Low-dose priming of gamma radiation enhanced cadmium tolerance in *Chlamydomonas reinhardtii* by modulating physio-biochemical pathways

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**Abstract**

Microalgae are natural biotic models for exploring the genotoxic effect of heavy metals, irradiation, other external stimuli and the toxicant elimination. The effective removal of heavy metals from the aquatic environment using microalgae has gained considerable attention. However, limited research was carried out on cadmium toxicity in microalgae and their use as bio-accumulants. Previous research suggested that low-dose priming with non-ionizing radiations, such as gamma radiation, increased heavy metal tolerance in plants and aquatic photosynthetic microalgae. In the present study, we have hypothesized the growth inhibitory physiochemical properties of cadmium (Cd) in *Chlamydomonas reinhardtii*, and analyzed the protective role of low-dose gamma radiations priming against Cd-induced growth inhibition by emphasizing mechanism of cell survival by antioxidant defence system. Experimentally, the gamma-primed *C. reinhardtii* exhibited higher cell survival and Cd tolerance with effective modulation of biochemical responses such as antioxidant enzymes. The current investigation revealed that low-dose priming of gamma radiation masks Cd-mediated oxidative stress and enhances cellular detoxification via intracellular antioxidant enzymes in *C. reinhardtii*.

**Keywords** Antioxidant enzymes · Cadmium · *Chlamydomonas reinhardtii* · Gamma radiation · Reactive oxygen species (ROS)

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**Introduction**

Heavy metal pollution causes serious effects in aquatic and terrestrial organisms. 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). Recently, heavy metals have caused adverse health effects worldwide (Patra et al. 2007). Cadmium (Cd) is a naturally occurring environmental pollutant present in the soil, water, air, and food. In addition to natural sources of Cd, anthropogenic sources, such as burning 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 quickly disperses into the environment through the aquatic medium and elicits serious threats to many living organisms (Thapa et al. 2012). Therefore, cadmium toxicity would impact microalgae that subsequently affect aquatic and terrestrial food chains (Gomes et al. 2017). Heavy metals are required as a co-factor in enzymatic activities in microalgae, but
high levels could be exceedingly hazardous (Travieso et al. 1999). Cd accumulates in algae and has been connected to a variety of living animals in both fresh and marine biota, including fish and other organisms (Expósito et al. 2021). Microalgae also has extracellular and intracellular systems that protect it from metal toxicity. Heavy metals are classified as either essential or harmful depending on their role in microalgae physiology and concentration. In general, metal contaminants have harmful effects in algal cells due to their high reactivity, which inhibits enzyme function and causes oxidative damage. Heavy metal ions are primarily bound in the cytoplasm due to these factors (Sirko and Gotor 2007). Algal cells have a variety of antioxidant defense systems, both enzymatic and non-enzymatic, that are designed to precisely control the concentration of reactive oxygen species (ROS). Antioxidant enzymes are essential in the cellular defense mechanism in microalgae (Sirko and Gotor 2007). C. reinhardtii is a eukaryotic green microalga species that has high tolerance and survival in environments including stressors such as heavy metal, high salinity, and dehydration (Samadani et al. 2018). Cd inhibits photosynthesis by decreasing electron transport in Photosystem II (Ran et al. 2015), replaces the Mg in chlorophyll, and interferes with the 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).

ROS, reactive nitrogen species (RNS), and reactive sulfur species (RSS) are thought to influence signaling and genes as well as trigger tolerance mechanisms in plants (Antoniou et al. 2016). ROS and RNS are molecules are known for their actions, such as low accumulation ROS and RNS is anticipated for optimum health where as higher accumulation limits the organism health (Mittler 2017). Hormesis promotes the appearance of a minor biological reaction to low doses of stress that is the polar opposite of the response to higher doses of stress, such as stimulation vs. inhibition (Agathokleous et al. 2019). Hormesis was also discovered to be induced by several environmental pollutants. As a result, priming is preconditioning, a component of hormesis (Agathokleous et al. 2019). Preconditioning has been shown to protect plants from environmental stress and is thus a promising method for increasing tolerance to environmental problems. Recent research in this area focuses on adaptive responses caused in the low-dose zone of the complete dose-response continuum (Agathokleous et al. 2019). However, the effects of high dosage stress on relationships with toxicity have been thoroughly investigated in the past. On the other hand, low-dose stress has yet to be well researched in the field of toxicology (Agathokleous and Kitao 2018). Given these developments, it is appropriate to concentrate on low-dose stress responses, improving superiority strain, guard against hostile situations, and increasing plant productivity and health.

Microalgae are key components of aquatic ecosystems as primary producers (Maharana et al. 2019; Dash et al. 2020; Dash et al. 2021; Behera et al. 2021; Pradhan et al. 2021a; Behera et al. 2020). Apart from this, they are termed effective bio-accumulants that reduce heavy metal toxicity in aquatic ecosystems. In the meanwhile, these organisms also exhibit metal tolerance. For example, C. reinhardtii, eukaryotic green microalgae, exhibited extraordinary metal tolerance 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 the removal of algae (Pradhan et al. 2020a). However, the priming effect of gamma irradiation on heavy metal tolerance and elimination in C. reinhardtii has not yet been elucidated to establish a hypothetical model of how gamma irradiation priming could protect the algae from subsequent heavy metal exposure. Ionizing radiations cause toxicity in biotic systems. Previous reports demonstrated that gamma radiation upsets algal growth and survival (Pradhan et al. 2020a). Gamma irradiation is widely used to mutate the organisms in the last couple of decades but needs to be in microalgae (Bala and Pal Singh 2013). 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, irradiation can improve the tolerance of microalgae to different abiotic stresses, such as heavy metals, cold, drought, temperature, and salinity via upregulation of ROS defense antioxidant enzymes. Moreover, it can also scavenge the free radicals generated due to ROS by elevated primary and secondary signalling molecules (Wang et al. 2018; Qi et al. 2015; Haleem 2012).

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 analyzed the protective role of low-dose gamma radiation priming against Cd-induced growth inhibition using the green algae C. reinhardtii. In addition, the mechanistic investigation on the mode of tolerance and survival of the species has focused on the antioxidant defence enzymes.

Materials and methods

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 \((NAD)\), 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.

### Microalgal culture conditions

*Chlamydomonas reinhardtii* (MJ11/33) was acquired from Berhampur University, Algal Biotechnology and Molecular Systematic Laboratory, Department of Botany. The microalgae were cultured in a 500-mL borosil flask. The flask containing 250 mL of Bold Basal Medium was incubated for 21 days for mass culture in algal culture room at 25 ± 2 °C with a light intensity of 7.5 W/m² of photoperiod 14: 10 (light: dark). The culture was shaken 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.

### Pre-treatment of gamma irradiation and cadmium treatment

#### Experimental setup

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. The control group did not receive gamma irradiation or cadmium supplementation. After one set of gamma irradiation treatment and 24 h 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 was 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 a 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 OD750 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 protecting antioxidant enzymes, such as CAT (catalase), APX (ascorbate peroxidase), GPX (glutathione peroxidase), GR (glutathione reductase), and SOD (superoxide dismutase). Furthermore, the TPC (total phenolics content), TFC (total flavonoid contents), and TAA (total antioxidant activity) were also calculated and the free radical scavenging activity was also estimated. The experimental conditions used for determining these parameters are detailed below.

### Measurement of algal growth, chlorophyll and carotenoid

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

### 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. 2018) with slight modification. Briefly, 10 mL of algal suspension was centrifuged at 5000xg 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 min at 100 °C and the EC$_2$ was measured. The electrolyte leakage was calculated as a percentage by the following equation:

$$EL(\%) = \left( \frac{EC1}{EC2} \right) \times 100$$

The membrane lipid peroxidation was evaluated by determining the MDA content as previously described (Pradhan et al. 2021d). 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. 2021d).

**Estimation of protein and antioxidant enzymes**

The estimation of protein content and antioxidant defence enzymes such as CAT, APX, GPX, GSR, and SOD was quantified as previously described by Pradhan et al. (Pradhan et al. 2020a, b). The superoxide assay was determined as previously described by Patra et al. (Patra et al. 2020a, b). The superoxide assay was based on the reduction of NBT in the presence of NADH and PMS, as already described (Pradhan et al. 2021c, b). 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.

**Phytochemicals screening and estimation of total phenolic, flavonoid content and free radical scavenging activity**

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

The free radical scavenging activity of *C. reinhardtii* was determined by DPPH assay as previously described (Pradhan et al. 2020a, b). The hydroxyl radical scavenging activity of *C. reinhardtii* was determined as previously described by Patra et al. (Patra et al. 2020a, b). The superoxide assay was based on the reduction of NBT in the presence of NADH and PMS, as already described (Pradhan et al. 2021c, b). 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.

**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. 2021d). 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). The p-value was defined as follows: $p$-value < 0.05 was considered significant. *$p$*-value < 0.05 is compared with control; **$p$*-value < 0.05, co-treatment is compared with Cd; ***$p$*-value < 0.05, co-treatment is compared with GR.

**Results**

**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 24 days with three days intervals (Fig. 1). In Cd-treated (0.1–1.0 mg/mL of Cd) *C. reinhardtii*, 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 (Fig. 1D).

Morphological characterization was also conducted to assess the strain’s growth and survival under these treatment conditions, revealing morphological alterations and cell wall disintegration (Fig. 2). As compared to Cd treatment, it was observed that LDGR reduced cell wall deterioration (Fig. 2A–E). There was also less cell death in radiation primed Cd-treated cells when compared to Cd or radiation treatment alone (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). We noticed an elevated palmonoid production in the Cd+GR treatment compared to the Cd and GR treatment alone because palmonoid stage is a marker of stress tolerance response (Fig. 2P–T).

**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, we looked at how much chlorophyll a was present after Cd, GR, and Cd+GR treatments. Our findings revealed that the synergistic treatment restored chlorophyll a content compared to the individual treatments and was significantly different from the control (Fig. 3A). The chlorophyll b content followed a similar pattern to chlorophyll a (Fig. 3B). Furthermore, we evaluated another stress response pigment, i.e., the carotenoid content. We observed a substantial reduction in its concentration in Cd-treated samples, which was restored when the algae were primed with low-dose gamma radiation (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 element, we also quantified the carbohydrate content in the strain under the treatment conditions, observing their increase after the synergistic treatment compared to Cd doping and control, although less than after GR treatment (Fig. 3D).

**LDGR treatment reduces Cd-related toxicity in C. reinhardtii via modulating the physicochemical parameter.**

The percentage of electrolyte leakage (EL) is a stress response signature. We observed a reduced electrolyte leakage in the Cd-treated cells, further enhanced in the gamma radiation primed cells (Fig. 4A). The highest electrolyte leakage was observed in the GR treated cells as compared to the control. These results suggest that low-dose primed cells are more responsive toward stress than the Cd-treated cells, which in turn allowed them to survive under stressful conditions. Similarly, the accumulation of proline is also a responsive marker of stress. We observed a decreased proline content in the Cd-treated cells (Fig. 4B). On the other hand, a higher accumulation of proline was observed in the GR-treated cells as compared to the control. The proline content was restored in the Cd+GR samples. A similar pattern was observed in the MDA contents (indicative of the degree of lipid peroxidation), higher in the Cd+GR group than the Cd-treated group (Fig. 4C).

After extraction in methanol, a qualitative screening of phytochemicals was performed (Table 1). Since polyphenols are crucial in stressful conditions, scavenging free radicals responsible for oxidative stress, we further...
We estimated the total phenolic (TPC) and flavonoid content (TFC). We observed a reduced TPC in the Cd-treated cells compared to the control (Fig. 4D and supplementary table 1). However, low-dose priming with gamma radiation raised their TPC content again, although less than GR alone. Similarly, a reduced TFC content was noted in the Cd-treated group compared to the control (Fig. 4E and supplementary table 1). Nevertheless, low-dose priming with gamma radiation enhanced the TFC, as confirmed by GR treatment alone. Finally, the total antioxidant activity followed a similar pattern (Fig. 4F and supplementary table 1).

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

We investigated the distinct free radical scavenging activity of *C. reinhardtii* that was responsible for the antioxidant defense mechanism in a stressed environment after measuring the TFC, TPC, and TAA. Our preliminary data revealed that at 500 g/mL, gamma radiation primed cells had the highest percentage of hydroxyl radical inhibition (79.23%), while GR (70.29%) and Cd (68.53%) treatments alone had much lower percentages (Fig. 5A). The IC₅₀ value of the CDGR sample of *C. reinhardtii* was found to
be more significant as compared to the individual treatments (Table 2). 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, which was further enhanced in the gamma radiation primed cells (Fig. 5B). The IC50 value of the hydrogen peroxide radical scavenging activity in the Cd+GR sample of C. reinhardtii was significantly lower than that of the Cd-treated strain (Table 2), suggesting the most potent radical scavenging activity in the radiation primed strains as compared to the Cd-treated strain. A similar inhibition pattern 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 (Fig. 5C). Consequently, the corresponding IC50 value was much lower in the Cd+GR-treated strain than that of the Cd-treated strain (Table 2). 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 than that of the strain primed with a low dose of gamma radiation (Fig. 5D). The IC50 of DPPH radical scavenging activity in the Cd+GR-treated sample was significantly less than that of the Cd-treated strain (Table 2).

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

To distinguish the oxidative stress response to Cd treatment, antioxidant enzyme activities were assessed. First, we determined the total soluble protein content in each treatment condition. With compared to the control, Cd treatment reduced total soluble protein concentration. The total soluble protein content in the strain was recovered after low-dose priming with gamma radiation, substantially higher than Cd treatment alone. The findings showed that low-dose gamma radiation priming reduced cadmium stress by increasing protein content (Fig. 6A). Furthermore, the activity of antioxidant enzymes such as CAT, APX, GPX, GR, and SOD, which play a significant role in combating oxidative stress, was assessed.

**Fig. 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 was defined as follows: p-value > 0.05 was considered not significant (ns); p-value < 0.05 was considered significant. a p-value < 0.05 is compared with control; b p-value < 0.05, co-treatment is compared with Cd; c p-value < 0.05, co-treatment is compared with GR.
Initially, we quantified the CAT activity and found reduced activity in the Cd-treated strain compared to the control (Fig. 6B). On the other hand, the strain treated by low-dose priming with gamma radiation exhibited an elevated CAT activity compared to that of the Cd treatment alone. The strain treated individually by GR also showed a higher CAT activity. In addition, we analyzed the APX enzyme activity, observing a reduced activity in the Cd-treated strain, which increased in the GR-treated sample (Fig. 6C). The control and the GR treatment alone exhibited respectively.

The GPX activity was then measured, showing a similar expression level in the treatment groups and control. At the same time, LDGR strain activity was somewhat higher (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, which was found to be recovered in the Cd+GR treated strain (Fig. 6C). The control and the GR treatment alone exhibited respectively.

The two principal components, i.e., PC1 and PC2, drawn taking all the physiological variables, represented 98.3% of the total variance (Supplementary Fig. 1). 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). Further, the TAA, protein content, and CAT were positively correlated. On the other hand, the PC2 only elucidated 1.5% of the total variance (Supplementary Fig. 1).

**Discussion**

Microalgae are valuable natural biotic models for studying the genotoxic effects of heavy metals and other external stressors in the aquatic environment (El-Din and Abdel-Kareem 2020). The aquatic ecosystem is preserved from radiation-related toxicities, radiation primed algal species can be developed which can be employed in the contaminated fields to work as bioaccumulants. However, developing such primed species will be useful for future applications. Heavy metals, such as Cd, cause oxidative stress, lipid peroxidation, and DNA damage in cells, resulting in decreased cell development and death (Ran et al. 2015). However, microalgae can tolerate heavy-metal associated oxidative stress by deploying antioxidant enzymes (Ganapathy et al. 2017). Priming microalgae with low doses of non-ionizing radiation boosts the metabolic responses to heavy metal damage (Bradshaw et al. 2019; Toghyani et al. 2020).
During heavy metal toxicity, cell death is accompanied by growth retardation, damage of cell walls, and degradation of the nucleus (Spiteller 2003). In the present study, our results indicated that either Cd or GR treatment retarded the growth of the *C. reinhardtii* as evidenced from broken cell walls, and deteriorated nuclei. However, in the LDGR group, we noticed increased palmonoid-stage development indicating the tolerance of stress (Pradhan et al. 2020a). Moreover, the photosynthetic parameters such as the content of chlorophyll a and b along with carotenoids that stabilize the plasma membrane against membrane lipid peroxidation and in scavenging free radicals during oxidative stress were shown to be increased in the co-treated strain as compared to the mono-treatment of Cd (Pérez-Pérez et al. 2012; Havaux 1998). This phenomenon is well supported by the previous findings (Pradhan et al. 2020a).

Additionally, under oxidative upset, membrane damage leads to leaking of electrolytes from the cell. Electrolyte leakage is thus an indicator of stress resistance in

### 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 IC$_{50}$ values of the free radical scavenging activities *Chlamydomonas reinhardtii*

| Parameter                        | Control | CD     | GR     | CDGR   |
|----------------------------------|---------|--------|--------|--------|
| DPPH scavenging activity         | 3.88± 0.33 | 5.47± 0.23$^a$ | 4.67± 0.45$^a$ | 4.22± 0.63$^{ab, ns}$ |
| H$_2$O$_2$ scavenging activity   | 3.21± 0.80 | 4.15± 0.53$^a$ | 4.1± 0.58$^a$ | 3.88± 0.77$^{ab, b, c}$ |
| Superoxide scavenging activity   | 4.59± 0.93 | 8.17± 0.41$^a$ | 5.9± 0.88$^a$ | 5.11± 0.91$^{ns, b, c}$ |
| Hydroxyl radical scavenging activity | 3.31± 0.73 | 4.01± 0.79$^a$ | 3.71± 0.49$^{ns}$ | 3.48± 0.22$^{ns, b, ns}$ |

The $p$-value was defined as follows: $p$-value > 0.05 was considered not significant (ns); $p$-value < 0.05 was considered significant. $^a$p-value < 0.05 is compared with control; $^b$p-value < 0.05, co-treatment is compared with Cd; $^c$p-value < 0.05, co-treatment is compared with GR.
algae to neutralize oxidative stress. 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 healing electrolytic leakage in the gamma primed group suggesting enhanced membrane stability (Anaraki et al. 2018; Aghaie et al. 2018). A high amount of proline content was manifested under abiotic stress (Zhang et al. 2008). We found that Cd treatment decreased the proline content, while there was higher proline accumulation in the gamma primed C. reinhardtii strain.

As oxidative stress is accompanied by lipid peroxidation, we evaluated the MDA content in the treated strain. The Cd-treated strain exhibited lower MDA concentration, while the gamma primed strain showed alleviated MDA content due to reduced lipid peroxidation to counteract oxidative stress for cellular homeostasis (Pradhan et al. 2021d). TPC, TFC, and TAA act as natural scavengers of free radicals under heavy metal stress (Pradhan et al. 2021d). We noticed a significant reduction in the TPC, TFC, and TAA content which is well reversed in the gamma radiation primed strain (Pradhan et al. 2020a, d).

Hydroxyl radicals, hydrogen peroxide radicals, and superoxide radicals are the main contributors to the induction of oxidative imbalance and are responsible for hormesis (Gill and Tuteja 2010; Agathokleous et al. 2019). Hence, scavenging of these free radicals is required for cellular redox homeostasis (Pradhan et al. 2020a). Hence, 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. The co-treated strains exhibited enhanced radical scavenging activity to protect the strain from oxidative stress related cell death.

Total soluble protein concentration, subcellular localization, intercellular metabolism, and mode of activity during stress tolerance are all important factors in maintaining a healthy cellular environment (Razi and Hasnain 2006). The total soluble protein, as a primary response to stress increases, 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, a reduced protein content in the Cd-treated cells was observed, which was significantly elevated in the low-dose gamma radiation primed strains, even after the Cd treatment (Pradhan et al. 2020c; Achary et al. 2008; Kováčik and Dresler 2018; Golari et al. 2018). During heavy metal stress, accumulation of ROS induces cell death (Zhao et al. 2020; Chokshi et al. 2020). Cellular antioxidant enzymes nullify the ROS accumulation to promote cell viability. Hydrogen peroxide radicals are mainly responsible for introducing cell damage, while APX and GPX catalyzes their detoxification (Daspute et al. 2017). In the present study, we observed an enhanced APX and GPX activity in the radiation-primed strain compared to the Cd-treated strain suggesting an effective detoxification of hydrogen peroxide radicals. In addition, an increased GSR suggested 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 homeostasis (Alschler et al. 2002).

Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were exploited to investigate the correlation among the assessed experimental parameters (physiological and biochemical) and to determine the multidimensional response under oxidative stress (Löw et al. 2012; Husseini et al. 2020; Toghyani et al. 2020). 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 defense mechanisms are all upregulated, thus providing an immediate quick response to counteract the oxidative stress produced by the heavy metal.

**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. It also nullifies the heavy metal contaminant-related oxidative damage. The present study demonstrated that Cd causes an oxidative imbalance in C. reinhardtii by downregulating the oxidative enzymes. The reduction of the photosynthetic and accessory pigment content was also responsible for the Cd-related toxicity, causing stunted cell growth and inducing cell death. However, low-dose priming with gamma radiation recovered the cell growth and inhibited cell death.
death by deploying the antioxidant enzymes, such as CAT, 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 mechanistic insight into heavy metal stress tolerance by \textit{C. reinhardtii}. This study inform a new model for further research in heavy metal-related toxicity and their removal to sustain species growth.

\textbf{Fig. 6} Low-dose priming with gamma radiation deploys antioxidant enzymes for enhanced Cd tolerance in \textit{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 \textit{C. reinhardtii} at 18\textsuperscript{th} 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 \textit{p}-value was defined as follows: \textit{p}-value > 0.05 was considered not significant (ns); \textit{p}-value < 0.05 was considered significant. \textit{a} \textit{p}-value < 0.05 is compared with control; \textit{b} \textit{p}-value < 0.05, co-treatment is compared with Cd; \textit{c} \textit{p}-value < 0.05, co-treatment is compared with GR.

\textbf{Fig. 7} Hierarchical cluster analysis (HCA) between the physiological and biochemical parameters and the treated strains of \textit{C. reinhardtii}.

\textbf{Fig. 8} Hierarchical cluster analysis (HCA) between the antioxidant enzymes and the treated strains of \textit{C. reinhardtii}.
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**Data availability** All data and material are available upon request.

**Declarations**

**Ethics approval** Not applicable.

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