UV-B induced alterations in composition of thylakoid membrane and amino acids in leaves of *Rhizophora apiculata* Blume

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Abstract

Seedlings of *Rhizophora apiculata* were exposed to UV-B radiation at four doses equivalent to 10, 20, 30, and 40 % ozone depletion. The seedlings irradiated with high doses of UV-B had characteristic decline in contents of specific proteins with molecular masses of 33, 23, and 17 kDa. On the contrary, proteins of 55, 33, 25, 23, and 17 kDa were accumulated in the seedlings exposed to low doses of UV-B. The UV-B, in general, enhanced formation of saturated fatty acids and reduced unsaturated fatty acids, to a maximum extent of 88 and 26 %, respectively. The low dose of UV-B increased content of oleic acid by 9 %, and the high dose reduced it by 34 %. The high dose of UV-B enhanced the lipid peroxidation by 48 %, whereas the low dose of UV-B did not show any significant effect. The contents of amino acids such as aspartate, glutamate, asparagine, serine, glutamine, threonine, and histidine were increased in low UV-B doses by 53, 86, 142, 72, 3, 119, and 32 %, respectively; while in high doses they were reduced significantly.

Additional key words: lipid peroxidation; mangrove; saturated and unsaturated fatty acids.

Introduction

Since the discovery of ultraviolet (UV) wavelength band, studies pertaining to the effects of UV on biological organisms have been intensified. Solar electromagnetic radiation falling on earth surface does not go below 290 nm, due to the presence of ozone in stratosphere, which characteristically absorbs shorter wavelength UV radiation. Hence, UV radiations which reach earth are UV-A (320-400 nm) and UV-B (280-320 nm). Recently, Blumthaler and Ambach (1994) indicate that since 1980 terrestrial UV-B fluxes have been progressively increasing by 1 % per year. Impacts

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of enhanced UV-B radiation on biological processes in plants have been studied extensively (Caldwell 1971, Teramura 1980). UV-B is deleterious to photosynthetic activities and other physiological functions in crop and tree species (Sullivan *et al.* 1994, Teramura *et al.* 1991, Ziska and Teramura 1991). However, some plants counter the damage by producing UV-absorbing compounds, by alterations in leaf anatomy (Mirecki and Teramura 1984) or by leaf thickening (Sullivan *et al.* 1994). Membranes are the prime target of UV-B radiation (Kramer *et al.* 1991). UV-B induced decrease in photosynthesis may involve damage of thylakoid membrane by reducing the contents of individual fatty acids, thus altering the ratio of polar lipids (Kramer *et al.* 1991), lipid peroxidation (Panagopoulos *et al.* 1990), and content of thylakoid proteins (Enami *et al.* 1989, Nedunchezhian and Kulandaivelu 1991), and lowering the size of photosynthetic unit (Lingakumar and Kulandaivelu 1996). Besides altering the chloroplast structure and function, UV might influence the concentration of individual amino acids which are important in nitrogen metabolism of mangroves (Bhosale 1985). All the parameters have been well explained in crop plants and tree species, but remain unknown in mangroves. Therefore, we tested the possible effects of UV-B radiation on thylakoid membrane structure and accumulation of free amino acids in leaves of the mangrove species *Rhizophora apiculata*.

Materials and methods

The culture of mangrove propagules and the UV-B treatments have been described by Moorthy and Kathiresan (1997). Thylakoid membranes were isolated according to Yoshihira and Ikehara (1988). The leaves freed from their midribs were homogenised in 0.05 M histidine-HCl buffer (pH 6.5). The homogenate was filtered through four layers of gauze to remove cell debris and centrifuged at 1800×*g* for 12 min. The resulted pellet was suspended in suspension medium containing histidine-HCl buffer (pH 6.5) devoid of *PEG 4000*, and centrifuged. The resulting pellet was regarded as thylakoid membranes.

Isolated thylakoids were then mixed with 10 % TCA and centrifuged at 3000×*g* for 3 min. The pellet was washed with cold diethyl ether and dried. After drying, protein was solubilised with sodium dodecyl sulphate (2 %). The protein profile was analysed using sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Protein samples equivalent to 300 µg each were mixed with equal volume of sample buffer and heated at 100 °C for 1 min. After cooling to room temperature, the sample was centrifuged at 1000×*g* for 2 min. The clear supernatant was loaded on gel and was run at 60 V till the sample passed through stacking layer. The voltage was monitored regularly and increased to 160 V. Separation was run at 20 °C. The gel was stained for 5 h with 0.25 % Coomassie brilliant blue *R 250* (*Sigma*) in 50 % methanol and 7 % acetic acid. Subsequently the gel was destained in the same mixture devoid of dye.

Methyl esters of fatty acids in thylakoid membrane were prepared according to Roughan and Batt (1968). Thylakoid membranes (100 µg total chlorophyll) were

mixed with methanol : chloroform (7 : 3 v/v) mixture and heated at 80 °C. The resulting mixture was filtered through *Whatman* No. *1* filter paper and incubated with 0.73 % NaCl at 10 °C for 12 h, with occasional shaking. Subsequently, chloroform layer was removed and dried. The dried extract was mixed with chloroform : methanol (2 : 1) mixture and saponified with 40 % alcoholic KOH and 0.01 % ethanolic pyrogallol. The mixture was hydrolysed at 90 °C for 20 min. After cooling, samples were extracted in hexane and acidified with 12 M HCl at 4 °C for 12 h. The resulted material was again extracted with hexane and evaporated under nitrogen.

Methyl esters were prepared by the addition of boron trifluoride in 14 % methanol and boiling for 15 min. The methylated fatty acids were extracted with hexane and evaporated under nitrogen. The methyl esters were determined qualitatively and quantitatively using a gas chromatograph *Hewlett Packard 5890* (USA) fitted with a flame ionizing detector. The chromatogram was obtained on an integrator (*Hewlett Packard 3380 A*). Methyl esters of fatty acids were quantified using standard fatty acids (*Sigma ME-14*).

Lipid peroxidation was quantified as residual malondialdehyde (MDA) (Heath and Packer 1968). Fresh leaf tissue was ground in 10 cm^3 of double distilled water. To 3 cm^3 of the aliquot, 5 cm^3 of thiobarbituric acid in 20 % trichloroacetic acid was added and incubated at 90 °C for 30 min. The sample was cooled and centrifuged at 2000×*g* for 4 min. The clear supernatant was used to measure the absorbances at 532 and 600 nm. After correction for non-specific absorption, the MDA content was estimated using the molar absorption coefficient of 155 mM^{-1}.

Concentrations of free amino acids in leaves were determined (Rajendra 1987) using high performance liquid chromatography, HPLC (*ISCO 2350*, USA). Leaf tissues were extracted in acetate buffer and derivatised in OPA (orthophthalaldehyde) containing mercaptoethanol. After 2 min of derivatisation, the samples were injected into HPLC fitted with a fluoresence detector (9 mm^3 flow cell *FL2, ISCO*, USA) for determination. As standards, OPA amino acids were run. Using the retention time and peak area, individual amino acids were identified and quantified. Three replicates of 10 propagules each were analysed for each treatment.

Different treatments were statistically analysed by Student's *t*-test. Least significant difference (LSD) was calculated and defined at 95 % confidence level.

Results and discussion

The UV-B tolerance of plants appears to be correlated with degree of unsaturation of fatty acids and extent of lipid peroxidation. Five fatty acids were quantified (Table 1) from the thylakoid membrane of mangroves, *viz.* myristic (14:0), palmitic (16:0), oleic (18:1), linoleic (18:2), and myristoleic (14:2). Of these, oleic acid, an unsaturated fatty acid, was found high in mangrove thylakoids. Dutta *et al.* (1985) also recorded a high content of oleic acid in mangroves such as *Avicennia officinalis*, *Acanthus ilicifolius,* and *Bruguiera gymnorrhiza*. In response to UV-B, the content of oleic acid was reduced, but content of palmitic acid was increased. Total unsaturated

Table 1. Changes in fatty acid content of thylakoid membranes isolated from *R. apiculata* seedlings exposed to UV-B radiation doses equivalent to 0, 10, 20, 30, and 40 % of stratospheric ozone depletion. *Significant at 5 % level. Values in parentheses are % increase or decrease over control.

Fatty acids		UV-B [%] 0	10	20	30	40
saturated	myristic	17.10	12.30 (-30)	23.73 (38)*	19.88 (16)	20.82 (22)
	palmitic	5.40	12.59 (133)*	19.74 (266)*	17.84 (230)*	10.76 (99)*
unsatutrated	myristoleic	12.46	19.19 (54)*	7.18 (-41)*	14.74 (18)*	21.92 (76)*
	oleic	55.76	50.34 (-9)	40.87 (-28)	38.92 (-30)	36.82 (-34)*
	linoleic	8.68	5.58 (-36)*	8.98 (4)	8.98 (4)	9.68 (12)
satur./unsatur.		3.32	3.02 (-9)	1.30 (-61)*	1.65 (-50)*	2.17 (-35)*

fatty acids were maximally reduced by 26 %, but saturated fatty acids were enhanced by 88 %, thus the ratio of unsaturated/saturated fatty acids decreased to 61 % at 20 % UV-B. An increase in unsaturated/saturated fatty acids is a pre-requisite for membrane rigidity (Hugly *et al.* 1989, Kramer *et al.* 1991), but the reverse was evident in mangroves under high UV-B irradiance. Against this, Predieri *et al.* (1993) could not trace any visible change in membrane lipids of *in vitro* grown pear exposed to UV-B. Decrease in unsaturated/saturated fatty acids with UV-B radiation could be due to accumulation of saturated fatty acids. Our study showed that the concentration of palmitic acid was enhanced up to 266 % at 30 % UV-B which is attributed to its synthesis or interconversion of unsaturated to saturated fatty acids.

Lipid peroxidation was increased (by 48 %) in seedlings treated with high doses of UV-B (40 %); but it was low in the plants exposed to low fluence of UV-B (Fig. 1).

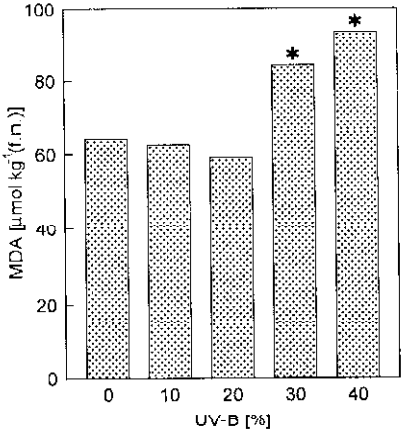

Fig. 1. Changes in lipid peroxidation in leaves of *R. apiculata* (expressed as malondialdehyde, MDA) as influenced by the UV-B radiation doses equivalent to 0, 10, 20, 30, and 40 % depletion of stratospheric ozone. *Significant at 5 % level.

Increased lipid peroxidation was already reported in sugar beet (Panagopoulos *et al.* 1990), cucumber (Kramer *et al.* 1991), or pear (Predieri *et al.* 1995) which would result in larger damage to the biological membrane system due to increased penetrability of UV-B into the leaves or reduced ability to scavenge free radicals produced by UV-B (Kramer *et al.* 1991).

To view the changes induced by UV-B on thylakoid organisation, SDS-PAGE analysis was done. The UV-B treatment did not produce any new protein in mangroves. Seven proteins with molecular masses of 55, 47, 33, 25, 23, 17, and 15 kDa were recorded (Fig. 2). At 10 % UV-B treatment, high accumulation of proteins of 55, 33, 25, 23, and 17 kDa was found. This accumulation of proteins at low flux of UV-B might have enhanced photosynthesis through reorganisation of thylakoid membrane polypeptides. The 55 kDa protein represents the larger subunit of ribulose-1,5-bisphosphate carboxylase, RuBPC (Lingakumar and Kulandiavelu 1993). Hence, we assumed that low UV-B fluence might stimulate biosynthesis of the RuBPC protein, but it was not quantified in the present work. However, as the dose of UV-B increased, the accumulation of proteins declined, though not prominently. Kulandaivelu *et al.* (1991) also reported altered contents of 55, 42, 38, 35, 28, 26, and 20 kDa proteins in C_3 and C_4 plants. Loss of proteins with molecular masses of 33, 23, and 27 kDa would result in impaired O_2 evolution (Shen *et al.* 1988, Enami *et al.* 1989). In *Vigna sinensis*, Nedunchezhian and Kulandaivelu (1991) quantified a reduction of the 33 kDa protein and total elimination of the 23 kDa protein. Lesser accumulation of the 25 kDa protein at high UV-B irradiance was attributed to degradation of the light-harvesting complex (LHC) (Thornber 1975), and it is also in agreement with the study where the amount of LHC-chlorophyll decreased (Moorthy 1996).

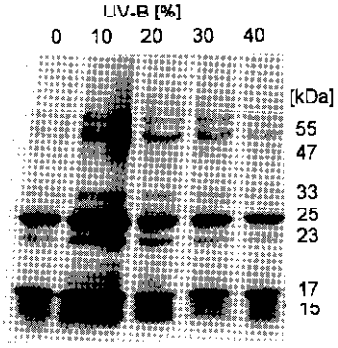

Fig. 2. Electrophoretic profiles of polypeptides in thylakoids isolated from leaves of *R. apiculata* as influenced by the UV-B radiation doses equivalent to 0, 10, 20, 30, and 40 % of stratospheric ozone depletion.

Amino acids are the vital compounds of nitrogen metabolism of mangroves (Bhosale 1985). Aspartate, glutamate, asparagine, serine, glutamine, threonine, and histidine were accumulated by 53, 86, 142, 72, 3, 119, and 32 %, respectively, in seedlings exposed to 10 % UV-B (Table 2). At the same fluence, tyrosine and lysine contents were decreased, and a specific induction of methionine and valine synthesis was also noticed. Methionine and valine could not be found either in control or other UV-B treatments. Increased synthesis of amino acids might be due to *de novo* synthesis (Rhodes *et al.* 1983). At 20 % UV-B irradiance, serine and alanine contents were enhanced by 6 %. Higher content of serine is due to inhibition in synthesis of glycine and cysteine, because serine acts as a precursor for these amino acids (Stryer 1981). Tyrosine was inhibited at all levels of UV-B. On the contrary, the precursor for flavonoid biosynthesis, phenylalanine (Tevini *et al.* 1981) was found only in the control and in the 30 % UV-B treatment. In leaf tissues of mangroves, glycine,

Table 2. Changes in contents of amino acids [mg kg^{-1}(f.m.)] in leaves of *R. apiculata* seedlings exposed to UV-B radiation doses equivalent to 0, 10, 20, and 30 % of stratospheric ozone depletion. Glycine, arginine, isoleucine, leucine, and ornithine were not detected in our samples. *Significant at 5 % level. ND - not detected. Values in parentheses are % increase or decrease over control.

	UV-B [%] 0	10	20	30
Aspartate	51	78 (53)*	34 (-33)*	23 (-55)*
Glutamate	155	289 (89)*	128 (-17)	150 (-4)
Asparagine	272	657 (142)*	89 (-69)*	88 (-67)*
Serine	87	150 (72)*	92 (6)	80 (-8)
Glutamine	6644	6812 (8)	1985 (-70)*	2599 (-61)*
Histidine	796	1105 (39)*	369 (-54)*	394 (-51)*
Threonine	27	59 (119)*	21 (-22)	14 (-48)*
Alanine	70	114 (63)*	74 (6)	83 (19)
Tyrosine	110	101 (-8)	57 (-48)*	93 (-15)
Methionine	ND	218	ND	ND
Valine	ND	21	ND	ND
Phenylalanine	28	ND	ND	12 (-57)*
Lysine	75	44 (-41)*	ND	ND

arginine, isoleucine, leucine, and ornithine were not found in detectable quantities. The reason for low contents of some amino acids under UV-B is probably the inhibition of amino acid synthesis or amino acid utilization for protein synthesis or oxidation of existing amino acids. Accumulation of protein in leaves (values not shown) or chloroplasts documented in this study was a possible reason for the decline in contents of some amino acids.

Considering the present scenario of global increases in UV-B incident on earth surface, marine plants have to be manipulated to combat the lethality of UV-B. In this context, the present results provide an ample basis for development of mangrove species tolerant to UV-B.

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