secreted some other toxins that inhibited the *B. subtilis* growth in our experiment. We suggest that some other toxins or compounds, such as enzymes, acted as the main virulence factors. We support the suggestion of López et al. (2016b) that multi-mycotoxin approach is needed to be able to assess the harmfulness of *Alternaria* contamination. The measuring should be extended to include other toxins, such as TEN and TeA (López et al., 2016b). The latter has been shown to induce more pathogenicity symptoms to tomato than AOH and AME (Meena et al., 2016). Because of the great variety of toxins secreted, techniques to measure the overall harmfulness of the mycotoxigenic fungal species should be further developed as well.

Most mycotoxins are chemically and thermally stable and thus impossible to be destroyed by pasteurization or boiling (Alshannaq and Yu, 2017). We observed the heat resistance of fungal spores in 18 out of 19 isolates. Only one of the isolates lost its ability to inhibit *B. subtilis* growth after the spores were boiled. Resistance of the fungi against heat poses a difficult challenge to food security.

We observed that different *Alternaria* species were the most common and abundant species contaminating stored wheat grain in Saudi Arabia, Riyadh. *A. compacta* was the most common *Alternaria* species, contrary to results elsewhere. *A. tenuissima* was the most common species isolated from Argentinean wheat (Patriarca et al., 2007). *A. alternata* and *A. infectoria* have been reported as the predominant species in cereals in several studies worldwide (Lee et al., 2015).

In conclusion, all *A. compacta* strains that contaminated stored wheat grain secreted harmful toxins. Moreover, the fungal spores and thus, their toxicity were not possible to be destroyed by boiling. Different *A. compacta* strains had different mycotoxin cocktails of which toxicity differed. We interpret that some other toxins than the most commonly studied AOH, AOL or AME acted as the main virulence factors. We suggest that the mycotoxins most commonly studied do not necessarily indicate the harmfulness of the fungi. The high variation in the amounts and toxins that the different *Alternaria* species and strains secrete pose a challenge to the food supply chain in many regions of the world.

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Competing interest

The authors declare that they have no competing interests.

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