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Do flavonoids and tannins have a role in photoprotection in mangroves?

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Introduction

Mangroves, as denizens of tropical intertidal zones, are subject to simultaneous environmental stresses including high light, high temperature, high salinity and low nutrient availability. In our previous studies, we have examined photosynthesis and photoprotection using a combination of gas exchange, fluorescence and biochemical techniques (Cheeseman et al., 1991, Cheeseman et al., 1997). Using *Rhizophora stylosa* mangroves from Western Australia, we reported excess electron flow through PSII when CO_2 fixation was limited by stomatal closure. Based on the behavior of C_i during stomatal closure, and our inability to open stomates or enable recovery of gas exchange once stomates had closed, we suggested that Rubisco itself was down regulated. We also found that the photochemical efficiency of PSII ($ø_{II}$) remained high up to 500 µmol photons m^{-2} s^{-1}, suggesting that PSII was not being down regulated. Fv:Fm recovered to ~0.8 within 20 min of darkening.

To examine the role of the Mehler-Ascorbate peroxidase (MAP) pathway in energy dissipation, we assayed ascorbate (Asc) and glutathione, and glutathione reductase, ascorbate peroxidase (APx) and superoxide dismutase (SOD) in field grown *R. stylosa* and greenhouse grown *R. mangle*, finding that SOD levels were nearly 40 times higher than APx in *R. stylosa* and more than 6 times higher in *R. mangle* (Cheeseman et al., 1997). At 8 mM on a tissue water basis, Asc seemed amply available as a substrate for APx. We concluded that the problem of $O_2^{\bullet}$ scavenging might be transferred to one of H_2O_2 scavenging. We also suggested that because of the diffusability of H_2O_2 and its involvement in tannin production, lignification and defense, this might be a good ecological strategy.

At about the same time, Yamasaki et al. (1997) presented a model for H_2O_2 detoxification that relied on the cyclic oxidation of flavonoids, and re-reduction of flavonoid free radicals by ascorbate (Asc) before oxidative decomposition occurred. Asc would, in turn, be re-reduced by dehydroascorbate reductase. Both the production of H_2O_2 and the re-reduction of Asc could serve as protective consumers of electrons.

Here, we examine photosynthesis, phenolic peroxidase (POX) activity and the phenolic composition of *R. mangle* to relate the Yamasaki model to mangroves in an ecologically relevant context.

Materials and methods

Trees were sampled at Twin Cays, a Smithsonian Institution research site 12 km off the coast of Belize (Central America). Initial acetone extraction of the tissue was performed either at

the Smithsonian lab on Carrie Bow Caye, or at Illinois using leaves harvested just before returning from the field. In this paper, we will emphasize data from the interior, "dwarf" zone (Rützler and Feller, 1996). POX was also measured in leaves from the "fringe zone", a nearby rookery island (Man O War Caye) and a mainland estuary (Sittee River). Gas exchange studies were performed *in situ* using a Licor 6400 gas analyzer. Fluorescence measurements, *in situ* and in the Carrie Bow lab, used an Opti-Sciences OS-500 PAM fluorimeter. Soluble POX was assayed at pH 5.3 in acetate buffer with 2.4 mM guaiacol (ε = 25.5 $mM^{-1}cm^{-1}$ at 436 nm) and 1.1 mM H_2O_2 as substrates. Dried tissue was resuspended with grinding in buffer at a concentration of 0.1 g/mL and allowed to reconstitute overnight; little additional activity resulted from re-extraction with 1 M NaCl. The enzyme was stable for several days with refrigeration. Preliminary studies indicated that there was no active APx in the assays.

Flavonoids and tannins were analyzed from the acetone soluble material using TLC (pre-coated silica gel 60 with ethyl acetate-methanol-water (79:11:10) as the solvent), and HPLC (C18 reverse phase column with a solvent gradient running from 0.5% MeOH in water to 100% acetonitrile). Individual constituents were identified using co-TLC chromatography with known standards, and by NMR and MALDI mass spectroscopy (Kandil, Seigler and Cheeseman, in preparation). Total condensed tannins in crude extracts were quantified according to Hagerman and Butler (1978).

Results

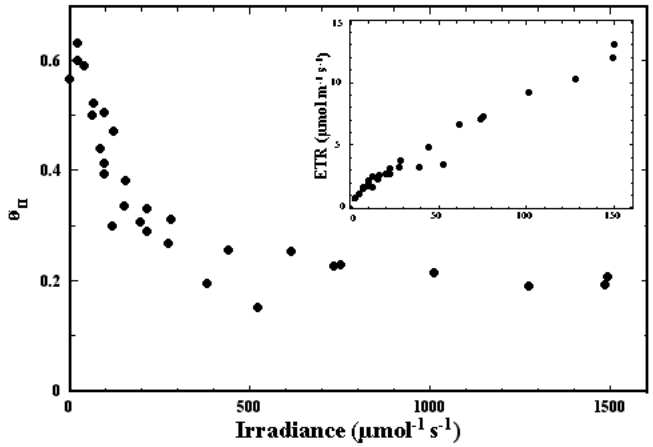

Figure 1. Response of $ø_{II}$ and electron transport through PSII to irradiance in dwarf *R. mangle* leaves. Data were collected using excised branches in the laboratory; collection was at mid-day and experiments were performed ca. 2 h later. Combined results of two experiments are shown.

Based on approximately 180 *in situ* measurements, net CO_2 assimilation in *R. mangle* leaves light saturated at approximately 500 µmol $m^{-2}s^{-1}$ both in the taller, more rapidly growing trees along channels (the "fringe" zone) and in the stunted trees of the dwarf zone (data not shown). The saturated rate was, however, 75% higher in fringe zone leaves (9.5 vs. 5.5 µmol CO_2 $m^{-2}s^{-1}$). Assimilation peaked in the early morning and declined after 1030 h. Unlike our previous report (Cheeseman et al., 1997) for *R. stylosa*, however, the photochemical efficiency of PSII ($ø_{II}$) declined with irradiance to the level at which assimilation saturated (Figure 1, see also Demmig Adams, 1998). This was reflected in the relationship between calculated electron transfer through PSII and irradiance as curvature at very low light (insert, Fig. 1). Thus, at higher irradiance, the magnitude of excess electron flow could increase, particularly if Rubisco activity were limited (Cheeseman et al., 1997).

The description of POX activity was problematic as no basis of expression (e.g. weight, area or chlorophyll) was unaffected by environmental factors which would also influence POX activity. For the present, we have selected an area basis because of the comparability it affords to measurements of photosynthesis and because, since overall POX was positively correlated to specific leaf mass, variations in activity per area would most reflect environmental influences.

Figure 2 shows POX activities, with site, age and nutrient availability effects. Enzymes from fringe and dwarf zones, and from Man O War Caye had similar activities. Phosphorous fertilization in the dwarf zone greatly stimulated growth and P–sun leaves had reduced activities. Comparable results were found in other rapidly growing leaves ("Dwarf – young") and leaves from the estuarine site (Sittee River) which receives nutrient enrichment from agriculture. The "Best Zone", an isolated area with anomalously good growth within the dwarf zones, also had reduced POX activity. Activity increased with age as seen from the comparison of young and old leaves from single dwarf trees.

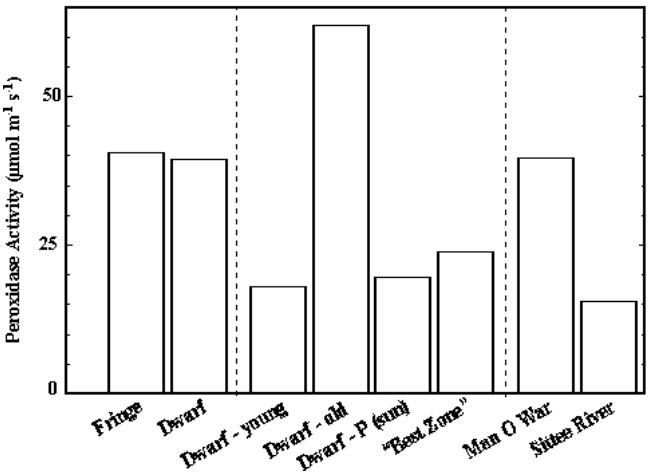

Figure 2. Phenolic peroxidase (POX) activity in leaves of *R. mangle* collected at Twin Cayes, Belize, at a nearby rookery island (Man O War Caye) and at a mainland estuarine site (Sittee River). Groups with high, intermediate and low activity are statistically different.

POX activity was also stable during leaf senescence (Figure 3), possibly reflecting its roles in functions other than photoprotection, but suggesting that the structure of the phenolic population may also change during the senescence process.

Based on the Hagerman-Butler assay, condensed tannins accounted for approximately 8% of the total dry weight, with no significant difference between habitats and treatments, though levels increased with age (data not shown). The dominant flavonoids were quercetin diglycosides, especially rutin, with significant quantities of monoglycosides and some triglycosides (see Fig. 4). Only in senescent leaves was the aglycone visible on TLCs. Condensed tannins were polymers of catechin and epicatechin (Kandil, in preparation). Of these, only the aglycone, quercetin, was a reasonably good substrate for peroxidase. However, crude acetone extracts were quite active in this role based on an assay in which ΔA_{436} was used as a measure of general browning (unpublished data). As expected (Yamasaki et al., 1997), H_2O_2 dependent oxidation could be stopped by KCN (which inhibits POX) and by Asc, putatively through re-reduction of the phenolic free radicals.

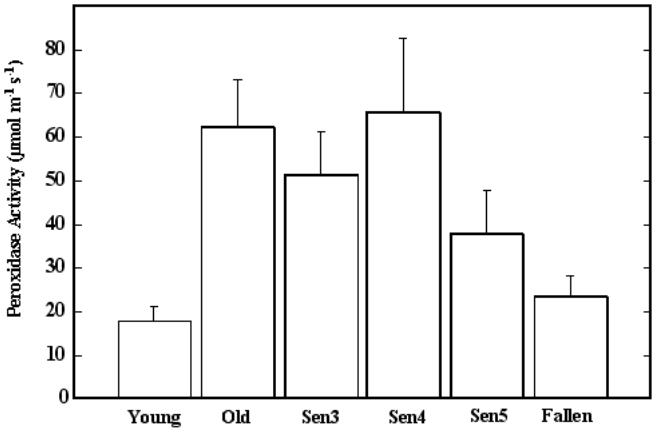

Figure 3. POX activity through leaf development and senescence. All leaves collected from the dwarf zone in January. Sen3 leaves had lost all visible green. Sen4 were ca. 50% reddish and Sen5 were quintessentially red with little other color. Fallen (floating) leaves were rinsed to remove surface slime before extracting.

TLC analysis (with vanillin HCl reactivity) showed progressive condensation of tannins during senescence; whether this is enzymatically mediated remains to be seen. At the same time, the flavonoid glycosides were partially hydrolyzed, and quercetin appeared (Kandil, Seigler and Cheeseman, in preparation).

Nevertheless, HPLC analysis showed few qualitative differences in the major peaks as leaves progressed from young and expanding, through yellowing, to floating face down in a pool of salty water (Figure 4). The major peaks in all stages correspond to quercetin

glycosides. We are currently in the process of further fractionating crude phenolic extracts and testing their suitability as substrates in order to put the role of the flavonoid oxidation and recycling system into the context of the overall photoprotective activities in mangrove leaves.

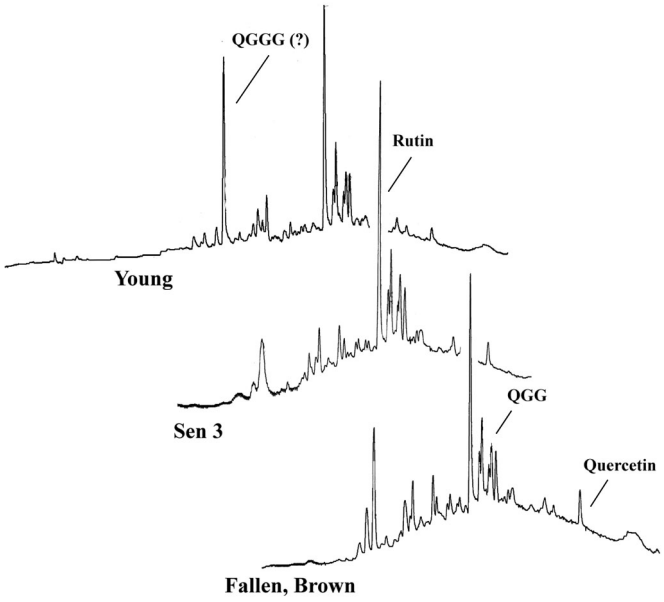

Figure 4. HPLC output showing major phenolic constituents of *R. mangle* leaves as they progressed from young, recently expanded, to yellowed during senescence, to fallen. QGG = quercetin diglycosides of which only rutin has been specifically identified. QGGG?= suspected quercetin triglycosides. Despite their quantitative importance, proanthocyanidins do not show up as major components with this solvent system.

Discussion

R. mangle exemplifies a group of plants which has limited opportunity for net CO_2 fixation because of environmental stresses that conspire to limit gas exchange. Moreover, during those few hours when conductance is positive, N and P limitations restrict the use of products. The strategy that mangroves appear to have adapted is to produce relatively non-palatable compounds built only from basic carbohydrate skeletons. These can be metabolized to recycle the C if N and P become available, but they can also be used as protection against herbivores, absorbers of UV light, antioxidants, and electron acceptors for detoxification of reactive oxygen species generated in photosynthesis and oxidative metabolism. From this study, it is clear that all the components required for operation of the Yamasaki model are present in mangroves, but that separating their function from other aspects of phenolic metabolism may be difficult at any time; the balance between them almost certainly shifts throughout the life cycle of a leaf, until in the end, maintenance of active peroxidase in fallen leaves may speed the recycling of those nutrients not removed during the remobilization processes of senescence.

Acknowledgements

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