Algal Research 32 (2018) 70–78

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

An investigation on involvement of the ascorbate-glutathione cycle in modulating NaCl toxicity in two cyanobacteria photoacclimatized to different photosynthetic active radiation

Jitendra Kumar, Vijay Pratap Singh[1], Sheo Mohan Prasad*

Ranjan Plant Physiology and Biochemistry Laboratory, Department of Botany, University of Allahabad, Allahabad 211002, India

ARTICLE INFO

Keywords:
Ascorbate-glutathione cycle enzymes
Cyanobacteria
Oxidative stress biomarkers
Photoacclimation
Salinity

ABSTRACT

In the present study, comparative responses of two cyanobacteria viz. *Nostoc muscorum* and *Phormidium foveolarum*, photoacclimatized at three distinct levels (sub-optimum; 25 ± 0.5, optimum; 75 ± 2.5 and supra-optimum; 225 ± 3.5 μmol photons $m^{-2} s^{-1}$) of photosynthetic active radiation (PAR), and subsequently treated with two doses (30 and 90 mM) of NaCl were determined by analyzing changes in growth pattern, oxidative stress markers, enzymes of the ascorbate-glutathione cycle i.e. ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), and associated metabolites (ascorbate and glutathione). PAR influences growth behavior of both cyanobacteria, as maximum growth observed in supra-optimum PAR acclimatized cells followed by optimum and sub-optimum PAR. Maximum superoxide radical (SOR) and hydrogen peroxide (H_2O_2) contents and their consequent damages to lipids in terms of MDA equivalents were observed in supra-optimum PAR acclimatized cells in individual as well as in combined treatment of NaCl. Further, NaCl-induced the least percent reduction in growth was observed in supra-optimum PAR acclimatized cells, which was in consistence with increased activities of the AsA-GSH cycle enzymes (APX, GR, MDHAR and DHAR) and its associated metabolites i.e. ascorbate (AsA) and glutathione (GSH) contents and reduced and oxidized ratios of ascorbate (AsA/GSH) and glutathione (GSH/GSSG). The results suggest that the AsA-GSH cycle might have associated with better growth in supra-optimum PAR acclimatized cells than optimum and sub-optimum PAR acclimatized cells under NaCl stress. Besides this, study also establishes fact that *P. foveolarum* is more tolerant than *N. muscorum* against these twin stresses, and may be inoculated in paddy fields under existing environmental conditions for increasing fertility of the soil.

1. Introduction

Salinity, a major environmental stress, has become a constraint to crop production worldwide. The increasing pressure of salinization on agrarian land is exerting negative effects, will result into shrinkage of approximately 30% agricultural land within next 25 years and up to 50% by the middle of 21st century [1,2]. The probable reason of soil salinity is thought to be the delivery of salt along with water which is being used for irrigation practices, and gets concentrated after long time in the soil. The enhanced level of salt in land may cause hyperionic and hyperosmotic stresses that can lead to serious metabolic impairments in plant cells [3–6]. Impairments include disruption in the cellular integrity, dysfunctioning of numerous enzymes, and altered nutrient acquisition and photosynthetic ability. Apart from this, over production of reactive oxygen species (ROS) during salt stress contributes significantly in degrading numerous vital macromolecules of the cell [7,8].

Furthermore, under ambient environment, aquatic autotrophs are always under the pressure of abiotic stresses. The ambient light environment is enormously diverse since the intensity of light and duration are so fluctuating between the equator and poles. Dependency of photoautotrophs on light to drive photosynthesis is seemingly under risk of new kind of stress, which limits growth and productivity of plant throughout the globe. Thus, any modifications in optimum light intensity (whether a scarce or surplus) would definitely hinder normal metabolism of cyanobacteria. Till date, researches showed that fluctuating light serves as a limiting factor that is responsible for distorted growth behavior of plants under numerous stress conditions like salinity [8], temperature [9] and UV-B radiation [10]. Thus, intensity and period of light definitely standardize basic metabolic processes, bearing

* Corresponding author.
E-mail address: profsmprasad@gmail.com (S.M. Prasad).
[1] Present address: Govt. Ramanuj Pratap Singhdev Post Graduate College, Baikunthpur 497335, Koriya, Chhattisgarh, India.

https://doi.org/10.1016/j.algal.2017.10.019
Received 5 October 2016; Received in revised form 25 October 2017; Accepted 25 October 2017
2211-9264/ © 2017 Published by Elsevier B.V.

capacity and locality assortment of the organism. In this way, photoautotrophs adapt various strategies to survive under fluctuating ambient light environment. Among different strategies, one of the unique strategies is modification in the structure and the function of the photosynthetic machinery [2,11,12]. Under scarcity of light, amount of light harvesting antenna complexes increases in order to absorb maximum light energy so that they can meet demands of cells for light energy. In contrast to this, these harvesting complexes get down-regulated against oversupply of light for avoiding absorption of excess light energy since it may cause the production of harmful ROS [13–16].

In oxygenic photoautotrophs including cyanobacteria, ROS i.e., $O_2\cdot^-$, H_2O_2, $\cdot OH$ and $^1O_2$ are produced as byproducts during transportation of electrons in the photosynthetic process. At the same time, well maintained antioxidant defense systems (enzymatic as well as non-enzymatic) keep the ROS level to threshold limit so that they can also contribute in signaling processes. Among a wide range of antioxidants, enzymes participating in the ascorbate-glutathione (AsA-GSH) cycle have an immense contribution in neutralizing unwanted oxygen derivative (H_2O_2) that is produced under aerobic environment [17]. In this cycle, for dissociation of H_2O_2 into H_2O, electrons do deliver to enzyme ascorbate peroxidase (APX) from the ascorbate (AsA) [18]. At the same time, AsA gets restored from instantaneous action of dehydroascorbate reductase (DHAR) on dehydroascorbate (DHA) at the charge of reduced glutathione (GSH), yielding oxidized glutathione (GSSG). Furthermore, GSSG is rehabilitated into GSH via accepting electrons from NADPH due to the action of a NADPH-dependent glutathione reductase (GR). Both AsA and GSH are interconnected with the AsA-GSH cycle and found abundantly in plant cells as non-enzymatic low molecular weight antioxidants. They can efficiently neutralize oxygen derivatives such as $O_2\cdot^-$, H_2O_2, $\cdot OH$ and $^1O_2$ and thus pay significant contribution in protecting plants against oxidative stress [19,20]. The proper functioning of the AsA-GSH cycle enzymes keep a balance between reduced and oxidized forms of ascorbate as well as glutathione in preferred limits. Thus, any shifting in the AsA-GSH cycle may seriously influence regular channel of ROS quenching process which could limit the growth of organism. Moreover, interaction of multiple stresses is very common in ambient environment. Evidences showed that risk of salinity stress in organisms may be modulated in the presence of other stresses [8,21]. The study pertaining to salinity induced alterations in the AsA-GSH cycle has not been given a full attention in cyanobacteria [22]. Besides, probable role of PAR in regulating the adverse effects of salt in cyanobacteria is remain to be investigated. Thus, there is a need to investigate the possible involvement of the AsA-GSH cycle enzymes in cyanobacteria against these twin stresses. Therefore, in the present study, modulations in the AsA-GSH cycle enzymes and corresponding metabolites were investigated in photoacclimatized cyanobacteria treated with two doses (30 and 90 mM) of NaCl. At the same time, present work has also determined the tolerance nature between the two cyanobacteria– *Nostoc muscorum* and *Phormidium foveolarum* against interaction of two stresses.

2. Materials and methods

2.1. Organisms and growth conditions

The cultures of filamentous, heterocystous form of cyanobacterium, *Nostoc muscorum*, and non-heterocystous form of cyanobacterium, *Phormidium foveolarum* were maintained in our laboratory. The axenic and homogenous cultures of *N. muscorum* and *P. foveolarum* were cultivated in BG-11 medium in temperature controlled culture room at 25 ± 2 °C under the exposure of 75 μmol $m^{-2} s^{-1}$ of photosynthetically active radiation (PAR, 400–700 nm) provided by white fluorescent tubes (Osram L 40W/25-1) with a 14:10 h of light: dark regime. In the case of *P. foveolarum*, BG-11 medium was externally supplemented with $NaNO_3$ (1.5 g/l) as nitrogen. All experiments were performed with exponential phase of culture.

2.2. Photoacclimatization and NaCl treatment

Exponentially grown cultures of *N. muscorum* and *P. foveolarum* were illuminated to three distinct photosynthetic active radiations (PAR): (sub-optimum; 25 ± 0.5 μmol photons $m^{-2} s^{-1}$, optimum; 75 ± 2.5 μmol photons $m^{-2} s^{-1}$ and supra-optimum; 225 ± 3.5 μmol photons $m^{-2} s^{-1}$) obtained by white fluorescent tubes (Osram L 40W/25-1). The 14:10 h light and dark photoperiod was taken for acclimatization by irradiating cultures to above mentioned PAR condition daily. Both cyanobacterial cultures were acclimatized by constant sub-culturing in respective culture medium for 25 days (5 generations comprising of 4 days each to avoid the lag phase) at 25 ± 2 °C.

Further, for NaCl treatment photoacclimatized *N. muscorum* and *P. foveolarum* cells were harvested by centrifugation at 4000*g* for 10 min and washed twice with sterile distilled water, and then pellets were re-suspended in respective culture media containing two concentrations (30 mM and 90 mM) of NaCl. Further, cultures of both cyanobacteria were allowed to grow under their respective growth condition in culture room. After 72 h of NaCl treatment, different parameters were analyzed by withdrawing treated and untreated cells.

2.3. Measurement of growth

For measurement of growth, treated and untreated cells were harvested by centrifugation at 4000*g* for 10 min, washed twice with sterile distilled water. Dry weight of each sample was determined by using digital electronic balance (Contech—CA 223, India), after drying sample at 80 °C for 48 h. Total amount of protein in dried cells of each sample was quantified with Folin phenol reagent as described by Lowry et al. [23] using bovine serum albumin as standard.

2.4. In vitro measurement of superoxide radicals and hydrogen peroxide

Superoxide radicals (SOR; $O_2\cdot^-$) in control and treated cyanobacterial cells were determined by following the method of Elstner and Heupel [24]. Hydrogen peroxide (H_2O_2) in control and treated cultures were recorded by following the method of Velikova et al. [25].

2.5. In vivo measurement of ROS production

The production of $O_2\cdot^-$and H_2O_2 was quantified by in vivo staining of different PAR acclimatized cyanobacterial cells with nitroblue tetrazolium (NBT; Sigma) and 3, 3′ diaminobenzidine (DAB)–HCl (Sigma), respectively [26]. Equal amounts of biomass from treated and untreated cultures were harvested by centrifugation at 2500*g* for 5 min. The pellets were re-suspended in medium containing 1 mM NBT and/or 5 mM DAB and incubated in dark for 10 min. Subsequently, cultures were transferred to their respective PAR condition for 1 h in culture room. The cultures were filtered onto glass microfiber filters (diameter 24 mm, GF/C; Whatman), and the filter paper containing cells was washed twice with 100% methanol to remove the pigments. NBT is directly reduced by $O_2\cdot^-$, which forms the water insoluble, deep blue formazan precipitate. DAB interacts with H_2O_2 forming a reddish-brown polymerization product. Thereafter, filters were imaged with the help of digital camera.

2.6. Measurement of lipid peroxidation

Lipid peroxidation in terms of malondialdehyde (MDA) equivalents content was assayed by following the method of Heath and Packer [27]. The absorbance of reaction mixture was read spectrophotometerically at 532 and 600 nm. The values of non-specific absorption read at 600 nm were deducted from the values read at 532 nm. The concentration of MDA equivalents content was quantified by using an extinction coefficient of 155 mM cm^{-1}.

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