
Salt Stress: A Biochemical and Physiological Adaptation of Some Indian Halophytes of Sundarbans

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Abstract

Experiment was conducted with five typical mangroves (*Bruguiera gymnorrhiza*, *Excoecaria agallocha*, *Heritiera fomes*, *Phoenix paludosa*, and *Xylocarpus granatum*) both from Sundarbans (*in-situ*) and grown in mesophytic condition (*ex-situ*, in the Indian Statistical Institute's premises) since 15–17 years. A comparative account on PAR utilization for maximum photosynthesis, stomatal conductance, total leaf proteins, and polymorphic expression of two antioxidative enzymes (peroxidase and superoxide dismutase) and two hydrolyzing enzymes (esterase and acid phosphatase) were estimated both qualitatively and quantitatively. The present work revealed that the net photosynthesis was higher in mangroves from mesophytic habitats than those of the native plants, but the PAR acquisitions for maximum photosynthesis were greater in most of the Sundarbans species, except *H. fomes* and *X. granatum*. At the same time, the stomatal conductance was remarkably depleted under salinity stressed habitats than those of the nonsaline counterparts and ranged between nearly 25 and 52%. Total leaf protein content from the above said taxa revealed that the increment of total protein occurred in mesophytic habitat and it was ranged between 156% (in *P. paludosa*) and 5.7% (in *X. granatum*). PAGE analysis revealed that in most of the cases there were extra numbers of protein bands expressed with relatively low molecular weight in saline habitat plants. In all salinity imposed plants, there were sharp increase in band intensity and number of isoforms of each enzyme. Peroxidase increment in

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saline plants was ranged between 257% (in *Bruguiera*) and 139% (in *Excoecaria*). Similarly, superoxide dismutase (SOD) was estimated as 247% (in *Heritiera*) to 147% (in *Excoecaria*) in saline habitats. Increments of esterase and acid phosphatase were varied from 287% (in *Phoenix*) to 154% (in *Excoecaria*) and 293% (in *Bruguiera*) to 139% (in *Excoecaria*), respectively. Salinity imposed increment of antioxidant enzymes proved their efficient scavenging ability to evolved reactive oxygen species (ROS), but these increments were relatively lower in *Heritiera* and *Xylocarpus* even though the net photosynthesis was higher. This might be related to their less adaptability in elevated salinity stress than those of the other three species investigated from the same regime. Among the plants grown in *in situ* condition, some taxa have the better ability of enzyme production, which might be correlated with the efficient stress management practice. A statistical relationship was observed between the total protein content and the investigated enzyme concentration, dependent on the habitat and discussed accordingly.

Introduction

Mangrove Vegetation: Global Context

The environment and ecosystem of tropical and subtropical coastal zones are marked with unique geophysical characteristics like frequent sea surges with tidal influences, upland discharges, rapid sedimentation, substrate erosion, and incidence of episodic cyclones. It is estimated that about 55% of the world's population lives in coastal areas. In East Asia, more than 70% of the population depends on coastal resources for food, employment, and generation of income (Kathiresan and Bingham 2001). In South Asia, demographic compulsion is considered as greatest threat on diminishing coastal resources. The Western Pacific region, due to unplanned developmental activities and climatic change, significant degradation occurred on coastal ecology. Moreover, the increasing human habitation in coastal areas globally, predicted to be 6 billion by 2030 provides the inevitable need for biodiversity conservation (Adeel and Caroline 2002). In most of the developing countries, due to demographic need for more food production and socioeconomic developmental activities which are mostly at the expense of coastal biodiversity loss.

Mangroves, a cosmopolitan assemblage of plant families representing a converging genetic adaptation to a typical saline environment and

are best developed on shorelines of tropical world particularly in vast areas of tidal influence. Mangroves are largely restricted to latitudes between 30°N and 30°S. Northern extensions of this limit occur in Japan (31°22' N) and Bermuda (32°20' N); southern extensions are in New Zealand (38°03' S), Australia (38°45' S), and on the east coast of South Africa (32°59' S) (Spalding et al. 1997). The cradle of mangrove ecosystem is the Indo-Malaysian area as the mangroves first developed here and spread afterward to other tropical and subtropical world (Alongi 2009). The luxuriant occurrence of mangrove composition is found in the Indo-Pacific region. In Southeast Asia, the mangrove forests are the richest in species diversity. The mangrove forests are distributed over the tropical and subtropical estuaries from mean sea level to highest spring tide (Alongi 2009). The current estimate of mangrove forests of the world is less than half of what it once was (Spalding et al. 1997; Spiers 1999) and maximum of which exists in a gaunt condition. Coastal habitats across the world are presently under constant demographic and developmental pressure and periodic natural calamities. The existence crisis of this important habitat starts after industrialization and development; conversion of agricultural land, aquaculture, human habitation, industrial runoff, and over exploitation are all considered as much threats on these estuarine vegetation (Alongi 2002; Giri et al.

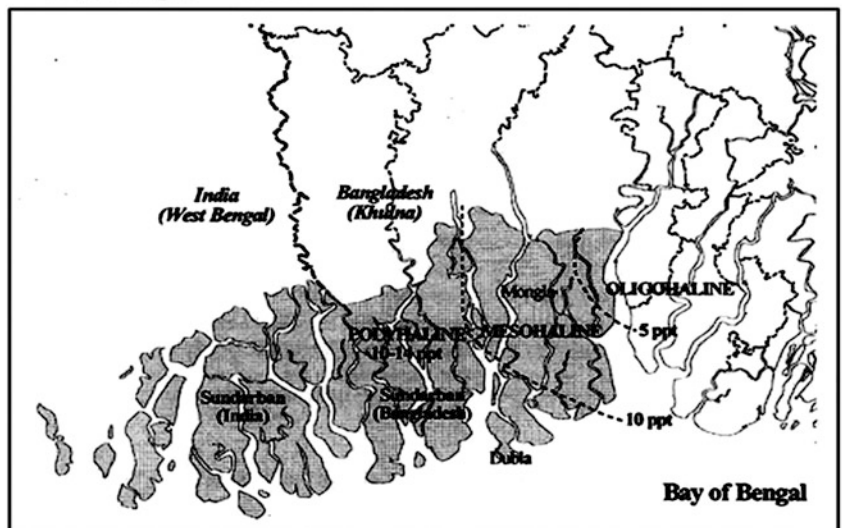
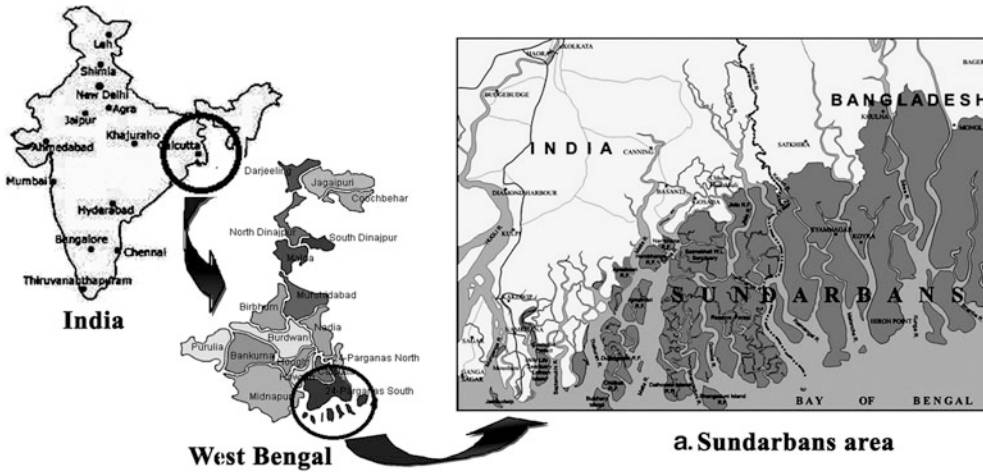
2008). Simultaneously, global climatic changes also have a great impact on mangrove vegetation. The destruction of mangrove forests is happening at a time when there are clear indications of potential changes in the climate (precipitation and temperature), sea level rise and incidence of UV- β radiation. Mangrove ecosystems are the front-line defense against these adverse consequences of change in the sea level. These plants are known to absorb the UV radiation to a considerable extent (Hogarth 2007). The earth's crust has warmed by 0.5–1.2°C since industrial revolution. Further warming of 0.8–2.0°C is to be expected owing to the inertia of the geosystem. Doubling of atmospheric CO₂ levels to about 700 ppm in the next 40–50 years expected to lead to a further increase in mean temperature of 1.5–4.5°C. Rise in relative sea level globally has been reported to be about 10 cm over the last 100 years, with an expected rise of 30–50 cm by 2005 and 100 cm by 2100 (Clough 1994). These climatic changes will result in changing patterns of rainfall, cloud cover and aridity. Duke et al. (2007) opined that the decline of the mangrove forest occurs at a faster rate than any other inland tropical forest. The relative sea level rise could be the greatest threat to mangrove formation (Gilman et al. 2008) and it is postulated that 30–40% of coastal wet lands and 100% of the mangrove forest could be lost in the next 100 years if the present rate of denudation continues (Duke et al. 2007). These losses represent about 2.0% per year since 1980–1990, and 0.7% per year within 1990–2000 (FAO 2007). These figures show the magnitude of mangrove loss and demand serious attention to the potentiality of mangrove restoration program. Indian coastline covers about 7,500 km² and it accounts for 8% of the world's mangrove area, and the eastern coast of India accounts for about 82% of the mangrove forest cover throughout India (Parida et al. 2002). Due to lack of any national plan for conservation and sustainable utilization, mangroves along the Indian coast have reached an alarming stage of depletion. It is reported that 25% reduction in mangrove forest cover has been estimated along the Indian region during the last 25 years (FAO

2007). Predictions of these features of global changes indicate an increase in frequency and intensity of episodic cyclones, hurricanes, tsunami, storms, and floods. Though mangroves do provide a protective buffer and minimize these impacts, they will have increasing impacts on mangrove ecosystem too.

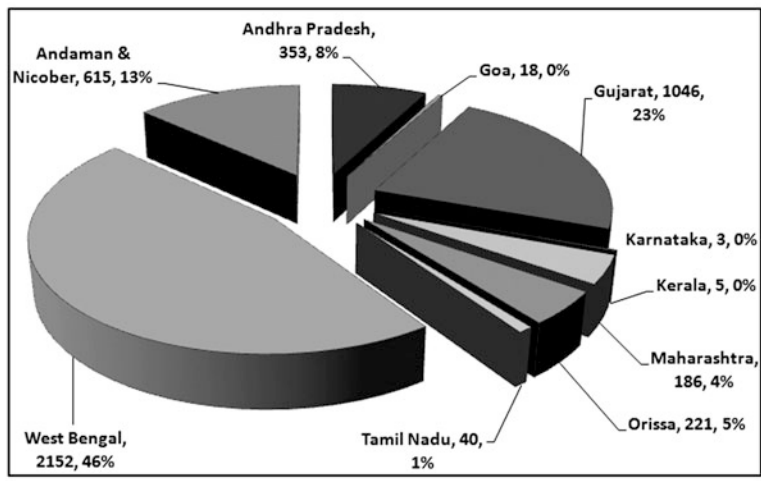
Mangrove ecosystems currently cover 146,530 km² of the tropical shorelines of the world (FAO 2007). This represents a decline from 198,000 km² of mangroves in 1980, and 157,630 km² in 1990 (FAO 2007). From the recent estimate done by Giri et al. (2011), it is revealed that the total mangrove forests in the world include 137,760 km², and it is 0.7% of the total tropical forest in the world. In India, the estimated mangrove forest area includes 4,844 km² (FAO 2007). Using Satellite data, FSI (2009) reported that the Indian mangrove area covers 4,639 km² (Fig. 6.1c) which is 58 km² increments (2.4%) in comparison with the earlier estimate in 2005 (4,581 km²). The largest mangrove forest area is covered in Asia (about 42%) followed by Africa (20%), North and Central America (15%), Oceania (12%), and South America (11%). Giri et al. (2011) confirmed that the mangrove area decreases with the increase of latitude, except 20°N and 25°N latitude, where the Sundarbans area is located and considered this area as the largest block of the mangrove forest in the world. Although the species diversity is the highest in this region, the conservation and sustainable management of this diversity presently need serious attention.

Sundarbans Mangrove: Ecosystem Scenario

Sundarbans delta, a mangrove swamp with world's richest species diversity in the Indian subcontinent (extends between 21°31'–22°30' N and 88°10'–89°51' E), is formed by two major rivers the Ganga and the Brahmaputra and their innumerable tributaries (Fig. 6.1a). The flora comprises 36 true mangroves, 28 associates, and seven obligatory mangrove species



b. Different salinity zones of totla Sundarbans area (Source: Karim, 1994)



c. Mangrove areas in India (Source: FSI, 2009)

Fig. 6.1 (a) Sundarbans mangrove area (included in India and Bangladesh); (b) Zone wise division according to soil salinity (after Karim 1994); (c) Mangrove area in India in terms of Km² (after FSI 2009)

representing 29 families and 49 genera (Naskar and Guha Bakshi 1983). The forest area (Indian Territory) covers approximately 2,152 km² (FSI 2009) excluding the anastomosing network of creeks and backwaters. But this scenario is changing rapidly. Due to tectonic upliftment, there has been a very slow tilting of the coast in the northwestern part (India) and subsidence in the east (Bangladesh); an increased salinity persists in the western part (India) of Sundarbans that has a direct impact on mangrove vegetation pattern. Since the twentieth century, damming on the lower Gangetic plane cut off an ample freshwater influx through Hooghly and its tributaries to the islands of Sundarbans. Moreover, urban sewage and industrial wastes expedite silt formation in the riverbed causing decreased runoff of sweet water influx through the river system leading to elevate the salinity level of water in the delta region. The soil salinity reaches between 15 and 27 PPT (Nandy (Datta) et al. 2007). Haq et al. (1999) estimated that salinity has gone up by 20% in the Sundarbans since 1990. These manual and environmental adversities have posed disastrous for some important plant species of Sundarbans forest including *Aegialitis rotundifolia*, *Heritiera fomes*, *Nypa fruticans*, *Xylocarpus granatum*, and *X. mekongensis* (Banarjee 1999; Upadhyay et al. 2002). These species are predominant in between the Raimangal and Matla rivers, where freshwater influx from the Ichamati River through 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 2 m in girth, but presently beyond 1 m girth is no longer common, and top dying of *H. fomes* is very frequent in the Sundarbans forest (Curtis 1993). However, rise in water level and increasing salinity in the rivers and creeks, human interference, and changed ecology pose the biggest threat to the Sundarbans and have caused rapid destruction to the mangrove forests and even caused extinction to a few of the locally existing species (Parani et al. 2000). The soil of the Sundarbans is saline due to tidal interactions,

although the salinity is low compared to soil salinity in other mangrove forests of the world (Karim 1994). 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. It is found that conductivity of subsurface soil is much higher than that of surface soil (Chaffey et al. 1985). Considering the salinity scale established by Walter (1971), the forest areas have been divided into three zones based on soil salinity (Karim 1994), such as (1) *oligohaline (ormiohaline) zone* – the zone is characterized by the soil containing less than 5 PPT of NaCl salt. The oligohaline zone occupies a small area of the northeastern part of the forest; (2) *mesohaline zone* – the zone is characterized by NaCl content within the concentration range of 5–10 PPT in soil. This zone covers the north-central to south-central part of the forest; and (3) *polyhaline zone* – NaCl content of the soil in this zone is higher than 10 PPT. This zone covers the western portion (Indian part) of the forest (Fig. 6.1b).

The Problems Addressed

The mangroves are specially suited for the inhospitable environmental condition and thus pose a lot of challenging problems to the biologists. Salinity is the major environmental factor that is limiting plant growth and productivity worldwide. It has been well established that they are considered as an ecologically essential component in protecting adjacent land by forming a front-line barrier against tidal waves and sea storm (Hogarth 2007). Salinity stress has long been considered as potential and important factor that are regulating different physiological and biochemical processes (Lin and Sternberg 1993). Mangroves growing in the areas of high salinity, excess irradiance, lower substrate osmotic potential, and anaerobic soil and thus imposed stress on them which were confirmed by decreased CO₂ assimilation, higher stomatal

conductance, and increased leaf water potential (Nandy Datta et al. 2005; Dasgupta et al. 2011). Differential photosynthetic gas exchange ability, extent of water potential, and stomatal conductance along with leaf anatomical characteristics (e.g., mesophyll ratio, nature of palisade cells) are reported as potential indicator of physiological status of a plant and well related to the relative adaptability of different mangrove species in the same regime (Ball and Farquhar 1984; Das 1999, Nandy Datta et al. 2007, 2009).

Unlike morphological markers, molecular markers are not prone to environmental influences and provide some vital information toward 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).

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 turgor 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). Cheeseman 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 polyphenol compounds accumulate in the cell sap of *Bruguiera parviflora* to restore the required 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, 2001; Swire-Clark and Marcotte 1999). 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 in the levels of the isoforms of antioxidative enzymes 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). An increased level of peroxidase and SOD accumulation was reported in water logging stress in *Kandelia candel* and *Bruguiera gymnorrhiza* (Ye et al. 2003). Antioxidative enzymes in relation to salt tolerance in different plant have been evaluated much, but it is still a paradox because enhanced accumulation of these not only associated with salt tolerance but also with salt sensitivity too (Abogadalla 2010). This paradox is due to the following: (1) it is difficult to estimate the evolved amount of ROS in a plant and (2) detoxification of ROS leading to efficient upregulation of genes responsible for all antioxidative enzymes (SOD, PRX, CAT, GR, etc.), though the efficient antioxidative activity never solely means the strong upregulation of all antioxidant enzymes.

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 lignification while other isoenzymes have the regulatory role in plant senescence or in the destruction of auxins (Frenkel 1972; Stonier and Yang 1973). Generation of reactive oxygen species (ROS) such as superoxide, hydroxyl, and peroxy radicals is inevitable under oxidative stress as does the level of ROS-induced oxidative damage to lipids,

proteins, and nucleic acids (Meloni et al. 2003). 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 upregulation of cellular ROS accumulation leading to destruction of membrane lipids, proteins, and nucleic acids has been reported by earlier works (Hernandez et al. 2000; Mansour et al. 2005; Ben-Amor et al. 2007; Eyidenan and Oz 2007).

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 aims 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 freshwater condition in the garden of ISI Kolkata. The comparative assessment, both gel electrophoresis study and quantitative estimation of total leaf protein and enzyme, would provide some important clues toward their reverse adaptability to mesophytic condition for postulating proper conservation technique in *ex-situ* condition.

Methodology

Five true mangroves species grown in Sundarbans mangrove forest (*Bruguiera gymnorrhiza*, *Excoecaria agallocha*, *Heritiera fomes*, *Phoenix paludosa*, and *Xylocarpus granatum*) were selected among which *Heritiera* and *Xylocarpus* are precarious in occurrence in western part of Sundarbans (Indian territory) and the remaining grown profusely in the same regime (considered as natural control). Leaf buds were collected

from *in-situ* (from Sundarbans forest, where salinity ranges from 15 to 27 PPT) plants (about 15–17 years old) and their replica from *ex-situ* (grown in mesophytic condition, in the premises of the Indian Statistical Institute, Kolkata) of all most same age and the soil salinity ranging from 2 to 2.5 PPT.

Estimation of Carbon Assimilation and Stomatal Conductance

The rate of net photosynthesis and stomatal conductance in different PAR were measured with an infrared CO₂ 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 CO₂ 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.

$$P_n = -W \times (C_o - C_I) \\ = -2005.39 \times \{(V \times P)/(T_a \times A)\} \\ \times (C_o - C_I)$$

where W = mass flow rate per leaf area ($\text{mmol m}^{-2} \text{s}^{-1}$); C_o (C_I) = outlet (inlet) CO₂ conc. ($\mu\text{mol m}^{-2} \text{s}^{-1}$); 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 ($^{\circ}\text{C}$).

$$C_{\text{leaf}} = W / \{[(e_{\text{leaf}} - e_o)/(e_o - e_I)] \\ \times \{(P - e_o)/P\} - R_b W\} \times 1,000$$

where e_{leaf} = saturated water vapor at leaf temperature (bar); R_b = leaf boundary layer resistance ($\text{m}^2\text{s/mol}$); P = atm. pressure (bar); and W = mass flow rate per leaf area ($\text{mmol m}^{-2} \text{s}^{-1}$).

The data were downloaded and computed through RS 232 Port.

Protein Estimation and SDS-PAGE Analysis

Total protein estimation was carried out for five mangrove taxa from both habitat following Lawrey et al. (1951). Extraction of protein for gel electrophoresis was done from 2 g of fresh leaf. Leaf samples were macerated in a mortar-pestle, add 5 ml of extraction buffer (containing 10% (w/v) SDS, 10 mM β -mercaptoethanol, 20% (v/v) glycerol, 0.2 M Tris-HCl (pH 6.8) and 0.05% Bromophenol blue). Centrifuge at 10,000 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 prestained protein marker, 10–200 kDa) with the Kodak MI software after documentation the gel slab with Gel-Doc system (Biostep GmbH – Germany).

Extraction of Enzymes Native Gel Electrophoresis

Two grams of young leaf buds were macerated to powder in 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 -mercaptoethanol; pH is adjusted at 8.5) were added to it and homogenized. The extractants were centrifuged at 10,000 rpm for 20 min at 4°C; supernatants were used as samples for gel electrophoresis. Isozyme analysis of four enzymes, viz., peroxidase, superoxide dismutase, esterase, and acid phosphatase, were done for the investigated five taxa. Equimolar amount of enzymes were loaded in each well. Samples from saline and nonsaline 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.

Enzyme Assay

Peroxidase (PRX, E.C.1.11.1.7): 200 mg fresh leaf sample was extracted in 1–1.5 ml of 0.9% KCl and centrifuged at 12,000 rpm for 15 min at 4°C; supernatant used as enzyme sample. Absorbance; was 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 200 mg of leaf in 1–1.5 ml of 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 μ g/ml) of the standard enzyme samples were also used for preparing the standard curve, and absorbance was measured at 550 nm (Keith et al. 1983).

Esterase (EST, E.C.3.1.1.1): Enzyme sample was prepared from 200 mg fresh leaf sample extracted with 1–1.5 of 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. Absorbance was noted at 322 nm with respect to the prepared standard curve (Balén et al. 2004).

Acid Phosphatase (ACP, E.C.3.1.3.2): 200 mg fresh leaf sample was extracted in 1–1.5 ml of 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 absorbance was taken at 322 nm (Huttová et al. 2002).

Results

The salinity ranged between 15 and 27 PPT throughout the year in Sundarbans, but in garden soil it never exceeds beyond 2 PPT. The Irradiance (PAR) that measured in two ecosystems is ranged between 428 and 2,110 $\mu\text{mol m}^{-2}\text{s}^{-1}$ in the saline-habitat (Sundarbans) and 600 and 1,880 $\mu\text{mol m}^{-2}\text{s}^{-1}$ in the nonsaline (ISI garden, Kolkata) environment.

Carbon Assimilation and Stomatal Conductance

The net photosynthesis was higher in mangroves of nonsaline soil habitat than that of the native ones (Fig. 6.2a), but the PAR acquisition for maximum photosynthesis was greater in most of the Sundarbans species except *H. fomes* and *X. granatum* (Fig. 6.2a). In *B. gymnorrhiza*, the maximum photosynthesis ($10.47 \mu\text{mol m}^{-2} \text{s}^{-1}$) was achieved only at $873 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR when grown in nonsaline soil, but as high as $1078.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR was utilized to obtain the highest assimilation rate ($9.19 \mu\text{mol m}^{-2} \text{s}^{-1}$) under saline condition (Fig. 6.2a). In *E. agallocha* the optimum PAR required for maximum photosynthesis were $1445.8 \mu\text{mol m}^{-2} \text{s}^{-1}$ in Sundarbans and $1402.6 \mu\text{mol m}^{-2} \text{s}^{-1}$ in garden, whereas the highest assimilation rates were 12.27 and $14.69 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively (Fig. 6.2a). Similarly, in *P. paludosa*, the optimum PAR value was $1662.3 \mu\text{mol m}^{-2} \text{s}^{-1}$ in Sundarbans forest beyond which photosynthesis started declining, whereas in garden, the highest rate of net photosynthesis ($6.92 \mu\text{mol m}^{-2} \text{s}^{-1}$) was recorded at a much lower PAR value ($1012.6 \mu\text{mol m}^{-2} \text{s}^{-1}$) (Fig. 6.2a). On the contrary, under salt stress, the rate of assimilation in *X. granatum* dropped just beyond $827.7 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, whereas in nonsaline condition, the optimum PAR was as high as $1557.6 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 6.2a). Among the studied species, photosynthesis rate was maximal in *H. fomes* under both the environmental conditions ($10.63 \mu\text{mol m}^{-2} \text{s}^{-1}$ in Sundarbans and

$12.63 \mu\text{mol m}^{-2} \text{s}^{-1}$ in garden) (Fig. 6.2a). Stomatal conductance was remarkably decreased under salinity stressed habitats than those of the sweet water counterparts (Fig. 6.2b). In *B. gymnorrhiza* and *E. agallocha*, the salinity imposed restriction of stomatal conductance was noticed about 44%; in *P. paludosa* and *X. granatum*, it was nearly 52% and in *H. fomes* 25%.

Protein Analysis

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 nonsaline replica and molecular weight ranged between 169.1 and 66.67 kDa (nonsaline) and 210.7 and 66.11 kDa (saline). *Excoecaria* showed the same number of bands in both habitats having molecular weight ranged between 205.8 and 65.55 kDa (nonsaline) and 213.2 and 77.72 kDa (saline). The highest number of protein bands appeared in *Heritiera* from both the environments, nine bands in each having molecular weight 211.2–26.71 kDa in nonsaline and 212.2–37.0 kDa in saline taxa. One extra band appeared in nonsaline *Phoenix* than its saline pair, and the molecular weight ranged between 201.3 and 46.43 kDa and 213.2 and 46.0 kDa, respectively. In *Xylocarpus*, one more band was expressed in saline plant, having 202.8–50.57 kDa (nonsaline) and 197.3–58.27 kDa (saline) (Fig. 6.2c).

Total Protein

Total leaf protein was estimated from the five enough mature taxa, grown in both saline and freshwater environment. In all five species, the total protein content showed higher amount in freshwater grown plants than those of their Sundarbans counterparts (salt stress environment).

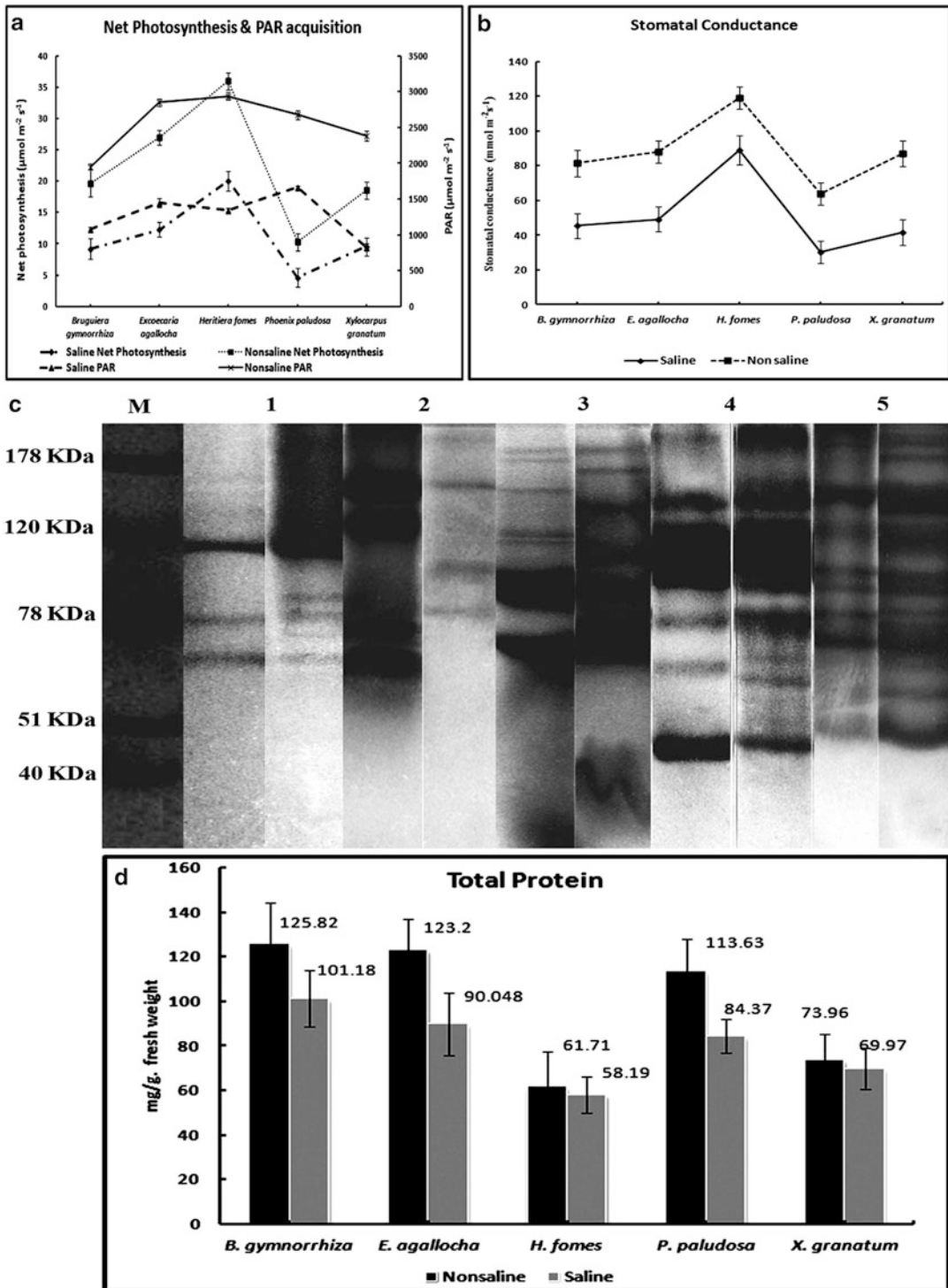


Fig. 6.2 (a) Net photosynthesis among the five investigated mangrove taxa in saline and nonsaline habitat; (b) Stomatal conductance in two different habitats; (c) SDS-

PAGE photograph of total leaf proteins; (d) Amount of leaf proteins in two different habitats along with standard error bars

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*, freshwater habitat showed 57% more protein content than that of the *in-situ* habitat (Fig. 6.2d).

Native Gel Electrophoresis

Peroxidase (PRX)

Band expression obtained from gel electrophoresis revealed that *H. fomes* and *X. granatum* showed the same number of isoforms in two different habitats, whereas in *B. gymnorrhiza*, *P. paludosa*, and *E. agallocha*, the numbers of isoforms were higher in Sundarbans species than those of their replicas from freshwater condition. 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}), whereas the freshwater individual showed five isoforms with highest OD 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 freshwater habitats highest OD values were 166.0 (0.07 R_{mf}) and 89.9 (0.07 R_{mf}), respectively. Nonsaline *Phoenix* and *Excoecaria* showed three and two isoforms of PRX, and saline partners expressed four and three isoforms, respectively (Fig. 6.3).

Superoxide Dismutase (SOD)

The experimental data showed that in all five species, isoforms of SOD expressed in less number from the freshwater grown individuals than those of their saline replicas. All four species expressed three isoforms in nonsaline 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 (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 nonsaline habitat. In *Xylocarpus* and *Phoenix*, saline and freshwater 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 nonsaline habitats as 170.07 OD (0.31 R_{mf}) and 163.0 OD (0.33 R_{mf}) respectively (Fig. 6.4).

Esterase (EST)

From the stained gel, it revealed that EST expression in all species from freshwater habitats were two isoforms, except *Xylocarpus* (single band) and *Excoecaria* (three bands). The comparative band intensities were 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 nonsaline 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 on 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 nonsaline it was 102.27 OD (0.24 R_{mf}) (Fig. 6.5).

Acid Phosphatase (ACP)

Among the five investigated taxa, all four species showed excess number of 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 nonsaline 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

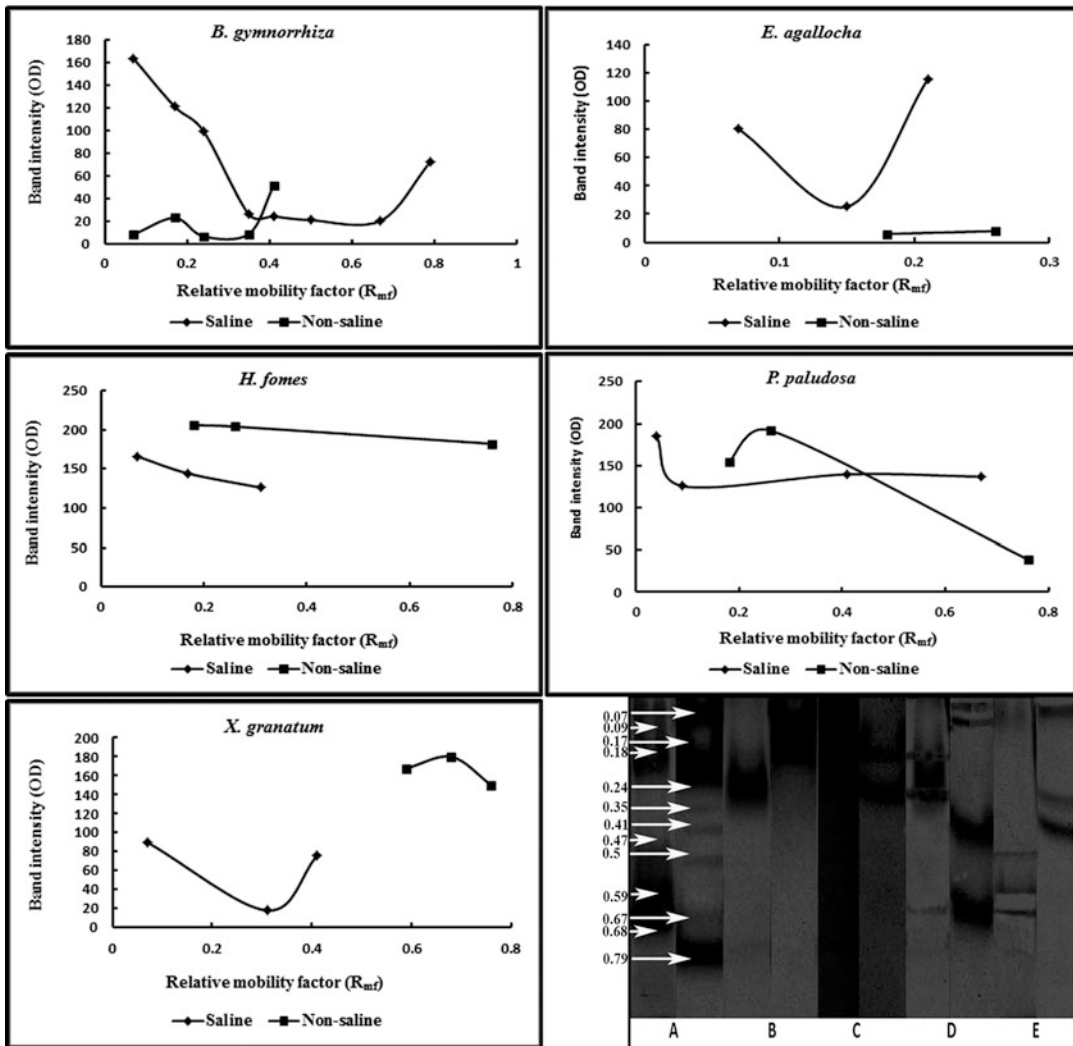


Fig. 6.3 Band intensities and relative mobility factors (R_{mf}) of peroxidase enzyme among the investigated taxa: a comparative graphical representation of two habitat plants

OD (0.32 R_{mf}), but the freshwater plant has only one band with 124.0 OD (0.53 R_{mf}). In both *Xylocarpus* and *Phoenix*, saline environment expressed one more isoforms than those of their reverse habitat (three isoforms were expressed in freshwater 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 R_{mf} value were almost same (248.0 OD and 0.46). In *ex-situ* plant of *Phoenix*, the highest intensity is 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 nonsaline plant (Fig. 6.6).

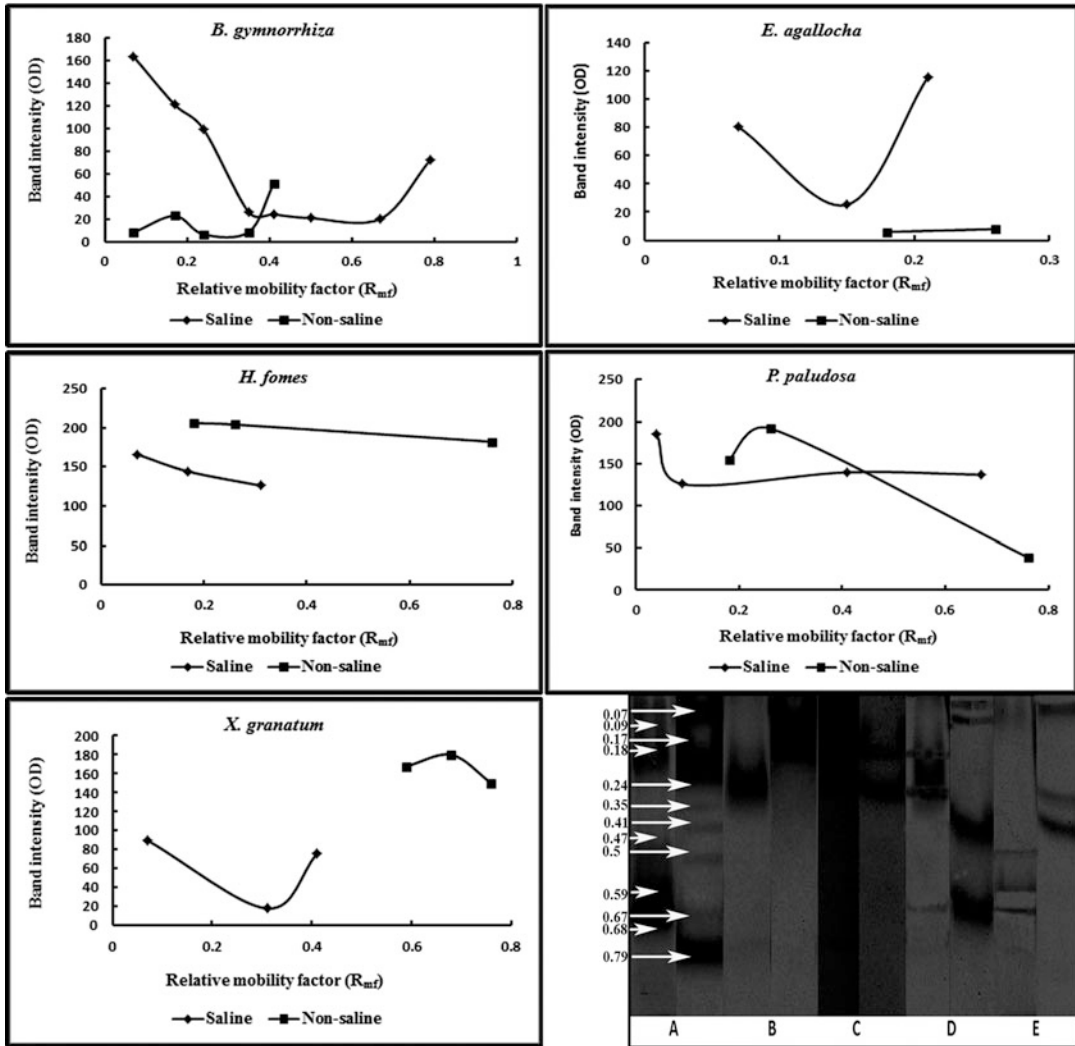


Fig. 6.4 Band intensities and relative mobility factors (R_{mf}) of superoxide dismutase (SOD) enzyme among the investigated taxa: a comparative graphical representation of two habitat plants

Quantitative Assay of Enzymes

The plant species from saline environment showed all four (PRX, SOD, EST, and ACP) investigated enzymes were in higher quantities than those of their freshwater 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. 6.7a).

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. 6.7b). Similarly, EST was highest increased in *Phoenix* (287%), *Bruguiera* (257%), and *Heritiera* (241%), and lowest in *Excoecaria* (154%) (Fig. 6.7c). ACP reached its maximum increment in *Bruguiera* (293%) and *Xylocarpus* (267%) and lower in *Excoecaria* (139%) (Fig. 6.7d).

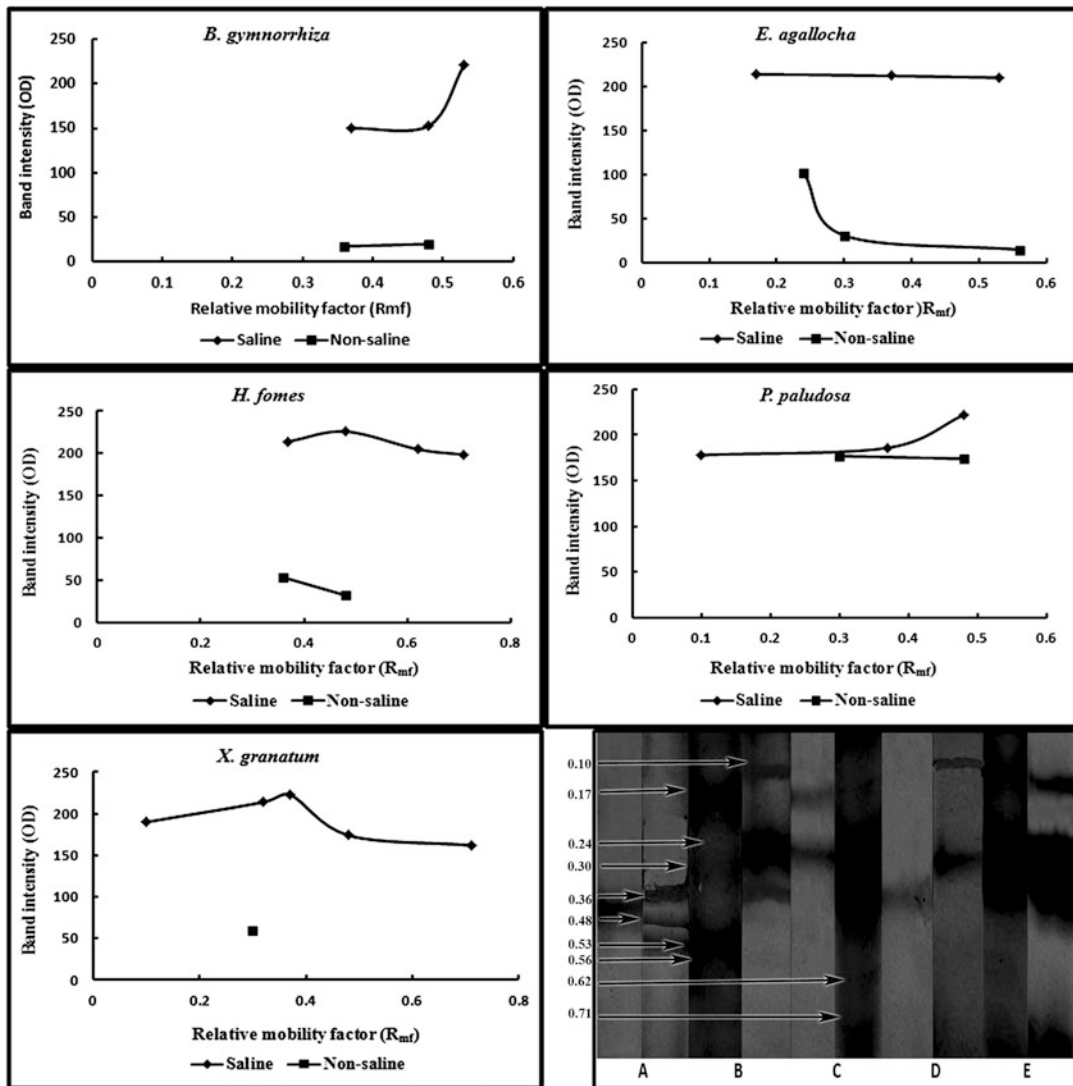


Fig. 6.5 Band intensities and relative mobility factors (R_{mf}) of esterase enzyme among the investigated taxa: a comparative graphical representation of two habitat plants

Statistical Analysis

Estimated total protein and four enzymes from two habitats were taken into account. A two-tailed bivariate correlation coefficient (Pearson coefficient) was calculated among each parameter (Table 6.1). The analysis showed that in case of the relationship between protein and SOD, all species in saline environment have inverse rela-

tionship (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

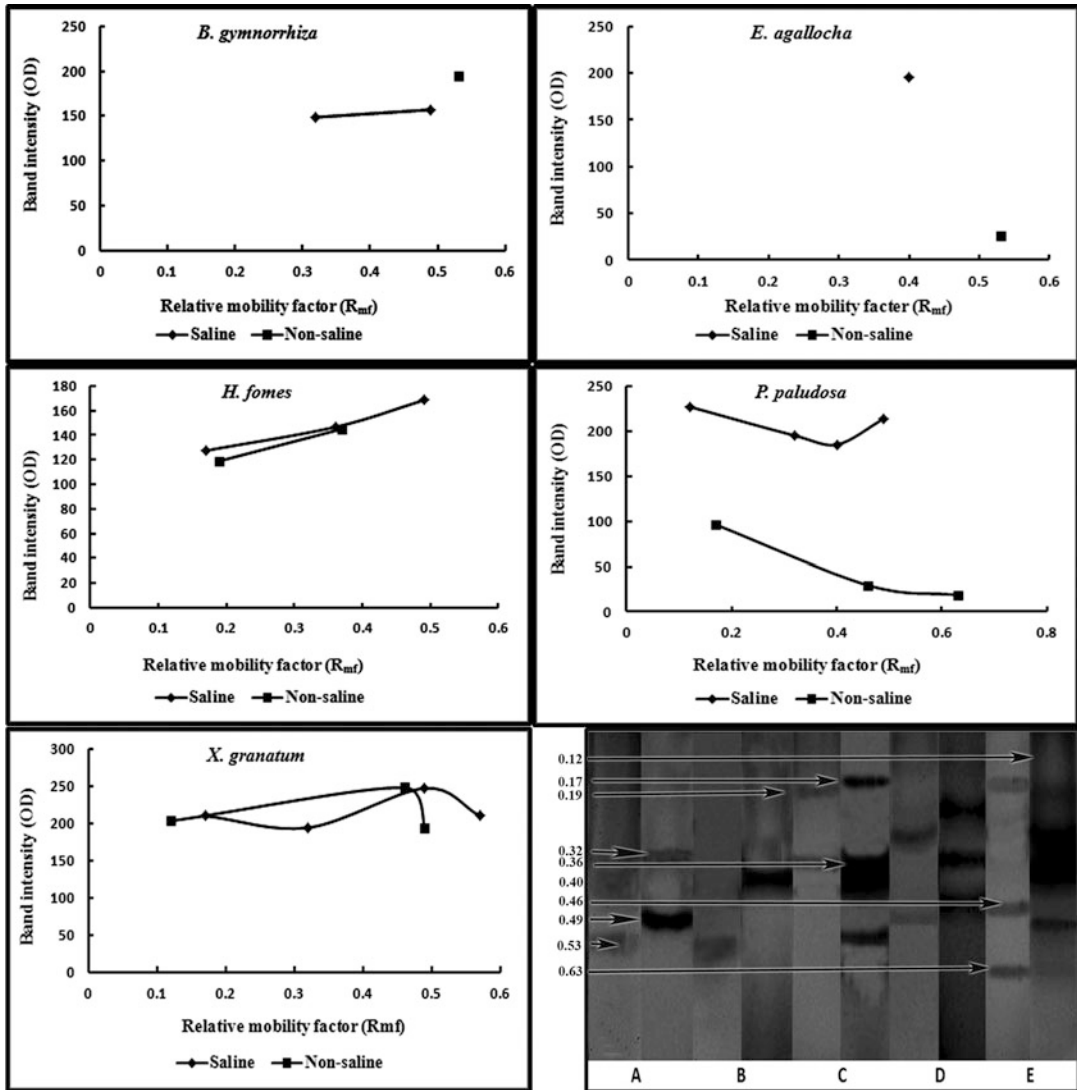


Fig. 6.6 Band intensities and relative mobility factors (R_{mf}) of acid phosphatase enzyme among the investigated taxa: a comparative graphical representation of two habitat plants

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, whereas in case of other plants, it showed no relationship.

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 approach of rate 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*, the optimum

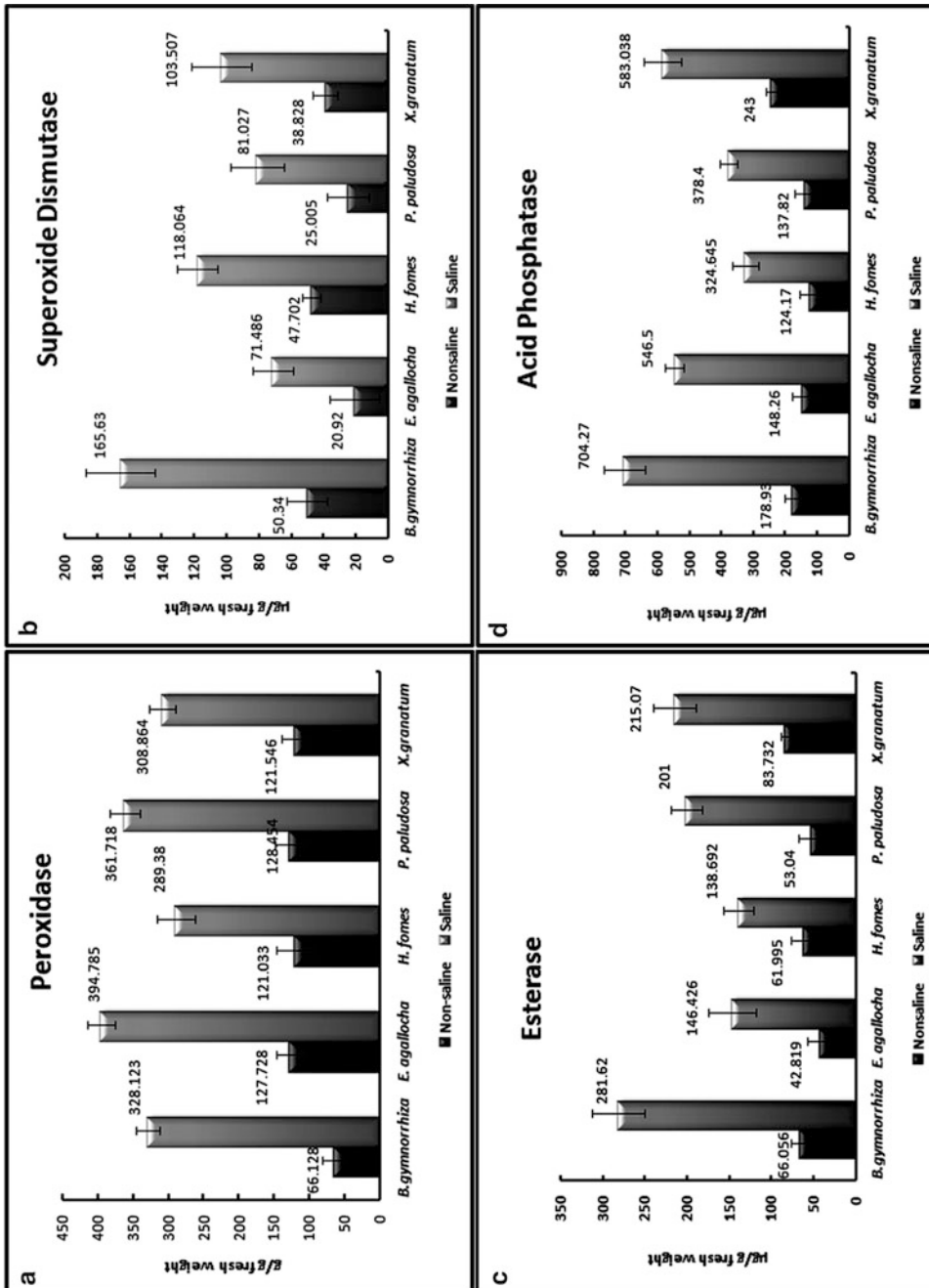


Fig. 6.7 Quantitative estimation of four (a, b, c, and d) enzymes among the taxa, in two habitats. Bars indicates as standard error

Table 6.1 Correlations among the different enzymes and total proteins in the plants of two habitats

Species Treatment	<i>B. gymnorhiza</i>		<i>E. agallocha</i>		<i>H. fomes</i>		<i>P. paludosa</i>		<i>X. granatum</i>	
	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*
	0.110	0.301	0.308	-0.442	0.795**	-0.348	0.698*	0.187	0.555	-0.013
	0.213	0.667*	0.603	-0.66*	0.468	0.099	0.554	-0.517	0.518	-0.363
	-0.206	0.430	-0.510	-0.65*	0.192	-0.9**	-0.254	0.495	-0.407	-0.403

Ns nonsaline, S saline

*Significant at 0.01%; **Significant at 0.05%

PAR requirements were higher for maximum photosynthesis in Sundarbans than those in the mesophytic taxa, whereas the peak photosynthesis rates were higher in the nonsaline 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 that *B. sexangula* prefers low salinity combined with low light intensity. Cheeseman and Lovelock (2004) experimentally proved that in *Rhizophora mangle* under low saline condition, net CO₂ exchange and photosynthetic electron transport become light saturated at less than 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. 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 (Datta) 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 25–52% under salinity stress that effectively limited CO₂ influx. Although reduced stomatal conductance imposed by high salinity restricts CO₂ diffusion, but may elevate the CO₂ partial pressure across the stomata, that utilized by mangrove leaves to maintain a consistently moderate rate of photosynthesis throughout the day, leading to avoid CO₂ starvation and photoinhibition. This result is well accord with Cowan (1982), Nandy (Datta) 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 1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) to the present data. The opposite phenomenon occurred in *H. fomes* and *X. granatum* can be explained as less affinity of these species toward 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 (Datta) et al. 2005). The effect of salinity stress on the photosynthetic enzyme activities pos-

tulated to be a secondary effect mediated by the reduced CO₂ partial pressure in the leaves caused by the stomatal closure (Lawlor and Cornic 2002; Meloni et al. 2003; DeRidder and Salvucci 2007). The present study also reveals that in all the mangroves grown in nonsaline soil, an increased rate of assimilation is coupled with increased stomatal conductance.

All the five investigated mangrove taxa from freshwater habitat showed an increase amount of total leaf protein than those of their saline 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, and 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 provides amino acids for synthesis of new proteins suited for growth or survival under the modified condition. Mansour (2000) reported that protein biosynthesis declines under salt stress condition, while cells preferentially synthesize 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). In the present investigation, the degradation of proteins in salt habitat *Heritiera* and *Xylocarpus* was lesser amount than the other three taxa investigated probably leading to 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 decreased 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, in this chapter we estimated two – peroxidase (PRX) and superoxide dismutase (SOD). Qualitative and quantitative study of two antioxidant enzymes (PRX and SOD) and two other important (hydrolyzing) enzymes (EST and ACP) from saline and freshwater grown plants revealed that in most of the cases number of isoforms, band intensity, and enzyme expression were higher in salt-stressed plant. 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 ($\cdot OH$) (Halliwell and Gutteridge 1985). These cytotoxic ROS may seriously affect the normal metabolism through oxidative damage of lipids, proteins, and nucleic acids (Fridovich 1986). During photosynthesis, the internal O_2 level becomes high and chloroplast is 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. 2004a, b). The present work resulted that both PRX and SOD expressions were high in saline plants and the increments were ranged between 139 to 257% in case of PRX and 147 to 241 in SOD. The present result was substantiated with the earlier works (Cheeseman et al. 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 those of the other three species of saline habitat. Parida et al. (2004b) 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 *Xylocarpus* (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 freshwater counterparts. Therefore, it is evident that the salt imposed production of toxic ROS is mostly regulated by upregulation of antioxidative enzymes like PRX and SOD. Sahu and 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 freshwater 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 nonsaline – 2) and *Xylocarpus* (saline – 5; in nonsaline – 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 and 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 acetyesterified 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 the magnitude of increment in saline grown plants occurred ranging from 139 to 293%. It may be due to the fact that under stressful conditions, 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 modulating osmotic adjustment by free phosphate uptake mechanism (Fincher 1989). Jain et al. (2004) also demonstrated that in 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 cotransported 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 gymnorhiza*, *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), 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 forces 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 toward the proper salt management mechanism for sustainable existence in the hostile environment. Therefore, the absence of it, might be one of the reasons toward less adaptability 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 baseline information and a system necessary to conduct future research in relation to the genetic basis of salt tolerance.

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