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Primary level of defense reaction vary upon challenged Arbuscular mycorrhizal fungal inoculation among the differently responsive rice varieties

[1] Ramkrishna Saha, [2] Bidisha Mondal

[1] Assistant Director of Agriculture, Department of Agriculture, Government of West Bengal, India.

[2] Scientist, Directorate of Research, Bidhan Chandra Krishi Viswavidyalaya, West Bengal, India.

Abstract
Rice having a varying level of responsiveness to Arbuscular Mycorrhizal Fungi (AMF) mostly High Yielding and hybrid varieties which have a high PO_4 demand with comparatively less root growth rate showed more responsiveness than the varieties having less PO_4 demand with comparatively higher root growth.

As Arbuscular Mycorrhiza inoculation trigger a primary level of defense mechanism the quantitative & qualitative changes in the whole root peroxidase studies at the early stage of AMF colonization showed a rise in the peroxidase activity in negatively or non-responsive rice varieties whereas declined the activity in highly responsive rice varieties. The zymogram of rice roots by Peroxidase polyacrylamide gel electrophoresis showed five distinct bands. Increased activity of peroxidase in negatively responsive variety may due to transitional defense reaction of the plant showing a level of incompatibility where as in responsive variety the activity may suppress. Densiometric estimation also supported the above results.

Keywords: Arbuscular Mycorrhiza, Rice, Densiometric, Peroxidase, Zymogram

1. Introduction

In response to microbial attack, plants elaborate an array of inducible defense reactions many of which involve the transcriptional activation of the corresponding defense genes [1]. These include the genes that encode enzymes involved in the phenylpropanoid pathway – synthesis of lignin and phytoalexins, PR-proteins, Cell wall hydrolases and Reinforcement of plant cell walls, such as hydroxyproline rich glycoproteins (HRGP).

During initial stages of AM colonization, only weak and transient increases in gene expression for cell wall hydrolases, HRGP, and those involved in phenylpropanoid pathway are observed [2]. Production of reactive O_2 intermediates through an oxidative burst is the hallmark of plant's defense responses, especially through the phenylpropanoid pathway. Peroxidases participate in a variety of defense mechanism [3] in which H_2O_2 is often supplied by the oxidative burst. The cell wall appears to be the site of defense related peroxidase polymerization reactions, such as lignification, suberization and cross linking of structural cell wall proteins [4, 5]. This peroxidase activity is utilized for studying the difference between AM responsive and non-responsive varieties of rice. Both quantitative and qualitative expression showed the same result.

2. Materials and Methods

Four selected rice varieties 2 non-responsive varieties – Black Gora (BG) and ARC- 12737 (ARC) and 2 responsive varieties – Jhingasal (Jhing), and MTU-7029 (MTU) [6] were grown in small volume sterile soil with or without AM inoculation for 10 and 25 days. Experimental plants were grown in pots over cemented racks in a partially covered net house under ambient light, temperature and humidity. For the purpose of biochemical analysis, small volume plastic cups were kept covered under transparent fine polythene nets. Irrigation was provided with fresh demineralized water.

For the purpose of this experiment rice seedlings were grown in low volume sterilized soil in 250 ml thermocol cups keeping 3 seedlings per cup for 10 days and 25 days. Sterilized soil was inoculated with pure root based AMF inoculum at the rate of 5 g per kg soil to raise the infective inoculum density to approx. 3.5 X 10^5 propagules per g soil. Control plants also inoculated with sterilized root inoculum at the same rate. Seedlings were raised from surface sterilized seeds in inoculated soil and control cups and whole plant harvests were made between 10 and 25 days of seedling growth. Roots taken from the harvested plants was processed and analyzed for peroxidase by colorimetry [7] at 10 and 25 days and polyacrylamide gel electrophoresis [8] at 10 days, keeping adequate number of both plant and replicates.

1g of plant root stored overnight at –20ºC, was extracted with 1 ml of phosphate buffer (pH 7) by grinding in a pre-cooled glass mortar and pestle at 0-4ºC. The homogenate was centrifuged at 18000 g at 5ºC for 15 minutes and the supernatant was used as enzyme source immediately. 0.2 ml enzyme extract and 0.1 ml freshly prepared O-dianosidine solution was added to 3.5 ml phosphate buffer in a dry cuvette. The temperature of the assay mixture was immediately brought to 28-30ºC and the cuvette was placed in spectrophotometer at 430 nm. 0.2 ml 0.2 Molar H_2O_2 was then added immediately. The initial absorbance at 0 time immediately after addition of H_2O_2 was recorded and followed further by recording at every 30 sec interval up to 3 minutes. A blank with reaction mixture minus enzyme extract was run parallel. Enzyme activity was expressed as rate of increase in absorbance per unit time per mg protein.

Soluble root protein was estimated by Lowry's colorimetric method. Whole root crude protein extract (1 g root in 1 ml phosphate buffer) (pH 6.8) was centrifuged at 10000 rpm for 15 minutes. The extract was diluted 20 times with buffer and

mixed with Reagent C (1 ml diluted extract + 5 ml Reagent C). 0.5 ml 1 Nomal Folin's reagent was added to the treated extract after 15 minutes shaking and incubated in dark for 30 minutes. OD of the resultant material was read at 660 nm using Spectronic 20 Spectrophotometer. Protein concentration per g dry root was determined from BSA standard curve.

Polyacrylamide gel mixture for 16 cm x 14 cm x 1.5 mm size was prepared with the constituents. Freshly prepared gel mixture solution was carefully poured into the chamber between the glass plates. A 13 well comb was placed on the top of the gel. A layer of small volume of distilled water was provided on top of the gel and kept for 45 minutes for polymerization. After proper polymerization the gel was put to pre-run for 60 minutes at 4ºC inside a cold chamber in a vertical slab gel apparatus (Biotech Model No. EBW-20) at a constant current of 30 mA. This was followed by washing of individual well with electrode buffer (pH 8.2-8.4). Chilled enzyme extract containing equal quantities of protein in sample buffer were loaded in each lane.

For gel running, initially 15-20 mA current was provided but later gel was allowed to run at a constant current of 30 mA for movement of the samples through the stacking gel. Gel was run until the Bromo-phenol blue dye reached the bottom of the gel. After complete run, the gel was stained in dark with freshly prepared staining solution for 10-15 minutes followed by a quick exposure to hydrogen peroxide solution. The reaction was stopped by washing with distilled water and observed for peroxidase bands.

Enzyme activity was measured as unit peroxidase per mg of root protein. Known amount of peroxidase (peroxidase unit per mg of root protein as analyzed) was loaded in the gel. Peroxidase bands obtained by PAGE were visualized, analyzed and documented by Ultra-Lum Inc. USA gel documentation system (Model EBW-20) with Total Lab gel analysis software. The presence of unit peroxidase in different bands were then calculated from the total unit of peroxidase loaded.

3. Results and Discussion

Quantitative estimation of peroxidase activity was done from whole root protein extracts after harvest of plants at 10 and 25 days of seed germination. Results of the analysis are presented in Fig. 1-3 and Table 1.

Results of the analysis (Table 1, Fig. 1 & 3) showed that there was a highly significant rise in whole root peroxidase activity due to AM inoculation at 10 days in the early negative or non-responsive varieties (BG, ARC). On the other hand, the varieties which responded positively to inoculation (Jhing, and MTU) showed a significant decline in the activity at the corresponding period. At 25 days the difference between the two differently responding variety groups in their changed peroxidase activity following infection narrowed down but remained still significant and perceptible.

These results showed that at the very early stage of AM infection there was differential response of whole root peroxidase activity in the roots of differently AM responsive varieties. While the activity may rise in the negatively or non-AM responding varieties, the same may decline in positively responding varieties. With progress of colonization the magnitude of both negative and positive changes might diminish. By repeated polyacrylamide gel electrophoresis of peroxidase from whole root crude protein extract and analysis of the zymograms using standard software, isozyme profiles of the roots of 4 rice varieties at 10 days was first standardized. The zymograms (Plate 1) revealed presence of 5 clearly identifiable peroxidae isozyme bands, excluding the unresolved band position at or below Rf 0.1.These bands were identified as follows:

Out of these 5 bands, the bands number 3 and 4 were present in significantly higher intensity in all the varieties than the rest of the bands. These two bands were considered as the most predominant peroxidase isozymes of rice roots at the early seedling stage.

Post-infectional qualitative and quantitative changes in peroxidase isozyme profile of the roots of differently AM responsive rice varieties were then analyzed at 10 days. Results of the analysis are presented as peroxidase zymogram diagrams together with densitometric analysis of the zymograms of four varieties (2 negatively or non-responding varieties, 2 positively responding varieties). Result of the analysis is presented in Plate 1. Summary of the analyzed results are presented in Table 3.

Results of the analysis showed significant differences in the titer values of whole root peroxidase activity among the varieties, both under inoculation and no-inoculation. Estimation of the peroxidase activity by densitometric analysis of the isozyme bands showed rise in whole root peroxidase titers at 10 days in the roots of the 2 rice varieties following infection (Black Gora and ARC 12737) both of which responded negatively to AM inoculation at the corresponding period (Table 3). Two varieties which responded positively at the same period (MTU 7029 and Jhingasail) showed a lowering of the titer value. This confirmed the results obtained by colorimetric analysis of whole root peroxidase of the rice varieties presented earlier (Table 1, Fig. 2).

Table 1: Whole root peroxidase activity of 4 differenty responsive rice varieties between 10 and 25 days after seed germination in AM inoculated soil (Rate of change of OD of O-Dianisidine reaction mixture per mg whole root crude protein per second)

Treatment	10 days				25 days			
	BG	**ARC**	**Jhing**	**MTU**	**BG**	**ARC**	**Jhing**	**MTU**
AM -	0.043±0.002	0.048±0.001	0.072±0.005	0.072±0.006	0.045±0.002	0.039±0.001	0.036±0.002	0.059±0.003
AM +	0.075±0.007	0.083±0.008	0.046±0.002	0.056±0.003	0.060±0.002	0.046±0.003	0.031±0.002	0.049±0.002
Est t	7.615**	7.521**	8.364**	4.132*	9.187**	3.835*	3.062*	4.805**
Table t	0.05 *p* 2.776				0.01 *p* 4.604			
% change	+ 74	+ 73	-36	-22	+ 33	+ 18	- 14	- 17
Mean of differently responsive varieties	+73.5		-29.0		+25.5		-15.5	

Based on 3 x 3 root sample analyses of each variety

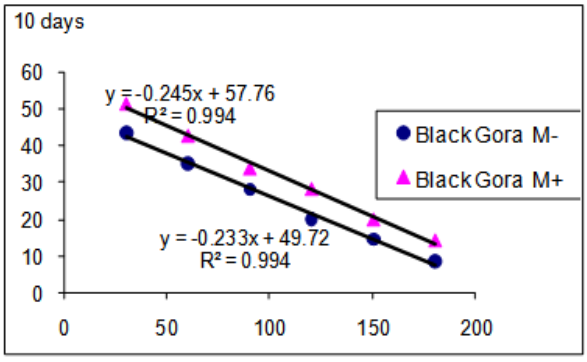
Table 2: Bands detected by Peroxidase Isozyme Zymogram

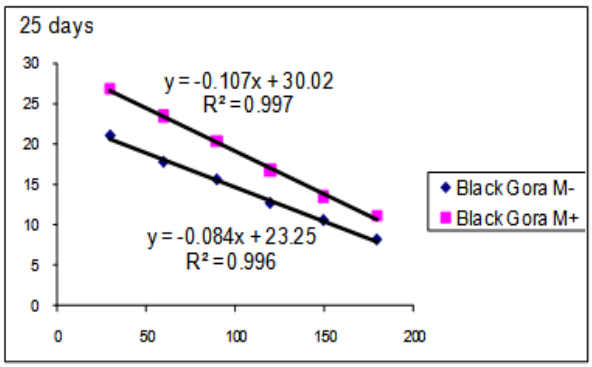
Band number	Band position (Rf)
1	Near 0.20 (0.18 – 0.23)
2	Near 0.37 (0.34 - 0.39)
3	Near 0.60 (0.56 – 0.60)
4	Near 0.65 (0. 61 – 0.68)
5	Near 0.85 (0.81 – 0.88)

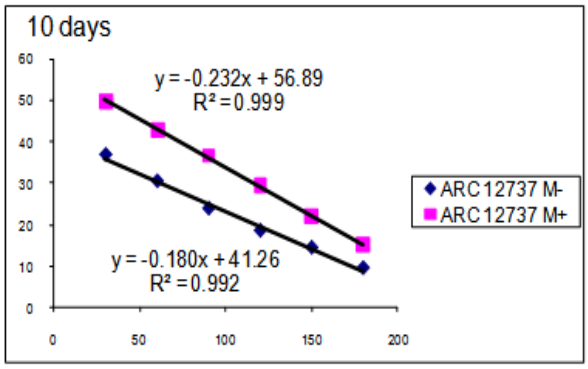
Table 3: Analysis of the changes in whole root peroxidase isozymes profile by gel electrophoresis (Based on densitometric analysis of zymograms shown in Plate I and 2)

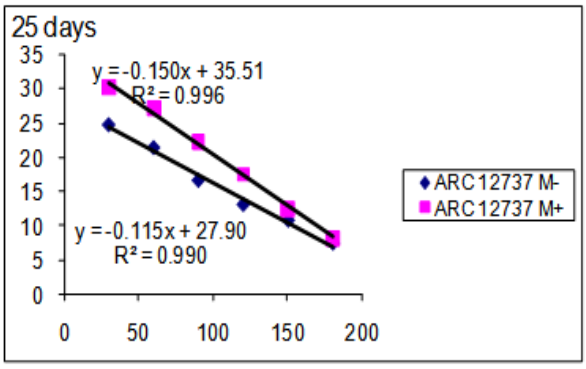
Variety	Treatment	Peroxidase enzyme (activity units / mg crude soluble root protein)						
		Total	**Band 1**	**Band 2**	**Band 3**	**Band 4**	**Bands 3+4**	**Band 5**
Black Gora (BG)	AM -	12.4	0.52	2.04	4.08	2.84	6.92	2.92
	AM +	20.4	1.28	2.08	4.86	8.59	13.45	3.59
ARC 12737 (ARC)	AM -	37.6	0.49	2.23	27.02	6.49	33.51	1.38
	AM +	40.8	0.29	0.75`	28.21	9.77	37.98	1.78
Jhingasail (Jhing)	AM -	19.4	0.29	0.25	8.10	10.07	18.17	0.69
	AM +	4.5	0.67	1.98	1.66		1.66	0.19
MTU 7029 (MTU)	AM -	69.8	0.92	10.92	18.62	34.98	53.60	4.36
	AM +	26.0	0.59	10.57	3.08	3.78	6.86	7.98

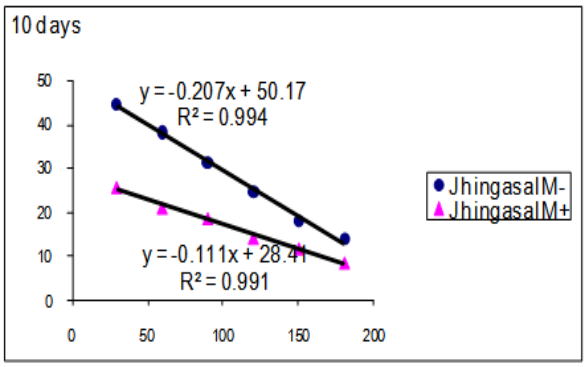
(Values in bold print show significant changes in activity by densitometry)

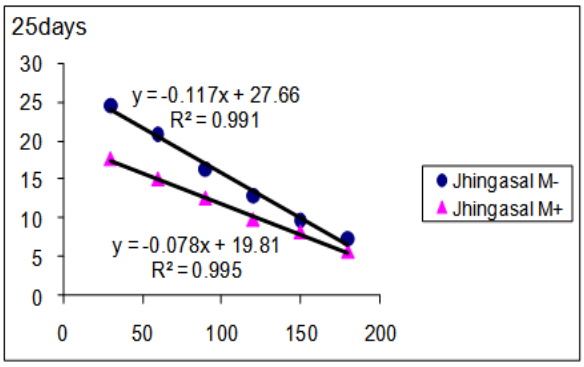


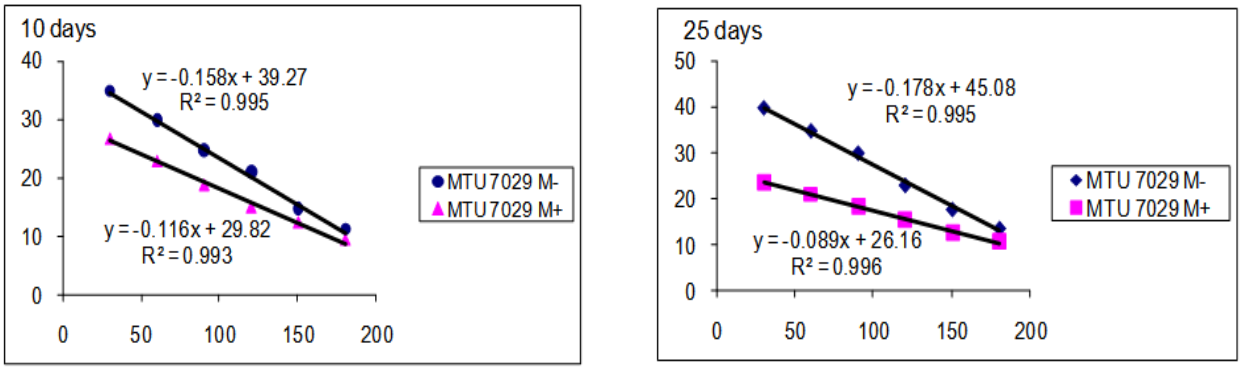












(y -axis: change in OD X 10^3 per microgram crude protein; x-axis: time interval in sec over 180 sec)

Fig 1: Whole root peroxidase activity of rice varieties at 10 and 25 days under AM inoculation and no-inoculation

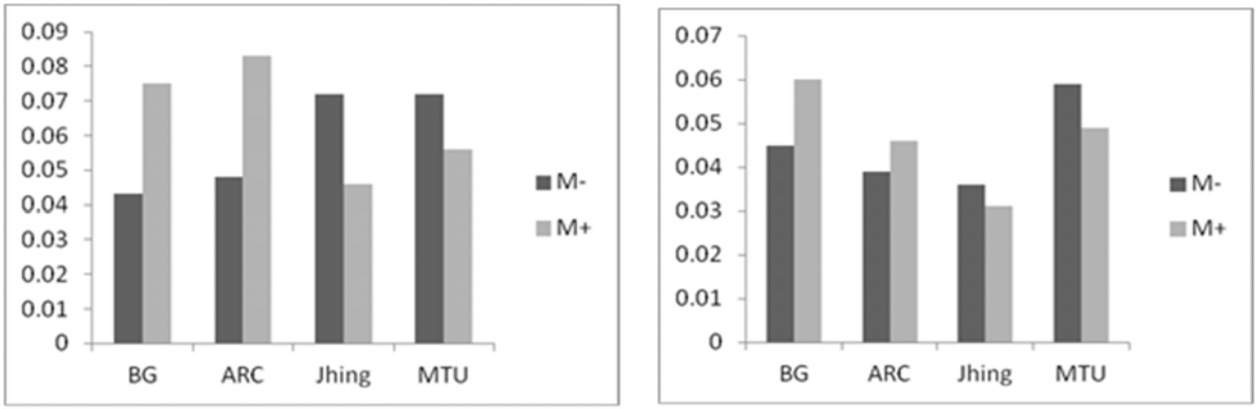


Fig 2: Changes in whole root peroxidase activity due to AM infection: Rate of change in OD of O-dianisidine per mg protein per sec over 3 minutes

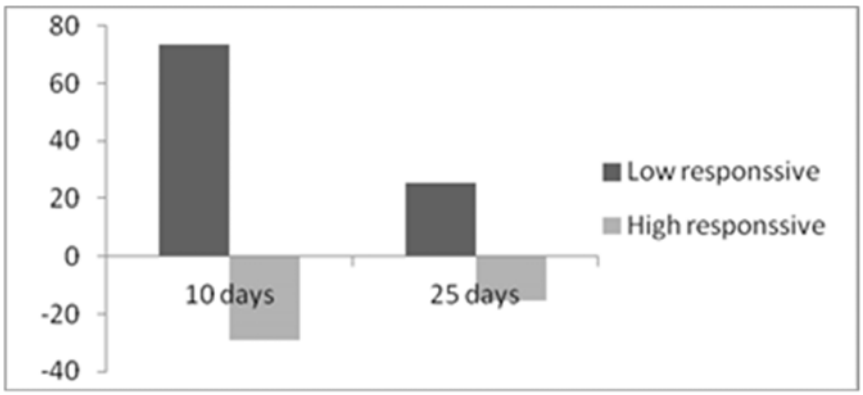


Fig 3: Comparison of changes in whole peroxidase activity of the differently responsive rice varieties following infection at 10 and 25 days.

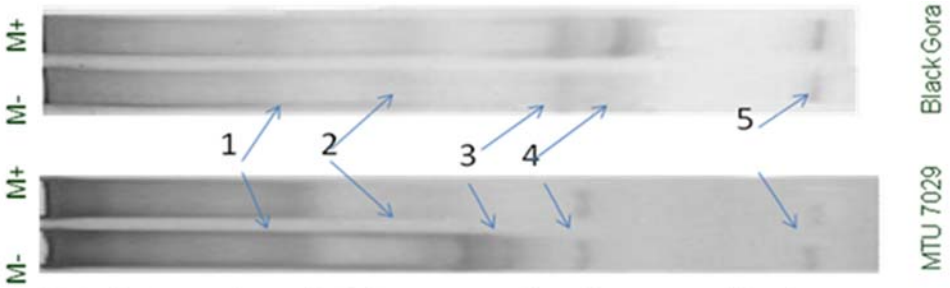
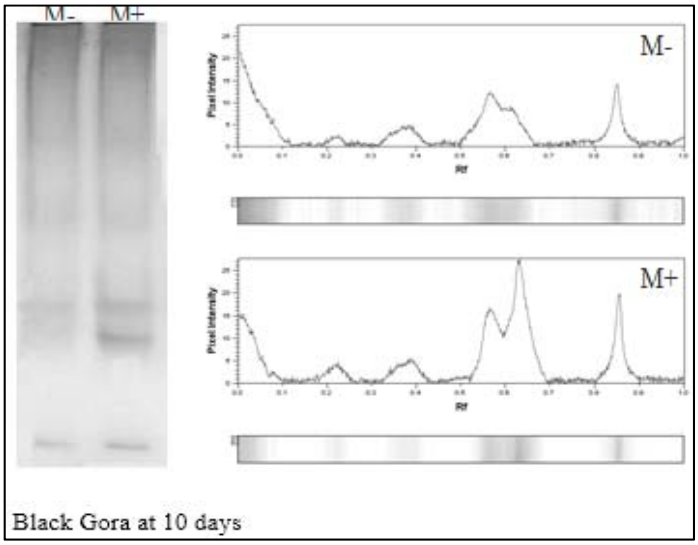
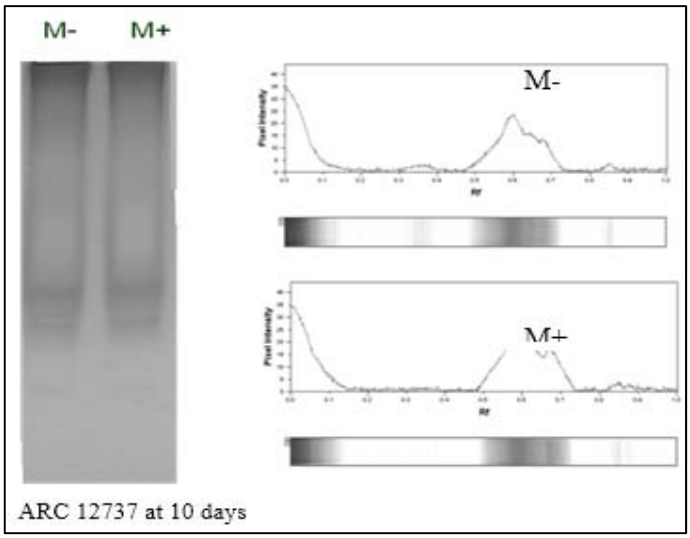


Plate 1: Comparison of whole root peroxidase isozyme profile of one negatively responding (Black Gora), and one responding (MTU 7029) variey of rice under AM inoculation and no inoculation at 10 days.



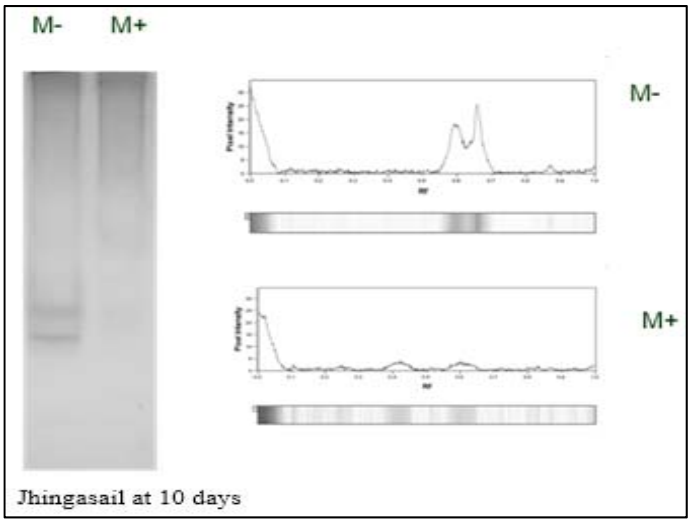
Black Gora at 10 days

Treatment	Band no.	Rf	Volume	Area	Band %
M-	1	0.223	6052.10	3740	4.28
	2	0.381	23106.00	8160	16.34
	3	0.561	46556.45	6800	32.93
	4	0.613	32380.82	5372	22.90
	5	0.848	33.281.48	7752	23.54
M+	1	0.229	14539.10	5236	6.26
	2	0.392	23725.05	7344	10.22
	3	0.567	55274.02	5440	23.81
	4	0.630	97794.19	7276	42.12
	5	0.852	40859.89	7140	17.60



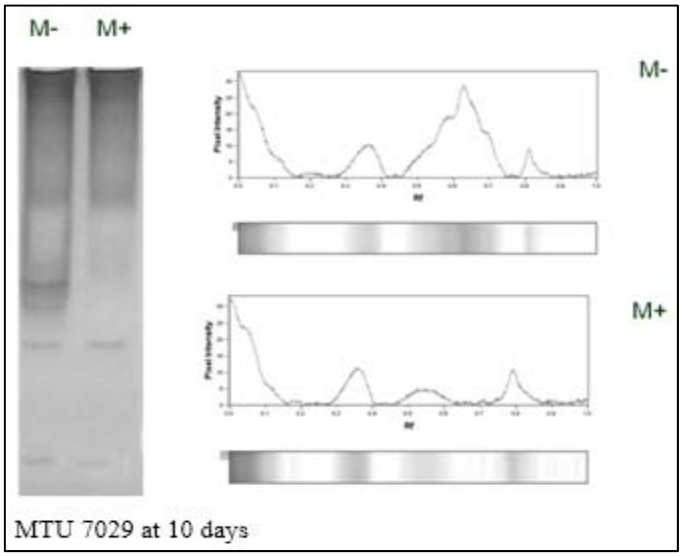
ARC 12737 at 10 days

Treatment	Band no.	Rf	Volume	Area	Band %
M-	1	0.200	2806.20	2736	1.31
	2	0.365	12712.20	5904	5.93
	3	0.599	154047.58	12384	71.87
	4	0.676	36894.97	4248	17.21
	5	0.852	7873.32	4320	3.67
M+	1	0.197	1687.50	1904	0.72
	2	0.353	4226.10	3536	1.81
	3	0.588	161009.09	10608	69.15
	4	0.670	55777.69	5032	23.95
	5	0.849	10147.27	4692	4.36



Jhingasail at 10 days

Treatment	Band no.	Rf	Volume	Area	Band %
M-	1	0.183	3334.00	3200	1.51
	2	0.338	2902.37	4320	1.32
	3	0.602	91976.08	8080	41.73
	4	0.664	114405.40	9600	51.91
	5	0.879	7791.67	5600	3.54
M+	1	0.245	7639.79	5688	14.86
	2	0.423	22590.50	8424	43.95
	3	0.604	18930.52	8064	36.83
	4				
	5	0.877	2236.26	1944	4.35



Treatment	Band no.	Rf	Volume	Area	Band %
M-	1	0.201	5236.85	4346	1.32
	2	0.361	62165.58	10332	15.64
	3	0.578	106066.68	10660	26.68
	4	0.630	199199.83	11562	50.11
	5	0.812	24.856.32	7380	6.25
M+	1	0.186	3365.00	3116	2.27
	2	0.358	60361.90	9512	40.63
	3	0.520	17593.62	5658	11.84
	4	0.556	21620.59	6888	14.55
	5	0.791	45623.20	11070	30.71

Plate 2: Whole root peroxidase isozyme profile together with densiometrc analysis of zymogram of 4 rice variety upon AM inoculation and non-inoculation at 10 days.

There was no perceptible qualitative change in the composition of the peroxidase isozyme profile (banding pattern) of the differently responsive rice varieties. Characteristically, however, percent composition of the 5 identified bands of the varieties changed due to inoculation. The most significant change was in bands number 3 and 4 at Rf positions 0.60 and 0.65 respectively which were found as predominant root peroxidase isozmes of rice at the particular growth stage. These two bands appeared in close proximity and their presence as percent of total of all bands increased in the negatively responding varieties, declined in the positively responding varieties. Out of the 4 varieties, largest significant changes in the percent occurrence of these two bands were observed in MTU 7029 and Jhingasal, both of which were positively responding varieties.

These results, confirming the previous results of colorimetric estimates of whole root peroxidase activity of rice roots due to AM inoculation, showed that apart from the quantitative change in whole root peroxidase activity due to AM infection at early seedling stage, extent of presence of the individual isozymes may also change significantly. The two isozymes which occur in largest amounts in rice roots were more prone to change in quantity due to infection. Moreover, their change was found to be differential – rise in the negatively responding varieties and decline in the positively responding varieties.

Based on the results of analysis of whole root peroxidase activity and of the peroxidase isozyme profile, it appeared that during the initial stages of AM colonization regulation of peroxidase enzyme (and isozymes) may change in roots suggesting for possible association/ involvement of the enzyme with the colonization process. Root peroxidase activity in the low AM responsive varieties which do not or negatively respond to AM infection at the early growth stage might rise significantly following initial attempts for colonization. With progress of time and success of colonization the rising trend might be reversed to show a decline in the increased activity. In the positively responding varieties the activity might be suppressed [9] right at the initial stage of colonization and such suppression might become more significant with time and progress of infection. Apparently, the regulation of the two most predominant peroxidase isozymes of rice roots was most involved in such differential response of peroxidase enzyme to AM colonization, which were either up or down in expression quantity depending upon the response character of the varieties.

Results of the studies on the changes in whole root peroxidase activity of the differently responding rice varieties become interesting in the context of both low or negative growth response and slower colonization rates of the traditional varieties. There are evidences of transitory increases of enzymes of post –recognition defense pathways of hosts [10] including the whole root peroxidase activity, at initial stages of colonization of host roots by the AMF [11, 3]. Our observations in the context showed a clearly different response of the two variety groups, responding negatively and positively to AMF colonization at the initial stage. While the early negatively responding varieties which were colonized at slower rate showed a significant rise in whole root peroxidase activity, the varieties which were colonized at a faster rate and had a positive growth response showed a decline in peroxidase activity. The two peroxidase isozymes which were present in rice roots at higher amounts at that age were observed to be up or down regulated in the negatively and positively responding varieties respectively. Accepting that activation of peroxidase enzyme in roots under initial AMF colonization is an early expression of defense reaction through possible reinforcements of cell wall [12, 13] the increased peroxidase activity in the negatively responding, and slowly colonized varieties can be interpreted as a possible activation of defense response to AMF colonization at the initial stage in these varieties. That would mean that root peroxidase activity in the high responding, rapidly colonized varieties were suppressed at the initial stage. Clearly, these were indications for some degree of incompatibility of the host-AMF combination in case of traditional varieties which responded negatively to AMF inoculation and such response may mean that the host perceives the invading AMF as a potential pathogen.

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