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Research Article

Oxidative Membrane Lipid Peroxidation and Accumulation of Redox-Sensitive Polyphenolic Compounds Serves as Sensitive Redox-Metabolic Biomarkers of Drought Stress of Rice

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Abstract

In this paper, an effort have been made to standardize parameters of oxidative membrane damage (membrane lipid) and elicitation ability of polyphenolic compounds for the assessment of drought stress tolerance of an Indigenous aromatic rice cultivar, commonly cultivated in West Bengal, India. Imposition of different magnitude of drought stress (-0.344 MPa, -0.851 MPa, -1.619 MPa) at germination stage (through Polyethyleneglycol-6000 treatment) to the experimental cultivar revealed a dose-dependent response in terms of accumulation of total ROS, thiobarbituric acid reactive substances, conjugated diene and hydroperoxide and activity of lipoxygenase. The level of accumulation of all these oxidative stress parameters further showed strong correlation with germination and early growth parameters. Treatment, which exhibited highest accumulation of hydroperoxide, thiobarbituric acid reactive substances and conjugated diene, exhibited lowest germination and early growth performances (T50 value, relative germination performance, relative growth index, germination rate index etc.). RP-HPLC of extractible phenolics from seedlings raised from different magnitude of drought stress, exhibited dose-dependent accumulation of phenolic acids (gallic acid, chlorogenic acid, caffeic acid, sinapic acid, p-coumaric acid) and flavonoids (rutin, kaempferol, myricetin, apigenin). The accumulation of flavonoids and phenolic acids in drought stress raised seedlings found to be inverse to the oxidative membrane lipid damages and has strong impact on subsequent germination.

Keywords: Drought; Lipid peroxidation products; Phenolics; Stress biomarker

Introduction

Drought is the single most important environmental constraint that limit plant growth and development and limit productivity of crops [1,2]. Monitoring the effect of drought stress is extremely important from the point of view of its important assessment and screening. Selection of judicious reliable metabolic parameters for the drought stress impact assessment and screening depends on their sensitivity, reproducibility and application.

Drought, depending its severity, duration and stage of impact, largely affect the redox homeostasis, of plants by altering ROS-antioxidant interaction at metabolic interface [3,4]. Decontrolled ROS generation always leads to oxidative deterioration of cellular macromolecules [3,5]. Membrane lipids are one of the most important immediate targets of the drought induced oxidative stress [3,4]. Membrane Lipid Peroxidation (MLP) is the single most important response that not only gets aggravated but also contributes further to the oxidative stress induced by drought [3,6].

Peroxidation of membrane lipid is one of the very few example of carbon centred ROS production event in plant cell which essentially involves initiation, prevention, termination and propagation events [3]. The mechanism is basically triggered by abstraction of hydrogen from fatty acids or addition of ROS of membrane lipid, causing breakdown of membrane based PUFAs. Subsequently the oxygenation of carbon centred lipid radical (L·) takes place forming peroxy radicals (LOO·). LOO· formed subsequently generates organic hydroperoxides. Alkyl radicals formed can be stabilized with the formation of conjugated diene. In several instances, the enzyme lipoxygenase found to mediate the process, although there are strong evidences of non-enzymatic ROS-mediated membrane lipid peroxidation [3,7].

The products of oxidative MLP often been used by several workers to assess environmental stress assessment [8,9,7, 10]. Infact several analytical techniques are in practice for the assessment of stress induced MLP as a contrivance of understanding changes in redox status of tissue or oxidative stress suffered by plant under stress.

Plant's ability to withstand drought stress largely depends on efficiency of antioxidative defense system that reflects ROS-antioxidant interaction at metabolic level and determine the redox fate of the tissue [11,12]. Out of an array of antioxidative defense system, some secondary metabolites like polyphenolic compounds exerts strong antioxidant functions through their ROS scavenging

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activity, metal chelating activity etc. [13,14]. In most of the cases an up-regulation of synthesis of poly phenolic compounds are found to be associated with environmental odds like drought [15-17]. Therefore, there are scattered evidences of drought stress induced accumulation of phenolic compounds but they are associated with redox regulation and mitigation of oxidative membrane damage and not yet been explored properly. Therefore, in the present work an effort have been made to explore the relationship between drought induced oxidative MLP and elicitation of polyphenolic compounds for the mitigation of oxidative stress. Further, the parameters of oxidative MLP and accumulation of redox-sensitive flavonoids and phenolic acids under drought were critically analysed for their suitability as redox biomarker of drought stress.

Materials and Methods

Plant growth and treatment of PEG-6000 to induce post imbibitional dehydration stress

As experimental material, seeds of the Indigenous Aromatic Rice Cultivar (IARC *Oryza sativa* L., Cultivar Badshabhog) was selected. It was collected from Crop Research and Seed Multiplication Farm (CRSMF), The University of Burdwan, West Bengal, India. Experimental seeds were washed with distilled water and then they were treated with 0.2% $HgCl_2$ for 5 minutes for surface sterilization and again washed thrice with sterile distilled water. Then the seeds were kept for imbibition in distilled water in darkness at 25°±2°C for 48 hours. Thereafter, they were sown in Petri plates on moist filter paper and were placed in seed germinator cum stability chamber in standardized conditions with 14 hour photo period (light intensity 270 µmol m^{-2} s^{-1}), 78±2% relative humidity and 25°±2°C temperature. PEG-6000 was used for imposing post imbibitional drought stress of different magnitude. Out of four different water-imbibed seed lots, three were treated daily with -0.344 MPa, -0.851 MPa and -1.619 MPa PEG-6000 and fourth seed lot was sown in petriplates containing moist filter paper absorbing sterile distilled water, representing untreated control set. All seed lots were allowed to grow for 7 days in aforesaid conditions and then used for biochemical analyses.

Estimation of membrane lipid peroxidation

Test for Accumulation of Thiobarbituric Acid Reactive Substances (TBARS) to estimate membrane lipid peroxidation was performed following the process of [18]. Seedling sample of 200 mg was homogenized with 0.1% Trichloroacetic Acid (TCA) and centrifuged at 10,000 rpm for 15 minutes. Supernatant was collected. 1mL of supernatant and 3 mL of 5% TCA containing 1% Thiobarbituric Acid (TBA) were mixed and was kept in hot water bath for 30 minutes. After cooling immediately in cold water bath, again centrifuged at 10,000 rpm for 10 minutes. The spectrophotometric estimation of the supernatant at 530 nm was done. The measurement of concentration of TBARS was calculated by its extinction coefficient (155 µM cm^{-1}) and expressed in n mol g^{-1} dry mass of seedling tissue.

Estimation of Hydroperoxide

The method of [19] was followed with some necessary modifications for estimation of hydroperoxide. Seedlings were homogenized with 150 mM tris-HCl (p^H-6.8) and supernatant was collected after proper centrifugation. The assay mixture containing an aliquot of sample extract, 0.25 mM H_2SO_4, 250 mM ammonium ferrous sulphate, 4 mM BHT (in 90% methanol) and 100 mM xylenol orange was incubation at room temperature for 30 minutes. Then triphenyl phosphine (100 mM) was added and the absorbance was taken at 560 nm.

Estimation of Conjugated diene

Conjugated diene was estimated by following the method of [20]. Seedling tissue was homogenized with chloroform: methanol mixture (2:1). After vigorous vortexing the homogenate was centrifuged at 2000 rpm for 10 minutes. From the supernatant the lower chloroform layer was collected and dried at 45°C under steam of nitrogen by rotary vacuum evaporator. The obtained residue was dissolved in cyclo-hexane and measured at 230 nm.

Estimation of Lipoxygenase activity

Lipoxygenase activity was measured by following the method of [21]. For the extraction of enzyme centrifugation at 5000 rpm was performed. Then re-centrifugation performed at 17000 rpm in cold condition by adding 50 mM sodium-phosphate buffer (p^H-6.5). Then the assay mixture was made containing enzyme extract, 1.3 mM linoleic acid and 1.65 mM sodium-phosphate buffer (p^H-6.5). The assay mixture was kept for incubation for 1 hour at 25°C. Finally absorbance was taken at 234 nm.

Estimation of total ROS generation

Total ROS generation was estimated by following the method of [22]. 30 mg of seedling tissue of cultivar Badshabhog was put in 40 mM TRIS–HCl buffer (p^H-7.0) containing lM 2', 7'-dichlorofluorescindiacetate at 30° C. After 60 min, supernatant was removed and fluorescence was monitored in a spectrofluorometer (Hitachi, Model F-4500 FL Spectrophotometer) with excitation at 504 nm and emission at 525 nm. Additional controls were performed to differentiate ROS from other long-lived substances able to react with DCFDA. For additional controls, tissues were incubated without DCFDA (60 min) and then tissues were removed. Then DCFDA was added. After 60 min, fluorescence was determined. To assess the fluorescence of ROS, this florescence values was subtracted from all readings.

Quantitative assessment of phenolic acids and flavonoids by RP-HPLC

Sample preparation: Samples were prepared from dry powdered seedlings through soxlet mediated hydro-ethanolic extraction. Further, the sample was concentrated by using rotary vacuum evaporator. For HPLC study, the volume of sample taken was 20µl [23].

RP-HPLC analysis of phenolic acids and flavonoids: Dionex Ultimate 3000 liquid chromatograph including a Diode Array Detector (DAD) with 5 cm flow cell and with Chromeleon system manager as data processor was used for HPLC analyses. Separation was achieved by a reversed-phase Acclaim C18 column (5 micron particle size, 250 x 4.6 mm). All extracted solutions were filtered through HPLC filter 0.45 mm membrane filter (Milipore). Methanol (Solvent A) and 0.5% aquous acetic acid solution (Solvent B) were used in mobile phase and the column was thermostatically controlled at 25°C. The injection volume was of 20 µl. A gradient elution was performed by varying the proportion of solvent A to solvent B. Phenolic acids and flavonoids in the sample extracts were quantified by the measurement of the integrated peak area. The contents were

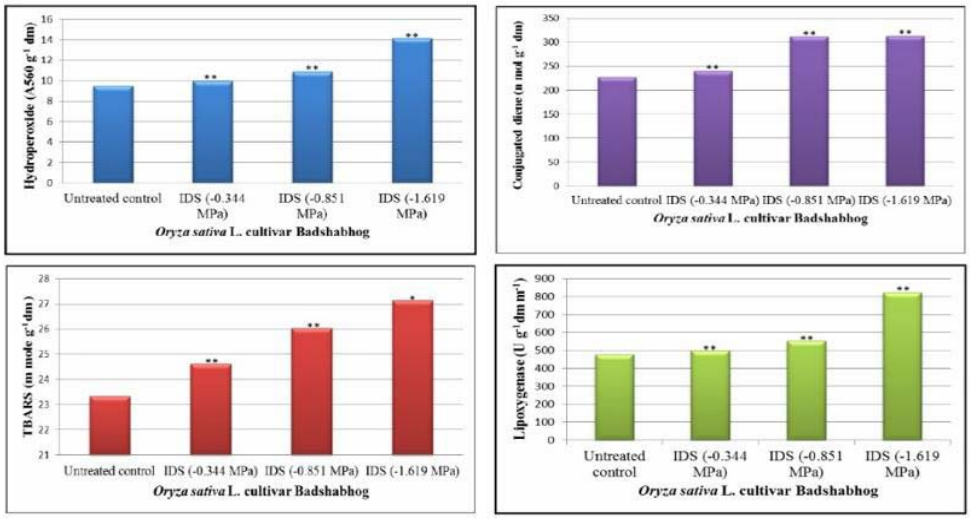

Figure 1: Impact of different magnitude of post imbibitional dehydration stress (PIDS: -0.344 MPa, -0.851MPa and -1.619 MPa) on enzymatic membrane lipid peroxidation (assessed in terms of accumulation of hydroperoxide, conjugated diene, TBARS and lipoxygenase activity) of Indigenous aromatic rice cultivar (IARC-Badshabhog). Results are mean of three replicates ± standard error. *Significant from control at 0.05 level (t-test). **Significant from control at 0.01 level (t-test).

calculated by using the calibration curve by plotting peak area against concentration of the respective standard sample. Standard stock solutions of eight flavonoids and forteen phenolic acids like Catechin, Naringin, Rutin, Myricetin, Quercetin, Naringenin, Apigenin and Kaempferol, Gallic acid, Protocatechuic acid, Gentisic acid, p-Hydroxy benzoic acid, Chlorogenic acid, Vanillic acid, Caffeic acid, Syringic acid, p-Coumaric acid, Ferullic acid, Sinapic acid, Salicylic acid, Ellagic acid were prepared in methanol (10 µg ml^{-1}). All standard solutions was filtered through HPLC filter 0.45 mm membrane filter (Milipore).

Determination of germination performances and post germinative growth performances

Germination and early growth performances [24,25,26] of PIDS-raised IARCs vis-a- vis their untreated control were assessed in terms of Germination Rate Index (GRI), T_{50} value of germination, Relative Growth Index (RGI) and Relative Germination Performance (RGP) by the formulae as follows:

Germination Rate Index (GRI)

$$\Sigma\left(\frac{Ni}{i}\right)$$

T_{50} value

Time (In hour) of 50% germination of seeds sown

Relative Growth Index (RGI)

$$\frac{\text{average dry mass of ten treated seedlings}}{\text{average dry mass of ten control seedlings}}\times 100$$

Relative Germination Performance (RGP)

$$\frac{\text{Percentage of germination under treatment}}{\text{Percentage of germination under control}}\times 100$$

Results and Discussion

The oxidative damage of membrane lipid that aggravates under PIDS of the experimental aromatic rice cultivars can be exploited as reliable index of secondary oxidative stress, which convey the status of internal redox cue. All the parameters pertaining oxidative lipid peroxidation like accumulation of ROS, TBARS, Hydroperoxide (HPOX), Conjugated Diene (CD) and activity of Lipoxygenase (LOX) were assessed in seedlings raised from different magnitude of PIDS (-0.344MPa, -0.851MPa, -1.619MPa) (Figure 1), (Table 1). The result in general showed a dose-dependent aggravation of MLP. When the extent of MLP was assessed in terms of accumulation of TBARS in the seedlings raised from different magnitudes of PIDS, a dose-dependent increament of MLP was observed (Figure 1). The same trend of result was observed when we assessed and compared the accumulation of other secondary MLP products like HPOX and conjugated diene (Figure 1). There is infact an increament of 5.53%, 49.74% and 37.39% TBARS, HPOX and CD in PIDS (-1.619MPa)-raised seedlings over their corresponding untreated control. The increament of lipoxygenase for -0.344MPa and -0.851MPa-raised seeling though found to be only marginal but -1.619MPa-raised seedlings exhibited significant increament (Figure 1). Therefore, the result in general exhibited significant dose dependent accumulation of all oxidative lipid peroxidation products (DCFDA oxidation, TBARS, HPOX and CD) with correspondingly enhanced activities of LOX in PIDS-raised sedlings.

In order to evaluate the role of polyphenolic compounds in the regulation of PIDS induced redox homeostasis in the experimental rice seedlings, RP-HPLC based identification and quantitative estimation important redox sensitive flavonoids and phenolic acids was done. The result in general showed significant dose-dependent accumulation of rutin, kaempferol, myricetin, apigenin, gallic acid, chlorogenic acid, caffeic acid, sinapic acid and p-coumaric acid in PIDS raised seedlings over untreated control, implying significant role in redox-regulation (Figure 2). Out of all these compounds, Rutin and p-coumaric acid showed maximum elicitation under drought.

For understanding whether PIDS-induced oxidative MLP has any impact on germination or early growth performances, several

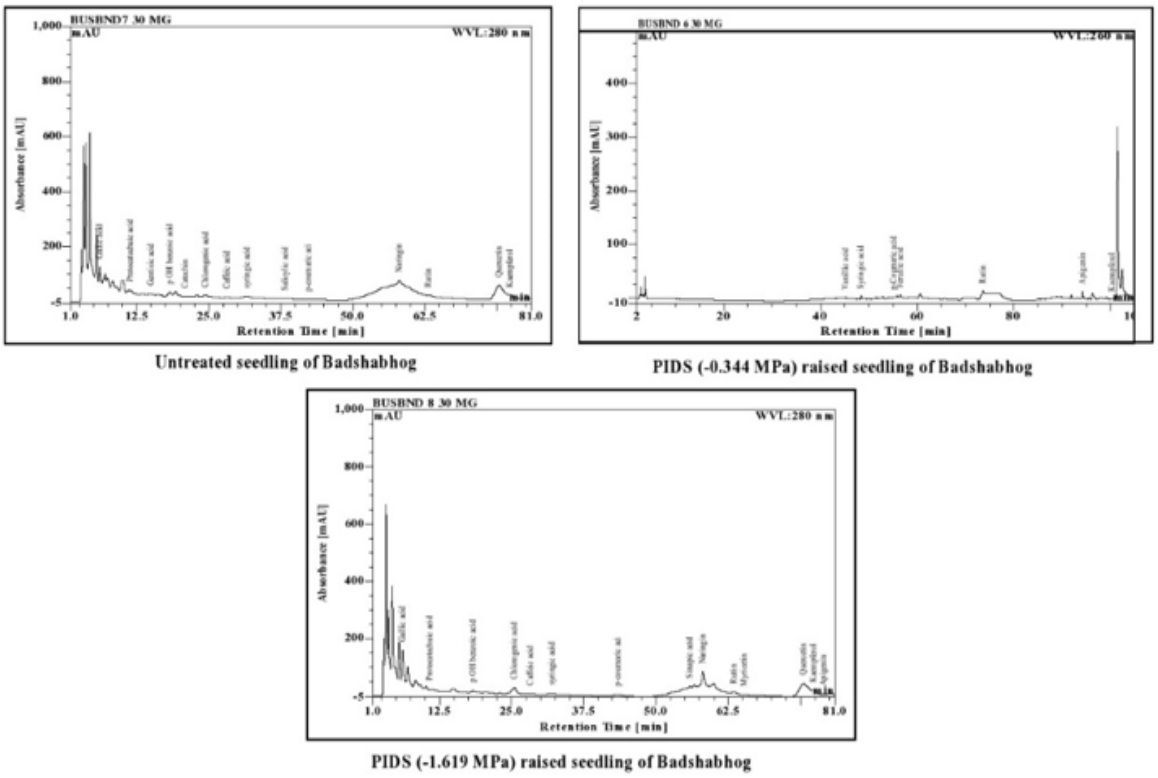

Figure 2: HPLC chromatogram of extracted polyphenolic compounds from seedlings of Indigenous aromatic rice cultivar (IARC-Badshabhog) raised from different magnitude of post imbibitional dehydration stress (PIDS; -0.344 MPa and -1.619 MPa) and untreated control.

Table 1: Impact of post imbibitional dehydration stress of different magnitude (PIDS: -0.344 MPa, -0.851 MPa and -1.619 MPa) on the synthesis of redox-sensitive polyphenolic compounds (rutin, kaempferol, myricetin, apigenin, gallic acid, chlorogenic acid, caffeic acid, sinapic acid and p-coumaric acid), oxidative membrane damage (total ROS, conjugated diene and hydroperoxide) and early growth performances of the Indigenous aromatic rice cultivar (IARC- Badshabhog). Results are mean of three replicates ± standard error. *Significant from control at 0.05 level (t-test). **Significant from control at 0.01 level (t-test).

Experimental cultivar	Treatment (MPa)	Accumulation of flavonoids (µg/100g dm)				Accumulation of phenolic acids (µg/100g dm)					Accumulation of total ROS (DCFDA oxidation) (AU mg^{-1} dm)	Accumulation of conjugated diene (n mol g^{-1}dm)	Accumulation of hydro-peroxide (A560 g^{-1} dm)	Germination and early growth performances			
														T_{50} (hour)	RGP	GRI (day^{-1})	RGI
		RUTIN	KAEMPFEROL	MYRICETIN	APIGENIN	GALLIC ACID	CHLOROGENIC ACID	CAFFEIC ACID	SINAPIC ACID	P-COUMARIC ACI							
Badshab-hog	Untreated control	4.8	1.6	-	-	73.2	57	1.77	-	0.03	98.68±0.16	227.63±0.54	9.47±0.02	75 ±0.23	100±0.00	43.15±0.07	100±0.00
	-0.344	6.07	2.60	-	0.46	-	-	-	-	-	53.28±0.10**	239.25±0.51**	9.95±0.04**	120±0.41	120.00±0.68	44.14±0.07**	104.12±0.68
	-0.851	-	-	-	-	-	-	-	-	-	92.30±0.11**	311.63±0.49**	10.91±0.05**	100±0.82	100.00±0.27	41.82±0.03**	101.03±0.41
	-1.619	16.77	2.70	21.67	**7.73**	86.33	65.27	1.87	3.83	9.57	113.41±1.40*	312.75±0.30**	14.18±0.06**	100±0.27	92.00±0.22**	36.74±0.05**	109.28±0.98

parameters like Relative Germination Performance (RGP), T50 value, Germination Rate Index (GRI) and Relative Growth Index (RGI) were assessed and compared. The result in general exhibited significant inverse correlation between oxidative MLP and early growth performances (Table 1). The greater the magnitude of oxidative MLP under PIDS more was the inhibitory impact on germination and early growth performances. Regression analysis between germination/early growth parameters (T50 value, GRI, RGI and RGP) and parameters

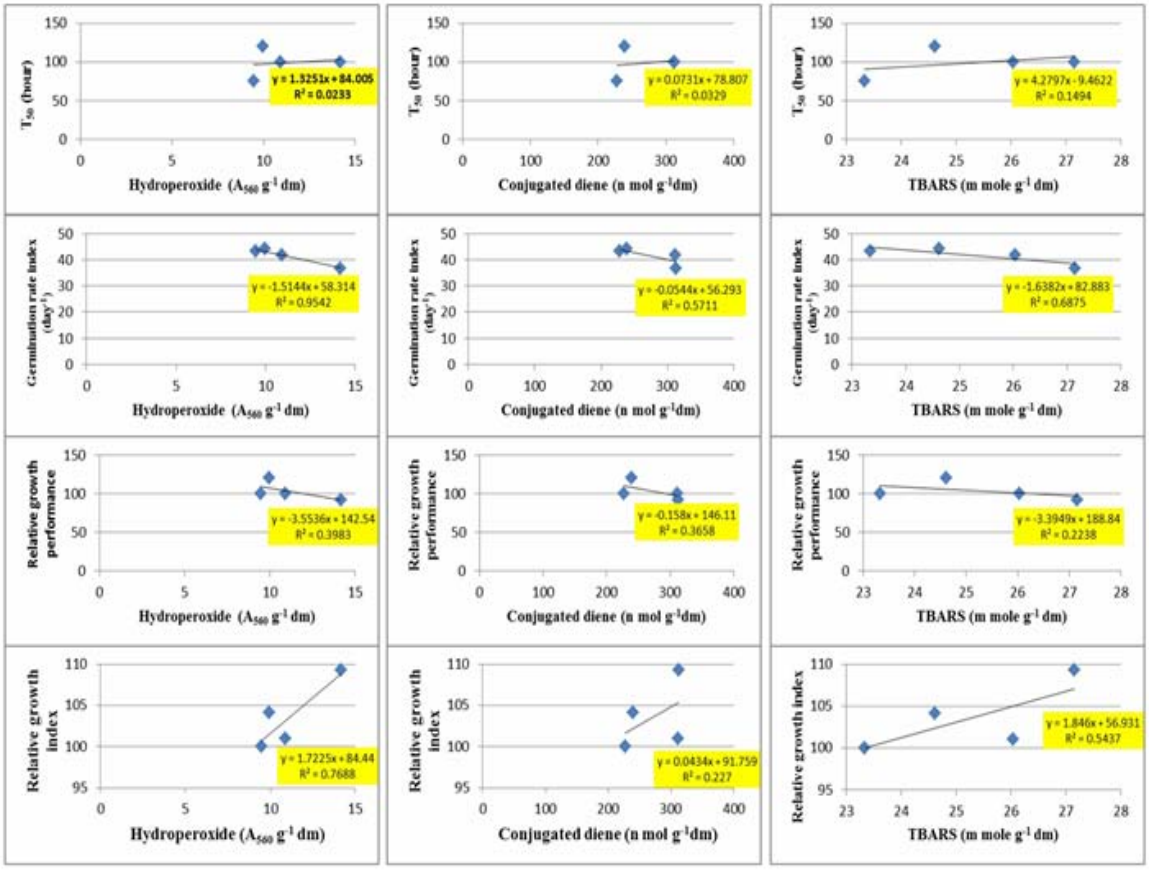

Figure 3: Regression curve showing the relationship between early growth parameters and the product of oxidative membrane lipid peroxidation (Hydroperoxide, TBARS and conjugated diene).

of oxidative MLP (HPOX, CD and TBARS) showed significant correlation (R^2 value) in most of the cases (Figure 3).

In the present investigation, osmotic stress was imposed to germinating seedling of experimental aromatic rice cultivar (Badshabhog) by using PEG-6000 in different doses in the growing media. PEG being inert, non-ioning, impermeable and water binding polymer mimic drought stress to the experimental plants [27,10]. Drought stress induced changes in internal redox homeostasis due to imposition of secondary oxidative stress is a well-recognized phenomenon that has direct connection with growth, development and yield of the crops [28,7,27].

Present experiment entitled a strong correlation between dose-dependent PIDS-induced oxidative membrane damage and early growth performances of germinating seeds. The oxidative membrane damage was assessed in terms of accumulation of ROS (DCFDA oxidation) and enzymatic MLP (assessed in terms of accumulation of HPOX, CD and TBARS and LOX activity). Oxidative MLP is one of the prime event under drought that possess several adverse and physiological consequences [29,7,6]. This process disrupts not only membrane architecture but also generates ROS and other toxic secondary products, further aggravating the membrane damage and cellular homeostasis [5,6,12,29].

Conjugated dienes, formed from oxidation of PUFAs as the intermediates of MLP along with Malondialdehyde and other hydroperoxides the end products of MLP are accepted markers of oxidative stress [6,12,30,31]. Therefore, in the present study, the assessment of byproducts of MLP under different magnitude of PIDS exhibited strong correlation with germination and early growth performances of the experimental rice cultivar. This result also strongly substantiated by other studies [27,32,33,7].

The role of non-enzymatic antioxidants like phenolic acids and flavonoids are tested in PIDS raised redox-regulation of experimental rice seedlings. The result in general showed up-regulation of synthesis of some redox sensitive phenolic acids and flavonoids (rutin, kaempferol, myricetin, apigenin, gallic acid, chlorogenic acid, caffeic acid, sinapic acid and p-coumaric acid). In fact, all these compounds are derived from their important metabolic routes viz. phenylpropanoid pathway, pentose-phosphate pathway and sikkimic acid pathway. There are instances of strong elicitation of several phenolic acids and flavonoids under environmental stress [34,35]. Apart from other important physiological roles both flavonoids and phenolic acids exert their strong antioxidative properties because of their strong reducing ability through H-donation to ROS, quenching of $^1O_2$ and metal chelation [36,37,12]. Flavonoids can also up-regulate antioxidative defence system and regulate redox homeostasis of the cell [38]. Flavonoids even can mitigate MLP by interfaring with chain propagation reaction [12]. Rutin in particular chelate iron (Fe),

necessary for MLP [38].

Conclusion

The susceptibility of newly assembled membrane system towards oxidative MLP and elicitation of redox-sensitive polyphenolic compounds to combat such deteriorative event under PIDS to the aromatic rice cutivar determine the redox status of the germinating tissue and early growth performances as well. Estimation of parameters of MLP (HPOX, TBARS, CD, LOX activity, DCFDA oxidation) along with redox sensitive flavonoids and phenolic acids can be used as sensitive biomarkers of drought stress.

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