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A Study on Cadmium uptake and Accumulation Potential of Some Selected Rice Cultivars Grown Locally in West Bengal, India

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ABSTRACT

Cadmium is the most common pollutants of the agricultural sector, contaminate through phosphate fertilizer affect crop yield. Rice being the major important crop is affected most and negatively affects human health, as Cd is mostly accumulated in the grains, due to its high mobility. The uptake and accumulation potential of Cd varies among the rice cultivars. Present study aimed to investigate Cd accumulation and tolerance potential in six locally grown cultivars grown under moderate Cd concentration (10 µM). Several physio biochemical parameters (root-shoot length, pigment, starch, reducing and non-reducing sugar, endogenous H_2O_2, proline, NPSH, flavonoid content and antioxidant activity) were studied from 14d treated seedlings and agronomic parameters were studied from mature plants. Results clearly revealed that, MTU-1010 is most Cd sensitive as flavonoid (9%), pigment (67%), NPSH content (9%), panicle weight (71.4%) and seed production (23.8%) decreased significantly with increase in membrane damage (72.8%) and electrolytic leakage (12%), while Swarnamasur, Sundari and Khitish are moderately sensitive. On the contrary Pankaj and Sabita showed tolerance towards Cd toxicity by 87% and 27% increase in proline, 56.26 % in phenol and 25.38% in NPSH contents. Being Cd tolerant, Pankaj is potential cancer risk elevator due to its considerable grain accumulation (0.4 mg/kg). Our study indicated that, Pankaj though incur less damage in terms of biomass and crop yield are not favorable for animal/ human consumption, while, Khitish, Swarnamasur and MTU-1010 can be utilized as allele donor for breeding Cd non-accumulating rice cultivars.

Keywords Accumulation, Antioxidants, Cadmium, Phenol, Pankaj.

INTRODUCTION

A rapid propagation of heavy metal pollution in agricultural fields due to overuse of sewage sludge, domestic waste and heavy metal infested fertilizers or pesticides are becoming a scar on the face of modern agriculture. Being the most consumed staple foods,

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rice (*Oryza sativa* L.) is enormously grown worldwide mainly in Asia (Honma *et al.* 2016) where rice cultivation takes up about 90% arable land (Fahad *et al.* 2019). Rapid urbanization leads to soil Cd pollution in rice fields putting a great threat to rice cultivation, and consumption of these rice-made products are becoming ample source of Cd exposure to our food chain (Zhang *et al.* 2018). Since last five decades West Bengal is the abode of mass rice cultivation. Almost 5000 different genotypic cultivars are grown here (Barman *et al.* 2020). These landraces vary not only in their yield parameters, but also in abiotic stress tolerance potential, especially towards heavy metal contamination depending on their metal uptake capacity (Grant *et al*. 2008). Absorption and bio accumulation of Cd are entirely age, dosage and genotype dependent among various rice cultivars (Cao *et al.* 2015). Cd content in arable soil is directly proportional to the amount of Cd present in phosphate fertilizers (Majumder *et al.* 2018). Moderate dose of soil Cd contamination ranges between 1.1 to 5.2 mg/kg of soil (Duan *et al.* 2017).

Low concentration of Cd also imparts toxicity due to its better water solubility and mobility (Song *et al.* 2015) as it is taken up easily by plants via essential nutrients transporter system. Due to this phytoaccumulation and subsequent trophic transfer, Cd gains easy access to the food chain resulting osteomalacia and kidney damage in human (Xue *et al.* 2014). Cd contamination in agricultural soil affects plants by inducing chlorosis, growth retardation, and yield reduction (Srivastava *et al.* 2014). The maximum allowable limit of Cd concentration in rice is 0.4 mg Cd/kg of polished rice (Majumdar *et al.* 2018). Cd imposes negative impact on growth, biomass (Barman *et al.* 2020), seed germination, chlorophyll biosynthesis, stomatal conductance and photosynthesis (Rehman *et al.* 2015), alpha -amylase activity and vigour index at early stage (Barman *et al.* 2020). Cadmium not only affects the uptake and translocation of essential nutrients (Fe, Zn, Cu) in rice but also disrupts antioxidant status in rice seedlings under contaminated soils (Rizwan *et al.* 2016). Plants have nominal methods of Cd toxicity avoidance so they modulate various enzymatic (SOD, GOPD, CAT) and non enzymatic antioxidant molecules to serve as cellular redox buffer. Depending on the tolerance and sensitivity of the cultivars (Barman *et al.* 2020), rice plant modifies antioxidative defense and detoxification strategies positively or negatively to combat Cd stress.

Phosphate fertilizer imposed Cd contamination of paddy fields are well known fact but deficiency of ample research incites our team to conduct a basic study regarding Cd accumulation in some selected rice cultivars, grown in the fields of N 24 Parganas district of West Bengal. Screening of Cd uptake and accumulation capacity of 6 different most available rice cultivars was of extreme necessity to execute our survey. The main focus of the present study is to investigate the varietal comparison of 6 rice cultivars regarding their Cd uptake, accumulation capacity to determine the tolerance potential of all these cultivars. The identification of Cd tolerant and sensitive verities would provide a clear idea about the safe cultivar to be grown for human consumption. Cultivar without any Cd accumulation in the straw can be used for fodder.

Depending on various stress parameters (root shoot length, root shoot starch content, reducing and non reducing sugar, photosynthetic pigment content, lipid peroxidation, endogenous H_2O_2 content, electrolytic leakage) antioxidants status (proline, NPSH, phenol, flavonoid contents and activities of antioxidant enzymes) and PCA analysis, cultivars were grouped in different clusters reflecting their Cd tolerance. We exposed seedlings to 10 µM and 12 µM $CdCl_2$, but higher concentration was found to be detrimental and all seedlings were suffering from chlorosis before 7 days duration of treatment. So we further continued with 10 µM treatment.

MATERIALS AND METHODS

Treatment of plant materials

Seeds of six local *kharif* rice cultivars Swarnamasur, Pankaj, Khitish, Sabita, Sundari and MTU 1010 of North and South 24 Parganas were collected from farmer of N 24 Parganas. For surface sterilization of seeds 5% sodium hypochlorite solution was used. The seeds were wiped thrice with deionized water and kept in dark to soak overnight.

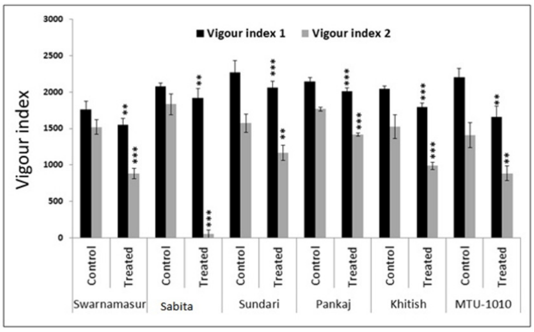

Fig. 1. Assessment of vigour index of cadmium treated (10 μM) rice seedlings. Data are mean ± SE of three independent replicates (n =3). Bars followed by (*), (**) and (***) show significant difference at P ≤ 0.05, P ≤ 0.01, P ≤0.001 level respectively, by using t-test.

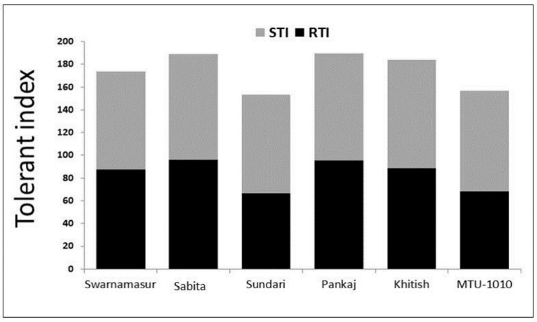

Fig. 2. Stress tolerant index (RTI and STI) of cadmium treated (10 μM) rice seedlings.

Hydroponic growth medium

To estimate all stress parameters the plants were grown in hydroponic solution. For germination 10-15 seeds were kept on separate petri dishes coated with moist filter paper under 25ºC ± 0.5ºC. After 7 days, germinated seedlings were shifted to Hoagland Solution (Hoagland and Snyder 1933) (1mM KH_2PO_4, 5mM KNO_3, Ca $(NO_3)_2$. $4H_2O$, $MgSO_4.7H_2O$,11.8μM $MnSO_4$. H_2O, 0.7μM $ZnSO_4.7H_2O$, 0.32 μM $CuSO_4.5H_2O$, 0.16μM $(NH_4)_6MO_7O_{24}$. $4H_2O$, 46.3μM H_3BO_3, 5μM $FeCl_3$), pH 5.8 and kept for next 7 days followed with the treatment of 10 μM $CdCl_2$. Untreated seedlings were considered as control. Seedlings were harvested 14 days post treatment (DPT) and following experiments were conducted with shoot and root tissue in triplicates.

Study of physiological parameters

Root tolerance index (RTI) was determined as Root

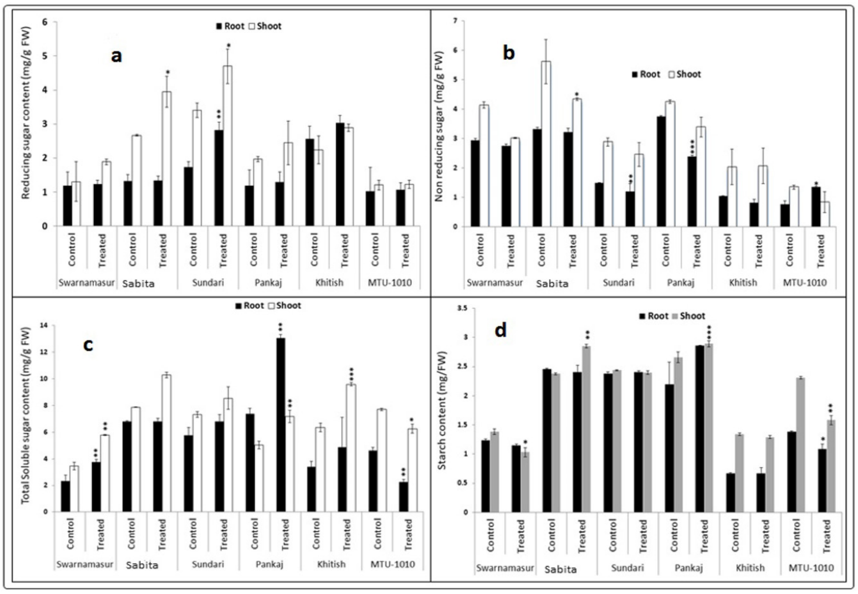

Fig. 3. Estimation of carbohydrate contents of cadmium treated (10 μM) rice seedlings, A: Reducing sugar content, B : Non reducing sugar content, C: Total soluble sugar content, D: Starch content. Data are mean ± SE of three independent replicates (n =3). Bars followed by (*), (**) and (***) show significant difference at P ≤ 0.05, P ≤ 0.01, P ≤0.001 level respectively, by using t-test.

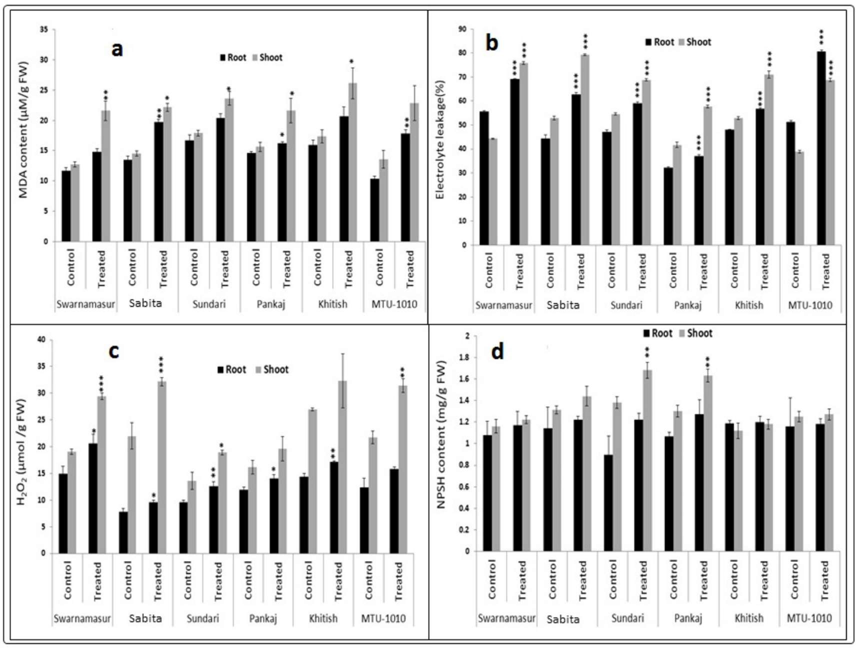

Fig. 4. Estimation of stress parameters of cadmium treated (10 µM) rice seedlings, A: MDA content, B: Electrolyte leakage, C: Endogenous H_2O_2 content, D: Non protein thiol content. Data are mean ± SE of three independent replicates (n =3). Bars followed by (*), (**) and (***) show significant difference at P ≤ 0.05, P ≤ 0.01, P ≤0.001 level respectively, by using t-test.

tolerance index (RTI)% = (maximum root length of treated plant/ maximum root length of untreated plant)×100, according to Mckendry *et al.* (1996).

Vigour index

Both vigour indices I and II were calculated following method of Anderson (1973) at 14 day time points. Vigour index I = Germination % × Seedling Length (Root + Shoot), Vigour index II = Germination % × Seedling Dry weight (Root + Shoot).

Assessment of photosynthetic pigment content

100 mg of fresh leaf tissue were incubated under dark condition with 2 ml of 80% acetone for overnight followed by measurement of the OD at 470 nm, 648.6 nm and 664.2 nm according to the protocol of Lichtenthaler (1987).

Estimation of total soluble sugar

Following the standard protocol of Dubois *et al.* (1956) total soluble sugar of all seedlings was estimated. The OD value of the mixture was taken at 490 nm. From standard curve of glucose, the amount of the total soluble sugar was estimated in mg g^{-1} fw.

Estimation of reducing and non reducing sugar

Standard protocol of Miller (1972) was followed to assess reducing and non reducing sugar. A standard curve of glucose was prepared from which the concentration of reducing sugar was estimated in mg g^{-1} fw.

Estimation of starch content

Quantity of starch of was determined following the protocol of McCready *et al*. (1950).

Measurement of stress markers

Estimation of MDA content

Lipid peroxidation was estimated by the assessment

Table 1. Growth parameters and pigment content of Cd treated seedlings of rice cultivars, data are mean ± SE of three independent replicates (n =3). (*), (**) and (***) show significant difference at P ≤ 0.05, P ≤ 0.01, P ≤0.001 level respectively, by using t-test.CK: control blank.

CV	Set	Biomass (mg) Root	Biomass (mg) Shoot	Plant relative water content (RWC)	Plant height (cm) Root length	Plant height (cm) Shoot length	Photosynthetic pigment content (µg/g FW) Chloro-phyll a	Chloro-phyll b	Carote-noid
Swarna	CK	0.028	0.06	35.54	6.5	11.1	764.83	563	32.73
masur		±0.002	±0.014	±1.92	±0.498	±1.019	±26.897	±14.678	±0.492
	10 µM	0.02	0.034	35.17	5.9	9.6	423.23	353.2	24.77
		±0.009*	±0.012*	±6.33	±0.432	±0.571	±5.64***	±10.064***	±3.446*
Sabita	CK	0.06	0.031	38.78	8.8	11.96	865.13	668	45.82
		±0.041	±0.034	±7.72	±0.498	±0.59	±4.053	±9.73	±0.625
	10 µM	0.035	0.027	38.93	8.5	10.6	554.43	347.8	34.97
		±0.014*	±0.012*	±12.33	±0.41	±0.927	±17.987***	±13.932***	±1.26***
Sundari	CK	0.019	0.028	30.7	8.3	14.36	959.4	459.16	41.48
		±0.008	±0.009	±7.75	±1.329	±1.02	±25.276	±13.908	±2.906
	10 µM	0.022	0.027	38.42	7.8	12.76	744.45	292.2	38.76
		±0.012*	±0.009	±5.48**	±1.014	±0.59	±4.476**	±19.234**	±6.069
Pankaj	CK	0.023	0.026	26.56	6.2	15.23	1112.3	758.05	71.4
		±0.006	±0.013	±1.04	±0.329	±0.249	±33.568	±3.292	±2.515
	10 µM	0.022	0.026	30.21	5.8	14.23	872.5	459.23	85.94
		±0.006	±0.009	±5.32**	±0.478*	±1.617	±44.873**	±24.88***	±2.515**
Khitish	CK	0.029	0.03	23.55	7	12.76	860.03	562.23	33.78
		±0.006	±0.014	±4.12	±0.368	±0.74	±26.398	±23.268	±2.204
	10 µM	0.023	0.04	33.67	6.9	11.03	458.78	292.53	23.6
		±0.003	±0.028	±6.34**	±0.244	±0.579	±44.873***	±12.77***	±2.373*
MTU-		0.015	0.046	22.68	8.3	13.66	751.38	451.5	39.89
1010	CK	±0.003	±0.017	±6.93	±1.329	±0.169	±8.357	±10.987	±1.855
	10 µM	0.016	0.034	25.37	5.7	10.8	341.3	149.1	23.16
		±0.008*	±0.006*	±12.05	±0.368*	±1.151	±15.072***	±18.572***	±1.61**

of MDA content according to the method of Heath and Packer (1968). Reaction mixture was prepared with 1 ml of extract and 4 ml of 0.5% thiobarbituric acid. Absorbance of the extract was measured at 532 and 600 nm and by using extinction coefficient of 155 mM^{-1} cm^{-1} final concentration of MDA was determined.

Electrolyte leakage

The measurement of electrolytic leakage in roots and leaves was conducted according to the protocol of Wang *et al*. (2008). 100 mg of both treated and untreated samples were washed repeatedly to remove the surface contamination and heated at 25ºC for 2 h in a water bath. For determination of initial electrical conductivity (EC_1) this suspension was used. Then to release all the electrolytes this sample was boiled for 15 min, followed by cooling and the final electrical conductivity (EC_2) was estimated. The electrolytes leakage was expressed in percentage (EC_1/EC_2) × 100.

Estimation of endogenous H_2O_2 production

Generation of hydrogen peroxide (H_2O_2) was assayed following the procedure of Loreto and Velikova (2001). Reaction mixture contains 0.75 ml of 10 mM phosphate buffer, 1.5 ml of 1 M freshly prepared potassium iodide (KI) and 0.75 ml of extract. The OD was measured at 390 nm. The quantity of endogenous H_2O_2 was deduced from a standard curve.

Estimation of proline content

According to the standard protocol of Bates *et al.* (1973) proline was estimated. Using D-proline

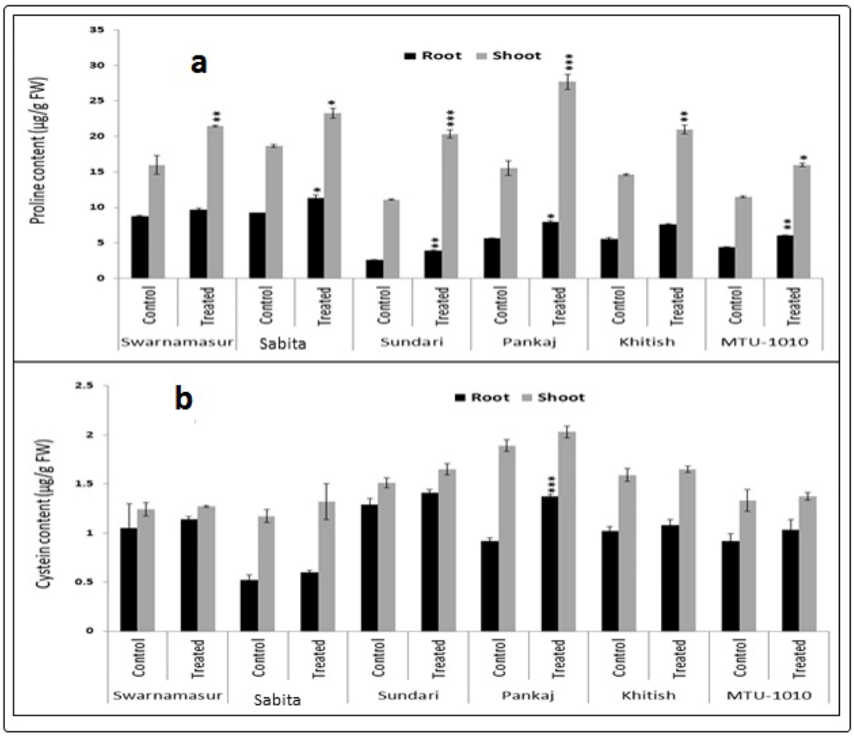

Fig. 5. Estimation of stress biomarkers of cadmium treated (10 μM) rice seedlings, A : Proline content, B: Cysteine content. Data are mean ± SE of three independent replicates (n =3).

standard curve final proline content of seedlings was calculated.

Antioxidative defense responses

Non-enzymatic antioxidants

Non protein thiol content (NPSH) was estimated according to Cakmak and Marschner (1992). Reaction mixture contains 0.5 ml extract, 2.5 ml 150 mM phosphate buffer (pH 7.4) with 5 mM EDTA and 0.5 ml of 6 mM 5-5- dithiobios 2 benzoic acid. Reduced glutathione was used as standard in the range of 0-100μg/ μl. Results was expressed as μg of non proteinthiol/mg of tissue. Total flavonoid content was assayed following the protocol of Chang *et al*. (2002). For the analysis ethanolic extract of the plant sample was mixed with 10% aluminium chlorides solution in 1:1 ratio and kept in room temperature for 30 mints. The golden yellow color of the sample was detected by 415 nm using quercetin as a standard. Total cysteine content was assayed following the method of RoyChoudhury *et al.* (2007).

Enzymatic antioxidants

Superoxide dismutase activity was assayed by estimating the capacity to stop the photochemical reduction of NBT following the standard protocol of Beauchamp and Fridovich (1971). Final volume of reaction mixture made upto 3 ml containing 50 mM of potassium phosphate buffer (pH : 7.8), 13 mM of methionine, 75 μm of NBT, 2μm riboflavin and 0.1 mM EDTA and the enzyme extract. The quantity of protein capable to cease 50% initial reduction of NBT under light is considered as one unit SOD activity. GR activity was measured according to Schaedle and Bassham (1977). 1 ml reaction mixture contains 50 mM of Tris HCL buffer (pH: 7.6), 0.15 mM NADPH, 1 mM GSSG, 3mM $MgCl_2$, along with 200 μl of enzyme extract. Decrease in OD value of NADPH at 340 nm was recorded.

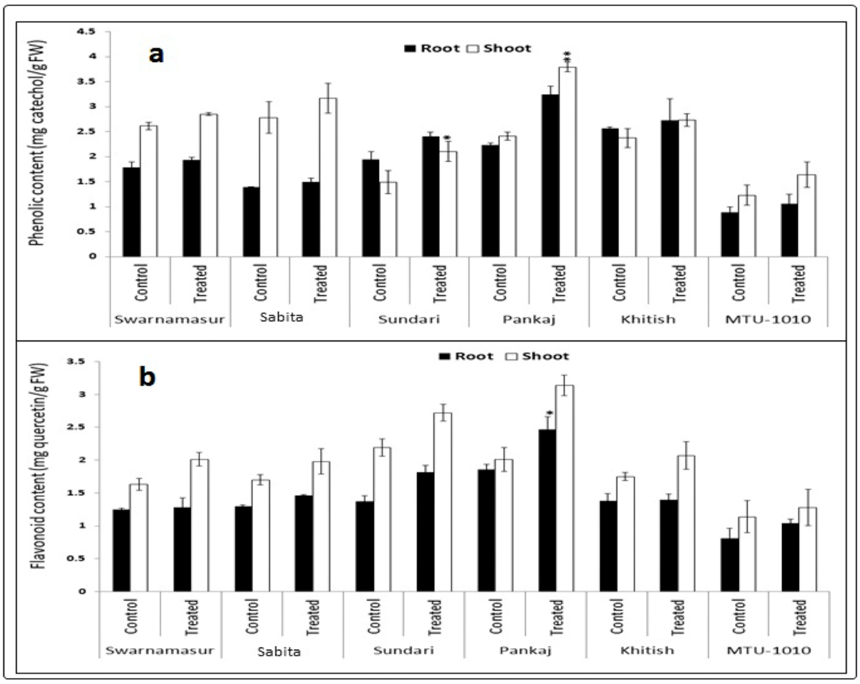

Fig. 6. Estimation of secondary metabolites of cadmium treated (10 µM) rice seedlings, A: Phenolic content, B: Flavonoid content. Data are mean ± SE of three independent replicates (n =3). Bars followed by (*), (**) and (***) show significant difference at P ≤ 0.05, P ≤ 0.01, P ≤0.001 level respectively, by using t-test.

Statistical analysis

All experiments were repeated thrice expressed as mean of triplicates. All data sets obtained from experiments were statistically analyzed by t test to determine the significant variation between control and cadmium treated plants. Significance level was compared at P < 0.05. Considering all stress parameters of cadmium contaminated seedlings hierarchical cluster analysis was conducted and heat maps were created. Principal component analysis (PCA) was conducted to determine the differential response patterns in the 6 different rice cultivars which were induced by cadmium treatment.

RESULTS AND DISCUSSION

Cd alters growth parameters, pigment content and photosynthetic products

The selected cultivars considered in the present study are mostly cultivated in the North 24 Pargana districts with rain-fed land having enough exposure of phosphate fertilizers resulting Cd contamination in arable land. In all the cultivars root and shoot of Cd contaminated plants showed considerable growth retardation in comparison with control plants (Table 1). Maximum root length was affected in Swarnamasur (9.2%) and MTU-1010 (31.3%) whereas Sabita (3.4%) and Pankaj (6.4%) showed least growth inhibition. Shoot length reduction found least in Pankaj. Surprisingly RWC remained least altered in most cases and increased in Pankaj (13.7%) and Sabita (4.18%) reflecting Cd tolerance (Table 1). The negative consequences of Cd toxicity on rice plant are evident by compromised dry biomass, shoot length and more acute in root length as root is the primary structure to face direct exposure of Cd in growth media resulting retardation of cellular activities of root apex (Zhao *et al.* 2019). This is supported by the various studies which revealed that maximum Cd accumulation in rice plant root causing drastic root length reduction (Khaliq *et al*. 2019; Barman, *et al.* 2020). Depending on growth parameters Pan-

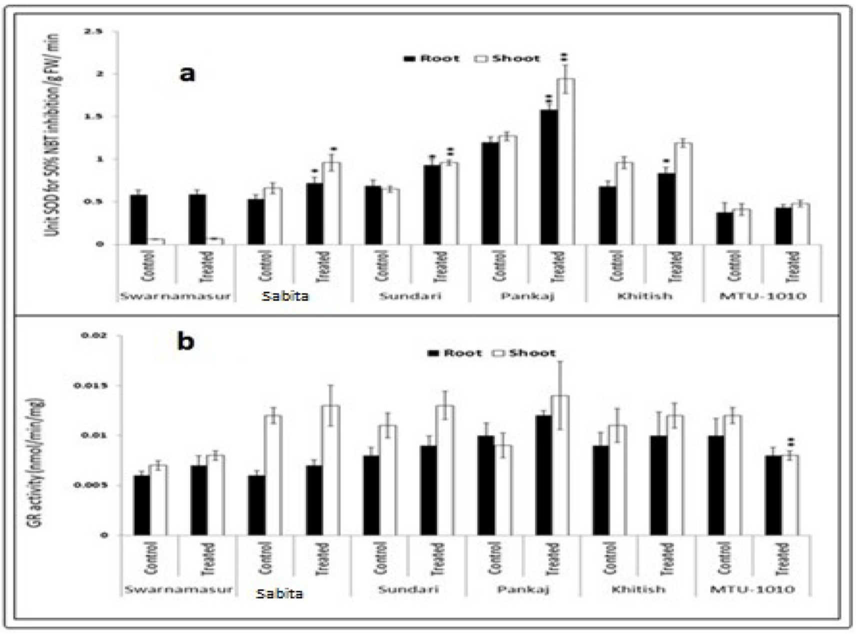

Fig. 7. Anti-oxidative enzymes (A: Superoxide dismutase SOD, B: Glutathione reductase GR) activity of cadmium treated (10 µM) rice seedlings. Data are mean ± SE of three independent replicates (n =3). Bars followed by (*), (**) and (***) show significant difference at P ≤ 0.05, P ≤ 0.01, P ≤0.001 level respectively, by using t-test.

kaj and Sabita were found to be less affected by Cd than Khitish, MTU-1010 and Swarnamasur. This was further corroborated by PCA analysis which revealed that Pankaj and Sabita being least affected by Cd formed a separate cluster representing Cd tolerance while MTU-1010 was out grouped proving its maximum sensitivity. RWC (relative water content) is the indicator of plant water status, overall health and reflects the balance between water supply and transpiration rate (Rucinska-Sobkowiak 2016). It is reported that treatment of cadmium and other metals decline RWC (Srivastava *et al.* 2014) by lowering of leaf water potential and impeding short-distance water transfer (Rucinska-Sobkowiak 2016). Our study revealed that under 10µM Cd toxicity all the cultivars had least unaltered RWC while Pankaj and Sabita had significantly (P≤0.01) higher RWC indicating better adaptability to Cd stress (Dey *et al.* 2019). Our result can be validated with the work of Kaznina *et al.* (2014) where Cd suppressed transpiration rate in *S. viridis* while maintaining high rate of photosynthesis by increase in RWC. Seedling vigour index is a measurement of the extent of damage that accumulates as viability declines. Better vigor index (Fig. 1) was noted in Pankaj and Sundari compared to others reflecting Cd tolerance. Vigour index comprises seedling length, health and growth rate which represents a wholesome good beginning.

Significantly unaltered vigour index of Pankaj and Sabita indicates optimum growth. Depending on the tolerant index (Fig. 2), Pankaj and Sabita were found to be better tolerant to Cd. Thereby in PCA analysis, cultivars are placed into three well defined separate groups; tolerant, sensitive and moderately tolerant which includes Pankaj, MTU-1010 and others respectively.

Cd treated seedlings showed a significant decline in photosynthetic pigment content with respect to control plants (Table 1). MTU-1010 showed maximum reduction in chl a (54.6%), chl b (67%), and carotenoid content (42%) while Pankaj showed least reduction of chl a (21.6%) and increased carotenoid (20%), indicating Cd tolerance. Except Pankaj, others showed reduction in pigments which is correlated with the reduced biomass attributed to the decreased supply of essential minerals important for seedling

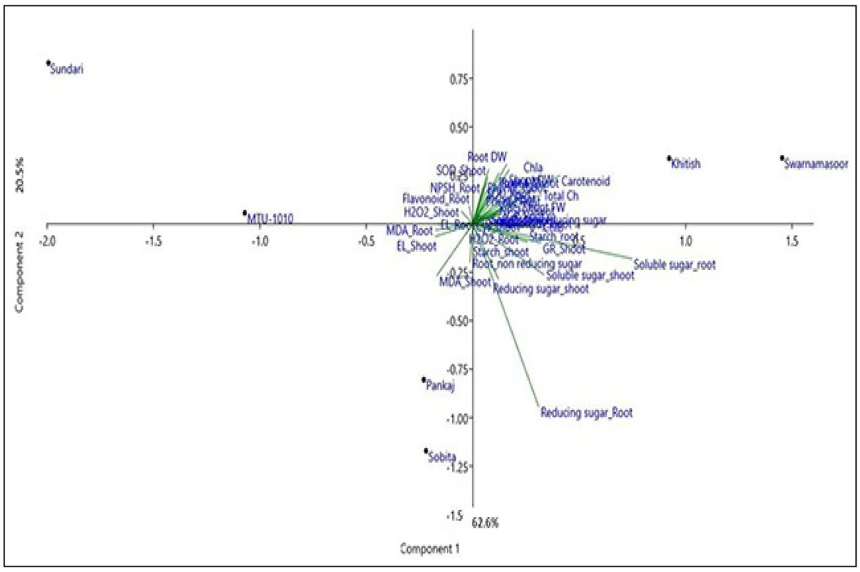

Fig. 8. PCA analysis of six rice cultivars considering all stress parameters and enzymatic, non enzymatic antioxidative properties.

growth (Wang *et al*. 2018, Bhuyan *et al*. 2020).

Depletion in pigment under Cd contamination is attributed to the hypo activity of few enzymes, affecting pigment biosynthesis (Bhuyan *et al.* 2020). Based on our findings and PCA clustering higher amount of chlorophyll depletion in cultivar MTU-1010, Khitish and Swarnamasur results decrease in seedling biomass.

Heavy metals affect two important aspects of crop productivity i.e., rate of photosynthesis and the source sink import export. To assess the photosynthetic efficiency under Cd stress estimation of total sugar, reducing sugar and starch is important. Though Sundari revealed noticeable enhancement (Fig. 3) in reducing sugar (62% in root, 37.35% in shoot) and Sabita had minimal decrease in non reducing sugar content but significantly higher production of total soluble sugar (80% in root, 31.25% in shoot) with starch content was recorded in Pankaj among all. Khitish, MTU-1010 and Swarnamasur showed lower accumulation of starch and sugar. Cd treated Sundari showed enhancement in reducing sugar corresponding reduction in non reducing sugar. This might be due to increase in acid invertase (sucrose degrading enzyme) and decrease in sucrose synthase which is linked to the change in osmopotential of the cell needed for osmotic adjustment under heavy metal induced osmotic stress (Rucinska-Sobkowiak 2016).

Profound increase in total sugar content of Cd treated Pankaj reflect its highest capacity of osmotic adjustment. Acc to Devi *et al*. (2007) Cd sensitive rice showed accumulation of starch due to less utilization or conversion into soluble sugar resulting in growth reduction (Asgharipour *et al.* 2011). On the contrary, here Cd treated Pankaj showed more increase in total sugar content (80% in root, 31.25% in shoot) than starch content (18% in root, 11.5% in shoot) proving its least affected growth under Cd stress.

Cd stress induces membrane damage, electrolyte leakage and H_2O_2 generation

Acute stress response of Cd toxicity is lipid peroxidation resulting membrane damage. Cd induced membrane damage was determined by MDA content and electrolytic leakage (Fig. 4). Max MDA was observed in MTU-1010 root (72.8%) and shoot (69.62%), followed by Sundari (47%- root, 52% - shoot) and Khitish, while least increase was noted in Pankaj root (10.95%) and shoot (38.46%). Electrolytic leakage is used as hallmark phenomenon to measure plant stress

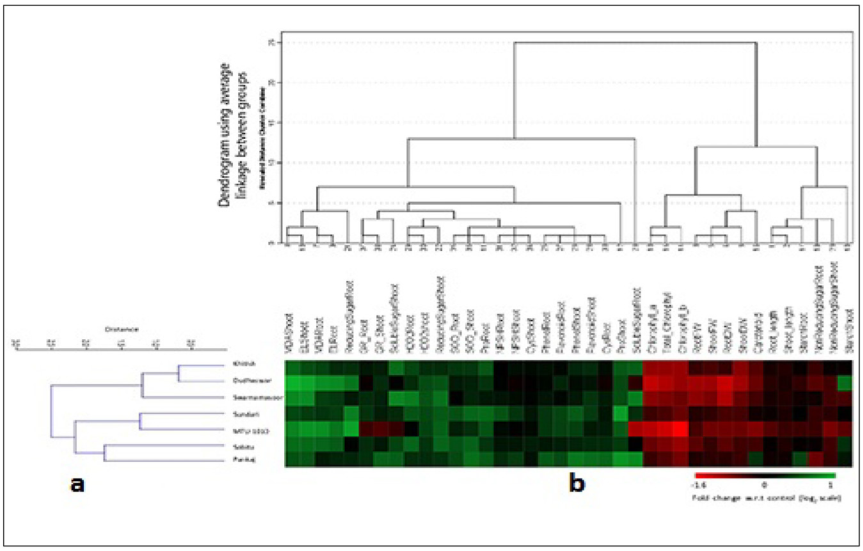

Fig. 9. a. Hierarchial clustering of treatment rice seedlings on the basis of biochemical and physiological parameters after 14 days exposure to 10 µM Cd. **b:** Cluster analysis and heat map showing fold change in physiological and biochemical parameters with respect to control.

tolerance (Fig. 4). MTU-1010 (57.6% in root; 76.8% in shoot), Swarnamasur (24% in root; 71% in shoot) and Sabita (42% in root, 79% in shoot) showed elevated electrolytic leakage compared to Pankaj (15% in root, 38.17% in shoot).

In mature plant Cd induced excessive H_2O_2 accumulation, lipid peroxidation and electrolyte leakage (EL) leads to oxidative and osmotic imbalance (Ekmekci *et al.* 2007). Here maximum EL was recorded in MTU-1010, Swarnamasur and Sabita indicating their Cd sensitivity. Among 6 cultivars Cd treated Pankaj showed minimal increase in H_2O_2 accumulation (Fig. 4). This is in accordance with the report of Bhuyan *et al.* (2020) where MDA and H_2O_2 content increased noticeably in rice under 1 mM $CdCl_2$ treatment. Differential tolerance of varieties was evident by difference in rise of MDA content among landraces which is possible due to their innate endurance to Cd. Proline accumulation in plants is a trademark of heavy metal induced physiological abnormalities. One of the most important osmolytes proline also behaves as a signaling molecule also (Saadati *et al.* 2019). Sundari (root- 49.4%, shoot- 82.89) and Pankaj (root- 40.74%, shoot-80.4%) showed significantly higher proline content than Sabita (27.7% in shoot) and their untreated counterpart indicating Cd tolerance while Khitish and MTU-1010 showed minimal increase (Fig. 5). Proline protects cell from electrolytic leakage, protein degradation and prevent oxidative damage by limiting ROS generation (Dey *et al.* 2019). All Cd treated cultivars showed more or less elevated proline production but significant rise was recorded in Pankaj ($P \leq 0.001$), Sabita ($P \leq 0.05$) and Sundari ($P \leq 0.001$) shoot. Maximum increase in proline content of Pankaj justified its better adaptibility to Cd stress and can be validated with the PCA analysis that clustered Pankaj, Sabita in a separate representing better tolerance.

Being a by-product of lipid peroxidation, MDA content represents membrane damage that can be mitigated by proline (Khanna-Chopra *et al.* 2019). Least increase in MDA content of Cd treated Pankaj is positively correlated to its highest production of proline whereas MTU-1010 and Khitish showed higher membrane damage owing to their lower proline content. This is because depletion in antioxidants due to elevation of superoxide radical resulted hyper accumulation of malondialdehyde in the cells (Majumdar *et al.* 2018).

Antioxidative defense system is altered by Cd

It is well established that for detoxification of ROS plants use phenolics and flavonids compounds as potential antioxidants (Granato *et al.* 2018), which acts as metal chelator, reduce lipid peroxidation by scavenging the lipid alkoxyl radical (Szabados and Savoure 2010) and metal homeostasis. Among all cultivars Pankaj had highest increase (Fig. 6) in phenolic (57.26% -shoot, 45.29% - root) and flavonoid (56.2% - shoot, 32.8% - root) owing to its better Cd tolerance, while Swarnamasur showed least increase (9.2% in shoot, 8.42% in root). This is further validated by PCA analysis where Pankaj is clustered in Cd tolerant group followed by Sabita having second highest phenol content. According to PC1 and PC 2 value, growth parameters are highly correlated with the non enzymatic antioxidant phenol and flavonoids in Pankaj and Sabita. Flavonoids are also a suitable antidote for heavy metal poisoning as metal-flavonoid chelates are much more powerful free radical scavengers than the parent flavonoids. Flavonoid mediated growth rescue under 500 µM Cd stress was proven by previous worker Kelig and Ludwig-Mueller (2009) in Arabidopsis, where growth reduction of flavonoid deficit mutant was withdrawn by externally applied flavones quercetin. Here highest flavonoid content was recorded in Pankaj while MTU-1010 had least increase both in phenol and flavonoid representing Cd susceptibility.

Being the precursor molecule, Cysteine is the rate limiting factor for the synthesis of glutathione (GSH), the chief non protein thiol that provides antioxidative protection against Cd toxicity in plant. Moreover GSH contributes the synthesis of phytochelatin (PCs), the thiolate peptide which detoxify heavy metals by vacuole sequestration (Anjum *et al.* 2008, Matraszek-Gawron and Hawrylak-Nowak 2019). So estimation of cysteine and NPSH is of great need. Significant elevation in NPSH content (Fig. 4) was recorded in Pankaj (18.7% in root, 25.38% in shoot) and Sundari (35.5% in root, 21.73% in shoot) compared to conrol while Khitish and MTU-1010 showed least increase. Similarly, only Cd treated Pankaj had significant increase in root cysteine content (49%) than control set, reflecting Cd tolerance (Fig. 5) which is in conformity with the fact that Cd endurance is positively correlated with enhanced Cys biosynthesis in Arabidopsis (Harada *et al.* 2002) and rice (Srivastava *et al.* 2014). Cys dependent Cd induced oxidative stress mitigation in plants (Guha *et al.* 2020) is well known. On contrary least increase of Cysteine and GSH in Khitish, Swarnamasur and MTU-1010 was associated with their Cd susceptibility as lesser quantity of GSH production is directly linked with Cd sensitivity, may be due to limited PCs synthesis (Mullineaux and Rausch 2005). Our results have been also further validated by PCA analysis that reflects significantly higher NPSH of Pankaj might have alleviated growth inhibition under Cd stress. This is in good accordance with the report of Cai *et al.* (2010) where exogenous GSH significantly ameliorated Cd induced growth suppression in two rice genotypes.

Antioxidative enzymes are the major way to deal with oxidative stress. These enzymatic antioxidants (SOD, CAT, GPOD, GR) function to detoxify ROS and serve as redox buffers of cell. Metalloenzyme SOD is responsible for dismutation of superoxide (O_2) into molecular O_2 and H_2O_2 followed by the reaction of CAT that converts H_2O_2 into H_2O for ultimate detoxification. Significantly enhanced SOD activity was recorded from Cd treated Pankaj root (29.46%) and shoot (47%) (Fig. 7). SOD activity and H_2O_2 accumulation under Cd stress (Barman *et al.* 2020) is positively correlated. Increased intracellular level of H_2O_2 due to enhanced SOD activity is detoxified primarily by the enzymes catalases, peroxidase (Srivastava *et al.* 2014), and non enzymatic antioxidants flavonoids (Hossain *et al*. 2015). Here rise in SOD activity of Pankaj with the least accumulation of H_2O_2, might be due to collective hyperactivity of non enzymatic antioxidants (flavonoids, phenolics). Cd treated MTU 1010 and Swarnamasur had negligible alteration in SOD level indicating their Cd sensitivity.

Elevation of GR activity (Fig. 7) under Cd stress also indicated the better Cd tolerance in Pankaj (shoot 41%, root 33%) and Sabita (17%) among others while MTU-1010 showed a prominent decline and Khitish, Swarnamasur had negligible change compared to control. Presence of Cd in growth medium always demands increases in GR activity to support the continuous renewing of GSSG to GSH which is the prerequisite for Cd sequestration and alleviation of Cd induced growth inhibition (Srivastava *et al.* 2014). Here, maximum GR activity of Pankaj followed by Sabita can be correlated with significant rise in non protein thiol content owes to their better Cd tolerance capacity with respect to other concerned cultivars. This is further validated with PCA analysis where closely associated Pankaj and Sabita are placed in separate cluster indicating that cadmium had the least deleterious effects on the growth of the respective cultivars.

PCA analysis

Euclidean distance algorithm based hierarchical clustering indicated generation of three separate clusters (Fig. 8) dependent on distinctive responses of the rice cultivars under cadmium contamination. Cultivars like Pankaj and Sabita showed tolerance towards Cd stress and they formed a separate cluster. The second cluster comprised of Sabita and Khitish showing moderate tolerance. However, cultivar MTU-1010 formed an outgroup since it was most sensitive. A clustered heat map (Fig. 9) was created with differential color intensity for visual perception of all experimental data obtained from all considered parameters where it was evident that in tolerant cultivars like Pankaj and Sabita showed least membrane damage and lower amount of H_2O_2 generation compared to other cultivars. At the same time, non-enzymatic antioxidants like proline, phenol and flavonoid contents were maximum in Pankaj. On the contrary, cultivar MTU-1010 lacked robust ROS scavenging system hence showed maximum growth impairment. PCA was carried out to understand the correlations between the selected rice cultivars and changes in physiological and biochemical parameters of the plants upon cadmium treatment. The value of PC1 along with PC2 comprehensively clarified 80.7% of data variability. Both PC1 and PC2 had positive values associated with growth parameters such as length of root, shoot and vigour of seedling, RWC, chlorophyll contents, starch and other parameters which combat against oxidative stress like, SOD activity, proline, non-protein thols, phenol and flavonoid contents indicated the correlation among these parameters.

These components formed a separate cluster and rice cultivars like Pankaj and Sabita were closely associated with these components which clearly indicate that cadmium had the least deleterious effects on the growth of the respective cultivars. In contrast stress indicating factors like H_2O_2, MDA, and electrolytic leakage levels were grouped in a different quadrant with reduced PC1 and PC2 values. This reflects negative correlation between these 2 clusters. MTU-1010 associated closely with the stress indicating factors and it represented the out group and can be considered to be the most sensitive cultivar. However, other cultivars like Sundari, Khitish and Swarnamasur was neither associated with growth factors or stress markers and hence can be considered to be moderately tolerant.

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