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Short Communication

Isolation of azole-resistant *Aspergillus fumigatus* from imported plant bulbs in Japan and the effect of fungicide treatment

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Increasing numbers of azole-resistant *Aspergillus fumigatus* (AR*Af*) in the environment have become a global public health issue. We surveyed tulip bulbs that were imported from the Netherlands and found that 6.3–15.8% of bulbs were contaminated by AR*Af* with a tandem-repeat mutation in the promoter region of the *cyp51A* gene. We also showed that fungicide treatment of the tulip bulbs by benomyl or prochloraz effectively reduced the rate of isolation. This is the first report demonstrating a method of eliminating human fungal pathogens from plant bulbs.

Keywords: *Aspergillus fumigatus*, azole resistance, plant bulb, fungicide treatment.

Electronic supplementary materials: The online version of this article contains supplementary materials (Supplemental Fig. S1, Table S1 and S2), which are available at http://www.jstage.jst.go.jp/browse/jpestics/.

Introduction

The human pathogenic fungus *Aspergillus fumigatus* is ubiquitously present in the environments, in the soil and air, and is a main causative agent of aspergillosis. This fungal infection can be treated by a limited number of antifungal drugs. Among them, triazole antifungals, itraconazole and voriconazole, are the first-line drugs for the treatment of aspergillosis. In the last decade, however, it has become a global issue that azole-resistant *A. fumigatus* (AR*Af*) strains have been increasingly isolated all over the world not only from patients but also from the environment.[1)] Molecular targets of the medical azoles are Cyp51 proteins, key enzymes in the fungal ergosterol biosynthesis pathway. The main resistance mechanism of AR*Af* is a tandem-repeat (TR) sequence inserted in the promoter region of the *cyp51A* gene. In general, the repeat sequences are 34 or 46 bp (called TR34 or TR46, respectively) in combination with certain amino acid substitutions in the protein.[2)] Recent studies have suggested that TR-type AR*Af* strains were derived from the environment, where *A. fumigatus* had been exposed to azole fungicide residues.[3)] Since the first isolation of TR34-type AR*Af*s in the Netherlands in 1998, TR-type AR*Af*s had been isolated in most Asian countries as well as the American continents.[4)]

In 2017, an Irish research group reported that TR-type AR*Af* was isolated from plant bulbs imported from the Netherlands.[5)] This important report indicated the possibility that problematic AR*Af* strains were being internationally transferred through the overseas trading of a garden plant. Given that plant bulbs produced by Dutch horticultural companies are at high risk for AR*Af* contamination, immediate international action was required to effectively prevent the spread of AR*Af* strains. As is the case for other countries, Japan has imported huge quantities of plant bulbs from the Netherlands. Therefore, it is essential to examine these for AR*Af* contamination. To this end, we purchased tulip bulbs imported from the Netherlands and investigated whether they were contaminated with TR-type AR*Af*. We also aimed to demonstrate that fungicide treatment could eliminate the isolation of AR*Af* strains from plant bulbs.

Materials and Methods

1. Plant bulbs

Several kinds of tulip bulbs were purchased in two different stores (Store D and Store Y) in 2018 and 2019 in Japan. All tulip bulbs were imported from the Netherlands.

2. Fungal isolation

For the isolation of *A. fumigatus* from tulip bulbs, Potato Dextrose Agar (PDA, Becton Dickinson, NJ, USA) supplemented with 10 μg/mL fluconazole (for preventing fungal growth other than *Aspergillus* species), 10 μg/mL chloramphenicol, and 100 μg/mL penicillin G (for preventing bacterial growth) were used. The

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bulbs were divided into two pieces by a disinfected knife. They were placed onto the PDA plate and incubated at 45°C for 3 days. The visible and apparent fungal colonies were transinoculated to new PDA plates and incubated at 45°C for 2 days.

3. *Sequencing of the* cyp51A *promoter region and ORF*

The promoter region (approximately 500 bp) of the *cyp51A* gene was amplified by colony PCR using a set of primers Pcyp51A-F(-500) and Pcyp51A-R (Supplemental Fig. S1 and Table S1). GoTaq Green Master Mix (Promega, Madison, WI, USA) or KOD One PCR Master Mix Blue (Toyobo, Osaka, Japan) was used for amplification. The amplified targeted fragments were purified and sequenced by the Sanger method, using either of the above primers. For full-length sequencing the *cyp51A* gene, a set of primers Pcyp51A-F(-500) and cyp51A-R were used to amplify the ORF, and cyp51A-SQ1, cyp51A-SQ2, and cyp51A-SQ3 were used for sequencing (Supplemental Table S1). The reference sequence was retrieved from the *A. fumigatus* A1163 genome.

4. *Antifungal susceptibility test*

MICs of each isolate against voriconazole were determined on PDA using an E-test strip (Biomerieux Japan LTD., Tokyo, Japan) in accordance with the manufacturer's instructions. Minimum inhibitory concentrations (MICs) for itraconazole (Tokyo Chemical Industry Co. Tokyo, Japan), prochloraz (Tokyo Chemical Industry Co.), and benomyl (Wako Pure Chemical Industries, Osaka, Japan) were investigated by the broth microdilution method using Potato Dextrose Broth (Becton Dickinson). The

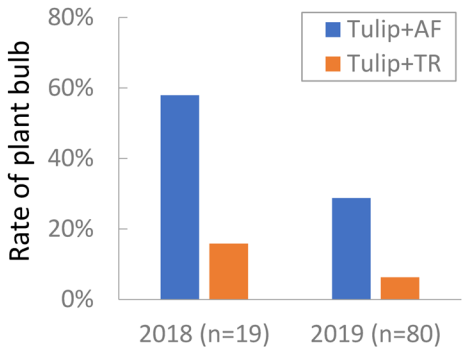

Fig. 1. The rate of tulip bulb contamination with *A. fumigatus* and TR-type AR*Af*. AF indicates *A. fumigatus* and TR indicates TR-type AR*Af*.

Table 1. The *A. fumigatus* strains isolated from tulip bulbs that were bought in 2018

Bulb ID	Strain ID	TR-type[a)]	AA substitutions in Cyp51A (vs A1163)[a)]	Microsatellite[a)]									MIC [mg/L][a)]			
				2A	2B	2C	3A	3B	3C	4A	4B	4C	VRCZ (E-test)	ITCZ	Prochloraz	Benomyl
1	1-1-B	TR46	Y121F, T289A, S363P, I364V, G448S	25	20	12	45	9	11	8	10	18	>32	>32	25	50
2	1-3-A	nd	nd	—	—	—	—	—	—	—	—	—	0.25	0.25	—	—
3	2-1-A	nd	nd	—	—	—	—	—	—	—	—	—	0.094	0.125	—	—
4	2-3-A	nd	nd	—	—	—	—	—	—	—	—	—	0.094	0.064	—	—
5	3-1-A	TR46	Y121F, T289A	10	20	8	44	9	10	8	10	7	>32	0.5	6.25	50
5	3-1-B	TR34	L98H, T289A, I364V, G448S	23	10	9	35	9	6	8	10	18	>32	>32	12.5	50
5	3-1-C	TR34	L98H, T289A, I364V, G448S	23	10	9	35	9	6	8	10	18	>32	>32	12.5	50
5	3-1-D	TR46	Y121F, T289A, S363P, I364V, G448S	24	20	12	45	9	11	8	10	18	>32	>32	12.5	50
5	3-1-E	TR46	Y121F, T289A	26	20	12	36	9	22	8	14	31	>32	>32	12.5	50
5	3-1-F	TR46	Y121F, T289A	25	20	12	45	11	6	10	12	18	>32	>32	6.25	50
5	3-1-G	TR46	Y121F, T289A	23	10	9	36	9	6	12	10	7	>32	>32	6.25	50
5	3-1-H	nd	nd	—	—	—	—	—	—	—	—	—	0.064	0.25	—	—
6	3-3-A	TR46	Y121F, T289A	25	20	12	44	9	6	12	10	7	>32	0.5	6.25	50
6	3-3-B	TR46	Y121F, T289A	25	21	12	45	9	6	12	10	7	>32	0.25	6.25	>50
6	3-3-C	TR46	Y121F, T289A	25	20	9	10	9	11	14	9	18	>32	0.25	3.125	50
7	4-2-A	nd	nd	—	—	—	—	—	—	—	—	—	0.125	0.064	—	—
8	5-1-A	nd	nd	—	—	—	—	—	—	—	—	—	0.064	0.064	—	—
9	5-3-A	nd	nd	—	—	—	—	—	—	—	—	—	0.125	0.064	—	—
9	5-3-B	nd	nd	—	—	—	—	—	—	—	—	—	0.125	0.064	—	—
9	5-3-C	nd	nd	—	—	—	—	—	—	—	—	—	0.064	0.064	—	—
10	6-1-A	nd	nd	—	—	—	—	—	—	—	—	—	0.094	0.064	—	—
11	7-1-A	nd	nd	—	—	—	—	—	—	—	—	—	0.094	0.064	—	—
	IFM 62541	—	—	—	—	—	—	—	—	—	—	—	0.094	0.25	0.1	>50

[a)] "nd" indicates not detected. "—" indicates not determined. VRCZ: Voriconazole; ITCZ: itraconazole

Table 2. The *A. fumigatus* strains isolated from tulip bulbs that were bought in 2019

Bulb ID	Store ID	Strain ID	TR[a)]
1	Y	Y1	nd
2		Y2-1	nd
2		Y2-2	nd
3		Y3-1	TR34
3		Y3-2	nd
4		Y4-1	nd
4		Y4-2	nd
5		Y5	nd
6		Y6	nd
7		Y7	TR46
8		Y8	nd
9		Y9	nd
10		Y10	nd
11		Y11-1	nd
11		Y11-2	nd
12	Y	R1	nd
13		R2	nd
14		R3	nd
15		R4	TR46
16		R5	nd
17		R6	TR34
18	Y	YY1-1	TR46
18		YY1-2	TR46
19		YY2	nd
20		YY3	nd
21	D	P1-1	nd
21		P1-2	nd
22		P2-1	nd
22		P2-2	nd
23	D	W1	nd

[a)] "nd" indicates not detected.

plates were incubated at 37°C for 20 to 24 hr before judging the growth inhibition. Tests were performed in triplicate. IFM 62541 was used as a control for drug susceptibility and was provided by the National BioResource Project.

5. Microsatellite genotyping

Nine microsatellite regions of approximately 400 bp were amplified by PCR using previously described primer sets and sequenced by the Sanger method.[6)] The repeat number in each locus was counted from the sequence.

6. Fungicide treatment

Three hundred tulip bulbs were purchased in 2019. Thirty tulip bulbs were sunk in 2 L of diluted Sportac (25% prochloraz; 1 : 200) (Nissan Chemical Corporation, Tokyo, Japan) or Benlate (50% benomyl; 1 : 500) (Sumika Agro Manufacturing LTD., Yamaguchi, Japan) at room temperature for 30 min. They were then placed on a pile of newspaper for 30 min to remove fungicide residues. After fungicide treatment, the bulbs were cut into two pieces and incubated on PDA supplemented with fluconazole, chloramphenicol, and penicillin G at 45°C for 3 days. The control was treated with water in the same manner. For the isolated strains, the *cyp51A* promoter region amplification and TR sequence were analyzed by the same method. Fungicide treatment was performed in triplicate.

Results and Discussion

From 11 of 19 tulip bulbs (57.9%) purchased in 2018, *A. fumigatus* was isolated and morphologically and genetically identified (Fig. 1, Table 1). In some bulbs, multiple strains were isolated. The promoter region of the *cyp51A* gene was sequenced for all isolates, and it was revealed that 3 bulbs were contaminated with *A. fumigatus* harboring TR (Fig. 1, Table 1). All TR strains showed resistance to voriconazole (VRCZ), while some strains were susceptible to itraconazole (ITCZ) (Table 1). Then, *cyp51A* ORF was sequenced for strains with TR. Either typical combinations of TR and amino acid substitution, TR34/L98H or TR46/Y121F/T289A, were detected in the strains (Table 1). It is also notable that T289A was present in all TR strains, and there were additional amino acid substitutions in some strains. To gain insight into genetic phylogeny, microsatellites were investigated in the TR-type strains. As a result, 3-1-B and 3-1-C strains showed identical patterns of repeat numbers, whereas 1-1-B and 3-1-D strains were closely related (Table 1). It was recently reported that TR-type AR*Af* strains are unevenly distributed around the world, which suggests a rapid distribution of specific lineages with the TR mutation.[7)] Notably, TR strains isolated from plant bulbs in the present study showed microsatellite patterns similar to those of previously reported clinical and environmental TR strains (Supplemental Table S2).[8)] These data suggest that AR*Af* strains from plant bulbs are not special and can be an infectious agents for humans.

In 2019, 80 tulip bulbs were purchased at two different stores

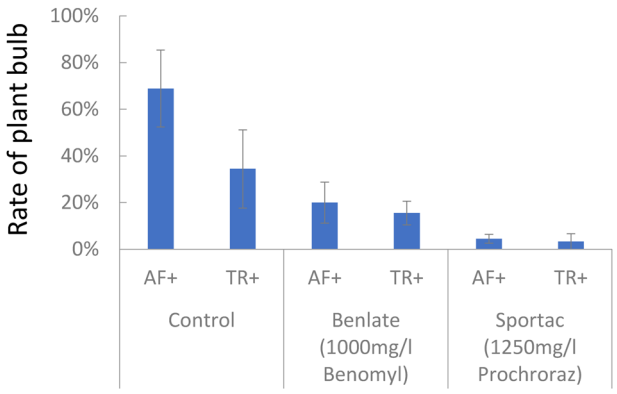

Fig. 2. The rate of tulip bulb contamination with *A. fumigatus* and TR-type AR*Af* after fungicide treatment. AF indicates *A. fumigatus* and TR indicates TR-type AR*Af*. The experiment was performed in triplicate. The error bar indicates the standard deviation.

and investigated for *A. fumigatus* contamination. Among them, 23 bulbs (28.8%) were contaminated with *A. fumigatus*, and 6 TR-type AR*Af* strains were isolated from 5 bulbs (Bulb IDs: 3, 7, 15, 17, and 18 in Table 2). A survey of two continuous years showed that TR-type AR*Af* was isolated from tulip bulbs that were imported from the Netherlands to Japan.

In tulip bulbs for planting, benomyl and prochloraz are approved for disinfection to prevent tulip bulb rot caused by *Fusarium oxysporum* f. sp. *tulipae*. Therefore, we tried to investigate whether fungicide treatment could minimize the risk of AR*Af* being disseminated into the soil when bulbs are planted. First, an *in vitro* drug susceptibility test for benomyl and prochloraz was performed with TR-type AR*Af* isolates (Table 1). All of the TR-type isolates showed a higher resistance to prochloraz (≧3.125 mg/L) as compared with the control strain IFM 62541 (0.1 mg/L), whereas the MICs for benomyl were largely comparable. When bulbs were treated with benomyl (Benlate) or prochloraz (Sportac), the number of bulbs with *A. fumigatus* isolation was reduced as compared with the mock treatment (Fig. 2). Accordingly, the bulbs with TR-type AR*Af* also decreased. As compared with Benlate, Sportac treatment seemed more effective in eliminating the fungal strains.

In our survey, TR-type AR*Af* was isolated from imported tulip bulbs at a specific rate. This is the second report pointing out that AR*Af* can be disseminated by international trade. As *A. fumigatus* can produce tons of hydrophobic conidia, conidial diffusion in the air may be regarded as one of the main channels for intercontinental transfer of the AR*Af* strain. A more intensive and wider survey is needed to understand the real situation and to act against AR*Af* contamination.

The present study demonstrated a potent way of reducing the urgent public health risk. Treatment with Sportac drastically reduced the rate of contaminated bulbs, although TR strains showed high MICs for prochloraz *in vitro* as compared with that for the control strains. The real concentration of prochloraz in diluted fungicide solutions used for treatment is calculated as 1250 mg/L, which is much higher than the MIC values in TR-type strains (3.125–25 mg/L). This could explain why AR*Af* was eliminated by fungicide treatment.

This study is the first demonstration of an effective method of disinfecting plant bulbs contaminated with human pathogenic fungi. However, the overuse of fungicides may lead to the emergence of resistance in *A. fumigatus* as well as the dry rot pathogen, *F. oxysporum* f. sp. *tulipae*. Protecting from both plant and human pathogenic fungi is important; thus, further studies are required to establish an appropriate fungicide-application strategy with low risk for resistance.

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