Rhizoctonia solani Kuhn の菌学的および病理学的研究

Mycological and Phytopathological Studies on *Rhizoctonia solani* Kühn

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キーワード：リゾクトニア菌，土壌伝染病，菌核形成，
Key Words : *Rhizoctonia*, soil-borne disease, sclerotium formation,

Summary

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 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

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Sci. Bull. Fac. Agr. Univ. Ryukyus 44 : 1～79 (1997)

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℃ 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℃ survived at a high ratio but died rapidly at 35℃; 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℃. 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 the 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 *Aspergillus* 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 arabinose 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 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 at several 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.

I INTRODUCTION

The genus *Rhizoctonia* was newly established by Candolle in 1815 and *Rhizoctonia solani* was first described by Kühn in 1858 as a causal agent of potato black scurf (Kuroazabyo)[67]. The fungus is distributed widely in the fields, paddy fields, meadows and forests of the world and is a parasite on over 250 plants: crops, vegetables, flowering plants, grasses *etc.* having various symptoms such as sheath blight, web-blight, damping-off, root rot *etc.* (Table 1, Plate 1). Furthermore, *R. solani* is not only parasitic on living plants but also lives on decaying plant residue and, symbiotically, on orchid plants[117,123-125,127,130].

Rhizoctonia species is classified in the family Agonomycetaceae (Sterele mycelia), Agonomycetales (Mycelia Sterilia), Hyphomycetes (Fungi imperfecti). The fungus is identified by: 1) the number of nuclei in nutrient hyphae, 2) the presence of dolipore septum, 3) the branching manner of the hyphae, 4) the color of the hyphae, 5) the formation of moniloid cell, 6) the formation of sclerotia, 7) the hyphal size and 8) the hyphal growth rate, because it does not produce conidia[100]. The perfect stage of this fungus had been unknown for a long time because of the difficulty of formation of sexual spore. Even after the perfect stage (basidiospore) was clarified by various techniques, the classification had been continuously discussed and the genus name was changed in *Hypochnus, Corticium, Pellicularia, Botrybasidium* and *Ceratobasidium*. At present, the name *Thanatephorus* is strongly supported. Parmeter *et al.*[100,101] have named *T. cucumeris* (Japanese name: Kumonosukouyakutake) as the perfect stage of *R. solani*. They also explained that there are 3 types known as "praticola," "sasakii" and "solani" of *T. cucumeris* and that it is difficult to distinguish them as different species by the morphology of the perfect stage[101]. On the other hand, it is well-known that *R. solani* involves morphologically, physiologically and pathologically different groups. Watanabe and Matsuda[130] examined 220 isolates of *R. solani* from 59 different field crops from various places in Japan and classified their ecological characters by cultural type, pathogenicity and saprophytic activity. Ogoshi[92-95] had classified *R. solani* by anastomosis which occurred in the same or related isolates and reported that anastomosis groups coincided well with pathogenetic or other classifications. The primary infection source of the diseases by *R. solani* is mostly by sclerotium (*pl.* sclerotia)[121,125,126]. Fungal sclerotia are asexual, multicellular, firm resting structures which become interwoven and aggregate. They are rich in stored nutrients so that they can persist for longer periods than the vegetative hyphae in adverse soil environments[29,119,138]. Sclerotia are classified according to the modes of germination into three types: 1) the myceliogenic type, in which individual hyphae or hyphal aggregates are developed directly, such as in *Helicobasidium purpureum, Macrophomina phaseoli, Mycosherella ligulicola, Phymatotrichum omnivorum, Rhizoctonia solani, Sclerotinia gladioli, S. delphinii, S. rolfsii and Verticillium dahliae* (as *V. albo-atrum*), 2) the sporogenic type, in which conidiophores and conidia are produced, such as *Botrytis convoluta, B. tulipae, Colletotrichum coccodes, Gloeocercospora sorghi and Helminthosporium oryzae,* and 3) the carporogenic type, in which fruit bodies such as ascocarps or basidiocarps are generated, such as *Claviceps microcephala, C. purpurea, Sclerotinia minor, S. polyblastis, S. sclerotiorum, S. trifoliorum, Typhula idahoensis and T. incarnata*[29]. These modes

correlate with the sclerotial size because of the necessity of a food-base for the germination. In general, the size of the myceliogenic sclerotia tends to small, whereas the carpogenic sclerotia are correspondingly larger because of the larger amounts of nutrients required for the formation of fruit bodies[37]. Furthermore, root and foliage infecting fungi are myceliogenic and carpogenic, respectively[37]. Sclerotial initials originate by hyphal branching, interweaving and aggregation. These then developed to white immature sclerotia with an increase in size by repeated branching, aggregation and granulation, and matured with pigmentation[22,119]. The manner of initial formation is different in different fungal species. Townsend and Willetts[119] divided the sclerotium forming fungi into 3 types: the terminal type, the strand type and the loose type. Mature sclerotia vary in layer structure from those without a differentiated rind to those with several well differentiated layers from rind to medulla. *Sclerotium rolfsii* produces sclerotia of most complex structure with 4 distinct layers: a) fairly thick skin or cuticle, b) a rind, two to four cells thick, made of broad and tangentially flattened cells. c) a thin-wall cortex with densely stained cytoplasm, and d) a medulla of loose filamentous hyphae also with dense contents[29].

The sclerotium produced by *R. solani* belongs to the myceliogenic type with regard to the manner of germination, and to the loose type with regard to the forming of the initials, thus the simplest of all fungi[29,103]. Although there is no definite pattern of organization of the hyphae in the initials, the sclerotial initial is originated by an irregular branching of the ordinary mycelium with production of much more numerous septa. The mature sclerotia are much less tightly interwoven than those of other fungi and there is no obvious differentiation into a rind and a medulla[119]. Although there are certain differences in morphology of sclerotia of *R. solani*, in general, they become dark pigmented and have spherical, irregular or flat shapes from 0.2 mm to 6 mm in size[125].

In recent years, with changing cultivation practices, damage caused by the fungus has been increasing annually. In this thesis the author describes the application of the zymogram analysis to clarify the relationships among the anastomosis groups of *R. solani*, and compares the structural changes during the morphogenesis, and survival of this fungus in various physical and microbiological environments, with those of another sclerotium forming fungi of different morphology. Also, an enzymatic study was undertaken to elucidate the process of sclerotium formation, the inducing and inhibiting factors were investigated as part of a fundamental research into methods for controlling the disease caused by *R. solani*.

Table 1-1. Rhizoctonia diseases in Japan

Host plants	Disease name	Fungus	Note
Food crops and special crops			
1. Rice	Sheath blight	*R. solani*	AG-1[a], IA[b]
2. Rice	Borded sheath spot	*R. oryzae*	
3. Rice	Brown sheath blight	*R. solani*	AG-2-2, IIIB
4. Barley	Sheath blight	*R. solani*	AG-1, IA
5. Foxtail millet	Sheath blight	*R. solani*	AG-1, IA
6. Sorghum	Sheath blight	*R. solani*	AG-1, IA
7. Maize	Leaf-sheath blight	*R. solani*	AG-1, IA
8. Common millet	Sheath blight	*R. solani*	AG-1, IA
9. Barnyard millet	Sheath blignt	*R. solani*	AG-1, IA
10. Potato	Foliage blight	*R. Solani*	
11. Potato	Black scurf	*R. solani*	AG-3, IV
12. Sweet potato	Charcoal rot	*R. bataticola*	
13. Soybean	Rhizoctonia rot	*R. solani*	AG-2-1, II
14. Adzuki bean	Rhizoctonia rot	*R. solani*	AG-2-1, II
15. Adzuki bean	Charcoal rot	*R. bataticola*	
16. Kidney bean	Rhixoctonia rot	*R. solani*	AG-2-1, II
17. Kidney bean	Charcoal rot	*R. bataticola*	
18. Cowpea	Charcoal rot	*R. bataticola*	
19. Pea	Rhizoctonia stenm rot	*R. solani*	AG-2-2
20. Broad bean	Rhizoctonia rot	*R. solani*	AG-2-1, II
21. Tobacco	Sore shin, Damping-off	*R. solani*	AG-4, IIIA
22. Tea	Web-blight	*R. solani*	AG-1, IB
23. Sugar beet	Leaf blight	*R. solani*	AG-2-2, IV
24. Sugar beet	Seedling damping-off	*R. solani*	AG-4, IIIA
25. Sugar beet	Root rot	*R. solani*	AG-2-2, IIIB
26. Sugar beet	Damping-off	*R. candida*	
27. Sugar cane	Banded sclerotial disease	*R. solani*	AG-1, IA
28. Mat rush	Stem rot	*R. solani*	AG-1, IA
29. Cotton	Sore shin	*R. solani*	AG-4, IIIA
30. Flax	Wilt	*R. solani*	AG-4, IIIA
31. Ramie	Stem rot	*R. solani*	AG-4, IIIA
32. China jute	Stem rot	*R. solani*	AG-4, IIIA
33. Jute	Seedling blight	*R. solani*	AG-4, IIIA
34. Amberi hemp	Stem rot	*R. solani*	AG-4, IIIA
35. Paper-bush	Web-blight	*R. solani*	AG-1, IB
36. Sesame	Foliage rot	*R. solani*	AG-2-2, IV
37. Sesame	Damping-off	*R. solani*	AG-4, IIIA
38. Wax tree	Damping-off	*R. solani*	
39. Lacquer tree	Web-blight	*R. solani*	AG-1, IB
40. Konnyaku	Root rot	*R. solani*	AG-2-2, IIIB
41. Ginseng	Foot rot	*R. solani*	AG-4, IIIA
42. Japanese mint	Stem rot	*R. solani*	
43. Castor bean	damping-off	*R. solani*	AG-4, IIIA
44. Digitalis	Stem rot	*R. solani*	Unknown
45. Camphor tree	Web-blight	*R. solani*	AG-1, IB
Vegetables, flowers and ornamental plants, forage grasses and legumes, turfgrasses			
46. Tomato	Damping-off	*R. solani*	AG-4, IIIA
47. Eggplant	Damping-off	*R. solani*	AG-4, IIIA
48. Red pepper, Sweet pepper	Damping-off	*R. solani*	AG-4, IIIA
49. Cucumber	Damping-off	*R. solani*	AG-4, IIIA
50. Oriental melon	Damping-off	*R. solani*	AG-4, IIIA
51. Oriental pickling melon	Damping-off	*R. solani*	AG-4, IIIA
52. Bottle gourd	Damping-off	*R. solani*	AG-4, IIIA
53. Japanese radish	Root rot	*R. solani*	AG-2-1, II
54. Chinese cabbage	Bottom rot	*R. solani*	AG-2-21, II, IB
55. Cabbage	Damping-off	*R. solani*	AG-4, IIIA
56. Wasabi	stem rot	*R. solani*	Unknown

[a] Anastomosis group, [b] Culture type.

Table 1-2. Rhizoctonia diseases in Japan

Host plants	Disease name	Fungus	Note
57. Onion	Damping-off	*R. solani*	AG-4, IIIA
58. Welsh onino	Dampihg-off	*R. solani*	AG-4, IIIA
59. Edible burbock	Black scurf	*R. solani*	AG-2-2, IIIB
60. Lettuce	Bottom rot	*R. solani*	AG-1, IB
61. Carrot	Crown rot, Damping-off	*R. solani*	AG-2-2, IIIB
62. Japanese hornwort	Damping-off	*R. solani*	AG-4, IIIA
63. Spinach	Foot rot	*R. solani*	AG-4, IIIA
64. Chinese yam	Root rot	*R. solani*	AG-2-2, IIIB
65. Ginger	Sheath blight	*R. solani*	AG-2-2, IIIB
66. Calceolaria	Damping-off	*R. solani*	AG-4, IIIA
67. Cineraria	Damping-off	*R. solani*	AG-4, IIIA
68. Sweetpea	Damping-off	*R. solani*	AG-4, IIIA
69. Madagascar periwinkle	Root and stem rot	*R. solani*	AG-4. IIIA
70. California-poppy	Root rot and stem rot	*R. sorani*	Unknown
71. Sunflower	Root and stem rot	*R. solani*	Unknown
72. Begonia	Stem rot	*R. solani*	Unknown
73. Lupine	Setm rot	*R. solani*	Unknown
74. Pink	Stem rot, Root rot	*R. solani*	Unknown
75. Bellflower	Root rot	*R. solani*	Unknown
76. Balloon flower	Damping-off	*R. sp.*	
77. Chrysanthemum	Root and stem rot	*R. sp.*	
78. Gladiolus	Collar rot, Leaf-base rot	*R. solani*	
79. Crocus	Root rot	*R. solani*	
80. Cyclamen	Seedling blignt	*R. solani*	
81. Dahlia	Damping-off	*R. solani*	AG-4, IIIA
82. Tulip	Gray bulb rot	*R. tuliparum*	
83. Tulip	Leaf rot	*R. solani*	AG-2-1, II
84. Red clover	Summer blight	*R. solani*	AG-1, IA, IB
85. Red clover	Black patch disease	*R. leguminicola*	
86. Red clover	Root rot	*R. solani*	
87. Red clover	Charcoal rot	*R. bataticola*	
88. White clover	Summer blight	*R. solani*	AG-1, IA, IB
89. White clover	Black patch disease	*R. leguminicola*	
90. White clover	Root rot	*R. solani*	
91. Alsike colver	Summer blight	*R. solani*	AG-1, IB
92. Alsike clover	Black patch disease	*R. leguminicola*	
93. Crimson clover	Summer blight	*R. solani*	AG-1, IA, IB
94. Crimson clover	Black patch diseasse	*R. leguminicola*	
95. Subterranean clover	Summer blight	*R. solani*	AG-1, IA, IB
96. Subterranean clover	Black patch disease	*R. leguminicola*	
97. Alfalfa	Summer blight	*R. solani*	
98. Alfalfa	Charcoal rot	*R. bataticola*	
99. Common vetch	Summer blight	*R. solani*	
100. Hairy vetch	Summer blight	*R. solani*	
101. Trefoil	Summer blight	*R. solani*	
102. Trefoil	Charcoal rot	*R. bataticola*	
103. Sweet clover	Summer blight	*R. solani*	AG-1, IB
104. Sweet clover	Black patch disease	*R. leguminicola*	
105. Kudzu	Summer blight	*R. solani*	AG-1, IB
106. Japan clover	Summer blight	*R. solani*	AG-1, IB
107. Wheatgrass	Summer blight	*R. solani*	AG-1, IA, IB
108. Redtop, Bentgrass	Summer blight	*R. solani*	AG-1, IA, IB
109. Meadow foxtail	Summer blight	*R. solani*	AG-1, IA, IB
110. Sweet vernalgrass	Summer blight	*R. solani*	AG-1, IA, IB
111. Tall oatgrass	Summer blight	*R. solani*	AG-1, IA, IB
112. Brome grass	Summer blight	*R. solani*	AG-1, IA, IB
113. Orchard grass	Summer blight	*R. solani*	AG-1, IA, IB
114. Fescues	Summer blight	*R. solani*	AG-1, IA, IB
115. Velvetgrass	Summer blight	*R. solani*	
116. Ryegrass	Summer blight	*R. solani*	AG-1, IA, IB

Table 1-3. Rhizoctonia diseases in Japan

Host plants	Disease name	Fungus	Note
117. Reed canarygrass	Summer blight	*R. solani*	AG-1, IA, IB
118. Timothy	Summer blight	*R. solani*	AG-1, IB
119. Bluegrass	Summer blight	*R. solani*	AG-1, IA, IB
120. Rhodes grass	Summer blight	*R. solani*	AG-1, IA, IB
121. Bermuda grass	Summer blight	*R. solani*	AG-1, IA, IB
122. Teosinte	Summer blight	*R. solani*	AG-1, IA, IB
123. Paspalum	Summer blight	*R. solani*	AG-1, IA, IB
124. Rutabaga	Summer blight	*R. solani*	AG-1, IA, IB
125. Asparagus	Root rot	*R. solani*	
126. Celery	Root rot	*R. sp.*	
127. Taro	Root rot	*R. sp.*	
128. Zoysia grass	Brown apatch	*R. solani*	
129. Zoysia grass	Dollar spot	*R. monteithianum*	
Fruit trees			
130. Pineapple	Wilt	*R. sp.*	
131. Durian	Leaf spot	*R. sp.*	
132. Papaya	Damping off	*R. solani*	
Conifers and bamboos			
133. Ginkgo	Web-blight	*R. solani*	
134. Fir	Black root rot	*R. bataticola*	
135. Japanese yew	Racodium snow blight	*R. sp.*	
136. White fir	Damping-off	*R. solani*	
137. Japanese hemlock	Racodium snow blight	*R. solani*	
138. Silver fir	Damping-off	*R. solani*	
139. Japanese larch	Damping-off	*R. solani*	
140. Japanese red pine	Web-blight	*R. solani*	AG-1, IB
141. Jspanese red pine	Damping-off	*R. solani*	
142. Japanese red pine	Root rot	*R. solani*	
143. Japanese black pine	Web-blight	*R. solani*	AG-1, IB
144. Japanese black pine	Damping-off	*R. solani*	
145. Japanese black pine	Root rot	*R. solani*	
146. Japanese cedar	Damping-off	*R. solani*	
147. Japanese cedar	Root tot	*R. solani*	
148. Japanese thuja	Damping-off	*R. solani*	
149. Japanese cypress	Web-blight	*R. solani*	AG-1, IB
150. Japanese cypress	Damping-off	*R. solani*	
151. Sawara cypress	Damping-off	*R. solani*	
Broad leaved trees			
152. Beefwood	Web-blight	*R. solani*	AG-1, IB
153. Beefwood	Damping-off	*R. solani*	
154. Poplar	Damping-off	*R. solani*	
155. Birch	Damping-off	*R. solani*	
156. Alder	Black root rot	*R. bataticola*	
157. Alder	Damping-off	*R. solani*	
158. Oak	Damping-off	*R. solani*	
159. Zelkova tree	Damping-off	*R. solani*	
160. Hortensia	Leaf rot	*R. solani*	
161. Indigobush	Damping-off	*R. solani*	
162. Azalea	Damping-off	*R. solani*	
163. Azalea	Web-blight	*R. solani*	AG-1, IB
164. Paulownia	Damping-off	*R. solani*	
165. Moluccan sau	Damping-off	*R. solani*	
166. Ipil-ipil	Damping-off	*R. solani*	
167. Mahogany	Damping-off	*R. solani*	
168. Kaatoan bangkal	Damping-off	*R. solani*	

II. Grouping *Rhizoctonia solani* Kühn with nonspecific esterase zymogram

Since Kühn's first report[67] in 1858, *R. solani* has been well documented as a causal agent of various plant diseases. In 1967, Parmeter *et al.*[99] proposed *Thanatephorus cucumeris* as the name of the perfect stage of this fungus. Furthermore, they reported that there are 3 types known as "praticola", "sasaki" and "solani" that are difficult to distinguish as different species by the morphology of the perfect stage[100]. On the other hand, it has been well known that *R. solani* involves morphologically, physiologically and pathologically different groups. There have been many attempts to classify them as strains, formae speciales, varieties or species. The usefulness of electrophoretic comparison of proteins and zymograms for the classification of microorganisms has been stressed[9,10,19,26-28,33,35,36,38-42,44-46,61-63,68,70,75-78,80,82,101,102,107,108,110-112,133]. In this chapter, the application of this technique is described to clarify relationships among the groups of *R. solani*.

Materials and Methods

Isolates. Forty eight isolates of *R. solani* selected from various amastomosis groups were used. All of them had been classified as to their anastomosis groups by Ogoshi's designation[94]. The details of these isolates are given in Table 2.

Culturing. Each isolate was cultured on a shaker in 100 ml liquid synthetic medium at 26℃ for 9 days.

Enzymes. Two volumes of cold acetone was added to chilled culture filtrates (100 ml). The precipitates were collected by centrifugation at 3,200×g for 10 min. The supernatant was discarded and remained acetone was eliminated *in vacuo*. The pellets were stored in deep freezer until needed. For electrophoresis the precipitates were dissolved in 1 ml of 1/60 M phosphate buffer (pH 7.4).

Thin layer electrophoresis. Cyanogum 41 (Sigma Co.) was used as a supporting medium with DMAPN and ammonium persulfate as a catalyst. A 4% gel was used with a discontinuous buffer system [gel buffer: 0.038 M Tris(hydroxy-methyl)aminomethame - 0.0026 M citric acid (pH 8.8), electrode buffer : 0.3 M boric acid - 0.05 M NaOH (pH 8.2)]. Ten ml of each sample was poured into the gel slots. Electrophoresis was carried out at ca. 5℃ for about 100 min at constant 130 V.

Detection. Nonspecific esterase zymograms were detected on the gel with 0.1% Fast Violet B salt and 0.05% 1-or 2-naphthylacetate in 1/60 M phosphate buffer (pH 6.8). The positions of bands were expressed in terms of the ratio of the respective distance to the distance of the front (Ef).

Table 2. *Rhizoctonia solani* isolates used in this study

Isolate	Host	Part	Anastomosis Group	Locality	Date	Note
13	Rice	Sheath	AG-1[a)]	Shizuoka	1974	IA[b)]
31	Rice	Sheath	AG-1	Shizuoka	1974	IA
34	Rice	Sheath	AG-1	Ishikawa	1974	IA
61	Rice	Sheath	AG-1	Iwate	1974	IA
66	Rice	Sheath	AG-1	Tochigi	1974	IA
71	Rice	Sheath	AG-1	Yamagata	1974	IA
C-14	Hamasuge	Leaf	AG-1	Fukuoka	1965	IA
C-124	Rice	Sheath	AG-1	Fukuoka	1974	IA
C-324	Sugar cane	Sheath	AG-1	Kagoshima	1975	IA
C-325	Rice	Sheath	AG-1	Fukuoka	1975	IA
C-326	Rice	Sheath	AG-1	Fukuoka	1975	IA
C-10-3-1	Rice	Sheath	AG-1	Hiroshima		IA
R-1-2-1	Wattle		AG-1	Tokyo	1950	IB
RI-86	Sugar beet	Sclerotia	AG-1	Shizuoka	1960	IB
№21	Orchard grass	Leaf	AG-1	Tochigi	1972	IB
CF-93-18	Soil		AG-1	Hokkaido	1962	IB
HK-616-23	Soil		AG-1	Hokkaido	1962	IB
SH-3	Soil		AG-2-1	Hokkaido	1962	II
SH-5	Soil		AG-2-1	Hokkaido	1962	II
F-15	Flax	Seedling	AG-2-1	Hokkaido	1960	II
TG-1	Tulip	Leaf	AG-2-1			II
C-112	Mat rush	Stem	AG-2-2	Kumamoto	1973	IIIB
C-319	Mat rush	Stem	AG-2-2	Fukuoka	1975	IIIB
C-321	Mat rush	Stem	AG-2-2	Fukuoka	1975	IIIB
C-323	Mat rush	Stem	AG-2-2	Fukuoka	1975	IIIB
C-328c)	Rice	Sheath	AG-2-2	Fukuoka	1975	IIIB
C-354	Rice	Sheath	AG-2-2	Fukuoka	1975	IIIB
R-1-2-6	Sugar beet	Petiole	AG-2-2	Ibaraki	1960	IIIB
BV-44	Sugar beet	Petiole	AG-2-2	Okayama	1959	
C-4	Sugar beet	Petiole	AG-2-2	Ibaraki	1960	IV
BV-28	Sugar beet	Root	AG-2-2	Hokkaido	1964	IV
C-34	Sugar beet	Root	AG-2-2	Fukuoka	1959	IV
C-3	Sugar beet	Leaf	AG-2-2	Aomori	1959	IV
BV-34	Sugar beet	Leaf	AG-2-2	Hokkaido	1956	IV
St-2	Potato	Tuber	AG-3	Hokkaido	1953	IV
St-7	Potato	Bud	AG-3	Hokkaido	1955	IV
St-9	Potato	Sclerotia	AG-3	Tokyo	1946	IV
GM-5	Soy bean	Root	AG-4	Saitama	1970	IIIA
GM-8	Soy bean	Root	AG-4	Nara	1970	IIIA
HI-822-29	Soil		AG-4	Hokkaido	1962	IIIA
SN-1	Soil		AG-4	Nagano	1969	IIIA
Lu-5	Flax	Stem	AG-4	Hokkaido	1967	IIIA
ST-8	Potato		AG-5	Hokkaido	1951	
P-3	Potato		AG-5	Hokkaido	1951	
SH-4	Soil		AG-5	Hokkaido	1962	
SH-25	Soil		AG-5	Hokkaido	1962	
SH-26	Soil		AG-5	Hokkaido	1962	
SH-29	Soil		AG-5	Hokkaido	1962	

All isolates are the stock cultures of NIAS(Tsukuba). Some of them originated from Hokkaido Univ., Ibaraki Pref. Agric Exp. Sta., Fukuoka Pref. Agti. Exp. Sta., Kyushu Natl. Agric. Exp. Sta., Kumiai Chem. Co..

a) Anastomosis groups (Parmeter et al. 1969)[139,150]

b) Cultural types (Watanabe and Matsuda 1966)[139,195]

c) This isolate came from the quasi-sheath blight of rice.-:Unknown.

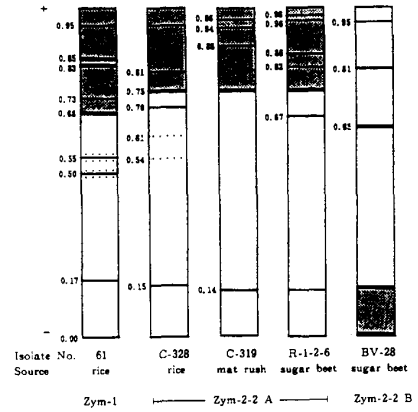


Fig. 1. Diagramatic representation of esterase Zymograms. Isolate No. 61 came from sheath blight and No. C-324 from quasi-sheath blighc of rice.

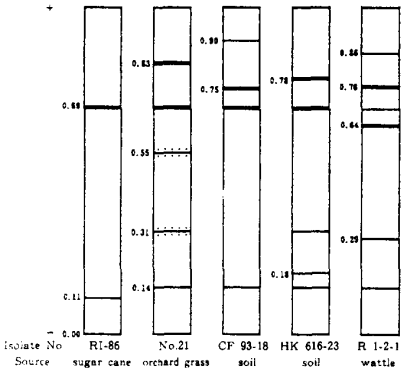


Fig. 2. Site of esterase activity for five isolates of anastomosis group AG-1 isolated from various plants other than rice and its rclatives. Though common isozymes around Ef 0.15 and 0.69 were detected. The patterns as a whole appear to be unrelated.

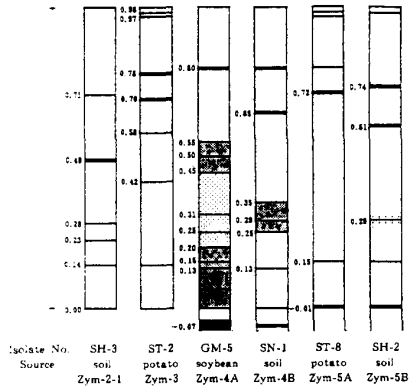


Fig. 3. Zymograms of the isolates of various anastomosis groups. Some isozymes moved to the cathode in Zym-4A, 4B, 5A and 5B.

Results

The zymograms of *R. solani* isolates obtained from various diseased plants and soil can be classified into several groups.

1). *Zym-1* : Isolates from the sheath blight of rice gave almost identical zymograms designated as *Zym-1*. The first lane of Fig. 1 illustrates a typical pattern (Plate 2, lanes A and B). This zymogram had one or two distinct bands around Ef 0.85. The dotted area between Ef 0.7 to 1.0 was lightly stained and was characteristic of the group. Some isolates from the leaf rot of Hamasuge (*Cyperus rotundus*) and the banded sclerotial disease of sugar cane also showed the *Zym-1* pattern. All of these isolates belonged to anastomosis group AG-1. However, AG-1 group isolates from various plants other than rice and its relatives (the web-blight type) showed the zymogrames which are quite different from *Zym-1* and varied according to their host plants (Fig. 2 and Plate 2, lane C).

2). *Zym-2-1* : The isolates in AG-2-1 showed the pattern *Zym-2-1* (Fig. 3). This was strikingly different from other zymograms. Most bands were detected in the middle and lower portions of the zymogram.

3). *Zym-2-2A* : This zymogram (Fig. 1) was given by all of the isolates from stem rot of mat rash (Igusa), the quasi-sheath blight of rice (Kasshokumongare-byo), some isolates from the petiole rot of sugar beet and one isolate from the sheath blight of rice. All of these isolates belonged to the anastomosis group AG-2-2. The zymogram of the isolates C-328 (the quasi-sheath blight), C-319 (the stem rot of mut rush) and R-1-2-6 (the petiole rot of sugar beet) were illustrated in Fig. 1. This zymogram resembled *Zym-1*. However, the bands around Ef 0.85 and Ef 0.5～0.55 were very faint or missing in Zym-2-2A.

4). *Zym-2-2B* : Some isolates from the root and leaf rot of sugar beet which also belonged to AG-2-2 showed the pattern *Zym-2-2B* (Fig. 1).

5). *Zym-3* : Three isolates from potato which belonged to AG-3 showed *Zym-3*. The zymograms had characteristic bands at Ef 0.70, 0.78 and 0.98 (Fig. 3 and Plate 2, lane D).

6). *Zym-4A, 4B* : AG-4 isolates exhibited patterns designated *Zym-4A* or *4B* (Fig. 3). The common bands of *Zym-4A* and *4B* were detected at Ef 0.80 and −0.07 (cathode side). However, isolates from soybean gave different patterns in the middle and lower portions of the zymogram.

7). *Zym-5A, 5B* : Two types of zymograms were given by isolates of AG-5 (Fig. 3). Common bands were detected at Ef 0.15 and −0.01 (cathode side). The isolates which gave *Zym-5A* resembling *Zym-3* (potato type) were also from potato.

Overall results were reproducible, except for some minor variation, when the experiment was repeated after one year.

Discussion

Sound classification of microorganisms should clarify the evolutionary relationships among them. There are several ways to approach this objective. The usefulness of biochemical techniques for describing and identifying microorganism has been emphasized. Although the most direct technique may be analysis of DNA, but it is not always

available at present without deciding target genes. Protein and zymogram analyses are relatively facile and reliable. Matsuyama *et al.*[75-78] have explored the use of this technique with *Pyricularia, Fusarium* and *Colletotrichum* spp. with thin layer polyacrylamide gel electrophoresis.

R. solani has many strains which are quite different from each other in physiological, ecological and pathological characteristics. The author tried to distinguish the strains of this fungus by comparison of zymograms.

The zymograms of isolates from the sheath blight of rice and its relatives (the sasaki type[130], IA[130], AG-1[94] (Fig. 1) and from the web-blight of black locust and various plants (the web-blight type, IB, AG-1) were quite different. Although these two groups belonged to the same anastomosis group (AG-1), their physiological characters were obviously different. Isolates from the sheath blight of mat rush (Igusa)[96] and the quasi-sheath blight of rice (Kasshokumongare-byo) (the rush type, IIIB, AG-2-2) showed *Zym-2-2A*. Interestingly, some isolates from sugar beet also showed this zymogram. Watanabe and Matsuda[130] reported that many isolates from sugar beet belonged to the rush type. Since sugar beet had sometimes been planted as a second crop after mat rush, part of the rush type isolates from sugar beet could perhaps have come from the sheath blight of mat rush.

The finding that isolates of AG-2-2 gave two kinds of zymograms *Zym-2-2A* and *2-2B,* one of which was so to speak the rush type, agrees with the observation that AG-2-2 includes two ecological types namely the rush and root rot[130]. All of the isolates which belonged to AG-2-1 showed *Zym-2-1.* Though both AG-2-1 and 2-2 belonged to Parmeter's AG-2 group, the zymograms indicate that these two groups are physiologically different from each other. The isolates of AG-3 came mostly from potato and showed a unique zymogram, *Zym-3.* It is interesting that some isolates of AG-5 from potato showed a zymogram similar to *Zym-3.*

These results show that *R. solani* includes strains of various zymogram types and that although these types agree roughly with anastomosis groups[92,94,100], they sometimes agree more closely with ecological types[130]. The morphological characteristics of the perfect stage of fungi are important taxonomic criteria. However, large parts of isolates of *R. solani* are not available to form the perfect stage. Furthermore, the morphological characteristics of imperfect stage are scarce. Therefore, physiological characteristics, especially the comparison of zymograms was conducted. The differences among zymograms of *R. solani* strongly indicated that the fungus involved a lot of strains and should be divided at species level.

For more consistent and reproducible patterns, harvesting time of culture filtrate would be needed since the zymogram patterns were variable at early culture stages.

Ⅲ. Morphology of sclerotia of *Rhizoctonia solani*

Sclerotia produced by certain soil-borne fungi vary in structure, size and pigmentation among genera or species. Townsend and Willetts[119] studied the development of sclerotia of 6 species of fungi and concluded that most of the species could be fitted into one of the 3 groups (the loose type, the terminal type and the lateral type) with different

modes of initial formation.

R. solani forms initials of the loose type which develop by increased branching and septation of the ordinary hyphae so that the mature sclerotia are loosely constructed of brown, barrel-shaped cells with dense contents[29,119].

In this chapter, the morphogenesis from hyphae to sclerotia of the fungus was observed and compared with that of *Sclerotinia sclerotiorum*, which produces sclerotia of the terminal type.

Materials and Methods

Fungi used. An isolate, C-324, of *R. solani* (NIAS) and, for comparisons, two isolates of *S. sclerotiorum* isolated from gayfeather (*Liatris spocata* Willd) and kidney bean (*Phaseolus vulgaris* L.) were used in this chapter.

Observation of each stage of sclerotium formation. Hyphae, sclerotial initials, white sclerotia, pigmenting sclerotia, and mature sclerotia were observed with a scanning electron microscope (JEM-25S). The edge of mycelial colony precultured on potato dextrose agar (PDA:200 g potato decoction, 20 g glucose, 20 g agar, 1,000 ml water) for 2～3 days cut with a cork borer. The seamless cellulose tubing (Visking Co.) for dialysis was previously rinsed to remove impurity, cut to 8.5 cm in diameter, were sheeted on a PDA medium, and mycelial discs (5 mm in diam.) were centered on the sheet and cultured at 25℃. The discs with cultures were cut into 5×5 mm tips at each of the stage of sclerotium formation. The tips were fixed with glutalaldehyde and osmium, dehydrated with 50～100% ethanol series, substituted for amyl acetate, and dried at the critical point with liquid CO_2. The samples were coated with 100 Å gold-palladium using the vacuum method.

Results

Sclerotia on the medium

Sclerotia of *R. solani* formed on PDA were 2～3 mm in diameter and produced about 80 to 100 in number in zones. The times to reach each stage were 54, 63 and 73 hr for initials, white sclerotia, and immature sclerotia, respectively. Also, about 10 of sclerotia of 4～6 mm in diameter formed by *S. sclerotiorum* was examined. The time to reach the initial was 81 hr, the white sclerotial stage 99 hr and the immature sclerotial stage 107 hr. The times were much slower than those of *R. solani*. As for the sectional structures, the white sclerotia of *S. sclerotiorum* was found to contain much water inside, but the medulla and rind being indistinguishable at this stage. Although, the mature sclerotia of *R. solani* wholly changed to dark brown, those of *S. sclerotiorum* had differentiated the medulla and rind (Plate 3-1, 3-2).

Observation with the scanning electron microscope

a. Hyphae : At the 48 hr after inoculation, at which stage the mycelia do not reach the edge of Petri dish, only hyphae had developed but initials had not yet been formed. The hyphae of *R. solani* developed linearly and branched at regular intervals at right angles, whereas those of *S. sclerotiorum* were forming curves (Plate 3-3, 3-4).

b. Initials : From 50 hr after inoculation, the leading hyphae began to branch

frequently and interweave, networked and developed into sclerotial initials. As for *S. sclerotiorum*, hyphal branching was not frequent, but a number of hyphae would gathered together to form initials (Plate 3-5, 3-6).

c. White sclerotia : White sclerotia are masses where initials developed, enlarged and granuled. The white sclerotia of *R. solani* are subspherical, in general, and covered with dense hyphae, the sections showing a honeycomb structure both inside and outside. On the surface structure of *S. sclerotiorum* the white sclerotia were covered with hyphae of varying densities so that there was no distinction between the medulla and rind (Plate 3-7, 3-8, 3-9, 3-10).

d. Immature sclerotia (just pigmenting sclerotia) : Normal hyphae were still observed on the immature sclerotia of *R. solani* and there was no difference in the sections with white sclerotia except for the pigmentation (Plate 3-11, 3-13). Conversely, the sclerotia of *S. sclerotiorum* were distinctly differentiated from medulla and rind (Plate 3-12). The outer layer consisted of two to several cells with numerous vacuoles and an inner layer of diffused cells with spaces between cells being narrow (Plate 3-14).

e. Mature sclerotia : Sclerotia of *R. solani* were colored to dark brown, hyphae still remained on the sclerotium surfaces and the section showed honeycomb structure (Plate 3-18). On the other hand, there were no normal hyphae on sclerotia of *S. sclerotiorum*. The surface structure showed armor-like characteristics fused hyphae forming a mass (Plate 3-15, 3-16, 3-17).

Discussion

Garrett[37] reported that sclerotia produced by air-borne fungal pathogens vary in size, attaining maximal diameter of 2 cm, and usually produce fruiting bodies such as apothecia or perithecia, whereas those of root-infecting fungi are smaller (1～2 mm diameter), more uniform in size and more regular in shape. It is well known that *Sclerotinia* species produce apothecia from sclerotia, though *Rhizoctonia* sclerotia elongate hyphae directly. Sclerotia by *R. solani* were smaller in size than those by *S. sclerotiorum*.

Since grouping the 6 sclerotial forming fungi into 3 types by Townsend and Willetts [119] in 1954, it has been reported that other sclerotium forming fungi are in one of those groups. Although, *R. solani* had been considered to be the only fungi of the loose type, Blakeman and Hornby[12] showed that *Mycosphaerella ligulicola* also belongs to this type. Fukano[36] reported that there was no distinction between the inner and outer layer in sclerotia of *R. solani* which was isolated from the rice plant. Contrary to this, Hashiba and Mogi[49] proposed that natural sclerotia which was forming on the surface of rice plant had a differentiated inner layer, consisting of living cells and an outer layer of empty cells. Such characteristics could not be recognized in the sclerotia grown on synthetic media *in vitro*. Furthermore, Hashiba *et al.*[51] suggested that this characteristic is related to the buoyancy of the sclerotia, an important factor in the infection of the rice plant by the fungi. On the other hand, the author has ascertained that all the small pieces of inner and outer layers of natural and cultured sclerotia of *R. solani*, *S. sclerotiorum* and *Sclerotium rolfsii* were able to germinate, and the whole body of sclerotia was constructed by living fungus cells. It could be considered, however, that in

sclerotia found in nature, e.g., in the soil, the outer parts in direct contact with the environment would die after being parasitized or lyzed by antagonistic microbes. On the other hand, *Sclerotinia* species differ in the types: *S. fructicola* (Willetts[134], 1968), *S. sclerotiorum* (Townsend and Willetts[119], 1954) and *S. trifoliorum* (Willetts and Wong[135], 1971) belong to the terminal type and *S. gladioli* (Townsend and Wong[119], 1954) to lateral type. Comparing with the sclerotia of *S. sclerotiorum*, those of *R. solani* are smaller and more numerous in vacuole cells. The sclerotia of *R. solani* are initiated with branching and network formation of leading hyphae, and are wholly stained. The inner layer of the sclerotia by *S. sclerotiorum* consisted of medulla whose hyphae diffused and the outer layer consisted of vacuole cells. This fact may indicate that sclerotia of *R. solani* have a lower degree of morphological differentiation than those of *S. sclerotiorum*.

IV. Effects of several physical factors on sclerotium longevity of *Rhizoctonia solani*

The sclerotia can survive longer periods in various environments than other fungal organs such as the hyphae and spores, because of their unique structure and pigmentation. Coley-Smith and Cooke[29] roported that the sclerotia of *S. sclerotiorum* survived for 2 years in the field conditions, and those of *R. solani* persisted for 6 years under dry conditions.

Although physical factors (e.g. temperature, humidity, oxygen, carbon dioxide and pH), chemical factors (e.g. soil components and pesticides), and biological factors (e.g. soil microbes) are listed as items affecting the longevity of sclerotia, their effects are complicated influencing one another under natural conditions[29,43].

In this chapter, the effects of the most fundamental factors, temperature, water, ultra-violet light and pH, on the longevity of *R. solani* were tested and the results were compared with those of *S. sclerotiorum*, which differs in the manner of the sclerotium formation and subsequent development.

Materials and Methods

Fungi used. An isolate, C-324, of *R. solani* and the 2 isolates of *S. sclerotiorum* described in Chapter III were used.

Culturing at different temperatures. The edges of mycelial colonies of fungi precultured on PDA were punched out with a cork borer. The discs were placed on PDA and cultured at 25℃ under dark conditions. When the culture reached the hypha, initial, and white sclerotium stages, the colonies were punched out with a cork borer, 6 mm in diameter. Immature and mature sclerotia were taken and put on wet discs of filter paper. These samples were placed in an incubator, set at 40～60℃, for 65 min and then transferred on PDA to determine whether they were alive or dead. After a week, the samples from which no hyphae had germinated were regarded as dead.

Test of the effects of water and temperature. Each fungus cultured on PDA at 25℃ and its mature sclerotia formed were collected 2 weeks after inoculation. The sclerotia were stored at 25℃ and 35℃ in sterilized water. Then the portions of the sclerotia were

put on PDA and incubated at 25℃. After a 5 days culture, the sclerotia that germinated were defined as being alive. When all sclerotia died within 7 days, the treatment conditions were subdivided.

Irradiation of ultraviolet light. The cultures were kept in a Petri dish each sheeted with a wet filter paper disc. Irradiation was performed at 0.1 mw/cm^2 for 5 min to 10 days by a 15 W ultraviolet lamp (Toshiba GL-15). To prevent the sclerotia from drying (the temperature became 30~35℃), distilled water was added to the filter paper.

Culturing at different pHs. Ten mature sclerotia produced by the culture on PDA for 2 weeks were each soaked for 1~2 weeks in 20 ml of the following sterile buffer. The buffers used were 0.1 M sodium acetate buffer at pH 4~5, 0.1 M sodium phosphate buffer at pH 6~7, and 0.1 M Tris-HCl buffer at pH 8~9.

Results

Changes of thermal resistance during the sclerotium formation

The thermal resistance of *R. solani* was increased with the development from hyphae to sclerotia (Fig. 4-1). The hyphae survived completely at 45℃, but became extinct at 46~47℃. The initials and white sclerotia died at 48℃. However, a few mature sclerotia were viable even at 51℃. Although, *S. sclerotiorum* also showed an increase of thermal resistance during development, it was more liabile than *R. solani* in each stage. Almost all of the hyphae of this fungus died at 42℃ (Fig. 4-2). There was no difference in the property between the 2 isolates.

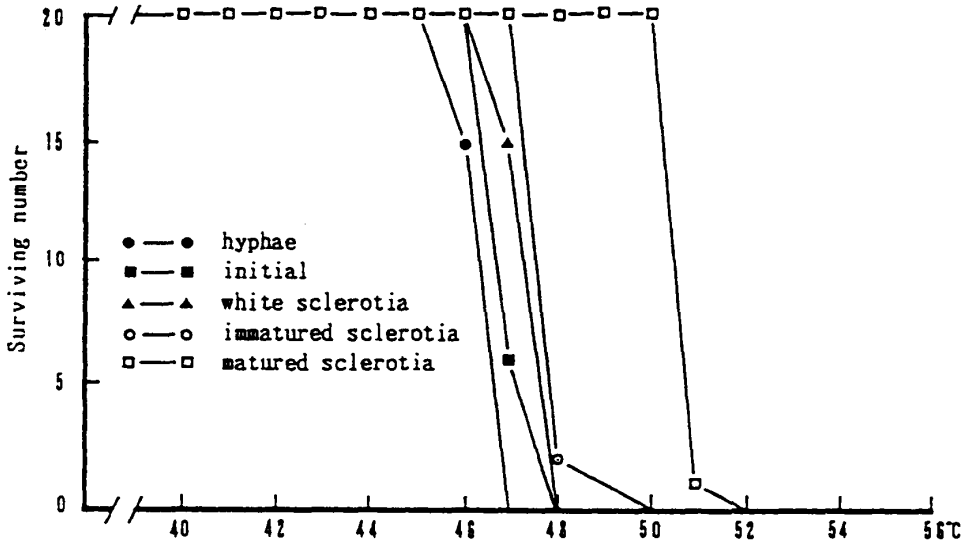


Fig. 4-1. The effect of temperature on survival of several sclerotial stages of *Rhizoctonia solani* (C-324).

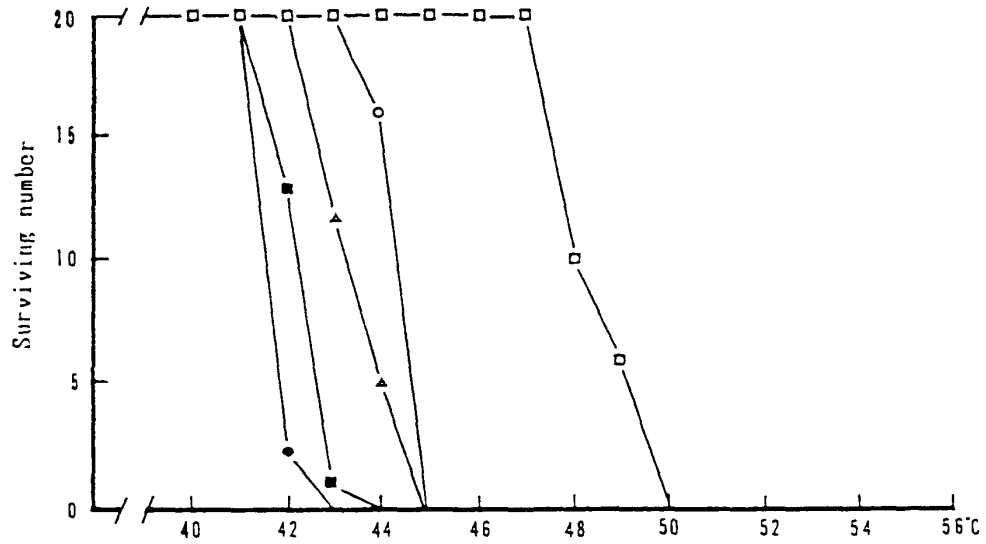


Fig. 4-2. The effect of temperature on survival of several sclerotial stages of *Sclerotinia sclerotiorum*.

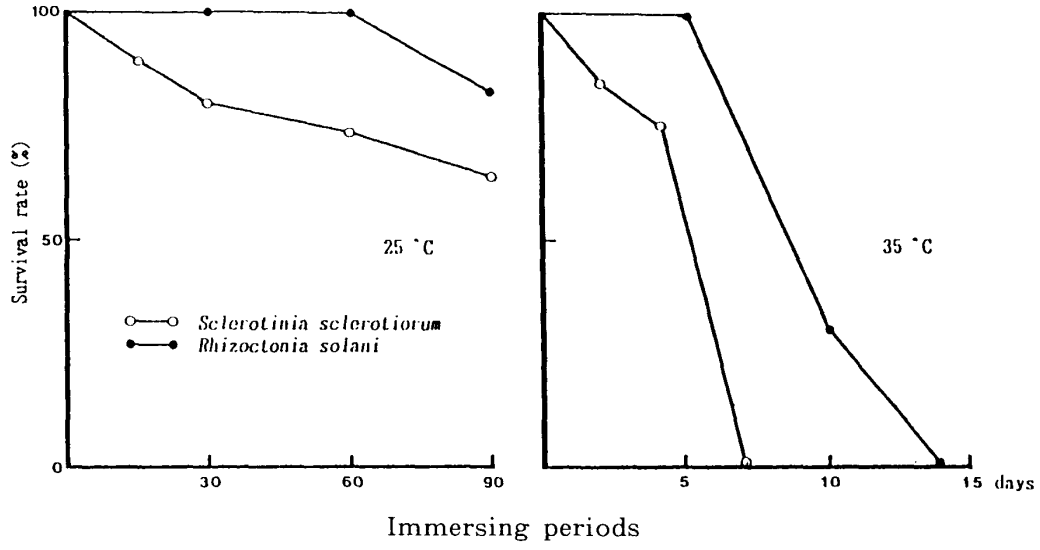


Fig. 5. Time-courses of survival rate of sclerotia in pure water.

Table 3-1. The effectc of ultraviolet irradiation on survival of several sclerotial stages of *R. solani* (C-324)

Time of irradiation[a]	Hyp.[b]	Init.	Wh. Scl.	Imm. Scl.	Mat. Scl.
5 min	+[c]	+	+	+	+
15 min	±	+	+	+	+
30 min	−	+	+	+	+
1 hr	−	+	+	+	+
6 hr	−	+	+	+	+
24 hr	−	+	+	+	+
2 days	−	+	+	+	+
3 days	−	±	+	+	+
5 days	−	−	+	+	+
10 days	−	−	+	+	+

a) Ultraviolet light was irradiated at 0.1 mw/cm^2.
b) Hyp.:Hyphae, Init.: Initial, wh. Scl.: white sclerotia, Imm. Scl.: Immatured sclerotia, Mat. Scl.: Matured sclerotia.
c) +: Germinated, −: Not germinated.

Table3-2. The effects of ultraviolet irradiation on survival of several sclerotial stages of *S. sclerotiorum*

Time of irradiation	Hyp.	Init.	Wh. Scl.	Imm. Scl.	Mat. Scl.
5 min	+	+	+	+	+
15 min	±	+	+	+	+
30 min	−	+	+	+	+
1 hr	−	+	+	+	+
6 hr	−	±	+	+	+
24 hr	−	±	+	+	+
2 days	−	−	+	+	+
3 days	−	−	+	+	+
10 days	−	−	+	+	+

Table 4-1. The effectc of pH on sclerotial survival of *R. solani* (C-324)

pH	1	2	3	4	5 days
4	0[a)]	0	0	0	0
5	0	0	0	0	0
6	80	100	100	100	100
7	20	100	100	100	100
8	20	60	60	100	100
9	30	100	100	100	100

[a)] Rate of germination (%)
Sclerotia were sudmerged for a week.

Table4-2. The effects of pH on sclerotial survival of *S. sclerotiorum*

pH	1	2	3	4	5 days
4	0	0	0	0	0
5	0	0	0	0	0
6	0	30	100	100	100
7	0	50	70	100	100
8	0	20	90	100	100
9	0	40	90	100	100

Effects of water and temperature

When the sclerotia of *R. solani* and *S. sclerotiorum* were kept at 25℃, 90% and 75% respectively were alive even after 90 days (Fig. 5). When kept at 35℃, however, the sclerotia of both fungi were completely inactivated within 7~14 days. Particularly, the survival rate for *S. sclerotiorum* was low, declining remarkably after 48 hr and reaching zero after 7 days.

Changes of ultraviolet resistance during the sclerotium formation

The hyphae of *R. solani* had undergone a sterilizing process of ultraviolet irradiation for more than half an hour. One half of the number of sclerotial initials persisted up to 3 days of irradiation, moreover, white sclerotia, immature sclerotia, and mature

sclerotia survived under 10 days of irradiation (Table 3-1). The initials of *S. sclerotiorum* survived 24 hr of irradiation, but the hyphae, only 30 min. After the white sclerotial stage, there was no effect on the viability even up to 10 days of irradiation (Table 3-2).

Effects of pH on the survival of sclerotia

The mature sclerotia of the 2 fungi, *R. solani* and *S. sclerotiorum*, perished at pH 4 and 5, but survived at pH 6 to 9 (Table 4-1, 4-2). At pH 7 to 9, germination of sclerotia was partially inhibited. This inhibition was considered to be caused by contamination of bacteria, but sterilization may occur on the surface of the sclerotia, bringing in the apparent recovery.

Discussion

The sclerotia produced by *R. solani* consists of a roughly interwoven hyphal mass and an uniformly colored, dark brown, sectional structure with an undifferentiated rind (outer layer) and medulla (inner layer). Characteristic of the white sclerotium of *S. sclerotiorum* is a colored rind of several layers of vacant cells, which appear to protect the medulla, and its sclerotium has a higher degree of morphological differentiation[87]. The present study showed that the sclerotium of *S. sclerotiorum* was larger than that of *R. solani*. The resistance to ultraviolet and pH shows a similar tendency in 2 fungi. Though, the thermal resistance was more prominent in *R. solani* than in *S. sclerotiorum*. Moreover, when the sclerotia of both fungi were stored in pure water, they survived for long periods at 25℃, but died rapidly at 35℃. Hashiba and Yamaguchi[50] reported that the germination rate of the natural sclerotia in *R. solani* decreased when they were preserved at a high temperature and high humidity, although the rate of sclerotium formation on the cultivation media was decreased at both low and high temperatures (particularly at the latter). The resistance to ultraviolet was increased with the advance of sclerotial morphogenesis in both fungi. This phenomenon may be explained by one or both of the following assumptions. (1) The sclerotia survived because their surface was sterilized by the ultraviolet irradiation, but their interior was not. This inference comes from the characteristic granulation; the sclerotial initials have a hyphal network that retains their shape but yet granulated and thus ultraviolet light may not affect strongly the initial components. (2) The hyphae consist of sclerotia that change physiologically to become more resistant to ultraviolet light. The increase in thermal resistance during the sclerotium differentiation may support the latter idea. On the other hand, the effects of pH were more significant in the acid region than in the alkaline. It may be assumed that the hydrogen ion concentration has a direct effect as does the chemical composition of the buffer used. The concentration of hydrogen ions strongly affects the microorganisms in natural soil, modifying the microflora probably resulting in a difference in microorganisms antagonistic to pathogenic sclerotia, thus indirectly influencing viability of the latter.

V. Enzymatic study on the sclerotium formation in *Rhizoctonia solani*

R. solani belongs to a translocating fungus, in which nutrients required for the sclerotium formation are carried through the hyphae from places that are apart[121]. In the formation of the fungus in a Petri dish, the initials are formed by hyphal branching and aggregation when the nutrient hyphae have grown to the edge of the dish. The sclerotial initials differentiated to white sclerotia with granulation. The white sclerotia further developed to mature sclerotia with melanization and with still enlargement[89]. There are some biochemical approaches to elucidate the process of fungal sclerotium formation. Wong and Willetts[136] and Chet *et al.*[24] discussed this process using electrophoresis in *S. sclerotiorum* and *Sclerotium rolfsii*, respectively.

In order to clarify the metabolic aspects during the sclerotium morphogenesis in *R. solani*, the author examined in this chapter the changes in activity of the enzymes, malate dehydrogenase (EC 1.1.1.37), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), phosphogluconate dehydrogenase (EC 1.1.1.44), acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1).

Materials and Methods

Fungus used. An isolate (C-324) of *R. solani* was used.

Culturing and extraction of crude enzyme solutions. The isolate was cultured on 15 ml of PDA in a Petri dish at 25℃ under dark conditions. During sclerotium formation, hyphae (48 hr after inoculation), initials, white sclerotia, pigmenting sclerotia and mature sclerotia (2 weeks after inoculation) were collected. At and after the white sclerotium stage, the hyphae and sclerotia were separately collected. Hyphae or sclerotia were homogenized in 7 volumes of 0.05 M sodium phosphate buffer (pH 6.5) and the homogenate was centrifuged at 3,000 g for 20 min. The supernatants were dialysed against 0.02 M sodium phosphate buffer (pH 6.5) for 12 hr and were concentrated to a 1/4 volume with a Minicon B-15 doncentrator (Amicon Ltd.). The samples were used for the measurements of enzyme activities.

Measurements of the activities of enzymes. Aliquots (50 μl) of the samples were each mixed with the substrate solution as specified later and incubated for 50 min at 37℃. Dehydrogenase activities were measured by the increase of $NADH_2$ or $NADPH_2$ at 340 nm, and phosphatase activities by the increase of p-nitrophenol at 400 nm. A Shimadzu UV 180 spectrophotometer was used. The results were expressed in terms of the activity per ml sample (relative activity).

The substrate solutions for the measurements of the enzyme activities were prepared as follows.

a) 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).

b) Glucose-6-phosphate dehydrogenase : 7 mg NADP and glucose-6-phosphate were dissolved in 0.05 M sodium phosphate buffer (pH 6.5).

c) Phosphogluconate dehydrogenase : 7.5 mg NADP and 6-phospho-D-gluconate were dissolved in 0.05 M sodium phosphate buffer (pH 6.5).

d) Acid phosphatase : 1 mg p-nitrophenylphosphate was dissolved in 5 ml of 0.1 M

sodium phosphate buffer (pH 5.5).

e) Alkaline phosphatase : 1 mg p-nitrophenylphosphate was dissolved in 5 ml of 0.1 M sodium borate buffer (pH 9.0).

Results

Developmental changes in relative activity of the 5 enzymes were investigated and the results are listed in Table 5.

Malate dehydrogenase

The activity of malate dehydrogenase from the hyphal to mature sclerotium stage was low in the hyphal parts, but high in the sclerotial parts at the white and mature sclerotial stages.

Table 5. The enzyme activities at the stages of sclerotial morphogenesis in *R. solani*

Enzymes	Stage of sclerotial morphogenesis							
	Hypha	Initial	White sclerotium		Pigmenting sclerotium		Matured sclerotium	
			Hypha	Sclerotium	Hypha	Sclerotium	Hypha	Sclerotium
Malate dehydrogenase	11.5[a)]	9.4	5.3	42.1	4.3	47.4	3.1	40.2
Glucose-6-phosphate dehydrogenase	50.5	2.9	2.6	3.9	2.4	6.1	20.4	6.3
Phosphogluconate dehydrogenase	56.3	20.3	3.9	5.4	3.6	7.2	25.1	40.6
Acid phosphatase	2.7	3.2	31.6	24.5	23.2	2.3	42.1	2.7
Alkaline phosphatase	86.7	70.6	67.5	27.4	28.6	18.5	4.6	12.2

[a)] Relative activity.

Clucose-6-phosphate dehydrogenase

This enzyme exhibited a high activity at the hyphal stage. The activity level was decreased at the initial stage and was recovered to some extent in the hyphae of the mature sclerotia (at 2 weeks after inoculation). The activity in the sclerotia was low as a whole, although increased slightly with the maturation of sclerotia.

Phosphogluconate dehydrogenase

The activity of phosphogluconate dehydrogenase was highest at the early stage, then decreased at the white sclerotium and pigmenting stages. The activity augmented at the maturation stage, particularly in the sclerotial parts. The high activity of the sclerotia persisted for 4 weeks.

Acid phosphatase

A very low activity of this enzyme was observed through the hyphal and initial stages, although the activity increased at the white sclerotial stage. The level remained high in the hyphal parts at the pigmenting and mature sclerotial stages, but again low in the sclerotial parts at the same stages.

Alkaline phosphatase

The pattern of this activity was the opposite of acid phosphatase. The highest activity was observed in the hyphae at the early non-sclerotial stage. Thereafter, the activity in the hyphal parts decreased. Moreover, the activity in the sclerotial parts was lower than in the hyphal parts until the pigmenting stage. After decreasing its activity at the mature stage it remained stable afterwards.

Discussion

At the early stage of sclerotium formation in *S. sclerotiorum*, the hyphae are reported to get energy both from the glycolysis and krebs cycle[136]. As the oxygen level is reduced with the compacting of sclerotium, the pentose phosphate pathway which requires less oxygen than the krebs cycle is of a greater importance in the metabolism of the medullary hyphae. In *S. sclerotiorum*, mitochondria of the sclerotium cells have fewer cristae than those of the vegetative hyphal cells and thus the aerobic respiration in the sclerotial cell may be low[4]. The results obtained in this study are contrary to the above situation. In *R. solani*, the activity of malate dehydrogenase, as an index of the krebs cycle activity, was at a higher level in sclerotia than in hyphae. This may indicate that the sclerotium produced by *R. solani* is not a resting organ, but one in which aerobic metabolism takes place vigorously. Glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase, both are the key enzymes of pentose phosphate pathway, were at their high activity levels in the hyphae at the early non-sclerotial stage. This fact shows that the reducing activity for biosynthetic process was necessitated in the young hyphae than in the aged hyphae and sclerotia. Some recovery of the once diminished activities in the hyphae at the mature sclerotial stage at 2 weeks after inoculation agrees with the development of new aerial hyphae. The activity of alkaline phosphatase was high at the early stages, while that of acid phosphatase was high at the later stages, in which the sclerotia are formed. In *S. rolfsii*, no change in activity of acid phosphatase isozymes has been detected during the sclerotium formation[24]. In *Aspergillus nidulans*, on the other hand, alkaline phosphatase was most active in the early phases of development and acid phosphatase in the later phases[32]. In addition, in *Neurospora*, alkaline phosphatase exhibited a strong activity in a short region behind the extreme tips, but that acid phosphatase at the tips was less active than the rest of the hyphae[139]. In the case of *R. solani*, it is suggested that the hyphae at the early stage take nutrients from the medium for the synthetic processes with the production of reducing power, and that after the initial stage some hyphal components produced by autolytic hydrolases (including phosphatases), as well as nutrients from the medium, were utilized as the substrates for the construction of sclerotia and newly developing hyphae.

VI. The longevity of sclerotium forming fungi in soil

Sclerotium forming pathogens are also capable of long term survival[29]. However, sclerotia in the natural soil are incapable of longer survival than those in sterilized soil. The factors affecting the sclerotial longevity could be described as physical, chemical

and biological ones[2,29-31,50,61]. Organisms that feed on, or parasitize, sclerotia include nematodes, earthworms, snails, centipedes, gall midge larvae, mites, bacteria, and fungi [8,57,91,131]. A number of microorganisms isolated from sclerotia can also destroy them under laboratory conditions. Above all, the fungus *Trichoderma* is best known as a sclerotium parasite which penetrates sclerotia or germinating hyphae[5,20,34,47,97]. However, the use of biological control with fungus has been declining inspite of its benefit giving neither soil pollution nor residual toxicity produced by the chemical fungicides. One reason is that if the *Trichoderma* antagonist is introduced into the soil, the population decreases shortly to its original level[65].

In this chapter, the effects of soil microorganisms on the viability of 4 types of sclerotia in 2 kinds of soils were examined. Furthermore, several factors affecting the distribution of the fungi *Trichoderma* were investigated. The soils used were kunigami Maaji and Jaagaru which are commonly distributed in Okinawa Prefecture (Table 6).

Table 6. The characteristics of the soil used in this chapter

Soil	Parent materials	Distribution	Characteristics	pH
Jaagaru	Mudstone of Shimajiri bed	Middle and southern districts of Okinawa Island	Heavy clay, Drainage failure	Alkali (7.5[a)])
Kunigami Maaji	Kunigami gravels Old alluvium sediments, pyllite, granite etc.	Middle and northern districts of Okinawa Island, Ishigaki and Iriomote Island	Drainage failure	Acidity (4.5)
Sea sand	Coral reef, foraminifer			Alkali (8.5)

[a)] The values tested in this study.

Materials and Methods

Fungi tested. The 4 typical sclerotium forming fungi *R. solani, S. sclerotiorum, Sclerotium delphinii* and *Sclerotium rolfsii*, which are different in the sclerotium forming manner and their morphological structures, were used.

Survival of sclerotia in soil suspensions. The test soil (50 g) was suspended in 10

volumes of sterilized water in an Erlenmeyer flask. One hundred of matured sclerotia, wrapped in nylon mesh, were submerged in the soil suspensions. After 7, 14, 30, 60 and 90 days, sclerotia were picked out, rinsed with sterilized water, dried and put on PDA. The viabilities of sclerotia were investigated by culturing at 25℃ for 5 days.

Survival of sclerotia in soil. The test soils were potted in porous pots, 8 cm in diameter and 7.5 cm in height, and sclerotia, wrapped in nylon mesh, were buried 3 cm below the soil surface. After 7, 14, 30, 60 and 90 days, sclerotia were picked out, rinsed with sterile water, dried and put on PDA. The viabilities of sclerotia were investigated by culturing at 25℃ for 5 days. Sclerotia buried in sterilzed sea sand were used as a control.

Antagonism of microorganisms in soil.

(1) *Microflora in test soil*: Soil microflorae in 2 test soils, Kunigami Maaji (weathered Kunigami gravel) and Jaagaru (marl), widely distributed in Okinawa Prefecture and differing in soil properties, were investigated using the soil dilution method. Kunigami Maaji was collected at a pineapple field in Nago City and Jaagaru, at a vegetable field in Tomigusuku Village. Samples were collected twice.

(2) *Antagonism of isolated fungi*: Antagonism of fungi isolated by the soil dilution method and the trap method was examined by confronting culture with 4 sclrotium forming fungi on PDA. Fungi *Trichoderma*, having strong antagonistic ability, were inoculated to the 4 fungi *in vitro*. And the antagonistic behavior was observed with a scanning electron microscope. *Aspergillus terreus* (RA-2) was cultured in a liquid medium and the effects of substances produced in the culture filtrates on 4 fungi were tested.

(3) *Inoculation of Trichoderma*: The culture of *Trichoderma lignorum* (RT-1) cultured on PDA for a week was thoroughly stirred in 10 ml of sterile water and this spore suspension was used to inoculate into the soil of the porous pots in which sclerotia were buried. The sclerotia were picked out 30 days after inoculation at 25℃, rinsed, dried and put on PDA to determine whether they were alive or dead.

(4) *Electron microscope observation of the infection sites: T. lignorum* (RT-1) and sclerotium forming fungi were cultured on PDA, which were covered with sterile nylon meshes (5×5 mm), by confronting each other, at 25℃ for 4 days. The nylon meshes with the developed hyphae were peeled off from culture, fixed with 3% glutalaldehyde for 12 hr, dehydrated with ethanol series (50~100%), and replaced with amyl acetate. The samples were coated with 100 Å platinum-palladium. A scanning electron microscope (JSM-25S) was used for the observation.

(5) *Effects of filtrates of Aspergillus terreus*: An isolate of *Aspergillus terreus* (RA-2) was shake-cultured in 200 ml of Czapek medium (30 g sucrose, 0.5 g $MgSO_4$, 2 g Na_2SO_4, 0.01 g $FeSO_4$, 0.1 g K_2HPO_4, 0.5 g KCl, 1,000 ml water) at 25℃ for 10 days. Paper disks were dipped in the fluid filtered through Miliporefilter (0.45 μm), 6 mm in diameter, and confronted with the sclerotium forming fungi on PDA.

Factors affecting on Trichoderma.

(1) *Effects of pH on the hyphal growth*: The isolates of *T. lignorum* (RT-1) was precultured on PDA at 25℃ for 2 days. The small discs of 5 mm in diameter were cut from the edge of the mycelial mat with a cork borer. Fifteen ml of each medium (PDA) in which pH was adjusted with 1 N phosphoric acid or 0.05 M phosphate buffer was

poured into a Petri dish and the isolate was inoculated. After culturing at 25℃ the diameter of mycelial colony was measured at every 12 hr.

(2) *Effects of pH on the sporulation*: Culturing was conducted for 14 days in 350 ml of PS liquid medium whose pH was adjusted with 1 N phosphoric acid or 0.05 M phosphate buffer for 14 days. At the end of culturing, pH values of the filtrates was mesured. Tween 20 (1/5,000) was added to the cultured filtrates and filtered through gauze. Spores were counted by a hemocytometer.

(3) *Alteration of Trichoderma population in soils*: An isolate (RT-1) was cultured on PDA. The test soil (1 kg) were potted in an Erlenmeyer flask (1 l). Spore suspension (ca. 10^6/g) were inoculated to the test soils. The number of spore was calculated by the soil dilution method at every 10 days.

(4) *Antagonism of microorganisms against Trichoderma*: To test antagonism to the fungi *Trichoderma*, microorganisms isolated by the soil dilution method were cultured by confronting with *T. lignorum* (RT-1) on PDA.

(5) *Effects of the culture filtrates of Aspergillus spp.*: Three isolates of *Aspergillus* were cultured in 350 ml of PS at 25℃ for 14 days. One ml of the culture filtrates was dropped on a paper disc, 8 mm in diameter. *T. lignorum* (RT-1) was inoculated on the PDA keeping distance form the disc.

Results

Survival in soil suspensions

When the sclerotia preserved in soil suspension of Kunigami Maaji and Jaagaru at 25℃, only the sclerotia of *S. rolfsii* survived at a high rate, even after 90 days (Fig. 6). Other fungal sclerotia had different survival rate. The survival rate of sclerotia of *R. solani* was 50% in both soil suspensions after 90 days, and 0% of those of *S. delphinii,* within 60 days. The sclerotia of *S. sclerotiorum* were weakest in soil suspensions, perishing within 14 days in Jaagaru and 30 days in Kunigami Maaji. Scarce visual changes were observed in the dead sclerotia of *R. solani*. The outer surfaces of sclerotia of *S. delphinii* maintained their original form, except for decomposed medullae. On the other hand, the outer layers of sclerotia of *S. sclerotiorum* separated quickly, breaking up beyond recognition (Plate 4-1). These sclerotia disappeared when immersed in the soil suspension for more than 30 days.

Effects of soil microbes on sclerotium survival

The microflorae in the soils used in this study was investigated with soil dilution method as in the previous experiments. The total number of soil microorganisms in Jaagaru and in Kunigami Maaji was about 1×10^8 and 3×10^7, respectively (Table 7). The number of bacteria in Jaagaru was larger than in Kunigami Maaji, whereas the number of fungi in Jaagaru was about half of that (Table 7). *Aspergillus* (22.6%), *Fusarium* (18.5%) and *Rhizopus* (11.7%) were well isolated from Jaagaru and *Penicillium* (42.5%), *Trichoderma* (18.0%) and *Chaetomium* (9.2%) from Kunigami Maaji (Table 8). Jaagaru is a heavy, gray, clay soil consisting of tertiary marl-like sediments showing alkalinity, and is distributed in the middle and southern parts of Okinawa Island[25]. Kunigami Maaji is a yellow-red soil whose mother materials such as granite, phyllite and gravel were weathered, exhibiting acid property and is distributed in the middle and northern mountainous parts of the Island[25].

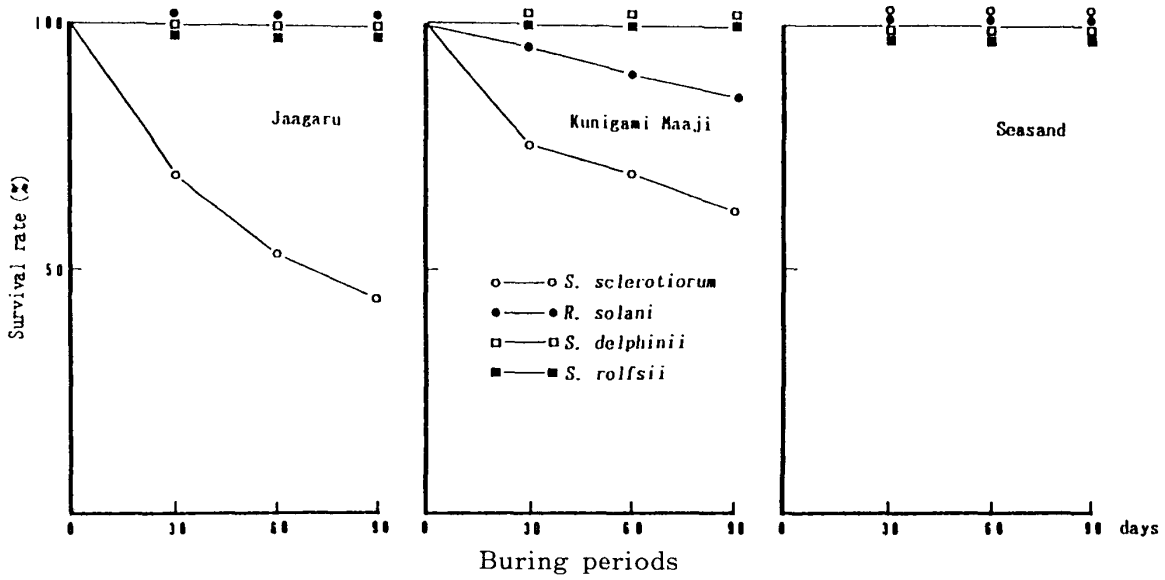


Fig. 7. The effect of differnt soil on sclerotial survival.

Table 7. The microorgenisms in the test soils

	Test soil	
	Kunigami Maaji	Jaagaru
Fungi	7.8×10^{6} *)	4.0×10^{6}
Bacteria	3.8×10^{7}	1.3×10^{8}
Total	4.6×10^{7}	1.34×10^{8}

* Isolated number per 1 g dry soil.
** Calculated by the soil dilution method.

Table 8. The fungal genus and the rate of isolation in test soil

Genus	Isolation rate	
	Kunigami Maaji	Jaagaru
Aspergillus	0[a)]	22.6
Cepharosporium	0	12.4
Chaetomium	9.2	0
Fusarium	1.5	18.5
Gongronella	6.5	0
Penicillium	42.5	6.8
Phoma	0	10.9
Rhizoctonia	0	1.2
Rhizopus	0	11.7
Trichoderma	18.0	0
Unknown	24.3	14.5

[a)] The ratio of isolation (%)

The survival rate of sclerotia buried in both soils and in sea sands was examined. Microorganisms which decompose and antagonize sclerotia were isolated from both test soils in order to determine the quantities of antagonists distributed in the soils and their effects on the sclerotium viability. The sclerotia of *S. rolfsii* and *S. delphinii* survived completely even 90 days after burial (Fig. 7). Although all the sclerotia of *R. solani* survived in Jaagaru, 80% of the sclerotia survived in Kunigami Maaji after 90 days (Fig. 7). The survival rate of sclerotia of *S. sclerotiorum* decreased gradually in Jaagaru to 45% after 90 days. While in Kunigami Maaji about 60% of the sclerotia of this fungus survived 90 days after burial (Fig. 7). The sclerotia in sea sand used as a check survived completely even after 90 days. *Trichoderma* spp. were continuously isolated from the destroyed sclerotia of *S. sclerotiorum* buried for 7~90 days in Kunigami Maaji. *Trichoderma* fungi were also isolated after 30 days from those of sclerotia of *R. solani*. On the contrary, different fungi such as *Fusarium, Aspergillus* and *Rhizopus* were isolated from those of sclerotia of *S. sclerotiorum* buried for 7~90 days in Jaagaru.

When the fungi isolated were cultured by confronting to the 4 sclerotium forming fungi, almost all *Trichoderma* isolates had strong antagonism, and many of *Aspergillus* (Plate 3-4) and *Fusarium* exhibited it to some degree. In the sites of *R. solani* contacted with *Trichoderma*, the hyphae of the *Trichoderma* intertwined around the hyphae of *R. solani* (Plate 4-2).

In general, the isolation ratio of bacteria depends on the type of the sclerotia and the soils. Bacteria were well isolated from Jaagaru during the early stage of burial. In the case of the sclerotia buried in the sea sands, the survival ratio was 100% even at 90 days (Fig. 7).

The rate of antagonistic fungi isolated by trap method was more than 70% for both Jaagaru and Kunigami Maaji but by the dilution method it was 20% for Jaagaru and 50% for Kunigami Maaji (Fig. 8).

Effect of Trichoderma lignorum (RT-1) on sclerotium survival

The fungi *Trichoderma* are recognized as invaders to the fungal sclerotium cells. The 4 sclerotium forming fungi were inoculated with the isolate (RT-1) of *Trichoderma* in Jaagaru, Kunigami Maaji and sea sand. RT-1 showed strong ability to suppress sclerotium viability, especially for *S. sclerotiorum* (Fig. 9).

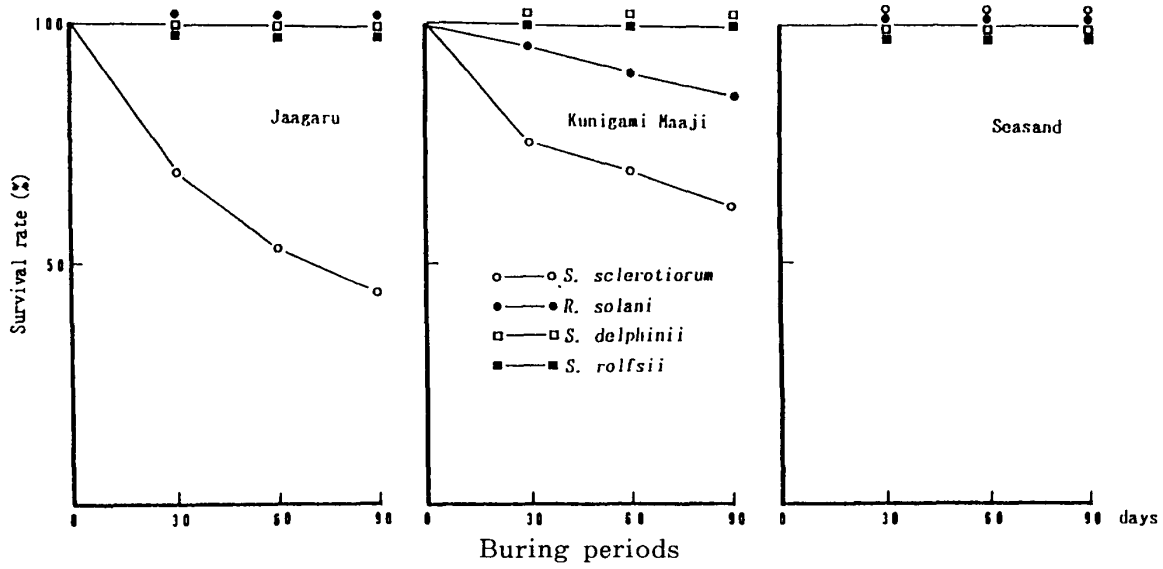


Fig. 7. The effect of differnt soil on sclerotial survival.

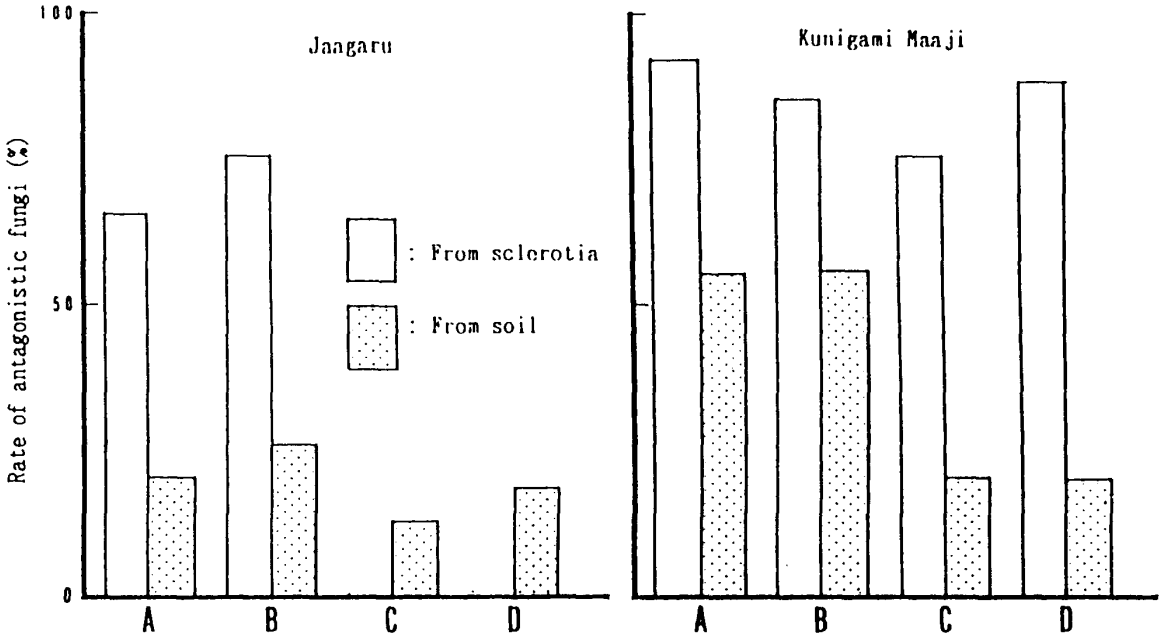


Fig. 8. The rate of antagonistic fungi isolated from different soil and sclerotia buried to sclerotium forming fungi.

A: *S. sclerotiorum*, B: *R. solani*, C: *S. delphinii*, D: *S. rolfsii*.

Effects of several factors on T. lignorum (RT-1)

There are no significant differences on the hyphal growth of *T. lignorum* (RT-1) at pH 4 and pH 6. However, the growth rate at pH 8 was inferior to at pH 4 and pH 6 (Fig. 10). Furthermore, after the 14 days culture, mycelial weight and the number of spores were most excellent at pH 6, mycelial dry weight at pH 6 being about 4 times as much as at pH 8 and the number of spores 6 times as many (Table 9).

Changes in the population of T. lignorum after inoculation

When the spore suspensions were inoculated to the natural and stelilized soils of Kunigami Maaji (pH 4.2) and Jaagaru (pH 7.8), the population of RT-1 was gradually decreased in natural soil of Jaagaru, but in stelilized soil of this, the fungal population at 50 days after was increased by about 10 times that of inoculation (Fig. 11). On the other hand, in Kunigami Maaji, a little increase of the fungal population was observed in stelilized soil but the population was maintained at $4\sim6\times10^6$ level during incubation in natural soil.

Antagonisms of microbe to T. lignorum

Fungi and bacteria isolated by a soil dilution method were cultured by confronting with RT-1. The antagonistic rate of the fungi to RT-1 was 24.7% in Kunigami Maaji and 41.1% in Jaagaru and the antagonistic rate of bacteria was 10.2% and 19.5%, respectively (Table 10). Many isolates of *Aspergillus* spp. showed strong antagonism to RT-1 (Plate 4-4). However, the culture filtrates of *Aspergillus* did not inhibit the RT-1.

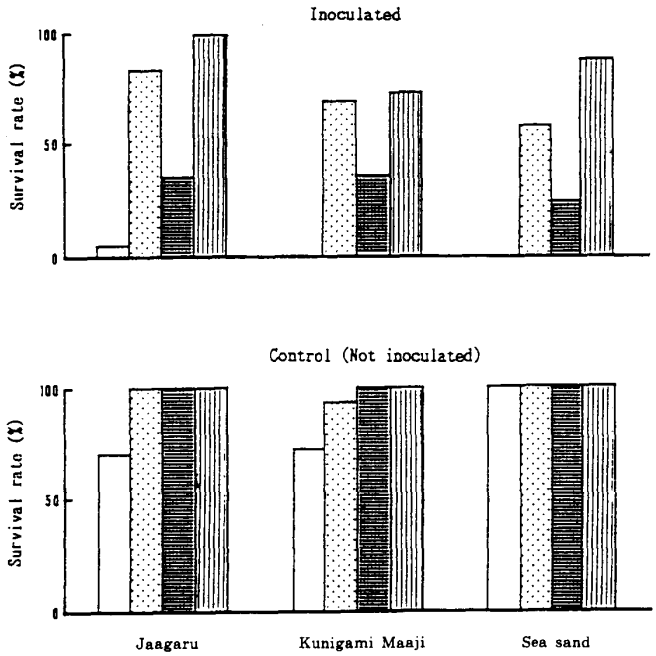


Fig. 9. The effect of *T. lignorum* (RT-1) on sclerotial viability of four sclerotium forming fungi.

: *S. sclerotiorum*, : *R. solani*, : *S. delphinii*, : *S. rolfsii*

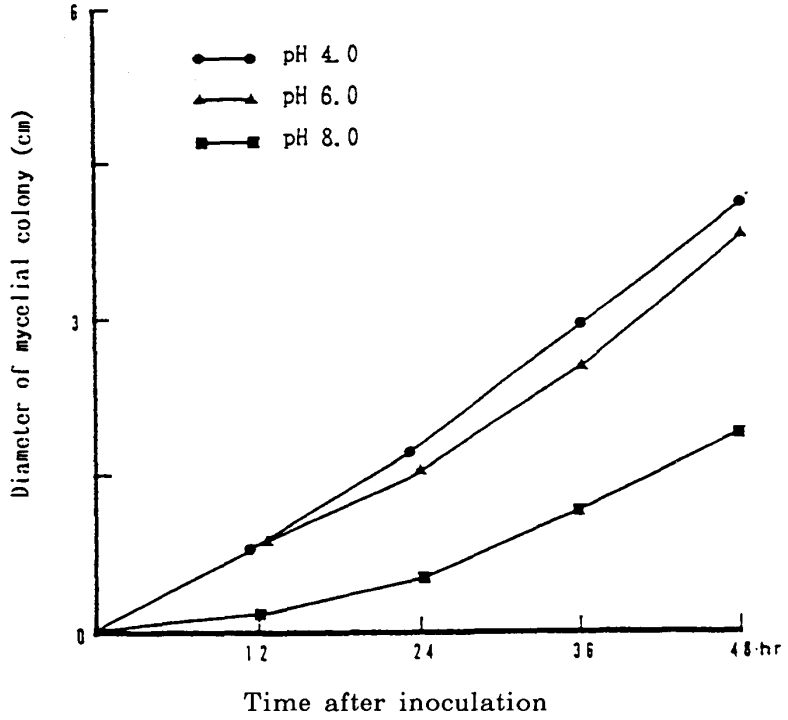


Fig. 10. The effect of pH on the hyphal growth of *Trichoderma lignorum* (RT-1).
* PDA was used as basal medium.

Table 9. The effect of pH on mycelial weight and spore formation of *T. lignorum*

	pH value		
	4	6	8
Mycelial fresh weight	13.68[a)]	22.72	5.28
Mycelial dry weight	1.68[a)]	2.88	0.72
Number of spore	1.16×10^8	1.96×10^8	0.32×10^8

a) g.

Table 10. Antagonisms of microorganism in soils to *T. lignorum*

Soil	pH	Antagonistic degree	Microorganisms	
			Fungi	Bacteria
Kunigami Maaji	4.2	++	18.7[a)]	4.2
		+	5.4	6.4
		±	18.3	13.6
		−	57.5	75.8
Jaagaru	7.8	++	28.8	11.8
		+	12.3	7.7
		±	17.3	43.1
		−	40.6	37.4

a) Ratio (%)

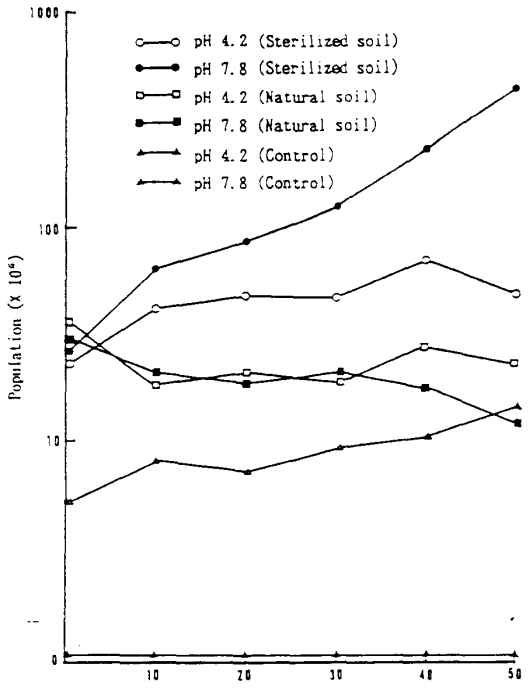


Fig. 11. The change of the population of *T. lignorum* (RT-1) in Kunigami Maaji and Jaagaru.

Discussion

The morphologically characteristic forms of sclerotia produced by plant pathogenic fungi depend on the species[29,119]. This may suggest that the resistance of the sclerotia to environmental factors also depends on the species. In the previous chapter, the author examined the tolerance of typical sclerotium forming fungi, *R. solani* and *S. sclerotiorum*, which are different in the manner of sclerotium formation. The effects of several physical factors on sclerotial viabilities were examined. It was demonstrated that the thermal resistance of sclerotia produced by *R. solani* was much better at each stage of the sclerotium formation than those by *S. sclerotiorum*. When the sclerotia of the 4 fungi were stored in pure water at 25℃ and 35℃, they survived long periods at 25 ℃, but died out at 35℃ after 28 days. The stability of sclerotial death differed in the fungal species. When kept in both natural soil suspensions of Jaagaru and of Kunigami Maaji at 25℃, the sclerotia of *S. sclerotiorum*, *R. solani* and *S. delphinii* died rapidly, especially those of *S. sclerotiorum*, within 15 days in Jaagaru and 30 days in Kunigami Maaji (Fig. 6). It seems that the soil microorganisms strongly affect the viability of sclerotia. Diversity and quantity of microorganisms in soils are principally represented by bacteria, protozoa, actinomycetes and fungi, all of which are compatible with various environmental factors. The soils distributed in Okinawa Island are different in geological features[25]. The differences of physical and chemical natures of the soils are due to the floral and microfloral differences. Bacteria isolated from Jaagaru were about 3 times as much as those from Kunigami Maaji, while the latter had twice as much fungi as compared with the former. Moreover, the fungi isolated from the soils were completely different in quality: the fungi *Trichoderma* obtained were 18.0% of the total fungi from Kunigami Maaji but 0% from Jaagaru. Comparing the sclerotial longevity in both soils, the rate of death in sclerotia of *S. sclerotiorum* was higher than that of other fungi. The antagonistic rate of fungi isolated from buried sclerotia to the fungi was higher in Jaagaru for *S. sclerotiorum* and *R. solani*, and in Kunigami Maaji for all the fungi. Antagonists were also isolated from fungi that had been buried and survived. More than 50% of the fungi obtained by soil dilution method from Kunigami Maaji showed antagonism to *S. sclerotiorum* and *R. solani* but at a generally lower level than in the rate of the trap method. This may indicate that the microorganisms adhering to the sclerotia take nutrients or may act as antagonists. Of the fungi isolated and antagonized to the sclerotium forming fungi, many isolates of *Trichoderma*, *Penicillium* and *Aspergillus* show antibiosis and a few isolates of *Fusarium* show fungistasis. The fungi isolated from sclerotia buried in the clay loam in Hokkaido were reported to be mainly *Penicillium*, *Aspergillus* and *Fusarium*[90, 91]. *Trichoderma* is recognized as an invader of fungal sclerotia, and *Penicillium, Aspergillus*, and *Fusarium* as producers of antibiotics[98]. It was demonstrated that several isolates of *Aspergillus terreus* inhibit the sclerotium formation of *R. solani* without hyphal growth. Comparing the tissues with 3 other fungal sclerotia, the sclerotia of *S. sclerotiorum* were large-sized and their surfaces were rough and medullae (inner parts) were composed of white watery tissues[87]. In contrast, the sclerotia of *R. solani* consist of uniformly melanized cells and those of *Sclerotium* were differentiated into several layers and whose surfaces were fine[4, 87, 119]. When the dead sclerotia of 4 fungi were examined, no visual differences were observed in

R. solani. On the other hand, the inner parts of the dead sclerotia in both species of *Sclerotium* had changed to a sponge like tissue and those of *S. sclerotiorum* had decomposed and broken. Possibly, the sclerotia of *S. sclerotiorum* are more susceptible to antagonistic microorganisms in soils.

On the other hand, best antagonists, the fungi *Trichoderma*, were not isolated from Jaagaru. It is well known that *Trichoderma* is one of the acidophilic fungi[97]. However, *T. lignorum* (RT-1) could grow and form spores at pH 8 to some degree *in vitro*. Moreover, the pH value of the medium ajusted at pH 8 was changed to pH 7. It may suggest that the fungi can regulate the pH value in environments to a favorable condition for them. When a certain number of spore (ca. $10^6/g$) of the fungi was inoculated to both natural and sterilized soils and investigated the change of population, in sterilized soils, a striking increase was recognized in alkaline soil rather in acidic one. The fact shows that *Trichoderma* can live fundamentally in a alkaline soil. The microflorae in soils could be given as one of the important factors influencing the distribution of *Trichoderma*. As the result of the antagonistic test, fungi and bacteria having antagonism to *T. lignorum* (RT-1) were obtained at a high rate from Kunigami Maaji than from Jaagaru. Certain *Aspergillus* spp. inhibited strongly the hyphal growth of RT-1, though those cultural filtrate did not affect. As described above, there are many natural enemies to *Trichoderma* in soils, and that those depend indirectly on the longevities of phytopathogenic sclerotia.

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 (EC 1.1.1.37) 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℃ 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℃ for 10 min) was poured into a Petri dish and inoculated. Culturing was carried out at 25℃ for 14 days under dark conditions. After 14 days, the sclerotia formed were taken out, dried at 60℃ 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 (200g) 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 medium.

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

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	51.18±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
Monosacdcharide			
Glucose[a)]	42.85±3.34[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.

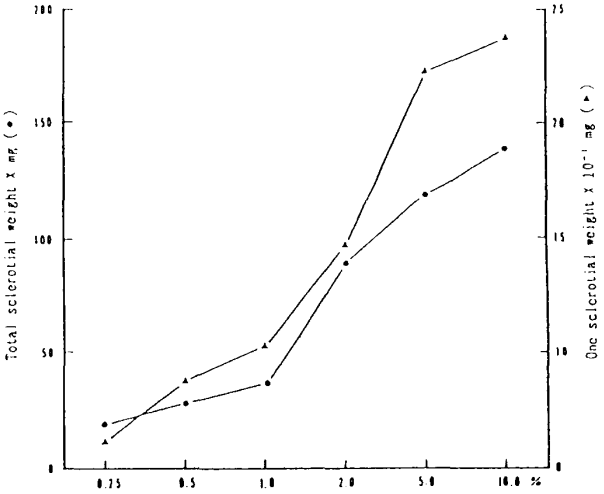


Fig. 12. The effect of glucose concentration on sclerotium formation of *R. solani*.
* C-324 was used.

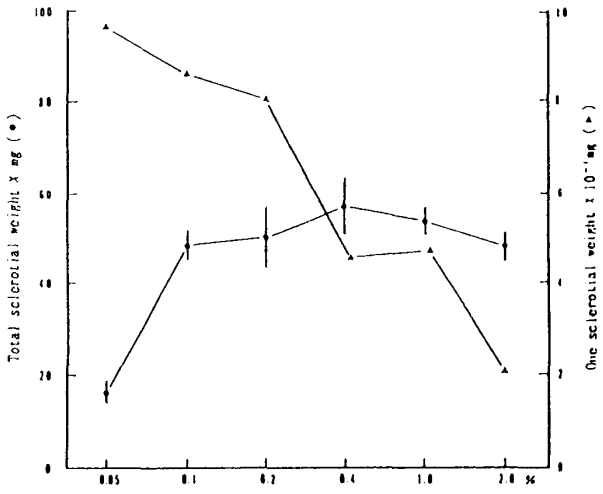


Fig. 13. The effect of KNO_3 concentration on sclerotium formation of *R. solani*.
* C-326 was used

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 welght 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 sclerotiorum*, 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℃ for 10 min.

Effects of carbon concentration on 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℃ 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.

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

Incorporation of carbon source form 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℃ 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 (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℃. 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℃. 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. After the culture for 14 days at 25℃, sclerotia formed on the membrane were collected, 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 mizer, 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℃. When the culture 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℃. 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 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.

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.

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 the 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). The optimal concentration of KH_2PO_4 for the sclerotium formation was in the region of 100 ppm at the presence and

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

Cocentration 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*

Cocentration 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.

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 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 those on the medium without the salt (Table 26).

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

KH_2PO_4	Hyphal stage[a)]		Sclerotium 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 phospgorus 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.

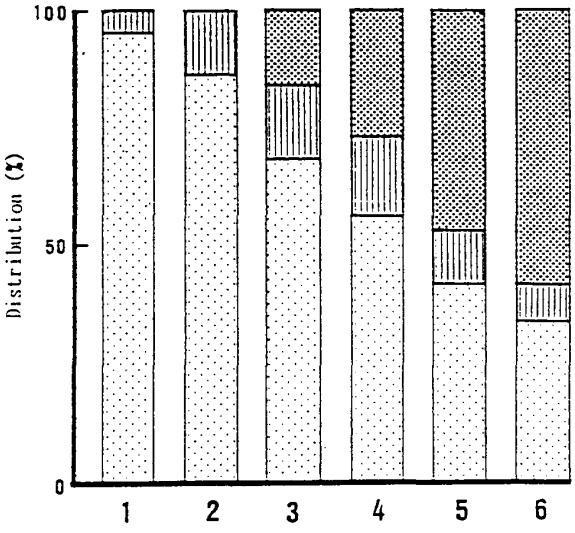


Fig. 15. Rate of ^{32}P distriburion in the medium, mycelium and sclerotium at different stage of sclerotial morphogenesis of *R. solani*.

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

: Medium, : Mycelium, : Sclerotium.

Discussion

In Exp. 1, the author showed that the quantity 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 phosphorus 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 prosphorus 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 the sclerotium 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℃ 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℃, sclerotia formed were taken, dried at 60℃ 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℃ 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℃ 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 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 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 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.

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.

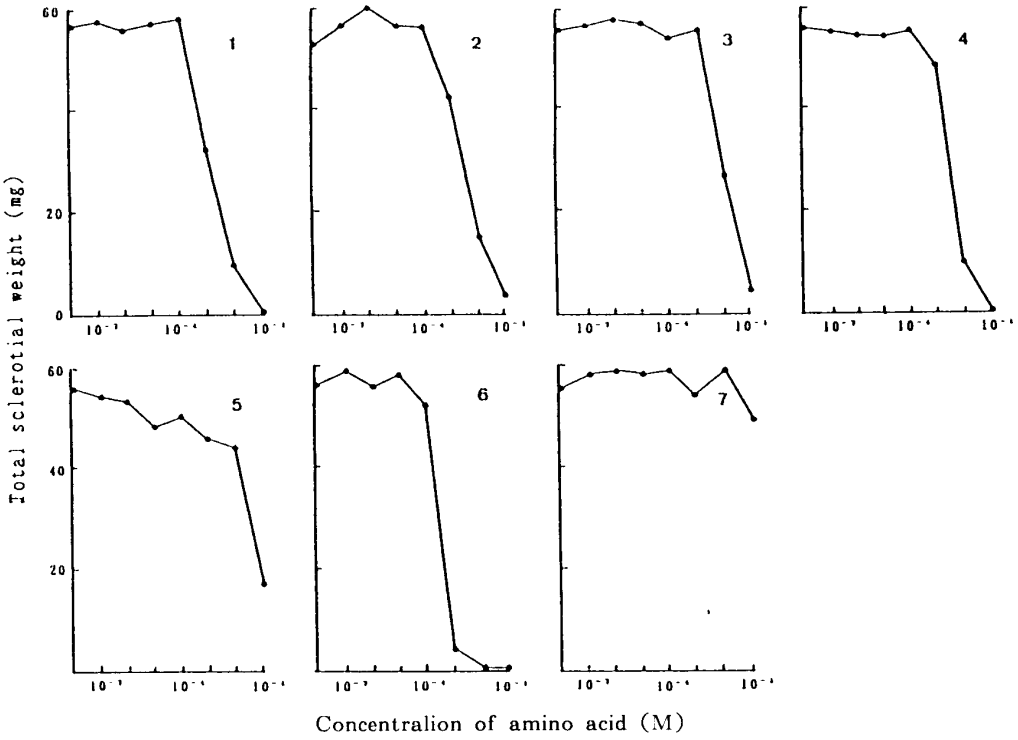


Fig. 16. The effect of concentralion of inhibitive anino acids on sclerotial formation of *R. solani*. 1. Cysteine, 2. Cystime, 3. Homocysteine, 4. Methiomime, 5. Taurime, 6. Histidime. 7. Leucine.

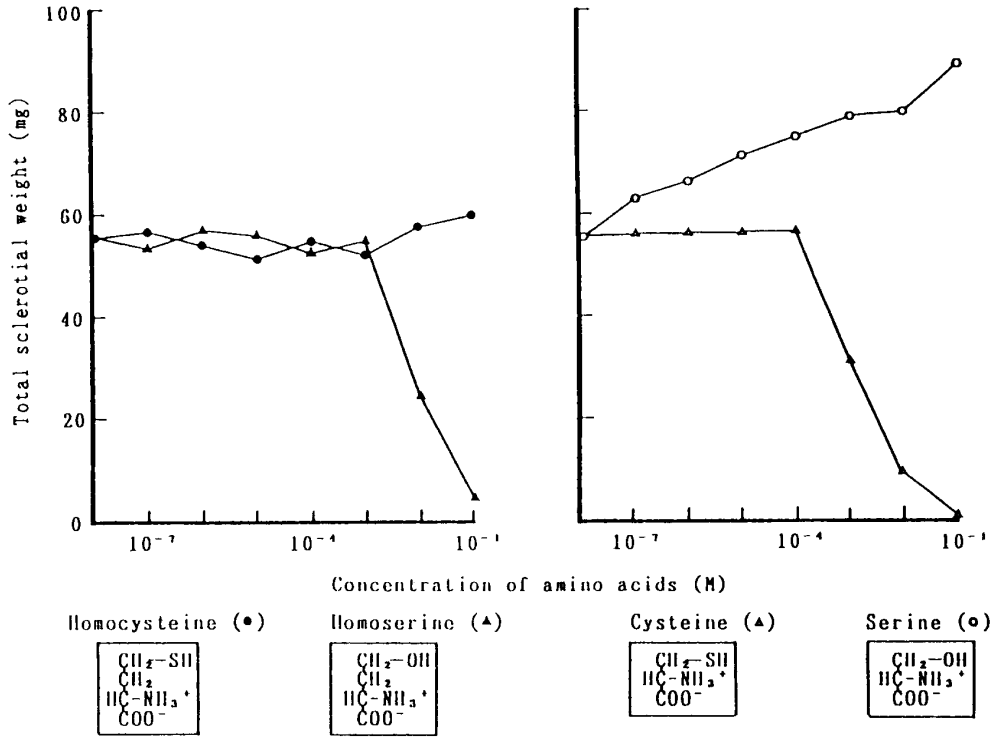


Fig. 17. The effect of sulfur containing and not containing amino acids on sclerotial formation of *R. solani* (C-324) as additionally amended Hopkins medium.

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	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	
	24ht	48hr
Basal+Met		
10^{-5}	3.76±0.24[a)]	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 basel 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	2	3
Basal+Met			
10^{-5}	2.35±0.42[a)]	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^{-2}	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 basel 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.

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

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 had been reported in *Selerotium 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 immature 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.

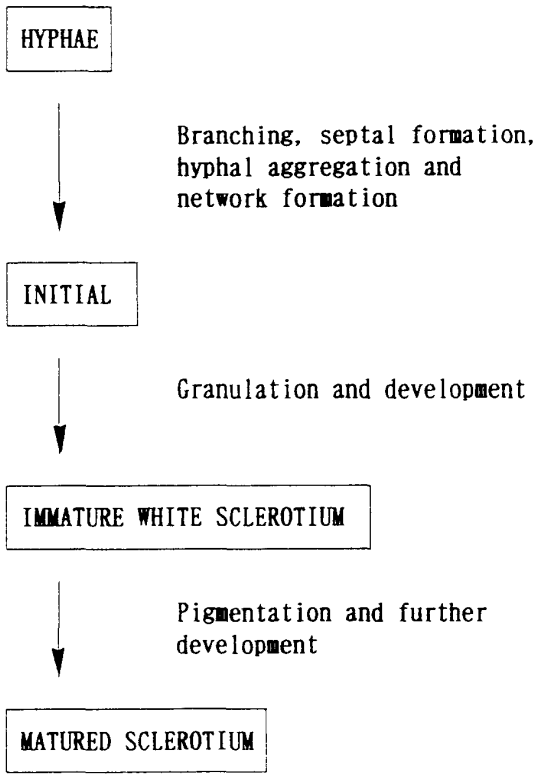


Fig. 18. Diaramatic representation of sclerotium formation of *R. solani*.

Materials and Methods

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

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 × 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 ℃. 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℃ 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 or 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 1～1.5 cm^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 branching 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[49] 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.

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	6days
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	6days
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	1	1
Ser	26.80	19.85	8.80	6.75
	44.90	47.00	27.50	15.20
Control	30.70	25.65	18.20	19.55
(the basal medium)	90.70	63.60	48.10	23.70

a) The weight (mg) of sclerotia (total weight per dish) differentiated fom hypgal 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.

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

In Chapters and , 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℃ 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℃ 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℃ 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℃ 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℃ 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℃). 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 sclerotia.

Table 35. The effects of continuous irradiation of wgite 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.

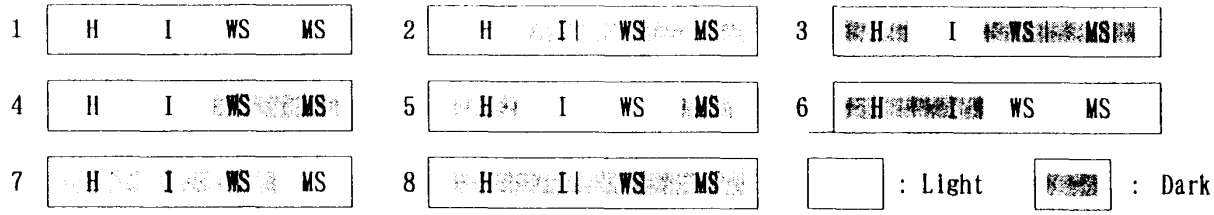
The effects of irradiation of wide-wave length light at a selected phases of sclerotial development as specified in Table 36 were examined. Although the irradiation at any 1 or 2 phase(s) stimulated the sclerotium formation, the stimulation was distinct when the irradiation was carried out in the early 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.

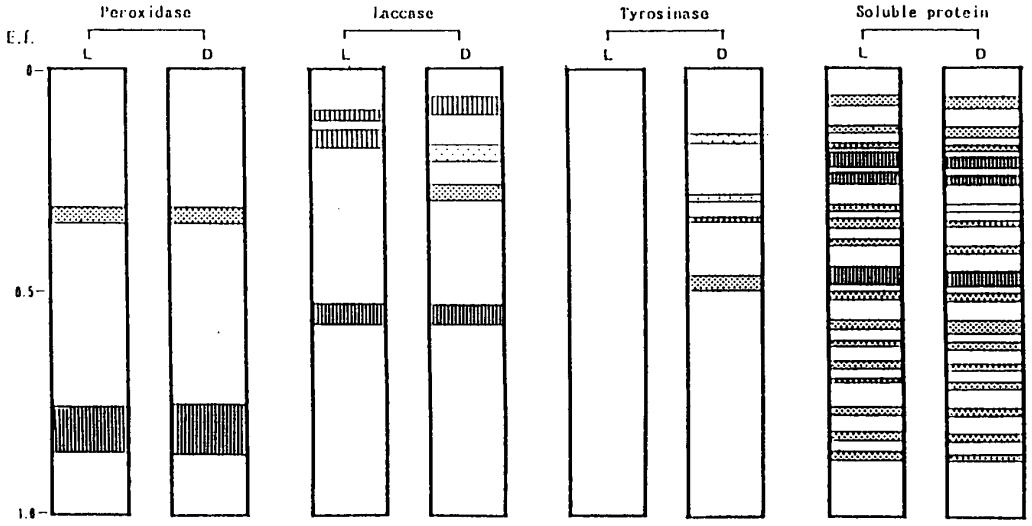


Fig. 19. Zymogramatic comparison of three enzymes and soluble protein of *R. solani* cultured under continuous irradiation and dark condition. L : Light, D : Dark.

Table 38. The effect of monochromatic light on mycelial growgh 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.

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} 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 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
	Peroxldase	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 and , 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 in the 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] described that 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 microsclerotial 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. Acknowledgments

The present work was started under the the direction of Professor Dr. Nobuaki Matsuyama of the Plant Pathology Laboratory, Faculty of Agriculture, Kyushu University from 1978. The author would like to gratefully thank him for suggesting this problem and for generous instruction throughout the present research and in the preparation of the manuscript. The author equally grateful to Emeritus Professor Dr. Satoshi Wakimoto for his helpful advice and encouragement. I am also pleased to acknowledge the considerable assistance of Dr. Masao Tamori of Professor of Plant Pathology,

Faculty of Agriculture, University of the Ryukyus. I am indebted to Dr. Cyoyu Shinjyo, Emeritus Professor of Faculty of Agriculture, University of the Ryukyus, who supported financially in part of the work. Thanks are due to the colleagues and students of the Laboratory of Plant Pathology, Department of Agriculture, Faculty of Agriculture, University of the Ryukyus, who cooperated with the author on various problems.

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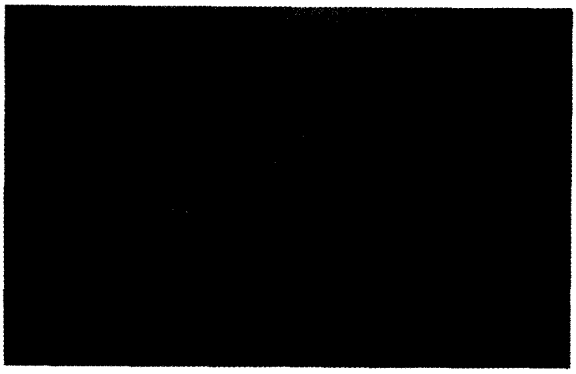
Explanation of Plates

Plate 1.

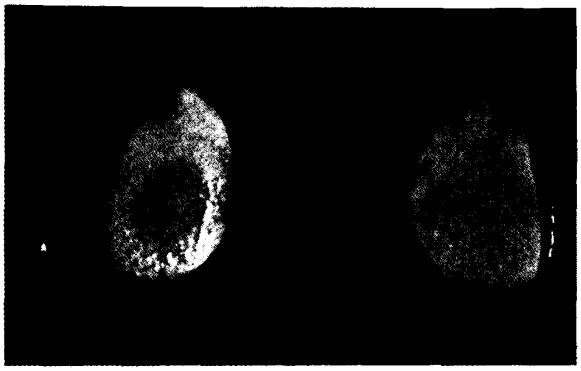
The symptoms of rice sheath blight caused by *Rhizoctonia solani* (AG-1, IA).

Plate 2.



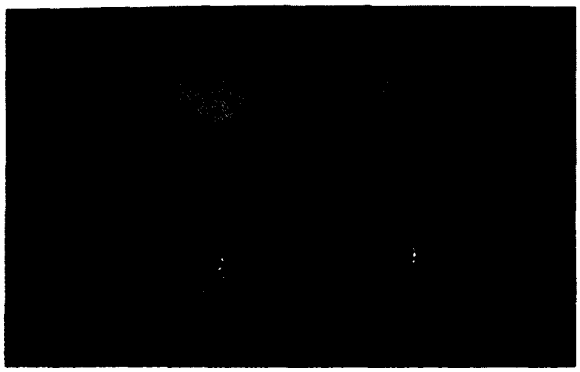
The zymograms of 4 isolates of *R. solani*. A : C-326, B : 34, C : HK-616-23, D : ST-7.

Plate 3−1.



The section of white sclerotium of *Sclerotinia sclerotiorum*.

Plate 3−2.

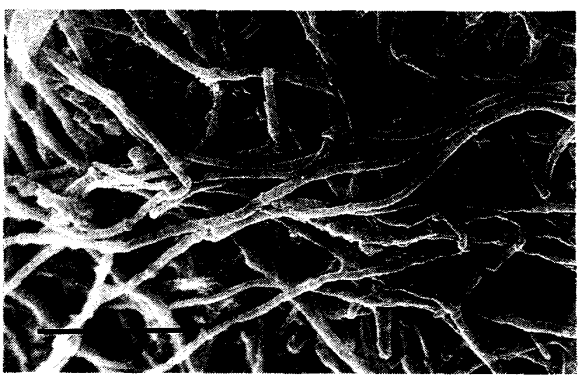


A comparison of matured sclerotium to *R. solani* and *S. sclerotiorum*.

Plate 3−3.

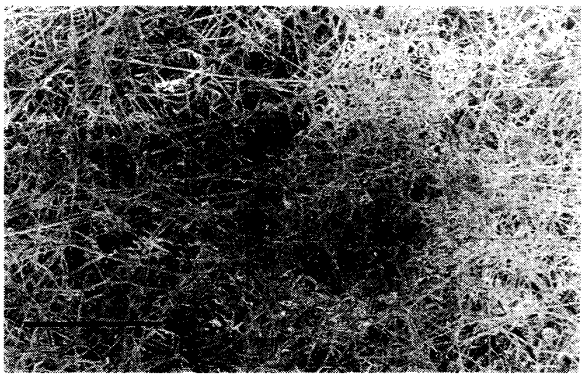
Hyphae of *R. solani* on the filter paper (bar=100 μm).

Plate 3−4.



Hyphae of *S. sclerotiorum* on the filter paper (bar=33 μm).

Plate 3–5.

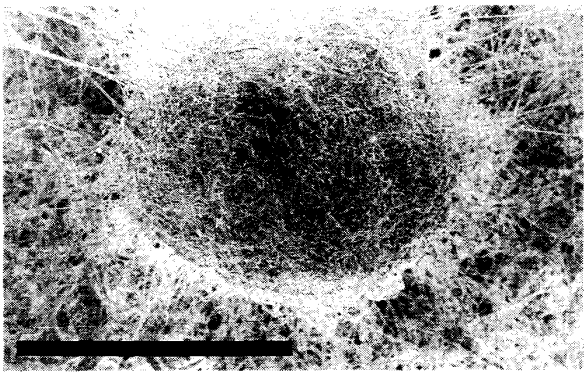


Initial of *R. solani* (bar=0.5 mm).

Plate 3–6.

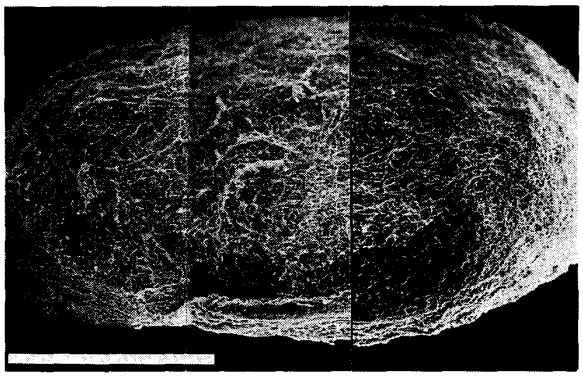
Initial of *S. sclerotiorum* (bar=0.5 mm).

Plate 3–7.



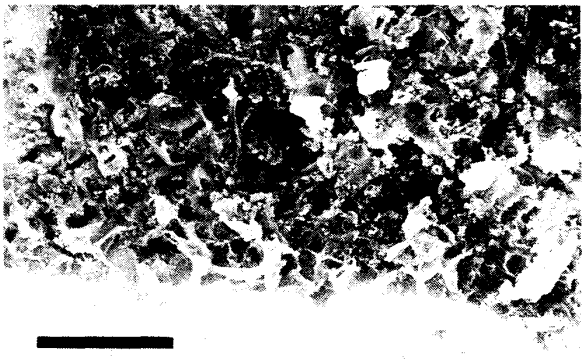
A white sclerotium of *R. solani* (bar=1 mm).

Plate 3–8.



A white sclerotium of *S. sclerotiorum* (bar=0.5 mm).

Plate 3–9.

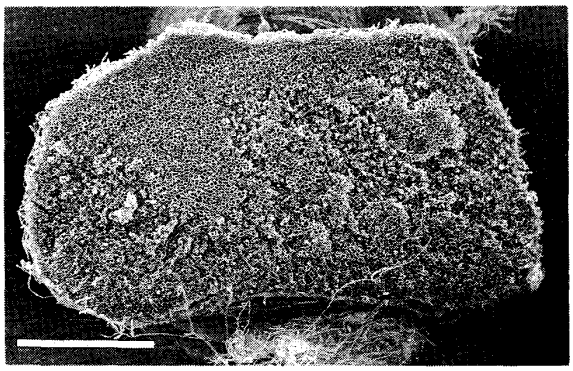


The outer part of white sclerotium of *S. sclerotiorum* (bar=50 μm).

Plate 3–10.

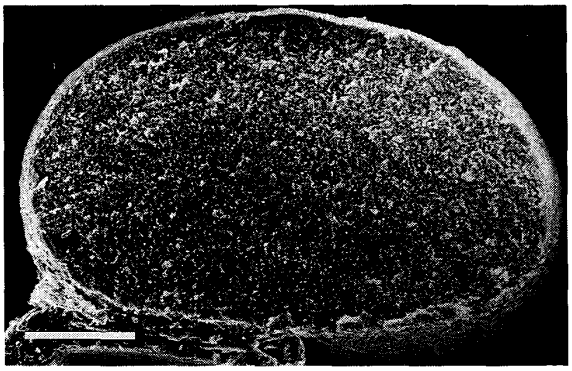
The inner part of white sclerotium of *S. sclerotiorum* (bar=20 μm).

Plate 3−11.



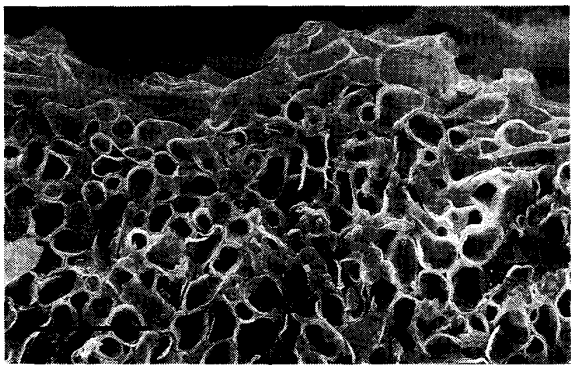
The sectional structure of immature sclerotium of *R. solani* (bar=0.5 mm).

Plate 3−12.



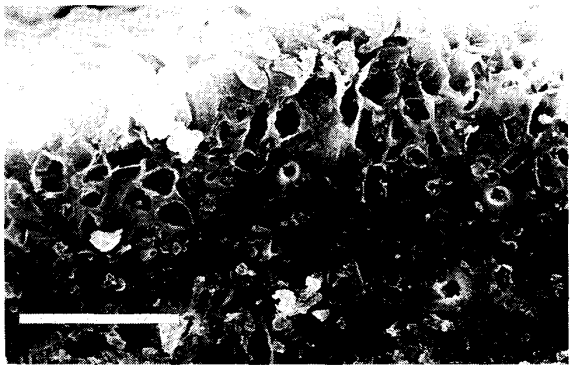
The sectional structure of immature sclerotium of *S. sclerotiorm* (bar=0.5 mm).

Plate 3−13.



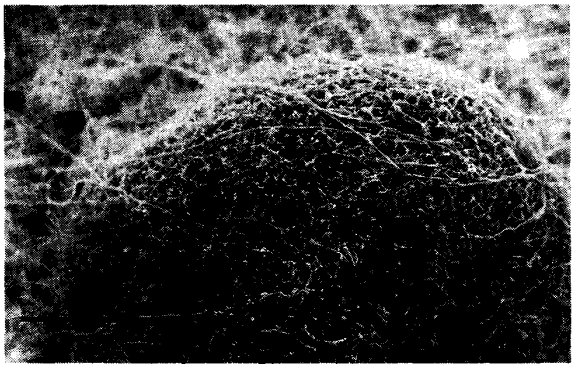
The sectional structure of outer part of immature sclerotium of *R. solani* (bar=50 μm).

Plate 3−14.



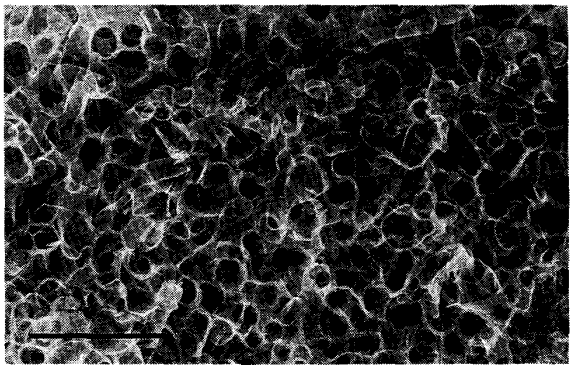
The sectional structure of outer part of immature sclerotium of *S. sclerotiorum* (bar=33 μm).

Plate 3−15.



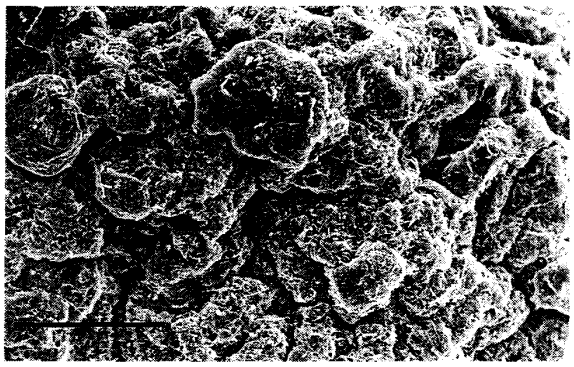
The surface of mature sclerotium of *S. sclerotiorum* (bar=0.5 mm).

Plate 3−16.



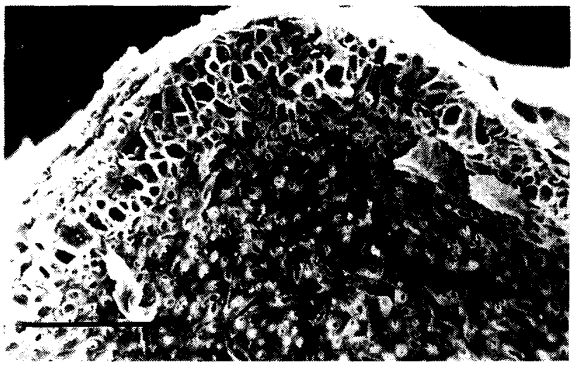
The sectional structure of mature sclerotium sclerotium of *S. sclerotiorum*(bar=50 μm).

Plate 3−17.



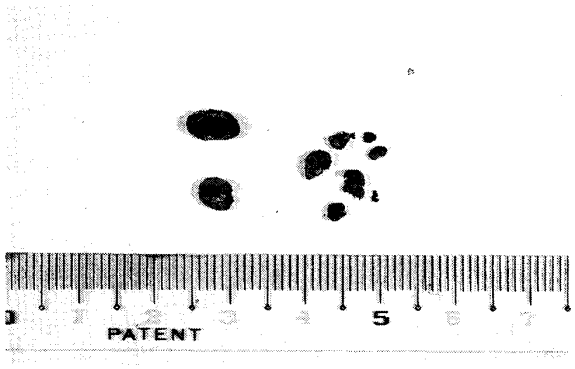
The surface of mature sclerotium of *S. sclerotiorum* (bar=0.5 mm).

Plate 3−18.



The sectional structure of mature sclerotium of *R. solani* (bar=100 μm).

Plate 4−1.

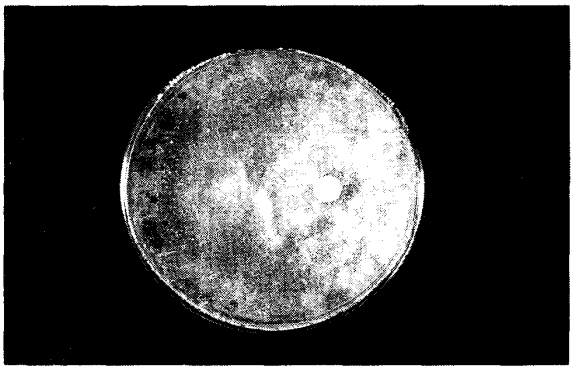


The decomposed sclerotia of S. sclerotiorum, which were buried in Kunigami Maaji.

Plate 4−2.

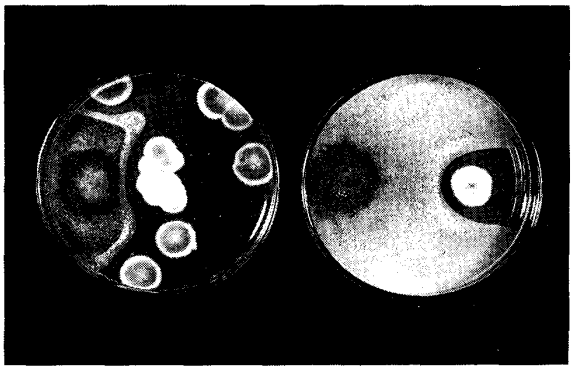
Hyphae of *Trichoderma lignorum* (RT-1) coiling around and penetrating C-324 isolate of *R. solani*.

Plate 4−3.



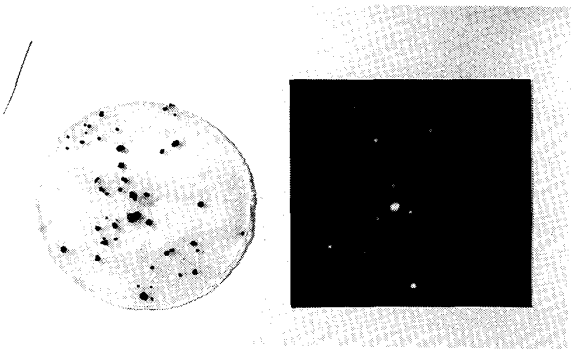
Effects of the filtrate of *Aspergillus terreus* (*RA-2*) *on the sclerotium formation of R. solani.*

Plate 4−4.



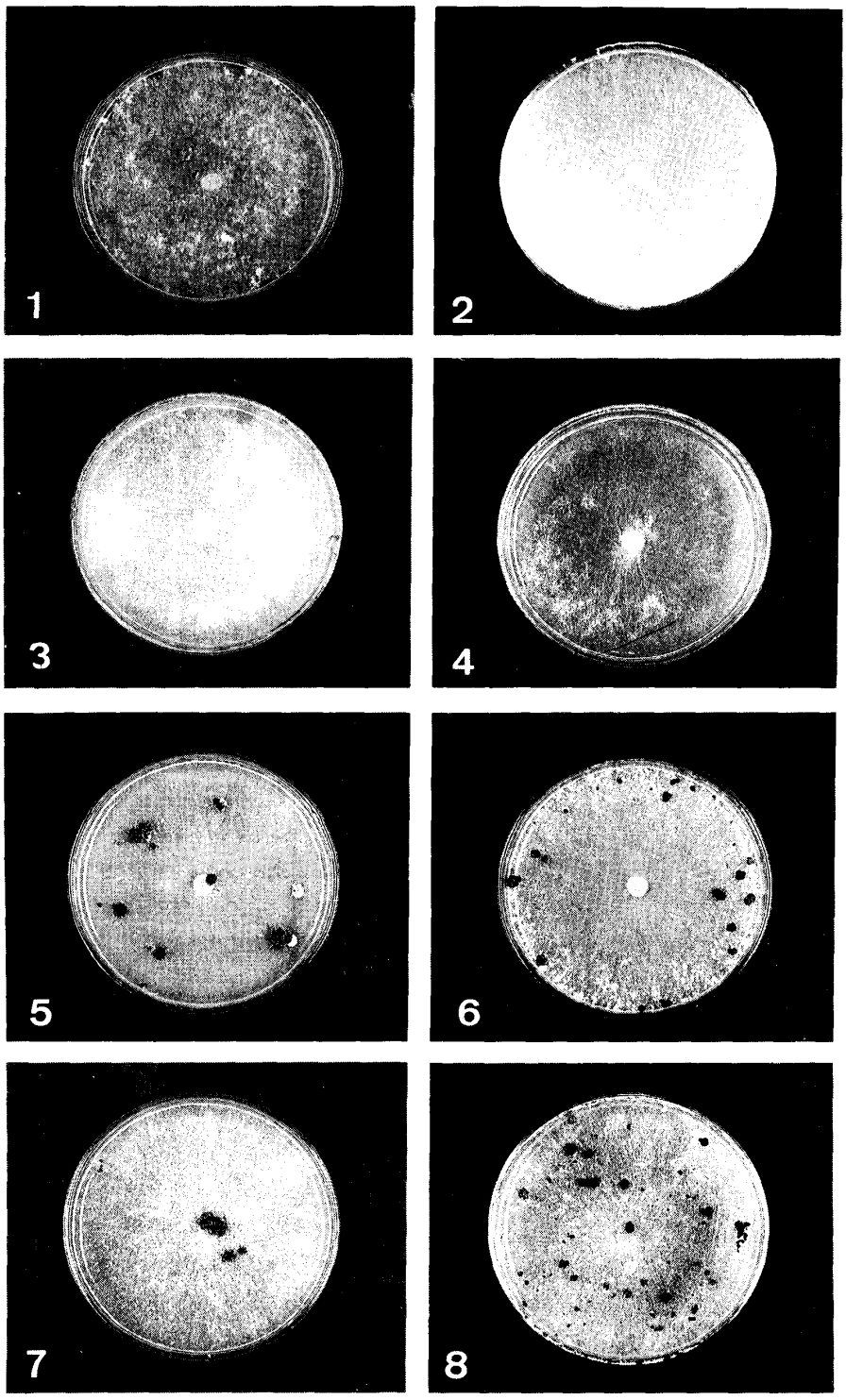
The antagonism of *Aspergillus* spp. to *T. lignorum* (RT-1).

Plate 5.



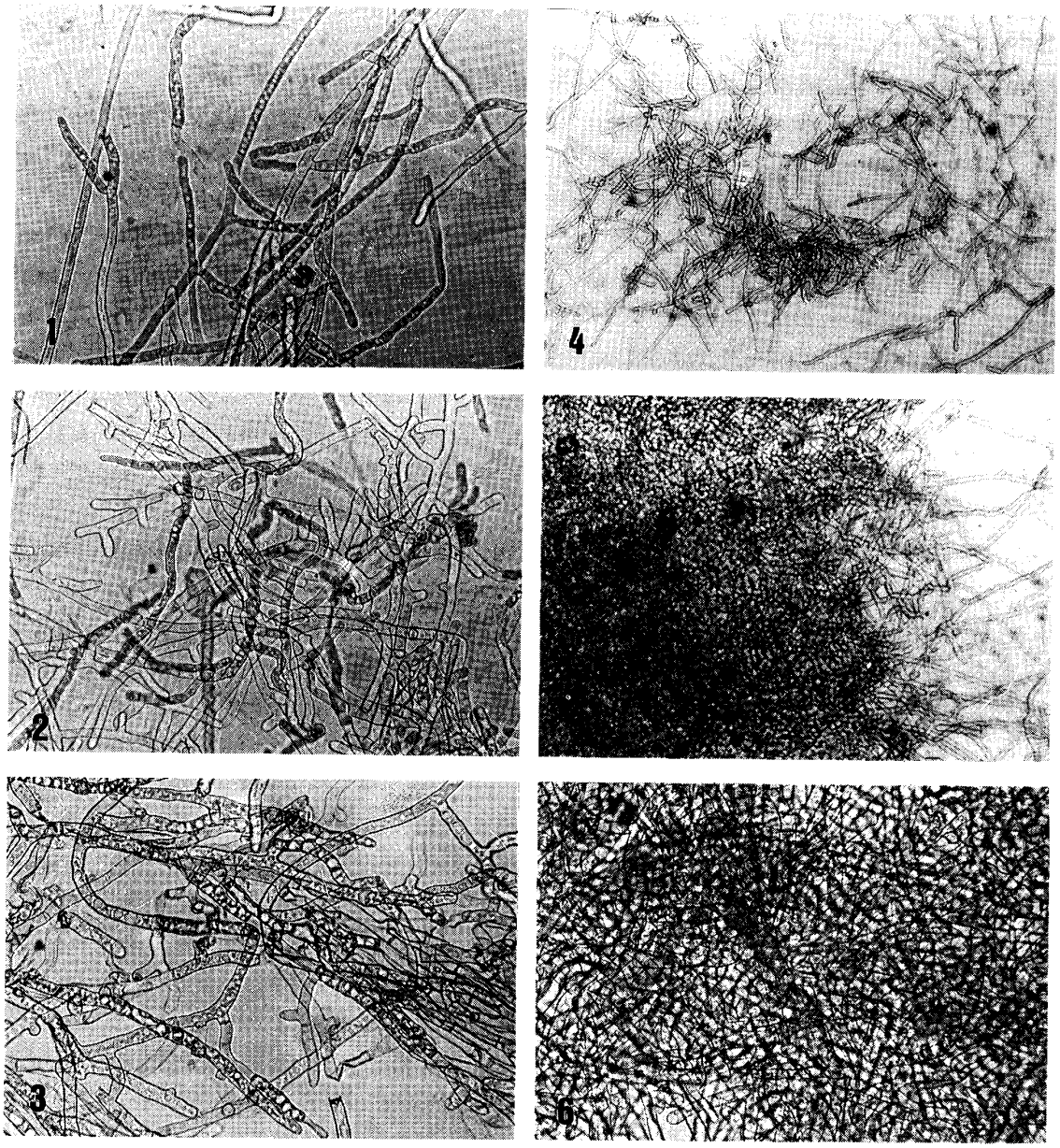
The sclerotial formation of *R. solani* at 14 days after culture on Hopkins medium and distribution of ^{32}P in the sclerotia formed.
Left : Culture on Hopkins medium, Right : Autoradiograph.

Plate 6.



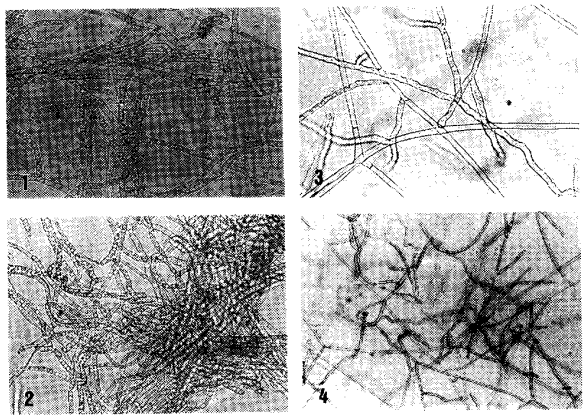
The sclerotium formation on the media, which are amended with various amino acids.
1: Val, 2: Cys, 3: Met, 4: His, 5: Pro, 6: Asp, 7: Ser, 8: Hopkins.

Plate 7.



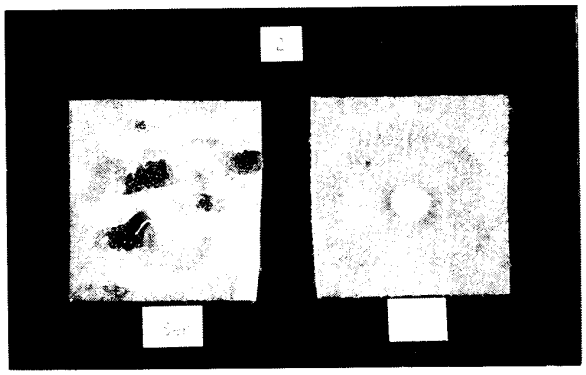
The process of sclerotium development of *R. solani*.
1 : Leading hypha,
2 : Hyphal branching,
3 : Aggregation,
4 : Aggregation and net work formation,
5, 6 : Further development.

Plate 8.



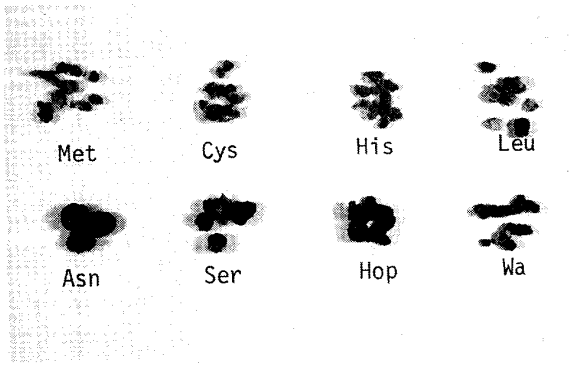
1, 2 : Hyphal branching and aggregation on sulfur amino acid-containing medium.
3, 4 : Hyphal branching on histidine -containing medium.

Plate 9.



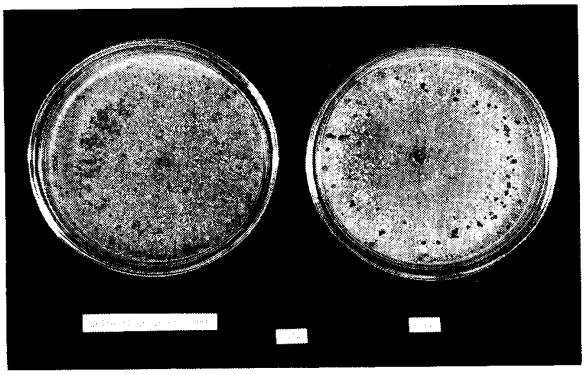
Effects of serine and systeine on the sclerotium formation of *R. solani*

Plate 10.



The further development from white sclerotium, and pigmentation on various media. Hop : Hopkins medium, Wa : Water agar.

Plate 11.



The comparison of the pigmentation and the manner of sclerotium formation of *R. solani* (C-14) cultured under irradiation and dark conditions.