Potential mycotoxin productivity of *Alternaria alternata* isolated from garden trees

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Summary

Potential mycotoxin productivity of *Alternaria alternata* isolated from leaves of *Mube* (*Stauntonia hexaphylla*), *Hanamizuki* (*Cornus florida*), and *Kobushi* (*Magnolia praecocissima*) was investigated with cultures growing on rice medium. By thin-layer chromatography and high-performance liquid chromatography, the presence of alternariol (AOH), alternariol monomethyl ether (AME), and altenuene (ALT) was confirmed in the rice culture extract. *A. alternata* isolate from *Mube* leaves produced AOH, AME, and ALT at concentrations of 22.99 mg/kg, 9.13 mg/kg, and 2.53 mg/kg, respectively. *A. alternata* isolate from *Hanamizuki* leaves produced AOH, AME, and ALT at concentrations of 1.50 mg/kg, 0.59 mg/kg, and 3.42 mg/kg, respectively. *A. alternata* isolate from *Kobushi* leaves produced AOH, AME, and ALT at concentrations of 20.37 mg/kg, 0.95 mg/kg, and 7.25 mg/kg, respectively. These results suggested that *A. alternata* on garden trees contaminate food and feed with the mycotoxins.

Key words : *Alternaria alternata*, *Alternaria* mycotoxins, garden trees

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Introduction

Fungi of the genus *Alternaria* are widely distributed in the environment and are commonly isolated from soil, living plants, decaying plant tissue and food¹⁾. Species in this genus include saprophytes and plant pathogens that are responsible for the spoilage of grain²⁻⁴⁾, fruits⁵⁻⁸⁾ and vegetables⁹⁾ as well as field crops of cereal.

Many *Alternaria* species including *Alternaria alternata* can produce several mycotoxins¹⁰⁾ that belong to three classes of compound: dibenzo- α -pyrones derivatives (alternariol, AOH; alternariol monomethyl ether, AME; and altenuene, ALT), tetramic acid derivatives (tenuazonic acid, TeA) and perylene derivatives (altertoxin I, ATX-I; and the related compounds altertoxins II and III). *Alternaria* mycotoxins have been detected at considerable concentrations in crops and fruits, crop and fruit products including apple juice and tomato products¹¹⁻¹⁴⁾, and animal feed¹⁵⁾.

Alternaria mycotoxins exhibit toxicity to mammalian cells, bacteria and experimental animals^{10,16-27)}. AOH, AME and ATXs are mutagenic to bacteria^{19,21,23,25-27)} and mammalian cells^{17,28,29)}, and AME and

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TeA induced precancerous changes in the esophagus mucosa in mice²⁴). It is suspected that the metabolites of *A. alternata* are associated with human esophageal cancer²⁸⁻³¹).

There have been many studies of the mycotoxigenic characteristics of *Alternaria* species isolated from crops, but few studies of the incidence of *Alternaria* species in garden trees planted in residential areas and their potential mycotoxin productivity. Because garden trees are sometimes planted in gardens near human and animal houses, the presence of mycotoxigenic *Alternaria* species growing on garden trees may lead to spore dispersal from these trees and then to mycotoxin production in food or feed. The objectives of this study are therefore to determine the potential mycotoxin productivity of *A. alternata* isolated from garden trees planted in a residential area.

Materials and methods

Leaf samples and isolation of *Alternaria* Leaves of *Mube* (Japanese staunton vine) (*Stauntonia hexaphylla*) were collected in November, leaves of *Hanamizuki* (Dogwood) (*Cornus florida*) in September and leaves of *Kobushi* (Northern Japanese magnolia) (*Magnolia praecocissima*) in June and November (Table 1). All leaves were collected from the same trees, which grow in a garden of a residential area located in Tsukuba, Japan.

The leaf segments of *Mube*, *Hanamizuki* and *Kobushi* were placed on 2 % agar (Wako Pure Chemicals Industries Ltd., Japan) plates. The plates were incubated at room temperature (22-25 °C) for 5-7 days. They were observed under a stereomicroscope, and spores of *Alternaria* growing from the leaf surface were transferred to a corn meal agar (Nissui, Tokyo, Japan) slant and incubated at room temperature (22-25 °C) for 5-7 days. Then, single-spore isolation was performed to establish isolates of *Alternaria* with 2 % agar plates, and these isolates were maintained on a corn meal agar medium at room temperature (22-25 °C) for morphological examination and metabolite profiling. Thirteen isolates, comprising 3 isolates from different colonies from leaves of *Mube*, and 4 and 6

Table 1. Origins, isolate numbers, collection months and mycotoxin productivities of *A. alternata* isolates studied.

Isolate	Month collected	Origin	Mycotoxin productivity		
			AOH	AME	ALT
M-1	November	<i>Mube</i>	+	+	+
M-2	November	<i>Mube</i>	+	+	+
M-3	November	<i>Mube</i>	+	+	+
H-1	September	<i>Hanamizuki</i>	+	+	+
H-2	September	<i>Hanamizuki</i>	+	+	+
H-3	September	<i>Hanamizuki</i>	+	+	+
H-4	September	<i>Hanamizuki</i>	+	+	+
K-1-1	June	<i>Kobushi</i>	+	+	+
K-2-1	November	<i>Kobushi</i>	+	+	+
K-2-2	November	<i>Kobushi</i>	+	+	+
K-2-3	November	<i>Kobushi</i>	+	+	+
K-2-4	November	<i>Kobushi</i>	+	+	+
K-2-5	November	<i>Kobushi</i>	+	+	+

AOH: alternariol, AME: alternariol monomethyl ether, ALT: altenuene

isolates from *Hanamizuki* and *Kobushi*, respectively, were selected and used in this study. For light microscopic morphological examination of hyphae and spores, these specimens were prepared with a 0.15 % aqueous gelatin solution or lactic phenol solution. Their sporulation pattern cultured on a corn meal agar for 7-14 days was observed with a stereomicroscope.

Culture conditions and extraction procedure For the examination of AOH, AME and ALT productivities of *Alternaria* isolates, each isolate was inoculated into 50 g of milled rice which had been moistened with 25 ml of water after soaked in water for 1 hour and autoclaved for 20 min at 121 °C, and incubated at room temperature (22-25 °C) for 21 days. To obtain a crude rice culture extract, the medium was homogenized with a spatula in 100 ml of methanol, extracted by soaking overnight with the methanol, and then filtered through filter paper (Advantec, Tokyo, Japan). The residues were re-extracted twice with 50 ml of methanol and filtered again. The combined methanol extract was mixed with 200 ml of 20 % ammonium sulfate in a separatory funnel and then filtered. The filtrate was transferred to another separatory funnel and extracted three times with 100 ml of chloroform. The combined chloroform extract was dried over anhydrous sodium sulfate and evaporated to near dryness using a rotary evaporator at 40 °C, and then, completely dried under a stream of nitrogen gas. The resulting residue was dissolved in 3 ml of methanol. The sample solution was analyzed for AOH, AME and ALT by thin-layer chromatography (TLC). Ten-fold dilutions of the sample solution were prepared for high-performance liquid chromatography (HPLC).

TLC The rice culture extracts were screened for AOH, AME, and ALT using a precoated silica gel TLC plate (silica gel 60 F254 Merck Ltd. Japan) by comparing the rate of flow (Rf) values of these mycotoxins with those standards. The solvent systems was toluene:ethyl acetate: formic acid (50:40:10, v/v/v) according to Seitz et al.³²⁾, and detected under UV light at a wavelength of 365 nm.

HPLC Analysis was performed using a Shimadzu LC-6A HPLC system equipped with a Shimadzu SCL-6A system controller, a Shimadzu SCL-6A pump, a Shimadzu SPD-6A UV detector, and a Shimadzu C-R3A integrator. The rice extracts were applied to a Phenomenex column (Prodigy ODS-3, 4.6 mm I.D. × 25 cm, 5 µm particle size, Shimadzu, Japan) with an ODS 3.0 mm I.D. × 4.0 mm Phenomenex guard cartridge column (Shimadzu, Japan) thermostatted at 30 °C in an oven (Shodex oven AO-30). The mobile phase was methanol: water (80:20, v/v) at a flow rate of 0.4 ml/min. Detection was performed at a wavelength of 260 nm, and 10 µL of a sample was injected by a Rheodyne 7125 injector.

The peak area of the extract of the toxin was quantitatively determined by comparing it with the peak area of a standard toxin of known concentration. The recovery of AOH, AME and ALT were 81 %, 81 %, and 88 %, respectively, from 50 g of rice culture spiking at a concentration of 0.5 mg/kg for each toxin. No AOH, AME and ALT were recovered from non-spiked rice culture.

Reagents Standard *Alternaria* mycotoxins including AOH, AME, and ALT were purchased from Sigma Co., Ltd., USA. The reagent-grade solvents used for TLC and the HPLC-grade solvents used for HPLC were purchased from Wako Pure Chemicals Industries Ltd., Japan.

Results and discussion

All the thirteen *Alternaria* isolates from the leaves of *Mube*, *Hanamizuki* and *Kobushi* were morphologically identified as *A. alternata*, and the results of TLC demonstrated that all the isolates of

A. alternata produced AOH, AME and ALT in the rice culture with some different characteristics in terms of the productivities of the metabolites.

Spores of an isolate grown on a corn meal agar are shown in Fig. 1. All isolates from *Mube*, *Hanamizuki* and *Kobushi* produced non-beaked conidia, with were ovoid or obclavate, pale brown to brown, usually branched and found in moderately long chains (approximately 4-10 spores). They exhibited the typical characteristics of *A. alternata*, as defined in the literature³³.

In our experiment, all the thirteen isolates of *A. alternata* produced AOH, AME and ALT during growth in the rice culture similarly to the isolates in other studies that were isolated from maize, oilseed and wheat^(2,4,34,35). Result of the TLC of rice culture extracts (Fig. 2) demonstrated that AOH (Rf,

Fig. 1. Morphology of *A. alternata* spores produced on corn meal agar. Spores of *Hanamizuki* isolate (H-4). Bar=100 μ m

Fig. 2. TLC of extracts from rice culture inoculated with each isolate and standard *Alternaria* mycotoxins, namely, AOH(0.2 μ g), AME(0.2 μ g) and ALT(0.2 μ g). AOH, AME and ALT were produced by all *A. alternata* isolates. Std, standard of *Alternaria* mycotoxins. A, B and C are extracts from the rice culture inoculated with *Mube* isolates of M-1, M-2 and M-3, respectively. D, E, F and G are extracts from the rice culture inoculated with *Hanamizuki* isolates of H-1, H-2, H-3, H-4, respectively. H, I, J, K, L and M are extracts from rice culture inoculated with *Kobushi* isolates of K-1-1, K-2-1, K-2-2, K-2-3, K-2-4 and K-2-5, respectively.

0.59) and AME (Rf, 0.72) were detected as blue-fluorescence spots and ALT (Rf, 0.40) was detected as a greenish-blue fluorescence spot under UV light at a wavelength of 365 nm. Three *Alternaria* isolates randomly selected from each tree isolate were quantitatively analyzed by HPLC. HPLC profiles of rice culture extracts from the three selected isolates are shown in Fig. 3. The peaks of AOH, AME and ALT appeared with retention times of 9.4, 15.4 and 7.7 min, respectively. The concentrations of *Alternaria* toxins in the rice culture are shown in Table 2. *A. alternata* isolate from *Mube* (M-2) had the ability to produce AOH, AME, and ALT at concentrations of 22.99 mg/kg, 9.13 mg/kg, and 2.53

Fig. 3. HPLC profiles of rice culture extracts of *A. alternata* isolated from *Mube*, *Hanamizuki* and *Kobushi*, and standard *Alternaria* mycotoxins, namely, AOH (0.1 mg/L), AME (0.1 mg/L), and ALT (0.1 mg/L). AOH, AME and ALT were detected in the extracts. Std, standard of *Alternaria* mycotoxins; M, extract from rice culture inoculated with *A. alternata* isolated from *Mube* (M-2); H, extract from rice culture inoculated with *A. alternata* isolated from *Hanamizuki* (H-4); K, extract from rice culture inoculated with *A. alternata* isolated from *Kobushi* (K-1-1).

Table 2. Toxin productions by *A. alternata* isolate in rice culture.

Isolates analyzed	Toxin content (mg/kg)			Origin
	AOH	AME	ALT	
M-2	22.99	9.13	2.53	<i>Mube</i>
H-4	1.50	0.59	3.42	<i>Hanamizuki</i>
K-1-1	20.37	0.95	7.25	<i>Kobushi</i>

mg/kg, respectively. *A. alternata* isolate from *Hanamizuki* (H-4) produced AOH, AME, and ALT at concentrations of 1.50 mg/kg, 0.59 mg/kg, and 3.42 mg/kg, respectively. *A. alternata* isolate from *Kobushi* (K-1-1) produced AOH, AME, and ALT at concentrations of 20.37 mg/kg, 0.95 mg/kg, and 7.25 mg/kg, respectively. The production of the toxins in the rice culture resulted in a lower AME concentration than the AOH concentration, and this was similar to the results of previous studies ^{2,4,34,35}.

These results indicate that the leaves of *Mube*, *Hanamizuki* and *Kobushi* are habitats of mycotoxigenic *A. alternata*. These results suggested that *A. alternata* on garden trees contaminate food and feed with the mycotoxins.

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庭木から分離した *Alternaria alternata* のマイコトキシン産生能

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ムベ、ハナミズキおよびコブシの葉から分離した *Alternaria alternata* の米培地上でのマイコトキシン産生能を調査した。薄層クロマトグラフィーと高速液体クロマトグラフィーにより、アルタナリオール、アルタナリオールモノメチルエーテル、アルタヌエンが米培地抽出物中に確認された。ムベから分離した *A. alternata* は米培地上で AOH, AME, および ALT をそれぞれ 22.99 mg/kg, 9.13 mg/kg, および 2.53 mg/kg 産生していた。ハナミズキの葉から分離した *A. alternata* は米培地上で AOH, AME, および ALT をそれぞれ 1.50 mg/kg, 0.59 mg/kg, and 3.42 mg/kg 産生していた。コブシの葉から分離した *A. alternata* は米培地上で AOH, AME, および ALT をそれぞれ 20.37 mg/kg, 0.95 mg/kg, および 7.25 mg/kg 産生していた。これらの結果は庭木由来の *A. alternata* が食物および飼料をマイコトキシンの汚染することを示唆している。

キーワード：*Alternaria*, *Alternaria* マイコトキシン, 庭木