Adaptation of tropical mangroves to high solar radiation

Thesis submitted by
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Summary

Mangroves grow in solar radiation environments that are high in visible and UV radiation. Both UV and visible radiation have been shown to be responsible for damage to the photosynthetic apparatus of plants (Caldwell, 1981; Powles, 1984). The general aim of this study was to investigate mechanisms that allow mangroves to tolerate their high solar radiation environment. Investigation into tolerance of visible light centered on the xanthophylls, while for UV radiation, emphasis was placed upon the accumulation of UV-absorbing phenolic compounds.

Mangroves have similar xanthophyll/chlorophyll ratios to other plant species. Xanthophyll/chlorophyll ratios were sensitive to the light environment in which leaves grow, decreasing as light levels decreased over a vertical transect through a forest canopy. The xanthophyll/chlorophyll ratio also varied between species. In sun leaves the xanthophyll/chlorophyll ratios over all species correlated with the proportion of leaf area displayed on a horizontal plane, which is determined by leaf angle. Thus leaf angle and xanthophyll/chlorophyll ratios may be equally important in providing protection from high light levels in mangrove species.

A canopy survey assessed whether xanthophyll/chlorophyll ratios could be correlated to species dominance of exposed positions in forest canopies. *Rhizophora* mangroves, with near-vertical leaf angles, and *B.parviflora*, with small, horizontal, xanthophyll-rich leaves, dominated the canopy, while *B.gymnorrhiza*, a species with large, horizontally arranged leaves, was less abundant at the top of the canopy. Thus, two different strategies for adapting to high solar radiation levels may exist in these two species. The first strategy is avoidance, through near vertical leaf angles, and the second is a large capacity to dissipate energy through zeaxanthin. The xanthophyll/chlorophyll ratio was also negatively correlated with the epoxidation state of the xanthophylls (the proportion present as violaxanthin and half that present as antheraxanthin) at midday. This suggested that the requirement for dissipation of excess light (represented by the midday epoxidation state) may influence the xanthophyll/chlorophyll ratio. Thus, an investigation into the factors that influence epoxidation state was undertaken.

Within a data set where solar radiation was greater than 750 $\mu mol.m^{-2}.s^{-1}$ photosynthetic rates accounted for a greater proportion of the variation in

epoxidation state than solar radiation. With a data set collected over a wide range of solar radiation levels, leaf temperatures, and photosynthetic rates, photosynthesis and solar radiation were found to account for similar proportions of the variation in epoxidation state. Leaf temperature was not significant in directly explaining the variation in epoxidation state, but exerted its influence indirectly through its effect on photosynthesis. As solar radiation influences photosynthesis, leaf temperature and epoxidation state, solar radiation is probably the most important factor in determining the epoxidation state, and thus the total xanthophyll concentration in mangrove leaves.

UV-absorbing phenolic compounds, that have been shown to be protective against the damaging effects of UV-B radiation (Tevini *et al.*, 1991) were found in the epidermis of mangrove species. An extensive field survey showed that the concentration of these compounds varied between species, sites and sun and shade leaves. Sun leaves have greater concentrations than shade leaves, and more saline sites have plants with greater concentrations in their leaves than less saline sites. It was concluded from this study that although these compounds form a UV screen in the epidermis of mangrove leaves, UV radiation may not be the only factor influencing their accumulation.

In an experiment to assess the adaptation of three species of mangroves to UV radiation, UV radiation resulted in a greater concentration of UV-absorbing compounds in *B.parviflora*, but not the other two species, and lower chlorophyll concentrations occurred in *R.apiculata*, but not the other two species. There was no difference in leaf morphology, carotenoid/chlorophyll ratios, or chlorophyll a/b ratios between UV treatments, although these varied between species; *B.parviflora* having the highest carotenoid/chlorophyll ratio and *R.apiculata* having the lowest. Chlorophyll a/b ratios were correlated with the concentration of UV-absorbing compounds over all species. Thus it was proposed that the effects of UV radiation measured in these experiments may be associated with the total energy absorbed by leaves, and that differences between species response to UV radiation may be associated with their abilities to dissipate the increased energy associated with UV radiation.

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DECLARATION

I declare that this thesis is my own work and has not been submitted in any other form for any other degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

C.E. Lovelock
28 September 1991

CHAPTER 1

General Introduction

Solar radiation is essential for photosynthesis in plants, but paradoxically it can also cause damage to the photosynthetic apparatus of plants. Damage to the photosynthetic apparatus of plants has been attributed to both high levels of visible (380-750 nm) and ultraviolet (UV) (280-380 nm) radiation. Wavelengths shorter than 280 nm (UV-C) are largely eliminated by the earths atmosphere and therefore do not occur in natural environments (Frederick *et al.*, 1989). This Chapter reviews the damage to plants that arises from solar radiation, and the mechanisms plants have to avoid or tolerate conditions that may result in damage from solar radiation.

1.1 Damage from visible radiation

The amount of visible solar radiation absorbed by plants is often greater than can be used in photosynthesis, due to limitations imposed on photosynthesis by leaf biochemistry, electron transport and CO_2 diffusion. Damage to plants from this excess visible light appears to be due mainly to oxygen dependent inhibition of Photosystem I (PS I), and oxygen independent damage to Photosystem II (PS II) (reviewed by Powles, 1984 and Asada and Takahashi, 1987). Another harmful effect of high levels of visible radiation may arise from the "over-excitation" of chlorophyll which results in the conversion to its triplet state. This high energy state is long lived, and therefore can react destructively with cell components, or with oxygen to produce singlet oxygen, which can also be involved in destructive reactions with chlorophyll, lipids and proteins (see review by Knox and Dodge, 1985). Triplet state chlorophyll, and singlet oxygen

could occur in the chlorophyll associated with either photosystem, their production being enhanced in chloroplasts where electron acceptors are limited (Asada and Takahashi, 1987). However, their contribution to damage to the photosynthetic apparatus has not been assessed due to the difficulty in detecting them (Asada and Takahashi, 1987). As triplet state reaction centres of Photosystem I have been shown not to react with oxygen (Takahashi and Katoh, 1984), this kind of damage is more likely to occur at the antenna chlorophyll associated with this photosystem.

1.1.1 Oxygen-dependent damage to PS I

Photooxidative damage to PS I has been attributed to active and destructive oxidizing species which are produced when PS I, or some component close to it, donates electrons to oxygen. These active oxygen species include super-oxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^\cdot$) (see reviews by Powles, 1984 and Asada and Takahashi, 1987). The production of active oxygen species occurs mainly via the Mehler reaction (Furbank and Badger, 1983). This involves the reduction of oxygen to super-oxide, probably through the oxidation of reduced ferredoxin (Furbank and Badger, 1983). Approximately 90% of the super-oxide produced is then dismuted by super-oxide dismutase to hydrogen peroxide, which can prevent photosynthesis at very low concentrations by reacting with enzymes of the photosynthetic carbon reduction pathway (Asada and Takahashi, 1987). Hydrogen peroxide can also react with metal complexes to produce hydroxyl radicals which also engage in oxidative reactions with chlorophyll, carotenoids, proteins and lipids (Asada and Takahashi, 1987).

Super-oxide, hydrogen peroxide and hydroxyl radicals are neutralized in the chloroplasts by reactions with a variety of compounds including carotenoids, superoxide dismutase, catalases and peroxidases, ascorbic acid, glutathione and flavonols (see reviews by Asada and Takahashi, 1987 and Salin, 1987). Ascorbate peroxidase, of the ascorbate-glutathione cycle, has been shown to have a high capacity to reduce hydrogen peroxide (Anderson *et al.*, 1983). Flavonols also reduce hydrogen peroxide in chloroplasts in the light with a similar K_m to ascorbate peroxidase of approximately 30 μmol H_2O_2 (Takahama, 1984).

It has been suggested that the Mehler reaction, rather than being wasteful or destructive, may have a protective function by allowing the continuation of non-cyclic electron transport through the oxidation of reduced ferredoxin, thus dissipating excess energy (see review by Powles, 1984). Presumably damage occurs when the active oxygen "scavenging" systems can no longer cope with the amount of active oxygen produced (Asada and Takahashi, 1987).

1.1.2 Oxygen-independent damage to PS II

The oxygen-independent damage to PS II that results from exposure to high levels of visible light is associated with a reduction in the apparent quantum yield of light-limited photosynthesis, and to a reduction in relative chlorophyll fluorescence yields (Cleland, 1988). This inhibition of photosynthesis or "photoinhibition" (Powles, 1984), is associated with inhibition of PS II photochemistry leading to the proposal that photoinhibition is due to the loss of function of P680 reaction centres (Cleland, 1988).

Photoinhibition is increased by water deficits (Osmond, 1983; Björkman and Powles, 1984), leaf temperatures that are not optimal for photosynthesis

(Greer *et al.*, 1986; Bongi and Long, 1987; Demmig-Adams *et al.*, 1989*a*; Demmig-Adams *et al.*, 1989*b*; Greer and Laing, 1989; Gamon and Pearcy, 1990; Bilger and Björkman, 1991) and low nitrogen status (Osmond, 1983; Henley *et al.*, 1991). These conditions all limit photosynthesis, and thus result in an increase in the light energy absorbed by chlorophyll that cannot be used in photosynthesis. It follows from this and the previous discussion that mechanisms which (1) maintain photosynthetic rates, (2) avoid the absorption of excess energy, or (3) dissipate excess energy absorbed by leaves through non-harmful processes, may prevent photoinhibition.

1.2 Protection from visible light-related damage

Numerous studies over a wide range of plant species grown at a variety of light levels provide evidence of adaptations to high irradiance that prevent photoinhibition (Björkman, 1981; Anderson and Osmond, 1987). Adaptation to high visible light levels occur at all levels of leaf organization, from canopy architecture to leaf biochemistry. Adaptation to high visible light levels can either lead to reductions in the light intercepted by leaves, or to tolerance of excess light absorbed. Changes in leaf morphology and anatomy in high photon flux density environments are usually associated with reducing light intercepted, while changes in leaf ultrastructure and biochemistry can be viewed as adaptations that generally lead to tolerance of excess light.

1.2.1 Reducing light absorbed

Leaves exposed to high photon flux densities can reduce incident solar radiation in a variety of ways. Steeply inclined foliage results in absorption of less light per area of leaf (Björkman, 1981; Ball *et al.*, 1988; Givnish, 1988). This has

the additional benefit of maintaining leaf temperatures close to that of the surrounding air, thus contributing to the maintenance of photosynthetic rates, which is important in protecting leaves against photoinhibition (Osmond, 1981; Krause and Cornic, 1987). For example, in mangroves, Ball *et al.*, (1988) showed that near-vertical leaf angles were important in avoiding leaf temperatures that were above the optimum for photosynthesis. Smaller leaf size may also be effective in preventing leaf temperatures that are above optimum for photosynthesis (Givnish, 1987), as smaller leaves allow more effective convective heat loss to the surrounding air compared to large leaves, due to the lower boundary layer resistance (Givnish, 1987).

Other adaptations that have been proposed to reduce light absorbed by leaves include reflective leaf surfaces (Osborne and Raven, 1986), and chloroplast movement, in which chloroplasts reorientate within the cell in order to decrease light interception by chloroplasts (Haupt, 1982; Osborne and Raven, 1986). There is yet no evidence that reflective leaf surfaces, or chloroplast movement (Haupt and Scheuerlein, 1990) can diminish photoinhibition when leaves are exposed to high solar radiation levels.

1.2.2 Tolerance of excess light

In addition to differences in leaf morphology and anatomy, adaptation to high light levels appears to involve reorganization of the chloroplast membrane and its components. Adaptation to high light environments include increases in chlorophyll a/b ratios, Rubisco concentrations, ATP synthase activities, electron transport components, carotenoids/chlorophyll ratios, a reduction in the amount of chlorophyll per chloroplast (Anderson *et al.*, 1988) and an increase in the xanthophyll/chlorophyll ratio (Thayer and Björkman, 1990). Most of these leaf

ultrastructural and biochemical adaptions to high light levels result in increased photosynthetic rates (Chow *et al.*, 1988), which is important in protecting leaves against photoinhibition (Osmond, 1981; Krause and Cornic, 1987). However, some of these adaptations are known to have specific protective functions. For example, carotenoids react with triplet state chlorophyll and singlet oxygen, dissipating excitation energy harmlessly as heat, thus preventing destructive oxidative reactions (Cogdell, 1978).

The degree of photoinhibition experienced by plants is also dependent on repair processes that require protein synthesis. Under experimental conditions that caused photoinhibition, photoinhibition was increased in leaves that were treated with a protein synthesis inhibitor compared to leaves that were not (Greer *et al.*, 1986). Thus, conditions that limit repair processes, like low temperatures, increase photoinhibition (Ogren and Öquist, 1984; Greer, 1988; Greer and Laing, 1989), while conditions that enhance repair processes, like optimal leaf temperatures, should decrease the degree of photoinhibition in leaves exposed to excess light.

Under conditions where leaves are absorbing more light than is needed for photosynthesis, the ability to tolerate these conditions without incurring photosynthetic damage is thought to be dependent on the dissipation of excessive light energy through pathways other than photosynthesis (Osmond, 1981; Powles, 1984; Cleland, 1988). This can occur by pathways that allow continued non-cyclic electron flow, for example, photorespiration (Powles and Osmond, 1979) and the Mehler reaction (Heber *et al.*, 1978), or, through dissipation of energy at the antenna chlorophyll (Demmig and Björkman, 1987; Björkman *et al.*, 1988; Demmig and Winter, 1988), or at the reaction centres (Weis and Berry, 1987)

1.2.3 Dissipation of excess visible light energy

Photorespiration, in which Rubisco is oxygenated and glycolate is formed, has been shown to contribute to protection from photoinhibition (Powles and Osmond, 1979). The cycle has a large energy demand which results in the regeneration of $NAD(P)^+$ and ADP which are necessary for continued non-cyclic electron transport (Osmond, 1981).

The Mehler reaction (see section 1.1.1, page 2) is also thought to prevent damage to the photosynthetic apparatus as it allows the continuation of non-cyclic electron transport when $NAD(P)^+$ concentrations are low, by reducing oxygen to super-oxide which is then detoxified. The effectiveness of this process would depend on the active oxygen detoxifying capacity of the chloroplast. Hodgson and Raison (1991) have shown that chilling resistant plants suffered less photoxidative damage under high light conditions than chilling sensitive plants. The tolerance of chilling resistant plants under high light conditions correlated with the super-oxide dismutase activity (see section 1.1.1, page 2, for a description of super-oxide dismutase and reactions associated with it). Similarly, Ostrovskaya *et al.* (1990) showed that chlorotic plants with low photosynthetic capacities have higher concentrations of super-oxide dismutase than non-chlorotic plants. These results provide indirect evidence that the Mehler reaction and the associated production of super-oxide is important in determining the tolerance of plants to excess light. In addition, the Mehler reaction contributes to the development of ΔpH between the lumen and the stroma which is in turn correlated with the thermal dissipation of excessive energy (Krause and Cornic, 1987; this is considered in more detail below). However, neither the Mehler reaction, nor photorespiration are thought to be capable of dissipating the large amount of excess energy that can occur at high light levels, and it is generally thought

(though not yet quantitatively shown) that thermal dissipation of excess energy is the major contributor to the dissipation of excess light energy (see review by Krause and Cornic, 1987).

Thermal dissipation of excess energy has been shown to result from phosphorylation of the light harvesting complex of PS II, resulting in the spill over of energy to PS I (Horton and Lee, 1985), and to factors correlated with the thylakoid ΔpH (Krause and Behrend, 1986; Krause, 1988; Foyer *et al.*, 1990). The ΔpH between the lumen and the stroma may be involved in the dissipation of excessive energy through facilitating the conversion of photochemically active PS II centres to PS II centres with a low photochemical yield which can accept excitation energy and then safely dissipate it (Weis and Berry, 1987; see review by Melis, 1991), and through promoting the conversion of the violaxanthin to zeaxanthin in the "xanthophyll cycle" leading to dissipation of excess energy at the antenna chlorophyll (Demmig *et al.*, 1987; Demmig *et al.*, 1988).

1.2.4 Xanthophyll cycle

The xanthophyll cycle involves the conversion, by de-epoxidation, of violaxanthin to zeaxanthin in the light, and a return to violaxanthin in the dark or under low light conditions (Yamamoto, 1979) (Fiure 1.1). The conversion of violaxanthin to zeaxanthin is thought to occur in the thylakoid lumen where excess light energy results in the acidification of the lumen, which activates violaxanthin de-epoxidase (Yamamoto, 1979). This reaction has maximum activity at pH 5 and requires ascorbate as a reductant (Yamamoto, 1979). The reverse reaction (epoxidation) is thought to occur in the stroma (Yamamoto, 1979).

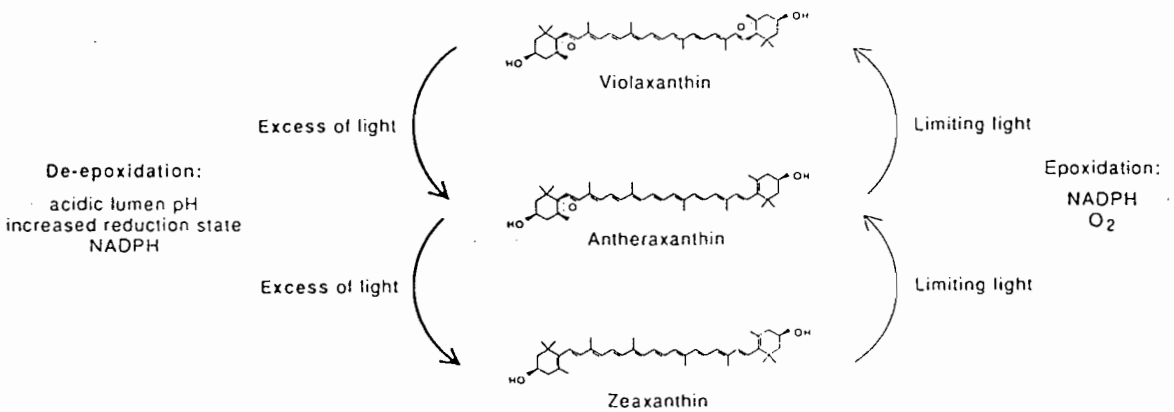


Figure 1.1. The "xanthophyll cycle" (from Demmig-Adams, 1990).

Dissipation of excess energy is proposed to occur through zeaxanthin rather than through the conversion of violaxanthin to zeaxanthin in the xanthophyll cycle (Demmig-Adams, 1990). The mechanism by which zeaxanthin results in the dissipation of excess energy is not understood (although it is known that the xanthophylls are mainly associated with the antenna chlorophyll (Siefermann-Harms, 1985)), nor is the relationship to other proposed mechanisms that dissipate excess energy. Bilger *et al.* (1989) showed that the artificial stimulation of the formation of zeaxanthin by infiltrating cotton leaf discs with ascorbate buffer at pH5 under low light conditions (10 μmol quanta.m^{-1}.s^{-1}) was not correlated with increased non-photochemical chlorophyll fluorescence quenching that is usually associated with increases in the thermal dissipation of

excess energy. This lead these researchers to conclude that some other unknown factor in addition to zeaxanthin is required for thermal dissipation of energy via zeaxanthin. Morales *et al.* (1990) came to the same conclusion when they found that chlorophyll fluorescence indicative of thermal dissipation of excess energy was not correlated with zeaxanthin contents in iron deficient sugar beet plants. In addition, Rees *et al.* (1989) showed that the presence of zeaxanthin increased the sensitivity of thermal dissipation of energy to the ΔpH. Thus, the dissipation of excess energy through zeaxanthin appears to be dependent on the ΔpH, and some other unknown cofactor (Bilger and Björkman, 1991). It has been suggested that this cofactor is associated with the structure of the thylakoid membrane (Demmig-Adams *et al.*, 1989*b*; Morales *et al.*, 1990; Bilger and Björkman, 1991) that may allow a closer association between zeaxanthin and the antenna chlorophyll (Demmig-Adams *et al.*, 1989*b*).

The evidence for the involvement of zeaxanthin in dissipating excess visible light comes from the correlation between thermal dissipation of excess energy measured by modulated chlorophyll fluorescence techniques and the zeaxanthin contents of leaves (Demmig *et al.*, 1987; Demmig *et al.*, 1988; Demmig-Adams *et al.*, 1989*a*; Demmig-Adams *et al.*, 1989*b*; Bilger and Björkman, 1991). It has been shown that lichens without the xanthophyll cycle are more prone to photoinhibition than those with a functional xanthophyll cycle (Demmig-Adams *et al.*, 1990*a*; Demmig-Adams *et al.*, 1990*b*), and in higher plants, that leaves of cotton, spinach and *Nerium oleander* infiltrated with violaxanthin de-epoxidase inhibitor, dithiothreitol, are more prone to photoinhibition than those with a functional enzyme (Bilger *et al.*, 1989; Winter and Koniger, 1989; Adams *et al.*, 1990).

Studies of species differences in the xanthophyll cycle of higher plants grown under similar conditions are few. Demmig *et al.* (1987), and more recently Thayer and Björkman (1990) have have shown that xanthophyll contents vary between species. For example, *Citrus* had 145 $\mu mol.m^{-2}$ while *Lycopersicon* had 72.5 $\mu mol.m^{-2}$ (Thayer and Björkman, 1990). In addition, Bilger and Björkman (1991) found differences in both the rate of de-epoxidation and the apparent activation energy of violaxanthin de-epoxidase between cotton and *Malva parviflora,* that may be associated with the fluidity of the thylakoid membrane. The implications of species differences in the concentration of xanthophyll cycle components in determining the adaptations of plants to their environment has not yet been investigated.

Other evidence that supports the view that the xanthophyll cycle is involved in the dissipation of excess light energy absorbed by leaves comes from studies of the response of xanthophylls to either long term growth conditions, or short term experiments that increase the amount of light that is in excess of what can be used in photosynthesis. It has been shown, over a variety of species, that sun leaves have more xanthophylls than shade leaves (Thayer and Björkman, 1990), and that *Nerium oleander* plants grown under water deficits have higher leaf xanthophyll contents than those grown under well watered conditions (Demmig *et al.*, 1988). Experiments that have assessed daily variations in the components of the xanthophyll cycle have found that daily reductions in the photochemistry of the crassulacean-acid-metabolism plant, *Clusia rosea* were associated with increases in the zeaxanthin contents of leaves (Winter *et al.*, 1990), and that the midday depression in photosynthesis of field grown lupins was associated with increases in leaf zeaxanthin contents (Demmig-Adams *et al.*, 1989*a*).

Short term experiments that assessed the response of the xanthophylls to conditions that increase the amount of excess light have shown that exposure to 100 μmol. quanta.$m^{-2}.s^{-1}$ in 0% CO_2 and 2% O_2 resulted in increased leaf zeaxanthin concentrations over a wide range of species, including the mangrove, *R.mangle* (Demmig *et al.*, 1989*b*). This pretreatment was subsequently shown to diminish the extent of inactivation of photochemistry on exposure of plants to high light levels and chilling temperature. Conversion of violaxanthin to zeaxanthin in spinach (Adams *et al.*, 1990), cotton, and *Malva parviflora* (Bilger and Björkman, 1991) has been shown to be temperature dependent, with optimal temperatures for zeaxanthin formation occuring at temperatures optimum for photosynthesis. However, the amount of zeaxanthin formed in steady state conditions was much greater at low leaf temperatures than at leaf temperatures optimal for photosynthesis (Bilger and Björkman, 1991).

1.3 Damage from UV radiation

Like high levels of visible radiation, high levels of UV radiation have also been shown to be detrimental to plants. Studies have largely concentrated on UV-B (280-320 nm) radiation with little attention given to UV-A (320-380 nm) spectral region (Coohill, 1989). High levels of UV-B radiation generally reduce plant growth rates, total biomass, leaf area, stem length and photosynthesis (see reviews by Caldwell, 1981 and Tevini and Teramura, 1989), usually in response to the duration of exposure to UV-B radiation (Sisson and Caldwell, 1977; Strid *et al.*, 1990). These damaging effects arise because of the high energy per photon (up to four times the energy of a visible photon), and also because some molecules in plants preferentially absorb UV-B radiation (Caldwell, 1981). These include nucleic acids, proteins, pigments and lipids (Caldwell, 1981). Such compounds can be directly damaged through destruction of their covalent bonds

(Caldwell, 1981), or can cause damage through their conversion to highly energetic triplet states which can directly react with nucleic acids, proteins, lipids or pigments, or react with oxygen to produce toxic active oxygen species (Larson and Berenbaum, 1988). Compounds that can be converted into toxic triplet states include chlorophyll (described in section 1.1, page 1), riboflavin, tryptophan, tyrosine and others. Both direct destruction of covalent bonds, and oxidative reactions result in the loss of pigments, proteins and enzyme activities, particularly Rubisco and the D1-protein of the core PS II complex, which have rapid turn-over rates (Strid *et al.*, 1990). UV-B radiation may also reduce photosynthesis by increasing stomatal closure (Teramura *et al.*, 1983; Negash, 1987).

UV radiation has also been shown to stimulate photosynthesis (Teramura *et al.*, 1980). This could be due to the stimulation of Rubisco (Daley *et al.*, 1978), or to the transfer of energy from a UV-absorbing chromophore to chlorophyll (shown in coral species by Schlicter *et al.*, 1985 and Schlicter *et al.*, 1986). In experiments to establish the UV action spectra of photosynthetic damage, Bornman *et al.* (1984) suggested that UV radiation energy may be transferred through some unknown chromatophore to the site of damage, a protein associated with PS II. If this occurs, UV radiation may contribute to photosynthetic damage in a similar way as high visible light levels.

Unlike the case for visible light, damage from UV-B radiation is not increased by additional environmental stress. Water deficits, high visible light levels or nutrient deficiencies, in conjunction with high UV-B radiation levels, lessen the effects of high UV-B radiation (Murali and Teramura, 1986; Murali and Teramura, 1987; Cen and Bornman, 1990). That is, UV-B radiation results in no further damage than is already observable under the stress treatments.

1.4 Protection from UV radiation

Although near-vertical leaf angles are a mechanism that reduce the absorbance of direct visible radiation by leaves (see section 1.2.1, page 4), 45-75% of UV radiation is present in the diffuse radiation component, substantially reducing the effectiveness of leaf angle in avoiding damaging UV radiation (Caldwell, 1981). Increases in leaf thickness of soybean grown at high visible or UV-B radiation levels relative to those grown at low visible or UV-B radiation levels, have been shown to be protective when they were exposed to enhanced UV-B radiation (Warner and Caldwell, 1983; Murali *et al.*, 1988). Protection from UV-B radiation is thought to arise through attenuation of UV-B radiation as it passes through the thickened leaf (Caldwell *et al.*, 1983). In addition to these physical means of protection from UV radiation, a wide range of plant species have been shown to have molecules that detoxify toxic oxygen species that are generated on exposure to UV radiation (Salin, 1987; Larson and Berenbaum, 1988) (see section 1.1, page 1), molecular repair mechanisms, where UV-A radiation stimulates enzymes that repair damaged DNA (Degani *et al.*, 1980), and UV-B absorbing compounds within their leaf epidermal layers that effectively screen UV-B radiation (Caldwell *et al.*, 1983; Robberecht and Caldwell, 1978; Les and Sheridan, 1990; Tevini *et al.*, 1991).

Tolerance of UV-B radiation varies between genotypes (Tevini *et al.* 1983; Flint *et al.*, 1985; Murali *et al.*, 1988; Larson *et al.*, 1990; Teramura *et al.*, 1990). For example, UV-B tolerant soybean cultivar Essex showed no reduction in its photosynthetic rates when exposed to enhanced UV-B radiation relative to its UV-B intolerant counterpart (Teramura *et al.*, 1990). The differences in genotype response to UV-B radiation have been attributed to differences in leaf thickness

and the composition and concentration of UV-absorbing compounds within the leaves (Caldwell *et al.*, 1983; Robberecht and Caldwell, 1978; Les and Sheridan, 1990), and also to differences in peroxidase activities (Murali *et al.*, 1988).

1.4.1 UV-screening compounds

UV absorbing compounds within leaf epidermal layers are mainly phenolic compounds (e.g. cinnamoyl esters, flavones, flavonols and anthocyanins esterified with cinnamic acids) (Tevini *et al.*, 1991). The production of UV-screening compounds has been shown to increase in parsley (Wellman, 1983) and rye (Tevini *et al.*, 1991) with increasing time of exposure to UV-B radiation. A correlation has been shown between the production and accumulation of flavonoids, and protection from photosynthetic damage in rye plants exposed to enhanced UV-B radiation (Tevini *et al.*, 1991).

It has been proposed that UV radiation stimulates the production of flavonols by activating key enzymes in the pathway that produce these compounds (Caldwell, 1981; Caldwell *et al.*,1983). These enzymes are 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase, which catalyses the condensation of erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) (the first step in the shikimic acid pathway), and phenolalanine lyase (PAL), which converts phenylalanine to cinnamate (a simple phenolic) (Jensen, 1985). Phytochrome, and perhaps some unknown blue light receptor, have been implicated in the induction of these processes (see review by Tobin and Silverthorne, 1985). Riboflavin has been shown to be involved in the UV-A and visible light stimulation of anthocyanin production (Jain and Guruprasad, 1990), and Cen and Bornman (1990) have shown that high levels of visible light increased the UV absorbance of bean leaf extracts, thus implicating visible light in

influencing the accumulation of UV-absorbing compounds in this species. In addition, developmental stage of the tissue, tissue type, wounding, and the presence of pathogens also influence the production of phenolics (see review by Hahlbrock and Grisebach, 1979).

1.5 This study

This study examines adaptation to high solar radiation in plants that naturally grow under these conditions. The "mangroves" are a group of approximately thirty tree species that inhabit 1.2 million hectares of intertidal area along the tropical and subtropical coastlines of Australia (Galloway, 1982). They are an ideal group of plants in which to study photoprotective mechanisms as in mangrove areas of northern Australia maximum visible solar radiation levels vary between 2000 to 2200 μmol quanta.$m^{-2}.s^{-1}$, while ultraviolet A and B radiation is approximately double that experienced by temperate species (UNEP Environmental Effects Panel Report 1989). In addition, photosynthetic rates in mangroves are low due to the high and variable salinities in which they grow (Andrews *et al.*, 1984; Ball *et al.*, 1988; Björkman *et al.*, 1988; Clough and Sim, 1989; Cheeseman *et al.*, 1991), and possibly due to low nutrient availability in their soils (Boto, 1982), further adding to the potential for photoinhibition in these species (see section 1.1.2, page 3).

Although there are numerous investigations of photosynthesis in mangroves (for example, Attiwell and Clough, 1980; Andrews *et al.*, 1984; Ball and Farquhar, 1984*a*; Ball and Farquhar, 1984*b*; Andrews and Muller, 1985) there have been few investigations into mechanisms that may protect mangroves from photosynthetic damage by solar radiation. Three studies have indicated that mangroves may have mechanisms that confer tolerance of high solar radiation

levels. The study of Björkman *et al.* (1988) found that quantum yields of sun leaves of the mangrove *Rhizophora stylosa* were lower relative to shade leaves, and that this was likely to be due to the reduced efficiency of energy conversion at PS II. These researchers suggested that the reduction in PS II efficiency was associated with prevention of photoinhibition through thermal dissipation of excess excitation energy, rather than being indicative of photosynthetic damage. Cheeseman *et al.* (1991) found that leaves of the mangrove *Bruguiera parviflora* that were exposed to high solar radiation levels, and had negligible photosynthetic rates at midday, recovered within 30 minutes, and thus did not seem to be photoinhibited. Demmig-Adams *et al.* (1989*b*) have also shown that the presence of zeaxanthin in leaves of the mangrove *R.mangle* diminished the extent of inactivation of photochemistry when plants were exposed to high light levels and low temperatures.

In addition to the three studies outlined above, Ball *et al.* (1988) found that near-vertical leaf angles were important in mangroves adaptation to their environment as they reduced the midday radiation incident on leaves, and thus helped maintain leaf temperatures within a range in which photosynthesis could continue. Therefore, near-vertical leaf angles may reduce the likelihood of photoinhibition by avoidance of high light levels and leaf temperatures, and maintaining photosynthetic rates. Preliminary tests on the mangrove *R.stylosa* (Appendix I, page 127) suggest that chloroplast movement is unlikely in this species, or, that if it does occur it has no observable effect on the light transmitted through leaves, and is therefore unlikely to be involved in avoidance of high light levels. The results of these studies suggest that zeaxanthin, and the associated dissipation of excess energy, and leaf angles may be important in the prevention of photoinhibition in mangroves. Thus, Chapter 2 of this thesis assesses the

xanthophyll cycle in mangroves over three species from the family Rhizophoraceae in which leaf angle and size vary.

Chapter 3 of this thesis deals with the more general question of how photosynthesis, solar radiation and leaf temperature influence the concentration of zeaxanthin within leaves in the field. Photosynthesis, solar radiation and leaf temperature have all been shown, or implicated, in having an effect on zeaxanthin concentration in other plant species (see section 1.2.4, page 8). Therefore, this Chapter is aimed at examining the interrelationship between these variables under natural conditions, and in doing so, assesses some of the assumptions made about zeaxanthin and its function.

The second major goal of this study is to assess the tolerance of mangroves to UV radiation. The influence of UV radiation on mangroves, and tolerance of mangroves to UV radiation have not previously been assessed. However, mangroves are known to have high concentrations of phenolic compounds in their leaves (Robertson, 1988). As similar types of compounds have been shown to function as UV screens in other plant species (see section 1.4.1, page 14), these compounds are investigated in mangroves. Chapter 4 assesses the UV-screening capabilities of these compounds, and also examines differences between species, and the effect of environment on the accumulation of these compounds. The fifth Chapter in this thesis examines the adaptive response of three mangrove species to UV radiation.

CHAPTER 2

The influence of light levels and leaf angle on xanthophyll concentrations

2.1 Introduction

Mangrove plants inhabit intertidal environments in tropical latitudes where solar radiation levels are high. The saline conditions in which they grow usually result in low stomatal conductance, generally low photosynthetic rates, and high water use efficiency of CO_2 fixation (Andrews *et al.*, 1984; Andrews and Muller, 1985; Clough and Sim, 1990). Low photosynthetic rates in conjunction with high solar radiation levels are likely to predispose mangroves to photoinhibition (see section 1.1.2, page 3).

Protection from photoinhibition depends on the avoidance of conditions that increase photoinhibition (e.g. high light levels and low photosynthetic rates), and also on the ability of the leaf to safely dissipate excess light energy. There is some evidence that mangrove species have a high capacity to safely dissipate excess light energy (Björkman *et al.*, 1988). Dissipation of excess light has been shown to correlate with the concentration of zeaxanthin within leaves (Demmig *et al.*, 1987). Thus, the first aim of this study was to assess the concentration of xanthophylls in mangroves, and how leaf xanthophyll concentrations vary over naturally occurring light gradients.

Demmig *et al.* (1987) and more recently Thayer and Björkman (1990) have found that the concentration of xanthophylls varies between species. In

addition Ball *et al.* (1988) have shown that near-vertical leaf angles are important in avoiding solar radiation and maintaining leaf temperatures within a range in which photosynthesis can continue. As leaf angles vary between mangrove species, the importance of leaf angle in influencing the concentration of xanthophylls over three species was examined. These three species occur together in mangrove forests. By examining the canopy dominance of each species, the influence of xanthophyll concentrations and leaf angle on the distribution of species through canopies was assessed.

2.2 Materials and Methods

2.2.1 Canopy study

To examine the response of xanthophyll concentrations of mangrove leaves to a natural occurring light gradient, leaves were sampled through the canopy of a well developed *Rhizophora apiculata* forest at a site 7 km upstream from the mouth of the Daintree River (Lat. 17°S, Long. 147°E). A scaffolding tower was constructed to the top of the canopy (22 m from ground level) allowing studies along a vertical profile through the canopy. 50 leaves were sampled at midday throughout the canopy profile and the heights were recorded for each leaf. Four leaf discs (1.37 cm^2) to be used in pigment analysis were punched from each leaf and immediately immersed in liquid nitrogen. On returning to the laboratory samples were transferred to a -80°C freezer. Light levels through the canopy were obtained by averaging measurements taken between 11:00 am and 1:00 pm over a five day period using small, visible light sensors held at the same orientation as the leaves. This data was kindly made available by Dr. B.F. Clough from the Australian Institute of Marine Science.

2.2.2 Species and sun/shade comparisons

The mangrove species, *Rhizophora stylosa*, *Bruguiera parviflora* and *Bruguiera gymnorrhiza* were selected for this study. They are closely related species (from the family Rhizophoraceae), but have differing leaf characteristics. Field observations of the leaf characteristics of these species are summarised in Table 2.1.

Four sun leaves from three trees of each of the three mangrove species were sampled between 9.00 and 11.00 am from: 1) an open 3m tall canopy at the southern side of the river mouth of the Daintree River, north Queensland (Lat. 17°S, Long. 146°E) in October 1989, and 2) at the Australian Institute of Marine Science (Lat. 19°S, Long 147°E) in December 1990 using plants grown in natural sun light and in nutrient culture adjusted to salinity of 25% seawater (see Appendix II, page 130). Sun and shade leaf comparisons were made by sampling four shade leaves at the Daintree River site from each of the same trees from which the sun leaf samples were obtained. Samples were taken by punching four 1.37 cm^2 leaf discs from each leaf and emersing them in liquid nitrogen.

Table 2.1. Field observations of leaf size and angle of three mangrove species from the family Rhizophoraceae.

Species	Approximate leaf size (cm^2)	Field Observation
B.parviflora	20	Predominantly horizontal leaf angles irrespective of position in the canopy
B.gymnorrhiza	80	Predominantly horizontal leaf angles irrespective of position in the canopy
R.stylosa	40	Leaves predominantly acutely angled at the top of the canopy, becoming increasingly more horizontally displayed with increasing depth in the canopy

Leaves of *Nerium oleander* that were used as a comparison to published data (Table 2.1) were picked from a Townsville garden at 8:00am in March, 1990.

2.2.3 Leaf angle experiment

This experiment was designed to test the hypothesis that the xanthophyll concentration of leaves increases with increasing exposure to solar radiation. Leaf angles of *Rhizophora stylosa* growing in Bowling Green Bay close to the Australian Institute of Marine Science were altered. The experiment was done in July (cool, dry season), so northerly facing leaves and the north side of trees were chosen to maximize solar radiation received. Four northerly facing leaves on the north side of nine trees were selected. Two of the four leaves from each tree were forced into different angles using a wire holder while the other two were left at their natural, near-vertical angle. Two weeks after leaf angles had been altered, leaf discs were punched from the leaves and placed in liquid nitrogen for subsequent analysis of xanthophyll and chlorophyll concentrations. Data presented in Figure 2.7 are means fitted to at least 6 measurements at that leaf angle. During this period over which the leaves were acclimatizing to their new orientation there was lower than average temperatures in Townsville (5°C minimum and 20°C maximum). Four control leaves on one tree were placed in the wire holder but their leaf angle was not altered. There was no significant difference ($p>0.05$) in xanthophyll/chlorophyll ratio between the control and the non-wired leaves.

Daily radiation received by the leaves was estimated for each leaf angle by integrating the direct solar irradiance incident on the upward facing surface of the

leaf between dawn and when the leaves came into deep shade because of the near-by hill (approximately a 10 hour period). This can be described by:

$$\text{Total daily solar radiation} = \int_0^x I_o \sin\beta \sin(\beta - \theta)\, d\beta$$

$$= \tfrac{1}{2} I_o \left[\left(\tfrac{2\pi}{3} - \theta \right) \cos\theta + \tfrac{1}{4} \sin\theta + \tfrac{\sqrt{3}}{4} \cos\theta \right]$$

where I_o is the solar constant (approximated by 2000 μmol quanta.m^2.s^{-1}, or 7.2 mol.m^{-2}.hr), x is the angle when the leaves are in deep shade, in this case at $2\pi/3$ (120°), ß is the solar angle and θ is the angle of the leaf relative to the horizontal.

Leaf area displayed on a horizontal plane, or the proportion of leaf area exposed to direct sunlight at noon was determined by measuring leaf angles with a hand held clinometer (Suunto Instruments, model PM-5/360 PC, Helsinki, Finland). The percentage of leaf area displayed on a horizontal plane was calculated as the cosine of the leaf angle multiplied by 100 (Ball *et al.*, 1988).

2.2.4 Canopy dominance surveys

To determine which mangrove species dominate the forest canopy, the area that a species foliage covers at the top of the canopy was compared to its abundance measured by counting trees at ground level. Two sites at the Daintree River were chosen, the south bank 7 km upstream of the river mouth, and the north bank 10 km upstream of the river mouth. Estimations of foliage cover for each species were made by taking aerial photographs of mangrove

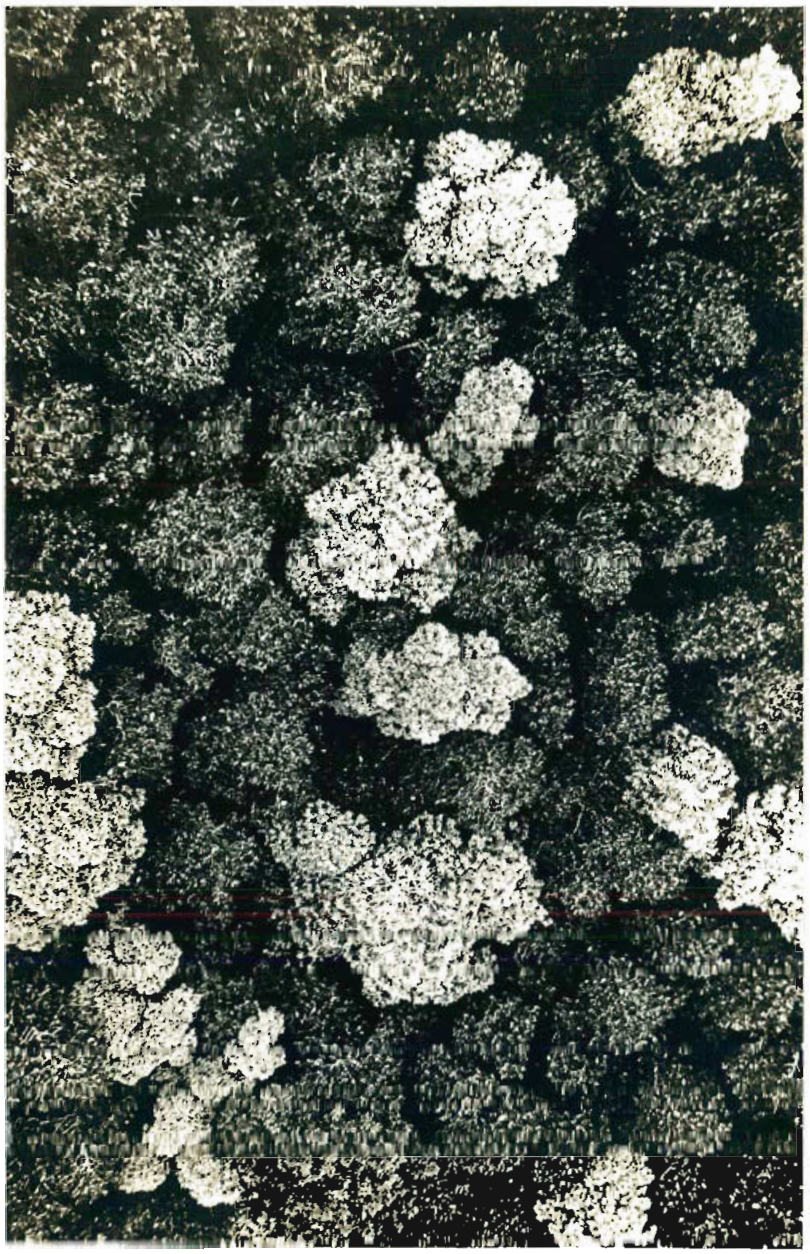


Plate 2.1. Example of the photographs taken of mangrove forest canopies that were used to quantify canopy dominance.

canopies from a helicopter hovering approximately 50 meters above the canopy (Plate 2.1 shows an example of the aerial photographs). Five replicate 25 m^2 areas of the canopy (approximated by one frame of the film) were assessed at each site. These were digitized (GP-8 Sonic Digitizer, Scientific Assessories Co., USA) using a digitizing table onto which the canopy photograph was projected. The area covered by each species was expressed as a proportion of the total, and then averaged over the five replicate photographs. Abundance of each species at ground level represents the proportion of trunks of that species in three 25 m^2 plots at each site (data provided by Dr. B.F. Clough, Australian Institute of Marine Science, Townsville). It was not possible to differentiate *Rhizophora stylosa*, *R. apiculata* and *R. mucronata* species in the aerial photographs as their foliage is similar, so these species were combined in both measures of species abundance.

2.2.5 Pigment Analysis

2.2.5.1 *Extraction procedure*

Leaf discs were removed from the -80°C freezer and ground to a powder in liquid nitrogen using a small porcelain mortar and pestle under a stream of nitrogen gas in a darkened laboratory. The leaf powder was transferred to a plastic screw cap test tube containing approximately 1mg of sodium bicarbonate (to prevent acidification of the carotenoids). 3 ml of 80% or 100% acetone was added. Tubes were sealed under nitrogen, and the contents shaken. The tubes were then left in a -5°C freezer for two hours. After two hours the samples were centrifuged for 5 minutes and the supernatant removed to another test tube. The ground leaf tissue was then resuspended in another 2 ml of the solvent for another two hours, again sealing under nitrogen. This was repeated a third time, the

samples were then combined and made up to 8 ml before they were ready to process by high performance liquid chromatography (HPLC). The 8ml volume was chosen as it provided a concentration of pigments adequate for HPLC analysis.

The efficiency of the extraction procedures was calculated (Dunlap and Chalker, 1986) for both 80 and 100% acetone (this is described fully in Appendix III, page 131). The extraction efficiency after three extraction steps was 97% for 100% acetone and 76% for 80% acetone. 100% acetone allowed quantification of ß-carotene that was not possible using 80% acetone.

2.2.5.2 *Pigment determination*

Either of two HPLC methods were used to determine the pigment contents of the leaf extracts. The HPLC equipment consisted of;

1) the solvent delivery system and pump, Spectra Physics (San Jose, CA, USA), model SP8700,

2) solvent mixer (Spectra Physics, model SP8300),

3) Rheodyne model 7125 injector (Rheodyne Incorperated, Cotati, CA, USA),

4) Waters (Waters Associates, Milford, MA, USA) fixed wavelength absorbance detector (Model 441) set at 436 nm, and

5) an integrator. This was either an LKB, model 2221 (LKB, Bromma, Sweden), or Spectra Physics, model SP4100 (depending on availability).

Solvents used were Waters HPLC grade. These were filtered (Whatman GF/F glass microfilters) under a vacuum before use and bubbled with helium during chromatography.

Method 1

Using a Brownlee (Applied Biosystems, Foster City, CA, USA) C-18 5µm, 25 cm steel column with a C-18 guard column preceding it, 50 to 100 µl (depending on the concentration of the sample) of each 8 ml extract was injected on to the column. The solvent gradient used was:

Time (min)	Solvent A	Solvent B
0	100	0
25	0	100
30	100	0

where Solvent A was acetonitrile water (9:1) and Solvent B was ethyl acetate. The flow rate was 1 $ml.min^{-1}$. 15 minutes of acetonitrile water was required to equilibrate the column. This gradient was generously supplied by Dr. George Britton (University of Liverpool, UK).

Method 2

Using a 10cm Bondapak (Waters Associates, Milford, MA, USA) C-18 column preceded by a guard column, 50 to 100 µl (depending on the concentration of the extract) of the sample was injected on to the column. The gradient was:

Time (min)	Solvent A	Solvent B
0	100	0
3	100	0
11	0	100
12	0	100
17	100	0

where Solvent A was 51% tetrahydrofuran in water and Solvent B was 90% tetrahydrofuran in water. The flow rate was 1 $ml.min^{-1}$. The column was reequilibrated over 5 minutes at the intial conditions. This gradient was modified from Juhler and Cox (1990) who used a Shandon Hypersil 5µm, 12 cm, C-18 column. Their original gradient was from 100% Solvent A to 100% Solvent B in 13 minutes, 3 minutes to revert to the initial conditions and 3 minutes to reequilibrate the column.

Zeaxanthin standard was obtained from Extrasynthase (France), ß-carotene from Roche, and chlorophyll a and b from Aldrich (USA). Standards for lutein, neoxanthin and violaxanthin were obtained by collecting pure fractions separated by HPLC, measuring their absorption spectra in 1ml of ethanol to confirm their purity and quantifying by comparison with published absorbance spectra (Davies, 1976). A known volume of the solution (i.e. known quantity of the pigment) was injected back on to the column and an integration obtained for this amount. This procedure is described in detail in Appendix IV (page 133). Quantification of antheraxanthin was done using the same calibration for violaxanthin as they have similar molar extinction coefficients (see Appendix IV, page 133).

The concentration of xanthophylls in leaves is expressed as a proportion of the total chlorophyll within a leaf, over the same leaf area. This ratio was chosen as xanthophylls are presumed to provide protection at the antenna chlorophyll, therefore the relationship between chlorophyll and xanthophylls is of primary interest. Furthermore, the use of this ratio corrects for differences between species, and leaves of different age or nutrient status, that may have different net chlorophyll concentrations. Epoxidation state is defined as the proportion of the xanthophylls present in the epoxidized state (Thayer and

Björkman, 1990). Violaxanthin (V) has two epoxide groups, antheraxanthin (A) has one epoxide group, while zeaxanthin (Z) has none. Thus;
Epoxidation state = (V + 0.5A)/(V+A+Z).

2.2.6 Statistical analysis

Statistical analysis used the computing package STATISTIX (NH Analytical Software, Roseville, MN, USA). When analysing data from vertical transects through the *R.apiculata* canopy it was necessary to fit non-linear curves (Figure 2.2). In this instance non-linear, exponential curves were fitted using the NLIN procedure of the SAS computing package (SAS Institute, NC, USA). In Figure 2.2, height in the canopy was transformed to (maximum height-height)/100 in order to obtain convergence of the model fitting process.

Analysis of variance of xanthophyll/chlorophyll ratios with sites, species, and sun/shade as treatments used a design in which species, sites, and sun/shade treatments were represented as crossed factors, with trees nested within the crossing of species and site, or species and sun/shade, and with leaves nested within trees. Sites, species and sun/shade were considered as fixed effects and leaves and trees as random effects. As variation in the xanthophyll/chlorophyll ratio increased with increasing size of the variable (i.e. non-constant variance) the data were logarithmically transformed to satisfy the constant variance requirements of analysis of variance models. The least significant difference (LSD) between any two means in Figure 2.4 and 2.5 was calculated by multiplying the critical t value at p=0.05 with the standard error of the difference between two means (i.e. $LSD = t_{crit} * (s/\sqrt{2n})$).

The relationship between: 1) total chlorophyll and lutein and neoxanthin contents within leaves, 2) midday epoxidation state and the xanthophyll/chlorophyll ratio and, 3) xanthophyll/chlorophyll ratio and leaf area displayed on a horizontal plane (Figure 2.6) was fitted using least squares linear regression and its significance tested using a t-test at p=0.05. Error bars in Figure 2.7 represent plus and minus the standard error of the mean (SEM=s/$\sqrt{n}$).

2.3 Results

The three mangrove species had similar xanthophyll/chlorophyll ratios to non-mangrove species (Table 2.2). The lower total chlorophyll and xanthophyll concentrations of the mangroves and of the Townsville grown *N.oleander* compared to published values may reflect the different conditions under which these plants were grown. The carotenoids neoxanthin and lutein increased proportionally with total chlorophyll in all species (linear regressions are: Neoxanthin=-0.83+0.0896*Tchl, r^2=0.77, p<0.001; Lutein=11.41+0.209*Tchl, r^2=0.76, p<0.001), whereas changes in concentration of the xanthophyll cycle components (i.e. V+A+Z) were less correlated with changes in chlorophyll concentrations (r^2=0.32) (Figure 2.1).

Table 2.2. A comparison of xanthophyll (V+A+Z) and chlorophyll concentrations in the leaves of mangrove species with other species. PFD is the photon flux density ($\mu mol.m^{-2}.s^{-1}$).

Species	Growth conditions	Total Chl $mg.cm^{-2}$	V+A+Z $ug.cm^{-2}$	(V+A+Z)/Chl $ug.ug^{-1}$	Author
P. balsamifera	deep shade	54.00	3.00	0.056	(a)
H. helix	"	79.80	2.96	0.037	"
M. deliciosa	"	74.40	1.66	0.022	"
N. oleander	200 PFD	87.17	7.72	0.087	(b)
	800 PFD	91.76	8.42	0.092	"
	1000 PFD	72.47	7.72	0.107	"
N. oleander	sun (natural)	34.00	1.77	0.052	this study
B. parviflora	"	19.32	2.18	0.116	"
B. gymnorrhiza	"	32.74	3.12	0.100	"
R. stylosa	"	26.24	2.62	0.104	"

(a) Demmig *et al.* (1987)
(b) Demmig *et al.* (1988)

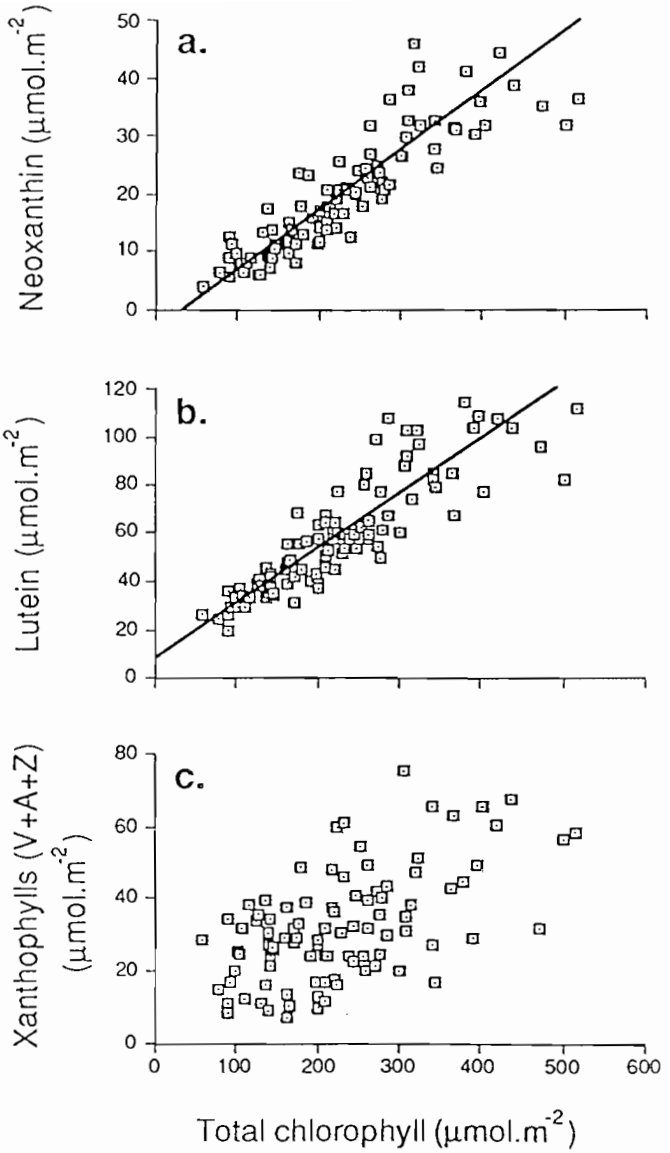


Figure 2.1. Relationship between neoxanthin (panel a), lutein (panel b) and xanthophylls (panel c) with total chlorophyll contents in the leaves of mangrove species. Lines of best fit are shown for neoxanthin and lutein. Regressions and r^2 values appear in the text.

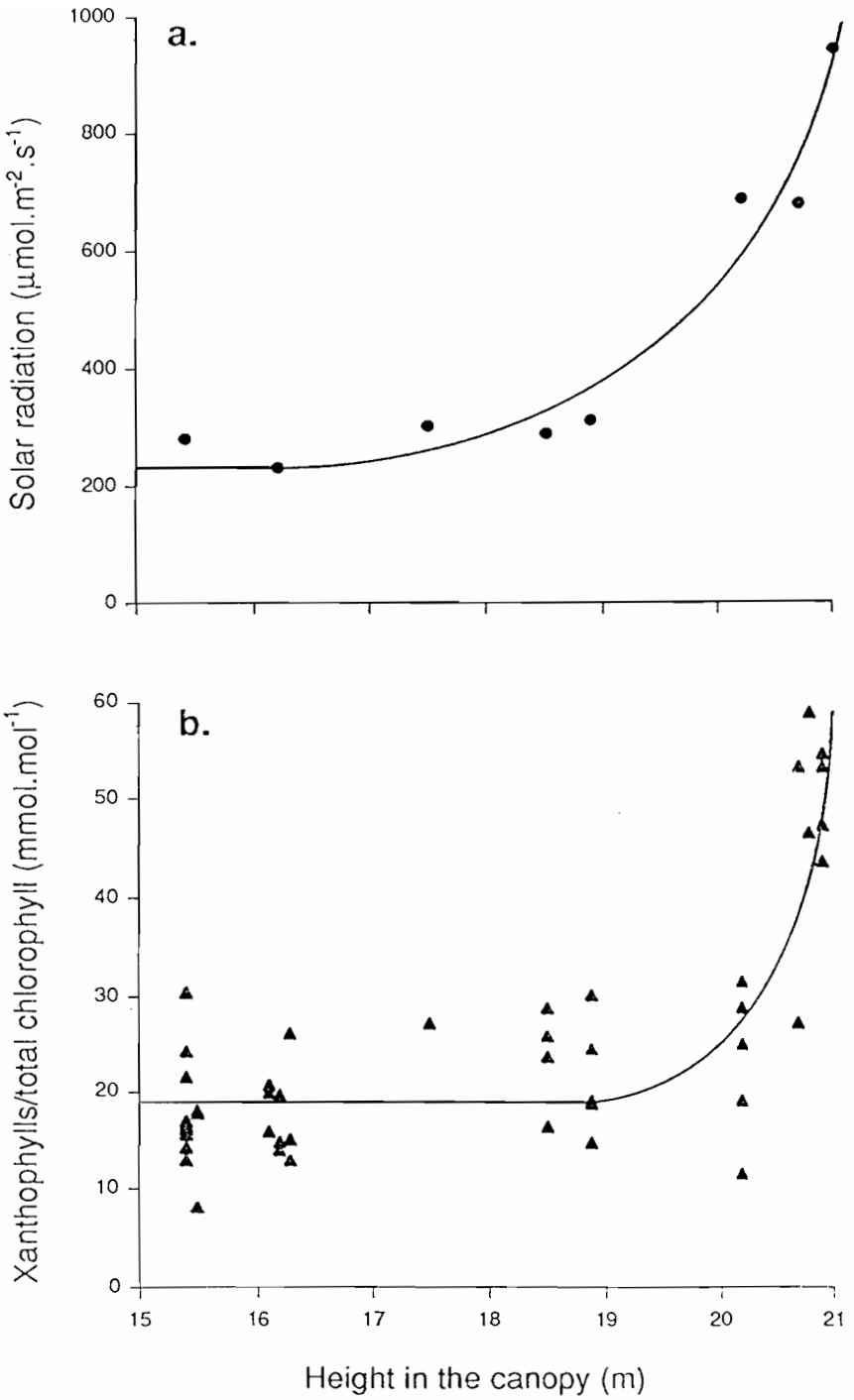


Figure 2.2. Variation in the light intercepted (panel a), and the xanthophyll/chlorophyll ratio (panel b) of leaves of the mangrove *Rhizophora apiculata* through a well developed forest canopy at the Daintree River, north Queensland. Curves are the lines of best fit and are described in the text.

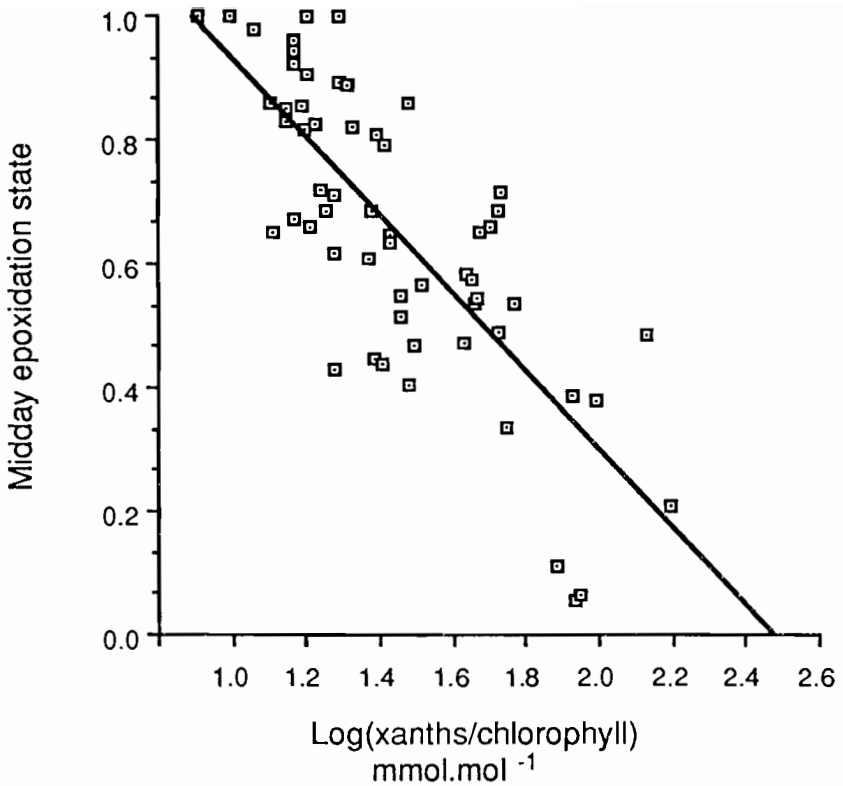


Figure 2.3. Variation in midday epoxidation state [i.e. (V+0.5*A)/(V+A+Z)] with the xanthophyll/chlorophyll ratio for *Rhizophora apiculata* leaves sampled through a well developed forest canopy. Regression is:
Midday Epox=1.564+-0.630*log(xanths/chl), r^2=0.605.

A vertical transect through a 22 meter high *R. apiculata* forest canopy in the Daintree River was used to test the hypothesis that light is an important factor in determining xanthophyll concentration in leaves. Light levels decreased exponentially from the top, down through the canopy, with much of the light being attenuated in the first meter of the canopy (Figure 2.2a). Leaf xanthophyll/chlorophyll ratios were reduced in a similar way (Figure 2.2 b). Curves fitted to Figure 2.2(a) and 2.2(b) have the form $Y=a+b\ exp(k*H)$, where Y is either the solar radiation or the xanthophyll/chlorophyll ratio, a, b and k are parameters and H=(21-height in the canopy)/100. The parameter estimates and their standard errors (in parenthesis) are for solar radiation: a=212.54 (79.71), b=698.18 (86.18), and k=-75.60 (29.12); and for the xanthophyll/chlorophyll ratio: a=18.18 (1.20), b=41.82 (5.34) and k=-231.62 (63.28). Xanthophyll/chlorophyll ratios of leaves also decreased with the midday epoxidation state (proportion of the xanthophylls in the epoxidated state, i.e. the proportion present as violaxanthin plus half that present as antheraxanthin) of the leaves (Figure 2.3).

Significant differences in xanthophyll/chlorophyll ratios were observed between mangrove species ($F_{(2,12)}$=7.76, p=0.0069) and between sites ($F_{(1,12)}$=5.83, p=0.0326) (Figure 2.4). There was also a significant interaction between site and species effects ($F_{(2,12)}$=4.76, p=0.0300) suggesting that site factors influence species to different degrees. For example, *R.stylosa* had a similar xanthophyll/chlorophyll ratio as the other species in 25% seawater nutrient culture, but had a lower xanthophyll/chlorophyll ratio at the Daintree site where salinities are higher.

Comparison between sun and shade leaves collected at the Daintree River mouth showed sun leaves had greater xanthophyll/chlorophyll ratios, but this

trend was not statistically significant ($F_{(1,17)}$=1.79, p=0.1983) (Figure 2.5). Given that leaf angle and azimuth is an important factor in determining the amount of light received by a leaf (Ball *et al.*, 1988), it was hypothesised that the differences in xanthophyll/chlorophyll ratios between mangrove species and sites, and the non-significant difference between sun and shade leaves of mangroves, could be explained by variations in leaf angles. The xanthophyll/chlorophyll ratios of sun leaves of all three mangrove species over two sites were plotted against the proportion of leaf area displayed on a horizontal plane (Figure 2.6). Xanthophyll/chlorophyll ratios increased with the proportion of leaf area displayed on a horizontal plane and the relationship between these two variables was significant ($F_{(2,48)}$=5.67,p<0.001).

To test directly whether leaf angles, and the corresponding light intercepted by leaves, are correlated with leaf xanthophyll/chlorophyll ratios, leaves of *R.stylosa* were constrained to different angles. Figure 2.7 shows the calculated light levels received by leaves with a northerly azimuth over a range of leaf angles and also the xanthophyll/chlorophyll ratios of the leaves held at those orientations. Leaves that were artificially constrained to horizontal leaf angles, and so received higher levels of solar radiation, had higher xanthophyll/chlorophyll ratios than those at their natural, near-vertical orientation. However, higher xanthophyll/chlorophyll ratios did not correlate with increased incident daily radiation when leaves were fixed at leaf angles between 0 and 40° from the horizontal.

The significance of protection from excessive visible light on community structure of mangrove forests was assessed by determining which species dominate the top of mangrove forest canopies and whether xanthophyll/chlorophyll ratios, leaf angles or leaf size could be correlated with

species abundance at the top of a forest canopy. The proportion of species visible at the top of the canopy was compared to the proportion of trunks of that species in 25 m^2 plots at the same site (Table 2.3). *Bruguiera parviflora* and the *Rhizophora* species occurred in similar proportions at both the canopy and ground level, while *B.gymnorrhiza* was less abundant in the canopy (4 and 7%) than it was for the on-the-ground measures (26% at both sites). Thus, the species with large horizontally arranged leaves were less abundant at the top of the canopy than species with small, horizontal leaves with high xanthophyll/chlorophyll ratios, or species with near-vertical leaf angles.

Table 2.3. Abundance of mangrove species at the top of the canopy (estimated using aerial photography) compared with abundance measured at ground level for three mangrove species at two sites at the Daintree River, north Queensland, Australia.

Species	% abundance at canopy level (std. error)	% abundance at ground level
7 km upstream (n=5)		
Bruguiera parviflora	15.35 (4.25)	4.70
Bruguiera gymnorrhiza	3.937 (3.77)	26.47
Rhizophora species	67.93 (2.71)	66.18
Unknown or minority species	0.194 (0.19)	2.65
10 km upstream (n=3)		
Bruguiera parviflora	71.09 (6.22)	62.97
Bruguiera gymnorrhiza	6.724 (2.37)	25.95
Rhizophora species	1.779 (0.95)	2.97
Unknown or minority species	2.832 (0.60)	3.86

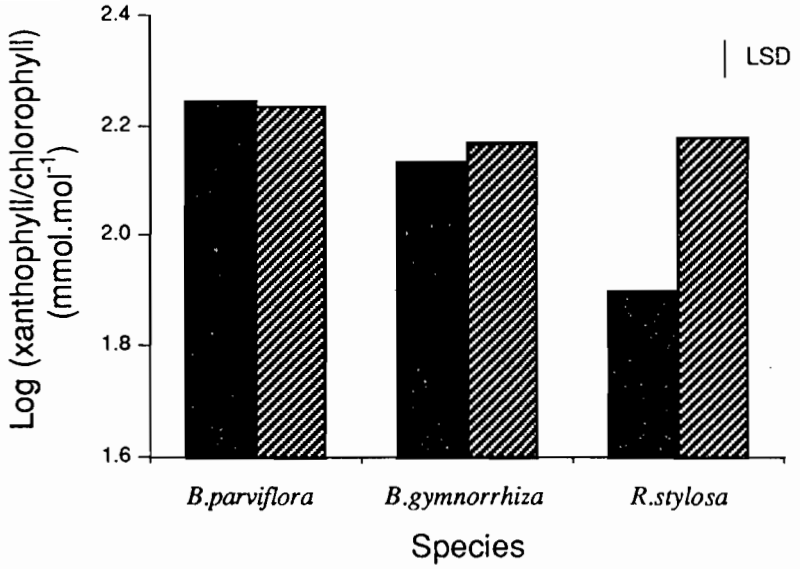


Figure 2.4. Xanthophyll/chlorophyll ratios of three mangrove species at the Daintree River (solid) and at the Australian Institute of Marine Science (shaded). LSD is the least significant difference ($p<0.05$) between any two means.

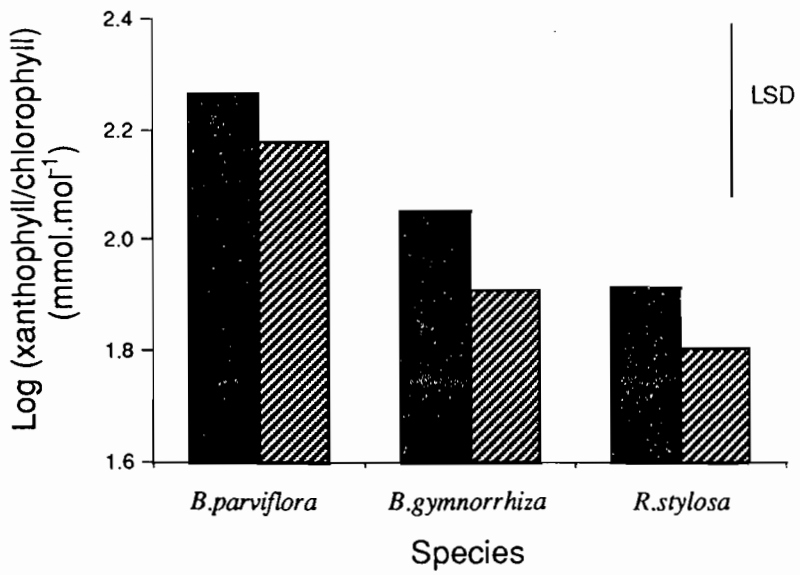


Figure 2.5. Xanthophyll/chlorophyll ratios of sun leaves (solid) and shade leaves (shaded) of three mangroves species growing in a 3 meter tall, open forest at the mouth of the Daintree River, north Queensland. LSD is the least significant difference ($p<0.05$) between any two means.

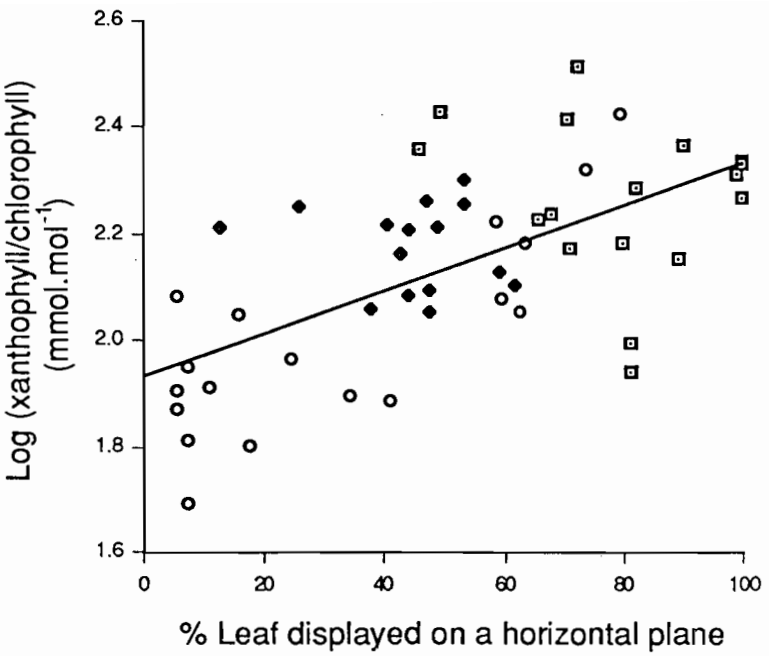


Figure 2.6. The variation in the xanthophyll/chlorophyll ratio of three mangrove species (*Rhizophora stylosa* (circles), *Bruguiera parviflora* (squares), and *B.gymnorrhiza* (diamonds)) with the proportion of leaf area displayed on a horizontal plane (i.e. cos (leaf angle)*100). Linear regression is: Log(xanths/chl)=1.929+0.004054*leaf display, r^2=0.350.

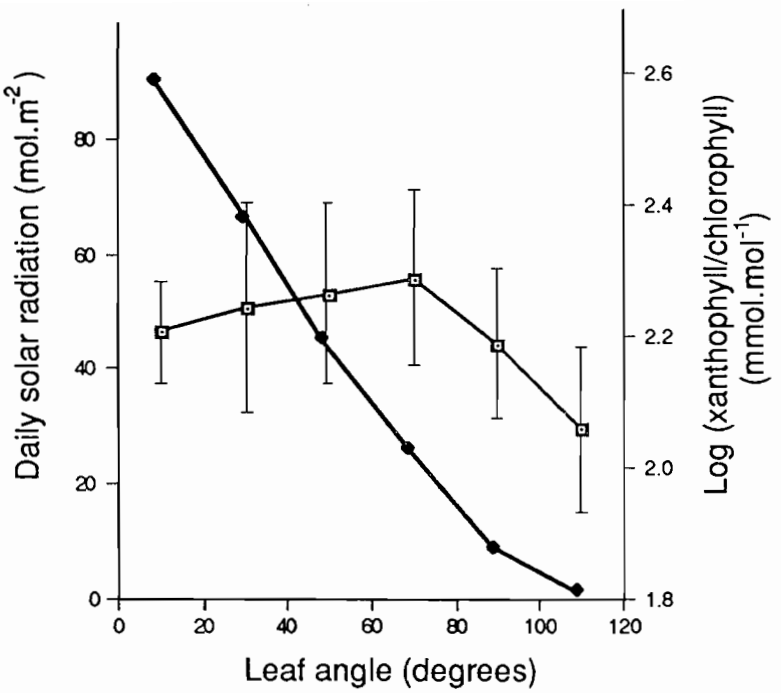


Figure 2.7. Xanthophyll/chlorophyll ratio (squares) and calculated solar radiation (diamonds) absorbed by *Rhizophora stylosa* leaves artificially constrained to leaf angles more horizontal than their usual, near-vertical leaf angles. Error bars are plus and minus one standard error of the mean.

2.4 Discussion

Mangroves have similar xanthophyll/chlorophyll ratios as other species (Table 2.2). This reflects the similarity of mangrove photosynthetic biochemistry to that of non-halophytic terrestrial species (Andrews *et al.*, 1984; Ball, 1988). The strong correlation between total chlorophyll concentrations in mangrove leaves and the lutein and neoxanthin concentrations (Fig. 2.1 a and b) is expected as lutein and neoxanthin are believed to be associated with the light harvesting pigment-protein complexes (Siefermann-Harms, 1985), and has been recently reported for other species (Morales *et al.*, 1990; Thayer and Björkman, 1990). There was a low correlation between the xanthophyll and total chlorophyll concentrations (Fig. 2.1c). This has also been found by other researchers (Morales *et al.*, 1990; Thayer and Björkman, 1990) and is consistent with the view that these pigments are not directly associated with light harvesting but have some other function within the chloroplast.

The correlation between position of a leaf within the canopy profile, the average light level to which it is exposed, and its xanthophyll/chlorophyll ratio shows that light is an important factor in determining xanthophyll/chlorophyll ratios in leaves (Fig. 2.2). The non-significant difference between sun and shade leaves at the Daintree River mouth site (Fig. 2.5) would seem to be inconsistent with this. However, the relatively open canopy at this site (compared to the well developed canopy where the vertical canopy transect was done) would have increased the level of light intercepted by shade leaves. In addition, for some species sun leaves have leaf angles that may effectively reduce the light intercepted by them to a value similar to that received by shade leaves.

Differences in the xanthophyll/chlorophyll ratio of sun leaves between the three mangrove species over two sites were shown to correlate with the proportion of leaf area displayed on a horizontal plane (Fig. 2.6). This was a function of their leaf angle, which influences light interception by leaves. The artificial changes in leaf angles of *R.stylosa* to more horizontal orientations also resulted in increased xanthophyll/chlorophyll ratios (Fig. 2.7). However, the xanthophyll/chlorophyll ratios of leaves did not correlate with increasing light levels absorbed by leaves when they were constrained to near-horizontal leaf angles. This may have been due to: 1) the abnormally low air temperatures (5°C minima) experienced during the experiment that may have limited xanthophyll biosynthesis, making it impossible for leaves to synthesise large amounts of xanthophylls, or 2) mangroves having long lived leaves (up to 18 months) which can limit the level of metabolic adjustment leaves can make to new environmental conditions (Osmond, 1987), or 3) the low nutrient environment in which they grow. Osmond (1983) found that shade plants grown under depleted nitrogen conditions never fully adapted when transfered to high light conditions.

The significant interaction between species and site (Fig. 2.4) largely reflects the ability of *R.stylosa* to grow leaves at nearly vertical angles in response to environment. This results in a decreased level of solar radiation intercepted by the leaf (calculated values shown in Fig. 2.7), and thus a corresponding decrease in leaf temperature under conditions in which water deficits are likely (i.e. in saline environments). It has been established that interactions between water deficits and high light levels lead to photoinhibition (Osmond, 1983; Björkman and Powles, 1984), and that high light level, high leaf temperature interactions may also result in photoinhibition (Bongi and Long, 1987; Gamon and Pearcy, 1990). *R.stylosa* may avoid conditions that predispose it to photoinhibition by having leaves that grow at angles that reduce solar

irradiance absorbed by leaves. This presumably results in carbon and energy savings due to the maintenance of photosynthetic rates (Ball *et al.*, 1988), avoidance of chloroplast repair costs (Greer *et al.*, 1986), and probably a reduced requirement for xanthophyll biosynthesis.

The level of visible radiation in excess of that which is used for photosynthesis increases with increasing solar radiation and decreasing photosynthetic rates (see section 1.1, page 1). Therefore, the correlation between xanthophyll/chlorophyll ratios and the level of solar radiation intercepted by a leaf (Figures 2.4 and 2.6) may also reflect a response to factors associated with high levels of incident solar radiation that limit photosynthetic rates. The increase in xanthophyll/chlorophyll ratios with lower midday epoxidation states (i.e. more zeaxanthin) (Fig. 2.3) supports this view.

Photosynthesis of mangroves has been shown to be highly sensitive to leaf temperature (Andrews *et al.*, 1984; Ball *et al.*, 1988). Increases in incident solar radiation on mangrove leaves are highly correlated with increases in leaf temperature (Ball *et al.*, 1988). Thus, vertical leaf angles, which reduce solar radiation absorbed by leaves, have been shown to be important in maintaining leaf temperatures within a range where photosynthesis can continue when water use was restricted by saline conditions (Ball *et al.*, 1988). The effectiveness of vertical leaf angles in maintaining productivity is a view which is supported by Smith and Ullberg (1989), who found that vertical and north-south orientation of leaves was correlated with increased photosynthetic rates of the prairie shrub *Silphium terebinthinaceum*. Thus, increases in xanthophyll/chlorophyll ratios in leaves exposed to high levels of solar radiation (Fig. 2.2 and 2.6) may be due, in part, to reductions in photosynthetic rates brought about by increased leaf temperatures.

Leaf size also influences leaf temperature (Givnish, 1987). Under the conditions in which mangroves in north Queensland grow (average summer air temperatures of 30-36°C), and due to their low stomatal conductances that restrict evaporative cooling (Ball *et al.*, 1988), large leaves would be expected to be hotter than small leaves, particularly when air movement is low and convective heat loss is correspondingly low. For this reason it could be expected that leaf size, leaf angle and xanthophyll content of leaves may influence the dominance of species in mangrove environments where photoinhibition is likely.

Bruguiera gymnorrhiza, a large leafed mangrove species with horizontally arranged leaves, rarely occurred at the top of the canopy, despite its abundance observed at ground level (Table 2.3). This supports the idea that leaf characteristics are important in determining mangrove community structure. *Bruguiera parviflora* and the *Rhizophora* species occurred in the same proportion at the top of the canopy compared to that at ground level (Table 2.3). These species have different leaf characteristics, which may provide contrasting photoprotective strategies. The *Rhizophora* species incline their leaves to angles that reduces the amount of high intensity midday solar radiation intercepted, which also results in leaf temperatures that are suitable for photosynthesis, while *B.parviflora*, has relatively horizontal leaf angles, but has high xanthophyll/chlorophyll ratios. The greater abundance of *B.parviflora* relative to *B.gymnorrhiza* may be due to the considerably smaller leaf size of *B.parviflora* compared to *B.gymnorrhiza*, which results in lower leaf temperatures that are more favourable for photosynthesis. These leaf characteristics may result in *B.parviflora* having an equivalent level of photoprotection from excessive visible light as the *Rhizophora* species over the sites studied.

Species from the family Rhizophoraceae that incline their leaves to near-vertical orientations (for example, *R.stylosa* and *Ceriops tagal* var. *australis*) often form mono-specific stands in highly saline regions (salinities close to, or greater than that of seawater) of tropical estuaries (Macnae, 1969). This ability to dominate in essentially arid conditions may be due to the avoidance of high solar radiation levels through near-vertical orientation of their leaves, thus leading to avoidance of conditions that have been shown to result in photoinhibition (i.e. high solar radiation levels, high leaf temperatures and low photosynthetic rates).

However, it is unlikely that the avoidance of photoinhibition is the only factor that determines the distribution of mangroves species within their estuarine environment. Differences in seed predation (Smith, 1987*a*), seed dispersal and mortality (Rabinowitz, 1978), salinity tolerance (Smith, 1988), and competition between species have all been proposed to influence the distribution of mangrove species. There is some evidence to suggest that competition between species is based upon differences in their shading tolerance or intolerance (Ball, 1984; Smith, 1987*b*). For example, Ball (1984) suggested that *Rhizophora mangle* out-competed *Languncularia racemosa* in intertidal regions in southern Florida. Because *L.racemosa* was unable to compete with *R.mangle* under these conditions, possibly due to its shade intolerance, its distribution was limited to drier regions where *R.mangle* could not compete due to the limited tidal influence. An additional factor that may also explain species distribution patterns, may be that avoidance of photoinhibition by species like *L.racemosa*, enables dominance in exposed, arid zones.

This study has found that xanthophyll/chlorophyll ratios in mangrove leaves vary between species, and with average light level of their growth environment, the difference between species largely reflecting the influence of leaf

angle in determining the light level absorbed by leaves. The canopy dominance survey described in this study showed that species with large, horizontally arranged leaves are diminished in abundance at the top of the canopy compared to species with near vertical leaf angles, or those with small leaves that have a high capacity to dissipate excess energy through zeaxanthin. These results point to the importance of solar radiation, leaf temperature and the ability to dissipate excess energy in influencing mangrove species interactions, and to the contrasting strategies for photoprotection that may occur in plants.

The level of zeaxanthin in leaves at midday, when solar radiation is at its most intense, correlates with the xanthophyll/chlorophyll ratio within leaves (Fig. 2.3). This suggests that the requirement for photoprotection by the xanthophylls, which should be related to the amount of excess light that is absorbed by a leaf, influences the total amount of xanthophylls within a leaf. The requirement for photoprotection should be a function of both the light level intercepted by the leaf and the photosynthetic rate. This is investigated further in the following Chapter where the relationship between light intercepted by leaves and photosynthesis is examined.

CHAPTER 3

The influence of photosynthetic rate, solar radiation and leaf temperature on zeaxanthin concentrations

3.1 Introduction

The xanthophylls, particularly zeaxanthin, are thought to be involved in protection against damage caused by excess light (i.e. the light absorbed by leaves that is in excess of what is used for photosynthesis) (Demmig *et al.*, 1987). This is supported by the observation that the light environment in which leaves develop is important in determining their xanthophyll content (see previous Chapter; Demmig *et al.*, 1987; Thayer and Björkman, 1990). However, the light environment in which a leaf grows may not be the only factor that determines its xanthophyll concentration. The negative correlation between the leaf xanthophyll content and the proportion of the xanthophylls present in the epoxidized form (largely as violaxanthin) (Fig. 2.3; Thayer and Björkman, 1990), suggests that the daily requirement for photoprotection (represented by their midday epoxidation states) may be an important factor in influencing the total xanthophyll content of leaves.

A number of other studies have shown that photoinhibition increases under conditions that reduce photosynthetic rates, for example, water deficits (Björkman and Powles, 1984), low nitrogen status (Osmond, 1983), and leaf temperatures not optimal for photosynthesis (Greer *et al.*, 1986; Bongi and Long,

1987). In addition, recent studies have shown that the zeaxanthin content of leaves increases at low CO_2 and O_2 concentrations (Demmig *et al.*, 1989), and at leaf temperatures that are below those optimal for photosynthesis (Bilger and Björkman, 1991). It is therefore possible that the likelihood of photoinhibition (or, in other words the requirement for photoprotection) may be influenced jointly by photosynthetic rates, leaf temperature and light regimes. The work described in this Chapter is directed towards quantifying the relationship between leaf zeaxanthin concentration and solar radiation, photosynthetic rate and leaf temperature in mangroves in their natural environment. In order to examine these relationships a statistical modelling approach was adopted in which functions representing the hypothesised relationships between variables were fitted to the data, and the adequacy of the fit assessed using statistical criteria (see 3.2).

3.2 Materials and Methods

3.2.1 Plant material

3.2.1.1 *Relationship between photosynthesis and epoxidation state*

In this experiment, sun leaves of *Rhizophora stylosa, Bruguiera parviflora* and *Bruguiera gymnorrhiza* were collected from an exposed, northerly aspect on the southern side of the mouth of the Daintree River, North Queensland (Lat. 16°S, Long. 146°E) on October 10, 1989, and from plants grown under natural sunlight at the Australian Institute of Marine Science (Lat. 19°S, Long. 147°E) in nutrient culture adjusted to salinity of 25% seawater (see Appendix II, page 130). At both sites four sun leaves were sampled from three trees of each species between 9:00 and 11:00 am.

3.2.1.2 *Interactions between photosynthesis, leaf temperature and solar radiation in determining epoxidation state*

Plant material was obtained at the Australian Institute of Marine Science from plants grown in nutrient culture at either 25 or 100% seawater, or collected at the Daintree River between October 1989 and December 1990. A total of 169 leaves were sampled from both sun and shade environments from four species of mangroves within the family Rhizophoraceae (*Rhizophora apiculata*, *R.stylosa*, *Bruguiera parviflora* and *B.gymnorrhiza*).

3.2.2 Sampling procedure

Photosynthesis, solar irradiance and leaf temperature were measured *in situ* using a Li-Cor LI-6200 portable photosynthesis measuring system (Li-Cor, Lincoln, Nebraska, USA) with a modified leaf chamber (Clough and Sim, 1990). The modified leaf chamber clamped on to the leaf, enclosing 5.7 cm^2 of the lower leaf surface. This arrangement is possible as the species investigated were hypostomatous. The volume of the chamber was 275 cm^3. Air inside the chamber was circulated with a small fan, giving a boundary layer conductance of 1.32 $mol.m^{-2}.s^{-1}$. Leaf temperature was measured with a 36-SWG chromel-constantan thermocouple pressed to the underside of the leaf during the measurement. Light levels received by leaves were measured using an average value calculated from 4 small light sensors set into the corners of the leaf clamp of the chamber. These small sensors were calibrated against a factory calibrated Li-cor quantum sensor. Relative humidity within the chamber was measured with a Vaisala Humicap 6061 RH sensor (Vaisala, Oy, Helsinki, Finland). Air temperature within the leaf chamber was measured with a YSI-44202 (Yellow

Springs Instrument Co., Yellow Springs, Ohio, USA) linearised thermistor. Magnesium perchlorate was used as a desiccant in the air line to the infrared gas analyser (IRGA). This was replaced daily. Before use the humidity sensor was calibrated using air streams of known humidity and the IRGA calibrated by injecting known volumes of gas that had previously been intercalibrated against gas mixtures generated with Wostoff pumps (H. Wostoff oHG, Bochum, Germany). K tests (described in the LI-6200 Primer booklet), to test for water adsorption and absorption within the system, and leak tests were also carried out. The IRGA zero was checked at half hourly intervals using CO_2 free air that had been passed through soda lime. The flow meter was also zeroed every half hour. Changes in CO_2 concentrations within the closed system were measured over a 20 second period.

Immediately after the photosynthesis, light level and leaf temperature measurement were completed, leaf discs 1.37 cm^2 in area were punched from the leaf and immediately (within 1 minute) immersed in liquid nitrogen. On return to the laboratory they were transferred to a -80°C freezer where they were stored until analysis. Pigment analysis and quantification was carried out using HPLC as described in section 2.2.5 (page 25). Epoxidation state was calculated as: $(V + 0.5\ A) / (V + A + Z)$ (Thayer and Björkman, 1990).

3.2.3 Statistical Analysis

The overall aim of the analyses described below was to create a hierarchial model that describes the influence of photosynthesis, solar radiation and leaf temperature on epoxidation state, and to assess the strength of the relationships between them. The proposed hierarchy has a structure where;

1) epoxidation is dependent on photosynthesis, solar radiation and leaf temperature,

2) photosynthesis is dependent on solar radiation and leaf temperature, and

3) leaf temperature is dependent on solar radiation.

In order to assess this structure the following progression of analyses was done.

3.2.3.1 *Modelling the control of epoxidation*

To assess the relationship between epoxidation state and photosynthetic rate, a logistic curve (which has a sigmoidal shape with an upper and lower asymptote) was fitted to the data collected in 3.2.1.1. This data set was collected and used in this analysis as solar radiation levels were constantly saturating for photosynthesis (i.e. greater than 750 μmol quanta.$m^{-2}.s^{-1}$), while photosynthetic rates declined over the sampling period. A logistic function was chosen due to the need to constrain the response variable (i.e. epoxidation state) between zero and one. The form of the logistic curve was:

$$EPOX = 1 / (1 + exp(a + b\, PS)), \qquad (1)$$

where *EPOX* is the epoxidation state, *PS* is photosynthetic rate, and *a* and *b* are location and slope parameters respectively. The significance of these parameters was evaluated using t-tests with a significance level of p=0.05.

The influence of solar radiation on the epoxidation state of this data set was also assessed by fitting a curve of the same form:

$$EPOX = 1 / (1 + exp(a + b\, PS + c\, QN)), \qquad (2)$$

where *EPOX*, *PS* and *a* and *b* are the same as above, and where *QN* is the solar radiation intercepted by the leaves, and *c* is the *QN* slope parameter. The importance of *PS* and *QN* in determining epoxidation state were evaluated using t-tests with a significance level of p=0.05.

In the field solar radiation levels, photosynthetic rates and leaf temperatures vary considerably. Thus, in order to describe how epoxidation state varied over a range of conditions a statistical model of the same logistic form was fitted to a larger data set (collection of which is outlined in 3.2.1.2):

$$EPOX = 1 / (1 + exp(a + b\,PS + c\,QN + d\,TL)), \qquad (3)$$

where *EPOX* is the epoxidation state, *QN* is the solar radiation level, *PS* is the photosynthetic rate, *TL* is the leaf temperature and *a*, *b*, *c* and *d* are parameters. The significance of each variable in the model was assessed using a t-test at a significance level of p=0.05. Non-significant (p>0.05) variables were removed from the model sequentially. After a variable was removed the model was refitted. This process was continued until a minimal model was established where all the explanatory variables were significant .

3.2.3.2 *Relationship of solar radiation and leaf temperature to photosynthesis*

The assessment of the relationship between solar radiation, leaf temperature and photosynthesis was evaluated by non-linear multiple regression using the function:

$$PS = (a + b\,TL + c\,TL^2) (1 - exp(d\,QN)), \qquad (4)$$

where *PS* is the photosynthetic rate, *TL* is the leaf temperature minus 35°C (this was used to facilitate convergence when fitting non-linear functions), *QN* is the solar radiation received by the leaf, *a*, *b* and *c* are parameters that determine the upper asymptote of the curve for a given leaf temperature, and *d* describes the rate of change of photosynthesis due to changes in solar radiation.

The form of equation 4 was chosen as it represents a family of photosynthetic light response curves which are exponential in shape, where the coefficient, $a + b\,TL + c\,TL^2$, determines the upper asymptote of the curve, which can be interpreted as the light saturated photosynthetic rate. The quadratic function for the asymptote was chosen as the variation in photosynthetic rate with leaf temperature approximately follows this form (Berry and Björkman, 1980). The importance of each of *TL*, TL^2 and *QN* in determining photosynthesis was evaluated using t-tests with a significance level of p=0.05. As described above, non-significant variables (p>0.05) were removed from the model sequentially and the model refitted, until a minimal model was established, where all the explanatory variables were significant.

3.2.3.3 *Leaf temperature and solar radiation*

Linear regression was used to assess the influence of solar radiation on leaf temperature. Curvature in the relationship was accounted for by fitting a quadratic term to the model:

$$TL = a + b\,QN + c\,QN^2, \qquad (5)$$

where *TL* is the leaf temperature, *QN* is the solar radiation incident on the leaf and *a*, *b* and *c* are constants.

Linear regression of leaf temperature on solar radiation was done using the computing package STATISTIX (NH Analytical Software, Roseville, MN, USA). All non-linear analyses described above used maximum likelihood estimation by means of the non-linear procedure (NLIN) of SAS statistical computing package (SAS Institute, NC, USA). Residual plots where used to assess the adequacy of the models. Coefficients of determination (R^2) for the models represent a measure of variation in the data that is explained by the model.

3.3 Results

3.3.1 Relationship between photosynthesis and epoxidation state under high solar radiation levels

Assuming (1) absorption of light in excess of that utilized in photosynthesis increases as light levels increase or as photosynthesis decreases, (2) an increase in excess light results in an increase in the proportion of the xanthophylls present as zeaxanthin, and (3) that xanthophylls are conserved (i.e. violaxanthin + antheraxanthin + zeaxanthin = a constant), at least in the short term, the state of the xanthophylls within a leaf at any time can be expressed as the epoxidation state (i.e. the proportion of the xanthophylls that are epoxidated), which varies between zero and one.

With these assumptions and under constant, high light conditions, epoxidation state should vary sigmoidally between zero and one with increasing photosynthetic rates. In other words, as photosynthesis increases and excess light decreases, epoxidation state should increase to an upper asymptote of one, or

conversely, as the amount of excess light increases due to decreases in photosynthesis, the epoxidation state should decrease to an asymptote of zero. Thus, a logistic (sigmoidal shaped) curve was chosen to represent the relationship between photosynthesis and epoxidation state as it is a curve that can be constrained between zero and one.

In this experiment light levels varied between 750 and 2300 μmol.quanta.m^{-2}.s^{-1} and leaf temperatures ranged between 34 and 43°C (Figure 3.1). Over this range of leaf temperatures and solar radiation levels, there was a significant ($t_{(67)}$= 5.329, $p<0.001$) relationship between photosynthesis and epoxidation state described by the logistic curve:

$$EPOX = 1 / (1 + exp(a + b\,PS)). \qquad (1)$$

Parameter values, standard errors of the parameters and R^2 values are shown in Table 3.1. Parameter *a* defines the location of the curve on the axis, while parameter *b* influences the slope of the curve.

Data for different species are more common in some regions along the curve than others (Figure 3.1). *B.gymnorrhiza* fell mainly on the lower part of the curve where epoxidation state is close to zero, *R.stylosa* fell mainly on the upper part of the curve where epoxidation state is close to one, and *B.parviflora* tended to lie along the steeper region in the middle of the curve.

When the level of incident solar radiation was included in the above model (see equation 2) it also had a significant effect on epoxidation state ($t_{(66)}$=3.09, p=0.003). Parameter values, standard errors of the parameters and R^2 values are shown in Table 3.2. By including the effect of solar radiation in the model, the

proportion of the variation in the data explained by the model increased by approximately 10% (i.e. $R^2 = 0.545$ compared to $R^2 = 0.435$).

Table 3.1. Parameter estimates, their standard errors and their associated t and p values, of the model [*EPOX = 1 / (1 + exp(a + b PS)*] that describes the relationship between epoxidation state (*EPOX*) and photosynthetic rate (*PS*) for mangroves of the family Rhizophoraceae at two sites in north Queensland, Australia in which solar radiation levels were greater than 750 µmol quanta.$m^{-2}.s^{-1}$.

Parameter	Parameter estimate	Standard error	t(67)	p
a	2.5295	0.4881	5.182	<0.001
b	-0.3704	0.0695	5.329	<0.001

$R^2 = 0.435$

Table 3.2. Parameter estimates, their standard errors and their associated t and p values, of the model [*EPOX = 1 / (1 + exp(a + b PS + c QN)*] that describes the relationship between epoxidation state (*EPOX*), photosynthetic rate (*PS*) and solar radiation (*QN*) for mangroves of the family Rhizophoraceae at two sites in north Queensland, Australia in which solar radiation levels were greater than 750 µmol quanta.$m^{-2}.s^{-1}$.

Parameter	Parameter estimate	Standard error	t(66)	p
a	0.6037	0.7319	0.825	0.414
b	-0.3428	0.0647	5.298	<0.001
c	0.001086	0.000351	3.091	0.003

$R^2 = 0.545$

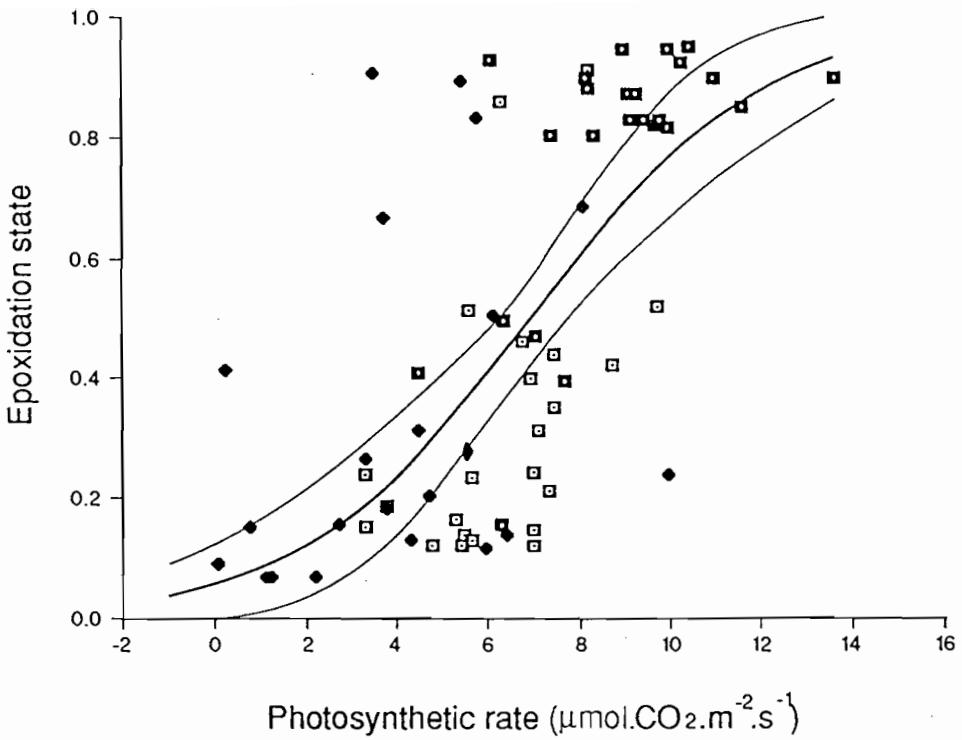


Figure 3.1. Variation in epoxidation state with photosynthesis for three mangrove species (*R.stylosa* (solid squares), *B.gymnorrhiza* (diamonds), and *B.parviflora* (open squares)). Leaf temperatures ranged between 34 and 43°C, and incident solar radiation levels between 750 and 2300 µmol quanta.$m^{-2}.s^{-1}$. The fitted line is described by the equation: *EPOX = 1 / (1 + exp(2.5295 - 0.3704 * PS))*, R^2=0.435 (see Table 3.1). The finer lines represent the 95% confidence intervals.

The fitted model (Table 3.2) therefore shows that as photosynthesis increases epoxidation state increases, and conversely as solar radiation increases, epoxidation state decreases. The t-values give some indication of the strength of each variable in determining the epoxidation state. In this case (where solar radiation was greater than 750 μmol quanta.$m^{-2}.s^{-1}$), changes in photosynthesis accounted for a greater proportion of the variation in epoxidation state than did changes in the photon flux density.

3.3.2 Relationship between epoxidation state, photosynthesis, solar radiation and leaf temperature

The relationship between epoxidation and photosynthesis, solar radiation and leaf temperature was assessed by fitting a similar model to that described in section 3.3.1 (i.e. a logistic curve bound between zero and one), using photosynthesis, leaf temperature and solar radiation as explanatory variables (equation 3). Variables were dropped from the initial model (equation 3) until all remaining ones were statistically significant ($p<0.05$).

Photosynthetic rate and solar radiation levels had a significant ($p<0.001$) effect in determining the epoxidation state of leaves, explaining 28.3% of the variation in the data (Table 3.3). The effect of leaf temperature was not significant ($p>0.05$) in directly determining epoxidation state and was dropped from the model. However, leaf temperature did have a significant effect on photosynthesis (see section 3.3.3 below). The final model was:

$$EPOX = 1 / (1 + exp(a + b\,PS + c\,QN)). \qquad (6)$$

Parameter values, standard errors and R^2 values are shown in Table 3.3.

The fitted model (Table 3.3) shows a similar result to that obtained in 3.3.1, that is, as solar radiation increases and photosynthetic rate decreases, epoxidation state decreases. However, under the wide range of conditions over which this data set was collected, solar radiation and photosynthetic rates explained a similar proportion of the variation in epoxidation state. In order to represent this three variable (and hence three dimensional relationship) a linearized version of the final model (equation 6) was obtained through rearranging equation 6 in terms of *PS* and *QN* and then taking natural logarithms. That is:

$$LEP = ln\,[EPOX / (\,1 - EPOX\,)] = -(\,b\,PS + c\,QN\,),$$

thus, *LEP* is a linear function of *QN* and *PS*. The surface shown in Figure 3.2 is the response of *LEP* to variations in solar radiation and photosynthesis. This surface indicates that epoxidation state is at its highest where photosynthesis is high and radiation levels are low, and at its lowest where solar radiation levels are high and photosynthetic rates are low. As *LEP* is a linear transformation of epoxidation state, the flat surface in Figure 3.2 represents a surface of sigmoidally shaped curves that follow the same pattern with respect to variations in photosynthetic rates and solar radiation levels, and are bound between zero and one.

Table 3.3. Parameter estimates and their standard errors of a model [$EPOX = 1 / (1 + exp(a + b\ QN + c\ PS))$] to describe the relationship between solar radiation, photosynthetic rate and epoxidation state for mangroves of family Rhizophoraceae over a wide range of environments.

Parameter	Parameter estimate	Standard error	t(166)	p
a	0.1241	0.1752	0.708	0.487
b	-0.1417	0.0167	8.505	<0.001
c	0.00082	0.00010	8.200	<0.001

$R^2 = 0.283$

3.3.3 Effect of leaf temperature and solar radiation on photosynthesis

A family of leaf light response curves, approximated by exponential functions, were used to describe photosynthetic response to solar radiation at different leaf temperatures. The quadratic term initially specified in the model ($a + bTL + cTL^2$, equation 2) represents the asymptote of the light response curves (i.e. the light saturated photosynthetic rate), and was chosen because it closely resembles the reported variation in photosynthesis with leaf temperature (Berry and Björkman, 1980). The TL^2 term was found to be non-significant ($p>0.05$) accounting for only a small proportion of the variation in the data and was subsequently removed. The final model, where all terms within the model were significant, was:

$$PS = (a + b\ TL) (1 - exp(d\ QN)). \qquad (7)$$

Parameter estimates and the coefficient of determination (R^2) are shown in Table 3.3. Parameters *a* and *b* determine the asymptote of the curves (i.e. the light saturated photosynthetic rate), while parameter *d* describes the rate of change of photosynthesis with increasing solar radiation levels.

Figure 3.3 illustrates the predicted photosynthetic light response curves (solid lines) at three leaf temperatures superimposed on the data for those leaf temperatures. As leaf temperature increases the asymptote of the curve decreases.

Table 3.4. Parameter estimates, their standard errors and associated t and p values, of the model [$PS = (a + b\,TL) (1 - exp(d\,QN))$], that describes the relationship between solar radiation (*QN*), leaf temperature (*TL*=leaf temperature-35°C) and photosynthetic rate (*PS*) for mangroves of the family Rhizophoraceae growing over a wide range of environments.

Parameter	Parameter estimate	Standard error	t(166)	p
a	14.44	3.794	3.803	<0.001
b	-1.806	0.5552	3.251	0.002
d	-0.00066	0.00026	2.538	0.012

$R^2 = 0.471$

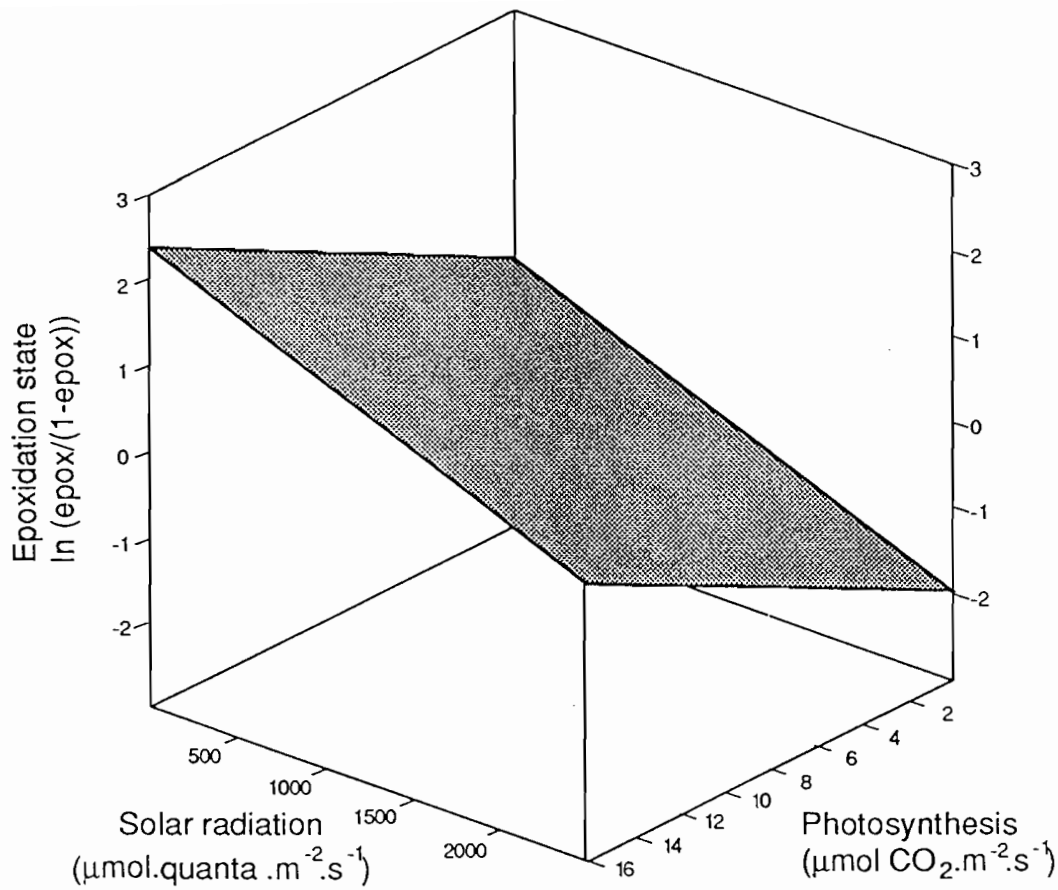


Figure 3.2. The linearized response surface of epoxidation state (see text) to photosynthesis and incident solar radiation in mangrove leaves of four species from the family Rhizophoraceae, collected in north Queensland over a wide range of environments. See Table 3.3 for a description of the model which the response surface represents.

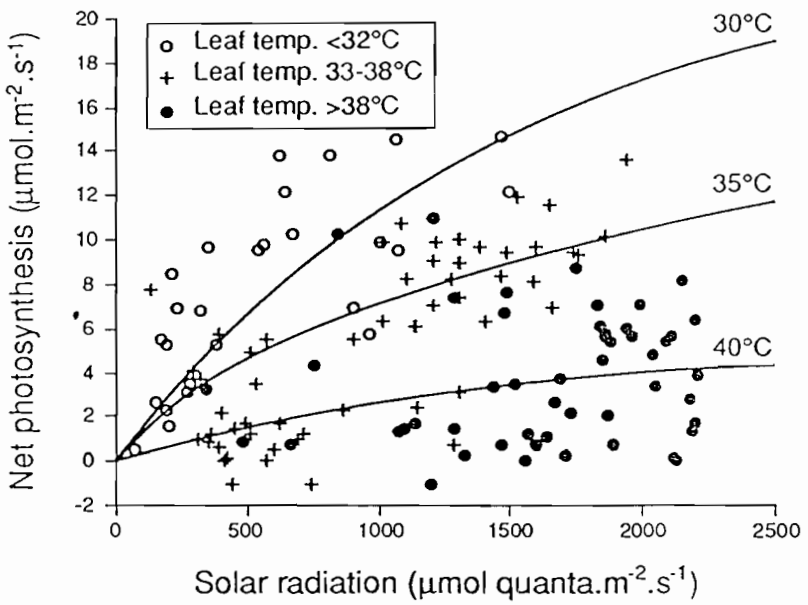


Figure 3.3. Comparison between the statistical model to describe the response of photosynthesis to solar radiation over varying leaf temperatures (see Table 3.3 for a description of the model) and data collected for four mangrove species from the family Rhizophoraceae over similar temperature ranges.

3.3.4 Leaf temperature and solar radiation

Leaf temperature increased significantly with increasing solar radiation intensity incident on leaves (Figure 3.4). The response of leaf temperature to solar radiation was described by equation 5 (section 3.2.3). Parameter estimates, standard errors and the correlation coefficient for the model are shown in Table 3.4.

Table 3.5. Parameter estimates, their standard errors and associated t and p values, of the statistical model [$TL = a + b\,QN + c\,QN^2$] that describes the relationship between leaf temperature (*TL*) and solar radiation (*QN*) for mangroves of the family Rhizophoraceae.

Parameter	Parameter estimate	Standard error	t(166)	p
a	28.602	0.642	44.55	<0.001
b	0.00956	0.00135	7.090	<0.001
c	$-2.115*10^{-6}$	$0.593*10^{-6}$	3.620	<0.001

$r^2 = 0.575$

3.3.5 The hierarchial model

A hierarchial model (Figure 3.5) was established from the above analyses. Figure 3.5 summarizes the control of epoxidation state by photosynthesis, solar radiation and leaf temperature in mangroves. Solar radiation had a direct effect on leaf temperature, photosynthetic rate and epoxidation state (section 3.3.3). Leaf temperature directly influenced photosynthetic rate, but had no direct influence on epoxidation state, while photosynthesis influenced epoxidation state directly.

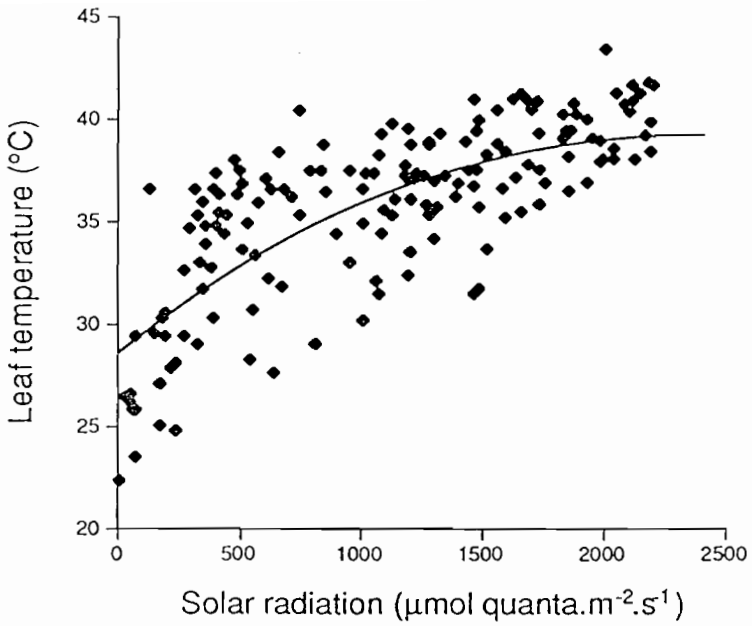


Figure 3.4. The relationship between incident solar radiation and leaf temperature of mangrove leaves from four species from the family Rhizophoraceae. See Table 3.4 for description of the fitted line.

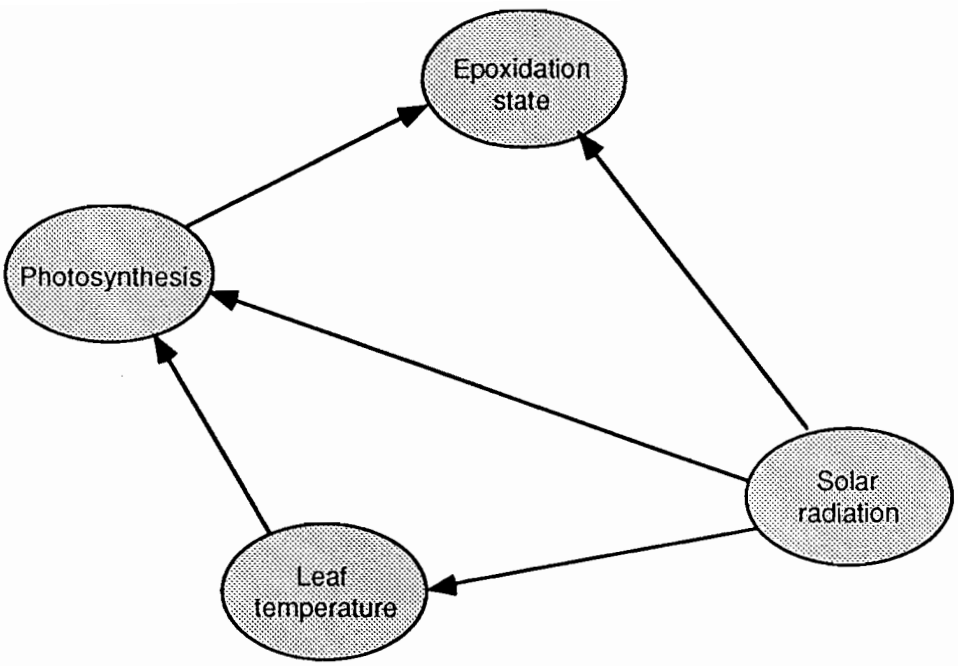


Figure 3.5. Hierarchial model to describe the response of leaf epoxidation state to incident solar radiation, leaf temperature and photosynthetic rate. Arrows represent statistically significant ($p<0.05$) relationships between variables.

3.4 Discussion

Within the framework of the assumptions made (see section 3.3.1) the relationship between photosynthesis and epoxidation state under high light conditions (Fig. 3.1, Table 3.1), and the relationship between photosynthesis, solar radiation and epoxidation state (Fig. 3.2, Table 3.2) is consistent with the idea that the formation of zeaxanthin responds to light absorbed by leaves that is not utilized in photosynthesis, which can be due to decreases in photosynthetic rates (Fig. 3.1), or increases in solar radiation levels (Table 3.2), or both (Fig. 3.2). Thus, increases in zeaxanthin contents observed in the experiments described here are consistent with the proposed protective, energy-dissipating role of zeaxanthin (Demmig *et al.*, 1987).

The analyses were also based on the assumption that solar radiation, photosynthesis and leaf temperature influenced epoxidation state, and that there was no effect (in the short term) of epoxidation state on any of these variables. In other words, there was no feedback control of photosynthesis, solar radiation and leaf temperature by epoxidation state. The analyses done in this chapter cannot determine whether this is true. Thus, although control of epoxidation state over incident solar radiation and leaf temperature would seem unlikely, this may not be the case for photosynthesis. Therefore, the relationship between photosynthesis and epoxidation state could be viewed as the response of epoxidation state to photosynthesis, or the response of photosynthesis to epoxidation state, or both.

In sun leaves under conditions where solar radiation levels were greater than 750 μmol quanta.m^{-2}.s^{-1}, decreases in photosynthesis accounted for a greater proportion of the variation in epoxidation state than increases in solar radiation incident on leaves (compare t-values in Table 3.2). It is known that

photosynthesis is protective against photoinhibition in a way that is not quantitatively linked to the light energy that is used in photosynthesis (Krause and Cornic, 1987). From the results presented here this also may be the case for mangroves, as increases in solar radiation had little effect on zeaxanthin contents, and presumably on the dissipation of excess energy associated with it, unless photosynthetic rates were reduced.

Decreases in photosynthetic rates are associated with a build up of the ΔpH between the lumen and the stroma. This activates violaxanthin de-epoxidase (Yamamoto, 1979) and may also enhance the dissipation of excess energy, either through zeaxanthin (Demmig-Adams *et al.*, 1989; Morales *et al.*, 1990; Bilger and Björkman, 1991), or through state transitions (Horton and Lee, 1985), or through the conversion of PS II reaction centres to energy dissipating forms with low photochemical yields (Weis and Berry, 1987). Results of other studies have found that conditions which limit photosynthesis (for example, water deficits, low leaf temperatures and nutrient deficiencies) increase the extent of photoinhibition (see review by Powles, 1984 and section 1.1.2, page 3), perhaps by increasing the amount of energy to be dissipated beyond the combined capacity of all energy dissipating processes. The dependence of the level of zeaxanthin within leaves on photosynthetic rates under high light conditions in mangroves is in agreement with studies that have shown photoinhibition is more sensitive to decreases in photosynthesis under high light conditions than to increases in light levels alone.

However, in leaves collected from four species, in both sun and shade environments and at varied times of the day, photosynthetic rate and solar radiation were of nearly equal significance in explaining the variability in the data (Table 3.3). The low R^2 of the model (Table 3.3) may be due to the data not being corrected for the maximum photosynthetic rate of each leaf, which is important in

determining the level of excess light a leaf may be absorbing, and also could be due to the influence of other energy dissipating processes that may be ocurring (see section 1.2.3, page 7). It is also evident from the results that solar radiation had a large effect on photosynthesis both directly (Fig. 3.3), and indirectly, through its effect on leaf temperature (Fig. 3.4). Thus, incident solar radiation is probably the most important variable included in this model in influencing the amount of zeaxanthin in leaves over a wide range of conditions. This supports the results of Chapter 2 which found that the level of xanthophylls within mangrove leaves was correlated with the light regime under which leaves grow (Fig. 2.2, page 32).

Leaf temperature did not affect epoxidation state directly but exerted its influence through its effect on photosynthetic rates (Fig. 3.5). Adams *et al.* (1990) found that the rate constant for non-radiative energy dissipation associated with the presence of zeaxanthin was lower at leaf temperatures optimal for photosynthesis than at leaf temperatures above, and below the temperature optimum for photosynthesis. Bilger and Björkman (1991) found that although the activity of violaxanthin de-epoxidase (the enzyme that converts violaxanthin to zeaxanthin) was optimal at temperatures similar to the temperature optimum for photosynthesis, steady state levels of zeaxanthin where higher at leaf temperatures below that optimal for photosynthesis. Both these reports are consistent with the finding that lower epoxidation states (i.e. high zeaxanthin concentrations) occurred in mangroves when photosynthetic rates were low and leaf temperatures were high.

Although leaf temperature did not have a direct effect on the epoxidation state in these experiments (Fig. 3.5), it did have a significant effect on photosynthetic rates of mangrove species (Fig. 3.3, Table 3.3). Ball *et al.* (1988)

demonstrated the importance of leaf temperature in optimizing photosynthesis and minimizing water loss in mangroves, while Clough and Sim (1990) have shown that high leaf to air vapour pressure deficit, which is largely influenced by leaf temperature, can limit photosynthetic rates in mangroves over a wide range of salinities. The importance of leaf temperature in this analysis reiterates the theme of the previous Chapter (section 2.4, page 39) showing that, for leaves exposed to full sunlight, the maintenance of leaf temperature within a range in which photosynthesis can continue is paramount in the acclimation of mangroves to their environment.

The sigmoidal shape of the photosynthesis-epoxidation state relationship found in this experiment (Fig. 3.1) shows a region where low epoxidation states are associated with low photosynthetic rates. That is, there is almost complete de-epoxidation of violaxanthin to zeaxanthin under high light conditions when photosynthesis approaches zero. *B.gymnorrhiza* is the most common species in this region of the photosynthesis-epoxidation curve (Fig. 3.1). This species was found in low abundance at the top of mixed forest canopies (Table 2.3). Therefore, it may be possible that the zeaxanthin-related dissipation of excess energy can be exceeded in this species, resulting in photoinhibition and repair that may reduce the ability of *B.gymnorrhiza* to compete with other species for exposed positions at the top of the canopy. However, *B.gymnorrhiza* is dominant in some mangrove forests. Therefore it is unlikely that the ability to avoid photoinhibition is the only factor influencing its distribution in mangrove areas (see discussion section 2.4, page 39, and a review by Ball, 1988).

The occurrence of energy dissipating processes other than through zeaxanthin may also reduce the likelihood of photoinhibition in mangroves which are absorbing high levels of solar radiation when photosynthetic rates are low.

The contribution of energy dissipating PS II centres, photorespiration, or the Mehler reaction, to the dissipation of excess light energy have not been determined in mangrove species, and may be important, either during, or when the capacity of xanthophyll protection is exceeded.

The level of zeaxanthin in mangrove leaves responds to solar radiation, photosynthesis, and leaf temperature in a way that is consistent with its proposed involvement in dissipating excess light. However, this result, like the results of other researchers (for example, Demmig-Adams *et al.* 1989*a*) is based on correlations without a clear understanding of the mechanisms by which zeaxanthin dissipates energy. Examining the dissipation of excess energy when the majority of xanthophylls are present as zeaxanthin may clarify the limits of zeaxanthin-related prevention of photoinhibition, and the consequences of the protective capacity of zeaxanthin being exceeded.

CHAPTER 4

Distribution and accumulation of UV-absorbing compounds

4.1 Introduction

The previous Chapters were concerned with protection from high levels of visible light. In addition to high levels of visible light, tropical mangroves receive high levels of UV radiation relative to plants inhabiting temperate regions (Frederick *et al.*, 1989). The depletion of the atmospheric ozone layer and the expected associated increases in UV radiation at the earths surface (Caldwell *et al.*, 1989) may also increase the flux of UV radiation in mangrove environments. Thus, this study is directed towards assessing the mechanisms which may protect mangroves from UV radiation.

UV radiation has been shown to be destructive to plants. Exposure of plants to enhanced UV-B radiation has been found to inhibit plant growth and depress photosynthesis (see reviews by Caldwell, 1981; Tevini and Teramura 1989; section 1.3). Reduced photosynthesis under enhanced UV-B radiation has been attributed to stomatal closure (Teramura *et al.*, 1983; Negash 1987), and to UV-B effects on protein synthesis and denaturation (Strid *et al.*, 1990). It has been shown that UV-absorbing compounds (usually phenolic compounds) form a protective, UV radiation screen within the leaf that could protect the chloroplasts and other UV-sensitive molecules from damaging UV-B radiation (Caldwell *et al*, 1983; Robberecht and Caldwell, 1978; Les and Sheridan, 1990).

Increases in leaf thickness which result in increased light attenuation through leaves, have also been proposed to be protective against UV radiation (Warner and Caldwell, 1983). More recently, Murali *et al.* (1988) found that tolerance to UV-B radiation in different soybean genotypes was correlated with leaf thickness, and the concentration and composition of UV-absorbing pigments in their leaves.

Mangrove species have high concentrations of phenolic compounds in their leaves (Robertson, 1988) and often have thick, succulent leaves (Ball *et al.* 1988, pers. field observation). In the past most of the studies of phenolics in mangrove leaves have been done by ecologists with an interest in the effects of tannins on consumption of leaf litter by crabs (e.g. Poovachiranon *et al.*, 1986). Very little consideration has been given to the possibility of a physiological role.

The aim of the experiments described here was to: 1) investigate phenolic compounds and leaf succulence in mangroves with a view to assessing their likely involvement in providing protection from UV radiation, 2) determine whether there are species differences in the accumulation of phenolic compounds in mangrove leaves, and 3) assess whether the light regimes under which leaves are grown, site, or nitrogen nutrition correlate with leaf succulence and phenolic contents of mangrove leaves.

4.2 Materials and Methods

4.2.1 Distribution of phenolic compounds in mangrove leaves

Sun and shade leaves of *B.parviflora*, *B.gymnorrhiza*, *R.stylosa* and *Xylocarpus granatum* were collected at the mouth of the Daintree River (Lat. 16°S, 146°E). These were stained using a saturated solution of ferrous sulphate in water with 5% formaldehyde, which selectively stains phenolic compounds (Johansen, 1940). Leaf material was left in the stain for 48 hours before being washed and stored in 70% ethanol. Leaf material was imbedded in paraffin before sectioning. Sections were cut 12 μm thick, mounted and photographed.

4.2.2 UV absorbance of fresh epidermal pieces

The epidermal absorption spectra of leaves was assessed in the mangrove species, *B.parviflora*, *B.gymnorrhiza* and *R.stylosa* which were growing in 100% seawater in natural sunlight at the Australian Institute of Marine Science (Lat. 19°S, Long. 147°E). Six epidermal pieces were peeled, using a scalpel, from the leaves of each species. These were mounted in a small holder and clamped in a quartz spectrophotometer cuvette to ensure that the epidermal pieces did not move during measurement of absorbance. An initial measurement of the absorption spectrum from 250 to 750 nm in distilled water using a distilled water blank was made using an Hitachi scanning spectrophotometer (model U-3200, Hitachi, Japan). Absorbance was integrated between the following wavelengths; 280-320 nm (UV-B), 320-400 nm (UV-A) and 400-750 nm (visible). Epidermal pieces were then washed in 100% methanol for two hours while still in the cuvette to remove UV-absorbing compounds (Caldwell *et al.*, 1983; Robberecht and Caldwell, 1978). During this period the methanol was changed half hourly and

stored. The four half hourly extracts for each epidermal peel were combined and the absorption spectrum of the extracted pigment was measured. After the two hour extraction the epidermal pieces were then rescanned in distilled water. The difference between the initial and final integrated values represents the amount of pigment removed. Values are expressed as a proportion of the initial absorbance, as epidermal thickness and the exact area of epidermis placed in the light path was not measured.

Using a spectrophotometer to measure the spectral qualities of the epidermis of leaves is less than ideal because it does not account for light reflected by the epidermis (Robberecht and Caldwell, 1978). The preferred equipment, consisting of an integrating sphere attached to a monochromator via a fibre optic cable (Robberecht and Caldwell, 1978) was not available for use in the experiments described in this Chapter. Hence, the epidermal absorption characteristics presented here are not absolute. However, they are adequate to allow comparisons between the three species investigated here.

4.2.3 Effect of sun, shade, site, and species on the phenolic contents of leaves

Leaves from sun and shade environments of mangrove species *B.parviflora, B.gymnorrhiza, C.tagal, R.stylosa, L.racemosa* and *X.granatum* were collected at four sites, three in the Daintree River and one in Bowling Green Bay close to the Australian Institute of Marine Science (Table 4.1). All six species were not present at each site. At each site two trees of each species present were selected and five replicate leaves from both sun and shade conditions were collected. Leaves were weighed, their area measured and then dried for seven days at 70°C. The dried leaves were ground to a fine powder in an agate ball mill

(KHD Humboldt Wedag, Germany), and 0.1g of the dried powder was assayed for phenolic content using the Folin-Denis colormetric assay for soluble phenolics (Allen *et al.*, 1974), with improvements recommended by Mole and Waterman (1987). Tannic acid was used as a standard. A preliminary test of variance within the trees sampled (i.e. between the 5 leaves of any tree) showed that variation between individual leaves from any one tree, in the sun or shade, was small. Therefore, the five replicate leaves for each tree were bulked for analysis. Leaf succulence was calculated as: (leaf fresh weight-leaf dry weight)/leaf area.

Table 4.1. Distribution of mangrove species at selected sites in north Queensland.

Site	Location and site description	Species present
1	Daintree River mouth, South -High rainfall (>2m.yr^{-1}) -Substrate salinity: approx. 350 mmol. NaCl	*Rhizophora stylosa* *Bruguiera parviflora* *B. gymnorrhiza* *Xylocarpus granatum*
2	Daintree River, 6km up-river -High rainfall (>2m.yr^{-1}) -Substrate salinity: 100-200 mmol. NaCl	*B. gymnorrhiza* *B. parviflora* *X. granatum*
3	Daintree River mouth creek North side -High rainfall (>2m.yr^{-1}) -Substrate salinity: approx. 350 mmol. NaCl	 *R. stylosa* *B. gymnorrhiza* *X. granatum*
4	Bowling Green Bay North -Low rainfall (1-1.4 m.yr^{-1}) -Substrate salinity: >350 mmol. NaCl	*R. stylosa* *Ceriops tagal* *Lumnitzera racemosa*

4.2.4 Nitrogen nutrition and phenolic accumulation

Seedlings of *R.stylosa* were grown for 18 months in nutrient culture in the shadehouse at the Australian Institute of Marine Science. Nutrient solutions were adjusted to 25% seawater (Appendix II, page 130). Nitrogen was supplied

as both ammonium and nitrate to make up nitrogen levels to 0, 5, 10, 20 or 180 ppm. Maximum visible light levels within the shadehouse were approximately 1000 μmol.quanta.m^{-2}.s^{-1}.

A leaf from the second leaf pair on the primary shoot of each of the four plants grown at each nitrogen level was harvested, weighed, and its area measured (Li-Cor planimeter, Li-Cor, Lincoln, Nebraska, USA). A 10 cm^2 disc was then punched from the leaf and used to determine the apparent quantum yield of photosynthesis, and photosynthetic rate at 160 μmol quanta.m^{-2}.s^{-1} on an incident light basis. This was done in a Hansetech Leaf Disc Electrode system (Hansetech, Kings Lyn, UK) illuminated with a tungsten halogen projector lamp (Sylvania, Japan) from which the infrared component was removed by a Schott infrared filter (Schott, Germany) and passed through a Schott cold mirror. Neutral density filters (Schott) were used to adjust the light levels. CO_2 was supplied to the leaf disc by plumbing a humidified stream of 5% CO_2 in air (prepared by CIG, Townsville, Australia), at a flow of 5 ml.min^{-1} (see Björkman and Demmig, 1987), into the chamber. Prior to measurements the instrument was zeroed using a stream of nitrogen gas, and calibrated by injecting 1ml of air from a gas tight syringe into the closed chamber. Before measurements began on a leaf disc, the leaf disc was exposed to 20 minutes at 160 μmol quanta.m^{-2}.s^{-1}. It was then placed in the dark to measure a dark respiration rate. Light levels were then increased in 5 steps to 70 μmol quanta.m^{-2}.s^{-1} with a final measurement at 160 μmol quanta.m^{-2}.s^{-1}. The chamber was closed and a measurement of the O_2 evolution made once a stable rate of O_2 evolution was reached at each new light level. The chamber was then reopened and flushed with the stream of 5% CO_2 in air until the O_2 concentration in the chamber had returned to normal (usually not longer than two minutes). The leaf disc was then dried at 70°C for one week, ground and assayed for soluble phenolics (Folin-Denis colormetric assay, see

4.2.3 above). 0.1g of the ground tissue was also used to determine carbon, nitrogen and hydrogen contents (CHN-600 Determinator, Leco Corp., USA).

4.2.5 Statistical analysis

All statistical analysis was done using the computing package STATISTIX (NH Analytical Software, Roseville, MN, USA). The proportion of UV-absorbing compounds leached from mangrove leaves of different species (4.2.2) was assessed using a one-way analysis of variance. Significance was assessed using an F-ratio test at p=0.05. The least significant difference (LSD), defined as the standard error of the difference between two means multiplied by the critical t-value (i.e. $LSD=t_{crit}*(s/\sqrt{2n})$), was used for comparison between means.

Comparison of phenolic compounds, and leaf succulence between species and sun/shade was assessed using analysis of variance on a site by site basis due to the unbalanced design (not all species appear at every site). Species and sun/shade were represented as crossed factors, with trees nested within the crossing of species and sun/shade factors. Sun/shade and species were considered fixed effects and trees as a random effect. Comparison between sites and sun/shade used data for *R.stylosa* over sites 1, 3 and 4. The design was the same as for species and sun/shade comparisons, except site replaces the species as one of the fixed effects. Significance of effects was assessed using an F-ratio test at p=0.05. LSD was calculated as described above.

The relationships between leaf nitrogen and each of phenolic contents, quantum yield and leaf specific weight (Figure 4.6) were determined using linear regressions, as was the relationship between specific weights of leaves and their soluble phenolic contents (Figure 4.7). Significance of the independent variable

(leaf nitrogen content or specific weight of leaves) was assessed using a t-test at a significance level p=0.05.

4.3 Results

4.3.1 Distribution of phenolic compounds within mangrove leaves

In the four species considered, phenolic compounds were present as a continuous band in the epidermis of the upper surface of the leaf (Plate 4.1). Phenolics are present in the outer epidermal layer of *B.parviflora* (panel 4.1a), *B.gymnorrhiza* (panel 4.1b) and *X.granatum* (panel 4.1d). In *R.stylosa* phenolics were not present in the upper hypodermis but in the cell layer directly below this (panel 4.1c.). In all species the cuticle did not stain, however its waxy nature may have prevented penetration of the stain. In all four species the lower epidermis contained phenolics. Stained cells were present throughout the spongy and palisade mesophyll of all four species. *X.granatum* displayed the least dense staining of the four species and this matched its lower phenolic content in the soluble phenolics assay (see 4.3.2 below). Shade leaves were less densely stained than sun leaves of all species (Plate 4.2 shows this for *B.parviflora*).

Succulence differed between species. Large cells were evident below the second epidermal layer and throughout the mesophyll of both *R.stylosa* and *X.granatum* (Figure 4.1c and d). In *R.stylosa* these were particularly well developed and irregularly shaped compared to the more regular arrangement in *X.granatum*.

4.3.2 Epidermal absorption characteristics

In fresh epidermal peels of the three mangrove species used in this experiment, absorbance in the UV range was approximately 30% higher compared to that in the visible portion of the absorption spectra. The epidermal peels of all species contained UV-absorbing pigments that could be leached from the tissue with methanol. Figure 4.1(a) shows an example of an epidermal absorption spectra before and after leaching in methanol. Epidermal absorbance decreased with a decrease in the energy of the light wavelength (Figure 4.1b). After washing in methanol for two hours the average proportion of the absorbance at 310 nm removed was 22.27% in *R.stylosa*, 15.27% in *B.parviflora* and 11.62% in *B.gymnorrhiza* (Figure 4.2). *R.stylosa* had a significantly larger ($F_{(2,17)}$=9.30, p=0.0024) proportion of UV absorbance that could be removed by methanol than *B.gymnorrhiza* or *B.parviflora*. The difference between the UV absorbance of the fresh epidermis (30%) and the extractable absorbance is probably due to either the cell structure or pigments remaining in the cells or cuticle (insoluble in methanol). The absorption spectrum of the methanol extract (Figure 4.3) was the same for all three species and resembled a flavonoid compound (Swain, 1976) with peaks at 348 nm and 268 nm.

4.3.3 Sun, shade, and site comparisons

Significant differences ($p<0.05$) in both soluble phenolic contents on a leaf area basis and in leaf succulence were evident between species at three out of the four sites (Figure 4.4 and 4.5, Table 4.2 and 4.3). Sun leaves were generally more succulent than shade leaves over all sites, but this was not statistically significant ($p>0.05$) at any site (Figure 4.5, Table 4.3).

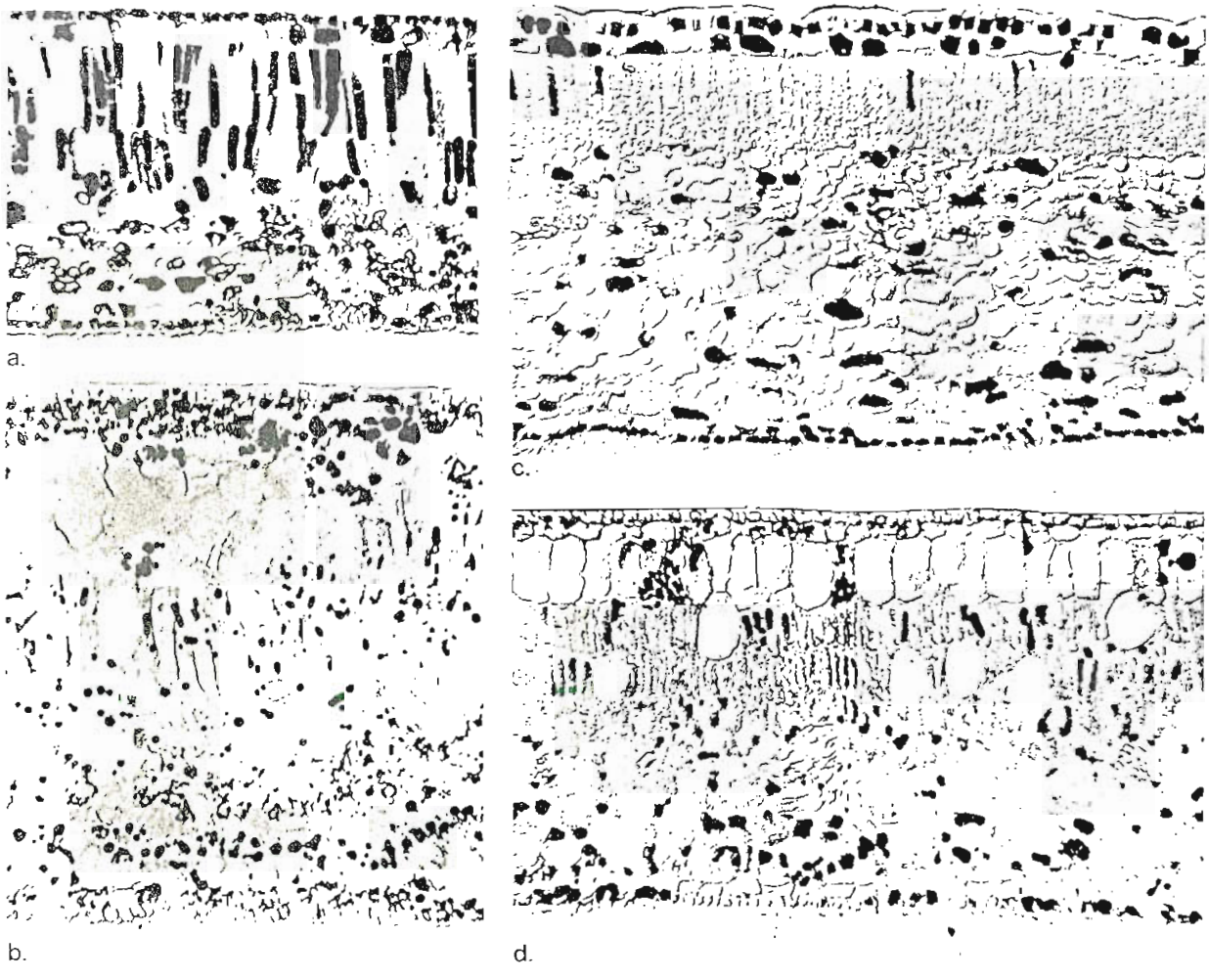
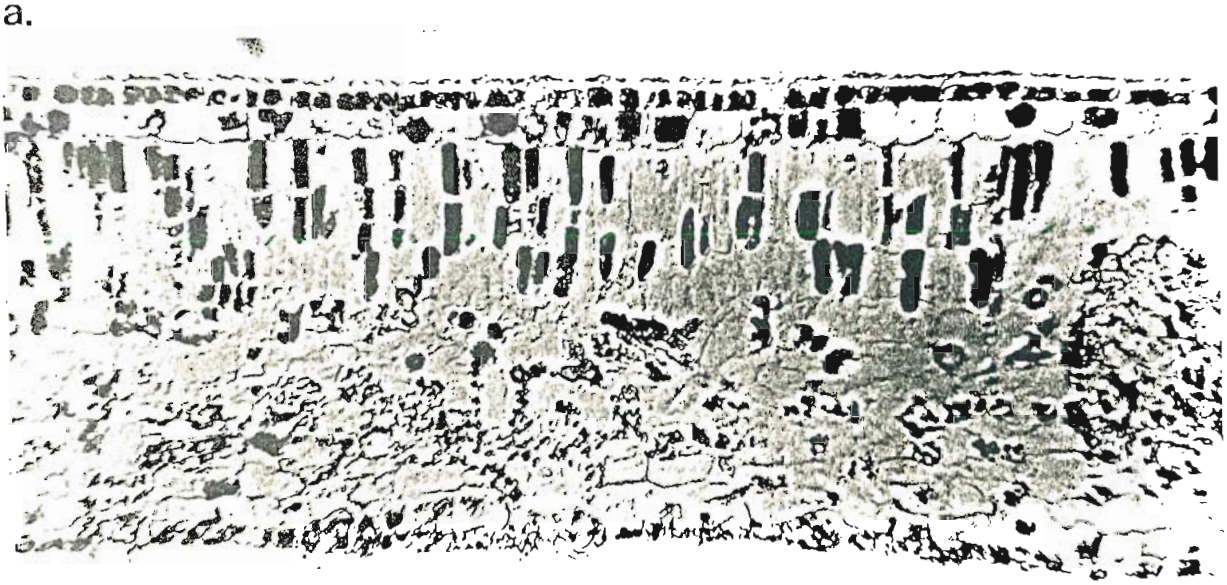


Plate 4.1. Transverse sections of leaves of the mangrove species *Bruguiera parviflora* (a), *B.gymnorrhiza* (b), *Rhizophora stylosa* (c) and *Xylocarpus granatum* (d) that were stained for phenolic compounds.

a.



b.

Plate 4.2. Transverse section of leaves of sun (a) and shade (b) leaves of *Bruguiera parviflora* that were stained for soluble phenolic compounds.

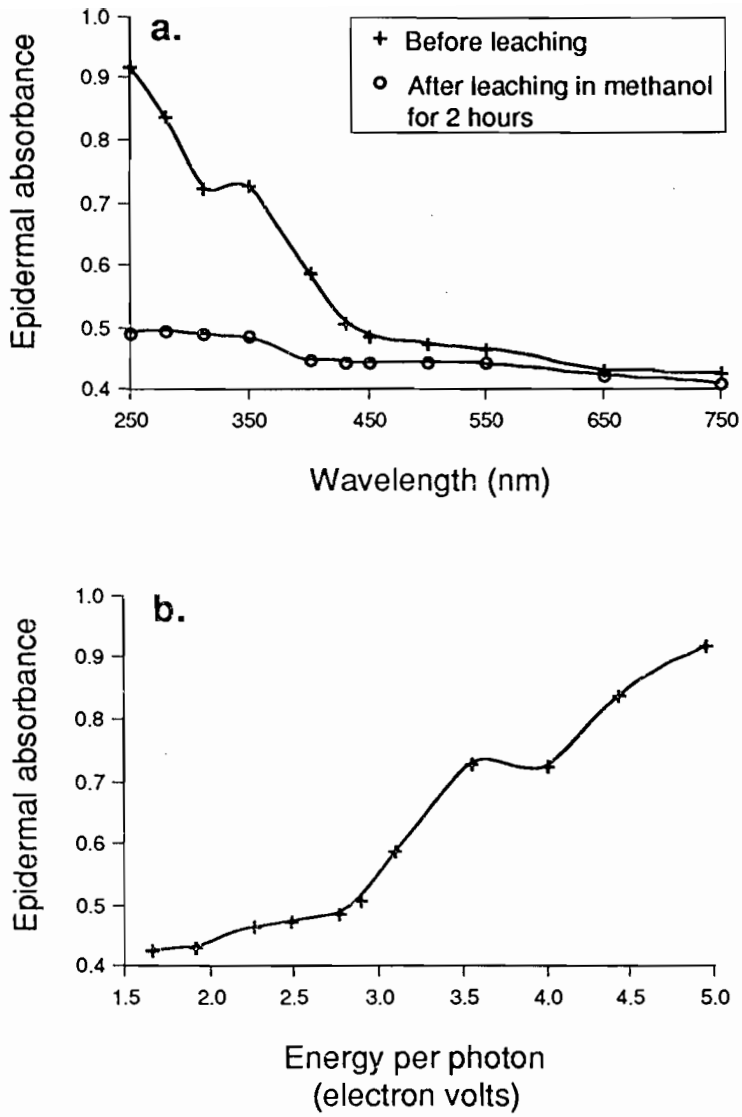


Figure 4.1. Absorbance spectra of an epidermal peel of *Rhizophora stylosa*, (a) before and after leaching the tissue in methanol for two hours, and (b) as a function of the energy per photon.

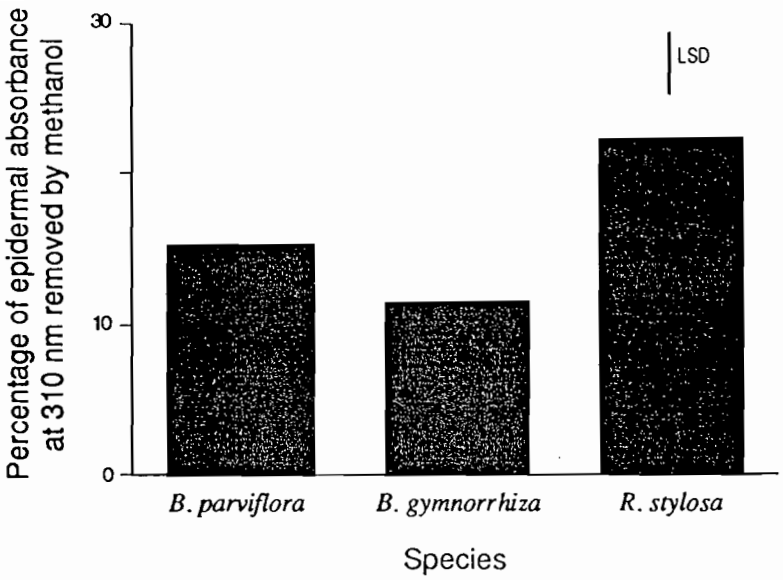


Figure 4.2. Percentage of the epidermal absorbance at 310 nm removed by leaching in methanol for three mangrove species. LSD is the least significant difference ($p<0.05$) between any two means.

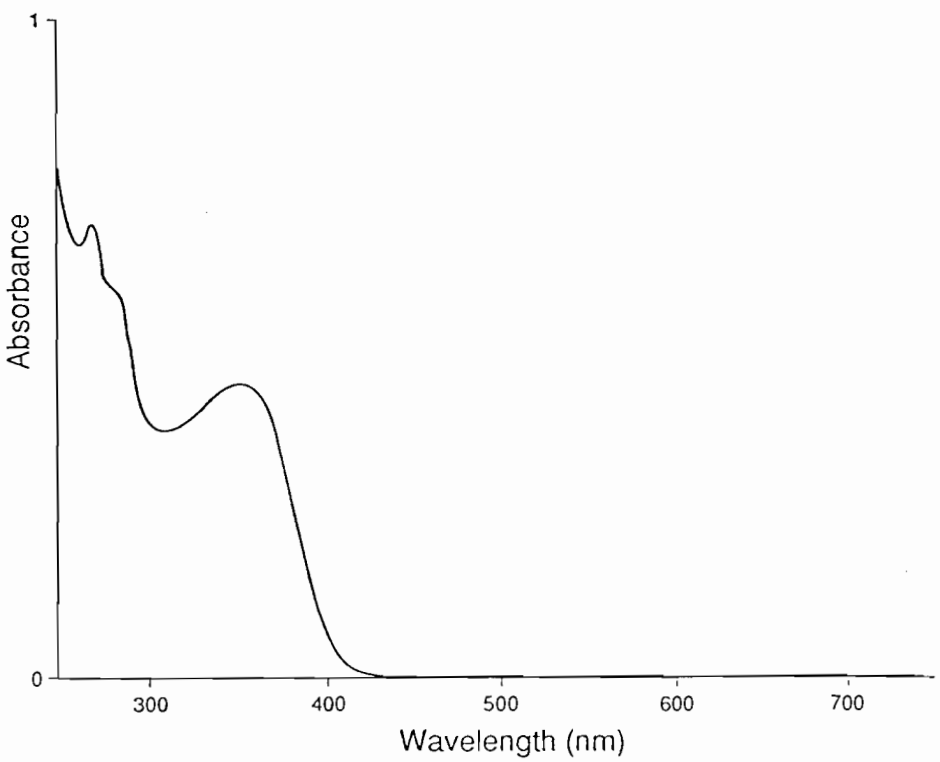


Figure 4.3. Absorbance spectra of the methanol extract obtained from leaching the epidermis of mangrove leaves for 2 hours.

Table 4.2. The significance (F ratios and p values) of sun/shade and species in determining the soluble phenolic contents on a leaf area basis in mangroves at four sites.

Site	Sun/shade		Species	
	F	p	F	p
1	$F_{(1,15)}=9.14$	0.0165	$F_{(3,15)}=12.38$	0.0023
2	$F_{(1,11)}=2.77$	0.1470	$F_{(2,11)}=0.900$	0.4560
3	$F_{(1,11)}=5.23$	0.0623	$F_{(2,11)}=24.10$	0.0014
4	$F_{(1,11)}=48.0$	0.0002	$F_{(2,11)}=48.53$	0.0004

Table 4.3. The significance (F-ratio and p values) of sun/shade and species in determining the succulence of mangrove leaves at four sites.

Site	Sun/shade		Species	
	F	p	F	p
1	$F_{(1,15)}=0.00$	0.9603	$F_{(3,15)}=14.05$	0.0015
2	$F_{(1,11)}=0.12$	0.7412	$F_{(2,11)}=25.42$	0.0012
3	$F_{(1,11)}=1.99$	0.2078	$F_{(2,11)}=0.050$	0.9559
4	$F_{(1,11)}=1.83$	0.2253	$F_{(2,11)}=15.36$	0.0044

A comparison of *R.stylosa* for soluble phenolics per leaf area and leaf succulence over sites (Figure 4.6) showed that sites were significantly different (i.e. for soluble phenolics $F_{(2,11)}=25.09$, $p=0.001$, and for leaf succulence $F_{(2,11)}=11.48$, $p=0.009$), with soluble phenolics and succulence increasing with the salinity of sites. Sun leaves had greater levels of soluble phenolics ($F_{(1,11)}=11.64$, $p=0.014$) and succulence ($F_{(1,11)}=7.28$, $p=0.036$) than shade leaves.

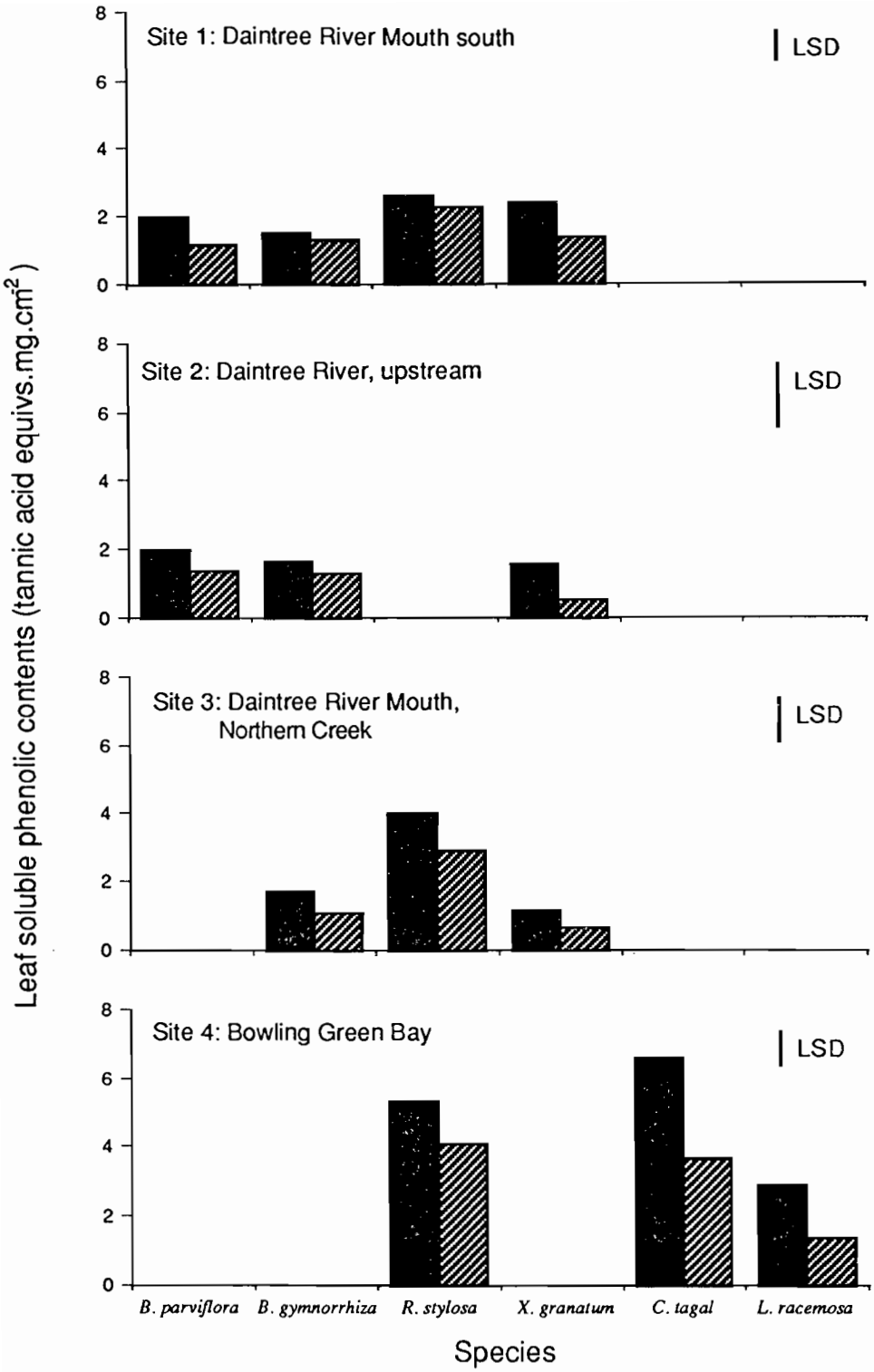


Figure 4.4. Variation in soluble phenolic contents between sun (solid), and shade (shaded) leaves of six mangrove species over four sites in north Queensland. LSD is the least significant difference ($p<0.05$) between any two means.

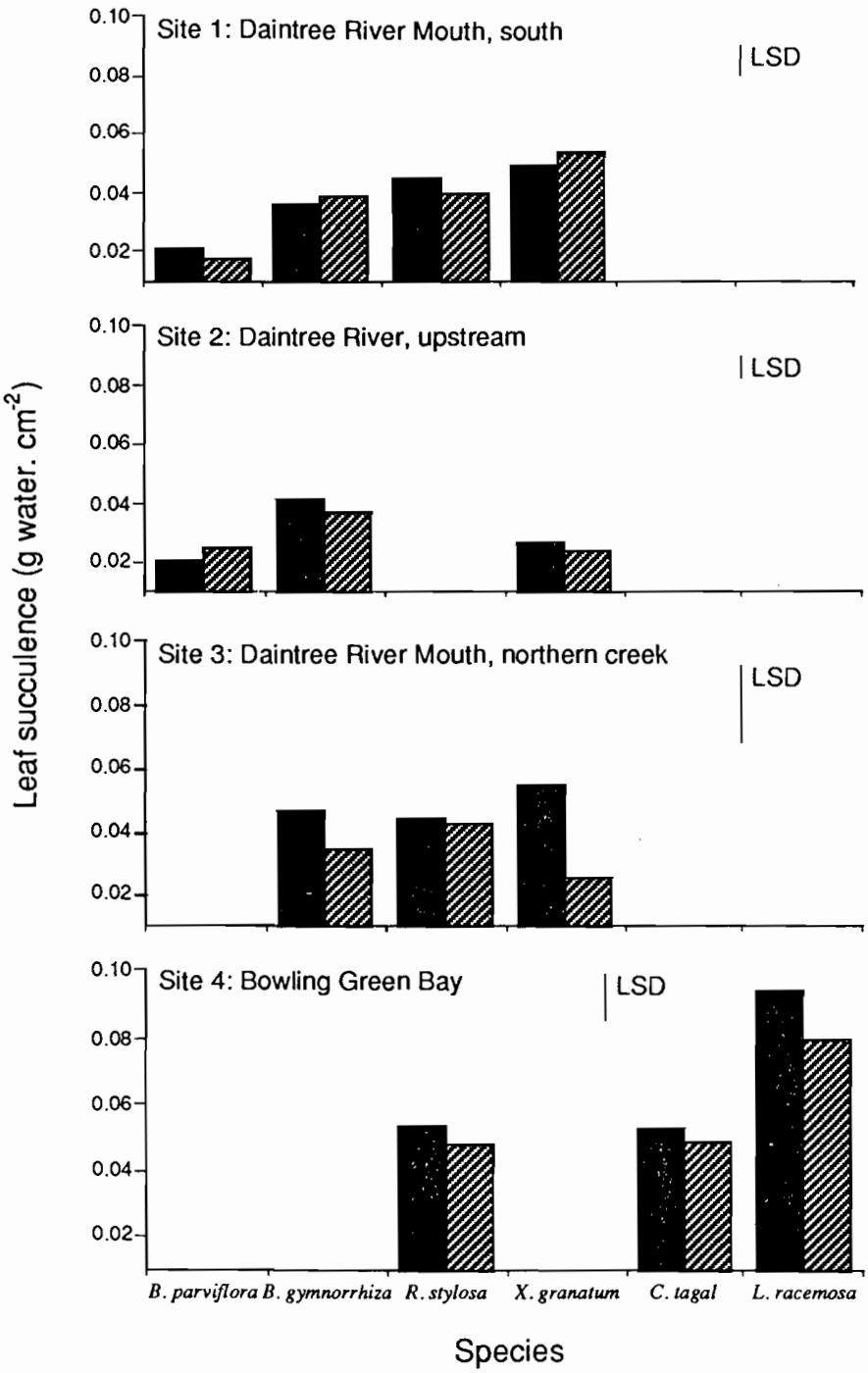


Figure 4.5. Variations in leaf succulence between sun (solid), and shade (stippled) leaves of six mangrove species over four sites in north Queensland. LSD is the least significant difference ($p<0.05$) between any two means.

4.3.4 Phenolic accumulation in response to nitrogen nutrition

In *R.stylosa* plants growing in a uniform low light environment (maximum solar radiation of 1000 μmol.quanta.$m^{-2}.s^{-1}$) increases in leaf nitrogen contents had no significant effect ($t_{(23)}$=-0.46, p=0.65) on phenolics accumulated per unit leaf area (Figure 4.7a), or specific leaf weight ($t_{(23)}$=0.41, p=0.68) (Figure 4.7b), but had a significant, positive effect on quantum yield of photosynthesis ($t_{(23)}$=3.46, p=0.002) (Figure 4.7c).

Over all species and sites it was found that soluble phenolics were accumulated as a constant proportion of dry weight per leaf area (Figure 4.8, soluble phenolics/leaf area = -1.941 + 0.228*specific leaf weight, r^2=0.74). Approximately 23% of the leaf tissue dry weights are phenolics (estimated from the slope of Figure 4.8).

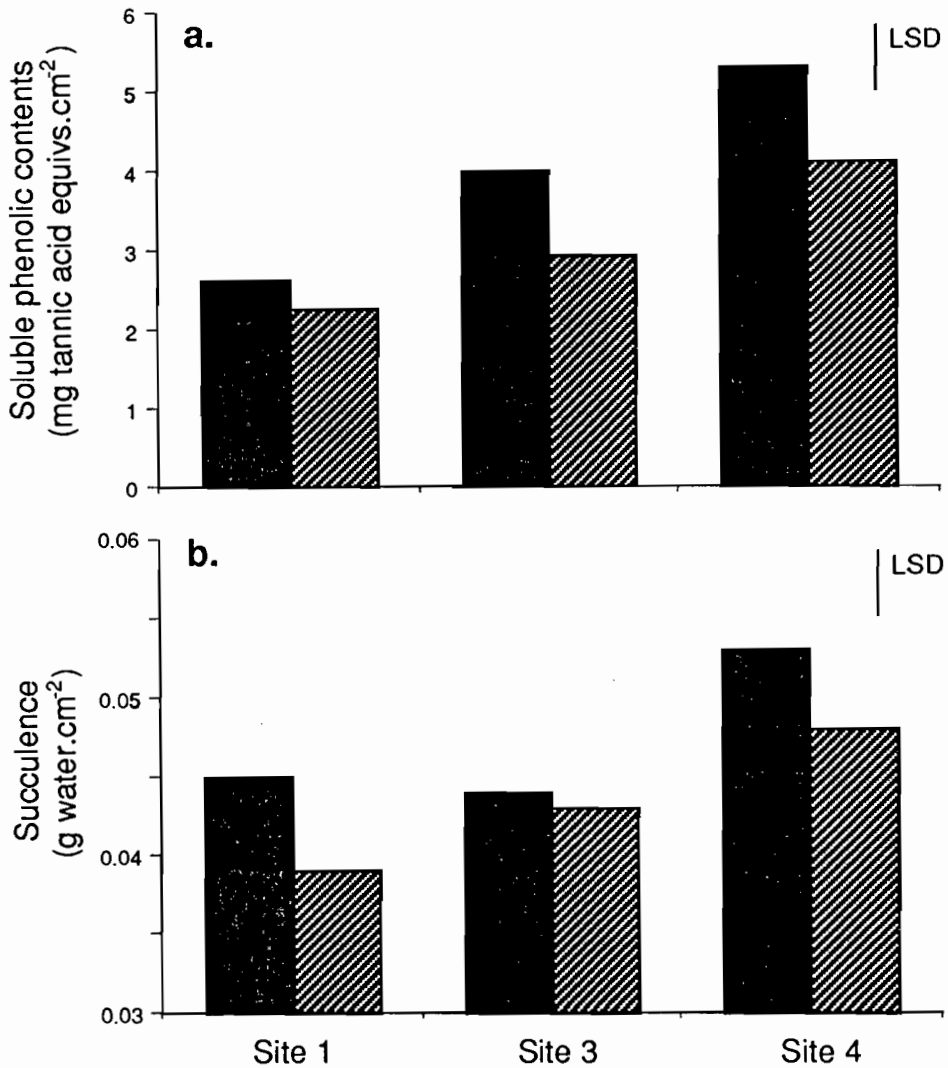


Figure 4.6. Variation in (a) soluble phenolic compounds, and (b) leaf succulence of sun (solid) and shade (shaded) leaves of *Rhizophora stylosa* growing at three sites in north Queensland. LSD is the least significant difference ($p<0.05$) between any two

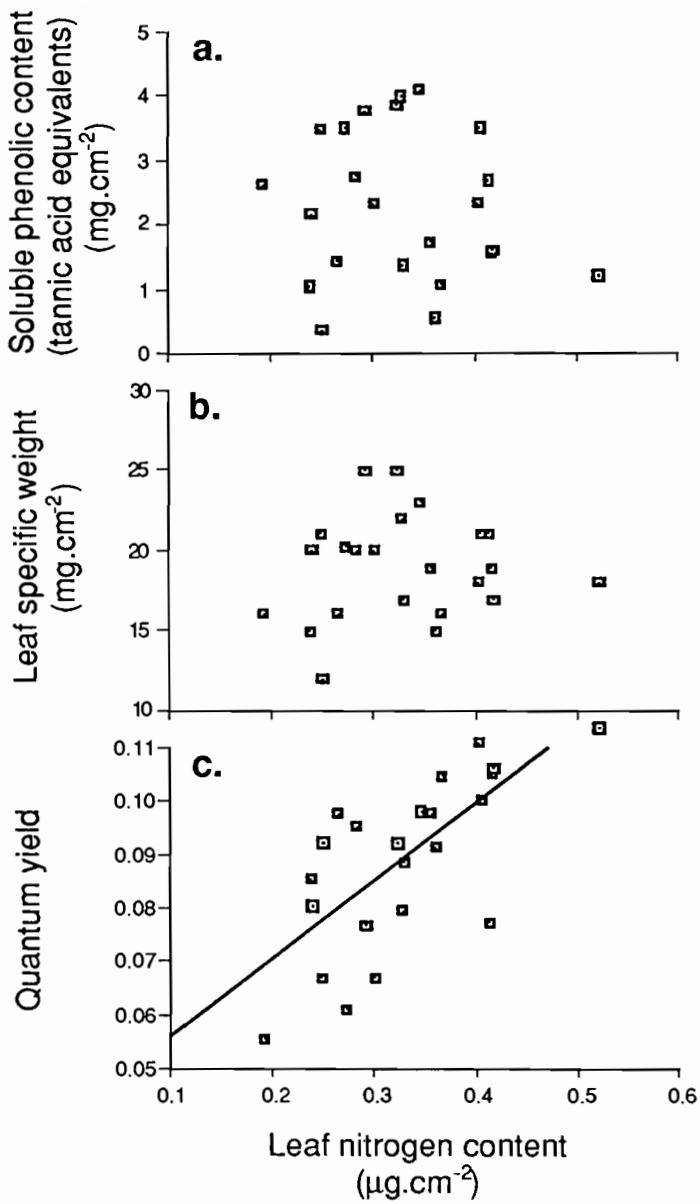


Figure 4.7. Variation in (a) soluble phenolic contents, (b) leaf specific weights, and (c) quantum yield with leaf nitrogen contents of leaves of *Rhizophora stylosa*. Linear regression for (c) is: quantum yield=0.0382+0.154*N content, r^2=0.491.

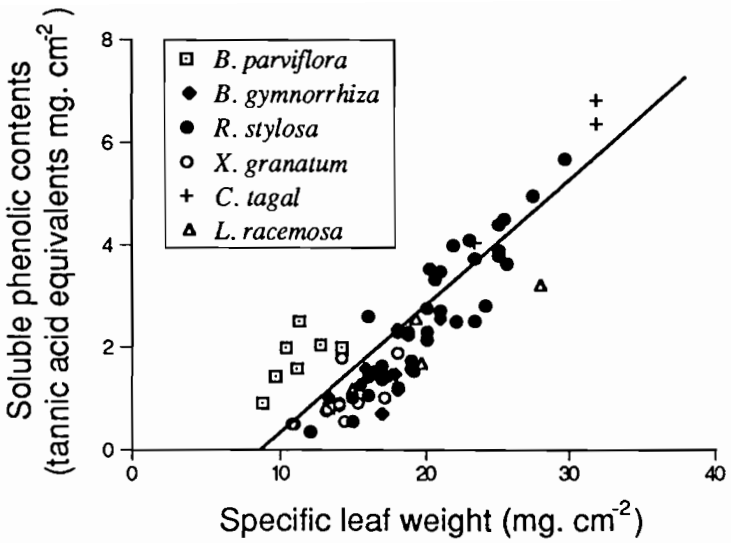


Figure 4.8. Correlation between leaf specific dry weight and soluble phenolic content/leaf area of leaves of six mangroves species in both sun and shade environments. Linear regression is:
soluble phenolics/leaf area=-1.941+0.228*spec. leaf weight, r^2=0.740.

4.4 Discussion

Caldwell *et al.* (1983) estimated that 75-95% of incident UV radiation on leaves is absorbed by the epidermis, so implicating it as a protective UV filter. The distribution of phenolic compounds in the epidermis of mangrove leaves (Plate 4.1, Plate 4.2), and the light absorption characteristics of phenolic compounds of mangrove leaves (Fig. 4.1 and 4.2) has provided evidence that these compounds could protect mangrove leaves from the damaging effects of UV radiation.

Sun leaves are exposed to higher levels of UV (Caldwell *et al.*, 1983) and visible radiation than shade leaves. Comparison between sun and shade leaves of mangroves over species and sites showed that sun leaves have greater levels of soluble phenolic compounds (Plate 4.2) and succulence than shade leaves (Fig. 4.3 and 4.4). The correlation between phenolic contents per leaf area and dry weights per leaf area (Fig. 4.7) was probably due to differences in the specific weight of leaves that occur between sun and shade leaves (Givnish, 1988). In soybean grown at high visible light levels, Warner and Caldwell (1983) showed that leaf phenolic contents and leaf thickness were higher than in plants grown at low light levels. Plants grown at high visible light levels showed no reduction in photosynthetic rates after five hours of high level UV-B irradiance, whereas those grown at low light levels suffered photosynthetic damage. The insensitivity of high light grown plants to UV-B radiation was attributed to higher phenolic contents in conjunction with thicker leaves. Tevini *et al.*, (1991) found no indication of photosynthetic damage from UV-B radiation in rye plants in which the accumulation of UV absorbing compounds had been stimulated by pretreatment with UV-B radiation. Thus, the presence of UV-absorbing compounds and probably leaf succulence could contribute substantially to the

attenuation of UV radiation and hence protection from UV radiation in mangrove species.

The large effect of site on the accumulation of phenolic compounds in sun leaves (Fig. 4.5), particularly between sites in the same river system (i.e. sites 1 and 3), indicate that factors, other than exposure to direct sunlight may also influence the accumulation of UV absorbing phenolic compounds. The Bowling Green Bay site (Site 4, Table 4.1, Fig. 4.3) at which plants had the highest levels of UV absorbing compounds is the most arid of the sites surveyed. High salinities result in highly conservative water use in mangroves which depresses photosynthetic rates during periods of high solar radiation and high leaf temperatures (Ball *et al.*, 1988; Clough and Sim, 1989). It is possible that decreased photosynthetic rates due to unfavourable water relations could influence the production of UV absorbing compounds in mangroves. Support for this view comes from Murali and Teramura (1986) who showed that enhanced UV-B radiation had no effects on photosynthetic rates of soybean experiencing water deficits, whereas photosynthesis in the well watered controls was sensitive to UV-B radiation. This suggests that water deficient plants were better protected from UV-B radiation than well watered plants, or that effects of UV-B radiation are small compared to the effects of water deficits on plants. The differences in phenolic contents of leaves between sites (Fig. 4.5), and the results of Murali and Teramura (1986) which showed that enhanced UV-B radiation had no additional detrimental effect on photosynthesis in water deficient soybeans plants, suggests that water deficits may be influencing the accumulation of phenolic compounds in mangrove leaves.

Differences in the concentration of UV-absorbing compounds between sites could also be due to other factors, for example, soil nutrient status or the degree of tidal innundation. The influence of nitrogen availability on the concentration of UV-absorbing compounds was assessed in *R.stylosa* growing in a shadehouse environment. There was no correlation between the accumulation of phenolics (Fig. 4.6a), or specific leaf weight (Fig. 4.6b) and the nitrogen content of leaves, but the quantum yield of photosynthesis (Fig. 4.6c) increased with leaf nitrogen content. Leaf nitrogen contents have been shown to correlate with increases in the quantum yield and net photosynthetic rates of plants (Evans and Terashima, 1987; Küppers *et al.*, 1988). As photosynthesis of mangrove leaves increased with no corresponding increase in the concentration of UV-absorbing compounds it is suggested that, in this experiment where plants were growing in a uniform and relatively low light environment, greater rates of photosynthesis were not associated with greater concentrations of UV-absorbing phenolic compounds.

Murali and Teramura (1987) showed that exposure of phosphate deficient plants to enhanced UV-B radiation had no additional effect on photosynthesis and growth. This suggests that phosphate deficient soybean plants, like those experiencing water deficits (Murali and Teramura, 1986), are either better protected from the effects of UV-B radiation, or that the effect of UV-B radiation on photosynthesis is secondary to those of phosphate deficiency or water deficits. Cen and Bornman (1990) also found that high visible light levels reduced the effects of UV-B radiation on photosynthesis in bean plants, relative to plants grown at low visible light and high UV-B radiation levels, and that this was associated with increases in the UV absorbance of leaf extracts.

Both phosphate deficiency and water deficits reduce photosynthetic rates, resulting in leaves absorbing light that is not used for photosynthesis (see section

1.1, page 1). Increases in visible light levels in the experiments of Cen and Bornman (1990) may have had a similar effect. Therefore, it is possible that the reduced effect of UV-B radiation, when UV-B radiation treatments are combined with treatments that increase the amount of excess visible light, may be due to increases in concentration of UV-absorbing compounds in the leaves (certainly this was observed in the experiments of Cen and Bornman (1990)), and that this may be associated with increases in the light absorbed that is not used in photosynthesis. Therefore, differences in the concentration of UV-absorbing phenolic compounds between sun and shade leaves of mangroves (Fig. 4.3) may be a response to the amount of light absorbed by the leaves that is in excess of what can be used in photosynthesis.

Succulence and the UV-absorbing phenolic compounds present in mangrove leaves would behave as selective filters, removing short and energetic wavelengths. The accumulation of these compounds in mangrove leaves varied between species and over sites. The variation between sites that would be experiencing similar UV radiation levels, suggested that UV radiation is not the only factor that influences the accumulation of UV-absorbing pigments in mangroves. Increases in leaf nitrogen contents and photosynthetic rates did not correlate with increasing concentrations of UV-absorbing phenolic compounds. Variations in the concentration of UV-absorbing compounds over sites, and between sun and shade leaves, may be linked to the amount of light absorbed by leaves that is in excess of what is used in photosynthesis. Excess light could be the result of water deficits, and associated reductions in photosynthetic rates that vary between sites, or, in the case of sun and shade leaves, due to higher levels of light absorbed by sun leaves compared to shade leaves. In the following Chapter the effect of UV radiation on the accumulation of UV-absorbing compounds in mangroves is assessed. Comparisons in the response of different mangrove

species to UV radiation are made with a view to determining whether adaptation to UV radiation differs between mangrove species.

CHAPTER 5

Effect of UV radiation on three mangrove species

5.1 Introduction

Tolerance of UV-B radiation has been shown to vary between genotypes (Flint *et al.*, 1985; Murali *et al.*, 1988; Teramura *et al.*, 1990). This variation has been attributed to the varying thickness of leaves (that attenuates UV radiation), to different abilities of genotypes to produce UV-absorbing compounds (Robberecht and Caldwell, 1978; Caldwell *et al.*, 1983; Murali *et al.* 1988; Les and Sheridan, 1990), and also to differences in leaf peroxidase activities and carotenoid contents (Murali *et al.*, 1988).

It has also been shown that the combination of enhanced UV-B radiation and other treatments that impose physiological stress, for example, high levels of visible light (Cen and Bornman, 1990), water deficits (Murali and Teramura, 1986) and phosphate deficiencies (Murali and Teramura, 1987), reduce the effects of UV-B radiation relative to treatments in which these additional stresses were not imposed. This suggests that additional physiological stress may in some way contribute to protection from the effects of UV radiation, either by stimulating the production of UV-absorbing compounds (shown by Cen and Bornman (1990)), by resulting in the alterations to leaf morphology, or through increased carotenoid contents.

The previous Chapter showed that mangroves have UV-absorbing compounds in their leaves and that the concentration of UV-absorbing compounds varied between mangrove species (Fig. 4.4, page 85). Thus, UV-absorbing compounds may be more important in providing protection from UV radiation in some species than others. The variation in the concentration of UV-absorbing compounds in leaves of *R.stylosa* between sites within the same river system (Fig. 4.6, page 88) suggested that environmental factors other than UV radiation, possibly those that reduce photosynthesis, may influence the production and accumulation of UV-absorbing compounds in mangrove leaves.

The aim of this Chapter was to determine the changes, either adaptive or otherwise, that three mangrove species undergo when they are exposed to UV radiation. Of particular interest was whether UV radiation stimulates the protective mechanisms that have previously been shown to occur in other plants. That is, whether there is: 1) increased production and accumulation of UV-absorbing compounds, 2) increased concentration of carotenoids, 3) change in leaf morphology, and 4) whether the responses to UV radiation differ between species. Different levels of UV radiation were obtained in natural sunlight by enclosing plants in growth cabinets made out of acrylic sheeting that either eliminated UV radiation (filtered-out wavelengths shorter than 380 nm), or removed wavelengths shorter than 300 nm.

5.2 Materials and methods

5.2.1 Growth cabinets

Four plants from the mangrove species *Rhizophora apiculata*, *Bruguiera gymnorrhiza* and *B. parviflora* (all from the family *Rhizophoraceae*) were grown between July and November in 100% seawater nutrient solutions (see Appendix II, page 130), in either ambient, or depleted UV radiation environments using natural sunlight as the light source. The two UV radiation treatments (depleted UV, or near-ambient UV radiation levels) were obtained by using ventilated growth cabinets made out of acrylic sheeting that either removed UV radiation (Shinkolite, Mitsui, Japan), filtering out wavelengths less than 380 nm, or allowed much of the UV radiation to penetrate the cabinet, filtering out wavelengths less than 300 nm (Acropoly, Korea). Absorption spectra of the two acrylic sheeting types are shown in Figure 5.1. Each growth cabinet was ventilated using two electric fans that each exhausted 1 $m^3.min^{-1}$ (part no. 508-021, RadioSpares Components, WA, Aust.). After four months under these conditions the three youngest, fully expanded leaves from each plant were used for analysis.

5.2.2 Sampling procedure

Before harvesting each leaf, a photosynthetic measurement was taken using a Li-Cor 6200 portable photosynthetic measuring system with a modified leaf chamber (see section 3.2.2, page 48 for description and operational procedures). Immediately after the leaf was harvested, four discs (1.37 cm^2in area) were punched from the leaf, frozen in liquid nitrogen and stored at -80°C until further analysis. The remainder of the leaf was weighed and its area

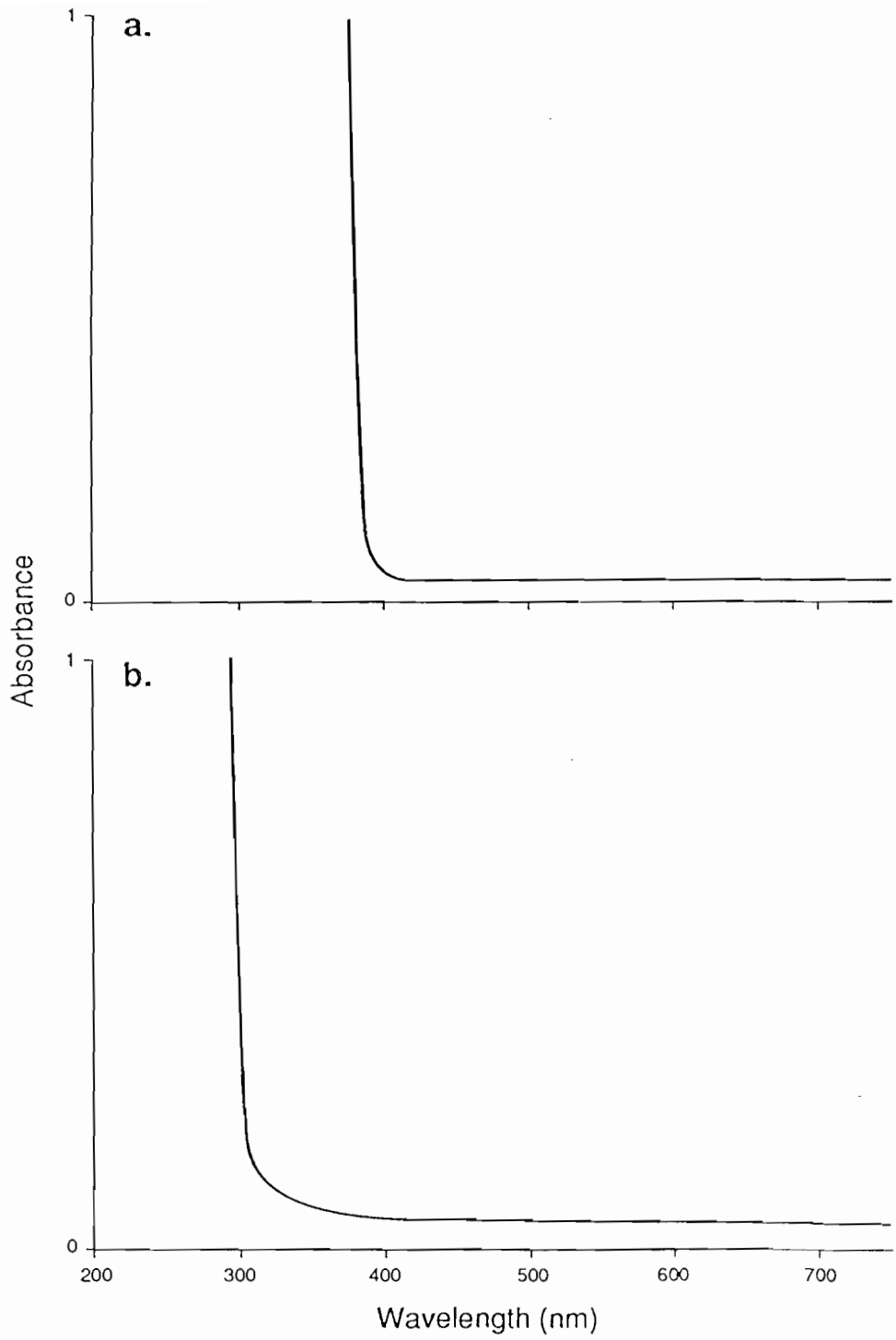


Figure 5.1. Absorption spectra of the acrylic sheeting used to construct growth cabinets. Panel a is Shinkolite (Mitsui, Japan) used for the UV depleted treatment, and Panel b is Acropoly (Korea), used for the near-ambient UV treatment.

measured (Li-Cor planimeter, Li-Cor, Lincoln, Nebraska, USA). Leaves were dried at 70°C for a week to determine leaf dry weights. Succulence was determined as [(fresh weight - dry weight)/leaf area]. Measurements of leaf area included the area of the leaf discs punched when the leaves were harvested.

5.2.3 Pigment analysis

Pigments from leaves were extracted in 100% acetone as described in section 2.2.5 (page 25). Carotenoid and chlorophyll concentrations were determined by HPLC using method 2, described in section 2.2.5.2 (page 26). Quantification of the UV absorbing compounds was obtained by HPLC. The HPLC equipment used was described in section 2.2.5.2 (page 26). UV-absorbing compounds within leaves were eluted on a 25 cm C-8 column (Brownlee, Applied Biosystems, Foster City, CA, USA) with a C-8 guard column preceding it with the detector set at 340 nm using a gradient modified for mangroves from Tevini *et al.* (1991). The gradient was linearly developed from 20% to 49% solvent B (5% ortho-phosphoric acid, 16% methanol and 18% tetrahydrofuran in water) in solvent A (5% ortho-phosphoric acid, 6% methanol, and 10% tetrahydrofuran in water) over 10 minutes with a flow rate of $1 ml.min^{-1}$. The gradient of Tevini *et al.* (1991) was developed over 14 minutes.

5.2.4 Statistical analysis

All statistical analysis used the general linear model procedure (GLM) of SAS (SAS Institute Inc., NC, USA). Analysis of variance of the plant response variables (e.g. pigment concentrations and leaf morphological characteristics) with UV treatment and species used a design in which species and UV treatments were represented as crossed factors, with trees nested within the crossing of species and UV treatment, and with leaves nested within trees. Significance of effects was

assessed using F-ratio tests. Logarithmic transformations of the data were made where the variability in the data was not constant, in order to satisfy the constant variance requirement of analysis of variance models.

An analysis of covariance, using the concentration of UV-absorbing compounds as the covariate, was used to determine whether the concentration of UV-absorbing compounds within leaves was correlated with any other of the plant response variables. Significance of the covariate was established using t-tests.

5.3 Results

5.3.1 UV and species effects

Representative HPLC chromatograms of extracts of leaves of three mangrove species are shown in Figure 5.2. The absorption spectra of the major peaks are shown in Figure 5.3. All species had a peak at 5 minutes with a maximum absorbance at 260nm. Each species had its own unique series of UV-absorbing compounds. The peak at 11 minutes was the largest component of the UV-absorbing compounds and was common to all three species. Its absorption spectrum matched that of the epidermal extracts described in Chapter 4 (maximum absorbance at 254 and 350 nm) (compare Figure 4.3 (page 83) with Figure 5.3). Because the 11 minute peak was the largest and most clearly resolved component of the UV-absorbing compounds in *all species*, and because its absorption spectra matched that of the UV-absorbing pigment extracted from leaf epidermal peels, the area of this peak was used to compare species. That the integral of the 11 minute peak represented a measure of the concentration of UV-absorbing

compounds within the leaves was assessed by comparing the integral of this peak with the sum of the integrals of all the peaks (excluding the 5 minute peak which was thought to be a DNA/protein signal, i.e. peak absorbance at 260 nm). A significant correlation was found between these variables with an r^2 of 0.77 (Figure 5.4). Time and other constraints did not permit identification of this compound. Therefore, results are presented as the integral of the 11 minute peak for 1.37 cm^2 leaf area (i.e. a relative measure of the concentration of the compound).

UV-absorbing compounds were present in all three species and in both UV treatments (Figure 5.5). Under near-ambient UV conditions all three species contained similar amounts of UV-absorbing compounds on a leaf area basis. However, the concentration of UV-absorbing compounds of *B.parviflora* was lower ($F_{(1,17)}$=7.66, p=0.0132) in the depleted UV treatment than in the near-ambient UV treatment (Figure 5.5). There was no significant difference in the UV-absorbing compounds between treatments for *B.gymnorrhiza* ($F_{(1,17)}$=0.36, p=0.555) or *R.apiculata*. ($F_{(1,17)}$=0.06, p=0.816).

Total chlorophyll concentrations were significantly lower ($F_{(1,17)}$=5.66, p=0.0293) in *R.apiculata* when grown in near-ambient UV conditions relative to depleted UV conditions (Figure 5.6). This contrasts with both the *Bruguiera* species which showed no significant difference in total chlorophyll concentrations between near-ambient and the depleted UV treatment (for *B.parviflora* $F_{(1,17)}$=0.77, p=0.393 and for *B.gymnorrhiza* $F_{(1,17)}$=0.78, p=0.391). *R.apiculata* had higher chlorophyll contents than the *Bruguiera* species in UV depleted conditions, and similar chlorophyll concentrations under near-ambient UV conditions.

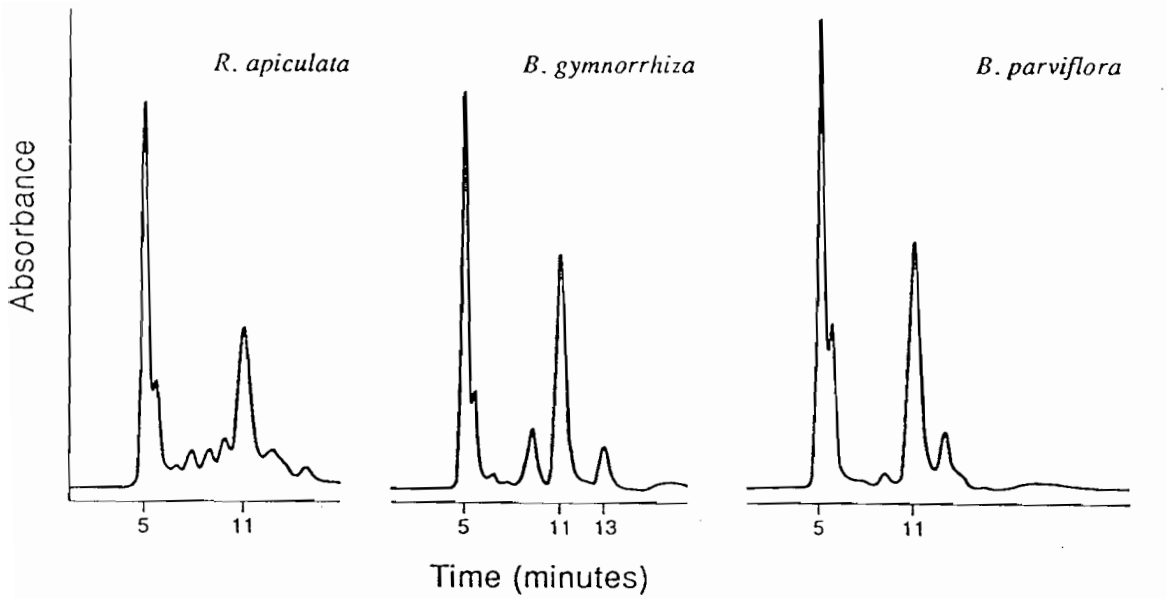


Figure 5.2. Representative HPLC chromatograms measured at 340 nm of extracts of leaves of *Rhizophora apiculata*, *Bruguiera gymnorrhiza* and *B.parviflora*. Each species has a unique series of UV-absorbing compounds shown by the different series of peaks along the chromatogram. However all three species have a major peak at 11 minutes.

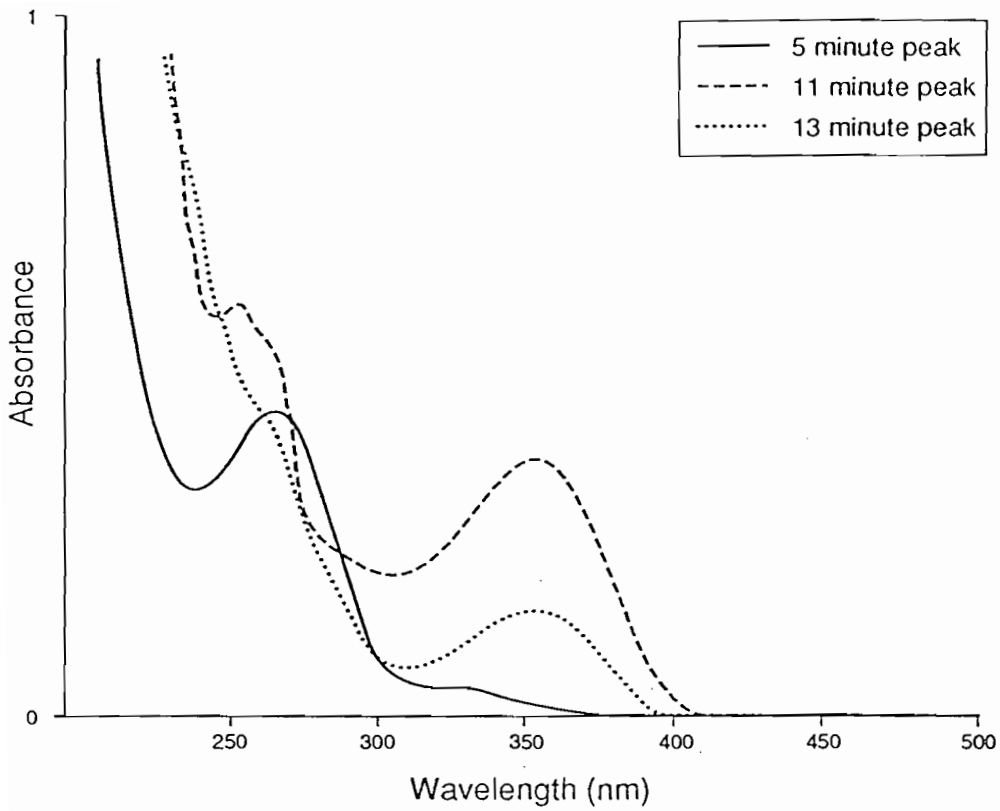


Figure 5.3. Absorption spectra of three of the UV-absorbing compounds eluted by HPLC. Solid line is the substance eluted at 5 minutes, dashed line is the compound eluted at 11 minutes for all species, and the dotted line is the compounds eluted at 13 minutes for *B.gymnorrhiza*.

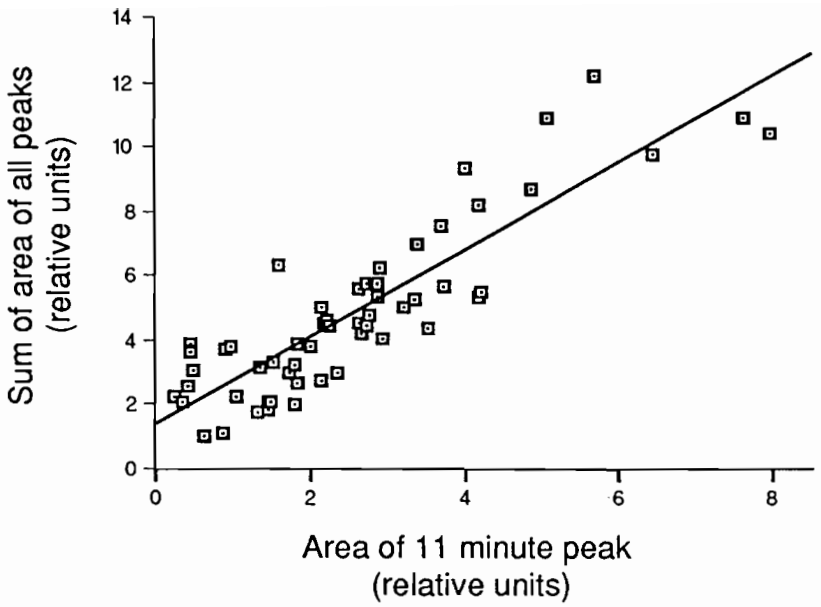


Figure 5.4. Relationship between the area of the 11 minute peak and the sum of the areas of all other UV-absorbing peaks (excluding the five minute peak, see text) obtained from HPLC of leaf extracts of three mangrove species. Linear regression is: Sum of the peaks=1.40 +1.34*area of 11 min. peak, r^2=0.77.

Chlorophyll a/b ratios where not significantly different between UV environments for any species ($F_{(1,17)}$=2.79, p=0.1041). However, chlorophyll a/b ratios differed significantly between species ($F_{(2,17)}$=15.03, p<0.001), *R.apiculata* and *B.parviflora* having higher chlorophyll a/b ratios than *B.gymnorrhiza* (Figure 5.7).

Despite *R.apiculata*'s lower chlorophyll concentrations under near-ambient UV conditions relative to depleted UV conditions there was no significant difference (p>0.05) in the average photosynthetic rates measured between treatments or species. The leaves sampled had net photosynthetic rates that varied between -1 and 7 μmol $CO_2.m^{-2}.s^{-1}$, with a uniformly low average photosynthetic rate of 2 μmol $CO_2.m^{-2}.s^{-1}$ (Figure 5.8). This low average reflects the harsh conditions under which the plants were grown, that is, in 100% seawater and in full sunlight, and the above average air temperatures (35°C) on the morning that the measurements were taken.

Table 5.1. Means (n=24 leaves) of leaf characteristics of three mangrove species grown in acrylic sheeting growth cabinets. F-ratio test statistic (df=(2,17)) and probability are shown for the comparison between species.

	SPECIES			
	B. parviflora	*R. apiculata*	*B. gymnorrhiza*	
leaf area cm^{-2}	20.49	37.35	39.26	F=52.05 p<0.001
Leaf display (% leaf area)	74.33	23.18	63.68	F=20.66 p<0.001
Succulence g $H_2O.cm^{-2}$	0.0291	0.0391	0.0520	F=26.70 p<0.001
Xanths/Chl $mmol.mol^{-1}$	215.8	98.40	130.6	F=11.71 p<0.001
Total carotenoids/Chl $mmol.mol^{-1}$	682.3	432.5	502.3	F=15.94 p<0.001

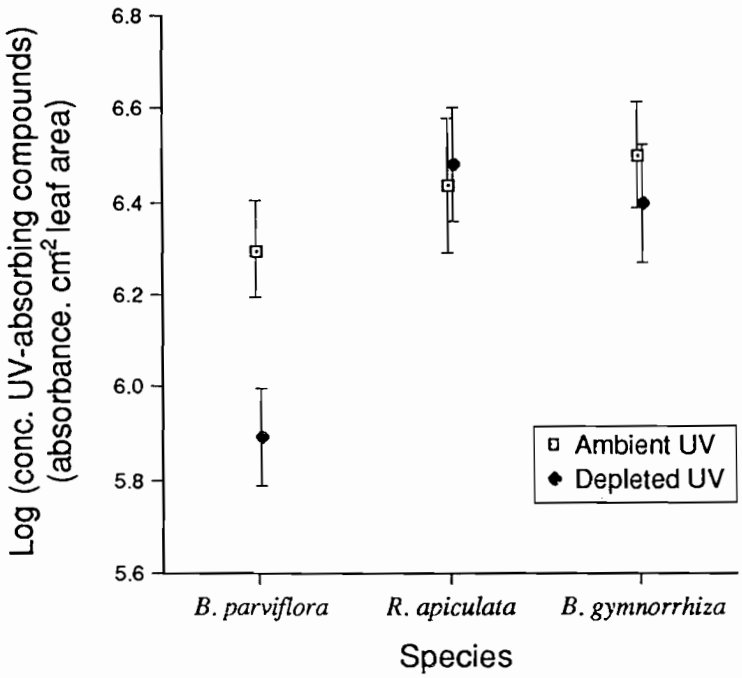


Figure 5.5. Concentration of UV-absorbing compounds (obtained by HPLC, see text) for three mangrove species, grown in either depleted or near-ambient UV conditions. Error bars are the standard errors about each mean (n=12).

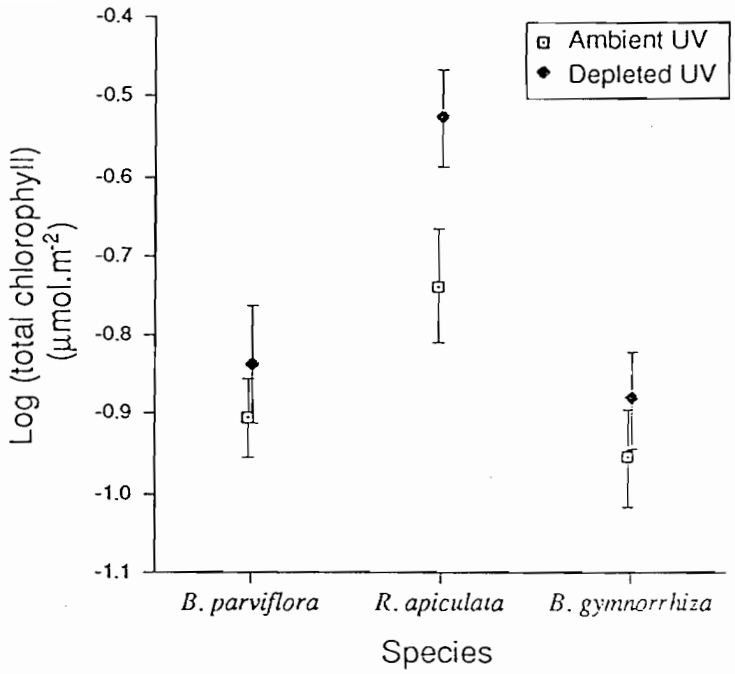


Figure 5.6. Total chlorophyll concentrations of three mangrove species grown in either depleted or near-ambient UV conditions. Error bars are the standard errors about each mean (n=12).

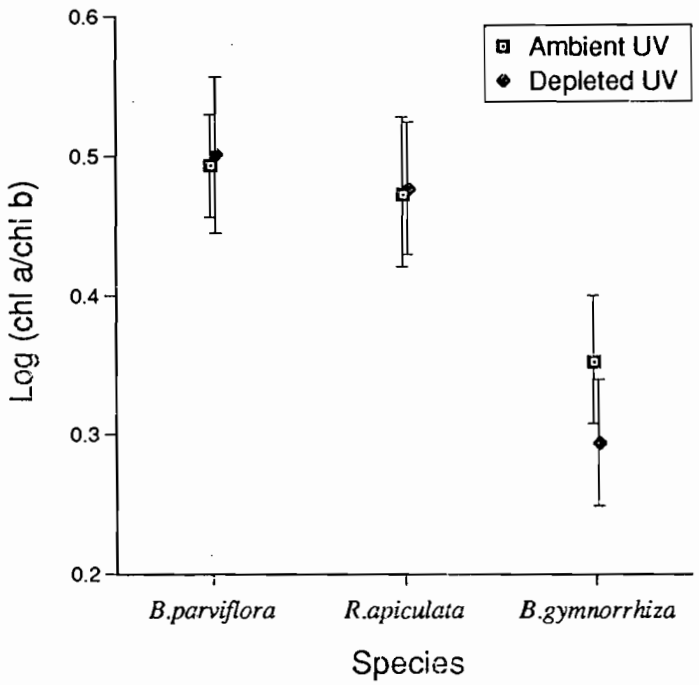


Figure 5.7. Chlorophyll a/b ratio for three mangrove species grown in either depleted or near-ambient UV conditions. Error bars are the standard errors about each mean (n=12).

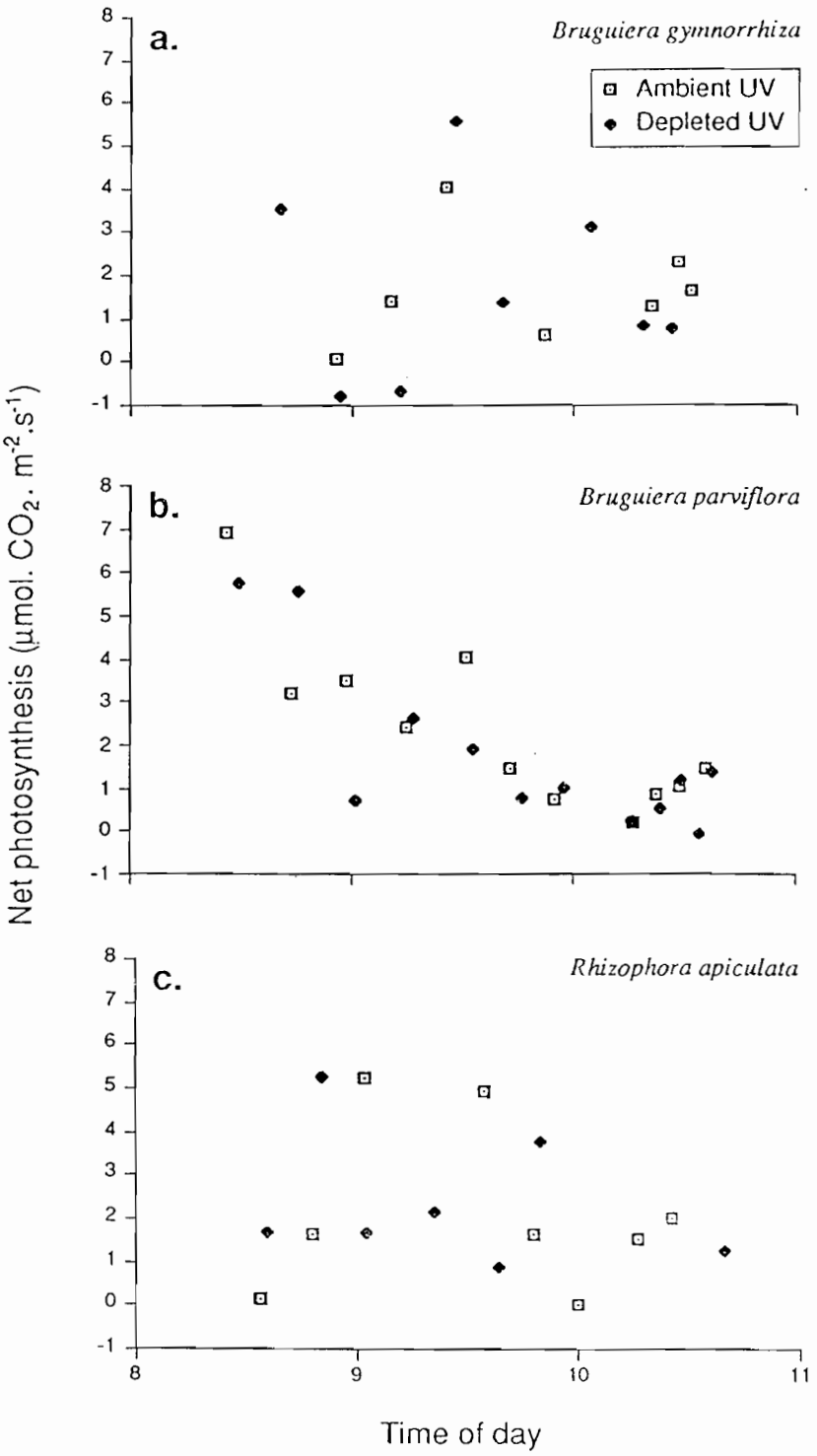


Figure 5.8. Net photosynthetic rates of *Bruguiera gymnorrhiza* (a), *B.parviflora* (b) and *Rhizophora apiculata* (c) grown in either depleted, or near-ambient UV conditions. Each point on the plots represent an individual leaf.

There were no significant differences ($p>0.05$) between UV treatments for leaf size, angle, succulence, total carotenoid/chlorophyll or xanthophyll/chlorophyll contents. However, there were significant ($p<0.05$) differences between the species in these leaf characteristics (Table 5.1). *B.parviflora* had the smallest leaves, the least succulence and the greatest leaf area displayed on a horizontal plane (most horizontal leaf angles). It also had the highest ratios of xanthophylls/chlorophyll and total carotenoids/chlorophyll. Conversely, *R.apiculata* had the most vertical leaf angles, lowest xanthophyll/chlorophyll ratio and lowest carotenoid/chlorophyll ratio. *B.gymnorrhiza* had intermediate characteristics for all variables, except for succulence levels where it has the highest value with an average of 0.0520 g $H_2O.cm^{-2}$.

5.3.2 Influence of UV-absorbing compounds

The concentration of UV-absorbing compounds within the leaves was fitted as a covariate in order to assess whether the concentration of these compounds was correlated with any other of the plant response variables measured. Using this procedure it was possible to assess whether the observed effects of UV radiation on leaves was influenced by the concentration of UV-absorbing compounds within the leaf. In all species the concentration of UV-absorbing compounds increased significantly with the chlorophyll a/b ratio (parameter estimate for the covariate=0.147, standard error=0.066, t=2.22, p=0.0334), leaf succulence (parameter estimate for the covariate=0.0149, standard error=0.0078, t=1.91, p=0.0649) and total leaf area (parameter estimate for the covariate=0.176, standard error=0.076, t=2.29, p=0.0283). The concentration of UV-absorbing compounds had no significant correlation with

any other variables measured. By combining the analysis of covariance results and the results of Table 5.1 (i.e. species differences), it follows, that the size of the chlorophyll a/b ratio, succulence, and leaf area vary with species, but within each species, the concentration of UV-absorbing compounds correlates with leaf area, succulence and the chlorophyll a/b ratio (Figure 5.9).

A comparison of species responses to UV radiation is summarized in Figure 5.10. Only the results for *R.apiculata* and *B.parviflora* are shown as these exhibited the largest responses to UV radiation (see Fig. 5.5 and 5.6). UV radiation increased the concentration of UV-absorbing compounds in *B.parviflora.* ($F_{(1,17)}$=7.66, p=0.0132), but not in *R.apiculata* ($F_{(1,17)}$=0.06, p=0.8165). In both species there was a positive correlation between the concentration of UV-absorbing compounds and the chlorophyll a/b ratio. However, this could not be statistically linked to the level of UV radiation in *R.apiculata.* UV radiation had no significant effect on the concentration of total chlorophyll in *B.parviflora* ($F_{(1,17)}$=0.77, p=0.3928), but had a negative effect on the total chlorophyll concentration in *R.apiculata.* ($F_{(1,17)}$=5.66, p=0.0293).

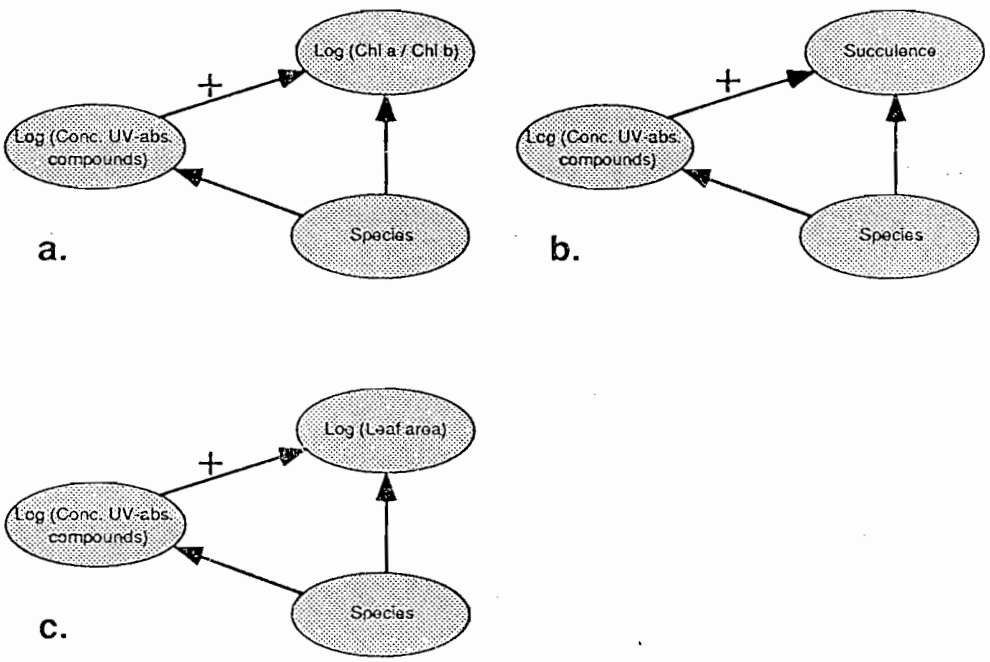


Figure 5.9. The influence of the concentration of UV-absorbing compounds in mangrove leaves on (a) chlorophyll a/b ratios, (b) leaf succulence, and (c) leaf area established by analysis of covariance. Arrows denote significant relationships. Signs show the direction of the relationship between the covariate [Log(conc. UV-absorbing compounds)] and the variable. Parameter estimates of the covariate, standard errors of the estimates and significance values appear in the text.

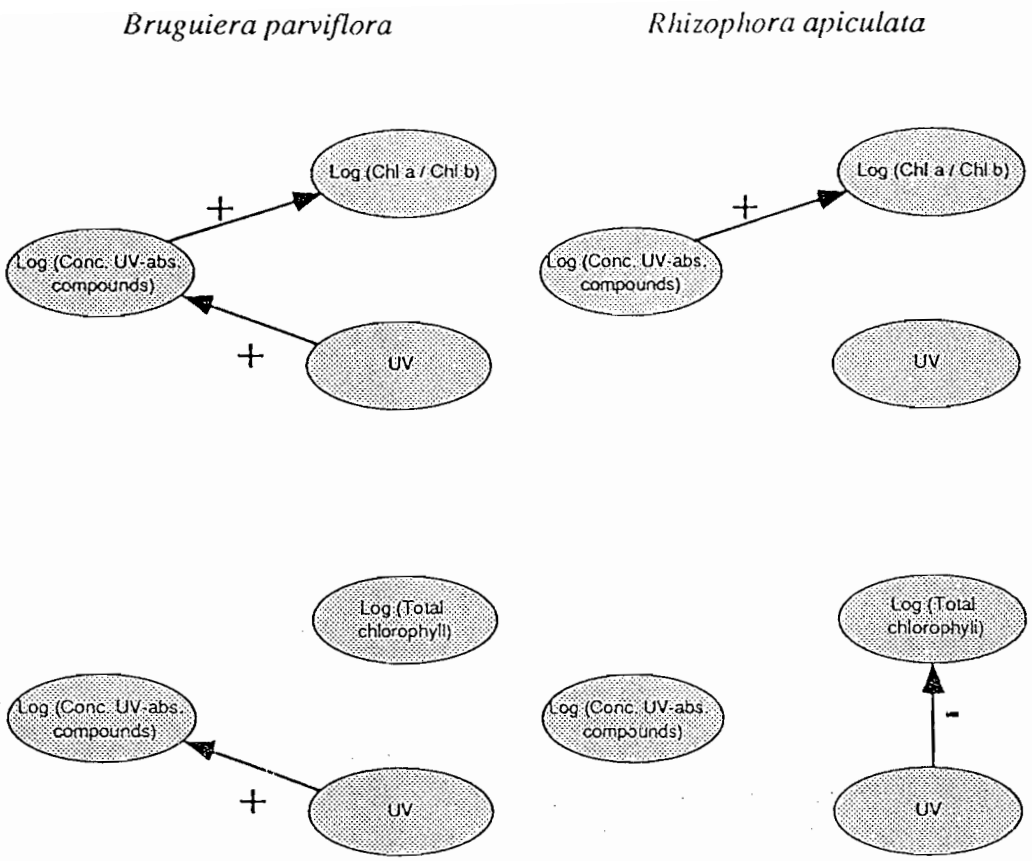


Figure 5.10. Relationship between UV radiation and the concentration of UV-absorbing compounds with total chlorophyll and chlorophyll a/b ratios in mangrove leaves of *Bruguiera parviflora* and *Rhizophora apiculata*. Significance of relationships between variables are denoted by arrows. Signs show the directions of the relationships. Parameter estimates of the covariate [log(conc. UV-absorbing compounds)], standard errors of the estimates and significance values appear in the text.

5.4 Discussion

Exposure to UV radiation significantly increased the concentration of UV-absorbing compounds in *B.parviflora* relative to the UV-depleted treatment (Fig. 5.5). This agrees with the results of Wellman (1983) and Tevini *et al.* (1991), who found that accumulation of UV-absorbing compounds was correlated with increasing UV-B dose, possibly due to the stimulation of the production of phenolics, by activation of key enzymes in their synthetic pathway (Wellman, 1983; Tobin and Silverthorne, 1985; Jain and Guruprasad, 1990). Other factors, like developmental stage of tissue, wounding and the presence of pathogens, have also been found to influence the synthesis of phenolic compounds (see review Hahlbrock and Grisebach, 1979). Thus, the presence of UV-absorbing compounds within the leaves of all three mangrove species, in the depleted UV treatment (Fig. 5.5), suggests that the accumulation of UV-absorbing phenolic compounds in mangroves is not influenced by UV radiation alone, although UV radiation did affect the concentration of UV-absorbing compounds in *B.parviflora*. This implies a complex response to UV radiation that differs between species, and is influenced by other environmental factors.

In Chapter 4 it was proposed that the amount of light energy leaves absorb that is in excess of what is used for photosynthesis may be important in determining the concentration of UV-absorbing compounds within mangrove leaves. This hypothesis was based upon: 1) results of other researchers who found that stress treatments which reduced photosynthetic rates also reduced the effect of UV-B radiation on photosynthesis (Murali and Teramura, 1987; Murali and Teramura, 1988; Cen and Bornman, 1990), possibly due to the increased concentration of UV-absorbing compounds within leaves (Cen and Bornman, 1990); 2) evidence that suggests that UV radiation may be used for

photosynthesis (Bornman *et al.*, 1984; Schlicter *et al.*, 1985; Schlicter *et al.*, 1986); and 3) the observation that the concentration of UV-absorbing compounds within mangrove leaves varied between sites (Fig. 4.6, page 88), generally increasing with the salinity of soil and correlated reductions in photosynthetic rates (Ball and Farquhar, 1984*b*). In this study chlorophyll a/b ratios increased with increasing concentration of UV-absorbing compounds within leaves over all three mangrove species (Fig. 5.9). As it is known that chlorophyll a/b ratios increase with increasing levels of visible radiation intercepted by leaves (Anderson *et al.* 1988), this provides further evidence that in these experiments with mangroves, the accumulation of UV-absorbing compounds may be linked to the total radiation energy being absorbed by leaves, and more specifically to the amount of excess radiation being absorbed by the leaves.

The photosynthetic rates of all three mangrove species were uniformly low and showed no significant difference between the near-ambient and depleted UV conditions (Fig. 5.8). Given the above hypothesis (that excess light energy influences the accumulation of UV-absorbing compounds), it would be expected that all species would have higher concentrations of UV-absorbing phenolic compounds in the near-ambient UV environment compared to the depleted UV environment. *B.parviflora* was the only species in which this occurred (Fig. 5.5). However, lower total chlorophyll contents in *R.apiculata* leaves were found in the near-ambient UV treatment compared to the depleted UV treatment (Fig. 5.6) while *B.gymnorrhiza* showed no significant increase in the concentration of UV-absorbing compounds (Fig. 5.5), or decrease in chlorophyll contents (Fig. 5.6) in the near-ambient relative to depleted UV treatment. This may be evidence that a more complex system of protection, involving factors other than the accumulation of UV-absorbing compounds in leaves is involved in determining the response of different species to UV radiation.

In all three mangrove species studied their was no difference in leaf succulence or leaf area between depleted and near-ambient UV treatments. However, these characteristics varied between species (Table 5.1), and for each species were found to be correlated with increases in the concentration of UV-absorbing compounds (Fig. 5.9). Under enhanced UV-B radiation, reduction in leaf areas have been reported (Flint *et al.*, 1985; Barnes *et al.*, 1990). The ability of the UV-absorbing compounds to prevent decreased cell division and/or expansion, reported in plants grown under enhanced UV-B radiation, has been attributed to the protective screen they provide (Caldwell, 1981; Tevini and Teramura, 1989). In these experiments, the correlation between the concentration of UV-absorbing compounds and leaf area in plants grown under both depleted and near-ambient UV radiation conditions suggest that an effect due to screening of UV radiation is unlikely. The correlation between leaf area and the concentration of UV-absorbing compounds may simply reflect a concurrent increase in the accumulation of these compounds and leaf expansion. In addition the correlation between the concentration of UV-absorbing compounds and leaf succulence may be a consequence of the need to store UV-absorbing compounds within the leaf, or may also be due to the concurrent increase in leaf succulence and the accumulation of UV-absorbing compounds with leaf age.

Carotenoids are known to be protective against the effects of UV and visible radiation damage (Knox and Dodge, 1985; Salin, 1987; see section 1.2.2, page 5). The concentration of xanthophylls and total carotenoids in leaves was not affected by the level of UV radiation in these experiments. However, species varied in their xanthophyll/chlorophyll and total carotenoid/chlorophyll ratios (Table 5.1). *B.parviflora* and *B.gymnorrhiza* had significantly greater xanthophyll/chlorophyll and total carotenoid/chlorophyll ratios than *R.apiculata*.

The species with high carotenoid concentrations showed no change in total chlorophyll contents in response to UV radiation. This suggests that the levels of carotenoids in mangroves may be crucial to avoiding any adverse effects of UV or excess radiation energy.

In addition to carotenoids, the photorespiratory pathway and the Mehler reaction can contribute to the dissipation of excess energy and thus can probably reduce the likelihood of damage to the photosynthetic apparatus (see section 1.2.3, page 7). In the Mehler reaction super-oxide and hydrogen peroxide are produced and then detoxified. UV-absorbing flavonols (Takahama, 1983; Takahama, 1984) have been found to have a high capacity to eliminate H_2O_2 in chloroplasts. Thus, it is possible that the UV-absorbing compounds within mangrove leaves are involved in the detoxification of H_2O_2.

Using values published in the literature it is possible to calculate approximately the likely rate of production of oxidized flavonols if they react with H_2O_2 produced via the Mehler reaction when photosynthesis is limited. Assuming an O_2 uptake of 200 μmol.mg Chl.hr^{-1} (Osmond, 1981), with a conservative estimate of 5% of this uptake going to the Mehler reaction (Furbank and Badger, 1983), it could be expected that 5 μmol O_2^-.mg Chl^{-1}.hr^{-1} would be produced, which on reaction with super-oxide dismutase would produce 2.5 μmol H_2O_2.mg Chl^{-1}.hr^{-1}. The results of Takahama (1984) show that 2.5 μmol H_2O_2 will oxidize 5 μmol flavonol (quercetin).mg Chl^{-1}.hr^{-1}, or approximately 1.7 mg quercetin.mg Chl^{-1}.hr^{-1}. Therefore, a 20 cm^2 mangrove leaf with a chlorophyll concentration of 200 mg Chl.m^{-2} that is generating H_2O_2 for one hour a day (an estimate of the duration of the midday depression of photosynthesis in plants experiencing water deficits), would be expected to accumulate 0.7 mg of quercetin a day. In most mangrove leaves approximately 20% of the leaf dry weight is phenolic

compounds (like quercetin) (Figure 4.8, page 90). For a leaf that is 300 mg dry weight in which there is no transport or turnover of these compounds (which is unlikely, see review by Jensen 1985), it would take approximately 90 days to accumulate the 60 mg of flavonols present in mangrove leaves. Although these calculations do not provide evidence for this process, they do suggest that the involvement of flavonols in detoxifying hydrogen peroxide within chloroplasts is plausible.

In these experiments with mangroves where photosynthetic rates are low, excess energy (either UV or visible) absorbed by chlorophyll may have exceeded the capacity of the carotenoids to detoxify triplet state chlorophyll and singlet oxygen, and of zeaxanthin, and other energy dissipation processes to dissipate energy safely. Under these conditions the Mehler reaction may contribute to the prevention of photosynthetic damage. When the capacity of the carotenoids and super-oxide dismutase, ascorbate peroxidase and flavonol scavenging of H_2O_2 is exceeded, damage may occur to both the enzymes of the photosynthetic carbon reduction cycle and chlorophyll itself. This may in turn exert further pressure on the protective and repair mechanisms within the chloroplasts, finally leading to irreversible photoxidative damage.

In terms of this study, species differences in the capacity for dissipation of excess energy and removal of destructive triplet chlorophyll and singlet oxygen may determine the sensitivity of species to UV radiation and the associated increase in total radiation energy absorbed by leaves. That is, *B.parviflora* may have withstood high radiation in the UV depleted environment without a large activation of the Mehler reaction and flavonol production due to the presence of high concentrations of xanthophylls and total carotenoids (Table 5.1). In the near-ambient UV treatment the excess radiation energy may have reached a level where

the Mehler reaction and flavonol production were increased to the level of the other species. In *R.apiculata*, protective processes may have been saturated in the UV depleted conditions. Thus, a further increase in radiation energy absorbed by the leaves under the near-ambient UV treatment may have resulted in lower chlorophyll concentrations due to photooxidation of chlorophyll. The difference between the response of *B.gymnorrhiza* and *R.apiculata* (which had the same concentration of UV-absorbing compounds in both ambient and depleted UV conditions) may lie in the greater carotenoid concentrations and greater succulence (which increases the attenuation of UV radiation) of *B.gymnorrhiza* leaves (Table 5.1).

Sensitivity to UV radiation varied between mangrove species in this study. This could be attributed to the concentration of UV-absorbing compounds within mangrove leaves, to the concentration of carotenoids, and possibly to leaf succulence. By looking at the effects of visible radiation in the absence of UV radiation it was shown that the accumulation of UV-absorbing compounds in mangrove leaves occurs in the absence of UV radiation, but that UV radiation stimulates the production of these compounds in some species. It is proposed that the accumulation of UV-absorbing compounds may be linked to the amount of radiation energy absorbed by leaves that is dissipated through the Mehler reaction. The involvement of UV-absorbing phenolic compounds in removing active oxygen species generated through the Mehler reaction deserves further investigation.

CHAPTER 6

Conclusions

The study of protection from visible radiation centered on a study of the newly proposed photoprotective mechanism provided by the xanthophyll, zeaxanthin. The xanthophylls have rarely been studied in plants growing in their natural environments. In this study light regimes under which leaves grow were shown to be important in determining the amount of xanthophylls in leaves (see also Demmig *et al.*, 1987 and Thayer and Björkman, 1990). However, in sun leaves leaf angle is important in determining how much light a leaf absorbs, and it was shown that mangrove species with different leaf angles had different levels of xanthophylls in their leaves. Therefore, in Rhizophoraceae mangroves, avoidance of excess light by near-vertical leaf angles, and the dissipation of excess light energy by zeaxanthin, may be of equal importance in preventing photoinhibition in mangroves.

The relative abundance of species at the top of the canopy compared to their on-the-ground abundance (Table 2.3, page 36) also suggested that either near vertical leaf orientations, or high leaf xanthophyll contents influenced top-of-the-canopy position. However, the importance of leaf temperature was also evident as the large leafed *B.gymnorrhiza* was diminished in abundance at the top of the canopy relative to the abundance of its tree trunks at the forest floor. Mangrove species with near-vertical leaf angles tend to dominate regions that are highly saline. In addition, hypersaline regions are dominated by species that have near-vertical leaf angles and small leaves. This tends to support the view that near-vertical leaf orientations and the maintenance of leaf temperature close to that optimal for photosynthesis, by either avoiding solar radiation, or by having

smaller leaves (that result in more efficient transfer of heat away from the leaf than is likely in larger leaves under the same conditions), are important factors in determining the distribution of species over the range of soil salinities found in mangrove environments.

Although the light regime of the leaf growth environment correlated with xanthophyll/chlorophyll ratio (Fig. 2.2, page 32) it was also found to correlate with lower midday epoxidation states (higher zeaxanthin contents) (Fig. 2.3, page 33). This suggests that not only light levels, but factors that give rise to the formation of zeaxanthin are influential in determining the total level of xanthophylls within leaves (see also Thayer and Björkman, 1990). Zeaxanthin is proposed to be photoprotective through the dissipation of light that is absorbed by leaves that is not used for photosynthesis. As excess light should increase with both increasing light levels and decreasing photosynthetic rates, part of this study was aimed at quantifying the response of zeaxanthin concentrations to photosynthesis and solar radiation levels.

In mangroves growing in their natural environment, solar radiation and photosynthetic rates had a direct effect on the zeaxanthin contents within leaves, with photosynthesis accounting for a larger proportion of the variation in epoxidation state than solar radiation when light levels were saturating for photosynthesis. Leaf temperature had no direct, significant effect on epoxidation state, and thus exerted its influence on zeaxanthin formation through its effects on photosynthetic rates. As solar radiation influences epoxidation state directly, and indirectly through its influence over leaf temperature and photosynthesis, solar radiation probably exerts the largest influence over epoxidation state, and therefore over xanthophyll/chlorophyll ratios. The correlation between light

regime under which leaves grow and the xanthophyll/chlorophyll ratio support this conclusion.

The second part of this study investigated protection from UV radiation in mangroves. Protection from UV radiation in other plant species is associated with the accumulation of UV-absorbing phenolic compounds in the epidermis of leaves (Tevini *et al.*, 1991). These compounds were found in the epidermis of mangrove leaves. The concentration of UV-absorbing phenolic compounds varied between species, and between sun and shade leaves, with sun leaves having higher concentrations than shade leaves. In addition, the concentration of UV-absorbing phenolic compounds within *R.stylosa* leaves varied between sites within the same river system (Fig. 4.6, page 88), with sites with higher salinities having trees with greater concentration of UV-absorbing compounds within their leaves. Thus, it was proposed that factors other than UV radiation may be influencing the accumulation of these compounds.

In order to assess directly the effects of UV radiation on three species of mangroves, plants were grown in natural sunlight either with, or without UV radiation. Accumulation of UV-absorbing compounds occurred in both depleted and near-ambient UV treatments, with two out of three species having similar concentrations of UV-absorbing compounds in both the near-ambient and depleted UV treatments, and one species (*B.parviflora*) having a higher concentration of UV-absorbing pigments in the near-ambient compared to the UV-depleted treatment (Fig. 5.5, page 107). In addition, chlorophyll contents of *R.apiculata* were lower in the near-ambient UV treatment relative to the depleted UV treatment (Fig. 5.6, page 108). This showed that under the conditions in this experiment, the accumulation of UV-absorbing phenolic compounds in mangroves is influenced by factors other than UV radiation, but that UV radiation

can influence the accumulation of UV-absorbing compounds, and chlorophyll concentrations in some species.

The concentration of the UV-absorbing compounds and the chlorophyll a/b ratio (which is know to increase with increasing incident radiation levels) correlated over all species. It was proposed that the effect of UV radiation in this study may be related to increases in the total radiation absorbed by leaves. This assumes that UV radiation is absorbed by chlorophyll, or some chromophore that transfers energy to chlorophyll, and is handled by the plants photosynthetic apparatus in the same way as visible light. The enhancement of photosynthesis by UV radiation has been shown in coral species (Schlicter et al., 1985, Schlicter et al., 1986) and in soybean (Teramura et al., 1980). Thus, the species differences in their response to UV radiation observed in these experiments may reflect the overall ability of species to respond to increases in excess radiation energy. That the concentration of carotenoids (which are known to protect chlorophyll against photooxidative damage) varied between species, with *R.apiculata* (the species with decreased chlorophyll under near-ambient UV conditions) having the least carotenoids, and *B.parviflora* (the species with the increased concentration of UV-absorbing compounds under near-ambient UV conditions) having the greatest concentration of carotenoids within its leaves tends to support this view.

The production of UV-absorbing compounds may be linked to the amount of radiation energy absorbed by leaves that is in excess of what can be used for photosynthesis, through the Mehler reaction. The idea that the Mehler reaction may be important in providing protection from photoinhibition in mangroves is based on the following evidence: 1) UV-absorbing phenolic compounds are oxidised by hydrogen peroxide in chloroplasts in the presence of light, thereby detoxifying it (Takahama, 1984), 2) hydrogen peroxide is produced via the

Mehler reaction at PS I when photosynthesis is limited (it is thought that this process provides a protective function, as it allows continued non-cyclic electron transport, and hence contributes to the dissipation of excess energy) (see review by Krause and Cornic, 1987), 3) high concentrations of UV-absorbing compounds are found within mangrove leaves (approximately 20% of leaf dry weight) (Fig. 4.8, page 90), and 4) UV-absorbing phenolic compounds were found in mangroves under both depleted and near-ambient UV treatments during exposure to high levels of visible radiation when photosynthetic rates were low (Fig. 5.5, page 107). Thus, the ability to produce and sequester UV-absorbing phenolic compounds within leaves may be vital in determining the relative tolerance of species to environmental stress in high radiation environments.

It was speculated that differences in species responses to UV radiation were due to the capacity of each species to provide protection from radiation energy. The xanthophylls and carotenoids are protective against the effects of excess light energy, but the capacity of these protective mechanisms may be exceeded, as may have been the case for *R.apiculata* in the UV depleted treatment and *B.parviflora* in the near-ambient UV treatment. When this occurs the Mehler reaction may become important in providing protection from excess light, with UV-absorbing phenolic compounds being involved in detoxifying hydrogen peroxide, being accumulated at a rate proportional to that of the Mehler reaction. It is also possible that the ability to detoxify hydrogen peroxide and other active oxygen species may also become limited, and when this occurs, oxidation of chlorophyll may exceed the rate at which it is synthesized, resulting in decreased chlorophyll concentrations and possible damage to other components of the photosynthetic apparatus.

In mangroves, as probably in other plant species, adaptation to conditions that would otherwise predispose plants to photoinhibition will be dependent on the ability to avoid excess light, through strategies like near-vertical leaf angles, and the "safe" dissipation of excess energy. The dissipation of excess energy could occur through many different pathways (see section 1.2.3, page 7). This thesis has concentrated on protection from solar radiation in mangrove species provided by leaf morphology, the xanthophylls and other carotenoids, accumulation of UV-absorbing phenolic compounds, and the potential protection provided by the Mehler reaction. In order to fully assess the relative importance of the photoprotective mechanisms investigated in this study, quantitative comparisons between the protective processes examined here and other mechanisms plants have to protect themselves from solar radiation damage (e.g. fluorescence, photorespiration, inactive PS II centres, and state transitions) need to be investigated over a range of environmental conditions.

APPENDIX I

Chloroplast movement in the mangrove *Rhizophora stylosa*

Introduction

It has been proposed that chloroplast movement in response to high light levels may be a means of avoiding light induced damage to the photosynthetic apparatus (Haupt, 1982; Powles, 1984; Osborne and Raven, 1986). The experiment described here was done to assess whether chloroplast movement occurs in the mangrove *Rhizophora stylosa*, and whether it might be a factor involved in photoprotection.

Materials and methods

Chloroplast movement was assessed by measuring changes in the light transmitted through leaves over time after the incident light level was increased. Intact leaves of whole, shade grown *Rhizophora stylosa* plants were taped to Delta T light sensors (Model QS-005, Delta T Devices, Burwell, Cambridge, England) in the laboratory. A tungsten halogen light source (Sylvania, Japan) screened by an infrared filter and neutral density filters (Schott, Germany) was used.

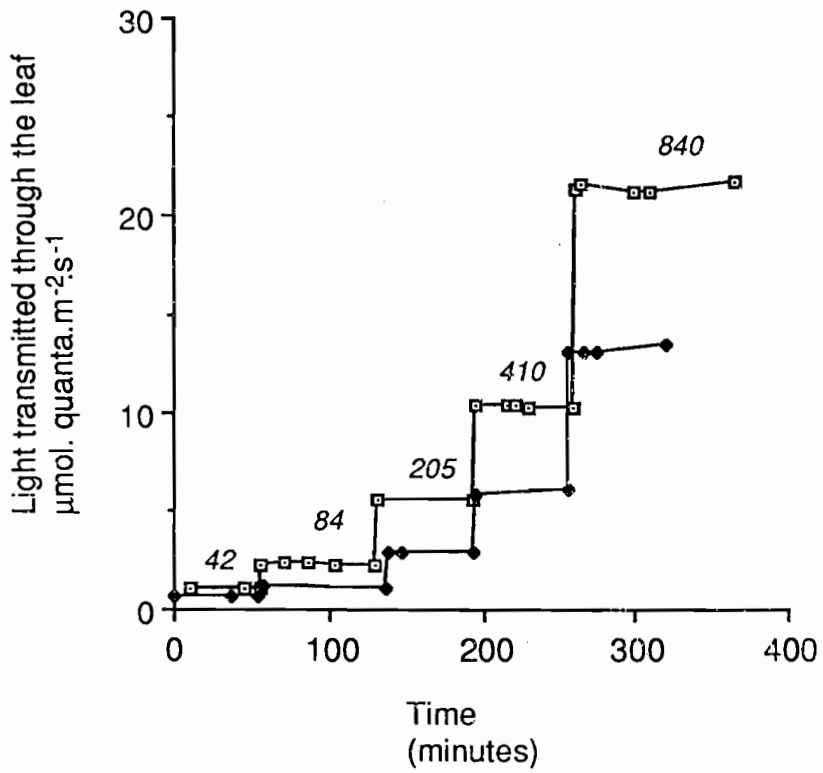
Experimental protocol

In order to maximize the likelihood of detecting any diurnal changes in leaf transmittance due to chloroplast movement the following procedure was used.

Plants were kept in the dark in the laboratory overnight. At 8.00 am lights were switched on and light levels were increased every hour (using neutral density filters) with maximum light level (approximately 1000 μmol quanta.m^{-2}.s^{-1}) reached at midday.

Results and conclusions

Transmittance of light through mangrove leaves was low (less than 5%). Exposure to light levels higher than those in which the leaf was previously acclimatized did not result in increased transmittance of light through the leaves (Appendix figure 1). This suggests that chloroplasts of *R.stylosa* do not move in response to increased light levels. Alternatively, it is possible that they do move, but without having any effect on the light that is transmitted through the leaf. In this case, chloroplast movement would probably be of little value in reducing the light absorbed by leaves, and would be unlikely to provide protection from high light levels. Haupt and Scheuerlein (1990) found no evidence to suggest that chloroplast movement contributed to protection from photoinhibition, and concluded that chloroplast movement was unlikely to provide protection from photoinhibition in plants where leaf absorbance is high.



Appendix figure 1. The light transmitted through two leaves of *R.stylosa* (leaf 1, squares; leaf 2, diamonds) as light levels are increased stepwise. The photon flux density leaves were exposed to appears in italics.

APPENDIX II

Nutrient culture of mangroves

Mangrove propagules were cultured in a similar way to that described in Clough (1984). Propagules were grown in washed beach sand in 240 mm tall, 2.4 litre PVC pots with a 7mm drainage hole. The pots were placed in troughs of sufficient depth to allow them to be completely flooded when the troughs were full of nutrient solution. Plants were irrigated daily for 1.5 hours by pumping nutrient solution into the trough from a reservoir beneath the trough, and then allowing it to drain back into the reservoir.

Saline nutrient solutions were made by mixing tapwater and seawater, to which supplementary nutrients were added. The composition and concentration of the nutrients in the nutrient solution are show in Appendix table 1. The concentration of individual nutrients in the irrigating solution was checked fortnightly and amended as required. Nutrient solutions were replaced monthly.

Appendix table 1. Elemental composition of nutrient culture solution in which mangroves were grown.

Major Nutrients		Minor nutrients	
Element	Concentration ($mol.m^{-3}$)	Element	Concentration ($mmol.m^{-3}$)
N (NH_4^+)	1.71	Fe	72.0
N (NO_3^-)	11.14	B	18.0
P	0.16	Mn	3.5
S	1.03	Zn	1.2
K	2.05	Cu	0.3
Ca	3.74	Mo	0.005
Mg	1.03		

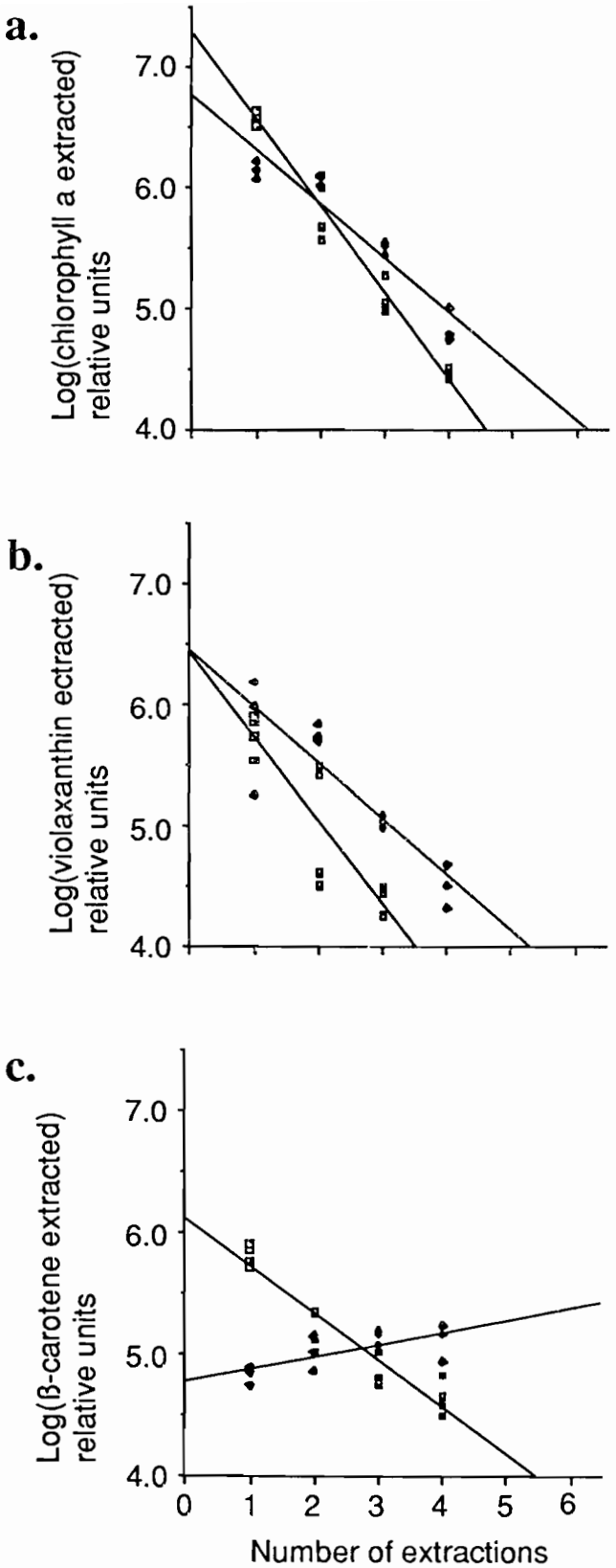
APPENDIX III

Efficiency of leaf pigment extraction procedure

The efficiency with which pigments can be extracted varies with the plant tissue used. The extraction efficiency must therefore be known in order to quantify accurately the pigment concentration (Dunlap and Chalker, 1986). Multiple extractions of the same piece of tissue was done, and the amount of pigment extracted after each extraction step is measured by HPLC (Appendix figure 2). A linear extrapolation of the regression of the logarithm of the pigment extracted after each extraction step gives the predicted number of extractions to remove all the pigment (X axis intercept), and also an estimate of the total pigment present (Y axis intercept).

A comparison of the extraction efficiency of 80% and 100% acetone (Appendix figure 2) showed that 100% acetone was a better solvent for carotenoids, extracting 96% of the xanthophylls and 76% of ß-carotene by the third extraction, compared to 76% of the xanthophylls and an unknown proportion of ß-carotene extracted when using 80% acetone. The ß-carotene content could not be estimated when 80% acetone was used as the quantity of ß-carotene increased with each successive extraction (Appendix figure 2c).

Many of the quantitative studies on carotenoid pigments appearing in plant physiological literature use 80% acetone for extractions with no reference to the efficiency of their extraction procedure. The results presented here show that for mangrove leaves, at least three extractions in 80% acetone are required for quantification of xanthophyll pigments, and that three extraction in 100% acetone are required to quantify ß carotene.



Appendix figure 2. Efficiency of multiple extractions of mangrove leaf tissue in either 80% (•) or 100% (▪) acetone for; a) chlorophyll a, b) violaxanthin, and c) ß-carotene.

APPENDIX IV

Quantification of violaxanthin, lutein, neoxanthin and antheraxanthin without commercially prepared standards

Pure fractions of violaxanthin, lutein and neoxanthin were obtained by HPLC. The same system outlined in Method 1 (2.2.5.2, page 26) was used. In order to obtain enough carotenoids for quantification, the 25cm C-18 column was replaced with a semi-preparative C-18 column (Brownlee, Applied Biosystems, Foster City, USA) and the flow rate adjusted to 2.5ml/min. Eluted peaks were collected as they passed through the detector. The collected fractions were freeze dried and then redisolved in 1 ml ethanol. The absorption spectra of these solutions were measured in a 1ml cuvette using a scanning spectrophotometer (Hitachi, model U-3200, Japan). Their identity was confirmed by comparing their absorption spectra with published values. They were quantified using published extinction coefficients ($E^{1\%}_{1cm}$) (Davies 1976). 50µl of the 1ml ethanol solution was then reinjected into the HPLC system so that the integral of the peak on the chromatogram could be attributed to a known quantity of the carotenoid. The following is an example calculation for violaxanthin:

$E^{1\%}_{1cm}$ = 2550 at 443 nm wavelength

$x\ g = E_y / E^{1\%}_{1cm} * 100$ where x is grams per ml and E_y is absorbance in a 1cm path length cuvette.

$= 0.2593 / (2550*100) = 1.0167\ \mu g.ml^{-1}$

50 μL injected has 0.05084 μg of pigment

Integrated peak area from this is 1 185 517

Conversion factor (integrated values converted to μg. cm^{-2} leaf area)

= integration * 0.05084 / 1 185 517 * dilution factor / leaf area

The conversion factor calculated for violaxanthin was used for antheraxanthin as a quantity of antheraxanthin sufficient for quantification in the manner described above was difficult to obtain. $E^{1\%}_{1cm}$ in ethanol for this compound is similar to violaxanthin, i.e. violaxanthin is 2550 at 443 nm, and antheraxanthin is 2500 at 444 nm.

APPENDIX V

Ideas for future research

1. Assessment of the significance of photoprotection (both morphological and biochemical) on long term growth of plants, and how photoprotection influences competition between species and community structure: Plant communities that inhabit extreme environments (like mangroves) would be good targets for this research as under conditions of physiological stress small adaptations may make large differences to competitive ability of species.

2. Investigation of the mechanism by which zeaxanthin dissipates absorbed light that is not used in photosynthesis.

3. A quantitative study of the energetics of photoprotection: For example, Dau and Hanson (1990) estimate that 10% of the light absorbed by leaves is emitted as fluorescence. The proportion of energy dissipated through fluorescence, carotenoids, photorespiration, the Mehler reaction, inactive PS II centres, and photoinhibition should be assessed over a wide range of photosynthetic rates, temperatures and light levels, and for different genotypes. Consideration should also be given to the time frame over which each of these mechanisms may be operating.

4. Examination of the role of photoprotective processes in transient light conditions (e.g. sunflecks): In this study with mangroves, leaves in deep shade had variable xanthophyll concentrations (Fig. 2.2, page 32). Whether this has significance for protecting the photosynthetic apparatus from photoinhibition

when shade leaves are exposed to short durations of high light levels remains to be investigated.

5. Investigation of the effects of UV-A (320-380 nm) radiation on plants: An action spectrum for damage and for photosynthetic enhancement is required, as is work on the interaction of UV-A radiation with visible light and other environmental stress.

6. Investigation of what function phenolics have in plant leaves, particularly their involvement in photosynthesis and with the products of the Mehler reaction: More generally, a physiological role for plant secondary metabolites should be investigated. There are interesting and noteworthy cases where secondary plant products may be implicated as an adaption to environment. For example, there are high concentrations of anthocyanins in newly emerged leaves of many plants, and alkaloid containing plants increase in abundance with decreasing latitude (Levin, 1976). Many of these compounds are formed through the shikimic acid pathway, and thus can be directly linked to carbohydrate metabolism of plants (Jensen, 1985). In addition, many of these aromatic compounds absorb light and are readily oxidized.

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