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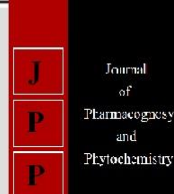

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Biochemical and molecular analysis of wild rice and maize (non-host) against *R. solani*

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Abstract
Sheath blight caused by *Rhizoctonia solani*, is a serious disease of rice crop grown worldwide. In rice, (*O. sativa* L.)], complete genetic resistance does not exist. For the identification of possible gene(s) involved in defense against this pathogen during infection, wild rice accession *Oryza rufipogan*, and nonhost maize cv. Kanchan has been showed moderately resistance. Total RNA and protein has been isolated from the control and inoculated plants leaves. Isolated mRNA was converted into cDNA and differentially expressed proteins were studied with RGA primers. The biochemical processes underlying the expression of resistance to *R. solani* infection were investigated and compared in wild rice, and non-host maize. Some important resistance gene analogue fragments and proteins were showed higher expression in wild rice (*Oryza rufipogan*) in comparison to maize cv. Kanchan. These induce RGA and biochemical signals will be further used for the investigation of resistance against sheath blight pathogen.

Keywords: host resistance, sheath blight, differentially expressed protein, wild rice

Introduction
Rhizoctonia solani Kuhn is a plant pathogenic fungus with a wide host range and worldwide distribution and has a significant economic impact in the development and production of a wide variety of crops [1]. To date, 13 Anastomosis Groups (AGs) have been recognized according to hyphal anastomosis behavior, cultural morphology, host range and pathogenicity [2]. Sheath blight (ShB), causes significant yield loss and reduction in grain quality for rice (*Oryza sativa* L.) in the southern U.S. and other regions of the world [3]. This disease has been described as crown and brace root rot on maize (*Zea mays* L.), caused by *R. solani* [teleomorph *Thanatephorus cucumeris* (Frank) Donk)], anastomosis group AG2-2IIIB [4]. Wild rice accessions have been used to successfully develop resistance against many rice diseases [5]. Over the years, a very large number of accessions from different species of *Oryza* have been tested at IRRI (International Rice Research Institute) to identify sources for ShB resistance. From a total of 233 accessions tested, 76 were found to contain a high level of resistance to ShB and 29 showed moderately resistance. The latter accessions belonged to the African rice, *O. glaberrima* (2n = 24 AA), a close relative of *O. sativa* (2n = 24 AA). Gene expression profiling is a promising approach to study the regulatory mechanisms and signaling networks that underlie plant defense responses and pathogenesis. Zhao *et al.* (2008) [6] found 50 genes of diverse function that were transcritpionally activated in rice after challenge by *R. solani*. Venu *et al.* (2007) [7] detected numerous up- and down-regulated rice genes after infection by *R. solani* using SAGE and microarray analysis. Silva *et al.* (2012) [8] conducted a research to exploit whole genome sequences of 13 rice (*Oryza sativa* L.) inbred lines to identify non-synonymous SNPs (nsSNPs) and candidate genes for resistance to sheath blight.

Over the last few years, genetic and molecular studied on the disease and pathogen have been reported in maize [9, 10]. These studies have revealed that resistance to BLSB is a typical quantitative trait controlled by polygenes and three significant QTL located on chromosomes two, six, and ten to be responsible for resistance to BLSB respectively [11]. In addition, many catalytic enzymes involved in response to *R. solani* infection were analyzed, including chitinase, glucanase and phenylamine ammonia lyase and few pathogenesis-associated genes and some potential defense pathways were involved in response to *R. solani* infection [12, 13].

In contrast to the aforementioned developments, there has been little progress toward understanding the resistance networks of genetic and proteomic molecules involved for sheath blight in different hosts including rice at a transcriptomics and proteomic levels. Thus, the present study is conducted for the comparative biomolecules for the understandings of defence mechanisms of different hosts against *R. solani*.

Materials and Methods

Seeds of wild rice *O. rufipogon* was collected from International Rice Research Institute, Philippines, Maize seed cv. Kanchan was collected from the Department of Genetics and Plant Breeding, N.D. University of Agriculture and Technology, Kumarganj, Faizabad, India. A multinucleate compatible, highly virulent strain of *R. solani,* D-14 belonging to AG1-IA anastomosis group was obtained from the Rice Pathology Laboratory, G. B. Pant University of Agriculture and Technology, Pantnagar, India. This isolate was grown on potato dextrose agar at 28 ± 1 °C for 6 days and used as the inoculation purpose. At five different time intervals 24 HAI, 48 HAI, 96 HAI and 120 HAI (HAI, hours after inoculation) the plants samples were collected for the biochemical analysis and compared with the control condition.

Assay of catalase (CAT)

The enzyme extract for measuring catalase activity in different samples [Fresh control and infected leaves samples (24 HAI, 48 HAI, 72 HAI 96 HAI and 120 HAI)] were prepared by homogenizing leaves (1 g) samples in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) at 4 °C. The homogenate was centrifuged at 15,000 x g at 4 °C for 30 min., pellets were discarded and supernatant was used as enzyme source. The standard reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0) and 15 mM H_2O_2. A 100 µ1 sample of the enzyme extract was added to 2.9 ml of the standard reaction mixture to initiate the reaction[14]. Catalase units were defined as mmol oxygen released min-1g fresh weight^{-1}.

Assay of peroxidase (PO)

1 g of leaves samples was ground in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) at 4 °C. The homogenate was centrifuged at 15,000 x g at 4 °C for 30 min., and supernatant was collected. Peroxidase activity was determined according to the procedure given by Srivastava (1987) [15]. In enzyme assay, pyrogallol oxidation was maintained in the mixture containing 50 mM phospate buffer (pH 6.5). In a sample cuvette, 1.5 ml of 0.05 M pyrogallol and 100 µl of enzyme extract were taken up. In reference cuvette, inactivated enzyme (by boiling) extract (100 µl) was taken along with 1.5 ml of 0.05 M pyrogallol. The reading was adjusted to zero at 420 nm in a spectrophotometer. To initiate the reaction, 100 µl of H_2O_2 (1%) (v/v) was added and the absorbance was read at 420 nm. The enzyme activity was expressed as ktkat g^{-1} tissue.

Inoculation of different hosts and non-hosts for RNA isolation

Inoculation of the different hosts plants for the total RNA isolation purpose were done in hot humid chamber. Leaves were harvested from the non inoculated (control), and inoculated different hosts plants after 24 hours, 36 hours, 48 hours and 72 hours of inoculation. The harvested leaves were briefly rinsed with in sterile distilled water, dried with paper towel, put immediately in liquid nitrogen and stored in the deep freezer (-80 °C). All the solutions and glassware were treated with 0.1 % DEPC water and sterilized for the RNA wok. Total RNA was isolated from the different hosts and non-hosts plants by using Qiagen RNeasy Plant Mini Kit. The quantity and quality of total RNA was checked using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The total-RNA was subjected to NanoDrop ND-1000 using RNase free water as blank: absorbance was recorded at 260/280 and 260/230. Total RNA (100 µg) from the control and different inoculated time period was reverse transcribed into first-strand cDNA with use of an oligo(dT) primer (Fermentos, Life Sciences, USA).

Semi-Quantitative RT PCR

For semi-quantitative RT-PCR, the first strand cDNA (1µg) was used a templates and the concerned gene was amplified by the different primers. Actin gene was used as internal control for all the treatments. Different gene-specific primers were used for the amplification of target cDNA (Table 1). Total RNA was isolated from non-inoculated (control) and inoculated (24HAI, 36HAI, 48HAI and 72HAI) and with *R. solani* isolates to cultivated rice cvs., *O. rufipogon,* and maize cv. Kanchan by using Qiagen RNeasy Plant Mini Kit. cDNA (complementary DNA) was synthesized by using first strand cDNA synthesis kit (Fermentas, Life Sciences, USA). The semi-quantification of different hosts and non-hosts were determined by four different RGA (resistance gene analogue) primers with the help of 100ng of first strand cDNA as the templates for the amplification of the target RGAs. Five different conditions cDNA namely; control (no inoculation with *R. solani*), 24HAI (24 hours after inoculation with *R. solani*), 36HAI, 48HAI and 72HAI were used for the amplification purpose.

Table 1: Different Resistance gene analogue (RGA) primers used in the semi-quantitative PCR

Sr. No.	Primer name	Sequence (5` to 3`)	Tm	Conserved motif/domain
1	RGA 1 F	AGGTCGGTGACAGAGACTCG	56	Probable serine/threonine protein kinase
	RGA 1 R	CTCAGAGACCCCAAGAAAGC		
2	RGA 2F	AGCATAATCTGCCGAAGAGC	55	Putative serine/threonine protein kinase
	RGA 2F	CTGGTCAATCCCAACTAGGC		
3	8A1 F	GTGGTCCAATAGAGTGATGC	55	Putative ubiquitin conjugating enzyme E2
	8A1R	AGAGACATACCAATTGCAGG		
4	PR1 F	CAGGACTACGTGAGGCTCCA	55	Pathogenesis related gene
	PR1 R	CCTCTGTCCGACGAAGTTGC		
5	Actin F	CTGCTGGAATGTGCTGAGAGAT	56	Housekeeping gene
	Actin R	CGTCTGCGATAATGGAACTGG		

Experimental design and statistical analysis

All experiments of enzymatic and defence signaling assay were performed in triplicates. For the enzyme analysis, the data obtained for each enzyme were analysed separately. Paired t test was performed in all the cases using GraphPad PRISM software [17]. Pearson correlation coefficients and *P* values were used to show correlations and their significance. Differences of $P<0.05$ were considered significantly.

Results and Discussion

The content of catalase in inoculated wild rice accession *O. rufipogon* and Maize cv. Kanchan increased after *R. solani* infection at 24 HAI to 48 HAI of inoculation and was

significantly higher (up to two- fold) in infected plants than the susceptible line and control plants. Although catalase content in infected susceptible plants of both hosts and non-hosts increased from 24 HAI to 48 HAI, and were significantly lower than those of control plants. Furthermore, the time course of change in the content of catalase in the infected rice and non-host maize cv. Kanchan was similar up to some level of induction. The wild rice accession *O. rufipogon* showed higher level of catalase induction after 24 HAI to 120 HAI of inoculation compared to other (Fig. 1). Plants have equipped with the endogenous defence mechanisms that can be induced after attacking by insects and pathogens. Inducing the plant's own defence mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy against the pathogens [18]. Plants have developed non-enzymatic and enzymatic mechanism to remove the excess ROS generated during the host-pathogen interaction [19].

Peroxidase activities increased (>2-3 fold) in the infected wild rice accession *O. rufipogon* and Maize cv. Kanchan from 24 HAI to 120 HAI. The time course of change in peroxidase among infected wild rice accession *O. rufipogon* and Maize cv. Kanchan to change in >3 to 6-fold. The peroxidase activity was found maximum in *O. rufipogon* after 96 HAI followed by Kanchan (at 48 HAI) after inoculation. Furthermore, peroxidase activity in infected wild rice O. rufipogon was highest from 24 HAI to 48 HAI of inoculation periods which was similar to control condition (Fig. 1). Superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, glutathione peroxidase and glutathione reductase are some common enzymes involved in the host defence against oxidative stress [20, 21].

The expression level of the selected RGA1 (probable serine/threonine protein kinase) gene was regulated upstream after infection of the *R. solani*. In wild rice *O. rufipogon*, the expression level of this selected gene increased; however after 48HAI the down regulation of the gene was observed. The maize cv. Kanchan showed the increased expression level of the RGA1 gene after *R. solani* infection (Fig 2a and 2b). Expression of the RGA2 gene (probable serine/threonine protein kinase), two wild rive accessions showed the up-regulation of the expression after 36 HAI, however after 48HAI the expression increased. The continue expression observed maize cv. Kanchan, the expression was not found after 24HAI and 36HAI respectively (Fig 2a and 2b). Expression of PR1gene (pathogenesis related gene) was found in all the hosts and non-hosts tested in control and after inoculation by *R. solani*, the wild rice *O. rufipogon* at 48HAI of inoculation and maize cv. Kanchan also showed up-regulation, however the regulation patterns was not high as in barley (Fig 2a and 2b). Expression pattern of 8A1 (putative ubiquitin conjugating enzyme E2) gene were found up regulated wild accession *O. rufipogon*. The 8A1 primer pair was not amplified by the other non-host cv., maize (Kanchan) (Fig 2a and 2b). Earlier, Zhao *et al*. (2008) [8] also reported that ubiquitin-conjugating enzyme E2 and transferase, transferring glycosyl groups were also successfully induced in the rice after *R. solani*. The non-host; maize cv. Kanchan also showed increased expression level of the RGA1, PR1 and PBZ1 gene (Pathogenesis related gene) gene after *R. solani* infection. The PR1gene was induced in the resistance reaction to rice blast (Mitsuhara *et al*., 2008), whereas PBZ1, an intracellular protein, was induced by probenazole (3-allyloxy-1, 2-benzisothiazole-1, 1-dioxide), an effective chemical inducer of host resistance against rice blast infection [22, 23].

The expression of the sub-domain gene after inoculation by *R. solani* was found down regulated in wild rice cv. *O. rufipogon* after inoculation by *R. solani*. The specific bands of ~400bp was also induced after the 48HAI in the three wild rice accessions *O. rufipogon*, however this specific bands was not amplified by maize cv Kanchan (Fig. 2a and 2b). The expression patterns of Ptokin½ and Ptokin¾ (protein kinase) varied in the different hosts and non-hosts after inoculation by *R. solani* [24]. The wild rice accessions *O. rufipogon* were showed one extra allele expression after 48HAI of inoculation. On the other hand, non-host maize cv. Kanchan amplified a single band which was down regulated in most of the time of inoculation. The analysis revealed the core disease resistance biomolecules and gene network operating in *R. solani* infection in both the studies wild rice accession *O. rufipogon* and non-host maize cv. Kanchan.

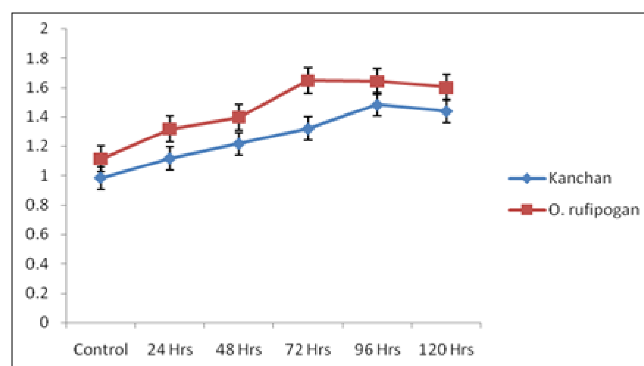

Fig1: Biochemical assay of catalase (CAT) and peroxidase (PO) after infection with *R. solani* in *Oryza rufipogan* and maize cv. Kanchan

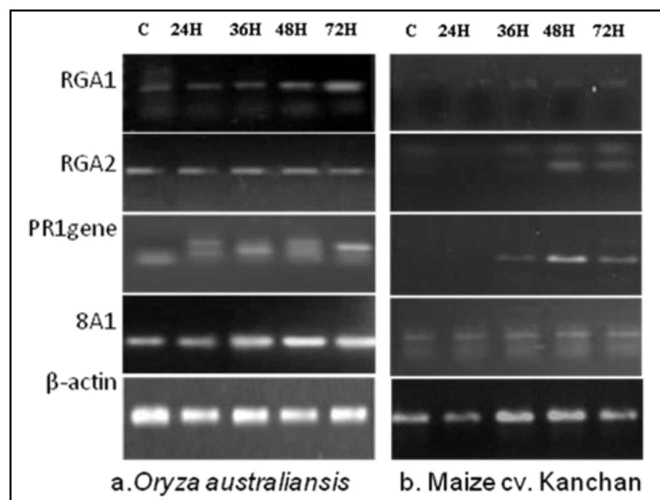

Fig 2: PCR products of different RGA (resistance gene analogue) amplified by primer pair on 1.5% agarose gel

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