SOME ENZYMES IN GERMINATING SCLEROTIA OF *SCLEROTIUM ROLFSII* SACC*

FU-KUEN LIN, and LUNG-CHI WU**

(Received July 28, 1967)

INTRODUCTION

Sclerotium rolfsii Sacc., the causal organism of the disease southern blight, is long known to be polyphagous (Weber, 1931) and to distribute generally in the warmer parts of the temperate zone and in the tropics around the world.

In Taiwan, a serious loss in soybean and peanut yield is frequently caused by this fungus every year through the growing stages of these crops when the weather is characterized by rainy season.

Since Peter Henry Rolfs described and reported the disease caused by this fungus in 1892, many papers have been reported about the physiology of this fungus (Higgins, 1927; Rosen and Shaw, 1929; Joham, 1943; Johnson and Joham, 1954; Abeygunawardena and Wood, 1957; Townsend, 1957; Husain, 1958; Bateman and Beer, 1965; Heins *et al.*, 1965). However, for the present, little is known about the enzymatic changes during the course of sclerotial germination of this fungus and even of other fungi. In order to extend information for better understanding of sclerotial germination, the present study was undertaken.

MATERIALS AND METHODS

Three isolates of *Sclerotium rolfsii* (#1, #5, and #10) isolated from infected soybean plants from different areas in the Ping-tong Prefecture were used. Isolates #1 (from Chiu-lou) and #5 (from Kaohsiung District Agricultural Improvement Station) were morphologically similar, producing small sclerotia; #10 (from Li-kaoung) produced large sclerotia, round or irregular. These fungi were carried in stock cultures on Joham's agar medium, and their 3-transfer generations on Joham's agar were used in the following experiments. Joham's solution contains: 0.0015 M $MgSO_4$, 0.004 M K_2HPO_4, 0.002 M KCl, 0.0125 M NH_4NO_3, 0.1 ppm thiamine chloride, 4.0% glucose, and 2.0 ppm each of iron, zinc, and manganese. When agar (1.9 %, W/V) was added, it was called

* Portions of an M. S. thesis. Research was partly supported by JCRR.

** Respectively, Research Assistant and Professor, Department of Plant Pathology and Entomology, National Taiwan University.

Joham's agar medium (Joham, 1943). Sclerotia were produced by growing the fungus on Joham's agar medium in 9-cm. Petri dishes which were incubated at 29–31°C. in the light. In this case, sclerotia were used as inocula. The sclerotia were removed after incubation period of 20 days by washing the surface of media with distilled water. The harvested sclerotia were collected and dried on filter paper in a Buechner funnel, then stored in a 4°C. refrigerator. The sclerotia stored at 4°C. for one week to 2 months were used for enzymic study. Joham's solution was employed for sclerotia germination of the three isolates. About 0.15 g. of sclerotia were incubated on a sterile filter paper containing 3 ml. of Joham's solution at 35°C for 0, 12, 18, and 24 hours. After various incubation periods, the sclerotia were collected and washed in three changes of distilled water. These sclerotia were immediately used for the enzyme assays. A portion of sclerotia (0.5 g.) from each incubation period was dried in an air oven at 85°C. for 24 hours, then placed in a desiccator and weighed. A sclerotium was considered to be germinated if the length of germ tube was visible with the aid of magnifying glass (×15).

To make cell-free extracts (CFE), 1 g. of sclerotia was placed in a pre-cooled mortar and ground with 2 g. of acid-washed sand in 8 ml. of 0.25 M sucrose solution. The enzyme preparation was prepared by centrifugation of the homogenate at 1000 g. for 30 minutes at 4°C. The supernatant was used in all enzyme assays. The supernatant was microscopically free of intact cells. Spectrophotometric determinations were made with a Unicam SP. 500 spectrophotometer at 26°C. The cuvettes used were of 1-cm. light path and 3-ml. capacity.

Water used was doubly (glass) distilled. Common chemicals used were of chemical purity. Special reagents indicated below were of reagent grade, and their sources were (i) Sigma Chemical Co: nicotinamide-adenine dinucleotide phosphate (NADP), glucose-6-phosphate, 6-phosphogluconate, glycylglycine, fructose-1, 6-diphosphate, nicotinamide-adenine dinucleotide (reduced form, NADH), (ii) Wakō Pure Chemical Industries Ltd.: sodium citrate, (iii) Pabst Laboratories: NADP.

Protein in cell-free preparation was determined by Biuret method (Layne, 1957), For enzyme assays, endogenous activity was measured by omitting the substrate and boiled CFE was served as a control.

The following assays were carried out:

1) Fructose-1,6-diphosphate aldolase (EC 4.1.2. b).—The methods of Bruns and Bergmeyer (1963) was adopted. The principle of this assay is as follows: The triose phosphates (dihydroxyacetone phosphate and D-glyceraldehyde phosphate) formed from fructose-1,6-diphosphate by the action of aldolase

are trapped with hydrazine. After deproteinization with trichloroacetic acid (TCA) they are hydrolyzed by NaOH. The free trioses are treated with 2,4-dinitrophenylhydrazine, yielding a mixture of methylglyoxal-2,4-dinitrophenylosazone and pyruvic acid-2,4-dinitrophenylhydrazine. Both compounds are dissolved in alkali, forming red color with an absorption maximum between 535 and 540 mμ.

The reaction mixture contained collidine-hydrazine solution 1.75 ml.; 0.1 M fructose-1.6-diphosphate (FDP), 0.25 ml.; CFE; and distilled water to 3 ml. The reaction mixture was incubated for 60 minutes at 37°C. (water bath), and the reaction was stopped by the addition of 3 ml. of 10% TCA. A blank was prepared and incubated in the same way except that FDP was added after the addition of TCA. The mixture was filtered and mixed 1 ml. of filtrate with 0.75 ml. of 0.75 N NaOH, thereafter, kept for 10 minutes at room temperature until alkali labile triose phosphate was hydrolyzed. One ml. of 2,4-dinitrophenylhydrazine solution (0.1% in 2N HCl) was added to the reaction mixture, mixed, and incubated for 10 minutes at 37°C. in a water bath. Between 3 and 15 minutes after adding 8.25 ml. of 0.75 N NaOH, optical density (brown-red color) was measured against the blank with a spectrophotometer at 540 mμ Thereafter the color intensity was found to be decreased. It was about 90% of its original value after 30 minutes and about 50% after 2 hours. The specific activity of aldolase was expresses as μ moles FDP cleaved per hour per mg. protein.

2) Aconitase (EC 4.2.1.3).—The assay is based on the transformation of citrate to cis-aconitate and water, and the absorbance at 240 mμ by cis-aconitate is measured (Anfinsen, 1955).

The assay system contained sodium citrate (0.03 M in 0.05 M potassium phosphate buffer, pH 7.4), 2.9 ml; CFE; and water to 3 ml. One unit of enzyme was defined as that amount which caused an initial rate of increase in optical density ($\triangle E_{240}$) of 0.001 per minute under the above conditions. Specific activity was expressed as units per milligram of protein.

3) Glucose-6-phosphate dehydrogenase (EC 1.1.1.49).—The assay depends on the direct oxidation of glucose-6-phosphate by $NADP^+$ (Kornberg *et al.*, 1955). The rate of NADPH formation is a measure of the enzyme activity and it is followed by means of the increase in absorption at 340 mμ.

The assay system contained 1.5×10^{-3} M NADP, 0.1 ml.; 0.04 M glycylglycine buffer (pH 7.5), 0.25 ml.; 0.1 M $MgCl_2$, 0.2 ml.; 0.02 M glucose-6-phosphate, 0.2 ml.; CFE; and water to 3 ml. A unit of enzyme was defined as that amount which caused an initial change in optical density of 1.000 per minute (room temperature 23° to 25°C) under the above conditions. Specific activity was

expressed as units per milligram of protein.

4) 6-Phosphogluconic dehydrogenase (EC 1.1.1.44).—The assay depends on the oxidation of 6-phosphogluconate by NADP. Its activity is assayed by measuring the rate of reduction of NADP at 340 mμ (Kornberg *et al.*, 1955).

The assay was made in the same manner as glucose-6-phosphate dehydrogenase), except that 6-phosphogluconate (0.02 M, 0.2 ml.) was the substrate. The definition of unit and expression of specific activity for this enzyme was the same as already described in the case of glucose-6-phosphate dehydrogenase.

Each treatment done in this report was triplicate and figures given were the mean of the triplicate samples of duplicate experiments.

RESULTS

(A) *Changes in percentage germination and weight.*

The periods required for visible length of germ tube were 12 hours for isolates #1 and #5, 15 hours for isolate #10. The percentage germination increasd gradually with the lapse of time. For 24-hour incubation, the percentage germination of the isolates #1, #5 and #10 was respectively 97, 98, and 88 (Fig. 1).

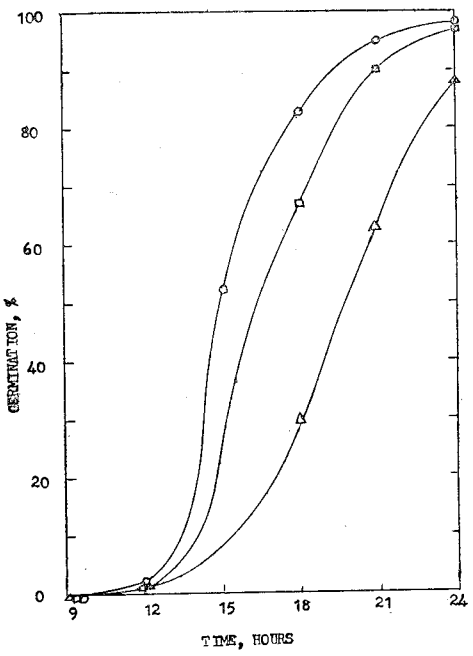

Fig. 1. Percentage germination of sclerotia of *Sclerotium rolfsii*, isolates #1 (□—□) #5 (○—○), and #10 (△—△), during different periods of incubation,

The dry weight of the sclerotia changed slightly during the course of germination, namely within the range of 1.02–1.06 times of the initial dry weight in 24-hour incubation (Table 1). No relationship between the dry weight change and percentage germination was found (Fig. 1 and Table 1).

Table 1. *Changes in dry weight of sclerotia of* ***Sclerotium rolfsii*** *during germination process at 35°C*

Isolate	Dry weight (g) of Sclerotia incubated for			
	0 hr.	12 hr.	18 hr.	24 hr.
# 1	0.2402*	0.2386	0.2401	0.2231
# 5	0.2286	0.2163	0.2321	0.2338
#10	0.2351	0.2256	0.2203	0.2489

* Five tenths gram sclerotia were dried at 85°C for 24 hours before weighing.

(B) *Changes in enzyme activity.*

Aldolase—The initial aldolase specific activity was low and about the same among three isolates (Table 2). The specific activity in three isolates increased slightly at the time of germ tube formation. As the time of incubation progressed, it changed in different ways (Fig. 2). For isolates #1 and #10, the increase in specific activity from initial to final assay was 1.01 and 2.45 times, respectiely. For isolate #5, the final specific activity decreased (Table 2). Total activity increased at the time when the germ tube formed (Fig. 3). However, the total activity was decreased in the isolates #1 and #5, whereas it was increased 2.58 times in the case of isolate #10 (Table 3) when the measurement was carried out with sclerotia incubated for 24 hours at 35°C.

Table 2. *Initial and final specific activity of enzymes in germinating sclerotia of* ***Sclerotium rolfsii***

Enzyme	Specific activity								
	#1			#5			#10		
	Initial (I)*	Final (F)*	F/I	Initial	Final	F/I	Initial	Final	F/I
Aldolase	0.089	0.090	1.01	0.078	0.036	0.46	0.067	0.154	2.45
Glucose-6-phosphate dehydrogenase	0.099	0.061	0.62	0.057	0.086	1.50	0.047	0.038	0.81
6-phosphogluconic dehydrogenase	0.020	0.012	0.60	0.029	0.062	2.14	0.058	0.037	0.64
Aconitase	4.891	12.807	2.62	4.858	10.319	2.13	2.619	13.916	5.31

* The enzyme activity was measured at 0 time and 24-hour incubation of sclerotia for the initial and final readings, respectively.

Glucose-6-phosphate dehydrogenase—Glucose-6-phosphate dehydrogenase specific activity varied from 0.047 to 0.099 among three isolates (Table 2). For isolate

#1 and #5, the specific activity showed a peak at 18-hour incubation, thereafter declined. In the case of isolate #10, specific activity decreased gradually with the lapse of time (Fig. 2). The pattern of change in total activity was not similar to that of specific activity (Fig. 3). The final total activity was decreased in isolate #1 and #10, while an increase up to 1.27 times was obtained in the case of isolate #5 (Table 3).

Table 3. *Initial and final total activity of enzymes in germinating sclerotia of* ***Sclerotium rolfsii****

Enzyme	Total enzyme activity								
	#1			#5			#10		
	Initital (I)**	Final (F)**	F/I	Initial	Final	F/I	Initial	Final	F/I
Aldolase	0.329	0.253	0.77	0.353	0.227	0.64	0.309	0.796	2.58
Glucose-6-phosphate dehydrogenase	0.629	0.392	0.62	0.333	0.424	1.27	0.344	0.260	0.76
6-phosphogluconic dehydrogenase	0.115	0.098	0.85	0.148	0.512	3.46	0.425	0.258	0.61
Aconitase	22.925	90.530	3.94	22.925	78.215	3.41	20.866	87.671	4.21

* Total enzyme activity was calculated by multiplying the specific activity by the total protein of 1 ml. cell-free extract of sclerotia.

** The enzyme activity was measured at 0 time and 24-hour incubation of sclerotia for the initial and final readings, respectively.

6-Phosphogluconic dehydrogenase—The initial specific activity of 6-phosphogluconic dehydrogenase was low in isolates #1 and #5, i. e. 0.02 and 0.029, respectively. However, isolate #10 had a higher specific activity, 0.058 (Table 2). In isolate #1, the specific activity increased with the lapse of time and culminated at 18-hour incubation, then decreased sharply. In isolate #5, there was an early decrease, then increased gradually. In isolate #10, contrarily, there was an early increase, then it decreased gradually (Fig. 2).

The changes in total activity were similar to the pattern of specific activity in both isolates #1 and #5. In the case of #10, total activity change was not in accordance with specific activity change (Fig. 3). It decreased during the course of germination. The final total activity was increased to 3.46 times the initial activity in isolate #5 while a peak was obtained at 18 hour-incubation in isolate #1 (Table 3).

Aconitase—The initial aconitase specific activity was low, ranging from 2.619 to 4.891, among the three isolates (Table 2). For all three isolates, it increased during the sclerotial germination. The increases were 2.62, 2.13, and 5.31 times the initial activity in isolates #1, #5, and #10, respectively. Changes in total activity were in the same way. The final increases were 3.94, 3.41, and 4.21 times the initial activity in the same isolates (Table 3). The patterns

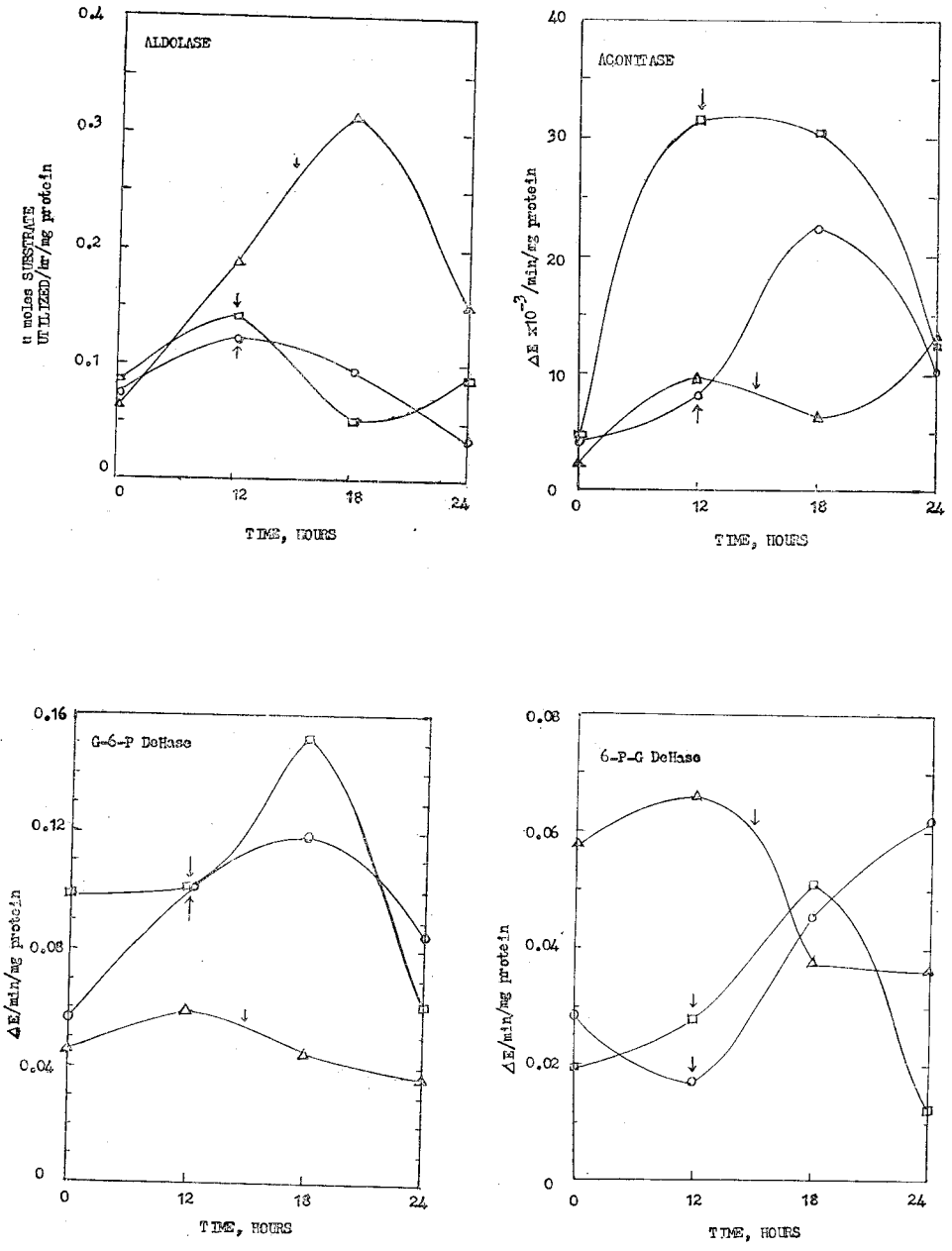

Fig. 2. Changes in enzyme specific activity during the germination of sclerotia of *Sclerotium rolfsii*, isolates #1 (□—□), #5 (○—○), and #10 (△--△). The arrow indicates the time when the germ tube becomes visible.

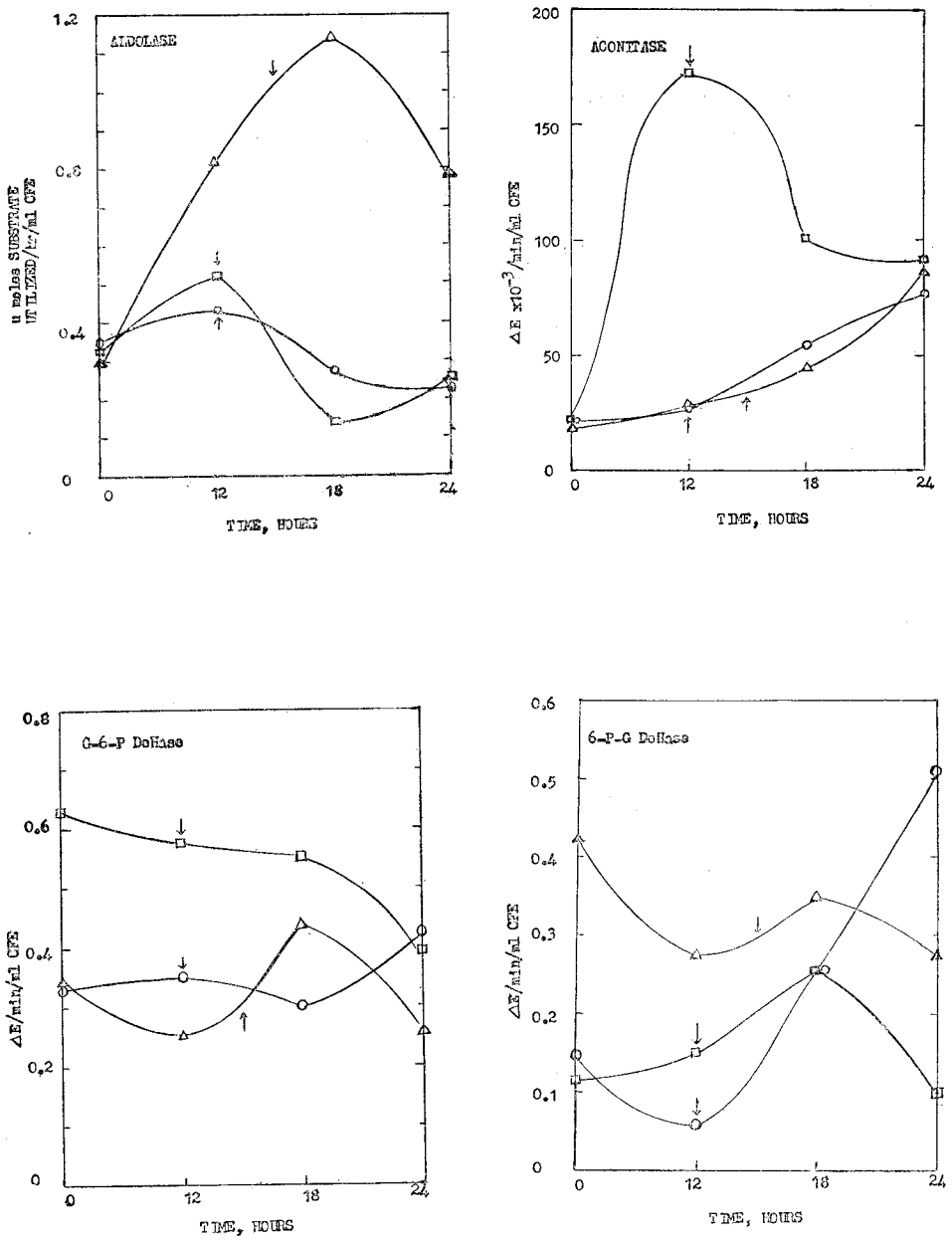

Fig. 3. Changes in enzyme total activity during the germination of sclerotia of *Sclerotium rolfsii* isolates #1 (□—□), #5 (○—○), and #10 (△—△). The arrow indicates the time when the germ tube becomes visible.

in the changes of aconitase activity varied among the isolates tested (Fig. 2 and 3).

DISCUSSION

Changes in specific activity and total activity of different enzymes during the course of sclerotial germination were observed. The total activity of enzyme was a measure of metabolic rate in the sclerotia. It might result from a small quantity of enzymes of high specific activity or a large amount of enzymes of low specific activity.

No obvious change in dry weight of sclerotia during the course of germination was observed. This might be due to the fact that mycelia produced by the sclerotial germination contributed little to the weight of large, 'heavy' sclerotia. Yet, the fluctuation of dry weight during the germination was puzzling.

Among the four enzymes detected (aldolase, aconitase, glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase), aldolase activity, specific or total, increased in all isolates tested when the germ tube sent out. The same was true for aconitase. On the other hand, the changes in specific and total activities of aldolase varied depending on the isolates examined. The activities of aconitase increased all the way during the course of germination. In the case of 6-phosphogluconic dehydrogenase and glucose-6-phosphate dehydrogenase, generally, there was a decrease in specific and total activities except isolate #5.

The relative pathogenicity of three pathogenic species of *Alternaria* was found to be associated with the corresponding change in the aldolase activity (Verma, 1965), but it was not found in the case of five races of *Piricularia oryzae* (Wu *et al.*, 1966). Since the relative pathogenicity of *sclerotium rolfsii*, (isolates #1, #5, and #10) was not established, the relation between the pathogenicity and aldolase activity was not known.

The present study showed that dormant sclerotia possessed low metabolic potentials which needed for repairing and maintainig their metabolic apparatus. This low metabolic activity might also account for its long longevity. When sclerotia germinated, they probably showed a shift in metabolic pathways, from hexosemonophosphate shunt to Krebs cycle. The increase in aconitase activity during the germination of sclerotia might probably show an active operation of Krebs cycle which resulted in a large amount of energy production that needed for the synthesis of more enzymes and other metabolites for new metabolic events to take place, as in the case of spore germination (Gottlieb, 1964; Ohmori and Gottlieb, 1965). Gottlieb stated that spore germination was a process by which a spore was transformed from a dormant stage, of low

metabolic activity, to one of high activity. Formation of the germ tube was the outward and visible sign that this metabolic change was complete. This definition for spore germination is probably also applicable to the case of sclerotial germination.

SUMMARY

Hexosemonophosphate shunt was found to be operated in ungerminating and germinating sclerotia of *Sclerotium rolfsii* (isolates #1, #5, and #10). Of these three isolates except #5, glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase decreased at 24-hour incubation. Aldolase and aconitase activities were also detected in the ungerminating and germinating sclerotia of these isolates. This showed that the Embden-Meyerhof pathway and Krebs cycle were operated in the sclerotia. Aldolase activity increased at the time when germ tube was visible. Aconitase activity increased during the course of germination of sclerotia. This might indicate that more energy was required for sclerotia to germinate. The changes in enzyme activity of the four enzymes mentioned above varied with the isolates tested.

白絹病菌菌核發芽過程之酵素研究

林 福 坤　吳 龍 溪

白絹病菌三菌株（#1, #5, 及 #10）之未發芽及發芽菌核內有 Hexosemonophosphate shunt, Embden-Meyerhof pathway 及 Krebs cycle 之存在。在所試三菌株中，除 #5 菌株外，Glucose-6-phosphate dehyrogenase 及 6-phosphogluconic dehydrogenase 在 24 小時培養之菌核內，活力降低；而 aconitase 之活力則增高。Aldolase 之活力在菌核形成發芽管時增加。Aconitase 在發芽過程活力之增高可能表示菌核在發芽時需要較多之能量。上述四酵素活力之變化隨菌株而異。

LITERATURE CITED

ABEYGUNAWARDENA, D. V. W. and R. K. S. WOOD. Factors affecting the germination of sclerotia and mycelial growth of *Sclerotium rolfsii* Sacc. Trans. Brit. Mycol. Soc. **40**: 221-231, 1957.

ANFINSEN, C. B. Aconitase from pig heart muscle, p. 695-698. In S. P. Colowick and N. O. Kaplan (ed.) Methods in enzymology. Vol. 1, Academic Press, New York. 1955.

BATEMAN, D. F. and S. V. BEER. Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotium rolfsii*. Phytopathology **55**: 204-211, 1965.

BRUNS, F. H. and H. U. BERGMEYER. Fructose-1,6-diphosphate aldolase, p. 724-731. In H.U. Bergmeyer (ed.) Methods of enzymatic analysis. Academic Press, New York. 1963.

GOTTLIEB, D. Germination of fungus spores. Endeavour **23**: 85-89, 1964.

HENIS, Y. I. and Z. A. HERSHENZON. Nutritional and mechanical factors involved in mycelial growth and production of sclerotia by *Sclerotium rolfsii* in artificial medium and amended soil. Phytopathology **55**: 87-91, 1965.

HIGGINS, B. B. Physiology and parasistism of *Sclerotium rolfsii*. Phytopathology **17**: 417-448, 1927.

HUSAIN, A. Production of cellulolytic enzymes by *Sclerotium rolfsii*. Phytopathology **48**: 388-340, 1958.

JOHAM, H. C. A nutritional study of the fungus *Sclerotium rolfsii*. M. S. thesis. A. and M. College of Texas. Jan. 1943 (Cited in G. M. Watkins, Physiology of *Sclerotium rolfsii* with emphasis on parasitism. Phytopathology **51**: 107-124, 1961).

JOHNSON, S. P. and H. C. JOHAM. Some physiological notes on *Sclerotium rolfii* I. Utilization of certain carbon compounds. Pl. Dis. Report. **38**: 602-606, 1954.

KORNBERG, A., B. L. HORECKER, and P. Z. SMYRNIOTIS. Glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, p. 323-327. In S. P. Colowick and N. O. Kaplan (ed.) Methods in enzymology. Vol. 1, Academic Press, New York. 1955.

LAYNE, E. Spectrophotometric and turbidimetric methods for measuring protein, p. 447-454. In S. P. Colowick and N. O. Kaplan (ed.) Methods in enzymology. Vol. 3, Academic Press, New York. 1957.

OHMORI, K. and D. Gottlieb. Development of respiratory enzyme activities during spore germination. Phytopathology **55**: 1328-1336, 1965.

ROSEN, H. R. and L. SHAW. Studies of *Sclerotium rolfsii*, with special reference to the metabolic interchange between soil inhibitants. Journ. Agr. Res. **39**: 41-61, 1929 (Abstr. in Rev. Appl. Mycol. **9**: 49-50, 1930).

TOWNSEND, B. B. Nutritional factors influencing the production of Sclerotia by certain fungi, Ann. Bot. Lond., N. S. 21: 153-166, 1957 (Abstr. in Rev. Appl. Mycol. **36**: 491, 1957).

VERMA, J. P. Studies in the enzyme make up of *Alternaria* V. Aldolase activity. Mycologia **56**: 909-919 1964.

WEBER, C. F. Blight of carrots caused by *Sclerotium rolfsii*, with geographic distribution and host range of the fungus. Phytopathology **6**: 1129-1140, 1931.

WU, L.-C., T.-C. Tseng, and Y.-S. Lee. Enzymatic studies on *Piricularia oryzae* Cav. Mem. Agri. Coll. National Taiwan University, 8: 86-96, 1966.