A bentazone-resistant mutant of cyanobacterium, *Synechococcus elongatus* PCC7942 adapts different strategies to counteract on bromoxynil- and salt-mediated oxidative stress

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Abstract A bentazone-resistant mutant of *Synechococcus elongatus* PCC7942, called Mu2, tolerated elevated NaCl concentrations. As bentazone and bromoxynil exhibit similar mechanism of action, we investigated whether the mutant also tolerated bromoxynil and found it to be true. The line of investigation was then whether the acclimation strategy for the three stressors, bentazone, bromoxynil and NaCl was same or different. The cellular contents of malondialdehyde, hydrogen peroxide and superoxide increased in wild type strain following all the treatments suggesting their toxicities due to oxidative response. Notwithstanding, there were apparently different anti-oxidative measures pertaining to the herbicide and salinity stress. Glutathione contents and activities of superoxide dismutase, catalase-peroxidase, glutathione S-transferase and glutathione reductase decreased under NaCl, whereas bromoxynil affected only glutathione S-transferase reductase. Moreover, in-gel assays revealed that bromoxynil promoted appearance of isozymes of catalase-peroxidase, while NaCl induced such response only for superoxide dismutase. On the other hand, in Mu2, glutathione peroxidase-reductase and glutathione showed upward trend after bromoxynil exposure, whereas NaCl raised peroxidase and superoxide dismutase. Proteome comparison revealed peroxiredoxin Q to be highly expressed in wild type strain under bromoxynil, whereas NaCl favoured flavodoxin over-expression. Their amounts were already high in Mu2. We suggest that Mu2 acclimatized to bromoxynil in a manner similar to bentazone by upgrading peroxiredoxin Q and glutathione peroxidase-

reductase. Conversely, for NaCl it devised another mechanism involving peroxidase and superoxide dismutase, and flavodoxin.

Keywords Antioxidant enzymes · Bromoxynil · Flavodoxin · Peroxiredoxin Q · Proteome · Co-tolerance · Salt (NaCl) · *Synechococcus elongatus* (cyanobacterium)

Introduction

A number of pesticide pollutants originating from agricultural watersheds, including the post-emergence herbicides bentazone and bromoxynil, adversely affect phytoplankton survival and dynamics (Belden et al. 2007). According to a “pollution-induced community tolerance” (PICT) concept, herbicide pre-exposure promotes tolerance to self (Garcia-Villada and Rebound 2007) and other herbicides with same mode of action (Knauer et al. 2010). Triazines and substituted ureas, the “classical” herbicides, inhibiting PS II-mediated electron flow (Nimbal et al. 1996), mutually show a concentration-dependent additive response (Porsbring et al. 2010), and promote joint tolerance. Studies on unicellular cyanobacterium, *Synechococcus elongatus* PCC7942 (hereafter *S. elongatus*) revealed that bentazone and bromoxynil inhibit PS II activity, evoke oxidative response by triggering reactive oxygen species (ROS), mainly H₂O₂, and promote cytosolic acidification (Das and Bagchi 2010). It was assumed that mutual tolerance is a common occurrence within the “phenolic-type” herbicides, too.

We have selected by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine treatment *S. elongatus* mutants surviving higher concentrations of bentazone. One such strain, termed Mu2, expressed at higher level the manganese stabilizing protein PsbO, PS I - core proteins - PsbA and PsbB, ferredoxin-NADP⁺ oxidoreductase (FNR) and ATP synthase subunit

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AtpA. Moreover, iron stress-induced protein A (IsiA), a component of PS I – IsiA supercomplex under iron starvation, was also found to be highly expressed under iron-replete conditions (Bagchi et al. 2007). A better organized PS II and PS I-cyclic electron flow rendered the mutant an ability to survive the oxidative stress. Moreover, later studies revealed that the anti-oxidant system involving, in particular, reduced glutathione (GSH) contents and glutathione peroxidase-reductase (GPX-GR) activities in Mu2 were enhanced to act on bentazone-derived ROS. The other modification revealed from proteome analysis was an up-regulation of bacterioferritin co-migratory protein-homologue peroxiredoxin-Q (PRX-Q) (Das and Bagchi 2011).

Salinity and temperature are reported to enhance herbicide toxicity (DeLorenzo et al. 2011). Consequently, herbicide resistance in a DCMU-resistant mutant of *Prophyridium* sp. and in bentazone resistant mutant Mu2 is accompanied with associated salt tolerance (Bagchi et al. 2007; Manandhar-Shrestha et al. 2009). In this paper we tried to answer whether pollution-induced community tolerance is a concept confined to only the chemical (pesticide) co-tolerance phenomenon among the phytoplankton, or it can be extended to understand acclimation to the environmental stressors, viz. salinity. Accordingly, we attempted to answer whether the aforementioned modifications could promote bromoxynil and salt tolerance in Mu2, or whether there are different strategies pertaining to each kind of stress tolerance. The proteomes of bromoxynil- and salt-treated cells and their untreated counterparts were compared in order to identify stress-specific and stress-induced proteins. We also report the changes in the enzymatic and non-enzymatic anti-oxidant system that might result in acquisition of co-tolerance(s) in the mutant.

Materials and methods

Chemicals

Bromoxynil (CAS registry number 1689-84-5, purity 98.0 %) was purchased from Accustandard Inc. (USA). 3' (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), baker's yeast GR, GSH and oxidized glutathione (GSSG), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), NaCl (A.R.) and other fine chemicals were purchased from Sigma Life Science, Himedia, Life Technologies and Qualigens, India. IPG strips were purchased from GE Healthcare Bio-Sciences Ltd., India.

Cyanobacterial strains and growth conditions

Axenic cultures of *S. elongatus* PCC7942 (Pasteur Culture Collection of Cyanobacteria, Institute Pasteur, Paris, France)

wild type (WT) and Mu2 (Bagchi et al. 2007) were grown in BG-11 medium at uniform light intensity of 60 $\mu\text{E photons m}^{-2} \text{ s}^{-1}$ (fluorescence lamps, Philips, India) and at 30 °C as described (Ray and Bagchi 2001; Das and Bagchi 2011). For growth in presence of NaCl, cells previously grown in BG-11 medium were harvested and transferred to fresh BG-11 medium to attain cell density of OD 0.2 at 750 nm (ME 802 Spectrophotometer, Systronics, India). Filter sterilized bromoxynil from ethanolic 0.5 M stocks and NaCl from aqueous 6 M stocks were added to autoclaved medium. Controls received equivalent ethanol (<2 % v/v) or none. Absolute growth was monitored as $\Delta\text{OD}_{750 \text{ nm}}$ between 4 d and zero time. The time kinetics of growth was determined and chlorophyll (chl) was estimated according to Mackinney (1941).

Determination of cellular H_2O_2 , O_2^- , malondialdehyde (MDA) and glutathione contents

For H_2O_2 determination, harvested cells from 1 L cultures were vortexed for 5 min with 1 mL 0.1 % trichloroacetic acid (TCA) in ice-bath and centrifuged at 3,000 $\times$ g. Then 1 mL of 50 mM K- PO_4 buffer pH 7.0 and 1.5 mL of 1 M KI were added to the supernatant and H_2O_2 content was monitored at 390 nm (Lee and Shin 2003). Superoxide radicals were measured according to Yang et al. (2008) as follows: harvested cells were suspended in 3 mL of 50 mM K- PO_4 buffer pH 7.8 and sonicated by MSE, Soniprep 150 sonicator (3 $\times$ 2 min, 5 min interval at 4 °C), centrifuged at 5,000 $\times$ g for 10 min at 4 °C. To 1 mL of supernatant 0.5 mL of K- PO_4 buffer, pH 7.8 and 1 mL of 1 mM hydroxylamine HCl were added, incubated at 25 °C for 1 h, and 1 mL each of 17 mM sulfanilamide and 7 mM α -naphthylamine were added. After incubation at 25 °C for 20 min, the absorbance was read at 530 nm. Lipid peroxidation in terms of MDA was determined as follows: cells were suspended in 10 mL of 0.25 % 2-thiobarbituric acid in 10 % TCA and heated at 95 °C for 30 min and quickly chilled in ice. After centrifuging at 3,000 $\times$ g, absorbance at 532 and 600 nm were recorded and $\epsilon=155 \text{ mM}^{-1} \text{ cm}^{-1}$ was employed (Lee and Shin 2003). Total glutathione and GSSG levels were determined by following the procedure of Griffith (1980). Cells were harvested, suspended in 1 mL 5 % sulphosalicylic acid and broken using a sonicator as above. After centrifuging at 10,000 $\times$ g for 10 min at 4 °C, 18 μL of 7.5 M triethanolamine was added to 300 μL of supernatant to bring pH to neutrality. Of this, 150 μL was taken for determination of total glutathione (GSH + GSSG) and remaining aliquot was used for GSSG, for which GSH interference was nullified by adding 3 μL 2-vinyl pyridine and incubating at 20 °C for 60 min. Aliquots of 50 μL from each preparation were mixed with 700 μL of 0.3 M NADPH, 100 μL of 10 mM of DTNB and 150 μL of 125 mM Na- PO_4 buffer pH 6.5 containing 6.3 mM Na_2EDTA . Later, 10 μL of Baker's yeast GR

(50 U mL⁻¹) in Na-PO₄ buffer was added and Δ OD at 412 nm was recorded; GSH calibration curve was used. GSH content was calculated from the difference between total glutathione and GSSG. The cellular contents were represented as per mg chl.

Estimation of antioxidant enzyme activities

For determining the activities of antioxidant enzymes spectrophotometrically, the harvested cells in 50 mM Na-PO₄ buffer pH 7.0 were sonicated as mentioned above. For all the assays given hereunder, the cell-free extract equivalent to 75 μ g protein measured according to Lowry et al. (1951) was used as enzyme source. Catalase (CAT; EC 1.11.1.6) activity was determined after Aebi (1984) as decomposition of H₂O₂ recorded at 240 nm in a reaction mixture containing 1.5 mL of 100 mM K-PO₄ buffer pH 7.0 and 1.2 mL of 150 mM H₂O₂. Peroxidase (POX; EC 1.11.1.7) activity was determined following the method of Hirsch et al. (1961), in a reaction mixture containing 500 μ L 26 mM H₂O₂, 1 mL 0.01 M freshly prepared pyrogallol and 1 mL 0.1 MK-PO₄ buffer pH 7.0. The change in absorbance at 420 nm was read up to 150 s. The method of Tripathi et al. (2006) was adopted for determining superoxide dismutase (SOD; EC 1.15.1.1) activity as photochemical reduction of nitrobluete-tetrazolium (NBT), measured in a reaction mixture containing 50 mM K-PO₄ buffer, pH 7.8, 100 μ L of each of 30 mM EDTA, 2.3 M NBT and 0.5 M riboflavin. After incubation under fluorescent light for 10 min, the absorbance was taken at 560 nm. GR (EC 1.6.4.2) activity was determined as NADPH oxidation recorded at 340 nm (Halliwell and Foyer 1978). The reaction mixture comprised of 1.8 mL of 50 mM K-PO₄ buffer pH 7.8, 300 μ L of 3 mM Na₂EDTA, 0.1 mM of NADPH and 1 mM of GSSG. For measuring GPX (EC 1.11.1.9) activity the reaction mixture had 500 μ L of 50 mM K-PO₄ buffer pH 7.0 containing 2 mM Na₂EDTA, 100 μ L of 0.28 μ M NADPH, 100 μ L of 0.13 μ M GSH, 40 μ L of 0.16 U GR and 100 μ L of 0.073 mM *t*-butyl hydroperoxide. Oxidation of NADPH was monitored at 340 nm (Hopkins and Tudhope 1973). Glutathione-S-transferase (GST; EC 2.5.1.18) assay was performed in a reaction mixture containing 500 μ L of 100 mM K-PO₄ buffer pH 6.2, 100 μ L of 0.75 mM CDNB and 50 μ L of 30 mM GSH. The increase in absorbance was monitored at 340 nm (Habig et al. 1974). The activities were expressed as per mg protein.

In-gel assays of antioxidant enzymes

For *in-gel* detection of antioxidant enzymes, extracts equivalent to 75 μ g protein were applied to native PAGE using 7.5 % separation gels according to Laemmli (1970). The gels were stained as follows: for CAT gel was incubated with 3.27 mM

H₂O₂ for 25 min, rinsed with water and shaken in freshly prepared solutions each of 1 % FeCl₃ and K₃Fe(CN)₆ until the achromatic bands on a prussian blue background appeared (Lee and Shin 2003); for POX the gel was pre-incubated with 50 mM Na-PO₄ buffer pH 7.0 for 15 min followed by an additional incubation with 4 mM H₂O₂ plus 20 mM pyragallol until dark brown bands appeared (Mittler and Zilinskas 1993); for SOD the gel was immersed in 50 mM Tris–HCl buffer pH 8.5 containing 10 mg methylthiazolyl tetrazolium, 6 mg phenazine methosulphate and 15 mg MgCl₂. The enzyme formed colorless zones on a dark blue background (Lee and Shin 2003). GPX activity was determined according to Das and Bagchi (2010). The gel was washed in sequence with 2.5 % Triton X-100 and distilled water for 15 min each. It was immersed in 10 mM K-PO₄ buffer pH 7.2 containing 2 mM *o*-dianisidine dihydrochloride for 1 h and shifted for 15 min to another solution containing the above buffer and 0.1 mM H₂O₂ until brown bands against pale yellow background appeared. GR activity was located by immersing the gel in 100 mL of 0.25 M Tris–HCl buffer pH 7.5 containing 0.24 mM MTT, 0.4 mM of NADPH, 10 mg of 2,6-dichlorophenolindophenol and 3.4 mM of GSSG. The gel was incubated in dark for 1 h to develop purple bands (Kang et al. 1999). For GST the gel was incubated for 10 min each in 0.1 MK-PO₄ buffer pH 6.5 and 0.1 MK-PO₄ buffer pH 6.5 containing 4.5 mM GSH, 1 mM CDNB and 1 mM NBT, followed by 0.1 M Tris–HCl buffer pH 9.6 containing 3 mM phenazine methosulphate. Achromatic zones were discernable against dark blue background (Ricci et al. 1984).

Two way-ANOVA was applied for testing statistical differences between treatments and between replicates with bromoxynil/NaCl and time as two fixed factors. Mean values were compared by using Tukey's HSD *post-hoc* test at threshold *p* value of 0.05 using Sigma Stat 5.1 software.

Two-dimensional gel electrophoresis (2-DE)

Total soluble proteins were extracted by adapting method of Bhargava et al. (2006). WT and Mu2 cells from 500 mL cultures (OD_{750 nm}=0.7–0.8), kept for 12 h without or with 0.15 mM bromoxynil and 0.55 M NaCl, were harvested, washed and dispersed in 3 mL extraction buffer 20 mM Tris–HCl pH 8.0; and then sonicated and centrifuged as above to obtain clear supernatants. The supernatant and 10 % TCA in acetone (1:3 v/v) were mixed and proteins precipitated overnight at 4 °C, centrifuged at 5,800 $\times$ g for 15 min at 4 °C, and washed at least three-times with chilled acetone. The pellet was air-dried and suspended in loading buffer containing 8 M urea, 2 % CHAPS, 1 % DL-dithiothreitol (DTT), 0.5 % (v/v) immobilized pH gradient (IPG) buffer (GE Healthcare Bio-Sciences Ltd, India) and 0.002 % bromophenol blue. The first dimension of IEF was performed in ETTan IPGphor 3 (GE Healthcare Bio-Sciences Ltd, India) at step gradients *viz.*

500 V for 1 h, 1,000 V for 1 h and at a linear gradient from 2,000 V to 8,000 V for 10 h. After IEF, the IPG gel strip was equilibrated twice, each time for 15 min in equilibration buffer containing 50 mM Tris–HCl buffer pH 8.8, 6 M urea, 30 % (v/v) glycerol and 2 % SDS in which during first equilibration 10 mg mL⁻¹ DTT, and in second, 40 mg mL⁻¹ iodoacetamide were added. Finally, the strips were rinsed briefly with de-ionized water. For second dimension, SDS-PAGE was carried out on 12 % gel at 50 V for 1 h and 100 V for 14 h (Laemmli 1970). The separated proteins then were visualized by Coomassie Brilliant Blue R250 staining and scanned using an image scanner (GE Healthcare Bio-Sciences Ltd, India). Protein spots of two independent experiments per treatment were analyzed for differential expression by Melanie software version7 (GeneBio, Switzerland). The differential expression of upregulated proteins between WT and Mu2 (bromoxynil and NaCl) were analyzed and significant differences were calculated by least significant difference (LSD) at $p < 0.05$. From three independent samples, protein spots were manually excised and sent to the Technoconcept India Pvt. Ltd. for MALDI-TOF/TOF analysis and homology search was carried out using MASCOT.

Results

In order to determine the relative susceptibility of WT and bentazone-resistant mutant, Mu2, towards bromoxynil, the cells were transferred to BG-11 medium with added bromoxynil (10–200 μ M). As shown in Fig. 1a, in terms of absolute growth between 4 and 0 d, 30 and 150 μ M herbicide inhibited growth of the respective strains by about 50 %. The results of the time-kinetics of growth indicate that in absence of bromoxynil (controls), growth of Mu2 was moderately (10–25 %) higher than WT, whereas upon adding 30 μ M bromoxynil to the cultures, WT continued to grow up to 2 days and thereafter ceased to grow, while Mu2 continued to grow normal (Fig. 1b, c). At higher concentration of 150 μ M, Mu2 initially grew like controls, but not beyond 2 days (Fig. 1c). When absolute growth of the two strains with addition of NaCl was compared, 0.35 M and 0.55 M NaCl inhibited WT and Mu2 growth by about 50 %, respectively (Fig. 1d). At 0.35 M, WT ceased to grow after 2 days, and even at higher concentration of 0.55 M WT could grow up to one-day albeit at lower rates than the

Fig. 1 a, d Absolute growth as ΔOD_{750nm} (0 $\rightarrow$ 4 day) of *S. elongatus* PCC7942 WT (solid bar) and Mu2 (open bar) as a function of increasing bromoxynil and NaCl concentrations, respectively, added to BG-11 medium. Time kinetics of growth of WT and Mu2, respectively, in b, c absence (filled square), or presence of 30 μ M (filled triangle) and 150 μ M (filled circle) bromoxynil, and e, f absence, or presence of 0.35 and 0.55 M NaCl in above order. The cells were

grown in BG-11 medium for 4 days, harvested and transferred at equal cell density corresponding to OD_{750nm} of 0.2 to medium containing or lacking graded concentrations of bromoxynil/NaCl. Bar represents mean $\pm$ SD of three independent experiments with triplicate samples each. Asterisk denotes significant difference from the controls at $p < 0.05$

controls; the corresponding growth rate in the mutant was nearly half that of controls (Fig. 1e, f).

The initial growth for 24 h between WT and Mu2 with either 0.55 M NaCl or 0.15 mM bromoxynil was not much different (*cf.* Fig. 1). We wanted to know, what could have been the difference in the acclimation process that enabled Mu2 to overcome the stress. As an indicator of oxidative damage, cellular concentrations of MDA were monitored. As shown in Table 1, incubation of WT for 12 h increased cellular contents of O_2^- , H_2O_2 and MDA by 4–6 fold. In comparison, in Mu2 the rise was negligible. From the above differences, it appeared that in the mutant, ROS produced from bromoxynil- or NaCl incubation were more efficiently detoxified.

To investigate the likely modifications in anti-oxidant enzymes, the activities of CAT, POX, SOD, GPX, GR and GST and cellular contents of GSH and GSSG were monitored under bromoxynil/NaCl incubations for 0-, 6- and 12-h. As shown in Fig. 2, WT growing in medium containing bromoxynil exhibited a time-dependent inactivation of GR and GST. In contrast, with added NaCl, except GPX, the other enzymes were inactivated by 25–50 %. The responses of Mu2 to added bromoxynil and NaCl was also different; with bromoxynil a significant rise (60–85 %) in the activities of POX, GST, GPX and GR was recorded, whereas with NaCl, along with POX, GPX and GR, SOD activity also showed an increasing trend, attaining after 12 h, 2.3-fold values. The cellular levels of GSH and GSSG were also monitored. It was found that in the mutant GSH level was significantly enhanced with added bromoxynil, whereas under all incubations GSSG levels were reduced.

The differences in the enzymatic activities were also revealed upon *in-gel* activity staining (Fig. 3). The assays clearly depicted a loss of band intensities of CAT, POX and GPX in WT indicating an inhibition of their activities by NaCl. Moreover, the GR and GST activity gels displayed four (I–IV) to five (I–V) bands respectively in controls, of which two bands of GR (I and II) and GST (II and III) were not observed under salt treatment. On the other hand upon NaCl treatment, despite SOD inactivation, two high

molecular weight bands, presumably of isozymes (SOD II; III), began to appear. Replacing salt with bromoxynil in WT resulted in appearance of an additional low molecular weight SOD form (SOD IV). Moreover, the effect on GR and GST was less pronounced, compared to NaCl, and only one band (GR I; GST III) was absent in bromoxynil treated cultures. Another significant difference was visualization of one/two high molecular weight isozymes of CAT and POX upon bromoxynil treatment. When compared with WT, Mu2 gels exhibited NaCl-promoted appearance of two high molecular weight SOD isozymes, and loss of GR II and GST III forms (Fig. 3). Visibly, bromoxynil did not impose significant change in the enzyme profiles in Mu2, except that one high molecular weight GPX (GPX II) appeared after 12 h.

To further identify the bromoxynil- and NaCl-“induced” proteins and to determine the changes in protein expression pattern, total soluble proteins of WT and Mu2 were resolved by 2-DE. From software analysis, a total of 214, 162 and 108 protein spots were detected in WT controls, WT 12 h bromoxynil and WT 12 h NaCl incubations, respectively (Fig. 4). The stress impact on total proteins was much less pronounced in Mu2, and the corresponding figures were 235, 223 and 193. Normalizing the data of three independent biological samples revealed that, at least two spots at *pI* 3.33; MW 15.5 kDa (termed *Bx-1*) and *pI* 4.9; MW 26.5 kDa (*Bx-2*) showed 1.8 to 2-fold higher amount in 12 h WT-bromoxynil samples, compared to zero-time. The corresponding NaCl-incubations led to an increased expression of two different spots, located at *pI* 3.95; MW 18 kDa (termed *N-1*) and *pI* 3.75; MW 27 kDa (*N-2*). Comparing WT and Mu2 control gels, spots *Bx-1* and *N-1* were observed at significantly higher amounts in Mu2 (4 to 5-fold; $p \leq 0.05$), and neither incubations increased the intensity further. There was, however, one additional spot of a protein discernable at *pI* 5.3; MW 36 kDa (*BxN-3*). The bromoxynil- and NaCl-induced proteins (*Bx-1*; *N-1*), commonly expressed in WT and Mu2 cells, were subjected to MALDI-TOF, followed by MASCOT analysis. From database search, *Bx-1* resembled bacterioferritin co-migratory protein (PRX-Q; sequence

Table 1 The effect of bromoxynil and NaCl incubations on cellular contents of H_2O_2 and MDA ($\mu\text{mol mg}^{-1}$ chl), and of O_2^- ($\mu\text{mol h}^{-1}$ mg^{-1} chl) in *S. elongatus* PCC7942 WT and mutant Mu2 strains

Parameter	WT-Bx (150 μM)			WT-NaCl (0.55 M)			Mu2-Bx (150 μM)			Mu2-NaCl (0.55 M)		
	0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h	0 h	6 h	12 h
H_2O_2 content	14 $\pm$ 2	55 $\pm$ 9 ^a	85 $\pm$ 13 ^a	25 $\pm$ 4	45 $\pm$ 7 ^a	84 $\pm$ 13 ^a	15 $\pm$ 2	25 $\pm$ 4 ^a	35 $\pm$ 5 ^a	28 $\pm$ 4	36 $\pm$ 5 ^a	47 $\pm$ 7 ^a
O_2^- content	11 $\pm$ 2	22 $\pm$ 3 ^a	44 $\pm$ 7 ^a	12 $\pm$ 9	38 $\pm$ 6 ^a	65 $\pm$ 10 ^a	18 $\pm$ 3	25 $\pm$ 4 ^a	27 $\pm$ 4 ^a	18 $\pm$ 3	15 $\pm$ 2	32 $\pm$ 5 ^a
MDA content	55 $\pm$ 8	166 $\pm$ 25 ^a	266 $\pm$ 40 ^a	23 $\pm$ 4	56 $\pm$ 8 ^a	126 $\pm$ 19 ^a	12 $\pm$ 2	25 $\pm$ 4 ^a	28 $\pm$ 4 ^a	28 $\pm$ 4	36 $\pm$ 5*	47 $\pm$ 7 ^a

Untreated cells in BG-11 medium (0 h) and those treated with 150 μM bromoxynil (Bx) and 0.55 M NaCl for 6 and 12 h were adjusted to 25–30 $\mu\text{g chl mL}^{-1}$ and used for measuring the metabolite contents

^a Denotes significantly different values from zero-time calculated separately for WT and Mu-2 at $p < 0.05$ by two-way ANOVA followed by Tukey's HSD *post hoc* test

Fig. 2 Response of bromoxynil (Bx) and NaCl incubations for 6–12 h and no treatments (0 h) on glutathione contents (GSH; GSSG), and on activities of antioxidant enzymes in *S. elongatus* PCC7942 WT and mutant Mu2 strains. Untreated cells in BG-11 medium (0 h) and those treated with 150 μM bromoxynil and 0.55 M NaCl for 6 and 12 h were adjusted to 25–30 μg chl mL⁻¹ and used for measuring the enzyme

activities and metabolite contents. The values are mean±SD of three independent experiments and duplicate samples each. Asterisk denotes significantly different values from zero-time calculated separately for WT and Mu2 at $p < 0.05$ by two-way ANOVA followed by Tukey's HSD *post hoc* test

coverage=62 %) and *N-1* matched with flavodoxin (sequence coverage=50 %), with the other details presented in

Table 2. The identities of the remaining proteins could not be established.

Fig. 3 In-gel activity staining of CAT, POX, SOD, GPX, GR and GST of *S. elongatus* PCC7942 WT and Mu2 upon bromoxynil and NaCl treatments after given time points showing several isoforms. The cells were incubated (cf. Fig. 2) and cell-free extracts prepared as in Materials and methods section. Extracts equivalent to 75 µg protein were resolved by native-PAGE and gels were stained. The isoforms are numbered in roman. Representative gels of two independent experiments are presented

Discussion

Collating the present and the previously reported data (Das and Bagchi 2011), it appears that acclimation mechanisms to bromoxynil- and bentazone in *S. elongatus* PCC7942 Mu2 are analogous—increased in GPX-GR activities and up-regulation of PRX-Q protein. The pathway could be that ROS, particularly peroxides, produced from incubating with herbicides were reduced by GPX, and GSSG was recycled to GSH by GR (Vassilakaki and Pflugmacher 2008). To support this assumption, we found that cellular contents of GSH that might be acting *per se* as non-enzymatic antioxidant or as GPX substrate, increased proportionate to GPX-GR. Bromoxynil showed no effect on CAT activity, though changed the isozyme patterns in WT, suggesting that *de novo* synthesis of CAT need not necessarily contribute to the enzyme activity. The same explanation holds true for SOD. Further, it appears from the activity rise that SOD and POX participate in quenching bromoxynil-derived ROS in WT. But as the activities also increased at a similar rate and to the same extent in Mu2, it can be assumed that there is no added advantage that the mutant is able to derive from these enzymes to grow under bromoxynil. Synthesis of a new GPX-II isoform and a rise in GST activity seemingly are the attributes exclusively promoted by bromoxynil. It can be assumed that GPX-II may have a direct role in nullifying the bromoxynil toxicity. The GST is associated with phase-II detoxification of non-target-site

herbicides (Powles and Yu 2010), and might be participating in bromoxynil metabolism in the mutant.

From our results, in *S. elongatus*, NaCl adaptation mechanisms are different from that of bromoxynil. An overall inactivation of WT antioxidant enzymes and parallel disappearance of the isoforms, *viz.* GR I, II and GST II and III, could be on account of ROS and/or hyper-osmotic (ionic and water) stress (Hagemann 2011). Conversely, in the mutant, except GR II and GST III, the other isoforms continued to show up under salt. In fact, most of the antioxidant enzymes displayed strong salt resistance and increase in activities, suggesting a possibility of their participation in alleviating oxidative stress arising from salt incubation. Salinity also caused enhancement of GPX and GR activities, yet we believe that these antioxidant enzymes have redundant role in salt acclimation, as the level of GSH substrate did not proportionately increase. We rather assume that SOD and POX, whose activity steadily increased in the mutant, as opposed to WT trend, may preferentially bring down the salt stress.

The results of 2-DE analysis in variously incubated Mu2 cells revealed differences in expression of total proteins. With added bromoxynil most of the proteins remained intact, whereas NaCl caused over 20 % decay; this was observed when both inhibited 50 % of growth. Relatively, WT exhibited higher sensitivity towards bromoxynil and NaCl, as the loss in total proteins was much greater. Notwithstanding, some stress-responsive proteins were found to be over-expressed in WT

Fig. 4 Two-DE images of the total soluble proteins of *S. elongatus* PCC7942 WT and Mu2 as a function of bromoxynil and NaCl incubations. Total soluble proteins (250 μ g) of untreated and variously treated cells at indicated time points (*cf.* Fig. 2) were separated by IEF and SDS-PAGE, and stained by Coomassie Brilliant Blue. Representative images

of two independent experiments are shown. From software analysis, the protein spots, whose intensity (volume) over controls increased in the respective treatments, is highlighted as being exclusive for bromoxynil- (Bx-) and NaCl- (N-) or present in common (BxN-). Note the highlighted spots are different between the strains and the treatments

and Mu2. Among them, bacterioferritin co-migratory protein homologue PRX-Q was unequivocally identified in WT under bromoxynil but not salt treatment. Conversely, flavodoxin, which was present in WT growing under regular conditions, was overexpressed upon NaCl treatment. The level of the proteins were already high in Mu2, suggesting their role in the organism's ability to withstand bromoxynil and salt stress. PRX-Q is reported to be responsible for quenching ROS arising from photosynthesis perturbations, such as from herbicide action (Tripathi et al. 2009). Flavodoxin (IsiB), on the other hand,

was shown to be up-regulated in *S. elongatus* under iron stress (Michel et al. 2003). Moreover, using immunoblotting methods, we have shown constitutive expression of IsiB in Mu2 under salt stress (Bagchi et al. 2007). We have also shown that PsaA, PsaB, FNR and AtpA were up-regulated in Mu2 (Bagchi et al. 2007). Together with this feature, NaCl-induced up-regulation of flavodoxin may enhance the photosynthetic energization capabilities at the level of PS I-cyclic electron flow. It has been shown that membrane energization has a vital role to play in alleviating salt stress (Hagemann et al. 1999).

Table 2 Identification of bromoxynil- (Bx-) and NaCl- (N-) induced proteins of *S. elongatus* PCC7942 WT and mutant Mu2 strains resolved by 2-DE

Spot ^a	Homologous protein	Mascot score	Mr (kDa)	pI	Accession number
Bx-1	Bacterioferritin co-migratory protein (<i>Synechococcus elongatus</i>)	765	15.7	4.52	Q31L59
N-1	Flavodoxin (<i>Synechococcus elongatus</i>)	1,006	18.7	3.99	P10340

^a *cf.* Fig. 4

To sum up, while selected on bentazone, *S. elongatus* mutant Mu2 seems to acquire distinct defense strategies – enhancement of GPX-GR, PRX-Q and GST to cope with bromoxynil, and of flavodoxin, SOD and POX to acclimatize to salt. In this context, future research should be to identify multi-stress responsive “global” regulators, like *S. elongatus* PCC6301 non-coding RNA, Yfr1 (Nakamura et al. 2007), possibly selected in one mutational event. Finally, broadening the purview of PICT, we propose that while compromising with one chemical stressor, the dominant freshwater picophytoplankton, e.g., *Synechococcus* sp. (Callieri 2007), co-ordinates different adaptive strategies to tackle anthropogenic and environmental adversities.

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