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Exogenous Application of 28-Homobrassinolide Modulates the Dynamics of Salt and Pesticides Induced Stress Responses in an Elite Rice Variety Pusa Basmati-1

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Abstract Brassinosteroids play an essential role in regulating various aspects of plant growth and development as well as adaptation to various environmental stresses. The present work provides an analysis of the response of various stress markers upon exogenous application of 28-homobrassinolide (HBL) on Pusa Basmati-1, a commercially important rice variety, under salt and pesticide (Chlorpyrifos and Imidacloprid) stress. Rice seeds treated with HBL were analyzed for various growth parameters, protein, proline and malondialdehyde content (MDA), antioxidant enzyme activities, and their gene expression analysis (*Cu/Zn-SOD*, *Fe-SOD*, *Mn-SOD*, *APX*, *CAT,* and *GR*) in the presence or absence of salt and pesticide stress. Stress-induced reduction in growth, protein, and chlorophyll content and enhancement of proline and MDA content of seedlings was observed. The exogenous application of HBL resulted in the improvement of growth parameters as well as protein and proline content. MDA content decreased significantly under the effect of HBL treatment both under stress and control conditions. HBL treatment also enhanced the activity of various antioxidant enzymes which corroborated with the reduced accumulation of $O_2^{\cdot-}$ and H_2O_2 under the effect of salt and pesticides. The differential response of various isoforms of SOD under the effect of HBL and stress treatments was observed under salt and among different pesticide treatments. From this study, the potent activity of HBL in stress mitigation in response to salt and pesticide treatment in rice is established.

Keywords Chlorpyrifos · 28-Homobrassinolide · Imidacloprid · Salinity · Oxidative stress · Reactive oxygen species

Introduction

Rice is an important staple crop which provides food security to many countries worldwide. The ever increasing global population has resulted in increased demand for rice cultivation. The demand to enhance its productivity is further aggravated due to shrinking cultivable land and adverse impacts of multiple stress factors. Soil salinity is the major abiotic stress encountered by rice which greatly reduces its productivity (Kumar and others 2013; Golldack and others 2014). It is particularly a major problem in coastal regions because of the intrusion of brackish water during the dry season and at the start of the wet season. Salt stress is also detrimental to inland areas owing to the buildup of salinity as a consequence of use of excessive irrigation water and/or the poor quality irrigation water (Ismail and others 2010). High salt content in the rhizosphere causes plants to undergo osmotic and ionic stress due to the accumulation of salts outside the roots and inside the plant cells, respectively. This generates a zone of low water potential creating hindrance for roots to uptake water and nutrients from the soil resulting in a physiological drought condition (Barkla and others 2013; Mahajan and others 2008). Thus, soil salinity is a complex phenotypic

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and physiological phenomenon causing ion disequilibration thereby disrupting the integrity of cellular membranes and adversely affecting the activities of various enzymes linked to plant metabolism, growth, and development. It has also serious bearing on plant nutrient acquisition and functioning of the photosynthetic apparatus (Kumar and others 2013; Barkla and others 2013).

Another growing concern in rice cultivation has been the superfluous use of pesticides. To counter the challenges posed by declining resources for crop cultivation and to increase crop productivity, the use of synthetic pesticides has been a routine practice in modern agriculture. Though pesticides help control crop pests, they pose several unwanted side effects to the environment and biodiversity (Sharma and others 2013a). Among several pesticides, chlorpyrifos (CPF) and imidacloprid (IMI) are the most commonly used insecticides worldwide. CPF is a broad spectrum systemic insecticide which has been used for more than a decade to control a number of important arthropod pests (Chen and others 2012; Farahat and others 2011). IMI, on the other hand, was the first commercialized neonicotinoid insecticide and enjoys the status of one of the largest selling insecticides possessing both contact and long-lasting systemic activity to control a wide range of domestic and agricultural pests (Starner and Goh 2012). Continuous application of pesticides to the crops results in disruption of various physiological and biochemical processes affecting the crop and its resistance to pests (Xia and others 2006; Sharma and others 2012). Further, its excessive application on crops raises concern toward safety and quality of the harvested products.

In the past, several efforts including genetic engineering, QTL mapping, and conventional breeding were undertaken to improve rice plants to withstand various abiotic stresses (Kumar and others 2013). However, in the era of sustainable agriculture, the major challenge is to evolve effective and acceptable crop stress management strategies. In this context, development of crops with an inherent capacity to withstand stresses has taken center stage. Recently, the potential of phytohormones for their anti-stress effects are being explored worldwide using rational scientific approaches (Dhaubhadel and others 1999, 2002; Zhang and others 2014). Brassinosteroids, a class of polyhydroxysteroids, are known for their versatile role in plants (Clouse and Sasse 1998). BRs are perceived by the cell surface receptor complex and the subsequent activation of downstream transcription factors and genes results in various cellular responses like stem elongation, vascular differentiation, the regulation of gene expression, nucleic acid, and protein synthesis and photosynthesis (Gruszka 2013). However, insights into the role of BRs in abiotic stress amelioration has recently gained momentum (Wang and others 2014; Zhang and others 2014; Fariduddin and others 2014). In continuation with these efforts, the present paper reports the behavior of various stress markers under the effect of salt (NaCl), pesticides (CPF and IMI), and HBL (28-homobrassinolide) treatment in a commercially important indica rice variety, Pusa Basmati-1. The present study also provides information on comparative study of two different types of stresses in rice employing physiological, biochemical, and molecular approaches.

Materials and Methods

Plant Material and Growth Conditions

Seed sterilization was performed as mentioned by Sharma and others (2013b). Based on previously published results (Sharma and others 2012, 2013b) and initial experiments conducted in the laboratory on HBL, 10^{-7} M HBL was found to be most effective in enhancing the growth of seedlings under stress and control conditions, and thus, in the present study, HBL treatment was given as a 10^{-7} M formulation. The stock solution (10^{-3} M) of HBL was prepared by dissolving HBL in ethanol (HPLC grade) and was stored at −20 °C. The working concentration of HBL was prepared by diluting the stock solution with double distilled water. The concentrations for treatment of pesticides and salt were chosen based on their IC_{50} values determined on the basis of rate of germination and seedling growth under a range of concentrations of pesticides and salt. Seeds were soaked for 8 h in distilled water (control) and in a solution of HBL (10^{-7} M). Afterward, seeds were sown in autoclaved sand moistened with a solution of distilled water/NaCl (100 mM)/CPF (0.04 %)/IMI (0.015 %) in plastic boxes. Seeds were kept for germination in each box under controlled conditions: 25 °C (day/night), 70–80 % RH (day/night), and 14 h photoperiod. Samples were collected after 12 days to assess the following parameters.

Harvesting of Samples

Seedlings were harvested after 12 days. They were removed from the boxes and were dipped in water to remove adhering sand particles. A representative lot of 15 seedlings was chosen for study of morphological parameters, whereas the remaining seedlings were flash frozen in liquid nitrogen and then stored at −80 °C for further analysis.

Study of Morphological Parameters

For morphological parameter analysis, root and shoot lengths were measured using a meter scale, and observations for fresh weight of seedlings were made. Root

number for each of the seedlings was recorded. The seedlings were then placed in an oven at 70 °C until a constant weight was achieved, and then observations for dry weight were recorded. The experiment was repeated thrice with three biological replicates.

Analysis of Plant Stress Indices

Chlorophyll content was estimated following the standard method given by Arnon (1949). Proline content in the seedlings was determined by the method suggested by Bates and others (1973). Lipid peroxidation in the samples was determined according to the method of Hodges and others (1999) by quantification of malondialdehyde (MDA) content which is an end-product of lipid peroxidation. The activity of antioxidative enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase, (DHAR) and glutathione reductase (GR) was determined by the standard methods as mentioned in Sharma and others (2013b).

Histological Determination of H_2O_2 and Superoxide Radical

Superoxide radicals ($O_2^{\cdot-}$) were visually detected in rice leaves according to the method suggested by Wu and others (2010). Plants were excised at the base of stems with a razor blade and supplied with 10 mM Na-citrate buffer (pH 6.0) containing 6 mM NBT for 8 h under light at 25 °C. Pale yellow NBT reacts with superoxide radicals and forms a dark-blue insoluble formazan compound. The leaves were then decolorized by immersing them in boiling ethanol (95 %) for 10 min to remove the green background of leaves except for the dark-blue insoluble formazan deposits produced by the reaction of NBT with $O_2^{\cdot-}$. After cooling, leaves were observed using a stereomicroscope and photographed.

Hydrogen peroxide (H_2O_2) production in rice leaves was visualized histochemically using 3,3-diaminobenzidine (DAB) as a substrate (Wu and others 2010). Plants were excised at the base of stems with a razor blade, and through the cut stems, 1 mg ml^{-1} solution of DAB (pH 3.8) was supplied under light at 25 °C for 6 h. The leaves were eventually immersed in boiling ethanol (95 %) for 10 min to remove the green background. The deep brown polymerization product formed after reaction of DAB with H_2O_2 was clearly visualized under a stereomicroscope and photographed.

Statistical Analysis

Data were subjected to one-way analysis of variance (ANOVA) for studying the interaction of stress (NaCl, CPF, and IMI) with HBL and expressed as mean ± SE of three independent replicates. The Fisher's LSD test was applied for multiple comparisons using Sigmastat version 3.5, and significance of difference between stress and HBL treatment was set at $p \leq 0.05$.

Expression Analysis of Key Antioxidant Genes

Semi-quantitative reverse transcriptase-polymerase chain reaction (semi-qRT-PCR) was performed to study the expression profile of selected genes (*Fe-SOD, Cu–Zn-SOD, Mn-SOD, CAT, GR, APX*) in response to various treatments. Total RNA was extracted using Trizol reagent (Invitrogen; www.invitrogen.com) according to the manufacturer's instructions. 5 µg of total RNA was reverse transcribed using the Super Script First Strand Synthesis System for RT-PCR (Invitrogen). The cDNA synthesized from mRNA of different samples was used as a template for PCR using gene specific primers (Table 1). The rice elongation factor 1α (EF1α) gene was used as a reference. All PCRs were repeated using at least three biological samples.

Results and Discussion

The adverse effects of salt and pesticides on morphological parameters such as shoot and root lengths, fresh and dry weight, and root number were clearly observed (Table 2). However, such physiological disturbances were substantially prevented by chemical pretreatment with HBL. In comparison to control (13.25 ± 0.24), the shoot length (cm) decreased to 38 % (8.16 ± 0.61), 51 % (6.43 ± 0.60), and 42 % (7.79 ± 0.17) in seedlings growing in soil solutions of NaCl, CPF, and IMI, respectively. On pre-soaking of seedlings with HBL, there was a significant enhancement in the shoot length in seedlings growing in distilled water as well as in NaCl, CPF, and IMI. An enhancement of 19 % (9.66 ± 0.38), 28 % (8.72 ± 0.43), and 34 % (10.43 ± 0.66) was observed in samples pre-treated with HBL and then grown in NaCl, CPF, and IMI, respectively. A similar decrease in root length was also observed in seedlings growing in stress conditions. However, pretreatment with HBL to the seedlings followed by application of NaCl, CPF, and IMI stress, resulted in an enhancement in root number by 34, 33, and 38 %, respectively, as compared to only stress conditions. Induction of stress conditions also led to a similar decrease in root number, fresh weight (g $seedling^{-1}$), and dry weight (g $seedling^{-1}$). However, co-treatment of seedlings with HBL resulted in significant enhancement in growth parameters under all the stress conditions. HBL-induced reversal of growth inhibition under stress and control conditions may be associated with the ability of BRs to enhance cell elongation, cell cycle progression, and

Table 1 List of primers for RT-PCR

S. No.	Name of the gene	Primer sequence
1.	EF1-α	F: 5′-GTACAAGATCGGTGGTATT-3′
		R: 5′-GGGTACTCAGAGAAGGTCT-3′
2.	Cu/Zn-SOD	F: 5′-CCTCAAGCCTGGTCTCCAT-3′
		R:5′-CAGCCTTGAAGTCCGATGAT-3′
3.	Fe-SOD	F: 5′-CTTGATGCCCTGGAACCTTA-3′
		R: 5′-GCCAGACCCCAAAAGTGATA-3′
4.	Mn-SOD	F: 5′-GCCATTGATGAGGATTTTGG-3′
		R: 5′-CAAGCAGTCGCATTTTCGTA-3′
5.	CAT	F: 5′-GTTCGGTTCTCCACAGTCGT-3′
		R: 5′-CCCTCCATGTGCCTGTAGTT-3′
6.	APX	F: 5′-CCAAGGGTTCTGACCACCTA-3′
		R: 5′-CAGTTCGGAGAGCTTGAGGT-3′
7.	GR	F: 5′-AACAGCCGATGGCATAAAAG-3′
		R:5′-CAACCACCAGTTTCATGACG-3′

Table 2 Effect of HBL on shoot length, root length, number of roots, fresh weight, and dry weight of 12-day-old *Oryza sativa* seedlings under NaCl, CPF, and IMI stress

Treatments	Shoot length (cm)	Root length (cm)	Root number	Fresh weight (g)	Dry weight (g)
Control	13.25 ± 0.24*	7.05 ± 0.34*	4.1 ± 0.07*	0.069 ± 0.001*	0.033 ± 0.001*
HBL (10^{-7} M)	14.63 ± 0.26**	7.64 ± 0.66**	5.3 ± 0.07**	0.080 ± 0.001**	0.037 ± 0.001**
NaCl (100 mM)	8.16 ± 0.61*	4.07 ± 0.11*	3.2 ± 0.31*	0.047 ± 0.001*	0.022 ± 0.001*
NaCl + HBL	9.66 ± 0.38**	5.3 ± 0.10**	4.3 ± 0.07**	0.055 ± 0.001**	0.030 ± 0.001**
CPF (0.04 %)	6.43 ± 0.60*	3.92 ± 0.29*	2.7 ± 0.17*	0.047 ± 0.001*	0.028 ± 0.001*
CPF + HBL	8.72 ± 0.43**	4.93 ± 0.19**	3.6 ± 0.11**	0.058 ± 0.001**	0.037 ± 0.001**
IMI (0.015 %)	7.79 ± 0.17*	4.67 ± 0.32*	3.0 ± 0.20*	0.042 ± 0.001*	0.023 ± 0.001*
IMI + HBL	10.43 ± 0.6**	6.22 ± 0.57**	4.13 ± 0.12**	0.055 ± 0.007**	0.027 ± 0.003**

Data represent mean ± SE ($n = 15$).

Asterisk (*,**) represents the significant difference with HBL application for individual stress (Fisher LSD, $p \leq 0.05$)

enhancement of mRNA transcripts for cellulose biosynthesis and cell wall modifying enzymes contributing to plant growth and enhanced photosynthesis (Zhang and others 2014; Jin and others 2014; Xie and others 2011; Dobrikova and others 2014). Moreover, BRs augment the activity and expression of pesticide metabolism and detoxification enzymes which degrade and reduce their residual level in plants resulting in promotion of growth of seedlings under pesticide stress (Xia and others 2009).

Salt and pesticide stress resulted in a significant decline in the chlorophyll content (mg gFW^{-1}) (Table 3). However, a significant enhancement of 21 % (1.61 ± 0.06), 22 % (0.66 ± 0.01), and 26 % (2.27 ± 0.04) was observed in chlorophyll a, chlorophyll b, and total chlorophyll content, respectively, in seedlings treated with salt and HBL as compared to salt treated samples only. Similarly, a significant elevation in the chlorophyll content was observed in seedlings supplemented with HBL and then treated with pesticides as compared to pesticide treatment alone. The enhancement in chlorophyll content could be due to BR-mediated regulation of transcription and/or translation of genes responsible for synthesis of chlorophyll pigments (Ahammed and others 2013) or due to the their role in reducing the degradation of chlorophyll (Honnerova and others 2010). Increased pigment content under the effect of HBL may be the result of enhanced photosynthetic efficiency leading to improved growth of seedlings (Xia and others 2009).

Under various stress conditions, protein content (mg gFW^{-1}) of rice seedlings decreased as compared to control (31.74 ± 1.84) (Table 3). It decreased to 43 % (18.01 ± 0.77), 31 % (21.91 ± 2.07), and 43 % (18.11 ± 1.25) under NaCl, CPF, and IMI stress as compared to control conditions (Table 4). However, pretreatment with HBL improved the protein content of seedlings. BRs are known to enhance the expression of some components of

Table 3 Effect of HBL on chlorophyll a, chlorophyll b, and total chlorophyll content of 12-day-old *Oryza sativa* seedlings under NaCl, CPF, and IMI treatments

Treatments	Chlorophyll a (mg g FW^{-1})	Chlorophyll b (mg g FW^{-1})	Total chlorophyll (mg g FW^{-1})
Control	1.64 ± 0.06*	0.70 ± 0.02*	2.34 ± 0.04*
HBL (10^{-7}M)	1.76 ± 0.06*	0.76 ± 0.02*	2.52 ± 0.01**
NaCl (100 mM)	1.26 ± 0.07*	0.54 ± 0.03*	1.80 ± 0.03*
NaCl + HBL	1.61 ± 0.06**	0.66 ± 0.01**	2.27 ± 0.04**
CPF (0.04 %)	1.34 ± 0.03*	0.52 ± 0.02*	1.86 ± 0.05*
CPF + HBL	1.69 ± 0.09**	0.96 ± 0.08**	2.89 ± 0.12**
IMI (0.015 %)	1.39 ± 0.06*	0.63 ± 0.01*	2.02 ± 0.12*
IMI + HBL	1.67 ± 0.09**	1.18 ± 0.07**	2.85 ± 0.08**

Values represent mean ± SE ($n = 3$)

Asterisks (*,**) represent the significant difference with HBL application for individual stress (Fisher LSD, $p \leq 0.05$)

Table 4 Effect of HBL on protein, proline, and malondialdehyde content of 12-day-old *Oryza sativa* seedlings under NaCl, CPF, and IMI treatments

Treatments	Protein content (mg g FW^{-1})	Proline content (μmoles g FW^{-1})	Malondialdehyde content (μmoles g FW^{-1})
Control	31.74 ± 1.84*	0.24 ± 0.012*	1.15 ± 0.01*
HBL (10^{-7} M)	36.1 ± 2.51**	0.28 ± 0.018*	0.99 ± 0.01*
NaCl (100 mM)	18.01 ± 0.77*	0.39 ± 0.014*	1.61 ± 0.07*
NaCl + HBL	30.13 ± 1.00**	0.49 ± 0.007**	1.08 ± 0.05**
CPF (0.04 %)	21.91 ± 2.07*	0.68 ± 0.01*	3.04 ± 0.11*
CPF + HBL	27.20 ± 1.62**	0.90 ± 0.02**	1.95 ± 0.04**
IMI (0.015 %)	18.11 ± 1.25*	0.79 ± 0.07*	2.98 ± 0.11*
IMI + HBL	25.27 ± 0.41**	1.12 ± 0.04**	2.15 ± 0.12**

Values represents mean ± SE ($n = 3$)

Asterisks (*,**) represents the significant difference with HBL application for individual stress (Fisher LSD, $p \leq 0.05$)

translational machinery, stimulate protein synthesis, and inhibit protein degradation (Dhaubhadel and others 2002; Chris and others 2011). On the other hand, stress treatment resulted in an enhanced proline content (μmoles gFW^{-1}) (Table 4). Proline content (μmoles gFW^{-1}) showed a marked increase under the effect of various stress conditions. An increase of proline content to 63 % (0.39 ± 0.014) in NaCl, 183 % (0.68 ± 0.01) in CPF, and 229 % (0.79 ± 0.07) in IMI stress conditions was observed compared to controls (0.24 ± 0.012). Treatment with HBL further elevated the level of proline content by 26 % (0.49 ± 0.007) under NaCl, 32 % (0.90 ± 0.02) under CPF, and 42 % (1.12 ± 0.04) under IMI stress as compared to seedlings growing in respective stress conditions only. Proline is a multifunctional molecule involved in osmoregulation, ROS scavanging, and serves as a source for carbon and nitrogen which is known to accumulate in cells under environmental stress (Hayat and others 2014). Elevation in proline content has been reported under salt and pesticide stress (Sharma and others 2013a, b; Wu and others 2010) which is further enhanced by HBL application indicating that proline accumulation might prevent salt and pesticide-induced production of ROS and protect plants from the oxidative damage. Salt- and pesticide-induced stresses were further manifested by the increase in the level of lipid peroxidation which is usually considered to be the initial step toward cellular membrane damage by pesticides and salts (Parween and others 2012; Sharma and others 2013b). In Table 4, the level of lipid peroxidation increased to 40 % in NaCl, 164 % in CPF, and 151 % in IMI-treated samples as compared to control plants which, however, decreased under the effect of HBL application. MDA content was found to be significantly reduced to 48 % (1.08 ± 0.05) under NaCl, 55 % (1.95 ± 0.04) under CPF, 39 % (2.15 ± 0.12) under IMI stress and HBL treatment, as compared to seedlings growing in stress conditions alone. This could be regarded as evidence for HBL-induced efficient scavenging of reactive oxygen species (ROS) (Hayat and others 2014; Fariduddin and others 2014). Thus, the increased pigment content, proline and protein content, and reduced membrane damage could be contributing to the enhanced growth of seedlings under control as well as under stress conditions.

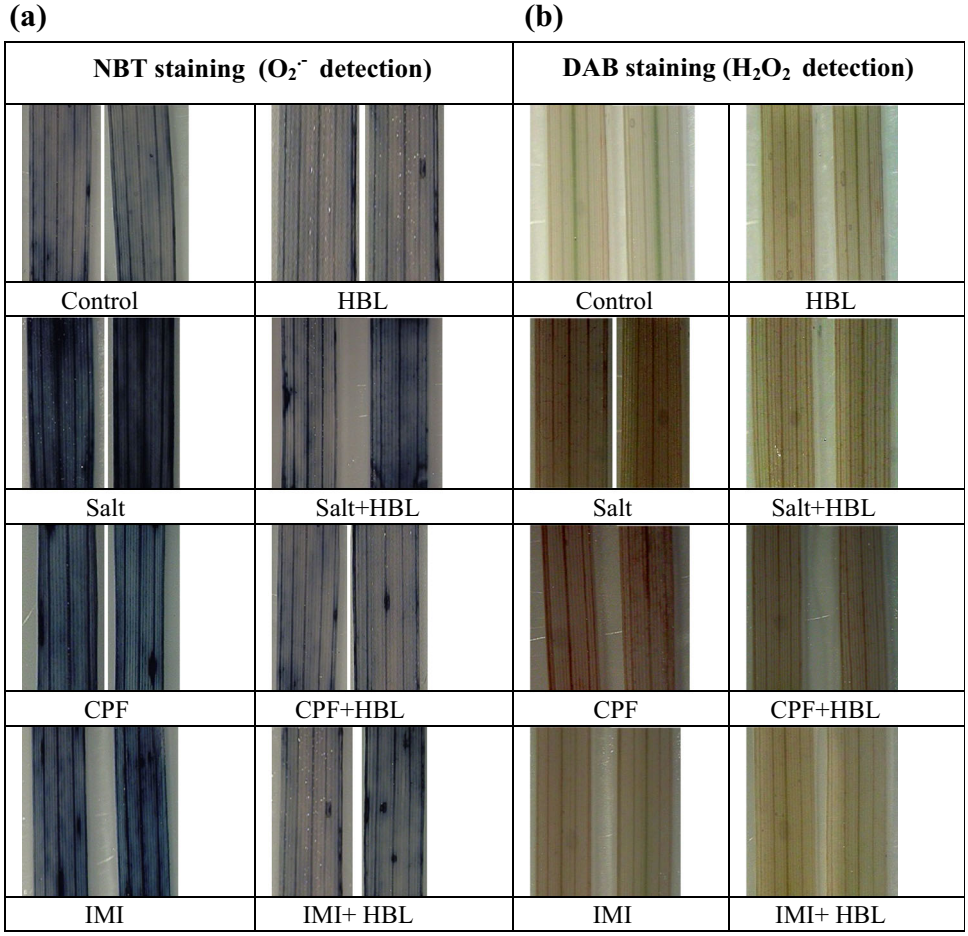


Fig. 1 O_2^{-} and H_2O_2 accumulation in leaves of rice exposed to different stress conditions (NaCl, CPF and IMI) and HBL for 12 days. Plants were excised at the base of stems and supplied through the cut stems with nitroblue tetrazolium (NBT) solution for 8 h (**a**) and 3, 3diaminobenzidine (DAB) solution for 6 h (**b**). Afterward, the leaves were photographed

Table 5 Effect of HBL on specific activity of SOD, APX, CAT, GR, GPX, MDHAR, and DHAR on 12-day-old *Oryza sativa* seedlings under NaCl, CPF, and IMI treatments

Treatments	SOD (units ug protein^{-1})	APX (μmol min^{-1} mg protein^{-1})	CAT (μmol min^{-1} mg protein^{-1})	GR (μmol min^{-1} mg protein^{-1})	GPX (μmol min^{-1} mg protein^{-1})	MDHAR (μmol min^{-1} mg protein^{-1})	DHAR (μmol min^{-1} mg protein^{-1})
Control	27.6 ± 0.7*	15.86 ± 0.91*	0.78 ± 0.04*	3.17 ± 0.03*	27.77 ± 1.11*	1.73 ± 0.12*	1.88 ± 0.08*
HBL (10^{-7} M)	30.5 ± 0.3**	21.74 ± 1.85**	0.90 ± 0.05**	3.68 ± 0.11**	29.13 ± 0.59*	1.81 ± 0.02*	2.35 ± 0.03**
NaCl (100 mM)	38.9 ± 1.2*	28.43 ± 0.47*	0.79 ± 0.04*	2.69 ± 0.09*	20.91 ± 1.02*	1.74 ± 0.07*	1.17 ± 0.15*
NaCl + HBL	43.8 ± 0.5**	33.38 ± 0.51**	0.99 ± 0.09**	3.43 ± 0.06**	23.47 ± 2.29**	1.73 ± 0.05*	1.28 ± 0.16*
CPF (0.04 %)	41.7 ± 1.0*	43.6 ± 1.2*	1.20 ± 0.02*	3.49 ± 0.15*	36.9 ± 0.41*	0.99 ± 0.02*	1.37 ± 0.04*
CPF + HBL	45.4 ± 1.2**	53.4 ± 1.4**	1.34 ± 0.05**	3.87 ± 0.13**	37.3 ± 0.5*	1.02 ± 0.07*	1.55 ± 0.05**
IMI (0.015 %)	37.73 ± 0.7*	28.9 ± 0.5*	0.79 ± 0.04*	4.03 ± 0.15*	22.3 ± 0.41*	1.87 ± 0.05*	1.22 ± 0.04*
IMI + HBL	41.05 ± 0.7**	35.8 ± 1.2**	0.81 ± 0.02*	4.06 ± 0.09*	27.5 ± 0.50**	1.80 ± 0.07*	1.21 ± 0.05*

Values represent mean ± SE ($n = 15$)

Asterisks (*,**) represent the significant difference with HBL application for individual stress (Fisher LSD, $p \leq 0.05$)

To monitor the production of ROS under the effect of stress conditions, histological determination of H_2O_2 and superoxide ions was done (Fig. 1). Figure 1 shows that in comparison to control leaves, leaves from the plants treated with stress showed a higher intensity of blue color pigment revealing superoxide ion accumulation. However, plants

co-treated with stress and HBL showed less accumulation of superoxide ions revealed by a lesser intensity of blue color as compared to those treated with stress alone. Samples treated with HBL alone had only a slight visual difference from that of control plants. Similarly, H_2O_2 levels were also observed to be elevated in stressed plants as compared to the control. Seeds supplemented with HBL and then grown in stress conditions showed less coloration as compared to plants treated with stress alone, showing the alleviation of H_2O_2 levels due to HBL treatment as compared to the stress condition alone. The reduced accumulation of ROS may be due to the role of BRs in enhancing the process of ROS scavenging (Fariduddin and others 2014). Interestingly, treatment with HBL alone resulted in a slightly enhanced accumulation of H_2O_2 levels as compared to controls. A similar indication of a BR-induced transient oxidative burst with H_2O_2 accumulation has been reported recently (Xia and others 2009; Jiang and others 2012a, b). This clearly indicates a possible link between H_2O_2 and BR signaling.

Modulation of the antioxidant system is believed to play a critical role in BR-mediated stress amelioration (Fariduddin and others 2014). In the present experiment, different stress conditions resulted in an alteration in the activity of various antioxidant enzymes. There was an enhancement in the activity of SOD under the effect of various stress conditions (Table 5). SOD activity increased to 41 % under NaCl (38.9 ± 1.2), 51 % under CPF

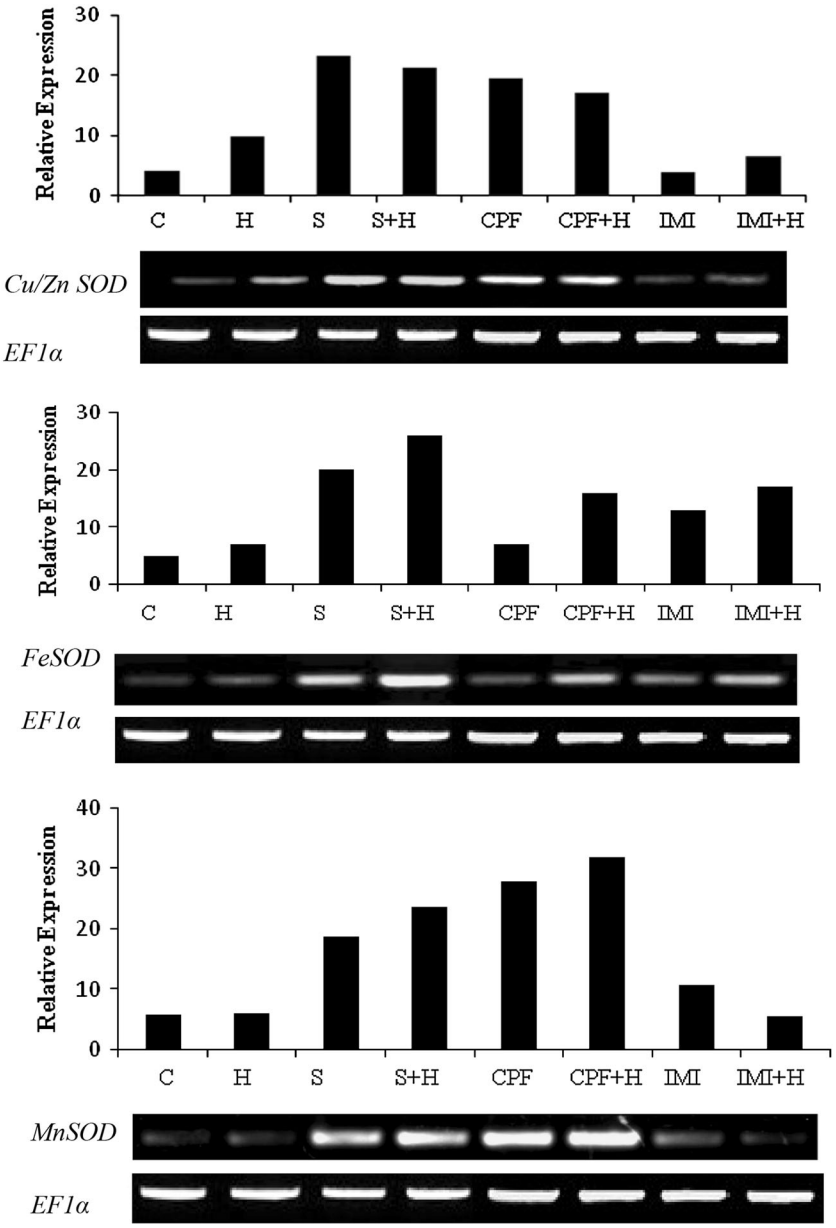
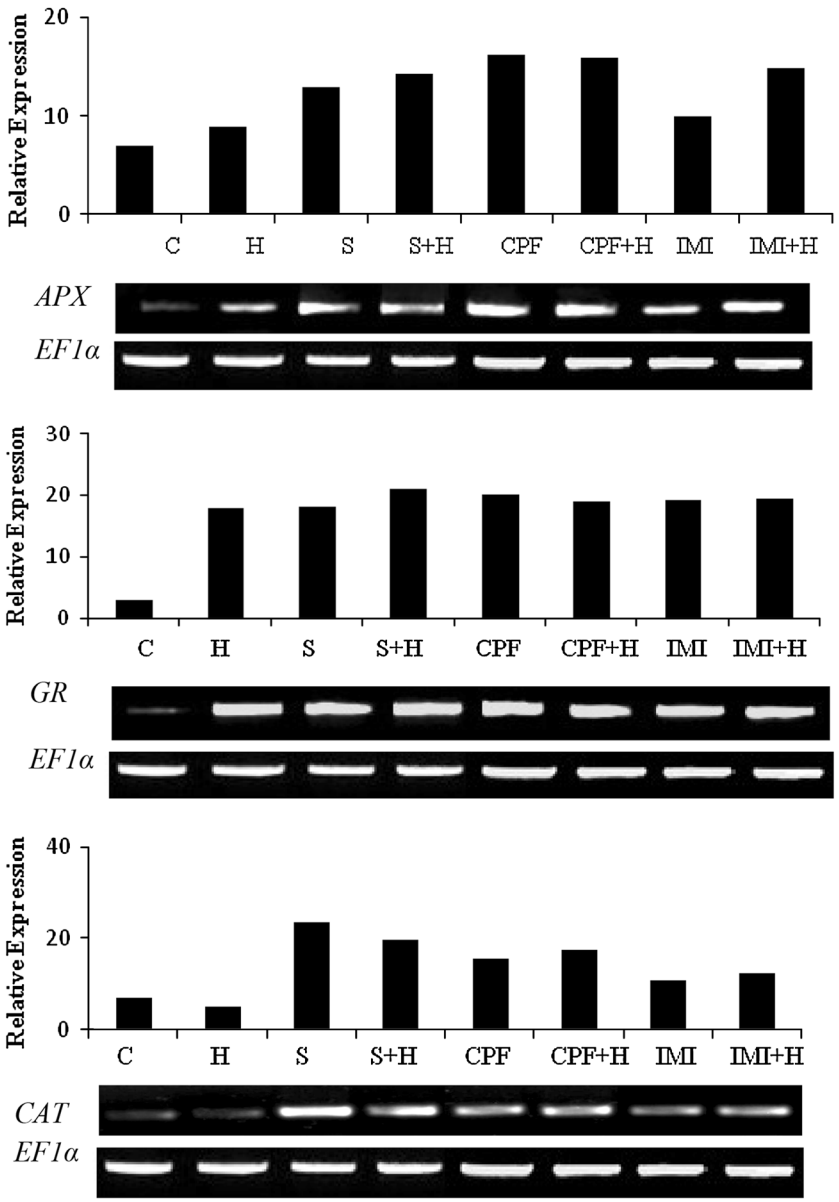


Fig. 2 An ethidium bromide–stained agarose gel harboring products from reverse transcriptase–PCR of 12-day-old rice seedlings exposed to distilled water (control, C), NaCl (S), chlorpyrifos (CPF), imidacloprid (IMI), and their combination with 10^{-7} M HBL (H), NaCl and HBL (S + H), CPF and HBL (CPF + H), IMI and HBL (IMI + H) for various key antioxidant genes. The gel shows the PCR products after 35 cycles of PCR

Fig. 2 continued



(41.7 ± 1.0), and 37 % under IMI stress (37.73 ± 0.7) as compared to control samples (27.6 ± 0.7). Co-treatment with HBL led to a significant elevation of 13 % under NaCl (43.8 ± 0.5), 8 % under CPF (45.4 ± 1.2), 9 % under IMI (41.05 ± 0.7) stress in SOD activity as compared to respective stress conditions alone. Similar results were obtained with other enzymes too. For efficient scavenging of ROS, the activity of APX and SOD should be high so as to remove H_2O_2 produced by superoxide ion dismutation (Pospíšil 2012). Similar to SOD activity, APX activity increased not only under stress conditions but HBL treatment further augmented the activity by 17.4 % (33.38 ± 0.51) under NaCl, 22 % (53.4 ± 1.4) under CPF, and 24 % (35.8 ± 1.2) under IMI stress as compared to respective stress conditions alone. Interestingly in the case of CAT, of all the stresses, only CPF treatment induced the activity as compared to control. Such a result could be hinting toward the CPF-led targeting of peroxisomes resulting in activation of CAT activity for scavenging ROS. Like APX, GR plays an essential role in the protection of chloroplasts against oxidative damage by maintaining a high reduced/oxidized glutathione (GSH/GSSG) ratio (Parween and others 2012). GR activity elevated to 29 % (3.49 ± 0.06) under CPF and 31 % (4.03 ± 0.13) under IMI stress as compared to control revealing pesticide-induced toxicity of chloroplasts. HBL treatment further boosted the activity of GR for enhanced ROS mitigation as well as inactivation of pesticides by GSH-conjugate

formation (Xia and others 2009; Parween and others 2012). In the case of GPX, HBL application elevated the activity under salt and IMI stress as compared to stress conditions alone. DHAR and MDHAR activity either decreased or did not show any change under stress conditions, though a slight increase in DHAR activity under the effect of HBL supplementation with CPF and NaCl was observed as compared to stress alone. Overall, BR application boosted the antioxidant defense system to protect plants against an oxidative burst. BR-induced changes in antioxidant enzyme activities could be due to enhanced protein synthesis or changed kinetic properties of the enzymes (Thao and Tran 2012).

To understand the transcriptional regulation of antioxidant genes under HBL and stress conditions, expression analysis of the key antioxidant genes was done (Fig. 2). The differential response of antioxidant genes under the effect of stress alone as well as in combination with HBL merits some attention. Treatment with salt stress led to the upregulation of all the *SOD* isoforms whereas CPF stress resulted in major upregulation in *Cu/Zn-SOD* and *Mn-SOD* and seedlings treated with IMI stress manifested a pronounced enhancement in the *Fe-SOD* transcript level. Samples co-treated with HBL and different stresses resulted in an enhanced expression of *SOD* isoforms, though to different levels. *Cu/Zn-SOD* expression increased to 1.7-fold in HBL + IMI samples as compared to only IMI-treated sample. In the case of *Fe-SOD*, HBL application resulted in an enhancement in the expression to 1.3-, 2.3-, and 1.3-fold under the NaCl, CPF and IMI stress conditions, respectively, as compared to respective stress conditions alone. Expression of *Mn-SOD* increased to 1.3- and 1.2-fold under the combined treatment of HBL with NaCl and with CPF, respectively, in comparison to stress conditions alone. The differential regulation of certain antioxidant enzymes of the seedlings in response to different types of stresses and hormone treatment could be linked to their cellular localization and targeted effect on specific cell organelles (Gomez and others 2004; Mylona and others 2007). Similarly, in the case of APX, the combined treatment of HBL and stress resulted in a selective upregulation (1.5 fold) under the effect of HBL and IMI as compared to samples treated with IMI alone. The expression of *GR* and *CAT* was enhanced under the effect of stress, whereas the combined treatment of stress with HBL did not result in a significant change in expression. The expression profile for most of the genes corroborated with the activity of the antioxidant enzymes except for CAT expression in samples treated with salt and HBL and *GR* expression, which either decreased or remained unchanged, though a significant increase in the activity was shown. These data indicate that the expression of these genes involves both transcriptional and post-translational regulation. Increased activity and expression of chloroplastic genes *APX*, *GR*, and *Cu/Zn-SOD* under various stresses points toward enhanced ROS generation in chloroplasts under these stresses. However, CPF stress showed marked enhancement in the expression of mitochondrial *Mn-SOD* revealing that CPF may have a more deleterious effect on mitochondrial function. Previously it has been reported that pesticides as well as their adjuvants affect chloroplastic membrane fluidity, inhibition of photosynthetic rate, and photosynthetic electron transport which can be connected to the damage caused by increased ROS production (Caux and Weingerger 1993; Xia and others 2009; Chris and others 2011). The diverse effects of the different stress conditions on mRNA abundance of various genes thus provide an insight into the mode of action of each treatment and their effect on the different subcellular compartments (Gomez and others 2004; Mylona and others 2007).

Conclusion

Based on the present study, it is concluded that HBL helps restore the homeostasis of rice plants under different stress conditions and makes the plant better equipped to fight stress conditions. Stress amelioration is facilitated by BR impacts on various regulatory mechanisms like accumulation of osmolytes, improved pigment content, reduction in lipid peroxidation, reduced accumulation of ROS and enhanced activity, and expression of antioxidative defense genes. All these factors contribute to the array of protective mechanism employed by plants for combating the cytotoxic levels of various stresses including salt and pesticides. In conclusion, this study showed potent activity of BRs in stress response which is important not only for understanding the essential role of this plant growth regulator to a variety of stresses but also for its futuristic application in agriculture.

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