International Journal of Food Microbiology, Volume 268, March 2018, pp. 44-52
DOI:10.1016/j.ijfoodmicro.2018.01.003

Mycotoxin production of *Alternaria* strains isolated from Korean barley grains determined by LC-MS/MS

Thuong T.T. Nguyen[a,1], Jueun Kim[b,1], Sun Jeong Jeon[a], Chul Won Lee[b], Naresh Magan[c], Hyang Burm Lee[a,*]

[a]*Division of Food Technology, Biotechnology & Agrochemistry, College of Agriculture & Life Sciences, Chonnam National University, Gwangju 61186, South Korea*
[b]*Department of Chemistry, Chonnam National University, Gwangju 61186, South Korea*
[c]*Applied Mycology Group, Cranfield University, Cranfield, Bedford MK43 0AL, U.K.*

*Corresponding author; e-mail: hblee@jnu.ac.kr
[1]These authors contributed equally to this work

Running title: Determination of *Alternaria* mycotoxins using LC–MS/MS

Abstract
Twenty-four *Alternaria* strains were isolated from barley grain samples. These strains were screened for the production of mycotoxins on rice medium using thin layer chromatography. All 24 strains produced at least one of the five mycotoxins (ALT, AOH, ATX-I, AME, and TeA). Three representative strains, namely EML-BLDF1-4, EML-BLDF1-14, and EML-BLDF1-18, were further analyzed using a new LC–MS/MS-based mycotoxin quantification method. This method was used to detect and quantify *Alternaria* mycotoxins. We used positive ion electrospray mass spectrometry with multiple reaction mode (MRM) for the simultaneous quantification of various *Alternaria* mycotoxins produced by these strains. Five *Alternaria* toxins (ALT, ATX-I, AOH, AME, and TeA) were detected and quantified. Sample preparation included methanol extraction, concentration, and injection into LC–MS/MS. Limit of detection ranged from 0.13 to 4 μg/mL and limit of quantification ranged from 0.25 to 8 μg/mL.

Keywords: *Alternaria* mycotoxins; *Alternaria* strains; simultaneous quantification; determination; analysis conditions; LC–MS/MS

1. Introduction

Genus *Alternaria* comprises some of the most common plant pathogenic and saprophytic fungi that cause pre- and postharvest damage to agricultural products, including cereal grains, fruits, and vegetables (Ostry, 2008; Storm et al., 2014). The genus includes more than 300 species (Lee et al., 2015). *Alternaria* species produce various secondary metabolites. Some of these metabolites are known to be toxic, while others have not yet been studied and may be potentially toxic. The most common *Alternaria* toxins found in cereals and food items are alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN), altenuene (ALT), and tenuazonic acid (TeA) (López et al.,2014; Ostry, 2008; Patriarca et al., 2008). These toxins are classified into three different structural groups: (1) dibenzopyrone derivatives (AOH, AME, and ALT); (2) perylene-derived altertoxins (ATX-I, ATX-II, and ATX III); and (3) tetramic acid derivatives (TeA) (Pinto and Patriarca, 2017). Cereals can be infected by several species of genus *Alternaria*, particularly by *A. alternata* (Broggi et al., 2007; Azcarate et al., 2008; Xu et al., 2016). *A. alternata* synthesizes several mycotoxins such as AOH, AME,

Published by Elsevier. This is the Author Accepted Manuscript issued with:
Creative Commons Attribution Non-Commercial No Derivatives License (CC:BY:NC:ND 4.0).
The final published version (version of record) is available online at DOI:10.1016/j.ijfoodmicro.2018.01.003
Please refer to any applicable publisher terms of use.

ALT, TeA, and ATX-I, which are the most frequently detected mycotoxins (Schade and King, 1984; Scott, 2001) (Fig. 1). These *Alternaria* mycotoxins are potential food contaminants and are found to be associated with agricultural products in nature.

Strong evidence suggests that AOH and AME may be mutagenic (An et al., 1989; Brugger et al., 2006). AOH and AME inhibit the secretion of progesterone by porcine granulosa cells *in vitro*. It has been reported that *in vivo* inhibition of progesterone production markedly affected reproductive performance in pigs and other mammals (Tiemann et al., 2009).

TeA is known to have acute toxic effects on human and animal health. It inhibits protein biosynthesis by ribosomes (Zhou and Qiang, 2008). It also shows several bioactivities, including antitumor, antibacterial, antiviral, and phytotoxic activities (Chelkowski and Visconti, 1992).

Altertoxins are responsible for causing acute toxicity in mice. They also cause mutagenicity and cytotoxicity in bacterial and mammalian cells (Solfrizzo et al., 2005).

Although these mycotoxins have a widespread occurrence, currently, there are no regulations governing *Alternaria* toxins in food and feed worldwide.

In a previous study, Lee and Yu showed that eight *Alternaria* species, namely *A. cucumerina*, *A. dauci*, *A. macrospora*, *A. porri*, *A. sesami*, *A. solani*, *A. tagetica*, and *A. zinnia*, that were isolated from red pepper fruits and sesame seeds in Korea produced AOH and AME (Lee and Yu, 1995a, 1995b). They also showed that potential mycotoxin-associated problems may exist in some agricultural products contaminated with *Alternaria* species, which produced large amounts of TeA, AOH, and AME. Their study demonstrated that 75 out of 280 isolates produced at least one of the five toxins, only *A. alternata* was toxic to rats, while the species, namely *A. sesami*, *A. sesamicola*, and *A. solani*, were nontoxic.

Therefore, *Alternaria* toxins have been receiving increasing attention in recent research, especially risk assessment studies. Accurate and rapid quantitative methods to measure *Alternaria* toxins would be very helpful for such studies. Recently, LC–MS/MS has been used to determine and confirm the presence of AOH and AME in apple juice and other fruit beverages at sub-nanogram per milliliter levels (Lau et al., 2003; Scussel et al., 2012). A multiphase method was developed by which 33 mycotoxins (including AOH and AME) in various products such as peanuts, pistachios, wheat, maize, cornflakes, raisins, and figs could be analyzed simultaneously (Lee et al., 2015). Although numerous mycotoxins have already been chemically characterized and classified in several studies, there are no data on *Alternaria* mycotoxins from Korean foodstuffs, which is a concern that needs to be addressed.

The purpose of this study was to develop a new quantitative method based on high performance liquid chromatography (HPLC)-tandem mass spectrometry (LC–MS/MS) for the detection and quantification of mycotoxins produced by *Alternaria* species isolated from Korean barley grain samples. The method established in this study was validated on 3 representative *Alternaria* strains producing mycotoxins.

2. Materials and methods

2.1. Isolation of Alternaria strains from Korean barley grain samples

Seeds of barley were collected from local growing areas of Jeonnam, Korea. A total of 100 barley seeds were placed in plates containing two layers of moist blotting paper, 20-25 seeds per plate. The plates were incubated at 25 °C for 7 days. Fungal spores on the seeds were examined under stereo microscope and transferred to potato dextrose agar (PDA) plates using

a capillary tube. Pure isolates were maintained in PDA slant tubes and stored in 20% (v/v) glycerol at −80 °C at the Environmental Microbiology Laboratory Fungarium, Chonnam National University, Gwangju, Korea.

2.2. Molecular identification of fungi

Fungal strains were grown for 5–7 days at 25 °C on PDA plates covered with cellophane. Genomic DNA was extracted from 50 mg fungal mycelia of each isolate using the HiGene Genomic DNA Prep kit (BIOFACT Corp., Daejeon, Korea). The internal transcribed spacer (ITS) region, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), and RNA polymerase II subunit 2 (*rpb2*) genes were amplified with the primer pairs ITS4, ITS5 (White et al., 1990); gpd1, gpd2 (Berbee et al., 1999); and rpb2-5F-Eur, rpb2-7cr-Eur (Hibbett, 2006; Houbraken et al., 2011), respectively. The PCR amplification mixture (total volume, 20 µL) contained 2 µL DNA template, 1.5 µL each primer (5 pM), 14 µL demineralized sterile water, and 1 µL Accuprep PCR Premix (containing *Taq* DNA polymerase, dNTPs, buffer, and a tracking dye; Bioneer Corp., Daejeon, Korea). PCR products were purified using the Accuprep PCR Purification kit (Bioneer Corp.) according to the manufacturer's instructions. DNA sequencing was performed on an ABI 3700 Automated DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA).
Sequence data obtained from the GenBank database (Table 1) were aligned using ClustalX v.1.83 (Thompson et al., 1997) and edited with Bioedit v.5.0.9.1 (Hall, 1999). Phylogenetic analyses were performed using MEGA 6 (Tamura et al., 2013). The neighbor joining (NJ) and maximum likelihood (ML) phylogenetic trees were constructed for individual datasets for ITS rDNA and combined datasets of the ITS, *gapdh*, and *rpb2* sequences. The sequences of *Dendryphiella salina*, *Cochliobolus heterostrophus*, and *Alternaria alternantherae* were used as outgroups.

2.3. Production and extraction of Alternaria mycotoxins

For inoculum preparation, 50 g rice grains were placed in 500-mL Erlenmeyer flasks containing 50 mL distilled water. The flasks containing the media were autoclaved at 121 °C for 30 min, and allowed to stand overnight. Each flask was inoculated with mycelial plugs obtained from a 7-day-old PDA culture and incubated at 25 °C for 2 weeks. The flasks were shaken once or twice daily, during the initial 2 d to ensure growth of fungal mycelia on rice grains. After 2 weeks, *alternaria* toxins were extracted from rice grains as described previously by Lee and Yu (Lee and Yu, 1995b). Briefly, 50 g rice grains were extracted with 250 mL methanol and filtered through a filter paper (Whatman No 2). The filtrate was clarified with 80 mL 20% ammonium sulfate, partitioned into methylene chloride, and divided into two parts. The organic phase was combined and the solvent was completely evaporated. 1 mL methanol was added to dissolve the residues. ALT, AOH, AME, and ATX-I were analyzed by TLC.
For TeA analysis, the water phase remaining after methylene chloride extraction was adjusted to pH 2 with 6 N HCl and extracted twice with 50 mL methylene chloride. Next, TeA was partitioned using 30 mL 5% sodium bicarbonate that was subsequently acidified to pH 2, and extracted twice with 2 mL methylene chloride. The methylene extracts were combined and evaporated to dryness. The residue was redissolved in methanol and TeA analysis was performed using TLC.

2.4. Thin layer chromatography (TLC) analysis

TLC was performed as described previously by Lee and Yu (1995ab). Briefly, 2 µL solvent extract was spotted onto 10 × 10 cm silica gel plates (Kiesel gel 60 F254; Merck, Darmstadt, Germany) precoated with fluorescen indicator for TLC separation. Subsequently, the TLC plates were developed in a glass chamber using 100 mL mobile phase containing a mixture of chloroform:acetone (88 : 12, v/v). The plate was removed from the chamber and air-dried. Mycotoxins were detected under UV light at 254 and 365 nm (Vilber Lourmat, Marne La ValleBe, France).

2.5. LC–MS/MS analysis

This method was developed using the Shimadzu LC-10ADvp system (Shimadzu, Japan) coupled to the API2000 (AB SCIEX, US) with a triple quadrupole. The Thermo Scientific BDS Hypersil C8 column (3 × 50 mm, 3-µm particle size; US) was used. The column temperature was set at 25 °C. The mobile phases were water (Milli-Q) containing 2 mM ammonium formate with 0.1% formic acid (mobile A) and acetonitrile with 0.1% formic acid (mobile B) at a flow rate of 0.4 mL/min. Acetonitrile (ACN, LC gradient grade) was purchased from Merck (Darmstardt, Germany), and formic acid (LC–MS grade) and ammonium formate (Optima® LC–MS grade) were obtained from Sigma–Aldrich and Thermo Scientific, respectively. B was held at 3% from 0 to 1 min, increased to 95% for 13 min, and brought back to 3% within 1 min. The system was equilibrated for 15 min at 3% B before the next run. Analytical grade calibrants for ALT, ATX-I, TeA, AOH, and AME were purchased from Sigma–Aldrich. Stock solutions were prepared by diluting with 100% methanol containing 0.1% formic acid. All stock solutions were stored in a refrigerator at –20 °C until further use. The extracts from EML-BLDF1-4 (2.3 mg), EML-BLDF1-14 (3.0 mg), and EML-BLDF1-18 (3.0 mg) were re-dissolved in 200 µL methanol containing 0.1% formic acid and injected in LC–MS/MS. The injection volume was 5 µL. The mass spectrometer was operated in the ESI positive ionization mode using multiple reaction monitoring (MRM) mode. Two ion transitions (Q1 and Q3) were scanned for each target toxin. The selected ion transitions were optimized with respect to declustering potential (DP), focusing potential (FP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE), and collision cell exit potential (CXP). The optimized mass spectrometry parameters were as follows: curtain gas, 30; spray voltage, 5,500; source temperature, 500 °C; and ion source gas, 50 psi.

2.6. Method validation

The analysis method was validated for EML-BLDF culture medium samples using LC–MS/MS system. The calibration curve was constructed over a concentration range of 0.25–256 µg/mL. Four different points were plotted. The limit of detection (LOD) and limit of quantification (LOQ) of the method were calculated as 3 and 10 × signal-to-noise ratio, respectively. The coefficient of correlation (R^2) was used to judge the linearity. Recovery rates were determined by adding 100 µL standard mixture to the sample before extraction, amounting to LOQ1 and LOQ2 of each toxin.

3. Results and discussion

3.1. Identification of fungi

Results of the ITS rDNA sequence analysis of 24 isolates from barley samples are shown in Fig. 2. 24 isolates were placed within section *Alternaria* as described by Woudenberg et al. (2013). BLASTn search revealed that the ITS sequences of isolates showed 99.4 %–100% to those of *Alternaria alternata*.

Within section *Alternaria*, three representative strains, namely EML-BLDF1-4, EML-BLDF1-14, and EML-BLDF1-18, were identified based on two additional genes, *gapdh* and *rpb2*. BLASTn results revealed that the *gapdh* gene sequences of EML-BLDF1-4, EML-BLDF1-14, and EML-BLDF1-18 showed 100% (562/562, 564/564, and 574/574 bp, respectively) identity with those of *A. alternata* (GenBank accession no. KP057228), *A. tenuissima* (GenBank accession no. KY290574), and *A. alternata* (GenBank accession no. KP057228), respectively. The *rpb2* gene sequences of strains EML-BLDF1-4, EML-BLDF1-14, and EML-BLDF1-18 showed 100% (824/824, 865/865, and 865/865 bp) identity with those of *A. tenuissima* (GenBank accession no. LT707523), *A. alternata* (GenBank accession no. KU738707), and *A. alternata* (GenBank accession no. KU738707), respectively. Phylogenetic analyses based on the combined dataset of ITS, *gapdh*, and *rpb2* sequences showed that the strains EML-BLDF1-4, BLDF1-14, and BLDF1-18 were placed within the *A. alternata* clade of sect. *Alternaria* (Woudenberg et al., 2015) (Fig. 3).

Our results showed that 24 strains from barley grains were identified as *Alternaria alternata*. Bruce et al. (1984) observed *Alternaria* infection in all 57 barley samples analyzed, and identified *A. alternata* as the dominant species. Sanchis et al. (1993) also reported that the most predominant genera were *Alternaria*, *Penicillium*, and *Aspergillus*, which were isolated from 60 samples of barley collected in Spain. Most *Alternaria* isolates were identified as *A. alternata* (90.2%). A recent study by Medina et al. (2006) showed that *Alternaria* species were the dominant fungal species isolated from malting barley samples.

3.2. TLC analysis

Solvent extracts of the 24 strains of *A. alternata* isolated from barley samples were developed on TLC plates for the detection of mycotoxins (Table 2). TLC analyses showed that all the 24 strains were able to produce at least one of the five mycotoxins in rice culture medium. 24 strains (100%) produced both ALT and AOH, 18 strains (75%) produced ATX-I, 22 strains (91.6%) produced AME, and 9 strains (37.5%) produced TeA (Fig. 4). Thus, we demonstrated and confirmed the presence of five mycotoxins, including ALT, AOH, AME, TeA, and ATX-I, using TLC analysis. AME, ALT, ATX-I, and TeA were identified on the basis of their *Rf* values using the TLC system constructed by Lee et al. (1995ab). AOH, AME, and TeA were also detected in rice culture medium (Mass et al.,1981; Bruce et al., 1984; Torres et al., 1998; Medina et al., 2006).

After TLC analysis, three *Alternaria* strains, namely EML-BLDF1-4, EML-BLDF1-14, EML-BLDF1-18, were selected for further analyses with optimization of LC–MS/MS conditions based on the ability to produce different mycotoxins. In the present study, EML-BLDF1-18, EML-BLDF1-4, EML-BLDF1-14 were assigned as TeA, ATX-I, and AOH and AME producing strains, respectively.

3.3. Optimization of LC–MS/MS conditions

LC–MS/MS analysis with multiple reaction monitoring (MRM) mode enables sensitive and specific detection of target toxins. MS/MS settings were optimized on the API2000 instrument using direct infusion of individual standard solutions and flow injection analysis. Ion transitions were tuned using 10 μg/mL standard solutions in 100% methanol containing 0.1% formic acid. MThe mass parameters were optimized to obtain the strongest signals for $[M + H]^+$ ion. The product ion values were determined from the results of the MS/MS analysis. The optimized precursor and product ions are summarized in Table 3. Changing the ion trace selection resulted in decreased noise peaks and enhanced selectivity for appropriate identification. The mobile phase was chosen based on the ionization and separation efficiency. Due to good separation performance, ACN was employed as strong elution mobile phase B and water was chosen as weak elution mobile phase A. The *Alternaria* toxins were separated by gradient conditions for 14 min (Fig. 5).

3.4. Method validation

The calibration equation, R^2, LOD, and LOQ values have been shown in Table 4. A linear calibration curve (concentration range, 0.25–256 μg/mL) was obtained for all five *Alternaria* toxins. The R^2 value ranged from 0.993 to 0.999. The LOD value ranged from 0.13 μg/mL for ALT to 4 μg/mL for ATX-I. The LOQ value ranged from 0.25 μg/mL for ALT to 8 μg/mL for ATX-I. These LOD and LOQ values were either similar or lower compared to those determined using LC–MS/MS in earlier studies (Meena et al., 2017; Prelle et al., 2013). Sample recovery calculated by external standard method using standard solutions for ALT, ATX-I, TeA, AOH, and AME ranged from 83.5 to 99.2% of their spiked concentrations. Precision of the method was determined by evaluating its reproducibility expressed in terms of RSD (%). RSD ranged from 0.59 to 12.06% (Table 5).

3.5. Analysis of Alternaria mycotoxins in EML-BLDF rice culture medium

The culture media extracts of EML-BLDF were concentrated and re-dissolved in 100% methanol containing 0.1% formic acid and analyzed by LC–MS/MS. The toxigenic profiles of mycotoxins produced by three *Alternaria* strains EML-BLDF1-4, EML-BLDF1-14, and EML-BLDF1-18 on rice culture medium have been shown in Table 6. Our data showed that ALT was detected in the extracts from all three strains. The highest concentration of ALT was 85.5 μg/mL in the EML-BLDF1-4 extract. TeA was detected only in the EML-BLDF1-18 extract. Its concentration was higher (201 μg/mL) than that of the other mycotoxins. In addition, ATX-I was detected only in the EML-BLDF1-4 extract. Interestingly, the benzopyrone derivatives (AOH, AME, and ALT) were detected in both the EML-BLDF1-4 and EML-BLDF1-14 extracts.

In our study, compared to the other toxins detected in rice culture medium extracts, TeA concentration was observed to be the highest. Notably, high TeA concentration was also reported by other authors in earlier studies. Logrieco et al. (1990) showed that 14 strains (100%) produced TeA and the concentration of this toxin was 6,000 mg/kg; 13 strains (93%) produced AOH, AME, and ALT and the concentrations of these toxins were 120, 59, and 37 mg/kg, respectively. Li et al. (2001) observed that among the 25 isolates obtained from the Chinese weathered wheat, 8 (32%) isolates produced TeA (1,369–3,563 mg/ kg), 25 (100%) produced AOH (2–178 mg/ kg), 25 (100%) produced AME (1–98 mg/kg), 21 (84%)

produced ALT (2–145 mg/kg), and 21 (84%) produced ATX-I (2–23 mg/kg). Sanchis (1993) observed that among the 176 *A. alternata* isolates obtained from barley collected in Spain, the percentage of species producing TeA, AOH, and AME was 88.6%, 15.3%, and 9%, respectively. Similar data were obtained by Patriarca et al. (2007). They reported that the highest concentration of TeA (up to 9,478 mg/kg) was observed in the *A. alternata* isolate from Argentinean wheat; however, AOH and AME were present at lower concentrations (4–622 and 21–2,352 mg/kg, respectively).

Results of the present study indicated that the amount of toxin produced by *Alternaria* isolates varied considerably among different isolates. Different growth conditions affected mycotoxin production, especially the production of TeA. Magan et al. (1984) and Sanchis and Magan (2004) showed that water activity played an important role in the production of AE, AME, AOH, and TeA in *A. alternata*. Temperature is also an essential factor in growth of *A. alternata* on toxin synthetic medium (Pose et al., 2010). Brzonkalik et al. (2011) showed that carbon and nitrogen sources affected mycotoxin production by affecting *A. alternata* growth on synthetic medium.

Our study showed that *A. alternata* strains isolated from barley seed grains in Korea produced different mycotoxins on rice culture medium. Especially, the strain EML-BLDF1-18 produced high amounts of the TeA toxin. In addition, among all the strains, the strain EML- BLDF1-4 was the best producer.

In future, studies evaluating the natural occurrence of ALT, AOH, ATX-I, AME, and TeA on different grains, including barley, produced by *A. alternata* should be performed.

4. Conclusion

Mycotoxin production by 24 *Alternaria* strains isolated from Korean barley grains was analyzed. Herein, we developed and validated a reliable LC–MS/MS method for the simultaneous determination of various *Alternaria* mycotoxins. Our results showed that ALT, ATX-I, AOH, AME, and TeA were detected in the rice culture medium extracts ranging from 0.25–8, 8–256, 4–64, 4–64, and 6.25–100 μg/mL, respectively.

Acknowledgments

This work was supported by a grant from the National Institute of Animal Science and from under Rural Development Administration, and in part by a fund from National Institute of Food and Drug Safety Evaluation under the Ministry of Food and Drug Safety, Republic of Korea.

References

An, Y.H., Zhao, T.Z., Miao, J., Liu, G.T, Zheng, Y.Z., Xu, Y.M., Van Etten, R.L., 1989. Isolation, identification, and mutagenicity of alternariol monomethyl ether. J. Agric. Food Chem. 37, 1341-1343.

Azcarate, M.P., Patriarca, A., Terminiello, L., Fernández Pinto, V., 2008. *Alternaria* toxins in wheat during the 2004 to 2005 Argentinean harvest. J. Food Prot. 71, 1262-1265.

Berbee, M.L., Pirseyedi, M., Hubbard, S., 1999. *Cochliobolus* phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. Mycologia. 91, 964-977.

Broggi, L.E., González, H.H.L., Resnik, S.L., Pacin, A.M., 2007. *Alternaria alternata*

prevalence in cereal grains and soybean seeds from Entre Ríos, Argentina. Rev. Iberoam. Micol. 24, 47-51.
Bruce, V.R., Stack, M.E., Mislivec, P.B., 1984. Incidence of toxic *Alternaria* species in small grains from the USA. J. Food Sci. 49, 1626-1627.
Brzonkalik, K., Herrling, T., Syldatk, C., Neumann, A., The influence of different nitrogen and carbon sources on mycotoxin production in *Alternaria alternata*. Int. J. Food Microbiol. 147, 120-126.
Brugger, E.M., Wagner, J., Schumacher, D.M., Koch, K., Podlech, J., Metzler, M., Lehmann, L., 2006. Mutagenicity of the mycotoxin alternariol in cultured mammalian cells. Toxicol. Lett. 164, 221-230.
Chelkowski, J., Visconti, A., 1992. *Alternaria*: Biology, Plant Diseases and Metabolites. Amsterdam, The Netherlands: Elsevier, 449-541.
Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95-8.
Hibbett, D.S., 2006. A phylogenetic overview of the Agaricomycotina. Mycologia. 98, 917-925.
Houbraken, J., Frisvad, J.C., Samson, R.A., 2011. Taxonomy of *Penicillium* section *Citrina*. Stud. Mycol. 70, 53-138.
Lau, B.P.-Y, Scott, P.M., Lewis, D.A., Kanhere, S.R., Cléroux, C., Roscoe, V.A., 2003. Liquid chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry of the *Altemaria* mycotoxins alternariol and alternariol monomethyl, ether in fruit juices and beverages. J. Chromatogr. A. 998,119-131.
Lee, H.B., Patriarca, A., Magan, N., 2015. *Alternaria* in food: ecophysiology, mycotoxin production and toxicology. Mycobiology. 43, 93-106.
Lee, H.B., Yu, S.H., 1995a. Incidence of *Alternaria* species in red pepper and sesame from Korea and their ability to produce mycotoxins. Plant Pathol. J. 11, 1-8.
Lee, H.B., Yu, S.H., 1995b. Distribution of mycotoxin-producing isolates in the genus *Alternaria*. Plant Pathol. J. 11, 151-7.
Li, FQ., Toyazaki, N., Yoshizawa, T., 2001. Production of *Alternaria* mycotoxins by *Alternaria alternata* isolated from weather-damaged wheat. J. Food Prot. 64, 567-571.
Logrieco, A., Bottalico, A., Solfrizzo, M., Mule, G., 1990. Incidence of *Alternaria* species in grains from mediterranean countries and their ability to produce mycotoxins. Mycologia. 82, 501-505.
Magan, N., Cayley, G.R., Lacey, J., 1984. Effect of water activity and temperature on mycotoxin production by *Alternaria alternata* in culture and on wheat grain. Appl. Environ. Microbiol. 47, 1113-1117.
Mass M.R., Woody M.A., Chu F.S., 1981. Production of alternariol and alternariol methyl ether by *Alternaria* spp. J. Food Saf. 3, 39-47.
Medina, A., Valle-Algarra, F.M., Mateo, R., Gimeno-Adelantado, J.V., Mateo, F. and Jiménez, M., 2006. Survey of the mycobiota of Spanish malting barley and evaluation of the mycotoxin producing potential of species of *Alternaria*, *Aspergillus* and *Fusarium*. Int. J. Food Microbiol. 108, 196-203.
Meena, M., Swapnil, P., Upadhyay, R.S., 2017. Isolation, characterization and toxicological potential of Alternaria-mycotoxins (TeA, AOH and AME) in different *Alternaria* species from various regions of India. Sci. Rep. 7, 8777.
López, P., Venema, D., de Rijk T, de Kok, A., Scholen, J., Mol, H., de Nijs, M., 2014. Occurrence of Alternaria mycotoxins in food products in the Netherlands. In: World

Mycotoxin Forum, Vienna, Austria
Ostry, V., 2008. *Alternaria* mycotoxins: an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. World Mycotoxin J. 1, 175-188.
Patriarca, A., Azcarate, M.P., Terminiello, L., Pinto, V.F., 2007. Mycotoxin production by *Alternaria* strains isolated from Argentinean wheat. Int. J. Food Microbiol. 119, 219-222.
Pinto, V.E.F., Patriarca, A., 2017. *Alternaria* species and their associated mycotoxins. Methods Mol. Biol. 1542, 13-32.
Pose, G., Patriarca, A., Kyanko, V., Pardo, A., Fernández Pinto, V., 2010. Water activity and temperature effects on mycotoxin production by *Alternaria alternata* on a synthetic tomato medium. Int. J. Food Microbiol. 142, 348-353.
Prelle, A., Spadaro, D., Garibaldi, A., Gullino, M.L., 2013. A new method for detection of five alternaria toxins in food matrices based on LC-APCI-MS. Food Chem. 140, 161-167.
Schade, J.E., King, A.D.Jr., 1984. Analysis of the major *Alternaria* toxins. J. Food Protect. 47, 978-995.
Sanchis, V., Magan, N., 2004. Environmental profiles for growth and mycotoxin production. Chapter 8. In: Magan N, Olsen M, editors. Mycotoxins in food: detection and control. Cambridge: Woodhead Publishing Ltd.; 174-189.
Sanchis, V., Sanclementele, A., Usall, J., Vinas, I., 1993. Incidence of mycotoxigenic *Alternaria alternata* and *A. flavus* in barley. J. Fd. Prot. 56, 246-248.
Solfrizzo, M., Girolamo, A.D., Vitti, C., Tylkowska, K., Grabarkiewicz-Szczesna, J., Szopińska, D., Dorna, H., 2005. Toxigenic profile of *Alternaria alternata* and *Alternaria radicina* occurring on umbelliferous plants. Food Addit. Contam. 22, 302-308.
Scott, P.M., 2001. Analysis of agricultural commodities and foods for *Alternaria* mycotoxins. J. AOAC Int. 84, 1809-1817.
Scussel, V.M., Scholten, J.M., Rensen, P.M., Spanjer, M.C., Giordano, B.N.E., Savi, G.D., 2012. Multitoxin evaluation in fermented beverages and cork stoppers by liquid chromatography–tandem mass spectrometry. Int. J. Food Sci. Technol. 48, 96-102.
Storm, I.M.L.D., Rasmussen, R.R., Rasmussen, P.H., 2014. Occurrence of pre- and post-harvest mycotoxins and other secondary metabolites in Danish maize silage. Toxins. 6, 2256-2269.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary Genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725-2729.
Thompson, J.D., Gibson, T.J, Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876-4882.
Tiemann, U., Tomek, W., Schneider, F., Müller, M., Pöhland, R., Vanselow, J., 2009. The mycotoxins alternariol and alternariol methyl ether negatively affect progesterone synthesis in porcine granulosa cells in vitro. Toxicol. Lett. 186, 139-145.
Torres, A., González, H.H., Etcheverry, M., Resnik, S.L., Chulze, S., 1998. Production of alternariol and alternariol mono-methyl ether by isolates of *Alternaria* spp. from Argentinian maize. Food Addit Contam. 15, 56-60.
White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: Academic Press. pp. 315-322.
Woudenberg, J.H.C., Groenewald, J.Z., Binder, M., Crous, P.W., 2013. *Alternaria* redefined. Stud. Mycol. 75, 171-212.
Woudenberg, J.H.C., Seidl, M.F., Groenewald, J.Z., de Vries, M., Stielow, J.B., Thomma,

B.P.H.J., Crous, P.W., 2015. *Alternaria* section *Alternaria*: Species, formae speciales or pathotypes? Stud. Mycol. 82, 1-21.
Xu, W., Han, X., Li, F., Zhang, L., 2016. Natural occurrence of *Alternaria* toxins in the 2015 wheat from Anhui province, China. Toxins. 8, 308.
Zhou, B., Qiang, S., 2008. Environmental, genetic and cellular toxicity of tenuazonic acid isolated from *Alternaria alternata*. Afr. J. Biotechnol. 7, 1151-1156.

Figure legends

Fig. 1. Chemical structures of *Alternaria* mycotoxins: (A) ALT; (B) ATX-I; (C) AOH; (D) AME; and (E) TeA.

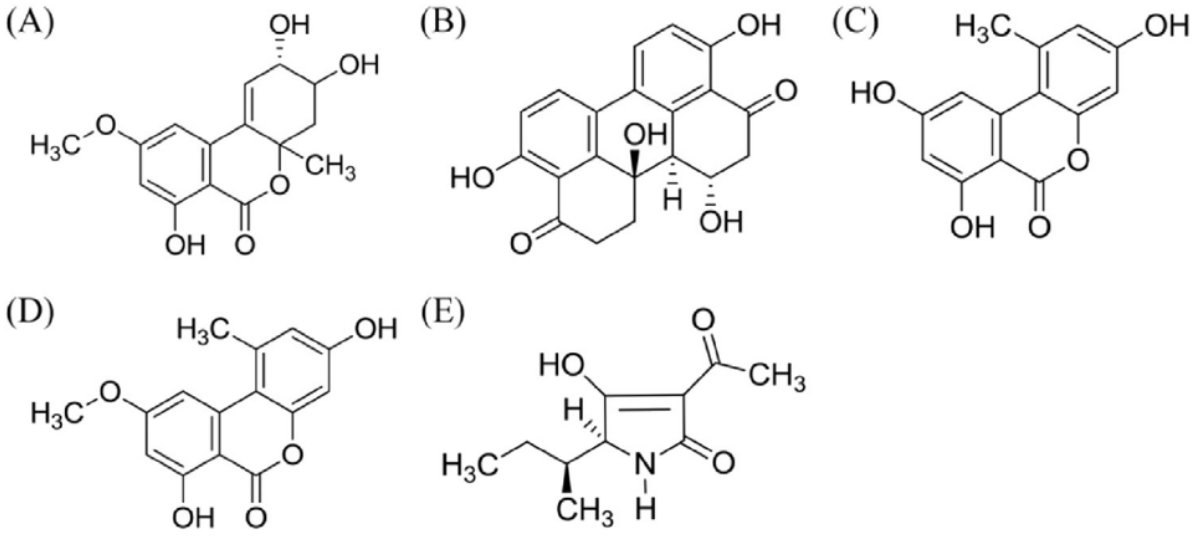

Fig. 2. Phylogenetic tree constructed based on the ITS rDNA sequences using neighbor-joining method. The sequences of *Dendryphiella salina* and *Cochliobolus heterostrophus* were selected as the outgroups. Numbers at the nodes indicate the bootstrap values (> 50%) of 1,000 replicates.

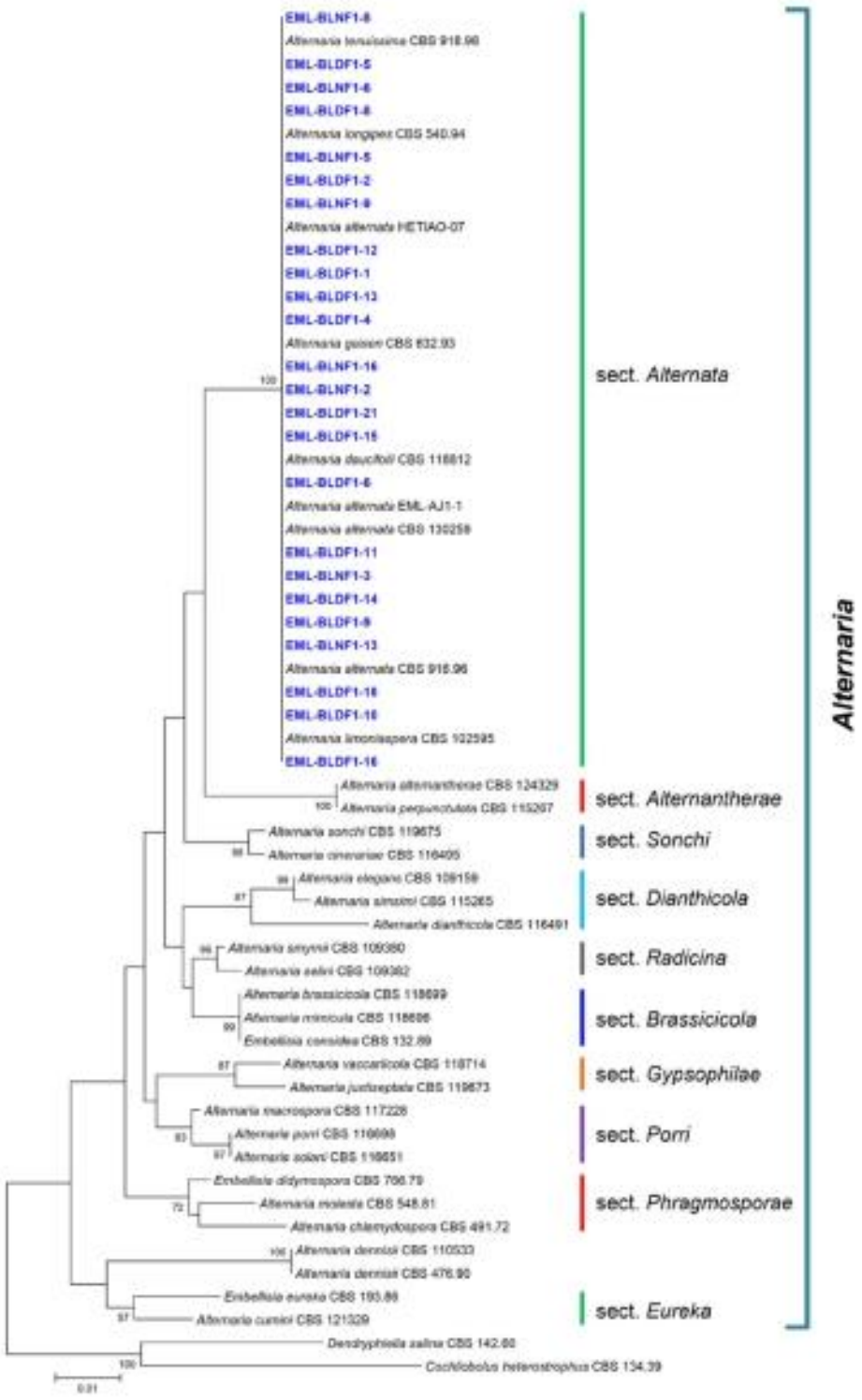

Fig. 3. Phylogenetic tree constructed based on the combined datasets of the internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), and *rpb2* gene sequences using maximum likelihood method. The sequence of *Alternaria alternantherae* CBS 124392 was selected as the outgroup. Numbers at the nodes indicate the bootstrap

values (>50%) from 1,000 replications.

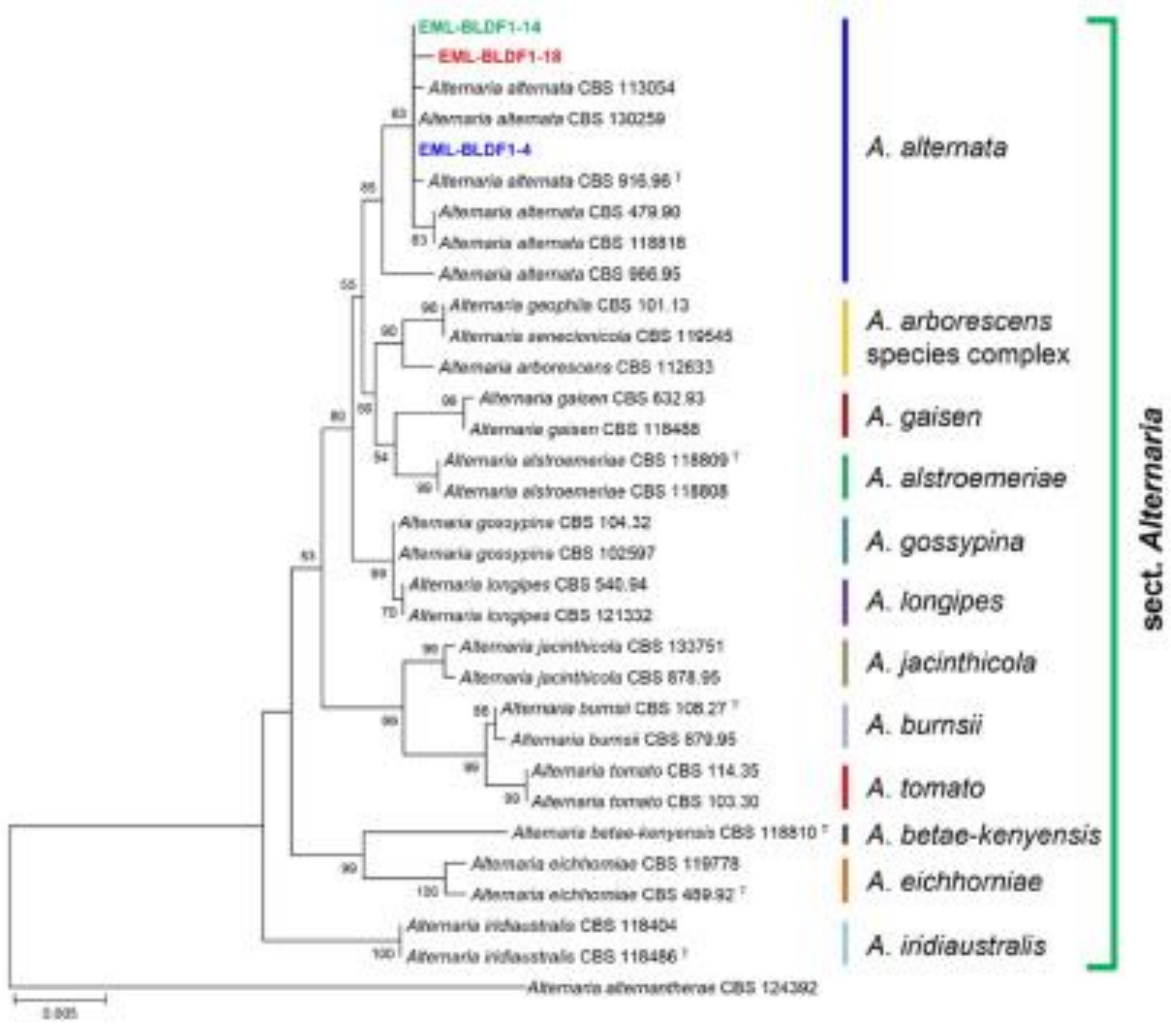

Fig. 4. Thin layer chromatography (TLC) analysis of *Alternaria* mycotoxins produced by different *Alternaria* strains. Different spots of *Alternaria* culture extracts on TLC plates under 254 nm UV (A) and 365 nm UV (B). 1-14 Sample (1, EML-BLDF1-1; 2, EML-BLDF1-1; 3, EML-BLDF1-4; 4, EML-BLDF1-5; 5, EML-BLDF1-6; 6, EML-BLDF1-8; 7, EML-BLDF1-

9; 8, EML-BLDF1-10; 9, EML-BLDF1-11; 10, EML-BLDF1-12; 11, EML-BLDF1-13; 12, EML-BLDF1-14; 13, EML-BLDF1-15; 14, EML-BLDF1-16; 15, EML-BLDF1-18; 16, EML-BLDF1-21; 17, EML-BLNF1-2; 18, EML-BLNF1-3; 19, EML-BLNF1-5; 20, EML-BLNF1-6; 21, EML-BLNF1-8; 22, EML-BLNF1-9; 23, EML-BLNF1-13; and 24, EML-BLNF1-16. TLCs on silica gel plates (Kiesel gel 60 F254) were developed in chloroform:acetone (88:12, v/v), air-dried, and then irradiated under short and long UV wavelength.

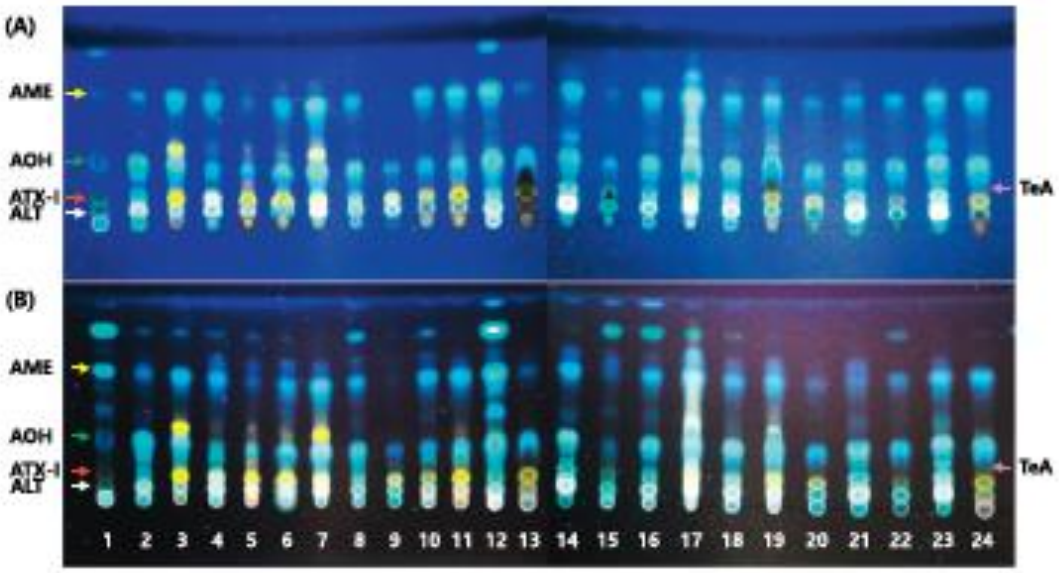

Fig. 5. Multiple reaction monitoring (MRM) chromatogram for selective ion chromatogram: (A) ALT, (B) ATX-I, (C) AOH, and (D) AME obtained from the culture of strain EML-BLDF1-4; (E) ALT, (F) AOH, and (G) AME obtained from the culture of strain EML-BLDF1-14; (H) TeA, and (I) ALT obtained from the culture of strain EML-BLDF1-18.

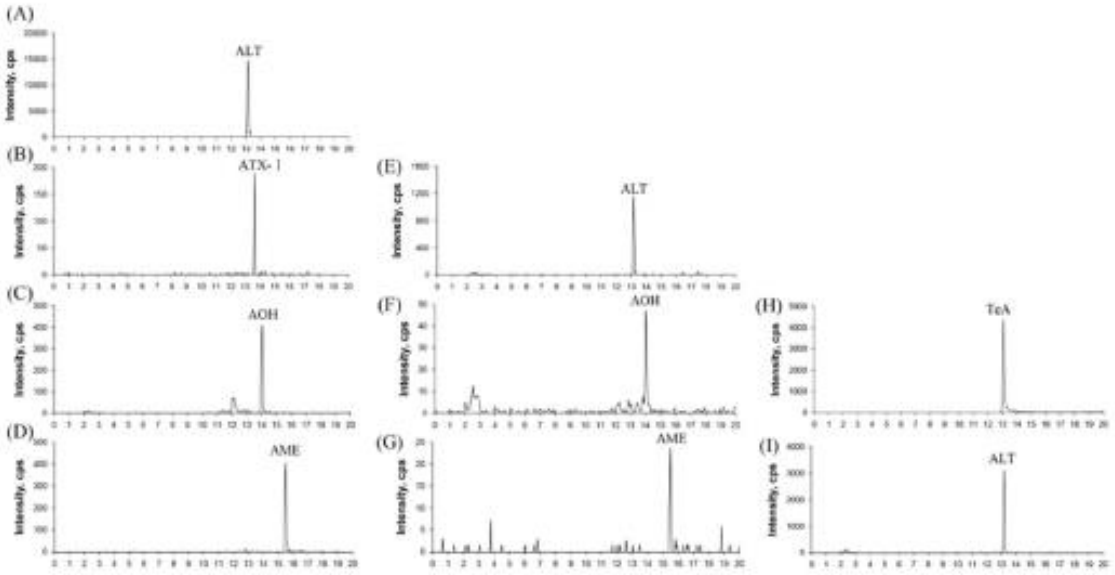

Fig. 6. Standard calibration curves for *Alternaria* mycotoxins: ALT, ATX-I, TeA, AOH, AME.

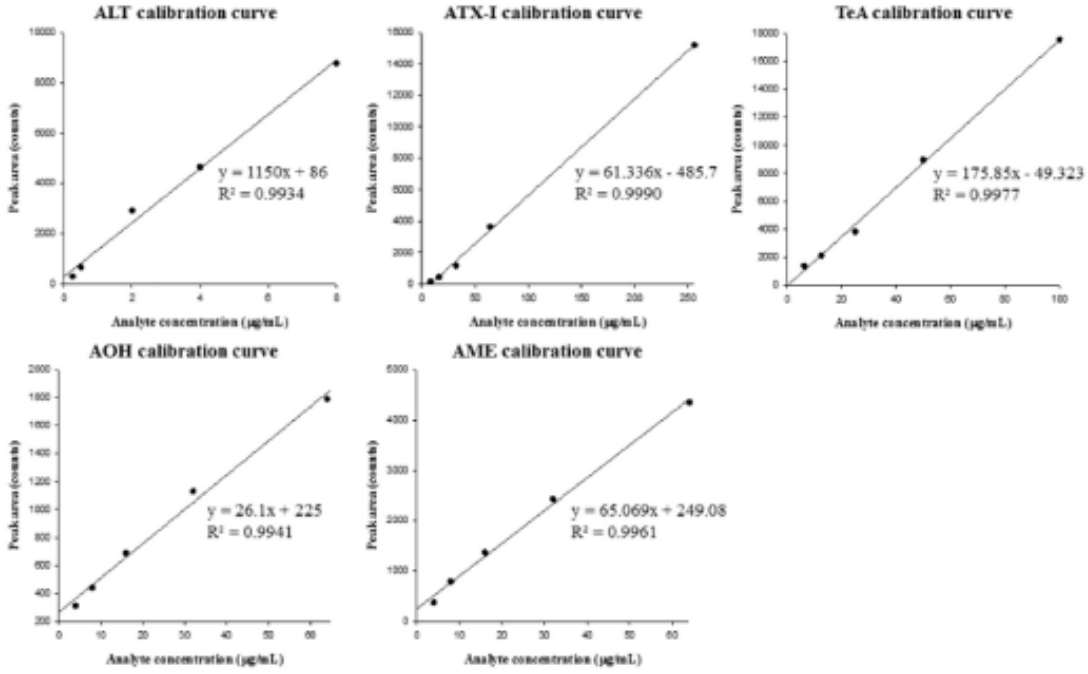

Table 1
Sequences used in this study, including GenBank accession numbers.

Taxon name	Collection no. (isolate no.)	GenBank accession no.		
		ITS	gapdh	rpb2
Alternaria alstroemeriae	CBS 118808	KP124296	KP124153	KP124764
A. alstroemeriae	CBS 118809^{T}	KP124297	KP124154	KP124765
A. alternantherae	CBS 124392	KC584179	KC584096	KC584374
A. alternata	CBS 479.90	KP124319	KP124174	KP124787
A. alternata	CBS 118818	KP124359	KP124213	KP124827
A. alternata	CBS 113054	KP124346	KP124200	KP124814
A. alternata	CBS 916.96^{T}	AF347031	AY278808	KC584375
A. alternata	CBS 130259	KP124386	KP124238	KP124856
A. alternata	CBS 966.95	KP124324	KP124179	KP124792
A. alternata	HETIAO-07	KJ002061		
A. alternata	EML-AJ1-1	KX765268		
A. alternata	**EML-BLDF1-4**	**MG041370**	**MG051340**	**MG051337**
A. alternata	**EML-BLDF1-14**	**MG041371**	**MG051341**	**MG051336**
A. alternata	**EML-BLDF1-18**	**MG041372**	**MG051339**	**MG051338**
A. arborescens	CBS 112633	KP124400	KP124252	KP124870
A. betae-kenyensis	CBS 118810^{T}	KP124419	KP124270	KP124888
A. burnsii	CBS 108.27^{T}	KC584236	KC584162	KC584468
A. burnsii	CBS 879.95	KP124422	KP124272	KP124891
A. brassicicola	CBS 118699	JX499031		
A. chlamydospora	CBS 491.72	KC584231		
A. cinerariae	CBS 116495	KC584190		
A. cumini	CBS 121329	KC584191		
A. daucifolii	CBS 118812	KC584193		
A. dennisii	CBS 110533	KC584232		
A. dennisii	CBS 476.90	JN383488		
A. dianthicola	CBS 116491	KC584194		
A. eichhorniae	CBS 489.92	KC146356^{T}	KP124276	KP124895
A. eichhorniae	CBS 119778	KP124426	KP124277	KP124896
A. elegans	CBS 109159	KC584195		
A. gaisen	CBS 632.93	KC584197	KC584116	KC584399
A. gaisen	CBS 118488	KP124427	KP124278	KP124897
A. geophila	CBS 101.13	KP124392	KP124244	KP124862
A. gossypina	CBS 104.32	KP124430	JQ646312	KP124900
A. gossypina	CBS 102597	KP124432	KP124281	KP124902
A. iridiaustralis	CBS 118404	KP124434	KP124283	KP124904
A. iridiaustralis	CBS 118486^{T}	KP124435	KP124284	KP124905
A. jacinthicola	CBS 878.95	KP124437	KP124286	KP124907
A. jacinthicola	CBS 133751	KP124438	KP124287	KP124908
A. juxtiseptata	CBS 119673	KC584202		
A. limoniaspera	CBS 102595	FJ266476		
A. longipes	CBS 540.94	AY278835	AY278811	KC584409
A. longipes	CBS 121332	KP124443	KP124292	KP124913
A. macrospora	CBS 117228	KC584204		
A. mimicula	CBS 118696	FJ266477		
A. molesta	CBS 548.81	KC584205		
A. perpunctulata	CBS 115267	KC584210		
A. porri	CBS 116698	DQ323700		
A. selini	CBS 109382	AF229455		
A. senecionicola	CBS 119545	KP124409	KP124260	KP124879
A. simsimi	CBS 115265	JF780937		
A. smyrnii	CBS 109380	AF229456		
A. solani	CBS 116651	KC584217		
A. sonchi	CBS 119675	KC584220		
A. tenuissima	CBS 918.96	AF347032		
A. tomato	CBS 103.30	KP124445	KP124294	KP124915
A. tomato	CBS 114.35	KP124446	KP124295	KP124916
A. vaccariicola	CBS 118714	KC584224		
Cochliobolus heterostrophus	CBS 134.39	DQ491489		
Dendryphiella salina	CBS 142.60	DQ411540		
Embellisia conoidea (currently *Alternaria conoidea*)	CBS 132.89	FJ348226		

Table 1 (*continued*)

Taxon name	Collection no. (isolate no.)	GenBank accession no.		
		ITS	gapdh	rpb2
E. didymospora (currently *Alternaria didymospora*)	CBS 766.79	FJ357312		
E. eureka	CBS 193.86	JN383490		

CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; EML, Environmental Microbiology Laboratory Fungarium, Chonnam National University, Gwangju, South Korea; T, ex-type strain. Bold letters indicate isolates and accession numbers determined in our study.

Table 2

Quantities of mycotoxins produced in rice kernel medium by 24 *Alternaria* strains isolated from barley seeds.

No.	Taxa	Isolate no.	Mycotoxin production[a]				
			ALT	ATX-I	TeA	AOH	AME
1	*Alternaria alternata*	EML-BLD01-1	+ −	+ −	ND	+ −	+ +
2	*A. alternata*	EML-BLD01-2	+ +	ND	ND	+ +	+ −
3	*A. alternata*	EML-BLD01-4	+	+ +	+ −	+ +	+
4	*A. alternata*	EML-BLD01-5	+ +	+ −	ND	+ −	+
5	*A. alternata*	EML-BLD01-6	+	+	+	+ −	+ −
6	*A. alternata*	EML-BLD01-8	+	+	+	+ −	+ −
7	*A. alternata*	EML-BLD01-9	+ +	+	ND	+ +	+ +
8	*A. alternata*	EML-BLD01-10	+	+ −	ND	+	+
9	*A. alternata*	EML-BLD01-11	+	+	ND	+ −	ND
10	*A. alternata*	EML-BLD01-12	+ −	+	+	+ −	+
11	*A. alternata*	EML-BLD01-13	+ −	+	+	+ −	+
12	*A. alternata*	EML-BLD01-14	+	+ −	ND	+ +	+ +
13	*A. alternata*	EML-BLD01-15	+ −	+	+	+	+ −
14	*A. alternata*	EML-BLD01-16	+ +	ND	ND	+ +	+ +
15	*A. alternata*	EML-BLD01-18	+	ND	+	+	ND
16	*A. alternata*	EML-BLD01-21	+	ND	ND	+ +	+ +
17	*A. alternata*	EML-BLNF1-2	+ +	+ +	+	+ +	+ +
18	*A. alternata*	EML-BLNF1-3	+ +	ND	ND	+ +	+ +
19	*A. alternata*	EML-BLNF1-5	+	+ +	+	+ +	+ +
20	*A. alternata*	EML-BLNF1-6	+	+	ND	+	+ −
21	*A. alternata*	EML-BLNF1-8	+ +	ND	ND	+ +	+ −
22	*A. alternata*	EML-BLNF1-9	+	+ −	ND	+ +	+ −
23	*A. alternata*	EML-BLNF1-13	+ +	+	ND	+ +	+ +
24	*A. alternata*	EML-BLNF1-16	+ −	+	ND	+ +	+ +

−: Not detected, + −: trace, +: moderate production, + +: high production.

[a] ALT: altenuene; ATX-I: altertoxin-I; TeA: tenuazonic acid; AOH: alternariol; AME: alternariol monomethyl ether.

Table 3.

Optimized LC-MS/MS instrumental parameters.

Analyte[a]	Retention time (min)	Molecular weight	MRM[b] (m/z, positive)	DP	FP	EP	CEP	CE	CXP
ALT	13.09	292.2	293.2 → 257.0 (Q) 293.2 → 239.3 (I)	11.0	360.0	6.5	15.85	19.0	12.0
ATX-I	13.53	352.3	353.2 → 317.1 (Q) 353.2 → 334.9 (I)	151.0	330.0	10.5	17.35	39.0	6.0
TeA	12.96	197.2	198.0 → 124.8 (Q) 198.0 → 138.8 (I)	16.0	270.0	11.5	68.0	33.0	44.0
AOH	13.96	258.2	259.2 → 213.1 (Q) 259.2 → 217.2 (I)	46.0	370.0	10.5	15.00	35.0	32.0
AME	15.37	272.2	273.1 → 257.9 (Q) 273.1 → 230.0 (I)	41.0	270.0	4.0	15.35	44.0	52.0

[a] ALT: altenuene; ATX-I: alterioxin-I; TeA: tenuazonic acid; AOH: alternariol; AME: alternariol monomethyl ether.

[b] MRM: (Q) transition used for quantification and (I) transition employed to confirm the identification. CE: collision energy. CEP: collision cell entrance potential. CXP: collision cell exit potential. DP: declustering potential. EP: entrance potential. FP: focusing potential.

Table 4

Calibration data, limit of detection (LOD) and limit of quantification (LOQ) values.

Analyte	Concentration range (μg/mL)	Calibration equation[a]	Correlation factor R^2	LOD (μg/mL)	LOQ (μg/mL)
ALT	0.25–8	Y = 1150x + 86	0.9934	0.13	0.25
ATX-I	8–256	Y = 61.336x − 485.7	0.9990	4.0	8.0
TeA	6.25–100	Y = 175.85x − 49.323	0.9977	3.13	6.25
AOH	4–64	Y = 26.1x + 225	0.9941	2.0	4.0
AME	4–64	Y = 65.069x + 249.08	0.9961	0.5	4.0

[a] Calibration equation; x is concentration of *Alternaria* toxin solution in μg/mL and y is peak area.

Table 5

Percentage of recoveries (n = 3) and RSDs for *Alternaria* toxins in rice kernel medium extracts.

Spike levels	ALT		ATX-I		TeA		AOH		AME	
	Recovery (mean, %)	RSD (%)	Recovery (mean, %)	RSD (%)	Recovery (mean, %)	RSD (%)	Recovery (mean, %)	RSD (%)	Recovery (mean, %)	RSD (%)
1 X LOQ	96.2	5.13	99.2	0.73	94.5	3.03	94.6	4.75	87.0	12.06
2 X LOQ	95.9	4.62	83.5	8.16	95.0	3.71	97.7	0.59	97.7	0.59

Table 6

Quantification of *Alternaria* mycotoxins produced by three *Alternaria* strains EML-BLDF1-4, EML-BLDF1-14, and EML-BLDF1-18 in rice culture medium based on LC-MS/MS analysis.

Strains	Detected concentration (μg/mL)				
	ALT	ATX-I	AOH	AME	TeA
EML-BLDF1-4	85.5	20.1	83.8	33.8	ND[a]
EML-BLDF1-14	5.39	ND	4.18	0.535	ND
EML-BLDF1-18	14.8	ND	ND	ND	201.0

[a] ND; not detected.

Cranfield University

CERES Research Repository **https://dspace.lib.cranfield.ac.uk/**

School of Water, Energy and Environment (SWEE) Staff publications (SWEE)

Mycotoxin production of Alternaria strains isolated from Korean barley grains determined by LC-MS/MS

Nguyen, Thuong T. T.

2018-01-03

Attribution-NonCommercial-NoDerivatives 4.0 International

Nguyen TT, Kim J, Jeon SJ, Lee CW, Magan N, Lee HB, Mycotoxin production of Alternaria strains isolated from Korean barley grains determined by LC-MS/MS, International Journal of Food Microbiology, Volume 268, 2 March 2018, pp. 44-52

http://dx.doi.org/10.1016/j.ijfoodmicro.2018.01.003

Downloaded from CERES Research Repository, Cranfield University