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# Phenotypic variations among wild-type strains of the ectomycorrhizal fungus *Tricholoma bakamatsutake* when associated with *Quercus serrata* and cultivated in a barley-based substrate

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**Key words**: Agaricales, mushroom cultivation, plant-microbe interactions, symbiosis

## Introduction

*Tricholoma bakamatsutake* is an ectomycorrhizal fungus that produces fruiting bodies, which resemble the prized mushroom *matsutake*, in association with trees in the Fagaceae family, such as *Quercus serrata* and *Quercus stenophylla* (Murata et al. 2023b). The colony morphology of *T. bakamatsutake* varies among wild-type strains when grown on agar media to produce "slow-growing brown" and "fast-growing white" mycelia (Fig. 1; Murata et al. 2023a). Herein, we examined how variable such wild-type *T. bakamatsutake* strains are when associated with *Q. serrata* seedlings in vitro and when cultured in barley-based substrates, which are used for producing spawn for fruiting of the ectomycorrhizal mushroom *Lyophyllum shimeji* (Ohta 1994, Murata et al. 2019).

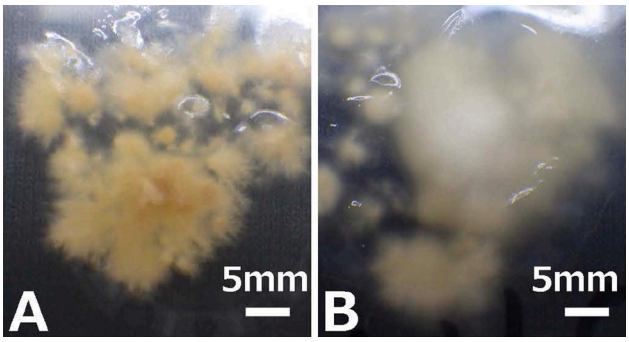


**Fig. 1. Variable mycelial morphology of *Tricholoma bakamatsutake* SF-Tf08 grown in semisolid MMN + V8 + 0.3% SeaKem® GTG agarose™ plates at 23°C for 65 days. (A.) Brown and (B.) white mycelia.**

## Materials and methods

*T. bakamatsutake* strains SF-Tf05 and SF-Tf08, and SF-Tf09 were obtained from a *Q. stenophylla* forest in Yogo-machi, Shiga on September 19th, 2005, and September 12th, 2008, respectively (Murata et al. 2013a, b, Ichida et al. 2023). These were cultured in MMN + V8 liquid medium or on MMN + V8 containing either 1.5% agar or 0.3% SeaKem® GTG agarose™ at 23°C (Murata et al. 1999, 2023a). SF-Tf05 strain solely exhibits brown mycelia on the MMN+V8 1.5% agar plate, and little segregates into white ones on the MMN + V8 semisolid 0.3% SeaKem® GTG agarose™ plate (Murata et al. 2023a). SF-Tf08 strain originally exhibited brown mycelia but tends to segregate into white ones on the media plate with 1.5% agar. SF-Tf08 strain segregates into brown and white mycelia at the highest frequency on the semisolid plate with 0.3% SeaKem® GTG agarose™ (Fig. 1; Murata et al. 2023a). SF-Tf09 strain tends to retain brown mycelia on a 1.5% agar plate but segregates into white mycelia to some extent on the semisolid plate with 0.3% SeaKem® GTG agarose™ (Murata et al. 2023a).

Dual cultivation using *Q. serrata* and *T. bakamatsutake* was axenically performed in vitro based on a protocol by Murata et al. (2013c), except for using vermiculate instead of granite soil. Briefly, axenic *Q. serrata* plants were generated from sterilized *Q. serrata* seeds followed by cottage propagation of branches in Murashge and Skoog (MS: Fujifilm) agar. An axenic *Q. serrata* plant and six 5 × 5-mm pieces of *T. bakamatsutake*

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mycelia grown in MMN + V8 agar were coinoculated into substrates containing vermiculite and 1/4 MS liquid medium with 0.1% glucose and 0.5% sucrose (Murata et al. 2013a). Cocultivation was performed for 27 months at 23°C under daylight for 16 h per day and at 16°C under darkness for 8 h per day. At least three replicates were prepared per treatment. No control plants were used due to the limited numbers of axenic plants. Fresh weight (g) and above-ground height (mm) were measured. Significant differences ($P < 0.05$) among plants associated with different *T. bakamatsutake* strains were statistically analyzed using the Tukey–Kramer test. Fresh weight was measured, rather than dry weight, as plants were acclimatized with *T. bakamatsutake* mycorrhizas in open pots.

*T. bakamatsutake* was axenically cultivated with barley-based substrate prepared in 450-mL capped jar; the cap had an 8-mm vent sealed with MilliSeal (0.45 mm in pore size; Merck) to enable aeration without contamination (Murata et al. 2019, 2020) and had originally been developed for fruiting of *L. symeji* (Ohta 1994). At least two replicates were prepared per treatment.

## Results and discussion

Three *T. bakamatsutake* strains conferred three different responses on *Q. serrata* plants (Fig. 2). SF-Tf05 allowed the *Q. serrata* plants to wilt completely (Fig. 2A). SF-Tf08 with brown mycelia allowed two *Q. serrata* plants to grow while one seedling to wilt (Fig. 2B). SF-Tf08 with white mycelia allowed all three plants to completely wilt (Fig. 2C). SF-Tf09 with both brown and white mycelia, however, allowed all *Q. serrata* plants to apparently show better growth than any other plants with SF-Tf08 or SF-Tf09, exhibiting no sign of wilting (Fig. 2D and E). As it is difficult to axenically propagate *Q. serrata* plants, we could not establish control plants without *T. bakamatsutake*, which would have allowed us to examine how the association influenced plants in comparison with uninoculated plants. However, *Q. serrata* plants clearly responded in different ways to the three *T. bakamatsutake* strains.

SF-Tf09 with white mycelia conferred the highest total shoot growth, whereas SF-Tf05 with brown mycelia conferred least total shoot growth, between which the statistically significant differences were observed (Fig. 3A). SF-Tf08 with both brown and white mycelia and SF-Tf09 with brown mycelia conferred no significant total shoot growth in the plants as compared with all the *T. bakamatsutake* strains tested (Fig. 3A). Although plant response to different *T. bakamatsutake* strains visually differed from each other (see above, Fig. 2), which also reflected the difference in the total shoot length of plants with different *T. bakamatsutake* strains (Fig. 3A), there was no statistical difference in the total plant fresh weight among

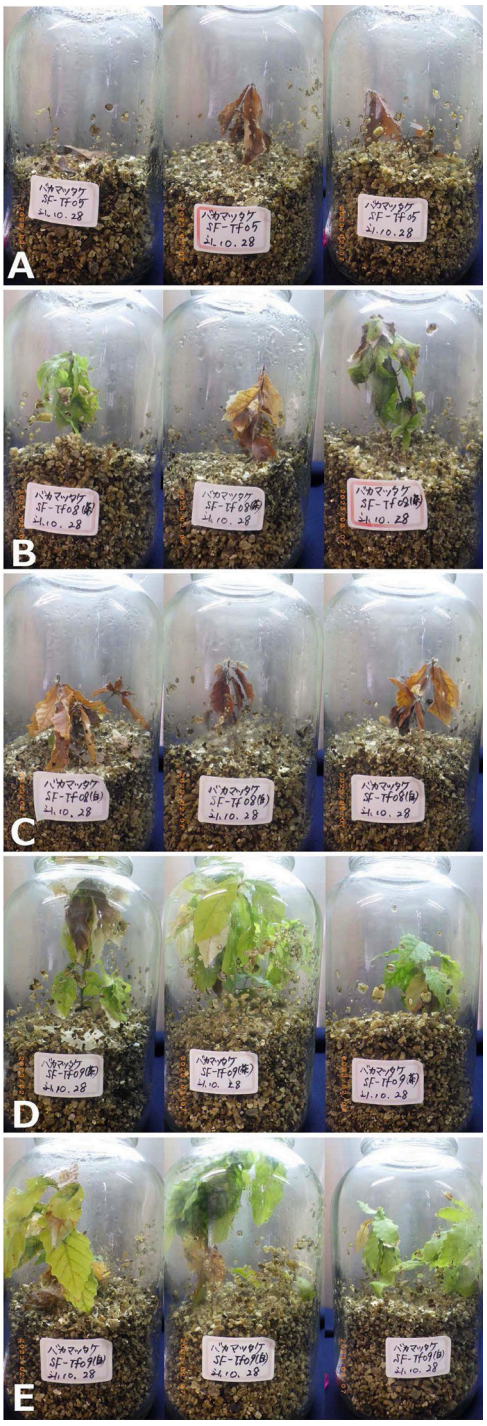


**Fig. 2. *Quercus serrata* plants axenically associated with *Tricholoma bakamatsutake* SF-Tf05, SF-Tf08 and SF-Tf09. SF-Tf05 only formed brown mycelial colonies and such mycelia were used for the experiment. SF-Tf08 and SF-Tf09 formed brown and white mycelia, which were separately inoculated in the rhizosphere of *Q. serrata* plants. *Q. serrata* plants with (A.) SF-Tf05, (B.) SF-Tf08 (brown mycelia), (C.) SF-Tf08 (white mycelia), (D.) SF-Tf09 (brown mycelia) and (E.) SF-Tf09 (white mycelia).**

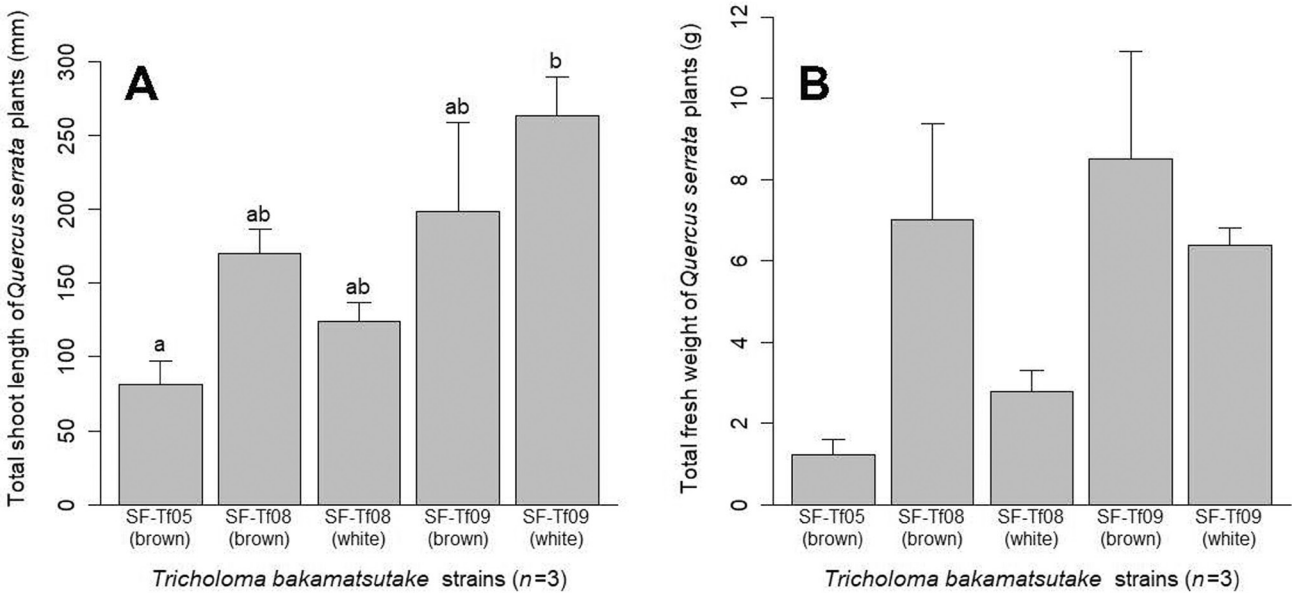


**Fig. 3. Total shoot length (mm) and total fresh weight (g) of *Q. serrata* plants axenically associated with *T. bakamatsutake* strains. Significant difference ($P < 0.05$) was determined using the Tukey–Kramer test. Fresh weight, rather than dry weight, was measured because of the acclimatization of *Q. serrata* plants associated with *T. bakamatsutake* SF-Tf09 in soil in the open air.**

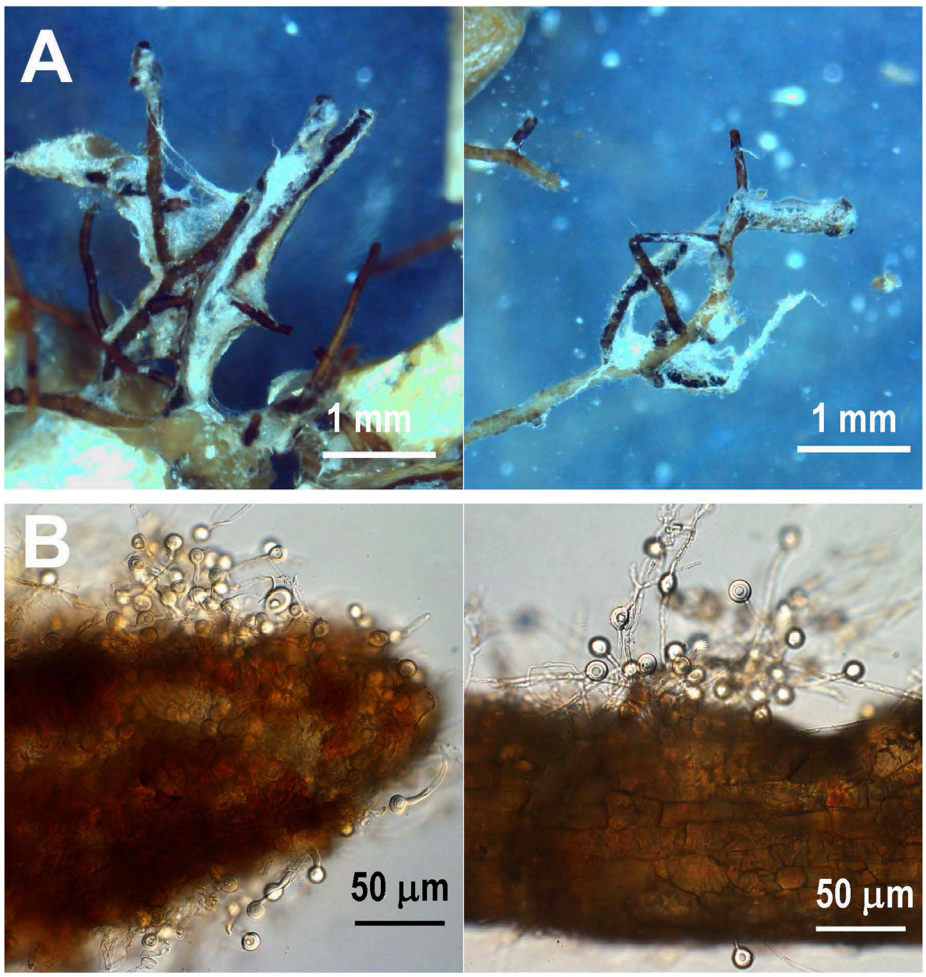


**Fig. 4. Dissecting and differential-interference-contrast micrographs of roots associated with *T. bakamatsutake* SF-Tf09. (A.) Dissecting micrographs, (B.) Differential-interference-contrast micrographs. A large number of chlamydospores were observed; however, the Hartig net was not found.**

the tested *T. bakamatsutake* strains (Fig. 3B). Although further open pot acclimatization experiment of plants with SF-Tf09 did not allow us to do so, dry weight analysis should be able to provide more statistically meaningful results.

Dissecting microscopic analysis revealed that the *Q. serrata* plants associated with SF-Tf09 contained carbonized mycorrhiza-like root tips without root hairs surrounded by fungal mycelia (Fig. 4A). By contrast, *Q. serrata* plants associated with SF-Tf05 and SF-Tf08 exhibited carbonized root tips but rare fungal association. Differential-interference-contrast microscopy revealed that most of the mycorrhizal-like tips with SF-Tf09 had a limited Hartig net, the hallmark structure of ectomycorrhizas, but did have many chlamydospores filled with mycorrhizal-like tips (Fig. 4B). We found a few ectomycorrhizas with a Hartig net in the plants associated with SF-Tf09, which indicates the occurrence of the ectomycorrhizal association between *Q. serrata* and SF-Tf09. Yamada et al. (2010, 2014) showed the occurrence of ectomycorrhizal association between *Q. serrata* seedlings and *T. bakamatsutake* B1 (NBRC 33138) in a soil condition with limited nutrients similar to that used in the present study. By observing the variability of such plant association profiles, we conclude that *T. bakamatsutake* could form a strain-dependent association with the native host *Q. serrata*. Notably, *T. matsutake* also differentially associates with the native hosts *Pinus densiflora* and *Pinus sylvestris* inhabiting Asia and Europe, respectively; a fungal isolate from Scandinavia can associate with both *P. densiflora* and *P. sylvestris*, and an isolate from Japan can associate with *P. densiflora* but not with *P. sylvestris* (Yamada et al. 2010, 2014).

Axenic cultivation of the fungal strains with the barley-based substrate showed that the thick brown mass of the SF-Tf05 mycelia grew rather irregularly or randomly in the substrate (Fig. 5A and B). By contrast, the thin white hyphae of *T. bakamatsutake* SF-Tf08 and SF-Tf09 grew more uniformly in the substrate (Fig. 5C and D). The brown mycelia of SF-Tf08 and SF-TF09 grew poorly in the substrate.

The behavior of *T. bakamatsutake* is complex and depends upon the fungal strains, association with a host plant, and growth in substrate cultivation, even with those from the same forest. Whether the phenomena presented in this study are relevant to the growth of phenotypic variants in an agar culture is unknown. The *T. bakamatsutake* strains SF-Tf05, SF-Tf08 and SF-Tf09 tested here did not exhibit any correlations between growth and morphology on an agar plate (Murata et al. 2023a) or with their behaviors in association with host plants or in substrate cultivation.

Our unpublished genomic analyses, using the nearly-complete *T. bakamatsutake* SF-Tf05 genome sequences as a reference (Ichida et al. 2023), revealed that a high number of point mutations and deletions could occur in the genome of *T. bakamatsutake*. Although these could enable differentiation between the SF-Tf05, SF-Tf08 and SF-Tf09 genomes, we cannot specify which mutations are responsible for variable phenotypes among these strains. The current study implies that the population of *T. bakamatsutake* is highly variable, and strains suitable for both field and substrate cultivation could exist.

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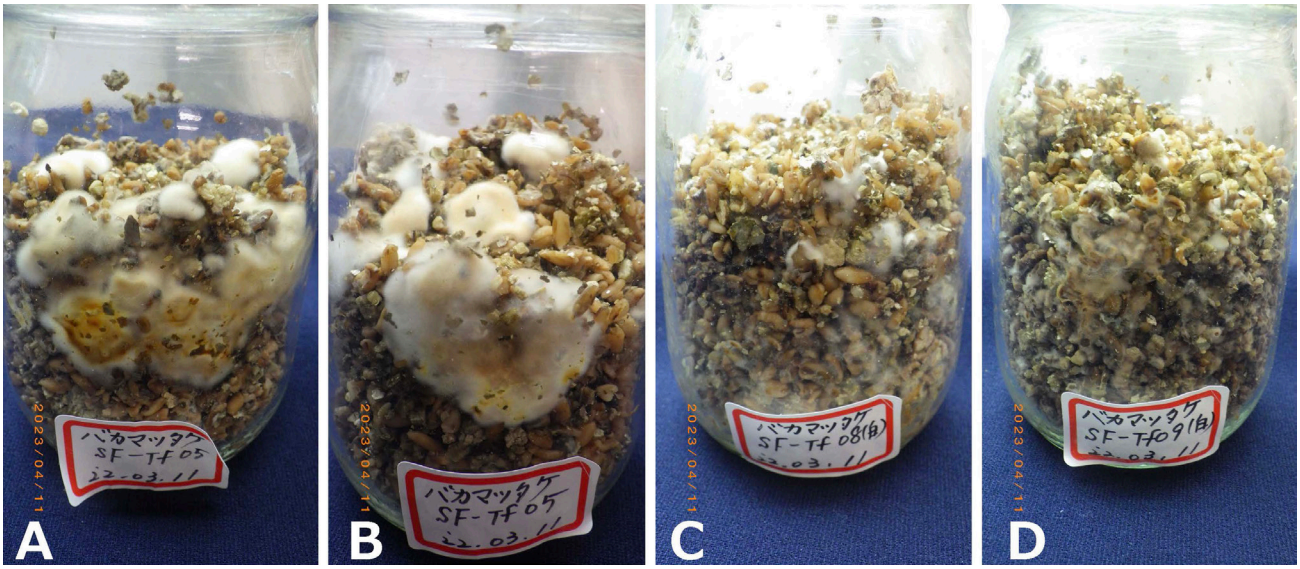


**Fig. 5. Mycelial growth of *T. bakamatsutake* strains with barley-based substrate prepared in a 450-mL capped jar as described by Murata et al. (2019). (A and B.) SF-Tf05, (C.) SF-Tf08 (white mycelia) and (D.) SF-Tf09 (white mycelia).**

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