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Salinity Dependent Photosynthetic Response and Regulation of Some Enzymes in Halophytes from Indian Sundarbans

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1. Introduction

The Environment and Ecosystem of tropical and subtropical coastal zone is marked with unique geophysical characters like sea surges with tidal waves, upland discharges, rapid sedimentation, substrate erosion and episodic cyclones. Mangroves are representing a genetic adaptation of a large variety of plant community of different families to a typical saline environment and are best developed on shorelines of tropical world particularly in vast areas of tidal influence. The mangroves are specially suited for the inhospitable environmental condition and thus pose a lot of challenging problems to the biologists. The main feature of mangroves is in their ability to successful colonization under constant physiological stress (Chaudhuri 1996). These plants grow by developing some morphological, physiological and reproductive adaptation (Zimmermann 1983; Das 1999). This vegetation provides a multidimensional beneficial impact on coastal ecosystem in the form of production and protection. This vast greenery nurses several estuarine habitats and mitigate the violence of cyclonic effect (Hogarth, 1999). Recently, these economic and ecologically utility plant communities are under severe threats world-wide. Hence, conservation and management of such ecosystem is a front-line issue to the scientific world. It is well established that biodiversity of the mangrove vegetation is getting degraded to a large extent all over the world due to human interference and tectonic activities. Mangrove ecosystems currently cover 146,530 km² of the tropical shorelines of the world (FAO 2003). This represents a decline from 198,000 km² of mangroves in 1980, and 157,630 km² in 1990 (FAO 2003). These losses represent about 2.0% per year since 1980–1990, and 0.7% per year within 1990–2000. These figures show the magnitude of mangrove loss, and hence the potentiality of mangrove restoration programme.

In the Indian subcontinent (extends between 21°31' - 22°30' N and 88°10′ - 89°51′ E), two important river systems, namely the Ganga and Brahmaputra, constitute the largest delta formation where the vast mangrove vegetation thrives with highest species diversity. Given the marked uniformity of zonation pattern, mangrove communities may be useful in interpreting minor changes in coastal conditions and serving as biological indicators. In the Sundarbans delta, there has been a very slow tilting of the coast due to tectonic uplift in the northwestern part (India) and subsidence in the east (Bangladesh). This has a major

impact on mangrove species distribution as increased salinity prevails in the western part (India). This forest area (Indian territory) covers approximately 2195 km^2 (Sanyal, 1996) excluding the anastomosing network of creeks and backwaters. Presently the soil salinity ranges between 15 – 27 PPT and maximum available irradiance was reported as approximately 2000 $\mu mol\ m^{-2}s^{-1}$ (Nandy (Datta) et al., 2007). The flora comprises 36 true mangroves, 28 associates and seven obligatory mangrove species representing 29 families and 49 genera (Naskar and Guha Bakshi, 1983). Unfortunately, excessive demographic pressure, over-harvesting for timber and fuel-wood, poaching, reclamation for aquaculture and industrial pollution are being detrimental for these coastal resources. The soil of the Sundarbans is saline due to regular tidal interactions, although the salinity is low compared to soil salinity in other mangrove forests of the world (Karim, 1988). Soil salinity, however, is regulated by a number of other factors including surface runoff and groundwater seepage from adjacent areas, amount and seasonality of rainfall, evaporation, groundwater recharge and depth of impervious subsoil, soil type and topography etc. It is found that, conductivity of subsurface soil is much higher than that of surface soil (Chaffey et al., 1985). Using the salinity scale established by Walter (1971), the forest areas have been divided into three zones based on soil salinity (Karim, 1994). These are:

i. Oligohaline (ormiohaline) Zone: The zone is characterised by the soil containing less than 5 ppt of NaCl salt. The oligohaline zone occupies a small area of the north-eastern part of the forest;
ii. Mesohaline Zone: The zone is characterized by NaCl content within the concentration range of 5 to 10 ppt in soil. This zone covers the north-central to south-central part of the forest; and
iii. Polyhaline Zone: The NaCl content of the soil in this zone is higher than 10 ppt. This zone covers the western portion of the forest (Figure 1).

These manual and environmental adversities proved disastrous for some important plant species like *Heritiera fomes*, *Nypa fruticans, Xylocarpus granatum* and *X. mekongensis* (Banerjee, 1999; Upadhyay et al., 2002). These species predominate in between the Raimangal and Matla rivers, where fresh water influx from the Ichamati river towards Raimangal is much better (in Bangladesh part). Especially *H. fomes* prefers slightly and/or moderately saline zone and the ridges of higher elevation that are inundated only during spring tide (Alim, 1979). Previously in West Bengal, these trees used to be 2m in girth, but over 1 m girth are no longer common and top dying of *H. fomes* is very frequent in the Sundarbans forest.

High irradiance and elevated salinity of intertidal swamps impose at least two potential restrictions on the photosynthetic rate of mangroves; water deficiency and low stomatal conductance enforce these plants to thrive under considerable stress. Generation of Reactive oxygen species (ROS) such as superoxide, hydroxyl and peroxyl radicals are inevitable under oxidative stress as does the level of ROS-induced oxidative damage to lipids, proteins, and nucleic acids (Meloni et al., 2003). Current strategies for improving salt tolerance rely primarily on the production of low-molecular weight solutes e.g. Flavonoids and polyphenols and radical-scavenging enzyme systems (Tarczynski et al., 1993; Kishor et al., 1995) in order to defend alteration in the cytosolic osmotic potential. Mangroves, being the well recognized halophytes have high ability of salt tolerance and these plants can be used as a potential source of several salt resistant genes and proteins.

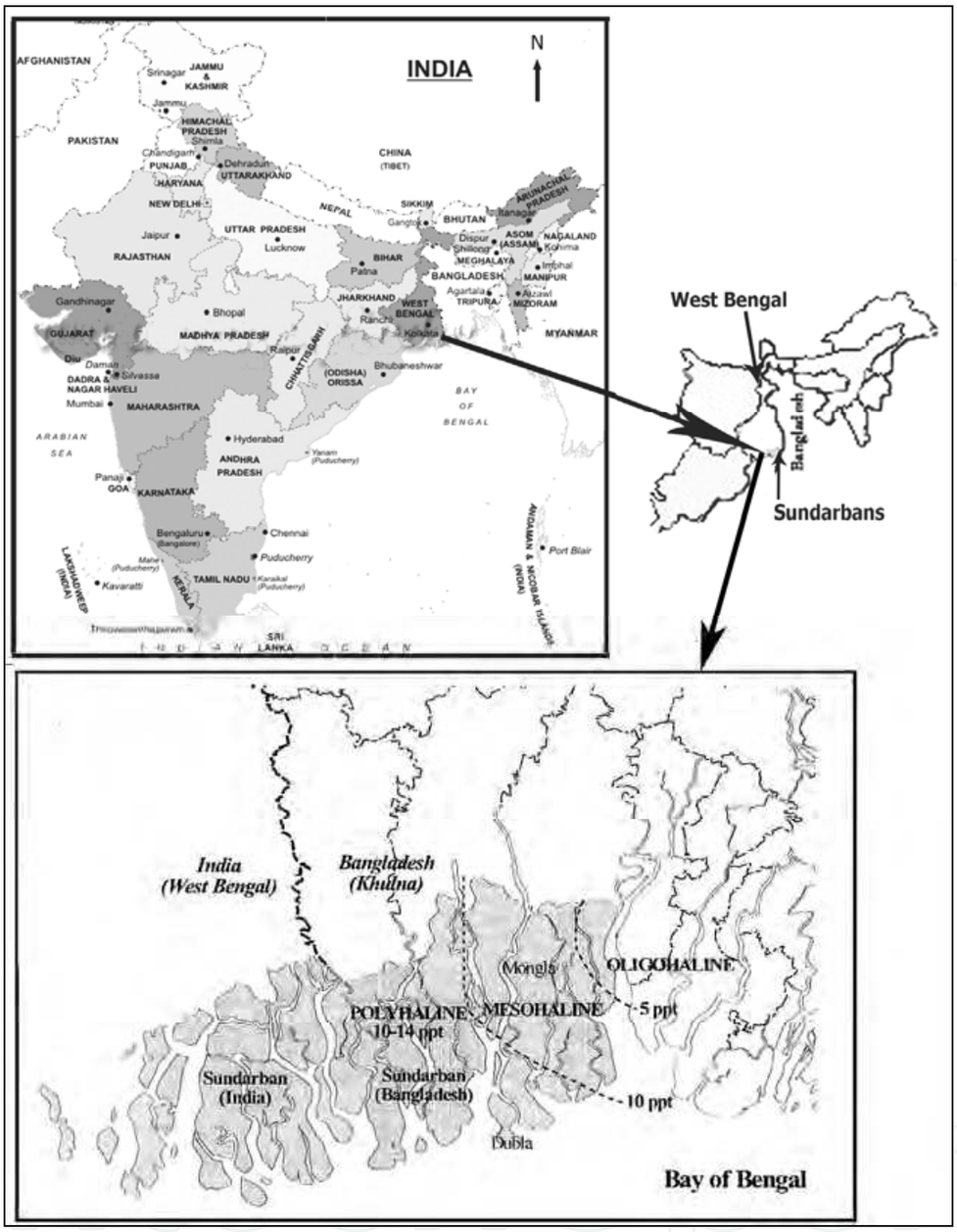

Fig. 1. Map of the study area. Differential salinity zone of undevided Sundarbans (in Bangladesh and India) area (After Karim, 1988).

To mitigate the extent of destruction of cellular components by ROS, a front line defense mechanism is developed in plants with complex antioxidant enzyme mechanisms like peroxidase (PRX), superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR). Salinity resistance is improved by elevated regulation of antioxidant enzymes leading to ROS scavenging (Alscher et al. 2002). Salinity imposed up regulation of cellular ROS accumulation leading to destruction of membrane lipids, proteins and nuclic acids have been reported by earlier works (Hernandez et al. 1999, 2000; Mansour et al. 2005; Ben-Amor et al. 2007; Eyidogan and Oz, 2007).

Due to regular tidal inundation, increased saline water makes the substrate physiologically dry and hence, mangroves have to combat with the problem of maintaining turgour pressure and protecting their metabolic activity from high NaCl concentration (Greenway and Munns, 1980). The cumulative effects of extreme microclimate (irradiance and temperature) and high salinity affect the rate of photosynthesis (Ball, 1988). To prevent photoinhibition, mangroves have to maintain considerably low assimilation rate throughout the day (Cowan, 1982; Nandy and Ghose, 2001). From a recent comprehensive study it was revealed that both salt and drought stress led to down-regulation of some photosynthetic genes, with most of the changes being small (ratio threshold lower than 1) possibly reflecting the mild stress imposed and compared with drought, salt stress affected more genes and more intensely, possibly reflecting the combined effects of dehydration and osmotic stress in salt-imposed habitats (Chaves et al., 2009). Desingh and Kanagaraj (2007) pointed out that photosynthetic rate and RuBP carboxylase activity decreased with increasing salinity but significantly increased for some antioxidative enzymes. An important consequence of salt stress is the excessive generation of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2) and the hydroxyl radicals ($OH^{\bullet}$) particularly in chloroplast and mitochondria (Asada, 1994; Prochazkova and Wilhelmova, 2007).

Unlike morphological markers, molecular markers are not prone to environmental influences and provide some vital information towards the priority areas for conservation strategies. Therefore, the use of molecular markers (enzymes, DNA) might enhance the understanding of such situation. Enzyme analysis is an added tool for detecting this diversity (Zeidler, 2000). The International Union for Protection of New Varieties of Plants (UPOV) have harmonized and adopted test guidelines and procedures for the use of isozyme electrophoresis as a characteristic for establishing uniqueness of plants (UPOV, 1997).

Scientific knowledge on the structural and functional characteristics of mangroves and the natural processes operating in these vulnerable fragile ecosystems is rather poor (Upadhay et al., 2002). Mangroves have to cope with considerably high soil salinity and consequently, a physiologically dry substrate. As such they are confronted with the problem of maintaining adequate turgour pressure within the cell sap because of high salt concentrations in the growth medium and thus protecting their metabolic activity (Flowers et al., 1977). This leads to accumulation and /or synthesis of organic substances in the form of compatible solutes within the vacuole (Hasegawa et al., 2000). Cheesman et al. (1997) experimentally showed that ascorbate peroxidase and SOD synthesis are much higher in field grown mangroves. Superoxide dismutase (SOD) and several antioxidant enzymes are potentially involved in H_2O_2 metabolism leading to photoprotection. Parida et al. (2002) reported that sugar, proline and some polyphenolic compounds accumulate in the cell sap of *Bruguiera parviflora* to restore the water potential more negative. Experimental works reported that in mangroves, the synthesis of these osmolytes, specific proteins and translatable mRNA induced and increased by salt stress (Hurkman et al., 1989; Bray, 1993; Xu et al., 1996; Swire-Clark and Marcotte, 1999; Xu et al., 2001). A Positive linear relationship between peroxidase activity and leaf tissue metal concentrations were reported in *Avicennia marina* (Macfarlane and Burchett, 2001). *In-vitro* experiment on *B. parviflora* resulted the differential changes of the isoforms of antioxidative enzymes in the levels due to NaCl treatment which may be useful as markers for recognizing salt tolerance in mangroves and suggested that the elevated levels of the antioxidant enzymes protect the plants against the reactive oxygen species (ROS) thus avoiding lipid peroxidation during salt stress (Parida et al. 2004a, b). Amirjani (2010) opined that the major ROS scavenging mechanism of plants involve over expression of antioxidant enzymes such as superoxide dismutase, catalase and glutathion peroxidase. The primary scavenger is SOD which convert singlet oxygen ($O_2^{\bullet -}$) to H_2O_2, which is finally scavenged by peroxidase. If this

antioxidative defense mechanism against ROS is somehow disrupted, the cellular homeostasis will be lost (Li, 2009). An increased level of peroxidase and SOD accumulation was reported in water logging stress in *Kandelia candel* and *Bruguiera gymnorrhiza* (Ye et al., 2003).

In obligate halophytes, reverse adaptation often provoke significant metabolic shifts that can be partially characterized by isozyme study. Peroxidase (in different isoforms) is widely distributed throughout the growing phase and has great biological importance. In plants, peroxidase is either bound to cell wall or located in the protoplast (Mader, 1976). Cell wall bound peroxidases are probably involved in lignifications while other isoenzymes have the regulatory role in plant senescence or in the destruction of auxins (Frenkel, 1972; Stonier and Yang, 1973). Beside the morphological adaptation, certain biochemical changes occur in halophytes. Depending on the efficient salt management strategies, mangroves show their differential suitability in elevated salinity level of the present day's Sundarbans forest. Much works on carbon assimilation and its attributes were done in *in vitro* condition under different salinity grdient (Cheeseman, 1994; Cheeseman et al., 1991; Kathiresan and Moorthy, 1993, 1994; Parida et al., 2004).

The literature review reveals that hardly any information is available on the physiological responses of 15-17 years old mangroves grown in fresh water conditions. The present study points to the reverse adaptation in five Indian 'true mangrove' species (*Bruguiera gymnorrhiza*, *Excoecaria agallocha*, *Heritiera fomes*, *Phoenix paludosa* and *Xylocarpus granatum*) with respect to their photosynthetic response in relation to their sustainable existence in Indian Sundarbans. Due to changed ecology, isoforms of these stress related enzymes were differentially expressed. There are hardly any report dealt with a comparative account of quantitative and qualitative analysis of antioxidant and hydrolyzing enzymes in Indian context. In view of above, this work aim to understand the extent of changes of isoforms of two antioxidant enzymes (peroxidase and superoxide dismutase) and two important hydrolyzing enzymes (esterase and acid phosphates) in five true mangrove species grown in the natural field condition (in Sundarbans) and their counterparts grown in the fresh water condition in the garden of ISI Kolkata. The comparative assessment of some physiological response (efficiency of PAR acquisition, Carbon assimilation and stomatal conductance) and biochemical characterization (enzimes and proteins) through both gel electrophoretic study and quantitative estimation of total leaf protein and enzyme, would provide some important clues towards their reverse adaptability to mesophytic condition for postulating proper conservation technique in *ex-situ* condition.

2. Methodology

Five species of true mangroves (*viz. Bruguiera gymnorrhiza*, *Excoecaria agallocha*, *Heritiera fomes*, *Phoenix paludosa* and *Xylocarpus granatum*) were selected for this experiment among which *Heritiera* and *Xylocarpus* are in very much stressed and rest three are profusely grown in western Sundarbans. The youngest leaf buds were collected in ice from properly identified and well matured *in-situ* (from Sundarbans forest, where salinity ranges between 15 – 27 PPT) plants (about 10-12 years old) and their counter parts from *ex-situ* (grown in fresh water condition – in the premises of Indian Statistical Institute, of all most same age, salinity ranges between 2 – 2.5PPT) conditions.

2.1 Measurement of photosynthesis and stomatal conductance

The rate of net photosynthesis and stomatal conductance in different PAR were measured with an infrared CO_2 gas analyzer (PS 301 CID, USA) that uses an electronic mass flow meter

to monitor airflow rate. Measurements were taken from the exposed surface of leaves from top, middle and bottom of each plant. The rate of net photosynthesis (P_n) was determined measuring the rate, at which a known leaf area assimilated CO_2 concentration at a given time. The data were taken from randomly 20 plants of almost same age in full sunshine condition. The average data and their standard error bars were presented in the graphs.

$P_n = -W \times (C_o - C_I) = -2005.39 \times \{(V \times P) / (T_a \times A)\} \times (C_o - C_I)$ …… [W = mass flow rate per leaf area (mmol $m^{-2}s^{-1}$); C_o (C_I) = outlet (inlet) CO_2 conc. (μmol $m^{-2}s^{-1}$); P = atm. pressure (bar); and T_a = air temp. (K)].

Stomatal conductance (C_{leaf}) was calculated from the rate of water efflux and leaf surface temperature (^{0}C).

$C_{leaf} = W / [\{e_{leaf} - e_o) / (e_o - e_I)\} \times \{(P - e_o) / P\} - R_b W] \times 1000$……[$e_{leaf}$ = saturated water vapour at leaf temperature (bar); R_b = leaf boundary layer resistance (m^2s / mol); P = atm. pressure (bar) and W = mass flow rate per leaf area (mmol $m^{-2}s^{-1}$)].

The data were downloaded and computed through RS 232 Port.

2.2 Protein estimation and SDS-PAGE analysis

Total protein estimation was carried out for five mangrove taxa from the both habitat following Lawrey et al. (1951). Extraction of proteins for gel electrophoresis was done from 2g of fresh leaf. Leaf samples were macerated in a mortar-pestle, add 5ml of extraction buffer (containing 10% (w/v) SDS, 10mM β- Marcaptoethanol, 20% (v/v) glycerol, 0.2 M Tris/HCl (pH 6.8) and 0.05% Bromophenol blue). Centrifuge at 10000 rpm for 20 min. Supernatants were used as samples. Protein samples were resolved in 12.5% SDS–PAGE gels following the procedure of Laemmli (1970) and stained with Coomassie Brilliant Blue R-250 (Sigma). Molecular weights of different protein bands were determined with respect to standard protein marker (Bioline Hyper Page pre-stained protein marker, 10kDa – 200kDa) with the Kodak MI software after documentation the gel slab with Gel-Doc system (Biostep GmbH – Germany).

2.3 Extraction of enzymes for native gel electrophoresis

Two grams of young leaf buds were macerated to powder with liquid Nitrogen with a mortar- pestle, then 0.1 g PVP and 5 ml of extraction buffer (consists of 1 M Sucrose, 0.2 M Tris-HCL and 0.056 M β - Marcaptoethanol; pH is adjusted at 8.5) was added to it and homogenized. The extractants were centrifuged at 10000 rpm for 20 minutes at 4°C; supernatants were used as samples for gel electrophoresis. Isozyme analysis of four enzymes *viz.* Peroxidase, Superoxide dismutase, Esterase and Acid phosphatase was done for the investigated five taxa. Equimolar amount of enzymes were loaded in each well. Samples from saline and non-saline environment were loaded side by side for precision of polymorphic band expression. Slab gels were stained for definite enzymes following Das and Mukherjee (1997). Gels were documented with a Gel-Doc System (Biostep GmbH – Germany) and analysis for band intensity and Relative Mobility Factor (R_{mf}) were estimated with Kodak-MI software.

2.4 Enzyme assay

Peroxidase (PRX, E.C.1.11.1.7): 200 mg fresh leaf sample was extracted in 1-1.5 ml 0.9% KCl and centrifuged at 12,000 rpm for 15 min at 4°C; supernatant used as enzyme sample. Absorbances were taken by Helios γ spectrophotometer (Thermo electron Corporation,

USA) at 460 nm in respect to the standard curve prepared following Shannon et al. (1966) with minute modification.

Superoxide dismutase (SOD, E.C.1.15.1.1): Cell sap was extracted from 200mg of leaf and 1-1.5 ml 50 mM Phosphate buffer, ph adjusted to 7.0; centrifuged at 12,000 rpm for 15 min at 4°C. Supernatants were used for enzyme samples. Different aliquots (50, 100, 150, 200, 250 µg/ml) of the standard enzyme samples were also used for preparing the standard curve and absorbance were measured at 550 nm following the protocol described by Keith et al. (1983) with minute modification.

Esterase (EST, E.C.3.1.1.1): Enzyme sample was prepared from 200 mg fresh leaf sample extracted with 1-1.5 ml ice cold 0.1 M Tris/HCl buffer adjusted pH 8.0. Extractants were centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant used as sample. Absorbances were noted at 322 nm with respect to the prepared standard curve following the procedure described by Balen et al. (2004).

Acid Phosphatse (ACP, E.C.3.1.3.2): 200 mg fresh leaf sample was extracted in 1-1.5 ml 40 mM succinic acid /NaOH buffer, pH adjusted to 4.0; centrifuged at 12,000 rpm for 15 min at 4°C. Supernatant was taken for enzyme assay. Prepared a standard curve with the known enzyme samples and absorbances were taken at 322 nm following Huttová et al. (2002).

The data presented was the average of 20 readings for each plant and standard errors were also depicted in the figures. SPSS 12.0 version was used for statistical analysis towards estimating the correlation value, if any, between the total protein amount and quantitatively assayed enzymes. For each enzyme, the pure samples (Sigma chemicals) were used for preparing the standard curves.

3. Results

The salinity ranged between 15-27 ppt throughout the year in Sundarbans, but in garden (mesophytic) soil it never exceeds beyond 2 ppt. The irradiances (PAR) were measured in two ecosystems and range obtained between 428 - 2110 µmol $m^{-2}s^{-1}$ in the saline habitat (Sundarbans) and 600 – 1880 µmol $m^{-2}s^{-1}$ in the mesophytic (ISI garden) environment.

3.1 Photosynthesis and stomatal conductance

The average net photosynthesis was generally higher in mangroves of non-saline habitat than that of the native ones (Fig. 2A), but the PAR acquisition for maximum photosynthesis were greater in most of the Sundarbans species except *H. fomes* and *X. granatum* (Fig. 2A). In *B. gymnorrhiza*, the maximum photosynthesis (10.47µmol $m^{-2}s^{-1}$) was achieved only at 873µmol $m^{-2}s^{-1}$ PAR when grown in non-saline soil, but as high as 1078.5µmol $m^{-2}s^{-1}$ PAR was utilized to obtain the highest assimilation rate (9.19µmol $m^{-2}s^{-1}$) under saline condition (Fig. 2A). In *E. agallocha* the optimum PAR required for maximum photosynthesis was 1445.8 µmol $m^{-2}s^{-1}$ in Sundarbans and 1402.6 µmol $m^{-2}s^{-1}$ in the garden, whereas the highest assimilation rates were 12.27 µmol $m^{-2}s^{-1}$ and 14.69 µmol $m^{-2}s^{-1}$ respectively (Fig.2A). Similarly, in *P. paludosa*, the optimum PAR value was 1662.3 µmol $m^{-2}s^{-1}$ in Sundarbans forest beyond which photosynthesis started declining, whereas in the garden, the highest rate of net photosynthesis (6.92 µmol $m^{-2}s^{-1}$) was recorded at a much lower PAR value (1012.6 µmol $m^{-2}s^{-1}$) (Fig. 2A). On the contrary, under salt stress, the rate of assimilation in *X. granatum* dropped just beyond 827.7 µmol $m^{-2}s^{-1}$ PAR, whereas in non-saline condition, the optimum PAR was as high as 1557.6 µmol $m^{-2}s^{-1}$ (Fig. 2A). Among the studied species, photosynthesis rate was maximal in *H. fomes* under both the environmental conditions (10.63 µmol $m^{-2}s^{-1}$ in Sundarbans and 12.63 µmol $m^{-2}s^{-1}$ in garden) (Fig. 2A).

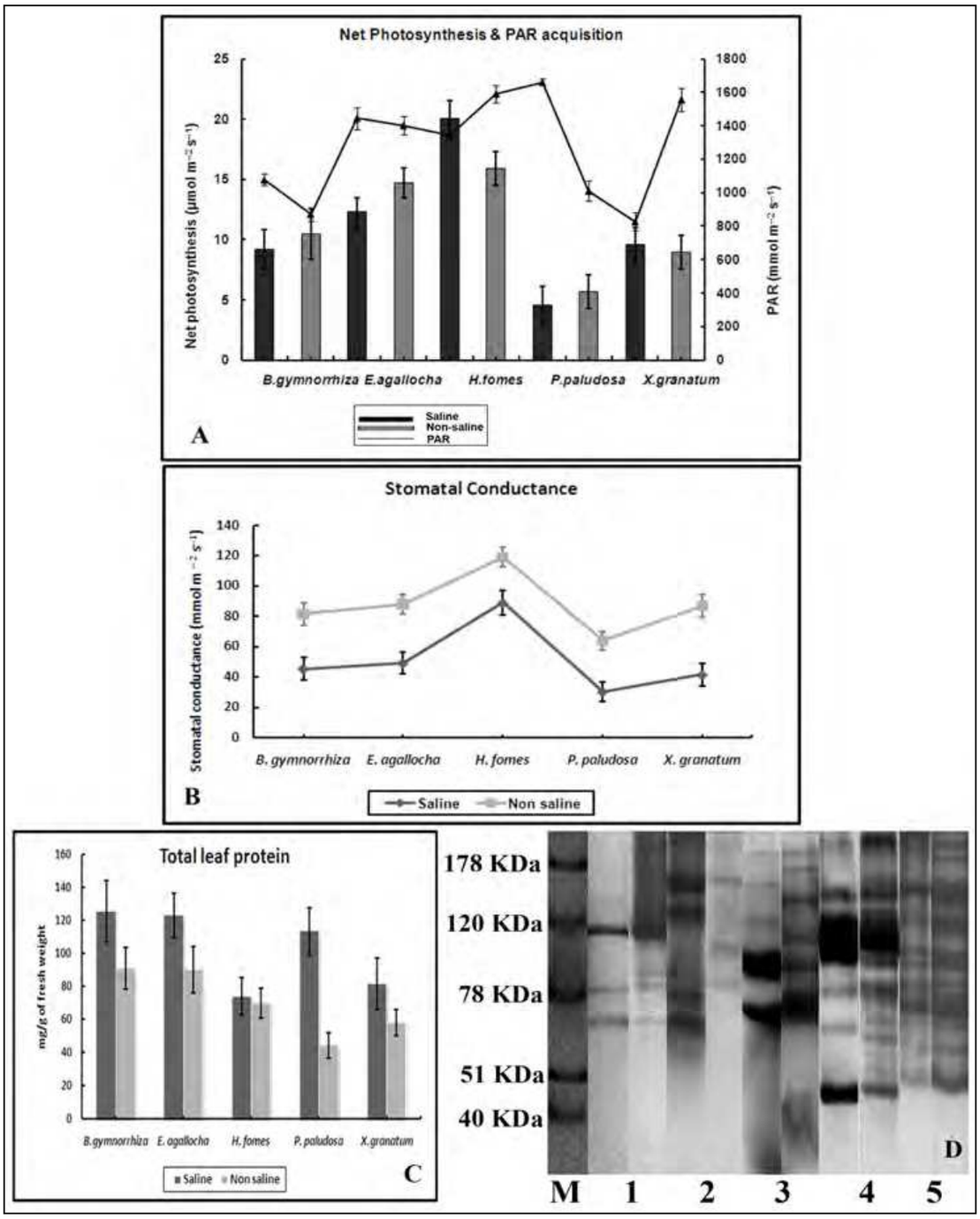

Fig. 2. **A.** Comparative account of net photosynthesis and PAR acquisition of the investigated mangroves in two different salinity zones. **B.** Graphical representation of stomatal conductance of five mangroves from two different environments. **C.** Total leaf protein content along with standard errors. **D.** Documentation of SDS-PAGE analysis of proteins along with standard marker (M - marker, **1.** – *B. gymnorrhiza*, **2.** – *E. agallocha*, **3.** – *H. fomes*, **4.** – *P. paludosa* and **5.** – *X. granatum*). Each consecutive two lanes representing one species; in left – non-saline and in right – saline environment grown plant species.

Stomatal conductance was remarkably decreased under salinity stressed habitats as compared to those of the sweet-water counterparts (Fig. 2B). In *B. gymnorrhiza* and *E. agallocha* the salinity-imposed restriction of stomatal conductance was about 44%, in *P. paludosa* and *X. granatum* it was nearly 52% and in *H. fomes* 25%.

3.2 Total protein

Total leaf protein was estimated from the five enough mature taxa, grown in both saline and fresh water environment. In all five species, the total protein content showed higher amount in fresh water grown plants than that of their Sundarban counterpart (salt stress environment). The highest amount was estimated in *B. gymnorrhiza* (125.82 mg/g fr. wt.) and *E. agallocha* (123.2 mg/g fr. wt.) and minimum was in *X. granatum* (73.96 mg/g fr. wt.) grown in *ex-situ* condition. The increment of total protein was estimated at highest in *P. paludosa* (156%) and lowest in *X. granatum* (5.7%). In *H. fomes*, fresh water habitat showed 57% more protein content than that of the *in-situ* habitat (Fig. 2C).

SDS – PAGE analysis: This analysis revealed that the numbers of protein bands were expressed differentially in the same species from two different habitats. The molecular weights of these bands were calculated with respect to standard marker, run in the same gel. The result revealed that in *Bruguiera*, the saline habitat individual showed one extra band than its non-saline replica and molecular weight ranged between 169.1 – 66.67kDa (non-saline) and 210.7 – 66.11kDa (saline). *Excoecaria* showed the same number of bands in both habitats having molecular weight ranged between 205.8 – 65.55kDa (non-saline) and 213.2 – 77.72kDa (saline). The highest number of protein bands appeared in *Heritiera* from both the environments, nine bands in each having molecular weight 211.2 – 26.71kDa in non-saline and 212.2 – 37.0kDa in saline taxa. One extra band appeared in non-saline *Phoenix* than it saline pair and the molecular weight ranged between 201.3 – 46.43kDa and 213.2 – 46.0kDa respectively. In *Xylocarpus*, one more band was expressed in saline plant, having 202.8 – 50.57kDa (non-saline) and 197.3 – 58.27kDa (saline) (Fig. 2D).

3.3 Native gel electrophoresis: Peroxidase (PRX)

Band expression obtained from gel electrophoresis revealed that in *H. fomes* and *X. granatum* showed the same number of isoforms in two different habitats, where as in *B. gymnorrhiza*, *P. paludosa* and *E. agallocha* the number of isoforms were higher in Sundarbans species than that of their replica from fresh water condition (Table 1). But the R_{mf} and band intensity were different to a large extent in all the five species. In *Bruguiera*, the saline plant showed eight isoforms with highest OD 163.5 (0.07 R_{mf}) where as the fresh water individual showed five isoforms with highest OD was 51.37 (0.68 R_{mf}). In *Heritiera* and *Xylocarpus*, the numbers of isoforms were same but highest OD obtained 206.0 (0.18 R_{mf}) and 180.0 (0.68 R_{mf}) from saline individual and from fresh water habitats highest OD values were 166.0 (0.07 R_{mf}) and 89.9 (0.07 R_{mf}) respectively. Non-saline *Phoenix* and *Excoecaria* showed three and two isoforms of PRX and saline partners expressed four and three isoforms respectively (Table 1; Fig. 3A).

Superoxide dismutase (SOD)

The experimental data showed that in all five species, isoforms of SOD expressed in less number from the fresh water grown individuals than that of their saline replica. All four species expressed three isoforms in non-saline environment, except *Phoenix*, where it was two. The plants from saline habitat, *Heritiera*, *Phoenix* and *Xylocarpus* showed five isoforms, *Bruguiera* and *Excoecaria* have two. The Densitometric scanning resulted that the band intensity of each isoforms were much higher in saline habitats. In *Heritiera*, highest intensity

Enzymes / Env. / Plants	Peroxidase				Superoxide Dismutase				Esterase				Acid phosphatase			
	Saline		Non-saline		Saline		Non-saline		Saline		Non-saline		Saline		Non-saline	
	R_{mf}	OD	R_{mf}	OD	R_{mf}	OD	R_{mf}	OD	R_{mf}	OD	R_{mf}	OD	R_{mf}	OD	R_{mf}	OD
B. gymnorrhiza	0.07	163.5	0.09	8.4	0.31	135.0	0.03	16.42	0.37	150.0	0.36	17.33	0.32	148.6	0.53	194
	0.17	121.1	0.18	23.02	0.42	134.0	0.11	25.75	0.48	153.0	0.48	20.0	0.49	157.0		
	0.24	99.38	0.47	6.76	0.66	127.0	0.18	41.53	0.53	221.0						
	0.35	26.12	0.59	8.96	0.73	142.0										
	0.41	24.32	0.68	51.37												
	0.5	21.11														
	0.67	20.64														
	0.79	72.51														
E. agallocha	0.07	80.12	0.18	6.03	0.02	129.38	0.18	113.0	0.17	214.0	0.24	102.27	0.40	196.0	0.53	26.15
	0.15	25.3	0.26	8.05	0.31	170.07	0.33	163.0	0.37	212.0	0.3	30.82				
	0.21	115.66			0.34	86.36	0.49	162.0	0.53	210.0	0.56	14.62				
					0.57	90.99										
					0.66	74.39										
H. fomes	0.07	166.0	0.18	206.0	0.06	63.0	0.33	5.43	0.37	214.0	0.36	53.6	0.17	127.4	0.19	189.0
	0.17	184.0	0.26	204.0	0.2	26.5	0.63	6.48	0.48	226.0	0.48	32.48	0.36	146.6	0.37	245.0
	0.31	196.0	0.76	182.0	0.31	43.45	0.75	5.42	0.62	205.0			0.49	168.4		
					0.42	50.31			0.71	198.5						
					0.78	138.7										
P. paludosa	0.04	184.94	0.18	154.0	0.06	160.0	0.11	40.0	0.1	178.0	0.3	177.0	0.12	227.0	0.17	96.03
	0.09	125.62	0.26	192.0	0.31	131.0	0.26	42.0	0.37	186.0	0.48	174.0	0.32	195.0	0.46	29.31
	0.41	139.67	0.76	39.0	0.34	128.2	0.39	39.6	0.48	222.0			0.4	185.0	0.63	18.6
	0.67	136.81			0.48	176.0							0.49	214.0		
					0.78	184.0										
X. granatum	0.07	89.91	0.59	168.0	0.14	44.0	0.24	5.5	0.10	190.0	0.3	59.5	0.17	210.0	0.12	203.0
	0.31	18.0	0.68	180.0	0.42	104.0	0.49	12.0	0.32	214.0			0.32	194.0	0.37	248.0
	0.41	75.43	0.76	150.0	0.57	41.0	0.63	5.87	0.37	223.0			0.49	247.0	0.46	194.0
					0.84	155.0			0.48	174.0				210.0		
					0.87	147.0			0.71	162.0						

Table 1. Band intensity (OD) and Relative Mobility Fraction (R_{mf}) of isozymes from mangroves in two environments.

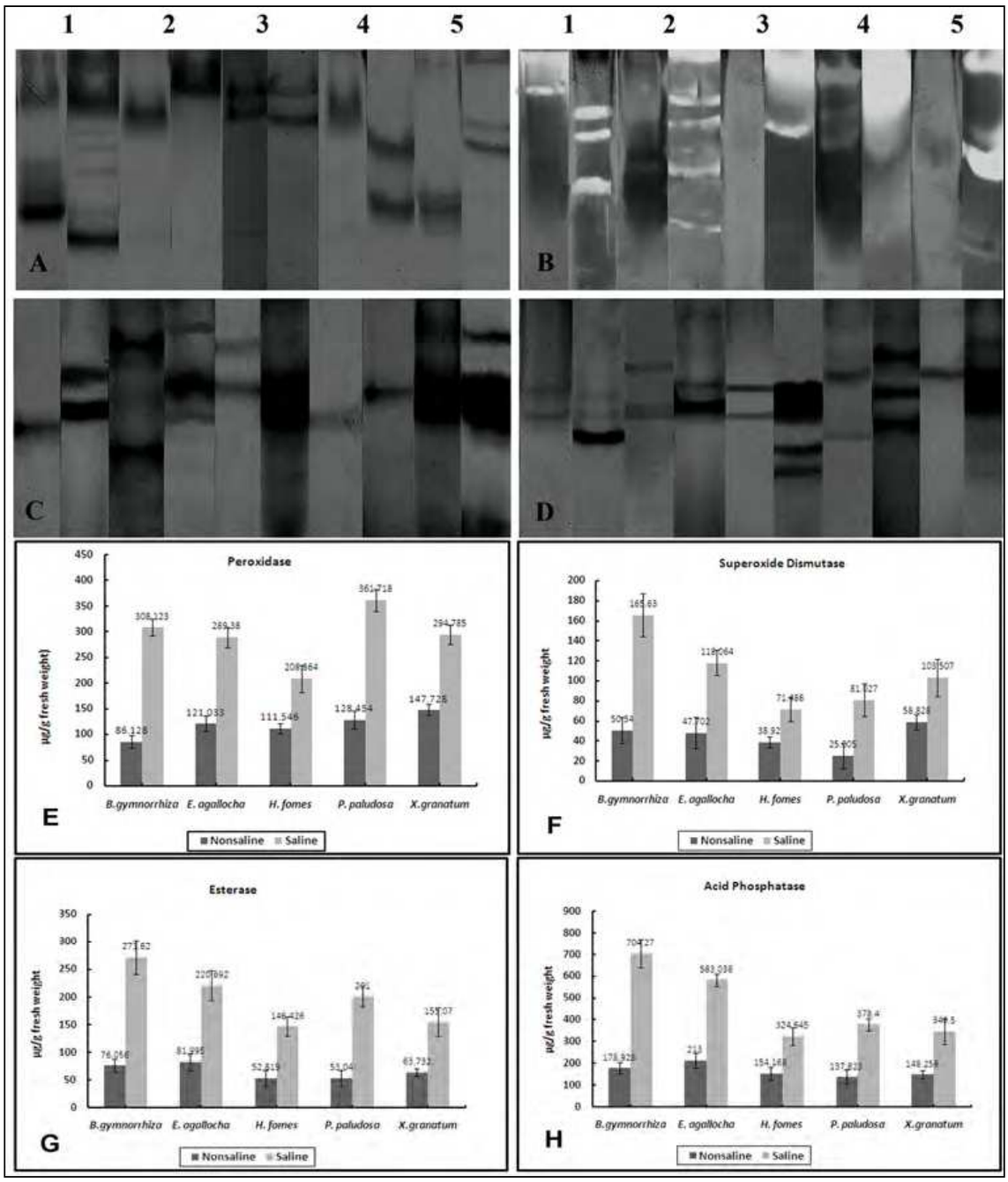

Fig. 3. A – D, Documentation of different enzymes; A – Peroxidase, B – Superoxide dismutase, C – Esterase and D – Acid phosphatase (*1 – B. gymnorrhiza, 2 – E. agallocha, 3 – H. fomes, 4 – P. paludosa and 5 – X. granatum*). Each consecutive two lanes representing one species; in left – non-saline and in right – saline environment grown plant species. E – H, Graphical representation of quantitative estimation of studied enzymes. Standard error bars were showed on each bar diagram.

(138.7 OD) occurred with R_{mf} value 0.78 in saline individual, where in reverse habitat it was much less (6.48 OD and 0.63 R_{mf}). Similarly, in *Bruguiera*, it was 142.0 OD with 0.73 R_{mf} in saline and 41. 53 OD at 0.18 R_{mf} in non-saline habitat. In *Xylocarpus* and *Phoenix*, saline and fresh water condition showed the highest peak as 147.0 OD (0.87 R_{mf}) and 12.0 OD (0.49 R_{mf}) and 184.0 OD (0.78 R_{mf}) and 42.0 OD (0.26 R_{mf}) respectively. In *Excoecaria* highest peak of intensity were observed in saline and non-saline habitats as 170.07 OD (0.31 R_{mf}) and 163.0 OD (0.33 R_{mf}) (Table 1; Fig.3B).

Esterase (EST)

From the gel staining it revealed that EST expression in all species from fresh water habitats were two isoforms, except *Xylocarpus* (single band) and *Excoecaria* (three bands). The comparative band intensity was also remarkably high from all saline habitat taxa except in *Phoenix*, where it was slightly higher (222.0 OD at 0.48 R_{mf} in saline plants and 177.0 OD at 0.3 R_{mf} in non-saline habitat). In *Heritiera*, among the four expressed bands in saline habitat, the highest band intensity occurred at 226.0 OD (0.48 R_{mf}) and it was 53.6 OD (0.36 R_{mf}) in reverse habitat. *Bruguiera* showed as high as 221.0 OD (0.53 R_{mf}) in saline (expressed number of isoforms was three) and 20.0 OD (0.48 R_{mf}). In *Xylocarpus*, out of five isoforms in saline condition, the highest OD was 223.0 (0.37 R_{mf}) in saline and the other side it was 59.5 OD (0.3 R_{mf}). Out of three isoforms, in saline species of *Excoecaria*, highest OD obtained 214.0 (0.17 R_{mf}) and in non-saline it was 102.27 OD (0.24 R_{mf}) (Table 1; Fig3C).

Acid phosphatase (ACP)

Among the five investigated taxa all four species showed excess number isoforms of ACP in saline individual except in *Excoecaria*, where it was single band in both the environment, though the band intensity was higher in saline plants (196.0 OD, 0.4 R_{mf}) than non-saline partner (26.15 OD, 0.53 R_{mf}). In *Bruguiera* the saline habitat expressed two isoforms of ACP with higher intensity of 157.0 (0.49 R_{mf}) and 148.6 OD (0.32 R_{mf}) but the freshwater plant have only one band with 124.0 OD (0.53 R_{mf}). In both Xylocarpus and Phoenix, saline environment expressed one more isoforms than that of their reverse habitat (three isoforms were expressed in fresh water habitat in each). The highest band intensity in *in-situ Xylocarpus* occurred with 247.0 OD (0.49 R_{mf}) and in reverse condition the highest band intensity and Rmf value were almost same (248.0 OD and 0.46). In *ex-situ* plant of *Phoenix* the highest intensity observed at 96.03 OD (0.17 R_{mf}) and in counterpart it was 227.0 at 0.12 R_{mf}. Among the three expressed bands, highest OD value occurred as 168.4 (0.49 R_{mf}) in *Heritiera* (saline) and 145.0 OD (0.37 R_{mf}) in non-saline plant (Table 1; Fig. 3D).

3.4 Quantitative assay of enzymes

The plant species from saline environment showed the all four (PRX, SOD, EST and ACP) investigated enzymes were in higher quantities than that of their fresh water grown individual. Increase in PRX quantity (µg/g) was highest in *Bruguiera* (257%), then *Xylocarpus* (209%), *Phoenix* (181%) and *Heritiera* (176%) while the increment was 139% in *Excoecaria* (Fig. 3E). In case of SOD, the highest increment occurred in *Heritiera* (241%), then *Bruguiera* and *Phoenix* (229 and 224% respectively) and lowest in *Excoecaria* (147%) (Fig. 3F). Similarly, EST was highest increased in *Phoenix* (287%), *Bruguiera* (257%) and *Heritiera* (241%), lowest in *Excoecaria* (154%) (Fig. 3G). ACP reached its maximum increment in *Bruguiera* (293%) and *Xylocarpus* (267%) and lower in *Excoecaria* (139%) (Fig. 3H).

3.5 Statistical analysis

Estimated total protein and four enzymes from two habitats were taken in account. A two-tailed bivariate correlation coefficient (Pearson coefficient) was calculated among the each parameter (Table 2). The analysis showed that in case of the relationship between Protein and SOD, all species in saline environment have inverse relationship (at 0.01% level) except of *Bruguiera*, wherein it was significant at 0.05% level. In PRO vs. PRX significant inverse relationship was observed only in *Bruguiera* (0.05%) and *Phoenix* (0.01%) whereas the other three plants (*Excoecaria, Heritiera* and *Xylocarpus*) showed no statistically significant relationship. Correlation between PRO and EST obtained a significant positive relationship at 0.01% level only in *Bruguiera* and *Excoecaria* in saline inhabitants and others did not show any relationship. The only inversely correlation was obtained in *Excoecaria* (saline plant) at 0.01% level, where as in case of other plants it showed no relationship.

Species / Treatment	*B. gymnorrhiza*		*E. agallocha*		*H. fomes*		*P. paludosa*		*X. granatum*		Treat-ment
	Ns	S	Ns	S	Ns	S	Ns	S	Ns	S	
PRO	0.186	-0.571**	0.380	-0.754*	0.045	-0.529*	-0.383	-0.731*	0.145	-0.705*	SOD
	0.110	0.301	0.308	-0.442	0.795**	-0.348	0.698*	0.187	0.555	-0.013	POX
	0.213	0.667*	0.603	-0.66*	0.468	0.099	0.554	-0.517	0.518	-0.363	EST
	-0.206	0.430	-0.510	-0.65*	0.192	-0.9**	-0.254	0.495	-0.407	-0.403	ACP

* Significant at 0.01%; ** Significant at 0.05%; Ns – non saline, S – saline.

Table 2. Correlations among the different enzymes and total proteins in the plants of two habitats.

4. Discussion

Five typical mangroves (*Bruguiera gymnorrhiza*, *Excoecaria agallocha*, *Heritiera fomes*, *Phoenix paludosa* and *Xylocarpus granatum*) from *in situ* grown where salinity level of the substrate was quiet high (15 - 27 ppt) and *ex situ* (mesophytic) habitat (salinity level was 1.8 - 2 ppt) were investigated with respect to their comparative rates of net photosynthesis, stomatal conductance, and expression of two antioxidative enzymes, both qualitative and quantitative estimation.

Among the five investigated taxa *B. gymnorrhiza*, *E. agallocha* and *P. paludosa* had optimum PAR requirements for maximum photosynthesis that were higher in Sundarbans than those in the mesophytic taxa, whereas, the peak photosynthetic rates were higher in the mesophytic soil. But *H. fomes* and *X. granatum* showed the reverse phenomenon, where at comparatively low PAR the highest net photosynthetic rate occurred. Krauss and Allen (2003) pointed out that *B. sexangula* prefers low salinity combined with low light intensity. Cheesman and Lovelock (2004) experimentally showed that in *Rhizophora mangle* under low salinity conditions, net CO_2 exchange and photosynthetic electron transport becomes light saturated at less than 500 μmol $m^{-2}s^{-1}$. In Sundarbans however, despite tidal influence, high salinity makes the substrate physiologically dry. In order to check desiccation and xylem embolism, mangrove leaves reduce the rate of water efflux (Nandy and Ghose, 2001) that may enhance the tendency to elevate the leaf temperature with subsequent decline in photosynthesis. The present observation revealed that in all five species, stomatal conductance was reduced ranged by 25% to 52% under salinity stress that effectively limited

CO_2 influx. Although reduced stomatal conductance imposed by high salinity restricts CO_2 diffusion, it may elevate the CO_2 partial pressure across the stomata that are utilized by mangrove leaves to maintain a consistently moderate rate of photosynthesis throughout the day, thus avoiding CO_2 starvation and photoinhibition. This result is in accordance with Cowan (1982) and Nandy and Ghose (2001). Naidoo et al. (2002) also measured the optimum PAR for highest photosynthesis in *B. gymnorrhiza* at Durban Bay site that is similar (around 1000 μmol $m^{-2}s^{-1}$) to the present data. The opposite phenomenon occurred in *H. fomes* and *X. granatum* can be explained as less affinity of these species towards high salinity, irradiance and temperature of the Sundarbans forest. Theoretically, high photosynthetic efficiency can increase water use efficiency as more carbon is assimilated per unit water transpired. In mangroves, a positive correlation was reported between photosynthesis and stomatal conductance – an important determinant of water use efficiency (Nandy et al., 2005, Dasgupta et al., 2011). The effect of salinity stress on the photosynthetic enzyme activities is postulated to be a secondary effect mediated by the reduced CO_2 partial pressure in the leaves caused by the stomatal closure (Lowler and Cornic, 2002; Meloni et al., 2003; DeRidder et al., 2007). Salt stress depletes the water potential of soil which in turns causes water stress in plants. This phenomenon triggers the signal for stomatal closure and consequently makes CO_2 deficit in the leaf cells. Tausz et al. (2004) reported that a breakage of proper coordination between CO_2 assimilation and chloroplast photo function electron transport chains ultimately leads to the transfer of electrons in an alternating electron acceptor like O_2.. The excess electron reduces molecular oxygen to produce ROS. During normal growth and development this pathway monitors the level of ROS, produced by aerobic metabolism, and controls the expression and activity of ROS-scavenging pathways. The basic ROS cycle may also perform fine metabolic tuning, e.g., suppression of photosynthesis, for reducing the production rate of ROS. There are many potential sources of ROS in plants. Some are reactions of normal aerobic metabolism, such as photosynthesis and respiration, while others belong to pathways enhanced during abiotic stresses, such as photorespiration. In the present work, though *H. fomes* and *X. granatum* showed relatively higher rate of net photosynthesis using less PAR in saline environment than those of the other three species, production of PRX and SOD were relatively low. Hence from the present work it can be postulated that these two species probably less ability to successful management of salt-stress than those of other three species investigated in the present day salinity level at Sundarbans. This study also reveals that in all the mangroves grown in non-saline soil, an increased rate of assimilation is coupled with increased stomatal conductance.

All the five investigated mangrove taxa from fresh water habitat showed an increase amount of total leaf protein than that of their saline habitat replicas. It was noted that the percent of increment varied in a wide range from 5% to 36%, in which the highest increment occurred in *Excoecaria* and *Phoenix* while lowest in *Heritiera* and *Xylocarpus* (6.05 and 5.7% respectively). This occurred probably as salinity imposed plants are adversely affected in their growth and metabolism due to osmotic effect of salt, nutritional imbalance, accumulation of incompatible toxic ions. The decreased protein content in saline environment might be due to enhance activity of protease (Parida et al., 2002). The present result was well accord to Rajesh et al. (1999), where they experimentally reported that in *Ceriops*, the total leaf protein decreased under higher concentration of saline treatment. Raymond et al. (1994) opined that stress induced protein degradation may be essential which provide amino acids for synthesis of new proteins suited for growth or survival under the modified condition. Mansour (2000) reported that protein biosynthesis decline

under alt stress condition, while cells preferentially synthesis some specific stress proteins. Stress induced proteins accumulated in the cell which might be synthesized *de novo* in response to salt or might be present constitutively at low level (Pareek et al., 1997). Reports from earlier works also confirm that enhanced cellular accumulation of ROS due to salinity stress can expedite the destruction of membrane lipids, proteins and nucleic acids (Hernandez et al., 1999, 2000; Mansour et al., 2005; Ben-Amor et al., 2007; Eyidogan and Oz, 2007). In the present investigation, it was evident that the degradation of proteins in *Heritiera* and *Xylocarpus* from saline habitat was lower amount than the other three taxa investigated might be pointed to the synthesis of lesser amount of compatible amino acids in salt habitat. Parida et al. (2002) reported that the total soluble leaf proteins decreased in *Bruguiera parviflora* under NaCl treatment. This decrease might have the outcome of adverse effect of NaCl treatment resulted synthesis of certain low molecular weight proteins which are yet to be elucidated.

Among the various antioxidant enzymes, qualitative and quantitative estimation of two antioxidant enzymes (PRX and SOD) and two other important (hydrolyzing) enzymes (EST and ACP) from saline and fresh water grown plants were made. The results revealed that in most of the cases number of isoforms, band intensity and enzyme expression were higher in salt stressed plants than those of their mesophytic counterparts. It has been proved that during electron transport in the mitochondria and chloroplasts some leakage of electrons occurs and these leaked electrons react with O_2 during aerobic metabolism to produce Reactive Oxygen Species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (^-OH) (Halliwell and Gutteridge, 1985). These cytotoxic ROS may seriously affect the normal metabolism through oxidative damage of lipids, proteins and nuclic acids (Fridivich, 1989). During photosynthesis, the internal O_2 level become high and chloroplast are prone to generate ROS at that time (Foyer and Mullineaux, 1994). Plants synthesize a number of antioxidative enzymes to counteract these ROS, especially SOD converts O_2^- into H_2O_2 and PRX catalyze H_2O_2 (Asada, 1994). In salinity imposed plants the balance between the production of ROS and the scavenging activity of the antioxidants becomes disrupted which ultimately results in oxidative damage. Plants with high levels of antioxidants, either constitutive or induced, have been reported to provide sufficient resistance against oxidative damage (Parida et al., 2004; Jithesh et al., 2006). The present work resulted that both PRX and SOD expressions were high in saline plants and the increment were ranged between 139 – 257% in case of PRX and 147 – 241 in SOD. The present result was substantiated with the earlier works (Cheeseman, 1997; Takemura et al., 2000). In both the cases the increments were lower in *Heritiera* (139% in PRX, 147% in SOD) and *Xylocarpus* (142% in PRX, 166% in SOD) than that of the other three species of saline habitat. Parida et al. (2004) opined that high salt concentration enhanced the accumulation of free amino acids and polyphenols. Thus, NaCl stress not only imposes alterations in antioxidative metabolism, but also accumulation of osmolytes as adaptive measures. The numbers of isoforms were also increased in case of PRX and SOD in saline habitat plants. In *Bruguiera* (saline) the highest numbers of isoforms were expressed in case of PRX, but it was unchanged in case of *Heritiera* and *Xylocarous* (three isoforms in each habitat). This might be due to the relatively less suitability of those plants in the saline environment. SOD showed the excess isoforms in all saline plants than their fresh water counterpart. Therefore, it is evident that the salt imposed production of toxic ROS is mostly regulated by up regulation of antioxidative enzymes like PRX and SOD. Sahu & Mishra (1987) reported changes in enzymatic activity of peroxidase during senescence of rice leaves when submitted to salt stress. They observed

that NaCl increased peroxidase activity which could be related to regulation of membrane permeability, cell wall formation and oxidation of accumulated substances due to salt stress. It was also proved that peroxidases are enzymes related to polymer synthesis in cell wall (Bowles, 1990), as well as with prevention of oxidation of membrane lipids (Kalir et al., 1984).

Biosynthesis of Esterase (EST) revealed that in all five species it is in higher amount in the *in-situ* taxa investigated. The fresh water grown plants synthesized esterase enzyme with less number of isoforms except Excoecaria, where the numbers of isoforms were same (3), but band intensity was more in saline plants. Highest number of isoforms occurred in Heritiera (saline – 4; in non-saline – 2) and Xylocarpus (saline – 5; in non-saline – 1). Still the percentage of increment was lower in the above two taxa than the other three from saline habitat (123 and 156% respectively), the other three species ranged between 241 – 287% of esterase increment. This result supplemented by Hassanein (1999), where he experimentally proved that nine different esterase isoenzymes were detected in embryos of seeds germinated in 105 mM NaCl, whereas only five of them were detected in the embryos of untreated seeds. Pectins are major components of the primary plant cell wall. They can be both methylesterified and acetylesterified and de-esterification occurs by specific esterases (Cécile et al., 2006). Al-Hakimi and Hamada (2001) reported that the contents of cellulose, lignin of either shoots or roots, pectin of root and soluble sugars of shoots were lowered with the rise of NaCl concentration. Hence, esterases play a major role to counteract the salt induced imbalance in cell wall formation.

Acid Phosphatases (ACP) are a group of enzymes that catalyze the hydrolysis of a variety of phosphate esters. These enzymes are widely distributed in plants and are related to phosphate supply and metabolism from a vast array of phosphate esters which are essential for normal growth and development of plant organs (Olczak et al., 2000). The present work revealed that amount of increment in saline grown plant occurred ranging from 139 – 293%. It may be due to fact that under conditions of stress, growth is restricted and delivery of phosphate is impaired, thus resulting in the activation of the cellular phosphatases that release soluble phosphate from its insoluble compounds inside or outside of the cells thereby modulates osmotic adjustment by free phosphate uptake mechanism (Fincher, 1989). Jain et al. (2004) also demonstrated that the endosperm acid and alkaline phosphatase activities were significantly higher after salt treatment, than that of the control in pearl millet. Olmos and Hellin (1997) observed that acid phosphatases are known to act under salt and water stress by maintaining a certain level of inorganic phosphate which can be co-transported with H^+ along a gradient of proton motive force. Hence, the plants in which the ACP increments were observed lower might be less suited in higher salt environment.

The present investigation revealed that a significant inverse correlation obtained between the concentration of the antioxidative enzymes, peroxidase and SOD with total protein in the case of *Bruguiera gymnorrhiza*, *Excoecaria agallocha* and *phoenix paludosa* in saline habitat. This elevation in the antioxidant enzyme concentration level may have taken place to scavenge more number of free radicals that are produced during stress (Davies, 2000, Dasgupta et al., 2010) and the decrease in protein concentration might be the result of formation of more compatible osmolytes to restore more negative water potential in cell sap. Both these phenomenon might provide some combat force to the plants against salinity stress. On the other hand no such statistical significant relationship between antioxidant enzymes and total protein concentration was found in case of *Heritiera fomes* and *Xylocarpus granatum.* This relationship, as discussed above may provide some important clue towards

the proper salt management mechanism for sustainable existence in the hostile environment. Therefore, the absence of it might be one of the reasons towards less adaptibility for the plants in present situation. Though there are scopes yet to elucidate in detail regarding the significance of increment of these enzymes in salt imposed plants, the present work might provide the base-line information and a system necessary to conduct future research in relation to the genetic basis of salt tolerance.

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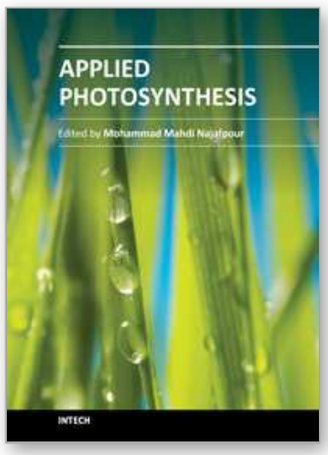

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Photosynthesis is one of the most important reactions on Earth, and it is a scientific field that is intrinsically interdisciplinary, with many research groups examining it. This book is aimed at providing applied aspects of photosynthesis. Different research groups have collected their valuable results from the study of this interesting process. In this book, there are two sections: Fundamental and Applied aspects. All sections have been written by experts in their fields. The book chapters present different and new subjects, from photosynthetic inhibitors, to interaction between flowering initiation and photosynthesis.

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