Full Paper

Title

Growth characteristics of *Aspergillus oryzae* in the presence of 2,4,6-trichlorophenol

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Names of authors

Michiko Endo,¹ Chika Matsui,¹ Naomi Maeta,¹ Yurina Uehara,¹ Ryoya Matsuda,¹ Yuzo Fujii,¹ Akiko Fujita,^{2,*} Tsutomu Fujii,^{2,3} and Osamu Yamada²

Institutions

¹ *Department of Materials Science, Yonago College, National Institute of Technology, Yonago, Tottori 683-8502, Japan*

² *National Research Institute of Brewing, Higashi-hiroshima, Hiroshima 739-0046, Japan*

³ *Present address: Faculty of Food and Agricultural Sciences, Fukushima University, Fukushima, Fukushima 960-1296, Japan*

Running head

Effect of TCP on growth of a fungus

*Corresponding author: Akiko Fujita, National Research Institute of Brewing, 3-7-1 Kagamiyama, Higashi-hiroshima, Hiroshima 739-0046, Japan.

E-mail: fujita@nrib.go.jp

Summary

During the making of rice-*koji* for sake production, 2,4,6-trichlorophenol (TCP) is *O*-methylated to 2,4,6-trichloroanisole (TCA) by the *koji*-mold, *Aspergillus oryzae*, resulting in a musty/moldy off-odor, which significantly reduces the quality of sake. Thus, we aim to develop *A. oryzae* strains with a less-efficient ability to produce TCA. TCP is a fungicide that suppresses the growth of fungi, whereas TCA does not. The exact effects of TCP on the growth of *A. oryzae* are unknown. However, it is assumed that a strain with low TCP conversion ability will be sensitive to TCP concentration. In this study, we investigated the effects of the different concentrations of TCP on the growth suppression of *A. oryzae*. As the TCP concentration in the media increased, the growth rate, and conidia formation of *A. oryzae* slowed down. No growth was observed in liquid culture (for 1 day at 30°C) containing more than 30 µg/mL of TCP and in agar culture (for 7 days at 30°C) containing more than 50 µg/mL of TCP. However, *A. oryzae* was able to grow on alpha rice containing higher concentrations of TCP. The results in agar culture are consistent with the effects of TCP on other *Aspergillus* species.

Key Words: *Aspergillus oryzae*; fungicide; methylation; 2,4,6-trichloroanisole; 2,4,6-trichlorophenol

Introduction

2,4,6-Trichlorophenol (TCP) is toxic to fungi and inhibits their growth. It was formerly used as a fungicide for wood, and it may also be produced during chlorination of drinking water and wood by chlorinated disinfectants, such as sodium hypochlorite (Karlsson et al., 1995; Iwata et al., 2007). In the environment, fungi are able to detoxify chlorophenols *via* *O*-methylation, producing chloroanisole (Cserjesi and Johnson, 1972); however, fungal

growth is inhibited at high concentrations of chlorophenols. The antifungal activity of chlorophenols may also depend on their chemical structures and the target species of fungi (Ruckdeschel and Renner, 1986).

2,4,6-Trichloroanisole (TCA), which is the main cause of cork taint of wines, is produced when fungi growing on cork stoppers *O*-methylate TCP in cork (Álvarez-Rodríguez et al., 2002). Sake is a traditional alcoholic beverage made from the following main components: steamed rice, cultured *koji*-mold grown on the steamed rice grains (rice-*koji*), water, and yeast. The development of a musty/moldy off-odor in sake is a serious problem for sake makers. The cause of such odor, TCA, is mainly produced by the biomethylation of TCP, which originates from the wooden tools utilized for making rice-*koji* (Miki et al., 2005). To prevent TCA formation, the use of TCP-containing wooden tools and chlorine disinfectants should be avoided (Iwata et al., 2007). In addition, a strain of *koji*-mold (*Aspergillus oryzae*) with a weakened ability to methylate TCP is desirable.

Previously, we found a primary *O*-methyltransferase gene (AO090010000551 (alias AO080521000231), *omtT*) involved in the conversion of TCP to TCA in rice-*koji* making with *A. oryzae*. We demonstrated that the ability to convert TCP to TCA in the *omtT* disruptant was much lower than that of a non-disrupted transformant (Endo et al., 2011). Strains with low TCP detoxification ability are assumed to be sensitive to TCP; however, the effects of TCP on the growth of wild type *A. oryzae* are unknown. In this study, we cultured *A. oryzae* in liquid, agar, and solid media containing TCP and observed the growth inhibition in the presence of TCP.

Materials and Methods

Strain and media. The strain used in this study, *A. oryzae* RIB 40, is a representative strain

whose genome has been previously sequenced (Machida et al., 2005) and stored at the National Research Institute of Brewing (Hiroshima, Japan). Czapek-Dox medium (0.3% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, 0.001% FeSO₄·7H₂O, 3% glucose; pH6.0) and rice-*koji* extract (RKE) were used as the culture media. RKE was prepared by mixing 1 kg of dry *koji* made from rice with a rice-polishing ratio of 70% (weight) (Tokushima Seikiku, Tokushima, Japan) with 3 L of tap water. The mixture was incubated at 60°C overnight followed by filtration through qualitative filter paper, No. 2 (Advantec, Tokyo, Japan). The Brix value (the percentage of sugar content) of RKE was measured using a PAL-J portable refractometer (ATAGO, Tokyo, Japan). To make liquid and agar media, RKE was diluted with water to Brix values of 2% and 10%, respectively. TCP (Sigma-Aldrich, MO, USA) was added to the media as necessary.

Liquid media cultures. To investigate the effects of high concentrations of TCP on growth of *A. oryzae*, 10⁶ conidia/mL of *A. oryzae* RIB 40 were added to a 100-mL baffled Erlenmeyer flask containing 30 mL of Czapek-Dox or RKE (Brix 2%) and different concentrations of TCP (0–40 µg/mL). The mixtures were shaken at 130 rpm for 24 h at 30°C. To investigate the effects of lower concentrations of TCP, 100 conidia/mL of *A. oryzae* RIB 40 were cultured in Czapek-Dox for 48 h or in RKE (Brix 2%) for 24 h. Both media contained 0–10 µg/mL of TCP. After the specified time, the culture media were filtered through a membrane filter (pore size, 0.45 µm), and the collected mycelia were washed with distilled water and dried at 60°C to achieve a constant dry weight; the dry weight was then measured.

Agar media cultures. Three thousand conidia of *A. oryzae* were inoculated onto the center of a petri dish (diameter, 5 cm) containing Czapek-Dox or RKE (Brix 10%) media with 2%

agar and 0–50 µg/mL of TCP. The cultures were grown at 30°C for 7 days, after which the mycelial growth and conidia formation of each culture were observed using photography.

Rice cultures (rice-koji making method). About 20 g of pregelatinized rice (α -rice) with a rice-polishing ratio of 70% (weight) (Tokushima Seikiku) was placed into a 200-mL Erlenmeyer flask, dry-heat sterilized at 95°C for 3 h, and then cooled. 2.9×10^6 conidia/mL of *A. oryzae* conidia were suspended in a 0.05% Tween 80 solution containing 0–300 µg/mL of TCP, and then 7 mL of the suspension was spread over the sterilized α -rice. The final concentration of *A. oryzae* conidia was approximately 1 million conidia per g of α -rice. The flask was placed in a CRB-14A incubator (Nihon Freezer, Tokyo, Japan) connected to an NP408-00 ultrasonic humidifier and an HS1-1 humidity sensor (Nippo, Saitama, Japan) and incubated at 35°C and 80% humidity for 44 h for rice-*koji* making. The flask was covered with aluminum foil to prevent TCP and TCA from scattering, and the lid was opened at 1, 6, 24, and 30 h to replenish the oxygen supply and agitate the mixture.

The enzymes were extracted from rice-*koji* according to the standard analytical method of the National Research Institute of Brewing, Japan (standard analytical method 111-5-2; <https://www.nrib.go.jp/bun/pdf/bun/nb111.pdf>). α -Amylase activity was measured using α -Amylase Assay Kit (Kikkoman Biochemifa, Tokyo, Japan). The grown mycelial content was calculated by first determining the amount of *N*-acetylglucosamine in rice-*koji* (Reissig et al., 1955). *N*-acetylglucosamine was extracted from rice-*koji* using a commercial cell wall lytic enzyme, Yatalase (Ozeki, Hyogo, Japan), according to a previously published protocol (Fujii et al., 1992).

Analysis of mineral concentrations in α -rice and RKE. The mineral concentrations in

α -rice and RKE (Brix 10%) were analyzed using inductively coupled plasma atomic emission spectrometry (ICP-AES, ICPS-9000E, Shimadzu, Kyoto, Japan) after wet-ashing them using a microwave digestion system (MLS-1200 MEGA, Milestone, Bergamo, Italy) according to the procedure described by Okuda et al. (2014, 2015).

Results and Discussion

Effect of TCP on the mycelial growth of A. oryzae in liquid media

The presence of TCP in the growth media inhibits the growth of *A. fumigatus* and *A. niger* (Ruckdeschel and Renner, 1986); however, the growth characteristics of *A. oryzae* in the presence of TCP is unknown. We first observed the effects of 0–40 $\mu\text{g/mL}$ of TCP in the liquid culture media on the growth of *A. oryzae* RIB 40 (Fig. 1A). Twenty-four hours after inoculating 10^6 conidia/mL, we observed that fungal growth inhibition was dependent on TCP concentration. Fungal growth was significantly inhibited in Czapek-Dox containing 10 $\mu\text{g/mL}$ of TCP and in RKE containing 30 $\mu\text{g/mL}$ of TCP. No mycelial growth was observed in both media containing more than 30 $\mu\text{g/mL}$ of TCP after 7 days of culture (data not shown). Álvarez-Rodríguez et al. (2002) reported that the growth of *Trichoderma longibrachiatum* (another fungus responsible for the cork odor in wine) was inhibited when cultured in liquid media containing 10 $\mu\text{g/mL}$ of TCP for up to about 30 h. However, growth resumed after the concentration of TCP was reduced as it was converted to TCA. It is likely that *A. oryzae* grows as it converts low concentrations of TCP to non-toxic TCA. However, high concentrations of TCP inhibit the growth of *A. oryzae*.

The effects of low concentrations of TCP on the growth of *A. oryzae* conidia (initial inoculum size = 10^6 conidia/mL) are presented in Fig. 1B and 1C. Compared with media without TCP, mycelial weights were significantly lower ($p < 0.05$) in Czapek-Dox containing more than 0.05 $\mu\text{g/mL}$ of TCP and RKE containing more than 0.5 $\mu\text{g/mL}$ of

TCP. The relative weight of mycelia growing in RKE containing 2 µg/mL of TCP was much lower than that in RKE containing 1 µg/mL of TCP.

Effect of TCP on mycelial growth and conidia formation of A. oryzae on agar media

The results in agar media were similar to those in liquid media, i.e., media with more TCP resulted in higher levels of inhibition of mycelial growth and conidia formation in *A. oryzae* (Fig. 2A). No mycelia were observed on Czapek-Dox and RKE containing 50 µg/mL of TCP after 7 days of culture. In *A. fumigatus* and *A. niger* growing on Mueller-Hinton agar, mycelial growth is completely inhibited at TCP levels of 0.25 µmol/mL (about 50 µg/mL) and 0.5 µmol/mL (about 100 µg/mL), respectively, at 22°C for 10 days (Ruckdeschel and Renner, 1986). Thus, our results are consistent with the effects of TCP on other species of *Aspergillus*.

When *A. oryzae* was cultured with levels of less than 10 µg/mL of TCP, we observed mycelial growth inhibition on media with as low as 2 µg/mL of TCP (Fig. 2B). At up to 6 µg/mL of TCP, more mycelial growth occurred on RKE than on Czapek-Dox. At greater than 8 µg/mL of TCP, smaller colonies were observed on RKE compared with those on Czapek-Dox. Previous research suggests that microbial *O*-methylation of TCP to TCA is affected by divalent metal cations (i.e., Mn^{2+} and Mg^{2+}), TCP concentration, temperature, and pH (Zhang et al., 2016). Czapek-Dox contains 49 µg/mL of Mg^{2+} , whereas RKE contains only 1 µg/mL of manganese and 9 µg/mL of magnesium. Thus, for *A. oryzae* growing on RKE with high concentrations of TCP, the supply of divalent metal cations is insufficient to *O*-methylate all the TCP to TCA, probably resulting in slow growth. In general, we observed more conidia formation on RKE than on Czapek-Dox, as usually experienced.

Effect of TCP on mycelial growth of A. oryzae on solid media

During rice-*koji* making for sake production, *A. oryzae* is cultured on solid steamed rice. Here, as a solid culture of *A. oryzae*, rice-*koji* was made in the presence of up to 105 µg of TCP per g of α -rice, and their effects are presented in Fig. 3. *A. oryzae* cultured on α -rice with up to 3.5 µg of TCP per gram of α -rice appeared similar to that cultured on rice without TCP, and in both cases, the mycelial contents were a little less than that of typical rice-*koji* (Fujii et al., 1992). Consistent with the results in liquid and agar media, the higher the level of TCP in α -rice, the higher the level of inhibition of mycelial growth of *A. oryzae*. The addition of 35 and 105 µg of TCP to α -rice resulted in mycelial reductions of 64% and 84%, respectively, compared with that on α -rice without TCP, after 44 h of culture. An amount of 35 µg of TCP on α -rice resulted in fungal growth, which was different from what was observed in liquid (Fig. 1A) and agar (Fig. 2A) media containing 30-40 µg/mL of TCP, where growth was strongly inhibited.

According to the Comprehensive *Aspergillus oryzae* Genome Database (<https://nribf21.nrib.go.jp/CAoGD/>), *omtT* is expressed more strongly in rice-*koji* than in other media, including liquid ones. We speculate that higher levels of *O*-methyltransferase, which converts TCP to TCA, are produced in rice-*koji*. Moreover, the concentration of magnesium in α -rice (45 µg/g) is similar to that in Czapek-Dox (49 µg/mL), and α -rice contains 8 µg/mL of manganese. All these factors may explain why the impact of TCP on mycelial growth is less in α -rice compared with those in liquid and agar media. Moreover, the more TCP was added to α -rice, the lesser the α -amylase activity and mycelial content of rice-*koji*.

Our results suggest that the effect of TCP on the growth of *A. oryzae* depends on the conditions of the culture medium. Delayed growth in response to the presence of TCP may be a useful trait to develop a strain that will not produce musty/moldy odor during the

production of sake. Moreover, it will be necessary to consider the culture conditions when screening for TCP-sensitive strains that have a reduced ability to *O*-methylate TCP.

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Figure legends

Fig. 1. Effect of 2,4,6-trichlorophenol (TCP) on the mycelial growth of *Aspergillus oryzae* RIB 40 in liquid media.

Conidia were cultured in Czapek-Dox or rice-*kaji* extract (RKE) media containing 0–40 µg/mL of TCP for 24 h (A), in Czapek-Dox containing 0–10 µg/mL of TCP for 48 h (B), and in RKE containing 0–10 µg/mL of TCP for 24 h (C). The relative mycelial weight means the ratio of mycelial dry weight in each medium containing TCP compared with that in the control medium without TCP. Data are expressed as means ± standard deviations of three independent experiments. Symbols: solid circles, Czapek-Dox; open circles, RKE.

Fig. 2. Effect of 2,4,6-trichlorophenol (TCP) on mycelial growth and conidia formation of *Aspergillus oryzae* RIB 40 growing on agar media.

Conidia were cultured on Czapek-Dox or rice-*kaji* extract (RKE) media (Brix 10%) containing 0–50 µg/mL (A) or 0–10 µg/mL (B) of TCP for 7 days.

Fig. 3. Effect of 2,4,6-trichlorophenol on mycelial growth and α-amylase production of *Aspergillus oryzae* RIB 40 growing on α-rice.

Conidia were cultured for 44 h. Solid and open bars show the mycelial content (mg/g *kaji*) and α-amylase activity (U/g *kaji*) of rice-*kaji*, respectively. Data are expressed as means ± standard deviations of three independent experiments.

Fig. 1

Fig. 2

Fig. 3