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DNA Base Composition Heterogeneity in Two *Avicennia* Species in Response to Nitrogen Limitation in the Sundarban Mangrove Forest, India

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ABSTRACT

The influence of ecological nitrogen limitation on the composition of plant genomes is still unclear. This study aimed to find the role of nitrogen source in the Sundarbans on the genomic nitrogen and base heterogeneity in the leaf and root DNA of two *Avicennia* species. DNA in mangrove leaf and roots were quantified by spectrophotometric method. The quantitative determination of individual bases in the DNA after acid-hydrolysis was accomplished by reverse phase high-pressure liquid chromatography with UV detection. DNA was digested with alkaline persulphate solution in an autoclave and P and N concentration in the digest were determined using spectrophotometric method. For total inorganic nitrogen, soil sample was extracted in 2 M potassium chloride solution and ammonia-nitrogen, nitrite and nitrate-nitrogen were determined in the extract by using spectrophotometric method. Inorganic nitrogen concentration in sediment was found low ($4.04 \pm 1.06 \mu\text{g g}^{-1}$) and it exhibited positive linear relation with DNA nitrogen extracted from leaf ($\text{DNA-N} = 0.005 \pm 0.0572 \text{ Sed-N}$, $R^2 = 0.85$) and root ($\text{DNA-N} = -0.113 \pm 0.0791 \text{ Sed-N}$, $R^2 = 0.9$). N:P ratios in DNA were <4 . In contrast to the roots (A-T 57.63-59.01%; G-C 40.89-42.34%), the percentage of G-C-base pairs in leaves (58.42-64.38%) was greater than the percentage of A-T-base pairs (35.58-41.57%). Low abundance of nitrogen in the soil results in the occurrence of N-poor nucleotides in root DNA. The mangroves seem to assimilate nitrogen from both soil and the atmosphere with atmospheric NO_x uptake playing a greater role in leaf protein content than soil inorganic nitrogen. This study supports the hypothesis that higher levels of DNA with N-rich nucleotides in leaves relative to roots are necessary for enhance synthesis of protein to offset damage by photochemical processes.

Key words: Nitrogen limitation, N-poor genome, base stoichiometry, HPLC, mangrove

INTRODUCTION

Plants under stress exhibit epigenetic variation as an adaptive response to environmental factors (salt stress, nutrient stress, induction of anti-herbivore and anti-pathogen) and susceptibility to methylation (Vanyushin and Ashapkin, 2011). Alteration of their physical structure and chemical content as well as genetic materials could be related to their exposure to pollutants (Agrawal and Agrawal, 1999). For example, *Posidonia oceanica* Delile showed loss of A-T rich DNA (66.73%) on exposure to mercury (Maestrini *et al.*, 2002). However, ct-DNA could be protected from

the destabilizing action of high concentration of osmolyte synthesized during salinity stress by a natural polyamine, cadaverine (Petraccone *et al.*, 2004). To maintain growth associated with high RNA content, reduction of plant genomic size could occur as a result of evolutionary pressure promoted by nutrient limitation and could be linked to the reallocation of N from DNA to RNA (Hessen *et al.*, 2009). DNA base pair composition could be changed from an ancestral value as a result of environmental change (Berg *et al.*, 2006). Nitrogen atoms required for the building block nitrogen bases in the plant genome are derived from the inorganic form of nitrogen (NH_4^+ , NO_3^- and NO_2^-) occurring in the sediment. Plants could favor the use of bases with fewer nitrogen atoms under nitrogen limiting condition (Elser *et al.*, 2007).

Mangroves are an example of a group of plants that face a number of stresses, ranging from salinity and nutrient limitations to environmental stresses due to human activities. Deficiency in nitrogen in shoot tissues could occur in plants exposed under salinity stress (Backhausen *et al.*, 2005). Structural and physiological adaptation for survival is the common phenomenon observed amongst plants under extreme environmental stress (Dodd and Rafii, 2002). To study the adaptive behavior of mangrove plant to environmental factors by means of natural epigenetic variations is of recent interest (Lira-Medeiros *et al.*, 2010). Isolation and identification of 126 salinity tolerant cDNA from the root of *Bruguiera cylindrica* from mangrove area at Morib, Malaysia (Wong *et al.*, 2007) indicates the importance of further study of the salinity tolerance in mangrove plants. Again, salinity stress could decrease nitrate reductase activity and nitrate uptake in plants (Shaaban *et al.*, 2008; Bybordi and Ebrahimian, 2011). Nitric Oxide (NO) uptake under nitrogen limiting condition could induce salt tolerance in plants (Hasanuzzaman *et al.*, 2010). Ruan *et al.* (2002) also showed less destruction of chlorophyll in NO treated wheat leaves. Dahmardeh (2011) observed Photosynthetic Active Radiation (PAR) absorption increase with increasing nitrogen uptake in Maize.

In general, nitrogen is limiting in estuaries and marine ecosystems and this is also true for many mangrove ecosystems (Alongi, 2009). Nitrogen limitation could promote mangroves to select N-poor nucleobases. The A-T base pair is N-poor (74%) compared to the G-C base pair (84%). Vanyushin and Ashapkin (2011) suggested that genomic modification based on plant DNA methylation could be species specific and was located in non-GC sites. The Sundarbans mangrove environment is dominated by *Avicennia* followed by other mangrove species like *Ceriops*, *Bruguiera* and *Rhizophora* (Ray *et al.*, 2011). To understand the ecology of *Mangrove* species and communities, it is important to characterize DNA structure and its relationship to environmental factors. The main objective of this study was to determine the role of nitrogen limitation on the genomic nitrogen and base heterogeneity in the two species of *Avicennia* leaf and root DNA. In formulating the hypothesis for this study following question were considered: (1) How is nitrogen resource availability reflected in the nitrogen concentration in leaf and root DNA of *A. marina* and *A. alba* and (2) Does it affect base composition heterogeneity in genomic DNA?

MATERIALS AND METHODS

Study area: The Indian Sundarbans mangrove forest (latitude 20°32'-20°40'N and longitude 88°05'-89°E), is located on the world's largest delta in the estuarine portion of the river Ganges. It is a unique bioclimatic zone with a great biodiversity of mangrove flora and fauna. It is the last remnant of Bengal flood plains, a sprawling archipelago of 102 islands, out of which 54 are

reclaimed for human inhabitation. Anthropogenic perturbation could change the riverine inputs of nitrogen to this mangrove ecosystem.

Sample collection method: Samples (leaves and roots) of *A. alba* and *A. marina* in triplicate were collected from three islands: Nayachar (riverine), Chemaguri (estuarine) and Lothian (marine) in three seasons: monsoon, post-monsoon (winter and spring) and pre-monsoon in 2009. Plant leaves were collected from a 10 m height and roots were collected from plant parts extruded from sediments. Samples were washed thoroughly with deionised water and stored in liquid nitrogen before extraction. A corer made of stainless steel (5.5 cm i.d.) was used to extrude sediment upward and section it at three depth intervals (0-5, 5-10 and 10-15 cm) to measure total inorganic nitrogen in soil.

Laboratory analyses: This study used a simple and rapid method (Kelly *et al.*, 2008) for the determination of purine and pyrimidine bases isolated from *A. alba* and *A. marina* by reverse phase high-pressure liquid chromatography (RP-HPLC) with UV detection.

All four bases including Herring sperm DNA were obtained from HIMEDIA. Deionised water with specific resistance of greater than 18.2 M Ω cm was used. The HPLC equipment consisted of a JASCO HPLC apparatus (JASCO, Japan) consisting of a pump and controller unit with an injection volume of 20 μ L and UV detector. The system was coupled to a ODS Hypersil column (Thermo electron Corporation, Part No. 30109-254630, 4.6 \times 25 mm, 5 μ particle size and a guard column (5 \times 4.6 mm). The column temperature was ambient (22.0 \pm 3 $^{\circ}$ C) and the detector wave length was set at 254 nm.

Ten grams of crushed leaves were added to 50 mL of extraction buffer containing 100 mM Tris-HCl, 10 mM EDTA and 500 mM NaCl, homogenized and then mixed with 5 mL of SDS (20%) and stirred for 15-20 min. The samples were incubated for 10 min at 65 $^{\circ}$ C. Genomic DNA was precipitated from the extract using isopropanol and separated by centrifuging at 12,000x g at 10 $^{\circ}$ C for 15 min (Deshmukh *et al.*, 2007). The supernatant was transferred to another tube. The residue was resuspended in 25 mL extraction buffer and the process was repeated. The supernatant obtained from the second cycle was added to first and mixed with 50 mL 5 M potassium acetate and incubated at 0 $^{\circ}$ C for 30 min to remove protein and polysaccharides. The Supernatant obtained after centrifugation for 15 min was added to 78 mL isopropanol and stored (-80 $^{\circ}$ C, 2 h). After centrifugation (10,000x g, 20 min) the precipitate was washed with 70% (v/v) ethanol. After the initial extraction and alcohol precipitation, additional treatments were made using DNA solution in a saline citrate buffer (0.15 M NaCl, 0.015 M sodium citrate). Separation of RNA was performed by adding 1/3 volume of cold 8 M LiCl to the DNA solution and incubating the mixture at 4 $^{\circ}$ C overnight followed by centrifugation. The supernatant was subjected to two sequential extractions with phenol/chloroform (100/50/50 μ L) and isopropanol precipitation of DNA. Plant DNA was dissolved in saline citrate buffer (0.15 M NaCl, 0.015 M sodium citrate) for quantification by spectrophotometric methods using diphenylamine as the reagent and herring sperm as the standard (Plummer, 1971). The detection limit for this process was 0.46 μ g.

One hundred to five hundred micrograms of DNA was hydrolyzed in 1.0 mL of 72% (v/v) perchloric acid (~12 N) over a water bath for 2 h in a sealed Teflon bomb. The hydrolysate was diluted to 2.0 mL with water. Then the mixture was neutralized with 5 (N) KOH and centrifuged

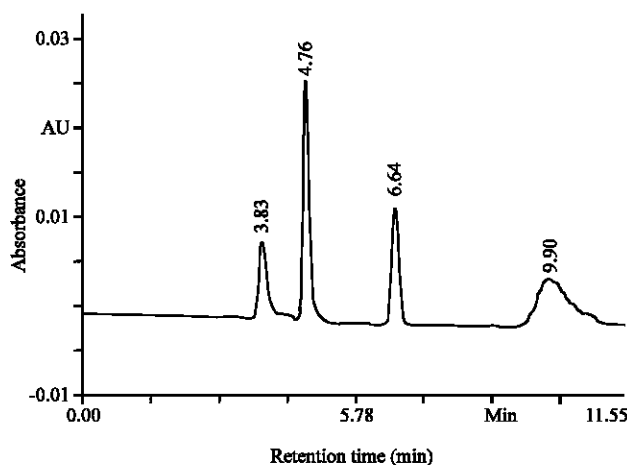


Fig. 1: Chromatogram (Absorbance, AU vs. Time (min)) for standard base mixture (order of elution, Cytosine, Guanine, Thymine and Adenine)

at 3000 rpm for 30 min. The supernatant was transferred to another tube. The solution was diluted 10 to 100 times with deionized water prior its injection.

Using a ODS Hypersil C18 reverse phase column, mobile phase constituting 3% (v/v) ACN, 50 mM acetic acid and 85 mM ammonium acetate at 1 mL min⁻¹ of flow rate was determined to be optimum for the separation of C, G, T and A. Injection of a 0.01 mM C, G, T, A individual and mixed standards into 1.0 mL min⁻¹ eluent stream resulted in retention times for the four purine and pyrimidine bases of 3.83 min (C), 4.76 min (G), 6.64 min (T) and 9.90 min (A) (Fig. 1). Acedolysis of DNA was accomplished by using 72% perchloric acid, with precipitation of excess perchlorate ion obtained through the neutralization of the mixture with KOH. The four bases in plant DNA were identified and quantified by comparing their retention times with those of the individual base solutions and a mixture of four bases from an acedolysed herring sperm standard. Recovery rates obtained by adding pre-determined amounts of individual bases to the diluted hydrolysates of herring sperm DNA were more than 98%. The method described here was found sensitive and applicable to the study of purine and pyrimidine bases in DNA of mangrove.

Thirty grams of the soil subsample was extracted in 75 mL of 2 mol L⁻¹ potassium chloride and ammonia-nitrogen and nitrite and nitrate-nitrogen were quantified in the extract using spectrophotometric method (Riley and Vitousek, 1995). Sediment samples were placed in screw capped centrifuge tubes, from which pore water was separated avoiding air contact by means of centrifugation (30 min, 5000 rpm). Salinity of the pore water samples was determined by Mohr-Knudsen titration (Strickland and Parsons, 1972). Phosphorus and nitrogen concentrations in sediment DNA were determined using an alkaline persulphate autoclave digestion (potassium peroxy disulphate, boric acid and sodium hydroxide) followed by quantification of the released orthophosphate and nitrate in a spectrophotometric assay (Grasshoff *et al.*, 1983).

Statistical analyses: An ANOVA analysis was performed for comparing the significance difference between leaf and root DNA concentration. To evaluate the relationship between sediment nitrogen and DNA nitrogen regression and correlation analysis was performed. All statistical analyses were performed using the MINITAB (Version 13.0) statistical package.

RESULTS

The most interesting patterns were found, concerning differences in DNA nitrogen content between leaf and root tissues. The mean DNA concentrations in *A. marina* and *A. alba* were found to be 32.14 ± 15.8 and 32.57 ± 6.12 in leaves and 15.2 ± 2.7 , 14.8 ± 3.0 $\mu\text{g g}^{-1}$ in roots, respectively (Table 1). The mean leaf and root DNA-nitrogen concentration were statistically different between the two species (0.20 ± 0.02 and 0.16 ± 0.05 $\mu\text{g g}^{-1}$ DNA in *A. marina* versus 0.24 ± 0.12 and 0.20 ± 0.16 $\mu\text{g g}^{-1}$ DNA in *A. alba*). However, leaf and root DNA-phosphorus levels did not differ significantly between the species (0.13 ± 0.02 and 0.13 ± 0.01 $\mu\text{g g}^{-1}$ DNA in *A. marina* versus 0.15 ± 0.06 and 0.14 ± 0.08 $\mu\text{g g}^{-1}$ DNA in *A. alba*). The mean nitrogen to phosphorus ratios was lower in mangrove leaves (3.4-3.5) and roots (2.7-3.2) than in Herring sperm (4.0).

Depth profile of nitrate, nitrite and ammonia in sediments from different sites are given in Table 2. Total inorganic nitrogen concentrations were highest (4.5 - 5.7 $\mu\text{g g}^{-1}$) at salinity 4 psu, intermediate (4.0 - 4.2 $\mu\text{g g}^{-1}$) at salinity 11-12 psu and lowest (2.7 - 3.0 $\mu\text{g g}^{-1}$) at salinity 15-17.5 psu in dry sediment.

Typical examples of chromatogram for *A. marina* and *A. alba* are given in Fig. 2a and b for leaves and in Fig. 3a and b for roots. Concentrations of four purine and pyrimidine bases in the sample were estimated comparing the area and retention times observed for C (3.83 min), G (4.76 min), T (6.64 min) and A (9.90 min) in the standard DNA sample (Fig. 1). In leaves, the

Table 1: Mean DNA concentration, AT and GC base percentages, total N and N: P ratios in DNA from various biotic sources

Name	Total DNA ($\mu\text{g g}^{-1}$)	Adenine + Thyamine (AT %)	Guanine + Cytosine (GC %)	Total N, P ($\mu\text{g g}^{-1}$ DNA)	N:P molar ratio
Herring sperm (Standard)	-	58.32	41.66	0.35, 0.18	4.0
<i>A. marina</i>				0.20 ± 0.02 , 0.13 ± 0.02	3.4
Leaf	32.14 ± 15.8	35.58	64.38	0.16 ± 0.05 , 0.13 ± 0.01	2.7
Root	15.20 ± 2.7	57.63	42.34	0.24 ± 0.12 , 0.15 ± 0.06	3.5
<i>A. alba</i>				0.20 ± 0.16 , 0.14 ± 0.08	3.2
Leaf	32.57 ± 6.12	59.01	40.89		
Root	14.80 ± 3.0				
Sea urchin*		64.90	36.10		
<i>Mycobacterium tuberculosis</i> *		29.70	70.30		

*Chargaff and Davidson (1955)

Table 2: Depth profiles for salinity and different forms of Nitrogen in the Sundarbans mangrove sediment. Reported values are means over several samples

Station name	Salinity (‰)	Nitrate ($\mu\text{g gm}^{-1}$ dry sediment)	Nitrite ($\mu\text{g gm}^{-1}$ dry sediment)	Ammonia ($\mu\text{g gm}^{-1}$ dry sediment)	Total inorganic nitrogen ($\mu\text{g gm}^{-1}$ dry sediment)
Chemaguri (0-5 cm)	12	0.097 ± 0.04	0.026 ± 0.003	4.016 ± 0.15	4.139
Chemaguri (5-10 cm)	11	0.166 ± 0.07	0.116 ± 0.03	3.878 ± 0.09	4.160
Chemaguri (10-15 cm)	12	0.232 ± 0.02	0.172 ± 0.011	3.754 ± 0.13	4.037
Nayachar (0-5 cm)	4	0.732 ± 0.06	0.051 ± 0.02	4.590 ± 0.09	5.373
Nayachar (5-10 cm)	4	0.233 ± 0.07	0.130 ± 0.04	4.121 ± 0.10	4.484
Nayachar (10-15 cm)	4	0.794 ± 0.10	0.105 ± 0.025	4.771 ± 0.07	5.670
Lothian (0-5 cm)	15	0.718 ± 0.11	0.035 ± 0.004	1.949 ± 0.11	2.700
Lothian (5-10 cm)	15	0.701 ± 0.07	0.099 ± 0.01	2.011 ± 0.12	2.801
Lothian (10-15 cm)	17.5	0.476 ± 0.06	0.141 ± 0.02	2.363 ± 0.10	2.980

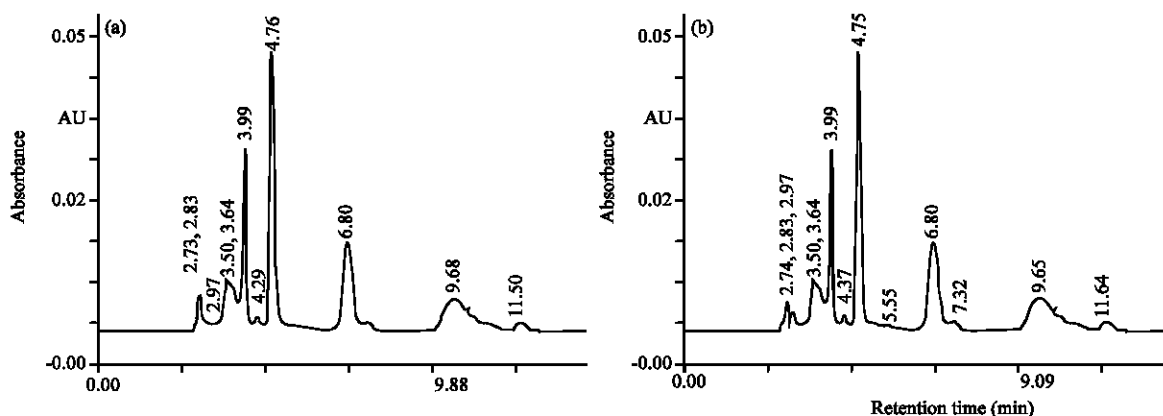


Fig. 2(a-b): Chromatogram (Absorbance, AU vs. Time (min)) for the acidolysed leaf DNA of (a) *A. alba* and (b) *A. marina*

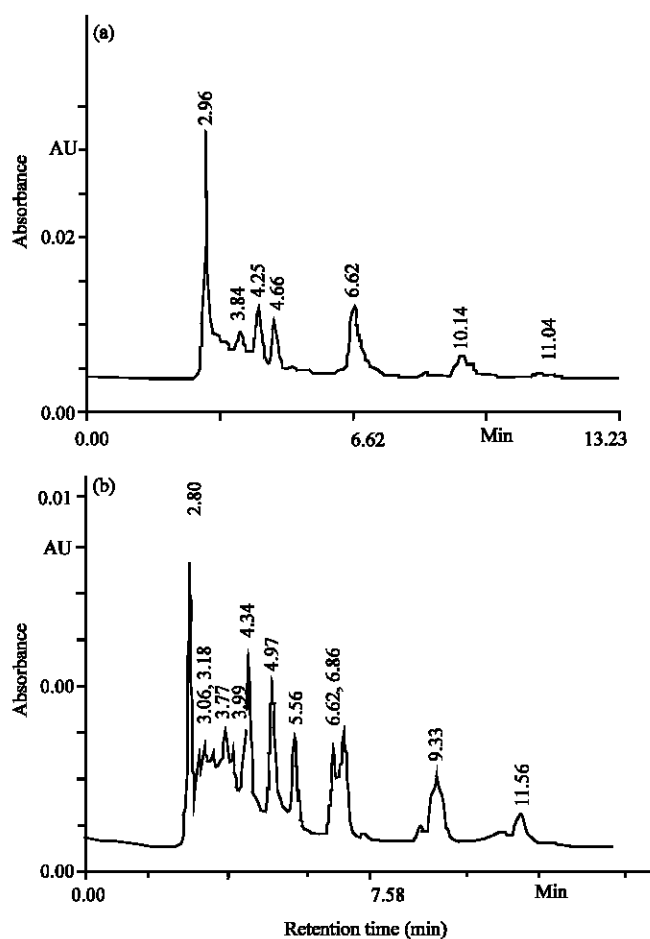


Fig. 3(a-b): Chromatogram (Absorbance, AU vs. Time (min)) for the acidolysed root DNA of (a) *A. alba* and (b) *A. marina*.

percentage of GC was found greater than for AT (64.38, 35.58% in *A. marina* and 58.42, 41.57% in *A. alba*, in contrast to the roots which showed greater percentages of AT than GC (57.63, 42.34 in *A. marina* and 59.01, 40.89% in *A. alba*). To evaluate the relationship between

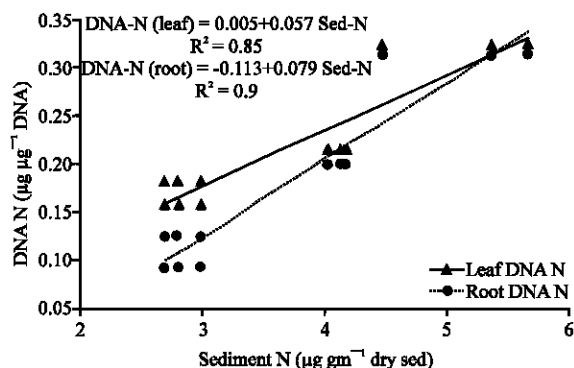


Fig. 4: Total inorganic nitrogen in the sediment (Sed-N) as a function of DNA- nitrogen (DNA-N) for leaves (closed triangles) and roots (closed circles). Significant linear regressions describing the relationship between Sed-N and DNA-N for leaves and roots are of the form $\text{DNA-N} = 0.005 \pm 0.057 \text{ Sed-N}$, $R^2 = 0.85$ and $\text{DNA-N} = -0.113 + 0.079 \text{ Sed-N}$, $R^2 = 0.9$, respectively

sediment- nitrogen and leaf/root DNA-nitrogen, a regression procedure was applied to the data for two species. Significant positive regressions ($p < 0.001$) were obtained and 85 and 90% of the variability in DNA nitrogen in leaves and roots were explained (Fig. 4) by the concentration of nitrogen in the sediment ($\text{DNA-N (leaf)} = 0.005 \pm 0.057 \text{ Sed-N}$, $R^2 = 0.85$ and $\text{DNA-N (root)} = -0.113 \pm 0.079 \text{ Sed-N}$, $R^2 = 0.9$)

DISCUSSION

The DNA concentration found in mangrove leaves is lower than the range that is reported for leaves of *Gossypium hirsutum* L. ($150\text{-}400 \mu\text{g g}^{-1}$) (Permingeat *et al.*, 1998) and tobacco ($67.3 \mu\text{g g}^{-1}$) (Ceccherini *et al.*, 2003). Occurrence of lower DNA concentration in mangrove roots than in leaves (ANOVA, $F = 16.66$, $p = 0.006$) could be responsible for lower ratios of Living Below-ground Biomass (LBGB) to Above-ground Biomass (AGB) ($0.08\text{-}0.46$) (Wong *et al.*, 2007). Alongi *et al.* (2003) suggested that environmental stress could produce differences in biomass allocation between roots and leaves even within the same species. In the two species of *Avicennia* studies here, GC and AT percentages in DNA were in the range reported in other organisms *M. tuberculosis*, 70.3% GC, sea urchin, 64.9% AT (Chargaff and Davidson, 1955); *Rickettsiae* 30-38.6% GC (Tyeryar *et al.*, 1973) and *Pinus*, 39.5% GC (Bogunic *et al.*, 2003). Kannan and Vincent (2011) isolated 20 *Streptomyces* from rhizosphere soil of Manakkudy mangroves, West Coast of India and GC content were found to be 58-59.6%. However, the occurrence of AT-rich root DNA in both species of *Avicennia* could presumably be due to environmental factors (Kapraun *et al.*, 1993) and root could be relatively more under nitrogen limitation and pollutant stress than leaf (Agrawal and Agrawal, 1999; Backhausen *et al.*, 2005).

Mean inorganic nitrogen levels found in the *Sundarbans mangrove* sediment are lower ($4.04 \pm 1.06 \mu\text{g g}^{-1}$) than in an Australian mangrove forest ($4.3\text{-}7.3 \mu\text{g g}^{-1}$) (Boto and Wellington, 1984). *Avicennia* is one of the species which responds to nitrogen resource constraints via adaptation. An ANOVA analysis indicated that there was no significant difference of leaf or root DNA concentration ($p < 0.05$) between *A. marina* and *A. alba*. This suggests that nitrogen resource variation could result in substantially different DNA-N content between plant organs

(Niklas *et al.*, 2005) and that this variation could be reflected in N:P ratios (Feller *et al.*, 2007). Both P and N are part of nucleic acid molecules which are more enriched in P than in N with an N:P stoichiometry of 4:1 (Reef *et al.*, 2010). We found N: P stoichiometry of DNA lower in *A. marina* and *A. alba* leaves and roots than in the standard Herring sperm DNA. Mangroves absorb nitrogen from both soil and the atmosphere, with NO_x uptake playing a greater role for leaf protein content compared to soil inorganic nitrogen in the Sundarbans mangrove ecosystem (Ganguly *et al.*, 2009) and salinity tolerance (Zhao *et al.*, 2007; Hasanuzzaman *et al.*, 2010). Compared to roots, mangrove leaves may need higher levels of DNA with N-rich nucleotides for transcription and translation to support protein synthesis (Hessen *et al.*, 2009) in order to offset damage caused by photochemical processes (Raven, 1989). Shi *et al.* (2005) suggested that NO uptake could effectively protect plants against UV-B radiation, possibly through the enhanced antioxidative enzyme activity.

CONCLUSION

The results support the hypothesis that higher level of DNA with N-rich nucleotides in the leaf in contrast to the root is necessary to increase the synthesis of protein for its damage by photochemical processes. Under limited supplies of nitrogen *Avicennia* sp. preferentially uses N-poor nucleotides for root DNA.

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