Short Research Article

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

ABSTRACT

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

Methodology: Seven strains of *T. matsutake* 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, the 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 in each vial. The mycelial densities in the soil medium in all the vials were analyzed using qPCR to determine the mycelial biomass.

Results: The mycelial growth of *T. matsutake* strains was mainly observed at 5–25 °C. The optimum temperatures for linear mycelial growth and for the increase in mycelial density 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 relationship with the mean air temperature at the site of strain collection.

Conclusion: It can be suggested that a temperature around 18 °C is suitable for cultivation of *T. matsutake* mycelia in the soil medium.

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

1. INTRODUCTION

Tricholoma matsutake is an ectomycorrhizal fungus in Agaricales, Basidiomycota, with the basidiomata of pileus being ~8–20 cm across and stipe length being ~10–20 cm [1]. *T. matsutake* is an economically important fungus and usually inhabits forests primarily composed of *Pinus densiflora* (Japanese red pine) in Japan [2]. It also grows in the pine forests in other countries [3,4], and substantial amounts of it are exported to Japan [5]. A stable artificial cultivation method for *T. matsutake* has not yet been developed [6]. The fruiting bodies of *T. matsutake* need to be collected from natural forests, which makes its production unsustainable because of the effects of environmental conditions that change annually [7]. For sustainable production of *T. matsutake* fruiting bodies, expanding the area of occurrence of this fungus by inoculation of mycelium or mycorrhizal nursery stock in forests using cultured mycelium is, therefore, essential [5].

In basidiomycetes, the mycelial growth rate, with regard to both the length and density, is affected by temperature [8]. Therefore, temperature is an important factor in determining the

culture conditions for basidiomycetes. The effect of temperature on mycelial growth has been reported for saprophytic fungi as well as for ectomycorrhizal fungi [9], including *T. matsutake*. For naturally growing *T. matsutake*, the expansion rate of the fairy ring formed by the fruiting bodies showed a positive relation with the air temperature [7]. This phenomenon suggests that air temperature affects the growth rate of underground mycelia by modulating the soil temperature. Notably, mycelial growth of *T. matsutake* is slower than that of some other ectomycorrhizal fungi, such as *Rhizopogon roseolus* and *Suillus bovinus* [10]. This slower mycelial growth is an obstacle for efficient experimentation involving *T. matsutake* cultivation.

The optimum temperature for mycelial colony expansion of *T. matsutake* on agar media was reported to be 23 °C [11]. However, in nature, *T. matsutake* mycelia grow in the soil [12], 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 [13] but also among strains [14]. However, a comparison of the mycelial growth of different *T. matsutake* strains, isolated from various areas of Japan, in the soil medium has not been reported. Therefore, in this study, we measured the mycelial growth, in terms of the linear growth and biomass increase, of different *T. matsutake* strains in the soil at different temperatures.

2. MATERIALS AND METHODS

2.1 Fungal strains

Seven *T. matsutake* strains isolated from different areas of Japan were used in this study (Table 1). Among these, two strains were isolated from the Hokkaido Island, three from the northern part of the mainland (e.g., Iwate prefecture), and two from the southern part of the 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	Hokkaido	43°24'N	142°36'E	6.2	20.6	17.8
Rin10	Hokkaido	43°22'N	143°59'E	3.9	17.1	17.3
I122	Iwate	39°56'N	141°14'E	9.4	20.8	17.7
I129	Iwate	39°56'N	141°14'E	9.4	20.3	17.3
I33	Iwate	39°56'N	141°14'E	9.4	20.5	17.7
Sakai	Kyoto	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 annual mean air temperature observed at the nearest weather station of Japan meteorological agency from each study site.*

2.2 Cultivation of mycelia

2.2.1 Isolation of mycelia

The strains used in this study were isolated from hymenia except strain NF2970 from lammella, using nutrient agar media, for example the modified MYPG medium [15] (2.5 g malt extract, 1.0 g yeast extract, 1.0 g peptone, 5.0 g glucose, 15 g agar, and 1000 mL deionized water), and stored at 4 °C until use.

2.2.2 Inoculation of mycelia

Mycelial growth rate of *T. matsutake* was determined using flat-bottom incubation vials (30 mm outer diameter and 120 mm height) containing the soil medium prepared as follows: loamy soil (Turf soil, Koujiya Co. Ltd., Japan), with a particle diameter of 1.4 mm, was soaked in modified MYPG liquid medium [15] (0.25% malt extract, 0.1% yeast extract, 0.1% peptone, 0.5% glucose, pH 5.0) 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. The B-horizon soil (brown forest soil) was collected from a depth of 10–20 cm at the *T. matsutake* growth site in the Japanese red pine forest [7], air dried, and passed through a sieve (7-mm mesh size) to remove any stones from the soil. Thereafter, the B-horizon soil was mixed with loamy soil at a ratio of 3:1 (v/v). The moisture content was adjusted to 25% (w/w) with tap water. This soil medium was then filled into the incubation vials to a height of 8 cm (48.5 g) and autoclaved at 121 °C for 60 min (Fig. 1).

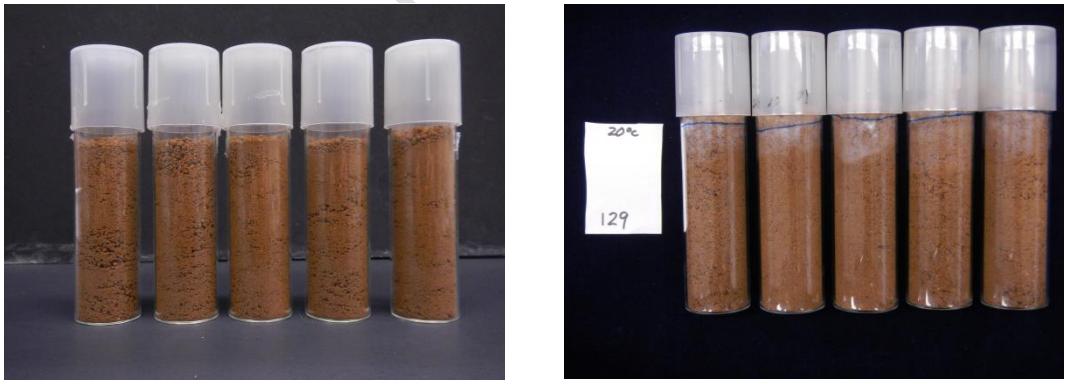

Fig. 1. Flat-bottom vials containing the soil medium used in this study.
The panel on the *left shows the soil medium before inoculation and that on the right shows the medium after 89 d of cultivation at 20 ℃ for strain I129.*

Each strain was pre-incubated on modified Norkrans' C agar medium [16] (1.0 g KH_2PO_4, 0.5 g $MgSO_4 7H_2O$, 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) at 23 °C. After 57 d of incubation, the growth zone of the

colony was bored with a cork borer (9-mm diameter), and five mycelial plugs were placed in 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 with Parafilm (Bemis Flexible Packaging, USA). The cultures were incubated at 5, 10, 15, 20, 25, and 30 °C for 89 d. Five replicates were kept 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 *T. matsutake* mycelia from the inoculum was measured on four vertical lines under a dissecting microscope. The observed values were averaged and divided by the cultivation day number to obtain the growth rate for each vial. Thereafter, the soil medium containing the inoculated and/or cultured mycelia was collected immediately and stored at -30 °C for further qPCR analysis. *T. matsutake* mycelial densities in the soil medium in all the incubation vials were analyzed by qPCR to determine the mycelial biomass [17]. The soil samples were lyophilized, pulverized with a Multi-beads Shocker (Yasui Kikai Corporation, Japan), and the sample 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 the *T. matsutake* mycelia [17]. DNA amplification and detection were performed in glass capillaries in a total volume of 20 µL, which contained 2 µL of LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Switzerland). The reaction mixture contained 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 or sample 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 known numbers of copies of the template plasmid containing the target DNA. The copy number in the serial dilutions of the template plasmid ranged from 10^7 to 10^1. The sample DNA 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 in each vial were fitted to quadratic and tertiary equation models to estimate the optimum temperature for the mycelial growth or increase in the density of *T. matsutake*, because the observed value of mycelial growth rate and density showed a single peak with a long tail for some strains (Fig. 2 and Fig. 3). 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 [18].

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. 2). The mean linear mycelial growth rate was highest at 20 °C for six of the seven strains. The daily linear growth rate at 20 °C varied from 0.12 mm/d (strain Rin10 and I33) to 0.19 mm/d (strain I129). Significant difference among the strains was observed for the mean daily linear growth rate at 20 °C (p = .01, ANOVA). The linear growth rate declined rapidly between 25 °C and 30 °C, which is in accordance with the results of a previous report [11]. The results of curve fitting showed that AIC was the smallest in the tertiary equation model for all the seven strains (Fig. 2). Using the tertiary equation model, the optimum temperature for linear mycelial growth was estimated to range from 17.1 °C (strain Rin10) to 20.8 °C (strain I122), with an average of 19.6 ± 1.3 SD °C (Table 1).

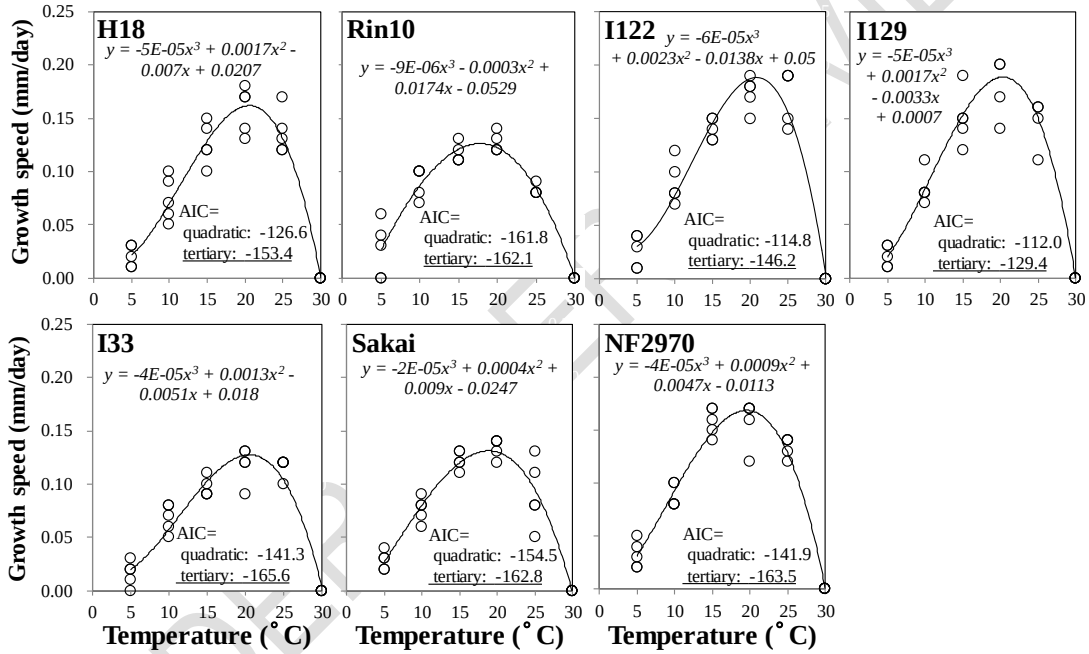

Fig. 2. Effect of temperature on linear mycelial growth of seven *Tricholoma matsutake* strains in the soil medium after 89 d of cultivation.

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

The mycelial density increased during the incubation period at each selected temperature (Fig. 3). Consistent with the linear mycelial growth rate, significant difference among strains was observed for the mycelial growth rate at 20 °C (p = .05, ANOVA). A decrease in mycelial density was observed between 25 °C and 30 °C as was observed for the linear mycelial growth rate. The curve fitting revealed the smallest AIC for the tertiary equation model for strain H18 and strain NF2970, and for the quadratic equation model for the other strains (Fig. 3). Thus, using a suitable growth model for each strain, the optimum temperature for mycelial density increase was estimated to range from 15.6 °C (for the strain Sakai) to 19.8 °C (strain NF2970), with the average being 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 relationship was observed between the optimum temperature for mycelial density increase and that for linear mycelial growth (r = 0.24, p = .05). Although the linear mycelial growth was not observed at 30 °C, the 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 response to the temperature.

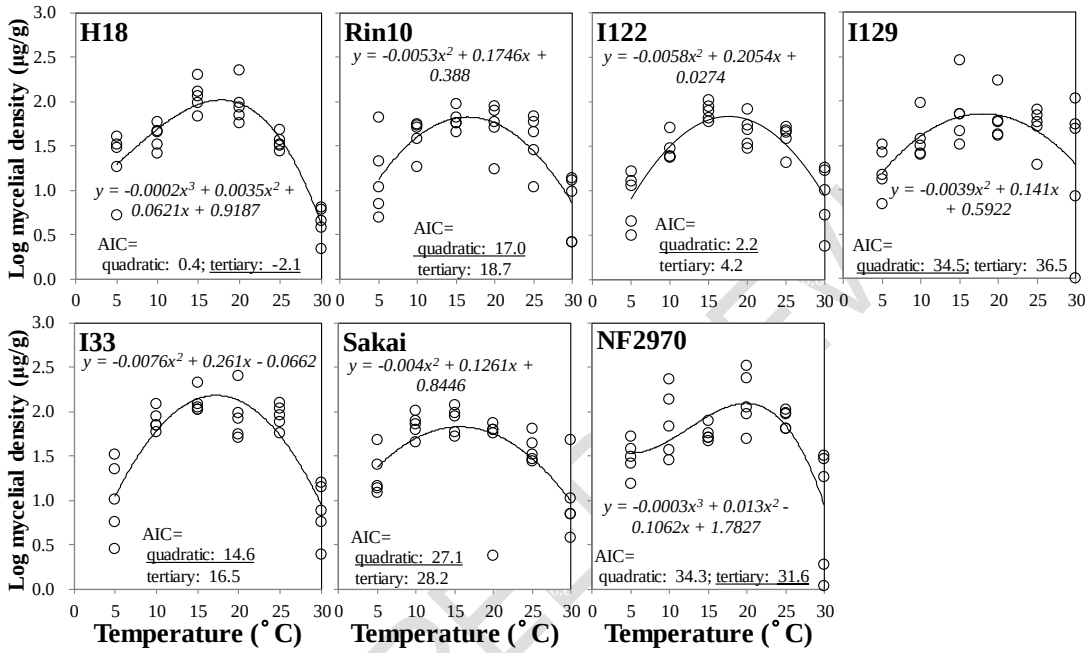

Fig. 3. Effect of temperature on mycelial density increase in seven *Tricholoma matsutake* strains in the soil medium after 89 d of cultivation.

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

No clear relationship was observed between the optimum temperature for linear mycelial growth or mycelial density increase and the mean air temperature at the collection site (r < 0.2, p = .05), suggesting that the growth rate of strains collected from the cold region might not always be lower than that of strains collected from the warm region, as reported previously [19]. On the other hand, the distribution area of *T. matsutake* is known to be remarkably larger compared to the area included for strain collection in this study, such as that from the northern Europe [4], with an annual mean temperature of 4.4–6.7 °C in Finland [20] to south-east Asia [3], with an annual mean temperature of 19.6 °C in Xien Khouang city, Laos [21]. The inclusion of a wider area may provide greater variance of ecological conditions, especially temperature, among the growth sites. Therefore, further studies comparing samples collected from a wider area are warranted to verify the relationship between the 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 for each of the strains tested were about 3–5 °C lower than those reported in previous studies on *T. matsutake* using agar media (such as 23 °C) [11] or using liquid media (such as 25 °C) [22]. The estimated optimum temperatures were also lower than those for most of the 62 species of ectomycorrhizal fungi grown in liquid medium [23] and for the two allied species of *T. matsutake*, namely *T. fuluvocastaneum* [24] and *T. bakamatsutake* [25], grown on agar media. The optimum temperatures lower than those reported previously are consistent with the climatic conditions prevailing at the isolation sites because the ground temperature does not always reach 20 °C, even in summers, especially in the northern mainland of Japan [26].

4. CONCLUSION

The optimum temperatures for linear mycelial growth and mycelial density increase of *T. matsutake* were lower than those reported previously using agar or liquid media. The determination of the optimum temperatures would contribute to the improvement of artificial cultivation methods for *T. matsutake*. Based on the results of this study, the optimum temperature for cultivation of *T. matsutake* for use as an inoculum for forest or nursery stock is expected to be 18 °C.

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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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