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Research Article

Expression Studies of Selected Gene Candidates and Isolation of Total Leaf Proteins of *Rhizophora mucronata* Lam. in Response to Salinity

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Abstract

Expression studies help to know the activity of genes and proteins in response to a set of conditions at a given time. In the study, the expression of selected genes, BADH (Betaine Aldehyde Dehydrogenase), NHX1, P5CS (Pyrroline-5-carboxylate synthase), Cytochrome P450, Abscisic acid (ABA) encoding gene, Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH), Salt Overly Sensitive (SOS1) and Aquaporin were analyzed in *Rhizophora mucronata* Lam., their expression analysis under different salt concentration was done by using real-time PCR technique and the genes showed early and delayed expression. The gene products were sequenced and the partial sequence was obtained. Total leaf proteins in *Rhizophora mucronata* Lam. at different saline conditions were isolated and analyzed using two - dimensional gel electrophoresis.

Keywords: *Rhizophora mucronata Lam., salinity, expression analysis, genes, proteins, tolerance.*

Introduction

Genes and proteins are involved in balancing salt uptake from the soil and their movement throughout the plant, ultimately bringing about salt tolerance. Understanding the genes involved in tolerance mechanism helps in improving the tolerance property of crop plants and it is seen that overexpression of a single gene can improve the salt tolerance property (Liang, *et al*., 2017). In this study, we are looking for the expression levels of some of the genes reported to be involved in plant salt tolerance in *Rhizophora mucronata* Lam., which is a salt-tolerant mangrove species. P5CS (Pyrroline-5-carboxylate synthase), NHX1, Betaine Aldehyde Dehydrogenase (BADH), Cytochrome P450, Abscisic acid (ABA) encoding gene, Nicotinamide Adenine Dinucleotide Phosphate (NADPH), Salt Overly Sensitive (SOS 1) and Aquaporin were the genes selected for the study.

BADH and P5CS genes synthesize glycine betaine and proline respectively, they are the important osmoprotectants in plants during salt stress. NHX1 is a Na^+/H^+ antiporter that pumps sodium ions into the vacuoles, whereas the Salt Overly Sensitive (SOS1) gene is involved in Na+ and K+ homeostasis (Shi, *et al*., 2000, Hassan, *et al*., 2015). ABA gene involved in the synthesis of the stress hormone, Abscisic acid. Cytochrome P450 functions in the production of secondary metabolites and NADPH oxidase is the key enzyme in superoxide production. Plant NADPH oxidase is also known as respiratory burst oxidase homologues (RBOHs) (Villa-Ruano, *et al*., 2015, Marino, *et al*., 2012). Reports by Krishnamurthy, *et al*., 2014 showed that gene encoding cytochrome P450 may control suberin biosynthesis and increase the efficiency of salt tolerance in *Avicennia officinalis*. Aquaporins are membrane channels that function in the transport of water during

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the plant's physiological processes. Gene activity changes with change in salinity (Munns, 2005), in the study we analyzed the expression of genes at different salt concentration. Proteins are also involved in bringing about salt tolerance in plants, their structure and activity level varies under different stress responses (Kosova, *et al*., 2013). Proteomic studies showed the presence of photosynthesis activation protein, defense-related proteins, ion transporters, proteins involved in the activation of a protective compound, etc. (Wang, *et al* ., 2009).

Materials and Methods

Plant Sample Collection

Rhizophora mucronata Lam. propagules were collected from Melur area [11°46'31.37" N 75°27'52.52" E], Thalassery and grown in polythene bags filled with potting mixture (soil, sand and organic manure in 1:1:1 ratio) in a greenhouse, watered with tap water for a year. Plants were treated with different concentrations of salt for experimental purpose and the non-salt treated plants were kept as control. In the experiment, the plants were watered with 0 to 30 % salt water (30% salt water equivalent to 5M sodium chloride) Leaves were used for the study.

RNA Isolation

Before RNA isolation, tips, tubes, mortar and pestle were treated with 0.1% DEPC (Diethyl pyro carbonate) water overnight and autoclaved to remove DEPC. Leaves were surface sterilized and frozen immediately. RNA isolation protocol followed was that of Rubio - Pina and Zapata – Perez, 2011. Leaf sample was powdered using liquid nitrogen and transferred to tubes, to this 900 µl of extraction buffer [2% (w/v) Cetyltrimethylammonium bromide (CTAB), 20mM Ethylene Diamine Tetra Acetic acid, 0.1 M Tris - HCl (pH: 8), 2% (w/v) Polyvinyl poly pyrrolidone (PVPP), 1.4 M NaCl] and 2-mercaptoethanol was added to make a final volume of 1ml, mixed well and kept at 65 ^{o}C for 10 min. Tubes were shaken 3 - 4 times during incubation. After incubation, added 800µl chloroform and mixed well. Centrifuged the samples at 10000 rpm for 10 min at 4 ^{o}C. The supernatant was transferred to a new tube and 800 µl phenol- chloroform in the ratio 1:1 was added, mixed well and centrifuged at 10000 rpm for 10 min at 4^{o}C. The supernatant was collected and mixed with an equal volume of chloroform – isoamyl alcohol (24:1) and centrifuged at 10000 rpm for 10 min at 4 ^{o}C. The supernatant was transferred to a new tube and RNA was precipitated by adding 1/3rd volume of Lithium chloride (8M), kept at -20 ^{o}C overnight. Samples were centrifuged at 10000 rpm for 20 mins at 4 ^{o}C. RNA was pelleted and the pellet was washed in ethanol (100% and 70% respectively). The RNA pellet was air-dried and dissolved in DEPC water. For the purification of RNA, phenol-chloroform extraction was repeated and RNA precipitated by adding 0.1 volume sodium acetate (3M) and 2 volume 100 % ethanol and incubated at -20 ^{o}C overnight. This was centrifuged at 13000 rpm for 15 mins at 4 ^{o}C. The RNA pellet was washed with 70 % ethanol, air-dried and dissolved in DEPC water.

RNA Quantification

RNA samples were quantified using Biophotometer (Eppendorf). The ratio of absorbance at 260 and 280 gives the purity of RNA samples. The quality of RNA samples was checked by 1% agarose gel electrophoresis.

cDNA Synthesis

The RNA samples were DNase treated and cDNA was synthesized using Oligo (dT) primer and reverse transcriptase (200 U/L). Total RNA (approximately 1µg) was mixed with 2 µl Oligo (dT) primer and 2 µl dNTP mix (10mM) and incubated at 65 °C for 5 minutes. The mix was immediately chilled on ice and added 8 µl of 5x first strand buffer, 1 µl of RNasin, and 2 µl RTase (reverse transcriptase enzyme). The reaction was adjusted to a total volume of 20 µl with molecular biology grade water and the mix was kept at 42 °C for 60 minutes for incubation, and followed by reaction inactivation at 72 °C (10 minutes). (All the reagents were purchased from Thermo scientific).

Primer Designing and Standardization

Genes involved in salt tolerance were selected and the sequence from a related mangrove species was used for primer designing. The raw sequence in FASTA format was loaded onto Primer3 plus software for primer designing. The most appropriate primer set was sent to Integrated DNA Technologies (IDT) primer synthesizing services for synthesis. The primer was diluted to 50 micromolar concentrations and amplified at different annealing temperatures within the range of primer melting temperature. PCR conditions: 95°C for 2 mins followed by 40 cycles of 95°C for 30s, primer annealing temperature for 30s and 72°C for 40s and72°C for 10 mins, PCR products were checked on 1 % agarose gel. The annealing temperature was selected based on absence of primer dimers and non-specific amplification.

Table 1: List of candidate genes used for the study

Gene	Function	Primer sequence
BADH	Glycine betaine synthesis	F:GAAGCGATCGTCGGTGTATT R:TCAACATTGGGATGAGATGC
P5CS	Proline synthesis	F:TCCAAGAGGAGTCCATCTGC R:GCAGGAAGCTGGGTTAAGAA
NHX1	Na+/H+ antiporter	F: CAGTTCACCAACAGGTGCAG R:ACCTCCATAGCACGCCTCT
Aquaporin	Water channel	F:TCGGCCTTTTCTTTGTCATC R:TGTTGGCACCCACTATGAAA
CytochromeP450	1^{o} and 2^{o} metabolism	F:ATGTCCTGCTCAGGCTCACT R:CAACCCCAAGACGTCTCATT
SOS1	Na^{+} and K^{+} homeostasis	F:CCACCATCCTCTGCAAACTT R:TCACATTGGTACCCTGCAAA
ABA	Plant developmental processes, Senescence	F:AACAGGGCCTTGGTAGGACT R:TGAAAGCAAACGCAAGATCA
NADPH Oxidase	Production of superoxide	F:AAGAGAGCACAGAGGGCAAT R:GACCTTGCGTCACCTTCTTC

Housekeeping gene	Function	Primer sequence
Actin	Cytoskeleton structural protein	F: CCTTCCAGCAGATGTGGATT R:ATGACAAGGGTGGAAAGCTG
TUB1	Component of microtubule	F:CGGCAGACGAGGATGAGTAT R:CGACCCAAAATAGGTGCGTA
GAPDH	Glycolysis	F:AGCAAGCAGAAGGATGACAAA R:TTGCCAACCCCTTCTTACAC
18srRNA	Ribosomal RNA	F: ACCTTTTAGGCCACGGAAGT R:GTACAAAGGGCAGGGACGTA
EF1	Translation	F: GATGGACAGACTCGTGAGCA R:TCCCTCAAAACCAGAAATGG

Gene Expression Analysis

a. Housekeeping Gene Normalization

geNORM Analysis

geNorm is an algorithm based computer program used to find out the most stable reference genes for real time experiments. The analysis involve the stepwise exclusion or ranking process followed by geometric averaging for the selection of most stable control gene from a list of candidate housekeeping genes. The method, whereby 'pairs of genes' are compared using a simple ΔCt approach similar to that described by Vandesompele, *et al.*, The relative gene expression values were calculated using qbase analyzer, in this software the expression stability calculations are automatically performed. Relative gene expression values

for gene-specific efficiency were calculated. The relative gene expression values were imported to the geNorm program and ranked according to their expression stabilities. The program automatically calculates gene expression stability measure (M) for all housekeeping genes and eliminates the worst scoring housekeeping genes (that is, one with the highest M values).

b. Real Time PCR

The Real-time PCR analysis of control and target gene transcripts in cDNA samples was conducted in a Real-Time PCR System (Quant 3 studio, Thermo Scientific). The PCR reaction contained a cDNA template (1µg), 2X SYBR green master mix (10 µl) (Thermo Scientific), forward and reverse primers (concentration 50mM) and the volume was made up to 20 µL with sterile water. All the samples were analyzed under the following conditions: 95°C for 2 min followed by 40 cycles of 95°C for 30s, primer annealing temperature for 30s and 72°C for 40s. The PCR products were then subjected to melt curve analysis. The melt curve analysis was done from 95°C for 15sec and 60°C for 1 min to confirm that the resultant fluorescence originated from a single PCR product and did not signify primer dimers formed during the PCR or a non-specific product. No-template controls were also included for each gene to detect any false signals arising from the amplification of any DNA contamination or primer dimer formed during the reaction. The passive reference dye (ROX) included in the PCR master mix standardizes the SYBR green fluorescent signal. qPCR results were analyzed using QuantStudio design and analysis software. Experiments were done in triplicates. Ct values obtained from the analysis were used to calculate the fold change. All the genes were sequenced and the sequences were subjected to BLAST analysis to check the similarity of gene sequences.

Total Leaf Protein Isolation

Various methods were followed to isolate total leaf protein from *Rhizophora mucronata* Lam. Due to the presence of high polyphenols in the plant, the extraction was very difficult. A protocol reported earlier by Wang, *et al*., 2007 was modified. Leaf powder was resuspended in 8ml of extraction buffer [100 mM Tris (pH 8),100 mM EDTA,50 Mm borax,50 mM Vitamin C,2% PVPP,1.5% Triton X-100, 30% Sucrose and 2% b-mercaptoethanol], 0.5% SDS was also added. Samples were mixed for 10 minutes at room temperature and added 2 volumes of Tris-saturated phenol (pH 8) and the sample was further mixed for 10 minutes. Centrifuged the samples at 15000 g for 15 minutes at 4°C. Transferred the upper phase to a new tube and mixed with an equal volume of extraction buffer. The mixture was mixed for 10 minutes and centrifuged at 15000 g for 15 minutes at 4°C. To a new tube upper phase was transferred and the protein was precipitated using 5 volumes of 0.1 M ammonium acetate in methanol and kept at -20°C overnight. Protein was pelleted and washed using ice-cold methanol followed by ice-cold acetone twice and dried the pellet and dissolved in phosphate buffer saline (PBS).Proteins were further quantified and loaded on 12% SDS-PAGE and electrophoresed at 100V.

Two Dimensional Gel Electrophoresis

The protein pellet was dissolved in rehydration buffer containing 8 M urea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM Dithiothreitol (DTT), 0.2% Bio-Lyte 3/10 ampholyte, 0.001% Bromophenol Blue (Bio RadRehydration/Sample Buffer). IPG strips (Bio Rad) of 7cm (pH 3-10) was used for first-dimension separation. Protein sample at a concentration of 300µg was applied by placing the IPG strip gel side down over the sample (125µl for 7cm strip) on the rehydration tray overlaid with mineral oil and kept overnight for passive rehydration. After rehydration, mineral oil was blotted and the strips were placed on focusing tray. Connect the positive and negative electrodes. Place the paper wicks at the end of each electrode and wet the same using distilled water. Position the IPG strips such that wicks are between the end of the strip and the electrodes. The tray was fixed on the Bio Rad IEF system for Iso Electric Focusing and the condition was standardized as 100V for 20 minutes in linear

mode, 250V for 20 minutes in linear mode, 4000V for 2 hours in linear mode, 4000V to 10,000 Volt hours in Rapid mode. IPG strips were equilibrated after IEF in equilibration buffer 1 & 2 (Ready Prep 2-D Starter Kit Equilibration Buffer) for 15-20 minutes each. The strip was loaded on 12% SDS stacking gel and electrophoresed at 100V. The gel was stained by coomassie staining. Experiments were done in triplicates.

Samples for the Study

Propagules were grown and leaves of plants at the four-leaf stage (1-year-old) were used for the study. Soil compositions were analyzed from Pepper Research station, Panniyur. The pH of the soil was found to be normal (pH 7) with essential macro and micronutrients. Leaves were used for the study and were surface sterilized using 70% alcohol and RNase Zap before RNA isolation.

Results and Discussion

Figure 1: *Rhizophora mucronata* Lam. propagules planted in soil

Figure 2: *Rhizophora mucronata* Lam. control plants

RNA Isolation

Total RNA from *Rhizophora mucronata* was isolated by lithium chloride precipitation method. The quality and quantity of RNA were checked using Biophotometer (eppendorf) and agarose gel electrophoresis respectively. To assess the quality of RNA samples, the samples were electrophoresed on 1% agarose gel stained with ethidium bromide and bands were documented using the documentation unit (BioRad Chemidoc system). All the RNA samples isolated were of good quality. rRNA bands were intact with little or no degradation.

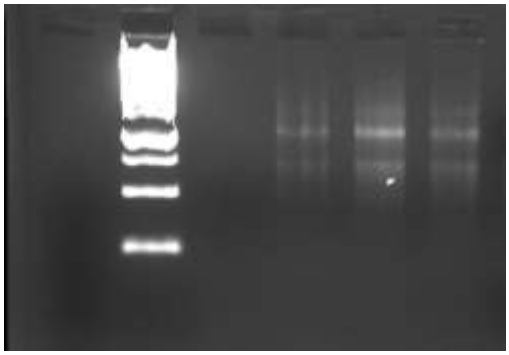


Figure 3: RNA bands on agarose gel electrophoresis

Quantification of total RNA

The quantity of total RNA in each sample was determined by measuring the absorbance using Biophotometer. The samples showed a A260/A280 ratio between 1.9 and 2.08 and A260/A230 ratio greater than 2.0 and the concentration of isolated RNA ranged from 50-200ng/µl.

cDNA Synthesis

Oligo dT primers and reverse transcriptase were used to synthesize cDNA from 1µg of DNase treated total RNA.

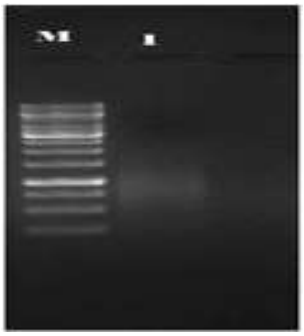


Figure 4: cDNA streak on agarose gel

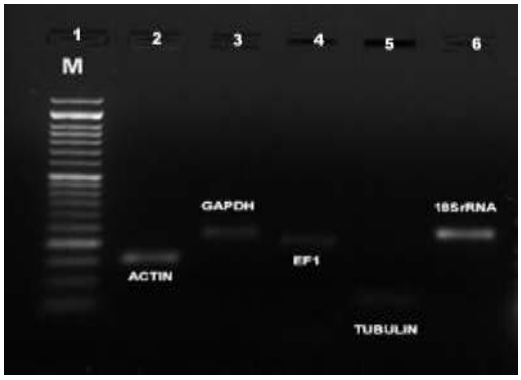
Primer Standardization

Optimal concentrations of forward and reverse primers were standardized for each gene. Annealing temperature which gave good amplicons without nonspecific products and primer dimers were selected.

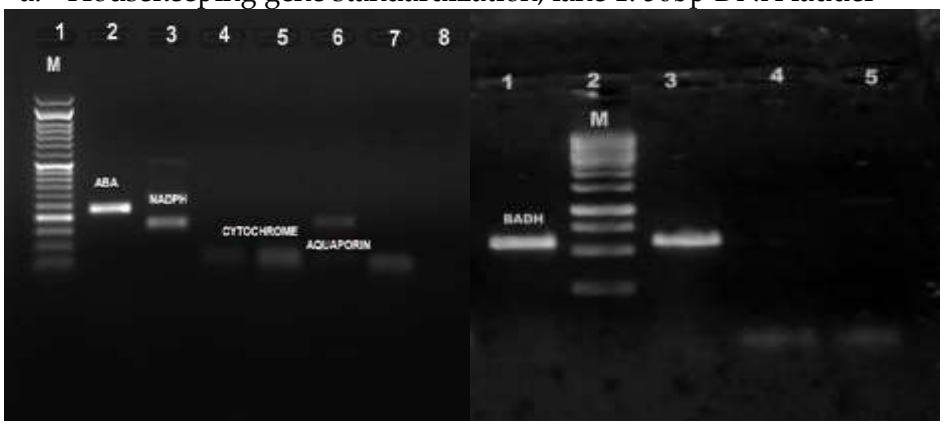
Table 2: List of genes and their annealing temperature

GENE	Annealing temperature(°C)
BADH	56
P5CS	52
NHX1	59
AQUAPORIN	56
CYTOCHROME P450	56
SOS1	58
ABA	60

NADPH OXIDASE	56
GAPDH	56
ACTIN	60
TUB1	59
18srRNA	60
EF1	52

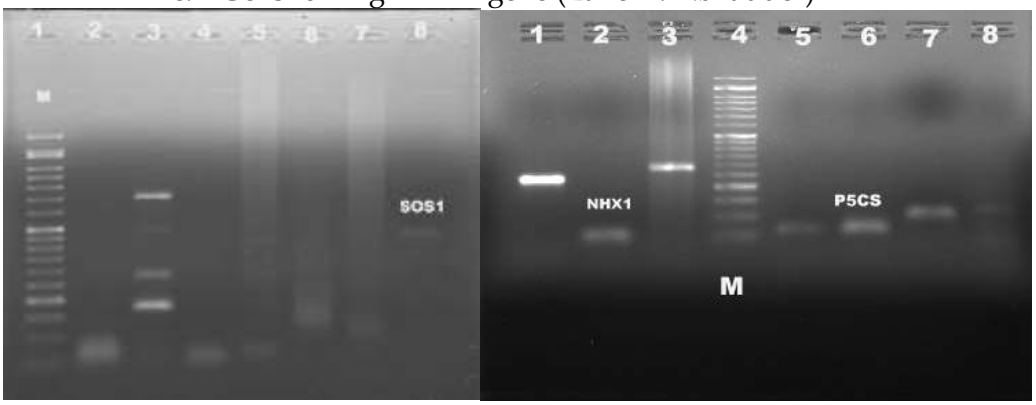


a. Housekeeping gene standardization, lane 1: 50bp DNA ladder



b. Gel showing ABA, NADPH Oxidase, Cytochrome P450,Aquaporin (lane 1 : 50bp DNA ladder)

c. Gel showing BADH gene (Lane 1 :1kb ladder)



d. Gel showing SOS 1 gene (lane 1 : 50bp DNA ladder)

e. Gel showing NHX1 and P5CS gene (lane 4 : 50bp DNA ladder)

Figure 5: Primer standardization

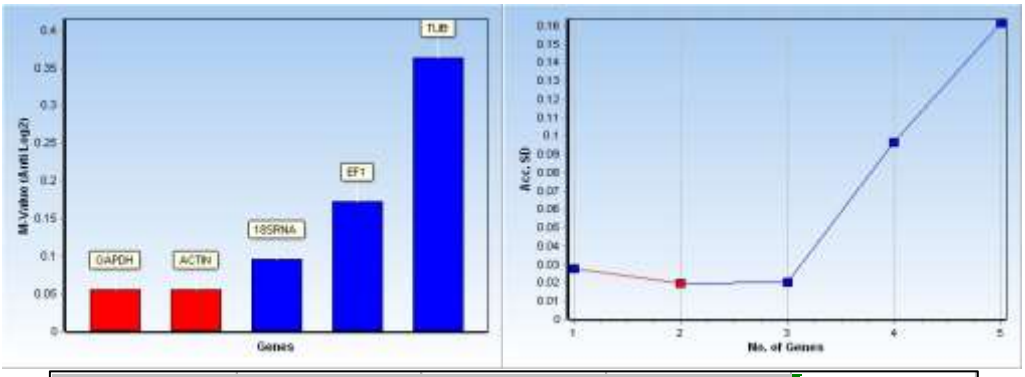
Gene Expression Analysis

a. Housekeeping Gene Normalization

geNORM Analysis

geNORM was used to analyze the expression stability of genes and ranked them accordingly. It is a statistical algorithm that determines the gene stability measure (M).

Genes with the lowest M values has the most stable expression. Analyzed the stability of five housekeeping genes [ACTIN, GAPDH, TUB, 18rRNA, EF1] with corresponding efficiency values of the genes. The average expression stability values of ACTIN and GAPDH were least for gene-specific efficiency calculation. Pairwise variations were calculated using geNORM to determine the optimal number of housekeeping genes for normalization. For this, first normalization factors (NF) were calculated for the most stable housekeeping genes (with lowest M value) and then for other genes by stepwise inclusion of the control gene that remains most stable. Accumulated standard deviation (Acc.SD) showed that ACTIN is most stable among the genes. Analysis by geNORM indicated that the most stable among the housekeeping genes was ACTIN and is sufficient for normalization of genes for expression studies.



Gene Name	SD	Acc. SD	SD	0.0279
GAPDH	0.0279	0.0197		
ACTIN	0.0279	0.0279	Best Gene	ACTIN
18SRNA	0.0468	0.0204		
EF1	0.3809	0.0964		
TUB	0.7095	0.1615		

Figure 6 geNorm analyses for housekeeping gene normalization

b. Real Time PCR

To study the role of the specific salt-tolerant gene, mRNA levels of genes at different salt concentrations and periods were monitored using RT-qPCR analysis. Target gene expression data was normalized with stable housekeeping gene ACTIN. The role of each gene was analyzed. Expressions were analyzed from the amplification plots, further confirmed by melt curve analysis and gel check. The fold change of the genes was calculated using the 2(-ΔΔCt) method and the results were statistically analyzed using ANOVA. Among the genes analyzed, NHX1 and SOS1 showed early expression, BADH, P5CS, and Aquaporin showed delayed expression whereas ABA and NADPH were active throughout the days analyzed. Cytochrome did not show much higher expression. The analysis suggested that late expressed genes may be involved in salt tolerance.

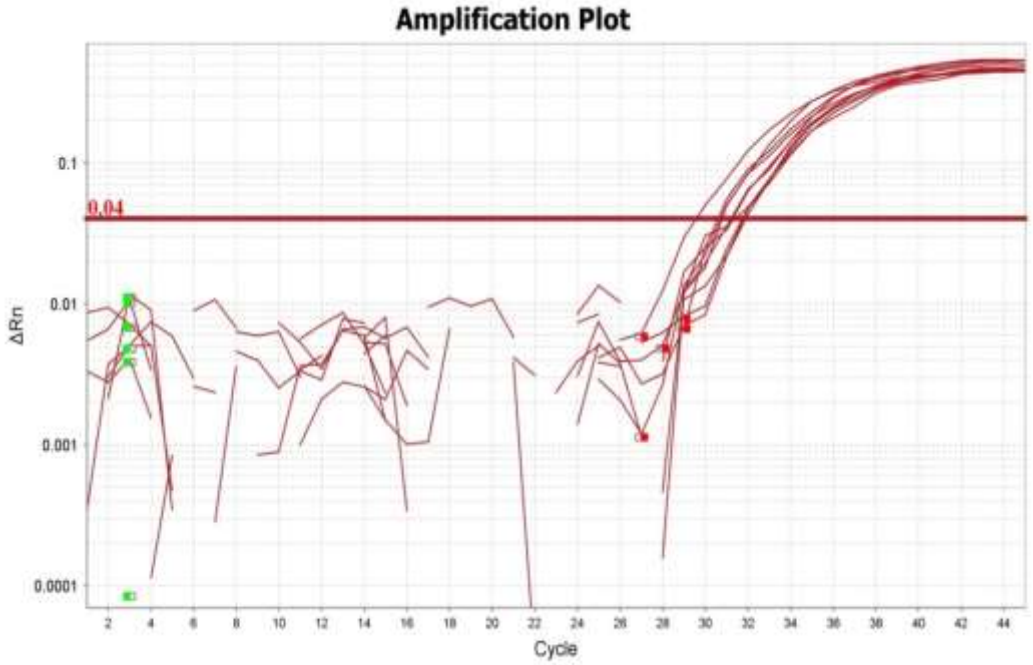


Figure 7: Representative image showing amplification plot

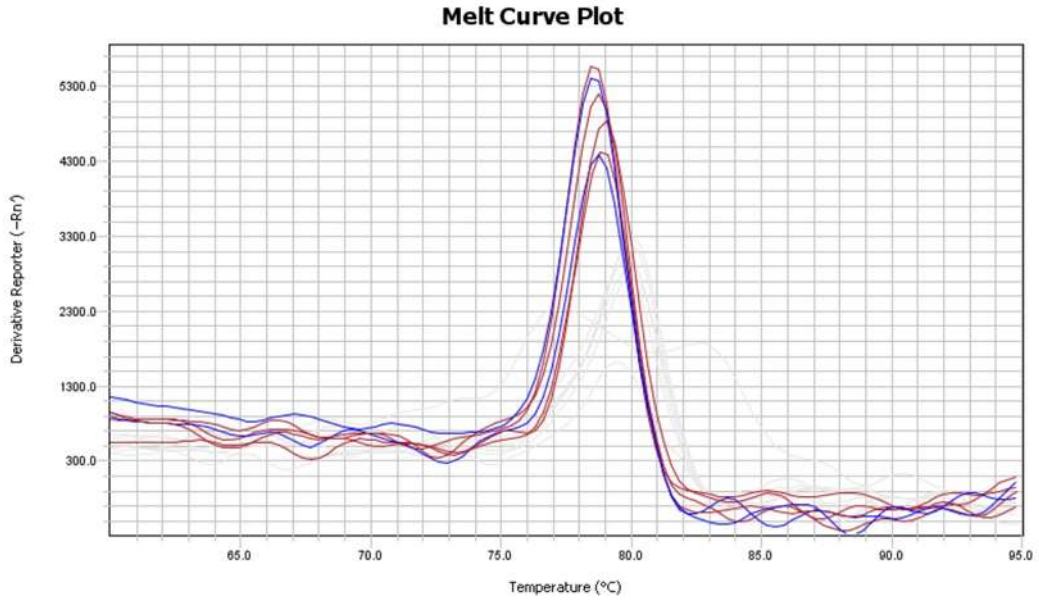


Figure 8: Representative image showing melt curve

From the genes analyzed ABA encoding gene showed an expression pattern as shown in figure 9. The gene showed higher fold change during the initial minutes of salt treatment. Later on, the fold change was observed at 500mM treated plants. Gene activity was observed throughout the study period. The amplified gene product was sequenced (211bp) and using the partial gene sequence, corresponding amino acid sequence (EMBOSS Transeq) of 64 residues and protein molecular weight of 6.94 kDa was predicted.

In plants Abscisic acid (ABA) is synthesized in response to various environmental stresses. Osmotic stress during high salinity leads to the synthesis of ABA and stomatal closure is the initial response to osmotic stress, in which ABA is involved (Sah, *et al*., 2016). Studies showed that ABA induces the production of hydrogen peroxide, which is essential for ABA signaling (Ren, *et al*., 2018). Studies in

Ceriops tagal showed that Gibberellic acid and abscisic acid are essential regulators of the salt stress response (Xiao, *et al*., 2016). Thus ABA plays a major role in mitigating the adverse effect of high salinity. This study also shows that the ABA encoding gene was activated in *Rhizophora mucronata* under the saline condition and helps in tolerating salt.

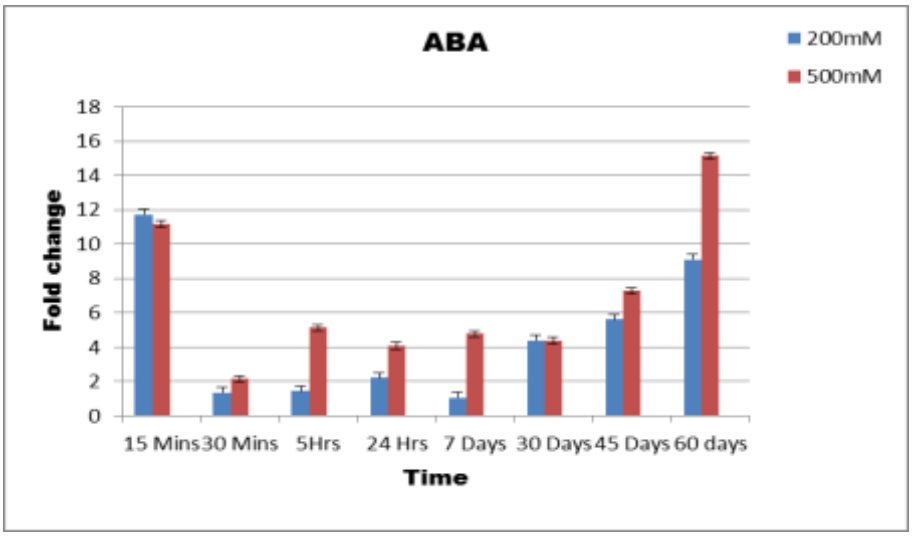


Figure 9: ABA encoding gene responses to salinity

The expression pattern of NHX1 gene was shown in figure 10. NHX1 is a Na^+/H^+ antiporter gene and these antiporters are important in ion homeostasis during salt stress (Fahmideh and Fooladvand, 2018). Studies report that in salt-tolerant plants, Na^+ ions get compartmentalized into vacuoles with the help of vacuolar Na^+/H^+ antiporter and balances the Na+ ion in the cytosol and retains osmotic balance (Apse, *et al*., 1999). In this study, the gene expression was observed in the early days of treatment, later on, the expression was declined. This may be because during the initial stage the plant maintains its ionic balance with the help of Na+ /H+ antiporter and once the homeostasis is established plant become tolerant to salt. The gene product was sequenced and a partial sequence of 400bp was obtained. Protein structure using 125 residue sequence was predicted using the bioinformatics tool and a protein of 14.60 kDa was predicted.

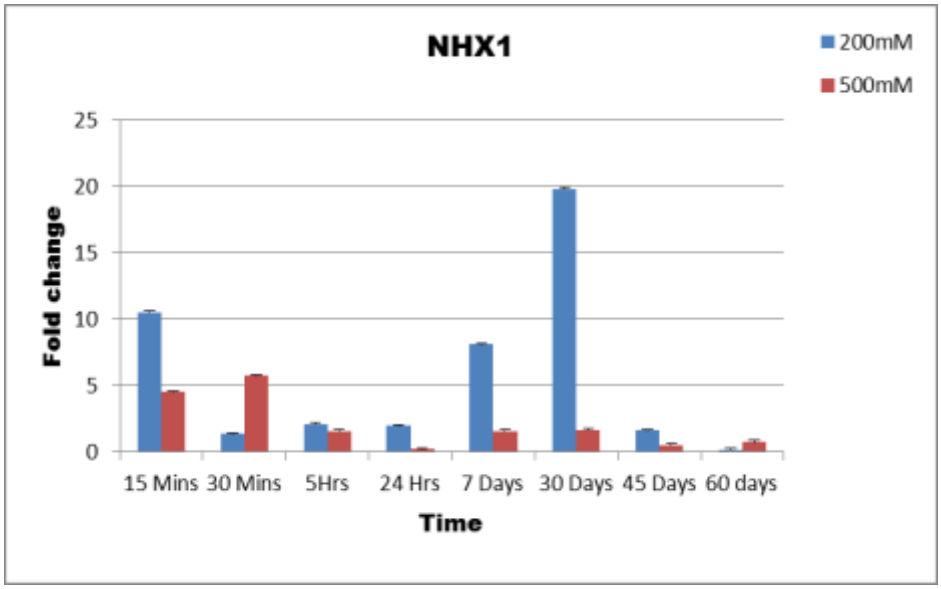


Figure 10: NHX1 gene responses to salinity

SOS 1 is also a Na^+ / H^+ antiporter located in the plasma membrane and ejects Na+ in apoplasts. To maintain ionic balance, SOS1 functions in loading Na+ to xylem or retrieving Na+ ions from xylem (Fahmideh and Fooladvand, 2018).SOS1 also functions in K+ ions homeostasis (Shi, *et al*., 2000). In the study, SOS 1 gene expression declined at later days of treatment and the fold change was higher in plants treated with 500mM salt. This may be due to the availability of more Na+ ion at a higher concentration of salt and once homeostasis is attained, gene expression declined. The gene product was sequenced (590bp) and a protein of 23.09 kDa (190 residues) was predicted using the bioinformatics tool.

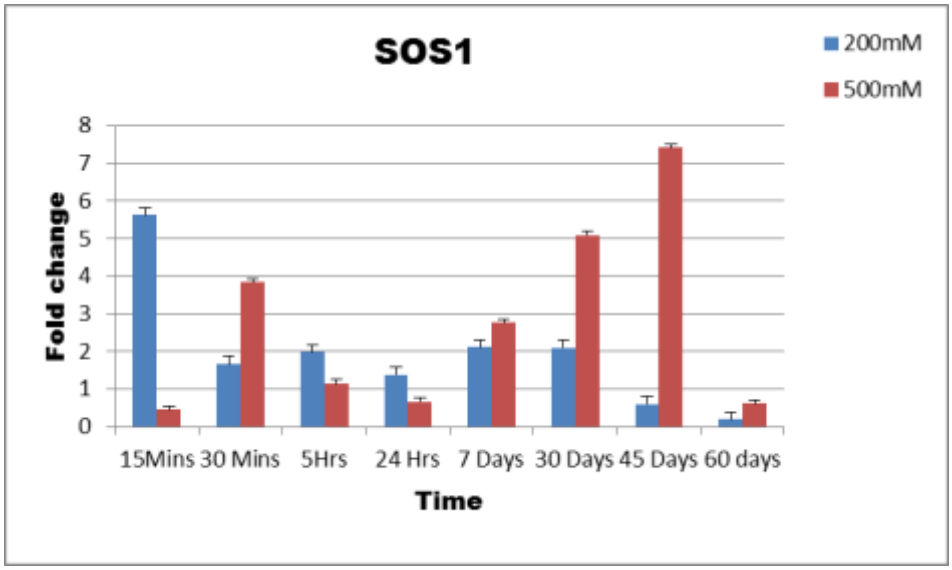


Figure 11: SOS1 gene response to salinity

BADH genes are plays a major role in the synthesis of glycine betaine, which is an important osmoprotectant in the plant in response to various environmental stresses. In response to salt stress BADH was first identified in spinach leaves (Weigel, *et al*., 1986). Studies reported that in plants, the BADH level was increased in response to salinity (Fitzgerald, *et al*., 2009). BADH gene was isolated and sequenced in *Rhizophora mucronata* Lam., as part this study (Sreeshan, *et al*., 2018) In the study, BADH gene expression was observed in later days of treatment and this may be due to the synthesis of glycine betaine at the later stage of treatment. In the sample collected from the plant's natural habitat, BADH expression was observed. The gene product was sequenced and a partial sequence of 515bp was obtained. From the sequence 164 residue amino acid sequence and a molecular weight of 19.31 kDa was predicted using the bioinformatics tool.

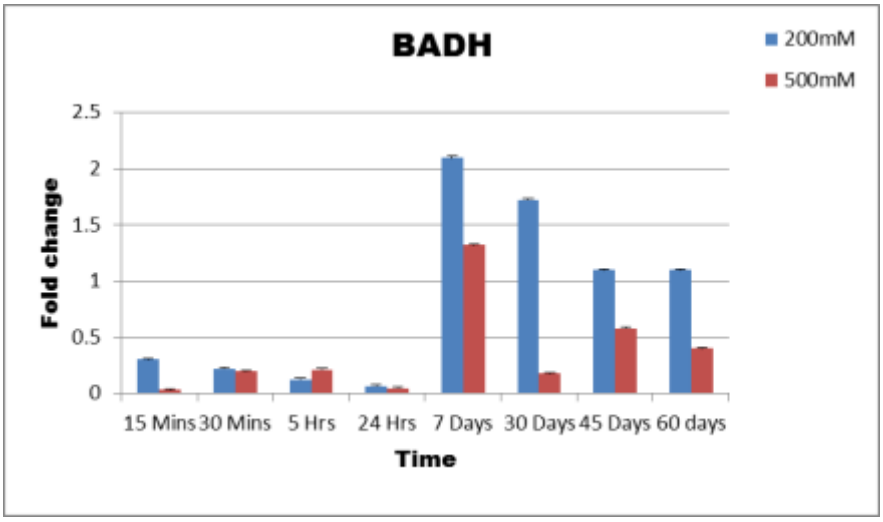


Figure 12: BADH gene response to salinity

NADPH Oxidase catalyzes the production of superoxide, which is involved in several processes in plants like growth and development, plant defense and cellular signaling (Qu, *et al*., 2017). In the present observation, NADPH Oxidase activity was observed throughout treatment. In plants treated with 500mM salt their expression was higher, this may be because at a higher salt concentration the plant has to produce more NADPH Oxidase to bring about tolerance. The gene product was sequenced and a partial sequence of 150bp was obtained. Protein sequence (50 residue) was predicted using online tools and was confirmed as NADPH Oxidase by comparing the sequence in UNIPROT. Protein molecular weight of 6.03 kDa was predicted using the bioinformatics tool.

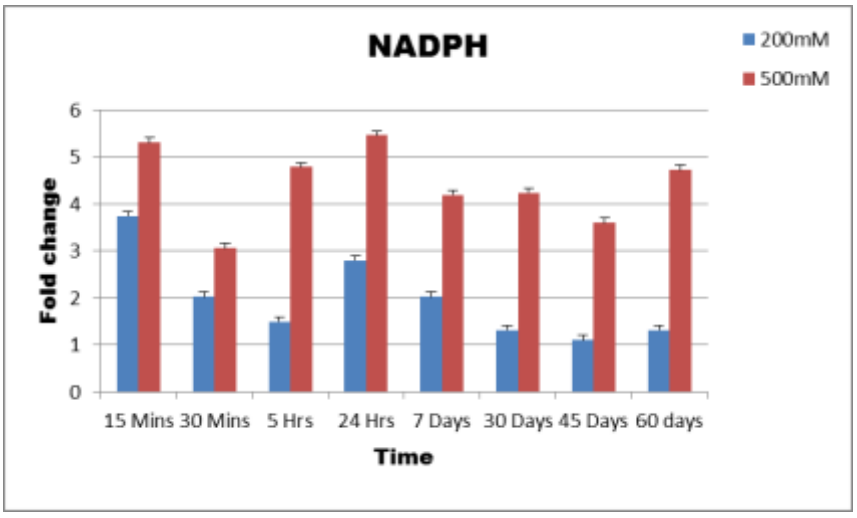


Figure 13: NADPH Oxidase gene response to salinity

P5CS is the enzyme involved in proline biosynthesis. Proline is an important osmoprotectant in plants and during abiotic stress by increasing their level proline provides tolerance (Singh, *et al*., 2014). Proline regulates expression of several genes related to antioxidant enzymes under salt stress, among which the gene Δ1-pyrroline-5-carboxylate synthetase is responsible for up-regulating the stress-induced proline accumulation under salinity stress (Amini, *et al*., 2015). In the study, the P5CS gene from *Rhizophora mucronata* Lam. was amplified and its expression showed higher fold change during the later period of salt treatment. On the 60th day of treatment, the gene expression was lower this may be due to tolerance acquired by the plant during the later days.

The gene product was sequenced and a product of 253 bp was obtained. Protein sequence (78 residues and 8.95 kDa) and its structure was predicted using bioinformatics tool.

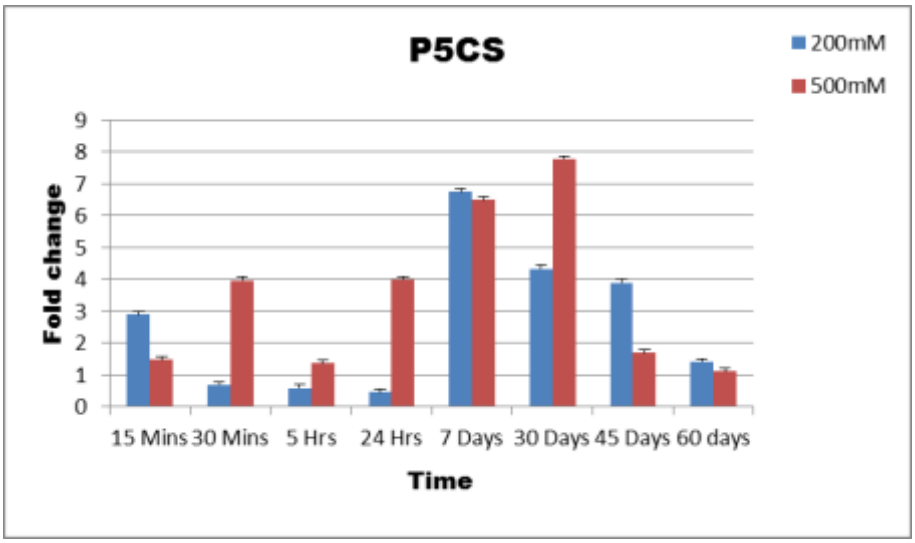


Figure 14: P5CS gene response to salinity

Aquaporins are membrane channel proteins located in plants which helps in the transport of water and small uncharged molecules. Aquaporins are involved in plant growth, development and several abiotic and biotic stresses (Kumar and Saddhe, 2018). Studies showed that the presence of aquaporin increases water permeability in the membranes and the role of aquaporin in mangrove species *Avicennia marina* and *Rhizophora stylosa* (Reef and Lovelock, 2015). In the present study, aquaporin was identified in *Rhizophora mucronata* Lam. and their expression showed an increase in fold change during a later stage of salt treatment as shown in figure 15. The gene product was sequenced and the partial sequence of 143bp was obtained. The amino acid sequence was predicted using the partial gene sequence and a 48 residue sequence of 5.25 kDa protein was obtained.

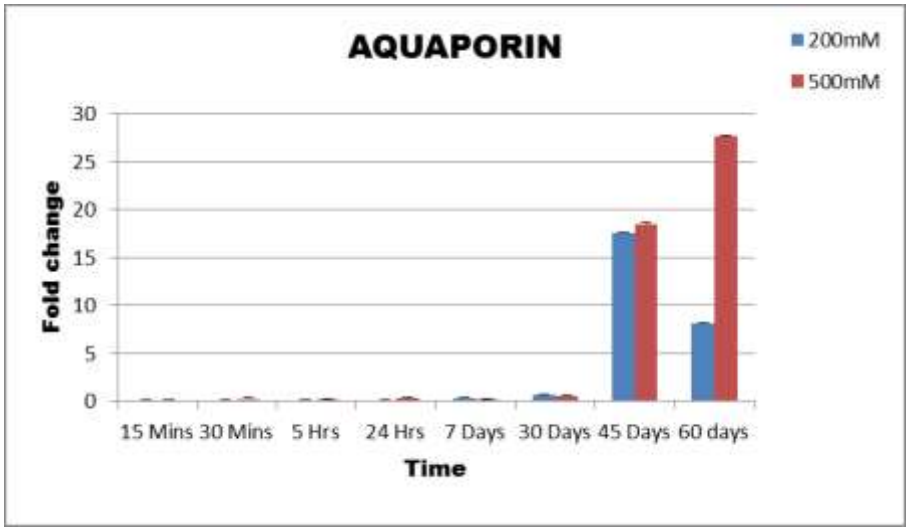


Figure 15: Aquaporin gene response to salinity

Cytochrome P450 genes are involved in the synthesis of secondary metabolites in plants (Mao, *et al*., 2013) and help in bringing about salt tolerance. In the study, the cytochrome P450 genes were amplified but the expression was not much higher. This may be because

the gene was not active during the period of treatment. The cytochrome P450 gene in *Rhizophora mucronata* Lam. was sequenced. A partial of sequence of 368bp was obtained. Amino acid residue of 118 and a protein of 14.76 kDa were predicted using the bioinformatics tool.

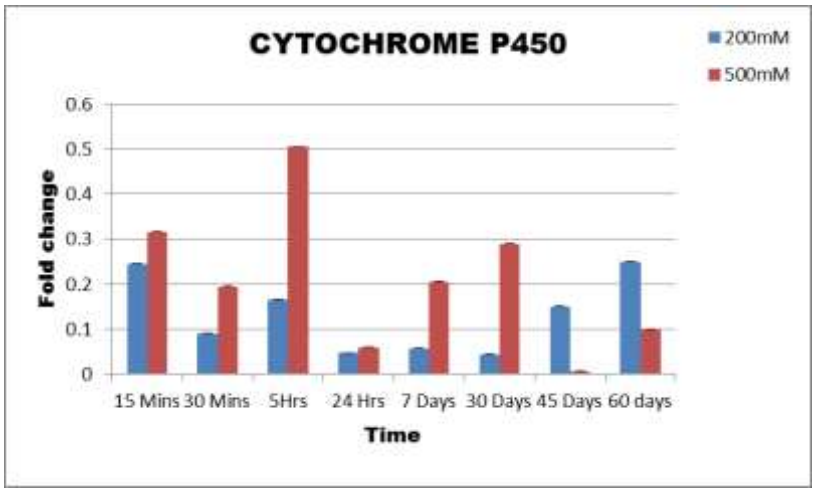


Figure 16: Cytochrome P450 gene response to salinity

In the study, the plant, *Rhizophora mucronata* Lam. was watered with 5M saltwater and got wilted within hours. The initial stage of salt treatment, leaves were collected and RNA was isolated and within days the plant dried off. The expression pattern of selected genes during the initial period of 5M treated plants showed the pattern as shown in figure 4.26. ABA, NHX1, SOS1 and NADPH Oxidase were highly expressed whereas BADH, P5CS, Aquaporin, and Cytochrome P450 were not significantly expressed which suggests that the less expressed genes may be essential for the plant to bring about salt tolerance and to survive in a saline environment.

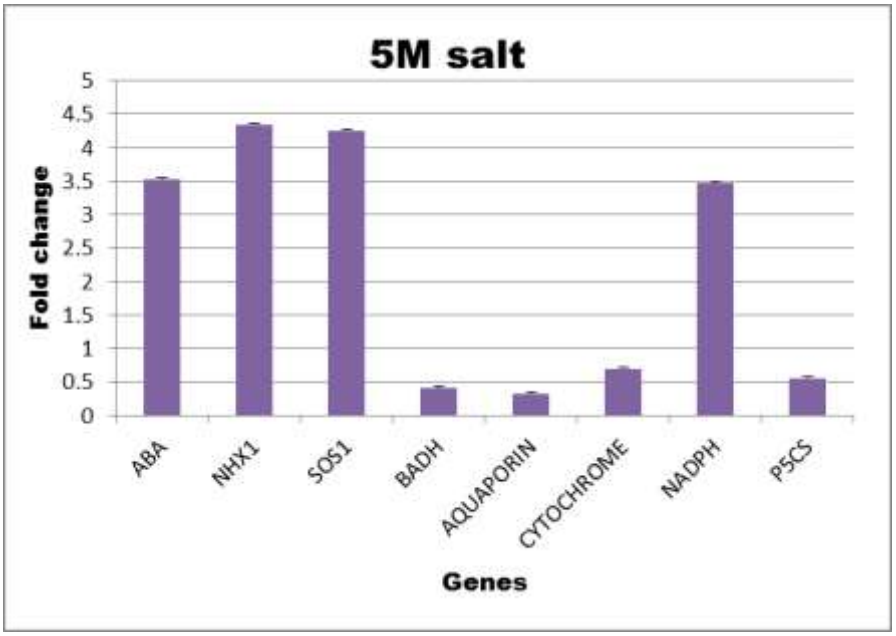


Figure 17: Gene response at high salinity

Total Leaf Protein Isolation

Total leaf protein was precipitated using modified BPP protocol (Wang, *et al*., 2007). Protein was dissolved, quantified and loaded on 12% SDS PAGE and the protein bands obtained were shown in figure 18. Protein bands were compared with Hi-range protein marker (Lane 3: 14-220kDa).

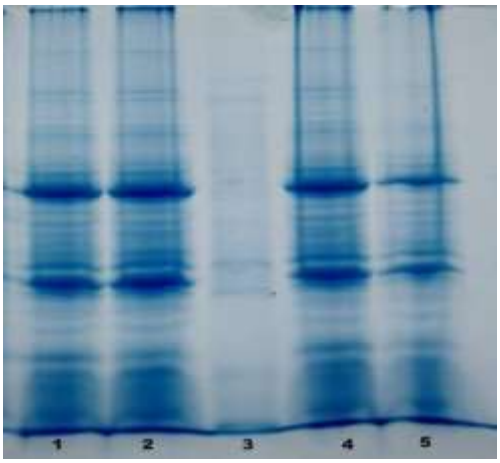


Figure 18: Protein bands on 12% SDS PAGE

2D Gel Electrophoresis

Two dimensional gel electrophoresis was performed to analyze the protein expression profiling in *Rhizophora mucronata* Lam. leaf. To identify the protein expressed in saline condition control and salt treated samples were loaded on IPG strips and electrophoresed. Gel image analyzed by PD quest Basic 2D Gel Analysis software. By comparing three gels of a sample, 90 protein spots were observed non-salt treated control protein sample,111 spots in 200mM salt treated sample and 82 protein spots in 500mM salt treated samples. Major spots were excised from the gel and performed LC Q TOF-MS analysis, which showed proteins with molecular weight of 228,272 and 316 Da. Further high throughput proteomic analysis will help to get a clear understanding about the proteins involved in salt tolerance in *Rhizophora mucronata* Lam.

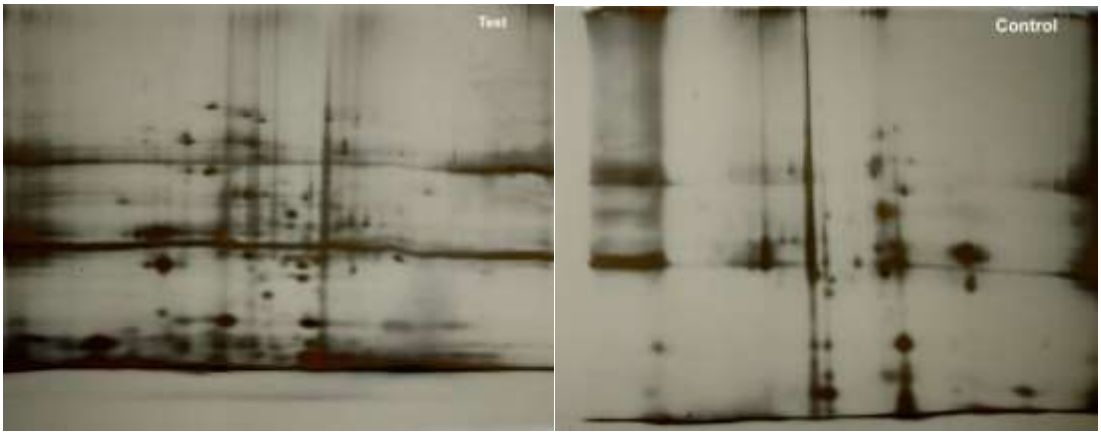


Figure 19: Two dimensional gel image of test and control protein sample

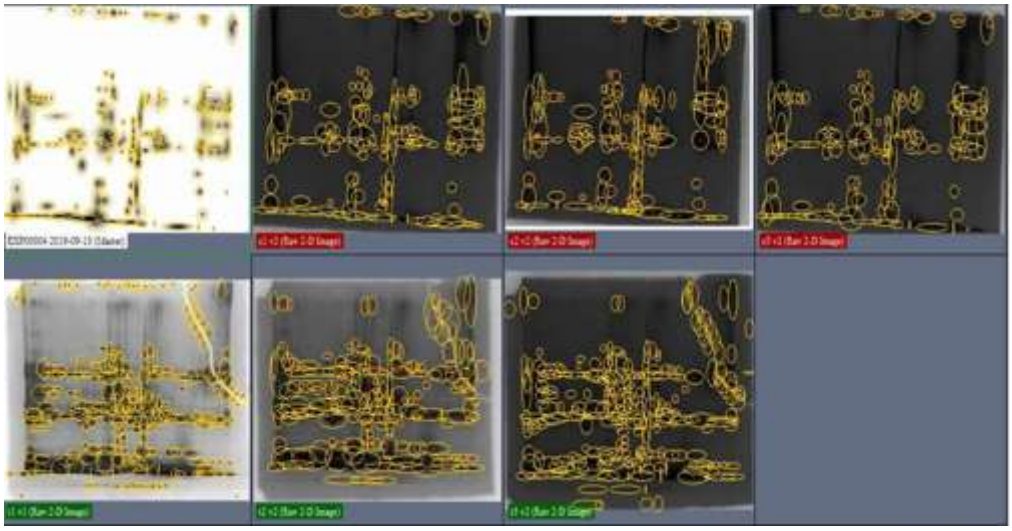


Figure 20: 2D spot analysis –PD quest Basic 2D Gel Analysis software

Table 3: Spot analysis report

	Total spots	Control vs. test	Intersection spots
Control	90	-	-
200mM	111	21	9
500mM	82	8	2

In mangroves like *Kandelia candel, Avicennia marina, Bruguiera gymnorhiza and Bruguiera parviflora* proteins were isolated to identify salt responsive ones. In *Kandelia candel* leaf protein was isolated and on 2D gel electrophoresis showed 53 differentially expressed protein spots (Wang, *et al*., 2014). Shen, *et al*., 2018 identified 49 differentially expressed protein spots in *Avicennia marina* and *Bruguiera gymnorhiza* leaves, one protein was identified as salt responsive protein (Tada and Kashimura, 2009). In the present study, 21 spots were differentially expressed in plants grown under optimum saline condition (200mM) when compared with control and 8 spots were expressed in high saline condition (500mM). Further studies are needed to identify these proteins and their role in salt tolerance in *Rhizophora mucronata* Lam.

Conclusion

Rhizophora mucronata Lam. leaves were used for the study. As the plant is rich in polyphenols, RNA and protein isolation was very difficult, several protocols were followed for their isolation. RNA was isolated by Lithium chloride precipitation method. In the study, we checked the expression of selected genes that are involved in plant salt tolerance. Among the genes analyzed NHX1 and S0S1 showed early expression, BADH, P5CS, and Aquaporin showed delayed expression whereas ABA and NADPH were active throughout the days analyzed. Cytochrome didn't show higher expression. The analysis showed that late expressed genes are involved in salt tolerance. Total leaf protein was isolated using BPP protocol and for the identification of protein involved in salt tolerance, two-dimensional gel electrophoresis was done. Protein spots were analyzed using PD Quest software and the analysis showed 90 spots in control sample (without salt) loaded gel and 111 spots and 82 spots in 200mM and 500mM salt-treated protein sample loaded gel respectively. Further studies are to be done to identify proteins involved in salt tolerance.

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