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STUDIES ON STRAINS OF *FUSARIUM VASINFECTUM* – IV. IN VITRO PRODUCTION OF PATHOGEN FACTORS ★

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An Indian strain (12) of *Fusarium vasinfectum* synthesized fusaric acid on susceptible host tissue, while an American strain (A 1) and non-pathogenic Indian (I1) did not. This capacity of the former strain may be of importance in its selective pathogenicity. None of the three strains exihibited any PME activity. All of them synthesized PG on living host tissue but no correlation could be found between virulence and PG synthesis. There was again no correlation between *in vitro* production of macerating enzymes or proteolytic enzymes of the strains and their virulence.

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INTRODUCTION

In the wilt disease of cotton caused by *Fusarium vasinfectum* Atk., pathogen factors such as pectic enzymes and wilt toxin fusaric acid have been assigned a role in pathogenesis (Sadasivan & Kalyanasundaram, 1965; Dimond, 1970). However, attempts at correlating virulence or avirulence of the pathogen with *in vitro* production of these pathogen factors have yielded contradictory results (Kern, 1952; Paquin & Coulombe, 1962; Horst, 1968; Charudattan 1970). The problem was therefore investigated using a medium nearest to the natural substrate of the pathogen, namely, living roots of the host.

MATERIALS AND METHOD

Three strains of *F. vasinfectum* designated I1, I2, A1 were used. I1 and 12 were isolated from the cotton fields of Palladam Tamil Nadu, India while A1 was obtained from Clemson University, Kershaw, South Carolina U. S. A. Strain A1 was pathogenic to *Gossypjum hirsutum* and strain 12 to *G. arboreum* while strain I1 was nonpathogenic to either of them (Venkataraman & Kalyanasundaram, in press). The media employed to grow the strains for the prodnction of fusaric acid as well as pectic enzymes were as follows : Richard's medium having 1/10 th of its salt concentration was the synthetic medium used. This was designated as the basal medium. To get an idea of the capcity of the strains to preferentially use the inorganic salts or the organic host substrate a series was set up incorporating living roots in the basal medium. A series was also set up incorporating steam, killed roots in the basal medium. In addition, a series was set up by having living roots suspended in dis illed water. To check on the possibility of enzymes/toxins being produced in the uninoculated host roots, uninoculated living roots controls were maintained. Only in the case of living roots incorporated in basal medium there was polygalacturonase activity. These values have been taken note of in calculation.

50 ml of sterile basal medium or sterile distilled water as the case may be, were taken in 250 ml Erlenmeyer flasks. The amendments living roots, or steam killed roots were added at the rate of 10 grams per flasks. Roots of one week old seedlings of *G. arboreum* were thoroughly washed and surface sterilized with 0.1% mercuric chloride, washed thrice with sterile distilled water and placed in the media under aseptic conditions to give the living root amendment. Similarly prepared steam killed roots were used for another series. The media were inoculated with a uniform concentration of spore suspension of the fungus incubated in dark at 27 ± 2°C and the activities of the culture filtrates were studied at the three age levels of 7, 14 and 21 days.

Extraction and Estimation of pathogen factros

Fusaric acid

Fusaric acid was extracted by the method of sandhu (1959) and its chemical and biological estimations were done according to the methods of Zahner (1954) and Kalyanasundaram (1955).

Pectolytic enzymes

The pectin methyl esterase (PME) and polygalacturonase (PG) activities were assayed according to the metheds of Kertesz (1937) and Bernfeld (1955).

Macerating enzyme

The fungi were grown in normal Richard's medium amended with 1% pectin. The enzyme activity was estimated by the methods of Brown (1915) and Bateman and Beer (1965)

Proteolytic enzymes

Richard's medium with glucose as carbon source was used as medium control. In series A 50% of the glucose was replaced by caesin (18.67g/lit.). In series B glucose was completely replaced by caesin (37.37 g/lit.). In series C glucose was completely replaced by host root (50 g/lit,). When protein was supplemented, the excess nitrogen, phosphorus and sulphur were equalised by ammonium nitrate, ammonium tripphosphate, and ammonium sulphate respectively. These were set up as controls for A and B. The following series were set up :

Medium control : Richaro s medium with glucose as carbon soeurce.

Series A	Control to series. A	
50% of glucose in the medium replaced by caesin 18.67 g	NH_4NO_3	7.575 g
	$(NH_4)_2SO_4$	0.555 g
	$(NH_4)_3PO_4$	0.639 g
Series B	Control to series B	
Glucose in the medium completely replaced by caesin 37.37 g	NH_4NO_3	15.150 g
	$(NH_4)_2SO_4$	1.109 g
	$(NP_4)_3pO_4$	1.277 g
Series C		
Glucose in the medium completely replaced by live host root 50.00 g.		

The enzyme was purified and estimated according to the method of Sampathnarayanan and Shanmugasundaram (1966).

Experimental results

Fusaric acid

Chemical estimation

The non-pathogenic Indian strain I1 and the pathogenic American strain A 1 produced fusaric acid in the basal medium. While the pathogenic Indian strain 12 did not. Of all the strains I1 produced maximum fusaric acid in this medium after 21 days of incubation. Strains I1 and A1, however, did not produce any fusaric acid on living roots or killed host roots. It is significant to point out that strain 12 produced toxin fusaric acid, only on the living roots of the host after 14 days incubation, the quantity synthesized being the highest (Table 1).

Biological estimation

Culture filtrates of the strains I1 and A1 obtained from the treatments at any of the age levels did not inhibit the growth of the test organism. Strain

12, however, was observed to produce zones of inhibition of the test organism, when the culture filtrate from treatments living roots or basal medium + living

Table 1 *Fusaric acid production by strains of F. vasinfectum grown on host roots and media amended with host roots, estimated by the chemical methods,*

Strains	Media	Fusaric acid (mg/l culture filtrate* of cultures in days) Age 7	14	21
I1	Live roots	—	—	—
	BM	8.00	7.00	18.00
	BM+live roots	—	—	—
	BM+killed roots	—	—	—
I2	Live roots	—	30.00(60.00)	—
	BM	—	—	—
	BM+Live roots	— (40-00)	—	—
	BM+killed roots	—	—	—
A1	Live roots	...	—	—
	BM	9·00	...	8.00
	BM+live roots	—	—	—
	BM+killed roots	—		=

BM=1/10th normal Richards medium

*=Values of estimation by bioassay method are given in brackets.

Table 2. *Polygalacturonase activity of filtrates from strains of F.* vasinfectum *cultivated on host roots and media with host roots amendment.*

Media	PG activity (Copy glucose / ml engymt) Strain I1 Age of culture in days			Strain I2 Age of culture in days			Strain A1 Agre of culture in days		
	7	14	21	7	14	21	7	14	21
Live roots	250	135	100	—	135	210	385	100	250
BM★	220	230	—	125	135	395	420	195	195
BM + live roots	245	60	.	—	200	655	400	75	150
BM + killed roots	245	505	145	—	250	630	370	285	—

★ BM 1/10th normal Richards medium

roots was tested, the concentration of fusarlc acid being 60 mg/1 and 40 Mg/1 respectivelyas recorded on the 14th day in the fermer and the 7th day in the latter (Teble 1)

Pectolytic enzymes

Pectin Methyl esterase (PME)

There was no detectable activity of PME by and of the three strains in any of the media tried.

Polygalacturonase (PG)

All the three strains produced PG regardless of the substrates used. Maximum activity by any one strein on any single day was by strain I2 when grown in Basal medium + leving/killed roots for 21 days. This strain however, initiated the production late (14th day) in ali the media. As opposed to this, strains A1 and I1 had an initial spurt of activity (7th day) which tapered off after the 14th day. The same trend of activity was exhibited by all the strains in all the media (Table 2).

Macerating enzyme

Brown's method

Strains I2 and A1 exhibited a fairly high degree of enzyme activity when grown with or without pectin in the Richard's medium. Sirain I1 dıd not have any activity (Table 3).

Table *3. Maceration of potato tuber discs by the culture filtrates of strains of* F. vasinfectum *grown in Richard's medium with and witnout pectin (Brown's method)*

Strains	Mebia	Age of cultures in days 7	14	21
I1	Nedium	—	—	—
	Medium + pectin	—	+	—
I2	Medium	—	+	+++
	Medium + pectin	+	+++	++++
A1	Medium	—	+++	+++
	Medium + pectin	—	+++	++++

—: no maceration; + : very low; ++ : high; ++++ : very high

Bateman and Beer's method

The turbidity values of the reaction mixtures indicated that all rhe strains

had maximum activity on the 14th day. All of them could macerate the tissue only after a 12 hour incubation period. Strain I2 had maximum activity in Richard's medium followed by A1 and I1 strains. The latter to had almost equal activity. With pectin amendment strains 12 and I2 showed appreciable increase in activity while A1 did not (Table 4).

Table 4. *Macerating activity * of culture filtrates of strains of F. vasinfectum grown in Richard's mediu with and without pectin.*

Strains Media	Age of culture in days								
	7			14			21		
	4 h	8 h	12 h	4 h	8 h	12 h	4 h	8 h	12
I1 Medium	6.70	—	2.30	4.00	4.00	40.15	3.60	3.60	—
Medium+pectin	4.50	—	1.20	2.50	2 50	34.35	2.00	3.05	--
I2 Medium	6.50	2.30	4.50	8.60	48.70	71.40	—	1.35	—
Medium+pectin	0.50	—	—	3.20	5.40	32.33	—	—	17.9
A1 Medium	12.90	4.50	—	6.37	8.40	44 20	3.83	5.68	—
Medium+pectin	1 46	—	3.00	—	—	—	0.64	—	—

* Maceration activity expressed as turbidity units for different reaction periods.

Proteolytic enzymes

The Indian strain I2 and tha American strain A1 showed maximum growth when the carbon source glucose was replaced fully or partially by the inducer protein caesin (Series A and B of Table 5). This was not the case with the non-pathogenic Indian strain I1, it showed maximum growth in media control A and B, i.e., when caesin replacced glucose fully or partially and when the added ammonium salts were also present. All the strains showed poor growth when the living host roots were replaced the glucose of the medium.

All the strains showed low activity in the absence of inducer substrate, i.e., in medium control. Strain I2 showed slightly higher activity then the others when casein replaced glucose in entirety (Series B). Strains A1 and I1 showed higher activity when glucose and casein were present in the

medium (Series A). when host roots replaced glucose, none of the strains produced this enzyme (Table 5).

Table 5. *Proteolytic enzyme activity * of culture filtrate of strains of* F. vasinfectum *grown in medium With casein or host roots as substrates.*

Strains	Age of culture in days	Media used for growing the strains: Medium control	Series A	Series B	Series C	Control of series A	Control of series B
I1	7	0.8	5.9	1.7	0.2	—	—
	14	0.2	4.8	3.1	0.3	—	0.4
	21	1.0	2.7	1.8	0.2	0.3	—
I2	7	0.3	2.7	4.0	0.4	—	0.4
	14	—	3.3	6.6	—	0.1	—
	21	0.8	3.8	3.3	—	—	0.1
A1	7	0.1	7.3	4.4	0.1	0.1	0.1
	14	0.4	1.1	3.6	—	—	—
	21	0.7	8.4	5.3	—	0.7	0.2

* Enzyme activity *expressed as* mg *tyrosine liberated by mg protein.*

DISCUSSION

In an attempt to correlate the virulence or avirulence of pathogegens with their *in vitro* production of toxins and pectolytic enzymes test strains were cultivated on living host roots and also in a synthetic medium with reduced nutrient concentration (Basal medium). Basal medium was supplemented with either killed or living roots.

In the present work the avirulent strains, I1 and A1 produced fusaric acid in the basal medium while the virulent strain I2 did not (Table 1.). Charudattan (1970) reported a high fusaric acid production by the avirulent strains A1 and I1 and low production by the virulent strain I2 in the complete Richard's medium. The concentration of the constituents of the synthetic medium appears to affect the quautity of fusaric acid synthesized. The point of interest is however, the ability of the virulent strain to synthesize the toxin when cultivated on

living host roots and the inability of the avirulent strains to do so. Fusaric acid production by the three strains evaluated by the dio-assay method has only confirmed the results of chemical estimation.

The low concentration of fusaric acid produced by strains A1 I1, in Basal medium could not be detected by the bioassay method as the concentration was too low for detection.

Although fusaric acid is one of the established *vivo toxins* (since it is not host specific) its role in pathogenicity was questioned (Kuo and Scheffer, 1964). The present study indicated that the toxin may have a role in selective pathogenicity, as its synthesis was found to be selective.

Pectic enzyme was not synthesized when pectin was replaced in the medium with host roots and this is in contrast to the results reported earlier on the adaptive nature of this enzyme (Lakshminarayanan, 1955).

There was no correlation between virulence and PG synthesis on the host roots ; however, in basal medium supplemented with host roots the virulent strain (I2) recorded higher activity than the avirulent ones Perhaps the virulent strain was better adapted use the native pectin available in the susceptible host tissue than the avirulent strains. But with the limited observations it is prematur to conclude that the host specificity can explained on the basis of selective action of this enzyme.

Studies on the macerating enzyme was warranted by the microscopic evidence of cell wall degradation observed at the prodrome stage in the cortical cells of arboreum cotton plants infected with 12 strain (Venkatarman, 1971). There was no correlation between virulence and macerating enzyme activity according to either of the methods followed here.

Strains 12 and A1 which are host specific appeared to utilize substrate casein better synthesize more of proteolytic enzyme than the avirulent strain I1. However, this enzyme activity of the strains I2 and A1 did not throw any light on their virulence and avirulence respectively to arboreum cotton. When host roots served as substrate the strains recorded negligible activity in regard to this enzyme.

The significance of extracellular proteolytic enzymes in the development of cotton wilt was not clear since the accessibility of host protein to the enzymes secreted by the pathogen is not known. It remains to be seen whether the structural protein of the cell wall will be readily ancessible to the action of proteolytic enzymes secreted by the pathogen.

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