Mycoscience 35: 153–159, 1994

Change in medium components and colony morphology due to mycelial growth of ectomycorrhizal fungus *Tricholoma bakamatsutake*

Yoshie Terashima

Chiba Prefecture Forest Experiment Station, Haniya 1887-1, Sanbu-machi, Sanbu-gun, Chiba 289-12, Japan

Accepted for publication 7 March 1994

Mycelia of *Tricholoma bakamatsutake* isolate No. 4 grew at temperatures ranging from 10 to 30°C, and the optimum was around 25°C. In well-buffered media of initial pH 5.0 and 6.0, No. 4 mycelia secreted gluconic acid and lowered medium pH. Mycelial growth then accelerated slightly; and with the exhaustion of glucose, growth and secretion of gluconic acid stopped. In 10 different media of initial pH 4.0–7.0, No. 4 mycelia showed higher gluconic acid secretion with higher initial pH. No. 4 mycelial grew best in pH 5.0 media, in which gluconic acid secretion was low. Mycelia of 29 isolates including No. 4 grew better in the media in which less glucose, total carbon and total nitrogen remained, and almost all isolates secreted gluconic acid. Most of the 29 isolates showed irregular colony shapes with rough mycelial fronts, brown pigmentation and aerial hypha on colony surfaces, and brown pigmentation of media under colonies. Dissimilarities were calculated with coded morphological characters on colonies, and similarity between isolates was found not to correlate with proximity of origin. Chlamydospores were observed on every colony of the 29 isolates. Chlamydospores were present on colonies of No. 4, reaching to 2 mm from the mycelial front, where brown pigmentation had not yet developed, and the numbers of chlamydospores incresed with mycelial aging.

Key Words——colony morphology; ectomycorrhizal fungus; medium component; mycelial growth; *Tricholoma bakamatsutake*.

Introduction

Mycelia of *Tricholoma bakamatsutake* Hongo and allied species, *T. matsutake* (S. Ito & Imai) Sing., *T. robustum* (Alb. & Schw.: Fr.) Ricken s. Imazeki, *T. fulvocastaneum* Hongo, all of which are ectomycorrhizal fungi, are relatively slow growers (Kawai and Abe, 1976; Ogawa, 1978). *T. bakamatsutake* has been reported to grow especially slowly (Ogawa and Ohara, 1978; Shimazono, 1979), and only a few studies have been reported on cultural characteristics of this species in comparison with other fungal species (Ogawa, 1978; Kiuchi, 1983; Iwase, 1992). Twenty-nine isolates of *T. bakamatsutake* were studied here to examine mycelial growth, change in medium components and colony morphology.

Materials and Methods

Media Well-buffered medium for *T. matsutake* (Ohta, 1990) was used as the basal medium, which consisted of 10 g glucose, 1 g citric acid, 1 g ammonium tartrate, 1 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.2 g $FeCl_3$, 1 mg $ZnSO_4 \cdot 7H_2O$, 1 mg $MnSO_4 \cdot 4H_2O$, 1 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg $CoSO_4 \cdot 7H_2O$, 1 mg $NiSO_4 \cdot 6H_2O$, 7 g HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 10 mg thiamine·HCl, 0.1 mg nicotinic acid, 0.03 mg folic acid, 0.3 mg biotin, 0.1 mg carnitine chloride, 0.03 mg adenine·$H_2SO_4 \cdot 2H_2O$ in 1 L of distilled water. pH was adjusted with 1 N KOH solution. The media were autoclaved at 120°C for 15 min before measuring initial pHs. For preparing plate media in Petri dishes, 1.5% of agar was added. GPY media, which contained 20 g glucose, 5 g polypepton, 5 g yeast extract, 0.5 g $MgSO_4 \cdot 7H_2O$ and 0.2 g $CaCl_2 \cdot 2H_2O$ in 1 L of distilled water, with 1.5% agar was also used for agar plate media.

Organism Twenty-nine isolates, No. 1–29, of *T. bakamatsutake* were examined (Table 1). Nineteen of them were prepared by explanting fruit-body tissues collected at Takeyama and Kimitsu, Chiba Pref., and kept by transferring several times. The rest were provided as stock cultures of mycelia: two originated from Tsukuimachi and Kiyokawa-mura, Kanagawa Pref., six from Ryujin-mura and Nakaheji-machi, Wakayama Pref., one from Totsukawa-mura, Nara Pref. and one from Yuzuhara-machi, Kouchi Pref.

Mycelial plugs of each stock were trasferred into agar plate media, incubated for up to two months to form adequate-sized colonies and used within three months. A mycelial plug of 8 mm in diam was taken from near the front margin of a colony and used as an inoculum for liquid medium, and one of 5 mm in diam was used for agar plate medium.

Cultivation Seven different tests were carried out: (1)

mycelia of isolate No. 4 were cultured on GPY agar plate media at temperatures ranging from 5 to 35°C, (2) No. 4 mycelia were cultured in liquid media of initial pH 5.0 and (3) 6.0 and harvested every 7 days for 84 days to examine time courses, (4) No. 4 was cultured in liquid media of initial pHs ranging from 4.0 to 7.0, (5) No. 4 was cultured on 60 agar plate madia, and five plates were examined every seven days for 84 days for pigmentation and chlamydospore formation on the colonies, (6) the 29 isolates including No. 4 were cultured in liquid media and (7) on agar plate media.

In all the tests, mycelia were incubated at 25°C statically in the dark in liquid media or on agar plate media of initial pH 5.0 for 42 or 56 days respectively, unless otherwise mentioned. Ten replications were examined in each test, which was repeated at least twice.

Measuring mycelial growth Mycelia in liquid media were collected by filtration, rinsed with distilled water, dried at 105°C for over 24 h, and dry weight was measured. Mycelial growth on agar plate media was measured in four radius lengths of a colony from the outer edge of the inoculum. To weigh mycelia grown on agar media, a mycelial colony was immersed in distilled water and autoclaved, and released mycelia were dried in the same way as those in liquid media.

Analyzing liquid media Final pH values of the cell-free extracts of media were measured. Amounts of total carbon and total nitrogen were analyzed with an NC analyzer (Sumigraph-90A, Sumika Chemical Analysis Service) after centrifugating the extracts at 1,200×g for 3 min. Glucose was assayed by the anthron method of Horikoshi (Fukui, 1990) after passage of the extracts through a column of IR-120B, a strong cation exchange resin, to eliminate amino acid (Asada et al., 1966). Gluconic acid was estimated by a UV-method with an F-Kit (Boehringer Mannheim) for D-gluconic acid (Iwase, 1992).

Analyzing morphologies of mycelial colonies The morphological characters of colonies of the 29 isolates were examined in terms of colony shape and form of mycelial growth front, brown pigmentation of the colony and inoculum, aerial hypha on the colony and inoculum, mycelial cords on the inoculum, radial striations of the colony, and brown pigmentation of the medium under the colony and inoculum. There were coded with the numbers of

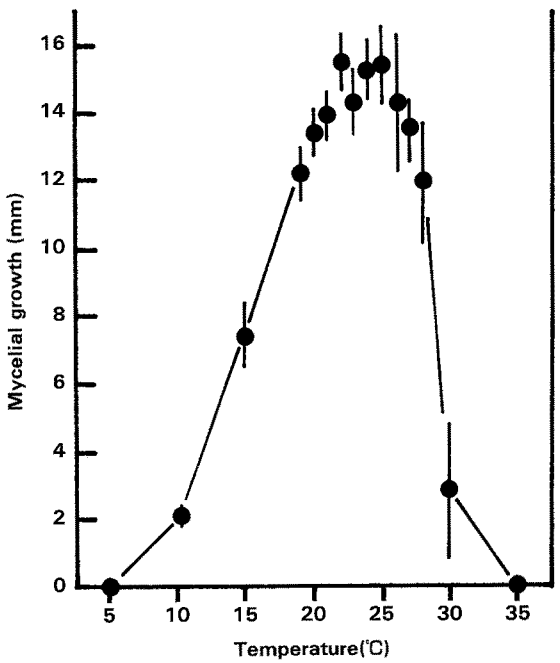

Fig. 1. Influence of temperature on mycelial growth of *T. bakamatsutake*.
Bars indicate standard deviations.

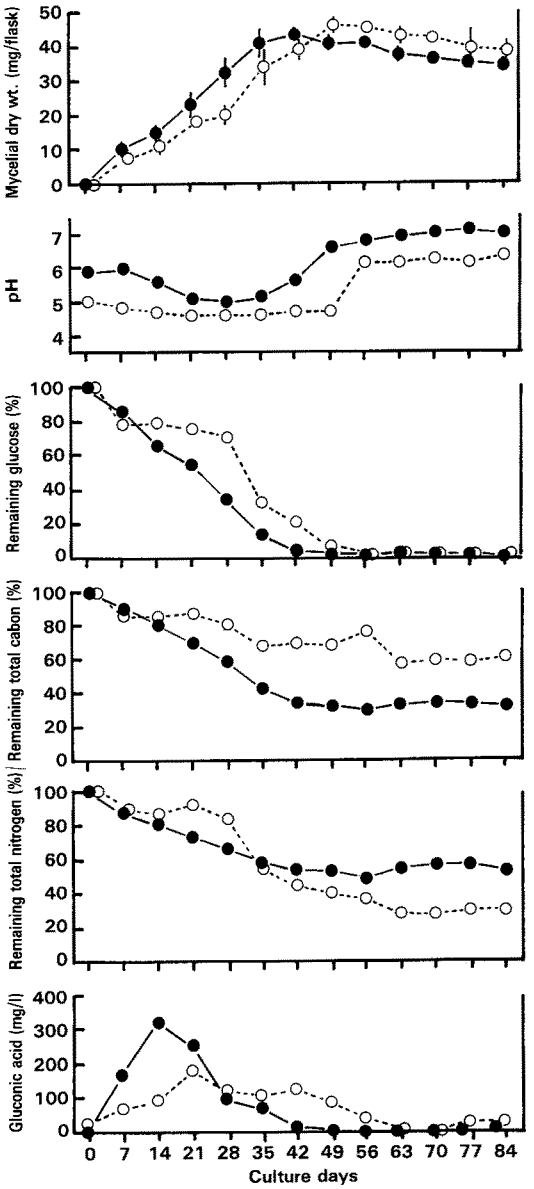

Fig. 2. Changes in mycelial dry weight of *T. bakamatsutake*, pH consumption of glucose, total carbon and total nitrogen, and amount of gluconic acid in media of initial pH 5.0 and 6.0.
Initial pH: ○···○, 5.0; ●—●, 6.0.
Bars indicate standard deviations.

plates out of ten that showed these characters. These colonies of the 29 isolates were also observed under a microscope for the presence or absence of chlamydospores and brown pigment.

Measuring pigmented zones and counting chlamydospores Pigmented zones of mycelial colonies were measured in the same way as mycelial growth was measured on agar plate media. To count chlamydospores, 2 mm (along a radius) × 1 mm mycelial plugs were continuously taken from an inoculum before colonization and after that from the edge of an inoculum to the front margin of a colony. Each mycelial agar plug was homogenized with 5 ml of distilled water in a 30-ml cup in a homogenizer (AM-5, Nihonseiki) at 10,000 rpm for 5 min and centrifugated at 1,200×*g* for 3 min. Chlamydospores were stained with congo-red and the number per μl of precipitate was counted under a microscope using a hemacytometer.

Results

Influence of temperature on mycelial growth Figure 1 shows mycelial growth of *T. bakamatsutake* isolate No. 4 at 5-35°C. Mycelia grew visibly at 10-30°C, and best at around 25°C.

Changes of mycelial dry weight and components in media of pH 5.0 and 6.0 Figure 2 shows changes of mycelial dry weight, pH, percentages of remaining glucose, total carbon and total nitrogen, and amounts of gluconic acid detected in media of pH 5.0 and 6.0 during cultivation of isolate No. 4. Mycelial dry weights reached the maximum on days 42 and 49 respectively in media of pH 5.0 and 6.0 and slightly decreased after that. Values of pH remained low during these growth periods, but rose rapidly in the seven days after the growth peaks. Percentages of remaining glucose decreased and were almost constant from the 7th day after the peaks. Percentages of total carbon and total nitrogen also decreased until the 14 day after the peaks, then increased slightly from the 21st day after the peaks. These increases might be due to the release of substances from dead mycelia into the media. Gluconic acid increased during the first 14 and 21 days, and decreased to near zero after 49 and 63 days in media of pH 5.0 and 6.0 respectively.

Influence of medium pH on mycelial dry weight and medium components Figure 3 shows the mycelial dry weight, final pH, percentages of remaining glucose, total carbon and total nitrogen, and amount of gluconic acid during cultivation of isolate No. 4 in media with 10 different initial pHs. Mycelia grew best in pH 5.0 media. In all media, final pH values were between 4.0 and 5.7. Remaining glucose, total carbon and total nitrogen were lower in media of pH 5.0-7.0. In media of pH 5.0 and 5.3, glucose remained and the mycelia might have grown more. In media of pH 6.5 and 7.0, glucose was not exhausted, but the increasing percentages of total carbon and nitrogen indicate that the self-digestion of mycelia might have begun. Gluconic acid secretion increased with the increase of initial pH.

Changes of mycelial dry weights and medium components due to mycelial growths of the 29 isolates As shown in Table 1, the maximum and minimum mycelial growth among the 29 isolates were observed for No. 29 and 21, respectively. pH values of all the isolates were between 4.1 and 4.8. Mycelia grew better in the media with lower percentages of remaining glucose, total carbon and total nitrogen. Amounts of gluconic acid produced had no relationship with either mycelial growth or pH.

Colonial morphologies of the 29 isolates Table 2 shows mycelial dry weights and coded morphological characters of colonies of the 29 isolates. Most of the isolates showed irregular colony shapes, rough mycelial fronts, brown pigmentation of colonies and inocula, aerial hypha on colonies and inocula, mycelial cords on inocula absent, radial striations of colonies absent and brown pigmentation of media under colonies and inocula. Mycelial dry weights were not correlated with either brown pigmentation of colonies or media.

Dissimilarities of the 29 isolates were calculated us-

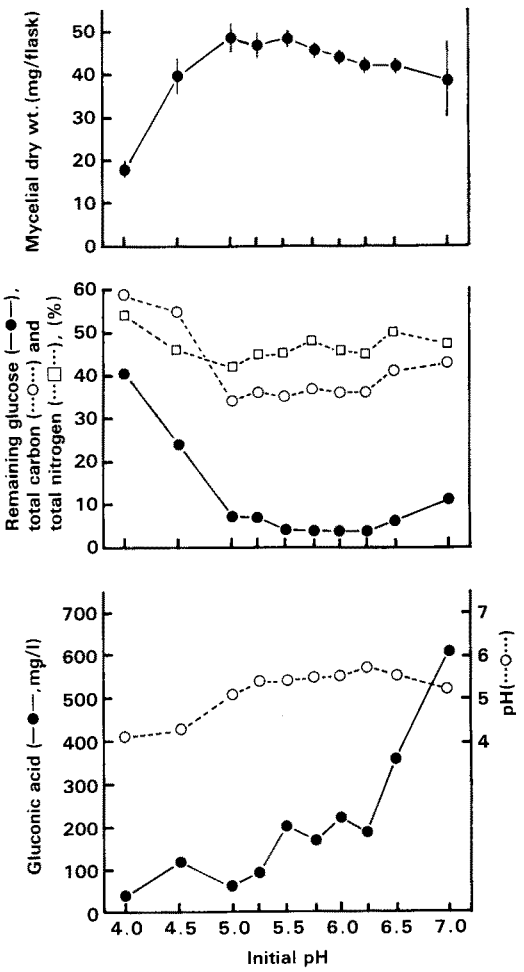

Fig. 3. Influence of initial pH of media on mycelial dry weights of *T. bakamatsutake*, final pH, consumption of glucose, total carbon and total nitrogen, and amount of gluconic acid.
Bars indicate standard deviations.

Table 1. Mycelial dry weight, pH, percentages of remaining glucose, total carbon and total nitrogen, and amount of gluconic acid in liquid media after cultivation of 29 isolates of *T. bakamatsutake*.

Isolate No.	Locality	Mycelial dry wt. (mg/flask)	pH	Remaining components (%)			Gluconic acid (mg/l)
				Glucose	Total carbon	Total nitrogen	
29	Yuzuhara, Kouchi[1]	43.8± 4.9[6]	4.2	15.7	44.7	61.6	34.0
23	Nakaheji, Wakayama[2]	43.6± 5.9	4.2	18.5	46.5	62.5	62.8
1	Tateyama, Chiba	42.1± 5.8	4.1	8.1	42.3	65.0	169.0
4	〃 〃	40.1± 3.3	4.5	20.5	48.5	65.9	165.1
5	〃 〃	38.2± 6.6	4.2	25.2	53.9	70.0	130.7
25	Nakaheji, Wakayama[3]	37.0±13.0	4.3	21.3	44.2	59.5	126.4
7	Tateyama, Chiba	34.8± 9.7	4.4	24.7	50.2	66.1	160.5
13	〃 〃	33.7±11.6	4.5	24.8	40.6	68.0	47.5
28	Totsukawa, Nara[4]	33.7± 6.4	4.5	29.8	54.3	68.0	47.5
14	Tateyama, Chiba	33.6± 7.1	4.3	25.8	52.0	66.4	62.8
20	Tsukui, Kanagawa[5]	33.5± 5.9	4.4	43.7	58.5	66.0	0
3	Tateyama, Chiba	32.7± 5.5	4.3	30.9	55.2	67.8	147.2
2	〃 〃	31.6± 4.9	4.5	26.2	53.5	70.0	155.9
10	〃 〃	31.1± 4.0	4.5	41.1	61.7	72.0	108.1
16	〃 〃	30.8± 4.8	4.3	40.3	60.8	69.0	139.5
19	Kimitsu, Chiba	29.2± 4.0	4.5	52.1	60.6	72.2	158.5
6	Tateyama, Chiba	29.2± 2.2	4.3	37.2	59.4	70.0	245.6
15	〃 〃	28.7± 7.3	4.2	35.0	59.8	65.3	47.5
22	Nakaheji, Wakayama[2]	28.1± 4.6	4.5	51.8	63.7	75.1	6.4
18	Kimitsu, Chiba	26.8± 9.6	4.4	44.7	63.0	72.1	218.4
8	Tateyama, Chiba	26.5± 4.5	4.5	50.8	64.7	70.5	13.5
12	〃 〃	26.0±10.4	4.3	47.7	62.4	68.2	254.2
26	Nakaheji, Wakayama[3]	24.8± 6.4	4.3	40.8	61.3	71.5	264.0
9	Tateyama, Chiba	23.3± 4.9	4.4	42.6	63.8	74.3	166.2
27	Nakaheji, Wakayama[3]	21.2± 6.6	4.5	56.4	69.8	74.2	172.3
17	Kimitsu, Chiba	20.4± 1.6	4.7	62.7	70.9	75.7	110.6
11	Tateyama, Chiba	17.5± 6.9	4.5	45.4	56.5	60.3	178.9
24	Ryujin, Wakayama[3]	15.6± 2.5	4.4	61.1	74.5	72.3	381.3
21	Kiyokawa, Kanagawa[5]	15.1± 3.9	4.8	60.1	75.9	77.9	125.5

Provided by [1] Mr. M. Arao, [2] Dr. K. Iwase, [3] Mr. S. Jyodo, [4] Mr. M. Kinuta, and [5] Mr. N. Kiuchi. [6] Means and standard deviations.

ing the squared Euclidean distances, and anlyzed by the nearest neighbor method as a dendrogram, shown in Fig. 4. Isolates No. 8, 13, 15, 22 and 26 were morphologically most similar to each other, followed by these five and No. 25, and by these six and No. 9 and 11; and No. 19 was the most dissimilar, followed by No. 12, 3, 18 and 20.

Brown-pigmentation and chlamydospore formation due to mycelial growth Chlamydospores were observed microscopically on every colony of the 29 isolates, and brown pigments on those of 26 isolates. Brown-pigmented mycelia and/or brown pigmented crystals were found microscopically on some plates.

Figure 5 shows lengths of brown-pigmented zones in relation to the whole mycelial growth lengths, and the numbers of chlamydospores on inocula or colonies of isolate No. 4. The mycelia began to colonize from day 14, and brown pigmentation was observed from day 35. Chlamydospores were found from day 42 on almost the whole colony except for the 2 mm zone of the mycelial front, where the pigmentation had not yet developed.

Discussion

In liquid media of initial pH 5.0 and 6.0, mycelia of *Tricholoma bakamatsutake* isolate No. 4 secreted gluconic acid and medium pH decreased during the early stages. Mycelial growth rates then increased to their maxima, after which secretion of gluconic acid decreased and pH rose. More gluconic acid was secreted at higher initial pHs. Mycelia grew best in media of pH 5.0–6.3, and gluconic acid secretions were comparatively low.

Organic acid secretion by ectomycorrhizal fungi has been reported for oxalic acid in connection with calcium accumulation (Lapeyrie et al., 1987; Cromack, Jr. et al., 1979; Malajczuk and Cromack, Jr., 1982) and disease suppression (Duchesne et al., 1989), and humic, fulvic and uronic acid (Tan et al., 1978). Gluconic acid synthe-

Table 2. Mycelial dry weights and coded morphological characters of colonies of 29 isolates of *T. bakamatsutake*.

Isolate No.	Mycelial dry wt. (mg/plate)	Character									
		I	II	III	IV	V	VI	VII	VIII	IX	X
1	28.2± 6.2	10	1	4	3	10	10	10	10	10	10
2	21.3± 4.5	8	3	10	4	10	10	10	10	10	10
3	16.3± 3.1	7	10	0	0	10	10	10	10	0	10
4	31.6± 2.7	6	10	10	7	10	10	10	10	0	10
5	23.1± 3.8	10	10	3	1	10	10	10	10	10	10
6	25.4± 2.3	10	10	2	4	10	10	10	10	10	10
7	19.4± 5.1	10	10	0	0	10	10	10	10	10	10
8	25.4± 2.6	10	10	10	10	10	10	10	10	10	10
9	13.0± 2.3	9	10	7	8	10	10	10	10	10	10
10	29.5± 3.3	10	10	5	8	10	10	10	10	2	10
11	22.4± 3.7	9	9	7	6	10	10	10	10	10	10
12	11.1± 1.1	0	10	10	0	0	10	10	10	10	10
13	13.7± 3.0	10	9	10	10	10	10	10	10	10	10
14	19.3± 2.8	2	10	10	10	10	10	10	10	10	10
15	16.2± 1.8	10	10	10	10	9	10	10	10	10	10
16	28.7±17.2	10	9	7	9	8	10	10	10	7	10
17	23.6± 8.9	10	8	10	8	7	10	10	10	10	10
18	38.5± 9.7	6	10	10	10	10	10	10	10	7	2
19	6.1± 1.4	8	10	10	10	0	0	10	10	0	10
20	33.9± 6.2	6	0	0	2	10	10	7	6	10	10
21	40.5± 7.2	6	5	3	9	8	10	10	10	10	10
22	29.2± 3.9	10	10	10	10	10	10	10	10	10	10
23	39.9± 4.8	10	10	10	2	10	10	10	10	10	10
24	15.7± 5.5	10	10	3	5	8	10	10	10	10	10
25	29.6± 9.3	9	10	8	10	10	10	10	10	10	10
26	18.8± 2.9	9	10	10	10	10	10	10	10	10	10
27	33.3± 8.5	9	10	9	2	7	10	10	10	10	10
28	20.9± 3.5	5	10	10	10	4	10	10	10	10	10
29	21.7± 7.0	7	10	1	3	9	10	10	10	10	10

I: Irregular colony shape, II: rough mycelial front, III: brown pigmentation of colony, IV: brown pigmentation of inoculum, V: aerial hypha on colony, VI: aerial hypha on inoculum, VII: mycelial cords absent, VIII: radial striation of colony, IX: brown-pigmented medium under colony, X: brown-pigmented medium under inoculum.

sis by *T. bakamatsutake* and *T. robustum* mycelia, two allied species, was reported to inhibit mycelial growth (Iwase, 1992). The finding here that *T. bakamatsutake* mycelia secreted gluconic acid and that mycelial growth accelerated after stabilization of pH suggested that this acid served as a pH stabilizer.

A morphological study of colonies of *T. bakamatsutake* was reported by Shimazono (1979). Irregular colony shape, rough mycelial front, brown pigmentation, aerial hypha, the characters of most of the 29 isolates of *T. bakamatsutake* here, agreed with those of the strain used by Shimazono. Statistical analysis of numerically coded characters has been reported for *Lyophyllum* species by Clémençon and Moncalvo (1990). Similarity between isolates was not correlated with proximity of origin. Isolates originating from Tateyama, Chiba Pref. did not show similarities to each other, whereas isolate No. 19 originating from Kimitsu, Chiba Pref. showed the highest dissimilarity.

Brown pigmentation was macroscopically observed on most of the mycelial colonies here. Pigmentation of *T. bakamatsutake* mycelial colonies has been reported (Ogawa and Ohara, 1978; Shimazono, 1979; Iwase, 1992). Iwase assumed that the brown pigment may be an inhibitor of mycelial growth. But the brown pigmentation of either mycelial colonies or medium had no obvious relationship with the mycelial dry weight measured here.

Chlamydospores, asexual, thick walled and perennial spores (Griffiths, 1974), have been observed on some ectomycorrhizal fungi; *Bankera fuligineo-alba* (Schmidt ex Fr.) Pauzar (Danielson, 1984), *Elaphomyces muricatus* Fries (Miller and Miller, Jr., 1984), *Sarcodon imbricatus* (L.: Fr.) Karst. (Agerer, 1991) and *Phellondon nigar* (Fr.: Fr.) Karst (Agerer, 1992). Chlamydospores formed by *T. bakamatsutake* in the field have been reported by Ogawa and Ohara (1978) and Terashima et al. (1993), and in

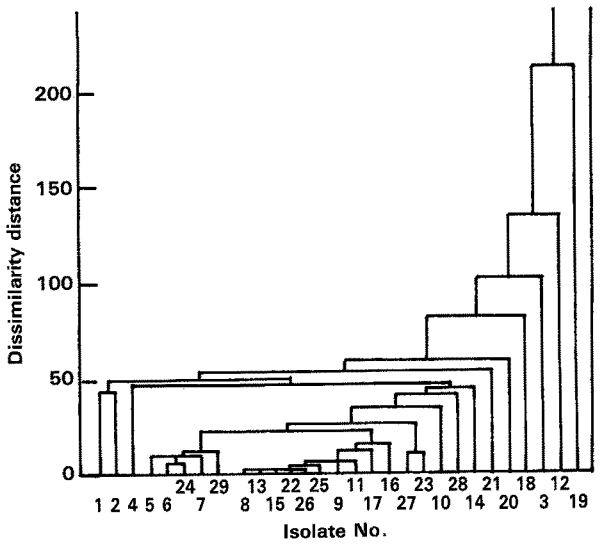

Fig. 4. Dendrogram of coded morphological characters of mycelial colonies of 29 isolates of *T. bakamatsutake*. Dissimilarities are calculated as squared Euclidean distances and analyzed by the nearest neighbor method.

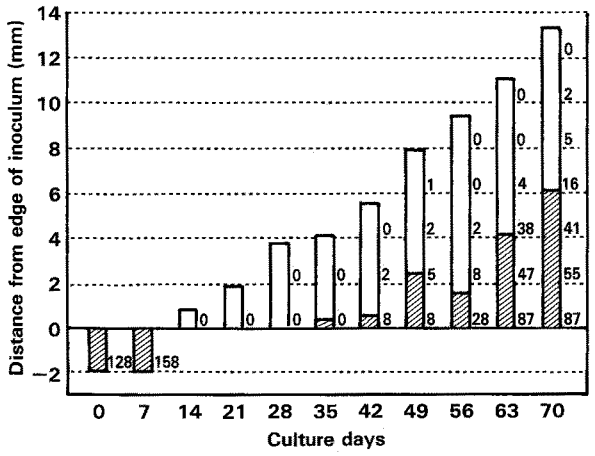

Fig. 5. Brown-pigmented zones and numbers of chlamydospores on mycelial colonies of *T. bakamatsutake*.
▨,□: Brown-pigmented and non-pigmented zones, respectively.
Numbers indicate numbers of chlamydospores ($\times 10^8$) on 2 mm (along a radius) $\times$ 1 mm mycelial plugs taken from inocula for the first 7 days and colonies from day 14.

vitro by Shimazono (1979) and Iwase (1990). The fact that chlamydospores were observed on every colony of the 29 isolates here characterized the mycelial culture of *T. bakamatsutake*. Chlamydospores, which are resistant forms originating from modification of hyphal segments (Griffiths, 1974), were observed to be formed near the front margins of active hypha.

Acknowledgements——The author is grateful to Dr. A. Suzuki, Assoc. Professor of Chiba University, for helpful suggestion and reviewing the manuscript; Dr. T. Terashita, Assoc. Professor of Kinki University, for valuable technical advice; and Dr. K. Yamanaka, Director of Mushroom Laboratory, Hokuto Sangyo Co., Ltd., for his suggestion on examining morphological characters. Thanks are also extended to Mr. N. Kiuchi, Kanagawa Prefectural Forest Experiment Station, Dr. K. Iwase, Kansai Environmental Engineering Center, Mr. S. Jyodo, Forest Division of Wakayama Prefectural Government, Mr. M. kinuta, Nara Prefectural Forest Experiment Station, Mr. M. Arao, Kouch Prefectural Forest Experiment Station for providing mycelial cultures of *T. bakamatsutake*.

Literature cited

Agerer, R. 1991. *Sarcodon imbricatus*. In: "Colour atlas of ectomycorrhizae," (ed. by Agerer, R.), plate 66. Einhorn-Verlag, Germany.

Agerer, R. 1992. Ectomycorrhizae of *Phellodon niger* on Norway spruce and their chlamydospores. Mycorrhiza **2**: 47–52.

Asada, K., Konishi, S., Shimokawa, K. and Saito, K. 1966. IV. Analysis with analyzers, Analysis by chromatography. Jpn. J. Soil Sci. Plant Nutr. **37**: 85–112 (in Japanese).

Clémençon, H. and Moncalvo, J. M. 1990. Taxonomic analysis of cultural characters in the group *Lyophyllum shimeji* (Agaricales, Basidiomycetes) from Japan. Trans. Mycol. Soc. Japan **31**: 479–488.

Cromack, Jr., K. et al. 1979. Calcium oxalate accumulation and soil weathering in mats of the hypogeous fungus *Hysterangium crassum*. Soil Biol. Biochem. **11**: 463–468.

Danielson, R. M.1984. Ectomycorrhizal associations in jack pine stands in northeastern Alberta. Can. J. Bot. **62**: 932–939.

Duchesne, L. C., Ellis, B. E., and Peterson, R. L. 1989. Disease suppression by the ectomycorrhizal fungus *Paxillus involutus*: contribution of oxalic acid. Can. J. Bot. **67**: 2726–2730.

Fukui, S. 1990. Quantitative analysis of sugar by sulfuric acid treatment. In: "quantitative analysis of reducing sugar, 2nd ed.," pp. 54–55, Gakkai Shuppan Center, Tokyo (in Japanese).

Griffiths, D. A. 1974. The origin, structure and function of chlamydospores in fungi. Nova Hedwigia **25**: 503–547.

Iwase, K. 1990. Nuclear distribution in hyphal cells of isolates from fruit-bodies and single basidiospores in ectomycorrhizal fungus *Tricholoma robustum* and allied species: Visualization of nuclei and septa by a double staining method, Mem. Fac. Sci., Kyoto Univ. (Ser. Biol.). **14**. 117–127.

Iwase, K. 1992. Gluconic acid synthesis by the ectomycorrhizal fungus *Tricholoma robustum*. Can. J. Bot. **70**: 84–88.

Kawai, M. and Abe, S. 1976. Studies on the artificial reproduction of *Tricholoma matsutake* (S. Ito et Imai) Sing. I. Effects of carbon and nitrogen sources in media on the vegetative growth of *T. matsutake*. Trans. Mycol. Soc. Japan **17**: 159–167 (in Japanese).

Kiuchi, N. 1983. Comparative studies on physiological characteristics of mycelia of *Lyophyllum shimeji* and *Lyophyllum decastes*. Rep. Kanagawa Forest Expt. Stn. **9**: 9–18.

Lapeyrie, F., Chilvers, G. A. and Bhem, C. A. 1987. Oxalic acid synthesis by the mycorrhizal fungus *Paxillus involutus* (Batsch ex Fr.) Fr. New Phytol. **106**: 139–146.

Malajczuk, N. and Cromack, Jr., K. 1982. Accumulation of calcium oxalate in the mantle of ectomycorrhizal roots of *Pinus*

radiata and *Eucalyptus marginata*. New Phytol. **92**: 527–531.

Miller, S. L. and Miller, Jr., O. K. 1984. Synthesis of *Elaphomyces muricatus*+*Pinus sylvestris* ectomycorrhizae. Can. J. Bot. **62**: 2363–2369.

Ogawa, M. 1978. "Biology of *Matsutake* mushroom," pp. 275–278. Tsukiji Shokan, Tokyo (in Japanese).

Ogawa, M. and Ohara, H. 1978. Microbial ecology of 'Shiro' in *Tricholoma matsutake* (S. Ito et Imai) Sing. and its allied species VIII *Tricholoma bakamatsutake* Hongo in *Quercus mongolica* var. *grosseserrata* forest and *Q. serrata* forests. Trans. Mycol. Soc. Japan **19**: 391–405 (in Japanese).

Ohta, A. 1990. A new medium for mycelial growth of mycorrhizal fungi. Trans. Mycol. Soc. Japan **31**: 323–334.

Shimazono, H. 1979. Comparative studies on morphological characteristics of the colonies of *Tricholoma matsutake*, *T. fulvocastaneum* and *T. bakamatsutake* on agar media. Trans. Mycol. Soc. Japan **20**: 176–184 (in Japanese).

Tan, K. H., Sihanonth, P. and Todd, R. L. 1978. Formation of humic like compounds by the ectomycorrhizal fungus, *Pisolithus tinctorius*. Soil Sci. Soc. Am. J. **42**: 906–908.

Terashima et al., 1993. Distribution and characteristics of shiros of *Tricholoma bakamatsutake* in a mixed forest of *Pasania edulis* and *Castanopsis cuspidata* var. *sieboldii* Trans. Mycol. Soc. Japan **34**: 299–288.