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Differential antioxidative responses of three different rice genotypes during bacterial blight infection

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Abstract

Using three rice genotypes exhibiting different disease symptoms towards bacterial blight (BB) disease, *O. longistaminata* (BB-highly resistant), IRBB21 (BB-resistant) and Pusa Basmati 1 (BB-sensitive), we investigated the variable antioxidant profile and oxidative damages resulting from bacterial blight infection to elucidate the antioxidative protective mechanism governing differential BB resistance. Rice genotypes were grown in growth chamber and after 45 days of transplantation in pots, they were inoculated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) isolate DX133 following the leaf clipping method. Different biochemical assays were performed with leaf samples collected on 5th day after infection (5th DAI), 10th DAI and 15th DAI. The results showed that *O. longistaminata* exhibited higher level of total chlorophyll (~4.42±0.09 mg/g fw) and carotenoid (~2.74±0.04 mg/g fw) pigments throughout the studied periods of infection when compared to other genotypes. At 5th DAI, the higher production of non-enzymatic antioxidants including total phenolic content (10.82±0.01 mg GAE/g fw) and flavonoid content (18.60±0.03 mg QE/G fw) was observed in highly resistant genotype *O. longistaminata*, compared to Pusa Basmati1 and IRBB21. The activity of catalase (CAT) was increased in all the three genotypes under BB infection. However, *O. longistaminata* showed highest CAT activity on later stages of infection (15th DAI). The level of total antioxidant and ferric reducing power (FRAP) increased in the infected plants compared to controls on the onset of infection.The present study clearly demonstrates higher level of antioxidative protection in the highly resistant wild genotype *O. longistaminata* and can significantly contribute to understand the physiological mechanisms in rice conferring BB resistance.

Keywords: Antioxidant enzymes, non-enzymatic antioxidants, Radical scavenging, *Oryza sativa*.
Abbreviations: BB-bacterial blight; DPPH-1, 1-diphenyl-2-picrylhydrazyl; FRAP-ferric reducing antioxidant potential; FW-fresh weight; GAE-gallic acid equivalents; GPX-Glutathione peroxidase; PWC-pot water holding capacity; ROS-reactive oxygen species; SWC- Soil water content; SOD-superoxide dismutase.

Introduction

Rice (*Oryza sativa* L.) is one of the most extensively cultivated food crop of the world, whose production is remain constrained by diseases of fungal, bacterial, viral and nematode origin. Bacterial blight (BB) of rice, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most noted and oldest recognized diseases of rice. BB is known to occur in epidemic proportions almost across the world and hampered productivity up to 80% (Singh et al., 1977). Plants encounter both biotic as well as abiotic stresses which adversely affect the growth and productivity. It also initiates a chain of anatomical, morphological, physiological, biochemical and molecular changes in plants. The chlorotic zone formed on the leaves of rice by bacterial blight lead to reduction in photosynthetic rate which inturn the yield loss in rice (Rajarajeswari and Muralidharan, 2006; Kumar et al., 2013). Plants produces singlet oxygen, perhydroxyl radical, hydroxyl radicals, hydrogen peroxide and alkoxy radical like reactive oxygen species upon encounter with biotic stresses. (Jaleel et al., 2006). The ROS generated following stresses reacts with proteins, lipids and DNA, causing oxidative damage and impairing the normal functions of cells (Wu et al., 2004). This effect of ROS is minimized in the plants by enzymatic and non-enzymatic detoxification systems and thereby protect the cells from oxidative damage. This protection is shown by a variety of antioxidant enzymes and lipid-soluble or scavenging molecules produced during different stresses (Walker et al., 1993). The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR) function in detoxification of superoxide and hydrogen peroxide (H_2O_2) (Asada et al., 1994). The primary scavenger is SOD, which converts O_2 to H_2O_2. The APX, dehydro ascorbate reductase (DHAR) and GR remove the toxic product of SOD reaction, (Manivannan et al., 2008). H_2O_2 is also scavenged by CAT though the enzyme which is less efficient than APOX-GR system (Zhao

et al., 2008). Cystein, reduced glutathione and ascorbic acid are components of non–enzymatic system (Jaleel et al., 2008a). In biotic stress tolerance, such as bacterial blight, high activities of antioxidant enzymes and high content of non-enzymatic constituents are important. Superoxide radicals and H_2O_2 are also scavenge by various POX and peroxiredoxins apart from CAT through ascorbate glutathione cycle. The production of enzymatic and non-enzymatic antioxidants during stress together causes the oxidative damage to plant tissue. Among them the most important include β-carotenes, ascorbic acid, α-tocopherol, GSH and enzymes including SOD, POX, APX, CAT, PPO and GR (Blokhina et al., 2003). Free radical-induced peroxidation of lipid membranes is reported to be a reflection of stress-induced impairment at the cellular level (Sankar et al., 2007). Different rice species exhibit variation in their sensitivity towards *Xoo* infection. Based on the phenotypic response of disease symptoms after *Xoo* infection, we had classified *Oryza longistaminata* (OL), IRBB21 and Pusa Basmati-1 (PB-1) into highly resistant (HR), resistant (R) and susceptible (S), respectively. Till date, not much is known about the BB resistance of these genotypes and their association with antioxidant profiles. There are very few reports regarding the difference in the biochemical responses of these cultivars to BB stress. At present, our ability to improve BB resistance is limited due to poor understanding of the stress physiology and their adaptation processes. Therefore, the aim of the present study was, to study various antioxidant constituents actively involved during BB disease and compare the antioxidant profile in three phenotypically variable rice genotypes towards *Xoo* infection and also to examine the relationship between imposition of BB stress and induction of oxidative stress in susceptible, resistant and highly resistant genotypes of rice.

Results

Effect of BB stress on photosynthetic pigments in three rice genotypes

Bacterial infection induces several physiological changes in rice leaves at different stages of infection. Bacterial blight disease led to decrease in Chl (a + b) content in all three genotypes due to lesion formation (Fig 1). The total chlorophyll content was significantly reduced in infected plants (Table 1). The change in total chlorophyll content of PB-1 was minimal at first two stages of infection, however, this genotype showed drastic reduction on 15^{th} DAI (1.53±0.10 mg/g fw). Almost similar trend was observed in IRRB21 wherein chlorophyll content depleted progressively, whereas in OL, we found that the cholorphyll content was maintained at the same level even at 15^{th} DAI. Similar trend was observed with that of carotenoid content in all the three genotypes.

Gradual decrease in total polyphenol and flavanoid content in infected rice

Leaf phenolics are important protective components of the plant system. In the present study, the phenolic contents were also found to be enhanced significantly due to BB disease. At the initial stages of infection, the higher level of non-enzymatic antioxidants such as phenolics and flavanoids were produced in highly resistant genotype OL, compared to PB-1 and IRBB21. Infected OL substantially produced double the amount of phenolics and flavonoids in comparison to control at 5^{th} DAI. During the progression of disease, the inhibitory effect of biotic stress on phenolic content was observed in all the three genotypes (Table 2). The phenolic content reduced dramatically at the later stage of infection in all the three genotypes. The highest rate of phenolic content reduced after infection being observed in OL (from 10.82±0.01 to 4.81±0.06 mg/g fw), followed by PB-1 (from 12.23±0.06 to 4.96±0.05 mg/g fw) and IRBB21 (from 11.28±0.05 to 4.31±0.04 mg/g fw). The flavonoid accumulations in infected plants were increased during the earlier stages of infection and subsequently declined at the later stages, thus flavonoids might play a protective role under stress conditions. Like phenolics, flavonoid content in OL showed highest production at the 5^{th} DAI (18.6±0.03 mg QE/g fw) in camparison to control plants (8.7±0.05 mg QE/g fw) and started to decrease and reached to (7.0±0.06 mg QE/g fw) at 15^{th} DAI. The other two genotypes, PB-1 and IRBB21 showed similar pattern (Table 2). The change in flavonoid content in stressed PB-1 was found 22.2±0.02 to 8.73±0.04 mg QE/g fw and in IRBB21, the reduction was from 19.1 ± 0.05 to 8.98±0.05 mg QE/g fw.

Total antioxidant activity shoot up during infection

Earlier studies have shown that stress in plants induce the oxidative burst followed by the activation of antioxidative system (Saleh et al., 2009). We conducted our studies on the antioxidative responses of rice genotypes to assess the redox potentials under BB stress condition. Our results showed that the total antioxidative capacity of three rice genotypes sharply increased during initial stage of stress period. The effect of BB stress on the activities of total antioxidants participating in the scavenging of ROS which showed gradual decrease in all the three genotypes is shown in Table 2. The level of total antioxidant and ferric reducing power increased in the infected plants in comparison to the control plants on the onset of infection. The maximum antioxidant level was observed in treated OL (16.19±0.02 mg AAE/g fw) compared to control (9.48 ±0.02 mg AAE/g fw). Similar patterns of ferric reducing power (increased activity during first stage followed by decreased activity at the later stages) were also observed for all the three genotypes (Table 2). OL was found to be more active as compared to other genotypes with maximum level of ferric reducing power value on 5^{th} DAI (10.09±0.02 mg AAE/g fw), while there was less significant change in the FRAP activity in other two genotypes, PB-1 and IRBB21, 9.23 ±0.02 and 10.48±0.01 mg AAE/g fw, respectively on the same day in comparison to control plants (8.53±0.06 and 9.16±0.01 mg AAE/g fw for PB-1 and IRBB21, respectively).

BB stress enhanced the accumulation of total free amino acid

The free amino acid pool did not change very much in control samples during the entire period of investigation,

Table 1. Bacterial blight stress induced changes in chlorophyll and carotenoid contents (mg/g fw) of three rice genotypes.

Sample	Chl (a+b)		Carotenoid	
	Untreated	Treated	Untreated	Treated
5th Day after Infection (5th DAI)				
OL	6.06±0.02	5.24±0.07*	3.09±0.07	3.03±0.03
PB-1	6.47±0.03	4.99±0.07*	3.08±0.05	2.99±0.04*
IRBB21	6.43±0.02	4.93±0.10*	3.08±0.02	3.00±0.04
10th Day after Infection (10th DAI)				
OL	5.43±0.08	5.00±0.09*	3.06±0.06	2.98±0.05
PB-1	5.66±0.08	4.81±0.08*	3.07±0.06	2.98±0.04
IRBB21	4.99±0.05	4.02±0.07*	3.03±0.08	2.65±0.03*
15th Day after Infection (15th DAI)				
OL	5.37±0.08	3.07±0.13*	3.04±0.04	2.22±0.06*
PB-1	5.62±0.08	1.53±0.10*	3.05±0.04	1.07±0.06*
IRBB21	5.83±0.08	2.21±0.07*	3.01±0.05	1.38±0.07*

Values are presented as mean of triplicate determinations ± standard deviation. (* = p≤0.001; ANOVA one way variance, Student's t test).

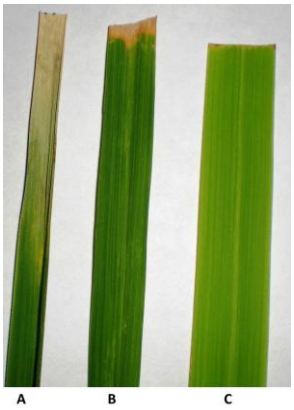

Fig 1. The effect of *Xoo* inoculation on the leaf blades of three rice genotypes (A) PB1; (B) IRBB21 and (C) *O. longistaminata* on 10th day after inoculation. Rice seedlings were inoculated with *Xoo* isolate DX133 by leaf clipping method.

while in stress induced plants, total free amino acid content increased with increasing time period of stress in all three genotypes particularly at 15th day of infection **(**Table 2). Mean TFA (total free amino acid) levels ranged from 21.82 to 29.21 µg/g fw at 15th DAI under stress conditions. The highest accumulation of the amino acid was recorded in PB-1 (29.21±0.25µg/g fw), followed by OL (26.89±0.78µg/g fw) and IRBB21 (21.82±0.48µg/g fw). During the early stages of stress, all the three genotypes evidenced the minimal accumulation of total free amino acids.

Higher DPPH free radical scavenging activityis related to resistance

Antioxidant potential of three genotypes was determined by employing 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging capacity of leaf tissues of plants grown under different days of treatments. The leaf tissues of OL under bacterial stress condition at the 5th DAI showed a significantly higher capacity to detoxify oxygen radicals (51.2±0.01%) over the antioxidant potential of the leaves of non-treated (18.06±0.01%). In other two genotypes, PB-1 and IRBB21, the activity was similar throughout the experimental period which shows that only OL had evident tendency to detoxify oxygen radicals to counter act the stress conditions (Fig 2).

Comparative scavenging activity of CAT and GPX

Catalase activity (CAT) was measured in three rice genotypes which steadily increased during infection period and highest activity was observed at later stage, *i.e*; 15th DAI (Fig 3). CAT activity increased significantly upon exposure to BB infection in all three genotypes. The highest activity was observed in IRBB21 at all time points followed by OL and PB-1 respectively. The peroxidase activity showed significant increase with regard to biotic stress in comparison to control plants (Fig 2). Peroxidase activity significantly increased with increasing stress period in OL at 10th DAI, however, slight decrease in activity was observed upon increasing exposure to bacterial stress. The resistant IRBB21 also showed an increased peroxidase activity with increasing stress. Similar results were observed in PB-21 genotype where activity started to increase from initial phase of the infection.

Discussion

BB is one of the most important biotic stresses limiting crop productivity. Oxidative stress is regarded as a major detrimental factor in plants exposed to large range of biotic and abiotic stresses. In order to improve stress tolerance in crops and specially BB tolerance, it is essential to identify salient components of antioxidative defence system which are induced under BB stress and may have role in conferring BB tolerance. In our experiments, a substantive increase in phenolics and flavonoids was observed in the leaves of highly resistant; OL compared to resistant IRBB21 and suceptible PB1. The induced accumulation of flavonoids and phenolics during infection might have occurred to protect the plant from further invasion and growth of the pathogens population. Increase in production of flavonoids after invasion by pathogens or pests is a well known phenomenon

Table 2. Effect of bacterial blight on total polyphenol content (TPC), total flavonoid content (TFC), total antioxidant capacity (TAC), ferric reducing power (FRAP) and free amino acid content (FAA) on three rice genotypes.

Sample	TPC[a]		TFC[b]		TAC[c]		FRAP[c]		FAA[d]	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
5th Day after Infection (5th DAI)										
OL	5.52±0.03	10.82±0.01†	8.70±0.05	18.60±0.03†	9.48±0.02	16.19±0.02†	6.47±0.05	10.09±0.02†	6.43±0.05	8.30±0.42†
PB-1	10.14±0.01	12.23±0.06†	19.32±0.07	22.20±0.02†	13.13±0.02	17.05±0.03†	8.53±0.06	9.22±0.02†	2.82±0.04	4.14±0.52†
IRBB21	9.75±0.03	11.28±0.05†	16.40±0.01	19.10±0.05	15.59±0.03	15.25±0.05	9.16±0.01	10.48±0.01†	7.92±0.02	9.54±0.34†
10th Day after Infection (5th DAI)										
OL	7.68±0.03	10.47±0.03†	11.90±0.09	18.03±0.03†	11.90±0.02	17.15±0.01†	8.32±0.05	9.75±0.06†	2.31±0.02	6.54±0.11†
PB-1	9.40±0.02	11.11±0.06†	14.28±0.03	17.73±0.05†	15.27±0.05	16.03±0.04	7.74±0.04	10.38±0.01†	4.42±0.01	11.34±0.98†
IRBB21	10.07±0.05	12.80±0.04†	16.28±0.02	19.85±0.06	14.85±0.01	15.18±0.02	8.45±0.05	9.83±0.06†	6.34±0.08	13.10±0.36
15th Day after Infection (15th DAI)										
OL	2.77±0.02	4.81±0.06†	5.45±0.03	7.0±0.06†	5.08±0.02	10.28±0.04†	5.04±0.01	6.02±0.02†	4.12±0.02	26.89±0.78†
PB-1	4.30±0.07	4.96±0.05†	7.40±0.08	8.73±0.04†	6.53±0.05	10.35±0.02†	5.64±0.03	5.93±0.05†	4.35±0.02	29.21±0.25†
IRBB21	4.94±0.04	4.31±0.02†	7.78±0.09	8.98±0.05†	8.91±0.04	9.03±0.02†	5.83±0.06	6.10±0.04†	8.65±0.06	21.82±0.48†

a: gallic acid; b: quercetin; c & d: ascrobic acid equivalents mg/g fw plant material respectively; Values are presented as mean of triplicate determinations ± standard deviation. († = p≤0.01; ANOVA one way variance, Bartlett's test).

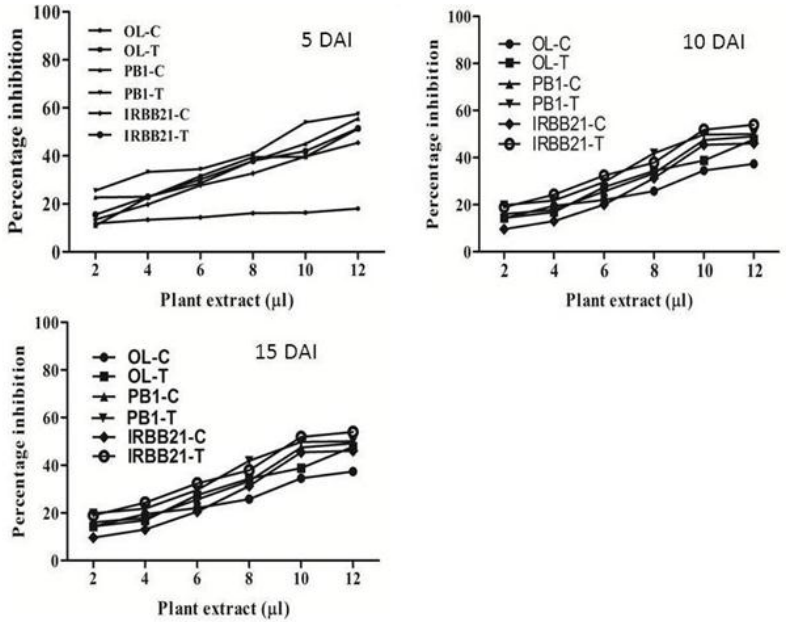

Fig 2. DPPH scavenging activity of extracts of different genotypes of *Oryza.* The leaf tissues of OL under bacterial stress condition at the 5th DAI showed a significantly higher capacity to detoxify oxygen radicals'when compared to IRBB21 and PB1.

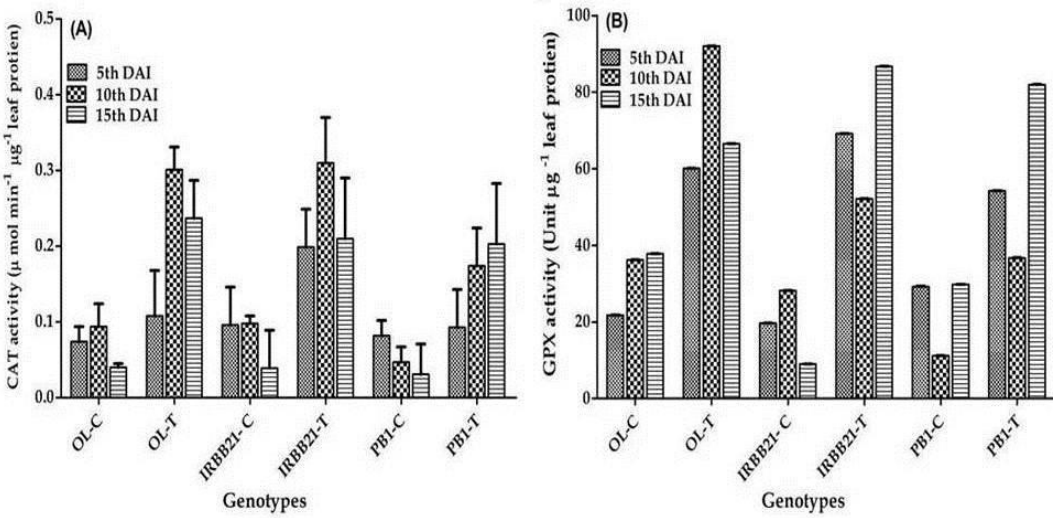

Fig 3. Activity of antioxidative enzymes, CAT (A) expressed as Units μmol min^{-1} $μg^{-1}$ protein and GPX (B) as Units $μg^{-1}$ protein. Values are mean ± SD for three observations (n=3). The highest activity was observed in IRBB21 at all time points followed by OL and PB-1 respectively.

(Gallet et al., 2004). In cotton leaves, epidermal anthocyanin production is an indicator of resistance to bacterial blight *Xanthomonas campestris* pv. *malvacearum* (Kangatharalingam et al., 2002). Higher levels of H_2O_2 have been detected under various stress conditions (Smirnoff, 1993; Menconi et al., 1995). H_2O_2 also predisposes the chloroplasts to photodisintegration of thylakoid membrane causing the observed loss of chlorophyll as in pea (Angelopoulos et al., 1996; Moran et al., 1994) and *Arabidopsis* (Jung et al., 2004). Therefore, resistant genotype OL might be able to hold the integrity of thylakoid membrane and hence, have maintained its level of chlorophyll pigments during the progression of the disease. Low molecular weight antioxidants like carotenoids effectively scavenge the harmful radicals and stabilize lipid oxidation (Strzalka et al., 2003). The carotenoid level was found to be higher in highly resistant OL during disease progression at all the three time periods. There was severe reduction of carotenoid content in susceptible PB-1 and resistant IRBB21 during the same time periods. Antioxidants and metabolites increase under various stresses, with their comparatively higher activity in stress-resistant cultivars, suggesting that higher antioxidant activity imparts tolerance (Polle, 1997; Sairam et al., 2000). The higher activities of antioxidants were observed in highly resistant OL which is in support of earlier studies, whereas the susceptible genotype maintained a lower protection from oxidants. Moreover, BB sensitive genotype has a lower antioxidant capacity than do the resistant genotypes. The reducing power ability of the antioxidants enable them to scavenge the DPPH radical by donating hydrogen and forming reduced DPPH**(** Duh, 1998; Sun et al., 1999). In our study, resistant OL has shown maximum scavenging activity during infection compared to rest of the two genotypes. The activation and increment of DPPH-radical scavenging activity also appeared to be correlated with BB induced tolerance in rice genotypes. The ability to donate a hydrogen atom to free anionic radicals so as to terminate the free radical reactions is measured by the reducing power of a sample. Increased oxidative damages might have led to the induction of reducing power ability, maximum being in OL, thus enabling their sustenance under BB stress conditions. The induced reducing power, due to BB stress, donating free H^+ ions, was a means to neutralize the excess free radicals, which are mostly negative ions, thus trying to restore overall homeostasis and overcome metabolic imbalance under BB infection. The total free amino acid content increased in all genotypes during disease progression. The highest free amino acid accumulation was found in the case of PB-1 (sensitive to BB), followed by OL and IRBB21. Significant differences was found between highly resistant OL and susceptible PB-1. The up-regulation of free amino acids during stress helps in renaturing or removal of denatured proteins which increased under stress conditions. H_2O_2 also functions as an intercellular signal to either stimulate or deactivate certain antioxidative enzymes like SOD, CAT and GPX (Lee et al., 2001). The induction of CAT activity was far greater in IRBB21 and OL compared to PB1. The observed less increase in CAT activity in BB susceptible PB1 could diminish the ability to scavenge harmful radicals favouring accumulation of oxygen radical species, which could cause membrane damages. The GPX activity was higher in OL and IRBB21 compared to PB1. The higher induction of GPX in highly resistant and resistant genotypes may enable these genotypes to limit the accumulation of harmful radicals.

Materials and methods

Plant material, growth conditions and stress treatments

Three rice genotypes; *Orzya longistaminata* (OL), near isogenic line (IRBB21) and Pusa Basmati 1 (PB-1) were grown in a growth chamber under 28ºC/22ºC, relative humidity of 85%, and photoperiod of 12 hrs. The germinated seeds were uniformly watered and fertilized with a half-strength Hoagland nutrient solution. After 30 days, healthy seedlings were transplanted to 20 Ltrs earthen pots. Six pots (3 control; 3 treated) were kept for each genotype. The pots were filled with a mixture of clay and peat (1:1; v/v). After 45 days of transplantation, all plants were challenged with DX133 (Indian virulent *Xoo* isolate) strain which was obtained from Department of Plant Pathology, Directorate of Rice Research, Hyderabad, India and cultured in modified Wakimoto's medium. The bacteria were then scraped and suspended in sterile distilled water and the concentration was adjusted to 0.1 - 0.2 OD ($1x10^8$-$1x10^9$ CFU/ml). Using this bacterial suspension, 5-6 uppermost leaves of plants at the

booting stage were inoculated following the leaf clipping method (Kauffman et al., 1973). The control plants were mock inoculated by clipping with scissors dipped in sterile water. PB-1 was used as a susceptible check and OL was included as a resistant check for the *Xoo*. Soil water content (SWC) was kept at 100% pot water holding capacity (PWC) and periodically measured (gravimetrically) in different points of the pot to check the homogeneity of moisture content in soil.

Sample collection and preparation

Leaf sample were collected on different time points such as 5^{th} day after infection (5^{th} DAI), 10^{th} DAI and 15^{th} DAI. The leaf samples were ground with 80% ethanol to fine pulp. The homogenates were centrifuged at 12000 g for 20 min and supernatants were collected and used for assays. For enzymatic assays, fresh leaves were homogenized (w/v) in 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% (w/v) polyvinylpolypyrrolidone at 4ºC and centrifuged at 15000 g for 15 min at 4ºC. Supernatants were used to determine different enzymatic activities.

Extraction and estimation of chlorophyll content

On three different days of experiment, leaf discs were taken from five fully expanded leaves of comparable physiological age. Leaf sections were ground in 80% acetone and the total chlorophyll concentration was determined (Arnon et al., 1949).

Determination of non-enzymatic antioxidants

Estimation of total phenolic content

The total phenolic content of the rice leaf extracts were estimated by the Folin- Ciocalteu method (Gul et al., 2011). In brief, 20 µl of the extracts were mixed with 180 µl of distilled water and after 5 min, 100 µl of Folin- Ciocalteu reagent was added. After 10 min of incubation, 300 µl of 20% sodium carbonate was added, thoroughly vortexed and the final volume was made to 1 ml. The reaction mixture was further incubated for 90 min in dark and thereafter the absorbance was measured at 765 nm using UV-Visible Spectrophotometer. The total phenolic content was expressed as mg gallic acid equivalents (GAE) per gram of fresh weight calculated from standard graph of gallic acid.

Estimation of total flavonoid content

A slightly modified version of the spectrophotometric method (Barreira et al., 2008) was used to determine the flavonoid content of samples. Plant extracts (20 µl each) were taken and diluted with 480 µl of double distilled water, followed by addition of 30 µl of 5% sodium nitrite. The solution was mixed well and kept at room temperature for 10 min. To this solution, 60 µl of 10% aluminium chloride was added. After 5 min, 350 µl of 1 M sodium hydroxide was added. The final volume was made to 1 ml with distilled water Samples were further incubated for 30 min at room temperature and subsequently, the absorbance was recorded at 510 nm. Flavonoid content was determined as mg quercetin equivalents (mg QE/g) of fresh weight using a standard curve of quercetin and the values were expressed as means of triplicate analysis.

Determination of total antioxidant potential

The antioxidant activity of leaf extracts were evaluated as per the protocol based on the reduction of Mo (VI) – Mo (V) by the extract, following formation of a green phosphate/Mo (V) complex at acidic pH (Prieto et al., 1999). Aliquots of the leaf samples were mixed properly with 1 ml of the reagent solution (0.6 M H_2SO_4, 28 mM Na_2HPO_4 and 4 mM $(NH_4)6Mo_7O_{24}$). The tubes were incubated in dry thermal blocks at 90ºC for 90 min. Further, absorbance of sample solutions were measured at 695 nm against methanol (blank). Total antioxidant activity was expressed as mg of ascorbic acid equivalents (mg AAE/g) of fw.

Determination of reducing activity by FRAP assay

The capability to reduce ferric ions was measured using the method reported by Oyaizu et al., 1986. About 10 µl of leaf sample were mixed with 0.25 ml of distilled water and 0.25 ml of 1% potassium ferric cyanide and reaction mixture was incubated in a water bath at 50ºC for 30 min. 0.25 ml of 10% tricholoro acetic acid was added and centrifuged at 8000 g for 10 min. 0.5 ml of supernatant were taken to which 100 µl of ferric chloride was added and volume was made to 1 ml. The increase in absorbance at 700 nm was measured after 30 min. The antioxidant capacity of the extract was expressed as mg ascorbic equivalents per gram of leaf material based on fresh weight. Ascorbic acid was used for standardization.

Measurement of the DPPH radical scavenging activity

The DPPH free radical scavenging activity was carried out by employing the standard protocol given by Braca et al., 2002. Different dilutions of the extract were incubated with 1 ml of DPPH solution (0.004% w/v) in dark for 45 min. The decrease in absorbance due to scavenging DPPH radicals by extract was determined at 517 nm using a spectrophotometer.

Estimation of total free amino acids

The total amino acids were quantified by the ninhydrin method (Moore et al., 1968). In 1 ml of ninhydrin solution, 0.1 ml of leaf extracts were mixed and volumes were made upto 2 ml with distilled water. The tubes were incubated in boiling water bath for 20 min followed by addition of 5 ml diluents solvents (1:1 ratio of 1-propanol and water). After 15 min, the intensity of the colour formed was read at 570 nm against a blank. Leucine was used for standardization.

Evaluation of enzymatic antioxidants

Determination of Catalase Activity

CAT activity was determined by monitoring the disappearance of H_2O_2 at 240 nm ($\varepsilon = 40 mM^{-}1\ cm^{-}1$) (Aebi et al., 1984). The reaction mixture comprised 50 mM sodium phosphate buffer (pH 7.0), 20 mM H_2O_2 and 50 µg of plant extract. Absorbance was taken at 470 nm. The molar extinction coefficient of hydrogen peroxide at 240 nm was taken as 0.04 sq. cm/µ mole. Enzyme activity was expressed as µmoles of hydrogen peroxide degraded/min g fw.

Determination of Guaiacol peroxidase activity

Glutathione peroxidase (GPX) activity was determined as per the standard protocol (Hemeda et al., 1990).

A reaction mixture containing 1% Guaiacol (v/v) 0.3% hydrogen peroxide and 80 ml of 50 mM phosphate buffer (pH 6.6) to which enzyme extracts (50 µl each) were added to a final volume of 3 ml. The extinction coefficient of guaiacol was considered 26.6/mM cm. Absorbance was taken at 470 nm. Enzyme activity was expressed as µmoles of guaiacol oxidized/min g fw.

Statistical analyses

The percentage inhibitions of free radicals were calculated using the formula:
Percentage Inhibition = $A_{control}$ - A_{sample} / $A_{control}$ × 100.
All the experimental data values were expressed as means of three measurements ± standard deviation (SD). Statistical differences between the samples were evaluated using ANOVA one way variance; treatments were compared using Bartlett's test and considered significantly different where *p* values were found to be equal to or less than 0.05. Statistical tests and graphical representation of the results was done using graphpad prism 5 software.

Conclusions

BB resistant genotype accumulates lesser amounts of peroxide and free radicals. Apparently OL seems to have potentially more tolerance to BB than the other two genotypes. The higher induction of major antioxidants, especially flavonoids and phenolics, and no-decrease in the activity of CAT in OL, probably enabled this genotype to limit H_2O_2 accumulation, thus warding off the irreversible damaging effects at the cellular and subcellular levels. IRBB21 behaved some what similarly to PB1 with respect to antioxidative nature suggesting that some other mechanism govern the resistance conferred by *Xa21* gene present in IRBB21. Although a short-term effect of BB stress on seedlings in the green house conditions may not always reflect exactly the behaviour of the plants exposed to a long term BB stress under field conditions, findings of such experiments could help to assess the plant genotypes for survival under BB stress conditions in the early stage of the life cycle. Comparison of biochemical responses, as undertaken in this study, might be useful to understand the mechanism of BB stress management, selection and development of rice genotypes resistant to BB stress.

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