Mycological and Phytopathological Studies on Rhizoctonia solani K?hn

諸見里, 善一

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VII. Effects of nutrients on sclerotium formation of *Rhizoctonia solani*

It is well known that fungal sclerotium production is affected by various physical and chemical factors[22]. In nutrients, especially, the quality and quantity of carbon and nitrogen sources and the C/N ratio play important roles in the sclerotium production[48, 137]. It was reported that microsclerotial formation of *Verticillium albo-atrum* was promoted by manganese ion and that certain isolates of *Sclerotinia sclerotiorum* did not produce sclerotia without zinc ion[128, 130]. In general, the fungal morphogenesis, i.e. spore, myxospore, perithecium and stroma formation is induced or promoted by various inorganic ions such as potassium, calcium, magnesium, manganese and phosphate ions[130].

In this experiment, the effects of nutrients on the sclerotium formation of 3 isolates of *R. solani* were tested (Exp. 1) and the strong effects of carbon, magnesium and phosphorus on the hyphal and sclerotium formation were demonstrated in detail (Exp. 2). The influence of carbon concentration on the activities of malate dehydrogenase and isocitrate dehydrogenase (EC 1.1.1.41) was also investigated.

Exp. 1

Materials and Methods

Fungi used. Three isolates of the *R. solani* (C-14 isolated from *Cyperus rotundus* in Fukuoka, C-324 from sugar cane in Kagoshima and C-326 from rice plant in Fukuoka) were used. These isolates belong to the anastomosis group AG-1 and the cultural type IA.

Culture. The isolates were precultured on PDA at 25°C for 2～3 days. The small discs of 5 mm in diameter were cut from the edge of the mycelial mat with a cork borer and used as inocula. To test the qualitative and quantitative effects of various nutrients on the sclerotium formation, various substances were replaced or added to amend to the basal medium. Hopkins medium (2 g KNO_3, 0.5 g $MgSO_4$, 0.1 g KH_2PO_4, 10 g glucose, 15 g agar, 1,000 ml water) was used as a basal medium because of its simple composition. Fifteen ml of each medium (autoclaved at 110°C for 10 min) was poured into a Petri dish and inoculated. Culturing was carried out at 25°C for 14 days under dark conditions. After 14 days, the sclerotia formed were taken out, dried at 60°C and weighed. Each value is the average of 5 dishes and every experiment was performed 3 times.

Results

Comparison of sclerotium formation among 3 isolates

To compare the ability of sclerotium formation, the 3 isolates were cultured on the basal medium for 14 days and sclerotia formed were weighed. As shown in Table 11, the C-14, C-324 and C-326 isolates produced about 47 mg, 53 mg and 37 mg sclerotia, respectively, per dish. The process of sclerotium formation of each isolate was similar, although location of sclerotial formation on the medium was slightly different.

Comparison of sclerotium formation among different media

The production of sclerotia by the 3 isolates on various media were studied. The composition of each medium is as follows: (1) PDA medium: 1,000 ml of potato (200 g) decoction, 20 g glucose, (2) Hopkins medium (the basal medium): 2 g KNO_3, 0.5 g $MgSO_4$, 0.1 g KH_2PO_4, 10 g glucose, (3) Asparagine medium: 5 g KH_2PO_4, 2.5 g asparagine, 0.2 g $MgSO_4$, 10 g sucrose, (4) Czapek medium: 0.5 g $MgSO_4$, 0.5 g $NaNO_3$, 0.01 g $FeSO_4$, 0.1 g K_2HPO_4, 0.5 g KCl, 50 g sucrose, (5) Richard medium: 10 g KNO_3, 5 g KH_2PO_4, 2.5 g $MgSO_4$, 50 g sucrose, 0.02 g $FeCl_2$. Each medium (total volume 1,000 ml) contained 2% agar and pH was adjusted at 6.0 with 1 N NaOH or 1 N HCl before autoclaving. As shown in Table 12, all the 3 isolates well produced sclerotia, especially on Richard and Czapek

Table 11. Sclerotium formation of 3 isolates of *R. solani*

Exp.	Isolates		
	C-14	C-324	C-326
1	44.10±4.12[a)]	51.15±3.55	35.45±3.86
2	47.00±3.89	52.05±2.41	38.00±3.77
3	49.25±4.67	51.10±3.88	36.55±2.56
Average	46.78	52.76	36.67

a) The mean sclerotium weight (mg) on 5 Petri dishes using Hopkins medium and standard error.

Table 12. Sclerotium formation of *R. solani* on various kinds of media

Media	Isolates		
	C-14	C-324	C-326
PDA	48.85±4.88	88.50±6.52	64.45±4.11
Hopkins	44.10±4.12	51.15±3.55	35.45±3.86
Asparagine	51.55±3.16	68.15±4.05	39.00±3.20
Czapek	79.31±5.33	125.20±6.38	93.65±4.77
Richard	195.10±8.86	213.35±10.03	266.30±12.60

Table 13. The effects of various inorganic ions on sclerotium formation of *R. solani*

Inorganic ions	Isolates		
	C-14	C-324	C-326
$CaCl_2$	35.00±4.98[a)]	59.60±0.53	55.90±0.79
CaH_2PO_4	32.40±6.28	62.10±2.20	56.00±4.52
$FeCl_3$	28.30±10.65	60.08±1.08	51.95±4.63
$FeSO_4$	38.65±9.63	58.50±1.51	47.70±2.96
$ZnSO_4$	1.43±1.10	45.75±6.38	31.75±6.92
Control[b)]	44.15±4.12	51.15±3.55	35.45±3.86

[a)] The mean sclerotial weight (mg) and standard error.
[b)] The basal (Hopkins) medium.

Table 14. The effects of nitrogen sources on sclerotium formation of *R. solani*

Nitrogen sources	Isolates		
	C-14	C-324	C-326
Ammonium citrate	57.50±5.61	59.75±1.41	22.70±3.76
Ammonium tartrate	49.90±1.19	34.55±3.18	39.55±2.45
Ammonium sulfate	18.65±7.12	17.40±8.88	25.15±2.16
Ammonium nitrate	42.80±8.89	36.10±2.16	28.40±4.09
Ammonium chloride	18.76±8.60	20.95±1.90	27.25±2.02
Sodium nitrate	48.50±5.28	60.50±2.74	44.58±0.99
Potassium nitrate[a)]	44.15±4.12	51.15±3.55	35.45±3.86

[a)] Nitrogen source in the basal (Hopkins) medium.

Table 15. The effects of saccharides as carbon source on sclerotium formation of *R. solani*

Saccharides	Isolates		
	C-14	C-324	C-326
Monosaccharide			
Glucose[a)]	42.85±4.34[b)]	51.30±3.56	37.45±2.70
Mannose	25.50±3.07	41.40±2.88	41.15±3.11
Galactose	57.35±4.72	57.45±3.26	36.30±2.19
Arabinose	1.50±0.87	7.70±1.20	0.80±0.67
Xylose	27.50±1.95	37.40±2.45	25.65±2.01
Levulose	33.20±2.70	46.70±3.70	27.25±1.59
Disaccharide			
Maltose	44.45±2.48	43.40±3.11	29.65±2.02
Lactose	1.05±0.56	30.70±4.29	12.50±1.50
Sucrose	53.95±4.71	44.55±3.31	30.45±2.99
Polysaccharide			
Starch	43.85±3.02	44.95±2.50	29.55±1.87
Inulin	1.90±0.84	4.35±1.41	1.60±0.18
Dextrin	43.40±3.19	45.95±2.20	23.55±1.63
Higher alcohol			
Mannitol	4.75±0.38	4.60±0.44	4.30±0.28
Glycerol	0.00±0.00	0.00±0.00	0.00±0.00

a) Carbon source in the basal (Hopkins) medium.

b) The mean sclerotial weight (mg) and standard error.

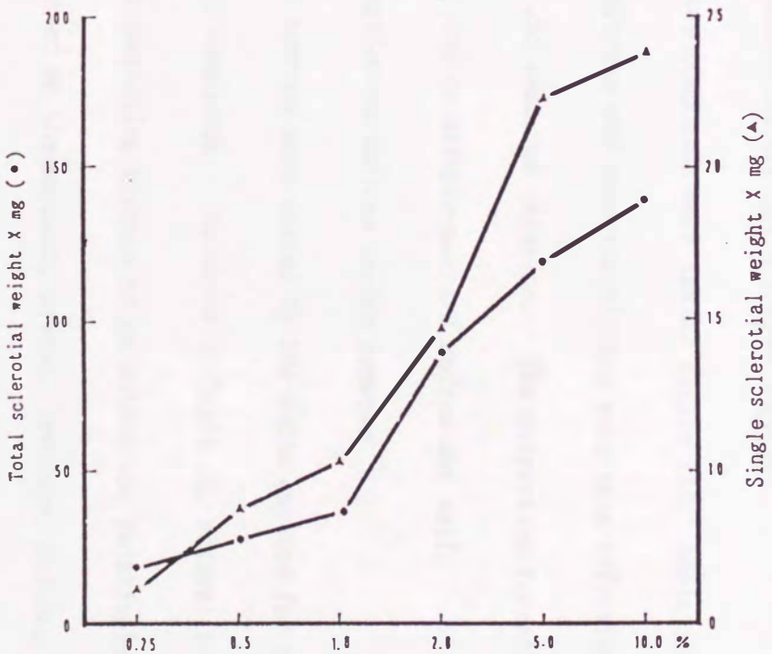


Fig. 12. The effects of glucose concentration on sclerotium formation of *R. solani*. * C-324 was used.

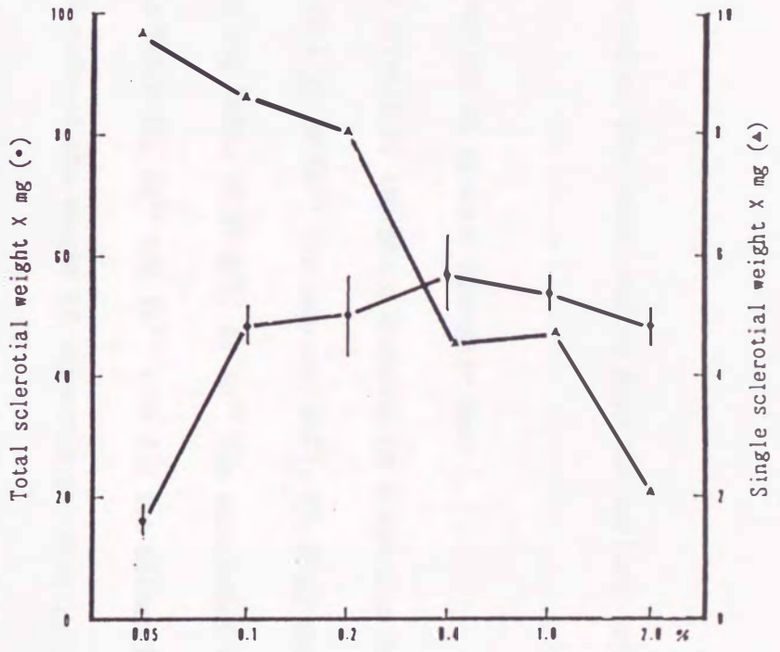


Fig. 13. The effects of KNO_3 concentration on sclerotium formation of *R. solani*. * C-326 was used.

medium.

Hopkins medium selected as the basal medium gave the smallest sclerotium formation.

Sclerotium formation on various inorganic ions

To examine the effects of inorganic elements on sclerotium formation, $CaCl_2$ (0.1 g) and $CaHPO_4$ (0.1 g) as Ca^{2+} ion sources, $FeCl_3$ (0.07 g) and $FeSO_4$ (0.07 g) as Fe^{2+} ion and $ZnSO_4$ (0.07 g/l) as Zn^{2+} ion were added to the basal medium. As shown in Table 13, Ca^{2+} and Fe^{2+} ions did not affect the sclerotium formation. Zn^{2+} ion reduced the weight of sclerotia per dish in the C-14 isolate.

Sclerotium formation on various nitrogen sources

Seven nitrogenous compounds were tested (Table 14). Additions of ammonium citrate, ammonium tartrate and ammonium nitrate were more effective than those of ammonium sulfate and ammonium chloride. The sclerotium formation on the potassium nitrate or sodium nitrate-amended medium was well.

Sclerotium formation on various carbon sources

Fourteen carbon sources were tested by the media amended for the carbon source with different compounds. As shown in Table 15, sclerotia were well formed on the medium containing glucose or galactose and relatively well production were observed on the mannose, xylose, levulose, maltose, lactose or

sucrose amended medium. However sclerotia were poorly formed on the arabinose medium. The linear hyphal growth of the 3 isolates on the lactose containing medium was almost the same but the poor sclerotium formation was observed in the C-14 isolate. Starch, inulin and dextrin were well utilized for the mycelial growth, while very little amount of sclerotia were produced on inulin medium. Higher alcohols such as glycerol and mannitol were used as carbon sources. However, hyphal growth and the sclerotium production were very poor, especially on glycerol amended medium.

Effects of carbon concentration on sclerotium formation

To examine the effects of concentration of the carbon source on sclerotium formation, glucose was added at various concentrations (0.25～10.0%). As shown in Fig. 12, the total as well as single sclerotial weight was increased with the glucose concentration. The single and total sclerotium weights of the C-326 isolate increased 4～5 times and 3～4 times, respectively, by a 10 times increase of the glucose concentration.

Effect of nitrogen concentration on sclerotium formation

The concentration of potassium nitrate was varied to examine the effects of the nitrogen concentration on sclerotium formation. Quite few amount of sclerotia were formed at a low concentration (0.05%). The weight of single sclerotium inversely decreased according with the increase of KNO_3 concentration

(Fig. 13).

Discussion

The effects of nutrients on fungal differentiation such as sporulation and sclerotium formation have been well documented[16, 22]. Townsend[118] reported that sclerotial initials were formed even on a relatively poor medium but further development requires nutrients. Moreover, unfavorable conditions may enhance the sclerotium formation.

The present experiment revealed several aspects of the sclerotium production by *R. solani* which were affected by changes of nutritional composition. Watanabe and Matsuda[130] studied the culture types of *R. solani* and reported that the size and productive ability of sclerotia were different between culture types. Well sclerotium production was obtained on Richard and Czapek medium, probably because the high concentration (50 g/l) of glucose in both media served as a good carbon source. Several minerals were tested but no significant difference was observed except for Zn^{2+} ion. In *Whetzelinea sclerotorum*, 1 mg/litre of Zn^{2+} caused maximal sclerotium formation, whereas higher amounts increased their total dry weight but in a smaller number of sclerotia[128]. In the present experiment, however, the addition of Zn^{2+} ion inhibited the

sclerotium formation, especially in the C-14 isolate. Several workers[7, 52, 85, 105, 129, 130] showed that the source and concentration of nutrient, particularly those supplying carbon and nitrogen, affected the sclerotium formation and that both NH_4^+ and NO_3^- can be utilized by sclerotium forming fungi for growth and sclerotium formation. No different effect of inorganic nitrogen sources on the sclerotium formation has been reported. However, in this experiment the formation on the ammonium sulfate or ammonium chloride containing medium was worse than that on the nitrate containing media. The effects of the carbon sources on sclerotium formation are well known. Heal and Issac[52] observed that the number of microsclerotium increased with the addition of carbohydrate, especially sucrose. Working on *Sclerotinia sclerotiorum*, Bedi[7] found that maltose was most suitable and that lactose and galactose were poor sources. Furthermore the author[7] reported that mannitol, although it was well utilized for mycelial growth, totally inhibited the sclerotium formation. On the other hand, Wang and LeTourneau[129] reported some different results, finding the highest sclerotium formation with raffinose, sucrose, maltose, lactose, mannose, glucose and fructose. In author's results, arabinose, lactose and inulin were the most suitable for the sclerotium formation. Mannitol and glycerol were unuseful for the mycelial growth and the sclerotium production. The concentration of carbon and nitrogen sources evidently affected the sclerotium

formation. The increase of glucose concentration augmented the total and single sclerotial weight. No obvious changes of total sclerotial weight in addition of potassium nitrate over 0.1% were observed and the decrease in size of each sclerotium occurred.

The dependence of sclerotium formation on the C/N ratio in the medium has been documented in several fungi[48, 137], the most suitable N/C ratio was found to be 1.4～7.0% in *R. solani*. The carbon source concentration may be a crucial factor for the total sclerotial weight and the nitrogen concentration for the sclerotial number.

Exp. 2

Materials and Methods

Fungus. An isolate (C-324) was used in this experiment.

Media. Preculture of the fungus was conducted on PDA. Hopkins medium was used as a basal medium. The pH of the media was adjusted to 6.5 before autoclaving at 110°C for 10 min.

Effects of carbon concentration of enzymes activities. A series (15 ml each) of the medium amended with the concentration of glucose (0～10%), was each poured into a Petri dish which was covered with cellulose membrane (8.5 cm in diam.). Mycelial discs precultured for 2～3 days were put on the centers of the media. When the mycelial tops reached the margin 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 3,000 *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 each mixed with the solution, and incubated at 37°C for 50 min for the measurement. Details were as described in Chapter V. The substrate solution 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.

$$\text{Protein concentration (mg/ml)} = 1.55D_{280} - 0.76D_{260} \quad ^{109)}$$

Incorporation of carbon source from medium. $^{14}C_6H_{12}O_6$ (1.85 MBq) was diluted with distilled water to 1/100. Aliquots (1 ml) of the solution were each filtered through a 0.2 μm cellulose nitrate filter membrane into 14 ml of the basal medium amended with the carbon concentration (0.25～10%). Cellulose membranes (8.5 cm in diam.) were sheeted on the media. Mycelial discs precultured on PDA were each placed on the center of the sheet and incubated at 25°C in an incubator for 14 days. The membranes were peeled off and the media were daily punched out at random with a cork borer to obtain discs of 6.5 mm in diameter. The discs were each melted in a water bath in 9 ml of distilled water per g of the sample. 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 300C scintillation counter.

Effects of carbon source on hyphal branching. The isolate was cultured on various media mixed with various concentrations of carbon source. At the stage when hyphae grew to 2 cm in diameter (Phase I), and at 6 (Phase II) and 12 hr (Phase III) after the hyphal tips reached to the edge of the plate, the hyphal internode (from one hyphal branch to another branch) was measured using a light microscope.

Effects of magnesium and phosphate on sclerotium formation. One or both of $MgSO_4$ and KH_2PO_4 were removed from the basal medium and additionally given at various concentrations (KH_2PO_4:1～1,000 ppm, $MgSO_4$:100～5,000 ppm) to the medium. For the test of the effects of different kind of phosphate, KH_2PO_4 of the basal medium was replaced with some of other phosphates with different cations. The edges of mycelial mats precultured on PDA were cut with a cork borer (5 mm in diam.), centered on the media and cultured at 25°C. The initials of sclerotia were counted at 4 days after inoculation and sclerotia formed on the media were collected, desiccated and weighed.

Effects of phosphate on sclerotial morphogenesis. The seamless cellulose dialysis bag was opened and the sheet was cut to round shape, 8.5 cm in diameter. The membranes were sheeted on the media with or without KH_2PO_4 and then mycelial discs were centered on the membranes and precultured at 25°C. The membranes which were covered with mycelia were transferred onto other media at

48 hr (the mycelial stage) or 96 hr (the initial stage) after inoculation. Aft er theculture for 14 days at 25°C, sclerotia formed on the membrane were collect ed,

counted, desiccated and weighed.

Incorporation and transportation of phosphorus. $KH_2{}^{32}PO_4$ (37 MBq) was diluted with distilled water to 1/200. One ml aliquots of the solution were added through a 0.2 μm cellulose nitrate filter, which were immersed in each 15 ml of the basal medium. The solutions were stirred well with a mixer, poured into Petri dishes (9 cm in diam.). After cooling, the discs were sheeted with cellulose membranes (8 cm in diam,). The mycelial disc precultured on PDA were placed on the centers and kept at 25°C. When the culturse reached the initial, white sclerotium or mature sclerotium stage, the membrane were peeled off, and media were punched out randomly with a cork borer to obtain discs of 6.5 mm in diameter. The discs were dried and counted the radioactivity at 1,200 V for 1 min by an Aloka GM counter.

Autoradiography. The isolate was cultured on the basal medium containing ^{32}P-labelled KH_2PO_4. After 14 days, an X ray film (Fuji FR) was attached tightly onto the culture for 6 hr.

Enzymatic activity. The edge of mycelial mat precultured on PDA was punched out. The discs were each placed on the center of the basal, KH_2PO_4-free

medium, on which cellulose membranes was sheeted, and cultured at 25°C. Hyphae were peeled off shortly before the initial formation and homogenized with 10 volumes of 0.05 M phosphate buffer (pH 6.5). The homogenate was centrifuged at 3,000 *g* for 20 min. The supernatants were concentrated to one 1/25 of its original volume by a Minicon B-15 concentrator (Amicon Ltd.), and measured for malate dehydrogenase and isocitrate dehydrogenase activities by the method as described above.

Results

Effects of carbon concentration in media on the fungal enzymatic activities

The activities of malate dehydrogenase and isocitrate dehydrogenase varied with the glucose carbon concentration. The activities were high when cultured with 0.5～5.0% of glucose. The optimal carbon concentration in the medium for the 2 enzymes was about 1%. Although highest mycelial growth was observed when the carbon concentration was adjusted around 10% in the medium, the enzyme activities per g hyphae or per mg protein were low under the same conditions (Table 16).

Changes of carbon source in medium during sclerotial morphogenesis

To elucidate the carbon consumption during the sclerotial morphogenesis of

Table 16. The effects of carbon concentration on the activities of dehydrogenases of *R. solani*

Concentration of glucose (%)	Hyphal weight (mg)	Protein per hyphae (μg/mg)	Malate dehydrogenase		Isocitrate dehydrogenase	
			Activity per g hyphae	Activity per mg protein	Activity per g hyphae	Activity per mg protein
0.00	1.1±0.5	1.2±0.0	5.1±0.4	4.3±0.3	5.0±0.4	4.3±0.4
0.25	1.2±0.4	1.6±0.0	8.5±0.3	5.3±0.3	7.2±0.4	4.5±0.3
0.50	1.1±0.2	1.7±0.0	13.4±0.5	7.9±0.3	11.9±0.4	7.0±0.2
1.00[a)]	1.0±0.4	1.6±0.1	44.0±0.7	28.1±1.7	41.1±0.4	26.2±2.0
2.00	1.6±0.3	1.0±0.0	18.5±0.4	19.6±0.7	17.1±0.5	18.1±0.5
5.00	2.9±0.7	1.4±0.0	14.6±0.2	10.6±0.4	13.8±0.3	10.0±0.3
10.00	8.3±1.2	1.2±0.0	6.8±1.3	5.7±1.1	7.6±1.0	6.4±0.9
Control	—	—	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

[a)] The standard carbon concentration in the basal (Hopkins) medium.

the fungus, periodical changes of the compound were examined using ^{14}C-labelled glucose. Fig. 14 shows that almost same amount of carbon sources to that at the inoculation were present at the hyphal stage (0～3 days). Then it was consumed within a few days during the sclerotial morphogensis stages from the initiation to the maturation. After the maturation, 4～5% of the compound remained. As shown in Table 17, the residual quantity of glucose was increased as the starting glucose was increased. When the fungus was cultured on a medium with 10% glucose, 80% of that was still left in the medium even 10 days after the inoculation.

Effects of carbon concentration in the medium on the hyphal branching

The frequency of branching, as measured by the lengths of internodes, was increased according to the carbon concentration and to the time of growth (Table 18). The branching at 10% glucose was 2～3 times more frequent compared to that at 0～0.5% glucose.

Effects of phosphorus and magnesium concentrations on sclerotium formation

To examine the effects of phosphorus and magnesium concentration on the sclerotium formation, one or both of KH_2PO_4 and $MgSO_4$ were omitted from the basal medium. Sclerotial initials and matured sclerotia were produced well on the $MgSO_4$-free media (Tables 19 and 20). Little sclerotium production was observed on the media in which both compounds or KH_2PO_4 were absent (Table 20).

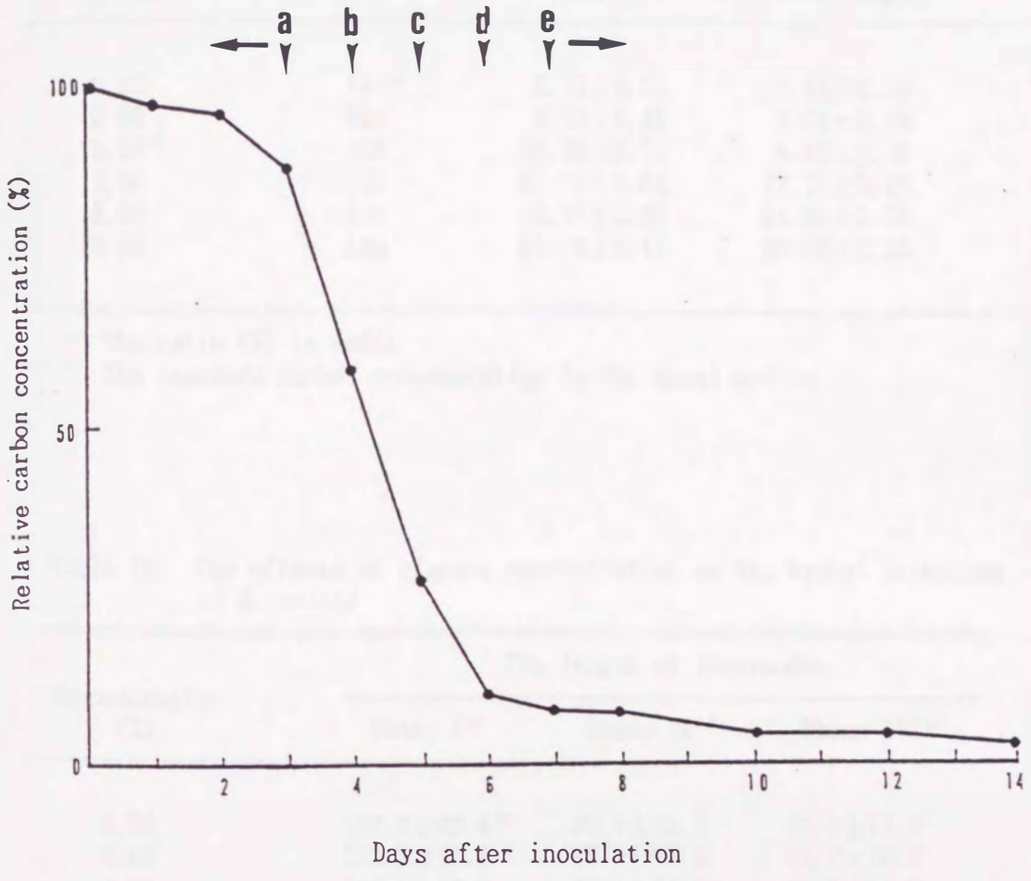


Fig. 14. Changes of glucose as a carbon source in medium during culture of *R. solani*. * The C-324 isolate was cultured on Hopkins medium at 25°C in the presence of $^{14}C_6H_{12}O_6$.
a : Hyphal stage, b : Initial stage, c : White sclerotial stage, d : Pigmenting stage, e : Matured stage.

Table 17. Changes of glucose concentration during the culture of *R. solani* in the media with different starting concentrations

Carbon concentration (%)	Day after inoculation		
	0	5	10 days
0.25	100[a]	6.73±0.51	5.42±0.59
0.50	100	5.93±0.41	4.64±0.34
1.00[b]	100	26.78±2.24	4.13±0.28
2.00	100	53.51±3.62	27.79±2.22
5.00	100	80.27±2.68	64.61±2.88
10.00	100	91.94±6.11	80.10±2.25

[a] The ratio (%) in media.
[b] The standard carbon concentration in the basal medium.

Table 18. The effects of glucose concentration on the hyphal branching of *R. solani*

Concentration (%)	The length of internodes		
	Phase I[a]	Phase II[b]	Phase III[c]
0.00	138.8±42.4[d]	99.7±29.5	82.0±17.9
0.25	132.3±49.6	87.7±24.5	64.6±16.2
0.50	112.1±18.3	97.1±14.7	65.6±15.5
1.00[e]	99.4±23.3	67.8±22.4	56.4±15.2
2.00	81.0±24.3	61.8±19.0	52.6±18.5
5.00	65.0±27.3	56.7±14.4	46.9±10.8
10.00	45.1±8.9	—[f]	—

[a] The phase when mycelia grow to 2 cm in diameter at 25°C.
[b] The phase at 6 hr after the mycelial tip reached the edge of the Petri dish.
[c] The phase at 12 hr after the mycelial tip reached the dish.
[d] The length (μm) of internode and standard error.
[e] The standard concentration in the basal (Hopkins) medium.
[f] Scarcely unmeasurable.

Table 19. The effects of $MgSO_4$ concentration on sclerotium formation of *R. solani*

Concentration of $MgSO_4$ (%)	Number of initials	Sclerotia formed	
		Number	Weight per dish (mg)
0	59.1±13.1	12.2±4.1	38.0±3.3
0.01	50.6±4.1	28.6±3.6	42.3±6.7
0.05	62.4±9.4	30.5±4.7	57.3±4.7
0.1	69.6±11.3	48.8±9.1	54.6±4.3
0.5	106.7±9.7	66.4±10.5	61.7±2.1

Table 20. Sclerotium formation of *R. solani* on various media

Media[a)]	Number of initials	Sclerotia formed	
		Number	Weight per dish (mg)
A	62.4±9.9	30.5±4.9	57.3±4.9
B	3.5±1.8	0.8±1.0	1.3±2.1
C	59.1±13.1	12.2±4.1	38.0±3.3
D	1.3±1.9	0.3±0.5	1.5±2.5

[a)] A: The basal medium, B: Without KH_2PO_4 and $MgSO_4$, C: Without $MgSO_4$, D: Without KH_2PO_4.

Table 21. The effects of KH_2PO_4 concentration on sclerotium formation of *R. solani*

Concentration of KH_2PO_4 (ppm)	With $MgSO_4$		Without $MgSO_4$	
	Number[a)]	Weight per dish (mg)	Number	Weight per dish (mg)
0	0.3±0.5	1.5±2.5	0.8±1.0	1.3±2.1
1	0.2±0.0	2.6±1.6	0.6±1.2	1.0±1.4
10	4.9±2.8	15.8±3.6	4.8±0.8	16.2±1.8
100	30.5±4.6	57.3±4.7	12.2±4.0	38.0±3.1
1,000	13.1±2.5	28.4±4.3	10.3±3.3	34.8±3.1

a) Sclerotial number formed in a dish.

Table 22. The effects of various phosphates on sclerotium formation of *R. solani*

Phosphates	Number of initials	Sclerotia formed	
		Number	Weight per dish (mg)
KH_2PO_4[a)]	62.4±9.4	30.5±4.7	57.3±4.0
K_2HPO_4	23.6±9.1	14.4±6.1	31.0±4.0
$(NH_4)_2HPO_4$	42.7±4.2	18.1±3.7	44.7±2.0
$CaHPO_4$	42.7±4.5	20.1±1.1	37.6±2.0

a) Phosphate in basal medium.

Table 23. The effects of KH_2PO_4 concentration on hyphal growth of *R. solani*

Concentration of KH_2PO_4 (ppm)	With $MgSO_4$		Without $MgSO_4$	
	Diameter[a]	Weight[b]	Diameter	Weight
0	8.9±0.4	7.8±1.4	7.8±0.6	10.2±2.6
10	8.7±0.3	31.8±5.1	7.5±0.8	28.5±5.4
100	8.7±0.2	63.9±4.8	8.2±0.3	34.5±1.6
1,000	8.6±0.3	55.6±2.4	8.2±0.5	32.6±3.0

[a] The diameter of mycelial colony (cm) was measured at 48 hr after inoculation.
[b] The hyphae (mg) were weighed at two weeks after inoculation.

Table 24. The effects of phosphorus on the frequency oh hyphal branching of *R. solani*

	With KH_2PO_4	Without KH_2PO_4
Primary internodes	194.2±74.5[a]	303.3±83.3
Secondary internodes	90.6±63.3	170.0±83.7

[a] Length (μm).
* Hopkins medium was used as a basal medium.

The optimal concentration of KH_2PO_4 for the sclerotium formation was in the region of 100 ppm at the presence and absence of magnesium (Table 21). The quality and quantity of sclerotia increased with $MgSO_4$ concentration (Table 19).

Effects of various kind of phosphates on sclerotium formation

When KH_2PO_4 in the basal medium was replaced with other phosphates such as K_2HPO_4, $(NH_4)_2PO_4$ and $CaHPO_4$, sclerotia were well produced with a certain variation on the number and total weight (Table 22). KH_2PO_4 had about twice as much the sclerotial forming effect than K_2HPO_4.

Effects of KH_2PO_4 concentration on hyphal growth and hyphal branching

Although there was no effect of KH_2PO_4 on the hyphal growth, the hyphal weight was increased with the KH_2PO_4 concentration (Table 23). As shown in Table 24, when the fungus was cultured on the phosphorus-free medium, the primary and secondary branching intenodes were longer than those of on phosphate containing medium.

Effects of phosphorus added at limited stages of sclerotium formation

No sclerotium was differentiated from hyphae on the medium without phosphate. When KH_2PO_4 phosphate was supplied at the hyphal phase (2 days after inoculation) and the hyphal mat was transferred afterward to the phosphate-free media, few sclerotia were differentiated (Table 25). Even when the phosphate was absent during the hyphal stage, sclerotia were well developed

Table 25. The effects of KH_2PO_4 on each stage of sclerotium formation of *R. solani*

KH_2PO_4	Hyphal stage[a]		Initial stage[b]	
	Number	Weight per dish (mg)	Number	Weight per dish (mg)
Present ↓ Present	14.0±1.5	38.9±5.9	11.2±1.5	42.5±5.9
Present ↓ Absent	2.3±1.0	6.4±2.1	4.4±1.2	16.0±5.5
Absent ↓ Present	12.6±1.5	37.0±6.6	20.9±2.3	42.2±5.9
Absent ↓ Absent	0.2±0.3	1.5±2.2	0.0±0.0	0.0±0.0

[a] Hyphal stage : 48 hr after inoculation.
[b] Initial stage : 96 hr after inoculation.

Table 26. The effects of phosphorus on the activities of enzymes of *R. solani*

Enzymes	KH_2PO_4		Control[a]
	Present	Absent	
Malate dehydrogenase	2.9[b]	2.0	0.0
Isocitrate dehydrogenase	1.8	0.8	0.0

[a] Includes no mycelial extract.
[b] Relative activity.

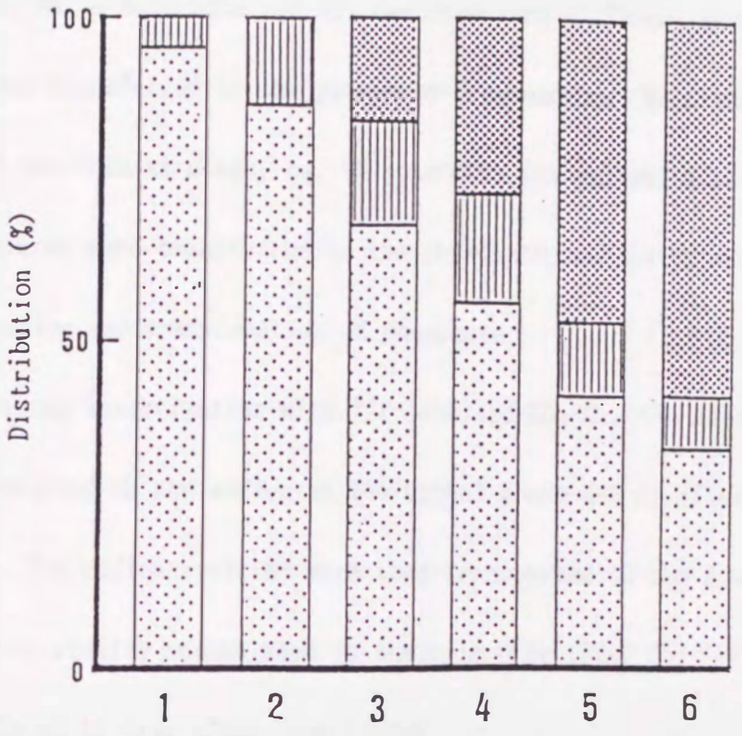


Fig. 15. Distribution of ^{32}P in the medium, hyphae and sclerotia at different stages of sclerotial morphogenesis of *R. solani*.

1 : Hyphal stage, 2 : Initial stage, 3 : White sclerotial stage, 4 : Pigmenting stage, 5 : Matured stage (10th day), 6 : Matured stage (14th day).

: Medium, : Hyphae , : Sclerotium

after the culture was transferred to the phosphate-containing medium. This phenomenon was also observed when the transfer was conducted at the sclerotial initial phase (4 days after inoculation). When phosphate was present at the initial phase, *ca.* 4 sclerotia (16 mg) per dish were differentiated even after the culture was transferred to the phosphate-free medium. When the compound was absent at the initial stage, *ca.* 20 sclerotia (42 mg) per dish were formed after the cultures were transferred to the phosphate-containing medium .

Incorporation and translocation of phosphate

By a tracing investigation with ^{32}P-labelled KH_2PO_4, the almost all of the isotope was detected in the medium at the hyphal phase (48 hr after inoculation). The radioactivities were then transported to the hyphae and about 60% of them were finally accumulated in the sclerotia (Fig. 25) Plate 5 shows a autoradiogram at 14 days after inoculation.

Enzymatic activity

The activities of malate dehydrogenase and isocitrate dehydrogenase in the hyphae developed on the media with phosphorus (KH_2PO_4) were higher than those on the medium without the salt (Table 26).

Discussion

In Exp. 1, the author showed that the quanity of carbon source is one of the important factors controlling the sclerotium formation of *R. solani*. A previous report[48] revealed that the N/C ratio and cyclic AMP play important roles for the sclerotium formation of the *R. solani*. Fungi are heterotrophic, in general and carbon sources are from media and/or from internal organic matters. As shown in Table 16, the activities of the 2 dehydrogenases were highest at 1% glucose. This result seemed not to agree with the quantities of sclerotia formed. However, considering that the hyphal weight on a membrane was increased as the starting carbon concentration was increased, the total activities per colony might also depend on the starting concentration. The consumption of carbon source was remarkable during the period of the sclerotial differentiation from the initial to the pigmenting sclerotium. This fact indicates that a small amount of carbon source allows hyphal growth but a large amount of it is essential to the sclerotium differentiation. The residual quantity of carbon source was increased with the starting concentration, though the total amount of consumption was also increased. Furthermore, the frequency of hyphal branching which is the first step of the sclerotium formation was significantly increased with the concentration. These facts could support the idea that the carbon

quantity is one of the important factors deciding the sclerotium quantity.

On the other hand, none or quite few sclerotia were formed on the medium from which phosphorus compound was removed. However, when only magnesium was removed from the basal medium, half in number and two-thirds in weight of sclerotia were formed. Some differences were observed when KH_2PO_4 in the basal medium was replaced with other phosphates such as K_2HPO_4, $(NH_4)_2PO_4$ and $CaHPO_4$. The optimal concentration of KH_2PO_4 was about 100 ppm regardless the presence of magnesium. For a large number of plants, it has been indicated that the optimal concentration of phosphate in soil is 1～10 μM, whereas its cytoplasmic concentration of a plant cell is 1～20 μM[11,83]. Compared with higher plants, the fungus required large amounts of phosphate. The number and total weight of the initial and matured sclerotia were increased with the magnesium concentration. Although there was no significant difference on the hyphal linear growth of the fungus by phosphate, the total weight of the mycelial mat after 2 weeks was obviously increased at 100 ppm phophorus in the presence of magnesium. It is suggested that magnesium ion is not always indispensable to the sclerotial morphogenesis but has an inductive or promotive effect. It is widely noted that sufficient growth of hyphae is essential to the subsequent sclerotium formation[22,48]. Wheeler and Sharan[132] showed that the number and weight of sclerotia of *Sclerotium rolfsii* were decreased in proportion to the

KH_2PO_4 concentration but concluded that phosphate has a lesser effect on the sclerotium formation than potassium. However, phosphorus is one of the essential compounds composing nucleic acids. If there was no phosphorus at all in a medium, no development of hyphae should be observed. A very small amount of phosphorus, which allows the hyphal development but not the initiation of the sclerotium formation, might be contaminated into the media as impurities of the ingredients and water used or from air during culture. The enzymatic activities concerning the TCA cycle in the hyphae grown on a medium with phosphorus were higher than in those grown on a phosphorus-free medium. Moreover, when KH_2PO_4 in Hopkins medium was replaced with $(NH_4)_2HPO_4$ or $CaHPO_4$, sclerotia were also well produced. Even if phosphorus was given during the hyphal and initial stages, few sclerotia were formed when the compound was taken away afterwards. Even though the chemicals were omitted during the hyphal and initial stages, well production was recognized when phosphorus was added later. These results may indicate that a large quantity of phosphorus may not always be necessary to the hyphal development, but indispensable to the sclerotial initiation, and the subsequent enlargement and maturation which required high energy. Thus, no sclerotia may be originated without phosphorus even when ample carbon or nitrogen as energy sources are present. Furthermore, it is indicated that a large amount of isotope-labelled phosphorus was accumulated into the sclerotial

tissues from media through hyphae.

An accumulation of ^{32}P in the sclerotia, hyphal tips and at branches of hyphae in *R. solani* has been reported[69]. A large proportion of phosphorus was absorbed during the trophophase migrated to the fungal spore in *Aspergillus nigar*[6,71,72]. It was proposed that fungal sclerotium is not only the hyphal mass but also the organ which is similar to perithecium and fruit body in morphology and physiology[48,49,51]. The fact observed in this experiment could support this proposal and could indicate that phosphorus is, at least, one of the essential constituents of the sclerotial tissue and that the manner of uptake of phosphorus closely resembles that seen in a seed of higher plants or a fungal spore.

On the other hand, sclerotia were normally initiated and matured without magnesium, though the weight of hyphae and mature sclerotia increased with the magnesium concentration. Magnesium ion, which is essential to higher plants for the chlorophyll and for the activators of various important enzymes, may not always be important to the fungal growth.

VIII. Effects of amino acids on sclerotium formation of *Rhizoctonia solani*

Part 1. Inhibition of sclerotium formation by various amino acids

R. solani involves many strains which have morphologically, physiologically, pathologically and ecologically different characteristics. However, most of them generally form the asexual structure *in vitro* and *in vivo*[92, 115, 123, 130].

The effects of physical and chemical factors on sclerotium formation of *Sclerotium rolfsii*, *S. delphinii*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, and *Verticillium albo-atrum* have been well-documented[1, 21, 23, 48, 60, 73, 74, 79, 84].

However, there are few reports in regard to the effect of nutrients on the sclerotium formation of *R. solani*[22]

In the previous chapter, the author reported that the nutrient factors, organic and inorganic substances have qualitative and quantitative effects on thesclerotium formation[86]. In this chapter the effects of amino acids, especially as inhibitory factors are studied.

Materials and Methods

Fungal isolates used. C-14 from *Cyperus rotundus*, C-324 from sugar cane

and C-326 from rice (AG-1, IA), were used in this study.

Culturing. Isolates were precultured on PDA plates at 25°C for 2～3 days. Small discs of 5 mm in diameter were cut from the edge of mycelial mat with a cork borer and used as an inoculum. Hopkins medium was used as a basal medium.

To examine the effects of amino acids on the sclerotium formation, potassium nitrate was replaced with various amino acids or their isomers so as to contain equivalent nitrogen with the control medium. After culturing for 14 days at 25°C, sclerotia formed were taken, dried at 60°C and weighed.

Amino acids which inhibited the sclerotium formation were additionally mixed at various concentrations (10^{-1}～10^{-7} M) to the basal medium which contains potassium nitrate.

The effects of cysteine and serine or homocysteine and homoserine, which have similar structures each other, were compared. Each of the amino acids were additionally given to the basal medium at various concentrations and C-324 was cultured at 25°C for 14 days. Sclerotia formed were weighed as stated above.

The effects of the inhibitory amino acids on mycelial growth was tested. C-324 isolate was cultured at 25°C for 14 days on amino acid-amended Hopkins media and then agar was melted. The mycelial mats were taken out with forceps, washed with hot water repeatedly, dried and weighed. When the sclerotia were

formed, these were separated and only the mycelial weight was measured. The linear mycelial growth was also measured at 24 and 48 hr after inoculation. Each value is average of 12 dishes and the experiments were repeated 3 times.

Results and Discussion

As shown in Table 27, sclerotia were well formed when the nitrogen source of Hopkins medium was replaced with various amino acids such as asparagine, aspartic acid, alanine, glutamic acid, serine, arginine and proline. The number of sclerotia formed on the valine, phenylalanine or threonine medium widely varied with the isolates. Quite few or no sclerotia were formed on the medium which contained methionine, homocycteine, taurine, cysteine, cystine, isoleucine, leucine, histidine, tryptophan, tyrosine, homocysteine or glycine. Plate 6 illustrates some of the features observable in the culture plates. To examine whether or not these amino acids directly inhibited the sclerotial morphogenesis, each of the amino acids was additionally given to the basal medium. As shown in Fig. 16, cysteine, cystine and histidine drastically inhibited the sclerotium formation at the concentrations more than 10^{-4} M. Methionine and homocysteine also inhibited the formation at their concentrations above 10^{-3} M. Distinct inhibition was observed at 10^{-1} M in taurine. Although

Table 27. The effect of amino acids on sclerotium formation of *R. solani*

Amino acids	Isolates		
	C-14	C-324	C-326
L-Asn	71.50±2.28[a)]	70.85±3.06	51.60±0.90
L-Asp	75.30±3.80	73.75±0.65	53.20±4.50
DL-Ala	65.65±1.82	60.05±3.63	45.20±2.20
L-Glu	73.00±5.49	75.30±2.40	25.35±4.79
L-Gly	4.25±3.50	9.40±1.19	1.25±1.23
DL-Ser	18.30±1.25	37.50±3.80	29.10±9.24
L-Thr	39.00±1.10	0.00±0.00	2.10±1.21
L-$(Cys)_2$	3.25±1.38	0.00±0.00	0.00±0.00
L-Cys	0.00±0.00	0.00±0.00	1.80±1.51
L-Met	0.00±0.00	0.00±0.00	0.00±0.00
L-HomoCys	0.00±0.00	0.00±0.00	0.00±0.00
L-Tau	0.00±0.00	0.00±0.00	0.00±0.00
L-Ile	0.00±0.00	0.00±0.00	0.00±0.00
L-Leu	0.00±0.00	0.00±0.00	0.00±0.00
L-Val	22.05±5.59	3.30±0.96	0.00±0.00
L-Arg	49.35±3.09	45.65±4.75	33.07±8.54
L-Pro	62.45±3.28	64.45±2.73	35.32±1.18
L-His	0.00±0.00	0.00±0.00	0.00±0.00
L-Phe	12.50±1.45	0.80±0.77	0.00±0.00
L-Trp	4.00±2.08	0.00±0.00	3.15±0.48
L-Tyr	0.00±0.00	0.00±0.00	1.05±1.44
Control (the basal medium)	41.60±4.19	51.15±3.55	34.45±3.86

[a)] Average of sclerotial weight (mg) formed in a Petri dish and standard error.

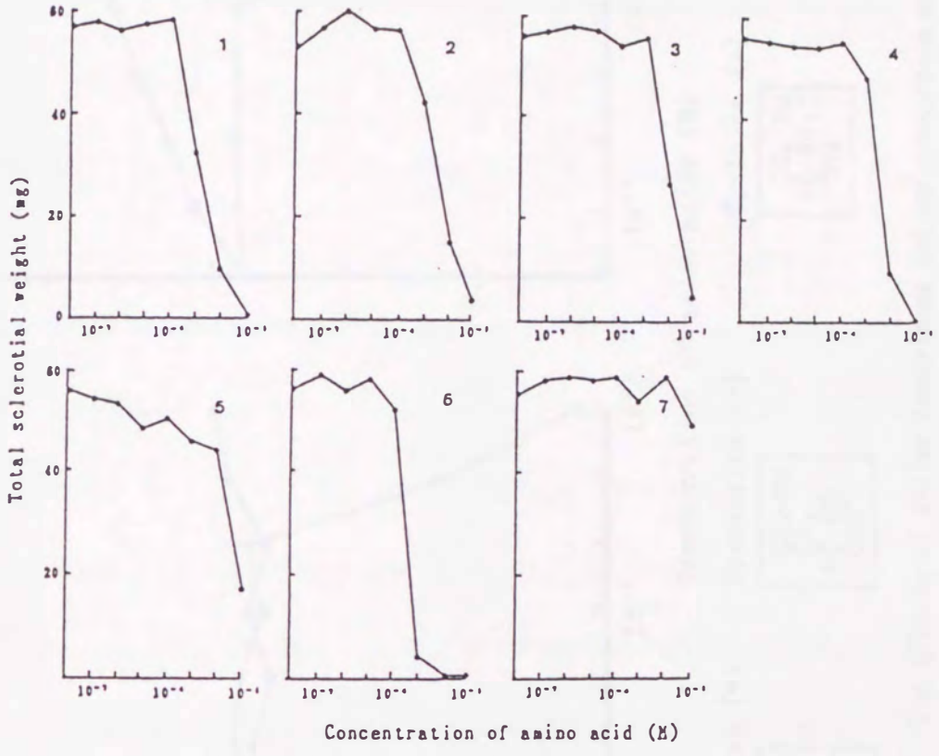


Fig. 16. The effects of concentration of inhibitory amino acids on sclerotium formation of *R. solani*.

1 : Cysteine, 2 : Cystine, 3 : Homocysteine, 4 : Methionine, 5 : Taurine, 6 : Histidine, 7 : Leucine.

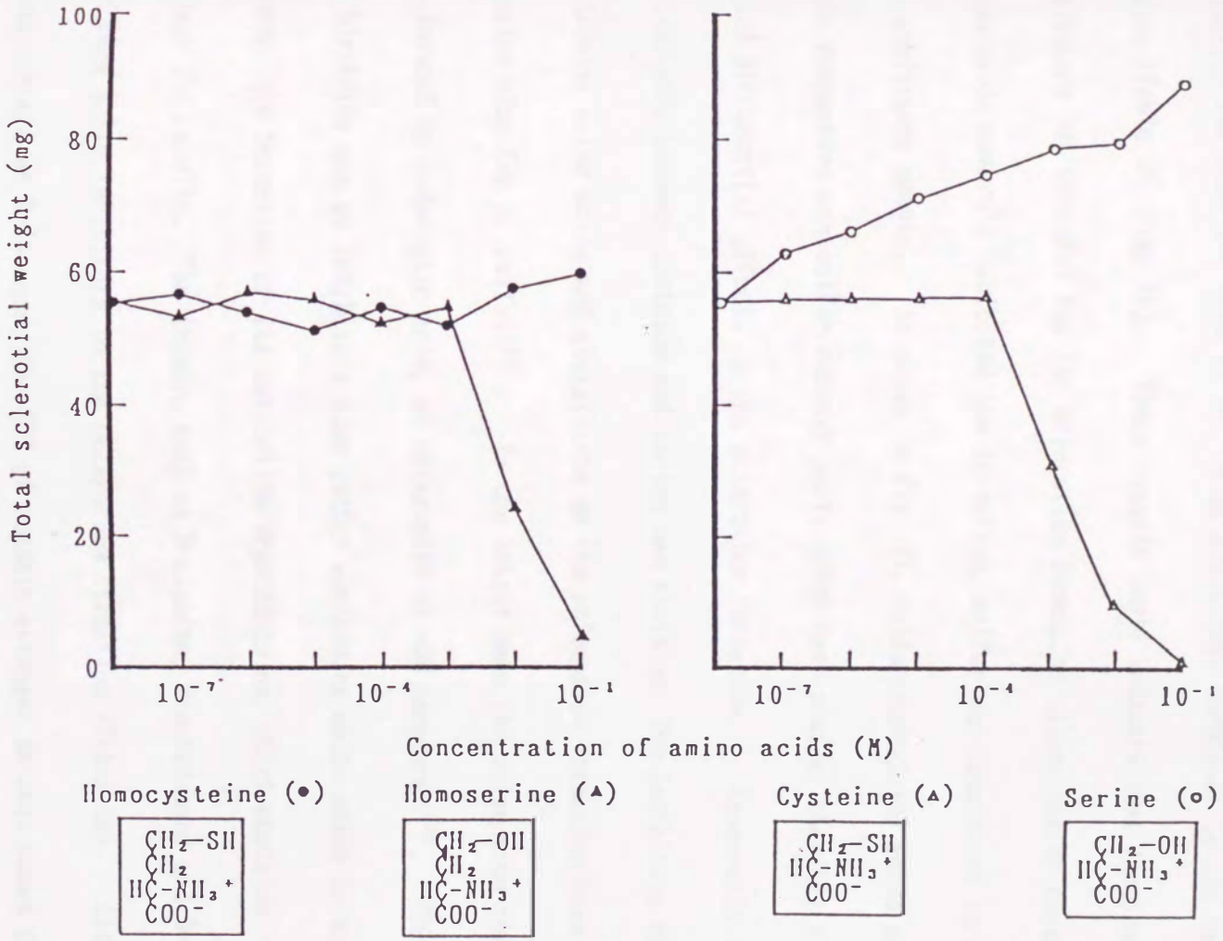


Fig. 17. The effects of sulfur containing and not containing amino acids on sclerotium formation of *R. solani* (C-324) as additionally amended Hopkins medium.

no sclerotia were formed when KNO_3 was replaced with leucine, sclerotium formation was observed even at 10^{-1} M as additional amendment to the basal medium (Table 27, Fig. 16). These results could indicate that leucine is not inhibitory but unuseful for the sclerotium formation. Since sulfur containing amino acids severely inhibited the formation, sulfur was considered to be one of the inhibitory agents. As shown in Fig. 17, sulfur-containing amino acids and their respective non-sulfur counter parts which have similar chemical structures showed differential effects on the sclerotium formation. Especially the distinctness between cysteine and serine was striking. The inhibition by sulfur-containing amino acids and glutathione on the sclerotium formation have been reported also for *S. rolfsii*[23]. In the latter case the sclerotium formation was induced by iodoacetic acid, an antagonist of -SH compounds[23]. The reason why histidine was so inhibitory like sulfur containing amino acids is uncertain. However, the formation of its catabolite ergothioneine, which contains -SH, may explain the results. The isomers such as D-alanine, D-methionine and D-aspartic acid were quite inhibitory to the sclerotium formation (Table 28). Although plants contain no D-isomers, 20～60% of organic nitrogen in soil comes from microorganisms and 10～20% of alanine and glutamic acid are D-isomers which could be originated from cell walls of bacteria and actinomycetes. Therefore, the inhibitory effects of these D-isomers on the sclerotium formationis of

Table 28. The effects of D and L form amino acids as nitrogen source on sclerotium formation of *R. solani*

Amino acids	Isolates	
	C-14	C-324
D-Ala	0.00±0.00[a)]	0.00±0.00
L-Ala	72.04±5.14	68.82±7.11
D-Asn	0.00±0.00	0.00±0.00
L-Asn	52.61±4.05	64.17±4.99
D-Met	0.00±0.00	0.00±0.00
L-Met	0.00±0.00	0.00±0.00

[a)] Average of sclerotial weight (mg) formed in 6 Petri dishes and standard error.

Table 29. The effects of amino acids on linear growth of *R. solani*[a)]

Media	Culture periods	
	24 hr	48 hr
Basal + Met		
10^{-5}	3.76±0.24[b)]	5.90±0.68
10^{-3}	2.92±0.59	4.73±0.81
10^{-1}	1.86±0.25	3.13±0.25
Basal + His		
10^{-5}	4.58±0.22	7.37±0.48
10^{-3}	2.45±0.35	4.13±1.03
10^{-2}	2.30±0.19	3.40±0.20
Basal + Ser		
10^{-5}	4.05±0.37	6.25±0.29
10^{-3}	4.74±0.72	7.63±0.63
10^{-1}	3.35±0.51	8.00±0.58
Control (the basal medium)	4.45±0.70	7.70±0.47

a) C-324 isolate from sugar cane was used.
b) Diameter (cm) of mycelial mat on agar plate.

Table 30. The effect of amino acids on mycelial weight of *R. solani*[a)]

Media	Culture periods		
	1	5	14 days
Basal + Met			
10^{-5}	2.35±0.42[b)]	10.65±5.07	12.81±2.10
10^{-3}	2.45±0.67	33.63±6.92	11.04±1.16
10^{-1}	0.95±0.38	37.37±5.72	31.75±3.44
Basal + His			
10^{-5}	2.97±0.71	2.83±0.43	8.37±1.87
10^{-3}	0.72±0.27	5.01±0.62	36.76±4.19
10^{-1}	0.95±0.37	10.83±1.20	47.05±3.81
Basal + Ser			
10^{-5}	2.86±1.14	8.60±1.16	8.49±0.68
10^{-3}	5.38±0.81	25.90±4.90	7.42±1.45
10^{-1}	4.10±0.34	39.00±6.33	9.90±1.02
Control (the basal medium)	2.34±0.33	9.04±2.48	6.00±0.45

a) C-324 isolate was used.
b) Weight (mg) of mycelial mat on agar plate.

interest from the ecological point of view .

The linear growth and the mycelial weight on the medium containing the inhibitory amino acids were reduced at first, and finally recovered (Table 29, 30). After 14 days, the mycelia had almost the same total weight to the control. It was reported that inhibitory substances to the sclerotial poduction reduced and stimulative substances induced the hyphal branching in *S. rolfsii*[55]. Taken together, these results imply that the inhibitory amino acids only limit the branching in the leading hypha, which in turn results in the inhibition of sclerotial production.

Part 2. Developmental process of sclerotium formation and its inhibition by several amino acides

In Part 1, the sclerotium formation of *R. solani* did not take place on the media with histidine and leucine or sulfur-containing amino acids. Similar observation has been reported in *Sclerotium rolfsii*[23], where the process of sclerotium formation was divided into the 3 phases, initiation, development and maturation phases. Inhibitory amino acids were shown to affect on all the 3 phases[23, 54]. In the present study, the morphological processes of the sclerotium formation of *R. solani* was divided into 5 stages, namely 1) the hyphal branching, 2) the hyphal aggregation and network formation, 3) the initial formation, 4) the formation of whitish immatured sclerotia, and 5) the maturation of pigmented sclerotia (Fig. 18, Plate 6).

The following experiments were done to determine which of the stages is affected by the inhibitory amino acids.

Materials and Methods

Isolate. An isolate (C-324) of the fungus from sugar cane was used in this experiment.

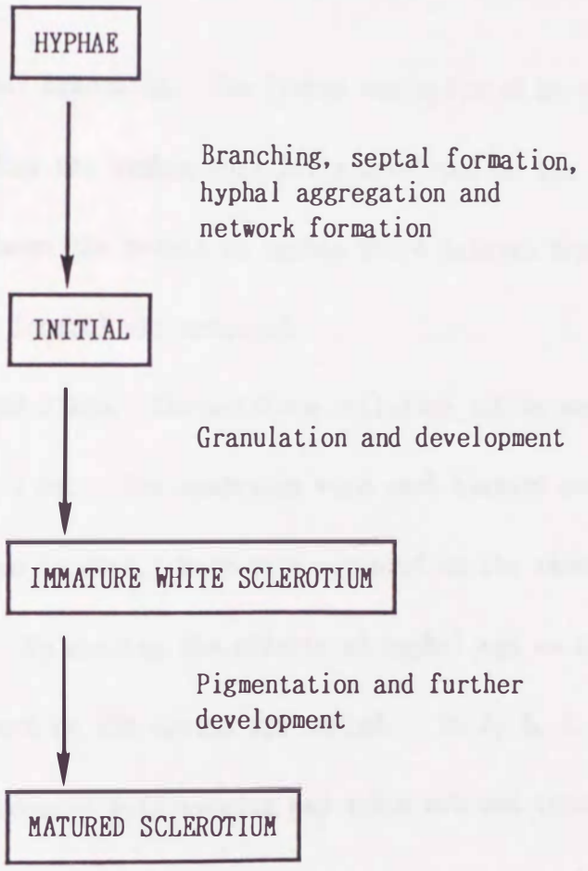


Fig. 18. Diagramatic representation of sclerotium formation of *R. solani*.

Media. PDA was used for preculture of this fungus. To examine the effects of amino acids on the various stages of sclerotial development, stimulative or inhibitory amino acids were added to the basal medium instead of potassium nitrate.

Test at the hyphal branching. The fungus was cultured on the amino acid-containing media. When the hyphae were fully developed on the surface of the media, the length between the points on hyphae where lateral branches were generated (internodal length) was measured.

Test at the hyphal stage. The seamless cellulose tubing was opened and cut to squares of 5 X 5 cm. The membranes were each sheeted on a PDA medium and mycelial disc (5 mm in diam.) were each centered on the sheets and precultured at 25°C. To examine the effects of hyphal age on differentiation, the period of preculture on PDA medium was varied. On 2, 3, 4, 5 and 6th day, each sheet which was covered with mycelia was taken out and transferred onto the test media. If the initials or sclerotia already appeared at this time, these places were marked on the reverse side of the Petri dish. After 14 days, sclerotia which were newly formed on unmarked areas were collected, dried at 60°C and weighed.

Test at the initial stage. Cellulose membranes (8 cm in diam.) were sheeted on PDA media and the mycelial discs were placed at the centers and

cultured for 3～6 days. At 3, 4, 5 and 6th days, the sheets were transferred to the test media and the initials were marked on the reverse side of a Petri dish. After 14 days, sclerotia differentiated from the initials were collected and weighed.

Test at the whitish sclerotium stage. Cellulose membranes (8 cm in diam.) were sheeted on PDA media and the mycelial discs was placed and precultured for 3～5 days. The areas of immature whitish sclerotia were cut together with the membranes in 1～1.5cm^2 pieces and 10 pieces were transferred on to the test medium. After incubation for 14 days, the dry weight of sclerotia was weighed and the degree of pigmentation was compared.

Results and Discussion

The hyphal branching followed by the aggregation and interweaving could be important through the morphological processes of sclerotium formation[119] (Plate 7). Also in *Sclerotium rolfsii*[55], inhibitory substances to sclerotium formation reduced lateral branching, whereas stimulatory substances induced the branching, indicating that the sclerotial morphogenesis and hyphal branching were closely related. As shown in Table 31 and Plate 8, methionine and histidine which were inhibitory to sclerotium formation reduced the lateral

Table 31. The length of branching internodes of *R. solani* on various media

	Media			
	His	Met	Leu	Hopkins[a]
Internodes (μm)	143.30±24.5[b]	100.11±5.96	87.56±11.23	51.40±13.10

[a] KNO_3 is used as nitrogen source.
[b] Average of 200 internodes measured at 72 hr after inoculation.

Table 32. The effects of amino acids on sclerotium differentiation from hyphae

Amino acids	Preculture periods				
	2	3	4	5	6 days
Cys	0[a]	0	0	0	0
Met	0	0	0	0	0
His	0	0	0	0	0
Leu	3.75	2.30	3.25	2.90	0
Ser	53.80	22.50	25.65	43.00	37.00
Control (the basal medium)	53.20	39.95	27.45	22.85	31.30

[a] The weight (mg) of sclerotia (total weight per dish) differentiated from hyphae on cellulose membrane.

Table 33. The effects of amino acids on sclerotium differentiation from initials

Amino acids	Preculture periods			
	3	4	5	6 days
Cys	2.60[a)]	0	0	0
	11.40[b)]	0	0	0
Met	2.80	0	0	0
	17.90	0	0	0
His	4.00	6.50	0	0
	26.30	4.20	0	0
Leu	6.35	8.85	0	0
	20.80	16.00	0	0
Ser	26.80	19.85	8.80	6.75
	44.90	47.00	27.50	15.20
Control (the basal medium)	30.70	25.65	18.20	19.55
	90.70	63.60	48.10	23.70

a) The weight (mg) of sclerotia (total weight per dish) differentiated from hyphal aggregates (initials) on cellulose membrane.
b) The Ratio (%) of sclerotia differentiated from hyphal aggregates.

Table 34. The effects of amino acids on sclerotium pigmentation and development from immature whitish sclerotium

Amino acids	Pigmentation	Final weight
Cys	Light brown	1.12±0.21[a)]
Met	Light brown	1.44±0.52
His	Light brown	2.06±0.48
Leu	Brown	3.12±0.33
Ser	Dark brown	8.01±1.24
Asn	Dark brown	11.66±1.39
Control (the basal medium)	Dark brown	3.10±0.25
Immature whitish sclerotium		0.86±0.08

a) Mean dry weight of sclerotia (mg) and standard error.

branchig from the leading hypha. Also leucine, which was not a strong inhibitor on the sclerotium formation, lightly reduced the branching. The frequency of branching on methionine and histidine-containing media was 1/2～1/3 of the control. The aggregation of hypha was observed even on inhibitive amino acid-amended media (Plate 8). Cysteine, methionine and histidine completely inhibited the differentiation from hypha to sclerotia (Table 32, Plate 9). Although leucine was also inhibitory, a few sclerotium formation occurred on the leucine-containing medium. While hyphae were well differentiated to sclerotia by 14th day after the transfer to the serine-containing medium or Hopkins medium. The rate of the differentiation was affected by the period of the preculture on PDA medium. The differentiation from the initials to the sclerotia were observed even on the medium which contains inhibitory amino acids such as cysteine, methionine and histidine. However, the rate of differentiation was low and sclerotia formed were small (Table 33). Since the process from the initials to sclerotia involves the interweaving of hypha, it is possible that the lateral branching were inhibited and resulted in the reduction of interweaving hypha and of sclerotium size. The reason why the differentiation from the initials only occurred at short preculturing is still uncertain. The development of sclerotia from whitish immatured sclerotia to pigmented matured sclerotia were also restricted by inhibitory amino acids

(Table 33, Plate 10). Sclerotia enlarged 1.2～1.3 times on the media containing cysteine, methionine or histidine and *ca.* 3.7 times on Hopkins medium. Hashiba and Mogi[19] reported that immature whitish sclerotia of *R. solani* reached full size in 40 hr and the degree of sclerotial enlargement was *ca.* 2.3 times in size. They also observed that 3 times increase of the width of sclerotium cells in the central mass occurred in parallel during this process. These results indicate that the enlargement from whitish sclerotia to matured sclerotia depends upon the sclerotial cell enlargement and the inhibition of cell enlargement could directly reflect the sclerotium size and weight. The pigmentation concomitantly occurred with the enlargement of sclerotia. However, it was quite inhibited on the methionine and histidine-containing media (Table 34, Plate 10). Thus, inhibitory amino acids could mainly reduce the lateral branching and result in the reduction of hyphal interweaving, which is necessary for the sclerotial development. Furthermore, inhibition of the sclerotium cell enlargement during the maturation of sclerotia resulted in the limitation of the final sclerotium size and weight. The effects of the inhibitory amino acids on hyphal fusion during the sclerotium formation will be the subject for a future study.

IX. Effects of lights on sclerotium formation of *Rhizoctonia solani*

In Chapters VII and VIII, the author showed that sclerotium formation of *R. solani* was affected by various chemical factors. However, there are fewer reports in regard to the effect of light on sclerotium formation of this fungus[3, 22, 48, 66, 86, 89, 106, 121, 122], compared to other fungi which has been well documented[13, 14, 17, 18, 22, 53, 58, 59, 62-64, 113, 114, 116, 120]. The microsclerotial development of *Verticillium albo-atrum* was completely inhibited by continuous irradiation with blue light[63]. The sclerotium formation of *Botrytis cinerea* was suppressed by blue light, although its sporulation was stimulated by near ultraviolet irradiation[114, 116]. On the other hand, sclerotial production by *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, and *S. delphinii* was enhanced by white light irradiation and stimulated most evidently under blue and near ultraviolet light[57].

This chapter deals with the effects of a wide wave-length light and 5 monochromatic lights, including near-UV, on the hyphal development and sclerotium formation of *R. solani*. Furthermore, the effects of lights on the activity levels and zymograms of oxidases, which may be associated with the sclerotium formation, were investigated.

Materials and Methods

Fungal isolates used. Two isolates of *R. solani*, C-14 and C-324, were used.

Light sources. A white fluorescent lamp (Toshiba FL-15) was used as a wide wave-length light source, and lamps with near-ultraviolet (National FL-20. BLB), blue light (FL-20.SB), green light (FL-20.SG), yellow light (FL-20S.SY) and red light (FL-20S.SR) were used as monochromatic light sources.

Culturing and measurements of hyphal growth and sclerotium formation. Each isolate was precultured on a PDA plate for 2 days and the edge of mycelial mat was cut off by a cork borer (5 mm in diam.). The mycelial discs were inoculated on PDA plates and each incubated at 25°C under the above described light sources at 1,000 lx. A plate incubated in the dark was used as the control. After 2 weeks of culture, the sclerotia formed were picked up with forceps, their number counted, dried at 80°C and weighed. The diameters of colonies of some other plates were measured after 24 hr of incubation, and then the culture media were melted out. The mycelial mats were repeatedly washed with hot water, dried at 80°C and weighed. Each value expressed as the average of 10 dishes, and each experiment was repeated 3 times.

Preparation of mycelial extracts. Each mycelial disc was inoculated on a

PDA plate, which was covered with a cellulose membrane and cultured at 25°C for 48 hr under the lamp at 1,000 lx. The mycelial mat was collected together with the cellulose membrane and homogenized with 0.05 M phosphate buffer (pH 6.5) in a homogenizer. The homogenate was centrifuged at 3,000 g for 20 min. The supernatant was dialyzed against 0.02 M phosphate buffer (pH 6.5) overnight, and then concentrated to 1/25 of its original volume by a Minicon B-15 concentrator (Amicon Ltd.), and used for the experiments described below. Absorbancies at 280 and 260 nm (A_{280} and A_{260}) were determined, as rough measures for the protein and nucleic acid concentration, respectively, by using a Simadzu UV 180 spectrophotometer. To determine the activities of peroxidase, laccase and tyrosinase, 50 µl aliquots of the extracts were each incubated with 50 ml of the following substrate solutions: 0.01 M acetate buffer (pH 4.5) containing 0.01% (v/v) hydrogen peroxide, 0.05% 3, 3'-diaminobenzidine tetrahydrochloride (for peroxidase), 0.2 M acetate buffer (pH 4.5) containing 0.48% dianisidine (for laccase) or 0.1 M phosphate buffer (pH 6.5) containing 0.4% L-dihydroxyphenyl-alanine (for tyrosinase). The mixtures were incubated at 37°C for 1 hr and for peroxidase, laccase and tyrosinase activities their absorbancies at 450, 420 and 475 nm were measured, respectively.

Zymogram and analysis of soluble proteins. The extracts mixed with glycerol (1:1) were subjected to disc electrophoresis by using polyacrylamide

gels at 3 mA per tube for about 1 hr in chilled room (5°C). After electrophoresis, the gels were each immersed in the above mentioned substrate solution for peroxidase, laccase or tyrosinase, to get the respective zymograms. Alternatively, the gels after electrophoresis were stained for proteins with 1% amido black in 7% acetic acid solution and destained for 2 days with 7% acetic acid.

Results

Effects of wide wave-length light

Table 35 shows the effects of continuous irradiation of light from a white fluorescent lamp on the mycelial growth, the number of sclerotia, and time required for the initial formation. Although the size of each sclerotium was decreased, the number of sclerotia in a colony was increased. As for the C-14 isolate, sclerotia were too small to count precisely. The sclerotia were formed in a peripheral zone of a plate incubated in the dark. By contrast, sclerotia were scattered over a plate incubated under light. This phenomenon was distinct at the initial phase of sclerotium formation. The color of sclerotium formed under light was brown and that of sclerotium formed under dark was dark brown (Plate 11), indicating that the irradiation suppressed the coloration of

Table 35. The effects of continuous irradiation of white fluorescent light on hyphal development and sclerotium formation of *R. solani*

Treatment	Isolates	Mycelia			Sclerotia			Time for initial formation
		Diameter (mm)	Fresh wt. (mg)	Dry wt. (mg)	Number	Fresh wt. (mg)	Dry wt. (mg)	
Continuous irradiation	C-14	21.79	—	—	196.58**	202.62	76.30	74.50
	C-324	23.53	78.20**	11.40**	72.55**	191.63	72.55	72.55
Control (Dark)	C-14	23.04	—	—	98.16	208.08	75.28	74.40
	C-324	21.52	100.45	16.30	54.50	211.07	82.59	75.45

Note : Mycelial growth was measured at 24 hr after inoculation. The total weight of sclerotia in a colony was measured at 14 days after inoculation.

** Significant at a 99% level.

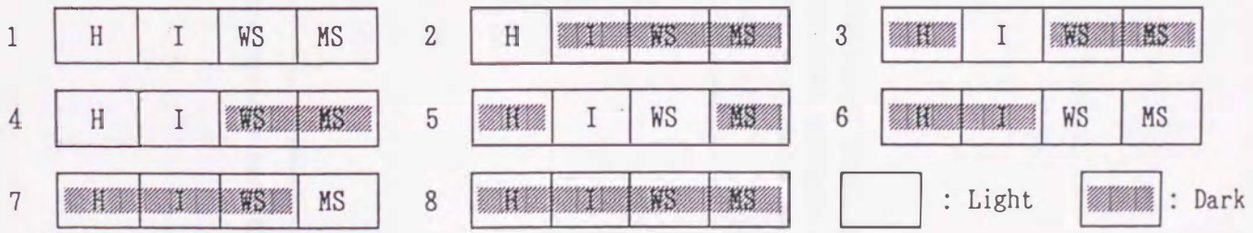
sclerotia.

The effects of irradiation of wide-wave length light at a selected phases of sclerotial development as specified in Table 36 were examined. Although the irradiation at any 1 or 2 phase(s) stimulated the sclerotium formation, the stimulation was distinct when the irradiation was carried out in the early phases, i.e. the hyphal and the initial stage (Table 36). No significant difference in the effect of irradiation on the average weight of sclerotia was observed when the irradiation was done continuously or at each phase of sclerotial development. No quantitative changes in A_{280} and A_{260} (as rough measure of soluble proteins and nucleic acids, respectively) and in the activities of peroxidase and laccase was recognized between the mycelia developed with and without continuous irradiation (Table 37). However, the activity of tyrosinase, which catalyzes melanization, decreased significantly by the irradiation. Along with this, drastic change of tyrosinase zymogram was brought about by the irradiation; the 3 major bands and 1 minor band observed in the tyrosinase zymogram of the colony grown in the dark were not detected in the zymogram of the irradiated colony (Fig. 19). No significant difference in electrophoretic patterns of soluble protein and peroxidase zymogram was observed. As for laccase zymogram, two bands vanished and a new one appeared.

Table 36. The effects of light on several phases of sclerotium formation of *R. solani*[a)]

Stages	Fresh weight of sclerotia (mg)	Dry weight of Sclerotia (mg)	Number of sclerotia
1. Continuous light	232.62	76.36	196.58**
2. Hyphal stage	234.00	77.22	144.64**
3. Initial stage	272.91	85.80	156.20**
4. Hyphal and initial stages	194.65	60.70	163.58**
5. Initial and white sclerotial stages	262.66	78.56	159.29**
6. White and matured sclerotial stages	190.04	68.56	128.67**
7. Matured sclerotial stage	259.17	82.21	117.56**
8. Continuous dark (Control)	208.08	75.28	98.16

a) C-14 isolate was used.



H : Hyphal stage, I : Initial stage, WS : White sclerotial stage, MS : Matured sclerotial stage.
** Significant at a 99% level.

Table 37. The effect of continuous irradiation of light on A_{280}, A_{260} and enzyme activities in the mycelial extracts of *R. solani*

	Continuous light	Control (Dark)
A_{280}	6.1	5.7
A_{260}	9.7	8.8
Peroxidase activity	1.96	2.02
Laccase activity	8.28	8.45
Tyrosinase activity	2.56*	3.65

Note : Each value represents the relative optical density.
* Significant at a 95 % level.

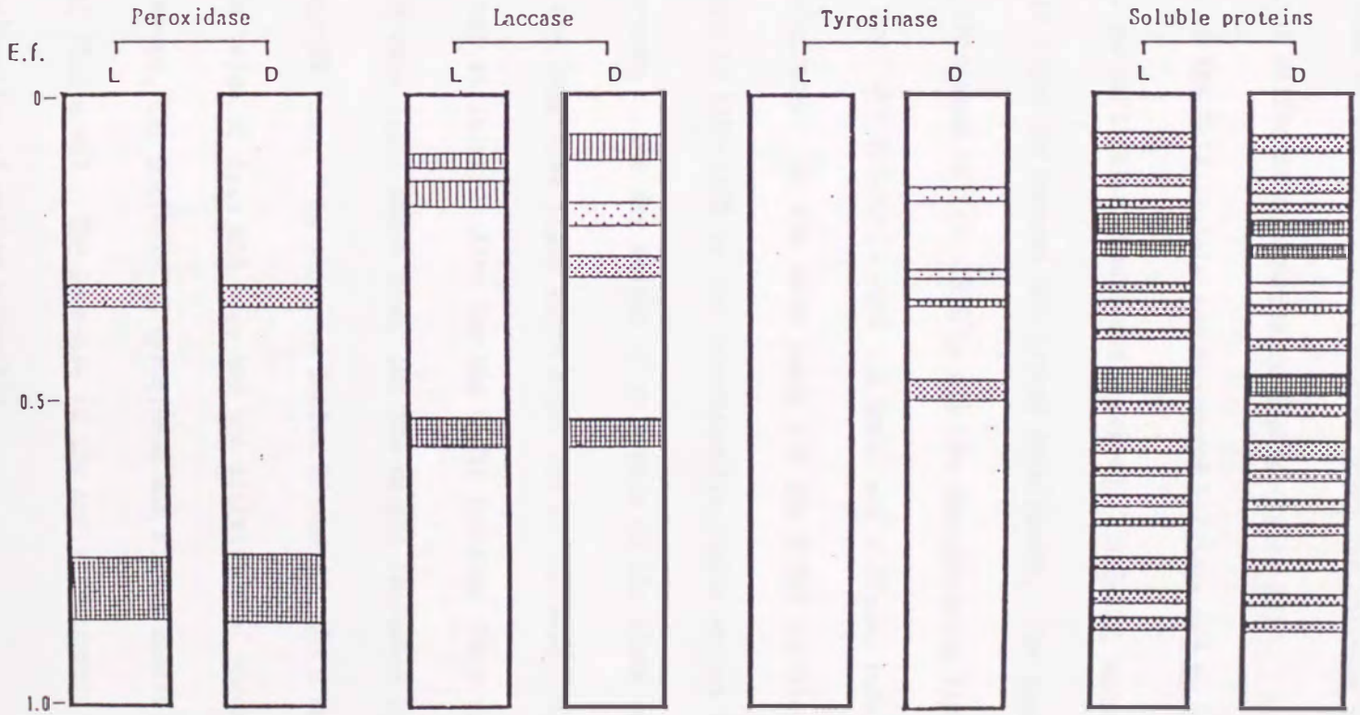


Fig. 19. Zymograms of 3 enzymes and soluble proteins of *R. solani* cultured under the continuous irradiation and dark conditions. L : Light, D : Dark.

Effects of monochromatic lights

The growth of the fungus was compared among the colonies grown under the irradiation of different monochromatic lights (Table 38). Although the hyphal development of the C-14 isolate was decreased by about 16% by near-UV irradiation, no particular change was observed. In C-324, none of the monochromatic light influenced the hyphal development. The number of sclerotium of C-14 was increased to 127～285% by all the monochromatic lights, especially the near-UV one; the sclerotia were too small and diffused into one another to count with accuracy. On the other hand, for the C-324 isolate, the sclerotia were increased to 116～163% by the monochromatic lights except the near-UV which caused a decrease. The dry weight of sclerotia on the plate varied by only 10～20% for the long-wave light above blue, but an increase (ca. 63%) by near-UV irradiation was striking. Also for the C-324 isolate, there were no changes from the long-wave light above blue, but the weight increased by about 120% under the near-UV lamp. As shown in Tables 39 and 40, there was not so much change in the value of A_{280} and A_{260} and the activities of peroxidase and laccase. However, the activity of tyrosinase was significantly decreased at the 99% level (Table 40). The decrease in the activity closely agreed with the failure in coloration of mature sclerotia.

Table 38. The effect of monochromatic light on mycelial growth and sclerotium formation of *R. solani*

Isolates		Continuous irradiation of monochromatic light					Control
		Near-UV	Blue	Green	Yellow	Red	Dark
C-14	Diameter of culture (mm)	33.20**	40.91	42.00	41.31	40.00	39.35
	Number of sclerotia	—	381.86**	296.68**	170.96**	316.04**	103.91
	Total dry weight of sclerotia (mg)	140.80**	102.93**	96.35	70.61**	100.09*	91.28
C-324	Diameter of culture (mm)	44.77	45.89	43.77	48.16	45.61	46.05
	Number of sclerotia	62.23**	79.32**	111.72**	98.96**	105.00**	68.37
	Total dry weight of sclerotia (mg)	132.29**	111.28	117.45	112.93	114.30	108.46

Note : Mycelial growth was measured at 24 hr after inoculation and the total dry weight of sclerotia in a colony was measured at 14 days.
** Significant at a 99% level. * Significant at a 95% level. — : Scarcely unmeasurable.

Table 39. The effect of monochromatic light on A_{280} and A_{260} of *R. solani*

Isolate	Constituents	Continuous irradiation of monochromatic light					Control
		Near-UV	Blue	Green	Yellow	Red	Dark
C-14	A_{280}	0.183	0.165	0.192	0.254	0.150	0.167
	A_{260}	0.309	0.284	0.307	0.449	0.256	0.279
C-324	A_{280}	0.157	0.213	0.264	0.246	0.137	0.192
	A_{260}	0.266	0.373	0.471	0.437	0.233	0.327

Note : Each value represents the relative optical density.

Table 40. The effects of monochromatic light on 3 enzyme activities of *R. solani*

Isolates	Enzymes	Continuous irradiation of monochromatic light					Control
		Near-UV	Blue	Green	Yellow	Red	Dark
C-14	Laccase	0.310	0.345	0.264	0.329	0.311	0.293
	Peroxidase	0.339	0.287	0.303	0.413	0.271	0.282
	Tyrosinase	0.053**	0.038**	0.055**	0.084**	0.035**	0.159
C-324	Laccase	0.276	0.332	0.267	0.292	0.297	0.311
	Peroxidase	0.263	0.376	0.381	0.302	0.205	0.318
	Tyrosinase	0.079**	0.133**	0.113**	0.097**	0.067**	0.479

Note : Each value represents the relative optical density.
** Significant at a 99 % level.

Discussion

In this experiment, the author obtained the results that the irradiation with a white fluorescent light and several kinds of monochromatic lamps stimulated the formation of the initials, which consisted of interwoven hyphal branches and resulted in the increase of the mature sclerotia, although the size and weight of each sclerotium were reduced. In Chapters VII and VIII, the effects of various nutrient factors on the sclerotium formation of *R. solani* were examined and the results demonstrated that the weight of sclerotia formed on the Petri dish was dependent on the amount of carbon sources in the culture medium. The pigmentation of sclerotia was suppressed and changed into light brown under the continuous light as opposed to dark brown in the absence of any light (Plate 11). The reduction in pigmentation by the light coincided with the reduction inthe activity of tyrosinase which catalyzes melanization. The vegetative growth and morphogenesis of fungi, such as hyphal development, sporulation and sclerotial formation, are affected by nutrients (*e.g.* carbon and nitrogen sources, the C/N ratio, minerals and vitamins) and physical factors (*e.g.* temperature, water or humidity, pH, and light)[22, 56]. According to the reactions to light in sclerotium formation, the fungi can be divided into four types; 1) Fungi inhibited by light (*Verticillium dahliae*[14]), 2) Fungi

stimulated by light (*Sclerotinia sclerotiorum*, *Sclerotium delphinii* and *S. rolfsii*[58,120]), 3) Fungi dependent on wavelength (*Botrytis cinerea* and *V. albo-atrum*[58,116]) and 4) Fungi neutral to light (some isolates of *Aspergillus*[104]). Kudo and Sakamoto[66] discovered that the hyphal growth of *R. solani* and the amount of sclerotia were decreased by short wavelength light irradiation. A previous report[63] describedthat microsclerotium formation was almost completely inhibited by exposure to blue light, in the case of *V. albo-atrum*, and that some isolates of the fungus produced an orange pigment and suggested that the pigment may play an inactive part in formation of microsclerotium. In several micro-sclerotial isolates of *V. albo-atrum*[14,15], white fluorescent and near-UV light delayed, or prevented, the formation of microsclerotia and the production of melanin in several microsclerotial isolates of *V. albo-atrum*, suggesting that there were at least two places in the chain of events leading to the melanin and microsclerotial production where near-UV could inhibit the process. *S. rolfsii* has been well studied with regard to its high responsibility to light; it was revealed in the fungus that the number of sclerotia per plate was increased, while the dry weight per sclerotium was decreased, by the irradiation, and that tyrosinase activity, which increased during the sclerotium formation, was suppressed by blue and white light[81]. Monochromatic lights, except the near-UV, results in an increase in the number of sclerotium of the C-14 and C-324

isolates. On the other hand, near-UV irradiation decreased the sclerotial number of the C-324 isolate but increased it for the C-14 isolate.

The present experiment demonstrated that the suppression of sclerotial pigmentation by wide-wave length light and each monochromatic light was related to the reduction in the activity of tyrosinase, which is contrary to the result in *S. rolfsii*.

X. CONCLUSIONS

This thesis deals with some subjects for the causal agent of soil borne disease, *Rhizoctonia solani* Kühn. The conclusions are summarized below.

1) Grouping with the zymogram

A collection of 48 isolates selected from various anastomosis groups of *R. solani* showed at least 9 distinct zymograms. Isolates from the rice (the sasaki type), or its relatives showed the zymogram pattern named *Zym*-1. However, isolates in the same anastomosis group (AG-1) from other host plants (the web-light type) exhibited several other different zymograms. Isolates of AG-2-2 from the mat rush (Igusa), rice (quasi-sheath blight) and sugar beet gave *Zym*-2-2A which resembled *Zym*-1. Other members of AG-2-2 from the sugar beet exhibited a different zymogram, *Zym*-2-2B. An isolate of AG-2-1 gave the pattern *Zym*-2-1 which was quite different from all the other zymogram patterns. Isolates from the potato (AG-3) showed a characteristic zymogram designated *Zym*-3. Similar zymograms were given by some isolates of anastomosis group AG-5 which also came from potato. The zymogram groups roughly agreed with the anastomosis groups, and sometimes correlated more closely with the ecological types. These results will serve as a clue to the revision of taxonomy of *R.*

solani.

2) Morphological study on sclerotium

The process of the sclerotial development of *R. solani* was compared with that of *Sclerotinia sclerotiorum* by a scanning electron microscope. The mature sclerotia of *R. solani* were 2～3 mm in diameter, and their outer and inner layers were dark brown in color, whereas those of *S. sclerotiorum* were 4～6 mm, their outer layer (rind) consisted of vacuous cells, and their inner layer (medulla) was composed of white watery cells. In the case of *R. solani*, the initials were formed by intertwining of several hyphae followed by branching of normal hyphae. The sclerotial surfaces from the white to mature sclerotial stages were covered with dense hyphae. Inner and outer parts of the sclerotia showed a honey-comb structure as observed by sectioning. On the other hand, initials of *S. sclerotiorum* were developed by intertwining of elongated, curved hyphae. No differentiation of inner and outer layers were observed at the white sclerotial stage. However, as the pigmentation proceeds, two or several layers vacuoled as rind and the inner parts consisted of hyphal fusion and anastomosis, which have few intercellular spaces, as medulla. The change of hyphae covering sclerotial surface was recognized during the maturation.

3) Effects of physical factors on sclerotial longevity

The order of high-temperature tolerance at the different morphological stages was hyphae < initials < white sclerotia < immature sclerotia < mature sclerotia. The mature sclerotia, in particular, resisted 3～7°C higher temperatures than the other stages. *R. solani* was more tolerant to high temperatures than *S. sclerotiorum*. On the other hand, *R. solani* at all the stages starting from the white sclerotia survived even under the UV irradiation applied for 10 days. The initials which are formed by hyphal branching followed by aggregation and interweaving became to be more tolerant to UV, suggesting that striking physiological changes took place at the initial stage. The mature sclerotia of the 2 fungi died at pH 4～5, but survived pH's above 6. The sclerotia immersed in pure water at 25°C survived at a high ratio but died rapidly at 35°C; especially the sclerotia of *S. sclerotiorum* died within 7 days.

4) Changes in enzymatic activity during the sclerotium formation

The activity of malate dehydrogenase was higher in sclerotia than in hyphae. On the other hand, glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase showed higher activity in young hyphae in which sclerotia were not formed. The activity of alkaline phosphatase was higher in younger hyphae, decreasing with hyphal aging, whereas that of acid phosphatase increased

with the aging of hyphae. These results may indicate that the sclerotium of *R. solani* is not resting but active in terms of vigorous metabolism, and that energy and substrate sources for the sclerotium formation are supplied from hyphal components, as well as directly from media.

5) Effects of soil microorganisms on sclerotial longevity

When the sclerotia of 4 fungi were soaked in two soil suspensions, such as Kunigami Maaji and Jaagaru, almost all died even at 25°C. As for *S. sclerotiorum*, the rind of dead sclerotia was peeled off and broken into pieces. When sclerotia were buried in Kunigami Maaji and Jaagaru soils, the survival rate of the sclerotia of *R. solani* decreased only in Kunigami Maaji, though the survival rate decreased in the case of *S. sclerotiorum* in both soils. *Trichoderma* and *Penicillium* were observed from the sclerotia of *R. solani* buried in Kunigami Maaji. On the other hand, *Fusarium*, *Aspergillus*, *Mucor* and *Rhizopus* were isolated from those of *S. sclerotiorum* in Jaagaru. Comparing the microflora in the 2 soils, bacteria were observed in Jaagaru and fungi in Kunigami Maaji. This may be due to the soil pH. By the soil dilution method, *Aspergillus*, *Mucor*, *Rhizopus* and *Fusarium* were well isolated from Jaagaru, and *Trichoderma*, *Penicillium* and *Chaetomium*, from Kunigami Maaji. As *Trichoderma lignorum* (RT-1) was introduced into the soils, all of buried sclerotia of *S. sclerotiorum* and 2/3 of *Sclerotium delphinii* were killed, whereas those of *R.*

solani and *S. rolfsii* were scarcely affected. Moreover, *Aspergillus* isolated from dead sclerotia also showed a high antagonistic ability; above all *A. terreus* (RA-2) specifically inhibited the sclerotium formation of *R. solani*. Comparing the structures of the 4 sclerotium forming fungi, the sclerotia of *S. sclerotiorum* were seen to be pigmented only on the rind, while the inner parts (medullae) were whitish and watery. Contrary to this, the sclerotia of *R. solani* were pigmented throughout. High susceptibility to the antagonists in th sclerotium of *S. sclerotiorum* may be due to its structure. On the other hand, when *T. lignorum* (RT-1) was inoculated to the soils differing in pH value, the isolate could increase in population in the sterile alkaline soil. Moreover, there were more antagonistic microorganisms to RT-1 in the alkaline soil than in the acidic soil. Many isolate of *Aspegrillus* from the alkaline soil strongly antagonized to RT-1 *in vitro*. Physical and biological factors in a complicated network may affect the sclerotial longevity, directly or indirectly.

6) Effects of nutrients on sclerotium formation

Most of the carbon sources tested were well utilized for the sclerotium formation. However, little sclerotium formation was observed on arabinoe or inulin as a carbon source, even though the hyphal growth was normal. The sclerotium formation was barely observable on lactose medium in the C-14

isolate. On mannitol or glycerol as a carbon source, the hyphal growth was very poor, and therefore little or no sclerotium formation was observed. The sclerotium formation on the medium containing ammonium sulfate or ammonium chloride amended as a carbon source was weaker than on the medium containing nitrate potassium. The sclerotial weight increased according to the carbon concentration. The addition of potassium nitrate as a standard nitrogen source above 0.1% scarcely affected the total sclerotial weight per dish, though induced a decrease in the size of each sclerotium. These results may indicate that the total weight of sclerotia of the fungus on the plate depends upon the carbon concentration, and the number of sclerotia, upon the nitrogen concentration.

The carbon concentration influenced the activities of enzymes related to the TCA cycle. Investigation by using ^{14}C-labelled glucose revealed that the carbon source was almost consumed during the sclerotium formation and the amount of caron consumption was correlated with the total sclerotial weight. From the morphological point of view, carbon quantity also depends on the hyphal branching of *R. solani*.

7) Effects of inorganic compounds on sclerotium formation

None or very few sclerotia were formed when potassium dihydrogen phosphate

was removed from the basal Hopkins medium, although hyphae grew to some degree. Also, the number and weight of sclerotia were decreased to a certain degree when magnesium sulfate was removed.

As potassium dihydrogen phosphate in the basal medium was replaced by other phosphate with different cations, the sclerotia were well formed, somewhat differing in quantity and weight. The optimal concentration of potassium dihydrogen phosphate was about 10 ppm for the sclerotium formation in number, weight and hyphal development. The addition of increasing amount of magnesium resulted in an increase in the number and weight of sclerotia. These results indicate that the phosphate ion, in a certain amount, is indispensable to the sclerotium formation, whereas the magnesium ion has a promotive effect.

The absence of phosphorus at the maturation stage resulted in the formation of fewer sclerotia, even when phosphorus was present at the hyphal and initial stages. Deficiency of phosphorus in the early stages of sclerotial development had no effect on the number and weight of sclerotia formed if phosphorus was supplied at the subsequent periods. The branching internodes of hyphae grown on phosphorus-free medium were longer than those grown on a medium containing phosphorus. This decrease in the hyphal branching with a decrease in the interweaving of hyphae may result in meager sclerotium formation. Moreover, the activities of malate dehydrogenase and phosphogluconate dehydrogenase were lower

in the hyphae grown on a phosphorus-free medium than in the hyphae grown on a phosphorus containing medium. Specific accumulation of ^{32}P in sclerotia was strikingly observed.

8) Effects of amino acids on sclerotium formation

The inhibitory effects of some amino acids on sclerotium formation of the fungus was tested by amending the basal medium with amino acids. Few or no sclerotia were formed on sulfur containing amino acids, histidine, leucine, isoleucine, tryptophan or tyrosine amended medium. Leucine was not inhibitory but was hardly utilized. Inhibitory amino acids limited the sclerotium formation, while scarcely affecting the mycelial growth. Although the structural differences between cysteine and serine, or between homocysteine and homoserine, was small, they had completely opposite effect on the sclerotium formation. D-isomers of stimulative amino acids inhibited the formation.

The morphological process of sclerotium formation could be divided into 5 stages. The inhibitory effects of amino acids, such as cysteine, methionine and homocysteine, on each of the developmental stages was studied. The lateral branching was quite limited with the amino acids, while the aggregation of hypha was not affected. The change of hypha to sclerotia was completely inhibited, but the changing of the initials was only slightly inhibited, although the

sclerotia formed were very small. The turning from the initials only occurred following a short preculturing on the PDA medium. The development of the whitish immature sclerotium to the pigmented mature sclerotium was also limited. The inhibition of sclerotium formation by some amino acids could be explained by the reduction of interweaving hypha, caused by the inhibition of lateral branching and the limitation of cell enlargement at sclerotial maturation.

9) Effects of lights on sclerotium formation

The irradiation by a wide wave-length light (fluorescent lamp) on the mycelia of the 2 isolates of the fungus increased the number of sclerotial initials and that of mature sclerotia, although mycelial growth and the total weight of sclerotia in a Petri dish were not affected. The irradiation was carried out atseveral stages of sclerotial differentiation, and the progression was observed at all the stages, with the most striking effects occurring at hyphal and sclerotial initial phases. The pigmentation of sclerotia during sclerotial maturation was inhibited to some extent by the light, and the tyrosinase activity, which catalyzes the melanization, decreased concomitantly.

As for the monochromatic lights, the hyphal linear growth of the isolates was not obviously affected by every kinds of light, except near-UV in C-14. On the other hand, the number of sclerotia in the C-324 isolate was slightly

reduced by near-UV, while it was increased by other lights, particularly in C-14. The total weight formed in a Petri dish was increased by near-UV in the 2 isolates. In the case of C-14 the degree of increase was about 60%. The pigmentation of sclerotia during maturation was also inhibited by every monochromatic light, in well agreement with the decrease of tyrosinase activity.

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