PRODUCTION OF PECTOLYTIC AND CELLULOLYTIC ENZYMES BY VIRULENT AND AVIRULENT ISOLATES OF *RHIZOCTONIA BATATICOLA* (TAUB.) BUTLER IN CULTURE AND IN ROOT EXTRACTS OF *ABELMOSCHUS* PLANTS

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Seven isolates of *Rhizoctonia bataticola* differing in the degree of virulence were studied with respect to the production of pectolytic and cellulolytic enzymes in culture and in diseased root extracts. Mycelial dry weight of isolates in culture was found to have no correlation with the virulence of these isolates. It was interesting to find that four isolates namely, BP, SF, KP and 481 which were virulent to *Abelmoschus* also produced pectolytic and cellulolytic enzymes in increased quantity inside the inoculated *Abelmoschus* roots. Enzymes produced in culture by different isolates did not seem to have any correlation with their pathogenic ability.

Polygalacturonase (PG), polygalacturonate trans-eliminase (PGTE), Pectin methylesterase (PME) were considered to play a definite role in pathogenesis by several workers. The results of the present study indicate that PG, PGTE and PME enzymes have a decisive role to play in the pathogenesis of *R. bataticola* on *A. esculentus*.

INTRODUCTION

Rhizoctonia bataticola is commonly found in several countries attacking an extremely wide range of host plants including oil seeds, cereals, vegetables, pulses and fibre-yielding plants causing seed decay, damping off, charcoal rot, stem cankers and root rots.

Field surveys revealed that the collar and root rot disease of *Abelmoschus esculentus* was of common occurrence in most of the 'Okra' growing areas of Punjab and Haryana States and took a toll of 25–35 per cent of the crop every year. The rotted plants on isolation gave *R. bataticola*. The pathogen is also responsible for pre- and post-emergent damping off of seedlings.

Pectolytic and cellulolytic enzymes have been implicated in pathogenesis by several workers (Bateman and Miller 1968; Goodman *et al.* 1967; Wood 1967; Chan and Sackston 1970). Pectolytic and cellulolytic enzymes were active in culture filtrates and in diseased root extracts of inoculated but not in uninoculated 'Okra' (*Abelmoschus*) plants (Goel and Mehrotra 1972). We therefore investigated the production of pectolytic and cellulolytic enzymes *in vitro* and *in vivo* of seven different isolates (differing in their degree of virulence) of *R. bataticola* and their possible role in pathogenesis.

Materials and Methods

Pusa sawani (Okra variety) was used for the experiments. Seeds were supplied by I.A.R.I., New Delhi. Plants for *in vivo* studies were grown aseptically in glass cylinders containing 150 g coarse and acid-washed sand. Sterile IN Hoagland's solution was added to each glass cylinder before autoclaving. Nutrient solution was added at intervals of 3–4 days. Surface-sterilized *A. esculentus* seeds were put on PDA medium in petri dishes and contamination-free healthy germinating seeds (20 in each cylinder) were transferred aseptically to the culture cylinders. The seedlings were inoculated with mycelial suspension of various isolates of *R. bataticola.*

The seven isolates of *R. bataticola* were as follows :

(*i*) 481 — Isolated from *Gossypium* sp., supplied by IARI.
(*ii*) 482 — Isolated from *Arachis hypogea,* supplied by IARI.
(*iii*) 1568 — Isolated from *Chorchorus capsularis,* supplied by IARI.
(*iv*) BP — Isolated from *Abelmoschus esculentus.*
(*v*) SP — Isolated from *Helianthus* sp.
(*vi*) KP — Isolated from *Kochia* sp.
(*vii*) RS — Isolated from *Abelmoschus* rhizosphere.

After 36 hr of growth in the cylinders the seedlings were inoculated with mycelial suspension of *R. bataticola* isolates. Twelve days after inoculation the plants showing hyp cotyl and root rot were harvested and washed with distilled water. Healthy and diseased root tissues were comminuted separately for 2 min at 0°C in a waring blendor with an equal volume, per fresh wt of tissues of 0.1 M NaCl solution, containing 0.02 M Na_2HPO_4 and KH_2PO_4 mixed buffer at *p*H 6.00 (Ayers *et al.* 1969). The preparations were clarified by centrifugation and then dialysed overnight against deionised water and the extracts thus obtained were utilized for enzyme assay.

Cultures were grown on PDA medium. *R. bataticola* isolates were grown in a synthetic medium for enzymic studies. The basal medium consisted of 2.5 g D–glucose, 2.5 g pectin, 0.75 g L–asparagine, 2.0 g KH_2PO_4, 0.05 g $MgCl_2.6H_2O$, 0.005 g $MnCl_2.6H_2O$, 0.005 g $FeCl_3$. 0.005 g $ZnCl_2$, and 0.095 g L–methionine per litre of distilled water (Haglund *et al.* 1959). All media were sterilized at 121°C for 20 min by autoclaving. L–methionine was sterilized by millipore filter separately and then added to each flask aseptically. Media were adjusted to *p*H 5.5 before and after autoclaving with sterile NaOH and HCl.

Mycelial discs 2 mm in size cut with the help of No. 2 cork borer from PDA plates were transferred to 40 ml of liquid medium in 250 ml flasks. Cultures were incubated at 30°C for 15 days. Culture filtrates were periodically checked at each assay for contamination by streaking on PDA plates and the filtrates were utilized for enzyme assays immediately or refrigerated and used within 24 hr.

Dry weights were determined by filtering the mycelium under vacuum through previously dried and weighed filter paper and washing the mycelial mats with deionised water, dried at 80°C and weighed.

Enzyme activity tests

Polygalacturonase — Polygalacturonase activity was assayed by the chromatographic method for exo-polygalacturonase (exo–PG) and by viscometric method for

endo-polygalacturonase (endo–PG) following the method of Bell *et al.* (1955).

Exo–PG activity was determined by descending paper chromatography. Culture filtrates or diseased root extracts were mixed in 1 : 1 ratio with (0.25 per cent) polygalacturonic acid in 0.1.M. citrate buffer at *p*H 4.5, 2 drops of toluene were added and incubated at 30°C. After incubation for 10 and 20 hr, 20 μl samples of the reaction mixtures were spotted on Whatman filter paper No. 1. The solvent consisted of equal volumes of butanolwater (1250 : 84) and propionic acid-water (587 : 74) mixed just before use. Mixtures with autoclaved culture filtrates or autoclaved diseased root extracts and uninoculated media served as control.

Endo–PG activity was assayed by the viscometric method of Bell *et al.* (1955). The substrate for endo–PG consisted of 1.2% solution of sodium polypectate or pectin prepared by mixing 50 ml of 0.1 M citrate buffer diluted with 50 ml of water in Waring blender at 4.5 *p*H. Six ml aliquots of reaction mixture were incubated for 1 hr at 30°C in a water bath. The activity was expressed in terms of relative activity (RA). Relative activity was defined as the reciprocal of the time in minutes for 50 per cent loss in viscosity multiplied by 10^3 (Bateman 1966).

Polygalacturonate trans-eliminase — PGTE activity was measured by the TBA and the standard viscometric method.

In the TBA method (Albersheim *et al.* 1960; Starr and Moran 1962) the reaction mixture consisted of 5 ml of 0.8 per cent polygalacturonic acid or pectin in 0.05 M tris buffer, 1 ml 0.05 M $CaCl_2$ and 2 ml dialysed enzyme preparation. The mixture was incubated at 30°C for 2 hr. Autoclaved filtrates were used as controls. After incubation 1 ml each of 9 per cent $ZnSO_4.7H_2O$ and 0.5 N NaOH were added to stop enzyme reaction and to precipitate enzyme protein and excess substrate (Wang and Pinchard 1971).

Five ml of the clarified reaction mixture were added to tubes containing 3 ml of 0.04 M TBA, 1.5 ml of: *N* HCl and 0.5 ml of distilled water. Tubes were placed in a boling water bath for 30 min and cooled (Ayers *et al.* 1966). Colour intensity was then measured at wave length between 400 and 590 nm.

The viscometric method used was similar to that used for endo–PG except that the filtrate or enzyme extracts were dialysed at *p*H 8.5 in 0.01 M Tris–HCl buffer and then added to 1.2 per cent solution of sodium polypectate in 0.05 M tris HCl buffer at *p*H 8.5.

Pectin methylesterase (PME) — PME activity was determined by a bromothymol blue indicator test of Smith (1958). The substrate consisted of 1.1 g pectin, 0.2 g phenol, 0.58 g NaCl and 2.5 ml of bromothymol blue (0.04 per cent) and distilled water (100 ml). One ml of culture filtrate or plant extract was added to 7.5 ml of substrate and solutions were brought to a blue colour by the addition of 0.1 N NaOH. Tube contents were thoroughly mixed during the titration. Reaction mixtures were incubated for 24 hr at 30°C and then titrated with standardized 0.025 N NaOH to their original blue colour and amount of 0.025 N NaOH used was multiplied by 0.775 which indicated the mg methoxyl groups released by the enzymes in culture filtrate within 24 hr, and then per ml calculated.

Cellulase — Cellulase activity was determined by viscometric method (Bell *et al.* 1955). The substrate consisted of 1.2 per cent solution of carboxymethyl cellulose prepared by mixing 50 ml of 0.5 M citrate buffer diluted with 50 ml of water in a

Waring blendor at *p*H 5.00. Six ml aliquots of reaction mixtures were incubated for 1 hr at 30°C. The activity was expressed in terms of relative activity (RA).

Results and Discussion

The seven isolates of *R. bataticola* differing in their degree of virulence towards *A. esculentus* responsible for root and collar rot were studied for the production of various pectolytic and cellulolytic enzymes both *in vivo* as well as *in vitro* and for their possible role in pathogenesis. RS isolate had the lowest, isolate 481 the maximum while all other isolates had a comparable mycelial dry wt on liquid basal medium (Table I). Some inoculated plants in the series kept to observe disease development died within a few days while others developed symptoms more slowly and died later. The uninoculated control plants remained free of symptoms. The more pathogenic isolates were BP, KP, SP and 481. Isolate 1568 and RS isolate were the least virulent and isolate 482 was midly virulent on *Abelmoschus* plants (Table II).

Table I

Net mycelial dry weight of different isolates of R. bataticola *on* 40 *ml liquid basal medium after* 15 *days of growth at* 30°C

Isolates	BP	SF	KP	RS	1568	481	482
Mycelial dry weight in terms of mg	101	105	102	65	109	140	111

Table II

Percentage mortality of Abelmoschus esculentus *seedlings in glass cylinders after* 12 *days of inoculation with different isolates of* R. bataticola

Isolates	BP	SF	KP	RS	1568	481	482	Control
% age mortality	100	90	100	20	18	88	27	nil

Pectolytic enzymes

Exo-polygalacturonase — Chromatographic studies indicate the production of galacturonic acid in culture filtrates and diseased root extracts with all the seven isolates of *R. bataticola.* Dialysed filtrates of all the isolates from 15 days old cultures cleaved polygalacturonic acid to galacturonic acid in 10–15 hr. Dialysed *Abelmoschus* root extracts taken from plants inoculated with all the seven isolates of *R. bataticola* showed exo–PG activity. Isolates 481, KP, BP and SF produced galacturonic acid in less than10 hr in mixtures of polygalacturonic acid and dialysed enzyme extracts, while with isolates 482, 1568 and RS, galacturonic acid was produced in between 10–20 hr There was no apparant production of di, tri. tetra galacturonides with increase in incubation periods. The absence of oligogalacturonides suggests that the exo–PG in the mixture rapidly converted any oligosaccharides

produced into galacturonic acid. There was no exo–PG activity in healthy root-extracts of uninoculated control plants.

Endo-polygalacturonase — Endo–PG activity of the culture filtrates and root extracts of *Abelmoschus* plants incubated with all the seven isolates of *R. bataticola* was assessed viscometrically and the results are presented in Table III. All the seven isolates produced endo–PG both in culture and in root extracts. The activity of endo-PG enzyme was maximum in culture filtrate of isolate 481 and lowest in isolate 1568.

TABLE III

Polygalacturonase activity in culture filtrates and root extracts with different isolates of R. bataticola *on pectin and sodium polypectate (NaPP) as the substrates*

Enzyme source	Substrate	Enzyme activity in terms of R.A. with different isolates of *R. bataticola*						
		BP	SF	KP	RS	1568	481	482
Culture filtrates	1.2% pectin	7.1	9.0	8.6	4.8	4.0	23.0	13.3
	1.2% NaPP	7.7	13.3	12.6	7.0	6.1	27.2	12.9
Diseased root extracts	1.2% pectin	16.2	20.6	30.6	0.3	11.3	16.7	7.5
	1.2% NaPP	19.0	23.6	29.5	2.6	11.2	22.9	11.6

In diseased root extracts endo–PG activity was high in isolates KP, SF, 481 and BP and comparatively low in 482, 1568 and RS isolates. The culture-filtrates and root extract studies (Table III) indicate that endo-polygalacturonase was viscometrically more active with sodium polypectate than pectin as the substrate.

There was no endo–PG activity in root extracts of uninoculated control plants. Production and activity of endo–PG thus appeared to be associated with virulence of the isolates. Similar close correlation between production of endo–PG *in vivo* and virulence of isolates has been reported by Goodman *et al.* (1967).

Polygalacturonate trans-eliminase — PGTE enzyme activity of the infected root tissues and from culture filtrates of all the seven isolates was assayed spectrophotometrically and the results are presented in Figs. 1 and 2. TBA method showed maximum light absorption between 545–550 nm which indicates the presence of trans-eliminase activity. High PGTE activity was recorded from the culture filtrates of isolates 481, 482 and BP while SF, KP, RS and 1568 showed comparatively low activity.

In diseased root extracts high PGTE activity was recorded with isolates KP, 481, SF and BP comparatively low with 1568 (While PGTE activity was nil in isolates RS and 482). Viscometric method (Table IV) showed high PGTE activity in culture filtrate of isolates 481 and 482 while it was comparatively low In other isolates. In

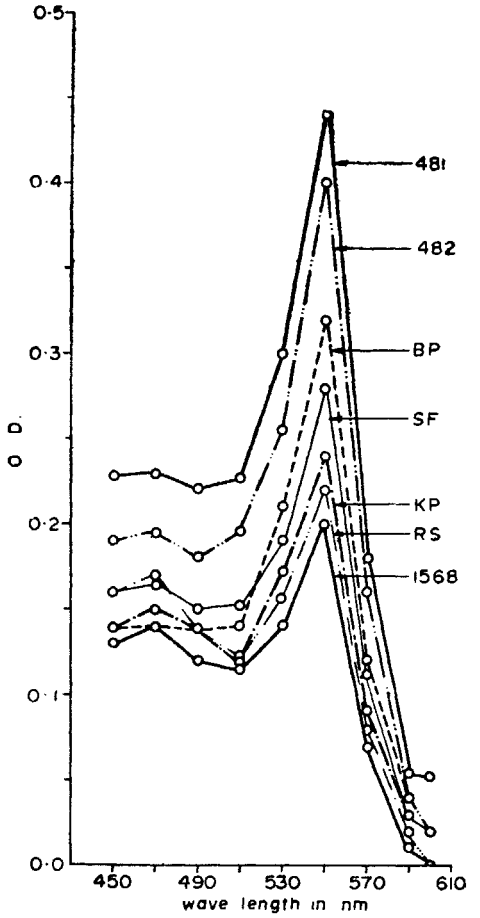

Fig. 1

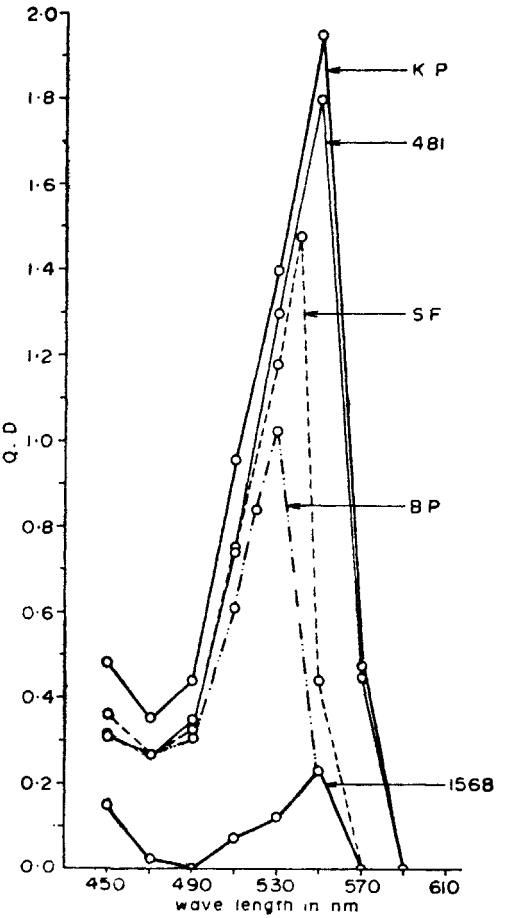

Fig. 2

FIGS. 1–2. 1, Polygalacturonate trans-eliminase activity in dialysed culture filtrates of *Rhizoctonia bataticola* isolates grown in basal medium by TBA method ; 2, Polygalacturonate trans-eliminase activity in extracts of diseased roots infected with different isolates of *R. bataticola* by TBA method.

diseased root extracts isolates KP, SF, 481 and BP showed high PGTE activity in comparison to other avirulent isolates.

No PGTE activity was detected with root extracts from healthy uninoculated control plants. Thus, production and activity of PGTE *in vivo*, seems to be closely correlated with virulence of the isolates.

Pectin methylesterase—PME activity from diseased root extracts and culture filtrates with seven isolates of *R. bataticola* was assayed by bromothymol-blue-indicator test of Smith (1958) and the results are presented in Table V. From this it is clear that the PME activity was maximum with isolate 481 and minimum with KP isolate in the culture filtrate. In root extracts maximum PME activity was recorded with KP isolate and minimum with RS isolate. There exists a close

TABLE IV

Polygalacturonate trans-eliminase activity in culture filtrates and root extracts with different isolates of R. bataticola *on sodium polypectate as the substrate at* pH 8.5

Enzyme source	Enzyme activity in terms of R.A. with different isolates of *R. bataticola*						
	BP	SF	KP	RS	1568	481	482
Culture filtrates	21.1	15.3	15.4	11.7	18.9	32.8	29.6
Diseased root-extract	27.6	23.6	26.6	3.7	4.3	21.5	11.0

TABLE V

Cellulase enzyme activity in culture filtrates and root extracts with different isolates of R. bataticola *on carboxymethyl cellulose as the substrate at* pH 5.00

Enzyme source	Enzyme activity in terms of relative activity (RA) with different isolates of *R. bataticola*						
	BP	SF	KP	RS	1568	481	482
Culture filtrate	16.4	21.2	13.3	16.8	19.4	29.0	23.1
Diseased root-extract	27.0	25.0	28.2	14.3	20.2	25.2	8.6

TABLE VI

Pectin-methyl-esterase enzyme activity in culture filtrates and root extracts with different isolates of R. bataticola *in terms of mg of methoxyl groups released within 24 hr*

Enzyme source	Amount of enzyme source taken (in ml)	mg of methoxyl groups released with different isolates of *R. bataticola*						
		BP	SF	KP	RS	1568	481	482
Culture filtrate	1	0.4	0.4	0.1	0.3	0.5	1.4	1.1
	2	0.7	0.4	0.2	0.7	0.7	1.7	2.1
Diseased root-extract	1	1.0	0.7	1.0	0.1	0.3	0.8	0.4
	2	1.5	1.1	1.9	0.1	0.7	1.3	0.7

correlation in the production of PME enzyme *in vivo* with virulence of the isolates thus PME enzymes might also be playing an important role in pathogenesis. Healthy root extracts showed very low PME activity.

Cellulase—Cellulase enzyme activity of all the seven isolates in culture filtrate and root extract was assayed by the standard viscometric method and the data is presented in Table V. In culture filtrate maximum cellulase activity was shown by isolates 481 and minimum with KP isolate. In root extracts, the cellulase activity was high in isolates SF, BP, KP and 481 and low with 482, RS and 1568. Hancock (1966)

found PGTE activity occasionally in host tissues infected with *Sclerotinia sclerotiorum* but not in culture filtrates. PG, PGTE and cellulase activities were shown to be correlated with the degree of virulence in inoculated sun flowers by Chan and Sackston (1970). Present work shows that the virulence of isolates of *R. bataticola* is not correlated with PG, PGTE, PME and cellulase enzyme production in culture, while the increased production of all these enzymes in inoculated plants with the virulence of the isolates confirm their definite role in pathogenesis.

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