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The biochemical constituents and pectinase activities associated with the virulence of *Rhizoctonia solani* isolates in rice in West Bengal, India

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Twenty five isolates of Rhizoctonia *solani* were isolated from infected rice fields from different agro-ecological regions of West Bengal. Cellulase and pectinase activities of the isolates were detected using carboxymethylcellulose (CMC) and pectin agar media, respectively. Diameter of the clearing zones on the respective media represented the level of enzyme activity of each isolate. Mycelial protein, carbohydrate and phenol contents were also recorded. The virulence of the isolates was studied on the rice cultivar (MTU 7029) under greenhouse conditions. Correlation of the biochemical constituents and enzyme activity with virulence of the *R. solani* isolates was performed. Pectinase activity found to be the important factor could predict the variation in the virulence of *R. solani* strains up to seventy six percent.

Key words: Pectinase, mycelial protein, carbohydrate, virulence, *Rhizoctonia solani.*

INTRODUCTION

Majority of the rice (90%) is produced in the Asian countries, with China and India being the major producers. Rice cultivation is often subjected to several biotic stresses of which diseases like blast, sheath blight, stem rot, and bacterial blight are the important ones. Among them, sheath blight ranks only after blast (Banniza and Holderness, 2001). The cosmopolitan fungus *Rhizoctonia solani* is an important plant pathogen worldwide. Plant tissue maceration and death of plant cells are the most dominant symptoms associated with R. *solani* and this is attributed to cell wall degrading enzyme activity of this pathogen. The management of *R. solani* is complicated due to the lack of sources of resistance and the ineffectiveness of fungicides in field trials (Cotterill, 1991). One strategy to engine resistance could therefore be the measures to reverse the virulence factor of the pathogen. A major barrier to host-penetration and colonization is the plant cell wall and all of the major groups of the plant pathogens are known to produce extra-cellular enzymes that can degrade cell wall polymers (Bateman and Basham, 1976). The enzymes produced by different pathogens are not always identical and within a single species of a pathogen, several enzymes and iso-enzymes are present. Constitutive pectinases are known to be virulence factor in *Aspergillus flavus* on cotton bolls and in *B. cinerea* on tomato (Shieh et al., 1997; ten Have et al., 1998). There is considerable evidence to suggest that cell wall degrading enzymes are

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determinants of virulence and therefore, represent potential targets for engineering resistance. Hence, the present research was aimed at studying the ability of *R. solani* isolates to produce phenol, protein, carbohydrate, cellulose and pectinase, and to study the correlation between the extracellular enzyme activity and mycelial biochemical constituents with the virulence of the isolates.

MATERIALS AND METHODS

R. solani isolates

Twenty five isolates collected from infected rice fields from different agro-ecological regions of West Bengal were maintained on potato dextrose agar (PDA- Hi media). Four days old pure culture of each isolate grown on PDA plates was used in the present investigation.

Inoculum preparation

Mycelial discs (4 mm diameter) from the margins of actively growing 4 days old culture were cut with a cork borer and used as the inoculum.

Study of the virulence of the isolates

The virulence of the 25 isolates of *R. solani* was determined by artificial inoculation on forty days old rice plant, under green house conditions (Figure 2).

Plant preparation

One month old rice seedlings (MTU 7029) were transplanted into 20 cm pots, filled with 2 kg of vermicompost amended soil (1:3 w/w). The soil was previously sterilized by autoclaving at 15 lbs for 30 min for three consecutive days. Pots were irrigated every alternate day, fertilized and hand weeded, whenever necessary ended is. Each isolate was considered as a separate treatment and three (replica) pots per treatment were maintained and kept on the greenhouse benches, 30 cm apart in a randomized complete block design containing three blocks.

Inoculation and disease rating

Rice plants were inoculated with *R. solani* thirty days after transplanting, by placing mycelial discs inside the rice sheath. The inoculum was held in place by wrapping absorbent cotton around the inoculated sheath (Figure 3). Water soaked lesions appeared 3 days after inoculation and disease severity was recorded at 20 days after inoculation by measuring both the height of the lesion and that of the plant height. Relative lesion height (RLH) was calculated using the following formula given by Sharma et al. (1990).

$$RLH = \frac{\text{Lesion height}}{\text{Plant height}} \times 100$$

0 = No infection; 1 = Lesion limited to lower 20% of the height of the plant; 3 = Lesion limited to lower 21-30% of the height of the plant; 5 = Lesion limited to lower 31-45% of height of the plant; 7 = Lesion limited to lower 46- 65% of height of the plant; 9 = Lesion more than 65% of the height of the plant; For evaluating the virulence of the isolates the 0-9 scale (Lal et al., 2012) was used.

1. DSV of isolates ≤5.0 = Low virulent isolates
2. 5.0 < DSV of isolates ≤ 6= Moderate to highly virulent isolates
3. DSV of isolates > 6.0 = High virulent isolates

Extracellular enzyme activity

All of the 21 isolates of *R. solani* were assayed for their ability to produce pectolytic and cellulolytic enzymes *in vitro* according to the method described by Godoy et al. (1990).

Cellulase activity

Isolates of *R. solani* were acclimatized by culturing them on cellulose agar plates before their transfer to the CMC (Carboxy methyl cellulose, Hi-media) growth media. Mycelial discs (5 mm) from the margin of actively growing 4-days-old cultures were aseptically transferred into the center of CMC-Na salt Petri-dish culture media (25 ml/plate) and incubated for 3 days at 24°C. Each isolate was replicated thrice. A control plate without the pathogen was maintained. After 3 days of incubation, culture plates were flooded with 1% w/v of Congo red (Hi-media) for 1 h at room temperature. The excess stain was discarded and the agar was de-stained with 1 M of NaCl solution. Plates were kept overnight at 4°C and examined for the clearing zone in the substrate around the point of inoculation. The diameter of the clear zone was measured and recorded. Cellulase activity was expressed as the ratio between the diameter of clear zone and the diameter of fungal growth in the control.

Pectin plate assay

Fungal isolates were grown on pectin agar media. The plates with 2 days old colonies were stained with 0.05% Ruthenium red (Hi-media) solution for 3 h and then the plates were thoroughly washed with distilled water twice. Plates without the pathogen were treated as control. Dark pink colour and a transparent zone suggested the utilization of pectin by the fungal isolates. The diameter of this zone was measured following the method of Hagerman (1985). The pectinase activity was expressed as the ratio between the diameter of the clear zone and the fungal growth in the control.

Collection of mycelial mats of *R. solani* isolates

The PDB (Potato dextrose broth, Hi-media) was inoculated with 2 days old fungal disc cut from the edge of the young growing colonies. They were allowed to grow for 4 days in an incubator (Orange Diagnostics) at 29 to 30°C and 99% relative humidity. At the end of the incubation, the mycelia mats were harvested, washed with sterile distilled water twice, dried in sterile blotting paper and kept at 50°C for overnight. Thereafter the dried fungal mat was used for further analysis.

Spectrophotometric estimation of total carbohydrate of the fungal mat

100 mg of fungal mycelia was taken in a boiling tube containing 5ml of 2.5 N HCl .The fungal mat was hydrolysed by keeping the tubes in a boiling water bath for three hours. The tubes were then cooled to room temperature and the slurry neutralized with solid sodium carbonate till the effervescence ceased. The volume was made up

Table 1. Distribution of different isolates of *Rhizoctonia solani* based on virulence.

Cluster No.	No. of isolates	Mean DSV	Name of the isolates
II (Low virulent)	4	3.975	Rs-80,91,22,17I
III (Moderate to highly virulent)	5	5.14	Rs-92,67,17II,12,14,6b
IV (Highly virulent)	11	7.67	Rs-36,10,49,56,6a,3,38,68,69,73,79

to 100 ml and centrifuged at 10,000 rpm for 15 min. The supernatant was collected and aliquots 0.5 and 1 ml were taken for analysis. The volume was made up to 1 ml by adding distilled water. Then 4 ml of anthrone reagent was added and heat for 8 min in a boiling water bath. The tubes were cooled rapidly and the green to dark green colour was appeared. The colour intensity was measured at 630 nm in a spectrophotometer (Zasco V-630). The amount of carbohydrate present in the samples was calculated from the standard curve prepared with different concentration of dextrose.

Estimation of total phenol from fungal mat

0.5 g of fungal mat was taken and ground in a mortar and pestle with 5 ml of 80% ethanol. The homogenate was centrifuged at 10, 000 rpm for 20 min. The supernatant was collected and evaporated to dryness. The residue was dissolved in 5 ml of distilled water. 1 ml aliquots were pipette out into test tubes and the volume made up to 3 ml with distilled water. 0.5 ml of Folin-Ciocaltue reagent (Merck) was added to each tube followed 3 min later by 2 ml of 20% sodium carbonate solution. The reagents were mixed thoroughly and placed in a boiling water bath for exactly 1 min., cooled and the absorbance measured in spectrophotometer (Zasco V-630) at 650 nm against a reagent blank. The standard curve was prepared using different concentrations of catechol (Malick and Singh, 1980).

Estimation of total protein from fungal mat

Fungal mat was washed with distilled water and then crushed in liquid nitrogen. 0.5 g fungal tissue dust was homogenized with 1ml 1M Sodium-phosphate buffer (pH-7) and centrifuged at 12000 rpm for 20 min at 4°C. The supernatant was collected and kept at 4°C and was used as the protein source for quantitative estimation in a spectrophotometer (Zasco V-630) at 660 nm following the method of Lowry (1951). Then reading was taken. The amount of protein present was calculated from standard curve prepared with different concentrations of bovine serum albumin.

RESULTS

Virulence of isolates

Variation in the virulence among the 21 isolates of *R. solani* was detected by their ability to infect and colonize the rice sheath under greenhouse conditions. Based on the relative lesion length, the isolates were grouped into three categories; low, moderate, and highly virulent (Table 1). There were differences also in disease severity caused by the isolates of *R. solani*. Four isolates, viz. Rs-17-I, 22, 80 and 91 belonging to low virulent group showed DSV value below 5, eight isolates from the moderate virulence group viz. Rs-12, 14, 67, 17II, 68, 69, 92 and 6b, exhibited DSV ranging from 5 to 6.9, while nine isolates Rs 36, 10, 49, 56, 6a, 3, 38, 73 and79 from the high virulent group showed DSV of above 7 and more (Table 2). Thus among the R. *solani* rice isolates, 19.05% belonged to low virulent group, 38.1% were moderately virulent and 42.86% were highly virulent. Similarly the virulence of the isolates could also be categorized into three groups. Variation in the virulence of isolates of *R solani* was reported by several workers (Shahjahan et al., 1987). Pascual et al. (2000) reported that fifty two isolates of *R. solani* belonged to anastomosis group AG1-1A and caused banded leaf and sheath blight in maize but they showed considerable variation in virulence.

Cellulase activity

Only some of the isolates exhibited positive activity for cellulase enzyme. Cellulase activity was indicated by clear zone created around the inoculation spot on CMC agar media (Figure 1A). Higher levels of cellulase activity were observed in the isolates of Rs-10, 14 and 91, while Rs-73, 79, 49 and 80 did not indicate cellulase activity in the plate. There was a negative correlation between the cellulase activity and the virulence of such isolates which was not significant. Hence some isolates showed high virulence even in the absence of cellulase activity and *vice versa*. 4.29% of the isolates collected from rice showed very high level of cellulase activity, 61.9% show moderately high level of cellulase activity while 4.76% of isolates showed low levels of activity (Table 2).

1. No Cellulase activity = 0
2. 0 < Cellulase activity ≤ 0.3 = low level of cellulase activity
3. 0.30< Cellulase activity ≤ 0.50 = medium level of cellulase activity activity
4. Cellulase activity > 0.50 = high level of cellulase activity

Pectinase activity

All isolates exhibited positive activity for pectinase enzyme. Pectinase activity was indicated by a clear zone created around the inoculation spot on pectin agar media (Figure 1B). Lowest levels (≤ 0.9) of pectinase activity was observed in the isolates of Rs-80, 92, 14, 17 II, 17I, 6b, 91, 67 and 22, whereas higher level (> 1.30) of it was

Table 2. Biochemical constituents and enzymatic activities of different *R. solani* isolates.

Name of the isolates	DSV	Carbohydrate content (mg g tissue^{-1})	Phenol content (mg g tissue^{-1})	Pectinae activity	Protein content (mg g tissue^{-1})	Cellulase activity
3	7.70^{c}	250.94	2.41	1.32	212.40	0.36
10	7.70^{c}	114.86	1.92	1.24	158.53	0.57
12	5.00^{b}	236.55	2.60	0.95	250.23	0.42
14	5.00^{b}	228.20	2.00	0.67	213.32	0.77
67	5.70^{b}	121.18	2.02	0.74	212.38	0.30
17 I	4.00^{a}	152.10	2.11	0.85	113.75	0.37
17II	5.00	160.94	2.18	0.60	121.23	0.41
22	4.50^{a}	154.53	2.27	0.65	179.33	0 39
36	8.00^{c}	269.67	2.39	1.40	219.47	0.37
38	8.80^{c}	266.49	2.53	1.45	138.68	0.39
49	8.80^{c}	241.20	2.15	1.48	134.03	0.00
56	8.20^{c}	260.44	2.40	1.24	205.30	0.39
68	6.00^{c}	150.92	1.97	1.12	202.99	0.42
69	6.00^{c}	230.39	1.95	1.02	287.46	0.41
73	7.30^{c}	235.29	2.67	1.10	190.33	0 00
79	7.70^{c}	239.39	2.51	1.20	269.96	0.00
80	3.70^{a}	150.49	2.28	0.85	149.46	0.00
92	5.00^{b}	157.65	2.12	0.71	151.26	0.38
6a	8.20^{c}	170.88	2.24	1.20	205.43	0.38
6b	5.70^{b}	152.84	2.27	0.65	120.44	0.45
91	3.70^{a}	131.58	2.07	0.75	102.33	0.59
SeM	0.184	5.66	0.046	0.011	3.418	NS
CD	0.53	16.69	0.135	0.030	10.082	NS

DSV = Disease severity Value; ^{NS}, non-significant; ^{a}, II (Low virulent); ^{b}, III (Moderate to highly virulent); ^{c}, IV (Highly virulent).

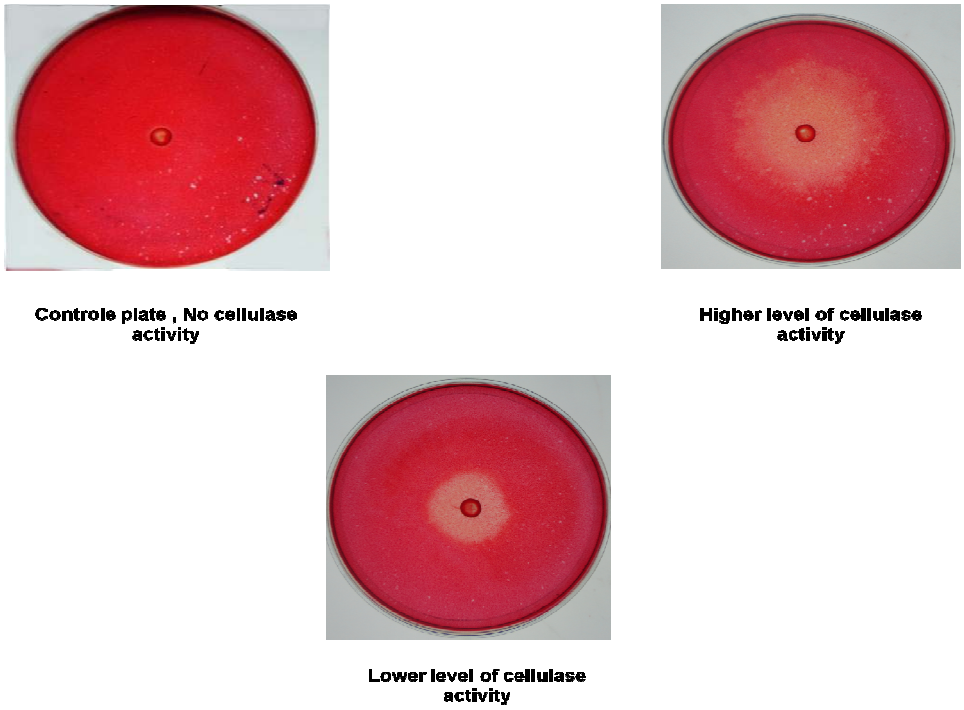

Figure 1A. Cellulase activity of *R. solani* isolates on plate.

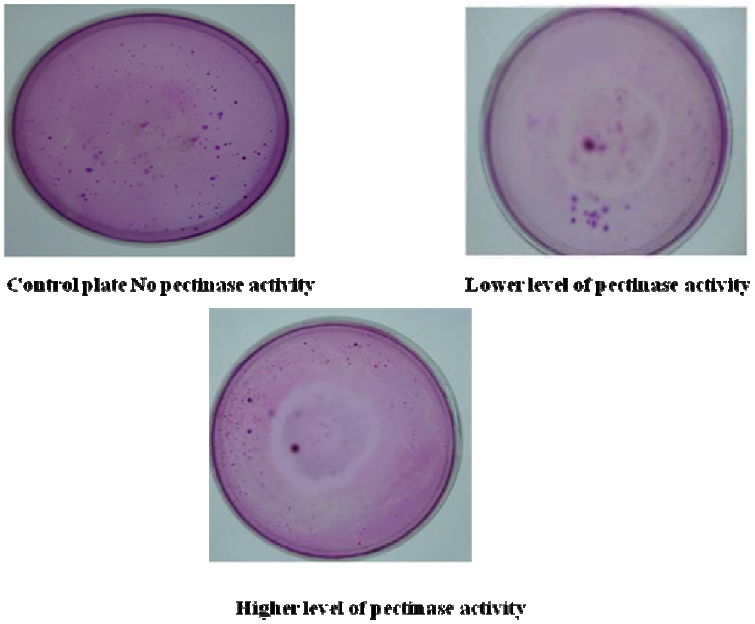

Figure 1B. Pectinase activity of *R. solani* isolates on plate.

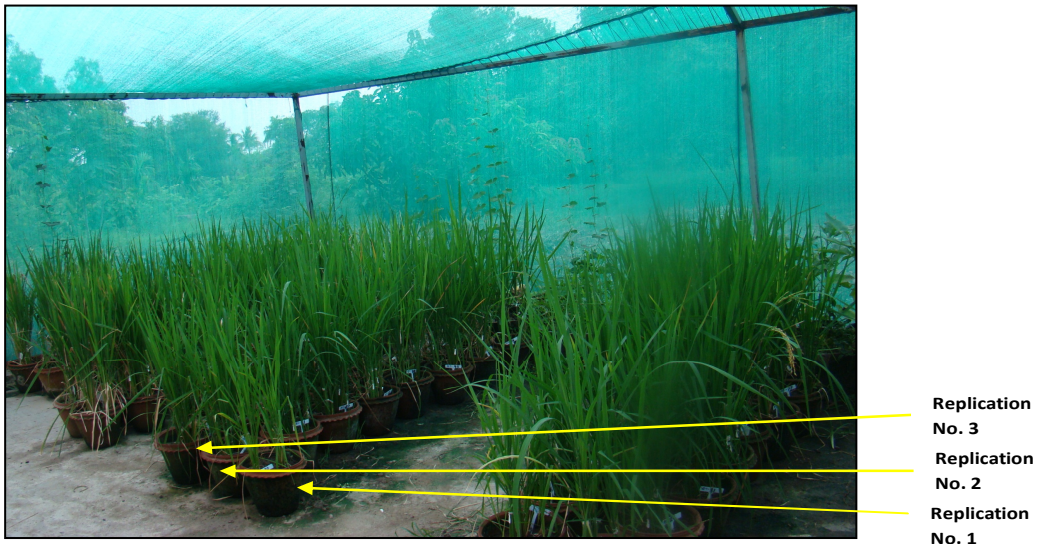

Figure 2. Virulence test was done under green house condition.

observed in the isolates of 38, 3, 49 and 36. 19.05% of the isolates collected from rice showed very high levels of pectinase activity, 38.1% showed moderately high levels of the enzyme activity, while 42.86% of isolates showed a low level of activity (Table 2). There was a significant positive correlation between the pectinase activity and the virulence of those isolates.

1. Pectinase activity ≤ 0.9 = low level of pectinase activity
2. 0.90 < Pectinase activity ≤1.30 = medium level of pectinase activity
3. Pectinase activity >1.30 = high level of pectinase activity.

Total carbohydrate content of fungal tissue

Mycelial carbohydrate content varied significantly among the different *R. solani* isolates. The highest range of mycelial carbohydrate content was recorded in Rs- 3, 12, 36, 38, 49, 56, 69, 73 and 79 isolates, whereas lower levels of mycelial carbohydrate was observed in the

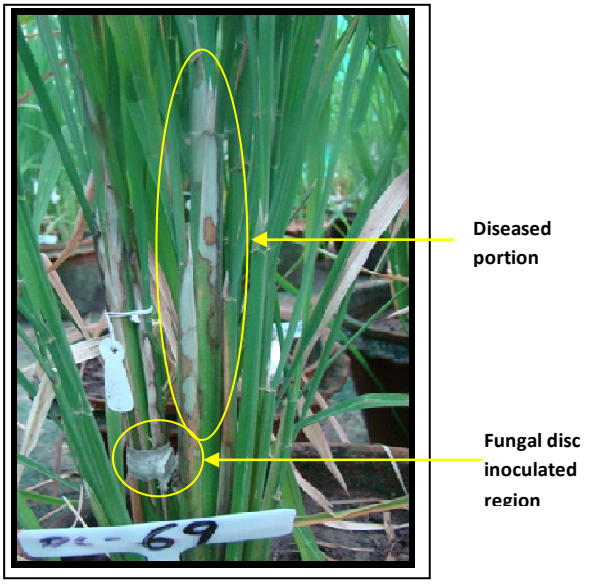

Figure 3. Picture shows the infected portions of Rice seedling.

isolates of Rs- 10, 67 and 91 (Table 2). Thus 42.86% of the isolates collected from rice contained high levels of carbohydrate and a percentage had medium level while 14.29% contained low levels of carbohydrate:

1. Carbohydrate content ≤150.0 = low level of carbohydrate content
2. 150.0 < Carbohydrate content ≤ 230.0 = medium level of carbohydrate content
3. Carbohydrate content > 230.0 = high level of carbohydrate content

There was a significant positive correlation between the mycelial carbohydrate content and the virulence of the isolates.

Mycelial phenol content of different *R. solani* isolates

Mycelial phenol contents varied significantly among the different *R. solani* isolates. The highest range of mycelial phenol content was recorded in Rs-73, 79 12, 38 and isolates, whereas lower level of the same was observed in the isolates of Rs-10, 14, 69 and 68 (Table 2). 19.05% of the isolates collected from rice showed very high levels of phenol, 61.90% show moderately high level of phenol while 19.05% of isolates showed low levels of phenol in the mycelia.

1. Phenol content ≤ 2.0 = low level of phenol content
2. 2.0 < Phenol content ≤ 2.5 = medium level of phenol content
3. Phenol content > 2.5 = high level of phenol content

Total protein content of fungal tissue

Mycelial protein contents varied significantly among the different *R. solani* isolates. The highest range of mycelial protein content was recorded in Rs-12, 69 and 79 isolates, whereas lower level of mycelial protein content was observed in the isolates of Rs- 10, 17I, 17II, 22, 38, 49, 73, 80, 92, 6b and 91 (Table 2). There was a positive correlation between the mycelial protein content and the virulence of those isolates. 14.29% of the isolates collected from rice showed very high level of protein, 33.33% showed moderately high levels of protein while 52.38% of isolates exhibited low levels of protein.

1. Protein content ≤ 200.0 = low level of protein content
2. 200.0< Protein content ≤ 250.0 = medium level of protein content
3. Protein content > 250 = high level of protein content

The biochemical constituents and extra-cellular enzymc activities of twenty one *R. solani* isolates with differences in virulence were statistically analysed for regression coefficient as independent variables and the disease severity, based on RLH, as a dependent variable. The result of the stepwise regression is presented in Equation 1. Correlation coefficient between all biochemical constituents and extra-cellular enzyme activities of the isolates with disease severity is presented in Table 3. A perusal of the data indicates that the virulence of *R. solani* was found to be significantly and positively correlated with pectinase activities and mycelia carbohydrate content of the fungi (at 1% level). There was also a positive correlation between the mycelial protein content and the virulence of the isolates (Table 3). Even some of the biochemical constituents and extra-cellular enzyme activities showed high correlation within themselves. Thus the independent variable considered here have certain amount of interdependence. So step wise regression was performed to eliminate the parameters having interdependence to find the most important biochemical constituents and extra-cellular enzyme activity contributing to the virulence of *R. solani*. Pectinase activity of the pathogen was regarded to be the most important predictor for virulence of *R. solani* and was found to predict the variation of virulence of *R. solani* up to seventy six percent.

DISCUSSION

The two tailed Pearson's correlation between virulence, pectinase activity and mycelial biochemical constituents of different *R. solani* isolates revealed that pectinase and mycelial carbohydrate content of the isolates was significantly and positively correlated with their virulence at 1% level. Based on the stepwise regression equation it was observed that extracellular pectinase activity and mycelial carbohydrate content were the two most

Table 3. Correlations correlation matrix including four biochemical constituents of *R. solani* isolates with the dependent variable (virulence of the pathogen).

	Virulence	Carbohydrate content	Phenol content	Pectinase activity	Total protein content	Cellulase activity
Virulence	1.000	0.597**	0.340	0.874**	0.273	-0.257

Equation 1: Y= 1.050 + 5.174** (Pectinase); R^2 = 0.764; $R^2_{(adj)}$= 0.752.

important predictors for evaluation of virulence of *R. solani* in rice system. It supported previous findings (Lumsden, 1976), in which they established the significant role of these enzymes in the pathogenic process. Virulence of *R. solani* is known to be dependent on nutritional status of the propagules (Weinhold et al., 1969). Thus from the above finding it may be concluded that pectinase activity and mycelial carbohydrate content were the two most important biochemical constituents which significantly contributed towards the virulence of *R. solani* isolates in rice system and only pectinase activity could determine the virulence of *R. solani* isolates by 76%. The pathogenic ability of *R solani* isolates with higher production of enzymes like pectic enzymes / polygalacturonase and cellulase was reported by Basu and Sengupta (1992); Banniza and Rutherford (2001). Asoufil et al., (2007) also reported that the cellulase and pectinase activities associated with the virulence of indigenous *Sclerotinia sclerotiorum* isolates in Jordan valley. However in the present investigation the role of cellulase in virulence appeared to be doubtful.

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