Nucleic Acid Metabolism in Developing Sclerotia of the Rice Sheath Blight Fungus (*Rhizoctonia solani* Kühn)

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SUMMARY

Sclerotia of the rice sheath blight fungus, *Rhizoctonia solani* Kühn, were examined for their capacity to synthesize RNA, DNA, polyadenylic acid, and for their ribonuclease and protease activities during development on a liquid medium. During maturation, sclerotia appeared to pass through three phases. The first, a period of rapid RNA synthesis, was accompanied by a decline in the content of polyA(+)-RNA and a decrease in the permeability of the sclerotia. These changes suggested that the first 2 h were a time of reorientation of cellular metabolism. This was followed by a period of general synthesis lasting for 30 h which was accompanied by a further decrease in permeability, increased protease and ribonuclease activities and a rapid build-up of ribosomes. The second period terminated when protease activity declined, the other activities stabilized and the sclerotia began to turn brown. The third period, maturation, lasted for 15 days at which time the sclerotia began to float.

INTRODUCTION

Fungi can survive adverse conditions or accomplish reproduction vegetatively through a variety of spore forms, such as conidia. In addition, many fungi produce storage organs called sclerotia. These are usually formed in the soil when dense masses of hyphae arise and the short cells enlarge laterally until a compact mass of pseudoparenchymatous tissue is formed, the cells of which become polyhedral by mutual pressure. The compact mass is filled with food materials, and the outermost cells form a protective cortex which may be light in colour or, more often, brown or black. Under certain conditions, new mycelia grow out from the sclerotia to initiate fungal growth.

The morphological changes associated with differentiation of sclerotia of the rice sheath blight fungus, *Rhizoctonia solani* Kühn, have been examined (Hashiba, Yamaguchi & Mogi, 1972; Hashiba & Mogi, 1975), but few studies of the biochemical mechanisms of initiation and development of the sclerotia have been reported (Trevethick & Cooke, 1971; Chet, Retig & Henis, 1972; Wong & Willetts, 1974). Since the disease is spread by the sclerotia floating on water (Hashiba *et al.*, 1972), it is important to clarify the biochemical mechanisms of sclerotial development. As a first step in the characterization of these mechanisms, we describe some of the macromolecular syntheses associated with sclerotial formation by *R. solani*.

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METHODS

Organism and media. The culture of *Rhizoctonia solani* Kühn used in this study was isolated from an infected rice plant as described previously (Hashiba *et al.*, 1972; Hashiba & Mogi, 1975). Cultures were maintained on modified Murashige & Skoog's (1962) medium which lacked kinetin, indole-2-acetic acid and inositol. Sclerotial initials were formed after 5 days at 25 °C. The time when recognizable initials appeared was taken as the zero time for observations and the ages of sclerotia were measured in relation to this. Sclerotia were similar to those formed on infected plants, including the development of buoyancy.

In experiments on the properties of sclerotia, 300 Petri dishes each containing 20 ml modified Murashige & Skoog's medium were inoculated centrally with 5 mm discs cut from the margin of a colony growing on Czapek's agar medium. At harvest, sclerotia were separated from the underlying mycelia, rinsed in ice-cold water and frozen in liquid nitrogen. The sclerotia were not stored for more than 3 h. Sclerotial dry weight was determined by drying at 110 °C for 10 h and weighing to the nearest mg. For grinding, 0.5 g sclerotia was homogenized for 2 to 3 min with 2 vols glass beads (1.0 mm) using a Braun MSK homogenizer (Bronwill Scientific, Rochester, New York, U.S.A.) cooled with liquid CO₂.

Analysis of RNA. RNA was extracted from sclerotia as described by Lovett & Haselby (1971). The incorporation of [2,8-³H]adenosine was studied by adding 0.1 µCi/ml medium 2 h before harvesting. Sclerotia were homogenized in 15 ml of a solution comprising 7.5 ml TIPS [100 ml 0.01 M-Tris pH 7.6, 1.0 g triisopropyl-naphthalenesulphonic acid, 6 g 4-aminosalicylic acid and 0.37 g KCl] and 7.5 ml phenol-cresol solution (50 ml redistilled phenol, 7 ml *p*-cresol, 20 ml water and 50 mg 8-hydroxyquinoline). The phases were separated by centrifuging for 5 min and the RNA was recovered as described by Lovett & Haselby (1971).

Extraction of adenosine. To extract [2,8-³H]adenosine which was incorporated by sclerotia but not utilized, the sclerotia were washed with cold distilled water, blotted dry and stirred for 10 min with 20 ml cold 5 % (v/v) trichloroacetic acid. After centrifuging, the supernatant solution was brought to volume and a sample was counted using liquid scintillation techniques.

Analysis of DNA. DNA was isolated by the procedure of Marmur (1961) as modified by Villa & Storck (1968). After labelling with [2,8-³H]adenosine, sclerotia were homogenized in 15 ml 0.15 M-NaCl/0.1 M-EDTA, pH 8.0, and then mixed for 30 min on a roller mill with an equal volume of chloroform/isoamyl alcohol/88 % (v/v) phenol (24:1:25, by vol.). The whole mixture was centrifuged briefly in a refrigerated centrifuge at 12 000 *g*, and the aqueous layer was precipitated with 14.7 mg potassium acetate and 2.3 ml ethanol per ml and stored at -20 °C. The precipitate was dissolved in 2 ml buffer (pH 7.6), containing, per ml, 10 µmol Tris, 10 µmol EDTA and 100 µmol NaCl (TEN buffer) and heated at 37 °C in the presence of 2.3 mg α-amylase, 5 µg RNAase A and 2 µg RNAase T1. After 2 h, 0.6 mg Pronase was added and the solution incubated for another 2 h. After cooling to room temperature, 3 ml chloroform/isoamyl alcohol/88 % phenol (24:1:25, by vol.) was added, and the DNA was deproteinized by rolling for 5 min. The aqueous layer formed after centrifugation was dialysed overnight at room temperature against TEN buffer.

Assay of polyadenylic acid. DNA-free RNA was extracted from sclerotia in a similar manner to that described previously (Edmonds & Caramela, 1969; Manahan, App & Still, 1973). Sclerotia were homogenized in buffer (pH 9.0) containing 100 mM-Tris-HCl, 10.0 mM KCl and 0.5 mM-MgCl₂, deproteinized with chloroform/88 % phenol (1:1, v/v), and the RNA was precipitated using potassium acetate and absolute ethanol. DNA was removed

from the RNA extract by hydrolysis with 20 μg RNAase-free DNAase. The RNA was deproteinized with an equal volume of 88 % phenol, and then re-precipitated. The polyadenylate content of the RNA was determined using the hydroxylapatite procedure of Greenberg & Perry (1972).

Ribosomes. Ribosomes were isolated by the procedure of Yaniv & Staples (1969), and included free as well as bound ribosomes. Sclerotia were homogenized in 15 ml buffer (pH 7.5) containing 0.45 M-sucrose, 50 mM-Tris, 5 mM-MgCl₂ and 5 mM-2-mercaptoethanol. The homogenate was centrifuged for 15 min at 20000 *g* and the supernatant solution (S 30) was stored at -20°C . Ribosomes were obtained from the S 30 solution by centrifuging for 60 min at 151000 *g*. The pellet was resuspended in buffer (pH 7.5) containing 50 mM-Tris, 15 mM-KCl, 5 mM-MgCl₂ and 5 mM-2-mercaptoethanol, and centrifuged briefly at 20000 *g* to remove aggregates.

Ribonuclease activity. Ribonuclease activity was assayed according to the procedure of Kalnitsky, Hummel & Dierks (1959) by measuring the amount of acid-soluble oligonucleotide liberated at pH 7.5, the optimum pH of the mycelial enzyme.

Protease activity. Protease was assayed using azure-hide powder as substrate (Rinderknecht *et al.*, 1968). Samples (2 ml) were incubated with 3 ml Tris-HCl buffer, pH 7.5, for 10 min at 39°C , and then poured into test-tubes containing 15 mg hide powder. The tubes were shaken gently to wet the powder and every 5 min thereafter. After 15 min, the suspension was filtered through Whatman no. 1 filter paper, and the extinction of the filtrate at 595 nm was determined against a water blank. The amount of protein hydrolysed was estimated using Pronase as a standard.

Special chemicals. The following solutions were obtained from Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.: α -amylase (42 mg ml⁻¹); pancreatic ribonuclease A (9 mg ml⁻¹) heated at 80°C for 10 min; RNAase T1 (1 mg ml⁻¹); and RNAase-free deoxyribonuclease (2 mg ml⁻¹). Azure-hide powder and Pronase (nuclease-free) were obtained from Calbiochem and diluted to 2 mg ml⁻¹ with water. [2,8-³H]Adenosine (100 μCi μg^{-1}) and [5-³H]polyuridylylate (10 μCi μmol^{-1}) were obtained from Miles Laboratories, Kankakee, Illinois, U.S.A.; and hydroxylapatite was from Bio-Rad Laboratories.

Replication. The data used in all figures are the average of three experiments each replicated twice.

RESULTS

Description of sclerotial formation

As described previously (Hashiba *et al.*, 1972; Hashiba & Mogi, 1975), young sclerotia were composed of compact masses of hyphal cells which increased in size for 30 h; at this time they began to turn brown (Fig. 1). The width of the cells in the central mass increased rapidly until browning was completed at about 40 h, while cell-wall thickness increased for 15 days.

Both fresh weight and dry weight increased steadily for 72 h after the appearance of sclerotial initials (Fig. 2a); but remained relatively constant (not shown) after the end of this period.

Fig. 1. Diagram showing structural changes during sclerotium formation in *Rhizoctonia solani*. (a) Initiation of sclerotium; 0 h old. (b) Sclerotium 10 h old. (c) Sclerotium 20 h old. (d) Sclerotium 30 h old; the browning process has begun, the sclerotium has reached its maximum size and many cells of the outer layer have emptied. (e) Sclerotium 40 h old; the cells have reached their maximum width. (f) Buoyant sclerotium, 15 days old; the cell-wall thickness has reached a maximum. Arrows indicate empty cells.

Changes in nucleic acids

Total RNA and DNA. The RNA content approximately doubled during the first 2 h after the appearance of sclerotia (Fig. 2*b*). Thereafter, the rate of increase was less rapid and was related to the increase in dry weight (Fig. 3*a*). The total DNA content increased steadily throughout sclerotial development (Fig. 2*b*).

Synthesis of RNA during the formation of sclerotia was followed by incorporation of [2,8-³H]adenosine for short periods (Fig. 2*c*). In spite of the steady increase in RNA content during the development of sclerotia, the amount of [2,8-³H]adenosine incorporated into RNA decreased markedly, suggesting that the sclerotia were becoming impermeable to the adenosine.

Free adenosine. The amount of ³H-labelled adenosine absorbed by sclerotia but not incorporated into RNA declined continuously after induction (Fig. 2*d*). After 20 h, very little adenosine was taken up, again suggesting that the sclerotia had become impermeable to the compound.

Polyadenylic acid. The polyadenylic acid (polyA) content was highest in RNA from mycelia (Fig. 2*e*). After induction, the amount of polyA rapidly declined from 26 µg/mg RNA to 13 µg/mg RNA in only 2 h. Thus sclerotia have a lower content of polyA(+)RNA than the mycelia from which they were derived. Possibly the polyA(+)RNA in sclerotia represents a stable form of mRNA as is typical of quiescent fungal propagules.

Ribosomes. The ribosome content of sclerotia increased steadily during the 72 h after the appearance of initials (Fig. 2*f*). It increased much more rapidly than dry weight during the first 30 h of sclerotial development, but then the rate declined (Fig. 3*b*).

Enzyme activities

Ribonuclease. The activity of ribonuclease approximately doubled during the first 2 h (Fig. 2*g*). Thereafter, it continued to increase in sclerotia and reached about 1.15 µg/100 sclerotia after 62 h.

Protease. After induction of sclerotia, protease activity increased about 4.5-fold and reached a maximum at 30 h (Fig. 2*h*). The enzyme activity then declined and could not be detected after 60 h.

Fig. 2. Changes in several parameters during the development of sclerotia of *R. solani*. (a) Fresh weight (○) and dry weight (●). (b) RNA (○) and DNA (●) contents. (c) Incorporation of adenosine into RNA (○) and DNA (●). (d) Content of free ^3H -labelled adenosine. (e) Content of polyA(+)RNA. (f) Ribosome content. (g) Ribonuclease activity. (h) Protease activity.

DISCUSSION

In *R. solani*, sclerotia first appeared as a more or less compact mass of hyphal cells (Hashiba *et al.*, 1972; Hashiba & Mogi, 1975). The cells in the central mass increased in diameter and many mitochondria and ribosomes were present. After 30 h, when the size of sclerotia and the cell width reached a maximum, the amount of mitochondria and ribosomes in individual cells appeared to be reduced. Cells in the central mass accumulated glycogen,

Fig. 3. Changes in (a) RNA and (b) ribosome contents, expressed on a dry weight basis, during the development of sclerotia of *R. solani*.

while cells in the outer layer began to lose their contents. After 15 days, the sclerotia became buoyant. Our studies were confined to the first 3 days after induction, and so did not involve the development of buoyancy.

Our data suggest that sclerotia pass through three characteristic periods of development after induction. First, there is an intense 2 h period of RNA synthesis during which the polyA(+)RNA content declines and the cells become impermeable. Secondly, there is a general period of RNA synthesis during which the activities of protease and RNAase increase. Protease activity reaches a maximum at 30 h; and then the third period begins as the cells in the outer layers begin to empty. The 30th hour also marks the time when impermeability reaches a maximum, and the rate of ribosome accumulation has slowed. The period after 30 h continues for 15 days until the sclerotia become buoyant. This buoyancy is an important property for the spread of the disease (Hashiba & Mogi, 1975). It will be of interest to learn how these three rather distinct periods of sclerotial development are co-ordinated when the sclerotia appear to be impermeable to the external environment.

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