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

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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.

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 > 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 (Lee et al., 2015; Zain, 2011). 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., 2007). 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* (Azcarate et al., 2008; Broggi et al., 2007; Xu et al., 2016). *A. alternata* synthesizes several mycotoxins such as AOH, AME, ALT, TeA, and ATX-I, which are the most frequently detected mycotoxins (Schade and King Jr, 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 (1995a, 1995b) showed that eight *Alternaria* species, *A. cucumerina*, *A. dauci*, *A. macrospora*, *A. porri*, *A. sesami*, *A. solani*, *A. tagetica*, and *A. zinnia*, that were isolated from red

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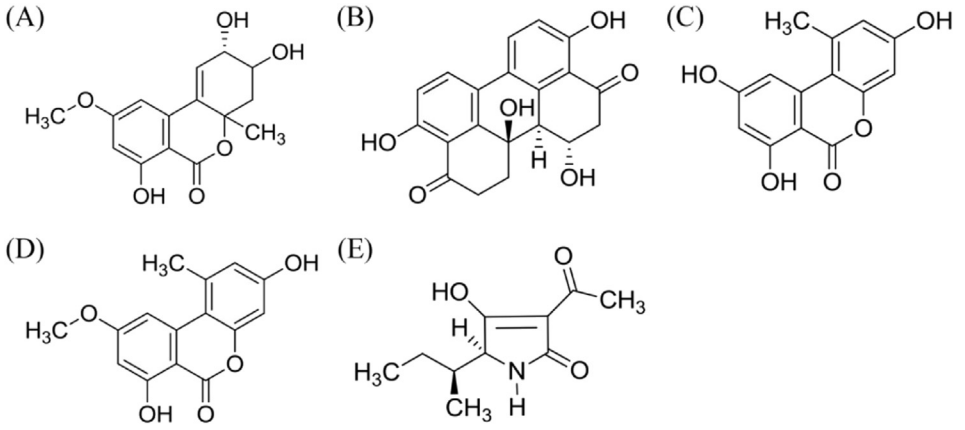

Fig. 1. Chemical structures of *Alternaria* mycotoxins: (A) ALT; (B) ATX-I; (C) AOH; (D) AME; and (E) TeA.

pepper fruits and sesame seeds in Korea produced AOH and AME. 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.