Conditions for Reversion of Sclerotial Formation in a Mutant of *Sclerotinia libertiana* Fuckel

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A mutant of *Sclerotinia libertiana* Fuckel sometimes though seldom, restored the ability to form sclerotia when grown at a low temperature, 15~20°C, on potato medium. There appeared to be a correlation between the regulation of vegetative growth and accumulation of precursors of melanine pigments. The effects of various factors on the morphogenesis were investigated using Czapek-Dox medium with or without addition of amino acids. A combination of Sclerin (SCL) and thiol reagents stimulated melanogenesis in the presence of amino acids at a low temperature. While, cyclic adenosine 3',5'-monophosphate (cyclic AMP) alone stimulated the melanogenesis without addition of the amino acids. Also, a combination of SCL and cyclic AMP or 3,4-dihydroxyphenylalanine (DOPA) induced the formation of sclerotium-like hyphal aggregates though not normal sclerotia.

Preservation of cultures of *Sclerotinia libertiana* Fuckel or *Sclerotinia sclerotiorum* (Lib) de Bary often causes a morphogenetic change. As reported previously, some IFO (Institute for Fermentation, Osaka) stock cultures and a culture maintained in our laboratory do not form any sclerotia under ordinary conditions (surface culture at 22~25°C, optimal growth temperature).¹⁾ These cultures differ in response to thiol reagents and SCL, a physiologically active metabolite, both affecting the formation of sclerotium or sclerotium-like hyphal aggregates.^{1,2)} There appears to be an involvement of other factors in the morphogenesis. Recently, cyclic AMP was reported to control the morphological change in *Neurospora*³⁾ and *Mucor*.⁴⁾ The effect of cyclic AMP is enhanced by thiol reagents and supposed to be conditioned by a metabolic balance for utilization of carbohydrate. The circumstances somewhat resemble the condition of the sclerotial formation in the above strains of *Sclerotinia*. In the present paper, the conditions for restoring the ability to form sclerotia in a mutant of *Sclerotinia libertiana* were

investigated, in combination with several factors and medium constituents.

MATERIALS AND METHODS

Strain and culture conditions. From a strain of *Sclerotinia libertiana* Fuckel (H. Yoshii), a mutant was previously obtained in our laboratory during preservation.²⁾ The mutant (Sm) which had lost the ability to form sclerotia was used. The original strain (Ss) had been transferred from the Faculty of Agriculture, Kyushu University, in 1946. These strains were cultured on agar slant or plate of the potato medium and Czapek-Dox medium prepared as reported previously.²⁾ The temperature and culture period were, unless otherwise specified, 22~25°C and 2~3 weeks, respectively.

Enzyme assay. Phenoloxidase was measured as previously reported,¹⁾ with pyrogallol as substrate. Isocitrate lyase was determined in accordance with the method of Daron and Gunsalus,⁵⁾ by preparing a mycelial extract as in the case with phenoloxidase, but using 10 volumes of M/15 phosphate buffer (pH 7.7) containing 0.005 M dithiothreitol. One unit (u) of enzyme activity was defined to be what produced 1 micromole of glyoxylate in 10 min at 30°C from isocitrate.

Chemicals. Cyclic AMP and DOPA were purchased from WAKO Pure Chemical Industries, Ltd. SCL was prepared^{6,7)} from the extract of a bran culture of the original strain (Ss) described above.

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RESULTS

Effect of temperature

With the original strain, Ss, it was previously reported that the formation of sclerotia in a potato medium showed little difference in a wide range of temperature, 15~27°C.²⁾ As seen in Photo. 1, though the Ss easily formed sclerotia on the potato medium at 23°C within 2 weeks, the mutant, Sm, did not but a white thick mycelium. The Sm, however, formed a few sclerotia only in a low temperature range, 15~20°C, changing periodically in day and night. The frequency was 30% with the potato slant culture. These cultures always produced a melanine pigments in the mycelia below 17°C, even when they did not form sclerotia. There was a close relation between the formation of a sclerotium-like hyphal aggregate and melanogenesis.

The effect of temperature on formation of phenoloxidase in the Sm cultured on bran is presented in Fig. 1. The enzyme formation at 17°C was at first very slow for about 1 week, as compared with that at 23°C, but, afterwards, rapidly increased and reached the same maxi-

FIG. 1. Effect of Temperature on Formation of Phenoloxidase in Bran Culture of Sm.

a) u/g dried mycelium

●—●, 23°C; ○—○, 17°C.

Bran culture was performed with 1.5 g of wheat bran and 2 ml of water in a 50 ml flask at 23°C for 5 days.

um level as at 23°C in nearly 2 weeks. This indicates that the activity of phenoloxidase has no direct relation with the effect of temperature on melanogenesis.

Effect of medium constituent

Effect of C/N ratio in medium constituents was tested at 17°C with Czapek-Dox medium, containing various amounts of ammonium nitrate in place of sodium nitrate as nitrogen source. As seen in Photo 2, optimal C/N ratio on melanogenesis was present in the range of 36~72, where sclerotial formation yet hardly occurred. On the other hand, the ratio in the potato medium as mentioned above was approximately 20. There may be involved a special constituent in potato tuber, promoting the formation or pigmentation of sclerotia. Then, two potato media were prepared from a variety called "Mayqueen," harvested in Hokkaido, which is known to have a high polyphenol content,⁸⁾ one extracted from the outer tissue (about 2 mm thick), and the other from the inner tissue, of the tuber. The test on the sclerotial formation was performed at 15~17°C for 2 weeks. As a result, the former favoured the formation more remarkably than the latter. The effect of the former may involve the polyphenols or their precursors contained in potato tuber, since polyphenols are abundant in the outer tissues.

PHOTO 1. Effect of Temperature on Formation of Sclerotia.

Culture periods, 2 weeks; medium, potato extract medium.

PHOTO 2. Effect of C/N Ratio on Melanogenesis. Culture periods, 2 weeks; medium, Czapek-Dox medium.

Effect of thiol reagents and SCL in the presence of amino acids or DOPA

It has already been shown that formation or pigmentation of sclerotia in *Ss* is stimulated by SCL and some thiol reagents, such as iodoacetic acid and indole-3-acetic acid (IAA).^{1,2)} IAA is known to be photo-oxidized, and produces a specific -SH reagent in plants and microorganisms in the presence of riboflavin.⁹⁾ Then, the effect of thiol reagents and SCL on *Sm* was reinvestigated at 15~17°C, in the presence of tyrosine, tryptophane or DOPA, with Czapek-Dox agar medium. As seen in Photo 3, thiol reagents induced the formation of dark spots or rings of melanine pigments in medium from the amino

PHOTO 3. Effect of SCL and Thiol Reagents on Melanogenesis in the Presence of Amino Acids in Czapek-Dox Medium.

Ty, 10^{-3} M tyrosine; Tr, 10^{-3} M tryptophane; M, 10^{-5} M moniodo-acetate; I, 5×10^{-5} M IAA; S, 10^{-3} M SCL; culture period, 2 weeks.

PHOTO 4. Effect of SCL on Hyphal Morphogenesis in the Presence of DOPA in Czapek-Dox Medium.

O, control; D, 10^{-3} M DOPA; S, 10^{-3} M SCL; temperature, 22~25°C.

acids, only when combined with SCL. It is supposed that while thiol reagents release the phenoloxidase, a Cu-enzyme, bound to -SH proteins, SCL represses biosynthesis of the -SH proteins, accumulating precursors of melanine pigment derived from the amino acids. Such effects did not occur at a temperature higher than 20°C. On the other hand, addition to the medium of DOPA produced melanine pigments at 22~25°C, without addition of the thiol reagents. Also, as seen in Photo 4, SCL stimulated the formation of a sclerotium-like hyphal aggregates in the presence of DOPA.

Effect of acetic acid and cyclic AMP in combination with SCL

Formation of sclerotia in *Sclerotium rolfsii* is known to be related with an increased activity of glyoxylate cycle.¹⁰⁾ SCL and acetic acid stimulate the formation of isocitrate lyase, a key enzyme of glyoxylate cycle, in

PHOTO 5. Effect of SCL and Acetate on Hyphal Morphogenesis.

A, 7.5×10^{-3} M Na-Acetate; S, 8.5×10^{-5} M SCL; medium, potato extract medium.

some microorganism.¹¹⁾ As shown in Table I, a combination of SCL and sodium acetate

PHOTO 6. Effect of c-AMP and Dibutyryl c-AMP on Hyphal Morphogenesis in Czapek-Dox Medium. 0, control; S, 10^{-5} M SCL; c-A, 10^{-5} M c-AMP; dc-A, 10^{-5} M dibutyryl c-AMP.

TABLE I. EFFECT OF ACETATE AND SCL ON FORMATION OF ISOCITRATE LYASE IN BRAN CULTURE OF Sm
Culture conditions were the same as in Fig. 1.

Na-Acetate (M)	SCL (M)	Activity ^{a)} $\mu \times 10^{-1}$
0	0	15.4
7.5×10^{-3}	0	17.2
7.5×10^{-3}	8.5×10^{-3}	19.1

^{a)} μ /g dried mycelium.

most enhanced the activity of isocitrate lyase in a bran culture of Sm, which did not form sclerotia. As seen in Photo 5, when SCL and acetate were added to agar media, ups and downs of growth of aerial hyphae occurred at 17~20°C, and gradually developed to straggled hyphal aggregates, but rather lacking melanogenesis. This may indicate the morphological change accompanied by a balance in the carbohydrate metabolism utilizing the glyoxylate cycle. Then, the effect of cyclic AMP was tested in place of acetate. As seen in Photo 6, although addition to the Czapek-Dox medium of cyclic AMP or dibutyryl cyclic AMP rather suppressed the mycelial growth, it remarkably stimulated the production of melanine pigments in the medium. Also, a combination of dibutyryl cyclic AMP and SCL formed sclerotium-like hyphal aggregates, but it did not develop to normal sclerotia.

DISCUSSION

Sclerotinia libertiana is a phytopathogen isolated from various plants such as rape, cabbage, bean, soybean, potato and tobacco in spring or autumn. As shown in the above experiments, it is of interest that a stock culture of the fungus which has lost the ability to form sclerotia restores the ability when grown at a low temperature, 15~20°C, on potato medium. The temperature corresponds to the field temperature in spring or autumn. Sclerotium is a hard hyphal aggregates persistent in nature over a year, and contains reserve materials. The effect of temperature may be related to the conserva-

tion of energy or metabolites necessary for sclerotial formation. Also, a diurnal change of temperature may disrupt a continuous elongation of mycelia, causing intertwining and aggregation of aerial hyphae. Such effects, however, are conditioned by medium constituents. The contents of carbon and nitrogen source in the potato medium are comparatively low, but it contains polyphenols and their precursors. There is a correlation between the regulation of vegetative growth and accumulation of precursors of melanine pigments. Though ammonium salt is a good nitrogen source for vegetative growth,¹⁾ it rather represses the melanogenesis unless concentration in medium is considerably low, as shown above. The increased protein synthesis and energy metabolism maintain a high level of -SH enzymes, probably inactivating the activity of phenoloxidase involved in melanogenesis. It seems reasonable that a combination of thiol reagents and SCL stimulates the melanogenesis in a synthetic medium containing amino acids at a low temperature. Since addition to the synthetic medium of DOPA does not need the -SH reagents and low temperature for melanogenesis, accumulation of DOPA may be controlled by a metabolic balance which is variable with temperature. A combination of DOPA and SCL induces the formation of the sclerotium-like hyphal aggregates in the synthetic medium, as shown above. SCL may stimulate the glyoxylate cycle involved in the hyphal morphogenesis, as in the case with *Sclerotinia rolfssii*.¹⁰⁾ While, DOPA is enzymatically changed to a quinone, which reacts with proteins and participates in the hardening or darkening of the hyphal aggregates through a tanning process. Cyclic nucleotides distinctly stimulate the melanogenesis in synthetic medium without adding the amino acids and thiol reagents, as shown above. Also, a combination of cyclic AMP and SCL further increases the melanogenesis and forms a sclerotium-like hyphal aggregates. Induction of conidium formation in *Neurospora* is also known to be related to increased activity of the glyoxylate cycle.¹²⁾ Since cyclic

AMP stimulates the conidiation in *Neurospora*,³⁾ it may increase the activity of glyoxylate cycle. It is supposed that, though SCL and cyclic AMP have a similar effect on regulation of carbohydrate metabolism, their modes of action are different.

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