Research Article

Antioxidant responses against aluminum metal stress in *Geitlerinema amphibium*

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Received: 4 February 2020 / Accepted: 25 March 2020 / Published online: 2 April 2020

Abstract
In the current scenario, aluminum is one of the major metals that cause environmental pollution and has ill impact on organism health. As around 40% of bauxite deposits of India are available in Odisha, aluminum toxicity in the soil has impacted as an intimidating problem of Odisha. This aluminum metal pollution is regarded as a primary factor for limiting crop productivity in acidic soils of tropic and subtropic countries. The presence of a high degree of aluminum metal in the soil leads to loss of beneficial microflora, i.e., nitrogen-fixing cyanobacteria that play a key role in soil fertility. Hence, this investigation was performed on a filamentous non-heterocystous cyanobacterium *Geitlerinema amphibium* isolated from the aluminum-polluted sites, i.e., NALCO, Ash pond, Angul to uncover the defense mechanism of the strain exhibited under such stress. The strain was treated with different doses of $AlCl_3$ (0–100 μM), and we observed redox imbalance leading to oxidative stress. Post-treatment of $AlCl_3$, a remarkable decline in growth was evident with possible modifications in pigments associated with the phenomena. Further to combat against the stress under the redox environment, the organism activated antioxidant enzymes CAT, APX, GR, GPX, and SOD which were measured to identify the key responses involved in it. All the stress responsive enzymes were analyzed to demonstrate the interlink between the induction of stress and their detoxification through cellular response. The outcome of the work explored the multifaceted role of oxidative upset caused by $AlCl_3$ pollutants and the cellular antioxidative strategies deployed by *G. amphibium* to nullify them.

Keywords Cyanobacteria · Aluminum · Antioxidants · Stress · Reactive oxygen species (ROS)

1 Introduction

Aluminum (Al) is one of the abundant metal pollutants found in the earth's crust. The major sources of Al pollutants are industrial mining, bauxite ore smelting, aluminum-made house appliances, food additives, wrapping materials, cosmetics and medicines waste [38]. As a result of the high demand for aluminum, the aluminum industries are steadily growing worldwide. In Odisha, Al pollution has been a looming problem since the large quantity of bauxite ore mines is being smelted and refined at VENDANTA mines and NALCO, respectively. The surroundings of these sites are contaminated by Al pollutants, and a sharp decline in crop production and human health has been observed in recent years. The contamination of Al pollutants heavily affects the terrestrial and aquatic ecosystem and drives a major concern toward their treatment. Cyanobacteria are autotrophic microorganisms regarded as the most prominent component of the aquatic and soil microbiota. These groups of bacteria contribute a major amount of fixed form of nitrogen to the soil and also act as primary producers of all aquatic ecosystems.

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SN Applied Sciences (2020) 2:800 | https://doi.org/10.1007/s42452-020-2599-1

Cyanobacteria are also widely regarded as the bio-indicators of pollution, and diversity of these organisms is commonly subjected to stress due to metal pollutants in the environment [7]. Further, cyanobacteria exhibit very specific and distinctive protective to manage the toxicity of metal stress and survive during such harsh environment [13]. Several studies have been carried out on the toxicity of different heavy metals stress (Cu, Zn, and Cd) on different microalgae and cyanobacteria [22, 31, 32, 34, 35]. In response to heavy metal stress, the cells undergo an oxidative burst, consequently producing huge amounts of reactive oxygen Species (ROS) [32]. In general, the reduced metal ions react with H_2O_2 and produce hydroxyl radicals ($HO^{\bullet}$) that damage different biomolecules [9]. Moreover, the noticeable phytotoxic symptoms exhibited by Al metal ions include defects in photosynthesis by change in the function of the associated pigment system and affect the cellular cytoplasmic-nuclear compartments to render the normal metabolism [1, 29]. However, the adaptability of cyanobacteria against the Al metal toxicity and antioxidant modulation in response to Al-induced oxidative stress mechanisms are yet to be addressed. Keeping the problems in mind, the present work was hypothesized to identify the key responses shown by cyanobacteria in Al-polluted sites of Odisha. Hence, a valiant attempt has been made to investigate the possible role of Al-mediated stress on cyanobacterium *G. amphibium* and the antioxidant defense mechanism enforced to eradicate it. This study will be helpful to monitor regular surveillance and management of necessary impact assessment for environmental pollution caused by aluminum.

2 Materials and methods

2.1 Isolation and purification of cyanobacterial strain

The soil and water samples were collected from aluminum-polluted sites the NALCO industry, Angul, Odisha (Ash pond, latitude and longitude 20° 50′ 57.36′ N, and 85° 9′ 14.97′ E). A non-heterocystous filamentous cyanobacterium strain was isolated by the serial dilution method. The organism was purified by repeated strike plate method and periodical microscopic observation. Finally, the pure strain has been identified as *G. amphibium* and the pure strain has submitted to Berhampur University algal culture collection by assigning strain no., i.e., *G. amphibium* BUACC21. The purified strain has been transferred to the flask containing the BG-11 nutrient medium with $NaNO_3$ and allowed to grow for 21 days.

2.2 Culture condition and aluminum treatment

The culture was treated with a different molar concentration of aluminum chloride ($AlCl_3$) (Merck, QK3Q632337) such as control, 1 µM, 10 µM, 50 µM, and 100 µM. The pH of all treated groups along with control was adjusted to 4.5. All the cultures were grown at 23 ± 2 °C and under 7.5 W m^2 light intensity in the culture room. The samples were harvested on the 21st day of the exponential phase for growth and further analysis.

2.3 Growth measurement

Post-21st day, the effect of $AlCl_3$ on the growth of *G. amphibium* under different micromolar (µM) concentration was measured spectrophotometrically (Eppendorf, Kinetic Bio-Spectrometer 6136GK603582) [25].

2.4 Pigments and protein analysis

2.4.1 Pigments analysis

The chlorophyll content of *G. amphibium* was estimated by hot extraction methods. Cyanobacterial culture (Control, 1–100 µM, 10 ml) was centrifuged (8000 rpm, 5 min, 4 °C) for chlorophyll-a estimation. Post-centrifugation, 90% methanol (10 ml) was added to the resultant pellet. After repeated vortex, the mixture was placed on the water bath for 30 min at 60 °C. Further, the samples were centrifuged (8000 rpm, 5 min, 4 °C) and the absorbance of the supernatant was measured by UV–Vis spectrophotometer at 665 and 650 nm, respectively, taking methanol as blank. The content of chlorophyll-a was calculated using the equation described by [37]. Phycobiliproteins such as phycocyanin (PC), phycoerythrin (PE) and allophycocyanin (APC) content were measured spectrophotometrically [18].

2.4.2 Protein extraction and estimation of antioxidant enzymes

Protein content was measured spectrophotometrically. Post-centrifugation (8000 rpm, 10 ml), the precipitated algal suspensions were homogenized using 5-mm stainless steel beads (Qiagen, 69989) for 10 min at 40 m s^{-1} in a Tissue Lyser (Model No LT No-23.1001/07581, Qiagen) in phosphate buffer (2 ml of 50 mM, pH 7.0), containing EDTA (0.1 mM), and polyvinyl-polypyrrolidone (1% (w/v)) and protease inhibitor at 4 °C. For ascorbate peroxidase assay, the grinding buffer additionally contained 5 mM of ascorbate (Himedia, Mumbai, CMS040-100G). The homogenates were centrifuged at 12,000 rpm for 15 min at 4 °C, and supernatant was stored at – 20 °C for the determination of

protein and enzyme activities. The soluble protein content was determined according to [8], and bovine serum albumin (Himedia. Cat No. GRM105-5G) was used for calibration of the standard curve. Further, different antioxidant enzymes, namely catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR), were estimated by a UV–Vis spectrophotometer.

Catalase (CAT) activity of the above strain was estimated according to the method of [12]. Catalase activity was analyzed by spectrophotometrically by adding the enzyme extracts into the 3 mL of the reaction mixture. The 3 mL of reaction mixture contains 50 mM of sodium phosphate buffer of (pH 7) 10 mM of H_2O_2 (Merck, CC3C630119) and 50 µl of the enzyme extracts. Catalase activity was determined by measuring the decreasing absorbance at 240 nm as a result of the degradation of H_2O_2. Enzyme activity was calculated using the extinction coefficient (0.036 mM^{-1} cm^{-1}). The CAT activity was expressed in nmol of H_2O_2 utilized mg^{-1} protein min^{-1}.

Ascorbate peroxidase (APX) activity of the above strain was estimated [20]. APX activity was measured spectrophotometrically by adding the enzyme extracts into the 3 mL of the reaction mixture. The 3 mL of reaction mixture contained 50 mM of sodium phosphate buffer of (pH 7), 0.5 mM of Ascorbate (Himedia, CMS040) in 10 mM of H_2O_2 (Merck, CC3C630119) and 50 µl of the enzyme extract. APX activity was determined by measuring the decreasing absorbance at 290 nm as a result of the degradation of H_2O_2. Enzyme activity was calculated using the extinction coefficient (2.8 mM^{-1} cm^{-1}). The APX enzyme activity was expressed in µmol of ascorbate oxidized mg^{-1} protein min^{-1}.

Guaiacol peroxidase (GPX) activity was determined [1] in the enzyme extract by measuring the increase in the absorbance at 470 nm due to formation of tetraguaiacol ($\varepsilon = 26.6$ mM^{-1} cm^{-1}) in the reaction mixture containing 50 mM of sodium phosphate buffer (pH 7.0), 8 mM H_2O_2 (Merck, CC3C630119), and 8 mM Guaiacol (Himedia, RM1118), and the activity was expressed as µ mol of tetraguaiacol formed mg^{-1} protein min^{-1}.

For the determination of glutathione reductase (GR) activity, an aliquot of the extract containing 50 µg protein was added to a 3 ml of reaction mixture containing 0.2 M of Tris-buffer (pH 7.8), 2 mM of EDTA (Himedia, PCT1002), 0.5 mM of GSSG (oxidized glutathione) (Himedia, RM550), and 0.2 mM of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH, Himedia, RM576). The reaction was initiated by the addition of NADPH at 25 °C. GR activity was followed by the decrease in absorbance spectrophotometrically at 340 nm due to NADPH oxidation ($\varepsilon = 6.2$ mM^{-1} cm^{-1}) according to [26] and expressed as µ mol of NADPH oxidized mg^{-1} protein min^{-1}.

Superoxide dismutase (SOD) activity was determined [20] by measuring inhibition of the photochemical reduction of NBT by the enzyme in the reaction mixture containing 50 mM of sodium phosphate buffer (pH 7.8), 0.1 mM of EDTA (Himedia, PCT1002), 75 µM of NBT (Himedia, RM578), 13 mM of methionine (Himedia, RM056), 0.3% (v/v) of Triton X-100 (Himedia, MB031), and 2 µM of riboflavin (Himedia, PCT0214). The reaction was initiated at room temperature by switching on the light (Phillips 40 W × 2 fluorescent tubes) for 8 min and stopped by switching off the light. The absorbance of formazan formed was recorded at 560 nm. One unit of SOD activity was defined as the amount of the enzyme that inhibited NBT reduction by 50% and expressed as units mg^{-1} protein min^{-1}.

2.5 Statistical analysis

All the experiments were done by taking the sample in triplicate, and pooled data were subjected to one-way analysis of variance (ANOVA) which is followed by the least significance difference (LSD). The $^{**}p$ value ≤ 0.01 was found to be statistically significant.

3 Results

3.1 Aluminum-induced stress affects the growth rate by affecting the photosynthetic pigments in *G. amphibium*

In response to different concentrations (0–100 µM) of $AlCl_3$, the growth rate of *G. amphibium* was decreased significantly. With an increase in the dose of $AlCl_3$, a substantial decline in growth was observed. As compared to control, we observed a 56.67% of retardation at 100 µM (Fig. 1a). Reduced content of the chlorophyll-a was evident during the progressive induction of stress. A (0.009 µg/mL) of chlorophyll-a was observed at control which is reduced during treatment of 100 µM (0.002 µg/mL) (Fig. 1b). A similar reduction of the three accessory light-harvesting phycobiliproteins was observed with an increase in the concentration of dose. Although a minimal deviation of reduction of PC was observed, a complete decline in APC and PE was evident at the highest dose. The PC content was reduced from control (0.0045 µg/mL) to 100 µM (0.0027 µg/mL), respectively (Fig. 1c), whereas APC declined from control (0.024 µg/mL) to 100 µM (0.007 µg/mL) (Fig. 1d) at same interval of dose. The PE too followed the same pattern as reduced 100 µM (0.0038 µg/mL) over control (0.0088 µg/mL) (Fig. 1e).

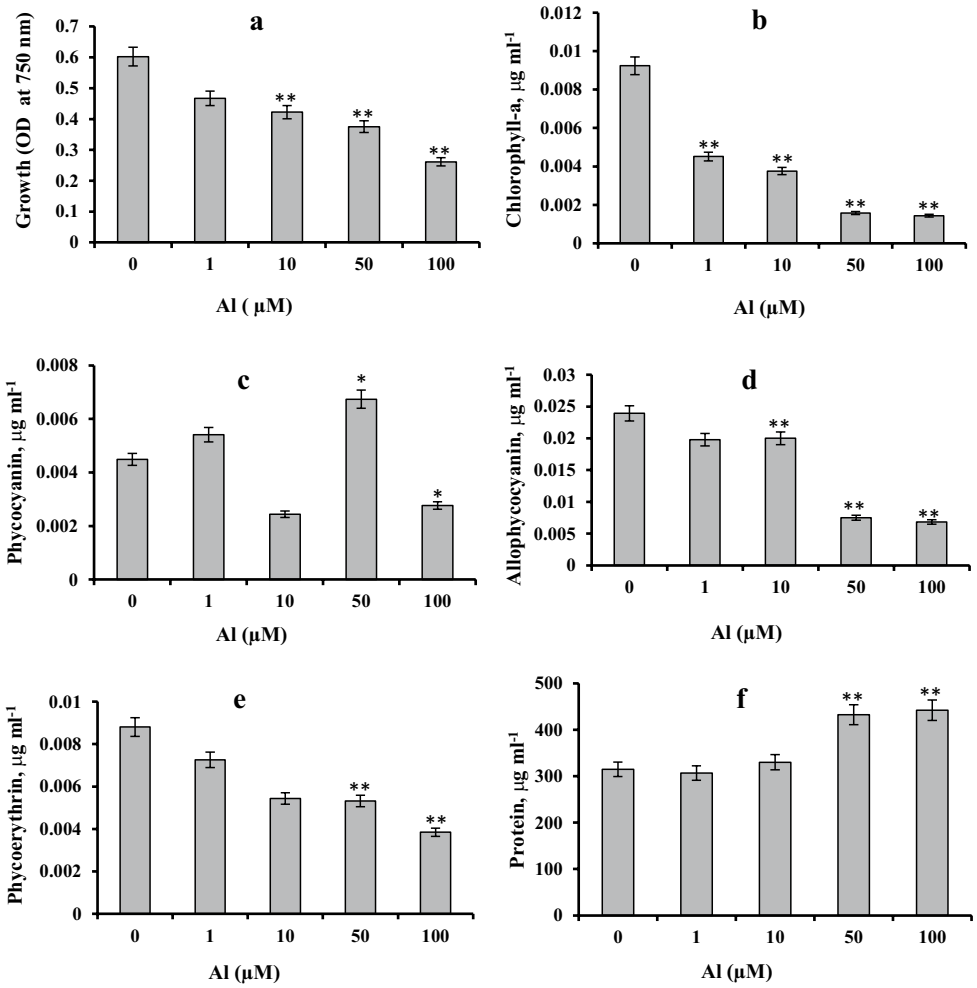

Fig. 1 Effect of aluminum ($AlCl_3$) on growth, change in pigment and protein content in *G. amphibium*. Post-treatment of different doses of $AlCl_3$ in the experimental strain *G. amphibium* for 21 days, the growth (**a**) of the strain was evaluated. Further investigation of photosynthesis-associated pigments such as chlorophyll-a (**b**), phycocyanin (**c**), allophycocyanin (**d**), and phycoerythrin (**e**) was performed. The total soluble protein content was determined to establish a link between stress and antioxidant response against it (**f**). All the experiments were done by taking the sample in triplicate, and pooled data were subjected to one-way analysis of variance (ANOVA) which is followed by the least significance difference (LSD). The $^{**}p$ value ≤ 0.01 was found to be statistically significant

3.2 *G. amphibium* promote antioxidant enzymes as a defense mechanism against aluminum-induced stress

The total soluble protein content of the strain was determined to mark the potential role of aluminum-induced stress as an increase in total soluble protein subsequently enhances the rate of abundance of stress-responsive proteins. A substantial increase in total soluble protein was observed with increasing doses. The results displayed an increase in protein content, i.e., (100 µM: 442.14 µg/mL) at highest as compared to the control (314.17 µg/mL) (Fig. 1f). Further determination of the antioxidative enzyme**s** like CAT, APX, GR, GPX and SOD activity was done that combats the aluminum-induced stress. The induction of stress marked an enhanced activity of the above-mentioned enzymes. The activity of CAT (control 74.07:100 µM 259.26) H_2O_2 utilized mg^{-1} protein (Fig. 2a) and APX (control 0.12: 100 µM 5.00) ascorbate consumed mg^{-1} protein min^{-1} were increased dose-dependently (Fig. 2b). In response to induction of stress the GR (control 1.12: 100 µM 3.93) NADPH oxidized mg^{-1} protein min^{-1} (Fig. 2c) and GPX (control 0.95: 100 µM 4.52) tetraguaiacol formed mg^{-1} protein min^{-1} also exhibited the similar pattern (Fig. 2d). Another prominent antioxidative enzyme SOD (control 2.4: 100 µM 5.24) inhibition of NBT reduced by 50% mg^{-1} protein too followed a noticeable hike with subsequent increase in the dose (Fig. 2e). The hypothesis uncovered the potential role of aluminum to induce oxidative stress on *G. amphibium* and the antioxidant mechanisms deployed by the strain to combat against such stress.

4 Discussion

Bioaccumulation and toxicity of heavy metals by cyanobacteria has often been discussed, as these photosynthetic organisms are regarded as the primary producers in the aquatic ecosystems. The effect of heavy metal toxicity has been explored recently with a limited notation on Al metal toxicity [2, 4, 30, 36]. Reported studies have

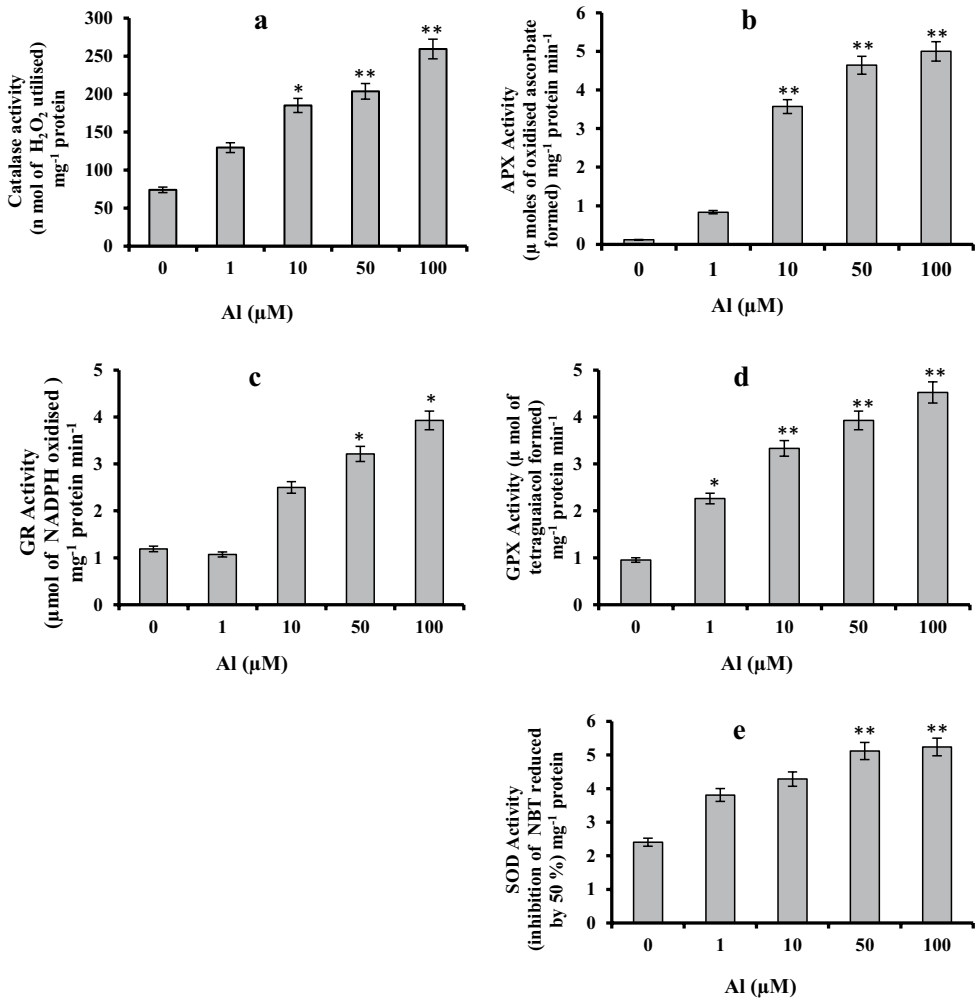

Fig. 2 Effect of aluminum ($AlCl_3$) on antioxidant enzymes as a defense mechanism against ROS generation in *G. amphibium*. After treatment of different doses of AlCl3, the antioxidant enzyme CAT (**a**) was analyzed to demonstrate the detoxification of free radicals under oxidative upset. Further quantification of APX (**b**), GPX (**c**) were done to clarify the mechanism of ROS detoxification. The GR (**d**) and SOD (**e**) were measured spectrophotometrically to clarify the overall antioxidant response exhibited by the strain to combat against stress. All the experiments were done by taking the sample in triplicate and pooled data were subjected to one-way analysis of variance (ANOVA) which is followed by the least significance difference (LSD). The $^{**}p$ value ≤ 0.01 was found to be statistically significant

remarked the colossal interlink between metal toxicity and pH of the soil as metal ions and H^+ at the cell surface get disturbed in the process [11, 15, 28]. The pH also regulates the growth and physiological parameters under induced stress environment. To get rid of such toxic phenomena and oxidative upset, the organisms deploy several antioxidative enzymes that perform as the inevitable comrades between stress and tolerance. At pH 4.5, the treatment of Al provoked associated stresses that regulate the physiochemical activities of *G. amphibium* and elicits an antioxidant defense mechanism.

Prolonged stress induced by $AlCl_3$ affects tolerance and adaptability by modulating biochemical responses; hence, a lower growth rate was evident. In addition to this, the induction of the negative feedback loop on photosynthetic pigments contributes toward the stunted growth rate of the strain. With our initial findings, we observed a dose-dependent decrease in the growth rate of the strain primarily contributed by the subsequent defects in the cell division. With an increase in dose, the inhibition of cell division resulted in stunted growth of the strain. A high dose treatment displayed maximal growth inhibition during prolonged stress induction. The results obtained are in agreement with that mercury, another heavy metal, affects the growth rate of *Chlamydomonas reinhardtii* [12]. Exposure to heavy metals generally affects the pigment profile of the strains. A sharp decline in the chlorophyll-a content was observed with increasing doses of Al. The maximal dose displayed the most effective reduction in chlorophyll-a content, hence disturbing the photosynthesis and associated pathways. Our study is well supported by the previous findings as a higher concentration of copper induces structural dysfunction and malfunctioning of several associated pigments [16]. Similarly, the chlorophyll-associated light-harvesting complex, i.e., phycobiliproteins (PC, APC, and PE), was marked an efficient reduction with increasing Al toxicity. The primary defection of photosynthesis associated pigments, it could be concluded the reduction of growth of a particular strain. Moreover, a reduced phycobiliprotein concentration was accountable for the reduction in antioxidant properties exhibited by the strain. As these complexes scavenge peroxyl, hydroxyl, and alkoxyl

radicals efficiently, the metal binding antioxidant phycocyanins prevent lipid peroxidation, resulting in reduced content of this displayed disrupt in the oxidative state. Mechanistically, the decline in phycobiliproteins under tremendous stress could address an enhanced requirement of metabolic energy for responses associated with adaptive mechanisms [14, 27].

Under selective macro-element-rich culture condition, usually there is an increase in the cellular protein content. This acts as a primary defense mechanism to combat stress through enzymatic regulation. This response maintains an equilibrium between stress tolerance and biomass productivity. Such supporting reports have been illustrated in different algae and cyanobacteria in the recent past [17, 23]. Several reports have been stated that under prolonged stress the strain elevates the expression of more proteins to combat it. Al-mediated stress stimulated expression of more soluble proteins and modified physiochemical metabolic pathways to regulate defense mechanisms [1]. Abiotic stresses disclose insightful impacts on proteomes including alterations in protein relative abundance and cellular localization. Those proteins which are usually related to the defensive mechanism of the organism are elevated with an elevation in total protein content. Stress adaptability reveals constitutively enhanced levels of several stress-related proteins subsequently associated with soluble protein. Hence, the soluble protein and cellular protection enzymes increase under abiotic stress [21, 24]. Our study exhibited a similar pattern of hike in the total soluble protein content with an increase in Al-mediated stress.

The mechanisms of cellular protection against biotic as well as biotic stresses are primarily modulated by ROS-mediated pathways. Exposure to Al is expected to elevate the intracellular production of ROS [1, 2, 19, 33]. The organisms display an adaptive response toward oxidative stress by regulating antioxidant enzymes. To nullify the oxidative imbalance caused and to mediate cellular detoxification of ROS, the synthesis of new isozymes occurs [1, 6]. During the peroxidative cycle, the enzyme peroxidase catalyzes the H_2O_2 dehydrogenation process. Further, another mechanism of H_2O_2 scavenging is mediated by CAT. Our findings demonstrated that, the Al-mediated abiotic stress exhibited a higher CAT activity at higher doses. Another mechanism deployed by this strain to counteract the H_2O_2-mediated oxidative imbalance is carried out by APX that acts as a specific negative charge donor to catalyze the reactions. The GR that functions as dimeric disulfide oxidoreductase and utilizes FAD to NADPH also scavenges hydroxyl radicals, singlet oxygen species and various electrophiles [10]. Our study revealed enhanced expression of GR that possibly acts as a threshold defense mechanism. The cellular degradation of H_2O_2 by GPX through the reduction of free hydrogen peroxide and release into water is another mechanism exhibited by this strain. We marked an enhanced GPX expression upon induction of stress which is in the line reported in several higher plant species [2]. Further SOD-mediated catalyses for dismutation reaction detoxify the cellular upset [3, 5]. Our study is in the path of similar finding reported above. A mutualistic correlation of metabolic phenomena and associated molecules are indispensable comrades to abrogate oxidative stress. Under oxidative upset, this strain triggers antioxidative enzymes to equilibrate the unfavorable cellular states.

5 Conclusion

The present study concluded that aluminum-mediated provocations of oxidative stress in *G. amphibium* influence the growth of the strain by altering both primary and associated photosynthetic pigments. To deal with such unfavorable phototoxic environment, this strain reinforces a variety of antioxidant enzymes that scavenge the free radicals produced and maintain an equilibrated intracellular state. These cellular detoxification responses can be elucidated as cellular defense mechanisms for adaptation to stress. Further, the study revealed the underlying mechanism of the stress response and proposed a predictive bioindicator of $AlCl_3$ pollution.

Acknowledgements The authors thank MoEF & CC, Govt. of India for providing fund to carry out this piece of research work. The authors also thank Berhampur University for providing necessary facilities.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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