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Variability in Isozyme Patterns for Virulence among the *Rhizoctonia solani* Isolates Causing Banded Leaf and Sheath Blight in Maize

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ABSTRACT

Isozyme analysis/isozyme patterns of esterases and peroxidase of twenty eight isolates of the pathogen *R. solani* collected from maize (27) and one isolate from rice were used to study the variability among the isolates of *R. solani* through Polyacrylamide Gel Electrophoresis (PAGE)) and polyphenol oxidases by Spectrophotometer.. The study revealed that the isolates of *R. solani* showed considerable diversity in the production of enzyme which plays a major role in pathogenicity. The electrophoretic patterns of isozymes provided a good indication of genetic diversity among the isolates. The results obtained from isozyme analysis in this study suggested that isozyme analysis could be useful in genetic diversity studies and identification of various *R. solani* isolates.

Introduction

Maize (*Zea mays* L.) is a C_4, and third most important cereal crop in the world's agricultural economy. It is used as a source of food, feed and industrial products. In spite of having high yield potential it is susceptible to several biotic stresses. The banded leaf and sheath blight (BLSB) caused by *Rhizoctonia solani f.* sp. *sasakii* Exner, (Tel: *Thanatephorus sasakii* (Shirai) Tu and Kimbro) is a very destructive disease of maize and is gaining economic importance especially in several hot and humid tropical maize growing areas worldwide (Akhtar *et al.,* 2009). The pathogen causes grain yield loss to an extent of 40.0%. Being variable and belongs to ubiquitous group, it had a wide host range. In recent years, the disease occurrence was wide spread in almost all major maize growing areas of Telangana and Andhra Pradesh states with different intensities in most of the popular cultivars especially in rice fallow maize (zero tillage) cropping system.

The disease has become a major constraint in breeding programme because of the non-availability of widely adapted and stable source of resistance to BLSB. The variability in the pathogen considerably complicates the host range and resistance screening for host material. Without a clear knowledge of strains present in a particular cropping ecosystem it is very difficult to conclude the etiology as well as to select varieties for areas under this ecosystem. The knowledge on existence of pathotypes/ physiological races can be achieved based on the distinct variations in the pathogen in relation to their pathogenicity. Since *R. solani* is a variable pathogen and had a wide host range and so far, no attempt has been made to define variability of *R. solani* in relation to spatial distribution in Telangana and Andhra Pradesh states, the differences in isozyme patterns underlying among *R. solani* populations collected from different maize growing districts of Telangana State will provide a useful information of the pathogen.

An isozyme, a direct expression of genotype can be used as an indicator of genetic relationship within related populations. One of the tools in studying mechanisms involved in genetic diversity in fungal populations is the use of isozymes. Isozyme analysis is a powerful biochemical technique with numerous applications in Plant Pathology. Mycologists and Plant Pathologists adopted the procedure to settle taxonomic disputes and analyze genetic variability among the plant pathogens (Micales and Bonde, 1995). It has a potential for resolving relationship among imperfect fungi (Bosland and Williams, 1987). Studies on isozyme patterns of polyphenol oxidase and esterase have been used to know the variability among the various plant pathogens (Horvath and Vargas, 2004). For fungal isolates with few or no readily distinguishable morphological features, such as *R. solani* biochemical probes may provide markers useful for classification.

Electrophoretic banding patterns of isozymes are usually predictable based on their genetic background. The selection of enzyme systems is very important in detecting the variability among plant pathogens. Any study that uses broad classes of enzymes such as esterases, peroxidases, alkaline / acid phosphotases and polyphenol oxidases will display disproportionally high levels of intra-specific variation. Hence, in the present study variability in electrophoretic isozyme patterns of esterases and peroxidises; and polyphenol oxidases by spectrophotometer among the population of BLSB fungus *Rhizoctonia solani* (RS) was assessed for easy breeding task.

Materials and Methods

Twenty seven samples of maize exhibiting BLSB symptoms were collected from nine major maize growing districts of Telangana and Andhra Pradesh states at the rate of three different mandals in each district. The pathogen *R. solani* isolates were isolated, identified and designated as RS1 to RS27. One isolate from rice collected from RangaReddy district was designated as RS28 (Table 1).

Isozyme patterns of peroxidases and esterases of all the twenty eight isolates of the pathogen *R. solani* were studied (Laemmli, 1970) through Polyacrylamide Gel Electrophoresis (PAGE) and polyphenol oxidases by Spectrophotometer.

Cultivation of fungus for electrophoretic studies

Agar discs of 5 mm diameter containing actively growing hyphae was used as inoculum. The discs were cut from the periphery of one week old culture on PDA with the help of a sterile cork borer. These culture discs were transferred to 250 ml conical flasks containing 50 ml of sterile

Potato Dextrose Broth (PDB) medium and incubated for 10 days at 27 +2 ºC.

Extraction of Intra- cellular mycelial enzymes

After incubation of the fungus on PDB for 10 days, the mycelial mats developed on liquid broth were separated by filtering through Whatman No. 1 filter paper. The mats were washed thoroughly with distilled water and homogenized with 0.1 M Tris- HCl buffer of pH 7.5 using pre-chilled mortar and pestle. The homogenates were centrifuged at 3,500 rpm for 20 minutes in a refrigerated centrifuge (Eppendorf AG, Germany). The clear supernatants were separated and used in electrophoretic studies. Enzyme extraction was improved when sterile sea sand was used to macerate the fungal mats.

Electrophoretic assay

Polyacrylamide gel was used as the supporting medium for separation of enzymes fractions. The electrophoretic method followed in the present investigation was Polyacrylamide slab gel electrophoresis (PAGE) system as given by Sambrook *et al.,* (1989). Native PAGE was employed to study the isozyme patterns in the collected isolates.

Staining for detection of enzyme bands

After performing the native PAGE the gels were taken off from the sandwiches carefully and stained with suitable stains to detect peroxidases and esterase isozyme bands.

a) Esterases (Shaw and Prasad, 1970)

The gel was immersed in the staining solution of Phosphate buffer (pH 6.0) containing 1% α-naphthyl acetate and β-naphthyl acetate and fast green (Shaw and Prasad, 1970) and incubated at 30^0C for 30 minutes in dark. The gels were destained in 7 per cent acetic acid for 30 minutes, photographed using gel documentation system and preserved in 2 % acetic acid solution.

b) Peroxidases (Mahmoud *et al.,*2007)

The gels were incubated in 0.05 M acetate buffer (pH 5.0) containing 65 mg of o-dianisidine dissolved in 5 ml of ethanol. After staining the gels were destained in 7 per cent acetic acid for 30 minutes, photographed through gel documentation system and stored in 2 per cent acetic acid solution.

c) Polyphenol oxidases (Erhan Astarci, 2003)

Polyphenol oxidase activity was measured spectrophotometrically. Culture supernatant was used as crude enzyme source. 100 mM catechol in 0.2 M phosphate buffer at pH 7.0 was used as substrate (Erhan Astarci, 2003). The reaction mixture contained 1 ml of 0.2 M phosphate buffer (pH 7.0), 0.5 ml culture supernatant and 0.5 ml substrate solution (100 mM) incubated at 50 ºC for 3 minutes. The reference cuvette contained buffer instead of enzyme and change in absorbance was followed at 410 nm and initial reaction rate was used to determine the enzyme activity. The enzyme activity was expressed as µg ppo/ mg proteins.

One unit of enzyme (U) is defined as the amount of the enzyme required to obtain 0.001 Optical Density (OD) change at 410 nm.

Zymograms and Similarity Index (SI) for esterases and peroxidase

The zymograms were prepared indicating the Relative mobility (Rm) values of isozyme bands. The differences in isozyme patterns of esterase and peroxidases enzymes were quantitatively expressed in terms of relative mobility (Rm) values.

Relative mobility (Rm) =

$$\frac{\text{Distance travelled by enzyme front}}{\text{Distance travelled by dye front}}$$

Similarity index (SI) and genetic distance values were calculated using the following formula.

$$SI = \frac{\text{No. of pairs of similar bands}}{\text{No. of different bands + No. of pairs of similar bands}} \times 100$$

Genetic distance = 100 – Similarity Index

Results and Discussion

Esterase

The banding pattern of esterase, their relative mobility, density and intensity were recorded by zymogram. The data presented in the Table 2, provides convenient means for comparing the banding patterns among all the *R. solani* isolates. The migration distances (from the origin to the cathode) of all bands from each isolate were compared with those of the bands from every other isolate. The comparison between the isolates was made based on relative mobility (Rm) values, density of bands, similarity index and genetic distance values. The *R. solani* isolates differed greatly with respect to esterase isozyme activity among them. A total of 57 esterase isozyme bands were produced in all the isolates including very faint bands (Plate 1).

The variation within these isozyme bands of different isolates usually involved in position of faint, minor bands or the comparative thickness of density of bands. Two bands numbered 7 and 10 were frequent, prominent and dense to medium and were found in most of the isolates. The Rm values of these isolates ranged from as low as 0.06 to as high as 0.55. Band number 1 with Rm value of 0.06 was observed by the isolates RS1(Medak), RS15(Warangal), RS24(West Godavari), RS25 and RS 27(Kurnool). The maize isolate RS11 from Khammam district and rice RS28 has produced their fourteenth band with Rm value of 0.44. But the final band with Rm value 0.55 was specific to the virulent isolate from the study i.e RS11 from Khammam district. However the isolate RS17 from Krishna district has produced maximum number of isozyme bands followed by the isolates RS12 and RS16 compared to rest of the isolates. Similarity index values between the isolates ranged from 0.43 to 1.00. Maximum similarity index values (1.00) were observed between the isolate combinations RS1 (Telangana) and RS27 (Rayalaseema); RS13 (Telangana) and RS21(Andhra); RS 24(Andhra) and RS25 (Rayalaseema). Minimum similarity index value of 0.43 was observed between the isolates RS12 (Telangana)-RS17 (Andhra Pradesh) (Table 3).

The esterase isozyme was estimated, depending on presence (1) or absence (0) of bands in each isolates by visual scoring and set in a binary matrix. Enzymatic similarities among the isolates were determined based on Jaccard's coefficient. A dendrogram was then constructed based on similarity levels generated from cluster analysis using the Unweighted Pair Group Method of the Arithmetic average (UPGMA) with a SAHN module of NTSYspl software version 2.1.

The zymogram constructed based on similarity levels generated from cluster analysis represents that all the 28 *R. solani* isolates were grouped into 2 main clusters at 0.67 coefficient distance (Fig.1). As per the dendrogram, maximum isolates showed higher similarity value *i.e* more than 85 % however isolates (RS1 and RS 27; RS5and RS7; RS4,

RS6 and RS9; RS13 and RS21; RS 24 and RS25) have shown 100 % similarity compared to rest of the isolates tested.

Genetic distance values was also more in the isolate combination RS12-RS17. Maximum genetic distance (99.44) was observed between the isolate combinations RS10-RS12; RS11-RS12; RS12-RS19; RS12-RS23; RS12-RS26; RS17-RS18 and RS17-RS25, while minimum genetic distance (99.0) values were noticed in the combinations given above for which similarity index values were maximum (100).

Peroxidase

The migration pattern of peroxidase among these *R. solani* isolates differed in their number, migration distance from the origin and width of each band along length of the gel. A total of 48 isozyme bands of peroxidase were observed among all the 28 isolates of *R. solani*. The band number 4 was medium to dense and found prominent among the isolates RS2(Medak), RS13, RS14, RS15 (Warangal) and RS17 (Krishna). The three bands 16, 18 and 19 were intense and thick on the polyacrylamide gel and were present in the maize isolates RS11(Khammam), RS16 (Krishna) and rice isolate RS28 (Plate 2).

The Rm values of peroxidase isozyme scored by the 28 *R. solani* isolates ranged from 0.26 to 0.94. Band number 1 with Rm value of 0.26 was specific to the isolate RS4, RS8, RS9 and RS22 (Table 4). The 4th band with 0.35 Rm value was present in the isolates RS13, RS14, RS15and RS17. The bands 6 and 20 were observed only in the isolates of RS24 (West Godavari) and RS16 (Krishna) with 0.39 and 0.69 Rm values respectively. The maize isolates RS11(Khammam), RS26 (Kurnool) and rice RS28 had band number 19 with 0.65 Rm value. Similarly, band 15th is common among the isolates RS18, RS19, RS20, and RS27 with 0.55 Rm value. The maize isolate RS12 from Khammam district has produced a unique dense band with 0.94 Rm value. However, the maize isolate RS17 from Krishna district has produced more number of peroxidise isozyme bands. The similarity index values between the isolates for peroxidase patterns ranged from 0.66 to 1.00. Cent per cent (1.00) similarity was observed between the isolate combinations RS8, RS9 and RS 22; RS5 and RS6; RS19 and RS20; RS13 and RS15; RS11 and RS26. The other isolates RS10-RS11; RS11-RS17; RS17-RS24; and RS17- RS26 shared minimum (0.66) similarity index (Table 5).

The phylogenetic tree resulting from cluster analysis based on isozyme data, revealed that the *R. solani* isolates separated into two major clusters which were separated at a distance of 0.79 on coefficient value scale. In cluster II, the isolate RS 10 (Khammam) had separated at a coefficient value of 0.79 (Fig.2).

The zymogram tree has further divided the cluster I with 27 isolates into 4 groups *i.e* IA, IB, IC and ID. The isolate RS24 from West Godavari in Group IA had separated at 0.80 coefficient value. In group IB, the virulent isolate, RS11 from Khammam district of Telangana shared cent percent similarity with the isolate RS 26 from Kurnool district of Andhra Pradesh and together these maize isolates shared 95.4% similarity coefficient with the rice isolate RS 28. The isolate RS17 from Krishna district of Andhra Pradesh showed similarity with the isolates, RS13-RS15; and RS14 from Warangal district of Telangana at 0.87 coefficient value.

Group ID is further divided into 2 sub groups *i.e.* ID-1 and ID-2.In group ID-1 isolates RS1(Telangana) and RS7 (Andhra Pradesh) are similar at 95.4 % similarity coefficient and shared similarity with the isolate RS21 at 0.93 coefficient value. Under ID-2 group, the isolates RS8, RS9 and RS22; RS5 and RS6 have shown 100% similarity. Similarly the

similarity index shared between the isolates RS2 and (RS8, RS9 and RS22); RS4 and (RS5, RS6) was 95.4%. Group ID-2 has two more minor clusters *i.e.* ID-2 (a) and ID-2(b). In group ID- 2(b) 100 % similarity was observed between the isolates, RS19 and RS20 and shared 95.4 % similarity with isolate RS18. Together, these isolates with isolate RS27 had shown 94 % similarity.

Genetic distance values were found to be maximum (99.34) in between the isolate combinations RS10 (Khammam) - RS17 (Krishna); RS11(Khammam) - RS17(Krishna); RS17(Krishna) - RS24 (West Godavari) and RS17 (Krishna) - RS26 (Kurnool). Least genetic distance values (99.0) were recorded in between the isolate combinations mentioned above for which the similarity index values were maximum (1.00).

Estimation of polyphenol oxidases

From the Table 6**,** it is evident that all the 28 isolates of *R. solani* increased the ppo activity significantly over time. Among the 27 maize isolates, significantly higher quantities were observed in isolate RS11 from Khammam district with 0.080 quantity of ppo followed by RS16 (0.074), RS 12 (0.071) RS17 (0.053) at OD value above 0.050 to 0.080 value and lowest quantity of (0.004) was produced by the isolate RS21 from Guntur district at below 10 OD value.

However the rice isolate RS28 has recorded highest (0.097) ppo activity compared to all the maize isolates and significantly differed from the maize isolates except for RS11, RS12 (from Khammam) and RS 16 (Vatsavai mandal of Krishna district) which were at par with each other.

The results showed that increase in absorbance and formation of brown colour in samples when assayed in catechol was due to the existence of the enzyme, not because of a compound that exists in the growth medium of fungus. In other words, the oxidation of catechol substrate was due to the existence of polyphenol oxidase. So, it can be suggested that all the *R. solani* isolates have produced polyphenol oxidase enzyme.

In the present study, diversity in isozyme patterns was evident within *R. solani* isolates obtained from various geographical locations in Telangana and Andhra Pradesh states. Based on isozyme patterns of peroxidase and esterase, each isolate could be separated based on the intensity and relative mobility of different isozymes.

The degree of relationship as reflected in the banding patterns of the peroxidise isozyme tested among the isolates of *R. solani* was depicted as zymogram. The isolates RS2, RS13, RS14, RS15 and RS17 from Doulatabad, Janagoan, Atmakur, Hasanparthy and Nuziveedu mandals have produced common band with relative mobility of 0.35.The minimum similarity index (66%) and maximum genetic distance value (34%) was observed in the isolate combinations of RS17-RS24**;** RS17-RS26.

It is evident from the results that the isolates of *R. solani* varied with respect to their esterase banding pattern. The band numbers 7 and 10 with relative mobility values were common to most of the isolates tested. But the fifteenth band was confined to two isolates RS11(maize) and RS28 (rice). The final band which is a low molecular weight band with relative mobility value of 0.55 was specific to the virulent isolate RS11 showing maximum / more enzyme activity compared to rest of the isolates. From the dendrogram obtained by cluster analysis of esterase banding patterns, which separated all the 28 isolates into two major clusters, in which cluster B had only one isolate *i.e.* RS12 from Yellandu mandal of Khammam distrct and the rice isolate RS28 clubbed with maize isolate RS11 in group A.

Table.1 Details *of Rhizoctonia solani* f sp *sasakii* isolates collected from major maize growing districts of Telangana and Andhra Pradesh States

S.No.	Isolates	Place of collection	District	State	Variety/Cultivar
1	RS1	Pragnyapur	Medak	Telangana	Kaveri-225
2	RS2	Doulatabad	Medak	Telangana	Pioneer-30V92
3	RS3	Gajwel	Medak	Telangana	Kanchana
4	RS4	Armur	Nizamabad	Telangana	C-Tex
5	RS5	Kamareddy	Nizamabad	Telangana	Kaveri
6	RS6	Mortad	Nizamabad	Telangana	Kanchana
7	RS7	Jagityal	Karimnagar	Telangana	C-Tex/Prince
8	RS8	Metpally	Karimnagar	Telangana	Pioneer
9	RS9	Raichal	Karimnagar	Telangana	Kaveri Gold
10	RS10	Bonakal	Khammam	Telangana	Kanchana
11	RS11	Chintakani	Khammam	Telangana	Pioneer
12	RS12	Yellandu	Khammam	Telangana	Kaveri /C-Tex
13	RS13	Janagoan	Warangal	Telangana	Yecca
14	RS14	Atmakur	Warangal	Telangana	Kanchana
15	RS15	Hasanparthy	Warangal	Telangana	Pioneer
16	RS16	Vatsavai	Krishna	Andhra Pradesh	Kaveri-50
17	RS17	Tiruvur	Krishna	Andhra Pradesh	Yecca
18	RS18	Nuziveedu	Krishna	Andhra Pradesh	Pioneer-30V 92
19	RS19	Tenali	Guntur	Andhra Pradesh	Pioneer/Kargil
20	RS 20	Mangalgiri	Guntur	Andhra Pradesh	Kaveri 255
21	RS 21	Kolipara	Guntur	Andhra Pradesh	Yecca
22	RS22	Eluru	West Godavari	Andhra Pradesh	Pioneer-30V 92
23	RS23	Jangareddygudem	West Godavari	Andhra Pradesh	Kanchana
24	RS24	Jeelugumilli	West Godavari	Andhra Pradesh	Kaveri-255
25	RS25	Nandikotkur	Kurnool	Andhra Pradesh	Kaveri Gold
26	RS26	Atmakur	Kurnool	Andhra Pradesh	Kargil
27	RS27	Thatipadu	Kurnool	Andhra Pradesh	Pioneer
28	RS28 (Rice)	Rajendranagar	RangaReddy	Telangana	BPT-5204

Table.2 Relative mobility (Rm) values of esterase of *R.solani* isolates

S No.	Rm	RS1	RS2	RS3	RS4	RS5	RS6	RS7	RS8	RS9	RS10	RS11	RS12	RS13	RS14	RS15	RS16	RS17	RS18	RS19	RS20	RS21	RS22	RS23	RS24	RS25	RS26	RS27	RS28
1	**0.06**	0.06														0.06									0.06	0.06		0.06	
2	**0.16**								0.16				0.16	0.2					0.16			0.16							
3	**0.19**			0.19	0.19		0.19			0.19							0.19	0.19						0.19					
4	**0.21**																												
5	**0.23**					0.23		0.23					0.23																
6	**0.24**																0.24	0.24						0.24					0.24
7	**0.27**		0.27	0.27					0.27						0.27					0.27					0.27	0.27			
8	**0.29**										0.29																0.29		
9	**0.31**															0.31		0.31		0.31	0.31								
10	**0.34**								0.34		0.34	0.34			0.34		0.34	0.34		0.34	0	0	0.34	0.34			0.34		
11	**0.35**												0.35						0.35										
12	**0.37**																										0.37		
13	**0.39**										0.39							0.39											
14	**0.4**												0.40				0.40												
15	**0.44**											0.44																	0.44
16	**0.55**											0.55																	

Table.3 Similarity index values of esterase patterns of *R. solani* isolates

	RS1	RS2	RS3	RS4	RS5	RS6	RS7	RS8	RS9	RS10	RS11	RS12	RS13	RS14	RS15	RS16	RS17	RS18	RS19	RS20	RS21	RS22	RS23	RS24	RS25	RS26	RS27	RS28
RS1	1.00																											
RS2	0.93	1.00																										
RS3	0.87	0.93	1.00																									
RS4	0.93	0.87	0.93	1.00																								
RS5	0.93	0.87	0.81	0.87	1.00																							
RS6	0.93	0.87	0.93	1.00	0.87	1.00																						
RS7	0.93	0.87	0.81	0.87	1.00	0.87	1.00																					
RS8	0.81	0.87	0.81	0.75	0.75	0.75	0.75	1.00																				
RS9	0.93	0.87	0.93	1.00	0.87	1.00	0.87	0.75	1.00																			
RS10	0.81	0.75	0.68	0.75	0.75	0.75	0.75	0.75	0.75	1.00																		
RS11	0.81	0.75	0.68	0.75	0.75	0.75	0.75	0.75	0.75	0.75	1.00																	
RS12	0.75	0.68	0.62	0.68	0.81	0.68	0.81	0.68	0.68	0.56	0.56	1.00																
RS13	0.93	0.87	0.81	0.87	0.87	0.87	0.87	0.87	0.87	0.75	0.75	0.81	1.00															
RS14	0.87	0.93	0.87	0.81	0.81	0.81	0.81	0.93	0.81	0.81	0.81	0.62	0.81	1.00														
RS15	0.87	0.81	0.75	0.82	0.81	0.81	0.81	0.68	0.81	0.68	0.68	0.62	0.81	0.75	1.00													
RS16	0.75	0.68	0.75	0.81	0.68	0.81	0.68	0.68	0.81	0.68	0.68	0.62	0.68	0.75	0.62	1.00												
RS17	0.68	0.62	0.68	0.75	0.62	0.75	0.62	0.62	0.75	0.75	0.62	0.43	0.62	0.68	0.68	0.81	1.00											
RS18	0.87	0.81	0.75	0.81	0.81	0.81	0.81	0.81	0.81	0.68	0.68	0.87	0.93	0.75	0.75	0.62	0.56	1.00										
RS19	0.81	0.87	0.81	0.75	0.75	0.75	0.75	0.87	0.75	0.75	0.75	0.56	0.75	0.93	0.81	0.68	0.75	0.68	1.00									
RS20	0.93	0.87	0.81	0.87	0.87	0.87	0.87	0.75	0.87	0.75	0.75	0.68	0.87	0.81	0.93	0.68	0.75	0.81	0.87	1.00								
RS21	0.93	0.87	0.81	0.87	0.87	0.87	0.87	0.87	0.87	0.75	0.75	0.81	1.00	0.81	0.81	0.68	0.62	0.93	0.75	0.87	1.00							
RS22	0.93	0.87	0.81	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.68	0.87	0.93	0.81	0.81	0.75	0.81	0.87	0.87	0.87	1.00						
RS23	0.81	0.75	0.81	0.87	0.75	0.87	0.75	0.75	0.87	0.75	0.75	0.56	0.75	0.81	0.68	0.93	0.87	0.68	0.75	0.75	0.75	0.87	1.00					
RS24	0.87	0.93	0.87	0.81	0.81	0.81	0.81	0.81	0.81	0.68	0.68	0.62	0.81	0.87	0.87	0.62	0.56	0.75	0.81	0.81	0.81	0.81	0.68	1.00				
RS25	0.87	0.93	0.87	0.81	0.81	0.81	0.81	0.81	0.81	0.68	0.68	0.62	0.81	0.87	0.87	0.62	0.56	0.75	0.81	0.81	0.81	0.81	0.68	1.00	1.00			
RS26	0.81	0.75	0.68	0.75	0.75	0.75	0.75	0.75	0.75	0.87	0.75	0.56	0.75	0.81	0.68	0.68	0.62	0.68	0.75	0.75	0.75	0.87	0.75	0.68	0.68	1.00		
RS27	1.00	0.93	0.87	0.93	0.93	0.93	0.93	0.81	0.93	0.81	0.81	0.75	0.93	0.87	0.87	0.75	0.68	0.87	0.81	0.93	0.93	0.93	0.81	0.87	0.87	0.81	1.00	
RS28	0.87	0.81	0.75	0.81	0.81	0.81	0.81	0.68	0.81	0.68	0.81	0.62	0.81	0.75	0.75	0.75	0.68	0.75	0.68	0.81	0.81	0.81	0.81	0.75	0.75	0.68	0.87	1.00

Table.4 Relative mobility (Rm) values of peroxidase of *R. solani* isolates

S No.	Rm	RS1	RS2	RS3	RS4	RS5	RS6	RS7	RS8	RS9	RS10	RS11	RS12	RS13	RS14	RS15	RS16	RS17	RS18	RS19	RS20	RS21	RS22	RS23	RS24	RS25	RS26	RS27	RS28
1	**0.26**				0.26				0.26	0.26													0.26						
2	**0.32**				0.32	0.32	0.32																						
3	**0.34**	0.34						0.34														0.34			0.34				
4	**0.35**		0.35											0.35	0.35	0.35		0.35											
5	**0.37**	0.37																								0.37			
6	**0.39**																								0.39				
7	**0.4**																	0.40											
8	**0.42**			0.42							0.42																		
9	**0.44**																											0.44	
10	**0.45**																	0.45											
11	**0.48**																					0.48							
12	**0.5**														0.50										0.50				
13	**0.52**										0.52																		
14	**0.53**													0.53		0.53		0.53											
15	**0.55**																		0.55	0.55	0.55							0.55	
16	**0.58**											0.58												0.58			0.58		
17	**0.6**										0.60								0.60										
18	**0.61**											0.61															0.61		0.61
19	**0.65**											0.65															0.65		0.65
20	**0.69**																0.69												
21	**0.94**												0.94																

Table.5 Similarity index values of peroxidase patterns of *R. solani* isolates

	RS1	**RS2**	**RS3**	**RS4**	**RS5**	**RS6**	**RS7**	**RS8**	**RS9**	**RS10**	**RS11**	**RS12**	**RS13**	**RS14**	**RS15**	**RS16**	**RS17**	**RS18**	**RS19**	**RS20**	**RS21**	**RS22**	**RS23**	**RS24**	**RS25**	**RS26**	**RS27**	**RS28**
RS1	1.00																											
RS2	0.85	1.00																										
RS3	0.85	0.90	1.00																									
RS4	0.80	0.85	0.85	1.00																								
RS5	0.85	0.90	0.90	0.95	1.00																							
RS6	0.85	0.90	0.90	0.95	0.01	1.00																						
RS7	0.95	0.90	0.90	0.85	0.90	0.90	1.00																					
RS8	0.90	0.95	0.95	0.90	0.95	0.95	0.95	1.00																				
RS9	0.90	0.95	0.95	0.90	0.95	0.95	0.95	0.01	1.00																			
RS10	0.76	0.90	0.90	0.76	0.80	0.80	0.80	0.85	0.85	1.00																		
RS11	0.76	0.80	0.80	0.76	0.80	0.80	0.80	0.85	0.85	0.71	1.00																	
RS12	0.85	0.90	0.90	0.85	0.90	0.90	0.90	0.95	0.95	0.80	0.80	1.00																
RS13	0.80	0.85	0.85	0.80	0.85	0.85	0.85	0.90	0.90	0.76	0.76	0.85	1.00															
RS14	0.80	0.85	0.85	0.80	0.85	0.85	0.85	0.90	0.90	0.76	0.76	0.85	0.90	1.00														
RS15	0.80	0.85	0.85	0.80	0.85	0.85	0.85	0.90	0.90	0.76	0.76	0.85	0.01	0.90	1.00													
RS16	0.85	0.90	0.90	0.85	0.90	0.90	0.90	0.95	0.95	0.80	0.80	0.90	0.85	0.85	0.85	1.00												
RS17	0.71	0.76	0.76	0.71	0.76	0.76	0.76	0.80	0.80	0.66	0.66	0.76	0.90	0.80	0.90	0.76	1.00											
RS18	0.80	0.85	0.85	0.80	0.85	0.85	0.85	0.90	0.90	0.85	0.76	0.85	0.80	0.80	0.80	0.85	0.71	1.00										
RS19	0.85	0.90	0.90	0.85	0.90	0.90	0.90	0.95	0.95	0.80	0.80	0.90	0.85	0.85	0.85	0.90	0.76	0.95	1.00									
RS20	0.85	0.90	0.90	0.85	0.90	0.90	0.90	0.95	0.95	0.80	0.80	0.90	0.85	0.85	0.85	0.90	0.76	0.95	0.01	1.00								
RS21	0.90	0.85	0.85	0.80	0.85	0.85	0.95	0.90	0.90	0.76	0.76	0.85	0.80	0.80	0.80	0.85	0.71	0.80	0.85	0.85	1.00							
RS22	0.90	0.95	0.95	0.90	0.95	0.95	0.95	0.01	0.01	0.85	0.85	0.95	0.90	0.90	0.90	0.95	0.80	0.90	0.95	0.95	0.90	1.00						
RS23	0.85	0.90	0.90	0.85	0.90	0.90	0.90	0.95	0.95	0.80	0.90	0.90	0.85	0.85	0.85	0.90	0.76	0.85	0.90	0.90	0.85	0.95	1.00					
RS24	0.85	0.80	0.80	0.76	0.80	0.80	0.90	0.85	0.85	0.71	0.71	0.80	0.76	0.85	0.76	0.80	0.66	0.76	0.80	0.80	0.85	0.85	0.80	1.00				
RS25	0.95	0.90	0.90	0.85	0.90	0.90	0.90	0.95	0.95	0.80	0.80	0.90	0.85	0.85	0.85	0.90	0.76	0.85	0.90	0.90	0.85	0.95	0.90	0.80	1.00			
RS26	0.76	0.80	0.80	0.76	0.80	0.80	0.80	0.85	0.85	0.71	0.01	0.80	0.76	0.76	0.76	0.80	0.66	0.76	0.80	0.80	0.76	0.85	0.90	0.71	0.80	1.00		
RS27	0.80	0.85	0.85	0.80	0.85	0.85	0.85	0.90	0.90	0.76	0.76	0.85	0.80	0.80	0.80	0.85	0.71	0.90	0.95	0.95	0.80	0.90	0.85	0.76	0.85	0.76	1.00	
RS28	0.80	0.85	0.85	0.80	0.85	0.85	0.85	0.90	0.90	0.76	0.95	0.85	0.80	0.80	0.80	0.85	0.71	0.80	0.85	0.85	0.80	0.90	0.85	0.76	0.85	0.95	0.80	1.00

Table.6 Variation in quantity of polyphenol oxidase content in the mycelium of *R. solani* isolates

S.No	Isolate	OD value at 410 nm
1	RS1	0.009
2	RS2	0.012
3	RS3	0.008
4	RS4	0.047
5	RS5	0.021
6	RS6	0.025
7	RS7	0.013
8	RS8	0.031
9	RS9	0.015
10	RS10	0.034
11	RS11	0.080
12	RS12	0.071
13	RS13	0.005
14	RS14	0.024
15	RS15	0.024
16	RS16	0.074
17	RS17	0.053
18	RS18	0.024
19	RS19	0.012
20	RS20	0.021
21	RS21	0.004
22	RS22	0.012
23	RS23	0.012
24	RS24	0.009
25	RS25	0.012
26	RS26	0.022
27	RS27	0.024
28	RS28 (Rice)	0.097
	CD 5%	0.026
	SE(d)	0.013
	SE(m)	0.009
	CV	54.961

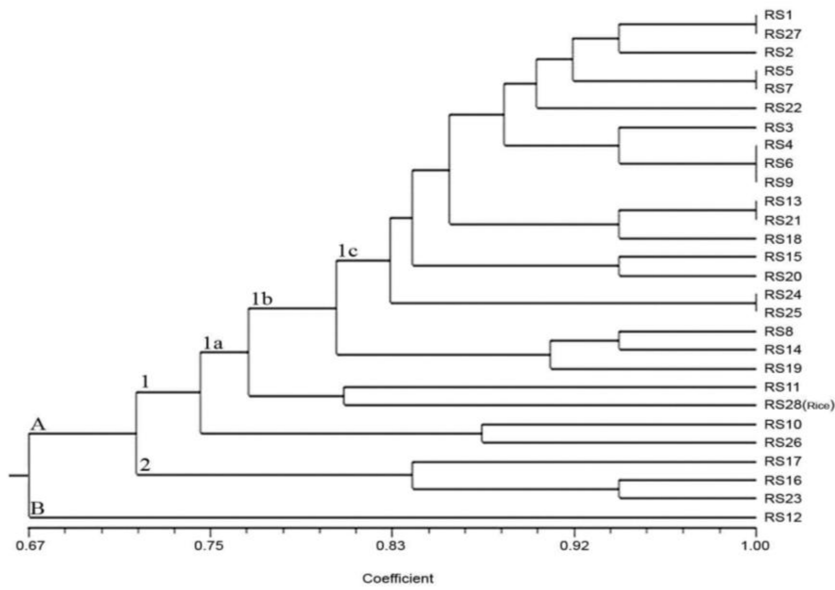

Figure 1. Grouping of *R. solani* isolates based on similarity index of esterase enzyme

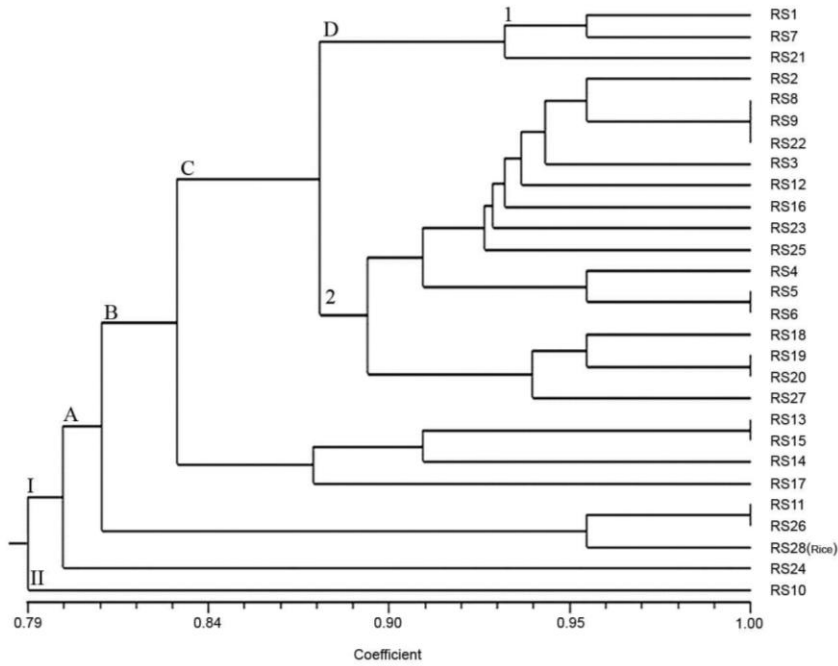

Figure **2** Grouping of *R. solani* isolates based on similarity index of peroxidase enzyme

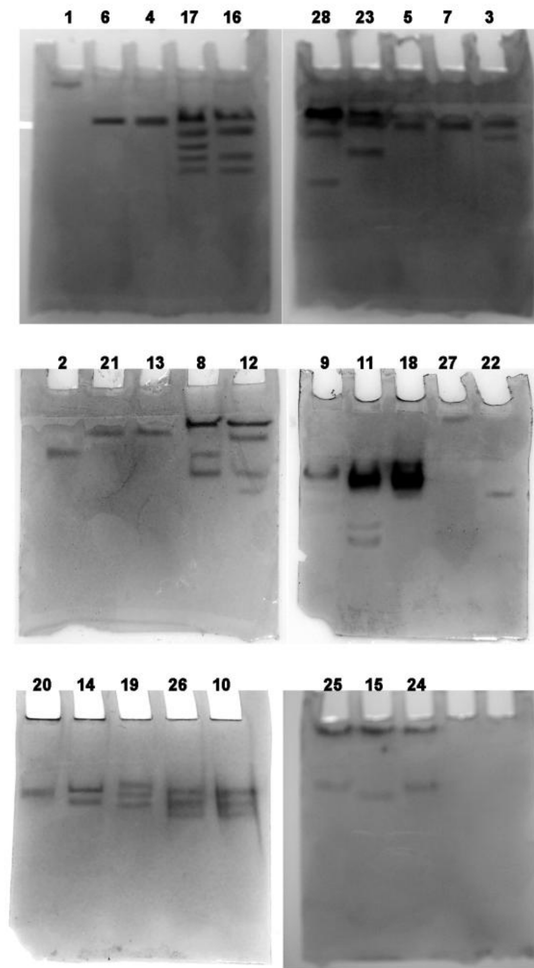

Plate 1 Esterase banding pattern of *R. solani* isolates

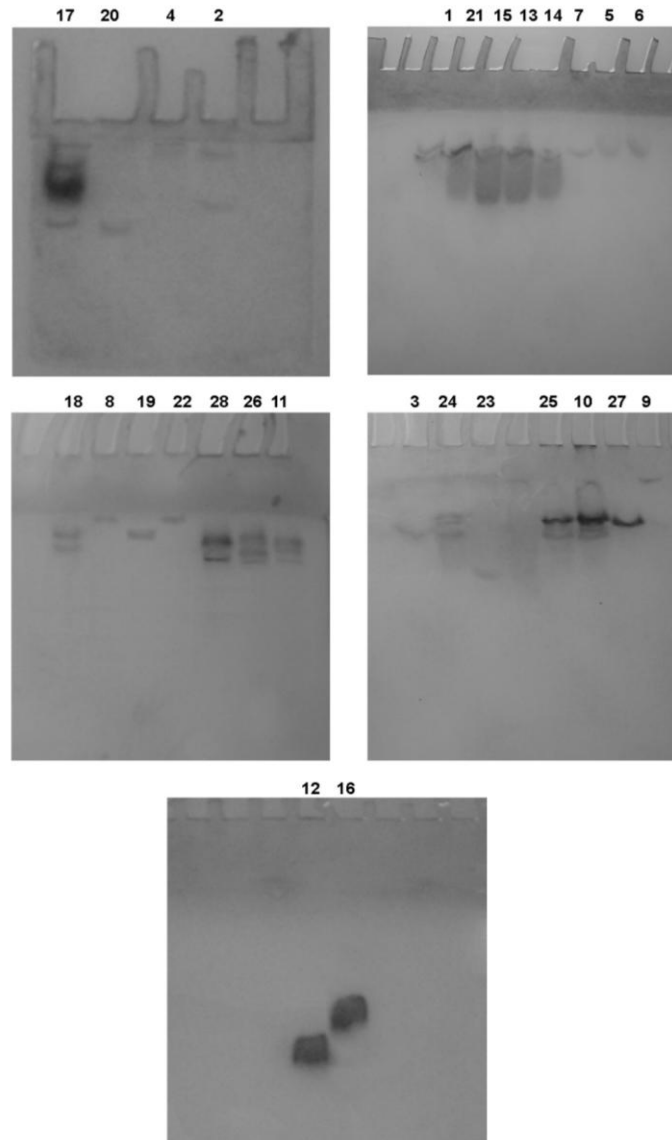

Plate 2 Peroxidase banding pattern of *R. solani* isolates

These results corroborate with the findings of Upamanyu *et al.,* (2005) who observed differences in esterase isozyme patterns of *R. solani* isolates from french beans in different parts of Himachal Pradesh. Esterases are often used to measure genetic variation, yet they may be influenced by external factors (Perrotey *et al.,*2002). Isozyme analysis revealed higher polymorphism among *R. solani* isolates using esterase (EST) and peroxidase (PER) isozymes (Mahmoud *et al.,*2007). Isozyme analysis using six enzymes including esterases, the *R. solani* isolates were divided into two genetically distinct groups, I and II. It is possible that isolates representing AG 1-IA subgroup may show a variation in pathogenicity because of diversity in isozyme profile (Mohammadi *et al.,*2003). Cluster analysis based on isozyme patterns resulted in one major cluster comprising 16 virulent

isolates, with two avirulent isolates loosely linked to this at 0.13 similarity. Isozyme systems of esterases (both α and β) and 6-phosphogluconic dehydrogenase could be used to fingerprint the individual isolates (Neeraja *et al.,*2002). Seema, *et al.,* (2013) reported that the isozyme profile of *R.solani* isolates collected from tobacco seedlings indicated the existence of variations among the isolates from different geographical area. Similarly, variations among different isolates of *Rhizoctonia bataticola* from chick pea were studied in respect of isozymes esterases and peroxidases by Pawar and Ingle (2014) and the *R.solani* isolates from cotton (Mikhail, *et al.,* 2009). Mondal *et al.,* (2013) **o**bserved marked variation in peroxidase activity among the *R. solani* isolates and among different isolates of *Fusarium ciceri* by Pawar and Mane (2014).

Electrophoresis results of present investigation revealed variation in position, width and intensity of bands and the result was in the line of (Amany and Ellil, 2005) who reported that analysis of esterase pattern of R1 has shown 4 distinct bands but for R4, 3 distinct bands were presented and a weak fourth band appeared, which is the only band similar to that of R1. Results from esterase isozymes analysis for *R. solani* suggested that the morphological traits are genetically based. This morphological variant may be due probably to one or more genetic blocks used. It was shown that the morphological trait was related to the absence or presence of one enzyme. Isozyme analysis has been widely used as a tool to study genetic diversity in AGs and within subgroups of *R. solani* (Micales *et al.,*1992). Jin and Korpradiskul (1998) differentiated 23 isolates of *R. solani* AG1 into 3 groups of I, II and III based on cluster analysis using data of 7 enzymes systems. Likewise, Laroche *et al.,* (1992) used enzymes analysis to distinguish AGs 3 and 9 in *R. solani*. Kaufman and Rothrock (1995) also differentiated AG11 isolates from Australia and Arkansas using isozymes analysis. Isozyme variation and genetic relatedness in binucleate *Rhizoctonia* species were studied by Damaj *et al.,* (1993). Mohammadi *et al.,* (2003) used isozymes analysis and total soluble protein profiles to measure the genetic diversity of the Iranian *R. solani* isolate AG1.

Using isozyme analysis sufficient variation within the asexual state of *R. solani* AG-2 was found to allow inter – isolate and inter - group comparisons (Liu *et al.,*1990). Isozyme analysis / isozyme patterns studied in this investigation revealed that the isolates of *R. solani* showed considerable diversity in the production of enzyme which plays a major role in pathogenicity and confirming the previous observations, where electrophoretic patterns of isozymes provided a good indication of genetic diversity among the isolates. The results obtained from isozyme analysis in this study suggested that isozyme analysis could be useful in genetic diversity studies and identification of various *R. solani* isolates. Similar results were observed by Mohammadi *et al.,* (2003, 2004), who used isozymes and total soluble protein in studying the genetic diversity of several isolates of *R. solani* and *Fusarium oxysporum* isolated from different locations in Iran.

Several studies on isozyme patterns have provided good indications of the genetic diversity among *Rhizoctonia* anastomosis groups and phenotypic subgroups, thereby reconfirming the genetic basis of the anastomosis grouping, (Meisong and Korpradiskul, 1999). Isozyme studies have also provided evidence for several genetically distinct subgroups (6 within AGI and 5 within AG2) and some isozyme alleles and loci have been identified as markers for each subgroup (Liu and Sinclair, 1992 and Liu *et al.,*1990).

In conclusion, the results revealed that Isozyme patterns provide good indications of the genetic diversity among *Rhizoctonia* anastomosis groups and phenotypic subgroups. The isolates of *R. solani* showed considerable diversity in the production of enzyme which plays a major role in pathogenicity and confirming the previous observations, where electrophoretic patterns of isozymes provided a good indication of genetic diversity among the isolates. Differences in electrophoretic isozyme patterns are an indication, in part, of genomic differences between isolates, but environmental circumstances affect the isozymes that are synthesized. The results obtained from isozyme analysis in this study suggested that that isozyme analysis could be useful in genetic diversity studies and identification of various *R. solani* isolates.

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