Mycoscience 40: 51–56, 1999

Carbon and nitrogen utilization and acid production by mycelia of the ectomycorrhizal fungus *Tricholoma bakamatsutake* in vitro

Yoshie Terashima

Chiba Prefectural Forest Experiment Station, 1887–1, Haniya, Sanbu-gun, Chiba 289–1223, Japan

Accepted for publication 25 December 1998

Mycelial growth of an isolate of *T. bakamatsutake* was tested in media with C/N ratio ranging from 0 to 50 and with 32 carbon and 12 nitrogen sources. The isolate grew best at the C/N ratio of 30. It utilized the monosaccharides D-glucose, D-mannose, and D-fructose, the disaccharide trehalose, and polysaccharide pectin among the carbon sources; and yeast extract, L-glutamic acid, and ammonium compounds among the nitrogen sources. The growth of ten isolates and secretion of gluconic and oxalic acids were compared in D-glucose, trehalose, and pectin media. The utilization of D-glucose, trehalose, and pectin differed among the ten isolates, but all the isolates secreted gluconic acid in the D-glucose media and oxalic acid in the pectin media.

Key Words——carbon source; ectomycorrhizal fungus; nitrogen source; *Tricholoma bakamatsutake*.

Among mycorrhiza-forming fungi, only ectomycorrhizal and ericoid mycorrhizal fungi can be grown in pure culture (Jakobsen, 1996). Study of the physiology of these fungal mycelia could be a key for analyzing the mechanism of their symbiosis with plants. Physiological studies of carbon and nitrogen utilization by an ectomycorrhizal fungus might reveal features of its survival and colonization in soil.

Tricholoma bakamatsutake Hongo is an allied species of *Tricholoma matsutake* (S. Ito & Imai) Sing. Kawai and Abe (1976) reported the optimum C/N ratio and the utilization of carbon and nitrogen sources for *T. matsutake*, but none has been reported for *T. bakamatsutake*.

Utilization of different carbon sources by mycelia of several ectomycorrhizal fungi has been reported (Palmer and Hacskaylo, 1970; Hacskaylo, 1973; Taber and Taber, 1987; Hutchison, 1990), and reviewed by Molina and Palmer (1982), Harley and Smith (1983), Jakobsen (1996), and Smith and Read (1996). Nitrogen utilization has been reviewed by Molina and Palmer (1982) and Smith and Read (1996).

This paper reports the optimum C/N ratio and the utilization of carbon and nitrogen sources for one isolate of *T. bakamatsutake*, and the utilization of D-glucose, trehalose, and pectin and the production of gluconic and oxalic acids in the media by 10 isolates. Gluconic acid was reported to be produced in the media during cultivation of the mycelia of *T. bakamatsutake* (Iwase, 1992; Terashima, 1994), and both acids were detected in the soil in colonies of *T. bakamatsutake* in the field (Terashima and Mizoguchi, 1995).

Materials and Methods

Tests were carried out to examine the effect on mycelial growth of one isolate of (1) C/N ratio, (2) carbon source, and (3) nitrogen source, and (4) the mycelial growth and contents of gluconic and oxalic acids secreted in the media by 10 isolates grown on three selected carbon sources, D-glucose, trehalose, and pectin.

Organism Isolate No. 4 was used for the C/N ratio, carbon and nitrogen source tests, and nine other isolates, Nos. 6, 10, 19, 20, 22, 23, 25, 26, 27 were also used for the selected carbon source test (Table 1). The isolates were described previously (Terashima, 1994). Nos. 4, 6, 10, 14, and 19 were prepared by explanting fruit-body tissues collected at Tateyama and Kimitsu, Chiba Prefecture and maintained by subculturing several times. The other isolates were provided as stock cultures of mycelia.

For inoculation, mycelial plugs of each stock were transferred onto agar plate media, incubated for up to 2 mo and used within 3 mo. Mycelial disks of 8 mm in diam were taken from the colonies on agar medium at equal distances and used as inocula for the C/N ratio, carbon and nitrogen source tests, and 5-mm disks from the 10 isolates showing different growth were for selected carbon source test.

Media GYP medium, 20 g D-glucose, 5 g yeast extract (Difco), 5 g polypepton (Nihon Pharm., Osaka), 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.2 g $CaCl_2 \cdot 2H_2O$ in 1 L of deionized and distilled water (distilled water hereafter), was used for the preparation of inocula on agar plates. As the basal medium, a well-buffered liquid medium for *T. matsutake* (Ohta, 1990) was used, which consisted of 10 g D-glucose, 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

Table 1. List of *T. bakamatsutake* isolates.

Isolate No.	Locality
4	Tateyama, Chiba
6	〃 〃
10	〃 〃
19	Kimitsu, Chiba
20	Tsukui, Kanagawa[a]
22	Nakaheji, Wakayama[b]
23	〃 〃
25	Nakaheji, Wakayama[c]
26	〃 〃
27	〃 〃

Provided by: [a]Mr. N. Kiuchi, [b]Dr. K. Iwase and [c]Mr. S. Jodo.

$ZnSO_4 \cdot 7H_2O$, 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 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 1 g citric acid, 10 mg thiamine·HCl, 0.1 mg nicotinic acid, 0.03 mg folic acid, 0.3 mg biotin, 0.1 mg carnitine chloride, and 0.03 mg adenine·$H_2SO_4 \cdot 2H_2O$ in 1 L distilled water. The pH was adjusted to 5.0 with 1 N KOH solution before autoclaving at 121°C for 5 min. Vitamin solutions were added to the autoclaved media after filtration through 0.2-μm membrane filters in all tests.

For the C/N ratio test, D-glucose at concentrations ranging from 0 to 50 times relative to 1 g/L ammonium tartrate was autoclaved separately from other components. For the carbon source test, the 32 carbon sources listed in Table 2 were used. Thirty g/L of a solid or liquid carbon source, or none (for control), was added to the basal medium in place of 10 g/L D-glucose. Each dissolved carbon source was filtered to avoid hydrolysis of the complex carbohydrates, except that insoluble or heavily colloidal compounds, soluble starch, dextrin, inulin, pectic acid, pectin, and arabic gum were autoclaved.

Twelve nitrogen sources were used, KNO_3, $NaNO_3$, NH_4NO_3, NH_4Cl, ammonium tartrate, glycine, casamino acids (Nihon Pharm., Osaka), urea, malt extract (Nihon Pharm., Osaka), yeast extract, polypepton and L-glutamic acid. For the nitrogen source test, 1 g/L of a nitrogen source or none (for control), was autoclaved separately from 30 g/L D-glucose solution and added to the basal medium in place of 1 g/L ammonium tartrate.

Cultivation conditions From 8 to 10 100-ml flasks containing 10 ml of medium were examined in each test, and each test was repeated at least twice. The mycelia were incubated at 25°C statically in the dark for 42 d for the C/N ratio test and 28 d for the others.

Measurements Mycelia in each flask were collected by filtration, rinsed with distilled water, dried at 70°C for over 48 h, and the dry weight was measured. Initial and final pH values were measured before and after cultivation, respectively, with a pH meter (F-12, Horiba, Kyoto). Gluconic and oxalic acids were determined by UV-methods with F-Kits (Boehringer Mannheim, Mannheim) following their manufacturer's instructions. The spectrophotometer used was a model UV-150 (Shimadzu, Kyoto).

Table 2. Carbon sources used.

- Carbohydrate
  - Monosaccharide
    - Pentose
      - L-Arabinose
      - D-Xylose
    - Hexose
      - D-Galactose
      - D-Glucose
      - D-Mannose
      - L-Sorbose
      - D-Fructose
    - Aldonic acid
      - Gluconic acid
    - Derivative
      - Sugar alcohol
        - Glycerol
        - D-Mannitol
        - D-Arabitol
      - Cyclitol
        - *myo*-Inositol
      - Amino sugar
        - *N*-Acetyl-D-glucosamine
  - Disaccharide
    - Glycosylglycose
      - Maltose
      - D-Cellobiose
      - Lactose
    - Glycosyl glycoside
      - Sucrose
      - Trehalose
  - Trisaccharide
    - Glycosylglycosyl glycoside
      - Raffinose
  - Polysaccharide
    - Homoglycan
      - Soluble starch
      - Glycogen
      - Dextrin
      - Inulin
      - Pectic acid
    - Heteroglycan
      - Pectin
      - Arabic gum
- Acid
  - L-Lactic acid
  - Acetic acid
  - n-Butyric acid
  - Oxalic acid
- Alcohol
  - Methanol
  - Ethanol

Results

C/N ratios Figure 1 shows the mycelial growth of isolate No. 4 of *T. bakamatsutake* in media with C/N ratio of 0–50. The optimum D-glucose ratio to 1 g/L ammonium tartrate was 30. Growth increased sharply at ratios from 0 to 10 and decreased slighly from 30 to 50.

Carbon sources Figure 2 shows the mycelial growth of isolate No. 4 of *T. bakamatsutake* on 32 carbon sources

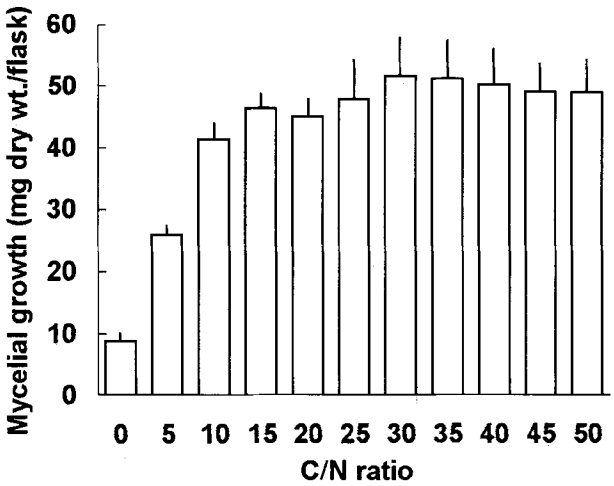

Fig. 1. Mycelial growth of isolate No. 4 of *T. bakamatsutake* after 42 d in media with different C/N ratios.
Bars indicate standard deviations.

and the initial and final pH values of the media. Among the carbon sources tested, the disaccharide trehalose showed the best growth, followed by the monosaccharide D-glucose and polysaccharide pectin. Among the monosaccharides and derivatives, D-mannose, D-fructose, glycerol, and D-mannitol supported relatively good growth. The disaccharides maltose, D-cellobiose, and lactose and the polysaccharides soluble starch and dextrin supported similarly good growth. The acids and alcohols tested induced poor growth.

Nitrogen sources Among the 12 nitrogen sources shown in Fig. 3, yeast extract showed the best growth of the mycelia of isolate No. 4 of *T. bakamatsutake*, followed by L-glutamic acid, NH_4NO_3, NH_4Cl, and ammonium tartrate. The pH values of NH_4NO_3, NH_4Cl, ammonium tartrate, urea and L-glutamic acid media decreased after cultivation, while those of the remaining media stayed the same or increased (Fig. 3).

Selected carbon source for ten isolates Figure 4 shows

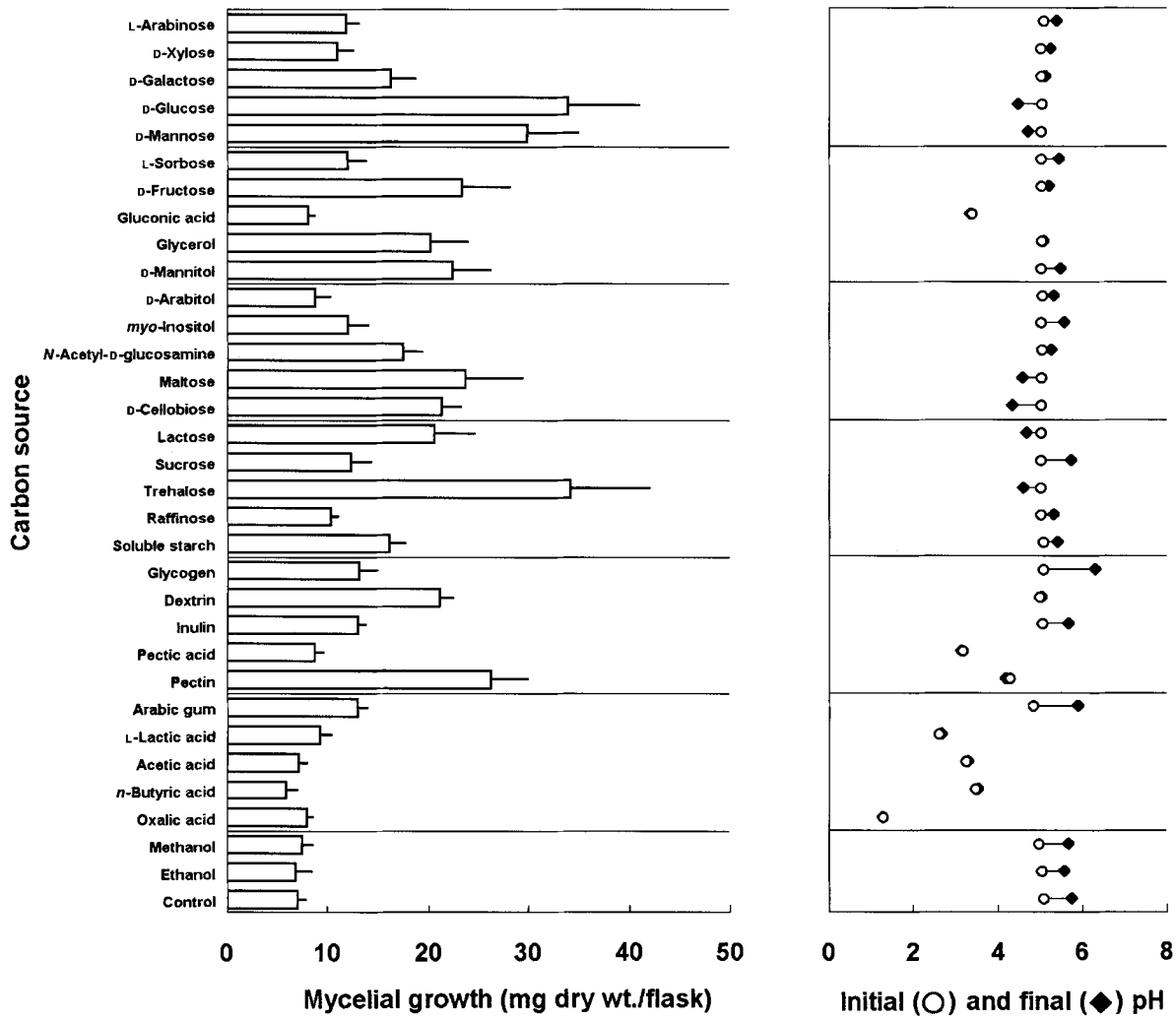

Fig. 2. Mycelial growth of isolate No. 4 of *T. bakamatsutake* after 28 d, and initial and final pH values of media with different 30% carbon sources.
Bars indicate standard deviations.

the mycelial growth of the ten isolates of *T. bakamatsutake* in D-glucose, trehalose, and pectin, the initial and final pH values of the media, and the contents of gluconic and oxalic acids secreted into the media. All of the isolates except for No. 4 grew better in the D-glucose medium than the trehalose medium. The seven isolates other than Nos. 22, 25 and 26 grew better in the D-glucose medium than the pectin medium. The pH values of media before and after cultivation showed similar tendencies for all isolates except No. 26 in trehalose medium, which grew the least. Ten and 5 isolates secreted gluconic and oxalic acids in the D-glucose medium, 9 and 2 in the trehalose medium, and 5 and 10 in the pectin medium, respectively. No. 27 secreted the greatest amounts of gluconic and oxalic acids in the D-glucose and trehalose media. In the trehalose medium, No. 23 secreted more gluconic acid than in the D-glucose medium.

Discussion

Among the carbohydrates tested, the monosaccharides D-glucose, D-mannnose, and D-fructose were utilized by *T. bakamatsutake*. Ectomycorrhizal fungi primarily utilize these monosaccharides (Harley and Smith, 1983), and the same was true of *T. matsutake* (Kawai and Abe, 1976). Norkrans (1950) also reported that four *Tricholoma* species could be grown only on simple sugars, mainly D-glucose. However, according to Palmer and Hacskaylo (1970), D-fructose utilization differed among six ectomycorrhizal fungal species tested.

All 10 isolates of *T. bakamatsutake* utilized the disaccharide trehalose, although there was considerable variation among them. Ectomycorrhizal fungi vary between and within species in the utilization of trehalose (Harley and Smith, 1983; Taber and Taber, 1987); and Palmer and Hacskaylo (1970) reported that only two of their six species grew on trehalose.

The *T. bakamatsutake* isolates utilized pectin to varying extents, and three isolates preferred pectin to D-glucose. Pectin was reported to be a satisfactory carbon source for ectomycorrhizal fungal species (Palmer and Hacskaylo, 1970), but some *Tricholoma* species do not utilize pectin (Hutchison, 1990). It is interesting to evaluate the ability to utilize pectin because of the symbiotic relation of fungi with plants. Smith and Read (1996) suspected that the potential of ectomycorrhizal fungi to use different carbon sources might principally be related to colonization and survival in the soil, but pectin utilization ability might be related not to use of forest litter as carbon sources but rather to penetration into the root tissues of the host plants.

Tricholoma bakamatsutake utilized the ammonium compounds NH_4NO_3, NH_4Cl, and ammonium tartrate; the organic nitrogen yeast extract; and the amino acid L-glutamic acid; but not the nitrates KNO_3 and $NaNO_3$, casamino acid, or urea. These fingings agreed broadly with the results reported for other ectomycorrhizal fungi (Molina and Palmer, 1982) and also *T. matsutake* (Kawai and Abe, 1976). A positive response to glutamic acid was observed by Norkrans (1953) in six *Tricholoma* species. Other ectomycorrhizal fungi were reported to grow on nitrates (Smith and Read, 1996). For casamino acids and urea, some isolates of *Tricholoma* species were found to utilize them (Hutchison, 1990) but others did

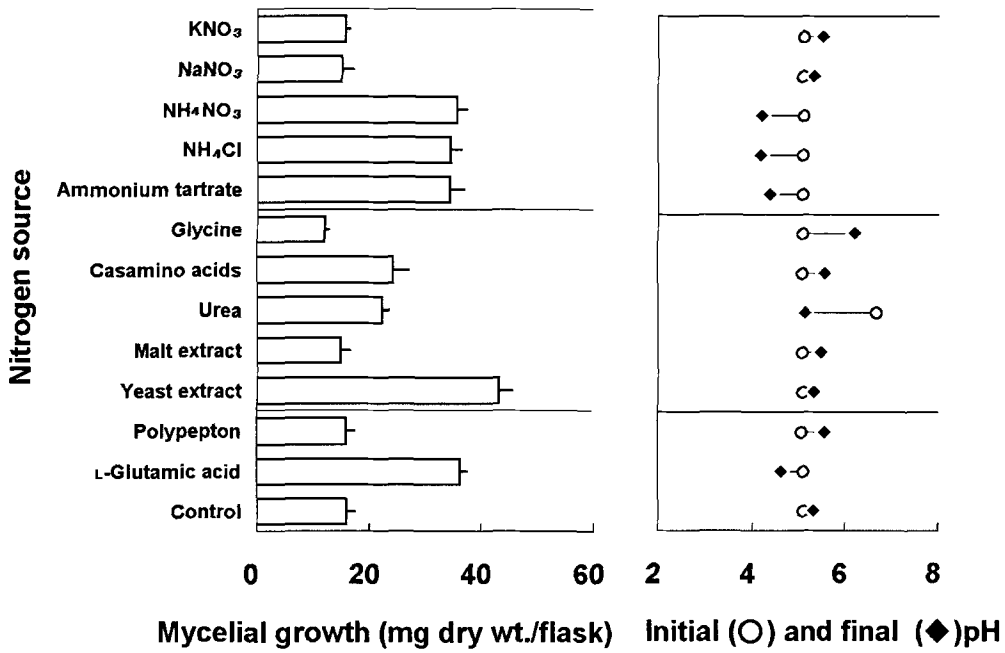

Fig. 3. Mycelial growth of isolate No. 4 of *T. bakamatsutake* after 28 d, and initial and final pH values of media with different 1% nitrogen sources.
Bars indicate standard deviations.

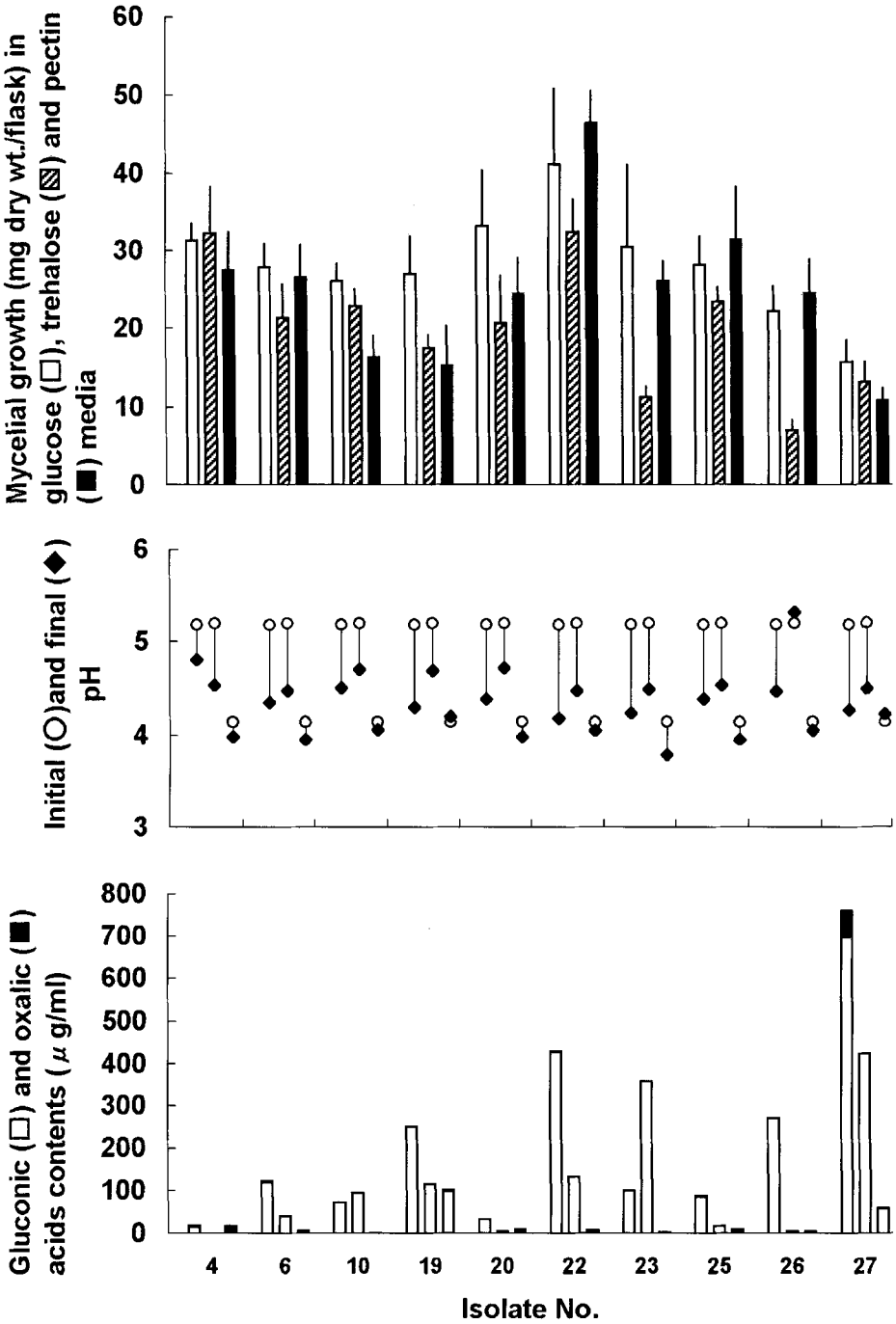

Fig. 4. Mycelial growth of ten isolates of *T. bakamatsutake* after 28 d, initial and final pH values of media, and contents of gluconic and oxalic acids in media with D-glucose, trehalose, and pectin as carbon sources. Bars indicate standard deviations.

not.

The production of gluconic and oxalic acids varied among the isolates and depended on the carbon source in the medium. It was interesting that isolate No. 27 produced both acids during cultivation on D-glucose, trehalose, and even pectin. Production of gluconic acid is peculiar to *T. bakamatsutake* and *T. robstum* (Alb. & Schw.: Fr.) Ricken s. Imazeki., another allied species. Gluconic acid was produced by *T. robstum* in D-glucose and trehalose media but not in D-fructose, D-mannitol, or sucrose media (Iwase, 1992).

Although the production and metabolism of oxalic acid by wood-decaying fungi has been reviewed by Shimada (1993), the significance of oxalic acid production by fungi remains unknown. However, several lines of evidence are suggestive: a plant disease fungus

penetrated plants by secreting oxalic acid, which degraded the pectic polysaccharides of the cell walls of the host plants (Mouly et al., 1992); ectomycorrhizal fungi secreted oxalic acid and released calcium and phosphorus in the soil (Cromack et al., 1979; Terashima and Mizoguchi, 1995); and oxalic acid released from an ectomycorrhizal fungus suppressed disease by *Fusarium* (Duchesne et al., 1989). Production of oxalic acid by ectomycorrhizal fungi was reported by Lapeyrie et al. (1987), and microscopic observation of crystals of calcium oxalate around the rhizosphere was reported by Cromack et al. (1979) and Malajczuk and Cromack (1982). Here, *T. bakamatsutake* utilized pectin and secreted oxalic acid. Oxalic and gluconic acid productions are suspected to have some relationship to the formation of mycorrhizas between the fungus and host plants.

Acknowledgements——The author is grateful to Mr. N. Kiuchi, Kanagawa Prefectural Government, Dr. K. Iwase, Kansai Environmental Engineering Center, and Mr. S. Jodo, Wakayama Prefectural Government for providing isolates of *T. bakamatsutake*. This study was financially supported by the Forestry Agency, Agriculture, Forestry and Fishery, Japan.

Literature cited

Cromack, K., Jr., Sollins, P., Graustein, W., Speidel, K., Todd, A. W., Spycher, G., Lie, C. Y. and Todd, R. L. 1979. Calcium oxalate accumulation and soil weathering in mats of the hypogeous fungus *Hysterangium crassum*. Soil Biol. Biochem. **11**: 463–468.

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**: 2776–2730.

Hacskaylo, E. 1973. Carbohydrate physiology of ectomycorrhizae. In: Ectomycorrhizae. Their ecology and physiology, (ed. by Marks, G. C. and Kozlowski, T. T.), pp. 207–230. Academic Press, New York.

Harley, J. L. and Smith, S. E. 1983. Mycorrhizal symbiosis, pp. 146–154. Academic Press, London.

Hutchison, L. J. 1990. Studies on the systematics of ectomycorrhizal fungi in axenic culture. II. The enzymatic degradation of selected carbon and nitrogen compounds. Can. J. Bot. **68**: 1522–1530.

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

Jakobsen, I. 1996. Carbon metabolism in mycorrhiza. In: Methods in microbiology, vol. 23. Techniques for the study of mycorrhiza, (ed. by Norris, J. R., Read, D. J. and Varma, A. K.), pp. 149–180. Academic Press, London.

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.)

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 K., Jr. 1982. Accumulation of calcium oxalate in the mantle of ectomycorrhizal roots of *Pinus radiata* and *Eucalyptus marginata*. New Phytol. **92**: 527–531.

Molina, R. and Palmer, J. G. 1982. Isolation, maintenance, and pure culture manipulation of ectomycorrhizal fungi. In: Methods and principles of mycorrhizal research, (ed. by Schenck, N. C.), pp. 115–129. Amer. Phytopathol. Soc., Minnesota.

Mouly, A., Rumeau, D. and Tugayé, M. T. E. 1992. Differential accumulation of hydroxyproline-rich glycoprotein transcripts in sunflower plants infected with *Sclerotinia sclerotiorum* or treated with oxalic acid. Plant Sci. **85**: 51–59.

Norkrans, B. 1950. Studies in growth and cellulolytic enzymes of *Tricholoma*. Symb. Bot. Upsal. **11**: 1–126.

Norkrans, B. 1953. The effect of glutamic acid, aspartic acid, and related compounds on the growth of certain *Tricholoma* species. Physiol. Plant. **6**: 584–593.

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

Palmer, J. G. and Hacskaylo, E. 1970. Ectomycorrhizal fungi in pure culture 1. Growth on single carbon sources. Physiol. Plant. **23**: 1187–1197.

Shimada, M. 1993. Oxalic acid metabolism of wood-destroying Basidiomycetes: Toward biochemistry of wood protection. Wood Res. Tech. Note **29**: 1–18. (In Japanese.)

Smith, S. E. and Read, D. J. 1996. Mycorrhizal symbiosis, 2nd ed., pp. 233–275. Academic Press, London.

Taber, W. A. and Taber, R. A. 1987. Carbon nutrition and respiration of *Pisolithus tinctorius*. Trans. Br. Mycol. Soc. **89**: 13–26.

Terashima, Y. 1994. Change in medium components and colony morphology due to mycelial growth of ectomycorrhizal fungus *Tricholoma bakamatsutake*. Mycoscience **35**: 153–159.

Terashima, Y. and Mizoguchi, T. 1995. Nutritional environment of soil and roots in and around mycelial blocks of an ectomycorrhizal fungus *Tricholoma bakamatsutake* in an evergreen Fagaceae forest. Mycoscience **36**: 167–172.