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Toxic Effects of Imidazolium Ionic Liquids on the Green Seaweed *Ulva lactuca*: Oxidative Stress and DNA Damage

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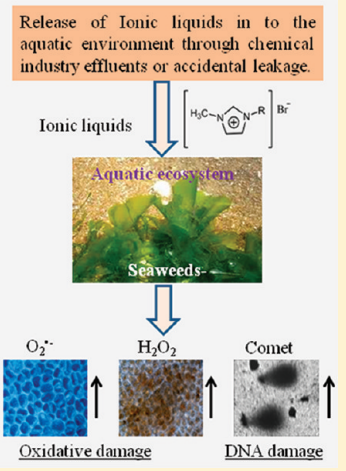
ABSTRACT: The green credentials of ionic liquids (ILs) are being increasingly questioned due to the growing evidence of their toxicity to aquatic ecosystems, although the mechanisms of toxicity are unknown. This study provides insights into the mechanism of toxicity and biological effects of 1-alkyl-3-methylimidazolium bromide [$C_n$mim]Br (n = 4 to 16) on the marine macroalga *Ulva lactuca*. The cell viability of this alga during IL exposure was found to be negatively correlated to the chain length of the alkyl group. The IL ([C_{12}mim]Br) exposure triggers the generation of reactive oxygen species (ROS viz. $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$), damage of the membrane and DNA, and inhibition of antioxidant systems in the alga. The enhanced production of ROS and lipid peroxidation in the alga subjected to LC_{50} concentration for 4 days was largely attributed to lipoxygenase (LOX) activity coupled with the induction of two LOX isoforms (~80 kDa and ~55 kDa). Pretreatment of the algal thallus with enzyme inhibitors such as diphenylene iodonium, sodium azide, cantharidin, and oxadiazoloquinoxalin-1-one, prior to [C_{12}mim]Br exposure showed the regulation of ROS by the activation of membrane bound NADPH-oxidase and cytochrome oxidase. The IL exposure resulted in the accumulation of n-3 and n-6 fatty acids at 0.5 LC_{50} concentration indicating the induction of desaturase enzymes. Furthermore, antioxidant enzyme activities such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR) were enhanced by 1.3−2.0-fold, while glutathione peroxidase (GSH-Px) diminished, together with a higher regeneration rate of reduced ascorbate and glutathione. The isoforms of antioxidant enzymes, namely, Mn-SOD (~85 kDa), APX (~125 and 45 kDa), and GR (~135 kDa) regulated differentially to IL exposure. The comet assay performed for the first time for seaweeds revealed the significant induction of DNA damage (>50−70% increase in % tail DNA over control) in alga exposed to ≥ LC_{50} concentration.



INTRODUCTION

Ionic liquids (ILs) are a group of molten salts consisting of asymmetrically substituted nitrogen or phosphorus cations together with an organic or inorganic anion moiety. The unique physiochemical properties of ILs, i.e., negligible vapor pressure, high thermal, chemical, and electrochemical stabilities and excellent solvent properties, are attributed to their functionality as green solvents.[1] Nevertheless, the green credentials of these ILs have been questioned for use in aquatic environments as the water solubility of majority of ILs is significant.[2] Since, the substitution of ILs for conventional solvents in a variety of chemical industries is on the rise, there is a possibility that ILs may find their way into water bodies. The release of ILs into aquatic environments through effluent discharge or accidental spillage could result in water pollution due to their poor biodegradability thereby negatively impacting the structure and function of the aquatic ecosystem.[3,4]

The 1-alkyl-3-methylimidazolium salts are some of the most common ILs used in industrial applications[5] and are reported to have poor biodegradability except for octyl imidazolium and other ILs.[4] Recently, there has been considerable effort to determine the toxicity of ILs on both terrestrial and aquatic organisms (see ref 2). The toxicological studies of ILs investigated so far have demonstrated that ILs cause membrane damage and disruption, coupled with increased generation of cellular reactive oxygen species (ROS) manifesting the inhibition of photosynthesis leading to oxidative stress.[2,6] However, the source for ROS origin and the precise mechanisms of IL induced toxicity have largely remained unexplored. The evidence of ILs toxicity necessitated the need for undertaking a detailed investigation addressing the effects of these compounds at various biological levels of organization.[3,6−13] However, to date, there is no study investigating the toxic effects of ILs on seaweeds, which are considered the main primary producers in coastal waters. These being an abundant component in coastal waters are recognized as alternate promising sources for biofuel and chemicals with the potential to reduce greenhouse gas emissions and global warming.[14] Any accidental release of ILs into an aquatic ecosystem may cause grave environmental concerns by perturbing the food-web dynamics and ecosystem structure and function. Seaweeds being of marine origin, the ILs could have different effects according to the salinity of the environment.[15] Therefore, IL toxicity mechanism based studies are of importance to mitigate the impact of IL mediated water pollution.

In this study, for the first time we report the toxicity and biological effects of ILs belonging to 1-alkyl-3-methylimidazolium bromide

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salts with different alkyl-chain lengths on the green seaweed *Ulva lactuca*. This alga was selected as it has a worldwide distribution with the potential to adapt and grow in diverse adverse environmental conditions.[16] We first determined the LC_{50} values of these ILs and their effect on ROS generation. Later, the regulation of enzymatic and nonenzymatic antioxidant systems, free fatty acids, damage to cell membranes and DNA, and the effects of different ROS scavengers were studied to elucidate the mechanism of IL toxicity.

MATERIALS AND METHODS

Test Organism and Treatment of ILs. The vegetative thalli of *U. lactuca* were collected from the intertidal region of the Veraval Coast (20° 54′ N, 70° 22′ E), Gujarat, India. Selected, clean juvenile thalli were brought to the laboratory in a cool pack. In order to initiate unialgal culture, the thalli were cleaned manually with a brush in autoclaved seawater to remove epiphytes and any superficial foreign matter. The cleaned thalli were acclimatized to laboratory conditions by culturing in aerated flat bottom, Erlenmeyer flasks in filtered natural seawater (NSW) of 30 psu salinity supplemented with provasoli enrichment seawater medium[17] and GeO_2 (5 mg L^{-1}) for 10 days. During the acclimatization period, the medium was replenished on alternate days and maintained under cool white fluorescent tubes at an irradiance of 50 μmol photons m^{-2} s^{-1} with a 12:12 h light/dark cycle at 22 ± 1 °C. For treatment, the acclimatized algal fragments were incubated in filtered NSW having 30 psu salinity with and without (control) ILs in aerated cultures.

The ILs used in this study included a series of 1-alkyl-3-methylimidazolium bromide ([$C_n$mim]Br) salts with alkyl chains which ranged from n = 4 to16, which were synthesized in the laboratory, as described in the literature.[18] The purities of ionic liquids were established to be >99% through proton nuclear magnetic resonance (^{1}HNMR) spectroscopy by integration of proton signals with respect to an internal standard of tetramethyl silane, and the NMR spectra were in agreement with the literature data. The water content of the ionic liquids was determined using a coulometric Karl Fischer autotitrator (Mettler Toledo DL39) with Combi-Coulomat fritless Karl Fischer reagent (Merck) and agreed within 120−300 ppm range. The stock solutions of ILs (15 mM) were prepared in distilled water and were found to be completely soluble. For treatment, the algal fragments were exposed to the tested concentrations while dissolving the required amount from the stock solutions in NSW having 30 psu salinity.

Cell Viability Assay. A 2,3,5-triphenyltetrazolium chloride (TTC) based method was used to determine the cell viability of the control and IL treated algal fragments according to Shiu and Lee.[19] The TTC cell viability assay is based on the tetrazolium salt reduction to formazan by dehydrogenase respiratory enzymes and thus indicates the resilience of the mitochondrial component of the cell machinery when challenged with IL-induced stress. After treatment, the algal fragments (50 mg fresh weight) were incubated at 25 °C in 3 mL of NSW containing potassium phosphate buffer (50 mM, pH 7.4) and 0.8% (w/v) TTC in darkness for 16 h. Thereafter, the algal fragments were washed three times in NSW, and the intracellular insoluble red formazon was extracted twice with 5 mL of 95% ethanol at 80 °C for 20 min. Extracts were combined, and the absorbance was determined at A_{530}. The cellular viability for IL treated thalli was calculated as a percentage, while considering the absorbance of the control algal fragments with 100% cell viability. The viability percentages were plotted against the concentration tested for each IL to obtain a linear regression and for estimating the LC_{50}. Each experiment was carried out in triplicate, and the mean values of the LC_{50} were calculated.

Determination, *In Situ* Localization of ROS, and Total Chlorophyll Estimation. The $O_2^{\bullet-}$ production rate was measured according to Liu et al.[20] Samples (500 mg fresh weight) were homogenized in 4 mL of 65 mM potassium phosphate buffer (pH 7.8) and centrifuged at 5,000*g* for 10 min. The incubation mixture contained 0.9 mL of 65 mM potassium phosphate buffer (pH 7.8), 0.1 mL of 10 mM hydroxylaminoniumchloride, and 1 mL of the supernatant. After incubation at 25 °C for 20 min, 17 mM sulphanilic acid and 7 mM α-naphthyl amine were added to the incubation mixture. After reacting at 25 °C for a further 20 min, the absorbance was read at 530 nm. A standard curve, with $NaNO_2$ was used to calculate the production rate of $O_2^{\bullet-}$. For the estimation of H_2O_2, 100 mg fresh samples were extracted in 200 μL of Na-acetate buffer (50 mM, pH 6.5) and incubated in reaction media containing 50 mM Na-acetate buffer, 1 mM 4-aminoantipyrine, 1 mM 2, 4-dichlorophenol, 50 mM $MnCl_2$, and 0.2 mM NADH for 24 h. The oxidation of aminoantipyrine was recorded at 510 nm, and the absorbance was compared to the standard curve prepared with H_2O_2 in the same reaction mixture. Determination of $OH^{\bullet}$ production was performed based on the degradation of 2-deoxyribose by $OH^{\bullet}$ radicals.[20] The $O_2^{\bullet-}$ and H_2O_2 were visually detected in the thalli using nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB) as the substrates respectively, according to Kumar et al.[21] Total chlorophyll (chl a+b) was extracted in 80% acetone and estimated in accordance with Kumar et al.[22]

Effect of ROS Inhibitors. To study the effect of inhibitors of ROS generation, the algal fragments were preincubated in NSW containing diphenylene iodonium (DPI, NADPH oxidase inhibitor, 10 μM), sodium azide (NaN_3, peroxidase inhibitor, 1 mM), cantharidin (CANT, protein phosphatase inhibitor, 10 μM), and oxadiazolo-quinoxalin-1-one (ODQ, guanylate cyclase inhibitor, 0.2 mM) for 24 h and then cultured in NSW supplemented with a lethal dose of [C_{12}mim]Br IL for 4 days. Thereafter, the thalli were washed with NSW twice and processed for ROS localization using NBT or DAB solutions.

Protoplast Isolation and Comet Assay. Protoplast isolation and purification were performed according to Gupta et al.[23] Briefly, following IL treatment and washing, the algal fragments (300 mg FW) were chopped into small pieces of tissue (≤1 mm thin) in natural seawater (NSW) in two replicates, each with 150 mg of tissue. The chopped tissues were then rinsed several times with NSW to remove debris. The tissues thus cleaned were incubated in an enzyme solution consisting of 0.5% dextron sulfate, 2% cellulase, 2% NaCl, and 0.8% mannitol in 50 mM MES buffer (pH 6.0) for 4−5 h on a rotary shaker (50 rpm) in the dark at 25 °C. The protoplasts were passed through a miracloth (Calbiochem Co., USA) and centrifuged at 300*g* for 10 min. The precipitates were suspended in saline phosphate buffer (PBS), at pH 7.4, and kept on ice at 4 °C. The protoplast yields were estimated by counting the cells using a hemocytometer under an inverted microscope.

For the comet assay, the protoplast solution (50 μL) containing approximately 1 × 10^3 protoplasts was mixed with 50 μL of 1% low-melting temperature agarose (LMPA) dissolved in phosphate-buffered saline. An 80 μL aliquot of the solution was layered onto a base slide, which was precoated with 1% agarose, and then covered with a coverslip. When the agarose gel solidified, the coverslip was gently slid off, and another agarose layer (90 μL, 0.5% LMPA) was layered while covering it with a new coverslip and then left for 10 min on a chilled metal plate in order for solidification of the agarose layer to occur. Following this, the coverslip was removed, and the slides were submerged in alkali lysis solution (2.5 M NaCl, 10 mM Trizma, and 100 mM EDTA) at pH > 13 overnight at 4 °C. Thereafter, the glass slide was incubated in fresh, cold electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, pH 13) in an horizontal electrophoresis tank (Model POWER PAC 200, Bio-Rad Co., USA) for 30 min at room temperature to allow for DNA unwinding. Electrophoresis was performed for 10 min at 25 V and 300 mA in a chamber cooled in an ice bath. After electrophoresis, the glass slides were neutralized in 0.4 M Tris−HCl (pH 7.5) buffer, washed twice in distilled water, and left overnight for drying at room temperature. The slides were stained following the silver staining method.[24] Stained slides were examined using an Olympus Microscope model-BX60 fitted with an Olympus-DP72 camera. The classification of comet category and their tail measurements were carried out according to Garcia et al.[25]

Table 1. LC_{50} Values for Seven Types of Imidazolium Ionic Liquids on *Ulva lactuca*

ILs	regression equation[a]	LC_{50} (mM) mean (n = 3) ± SD
[C_4mim]Br	(1) $Y = -0.0042x + 93.53$	9.80 ± 0.51
	(2) $Y = -0.0043x + 90.32$	
	(3) $Y = -0.0042x + 90.58$	
[C_6mim]Br	(1) $Y = -0.0415x + 104.25$	3.71 ± 0.37
	(2) $Y = -0.0136x + 99.91$	
	(3) $Y = -0.0124x + 96.18$	
[C_8mim]Br	(1) $Y = -0.0361x + 102.33$	1.42 ± 0.22
	(2) $Y = -0.0324x + 95.67$	
	(3) $Y = -0.0321x + 96.32$	
[C_{10}mim]Br	(1) $Y = -0.1957x + 78.82$	0.15 ± 0.015
	(2) $Y = -0.1654x + 74.12$	
	(3) $Y = -0.1414x + 70.39$	
[C_{12}mim]Br	(1) $Y = -0.3357x + 76.28$	0.07 ± 0.006
	(2) $Y = -0.2532x + 69.76$	
	(3) $Y = -0.2862x + 69.08$	
[C_{14}mim]Br	(1) $Y = -0.5115x + 75.31$	0.04 ± 0.001
	(2) $Y = -0.6233x + 80.67$	
	(3) $Y = -0.5572x + 76.38$	
[C_{16}mim]Br	(1) $Y = -0.7352x + 64.01$	0.02 ± 0.001
	(2) $Y = -0.7673x + 64.64$	
	(3) $Y = -0.9099x + 66.31$	

[a] Y stands for probit of percent inhibition, and x stands for natural logarithm of the concentration of ionic liquid.

Analysis of Fatty Acids and Determination of Enzymatic and Nonenzymatic Antioxidants. Fatty acids from lipids were converted to the respective methyl esters and analyzed by GC-2010 coupled with GCMS-QP2010.[21] The water-soluble antioxidants such as glutathione (oxidized glutathione, GSSG; reduced glutathione, GSH) and ascorbate (oxidized ascorbate, DHA; reduced ascorbate, AsA) were determined as described by Wu and Lee.[26] The activities of antioxidant enzymes, namely, superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and glutathione peroxidase (GSH-Px) were determined by the method of Wu and Lee[26] and lipoxygenase (LOX) by the method of Kumar et al.[22] The isoenzyme profile of antioxidative enzymes (SOD, APX, GR, and GSH-Px) and LOX and their molecular mass were determined with 10 or 12% nondenaturing polyacrylamide gels using their specific activity staining procedures.[22]

Data Analysis. Results are expressed as the mean of three replicates with standard deviation. Statistical analyses were performed by one way analysis of variance (ANOVA). Significant differences among the mean values were determined by the least significant difference (LSD) at $p < 0.05$.

RESULTS

LC_{50} Values. The LC_{50} values for seven types of 1-alkyl-3-methylimidazolium bromide ILs on *U. lactuca* exposed for 4 days are presented in Table 1. The results demonstrated that the LC_{50} value decreased with the increasing alkyl-chain length and duration of exposure. It was observed that the ILs [C_{14}mim]Br and [C_{16}mim]Br were highly toxic to alga even at LC_{50} values and showed complete bleaching of cells indicating the death of the tissue fragment within 36 h as inferred from the TTC cell viability assay. However, the fragments exposed to ILs-[$C_n$mim]Br (n = 12) were quite healthy and showed symptoms of bleaching only after 4 days at LC_{50} value, while at 2× LC_{50} concentration, they exerted significant damage to the cell membrane followed by bleaching at 4 days and eventually death after 5 days of exposure, as confirmed by the TTC cell viability assay. Therefore, in the subsequent experiment the thallus fragments were treated with [C_{12}mim]Br for 0.5 LC_{50}, LC_{50}, and 2× LC_{50} concentrations for a duration of 4 days only in order to examine the effects on ROS generation, lipo-peroxidation of cell membranes (while measuring the MDA level by TBARS activity assay), and DNA (assessed by COMET assay). However, to study the regulation of the antioxidant system and free fatty acid contents the exposure doses were restricted to 0.5 LC_{50} and LC_{50} only because at 2× LC_{50}, excessive ROS generation and maximum DNA damage was observed, which in turn reflected the total failure of the defense system of the cell and consequently the death of cells exposed to IL at 5 days.

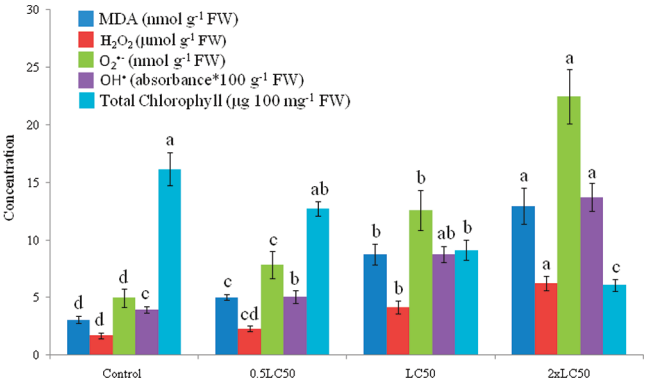


Figure 1. Effect of ionic liquid [C_{12}mim]Br exposure at concentrations ranging from 0.5 LC_{50}, LC_{50}, 2× LC_{50}, on reactive oxygen species ($O_2^{\bullet-}$, $OH^{\bullet}$, and H_2O_2) generation, lipid peroxidation (MDA level), and total chlorophyll (chl a+b) in *Ulva lactuca*.

Generation, Histochemical Localization of ROS, and the Effect of Their Inhibitors. The oxidative damage caused by [C_{12}mim]Br IL was determined by measuring the lipid peroxidation (MDA level) and ROS (including $O_2^{\bullet-}$, $OH^{\bullet}$, and H_2O_2) content in *Ulva* (Figure 1). IL exposure to concentrations 0.5 LC_{50}, LC_{50}, and 2× LC_{50} for 4 days inevitably induced oxidative stress with a significant increase in membrane lipid peroxidation by 1.63-, 2.85-, and 4.20-fold, respectively, over the control (3.08 nmol g^{-1} FW). Following the exposure to IL, the ROS level was also elevated to surprisingly high levels in plants by 1.25−5.25-fold, and accumulation was more evident in plants treated with the sublethal and lethal doses of [C_{12}mim]Br IL. The enhanced level of oxidative stress biomarkers resulted in a significant decrease in total chlorophyll content by almost 21% (0.5 LC_{50}), 43% (LC_{50}), and 62% (2× LC_{50}), respectively, when compared with that of the control (16.16 $\mu g \cdot 100$ g^{-1} FW) (Figure 1). Histochemical staining (Figure 2A and B) employed for *in situ* localization of $O_2^{\bullet-}$ and H_2O_2 radicals made apparent the distribution of reduced NBT dependent dense blue formazone and H_2O_2 dependent brown precipitate, respectively, all over the tissue in plants exposed to 0.5 LC_{50}, LC_{50}, and 2× LC_{50} concentrations of [C_{12}mim]Br IL. The accumulations of blue formazone and brown precipitate were more evident with increasing concentration of IL. The effect of different ROS scavengers was tested in thalli pretreated with diphenylene iodonium (DPI), sodium azide (NaN_3), cantharidin (CANT), and oxadiazoloquinoxalin-1-one (ODQ) and later exposed to the [C_{12}mim]Br IL (2× LC_{50}). Both the blue formazone and brown precipitate

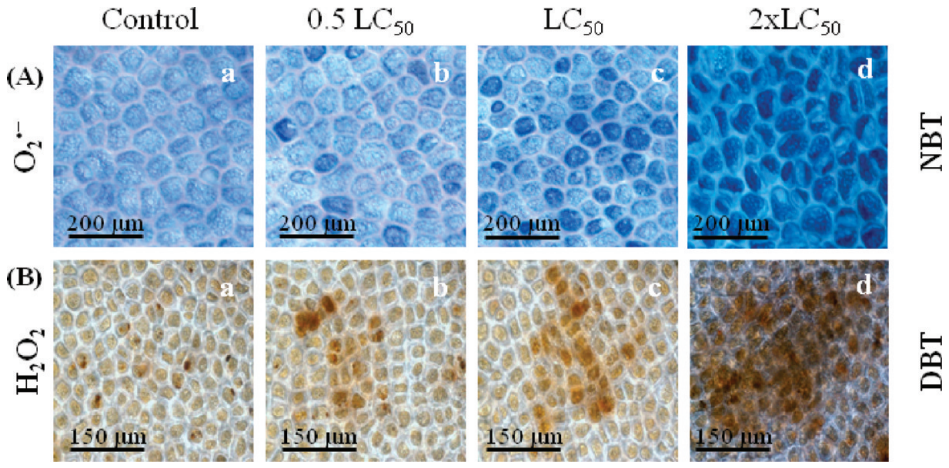


Figure 2. Reactive oxygen species generation in *Ulva lactuca* following exposure to the ionic liquid [C_{12}mim]Br for 4 days. (A) $O_2^{\bullet-}$ and (B) H_2O_2 radicals were detected with nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB) staining, respectively. a−d inside figures represent the various treatments, viz. control, 0.5 LC_{50}, LC_{50}, and 2× LC_{50}, respectively.

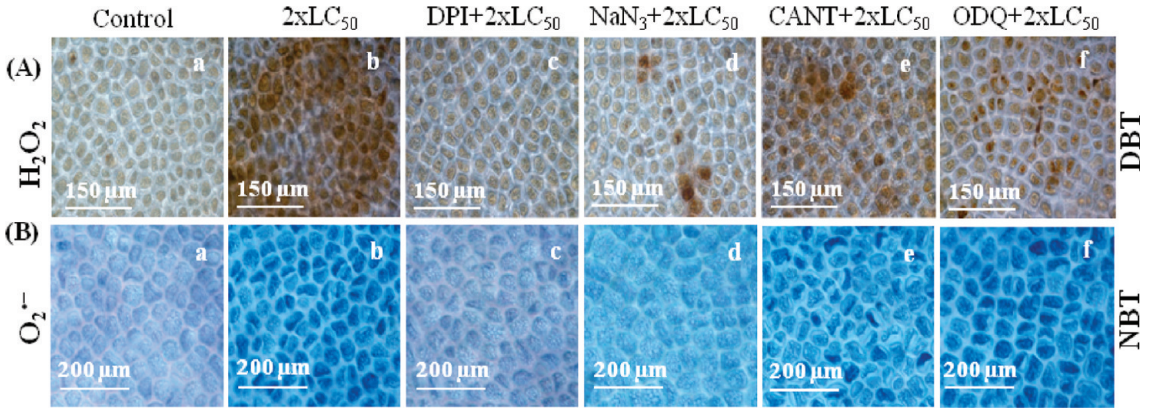


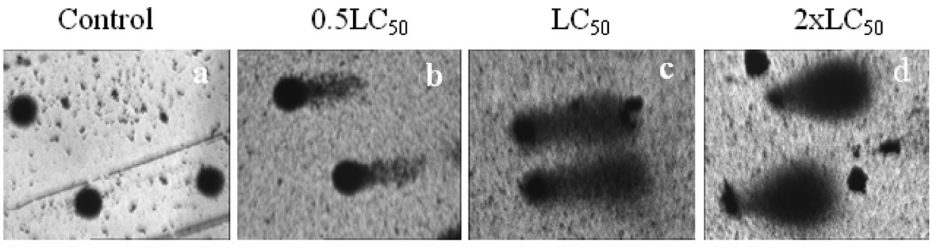
Figure 3. Effect of pretreatment by inhibitors (e.g., DPI, NaN_3, CANT, and ODQ) on the production of reactive oxygen species in *Ulva lactuca* following exposure to the ionic liquid [C_{12}mim]Br at 2× LC_{50} concentration. (A) H_2O_2 and (B) $O_2^{\bullet-}$ radicals were detected with 3,3-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining, respectively.

formed due to the generation of $O_2^{\bullet-}$ and H_2O_2 radicals, respectively, were almost abolished with DPI and NaN_3 treatment (Figure 3A and B) and also reduced partially in plants pretreated with CANT and ODQ when compared with the plants treated with 2× LC_{50} concentration.

DNA Damage. From the Comet assay, it was clearly evident that the ionic liquid induced DNA damage responded in a dose-dependent manner (Figure 4). Following exposure to ionic liquid [C_{12}mim]Br, the number of comets belonging to categories 3 (high damage, 45−70% of DNA in tail) and 4 (very high damage, >70% of DNA in tail) increased significantly ($p < 0.01$) in plants treated with LC_{50} and 2× LC_{50} concentrations, respectively. However, the plants treated with the 0.5 LC_{50} concentration showed significantly less DNA damage with the majority of the comets falling into categories 1 (low damage, 5−25% of DNA in tail) and 2 (medium damage, 25−45% of DNA in tail).

Fatty Acid, Enzymatic, and Nonenzymatic Antioxidants. Table 2 summarizes the variation in fatty acid composition of *U. lactuca* in response to IL-induced oxidative stress in terms of the percentage of total fatty acids (%TFA). Plants exposed to the IL (LC_{50}) exhibited a significant decrease ($p < 0.05$) in n-3 and n-6 polyunsaturated fatty acids (PUFAs), with a concomitant increase in saturated fatty acids (SFAs). Following the IL (LC_{50}) exposure, the content of SFAs, namely, palmitic, stearic, and behenic acids increased by 60−70%, when compared to their corresponding values of 24.42%, 3.05%, and 1.67% TFA in the control, while the content of most of the PUFAs under this treatment decreased considerably by ∼50%. On the contrary, the contents of both SFA and PUFAs in plants cultured with the supplementation of IL (0.5 LC_{50}) were found to be either similar or marginally higher than that of their contents in control, with the exception of palmitic acid (which increased by 30%) and docosahexaenoic acid contents (which decreased by 30%).

Superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and glutathione peroxidase (GSH-Px) were selected as biomarkers to determine the oxidative stress caused by the IL on the enzymatic antioxidant defense system of *Ulva*. Exposure to IL (0.5 LC_{50}) significantly enhances the enzyme activities of SOD, APX, and GR by 1.3−1.8-fold ($p < 0.01$) over the control activities with 87.45, 0.44, and 2.63 U mg^{-1} protein, respectively (Figure 5A). Exposure to even higher concentration (LC_{50}) decreased the enzyme activities. It is noteworthy that the enzyme activity of GR, contrary to GSH-Px, was quite indifferent



Comet category	%DNA in Tail	Tail length (μM)	Number of comets			
			Control	$0.5LC_{50}$	LC_{50}	$2xLC_{50}$
0	0-5	4.2-8.4	48	16	10	1
1	5-25	8.5-24.8	9	15	4	-
2	25-45	25.0-38.7	3	29	3	3
3	45-70	39.0-55.8	-	-	37	7
4	>70	56.0-96.3	-	-	6	49

Figure 4. Comets showing tails of different length induced by various concentrations of ionic liquid [C_{12}mim]Br exposure in *Ulva lactuca*: (a) control; (b) 0.5 LC_{50}; (c) LC_{50}; and (d) 2× LC_{50}. A total of 60 comets were examined for each treatment with two replicates.

Table 2. Effect of [C_{12}mim]Br Ionic Liquid on Fatty Acid Composition (% of Total Fatty Acids) in *Ulva lactuca* (Mean of Three Independent Experiments ± SD)

fatty acids	common name	control	0.5 LC_{50}	LC_{50}
C 12:0	lauric acid	0.71 ± 0.09 a	nd	0.48 ± 0.06 b
C 14:0	myristic acid	2.81 ± 0.19 a	2.21 ± 0.21 b	1.63 ± 0.02 c
C 15:0	pentadecanoic acid	0.92 ± 0.05 a	0.37 ± 0.06 c	0.54 ± 0.05 b
C 16:0	palmitic	24.42 ± 1.21 c	31.98 ± 2.25 b	40.51 ± 0.72 a
C 17:0	heptadecenoic acid	0.24 ± 0.00 a	0.17 ± 0.02 b	0.10 ± 0.00 c
C 18:0	stearic acid	3.05 ± 0.17 b	2.83 ± 0.19 b	4.82 ± 0.11 a
C 20:0	arachidic acid	0.15 ± 0.01 c	0.20 ± 0.02 b	0.35 ± 0.03 a
C 22:0	behenic acid	1.67 ± 0.01 b	1.80 ± 0.17 b	2.87 ± 0.16 a
C 24:0	lignoceric acid	0.27 ± 0.00[ab]	0.15 ± 0.01 b	0.30 ± 0.04 a
C 16:1(n-7)	9-hexadecenoic acid	1.51 ± 0.13 a	1.16 ± 0.16 c	1.23 ± 0.03 bc
C 16:1(n-9)	7-hexadecenoic acid	5.23 ± 0.62 a	4.01 ± 0.28 b	2.14 ± 0.21 c
C 17:1(n-7)	heptadecenoic acid	0.71 ± 0.07 a	0.54 ± 0.09 b	0.79 ± 0.09 a
C 18:1(n-9)	oleic acid	11.15 ± 0.29 a	11.94 ± 1.11 a	8.94 ± 0.54 b
C 18:2(n-6)	linoleic acid	6.72 ± 0.64 a	5.95 ± 0.54 b	5.06 ± 0.11 c
C 18:3(n-6)	γ-linolenic acid	1.10 ± 0.11 a	0.97 ± 0.10 b	0.50 ± 0.01 c
C 18:3(n-3)	α-linolenic acid	19.08 ± 0.73 b	21.40 ± 0.74 a	14.08 ± 0.67 c
C 18:4(n-3)	stearidonic acid	16.01 ± 0.88 a	13.49 ± 1.06 b	10.29 ± 0.45 c
C 20:3(n-6)	dihomo- γ-linolenic acid	0.18 ± 0.01 a	0.15 ± 0.01 b	0.14 ± 0.01 b
C 20:4(n-6)	arachidonic acid	0.84 ± 0.05 b	0.96 ± 0.06 a	0.56 ± 0.03 c
C 20:5(n-3)	eicosapentaenoic acid	0.85 ± 0.07 b	1.17 ± 0.15 a	0.42 ± 0.05 c
C 22:6(n-3)	docosahexaenoic	2.40 ± 0.14 a	1.68 ± 0.23 b	1.15 ± 0.05 c
ΣUFA/SFA[a]		1.92 ± 0.18 a	1.60 ± 0.14 b	0.89 ± 0.12 c

[a] UFA and SFA represent unsaturated and saturated fatty acids, respectively.

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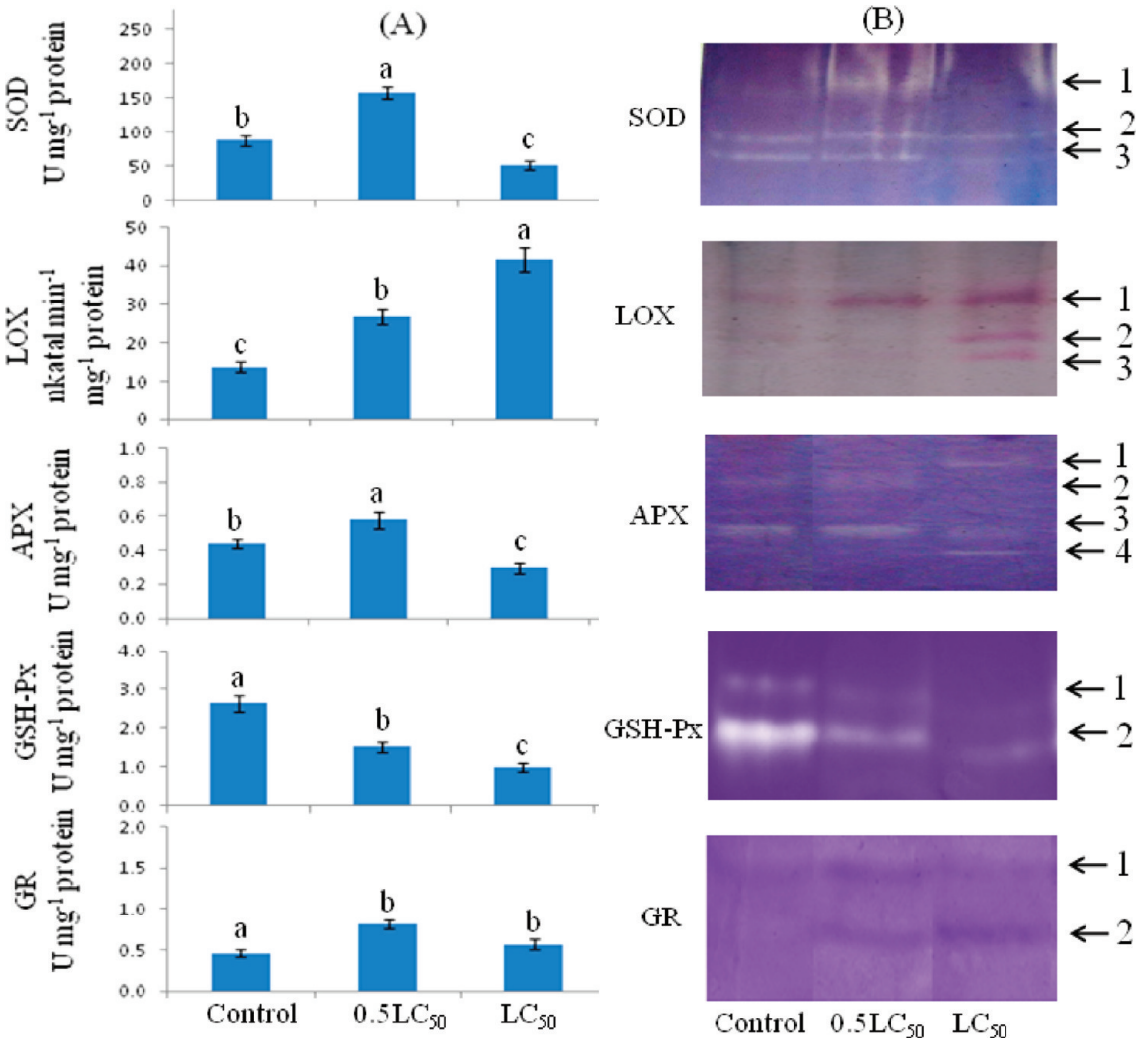

Figure 5. Effect of ionic liquid [C_{12}mim]Br exposure on (A) the activity of antioxidative enzymes (U mg^{-1} protein) and (B) their isoforms in *Ulva lactuca* in various treatments, viz. control, 0.5 LC_{50}, and LC_{50}.

to IL toxicity and showed higher activity (25%) even at LC_{50} over the control activity (0.46 U mg^{-1} protein). Furthermore, the IL treatment markedly enhanced the lipoxygenase (LOX) activity by 1.9- and 3.0-fold ($p < 0.01$) in plants exposed to 0.5 LC_{50} and LC_{50} concentrations, respectively.

Native PAGE analysis supported the spectrophotometric measurements of enzymatic activities (Figure 5B). The apparent higher activity of SOD observed in 0.5 LC_{50} treatments were solely attributed to Mn-SOD (SOD-1, ~150 kDa), confirmed by using H_2O_2/KCN as inhibitors. In the control, one Fe-SOD and two Zn-SOD (SOD-3, ~20 kDa, and -4, ~35 kDa) were observed. Exposure to LC_{50} resulted in partial inhibition of Fe and Zn-SOD isoforms. The enzyme APX showed differential regulation in response to IL (LC_{50}) with induction of APX-1 (~125 kDa) and APX-4 (~45 kDa), partial inhibition of APX-3 (~65 kDa), and complete inhibition of APX-2 (~100 kDa). However, the control and IL (0.5 LC_{50})-treated fragments exhibited only two APX isoforms (i.e., APX-2 and -3). The activity gel of GSH-Px showed only two isoforms GSH-Px-1 (~80 kDa) and GSH-Px-2 (~55 kDa) in the control, while their intensity got diminished with increasing IL doses (Figure 5B). Two forms of GR (GR-1, ~180 kDa; GR-2, ~135 kDa) were visualized in a 10% activity staining gel at 0.5 LC_{50}, while only a single isoform (GR-1) was seen in the control. The exposure of IL (LC_{50}) markedly inhibited the activity of GR-1 with the induction of the GR-2 isoform. Further, IL (LC_{50})-treatment induced two new isoforms of LOX (i.e., LOX-2 and -3) with molecular weights of nearly ~80 kDa and ~55 kDa, respectively, in addition to an isoform with a molecular weight of ~125 kDa recorded in the control. The content of water-soluble antioxidants such as reduced ascorbate (AsA), oxidized ascorbate (DHA), reduced glutathione (GSH), and oxidized glutathione (GSSG) were greatly influenced by IL exposure (Table 3). The content of the reduced antioxidants increased significantly by 2−3-fold at 0.5 LC_{50} concentration over the control but decreased markedly at higher concentration (i.e., LC_{50}). To determine the regeneration of these antioxidants, the ratios of AsA/DHA and GSG/GSSG were calculated and found to increase with values of 2.55 and 2.35 at 0.5 LC_{50} over their corresponding values in the control of 1.69 and 1.58.

DISCUSSION

ILs are stable salts with poor biodegradability and may maintain their properties intact in contaminated water bodies.[3]

Table 3. Effect of [C_{12}mim]Br IL on Water-Soluble Antioxidants[a] in *Ulva lactuca* (Mean of Three Independent Experiments ± SD)

treatments	GSSG	GSH	GSH/GSSG	DHA	AsA	AsA/DHA
control	0.31 ± 0.03 b	0.47 ± 0.10 bc	1.58 ± 0.45 b	0.48 ± 0.04 c	0.80 ± 0.11 c	1.69 ± 0.36 b
0.5 LC_{50}	0.47 ± 0.07 a	1.08 ± 0.08 a	2.35 ± 0.39 a	0.65 ± 0.07 b	1.64 ± 0.14 a	2.55 ± 0.42 a
LC_{50}	0.41 ± 0.07 a	0.35 ± 0.07 c	0.89 ± 0.28 c	0.80 ± 0.05 a	0.61 ± 0.05 b	0.76 ± 0.10 c
LSD (5%)	0.08	0.23	0.50	0.08	0.13	0.72

[a] GSH and GSSG represent reduced and oxidized glutathione, respectively; AsA and DHA represent reduced and oxidized ascorbate, respectively.

Their bioaccumulation in aquatic ecosystems may adversely affect the productivity of primary producers such as seaweeds that might eventually bring about environmental perturbations leading to the alteration of the delicate balance between the structure and function of ecosystems. In the present study, the toxicity mechanism of ILs was investigated for the first time using the most common green seaweed *U. lactuca* and was found to be greatly influenced by the alkyl chain lengths of ILs with the greatest toxicity associated with the longer alkyl chains. The results showed that the toxicity of ILs with alkyl chains ranging from $C_{12}-C_{16}$ was 150−450-fold greater than that of the least toxic IL (e.g., [C_4mim]Br). In this study, the LC_{50} values for [$C_{4,\ 6,\ 8,\ 10,12\ mim}$]Br were found to be 9.80, 3.71, 1.42, 0.15, and 0.07 mM, respectively. These values are quite higher than that of LC_{50} values (1.02−0.008 μM) reported for the marine green microalga *Oocystis submarina* and diatoms *Cyclotella meneghiniana* and *Skeletonema marinoi*.[7,27] The reason for these differences could be mostly due to the physical structure of *U. lactuca* having an expanded blade of two cell thickness (distromatic) with a large cell size (20−50 μm) and complex polysaccharides of water-soluble ulvans, insoluble cellulose, and traces of xyloglucans and glucuronan as cell wall materials. Ulvan is mainly built on disaccharide repeating units of sulfated rhamnose and glucuronic acid, iduronic acid, or xylose. The significance of cell size and the cell wall components has also been highlighted for mediating the toxic action of ionic liquids.[27,28] The adverse effects of [C_{14}mim]Br and ([C_{16}mim]Br ILs were apparent within in 36 h of incubation in the algal tissues, which turned to pale white from their original dark green color suggesting the damage of the photosynthetic apparatus. This effect could be attributable to stress-induced damage to membranes or electron transport chains with the excessive generation of ROS, which could have affected the redox state of some enzymes (regulate or inhibit) involved in photosynthesis, in turn decreasing or inhibiting the photosynthetic activity of the organism.[29] The relationship of longer alkyl chain length IL with greater lipophilicity and cell membrane disruption in aquatic organisms has been well documented.[30,31]

The findings obtained in the present study provide clear evidence for the narrow tolerance of *U. lactuca* against ILs. The histochemical localization investigation showed considerable accumulation of $O_2^{\bullet-}$ and H_2O_2 together with greater membrane damage, inhibition of antioxidant systems, and DNA damage in the alga following the exposure to [C_{12}mim]Br for 4 days, confirming the state of oxidative stress even at the LC_{50} concentration. These results are in agreement with the previous findings[6] reporting the production of ROS with the disturbance of the antioxidant system of *Daphnia magna*. Superoxide radicals inactivate several crucial enzymes pertaining to energy production and amino acid metabolism following Fenton chemistry. In order to get some insights into the sources of ROS production and the signal transduction pathway involved in the macroalgal responses to IL, the effect of various enzyme inhibitors were studied. Results showed that DPI (specific inhibitor for NADPH oxidase) in particular totally eliminated the formation of both NBT and DAB precipitates, thus strongly indicating that the IL induced $O_2^{\bullet-}$ and H_2O_2 production originated, at least in part, from plasma membrane bound NADPH oxidase. The possible involvement of NADPH oxidase in H_2O_2 generation indicates that ILs may mediate the toxicity and oxidative stress in algae in a mechanism similar to that reported for toxic heavy metals.[32,33] Apart from NADPH oxidase, there are several other potential enzymatic sources for ROS generation.[34] In the present study, the thalli of *U. lactuca* also showed their susceptibility to NaN_3 (a peroxidase and cytochrome oxidase inhibitor) with a significant inhibition of $O_2^{\bullet-}$ and H_2O_2 production during IL exposure at its lethal concentration. These results possibly suggest the activation of POD and the IL induced ROS production in *Ulva*. Furthermore, a partial accumulation of IL induced $O_2^{\bullet-}$ and H_2O_2 was restricted by CANT (protein phosphatase inhibitor) and ODQ (guanylate cyclase inhibitor), which hinted that the initial control point in ROS regulation is at the level of phosphorylation/dephosphorylation of proteins together with the involvement of cyclic GMP for the production of ROS through the elevation of Ca^{2+} concentration; however, further in-depth investigations are required to elucidate these signaling pathways.

The volatile aldehyde-like MDA and specific LOX isoenzymes are suitable markers for membrane lipid peroxidation. As compared with the control, the *Ulva* thalli exposed to [C_{12}mim]Br for 4 days at LC_{50} concentration showed a considerable rise in the MDA level over the control. The reason for an increased MDA level could be attributed to the incorporation of the alkyl chain of [C_{12}mim]Br into the polar head groups of the phospholipid bilayer, which in turn led to the disruption of membrane bound proteins.[35] The enhanced MDA level was also positively correlated to the increased activity of LOX. This enzyme generated singlet oxygen and superoxide anions while incorporating molecular oxygen into linoleic and linolenic fatty acids, to form lipid hydroperoxides. In the present study, the enhanced LOX activity, together with the induction of two new isoforms, i.e., LOX-2 and LOX-3, in the *Ulva* thalli treated with [C_{12}mim]Br for 4 days, could be ascribed to the decreased levels of C18 and C20 PUFAs, which had been utilized as the substrata for the catalytic reaction. Moreover, the induced LOXs isoform could also be categorized to Type II lipoxygenases, as these isoforms were induced during LC_{50} of [C_{12}mim]Br exposure when the content of the primary photosynthestic pigments (Chl a+b) were diminished. Type II lipoxygenases are known to be widespread in plants, and have a neutral pH optimum and a strong tendency to show co-oxidation reactions (e.g., with chlorophyll, carotenoids, lipophilic vitamins, etc.) caused by free radicals liberated during the catalytic process.[36] Recently, the inhibition of photosynthetic activity with decreased chlorophyll contents in *Pseudokirchneriella subcapitata*, *S. obliquus*, and *Chlorella ellipsoidea* after exposure to imidazolium- and/or pyridinium-derived ILs at different concentrations ranging from 10 μM−10 mM has also been observed.[10,30]

The ability to adjust the membrane fluidity by modulating the unsaturated fatty acids content with enhanced activity of fatty acid desaturases is a sign of stress acclimatization. Fluidity in membranes is required to maintain the diffusion of lipophilic compounds and the activities of membrane bound enzymes.[37] A consistent higher level of n-3 and n-6 PUFAs and a UFA/SFA ratio at 0.5 LC_{50} concentration implies their role in modulation and protection of desaturase from fatty acid peroxidase activities during oxidative stress. Enhanced desaturase activity and higher lipid unsaturation has also been demonstrated in *U. lactuca* as a means of combating the cadmium induced oxidative stress.[22] On the contrary, the declined n-6 PUFAs and UFA/SFA ratio at LC_{50} suggest the IL induced activation of fatty acid peroxidase activities including dioxygenases, peroxidases, and lipoxygenases, leading to ROS accumulation and loss of membrane integrity.

Among the relatively few reported mechanisms suggested for ILs toxicity, the most common is membrane disruption.[30,31] However, the specific component of the cells that regulated toxicity and/or is maximally affected by the ILs is still a topic for debate. As an indispensible strategy for overcoming ROS attack, plants in general deploy detoxifying enzymes to control ROS levels, thereby preventing the cells from oxidative stress. Of the detoxifying enzymes, SOD plays a key role in scavenging ROS as it is the first line of defense against ROS. In this study, it is interesting to note that higher activities of SOD, particularly Mn-SOD in the treatments of $[C_{12}mim]Br$ 0.5 LC_{50}, coincided with the higher activities of other antioxidant enzymes. This suggests a rapid breakdown of $O_2^{\bullet-}$ radicals by SOD to keep their levels in control at the place of their generation and was subsequently followed by the action of APX, GR, and GSH-Px that might have allowed green algae to effectively fight off oxidative stress. A similar increased SOD activity attributed to Mn-SOD had also been established in *U. lactuca* and *G. corticata* confirming its potential to scavenge $O_2^{\bullet-}$ more efficiently.[21,22] However, a significant decrease in SOD and APX activity in plants exposed to LC_{50} concentrations suggested the production of ROS, to such an extent that overwhelmed the antioxidant system, leading to cell and tissue damage.

The specific responses of the APX isoforms, APX-3 and APX-4, in this treatment revealed their greater ability for H_2O_2 detoxification, while at the same time inhibition of APX-2 during $[C_{12}mim]Br$ LC_{50} exposure revealed its sensitivity to H_2O_2. The higher activity of APX compared to that of other enzymes (i.e., GSH-Px and GR) involved in H_2O_2 detoxification could be due to its ease of availability throughout the cell and higher substrate affinity in the presence of AsA as a reductant.[22] Furthermore, increased AsA content together with a higher regeneration rate in *Ulva* treated with 0.5 LC_{50} would have modulated the gene transcription or may have acted as an antioxidant to impede the processes regulated through ROS-mediated signaling.[38] A significantly higher activity of enzymatic and nonenzymatic antioxidative systems in *U. lactuca* has also been observed for its better acclimatization to changing environments prevailing in subtidal habitats.[22] On the one hand, a significant higher content (2.5−4.0-fold) of GSH during 0.5 LC_{50} exposure was maintained via GR, while on the other, it was used to detoxify the H_2O_2 via GPX activity. Further, high GSH/GSSG ratios would enable *Ulva* to maintain the redox state of cells while also keeping the sulphydryl groups of soluble and membrane proteins in a reduced state. Enhanced activity of either individual or combined SOD, APX, GR, and GSH-Px enzymes together with AsA and GSH has also been observed in *U. fasciata*,[39] *Grateloupia turuturu*, *Palmaria palmata*,[40] and *Gracilaria corticata*,[41] when subject to oxidative stress resulting from sun-exposure and emersion, hypo/hyper-salinity, heavy metals, and/or other adverse environmental conditions.

In this study, we used the Comet assay as a sensitive biological marker for measuring DNA damage in cells exposed to oxidative stress representing the disproportion between free radical production and functions of the antioxidant system. For the first time, we demonstrated a dose-dependent induction of DNA damage induced by $[C_{12}mim]Br$ in the protoplasts isolated from *U. lactuca* which was most probably mediated through ROS, triggered by IL. The comets obtained from the group treated with a 2× LC_{50} concentration of $[C_{12}mim]Br$ showed complete DNA damage with significant amounts of tail DNA as compared to the control. The increase in SOD, APX, and GR activity together with higher redox level of nonenzymatic antioxidants could prevent the increase of IL-induced ROS level and consequently limit the IL-induced DNA damage in plants exposed to 0.5 LC_{50}.

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ABBREVIATIONS

$[C_n mim]Br$, 1-alkyl-3-methylimidazolium bromide; NSW, natural seawater; ROS, reactive oxygen species; SOD, superoxide dismutase; APX, ascorbate peroxidise; GR, glutathione reductase; GSH-Px, gluthathione peroxidise; LOX, lipoxygenase; AsA, reduced ascorbate; DHA, oxidized ascorbate; GSH, reduced glutathione; GSSG, oxidized glutathione; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; TTC, 2,3,5-triphenyltetrazolium chloride; NSW, natural seawater; NBT, nitroblue tetrazolium; DAB, 3,3-diaminobenzidine; DPI, diphenylene iodonium; CANT, cantharidin; ODQ, oxadiazolo-quinoxalin-1-one; LMPA, low melting point agarose

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