The Morphogenetic Effect of Sulphur-Containing Amino Acids, Glutathione and Iodoacetic Acid on *Sclerotium rolfsii* Sacc.

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SUMMARY

The sulphur-containing amino acids L-cysteine, L-cystine, L-methionine, L-homocysteine thiolactone, L-homocystine and glutathione at 10^{-4} to 10^{-5} M inhibited sclerotial formation without inhibition of mycelial growth and induced basidia formation in the fungus *Sclerotium rolfsii* Sacc. Higher concentrations inhibited linear growth and production of sclerotia. The effect of L-cysteine was competitively antagonized by iodoacetic acid at a molar ratio of 30:1. Iodoacetic acid induced sclerotial formation at 3×10^{-5} to 10^{-4} M, sclerotia being produced in circles around inoculation point. The possible role of -SH groups in the morphogenesis of *S. rolfsii* is discussed.

INTRODUCTION

Sclerotia are bodies produced by some septate fungi, they consist of an outer layer of thick-walled usually darkly pigmented cells which surround a mass of thinner-walled closely interwoven hyphae (Townsend & Willets, 1954). Because of their high resistance to chemical and biological degradation, many pathogenic fungi survive in soil as sclerotia. In spite of their biological and ecological significance, little is known about the factors which affect the production of sclerotia. The formation of microsclerotia by *Verticillium albo-atrum* is affected by light and an unidentified diffusible factor (Brandt, 1964). The production of sclerotia by *Sclerotium rolfsii* has been correlated with genetic processes (Higgins, 1927), growth under suboptimal conditions (Boyle, 1961) or inhibition of the growth of mycelial tips (Henis, Chet & Avizohar-Hershenzon, 1965; Wheeler & Waller, 1965). Attempts to control the production of sclerotia by *S. rolfsii* by sulphur-containing amino acids and related compounds led to the finding that these compounds not only affect sclerotial production, but may induce the appearance of the sexual stage of this fungus, which is rather rare (West, 1947).

METHODS

The strain of *Sclerotium rolfsii* Sacc. used throughout these experiments was isolated from sugar beet. It was grown on a chemically defined medium solidified with agar prepared according to Joham (1943) and was composed of (g./l.): glucose,

40; K_2HPO_4 , 0.7; NH_4NO_3 , 1; KCl, 0.15; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4$, 0.002; $MnCl_2$, 0.002; $ZnSO_4$, 0.002; thiamine chloride, 0.0001; Bacto agar (Difco), 20; in distilled water 1 l.; adjusted to pH 7.0. The medium autoclaved for 15 min. at 121°.

The compounds to be tested were prepared at tenfold concentrations, sterilized by filtration through a Seitz filter and 1 ml. samples mixed with 9 ml. melted agar medium which was kept at 45°. Inocula were prepared from 3-day cultures, grown on defined medium agar at 30°. Agar discs 0.5 cm. in diameter, covered with fungal mycelium, were cut by a sterile cork borer and placed at the centre of the defined medium agar plates containing the tested compounds. The incubation temperature was 30°. After incubation, sclerotia were collected and counted. Hyphal weight was determined by melting the agar, washing the remaining mycelium with hot water, drying it in the oven to constant weight in aluminium planchets at 60°, and weighing. Linear growth was determined by measuring the diameter of the growing colonies.

RESULTS

Effect of sulphur-containing amino acids and related compounds on the formation of sclerotia and mycelial growth of Sclerotium rolfsii

Plates containing defined medium agar with the tested compounds were inoculated with mycelial discs and incubated for 2 weeks at 30°. The sclerotia were collected and counted, hyphal weight and linear growth determined as described. The results are summarized in Fig. 1 and Table 1. L-Cysteine, L-cystine, L-methionine, L-homocysteine thiolactone, L-homocystine and glutathione at 10^{-4} to $10^{-5}M$ inhibited sclerotia formation without affecting mycelial weight and its linear growth. Although mycelial weight was not affected significantly at concentrations greater than $10^{-4}M$, both sclerotial formation and linear growth of mycelium were inhibited, the mycelium being denser and thicker than that of the control (Pl. 1, figs. 1, 2). Little inhibition of sclerotial formation and no effect on mycelial weight and linear growth were observed with β -mercaptoethanol, 2,3-dimercaptopropanol and thioglycollic (mercapto-acetic) acid, at 10^{-4} to $10^{-5}M$. Higher concentrations (up

Table 1. *The effect of some sulphur-containing compounds on linear growth and mycelial weight of Sclerotium rolfsii*

Incubation time: 7 days. Each value represents an average of 10 replicates.

Compound	Concentration of sulphur compound (M)					
	10^{-4}		10^{-3}		10^{-2}	
	Colony diameter (mm.)	Dry weight (mg./plate)	Colony diameter (mm.)	Dry weight (mg./plate)	Colony diameter (mm.)	Dry weight (mg./plate)
L-Methionine	90*	85	85	27	65	25
L-Cysteine	90	130	71	245	42	117
L-Cystine	90	25	74	69	46	60
Glutathione	90	155	90	126	57	60
L-Homocystine	90	93	90	55	—	—
L-Homocysteine thiolactone	90	154	90	130	71	137
Control	90	145				

* Colony diameter equal to that of the Petri dish at time of estimation.

to 10^{-2} M) inhibited both linear growth and sclerotial formation. No inhibition was observed at these concentrations with L-histidine, L-tyrosine, glycine, L-threonine, L-alanine, L-phenylalanine, L-asparagine, L-lysine or L-serine. In effective treatments at concentrations of 10^{-5} to 10^{-3} M formation of sclerotia was only temporarily arrested, lasting for 2 or 3 weeks; at 10^{-2} M both restriction of mycelial linear growth and permanent inhibition of sclerotial formation occurred.

Fig. 1. Effect of some sulphur-containing compounds on production of sclerotia by *Sclerotium rolf sii*. ---●---, L-Cysteine; ---○---, L-Cystine; ---■---, L-homocysteine; ---□---, L-methionine; ---△---, L-homocysteine thiolactone; ---▲---, glutathione. Control: 490 sclerotia. Incubation: 7 days at 30°. Each value represents an average of 10 replicates.

*Effect of sulphur-containing amino acids and related compounds
on the formation of the sexual stage of Sclerotium rolf sii*

In general, compounds which inhibited sclerotial formation without inhibiting linear mycelial growth stimulated the appearance of the sexual stage of *Sclerotium rolf sii* (Fig. 2). Basidia were formed mainly on side walls of the Petri dishes, the mycelium in these areas becoming very dense, yellow-cream in colour and typically

odorous. The cream-coloured dense mycelium was sectioned by means of an ice microtome (Reichert Optical Works, Austria) to a width of $10\ \mu$ and examined by a phase-contrast microscope. Basidia, sterigmata and basidiospores typical of the perfect stage of *S. rolfii* (West, 1947) were observed.

Fig. 2. Effect of some sulphur-containing compounds on production of basidia by *Sclerotium rolfii*. Index of basidia is based on an arbitrary scale: from zero, no basidia, to 4, maximal amount of basidia. Legend, conditions and replicates, as for Fig. 1. Control: no basidia.

Effect of iodoacetic acid on the production of sclerotia by Sclerotium rolfii

Because of the inhibitory effect of sulphhydryl-containing amino acids on the production of sclerotia by *Sclerotium rolfii*, it seemed of interest to examine the possible stimulatory effect of -SH antagonists on production of sclerotia. The effect of iodoacetic acid, L-cysteine and of mixtures of these compounds on sclerotial production by *S. rolfii* is shown in Pl. 1. The optimal concentration of iodoacetic acid for sclerotial formation was 10^{-4}M . Higher concentrations ($5 \times 10^{-3}\text{M}$ or more) inhibited mycelial growth. Whereas the total numbers of sclerotia produced on plates containing iodoacetic acid were not significantly higher than that of the controls, the sclerotia were produced earlier (30 hr as compared with 4–5 days), and at the mycelial edges only, in concentric circles around the initial inoculum (Pl. 1, fig. 3), several circles of sclerotia, produced at intervals, being formed around the inoculum.

Both inhibition of mycelial growth and concentric production of sclerotia by iodoacetic acid was prevented by the addition of L-cysteine to the medium. At effective concentrations, a molar ratio of 30:1 of L-cysteine:iodoacetic acid was required for the complete mutual elimination of their effects.

DISCUSSION

Environmental factors which have been reported to affect sporulation, spore germination and sclerotial formation in fungi, include CO₂, N₂, vitamins, pH value, light, temperature, humidity, and 'atmospheric conditions' (Baldwin & Rusch, 1965). The effect of sulphur-containing amino acids on vegetative form changes in fungi has been shown by Nickerson & Falcone (1956), Nickerson (1963) and Nickerson & Bartnicki-Garcia (1964). A stimulatory effect of sulphur-containing amino compounds on the formation of the perfect stage of *Aphanomyces* root rot has been also shown (Papavizas & Davey, 1963). However, no information is available about the effect of sulphur compounds on the formation of sclerotia and basidia in fungi.

In testing the effect of amino acids on the formation of sclerotia and basidia in *Sclerotium rolf sii* only the sulphur-containing amino acids, especially L-cysteine, were active. Their addition to the medium at 10⁻⁴ to 10⁻⁵ M resulted in decreased formation of sclerotia by *S. rolf sii*, without affecting mycelial weight and its linear growth, and in the induction of the production of basidia. However, other -SH-containing compounds such as β -mercaptoethanol, 2,3-dimercaptopropanol and thioglycollic acid were much less active. The morphogenetic effect of sulphur-containing amino acids and glutathione on the formation of sclerotia and basidia, does not necessarily mean that the same mechanism is involved.

If the intracellular concentration of -SH groups is a critical factor in the morphogenesis of *Sclerotium rolf sii* then it might be possible to induce sclerotial formation by a -SH antagonist such as iodoacetic acid. Indeed, the addition of 10⁻⁴ M iodoacetic acid resulted in stimulation of sclerotial formation within 30 hr from inoculation time. However, induction was limited to hyphal tips in the periphery of the fungal colony, the sclerotia forming a circle around the inoculation point. Moreover, the linear growth of the vegetative mycelium continued and new circles of sclerotia were formed around the first one (Pl. 1, fig. 3). Thus, iodoacetic acid enhanced sclerotial formation and changed the distribution of sclerotia on the agar plate. These phenomena may be connected with specific concentrations of iodoacetic acid at the mycelial tips, which result in a lower intracellular concentration of -SH groups. It seems now that inhibition of linear growth *per se* does not induce sclerotial formation, since both L-cysteine and iodoacetic acid inhibited linear mycelial growth at 10⁻² M and 5 $\times$ 10⁻³ M, respectively; but whereas L-cysteine inhibited sclerotial formation as well, iodoacetic acid induced it.

Iodoacetic acid and L-cysteine competitively antagonized each other at a ratio of 1:30. This high ratio may be due to differences in permeability or in reaction rate inside the hyphal cells. The rate of reaction between these compounds at the pH value of the growth medium (7.0) is relatively slow (Cecil & McPhee, 1959).

The perfect stage of *Sclerotium rolf sii* Sacc. (*Pellicularia rolf sii*) is rather rare (West, 1947) and no information is available about the factors involved in its formation. We believe that this is the first instance of a stimulation of the appearance of the perfect basidial stage of this fungus on an artificial growth medium.

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EXPLANATION OF PLATE

PLATE 1. Effect of L-cysteine and iodoacetic acid on *Sclerotium rolfsii*.

Fig. 1. Normal growth of *S. rolfsii* on defined medium agar after incubation for 10 days at 30°. Brown sclerotia are especially abundant at the edges of the Petri dish. $\times 0.66$.

Fig. 2. Growth of *S. rolfsii* in the presence of 10^{-2} M-L-cysteine. Mycelium is more dense than that seen in fig. 1. Only two sclerotia are seen; linear growth is significantly restricted. $\times 0.66$.

Fig. 3. Effect of 10^{-4} M-iodoacetic acid on growth pattern of *S. rolfsii*. Mycelium not affected, but sclerotia are formed in circles around the inoculum and are bigger than those seen in fig. 1. $\times 0.66$.

Fig. 4. Mutual inhibition of the effects of L-cysteine (10^{-4} M) and iodoacetic acid (3×10^{-5} M). A normal growth of *S. rolfsii* is seen. $\times 0.66$.