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Functional role of anthocyanins in the leaves of *Quintinia serrata* A. Cunn.

Kevin S. Gould[1,3], Kenneth R. Markham[2], Richard H. Smith[1] and Jessica J. Goris[1]

[1] *Plant Science Group, School of Biological Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand*

[2] *NZ Institute for Industrial Research and Development, PO Box 31–310, Lower Hutt, New Zealand*

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Abstract

The protective functions that have been ascribed to anthocyanins in leaves can be performed as effectively by a number of other compounds. The possibility that anthocyanins accumulate most abundantly in leaves deficient in other phytoprotective pigments has been tested. Pigment concentrations and their histological distribution were surveyed for a sample of 1000 leaves from a forest population of *Quintinia serrata*, which displays natural polymorphism in leaf colour. Eight leaf phenotypes were recognized according to their patterns of red coloration. Anthocyanins were observed in almost all combinations of every leaf tissue, but were most commonly located in the vacuoles of photosynthetic cells. Red leaves contained two anthocyanins (Cy-3-glc and Cy-3-gal), epicuticular flavones, epidermal flavonols, hydroxycinnamic acids, chlorophylls, and carotenoids. Green leaves lacked anthocyanins, but had otherwise similar pigment profiles. Foliar anthocyanin levels varied significantly between branches and among trees, but were not correlated to concentrations of other pigments. Anthocyanins were most abundant in older leaves on trees under canopies with south-facing gaps. These data indicate that anthocyanins are associated with photosynthesis, but do not serve an auxiliary phytoprotective role. They may serve to protect shade-adapted chloroplasts from brief exposure to high intensity sunflecks.

Key words: *Quintinia serrata*, leaf, anthocyanin, flavonoid, chlorophyll, carotenoid.

Introduction

The anthocyanins are a group of water-soluble flavonoids that impart pink to purple colours in leaves and other organs (Harborne, 1988). Synthesized in the cytoplasm and actively sequestered into cell vacuoles by a glutathione pump (Mars *et al*., 1995), anthocyanins are especially prominent in the flushing leaf primordia of tropical rainforest species (Richards, 1952), and in the senescing autumn foliage of deciduous trees (Chang *et al*., 1989).

There is no accepted explanation for the presence of anthocyanins in leaves. Indeed, it is entirely possible that anthocyanins do not contribute to leaf function. Synthesized at an end-point in the flavonoid pathway, anthocyanins might arise simply as the by-products of a saturated metabolism which are shunted into the cell vacuole for ergastic storage. However, the induction of anthocyanins by environmental stresses (Chalker-Scott, 1999), the appearance of red leaves at predictable times of the year and at specific stages in leaf development, and their prominence in particular environmental niches (Lee *et al*., 1979), have prompted many workers to postulate roles for anthocyanins in leaves. Among the various functional hypotheses that have been debated in recent years are: (i) modification of the quantity and quality of captured light (Lee, 1986; Barker *et al*., 1997); (ii) protection from the effects of UV-B (Burger and Edwards, 1996; Klaper *et al*., 1996); (iii) defence from herbivory (Coley and Kusar, 1996); (iv) protection from photoinhibition (Gould *et al*., 1995; Dodd *et al*., 1998); and (v) scavenging of reactive oxygen intermediates under stressful environments (Yamasaki, 1997; Sherwin and Farrant, 1998).

[3] To whom correspondence should be addressed. Fax: +64 9 373 7416. E-mail: k.gould@auckland.ac.nz

Abbreviation: Cy, cyanidin.

Two features of these hypotheses are of interest here. First, none of the proposed functions is unique to the anthocyanins; other compounds in the leaf can perform these tasks efficiently. For example, the presence of carotenoids and the distribution of chlorophylls can markedly modify light capture (Gausman, 1982; Smith *et al*., 1997; Tardy *et al*., 1998), and many phenolic compounds are more effective than anthocyanins as absorbers of UV-B (Shirley, 1996). Terpenes and other secondary metabolites serve as anti-herbivore defence compounds in many plants (Gershenzon and Croteau, 1991), the xanthophylls protect leaves from photoinhibition (Long *et al*., 1994), and certain flavonols, flavones, flavan-3-ols, theaflavins, and hydroxycinnamates have a proven antioxidant capacity (Rice-Evans *et al*., 1997). It is possible, therefore, that anthocyanins do not confer a unique function in leaves. Rather, they may act in concert with other protective molecules in the plant cell, perhaps compensating for deficiencies in concentrations of such molecules during periods of stress.

Second, the hypotheses require that anthocyanins reside at specific locations within the leaf for optimal effectiveness. For example, anthocyanins must be held in the upper epidermis and/or hypodermis to screen UV-B, in the mesophyll to protect chloroplasts from photoinhibition, or in the lowermost tissues to enhance light capture by internal reflection. The applicability of each hypothesis can, therefore, be tested by recording the histological distribution of anthocyanins for leaves facing specific environmental challenges. Leaves from a population of plants might well vary in anthocyanin distribution according to the type of protection required. Surprisingly few studies have described the locations of anthocyanins in leaves (Gould and Quinn, 1999), and none has examined for possible variation among natural populations.

Here, it is examined how levels of anthocyanins vary relative to those of other protective molecules in leaves from a natural population of *Quintinia serrata* A. Cunn. (Escalloniaceae), an evergreen canopy tree from lowland to montane forests in New Zealand (Allan, 1961). This species is especially polymorphic for leaf colour. The range of leaf phenotypes is described, the principal flavonoids present are identified and possible topophytic and environmental factors that may account for this polymorphism are explored. Finally, the leaves are surveyed for histological locations of anthocyanic cells in order to establish which of the putative protective hypotheses may be most applicable.

Materials and methods

Trees were sampled in January 1998 from a 2.5 km stretch of the Huia Ridge Track (latitude 36°59′ S, longitude 174°31′ E) in the Waitakere Ranges, 25 km west of Auckland, New Zealand. At an elevation of 340–470 m, the area holds a mixed, low-canopy broadleaf-podocarp forest, and is dominated on the ridges by *Agathis australis*. The first 100 *Q. serrata* trees encountered that bore at least two first-order branches were examined. The five youngest, fully-expanded leaves were removed from each of two, randomly selected branches on every tree. Tree height, branch height, leaf position (numbered basipetally from the youngest, fully expanded leaf), and direction of the nearest canopy gap were noted.

Images of both surfaces of each leaf were captured on a Macintosh computer using a JVC video camera. Leaves were sorted by phenotype according to the proportionate size and distribution of red pigmented areas as measured from the captured images.

Pigment quantification

Two 10 mm diameter discs from the distal half of each leaf lamina were agitated gently in the dark for 24 h at 4 °C in 1 ml of acetone : H_2O (4 : 1, v : v), and in 1 ml of 3 M HCl : H_2O : MeOH (1 : 3 : 16, by vol.), respectively. The extracts were centrifuged, and absorbances measured with a Pharmacia LKB UV/visible spectrophotometer. From the acetone extracts, measurements of A_{470}, A_{647} and A_{663} were taken to calculate concentrations of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and total carotenoids according to the equations given by Lichtenthaler (Lichtenthaler, 1988). Anthocyanin levels were estimated from the methanolic extracts as A_{530}–0.24 A_{653}. The anthocyanins in this solution absorbed maximally at 530 nm; subtraction of 0.24 A_{653} compensated for the small overlap in absorbance at 530 nm by the chlorophylls (Murray and Hackett, 1991). Concentrations of 'total UV-absorbing compounds' (Day, 1993) were estimated from 50-fold dilutions of the methanolic extracts as A_{300} (UV-B) and A_{350} (UV-A).

Flavonoid localization

Transverse hand-sections of the fresh material were taken from three regions within the proximal third of every leaf lamina. Where present, red regions of the laminae were preferentially sectioned. Sections were mounted in 10% sucrose, and the histological location of red pigment noted under bright field microscopy. The analysis assumed that the distribution of red coloration within a leaf correlated to that of anthocyanins. The possibility was tested that green or colourless cells might still contain anthocyanin, but which is rendered colourless because of local variation in the vacuolar pH (Brouillard, 1988). Selected sections were observed mounted in 10% sucrose, then infiltrated with 1 M HCl and examined for possible colour changes.

Transverse sections of selected red and green leaves were examined under epifluorescence using Zeiss filterset 09 (excitation: 450 nm; beamsplitter: 510 nm; emission 520 nm) in a Zeiss Axioplan 2 microscope. Sections were then stained with 0.2% Naturstoffreagenz A (diphenylboric acid 2-aminoethylester, Sigma) in water for 5 min, washed with distilled water, and re-examined. Stain-specific production of green-yellow fluorescence was attributed to the presence of flavonols and flavones (Schnitzler *et al*., 1996).

Flavonoid/hydroxycinnamate isolation, identification and quantification

Epicuticular flavonoids were extracted with diethyl ether (12–15 ml) at ambient temperature for 2 h from 0.6–1.0 g samples of intact leaves of types I, II, IV, V, VII, and VIII. The ether was decanted and evaporated, and the residue made up to 200 ml with MeOH. Absorption spectroscopy indicated the presence of flavones (band I maximum *c*. 330 nm), and

optical densities of these extracts at 330 nm were used to calculate flavone levels as apigenin equivalents, using 19 050 as the molar extinction coefficient.

The ether-treated leaves were finely ground and each sample extracted with 8 ml of HOAc : H_2O : MeOH (7 : 23 : 70, by vol.) for 2 h. Clarified solutions were obtained for analysis by centrifugation. The structures of anthocyanins and flavonol glycosides were determined after separation by 2D-PC in (i) t-BuOH : HOAc : H_2O (3 : 1 : 1, by vol.) and (ii) HOAc : H_2O (3 : 17, v : v). Flavonoid spots were detected by viewing the 2D-PCs in visible and UV (366 nm) light. Further structure information was obtained by treatment with ammonia vapour, spraying with Naturstoffreagenz A (Markham, 1982), and by comparing R_f values with those in the literature (Mabry *et al.*, 1970). Individual compounds were isolated from spots by elution with HOAc : H_2O : MeOH (7 : 23 : 70, by vol.), and the extracts were used for absorption spectroscopy studies as described previously (Markham, 1982). Individual components were co-chromatographed with authentic samples on HPLC using the solvents and methodology of Markham *et al.* (Markham *et al.*, 1998).

Anthocyanin levels were determined from optical density readings at λ_{max} 524 nm after the addition of 0.03 ml of 3 M HCl to 0.6 ml of clarified extract. Levels were calculated as cyanidin-3-glucoside equivalents (extinction coefficient 33 000). Internal flavonols and hydroxycinnamic acids were quantified from HPLC profiles measured at 352 nm on 10 µl aliquots of the clarified extracts. Flavonol concentrations were estimated as rutin equivalents by integration of all flavonol peaks in the HPLC, and converting the sum to µg using a conversion factor of 2 292 671 μg^{-1} (derived from a standard rutin solution). For the hydroxycinnamic acids, most HPLC peaks from RT 0–11.6 min gave on-line spectra approximating that of caffeic acid. The integrals were summed and converted to caffeic acid equivalents using the 5 437 000 μg^{-1} conversion factor established from a standard solution of caffeic acid.

Results

The leaf laminae of *Q. serrata* were lanceolate to oblanceolate, 5–15 cm long and 1–3 cm wide. Leaves were exstipulate, coriaceous, and hypostomatous, and typically had wavy, glandular margins.

Superficial pigmentation patterns, and proportions of red and green markings on leaf laminae were highly variable (Fig. 1). Anthocyanins were evident on both leaf surfaces, though the red coloration was usually more intense and more expansive on the adaxial surface. The sample of 1000 leaves was grouped into eight phenotypic categories according to the size and frequency of red areas on their adaxial surfaces (Table 1). At least two leaf types were present on every tree sampled. Leaves that bore mixtures of large and small red areas across the lamina (types IV and V) were the most common (Table 1). The larger red patches were most frequently contiguous to the midrib; smaller red markings were irregularly distributed over the lamina. Younger leaves had fewer and smaller red areas than older leaves.

Leaf anatomy

All laminae had a heavily cutinized adaxial epidermis, 1–3 layers of palisade mesophyll, 6–8 layers of spongy mesophyll, and abaxial stomata (Fig. 2A). Differences in the numbers of cell layers were not related to pigmentation pattern (data not shown). Druses were common throughout the mesophyll tissues, and peltate, glandular trichomes were present on both epidermises (Fig. 2A). These trichomes held an unidentified orange pigment in the vacuoles of their basal cells.

Anthocyanins were evident as red solutions inside cell vacuoles of the epidermises, the palisade and spongy mesophyll, and vascular parenchyma at the midrib (Fig. 2B). The intensity of red coloration increased when sections were infiltrated with 1 M HCl. However, those cells that had appeared green or colourless in cross section did not turn red upon acid treatment, indicating that they lacked anthocyanins and leucoanthocyanins. Red coloration was thus a reliable indicator of anthocyanin distribution.

The histological locations of anthocyanic cells varied both among leaves and between different regions of any one lamina. They were present as entire cell layers (Fig. 2 B), as discrete cell clusters (Fig. 2C), or as isolated cells (Fig. 2D). Variation in the intensity and hue of anthocyanic pigmentation was evident, often among cells within the same leaf section (Fig. 2B). Red markings on the adaxial surface could be attributed to anthocyanins in cells located above the third uppermost spongy mesophyll layer. These same layers occasionally also coloured the abaxial surface. For most leaves, however, abaxial surface coloration was associated with anthocyanins in the lower spongy mesophyll and/or the lower epidermis.

If the lamina be considered to consist of four tissues (upper epidermis, palisade mesophyll, spongy mesophyll, lower epidermis), then there are 15 possible tissue combinations in which anthocyanins might reside (Fig. 3). Anthocyanins were observed in all but one of these combinations across the sample as a whole (Fig. 3). Most leaves (57%) each had between two and five different combinations, each specific to a particular location on the lamina (a further 33% had a single anthocyanin distribution, and 10% were non-anthocyanic). The various combinations did not, however, occur with similar frequency. Anthocyanins were most prevalent in the palisade mesophyll, either alone, or in conjunction with spongy mesophyll and epidermal tissues (Fig. 3). Frequency distributions of the various combinations were comparable between branches on any one tree (χ^2; $P > 0.8$), among leaf types II to VII ($P > 0.1$), and among leaves older than one phyllochron ($P > 0.7$).

The youngest fully-expanded leaves on a branch, and the type VIII leaves (entirely green) had a disproportionately high frequency of non-anthocyanic tissues relative to other leaves. However, of the 40 leaves that had been categorized as type VIII according to their superficial appearance, 17 were found to hold anthocyanin pigment.

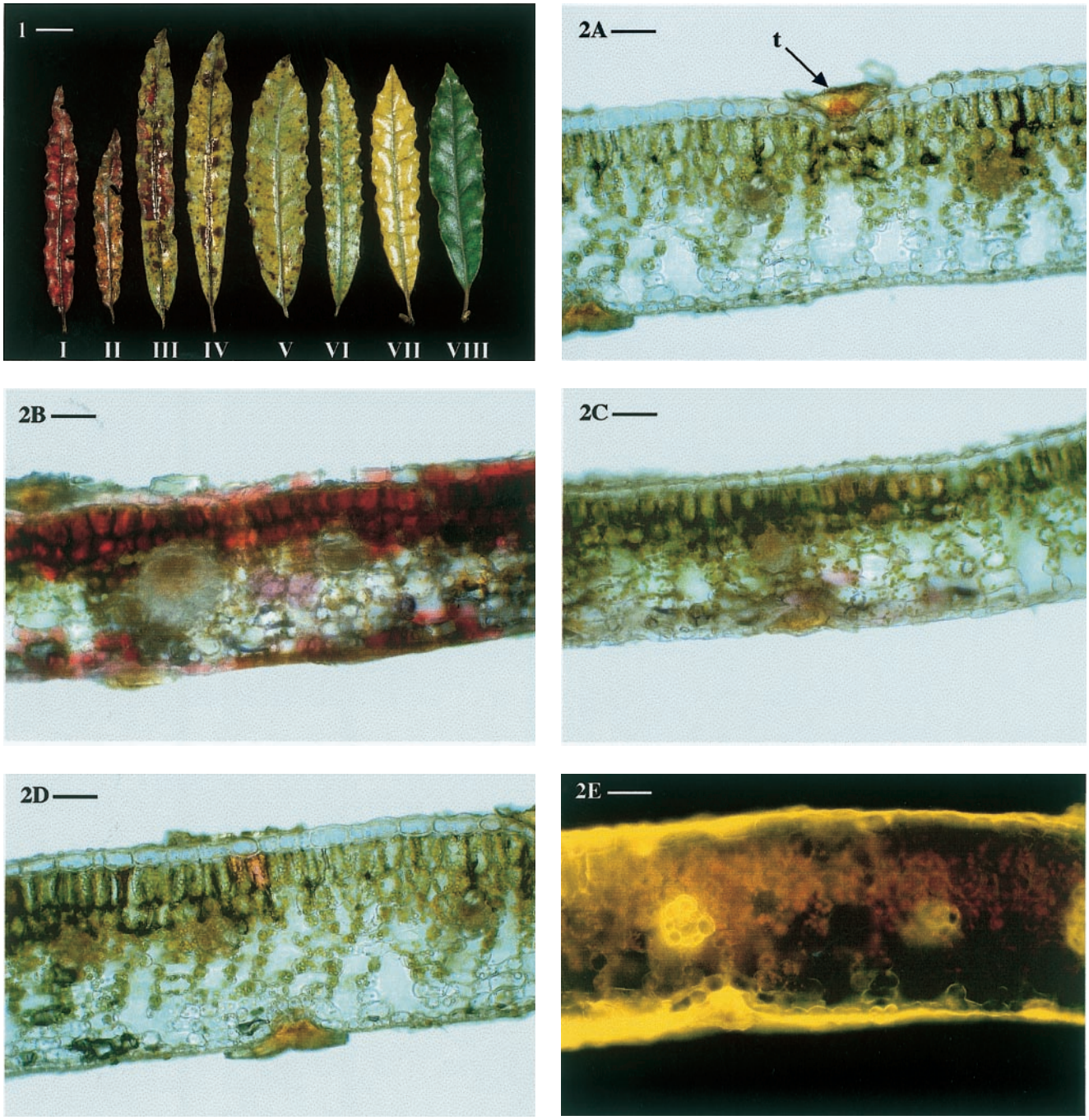


Fig. 1. Photograph of leaves from a natural population of *Quintinia serrata* showing eight phenotypic categories. Bar = 10 mm.

Fig. 2. Light micrographs of transverse sections through fresh *Q. serrata* leaves. (A) Green leaf with peltate trichome (t); (B) anthocyanins present in all tissues, showing variation in hue and intensity; (C) anthocyanins present in clusters of mesophyll; (D) Anthocyanins restricted to individual cells in palisade mesophyll; (E) fluorescence micrograph of section stained with Naturstoffreagenz A. Bars = 50 μm.

Anthocyanins in these leaves were restricted to isolated cells or small clusters of cells in the palisade and spongy mesophyll (Fig. 2C, D). Type I leaves (entirely red) were invariably characterized by an intense pigmentation in all palisade mesophyll layers, usually in combination with the spongy mesophyll (Fig. 2B).

The cuticles and cell walls of both upper and lower epidermises fluoresced strongly yellow-green with Naturstoffreagenz A, indicating the presence of flavonols and/or flavones (Fig. 2E). Fluorescence was also detected in the vacuoles of some epidermal cells, and in the lignified secondary walls of the vascular bundles. There were no differences in the location of fluorescence among leaf types I to VIII.

Table 1. *Leaf phenotype categories and frequency of occurrence in 1000 leaves from a forest population of* Quintinia serrata

Leaf type	Description of lamina	Frequency (%)
I	Entirely red	6
II	Mottled red over entire lamina	2
III	Discrete red region(s) contiguous to both margins	4
IV	Large red patches (each 25–75% lamina width) and small spots	24
V	Small red patches (each 10–25% lamina width) and small spots	35
VI	Frequent red spots, each $<10\%$ lamina width	18
VII	Predominantly green, 1–5 red flecks	7
VIII	Entirely green	4

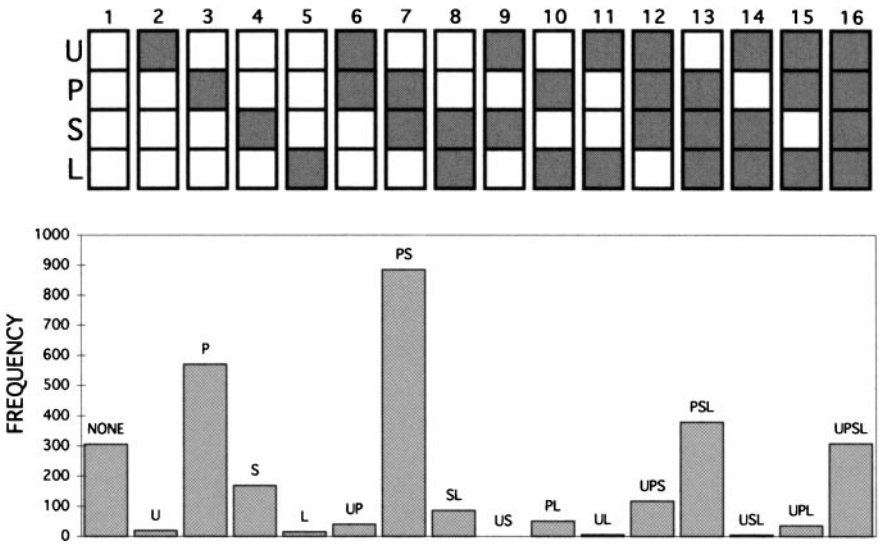


Fig. 3. Possible combinations of anthocyanin-containing tissues (stippled boxes), and frequency of occurrence in 3000 observations of sections through 1000 leaves of *Quintinia serrata*. U, upper epidermis; P, palisade mesophyll; S, spongy mesophyll; L, lower epidermis.

Flavonoid identification

The red leaves and red portions of variegated leaves of *Q. serrata* accumulated two anthocyanins, Cy-3-glc and Cy-3-gal in approximately equal amounts. These were tentatively identified from their absorption spectra and their 2D-PC and HPLC mobilities. The structures were subsequently confirmed by HPLC co-chromatography with authentic samples. Anthocyanins were not detected in the green leaves of types VII and VIII (Table 2).

Flavones, flavonols and hydroxycinnamates were present in all leaves of *Q. serrata*. The flavones, identified from their absorption spectra and ether solubility as apigenin-related, were lipophilic and occurred exclusively in epicuticular waxes. The flavonols occurred in cell vacuoles, and were identified as quercetin derivatives from their absorption spectra and 2D-PC properties. Specific identifications achieved by subsequent HPLC comparisons with authentic samples were: quercetin-3-gal with lesser amounts of quercetin-3-glc; isorhamnetin-3-gal with lesser amounts of isorhamnetin-3-glc; quercetin-3-[rha (1-6) gal] with lesser amounts of quercetin-3-[rha (1-6) glc]; isorhamnetin-3-[ara (1-6) gal]; and (possibly) quercetin-3-[ara (1-6) gal]. Levels of the epicuticular flavones and internal flavonols were calculated in apigenin and rutin equivalents, respectively, in view of the range of structural types identified and the absorption maxima observed. These levels were unrelated to the levels of anthocyanins. However, total flavonoid content did vary in concert with the anthocyanins (Table 2).

Pigment concentrations

Anthocyanin levels (A_{530} values), which varied 74-fold across the sample as a whole, did not correlate strongly with levels of chlorophylls, carotenoids or the estimates of total UV-absorbing compounds in the leaves. For example, the anthocyanins and UV-B-absorbing pigments (A_{300} values) shared only 3% of their variance (Fig. 4). A principal components analysis of pigment levels revealed three clear associations: A_{300} values were strongly correlated to A_{350} values; chlorophyll levels were correlated to carotenoid levels; and the anthocyanins formed a distinct third group (Fig. 4).

Multivariate analysis established that the following topophytic and environmental factors, listed in order of diminishing importance (smaller type III mean squares), best explained variability in anthocyanin levels: leaf posi-

Table 2. *Flavonoid and hydroxycinnamic acid levels (mg g^{-1} fresh weight) in leaves of six phenotypes of* Quintinia serrata

Levels were calculated from HPLC spectra of purified extracts.

Leaf type[a]	Anthocyanins[b]	Hydroxy-cinnamates[c]	Cuticular flavones[d]	Internal flavonols[e]	Total flavonoids[f]
I	1.8	6.0	8.0	8.4	16.4
II	0.34	2.0	10.0	7.3	17.3
IV	0.06	1.0	4.6	9.6	14.2
V	0.01	0.1	11.0	1.4	12.4
VII	0	0.1	4.0	5.5	9.5
VIII	0	5.0	3.4	4.1	7.5

[a]Described in Table 1.
[b]Determined as cyanidin-3-glucoside equivalents.
[c]Determined as caffeic acid equivalents.
[d]Determined as rutin equivalents.
[e]Determined as apigenin equivalents.
[f]Excluding anthocyanins.

tion; leaf type; tree number; canopy gap azimuth; and branches nested within trees. As discussed below, each factor had a statistically significant effect on anthocyanin accumulation (Pillai's trace; $P<0.001$).

The anthocyanin content of leaves increased basipetally along a branch. The two most apical, fully-expanded leaves held significantly less anthocyanin than all older leaves (Fig. 5). This trend was common to all trees that bore anthocyanic leaves, and occurred irrespective of leaf type, branch height or branch order number. However, differences in anthocyanin levels were not mirrored by changing levels of other pigments; A_{300} and A_{350} values did not vary significantly with respect to leaf position, and the chlorophylls and carotenoids were most concentrated in those leaves located at central regions of the branch samples ($P<0.05$; Fig. 5).

As expected, anthocyanins were most abundant in the reddest leaves, and levels decreased progressively from leaf type I to type VIII (Fig. 6). Levels of other pigments bore little relationship to the leaf colour classification (Fig. 6). These data were consistent with the absolute concentrations of individual flavonoids calculated from extractions and HPLC profiles of pooled samples (Table 2).

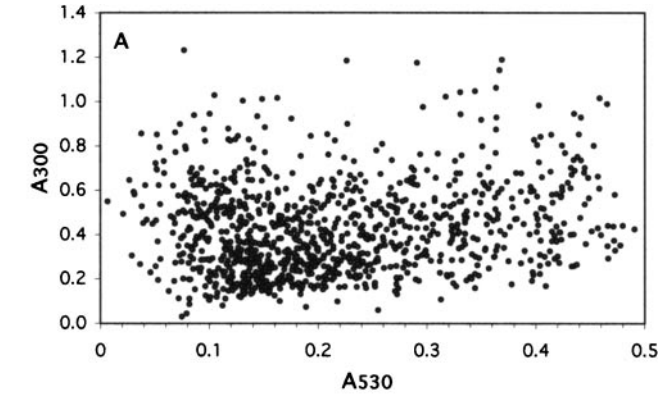
Strong differences in levels of foliar anthocyanins among trees and between branches within a tree could not be explained on the basis of tree height ($r=0.03$; $P>0.1$) or branch height ($r=-0.04$; $P>0.1$). However, there was a statistically significant relationship between anthocyanin levels in the leaves of sub-canopy trees and the direction of the largest canopy gap (Pillai's trace; $P<0.005$). South-facing gaps were associated with the highest anthocyanin values, and north-facing gaps with the smallest (Fig. 7). Interestingly, trees growing under exposed situations and those under dense, closed canopy held similar low levels of anthocyanins (Fig. 7).

Anthocyanin levels were unrelated to their histological location with leaf tissues. Leaves for which anthocyanins were located in multiple tissues did not consistently hold more anthocyanin than those for which the pigment was in a single tissue (Student's t-test; $P>0.05$). Mean values of A_{300} and A_{350} did not vary significantly with respect to the histological groups.

Discussion

These data are not consistent with the hypothesis that anthocyanins serve an auxiliary phytoprotective role in leaves. Levels of foliar anthocyanins vary significantly both within and among trees of the *Quintinia serrata* forest population. However, this variation is independent of the concentrations of flavonols, flavones, hydroxycinnamic acids, chlorophylls, carotenoids, or 'total UV-absorbing pigments' in the leaves (Table 2; Fig. 4). Both red and green leaves can hold comparable levels of these other pigments (Figs 5, 6). It is unlikely, therefore, that anthocyanin accumulation serves to compensate for deficiencies in levels of other protective pigments.

Although anthocyanin concentrations are unrelated to levels of the individual flavonoid types, they do increase in concert with the total leaf flavonoid content (Table 2). The different flavonoid types share some phytoprotective properties, and it may be argued that total flavonoid content is more important to a leaf than are levels of individual anthocyanins, flavones and flavonols. However, each flavonoid type has a unique location within *Q. serrata* leaves: the flavones are found exclusively in the epicuticular waxes; flavonols occur in the vacuoles of epidermal cells; and anthocyanins are associated predominantly with leaf mesophyll. Their spatial separation indicates that the different flavonoid types have distinct roles. For example, the flavones are optimally located to intercept UV-B radiation, but could play no part in the scavenging of organelle-generated reactive oxygen species. Similarly, the location of anthocyanins is unsuitable for screening UV-B, but is ideal for the scavenging of oxygen radicals produced by chloroplasts. Spatial separation of anthocyanins from certain other flavonoids has been



B

	A_{300}	A_{350}	Chl *a* +*b*	Carotenoids
Anthocyanin	0.17	0.19	-0.04	0.12
A_{300}		0.98	0.28	0.44
A_{350}			0.28	0.44
Chl *a* +*b*				0.89

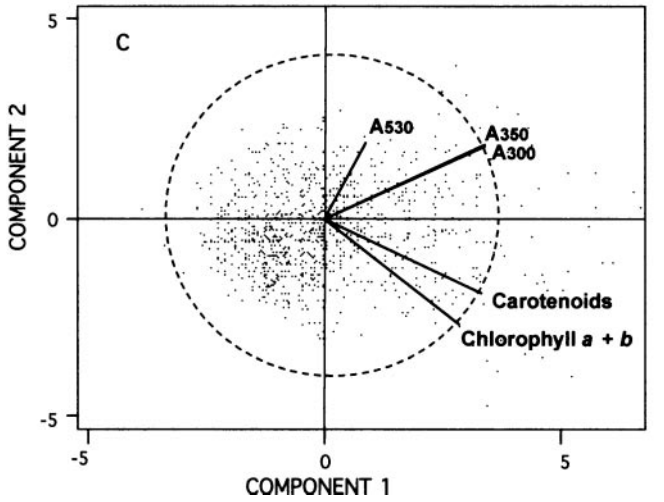


Fig. 4. Pigment composition among 1000 leaves of *Quintinia serrata*. (A) A_{300} values as a function of anthocyanin levels (A_{530}); (B) Pearson correlation coefficients for pigment levels; (C) H-plot of correlation matrix for pigment levels. Variance explained by component 1 = 54%, by component 2 = 26%, total for plot = 80%. The unit circle represents the extent of those h-plot vectors that are perfectly represented in the two-dimensional space. Vectors that approach the circle are well represented in this space, and can be interpreted reliably. Those that are short relative to the circle project away from the plotted space and are difficult to interpret reliably. Acute angles between vectors signify strong positive correlations. All vectors scaled 4-fold.

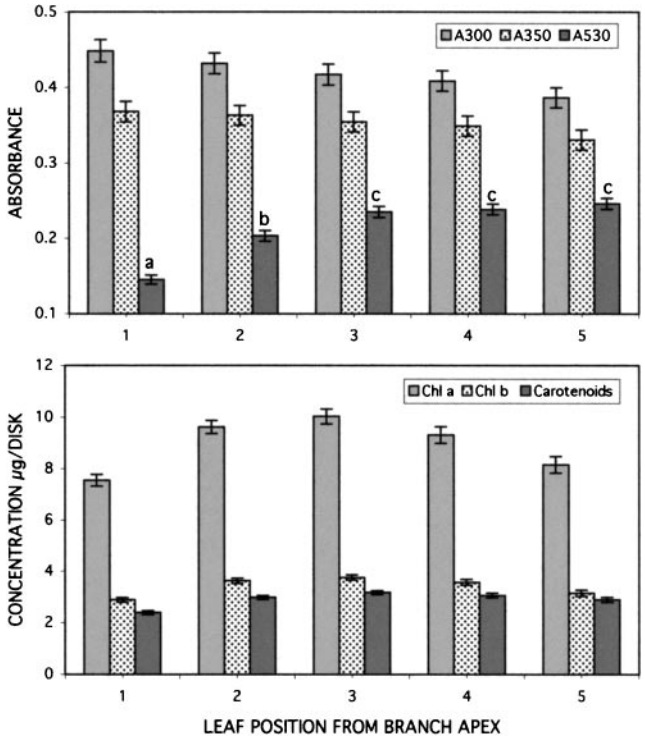


Fig. 5. Mean levels (±s.e.) of anthocyanins (A_{530}), UV-absorbing compounds (A_{300} and A_{350}), chlorophylls *a* and *b*, and carotenoids as a function of leaf position on a branch. Leaves were numbered basipetally from the youngest, fully-expanded leaf. Different letters above bars indicate significant differences among anthocyanin concentrations ($P < 0.05$).

reported previously for the primary leaves of rye (Strack *et al*., 1982; Schulz and Weissenböck, 1986), and suggests that anthocyanins are not merely the default products of a saturated flavonoid metabolism.

Anthocyanins are produced later in the course of leaf development than the UV-absorbing compounds. The phenolic compounds represented by A_{300} and A_{350} values are already present in significant amounts in the youngest (most apical) fully-expanded leaf on a branch, whereas anthocyanins do not reach maximal levels until the third phyllochron (Fig. 5). Although numerous phenolics can absorb ultraviolet radiation, the values for A_{300} and A_{350} would have been determined largely by the concentrations of hydroxycinnamic acids in the leaf extracts. This is because caffeic acid, which in acidified methanol has a peak absorbance at 327 nm and a shoulder at *c*. 295 nm, has a considerably higher molar extinction coefficient in the UV than any of the accompanying flavonoids. These data indicate that hydroxycinnamic acids, rather than anthocyanins, could protect nascent leaf primordia from the effects of UV-B. They are consistent with the evidence from studies in *Arabidopsis*, in which sinapate esters were shown to be more important UV-protectants than flavonoids (Landry *et al*., 1995; Li *et al*., 1993). The data contrast sharply with published observations of certain tropical rainforest species, for which both anthocyanins and total phenolics are most abundant in the youngest leaves, and levels decline in concert as the leaves age (Lee and Lowry, 1980). The functional significance of interspecific differences in the timing of phenolic production remains unknown.

Any unified explanation for anthocyanin function in *Q. serrata* leaves needs to account for the often highly localized pigment distribution, both across the lamina (Fig. 1) and among the various tissues (Fig. 2).

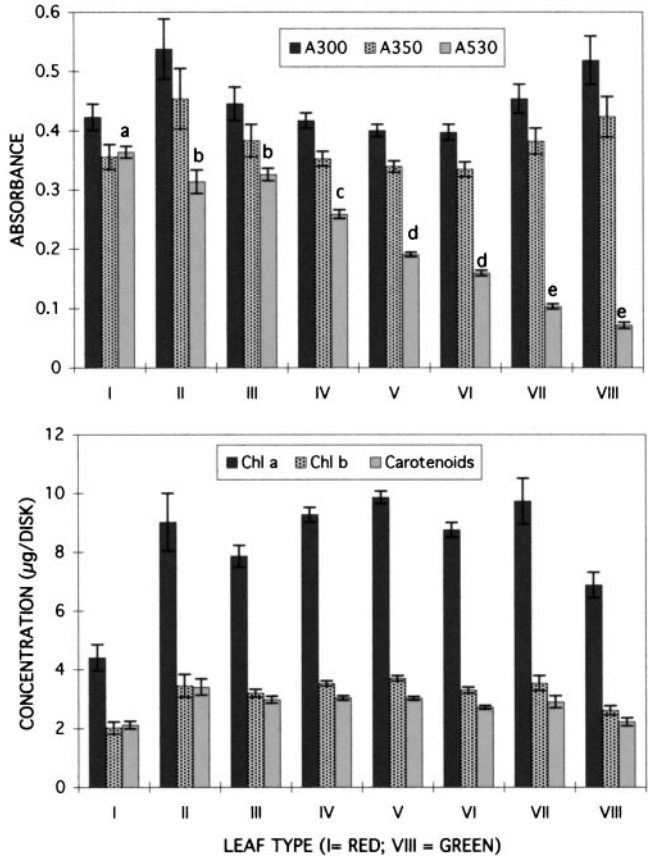


Fig. 6. Mean levels (±s.e.) of anthocyanins (A_{530}), UV-absorbing compounds (A_{300} and A_{350}), chlorophylls *a* and *b*, and carotenoids as a function of leaf phenotype for *Quintinia serrata*, according to descriptions in Table 1. Different letters above bars indicate significant differences among anthocyanin concentrations ($P<0.05$).

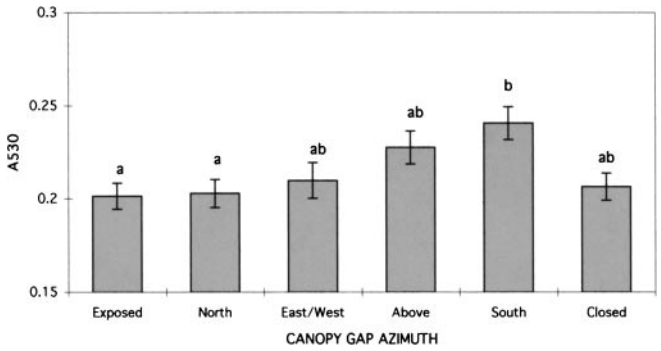


Fig. 7. Mean levels (±s.e.) of anthocyanin (A_{530}) per leaf of *Quintinia serrata* as a function of canopy gap azimuth.

Anthocyanins can occur in almost all combinations of all leaf tissues (Fig. 3); however, the strong association of anthocyanins with chlorophyllous cells indicates a primary role in photosynthesis, perhaps by protecting chloroplasts from photoinhibition during periods of high photon flux (Gould *et al.*, 1995). Numerous workers have reported a reduction in photosynthetic efficiency associated with anthocyanin production (Burger and Edwards, 1996; Dodd *et al.*, 1998; Pietrini and Massacci, 1998; Choinski and Wise, 1999), although the mechanism by which this is achieved remains obscure. However, one postulate, that the absorption of UV-A/blue light by anthocyanin reduces chlorophyll and carotenoid synthesis (Rau and Schott, 1987), clearly does not apply to *Q. serrata*. Concentrations of the chlorophylls and carotenoids are not diminished in those leaves that hold the highest levels of anthocyanins (Fig. 6).

The extent to which genetic and environmental factors each control the polymorphism in leaf colour of *Q. serrata* requires further investigation. However, the observation that anthocyanin levels vary predictably in relation to canopy gap azimuth, offers some insight. The bars in Fig. 7 have been presented in order of what is anticipated to be diminishing annual irradiance for trees in the southern hemisphere. The data indicate that anthocyanin biosynthesis requires a threshold light exposure (levels were minimal in those trees under dense canopy). For those trees growing under only partially covered canopies, anthocyanin levels increase with diminishing annual exposure to irradiation. The statistical significance of these observations was surprising to us given the subjective method used to determine canopy gap (i.e. with a hand-held compass), and the data require confirmation using a more refined technique such as hemispherical photography to estimate light interception. However, the data are consistent with the hypothesis that anthocyanins protect shade-adapted chloroplasts from brief exposures to high intensity sunflecks (Gould *et al.*, 1995).

The observations that anthocyanins are largely absent from green leaves, and from colourless or green cells within red leaves, have significant implications for future research. Green leaves would not serve as useful controls if they were found to contain equivalent amounts of anthocyanins as in red leaves. The sensitivity of the colour expression of anthocyanins to variation in vacuolar pH (Brouillard, 1988) means that it cannot be implicitly assumed that non-red tissues lack anthocyanins. In this regard, *Quintinia serrata* serves as an ideal system for evaluating the effect of anthocyanin distribution on leaf physiology.

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