ノート (Note)

A semisolid plate method to isolate mycelia maintaining chlamydospore formation in *Tricholoma bakamatsutake*

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Introduction

Tricholoma bakamatsutake is an ectomycorrhizal agaricomycete with fruiting bodies resembling those of *Tricholoma matsutake*, the prized mushroom known as *matsutake*, in Fagaceae forests (Ogawa 1978, Yamada et al. 2014, Herrera et al. 2022). Certain *T. bakamatsutake* isolates change their mycelial morphology in the course of mycelial transplantation to agar media, generally from “slow-growing brown” to “fast-growing white” mycelia, the latter being phenotypically stable on agar media, and do not become the brown ones in general. Brown mycelia display numerous chlamydospores generated asexually from hyphae (Fig. 1; Kües

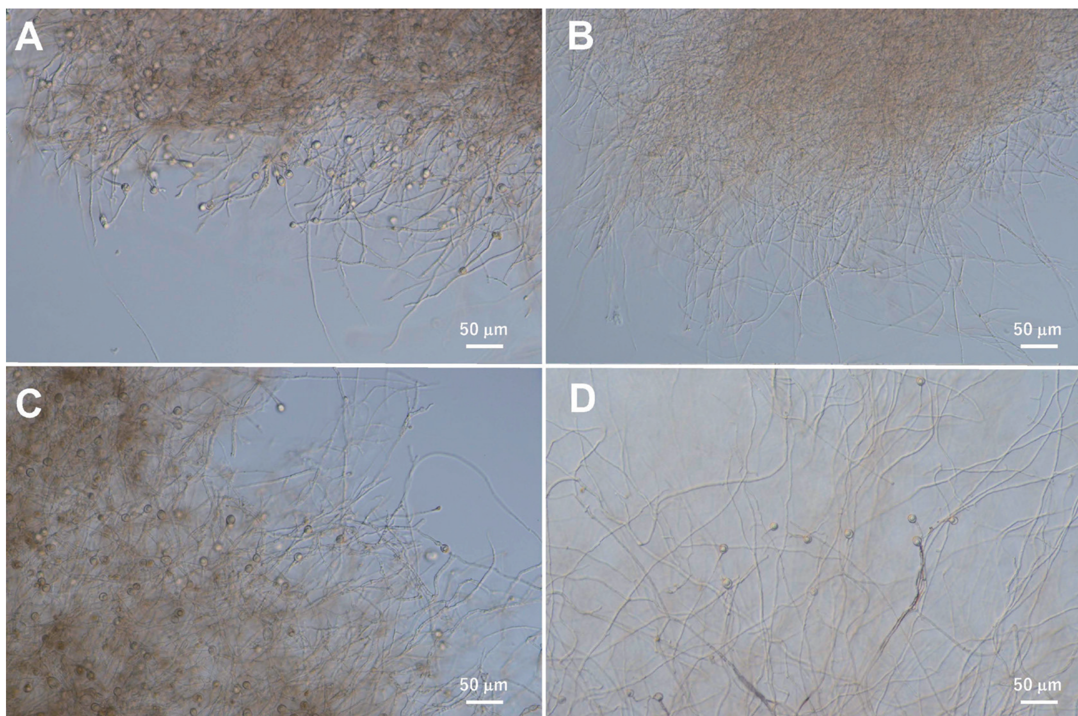

Fig. 1. *Trichloma bakamatsutake* hyphae and chlamydospores observed under a light microscope. A. Brown mycelia of SF-Tf08 with numerous chlamydospores. These brown mycelia were regenerated from the white mycelia using MMN+V8+0.3% SeaKem® GTG agarose™. B. White mycelia of SF-Tf08 without chlamydospores. C. Brown mycelia of SF-Tf09. D. Chlamydospores present in fast growing hyphal sector area of SF-Tf09.

バカマツタケ菌糸体から厚壁胞子形成能を維持した菌株を選抜する半流動培地
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2000). In this study, we developed an agar culture method that allows for distinguishing brown and white mycelial colonies in a single white mycelium of *T. bakamatsutake*. This approach allowed for reisolating strains with the original phenotype, i.e., slow-growing brown mycelia, from spontaneously occurring variants, the fast-growing white mycelia, of *T. bakamatsutake*.

Materials and methods

We used *T. bakamatsutake* strains SF-Tf05, SF-Tf08, and SF-Tf09 as described previously (Murata et al. 2013a, b). Unless stated otherwise, these fungal strains were cultured in the MMN+V8 liquid medium or on MMN+V8 containing 1.5% agar at 23°C (Murata et al. 1999). Semisolid agar plates to differentiate the original strains from phenotypic *T. bakamatsutake* variants were made of the following components: MMN+V8 medium or the half-strength version of the medium, and 0.3% SeaKem® GTG agarose™.

T. bakamatsutake variants were isolated as follows. The mycelia were cultured in MMN+V8+0.3% SeaKem® GTG agarose™ medium at 23°C until reaching a decent size, i.e., a diameter of 15 mm. The mycelia were then cut into pieces with a sterile surgical scalpel (Feather No. 21) and observable mycelial pieces were removed from the agar medium. The medium was then supplemented with fresh MMN+V8+0.3% SeaKem® GTG agarose™ to fill the agar plate and incubated further at 23°C until mycelial colonies appeared.

The DNA analysis-based taxon identification was performed using an rRNA gene internal transcribed spacer region (ITS1-5.8S-ITS2) as described previously (Gardes and Bruns 1993, Murata et al. 2013a, Aoki et al. 2022).

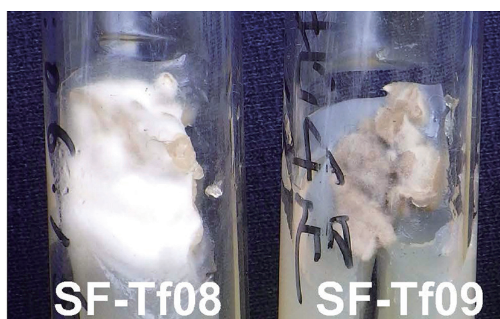

Fig. 2. *Trichloma bakamatsutake* mycelial morphology grown in the MMN+V8+1.5% agar slants.

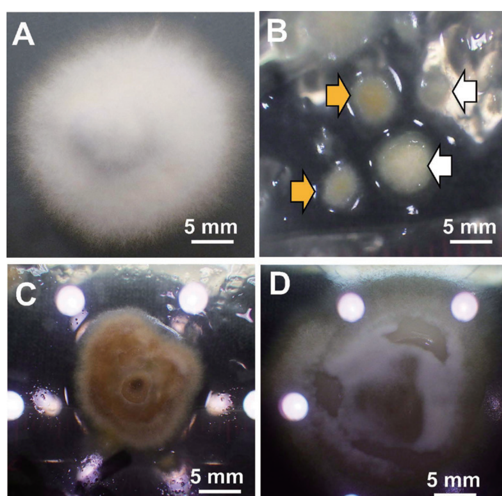

Fig. 3. *Trichloma bakamatsutake* SF-Tf08 mycelial morphology. A. SF-Tf08 strain grown in the MMN+V8+1.5% agar plate. B. SF-Tf08 strain grown in the MMN+V8+0.3% SeaKem® GTG agarose™ plate. Note the appearance of brown (yellow arrows) and white (white arrows) colonies. C-D. Strain SF-Tf08 reisolated from the MMN+V8+0.3% SeaKem® GTG agarose™ plate; stable growth of brown (C) and white (D) mycelia.

Results and discussion

T. bakamatsutake SF-Tf05 grew in the form of fully brown mycelia, exhibiting the same apparent colony morphology as that of the first fruiting body isolate in the MMN+V8 agar. In contrast, SF-TF08 and SF-Tf09 grew as white mycelia and brown mycelia with white aerial hyphae, which had originally grown in the form of totally brown mycelia (Fig. 2, 3A, 4A). *T. bakamatsutake* SF-Tf08 formed white mycelia with uniform appearance and has never formed brown mycelia either on MMN+V8+1.5% agar slants or plates (Fig. 2, 3A). Therefore, the original strain SF-Tf08 exhibiting brown colony morphology isolated from the fruiting body of SF-

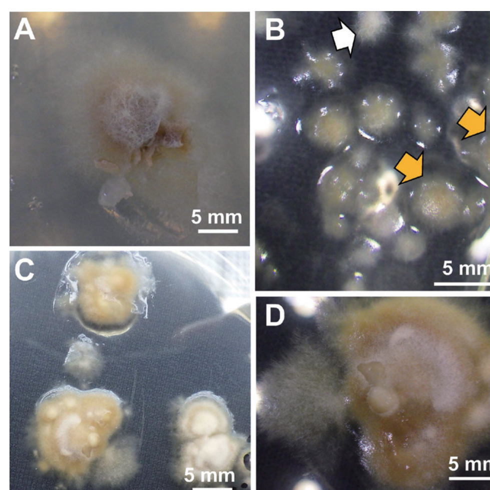

Fig. 4. *Trichloma bakamatsutake* SF-Tf09 mycelial morphology. A. SF-Tf09 strain grown in the MMN+V8+1.5% agar plate. B. SF-Tf09 strain grown in the MMN+V8+0.3% SeaKem® GTG agarose™ plate. Note the appearance of brown (yellow arrows) and white (white arrows) colonies. C-D. Growth of strain SF-Tf09 in the MMN+V8+0.3% SeaKem® GTG agarose™ plate.

Tf08 has no longer exists (Fig. 2). However, when culturing MMN+V8+0.3% SeaKem® GTG agarose™ as described in the materials and methods section, SF-Tf08 generated various mycelial colonies that appeared to grow from chlamydospores (Fig. 3B). Both brown and white mycelia isolated from MMN+V8+0.3% SeaKem® GTG agarose™ stably grew on the standard MMN+V8 with 1.5% agar with seldom generating variants (Fig. 3C, D).

Microscopic observations did not allow for detecting any chlamydospores in the white mycelia unlike the brown ones (Fig. 1). However, based on variant isolation from *T. bakamatsutake* SF-Tf08 mycelia using MMN+V8+0.3% SeaKem® GTG agarose™, a small number of chlamydospores could have potentially scattered in the white mycelia, giving rise to both brown and white mycelial colonies. Notably, the hyphae of *T. bakamatsutake* SF-Tf08 with brown mycelial colony are quite thicker than that with white mycelial colony (Fig. 5).

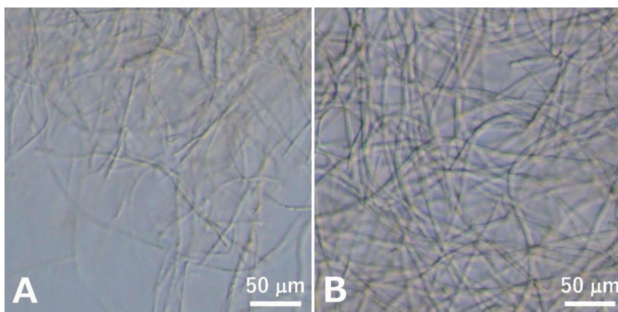

Fig. 5. *Trichloma bakamatsutake* SF-Tf08 hyphae observed under a light microscope showing that hyphae of white mycelia are thinner than those of brown ones. A. Hyphae of white mycelia. B. Hyphae of brown mycelia.

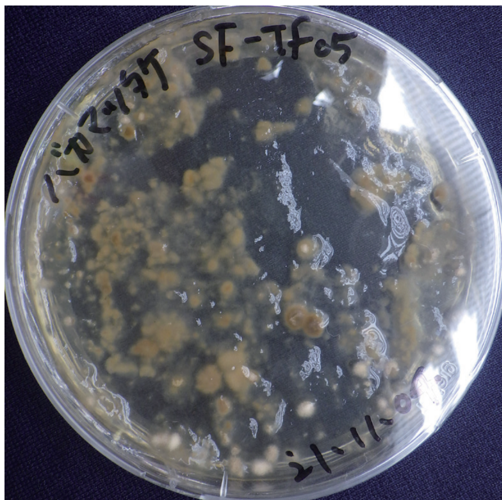

Fig. 6. *Trichloma bakamatsutake* SF-Tf05 mycelial morphology on the MMN+V8+0.3% SeaKem® GTG agarose™ plate. Note that brown colonies dominate the population, in which a few darker brown and white colonies appeared.

When *T. bakamatsutake* SF-Tf05 was cultured on the MMN+V8+0.3% SeaKem® GTG agarose™ plate, the brown mycelial colonies predominantly appeared with a few white colonies (Fig. 6). *T. bakamatsutake* SF-Tf09 exhibited the same trend as SF-Tf08 by culturing MMN+V8+0.3% SeaKem® GTG agarose™ (Fig. 4B–D). DNA analysis confirmed that all representing isolates with the above-described traits were *T. bakamatsutake*.

When isolating mycelial colonies in 0.3% SeaKem® GTG agarose™, the various observed *T. bakamatsutake* morphological phenotypes could be attributed to an agar plate with pure agar that loosely solidified the medium, allowing the fungus to better form chlamydospores that spread that medium. The described protocol is thus useful in isolating variants from *T. bakamatsutake* strains and reisolating the original isolate from a subculture that changed its phenotypic trait. Henceforth, the semisolid agar culture method may be useful in reisolating *T. bakamatsutake* strains from the single mycelial colony that might behave differently among the population in association with host plants and substrate cultivation for fruiting. In fact, we are currently examining the difference between *T. bakamatsutake* strains exhibiting brown mycelial colonies and those exhibiting white ones on agar plate culture in terms of mycorrhiza synthesis and spawn cultivation for fruiting. Furthermore, we isolated and characterized *Tricholoma matsutake* mutants generated by irradiation mutagenesis (Murata et al. 2019, 2021). The agar plate screening system could also be useful in such analyses.

References

Aoki, W., Bergius, N., Kozlan, S., Fukuzawa, F., Okuda, H., Murata, H., Ishida, T.A., Vaario, L.-M., Kobayashi, H., Kalmiş, E., Fukiharu, T., Gisusi, S., Matsushima, K., Terachima, Y., Narimatsu, M., Matsushita, N., Ka, K.-H., Yu, F., Yamanaka, T., Fukuda, M. and Yamada, A. (2022) New findings of the fungal species *Tricholoma matsutake* from Ukraine, and revision of its taxonomy and biogeography based on multilocus phylogenetic analyses. Mycoscience, 63(5): 197–214. https://www.doi.org/10.47371/mycosci.2022.07.004.

Gardes, M. and Bruns, T. D. (1993) ITS primers with enhanced specificity for basidiomycetes –application to the identification of mycorrhizae and rusts. Mol. Ecol., 2(2), 113–118. https://www.doi.org/10.1111/j.1365-294x.1993.tb00005.x.

Herrera, M., Wang, R., Zhang, P. and Yu, F.-Q. (2022) The ectomycorrhizal association of *Tricholoma matsutake* and two allied species, *T. bakamatsutake* and *T. fulvocastaneum*, with native hosts in subtropical China.

Mycologia, 114(2), 303–318. https://www.doi.org/10.1080/00275514.2022.2025563.

Kües, U. (2000) Life history and developmental processes in the basidiomycete *Coprinus cinereus*. Microbiol. Mol. Biol. Rev., 64(2), 316–353. https://www.doi.org/10.1128/MMBR.64.2.316-353.2000.

Murata, H., Nakano, S., Yamanaka, T., Shimokawa, T., Abe, T., Ichida, H., Hayashi, Y., Tahara, K. and Ohta, A. (2019) Conversion from mutualism to parasitism: a mutant of the ectomycorrhizal agaricomycete *Tricholoma matsutake* that induces stunting, wilting and root degeneration in seedlings of its symbiotic partner *Pinus densiflora* in vitro. Botany, 97(8), 463–474. https://www.doi.org/10.1139/cjb-2019-0060.

Murata, H., Nakano, S., Yamanaka, T., Shimokawa, T., Abe, T., Ichida, H., Hayashi, Y. and Tahara, K. (2021) Argon-ion beam induced mutants of the ectomycorrhizal agaricomycete *Tricholoma matsutake* defective in β-1,4-endoglucanase activity promote the seedling growth of *Pinus densiflora* in vitro. Botany, 99(3), 139–149. https://www.doi.org/10.1139/cjb-2020-0076.

Murata, H., Ota, Y., Yamada, A., Ohta, A., Yamanaka, T. and Neda, H. (2013a) Phylogenetic position of the ectomycorrhizal basidiomycete *Tricholoma dulciolens* in relation to species of *Tricholoma* that produce “matsutake” mushrooms. Mycoscience, 54(6), 438–443. https://www.doi.org/10.1016/j.myc.2013.02.003.

Murata, H., Ota, Y., Yamaguchi, M., Yamada, A., Katahata, S., Otsuka, Y., Babasaki, K. and Neda, H. (2013b) Mobile DNA distributions refine the phylogeny of “matsutake” mushrooms, *Tricholoma* sect. *Caligata*, 54(6), Mycorrhiza, 23(6), 447–461. https://www.doi.org/10.1007/s00572-013-0487-x.

Murata, H., Yamada, A. and Babasaki, K. (1999) Identification of repetitive sequences containing motifs of retrotransposons in the ectomycorrhizal basidiomycete *Tricholoma matsutake*. Mycologia, 91(5), 766–775. https://www.doi.org/10.1080/00275514.1999.12061082.

Ogawa, M. (1978) Biology of Matsutake mushroom. Tsukiji Shokan, Tokyo, 333pp.

Yamada, A., Endo, N., Murata, H., Ohta, A. and Fukuda, M. (2014) *Tricholoma matsutake* Y1 strain associated with *Pinus densiflora* shows a gradient of in vitro ectomycorrhizal specificity with Pinaceae and oak hosts. Mycoscience, 55(1), 27–24. https://www.doi.org/10.1016/j.myc.2013.05.004.