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PROTEIN AND ISOZYME PATTERNS OF *RHIZOCTONIA BATATICOLA* ISOLATES CAUSING CHICKPEA ROOT ROT

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ABSTRACT
Marked variations among different isolates of *Rhizoctonia bataticola* collected from various locations of Maharashtra were studied in respect of protein and isozyme analysis on the basis of pathogenecity. Protein content in different isolates of *R. bataticola* ranged from 0.756 mg to 1.021 mg/100 mg mycelium and maximum protein content was observed in R_{26} highly pathogenic isolate. The peroxidase activities was ranged between 39.35 to 69.79 U min^{-1} mg^{-1} protein and polyphenol oxidase activities from 0.17 to 1.10 U min^{-1} mg^{-1} protein among different isolates of *R. bataticola*. The maximum PPO activities was recorded in highly pathogenic isolate R_{34} of Aurangabad. Moreover, the maximum esterase activities among *R. bataticola* isolates was observed in moderately pathogenic R_{10} isolate of Buldhana district (9.92 μm min^{-1} mg^{-1} protein). Electrophoretic studies by using SDS-PAGE revealed that, seven soluble proteins bands between molecular weights 58.88 to 102.3 KDa and 0.05 to 0.25 Rf values were detected among the isolates of *R.bataticola*, however, weakly pathogenic isolates (R_{14}, R_{19} and R_{24}) are identical for expressing a common protein band of 0.15 Rf. The only common band of esterase (0.045 Rf) was noticed in slightly pathogenic isolates (R_8, R_{41} and R_{47}) which was absent in other isolates and the presence of common PPO band 0.024 Rf in all isolates of highly pathogenic isolates (R_{26}, R_{34} and R_{54}), indicates variability among isolates. Peroxidase as molecular marker also differentiates variability but existence was in traces *i.e.* maximum one band in most of the isolates, except isolates R_{41} (0.034 and 0.103 Rf) and R_{34} (0.034 and 0.092) which forms two bands, while no band was noticed in R_{54} isolate from Satara district.

INTRODUCTION

The cultivated chickpea (*Cicer arietinum* L.), was one of the first grain legumes domesticated in the old world. It is now widely cultivated in Europe, Persia, Egypt, India, Central and Southern America, Australia and parts of Africa and in India chickpea is primarily grown as *Rabi* (post rainy) season crop and occupies about 37% of the area under pulses and contributes almost 50 percent of total pulse production. Chickpea productivity however, remained virtually stagnant over recent decades because of its susceptibility to the insect pests and diseases besides other factors. Chickpea is prone to several fungal diseases, amongst them, dry root rot caused by *Rhizoctonia bataticola* (Taub) Briton-Jones [=*Macrophomina phaseolina* (Tassi) Goid] is one of the major production constraints that causes 10-20 percent annual loss (Vishwadhar and Chaudhary, 2001). The information is not available in respect of virulence/pathotypes of *R. bataticola* prevalent across the country and its correlation with morphological variability, if any. SDSPAGE fingerprinting is also an ideal, economical and less time consuming method for studying microorganism variation and identification (Panda *et al.*, 2013). Moreover, Gel electrophoresis of proteins has been widely used for studying variation in fungal populations. Hussein *et al.* (2000) used cluster analysis to compare protein banding patterns obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from 17 isolates of *R. solani*. Keeping this in view, investigation was undertaken to co-relate pathogenic variability among the isolates of *R. bataticola* collected from different chickpea-growing regions of Maharashtra with their biochemical variation (SDS-PAGE), isozyme analysis (Esterase, PPO and PO).

MATERIALS AND METHODS

Chickpea plants showing typical root rot symptoms were collected from different locations of Maharashtra and isolations were made on PDA medium. *R. bataticola* isolates were tested for their virulence by sick soil method on susceptible variety JG-62 according to the pot-culture inoculation method of Nene and Haware (1980). The isolates of *R. bataticola* were tentatively divided into five groups on the basis of virulence (Table 1).

Quantitative Assay of Protein and Isozymes

Protein and enzyme extraction

Pure culture of *R. bataticola* isolates was inoculated on PDA medium and seven days old growth were scrapped with the help of scrapper. Mycelium (100 mg) was homogenized in 1 mL sodium phosphate buffer by motorized homogenizer for 8 minutes. The mycelial extract was transferred to 1.5 mL centrifuge tubes. The samples in the tubes were centrifuged at 10000 rpm for 45 minutes at 4°C using Remi (C-24). The clear supernatant was collected and used as protein and enzyme source for electrophoretic studies. Entire extraction procedure was done under cold condition. A protein concentration of mycelial homogenate was determined by Bradford method (1976) by using Bovine Serum Albumin (BSA) as a standard

protein to construct the standard curve.

Esterase activity

Esterase activity was measured by microplate assay as per procedure of Dary *et al*. (1990). Three sets of 50 mL of different concentration of a napthol (5-50 nm) per well were loaded followed by 100 ml phosphate buffer (100 mM, pH 7). After gentle manual shaking, 100 mL of staining solution (0.8 mg Fast Brilliant Blue/ml of phosphate buffer (100 mM, pH 7) with 0.5% sodium dodocyl sulfate) was added in each well. The plates were incubated in dark at 20ºC for 30 min and were read in microplate reader (Metertech S960, USA) equipped with 600 nm filter. A standard graph was drawn by plotting mean values of concentration verses mean values of optical density.

Polyphenol oxidase assay

Polyphenol oxidase (PPO) activity was determined as per the procedure given by Mayer *et al*. (1965). Enzyme extract was used as enzyme source. The reaction mixture consisted of 200 mL of enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). For reaction, 200 mL of 0.01 M catechol was added and the enzyme activity, i.e. change in absorbance of reaction mixture at 495 nm were recorded. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.01 /min. (Coseteng and Lee, 1987).

Peroxidase assay

Peroxidase activity was determined according to Retig (1974) method. The reaction mixture contained 4.0 ml; 0.2 M phosphate buffer (pH 5.8), 0.1mL, 0.2 M guaicol, 1.0mL, 0.38 M hydrogen peroxide (H_2O_2) and enzyme extract 500 μL. The reaction was carried out at 30ºC. Absorbance readings, with U 2000 spectrophotometer (Hitachi make) at 470 nm were noted. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.01 / min (Deepa and Arumughan, 2002).

Electrophoresis

Electrophoresis of protein and isozyme in polyacrylamide gel was carried out in buffer gel (native PAGE) in vertical gel electrophoresis using the procedure given by Sadasivam and Manickam (1996). After the complete run the gel was removed carefully between the plates, subsequently the gel was stained in appropriate staining solution. After visualization of bands, the gel was photographed. The migration distance of each band and tracking dye was recorded. The migration was expressed as the ratio of the distance traveled by the protein/ isozyme to the tracking dye from the point of application. This factor is known as relative mobility. Using the Rf values, zymograms were prepared pictorially.

$$\text{Relative front (Rf)} = \frac{\text{Distance traveled by protein / isozyme}}{\text{Distance traveled by tracking dye}}$$

Native PAGE for protein was carried out using 5% stacking and 10% resolving gel and for isozyme 5% stacking and 6% resolving gel was used.

Staining and destaining for protein

First, dye was dissolved in methanol and staining solution was prepared for observation of protein bands. The gels were immersed in staining solution for overnight. Then stained gels were transferred to a suitable container containing destaining solution for 12 hours. Dye that is not bound to proteins was thus removed and proteins fractionated into bands were seen blue coloured.

Staining of esterase isozyme (Vallejos, 1983)

The gel was incubated in a staining solution of ?-naphthyl acetate (30 mg in 3 ml acetone) was first dissolved in acetone and then solution of fast blue BB salt (100 mg in 100 ml, of 0.1 M SPB pH7) was added. The blackish/ brown esterase bands were developed after 15 minutes.

Staining of polyphenol oxidase isozyme

The isozyme of polyphenol oxidase was localized on polyacrylamide gel as per the procedure suggested by Park *et al*. (1980). The gel was incubated in 0.03 m catechol containing 0.25 per cent phenylene diamine in citrate phosphate buffer, pH 6 (0.1 m citric acid solution, 2.10g in 100mL) + 0.2m solution of dibasic sodium phosphate, 3.56 g in 100 ml) for one hour.

Staining of peroxidase isozyme

The isozyme bands of peroxidase (Nov Acky and Hampton, 1968) were localized by first incubating the gel in 0.25 per cent guacicol for 30 minutes followed by incubation in 0.3 per cent hydrogen peroxide for 15 minutes which showed the appearance of reddish brown bands of peroxidase.

RESULTS AND DISCUSSION

Protein content in different isolates of *R. bataticola* ranged from 0.756 ± 0.003 to 1.021 ± 0.017 mg/100 mg mycelium with maximum protein (1.021 mg /100 mg mycelium) in highly pathogenic isolate R_{26} while minimum in moderately pathogenic isolate R_{22} (Table 2) and the higher peroxidase activities among *R. bataticola* isolates was recorded in highly pathogenic isolate R_{34} (69.79 U min^{-1} mg^{-1} protein) and low in moderately pathogenic isolate R_{22} (39.35 U min^{-1} mg^{-1} protein). However, the PPO activities in *R. bataticola* isolates were ranged from 0.17 to 1.10 U min^{-1} mg^{-1} protein with maximum activities in highly pathogenic isolate R_{34} and minimum in strongly pathogenic isolate R_{47}, whereas maximum esterase activities was observed in moderately pathogenic isolate R_{10} (9.92 μm min^{-1} mg^{-1} protein) and minimum in highly pathogenic isolate R_{26} (3.01 μm min^{-1} mg^{-1} protein). The quantitative estimation of protein, peroxidase, esterase and polyphenol oxidase in mycelial extract of *R. bataticola* isolates, revealed that their content varies from isolate to isolate. The marked variation in protein content and highly pathogenic ability of *R. bataticola* isolates with higher content of protein in R_{26} isolate and enzymes (peroxidase and polyphenol oxidase) in R_{34} isolates were on the same line as reported by Asoufil *et al.,* (2007) and Mondal *et al*. (2013) while working on the *R. solani* and *Sclerotinia sclerotirium* isolates, respectively.

Protein and isozyme studies by Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide Gel Electrophoresis (PAGE) was used to to study the variation in protein and isozyme patterns of different isolates of *R. bataticola*.

Table 1: Chickpea root rot isolates collected from different locations in Maharashtra state

Virulence group	Isolate No	(% Mortality)	Locality (District and location)	
I) Non-Pathogenic(0 %)	-	-	-	-
II) Weakly Pathogenic(1-20 %)	R_{14}	12	Buldhana	Khamgaon
	R_{19}	09	Washim	Washim Road
	R_{24}	16	Latur	Nilanga
III) Moderately Pathogenic(21-50 %)	R_{10}	45	Buldhana	Nandura Seed Farm
	R_{22}	43	Buldhana	A.R.S.(Buldhana)
IV) Strongly Pathogenic(51-70 %)	R_{8}	64	Buldhana	Manasgaon (Shegaon)
	R_{41}	63	Washim	A.R.S. (Washim)
	R_{47}	66	Dhule	College of Agril., Dhule
V) Highly Pathogenic(>70 %)	R_{26}	77	Khandwa (MP)	Tukaithod
	R_{34}	78	Aurangabad	NARP (Aurangabad)
	R_{54}	74	Satara	Gulumb (Wai)

Table 2: Quantitative estimation of protein, peroxidase, esterase and polyphenol oxidase among different isolates of *R. bataticola*

Isolates	Protein (mg 100 mg-1 mycelial mat)	Peroxidase(U min^{-1} mg^{-1} protein)	Polyphenol oxidase (U min^{-1} mg^{-1} protein)	Esterase(μm mg-1 Protein min^{-1})
Weakly pathogenic isolate				
R_{14}	0.908 ± 0.005*	46.26 ± 0.37	0.46 ± 0.09	5.85 ± 0.12
R_{19}	0.852 ± 0.017	44.20 ± 1.72	0.41 ± 0.08	4.23 ± 0.04
R_{24}	0.996 ± 0.016	53.97 ± 1.42	0.42 ± 0.08	5.82 ± 0.20
Moderately pathogenic isolate				
R_{10}	0.881 ± 0.013	48.43 ± 0.19	0.57 ± 0.00	9.92 ± 0.44
R_{22}	0.756 ± 0.003	39.35 ± 1.65	0.44 ± 0.00	9.20 ± 0.06
Strongly pathogenic isolate				
R_{8}	0.900 ± 0.008	53.80 ± 0.28	0.46 ± 0.09	6.67 ± 0.08
R_{41}	0.958 ± 0.009	58.11 ± 1.04	0.43 ± 0.09	6.94 ± 0.24
R_{47}	0.990 ± 0.021	58.07 ± 0.41	0.17 ± 0.00	3.81 ± 0.03
Highly pathogenic isolate				
R_{26}	1.021 ± 0.009	61.13 ± 0.68	0.98 ± 0.20	3.01 ± 0.09
R_{34}	0.906 ± 0.049	69.79 ± 0.64	1.10 ± 0.37	8.60 ± 0.11

*Standard error

Table 3: Rf values and estimated molecular weights of protein banding in different isolates of *R. bataticola*

Isolate/Rf values (mol. wt)	Protein bands 0.05 *(102.3)	0.08 (95.05)	0.1 (91.2)	0.12 (85.11)	0.15 (79.43)	0.21 (72.44)	0.25 (58.88)	Total no. of bands
WPI								
R_{14}	-	+	-	+	+	-	-	3
R_{19}	-	-	+	-	+	-	-	2
R_{24}	-	+	-	-	+	-	+	3
MPI								
R_{10}	-	-	+	-	-	+	-	2
R_{22}	+	-	-	+	-	-	-	2
SPI								
R_{8}	-	-	+	+	-	-	-	2
R_{41}	-	-	-	+	-	-	+	2
R_{47}	+	-	+	-	-	+	-	3
HPI								
R_{26}	-	-	+	-	+	-	+	3
R_{34}	-	-	-	-	+	+	-	2
R_{54}	+	-	+	-	-	-	+	3

* Figures in the parenthesis are mol. wt. of protein (KDa) of respective Rf value band; + = Presence of bands; — = Absence of bands

Protein profile

The differences in protein banding pattern of *R. bataticola* varied from 0.05 to 0.25 Rf value (Table 3 and Fig. 1) and the minimum Rf value band (0.05) was noticed in isolates of different pathogenic groups *i.e.* R_{22} (MPI), R_{47} (SPI) and R_{54} (HPI). The protein band, 0.1 Rf was the major common band characteristic to WPI (R_{19}), MPI (R_{10}), SPI (R_8 and R_{47}) and highly pathogenic isolate (R_{26} and R_{54}). The weakly pathogenic isolate (R_{14}, R_{19} and R_{24}) are identical for expressing a common band of 0.15 Rf and presence of characteristic band of 0.12 Rf was noticed in WPI (R_{14}), MPI (R_{22}) and SPI (R_8 and R_{41}).The uncommon band of 0.21 Rf was expressed in R_{10} (MPI), R_{41}

Table 4: Rf values of esterase banding pattern in *R. bataticola* isolates

Isolate/ Rf values	Isozyme bands																Total no. of bands
	0.034	0.045	0.099	0.315	0.341	0.352	0.364	0.371	0.438	0.466	0.477	0.483	0.494	0.506	0.539	0.944	
WPI																	
R_{14}	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	3
R_{19}	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	3
R_{24}	-	-	+	-	-	-	+	-	-	-	-	+	-	-	+	+	5
MPI																	
R_{10}	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	3
R_{22}	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	2
SPI																	
R_{8}	-	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	3
R_{41}	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	3
R_{47}	-	+	-	-	-	-	-	+	-	-	-	-	+	-	-	-	3
HPI																	
R_{26}	-	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	3
R_{34}	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	3
R_{54}	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	3

+ = Presence of bands, — = Absence of bands; WPI- Weakly Pathogenic Isolate; MPI- Moderately Pathogenic Isolate; SPI- Strongly Pathogenic Isolate; HPI- Highly; Pathogenic Isolate

Table 5: Rf values of polyphenol oxidase banding pattern in *R. bataticola* isolates

Isolate / Rf values	Isozyme bands										Total no. of bands
	0.024	0.036	0.044	0.056	0.131	0.144	0.202	0.222	0.226	0.244	
WPI											
R_{14}	-	-	+	-	+	-	-	-	-	+	3
R_{19}	-	+	-	-	-	+	-	+	-	-	3
R_{24}	+	-	-	-	+	-	-	-	+	-	3
MPI											
R_{10}	-	-	+	-	-	+	-	-	-	+	3
R_{22}	-	-	+	-	+	-	-	+	-	-	3
SPI											
R_{8}	-	-	-	+	-	+	-	-	-	+	3
R_{41}	-	+	-	-	+	-	-	-	-	+	3
R_{47}	-	+	-	-	+	-	-	-	+	-	3
HPI											
R_{26}	+	-	-	-	+	-	-	-	+	-	3
R_{34}	+	-	-	-	+	-	-	-	-	+	3
R_{54}	+	-	-	-	+	-	+	-	-	-	3

+ = Presence of bands; — = Absence of bands

(SPI) and R_{34} (HPI). The findings were in accordance with the work of Martin and Pagare (2012) showed several bands of water soluble fractions of lens protein ranging from 70-13.5 kDa and 74-13.5 kDa in *P. ticto* and *R. daniconus*, respectively by SDS-PAGE.

Molecular weight (KDa)

In the present study Fermentas make unstained protein molecular weight marker with sharp bands in the range of 14.4 KDa to 11.6 KDa was loaded in the electrophoresis assembly along with samples of *R. bataticola* for estimation of molecular weight (KDa). The molecular weight proteins in *R. bataticola* isolates varied from 58.88 to 102.3 KDa (Table 3). Among WPI lower mol. wt. protein, 58.88 was noticed in R_{24} isolate and higher of 95.05 KDa in R_{14} and R_{24} isolates, while 79.43 KDa proteins was observed in all isolates (R_{14}, R_{19} and R_{24}). Strongly pathogenic isolate (R_{8} and R_{41}) were similar for expressing 85.11 KDa proteins and R_{8} and R_{47} for 91.20 KDa proteins and highly pathogenic isolate (R_{26} and R_{54}) having two equal bands of 58.88 and 91.20 KDa proteins and 79.43 KDa in both R_{26} and R_{34} isolates, however HPI R_{54} having higher mol. wt. protein of 102.3 KDa. Thus, variation in the protein concentration among the isolates was observed in the present study. Similar observations on variations in the protein content were made on *F. oxysporum* f. sp. *carthami* (Raghuwanshi and Dake, 2005) and *Fusarium oxysporum* infecting FCV Tobacco in Karnataka (Sumana and Devki, 2014).

Esterase

The results presented in Table 4 revealed that the esterase banding pattern in all isolates of *R. bataticola* varied from 0.034 to 0.944 Rf and maximum five esterase bands was noticed in WPI R_{24} (Rf value range from 0.099 to 0.944 Rf) (Fig. 2). The band of 0.0341 Rf was exhibited in WPI (R_{14} and R_{19}), MPI (R_{10}) and HPI (R_{54}) and one common band of 0.045 Rf was noticed in SPI (R_{8}, R_{41} and R_{47}) which was absent in other isolates. The presence of 0.364 Rf band in WPI (R_{14}, R_{19} and R_{24}) and 0.477 Rf band in WPI (R_{14} and R_{19}) showed the uniqueness in pathogenic groups. However, the bands 0.352,

Table 6: Rf values of peroxidase banding pattern in *R. bataticola* isolates

Isolate / Rf values	Isozyme bands					Total no. of bands
	0.023	0.034	0.046	0.092	0.103	
WPI						
R_{14}	-	+	-	-	-	1
R_{19}	-	-	+	-	-	1
R_{24}	-	-	+	-	-	1
MPI						
R_{10}	-	+	-	-	-	1
R_{22}	-	+	-	-	-	1
SPI						
R_{8}	+	-	-	-	-	1
R_{41}	-	+	-	-	+	2
R_{47}	-	-	+	-	-	1
HPI						
R_{26}	-	-	+	-	-	1
R_{34}	-	+	-	+	-	2
R_{54}	-	-	-	-	-	0

+ = Presence of bands; — = Absence of bands

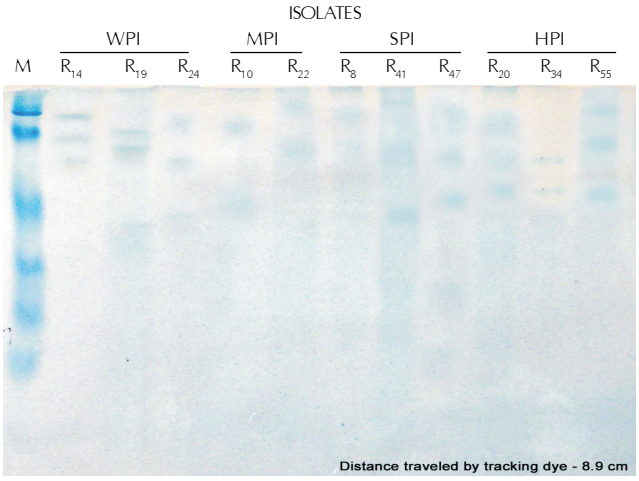

Figure 1: Protein electrophoregram of Rhizoctonia bataticola is native page

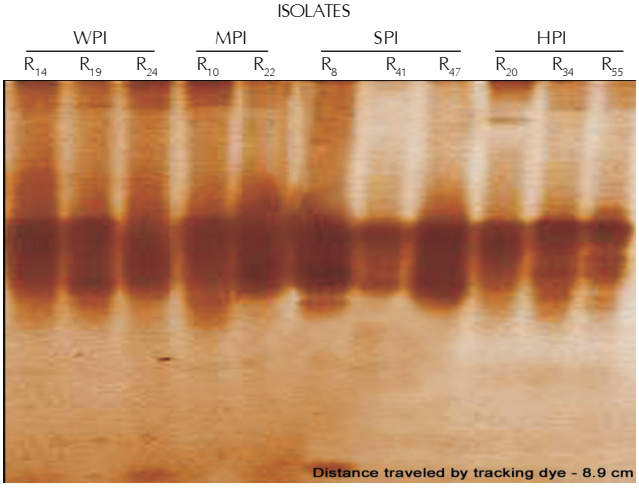

Figure 2:Esterase is ozymes electrophoregrem in the is olates os Rhizo Rhizoctonia bataticola is olates

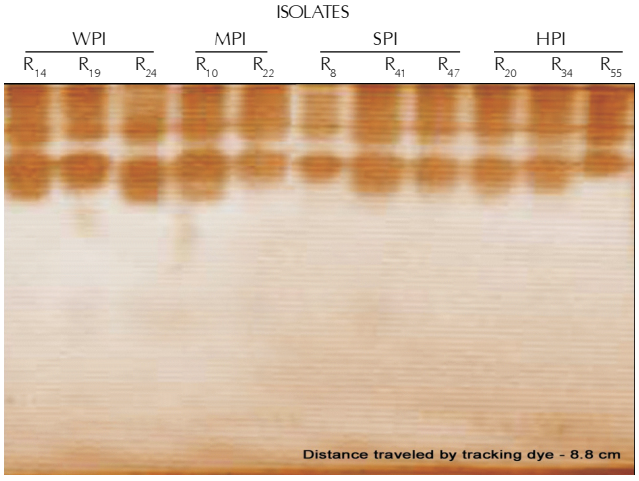

Figure 3: Polyphenol oxidas is ozymes electrophoregram in the is olates of rhizoctonia bataticola is olates

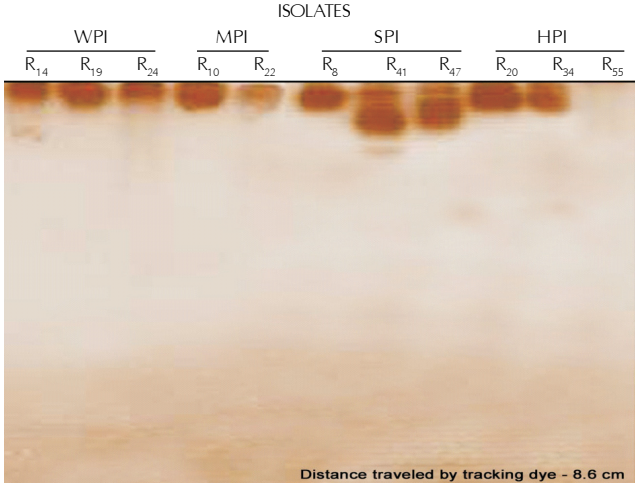

Figure 4: Peroxidase is ozymes electrophoregram in the is olates of rhizoctonia bataticola is olates

0.371, 0.438, 0.483 and 0.539 Rf expressed in one isolate only and absent in other isolates. Thus, no consistent appearance of esterase isoenzyme bands was visualized in the different pathogenic groups, which indicates variability among isolates of *R. bataticola*.

Polyphenol oxidase

Ten PPO bands within range of 0.024 to 0.244 Rf were present in 11 isolates of *R. bataticola* with most common band of 0.131 Rf in MPI (R_{14} and R_{24}), MPI (R_{22}), SPI (R_{41} and R_{47}) and HPI (R_{26}, R_{34} and R_{54}) isolates (Table 5 and Fig. 3). The 0.024 Rf band was present in all isolates of HPI and 0.044 Rf band in MPI (R_{10} and R_{22}) support the uniqueness in *R. bataticola* isolates based on their pathogenic ability. However, the band of maximum 0.244 Rf was noticed in MPI (R_{10}), SPI (R_{8} and R_{41}) and HPI (R_{34}). The banding pattern of PPO showed variation with respect to isolates tested and also no consistency in the appearance of band was noticed.

Peroxidase

The peroxidase banding pattern in case of *R. bataticola* revealed that may isolates produced only one band, except

isolates R_{41} (0.034 and 0.103 Rf) and R_{34} (0.034 and 0.092) forms two bands, while no band was noticed in R_{54} isolate (Table 6 and Fig. 4). The band of Rf value 0.034 was present in R_{14}, R_{10}, R_{22}, R_{41} and R_{34} isolates and 1.046 Rf band in R_{19}, R_{24}, R_{26} and R_{47} isolates. The isolates of *R. bataticola* do not showed any variation in respect of peroxidase banding pattern as per virulence of isolates.

Thus, marked variation among different pathogenic groups in respect of protein profile relates the existence of variability among the isolates of *R. bataticola* causing chickpea root rot. The findings of Mandal *et al*. (1994) and Giri (2003) are in the similar line of present results; while working on protein and isozyme pattern of *Fusarium* spp. Yehia *et al*. (2007) also revealed the polymorphism among *R. solani* isolates using esterase and peroxidase enzyme. Mahmoud *et al*. (2012) electrophoresed soluble proteins of the 21 isolates of *Rhizoctonia solani* by using SDS-PAGE and these isolates were categorized into three anastomosis groups base on protein banding patterns. Similarly, variations among different isolates of *Fusarium ciceri* were studied in respect of protein and isozyme analysis by Pawar and Mane (2014).

Although our electrophoresis study was insufficient to make any inferences concerning the contribution of the observed genetic diversity to population structure in this study, but several relationships between protein and isozyme profiling data were used as to tool to assess the extent of variability.

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