Short Research Article

Comparison of mycelial growth of different *Tricholoma matsutake* strains in soil medium at varying temperatures

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ABSTRACT

Aims: To investigate the effect of temperature on the mycelial growth of the highly prized mushroom *Tricholoma matsutake* in soil medium.

Methodology: Seven different *T. matsutake* strains collected from different areas of Japan were incubated in soil medium at 5, 10, 15, 20, 25, and 30 °C for 89 d. After incubation, linear growth of mycelia from the inoculum was measured on vertical lines under a dissecting microscope and classified by the cultivation day number to obtain the growth rate of each vial. *T. matsutake* mycelial densities in the soil medium of all incubated vials were analyzed using qPCR to determine the mycelial biomass.

Results: Mycelial growth of *T. matsutake* strains was mainly observed at 5–25 °C. The optimum temperatures for linear mycelial growth and mycelial density increase were 19.6 °C and 17.6 °C, respectively. These values were lower than those reported previously for *T. matsutake* grown on agar medium or in liquid medium. These optimum temperatures showed no clear relation to the mean air temperature of strain collection site.

Conclusion: These results will contribute to the improvement of an artificial cultivation method for *T. matsutake*.

Keywords: Tricholoma matsutake, linear mycelial growth, mycelial density, optimum temperature

1. INTRODUCTION

In basidiomycetes, mycelial growth rate, with regard to both length and density, is affected by temperature [1]. Therefore, temperature is an important factor to determine the culture condition for basidiomycetes. The effect of temperature on mycelial growth has been reported in saprophytic fungi as well as ectomycorrhizal fungi [2], including the highly prized mushroom *Tricholoma matsutake*. For naturally growing *T. matsutake*, the expansion rate of the fairy ring formed by fruiting bodies showed a positive relation with air temperature [3]. This phenomenon suggests that air temperature affects the growth rate of underground mycelia via soil temperature. Notably, mycelial growth of *T. matsutake* is slower than that of some other ectomycorrhizal fungi such as *Rhizopogon roseolus* and *Suillus bovinus* [4]. This slower mycelial growth is an obstacle for efficient experimentation involving *T. matsutake* cultivation.

Optimum temperature for mycelial colony expansion of *T. matsutake* was reported to be 23 °C [5] on agar media. However, in nature, *T. matsutake* mycelia grow in the soil [6], and the effect of temperature on mycelial growth in soil remains poorly understood. Furthermore, the

optimum temperature for mycelial growth of several saprophytic fungi is known to differ not only among species [7] but also among strains [8]. Nevertheless, a comparison of the mycelial growth of different *T. matsutake* strains, isolated from various areas of Japan, in soil medium has not yet been reported. Therefore, in this study, we measured mycelial growth as a linear growth and biomass increase and investigated the mycelial growth of different *T. matsutake* strains in soil at different temperatures. The optimum temperatures determined in this study will contribute to the improvement of an artificial cultivation method for *T. matsutake*.

2. MATERIAL AND METHODS

2.1 Fungal strains

Seven *T. matsutake* strains isolated from different areas of Japan were used in the study (Table 1). Of these, two strains were isolated from the Hokkaido island, three from the northern part of mainland (e.g., Iwate prefecture), and two from the southern part of mainland (e.g., Kyoto prefecture and Nara prefecture). The geographic distance between the most distant sites is approximately 1238 km. Living cultures of all the samples have been deposited in the institutions of the authors.

Table 1. Study sites and optimum temperature for linear mycelial growth and mycelial density increase.

Strain	Site	Latitude	Longitude	Mean air temperature[(a)]	Optimum temperature (˚C)	
					Linear mycelial growth	Mycelial density increase
H18	Kamifurano	43°24'N	142°36'E	6.2	20.6	17.8
Rin10	Meakan	43°22'N	143°59'E	3.9	17.1	17.3
I122	Yokkaichi	39°56'N	141°14'E	9.4	20.8	17.7
I129	Yokkaichi	39°56'N	141°14'E	9.4	20.3	17.3
I33	Yokkaichi	39°56'N	141°14'E	9.4	20.5	17.7
Sakai	Sakai	35°11'N	135°20'E	14.3	18.9	15.6
NF2970	Nara	34°10'N	135°41'E	14.6	19.6	19.8
mean±SD					19.6±1.3	17.6±1.2

[(a)] *Normal mean air temperature observed at the nearest weather station of Japan meteorological agency from each study site.*

2.2 Cultivation of mycelia

Mycelial growth rate of *T. matsutake* was determined using flat-bottom incubation vials (30 mm outer diameter and 120 mm height) containing soil medium prepared as follows: loamy soil, with a particle diameter of 1.4 mm, was soaked in MYPG liquid medium (0.25% malt

extract, 0.1% yeast extract, 0.1% peptone, 0.5% glucose, pH 5.0) [9] for 16 h to allow the soil to absorb the MYPG medium. Consequently, the soil was set onto a plastic mesh basket for 30 min to remove the extra medium. B-horizon soil (forest brown soil) collected from *T. matsutake* growth site in the Japanese red pine forest [3] was mixed with loamy soil at a ratio of 3:1 (v/v). Moisture content was adjusted to 25% (w/w) using tap water. This soil medium was then filled into incubation vials at the height of 8 cm (48.5 g) and autoclaved at 121 °C for 60 min.

Each strain was pre-incubated on modified Norkrans' C agar medium (1.0 g KH_2PO_4, 0.5 g $MgSO_47H_2O$, 0.5 mL of 0.2% $ZnSO_4$, 0.5 g NH_4-tartrate, 0.5 mL of 1.0% Fe-citrate, 50 µg thiamine, 0.23 g casein hydrolysate, 0.5 g yeast extract, 10 g glucose, 15 g agar, and 1000 mL deionized water; pH 5.0) [10] at 23 °C. After 57 d of incubation, the growth zone of the colony was bored by a cork borer with 9 mm diameter, and five mycelial plugs were placed into 125-mL flasks containing the MYPG liquid medium and incubated at 23 °C for 34 d under shaking at 35 rpm. Consequently, the mycelia were homogenized in a flask using Physcotoron homogenizer (Microtec Co., Ltd., Japan) at 1800 rpm for 5 s to prepare a uniform mycelial suspension for the next inoculation and incubated under static conditions continuously in the same flask. After 23 d of culture, 2 mL of the liquid medium including small pellets of *T. matsutake* mycelia was inoculated onto the soil medium, and the vial was capped and sealed by Parafilm (Bemis Flexible Packaging, USA). The cultures were incubated at 5, 10, 15, 20, 25, and 30 °C for 89 d. Five replicates were performed for each strain at each temperature. All the incubations were carried out in the dark and under aseptic conditions.

2.3 Measurement of mycelial growth and biomass

After 89 d of cultivation, linear growth of the mycelia from the inoculum was measured on 4 vertical lines under a dissecting microscope. The observed values were averaged and divided by the cultivation day number to obtain the growth rate of each vial. Then, *T. matsutake* mycelial densities in the soil medium of all incubation vials were analyzed by qPCR to determine the mycelial biomass [11]. The soil samples were lyophilized, pulverized with a Multi-beads Shocker (Yasui Kikai Corporation, Japan), and the DNA was extracted using the cetyltrimethylammonium-bromide (CTAB) lysis buffer. qPCR was performed using a LightCycler System (Roche Life Science, Germany) and the primer set MY201f: 5'-GAGACACAACGGCGAGATT-3' and MY101r: 5'-ACCCTTACCCGCTCAGT-3', to amplify a 202-bp DNA fragment used to specifically quantify *T. matsutake* mycelia. DNA amplification and detection were performed in glass capillaries in a total volume of 20 µL containing 2 µL of LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Switzerland). The reaction mixture composed of 3.2 µL (5 mM) of $MgCl_2$, 1 µL (0.25 µM) of each primer, 10.8 µL of H_2O (sterile PCR grade), and 2 µL of template DNA. To detect the signal derived from *T. matsutake* mycelia, qPCR was performed using the following program, as recommended by the manufacturer: 1 cycle at 95 °C for 10 min, followed by 50 cycles at 95 °C for 10 s, 67 °C for 2 s, 72 °C for 1 s, and 83 °C for 1 s, followed by signal detection. Unless stated otherwise, the standard curve was constructed using a 1.0 ng/µL sample of the respective plasmid containing the target DNA; the sample was serially diluted and added to the 20-µL reaction mixture. The PCR products with a single melting curve that fitted the respective standard curve were considered as authentic, quantified PCR products.

2.4 Data analysis

The mycelial growth rate and density of each vial were fitted to quadratic and tertiary equation models to estimate the optimum temperature for *T. matsutake* mycelial growth or

density increase. Next, the models were compared using the Akaike information criterion (AIC) (1) to select the most rational model for each strain:

AIC = residual deviance + 2(the number of variances) (1)

Finally, the selected models were differentiated to estimate the optimum temperature. All the above-mentioned analyses were performed using R 2.14.0 [12].

3. RESULTS AND DISCUSSION

For all the *T. matsutake* strains tested, a linear mycelial growth was observed at 5, 10, 15, 20, and 25 °C on the soil medium, but not at 30 °C (Fig. 1). The mean linear mycelial growth rate was largest at 20 °C for six of the seven strains. Daily linear growth rate at 20 °C varied from 0.12 mm/d (strain Rin10 and I33) to 0.19 mm/d (strain I129). The linear growth rate declined rapidly between 25 °C and 30 °C, which is in accordance with a previous report [5]. Curve fitting results showed that AIC was the smallest in the tertiary equation model for all the seven strains (Fig. 1). Using the tertiary equation model, the optimum temperature range for linear mycelial growth was estimated as 17.1 °C (strain Rin10) to 20.8 °C (strain I122) with an average of 19.6 ± 1.3 SD °C (Table 1).

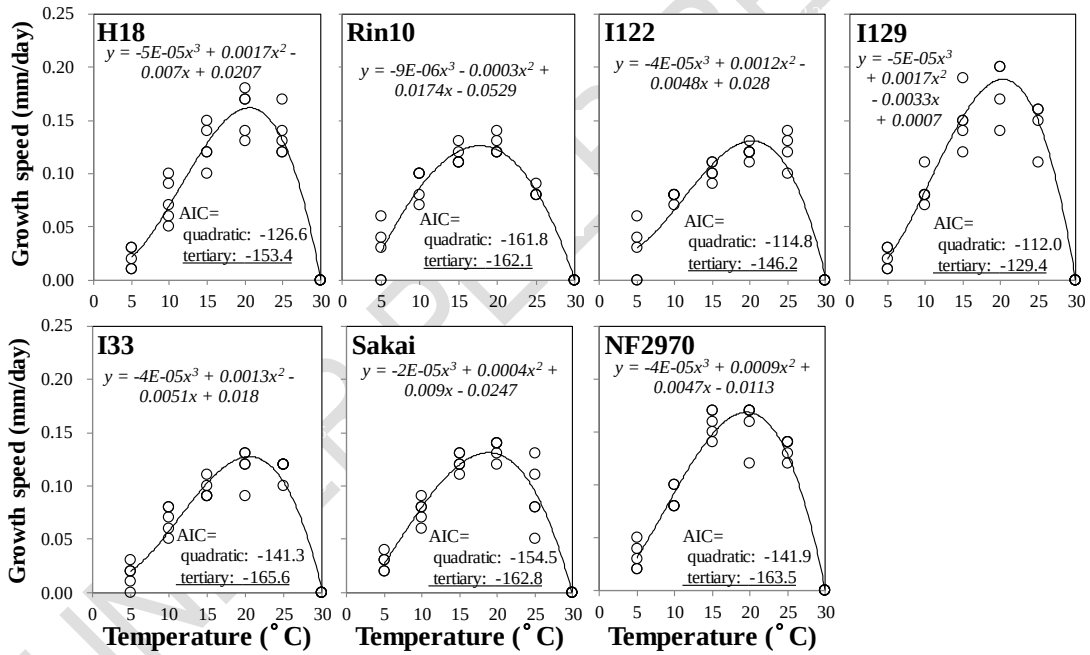

Fig. 1. Effect of temperature on linear mycelial growth of seven *Tricholoma matsutake* strains in soil medium after 89 d of cultivation

Circles indicate the observed values, and lines indicate the approximate curve. Fitted model was selected from quadratic or tertiary equation by comparing the Akaike information criterion (AIC) for each strain (underlined).

Mycelial density increased during the incubation period at each selected temperature (Fig. 2). Consistent with the linear mycelial growth rate, a decrease in mycelial density was observed between 25 °C and 30 °C. Curve fitting revealed the smallest AIC for the tertiary equation model for strain H18 and strain NF2970, and for the quadratic equation model for other strains (Fig. 2). Thus, using the suitable growth model for each strain, the optimum

temperature for mycelial density increase was estimated as 15.6 °C (strain Sakai) to 19.8 °C (strain NF2970) with an average of 17.6 ± 1.2 °C (Table 1). The mean optimum temperature for the mycelial density increase was slightly lower than that required for linear mycelial growth (p = .01, t-test). No significant relation was observed between the optimum temperature for mycelial density increase and that for linear mycelial growth (r = 0.24, p = .05). Although linear mycelial growth was not observed at 30 °C, mycelial density increased at 30 °C; this may be attributed to the inclusion of the mycelia present in the inoculum in the measurement of mycelial biomass. However, the reason for the difference between optimum temperatures for linear mycelial growth and mycelial density increase is unclear; the difference in the mechanism underlying linear mycelial growth and mycelial density increase probably affects the reaction to temperature.

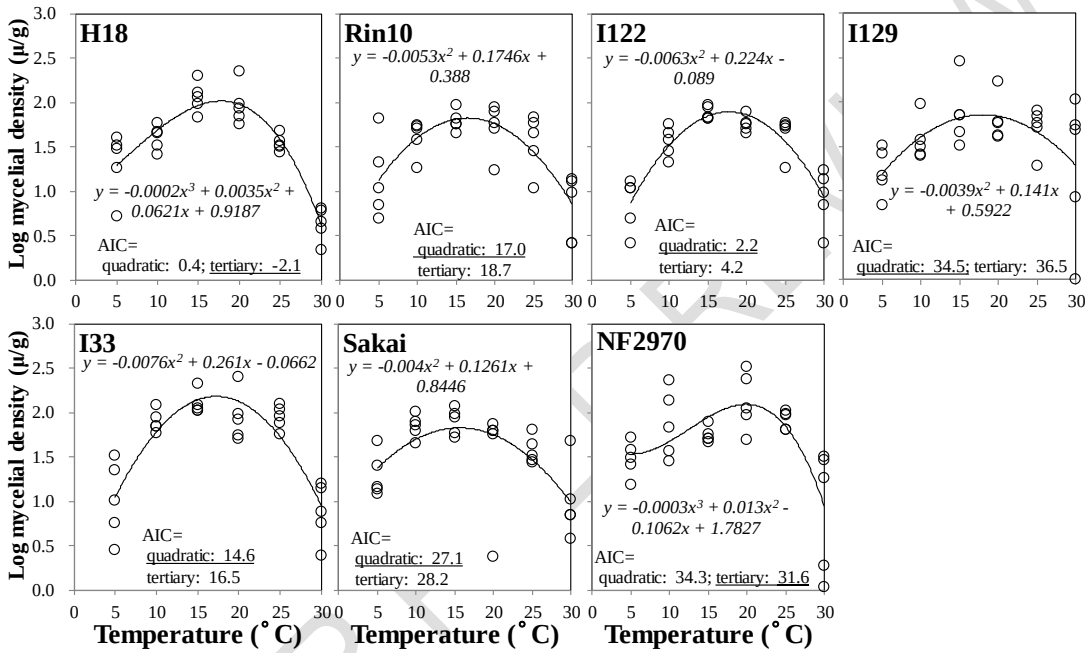

Fig. 2. Effect of temperature on mycelial density increase in seven *Tricholoma matsutake* strains in soil medium after 89 d of cultivation

Circles indicate the observed values, and lines indicate the approximate curve. Fitted model was selected from quadratic or tertiary equation by comparing the Akaike information criterion (AIC) for each strain (underlined).

No clear relation was observed between the optimum temperature for linear mycelial growth or mycelial density increase and the mean air temperature of the collection site (r < 0.2, p = .05), suggesting that the growth rate of strains collected from the cold region may not always be lower than that of strains collected from the warm region, as reported previously [13]. On the other hand, the distribution area of *T. matsutake* is known to be remarkably large, from northern Europe [14] to south-east Asia [15], compared to the area included for strain collection in this study. Including a wider area may provide greater variance in ecology among growth sites, especially temperature. Therefore, further studies comparing samples collected from a wider area are warranted to verify the relation between temperature of the natural growth site and the optimum temperature for mycelial growth.

Thus, in this study, we cultivated the *T. matsutake* mycelia in a soil medium with conditions similar to those in the natural habitat and verified the optimum temperature for *T. matsutake* growth with respect to linear mycelial growth and mycelial density increase. The optimum temperatures for linear mycelial growth and mycelial density increase of each strain tested were about 3–5 °C lower than those reported by previous *T. matsutake* studies using agar media [5] or liquid media [16]. The estimated optimum temperatures were also lower than those for most of the 62 species of ectomycorrhizal fungi grown in liquid medium [17] and two allied species of *T. matsutake*, including *T. fuluvocastaneum* [18] and *T. bakamatsutake* [19], grown on agar media. The lower optimum temperatures than those reported previously is consistent with the climatic condition of the isolation sites because the ground temperature does not always reach 20 °C, even in summer, especially in the northern mainland of Japan [20].

4. CONCLUSION

The optimum temperatures for linear mycelial growth and mycelial density increase of *T. matsutake* were lower than those reported previously using agar medium or in liquid medium. The optimum temperatures determined in this study will contribute to improving the cultivation efficiency of *T. matsutake* mycelia in soil medium.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS’ CONTRIBUTIONS

Narimatsu M., Yamaguchi M. and Yamanaka T. designed the study. Narimatsu M. and Yamaguchi M. carried out the experiment and performed the statistical analysis, wrote the protocol. Narimatsu M., Gisusi S., Tamai Y., Fujita T. and Kawai M. prepared strains for this study. All authors contributed to the interpretation of the results and approved the final manuscript.

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