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Antifungal activity of commercial disinfectants against a benomyl-tolerant strain of *Trichoderma harzianum*

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Abstract A benomyl-tolerant strain (94110Tr) of *Trichoderma* spp. was isolated from airborne fungi in a growing room for sawdust-based cultivation of *Lentinula edodes* (Berk.) Pegler in Hokkaido. The strain was identified as *T. harzianum* Rifai, so the antifungal activity of benomyl (BEN), thiabendazole (TBZ), benzalkonium chloride (BAC), chlorhexidine digluconate (CHG), and glutaraldehyde (GLA) were tested against six strains of *T. harzianum*, including 94110Tr. It was found that 94110Tr was more resistant than the others not only to BEN but also to TBZ, but it had no explicit tolerance to BAC, CHG, or GLA, similar to the other strains. CHG and GLA showed potent antifungal activity against most of the six strains. It is estimated that CHG and GLA are available for disinfection of the benomyl-tolerant strains of *T. harzianum* found in edible-mushroom factories.

Key words *Trichoderma harzianum* · Benomyl tolerance · Antifungal activity · Commercial disinfectant

Introduction

We reported that a strain of *Trichoderma* spp. (94110Tr) was tolerant to benomyl (BEN).¹ The strain was isolated from airborne fungi in a growing room for sawdust-based cultivation of *Lentinula edodes* (Berk.) Pegler in Hokkaido. The strain 94110Tr was identified as *T. harzianum* Rifai (M. Komatsu, 1996, personal communication). BEN was

an excellent fungicide for disinfecting *Trichoderma* spp. which is a pathogenic fungus found during edible-mushroom cultivation.²⁻⁶

This study was undertaken to investigate the antifungal activities of several commercial disinfectants against six strains of *T. harzianum* including 94110Tr. The purpose of this investigation was to screen the fungicides available for controlling benomyl-tolerant fungi such as 94110Tr. The disinfectants tested in this study were BEN, thiabendazole (TBZ), benzalkonium chloride (BAC), chlorhexidine digluconate (CHG), and glutaraldehyde (GLA). TBZ and BEN were classified as the benzimidazole fungicide group, and TBZ was available for control of *Trichoderma* spp. as was BEN.^{4,6} BAC, CHG, and GLA were classified into fungicide groups different from benzimidazole according to their chemical structures. BAC, CHG, and GLA are commonly used today in the food industry and medical institutions. In particular, CHG is widely used to disinfect not only skin but also medical treatment apparatuses and the hospital environment.⁷

Experimental

The six strains of *T. harzianum* (93130Tr, 93136Tr, 93156Tr, 94110Tr, IFO 30718, and IFO 31292) were used in the assays. The 93130Tr and 93136Tr strains were isolated from sawdust-based beds of *L. edodes* in Hokkaido. The 93156Tr and 94110Tr strains were isolated from airborne fungi in the growing rooms of *L. edodes*. These four strains were identified as *T. harzianum* (M. Komatsu, 1996, personal communication). All of the strains were maintained on PDA plates sealed with Parafilm to protect them from contamination and excess drying at 5°C.

The following commercial disinfectants were used: BEN (50%, w/w, wettable powder, Benlate; Du Pont Japan, Tokyo), TBZ (10%, w/v, solution, Bioguard; Hokukoh Chemical Industries, Tokyo), BAC (10%, w/v, solution, Osuban; Nihon Seiyaku K.K., Tokyo), CHG (5%, w/v, solution, Hibitane; Zeneca Yakuhin K.K., Osaka), and GLA

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(25%, w/v, solution, extra pure reagent; Nakalai Tesque, Kyoto). Appropriate amounts of the commercial disinfectants were mixed with potato-dextrose-agar (PDA), autoclaved, and cooled below 60°C; assay agar plates were prepared in petri dishes 90mm in diameter. *T. harzianum* inocula (mycelium plugs 5 mm in diameter) were obtained from the periphery of 4-day-old colonies incubated on PDA plates 9cm in diameter at 25°C. An inoculum then was placed in the center of each assay plate with or without (control) disinfectants. All dishes were sealed with Parafilm, and the mycelial growth from the edge of the inoculum to the front of the fungal colony was measured after 1–10 days of incubation at 25°C in 200–350lx with three replicates.

Results and discussion

The antifungal activity of the benzimidazole fungicides BEN and TBZ against *T. harzianum* are shown in Table 1. The mycelial growth areas on control PDA plates were at least 24.4mm for 2 days. The mycelial growth of 94110Tr exceeds the edge of the PDA plate amended with 0.01% (w/v) BEN for 5 days, and the mycelial growth was 36.8mm on the PDA plate amended with 0.01% (w/v) TBZ for 10 days. The mycelial growth of the other strains was clearly inhibited by BEN and TBZ, with growth ranges of 0–5.1 mm and 0–3.3mm for 10 days, respectively. The mycelial growth of 93156Tr, IFO 30718, and IFO 31292 was completely inhibited by BEN, TBZ, or both. These findings suggest that 94110Tr has crossover tolerance, and the other strains were susceptible to BEN.

The antifungal activity of commercial disinfectants against *T. harzianum* are shown in Table 2. The concentra-

tion of the mixture of BAC (0.05%, w/v) and BEN (0.05%, w/v) corresponds to that of environment disinfection during sawdust-based mushroom cultivation. CHG at a concentration of 0.05–0.1% (w/v) is used to disinfect hospital environments and medical treatment apparatuses. A GLA concentration of 0.2% (w/v) is approximately equal to the minimum inhibitory concentration (MIC) of *Penicillium* spp. isolated from mushroom cultivation factories in Hokkaido.⁸ The mycelial growth ranges of the six strains on the plate amended with 0.05% (w/v) BAC were 13.2 mm or less for 7 days. BEN inhibited the mycelial growth of all strains (except 94110Tr) more than BAC, but BAC inhibited that of 94110Tr more than BEN at 0.05% (w/v). The mycelial growth of 94110Tr exceeded the edge of the plate amended with 0.05% (w/v) BEN as well as 0.01% (w/v) BEN for 5 days. On the other hand, the mycelial growth of 94110Tr was inhibited by the mixture of BAC and BEN; the mycelial growth range was 7.7mm on the plate amended with the mixture for 7 days. Furthermore the antifungal activity of the mixture against 93130Tr and 93136Tr was stronger than that of independent utilization. Therefore, it can be considered that there is synergism of the antifungal activity in the mixture. CHG exhibited potent antifungal activity against all strains; the mycelial growth ranges were 1.8–3.8mm at 0.05% (w/v) and 1.3–2.2mm at 0.1% (w/v) for 7 days. GLA strongly inhibited the mycelial growth of most of the six strains; inhibition of 93156Tr and 94110Tr was not observed.

The 94110Tr strain had no explicit tolerance to BAC, CHG, GLA, or others. CHG and GLA exhibited a clear inhibitory effect on the strain. Therefore, it is estimated that CHG and GLA are effective for controlling benomyl-tolerant strains of *T. harzianum* distributed in edible-mushroom factories. In general, the action points and

Table 1. Effects of benzimidazole fungicides on mycelial growth of *Trichoderma harzianum*

Fungicide (concentration) and incubation period	Mycelial growth (mm), mean ± SEM, by strain of <i>Trichoderma harzianum</i>					
	93130Tr	93136Tr	93156Tr	94110Tr	IFO 30718	IFO 31292
Control						
1 day	7.7 ± 0.34	5.5 ± 0.27	9.4 ± 0.31	8.0 ± 0.21	16.2 ± 0.29	14.5 ± 0.04
2 days	30.8 ± 0.33	24.4 ± 0.97	33.1 ± 0.62	26.1 ± 0.90	36.1 ± 0.19	35.4 ± 0.41
3 days	F	F	F	F	F	F
5 days	F	F	F	F	F	F
7 days	F	F	F	F	F	F
10 days	F	F	F	F	F	F
Benomyl (0.01%, w/v)						
1 day	1.5 ± 0.00	1.2 ± 0.15	0	7.7 ± 0.19	0	0
2 days	–	–	–	21.7 ± 0.44	0	0
3 days	3.5 ± 0.08	3.0 ± 0.13	0.6 ± 0.26	37.8 ± 0.18	0	0
5 days	4.2 ± 0.15	3.8 ± 0.04	0.6 ± 0.26	F	0	0
7 days	4.6 ± 0.06	4.3 ± 0.00	0.8 ± 0.29	F	0	0
10 days	5.1 ± 0.05	4.9 ± 0.07	0.8 ± 0.29	F	0	0
Thiabendazole (0.01%, w/v)						
3 days	0	0	0	17.3 ± 0.44	0	0
5 days	0	0	0	27.0 ± 0.78	0	0
7 days	1.8 ± 0.12	1.2 ± 0.36	0	31.7 ± 0.87	0	0
10 days	2.5 ± 0.36	3.3 ± 0.36	0	36.8 ± 0.70	0	0

F, mycelial growth exceeds the edge of the plate (90mm in diameter) and was not measured; O, no growth; –, not measured.

Mycelial growth is that of six strains (three replicates per strain) at 25°C on PDA (potato-dextrose-agar) amended with the respective fungicides

Table 2. Effects of commercial disinfectants on mycelial growth of *Trichoderma harzianum* for 7 days at 25°C

Disinfectant (concentration)	Mycelial growth (mm), mean \pm SEM, by strain of <i>Trichoderma harzianum</i>					
	93130Tr	93136Tr	93156Tr	94110Tr	IFO 30718	IFO 31292
BAC (0.05%, w/v)	13.2 \pm 0.42	13.1 \pm 0.25	12.4 \pm 0.57	12.4 \pm 0.00	2.6 \pm 0.15	5.9 \pm 0.07
BAC (0.05%, w/v) + BEN (0.05%, w/v)	0	0	0	7.7 \pm 0.22	0	0
BEN (0.05%, w/v)	2.8 \pm 0.11	2.6 \pm 0.26	0	F	0	0
CHG (0.05%, w/v)	3.6 \pm 1.15	1.8 \pm 0.33	2.9 \pm 0.04	3.8 \pm 0.25	1.8 \pm 0.11	2.2 \pm 0.11
CHG (0.1%, w/v)	2.0 \pm 0.12	1.6 \pm 0.27	1.3 \pm 0.04	2.2 \pm 0.04	1.7 \pm 0.17	2.1 \pm 0.29
GLA (0.2%, w/v)	3.5 \pm 0.26	4.5 \pm 0.04	0	0	3.9 \pm 0.27	13.8 \pm 0.18

BAC, benzalkonium chloride; BEN, benomyl; CHG, chlorhexidine digluconate; GLA, glutaraldehyde

See Table 1 for explanations

antifungal reactions of benzimidazole (BEN and TBZ), BAC, CHG, and GLA are different. Whereas benzimidazole inhibits DNA synthesis of fungi, BAC and CHG attack the cell membrane, and GLA attacks the cell wall of microorganisms. It is thought that 94110Tr has a resistance to benzimidazole.

A benomyl-tolerant strain of *T. harzianum* has been reported.⁹ The strain was a ultraviolet-irradiated mutant, whereas 94110Tr was isolated from a growing room for *L. edodes*. The growing room had been disinfected with benzimidazole (BEN and TBZ) for more than 3 years. These findings suggest that benzimidazole influences the development of the benomyl-tolerant strain 94110Tr.

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