Low-dose Priming of Gamma Radiation Enhanced Cadmium Tolerance in Chlamydomonas Reinhardtii by Modulating Physio-biochemical Pathways

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Abstract

Microalgae, the key components of aquatic environments, are executive natural biotic models for exploring genotoxic effect of heavy metals, irradiation and other external stimuli and the toxicant elimination. In the current scenario, removal of metal contaminants is a significant challenge. Recently, the effective removal of heavy metals from the aquatic environment using microalgae has gained immense intention. However, the concurrent toxicity has limited their use as bio-accumulants to reduce the concentration of heavy metals and promote heavy metal tolerance. Few studies opined that low dose priming with non-ionizing radiations, such as gamma radiation, increased heavy metal tolerance in plant as well as aquatic photosynthetic microalgae. In the present study, we have hypothesized the growth inhibitory physio-chemical properties by cadmium (Cd) in the green algae *Chlamydomonas reinhardtii*, and analysed the protective role of low-dose gamma radiations priming against Cd induced growth inhibition. In addition, we have specially emphasized on the mechanism of cell survival in the experimental species with effective notation to antioxidant defence system during Cd induced toxicity. Experimentally, we primed the *Chlamydomonas reinhardtii* with low-dose gamma radiation prior to Cd treatment. On the other hand, gamma-radiated and Cd-treated organisms were considered as positive controls. We calculated the rate of cell death, the deployment of antioxidant enzymes such as catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GSR), and superoxide dismutase (SOD). Furthermore, the role of oxidative stress related genes was analysed computationally to delineate their involvement in cell death/survival, suggesting that the low-dose priming of gamma radiation enhances the Cd tolerance by alterating cell/death pathways and other biochemical responses.

1. Introduction

Heavy metal pollution causes serious ecological imbalance that has a deleterious health effect. Heavy metals are accumulated in aquatic micro and macro organisms and are transferred to humans through the food chain (Hassan and Aarts 2011; Uraguchi and Fujiwara 2012). In the last years, heavy metals such as cadmium (Cd) have caused serious adverse health effect around the world (Patra et al. 2007). Cd is a naturally occurring environmental pollutant present in soil, water, and air, as well as food. The common sources of Cd are natural. Anthropogenic sources, such as burning of coal and inorganic lubricant, smelters, alloy and paint industries, also liberate Cd to the environment (Patra et al. 2007). Cd is a water-soluble contaminant that easily disperses into the environment through the aquatic medium and elicit serious threat to all living organisms (Thapa et al. 2012). Cd inhibits photosynthesis by decreasing electron transport in Photosystem II (Ran et al. 2015), replaces the Mg in chlorophyll and also interferes in xanthophyll cycle (Bertrand et al. 2001). Long-term exposure to Cd causes toxic effects which lead to increase in lipid peroxidation and production of reactive oxygen species (ROS), such as hydroxyl and superoxide radicals, hydrogen peroxide, and singlet oxygen (Kumari et al. 2010). These ROS damage cellular macromolecules, such as DNA, RNA, lipids, and proteins, thus leading to cellular dysfunction, chromosomal aberrations, and lethal gene mutations (Choi et al. 2015).

Ionizing radiations cause toxicity in biotic systems. Previous reports demonstrated that gamma radiation upsets algal growth and survival (Pradhan et al. 2020a). However, low-dose gamma irradiation enhances cell proliferation, germination and growth of algae by increasing the activity of antioxidant enzymes (Pradhan et al. 2020a). In addition, the irradiation can improve the tolerance of microalgae to different abiotic stresses, such as heavy metals, cold, drought, temperature, and salinity. (Wang et al. 2018; Qi et al. 2015; Haleem 2012).

Microalgae are the key components in the aquatic ecosystems as primary producers (Maharana et al. 2019; Dash et al. 2020; Dash et al. 2021; Behera et al. 2020). Apart from this, they are termed as effective bio-accumulants that reduce heavy metal toxicity in the aquatic ecosystems. In the meanwhile, these organisms also exhibit metal tolerance. For example, *Chlamydomonas reinhardtii*, an eukaryotic green microalgae, exhibited extraordinary metal tolerance and enhanced sustainability under stress circumstances, such as heavy metals, salinity, and dehydration (Samadani et al. 2018). Several reports have demonstrated the role of ionizing radiations in metal tolerance and removal of algae (Pradhan et al. 2020a). However, the priming effect of gamma irradiation on heavy metal tolerance and riddance in *Chlamydomonas reinhardtii* has not yet been elucidated.

In the present study, we hypothesized the role of low dose priming of gamma radiation in maintaining the Cd-related metal toxicity through bioaccumulation and tolerance. Hence, we analysed the protective role of low-dose gamma radiations priming against Cd induced growth inhibition using the green algae *Chlamydomonas reinhardtii* collected from Balasore pond, Odisha. In addition, the mechanistic investigation on the mode of tolerance and survival of the species has been decrypted with a special notation on the antioxidant defence enzymes. The possible outcome of the present study is that low-dose priming of gamma radiation can modulate the physio-biochemical process to overcome Cd toxicity.

2. Materials And Methods

2.1. Chemicals and reagent used

1,1-Diphenyl-2-picrylhydrazyl (DPPH), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), nitro blue tetrazolium chloride (NBT), hydrogen peroxide (H_2O_2), nicotinamide adenine dinucleotide phosphate (NADPH), riboflavin, nicotinamide adenine dinucleotide (NADH), methionine, phenazine methosulfate (PMS), Triton X-100, guaiacol, glutathione, ascorbic acid, gallic acid, rutin, and sodium salicylate were provided by Sigma-Aldrich, Merck. Ferrous sulfate ($FeSo_4$), Tris HCl, sodium phosphate, ammonium molybdate, ferric chloride ($FeCl_3$), sodium carbonate, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), ammonia solution (NH_3), sulfuric acid (H_2SO_4), hydrochloric acid (HCl), Fehling's solutions A and B, aluminum chloride ($AlCl_3$), mercury potassium iodide, sodium hydroxide (NaOH), isoamyl alcohol, glacial acetic acid, nitric acid, methanol, chloroform, ethanol and hexane were purchased from Himedia.

2.2. Microalgal culture conditions

Chlamydomonas reinhardtii (MJ11/33) was acquired from Berhampur University, Algal Biotechnology and Molecular Systematic Laboratory, Department of Botany. The microalgae was cultured in 500 mL borosil flask. The flask containing 250 mL of Bold's Basal Medium (Bold and Wyne, 1985) was hatched for 21 days for mass culture in algal culture room at 25 ± 2°C with a light intensity of 7.5 W/m^2 of photoperiod 14 : 10 (light : dark). The culture was shacked at 100 rpm for 15 min every day in an electric shaker. After the exponential phase, the algal mass was collected for pre-treatment with gamma irradiation and subsequent experiments, as indicated below.

2.3. Pre-treatment of gamma irradiation and cadmium treatment

2.3.1. Experimental set up

The microalgal cell were centrifuged at 5000 rpm (10 min) and re-suspended in 10 mL BBM medium. The 500 mL flasks containing 250 mL of BBM medium with algal suspension were pre-treated with a gamma irradiation dose of 0.01 kGY and cadmium 1.0 mg/mL. Control experiments were performed either without cadmium and gamma irradiation treatment, or with just 1.0 mg/mL of cadmium. After one set of gamma irradiation treatment and 24 hour of inoculation of the cells, the algal suspension was centrifuged at 5000 rpm, washed three times with double distilled water, and then re-suspended in 10 mL of double distilled water. The post-inoculation of pre-treated gamma radiation of 0.01 kGy at dose rate 3.512 kGy h^{-1} group were treated with cadmium of 1 mg/mL. All the experiments were repeated three times. The gamma irradiation and cadmium free group was retained as the control group. The amount of gamma irradiation (0.01 kGy) and the dose of Cd (1.0 mg/mL) were selected from previous tests with a range of concentrations, i.e. 0.01, 0.05, 0.075, 0.1, 0.125, 0.150, and 0.2 kGy and 0.1, 0.5, 1.0, and 2.0 mg/mL, respectively. The gamma irradiation was achieved by using ^{60}Co (Cobalt 60) source (GC-5000, Mumbai) at Gamma house Department of Botany, Berhampur University, founded by BARC India. The irradiated samples were re-cultured in 500 mL cultured flask containing 250 mL of BBM medium and incubated for 18 days in the culture room under same condition described earlier. The cell growth was monitored by measuring the OD_{750} throughout the incubation period within an interval period of three days.

Several biochemical, antioxidant, and physiological parameters were evaluated at the end of the incubation period. The physiochemical and biochemical experiments included pigment profiling, lipid peroxidation, and determination of the concentrations of carotenoids, proteins, proline, and different ROS shooting antioxidant enzymes, such as CAT, APX, GPX, GSR and SOD. Furthermore, the TPC, TFC, and TAA were also calculated and the free radical scavenging activity was also estimated. The experimental conditions used for determining these parameters are detailed below.

2.3.2. Measurement of algal growth, chlorophyll and carotenoid

The algal growth curve indicates the capacity of the algae to improve themselves in stress circumstances. The growth curve was plotted by measuring the optical density (OD) at 750 nm (Pradhan et al. 2020a) throughout the incubation period with an interval of 3 days up to 24 days. The morphological variation of the cells was analysed in the microscopic photographs (Olympus BX-53 microscope) after the incubation period by using cellSens Image software (version 2.2, Olympus). The aliquots Cell were harvested by centrifugation after the incubation period for experimental analysis. Post centrifugation, chlorophyll a and b and the total carotenoids' content were estimated as previously described by Pradhan et al. (Pradhan et al. 2020a). The content of chlorophyll a and b and the total carotenoid content were calculated via the equation defined by (Wellburn 1994).

2.3.3. Estimation of electrolyte leakage, lipid peroxidation and proline content

In order to determine the cell membrane stability, electrolyte leakage was estimated by the previously described protocol of Aghaie et al. (Aghaie et al. 2018) with slight modification. Briefly, 10 mL of algal suspension was centrifuged at 5000×g for 10 min. The algal pellet was washed 2–3 times in 10 mL of double distilled water. After being properly washed, it was stored in dark condition at 25°C. After 24 h, the initial electrical conductivity (EC_1) was measured with a conductivity meter. The samples were then heated for 20 minutes at 100°C and the EC_2 was measured. The electrolyte leakage was calculated as a percentage by the following equation:

EL (%) = (EC1/EC2) × 100

The membrane lipid peroxidation was evaluated by determining the MDA content as previously described (Pradhan et al. 2021c). The MDA content was calculated by using the extinction coefficient of 155/(mM cm) and expressed as nmol/g of fresh weight (F.W.). The free proline content was estimated and expressed on an algal fresh weight as μmoles proline/g of F.W by using L-proline (20–100 μg/mL) as standard, as already described (Pradhan et al. 2021c).

2.3.4. Estimation of protein and antioxidant enzymes

The estimation of protein content and antioxidant defence enzymes such as CAT, APX, GPX, GSR, and SOD were quantified as previously described by Pradhan et al. (Pradhan et al. 2020a; Pradhan et al. 2020c).

2.3.5. Qualitative screening of phytochemicals

The qualitative investigation of phytochemicals in *C. reinhardtii* was carried out as previously described by Pradhan et al. (Pradhan et al. 2020a).

2.3.6. Estimation of total phenolic, flavonoid content and total antioxidant activity

The total phenolic content was measured by Folin-Ciocalteu as previously described (Pradhan et al. 2020a). The results are reported as mg of gallic acid equivalents (GAE) per g of dry weight of the sample. The total flavonoid content was measured as already described (Pradhan et al. 2020a). The results are reported as mg of rutin equivalents (RUE) per g of dry weight of the sample. The determination of total antioxidant activity was carried out by the phosphomolybdenum method as previously described (Pradhan et al. 2020a). The results are reported as mg of ascorbic acid per g of dry weight of the sample.

2.3.7. Estimation of DPPH, hydroxyl ion, superoxide, and hydrogen peroxide free radical scavenging activity

The free radical scavenging activity of *C. reinhardtii* was determined by DPPH assay as previously described (Pradhan et al. 2020a; Pradhan et al. 2021a). The hydroxyl radical scavenging activity of *C. reinhardtii* was determined as previously described by Patra et al. (Patra et al. 2020a; Patra et al. 2020b). The superoxide assay was based on the reduction of NBT in the presence of NADH and PMS, as already described (Pradhan et al. 2021b; Pradhan et al. 2020b). The ability of the extract of *C. reinhardtii* to scavenge hydrogen peroxide was determined according to Nabavi et al. (Nabavi et al. 2008) and modified as previously described (Pradhan et al. 2020a). The free radical scavenging activity in all assays was expressed as % of inhibition.

2.3.8. Computational work of homology modelling and validation via molecular docking

According to the hypothesis, we performed a computational study (molecular docking) with cadmium chloride ($CdCl_2$) and two oxidative regulator enzymes, glutathione peroxidase (GPXH) and glutathione-*S*-transferase1 (GSTS1), of *C. reinhardtii.* Primarily, for molecular docking study, we needed a target and ligand structure. However, due to the unavailability of NMR or X-ray structures of GPXH and GSTS1 in the protein data bank (https://www.rcsb.org/), we employed homology modelling (template-based modelling) to generate the 3D-structure from their sequence. Consequently, we retrieved the amino acid sequence of both enzymes, i.e. GPXH (id: O22448, consisting of 162 amino acids) and GSTS1 (id: A8JBA7, consisting of 218 amino acids), from the UniProtKB database (https://www.uniprot.org/). We then run BLASTp (https://blast.ncbi.nlm.nih.gov/) against the protein data bank structure for a suitable template identification. Finally, the tool SWISS-MODEL (https://swissmodel.expasy.org/) was used to model with the highest identified template. After modelling, the SAVES v 6.0 server (https://saves.mbi.ucla.edu/) was used for the validation, followed by Ramachandran plot analysis.

The newly generated 3D structures of both GPXH and GSTS1 enzymes, used as targets, and that of $CdCl_2$, used as ligand, were saved in a PDB (.pdb) file format. Auto Dock 4.1 software was used for the molecular docking study, followed by defaulter AutoDock parameter setting with newly generated grid-boxes, 70x64x60 with a spacing of 0.375 Å for the GPXH and 71x72x72 with a spacing of 0.486 Å for the GSTS1 (Swain et al. 2020; Swain et al. 2021). During the docking, ten $CdCl_2$ poses were generated against each target enzyme, from which the lowest energy occupied docking score was selected as the

most potential pose. Then protein-ligand docking complex visualization was carried out by the Discovery Studio Visualizer-2019 client version.

2.3.17. Statistical analysis

All the experiment was carried out independently in triplicate. The statistical significance of the data was evaluated by one-way analysis of variance (ANOVA) followed by least significance difference (LSD) test. Windows-10 Microsoft Excel software was used for data analysis and graphics. Furthermore, the multiple data were scaled in BioStatFlow (v.2.9) and ClustVis software was employed for the hierarchical clustering analysis (HCA) (Metsalu and Vilo 2015; Pradhan et al. 2021c). All the treatments were clustered via using heatmap function through row-wise scaling and achieved by way of correlation-based clustering. Finally, the principal component analysis (PCA) analysis was carried out according to Xie et al. (Xie et al. 2019).

3. Results

3.1 Low dose priming with gamma radiation (LDGR) sustains growth kinetics, cell death and stress tolerance of C. reinhardtii during Cd-induced toxicity

The growth kinetics of *C. reinhardtii* was evaluated in a 24 days period with 3 days interval (Fig. 1). In Cd treated (0.1-1.0 mg/mL of Cd) *Chlamydomonas* sp., we observed a dose dependent as well as time dependent retardation in growth rate (Fig. 1A). Similarly, in a separate set of experiments with gamma radiation treatment (GR; 10–100 Gy), a dose as well as time dependent decrease in growth rate was evident in *C. reinhardtii* (Fig. 1B). Furthermore, we primed the algal strain with the lowest dose of gamma radiation tested (10 Gy) and exposed it to Cd elated toxicity. Interestingly, we found that the synergism of both treatments (Cd + GR) induced a higher growth rate than each one individually in a time dependent manner, suggesting that LDGR sustained the growth of the strain even in Cd related toxicity (Fig. 1C). The specific growth rates at 18 days incubation (doubling time) were also investigated, and showing (Fig. 1D).

A morphological characterization was also performed to evaluate the strain growth and survival under these treatment conditions, evidencing morphological changes and cell wall degradation in the strain (Fig. 2). The images confirmed that LDGR reduced the cell wall degradation as compared to Cd treatment (Fig. 2A-E). Reduced cell death in radiation primed Cd-treated cells, as compared to either Cd or radiation treatment alone, was also found (Fig. 2F-J). On the other hand, we noticed a reduced % of cells with degraded nuclei after the combined treatment compared to each one individually (Fig. 2K-O). As palmonoid stage is a representative of stress tolerance response, we observed the same under the treatment conditions detecting an enhanced palmonoid formation in the Cd + GR treatment as compared to Cd and GR treatment alone (Fig. 2P-T).

3.2 LDGR restrains the photosynthetic pigments of *C. reinhardtii* associated with growth and survival

As photosynthesis is a key mechanism for cell survival and growth, we evaluated the photosynthetic pigments under the treatment conditions in *C. reinhardtii* (Fig. 3). First of all, we evaluated the chlorophyll a content after Cd, GR, and Cd + GR treatment. Our results showed that the chlorophyll *a* content was restored in the synergistic treatment (Cd + GR: 17.77 ± 0.65 µg/mL) as compared to the individual treatments (CD: 11.85 ± 0.13 µg/mL and GR: 16.20 ± 0.13 µg/mL) and found to be significant as compared to the control (19.21 ± 0.33 µg/mL) (Fig. 3A). Similarly, the chlorophyll b content followed a similar pattern to that of chlorophyll a (CD: 11.68 ± 0.09, GR: 13.36 ± 0.21, and Cd + GR: 21.29 ± 0.32 µg/mL) (Fig. 3B). Furthermore, we evaluated another stress response pigment, i.e. the carotenoid content. We observed a massive reduction in its concentration in Cd treated samples (3.28 ± 0.51 µg/mL), which was restored when the algae were primed with low dose gamma radiation (5.79 ± 1.13 µg/mL) (Fig. 3C). GR-primed cells with Cd treatment induced a significant increase of carotenoids, resulting in a higher cell survival under stress. As carbohydrates are another primary stress responsive elements, we also quantified the carbohydrate content in the strain under the treatment conditions, observing their increase after the synergistic treatment (Cd + GR: 185.33 µg/mL) as compared to Cd doping (104.58 µg/mL) and) and control (170 µg/mL), although less than after GR treatment (209.31 µg/mL) (Fig. 3D).

3.3 LDGR treatment reduces Cd-related toxicity in *C. reinhardtii* via modulating the physiochemical parameters.

The percentage of electrolyte leakage (EL) is a signature of stress response. We observed a reduced electrolyte leakage in the Cd-treated cells (15%), which was further enhanced in the gamma radiation primed cells (22%) (Fig. 4A). The highest electrolyte leakage was observed in the GR treated cells (25%) as compared to control (19%). These results suggest that low dose primed cells are more responsive toward stress as compared to the Cd-treated cells, that in turn healed themselves to survive under stressful conditions. Similarly, accumulation of proline is also a responsive marker of stress. We observed a decreased proline content in the Cd-treated cells (4.43 µmoles proline/g of fresh weight) (Fig. 4B). On the other hand, a higher accumulation of proline was observed in the GR-treated cells (8.54 µmoles proline/g of fresh weight) as compared to control (6.21 µmoles proline/g of fresh weight). The proline content was restored in the Cd + GR samples (7.34 µmoles proline/g of fresh weight). A similar pattern was observed in the MDA contents (indicative of the degree of lipid peroxidation), that was higher in the Cd + GR group (8.5 nmoles 10^6 $cell^{-1}$) as compared to the Cd-treated group (5.0 nmoles 10^6 $cell^{-1}$) (Fig. 4C).

After extraction in methanol, a qualitative screening of phytochemicals was performed (Table 1). Since polyphenols are crucial in stressful conditions as they are able to scavenge free radicals responsible for oxidative stress, we further estimated the total phenolic (TPC) and flavonoid content (TFC). We observed a reduced TPC in the Cd-treated cells (2.9 mg of GAE/g) compared to control (4.33 mg of GAE/g) (Fig. 4D). However, low dose priming with gamma radiation raised again their TPC content (5.0 mg of GAE/g),

although less than GR alone (6.5 mg of GAE/g). Similarly, a reduced TFC content was noted in the Cd-treated group (193.33 mg RUE/g) compared to control (252 mg RUE/g) (Fig. 4E). Nevertheless, low dose priming with gamma radiation enhanced the TFC (290 mg RUE/g), as confirmed by GR treatment alone (364.33 mg RUE/g). Finally, the total antioxidant activity also followed a very similar pattern (control: 198.67, Cd: 151.67, GR: 265.33, and Cd + GR: 203.33 mg of AAE/g) (Fig. 4F).

3.4 LDGR stimulates Cd tolerance via enhancement of free radical scavenging activity of *C. reinhardtii*

After estimating the TFC, TPC, and TAA, we examined the different free radical scavenging activity of the *C. reinhardtii* that were responsible for the antioxidant defence mechanism in a stressful environment. Our initial findings suggested a maximum percentage of inhibition of the hydroxyl radicals at 500 µg/mL in the gamma radiation primed cells (79.23%), while it was significantly lower in the GR (70.29%) as well as Cd (68.53%) treatment alone (Fig. 5A). The IC_{50} value of the CDGR sample of *C. reinhardtii* (3.48 µg/mL) was found to be more significant as compared to the individual treatments (Table 3). This result advocated that priming with low dose gamma radiation stimulates the scavenging of hydroxyl radicals. Furthermore, we evaluated the hydrogen peroxide radical scavenging activity. In the Cd treated strain, we found a reduced percentage of inhibition of hydrogen peroxide radicals at 500 µg/mL (75.22%) which was further enhanced in the gamma radiation primed cells (79.40%) (Fig. 5B). The IC_{50} value of the hydrogen peroxide radical scavenging activity in the Cd + GR sample (3.88µg/mL) of *C. reinhardtii* was significantly lower than that of the Cd-treated strain (4.15) (Table 3), suggesting a strongest radical scavenging activity in the radiation primed strains as compared to the Cd-treated strain. A similar pattern of inhibition was observed for the superoxide radical scavenging activity, with a value for Cd + GR sample significantly higher than that obtained in the Cd-treated sample alone (56.14 and 29.47%, respectively) (Fig. 5C). As a consequence, the corresponding IC_{50} value was much lower in the Cd + GR-treated strain (5.11) compared to that of the Cd-treated strain (8.17) (Table 3). Finally, the DPPH radical scavenging activity also exhibited a similar pattern. In Cd-treated strain the DPPH radical scavenging activity at 500 µg/mL was lower (41.90) than that of the strain primed with low dose of gamma radiation (58.32) (Fig. 5D). The IC_{50} of DPPH radical scavenging activity in Cd + GR-treated sample (4.22) was significantly less compared to that of the Cd-treated strain (5.47µg/mL) (Table 3).

Table 1: Preliminary phytochemical screening of *Chlamydomonas reinhardtii.*

Bioactive compounds	(+) Present or (-) Absent			
	Control	CD	GR	CDGR
Alkaloids	+	+	+	+
Glycosides	+	+	+	+
Reducing sugars	-	-	-	-
Proteins	+	+	+	+
Terpenoids	+	+	+	+
Phenols and Tannins	+	+	+	+
Steroids	-	-	-	-
Saponins	+	-	-	+
Anthocyanins	-	-	-	-
Coumarin	+	+	+	+

Table 2
Total phenolic, flavonoid and antioxidant activities of *Chlamydomonas reinhardtii.*

Bioactive compounds	**(+) Present or (-) Absent**			
	Control	**CD**	**GR**	**CDGR**
Alkaloids	+	+	+	+
Glycosides	+	+	+	+
Reducing sugars	-	-	-	-
Proteins	+	+	+	+
Terpenoids	+	+	+	+
Phenols and Tannins	+	+	+	+
Steroids	-	-	-	-
Saponins	+	-	-	+
Anthocyanins	-	-	-	-
Coumarin	+	+	+	+

Table 3
IC_{50} values of the free radical scavenging activities *Chlamydomonas reinhardtii*.

Parameter	Control	CD	GR	CDGR
Total phenolic content (mg of GAE/g)	4.33 ± 0.6	2.9 ± 1.0	6.5 ± 1.0	5 ± 1.0
Total flavonoid content (mg of RUE/g)	252.00 ± 1.00	193.33 ± 1.15	364.33 ± 1.16	290.00 ± 2.16
Total antioxidant activity (mg ofAAE/g)	198.67 ± 4.51	151.66 ± 5.59	265.33 ± 5.45	203.33 ± 3.45

Parameter	Control	CD	GR	CDGR
DPPH scavenging activity	3.88	5.47	4.67	4.22
H_2O_2 scavenging activity	3.21	4.15	4.1	3.88
Superoxide scavenging activity	4.59	8.17	5.9	5.11
Hydroxyl radical scavenging activity	3.31	4.01	3.71	3.48

3.5 LDGR deploys antioxidant enzymes for enhanced Cd tolerance in *C. reinhardtii*

The antioxidant enzyme activities were measured to demarcate the oxidative stress response to Cd treatment. Firstly, we quantified the total soluble protein content in the different treatment conditions. The data confirmed that Cd treatment reduced the total soluble protein content (79.33 µg/mL) as compared to the control (110.00 µg/mL). However, low dose priming of gamma radiation recovered the total soluble protein content (115.69 µg/mL) in the strain, yielding a significantly higher amount than that obtained after the Cd treatment alone. The results demonstrated that the low dose priming of gamma radiation neutralized the cadmium stress via increasing the protein content (Fig. 6A). Furthermore, the activity of antioxidant enzymes that play an important role to combat against the oxidative stress, i.e. CAT, APX, GPX, GR, and SOD, were determined.

Initially we quantified the CAT activity and found a reduced activity in the Cd-treated strain (60.52 nmoles of H_2O_2 utilised mg^{-1} protein min^{-1}) as compared to the control (82.23 nmoles of H_2O_2 utilised mg^{-1} protein min^{-1}) (Fig. 6B). On the other hand, the strain treated by low dose priming with gamma radiation exhibited an elevated CAT activity (82.74 nmoles of H_2O_2 utilised mg^{-1} protein min^{-1}) as compared to

that of the Cd treatment alone. The strain treated individually by GR also exhibited a higher CAT activity (90.86 nmoles of H_2O_2 utilised mg^{-1} protein min^{-1}). In addition, we analysed the APX enzyme activity, observing a reduced activity in the Cd-treated strain (0.3 µmoles of oxidised ascorbate formed mg^{-1} protein min^{-1}) which was found to be recovered in the Cd + GR treated strain (0.56 µmoles of oxidised ascorbate formed mg^{-1} protein min^{-1}) (Fig. 6C). The control and the GR treatment alone exhibited values of 0.5 and 0.81 µmoles of oxidised ascorbate formed mg^{-1} protein min^{-1}, respectively.

The GPX activity was then measured, showing a similar level of expression in the treatment groups and control, while LDGR strain activity was somewhat higher (0.05, 0.04, 0.09, and 0.05 µmoles of tetraguaiacol formed mg^{-1} protein mg^{-1} for the CDGR, Cd + GR, and control, respectively). Along with GPX, the GSR activity also followed a similar pattern (Fig. 6D). A significant increase in its activity was found in the gamma radiated strain (0.18 µmoles of NADPH oxidised mg^{-1} protein min^{-1}) which was reduced by the Cd-induced stress (0.09 µmoles of NADPH oxidised mg^{-1} protein min^{-1}). The low dose priming with gamma radiation elevated the GSR content (0.13 µmoles of NADPH oxidised mg^{-1} protein min^{-1}) as compared to the Cd treatment alone (Fig. 6E). Similarly, the SOD activity was found to be significantly reduced in the Cd-treated strain (6.12 units mg^{-1} protein min^{-1}), which however increased in the GR-treated sample (13.45 units mg^{-1} protein min^{-1}) (Fig. 6F). The SOD activity was also found to be well elevated as compared to Cd and control (9.96 units mg^{-1} protein min^{-1}) in the Cd + GR treatment (10.86 units mg^{-1} protein min^{-1}).

In general, the results indicated that low dose priming with gamma radiation elevated the activity of the antioxidant enzymes in the strain to rescue it from the oxidative stress related toxicity and cell death post Cd treatment.

3.6 GPXH and GSTS1 are crucial targets for Cd-related oxidative stress and toxicity as validated through computational approaches

During ideal template search through BLASTp, the crystal structure of glutathione-dependent phospholipid peroxidase of *Saccharomyces cerevisiae* (PDB ID: 3CMI) for GPXH and the crystal structure of the glutathione *S*-transferase of *Ascaris lumbricoides* (PDB ID: 4Q5F) for GSTS1 were selected as most suitable template structures modelling. Briefly, the 3CMI structure exhibited a 96% of query coverage with 60.06% of sequence identity with GPXH, and similarly the 4Q5F one showed 91% of query coverage with 36.95% of sequence identity with GSTS1 from blast search (see the target-template alignment presented in Figure S1 A and B). Subsequently, we modelled the respective 3D structures (Fig. 7A1 and 7B1) using these templates and validated them by the Ramachandran plot. Based on the phi (φ) and psi (ψ) torsion angle distributions, the GPXH model showed 93.55% of amino acid residues in the favourable region and only 1.9% of residues in outer regions, while the GSTS1 one 92.40% of residues in the favourable region and only 3.19% of residues in outer regions of the retrieved Ramachandran plots (Fig. 7A2 and 7B2). In addition, the MolProbity, ERRAT, Verify3D, Prove, GMQE, and QMEAN analyses confirmed that the

generated models were the most reliable and stable protein structures and they were employed in the subsequent docking study.

Based on the docking studies, $CdCl_2$ showed a docking score of -3.69 with ligand efficacy − 0.42 kcal/mol against GPXH and − 3.43 kcal/mol with ligand efficacy − 0.39 against GSTS1. As a heavy metal, the docking score > 3 kcal indicated that the $CdCl_2$ strongly interacted with the active site of the enzymes, thus inhibiting them and inducing cell death. The molecular interaction of the docking complexes are visualized in Fig. 7A3 and 7B3. The computational work supported our hypothesis, suggesting the beneficial effect of the LDGR treatment in avoiding the oxidative damage caused by $CdCl_2$.

3.7 Analysis of titanic interlink between physiological and biochemical parameters post treatment through HCA and PCA

The colossal interlink between physiological and biochemical parameters post Cd and Cd + GR treatment were evaluated by hierarchical clustering analysis (HCA). The HCA, visualized through heatmap, grouped the growth and physiological parameters previously determined into three main clusters (Fig. 8). Four parameters, i.e. the IC_{50} of hydrogen peroxide, superoxide, hydroxyl, and DPPH radical scavenging activity, were grouped in the first cluster (Fig. 8). The other parameters were clustered in two subgroups, one containing cell growth and chlorophyll a and b, the main indicators of stress response, while the remaining ones, e.g. lipid peroxidation, SOD, TAA, TPC, and APX, together with other physiological characters such as GPX, GSR, and electrolyte leakage, comprised the third group. From the HCA analysis we concluded that the control and the Cd + GR treated strain were closely related as the low dose priming had retrieved the Cd related toxicity and the strain behaved similarly to the control one. The protein content and all the antioxidant enzymes' parameters were additionally clustered into three groups, the first one containing protein and CAT, the second GPX and GSR, while the third comprised APX and SOD (Fig. 9). The HCA analysis demonstrated that low dose priming of the strain with gamma radiation increased the protein content as well as the anti-oxidative enzymes, e.g. CAT and, especially, SOD, APX, GPX, and GSR, thus reducing the oxidative upset caused by the Cd treatment.

The two principal components, i.e. PC1 and PC2, drawn taking all the physiological variables, were able to represent 98.3% of total variance (Supplementary Fig. 2). All the variables related to the PC1 did not show a clear separation between the doses of stress and elucidated 98.3% of the total variance. For the PC1, the growth and chlorophyll content were reduced in relation to Cd stress, but gamma radiation triggered cell growth and chlorophyll restoration. However, the TFC, APX, GPX, lipid peroxidation, proline, TPC, and growth were closely associated with the specific treatments (Cd, GR, and Cd + GR). Furthermore, the TAA, protein content, and CAT were positively correlated. On the other hand, the PC2 only elucidated 1.3% of the total variance (Supplementary Fig. 2).

4. Discussion

Microalgae, the key components of the aquatic environment, are executive natural biotic models for exploring genotoxic effect of heavy metals, irradiation, and other external stimuli. Heavy metals, such as Cd, causes reduced cell growth and death via induction of oxidative stress, lipid peroxidation, and DNA damage (Ran et al. 2015). However, these photosynthetic primary producers are able to tolerate these metals for surviving the oxidative stress by producing several biochemical responses and deploying antioxidant enzymes (Ganapathy et al. 2017). Nevertheless, priming of the strains with low doses of a non-ionizing radiation stimulates the biochemical responses against heavy metal toxicity. With high doses, the non-ionizing radiation can be lethal, however at lower doses they stimulate metal tolerance (Bradshaw et al. 2019; Toghyani et al. 2020b). With limited evidences available on this aspect, we tried to decode the mechanism of heavy metal tolerance post low dose priming of gamma radiation.

During heavy metal toxicity, cell death is accompanied by growth retardation, damage of cell wall, and degradation of nucleus (Spiteller 2003). In the present study, our results indicated that either Cd or GR treatment retarded the growth of the *C. reinhardtii*. However, the LDGR induced an enhanced growth rate even in the Cd-treated conditions. Furthermore, we observed a significant decrease in the percentage of cell death, of cells with damaged cell wall, and degraded nucleus, suggesting a minimal death of the radiation-primed strain in the metallic environment. As a measure of stress tolerance, we also observed enhanced palmonoid-like formation. This study is well supported by previous findings (Pradhan et al. 2020a).

We evaluated the photosynthetic parameters and provided a detailed mechanistic model of how cell growth is stunted and recovered after the radiation priming. With much of our interest, we observed a reduced chlorophyll a and b content in the Cd-treated strain, which led to inhibition of photosynthesis and subsequent cell death. On the other hand, low dose priming increased the content of chlorophyll a and b, suggesting their sustainability. Carotenoids play a defensive role in stabilizing the plasma membrane against membrane lipid peroxidation and in scavenging free radicals during oxidative stress situations (Pérez-Pérez et al. 2012; Havaux 1998). As carotenoids are also responsible for photosynthesis, we evaluated the carotenoids content. Interestingly, we found that Cd reduced the carotenoids content, which however was well recovered in the low dose radiation-primed strain, even in the Cd toxic environment. This phenomenon is well supported by the previous findings (Pradhan et al. 2020a).

Additionally, under oxidative upset, cellular ingredients are dripped out of the cell because of membrane damage and they can be enumerated by measuring the electrolytes leaked from the cell. Electrolyte leakage is thus an indicator of stress resistance in algae to neutralize the oxidative stress (Anaraki et al. 2018; Aghaie et al. 2018). As the carotenoids, together with proline and other antioxidant enzymes such as CAT, stabilize the xanthophyll cycle to protect the cell membrane under oxidative stress, the electrolytic leakage is reduced. We observed a reduced electrolytic leakage after Cd treatment, finally leading to membrane rupture. The priming of low doses gamma radiation enhanced the membrane stability, as evidenced by an enhanced percentage of electrolytic leakage (Anaraki et al. 2018; Aghaie et al. 2018). Similarly, we found that Cd treatment decreased the proline content, that plays a key role as protectant and stabilizer of cellular structures and macromolecules under oxidative stress, which was further

increased in the radiation-primed strains. The results showed that a high amount of proline content was manifested under abiotic stress, as already reported by Zhang and colleagues, who observed proline accumulation under abiotic stresses in *Chlamydomonas reinhardtii* (Zhang et al. 2008).

As oxidative stress is accompanied by lipid peroxidation, we also evaluated the MDA content in the treated strain demonstrating that the Cd treatment decreased its concentration due to lipid peroxidation. On the other hand, a higher MDA content was present in the radiation-primed strain, probably to counteract oxidative stress and to maintain cellular homeostasis, in accordance with previous findings (Pradhan et al. 2021c). Under heavy metal stress, the TPC, TFC, and TAA content are significantly increased to neutralize the oxidative upset, acting as natural scavengers of radicals (Pradhan et al. 2021c). In the present study, we noticed a significant reduction in the TPC, TFC and TAA content in the Cd-treated strain. However, in the gamma radiation primed strain, higher amounts of TPC, TFC and TAA were found, suggesting that the radiation-primed cells deploy the natural radical scavengers, such as phenolic and flavonoid compounds, to eliminate free radicals and maintain the oxidative balance in the cellular environment, as already reported (Pradhan et al. 2020a; Pradhan et al. 2021c).

Reactive oxygen species, singlet oxygen species, and other free radicals are the main culprits that causes oxidative imbalance in cells (Gill and Tuteja 2010). Hydroxyl radicals, hydrogen peroxide radicals, and superoxide radicals are the main contributors to the induction of oxidative imbalance (Daspute et al. 2017). Hence, scavenging of these free radicals is a primary requirement for maintaining the cellular redox homeostasis. With this in mind, we evaluated the hydroxyl radicals, hydrogen peroxide radicals, superoxide radicals, and DPPH radical scavenging capacity. In the Cd-treated strain, we observed a reduced radical scavenging activity, suggesting the accumulation of these free radicals and thus leading to oxidative imbalance and cell death. Interestingly, in the low dose gamma radiation primed strain, an enhanced scavenging of the hydroxyl radicals, hydrogen peroxide radicals, and superoxide radicals was noted, in accordance with previous findings (Pradhan et al. 2020a).

The total soluble protein content, their localization in the subcellular spaces, intercellular metabolism, and mode of function are critical points during stress tolerance for maintaining a healthy cellular environment (Razi and Hasnain 2006). The total soluble protein content seems to be elevated as a primary response to stress, with a subsequent increase in the concentration of antioxidant enzymes (Achary et al. 2008; Rodríguez-Serrano et al. 2009; Achary et al. 2012). In this study, our data confirmed a reduced protein content in the Cd-treated cells, which was significantly elevated in the low dose gamma radiation primed strains, even after the Cd treatment. These results are well supported by previous findings (Pradhan et al. 2020c; Achary et al. 2008; Kováčik and Dresler 2018; Golari et al. 2018). Several mechanistic investigations have suggested that ROS induces cell death post heavy metal stress (Zhao et al. 2020; Chokshi et al. 2020). In response to such stressed environment, antioxidant enzymes nullify the ROS and promote cell viability. It is well-known that hydrogen peroxide radicals are responsible for introducing cell damage, while APX catalyses their detoxification. In the present study, we observed an enhanced APX activity in the radiation-primed strain as compared to the Cd-treated strain. We also observed an increased content of GPX in the radiation-primed strain compared to the Cd-treated cells, suggesting an

effective detoxification of hydrogen peroxide radicals (Daspute et al. 2017). In addition, a similar trend in the GSR activity was observed, suggesting an efficient elimination of singlet oxygen species, such as hydroxyl radicals and other electrophiles in the course of oxidoreduction of FAD to NADPH (Daspute et al. 2017). Additionally, an increased SOD content in the radiation-primed strain indicated catalysis of the dismutation reaction of free radicals to maintain oxidative balance in the cellular system (Alscher et al. 2002). In conclusion, our investigations provided a mechanistic insight into the stress response through the use of antioxidant enzymes that in turn promoted cell growth and viability.

Furthermore, an *in silico* study was conducted to discover the genetic basis of the Cd-related toxicity. GSTS1 and GPXH are the main responsive genes under the oxidative stress (Swain et al. 2020). To validate the mode of binding of GSTS1 and GPXH with $CdCl_2$, homology modelling was performed. The 3D structures of the enzymes were derived and validated by the Ramachandran plot, showing a strong binding with $CdCl_2$. In addition, MolProbity, ERRAT, Verify3D, Prove, GMQE, and QMEAN analyses confirmed that the generated models were the most reliable and stable protein structures, and they were exploited for the subsequent docking studies (Swain et al. 2021). The computational work supported our previous hypothesis and the need of further treatment to avoid oxidative damage caused by $CdCl_2$.

Hierarchical cluster analysis (HCA) and principle component analysis (PCA) were exploited to investigate the correlation among the assessed experimental parameters and to determine the multidimensional response under oxidative stress, providing a multivariate approach (Löw et al. 2012; Husseini et al. 2020; Toghyani et al. 2020a). In particular, HCA and PCA were employed to reveal the aberrations in the physiological and biochemical parameters in the Cd-, GR-, and LDGR-treated strains. Accountably, our results indicated a close association of the physiological and biochemical parameters under the treatment conditions. The correlation of the physiological and biochemical parameters confirmed that priming of the algal strain with low dose gamma radiation induced an enhanced Cd tolerance. Upon priming, the primary stress responsive parameters are strongly correlated with the investigated defence mechanisms are all upregulated, thus providing an immediate quick response to counteract the oxidative stress produced by the heavy metal.

5. Conclusion

In the current scenario, heavy metal contaminants, such as Cd, have been a major limiting factor for crop production because of their toxicity in soil and water. Low dose priming with non-ionizing radiations, such as gamma radiation, proved to be a successful approach for maintaining a healthy redox environment and to nullify the heavy metal contaminant related oxidative damage. In the present study, we have demonstrated that Cd causes oxidative imbalance in *C. reinhardtii* by downregulating the oxidative enzymes. The reduction of the photosynthetic and accessory pigments content was also responsible for the Cd-related toxicity, causing stunted cell growth and inducing cell death. Low dose priming with gamma radiation, however, recovered the cell growth and inhibited cell death by deploying the antioxidant enzymes, such as GPX, GSR, APX and SOD. In addition, it has enhanced the concentration of photosynthetic and accessory pigments. Multivariate analysis and docking studies supported this

hypothesis, thus providing a mechanistic insight into heavy metal stress tolerance by *C. reinhardtii*. The low dose priming with gamma radiation approach could be easily implemented for treating higher plants, thus improving their safety and allowing to obtain better yields even from contaminated crop fields.

Declarations

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Declarations

Ethics approval

Not applicable.

Consent for Participate

Not applicable.

Consent for publication

Not applicable.

Authors' contributions

Biswajita Pradhan: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, figure editing, Visualization, Supervision, Proof correction. **Srimanta Patra:** Conceptualization, Formal analysis, Writing - review & editing, Visualization, Proof correction. **Rabindra Nayak:** Conceptualization, Methodology, Validation, Writing - review & figure editing. **Shasank S. Swain:** Validation, doing molecular tools. **Bimal Prasad Jit:** Formal analysis. **Chhandashree Behera:** Formal analysis. **Andrea Ragusa:** Formal analysis, review & editing, correction. **Jang-Seu Ki:** Formal analysis, review & editing, correction. **Mrutyunjay Jena:** Conceptualization, Methodology, Validation, Investigation, Resources, Review & editing, Supervision, Proof correction.

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Availability of data and materials

All data and material are available upon request.

Conflicts of interest

The authors have no conflicts of interest to disclose.

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Figures

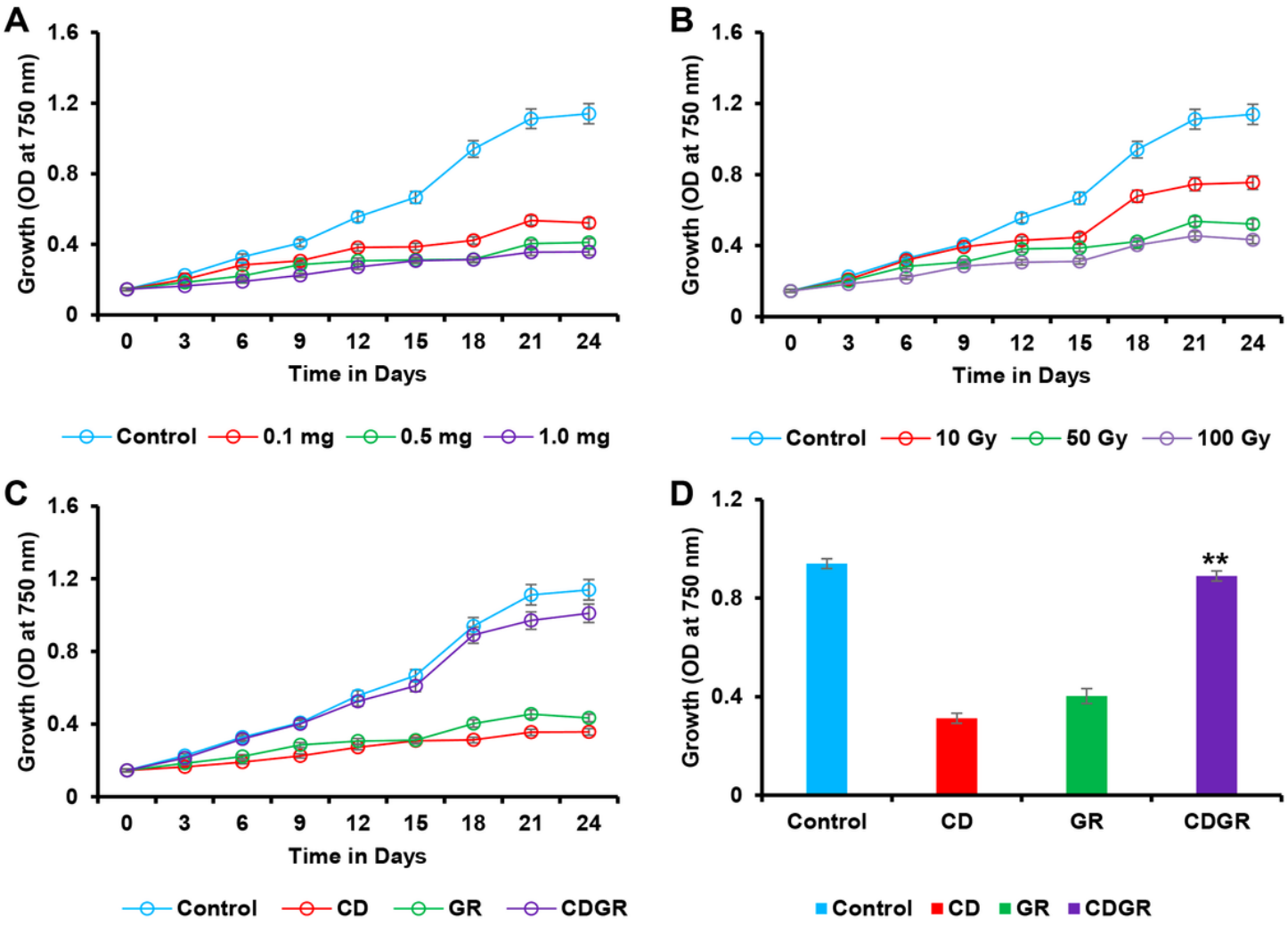

Figure 1

Low dose priming with gamma radiation sustains growth kinetics and specific growth in cell death C. reinhardtii during Cd toxicity. Growth kinetics of C. reinhardtii strains over a period of 24 days, with 3-day interval, upon different treatments. Cd (0.1-1.0 mg/mL) (A) and gamma radiation (10-100m Gy) (B) treatments. Low dose of gamma radiation (10 Gy) primed strain in Cd toxicity exhibited a higher growth rate (C) and specific growth rate at 18th days culture (D) of the strain.

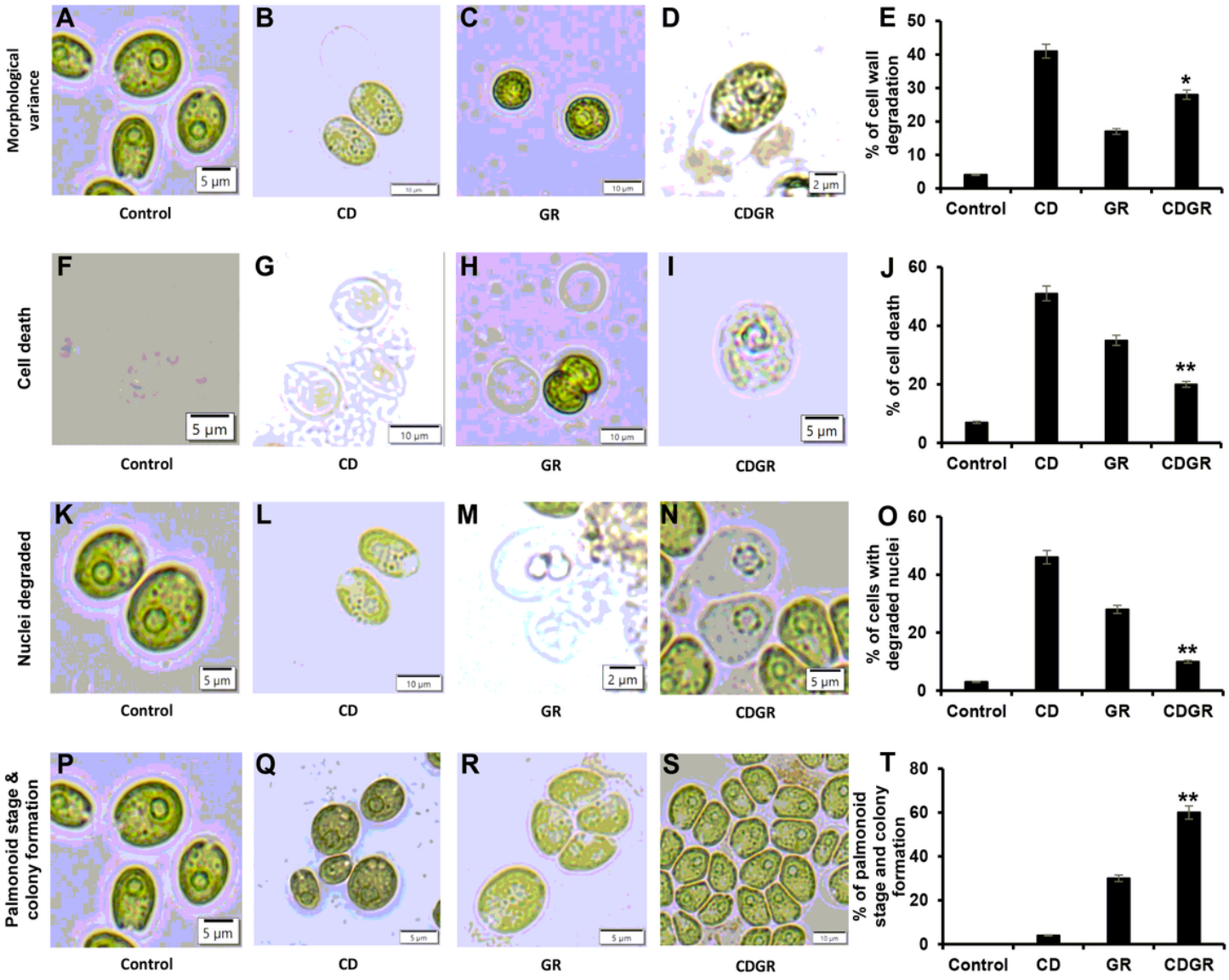

Figure 2

Low dose priming with gamma radiation morphological changes sustains cell death and stress tolerance in C. reinhardtii during Cd toxicity. Optical images of morphological changes (A-D) and % of cell wall degradation (E) of the strain; cell death (F-I) and % of cell death (J); nuclear degradation (K-N) and % of nuclear degradation (O); palmonoid or colony formation (P-S) and % palmonoid or colony formation (T). The * p-value ≤0.05 in histograms represent the statistically significance.

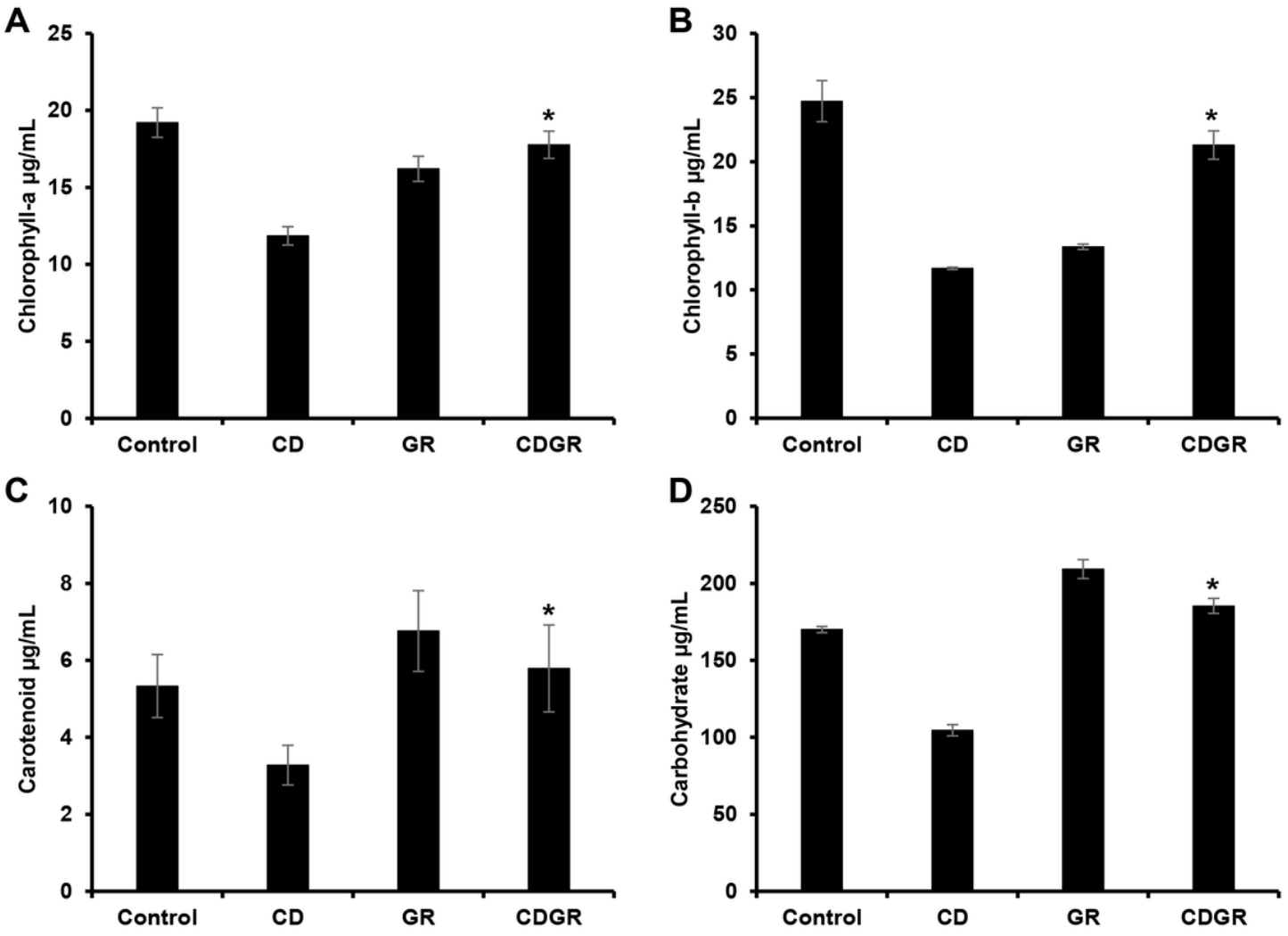

Figure 3

Low dose priming with gamma radiation restrains the photosynthetic pigments in C. reinhardtii associated with growth and survival. Concentrations of photosynthesis-associated pigments, i.e. chlorophyll a (A), chlorophyll b (B), carotenoids (C), and carbohydrate (D), after Cd, GR, and CdGR treatment of C. reinhardtii at 18th days culture. Control represents the untreated strain. All the experiments were performed in triplicate and pooled data were subjected to one-way analysis of variance (ANOVA) followed by the least significance difference (LSD). The * p-value ≤ 0.05 was found to be statistically significant.

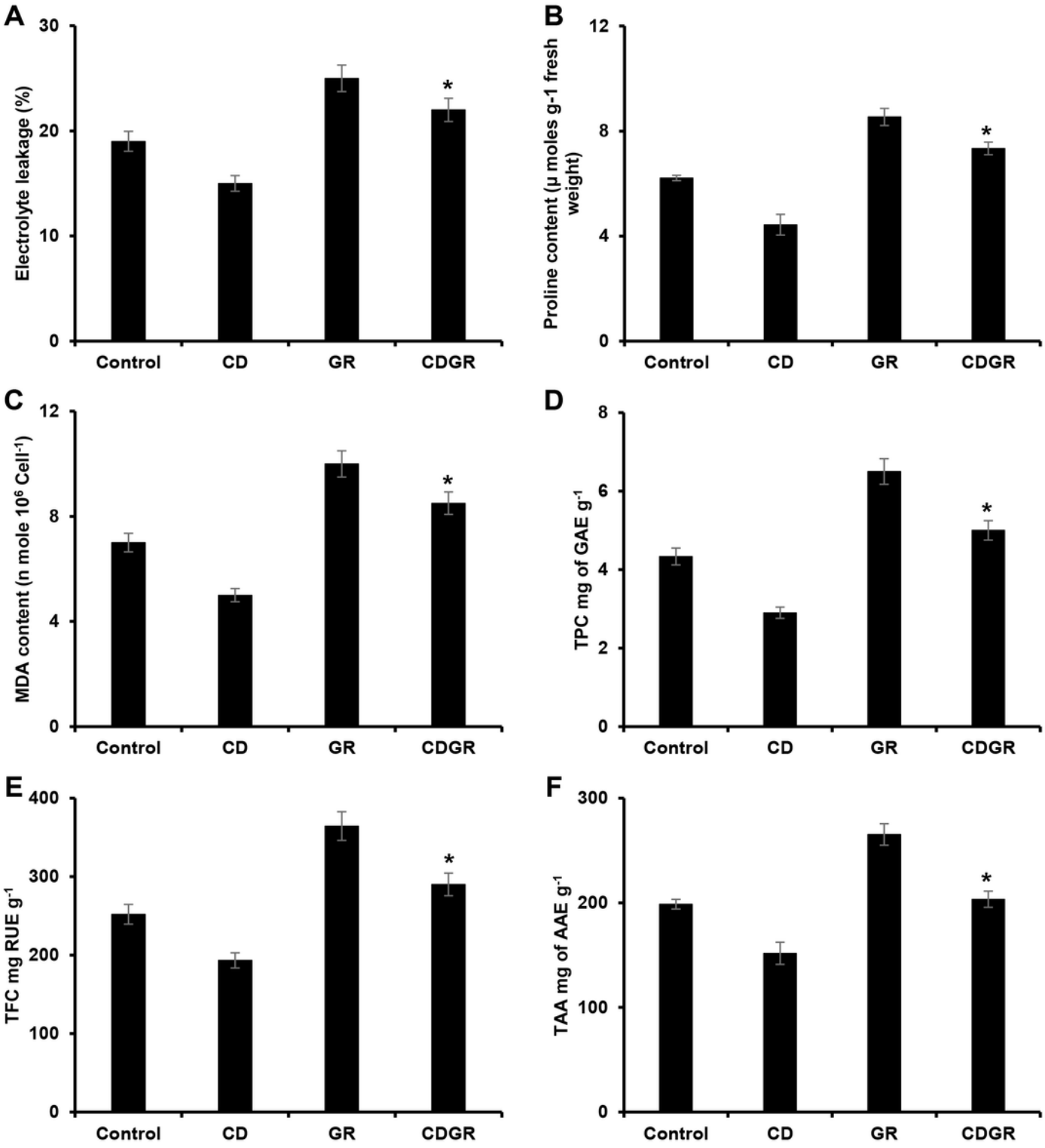

Figure 4

Low dose priming with gamma radiation enhances the electrolyte leakage, proline, malondialdehyde, phenolic, flavonoids and total antioxidant activity surpass Cd related toxicity in C. reinhardtii. Electrolyte leakage (A), proline (B), MDA (C), TPC (D), TFC (E), and TAA (F) concentrations after Cd, GR, and CdGR treatment of C. reinhardtii at 18th days culture. Control represents the untreated strain. All the experiments were performed in triplicate and pooled data were subjected to one-way analysis of variance

(ANOVA) followed by the least significance difference (LSD). The * p-value ≤0.05 was found to be statistically significant.

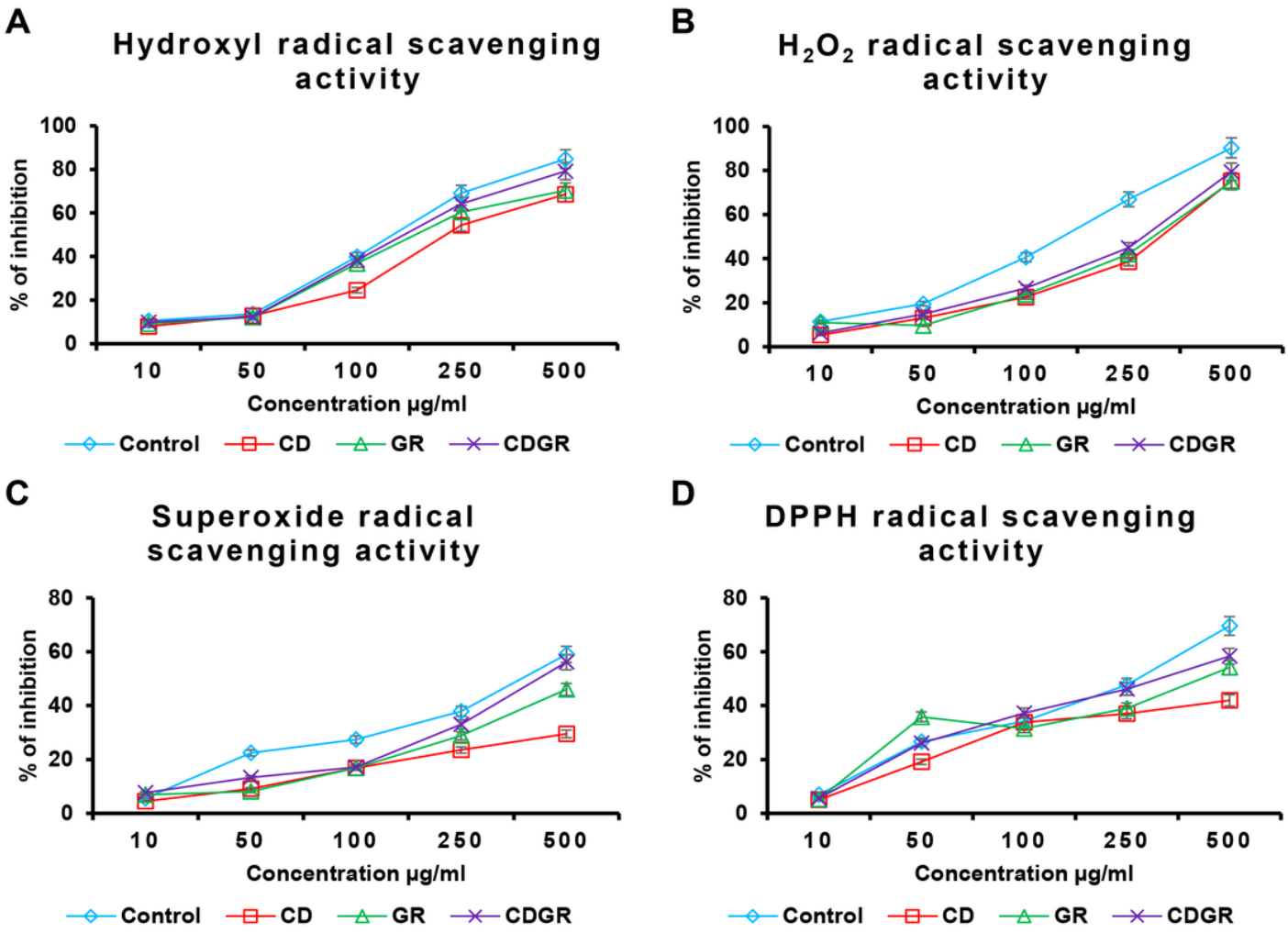

Figure 5

Low dose priming with gamma radiation stimulates Cd tolerance via enhancement of free radical scavenging activity in C. reinhardtii. Percentage of inhibition of the hydroxyl ion (A), hydrogen peroxide (B), superoxide (C), and DPPH (D) radical scavenging activity after Cd, GR, and CdGR treatment of C. reinhardtii at 18th days culture. Control represents the untreated strain.

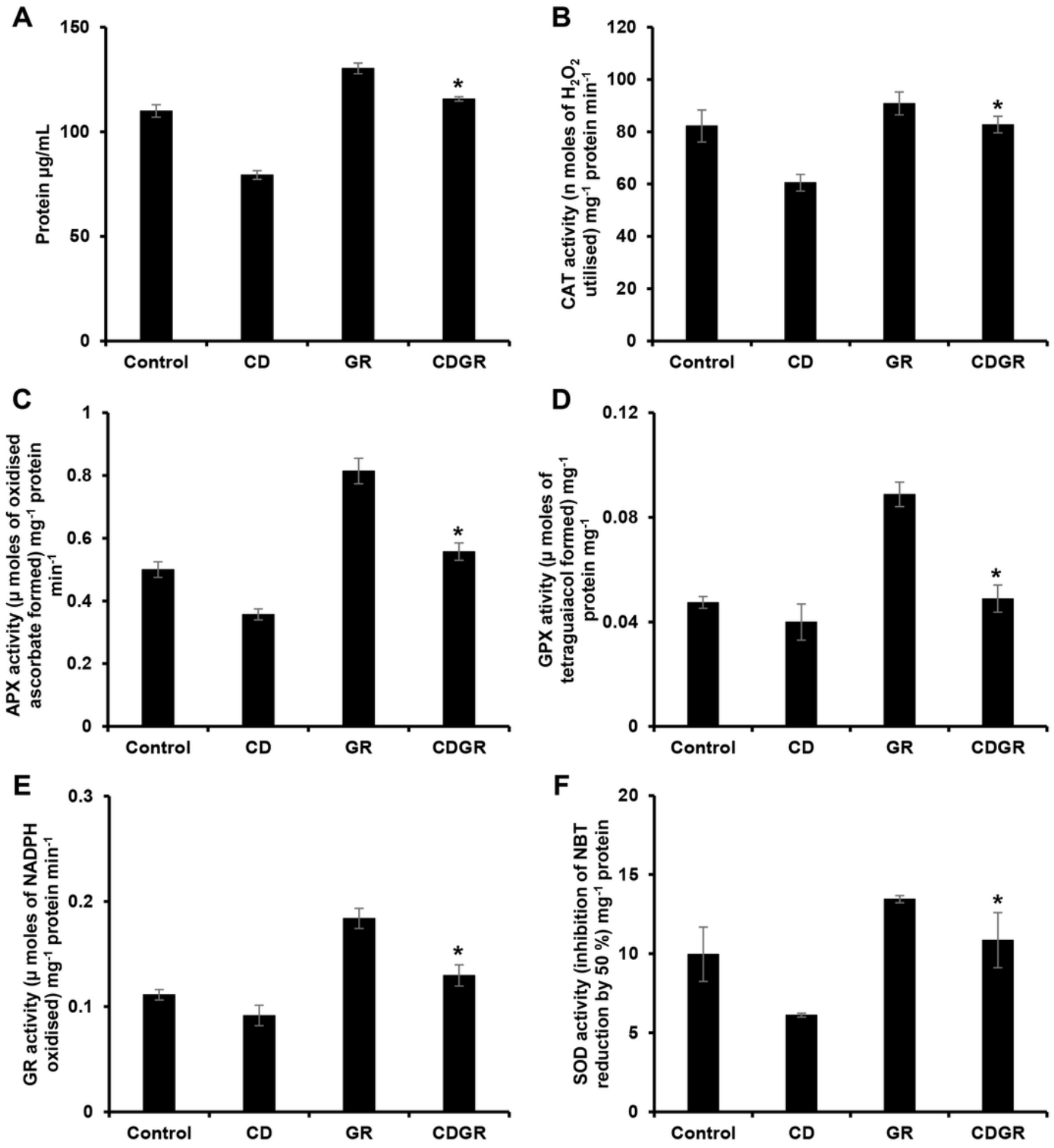

Figure 6

Low dose priming with gamma radiation deploys antioxidant enzymes for enhanced Cd tolerance in C. reinhardtii. Total soluble protein (A), CAT (B), APX (C), GPX (D), GR (E), and SOD (F) concentrations after Cd, GR, and CdGR treatment of C. reinhardtii at 18th days culture. Control represents the untreated strain. All the experiments were performed in triplicate and pooled data were subjected to one-way analysis of

variance (ANOVA) followed by the least significance difference (LSD). The * p-value ≤0.05 was found to be statistically significant.

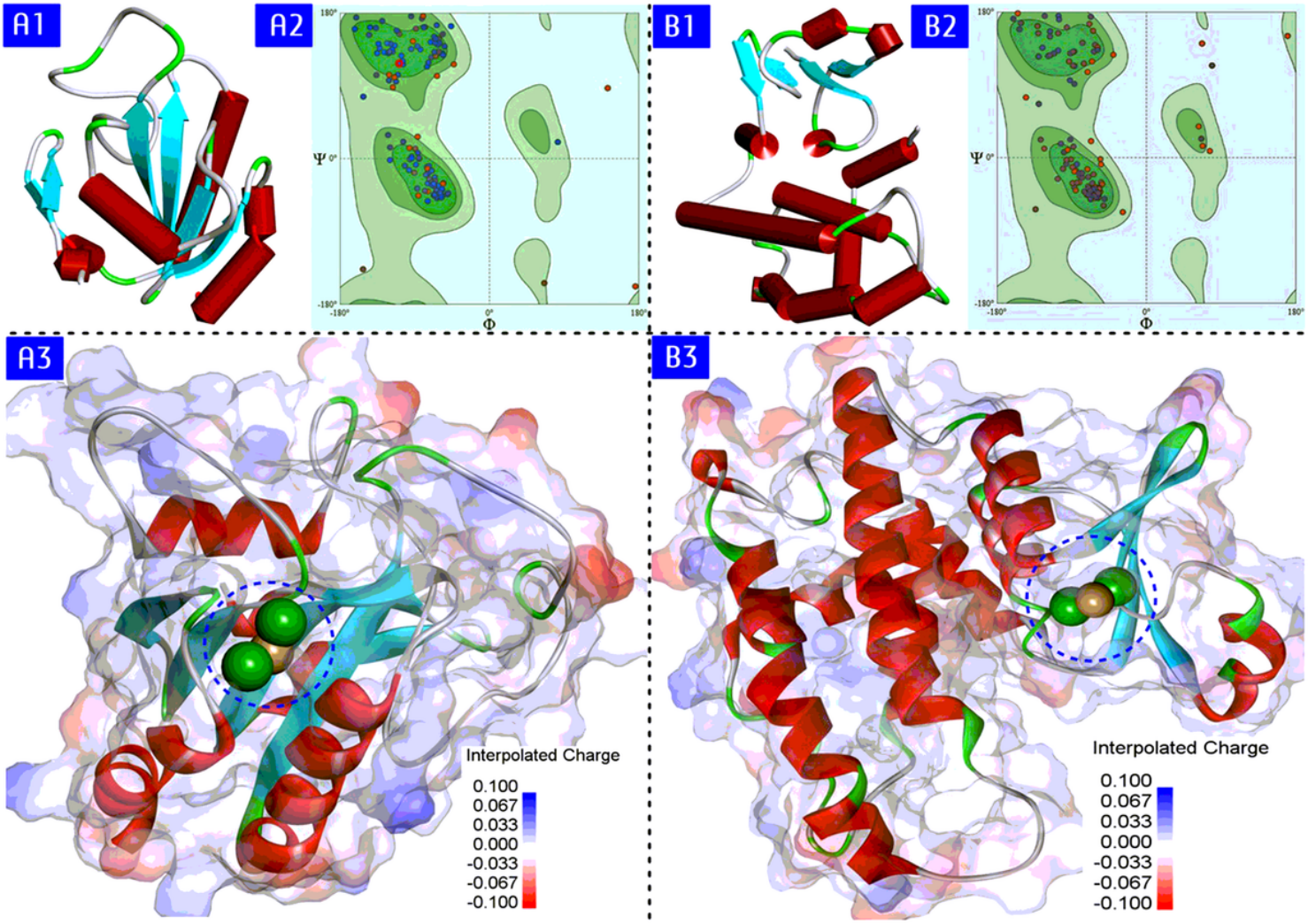

Figure 7

GPXH and GSTS1 are crucial targets for the Cd-related oxidative stress and toxicity. Generated 3D structures (A1 and B1) by homology modelling with their respective Ramachandran plots (A2 and B2) of the modelled GPXH (A) and GSTS1 (B). Mode of binding of the active site of the protein with CdCl2, i.e. GPXH-CdCl2 (A3) and GSTS1-CdCl2 (B3).

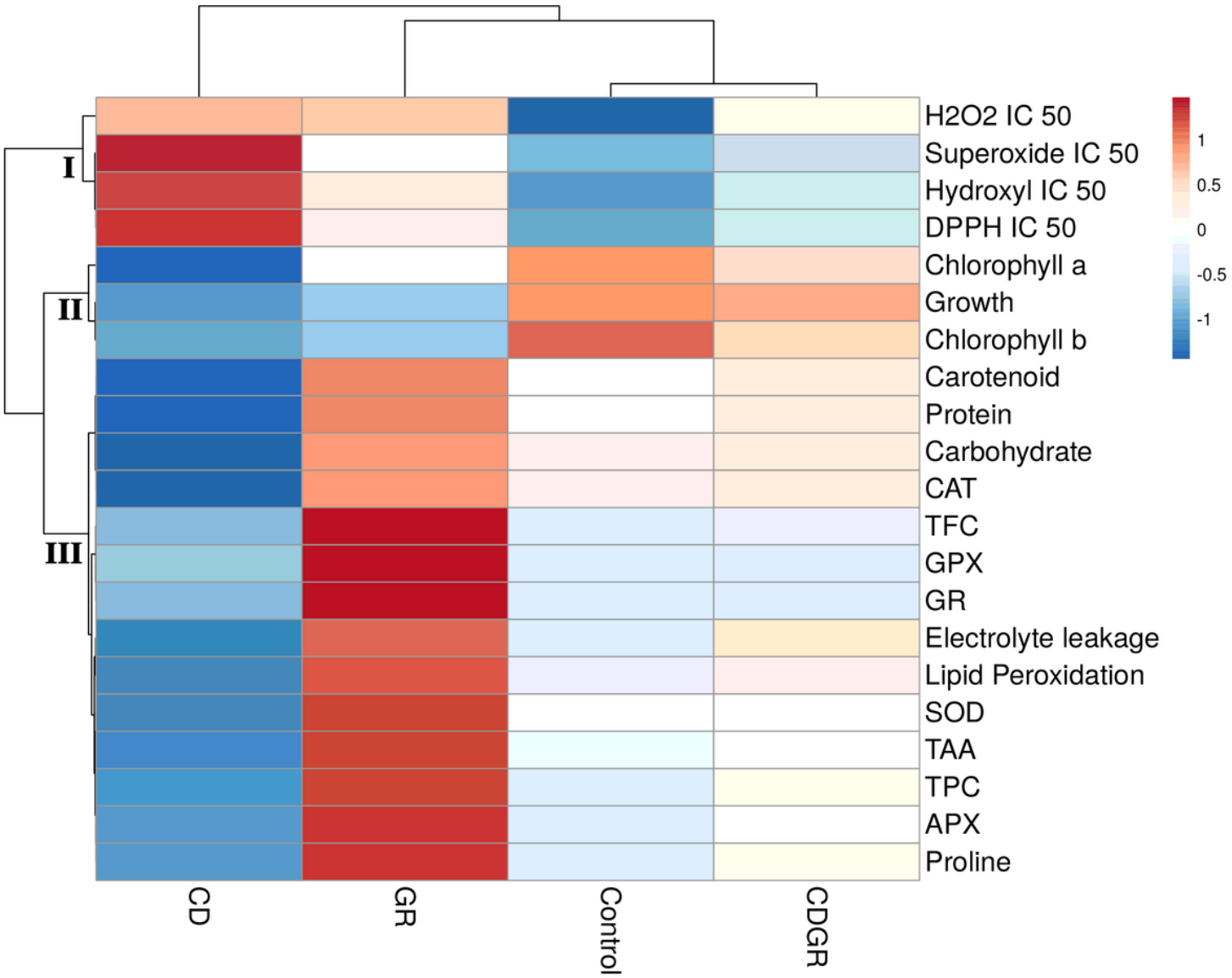

Figure 8

Hierarchical cluster analysis (HCA) between the physiological and biochemical parameters and the treated strains of C. reinhardtii.

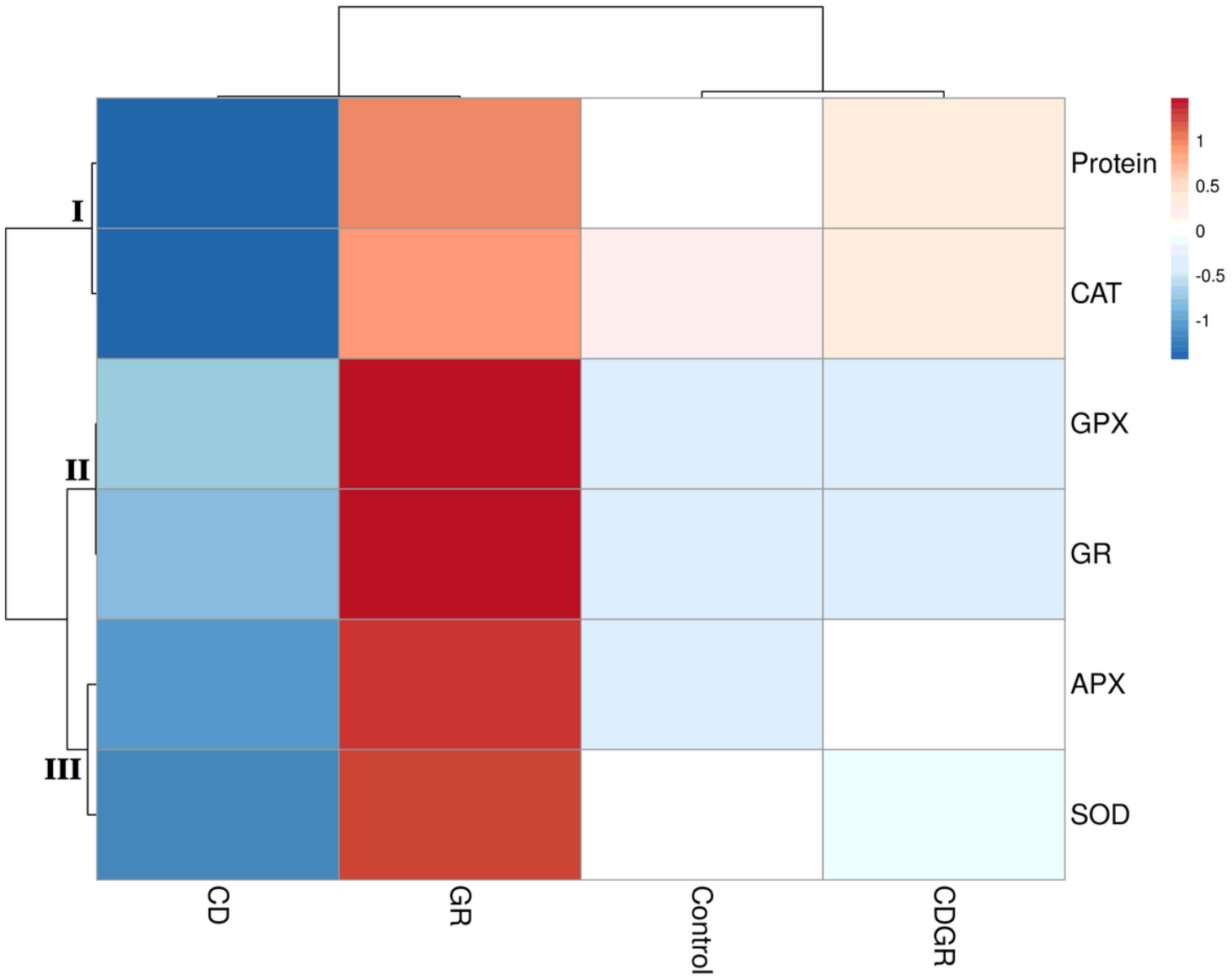

Figure 9

Hierarchical cluster analysis (HCA) between the antioxidant enzymes and the treated strains of C. reinhardtii.

Supplementary Files

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- GraphicalAbstract.tif
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