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Effect of total protein, total phenol, polyphenoloxidase, and peroxidase activity in rice (*Oryza sativa*) genotypes against *Magnaporthe grisea*

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ABSTRACT

The objective of our study was to know the differences in expression of total protein, total phenol, peroxidase and polyphenoloxidase activities in self-defense in plant tissues against rice blast diseases. The activities of these compounds were studied in different genotypes of rice (*Oryza sativa* L.) Ngoba, Bhalum-1, Lampnah and IET 7614 with varying degrees of susceptibility to *Magnaporthe grisea*. Observation was taken after two successive days interval up to 15 days repeatedly (eight times) and the data was subjected to repeated measure analysis. Gradual declination was observed for total protein, whereas total phenol, peroxidase and polyphenoloxidase activity has shown increasing trend. Rice genotypes have shown considerable difference in expression which was well-correlated with the degrees of resistance in specificity of pathogen. Univariate test for between and within subject factors were calculated and found to be significant ($P<0.001$). Since sphericity assumption was violated, MANOVA test was also done and significant difference among characters and genotypes were observed in due course of time.

Key words : Peroxidase, Polyphenoloxidase, Repeated measure analysis, Rice blast, Total phenol, Total protein

Rice (*Oryza sativa* L.) is one of the most important staple foods for the increasing world population, especially in Asia. Diseases are among the important limiting factors that affect rice production. More than 70 diseases caused by fungi, bacteria, viruses or nematodes have been recorded on rice among which rice blast is one of the most serious constraint. Blast is considered the principal disease of rice because of its wide distribution and high incidence under favourable conditions. Rice blast, caused by *Pyricularia grisea* (teleomorph, *Magnaporthe grisea*) has the potential to cause severe crop yield losses in rice as high as 50% where environmental conditions are favorable for disease development and thus the disease occurs in epidemic proportions (Babujee and Gnanamanickam 2000). Like many plant species, rice employs a diverse array of defenses that minimize losses during pathogen attack. Changes in biochemical and enzymatic activities like total protein, total phenol, peroxidase, and polyphenoloxidase are often related with resistant/susceptible reaction of plants against blast infection (Peng and Kuc 1992, Maraite 1973, Sridhar 1972).

In view of these reports, the present investigation was designed to assay these enzymes in leaves of a susceptible and a resistant variety of rice to study the effects of infection by *Pyricularia oryzae* on the biochemical changes.

Repeated measures are consecutive data obtained over time from the same experimental units such as plants or animals (Littell *et al*. 1996). The objectives of repeated measures data analysis are to examine and compare response trends over time. This can be comparisons of treatments at specific times, or averaged over time. It also can involve comparisons of times within a treatment (Littell *et al*. 1998). Repeated ANOVA is applied traditionally for statistical analysis of data including between-subject and within-subject factors. Repeated ANOVA is used safely when spherity assumption is provided (Eyduran and Akbaº 2010). The sphericity assumption is an assumption about the structure of the covariance matrix in a repeated measures design. It was observed that multivariate methods gave more reliable results than univariate methods (Tabachnick and Fidel 2001, Gurbuz *et al*. 2003).

MATERIALS AND METHODS

Seeds of a four variety of rice genotypes, viz. Ngoba, Bhalum 1, Lampnah and IET 7614 were grown in field

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conditions. Among them two were of upland (Bhalum 1 and IET 7614) and other two were of lowland ecology (Ngoba and Lampnah). Bhalum 1 and Lampnah were blast resistant varieties, whereas Ngoba and IET 7614 were found to be moderately susceptible and susceptible respectively in conditions of Meghalaya.

Leaves from both healthy and infected plants (50 days old) of same aged plant were collected from field in three replications. Leaves from healthy plants were used as control whereas infected plants were used as test. Seven-centimeter tips from fully expanded and matured leaves were washed in distilled H_2O, randomized, and floated in 30 ml of distilled H_2O containing kinetin (5μM) in 10 cm petri dishes in the dark at room temperature (25 ± 3°C). Leaves were homogenized with cold 0.1 M phosphate buffer (pH 6.8) (100 mg fresh tissue, 2 ml buffer) in a prechilled mortar and pestle. Filtered samples were taken initially and at two days intervals for biochemical and enzymic analyses (Kar and Mishra 1976).

The 5 ml portion of the homogenate was mixed with four parts of ethyl alcohol so that the final concentration of ethyl alcohol became 80% (v/v). This was boiled in a water bath for 10 min, cooled, and centrifuged for 15 min at 3 000g (relative centrifugal force). The pellet was extracted three times with boiled ethyl alcohol and centrifuged. The supernatants were combined and made to volume. Total phenol was determined from the alcoholic extract by the phenol reagent method (Sridhar 1972). The pellet was washed successively with 10% (w/v) cold trichloroacetic acid (twice), ethyl alcohol (once), ethyl alcoholchloroform (3:1, v/v, twice), ethyl alcohol-ether (3:1, v/v, once), and finally with ether (once). The pellet was allowed to dryness. The protein was solubilized by boiling with 1 N NaOH for 15 min in a water bath. It was centrifuged and an aliquot was taken for protein estimation (Lowry *et al*. 1951).

Five milliliters of the assay mixture for the peroxidase activity comprised: 125 μmoles of phosphate buffer (pH 6.8), 50 μmoles of pyrogallol, 50 μmoles of H_2O_2, and 1 ml of the 20 times-diluted enzyme extract. This was incubated for 5 min at 25°C after which the reaction was stopped by adding 0.5 ml of 5% (v/v) H_2SO_4. The amount of purpurogallin formed was determined by taking the absorbancy at 420 nm. (Kar and Mishra 1976).

Five-milliliter assay mixture for polyphenoloxidase activity consisted of the same assay mixture as that of peroxidase without H_2O_2. The absorbancy of the purpurogallin formed was taken at 420 nm (Kar and Mishra 1976).

The data was observed at 1, 3, 5, 7, 9, 11, 13 and 15 day in three replications with three plant leaves for each replication. Repeated measure analysis was done using GLM procedure of SAS 9.2. The results provided by the REPEATED statement in GLM procedure were based on univariate and multivariate analyses of contrast variables computed from the repeated measures variables. This approach basically bypassed the problems of covariance structure rather than addressing them directly. The REPEATED statement enabled users to obtain statistical tests for effects involving time trends. Correlation was calculated for each trait using CORR procedures of SAS 9.2 software (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Performances of different genotypes

For total protein content, gradual decline was observed over time in both control (without infection) and test leaves (infected). However increased content was observed at day 5, 7 and 11. This suggests that some classes of protein might be expressed during blast infection but overall protein content goes down. Total phenol content has increased as day progresses. The increased content of total phenol is more pronounced in test leaves, when compared to control leaves, showing its importance in imparting resistant reaction to rice blast disease. For peroxidase activity increase trend of activity was observed up to day 11, afterwards decreased activity was shown by both control and test genotypes. Coordinated increases in peroxidase activities and phenolic compounds are associated with the colonization of rice leaves by *Magnaporthe grisea*, the blast pathogen (Sridhar and Ou 1974, Toyoda and Suzuki 1960). The increase in the activity of peroxidase is one of the most reliable indicators of maturity and senescence (Doke 1983, Lamb and Dixon 1997). polyphenoloxidase activity has shown increasing trend up to 13 day afterwards declination was observed. Increase in Polyphenoloxidase activity during senescence of detached leaves or leaf disks as well as with the physiological age of the attached leaves was also observed by Maraite (1973) and Farkas *et al*. (1963) (Fig 1).

Pearson's correlation coefficients among repeated characters are presented in Table 1. For total protein content positive correlations were found for Day 1 with Day 5 and Day 13 with Day 15. Highest negative correlations were observed for Day 11 with 15 and 7. For total phenol content all the correlation coefficients were significant except for Day 1 and 3 with Day 11, 12, and 15. As far as polyphenoloxidase activity is concerned positive correlation coefficients were observed except Day 9 with 5 and 13. For Peroxidase activity negative significant correlation coefficients were observed for different days along with positive correlations as depicted in Table 1.

Repeated measures analysis

Repeated Measures analysis is a tool to know the effects of interventions in situations in which the same subject (genotype) receive sequential measurements over time.

Between-Subject and Within-Subject effect

In a repeated measure study, it is important to distinguish

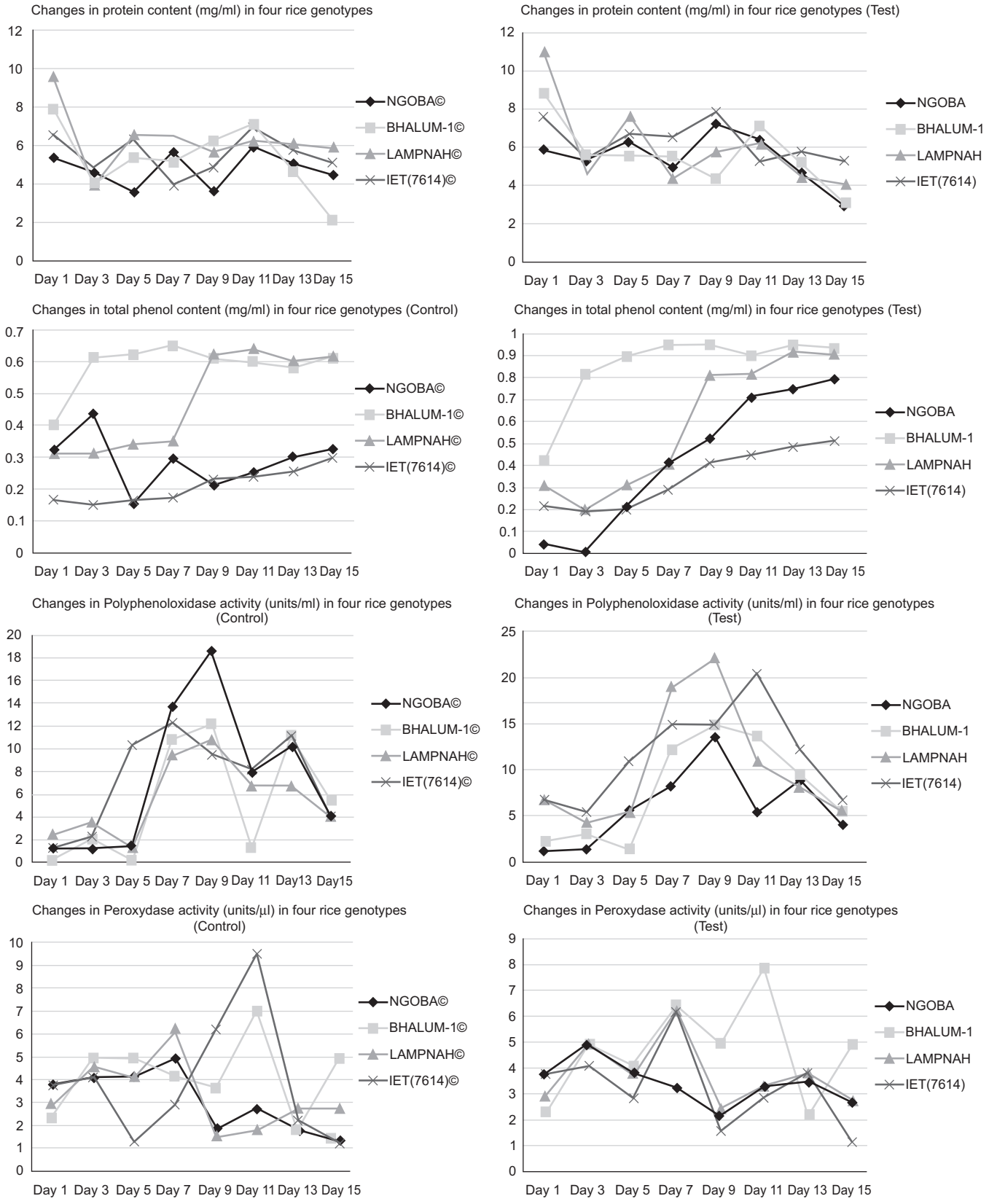

Fig 1 Performance of different genotypes

Table 1 Pearson's correlation coefficient between repeated characters

	Variables	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13	Day 15
Protein(mg/ml)	Day 1	1	–0.271	0.639	–0.217	–0.065	0.018	0.034	0.248
	Day 3		1	–0.220	0.264	0.351	–0.067	–0.113	–0.059
	Day 5			1	–0.297	0.277	–0.011	0.107	0.338
	Day 7				1	0.376	–0.563	0.293	0.332
	Day 9					1	–0.298	–0.193	–0.003
	Day 11						1	–0.001	–0.579
	Day 13							1	0.552
	Day 15								1
Phenol (mg/ml)	Day 1	1	0.843	0.686	0.564	0.450	0.239	0.242	0.259
	Day 3		1	0.844	0.781	0.409	0.230	0.213	0.241
	Day 5			1	0.954	0.758	0.628	0.570	0.596
	Day 7				1	0.772	0.711	0.672	0.701
	Day 9					1	0.955	0.932	0.928
	Day 11						1	0.974	0.977
	Day 13							1	0.992
	Day 15								1
Polyphenol oxidase	Day 1	1	0.872	0.400	0.743	0.577	0.637	0.133	0.478
(units/ml)	Day 3		1	0.353	0.471	0.262	0.692	0.003	0.350
	Day 5			1	0.234	-0.122	0.494	0.396	0.162
	Day 7				1	0.809	0.448	0.184	0.405
	Day 9					1	0.303	–0.062	0.376
	Day 11						1	0.305	0.555
	Day 13							1	0.692
	Day 15								1
Peroxidase activity	Day 1	1	–0.590	–0.700	–0.423	–0.123	–0.202	0.273	–0.877
(units/µlitre)	Day 3		1	0.529	0.247	0.009	0.060	0.273	0.754
	Day 5			1	0.417	–0.398	–0.280	–0.219	0.684
	Day 7				1	–0.380	–0.432	0.226	0.199
	Day 9					1	0.951	–0.407	0.260
	Day 11						1	–0.504	0.385
	Day 13							1	–0.289
	Day 15								1

Values in bold are significant at 5% level of significance

between two types of factors: (a) between-subject factors (BSFs) and (b) within-subject factors (WSFs). BSF is a factor whose levels are represented by independent subjects which provide unrelated observations. Observations across levels of WSFs are not independent. In contrast, a within-subject factor (WSF) has levels that represent repeated measurements observed on the same persons or in combination with others related to them. Repeatedly assessed subjects give rise to correlated measurements across the levels of any WSF. Between-subject effects which provide univariate tests of hypotheses are presented in Table 2. The effect of character, genotype, Con/test and their interactions were found to be significant.

Within subjects effects include a parameter time in addition to the parameters described above. The results of within subject effects are given in Table 3. Greenhouse-Geisser (G-G) and Huynh-Feldt (H-F) Epsilon adjusted F test approaches can practically use when validity of spherity assumption was not provided. Sphericity is an important assumption of a repeated measure ANOVA. It refers to the condition where the variances of the differences between all possible pairs of groups (i.e. levels of the independent variable) are equal. The violation of sphericity occurs when the variances of the differences between all combinations of the groups are not equal. If sphericity is violated, then the variance calculations may be distorted, which would result in an F-ratio that would be inflated. When these two approaches were taken into consideration, time effect and its interaction effect was found to be significant (P<0.001).

Mauchly's sphericity test and epsilon adjustment values

Mauchlys's sphericity test (Table 4) examines the form of the common covariance matrix. If the value is less than the alpha level of 0.05, the data do not meet the sphericity

Table 2 Repeated measures analysis of variance for between subject effects (Univariate Tests of Hypotheses)

Source	DF	SS	Mean Square	F Value	Pr > F
Character	3	5 796.221	1 932.074	18 398.500	<0.0001
Genotype	3	84.523	28.174	268.300	<0.0001
Control vs Test	1	104.984	104.984	999.730	<0.0001
Replication	2	7.781	3.891	37.050	<0.0001
Character*Genotype	9	208.231	23.137	220.320	<0.0001
Character* Con/Test	3	179.391	59.797	569.430	<0.0001
Genotype*Contest	3	47.439	15.813	150.580	<0.0001
Character*Genotype* Control vs Test	9	238.330	26.481	252.170	<0.0001
Error	62	6.511	0.105		

Table 3 Repeated measures analysis of variance for within subject effects (Univariate Tests of Hypotheses)

Source	DF	SS	Mean Square	F Value	Pr > F	Adj G - G	Pr > F H-F-L
Time	7	932.180	133.169	1 813.340	<0.0001	<0.0001	<0.0001
Time*Character	21	2 735.129	130.244	1 773.520	<0.0001	<0.0001	<0.0001
Time*Genotype	21	158.199	7.533	102.580	<0.0001	<0.0001	<0.0001
Time* Con/Test	7	29.302	4.186	57.000	<0.0001	<0.0001	<0.0001
Time*Replication	14	1.506	0.108	1.460	0.121	0.144	0.135
Time*Character*Genotype	63	662.981	10.524	143.300	<0.0001	<0.0001	<0.0001
Time*Character* Con/Test	21	209.212	9.962	135.660	<0.0001	<0.0001	<0.0001
Time*Genotype*Contest	21	113.742	5.416	73.750	<0.0001	<0.0001	<0.0001
Time*Charac*Genoty*Con/Test	63	520.680	8.265	112.540	<0.0001	<0.0001	<0.0001
Error(time)	434	31.872	0.073				

Greenhouse-Geisser Epsilon= 0.778; Huynh-Feldt-Lecoutre Epsilon= 0.861

assumption. For practical purposes, these issues are important only to decide which output to use. If we use the univariate output, we may have more power to reject the null hypothesis in favour of the alternative hypothesis. However, the univariate approach is appropriate only when the sphericity assumption is not violated. If the sphericity assumption is violated, then in most situations multivariate analysis is better. In our experiment probability of Mauchly's test for spherity assumption was significant (P<0.001). This means that multivariate approach should be prefferred instead of univariate approach.

Multivariate approach to repeated measures design (MANOVA) is one of the important multivariate approaches. A main concern of MANOVA is the examination of mean differences across several groups when more than one dependent variables are considered simultaneously. When variables are not independent, there are chances of strong potential interactions. This inflates the error even more highly and thus MANOVA will be more appropriate test as it may reveal differences not shown by separate ANOVAs. Results on time, time × character, time x genotype and time x character × genotype are presented in Table 5. We will reject the null hypothesis if value of Wilk's lambda is small (close to zero). Likewise if value of Pillai's Trace, Hotelling-Lawley Trace and Roy's Greatest Root statistic is large we will reject the null hypothesis.

Table 4 Sphericity test results

Mauchly's variables	DF	Criterion	Chi-Square	Pr > ChiSq
Transformed variates	27	0.331	65.785	<0.0001
Orthogonal components	27	0.331	65.785	<0.0001

As seen from Table 5, the value of Wilks' Lambda is small and value of Pillai's Trace, Hotelling-Lawley Trace and Roy's Greatest Root statistic is large, null hypothesis on the time effect was rejected at 1% level. This is also reflected in terms of probability of F value (<0.0001). Thus significant effects of time, time × character, time × genotype and time × character × genotype interaction were found. This shows that different compounds and genotypes had significantly varied over time. Significant effect of time × character × genotype interaction shows that inclination or declination of total protein, total phenol, peroxidase and polyphenoloxidase varies significantly among different genotypes and also with time. Some genotypes had shown more changes when compared to the others. This change might be responsible for a resistance or susceptible reaction against blast infection.

In this experiment for total protein, resistant genotypes likewise Lampnah followed by Bhalum 1 had shown more fluctuation. Also decrease in total protein in infected leaves

Table 5 MANOVA test criteria and exact F statistics for the hypothesis

	Effect	Value	F Value	Num, Den DF	Pr> F
No Time effect	Wilks' lambda	0.003	2 382.28	7, 56	<0.0001
	Pillai's trace	0.997	2 382.28	7, 56	<0.0001
	Hotelling-Lawley trace	297.785	2 382.28	7, 56	<0.0001
	Roy's greatest root	297.785	2 382.28	7, 56	<0.0001
No Time*Character effect	Wilks' lambda	0.000001	930.13	21, 161.35	<0.0001
	Pillai's trace	2.935	376.09	21, 174	<0.0001
	Hotelling-Lawley trace	904.508	2 368.71	21, 110.61	<0.0001
	Roy's greatest root	831.775	6 891.85	7, 58	<0.0001
No Time*Genotype effect	Wilks' lambda	0.0007	86.57	21, 161.35	<0.0001
	Pillai's trace	2.663	65.41	21, 174	<0.0001
	Hotelling-Lawley trace	36.766	96.28	21, 110.61	<0.0001
	Roy's greatest root	21.066	174.55	7, 58	<0.0001
No Time*Character*	Wilks' lambda	0.00000001	133.97	63, 321.5	<0.0001
genotype effect	Pillai's trace	6.000	41.36	63, 434	<0.0001
	Hotelling-Lawley trace	169.825	147.09	63, 193.11	<0.0001
	Roy's greatest root	80.710	556	9, 62	<0.0001

(test) was more fluctuated as compared to control leaves. This suggests that several classes of proteins might be activated and deactivated during blast infection. In a study, different patterns of gene expression and key metabolic processes activated during appressorium formation by blast fungus were identified (Soanes *et al*. 2012). Bhalum-1 and Lampnah had also shown more increase of phenol content whereas IET 7614, a susceptible variety had shown least response in both infected and control leaves. In another study it was observed that phenolic-like compounds or phytoalexins played a primary role in rice defense response against infection by *M. grisea* (Rodrigues *et al*. 2003). The more increase in phenol content may be an indirect way of selection of resistant genotypes but it need to be studied in detail. For peroxidase, susceptible variety IET 7614 had shown more increase in control leaves whereas Bhalum 1 followed by Lampnah had shown increased activity in test leaves. The increase in peroxidase activity is well correlated with the resistance. Many reports have suggested that peroxidase activities play roles in resistance to pathogens such as lignification and suberization, cross linking of cell wall proteins, xylem wall thickening, generation of reactive oxygen species, hydrogen peroxide scavenging, phytoalexin synthesis, antifungal activity of peroxidase activity itself and auxin metabolism (Sasaki *et al.* 2004 and Tanabe *et al.* 2011). Likewise for polyphenoloxidase less fluctuated behavior in control leaves as compared to infected leaves was shown by resistant genotypes. A negative correlation was observed for polyphenoloxidase activity with chlorophyll content in detached leaves of rice (Kar and Mishra 1976) whereas chlorophyll content in infected rice leaves was highly correlated with disease severity under leaf blast infection (Yun *et al.* 2000).

Further work needs to be done with blast resistant and susceptible rice varieties to establish the role of various biochemical constituents in disease resistance at both biochemical and molecular level.

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