PROTEINS AND CATALASE ISOENZYMES FROM *FUSARIUM SOLANI* AND THEIR TAXONOMIC SIGNIFICANCE

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Summary

Proteins and catalase isoenzymes from saprophytic and parasitic forms of *Fusarium solani* were separated electrophoretically in starch gels. Protein patterns of an isolate of *F. solani* f. *pisi* showed little response to altered cultural conditions but catalase patterns altered with culture age, illumination of culture, number of conidia in the inoculum, and portion of growth harvested. Under optimum conditions a maximum of four catalase bands was detected. Evidence is presented that hyphae and spores of this isolate have different catalase patterns and that these patterns are influenced by light intensity.

Under conditions optimum for the above isolate of *F. solani* f. *pisi*, culturally and pathogenically distinct isolates of *F. solani* produced essentially identical protein patterns but markedly different catalase patterns. The taxonomic implications of this are discussed.

I. INTRODUCTION

Identification of species of *Fusarium* and their pathogenic forms can be time-consuming and difficult. Rapid and reliable methods of identification would therefore be very useful. There is increasing interest in the application of protein analysis to taxonomy. Since the initial investigations of Chang, Srb, and Steward (1962) on *Neurospora* spp. and of Clare (1963) on *Pythium* spp. several investigators have examined the possibility of separating proteins or enzymes of fungi by electrophoresis in starch or acrylamide gels and using the resultant patterns as taxonomic criteria (McCombs and Winstead 1963; Meyer, Garber, and Shaeffer 1964; Durbin 1966).

Present systems for classifying fungi are based largely on morphological criteria. To some extent such systems may simply be ones of convenience, but ideally the arrangement of species should reflect genetic similarities and differences. Sibley (1962) points out that the two activities of genes are to replicate themselves and to direct protein synthesis. It follows that the genotype of an organism is reflected in its protein complement and that genetic control of morphology must be exerted through protein metabolism. It seems reasonable then to assume that a close relationship between the protein complement of an organism and its morphology.

The sequence of amino acids is, as far as we know, the only genetically determined property of a protein and this sequence determines a protein's size, shape, and amphoteric properties. As these are the characters that determine electrophoretic mobility (Smithies 1959) there is a theoretical basis for attempting to apply gel electrophoresis of proteins to taxonomic studies. This paper examines the protein and catalase patterns of *F. solani* isolates.

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II. Materials

Isolates of *F. solani* examined are listed in Table 1 and named according to the scheme of Snyder and Hansen (1941). Isolates without a form designation are considered to be saprophytic.

III. Methods

(*a*) *Cultural Methods*

Erlenmeyer flasks (250 ml capacity) containing 30 ml of Czapek Dox liquid medium plus 0·05% yeast extract (NDY) were inoculated as described below and incubated with continuous rotary shaking (103 r.p.m.) at 25°C at various light intensities. The intensity of light received by the cultures was adjusted by varying the distance between the light source (Philips 40-W Warm White fluorescent tubes) and the cultures. Cultures not to be exposed to light during incubation were grown in flasks wrapped in aluminium foil.

Table 1

NAME AND SOURCE OF ISOLATES USED IN THE INVESTIGATION

Isolate No.	Name	Origin
1	*Fusarium solani*	*Phaseolus* sp. stem, Queensland
2	*F. solani*	*Pisum sativum* seed, New Zealand
3	*F. solani* f. *phaseoli*	*Phaseolus* sp., California
4	*F. solani* f. *cucurbitae*	*Cucurbita* sp., California
5	*F. solani* f. *pisi*	*P. sativum*, Port Pirie, S. Aust.
6		*P. sativum*, Washington, Berkeley Collection No. 32
7		Virgin soil, Renmark, S. Aust.
8		*P. sativum*, Renmark, S. Aust.
9		*P. sativum*, Renmark, S. Aust.
10		*P. sativum*, Waite Institute, S. Aust.
11		*P. sativum*, Berkeley Collection No. 34, −mating type
12		*P. sativum*, Berkeley Collection No. 30, +mating type

Hyphae and conidia were produced by *F. solani* in liquid culture. The conidia varied in shape, size, and septation. In this account, conidia without septa are termed microconidia and conidia with one or more septa are termed macroconidia.

(*b*) *Preparation and Electrophoresis of Extracts*

The resultant growth was collected by filtration on Whatman No. 1 filter pads, washed with 400 ml of distilled water, blotted dry, and weighed. A portion (1–2·5 g fresh weight) of the harvested growth was broken at −20°C in an Eaton press (Eaton 1962), buffered with one-tenth its weight of 0·76M Tris–citrate–EDTA buffer at pH 9·0, and centrifuged in the cold at 40,000 r.p.m. (*c.* 175,000 *g*) for 60 min in a Spinco model L ultracentrifuge. The supernatant was collected and used without further treatment. Kjeldahl analyses at intervals throughout the period of investigation showed that the supernatant contained *c.* 5 mg nitrogen/ml and the pellet contained *c.* 5 mg nitrogen/g fresh weight. Portions (20–40 μl) of the supernatant

were fractionated electrophoretically at pH 9·0 over a distance of 7·5 cm in starch gels (Clare 1963). At this pH all protein and catalase bands moved towards the anode.

(*c*) *Staining of Gels*

Protein was detected with Amido Black (Smithies 1959) and isoenzymes of catalase were detected by their catalatic and peroxidatic activity. To detect catalatic activity, gels were soaked in 0·067M phosphate buffer (pH 5·6) for 3 hr at room temperature, flooded with 0·4% hydrogen peroxide in phosphate buffer for 45 sec, rinsed with water, and soaked in 1% potassium iodide in phosphate buffer until white bands developed on a blue background. Peroxidatic activity was detected, without adjusting the pH of gels, by flooding gels successively with aqueous solutions of pyrogallol (1%) and hydrogen peroxide (0·4%). The two procedures gave similar patterns but better resolution of bands was achieved by detecting peroxidatic activity. No peroxidatic activity was detected when pyrogallol was replaced with guaiacol. By analogy with the results of Rudolph and Stahmann (1964), this observation supports the contention that the enzyme detected was catalase and not peroxidase.

(*d*) *Recording of Results*

Gels stained for protein were soaked overnight in an aqueous solution of glycerol (15%) and acetic acid (3%) at room temperature. They were then wrapped in Cellophane sheets and cleared by heating at 60°C for 2 hr. Densitometer tracings of the cleared gels were obtained on a Beckman Analytrol film densitometer using a B-5 cam.

Gels stained for catalase activity were photographed 10–15 min after rinsing off the staining reagents. Photographs were taken on Ilford Pan F film without a filter.

IV. Results

(*a*) *Cultural Studies*

A series of cultural studies was undertaken to determine conditions suitable for determining the protein and catalase patterns of *F. solani*. Isolate 6 was chosen as the test organism.

(i) *Age and Illumination of Culture*

Cultures were grown from single germinated conidia in darkness or in light (50–100 f.c.). Protein and catalase patterns of the total harvested growth, fresh weight per flask, and number of spores per flask were determined at intervals after inoculation. The latter two sets of results (Fig. 1) are the means of two experiments and six replicates per treatment.

Illumination had no significant effect on the fresh weight or number of spores per flask (Fig. 1). The number of spores per flask reached a maximum at day 5 but fresh weight per flask was still rising when the experiments were discontinued at day 9. Protein patterns were not influenced by light and changed only slightly as cultures aged. Some proteins without enzymatic activity were present in such low concentration in the extracts that the bands were difficult to see and record objectively. Thus the

number of bands recorded differed slightly in separate extracts. Under optimum conditions 17 protein bands were regularly detected (Fig. 2). The maximum number of bands was detected between day 3 and day 5. At day 9 protein patterns were weak and poorly resolved.

A maximum of four major catalase bands, designated 1, 2, 3, and 4 (with R_F values of 0·33, 0·40, 0·49, and 0·60 respectively) was detected. Bands 1 and 2 tended to overlap, especially when detected by their catalatic activity (Plate 1, Pattern 1), but could be distinguished by the greater resolving power of the peroxidatic reaction

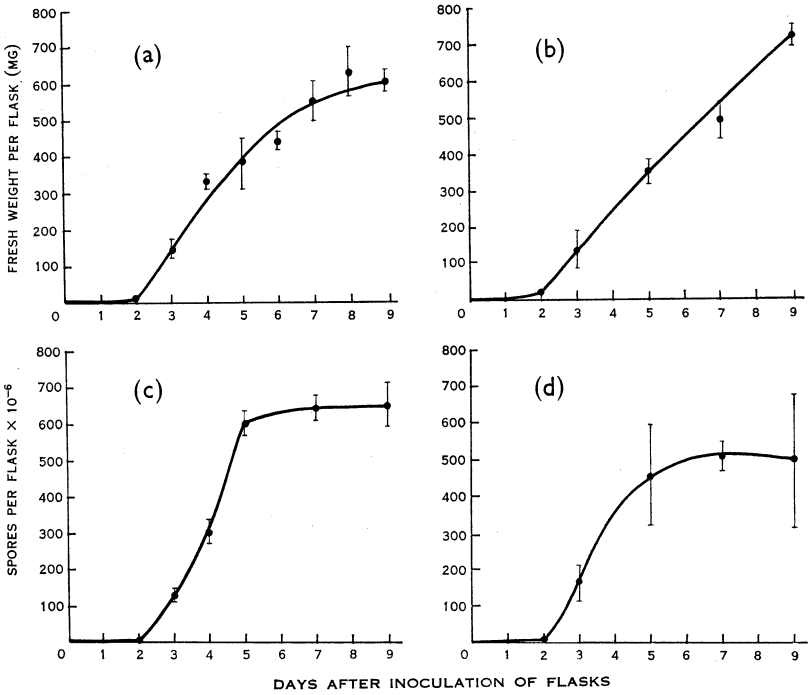

Fig. 1.—Increases in fresh weight and number of spores per flask in illuminated and unilluminated cultures of *F. solani* f. *pisi*, isolate 6. (*a*) Fresh weight, illuminated cultures; (*b*) fresh weight, unilluminated cultures; (*c*) number of spores, illuminated cultures; (*d*) number of spores, unilluminated cultures. The vertical lines through the experimental points indicate twice the standard error of the mean.

(Plate 1, Pattern 5) and by their different responses to light. A band less mobile than band 1 was sometimes detected and represented the least mobile area of a smear which, in more concentrated extracts, reached to band 1.

The number of catalase bands detected increased as cultures aged. Irrespective of illumination the appearance of catalase bands 1 and 3 between day 2 and day 3 (Plate 1, Patterns 2, 3, 7, and 8) was associated with a marked rise in the number of spores per flask and a three-fold increase in the number of spores per gram fresh weight (Fig. 1). However, the appearance of catalase band 2 in older cultures (Plate 1, Patterns 4, 5, and 6) depended on illumination of the cultures.

(ii) *Proteins and Catalase Isoenzymes from Conidia*

The following experiment was designed to determine, at three light intensities, the contribution of conidia to the total protein and catalase patterns. Cultures were grown from single conidia for 5 days at 0, 50–100, and 500–600 f.c. The conidia floating freely in the medium were separated from the residual growth, consisting

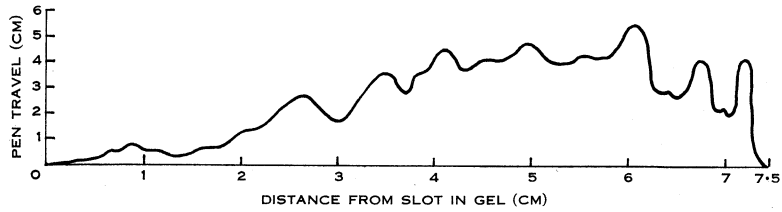

Fig. 2.—Protein pattern produced by 5-day-old cultures of *F. solani* f. *pisi*, isolate 6, in starch gel and recorded by a Beckman Analytrol densitometer. This pattern was typical of patterns obtained from 12 isolates of *F. solani*.

of an intimate mixture of conidia and hyphae, by filtration through two layers of muslin. It was not possible to completely separate the residual growth into hyphae and conidia. The proportions of microconidia and macroconidia in each sample of conidia (Table 2) and the protein and catalase patterns of conidia free in the medium and of the residual growth were determined.

TABLE 2

INFLUENCE OF LIGHT INTENSITY ON FRESH WEIGHT, NUMBER, AND TYPE OF SPORES PER FLASK AND CATALASE ISOENZYME COMPLEMENT OF SPORES OF *F. SOLANI* f. *PISI*

Light Intensity (f.c.)	Fresh Weight per Flask (mg)*	No. of Spores per Flask*	Analysis of Spore Sample		
			Percentage of Macroconidia	Catalase Bands Detected	Pattern No.†
0	398(64)	$372(54) \times 10^6$	0	1, 3, 4	13
50–100	400(35)	$390(62) \times 10^6$	18	2, 3, 4	15
500–600	333(26)	$305(52) \times 10^6$	52	2, 3	17

* Standard error of mean given in parentheses. † Refers to Plate 1.

Irrespective of the light intensity received by the cultures the conidial fraction and residual growth fraction each produced protein patterns similar to the pattern reproduced in Figure 2. Similarly, fresh weight per flask and total number of spores per flask (Table 2), determined from separate sets of cultures, were again not significantly affected by light intensity. However, the proportion of macroconidia in the population of spores rose as light intensity was increased (Table 2). Both the spores (microconidia only) and the residual growth produced in the dark contained catalase

bands 1, 3, and 4 (Plate 1, Patterns 12 and 13). Spores (microconidia and macroconidia) produced at 50–100 f.c. illumination contained catalase bands 2, 3, and 4 (Plate 1, Pattern 15) while the residual growth contained readily detected amounts of catalase bands 1 and 4 but only traces of bands 2 and 3 (Plate 1, Pattern 14). Thus, at 50–100 f.c. illumination bands 2 and 3 of the total catalase pattern are derived predominantly from conidia originally free in the medium. At 500–600 f.c. illumination, only catalase bands 2 and 3 were detected in the population of conidia free in the medium whereas the residual growth contained all four catalase bands.

(iii) *Size of Inoculum*

Flasks inoculated with single germinated conidia were incubated in darkness for 5 days and flasks inoculated with $1 \cdot 8$ by 10^6 ungerminated conidia suspended in 1 ml of distilled water were incubated for 5 days in darkness or in light (50–100 f.c.). Catalase activity of the extracts was determined by the peroxidatic reaction (Plate 1, Patterns 18–20). Fresh weight and numbers of microconidia and macroconidia per flask were determined from separate sets of cultures and the mean values, determined from six bulked replicates, are presented in Table 3.

TABLE 3

FRESH WEIGHT, NUMBER, AND TYPE OF SPORES OF *F. SOLANI* f. *PISI* IN RELATION TO SIZE OF INOCULUM AND ILLUMINATION OF CULTURE

No. of Spores in Inoculum	Light Intensity (f.c.)	Fresh Weight per Flask (mg)	No. of Spores per Flask		Spores per 1 mg Fresh Weight
			Microconidia	Macroconidia	
$1 \cdot 8 \times 10^6$	0	508	1945×10^6	55×10^6	$3 \cdot 9 \times 10^6$
1	0	272	402×10^6	8×10^6	$1 \cdot 5 \times 10^6$
$1 \cdot 8 \times 10^6$	50–100	635	1500×10^6	313×10^6	$2 \cdot 9 \times 10^6$

The proportion of macroconidia was higher in illuminated cultures than in unilluminated cultures and catalase band 2 was again only detected in illuminated cultures. In the absence of illumination catalase band 3 was more readily detected in cultures derived from the higher inoculum level and this was associated with a greater number of spores per milligram fresh weight of harvested growth.

(*b*) *Taxonomic Studies*

The study of isolate 6 suggested that the maximum number of protein and catalase bands from isolates of *F. solani* would be detected by growing cultures from a large number of conidia in the light. Protein and catalase patterns of the isolates listed in Table 1 were determined in the following way. Conidia taken from single-spore cultures on potato dextrose agar slopes were spread over NDY agar in petri dishes and incubated under room conditions for several days until a lawn of sporulating mycelium formed. Disks of agar 8 mm in diameter and bearing masses of conidia were then removed and placed singly into flasks. In this way a large (*c.* 2×10^6) but

undefined number of conidia was rapidly and conveniently added to each flask. Cultures were harvested after 5 days' incubation at a light intensity of 50–100 f.c. and protein and catalase patterns were determined.

The 12 isolates produced identical protein patterns similar to Figure 2. In repeated tests the protein patterns differed quantitatively but not qualitatively. However, marked qualitative differences between catalase patterns of culturally and pathogenically different isolates of *F. solani* were apparent (Plate 1, Patterns 21–32). Each isolate produced a characteristic pattern but no catalase band was common to the species and no distinction between saprophytic and parasitic forms on the basis of a characteristic band or pattern of bands was possible.

The eight isolates of *F. solani* f. *pisi* could be divided into four culturally distinct groups. Members of the first group, viz. isolates 5, 6, 7, and 8, produced identical catalase patterns comprising bands 1, 2, 3, and 4. Members of the second group, comprising isolates 9 and 10, produced similar patterns consisting of three bands referable to bands 1, 2, and 4 of isolate 6.

Isolates 11 and 12 were culturally distinct, of opposite mating type, and produced distinctive catalase patterns. Isolate 11 produced three bands, the least and most mobile bands corresponding to bands 1 and 4 respectively of isolate 6. The band of intermediate mobility varied in position in different gels, corresponding either with band 2 or band 3 of isolate 6. In Plate 1, Pattern 31, it is shown corresponding with band 3 of isolate 6. Isolate 12 produced two catalase bands, referable to bands 1 and 4 of isolate 6.

V. Discussion

Study of isolate 6 showed that, of a total of four catalase bands, the number readily detected depended on age of the culture, illumination of the culture, size of inoculum, and portion of growth extracted. It is common to find that isoenzyme patterns change as organisms age and undergo morphological changes. For example, esterase and catalase patterns of *Bacillus cereus* undergo changes during sporulation (Baillie and Norris 1963). The acid phosphatase and esterase patterns of *Dictyostelium discoideum* alter as the vegetative myxamoebae differentiate into spores (Solomon, Johnson, and Gregg 1964). Similarly, in many higher organisms, losses and gains of molecular forms of particular enzymes occur during differentiation (Markert and Møller 1959; Lindsay 1963).

One of the most obvious morphological changes occurring in fungi is the production of spores after a period of hyphal elongation. There is considerable information on changes in enzyme activity during germination of spores but comparative studies on enzymic changes in hyphae, conidiophores, and spores during sporulation seem to be lacking.

Before the onset of sporulation only catalase band 4 was detected in the mycelium of isolate 6 but there was evidence that an additional catalase isoenzyme (band 1) appeared in the hyphae during sporulation. Catalase band 1 was absent from conidia free in the medium but was present in the residual mixture of conidia and hyphae. This suggests either that catalase band 1 is produced in the hyphae

during sporulation or that the spores retained in the mycelial mat contain catalase band 1 and therefore differ enzymically from spores free in the medium.

Catalase band 2 was detected only in illuminated, sporing cultures. Light has a marked effect on the morphology and growth of many fungi. It is not surprising, then, to observe an effect of light at the molecular level. In fact, Domnas and Cantino (1963) demonstrated an effect of light on the amounts of enzymes concerned with arginine metabolism produced by *Blastocladiella emersonii*. However, the present report appears to be the first in which light is shown to affect the number or concentration of isoenzymes which develop in an organism.

There are several reports (Marsh, Taylor, and Bassler 1959) that light influences the types of spores produced by *F. solani*. In this study also, light affected the type but not the number of spores produced. Spores formed in unilluminated cultures were predominantly microconidia. Macroconidia and microconidia were produced in cultures exposed to light and the proportion of macroconidia rose as light intensity was increased. The presence of catalase band 2 in illuminated cultures could therefore be entirely due to the presence of macroconidia. An unequivocal answer was not obtained. However, catalase bands 2, 3, and 4 were present in a sample of spores produced in the light and comprising 82% microconidia, while catalase bands 1, 3, and 4 were present in microconidia produced in the dark. Thus catalase band 1 was absent from microconidia produced in the light and catalase band 2 was absent from microconidia produced in the dark but may have been present in both microconidia and macroconidia produced in the light.

Catalase band 4 could not be detected in spores from cultures receiving 500–600 f.c. of light. Whether this was due to a high proportion of macroconidia in the sample and the absence of band 4 from macroconidia or to a direct effect of light in reducing the concentration of band 4 *per se* was not determined.

In discussing the taxonomic significance of the results it must be emphasized that the work was done on representatives of one species. Saprophytic and parasitic forms of *F. solani* produced essentially identical protein patterns in starch gels. The idea that a particular fungus species may produce a characteristic protein pattern in starch gels (Clare 1963) is therefore not challenged by the present results. Below the species level there is increasing interest in using enzyme patterns produced electrophoretically in starch or acrylamide gels as taxonomic criteria for microorganisms. Classification of isolates of *Bacillus thuringiensis* based on esterase patterns was closely correlated with serological classification of the isolates (Norris 1964). Similarly, strains of four species of *Mycobacterium* could be identified by their characteristic esterase patterns (Cann and Willox 1965).

A survey of catalase patterns of 12 isolates of *F. solani* showed that it was not possible to correlate particular patterns or bands with the saprophytic or parasitic habit. However, when grown under conditions optimum for *F. solani* f. *pisi* each isolate produced a characteristic catalase pattern and culturally and pathogenically identical isolates from different localities produced identical catalase patterns. Rapid identification of cultural or pathogenic forms of *F. solani* by catalase or other enzyme patterns may therefore prove to be feasible.

Meyer, Garber, and Shaeffer (1964) studied esterase and phosphatase patterns of *Fusarium oxysporum* under one set of conditions and suggested that forms of this species may be characterized by the patterns they produce in starch gels. The present study shows that morphogenesis in liquid culture profoundly affects catalase patterns of *F. solani* and may therefore also affect other enzyme patterns of *Fusarium* spp. To evaluate the taxonomic use of enzyme patterns in mycology it is apparent that cultural conditions which provide a wide range of morphological forms of the fungus may be necessary. In fungi that do not undergo morphological changes as marked as spore production enzyme patterns may show less response to altered cultural conditions and may therefore prove to be rapid and reliable indicators of a taxonomic group.

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EXPLANATION OF PLATE 1

Patterns 1–32 are catalase patterns of the *F. solani* isolates listed in Table 1. Bands referred to in the text are labelled in Pattern 1. Direction of migration is towards the anode. Patterns 2–20 are catalase patterns of isolate 6 detected by peroxidatic activity, Patterns 2–17 being from cultures derived from single conidia. Patterns 21–32 are catalase patterns of isolates 1–12 detected by peroxidatic activity

Pattern 1.—Four catalase bands of isolate 6 detected by catalatic activity.
Patterns 2–6.—Illuminated cultures harvested at days 2, 3, 4, 5, and 9, respectively.
Patterns 7–11.—Unilluminated cultures harvested at days 2, 3, 5, 7, and 9, respectively.
Pattern 12.—Residual growth, produced in darkness.
Pattern 13.—Conidia, produced in darkness.
Pattern 14.—Residual growth, produced in light (50–100 f.c.).
Pattern 15.—Conidia, produced in light (50–100 f.c.).
Pattern 16.—Residual growth, produced in light (500–600 f.c.).
Pattern 17.—Conidia, produced in light (500–600 f.c.).
Pattern 18.—Single spore as inoculum, no illumination.
Pattern 19.—$1 \cdot 8 \times 10^6$ spores as inoculum, no illumination.
Pattern 20.—$1 \cdot 8 \times 10^6$ spores as inoculum, illumination of 50–100 f.c.
Patterns 21–32.—Catalase patterns of isolates 1–12, respectively.

ISOENZYMES FROM *FUSARIUM SOLANI*