

Rhizoctonia solani Kuhn AG-1 (IA)の菌核形成過程における炭素源の利用

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Carbohydrate Utilization during the Sclerotium Formation of *Rhizoctonia solani* Kühn AG-1 (IA)

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Abstract

The manner of use of the carbon source during the sclerotium formation of *Rhizoctonia solani* Kühn AG-1 (IA) was examined. Both the hyphal weight and the number and weight of sclerotia increased with carbon concentration. When the shift of the carbon source during the formation on the check medium (1% glucose) was investigated with ^{14}C -labeled glucose, almost the entire carbon source was consumed in the time interval from the initial to the maturing phases. CO_2 evolution also showed a similar tendency. The residual carbon was detected at concentrations of carbon source above 1%, increasing linearly with the concentration. The branching internodes and cell lengths of hyphae were shorter at higher concentrations, meaning that a certain amount of carbon is essential for hyphal branching and septation, the first steps in the sclerotium morphogenesis. The activities of enzymes, malate dehydrogenase and isocitrate dehydrogenase, associated with TCA cycle became more vigorous with the carbon concentration. The energy estimated from the CO_2 evolution, required to produce sclerotia, was 4-10 kcal/g. These results show that sclerotium morphogenesis requires a large amount of the carbon source because of the substrates and energy needed for enlargement, development and maturation. The hyphae, once developed, were hydrolyzed and reused for the sclerotium formation if the carbon source in the media had become depleted during the hyphal development.

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Key words: carbon utilization, *Rhizoctonia solani*, sclerotium formation.

INTRODUCTION

Rhizoctonia solani is one of the important plant pathogen with world-wide distribution and an extensive host range^{5,11}. The fungus produces sclerotia as primary infection sources. Sclerotia have the ability to survive under adverse conditions in the soil for longer periods than the ordinary hyphae^{22,24}.

Plant pathogenic fungi infect living organisms and establish a nutritious relationship with them. More than any other nutrients, the carbon source is essential for the pathogenic fungi undergoing heterotrophism. It is needed for the synthetic compounds which make up the living cells, and it is the sole source of appreciable amounts of energy. Depending on the cultural conditions or age, 43-53% (per dry weight) of the cells of most fungi are composed of carbon¹⁵.

It is generally accepted that sexual and asexual reproductions are most likely to occur when a vigorous mycelium has exhausted its nutrients or has been transferred to a medium with low nutrients^{10,25}. It is also supposed that sufficient nutrients are required to develop hyphae, but that sclerotium initiation occurs when the

sources are consumed²⁰. However, there is little knowledge of the effect of respective nutrients.

The study was carried out to evaluate the effects of the quantity of carbon source and clarify the manner of ingestion during the sclerotium formation of *Rhizoctonia solani*. A brief report of this experiment has been published elsewhere¹⁴.

MATERIALS AND METHODS

Fungus. An isolate (C-324) of *Rhizoctonia solani* AG-1 (IA) was used in this study.

Media. The fungus was precultured on potato dextrose agar (PDA). Hopkins medium (2 g KNO_3 , 10 g glucose, 0.5 g MgSO_4 , 0.1 g KH_2PO_4 , 1000 ml water) was used as a basal medium and to amend the glucose concentration.

Effects of the carbon source on sclerotium formation. The basal medium was additionally amended at various concentration of glucose (0-10%). The edge of colony precultured on PDA was cut with a cork borer, put it on the center of various media and incubated at 25°C for 2 weeks. Sclerotia collected from the media and mycelia were repeatedly washed with hot water, desic-

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cated and weighed.

Effects of the carbon source on the hyphal branching and septation. The isolate was incubated on the media amended with various amount of glucose to the basal medium. The hyphal internode, i.e., the distance between two hyphal branches, and the cell lengths were measured with a light microscope at three phases; Phase I: the time when mycelia grow by 2 cm in diameter, Phase II: 6 hr after the mycelial tip had reached the edge of the petri dish, and Phase III: 12 hr after the mycelial tip had reached the edge of the petri dish.

Effects of the carbon source on dehydrogenases. A series (15 ml) of the medium amended with the concentration of glucose (0–10%) was poured into a petri dish and solidified. Then, it was covered with a cellulose membrane (8.5 cm in diameter). Mycelial discs precultured for 2–3 days were placed on the center of the media. When the mycelial tips reached the edge of the plates, the colonies were peeled off, weighed and homogenized in 25 volumes of 0.05 M sodium phosphate buffer (pH 6.5). The homogenates were centrifuged at $3000 \times g$ for 20 min twice. The supernatants were dialyzed against 0.02 M sodium phosphate buffer (pH 6.5) for 12 hr. Aliquots (0.5 ml) of the samples were all mixed with the solution, and incubated at 37°C for 50 min for the measurement. The relative activities were measured at 340 nm by an optical photometer (Hitachi-101) as an increase in NADP_2 . The substrate solutions were prepared as follows. 1) Malate dehydrogenase: 7.5 mg NAD and 7 mg L-malate were dissolved in 5 ml of 0.05 M sodium phosphate buffer (pH 6.5). 2) Isocitrate dehydrogenase: 7.5 mg NAD and 7 mg DL-isocitric acid

trisodium salt were dissolved in 5 ml of 0.05 M sodium phosphate buffer (pH 6.5). The concentration of total soluble protein was tentatively calculated using the following formula^{18,21)}.

$$\text{Protein concentration (mg/ml)} = 1.55D_{280} - 0.76D_{260}$$

Incorporation of carbon source from the medium. ^{14}C -glucose (1.85 MBq) was diluted with distilled water to 1/100. An aliquot (1 ml) part of the solution was filtered through a $0.2 \mu\text{m}$ cellulose nitrate filter membrane into 14 ml of the basal medium amended with the carbon concentration (0.25–10%). Cellulose membranes were sheeted on the media. A mycelial discs from the preculture on PDA was placed on the center of the sheet and incubated at 25°C in an incubator for 14 days. The membranes were peeled off, and then the media were punched out daily at random with a cork borer to obtain 6.5 mm diametral discs. Ten ml of ACS II (Amersham) was added to 1 ml of the properly diluted sample, and the radioactivity was counted by a Packard Tri-Carb 300 C scintillation counter.

Autoradiograph. The isolate was cultured on the medium containing ^{14}C -glucose. After 48 hr and 2 weeks, X ray film (Fuji FR) was placed in close contact with the culture for 2 weeks in a chilled room.

RESULTS

Effect of carbon source on sclerotium formation

To examine the effects of concentration of the carbon source on the sclerotium formation, glucose was added to the basal medium at various concentration (0–10%). Sclerotium differentiation from ordinary hyphae was observed at a concentration above 0.25%, although

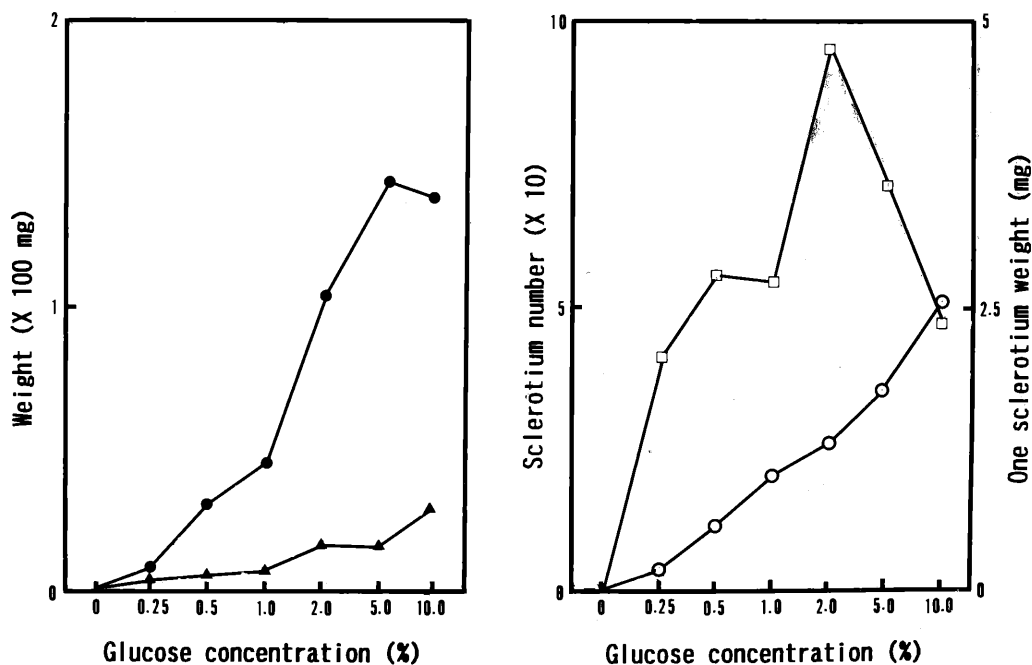


Fig. 1. The effect of glucose concentration on the hyphal growth and sclerotium formation of *Rhizoctonia solani* AG-1 (IA). ▲: Hyphal weight, ●: Total sclerotium weight, □: One sclerotium weight, ○: Sclerotium number.

Table 1. The effect of carbon concentration on the hyphal branching and septation of *R. solani* AG-1 (IA)

Glucose concentration (%)	Internode (μm) ^{a)}			Cell length (μm)		
	I ^{b)}	II	III	I	II	III
0.00	138.8 A ^{c)a} d)	99.7 A b	82.0 A c	205.5 A a	198.4 A a	186.3 A b
0.25	132.3 A a	87.7 A b	64.6 B c	201.5 A a	194.3 A a	173.4 B b
0.50	112.1 B a	97.1 B b	65.6 B c	199.9 AB a	186.5 AB b	142.7 D c
1.00 ^{e)}	99.4 C a	67.8 C b	56.4 C c	190.3 AB a	178.2 B c	153.3 CD c
2.00	81.0 D a	61.8 CD b	52.6 C c	206.6 B a	174.4 B b	138.4 D c
5.00	65.0 E a	56.7 D b	46.9 D c	168.4 B a	177.1 B a	156.5 CD b
10.00	45.1 F a	35.9 E b	— ^{f)}	188.6 B a	140.6 C b	—

a) Internode: distance between two hyphal branches.

b) I: The phase is the time when mycelia grow by 2 cm in diameter at 25°C. II: The phase is at 6 hr after the mycelial tip had reached the edge of the petri dish. III: The phase is at 12 hr after the mycelial tip had reached the edge of the petri dish.

c) Different signatures show significant between the concentrations.

d) Different signatures show significant between the phase ($p < 0.01$ Kruskal-Wallis and Scheffe's multiple comparison tests).

e) Standard concentration in basal Hopkins medium.

f) Scarcely unmeasurable.

Table 2. The effect of carbon concentration on the activities of dehydrogenases of *R. solani* AG-1 (IA)

Glucose concentration (%)	Mycelial weight (mg)	Protein per mycelia ($\mu\text{g}/\text{mg}$)	Malate dehydrogenase		Isocitrate dehydrogenase	
			A ^{a)}	B ^{b)}	A	B
0.00	1.04 B ^{c)}	1.14 C	5.09 G	5.51 B	4.95 F	5.49 B
0.25	1.18 B	1.59 A	8.49 E	10.50 AB	7.22 E	7.02 B
0.50	1.05 B	1.61 A	13.41 D	14.45 AB	11.89 D	12.62 B
1.00 ^{d)}	1.12 B	1.49 AB	43.97 A	41.96 AB	41.07 A	36.26 AB
2.00	1.61 B	0.96 D	18.48 B	30.28 AB	17.07 B	26.75 AB
5.00	3.04 B	1.38 A	14.58 C	43.13 AB	13.79 C	40.49 AB
10.00	8.03 A	1.16 C	6.79 F	54.89 A	7.63 E	61.71 A
Control	—	—	0.00 H	0.00 C	0.00 G	0.00 C

a) Activity per 1 g mycelia.

b) Activity per dish.

c) Significant between different signatures ($p < 0.01$ Kruskal-Wallis and Scheffe's multiple comparison tests).

d) Standard carbon concentration in basal Hopkins medium.

growth was discernible even at 0% (Fig. 1). The total sclerotium weight, as well as individual one, increased with glucose concentration.

Effects of carbon source on hyphal branching and septation

As a result of the measurement of the hyphal internodes and cell lengths before the initial formation, these dimensions diminished with advancing age (Table 1). The differences between glucose concentrations at the early phase (Phase I) were especially remarkable; the dimensions of hyphal internodes at 10% glucose concentration was around one thirds of those at a concentration of 0.25%. The effect of glucose on the cell lengths was also similar to that on hyphal internodes.

Effects of carbon source on dehydrogenases

The protein content per mycelia was somewhat greater at 0.25-1.0% glucose than that at the higher concentrations of glucose (Table 2). Moreover, the activities of two enzymes per 1 g of mycelia were highest at 1% glucose, and the activities at 10% glucose were 1/5-1/6 of those at 1% glucose. However, if the activity was

assessed as a total value per dish, then these enzymes showed higher activity at higher concentration because the mycelial weights would increase with carbon concentration.

Transfer of carbon during the sclerotium formation

Changes in carbon sources with the passage of time were examined with the tracing method using ^{14}C -labeled glucose at 1%. Although only 12% of carbon was consumed within 3 days during the hyphal phase, 80% of the compound was consumed in 3 days during the sclerotium morphogenesis from the initial to the pigmented sclerotium phase (Fig. 2). At the maturation stage (7-14 days), almost no transference of carbon from the media was observed. The change with time of released CO_2 , which subtracted from the total radioactivity in media, mycelia and sclerotium, showed that 11.5% of CO_2 was released during the hyphal phase, whereas 84.5% was released during the initial to matured phases. This behavior closely coincides with the carbon consumption as described above.

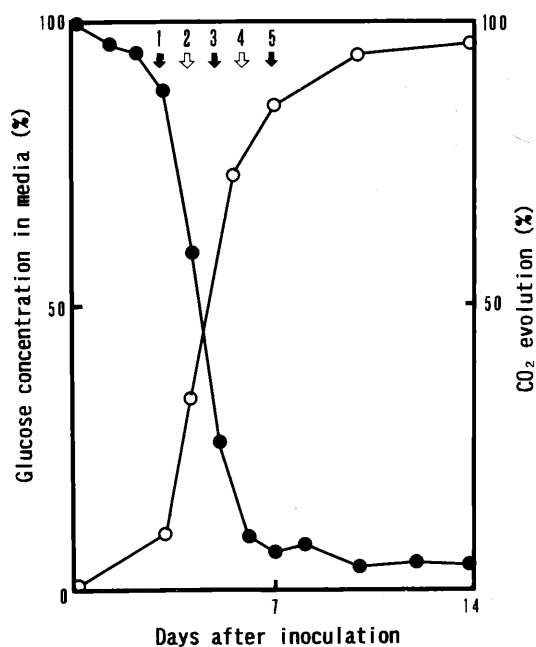


Fig. 2. Consumption of carbon source and CO₂ evolution during the sclerotium formation of *R. solani* AG-1 (IA). The basal Hopkins medium with 1% glucose was used. The residual carbon (●) was estimated from the amount of isotope (¹⁴C) in medium. The evolved CO₂ (○) was estimated from the formula, CO₂ evolution = 100 - (media + colony). 1: Hyphal phase, 2: Initial phase, 3: White sclerotium phase, 4: Pigmenting phase, 5: Matured phase.

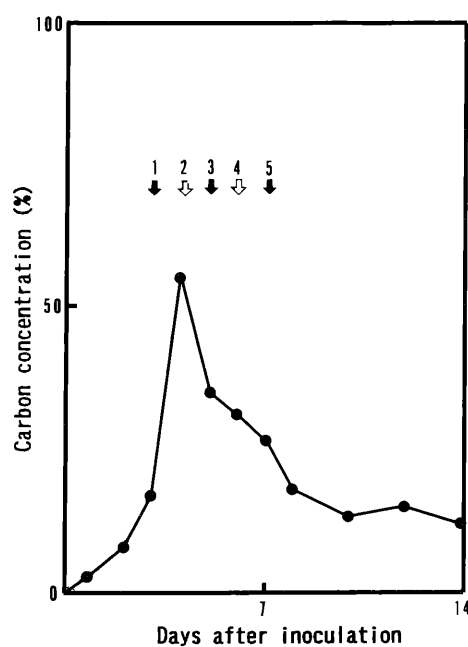


Fig. 3. The shift of carbon amount in hyphae during the sclerotium formation of *R. solani* AG-1 (IA). The basal Hopkins medium with 1% glucose was used. The carbon amount was estimated from the amount of the isotope (¹⁴C) in hyphae. 1: Hyphal phase, 2: Initial phase, 3: White sclerotium phase, 4: Pigmenting phase, 5: Matured phase.

Table 3. The shift of carbon amount in the media containing different carbon concentration during the sclerotium morphogenesis of *R. solani* AG-1 (IA)

Glucose concentration (%)	Amount of residual carbon source (%)		
	0 ^{a)}	5 ^{b)}	10 ^{c)} days
0.25	100 a ^{d)}	6.73 E ^{e)} b	5.42 C c
0.50	100 a	5.93 E b	4.64 C c
1.00 ^{f)}	100 a	26.71 D b	4.13 C c
2.00	100 a	53.51 C b	27.79 C c
5.00	100 a	80.27 B b	64.61 B c
10.00	100 a	91.94 A b	80.10 A c

a) Before culturing.

b) From initial to white sclerotium phases.

c) Matured sclerotium phase.

d) Different signatures show significant between the phases.

e) Different signature show significant between the concentrations ($p < 0.01$ Kruskal-Wallis and Scheffe's multiple comparison tests).

f) Standard concentration in Hopkins medium.

As a result of the culturing on different concentrations of the carbon sources, most of the glucose was immediately consumed at a concentration level 0.5% and below,

although the consumption process and the residual carbon in the media increased as the concentration became higher; 80% of the glucose remained unused on the medium containing 10% glucose (Table 3). The total quantity of carbon consumed, however, increased linearly with the concentration and the total amount of sclerotia formed.

The tracing method indicated that the carbon amount in the hyphae growing on a medium with 1% glucose was highest during the initial phase. It decreased gradually as the glucose concentration exceeded this level (Fig. 3). The isotope distribution was investigated by autoradiography; little carbon source was detected in the hyphae around sclerotia after 2 weeks, rather it was observed in the hyphae during the early hyphal phase (Fig. 4).

Energy consumption during the sclerotium formation

On the basis of the experimental results above, the amount of energy consumed during the sclerotium morphogenesis was estimated by the quantity of glucose converted to CO₂. The energy per dish was increased in proportion to the quantity of carbon source, however, the energy required to form 1 g sclerotia ranged from 4 to 10 kcal, and was inversely proportional to carbon quantity (Table 4). High correlation was observed between the total amount of sclerotia and the energy consumption.

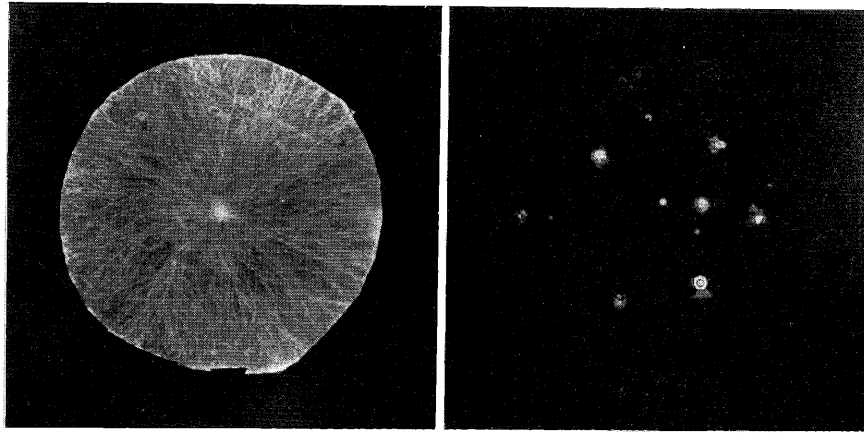


Fig. 4. Accumulation of ^{14}C at hyphal and matured sclerotium phase of *R. solani* AG-1 (IA). Left : Hyphal phase at 48 hr after inoculation, Right : Matured sclerotium phase at 2 weeks after inoculation.

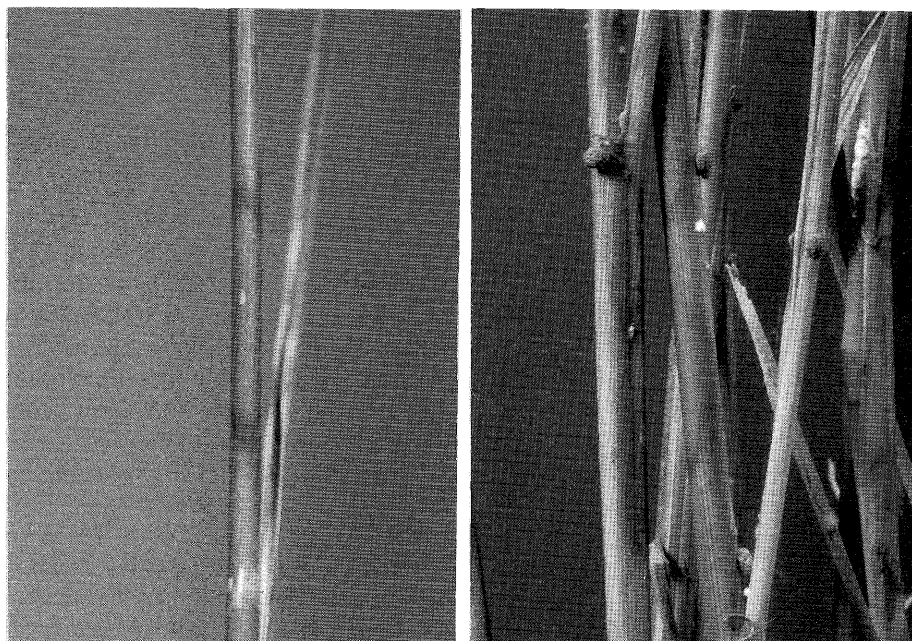


Fig. 5. The symptoms of rice sheath blight caused by *R. solani* AG-1 (IA). Left : Early stage, Right : Sclerotium-formed stage.

Table 4. The energy consumption during the sclerotium morphogenesis of *R. solani* AG-1 (IA)

Glucose concentration (%)	Glucose consumption (%)	Glucose converted to CO_2 (%)	Energy per dish ^{a)} (cal)	Energy per 1 g sclerotia (kcal)
0.25	94.58 ^{b)} E ^{c)}	91.87 F	131.60 F	10.24 A
0.50	95.36 D	91.49 E	262.12 E	8.05 AB
1.00 ^{d)}	95.87 C	85.37 D	489.17 D	9.62 A
2.00	72.21 B	61.46 B	704.33 B	6.07 BC
5.00	35.39 A	21.00 C	601.65 C	3.78 C
10.00	19.90 A	15.21 A	871.00 A	5.47 BC

a) Estimated from evolved CO_2 .

b) Consumption at 10 days after inoculation.

c) Different signatures show significant between the concentrations ($p < 0.01$ Kruskal-Wallis and Scheffe's multiple comparison tests).

d) Standard concentration in Hopkins medium.

DISCUSSION

It is well known that fungal morphogenesis, such as spore, fruit body, sclerotium and other organ formations, is susceptible to various nutritional factors^{1-4,6,7,9,12,23)}. Among the nutrients, the quality and quantity of the carbon sources are usually more significant than that of other nutrients.

Klebs¹⁰⁾ pointed out that the sexual and asexual production of most fungal spore is likely to occur when a vigorous mycelium has exhausted its nutrient or has been transferred to a medium that is low in a nutrient with very specific requirements as regards its concentration. Townsend²⁰⁾ showed that nutritional requirements for the initiation of sclerotia differ from those associated with their further development or maturation; high

amount of carbohydrate favors the initiation of large numbers of sclerotia, but retards or entirely inhibits their maturation in various fungi. On the other hand, it has been proposed that lower amounts of nutrients are required for hyphal growth and initial formation than are needed for further enlargement and maturation during the sclerotium formation of *R. solani*, ranging from the activities of several enzymes to the incorporation and translocation of phosphate¹³⁾.

When hyphal growth and sclerotium formation of *R. solani* on media with various concentrations of glucose (0–10%) were quantitatively examined, the hyphae were seen to grow even at 0% whereas sclerotia are formed only above 0.25%, the weight increasing proportionately with the concentration up to 5%. Although the number of sclerotia formed in a dish increased with the concentration, the optimal concentration for one sclerotium weight was 2%. A result that the fungal hyphae could grow linearly to some extent even on a medium with 0% glucose might be due to contamination during culture and impurities of the ingredients and water. This suggests that a very small amount of carbohydrate allows hyphal development, but that a large amount is required for the sclerotium development or maturation.

Hashiba *et al.*³⁾ indicated that the numbers of sclerotia of *Pellicularia sasakii* (*R. solani*, AG-1) were greatest on the media with nitrogen:carbon ratio of 1.4–7%, although the best N/C ratios for mycelial growth were at 0.03–7% using Czapek medium as a basal one. Their results coincide with those in this study; 10% glucose, which is optimal for the sclerotium formation, could be estimated as an N/C ratio of 0.7% and 5% for the total sclerotium weight 1.4% (Fig. 1). Although in the basal medium (1% glucose) the amount of carbon source consumed from the medium was 10% during hyphal phase and 30% at the phase of initial formation, 50% was consumed during the few days when the sclerotia developed and matured (Fig. 2). The residual quantity of glucose in mycelia, sclerotia and media out of the consumed amount was 4%, with an estimated 96% of glucose being released into the atmosphere as carbon dioxide. The result suggests that a large amount of energy is required for the sclerotium production.

When the manner of consumption was tested at various concentrations, almost all glucose was quickly used up at lower concentrations of glucose, although the chemical was used up slowly in the media containing higher concentrations and the residual amounts in the medium increased with the concentration (Table 3).

There was a tendency for the hyphal internodes and cell length to become shorter with the concentration (Table 1). As the sclerotium initially start from the frequent branching and septation of the normal hyphae, the carbon source is considered to be one of the important factors in sclerotium morphogenesis. Furthermore, the activities of malate dehydrogenase and isocitrate dehydrogenase per 1 g hyphae were maximal at 1% glucose, but the activities per dish increased with the

concentration (Table 2). It is also clear that carbohydrate is as essential as energy source because the chemical is for the cell components and the enzymes take part in the TCA cycle.

In higher plants, a large amount of energy is required for reproductive growth as well as for vegetative growth. Inaba *et al.*⁸⁾ used a rice plant to establish that the respiration rate of kernels after flowering increased with the progress of ripening and reached a maximum in 10–12 days. Furthermore, Seo *et al.*^{16,17)} showed that ATP content kept increasing during the entire ripening period in rice embryo and endosperm. These studies and our present results suggest that the sclerotium formation in fungi and seed formation in higher plants closely resembles in the action of carbon utilization.

Even if almost all of the carbon source had been used up during the hyphal growth at 0.25–0.5% glucose, sclerotia appeared. The tracing method and autoradiography showed that ¹⁴C in vegetative mycelia gradually decreased at low carbon concentration (Fig. 4). Takenouchi¹⁹⁾ established that the activity of acid phosphatase in mycelium increased with incubation time. It is suggested that the hyphae, once developed, are hydrolyzed and consumed as the substrates and energy sources for the subsequent sclerotium formation.

If the energy is estimated in calories from the quantity of released CO₂, ca. 4–10 kcal was dissipated for the 1 g sclerotia (Table 4). As a symptom of rice sheath blight caused by *R. solani* AG-1 (IA), sclerotia are formed at the last stage of the disease progress (Fig. 5). This suggests that a large amount of energy is needed for the sclerotium formation *in vivo* as well as *in vitro*, reflecting the severity of the symptom.

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和 文 摘 要

諸見里善一・小山田耕作・田盛正雄： *Rhizoctonia solani* Kühn AG-1 (IA) の菌核形成過程における炭素源の利用

炭素源濃度の異なる培地で *Rhizoctonia solani* Kühn AG-1 (IA) の菌糸の生育と菌核形成を比較した結果、菌糸重量および菌核の数・量ともにその濃度に比例して増加した。¹⁴C でラベルしたグルコースを用いて標準区(1%)における炭素源の行動を検討した結果、ほとんどの炭素源は菌核原基形成から成熟期までの極めて短期間に消費された。その96%はCO₂として放出され、また、2%以上の濃度区では炭素源の残存が認められ、残存量は濃度に比例して増加した。菌糸の分岐化や隔膜化は炭素源濃度に比例して促進されたが、これは菌糸から菌核原基への段階ではある程度以上の炭素源が必要であることを示している。さらに菌糸中のリンゴ酸脱水素酵素とイソクエン酸脱水素酵素などTCA回路中の酵素類の活性も炭素源濃度に比例して高くなった。放出されたと考えられるCO₂の量から菌核を産生するのに消費されたエネルギーを算出した結果、培地中の炭素源濃度により差異は認められたが、その範囲は4~10 kcal/gであった。以上のことから原基形成以後の発達・肥大および成熟には菌核の基質やエネルギー源として多量の炭素源が必要であることが明らかとなった。また、培地中の炭素源が微量で、菌糸生育期間中に枯渇した場合には、いったん生育した菌糸が加水分解され、その後の菌核形成に再利用されると考えられる。