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ORIGINAL ARTICLE

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Polyphenolics in *Rhizophora mangle* L. leaves and their changes during leaf development and senescence

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Abstract The chemical defenses in *Rhizophora mangle* L. are largely carbon based. The family has long been exploited for the high proanthocyanidin (condensed tannin) content of its wood, bark and leaves. In this paper, we quantify the overall pools of plant phenolics in *R. mangle* leaves, identify the major constituents of these pools and document their changes during leaf maturation and senescence. Overall, polyphenolics account for approximately 23% of the total leaf dry weight. The leaves contain at least seven flavonoid glycosides, five of them based on quercetin. Additional minor constituents are myricetin and kaempferol diglucosides. The aglycone, quercetin, was found only in senescing leaves. Also during senescence, a new compound, 5,4′-dimethoxy-7,3′,5′-trihydroxyflavone, appeared. The flavonoids were accompanied by a complex mixture of condensed tannins based mainly on (+)-catechin and (−)-epicatechin with A-type and B-type linkages; this pool is also distinguished by having previously unreported, high contributions of (+)-catechin and (−)-epicatechin glycosides. During senescence, but prior to leaf abscission, the polyphenolic pools become simplified: flavonol glycosides and low oligomeric tannins largely disappear, leaving only the largest tannin polymers. The ecological and physiological significance of these compounds as they appear in *R. mangle* is discussed.

Keywords Flavonoids · Quercetin glycosides · Condensed tannins · Proanthocyanidin glycosides · 5,4′-Dimethoxy-7,3′,5′-trihydroxyflavone

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Introduction

Mangroves are a diverse group of trees that comprise intertidal tropical forests. Mangrove communities are, in general, oligotrophic, limited at least by availability of N and P. Under these circumstances, defense of leaves, as the organs of photosynthesis, is of paramount importance. Overall, mangroves accomplish this effectively; Feller (1995), for example, showed that folivory resulted in less than 5% loss of leaf area by *Rhizophora mangle* in a Belizean community.

Chemical defenses in the Rhizophoraceae are largely carbon based (e.g., polyphenolics), although some species also contain tropane alkaloids (Hegnauer 1973,1990; Romeike 1978). In leaves, proanthocyanidins (condensed tannins) and other flavonoids may comprise more than 20% of the total dry weight (Robertson 1988; Benner et al. 1990a). These are known to deter feeding by insects and other herbivores (McKee and Feller 1995; Feller et al. 1999), but they also show a diversity of other biological activities of historic and potential importance to humans (Mainoya et al. 1986). In addition to uses in leather tanning, mangrove extracts have been used for diverse medicinal purposes and have a variety of antibacterial, antiherpetic and antihelminthic activities (Martínez 1969; Pittier 1978; Lemmens and Wilijarni-Soetjipto 1991). Recently, catechin and related compounds capable of forming *o*-quinones on oxidation have also indicated promise for antimelanoma therapies (Moridani et al. 2001).

Nevertheless, from the standpoint of a plant, polyphenolics represent a substantial diversion of carbon from growth and maintenance uses. Thus, understanding the nature, metabolism, and consequences of these compounds—not limited to defense—is important to understand the biology of the trees and the mangrove ecosystem more generally. The identification and characterization of polyphenolics from the Rhizophoraceae is as yet incomplete, but a number of studies have identified interesting and novel constituents (Bandaranyake 1995). For example, although the most common condensed tannins are

comprised of (+)-catechin and (−)-epicatechin, two unusual proanthocyanidin dimers, kandelins A-1 and A-2, were reported from the bark of *Kandelia candel* (Hsu et al. 1985) and a new A-type proanthocyanidin was reported from the bark of *Cassipourea gerrardii* (Drewes et al. 1992). Proanthocyanidin glycosides, now established to occur in a number of other plants (Ishimaru et al. 1987; Zhang et al. 1988; Kolodziej et al. 1991; Bae et al. 1994; Hatano et al. 2002), have also been reported from mangrove bark. For example, Achmadi et al. (1994) reported catechin-3-*O*-rhamnoside chain extending units in polymeric proanthocyanidins of *Bruguiera gymnorrhiza*.

The present study was performed to characterize the polyphenolics in *R. mangle* leaves, to document their changes during development and senescence, and to begin consideration of their roles at levels ranging from the individual organism to the community. The mangrove ecosystem used in these studies is located 19 km off the coast and approximately 3 km inside the barrier reef at Twin Cays, Belize (Rützler and Feller 1996; Middleton and McKee 2001). It is a highly oligotrophic, peat-based island group lacking terrigenous nutrient input (Feller et al. 1999; Middleton and McKee 2001). Like many Caribbean mangroves (Lugo and Snedaker 1974), these islands have distinct zonation, with *R. mangle* (red mangrove) dominating in all zones. The fringe zone, adjacent to the ocean or waterways, is relatively narrow (3–15 m), with trees that may reach 7 m tall, whereas in the interior dwarf zones, trees are seldom more than 1.5 m tall (Feller et al. 2003).

Materials and methods

Plant material collection

Leaves of *R. mangle* L. were collected at Twin Cays, Belize in January 2001. A voucher specimen has been deposited at the University of Illinois Herbarium (ILL). The collection site was the dwarf zone at Boa Flats on the East Island (88.10500° W, 16.82556° N). Because of the small numbers of leaves present on any tree, each sample was the combined harvest of up to ten trees. "Young" leaves were the most recently fully expanded leaves, with an average specific leaf mass (SLM) of 530±20 g m^{-2}. Old leaves were the oldest remaining, fully green leaves—usually the third or fourth pair—with an average SLM of 910±20 g m^{-2}. "Fallen" leaves were senescent leaves recovered from the surface of the peat. They were intact, i.e., showing only the preliminary stages of fungal and bacterial colonization prior to fragmentation. The leaves were rinsed in fresh water and surfaces were allowed to dry in air in a dark room. Other senescent leaves, i.e., showing at least the preliminary stages of yellowing and loss of chlorophyll, were still attached to the trees prior to collection. They were separated into senescence "classes": Sn1 were green with incipient chlorosis, Sn2 were chlorotic with approximately 10–20% remaining green, Sn3 were clearly and uniformly yellow, Sn4 were yellow with ca. 50% reddish coloration, Sn5 were quintessentially red with little other color, and Sn6 were totally brown. These classes are similar to those used by Benner et al. in their study of chemical changes during leaf decay in *R. mangle* in the Bahamas (Benner et al. 1990a,b), although their leaves had fallen prior to collection.

General

Silica Gel Type G, 10–40 μm, $CaSO_4$ binder (S-6503, Sigma, St. Louis, MO) was used for vacuum chromatography. Thin-layer chromatography (TLC) was carried out on pre-coated plates (Merck 1.05554, DC-Alufolien, Silica Gel 60 with F254 fluorescent indicator, 0.2 mm; Sigma, St. Louis, MO, USA) using ethyl acetate/methanol/water (79:11:10) as the solvent system. Fractionations were monitored by TLC, visualizing results with UV illumination, vanillin-HCl reagent and chromic acid solution (parallel plates). Vanillin–HCl reagent was a 10% solution of vanillin in methanol, acidified with 5–10 drops of concentrated HCl per liter (Kandil et al. 2000). Chromic acid solution was prepared by dissolving 5 g $K_2Cr_2O_7$ in 100 ml of 40% sulfuric acid solution (Stahl 1969). After spraying, plates were heated at 100°C for 10 min.

^{1}H-NMR and ^{13}C-NMR spectra were measured on a Varian 400 at 400 and 100 MHz, respectively, in DMSO-*d6* (Varian, Palo Alto, CA, USA). Mass spectra were determined by fast atom bombardment (FAB) on a Micromass ZAB-SE Spectrometer (Waters, Beverly, MA, USA), and matrix-assisted laser desorption/ionization (MALDI) on a Voyager-DE STR Spectrometer (Framingham, MA, USA) in the Mass Spectrometry Laboratory of the School of Chemical Sciences, University of Illinois, Urbana-Champaign.

Extraction, quantification and fractionation

For estimation of total tannins and phenolics, samples were collected from dwarf zone trees, and immediately placed in vials with 80% ethanol (EtOH). Each sample consisted of five, #3 cork-borer disks with a total area 2.04 cm^2. Parallel samples were taken for dry weight determination. For extraction, the EtOH was removed and the disks were crushed in a glass homogenizer in 80% EtOH, then extracted at 60°C for 24 h with three changes of solvent. The extracts, including the original collection solution, were combined and the volume adjusted to 10 ml. Subsamples of the extract were precipitated with BSA according to Hagerman and Butler (1978), but using a BSA-to-sample ratio of 10 rather than 2; this change was required because of the high tannin concentrations in the leaves. Following centrifugation of the tannin-containing pellet, the supernatant was analyzed for non-tannin phenolics using the $FeCl_3$/$KFe(CN)_6$ (Prussian blue) assay according to the original procedure of Price and Butler (1977). For tannins, the standard curve was based on a highly purified fraction from "young" leaves, consisting largely of highly polymeric (+)-catechin and (−)-epicatechin subunits (see below). For phenolics, the standard curve was based on an equal mass mixture of the two most dominant non-tannin polyphenolics, rutin and chlorogenic acid. It should be noted that the tannin and phenolic levels reported here are somewhat higher than those reported by McKee (1995), Feller (1995) and Hernes et al. (2001), also for *R. mangle*. This may reflect the more extensive extraction protocol in the current study, or the choice of the compounds—in the present case, the major components of the leaves—used as calibration standards (K. McKee, personal communication). The differences may also reflect the growth conditions of the trees; McKee (1995), for example, used fertilized, greenhouse plants.

For identification of the individual components, approximately 300 g of frozen, fresh leaves (young, old, senescence stages 2, 3, 4, 5 and 6, and fallen leaves) were extracted in a Waring blender with 70% aqueous acetone (three aliquots of 1 l of solvent for 30 min each). The extracts were combined, concentrated under vacuum to remove the acetone, and freeze-dried. Portions (15 g) of the freeze-dried material from each sample were dissolved in a minimum amount of methanol (MeOH), mixed with 10 g silica gel, and air dried. These mixtures were placed on the top of silica gel columns [GF-254, type 60 (Merck, Darmstadt); 300 g, column diameter 7 cm, length approximately 10 cm] that had been washed with petroleum ether. The columns were again washed with petroleum ether (150 ml). Subsequent fractions were eluted by vacuum chromatography with ethyl acetate (solvent A); increasing amounts

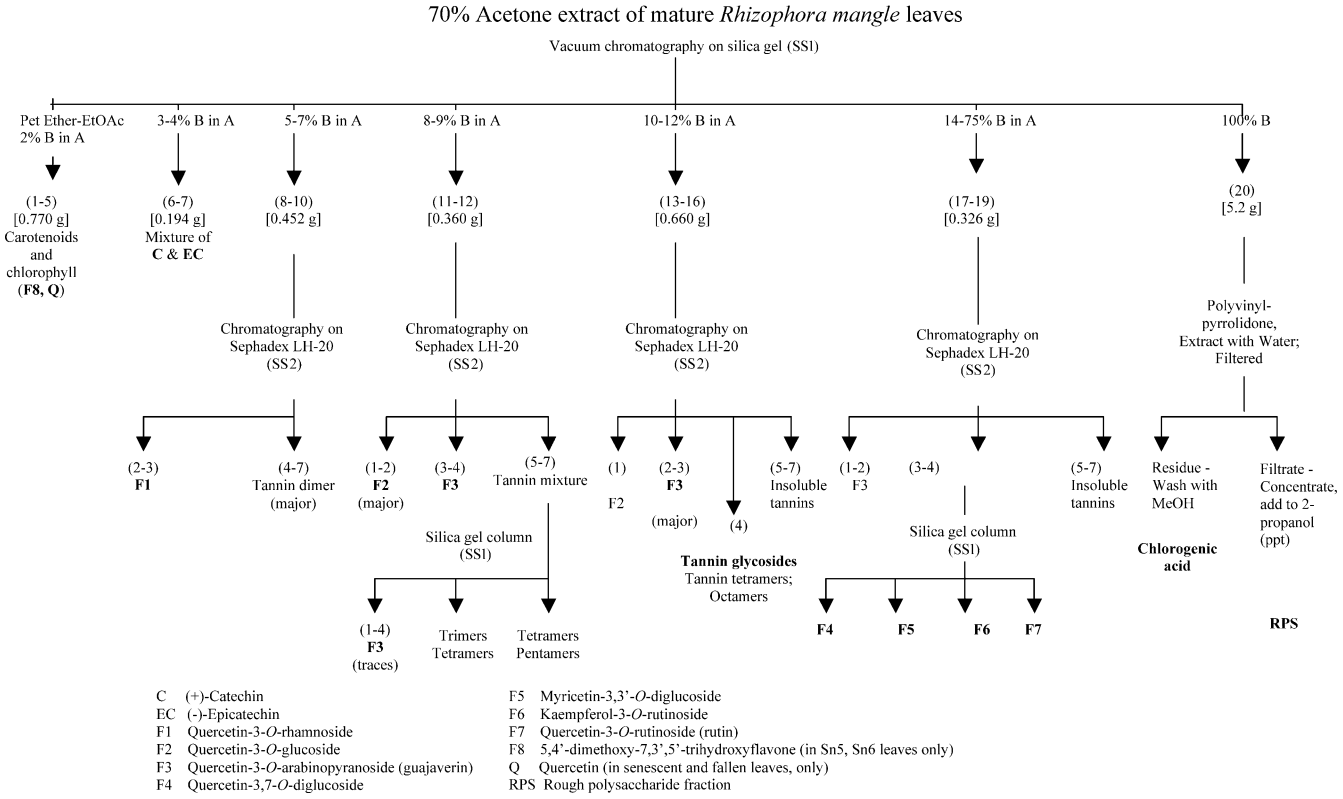

Fig. 1 Flow chart showing the protocol for separation of polyphenolics from *Rhizophora mangle* leaves beginning with a 70% acetone extract. Solvent systems were: (SS1) ethyl acetate (A) with increasing portions of 1:1 MeOH:H_2O and (SS2) (B)100% EtOH with increasing proportions of 40% acetone in water. Flavonoids (F1–F8) were recovered where indicated; their structures are shown in Fig. 2

of MeOH/water (1:1; solvent B) were added up to 100% B. At this point, all colored materials were removed from the column. Twenty fractions of 100 ml each were collected, concentrated under vacuum to remove volatile solvents, and the remaining aqueous solutions were freeze-dried. An outline of the separation and characterization protocol indicating both initial and secondary fractionation steps is shown in Fig. 1.

Based on TLC results, several fractions were recombined and further fractionated by vacuum chromatography, varying solvent composition to achieve optimal separation. Flavonoid glycosides from combined initial fractions 8–10, 11–12, 13–16, and 17–19, were separated from the co-occurring proanthocyanidins on Silica Gel G (Merck, Darmstadt) or Sephadex LH-20 (Pharmacia, Peapack, NJ, USA), by first eluting flavonoids with ethyl alcohol, and then proanthocyanidins with 40% acetone in water (Fig. 1). Solvent ratios for chromatography (solvent system 2, SS2) were adjusted to effect optimal resolution of the compounds or to produce mixtures of proanthocyanidins simpler in composition. If necessary, individual subfractions were again fractionated. When individual compounds were adequately resolved (as judged by TLC), they were purified by vacuum chromatography with Sephadex LH-20, eluted with 70% MeOH, and characterized by ^{1}H-NMR, ^{13}C-NMR and MALDI MS. When possible, identifications were verified by comparison with authentic standards, including (+)-catechin, (−)-epicatechin, quercetin, chlorogenic acid and caffeic acid (Sigma, St. Louis, MO, USA).

The isolated flavonoid glycosides were hydrolyzed and the resulting aglycones and sugars were characterized by standard procedures (Mabry et al. 1970). Sugars were determined on the basis of ^{1}H-NMR and ^{13}C-NMR signal patterns. This assignment was substantiated by acid hydrolysis, followed by paper chromatography and comparison with authentic sugars (arabinose, glucose and rhamnose; Sigma, St. Louis, MO, USA). Aglycones were identified by chemical and spectral analyses, and by comparison with authentic samples (quercetin, myricetin and kaempferol; Sigma, St. Louis, MO, USA) and published data (Mabry et al. 1970). The structures of major compounds discussed in this study are shown in Fig. 2. ^{1}H-NMR parameters characterizing the flavonoids and their glycosides are summarized in Table 1.

Identification of a new compound in red senescent leaves (Sn5)

Fraction 2 of the initial extraction of red senescent leaves, contained a novel compound, initially identified by its strong fluorescence on the TLC plates under UV light. After repeated chromatography on Sephadex LH-20, this was purified as a faintly yellow, amorphous powder [R_f 0.62 in EtOAc–MeOH–H_2O (79:11:10); F8 in Table 1]. This substance fluoresced blue under UV light and changed to a highly fluorescent yellow color on exposure to ammonia vapors, suggesting either a flavone or flavonol lacking a free 5-OH (Mabry et al. 1970). The UV spectrum of F8 in MeOH had absorption maxima at 350 and 250 nm, characteristic of a flavone skeleton. Addition of sodium methoxide solution produced a bathochromic shift of 55 nm in Band I with a decrease in intensity, pointing to a substituted 4′-position. UV spectra with $AlCl_3$, $AlCl_3$/HCl and NaOAc/H_3BO_3 exhibited shifts that supported the presence of a free 7-OH group and ruled out a dihydroxyl system in either the A or B ring (Mabry et al. 1970). The FAB-MS had an M+1 peak at *m/z* 331, corresponding to an aglycone with a molecular formula $C_{17}H_{14}O_7$. A sharp singlet at 7.039 ppm in the ^{1}H-NMR spectrum of F8 (DMSO-*d6*) corresponded to H-2′,6′ and indicated the presence of a 3′, 4′, 5′-substituted B-ring. Signals at 6.35 (d, *J*=1.5 Hz) and 6.51 ppm (d, *J*=1.5 Hz) were compatible with those expected for the 6 and 8 positions of the A ring, respectively. A singlet at 6.5 ppm corresponded to H-3, and singlets at 3.76 and 3.83 ppm, each of which each integrated for three protons, corresponded to –OMe

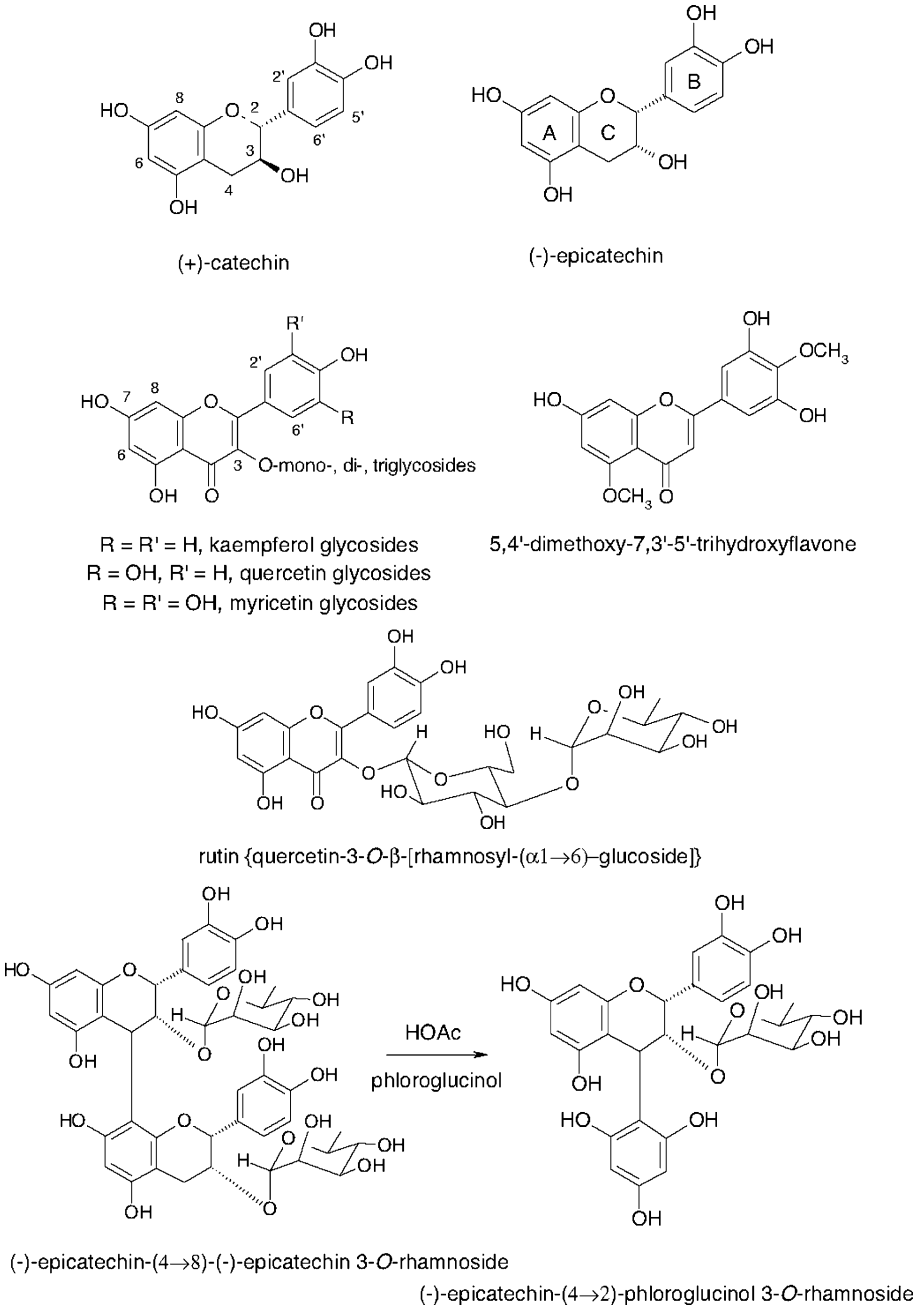

Fig. 2 Chemical structures for the major compounds isolated from *R. mangle* and discussed in this paper

groups at the 5 and 4′ positions. Further confirmation of structure was obtained from analysis of the ^{13}C-NMR spectrum. ^{13}C-NMR assignments (δ, ppm) for F8 in DMSO-*d6* were: C-2, 161.18; C-3, 107.61; C-4, 176.37; C-5, 160.59; C-6, 95.84; C-7, 163.26; C-8, 97.13; C-9, 159.63; C-10, 102.28; C-1′, 121.42; C-2′, 107.01; C-3′, 149.275; C-4′, 136.00; C-5′, 149.27; C-6′, 107.01; O-Me, 56.48; O-Me, 56.78.

Characterization of proanthocyanidins

Fraction 20 from the initial fractionations was used to characterize the proanthocyanidins. In the initial separation, this fraction appeared as one spot on the base line on silica gel plates when visualized with chromic acid solution. Polysaccharides were separated from this fraction according to Deters et al. (2001). Briefly, a portion of the fraction (1.5 g) was added to 0.3 g polyvinylpolypyrrolidone (Sigma, St. Louis, MO, USA), extracted three times with 20 ml of water, and filtered. The combined filtrates were concentrated to 2 ml by rotary evaporation under vacuum, and slowly added to 20 ml 2-propanol. The precipitated crude polysaccharides were centrifuged at 3,600*g*, and dialyzed (Spectro-por membrane, 12,000–14,000 cutoff, Spectrum Medical Industries, Los Angeles, CA, USA). The dialysate was lyophilized to yield a nearly white powder (0.12 g). The residue, after washing with water, was extracted with MeOH and separated on a Sephadex LH-20 column, eluting first with EtOH, then with 40% acetone in H_2O, to yield three fractions. These were subsequently identified as a flavonoid (F7, Fig. 1), chlorogenic acid (verified by comparison with authentic standard, Sigma, St. Louis, MO, USA), and a polymeric tannin fraction. The last subfraction of fraction 20 from young leaves was used as the tannin standard for the Hagerman–Butler assays.

Because of material limitations in the polymeric tannin fraction, more detailed analysis of the condensed tannins was limited to the

Table 1 ^{1}H-NMR of isolated flavonoids including both the aglycone and sugar components. With the exceptions of quercetin and a new compound (F8), all compounds were found in young, old and senescent leaves

	Quercetin	Quercetin-3-*O*-rhamnoside	Quercetin-3-*O*-arabinopyranoside (guajaverin)	Quercetin-3-*O*-glucoside	Quercetin-3,7-*O*-diglucoside	Kaempferol-3-*O*-rutinoside	Myricetin-3,3′-*O*-diglucoside	Quercetin-3-*O*-rutinoside (rutin)	5,4′-dimethoxy-7,3′,5′-trihydroxyflavone (F8)
H-6′	7.55 (dd, *J*=2, 8.5 Hz)	7.635 (d, *J*=8.5 Hz)	7.635 (dd, *J*=2, 8.5 Hz)	7.5–7.504 (*m*)	7.574–7.526 (*m*)	7.952–7.935 (dd, *J*=2, 8.5 Hz)	7.18 (d, *J*=9.4 Hz)	7.529–7.504 (*m*)	7.039 (*s*)
H-2′	7.69 (d, *J*=2 Hz)	7.505 (d, *J*=2 Hz)	7.505 (d, *J*=2 Hz)						
H-3′	–	–	–	–	–	6.859-6.842	–	–	–
H-5′	6.90 (d, *J*=8.5Hz)	6.830 (d, *J*=8.5 Hz)	6.835 (d, *J*=8.5 Hz)	6.826 (d, *J*=8.5 Hz)	6.825 (d, *J*=8.5 Hz)	(dd, *J*=2, 8.5 Hz)	–	6.826 (d, *J*=8.5 Hz)	–
H-8	6.42 (d, *J*=2 Hz)	6.427 (d, *J*=2 Hz)	6.427 (d, *J*=2 Hz)	6.367 (d, *J*=2.5 Hz)	6.375 (d, *J*=2 Hz)	6.266 (*bs*)	6.26 (*bs*)	6.367 (d, *J*=2.5 Hz)	6.35 (d, *J*=1.5 Hz)
H-6	6.2 (d, *J*=2 Hz)	6.194 (d, *J*=2 Hz)	6.194 (d, *J*=2 Hz)	6.171 (d, *J*=2.5 Hz)	6.157 (d, *J*=2 Hz)	6.070 (*bs*)	6.076 (*bs*)	6.171 (d, *J*=2.5 Hz)	6.51 (d, *J*=1.5 Hz)
H-3	–	–	–	–	–	–	–	–	6.508 (*s*)
H-1″-glucosyl	–	–	–	5.325 (d, *J*=8.4 Hz)	5.34 (d, *J*=8 Hz) 5.23 (d, *J*=8 Hz)	5.234 (d, *J*=8 Hz)	5.42 (d, *J*=8 Hz) 5.28 (*d J*=8 Hz)	5.325 (d, *J*=8.4 Hz)	–
H-1″-rhamnosyl	–	5.25 (d, *J*=5 Hz)	–	–	–	6.070 (*s*)	–	4.3 (*s*)	–
H-1″-arabinosyl	–	–	5.248 (d, *J*=5 Hz)	–	–	–	–	–	–
Sugar protons	–	3.0–3.8 (*m*)	3.482–3.140 (*m*)	3.60–3.20 (*m*)	3.6–3.2 (*m*)	3.50–3.00 (*m*)	3.620–3.144 (*m*)	3.6–3.2 (*m*)	–
Me-rhamnosyl	–	0.88–0.97 (*m*)	–	–	–	0.98 (d, *J*=6 Hz)	–	0.968 (d, *J*=6 Hz)	–
5-*O*-methyl	–	–	–	–	–	–	–	–	3.76 (*s*)
4′-*O*-methyl	–	–	–	–	–	–	–	–	3.83 (*s*)

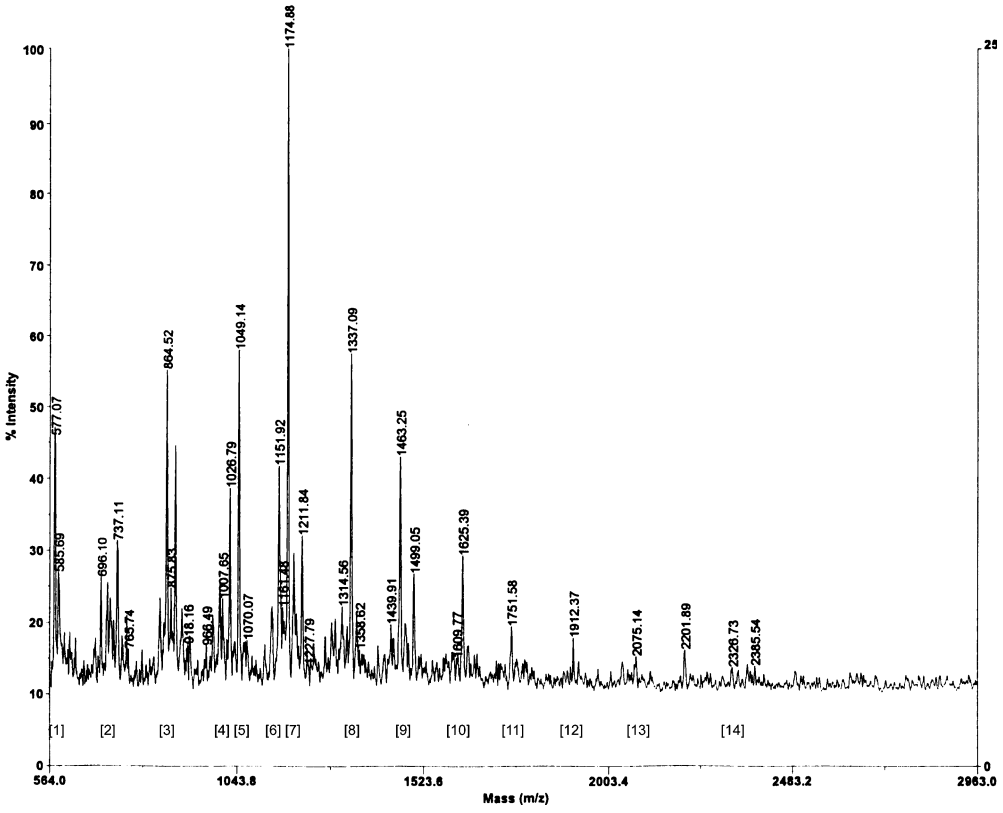

Fig. 3 MALDI-MS spectrum of fractions 13–16, subfraction 4 (see Fig. 1) showing the relative abundance of condensed tannin oligomers (A-type dimers to octamers) and their glycosides. Peaks at *m/z* 577.07 [1] and 737.11 [2] represent the protonated dimer type-A $[M+1]^+$, and its glycoside $[M+glycoside+1]^+$. A type-A trimer [3] and the corresponding glycoside [4] and its sodium adduct [5] appear at *m/z* 864.55 $[M+1]^+$, 1026.79 $[trimer+glycosyl+1]^+$; and 1049 $[M+glycosyl+Na]^+$. A tetramer [6–7] and corresponding glycoside [8], with two A-linkages, appear at *m/z* 1174 $[M+Na]^+$ and *m/z* 1337.09 $[M+glycosyl+Na]^+$. Other peaks are a pentamer ([9] *m/z* 1463.25) and pentamer glycoside ([10] *m/z* 1625.39), hexamer ([11] *m/z* 1751.68) and hexamer glycoside ([12] *m/z* 1912.37), the corresponding diglycosides ([13] *m/z* 2075.14), and an octamer ([14] *m/z* 2326.73), all with a single A-type linkage

fallen leaves. Fifty milligrams of this fraction was reacted with an equal weight of phloroglucinol in EtOH (0.5 ml) in the presence of acetic acid (0.1 ml) at 105° for 12 h in a sealed ampoule (Achmadi et al. 1994) (Fig. 2). After cooling, the product was diluted with H_2O (20 ml) and extracted with an equal volume of ethyl acetate (EtOAc) five times, to recover an EtOAc-soluble fraction (0.076 g). This was fractionated on a silica gel vacuum column, eluting with EtOAc and increasing amounts of MeOH/water (1:1) to collect 20 fractions (30 ml each). The fractionation was monitored by TLC. Fractions 15–16 containing the purest proanthocyanidins, were subfractionated, and subfractions 2–4 were combined for ^{1}H-NMR and mass spectrometry.

Results

Quantification of phenolic substances

Condensed tannins and other polyphenolics were quantified in *R. mangle* leaves from the most highly stressed area of the dwarf-zone. In that area, the surface was flooded throughout the year and salinity was equal to that of seawater or hypersaline. Substrate P and N levels were below 1 μM (M. Jacobson, personal communication). Individual branches had at most six active leaves (three pairs) oriented nearly vertically; any additional leaves were in the process of senescing. Leaf scars were tightly packed without internode elongation beyond that needed to support the next leaves (McKee et al. 2002; Cheeseman and Lovelock 2004). Because the trees were short (<1.5 m) and widely spaced, all leaves were unshaded.

Together, polyphenolics accounted for 23±1% of the leaf dry weight. Total condensed tannins, assayed using the method of Hagerman and Butler (1978), accounted for 79% of this. Other flavonoids, i.e., those not precipitated by BSA, accounted for the remaining 21%. These were assayed according to Price and Butler (1977), using the supernatant from the tannin assay with a rutin/chlorogenic acid mixture as the standard.

Fractionation and identification of individual components

The protocol used to separate individual compounds into 20 fractions is outlined in Fig. 1. Based on their UV appearance and high R_f values on TLC plates, early fractions contained mainly lipophilic compounds, including carotenoids and chlorophylls (Harborne 1973). Later fractions contained largely flavonoid glycosides and a mixture of proanthocyanidins. Fractions judged similar by TLC were combined for further analysis (Fig. 1). After repeated vacuum chromatography, three major and four minor flavonoid glycosides were isolated and identified (Fig. 2). The major compounds were quercetin-3-*O*-arabinopyranoside, quercetin-3,7-*O*-diglucoside and rutin (quercetin-3-*O*-rutinoside). Quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside, myricetin-3-3′-*O*-diglucoside, and kaempferol-3-*O*-rutinoside were identified as minor flavonoids.

The proanthocyanidins (condensed tannins) were based mainly on (+)-catechin and (−)-epicatechin units (Fig. 2), as indicated by the fact that complete acid hydrolysis with HCl gave a pink solution with a characteristic cyanidin

λ_{max} of 540 nm (Haslam 1982), by the characteristic ^{1}H-NMR and ^{13}C-NMR spectra of the lower oligomeric fractions, and by comparison with authentic standards. Individual fractions contained multiple components ranging from monomers, in fractions 6 and 7, to octamers in later fractions. MALDI-MS, however, showed that the oligomers were not composed only of unmodified (+)-catechin and (−)-epicatechin. For example, Fig. 3 shows analysis of a complex mixture from combined fractions 13–16, with a mixture of proanthocyanidins of A-type (i.e., compounds in which the oxygen at position 7 has formed an acetal linkage to carbon 2 in a second monomeric unit), and their glycosides (Porter et al.1985; Lazarus et al. 1999).

The last fraction, which accounted for approximately 35% of the mass of the acetone extract, was further fractionated, yielding rutin (Fig. 2), chlorogenic acid, and a highly polymeric tannin fraction (PTF, Fig. 1). Reaction of the polymeric tannin fraction with phloroglucinol and further purification (see Materials and methods) allowed identification of individual proanthocyanidin components using mass spectroscopy (negative ion mode). In addition to the phloroglucinol adducts of catechin/epicatechin [*m/z* 413 (M−1)], smaller peaks at *m/z* 397 (M−1) for the afzelachin/epiafzelachin-phloroglucinol adduct, and *m/z* 429 (M−1) corresponding to the gallocatechin/epigallocatechin-phloroglucinol adduct were identified. Moreover, corresponding glycosidic adducts of each of these was also present, at yet lower concentrations, as indicated by peaks at *m/z* 543.1 (M−1), 559.2 (M−1) and 575.3 (M−1). Although Achmadi et al. (1994) have previously reported (+)-catechin-3-*O*-rhamnoside as extender units in polymeric proanthocyanidins from *B. gymnorrhiza* bark, the corresponding (−)-epicatechin glycoside and flavan-glycosides other than (+)-catechin rhamnoside have not, to the best of our knowledge, been previously reported from this family.

Senescence related changes in leaf polyphenolics

The polyphenolic composition of young leaves, old leaves, and leaves in progressive stages of senescence (Sn2–Sn6) was compared using the techniques applied in the above analyses. Overall, the composition was similar, though the relative representation of individual compounds changed. TLCs of the initial acetone extracts, one visualized under UV light, a second with vanillin-HCl reagent, and a third with chromic acid solution, are shown in Fig. 5. As the youngest fully-expanded leaves aged, but while they still appeared fully green, the total quantity of aqueous acetone soluble organic materials increased (Fig. 5c). This corresponded to thickening of leaves, primarily by expansion of cells in the upper epidermis and hypodermis (data not shown).

The monomeric flavan-3-ols (+)-catechin and (−)-epicatechin, were significant components of non-senescent leaves, but disappeared rapidly during senescence. Also as senescence progressed, the amounts of quercetin mono-glycosides and diglycosides (compounds 4, 5, 6, 8, and 10, Fig. 5a) on the chromatograms diminished, and a yellow spot (R_f 0.9) appeared. This spot was particularly visible under UV light; spraying with $AlCl_3$ in methanol enhanced this effect (not shown), strongly suggesting the presence of a flavonol, which was subsequently identified as the aglycone, quercetin. Whether this compound arose by degradation of the glycosides or interruption of glycosylation is not known.

Leaves just prior to abscission (Sn5 and Sn6) yielded a compound appearing as a blue spot under UV light (compound three at R_f 0.8 in Fig. 5a). Based on its ^{1}H-NMR spectrum and comparison with an authentic sample, this was identified as caffeic acid. Caffeic acid was not found in any non-senescent leaves nor has it been previously reported from *R. mangle*. It may have appeared due to degradation of chlorogenic acid, or as the result of oxidative degradation of flavonoid carbon skeletons during senescence. Again, the actual source remains to be determined.

As leaves browned (Sn6), but before they fell, most of the polyphenolic compounds disappeared. In the fallen leaves, only quercetin, chlorogenic acid and a large spot at the origin (R_f 0.03) remained. Based on treatment with chromic acid (Fig. 5c), this large spot contained almost all the soluble organic material, largely as condensed tannins (Fig. 5b). The progressive loss of condensed tannins and organic material not at the origin (Fig. 5b,c) suggests that the low molecular weight tannins were either degraded or further polymerized during senescence.

Finally, fractionation of red, senescing leaves (Sn5) using the protocol in Fig. 1 revealed a compound not detected in either the young or old leaves. This compound occurred in fraction 2, eluting with ethyl acetate (Fig. 4), and was immediately noticeable because of its bright blue fluorescence under UV light. The compound was purified by repeated fractionation, and characterized by UV spectroscopy, FAB-MS, and ^{1}H and ^{13}C-NMR (see Materials and methods). Combined, these results allowed us to identify this as a new compound, 5,4′-dimethoxy-7,3′,5′-trihydroxyflavone (Fig. 2). Subsequently, we have found this compound in two other members of the Rhizophoraceae, *B. gymnorrhiza* and *B. parviflora*,

Fig. 4 Thin layer chromatogram of flavonoids from leaves at senescence stage 5 (Sn5). *Left lane* is the initial acetone extract visualized under UV light. *Right lane* is the newly characterized compound, 5,4′-dimethoxy-7,3′,5′-trihydroxyflavone (F8). TLC was carried out on pre-coated Silica Gel 60 plates with F254 fluorescent indicator using ethyl acetate/methanol/water (79:11:10) as the solvent system (see Materials and methods)

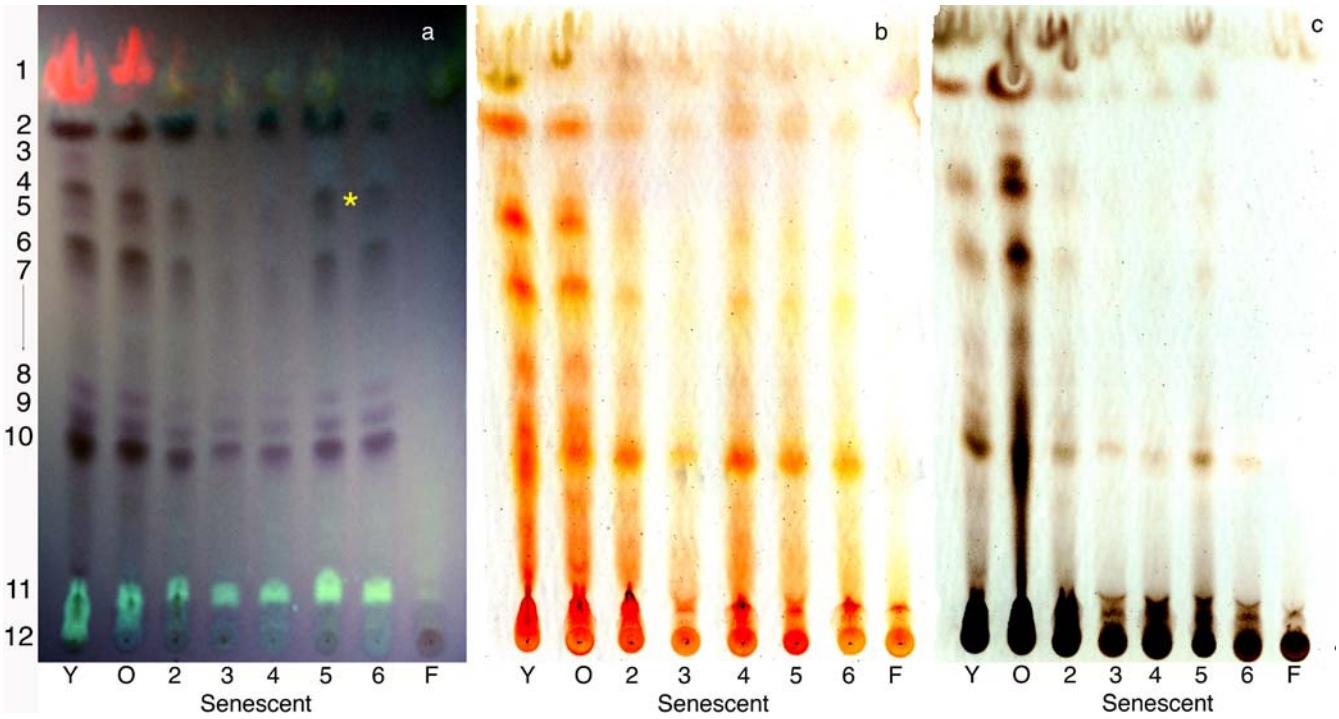

Fig. 5 Comparison of flavonoid, condensed tannin, and total organic composition of acetone extracts from *R. mangle* leaves during development and senescence. Extracts were from young (Y), old (O), senescent, and fallen (F) leaves. The definition of the progressive stages of leaf senescence (Sn2–Sn6) is given in the Materials and methods. TLC conditions were as in Fig. 4. **a** TLC viewed under UV light to visualize phenolics and flavonoids; **b** plate sprayed with vanillin–HCl reagent, heated at 100°C for 10 min to visualize monomeric flavan-3-ols and condensed tannins; **c** plate sprayed with chromic acid solution and heated at 100° for 5 min to visualize total organic material. Compounds indicated on the vertical axis were identified based on analyses of the previous sections and include [1] chlorophyll, carotenoids and quercetin; [2] monomeric flavan-3-ols (catechin and epicatechin); [3] caffeic acid; [4] F1 and F8 (see text, "New compounds"); [5] F3 and dimeric proanthocyanidins; [6] F3; [7] tetrameric and pentameric proanthocyanidins; [8] F4; [9] F5 and F6; [10] rutin (F7) and multimeric proanthocyanidins; [11] chlorogenic acid; [12] high molecular weight proanthocyanidins. It should be noted that compounds with similar R_f values are not resolved in this comparison of fractions before subfractionation. *Asterisk* denotes position of F8 in Sn5 leaves. TLC was carried out on pre-coated Silica Gel 60 plates with F254 fluorescent indicator using ethyl acetate/methanol/water (79:11:10) as the solvent system (see Materials and methods)

where it is present in non-senescent leaves (unpublished data).

Discussion

The condensed tannins and flavonol glycosides in *R. mangle* leaves are diverse groups of compounds (Fig. 2), and together, accounted for one quarter of the leaf dry weight. On a molecular basis, production of each flavan-3,4-diol precursor unit consumes 121 ATP (Lewis and Yamamoto 1989); on a mass basis, this cost is slightly higher than that of proteins and 60% higher than that of cellulose (Atkinson 1977). Clearly, this represents a significant investment on the part of the leaves, one that demands attention as part of the overall biology and ecological placement of the trees.

In putting our results into broader physiological or ecological contexts, it is important to examine, first, the chemical nature and possible functions of mangrove polyphenolics, individually and as a class. Second, the resource investment should be considered both for its implications for growth, maintenance or reproduction (costs), and for physiological and ecological benefits that might accrue. Finally, changes accompanying leaf senescence should be considered, albeit briefly because of the sparse literature available on the topic.

Nature and functions of the polyphenolics

Within the mangrove proanthocyanidins (+)-catechin and (−)-epicatechin were the most common monomeric units, although four other groups—afzelachin, epiafzelachin, gallocatechin and epigallocatechin—were identified in smaller quantities. As noted above, *R. mangle* appears to be unusual in that each of these groups also occurred as a 3-*O*-rhamnoside. Quercetin was the most prevalent flavonol, but appeared as an aglycone only in senescent leaves. Of the four major flavonol glycosides, rutin was most abundant (Fig. 5).

The diversity of mangrove polyphenolics reflects factors ranging from the biochemical to the evolutionary scales. Biochemically, for example, end-product diversity has been attributed in part to the non-specificity, or regiospecificity of modifying enzymes, i.e., glucosyltransferases involved in the late steps of glycoside synthesis (Vogt and Jones 2000). This implies that only if a

particular result were deleterious to long-term survival would there be selective pressure to eliminate it. Otherwise, the diversity of flavonoids may help ensure that plants maintain their defense capabilities over evolutionary time, in response to new capabilities of, for example, the digestive systems of potential specialist herbivores (Williams et al. 1989).

Qualitatively and quantitatively, the mangrove leaf polyphenolics are well-suited for protection against ultraviolet radiation, defense against herbivores and pathogens, and antioxidant activity. In the case of UV protection, these compounds are undoubtedly present at much higher levels than required, consistent with the findings by Lovelock et al. (1992) for mangroves in Queensland, and by McKee (1995) of the comparative levels of phenolic compounds in co-occurring but unrelated mangroves in the neo-tropics. With respect to herbivore and pathogen defense, tannins in general have potent and specific effects as Fe-chelators (Scalbert 1991), protein kinase inhibitors (Wang et al. 1996) or (after activation in insect guts) cytotoxic *o*-quinones (Moridani et al. 2001), all of which could be adequate against less well-adapted herbivores. Where folivory is found on *R. mangle*, however, it is more commonly associated with feeding by generalists such as the puss moth caterpillar, *Megalopyge dyerii* (I.C. Feller, personal communication). In that case, based on the shear volume of material that an individual can consume, the reduction of the overall tissue nutritional quality is undoubtedly paramount (Zucker 1983; Waterman and Mole 1994); tannins accomplish this by reducing digestibility through protein precipitation and by increasing leaf C/N ratios.

The antioxidant activities of polyphenolics have received considerable attention because of their links to human health (e.g., Rice-Evans et al. 1996), and in plants, for their association with multiple stress responses. Quercetin, the major flavonol on which the mangrove non-tannin flavonoids are based, is particularly effective in this function. Interestingly, however, the aglycone itself was detectable only late in senescence, possibly because its antioxidant activity is too high, i.e., it may be too susceptible to oxidation by peroxidases (Hodnick et al. 1988). Indeed, we have found that an acidic peroxidase from *R. mangle* leaves is more than 100 times more active as a H_2O_2 scavenger with quercetin as the phenolic substrate than with rutin (Pearse, Heath and Cheeseman, unpublished data). Quercetin also autoxidizes readily (Hodnick et al. 1988), producing semiquinones, or, on further oxidation, *o*-quinones. While these are toxic to herbivores and fungi as Cu and Fe chelators (Takahama and Hirota 2000), they are similarly dangerous to the plant itself.

Glycosylation reduces quercetin reactivity, detoxifying and stabilizing the compound (Morales et al. 1993), and the associated reduction of peroxidase substrate suitability may, in practice, be of little consequence: as scavengers of superoxide and singlet oxygen, both of which may present greater problems in leaf tissue than the more stable H_2O_2, the primary polyphenolics in *R. mangle*, i.e., rutin, other quercetin glycosides and catechin, are all more or less as equally effective as quercetin (Wollenweber and Dietz 1981; Waterman and Mole 1994; Rice-Evans et al. 1996). Moreover, recent studies suggest that quercetin can be mobilized by glycosidases, enabling maintenance of a small pool of the aglycone (Takahama and Hirota 2000; Suzuki et al. 2002). This aspect of antioxidant activity in mangroves and other plants deserves greater consideration.

Costs and benefits

Although the polyphenolic components of *R. mangle* leaves are well-suited for their functions, and diverse enough to ensure flexibility with changing biotic and abiotic environments, the high investment in their accumulation has potentially significant implications for the growth, maintenance and reproduction of the trees. The critical question here is whether the abiotic environment itself would allow greater productivity if the investment were reduced. Because the Twin Cays environment is so extremely nutrient limited (Feller et al. 2003), based on greenhouse studies which show decreased investment in flavonoids with increased nutrient availability (McKee 1995), and based on short-term physiological and growth responses of *R. mangle* to fertilization in the field (Cheeseman and Lovelock 2004), this seems unlikely to be the case. Instead, under these circumstances, the diversion of C to secondary compounds may be essentially cost-free with respect to growth or reproduction (Bryant et al. 1983). Indeed, Haslam (1985; 1986), argued that when utilization of carbon and energy for growth or maintenance is limited by lack of other resources, a potential metabolic imbalance between carbon supply and utilization could be avoided by shunting key intermediates, e.g., pyruvate, PEP, acetyl-CoA and 3-phosphoglycerate, into metabolically harmless, but ecologically useful products, including the flavonoids. In the case of *R. mangle*, however, this has the consequence that nearly 10% of all the carbon in the leaves has passed through phenylalanine and phenylalanine ammonia lyase. The control and significance of this also deserves further consideration.

Polyphenolic turnover and senescence

During leaf maturation and senescence, there are changes and simplifications in both the flavonoid and proanthocyanidin components of *R. mangle* (Fig. 5). Quercetin appears as the aglycone (Fig. 5); a new, methylated flavone (Fig. 4) appears which, in *Bruguiera* mangroves occurs in green leaves; and, at the final stages, there is a nearly total disappearance of all but the most recalcitrant polymeric tannins. At this point, unequivocal interpretation of these results is not possible; other than the ^{14}C-labelling study of *Populus deltoides* by Kleiner et al. (1999) which showed rapid turnover of both phenolic

glycosides and tannins even in non-senescent tissues, polyphenolic turnover has received little attention.

In *Rhizophora* mangroves, turnover and degradation during senescence may be critical for persistence in their ecosystem. For example, as long-lived individuals, mangroves depend heavily on internal and external recycling of nutrients for growth. However, previous studies have found only 80% of the N and 75% of the P to be recycled from leaves prior to abscission (Feller et al. 1999). Detritivores and decomposers are critical to recycling of the remainder, and compounds that would discourage this must be considered a liability at senescence. At that point, the "objective" of a leaf should be to be eaten, not to enter a refractory pool of non-decomposing leaves and exacerbate the already low nutrient availability with additional limitations on O_2 diffusion. Consistent with this, previous studies have shown that the leaf contribution in mangrove peats is less than 0.5% of the root debris (Cohen and Spackman 1977). In addition, Middleton and McKee (2001) reported that *R. mangle* leaves decompose rapidly, disappearing entirely within 5 months in the lower intertidal zone.

In conclusion, the polyphenolics in *R. mangle* are diverse, and well-suited for defensive roles against biotic, abiotic and internal biochemical stresses. Their production and accumulation is favored in the high light, high temperature, high salinity and low nutrient environment in which the trees occur, particularly when carbon use for growth or reproduction is precluded by lack of nutrient resources. Finally, changes in polyphenolic pools during leaf development and senescence indicate that these are dynamic components of the leaves whose functional significance, at both physiological and ecological levels is, as yet, only incompletely understood.

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