

Screening of certain mangroves for photosynthetic carbon metabolic pathway

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Abstract

The mangroves *Rhizophora lamarkii*, *Ceriops roxburghiana*, *Bruguiera gymnorhiza*, *Aegiceras corniculatum*, and *Lumnitzera racemosa* were screened for their carbon metabolic pathways by measuring net photosynthetic rate (P_N), ^{13}C discrimination rate, leaf anatomy, titratable acidity, and activities of phosphoenolpyruvate carboxylase, NADH-malate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, and pyruvate phosphate dikinase. The tested mangroves had a well developed succulence, opening of stomata during day time and closure in the night hours, and absence of diurnal fluctuation of organic acids in their leaves which excludes the possibility of these species being CAM plants. Moreover, the leaf anatomy had not exhibited Kranz syndrome. The high values of discrimination against ^{13}C , low P_N , high CO_2 compensation concentration, and the activities of aminotransferases in the direction of alanine formation suggest that the species may follow C_3 mode of carbon metabolic pathway.

Additional key words: alanine aminotransferase; aspartate aminotransferase; ^{13}C discrimination; C_3 carbon metabolic pathway; Kranz syndrome; NADPH-malate dehydrogenase; net photosynthetic rate; phosphoenolpyruvate carboxylase; pyruvate phosphate kinase; stomata opening.

Mangroves are inter-tidal forests of tropical and subtropical regions that function in a hoarse intertidal tropical environment. Salinity constituents are the main stressor and regulator of their development and productivity. Tolerance of these plants to salinity has been associated with ion inclusion or exclusion, ion compartmentation, and favourable ion balance (Naidoo 1994). The specific morphological and biochemical modifications which occur in each species might reflect the individual adaptive capacity of the plants in saline environment (Rais *et al.* 1993).

Responses of salt tolerance associated with salt effects include development of succulence in leaves (Waisel 1972, Flowers *et al.* 1986), changes in osmotic pressure (Popp 1984), salt exclusion on secretion (Naidoo 1994), and accumulation of low molecular mass compounds (Popp and Albert 1995). Among the various metabolic processes influenced by salt stress, photosynthesis is the key to plant productivity. Plant functional types mainly, including photosynthetic pathways (C_3 , C_4 , and CAM) and

morphological functional types (Liu and Wang 2006) are popular means for studying the logical links between physiological and life history strategies at plant level, as well as ecological processes at ecosystem and global levels (Chapin 1993, Paruelo and Lauenroth 1996, Wang 2003). Plant functional types are defined on the basis of plant morphological and physiochemical traits, life history, and bioclimatic tolerance, relying on the research intentions and studying scales (Wang 2003, 2007). Most of the studies on plant functional types were focused on photosynthetic pathway identification (Williams and Markley 1973, Downton 1975, Raghavendra and Das 1978, Waller and Lewis 1979, Redmann *et al.* 1995, Wang 2002, Liu *et al.* 2004). We tried to define the carbon assimilation pathways in certain mangroves to classify them to C_3 , C_4 , or CAM plants and on the photosynthetic enzymes to confirm the carbon assimilation pathways in these mangroves.

The mature and fully developed leaves of *Rhizophora lamarkii* Montr., *Ceriops roxburghiana* Arn., *Bruguiera*

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gymnorhiza (L.) Lamk. (Rhizophoraceae), *Aegiceras corniculatum* Blanco (Myrsinaceae), and *Lumnitzera racemosa* Willd. (Combretaceae) were collected from Vellar-Coleroon estuarine complex of Pichavaram mangrove forest, which is located on the South east coast of India (11°24'N, 79°44'E), brought to the laboratory, surface washed with distilled water, and blotted dry.

Net photosynthetic rate (P_N), intercellular CO_2 concentration (C_i), and stomatal conductance (g_s) were monitored using a *LI-6200* portable infra-red gas analyzer. All the measurements were made during the noon when the stomata were fully open. Precautions were taken to avoid any water vapour on the leaf surface during the measurements. Always, a 1 000 cm^3 leaf chamber was used. Readings were taken at 5-s intervals and 10 readings were computed for each measurement. Five to six such measurements were analysed. Only natural radiation was used during these measurements. For ^{13}C discrimination, leaf tissue was dried in an oven at 80 °C for 48 h, the dried tissue (5–10 mg) was ignited at 80 °C in an excess of oxygen, and isotope ratios of the CO_2 evolved were measured on a mass spectrophotometer (*Varian, SATURN-2200*) (Meirong 1993);

$$\delta^{13}\text{C} = R_{\text{sample}} - R_{\text{standard}} (R_{\text{standard}})^{-1} \times 1000,$$

where $R = \text{mass}_{44}/\text{mass}_{45}$ and the standard in carbonate from the fossil skeleton of *Belemnitellam americana* from the Peedee formation of South California (PDB). CO_2 compensation concentration (Γ) was determined based on pH measurement of bicarbonate buffer. Fully expanded leaves were cut and re-cut under water and transferred to a 5-cm³ vial of water. 20 cm³ of potassium bicarbonate solution (5×10^{-4} M) containing 1% universal indicator was put into a 250-cm³ conical flask. The vial containing plant was suspended with the help of a string in the flask 1 cm above the level of bicarbonate solution. The other end of the string was pulled out of the flask and the mouth of the flask was sealed with a double layer of plastic. The whole set-up was made absolutely airtight. Care was taken that the plant material did not contact the assay solution. The whole set-up was exposed for 1 h to irradiance of 160 W m⁻². The change in the pH of the bicarbonate solution was used to calculate carbon dioxide concentration and then Γ (Coombs 1978): $\Gamma = 22.4 [\text{CO}_2] \alpha^{-1}$ where α is the solubility of CO_2 at given temperature.

For determining Kranz syndrome, leaf transections 10 μm thick were mounted in the water film and first examined for the presence of a clearly defined parenchyma bundle sheath and for the arrangement of mesophyll cells and then potassium iodide was applied to detect starch. The starch distribution in the late afternoon and evening assured high content in all stages. Fresh leaf material was placed in 80% boiling ethanol until chlorophyll was fully extracted and then it was placed in 10% NaOH to attain clarity. The material was rinsed with distilled water and stained with potassium iodide solution.

To measure titratable acidity, 1 g of fresh leaf tissue was ground in 10 cm³ of distilled water with pestle and mortar and then boiled in a water bath of 80 °C for 10 min. The filtered extract was centrifuged at 600 rpm for 10 min and the supernatant was titrated against 0.2 M KOH to an obituary end point of pH 7.5 (Spalding and Edwards 1978).

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) activity was assayed by the oxidation of NADH at 340 nm. The reaction mixture contained a total volume of 3 cm³ of Tris-HCl buffer (1 mM, pH 7.8), 10 mM sodium bicarbonate, 5 mM phosphoenolpyruvate, and 0.4 mM NADH. A blank was simultaneously run with reaction mixtures without the addition of NADH. Crude malate dehydrogenase (NADH-MDH, EC 1.1.1.37) extract was obtained from the homogenized plant material which was ground with acid-washed sand in a pre-chilled pestle and mortar with a grinding medium containing 50 mM Tris-HCl buffer (pH 8.0), 50 mM MgCl₂, 5 mM 2-mercaptoethanol, and 1 mM EDTA. The homogenate was passed through four layers of cheese cloth and the filtrate was centrifuged at 3 000 rpm for 20 min at 5 °C. The supernatant was saved as enzyme source for MDH. The enzyme activity of MDH was assayed by the oxidation of NADH at 340 nm. The reaction mixture contained a total volume of 3 cm³ of 5 mM oxaloacetic acid, 10 mM of MgCl₂, 0.1 mM Tris-HCl buffer (pH 7.8), and 0.4 mM NADH. The aspartate aminotransferase (EC 2.6.1.1) source was extracted by grinding the plant tissue in 0.2 M potassium phosphate buffer (pH 7.5) in a homogenizer for 2 min. The slurry was passed through eight layers of cheese cloth and then centrifuged at 25 000 rpm for 15 min to get the enzyme fraction. The enzyme was assayed in a reaction mixture containing DL-aspartic acid, 2,4-dinitrophenyl hydrazine (DNPH), and 0.4 M sodium hydroxide. The activity was measured at 510 nm in a UV-VIS spectrophotometer (*Hitachi U-2001*). The alanine aminotransferase (ALT, EC 2.6.1.2) source was extracted by grinding the plant tissue in 0.2 M potassium phosphate buffer (pH 7.5) in a homogenizer for 2 min. The slurry was passed through eight layers of cheesecloth and then centrifuged at 25 000 rpm for 15 min to get the enzyme fraction. The enzyme was assayed in a reaction mixture containing DL-alanine, 2,4-dinitrophenyl hydrazine (DNPH), and 0.4 M sodium hydroxide. The activity was measured at 510 nm in a UV-VIS spectrophotometer (*Hitachi U-2001*). All these enzymes were measured according to Sadasivam and Manickem (1981). Pyruvate phosphate dikinase (PPD, EC 2.7.9.1) was extracted at 22±1 °C. The extraction medium contained 50 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 1 mM EDTA, 40 mM mercaptoethanol, 1.5% polyvinylpyrrolidone, and 10 mM sodium metabisulphite. PPPD was assayed in a reaction mixture (1 cm³) containing 50 mM Tris-HCl (pH 7.5), 1 mM pyruvate, 10 mM ATP, 1.3 mM sodium glutamate, and 0.1 cm³ crude extract. Reaction was started with the addition of 10 mm³ NaH¹⁴CO₃ and was terminated by the

Table 1. Characteristics of the tested mangrove species: net photosynthetic rate (P_N) [$\text{mg}(\text{CO}_2) \text{ m}^{-2} \text{ s}^{-1}$], intercellular CO_2 concentration (C_i) [$\mu\text{mol mol}^{-1}$], stomatal conductance (g_s) [cm s^{-1}], and enzyme activities: ALT – alanine transferase, AST – aspartate transferase, PEPC – phosphoenolpyruvate carboxylase, PPDK – pyruvate phosphodikinase, and NADH-MDH – NADH-malate dehydrogenase [$\text{mmol kg}^{-1} \text{ s}^{-1}$].

Plant species	P_N	C_i	g_s	$\delta^{13}\text{C}$	Γ	PEPC	NADH-MDH	ALT	AST	PPDK
<i>Rhizophora lamarkii</i>	9.1	0.681	0.12	-29.8	66	0.132	0.008	2.62	0.69	0.039
<i>Ceriops roxburghiana</i>	6.8	0.642	0.12	-26.5	46	0.072	0.005	1.89	0.57	0.052
<i>Bruguiera gymnorhiza</i>	8.3	0.566	0.13	-29.3	61	0.066	0.010	1.92	0.52	0.007
<i>Aegiceras corniculatum</i>	8.1	0.534	0.13	-28.2	47	0.043	0.002	2.02	0.37	0.021
<i>Lumnitzera racemosa</i>	6.4	0.642	0.11	-27.6	47	0.051	0.004	2.00	0.32	0.033

Table 2. Diurnal measurement of titratable acidity [meq kg^{-1} (fresh mass)] in the leaves of some mangrove species.

Plant species	07:00	09:00	11:00	13:00	15:00	17:00
<i>Rhizophora lamarkii</i>	49	47	47	44	43	40
<i>Ceriops roxburghiana</i>	44	42	42	40	39	37
<i>Bruguiera gymnorhiza</i>	39	37	35	31	58	26
<i>Aegiceras corniculatum</i>	43	42	40	34	32	30
<i>Lumnitzera racemosa</i>	42	40	40	39	37	35

addition of 0.05 cm^3 of 20 % trichloroacetic acid after 2 min at $25 \pm 1^\circ\text{C}$. The activity was measured by counting the radioactivity in a liquid scintillation counter (*LKB RACKBETA*, model 6010, UK). The amount of enzyme required to fix 1 μmol of CO_2 in 1 min of experimental conditions was defined as a unit (Das and Raghavendra 1976).

The mangrove species tested showed low P_N of $6.4\text{--}9.1 \text{ mg}(\text{CO}_2) \text{ m}^{-2} \text{ s}^{-1}$ and C_i between $0.534\text{--}0.681 \mu\text{mol mol}^{-1}$ (Table 1). $\delta^{13}\text{C}$ ranged between -26.5 and $-29.8 \text{ \textperthousand}$. Γ was high, 47–66 (Table 1). The tested mangrove species were without Kranz syndrome. The mesophyll cells surrounding the vascular bundles did not contain chloroplasts, there were no starch grains in the cells, and the cells surrounding the vascular bundles did not stain for starch. There was no diurnal fluctuation of organic acids (Table 2). The stomata opened during the day and closed during night.

The activity of PEPC ranged between 0.043 and 0.132 $\text{mmol kg}^{-1}(\text{protein}) \text{ s}^{-1}$, the activity of NADH-MDH was $0.002\text{--}0.010 \text{ mmol kg}^{-1}(\text{protein}) \text{ s}^{-1}$ (Table 1). The activities of ALT, AST, and PPDK were $1.89\text{--}2.62$, $0.32\text{--}0.69$, and $0.007\text{--}0.052 \text{ mmol}(\text{substrate}) \text{ kg}^{-1}(\text{protein}) \text{ s}^{-1}$, respectively. There was a highly uniform action of ALT in all the species tested. The AST activities were very low.

Plant matter produced during photosynthesis is depleted in ^{13}C (low $^{13}\text{C}/^{12}\text{C}$) compared to atmospheric CO_2 (Craig 1954, Bender 1968). The magnitude of this depletion mainly depends on the photosynthetic pathway of CO_2 fixation (Smith and Epstein 1971, Osmond *et al.* 1973). $\delta^{13}\text{C}$ in higher plants fall in three categories which are associated with the pathway of carbon assimilation, the conventional C_3 pathway, the dicarboxylic acid C_4 pathway, and the pathway associated with CAM. Discrimination against ^{13}C occurs during diffusion of CO_2 into the leaf and during the enzymatic convention of dis-

solved CO_2 in the cytoplasm to saccharides. C_3 and C_4 plants possess diagnostic stable carbon isotope ratios (Deines 1980): C_3 plants are characterized by $\delta^{13}\text{C}$ from -22 to $-38 \text{ \textperthousand}$ and C_4 plants from -8 to $-15 \text{ \textperthousand}$ (Ye and Wang 2001), whereas $\delta^{13}\text{C}$ of the atmospheric CO_2 is around $-8 \text{ \textperthousand}$ (Farquhar *et al.* 1989). The variation within C_3 plants can result mainly from canopy effects and moisture stress either driven by salinity or aridity (O'Leary 1988, Lin and Strenberg 1992). The $\delta^{13}\text{C}$ values for all tested mangrove species were high.

A capacity of CAM is usually associated with the photosynthetic leaves or stem which are succulent. The CAM species are characteristic by diurnal fluctuation of organic acids, night time stomata opening, and CO_2 uptake leading to formation of free malic acid. We found that the carbon pathway had not shifted to CAM, because the stomata were predominately open during day time and there was no diurnal fluctuation of organic acids. Activities of enzymes involved in the photosynthetic CO_2 assimilation indicated that AST activity was low which indicated that the transfer of amino group from aspartate is restricted, although the enzyme is versatile with respect to substrate (Joshi *et al.* 1975). Mangroves are aspartate formers. Moreover, under salinity transaminases are more active than dehydrogenases yielding massive amino acid synthesis, more in the direction of alanine formation than in aspartate formation. The low activity of AST indicated the possibility of aspartate formation by some other mechanism than transamination. Similarly, the pyruvate P_i kinase activity was low in the leaves of all the mangroves studied. PEPC is regenerated from pyruvate by the action of pyruvate P_i kinase (Hatch 1976), the only enzyme unique to C_4 plants. This enzyme has been found in some mangroves (Joshi *et al.* 1980). The activity

of NADH-MDH was not high enough to be detected in these plants, being rather low when compared to other malate formers.

The mangroves studied had low P_N and well developed succulence, opened stomata during day time and closed them in the night. Besides, absence of diurnal fluctuation of organic acids in the leaves of all mangrove species studied excluded the possibility of these species being CAM plants. Moreover, the leaf anatomy

did not exhibit Kranz syndrome and all the species showed high CO_2 compensation concentration. The activity PEPC was very low in all the mangroves studied. Similarly, ALT activity was higher than that of AST. The high ^{13}C discrimination, the low P_N , low activity of PEPC, high CO_2 compensation concentration without Kranz syndrome, and the activity of ALT in the mangrove species tested suggest that these species follow the C_3 mode of carbon assimilation.

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Hell, R., Dahl, C., Knaff, D., Leustek, T. (ed.): **Sulfur Metabolism in Phototrophic Organisms**. – Springer, Dordrecht 2008. ISBN 978-1-4020-6862-1 (hard bound), ISBN 978-1-4020-6863-8 (e-book). 516 pp., € 229.00, CHF 399.00, USD 329.00, GBP 176.00.

This book is volume 27 of the series “Advances in Photosynthesis and Respiration”. Two of its editors work in Germany, the other two in the U.S.A. This is reflected in the relatively high amount of chapter authors working in these two countries: out of the 55 authors, 18 are from Germany and 16 from the U.S.A. As concerns the remaining 21 authors, they are from the U.K. (5), Australia (4), Canada, Italy, and Japan (3 each), and Denmark, Israel, and the Netherlands (1 each). Only four chapters were prepared by one author. The majority of authors are scientists well known from the literature. One of them comes even from the Czech Republic (Stanislav Kopřiva), even if he works now in the U.K.

The relatively narrow research field enables to discuss all possible questions connected with sulfur in relation to photosynthesis. Twenty four chapters are divided into four parts. Seven chapters of Part I are dedicated to general questions such as sulfur uptake, distribution, and sub-cellular transport, phylogeny of sulfate assimilation, and biosynthesis and metabolic roles of the most important sulfur substances (cysteine and methionine) and enzymes (sulfotransferases and cysteine desulfurase) in plants and phototrophic bacteria. They deal also with the respective signal transduction pathways and regulation, genetics, differences among classes and species, and related substances (iron-sulfur assembly, thiamine, molybdenum co-factor).

The six chapters of Part II (Sulfur in Plants and Algae) are dedicated to molecular biology of plant sulfate uptake and assimilation, compartmentation and cellular functions of sulfur containing substances, sulfolipids, secondary sulfur metabolites and their function in plant defence, sulfur cycling, sulfate reduction, sulfite oxidation, their ecology, *etc.* Part III (Sulfur in Phototrophic Prokaryotes; 5 chapters) overviews the systematics of anoxygenic phototrophic bacteria, analyzes the function of inorganic sulfur compounds as electron donors, deals with sulfide oxidation in the wide organism range from

cyanobacteria to humans, *etc.* Green and purple sulfur bacteria are the most discussed organisms in this part. Part IV (4 chapters) is on sulfur ecology and biotechnology in nature, namely in sulfur bacteria, algae, and agricultural ecosystems. Sulfur requirements in nutrition of agrosystems, sulfur and food quality, adaptations to stress and pests are among the discussed questions. Molecular fossils containing sulfur are also included. A special chapter is on the use of anoxygenic photosynthetic bacteria for the removal of sulfide from wastewater (suitable bacteria, reactor concepts and models, direction of future research). The last part contains a chapter on using X-ray absorption spectroscopy for the detection and identification of sulfur compounds, and a chapter on imaging thiol-based redox processes in live cells.

I understand why the scientists working in systematics continuously change the genus and species names of plants and bacteria, but in case of well known organisms it does not help understanding in other fields of research. Thus in 2003 the often used genus name *Chlorobium* was re-named to *Chlorobaculum* (cf. Chapter 18).

The book is supplemented by many instructive tables and figures (pages CP1-CP4 present them in colour) and by a detailed numerical and alphabetical subject index. I found only one inconsistency in editorial work: the form of references in Chapter 13 differs from the standard one used in other chapters. It also seems strange that the position of acknowledgements is included in the list of contents.

The advantage of the recently published volumes of this book series is that they are produced in two forms – in printed and in electronic ones; the last one has some advantage for rapid finding the respective topic or reference according to authors’ names. One of these forms of volume 27 should certainly be on bookshelves of photosynthesis laboratories and university or research libraries.

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