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Nitrogen and Redox Metabolism in Cyanobacterium *Anabaena* sp. PCC 7120 Exposed to Different Sulfate Regimes

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Abstract

Sulfur is an important key nutrient required for the growth and development of *cyanobacteria*. Several reports showed the effect of sulfate limitation in unicellular and filamentous *cyanobacteria*, but such studies have not yet been reported in heterocytous *cyanobacteria* to ascribe the mechanisms of nitrogen and thiol metabolisms. Thus, the present work was carried out to appraise the impacts of sulfate limitation on nitrogen and thiol metabolisms in *Anabaena* sp. PCC 7120 by analyzing the contents as well as enzymes of nitrogen and thiol metabolisms. Cells of *Anabaena* sp. PCC 7120 were exposed to different regimes of sulfate, i.e., 300, 30, 3, and 0 µM. Application of reduced concentration of sulfate showed negative impact on the cyanobacterium. Sulfate-limiting conditions reduces nitrogen-containing compounds in the cells of *Anabaena*. Additionally, reduced activities of nitrogen metabolic enzymes represented the role of sulfate in nitrogen metabolism. However, decreased activities of thiol metabolic enzymes indicated that sulfate-limited cyanobacterial cells have lower amount of glutathione and total thiol contents. Reduced accumulation of thiol components in the stressed cells indicated that sulfate-limited cells have lower ability to withstand stressful condition. Hence, *Anabaena* displays differential response to different concentrations of sulfate, and thus, stipulated that sulfur plays an important role in nitrogen and thiol metabolisms. To the best of our knowledge, this is the first report demonstrating the impact of sulfate stress on nitrogen and redox metabolisms in heterocytous *cyanobacteria*. This preliminary study provides a baseline idea that may help improve the production of paddy.

Abbreviations

APR	APS-reductase
APS	Adenosine-5'-phopsphosulfate
ATPS	ATP sulfurylase
DO	Degree of oxidation
FAO	Food and agriculture organization
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
NiR	Nitrite reductase
NR	Nitrate reductase
OAS-TL	O-acetyl serine (thiol) lyase
SAT	Serine acetyltransferase
γ-GCS	γ-Glutamyl cysteine synthetase

Introduction

According to the FAO (Food and Agriculture Organization) 2020 report, more than 50% of people worldwide eat rice as their primary food [1]. The area of paddy fields accounts for the major cultivated area of kharif grain crops in India and ranks second in the area of rice fields planted in the world (https://worldpopulationreview.com/country-rankings/rice-production-by-country). Planting rice is a common and effective strategy to meet the demands of the growing population. Sulfur is one of the essential nutrients for the growth of the plants, it enters the paddy ecosystem through the application of sulfur fertilizers and gaseous sulfur dioxide.

In the current scenario, decrease in the emission of sulfur dioxide gas escalated the sulfur-limiting environment and posed adverse effects on agriculture as well as agriculturally important microbes [2]. Since sulfur is also an essential nutrient required for the growth and development of *cyanobacteria* [2]. Sulfur is needed for the proper functioning of several metabolic processes such as photosynthesis, respiration, nitrogen metabolism, redox metabolism, and many more [3, 4]. The decrease in sulfur in the paddy fields disturbs the physiology and metabolic processes of the plant,

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resulting in less productivity. In order to obtain a good and productive yield, the application of sulfur in the paddy fields is quite significant.

Cyanobacteria are used as bio-inoculants in the paddy fields. They have the potential to fix atmospheric nitrogen into ammonia and other related nitrogenous compounds [5]. Most of the studies focused on the fact that sulfur limitation severely affects the morphology, physiology, and metabolic attributes of rice and other plants, which results in reduced yields [6, 7]. In the paddy field, cyanobacterial growth could also face sulfate stress condition, which might affect nitrogen-fixing capability, thereby reducing agricultural productivity [3, 5–7]. Therefore, an understanding of the response of agriculturally important nitrogen-fixing *cyanobacteria* to sulfate stress condition is particularly relevant for improving soil fertility and paddy field crop production.

The growth of *cyanobacteria* is hampered by sulfate limitation and produces reactive oxygen species (ROS) that lead to electrolyte leakage and membrane damage resulting programmed cell death [3]. Previous studies have reported the responses of *cyanobacteria* under sulfate stress condition which are restricted to very few unicellular and filamentous strains [3–6, 8–10]. Sulfate stress causes pigment reduction and lowers photosynthetic processes in *Anabaena* sp. PCC 7120 (hereafter *Anabaena* 7120) [3]. Recently, Kharwar and Mishra reported carbon allocation and reduced nitrogen content in the cyanobacterial cells of *Anabaena* under sulfate stress conditions [3].

Previously, it was reported that cells of *Nostoc* experiences sulfate stress showed decreased activity of nitrogen metabolic enzymes such as nitrogenase [11, 12]. On the other hand, the effect of sulfate stress on nitrate and nitrite metabolism has been relatively scant. Besides, only meagre information is available on the effect of sulfur deficiency on the thiol metabolism. So far, there have been very few studies in connection with the relationship of sulfate to thiol redox metabolism in *cyanobacteria*. Hence, the present study was performed to assess the impacts of sulfate limitation on nitrogen and redox metabolisms in the heterocytous cyanobacterium *Anabaena* 7120. For the first time, we have studied the effect of sulfate stress on the nitrogen and redox metabolisms of the heterocytous cyanobacterium *Anabaena* 7120. The data will explain how heterocytous *cyanobacteria* can respond to changing concentrations of sulfate under the stressed conditions. The study also provides information that the sulfur amendment in the rice field should be taken into consideration while increasing productivity, flourishing the growth of *cyanobacteria*, and further suggesting ways to help in sustain the paddy yield and their productivity.

Materials and Methods

Cyanobacterium Culture Conditions and Experimental Design

Cyanobacterium *Anabaena* 7120 was a generous gift from Prof. C.P. Wolk (Michigan State University, USA). The cyanobacterium was grown and maintained in the 250 mL Erlenmeyer flask containing 100 mL of BG-11 (pH 7.4) liquid medium at 28°C ± 2°C under continuous illumination of 50–55 µM photons m^{-2} s^{-1} with a 14/10h light/dark cycle. The cyanobacterium was manually shaken twice a day.

Magnesium sulfate ($MgSO_4$) salt as a source of sulfate was used in the present study. Various concentrations of $MgSO_4$ were prepared from the stock solution (1 M $MgSO_4$) by dissolving it into the BG-11 medium. The stock solution was prepared in deionized Milli-Q water. On the basis of cell density experiment performed in our previous study [3], the effective concentrations of 300, 30, 3, and 0 µM $MgSO_4$ were selected for the present study. For each experiment, the stock solution of $MgSO_4$ was freshly prepared and high-pressure steam sterilization was done by autoclave. In an attempt to study sulfate stress responses in the cyanobacterium, exponentially grown cultures of *Anabaena* 7120 were harvested by centrifugation at 10,000 × *g* for 10 min. Pellets were washed with sterile sulfate-free medium to remove adherent salts. Further, the obtained pellets were dissolved in Milli-Q water and inoculated in the medium containing different sulfate concentrations such as 300, 30, 3, and 0 µM. The experiment was conducted in Erlenmeyer flasks of 250 mL filled with 100 mL of BG-11 culture medium. All the experiments were performed in triplicate. Table 1 describes all the assays performed in the present study and their importance. A comparative account was made between the stressed cyanobacterial cells and control (300 µM sulfate, i.e., normal BG-11 medium) cells with respect to nitrogen-containing compounds and nitrogen metabolic enzymes, components of thiol metabolism (total thiol, oxidized and reduced glutathione), and thiol metabolic enzymes.

Assay of Nitrogen-Containing Compounds and Nitrogen Metabolic Enzymes

Cyanobacterial cells exposed to different concentrations of sulfate were homogenized in the phosphate buffer (pH 7) and centrifuged at 10,000 × *g* at 25°C for 15 min. The collected supernatants were further used for quantification of nitrate (NO_3^-) and nitrite (NO_2^-) as per the protocols of Patterson et al. [13] and Ogawa et al. [14], respectively,

Table 1 The table describing all the assays performed in the present study and their importance

S. No	Assay	Method and protocol used in the present study	Importance
1	Nitrate (NO_3^-)	Patterson et al. (2010)	In order to understand the effect of sulfate stress on the nitrogen metabolism
2	Nitrite (NO_2^-)	Ogawa et al. (1999)	
3	Intracellular content ammonium	Molins-Legua et al. (2006)	
4	Nitrate reductase (NR) activity	Herrero et al. (1981)	
5	Nitrite reductase (NiR) activity	Herrero and Guerrero (1986)	
6	Glutamine synthetase (transferase) (GS) activity	Shapiro and Stadtman (1970)	
7	Total thiol contents	Sedlak Lindsay (1968)	To assess the thiols of the *cyanobacteria* under sulfate stress condition
8	Glutathione	Anderson (1985)	
9	Serine acetyltransferase (SAT) activity	Nakamura et al. (1987)	To gain insight into the effect of sulfate stress on the sulfate assimilation and redox metabolism
10	O-acetylserine (thiol) lyase (OAS-TL) activity	Kharwar and Mishra (2020)	
11	γ-glutamyl cysteine synthetase (γ-GCS) activity	Orlowski and Meister (1971)	
12	Glutathione reductase (GR) activity	Schaedle and Bassham (1977)	
13	Glutathione peroxidase (GPX) activity	Lawrence and Burke (1976)	

and expressed in nmoL mg protein^{-1}. Additionally, intracellular ammonium content was estimated using protocol described by Molins-Legua et al. [15] with the addition of Nessler's reagent. The absorbance was recorded at 420 nm and expressed in nmoL mg protein^{-1}.

Nitrate and nitrite reductase activities were assayed in cyanobacterial cells treated with different levels of sulfate using modified protocols of Herrero et al. [16] and Herrero and Guerrero [17], respectively. Enzyme activities were expressed in the activity units (U) corresponding to µmoL NO_3^- removed and/or NO_3^- produced min^{-1}. While glutamine synthetase (transferase) activity (GS) was assayed as per the protocol of Shapiro and Stadtman [18] and expressed in terms of µmoL γ-glutamyl hydroxamate mg^{-1} protein min^{-1}.

Assay of Components of Thiol Metabolism

Quantification of total thiol contents has been done according to the protocol of Sedlak and Lindsay [19]. Whereas oxidized and reduced glutathione contents were estimated using the method of Anderson et al. [20]. Oxidized glutathione was calculated by subtracting total glutathione from the reduced glutathione and expressed as µmoL protein^{-1}.

Assay of Thiol Metabolic Enzymes

Cyanobacterial cells were centrifuged (10,000 × *g*) for 30 min at 4°C, and the obtained pellets were homogenized in 1 mL of phosphate buffer (pH 7.5). Further, homogenates were sonicated for 1 min and recentrifuged at 10,000 × *g* for 30 min at 4°C. The collected supernatants were further used for enzymatic assays.

SAT activity was estimated by the calorimetric method as given by Nakamura et al. [21]. Whereas OAS-TL activity was determined by measuring the production of cysteine at 560 nm as per the protocol of Kharwar and Mishra [3], and activity was represented in nmoL min^{-1} mg protein^{-1} using the standard curve of cysteine. In addition, γ-GCS activity was assayed using the protocol of Orlowki and Meister [22] and expressed in U mg protein^{-1}. Moreover, glutathione reductase (GR) was performed using the modified method of Schaedle and Bassaham [23]. An assay of glutathione peroxidase (GPX) was carried out according to Lawrence and Burke [24] and expressed in terms of U mg protein^{-1}.

Statistical Analysis

At first, all the parameters were checked for analysis of variance, i.e., the normal distribution of residuals, by the Shapiro–Wilk test and the Kolmogorov–Smirnov test, and were found to be satisfactory. Further, one-way ANOVA (analysis of variance) was performed to assess the significant differences between each treatment. The degree of correlation among all the parameters was also analyzed. A matrix dendrogram utilizing the unweighted pair group method with arithmetic mean (UPGMA) algorithm along with Euclidian similarity indices was constructed using PAST 3.0 software for cladistic representation of parameters. Moreover, principle component analysis (PCA) was performed to analyze the correlation between all the studied parameters. All the data analyses were carried out by Sigma 14 and SPSS software

(SPSS Inc. Version 21.0, IBM Crop, Armonk, NY) and are represented as mean ± standard error.

Results

Nitrogen-Containing Compounds and Nitrogen Metabolic Enzymes

In order to understand the effect of sulfate stress in the heterocytous *cyanobacteria* on the nitrogen metabolic pathway, we have measured nitrogen-containing compounds and nitrogen metabolic enzymes. The influence of selected different concentrations of sulfate on nitrogen-containing compounds and nitrogen metabolic enzymes is shown in Fig. 1. Supplementation of 30, 3, and 0 µM sulfate substantially affect nitrogen metabolism by decreasing NO_3^-, NO_2^-, and NH_4^+ contents with respect to their control (300 µM sulfate) cells (Fig. 1a). Similarly, limited supplies of sulfate causes significant decrease in the enzyme activities of NR (4.8, 65.2, and 76.8%), NiR (16.2, 47.8, and 57.5%), and GS (7.4, 26.2, and 52%) at 30, 3, and 0 µM sulfate treated cells, respectively, compared to the control, i.e., 300 µM sulfate (Fig. 1b). Reduction in the nitrogen-containing compounds and nitrogen metabolic enzymes suggested that cyanobacterium was difficult to perform nitrogen assimilation efficiently in the limiting condition of sulfate.

Thiols and Thiol Metabolic Enzymes Confer Redox Status

Cyanobacteria withstand different types of stressors, including light, nutrients, and oxidative stress. Their regulation of thiol metabolic process is a characteristic of the ability to tolerate such stressful conditions. However, there is few experimental reports of the thiol metabolic components conferring redox status identity in *cyanobacteria*, and none of the studies were performed in heterocytous *cyanobacteria* under sulfate stress conditions.

In order to fill the lacuna, we study the thiol metabolic components and enzymes in the heterocytous cyanobacterium. Figure 2a shows that high thiol content was achieved by the cyanobacterial cells at the 300 µM sulfate concentration. The results pertaining to total thiol contents depicted a significant reduction at 30, 3, and 0 µM sulfate concentrations. The lowest (15.8%) and highest (35%) thiol contents were noticed at 0 and 30 µM sulfate, respectively, compared to control (300 µM sulfate) cells (Fig. 2a). Additionally, reduction in the reduced glutathione (GSH) content, i.e., 1.8, 1.7, and 1.5 µM mg protein^{-1} in the 30, 3, and 0 µM of sulfate treated cells compared to the control (300 µM sulfate) cells, i.e., 2.9 µM mg protein^{-1} were detected, while increased oxidized glutathione (GSSG) content was noticed upon treatment of cyanobacterial cells with 30, 3, and 0 µM of sulfate as compared to the control (300 µM sulfate) cyanobacterial cells of *Anabaena* 7120 (Fig. 2b). Thus, the inferred percent increment in the degree of oxidation was 8.7, 9.04, and 10% at 30, 3, and 0 µM of sulfate treatments, respectively, as compared to control (300 µM sulfate) cyanobacterial cells of *Anabaena* 7120 (Fig. 2c). Changing

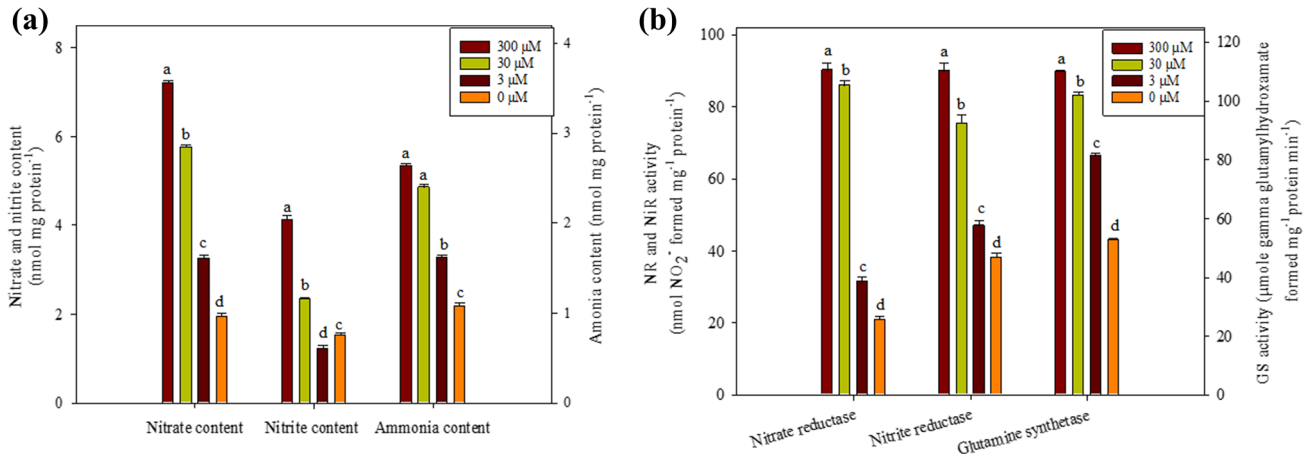

Fig. 1 **a** Nitrogen containing components; **b** Activities of nitrate reductase, nitrite reductase, and glutamine synthetase of the cyanobacterium *Anabaena* 7120 cells exposed to different concentrations of sulfate. 300 µM sulfate concentration indicated control condition, whereas 30, 3, and 0 µM sulfate concentrations represented as different treatments of sulfate in the present study. Each value is mean of three independent replicates, with bars indicating SE. Bars with different letters indicate that differences were statistically significant at $P < 0.05$

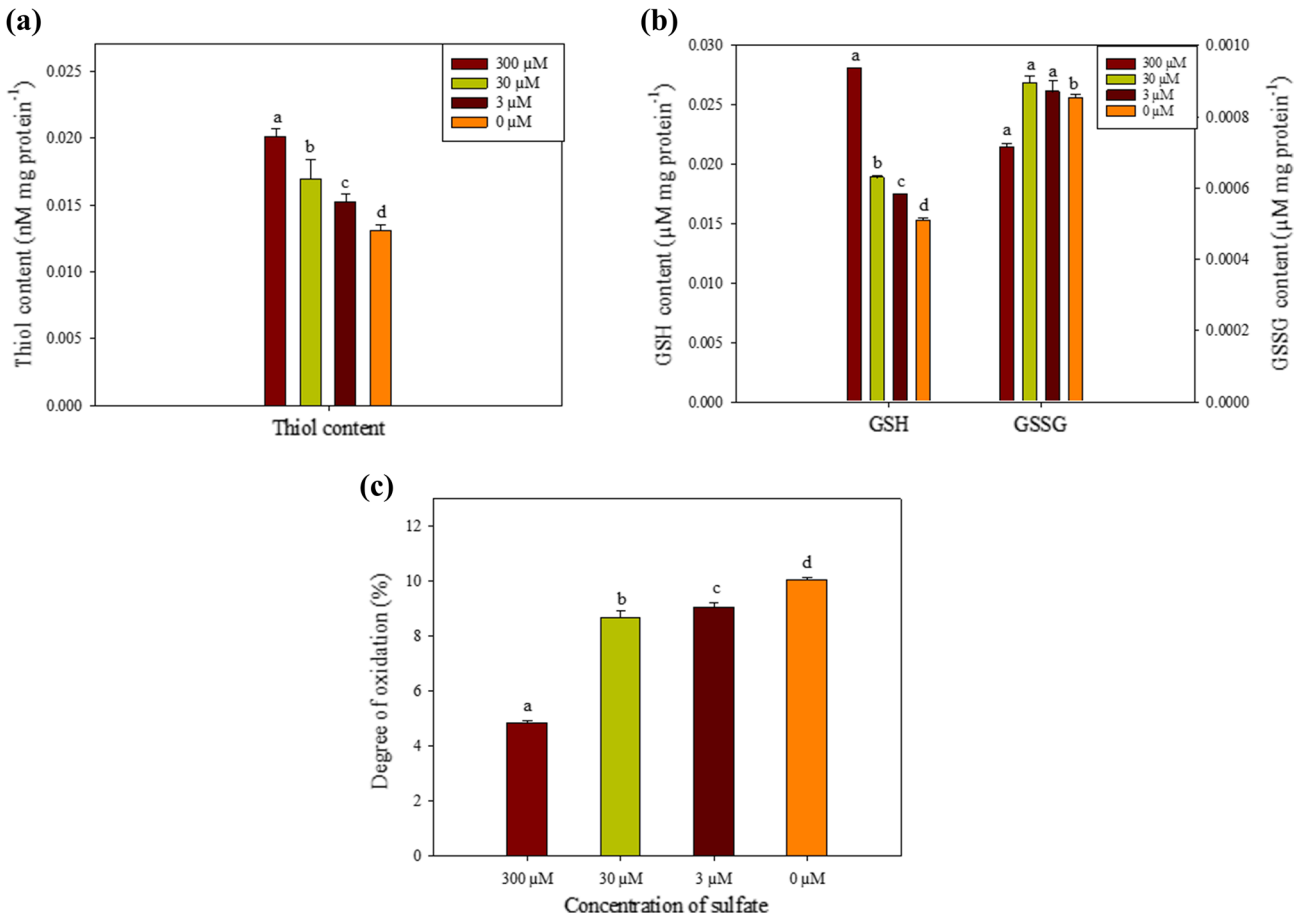

Fig. 2 **a** Thiol content; **b** Oxidized and reduced content of glutathione; **c** Degree of oxidation of glutathione of the cyanobacterium *Anabaena* 7120 cells exposed to the different concentrations of sulfate. 300 µM sulfate concentration indicated control condition, whereas 30, 3, and 0 µM sulfate concentrations represented as different treatments of sulfate in the present study. Each value is mean of three independent replicates, with bars indicating SE. Bars with different letters indicate that differences were statistically significant at $P < 0.05$

the concentrations of sulfate from 300 to 0 µM affects the accumulation of total thiol contents and glutathione content. Increasing the GSSG of the cyanobacterial cells treated with 30, 3, and 0 µM sulfate led to the oxidation of glutathione.

We first analyzed the enzymes involved in cysteine biosynthesis, such as SAT and OAS-TL. The negative effect of sulfate limitation was evident on the enzymes involved in cysteine biosynthesis. Percentage reduction in the activity of SAT was 7, 49, and 53.4%, while OAS-TL activity was reduced by 7.5, 11.3, and 14.5% at 30, 3, and 0 µM sulfate supplemented cells, respectively, as compared to the control, i.e., 300 µM sulfate (Fig. 3a and b). Hence, our results indicated that sulfate is important for maintaining cysteine biosynthesis in the test *cyanobacterium*.

Then, we analyzed the response of *Anabaena* 7120 on the thiol metabolic enzymes to different concentrations of sulfate. γ-GCS activity was detected in all the cells of *cyanobacteria* treated with different sulfate concentrations, and it was decreased during the stress condition. The activity of γ-GCS decreased by 8.5, 39.4, and 35.4% at 30, 3, and 0 µM sulfate treated cells, respectively, compared to control (300 µM sulfate) cells (Fig. 3c).

Next, the activity of GR in the cyanobacterium *Anabaena* 7120 under stress conditions was determined by harvesting the cyanobacterial cells from different concentrations of sulfate. As shown in Fig. 3d, there was a significant difference ($P < 0.05$) in the activity of GR for the tested concentrations of sulfate. Sulfate deficiency significantly reduced the activity of GR by 26.8, 91.5, and 87.1% in cells treated with 30, 3, and 0 µM sulfate, respectively, compared to control (300 µM sulfate) cyanobacterial cells (Fig. 3d).

Furthermore, the influence of sulfate stress on the activity of GPX in the test cyanobacterium was investigated. A reversed trend was observed for GPX activity, which was increased from 0.04 U mg protein^{-1} in control (300 µM sulfate) cells to 0.13, 0.25, and 0.37 U mg protein^{-1} in 30, 3,

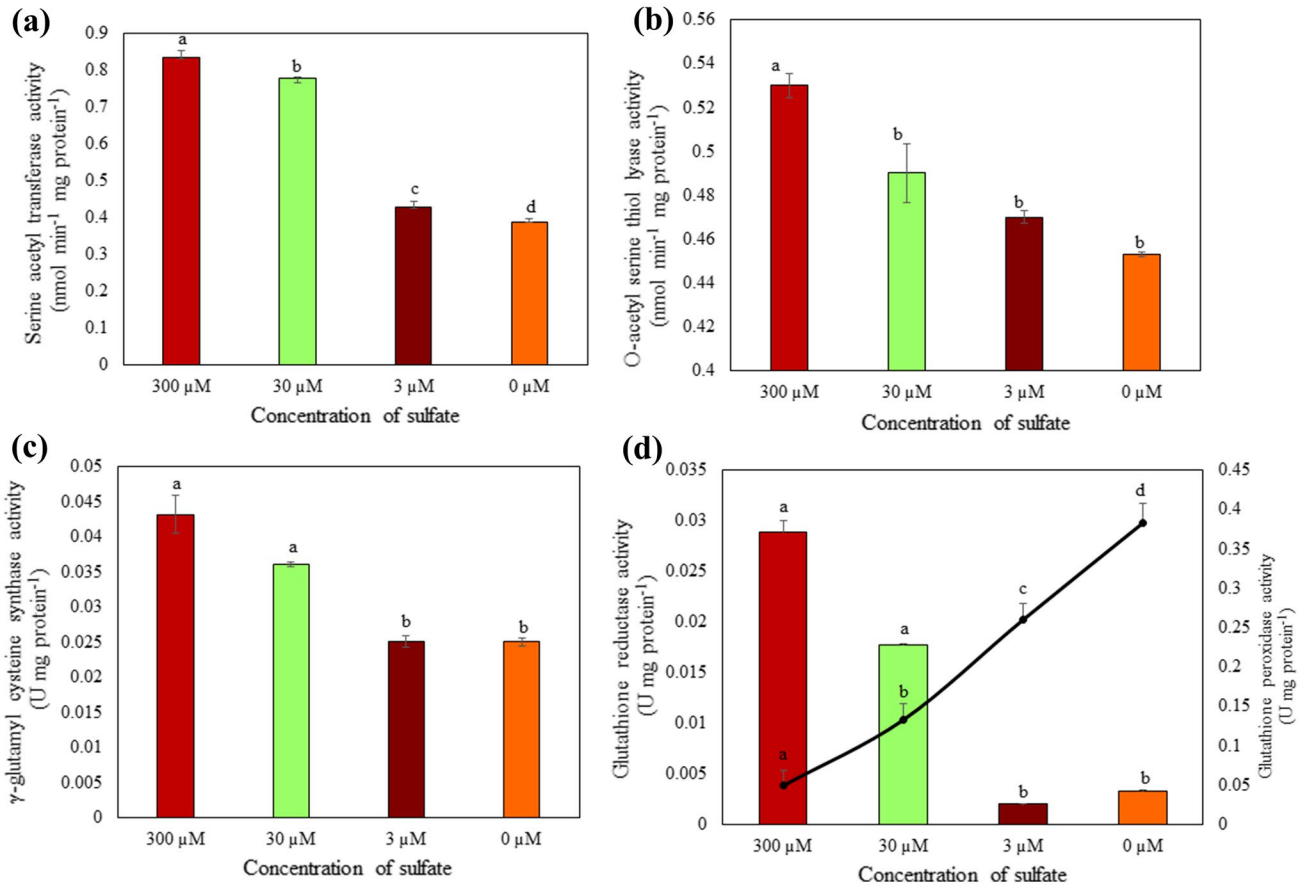

Fig. 3 Activities of thiol metabolic enzymes of the cyanobacterium *Anabaena* 7120 cells exposed to different concentrations of sulfate: **a** serine acetyl transferase; **b** O- acetyl serine thiol lyase; **c** γ-glutamyl cysteine synthetase; **d** glutathione reductase is represented with the bars and glutathione peroxidase is represented with the line in the diagram. 300 µM sulfate concentration indicated control condition, whereas 30, 3, and 0 µM sulfate concentrations represented as different treatments of sulfate in the present study. Each value is mean of three independent replicates, with bars indicating SE. Different letters indicate that differences were statistically significant at $P < 0.05$

and 0 µM sulfate treatments, respectively (Fig. 3d). Therefore, we suggested that the different levels of GPX activity detected in each concentration of sulfate could be due to a differential expression of the gene encoding GPX in the *cyanobacteria* under sulfate stress conditions.

In summary, changing the concentration of sulfate led to changes in the activities of thiol metabolic enzymes, conferring redox status in the cells of the cyanobacterium (Fig. 3).

Statistical Analysis

A principal component analysis (PCA) was generated by SPSS software to interpret the correlation between the studied parameters under sulfate stress conditions. Results of PCA analysis for all the parameters used in the present study showed that PC1 and PC2 explained 86.258% and 9.033% of the variance, respectively, and thus cumulatively represent 95.291% of the total variance. For maximum variation in PC1, the parameters responsible were ammonia, NR, SAT, GS, nitrate, NiR, thiol, GCS, GR, nitrite, GSH, degree of oxidation (DO), and GPX (Fig. 4a, c), whereas PC2 identified GSH, OAS-TL, nitrite, GR, thiol, GCS, DO, and GSSG in the cyanobacterial cells as dominant variables (Fig. 4a, c). However, these components of the PCA plot are grouped into two clusters, i.e., minor cluster I and major cluster II, sharing an inverse relationship (Fig. 4a, c). Cluster I contained OAS-TL, GSH, and nitrite. Whereas thiol, GR, GCS, NiR, nitrate, SAT, ammonia, GS, and NR were subsumed into cluster II (the major cluster) and positioned closer to cluster I. However, GPX, DO, and GSSG form clusters, i.e., III, IV, and V, respectively, are distinct from both the cluster I and cluster II. PCA showed that all the studied parameters were differentially affected by sulfate, hence, they formed combined as well as independent clusters. Parameters that were similarly affected by sulfate were thiol, GR, GCS, NiR, nitrate, SAT, ammonia, GS, and NiR (cluster I). In the PCA, the studied parameters demonstrated segregation

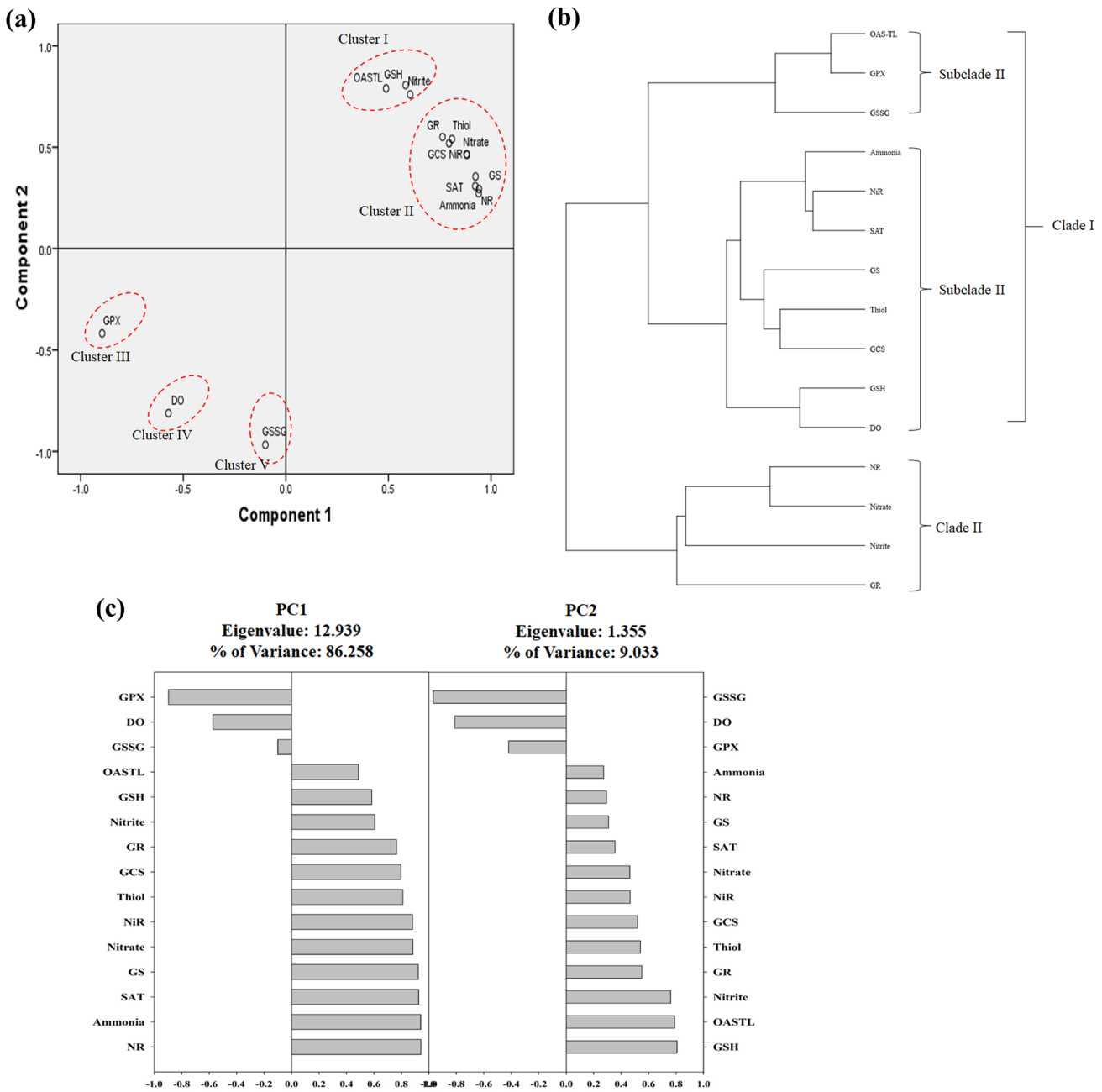

Fig. 4 Statistical relationship among various parameters in control and sulfate limited treated cells of *Anabaena* 7120. **a** Biplot of principal component analysis of the parameters in control and sulfate limited cells. 300 µM sulfate concentration indicated control condition, whereas 30, 3, and 0 µM sulfate concentrations represented as different treatments of sulfate in the present study. The contribution to PC 1 is shown on the x-axis, while the contribution to PC 2 is on the y-axis; **b** Matrix dendrogram showing clustering of studied parameters into clades; **c** Contributions of the studied parameters to PCs. Parameters are shown on the y-axis, while the degrees of contribution to PCs are on the x-axis. (*DO* degree of oxidation; *GPX* glutathione peroxidase; *GR* glutathione reductase; *GSH* reduced glutathione; *GSSG* oxidized glutathione; *NiR* nitrite reductase; *NR* nitrate reductase; *OAS-TL* O-acetylserine (thiol) lyase; *SAT* serine acetyltransferase; *γ-GCS* γ-glutamyl cysteine synthetase)

of the different sulfate concentrations with significant variability (Fig. 4a). A clear separation was observed in the parameters of clusters I and II from those clusters III, IV, and V. Cluster I and cluster II were presented in close proximity, showing their similar effects in response to different concentrations of sulfate. Additionally, cluster III, IV, and V were demarcated in close proximity, revealing that they respond to various sulfate concentrations with similar trends.

Table 2 Pearson's correlation coefficients of all the studied parameters

Parameters	Nitrate	Nitrite	Ammonia	Nitrate reductase	Nitrite reductase	Glutamine synthetase	SAT	OAS-TL	Thiol	GSSG	GSH	Degree of oxidation	γ-GCS	GR	GPX
Nitrate	1														
Nitrite	**0.877**	1													
Ammonia	**0.968**	0.75	1												
Nitrate reductase	**0.967**	**0.826**	**0.958**	1											
Nitrite reductase	**0.994**	**0.904**	**0.954**	**0.98**	1										
Glutamine synthetase	**0.971**	0.749	**0.982**	**0.939**	**0.948**	1									
SAT	**0.976**	**0.859**	**0.953**	**0.993**	**0.988**	**0.934**	1								
OAS-TL	**0.8**	**0.851**	0.667	0.666	0.778	0.72	0.713	1							
Thiol	**0.97**	**0.862**	**0.928**	**0.89**	**0.95**	**0.941**	**0.921**	**0.825**	1						
GSSG	− 0.547	− 0.804	− 0.389	− 0.396	− 0.552	-0.4	− 0.44	− 0.755	− 0.603	1					
GSH	**0.892**	**0.952**	0.778	0.777	**0.886**	**0.805**	**0.816**	**0.918**	**0.926**	− 0.844	1				
Degree of oxidation	− 0.885	− 0.943	− 0.771	− 0.763	− 0.876	-0.8	− 0.804	− 0.919	− 0.925	0.846	− 1	1			
γ-GCS	**0.921**	**0.918**	**0.838**	**0.909**	**0.941**	**0.833**	**0.947**	**0.81**	**0.903**	− 0.536	**0.863**	− 0.853	1		
GR	**0.904**	**0.936**	**0.809**	**0.894**	**0.932**	**0.813**	**0.929**	**0.8**	**0.883**	− 0.565	**0.871**	− 0.86	**0.991**	1	
GPX	− 0.994	− 0.833	− 0.972	− 0.955	− 0.979	-0.988	− 0.96	− 0.778	− 0.97	0.503	− 0.872	0.866	− 0.889	− 0.872	1

Bold values indicate significant correlation between two parameters

ATPS ATP sulfurylase; *GPX* glutathione peroxidase; *GR* glutathione reductase; *GSH* reduced glutathione; *GSSG* oxidized glutathione; *OAS-TL* O-acetylserine (thiol) lyase; *SAT* serine acetyltransferase; *γ-GCS* γ-glutamyl cysteine synthetase

Further, a correlation matrix for all the parameters defining the degree of correlation among various parameters is shown in Table 2. However, cladistic representation of all the data from treatments and control (300 µM sulfate), by UPGMA paired matrix dendrogram using Euclidian similarity indices was analyzed. All the parameters were clustered into two major clades, i.e., I and II. Clade I comprises of two minor subclades, i.e., subclade I and subclade II. The subclade I consisted of ammonia, NiR, SAT, GS, thiol, GCS, GSH, and DO, while subclade II consisted of OAS-TL, GPX, and GSSG. Moreover, parameters like NR, nitrate, nitrite, and GR were subsumed into clade II (Fig. 4b).

Discussion

Sulfate stress is very much expected, as it is actually occurring in the nature because of the decrease in sulfur dioxide gas emissions in the atmosphere. Although high concentrations of sulfate (approximately 28 mM) have been reported from marine ecosystems due to the presence of sulfate salts, while freshwater and agricultural soils contained lower amounts of sulfate [2]. In freshwater ecosystems and agricultural soils, sulfate concentrations range from 10 to 50 µM ensuring the occurrence of sulfur deficiency in common cyanobacterial habitats [2]. Since the *cyanobacteria* are used as biofertilizer in the paddy field [5–7]. It has been reported that the growth and productivity of *cyanobacteria* were adversely affected due to stress conditions. Thus, the *cyanobacteria* thriving in such niche might also face sulfate stress. Hence, it is interesting to understand the impact of sulfate stress on the paddy field cyanobacterium and how the *cyanobacteria* respond in case of sulfate stress. The components and enzyme activities of nitrogen and redox metabolisms of the cyanobacterium *Anabaena* 7120, were monitored in the current study under selected different concentrations of sulfate, namely 300, 30, 3, and 0 µM, in order to understand how the cyanobacterium responds to each concentration of sulfate and how the sulfate stress affects the paddy field production.

Regulation of the Nitrogen Metabolic Pathway Upon Sulfate Limitation in the cyanobacterium *Anabaena 7120*

Sulfate limitation alters the nitrate assimilation process of the cyanobacterium *Anabaena* 7120. Significant decrease in the nitrate and nitrite contents were noticed in the cyanobacterial cells supplemented with 30, 3, and 0 µM as compared to the control (300 µM sulfate) cells. Furthermore, NH_4^+ content was reduced by limiting sulfate supplies. The gradual decrease in the NH_4^+, NO_2^-, and NO_3^- contents in response to sulfate limitations suggested that reduced sulfate availability alters nitrate metabolism. Flores and Herrero demonstrated that the ABC-type transporter proteins, which likely use ATP as a source of energy, facilitate NO_3^- and NO_2^- uptake into the cyanobacterial cells [25, 26]. Reduction in the NO_3^- and NO_2^- uptake rates as the result of the disrupted photosynthetic electron transport chain perhaps associated with lower pool of ATP [27]. There was significant decrease in the NO_3^- and NO_2^- contents in sulfate-limiting cyanobacterial cells, possibly due to damage in the membrane bound transporter proteins [28], whereas cells supplemented with 300 µM sulfate showed higher enzyme activity, which was supported by increased photosynthetic activity and lower production of ROS. With decreased transportation of nutrients under different treatments of sulfate, i.e., 30, 3, and 0 µM, the MDA content was found to be increased in the cyanobacterial cells along with the high ROS production, which clearly indicated stressful condition in the treated (30, 3, and 0 µM sulfate) cyanobacterial cells of *Anabaena* 7120 that likely had not occurred in the control cells, i.e., 300 µM sulfate [3]. Moreover, significant decrease in the activities of NR and NiR were observed in the sulfate-limiting cells. Additionally, GS activity was also significantly reduced at 30, 3, and 0 µM sulfate treatments with respect to the control (300 µM sulfate) cells. Decrease in the NiR activity was associated with reduced availability of NO_2^- ions (as observed in our study). Furthermore, higher NR, NiR, and GS activities at 300 µM sulfate concentrations were connected with higher growth and better photosynthetic activity in terms of chlorophyll *a*, carotenoids, and phycobiliproteins. Since sulfate stress could strongly inhibit chlorophyll biosynthesis [3], one of the first visible consequences of stress conditions in *cyanobacteria* is a decrease in the photopigments. The chlorophyll content in the control cyanobacterial cells (300 µM sulfate) was relatively higher than that of cyanobacterial cells treated with 30, 3, and 0 µM sulfate concentrations. Since *cyanobacteria* require significant amount of sulfur, which plays various roles in the photosynthetic complex, it is present in the Fe-S cluster of *cyanobacteria*. Enhanced photosynthetic processes that generate ATP in *Nostoc muscorum* and *Phormidium foveolarum* have been reported by researchers [28, 29]. At lower concentrations of sulfate, i.e., 3 and 0 µM, an inhibition of growth and lower photosynthetic activities [3] might be the reason for the lesser activity of these enzymes. This decreased activity was probably caused by repression of the enzyme. Sulfate stress modulates NR and NiR activities through nitrate and nitrite uptake, respectively, since the enzyme activity is perhaps determined by nitrate and nitrite flux into the metabolic pool. Different authors reported that excess copper disturbs photosynthesis and redox equilibrium of the cells, disrupting the cells ultrastructure, and finally leading to cell death [30–32]. However, under high cadmium and zinc stress, activation of

metallothionein (small cysteine-rich proteins) was noticed, which chelates these heavy metals and helps in the survival of *cyanobacteria* [33]. In accordance with our results, Singh et al. also showed that heavy metals, pesticides, and UV-B stress hampers photosynthetic process of the *cyanobacteria*, resulting decreased activities of NR and NiR [28, 29]. The GS enzyme plays a key role in nitrogen metabolism. One of the 20 amino acids that make up the standard genetic code, glutamine, is involved in number of biochemical processes, such as synthesis of protein, production of ammonium to control the acid–base balance, providing energy to cells in addition to glucose, donating nitrogen for various anabolic processes, and replenishing citric acid cycle. GS catalyzes the ATP-dependent condensation of NH_4^+ with glutamate to produce glutamine, making it a crucial enzyme in ammonia assimilation [28, 29]. Hence, reduction in the activities of GS in the cyanobacterial cells treated with 30, 3, and 0 μM sulfate concentrations was presumed with lesser heterocyte frequency as compared to control cells (300 μM sulfate), since GS is located in the heterocyte of the cyanobacterial cells. Another possible explanation for decrease in GS activity is that the C/N balance disrupting cyanobacterial growth. Thus, changes in the status of the above-mentioned parameters reduce the nitrate assimilation process as well as the nitrogen content of the cell. This decrease in nitrogen content indicated that sulfate limitation also inhibits nitrogen uptake by *Anabaena* 7120 [3]. In the previous study, authors reported a significant impact of salt stress on the cyanobacterial nitrogen metabolism [34].

Sulfate Limitation Leads to Changes in the Sulfur-Containing Metabolites and Enzymes of the Thiol Metabolic Pathway, Indicating an Alteration in the Thiol-Based Redox Buffer of the Cyanobacterium *Anabaena* 7120

Glutathione is a key regulator of the cellular thiol redox buffer which regulates redox homeostasis and the cell cycle [35, 36]. Therefore, the present study is designed to understand the connection between sulfur, ROS, glutathione formation, and redox equilibrium under different sulfate concentrations in *Anabaena* 7120. The results showed that the pool of reduced GSH was lower in the sulfate treatment cells as compared to the control, which coincides with the lower activity of γ-GCS, whereas GSSG showed varied response. This results in a smaller GSH/GSSG redox couple in the sulfate stressed cells which favors an oxidize environment. The precise control of intracellular redox status, i.e., maintenance of physiological levels of ROS for mediating normal cellular function (oxidative eustress) while evading excess ROS (distress), is central player for the concept of redox system [36]. Intracellular redox changes affect cell signaling, gene transcription, translation, and cell death [37, 38]. A smaller value of the redox couple, i.e., GSH/GSSG, is reflected by rapid intracellular glutathione oxidation. The higher degree of oxidation in the stressed cells was determined by the increasing oxidized glutathione production. This indicated lower turnover of GSH/GSSG in the stressed cells relative to the control (300 μM sulfate) cells and changes in the glutathione redox potential. Thus, cyanobacterial cells at 30, 3, and 0 μM sulfate are experiences oxidative stress, reflecting that glutathione and sulfate are important for acclimating under the adverse conditions in *cyanobacteria*. Cameron and Pakrasi demonstrated an increase in GSSG level from the glutathione pool during sulfate starvation in *Synechocystis* sp. PCC 6803 [35]. Li et al. showed that glutathione is involved in the regulation of several processes through glutathionylation in *cyanobacteria* [39]. It has been demonstrated that glutathione and cysteine contents are reduced in *Synechocystis* 6803 during sulfate deficiency [40]. Another probable reason for reduced glutathione content in the stressed cyanobacterial cells was the reduction in NADPH content at 30, 3, and 0 μM sulfate concentrations, as observed in our previous study [3]. Adams et al. reported that the ratio of the reduced and total pyridine nucleotide pools is an index of cellular redox status [41]. NADPH is essential for recycling of glutathione and is related to its antioxidant functions [41]. Hence, there was disturbance in the redox homeostasis of the cyanobacterial cells treated with 30, 3, and 0 μM sulfate concentrations as compared to the control cells (300 μM sulfate). In addition, dramatic decline in the thiol contents was found in the cyanobacterial cells at 30, 3, and 0 μM sulfate as compared to the control (300 μM sulfate). This rapid decrement in the thiol contents also confers oxidative constraints in the cells.

In addition, the gradual reduction in SAT activities was noticed in the cyanobacterial cells at 30, 3, and 0 μM sulfate concentrations, whereas only meagerly significant alteration in OAS-TL enzyme activities was evident at 30, 3, and 0 μM sulfate supplementation as compared to the control (300 μM sulfate) condition, conferring modulations in OAS-TL activity. Sulfate limitations affect cysteine biosynthesis which results in reduced methionine and glutathione contents [40]. Concentration of these sulfur-containing metabolites decreases when sulfate limitations occur in the cells. This might have two reasons: first, upon sulfate limitations, sulfide rather than OAS becomes limiting for cysteine biosynthesis. Secondly, the cell might try to keep the thiol concentration at a certain level. Lower activities of SAT and OAS-TL were related to oxidative stress in sulfate-limiting conditions. OAS-TL and SAT scavenge ROS under stress condition, conferring oxidative stress tolerance [42]. A reduction in intracellular sulfate content is a typical consequence of sulfate deficiency [3]. Sulfate uptake and assimilation are functions of organisms' demand for sulfur metabolites, viz. sulfide, cysteine, and glutathione, which

are involved in arrays of signaling pathways and redox sensors [4].

GR serves as a marker for oxidative stress because it converts glutathione disulfide (GSSG) back to glutathione. Prior research revealed that *cyanobacteria* that can withstand any kind of stress typically exhibit higher GR activity [35]. Less significant reductions of γ-GCS and GR were observed in the sulfate stressed cells of *Anabaena* 7120. This reduction in the activities of γ-GCS and GR in the cyanobacterial cells treated with 30, 3, and 0 μM sulfate concentrations was thought to be due to decreased glutathione synthesis. In view of our results in *Anabaena* 7120, it could be suggested that the gene coding for GR (*gor*) is differentially transcribed as a function of the sulfur concentration, and the GR activity also depends on the sulfur concentration. γ-GCS is a rate-limiting enzyme in glutathione biosynthesis and plays a central role in glutathione homeostasis. The rapid changes in the glutathione and γ-GCS levels at the 30, 3, and 0 μM sulfate concentrations indicated that glutathione and γ-GCS were perhaps catabolized as a source of sulfur, which is limited during the sulfate-limiting condition in *cyanobacteria*. These decrease in γ-GCS levels in the stressed cyanobacterial cells represent decrease in the efficiency of cells to maintain the redox poise. Researchers have reported that reduction in glutathione and γ-GCS activity in yeast under nitrogen and sulfur stress conditions, which could be catabolized for amino acids [43, 44]. However, changes in the cellular metabolism were evident in plants during sulfur-limiting conditions [45]. GR was found to regenerate glutathione; thus, reduced levels of GR in the cyanobacterial cells at 30, 3, and 0 μM sulfate concentrations indicated decrease in the glutathione amount. It is one of the most abundant reducing thiol content which catalyzes reduction of glutathione disulfide (GSSG) to the reduced form of glutathione (GSH) by an electron donor, NADPH [46]. However, the lowered activities of these above-mentioned enzymes in the stressed cells of *Anabaena* 7120 might not necessarily scavenge ROS below their thresholds since the adverse effects of oxidative stress were exhibited by rapid lipid peroxidation in our previous study [3].

By contrast to GR, significant increment of GPX activities was evident in stressed cells of *Anabaena* 7120 at 30, 3, and 0 μM sulfate concentrations. It is an important enzyme that catalyzes the detoxification of hydrogen peroxide to water and GSSG by using GSH as a reductant. This antioxidative enzyme provides the most vital defense against peroxidative damage to membrane, which are reported to play an important and fundamental role in cellular homeostasis [47]. Thus, cyanobacterial cells at 30, 3, and 0 μM sulfate concentrations experience more oxidative damage. Overall, these results showed that the balance of the GSH/GSSG ratio and ROS homeostasis are regulated by GPX, which represents modulation in the oxidation–reduction system and culminates in altered growth and other processes. In our study, it was observed that the sulfate stressed cyanobacterial cells of *Anabaena* 7120 showed lesser growth as compared to the control (300 μM sulfate) cells. In general, light and nutrients stress exert comparable effects on cyanobacterial cells [35, 40, 48]. These stresses alter structural characteristics, cellular growth, and metabolism of *cyanobacteria* and activates the antioxidant system to detoxify ROS as well as regulate redox buffer of the cells [49], but in comparison to sulfate stress, *cyanobacteria* are fails to activate the antioxidant system due to the lower availability of sulfur for the synthesis of proteins involved in this mechanism. The breakdown of the antioxidant system is one of the critical aspects for the *cyanobacteria* to maintain their homeostasis under sulfate stress condition. The inability of *cyanobacteria* to endure disturbed homeostasis exemplifies their poor tolerance mechanisms under such condition. Since heterocytous nitrogen-fixing *cyanobacteria* are integral components of paddy fields and thus play a crucial role in improving the productivity of rice plants, it is very important that the paddy field was amended with an optimal amount of sulfur, which favors the growth of *cyanobacteria* and thereby enhances the yield and productivity of rice. The flourishing growth of *cyanobacteria* in the paddy field helps in bringing about changes in the fertility of the soil, its mineral composition, and many more factors that facilitates the productivity of rice plant [5]. Thus, our findings suggested that sulfate stress alters the nitrogen and redox processes of *Anabaena*. The present study also demonstrated that the optimal amount of sulfate should be taken into consideration when improving of rice production.

Conclusion

In conclusion, our results showed that sulfur plays a pivotal role in cyanobacterial metabolism (Fig. 5). Alterations in the activities of nitrogen metabolic enzymes as well as nitrogen-containing compounds were noticed, indicating that sulfur is an important nutrient regulating nitrogen metabolism in *Anabaena* 7120. Additionally, components of thiol and the activities of thiol metabolic enzymes in the cyanobacterium were also reduced, suggested the crucial role of sulfur in maintaining redox status of the cells. Overall, sulfate limitation alters cyanobacterial metabolism and therefore provides evidence for the role of sulfate in redox regulation. It also highlights the cross-talk among the thiol-based redox buffer and nitrogen metabolism of the cyanobacterium *Anabaena* 7120. This study provides a preliminary idea and expands our knowledge about how the cyanobacterium alters its metabolic pathways during sulfate stress, which subsequently affects the productivity of rice fields. This type of analysis

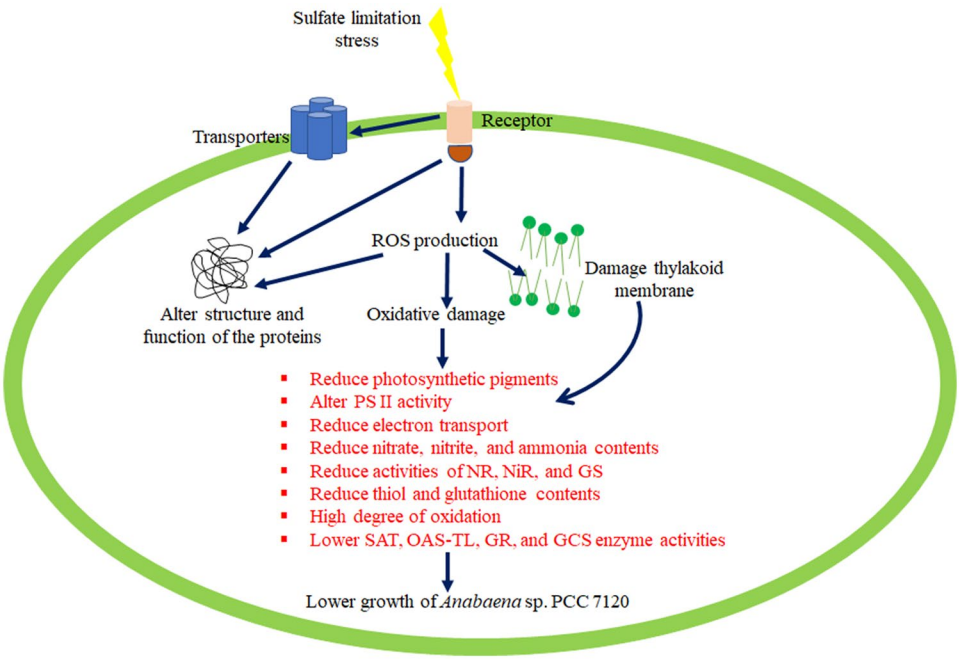

Fig. 5 Schematic diagram deciphers the adverse impacts of sulfate limitation inside the cyanobacterial cells of *Anabaena* 7120

gives an indirect assessment and a first-step study on the impacts of sulfate stress condition in *cyanobacteria*, which accelerate the growth of agronomically important plants. The task remains to determine the impacts of sulfate stress on the cyanobacterial cells grown in the paddy field in a natural environment.

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Data Availability The datasets of the present study are available from the authors.

Code Availability Not applicable.

Declarations

Conflict of interest Authors declared they have no conflict of interest.

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