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Investigation of Salinity Tolerance Mechanisms in the Halophyte, *Clerodendrum inerme*

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Diverse ecotypes of saline tolerant *Clerodendrum inerme* were studied to investigate the morphological, physiological and genetic variations. Seashore, backwater and inland ecotypes of *C. inerme* recorded significant difference in morphological characteristics viz., leaf area, leaf thickness and midrib thickness. Physiological studies revealed chlorophyll a/b ratio, total chlorophyll and proline content was higher in seashore ecotype than backwater and inland ecotype. The genetic diversity analysis using Randomly Amplified Polymorphic DNA primers showed that seashore ecotype remained as a separate cluster whereas backwater and inland were clustered together. DD-RT-PCR strategy was adapted using in vitro derived plantlets for understanding the molecular basis of salinity tolerance in *C. inerme*. The molecular studies showed differential expression of nine cDNA fragments of which, 300 bp and 100 bp cDNA were expressed in leaves of control plants (without NaCl) and 500 bp cDNA was expressed in leaves of 100 mM NaCl stressed plant. Sequencing of the differentially expressed cDNA and its *in silico* analysis using BLASTN revealed that there is significant similarity with *Gossypium hirsutum* clone MX152H12-jol, *Theobroma cacao* Ammonium transporter and *Solanum lycopersicum* chromosome ch10, respectively.

(***Key words***: *Clerodendrum inerme, Genetic diversity analysis, Randomly amplified polymorphic DNA, DD-RTPCR, in silico analysis*)

Environmental stresses such as low and high temperature, drought, alkalinity, salinity are potentially harmful to the plant. Salinity is one of the chronic stresses that poses serious problem that can affect worldwide crop productivity. It can inhibit plant growth through a range of mechanisms, including low external water potential, ion toxicity and interference with the uptake of nutrients, particularly K^+ (Munns and Tester, 2008, Zhang *et al*., 2010). This problem is most serious in tropical, semi-arid regions, where sodic saline soils are found in large proportions. The major saline ions Na^+ and Cl^+ affect nutrient uptake through competitive interaction or by affecting membrane selectivity (Tester and Davenport, 2003). Salt stress affect photosynthesis directly or indirectly by decreasing CO_2 availability caused by diffusion limitations (Flexas *et al.*, 2007) alterations in photosynthetic metabolism (Lawlor and Cornic, 2002) or restrictions in the photochemical system apparatus under severe stress conditions (Souza *et al.*, 2004).

Depending upon the behavior of plants in saline environments, they have been categorized into halophytes (salt tolerant) and glycophytes (salt sensitive) (Flowers *et al.*, 1977). Most of the water on Earth is seawater, each kilogram of which contains about 35 g of salts, and most plants cannot grow in this solution; less than 0.2% of species can develop and reproduce with repeated exposure to seawater (Flowers and Colmer, 2015). These 'extremophiles' are called halophytes. Halophytes are remarkable plants that tolerate salt concentrations that kill 99% of other species. The basic physiology of salinity tolerance in halophytes is osmotic adjustment, ion compartmentalization and accumulation of compatible solutes. Ecologically, halophytes are the native flora of saline soils, which survive completing their whole life cycle in such environments (Waisel *et al.*, 1972). Halophytes will play increasingly important roles as models for understanding plant salt tolerance, as genetic resources contributing towards the goal of improvement of salt tolerance in crops.

Halophytes encompass mangroves and many species of mangrove associates which are highly salt tolerant. The most striking feature of mangroves is their ability to tolerate NaCl up to and above the concentration in seawater (500 mM) (Khan and Aziz, 2001), while mangroves associates represent traditional lifestyle between terrestrial plants and true mangroves and are ideal to study the mechanism of adaptive evolution in plants. Mangrove associates include species of plants typically occurring on the landward margin of mangrove habitats, often in non-mangrove habitats such as salt marshes. Many species of mangrove associates are classified as halophytes because of their ability to tolerate high salinity.

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Clerodendrum inerme commonly known as seaside clerodendrum, glory bower, wild jasmine and Indian Privet (Hindi: Chhoti-ari and sankuppi; Tamil: Sangankuppi and Peechangan) is found abundantly both on the landward edge as well as deep inside the mangrove environment (Parani *et al*., 1998). *C. inerme* tolerates salinity up to the limit of 22 dS m^{-1} (Dagar and Singh, 2007) and thereby it is well established in coastal areas. Wang *et al*. (2010), classifed *C. inerme* as a controversial species based on the the characteristics of specific leaf area (SLA), leaf nitrogen concentration on a leaf mass (Nmass), succulence, sodium content, chloride content, osmolarity and sodium potassium ratio. The study revealed that *C. inerme* behaved both like a true mangrove and mangrove associate. With this overview, the present study is carried out to identify the best ecotype with higher level of salinity tolerance and to explore the mechanisms of salinity tolerance in *C. inerme.*

MATERIALS AND METHODS

Analysis of morphological and physiological traits

C. inerme plants were collected from three ecosystems *viz.,* seashore ecosystem (east coastal region of Mahabalipuram, Golden beach, Chennai), backwater ecosystem **(**backwaters of Bay of Bengal, in the east coastal region of Adiramapattinam, Pattukkotai (TK), Thanjavore (Dt.)**)**, and inland ecosystem (Department of Environmental Sciences, TNAU campus). These plants were maintained in the greenhouse at Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore. These plants were used for morphological, physiological and molecular analysis. The morphological traits *viz.,* leaf characteristics, which included leaf area, leaf thickness and midrib thickness (6th leaf from the tip from five different plants) of each ecotype were measured. Leaf thickness and midrib thickness were measured using digital calliper-carbon fibre composite. Leaf area was measured from the leaf impression on graph paper followed by counting the divisions covered by the leaf. The physiological parameters *viz*., chlorophyll and proline content were estimated using standard procedures. All the recorded observations were analysed statistically (completely randomised design) using AGRES software.

Genetic diversity analysis of *C. inerme* ecotypes

For analysis of genetic diversity among the ecotypes Random Amplified Polymorphic DNA (RAPD) markers were used. The genomic DNA of three ecotypes was isolated by using a modified Gawal and Jarret (1991) method. The DNA quality and quantity were assessed using Nano Drop. For analysis of the DNA profiles of these ecotypes, the reaction mixture consisting of 50 ng of genomic DNA, 20 M dNTPs, 15 pmoles of random primers (GeNei RPI-1 to GeNei RPI-25), 1x assay buffer and 0.3 units of taq polymerase (Bangalore Genei) were used. The amplification was done in thermocycler (Cyberlab, USA) and profile used for amplification was initial denaturation at 94°C for 5 min., followed by 10 cycles of denaturation at 94°C for 1 min, primer annealing at 37°C for 1 min., extension at 72°C for 1 min. which was further followed by denaturation at 94°C for 45 s., primer annealing at 37°C for 45s., extension at 72°C for 1 min. for 30 cycles and final extension for 10 min. The amplified products were electrophoresed on agarose gel and documented (GELSTAN 1300). RAPD bands were scored as 1 for presence of amplification product and as 0, for its absence for each primer. Estimates of genetic similarity (F) were carried out according to Nei and Li (1979) and the relationship among ecotypes was estimated by cluster analysis using Unweighted Pair Group Method with Arithmetic Averages (UPGMA). The analysis was plotted in the form of a dendrogram. All computations were carried out using the NTSYS-pc, Version 2.2 package (Rohlf, 2005).

In vitro screening for salinity tolerance

Nodal explants were collected from the ecotypes maintained in green house plants for *in vitro* screening. The explants were surface sterilized with mercuric chloride (0.1%) for 5 min followed by ethanol (70%) for 30 s. The treated explants were then washed three to four times with sterile water. A final trim was given and the explants were inoculated in MS (Murashige and Skoog, 1962) + 2 mg l^{-1} Benzyl Amino Purine + 2 mg l^{-1} Kinetin supplemented with NaCl (0 mM, 50 mM, 100mM, 150 mM, 200mM, 250 mM and 300mM). After inoculation, all these cultures were incubated at 25 ±2°C at a relative humidity of 60-70 percent with a light intensity of 2000 Lux using white fluorescent lamps. A photoperiod of 16 h light and 8 h darkness was maintained. Each treatment (NaCl levels of 0 mM, 50 mM, 100mM, 150 mM, 200mM, 250 mM and 300 mM) was replicated adequately and shoot length was recorded after 10, 20 and 30 days of inoculation in four different biological replications for 0 mM, 50 mM, 100mM, 150 mM, 200mM and 250 mM NaCl level.

Molecular characterization for sanility tolerance

Two and half year old *C. inerme* (inland ecotype) plants developed through plant tissue culture, and maintained under different salt concentration (control, 50 mM and 100 mM) were used for molecular studies. Total RNA was extracted from leaves of control and salt stressed plants (50 mM and 100 mM) of *C.inerme* (inland ecotype) using one step RNA Trizol reagent (BioBasic, Canada). Samples were ground in liquid nitrogen and transferred immediately to microcentrifuge tube containing 1 mL of Trizol reagent. Samples were incubated at room temperature for 5 min. and chunks of tissues from the samples were removed by centrifugation at 12,000 rpm at 4 ºC and the supernatant was transferred to fresh tube. Equal volume of chloroform was added and aqueous phase was separated by centrifugation at 12,000 rpm, at 4 ºC. The aqueous phase containing the RNA was transferred to fresh sterile micro centrifuge tubes and precipitated by adding 500 μl isopropanol. The precipitated RNA samples were pelletized and washed with 1 mL of 75% ethanol (prepared ethanol with DEPC water). The RNA pellets were air dried for 15 min. and resuspended in 50 μl sterilized DEPC treated water and stored at -80 °C for further analysis. To remove residual DNA, it was treated with 1 unit RNAse-free DNase (Fermantas, USA). 1µg of RNA sample and was incubated for 15 min. at 37 ºC, and then the reaction was stopped by adding 25 mM EDTA for 15 min. at 65 ºC. The quality and quantity of RNA was assessed using Nano drop. RNA samples were converted into single stranded cDNA using First Strand Thermo scientific cDNA Synthesis Kit as per the manufacturer's instructions. Each 20 µl reaction mixture contained 1µg of RNA, 1 μl primer (oligo $(dT)_{18}$), 4 μl 5X reaction buffer, 1 μl Ribolock RNase Inhibitor (20/ μl), 2 μl 10 mM dNTP mix, 2 μl M-MuLV Reverse Transcriptase. Complete reaction mix was incubated 120 minutes at 37 ºC, followed by incubation at 70 ºC for 5 minutes to stop the reaction. The cDNA synthesized were stored at -80 ºC. The first strand cDNA was used as template for the second strand synthesis. The cocktail for PCR amplification consisted of cDNA (80 ng), dNTPs (1 mM), Random Primer (GeNei RPI-1 to GeNei RPI-25) 20 p moles, Taq DNA polymerase (0.3 units), Assay Buffer (1X), 1% Polyvinylpyrrolidone (PVP) and sterile distilled water. The cycling conditions used in thermal cycler (Cyberlab, USA) were initial denaturation at 94 ºC for 5 min., 40 cycles of denaturation at 94 C for 1 min, primer annealing at 37 ºC for 1 min., extension at 72 ºC for 1 min. followed by final extension for 5 min. The amplified products were electrophoresed in agarose gel and the differentially expressed bands were eluted (Bio Basic Canada), reamplified using random primers and were send for sequencing. The sequencing was done with M13 forward and reverse primers (SciGenom Labs Private Ltd Cochin, Kerala). Further, the nucleotide sequence obtained was analyzed using BLASTN and BLASTX (Altschul *et al.*, 1997).

RESULTS AND DISCUSSIONS

C. inerme ecotypes collected from three ecosystems *viz*., seashore, backwater and inland ecosystem and were grown under greenhouse condition and analyzed for various morphological parameters *viz.,* leaf area, leaf thickness and leaf midrib thickness. The seashore ecotype recorded highest leaf area (10 cm^2) than inland ecotype (7 cm^2) and backwater ecotype (5 cm^2) (Fig. 1). The seashore ecotype recorded highest leaf thickness (0.4 mm) followed by backwater ecotype (0.3 mm) and inland ecotype (0.2 mm) respectively. In the case of midrib thickness, seashore (1.2 mm), backwater (0.9 mm) and inland (0.8 mm) ecotypes showed significant difference (Table 1). Similar results of decline in leaf area were observed in salinity sensitive and tolerant species *Cotoneaster lacteus* and *Eugenia myrtifolia* respectively (Cassaniti *et al.,* 2009). The salinity reduces the soil water potential and the ability of water uptake which causes reduction in the rate of cell expansion in growing tissue leading to slower formation of photosynthetic area (Munns and Sharp, 1993). Another common response of plants towards the high salinity is thickening of leaf that can reserve abundant water. Increase in the leaf thickness due to high salt level was reported in *Coleus blumei, Salvia splendens* and *Lumnitzera racemosa* (Sobrado and Ewe, 2006; Ibrahim *et al.,* 1991).

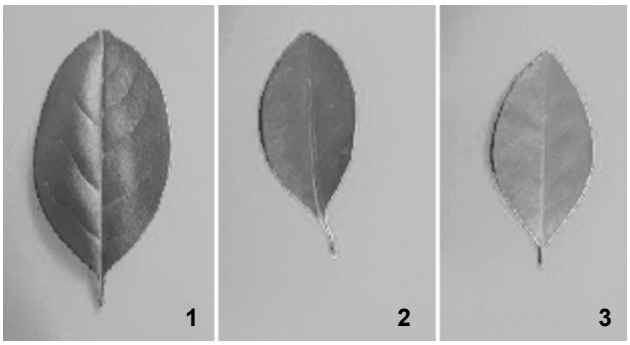

Fig. 1. *Variation in C. inerme ecotypes for leaf morphological traits (1- Seashore ecotype, 2- backwater ecotype and 3- inland ecotype)*

Table 1. *Variations for leaf characteristics in C. inerme ecotypes (mean of 5 observations from 5 plants)*

Ecotypes	Leaf area $(cm)^2$	Leaf thickness (mm)	Midrib thickness (mm)
Seashore ecotype	9.30	0.40	1.16
Backwater ecotype	7.66	0.30	0.82
Inland ecotype	5.00	0.30	0.80
SED	0.60	0.04	0.06
CD	1.42	0.09	0.14

The seashore ecotype recorded highest Chlorophyll a content (1.81 mg g^{-1}) followed by backwater ecotype (1.09 mg g^{-1}) and inland ecotype (0.95 mg g^{-1}) respectively showing significant difference among the ecotypes (Fig. 2). Whereas, the chlorophyll b, total chlorophyll and ratio between chlorophyll a and chlorophyll b, showed non-significant difference among the ecotypes. Chlorophyll a analysis revealed salt induced structural and functional destruction of photosynthesis apparatus and damage to the PS ll (Baker, 2008). Murkute *et al., (*2006) has concluded that Chlorophyll a concentration is reduced by salinity because of suppression of specific enzymes responsible for synthesis of photosynthetic pigments. Estimation of proline in this study showed that seashore ecotype recorded highest proline followed by backwater ecotype and inland ecotype respectively (Fig. 3). Ashraf (1994) and Rains (1989) reported that in higher plants normally higher concentration of proline accumulate as response towards the salinity. Higher proline concentration in salinity tolerant plants has been reported in *Bruguinera parviflora* (Parida *et al.,* 2002). Higher concentration of chlorophyll a and proline in seashore ecotype reveals better adaptation of this ecotype to salinity.

In this study, RAPD markers were used to estimate genetic similarity among the ecotypes of *C. inerme*. The polymorphism per cent in RAPD markers used in this study was 51.02%. The RAPD primers *viz*., RPI 16, RPI 17 and RPI 18 showed high polymorphism among the ecotypes. The genetic diversity analysis recorded the similarity coefficient as 64% between seashore ecotype and backwater ecotype, and highest similarity coefficient of 79% between backwater and inland ecotype. The dendrogram showed two clusters A and B. Cluster A contained seashore ecotype and cluster B contained backwater ecotype and inland ecotype (Fig. 4). The variations at genetic level in seashore ecotypes for its adaptability to high salt percentage in the beach soils would have contributed to separate clustering of seashore ecotype. A phylogenetic relationship between mangroves species using RAPD and RFLP has been reported (Parani *et al.,* 1998).

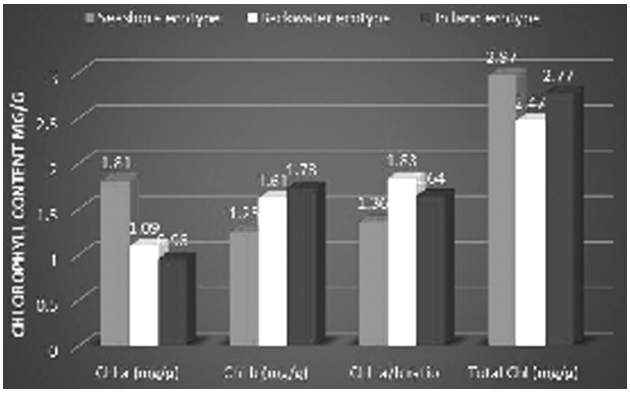

Fig. 2. *Chlorophyll a/b ratio and total chlorophyll content in leaves of C. inerme ecotypes*

In vitro screening for salinity tolerance

Nodal explants of *C. inerme* ecotypes were used to study the *in vitro* responses under different salt stress conditions. Observations of length of shoot were

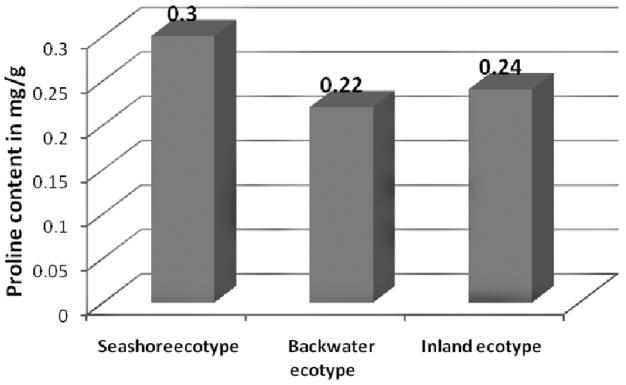

Fig. 3. *Proline content in leaves of C. inerme ecotypes*

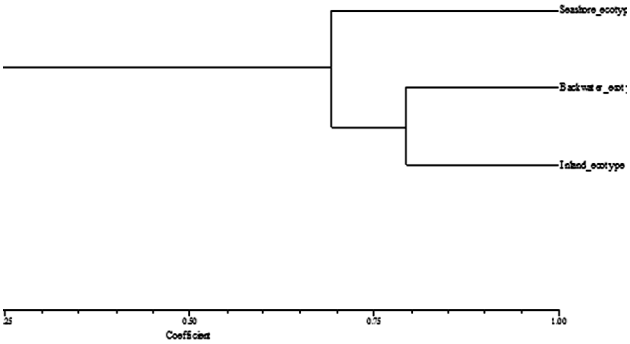

Fig. 4. *Dendrogram based on RAPD marker data*

recorded until transfer of micro shoots to the rooting media under *in vitro* conditions for backwater and inland ecotypes. However, seashore ecotype showed poor response for regeneration under *in vitro* conditions. After 10, 20 and 30 days of inoculation shoot length showed highly significant difference among the treatments in both backwater and inland ecotype. In backwater ecotype, Treatment T6 (250 mM NaCl stress) recorded lowest shoot length after one month of inoculation, while in inland ecotype the shoots were able to withstand salinity stress upto 200 mM NaCl only (Table 2). The *in vitro* developed microshoots were rooted and later hardened in paper cups followed by transfer to the pots. The tissue cultured plants of backwater and inland ecotypes were maintained at greenhouse under induced salinity stress conditions. After two and half years of maintenance in green house conditions under salinity stress condition (0 mM, 50 mM and 100 mM NaCl), tissue cultured plants of inland ecotype survived. In case of backwater ecotype, after one year of maintenance in green house conditions under salinity stress condition 0 mM, 50 mM, 100 mM and 150 mM NaCl stressed backwater ecotype plants survived.

Molecular characterization for salinity tolerance

Molecular technique such as differential display reverse transcription-polymerase chain reaction (DD-RTPCR) was used to study differential expression under varied environments. Differential display is a powerful tool for the isolation of plant genes regulated by salt stress (Zhang and Chen 1996). In this study, differential display was performed to isolate salt-responsive genes from leaf of *C. inerme* plant grown under control, 50 mM, and 100 mM NaCl stress conditions. For DD-RTPCR, total RNA was extracted from the two and half year old tissue cultured *C. inerme* under control, 50 mM and 100 mM NaCl induced salinity stress conditions from leaf sample. The quantity of RNA in different samples varied from 200-800 ng per µl. The total RNA of leaf (control, 50 mM and 100 mM) were used as template (equal volume) for cDNA synthesis. The second strand synthesis was carried out with RAPD primers with first strand cDNA as the template. Among them RPI 5, RPI 7, RPI 12, RPI 13, RPI 18, and RP I20 showed differential expression banding pattern under control, 50 mM and 100 mM (nine cDNA fragments). DD-RTPCR analysis using RPI 5 random primer showed down regulation of 300 bp cDNA in control, 50 mM salt stress to 100 mM stressed samples. Similarly, RPI 7 primer produced 100 bp differential expressed cDNA in control and same was down regulated in salt stress conditions (50 mM and 100 mM). In the case of RPI 12 RAPD marker amplification differential expression was documented in 500 bp DNA which was expressed in 100 mM NaCl stressed condition but was absent in control and 50 mM stress condition (Fig. 5).

The sequencing of the differentially expressed band (~ 300 bp) in control with RPI 5 primer yielded 205 bp nucleotide sequence. The analysis of the 205 bp nucleotide sequence using BLASTN revealed sequence similarity with *Gossypium hirsutum* clone MX152H12-jol, complete sequence and with retrotransposon RC

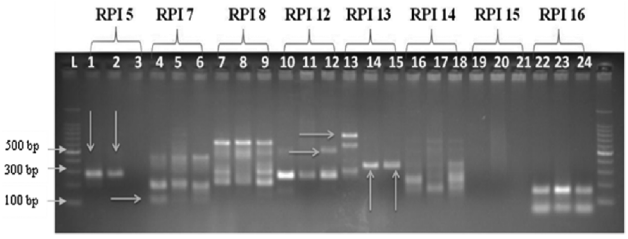

Fig. 5. *Amplification of cDNA using random primers (RPI 5 – RPI 16). Arrow indicating differetional expressed cDNA fragments*

Control = lanes 1, 4, 7, 10, 13, 16, 19, 22 L= 100 bp Ladder
50 mM = lanes 2, 5, 8, 11, 14, 17, 20, 23
100 mM =lanes 3, 6, 9, 12, 15, 18, 21, 24

Table 2. *Effect of salinity stress on shoot length in C. inerme (backwater ecotype) after 10, 20 and 30 Days (mean of 4 different biological replications)*

Treatments (NaCl levels)	After 10 days (cm)	After 20 days (cm)	After 30days(cm)	Grand mean
Control	1.68	2.96	4.80	3.14
50 mM	1.44	2.08	3.64	2.38
100 mM	1.16	1.84	2.6	1.86
150 mM	1.00	1.20	2.00	1.41
200 mM	1.00	1.04	1.20	1.08
250 mM	0.68	0.72	0.80	0.73
SED	0.06	0.18	0.18	
CD	0.12	0.37	0.39	

Tekay complete sequence of *Ricinus communis* 80% and 70%, respectively. BLASTX analysis of 205 bp nucleotide sequence revealed 69 % similarity with predicted low quality protein: uncharacterized protein LOC105167812 (*Sesamum indicum*) and 66 % similarity with predicted: uncharacterized protein LOC105634770 (*Jatropha curcas*). The sequence has been submitted in NCBI EST database (Accession: JZ896661.1)

The sequencing of differentially expressed DNA (~100 bp) under control condition using RPI 7 yielded 35 bp nucleotide sequences. The analysis using BLASTN in plants (taxid:3193) showed 100% similarity with 60% sequence coverage with *Theobroma cacao* Ammonium transporter 1,5 (TCM_021045) mRNA, complete cds. Song *et al.*, 2011, reported up regulation of ammonium transporter under control condition and down regulation under salt stress in algae *Dunaliella viridis*.

The differentially expressed band of 500 bp amplified in100 mM NaCl stressed condition was sequenced and this yielded 328 bp nucleotide sequences. The similarity analyzed using BLASTN with plants taxid, revealed the similarity of 93% and query coverage of 26% with *Solanum lycopersicum* chromosome ch10, complete genome. Further BLASTX analysis with plants taxid (3193), showed 32% similarity with uncharacterized protein LOC104230625 of *Nicotina sylvestris* isoforms X2 and X1. Similar results were recorded under salinity stress conditions in many species. The sequence has been submitted in NCBI EST database (Accession: JZ896660.1)

CONCLUSION

Analysis of morphological and physiological indices among diverse ecotypes of *C. inerme* revealed that seashore ecotype is found to possess desirable attributes to overcome salinity than the backwater and inland ecotypes. *In silico* analysis of differentially expressed cDNA revealed sequence similarity with uncharacterized proteins and ammonium transporter. This study shows that *C. inerme* exhibits morphological, physiological and molecular adaptations to tolerate salinity. Further molecular studies will lead to isolation of potential candidate genes for salinity tolerance which can be employed for crop improvement.

REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25:** 3389-3402.

Ashraf, M. (1994). Organic substances responsible for salt tolerance in *Eruca sativa*. *Biologia Plantarum* **36:** 255-259.

Baker, N. R. (2008). Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. *Annual Review of Plant Biology* **59:** 89-113.

Cassaniti, C., Leonardi, C. and Flowers, T. J. (2009). The effects of sodium chloride on ornamental shrubs. *Scientia Horticulturae* **122:** 586-593.

Dagar, J. and Singh. G. (2007). *Biodiversity of Saline and Waterlogged Environments: Documentation, Utilization and Management,* National Biodiversity Authority, Chennai, India. 76p.

Flexas, J., Diaz Espejo, A., Galmes, J., Kaldenhoff, R., Medrano, H. and Ribas-Carbo, M. (2007). Rapid variations of mesophyll conductance in response to changes in CO_2 concentration around leaves. *Plant, Cell & Environment* **30**: 1284-1298.

Flowers, T., Troke. P. and Yeo, A. (1977). The mechanism of salt tolerance in halophytes. *Annual Review of Plant Physiology* **28**: 89-121.

Flowers, T. J. and Colmer, T. D. (2015). Plant salt tolerance: adaptations in halophytes. *Annals of Botany* **115**: 327-331

Frank, D., Reichstein, M., Bahn, M., Thonicke, K., Frank, D., Mahecha, M. D., Smith, P., Velde, M., Vicca, S. and Babst, F. (2015). Effects of climate extremes on the terrestrial carbon cycle: concepts, processes and potential future impacts. *Global Change Biology* **21**: 2861-2880.

Gawal, N. and Jarret, R. (1991). A modified CTAB DNA extraction procedure forMusa andIpomoea. *Plant Moloecular Biology Reporter* **9**: 262-266.

Ibrahim, K., Collins, J. and Collin, H. (1991). Effects of salinity on growth and ionic composition of *Coleus blumei* and *Salvia splendens*. *The Journal of Horticultural Science and Biotechnology.* **66**: 209-214.

Khan, M.A. and Aziz, I. (2001). Salinity tolerance in some mangrove species from Pakistan. *Wetlands Ecology and Management* **9**: 229-233.

Lawlor, D. and Cornic, G. (2002). Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. *Plant, Cell & Environment* **25**: 275-294.

Munns, R. and Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology* **59**: 651-681.

Munns, R. and Sharp, R. (1993). Involvement of abscisic acid in controlling plant growth in soil of low water potential. *Functional Plant Biology* **20**: 425-437.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473-497.

Murkute, A., Sharma, S. and Singh, S. (2006). Studies on salt stress tolerance of citrus rootstock genotypes with arbuscular mycorrhizal fungi. *Horticultural Science* **33**: 70-76.

Nei, M. and Li, W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences* **76**: 5269-5273.

Parani, M., Lakshmi, M., Senthilkumar, P., Ram, N. and Parida, A. (1998). Molecular phylogeny of mangroves V. Analysis of genome relationships in mangrove species using RAPD and RFLP markers. *Theoretical and Applied Genetics* **97**: 617-625.

Parida, A., Das, A.B. and Das, P. (2002). NaCl stress causes changes in photosynthetic pigments, proteins, and other metabolic components in the leaves of a true mangrove, Bruguiera parviflora, in hydroponic cultures. *Journal of Plant Biology* **45**: 28-36.

Rains D.W. (1989). Plant tissue and protoplast culture: application to stress physiology and biochemistry. In: *Plants under Stresses. Biochemistry, Physiology and Ecology and Their Application to Plant Improvement*, H.G. Jones, T. J. Flowers and M.B. Jones (eds.), Cambridge University press, Cambridge. pp 181-196.

Rohlf, F. J. (2005). NTSYS-pc. Numerical Taxonomy and Multivariate Analysis System. Version 2.2. Exeter Software, New York.

Sobrado, M. and Ewe, S. M. (2006). Ecophysiological characteristics of Avicennia germinans and Laguncularia racemosa coexisting in a scrub mangrove forest at the Indian River Lagoon, Florida. *Trees* **20**: 679-687.

Song, T., Gao, Q., Xu, Z. and Song, R. (2011). The cloning and characterization of two ammonium transporters in the salt-resistant green alga, *Dunaliella viridis*. *Molecular Biology Reports* **38**: 4797-4804.

Souza, R., Machado, E., Silva, J., Lagôa, A. and Silveira, J. (2004). Photosynthetic gas exchange, chlorophyll fluorescence and some associated metabolic changes in cowpea (*Vigna unguiculata*) during water stress and recovery. *Environmental and Experimental Botany* **51**: 45-56.

Tester, M. and Davenport, R. (2003). Na^+ tolerance and Na^+ transport in higher plants. *Annals of Botany* **91**: 503-527.

Waisel, Y., Liphschitz, N. and Kuller, Z. (1972). Patterns of water movement in trees and shrubs. *Ecology* **53:** 520-523.

Wang, L., Mu, M., Li, X., Lin, P. and Wang, W. (2010). Differentiation between true mangroves and mangrove associates based on leaf traits and salt contents. *Journal of Plant Ecology* **1**: 10.

Zhang, C. and Chen, S. (1996). Analysis of genes specifically expressed under salt stress in salt-tolerant mutant of rice by using DDRT-PCR technique. *Scientia Sinica Vitae* **39**: 385-394.

Zhang, J. L., Flowers, T. J. and Wang, S. M. (2010). Mechanisms of sodium uptake by roots of higher plants. *Plant and Soil* **326**: 45-60.