Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; SP1: 898-907

Sareeta Nahakpam
Department of Biochemistry and Crop Physiology, Bihar Agricultural University, Sabour, Bhagalpur, Bihar, India

Correspondence
Sareeta Nahakpam
Department of Biochemistry and Crop Physiology, Bihar Agricultural University, Sabour, Bhagalpur, Bihar, India

Combined effect of cadmium toxicity and heat alter protein, lipid peroxidation and production of reactive oxygen species in growing rice seedlings of *cvs*. DR-92 and Bh-1

Sareeta Nahakpam

Abstract
Results suggest variation in stress response with the type of stress is given either singly or in combination of Cd^{2+} and heat to seedlings of rice *cvs*. DR-92 and Bh-1. Increased production of H_2O_2 and $O_2^{\cdot-}$ levels showed higher under combined stress. Elevated levels of TBARs indicated membrane damage in both roots and shoots. Maximum level of total soluble protein were noted under 100 µM Cd^{2+} + heat stress in *cv*. Bh-1 and in 500 µM Cd^{2+} + heat stress in *cv*. DR-92 at 15 day of growth. The study revealed an altered protein level under Cd^{2+} toxicity alone and Cd^{2+} + heat stress. Synthesis of stress specific LMW (20 to 60 kDa) and HMW (90 to 200 kDa) proteins under Cd^{2+}, heat and Cd^{2+} + heat stress were recorded in both the rice cultivars. In *cv*. DR-92 five new protein bands (S1-S5) were present in shoots under 500 µM Cd^{2+} + heat treatments. In roots of *cv*. DR-92 two new protein bands R1 (HMW, ~90-100 kDa) and R2 (LMW, ~55-58 kDa) were present under 10 µM Cd^{2+} + heat treatments. The induction of both HMW and LMW and an increase in the intensity of already existing proteins under combined effect of Cd^{2+} and heat stress suggest the involvement of both constitutive and inducible cytosolic proteins in stress combating phenomenon. Of the two rice cultivars *cv*. Bh-1 appears to better adapted to Cd^{2+} stress alone as well as in combination with heat stress, than *cv*. DR-92.

Keywords: Cadmium, heat, reactive oxygen species, TBARs, soluble protein.

Introduction
Changes in environmental conditions make plants suffer from various physiological and biochemical alterations [1, 2]. Various abiotic stresses like drought, salinity, extreme temperatures, high irradiance, UV light, nutrient deficiency, air pollutants, etc. cause molecular damage to plants either directly or indirectly through the formation of ROS [3, 4]. Organisms showing aerobic metabolism face constant risk from reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2), which are inevitably generated via number of metabolic pathways [5, 6]. Although ROS can induce considerable cellular damage, these molecules are also important signaling compounds, controlling the expression of stress-tolerance genes [7]. The fine line distinguishing between ROS as a threat to the cell and ROS as signaling molecules is a grey zone given the apparently well orchestrated bursts of ROS being produced during stress exposure [7].
Heavy metal cadmium is not known to have a biological or physiological function and therefore gets accumulated in plants [8] inducing a number of deleterious effects at morphological level. Cadmium brings about changes in the biochemical and physiological processes that lead to cellular damage [9, 10, 11, 12, 13] caused due to oxidative stress [14] by direct or indirect formation of active oxygen species [14]. There are recent reports that Cd^{2+} causes series of active oxygen species production namely NADH oxidase-dependent accumulation of hydrogen peroxide, followed by the accumulation of superoxide anion in mitochondria and subsequently fatty acid hydroperoxide accumulation in the tissues [15]. Also accumulation of ROS due to the presence of Cd^{2+} may damage different cell structure and function through oxidation of several macro-molecules [16].
Uptake and accumulation of Cd^{2+} in plant tissues is associated with/or bound to proteins as in *Phragmites australis* [17] the binding thereby leading to the denaturation of proteins. However, an increase in protein levels is also reported in rice [8] with the possibility of synthesis of new stress specific proteins [18] and inhibition of the activity of proteolytic and/or other antioxidative enzymes [19, 20, 21].
During summertime, soil systems may increase in temperature beyond the upper extreme of the organism tolerance [22]. Heat stress singly or in combination with other stress is a common constraint in many of the cereal crops [23]. High temperature induces severe cellular

injury and even cell death [24]. Direct heat injuries lead to protein denaturation and aggregation, and increased fluidity of membrane lipids. Indirect or slower heat injuries include inactivation of enzymes in chloroplast and mitochondria, inhibition of protein synthesis, protein degradation and loss of membrane integrity [25]. Dehydration of tissues and autocatalytic peroxidation of membrane lipids and pigments leading to the loss of membrane semi-permeability and modifications in membrane functions as a result of heat imposition is also reported [26, 18].

As Cd^{2+} and heat are two major abiotic stresses and studies related to a combined effect of the two stresses on the metabolic alterations in plants are very few, the present investigation was undertaken to examine the levels of total soluble protein, production of reactive oxygen species, extent of lipid peroxidation and study the Cd^{2+} and/or heat stress induced changes in protein profile in the seedlings of the two rice cultivars DR-92 and Bh-1 growing under increasing levels of $Cd(NO_3)_2$ in the growth medium and subjected to heat injury.

Materials and Methods

Plant material and stress treatments

Seeds from two rice *cvs*. DR-92 and Bh-1 were surface sterilized with 0.1 percent sodium hypochlorite solution and then imbibed in water for 24 h. Seedlings were raised in sand cultures in plastic pots either with Hoagland nutrient solution [27] that served as control or nutrient solution supplemented with 10 µM, 50 µM, 100 µM and 500 µM $Cd(NO_3)_2$ as treatment solutions [13]. The Cd^{2+} treatment levels were ascertained as low toxic (10 µM, 50 µM) and high toxic (100 µM, 500 µM) concentrations. Pots were maintained at field saturation capacity at pH 7.0 and irrigation was done when required. Seedlings were uprooted at 5 day intervals and roots and shoots served as Cd^{2+} treated plant samples. For heat treatments the control as well as Cd^{2+} treated seedlings were uprooted at 5-day intervals and subjected to 40°C temperatures for 2 h. All the experiments were performed in triplicate.

Measurement of reactive oxygen species

Estimation of hydrogen peroxide (H_2O_2)

The H_2O_2 level in root and shoot samples of rice from the two rice cultivars DR-92 and Bh-1 were determined according to the method of Jana and Chaudhuri [28]. Extraction was done by homogenizing 150 mg tissues with 5 ml of 50 mM phosphate buffer (pH 6.5). The homogenate was centrifuged at 6000 x g for 25 minutes. 3 ml of the supernatant was mixed with 1 ml of 0.1 percent titanium sulphate in 20 percent sulphuric acid. The mixture was then centrifuged at 6000 x g for 15 minutes. Intensity of yellow color developed was measured at 410 nm. The amount of H_2O_2 was calculated using an extinction coefficient of 0.28 μM^{-1} cm^{-1} and expressed as µmol g^{-1} dry wt.

Measurement of superoxide anion ($O_2^{\cdot-}$)

The formation of superoxide anion was measured spectrophotometrically by the method of Chaitanya and Naithani [29]. A 200 mg fresh root and shoot tissues were homogenized under a N_2 atmosphere in cold condition (0-4°C) in 100 mM sodium phosphate buffer (pH 7.2) containing 1 mM diethyl dithiocarbamate to inhibit SOD activity. The homogenate was then centrifuged at 22,000 x g for 20 minutes and supernatant was used for assay. Assay mixture in a total volume of 3 ml contained 100 mM sodium phosphate buffer (pH 7.2) with 1 mM diethyl dithiocarbamate, 0.25 mM NBT and the supernatant. Absorbance was read at 540 nm and was expressed as ΔA_{540} min^{-1} mg^{-1} protein.

Estimation of lipid peroxidation

Lipid peroxidation in root and shoot samples of both the rice *cvs*. DR-92 and Bh-1 were estimated in terms of thiobarbituric acid reactive substances (TBARS) by the method of Heath and Packer [30]. About 200 mg fresh tissues were homogenized in 0.25 percent 2-thiobabituric acid (TBA) in 10 percent trichloroacetic acid (TCA) using a mortar and pestle. After heating on a boiling water bath for 30 minutes the mixture was cooled on ice. The contents were centrifuged at 10,000xg for 10 minutes. The absorbance of the supernatant was read at 532 nm and adjusted for nonspecific absorbance at 600 nm. The blank was 0.25 percent TBA in 10 percent TCA. The concentration of lipid peroxides together with oxidatively modified proteins of plant were quantified in terms of TBARS using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} dry wt.

Extraction and estimation of total soluble protein

The root and shoot samples from the two rice *cvs*. DR-92 and Bh-1 were used. The samples were homogenized in 5 ml of 0.2 M sodium phosphate buffer (pH 6.5) containing 0.5 M NaCl. After centrifugation at 10000x g, the soluble proteins were precipitated from the supernatant with 1 ml of 10 percent TCA and dissolved in 0.1 N NaOH. The absorbance of the color developed was recorded at 660 nm according to the method of Lowry *et al*. [31] using BSA (Sigma) as standard.

SDS-PAGE analysis for protein profile under Cd^{2+} toxicity and heat

Protein extracts from roots and shoots of 15 day old rice seedlings from control, 10, 50, 100 and 500 µM Cd^{2+} treated seedlings and Cd^{2+} treated seedlings subjected to heat treatments were run on SDS-polyacrylamide gel electrophoresis according to the method of Laemmli [32]. Equal amounts of protein (40 µg) were separated in 12.5 percent polyacrylamide gels (7.5 percent stacking) during 3 h at 4ºC with a constant current of 30 mA per gel using Mini-PROTEAN 3 Electrophoresis system (Biorad, Hertz, UK). The gel was run using an electrode buffer (pH 8.3) containing 25 mM Tris-base, 192 mM glycine and 0.1 percent SDS. After the run, gel were stained with 0.125 percent Coomassie Blue R-250 in a mixture of methanol:acetic acid:water. After destaining protein were located as dark blue bands on the gel. Standard protein markers (10-200 kDa ladder) were used to ascertain the molecular mass of the proteins on the gel.

Statistical analyses

All the experiments were performed in triplicate. Values in the figures indicate mean ± SD based on three independent experiments. The significant differences were assessed by the analysis of variance (ANOVA) test, taking $P<0.05$ as significant according to Turkey's multiple range.

Results

Effect of Cd^{2+} toxicity and heat on hydrogen peroxide level

Effect of Cd^{2+} toxicity and heat stress on the levels of hydrogen peroxide in the two rice *cvs.* DR-92 and Bh-1 is shown in Figure 1. The values of H_2O_2 were always higher under abiotic stress conditions and showed a gradual increase with increasing levels of Cd^{2+} as and when compared with

controls. The concentration of H_2O_2 increased by 40 percent at day 10 in roots and shoots of *cv*. DR-92 grown under 50 µM Cd^{2+} treatments as compared to controls and a 45 percent increase was noted under combined effect of 50µM Cd^{2+} and heat stress (Figure 1). In *cv*. Bh-1, a 50 percent increase in H_2O_2 levels were noted earlier at day 5 of growth period in controls as well as in 10 and 50 µM Cd^{2+} treatments with heat stress.

µM Cd^{2+} treatments alone, the content of H_2O_2 in both roots and shoots were significantly higher at day 15 in *cv*. DR-92 which further increased under Cd^{2+} + heat treatments at the same day of growth period. Maximum level of H_2O_2 were observed in roots of 100 µM Cd^{2+} and heat treatments in *cv*. DR-92, whereas in *cv*. Bh-1 maximum H_2O_2 generation were observed in 500 µM Cd^{2+} + heat treatments in roots and 100 µM Cd^{2+} + heat treatments in shoots at 20 and 10 day respectively of the growth period. A higher production of H_2O_2 is observed later (i.e at day 15) in *cv*. DR-92 in both roots and shoots when compared to *cv*. Bh-1 where the significant increase in H_2O_2 levels were noted at day 10 of growth period. Moreover, beyond day 10 of growth period a concomitant increase in H_2O_2 levels were observed with increasing days of growth and under all stress treatments in the seedlings of both the rice cultivars.

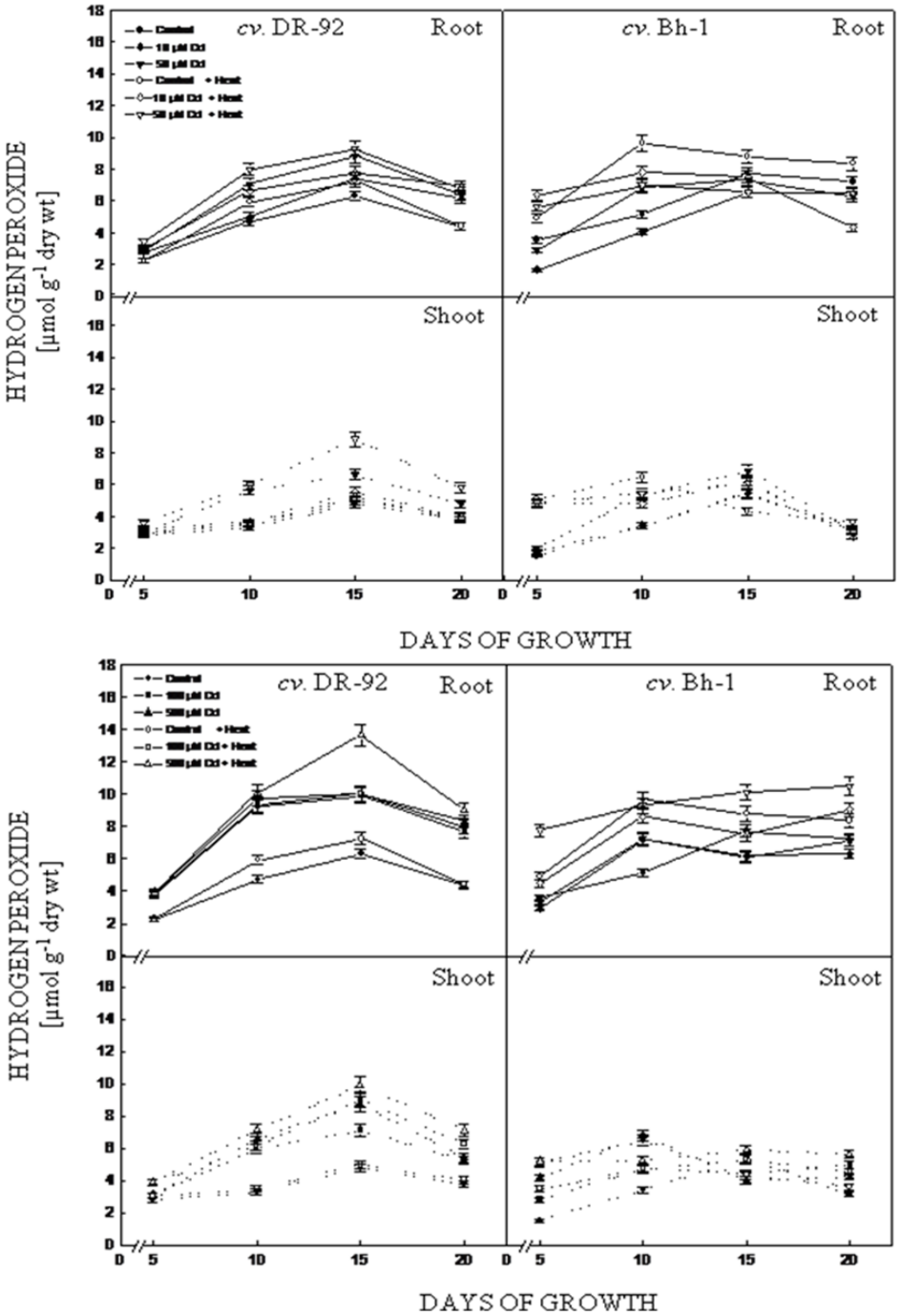

Fig 1: Effect of 10, 50, 100 and 500 µM cadmium toxicity and heat stress on levels of H_2O_2 in the roots and shoots of rice cvs. DR-92 and Bh-1 grown in sand cultures. Values are mean of triplicates ± SD.

Effect of Cd^{2+} toxicity and heat on generation of superoxide anion

The effect of Cd^{2+} and heat injury on generation of superoxide anion ($O_2^{\cdot-}$) in rice *cv*. DR-92 and *cv*. Bh-1 under low and high Cd^{2+} toxicity and heat stress during 5-20 day growth period were shown in Figure 2. A concomitant increase in superoxide anion production is observed in stressed seedlings of both the rice cultivars and throughout the growth period as

compared to non-stressed plants with shoots having more generation of anion than roots. Under 50 µM Cd^{2+} and heat stress a 3 fold increase in superoxide anion levels were noticed in *cv*. DR-92 at day 10 of growth period whereas a 2.5 fold increase at day 15 was noted (Figure 2). A similar trend was also noticed in *cv*. Bh-1 where 15-20 percent increase in anion generation at higher toxic level of 100 and 500 µM Cd^{2+} and heat stress was observed as and when compared to controls. Under 100 µM Cd^{2+} treatment alone *cv*. DR-92 showed more anion generation in roots whereas a significant increase in anion level in shoots were seen as a combined effect of 500 µM Cd^{2+} and heat treatments than that in controls. A combination of 100 and/or 500 µM Cd^{2+} and heat treatments revealed a more pronounced generation of superoxide anion in both the *cvs.* DR-92 and Bh-1, during 10-15 days of growth period that either remained constant or gradually declined thereafter.

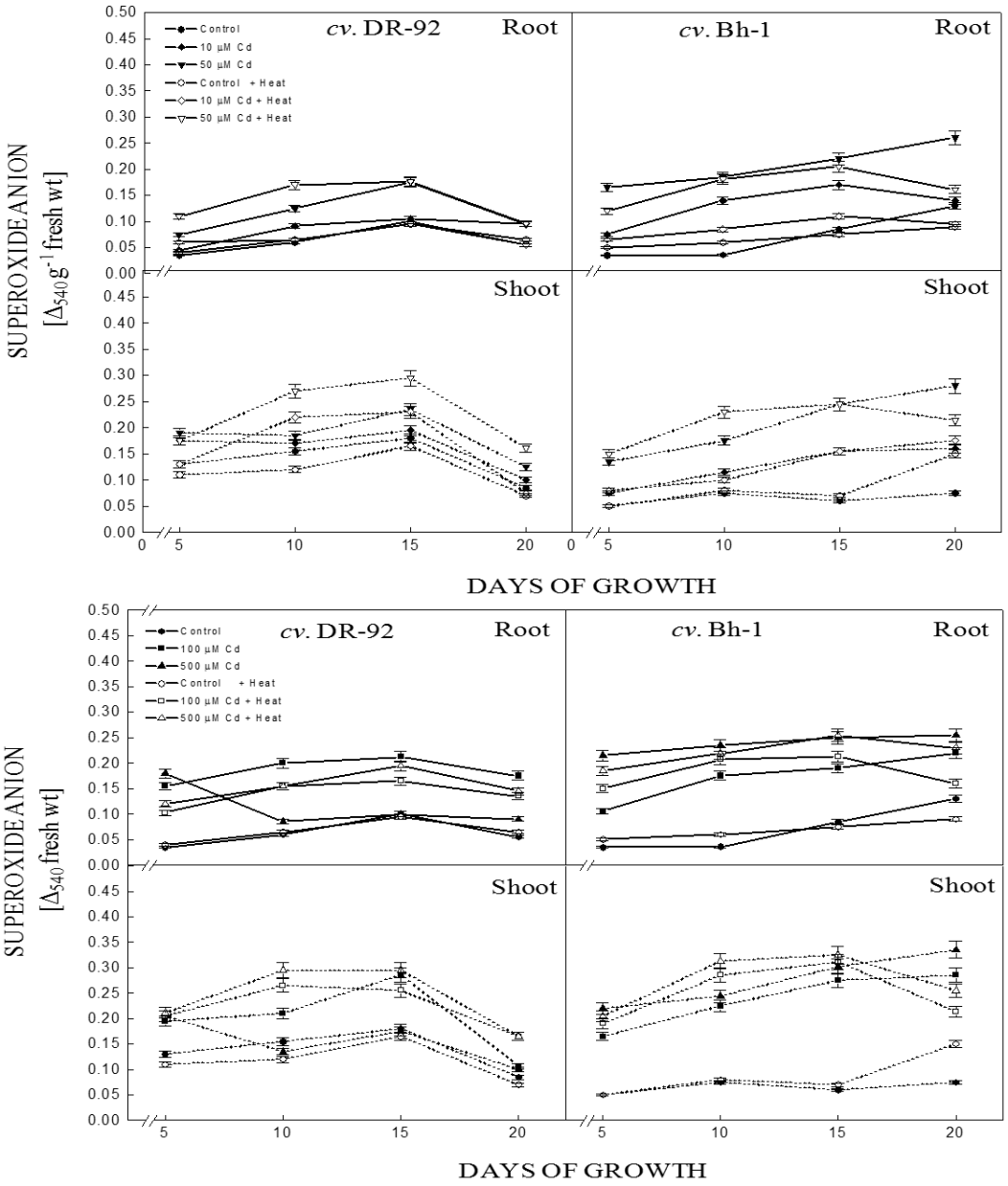

Fig 2: Effect of 10, 50, 100 and 500 µM cadmium toxicity and heat stress on production of $O^{2.-}$ in the roots and shoots of rice cvs. DR-92 and Bh-1 grown in sand cultures. Values are mean of triplicates ± SD.

Effect of Cd^{2+} toxicity and heat on level of thiobarbituric acid reactive substances

Figure 3 show the effect of Cd^{2+} and heat stress on the level of thiobarbituric acid reactive substances as a measure of lipid peroxidation in rice seedlings of *cv*. DR-92 and *cv*. Bh-1 at increasing days of growth. Similar to the observation for H_2O_2, the TBARS level in both the *cvs.* DR-92 and Bh-1 increased during 5 to 20 days of growth. In *cv*. Bh-1 more TBARS derivatives were observed at day 10 of growth period. A 10 µM Cd^{2+} and 50 µM Cd^{2+} levels + heat treatments led to a 50 percent increase in TBARS level in shoots of *cv*. Bh-1 whereas in roots the TBARS were maximum at day 15 followed by a thereafter.

A gradual increase in the levels of TBARS were observed under high toxic levels of Cd^{2+} in both the roots and shoots of *cv*. DR-92 in controls as well as in all stress treatments. The roots and shoots of Cd-treated seedlings of *cv*. DR-92 showed a significant rise under 500 µM Cd^{2+} levels at day 15 of the growth period, but a similar increase in TBARS levels were noted at day 10 in *cv*. Bh-1 grown under same level of Cd-toxicity. A combination of 100 and 500 µM Cd^{2+} and heat stress resulted in maximum level of TBARS in roots of 15 day old seedlings from rice *cv*. Bh-1 with a ~1.5 time increase in TBARS, when compared with controls.

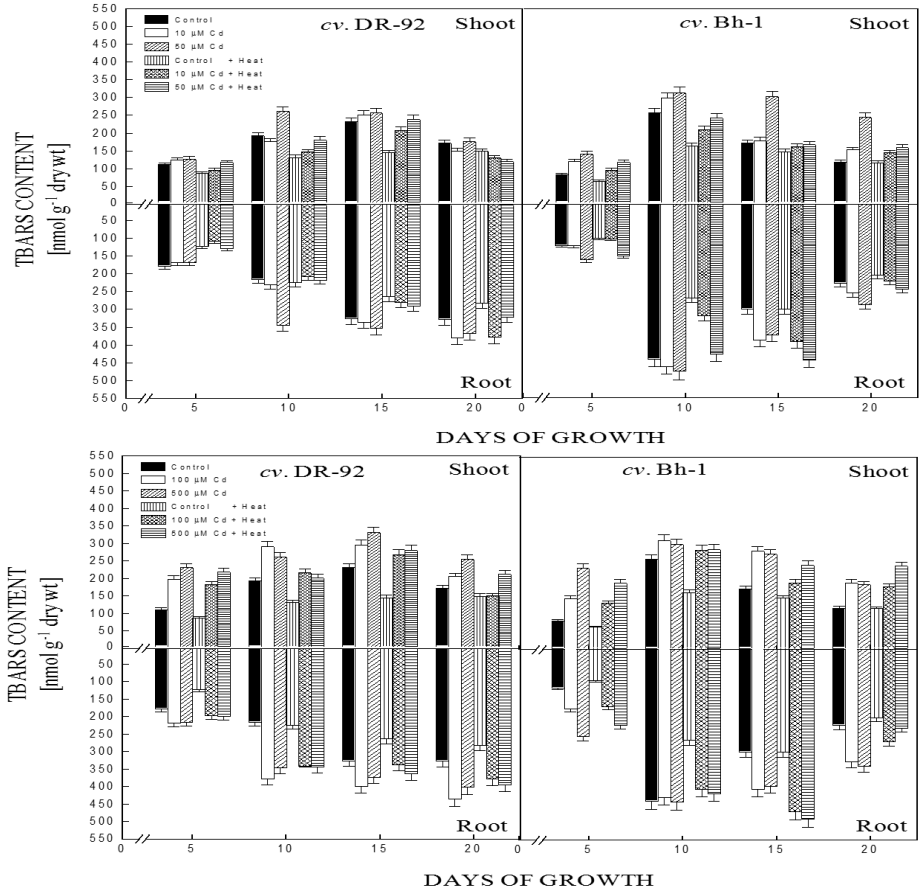

Fig. 3. Effect of 10, 50, 1000 and 500 µM cadmium toxicity and heat stress on levels of TBARS in the roots and shoots of rice *cvs.* DR-92 and Bh-1 grown in sand cultures. Values are mean of triplicates ± SD.

Effect of Cd^{2+} toxicity and heat on total soluble protein

Table 1 show the effect of Cd^{2+} and heat on total soluble protein. The total soluble protein increased in both roots and shoots of the two rice cultivars DR-92 and Bh-1 with increasing days of growth and Cd^{2+} levels. At day 10 in *cv*. DR-92, Cd^{2+} treatments led to 1.5 fold increase in soluble protein levels in shoots, whereas in *cv.* Bh-1 the increase was 2.0 fold in the same tissues as compared to controls. In both the roots and shoots of *cv*. Bh-1 highest soluble protein levels were seen under 10 µM Cd^{2+} concentration at day 15 and under 100 and 500 µM Cd^{2+} + heat stress at day 15 of growth period. Rice *cv*. DR-92 however, showed high protein levels under 50 µM Cd^{2+} and 500 µM Cd^{2+} in shoots and roots respectively at day 10 of growing rice seedlings. A 50 µM Cd^{2+} and heat injury led to a further increase in protein level at 15 day of growth in *cv*. Bh-1 (Table 1). In *cv*. DR-92 however, the levels of protein under Cd^{2+} and heat stress did not change significantly and maintained almost a similar level of protein as that of Cd-treatments alone.

Table 1: Soluble protein concentration of rice cvs. DR-92 and Bh-1 under combined effect of 0, 10, 50, 100 and 500 cadmium and heat stress. The values are mean of three replicate ± SD. Values with different alphabets are significantly different at $P \leq 0.05$ as obtained by ANOVA

Stress Treatments	Days of growth	Soluble protein mg g^{-1} dry wt			
		cv. DR-92		*cv*. Bh-1	
		Root	Shoot	Root	Shoot
Control	10	11.24 ± 0.562^{a}	21.01 ± 0.851^{b}	18.04 ± 0.902^{b}	21.31 ± 0.866^{b}
	15	22.40 ± 1.220^{c}	25.19 ± 0.759^{c}	25.63 ± 1.282^{c}	25.71 ± 0.786^{c}
	20	08.89 ± 0.445^{a}	22.69 ± 0.535^{c}	21.26 ± 1.063^{b}	22.32 ± 0.516^{c}
10 µM Cd	10	20.60 ± 1.030^{b}	27.46 ± 0.923^{c}	17.47 ± 0.874^{b}	25.03 ± 0.752^{c}
	15	22.98 ± 1.149^{c}	25.18 ± 0.759^{c}	28.98 ± 1.449^{c}	30.33 ± 0.917^{c}
	20	15.09 ± 0.755^{a}	17.59 ± 0.379^{b}	20.29 ± 1.015^{b}	20.55 ± 0.528^{b}
50 µM Cd	10	19.63 ± 0.982^{b}	29.24 ± 0.962^{c}	17.61 ± 0.881^{b}	24.89 ± 0.745^{c}
	15	21.92 ± 1.096^{b}	26.73 ± 0.837^{c}	22.81 ± 1.141^{c}	27.61 ± 1.481^{c}
	20	12.18 ± 0.609^{a}	15.94 ± 0.297^{b}	21.24 ± 1.062^{b}	19.13 ± 0.457^{b}
100 µM Cd	10	21.80 ± 1.090^{b}	25.64 ± 1.086^{c}	14.59 ± 0.729^{a}	22.74 ± 0.637^{c}
	15	20.19 ± 1.009^{b}	20.38 ± 0.819^{b}	17.18 ± 0.859^{b}	24.18 ± 0.709^{c}
	20	18.53 ± 0.927^{b}	18.41 ± 0.421^{b}	21.51 ± 1.075^{b}	21.64 ± 0.582^{b}
500 µM Cd	10	24.00 ± 1.200^{c}	21.20 ± 1.060^{b}	14.44 ± 0.722^{a}	23.16 ± 0.658^{c}
	15	21.27 ± 1.064^{b}	25.86 ± 0.793^{c}	20.27 ± 1.014^{b}	24.09 ± 0.705^{c}
	20	14.13 ± 0.707^{a}	17.69 ± 0.585^{b}	20.30 ± 1.015^{b}	21.10 ± 0.555^{b}
Control + Heat	10	21.81 ± 1.141^{b}	25.84 ± 0.992^{c}	17.83 ± 0.892^{b}	18.12 ± 0.806^{b}
	15	19.82 ± 0.991^{b}	25.08 ± 0.754^{c}	24.36 ± 1.218^{c}	25.60 ± 0.780^{c}

	20	08.55 ± 0.428[a]	18.81 ± 0.441[b]	21.36 ± 1.085[b]	20.08 ± 0.504[b]
10 µM Cd + Heat	10	20.30 ± 1.105[b]	28.08 ± 0.904[c]	16.92 ± 0.846[b]	19.17 ± 0.759[b]
	15	21.06 ± 1.053[b]	25.90 ± 0.795[c]	27.72 ± 1.386[c]	26.92 ± 0.846[c]
	20	14.81 ± 0.741[b]	17.74 ± 0.387[b]	20.19 ± 1.009[b]	20.04 ± 0.502[b]
50 µM Cd + Heat	10	19.25 ± 0.963[b]	29.32 ± 0.916[c]	17.61 ± 0.881[b]	20.17 ± 0.759[b]
	15	21.92 ± 1.096[b]	26.61 ± 0.831[c]	22.81 ± 1.141[c]	27.61 ± 1.481[c]
	20	15.71 ± 0.786[b]	26.89 ± 0.345[c]	21.24 ± 1.062[b]	24.13 ± 0.457[c]
100 µM Cd + Heat	10	21.90 ± 1.095[b]	28.20 ± 0.910[c]	14.11 ± 0.706[a]	22.79 ± 0.639[c]
	15	17.74 ± 0.887[b]	27.23 ± 0.862[c]	16.72 ± 0.836[b]	29.60 ± 0.697[c]
	20	13.85 ± 0.693[a]	17.08 ± 0.354[b]	21.50 ± 1.076[b]	21.64 ± 0.582[b]
500 µM Cd + Heat	10	24.00 ± 1.100[c]	27.67 ± 0.884[c]	14.72 ± 0.736[a]	23.86 ± 0.693[c]
	15	13.64 ± 0.682[a]	30.40 ± 0.734[c]	19.55 ± 0.978[b]	33.69 ± 0.685[c]
	20	10.81 ± 0.541[a]	20.69 ± 0.535[b]	20.30 ± 1.015[b]	21.10 ± 0.555[b]

Values with different alphabets are significantly different at $P \leq 0.05$

Effect of Cd^{2+} toxicity and heat on soluble protein profiles in rice seedlings

The soluble protein profile from roots and shoots of 15 day grown rice seedlings of *cv*. DR-92 as obtained on 12.5 percent SDS-PAGE (Figure 4). Both low molecular weight (LMW) and high molecular weight (HMW) protein bands were obtained in controls as well as in 10 µM or 50 µM Cd^{2+} stressed seedlings in the roots and shoots, with increase in band intensities under increasing Cd^{2+} toxicity levels.

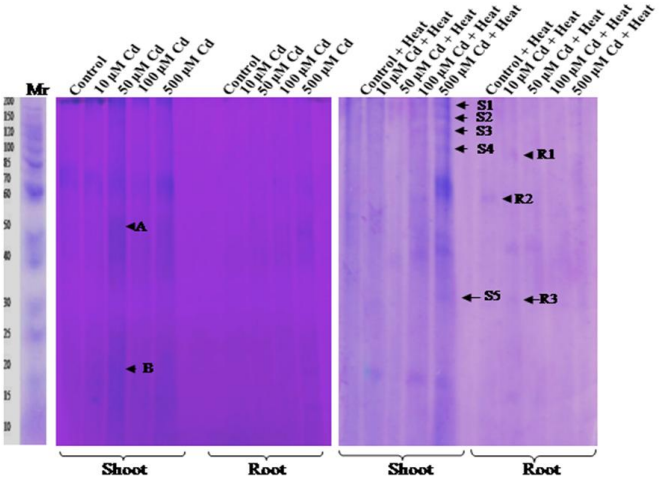

Fig 4: Soluble protein profile from roots and shoots of 15 day grown rice seedlings *cv*. DR-92 under increasing concentrations of cadmium alone and cadmium + heat treatments as revealed by 12.5 percent SDS-PAGE. Mr- Standard protein markers (10-200 kDa ladder). Arrows indicate additional protein bands under stress treatments. High Molecular Weight (HMW) protein bands-S1~ 200kDa, S2 ~ 150kDa, S3 ~ 120kDa, S4 ~ 100kDa and R1~ 95-100; Low Molecular Weight (LMW) protein bands- S5~32-35 kDa, R2 ~ 55-58 kDa and R3 ~ 32-35 kDa. Under 50 µM Cd^{2+} stress HMW protein band A and LMW protein band B decrease in intensity upon imposition of heat stress in shoots

A significant change in protein banding pattern of both LMW and HMW proteins were noted under combination of Cd^{2+} and heat injury in 15 day rice seedlings. In *cv*. DR-92 five new protein bands (S1-S5) were observed under 500 µM Cd^{2+} + heat treatments in shoots which were otherwise absent under Cd^{2+} treatments alone or in shoots of plants from control, 10 µM Cd^{2+} + heat and 50 µM Cd^{2+} + heat treatments. Protein bands S1, S2, S3 and S4 corresponded to the molecular mass of ~200 kDa, ~150 kDa, ~120 kDa and ~100 kDa, respectively. Band S5 corresponded to a LMW protein of ~32-35 kDa. Protein bands (S1-S4) were also observed under 100 µM Cd^{2+} + heat stress, however band S5 was exclusively observed under 500 µM Cd^{2+} + heat treatments in shoots of *cv*. DR-92. Heat treatment alone revealed the presence of a new LMW protein band R2 (~55-58 kDa) in control plants from *cv*. DR-92 which was absent in controls and all stress treatments. One new HMW protein band R1 (~95-100 kDa) was also noticed in 10 µM Cd^{2+} + heat treated 15 day old seedlings of *cv*. DR-92. The LMW protein band R3 (~32-35 kDa) observed under 500 µM Cd^{2+} + heat in roots corresponded to band S5 (~32-35 kDa) seen under 500 µM Cd^{2+} + heat treatments in shoots of *cv*. DR-92.

Figure 4 shows the isoenzyme profile in shoots of *cvs*. Bh-1 and DR-92 under a combination of 100 µM Cd^{2+} + heat stress. An additional LMW protein band S6 (25 kDa) along with band S5 (32-35 kDa) were observed under 100 µM Cd^{2+} + heat stress. The intensity of the LMW and HMW protein bands A and B (Figure 4) observed under 50 µM Cd^{2+} treatments alone decreased significantly under 100 µM Cd^{2+} + heat stress. Similarly the band intensities of HMW protein observed under Cd^{2+} toxicity also decreased upon imposition of heat in *cv*. DR-92.

Discussion

Results of the present study indicate that Cd^{2+} in the growth medium as well as heat stress elevates the production of ROS, mainly H_2O_2 and superoxide anion, alters levels of proteins, as well as affects the lipid peroxidation in rice. Diverse environmental stresses differentially affect plant processes that lead to loss of cellular homeostasis accompanied by the formation of reactive oxygen species (ROS), which causes oxidative damage to membrane, lipids, proteins and nucleic acids [33]. The oxidative damage to cellular components is limited under normal growing conditions due to efficient processing of ROS through a well-coordinated and rapidly responsive antioxidant system. However, under various abiotic stresses the extent of ROS production exceeds the antioxidant defense capability of the cell, resulting in cellular damages [34]. The results show an increase in H_2O_2 level at day 15 in *cv*. DR-92 and much earlier (at day 10) under Cd^{2+} + heat treatments in *cv*. Bh-1 followed by an almost steady level or decline thereafter. Over accumulation and an increased level of H_2O_2 has been reported in plants in response to Cd^{2+} treatments [35, 36] thereby inducing changes in the antioxidant status of plants [37]. The reports on the level of H_2O_2 and antioxidant enzyme activities are largely discordant with the plant response to Cd^{2+} or heat exposure [8, 38, 39].

Doke *et al.* [40] have reported that accumulation of H_2O_2 is a general stress response, which has been observed in plants exposed to Cd ions, low temperature, heat, pathogens and chilling. In contrast to superoxide anion, H_2O_2 is relatively stable and diffusible through membranes and so is considered as a signal molecule for selective induction of defense mechanisms in plant cells [41]. An H_2O_2 treatment is also shown to enhance tolerance to chilling in mung [42], heat, salt

and drought stresses [43, 44, 45].

The results indicate that a combination of Cd^{2+} and/or heat treatments cause a more enhanced production of superoxide radicals resulting in its elevated levels in both the rice *cv*s. DR-92 and Bh-1 than Cd^{2+} treatments alone suggesting a synergistic effect of the two stressors. A concomitant increase in the rate of superoxide anion generation in the Cd-stressed plants has also been reported earlier by Shah *et al*. [18]. The present results also indicate a cumulative effect of Cd^{2+} and heat stress on $O_2^{\cdot-}$ generation in rice. A gradual increase in generation of anion with increasing days of growth and high $O_2^{\cdot-}$ generation till day 15 of growth period followed by a decline in both roots and shoots of the two rice cultivars under increasing Cd^{2+} toxicity and heat injury could perhaps be the adaptation of the growing rice seedlings to combat multiple abiotic stresses.

The increased accumulation of lipid peroxides is indicative of enhanced production of toxic oxygen [46]. The results herein indicate a general increase in the level of TBARS with increasing days of growth under Cd^{2+} and heat stress. The results also show a profound increase in TBARS levels in *cv*. DR-92 under Cd^{2+} stress alone which further increased under Cd^{2+} + heat treatments. A 40-57 percent enhancement in lipid peroxide level in rice and a considerable increase in TBARS content in leaves of *Brassica juncea* under increasing Cd^{2+} concentrations has also been reported earlier [18, 46]. The levels of TBARS increased significantly and earlier in rice *cv*. Bh-1 than in *cv*. DR-92 grown under high Cd^{2+} toxicity. This was followed by a decline suggesting a controlled lipid peroxidation in rice *cv*. Bh-1 against Cd^{2+} stress. At 10 and 50 µM Cd^{2+} levels a lower or nearly constant TBARS levels as observed in *cv*. Bh-1 suggest a low membrane damage due to efficient removal of ROS by antioxidant defense system in rice *cv*. Bh-1. Therefore, it is quite likely that a more controlled lipid peroxidation level makes an important characteristic of Cd^{2+} tolerance in rice *cv*. Bh-1 which might be due to a controlled production of $O_2^{\cdot-}$ and H_2O_2 and low lipoxygenase activity in addition to the efficient removal of ROS by rice defense systems. TBARS content was used as indicator for lipid peroxidation in this study and represents a balance of oxidative stress that induced the production of TBARS in relation to Cd^{2+} and heat treatments. Therefore, TBARS can be regarded as a sink for oxidative radical [47]. Cd^{2+} induced generation of oxidative stress causing membrane injury as indicated by 10 to 11 fold increased electrolyte leakage in leaves and roots of sunflower exposed to 100 µM Cd^{2+} is reported [47]. Ouzounidou and coworkers [48] proposed that heavy metals indirectly induce depolarization of the membrane potential due to depletion of electrogenic H^+-pump caused by proton uptake into the cellular space. In support of the above Mishra *et al*. [49] suggested that Cd-metal has strong affinity towards nitrogen and sulfur-containing ligands and proteins causing distorted membrane ion channels and leakage of ions in *Bacopa monnieri*.

In this study, heat treatment caused significant increase in TBARS content associated with membrane thiol level suggesting an increased lipid peroxidation under heat stress. A cumulative effect of heat and cadmium stress in rice *cv*. DR-92 in all stress conditions as well as in *cv*. Bh-1 at high Cd^{2+} + heat stress suggest a modified membranal function at different stress levels specially under heat stress. Tissue dehydration with autocatalytic membrane lipid peroxidation leading to altered membrane fluidity and functions due to heat stress have been reported by Xu and coworkers [26]. Slight tissue dehydration was also observed as fold like structures in this work as reported earlier by Shah *et al*. [18].

Results also indicate an increase in total soluble proteins in Cd^{2+}stressed seedlings. It has been reported that under various types of stresses like heat, salinity, heavy metals, etc. normal plant metabolism is affected that alters the level of extractable proteins. Synthesis of stress specific proteins that enable plants to overcome, avoid or combat effect of stressors are also reported [8, 50]. A higher protein level under 50 µM Cd^{2+} and heat treatments in *cv*. Bh-1 at day 15 of growth as observed in this study suggest *cv*. Bh-1 to have high capability for Cd^{2+}- accumulation which is further enhanced upon exposure to heat. It is shown that symptoms of oxidative stress such as lipid peroxidation are a consequence of GSH depletion due to binding of Cd^{2+} to GSH and formation of GSH-derived low molecular wt. proteins or phytochelatins (PCs) [39]. The PCs pathway plays an important mechanism of metal detoxification in plants. Other than heavy metals, no other environmental factors are known to induce PC accumulation in plants. Accumulation and increased level of proteins therefore, could possibly be due to their increased synthesis and decreased utilization under Cd^{2+} stress. Presence of new LMW and HMW protein bands under Cd^{2+} + heat stress in the range 20 to 60 kDa and 90-150 kDa is in accordance with the reports in *Vigna mungo* seedlings raised under $Cd^{2+}$and heat treatments [51].

A well-known biochemical response to heat is the induction of a class of proteins called heat shock proteins (HSPs) [52]. Recruitment of these proteins is rapid and has served as an important model of gene induction. In addition to appearance of HSPs, severe heat shock involves the suppression of protein synthesis other than Hsp production as well. Kultz [52] reported the heat shock proteins to be a part of minimal stress proteome present in all living organisms, indicating that Hsps occupy a fundamental role in stress defence at cellular level. The involvement of LMW HSPs has been shown in soybean and in other plants [53, 25]. New HSPs (both LMW and HMW) under combined effects of heavy metal Cd^{2+} and heat indicate a cross-talk between the response pathways of individual stresses leading to production of new HSPs. Unfolding of proteins by heat is known and HSPs acting as molecular chaperons are believed to prevent thermal aggregation of proteins and helping in their refolding [54].

Several classes of heat shock protein (HSPs) have been described in plants, that include HSP 110, HSP 90, HSP 70, HSP 60 and low mol-wt. HSPs [53] which function to alter the conformation or assembly of other protein structures [54]. Kosakivska *et al*. [50] showed significant synthesis of HSP 50 and 60 kD a family protein in *B.campestris* and *A. caudate* under heat stress. At the genomic level Queitsch *et al*. [56] demonstrated in plants that it is HSP101 that is the upregulated and principally induced in *Arabidopsis thaliana* which was vital for heat stress acclimation. Pierce *et al*. [57] reported that gene activation and expression depend on the ecological strategy, therefore the differences obtained in protein synthesis patterns as seen here in this study and as is reported by Kosakivska *et al*. [50] could be due to such ecological strategy points that are different at molecular levels bringing about fast adaptive reactions in stressed plants. Baniwal *et al*. [58] reported that under normal conditions the cytoprotection by HSPs is achieved without an extensive *de novo* protein synthesis in plants owing to the presence of heat shock factors (HSFs), HsfA2 in particular that sustains HSP production at a continuous high levels. The increased protein levels under low Cd^{2+} + heat stress observed in this study

therefore could largely be a result of Cd-induced proteins however, under high toxic levels of 100 and 500 μM Cd^{2+} + heat stress the elevation in protein levels would be a contribution from both Cd-induced proteins as well as HSPs. This is further supported by the observation that under Cd^{2+} stress alone both LMW and HMW protein bands were observed in this study with increase in intensity of protein bands under increasing levels and duration of Cd^{2+} toxicity. Upon imposition of 500 μM Cd^{2+} + heat stress, new LMW and HMW protein bands appeared in both roots and shoots of *cv*. DR-92. An additional protein band in *cv*. Bh-1 under 100 μM Cd^{2+} + heat, which was otherwise absent in control plants was also noted. Results strongly suggest involvement of both constitutive and inducible stress proteins in ameliorating oxidative stress as a response of *Oryza sativa* to combination of stress of Cd^{2+} + heat. The level of stress response varies with the type of stress and if the stress was given singly or in combination with other stresses. A decrease in the intensity of protein bands A and B in *cv*. DR-92 under 50 μM Cd^{2+} + heat as compared to 50 μM Cd^{2+} alone indicate a cross protection by heat in rice plants against Cd^{2+} stress. It is also likely that other endogenous protective cellular mechanism(s) could be activated upon imposition of heat stress and convey protection against 50 μM Cd^{2+} treatment. Similar reports are there in *Vigna mungo* seedlings subjected to Cd^{2+} and heat stress [51]. The observed distinction in protein patterns thus suggest that stress response proteins could act as a biomarker language of different stressors and of different ecological strategies in rice. An important question arises whether a common environmental stress response (ESR) as observed in this study with Cd^{2+} + heat stress in rice plants is sufficient to provide protection against combined stresses received at the same time. Infact, Rizhsky *et al*. [59] suggest an element of collision in the tolerance pathways to different stress factors. They showed that to combat drought and heat stress, plants deploy a partial combination of two multigene defence pathways in addition to the specific 454 genes that are expressed during a combination of drought and heat. This suggests that combination of drought and heat imposes a different kind of stress to plant cells compared to drought or heat alone. The antagonistic approaches in gene expressions are also noted by Tamaoki *et al*. [60]. Therefore it can be concluded that though there might be an element of commonality between different stress combinations like in drought and heat stresses yet it cannot be extrapolated to other stress combinations for sure unless the defense pathway genes for individual stresses and in combination are studied separately in plants.

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