

Evaluation of the Nutritional Requirement and
Wood Decay Properties of a Termite Mushroom,
Termitomyces eurrhizus

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General introduction

The fungus *Termitomyces* belongs to the family Lyophyllaceae of the order Agaricales in the fungal phylum Basidiomycotina. It is well known that this genus is closely related to the genus Lyophyllum, including both saprophyte and mycorrhizae [1]. *Termitomyces* fungi are categorized as white-rot [2], and have a unique biology. They are cultivated by fungus-growing termites (Macrotermitinae, Isoptera) in special rooms called “fungus gardens” inside their nests. The gardens are provided with plant substrates collected by the host termites. The host termites eat the mycelia of *Termitomyces*, and the plant substrates are decomposed by the fungi [3]. Thus, the fungi and the termites establish a mutualistic relationship. Macrotermitinae termites are dominantly distributed in the rainforests and savannas of tropical and sub-tropical regions with the exception of North and South America [4-6]. On the other hand, the fruit bodies of many *Termitomyces* species are known to be favorable for human consumption and have commercial value in some regions [4].

Eleven genera and 330 species of Macrotermitinae and 40 species of *Termitomyces* are known at present, and each fungus species is shared by multiple termite species [7]. However, a single strain of *Termitomyces* is cultured by a single nest of Macrotermitinae termites [8,9]. All genera of Macrotermitinae are present in Africa, and the symbiosis between fungus-growing termites and *Termitomyces* is thought to have originated in Africa [6]. Several hypotheses regarding the function of the symbiosis have been proposed. The fungi are a rich protein source for the termites [10,11]. Lignin degradation by the fungi enhances the digestibility of cellulose [12,13]. Cellulases and xylanases originating from the fungus work synergistically for termite

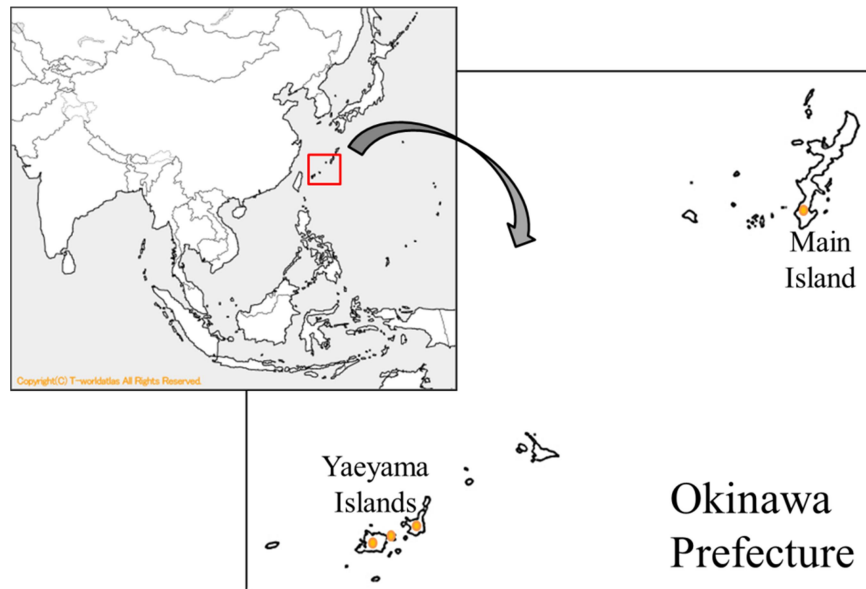


Fig. 1 Distribution of *T. eurrhizus* in Japan

digestion [14-16]. The C/N ratio of foraged lignocelluloses is decreased by the fungi [17]. The fungi are thought to be the main decomposer of lignocellulose for termite digestion [7]. Still, the exact roles of the symbionts are unclear and seem to differ across genera and species of termite [7,18-21].

Two *Termitomyces* species, *Termitomyces eurrhizus* and *Termitomyces clypeatus*, are reported to be distributed in Japan. *Termitomyces eurrhizus* is found in the Yaeyama Islands and the periphery of Shuri Castle on Okinawa Main Island (Fig.1) [22,23]. *Termitomyces clypeatus* is found only on Iriomote Island, in the Yaeyama Islands of Okinawa Prefecture [24]. Okinawa Prefecture is regarded as the eastern limit of *Termitomyces* distribution [23,25]. The host termite of these fungi in Okinawa is *Odontotermes formosanus* [26-28]. This termite species is known as a pest of many crops such as sugar cane and coffee [29,30]. The termite is subterranean and sometimes



Fig. 2 Fruit bodies of *Termitomyces eurrhizus*

(Ishigaki Island, Okinawa, 2012)

builds large cavities inside earthen dikes and dams, often causing collapse of these important human constructions [29]. Interestingly, there is no distribution of the fungus and the termite on the other islands between the Yaeyama Islands and the Okinawa Main Island. This separated and spotty distribution of *T. eurrhizus* and

O. formosanus in the region is thought to be due to artificial transportation [31]. *Termitomyces eurrhizus* also produces a delicious but highly perishable mushroom, which was used as a precious offering to the royal court of Ryukyu Dynasty [31].

The fruit body of *T. eurrhizus* is approximately 3–13 cm in height (Fig. 2). The pileus is cuspidate but narrowly bell-shaped as a button, 4–14 cm in diameter, yellowish brown/gray and darker at the center, with a conical sharp peak in the center, and radial shallow lines on the surface. The stalk is columnar, 1.0–1.8 cm in diameter, with no annulus and a slightly expanded base. Long and thin pseudorhiza extend to the comb in the underground termite nest (Fig. 3) [22,23]. In addition, it is noted that this fungus has a remarkably wide distribution, in Africa [32–34], South Asia [35–38], South-east Asia [39,40] and East Asia [41,42].

In “Konjaku Monogatari Shu” (The Tales of Times Now Past) which was compiled at the end of the Heian period (AD 794–1192), there are some well-known

stories of eating mushrooms [42], evidencing that Japanese people have been familiar with eating mushrooms since early times. While mushrooms were seasonal gifts from nature in the past, today they appear on the tables of even ordinary households year-round, due to the development of artificial cultivation techniques. Mushrooms are low in calories and rich in dietary fiber; therefore, they are gaining attention as a highly valued foodstuff. Supplementing fermented foods and probiotic fungi into a regular diet is called “Kin-katsu”. This practice is thought to be the engine that is boosting the current

consumption of mushrooms. The production of mushrooms in 2013 amounted to 225 billion yen in Japan [43], and the total exports of mushrooms, shiitake (*Lentinula edodes*), buna shimeji (*Hypsizygus marmoreus*), maitake (*Grifola frondosa*) etc., was 1448 tons valued at 0.4 billion yen in the same year, for Taiwan, Hong Kong, Malaysia, Singapore etc. [44]. A further increase of exports is expected. Currently, more than 20 species of mushrooms are cultivated, most of them saprophytic fungi. The fruit body of *T. eurrhizus* appears during a very short period at the end of rainy season, early June, in Okinawa [45]. Currently there is not much understanding or awareness of this mushroom among Japanese people. Nonetheless, it is crunchy and quite tasty, and could



Fig. 3 The fruit bodies of *T. eurrhizus*
from the comb in the termite nest

likely command a high market value if cultivated.

In order to provide a safe and steady supply of mushrooms to market, it is necessary to establish an artificial cultivation technology for the mushroom. Kinjo pointed out various issues for the development of artificial cultivation of *Termitomyces* fungi: 1) how to prevent fungus combs from contamination in the termite nests, 2) what status of combs is necessary to produce fruit bodies, 3) what factors determine the timing and frequency of production of fruit bodies, and 4) what location in the combs is suitable for forming the fruit body primordium [46]. Answering these questions for *T. eurhizus* will lead us into a greater understanding of the properties of this fungus, including the roles in its symbiosis with termites.

In this study, nutritional requirements and decay properties of selected *T. eurhizus* strains were investigated toward the goal of establishing of artificial cultivation method. First, some manageable strains were selected from *T. eurhizus* strains collected in Okinawa Prefecture (Chapter 1). These selected strains were used in the subsequent experiments. Second, the effects of carbon sources on the mycelial growth of *T. eurhizus* using 13 carbohydrates were evaluated (Chapter 2). Third, some strains of *T. eurhizus* were surveyed for their wood-decaying properties with three softwood and two hardwood species to discuss the possibility of using wood resources as a matrix for artificial cultivation (Chapter 3). Fourth, we surveyed the decay properties of *T. eurhizus* against moso bamboo, for which an effective and sustainable utilization has long been sought (Chapter 4). Finally, existence of one of lignin degradation enzymes, laccase, was detected using the Bavendamm test (Chapter 5).

Chapter 1

Screening of strains and media of *Termitomyces eurrhizus* collected in Japan

1.1 Introduction

Generally, the selection of strains is crucial in the mushroom cultivation, because the yield and quality of mushroom vary considerably with strains even under the same culture condition. In edible and herb mushrooms, selection of strains with desirable characters, for example suitable for mushroom bed cultivation, is a common procedure [47-52].

In order to artificially produce *Termitomyces* mushrooms, we have begun investigation of the properties of these species. Revealing the cultural characteristics would be conducive to clarify symbiosis of the fungi and the termites. However, it is known that *Termitomyces* fungi distributed in Japan generally show very slow-growing [46]. In this chapter, Chapter 1, many strains of *Termitomyces* were collected in Japan and tried to select strains for nutritional analyses in combination with artificial media so as to determine optimal culture conditions.

1.2 Materials and methods

Fungal strains

Twenty-seven strains of *Termitomyces* were served for the screening. All strains were isolated from fruit bodies. Firstly, all the strains were identified as *Termitomyces*

eurrhizus based on the morphological characteristics of their fruit bodies [6], and were confirmed to be the same species with DNA sequence analyses of internal transcribed spacer (ITS) regions 1 and 4 [53]. They were collected in Okinawa Prefecture, Japan, in 2004, 2005, 2007 and 2009. Six strains (T1-6) were from the Main Island of Okinawa, nine strains (T7-15) were from Ishigaki Island, eleven strains (T16-22 and T24-27) were from Iriomote Island, and one strain (T28) was from Kohama Island.

Media

Eight media were used in this experiment: (1) potato dextrose agar (PDA, Nissui Pharmaceutical Co. Ltd., Tokyo, Japan); (2) half concentration of PDA (1/2PDA); (3) corn meal agar (CMA, Becton, Dickinson and Company, Sparks, USA); (4) malt extract agar 1 (MA-1, malt extract: 2% [W/V], agar: 2% [W/V]); (5) malt extract agar 2 (MA-2, malt extract: 2% [W/V], peptone: 0.5% [W/V], agar: 2% [W/V]); (6) Matsutake (EBIOS® [Asahi food & healthcare Co. Ltd., Tokyo, Japan]: 0.5% [W/V], glucose: 2% [W/V], agar: 2%[W/V]); (7) potato carrot agar (PCA); and (8) hay extract agar (HA). All media were fashioned as ϕ 90-mm plates. The methods for PCA and HA preparations were as follows [54]:

PCA: 20 g potato and 20 g carrot were grated and boiled in 1000 ml distilled water for 1 h. The filtrate was adjusted to 1000 ml with 2% (W/V) agar.

HA: 50 g hay (Timothy Grass [*Phleum pratense*]) in 1000 ml distilled water was autoclaved at 121°C for 30 min. The filtrate was adjusted to 1000 ml with 0.2%(W/V) K_2HPO_4 and 2%(W/V) agar.

Mycelial growth

All the strains were pre-cultured on the same media, and 7-mm diameter pieces were taken with a cork-borer and inoculated to the plate media. The plate media were cultured in the dark at 26°C for a maximum 50 days. Diameter of the mycelium mats was measured at 5-day intervals in two vertical directions, and was averaged. The growth rate was calculated as an average speed (mm/day) over the period from day 15-30 post-inoculation, when the most of strains showed a linear growth. When mycelium mats did not grow over 7-mm diameter on the pre-culture media by day 60 post-inoculation, these strains were not tested on those media. The tested strains were slow-growing, and some of them exhibited frequent stagnation or sudden suppression of growth. In some cases, they died during cultivation. From these characteristics, the strains were rated as: A - easy to handle; B - medium; and, C - difficult to handle. The test was repeated 3 times for each medium ($n=3$).

Data analysis

The mycelial growth rates were analysed using an analysis of variance (ANOVA) and Tukey-Kramer tests for each medium, respectively ($p < 0.05$).

1.3 Results and discussion

The growth rates of all the strains and handling rates are shown in Table 1 and the growth lines on 8 media are shown in Fig. 1.1. There was considerable variety in the growth of the strains on each medium. All the strains grew very slowly and were sensitive to contamination; some strains stopped growing suddenly and died. Most of

the strains had dense and very short aerial hyphae on the mycelial mats, and some strains had little aerial hyphae. The surface of the mycelial mats showed white color in the early growing stage (Fig. 1.2-A), but changed to pale brown after long-term cultivation. In addition, some strains often showed radial or irregular folds on their mycelial mats (Fig. 1.2-B).

All *Termitomyces* strains have been kept on the PDA after collection, and we used PDA as the standard medium. Other than the PDA, only the Matsutake medium showed sufficient growth in all strains. The other media showed poor growth in 1-3 strains. For the quantitative comparison, a 0.2 mm/day difference was set up as the criterion. Seven strains showed faster growth on 1/2 PDA than on PDA. However, there was no strain representing faster growth on the PDA than on 1/2 PDA. Furthermore, twelve strains displayed a higher growth rate on MA-1 than on MA-2. However, only one strain recorded faster growth on MA-2 than on MA-1. These results suggested that *T. eurhizus* tended to prefer nutritionally poor media to rich media.

Eight strains exhibited faster growth on CMA among all the media, and the growth of 22 strains on CMA was faster than on PDA. The mycelial mats of many strains on CMA, however, were clearly thin in comparison with those on the other media. Seventeen strains showed the lowest growth rates on MA-2, and 15 strains demonstrated the fastest growth rates on HA among all the media. The mean growth rate on CMA and on HA were 1.4 mm/day, those were most fast than on other media. But there were some strains that did not show sufficient growth on those media.

From these results, we selected 4 strains for further study. T3, from the strains of the Main Island of Okinawa, had low media specificity (0.9-1.5 mm/day growth rates on all media) and grew tractably. From the strains of Ishigaki Island, T11 was easily

handled with similar growth rates to the other strains. In addition, this strain comparatively started to grow active after inoculation. T25 and T26 from the strains of Iriomote Island were also selected. These two strains grew very fast on any media. T25 was delicate and fragile, but had low media specificity (1.4-1.8 mm/day growth rates on all media), whereas T26 was easy to handle. For the selection of media, the Matsutake medium was evaluated as a good candidate for future experiments and keeping *Termitomyces* strains due to its availability to all the strains.

Table 1.1 Mycelial growth rates of all strains of *Termitomyces eurhizus*

Collecting place	Strains	Growth rates (mm / day)*								Data range	Handling***
		PDA	1/2PDA	CMA	MA-1	MA-2	Matsutake	PCA	HA		
Mam Island	T1	0.8 c	0.8 c	1.0 c	0.9 c	0.5 d	1.0 c	0.9 cd	1.3 c	0.5-1.3	B
	T2	1.1 b	1.0 bc	1.6 a	1.0 b	0.9 c	1.3 b	1.1 bc	1.6 b	0.9-1.6	C
	T3	1.1 b	1.1 bc	1.4 b	0.9 c	0.9 bc	1.4 b	1.1 bc	1.5 b	0.9-1.5	B
	T4	0.8 c	0.7 c	1.0 c	1.1 b	0.5 d	0.9 cd	1.1 bc	1.2 c	0.5-1.2	B
	T5	1.0 b	1.2 bc	1.2 bc	1.1 b	1.0 bc	1.1 c	0.6 d	0.5 d	0.5-1.2	B
	T6	1.0 b	1.0 bc	1.2 bc	0.7 c	0.9 c	1.0 cd	1.0 cd	1.7 ab	0.7-1.7	C
Ishigaki Island	T7	1.4 a	1.7 a	1.6 ab	1.4 a	0.7 d	1.6 a	1.7 a	1.1 c	0.7-1.7	C
	T8	1.0 b	1.1 bc	1.8 a	1.1 b	1.0 b	1.1 cd	1.1 bc	1.3 c	1.0-1.8	B
	T9	1.1 b	1.4 ab	1.3 b	1.1 ab	0.8 cd	1.4 b	1.1 bc	1.8 a	0.8-1.8	C
	T10	0.9 b	0.9 bc	1.2 bc	1.3 ab	1.2 b	1.3 b	1.1 bc	1.1 c	0.9-1.3	B
	T11	1.3 a	1.5 a	1.6 a	1.1 ab	0.7 cd	1.1 c	1.7 a	1.5 b	0.7-1.7	B
	T12	0.8 bc	0.8 c	1.5 ab	1.1 b	0.8 cd	0.9 d	1.5 a	0.8 d	0.8-1.5	C
	T13	1.1 b	1.2 bc	1.5 ab	1.2 ab	1.2 b	1.2 c	1.4 b	1.5 b	1.1-1.5	B
	T14	1.0 b	1.1 bc	***	0.9 bc	0.9 bc	1.2 c	***	1.4 b	0.9-1.4	B
	T15	0.7 c	***	***	***	***	0.7 d	***	1.1 c	0.7-1.1	C
	T16	1.3 ab	1.2 ab	1.7 a	0.6 c	0.6 cd	1.1 c	1.5 a	***	0.6-1.7	C
Iriomote Island	T17	0.9 bc	1.0 bc	0.9 c	0.9 c	0.9 c	1.1 c	1.0 cd	1.7 ab	0.9-1.7	A
	T18	1.2 b	1.1 bc	1.4 ab	1.3 ab	1.4 a	0.9 cd	1.1 bc	2.1 a	0.9-2.1	C
	T19	0.9 bc	0.9 bc	***	1.1 b	0.7 cd	1.0 cd	***	1.2 bc	0.7-1.2	C
	T20	0.8 c	0.9 bc	1.3 bc	0.9 bc	0.8 cd	1.1 c	1.2 bc	0.6 d	0.6-1.3	B
	T21	1.4 a	1.6 a	1.5 ab	1.4 a	1.3 ab	1.4 ab	1.7 a	1.4 b	1.3-1.7	A
	T22	1.2 ab	1.3 ab	1.3 bc	1.1 b	0.9 bc	1.1 c	1.7 a	0.8 d	0.8-1.7	B
	T24	1.0 b	1.2 bc	1.2 bc	1.1 b	0.8 cd	1.1 cd	1.3 bc	1.4 b	0.8-1.4	C
	T25	1.5 a	1.6 a	1.8 a	1.4 a	1.4 a	1.7 ab	1.7 a	1.5 b	1.4-1.8	C
	T26	1.4 a	1.6 a	1.7 a	1.5 a	1.3 ab	1.7 a	1.9 a	2.3 a	1.3-2.3	A
	T27	1.1 b	1.1 bc	1.3 bc	1.1 b	1.0 bc	1.0 cd	1.0 bc	1.7 b	1.0-1.7	B
	T28	1.0 b	0.9 bc	1.3 bc	***	***	1.4 b	0.9 cd	1.3 b	0.9-1.4	C
	Data range	0.7-1.5	0.7-1.7	0.9-1.8	0.6-1.5	0.5-1.4	0.7-1.7	0.6-1.9	0.5-2.3		
	Mean	1.1	1.2	1.4	1.1	0.9	1.2	1.3	1.4		

The values labeled with different letters are significantly different by Tukey-Kramer ($p < 0.05$), $n=3$.

*: Averages of day 15-30 post-inoculation

**: Did not grow over ϕ 7 mm by day 60 post-inoculation on the pre-culture media

***: Handling rates: A - easy to handle; B - medium; C - difficult to handle

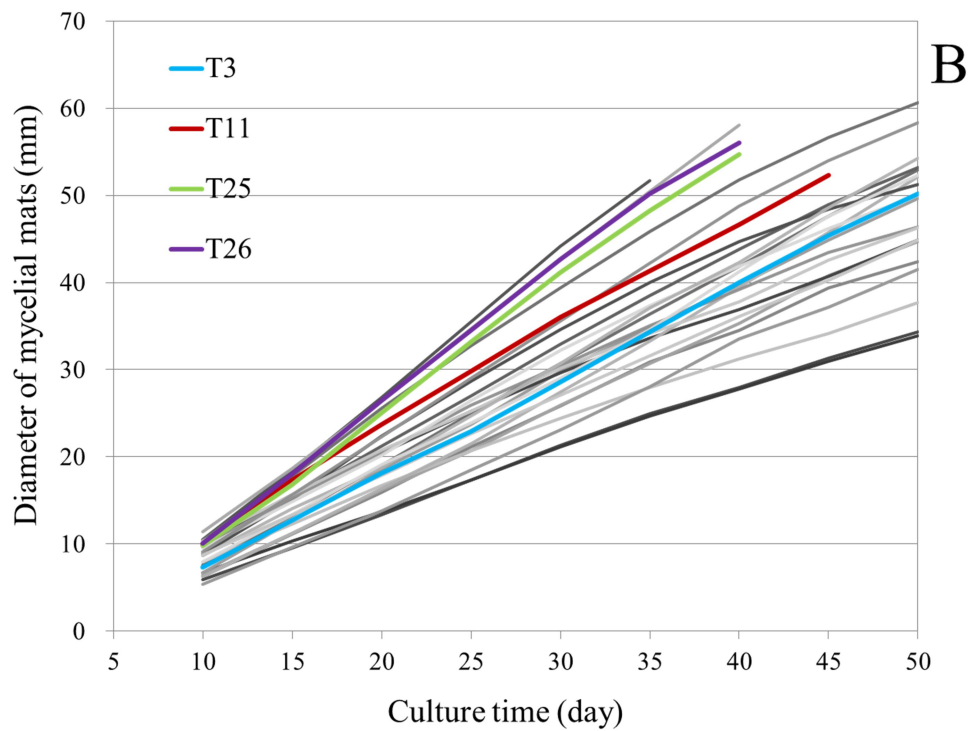
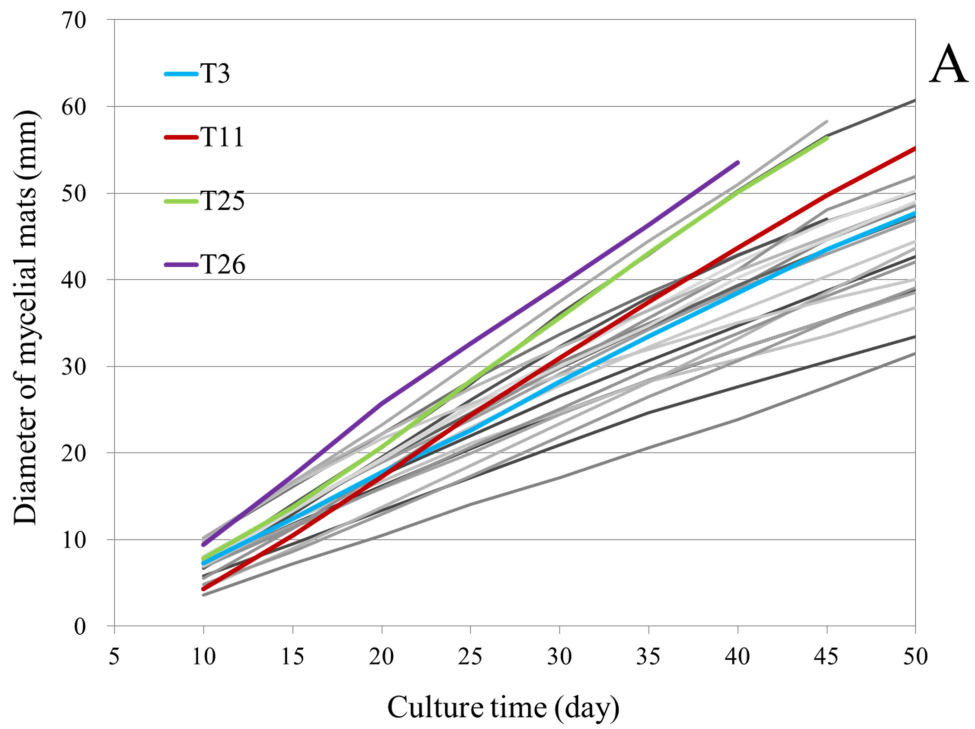


Fig. 1.1 Growth of *T. eurhizus* strains on 8 media

A: PDA, B: 1/2PDA, C: CMA, D: MA-1, E: MA-2, F: Matsutake, G: PCA, and H: HA.

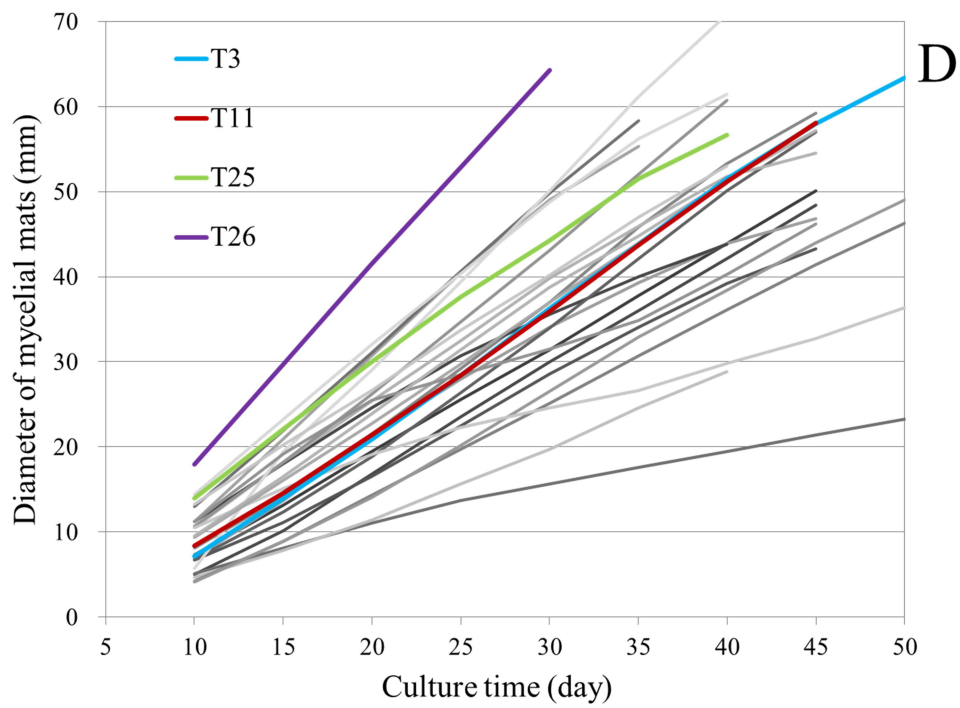
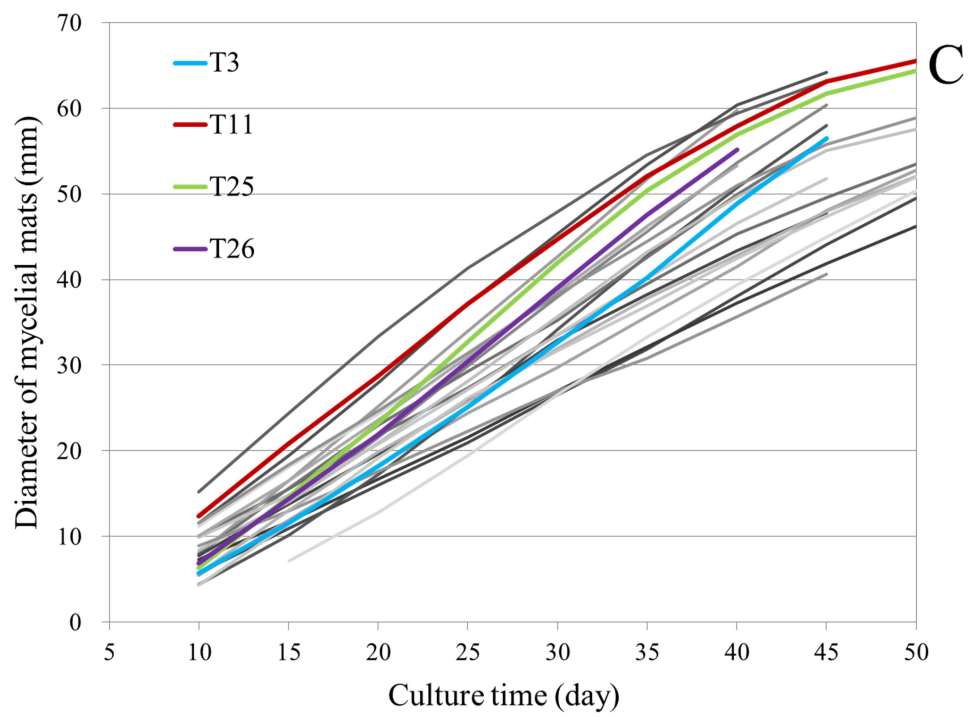


Fig. 1.1 Growth of *T. eurhizus* strains on 8 media (continued)

A: PDA, B: 1/2PDA, C: CMA, D: MA-1, E: MA-2, F: Matsutake, G: PCA, and H: HA.

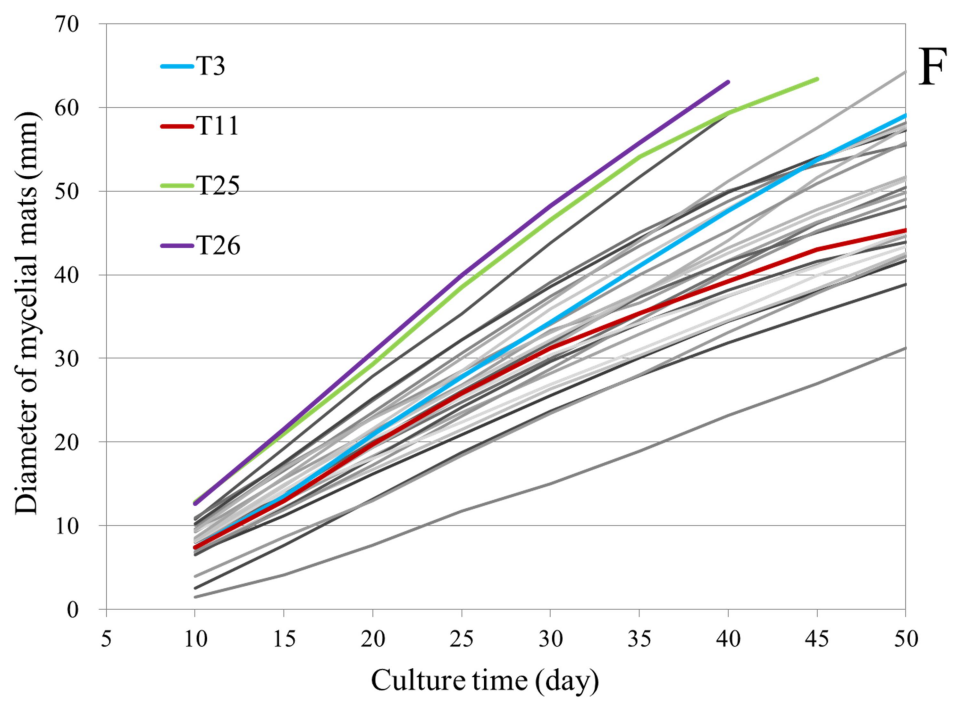
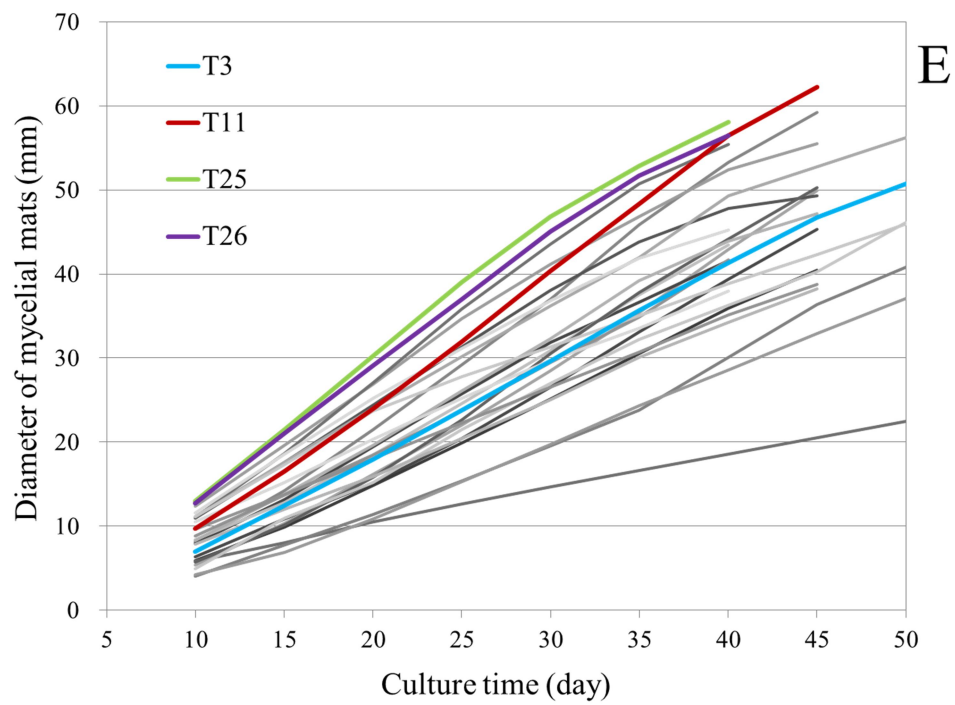


Fig. 1.1 Growth of *T. eurrhizus* strains on 8 media (continued)

A: PDA, B: 1/2PDA, C: CMA, D: MA-1, E: MA-2, F: Matsutake, G: PCA, and H: HA.

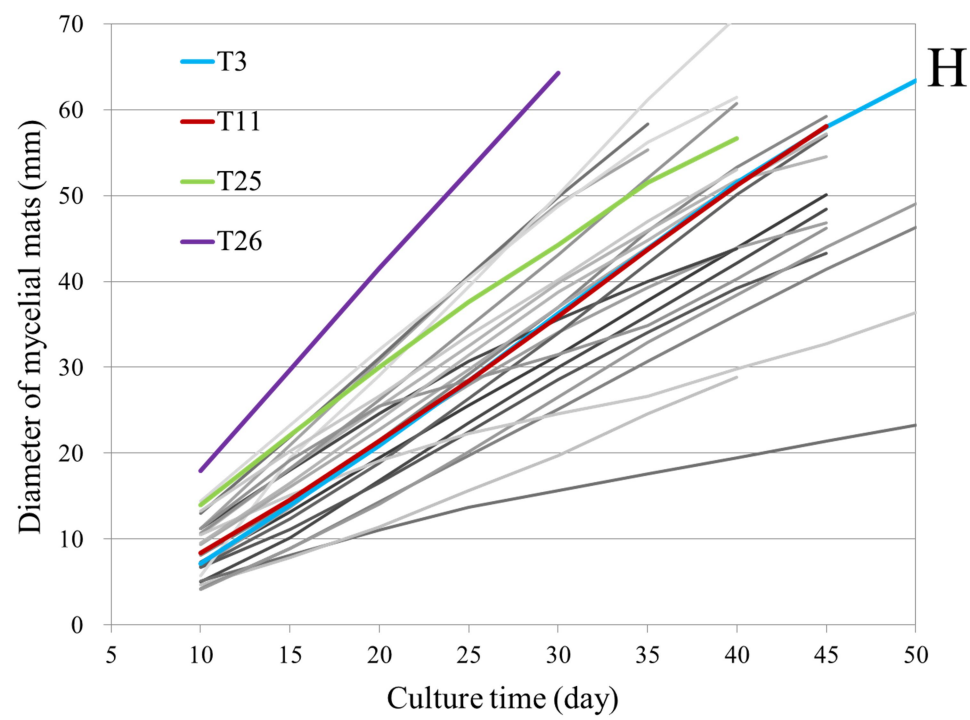
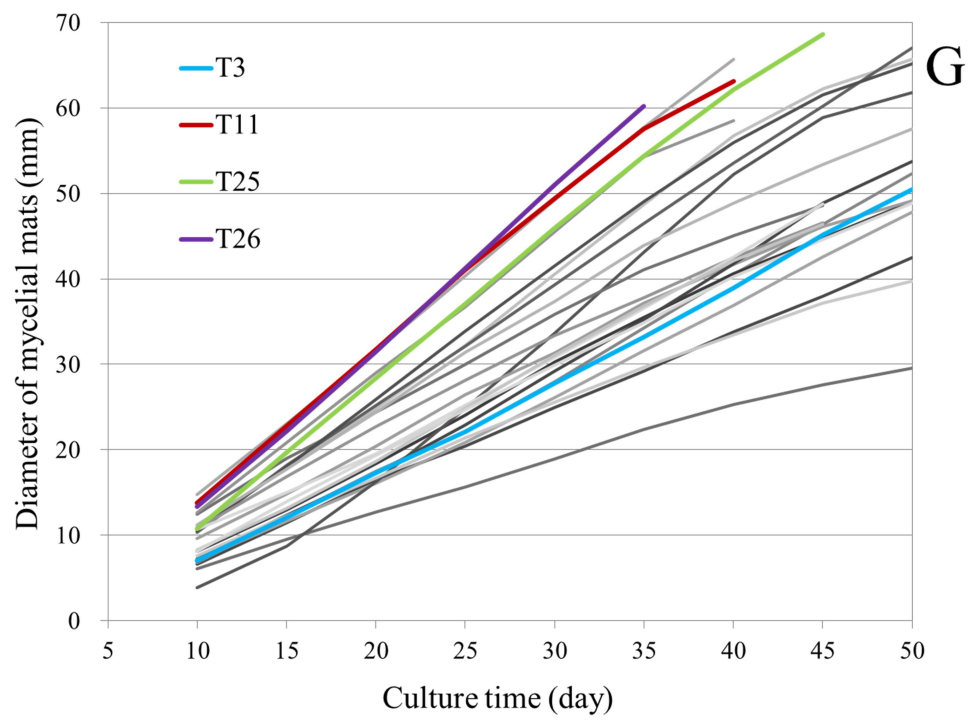


Fig. 1.1 Growth of *T. eurrhizus* strains on 8 media (continued)

A: PDA, B: 1/2PDA, C: CMA, D: MA-1, E: MA-2, F: Matsutake, G: PCA, and H: HA.

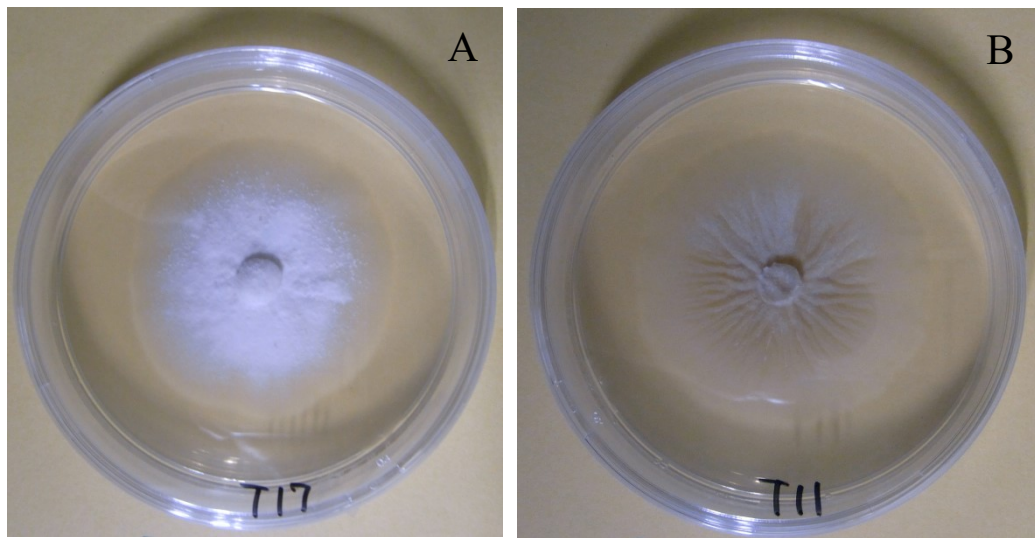


Fig. 1.2 Mycelial mats of *T. eurrhizus* on PDA

A: strain T17, 30 days post inoculation; B: strain T11, 30 days post inoculation.

1.4 Summary

Twenty-seven strains of *Termitomyces eurrhizus*, all from Okinawa Prefecture, Japan, were served for screening with eight artificial media. Four strains were selected for their growth, media-specificity and handling for future experiments on nutritional analyses. In addition, the Matsutake medium was found to have a good affinity to these strains.

Chapter 2

Effects of carbohydrate substrates on vegetative mycelial growth of *Termitomyces eurrhizus*

2.1 Introduction

There are many environmental factors affecting vegetative growth and fruit body formation of Basidiomycetes. Some edible mushroom species have problems in artificial cultivation, resulting in impeding the popularization of the cultivation of the target mushroom. To resolve these problems, clarification of physiological properties of the mushroom is indispensable. There have been many reports of nutritional requirements, especially carbon source and nitrogen source, of various edible mushrooms [55-61].

In Chapter 1, four strains of *T. eurrhizus* were selected from 27 strains collected from Okinawa Prefecture. In this chapter, Chapter 2, the author examined 13 carbohydrates for the artificial media to elucidate the nutritional preferences of *T. eurrhizus* selected strains.

2.2 Materials and methods

Fungal strains

Four strains of *T. eurrhizus* were used in this experiment (T3, T11, T25 and T26). The strains were screened from 27 strains collected in Okinawa Prefecture, Japan, in terms of rapid growth rate and low media-specificity (Chapter 1). The selected strains

were as follows: T3 from Okinawa's Main Island in 2005, T11 from Ishigaki Island in 2005, and T25 and T26 from Iriomote Island in 2004. They were identified as *T. eurrhizus* based on the morphological characteristics of their fruiting bodies [22] and DNA sequence analyses of internal transcribed spacer (ITS) regions 1 and 4 [47].

Media

Matsutake medium was employed as the base medium, because many strains of *Termitomyces* showed faster growth on this than on any other simple media in previous experiments (Chapter 1). The medium was prepared according to the media constitution guidelines of the Biological Resource Center of the National Institute of Technology and Evaluation (NBRC)[61]; EBIOS[®] (Asahi Food and Healthcare, Tokyo, Japan): 0.5% (W/V); glucose: 2% (W/V); agar: 2% (W/V). The glucose was replaced with 12 other carbohydrates: four monosaccharides (xylose, galactose, fructose, and mannose), five disaccharides (sucrose, maltose, cellobiose, lactose, and trehalose), two starches (soluble starch [s-starch] and corn starch [c-starch]), and cellulose. Insoluble carbohydrates, c-starch and cellulose, were suspended in the media.

Mycelial growth

Agar plugs (7 mm in diameter) of each strain were removed from mycelial cultures (Matsutake medium), transferred to new agar plates, and then incubated at 26°C in the dark. The diameters of mycelial colonies were measured bi-directionally at 10, 15, 20, 25, 30, 35 and 40 days post-inoculation. Mycelial lengths were an average of the bi-directional value. Daily mycelial growth rates were calculated from the mycelial length data of 15 days when the growth was linear: from 20–35 days for T3 and T11,

and from 15–30 days for T25 and T26. Fifteen repetitions of each carbohydrate were employed for each strain.

Data analysis

The mycelial growth rates were analyzed using an analysis of variance (ANOVA) and Tukey-Kramer tests ($p < 0.05$)[62].

2.3 Results and Discussion

The mycelial colonies of all four *T. eurhizus* strains were white in color. While the mycelia grew into the medium, the aerial mycelia were extremely short or absent depending on the strain. The color and appearance of mycelial colonies in each strain were similar, regardless of carbohydrate employed.

Table 2.1 shows the daily mycelial growth rates for all 4 strains/13 carbohydrate combinations. Growth lines of 4 strains on each carbohydrate medium show in Fig. 2.1. Among the 13 carbohydrates, T3 showed the most rapid growth on fructose, sucrose and maltose media (1.5–1.6 mm/day), followed by glucose, mannose, and trehalose (1.4 mm/day). However, the growth rates on the latter three did not significantly differ from that on fructose. This strain showed inferior growth on the galactose and lactose media (0.5 mm/day), these trends were also found on growth lines (Fig. 2.1-A). The other carbohydrates showed medium growth rates (1.0–1.3 mm/day). For T11, rapid growth was observed on seven carbohydrate media: fructose, mannose, sucrose, maltose, cellobiose, trehalose and s-starch (1.4–1.6 mm/day). Xylose and lactose suppressed the growth of this strain (0.5–0.7 mm/day) compared to the other carbohydrates (Fig. 2.1-B).

Glucose, c-starch and cellulose showed medium growth rates (1.1 mm/day). T25 exhibited rapid growth on six carbohydrate media: glucose, fructose, mannose, maltose, trehalose and s-starch (2.0–2.2 mm/day). The growth on sucrose (1.9 mm/day) did not differ significantly from that on the above carbohydrates except fructose. Galactose, cellobiose and lactose showed lower growth rates (0.9–1.0 mm/day) than the other carbohydrates. Xylose, c-starch and cellulose showed medium growth rates (1.2–1.5 mm/day). T26 displayed increased growth with the addition of fructose, mannose and maltose into the media (1.9–2.2 mm/day). Growth on galactose, sucrose, trehalose and c-starch media did not significantly differ from fructose and maltose (1.8 mm/day). This strain demonstrated lower growth rates on four carbohydrate media: xylose, cellobiose, lactose and cellulose (1.0–1.3 mm/day). Glucose and s-starch showed medium growth rates (1.7 mm/day).

In general, saprophytic fungi utilize many carbohydrates. The degree of catabolism, however, differs with the species or strain [58,63-66]. In regard to the *T. eurrhizus* used in this study, all the strains showed the most rapid growth on fructose and maltose media. In addition, all strains showed positive growth on mannose, sucrose and trehalose media.

A previous study reported that many *Termitomyces* strains collected in Africa utilized fructose and mannose as the sole carbon source, but did not grow on maltose and sucrose [67]. Additionally, the optimal carbohydrate sources of a strain of *T. albuminosus* in China were reported to be glucose and sucrose, although this strain could catabolize maltose and soluble starch to a similar extent [68]. Taking these results into account, it is likely that fructose and mannose in Matsutake medium are the best carbohydrates for the growth of *T. eurrhizus* strains T3, T11, T25 and T26, obtained in

Okinawa Prefecture. Our findings suggest that waste materials rich in fructose or mannose, such as wheat straw with fructan [69] or konjac foods by-products with mannan [70], are potential productive substrates for the artificial cultivation of *Termitomyces* fungi.

Sucrose and maltose seem to be favorable but strain-dependent carbohydrates. In Okinawa, *O. formosanus* is known as a pest of sugarcane and sweet potato containing sucrose and starch. Three strains (T3, T11 and T25) showed no differences in growth rates between c-starch and cellulose media, and T26 demonstrated more rapid growth on c-starch medium than on cellulose medium. In addition, the *O. formosanus* termite attacks agronomic crops such as wheat, rice and cotton and numerous trees in China [29]. The *T. eurhizus* used in this study were able to catabolize sucrose and maltose, and might utilize starch and cellulose similarly.

According to a study published by Itakura et al., *O. formosanus* workers collected in Ishigaki Island, Okinawa Prefecture, contained 3.47% trehalose [71]. Trehalose, a main carbohydrate in insect blood sugar, might be supplied from the termite to the fungal comb.

The results of this study reveal that *T. eurhizus* from Japan is likely to be characterized by high catabolism of fructose, mannose, sucrose, maltose and trehalose, and by poor catabolism of lactose. The former carbohydrates are potential good candidate additives in the artificial cultivation/ mushroom production of *T. eurhizus*.

Table 2.1 Mycelial growth rate (mm/day)

Carbohydrate	Fungal strain								
	T3			T11			T25		
	Mean	SD		Mean	SD		Mean	SD	
Glucose	1.4	0.12	bc	1.1	0.13	c	2.0	0.17	ab
Xylose	1.0	0.08	e	0.5	0.16	d	1.5	0.20	c
Galactose	0.5	0.09	f	1.2	0.26	bc	0.9	0.12	f
Fructose	1.5	0.09	ab	1.5	0.10	ab	2.2	0.07	a
Mannose	1.4	0.14	bc	1.5	0.06	ab	2.1	0.15	ab
Sucrose	1.6	0.19	a	1.6	0.07	a	1.9	0.35	b
Maltose	1.5	0.13	a	1.5	0.14	ab	2.0	0.20	ab
Cellobiose	1.1	0.06	e	1.4	0.06	ab	1.0	0.14	ef
Lactose	0.5	0.12	f	0.7	0.59	d	1.0	0.16	ef
Trehalose	1.4	0.10	bc	1.4	0.15	ab	2.1	0.27	ab
S-starch	1.3	0.15	cd	1.4	0.15	ab	2.0	0.14	ab
C-starch	1.2	0.11	de	1.1	0.17	c	1.3	0.48	cd
Cellulose	1.2	0.08	de	1.1	0.15	c	1.2	0.22	de

*Mycelial growth rates were calculated at 20–35 days post-inoculation for T3 and T11, and at 15–30 days for T25 and T26.

The values labeled with different letters are significantly different ($p < 0.05$).

$n=15$

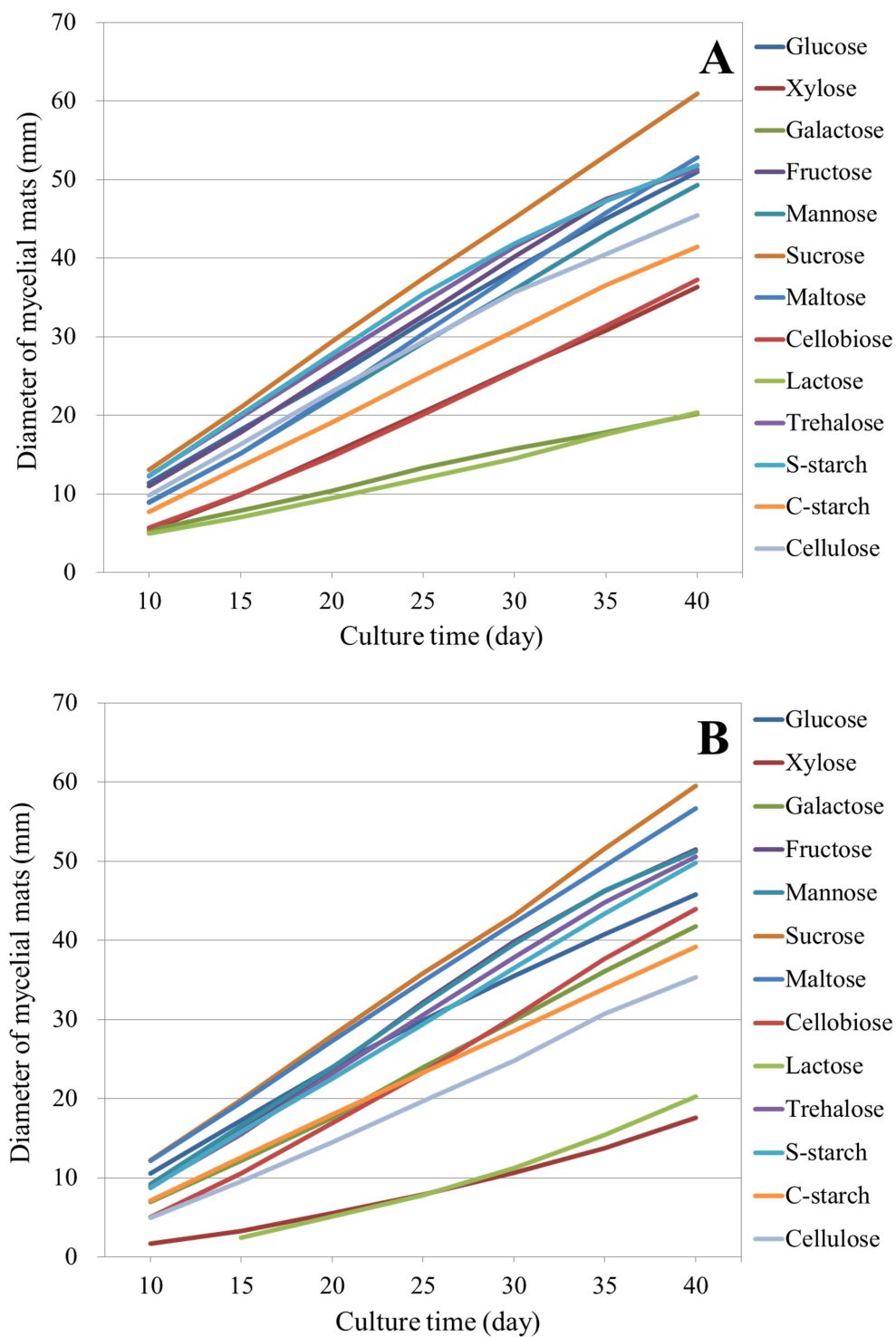


Fig. 2.1 Growth of 4 *T. eurhizus* strains on 13 carbohydrates media

A: T3, B: T11, C: T25, and D: T26

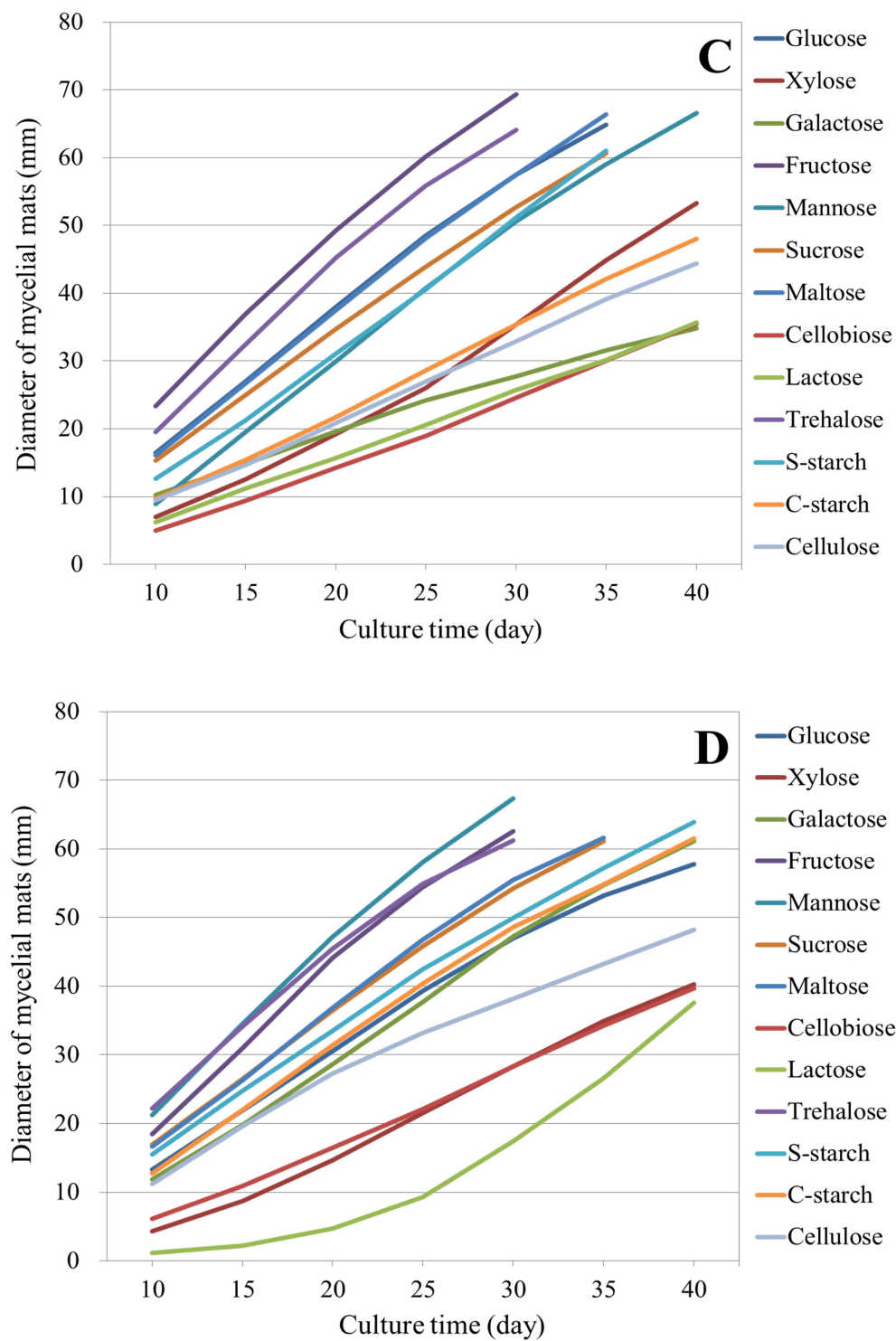


Fig. 2.1 Growth of 4 *T. eurhizus* strains on 13 carbohydrates media (continued)

A: T3, B: T11, C: T25, and D: T26

2.4 Summary

We studied the effect of carbohydrates on the growth rate of *Termitomyces eurrhizus* collected from Okinawa Prefecture, Japan. Four test strains were grown on the base media with the addition of 13 carbohydrates, respectively. These strains showed high catabolism of fructose, mannose, sucrose, maltose and trehalose. Two strains showed relatively high catabolism of soluble starch. However, all strains tended to grow poorly on lactose medium.

Chapter 3

Wood decaying properties of *Termitomyces eurhizus*

3.1 Introduction

In the preceding chapters, Chapter 1 and Chapter 2, four strains of *T. eurhizus* were selected from 27 strains collected in Okinawa Prefecture, Japan, for their rapid growth and easy handling [72], and the feasibility of many carbohydrate substrates was evaluated for the artificial media, respectively.

In this chapter, Chapter 3, the wood-decaying properties of four strains of *T. eurhizus* was surveyed. The major aim of this chapter was to evaluate wood decaying ability of *T. eurhizus* with four strains under the same culture condition. In addition, the feasibility of wood materials to artificial media was also discussed. It would also reveal some unknown relationships between host termites and *Termitomyces*. Among the five wood species used in the present study, *Pinus densiflora* is generally preferred by termites. *Cryptomeria japonica* and *Chamaecyparis obtusa* are dominant softwood plantation trees in Japan. *Fagus crenata* is commonly used in artificial cultivation of mushrooms in Japan. Finally, *Quercus miyagii* is a hardwood species distributed in the southern islands of Japan, where *T. eurhizus* and *O. formosanus* are also present [73].

3.2 Materials and methods

Fungal strains

Four strains of *Termitomyces eurhizus* (T3, T11, T25 and T26) were used in this study. They were collected in Okinawa Prefecture, Japan in 2004 and 2005, and isolated from fruit bodies. As controls, a white-rot fungus, *Trametes versicolor* (FFPRI 1030), and a brown-rot fungus, *Fomitopsis palustris* (FFPRI 0507), were also employed. These two strains were designated as decay test fungi according to the Japan Industrial Standard [74] (JIS K 1571-2010).

Wood samples

Heartwood and sapwood of 3 softwood species, *Pinus densiflora*, *Cryptomeria japonica*, and *Chamaecyparis obtusa* and one hardwood species, *Fagus crenata*, were employed for an 8-week decay test. *C. obtusa* is highest decay durability, followed by *C. japonica*, *P. densiflora* and *F. crenata* [75]. For the 12-week decay test, heartwood and sapwood of the above 4 wood species and the hardwood *Quercus miyagii* were used. Wood species was same, but samples for the 8-week test were taken from different logs than samples for 12-week test. The sample size was 10 mm (R) × 10 mm (T) × 5 mm (L). This small size of samples was determined by the slow growth of *T. eurhizus*, 1.1-1.2 mm/day on cellulose containing medium (Chapter 2)[76]. They were dried at 60°C for 48 h to determine the oven-dried weight before the tests according to the methods of JIS K 1571-2010 [74].

Decay tests

Fungal inoculums were prepared with potato dextrose agar (PDA; Nissui, Tokyo, Japan) plates. A 7-mm-diameter inoculum was taken from the plate, and was inoculated on 100 ml of agar medium (glucose 4% [w/v], malt extract 1.5% [w/v], peptone 0.3%

[w/v], agar 2% [w/v]) in a 450-ml screwcap glass bottle. After the mycelia grew over the media surface, three test wood blocks were set on the mycelial mat. The bottle-caps were tightly sealed because *T. eurrhizus* is extremely sensitive to contamination, then kept for 8 or 12 weeks at 26°C in the dark. After exposure, the mycelia that covered the samples were removed gently, and the samples were dried at 60-°C for 48 h and weighed to determine the mass loss. The numbers of repetitions were 6 and 9 for the 8-week and 12-week trials, respectively.

Scanning electron microscopic (SEM) observation

The 8-week decay samples were subjected to microscopic observation. The samples were dehydrated using a solvent displacement method by soaking in an ethanol series (10%, 20%, 40%, 60%, 80%, 90% and 100% [v/v]), acetone and pentane for 10 minutes, respectively. Each sample was sliced with a fresh razor and coated with gold for observation under a SEM (JSM-5310; JEOL, Tokyo, Japan) at 10 kV. Both surfaces and core portions of the samples were observed (Fig. 3.1).

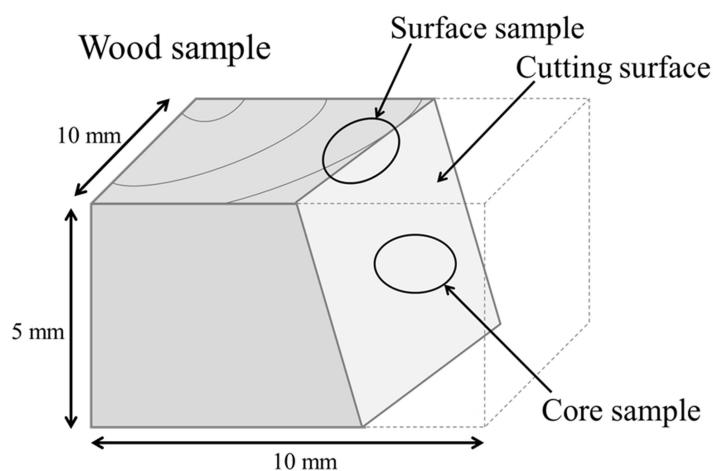


Fig. 3.1 Objects of SEM observation

Chemical analyses

For chemical analyses, 12-week decay samples were ground into 40-mesh-pass size by a high-speed blender (VM0113; Vitamix, Cleveland, OH). Lignin content was determined by the Klason method [77]. Using the filtrates from the Klason lignin analysis, holocellulose was quantitated as a total sugar by the phenol-sulfuric acid method [78]. Glucose was used as a standard. The lignin decrease rates (LDRs) and total sugar decrease rates (SDRs) were calculated from these results using the following pair of equations:

$$\text{LDR} = [L_1 - (100 - \text{MLR}) / 100 \times L_2] / L_1 \times 100$$

Where L_1 is the average lignin content of sound wood samples ($n=3$), L_2 is the lignin content of the individual decayed wood sample, and MLR is the mass-loss rate of the sample, and

$$\text{SDR} = [S_1 - (100 - \text{MLR}) / 100 \times S_2] / S_1 \times 100$$

Where S_1 is the average total sugar content of sound wood samples ($n=3$), S_2 is the total sugar content of the individual decayed wood sample, and MLR is the mass-loss rate of the sample.

Statistical analysis

The mass-loss rates (MLRs) of the decay tests, LDRs and SDRs were analyzed using analysis of variance (ANOVA) and Tukey-Kramer tests ($p < 0.05$).

3.3 Results and discussion

The MLRs of the wood samples after the 8-week decay tests are displayed in Table 3.1.

In the heartwood of *P. densiflora*, there were no significant difference in MLRs between the strains of *T. eurhizus* and the control strain *T. versicolor*, with the exception of T25. T25 showed lower MLRs in both heartwood and sapwood than those of the other *T. eurhizus* strains. In all the *T. eurhizus* strains, the MLRs of sapwood (8.7%, 7.6%, 6.0% and 8.3%) were significantly higher than those of heartwood (6.7%, 6.6%, 3.5% and 6.8%).

There was a wide variety in the strains of *T. eurhizus* regarding decay in the heartwood of *C. japonica*. The MLR by T11 (10.1%) was not significantly different from that of *T. versicolor*, which had the highest rate (11.5%). Meanwhile, T25 showed the lowest MLR (4.4%), and those of T3 and T26 were not significantly different. The MLR of T26 (5.6%) was not significantly different from that of the brown-rot fungus *F. palustris* (8.0%). In sapwood, *T. eurhizus* exhibited lower MLRs than those of *T. versicolor* and *F. palustris*. In addition, T11 and T25 did not display a significant difference in MLRs between heartwood and sapwood. However, T3 and T26 showed lower MLRs in heartwood than in sapwood.

The various strains of *T. eurhizus* also showed a variety of MLRs for heartwood of *C. obtusa*. T11 had the highest MLR (10.2%), while T25 had the lowest MLR (5.2%). There was no significant difference between the MLRs of T25 and *F. palustris* (5.2%), but both were significantly lower than the MLRs of the other 3 strains. In sapwood, two strains (T3 and T26; 11.7% and 12.0%, respectively) showed no significant difference from *T. versicolor* (14.5%) and other 2 strains (T11 and T25; 8.7% and 7.2%) showed

Table 3.1 Mass-loss rates of 4 wood samples after 8-week decay tests

Strains	<i>P. densiflora</i>				<i>C. japonica</i>			
	Heartwood		Sapwood		Heartwood		Sapwood	
	Mass loss (%)	SD	Mass loss (%)	SD	Mass loss (%)	SD	Mass loss (%)	SD
T3	6.7	0.4 B	8.7	0.2 A	4.7	1.8 D	8.8	2.2 CD
T11	6.6	0.7 B	7.6	0.1 AB	10.1	1.6 AB*	7.5	3.0 CD
T25	3.5	0.4 C	6.0	0.3 B	4.4	1.3 D*	5.5	1.5 D
T26	6.8	0.5 B	8.3	0.4 A	5.6	0.7 CD	10.8	2.6 C
<i>T. versicolor</i>	8.3	0.8 B*	7.0	1.3 AB	11.5	1.1 A	16.7	0.6 B
<i>F. palustris</i>	16.3	2.0 A	5.5	2.0 B	8.0	1.4 BC	22.7	0.7 A

Strains	<i>C. obtusa</i>				<i>F. crenata</i>			
	Heartwood		Sapwood		Heartwood		Sapwood	
	Mass loss (%)	SD	Mass loss (%)	SD	Mass loss (%)	SD	Mass loss (%)	SD
T3	7.8	0.5 B	11.7	0.4 BC	2.3	0.2 C	5.3	0.2 B
T11	10.2	0.4 A*	8.7	1.2 C	2.3	0.5 C	3.7	0.3 B
T25	5.2	1.1 C	7.2	0.8 D	0.9	0.5 C	3.2	0.5 B
T26	9.4	0.8 AB	12.0	0.8 B	2.4	0.4 C	4.7	0.2 B
<i>T. versicolor</i>	7.8	1.5 B	14.5	2.2 B	15.3	3.2 A*	17.9	3.6 A
<i>F. palustris</i>	5.2	1.1 C	21.1	2.2 A	8.5	6.6 B*	15.2	1.0 A

SD: Standard deviation of mass-loss rate

*: There was no significant difference between heartwood and sapwood (t-test $p>0.05$).

The same characters indicate no significant difference by Tukey-Kramer test ($p>0.05$).

lower than *T. versicolor*. All the strains had lower MLRs than that of *F. palustris* (21.1%).

In the case of the hardwood *F. crenata*, all strains of *T. eurrhizus* showed unexpected results. This wood is known to be susceptible to decay fungi [79], yet the MLRs of the 4 strains were 2.3%, 2.3%, 0.9% and 2.4% in heartwood, and 5.3%, 3.7%, 3.2% and 4.7% in sapwood, respectively. These values were significantly lower than those of the two control fungi, *T. versicolor* and *F. palustris*, in both heartwood (15.3% and 8.5%) and sapwood (17.9% and 15.2%).

In this test, strain T25 showed the lowest MLRs in all wood samples with the exception of *P. densiflora* sapwood. In many cases, the MLRs of *T. eurrhizus* in sapwood were higher than those of heartwood, and in some cases, there was no significant difference in MLRs between heartwood and sapwood.

The MLRs in 12-week decay tests are also shown in Table 3.2. In all test bottles, the mycelia of strain T25 died before finishing the test period; therefore, the results in T25 were omitted.

For *P. densiflora*, all fungi showed similar MLRs in the heartwood; in the sapwood; only T11 had lower MLR (4.8%) than the others. T11 had a lower MLR in sapwood than in heartwood, but the others showed no significant difference between heartwood and sapwood.

For *C. japonica* heartwood, T11 had a significantly lower MLR (1.1%) than the other 2 strains of *T. eurrhizus* (2.2% and 4.9%). All *T. eurrhizus* strains showed lower MLRs than that of *F. palustris* (9.4%), but there was no significant difference from that of *T. versicolor*. T26 (8.4%) showed no significant difference from *F. palustris* (9.9%) in sapwood, and both were higher than those of the other strains. The MLRs of T3 and

Table 3.2 Mass-loss rates of 5 wood samples after 12-week decay tests

Strains	<i>P. densiflora</i>				<i>C. japonica</i>				<i>C. obtusa</i>			
	Heartwood		Sapwood		Heartwood		Sapwood		Heartwood		Sapwood	
	Mass loss (%)	SD	Mass loss (%)	SD	Mass loss (%)	SD	Mass loss (%)	SD	Mass loss (%)	SD	Mass loss (%)	SD
T3	8.2	1.5 a*	7.3	0.6 a	2.2	3.5 b*	1.8	2.2 b	7.0	1.0 c*	7.9	0.9 b
T11	7.6	1.2 a	4.8	1.0 b	1.1	2.8 c*	4.4	1.8 b	7.7	0.7 bc*	6.4	1.6 b
T26	8.3	0.9 a*	7.9	0.7 a	4.9	2.2 b	8.4	0.7 a	10.2	0.5 a*	10.0	0.6 a
<i>T. versicolor</i>	8.5	2.9 a*	8.0	2.0 a	-5.2	9.7 bc*	3.7	2.7 b	-7.8	3.9 d	6.5	6.8 ab
<i>F. palustris</i>	9.0	1.3 a*	8.1	0.8 a	9.4	1.8 a*	9.9	1.1 a	8.6	0.4 b	10.0	0.5 a

Strains	<i>F. crenata</i>				<i>Q. miyagii</i>			
	Heartwood		Sapwood		Heartwood		Sapwood	
	Mass loss (%)	SD	Mass loss (%)	SD	Mass loss (%)	SD	Mass loss (%)	SD
T3	4.4	0.3 b*	4.2	0.4 b	0.5	0.3 b	2.1	0.7 c
T11	1.4	1.0 c*	0.9	1.7 c	1.2	0.6 ab	2.4	0.2 c
T26	4.2	0.3 b*	4.8	0.4 b	1.0	0.3 a	3.0	0.2 b
<i>T. versicolor</i>	17.9	5.0 a*	26.1	12.3 a	1.1	2.4 abc	5.1	1.9 a
<i>F. palustris</i>	5.2	0.5 b	4.5	0.1 b	-0.3	0.2 c	2.2	0.3 c

SD: Standard deviation of mass-loss rate

*: There was no significant difference between heartwood and sapwood (t-test $p>0.05$).

The same characters indicate no significant difference by Tukey-Kramer test ($p>0.05$).

T11 were not significantly different between heartwood and sapwood, but the MLR of T26 in heartwood was significantly lower than that in sapwood.

In *C. obtsa* heartwood, T26 showed a significantly higher MLR (10.2%) than all the other test fungi. There was no significant difference in the MLRs of T3 (7.0%) and T11 (7.7%), T11 and *F. palustris* (8.6%), respectively. In sapwood, T26 had a higher MLR (10.0%) than the other *T. eurrhizus* strains (7.9% and 6.4%). In addition, T26 and the control fungi did not have significantly different MLRs. No *T. eurrhizus* strains showed a significant difference in MLRs between heartwood and sapwood.

In the heartwood and sapwood of *F. crenata*, the MLRs in T3 (4.4%, 4.2%) and T26 (4.2%, 4.8%) were significantly lower than those of *T. versicolor* (17.9%, 26.1%), but they were not significantly different from those of *F. palustris* (5.2%, 4.5%), while the MLRs of T11 (1.4%, 0.9%) were significantly lower than those of the other fungi. There was no significant difference between heartwood and sapwood MLRs for any *T. eurrhizus* strain.

All the fungi showed the lower MLRs for the heartwood of *Q. miyagii* than for other wood species. Three *T. eurrhizus* strains showed low MLRs (0.5%, 1.2% and 1.0%), without any significant difference from that of *T. versicolor* (1.1%). In contrast, for sapwood, *T. versicolor* had a higher MLR (5.1%) than those of the *T. eurrhizus* strains (2.1%, 2.4% and 3.0%). All *T. eurrhizus* strains displayed significantly lower MLRs in heartwood than in sapwood.

In SEM observation, wood samples decayed by the white-rot fungus *T. versicolor* were employed as comparative control, because *Termitomyces* fungi are classified as white-rot fungi [80]. The observation positions in the sample are indicated in Fig. 1. The results of SEM observations are shown in Figs. 3.2-3.5.

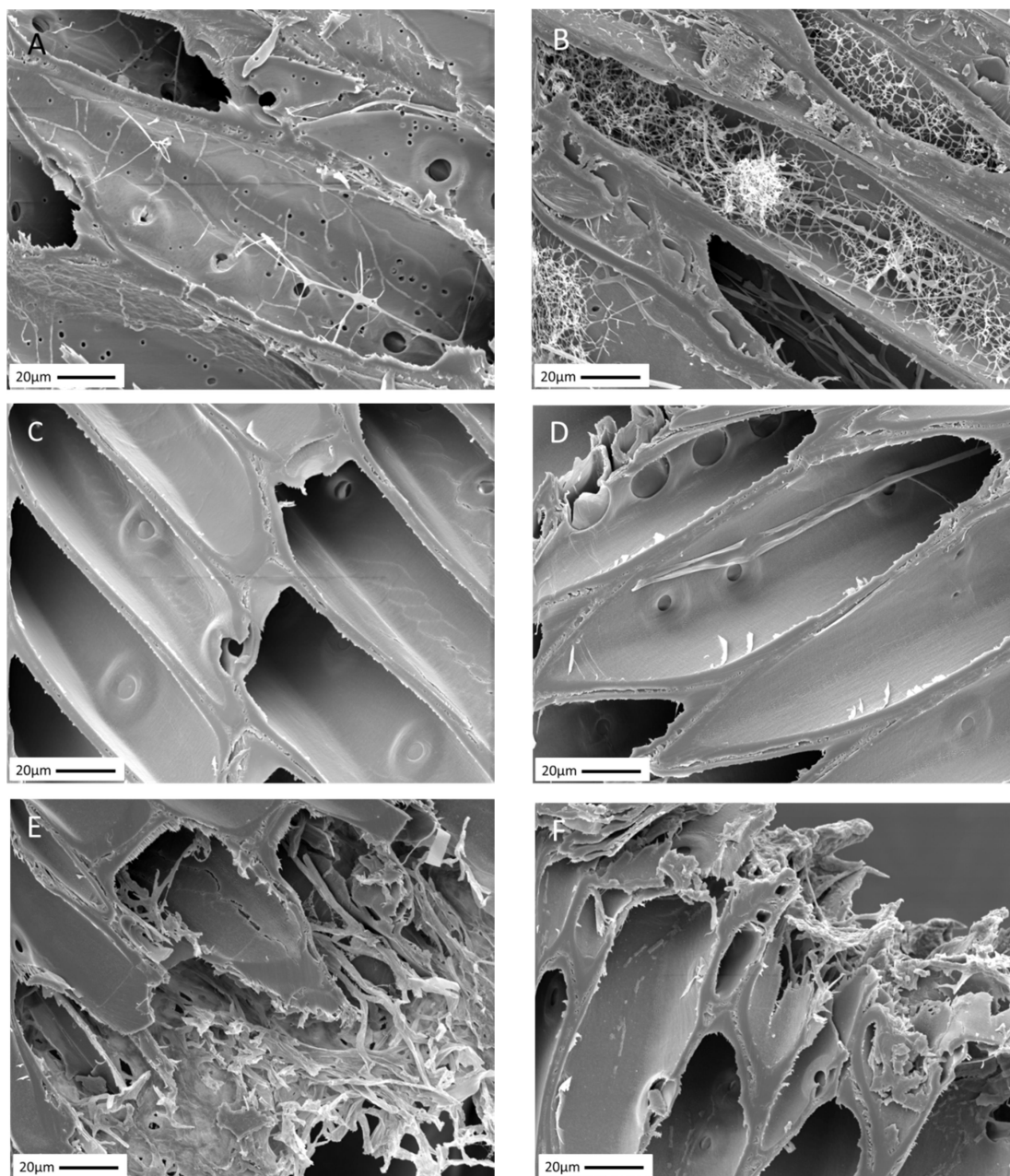


Fig. 3.2 SEM images of decayed wood samples of *Pinus densiflora*

A: Heartwood core sample decayed by *T. versicolor* (FFPRI1030)

B: Sapwood core sample decayed by *T. versicolor* (FFPRI1030)

C: Heartwood core sample decayed by *T. eurrhizus* (T3)

D: Sapwood core sample decayed by *T. eurrhizus* (T26)

E: The surface of a heartwood sample decayed by *T. eurrhizus* (T11)

F: The surface of a sapwood sample decayed by *T. eurrhizus* (T3)

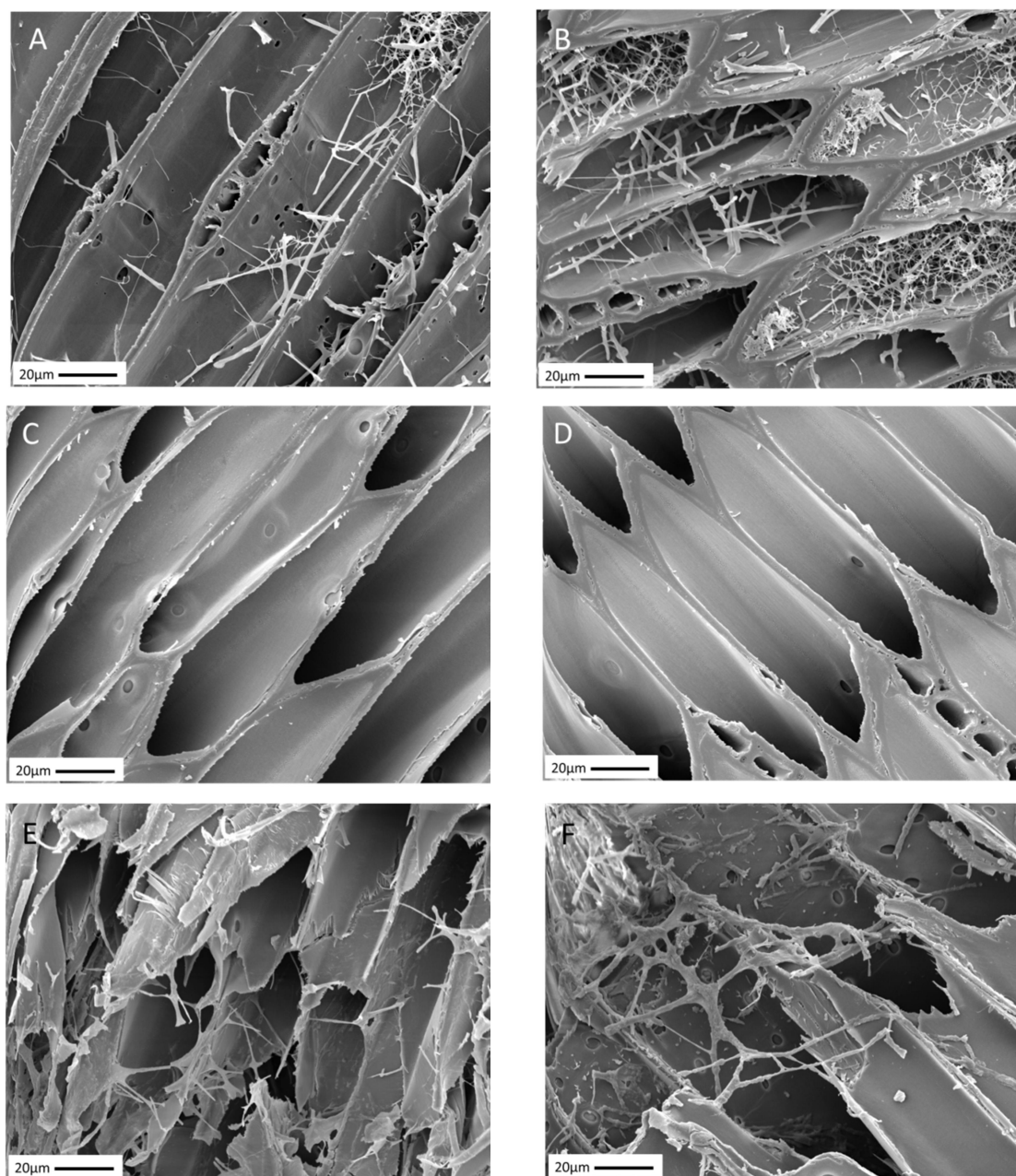


Fig. 3.3 SEM images of decayed wood samples of *Cryptomeria japonica*

A: Heartwood core sample decayed by *T. versicolor* (FFPRI1030)

B: Sapwood core sample decayed by *T. versicolor* (FFPRI1030)

C: Heartwood core sample decayed by *T. eurrhizus* (T3)

D: Sapwood core sample decayed by *T. eurrhizus* (T11)

E: The surface of a heartwood sample decayed by *T. eurrhizus* (T26)

F: The surface of a sapwood sample decayed by *T. eurrhizus* (T25)

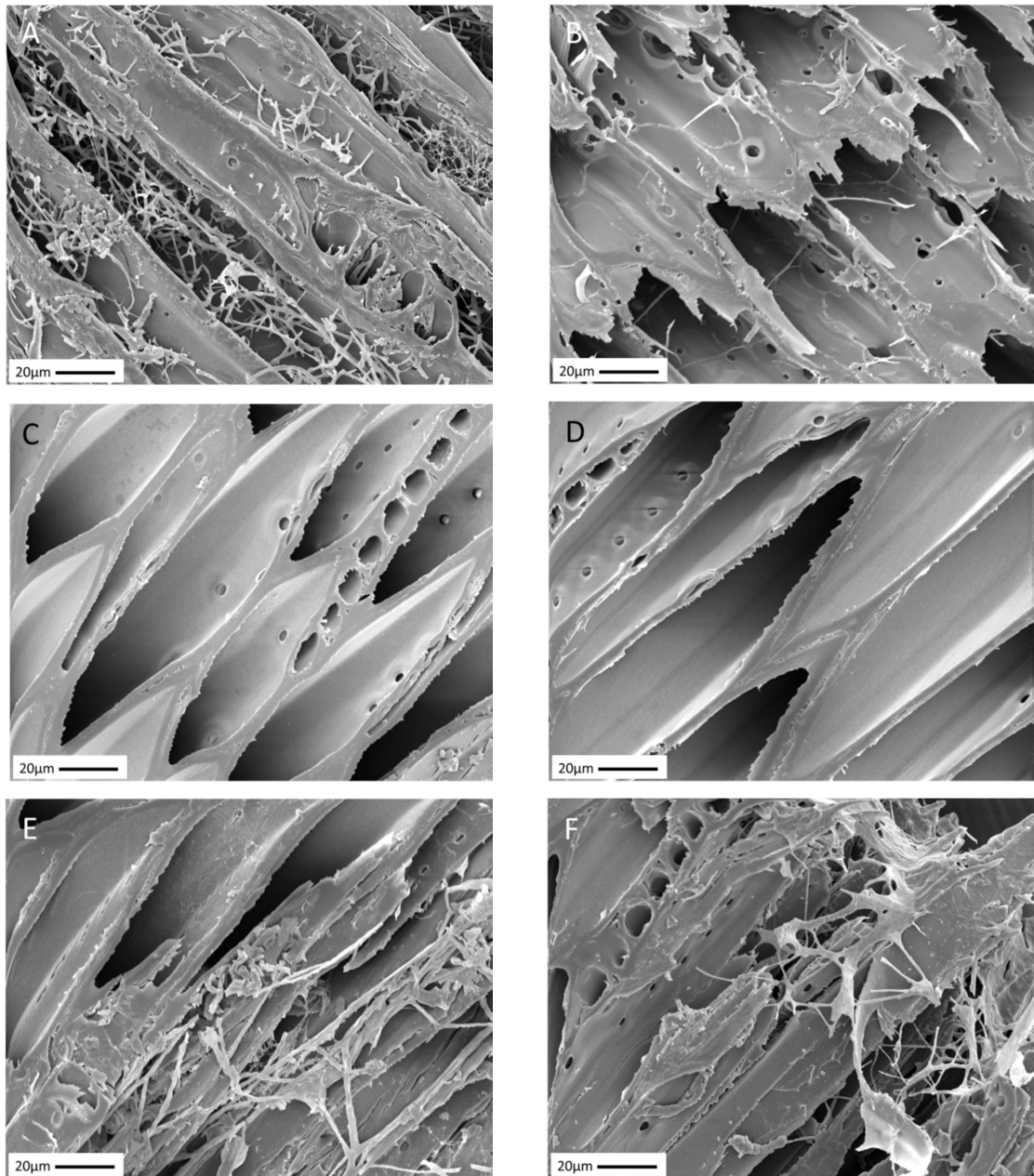


Fig. 3.4 SEM images of decayed wood samples of *Chamaecyparis obtusa*

A: Heartwood core sample decayed by *T. versicolor* (FFPRI1030)

B: Sapwood core sample decayed by *T. versicolor* (FFPRI1030)

C: Heartwood core sample decayed by *T. eurhizus* (T25)

D: Sapwood core sample decayed by *T. eurhizus* (T26)

E: The surface of a heartwood sample decayed by *T. eurhizus* (T11)

F: The surface of a sapwood sample decayed by *T. eurhizus* (T26)

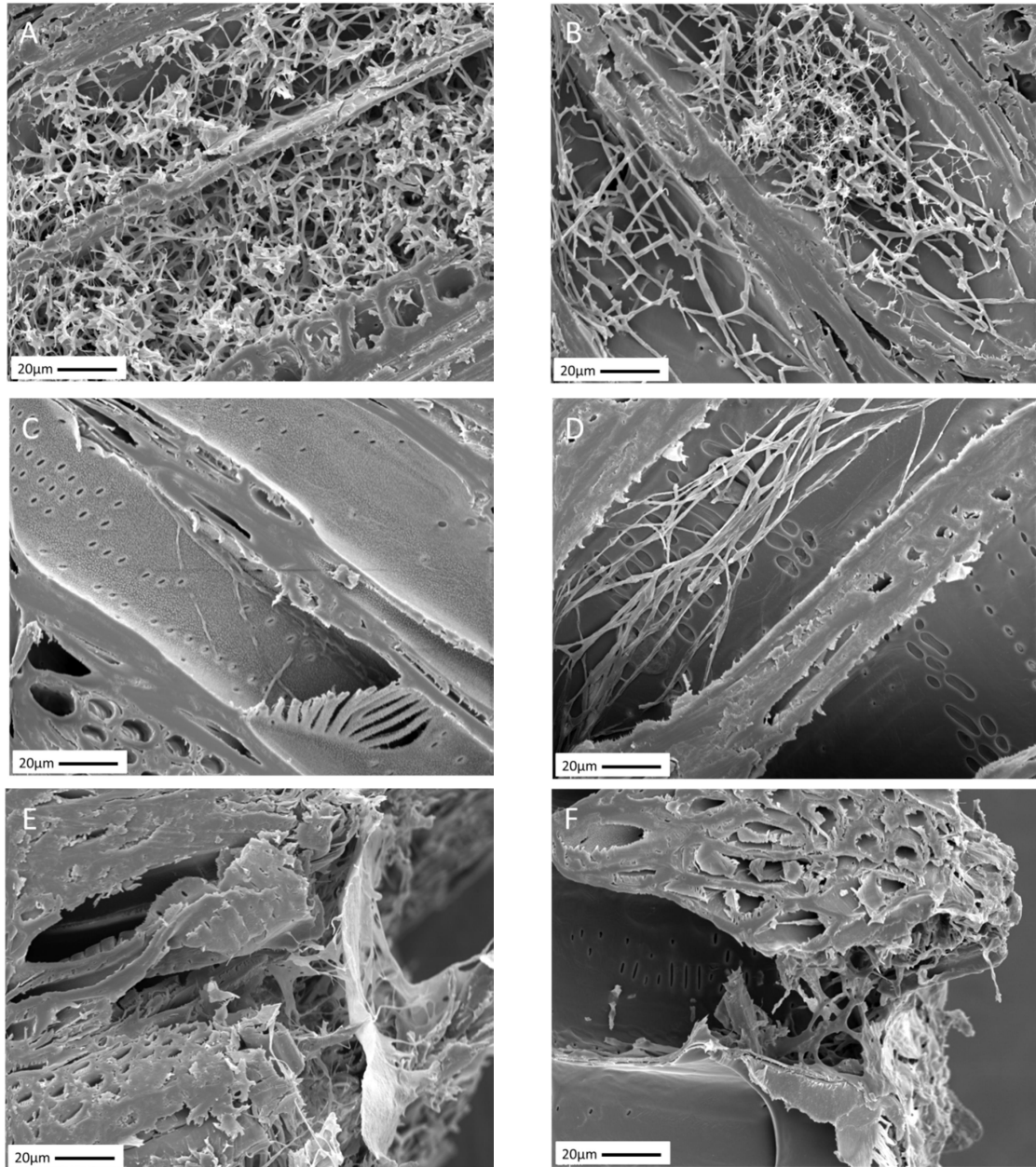


Fig. 3.5 SEM images of decayed wood samples of *Fagus crenata*

A: Heartwood core sample decayed by *T. versicolor* (FFPRI1030)

B: Sapwood core sample decayed by *T. versicolor* (FFPRI1030)

C: Heartwood core sample decayed by *T. eurhizus* (T11)

D: Sapwood core sample decayed by *T. eurhizus* (T3)

E: The surface of a heartwood sample decayed by *T. eurhizus* (T3)

F: The surface of a sapwood sample decayed by *T. eurhizus* (T11)

In all samples exposed to *T. versicolor*, there were many mycelia in almost all tracheids and vessels, with serious decay symptoms (*P. densiflora*, Fig. 3.2 A-B; *C. japonica*, Fig. 3.3 A-B; *C. obtusa*, Fig. 3.4 A-B; and *F. crenata*, Fig. 3.5 A-B). However, mycelia of *T. eurhizus* were rarely found in the tracheids of softwood (*P. densiflora*, Fig. 3.2 C-D; *C. japonica*, Fig. 3.3 C-D; and *C. obtusa*, Fig. 3.4 C-D). Additionally, there were no decay symptoms around the mycelia at all. In *F. crenata*, the mycelia of *T. eurhizus* were found inside vessels in the hardwood more frequency than tracheids in softwood (Fig. 3.5 C-D), but no decay symptoms were observed. In addition, there were no mycelia in other woody tissues, such as xylem rays and fibers. There were many *T. eurhizus* mycelia covering the sample surfaces and edges (*P. densiflora*, Fig. 3.2 E-F; *C. japonica*, Fig. 3.3 E-F; *C. obtusa*, Fig. 3.4 E-F; and *F. crenata*, Fig. 3.5 E-F).

LDRs and SDRs of 3 *T. eurhizus* strains (T3, T11 and T26), *T. versicolor* and *F. palustris* are shown in Tables 2 and 3, respectively.

There was no significant difference among all fungal strains in either the LDRs or the SDRs of heartwood and sapwood for *P. densiflora* (Tables 3.3 and 3.4).

In *C. japonica*, none of the strains showed any significant difference in LDRs between the heartwood and sapwood samples (Table 3.3). They also showed no difference in SDRs in the heartwood of *C. japonica*. In the sapwood, there was no significant difference in SDRs among the three *T. eurhizus* strains and *T. versicolor*. T3 exhibited a significantly lower SDR than that of *F. palustris*, but the other 2 strains showed no significant difference from *F. palustris* (Table 3.4).

Interestingly, all the fungi exhibited negative LDRs in heartwood of *C. obtusa*, and T3 and T11 also showed negative LRDs in sapwood (Table 3.3). LDRs of all the fungal strains were not significantly different in sapwood. It is suggested that *T.*

eurrhizus has low lignin degradation ability in *C. obtusa*. In heartwood, the SDRs of T11 and T26 were not significantly different from that of *F. palustris*; in sapwood, only the SDR of T26 was not significantly different from that of *F. palustris* (Table 3.4). With respect to the LDRs and the SDRs of *C. obtusa*, there was no significant difference among *T. eurrhizus* strains.

In the heartwood of *F. crenata*, the LDRs of T3 and T11 were significantly lower than that of *T. versicolor*, while the LDR of T26 was not significantly different. On the other hand, the LDRs of all the *T. eurrhizus* strains were significantly lower than that of *T. versicolor* in sapwood. The SDRs in heartwood of all fungal strains were not significantly different. But, in sapwood, the SDR of T11 was significantly lower than that of *F. palustris*.

Lignin degradation of *Q. miyagii* was not significantly different among all the fungal strains in heartwood, and in sapwood, the only significant difference was significantly lower LDR in T11 compared to *T. versicolor*. In heartwood, no fungal strains had significantly different SDRs. T26 did exhibit a significantly higher SDR than those of other *T. eurrhizus* strains in sapwood, but it was not significantly different from that of *F. palustris*.

Regardless of wood species and sample location, all *T. eurrhizus* strains showed similar mass losses after 8-week exposure. The results suggested that *T. eurrhizus* can degrade heartwood as well as sapwood. Heartwood generally exhibits higher durability than that of sapwood, because heartwood has more extractives than that in sapwood. *T. eurrhizus* may have some resistance to extractives. We propose that a mixture of heartwood and sapwood may be used as a medium for artificial cultivation of *T. eurrhizus*, e.g., a whole small log produced by forest thinning.

Table 3.3 Average of lignin content rates and lignin decrease rates (LDRs) of 5 wood species after 12-week decay tests

	Lignin											
	<i>P. densiflora</i>				<i>C. japonica</i>				<i>C. obtusa</i>			
	heart wood		sap wood		heart wood		sap wood		heart wood		sap wood	
	Content (%)	LDR (%)	Content (%)	LDR (%)	Content (%)	LDR (%)	Content (%)	LDR (%)	Content (%)	LDR (%)	Content (%)	LDR (%)
Sound wood	27.0		26.7		33.9		31.1		26.4		28.9	
T3	29.3	0.6	27.9	3.2	35.9	-4.9	31.2	0.8	32.1	-11.6	31.8	-1.2
T11	29.4	-1.0	28.6	-1.1	33.6	2.4	31.2	3.9	31.6	-9.9	31.5	-1.2
T26	29.3	1.1	28.9	0.2	35.0	1.5	34.2	-0.5	30.4	-3.1	31.7	1.5
<i>T.versicolor</i>	28.6	4.2	28.9	0.5	32.1	6.9	28.8	10.3	29.8	-22.8	29.1	3.5
<i>F.palustris</i>	34.1	-4.9	30.4	-4.7	44.0	-7.4	40.3	6.5	32.5	-10.1	35.1	4.9

	Lignin							
	<i>F. crenata</i>				<i>Q. miyagii</i>			
	heart wood		sap wood		heart wood		sap wood	
	Content (%)	LDR (%)	Content (%)	LDR (%)	Content (%)	LDR (%)	Content (%)	LDR (%)
Sound wood	27.7		28.1		28.6		25.7	
T3	27.8	3.9	27.1	7.6	29.7	-3.1	26.4	-1.1
T11	27.5	1.7	27.5	2.8	30.4	-4.8	27.1	-3.3
T26	25.3	12.2	27.0	8.5	30.2	-4.1	26.7	-0.8
<i>T.versicolor</i>	23.3	31.9	21.3	45.8	28.8	-0.6	23.5	12.3
<i>F.palustris</i>	29.9	6.8	36.2	14.3	30.3	-1.9	27.8	6.7

LDR: Lignin decrease rate

SD: Standard deviation of lignin decrease rate

The same characters indicate no significant difference by Tukey-Kramer test ($p>0.05$).

Table 3.4 Average of total sugar content rates and total sugar decrease rates

	Total sugar											
	<i>P. densiflora</i>				<i>C. japonica</i>				<i>C. obtusa</i>			
	heart wood		sap wood		heart wood		sap wood		heart wood		sap wood	
	Content (%)	SDR (%)	Content (%)	SDR (%)	Content (%)	SDR (%)	Content (%)	SDR (%)	Content (%)	SDR (%)	Content (%)	SDR (%)
Sound wood	65.0		65.7		62.9		65.2		62.0		62.4	
T3	67.9	4.5	65.9	7.3	67.0	-5.6	71.2	-8.0	66.3	1.6	59.7	12.0
T11	73.6	-5.1	73.5	-5.7	64.2	-0.5	66.2	2.7	63.8	5.6	66.9	0.5
T26	58.1	18.5	62.9	11.8	54.4	17.0	50.3	29.6	60.8	12.1	58.4	15.9
<i>T.versicolor</i>	65.5	8.9	67.0	6.2	61.1	4.6	66.5	1.3	61.3	-7.5	64.5	1.1
<i>F.palustris</i>	60.1	21.9	65.6	4.3	54.9	23.4	55.4	36.2	50.0	27.8	51.9	34.8

	Total sugar											
	<i>F. crenata</i>				<i>Q. miyagii</i>							
	heart wood		sap wood		heart wood		sap wood					
	Content (%)	SDR (%)	Content (%)	SDR (%)	Content (%)	SDR (%)	Content (%)	SDR (%)				
Sound wood	66.6		66.7		64.1		69.0					
T3	67.1	3.6	62.5	10.3	68.7	-6.6	74.3	-5.9				
T11	71.4	-6.1	68.1	-1.2	63.8	1.9	72.2	-2.2				
T26	70.6	-1.7	65.3	6.7	60.8	6.4	62.3	12.5				
<i>T.versicolor</i>	69.9	15.1	70.3	23.5	64.7	-0.9	69.4	3.9				
<i>F.palustris</i>	63.3	17.3	59.3	39.5	71.1	-6.8	67.5	15.8				

SDR: Total sugar decrease rate

SD: Standard deviation of total sugar decrease rate

The same characters indicate no significant difference by Tukey-Kramer test ($p>0.05$)

From the results of 8-week decay test, T25 showed the lowest values of MLRs in all wood species both heartwood and sapwood. This strain has low degradation potential of wood. On the other hand, T26 showed the highest MLRs both decay tests in most of wood species. T26 is comparatively high wood degradation ability among 4 strains.

Meanwhile, the LDRs of *T. eurrhizus* were not significantly different from that of the brown-rot fungus *F. palustris*. Since brown-rot fungi generally decompose little lignin [81], this might indicate that *T. eurrhizus* has low lignin degradation ability. *Termitomyces* fungi are symbionts of Macrotermitinae termites, and one of the roles of the fungi is thought to be decomposition of lignin for termite digestion [12]. Other scientists have reported that the roles of symbiotic fungi are unclear and differ depending on their host termites [18,20]. The host termite *Odontotermes formosanus* in Japan might not depend on high lignin degradation by *T. eurrhizus*. The lignin contents of the fungus comb collected in Japan were 3–10% [45], significantly lower than those of plants. *Odontotermes formosanus* may collect lignin-decomposed material for the comb. Alternatively, *Xylaria* fungi are known to settle in fungus combs of Macrotermitinae termites [43,82,83]. *Xylaria* species have a solitary habit and the ability to degrade lignin [84]. Hence, the lignin of the comb may be decomposed by these *Xylaria* fungi. The termites are also known to feed on the fungal nodules, balls of *Termitomyces* mycelia, on the fungus comb [7]. *T. eurrhizus* is likely to be a source of protein for *O. formosanus*. Although we found wide variation in the SDRs, those of *T. eurrhizus* and control fungi were not significantly different. It might indicate the similar degradation abilities of *T. eurrhizus* to holocellulose. Chapter 2 indicated that *T. eurrhizus* could grow sufficiently on cellulose-containing medium as a carbon-source [76].

In general, *F. crenata* is known to be susceptible to wood-decaying fungi, and is used in the cultivation of mushrooms in Japan [85]. But our *T. eurrhizus* strains induced little decay in either the 8- or the 12-week exposure. In addition, these strains also could not degrade *Q. miyagii*. These two species belong to the family Fagaceae. Fagaceae has tannin (0.2-8.8%) in wood [86], and tannin was reported to inhibit fungal decomposition [87]. Tannin could inhibit wood degradation of *T. eurrhizus*. Timbers of *Q. miyagii* have been known as hard and durable materials since early times [88,89]; the heartwood is especially known for its high decay durability.

Since T25 did not survive through the end of the test period of 12 weeks, *T. eurrhizus* might be unfit for long-term cultivation on artificial media. This was supported by the results that the MLRs of T3, T11 and T26 after 12 weeks were the same as those of the 8-week tests. We incubated all the bottles hermetically because *T. eurrhizus* seemed to be sensitive to bacterial and fungal contamination in our preliminary experiments (Ono, unpublished data). On the other hand, the nests of the host termites are well ventilated [90,91]. Fungus combs have a sponge-like appearance with a large surface area, meaning that *Termitomyces* fungi may prefer a well-ventilated condition. Moreover, fungus combs are fed from the bottom, and are maintained by termites by adding fresh media [21,25]. It is assumed that some metabolic products by the fungus might be stored in the agar media, and inhibit the growth of *T. eurrhizus*.

The results of SEM observation clearly showed that the mycelia of *T. eurrhizus* could not penetrate even the smaller wood blocks (1 cm[R] × 1 cm[T] × 0.5 cm[L]). It is presumed that *T. eurrhizus* can only decay the surfaces of the samples. Wood materials of small particle size such as saw-dust are strongly recommended for the artificial media. However, mycelia of *T. eurrhizus* were often found in the vessels of *F. crenata*. The

size of vessels of *F. crenata* is larger than those of the softwood tracheids (Fig. 2-5). If the mycelia of *T. eurhizus* prefer a well-ventilated condition, they might not penetrate deep into tracheids.

This study shows that *T. eurhizus* collected in Japan does not have high lignin-degradation ability. The low lignin content wood species may be favorable to *T. eurhizus*. In addition, the fungus can only decay the surfaces of wood block samples. There is a possibility that the fungus prefers softwood to hardwood, suggesting that in the future artificial cultivation with forest-thinning resource such as Japanese cedar (*C. japonica*) and cypress (*C. obtusa*) would be targeted. The presence of extractives might be one of factors relating to from this phenomenon.

3.4 Summary

Four strains of the termite mushroom *Termitomyces eurhizus* collected in Japan were surveyed for their wood decaying properties in three softwood and two hardwood species, in comparison with the white-rot fungus *Trametes versicolor* and the brown-rot fungus *Fomitopsis palustris*. All strains of *T. eurhizus* degraded only the surfaces of the wood samples, and differences in mass-loss rates between heartwood and sapwood were generally not significant. Higher mass-loss rates were generally obtained in softwood than in hardwood. The results of chemical analyses of decayed wood samples indicated that *T. eurhizus* does not have high lignin-degradation ability, even though it is categorized as a white-rot fungus. These results clearly suggest the unique physiological characteristics of *T. eurhizus*.

Chapter 4

Decay properties of *Termitomyces eurhizus* on moso bamboo (*Phyllostachys edulis*)

4.1 Introduction

In Japan, disordered proliferation of moso bamboo due to the lack of management has become a major environmental issue [92,93]. Moso bamboo is easily available and inexpensive, therefore, various researches have been performed using it as a medium for cultivation of edible mushrooms [94-96]. If moso bamboo were found to be an ideal medium for cultivation of *T. eurhizus*, a productive use would be found for this overabundant natural resource.

In this chapter, Chapter 4, the author surveyed the decay properties of *T. eurhizus* on moso bamboo (*Phyllostachys edulis*) by a no-choice test. Moso bamboo is known to contain 1.5-4.5% starch and 0.4-1.7% free sugars [97], and *T. eurhizus* has been reported to catabolize starch and sugars (Chapter 2) [76].

4.2 Materials and methods

Fungal strains

Three strains of *Termitomyces eurhizus* (T3, T11 and T26) were used in this study. They were collected in Okinawa Prefecture, Japan in 2004 and 2005, by isolation from fruit bodies. These strains were selected from 27 strains for their rapid growth and easy handling [72]. As controls, a white-rot fungus, *Trametes versicolor* (FFPRI 1030),

and a brown-rot fungus, *Fomitopsis palustris* (FFPRI 0507), were also employed. These two strains were designated as the fungi for testing wood preservatives by the Japan Industrial Standard [74].

Decay test

Samples of moso bamboo were harvested in August 2013 and February 2014 from Asiu Research Forest, Kyoto University, Miyama-Cho, Nantan-City, Kyoto. Moso bamboo was previously reported to have high starch content in February, but low starch content in August [97]. The surface of the bamboo samples were wiped with acetone to remove the wax after air drying. The sample size was the thickness of the bamboo culm (R) $\times$ 20 mm (T) $\times$ 10 mm (L). They were dried at 60°C for 48 h to determine the oven-dried weight before the tests.

Fungal inoculums were prepared with potato dextrose agar (PDA; Nissui, Tokyo, Japan) plates. A 7-mm-diameter inoculum was taken from the plate, and was inoculated on 100 ml of medium (PDA) in a 450-ml screwcap glass bottle. After the mycelia grew over the media surface, three test samples were separately set on the mycelial mat. The bottles were tightly sealed because *T. eurhizus* is extremely sensitive to contamination, then kept for 8 weeks at 26°C in the dark. After exposure, the mycelia that covered the samples were removed gently, and the samples were dried at 60°C for 48 h and weighed to determine the mass loss. The numbers of repetitions were 12 ($n=12$).

These decay tests were conducted within 3 months after the harvest, and the dried samples after the tests were kept in sealed plastic bags until the chemical analysis.

Chemical analysis

For chemical analyses, the bamboo samples were ground into 60-mesh-pass size by a high-speed blender (VM0113; Vitamix, Cleveland, OH). Lignin content was determined by the Klason method [77]. Starch and free sugar contents were quantified by the alkaline extraction-glucoamylase hydrolysis method [98]. These analyses were conducted on July 2016 after more than 2 years' custody.

4.3 Results and discussion

The mass loss rates (MLRs) of the bamboo samples after the decay tests are shown in Fig. 4.1.

All three *T. eurhizus* strains showed significantly higher MLRs for the bamboo specimens from August (approximately 10% on average) than those from February (7.4-7.6% on average). There was no significant difference among the three *T. eurhizus* strains for either August or February specimens. On the August specimens, T3 and T11 showed significantly higher MLRs than that of *F. palustris* (8.5% on average), whereas all three strains exhibited significantly lower MLRs than that of *T. versicolor* (12.6% on average). On February specimens, there was no significant difference among the three *T. eurhizus* strains and *F. palustris* (7.3% on average), and significantly lower MLRs than that with *T. versicolor* (12.3% on average) were obtained with the three strains of *T. eurhizus*.

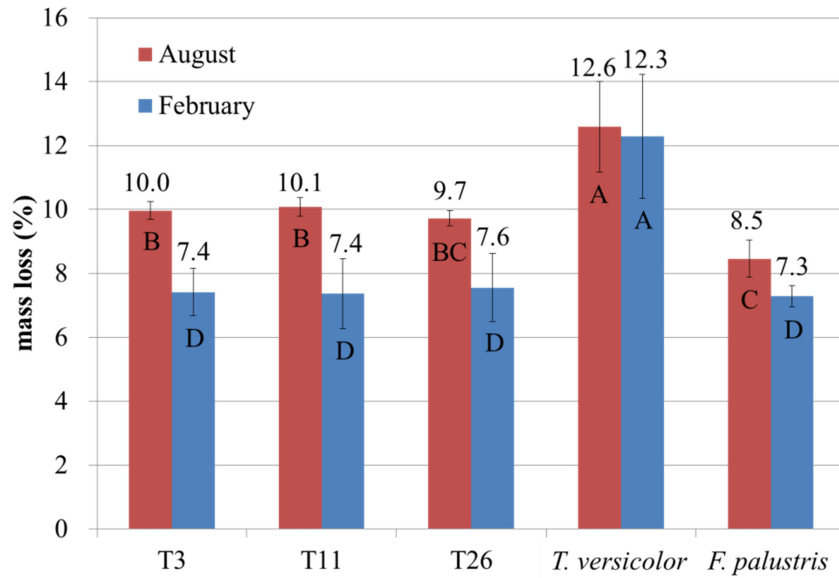


Fig. 4.1 The mass loss of moso bamboo from decay test

The same characters indicate no significant difference by the Tukey-Kramer test ($p < 0.05$).

On both the August and February specimens, *T. eurrhizus* strains showed lower mass loss than those of the white-rot fungus *T. versicolor*, but similar or higher mass loss than those of the brown-rot fungus *F. palustris*. These results suggest that this fungus behaves like brown-rot fungi on moso bamboo even it is categorized as a white-rot fungus [80]. Our recent results have shown that the decay characteristics of *T. eurrhizus* against many timber species are similar to those of *F. palustris* (Chapter 3).

MLRs of the August specimens decayed by all three strains were significantly higher than those of February. From the present results, *T. eurrhizus* appears to prefer bamboo harvested in summer over that in winter. It was previously reported that moso

bamboo had high starch content in February, but low starch content in August [97]. It is conventional wisdom that good-quality bamboo is harvested during the fall and winter due to the lower concentration of starch, which is an important nutrient for many pest insects, such as the bamboo powder-post beetle, *Dinoderus minutus*. We have recently showed that *T. eurhizus* can catabolize starch as well as free sugars (Chapter 2) [76]. It, therefore, must be considered why the fungus preferred the samples with the lower starch concentration. Other components such as extractives may have a role on the decay ability of *T. eurhizus*. For example, moso bamboo extracts contain quinone derivatives and have a broad spectrum of activity against bacteria and fungi [99].

The lignin, starch and free sugar (as glucose) contents of the specimens are shown in Table 4.1. As mentioned above, the samples were subjected to the decay tests just after the harvest, but the chemical analyses were conducted after 2 years' custody in the laboratory. The results must be interpreted with this time course in mind.

As shown in Table 1, lignin and starch contents in sound specimens are similar in both the August and February specimens. This suggests that starch contents would become stable at approximately 1% after 2 years' custody under laboratory conditions. Okahisa et al. reported the starch contents in moso bamboo samples as 3-4% in February and 1-2% in August [98]. On the other hand, the August specimens still have twice the free sugars after 2 years than do the February specimens. Okahisa et al. reported that the free sugar (as glucose) contents in moso bamboo samples had ranges of 1-1.5 % in February and 0.5-2% in August [98].

The lignin contents of the decayed specimens were a bit higher than those of the sound specimens not only with *T. eurhizus*, but also with *T. versicolor* and *F. palustris*. This may suggest that bamboo lignin, which consists of guaiacyl (G), syringyl (S) and

Table 4.1 Percentage of lignin content, starch content and free sugar (as glucose) content of sound and decayed bamboo samples

		Lignin	SD	Starch	SD	Free sugar	SD
Sound bamboo	Aug.	29.9	0.5	1.11	0.69	1.07	0.19
	Feb.	30.8	0.5	0.92	0.62	0.57	0.08
T3	Aug.	32.3	0.5	1.20	0.44	0.02	0.00
	Feb.	32.5	1.1	1.05	0.10	0.01	0.01
T11	Aug.	32.2	0.4	1.09	0.11	0.04	0.01
	Feb.	32.2	0.6	1.43	0.28	0.03	0.02
T26	Aug.	31.4	0.3	1.47	0.14	0.02	0.01
	Feb.	32.1	1.0	1.53	0.16	0.03	0.01
<i>T. versicolor</i>	Aug.	32.1	0.8	1.15	0.20	0.02	0.01
	Feb.	33.0	1.0	1.12	0.27	0.02	0.01
<i>F. palustris</i>	Aug.	31.7	1.1	1.13	0.42	0.02	0.00
	Feb.	32.5	0.9	0.79	0.21	0.06	0.04

p-hydroxyphenyl (H) units [100], is resistant to white-rot fungi. However, it is well known that bamboo is easily decayed under outdoor exposure. Attacks by pest beetles and/or bacteria may have a collaborative role in terms of fungal decay of bamboo.

Starch contents after the decay tests with *T. eurhizus* were similar or a bit higher than those of the sound specimens. These results clearly indicate that starch in moso bamboo is difficult for decay fungi to use. Starch is stocked as granules in the parenchyma cells of moso bamboo, and is difficult to extract. Therefore, the 8-week exposure was not long enough for the fungi to access and utilize the starch granules. Some physical/chemical pretreatments would be needed for the effective utilization of bamboo starch by fungi.

On the contrary, the free sugars content, measured as glucose, was drastically

decreased by the 8-week exposure to all the fungi. In most cases the free sugar contents after the decay tests was less than 0.05%, suggesting that free sugars were easily consumed by all the fungi. Free sugars of moso bamboo consist of glucose, fructose and sucrose [101], and *T. eurhizus* showed high catabolism of fructose and sucrose in our previous study [76]. Free sugars are also located in parenchyma cells in moso bamboo, but they are easily dissolved by water to become accessible to the fungi.

From the present experiments, the following conclusions were obtained. a) *Termitomyces eurhizus* preferentially catabolizes free sugars in moso bamboo. b) The starch in moso bamboo is difficult for decay fungi to utilize. c) Structural characteristics of lignin in moso bamboo contribute to decay resistance. d) Some physical/chemical pretreatments would be needed for the efficient utilization of moso bamboo as an artificial medium for fungi.

4.4 Summary

Moso bamboo (*Phyllostachys edulis*) specimens harvested in August and February were subjected to no-choice decay tests with the “termite-mushroom” *Termitomyces eurhizus*. The results showed that the August specimens, which had higher free-sugar content, were more highly decayed by *T. eurhizus* than those of February. *T. eurhizus* preferentially catabolized free sugars in moso bamboo. Starch in the bamboo was harder for the fungi to utilize. Some physical/chemical pretreatments are needed for the effective utilization of bamboo as an artificial media for fungi.

Chapter 5

Laccase activity in *Termitomyces eurhizus*

5.1 Introduction

Termitomyces fungi are classified as white-rot fungi [80], a species of this genus, *T. eurhizus*, does not have high lignin degradation ability, as discussed in Chapters 3 and 4.

The Bavendamm test is known as a simple detection method for the lignin-decomposing activity, especially the laccase activity, of fungi. When wood-rotting fungi are cultured on media containing gallic acid, they can be classified as white- or brown-rot fungi depending on the presence or absence of a colored zone, respectively [102, 103]. In this chapter, Chapter 5, the Bavendamm test was conducted on strains of *T. eurhizus* to confirm the existence of laccase activity in this species.

5.2 Materials and methods

Fungal strains

Four strains of *T. eurhizus* were used in this experiment (T3, T11, T25 and T26), as shown in Chapter 1. As controls, a white-rot fungus, *Trametes versicolor* (FFPRI 1030), and a brown-rot fungus, *Fomitopsis palustris* (FFPRI 0507), were also used. These two strains of wood-rotting fungi were designated for the standardized decay test in the Japan Industrial Standard [74] (JIS K 1571-2010).

Media

Potato dextrose agar (PDA, Nissui, Tokyo, Japan) was employed as a base medium. Three phenolic compounds, 1-naphthol, guaiacol and gallic acid, were added at 0.01% (w/v) into the medium. All media were prepared as ϕ 90-mm plates.

The Bavendamm test

All the strains were pre-cultured on the PDA media without the phenolic compounds, and 7-mm diameter pieces were taken with a cork-borer from the media and inoculated to the plate media with the phenolic compounds. The plate media were cultured in the dark at 26°C. The Bavendamm reactions of these plates were observed daily for 15 days. If these strains are capable of decomposing these phenolic compounds, the media are colored. The degree of the reaction was rated as follows:

- : no reaction for 10 days after inoculation
- +: the sizes of the colored zones were smaller than those of mycelial mats for 10 days after inoculation
- ++: the sizes of the colored zones were the same as those of mycelial mats for 10 days after inoculation
- +++: the sizes of the colored zones were bigger than those of mycelial mats for 10 days after inoculation

In addition, the diameters of the mycelial mats for the two vertical directions were measured every 5 days for 40 days post inoculation. The number of repetitions was 3 for each strain and medium ($n=3$).

5.3 Results and discussion

The results of the Bavendamm tests of all the fungal strains are summarized in Table 5.1.

As shown in Table 5.1, *T. versicolor*, a white-rot fungus, exhibited a clear colored zone beyond the mycelial mats in response to all three phenolic compounds. On the other hand, as expected, a brown-rot fungus, *F. palustris*, did not show any reaction to any of the media. None of the *T. eurrhizus* strains except T11 showed any colorization on any of the media. Only T11 exhibited slight colored zones on the media with 1-naphthol and gallic acid, but not on the guaiacol medium.

The growth of *T. eurrhizus* strains on the media with the phenolic compounds is shown in Fig.5.1.

None of the *T. eurrhizus* strains grew on the 1-naphthol medium, and they were conclusively shown to have died within several days after inoculation. However, *T. eurrhizus* strains showed varied growth on the guaiacol and gallic acid media. The growth of T3, especially in the early phase of the culture, was inhibited by guaiacol, but the strain grew faster on the gallic acid medium than on the additive-free (normal) PDA medium (Fig. 5.1-A). T11 and T25 showed rapid growth on the guaiacol and gallic acid media than on the normal PDA medium, and both strains grew equally on those two phenolic media (Figs. 5.1-B and 5.1-C). Meanwhile, nearly overlapping growth lines were obtained on the three media for T26 (Fig. 5.1-D).

Table 5.1 Results of Bavendamm tests

Strains	Reagents		
	1-naphthol	Guaiacol	Gallic acid
T3	–	–	–
T11	+	–	+
T25	–	–	–
T26	–	–	–
<i>T. versicolor</i>	++	+++	++
<i>F. palustris</i>	–	–	–

–: no reaction for 10 days after inoculation

+: the sizes of the colored zones were smaller than those of mycelium mats for 10 days after inoculation

++: the sizes of the colored zones were the same as those of mycelium mats for 10 days after inoculation

+++: the sizes of the colored zones were bigger than those of mycelium mats for 10 days after inoculation

T. versicolor and *F. palustris* were observed for 5 days after inoculation.

Among the enzymes relevant to lignin decomposition from white-rot fungi, namely laccase, lignin peroxidase and manganese peroxidase, laccase is responsible for the Bavendamm reaction. The fungi that showed high Bavendamm reactions are likely to have high lignin decomposition abilities [103]. As indicated in Chapters 3 and 4, *T. eurrhizus* does not have high lignin decomposition activity, a fact that supports the results obtained here. Because T11 indicated weak coloring, *T. eurrhizus* does not seem to principally lack laccase. Although the strain ATCC 42010 *T. aluminosus* (a synonym

for *T. eurrhizus*) was reported to have phenol oxidase [104], unfortunately, our previous experiment revealed that this strain is not in the *Termitomyces* genus (Ono et al., unpublished data). The species name of the strain has been changed in the American Type Culture Collection (ATCC) as a result of the information.

Only laccase activity was detected from fungus combs of *Microtermes* sp., *Odontotermes* sp. and *Macrotermes gilvus* in Thailand among the above-mentioned lignin-decomposing enzymes [13]. However, the origin of the enzymes was not clear in this report. Similarly, only laccase activity was detected from fungus combs of *O. formosanus* in China [105]. The optimal conditions of this laccase were pH 4.0 and 10°C, and the activity was stable under acidic conditions and between 0°C and 30°C [106]. Our *T. eurrhizus* strains were cultured in pH 5.6±0.2 (PDA) and 26±1°C, and so laccase should have been able to be fully active, but the coloring reactions were weak. The laccase of *Termitomyces* might have some special requirements to be activated, for example, the care by the host termites. Also, there are *Xylaria* fungi living with combs [44,83], and so there might be a possibility that these laccase activities originated from *Xylaria* fungi.

Bose et al. reported that *T. clypeatus* produced active laccase at the late phase of growth: after 10 days [107]. We made a daily observation of the Bavendamm reaction for 15 days with four strains of *T. eurrhizus*, but only weak activity was found in one strain. Laccase originating from *T. eurrhizus* may differ from that originating from *T. clypeatus*.

Phenol compounds generally were toxic for living organisms to a greater or lesser extent. *T. eurrhizus* has only a weak decomposition activity against phenolic compounds, so all the strains were killed by the highly poisonous phenolic compound, 1-naphthol.

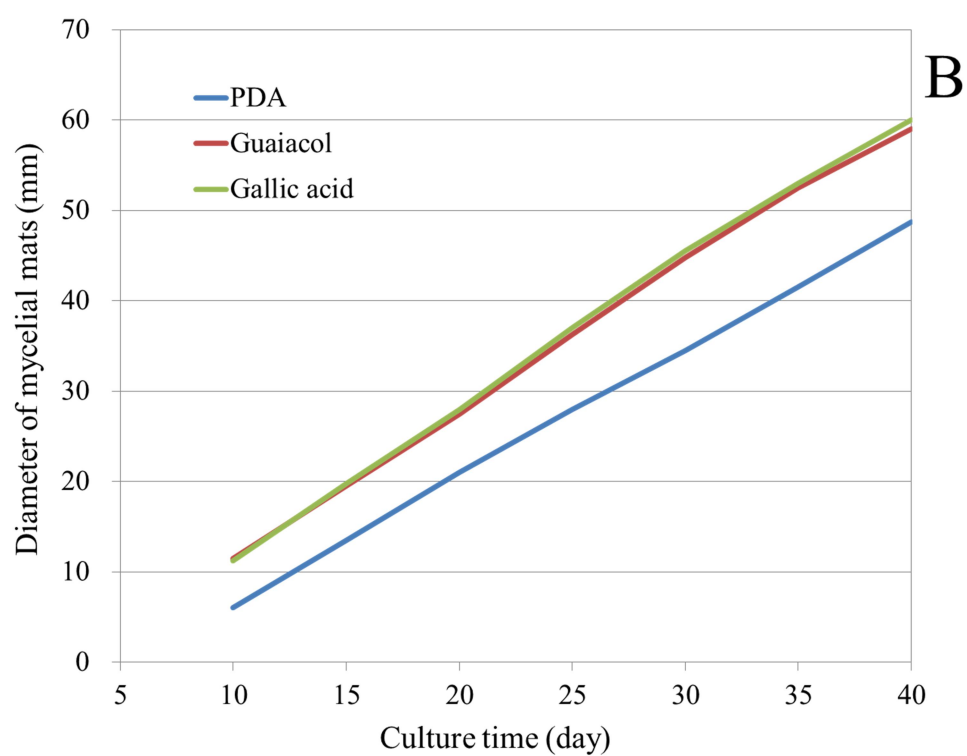
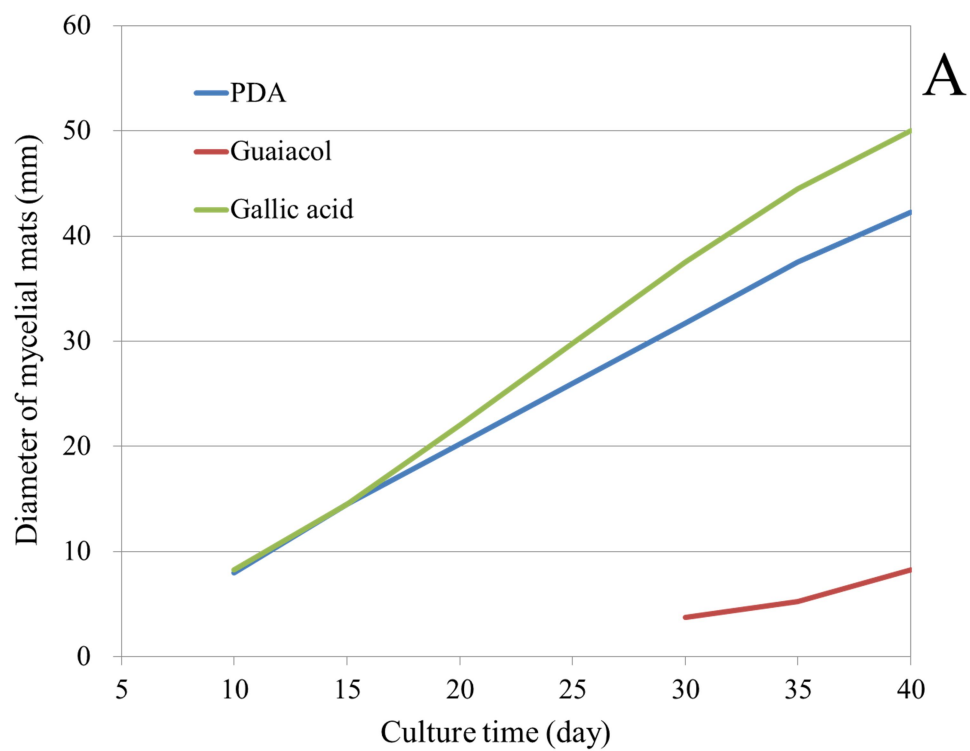


Fig. 5.1 Growth of four strains on phenol compounds media

A: T3, B: T11, C: T25, D: T26.

None of the strains grew on 1-naphthol medium.

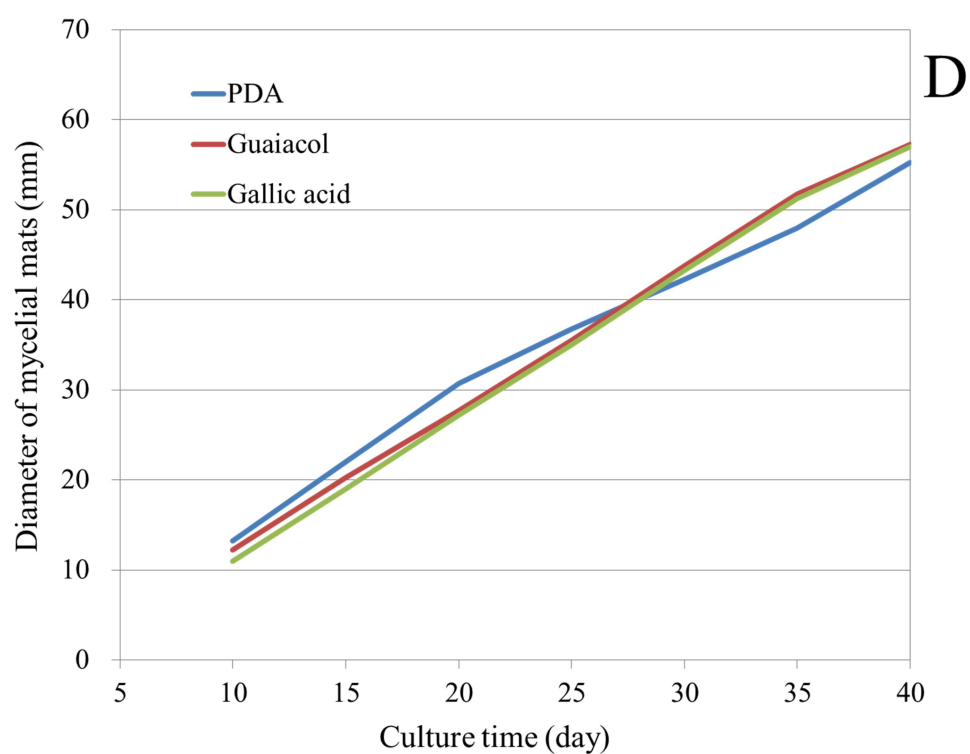
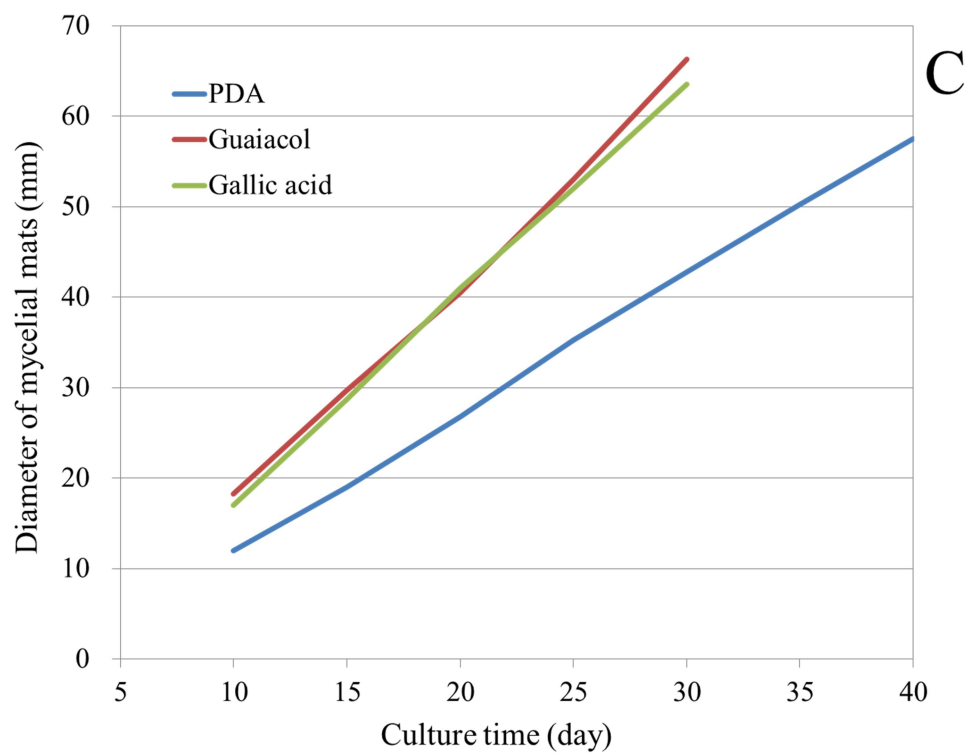


Fig. 5.1 Growth of four strains on phenol compounds media (continued)

A: T3, B: T11, C: T25, D: T26.

None of strains grew on 1-naphthol medium.

The growth of mycelia was also inhibited by guaiacol in one strain. On the other hand, adding guaiacol and gallic acid encouraged the growth of two strains. From these results, it is suggested that the lignin-decomposing activity, especially laccase activity, of *T. eurhizus* is generally weak but varies among strains, and the existence of lignin or lignin-originated compounds may have a role in the nutritional physiology of the fungus.

5.4 Summary

Termitomyces fungi are known for their unique ecology, habing a symbiotic relationship with termites. One of the suggested roles of the fungi in this symbiosis is to decompose lignin. Weak laccase activity was found in one of four strains of *T. eurhizus* by means of Bavendamm tests with 1-naphtol, guaiacol and gallic acid, suggesting that *T. eurhizus* supposedly has laccase but variety widely in terms of laccase activity.

Conclusions

The *Termitomyces eurhizus* mushroom has high potential market value. The fungi in this genus are called “termite mushrooms”, and have a symbiotic relationship with Mactotermitinae termites. Since to date, most researchers have been focused on this symbiotic relationship, the biological and physiological properties of the fungi themselves are less well understood. The purpose of this study was to evaluate the possibility of artificial cultivation of *T. eurhizus* by examining the nutritional characteristics of the fungus.

In Chapter 1, strains of *T. eurhizus* collected in Okinawa Prefecture were screened for the following experiments. Four strains were selected from 27 strains. Strain T3, collected from Okinawa Main Island, had low media specificity and grew tractably. Strain T11 from Ishigaki Island was easy to handle, with a medium growth rate on any media. Strain T25, collected from Iriomote Island, had low media specificity and grew fast, while T26 from Iriomote Island grew very fast on any media.

The author surveyed the effects of carbohydrate substrates on the mycelial growth of *T. eurhizus* in Chapter 2. Thirteen carbohydrates were tested in this experiment, and all 4 strains showed the most rapid growth on the fructose- and maltose-containing media. In addition, the 4 strains grew positively on mannose-, sucrose- and trehalose-containing media. *Termitomyces eurhizus* might utilize starch and cellulose similarly, and demonstrated a poor ability to catabolize lactose.

The decay properties of *T. eurhizus* were examined in Chapters 3 and 4. In Chapter 3, three softwood species, *Pinus densiflora*, *Cryptomeria japonica* and *Chamaecyparis obtuse*, and two hardwood species, *Fagus crenata* and *Quercus miyagii*,

were used as wood specimens. Scanning Electron Microscope (SEM) observations suggested that *T. eurhizus* could only attack the surfaces of small wood samples (1.0 (R) × 1.0 (T) × 0.5 (L) cm). There was no significant difference in mass-loss rate between heartwood and sapwood specimens exposed to *T. eurhizus* for 12 weeks. Moreover, the mass loss rates of softwood specimens were generally higher than those of hardwood specimens. Chemical analyses of decayed wood specimens suggested that *T. eurhizus* does not have high lignin-degradation ability, even though it has been categorized as a white-rot fungus.

In Chapter 4, decay property of the fungus against moso bamboo, *Phyllostachys edulis*, was investigated. *Termitomyces eurhizus* preferentially catabolized free sugars in the bamboo. The bamboo showed a high concentration of starch (approx. 1%), which the fungus found difficult to utilize.

From the results of Chapter 5, the existence of laccase, one of lignin decomposing enzymes, was detected using Bavendamm test. This method is a simple detection of phenol-oxidase. *Termitomyces eurhizus* strains did not show positive results except for one strain. The results suggested that *T. eurhizus* have laccase but its activity is also weak.

From the results of these experiments, the use of fine wood chips or sawdust is strongly recommended as a media matrix for the artificial cultivation of *T. eurhizus*. Wood species with lower lignin content are more favorable. Fagaceae trees such as Japanese beech (*Fagus crenata*), which are preferentially used for the media matrix of mushroom production in Japan, would not be suitable for *T. eurhizus*. On the other hand, sugi (*Cryptomeria japonica*) and moso bamboo (*Phyllostachys edulis*), which are the most easily obtained forest resources in Japan, have been suggested as possibilities

for use as a media matrix for *T. eurrhizus*.

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