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FULL PAPER

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Mycelial growth characteristics in a split-plate culture of four strains of the genus *Suillus*

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Abstract A split-plate method with two media in different concentrations in each compartment was applied for the mycelial growth of four strains of *Suillus luteus, S. grevillei, S. granulatus*, and *S. bovinus*. As the glucose concentration in the A-side (the side containing higher concentrations of glucose) increased, the mycelial growth in both A- and B-sides (the side containing lower concentrations of glucose) increased. The mycelial density in both sides and B/A ratio (the ratio of the mycelial growth in the B-side to that in the A-side) also increased, and the colony morphology changed. In both A- and B-sides, the colony area reached maximum at 10 g/l glucose in the A-side in most cases and at 33.3 g/l in several cases. The results indicated nutrients are translocated from mycelia in the A-side to those in the B-side. High concentration of phosphate or fructose + glucose in the A-side induced better mycelial growth in the B-side. Addition of high concentrations of phosphate to one side enhanced mycelial growth in the other side. Low-temperature incubation promoted the growth in the B-side. Our split-plate culture method will be useful for qualitative study of translocation in ectomycorrhizal fungi.

Key words High glucose concentration · Mycelial growth · Split-plate culture · *Suillus* · Translocation of nutrients

Introduction

Suillus species are popular in mountainous regions of central Japan, and some of them stimulate the growth of seedlings of *Larix Kaempferi* (Lamb.) Carriere (Murata 1991). We have been developing culture techniques for five species of *Suillus* Mich. ex S.F. Gray and *Boletinus* Kalchbr., which are adapted to media with relatively high concentration of carbon sources (Hatakeyama and Ohmasa, 2004). These fungi forming ectomycorrhizae mainly with conifers uptake most of their carbon sources from roots of host trees and translocate them to extraradical mycelia in soil where carbon sources are lacking. This process is important for redistribution of nutrients and fructification (Lewis and Harley 1965; Harley and Smith 1983; Soderstrom et al. 1988; Lamhamedi et al. 1994; Smith et al. 1994; Simard et al. 1997; Smith and Read 1997; Wu et al. 2001). In mycorrhizae, ectomycorrhizal fungi use a carbon source such as glucose or fructose, which has been converted from sucrose transported to the root of the host (Salzer and Hager 1991, 1993; Hampp et al. 1995; Schaeffer et al. 1995). Concentrations of carbon sources in the host roots vary two to three times with seasons (Shiroya et al. 1966; Fisher 1983; Niederer et al. 1992; Kleinschmidt et al. 1998). These facts indicate that ectomycorrhizal fungi experience relatively large change in carbohydrate concentrations in mycorrhizae with the change of season.

Many studies have been performed on the translocation of solutes in mycorrhizal fungi (Harley and Smith 1983; Jennings 1994; Smith and Read 1997). Most studies use dual organic experimental systems including mycorrhizae and host plants (Melin and Nilsson 1950; Lewis and Harley 1965; Skinner and Bowen 1974; Duddridge et al. 1980, 1988; Finlay and Read 1986a,b; Colpaert and VanLaere 1996; Wu et al. 2001). Pure culture is essential to clarify the characteristics of translocation of carbohydrates in these fungi. Although the phosphate uptake has already been studied with pure culture (Orlovich and Ashford 1993; Shepherd et al. 1993; Ashford et al. 1994), it has been rarely employed to study the uptake of carbon sources.

The split-plate culture method has been frequently used in translocation studies of solutes in saprophytic, vesicular-arbuscular mycorrhizal, and orchid mycorrhizal fungi. Studies on translocation in ectomycorrhizal fungi using the split-plate culture have been rather scarce, although the method has been applied for the formation of sclerotia of *Morchella esculenta* and spores of *Glomus intraradices* (Lundeberg 1970; Cooper and Tinker 1978; Amir et al.

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1992, 1994; ST-Arnaud et al. 1996; Smith and Read 1997; Douds et al. 2000; Tibbett et al. 2000).

In the previous report, we studied the dependence of mycelial growth of nine strains of the genera *Suillus* and *Boletinus* on concentrations of glucose and ammonium tartrate and found that both factors affected the mycelial growth seriously (Hatakeyama and Ohmasa, in press).

The first aim of this study was to examine mycelial growth characteristics in the split-plate culture, varying the concentration gradient of glucose and nitrogen sources. For the first aim, we further studied the effects of the kinds of the carbon sources, high concentrations of the phosphate ion in media, and other factors on mycelial growth. The second aim of this study was to establish the split-plate culture system, which consisted of high-nutrient and low-nutrient portions. For the second aim, we examined the inoculating positions of the split-plate and the amount of glucose that passed through the inoculum agar block. The third aim was to find evidence of nutrient translocation through the mycelium in split-plate culture. For the third aim, we examined the relation of mycelial growth characteristics of both sides of split-plates.

We used *Suillus* strains in this study because we possessed much information about *Suillus* strains and they showed usage of rather high concentration glucose in a previous report (Hatakeyama and Ohmasa, in press), which indicated the possibility of nutrient translocation from the high-nutrient portion to the low-nutrient portion through the mycelium. *Boletinus* strains showed usage of rather lower concentration glucose than *Suillus* strains. Furthermore, mycelial growth of *Suillus* strains was higher than that of *Boletinus* strains. Therefore, in this report, we studied the mycelial growth characteristics of four strains of the genus *Suillus* using the split-plate culture method.

Materials and methods

Fungal strains, media, and preparation of the inoculum

Suillus luteus (L.: Fr.) S.F. Gray SA50, *Suillus grevillei* (Klotz.) Sing. SA56, *Suillus granulatus* (L.: Fr.) O. Kuntze SA531, and *Suillus bovinus* (L.: Fr.) O. Kuntze SA532 are stock cultures of the Laboratory of Applied Mushroom Science, Faculty of Agriculture, Shinshu University.

The synthetic culture medium developed by Ohta (1990) was used for evaluation of mycelial growth as the basic culture medium. The agar medium was used with modifications by the addition of different concentrations of various carbon sources, ammonium tartrate (AT), and potassium dihydrogenphosphate (P).

Each strain was inoculated onto a modified basic agar medium with 1 g/l glucose (G) and 2% agar in a Petri dish, and was cultured for 30 days at 25°C in the dark. Inocula for the split-plate culture were excised with a scalpel of 5×25 mm.

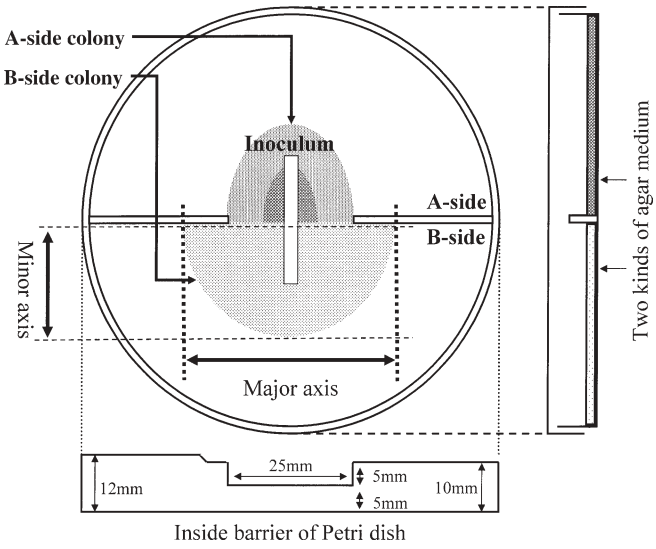

Fig. 1. Diagrams of split-plate culture

Preparation of media for split-plate culture

The internal barrier of the split-plates (KORD-Valmark, Brampton, Canada) was removed as shown in Fig. 1. Then, 14 ml of two different media with 1% agar (Nacalai Tesque, Kyoto, Japan; gelling temperature, 30°–31°C) were each poured into two sides (A- and B-sides) of the split-plate, in which the upper surfaces of both media reached the upper edge of the cut internal wall (5 mm from the bottom of the plate) but were not mixed with each other. The A-side contained media with higher concentrations of G and AT, and, unless otherwise indicated, these were 33.3 g/l G, 5 g/l AT, and 1 g/l P (standard split-plate medium). The B-side contained media with lower concentrations of G and AT, 1 g/l for both compounds, and also 1 g/l P.

Examination of the method of inoculation

Because in a preliminary experiment the growth of ectomycorrhizal fungi was affected severely by the central barrier and by the method of inoculation, it was necessary to find a better method of inoculation.

Using SA56, four inoculation methods were tested to detect the effect of inoculating position and the shape of inocula (Fig. 2): (1) inoculated on the A-side only; (2) inoculated on the B-side only; (3) inoculated on both sides separately; and (4) inoculated to connect both sides. The inoculum disks used for methods 1 through 3 were punched out from colonies with a cork borer 5 mm in diameter. The inoculum for method 4 was prepared as described in the section of strains, media, and preparation of inoculum.

Estimate of the amount of glucose passing through the agar block of inocula

The amount of G that passed through the agar block of inocula, but not by mycelia, was estimated by the following

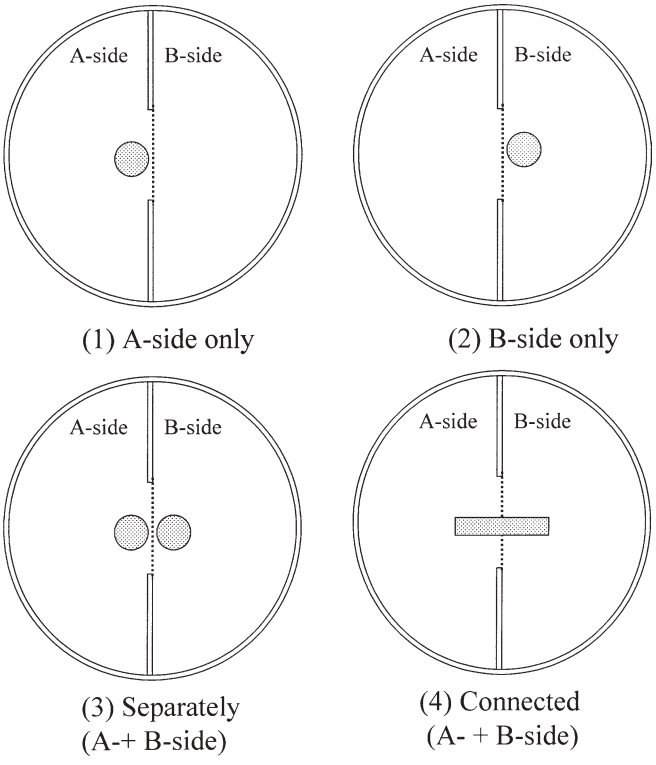

Fig. 2. Inoculating position and shape of inoculum in split-plate culture. (*1*) A mycelial disk 5 mm in diameter was inoculated on A-side only. (*2*) A mycelial disk was inoculated on the B-side only. (*3*) Two mycelial disks were inoculated on both sides separately. (*4*) A mycelial block 5 × 25 mm was inoculated to connect A- and B-sides

two methods. In the first experiment, the agar block with the same composition and size as the inoculum, but not inoculated with mycelium, was placed instead of inoculum as shown in (4) in Fig. 2. The B-side medium contained 1 g/l G and 1 g/l AT, and the A-side medium contained 10, 33.3, 100, or 200 g/l G and 5 g/l AT. Two replicates were prepared. After the split-plate was incubated at 25°C for 40 days in the dark, two agar disks were punched out from one split-plate with a 10-mm-diameter cork borer in zones of 0–10 mm and 20–30 mm (one disk for one zone) from the internal barrier in the B-side, and their G contents were measured. From the concentration of the two zones, the average concentration of the B-side was calculated on the assumption of a stepwise distribution of G concentration by the following method. In control experiments, no agar block was placed between the A-side and the B-side. We assumed that the G concentration of the B-side decreased in a stepwise mode according to the distance from the center of the split-plate. We classified the agar media of the B-side into the following four sections according to the distance from the center: (1) 0–10 mm; (2) 10–20 mm; (3) 20–30 mm; (4) 30–42 mm. We also assumed G concentrations of each section as follows: (1) Conc. 1; (2) (Conc. 1 + Conc. 2)/2; (3) Conc. 2; (4) (3 × Conc. 2 − Conc. 1)/2, where Conc. 1 and Conc. 2 meant G concentration measured in zones 0–10 and 20–30 mm, respectively. Then, we obtained the following formula:

$$\text{Average concentration of B-side (g/l)} \fallingdotseq [1.57 \times (\text{Conc. } 1) + 4.71 \times (\text{Conc. } 1 + \text{Conc. } 2)/2 + 7.85 \times (\text{Conc. } 2) + 13.57 \times \{3 \times (\text{Conc. } 2) - (\text{Conc. } 1)\}/2]/\{(4.2)^2 \times 3.14/2\}$$

Because passing of the sugar through the agar block on which the mycelium grew might differ from the agar block without mycelium, the second experiment was performed. *S. grevillei* SA56 was inoculated on agar media in four standard split-plates and incubated at 25°C in the dark. After 20 days, these plates were incubated for 1 h at 60°C to kill the mycelia. From the two plates, two agar disks 10 mm in diameter were punched out using the cork borer from a zone 20–30 mm away from the internal barrier in the B-side, respectively. The other two dishes were incubated 20 days at 25°C in the dark again, and agar disks were prepared as described above. Sampling from the 0–10 mm zone was not performed because mycelia had already grown to that area.

After each agar disk was incubated in 2 ml distilled water in a vial at room temperature for more than 2 h, G concentration in the water was determined by the enzymatic method (Chaplin 1994). The glucose content of the agar disk was calculated from the G concentration of the water.

Mycelial culture and determination of mycelial growth characteristics

In all experiments except one on the method of inoculation, the inoculum of each strain was placed on media in the split-plate so that it interconnected the two media in the plate. These plates were incubated for 40 days at 25°C in the dark. Only in the experiment on the effect of higher concentrations of G (100–200 g/l) on SA56 was the incubation period 30 days. In all experiments, inoculation was performed in seven replicates for each strain.

After incubation, the lengths of the major and the minor axes of colonies in A- and B-sides were measured (see Fig. 1). Then, the colony of each side was cut out from the split-plate separately, immersed in boiling water to melt and remove agar completely, and washed two times with distilled water. Dry weight of samples was measured as in Hatakeyama and Ohmasa (in press). Mycelial growth was expressed as dry weight. The colony area of the A-side or the B-side was calculated by assuming that each colony had a hemiellipsoidal shape (Fig. 1). The density of colonies and the ratio of mycelial growth in the B-side to that in the A-side (abbreviated as B/A ratio) were calculated. These data were analyzed statistically using Fisher's protected least squares difference (PLSD) test with $P < 0.05$.

Effects of components of media on mycelial growth characteristics

Because in the previous report (Hatakeyama and Ohmasa, in press) we found the growth peak of SA50 and SA56 to be below 100 g/l G, we first tested the growth of the split-plate culture to 100 g/l G in the A-side. The growth of mycelium

Table 1. Effects of inoculating position on mycelial growth characteristics

Inoculating position	Colony side[a]	Mycelial growth (mg DW): mean ± SE[c]	Mycelial density (mg DW/cm^2): mean ± SE[c]	B/A ratio[b]: mean ± SE[c]
A-side	A	70.13 ± 6.12a	3.76 ± 0.42a	0.14 ± 0.01a
B-side	A	83.76 ± 11.96a	4.09 ± 0.67a	0.20 ± 0.02a
Separate[d]	A	87.83 ± 7.11a	5.07 ± 0.68a	0.20 ± 0.04a
Connected[e]	A	87.81 ± 3.73a	3.93 ± 0.71a	0.34 ± 0.04b
A-side	B	10.16 ± 1.05a	0.77 ± 0.04a	–
B-side	B	15.51 ± 2.45a	0.61 ± 0.09a	–
Separate[d]	B	18.07 ± 3.84a	0.90 ± 0.16ab	–
Connected[e]	B	30.40 ± 4.64b	1.41 ± 0.35b	–

DW, dry weight
[a] The side in which measurement was performed
[b] B/A ratio is ratio of mycelial growth in the B-side to that in the A-side
[c] Mean ± SE labeled with different letters (a, b) are significantly different in each section ($P < 0.05$)
[d] The inoculum on each side of medium was separate
[e] The inoculum on each side of the medium was connected

in the B-side, however, increased monotonically until 100 g/l; therefore, we further tested a G concentration greater than 100 g/l.

We tested the mycelial growth in both A- and B-sides by changing the G concentration of the media in A-side from 1 to 100 g/l, with 1 or 5 g/l AT. To detect the effect of G and AT concentrations in A-side media on mycelial growth characteristics, two experiments were done. In one experiment, eight combinations of G and AT were employed: (1) G: 1 g/l, AT: 1 g/l; (2) G: 10 g/l, AT: 1 g/l; (3) G: 33.3 g/l, AT: 1 g/l; (4) G: 100 g/l, AT: 1 g/l; (5) G: 1 g/l, AT: 5 g/l; (6) G: 10 g/l, AT: 5 g/l; (7) G: 33.3 g/l, AT: 5 g/l; (8) G: 100 g/l, AT: 5 g/l.

In another experiment, G and AT concentrations were varied in the following six blocks: (1′) G: 100 g/l, AT: 1 g/l; (2′) G: 150 g/l, AT: 1 g/l; (3′) G: 200 g/l, AT: 1 g/l; (4′) G: 100 g/l, AT: 5 g/l; (5′) G: 150 g/l, AT: 5 g/l; (6′) G: 200 g/l, AT: 5 g/l.

In the experiment on the effects of the kind of carbon source on mycelial growth characteristics, G in A-side of the standard medium was changed to the following seven carbon sources: sucrose (abbreviated as S), fructose (F), soluble starch (SS), sucrose + glucose (S + G), fructose + glucose (F + G), soluble starch + glucose (SS + G), and glucose (G, control). In all blocks, total concentration of carbon sources was set to 33.3 g/l. In S + G and SS + G media, G concentration was set to 1 g/l, and in F + G, the concentration of the two carbon sources was set to be equal.

For the evaluation of effects of the P concentrations on mycelial growth characteristics, the concentration of P in the standard media in A- or B-sides was changed in three conditions, 7.35 mM (control), 50 mM, and 100 mM, to produce the following five blocks: (1) 7.35 mM (control); (2) A-side: 50 mM (AP50mM); (3) A-side: 100 mM (AP100mM); (4) B-side: 50 mM (BP50mM); and (5) B-side: 100 mM (BP100mM).

Mycelial growth characteristics of the four strains were studied at 15°C and 25°C to examine the effect of temperature.

Results

Effect of the position of inoculation on mycelial growth characteristics

Effects of inoculating position on mycelial growth characteristics of the strain *S. grevillei* SA56 are shown in Table 1. There was no significant difference of mycelial growth, mycelial density, and colony area in the A-sides among four methods of inoculation. In the B-sides, A+B-connected inoculation stably provided the best results of these three characteristics and B/A ratio, and thus we used this inoculation method in the following experiments.

Amount of glucose passing through the inoculum agar block

A certain amount of G was found to be passed through the agar block (Table 2). Difference in G concentration in different points of measurement was observed in every plate. The amount of G that passed through the agar block varied according to G concentration in the A-sides. In control plates, where no agar block was placed, the translocation of only small amounts of G was observed. Therefore, we neglected this translocation.

The glucose concentration in the B-side of the split-plate medium changed because of the transport of G from the A-side through the inoculum. In split-plates only with agar blocks of the same size as inocula, the average concentration of G after 40 days incubation at 25°C was calculated to be 3.57, 9.05, 27.48, and 68.99 g/l, respectively, for A-side G

Table 2. The amount of glucose passed through the inoculum agar block

Kind of inocula	A-side glucose concentration (g/l)	Incubation period (days)	B-side glucose concentration after incubation (g/l)		
			0–10 mm from the wall (Conc.1)	20–30 mm from the wall (Conc.2)	Average concentration
Agar block	10	40	4.61	3.67	3.57
	33.3	40	15.03	9.61	9.05
	100	40	36.51	28.32	27.48
	200	40	89.02	70.87	68.99
Mycelium[a]	33.3	20	–	5.66	–
	33.3	40	–	5.87	–

Conc., concentration
[a] The mycelium was killed at 60°C after the 20-day incubation

concentration of 10, 33.3, 100, and 200 g/l. The average G concentration in the A-side at the 40th day was calculated to be 6.43, 24.25, 72.52, and 131.01 g/l, respectively. Because these values were obtained at the 40th day, G concentration in the B-side before the 40th day was always lower than these values and was 1 g/l at time 0. The glucose concentration in the A-side as calculated above was considered to be the lower limit. The glucose concentration in the B-side was reduced to about 60% in the split-plate inoculated with the mycelium of SA56 compared to that in the agar plate with the corresponding G concentration, 33.3 g/l. If the same rate applies to other concentrations in the A-side and in the other strains, the concentration of G in the B-side would be about 60% of values mentioned above, that is, 2.14, 5.43, 16.49, and 41.40 g/l, and that in the A-side, 7.86, 27.87, 83.51, and 158.60 g/l, respectively.

In SA56, the difference of G concentrations between the 20th and 40th day was very small. Because mycelia were killed at the 20th day, this fact indicated that the diffusion of G through the inoculated agar block, which contained a large amount of mycelia, was very small during the latter half of the 40 days.

Morphology of mycelial colony

As shown in Fig. 3, the mycelial density of SA56 became very great as the G concentration in A-side increased. In the surface view, the colonies in both A- and B-sides resembled each other and formed a single colony at higher concentrations of G. In the underside view, the color of each side was different in general, which might reflect the physiological differences in the two sides. These characteristic morphological features were observed in all the four strains used in this study. To analyze the characteristics of the colony, we divided the colony along the internal barrier of the split-plate and measured mycelial growth characteristics separately.

Effect of glucose concentration (less than 100 g/l) and ammonium tartrate concentration on the mycelial growth characteristics

The mycelial growth in both A- and B-sides increased as G concentration increased (Fig. 4). In the A-side, saturation of mycelial growth was observed above 33.3 g/l with 1 g/l AT in all strains. With 5 g/l AT, only SA531 showed growth saturation above 33.3 g/l. For mycelial growth in the B-side, no growth saturation was observed under these conditions and the mycelial growth increased greatly until G concentration reached 100 g/l. In all strains, the maximum mycelial growth in the B-side was observed with 100 g/l G and 5 g/l AT. In many cases, at G concentration 100 g/l, the mycelial growth in the B-side was almost as great as that in the A-side.

In most cases, the colony area in A-side showed the maximum with 10 g/l G and decreased as G concentration increased (Fig. 5). The colony area in the B-side showed the maximum at 10–33.3 g/l or 1–10 g/l and the minimum at 100 g/l or 1 g/l, depending on the strain and AT concentration.

Mycelial density in the A-side increased in all strains as G concentration increased at both AT concentrations (Fig. 6), and became highest with 100 g/l G and 5 g/l AT. In all strains, mycelial density in the B-side also increased as G concentration increased at both concentrations of AT, and showed the highest values with 100 g/l G and 5 g/l AT. The mycelial density in the B-side was generally lower than that in the A-side, but it increased greatly with 100 g/l G. In SA50, the mycelial density in the B-side was as much as that in the A-side at 100 g/l.

The B/A ratio was close to 1 with 1 g/l G, when the G concentration of both sides was equal. As G concentration increased, B/A ratio decreased at first, then increased and became close to 1 in many combinations of strains and AT concentrations with 100 g/l G (Fig. 7).

Effect of glucose (100–200 g/l) and ammonium tartrate concentrations on mycelial growth characteristics

Mycelial growth in the A-side with 1 g/l AT showed the maximum with 100 g/l G in SA50, SA56, and SA532 and remained almost constant in SA531 (Table 3). Mycelial growth in A-side with 5 g/l AT showed the maximum with 100 g/l G in SA56 and SA532. At G concentration 200 g/l, the A-side mycelial growth was generally fairly good.

With 1 g/l AT, change of the B-side mycelial growth was small in all strains except SA532. With 5 g/l AT, the B-side

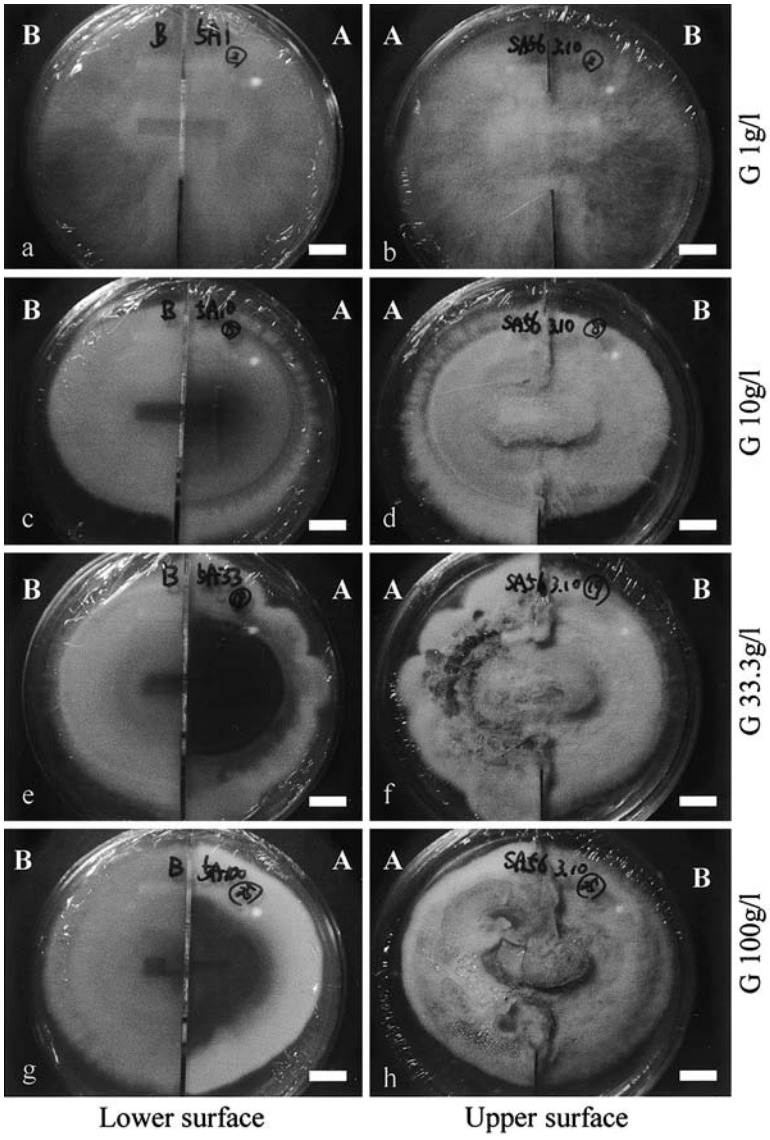

Fig. 3. Upper and lower surfaces of the mycelial colony of *Suillus grevillei* SA56 after 40 days incubation. Capital letters (*A*, *B*) indicate the side of the split-plates. Glucose (*G*) concentrations of the A-sides were 1 g/l (**a**, **b**), 10 g/l (**c**, **d**), 33.3 g/l (**e**, **f**), and 100 g/l (**g**, **h**). Ammonium tartrate concentration of the A-side was 5 g/l. *Bars* 1 cm

mycelial growth increased in SA50, SA531, and SA532, and the change was relatively small in SA56. In all strains, at G concentration 200 g/l, mycelial growth of the B-side was very good at both AT concentrations.

Mycelial density of both A- and B-sides generally remained relatively high (more than 5 mg/cm^2) with 100–200 g/l G concentration of the A-side. With 1 g/l AT, mycelial density in A-side increased as G concentration increased from 100 to 200 g/l or remained constant. With 5 g/l AT, the A-side mycelial density increased in SA50 and SA531, although it remained constant in SA56 and decreased in SA532. The B-side mycelial density increased or remained constant in all strains as G concentration increased at both AT concentrations. In SA50 and SA532, the B-side mycelial density was higher or equal to that of the A-side at G concentrations 150 and 200 g/l.

In the range 100–200 g/l of G concentration in the A-side, the B/A ratio was greater than 1 in 20 cases of 24 and close to 1 in 4 cases.

Effects of the kind of carbon sources, potassium dihydrogenphosphate concentration, and temperature on mycelial growth characteristics

Table 4 shows the effect of the kind of carbon sources on mycelial growth characteristics of SA50. The A-side mycelial growth was generally best with G and second best with F + G. Fructose was the third best carbon source in SA50, SA56, and SA531. For SA56 and SA531, F + G showed a similar result to that of G. In SA50 and SA531, the B-side mycelial growth with F + G was better than that with G and was best. The B-side mycelial growth with F was as good as F + G and better than G for the two strains. For SA56 and SA532, the B-side mycelial growth was best with G and second best with F + G, but the B-side mycelial growth with F + G was almost as good as with G.

Mycelial density in the A-side was generally high with G and F + G: G was best for SA50 and SA56, and F + G was best for SA531 and SA532. Fructose was the third best

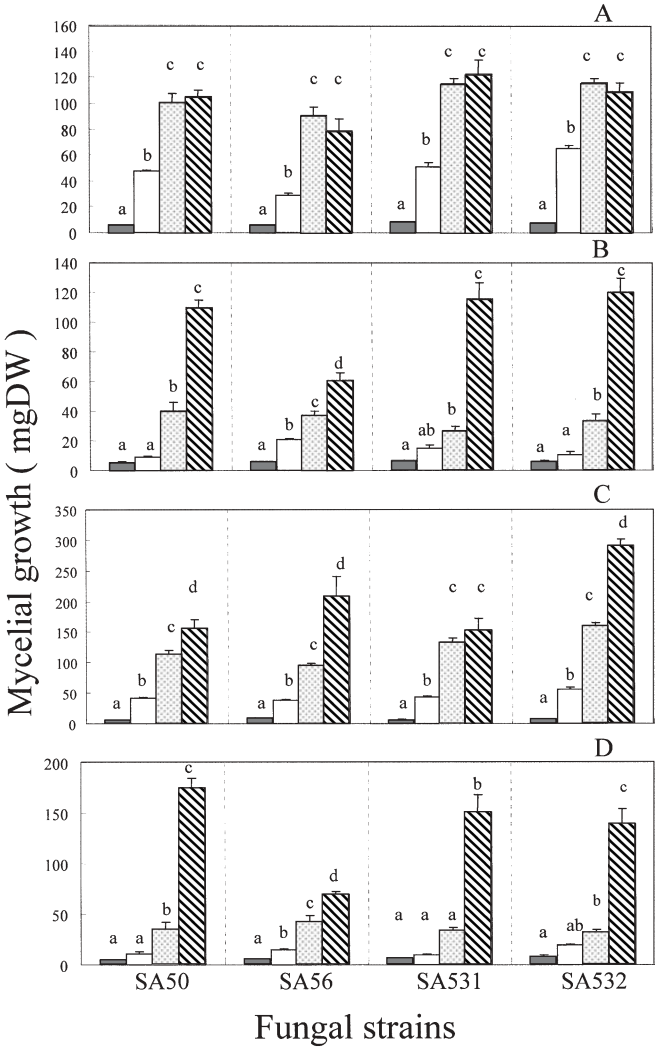

Fig. 4. Changes of mycelial growth of *Suillus* strains according to glucose concentration of the A-side medium. *Vertical axis* shows amount of mycelial growth (dry weight, *DW*); *horizontal axis* shows the strain tested. The following symbols (■, 1 g/l; □, 10 g/l; ⊠, 33.3 g/l; ⊠, 100 g/l) indicate the concentration of glucose in the medium. *Bar* shows SE; *columns* labeled with different letters (*a–d*) are significantly different ($P < 0.05$). In the following figure (Fig. 5), the meaning of symbols is the same. Side of mycelia measured and ammonium tartrate (AT) concentrations in media of the A-sides were as follows: **A** growth in A-side under 1 g/l AT; **B** growth in B-side under 1 g/l AT; **C** growth in A-side under 5 g/l AT; **D** growth in B-side under 5 g/l AT

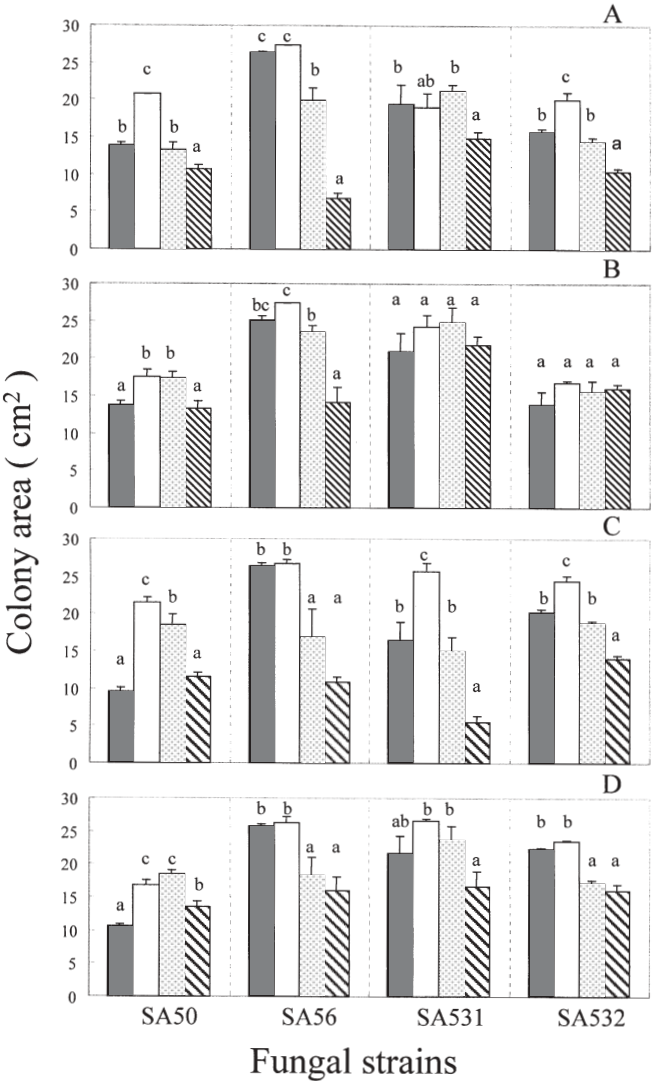

Fig. 5. Changes of colony area of *Suillus* strains according to glucose concentration of the medium. *Vertical axis* shows colony area [colony area (cm^2) = {major axis of the colony/2 (cm) × minor axis of the colony (cm) × 3.14} × 1/2]; *horizontal axis* shows the strain tested. Symbols and figures are as in Fig. 4; **A–D** are same as in Fig. 4

carbon source for SA50, SA56, and SA531. The B-side mycelial density was generally high also with G and F + G: G was best for SA50, SA56, and SA531, and F + G was best for SA50, SA531, and SA532. Fructose was the third best for SA50, SA56, and SA531. B/A ratio was rather high in media with F + G, F, SS, or S.

The effect of P concentration on mycelial growth characteristics were observed in all strains. In many cases, the A-side mycelial growth was best in control (7.35 mM P) or BP100mM and worst in AP100mM. In all strains, the B-side mycelial growth was best in AP100mM. For three strains, the growth was worst in BP100mM, for SA50, control was worst and BP100mM was second worst (Table 5).

The effect of the concentration of P on the A-side mycelial density depended on the strain. The B-side mycelial density remained generally lower than 5 mg DW/cm^2 in all strains. The B/A ratio was best in AP100mM and worst in BP100mM in all strains.

Results of temperature dependence of mycelial growth characteristics showed that the B-side mycelial density and B/A ratio increased as temperature decreased from 25° to 15°C in SA50, SA56, and SA532, and that B/A ratio was greater than 1 at 15°C for SA532.

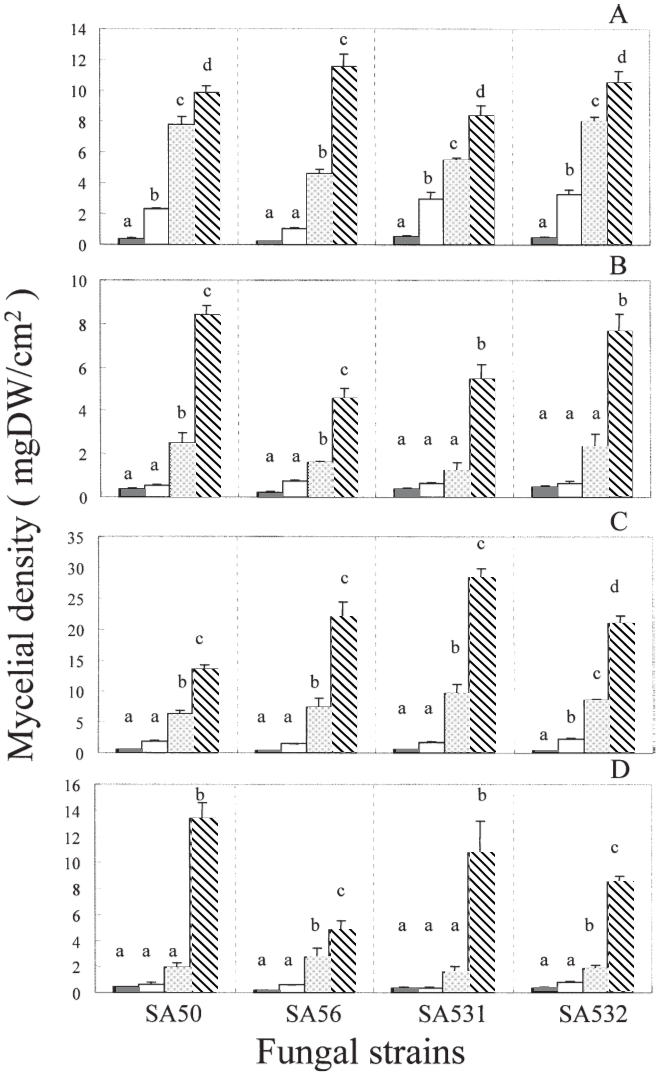

Fig. 6. Changes of mycelial density of *Suillus* strains according to concentration of AT in the culture medium. *Vertical axis* shows mycelial density [mycelial density (mg/cm^2) = dry weight (mg)/colony area (cm^2)]; *horizontal axis* shows the strain tested. Symbols and figures are as in Fig. 4; **A–D** are same as in Fig. 4

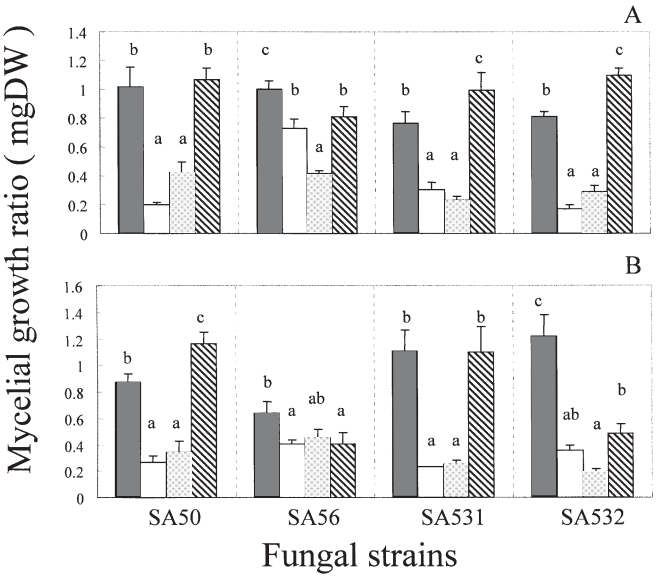

Fig. 7. Changes of B/A ratio of *Suillus* strains according to glucose concentration in the culture medium. *Vertical axis* shows B/A ratio [B/A ratio = dry weight of the B-side (mg)/dry weight of the A-side (mg)]; *horizontal axis* shows the strain tested. Symbols are as in Fig. 4. **A** 1 g/l AT in A-side; **B** 5 g/l AT in A-side

Discussion

Growth in high concentration of glucose

Although many strains of *Suillus* and *Boletinus*, including *S. grevillei* SA56, grew very slightly in Ohta liquid medium containing 100 g/l G (Hatakeyama and Ohmasa, in press), this study shows that the culture system of this work allows mycelial growth in the A-side of the four strains of *Suillus* at higher concentrations of G, such as at least 131 g/l in the A-side. Under these conditions, good mycelial growth of the four strains in the B-side was also observed. The effect of AT concentration on mycelial growth was also important at higher G concentrations in both A- and B-sides. Generally, mycelial growth of fungi is restricted in higher concentrations of solutes because of osmotic pressure. In our preliminary experiments, ectomycorrhizal fungi were not as tolerant to high osmotic pressure. As mentioned earlier, however, our *Suillus* strains could utilize rather high concentrations of G, such as 131 g/l. This result may be compared with the report of Saltarelli et al. (2003), which stated that the growth of a strain of *Tuber borchii* was inhibited by 50 g/l G and that the growth was about half of that in the media with 10 g/l G.

Mycelial density in both A- and B-sides became high with higher concentrations of G. The effect of AT concentration on mycelial density was also observed and was evident with higher concentrations of G. Several ectomycorrhizal fungi increase their tissue density as the nitrogen concentration of media increases and alter their growth forms according to N concentration (Dickie et al. 1998). Our results newly showed that G concentration changed the growth form of the ectomycorrhizal fungi in addition to the effect of N concentration (see Fig. 3). There was an interaction between G concentration and AT concentration on their effect on the mycelial growth and the growth form of our fungi, as seen by Hatakeyama and Ohmasa (in press).

Occurrence of translocation from A-side to B-side

Several results indicate that the translocation of growth substances would have actively occurred from A-side to B-side through the mycelia when the A-side contains higher concentrations of G: (1) B/A ratio was larger than 1 in more than 80% of cases and almost 1 in the other cases at 100–200 g/l G concentrations in the A-side, in which the mycelial growth was good in many cases in both A- and B-sides; and

Table 3. Effects of glucose concentrations (100–200 g/l) and ammonium tartrate concentrations on mycelial growth characteristics

Strains (species)	AT (g/l)	G (g/l)	Side[a]	Mycelial growth (mg DW): mean ± SE[b]	Mycelial density (mg DW/cm^2): mean ± SE[b]	B/A ratio: mean ± SE[b]
SA50 (*Suillus luteus*)	1	100	A	63.03 ± 6.02b	7.37 ± 0.52a	1.12 ± 0.11a
	1	150	A	29.79 ± 3.36a	7.54 ± 0.76a	3.06 ± 0.32b
	1	200	A	17.82 ± 3.70a	7.31 ± 1.53a	4.53 ± 1.10b
	1	100	B	68.30 ± 5.89a	5.62 ± 0.66a	–
	1	150	B	88.57 ± 10.38a	8.69 ± 0.46b	–
	1	200	B	65.90 ± 11.76a	7.77 ± 0.53b	–
	5	100	A	31.61 ± 2.16a	5.23 ± 0.37a	3.20 ± 0.29a
	5	150	A	49.26 ± 15.28a	10.61 ± 1.58b	2.87 ± 0.80a
	5	200	A	34.98 ± 4.31a	9.95 ± 1.08b	4.76 ± 0.83a
	5	100	B	98.96 ± 7.49a	8.79 ± 0.51a	–
	5	150	B	87.37 ± 9.99a	11.34 ± 0.89b	–
	5	200	B	153.88 ± 16.20b	13.02 ± 0.86b	–
SA56 (*S. grevillei*)	1	100	A	75.00 ± 14.01b	10.44 ± 0.65a	0.97 ± 0.08a
	1	150	A	40.25 ± 8.61a	12.12 ± 1.42a	1.62 ± 0.20b
	1	200	A	34.38 ± 8.54a	13.41 ± 2.40a	2.04 ± 0.40b
	1	100	B	68.53 ± 9.79a	4.91 ± 0.46a	–
	1	150	B	57.42 ± 6.50a	5.76 ± 0.51a	–
	1	200	B	60.25 ± 4.65a	5.09 ± 0.41a	–
	5	100	A	95.65 ± 49.29a	12.60 ± 2.1a	0.78 ± 0.17a
	5	150	A	28.55 ± 4.62a	13.05 ± 1.03a	1.65 ± 0.12a
	5	200	A	21.53 ± 5.01a	11.66 ± 1.87a	4.05 ± 0.67b
	5	100	B	65.05 ± 31.44a	5.61 ± 0.76a	–
	5	150	B	46.38 ± 6.29a	5.79 ± 0.27a	–
	5	200	B	71.63 ± 7.98a	6.88 ± 1.01a	–
SA531 (*S. granulatus*)	1	100	A	75.77 ± 13.68a	7.93 ± 0.50a	1.72 ± 0.34a
	1	150	A	77.84 ± 17.04a	7.61 ± 0.43a	1.77 ± 0.41a
	1	200	A	78.07 ± 15.57a	15.15 ± 3.28b	1.45 ± 0.36a
	1	100	B	103.49 ± 4.39a	5.94 ± 0.46a	–
	1	150	B	106.56 ± 14.00a	6.51 ± 0.59a	–
	1	200	B	89.14 ± 16.48a	6.88 ± 0.46a	–
	5	100	A	113.23 ± 30.14a	15.41 ± 2.22a	1.15 ± 0.35a
	5	150	A	143.30 ± 27.42a	25.11 ± 3.81b	0.84 ± 0.23a
	5	200	A	155.59 ± 19.04a	23.59 ± 2.39ab	0.87 ± 0.17a
	5	100	B	83.91 ± 14.53a	9.76 ± 2.19a	–
	5	150	B	108.94 ± 23.77a	8.93 ± 1.64a	–
	5	200	B	132.66 ± 26.77a	9.65 ± 1.17a	–
SA532 (*S. bovinus*)	1	100	A	57.83 ± 7.18b	6.25 ± 0.98a	1.38 ± 0.27a
	1	150	A	32.86 ± 8.06a	8.53 ± 1.83ab	4.41 ± 1.54a
	1	200	A	44.74 ± 7.19ab	11.59 ± 1.92b	3.71 ± 1.10a
	1	100	B	70.36 ± 7.64a	3.58 ± 0.29a	–
	1	150	B	82.84 ± 5.95a	7.45 ± 1.04b	–
	1	200	B	123.74 ± 5.07b	12.41 ± 0.61c	–
	5	100	A	67.38 ± 18.23b	11.64 ± 2.39b	1.16 ± 0.41a
	5	150	A	18.54 ± 14.08a	6.99 ± 2.07ab	29.91 ± 15.17a
	5	200	A	7.48 ± 4.01a	5.53 ± 1.30a	32.75 ± 12.84a
	5	100	B	54.06 ± 10.38a	8.94 ± 2.02a	–
	5	150	B	108.28 ± 5.68b	10.77 ± 0.55a	–
	5	200	B	103.78 ± 9.65b	9.74 ± 1.60a	–

AT, ammonium tartrate; G, glucose

[a] The side where measurement was performed

[b] Mean ± SE labeled with different letters (a, b) are significantly different in each section ($P < 0.05$)

(2) in experiments on the effect of P concentration and the kinds of carbon source on mycelial growth characteristics, some combinations in the A-side gave better growth in the B-side than the control, indicating the translocation of growth substance. The second point is be discussed later. (3) Mycelial density in the B-side was equal to or higher than that in the A-side in many cases for SA50 and SA532; and (4) the colonies of A- and B-side appeared completely con-

Table 4. Effects of the kinds of carbon sources on mycelial growth characteristics of *S. luteus* SA50

Kind of carbon sources[a]	Side[b]	Mycelial growth (mg DW): mean ± SE[c]	Mycelial density (mg DW/cm^2): mean ± SE[c]	B/A ratio: mean ± SE[c]
G	A	62.44 ± 6.63d	7.14 ± 0.77d	0.54 ± 0.10a
S	A	10.63 ± 1.95a	1.30 ± 0.27a	0.62 ± 0.07ab
S + G	A	12.63 ± 0.61ab	1.38 ± 0.11a	0.52 ± 0.04a
F	A	23.31 ± 3.96b	3.21 ± 0.60b	1.90 ± 0.37c
F + G	A	37.34 ± 6.95c	4.59 ± 0.65c	1.37 ± 0.33bc
SS	A	8.53 ± 1.03a	0.77 ± 0.12a	1.11 ± 0.10b
SS + G	A	18.41 ± 1.42ab	1.79 ± 0.14a	0.47 ± 0.06a
G	B	30.57 ± 3.20b	3.55 ± 0.57b	–
S	B	5.83 ± 0.21a	0.79 ± 0.11a	–
S + G	B	6.44 ± 0.35a	0.75 ± 0.07a	–
F	B	36.40 ± 2.58c	3.44 ± 0.26b	–
F + G	B	37.79 ± 2.38c	4.13 ± 0.57b	–
SS	B	8.84 ± 0.47a	0.88 ± 0.11a	–
SS + G	B	8.23 ± 0.55a	0.92 ± 0.09a	–

[a] G, glucose; S, sucrose; F, fructose; SS, soluble starch
[b] The side where measurement was performed
[c] Mean ± SE labeled with different letters (a–d) are significantly different in each section ($P < 0.05$)

Table 5. Effects of potassium dihydrogenphosphate concentrations on mycelial growth characteristics of *S. luteus* SA50

Potassium concentration (mM)	Side[a]	Mycelial growth (mg DW): mean ± SE[b]	Mycelial density (mg DW/cm^2): mean ± SE[b]	B/A ratio: Mean ± SE[b]
7.35	A	97.87 ± 10.71b	6.18 ± 1.16b	0.20 ± 0.01a
AP50	A	100.63 ± 11.41b	8.01 ± 0.49bc	0.30 ± 0.06a
AP100	A	24.79 ± 6.08a	3.87 ± 0.36a	2.20 ± 0.50b
BP50	A	96.70 ± 11.11b	8.79 ± 0.66c	0.41 ± 0.09a
BP100	A	125.85 ± 12.50b	6.95 ± 0.50bc	0.21 ± 0.04a
7.35	B	18.72 ± 1.68a	1.08 ± 0.10a	–
AP50	B	25.81 ± 2.61a	1.74 ± 0.34ab	–
AP100	B	40.27 ± 3.27b	3.54 ± 0.47c	–
BP50	B	33.76 ± 2.26b	2.34 ± 0.27b	–
BP100	B	24.40 ± 3.02a	1.45 ± 0.11ab	–

[a] The side where measurement was performed
[b] Mean ± SE labeled with different letters (a–c) are significantly different in each section ($P < 0.05$)

nected in the front surface of the colony when the A-side G concentrations were high.

In the experiment on the temperature dependence of mycelial growth, B/A ratio at 15°C was close to 1 and larger than that at 25°C in all strains except SA531. In SA532, B/A ratio was more than 1. These facts indicate that more active translocation of growth substances occurred at 15°C.

Olsson (1995) reported active translocation of all major nutrients in both directions when the mycelial density of both sides of interconnected media is high. In our study, at high concentrations of G in the A-side, mycelial densities in both A- and B-sides became high, which suggest the active translocation of major nutrients. Because our experimental conditions were quite different from Olsson's system and the mycelial growth of our fungi was slow, further experiments are required to prove these findings rigorously.

Effect of the kind of carbon source

The kind of carbon sources in the A-side considerably affected the growth in both A- and B-sides. Although F was not so good as G for A-side mycelial growth, G, F + G, and F gave good mycelial growth in the B-side. G and F may be supplied in the apoplast of mycorrhizal roots to ectomycorrhizal fungi by the hydrolysis of S by plant invertase (Salzer and Hager 1991, 1993; Hampp et al. 1995; Schaeffer et al. 1995). The strains used in this study would not produce invertase because S gave poor mycelial growth in both A- and B-sides and it was insufficient for the mycelial growth of *Suillus* and *Boletinus* (Hatakeyama and Ohmasa, 2004). F might contribute to translocation in *Suillus*, because F + G and F gave better mycelial growth in the B-side than G in SA50 and SA531 and rather good

results in the other two strains. Taber and Taber (1987) reported that *Pisolithus tinctorius* used F for respiration, but not for growth. The energy obtained by respiration might be used for translocation, if a similar situation was present in strains used in this study.

Effect of concentration of potassium dihydrogenphosphate

The effect of P concentration was not simple: 100 mM of P in the A-side decreased mycelial growth in the A-side and increased that in the B-side, while the same P concentration in B-side increased the mycelial growth of the A-side and decreased that of the B-side. The effect of 100 mM of phosphate in the A-side was especially clear. The result is reflected in the maximum value of B/A ratio in AP100mM and the minimum in BP100mM. This fact indicates the special role of a high concentration of phosphate ions in the regulation of growth of ectomycorrhizal fungi. Giltrap and Lewis (1981) reported that a high concentration of phosphate ion inhibited the growth of ectomycorrhizal fungi, and Harley and McCready (1952) showed that phosphates were accumulated in the fungal sheath of beech mycorrhiza. Our results suggest the possibility that the phosphate accumulated in the fungal sheath may be used to regulate the growth of mycelia in soil where the carbon source is poor.

Use of two-phase media in split-plate for the study of ectomycorrhizal fungi

Ectomycorrhizal fungi have been considered to accept nutrients from plants in apoplasts of roots (Hampp et al. 1995). Split-plate culture of ectomycorrhizal fungi can be regarded as a simple model of the mycorrhizal system in nature, if we simplify the role of host plants in ectomycorrhizae as providing a suitable medium for the fungi: that is, considering one side contains the medium with the nutrient composition close to the inside of mycorrhizae and the other contains medium resembling that of the outside of mycorrhizae. This method would allow the clarification of some fundamental characteristics of solute translocation in ectomycorrhizal fungi and also may allow us to clarify the potential of the mycelium to translocate nutrients.

In this study, we used the split-plate culture method with two-phase agar media, in which the concentration of nutrients in both phases are different, to study mycelial growth and translocation in *Suillus* spp., and could reveal some of the fundamental features of growth and translocation. The split-plate culture method has been frequently used to study translocation of fungi, such as *Morchella esculenta* and *Glomus intraradices* (Cooper and Tinker 1978; Harley and Smith 1983; Amir et al. 1992, 1994; ST-Arnaud et al. 1996; Smith and Read 1997; Douds et al. 2000). However, this method has rarely been used for studies on ectomycorrhizal fungi (for examples, Lundeberg 1970; Tibbett et al. 2000), especially on translocation study using pure cultures. The split-plate culture method could be useful to analyze effects of chemical factors and simple physical factors qualitatively in pure-cultured ectomycorrhizal fungi.

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