**A STUDY OF DEFENSIVE ENZYMES AGAINST LEAF RUST
(*PUCCINIA TRITICINA* ERIKS) INFECTION AND MOLECULAR
SCREENING FOR LEAF RUST RESISTANT GENES IN DICOCCUM
WHEAT (*TRITICUM DICOCCUM*) GENOTYPES**

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ABSTRACT

Wheat leaf rust caused by *Puccinia triticina* Eriks is the most destructive and prevalent rust disease among global wheat cultivars. The best early prevention method for avoiding rust diseases or minimizing their impact is to choose a variety with known resistance. In our present study, four dicoccum wheat genotypes DIC GPM 66 and DIC GPM 64 (susceptible), HW 1098 and DDK 1029 (resistant) were screened for leaf rust resistance genes such as Lr 28, Lr 46 and Lr 68. Antioxidative and nitrogen assimilatory enzymes activity were studied in healthy and leaf rust inoculated leaves. Lr 28, Lr 46 and Lr 68 genes were present in HW 1098 and DDK 1029. The activity of antioxidative enzymes such as superoxide dismutase, catalase, peroxidase, phenyl ammonia lyase activity was found higher in treated leaves compared to healthy leaves and resistant genotypes showed higher activity compared to susceptible genotypes, suggesting that higher activity was induced to detoxify the ROS production during leaf rust infection that made the plant to protect from oxidative damage in case of resistant genotypes. Minimum decrease in the nitrate and nitrite reductase activity was observed in case of resistant genotypes compared to susceptible genotypes, that shows susceptible genotypes lack the ability to maintain the nitrogen metabolism under leaf rust infection. Therefore, our study revealed the significant difference in defense related enzyme between resistant and susceptible genotypes along with molecular screening could be the promising tool for selecting the resistant cultivar.

KEYWORDS: Dicoccum Wheat, Antioxidant Enzymes & Molecular Markers

Original Article

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INTRODUCTION

Wheat is the world's most cultivated cereal food crop is known as "king of cereals" as its cultivation is easier, ecologically suitable and contain high amount of nutrients [32]. Three different wheat species cultivated in different parts of the world are *Triticum aestivum*, *Triticum durum* and *Triticum dicoccum*, which is mainly grown in Morocco, Spain, the Carpathian Mountains, on the border of the Czech and Slovak republics, Albania, Turkey, Switzerland, and Italy. However, in India and Italy, its cultivation is well established and expanding. In India, it is produced in the states of Maharashtra, Karnataka, Gujarat, and in some parts of Andhra Pradesh and Tamil Nadu [11]. There is a renewed interest in dicoccum wheat largely by producers and consumers because of its nutritional value and high therapeutic properties due to increased dietary fiber that effectively reduce the cardiovascular risk factors. The pyrolysis fragments derived from the polysaccharide fraction were significantly more abundant in

dicoccum than in other species [8].

Diseases are the major threats to wheat production, leaf rust (*Puccinia triticina* Eriks) is one of the most devastating and persisting disease causing yield losses greater than 50 per cent [14]. The best early prevention method for avoiding rust diseases or minimizing their impact is to choose a variety with known resistance. For which, identification of sources of resistance and a better understanding of the mechanisms involved in defense to *P. triticina* infection are necessary.

Upon pathogen invasion, an oxidative burst is one of the most rapid defense reactions elicited in the plant, which in turn leads to the transient production of high levels of reactive oxygen species (ROS) that include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH). In order to avoid deleterious effect of ROS, the plant has evolved efficient scavenging system. Such a system involves both enzymatic and nonenzymatic antioxidants. The enzymatic protective mechanism operates by sequential and simultaneous activation of several antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione reductase (GR) and polyphenol oxidase (PPO). Generally, the levels of ROS and the extent of oxidative damage depend largely upon the level of coordination among the ROS scavenging enzymes [7].

It has been reported that DNA markers that co-segregate with the resistant gene are a powerful method to accelerate development of a resistant cultivar. More than 120 resistance genes for leaf, stem and stripe rust have been found in wheat [15]. Molecular markers such as STS or SCAR and CAPS are available for leaf rust resistance genes *Lr1*, *Lr9*, *Lr10*, *Lr19*, *Lr21*, *Lr24*, *Lr 25*, *Lr28*, *Lr29*, *Lr34*, *Lr35*, *Lr37*, *Lr39*, *Lr47* and *Lr51*. SSR and AFLP markers are available for some *Lr* genes such as *Lr18*, *Lr40*, *Lr46* and *Lr50*. Screening of wheat genotypes for leaf rust resistance using gene specific molecular markers has prime importance for selecting rust resistant cultivars.

In this study, the genomic DNA of two resistant and two susceptible genotypes were amplified using SSR and STS markers. Defense related enzymes such as superoxide dismutase, catalase, peroxidase, phenyl ammonia lyase, nitrate reductase and nitrite reductase activity was studied for better understanding of resistance mechanism involved against *P. triticina* in dicoccum wheat genotypes.

MATERIALS AND METHODS

Plant Material and Experimental Design

In the present study, four dicoccum wheat genotypes HW 1098, DDK 1029 (resistant) and DIC GPM 66 and DIC GPM 64 (susceptible) were grown in separate control and inoculated plots and followed a randomized complete block design with three replications [21]. To ensure the maximum disease pressure, the artificial inoculation was done by spraying with rust inoculums on the 45 days old plants and sample was collected after 48 hours of infection for determining activities of SOD, CAT, POX, PAL, NR and NiR.

Extraction of Enzyme and Determination of Protein Content

Enzyme extract was prepared by grinding 0.5 g of healthy and infected leaf sample with liquid nitrogen and extracted with 0.05M sodium phosphate buffer of pH 7.8 and pH 7.0 for SOD and CAT respectively. POX were extracted in 0.1 M potassium phosphate buffer (pH 7.0). Grinding buffer for GR included 0.1M Tris-HCl pH 7.8 and 2mM dithiothreitol (DTT). PAL was extracted in 0.1 M Tris buffer (pH 8.5). Nitrite reductase (NiR) enzyme extracts was prepared in 0.1 M phosphate buffer, pH 7.5, containing 10 mM cysteine. Then homogenate was centrifuged at 14,000 rpm

for 20 min at 4 °C, the supernatant was used as enzyme source for assay. Protein content was estimated using Lowry's method.

Assay of Defense Related Enzymes

Superoxide Dismutase

The activity of SOD (EC 1.15.1.1) in dicoccum wheat leaves was assayed photochemically at 560 nm by the Beauchamp and Fridovich method [2]. 3 mL of reaction mixture contained 20 µL of enzyme extract, 10 mM of L-methionine, 33 µM of p-nitrobluetetrazolium chloride, 0.66 µM of ethylenediaminetetraacetic acid (EDTA), 3.3 µM of riboflavin in a 50mM potassium phosphate buffer of pH 7.8. The assay was initiated by adding riboflavin and took place in a glass tube illuminated by a 15W fluorescent lamp at 25°C for 20 minutes. The increase in absorbance of the blue formazan produced by NBT photo reduction was measured at 560 nm. A blank was maintained with all the constituents but in the dark. One unit of SOD is defined as the amount of enzyme required to inhibit 50% of the NBT photo-reduction per minute and specific activity is expressed as IU per mg protein.

Catalase

CAT (EC 1.11.1.6) activity in dicoccum wheat leaves was spectrophotometrically determined by Beers and Sizess method [3]. The reaction mixture contained 2.98 mL of 16.65 mM hydrogen peroxide in 50mM phosphate buffer, pH 7.0 and 20µL of enzyme extract was used to initiate the reaction. The decrease in absorbance at 240 nm was measured for 5 minutes using the substrate blank. One unit of CAT is defined as the one µmole of H₂O₂ decomposed per minute at pH 7.0 at 25°C and specific activity was expressed as µmole min⁻¹ mg⁻¹ protein.

Peroxidase Activity

The peroxidase (POX, EC 1.11.1.7) activity in dicoccum wheat leaves was spectrophotometrically determined [4]. 20 µL of the enzyme extract was added to reaction mixture consisting 2.88 mL of 0.1 M potassium phosphate buffer (pH 7.0), 50 µL of 0.02 M guaiacol, 50 µL of 0.042 % H₂O₂ and increase in optical density was measured at 436 nm for 5 minutes. One unit of POX is defined as the amount of enzyme which catalyses the formation of one micromole of oxidized guaiacol per minute at 25°C and the specific activity was expressed as µmole min⁻¹ mg⁻¹ protein.

Glutathione Reductase

GR (EC 1.8.1.7) activity in dicoccum wheat leaves was determined spectrophotometrically by Mavis and Stellwagen method [22]. The reaction mixture contained 100 µL of 30 mM oxidized glutathione, 1.5 mL of 100 mM potassium phosphate buffer with 3.4 mM EDTA, pH 7.6, 350 µL of 0.8 mM β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and 950 µL of water. The decrease in absorbance at 340 nm on addition of 100 µL of enzyme to reaction mixture was recorded for 5 minutes. One GR unit is defined as the amount of enzyme that oxidises 1.0 µmole of NADPH per minute at pH 7.6 at 25°C and specific activity is expressed as µmole min⁻¹ mg⁻¹ protein.

Phenylalanine Ammonia Lyase

The PAL (EC.4.3.1.5.) activity in dicoccum wheat leaves was determined spectrophotometrically by the method of Paltonen and Karjalainen [26]. Assay mixture included 0.5 ml of enzyme extract and 2.5 mL of 0.2% L- phenylalanine in 0.1 M Tris buffer (pH 8.5) which was incubated at 40 °C for 1 hr. The reaction was stopped by 0.2M HCl and the

absorbance was measured at 290 nm at periodic 5 min intervals for 30 minutes against substrate blank. One unit of PAL is defined as the amount of enzyme required to liberate 1 micromole of transcinna~~m~~te from L-Phenylalanine per minute and the specific activity was expressed as $\mu\text{mol}/\text{min}/\text{mg}$ protein.

Nitrate Reductase Assay

NR (EC 1.7.1.1) activity in dicoccum wheat leaves was spectrophotometrically determined by the method of Hageman and Reed [13]. A known weight (0.5 g) of fresh tissue was cut into pieces and suspended in screw cap vials containing 3.5 ml of incubation mixture (20 ml of 0.1M phosphate buffer, 20 ml of 5 per cent propanol and 10 ml of 0.2 per cent KNO_3). The vials were sealed and kept in dark condition at 30°C for 2h. Nitrite released into the medium was determined by treating 1 ml aliquot with 1 ml each of 1 % sulphanyl amide and 0.02 % N-1- naphthyl ethylene diamine hydrochloride. After 20 min, solution is diluted to 5 ml with water and absorbance is measured at 540 nm. Standard curve is prepared by using reagent grade concentrations of nitrite (KNO_2) solution. The nitrate reductase activity is expressed as nmoles of nitrite formed per hour per gram fresh weight.

Nitrite Reductase Assay

NiR (EC 1.7.7.1) activity in dicoccum wheat leaves was determined spectrophotometrically by the method of Wray and Fido [33]. The 0.8 ml of reaction mixture contained 0.2 ml of 0.1M phosphate buffer, 0.1 ml of 5mM sodium nitrite, 0.1ml of 1.5 mM methyl viologen, 50 μl of enzyme and distilled water. The reaction was started by adding 0.2 ml of the 2.5% sodium dithionite reagent and incubated for 10 minutes. The reaction was stopped by vigorously shaking the mixture until the dithionite was completely oxidized and the dye colour disappeared. For determination of nitrite consumed by enzyme 50 μl aliquot of above mixture was made to 2.0 ml using distilled water and 1.0 ml of 1 % sulphanilamide followed by 1 ml of 0.02 % NNED was added and incubated for 15 minutes. Blank was also processed in the similar way except for 50 μl of enzyme was added after the addition of sulphanilamide and NNED and read at 540 nm. The nitrite consumed by the action of enzyme was estimated from the nitrite standard curve. NiR activity is expressed as μmol of nitrite consumed /min and the specific activity as enzyme activity/mg protein.

DNA Isolation and PCR Protocol

The DNA was extracted from the dicoccum wheat genotypes by following CTAB (N, N, N, N-Cetyl Trimethyl Ammonium Bromide) [6], extraction method with few modifications as described. 0.1g fresh leaves from 6-8 days old seedlings were collected. The sample was grinded to fine powder in liquid nitrogen with micro pestle in 2 ml centrifuge tube. 1ml extraction buffer (10 % CTAB, 1M Tris base, 4M NaCl) added to the powdered sample and mixed properly. Samples were incubated for 30 min at 65°C in water bath with intermittent mixing. Equal volume of chloroform isoamyl alcohol (24:1 v/v) was added and gently agitated for 10 min to form an emulsion. The tubes were centrifuged for 10 min at 10,000 rpm at room temperature. The supernatant was transferred to sterile tubes and 1ml chilled isopropanol was added to each of tube, mixed by inverting and incubated at -20°C over night. The contents were centrifuged again for 10 min with 10,000 rpm at 4°C and the pellet retained by discarding the supernatant. The DNA pellet obtained was washed with 70 per cent ethanol and tubes were inverted on blotting paper to dry the pellet. Later, DNA was suspended in 50 μl T_{10}E_1 (10 mM Tris HCl, 1mM EDTA) buffer and stored at -20°C .

PCR reactions were carried out for the genomic DNA with the total reaction mixture of 20 μL consisting of 2.0 μL of 40 ng genomic DNA, 2 μL of 10X Taq buffer, 1 μL of 10 mM dNTPs, 1.0 μL each of forward and reverse primers (5

pmoles), 0.20 µL of 3U Taq DNA polymerase and 12.75 µL sterile distilled water. Total reaction mixture was subjected to PCR amplification with the cycling parameters of: 95°C for 2 minutes followed by 94°C for 1 minute, annealing temperature (table 1) for 45 seconds, 72°C for 60 seconds for 36 cycles and final extension at 72°C for 4 minutes. The PCR product was mixed with 2 µL of loading dye (Bromophenol blue) and loaded in 2 per cent agarose gel of 1x TAE buffer containing ethidium bromide. Gel was run at 90 volts. The gel was photographed by using gel documentation system.

RESULTS

Superoxide Dismutase (SOD)

Superoxide dismutase activity in the leaves of dicoccum wheat genotype differed significantly in control and 48 hr after inoculation. Activity of SOD in all the genotypes was significantly increased in inoculated leaves (11.38 U/mg protein) as compared to control (6.30 U/mg protein). In case of treatment, inoculated leaves of resistant HW 1098 and DDK 1029 genotypes recorded highest SOD activity (13.62 U/mg protein and 14.32 U/mg protein respectively) as compared to control leaves (7.40 U/mg protein and 8.66 U/mg protein respectively). Inoculated leaves of susceptible DIC GPM 66 and DIC GPM 64 genotypes showed least SOD activity (9.52 U/mg protein and 8.08 U/mg protein respectively) as compared to control leaves (5.33 U/mg protein and 3.83 U/mg protein respectively).

Catalase

Catalase activity in the leaves of wheat genotype differed significantly in control and 48 hr after inoculation. Levels of catalase activity in all the genotypes were significantly increased in inoculated leaves (21.55 U/mg protein) as compared to control (11.47 U/mg protein). In case of treatment, inoculated leaves of resistant HW 1098 and DDK 1029 genotype recorded Catalase activity (19.00 U/mg protein and 35.60 U/mg protein respectively) as compared to control leaves (11.46 U/mg protein and 18.46 U/mg protein respectively). Inoculated leaves of susceptible DIC GPM 66 and DIC GPM 64 genotypes showed least catalase activity (14.70 U/mg protein and 16.91 U/mg protein respectively) as compared to control leaves (9.43 U/mg protein and 6.53 U/mg protein respectively).

Peroxidase (POX)

Peroxidase activity in the leaves of wheat genotype differed significantly in control and 48 hr after inoculation. Levels of peroxidase in all the genotypes were significantly increase in inoculated leaves (0.40 U/mg protein) as compared to control (0.20 U/mg protein). In case of treatment, inoculated leaves of resistant genotype HW 1098 and DDK 1029 recorded highest peroxidase activity (0.53 U/mg protein and 0.51 U/mg protein respectively) as compared to control leaves (0.21 U/mg protein and 0.19 U/mg protein respectively). Inoculated leaves of susceptible genotypes DIC GPM 66 and DIC GPM 64 showed least peroxidase activity (0.23 U/mg protein and 0.31 U/mg protein respectively) as compared to control leaves (0.13 U/mg protein and 0.16 U/mg protein respectively).

Glutathione Reductase (GR)

Glutathione reductase activity in the leaves of dicoccum wheat genotype differed significantly in control and 48 hr after inoculation. Levels of Glutathione reductase in all the genotypes were significantly increased in inoculated leaves (1.51 U/mg protein) as compared to control (2.14 U/mg protein). In case of treatment, inoculated leaves of resistant HW 1098 and DDK 1029 genotype recorded highest Glutathione reductase activity (2.42 U/mg protein and 3.01 U/mg protein

respectively) as compared to control leaves (1.56U/mg protein and 1.91 U/mg protein respectively). Inoculated leaves of susceptible DIC GPM 66 and DIC GPM 64 genotypes showed least glutathione reductase activity (1.78 U/mg protein and 1.33 U/mg protein respectively) as compared to control leaves (1.36 U/mg protein and 1.24 U/mg protein respectively)

Phenylalanine Ammonia Lyase (PAL)

PAL activity in the leaves of wheat genotype differed significantly in control and 48 hr after inoculation. Levels of PAL activity in all the genotypes were significantly increased in inoculated leaves (4.00 U/mg protein) as compared to control (2.59 U/mg protein). In case of treatment, inoculated leaves of resistant HW 1098 and DDK 1029 genotype recorded highest PAL activity (4.80 U/mg protein and 5.12 U/mg protein respectively) as compared to control leaves (2.51 U/mg protein and 3.08 U/mg protein respectively). Inoculated leaves of susceptible DIC GPM 66 and DIC GPM 64 genotypes showed least PAL activity (3.52 U/mg protein and 2.57 U/mg protein respectively) as compared to control leaves (2.83 U/mg protein and 1.92 U/mg protein respectively).

Nitrate Reductase

Nitrate reductase activity in the leaves of wheat genotype differed significantly in control and 48 hr after inoculation. Levels of nitrate reductase activity in all the genotypes were significantly lower in inoculated leaves (0.66 U/mg protein) as compared to control (1.02 U/mg protein). In case of treatment, inoculated leaves of resistant HW 1098 and DDK 1029 genotype recorded less difference in nitrate reductase activity (0.92 U/mg protein and 1.07 U/mg protein respectively) as compared to control leaves (1.16 U/mg protein and 1.41 U/mg protein respectively). Inoculated leaves of susceptible DIC GPM 66 and genotypes DIC GPM 64 genotypes showed significant difference in nitrate reductase activity (0.47 U/mg protein and 0.17 U/mg protein respectively) as compared to control leaves (0.62 U/mg protein and 0.88 U/mg protein respectively).

Nitrite Reductase

Nitrite reductase activity in the leaves of wheat genotype differed significantly in control and 48 hr after inoculation. Activity of nitrite reductase in all the genotypes was significantly lower in inoculated leaves (0.23 U/mg protein) as compared to control (0.54 U/mg protein). In case of treatment, inoculated leaves of resistant HW 1098 and DDK 1029 genotype recorded least nitrite reductase activity (0.28 U/mg protein and 0.36 U/mg protein respectively) as compared to control leaves (0.65 U/mg protein and 0.58 U/mg protein respectively). Inoculated leaves of susceptible DIC GPM 66 and DIC GPM 64 genotypes showed highest nitrite reductase activity (0.14 U/mg protein and 0.16 U/mg protein respectively) as compared to control leaves (0.44 and 0.50 respectively).

To Screen for Leaf Rust Resistance Genes through Gene Specific Markers

The genomic DNA of two leaf rust resistant (HW 1098 and DDK 1029) and susceptible (DIC GPM 66 and DIC GPM 64) genotypes were amplified using above mentioned markers. In amplification pattern, it was observed that *Xwmc44* marker with allele size of 220 bp was present in HW 1098 and DDK 1029 and 200bp allele was present in DIC GPM 66 and DIC GPM 64. It showed that HW 1098 and DDK 1029 contained *Lr46* gene and DIC GPM 66 and DIC GPM 64 were devoid of *Lr46* gene.

Wmc 313 marker with fragment size of 320 bp was present in HW 1098 and DDK 1029 and with fragment size of 309 bp was present in DIC GPM 66 and DIC GPM 64. It showed that *Lr28* gene was present in HW 1098 and DDK 1029

Marker *csGS* with fragment size of 385 bp was present in HW 1098 and DDK 1029 and absent in DIC GPM 66 and DIC GPM 64, showing that *Lr68* was present in HW 1098 and DDK 1029 and absent in DIC GPM 66 and DIC GPM 64.

DISCUSSIONS

PCR based markers have been widely used to screen, characterize and evaluate genetic diversity in cereal species. Markers like STS (sequence tagged site), RAPD (randomly amplified polymorphic DNA), SSR (simple sequence repeats) and others have been proved useful for identifying resistance among different genotypes. Various researchers have utilized these markers for the investigation of wheat cultivars for different traits. In the present investigation four markers were used to screen for leaf rust resistance genes viz. *Lr46*, *Lr68* and *Lr28* in two susceptible and two resistant genotypes.

Leaf rust resistance gene *Lr28* located on chromosome 4AL of wheat provide higher resistance with other resistance genes. A microsatellite marker *wmc313* linked to *Lr28* at a distance of 5.0 cM was used for screening of wheat genotypes. HW 1098 and DDK 1029 showed presence of this gene carrying fragment size of 320bp and DIC GPM 64 and DIC GPM 66 are devoid of this gene carrying fragment size of 309bp.

It was reported that *Lr68* carrying lines had lower severities to leaf rust infection [8], *Lr68* located on chromosome 7BL, for this marker *csGS* linked at a distance of 1.2cM was used for screening. The amplification was observed only in DDK 1029 with fragment size of 385bp, showing that it was present in DDK 1029 and HW 1098 genotypes and absent in DIC GPM 66 and DIC GPM 64 genotypes.

Gene *Lr46* is adult plant resistant gene, involved in a resistance response of fewer and smaller uredinia, but with varying amounts of chlorosis in adult plants [21]. *Lr46* gene located on chromosome 1B, was screened by using marker *Xwmc 44*. The amplification was observed in all four genotypes, HW 1098 and DDK 1029 carrying fragment size of 242bp showing that *Lr46* gene was present in these two genotypes and DIC GPM 64 and DIC GPM 66 carried fragment size of 200b showing absence of the *Lr46* gene.

It was observed that genotype DDK 1029 and HW 1098 showed positive result for the presence of leaf rust resistant genes and also these genotypes showed well built antioxidative enzyme mechanism and higher lignin and phenol content after inoculation with *Puccinia triticina* compared to the DIC GPM 64 and DIC GPM 66. It provides better understanding of mechanism involved at molecular level in developing resistance to leaf rust disease.

Changes in the Activity of Stress Response Coccum Wheat under Leaf Rust Infection (*Puccinia Triticina*) Superoxide Dismutase

The enzyme SOD is metalloenzyme, constitutes the first line of defence against ROS by catalyzing the dismutation of O_2^- to O_2 and H_2O_2 [10]. As per the results obtained (Table 1) among all the wheat genotypes, DDK 1029 recorded highest activity in the control and inoculated leaves, where as lowest SOD activity was observed in DIC GPM 64 in the control and inoculated leaves. Leaf rust induced increase in SOD activity in leaves of both resistant and susceptible genotypes. SOD activity in the leaves in HW 1098 and DDK 1029 genotypes was higher in control compared to susceptible genotypes and increased (83.99 % and 65.26 % respectively) under inoculation. Susceptible genotypes DIC

GPM 66 and DIC GPM 64 showed low SOD activity in control and significantly increased (78.63 % and 110.94 % respectively) upon inoculation of *Puccinia triticina*. Similar results were observed in the study conducted by Anushree, *et al.* [1] in resistant and susceptible rice genotypes for rice blast disease, where SOD activity was significantly induced in infected leaf of both resistant and susceptible rice genotypes suggesting that their activation might be due to generation of ROS there by activating other defence cascade or display direct toxicity toward invading pathogen [32].

Catalase

Catalases are tetrameric heme containing enzymes with the potential to dismutate H_2O_2 into H_2O and O_2 . Up to a certain level of H_2O_2 production under stress may work as a signal for triggering defence responses via transduction pathways, which have H_2O_2 as secondary messenger [23] but high concentration of H_2O_2 leads to cell death. H_2O_2 which also resulted from the action of SOD is toxic to cells. Therefore, it is important that H_2O_2 be scavenged rapidly by the CAT to water and oxygen [12]. This excess H_2O_2 removed by catalase enzyme and provides protection against cellular damage.

As per the results obtained (Table 2) among all the wheat genotypes, DDK 1029 recorded highest activity in the control and inoculated leaves, where as lowest catalase activity was observed in DIC GPM 64 in the control leaves. Leaf rust induced increase in catalase activity in leaves of both resistant and susceptible genotypes. Catalase activity in the leaves in HW 1098 and DDK 1029 genotypes was higher in control compared to susceptible genotypes and increased (65.74 % and 92.80 % respectively) under inoculation. Susceptible genotypes DIC GPM 66 and DIC GPM 64 showed low SOD activity in control and significantly increased (55.97 % and 158.95 % respectively) upon inoculation with *Puccinia triticina*.

Similar results were observed in the study conducted in stripe rust resistant and susceptible wheat genotypes, where catalase activity was significantly increased in resistant genotypes compared to susceptible genotypes after stripe rust infection [5]. The same trend also followed in the study conducted in rice genotypes where catalase activity was increased stably in both mutant and wild type cultivar, the relative catalase activity was significantly higher in the Pooya (mutant variety) than in the Mosatarom (wild type cultivar) after inoculated with *Magnaporthe oryzae* [7]. It was observed that higher catalase activity is associated with resistance of the plant to leaf rust infection.

Peroxidase (POX)

Peroxidase (POX) is a heme containing protein which preferably oxidizes aromatic electron donor such as guaiacol and pyragallol at the expense of H_2O_2 . POX catalyses conversion of cinnamyl alcohol to lignin by oxidative polymerization [17]. Vascular plant posses several genes encoding different types of POXs, and there might be some distinct physiological function for each class in protecting the cell membrane against oxidative damage, which occur in plant under biotic and abiotic stresses [20].

As per the results obtained (Table: 3) among all the wheat genotypes, HW 1098 recorded highest activity in the control and inoculated leaves, where as lowest POX activity was observed in DIC GPM 66 in the control leaves. Leaf rust induced increase in POX activity in leaves of both resistant and susceptible genotypes. POX activity in the leaves in HW 1098 and DDK 1029 genotypes was higher in control compared to susceptible genotypes and increased significantly (152.3 % and 194.7 % respectively) under inoculation. Susceptible DIC GPM 66 and DIC GPM 64 show low SOD activity in control and increased (76.92 % and 93.75 % respectively) upon inoculation of *Puccinia triticina*.

Some studies reported that POX shows antifungal activity against a variety of fungal species, including *Trichosporium vesiculosum*, *Macrophomina phaseolina*, *Coprinus comatus*, *Mycophaearella arachidicola*, *Fusarium*

oxysporium and *Botrytis cinerea* [35]. This increase in POX activity in resistant wheat genotypes may suggest a possible role of POX in lignin biosynthesis to restrict pathogen infection. POX could use H_2O_2 as a substrate for the oxidative polymerisation of hydroxycinnamyl alcohol monolignols in the phenylpropanoid pathway to yield lignin, similar study where *Gossypium barbadense* cv. 71224 (resistant) and *G. Hirsutum* cv. YZ-1 (susceptible) were infected with the highly aggressive defoliating fungus *verticillium dahlia* strain V991, the resistant plant accumulate higher level of POX activity than susceptible in both leaves and root [34].

Glutathione Reductase (GR)

GR is a flavo-protein oxidoreductase, plays an essential role in defence system against ROS by sustaining the reduced status of GSH. It is localized predominantly in chloroplasts, but small amount of this enzyme has also been found in mitochondria and cytosol. GR catalyses the reduction of glutathione disulphide (GSSG) to the sulphhydryl form GSH. This enzyme employs NADPH as a reductant (Shigeoka *et al.*, 2002).

As per the results obtained (Table: 4) among all the wheat genotypes, DDK 1029 recorded highest activity in the control and inoculated leaves, where as lowest GR activity was observed in DIC GPM 64 in the control leaves. Leaf rust induced increase in GR activity in leaves of both resistant and susceptible genotypes. GR activity in the leaves in HW 1098 and DDK 1029 genotypes was higher in control compared to susceptible genotypes and increased significantly (55.11 % and 57.79 % respectively) under inoculation. Susceptible DIC GPM 66 and DIC GPM 64 show low GR activity in control and increased but not significantly (30.88 % and 7.70 % respectively) upon inoculation with *Puccinia triticina*.

This suggests that plant species use different enzymes as primary defences against ROS and these may require different regulatory systems. Similar result shown by Kumar *et al.* [16] in their studies on *Piriformospora indica* against *fusarium verticillioides*, GR activities were found to be higher in *F. verticillioides* colonized plants than in noncolonized plants. The changes in the levels of a particular GR isoform may be of more significance than changes in total GR activity in stressed plants.

Phenylalanine Ammonia Lyase (PAL)

Phenylalanine ammonia lyase is the key enzyme in the plant phenyl propanoid pathway catalyzing synthesis of secondary metabolites from L-phenylalanine including lignin, flavanoid and phytoalexins. Phenylalanine ammonia-lyase catalyzes the nonoxidative deamination of L-phenylalanine to form trans-cinnamic acid and a free ammonium ion. The conversion of the amino acid phenylalanine to trans-cinnamic acid is the entry step for the channeling of carbon from primary metabolism into phenylpropanoid secondary metabolism in plants.

As per the results obtained (Table 5) among all the wheat genotypes, DDK 1029 recorded highest activity in the control and inoculated leaves, where as lowest PAL activity was observed in DIC GPM 64 in the control leaves. Leaf rust induced increase in PAL activity in leaves of both resistant and susceptible genotypes. PAL activity in the leaves in HW 1098 and DDK 1029 genotypes was higher in control compared to susceptible genotypes and increased significantly (91.31 % and 65.86 % respectively) under inoculation. Susceptible DIC GPM 66 and DIC GPM 64 show less PAL activity in control and increased but not significantly (24.13 % and 33.54 % respectively) upon inoculation with *Puccinia triticina*.

It was reported that increased PAL activity in wheat leaves infected with *Botrytis cinerea* which was succeeded by lignin deposition at the site of infection. Peltonen and Karjalainen [26] observed increase in PAL activity in resistant barley

and wheat, in response to *Bipolaris sorokiniana*.

Nitrate Reductase (NR)

The primary role of nitrate reductase in plants is nitrogen assimilation, through the NAD(P)H dependent reduction of nitrate to nitrite, which is subsequently reduced to ammonium by the nitrite reductase. The ammonium is then incorporated into amino acids and other nitrogen-derived compounds through the glutamine synthetase/glutamine-2-oxoglutarate transaminase system [18].

As per the results obtained (Table: 6) among all the wheat genotypes, DDK 1029 recorded highest NR activity in the control and inoculated leaves, where as lowest NR content was observed in DIC GPM 66 control leaves. Leaf rust induced decrease in NR activity in the leaves. HW 1098 and DDK 1029 genotypes showed higher NR activity in control and decreased (20.44 % and 23.94 % respectively) under inoculation. Susceptible DIC GPM 66 and DIC GPM 64 showed significant decrease in NR activity (53.65 % and 60.4 % respectively) under inoculation.

In resistant genotypes decrease in nitrate activity is significantly lesser than susceptible genotypes and may suggests better nitrogen assimilation mechanism, similar result was obtained by Sarsenbaev *et al.* [27] in their studies on effect of leaf rust infection on the activity of molybdenum-containing enzymes nitrate reductase (NR) of the spring wheat varieties. It was shown that the NR activity decreases in the beginning of the infection process and then increases during progression of leaf rust infection process, up to the time occurrence of necrotic spots in the leaves it decreases again.

Nitrite Reductase (NiR)

The primary role of Nitrite reductase in plants is reduction of nitrite to ammonium. The ammonium is then incorporated into amino acids and other nitrogen derived compounds through the glutamine synthetase/glutamine-2-oxoglutarate transaminase system [18].

As per the results obtained (Table: 6) among all the wheat genotypes, HW 1098 recorded highest NiR activity in the control and inoculated leaves, where as lowest NiR content was observed in DIC GPM 66 control and inoculated leaves. Leaf rust induced decrease in NiR activity in the leaves. HW 1098 and DDK 1029 genotypes showed higher NR activity in control and decreased (57.18 % and 37.95 % respectively) under inoculation. Susceptible DIC GPM 66 and DIC GPM 64 showed significant decreased in NiR activity (67.47 % and 67.48 % respectively) under inoculation.

Nitrite is a particularly interesting and strong candidate for feedback regulation because it does not accumulate in the plant cells due to its toxicity and the rate limiting nature of NR [24]. Coordinated regulation of NR and NiR also ensures that nitrite is not accumulated in the cell [25]. The reduction of nitrite to ammonium requires ferredoxin (a product of photosynthesis). An abrupt decrease in photosynthesis would limit further assimilation and could lead to the accumulation of nitrite unless NR activity was down regulated. It was shown that the NR activity decreases in the beginning of the infection process and then increases during progression of leaf rust infection process, up to the time occurrence of necrotic spots in the leaves it decreases again.

CONCLUSIONS

Overall study revealed that the higher amount of antioxidant enzyme and nitrogen assimilation enzymes activity play an important role in defence mechanism of plants against wheat leaf rust infection in case of resistant varieties. These biochemical factors and molecular screening could be novel tool that can be used for selection of resistance lines against

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Figure 1

Table 1: Details of the Primers used in the Study

Target Gene	Molecular Markers	Forward Primer Sequence	Reverse Primer Sequence	Marker Type	Annealing Temperature	Chromosome	Amplicon Size (bp)	Reference
<i>Lr46</i>	<i>Xwmc 44</i>	5'GGTCTTCTGGGCT TTGATCCTG3'	5'TGTTGCTA GGGACCCGT AGTGG3'	SSR	57	1B	242	Lilemo <i>et al.</i> (2008)
<i>Lr28</i>	<i>Wmc 313</i>	5'TAGCAGTAGCAG CAGCAGGA 3'	5'GCAAGGAA GAGTGTTC GCC3'	SSR	62	4AL	320	Foessel <i>et al.</i> (2012)
<i>Lr68</i>	<i>csGS</i>	5'AAGATTGTTCAC AGATCCATGTCA 3'	5'GAGTATTC CGGCTCAAA AAGG 3'	STS	59	7BL	385	Foessel <i>et al.</i> (2012)

Table 2: Activity of Superoxide Dismutase in Leaves of Different Dicocum Wheat Genotypes in Response to Leaf Rust Infection

Genotype	Superoxide Dismutase Units per mg Protein		
	Control	Inoculated	% Increase
DIC GPM 66 (S)	5.33	9.52	78.63
DIC GPM 64(S)	3.83	8.08	110.94
Mean (S)	4.58	8.80	92.14
HW 1098 (R)	7.40	13.62	83.99
DDK 1029 (R)	8.66	14.32	65.26
Mean (R)	8.03	13.97	74.63
Grand mean	6.30	11.38	84.26
	S. Em. $\pm$		C.D. @ 5%
Factor G	0.04		0.188
Factor T	0.063		0.27
G $\times$ T	0.03		0.13

G: Genotype; T: Treatment

R: Resistant genotype, S: Susceptible genotype

Table 3: Activity of Catalase in Leaves of Different Dicocum Wheat Genotypes in Response to Leaf Rust Infection

Genotype	Catalase Activity Units per mg Protein.		
	Control	Inoculated	% Increase
DIC GPM 66 (S)	9.43	14.70	55.97
DIC GPM 64(S)	6.53	16.91	158.95
Mean (S)	7.98	15.81	98.11
HW 1098 (R)	11.46	19.00	65.74
DDK 1029 (R)	18.46	35.60	92.80
Mean (R)	14.96	27.30	82.43
Grand mean	11.47	21.55	87.89
	S. Em. $\pm$		C.D. @ 5 %
Factor G	0.27		1.14
Factor T	0.38		1.62
G $\times$ T	0.18		0.77

G: Genotype; T: Treatment

R: Resistant genotype, S: Susceptible genotype

Table 4: Activity of Peroxidase in Leaves of Different Dicocum Wheat Varieties in Response to Leaf Rust Infection

Genotype	Peroxidase Units per mg Protein		
	Control	Inoculated	% Increase
DIC GPM 66 (S)	0.13	0.23	76.92
DIC GPM 64(S)	0.16	0.31	93.75
Mean (S)	0.145	0.27	86.2
HW 1098 (R)	0.21	0.53	152.3
DDK 1029 (R)	0.19	0.51	194.73
Mean (R)	0.4	0.52	173.51
Grand mean	0.20	0.39	129.56
	S. Em. $\pm$		C.D.@ 5 %
Factor G	0.06		0.027
Factor T	0.09		0.04
G $\times$ T	0.04		0.02

G: Genotype; T: Treatment

R: Resistant genotype, S: Susceptible genotype.

Table 5: Activity of Glutathione Reductase in Leaves of Different Dicoccum Wheat Genotypes in Response to Leaf Rust Infection

Genotype	Glutathione Reductase Units per mg Protein		
	Control	Inoculated	% Increase
DIC GPM 66 (S)	1.36	1.78	30.88
DIC GPM 64(S)	1.24	1.33	7.70
Mean (S)	1.30	1.56	19.85
HW 1098 (R)	1.56	2.42	55.11
DDK 1029 (R)	1.91	3.01	57.79
Mean (R)	1.73	2.71	56.59
Grand mean	1.51	2.14	40.83
	S. Em. ±		C.D. @ 5%
Factor G	0.21		0.91
Factor T	0.3.		1.3
G × T	0.14		0.61

G: Genotype; T: Treatment

R: Resistant genotype, S: Susceptible genotype

Table 6: Activity of Phenylalanine Ammonia Lyase in Leaves of Different Dicoccum Wheat Genotypes in Response to Leaf Rust Infection

Genotype	Phenylalanine Ammonia Lyase Units per mg Protein		
	Control	Inoculated	% Increase
DIC GPM 66 (S)	2.83	3.52	24.13
DIC GPM 64(S)	1.92	2.57	33.54
Mean (S)	2.38	3.04	27.93
HW 1098 (R)	2.51	4.80	91.31
DDK 1029 (R)	3.08	5.12	65.86
Mean (R)	2.80	4.96	78.58
Grand mean	2.59	4.00	53.56
	S. Em. ±		C.D. @ 5%
Factor G	0.39		1.66
Factor T	0.55		2.35
G × T	0.26		1.11

G: Genotype; T: Treatment

R: Resistant genotype, S: Susceptible genotype

Table 7: Activity of Nitrate Reductase in Leaves of Different Dicoccum Wheat Genotypes in Response to Leaf Rust Infection

Genotype	Nitrate Reductase Activity (U/mg Protein)		
	Control	Inoculated	% Decreased
DIC GPM 66 (S)	0.62	0.47	24.37
DIC GPM 64(S)	0.88	0.17	80.68
Mean (S)	0.75	0.32	57.52
HW 1098 (R)	1.16	0.92	20.44
DDK 1029 (R)	1.41	1.07	23.94
Mean (R)	1.28	1.00	22.35
Grand mean	1.02	0.66	35.35
	S. Em. ±		C.D. @ 5 %
Factor G	0.08		0.37
Factor T	0.12		0.53
G × T	0.05		0.25

G: Genotype; T: Treatment

R: Resistant genotype, S: Susceptible genotype

Table 8: Activity of Nitrite Reductase in Leaves of Different Dicoccum Wheat Genotypes in Response to Leaf Rust Infection

Genotype	Nitrite Reductase Activity (U/mg Protein)		
	Control	Inoculated	% Decreased
DIC GPM 66 (S)	0.44	0.14	67.47
DIC GPM 64(S)	0.50	0.16	67.48
Mean (S)	0.47	0.15	67.48
HW 1098 (R)	0.65	0.28	57.18
DDK 1029 (R)	0.58	0.36	37.95
Mean (R)	0.61	0.32	47.57
Grand mean	0.54	0.23	57.52
	S. Em. $\pm$		C.D. @ 5 %
Factor G	0.01		0.04
Factor T	0.014		0.06
G $\times$ T	0.06		0.03

G: Genotype; T: Treatment

R: Resistant genotype, S: Susceptible genotype