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Research Article

Differential Changes of Protein and Isoforms of Some Antioxidative Enzymes under NaCl Salinity in a Mangrove, *Xylocarpus granatum* Koen

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Abstract

The effect of NaCl stress on protein content and antioxidant enzyme activity of *Xylocarpus granatum* was investigated. Salt stress was imposed on 60-days old plants with five different concentrations of NaCl (0, 50, 100, 150, 200 and 250 mM). NaCl induced significant differences in quantities of proteins. Nacl stress enhanced activities of SOD, CAT and POX. Further, in isoenzyme studies, 3 SOD isoenzymes (SOD1, SOD2 and SOD3), and 7 POX isoenzymes were detected with the treatment. Increase in antioxidant enzyme activity could be a response to an increased levels of free radicals induced by NaCl. This might have reduced the stress severity and allowing the plant to grow in a mangrove saline habitat.

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INTRODUCTION

Mangrove forests are widely distributed in the inter-tidal zones of the tropical and subtropical areas of the globe [1]. The mangrove plants in the forest adapt to harsh environments like marshy anoxic anaerobic soil and fluctuating salinity of the water bodies with several morphological and anatomical features[2]. The mangroves are divided in to secretor (possess salt gland or salt hairs) and non-secretor (do not posses such salt secreting features) groups, on the basis of their salt management strategies. They manage salt stress in a variety of ways, like ultrafitration by the roots, Na^+/ H^+ exchange and sodium accumulation in the xylem sap [3]. Salinity tolerance is defined as the ability of plants to continuously grow under salt stress conditions [4]. Out of several cosmopolitan species of mangrove, *Xylocarpus granatum* is an important species because it withstands varying soil

conditions, high levels of salinity and lack of freshwater inflow for a considerable period of time and pollution of varying forms [5].

In the present investigation an attempt has been made to study the leaf protein profile and behavior of some antioxidative enzyme systems in *Xylocarpus granatum* grown under saline conditions. On the basis of results the probable mechanism of salt tolerance in the species has been discussed.

MATERIAL AND METHODS

Plant Material

Seeds of *Xylocarpus granatum* were collected from Achara mangrove forest (16^0 13' N and 73^0 26' E) of Maharashtra, India. Seeds were sown in the pots. Seedlings were raised from seeds in clay pots containing a mixture of sand, Farm Yard Manure and red soil in the proportion of 1:1:1. They were irrigated regularly. Two months old seedlings were subjected to salt stress with different NaCl concentrations (0, 50,100,150, 200 and 250 mM)

Protein estimation and SDS-PAGE analysis

Total protein content was determined following the method of Lowry *et al*. [6]. Extraction of proteins for gel electrophoresis was done from 2 g of fresh leaf. Leaf samples were macerated in a mortar with pestle, and extracted in 5 mL of extraction buffer containing 10% (w/v) SDS, 10 mM β- Marcaptoethanol, 20% (v/v) glycerol, 0.2 M Tris/HCl (pH 6.8) and 0.05% Bromophenol blue. The extracts were centrifuged 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 [7] and stained with Coomassie Brilliant Blue R-250 (Sigma). Molecular weights of different protein bands were determined with the help of standard protein markers (Sigma, pre stained 46 – 205 kDa).

Assays of Antioxidant enzymes

Two grams of young leaf buds were macerated to powder with liquid nitrogen in a mortar with pestle, 0.1 g PVP and 5 mL of extraction buffer (consisting of 1 M Sucrose, 0.2 M Tris-HCl; pH adjusted at 8.5) were added and homogenized. The extracts were centrifuged at 10,000 rpm for 20 min at 4°C. Supernatants were used as samples for enzyme assay and gel electrophoresis.

Catalase (CAT; E.C.1.11.1.6) activity was measured by change in absorbance at 240 nm [8]. An assay mixture contained 3 ml H_2O_2 - phosphate buffer (0.64 mL of H_2O_2 diluted to 100 mL with 0.1M phosphate buffer pH 7.0) and 0.2 mL enzyme extract. The amount of enzyme required to change the absorption (Δ OD) by 0.01 min^{-1} mg^{-1} protein was taken as unit enzyme activity.

Peroxidase (POX; E.C. 1.11.1.7) activity was measured by the change in absorbance at 470 nm due to guiacol oxidation in the presence of H_2O_2 and enzyme [9]. The assay mixture contained 2 mL 0.1M phosphate buffer (pH 7.0), 1 mL 20 mM guaiacol, 0.05 mL H_2O_2 (20 mM) and 0.5 mL enzyme extract. The amount of enzyme required to change the absorption (Δ OD) by 0.01 min^{-1} mg^{-1} protein was taken as unit enzyme activity.

The activity of superoxide dismutase (SOD; E.C. 1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Beauchamp and Fridovich [10]. The reaction mixture (3 mL) contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, 2 µM riboflavin and 0.1 mL enzyme extract. Samples were illuminated using two 15 W fluorescent lamps for 10 min. The absorbance of reaction mixture was read at 560 nm on UV-VIS double beam spectrophotometer (HITACHI U-2910, America). A non-irradiated reaction mixture served as control. Log A560 was plotted as function of the volume of enzyme extract used in the reaction mixture [11]. In the resultant graph the volume of enzyme extract corresponding to 50% inhibition of the reaction was considered as one enzyme unit.

Analysis of Isoenzymes of Superoxide dismutase and Peroxidase

Separation of isoenzymes of SOD and POX were performed by Native PAGE (12.5 % polyacrylamide) by loading 50 µg of protein for POX and 100 µg for SOD. The gels were run in an electrode buffer composed of 0.025 M Tris and 0.192 M glycine (pH 8.8) for 3 h at 4°C at a constant current of 30 mA. After electrophoresis, the gels were stained for SOD by solution containing 2.5 mM NBT for 20 min. in dark and followed by incubating in 50 mM potassium phosphate buffer (pH 7.8) containing 28 µM riboflavin and 28 mM EDTA, for 20 min in dark and then exposed to light for 15 min [12]. The gels were stained for POX by incubating them in a freshly prepared solution of 10 mM O-dianisidine in 0.1 M Acetate buffer (pH 4.5) for 30 min. and then transferred to Acetate buffer containing 3% H_2O_2 until peroxidase bands were visible [13].

Statistical Analysis

The data presented here is based on mean of six independent experiments. All the data was subjected to one-way analysis of variance and significance was determined at 95% confidence level using SPSS-11.5 version.

RESULTS

Effect of NaCl on proteins

NaCl stress resulted in the reduction of amount of proteins. It was decreased to 80.17, 63.26, 50.36, 42.09 and 45.72% over control at 50, 100, 150, 200 and 250 mM NaCl respectively. A significant decrease was noticed with increasing salt concentration (Table 1).

Effect of NaCl on protein banding pattern

Qualitative differences in the expression of proteins were observed with increase in the level of salt stress (Figure 1A). The intensity of 45, 48, 73 and 97 kDa proteins was increased in NaCl treated plants as compared to control, whereas, the intensity of 117 kDa protein was increased with increasing salt concentrations upto 150 mM and later decreased at 200 and 250 mM NaCl. A protein of 97 kDa showed higher intensity with increasing the level of salinity stress.

Effect of NaCl on the activity and isoenzyme pattern of antioxidant enzymes

An increase in the activity of SOD, POX and CAT was observed as a function of external NaCl concentration (Table 1). According

Table 1: Effect of different concentrations of NaCl on the activity of enzymes SOD, POX and CAT and protein content of the leaves of *Xylocarpus granatum*.

NaCl (mM)	Protein content	Activity of enzymes		
		SOD	POX	CAT
	(mg 100^{-1}g FW)	(ΔOD min^{-1} mg^{-1} protein)		
Control (0)	17.91 ± 0.195 [a]	0.61 ± 0.001 [a]	1.18 ± 0.074 [a]	0.13 ± 0.005 [a]
50	14.36 ± 0.058 [b]	0.68 ± 0.001 [a]	0.88 ± 0.033 [a]	0.32 ± 0.018 [b]
100	11.33 ± 0.070 [c]	0.82 ± 0.001 [b]	1.05 ± 0.098 [a]	0.77 ± 0.022 [b]
150	9.02 ± 0.160 [d]	1.21 ± 0.003 [c]	1.37 ± 0.166 [b]	2.32 ± 0.091 [d]
200	8.19 ± 0.403 [e]	2.28 ± 0.002 [d]	2.16 ± 0.130 [c]	2.49 ± 0.019 [d]
250	7.54 ± 0.461 [f]	1.61 ± 0.002 [c]	2.62 ± 0.213 [c]	1.78 ± 0.030 [c]

Different letters indicate statistically different values at p≤0.05, ± S.E., n=6.

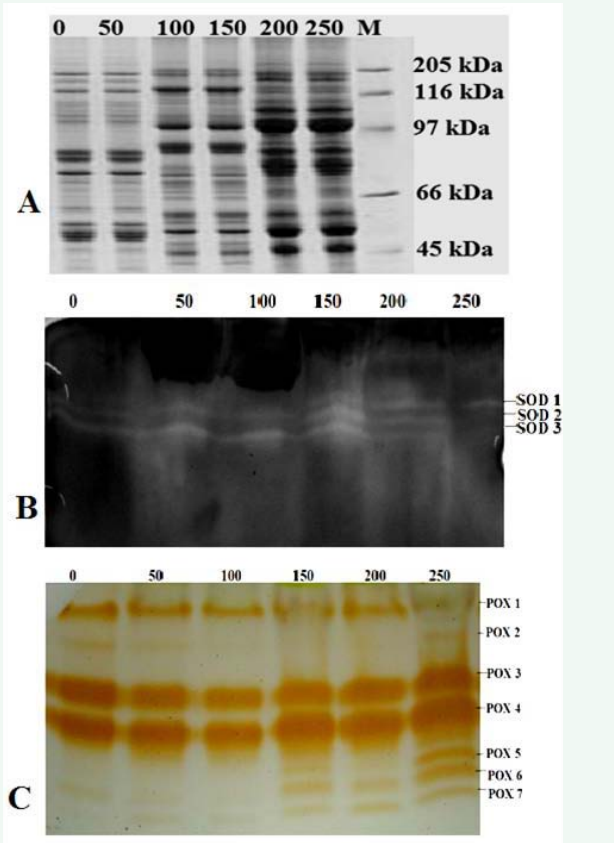

Figure 1 Changes in Protein expression (A), SOD isozymes (B) and POX isozymes (C) in *Xylocarpus granatum* under salinity stress.

to PAGE analysis the two SOD isozymes were observed in control as well as salt treated plants (SOD1 and SOD2). Induction of an additional isoenzyme (SOD3) due to salinity (Figure 1B) and all the isoenzymes were more prominent in salt treated plants which is in agreement with spectrophotometric assay. As compared to five isoenzymes (POX1, POX2, POX3, POX4 and POX5) in control, seven isoenzymes of peroxidase (POX1, POX2 POX3, POX4, POX5, POX6 and POX7) were observed in NaCl treated plants (Figure 1C). At 150, 200 and 250 mM NaCl treatment the isoenzyme bands appeared more prominent.

DISCUSSION

The decrease in protein content observed at higher concentrations of NaCl may be due to decrease in the synthesis of protein [14] or stimulation of protein hydrolysis [15]. The reduction in protein content due to salinity or water stress may be the result of proteolysis or inhibition of protein synthesis [16]. Several authors have reported a considerable decrease in the protein content of leaves in glycophytes [17-19] and in a mangrove species *Bruguiera parviflora*, in response to salinity [20]. The present results are in accordance with Rajesh *et al*. (1999), who experimentally reported that in *Ceriops,* the total leaf protein content was decreased under higher concentrations of salt in the medium [21]. SDS-PAGE analysis revealed the differential expression of proteins in the *Xylocarpus granatum*. The expressed proteins are having the molecular weight ranging from 45 - 190 kDa. The bands of low molecular weight proteins such as 45, 48, 73 and 97 kDa have shown higher intensities under salinity treatment. Several authors have reported that low molecular weight proteins ranging from 22-127 kDa accumulate in the plants like *Raphanus sativus* [22], *Arachis hypogea* [23]) and Maize [24]) under saline conditions. The intensity of 117 kDa protein in *Xylocarpus granatum* was decreased at higher salt concentrations. It has been reported that salinity causes decrease in the intensity of several proteins in *Bruguiera parviflora* [25]. A qualitative study of two antioxidant enzymes, POX and SOD, showed their higher expression under saline environment. 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^- during aerobic metabolism to produce Reactive Oxygen Species (ROS) such as superoxide(O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-) [26]. These cytotoxic ROS may seriously affect the normal metabolism through oxidative damage of lipids, proteins and nucleic acids [27]. During photosynthesis, the internal O_2 level becomes high and chloroplasts are prone to generate ROS at that time [28]. Plants synthesize a number of antioxidative enzymes to counteract these ROS, especially SOD converts O_2^- into H_2O_2 and POX degrades H_2O_2 [29]. In the salinity stress imposed plants, the balance between the production of ROS and the scavenging activity of the antioxidants becomes disrupted, which ultimately results in oxidative damage. Mangroves with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to such oxidative damage [30-33].

CONCLUSION

On the basis of experimental results it may be concluded that, under saline conditions, *Xylocarpus granatum* can activate antioxidant defense mechanism to resist the salt induced oxidative stress, which might be an effective means of *Xylocarpus granatum* that grows in a mangrove saline habitat.

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