THE FUNCTIONAL ROLE OF ANTHOCYANINS IN LEAVES

SAMUEL OLIVER NEILL

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FRONTISPIECE

Anthocyanic and acyanic leaves of *Elatostema rugosum* (Urticaceae) photographed in the Waitakere Ranges, New Zealand (Source: K. S. Gould).

ABSTRACT

The anthocyanins, a relatively small group of pigments in the diverse flavonoid family, are largely responsible for the red-blue colouration in a large number plant species worldwide. Their occurrence in fruits and flowers seems to offer clear benefits in attracting pollinators and aiding seed dispersal, however their presence in the vacuoles of leaves remains obscure.

The accumulation of anthocyanin pigments in leaves can be induced by a host of disparate environmental and anthropogenic stressors, such as UV-exposure, wounding, pathogen infection, high light, chilling, pollution, osmotic stress, and nutrient deficiency. Anthocyanins are in some species expressed throughout leaf development; in others the pigments are found exclusively in the young, rapidly expanding leaves, or at later stages of leaf senescence. Many researchers have explored the possible functional role(s) of anthocyanins within leaves, although none of the hypotheses provides a unified explanation for the diverse range of environmental triggers, or for the variability in the pigment's location and expression at particular stages of development.

Differences in the cellular location of cyanic pigments had little effect on the optical properties of leaves from several native New Zealand species. Instead, the absorptance of green-yellow light was strongly proportional to the concentration of anthocyanin. Red leaves absorbed up to 17 % more PAR (400-700 nm) than green leaves of same species. The reflectance of red light was independent of leaf anthocyanin content.

In *Lactuca sativa*, the absorption of light by anthocyanic cell vacuoles in the upper epidermis led to a reduction in the light incident on subjacent chlorenchyma. Under high irradiances, the dissipation of excess energy through non-photochemical processes (qNP) was lower in the red regions of the leaves than in the green regions. Red *L. sativa*, maintained higher photochemical efficiencies (ΦPSII), and had greater rates of photochemical quenching (qP) than the green tissues. Moreover, the cyanic areas were photoinhibited significantly less (7 %), as measured by levels of maximum PSII efficiency (Fv/Fm), than green areas during a high light treatment of 1300 μmol m^{-2} s^{-1}. Chloroplast suspensions from shade adapted *L. sativa* generated less superoxide ($O_2^{\cdot -}$) through the Mehler reaction and had reduced rates of

chlorophyll bleaching, when irradiated with 300 μmol m^{-2} s^{-1} of red light rather than white light. These data demonstrated the impact of the light-filtering effects of anthocyanins on the photophysiology of the leaves.

Complementary to their light-shielding functions, anthocyanins also demonstrated potent antioxidant capabilities at pH values typical of both the cytoplasm and the vacuole. The addition of cyanidin-3-malonylglucoside, which was colourless at the cytoplasmic pH, to a chloroplast suspension receiving high irradiance resulted in the significant scavenging of $O_2^{\cdot -}$. The red, flavylium form of anthocyanin was also oxidised by $O_2^{\cdot -}$. After 15 minutes, this oxidation equated to a 40 % reduction in antioxidant potential of the anthocyanin, as measured by cyclic voltammetry. The data suggested that anthocyanins could provide widespread cellular protection to cellular membranes, organelles, and DNA.

Analysis of the overall antioxidant defence in red and green leaves of *Elatostema rugosum*, a shade-adapted herb native to New Zealand, and *Quintinia serrata*, a native tree found on exposed ridges, provided evidence for a photoprotective role of anthocyanins. Red leaves of *E. rugosum* were on average five-times more effective at scavenging DPPH radicals than were green leaves. The anthocyanins constituted the most active phenolic component, providing a greater relative contribution to the antioxidant pool than the flavones, flavonols, and hydroxycinnamic acids. In contrast, red and green leaves of *Q. serrata* exhibited comparable ranges in antioxidant activities. The data suggest that for some species, anthocyanins can supplement the pool of low molecular weight antioxidants but are not a prerequisite for protection from oxidative stress. It is likely that the localised accumulation of anthocyanins in the leaves of *Q. serrata* serves to shield photosynthetic tissues that are the more susceptible to photodamage.

In conclusion, the accumulation of anthocyanins represents a multifunctional mechanism to; i) directly reduce ROS through scavenging and possibly metal chelation, acting in conjunction with other antioxidants, and ii) to shield photosynthetic processes experiencing excessive irradiances, thereby reducing the extent of photooxidation, photoinhibition, and wasteful dissipatory systems. Such protection may be vital for leaves experiencing stressful environments.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PREFACE

The chapters in this thesis have been written in the format of individual papers that either have been recently submitted for publication or have already been published. As such, each chapter introduces its particular topic separately, describes the methods used, and discusses the results in detail as independent entities. In contrast, the general introduction, background sections, and conclusions refer to the thesis as a whole.

CHAPTER 1 - General Introduction

Occurrence of anthocyanins in plants

Anthocyanins are water-soluble pigments that can provide red to blue colours in flowers, fruits, leaves, and storage organs (Harborne and Grayer, 1988). Anthocyanins are widely distributed throughout higher plants, ferns and mosses, but are seldom seen in liverworts, and are absent from the algae (Brouillard and Delaporte, 1977). Anthocyanin pigments are responsible for the visually striking swathes of colour in temperate deciduous forests during autumn, the crimson flushes of emerging leaves in tropical species, and the predominant hue in plants from tundra and mountainous areas (Chalker-Scott, 1999). They are most conspicuous in the sterile organs of angiosperm flowers, for which their intense colours have a biological role in attracting pollinators and agents for seed dispersal (McClure, 1975). However, the presence of anthocyanins in leaves of taxa predating flowering and fruiting plants suggests an earlier and at present unidentified function (Lee and Collins, 2001).

Anthocyanin expression is remarkably diverse. For some species, anthocyanins are permanently present in all leaves; for others, they occur at specific times of the year, or at particular stages in development (Harborne, 1967; Chalker-Scott, 1999). Anthocyanin production may also be induced by environmental factors, such as light quality and quantity, extremes of temperature, osmotic stress, mineral and pH imbalances, mechanical damage, herbivory and pathogen attack, pollution, and herbicides (McClure, 1975; Harborne, 1988; Chalker-Scott, 1999). Anthocyanin biosynthesis can also vary among individual plants within the same population, among leaves within a canopy, and among tissues within a leaf (Gould and Quinn, 1999; Gould *et al.*, 2000; Lee and Collins, 2001). Anthocyanins in most species are associated with the vacuoles of photosynthetic tissues, the palisade, and spongy mesophyll (Woodall *et al.*, 1998; Gould and Quinn, 1999; Gould *et al.*, 2000; Lee and Collins, 2001); however, in some species they may also occur in the leaf undersurfaces (Lee *et al.*, 1979), and/or in the upper epidermis (Burger and Edwards, 1996).

The occurrence of anthocyanins in leaves at predictable stages of development, and their induction by an array of environmental, biotic, and anthropogenic stressors, indicate that they

probably serve a specific function within the leaf. Anthocyanins are highly modified compounds, normally attached to one or more glycoside groups, which are produced at a biochemical cost to the plant. Thus, the question arises-'why should leaves expend energy in the synthesis of these compounds unless they confer a biological benefit'?

Anthocyanin biosynthesis

Anthocyanins are secondary metabolites and are part of the large phenolic family collectively known as the flavonoids (Fig. 1.1). (Phenolics are a class of compounds that possess one or more –OH group(s) attached to a benzene ring). Flavonoid biosynthesis is unique to plants and has been argued to play an essential part in their success in adapting to life as sedentary organisms in an ever-changing environment (Shirley, 1996). The different classes of flavonoids are assumed to have appeared sequentially during plant evolution (Swain, 1986; Stafford, 1990) reviewed by Cooper-Driver and Battacharya (1998) and Koes *et al*. (1994). The now widespread chalcones, flavanones, and flavones first appeared over 500 million years ago (mya) in the Bryophyta, with hydroxy-flavanones, lignins, and cinnamyl alcohols appearing in the Psilotophyta. Proanthocyanins, flavonols, and catechins are believed to have arisen around 370 mya in the ferns, the first vascular plants. It has been proposed that the evolution of angiosperms was accompanied and propelled by the synthesis of coloured flavonoids, (anthocyanins, chalcones, and aurones) which acted as pollinator attractants and fruit and seed dispersers (Swain, 1986). However, recent evidence indicates that lower plants can also contain complex flavonoid compounds. Some members of the Bryophyta produce aurones, isoflavones, and biflavonoids (Markham, 1988), and anthocyanins have been found in liverworts (Post and Meret, 1992; Kunz *et al.*, 1994) and mosses (Post, 1990).

Over 4000 different flavonoids have been chemically characterised (Strack and Wray, 1993; Das, 1994). They share the same carbon skeleton arranged in a C6-C3-C6 configuration, comprising two aromatic rings (A and B) joined to a C ring or, in the case of the chalcones, a three carbon unit (Markham, 1982; Davies, 2000). Based on the oxidation level of this central C ring, the flavonoids have been classified into twelve classes (Harborne, 1988; Fig. 1.1).

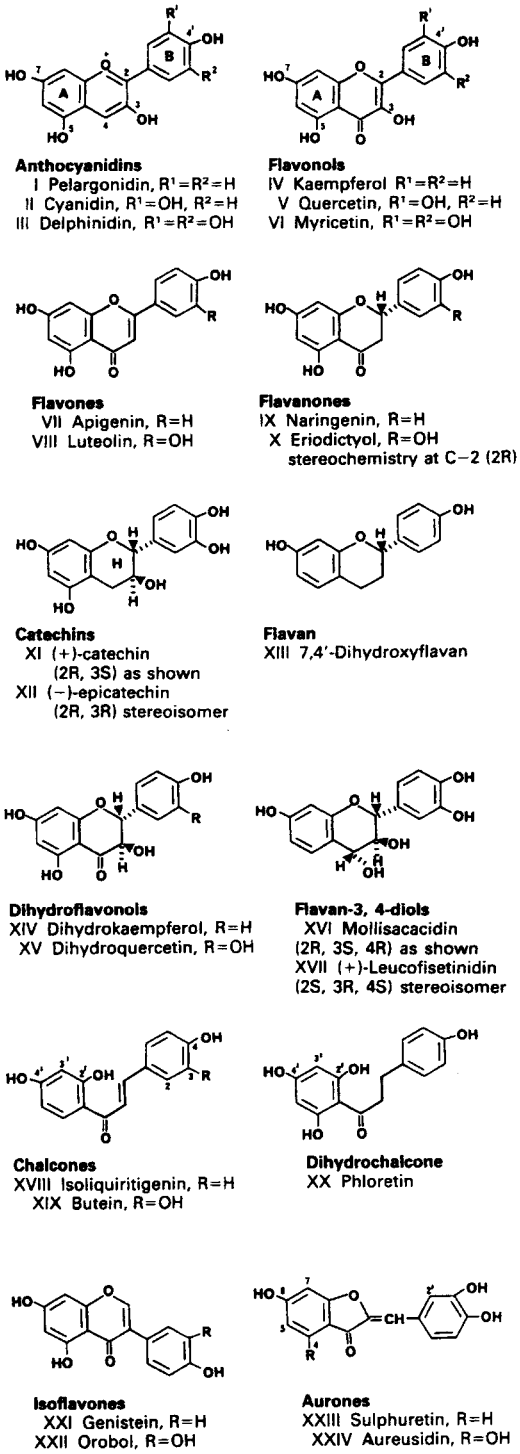


Fig. 1.1 Commonly occurring flavonoid aglycone structures (Source: Harborne, 1988).

Flavonoids are synthesised from the products of two major pathways: the phenylpropanoid pathway via the shikimic acid pathway and the malonic acid pathway (Fig. 1.2). The shikimic acid pathway produces aromatic acids derived from simple carbohydrate precursors of glycolysis and the pentose phosphate pathway (Herrmann, 1995). Only plants, fungi, and bacteria are able to utilise the shikimic acid pathway, and hence the products phenylalanine, tyrosine, and tryptophan are all essential nutrients in the animal diet.

Phenylalanine marks the branch point of primary and secondary metabolism from which the phenylpropanoid pathway can lead to the synthesis of all phenolic compounds. Phenylalanine ammonia lyase (PAL) catalyses the conversion of phenylalanine to trans-cinnamic acid, and is regulated by several PAL-encoding genes, some of which are only expressed in specific tissues or under certain stresses (Hahlbrock and Scheel, 1989). These simple cinnamic acids are converted by a sequence of hydroxylation and methylation reactions to form a number of substituted acids, which can ultimately be used for the synthesis of lignins, coumarins, and benozoic acids. They can also, by conversion to their corresponding coenzyme esters, enter the flavonoid pathway (Grisebach, 1982). Chalcone synthase (CHS) catalyses the stepwise condensation of one molecule of 4-coumaroyl-CoA (derived from the phenylpropanoid pathway), and three molecules of malonyl-CoA (which is a central intermediate in the Krebs tricarboxylic acid cycle) to yield naringenin chalcone (2',4,4',6'-tetrahydroxy-chalcone) (Koes *et al.*, 1994). This step marks the start of the flavonoid biosynthetic pathway.

The yellow naringenin chalcone can be converted to a brighter yellow aurone compound, but more commonly, they are converted to the colourless naringenin (a flavanone) by chalcone isomerase (CHI). This compound is a direct precursor for the large class of isoflavones and flavones catalysed by flavone synthase (FNS) (Heller and Forkmann, 1993). Flavone 3-hydroxylase (F3H) hydroxylates naringenin in position 3 to yield dihydrokaempferol which can then be transformed to dihydroquercetin by flavonoid 3'-hydroxylase (F3'H) or to dihydromyricetin by flavonoid 3',5'- hydroxylase (F3',5'H). F3',5'H can also convert dihydroquercetin to dihydromyricetin (Koes *et al.*, 1994).

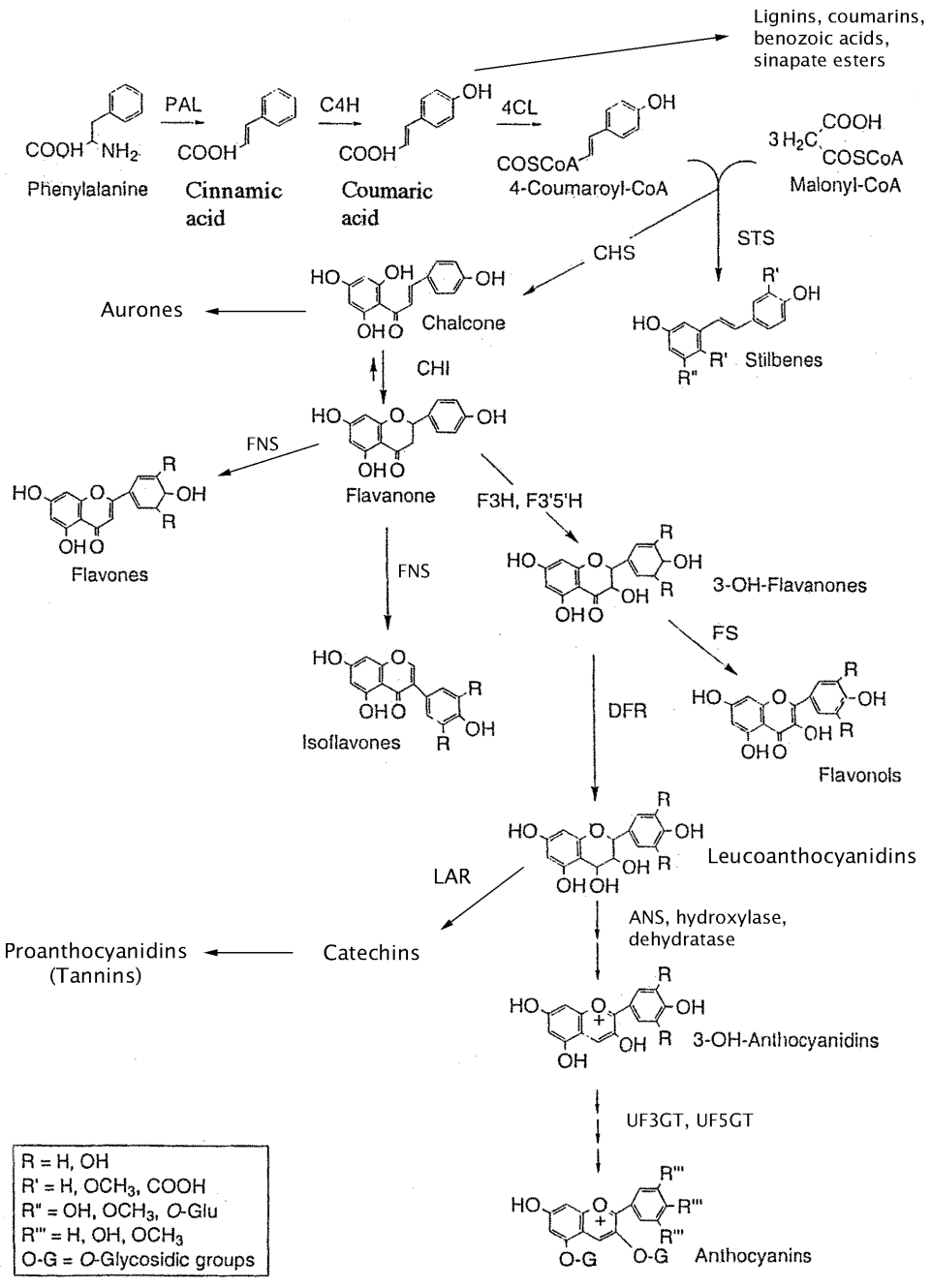


Fig. 1.2 The flavonoid pathway. The structures of the major classes of flavonoids and related compounds are illustrated. Abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; CHS, chalcone synthase; STS, stilbene synthase; CHI, chalcone isomerase; FNS, flavone synthase; F3H, flavanone 3-hydroxylase; F3'5'H, flavanoid 3'5'-hydroxylase; FS, flavonol synthase; DFR, dihydroflavonol reductase; LAR, leucoanthocyanidin 4-reductase; ANS, anthocyanidin synthase; UF3GT and UF5GT, UDP-glucose 3- and UDP-glucose 5-O-glucosyl transferase, respectively (Modified from: Shirley, 1996).

The dihydroflavonols are biosynthetic intermediates in the formation of the flavonols through the insertion of double bond between C-2 and C-3 catalysed by flavonol synthase (FS). The reduction of all three dihydroflavonols in position 4 leads to the formation of the corresponding leucoanthocyanidins (flavan-3,4-cis-diols) and is catalysed by dihydroflavonol 4-reductase (DFR). Further reduction at position 4 is achieved by the enzyme leucoanthocyanidin 4-reductase (flavan-3,4-cis-diol 4-reductase or LAR) and leads to the formation of catechins. From here proanthocyanidins, (the condensed tannins) are synthesised, however the enzyme involved is obscure (Heller and Forkmann, 1993).

Leucoanthocyanidins are also the direct precursors of the anthocyanidins, though the precise enzymatic steps catalysing the conversion are not well understood (Heller and Forkmann, 1993; Davies, 2000). Anthocyanin synthase (ANS) is believed to catalyse the 2-oxoglutarate-dependent oxidation of leucoanthocyanidin to 2-flavan-3,4-diol, which can then be readily converted to anthocyanidin by acidification without additional enzymes (Saito *et al.*, 1999; Nakajima *et al.*, 2001).

Characteristics of the anthocyanin molecule

The anthocyanin molecule consists of an anthocyanidin (the aglycone chromophore), bonded to one or more glycosides. The molecules can undergo further acylation, malonylation, and/or sulphation (Figueiredo *et al.*, 1999). To date, eighteen naturally occurring anthocyanidins have been discovered, all of which are based on the flavylium cation structure (Fig. 1.3) (Harborne, 1967; Timberlake and Bridle, 1975; Strack and Wray, 1993; Jackman and Smith, 1996). Six anthocyanidins are common: i) cyanidin, ii) peonidin, iii) delphinidin, iv) petunidin, v) pelargonidin, and vi) malvidin. Cyanidin is the most widespread among leaves (Fig. 1.4; Ribereau-Gayon, 1972). Although anthocyanidins are usually depicted as having their positive charge associated with the hetrocyclic ring, it is recognised that the charge is delocalised over the whole structure (Timberlake and Bridle, 1975). These flavylium salts are most susceptible to nucleophilic attack, principally at carbon positions 2 and 4.

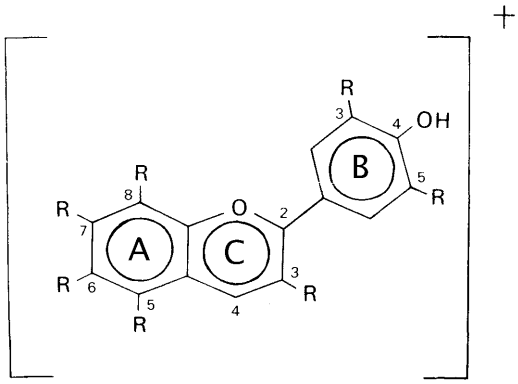
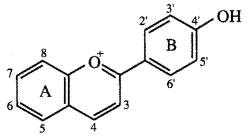


Fig. 1.3 The flavylium cation (Source: Timberlake and Bridle, 1975).



	Substitution pattern					
Anthocyanidin	*3*	*5*	*6*	*7*	*3′*	*5′*
Common basic structures						
Pelargonidin (Pg)	OH	OH	H	OH	H	H
Cyanidin (Cy)	OH	OH	H	OH	OH	H
Delphinidin (Dp)	OH	OH	H	OH	OH	OH
Common methylated structures						
Peonidin (Pn)	OH	OH	H	OH	OMe	H
Petunidin (Pt)	OH	OH	H	OH	OMe	OH
Malvidin (Mv)	OH	OH	H	OH	OMe	OMe
Rare 3-desoxy structures						
Apigeninidin (Ap)	H	OH	H	OH	H	H
Luteolinidin (Lt)	H	OH	H	OH	OH	H
Tricetinidin (Tr)	H	OH	H	OH	OH	OH
Rare hydroxylated structures						
Aurantinidin (Au)	OH	OH	OH	OH	H	H
6-Hydroxy-Cy (6OHCy)	OH	OH	OH	OH	OH	H
6-Hydroxy-Dp (6OHDp)	OH	OH	OH	OH	OH	OH
Rare methylated structures						
5-Methyl-Cy (5MCy)	OH	OMe	H	OH	OH	H
Rosinidin (Rs)	OH	OH	H	OMe	OMe	H
Pulchellidin (Pl)	OH	OMe	H	OH	OH	OH
Europinidin (Eu)	OH	OMe	H	OH	OMe	OH
Hirsutidin (Hs)	OH	OH	H	OMe	OMe	OMe
Capensinidin (Cp)	OH	OMe	H	OH	OMe	OMe

Fig. 1.4 Structures of naturally occurring anthocyanidins. Substitution pattern corresponds to the anthocyanidin carbon numbering system shown above. (Source: Strack and Wray, 1993).

Diversity within the anthocyanins is achieved by substitutions of hydroxyl groups, methylation, acylation, and glycosylation (Harborne, 1967) and gives rise to around 300 naturally occurring structures (Strack and Wray, 1993). Glycosylation is achieved enzymatically by the action of UDP-glucose:flavonoid 3-O-glucosyltransferase (UF3GT) and UDP-glucose:flavonoid 5-O-glucosyltransferase (UF5GT), usually at the 3 and/ or 5-position (Fig. 1.2; Davies, 2000). Glycosylation is thought to increase water solubility, and is essential for the stability of anthocyanin compounds in the acidic aqueous environment of the vacuole (Ribereau-Gayon, 1972; Markham, 1982; Strack and Wray, 1993). The most common monosaccharides involved in anthocyanin glycoslyaltion are glucose, galactose, rhamnose, arabinose, rutinose and xylose. Disaccharides and trisaccharides are also attached in some cases (Strack and Wray, 1989). Glycosides are also often acylated with organic acids such as p-coumaric, caffeic, and ferulic acids via ester bonds usually to the 3-position of the sugar group (Ribereau-Gayon, 1972; Markham, 1982). Cyanidin-3-glucoside is the most common anthocyanin found in leaves (Harborne, 1967).

A common feature of all flavonoids is their ability to absorb radiation (Fig. 1.5). The absorbance shifts to longer wavelengths with increasing conjugation of flavonoids and decreasing saturation of the three planar ring structures. Since anthocyanins undergo the greatest amount of modification through the flavonoid pathway, they are the only members to absorb appreciable radiation in the visible spectrum (500-550 nm; Fig. 1.5) (Harborne, 1967; Shirley, 1996). The wavelength of maximum absorption is dependent on the extent of acylation, vacuolar pH, and co-pigmentation with other flavonoids, and/or chelation to metal ions (Markham, 1982).

Anthocyanin stabilisation

Anthocyanins most commonly accumulate as solutions in the cell vacuole. Extensive research has found that H^+, OH^-, and H_2O are extremely reactive towards anthocyanins. Purified solutions of anthocyanins in an acidic (pH 4-6) aqueous environment typical of the plant vacuole can undergo complex hydration, acid-base, and ring-chain tautomeric modifications.

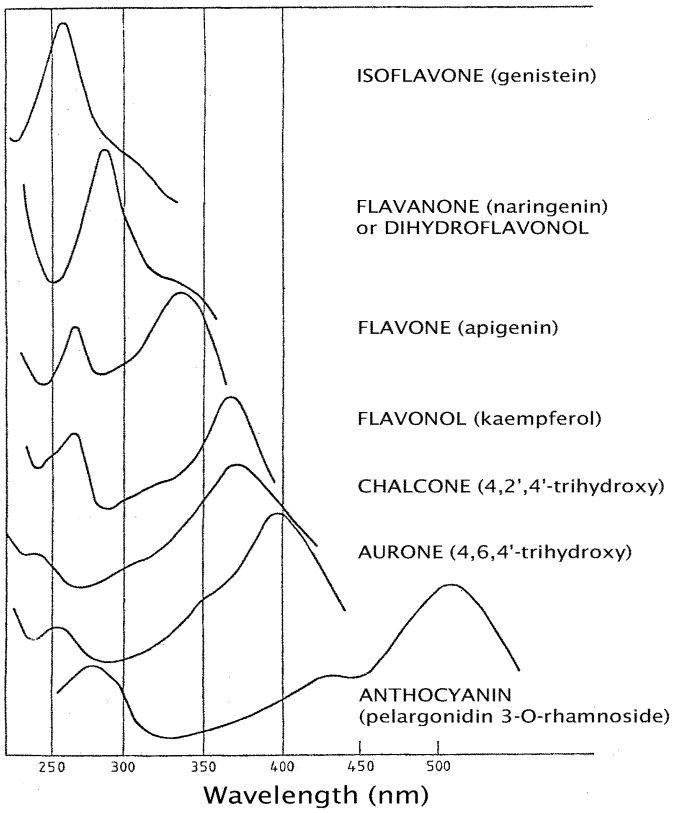


Fig. 1.5 Ultraviolet / visible absorption spectra of different flavonoid types with equivalent hydroxylation patterns (Source: Markham, 1982).

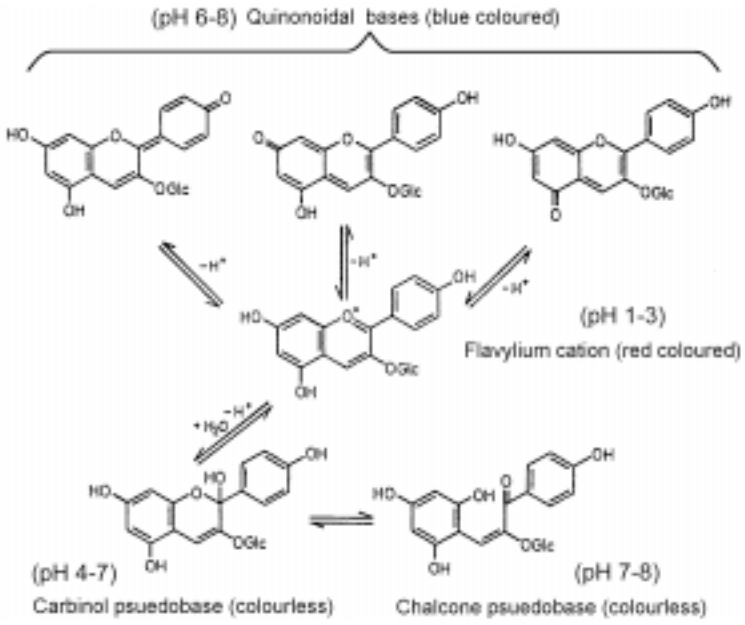


Fig. 1.6 Structural transformations of the anthocyanins (shown for pelargonidin 3-glucoside) in aqueous solution at varying pH. (Modified from: Brouillard, 1988).

As a result of the hydration reactions, anthocyanins are thought to exist as a mixture of four secondary structures in equilibrium (Fig. 1.6): the flavylium cation, the quinoidal base, the carbinol pseudobase, and the chalcone pseudobase (Brouillard, 1982). At low pH values (pH 1-3) the red coloured flavylium structures are dominant. Loss of a proton from either the 5, 7, or 4' position of the flavylium cation leads to the formation of one of three blue coloured quinoidal pseudobases in slightly acidic aqueous solutions (pH 4-6) (Brouillard, 1988). Hydration at the 2-position of the C ring produces colourless carbinol and chalcone pseudobases usually towards pH 7.0 (Brouillard, 1982; Strack and Wray, 1989). At mid pH values (i.e. above pH 3 and below pH 8), a number of different forms can exist, the proportions of which change as a function of pH.

The plant cell vacuole is maintained at approximately pH 5.5, at which 90 % of the total anthocyanin content would be expected to exist in the colourless carbinol pseudobase form, and a smaller proportion in the quinoidal base form (Brouillard, 1982; Brouillard, 1988; Brouillard and Dangles, 1993; Dangles *et al.*, 1994). However, anthocyanins normally exhibit red colouration *in situ*, and the occurrence of natural colourless pseudobases is extremely rare (Timberlake and Bridle, 1975). The reason for this has been postulated to be due to various stabilisation mechanisms, which allow the formation of the following stable, and sometimes highly complex, tertiary structures:

Self-association: At higher concentrations, anthocyanin molecules self-associate, effecting colour changes greater than would be expected from their absolute concentration (Timberlake, 1980; Brouillard, 1982). These interactions effectively form a core of stable coloured anthocyanins that are isolated from hydration events. Self-association may take place between anthocyanidins with the same or different secondary structures (i.e. between AA or AA^- or A^-A^-, where A = neutral quinoidal base and A^- = ionised quinonoidal base) (Brouillard and Dangles, 1994). It is thought that the presence of both the 4'-hydroxyl and 5 glycosyl groups are essential for this kind of self-association (Jackman and Smith, 1996).

Intermolecular co-pigmentation: Anthocyanins can form molecular complexes with other molecules, such as colourless polyphenols (flavones, flavonols, cinnamic and benzoic acid esters, and tannins). In the aqueous environment of the vacuole, these colourless co-pigments compete with water for association with anthocyanin molecules, thereby reducing the number

of sites on anthocyanin molecules available for hydration. Such interactions are believed responsible for a bathochromic shift in absorbance, and increased light absorption (Asen *et al.*, 1976).

Intramolecular co-pigmentation: Anthocyanins possessing at least two aromatic acyl groups usually display a bathochromic shift and extraordinary colour stability (Asen *et al.*, 1972; Brouillard, 1982). Stability results from individual anthocyanins being able to fold in such a way that the hydrophilic sugar/acyl moieties protect the central anthocyanin chromophore from hydration and nucleophilic attack (Goto and Kondo, 1991; Dangles *et al.*, 1993). Such interactions allow coloured forms of anthocyanins to exist in a wide range of slightly acidic to neutral aqueous media (Figueiredo *et al.*, 1999). The net result of stacking is to shift the secondary structure equilibrium in favour of the coloured flavylium and quinoidal forms (Brouillard and Dangles, 1994).

Metal chelation: Metal ions such as Al^{3+}, Mg^{3+}, Fe^{3+}, and Ga^{3+} can form deeply-coloured coordination complexes with anthocyanins possessing an ortho-dihydroxy aromatic moiety or catechol group in their B-ring (Brouillard, 1982; Brouillard and Dangles, 1993). Anthocyanins have been shown to shift from the red flavylium form to the deep purple quinoidal form upon coordination with Al^{3+} (Dangles *et al.*, 1994). It has been proposed that metal ions are able to chelate with anthocyanin molecules, which in turn stack with flavonoid molecules to form a cyclic complex (six anthocyanins and six flavones) around a nucleus of two metal ions (Goto and Kondo, 1991). Thus, it seems metal ions would serve to strengthen existing intramolecular associations (Brouillard and Dangles, 1993).

Cellular location and transport of the anthocyanins

Most flavonoids occur predominantly in the cell vacuole. However, they have also been found in cuticular waxes and cross-linked to cell walls (McClure, 1975; Stafford, 1990; Markham *et al.*, 2000 b). Certain flavonoids have also been noted to occur in cytoplasmic organelles such as the nucleus (Ibrahim, 1992; Grandmaison and Ibrahim, 1996; Hutzler *et al.*, 1998).

Over 90 % of the enzymes involved in anthocyanin biosynthesis, viz chalcone synthase (CHS), chalcone isomerase (CHI) and anthocyanidin 3-O-glucosyl-transferase (UF3GT) activity, were found to be located in the cytosol fraction of protoplasts from *Hippeasrum* and *Tulipa* petals and leaves (Hrazdina *et al.*, 1978). This led to the hypothesis that flavonoid biosynthesis occurs in the cytoplasm. The enzymes have a pH optimum of 7 or above consistent with the pH of the cytoplasm, and enzyme activity could not be detected in isolated vacuoles. Other work has shown that cinnamate hydroxylases and phenylalanine ammonia lyase (PAL) are associated with the endoplasmic reticulum (ER) (Czichi and Kindl, 1975), and it seems, therefore, that anthocyanin synthesis occurs near the ER by cytoplasmic and ER-bound enzymes (Hrazdina *et al.*, 1978). Thus, anthocyanin biosynthesis almost certainly occurs in the cytoplasm before incorporation into the central vacuole. Anthocyanins are probably present in the cytoplasm as the colourless pseudobase at the neutral pH (Stafford, 1990).

Transport of cytosolic anthocyanins into the vacuole has been postulated to involve the covalent linkage of an anthocyanin molecule to glutathione through the action of a type III glutathione S-transferase (GST) enzyme (Marrs *et al.*, 1995). This tripeptide allows a specific glutathione conjugate pump (GS-X pump), a type of ABC (ATP-binding cassette) transporter in the vacuolar membrane to recognise the glutathione S-conjugate and actively (requiring Mg^{2+} and ATP) sequester tagged anthocyanins (Martinoia *et al.*, 1993; Marrs *et al.*, 1995; Coleman *et al.*, 1997; Lu *et al.*, 1997; Marrs and Walbot, 1997). The glutathione pump is a common mechanism for sequestering toxic compounds; flavonoid aglycones may also be toxic compounds which need to be either sequestered into the vacuole and /or cell walls, or deactivated through covalent linkage to a hydrophilic molecule (e.g. glucose, malonate, glutathione, or glucuronate) (Debeaujon *et al.*, 2001). Indeed, Marrs *et al.* (1995) found that when anthocyanins were unable to be sequestered into the vacuole as in the case of Bz2 mutants, plants experienced an overall reduction in vigour, probably due to the toxic effects of oxidised and condensed anthocyanins (Harborne *et al.*, 1975; Stafford, 1990). The diverse GST family has a general role in detoxifying the cytoplasm of toxic natural and xenobiotic compounds such as heavy metals and herbicides (Sandermann, 1992; Coleman *et al.*, 1997). This seems to fit the model proposed by Marrs and co-workers.

However, alternative methods of anthocyanin sequestration have recently been proposed. Mueller *et al.* (2000) found that An9, a Bz2-related gene in *Petunia hybrida*, also encodes for a glutathione S-transferase, which may act as an 'escort' protein instead of catalysing the conjugation of a glutathione tag. It is thought that such a GST/anthocyanin complex would prevent oxidation (Alfenito *et al.*, 1998), cellular toxicity (Ahmed *et al.*, 1994) and may assist in specific cellular transport (Mueller *et al.*, 2000). Both Bz2 and An9 probably evolved independently from distinct types of GSTs, but both function to sequester anthocyanin into the vacuole (Alfenito *et al.*, 1998).

In *Arabidopsis thaliana*, a TT12 protein has also been postulated for vacuolar transport of anthocyanin precursors (Debeaujon *et al.*, 2001). The TT12 gene shows homology to a class of secondary transporters known as MATE (multi-drug and toxic compound extrusion) family. Recognition of anthocyanins for transport through such a channel may involve both glutathione or glycosyl moieties, and also the basic C6-C3-C6 skeleton (Klein *et al.*, 2000). In addition, unmodified flavonoid glycosides have been observed to pass readily into the vacuoles of isolated barley cells without the involvement of GSTs or glutathione (Klein *et al.*, 1996). Hopp and Seitz (1987) noted that acylated anthocyanins were transported into carrot cell vacuoles via a high affinity carrier, with a pH gradient across the tonoplast being important in sequestration. It seems, therefore that a number of different mechanisms could serve to transport anthocyanins across the vacuolar membrane. Such mechanisms could be species specific (Mueller *et al.*, 2000).

In addition to anthocyanins existing in solution within the vacuole, they have also been noted in association with membrane bound, intensely coloured structures called 'anthocyanoplasts'. Anthocyanoplasts were evident in the cytoplasm of epidermal cells of cabbage leaves and a variety of other plants (Pecket and Small, 1980). It was suggested that they were vesicles arising from the ER, and were crucial in the formative stage in the process of anthocyanin accumulation, given that pigmentation appeared in the vesicles before it was apparent in the vacuole. Recent work indicates that these 'anthocyanoplasts' do not possess a membrane or internal structure (Nozzolillo, 1994; Cormier, 1997; Nozue *et al.*, 1997). Similar structures, termed 'anthocyanic vacuolar inclusions' (AVIs) have been observed in the vacuoles of petal cells in various species (Markham *et al.*, 2000 a). AVIs are complexes of anthocyanins attached (probably via hydrogen bonding) to specific protein matrixes (Markham *et al.*, 2000

a; Xu *et al.*, 2001). AVIs may allow anthocyanins to exist at much higher levels than would normally occur in the vacuole and effectively enhance colour intensity. Curiously, these AVIs are specific for anthocyanin diglycosides (Markham *et al*., 2000 a).

Regulation of anthocyanin biosynthesis

Activation of the anthocyanin pathway and accumulation of the pigment in cell vacuoles is a complex process involving a wide array of environmental and developmental signals. Virtually all the anthocyanin biosynthesis genes isolated to date have come from *Arabidopsis*, maize, snapdragon, and petunia (Table 1.1) (Holton and Cornish, 1995). There are over 35 genes known to affect flower colour in petunia, (Wiering and De Vlaming, 1984), thus highlighting the committed genetic control of these metabolically expensive compounds.

Regulatory genes code for proteins (e.g. transcription factors) that control the expression of many of the structural genes, which themselves encode for anthocyanin biosynthetic enzymes (Table 1.2). They are thought to control timing, location, and level of anthocyanin biosynthesis (Holton and Cornish, 1995; Mol *et al.*, 1996). Two large and classes of regulatory genes were originally identified and classified according to their homology to transcription factors from animals and yeast; these are the diverse Myb and Myc/bHLH families (Dooner and Robbins, 1991; Forkmann, 1994; Mol *et al.*, 1998; Davies, 2000; De Majnik *et al.*, 2000). However, many more have been recently discovered, for example the WRKY, homeobox, and WD-40 regulatory genes (Kubo *et al.*, 1999; Walker *et al.*, 1999; Debeaujon *et al.*, 2001).

Various environmental signals have also been proposed to regulate anthocyanin biosynthesis (Mol *et al.*, 1998; Weiss, 2000). Deficiencies in minerals, especially phosphate (Bongue-Bartelsman and Phillips, 1995; Dedaldechamp *et al.*, 1995; Trull *et al.*, 1997), water stress (Balakumar *et al.*, 1993; Sherwin and Farrant, 1998), treatment with cytokinins (Deikman and Hammer, 1995), exposure to lowered pH (Suzuki, 1995), methyl jasmonate (Weiss and Halavely, 1989; Franceschi and Grimes, 1991), wounding (Creelman *et al.*, 1992; Ferreres *et al.*, 1997), and pathogen infection (Dixon *et al.*, 1994) have all been shown to induce anthocyanin production. Cold temperatures have been shown to induce anthocyanin synthesis in many plant species (Chalker-Scott, 1999; Hasegawa *et al.*, 2001), and it has been suggested

Table 1.1 The genetic loci of structural genes that encode the enzymes responsible for anthocyanin biosynthesis (Source: Holton and Cornish, 1995 and Debeaujon *et al*., 2001).

Enzymes	Arabidopsis	Maize	Snapdragon	Petunia
Chalcone synthase (CHS)	*Tt4*	*c2* *Whp*	*nivea*	NA
Chalcone isomerase (CHI)	*Tt5*	NA	NA	*Po*
Flavanone 3-hydroxylase (F3H)	*Tt6*	NA	*Incolorata II*	*An3*
Flavonoid 3'-hydroxylase (F3'H)	*Tt7*	Pr	*eosina*	*Ht1/Ht2*
Flavonoid 3'5'-hydroxylase (F3'5'H)	NA[a]	NA	NA	*Hf1* *Hf2*
Dihydroflavonol reductase (DFR)	*Tt3*	*A1*	*pallida*	An6
Anthocyanin synthase (ANS)	NA	*A2*	*Candica*	NA
Flavonoid 3-glucosyltransferase (3GT)	NA	*Bz1*	NA	NA
UDP rhamnose:anthocyanidin-3-glucoside rhamnosyltransferase (3RT)	NA	NA	NA	*Rt*
Anthocyanin acyltransferase (AAT)	NA	NA	NA	*Gf*
Anthocyanin 5-O-glycosyltransferase (5GT)	NA	NA	NA	NA
Anthocyanin methyltransferase (AMT)	NA	NA	NA	*Mf1/Mf2* *Mt1/Mt2*
Glutathione S-transferase (GST)[b]	NA	*Bz2*	NA	*An13*

[a] NA, a genetic locus encoding the structural gene has not been identified.
[b] Enzyme identity is inferred from sequence homology.

Table 1.2 Regulatory genes that control the anthocyanin biosynthesis (Source: Holton and Cornish, 1995, Shirley *et al*., 1995, and Debeaujon *et al*., 2001).

Species	Locus	Genes regulated[a]	Product type
Arabidopsis	Tt8	DFR	bHLH
	Ttg1	DFR	WD-40
	Ttg2	DFR	WRKY
	Anl2	——[b]	GL2
Maize	R	CHS, DFR, 3GT	bHLH
	R(S)	CHS, DFR, 3GT	bHLH
	R(Sn)	CHS, DFR	bHLH
	R(Lc)	CHS, DFR	bHLH
	B	DFR, 3GT	Myb
	C1	CHS, DFR, 3GT	Myb
	Pl	CHS, DFR, 3GT	Myb
	Vp1	C1	——
Snapdragon	Delila	F3H, DFR, ANS, 3GT	bHLH
	Eluta	F3H, DFR, ANS, 3GT	——
	Rosea	F3H, DFR, ANS, 3GT	Myb
Petunia	An1	ChsJ, DFR, ANS, 3GT, 3RT, AMT, F3'5'H, GST	bHLH
	An2	ChsJ, DFR, ANS, 3GT, 3RT, AMT, GST	Myb
	An4	ChsJ, DFR, ANS, 3GT, 3RT, AMT, GST	Myb
	An11	ChsJ, DFR, ANS, 3RT, AMT, GST	WD-40

[a] See Table 1.1 for the full names of enzymes
[b] ——, unknown gene or product type.

by Christie *et al.* (1994) that *cor* (cold regulation) genes are involved in their synthesis. It also seems that UV-B (Oren-Shamir and Levi-Nissim, 1997) and/or visible radiation (Leyva *et al.*, 1995; Janda *et al.*, 1996) is needed to trigger anthocyanin production at cold temperatures, indicating both complexity and probable overlapping pathways.

A common environmental cue for anthocyanin induction is solar radiation, which both produces energy through photosynthesis to drive anthocyanin biosynthesis and provides external signals for regulating developmental processes. Visible and ultraviolet radiation is perceived in plants through several distinct photoreceptors, including the phytochromes, cryptochromes, phototropins, and UV-B photoreceptors (Kendrick and Kronenberg, 1994; Neff *et al.*, 2000). Although photoinduction of anthocyanins has been demonstrated many times, both in the laboratory and in the field, it is unclear which photoreceptors are involved in the induction of anthocyanin biosynthesis (Mancinelli, 1985; Mancinelli, 1990). It was suggested in a review by Chalker-Scott (1999) that UV-B is the photoinducer, probably mediated by cryptochrome, whereas the extent of anthocyanin production is controlled by the phytochromes (Tekeda and Abe, 1992; Reddy *et al.*, 1994). The promotion of anthocyanin pigmentation in petunia corollas and *chs* gene expression is photon-flux dependent (Weiss and Halavely, 1991). In *Arabidopsis*, *chs* expression involves complex interactions with signals generated from UV-B, UV-A (cryptochromes), and phytochrome receptors (Wade *et al.*, 2001). The spectral sensitivity of anthocyanin production apparently varies with species; however, the most effective light regime in biological systems studied appears to involve blue light, and cryptochrome receptors for UV (Sponga *et al.*, 1986). Work by Neuhaus *et al.* (1993) found that the pathway for anthocyanin biosynthesis involves the activation of one or more heterotrimeric GTP-binding proteins (a type of molecular switch) located in the plasma membrane by active phytochrome (Pfr) proteins. Recent evidence indicates that phytochromes may regulate gene expression more directly, by translocating to the nucleus and binding to transcription factors (reviewed by Smith, 2000).

Light-inducible regulatory factors have been noted to bind to cis-acting elements of the *chs* promoter, which in turn are responsible for the activation of the *chs* gene (Weisshaar *et al.*, 1991; Mol *et al.*, 1998). *In situ* hybridisation of *chs* in irradiated mustard cotyledons has shown that anthocyanin synthesis occurs in cells which had previously expressed *chs* mRNA (Nick *et al.*, 1993). However, CHS was also detected in cells where, under the same

conditions, little or no anthocyanins were detected. Thus, it seems that transcription of the genes for CHS is necessary but not sufficient for anthocyanin synthesis; other factors also have a role in anthocyanin production.

Sugar levels may play an important regulatory role in anthocyanin biosynthesis as anthocyanins commonly accumulate in cell cultures (Cormier *et al.*, 1989; Suzuki, 1995) and whole plants (Murray and Hackett, 1991) experiencing osmotic stress. Other studies have shown that sugars, usually phosphorylated by hexokinases directly initiate signal transduction (Jang and Sheen, 1997). Sugars can regulate *chs* expression directly in *Arabidopsis* and soybean leaves (Tsukaya *et al.*, 1991; Sadka *et al.*, 1994). These compounds may also be involved in long distance communication for anthocyanin biosynthesis throughout a plant. Lewis *et al.* (1998) found that anthocyanins accumulated in foil covered potato tubers when their leaves where illuminated, but at a slower rate than when the tubers were irradiated directly. It was suggested that the initiation of anthocyanin production is controlled in the tuber, rather than in the shoot, even though an effector molecule, probably a sugar, from the shoot may be required to initiate the synthesis. Similarly, Biran and Halevy (1974) found that covering rose flowers did not inhibit growth and pigmentation, whereas covering or removing illuminated leaves decreased fresh flower weight and anthocyanin content.

Functional significance of anthocyanic leaves

Despite more than a century of research into the possible function of anthocyanins in leaves, a unified explanation remains obscure. Several different hypotheses have been put forward to explain the role of anthocyanins in leaves. However, none accommodates the extent of variability in pigmentation patterns over time, space, nor the diverse range of environmental cues.

It is possible that anthocyanins are incidental by-products of the flavonoid pathway, which are shunted into the vacuole as ergastic waste products. However, various sets of evidence point to functional role for anthocyanins in leaves: i) they are not usually found at the same histological location as other flavonoids; ii) their accumulation patterns do not match those of other pigments; and iii) there is usually a strong association with chlorophyllous cells, indicating a primary role in photosynthesis (Gould *et al.*, 2000). Moreover, the complex and

energetically expensive process of anthocyanin biosynthesis would appear to be an extravagant waste of resources if they served no function. The genetic control and regulation of anthocyanins requires specific environmental stimuli, which suggests that they play some kind of role in the response to the stimuli. Several families of the Caryophyllales (Stafford, 1994) hold flavonol and flavone glycosides similar to those in other plants but do not produce anthocyanin. However, their leaves have red pigmentation provided exclusively by an unrelated group of compounds, the betacyanins. Betacyanins are triggered by similar physiological factors as the anthocyanins and they accumulate at comparable tissue locations, yet they are synthesised by a very different pathway. This could indicate a universal role for red, water-soluble pigments (McClure, 1975; Stafford, 1994; Lee and Collins, 2001). Such a function may be satisfied by the anthocyanins in the majority of plant families and by betacyanins in others. A summary of the contemporary hypotheses for anthocyanin function in leaves is presented below.

Elevation of leaf temperature

Quanta absorbed by anthocyanins in leaves may serve to elevate leaf temperatures. Sturgeon and Mitten (1980) surveyed cone colour polymorphism in *Abies concolor* and proposed that anthocyanins have a thermoregulatory function. Anthocyanic forms were more frequent at higher elevations, and attained higher internal temperatures than the green forms. They suggested that purple cones have a selective advantage over green cones when exposed to low temperatures at high elevations, by permitting greater rates of biochemical activity. Other workers have also suggested that anthocyanins play an important role in alpine and arctic regions by absorbing light and warming leaf tissue (McClure, 1975). The potential thermoregulatory benefits are not confined to species in cold climates. Smith (1909) found that young developing anthocyanic leaves of tropical tree species were significantly warmer than adult green leaves and speculated that anthocyanic leaves might develop more rapidly.

Conflicting evidence for the thermoregulatory hypothesis was presented by Lee (1987) who found no significant difference in temperature between flushing (red) and mature (green) leaves of Mango (*Mangifera indica*) and Cacao (*Theobroma cacao*), either on cloudy or bright days. Moreover, the mature leaves from both species had higher absorptances than the flushing leaves, and would be expected to absorb a greater amount of thermal energy than the expanding leaves.

Defence, camouflage, and anti-fungal properties

Since fungal attack is an important source of mortality in young expanding leaves from the tropics, Coley and Aide (1989) suggested that anthocyanins, which are present in 20-40% of expanding leaves from tropical woody tree species, might confer anti-fungal defence. Leaf-cutting ants (*Atta columbica*), which cultivate and rely on fungi for their primary food source were deterred when purified anthocyanins were presented to them on oats. Deterrence was greater when ants were presented with higher anthocyanin concentrations. It has been shown that specific flavonoids, such as the isoflavonoid maackiain and the isoflavan mucronulatol, can protect plants from microbial invasion (Grayer and Harborne, 1994; Harborne and Williams, 2000).

Anthocyanins may also deter potential herbivores. Anthocyanins produced in response to aphids (*Sipha flava)* feeding on mature leaves of *Sorghum halepense* have been hypothesised to thwort subsequent attacks (Costa-Arbulú *et al*., 2001). However, it was found that aphids were not deterred by the anthocyanins themselves, but rather by other compounds associated with the increase in anthocyanins. It has also been postulated that the red colouration may function aposematically through cryptic coloration, warning herbivores of possible toxic compounds present within expanding (Janzen, 1979) and senescing leaves (Hamilton and Brown, 2001). However, work has revealed that young red leaves show equivalent herbivore damage to non-pigmented leaves (Coley and Aide, 1989). There are no convincing reports of anthocyanins imparting toxic or deterrent properties against insects or mammals (McClure, 1975; Lee, 1987). Indeed anthocyanins are common in the human diet (Brouillard, 1982) and are considered greatly beneficial to human health.

Stone (1979) noted that anthocyanins in young developing leaves of *Iguanura geonomaeformis* and *Pinganga* species appear brown due to the masking effect of chlorophyll. This was suggested to be of importance in camouflaging young, palatable leaves by conferring the appearance of dead or dying leaves, potentially reducing herbivore predation. Likewise, Givnish (1990) suggested that the mottling caused by anthocyanins in certain understorey plants might prevent grazing by disrupting their outline to colour-blind vertebrate herbivores. Such hypotheses certainly merit further investigation, but they would be difficult to test experimentally. However, recent work suggests that 'trichromatic'

primates are able to distinguish the younger, tender, digestible and protein rich leaves purely from the presence of the intense red colours displayed in about one half of Africa's, and one third of South and Central America's new foliage (Dominy and Lucas, 2001).

Osmotic protection

Desiccation and freezing impose similar stresses in that they both involve cytoplasmic dehydration. Exposure to mild water deficiencies impairs photosynthesis, carbon fixation, and membrane function, and translates to reductions in growth rates, leaf expansion, and general productivity (Hsiao, 1973). Dehydration can occur in numerous situations, such as when evapotranspiration is high, or when water uptake is restricted due to flooding, or during freezing. Freezing temperatures can induce the formation of ice crystals in the intercellular spaces and cell walls. This extracellular ice formation results in a sudden drop in water potential outside the cell, consequently water is drawn from the cytoplasm by osmosis and leads to cellular dehydration. The extent of water movement is dependent on the concentration of solutes in the cell and the freezing temperature (Xin and Browse, 2000).

Anthocyanins often accumulate in plants experiencing osmotic stress. For example, in the resurrection plants *Craterostigma wilmsii* and *Xerophyta viscose*, a three- and four-fold increase in anthocyanin content was observed during dehydration respectively (Sherwin and Farrant, 1998). It has also been found by (Suzuki, 1995), Sato *et al.* (1996), and Tholakalabavi *et al.* (1997) that anthocyanin levels increase in cell cultures when suspended in high osmotic sucrose and/or mannitol mediums. Such increases in anthocyanin content are associated with an enhanced resistance to water loss. Green-leafed coleus plants that were pre-treated with high light accumulated more anthocyanin and showed increased resistance to 5 ^{o}C chilling than plants that were not exposed to the light pre-treatment (Krizek *et al.*, 1995).

A potential mechanism for this protection was suggested by Chalker-Scott (1999). The glycosides attached to anthocyanins in the vacuole might modify the osmotic potential of the cells, thereby minimising water loss through evapotranspiration and/or freezing. Such protection was postulated to be sufficient to protect young tissues from frost damage (Chalker-Scott, 1999) or the leaves of deciduous species during the autumn mobilisation of substances for winter storage (Robinson, 1991).

Although, despite numerous reports of associatations between anthocyanin accumulation and cold hardiness / desiccation tolerance, there seems to be little evidence for the actual causality. Moreover, the question remains, why anthocyanins should be used by plants to provide osmotic protection when there are numerous other established organic solutes that could also produce similar effects at a lower metabolic cost (e.g. soluble carbohydrates, amino acids, sugar alcohols, and organic acids).

Transport of sugars

Anthocyanins have been suggested to assist in the transport of soluble sugars in senescing leaves of temperate plants and in developing leaves of montane plants (Wagner *et al.*, 1969). Although anthocyanins are comprised of an aglycone complexed to one or more sugar groups, a mechanism for their role in sugar transport has never been established (Lee *et al.*, 1987). Senescence is a complex, metabolically active process where nutrients released from the dismantling of cellular components are subsequently remobilised to storage organs or growing tissue (Matile, 2000). However, it is now regarded that anthocyanins do not participate in sugar remobilisation. Instead, it is thought that they permit the critical reabsorption of nutrients by absorbing excess light and protecting the unstable photosynthetic apparatus from photoinhibitory damage (Feild *et al.*, 2001; Hoch *et al.*, 2001), as discussed later.

The role of anthocyanins in relation to sugar transport recently received attention in relation to a sucrose-export-defective (SXD-1) mutant of maize (Russain *et al.*, 1996; Botha *et al.*, 2000). Structural blockages in the plasmodesmata prevented the symplastic export of sucrose, leading to an accumulation of starch in the leaf tips. It was noted that anthocyanin pigments also accompanied the accumulation of starch. It was proposed that the increased osmotic stress imposed by the starch caused these cells to accumulate anthocyanins. However, anthocyanins played no part in sucrose loading to the bundle sheath-vascular parenchyma (Russain *et al.*, 1996).

Protection against UV-B radiation

UV-B radiation (280-320 nm) can lead to the degradation of proteins, peroxidation of lipids, inhibition of photosynthesis, as well as diminished biomass and plant growth. DNA, which absorbs radiation in the 240 to 310 nm waveband, is oxidised by UV-B. Gene expression can

be altered through the formation of pyrimidine dimers (Tevini *et al.*, 1991). Plants occupying high elevations and lower latitudes are exposed to relatively high levels of UV-B radiation, and as a result show specific tolerance and/or avoidance mechanisms that may have assisted their survival. Such mechanisms include thickened cuticles, steeply inclined leaves, epidermal hairs, and the production of epidermal flavonoids, all of which act to diminish UV-B transmittance through the leaf (Greenberg *et al.*, 1997). Plants may also up regulate their DNA repair mechanisms, xanthophylls cycle activity, and reactive oxygen detoxification processes in response to elevated UV-B.

The flavonoids, including the anthocyanins, are induced and accumulated in plant tissue in response to visible and UV radiation (Tevini *et al.*, 1991; Beggs and Wellmann, 1994; Lois and Buchanan, 1994). They are most frequently found in the vacuoles of epidermal cells (Schemelzer *et al.*, 1988), and absorb strongly in the critical range of 230-380 nm (Markham, 1982). Flavonoid-deficient mutants suffer intense UV damage when illuminated by natural levels of solar radiation (Li *et al.*, 1993; Lois and Buchanan, 1994; Landry *et al.*, 1995). Rueber *et al*. (1996) studied rye (*Secale cereale*) seedlings grown under high light with or without 13 kJ m^{-2} day^{-1} UV-B radiation. They found that epidermal flavonoids were two fold higher in plants grown under the UV treatment than in the plants grown without UV. They also found that the levels of epidermal hydroxycinnamic acids showed little difference in the two treatments and are likely to form a constitutive shield against UV-B radiation, in contrast to the inducible epidermal flavonoids. According to Burchard *et al.* (2000), the relative contribution of these compounds to the UV-B protective function shifts from the hydroxycinnamic acids in young primary leaves to the epidermal flavonoids during later leaf development and acclimation.

Anthocyanins have generally been included as UV-B protectants along with other flavonoids (Gorton and Vogelmann, 1996; Klaper *et al.*, 1996). Lee and Lowry (1980) noticed that young, anthocyanic leaves found in tropical species had markedly lower reflectances in the UV-B region than corresponding mature leaves. Takahashi *et al.* (1991) observed that anthocyanins cell suspension cultures of *Centaurea cyanus* protected the cells from the damaging effects of UV-B and UV-C radiation. They showed that the formation of pyrimidine dimers was significantly negatively correlated in cells containing greater amounts of anthocyanin. Burger and Edwards (1996) found cyanidin-3,5-diglucoside, acylated with *p*-

coumaric acid, located within the upper epidermis of coleus leaves. This anthocyanin significantly reduced UV-B and UV-C damage (expressed as reduced quantum yields) compared with non-pigmented varieties. Similar findings were reported by Koostra (1994) and Stapleton and Walbot (1994). Work by Stapleton and Walbot (1994) showed that acylated anthocyanins (along with other flavonoids) protect against the formation of cyclobutane pyrimidine dimers of DNA in *Zea mays* when irradiated with UV radiation relative to similar plants that were genetically deficient in flavonoid compounds. It must be noted, however, that anthocyanins which lack acylatation have a much reduced absorptance maxima in the 310-320 nm range relative to acylated anthocyanins, and as a result are not as effective at blocking UV-B transmittance through the leaf tissue (Woodall and Stewart, 1998).

Recent data dismiss the wholesale application of the UV shielding hypothesis. First, non-acylated anthocyanins do not absorb UV-B as effectively as other flavonoids and hydroxycinnamic acids (Beggs and Wellmann, 1985; Brandt *et al.*, 1995). Indeed, work using *Arabidopsis thaliana* mutants indicates that the hydroxycinnamic acids are more effective at protecting leaves from UV-B than are flavonoids (Landry *et al.*, 1995). Second, flavonols and flavones usually occur in much greater concentrations than anthocyanins (Lee *et al.*, 1987; Woodall and Stewart, 1998). Woodall and Stewart (1998) found that levels of malvidin-3,5-diglucoside constituted only 3.4 and 5.5 % of the total phenols located in the epidermal and mesophyll tissues of expanding *Syzygium luehmannii* and *Syzygium wilsonii* leaves, respectively. Third, in order for anthocyanins to protect underlying tissue and serve as effective UV-B filters they must reside in the epidermal tissue of plant leaves (Caldwell *et al.*, 1983; Day, 1993; Gould and Quinn, 1999). However, in a survey of 25 anthocyanic native New Zealand plants, only four species held anthocyanins within the uppermost tissues (Gould and Quinn, 1999). In a more extensive survey of anthocyanin location in nearly 400 tropical angiosperms, Lee and Collins (2001) found that only 24 % had anthocyanins located within their epidermal tissue. These findings suggest that UV-B protection cannot be accepted as a unified explanation for the role of anthocyanins in leaves.

Modification of the light environment within leaves

Anthocyanins are the only members of the flavonoid family to absorb appreciable radiation in the visible spectrum (Shirley, 1996; Fig. 1.5). As both the absorption spectrum of isolated chlorophyll (Fig. 1.7) and the action spectra of photosynthesis are maximal in the red and blue

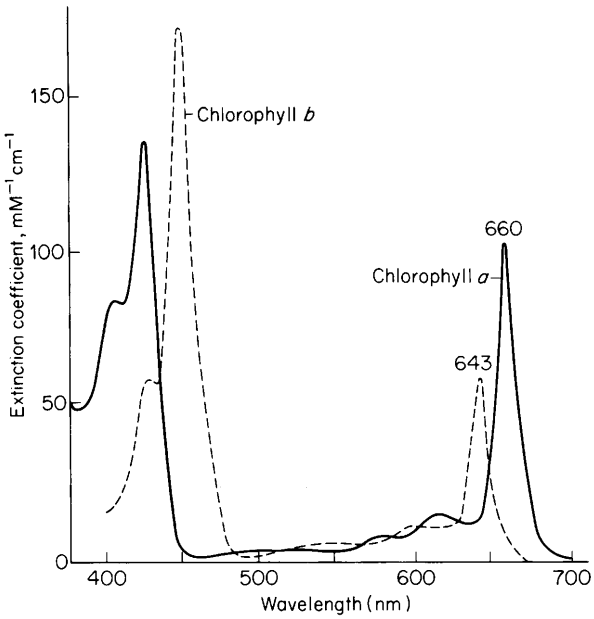


Fig. 1.7 Absorption spectra of chlorophylls extracted in ether (Source: Hall and Rao, 1994).

wavebands (Inada, 1976; Nishio, 2000) there is an apparent lack of absorption within the green region of the visible spectrum (Nishio, 2000). This 'green window' corresponds to the wavelengths at which anthocyanins have maximum absorptance (500-550 nm) (Harborne, 1967).

The impact of a red-pigmented layer on the physiology of chloroplasts, which are responsible for light capture and subsequent conversion to chemical energy, is uncertain. The presence of anthocyanins has been postulated either to boost or diminish light capture, depending upon its location. For instance, anthocyanins in the lower tissue layers of leaves have been postulated to increase light capture by reflecting the transmissible red light back to chloroplast-rich layers. Enhanced absorption would be of importance to plants growing in shaded environments (Lee *et al.*, 1979; Lee, 1986). Conversely, anthocyanins present in upper epidermal and palisade layers may act to reduce light capture by underlying chloroplasts, possibly indirectly reducing the extent of photoinhibition and photooxidation (Gould *et al.*, 1995). As such, the impact of anthocyanins on light capture requires further investigation and is therefore, a central theme of this thesis (Chapters 2, 3, and 4).

Anthocyanins as antioxidants

Due to the sessile nature of terrestrial plants, plant cells are prone to oxidative stress, often as a result of perturbations in environmental conditions and/or biotic stressors, such as herbivore grazing. Fortunately, plants are equipped with a range of protective antioxidant compounds that help minimise the damage experienced in such events. Recent evidence suggests that anthocyanins, which are often up regulated during periods of stress, may also confer effective antioxidant protection *in vivo*.

Phenolic compounds, including the anthocyanins, which are found in fruit, vegetables, and other plant-based products, have been recognized to be beneficial to human health (Halliwell and Gutteridge, 1998). Anthocyanins in red wine exert considerable antioxidant activity *in vitro*, inhibiting both low-density lipoproteins (LDL) oxidation and platelet aggregation (Frankel *et al.*, 1995; Ghiselli *et al.*, 1998). In addition, cyanidin was demonstrated to reduce lipid peroxidation in rabbit erythrocyte membrane and rat liver microsomal systems (Tsuda *et al.*, 1994). Other related compounds such as catechin, epicatechin, and gallic acid are also significant contributors to the total antioxidant activity of red wine (Rice-Evans *et al.*, 1995

b). These findings offer an explanation for the 'French paradox' where, despite a high polyunsaturated fat intake and smoking, the French have a low incidence of heart disease. Moreover, the extensive epidemiological study carried out in the town of Zutphen (Netherlands) established that the incidence of coronary heart disease in elderly men was inversely related to the dietary intake of flavonoids from vegetables and beverages including tea (Keli *et al.*, 1996).

There is now compelling evidence from medicine, food, and wine research that phenolic compounds, including the anthocyanins, have broad pharmacological benefits (Van Acker *et al.*, 1996), including potent antioxidant (Rice-Evans *et al.*, 1997), metal chelating (Morel *et al.*, 1993; Deng and Berkel, 1998) as well as antimutagenic, antiallergic, anti-inflammatory, antiviral, and anticarcinogenic properties (Pathak, 1991; Middleton, 1996; Fergusson, 2001). However, despite the economic consequences of these findings, it is still not clear whether anthocyanins confer a similar protective role in plants, where they are naturally synthesised. Therefore, further investigation is required as to whether anthocyanins function as antioxidants within plant cells. This theme is extensively explored in Chapters 5, 6, and 7.

Plant material

Two native New Zealand plant species have been used in this thesis for the analysis of anthocyanin function in leaves. These are *Quintinia serrata* A. Cunn. Escalloniaceae, and *Elatostema rugosum* A. Cunn. Urticaceae. Both species are remarkably polymorphic for foliar anthocyanin content. For each species, the red and green morphs are anatomically identical, and are found in similar locations. As such, these plants present an ideal opportunity to study the effects of anthocyanins in experimental systems, as the green morphs provide a naturally occurring control. In order to further test findings from natural populations, anthocyanic and acyanic areas of *Lactuca sativa* cv. Dark Lollo Roso leaves were employed due to the availability of abundant leaf material and the ease with which they could be grown under environmentally controlled conditions.

Quintinia serrata, A. Cunn. Escalloniaceae

This is a hemi-epiphytic endemic tree found on exposed ridges in northern lowland to montane forests from lat. 35^{o} to 39^{o} 30' of New Zealand. *Q. serrata* grows up to 9 m tall,

with a trunk diameter of up to 40 cm. Leaves show typical sun-leaf architecture and are lanceolate to oblanceolate, 5 - 15 cm long and 1 - 3 cm wide, on petioles up to 2 cm (Gould *et al.*, 2000). The leaves are coarsely and irregularly serrate, with waxy, glandular margins (Allen, 1961). The youngest leaves are acyanic. In some populations, older leaf laminae are blotched with red-pigmented patches.

***Elatostema rugosum*, A. Cunn. Urticaceae**

This is an endemic New Zealand sprawling herb found in damp shaded areas, especially streamsides and gullies, in lowland to montane forests from near North Cape (34^{o} 30' S) to Tararua Range (41^{o} 00'). Stems are usually unbranched and are fleshy and decumbent. *E. rugosum* produces distichous, chartaceous, and sharply and coarsely serrate leaves, which are 8 – 25 cm long and 2.5 – 6 cm wide. All leaves show shade-leaf anatomy, and are rugose with minute asperites (Allen, 1961). In some populations, the youngest leaves close to the meristem are pigmented intensely red, which gradually decreases with leaf age.

Aims of this thesis

This thesis attempts to elucidate the potential function of anthocyanins in leaves, and to discover a unified explanation for their occurrence in nature. Two key hypotheses have been identified which have the potential to provide an explanation for the presence of anthocyanins in leaves, yet require further investigation. The impact of anthocyanins on light capture and the potential of anthocyanins to act as antioxidants are both described in greater detail in the remainder of this thesis.

The specific objectives of this thesis are:

Does the cellular location of anthocyanins affect the absorptance of light by altering the quality and/or quantity? If so, does the presence of anthocyanins in abaxial cell layers reduce light capture in subjacent chloroplasts or do abaxially located anthocyanins enhance light capture through the 'back-scattering' of red light?

Does the absorption of green wavelengths correspond to a reduced occurrence of photoinhibition and photooxidation in leaves experiencing high irradiances? Do chloroplasts developing under an anthocyanic layer exhibit a shade-adapted physiology?

If anthocyanins are scavengers of reactive oxygen species (ROS), do red leaves hold an antioxidant advantage over green leaves? Does the relative concentrations of other antioxidants in red leaves correspond to the amount of anthocyanins present?

Is the role of anthocyanin as an antioxidant a primary one or does it confer other beneficial roles? Are anthocyanins, which are predominantly located within the cell vacuole, spatially near to biomolecules that require antioxidant protection? Do anthocyanins act by interfering with radical formation, by chelating transitional metals, repair damage, directly scavenge ROS, or a combination of the four?

CHAPTER 2 – Interactions with photosynthetic active radiation (PAR)

A key component of the work to be presented in this thesis concerns how anthocyanins interact with light quality and quantity within leaves. Therefore, background information on the various mechanisms plants use to moderate the amount of light they capture, and the processes of photoinhibition, and photooxidation are required to facilitate understanding of the following two chapters (Chapters 3 and 4).

Light capture: a dynamic equilibrium

Plants constantly require to balance the beneficial and potentially destructive consequences of light absorption. On the one hand, plants need to ensure adequate capture of quanta for photosynthesis. On the other hand, however, the absorption of excess radiant energy can lead to a decline in photosynthetic efficiency (photoinhibition and/or photorespiration) as well as photooxidative damage. Thus, light acclimation of the photosynthetic apparatus involves the coordinated allocation of resources to achieve and maintain productivity effectively under both limiting and excess irradiance (Anderson *et al.*, 1995).

Plants growing in higher light environments often have greater maximal capacities for carbon fixation, facilitated by larger cytochrome b/f complexes, ATP synthase, plastoquinone, and ferredoxin. These allow them to utilise a greater proportion of incident light in photosynthesis (Anderson and Osmond, 1987). Shade-adapted plants by contrast, strive to maximise light absorption by increasing both the ratio chlorophyll *b:a*, and the light-harvesting proteins of PSII and PSI. Such a strategy is at the expense of electron transport, photophosphorylation, ribulose bisphosphate carboxylase (RuBisCO) levels, and carbon fixation. Shade plants have a lower maximum photosynthetic rate, which saturates under relatively low irradiances, than have sun plants (Anderson *et al.*, 1995).

Photosynthetic energy capture and chlorophyll fluorescence

Absorption of the energy from a photon of light promotes electrons in chlorophyll molecules to higher energy orbitals, which can then emit energy to adjoining molecules via resonance transfer. Excitation energy captured in the antenna complexes is ultimately channelled into

the reaction centres for conversion to chemical energy via photosynthesis (Φ_P) (Krause and Weis, 1991; Maxwell and Johnson, 2000).

Light energy which is absorbed in excess of that used for photochemistry must be effectively dissipated by non-photochemical processes to avoid photooxidative damage. When excited chlorophyll pigments cannot transfer their energy onto other photosynthetic acceptors, for example when the primary quinone acceptors are fully reduced, they can emit their energy safely as heat (thermal dissipation or radiation-less deactivation Φ_D), or as fluorescence at longer wavelengths (685-800 nm) (Φ_F) (Fig. 2.1). Under a given light flux these dissipation processes operate in competition for a finite pool of absorbed energy:

$$\Phi_P + \Phi_D + \Phi_F = 1$$

Therefore, any change in energy utilisation by one process produces a complementary change in the others (Schreiber *et al.*, 1998). Measurements of chlorophyll fluorescence can therefore be used non-invasively to determine the state of photosynthetic processes (Krause and Weis, 1991; Maxwell and Johnson, 2000) and are regarded as a sensitive, *in vivo* probe of photosynthetic function (Bolhar-Nordenkampf and Oquist, 1993). Chlorophyll fluorescence is exclusive to chlorophyll *a* of PSII and the yield is relatively minor (2.5-5.0 %) relative to the amount of light absorbed (Bolhar-Nordenkampf and Oquist, 1993).

Following a sufficient period of dark adaptation, the primary quinone acceptors of PSII (Q_A) are fully oxidised and are ready to accept excitation energy. Upon irradiance with weak light, leaves emit minimal fluorescence (Fo), as the potential for photochemical quenching is maximal (Fig. 2.2). When then exposed to saturating light, Q_A are rapidly reduced and are unable to accept further excitation energy. This produces maximum fluorescence (Fm) (Kautsky *et al.*, 1960). The difference between Fm and Fo is termed the maximum variable fluorescence (Fv), which reflects the oxidation state of Q_A. The maximum efficiency of PSII correlates to the ratio Fv/Fm (Genty *et al.*, 1989; Schreiber *et al.*, 1998). This characteristic pattern of chlorophyll fluorescence in response to a pulse of saturating light is known as the Kautsky curve (Bolhar-Nordenkampf and Oquist, 1993). Depending upon the conditions

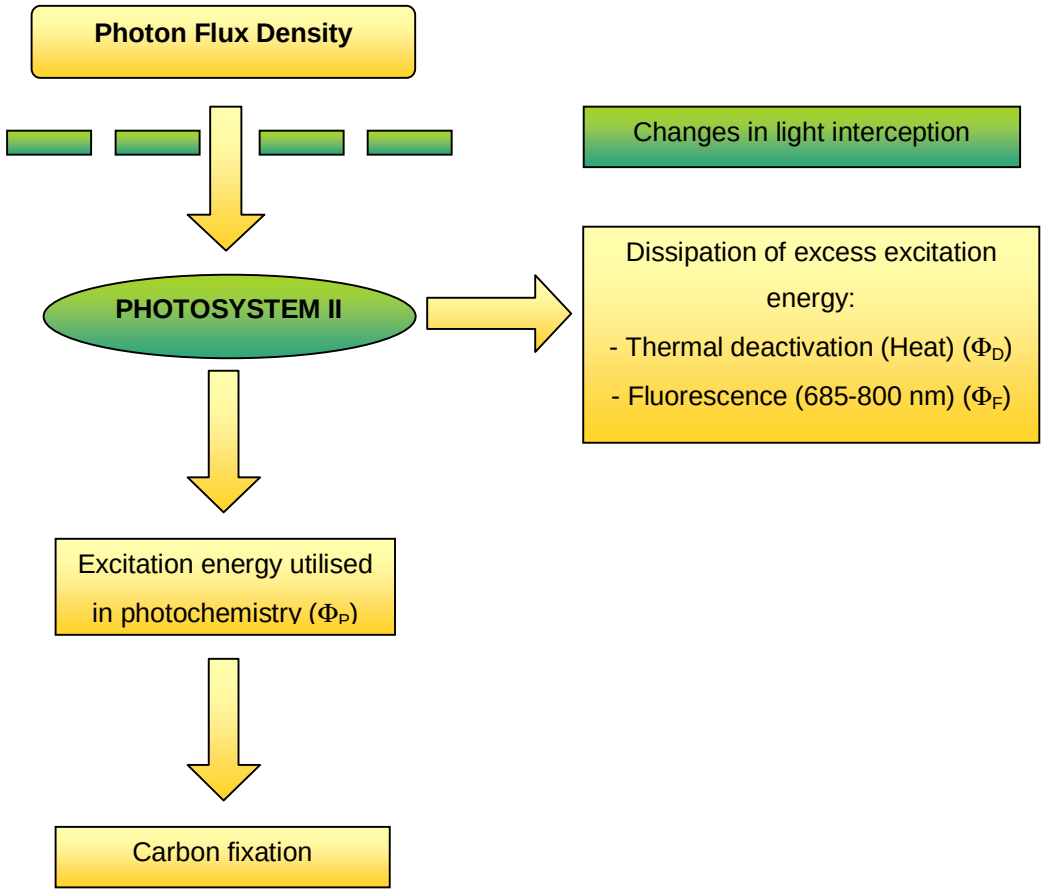


Fig. 2.1 The fate of excitation energy in chlorophyll pigments from light capture within the reaction centres of photosystem II (PSII) (Modified from: Demmig-Adams, Adams, and Grace, 1997).

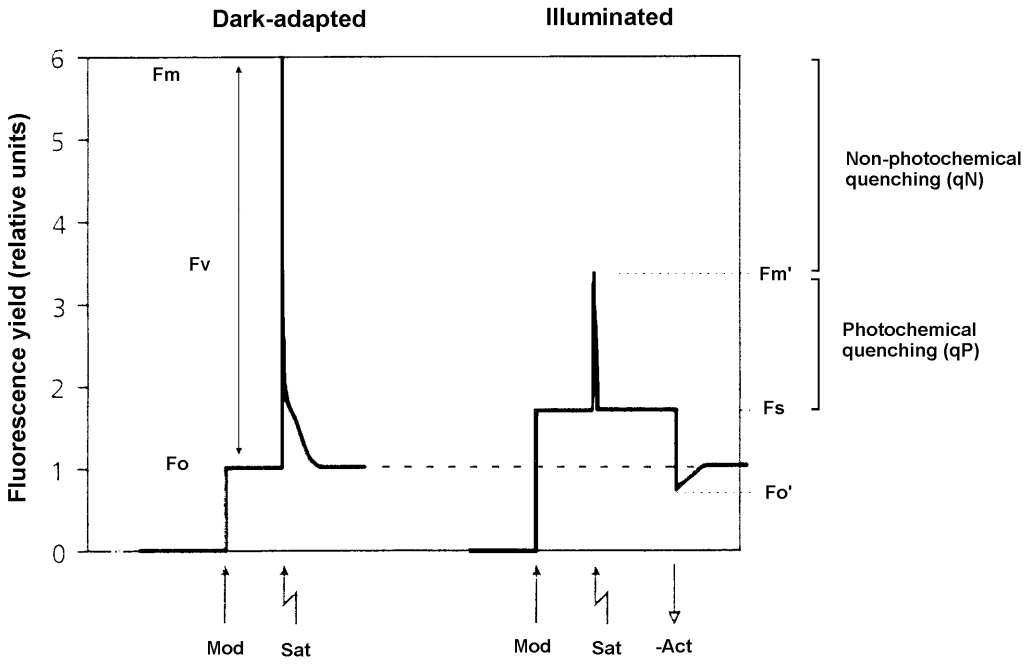


Fig. 2.2 A typical fluorescence curve of dark-adapted and illuminated leaves in response to a saturating pulse of white light. Mod = modulation measuring light; Sat = saturating pulse; -Act = actinic light off. Fluorescence symbols are explained in the text (Modified from: Schreiber *et al*., 1995).

experienced by the photosynthetic apparatus, healthy leaves generate, an Fv/Fm ratio of around 0.85 in (Bolhar-Nordenkampf and Oquist, 1993). When leaves are photoinhibited by excess excitation energy, values of Fv/Fm decline. As a result Fv/Fm provides a useful measure of photoinhibition (Powles, 1984).

It is also useful to obtain information about photochemical processes that occur under light-adapted conditions, for example in the presence of background illumination, or in full sunlight (Maxwell and Johnson, 2000). Such information can be collected using a weak, modulated light pulse of low enough intensity that re-oxidation maintains the open state of the reaction centres with minimal fluorescence generation (Quick and Horton, 1984; Van Kooten and Snel, 1990).

After a period of non-saturating actinic illumination, the photosynthetic apparatus gives a steady emission of fluorescence termed Fs, which is usually slightly higher than Fo (Lambers *et al.*, 1998). When a saturating light pulse is given under these steady-state conditions, fluorescence rises significantly (Fm'), although not as high as the initial dark-adapted Fm value (Lambers *et al.*, 1998). The reason for this reduction in maximum fluorescence is that competing non-photochemical quenching processes dissipate the excess excitation energy in the saturated reaction centres. Photochemical quenching (qP) indicates the proportion of photons absorbed by PSII that are used by the open reaction centres for photosynthesis, and is increased by the light induced activation of enzymes used in carbon metabolism and the opening of stomata (Maxwell and Johnson, 2000). Non-photochemical (qNP) quenching represents the proportion of photons absorbed by PSII that are instead non-radiatively dissipated as heat (Schreiber *et al.*, 1998). When the actinic light source is turned off and a far red light pulse applied, PSI is preferentially excited, effectively 'draining' electrons from PSII and allowing the measurement of Fo' to be made (Maxwell and Johnson, 2000). These light adapted parameters allow calculation of the efficiency with which excitation energy is passed from the antennae to the reaction centres (antenna efficiency of PSII = Fv'/Fm'), and the efficiency at which the photosystem operates as a whole (photochemical efficiency of PSII = ΦPSII) (Genty *et al.*, 1989). A number of other useful measurements can be obtained, knowing the irradiance incident on a leaf's surface, using the following equations:

1) Maximum efficiency of PSII (Fv/Fm) = (Fm-Fo)/Fm (Genty *et al.*, 1989).

2) Antenna efficiency of PSII (Fv'/Fm') = (Fm' – Fo')/Fm' (Genty *et al.*, 1989).

3) Photochemical quenching (qP) = (Fm' – Fs)/(Fm' – Fo') (Van Kooten and Snel, 1990).

4) Non-photochemical quenching (qNP) = 1-(Fm' – Fo')/(Fm – Fo) (Van Kooten and Snel, 1990).

5) Photochemical efficiency of PSII (ΦPSII) = Fv'/Fm' × qP (Genty *et al.*, 1989).

6) Electron transport rate (ETR) = ΦPSII × PFD absorption by the light harvesting centres of PSII (LHCII) (Genty *et al.*, 1989).

The last equation is problematic because it requires an estimation of the number of quanta absorbed by PSII. Variations in a leaf absorptance affect calculated rates. Researchers often multiply the product of the equation by a "typical" absorption value such as 84 % (Schreiber *et al.*, 1998). The absorptance of non-photosynthetic pigments, such as the anthocyanins must be taken in to account when calculating ETRs.

Photoinhibition

Photoinhibition is characterised as a reduction in Fv/Fm, and can be classified as one of two categories based on severity (Osmond, 1994). First, dynamic photoinhibition is characterised as a reversible depression of the Fv/Fm ratio. Plants experience such photoinhibition at midday when light intensities are at their greatest, and usually normal photosynthetic rates are restored as the irradiance declines. A more severe response, termed chronic photoinhibition, is non-reversible and leads to the sustained depression of Fv/Fm. Chronic photoinhibition is characterised by damage to the photosynthetic apparatus through the bleaching of chlorophyll pigments and ultra-structural damage of the thylakoid membrane via photooxidation (Hall and Rao, 1994). Chronic photoinhibition can occur when a shade-adapted plant is suddenly exposed to high irradiances from sun flecks or when a tree falls creating a canopy gap. The susceptibility of plants to photoinhibition is exacerbated by adverse environmental conditions

such as low temperature, water stress, and mineral deficiency (Powles, 1984). Plants under low irradiances can still experience reduced photosynthetic efficiencies under these conditions.

Photooxidation

Photoinhibition is thought to occur when reductants generated from photosynthesis are in excess of a plants capacity to utilise them in carbon fixation (Osmond, 1981; Foyer *et al.*, 1994). Over-reduction of the quinone acceptor molecules results in the excitation of the chlorophyll *a* dimers (P680) located in the PSII reaction centre to their triplet states. These $^3P680^*$ dimers are able to react with oxygen, forming singlet oxygen ($^1O_2$). Although $^1O_2$ has a relatively short lifetime in an aqueous environment, it exists about 30 times longer in non-polar environments such as the lipid bilayer, where it can readily react with unsaturated fatty acids in the thylakoid membranes (Knox and Dodge, 1985). It is also responsible for deactivating the function of reaction centres by attacking amino acid residues on the D1 protein, the principle quinone-binding site (Demmig-Adams and Adams, 2000).

Another process favouring the production of reactive oxygen species (ROS) is termed psuedocyclic electron transport or the Mehler reaction. During photoinhibitory conditions reduced ferredoxin (and other PSI electron acceptors) react with molecular O_2, rather than with $NADP^+$, forming superoxide radicals ($O_2^{\cdot-}$) and ATP (Hall and Rao, 1994). Such non-cyclic electron transport is primarily brought about by intense light, when levels of NADPH are high. It is thought that this process prevents the over-reduction of the linear electron transport chain proteins, thereby allowing cyclic phosphorylation to continue (Robinson, 1988; Schreiber and Neubauer, 1990; Osmond and Grace, 1995). $O_2^{\cdot-}$ can oxidize various cellular components directly, but it is thought to exert greater biological damage through chain reactions, which generate more reactive and destructive species (Halliwell and Gutteridge, 1998). The protonated form of $O_2^{\cdot-}$, $HO_2^{\cdot}$, is thought to co-exist at physiological pHs, is more reactive, can initiate the peroxidation of fatty acids, and is thought to be able to cross membranes as easily as H_2O_2 (Halliwell and Gutteridge, 1998). In addition, $O_2^{\cdot-}$ can either be hydrated directly (non-enzymatically), or converted via superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). H_20_2 is a potent inhibitor of photosynthetic processes and as a result, its removal is vital for chloroplast function (Kaiser, 1979). In the presence of iron salts

and certain other transition metals, H_2O_2 can react with $O_2^{\cdot-}$ to form the hydroxyl radical ($OH^{\cdot}$) through Haber-Weiss-Fenton reactions (Asada and Takahashi, 1987; Saran *et al.*, 1998). This is the most reactive species in biology with a lifetime of about 1 μs, which can attack and damage almost every molecule in living cells (Halliwell, 1984).

Alternative ways to reduce photoinhibitory stress

Light capture can be adjusted through a number of mechanisms to match the photochemical capacity and reduce the occurrence of photoinhibition (Anderson, 1986) or repair the associated damage (Foyer *et al.*, 1994). Such strategies can be divided into two main categories:

Avoidance

Avoidance of light stress may include leaf movements such as paraheliotropism (Kroller, 1990), changes in chloroplast distribution within cells (Haupt and Scheuerlein, 1990), or morphological adaptations which increase reflectance, such as the presence of leaf hairs (Ehleringer and Björkmann, 1978), waxes (Barker *et al.*, 1997), or salt cover (Mooney *et al.*, 1977).

It has also been postulated that the presence of anthocyanins in the upper epidermis of leaves can reduce light penetration into underlying photosynthetic cells by absorbing green light. Such attenuation is thought to be of importance under high irradiation to permit the maintenance of higher levels of oxidised of PSII acceptors, thereby minimising photoinhibition, and photodamage (Gould *et al.*, 1995; Krol *et al.*, 1995; Burger and Edwards, 1996; Smillie and Hetherington, 1999).

Tolerance

A common mechanism in which plants can tolerate long-term changes in light conditions are changes in their light harvesting ability, by alteration of both the antenna size and the proportion of accessory pigments (Demmig-Adams *et al.*, 1997). In addition to their ability to rapidly repair PSII reaction centres and re-activate some enzymes (Halliwell and Gutteridge, 1998), plants have evolved various photoprotective mechanisms to help them dissipate excess energy and avoid photooxidation. The conversion of excess light energy to heat is believed to be one of the most important non-photochemical quenching (NPQ) strategies. Such thermal

dissipation is brought about by the reversible conversion of violaxanthin to zeaxanthin (and antheraxanthin) in the xanthophyll cycle (Demmig-Adams and Adams, 1991; Demmig-Adams and Adams, 2000), which accounts for around 70 % of total NPQ protection (Niyogi *et al.*, 1998).

Under certain environmental stresses, it may be advantageous to dissipate excess captured energy through the process of photorespiration (Osmond, 1981; Steffen, 1991). Photorespiration is sometimes considered a futile cycle as RuBisCO uses excitation energy to catalyse the fixation of oxygen instead of carbon, yielding phosphoglycolate with the associated usage of ATP and reducing agents (Heber and Krause, 1980). It is also potentially damaging; in C_3 plants, photorespiration produces high levels of H_2O_2 through the action of peroxisomal glycollate oxidase as part of the glycerate salvage pathway (Noctor *et al.*, 2000).

CHAPTER 3 - Anthocyanins increase light capture

Abstract

We have tested the tacit assumption that the location of anthocyanic cells within a leaf affects the quality and quantity of light that is absorbed. Leaf optics are described for *Quintinia serrata*, a native New Zealand tree that is polymorphic for anthocyanin distribution and concentration. Anthocyanin production enhanced the absorptance of green-yellow wavelengths in proportion to pigment concentration. The reflectance of red light was independent of leaf anthocyanin content. Variation in the location of pigmented cells could not account for differences in leaf optics. The effects of anthocyanin on PAR absorptance were consistent across six further, unrelated species that bore red leaves. We conclude that anthocyanin concentration, rather than its histological distribution, has the greater impact on leaf optical properties.

Key words: anthocyanin, leaf optics, reflectance, absorptance, *Quintinia serrata*.

Introduction

The anthocyanins are a group of water-soluble, pigmented flavonoids present in a wide range of organs across the plant kingdom (Harborne, 1967). Anthocyanins are common in leaves, more so than is evident from the inspection of leaf surfaces because their red to purple colouration may be masked by chlorophylls, carotenoids, or pubescence (Moore, 1965; Sanger, 1971; Woodall *et al.*, 1998). There is no unified explanation for the presence and function of anthocyanins in leaves.

Anthocyanins differ from most other flavonoids in that they interact with visible wavelengths of the solar spectrum. Purified extracts of anthocyanins in acidified methanol typically absorb green-yellow light between 500 and 550 nm (Harborne, 1967). If anthocyanins can modify the light environment within a leaf, then they have the potential to regulate photosynthesis and limit photoinhibition (Gould *et al.*, 1995; Burger and Edwards, 1996). The optical properties of anthocyanic leaves appear to vary among plant taxa. Many workers have

reported an increased absorptance of green-yellow light in anthocyanic leaves over green leaves, although the magnitude of this increase differs considerably across species (Eller *et al.*, 1981; Gausman, 1982; Burger and Edwards, 1996; Woodall *et al.*, 1998). Others have noted that anthocyanins enhance the reflectance of red light from the leaf surface, which can either reduce overall absorptance (Boyer *et al.*, 1988; Ntefidou and Manetas, 1996; Barker *et al.*, 1997) or else increase the capture of red light by back-scattering to the chlorenchyma quanta that would otherwise be transmitted through the leaf (Lee *et al.*, 1979; Lee and Graham, 1986).

Variation in the optical properties of anthocyanic leaves may be related to differences in location of the pigment within leaf tissues. Anthocyanins reside most commonly in the vacuoles of palisade and spongy mesophyll cells, but in certain species, they occur in the epidermises, hypodermis, and/or vascular parenchyma (Gould and Quinn, 1999). Anthocyanins located in superficial, adaxial cell layers may reduce light capture by reflecting red light from the leaf surface, whereas those in the mesophyll may enhance light capture by absorbing green-yellow wavelengths directly. Abaxial anthocyanic tissues might enhance absorptance of red light by back-scattering.

No study has explicitly addressed the role of anthocyanin location on the quality of light capture. It is difficult to evaluate related studies because many anatomical and biochemical properties of leaves other than anthocyanin content can affect their optics; these factors vitiate comparisons across species. Moreover, the production of anthocyanin is commonly associated with the accumulation of cuticular wax (Barker *et al.*, 1997) or the development of pubescence (Ntefidou and Manetas, 1996), so that comparisons between red and green leaves even within a species often cannot resolve exclusive effects of anthocyanin.

We have made use of the remarkable colour polymorphism in leaves of *Quintinia serrata* A. Cunn. (Escalloniaceae), a canopy tree from montane forests in the North Island of New Zealand, to test the hypothesis that the location of anthocyanins impacts on leaf optics. The fully-expanded laminae of *Q. serrata* display a wide range of colour patterns, from entirely red to entirely green. Anthocyanins can be found in many combinations of the leaf tissues, although other anatomical features remain constant. We provide evidence that anthocyanin concentration, rather than histological location per se, has the greater impact on light capture.

We show that our conclusions are equally applicable to the anthocyanic leaves from six other native New Zealand species.

Materials and Methods

Plant material

Sixty fully expanded leaves of *Quintinia serrata* A. Cunn. (Escalloniaceae) were randomly collected from fifteen trees from the Huia Ridge Track in the Waitakere Ranges, 25 km west of Auckland, New Zealand. The sample of leaves varied in appearance from entirely red through to entirely green. Ten leaves from five trees of six other native New Zealand species were also collected along various tracks in the Waitakere Ranges. The other species were: *Aristotelia serrata* (J.R. et G. Forst.) W.R.B. Oliver (Elaeocarpaceae), *Dracophyllum latifolium* A. Cunn. (Epacridaceae), *Myrsine salicina* Hew. (Myrsinaceae), *Lophomyrtus bullata* (Sol. ex A. Cunn.) Burret (Myrtaceae), *Olearia rani* (A. Cunn.) Druce (Asteraceae), and *Pseudowintera colorata* (Raoul) Dandy (Winteraceae). Leaves were stored overnight in sealed moist plastic bags in a refrigerator.

Leaf optical properties

All leaves were washed with distilled water and then gently blotted with tissue paper. Reflectance and transmittance of each of the freshly collected leaves were measured using an external integrating sphere connected via a fibre optic cable to a Li-Cor 1800 spectroradiometer (Lincoln, NE) and compared to that of a barium sulphate standard. Leaf reflectance and transmittance are defined as the proportion of incident, diffuse light that is reflected from, and transmitted through the leaf, respectively. Absorptance was calculated as:

Absorptance = 1 – reflectance - transmittance (Lee and Graham, 1986).

The experimental set-up provided a spectral range of 350-1100 nm, scanning at intervals of 2 nm for both the adaxial and abaxial surfaces. Integrated absorptances of PAR (400-700 nm) and of wavebands from 500 to 600 nm and 600 to 700 nm were calculated as percentages of the incident light. Correlations between pigment concentration and leaf optics were analysed using the Pearson linear correlation coefficient.

Pigment assay

Two 10 mm diameter discs were taken from the regions of each leaf for which optical properties had been measured. They were placed in 1 ml of 100 % acetone, and in 1 ml of 3 M HCl-H_2O-MeOH (1:3:16), respectively. These were agitated gently in the dark for 24 h at 4°C. Extracts were centrifuged for 5 mins at 10,600 g, and then absorbances measured in reduced volume, glass cuvettes (1cm path length) using a Hitachi U-2001 dual beam spectrophotometer.

For the acetone extracts, absorbances were measured at 662 and 645nm. Total chlorophyll concentrations were estimated using the formula by Lichtenthaler (1987):

$$\text{Total Chlorophyll } (\mu g/cm^2) = 7.05\ A_{662} + 18.09\ A_{645}$$

Anthocyanin levels were estimated from the methanolic extracts as $A_{\lambda max} - 0.24\ A_{653}$ (Murray and Hackett, 1991). $A_{\lambda max}$, the wavelength of peak absorbance, was determined for the anthocyanins of each species from purified extracts. Fresh leaves were ground in 7% acetic acid or 3 M HCl-H_2O-MeOH (1:3:16). Extracts were filtered, centrifuged, concentrated to dryness *in vacuo* at 35°C, and redissolved in 1 ml of 7% acetic acid. Anthocyanins were separated from other pigments by reverse-phase chromatography using Isolute C18 500 mg/3 ml columns (IST Ltd., Hengoed, U.K.), and eluted in 3 M HCl-H_2O-MeOH (1:3:16).

Histological distribution of anthocyanins

Fresh hand sections were taken from close to the region of each lamina for which optical properties had been recorded. The cellular location of anthocyanin within each leaf was recorded using bright field microscopy. Five sections were examined per leaf. Anatomical comparisons were made by fixing fresh portions of red and green *Q. serrata* leaves overnight in a solution of 2.5 % glutaradehyde and 2% paraformaldehyde in 25 mM phosphate buffer at pH 6.8, under vacuum at room temperature. The specimens were rinsed in 25 mM phosphate buffer, dehydrated using a methyl cellosolve series, and infiltrated and embedded in glycol methacrylate resin 'Technovit 7100' (Kulzer and Co., Wehrheim, Germany). Transverse sections, 2 μm thick, were cut with glass knives on an LKB 2218 Historange microtome.

Sections were stained with 1 % toluidine in sodium benzoate buffer at pH 4.4 (Feder and O'Brien, 1968) and photographed with Kodak Ektachrome film.

Results

There were no obvious structural differences between red and green leaves of *Quintinia serrata*. Each had a heavily cutinised adaxial epidermis, 2-3 layers of palisade mesophyll, 6-8 layers of spongy mesophyll, and abaxial stomata (Fig. 3.1). Recessed, peltate trichomes were present on both upper and lower epidermises.

Anthocyanins were present in seven tissue combinations within 48 of the 60 *Q. serrata* leaves. The palisade mesophyll was most frequently pigmented red; all of the red leaves bore anthocyanins in this tissue either alone (19%), or in combination with spongy mesophyll (46%) and/or epidermis (35%). Distribution across the lamina was variable, ranging from isolated clusters of anthocyanic cells to discrete layers. Anthocyanins were not evident in the 12 green leaves, either from observations of transverse sections or from spectrophotometric measurements of A_{530}.

Red leaves absorbed up to 17% more PAR (400-700 nm) than green leaves (Fig. 3.2A). This increase was largely restricted to the green-yellow wavelengths (500-600 nm); absorptance beyond 600 nm was greater in the green leaves. Between 7 and 15% of the incident PAR was reflected from the adaxial surfaces of *Q. serrata* leaves. Red leaves reflected up to 62% less light than green leaves between 500 and 600 nm (Fig. 3.2B).

The shapes of absorptance and reflectance spectra varied among the red leaves. This variation could not be attributed to differences in the location of anthocyanins within leaf tissues. Those leaves for which anthocyanins were located exclusively in the palisade mesophyll generally had the highest overall absorptance (Fig. 3.2A) and the lowest surface reflectance (Fig. 3.2B). However, leaves for which anthocyanins were present in combinations of two or more tissues occupied unpredictable positions within the range of possibilities.

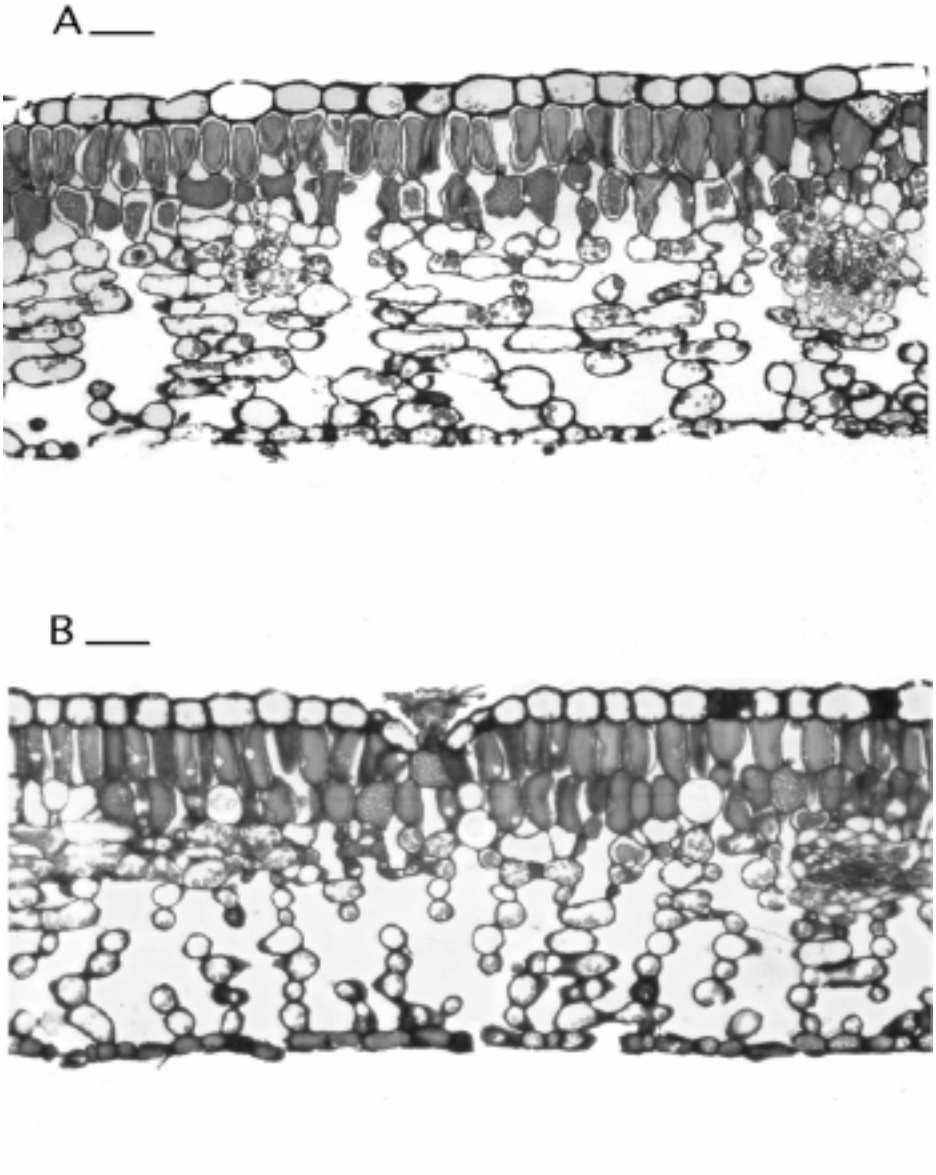


Fig. 3.1 Light micrographs of transverse sections through red (A) and green (B) leaves of *Quintinia serrata*. Bars = 50 µm.

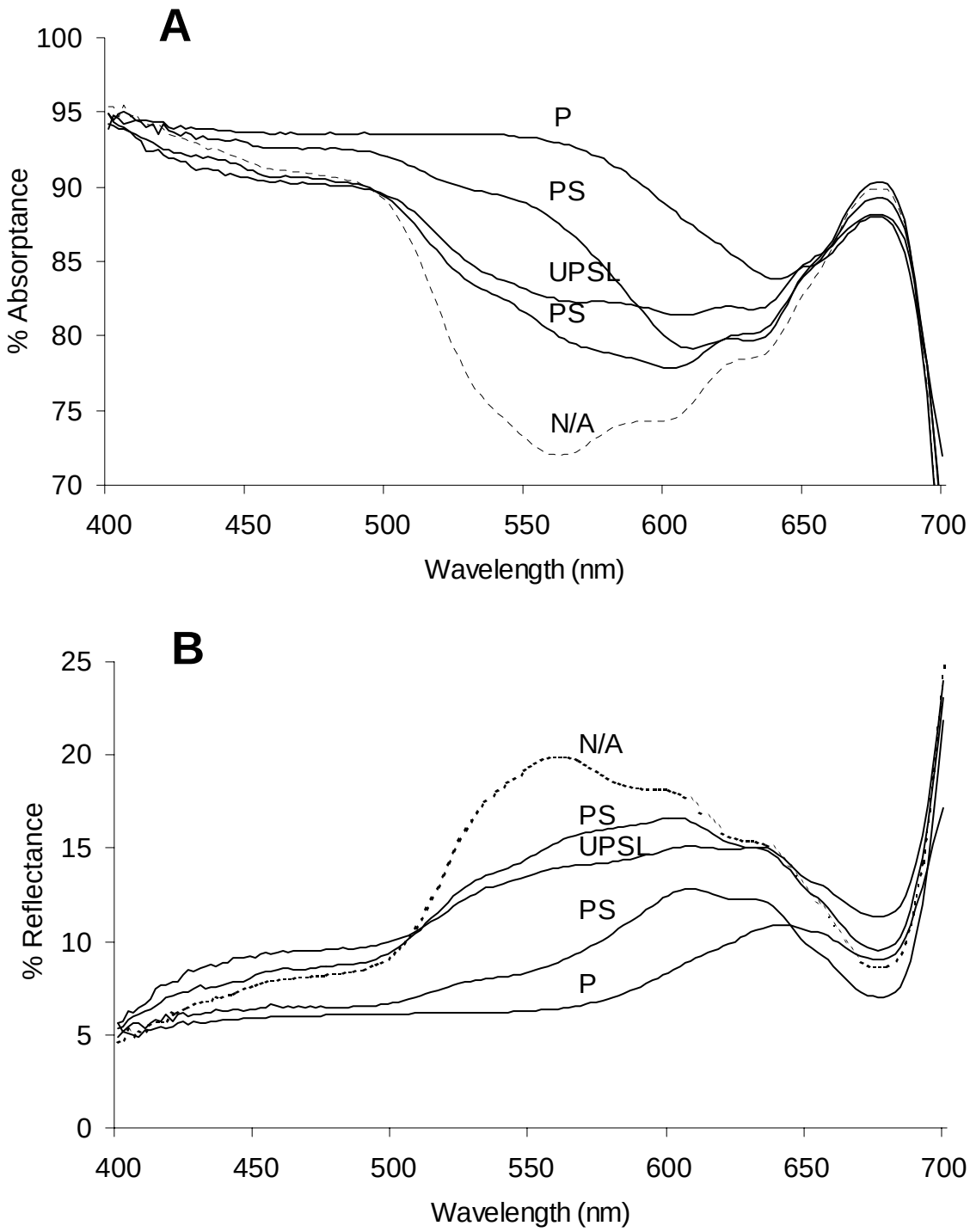


Fig. 3.2 Absorptance (A) and reflectance (B) spectra for the adaxial surfaces of five randomly selected *Quintinia serrata* leaves with different combinations of tissues. Anthocyanins located in: U = upper epidermis, P = palisade mesophyll, S = spongy mesophyll, L = lower epidermis. N/A = non-anthocyanic leaf.

There was a clearer relationship between leaf optics and anthocyanin concentration. Higher concentrations were associated with enhanced absorptance (Fig. 3.3A) and reduced reflectance (Fig. 3.3B) of the green-yellow wavelengths.

Absorptance between 500 and 600 nm increased logarithmically with anthocyanin concentration ($r = 0.77$; $P<0.001$). This relationship was independent of anthocyanin location (Fig. 3.4).

The reflectance of red light (600-700 nm) was poorly correlated to anthocyanin concentration per se (Fig. 3.3B). Because red wavelengths are absorbed by the chlorophylls, we hypothesised that differences in the reflectance properties among red leaves might be masked by variation in chlorophyll content, which differed across the sample of *Q. serrata* leaves by five-fold. Reflectance of red wavelengths was negatively correlated ($r = -0.64$; $P<0.001$) to chlorophyll content (Fig. 3.5), but was not related ($r = 0.09$; $P > 0.5$) to the concentration ratios of anthocyanins: chlorophyll (Fig. 3.6). Reflectance was independent of anthocyanin location within the leaf (Fig. 3.6).

The location of anthocyanins varied across the six further species examined, but was consistent for leaves within each species (Table 3.1). Extracts of these anthocyanins in acidified methanol all absorbed maximally in the green-yellow wavelengths (Table 3.1). Those species that held anthocyanins in the greater number of tissue layers generally had the higher integrated absorptances of PAR and of 500-600 nm (Table 3.1). Differences in absorptance were again attributable to variation in anthocyanin concentration rather than histological location. Absorptance of green-yellow wavelengths was strongly correlated to the logarithm of anthocyanin concentration ($r = 0.77$; $P<0.001$) across all species (Fig. 3.7). There was no clear relationship between reflectance of red light and anthocyanin location (Table 3.1). Red reflectance was only poorly correlated ($r = 0.33$; $P<0.001$) to the anthocyanin: chlorophyll ratio across species (Fig. 3.8).

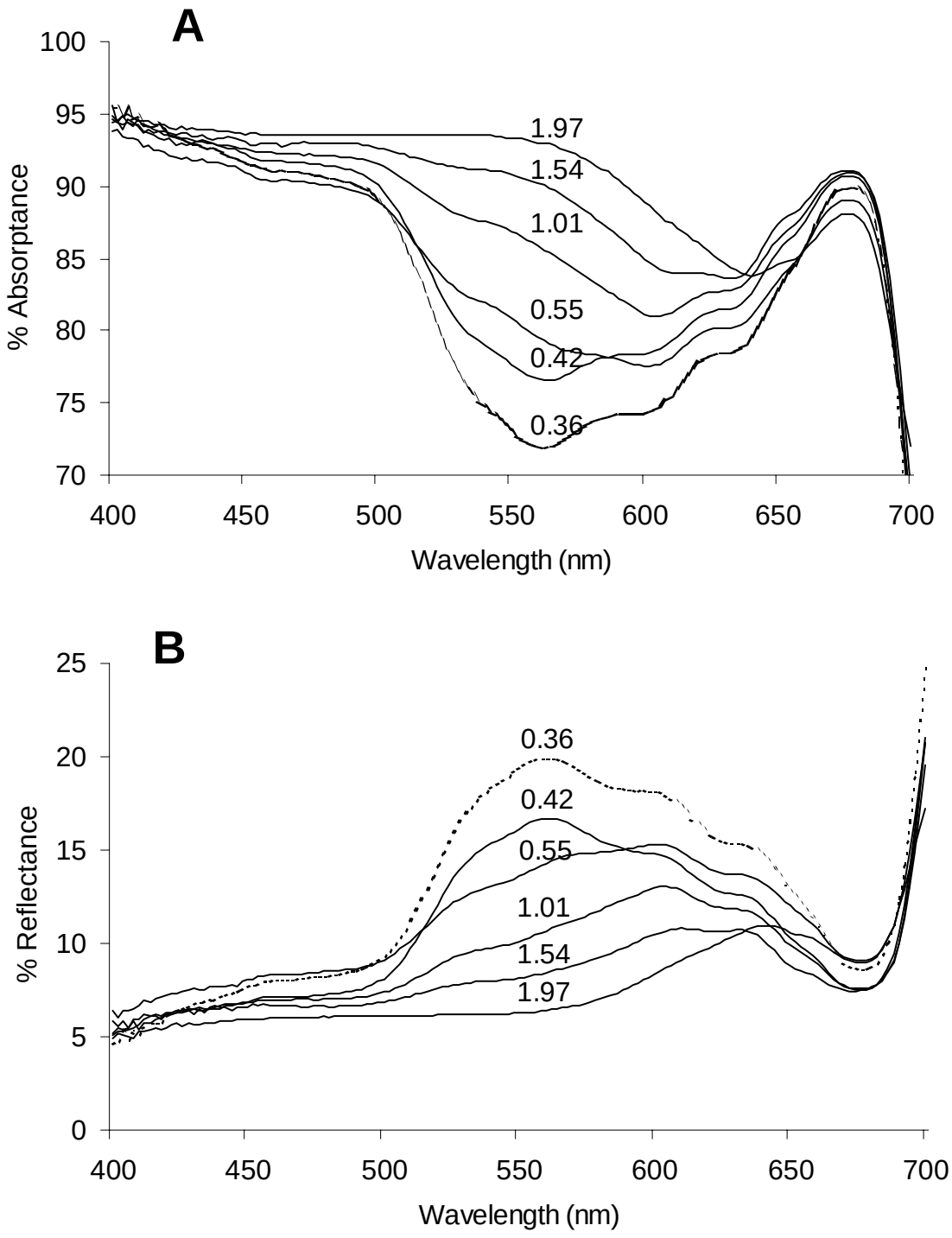


Fig. 3.3 Absorptance (A) and reflectance (B) spectra for the adaxial surfaces of five randomly selected *Quintinia serrata* leaves varying in concentration of anthocyanins. Numbers show anthocyanin content (A_{530} values).

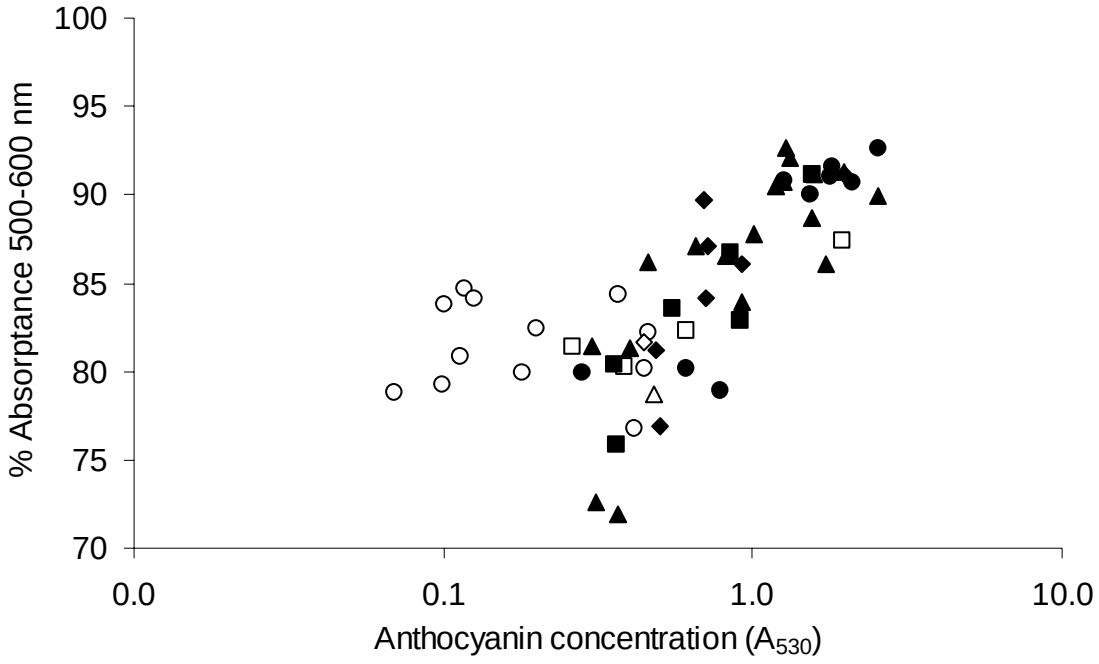


Fig. 3.4 Absorptance of incident green-yellow light (500-600 nm) as a function of the natural logarithm of anthocyanin concentration (A_{530}) and histological distribution in leaves of *Quintinia serrata*. Anthocyanins located in: U = upper epidermis, P = palisade mesophyll, S = spongy mesophyll, L = lower epidermis. Leaf tissue combinations: P (●), UP (△), PS (▲), UPS (□), PSL (■), UPL (◇), UPSL (◆). Anthocyanins absent (○).

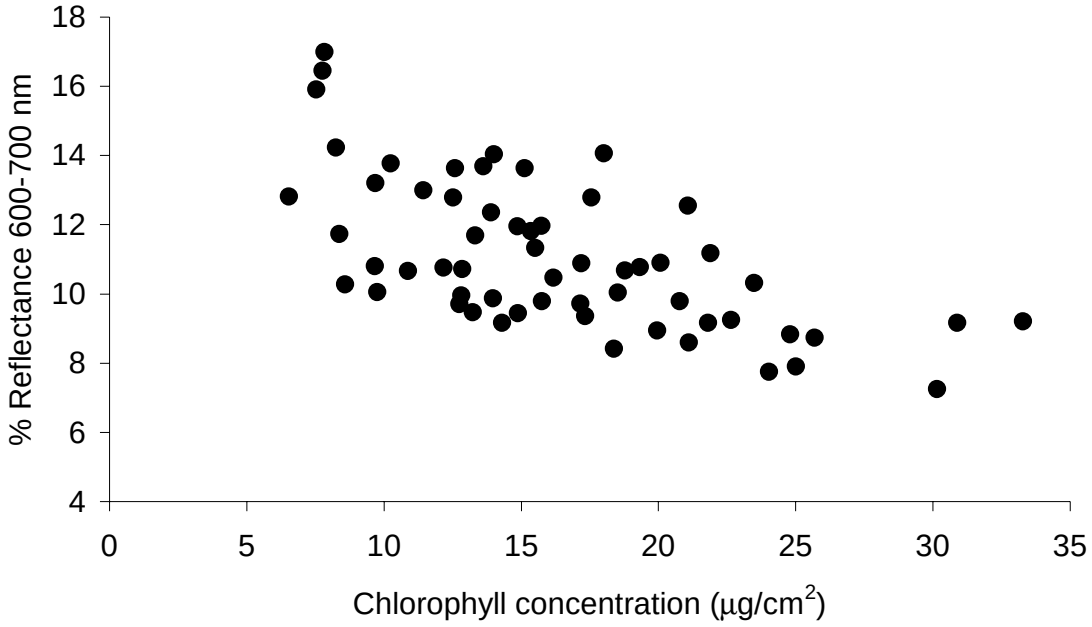


Fig. 3.5 Reflectance of incident red light (600-700 nm) as a function of chlorophyll concentration in leaves of *Quintinia serrata*.

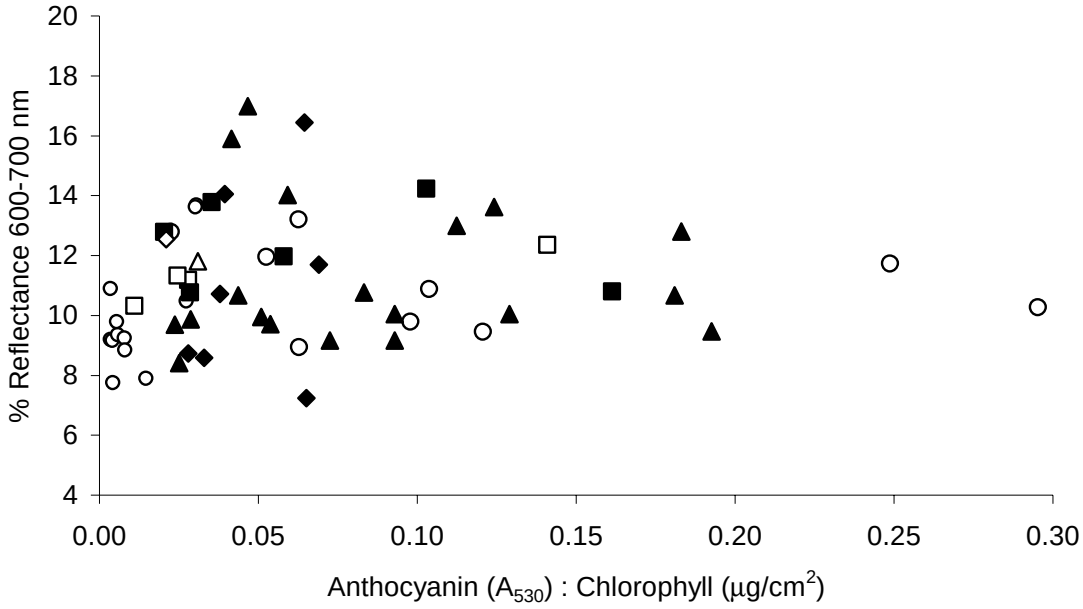


Fig. 3.6 Reflectance of incident red light (600-700 nm) as a function of anthocyanin: chlorophyll concentration ratios in leaves of *Quintinia serrata*. Anthocyanins located in: U = upper epidermis, P = palisade mesophyll, S = spongy mesophyll, L = lower epidermis. Leaf tissue combinations: P (●), UP (△), PS (▲), UPS (□), PSL (■), UPL (◇), UPSL (◆). Anthocyanins absent (○).

Table 3.1 Anthocyanin distribution, absorbance maxima, and absorptance and reflectance of adaxial surfaces of leaves from seven New Zealand species. Data show mean values ± s.e. for 60 leaves of *Q. serrata*, and for 10 leaves of each of the other species. U = upper epidermis, P = palisade mesophyll, S = spongy mesophyll, L = lower epidermis. ● = red cell(s) observed.

Species	Anthocyanin location U	P	S	L	λ_{max} (nm)	Mean % absorptance 400-700 nm	Mean % absorptance 500-600 nm	Mean % reflectance 600-700 nm
A. serrata				●	524	81 ± 0.8	71 ± 1.2	6 ± 0.2
M. salicina				●	525	85 ± 0.8	77 ± 1.3	8 ± 0.3
O. rani		●		●	526	81 ± 1.7	78 ± 3.0	11 ± 0.4
D. latifolium			●	●	524	87 ± 0.5	84 ± 1.1	8 ± 0.3
L. bullata		●	●	●	527	93 ± 0.7	90 ± 1.3	7 ± 0.3
P. colorata		●	●	●	526	90 ± 0.6	89 ± 1.2	12 ± 0.4
Q. serrata[1]	●	●	●	●	530	88 ± 0.4	85 ± 0.7	11 ± 0.3

[1] Highly variable anthocyanin distribution.

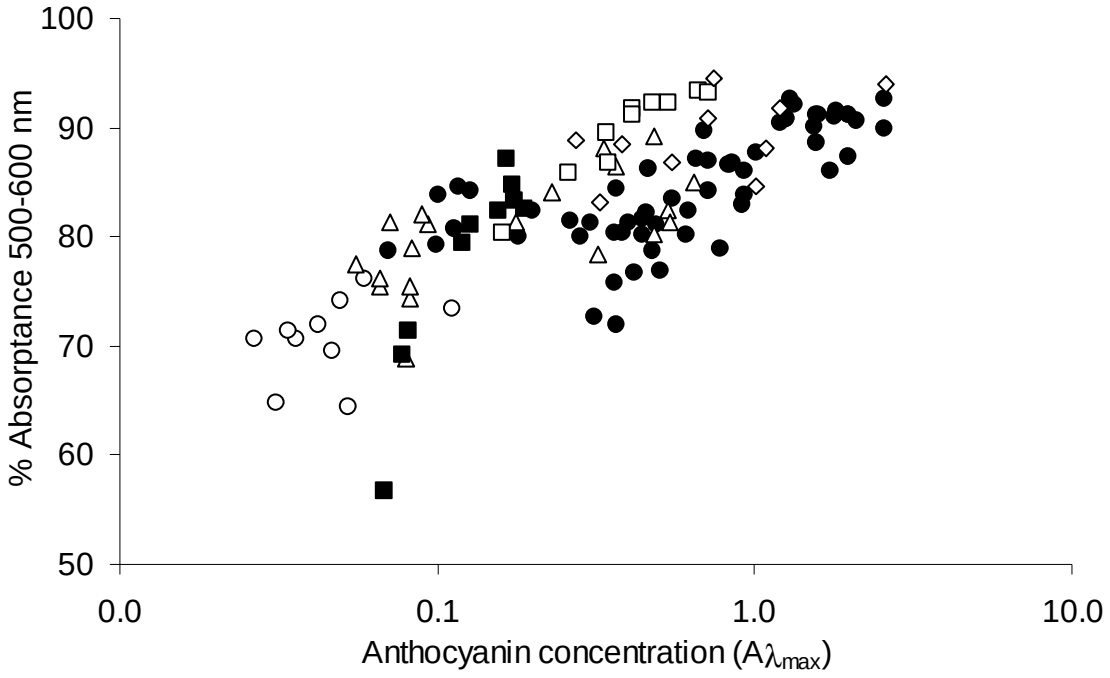


Fig. 3.7 Absorptance of incident green-yellow light (500-600 nm) as a function of the natural logarithm of anthocyanin concentration ($A_{\lambda max}$) for the adaxial surfaces of leaves from seven New Zealand plant species: *Q. serrata* (●), *A. serrata* (○), *D. latifolium* (△), *M. salicina* (▲), *L. bullata* (□), *O. rani* (■), *P. colorata* (◇).

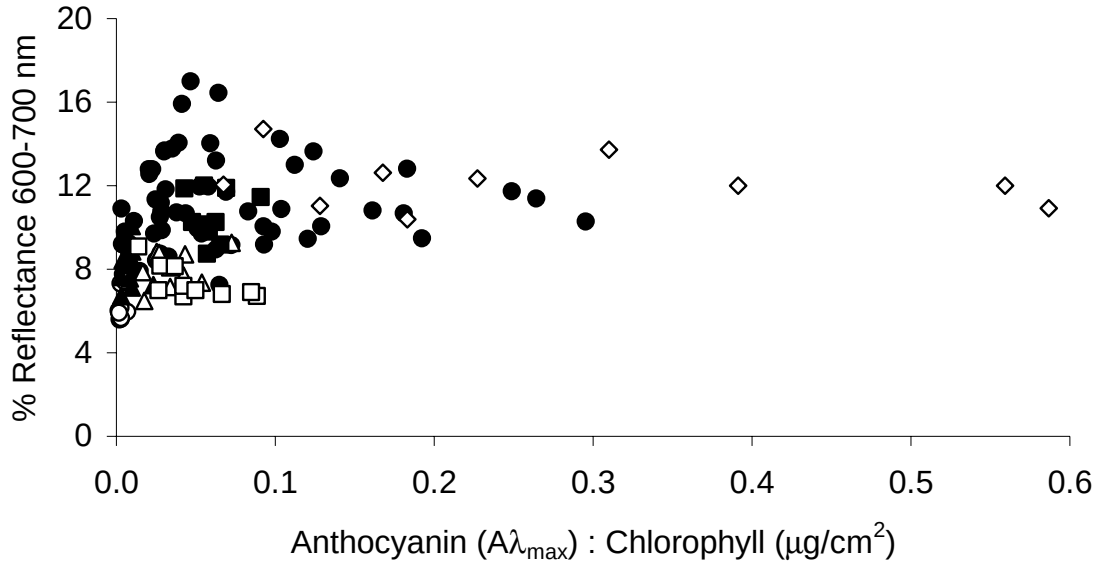


Fig. 3.8 Reflectance of incident red light (600-700 nm) against anthocyanin : chlorophyll concentration ratios for the adaxial surfaces of leaves from seven New Zealand plant species: *Q. serrata* (●), *A. serrata* (○), *D. latifolium* (△), *M. salicina* (▲), *L. bullata* (□), *O. rani* (■), *P. colorata* (◇).

Discussion

Anthocyanins impact significantly on both the quality and quantity of light that is absorbed by a leaf. All anthocyanic leaves absorbed more green and yellow light than non-anthocyanic leaves, but interception of red wavelengths was largely unaffected (Fig. 3.2 A). The extent to which green-yellow light was absorbed was proportional to the logarithm of anthocyanin concentration (Fig. 3.4). This relationship was consistent across seven species (Fig. 3.7), a remarkable observation given the extent to which those species differ in leaf anatomy and morphology (Allen, 1961).

Leaf optics are governed more by the overall concentration of anthocyanin than by the location of anthocyanins within leaf tissues. None of the seven combinations of anthocyanic tissues observed among leaves of *Q. serrata* could have been identified on the basis of leaf optical properties alone. Both reflectance and absorptance were independent of anthocyanin distribution. The highest absorptances were recorded for leaves in which anthocyanins were present exclusively in the palisade mesophyll, or in both palisade and spongy mesophyll tissues (Fig. 3.4); however, those leaves also held the highest concentrations of anthocyanins.

There was no evidence of enhanced light capture resulting from the back-scattering of quanta from red abaxial tissues (Lee *et al.*, 1979; Lee and Graham, 1986). Leaves of *Q. serrata* that bore an anthocyanic lower epidermis had absorptances (500-600 nm) comparable to those with the pigment in other tissues (Fig. 3.4). *Aristotelia serrata* and *Myrsine salicina*, for which anthocyanins resided at low concentrations exclusively in the lower epidermis, had the lowest absorptance (500-600 nm) values of all species tested (Table 3.1).

The majority of anthocyanic leaves reflected similar proportions of red light as the non-anthocyanic leaves (Fig. 3.6). Their colour, therefore, could not be ascribed to an enhanced reflectance of red wavelengths. Rather, red colouration was attributable to the subtraction of green-yellow wavelengths from the reflected spectrum (Fig. 3.2 B). The human eye responds with maximal sensitivity to radiant energy at 550 nm, the same wavelength for which differences in reflectance between red and green leaves were greatest. Thus, small reductions in reflected green light would be perceived as large increases in red colouration.

Our data are not consistent with the hypothesis that anthocyanins act as photoprotectants by reflecting incident red light (Barker *et al.*, 1997). Red reflectance was better correlated to declining chlorophyll concentrations (Fig. 3.5) than to anthocyanin levels. The correlation remained weak even when anthocyanin concentrations were normalised for variation in chlorophyll content (Figs. 3.6 and 3.8).

Although anthocyanins enhance light capture in leaves, it is still possible that they serve a photoprotective function. The abaxial leaf tissues are less tolerant of high light than the upper tissues (Evans *et al.*, 1993; Sun *et al.*, 1996). Because anthocyanins are most commonly located in the vacuoles of the palisade mesophyll (Gould and Quinn, 1999), they may protect the more sensitive, subjacent cells from photoinhibition by intercepting the high-energy quanta of incident green light (Gould *et al.*, 1995). Thus, the descending gradient of green light may be steeper within anthocyanic leaves than in green leaves. Energy that is intercepted by the anthocyanic cells might then be stored in the vacuole in photochemical form, re-emitted as lower energy photons, or else dissipated as heat. The distribution of light energy within anthocyanic leaves requires investigation.

CHAPTER 4 - Protection from photoinhibition and photooxidation: the shielding effects of anthocyanin

Abstract

The absorption of light by anthocyanins in leaves acts to reduce the numbers of quanta incident on chloroplasts. We have tested the hypothesis that light attenuation by anthocyanins in leaf laminae of *Lactuca sativa* cv. Dark Lollo Roso reduces the extent of photoinhibition and photooxidation under high irradiances. The presence of anthocyanin in the upper epidermal layer reduced total PAR absorptance by 17 % relative to that of acyanic regions, largely in the green waveband (500–600 nm). Anthocyanic and acyanic regions of the leaves, and chloroplast suspensions derived from each, were subjected to saturating light at 1300 μmol m^{-2} s^{-1} for 20 mins, and the extent photoinhibition quantified by chlorophyll fluorometery. Ratios of variable to maximal fluorescence (Fv/Fm) were reduced significantly more (7 %) in acyanic regions than in anthocyanic regions following photoinhibitory treatment. Chloroplast suspensions from anthocyanic areas demonstrated a comparable reduction in Fv/Fm as those from acyanic areas, but they recovered more rapidly after photoinhibitory treatments. Under low irradiances, chloroplasts isolated from anthocyanic regions had a 30 % greater photochemical efficiency of PSII (ΦPSII) than those from acyanic regions. These differences in chloroplast physiology are attributable to differences in the degree to which chloroplasts were exposed to light during development. Superoxide ($O_2^{\cdot-}$) production and chlorophyll bleaching were significantly reduced when chloroplast suspensions were irradiated through a red cellulose filter compared to those irradiated with white light of comparable flux. We suggest that an anthocyanic filter provides an efficient mechanism to minimise photoinhibition and photooxidative damage without impacting significantly on the light requirements for photosynthesis.

Key words: anthocyanins, photoinhibition, photooxidation, chloroplasts, *Lactuca sativa.*

Introduction

Light in excess of that which can be used for CO_2 fixation can reduce quantum efficiencies of plants through the processes of photoinhibition, photooxidation, and photorespiration (Osmond, 1994; Osmond and Grace, 1995). These processes are accompanied by the loss of photosynthetic pigments, enzyme deactivation, lipid peroxidation, and protein degradation (Powles, 1984). It follows that any mechanism that would act to reduce quantum fluxes incident on chloroplasts or else assist in the dissipation of excess excitation energy would effectively maintain high rates of photosynthesis under strong irradiance.

The anthocyanins, which are commonly associated with the photosynthetic tissues in leaves (McClure, 1975; Gould and Quinn, 1999; Lee and Collins, 2001), have been postulated to protect functioning chloroplasts by reducing incident light under high irradiances (Sherwin and Farrant, 1998; Neill and Gould, 1999; Feild *et al.*, 2001; Hoch *et al.*, 2001). Because anthocyanins absorb predominantly green light (500-550 nm) (Harborne, 1967; Markham, 1982, Neill and Gould, 1999), they have the potential to affect both quality and quantity of light incident on chloroplasts. Red leaves commonly have lower photosynthetic efficiencies than do green leaves (Burger and Edwards, 1996; Barker *et al.*, 1997; Dodd *et al.*, 1998). Green light is important for photosynthesis in the lower mesophyll layer (Jeje-A and Zimmermann, 1983; Nishio *et al.*, 1993; Sun *et al.*, 1996) and can efficiently stimulate electron transport (Ghirardi and Melis, 1984). Thus, anthocyanins could reduce the overall quantum efficiencies of leaves by depriving the lower mesophyll of green light.

The attenuatation of light by anthocyanins is thought to reduce photoinhibition under short periods of high irradiance (Gould *et al.*, 1995; Krol *et al.*, 1995; Pietrini and Massacci, 1998; Smillie and Hetherington, 1999) by maintaining the extent of light capture and chlorophyll excitation within the boundaries of photochemical utilisation (Osmond, 1994). Consistent with a photoprotective role of anthocyanins in leaves are observations that photoinhibition, as indicated by reductions in ratios of variable to maximum chlorophyll fluorescence (Fv/Fm), is less marked in anthocyanic leaves than in acyanic morphs (Gould *et al.*, 1995; Feild *et al.*, 2001). Tolerance to photoinhibition was correlated to anthocyanin levels in *Pinus banksiana* seedlings exposed to a low temperature and high irradiance (Krol *et al.*, 1995). However, in contrast to those findings, red and green leafed varieties of coleus showed no difference in quantum yield of O_2 evolution after a photoinhibitory treatment (Burger and Edwards, 1996).

Attempts to resolve the photoinhibitory hypothesis using leaves of *Syzygium* species yielded ambiguous data because of the differences in developmental age between anthocyanic and acyanic leaves (Dodd *et al*., 1998).

Measurements of chlorophyll fluorescence in *Cornus stolonifera* provided compelling evidence that anthocyanins can reduce the impact of photoinhibitory light treatment (Feild *et al.*, 2001). Exposure of 1500 μmol m^{-2} s^{-1} of white light resulted in the significantly greater depression of dark-adapted photosystem II photon efficiency (ΦPSII) in green leaves relative to that displayed in anthocyanic leaves. In addition, red leaves were shown to recover faster and to a greater extent of original values after the photoinhibitory treatment. There are two possible explanations for these differences between red and acyanic leaves. First, the chloroplasts in anthocyanic leaves may be responding to lower irradiances effected by the anthocyanic filter. Second, there may be inherent differences in chloroplast physiology, possibly as a result of developing under the red filter.

Anthocyanins can be induced, or else their production enhanced under conditions of environmental stresses (Chalker-Scott, 1999). Conditions such as high irradiance and low temperature are also known to lead to the generation of potentially damaging reactive oxygen species (ROS) (Powles, 1984). It is possible that in addition to their conferring protection from photoinhibition, anthocyanins also reduce the generation of ROS under high light situations. This could be achieved by i) shielding the light harvesting complexes from quanta in excess of those that can be used by non-cyclic electron transport, thereby reducing the formation of singlet oxygen ($^1O_2$); ii) maintaining higher levels of oxidised PSI acceptors, thus reducing the generation of superoxide radicals ($O_2^{\cdot-}$) through the Mehler reaction; or iii) minimising photorespiration and the associated production of H_2O_2. Thus, anthocyanins have the potential to reduce both photoinhibition and oxidative stress purely by reducing incident light upon chloroplasts under high irradiances. It must be noted, that anthocyanins may also play a more direct role in reducing oxidative stress by conveying antioxidant protection through the scavenging of ROS (Yamasaki *et al*., 1996; Yamasaki, 1997).

Efforts to test for putative multifunctional activities of anthocyanins *in vivo* are inherently fraught with complications. For example, comparisons of photoinhibition in red and green leaves are often confounded by differences in developmental age and their associated

photosynthetic competences. Leaf maturation is frequently accompanied by alterations in chloroplast ultrastructure, stomatal conductance, and reflective properties stemming from the accumulation of waxes, carotenoids, and/or leaf hairs. All of these have the potential to reduce the severity of photoinhibitory and photooxidative responses (Choinski and Johnson, 1993; Barker *et al.*, 1997; Dodd *et al.*, 1998; Sherwin and Farrant, 1998; Woodall *et al.*, 1998). In addition, because most ROS are extremely unstable (Halliwell and Gutteridge, 1998) it is also difficult to distinguish the effects of anthocyanins on ROS generation from those of ROS scavenging.

Here, we use intact leaves and chloroplast suspensions isolated from leaf laminae of *Lactuca sativa* to investigate the putative roles of anthocyanins on photoinhibition and photooxidation. By measuring fluorescence parameters of chloroplasts isolated from red and green areas of the same leaf we examine for inherent differences in physiology without the confounding effects of differences in developmental age. In addition, we compare the efficacies of a red filter to those of other colours as attenuators of photooxidative stress. It might be expected that because superoxide radicals are formed largely as a by-product of photosynthesis, any filter that attenuates red light would be the most effective in reducing photooxidative damage.

This paper aims to answer three questions: i) does the light-filtering property of anthocyanin protect chloroplasts from photoinhibitory damage; ii) do the chloroplasts found both within and subjacent to anthocyanic tissues exhibit the physiology of those from shade-adapted plants; and iii) does the photoabatement by anthocyanins reduce the extent of superoxide radical generation by chloroplasts?

Our data indicate that the absorption of light by anthocyanins confers protection to underlying chloroplasts from both photoinhibition and photooxidative stress under high irradiances.

Materials and methods

Plant material

Lactuca sativa cv. Dark Lollo Roso plants were grown for eight weeks from seed under controlled conditions (20 ^{o}C day, 16 ^{o}C night) under either low (150 μmol m^{-2} s^{-1}) or high (800 μmol m^{-2} s^{-1}) irradiances with a 16 hr photoperiod. Plants grown under 800 μmol m^{-2} s^{-1}

were utilised for the light response and recovery from photoinhibition experiments, and those grown under 150 μmol m^{-2} s^{-1}, which were sensitive to high light, were used for measurements of superoxide production in irradiated chloroplast suspensions. Irradiance was provided by a bank of 250 W Clean Ace metal halide bulbs.

Optical properties and spectral distribution of radiation

Reflectance and transmittance of PAR were measured for red and green areas of the adaxial surface of *L. sativa* leaves using an external integrating sphere connected via a fibre optic cable to a Li-Cor 1800 spectroradiometer (Lincoln, Nebraska) and compared to those of a barium sulphate standard. Absorptance was calculated as:

Absorptance = 1 – reflectance – transmittance (Lee and Graham, 1986).

The spectral distribution of light from the growth chambers incident on red and green areas of leaves were measured using a remote cosine receptor connected via a fibre optic cable to a Li-Cor 1800 spectroradiometer (Lincoln, Nebraska). Measurements were recorded for the 400-700 nm waveband at a scanning interval of 2 nm. Total PAR was measured using a Li-Cor (185B) photometer (Lincoln, Nebraska) .

Pigment assay

Two 10 mm diameter discs were taken from a red and an acyanic region of eight *L. sativa* leaves from different plants. They were extracted in 1 ml of 100 % acetone, and in 1 ml of 3 M HCl-H_2O-MeOH (1:3:16) in the dark for 24 h at 4°C. Extracts were centrifuged for 5 mins at 10,600 g, and then absorbances measured in reduced volume, quartz cuvettes (1cm path length) using a Hitachi U-2001 dual beam spectrophotometer.

For the acetone extracts, absorbances were measured at 662 and 645 nm. Concentrations of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and total carotenoids were estimated in μg/cm^2 using the equations described by Lichtenthaler (1987):

$$\text{Chlorophyll } a = 11.24\ A_{662} - 2.04\ A_{645}$$

$$\text{Chlorophyll } b = 20.13\ A_{645} - 4.19\ A_{662}$$

$$\text{Total Chlorophyll} = 7.05\ A_{662} + 18.09\ A_{645}$$

$$\text{Total carotenoids} = \frac{1000\ A_{470} - 1.90\ \text{Chlorophyll } a - 63.14\ \text{Chlorophyll } b}{214}$$

Red areas of fresh leaves were ground in 7% acetic acid or 3 M HCl-H_2O-MeOH (1:3:16), filtered, centrifuged, concentrated to dryness *in vacuo* at 35°C, and redissolved in 1 ml of 7% acetic acid. Anthocyanins were purified by reverse-phase chromatography using Isolute C18 500 mg/3 ml columns (IST Ltd., Hengoed, U.K.), and eluted in 3 M HCl-H_2O-MeOH (1:3:16). The wavelength of peak absorbance was determined for the anthocyanins from purified extracts. Anthocyanin levels were estimated from the methanolic extracts as $A_{530} - 0.24\ A_{653}$ (Murray and Hackett, 1991) and expressed as A_{530} values.

Chloroplast isolation

Intact chloroplasts were mechanically isolated as described by Leegood and Walker (1993). Briefly, 10 g of leaf laminae from six plants were homogenised in 50 mL of 0.33 M sorbitol, 10 mM $Na_4P_2O_7$, 5 mM $MgCl_2$, and 2 mM EDTA at pH 6.5. The suspension was filtered through muslin, centrifuged at 600 g for 3 mins, and resuspended in 2 mL of buffer containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes, 10 mM $NaHCO_3$, and 5 mM $Na_4P_2O_7$ at pH 7.6. The chloroplast suspension was further purified using a 40 % (v/v) Percoll gradient. The pellet was resuspended in 10 mL of buffer and held on ice for the duration of the experiments. All the purification steps were undertaken at 0-4 ^oC under low light. Chloroplast integrity was examined with a Zeiss Axioplan 2 microscope under bright field and epifluorescence using Zeiss filterset 02 (excitation: 365 nm; beamsplitter: 395 nm; emission: 420 nm).

Estimation of chloroplast frequencies

Chlorophyll concentrations were estimated as A_{680} values of the suspensions. Numbers of isolated chloroplasts were estimated using a calibration curve of chloroplast numbers, as counted using a haemocytometer, versus A_{680} values.

Light response curves and recovery from photoinhibition

Chlorophyll fluorescence parameters were recorded from continually agitated chloroplast suspensions in a quartz cuvette and from areas of intact red and green leaves using a pulse modulated chlorophyll fluorometer (FMS 2, Hanastech, Kings Lynn, UK) at 23^oC. Actinic

illumination with white light in each case was provided by the fluorometer, and was calibrated using a Li-Cor (185B) photometer (Lincoln, Nebraska).

To obtain light response curves, whole leaves and chloroplasts were dark-adapted for at least 30 mins, prior to an initial measurement of Fv/Fm. Measurements of Fs, Fm', and Fo' were taken after 10 mins of irradiance at 10, 30, 78, 150, 250, 390, or 550 μmol m^{-2} s^{-1}. Photochemical efficiency of PSII (ΦPSII) photochemical quenching (qP), and non-photochemical quenching (qNP) were calculated as described by Genty *et al*. (1989) and Van Kooten and Snel (1990). Photoinhibition was quantified as reductions in dark-adapted Fv/Fm after 20 min irradiance at 1300 μmol m^{-2} s^{-1}. The recovery of Fv/Fm was followed for 30 mins in the dark at room temperature.

Superoxide production

Superoxide production by chloroplast suspensions was measured using nitroblue tetrazolium (NBT), which yields a dark blue insoluble diformazan pigment in proportion to the number of superoxide ($O_2^{\cdot -}$) radicals present (Auclair and Voisin, 1985; Halliwell and Gutteridge, 1998). Precisely 0.5 ml of the chloroplast suspension from leaves grown under 150 μmol m^{-2} s^{-1} was inserted into a quartz cuvette containing 0.1 ml of 3 mM nitroblue tetrazolium (NBT) in the chloroplast resuspension buffer and 2.4 ml of 50 mM MOPS buffer (pH 7.0). The chloroplast suspensions were constantly agitated at 23 ^{o}C and irradiated with white light ranging from 75 to 850 μmol m^{-2} s^{-1} using a Schott (KL 1500) halogen collimated white light source (Mainz, Germany). The formation of the dark blue formazan pigment (estimated as A_{560} values) was recorded over 20 min at 2-min intervals using a Hitachi U-2001 dual beam spectrophotometer.

Chloroplast suspensions at room temperature were irradiated for 8 mins with 300 μmol m^{-2} s^{-1} of white, red, green and blue light to compare the effectiveness of an anthocyanic filter in relation to those of other colours on rates of superoxide production. Supergel Rosco (Sydenham, UK) cellulose 'medium pink' # 36 (absorptance λ_{max}: 524 nm); 'gaslight green' #338 (λ_{max}: 406 nm); 'daylight blue' # 65 (λ_{max}: 634 nm); and neutral density filters were used. Rates of chlorophyll *a* bleaching were recorded as reductions in A_{680} for each filter. The data presented are the means of three replicates.

Results

Anthocyanin accumulation

Lactuca sativa grown under the high light treatment (800 μmol m^{-2} s^{-1}) accumulated anthocyanins in the distal third of the leaf laminae; more proximal regions were acyanic. By virtue of their positions in the crown, the red areas received approximately 88 % more PAR than did the green, acyanic areas (Fig. 4.1). Anthocyanins were located exclusively in the upper and lower epidermises, and affected a 17 % increase in absorption of PAR (400-700nm) by the adaxial surface relative to those of acyanic regions. The enhanced absorption occurred primarily in the green wavebands (Fig. 4.2). Red areas of *L. sativa* leaves absorbed approximately 130 % more light at 550 nm than did adjacent acyanic areas. Leaf laminae from *L. sativa* grown under the low light treatment (150 μmol m^{-2} s^{-1}) did not produce anthocyanins.

Chloroplasts from under both red and acyanic areas were successfully isolated from lettuce leaves (Fig. 4.3 A). Structural integrity was confirmed by the emission of autofluorescence (685 nm) under UV-epifluorescence (Fig. 4.3 B).

Pigment composition

Red areas had significantly higher anthocyanin contents (t-test; $P<0.001$) than did acyanic regions. Levels of chlorophyll *a* and *b*, total chlorophyll, and total carotenoids were similar (Table 4.1).

Effects of anthocyanins on light utilisation and photoinhibition

Photochemical efficiencies (ΦPSII) and estimates of photochemical quenching (qP) were higher for intact leaves than for isolated chloroplasts (Fig. 4.4 A - D). Levels of ΦPSII and qP declined with increasing irradiance; this decline was more marked in acyanic tissue than in red areas. Differences between red and acyanic areas in ΦPSII were greatest (47 %) under the highest irradiance of 550 μmol m^{-2} s^{-1}. For the chloroplast suspensions isolated from red and acyanic areas, the greatest difference in ΦPSII (30 %) occurred under 10 μmol m^{-2} s^{-1}. Estimates of non-photochemical quenching (qNP) increased under higher irradiance and were greater in acyanic tissue than from red tissue for both intact leaves and chloroplast suspensions (Fig. 4.4 C, F). Mean dark-adapted values of minimal fluorescence (Fo) in the

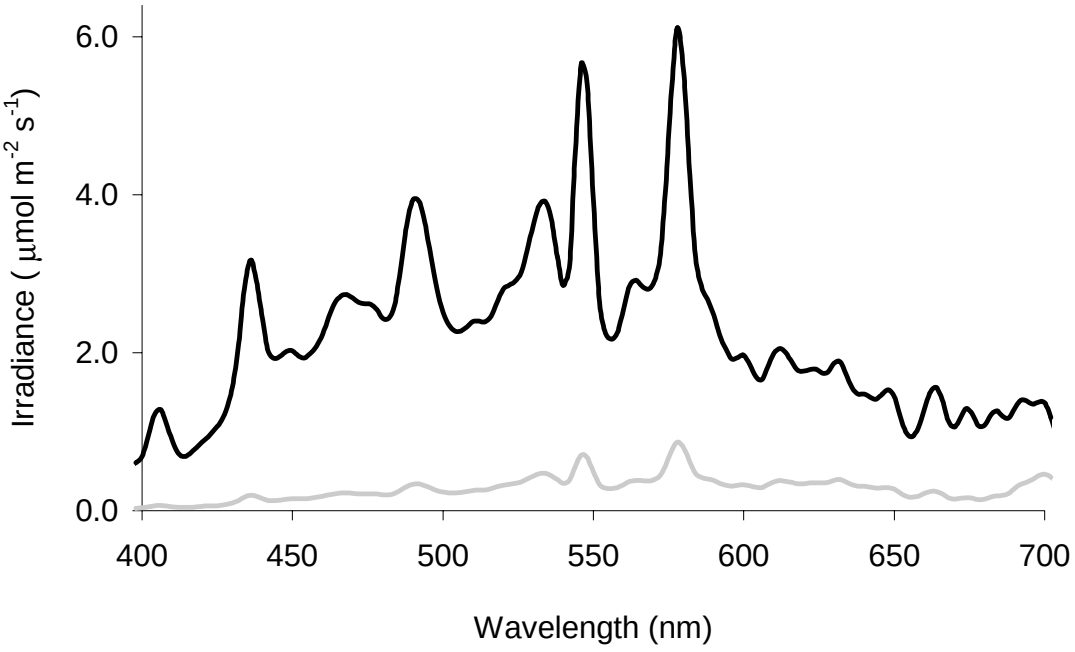


Fig 4.1 Mean spectral distribution of radiation at three red (black line) and green (grey line) areas of intact *Lactuca sativa* leaves grown at 800 µmol m^{-2} s^{-1}.

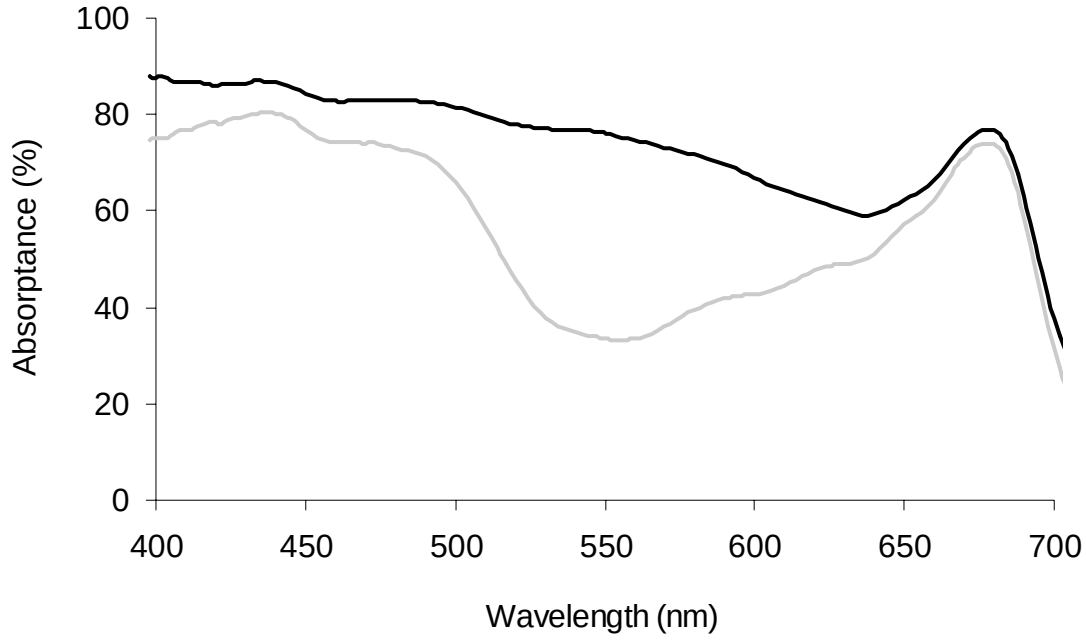


Fig 4.2 Absorptance spectra for the adaxial surfaces of red (black line) and green (grey line) regions of intact *Lactuca sativa* leaves grown at 800 μmol m^{-2} s^{-1}.

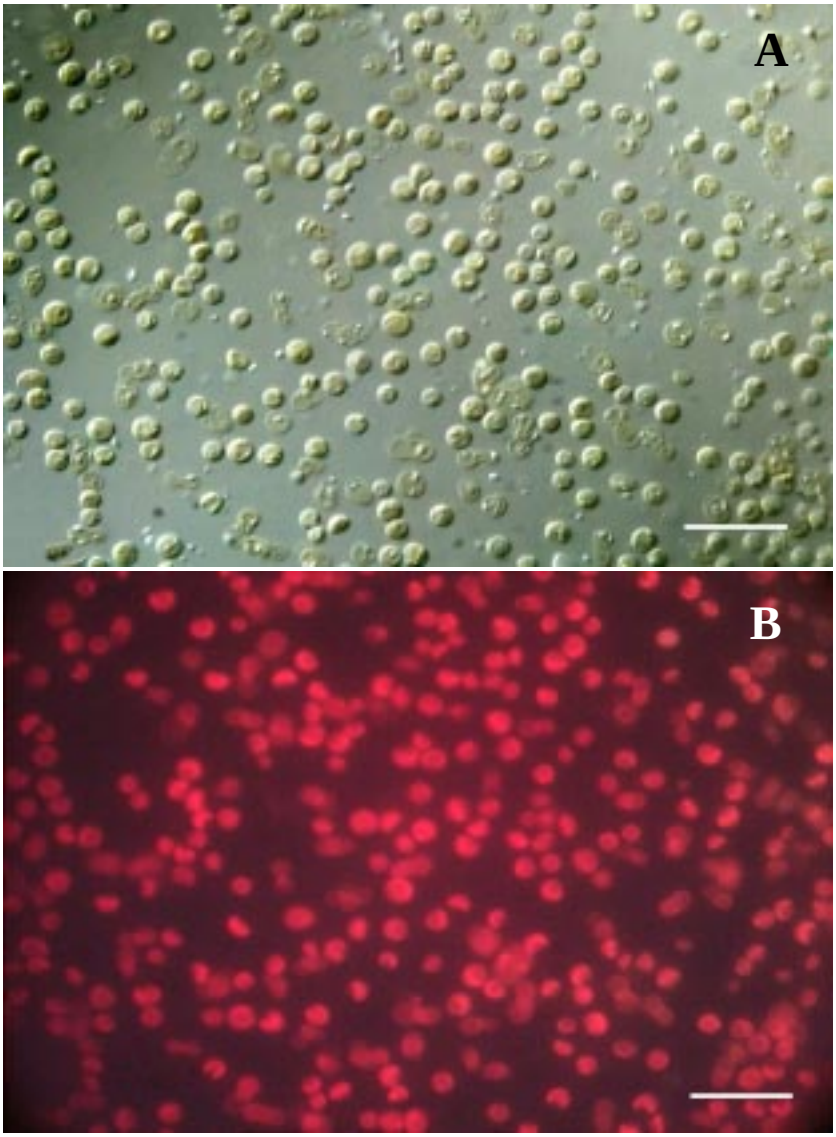


Fig 4.3 Light micrograph with DIC (A), and UV-fluorescence micrograph of chloroplasts isolated from leaves grown at 150 µmol m^{-2} s^{-1}. Bar = 20 µm.

Table 4.1 Pigment composition of red and acyanic areas of *Lactuca sativa* leaves grown under 800 µ mol m^{-2} s^{-1}. Values shown are the means (± s.e.) of 8 leaves. *** = P<0.001; n.s. = not significant (Student's t-test).

Pigment	Red	Acyanic
Anthocyanin (A_{530})	14.0 ± 1.4	0.0 ***
Chlorophyll a (µg cm^{-2})	1.4 ± 0.3	1.1 ± 0.2 n.s.
Chlorophyll b (µg cm^{-2})	0.5 ± 0.1	0.4 ± 0.1 n.s
Total chlorophyll (µg cm^{-2})	2.0 ± 0.5	1.5 ± 0.3 n.s.
Total carotenoid (µg cm^{-2})	0.6 ± 0.1	0.4 ± 0.1 n.s.
Chlorophyll a/b ratio	2.9	3.0 n.s

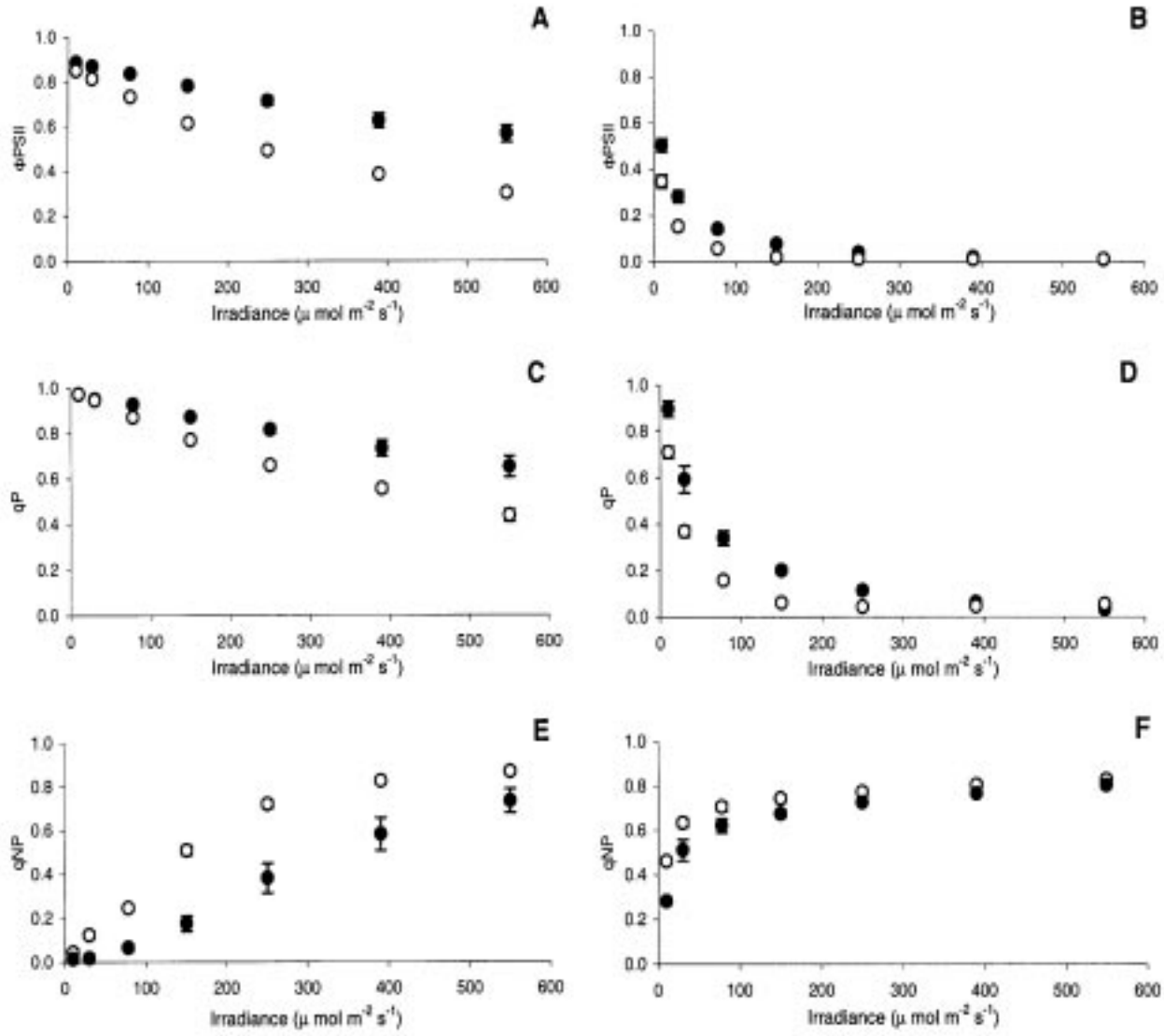


Fig 4.4 Photosynthetic responses of (A, C, E) whole leaves and (B, D, F) chloroplast suspensions from red (●) and (○) green areas of *Lactuca sativa* leaves grown at 800 μmol m^{-2} s^{-1}. Photochemical efficiency of PSII (ΦPSII) (A, B), photochemical quenching coefficients (qP) (C, D), and non-photochemical quenching (qNP) coefficients (E, F) are shown as means (± s.e.) of three replicates for increasing irradiances. Chloroplast yields per μL for red and green areas were 11,500 and 11,160 respectively.

chloroplast suspensions were significantly greater (t-test; $P<0.001$) in red areas (154) compared to acyanic areas (103).

Values of Fv/Fm were reduced by 37 % in red, and by 44 % in acyanic areas of intact leaves following the photoinhibitory treatment (Fig. 4.5 A). The red areas recovered to ca. 90 % of their original values, and the green areas to ca. 80 %, within 5 minutes after the photoinhibitory treatment. The chloroplast suspensions from red and acyanic areas each showed an 83 % reduction in Fv/Fm after photoinhibitory treatment (Fig. 4.5 B). Chloroplasts from red areas had a 7.5 % greater rate of recover of their original Fv/Fm values (70 % recovery of original) than did those from acyanic areas (64 % recovery of original) during the first 2.5 minutes. This difference in recovery rate was statistically significant (ANOVA; $P<0.05$).

Superoxide generation

Superoxide concentrations in the chloroplast suspension increased asymptotically over time (Fig. 4.6 A). Readings of A_{560} at 8 min were found to be linearly correlated to the applied irradiance and were subsequently used for comparisons across the treatments (Fig. 4.6 B). There was no evidence of NBT conversion in the absence of light, nor in the presence of light without chloroplasts.

The absorptance spectrum of the red cellulose filter used to simulate the light-screening effect of anthocyanins was similar to that of purified anthocyanins at pH 1.0 (Fig. 4.7 A). The green, red, and blue cellulose filters had absorptance peaks at 406, 524, and 634 nm, respectively (Fig. 4.7 B).

Chloroplasts shielded by all four cellulose filters received 25 % less total PAR than did the unshielded chloroplast suspensions. They generated significantly less $O_2^{\cdot -}$ (Fig. 4.8 A) and exhibited significantly lower levels of chlorophyll bleaching (Fig. 4.8 B).

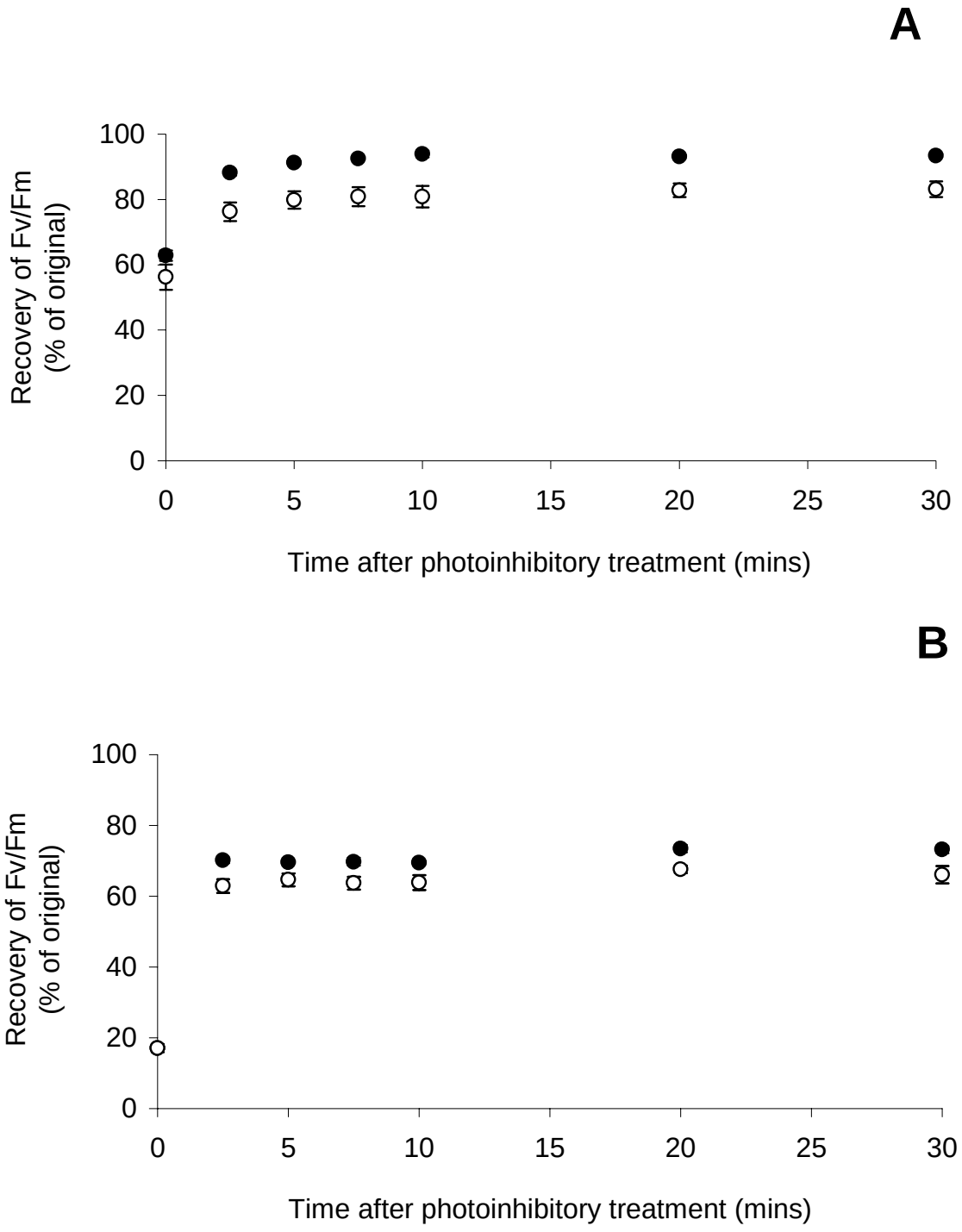


Fig 4.5 Recovery of maximum PSII efficiency (Fv/Fm) in (A) whole leaves and (B) chloroplast suspensions from red (●) and (○) green areas of *Lactuca sativa* leaves following a photoinhibitory light treatment. Values shown are means (± s.e.) of three replicates.

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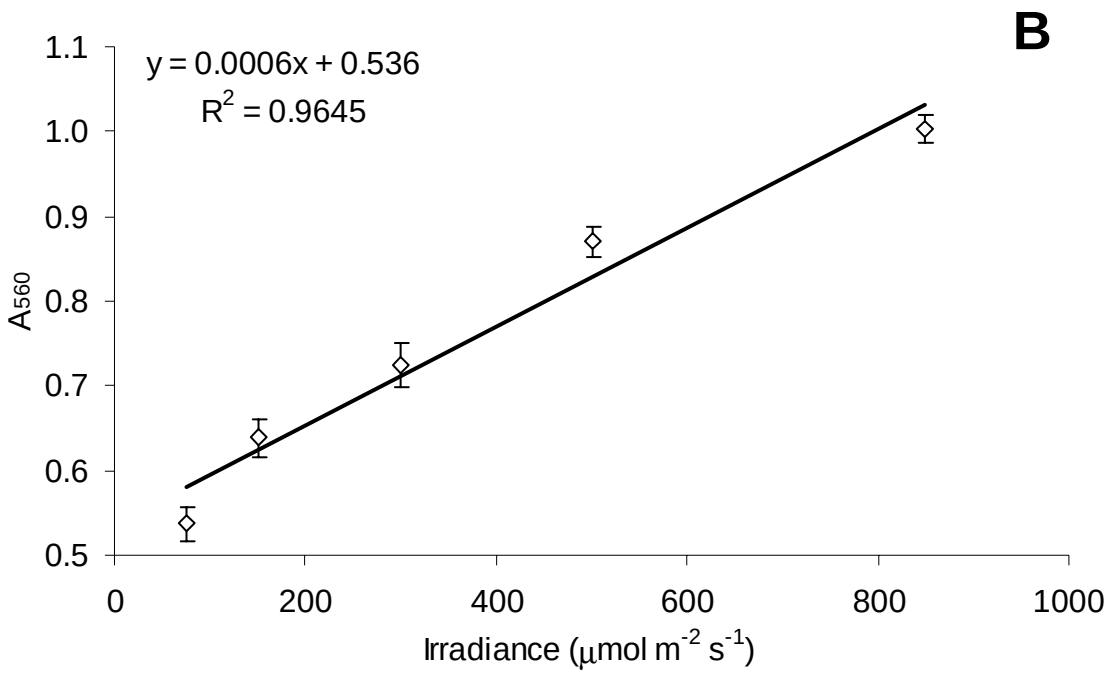


Fig. 4.6 Superoxide concentrations in chloroplast suspensions from *Lactuca sativa* leaves as measured by NBT conversion at A560. (A) Time course of superoxide generation in chloroplasts suspensions irradiated with 75 (●), 150 (○), 300 (▲), 500 (△), or 850 □mol m-2 s-1 (■) of white light at 23 oC. (B) Plot of superoxide generation after 8 mins against irradiance. Values shown are the means (± s.e.) of 3 replicates.

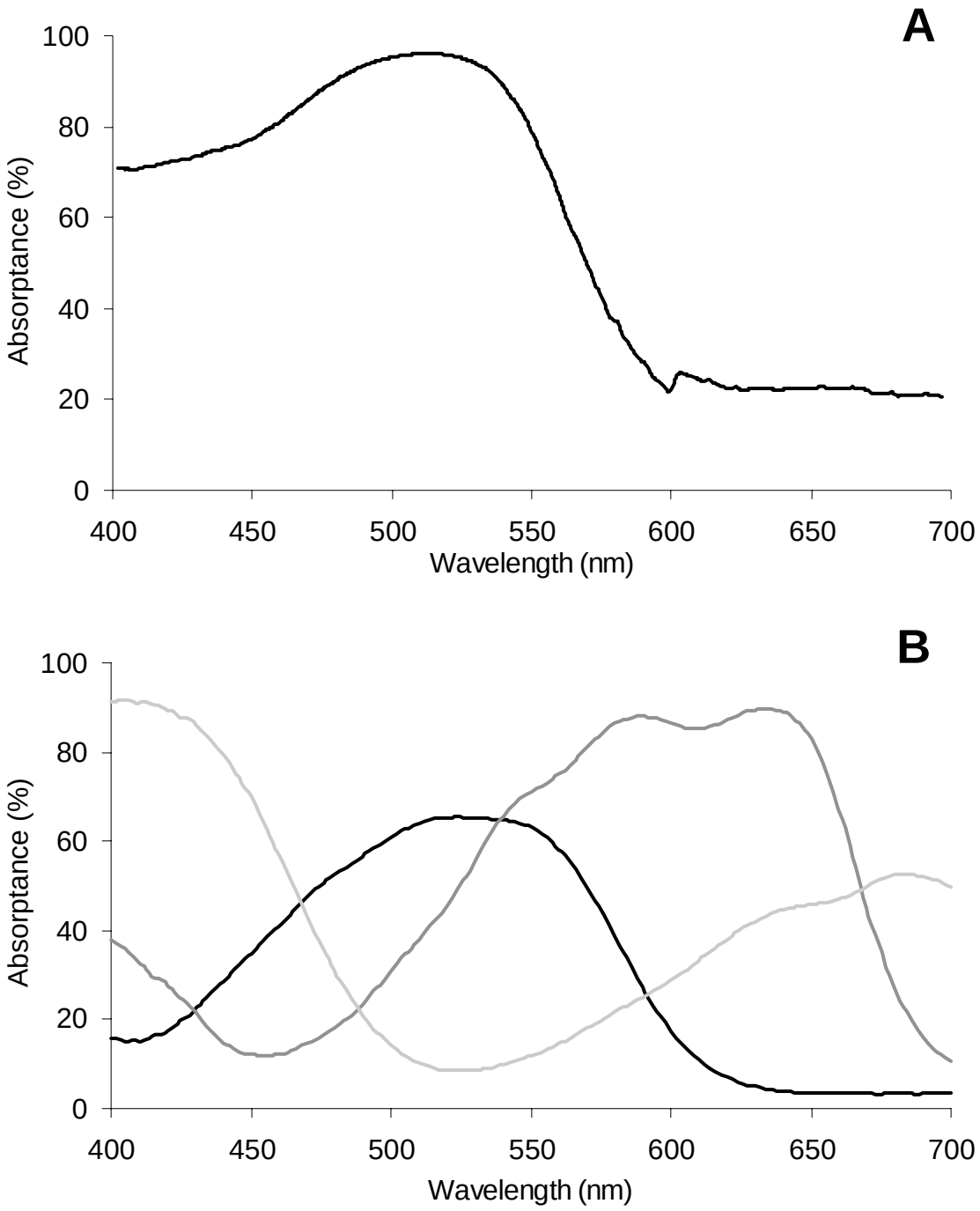


Fig. 4.7 Absorptance spectra of purified anthocyanins (A) and of red (black line), green (light grey line), and blue (dark grey line) cellulose filters (B).

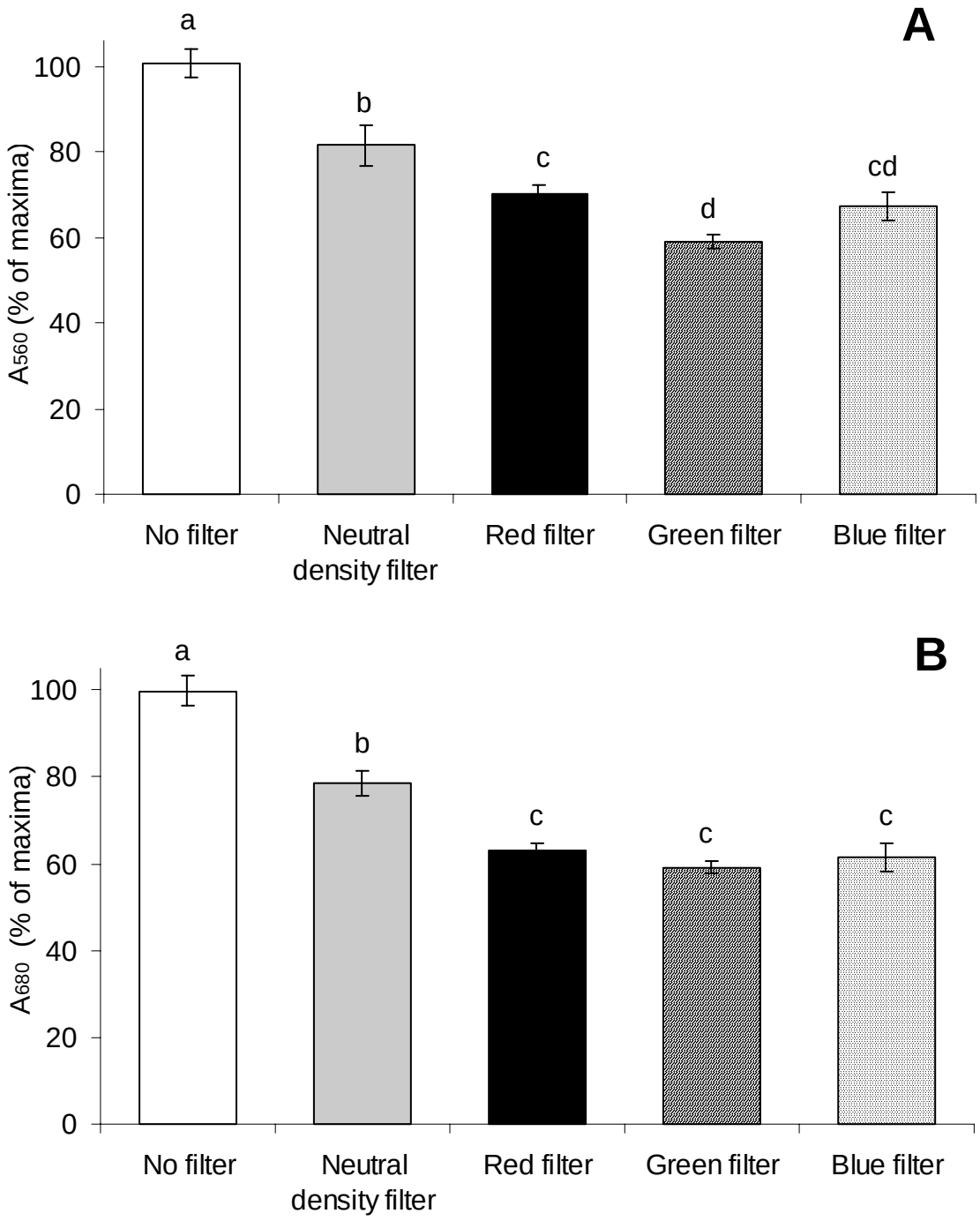


Fig. 4.8 Mean superoxide generation (A) and chlorophyll bleaching (B) (± s.e.) in chloroplast suspensions from *Lactuca sativa* leaves irradiated with 300 μmol m^{-2} s^{-1} at 23 °C through either neutral density, red, green, or blue filters. The effect of unfiltered light (400 μmol m^{-2} s^{-1}) on superoxide production is also shown. Values that are significantly different ($P<0.05$) are indicated by different letters (Tukey-Kramer HSD). N = 3 for each treatment.

For a given irradiance of the chloroplast suspensions there were also differences in $O_2^{\cdot -}$ generation and chlorophyll bleaching with respect to quality of light. The green filter, which absorbed maximally in the red and blue regions of the visible spectrum (Fig. 4.7 B), was the most effective chromatic filter in reducing $O_2^{\cdot -}$ production. Attenuation of green light by the red filter also significantly reduced generation of $O_2^{\cdot -}$ and chlorophyll bleaching compared to chloroplasts irradiated through the neutral density filter.

Discussion

Our data from both intact leaves and chloroplast suspensions demonstrate that anthocyanins confer two protective functions in leaves. First, they protect photosynthetic tissue from the effects of high irradiances by reducing the extent of photodamage and photoinhibition. Secondly, by reducing the quanta incident on underlying chloroplasts, anthocyanins reduce the generation of superoxide radicals through photooxidative processes.

Light filtering

Red leaf laminae exhibited greater photochemical efficiencies, utilised a greater proportion of absorbed quanta for photochemical processes, dissipated less energy through non-photochemical processes (Fig. 4.4 A-C), and were photoinhibited less by saturating irradiances (Fig. 4.5 A), relative to acyanic areas. Isolated chloroplasts from *L. sativa* were less tolerant of high light, and more susceptible to photoinhibition relative to intact leaves (Fig. 4.4 A-F, 4.5 A and B). However, the chloroplasts from red and acyanic regions exhibited similar differences in chlorophyll fluorescence kinetics as those in intact leaves (Fig. 4.4 D-F). However, unlike the in intact leaves, chloroplasts isolated from red and acyanic areas were photoinhibited to a comparable degree (Fig 4.5 B), indicating the importance of green light attenuation by the anthocyanic shield. Work by Krol *et al*. (1995) also implied that attenuation of light by anthocyanins could reduce photoinhibition. It was reported (although the data were not shown) that the presence of an artificial anthocyanic filter, which reduced incident PAR by 35 %, protected non-pigmented *Pinus banksiana* seedlings against low temperature photoinhibition to a level equivalent to that found in hardier, red-pigmented seedlings. Our data suggest that the absorption of high-energy quanta by anthocyanins diminishes the requirement for non-photochemical quenching i.e. heat

dissipation through the xanthophyll cycle (Demmig-Adams *et al.*, 1997), and reduces wastage associated with photoinhibition.

Anthocyanins in *Lactuca sativa* have been previously identified as cyanidin-3-malonylglucoside (Ferreres *et al.*, 1997), and reside exclusively in the adaxial epidermis. The enhanced absorption of PAR by these anthocyanins, allows subjacent chlorenchyma to maintain a greater proportion of their PSII reaction centres in the oxidised state, so that the photosynthetic apparatus can function under higher irradiances, as shown by higher qP values of red versus acyanic lamina regions (Fig. 4.4 B). The extent to which PAR absorptance was increased in the red regions of is comparable to those measured in pigmented native New Zealand species (Chapter 3). Similar effects of anthocyanic pigmentation were noted for purple and green pods of *Bauhinia variegata* in response to blue-green light (Smillie and Hetherington, 1999). However, irradiation of purple pods with red wavelengths, which are not absorbed by anthocyanins, resulted in photochemical efficiencies equivalent to values observed in green pods.

There were also inherent differences in the physiology of chloroplasts isolated from red and acyanic regions of *L. sativa* over and above those attributable to photoabatement by epidermal anthocyanins. Chloroplasts from the red areas of leaves recovered from photoinhibitory treatment at a significantly greater rate than those from acyanic areas (Fig. 4.5 B), indicating a greater physiological tolerance of high irradiances. Subtraction of the 17 % extra absorption of total PAR by epidermal anthocyanins from the irradiances applied to the intact on intact red areas of leaves failed to eliminate this difference. Photochemical efficiencies and photochemical quenching are inherently higher, and non-photochemical quenching coefficients lower in the chloroplasts of red regions than those in acyanic areas.

It is probable that red areas of the leaf laminae arose as a consequence of positional differences in their exposure to light. Those distal areas which accumulated anthocyanins, experienced 88 % more PAR than the proximal regions of leaves, which remained acyanic (Fig. 4.1). Levels of chlorophyll *a*, *b* and the carotenoids were not significantly different between red and acyanic areas (Table. 4.1) and hence cannot account for the differences in physiology. An indication of the total number of antenna complexes present within photosynthetic tissue can be obtained from dark-adapted measurements of minimal

fluorescence (Fo) (Krause and Weis, 1991). Comparison of the average Fo values from *L. sativa* chloroplasts indicates that tissue from anthocyanic areas possessed approximately 33 % more antenna complexes than did acyanic areas. It was similarly found by Field *et al*. (2001) that senescing leaves of *Cornus stolonifera* from shaded conditions did not accumulate anthocyanins, in contrast to those experiencing higher PPFDs. It was postulated that anthocyanin induction is facultative, i.e. if a leaf experiences irradiances that are high enough and are prolonged to cause photodamage, then anthocyanin is accumulated to provide protection. Indeed, variations in light quantity and quality, such as those experienced in exposed and shaded areas of the same leaf, or indeed leaves of the same plant under different microenvironments, dictate the physiological development of photosynthetic apparatus (Anderson *et al.*, 1995). Such elevated light exposure incident on red areas of *L. sativa* could lead to an enhanced pool of carbon fixation enzymes, electron acceptors such as plastoquinone, ferredoxin, and $NADP^+$, as well as greater levels of antioxidants such as superoxide and ascorbate, all of which help maintain the capacity to utilise captured excitation energy and recovery from photoinhibition (Anderson and Osmond, 1987; Foyer *et al.*, 1994; Osmond and Grace, 1995).

Superoxide generation

By maintaining a greater proportion of quinone acceptor molecules (Q_A) in their oxidised forms under high irradiances (Fig. 4.4 B, E), anthocyanins have the potential to reduce $O_2^{\cdot-}$ generation through the Mehler reaction (Foyer *et al.*, 1994). The Mehler reaction has been shown to play an important part in the dissipation of excess excitation energy when levels of terminal electron acceptors such as $NADP^+$ are low, thereby preventing over-reduction of the electron transport chain (Robinson, 1988; Schreiber and Neubauer, 1990; Osmond and Grace, 1995). However, $O_2^{\cdot-}$ is also a major contributor to the degradation of the PSII D1 reaction centre protein during photoinhibition (Henmi, 1997) and has been postulated to be involved in the enzymatic bleaching chlorophyll *a* (Adachi and Shimokawa, 1995).

Our experimental system demonstrated that imposition of a red cellulose filter, the absorption of which was comparable to that of isolated anthocyanin, significantly reduced $O_2^{\cdot-}$ generation and chlorophyll bleaching in chloroplast suspensions relative to those subjected to white light of comparable fluxes (Fig.4.8 A, B). However, in our study a red filter was not the best amongst all potentialities for the inhibition of $O_2^{\cdot-}$ generation. Filters that reduced the

transmittance of blue and red wavebands effected significantly greater photoprotection from $O_2^{\cdot-}$ generation than did the red filter. The action spectrum for $O_2^{\cdot-}$ generation recapitulates that for photosynthetic oxygen evolution (Englemann, 1882; Shibata *et al.*, 1954). Thus, the optimal colour filter for reducing $O_2^{\cdot-}$ production would be green, followed by a blue filter, but these would also have the greatest impact on CO_2 fixation by reducing the absorption of quanta by chlorophyll *a* and *b* (Nishio, 2000). Therefore, an anthocyanic epidermis, which strongly attenuates green light, may represent an evolutionary compromise that leads to the beneficial reduction of $O_2^{\cdot-}$ generation whilst impacting the least on the action spectrum of photosynthesis.

In conclusion, it appears that the presence of epidermal anthocyanins have the potential to finely adjust the quantity and quality of irradiation incident upon underlying chloroplasts. Photoabatement by anthocyanins permits the maintenance of higher levels of oxidised PSII acceptors, and therefore reduces the extent of photoinhibition, and photooxidation negating the need for wasteful photodissipatory processes.

CHAPTER 5 – Protection against reactive oxygen species (ROS)

Anthocyanins have been suggested to act as potent antioxidants. The purpose of this chapter therefore is to define reactive oxygen species (ROS) and free radicals, to describe the conditions under which they are produced, and to discuss ways in which plants can protect themselves from these damaging species. Here, the complex, integrated antioxidant defence system of plants is reviewed, and contemporary evidence detailing why anthocyanins are considered to act as low molecular weight antioxidants is presented. Such background information is crucial for the interpretation of the two subsequent chapters (Chapters 6 and 7).

Common factors in environmental stress

Anthocyanins are frequently produced in leaves during stressful periods, when either biotic or environmental stimuli are in deficiency or excess. Such conditions have been extensively documented to trigger the formation of harmful ROS, and free radicals.

What are free radicals and ROS?

A free radical is any species capable of independent existence that contains one or more unpaired electrons (an unpaired electron is one that occupies an atomic or molecular orbital by itself) (Halliwell and Gutteridge, 1998). Radicals can be formed when a non-radical loses an electron ($X \rightarrow e^- + Y^{+\cdot}$), when a non-radical gains an electron ($X + e^- \rightarrow Y^{\cdot}$), or when a covalently bonded molecule undergoes 'homolytic fission' ($H\text{-}OH \rightarrow H^{\cdot} + OH^{\cdot}$). The presence of an unpaired electron(s) can cause the species to be highly reactive, and thus they usually have short, transient lifetimes (Halliwell and Gutteridge, 1998).

Since diatomic oxygen possesses two unpaired electrons in its outer orbitals, it has a high electron affinity and can give rise to a wide variety of potentially damaging intermediates. These include free radicals, and a number of other compounds that behave as oxidants, yet do not posses unpaired electrons (Halliwell and Gutteridge, 1998; Mallick and Mohn, 2000). Such compounds are collectively termed reactive oxygen species (ROS), and include the oxygen radicals; superoxide ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), and peroxyl radicals ($ROO^{\cdot}$), and non-radical oxygen intermediates, such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and ozone (O_3) (Halliwell and Gutteridge, 1998; Rice-Evans *et al.*, 1991).

The presence of even low concentrations of ROS can be deleterious to the function of DNA, RNA, proteins, and polyunsaturated lipids. They cause mutations, inhibition of protein synthesis, altered enzyme activity, and increased membrane permeability. Such damage often takes place in localised cellular compartments through an autopropagating chain reaction (Saran *et al.*, 1998). However, it must also be noted that not all ROS are deleterious. Indeed, the generation of $O_2^{\cdot-}$ by NADPH oxidase in phagocytes is crucial for the elimination of foreign cells in the body (Babior *et al*., 1973). H_2O_2 in conjunction with peroxidases are required for the oxidation of phenolics, especially flavonols and phenylpropanoids to produce precursors for lignin formation (Takahama and Oniki, 2000). In addition, nitric oxide ($NO^{\cdot}$) an active nitrogen species (ANS) plays an important role in plant cell signalling (Yamasaki, 2000).

Generation of ROS in plant cells

Even under optimal conditions, plant cells unavoidably produce ROS from the leakage of electrons during electron transport activities of the chloroplasts, mitochondria, and plasma membrane (Fridovich, 1995; Foyer, 1997). Oxidative stress is further increased by various environmental and biotic stressors (Fig. 5.1) such as UV radiation (Barabas *et al.*, 1998); extremes of temperature (Doke *et al.*, 1994; Rao *et al.*, 1995); water stress (Smirnoff, 1993); heavy metals (Weckx and Clijsters, 1996); high salt concentrations (Meneguzzo *et al.*, 1999); air pollutants and herbicides (Mehlhorn, 1990, Tanaka, 1994 #600); mechanical injury (Legendre *et al.*, 1993); and by the invasion of pathogens (Low and Merida, 1996). The most frequent source of ROS are those derived from light capture in the process of photosynthesis, especially under conditions of excess illumination. Photosynthetic cells are particularly prone to oxidative stress as they contain an array of photosensitive pigments, and they both consume and produce oxygen (Foyer *et al.*, 1994).

Protection against ROS through the possession of antioxidants

High levels of antioxidants are crucial for the protection of plants cells from the damaging affects of ROS (Asada and Takahashi, 1987; Larson, 1988; Halliwell and Gutteridge, 1998; Foyer *et al.*, 1994; Alscher *et al.*, 1997; Polle, 1997). Under 'normal' conditions, a plant's antioxidant system provides adequate protection against ROS (Foyer *et al.*, 1994). However,

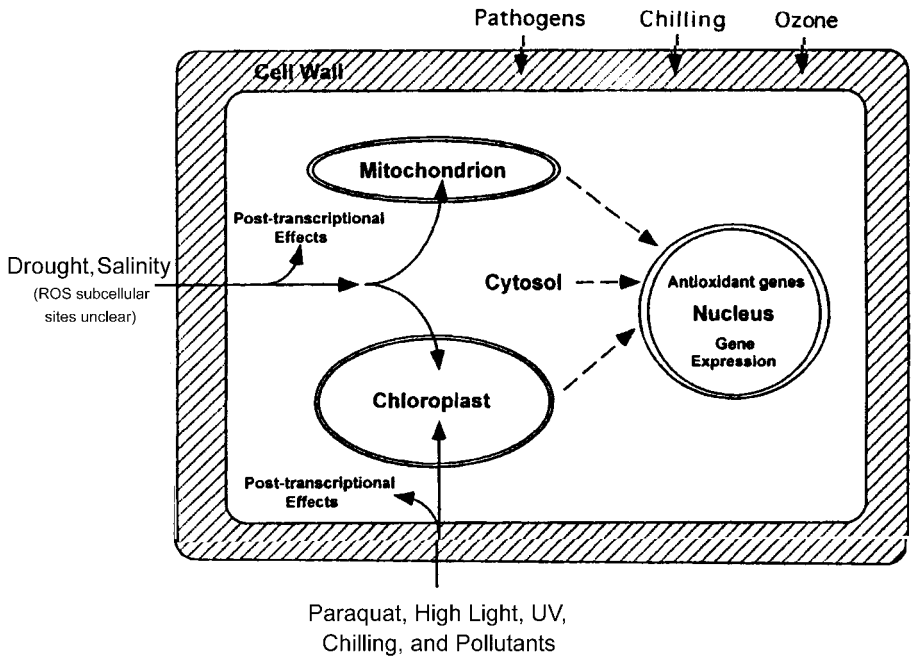


Fig. 5.1 Sites of reactive oxygen species (ROS) production and detoxification in green leaf cells. Environmental conditions give rise to ROS in the locations shown (solid arrows). The cytosol, mitochondria and chloroplast are sites of ROS detoxification. The cell wall may be one of these sites. Exposure to the environmental factors is proposed to affect gene expression and post-transcriptional event. Areas acted upon may signal to the nucleus resulting in expression of the nuclear-encoded antioxidant genes (dashed arrows). (Modified from: Alscher, Donahue, and Cramer, 1997).

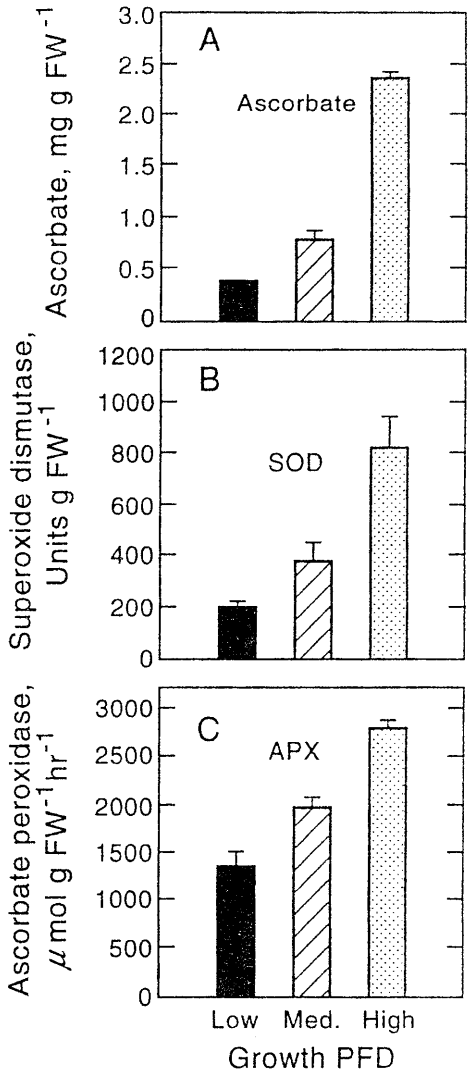


Fig. 5.2. Levels of antioxidant enzymes in the leaves of *Vinca major* grown at low (15 μmol m^{-2} s^{-1}), medium (85 μmol m^{-2} s^{-1}), and high (880 μmol m^{-2} s^{-1}) light intensities. A = levels of ascorbate, B = levels of superoxide dismutase, C = levels of ascorbate peroxidase (From: Demmig-Adams, Adams, and Grace, 1997).

when plant cells are stressed, further antioxidant defences are called upon or existing protection is up regulated. ROS themselves may be responsible for up regulating gene transcription, indirectly modifying metabolism of antioxidant enzymes through signal-transduction pathways (Levine *et al.*, 1994; Foyer *et al.*, 1997). For example, *Vinca major* plants grown at increasing irradiances have progressively higher levels of ascorbate, superoxide dismutase (SOD), and ascorbate peroxidase (APX) (Fig. 5.2) (Demmig-Adams *et al.*, 1997).

The integrated antioxidant defence system

The definition of an antioxidant is any substance which when present at low concentrations compared to those of the oxidisable substrate, significantly delays, or inhibits the oxidation of that substance (Halliwell and Gutteridge, 1998). Many antioxidants are able to 'scavenge' ROS by either donating or accepting an electron, and due to structural peculiarities, are able to stabilize their own radical state by mesomerism (e.g. ascorbate) (Bors *et al.*, 1990). 'Quenching', on the other hand is the process of excitation energy transfer from the ROS (e.g. $^1O_2$) to another molecule allowing the excited species to return to its ground state (Halliwell and Gutteridge, 1998). A large number of compounds exist to deactivate and reduce the reactivity of ROS, thus providing an integrated defence system (Fig. 5.3) (Noctor and Foyer, 1998). This defence system can be divided into three main categories: i) the enzymic antioxidants that directly scavenge ROS, ii) the low molecular weight antioxidants (LMWAs), and iii) enzymes that recycle reduced antioxidants and help maintain their levels.

Enzymatic antioxidants

Several antioxidant enzymes are involved in the scavenging of $O_2^{\cdot -}$ and H_20_2. The most important are superoxide dismutase (SOD), catalase (CAT), and various peroxidases (PX), which act in a concerted way depending on subcellular location (Fig. 5.3; Alscher *et al.*, 1997).

Superoxide dismutase (SOD)

Three types of SOD metalloisoenzymes have been discovered, each defined by the use of a particular metal as their prosthetic group (Mn, Fe, and Cu/Zn) (Alscher *et al.*, 1997). SOD converts the highly reactive superoxide radical to oxygen and hydrogen peroxide.

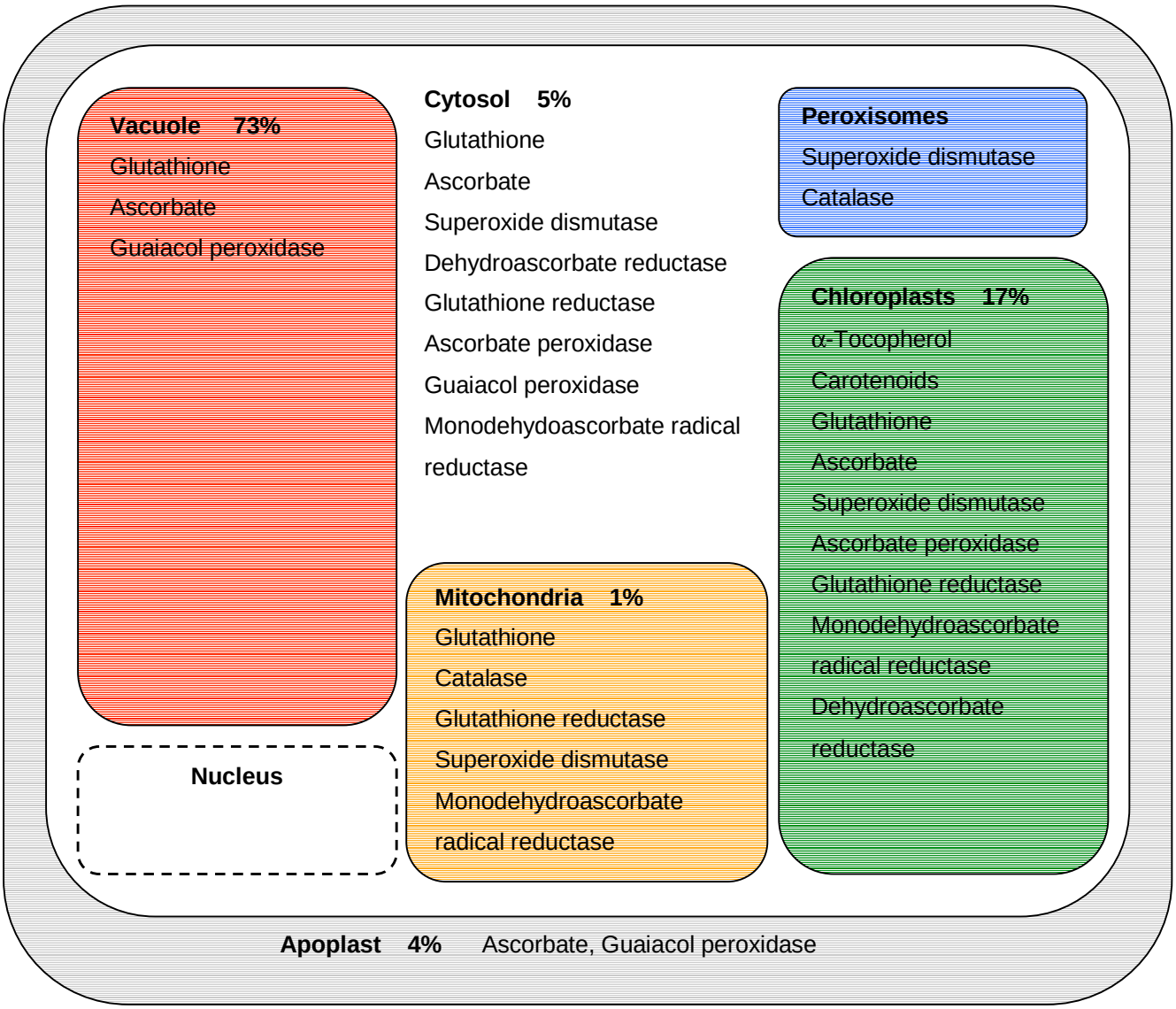


Fig. 5.3 Subcellular localisation of the major antioxidants and regenerative enzymes in plant cells. Relative volumes of the respective compartments are illustrated. (From: Polle and Junkermann, 1994; as modified by Polle, 1997).

$$2\ O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

The major SOD is a copper-zinc-containing protein (Cu/ZnSOD), which is predominantly associated with the chloroplast, but is also found in the cytosol, and peroxisomes (Asada, 1994; Bueno *et al.*, 1995; Ogawa *et al.*, 1995). The others consist of a manganese-containing isoenzyme (MnSOD) associated with the mitochondrial matrix of all species analysed (Bowler *et al.*, 1994), and a less common iron-containing enzyme (FeSOD) found in the chloroplasts of some plant species (e.g. *Glycine max, Arabidopsis thaliana, and Lycopersicon esculentum*) (Halliwell and Gutteridge, 1998; Van Camp *et al.*, 1991; Bowler *et al.*, 1994). Analysis of SOD sequence data, indicates that Cu/Zn-containing SODs, which are present in all eukaryotes, are structurally unrelated to the Fe and Mn SODs, which occur in pro- and ekaryotes (Bowler *et al.*, 1994), indeed FeSOD likely has an endosymbiotic origin in plants (Smith and Doolittle, 1992).

Catalase (CAT)

The action of SOD coverts $O_2^{\cdot -}$ to the equally damaging H_2O_2, therefore further antioxidants are needed to neutralise such ROS (Foyer *et al.*, 1994). CAT is a tetrameric, heme-containing enzyme found in the peroxisomes and mitochondria (Fig. 5.3; Scandalios, 1994) in most aerobic organisms. It converts H_2O_2 to oxygen and water (Mallick and Mohn, 2000).

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

CAT plays a crucial role in the peroxisomes, by neutralising the high flux of H_2O_2 produced under photorespiration (Foyer and Noctor, 2000). CAT is unique among the enzymatic antioxidants in that it removes H_2O_2 without producing another harmful ROS, or consuming reducing equivalents like the other H_2O_2-degrading enzymes, such as the peroxidases. It therefore provides an energy-efficient way to remove H_2O_2 from the cell (Mallick and Mohn, 2000).

Peroxidases (PX)

Peroxidase enzymes function to remove H_2O_2 by using it to oxidize another substrate (for example, SH_2)

$$SH_2 + H_2O_2 \xrightarrow{PX} S + 2H_2O$$

They can be specific for a particular substrate, such as ascorbate for ascorbate peroxidase (APX), or able to utilise a much broader range of compounds. Horseradish peroxidase (HRP) is an example of a 'non-specific' peroxidase that can oxidise a variety of compounds including guiacol, pyrogallol, CN^- ion, NADH, thiol compounds, plant hormones, and phenols (Halliwell and Gutteridge, 1998). Another example of a 'non-specific' peroxidase is guaiacol peroxidase that occurs in the vacuole, cytoplasm, and apoplast. It is able to use a broad range of phenolic substrates and is involved in the production of lignin, wound healing, and defence against pathogens (Polle, 1997).

H_2O_2 is a potent inhibitor of photosynthesis and its removal is vital to the function of the chloroplast (Kaiser, 1979; Foyer, 1993). 'Non-specific' peroxidases, and CAT are absent from the chloroplast (Fig. 5.4), and the degradation of H_2O_2 is achieved solely through the action of the haem-containing APX enzyme.

As APX relies on ascorbate for its antioxidant activity, it marks an important link between the enzymatic antioxidant defence system and the LMWAs (Fig. 5.4). The ability to recycle these oxidised LMWAs through the action of dehydroascorbate reductase (DHAR), and NADPH-dependent glutathione reductase (GR) is crucial to the continued function of the enzymic antioxidants (Law *et al.*, 1983; Foyer *et al.*, 1994).

$$H_2O_2 + \text{Ascorbate} \xrightarrow{APX} H_20 + \text{Dehydroascorbate}$$

$$\text{Dehydroascorbate} + GSH \xrightarrow{DHAR} \text{Ascorbate} + GSSG$$

$$GSSG + NAPH \xrightarrow{GR} GSH + NADP^+$$

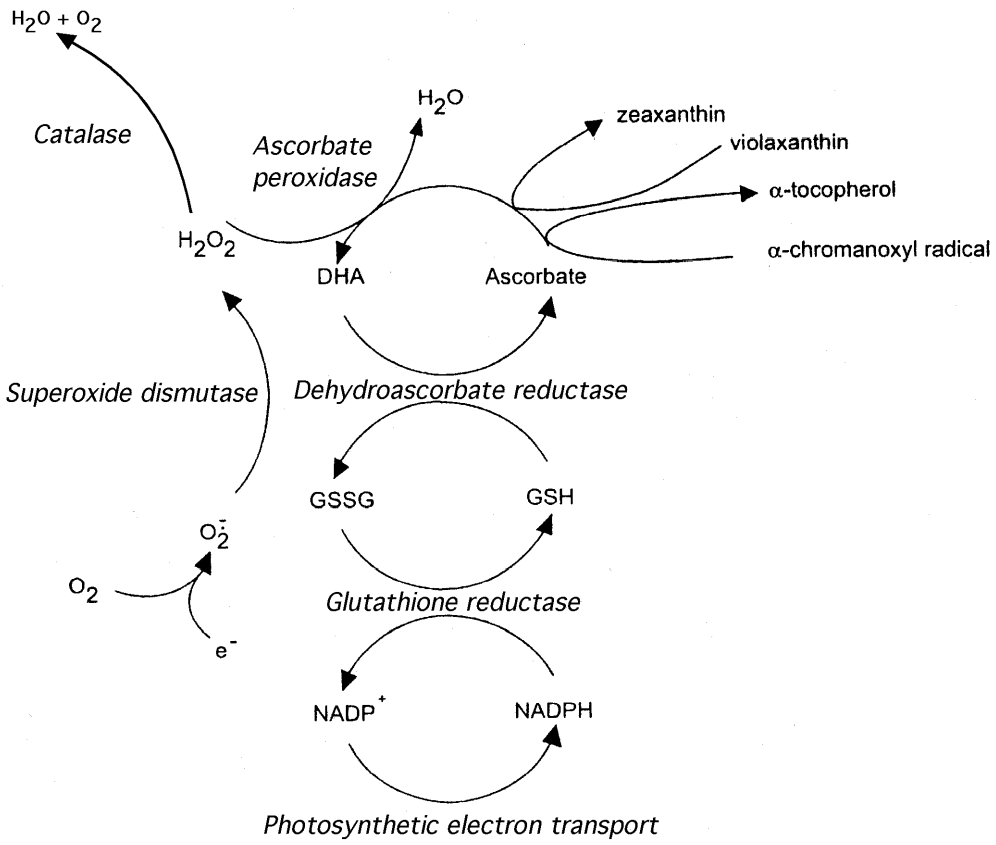


Fig. 5.4 Reactive oxygen species (ROS) scavenging system of the chloroplasts. Enzymatic antioxidants are shown in italics. Abbreviations: DHA, dehydroascorbate; GSH, reduced glutathione; GSSG, oxidised glutathione; $NADP^+$, reduced nicotinamide adenine dinucleotide phosphate; and NADPH, oxidised nicotinamide adenine dinucleotide phosphate (Modified from: Creissen, Edwards, and Mullineux, 1994).

Low molecular weight antioxidants (LMWAs)

The quenching of $^1O_2$ scavenging of $OH^{\cdot}$, and $O_2^{\cdot -}$ are undertaken by numerous small, hydrophilic molecules, including ascorbate, glutathione, and the flavonoids, along with the lipophilic α-tocopherol and carotenoid pigments (Foyer, 1993; Hess, 1993; Foyer *et al.*, 1994; Winkler *et al.*, 1994). The ROS scavenging system of the chloroplast and other cytoplasmic components involves successive oxidations and reductions of ascorbate, glutathione, and ultimately NADPH by the enzymes of APX, GR, and DHAR, the process of which is collectively known as the ascorbate-glutathione cycle (Fig. 5.4) (Halliwell and Gutteridge, 1998; Foyer *et al.*, 1994; Alscher *et al.*, 1997).

Ascorbate

Levels of ascorbate (Vitamin C) in the chloroplast can be as high as 12 to 25 mM and as such constitutes 30-40 % of the total cellular ascorbate content (Law *et al.*, 1983), the remainder is found in the cytosol, vacuole, and apoplast (Polle, 1997). Ascorbate plays a fundamental role in the ascorbate-glutathione cycle, and is central to the regeneration of membrane-bound xanthophylls and tocopherols (Fig. 5.4) (Foyer, 1993). It can also quench $^1O_2$, reduce $O_2^{\cdot -}$, $OH^{\cdot}$ (Asada and Takahashi, 1987; Smirnoff, 2000), and serves as a substrate in APX catalysed reactions to detoxify H_2O_2 (Groden and Beck, 1979; Hossain *et al.*, 1984). Sequential oxidation of ascorbate yields monodehydroascorbate (MDHA) and then dehydroascorbate (DHA). If it is not rapidly reduced back to ascorbate by monodehydroascorbate reductase (MDHAR), the monodehydroascorbate radical spontaneously disproportionates to yield ascorbate and dehydroascorbate, which can subsequently be recycled through the action of dehydroascorbate reductase (DHAR) (Mallick and Mohn, 2000; Smirnoff, 2000).

Glutathione

Around 10-50 % of cellular glutathione (GSH) is localised within the chloroplasts (Smith *et al.*, 1985); however, significant levels are also found in the vacuole, cytoplasm, and mitochondria (Fig. 5.3; Polle, 1997). GSH serves primarily as a reductant for the recycling of dehydroascorbate in the ascorbate-glutathione cycle, but can also reduce $O_2^{\cdot -}$ (Asada and Takahashi, 1987), and protects thiol-containing enzymes such as membrane-bound ATPases from oxidation (Halliwell, 1984; Krauss *et al.*, 1987). Maintenance of the GSH pool is achieved through the action of glutathione reductase (GR), which reduces the oxidised form,

glutathione disulphide (GSSG) back to GSH in a NADPH-dependent reaction (Noctor and Foyer, 1998; Mallick and Mohn, 2000).

Tocopherols

Tocopherols, of which α-tocopherol (Vitamin E analogue) is the most important, are located within the lipid membrane of the chloroplast, mitochondria, and endoplasmic reticulum. They are able to quench $^1O_2$, reduce $O_2^{\cdot -}$, and terminate lipid peroxidation (Halliwell and Gutteridge, 1998; Takenaka *et al.*, 1991). Their ability to scavenge lipid peroxyl ($LO_2^{\cdot}$) radicals is about four orders of magnitude faster than these radicals can react with fatty acids or with membrane proteins (Halliwell and Gutteridge, 1998). This is especially important, as most of the lipids of the chloroplast membrane are unsaturated C_{18} fatty acids, and are prone to oxidative damage (Winston, 1990). It should also be noted that α-tocopherol in combination with ascorbate and GSH can result in synergistic inhibition of oxidation damage to cell membranes (Niki, 1987).

Carotenoids

The carotenoids present within plant cells are primarily associated with the chloroplast and are of two main types; i) the carotene accessory pigments (e.g. β-carotene) present within the thylakoid membranes of the light harvesting complexes, which serve to quench chlorophyll triplet states ($^3Chl^*$) and $^1O_2$ (Schoner and Krause, 1990); and ii) the xanthophylls which protect the photosynthetic apparatus from excess irradiances by dissipating energy in the form of heat thereby reducing the potential for ROS formation (Demmig-Adams, 1990).

Metal binding proteins

Much of the damage caused by $O_2^{\cdot -}$ and H_2O_2 *in vivo* is thought to be due to their conversion into the highly reactive $OH^{\cdot}$ through complex Haber-Weiss-Fenton reactions with catalytic transition metals (Asada and Takahashi, 1987; Halliwell, 1990; Wardman and Candeias, 1996). Therefore the capture of free iron and to a certain extent copper ions, by biological chelators, such as phytic acid, phytoferritin and the phytochelatins is of great importance the prevention of ROS generation (Halliwell, 1990; Foyer *et al.*, 1994; Saran *et al.*, 1998).

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{\text{Metal catalyst}} O_2 + OH^{\cdot} + OH^-$$

Polyphenolics as antioxidants

Naturally occurring polyphenolics are another group of compounds that may be important antioxidants in plants. However, relatively little information exists on their function in plant cells, though their role in human health is undisputed (Pathak, 1991; Middleton, 1996; Fergusson, 2001).

Work by Bors *et al*. (1990), highlighted three structurally important groups that govern the relative antioxidant activities of the phenolics. Maximal scavenging is conferred when a) the ortho 3',4'-dihydroxy moiety in the B ring, b) the 2,3-double bond in the C ring in conjugation with a 4-oxo function for electron delocalisation from the B ring, and c) the presence of both a 3-hydroxyl group in the C ring and a 5-hydroxyl group in the A ring are present. Other workers have demonstrated that phenolic compounds possessing ortho-hydroxylation and/or multiple hydroxylation such as quercetin, robinetin, and myricetin, are particularly effective antioxidants (Pratt and Hudson, 1990; Larson, 1995), whereas compounds lacking these features, such as naringenin and hesperetin are usually not as efficient (Pratt and Hudson, 1990).

Work by Rice-Evans *et al.* (1995 a) has shown that only the first two structural requirements were present in effective antioxidants of eighteen plant-derived polyphenols, as assessed using the ferryl myoglobin/ABTS assay. Rice-Evans *et al.* (1995 b) compiled a league of phenolic antioxidants as Trolex equivalents (similar to Table 5.1) which highlighted the pattern of antioxidant activity in relation to structure. Moreover, polyphenolic compounds such as epicatechin gallate, epigallocatechin gallate, quercetin, delphinidin, and cyanidin showed antioxidant potentials at least four times more effective than that of vitamin E and C (Rice-Evans *et al.*, 1995 a; Rice-Evans *et al.*, 1997). However, the league probably overestimated the importance of phenolics as antioxidants *in vivo*, as most flavonoids and hydroxycinnamic acids are glycosylated in plant cells and glycosylation has been found to reduce the overall antioxidant activity (Rice-Evans *et al*., 1997).

Recent work by Repka and Fischerova (2000) illustrated the importance of flavonoids as antioxidants in plants. Greater levels of H_2O_2 were detected within flavonoid-deficient petunia petals compared with wild type petunia. Interestingly, the mutants expressed elevated levels of peroxidases, which may have substituted for flavonoid scavenging of H_2O_2.

Table 5.1 Relative total antioxidant activities of polyphenols and selected antioxidant vitamins. Anthocyanidins and anthocyanins are highlighted in red, vitamins are highlighted in grey (Modified from: Rice-Evans, Miller, and Paganga, 1995; and : Rice-Evans, Miller, and Paganga, 1997).

Antioxidant	Family	Antioxidant activity[a] (mM)
Epicatechin gallate	Flavonol	4.90 ± 0.02
Epigallocatechin gallate	Flavonol	4.80 ± 0.06
Quercetin	Flavonol	4.70 ± 0.10
Delphinidin	Anthocyanidin	4.44 ± 0.11
Cyanidin	Anthocyanidin	4.40 ± 0.12
Epigallocatechin	Flavonol	3.80 ± 0.06
Keracyanin	Anthocyanin	3.25 ± 0.10
Myricetin	Flavonol	3.10 ± 0.30
Gallic acid	Hydroxybenzoate	3.01 ± 0.05
Ideain	Anthocyanin	2.90 ± 0.03
Morin	Flavonol	2.55 ± 0.02
Epicatechin	Flavonol	2.50 ± 0.02
Gallic acid methyl ester	Hydroxybenzoate	2.44 ± 0.03
Catechin	Flavonol	2.40 ± 0.05
Rutin	Flavonol	2.40 ± 0.06
Apigenidin	Anthocyanidin	2.35 ± 0.20
Peonidin	Anthocyanidin	2.22 ± 0.20
p-Coumaric acid	Hydroxycinnamate	2.20 ± 0.06
Luteolin	Flavone	2.10 ± 0.05
Malvidin	Anthocyanidin	2.06 ± 0.10
Taxifolin	Flavanone	1.90 ± 0.03
Ferulic acid	Hydroxycinnamate	1.90 ± 0.02
Oenin	Anthocyanin	1.78 ± 0.02
Naringenin	Flavanone	1.53 ± 0.05
Apigenin	Flavone	1.45 ± 0.08
Chrysin	Flavone	1.43 ± 0.07
Hesperitin	Flavanone	1.37 ± 0.08
Kaempferol	Flavonol	1.34 ± 0.08
Pelargonidin	Anthocyanidin	1.30 ± 0.10
Chlorogenic acid	Hydroxycinnamate	1.30 ± 0.02
Caffeic acid	Hydroxycinnamate	1.30 ± 0.01
Hesperidin	Flavanone	1.08 ± 0.04
Vitamin C	Vitamin	1.00 ± 0.02
Vitamin E	Vitamin	1.00 ± 0.03
Narirutin	Flavanone	0.76 ± 0.05

[a] Measured as the TEAC (Trolox equivalent antioxidant activity) – the concentration of Trolox with the equivalent antioxidant activity of a 1 mM concentration of the experimental substance.

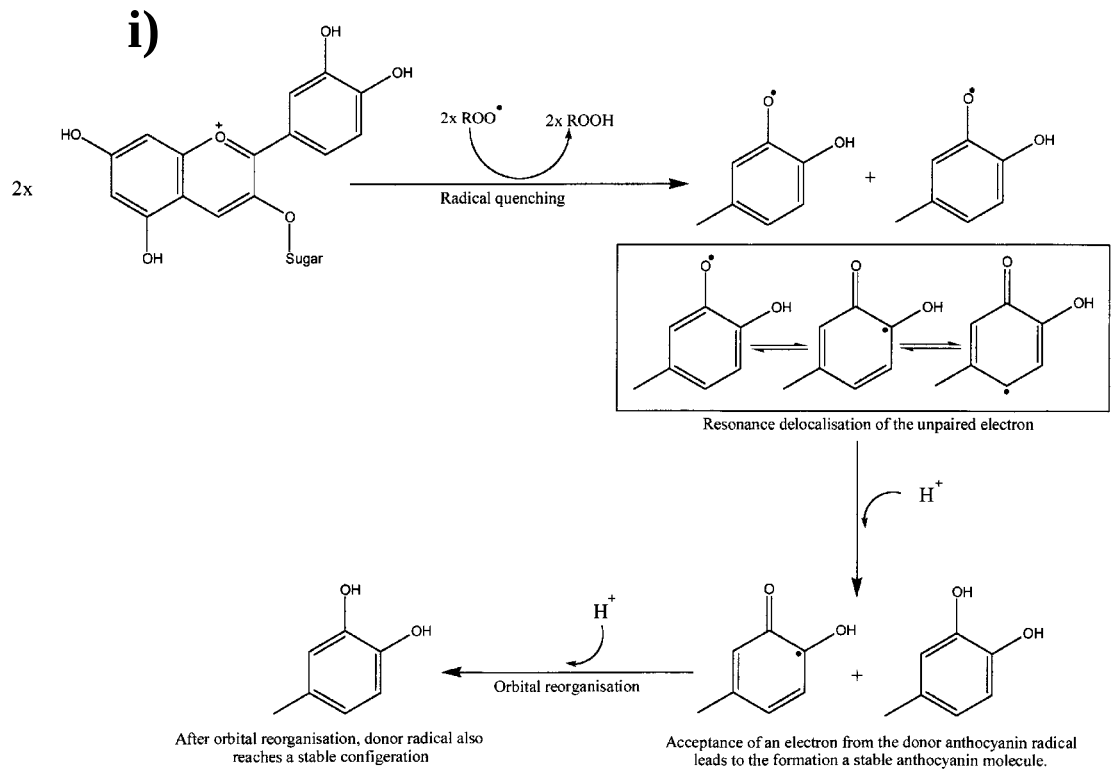
The potent antioxidant activities of anthocyanins are related to their unique structures. The O^+ (oxonium ion) in the C-ring, and their capacities to conjugate facilitate stable radical products after interrupting chain reactions (Bors *et al.*, 1990; Van Acker *et al.*, 1996; Larson, 1997).

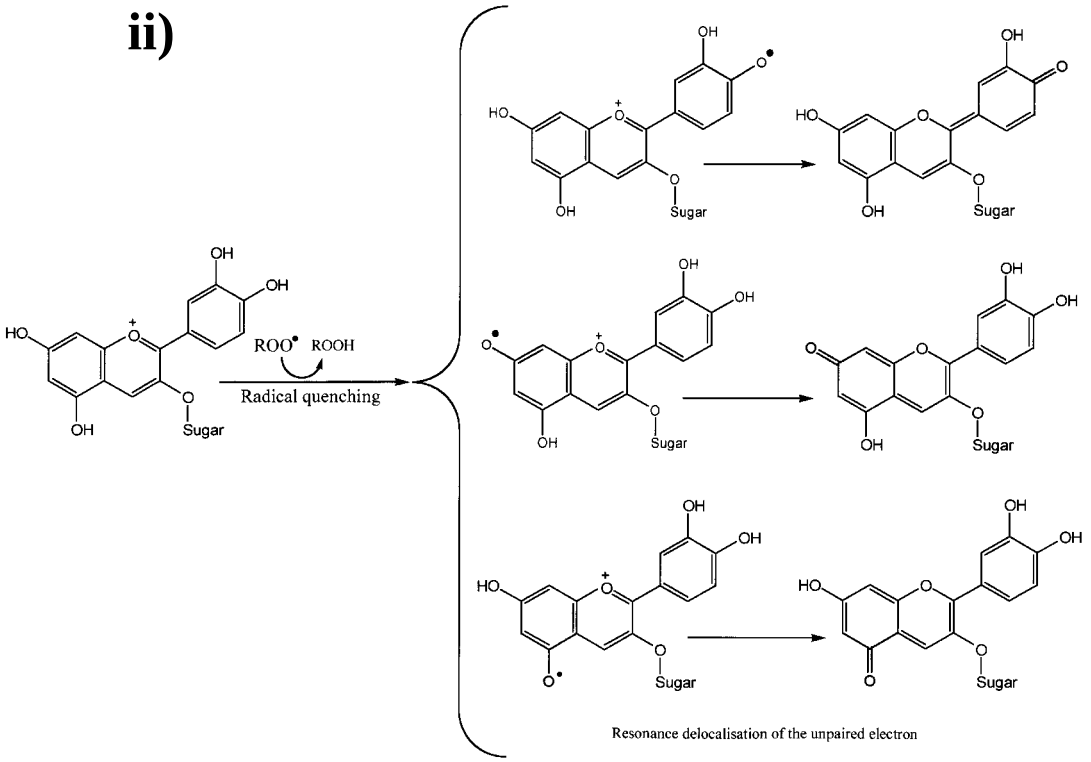
There are a number of ways anthocyanins can stabilise their own radical state. These include self stabilisation through interactions with other anthocyanin radicals and numerous electron delocalisation possibilities (Fig. 5.5 i-v), by further oxidation with another radical species, or by their reduction with compounds such as ascorbate (Bors *et al.*, 1990). Anthocyanins are also thought to act as important biological chelators able to indirectly reduce the oxidation of other biological antioxidants such as ascorbate (Sarma *et al.*, 1997).

By forming complexes with transition metals, anthocyanins have been demonstrated to prevent the conversion of H_2O_2 and $O_2^{\cdot -}$ to destructive $OH^{\cdot}$ radicals through Haber-Weiss-Fenton reactions (Van Acker *et al.*, 1996). The possession of an ortho 3',4'- dihydroxy substitution in the B-ring is thought to be important in chelation (Van Acker *et al.*, 1996; Brown *et al.*, 1998) and allows anthocyanins to covalently bind to trivalent cations such as Al^{3+}, Fe^{+3}, Ga^{3+} (Noda *et al.*, 1998; George *et al.*, 1999), and divalent cations Fe^{2+}, and Cu^{2+} (Van Acker *et al.*, 1996; Brown *et al.*, 1998).

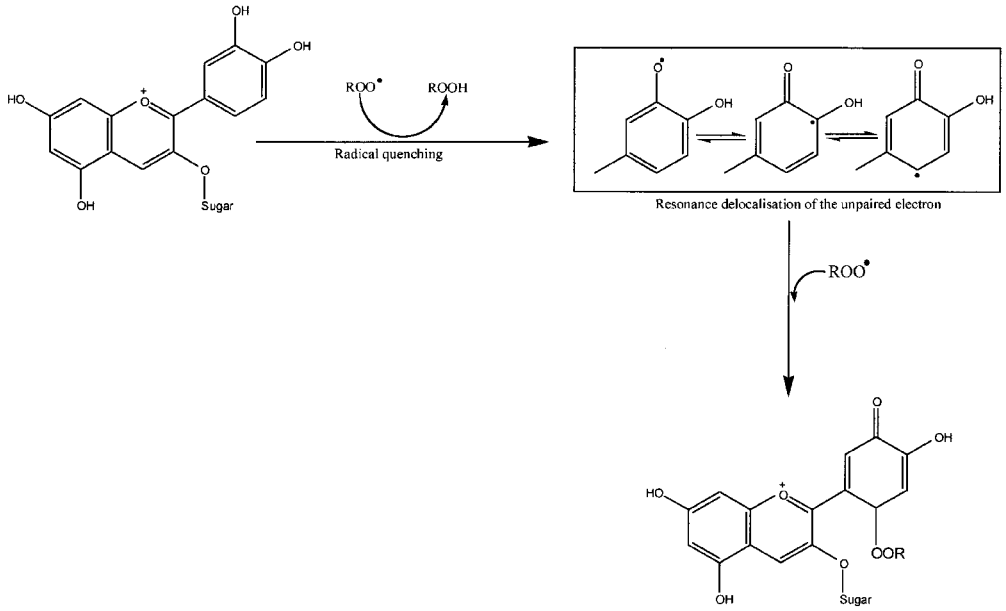
Most investigations of anthocyanins as antioxidants have used plant extracts. For example, pelargonidin, delphinidin, and cyanidin isolated from the seed coat of *Phaseolus vulgaris* was found to scavenge $O_2^{\cdot -}$ and reduce the formation of $OH^{\cdot}$ radicals (Tsuda *et al.*, 1996 b). Cyanidin extracted from Hibiscus petals was oxidised by $O_2^{\cdot -}$ generated from an EDTA-riboflavin system, indicating that anthocyanins can scavenge $O_2^{\cdot -}$ directly (Yamasaki *et al.*, 1996). Furthermore, H_2O_2 in the presence of horseradish peroxidase could induce anthocyanin oxidation, in a similar manner to other LMWAs such as ascorbate and glutathione (Yamasaki, 1997). It is unclear whether this bleached form of anthocyanin has a diminished antioxidant potential, or indeed if the potential can be restored.

However, the potential of anthocyanins to function as antioxidants in situ remains untested. There is scant information on their ability to act as antioxidants in leaves.

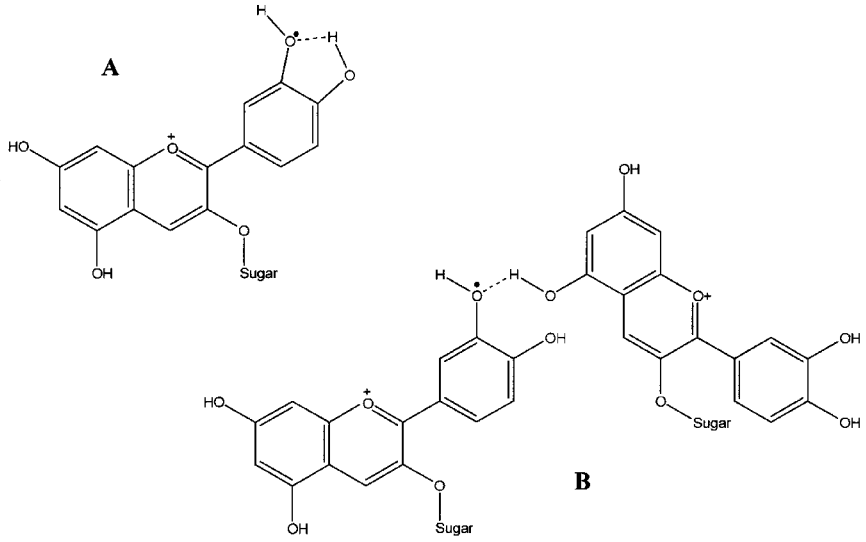

i)
2x
2x ROO•
2x ROOH
Radical quenching
Resonance delocalisation of the unpaired electron
H+
Orbital reorganisation
After orbital reorganisation, donor radical also reaches a stable configeration
Acceptance of an electron from the donor anthocyanin radical leads to the formation a stable anthocyanin molecule.


ii)
ROO• ROOH
Radical quenching
Resonance delocalisation of the unpaired electron

iii)


OH
OH
HO
O
O
Sugar
OH
ROO•
ROOH
Radical quenching
O•
OH
O
OH
O
OH
Resonance delocalisation of the unpaired electron
ROO•
O
OH
HO
O
OOR
O
Sugar
OH

iv)


A
H
O
H
O
HO
O
O
Sugar
OH
OH
H
O
H
O
O+
OH
OH
HO
O
O
Sugar
OH
B
OH
O
Sugar
OH

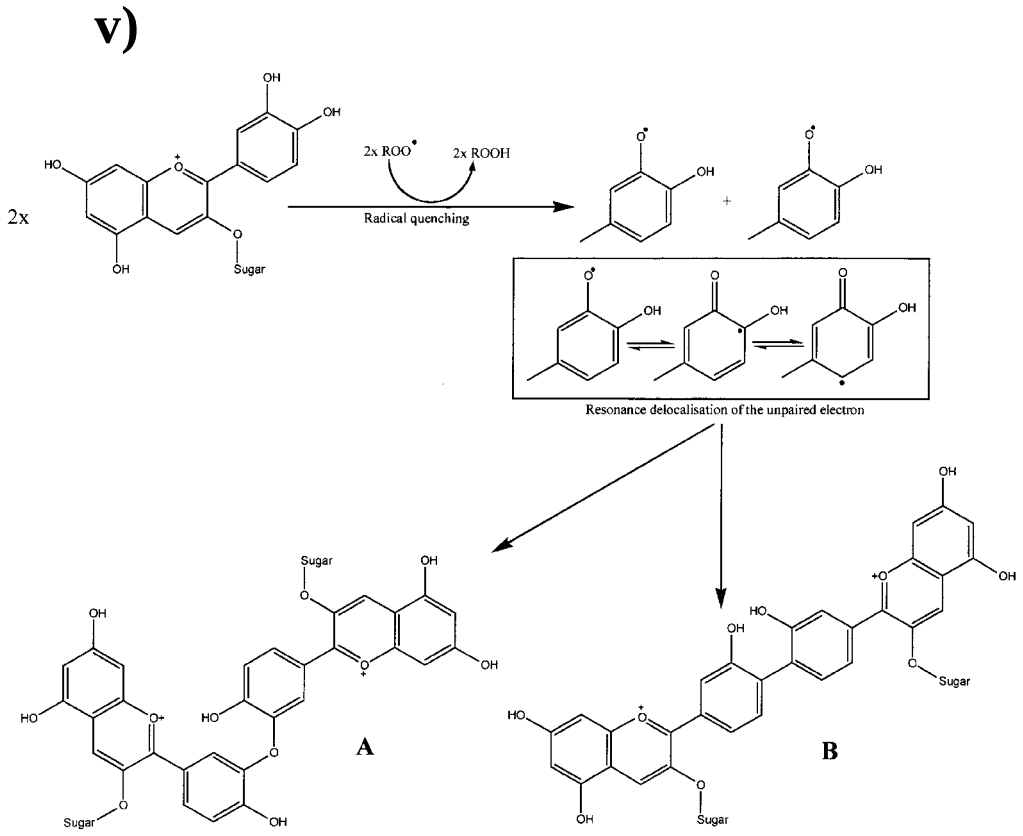


Fig. 5.5 Potential stabilisation mechanisms of anthocyanin radicals after a scavenging event within a plant cell:

i) One electron disproportionation reactions of anthocyanin radicals, which undego self-redox conversion to even electron products.

ii) Structural electron delocalisation of anthocyanin radicals resulting in stable cation formation.

iii) Radical addition to an existing anthocyanin radical. In this example, a peroxyl radical is added to the existing anthocyanin radical to from an even-electron product. This form of scavenging represents a particularly efficient process for deactivating a radical chain reaction.

iv) Anthocyanin radical stabilisation by hydrogen bonding from either (A) an adjacent hydroxyl bond, or (B) a hydroxyl bond from another anthocyanin molecule.

v) Anthocyanin radicals that have undergone dimerisation (intramolecular co-pigmentation) to produce (A) C-O or (B) C-C bonded hydroxydianthocyanin ethers or hydroxylated bianthocyanins. (Modified from: Larson, 1997).

Measurements of 'total antioxidant capacity'

Rather than measuring the individual components of the antioxidant defence system, it is sometimes particularly useful to measure the total antioxidant activity of an extract. Techniques using specific compounds, such as Trolox, ABTS, and TRAP, allow an overall estimate of a substances scavenging capacity.

In order to quantify the total antioxidant activity in red and acyanic leaves (Chapter 6) we have made use of two such total antioxidant assays. The first assay utilises the unique colour change of the α,α-diphenyl-β-picrylhydrazyl (DPPH) radical upon stabilisation, and allows the comparison of a pooled sample of antioxidants to scavenge the stable radical species. However, the DPPH assay is limited to the detection of certain antioxidants such as ascorbate, Vitamin E, and the flavonoids. Thus enzymatic antioxidants such as SOD, CAT, and glutathione will escape detection (Løvaas and Olsen, 1998) as will antioxidants which work by chelating pro-oxidant metal ions. The second total antioxidant technique called cyclic voltammetry (CV) provides information about a extracts ability to donate electrons and the gives a measure of total antioxidant concentration. Such measurements are limited by the fact that antioxidants have to be electrochemically active at an electrode surface. Unfortunately, many sulphur-containing antioxidants, such as glutathione, are slow to react at specific electrodes (Kilmartin, 2001).

Both assays can give misleading information about the concentration of specific antioxidant compounds. For example, rises in one antioxidant can obscure the depletion of another. Furthermore, total antioxidant activity is a simplification of what actually happens *in vivo*. Plant cells possess antioxidants in specific compartments, separated by boundaries that are not equally permeable to all molecules (Saran *et al*., 1998).

CHAPTER 6 - Antioxidant activities of red versus green leaves

Abstract

Anthocyanin biosynthesis in leaves increases under stresses which also generate reactive oxygen species (ROS). We have tested the hypothesis that red leaves are better equipped to scavenge ROS than green leaves. Antioxidants in leaf extracts from red and green morphs of *Quintinia serrata* and *Elatostema rugosum* were identified, and activities quantified using enzymatic and DPPH assays and cyclic voltammetry.

Red leaves from *E. rugosum* held greater amounts of superoxide dismutase, catalase, anthocyanins, and hydroxycinnamic acids, were significantly more effective at scavenging DPPH radicals, and produced higher voltammetric currents than green leaves. Anthocyanins contributed to the antioxidant pool more than all other constituent phenolics. Anthocyanin concentrations, and antioxidant activities declined with leaf age.

By contrast, leaves from red and green morphs of *Q. serrata* exhibited comparable ranges in antioxidant activities. Anthocyanin levels and associated antioxidant activities in red morphs increased as leaves aged. In green morphs, caffeic acid derivatives apparently substituted for anthocyanin antioxidants. Levels of enzymatic antioxidants were similar for both morphs.

The pooled phenolic content rather than composition is important in the protection from ROS. Purified anthocyanin fractions from both species displayed oxidative activities at both pH 7.0 and pH 5.5. Implications of the antioxidant potential of anthocyanin in its cytoplasmic and vacuolar locations are discussed.

Key words: *Quintinia serrata*; *Elatostema rugosum*; anthocyanin; antioxidant; reactive oxygen species; superoxide dismutase; catalase; ascorbate peroxidase; cyclic voltammetry.

Introduction

For many land plants, the production of anthocyanins in leaves can be induced or up-regulated through their exposure to stressful conditions. These include environmental stresses, such as high irradiances (Grace *et al.*, 1998 a), extremes of temperature (Christie *et al.*, 1994; Pietrini and Massacci, 1998), UV radiation (Mendez *et al.*, 1999), mineral imbalances (Bongue-Bartelsman and Phillips, 1995; Trull *et al.*, 1997), water stress (Balakumar *et al.*, 1993), and mechanical injury (Ferreres *et al.*, 1997), as well as biotic stresses such as herbivory and pathogen attack (Dixon *et al.*, 1994). In addition, anthocyanins are often most prominent during potentially stressful periods of a leaf's ontogeny, such as in the young, rapidly expanding leaves (Lee *et al.*, 1987; Woodall and Stewart, 1998), and during senescence (Hoch *et al.*, 2001).

These same stresses are known to cause the formation of free radicals and reactive oxygen species (ROS), which, if left unchecked, can result in cellular damage (Polle, 1997). It is at least possible that anthocyanins confer a protective role in leaves by scavenging those ROS. Purified solutions of anthocyanins have demonstrable antioxidant properties *in vitro*. For example, cyanidin, peonidin, malvidin, pelargonidin, and delphinidin-based anthocyanins extracted from fruits and seed coats reduce lipid peroxidation in model systems in a concentration-dependent manner (Tsuda *et al.*, 1996 a; Gabrielska *et al.*, 1999). Similarly, the oxygen radical absorbance capacity of different blueberry varieties was linearly correlated to anthocyanin concentration (Prior *et al.*, 1998). Anthocyanins, along with other flavonoids have been shown to scavenge H_2O_2 (Yamasaki, 1997), O_2^- (Yamasaki *et al.*, 1996), $ONOO^-$ (Tsuda *et al.*, 2000), and may also scavenge $\cdot OH$ and $^1O_2$ (Bors *et al.*, 1990; Chauhan *et al.*, 1992; Bors *et al.*, 1994).

The ability of purified anthocyanins to scavenge ROS *in vitro* does not necessarily translate to an enhanced antioxidant status of red versus green leaves *in vivo*. This is because leaves normally hold a suite of enzymatic antioxidants and low molecular weight antioxidants (LMWAs), any combination of which can serve to protect lipids, protein, and DNA from the effects of ROS (Foyer *et al.*, 1994; Alscher *et al.*, 1997). Stresses that stimulate anthocyanin production have been shown also to enhance levels of enzymic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) in leaves

(Grace and Logan, 1996; Logan *et al.*, 1998 a; Logan *et al.*, 1998 b; Sherwin and Farrant, 1998). LMWAs such as the anthocyanins and other flavonoids are often neglected major contributors to the total antioxidant capacity of biological tissues (Halliwell and Gutteridge, 1998). The relative contribution of anthocyanins to the antioxidant pool in leaves is not known.

We have exploited the natural polymorphism in leaf colour within populations of two native New Zealand species to test the hypothesis that anthocyanin-accumulating leaves are better protected against ROS than are green leaves. *E. rugosum* (Urticaceae) is a sprawling, understorey plant found in damp, sheltered gullies (Allen, 1961). Leaves from the 'red' morphs of this species are anthocyanic during expansion; the degree of red pigmentation declines as the leaves age (Fig. 6.1 A). *Quintinia serrata* (Escalloniaceae) is a hemi-epiphytic tree, found on sun-exposed ridges, which shows increasing red pigmentation with leaf age (Gould *et al.*, 2000). Both species also display 'green' morphs, which show no appreciable anthocyanic pigmentation through all stages in leaf development. The red and green morphs are anatomically comparable, and occur at similar locations.

Because any estimate of 'total antioxidant capacity' is inherently restricted to the limitations of the technique employed, we have taken two different approaches to compare antioxidant activities in leaves from the red and green morphs. First, standard enzymatic assays and the DPPH assay were used to identify and measure levels of SOD, CAT, APX, and methanol-extractable LMWAs. Second, leaf extracts were subjected to cyclic voltammetry, in which oxidation of the phenolic antioxidants produces a current at an electrode proportional to the amount present. Antioxidants which are the more powerful reducing agents are seen at the lower oxidation potentials (Chevion *et al.*, 1999; Kilmartin *et al.*, 2001). These two approaches provided complementary information on the antioxidant status of the red and green leaves. Our data indicate that anthocyanins can indeed confer a significant antioxidant advantage over green leaves in *E. rugosum*, whereas in *Q. serrata* they may act in concert with other phenolics to supplement protection from oxidative stress.

Materials and methods

Plant collection

Fully expanded leaf laminae were collected from four shoots on each of five red and five green *Quintinia serrata* A. Cunn (Escalloniaceae) and *Elatostema rugosum* A. Cunn (Urticaceae) plants growing in the Waitakere Ranges, New Zealand (latitude 36^{o} 59' S, longitude 174^{o} 31' E). Extreme examples of red and green morphs were preferentially selected. Analyses were undertaken on the first four most apical leaves of each shoot. All leaves were washed prior to analysis. Leaves were numbered basipetally such that leaf 1 corresponded to the youngest fully-expanded leaf.

Quantification and localisation of phenolic compounds

Concentrations of flavonoids and hydroxycinnamic acids were determined for 5 g of leaves from each age class. Each was a pooled sample from five plants. Cuticular waxes were removed in diethyl ether. The leaves were freeze-dried, ground, extracted with HOAc:H_20:MeOH (7:23:70) for 2 h, and clarified by centrifugation. The methanolic extracts were diluted with 3M HCl in MeOH for the determination of anthocyanin levels using absorption spectroscopy with optical density readings at the anthocyanin λ_{max} of 532 nm. Levels were calculated as cyanidin-3-glucoside equivalents (molar extinction coefficient 33,000). Anthocyanin aglycones were identified from the extracts as described by Markham *et al*. (1998). The colourless flavonoids and the hydroxycinnamic acids were quantified from HPLC profiles on 10 μL aliquots of the clarified extracts, using the solvents and methodology of Markham *et al*. (1998). Flavonol concentrations were estimated as rutin equivalents by integration of all the flavonol peaks in the HPLC and comparison of this integral with those of standard rutin solutions. Flavone levels were calculated as apigenin-7-O-neohesperidoside equivalents. Peaks representing caffeic and p-coumaric acid derivatives were identified from their absorption spectra and were quantified as caffeic and p-coumaric equivalents by comparison with standard solutions. All phenolic levels were expressed as μMol per g dry leaf material

Flavonoid locations were determined from transverse sections of fresh leaves from red and green morphs under bright field and epifluorescence in a Zeiss Axioplan 2 microscope, using Zeiss filtersets 02 (excitation: 365 nm; beamsplitter: 395 nm; emission: 420 nm) and 09

(excitation: 450 nm; beamsplitter: 510 nm; emission: 520 nm). Sections were stained with 0.2 % Naturstoffreanz A (diphenylboric acid 2-aminoethylester, Sigma) in water for 5 mins, washed, and re-examined. Stain-specific production of green-yellow fluorescence was attributed to the presence of flavonols and flavones (Schnitzler *et al.*, 1996).

DPPH scavenging activity

Individual leaves were freeze-dried, and 25 mg extracted in HOAc:H_20:MeOH (7:23:70) for 24 h at 4ºC in the dark. Anthocyanin levels were determined as $A_{530} - 0.24\ A_{653}$ (Murray and Hackett, 1991). Antioxidant activities of the extracts were assessed on the basis of their abilities to scavenge the stable α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical (Blois, 1958). DPPH has an intense violet colour with a maximum absorbance at 517 nm, but turns colourless as unpaired electrons are sequestered by antioxidants. Reaction mixtures containing 0 to 100 μL leaf extract and 1.5 mL of 18 μM DPPH in MeOH were diluted with MeOH to a final volume of 1.6 mL, vortexed, and then held at room temperature for 30 mins. Absorbances of the reaction mixtures were measured at 517 nm and compared with those from a 2.3 mM ascorbic acid standard in MeOH. Scavenging activities of DPPH were expressed as IC_{50} values, which denote the concentration of leaf extracts (μg dry wt. mL^{-1}) required to give a 50 % reduction in A_{517} relative to that of the control (the reaction mixture minus leaf extract). The lower IC_{50} values denote the higher antioxidant activities.

Relative contributions of various phenolics to the total LMWA pool were estimated for both red and green morphs. Approximately 5 g of leaves were ground in liquid nitrogen, resuspended in 40 mL of HOAc:H_2O:MeOH (7:23:70), agitated for 12 hrs at 4°C in the dark, then centrifuged at 5,000 g for 15 mins. Extracts were filtered, concentrated under vacuum, and phenolic components separated by 2D paper chromatography using t-BuOH:HOAc:H_2O (3:1:1) for 24 hrs, then HOAc:H_20 (15:85) for 12 hrs. Anthocyanins, the colourless flavonoids (flavonols plus flavones), and the hydroxycinnamic acids were eluted separately in HOAc:H_2O:MeOH (0.1:15:85). Locations of these phenolic types were determined by applying NH_3 vapour to similar chromatograms and comparing chromatographic and fluorescence characteristics with those previously described (Markham, 1982; Markham and Bloor, 1998). Samples were reduced to 5 mL, up to 400 μL of which was used for assay with

9 μM DPPH in MeOH. Relative contributions of the phenolic types to the total antioxidant potential were calculated as reciprocals of IC_{50} values expressed as percentages of the totals.

Extraction and activities of antioxidant enzymes

For all enzyme assays, 0.4 g of leaf tissue was ground in liquid nitrogen and extracted in their own specific buffer on ice. Extracts were vortexed for 30 s, centrifuged, and the supernatants passed through PD-10 columns equilibrated with 100 mM of buffer containing 0.1% (v/v) Triton X-100, all at 4^{o}C. Superoxide dismutase (SOD) was extracted in 10 ml of a 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM diethylenetriaminepentaacetic acid (DETAPAC), 1 % (w/v) insoluble polyvinylpolypyrrolidone (PVP), and 1 % (v/v) Triton X-100, and was centrifuged at 17, 000 g for 10 mins at 4 ^{o}C. Catalase (CAT) was extracted in 8 ml of 50 mM Tris- HCl buffer (pH 7.5), containing 4 % (w/v) PVP, 0.1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.3% (v/v) Triton X-100, and was centrifuged at 20, 000 g for 15 mins at 4 ^{o}C. Ascorbate peroxidase (APX) was extracted in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) with 1 mM EDTA, 5 mM ascorbic acid, 0.5% (v/v) Triton X-100, and 1 % (w/v) PVP, and centrifuged at 100,000 g for 20 mins at 4^{o}C. The total soluble protein content of 10 μl of the extracts was determined by using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce Ltd, Rockford, USA). Assay mixtures were incubated at 37^{o}C for 30 mins and measured at 562nm as described by (Lowry *et al.*, 1951).

SOD (EC 1.15.1.1) activity was determined spectrophotometrically using the method of Beauchamp and Fridovich (1971) as modified by Oberley and Spitz (1985). Briefly, xanthine and xanthine oxidase were used to generate a reproducible flux of $O_2^{\cdot -}$ which then reduced nitroblue tetrazolium (NBT) to the formazan, measured as an increased absorbance at 560 nm. Exactly 0.5 ml of extract was added to 0.9 ml of assay mixture containing 50 mM potassium phosphate buffer (pH 7.8), 1.8 mM xanthine, 2.24 mM NBT, 1.33 mM DETAPAC, 40 U/ml catalase and 10^{-2} xanthine oxidase at 25^{o}C. SOD activity was calculated as the amount of extractable protein required to inhibit NBT reduction by 50 % in the $O_2^{\cdot -}$ competing reaction, and was expressed as units of SOD per mg of total leaf protein.

CAT (EC 1.11.1.6) activity was monitored polarographically using a DW1 liquid phase oxygen electrode (Hansatech, UK), by a method similar to that described by Del Rio *et al*. (1977). Nitrogen was bubbled through 2.95 ml of potassium phosphate buffer (pH 7.0) to

displace oxygen. The solution was equilibrated with 100 µl of 33.5 mM H_2O_2, and then 50 µl of the leaf extract was added. Rates of O_2 evolution were measured over three minutes at 25^{o}C. Rates of oxygen production from the extracts were corrected for the non-enzymatic photodecomposition of H_2O_2 during the assay. Activities were expressed as units of CAT per mg of total leaf protein.

APX (EC1.11.1.11) activity was determined using the method of Nakano and Asada and Takahashi (1987) which relies on a decrease in absorbance at 290 nm as ascorbate is oxidized. To prevent inactivation of the enzyme during the extraction and purification procedure 5 mM ascorbate was added to the extraction buffer. The reaction was started by adding 200 µl of 0.5 mM hydrogen peroxide to a 1 ml reaction mixture consisting of 50 mM potassium phosphate buffer (pH 7.0), with 1 mM ascorbate and the purified extract at 25^{o}C. Changes in absorbance were recorded from 10 to 30 s after this addition. This rate was corrected for the non-enzymatic oxidation of ascorbate by hydrogen peroxide in the absence of the enzyme sample. APX activity was expressed as µM of ascorbate oxidized per minute per mg of total leaf protein.

Cyclic voltammetry analyses

Cyclic voltammetry was used to characterise the total antioxidant capacity of extractable LMWAs at pH 5.5 and pH 7.0. Exactly 5g of fresh leaf material from *Q. serrata* and *E. rugosum* was homogenised in 60 ml of HOAc:H_2O:MeOH (0.5:20:80). The homogenate was filtered through nylon mesh, and centrifuged at 5,000 g for 15 mins at 4^{o}C. The supernatant was collected, diluted ten fold in 50 mM potassium phosphate buffer at either pH 5.5 or 7.0. Cyclic voltammetry measurements were made using a 100A electrochemical analyser (Bioanalytical systems, West Lafayette, USA), with a BAS C2 cell stand (Kilmartin, 2001). A glassy carbon disk electrode (BAS M-2012) used for the measurements was cleaned with 3 µm alumina powder (PK-4) between runs. All measurements were recorded at a scan rate of 100 mVs^{-1} with a potential range of –100 mV to approximately 100 mV past the first anodic peak, and compared with a silver/ silver chloride reference electrode and a platinum counter electrode. Further scans were taken to 1200 mV to record subsequent redox processes at more positive potentials. Measurements were obtained by subtracting the current from blank scans made with HOAc:H_2O:MeOH (0.5:20:80) at either pH 5.5 or 7.0. A linear correlation

between current and leaf extract concentration was obtained only for currents smaller than 2 μA within the range of a 10 fold dilution of the leaf extracts in potassium phosphate buffer.

To determine the contributions of anthocyanins to the voltammetric characteristics of whole leaf extracts, anthocyanins were purified and also measured using cyclic voltammetry. Pooled samples of red leaves were homogenised in HOAc:H_2O:MeOH (0.5.20:80), filtered through nylon and centrifuged at 5,000 g for 15 mins. The supernatants were concentrated under vacuum and freeze-dried. They were re-dissolved in a minimal volume of HOAc:$H_2$0:MeOH (0.5:20:80) and run through a column of microcrystalline cellulose (Merck, Damstadt, Germany) equilibrated in the same solvent. The anthocyanic eluents were collected and again concentrated under vacuum, freeze-dried, then re-dissolved in t-BuOH:$H_2$0:MeOH (3:1:1) (TBA) and passed through a second column equilibrated with TBA. Purity of the extracts was confirmed by measuring absorbance spectra at 200 to 700nm. The purified solutions were then dried and re-dissolved in HOAc:H_2O:MeOH (0.5:20:80) adjusted to pH 5.5 or 7.0 using potassium phosphate buffers, and subjected to cyclic voltammetry. The behaviour of antioxidant standards ascorbic acid, caffeic acid, catechin, rutin and p-coumaric acid were determined as 0.05 mM solutions in the pH 5.5 buffer with 8% MeOH.

Results

Flavonoid profiles

Anthocyanin levels in leaves of *Q. serrata* and *E. rugosum* leaves mirrored their visual appearance (Fig. 6.1 A, B); leaves from the red morphs of both species held significantly more anthocyanins (ANOVA; $P<0.001$) than those from the green (i.e. acyanic) morphs at each developmental stage (Fig. 6.2 A, B). For the red morphs of *Q. serrata*, a 5.4 fold increase in anthocyanin level was observed with increasing leaf age (Fig. 6.2 A); green morphs showed a less marked (1.6 fold) increase in anthocyanin content. By contrast, for *E. rugosum*, anthocyanin levels were maximal in the youngest, fully expanded leaves, and declined significantly as leaves aged (Fig. 6.2 B). The aglycone complement of the anthocyanins extracted from red *E. rugosum* was malvidin (44%), peonidin (23%), cyanidin (17%), petunidin (11%), and delphinidin (5%). As determined previously, red leaves of *Q.*

serrata hold approximately equal amounts of cyanidin-3-glucoside, and cyanidin-3-galactoside (Gould *et al.*, 2000).

Caffeic acid derivatives were the major phenolic component in *Q. serrata* leaf laminae, and the flavonols were the principal flavonoid group (Fig. 6.3 A). Flavonols were present in the red morphs at approximately twice the concentration as in the green morphs. Total flavonoid levels in the red morph increased 1.2 fold over the four-plastochron increases in leaf age, as did the levels of caffeic acid derivatives (1.3 fold) (Fig. 6.3 A). For the leaves of *E. rugosum*, the flavones were the major flavonoid component; the green morphs had greater levels of both flavonols and flavones than the red morphs (Fig. 6.3 B). Total flavonoid levels in the red morph of this species decreased with leaf age (1.3 fold) (Fig. 6.3 B). However, red morphs held higher levels of hydroxycinnamates than the green morphs (3.7 fold); these were mainly caffeic derivatives, but also trace amounts of p-coumaric derivatives were present. Total flavonoid and total hydroxycinnamic acid levels in the red morphs of *E. rugosum* generally decreased with leaf age (1.3 fold and 1.1 fold respectively, over four plastochrons), although a small increase was evident between leaf 3 and 4. Levels remained relatively static in the green morphs (Fig. 6.3 B).

The red morphs of *Q. serrata* accumulated anthocyanins predominantly within the palisade and spongy mesophyll cells (Fig. 6.4 A). Flavones and/or flavonols were present in the cuticles and epidermal cells, as detected by strong yellow-green fluorescence after staining with Naturstoffreagenz A (Fig. 6.4 B). There were no differences in the location of fluorescence between red and green morphs, although fluorescence was stronger in the red morphs. Other phenolic compounds were evident in the mesophyll parenchyma of *Q. serrata*, apparently bound to the cell walls as distinguished by light blue UV-autofluorescence (Fig. 6.4C). For *E. rugosum*, anthocyanins were also located in the palisade and spongy mesophyll layers (Fig. 6.4 D). The flavones and flavonols were confined to the fine upper cuticle and trichomes (Fig. 6.4 E). Fluorescence was marginally stronger in the green morph. UV-autofluorescence (Fig. 6.4 F) also indicated the presence of other phenolic compounds associated with the upper and lower epidermal cell walls, and the leaf hairs.

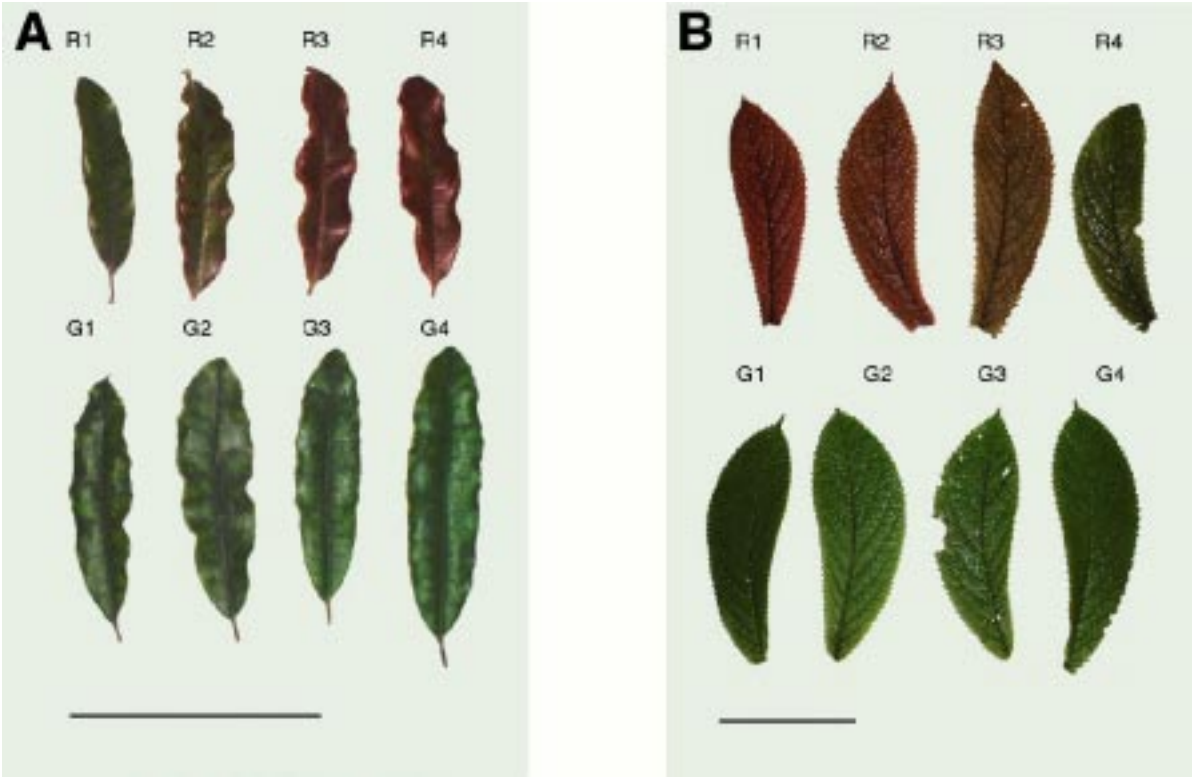


Fig. 6.1 Photographs of *Q. serrata* (A) and *E. rugosum* (B) leaves, showing the four youngest fully-expanded leaves from red (R) and (G) morphs. Bars = 10 cm.

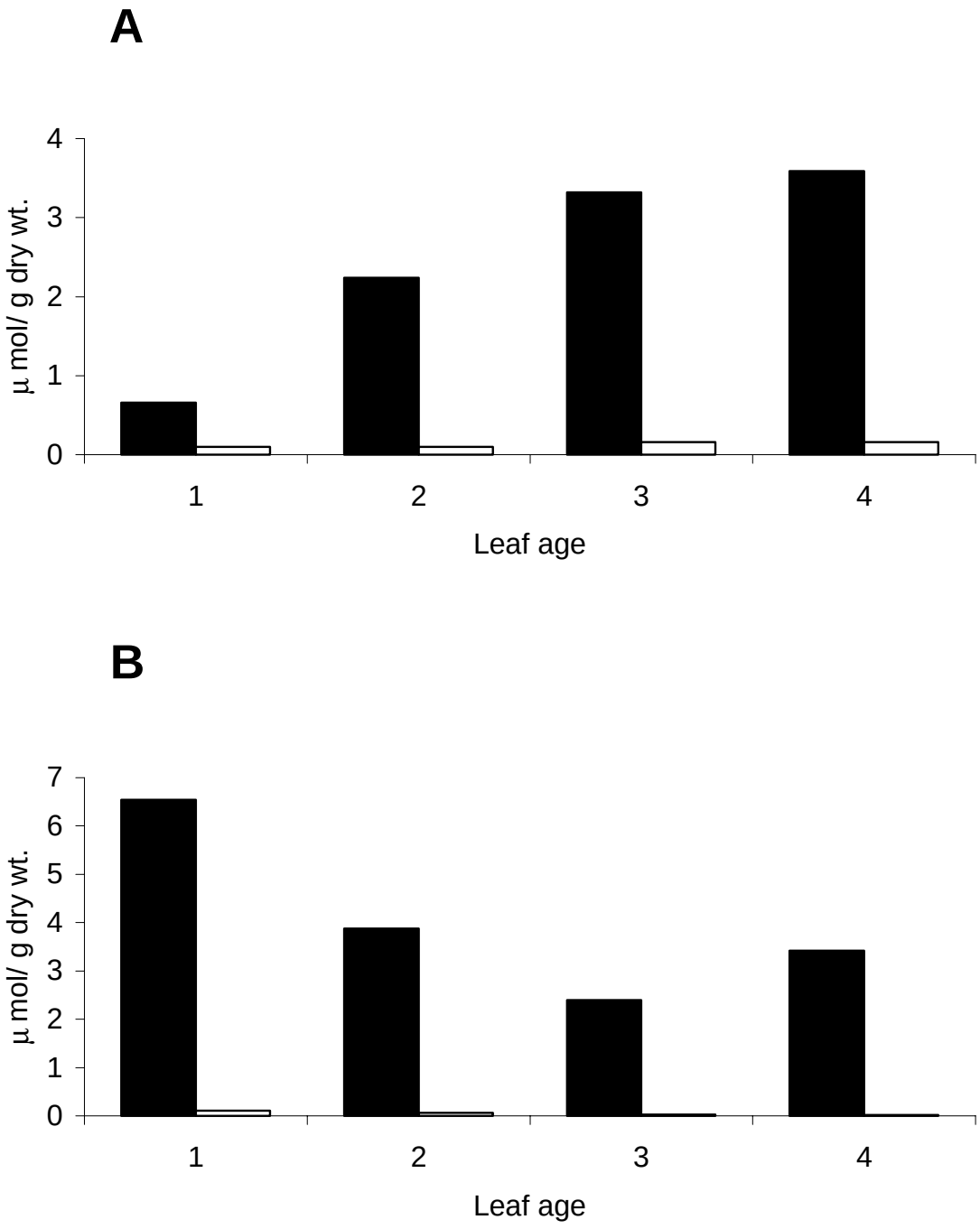


Fig. 6.2. Anthocyanin concentrations in pooled samples of successively older leaves from red (black bars) and green (white bars) morphs of *Q. serrata* (A), and *E. rugosum* (B).

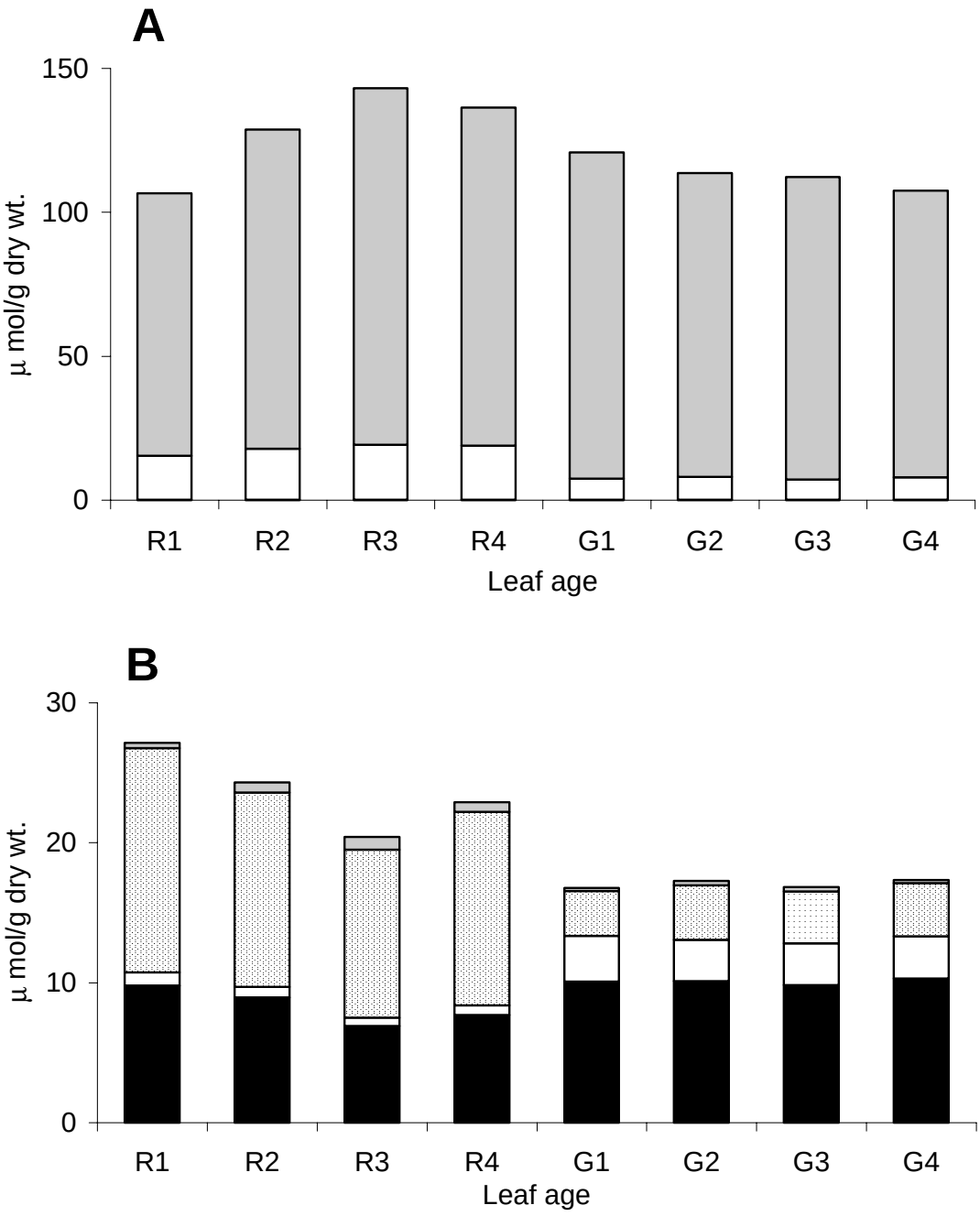


Fig. 6.3 Flavonoid and hydroxycinnamic acid concentrations in pooled samples of successively older leaves from red (R) and green (G) morphs of *Q. serrata* (A) and *E. rugosum* (B). Concentrations of flavones (black bars), flavonols (white bars), caffeic acid derivatives (grey bars), and p-coumaric derivatives (dotted bars) are shown.

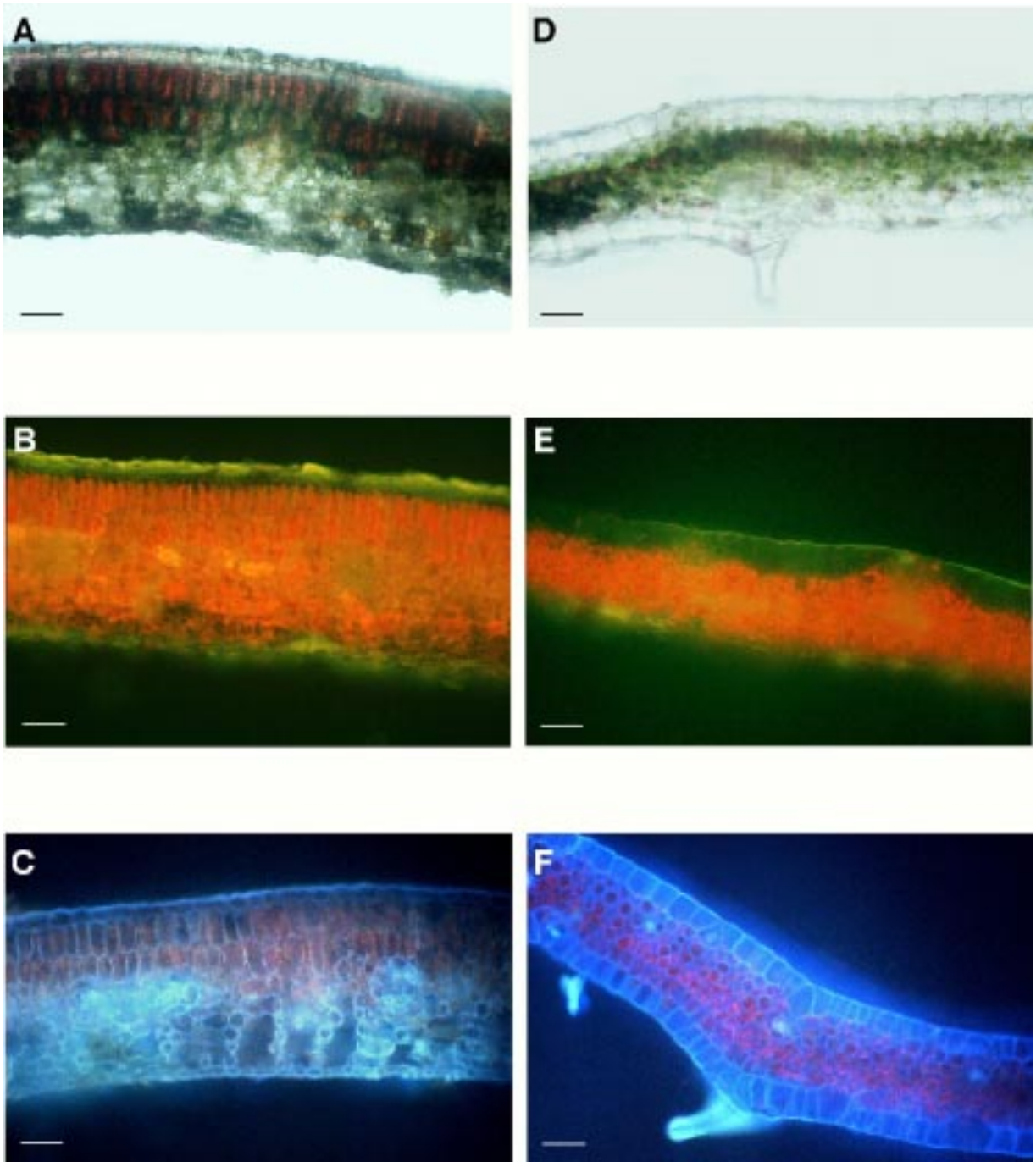


Fig. 6.4 Micrographs of transverse sections through fresh leaves of *Q.serrata* (A – C) and *E. rugosum* (D - F). A, D: Bright field micrographs showing anthocyanins within palisade mesophyll; B, E: Fluorescence micrographs of sections stained in Naturstoffreagenz A under Zeiss filterset 09; C, F: fluorescence micrographs under Zeiss filterset 02. Bars = 50µm.

Antioxidant assays

All leaves of *Q. serrata* and *E. rugosum* had measurable antioxidant activities as determined by the capacities of methanolic extracts to scavenge the DPPH radical (Fig. 6.5 A, B). For the leaves from the red morphs of both species, antioxidant capacity increased linearly with anthocyanin concentration. IC_{50} values were strongly negatively correlated to anthocyanin content ($r = -0.91$; $P<0.001$ for both species). Green leaves from both species showed no correlation between IC_{50} value and anthocyanin content ($r = -0.22$; $P>0.05$ for *E. rugosum* and $r = 0.13$; $P>0.05$ for *Q. serrata*).

The two species varied with respect to the antioxidant characteristics of leaves from green morphs. In *E. rugosum*, leaves from the green morphs always-held significantly lower antioxidant capacities (ANOVA; $P<0.001$) than did the leaves from the red morphs (ca 5 fold difference; Fig. 6.5 B). Variation among leaves from the green morphs was unrelated to leaf age or anthocyanin concentration. However, for *Q. serrata*, antioxidant capacities of green leaves encompassed the full range of values as those for anthocyanic leaves (Fig. 6.5 A). Variation in IC_{50} values was attributable to both leaf age and the individual tree from which green leaves were collected (ANCOVA; $P<0.001$ in both cases). Clearly, the green morphs of *Q. serrata* also contained colourless, methanol-soluble antioxidants, which were as effective as the anthocyanins.

For both red and green morphs of *Q. serrata* there was a significant increase in antioxidant activity (lower IC_{50} values) with increasing leaf age (Fig. 6.6 A). For *E. rugosum*, antioxidant activity decreased slightly with leaf age, although the differences were not statistically significant (ANOVA; $P>0.05$) (Fig. 6.6 B). Leaves from the green morphs of *E. rugosum* maintained a consistently low activity throughout leaf development (Fig. 6.6 B).

The relative contributions of extractable phenolic compounds as reductants of the DPPH radical differed for the two species (Fig. 6.7). For *Q. serrata*, the principal LMWAs were the hydroxycinnamates, followed by the anthocyanins. The relative contribution of hydroxycinnamic acids was 27% greater in the green morphs than in the red morphs (Fig 6.7).

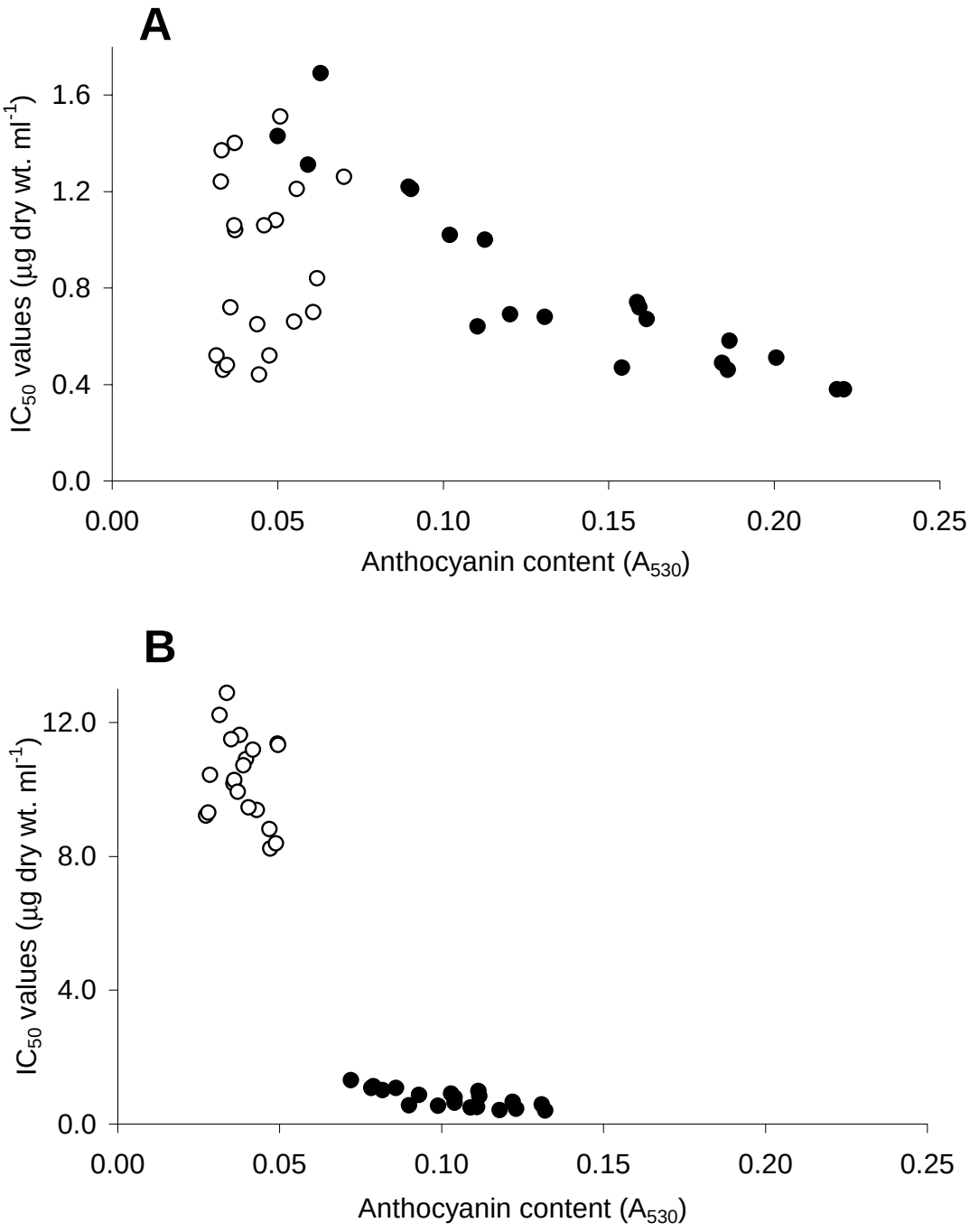


Fig. 6.5 Relative DPPH scavenging efficiencies (IC_{5O} values) as a function of anthocyanin content in red (●) and green (○) morphs of *Q. serrata* (A) and *E. rugosum* (B). Lower IC_{50} values signify the higher antioxidant activities. The equivalent IC_{50} value for ascorbic acid was 40 µg/ml (0.23 mM).

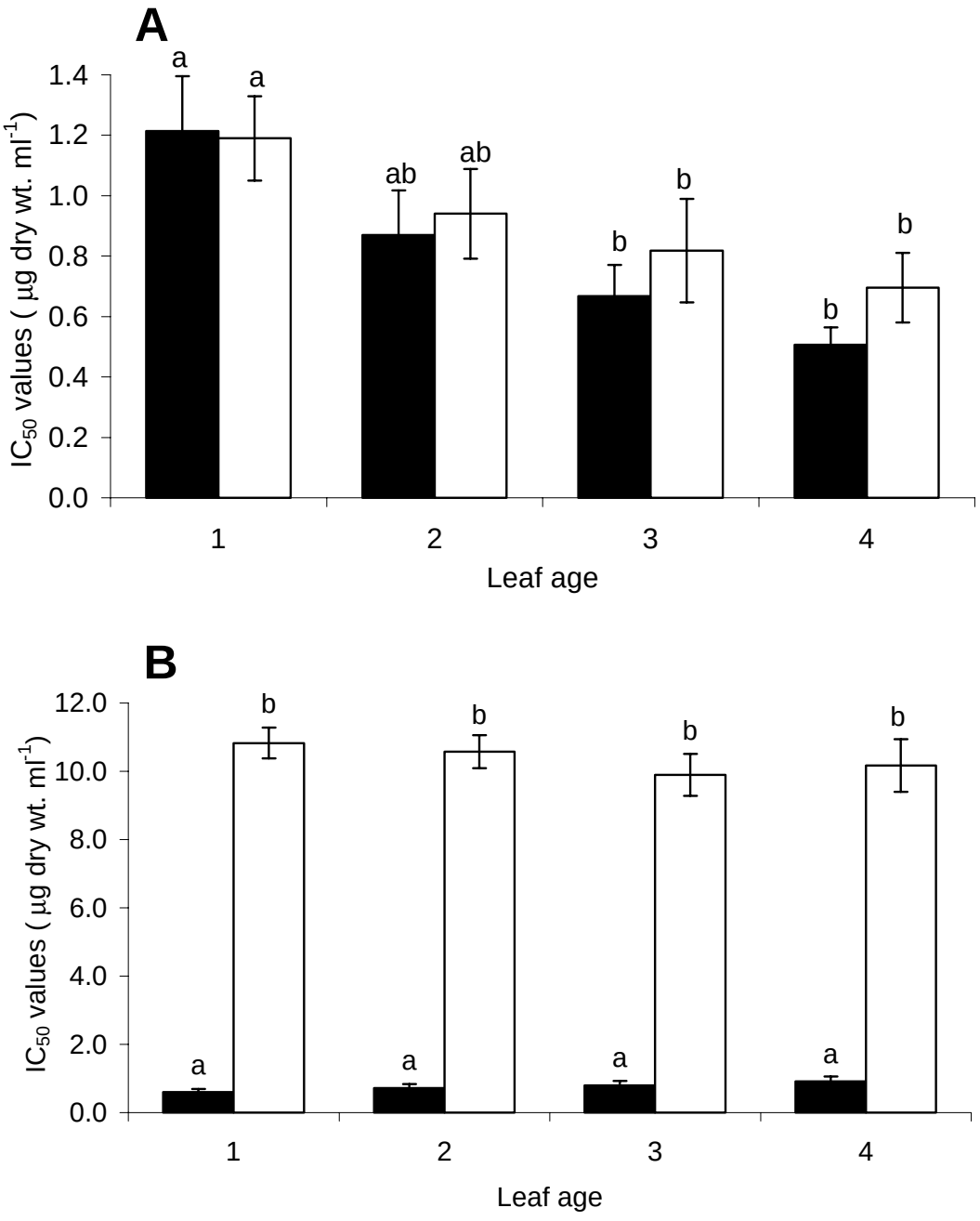


Fig. 6.6 Mean ± s.e. DPPH scavenging efficiencies (IC_{50} values) as a function of leaf age from red (black bars) and green (white bars) morphs of *Q. serrata* (A), and *E. rugosum* (B). Values that are significantly different ($P<0.005$) are indicated by different letters (Tukey-Kramer HSD). N = 5.

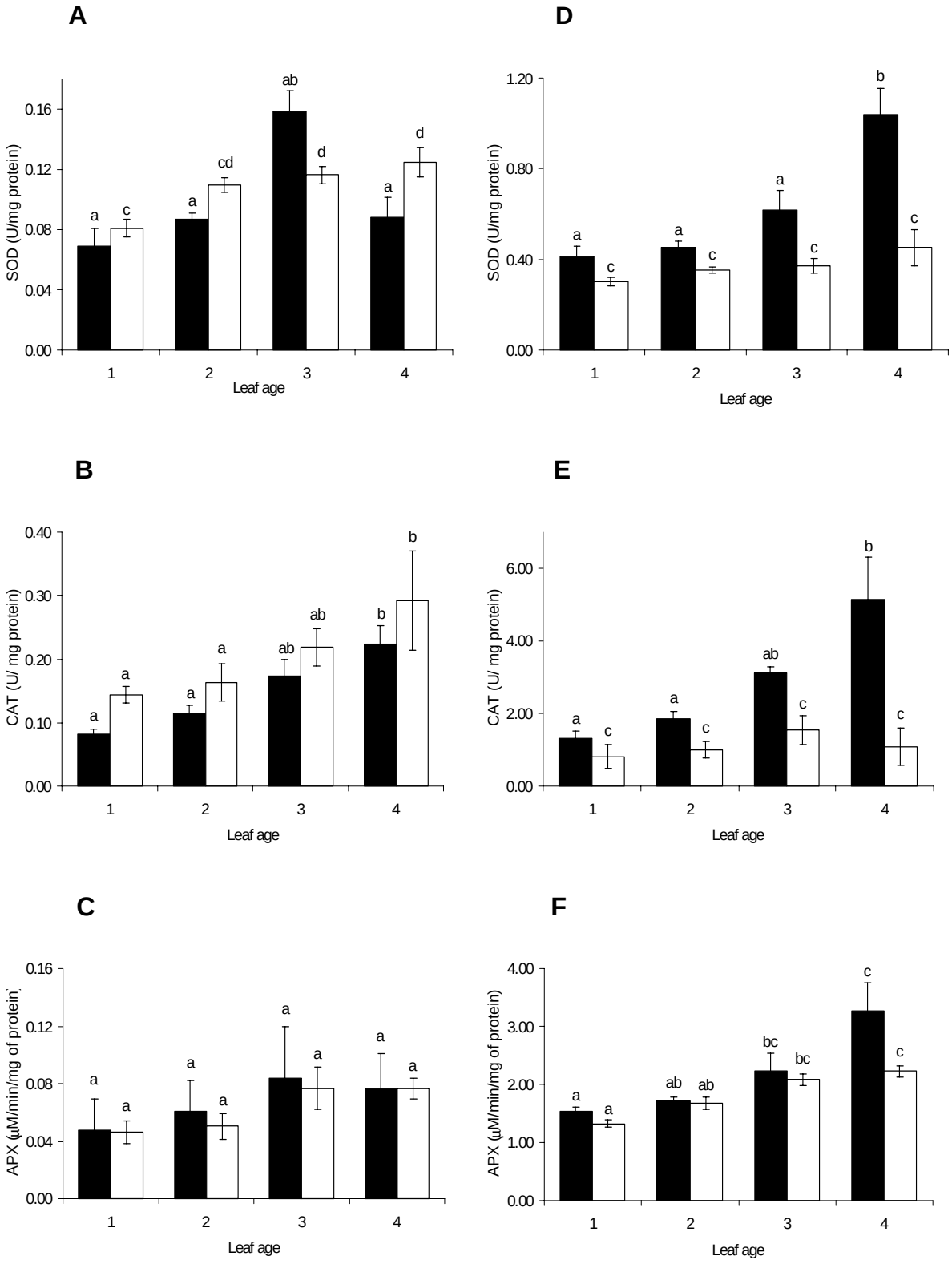


Fig. 6.7 Mean ± s.e. enzymic antioxidant activities of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) in leaves of red (black bars) and green (white bars) morphs of *Q. serrata* (A - C) and *E. rugosum* (D - E). Values that are significantly different ($P<0.005$) are indicated by different letters (Tukey-Kramer HSD). N = 4 for each analysis.

The anthocyanins contributed most significantly to the LMWA antioxidant pool in the red morphs of *E. rugosum*. The extremely low antioxidant capacity of green morphs comprised approximately equal contributions from the anthocyanins, flavonols and flavones, and hydroxycinnamic acids (Fig. 6.7).

The enzymatic antioxidants SOD, CAT, and APX were present in all leaves of both species (Fig. 6.8). The activities of these enzymes were significantly lower in the leaves of *Q. serrata* (Fig. 6.8 A – C), than in those of *E. rugosum* (Fig. 6.8 D – F). Leaves from the red morphs of *E. rugosum* had greater enzymatic activities than those from the green morphs (with the exception of APX), and this differential increased as leaves aged (Fig. 6.8 D – F). For *Q. serrata*, the activities of SOD, CAT and APX increased only marginally as leaves aged, and were comparable among leaves from the green and red morphs (Fig. 6.8 A – C).

Cyclic voltammetry

Positive sweep (linear) voltammograms of extracts from *Q. serrata* and *E. rugosum* (Fig. 6.9) yielded positive currents due to antioxidants which acted as reducing agents at the inert carbon electrode. Peaks in the voltammograms at lower potentials indicate those antioxidants which are more powerful reductants. The magnitude of the current is a measure of the antioxidant concentration. A voltammogram peak may represent a single compound, but the broad peaks that were observed with the crude leaf extracts are more likely to indicate groups of compounds with similar redox potential.

Total antioxidant levels were estimated from these peaks as the integrals of current (Q) to 500 mV (most active antioxidants) or to 1200 mV. Peak integrals were higher in red leaves than in green leaves, and higher for *Q. serrata*, where the values tended to increase with leaf age, than for *E. rugosum*, where the values decreased with leaf age (Table 6.1). The highest currents for each leaf series (expressed as integrals in Table 6.1) were given by those extracts which also showed the lowest IC_{50} values in the DPPH assay (Fig. 6.6), and they showed similar declines (*E. rugosum*) or increases (*Q. serrata*) with leaf age (Fig. 6.9). The voltammograms for both species had three peaks or shoulders at pH 5.5 (*Q. serrata*: 372, 820, 1038 mV; *E. rugosum*: 372, 840, 990 mV); the second peak was less discernible for the same extracts at pH 7.0.

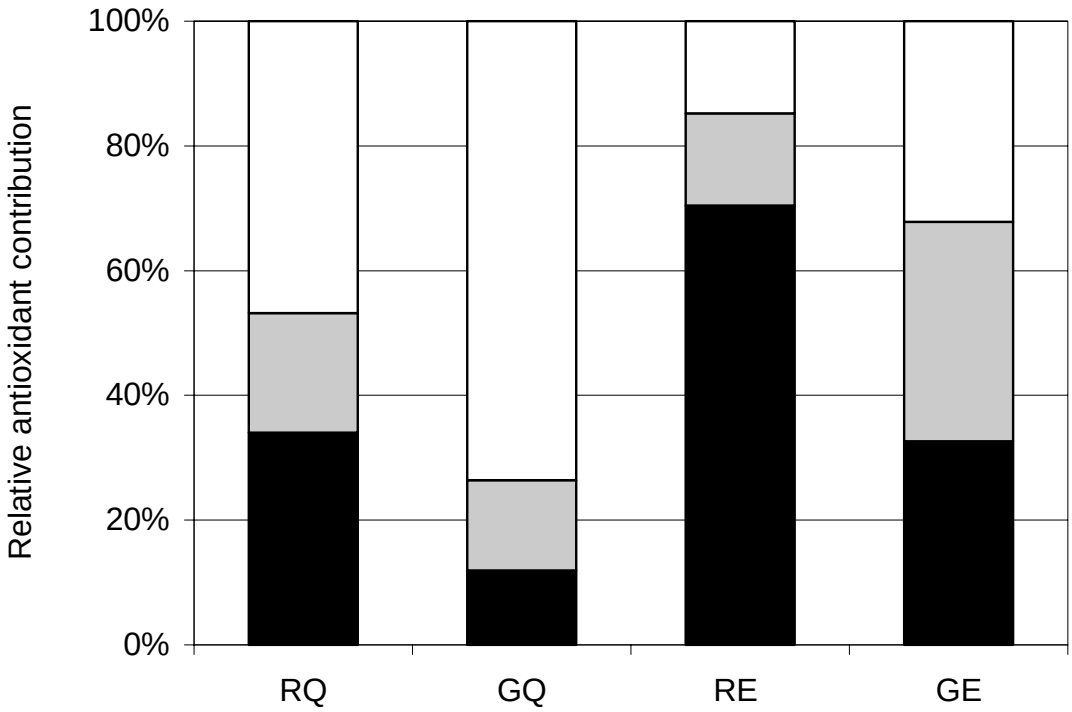


Fig. 6.8 Relative contributions of major methanol-soluble phenolics to the total antioxidant potential of leaves from red (R) and green (G) morphs of *Q. serrata* (Q) and *E. rugosum* (E). Data for anthocyanins (black bars), flavonols and flavones (grey bars), and hydroxycinnamic acids (white bars) are presented as reciprocals of IC_{50} values expressed as percentages of the total.

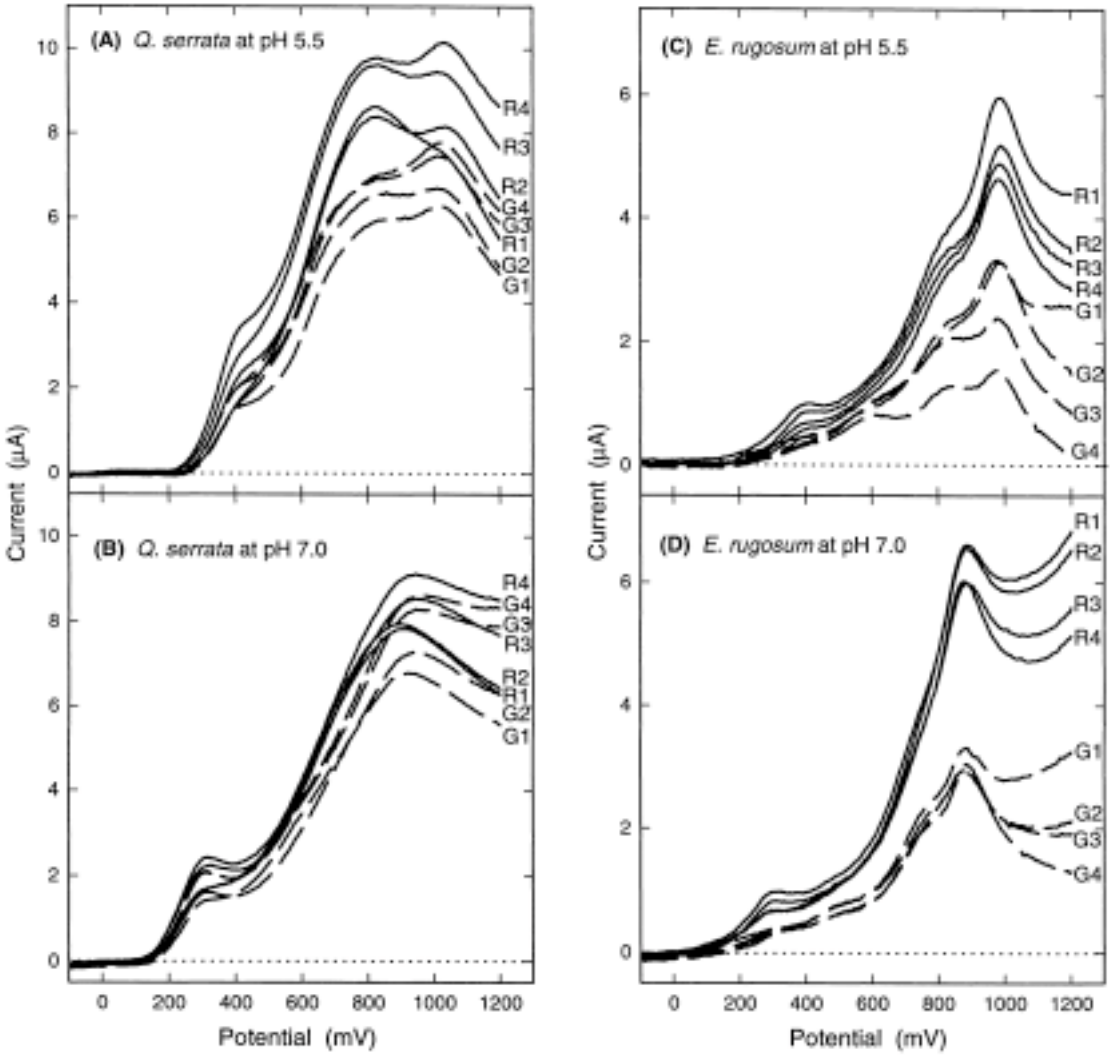


Fig. 6.9 Linear voltammograms of methanol-soluble compounds from red (solid lines) and green (broken lines) leaves of *Q. serrata* and *E. rugosum* at pH 5.5 (A, C) and pH 7.0 (B, D). Scans were measured at 100 mVs^{-1} at a 3 mm glassy carbon electrode, and taken to 1200 mV. Numerals indicate leaf age (red morph leaf extracts: R1 – R4; green morph leaf extracts: G1 – G4).

Table 6.1 Summarising data for linear voltammograms of methanol-soluble compounds from red and green leaves of *Q. serrata* and *E. rugosum* at pH 5.5 and pH 7.0 (Fig. 6.9). Integrals of current passed up to 500 mV and 1200 mV are shown as units of charge (Q) in μC. Scans were measured at 100 mVs^{-1} at a 3 mm glassy carbon electrode. Data are the means of five replications.

			pH 5.5		pH 7.0	
	Morph	Leaf age	Charge (Q) to 500 mV (μC)	Charge (Q) to 1200 mV (μC)	Charge (Q) to 500 mV (μC)	Charge (Q) to 1200 mV (μC)
Q. serrata	Red	1	4.0	52.7	5.1	48.7
		2	2.9	50.4	5.2	49.9
		3	5.0	62.3	6.3	53.5
		4	6.1	66.6	6.7	57.4
	Green	1	2.6	38.0	4.0	41.4
		2	2.7	42.0	4.3	43.1
		3	3.6	46.9	5.6	50.6
		4	3.5	47.3	5.6	52.4
E. rugosum	Red	1	2.4	27.5	2.3	34.6
		2	1.8	23.8	3.1	35.2
		3	1.5	22.2	2.4	31.1
		4	1.3	20.4	2.3	29.8
	Green	1	1.3	15.7	1.4	17.7
		2	1.0	14.2	1.0	14.0
		3	0.7	11.6	1.1	14.4
		4	0.6	7.0	0.8	12.9

An indication of the chemical nature of the voltammogram peaks was given by the behaviours of antioxidant standards and the purified anthocyanin fractions (Fig. 6.10). In these situations the scan was stopped at 500 mV and the direction of the potential sweep reversed. The cyclic voltammogram for 0.05 mM ascorbic acid (Fig. 6.10 A) had a broad peak at around 190 mV, corresponding to its low oxidation potential and high reactivity as an antioxidant. Caffeic acid and catechin both peaked at 310-320 mV, followed by rutin at 380 mV; each of these phenolic antioxidants possesses an ortho-dihydroxyl group which is easily oxidised, a process which is reversible at the carbon electrode (unlike those for ascorbic and gallic acids), generating the negative current peak on the reverse scan.

Purified anthocyanin fractions from *Q. serrata* produced a higher current response than those from *E. rugosum* (2.6 fold higher at pH 5.5, and 3.3 fold higher at pH 7.0), indicating that they have the higher amounts of low oxidation-potential antioxidants. The oxidation processes were reversible for *Q. serrata* anthocyanins, with significant negative current peaks seen on the reverse scans. *Q. serrata* anthocyanins gave at pH 5.5 a single peak at 375 mV (Fig. 6.10 B); the position of this peak was comparable to that of the first peak in the crude leaf extract from both red and green morphs (Fig. 6.9 A), and to that of rutin (Fig. 6.10 A). Thus, anthocyanins are likely to contribute to the first peak in the crude extracts of the red morph, either alone or in conjunction with rutin-type flavonoids. For the green morphs, the first peak would be given by rutin-type compounds alone.

Purified anthocyanin fractions (at pH 5.5) from *E. rugosum* leaves gave two peaks, at 240 and 370 mV (Fig. 6.6 C). The first peak could be tentatively assigned to glycosides of the triphenol delphinidin, and the second to glycosides of the ortho-diphenols cyanidin and petunidin (Kilmartin, 2001). Peonidin and malvidin, which have isolated phenol groups, would be expected only at potentials greater than 500 mV. Authentic solutions of caffeic acid and catechin, both of which possess ortho-diphenol groups, peaked at 310-320 mV (Fig. 6.10 C). By comparison, ascorbic acid had a peak at around 190 mV (the low potential reflecting its exceptionally high antioxidant activity), and rutin peaked at 380 mV (Fig. 6.10). Anthocyanins are likely to confer the first peak of activity in the crude extracts of red morphs.

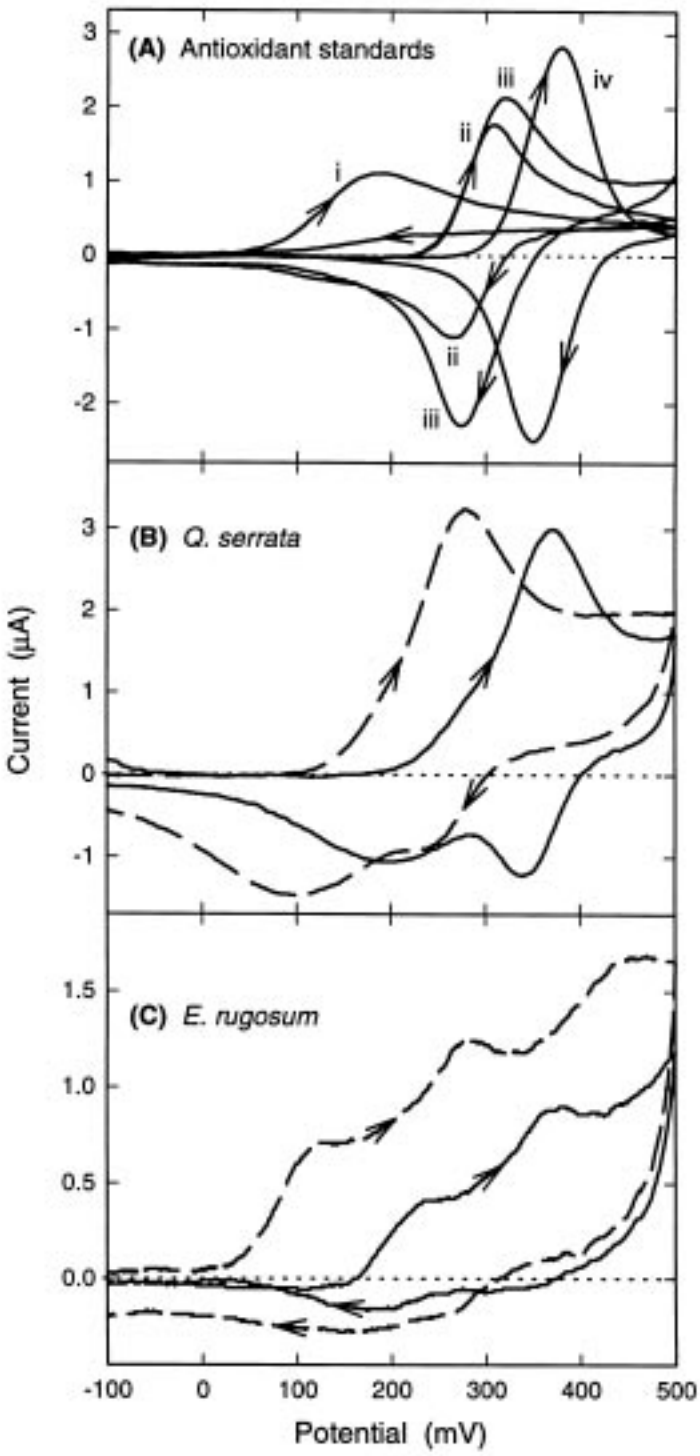


Fig. 6.10 Cyclic voltammograms for (A) 0.05 mM antioxidant standards at pH 5.5 (A): (i) ascorbic acid, (ii) caffeic acid, (iii) catechin and (iv) rutin; (B) purified anthocyanins from *Q. serrata*; (C) purified anthocyanins from *E. rugosum* at pH 5.5 (solid line) and pH 7.0 (broken line). Scans were measured at 100 mVs^{-1} at a 3 mm glassy carbon electrode, and taken to 500 mV.

Locations of peak voltammetric potentials were lower at pH 7.0 than at pH 5.5 for both the leaf extracts and the purified anthocyanins (Figs. 6.9, 6.10). This pH-related shift is consistent with the Nernst equation for the expected oxidation of the phenolic antioxidants, and does not signify changes in antioxidant strength.

Discussion

Our data indicate that for certain plant species the presence of anthocyanins in leaves can indeed confer a significant antioxidant advantage. In the shade plant, *E. rugosum*, leaves from the red morphs were on average five times more effective at scavenging DPPH radicals than were green leaves (Fig. 6.5 B), and had substantially greater antioxidant activities as measured by cyclic voltammetry (Fig. 6.9 C, D). Anthocyanins constituted the most active components of the extractable phenolic compounds, providing a higher relative contribution to the antioxidant pool than all other flavonoids and the hydroxycinnamic acids (Fig. 6.8).

By contrast, leaves from red and green morphs of the sun plant, *Q. serrata*, exhibited comparable ranges in LMWA activity, both as determined by the DPPH assay (Fig. 6.5 A) and by cyclic voltammetry (Fig. 6.9 A, B). For the red morphs of this species, antioxidant activities were greater in those leaves that held the higher concentrations of anthocyanins (Fig. 6.5 A). However, some leaves from the green morphs achieved similarly high antioxidant potentials by employing other phenolic compounds, predominantly the caffeic acid derivatives (Fig. 6.8). Thus, the pooled phenolic content, rather than the individual compound types, determines antioxidant activity in these leaves. Anthocyanin biosynthesis can enhance, but is not a prerequisite for, the protection from oxidative stress.

The elevated LMWA status of red versus green leaves in *E. rugosum* was further reinforced by their higher levels of antioxidant enzymes SOD and CAT (Fig. 6.7 D-F). Clearly, the red leaves are the better equipped to combat oxidative stress. Green morphs of this species occur less frequently than the red morphs, and tend to inhabit especially shaded and moist environments. We do not have data on the fluxes or quality of light incident on these leaves. However, the higher content of UV-absorbing phenolics in red leaves (Fig. 6.3 B), and the locations of these chemicals primarily within epidermal cell walls (Fig. 6.4 E, F), are consistent with the hypothesis that the red morphs experience more radiation than do the

green morphs (Tevini *et al.*, 1991; Day, 1993; Lois and Buchanan, 1994). The exposure of leaves to higher solar fluxes has been shown to increase levels of LMWAs and antioxidant enzymes in other species (Logan *et al.*, 1998 a; Logan *et al.*, 1998 b; Garcia-Plazaola *et al.*, 1999).

Red and green leaves of *Q. serrata*, by contrast, did not show appreciable differences in levels of antioxidant enzymes (Fig. 6.7 A-C). This species is exceptionally polymorphic for anthocyanin expression; pigmentation patterns vary across populations, among branches on an individual tree, and between different regions of a leaf lamina (Gould *et al.*, 2000). The red morphs are commonly found growing alongside green morphs. It is quite possible, therefore, for both leaf types to experience comparable degrees of photooxidative stress, and thus require similar levels of antioxidant enzymes. Activities of antioxidant enzymes were apparently lower than those for *E. rugosum*, although this difference was probably artefactual, a consequence of expressing values per unit total protein content; leaves of *Q. serrata* are thicker, structurally more complex (Fig. 6.4 A, D), and are likely to hold the more total protein. Maximum LMWA activities were comparable for *Q. serrata* and *E. rugosum* (Fig. 6.5).

The size and composition of the antioxidant pool changed over the course of leaf development. Activities of SOD, CAT, and APX increased over time for the red and green morphs of both species (Fig. 6.7). These observations are consistent with the data from many other species (reviewed by Polle (1997)). Levels of LMWAs decreased in the red morphs of *E. rugosum*, increased in the red morphs of *Q. serrata*, and remained relatively stable in the green morphs of both species (Figs. 6.2, 6.3). The changes may reflect developmental shifts in antioxidant requirements. Levels of antioxidants are governed as much by intrinsic determinants, such as the state of chloroplast maturation, as by external factors such as light flux. During leaf development there is a shift in the requirement for ROS removal, from the mitochondria and the cytosol (where anthocyanins and other LMWAs could play a dominant role) to the chloroplast, where organelle-bound enzymatic antioxidants scavenge superoxide and hydrogen peroxide produced during photosynthesis and photorespiration (Polle, 1997). The anthocyanins in *E. rugosum* could thus provide short-term antioxidant protection for the nascent leaves until a sufficient pool of antioxidant enzymes is synthesised. Anthocyanins in

Q. serrata would supplement the antioxidant requirement of older leaves that are subject to greater rates of photosynthesis than younger leaves.

Although the extracted flavonoids have measurable antioxidant activities *in vitro*, their potential to function as antioxidants in situ would be determined both by their cellular location and the pH environment. For both species, the flavonols and flavones reside predominantly in the epidermal cells (Fig. 6.4 B, E), a location that is optimal for attenuating UV-B radiation but which is unsuitable for the wholesale scavenging of cytosolic ROS. Anthocyanins are associated primarily with the photosynthetic tissues, both in this study (Fig. 6.4 A, D), and in the leaves of many other species (Gould and Quinn, 1999; Lee and Collins, 2001). The anthocyanins are prominent as red or purple solutions within the cell vacuole, a location that again is suboptimal for scavenging organelle-derived radicals such as $OH^{\cdot}$, which cannot permeate the tonoplast. However, they are synthesised in the cytoplasm as an equilibrium of colourless forms (Hrazdina *et al.*, 1978; Brouillard and Dangles, 1993). Thus, they have the potential to scavenge cytosolic ROS before their transfer into the vacuole via a glutathione pump (Marrs *et al.*, 1995). The vacuole is normally more acidic (ca. pH 5.5) than the cytoplasm (pH 7) (Davies, 1997), yet the cyclic voltammograms indicated that purified anthocyanins could effect significant antioxidant potentials at both of these pH values (Fig. 6.10 B, C). Lapidot *et al.* (1999) have also demonstrated that red and colourless forms of anthocyanins can achieve comparable antioxidant activities, as determined by lipid oxidation assays. The colourless forms of anthocyanins are, therefore, potentially useful scavengers of organelle-generated ROS.

In addition to their antioxidative capacities, the foliar phenolic compounds have the potential to further reduce oxidative stress by reducing radiation levels incident on the chloroplast. Hydroxycinnamic acids and the colourless flavonoids are strong attenuators of UV-B radiation (Shirley, 1996). Anthocyanins can absorb up to 17% of the incident green light (Neill and Gould, 1999), which is a key driver of photosynthesis in the lower spongy mesophyll tissue (Neill and Gould, 1999). It is also possible that the antioxidant activities of these phenolic compounds are ancillary, rather than primary functions in leaves; numerous and disparate phytoprotective functions have been attributed to the anthocyanins (reviewed by Chalker-Scott, 1999). We require the demonstration of ROS scavenging by red cells in situ to confirm that the antioxidant potential of anthocyanins is realised.

CHAPTER 7 - Subcellular scavenging by anthocyanins

Abstract

Anthocyanins apparently protect leaves by shielding chloroplasts from the effects of high irradiances. They may also function as antioxidants in the cell. Using chloroplast suspensions from *Lactuca sativa* and an EDTA-riboflavin system to generate superoxide radicals ($O_2^{\cdot-}$) we have quantified the scavenging activity of cyanidin-3-malonylglucoside at pH values representative of the vacuole and cytoplasm.

In the presence of colourless forms of anthocyanins at neutral pH, levels of chloroplast permeable $O_2^{\cdot-}$ generated by photooxidative processes decreased significantly by 17 %, as measured by extrachloroplastic formazan production. $O_2^{\cdot-}$ generation was neglible in darkness and was significantly reduced in the presence of SOD but not CAT.

Artificial generation of $O_2^{\cdot-}$ through the light activated EDTA-riboflavin system, resulted in the significant loss of colour at 525 nm of the flavylium forms of cyanidin-3-malonylglucoside, which after 15 minutes equated to a 40 % reduction in antioxidant potential as measured by cyclic voltammetry. We discuss the overall subcellular implications of our findings in relation to the potential of anthocyanins to confer antioxidant protection within the cell.

Key words: anthocyanin, superoxide, subcellular location, antioxidant.

Introduction

Free radicals and reactive oxygen species (ROS) are the inevitable by-products of metabolic processes within the cell. Exposure to biotic and abiotic stressors often leads to the production of elevated levels of radicals and/or ROS that have the potential for cellular damage (Foyer *et al*., 1994; Polle, 1997).

Chloroplasts are particularly vulnerable to photooxidative damage because of their high internal oxygen concentrations, high proportion of polyunsaturated fatty acids in the thylakoid

membrane, and the presence of excitable chlorophyll molecules (Halliwell and Gutteridge, 1998; Foyer *et al.*, 1994; Tsuda *et al.*, 1996 b). Excess excitation of chlorophyll results in the formation of unstable triplet states ($^3Chl^*$), allowing energy transfer to O_2 to yield singlet oxygen ($^1O_2$) (Asada, 1999). Photoreduction of O_2 via the Mehler reaction leads to the generation of superoxide radicals ($O_2^{\cdot-}$), which can then hydrate to produce hydrogen peroxide (H_2O_2) (Kaiser, 1979). H_2O_2 may also be generated via photorespiration in the peroxisomes (Smirnoff, 2000). Moreover, the accumulation of these radicals/ROS, in the presence of transitional metals, favours the production of the highly reactive hydroxyl radical ($OH^{\cdot}$) through Haber-Weiss-Fenton reactions (Van Acker *et al.*, 1996; Brown *et al.*, 1998; Noda *et al.*, 1998).

Chloroplast-derived radicals and ROS are normally swiftly scavenged through the actions of enzymatic antioxidants and low molecular weight antioxidants (LMWAs) (Foyer, 1993; Alscher *et al.*, 1997; Polle, 1997). However, under conditions of stress, such as high irradiances and/or water stress, the scavenging capacities of antioxidants may be exceeded, particularly if the available pools of ascorbate are oxidised (Yamasaki *et al.*, 1995). The radicals $O_2^{\cdot-}$, $OH^{\cdot}$, and $^1O_2$ do not normally traverse membranes, and are restricted to their local sites of generation (Alscher *et al.*, 1997). However, the relatively stable H_2O_2 species, and the protonated form of superoxide ($HO_2^{\cdot}$) are able to cross the chloroplast envelope, presenting the cytoplasm with an additional oxidative burden (Asada and Takahashi, 1987; Takahama, 1989; Yamasaki, 1997; Yamasaki *et al.*, 1997; Halliwell and Gutteridge, 1998). $HO_2^{\cdot}$ has recently been demonstrated to inactivate extrachloroplastic catalase (Shang and Feierabend, 1999).

These same environmental stresses often lead to the up-regulation of the general phenylpropanoid and flavonoid pathways (Dixon and Paiva, 1995). Indeed, anthocyanins in the leaves of many species are commonly associated with chlorophyllous cells (Lee and Collins, 2001), which are the most susceptible to oxidative damage. There is extensive evidence from *in vitro* studies that phenolic compounds, including the anthocyanins, are highly effective scavengers of free radicals and ROS. Flavonoid aglycones including quercetin, delphinidin, and cyanidin have scavenging capacities up to four times higher than those of ascorbic acid and α-tocopherol (Rice-Evans *et al.*, 1995 b; Rice-Evans *et al.*, 1997). Extracts of anthocyanins have been shown to scavenge $OH^{\cdot}$, $O_2^{\cdot-}$, and H_2O_2 (Bors *et al.*, 1990;

Tsuda *et al.*, 1996 b; Yamasaki *et al.*, 1996; Yamasaki, 1997), and may provide protection from $^1O_2$, nitric oxide (NO), peroxynitrite ($ONOO^-$), and lipid peroxyl ($ROO^\cdot$) species (Chauhan *et al.*, 1992; Jovanovic *et al.*, 1994; Grace *et al.*, 1998b; Haenen and Bast, 1999). In addition, anthocyanins can chelate metal ions, potentially reducing ROS that would be generated *in vivo* through Haber-Weiss-Fenton reactions (Van Acker *et al.*, 1996).

However, the importance of anthocyanins as antioxidants *in planta* remains uncertain. For most plants, the anthocyanins are predominantly located in the cell vacuole, a location that is remote from the typical source of ROS, such as the chloroplasts and mitochondria. In the acid pH of the vacuole, anthocyanins are present largely in the red-purple flavylium form (Brouillard, 1982; Harborne and Grayer, 1988). It is this form of anthocyanin that has been employed in most antioxidant assays (Takahashi *et al.*, 1991; Sarma *et al.*, 1997; Prior *et al.*, 1998; Sarma and Sharma, 1999). However, at their point of synthesis in the cytoplasm (Hrazdina *et al.*, 1978; Hrazdina, 1992; Saito *et al.*, 1999; Nakajima *et al.*, 2001), anthocyanins exist primarily as a mixture of colourless carbinol and chalcone pseudobase forms (Brouillard, 1982). It is possible that these colourless anthocyanins have the potential to provide antioxidant protection in the cytoplasm. Cytosolic anthocyanins would be ideally located to scavenge permeable ROS generated by mitochondria and chloroplasts under conditions of stress (Chapter 6).

No previous work has directly related ROS scavenging activity to the various molecular forms of anthocyanins, as they exist in their particular subcellular locations. Yamasaki *et al.* (1996) observed that anthocyanins isolated from *Hibiscus rosa-sinensis* were bleached in the presence of $O_2^{\cdot-}$ at neutral pH values. This 'bleaching' was postulated to be a consequence of anthocyanin oxidation by $O_2^{\cdot-}$, though this was never confirmed. In addition, the $O_2^{\cdot-}$ scavenging activities of the anthocyanin was interpreted as being representative of anthocyanins in their flavylium forms instead of the characteristic forms present in the neutral solution analysed.

Here we further investigate the findings of Yamasaki *et al.* (1996) and aim to test if the loss of colour associated with $O_2^{\cdot-}$ scavenging corresponds to a reduction in antioxidant capacity of anthocyanins in the flavylium form thought to be characteristic of the natural forms in the vacuole. In addition, as colourless forms of anthocyanins exist in solution in the cell

cytoplasm, we also aim to assess the importance of anthocyanins relative to other flavonoids in scavenging membrane-permeable radicals generated through photooxidative processes such as the Mehler reaction. In order to compare the antioxidant activities of colourless anthocyanins and related flavonoid structures we utilise nitroblue tetrazolium (NBT) as a detector for $O_2^{\cdot -}$ generated by irradiating chloroplast suspensions isolated from the leaves of shade adapted *Lactuca sativa*. The use of chloroplast suspensions in neutral pH buffer effectively simulates the cell cytoplasm providing the means to study the effects of anthocyanins on photooxidation and radical scavenging under high irradiances.

We demonstrate that the colourless forms of anthocyanin are able to scavenge $O_2^{\cdot -}$ generated via photooxidation of lettuce chloroplasts and that the basic flavonoid structure can affect the efficacy of radical scavenging. Additionally, we confirm that scavenging by flavylium forms of anthocyanin results in a loss of colour and a reduction in scavenging ability.

Materials and methods

Plant material

Lactuca sativa cv. Dark Lollo Roso plants were grown from seed for 8 weeks under controlled conditions (20 ^{o}C day, 16 ^{o}C night) with either low (150 μmol m^{-2} s^{-1}) or high (800 μmol m^{-2} s^{-1}) irradiances and a 16 hr photoperiod.

Chloroplast isolation

Intact chloroplasts were mechanically isolated from the low light grown plants by a method described by Leegood and Walker (1993). Approximately 10 g of leaves were harvested, washed, the midribs removed, and the laminae ground in 50 mL of semi-frozen grinding medium containing 0.33 M sorbitol, 10 mM $Na_4P_2O_7$, 5 mM $MgCl_2$, and 2 mM EDTA at pH 6.5. The homogenates were filtered, centrifuged at 600 g for 3 mins, resuspended in 2 mL of buffer containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes, 10 mM $NaHCO_3$, and 5 mM $Na_4P_2O_7$ at pH 7.6, and further purified using a 40 % (v/v) Percoll gradient. The pellet was resuspended in 10 mL of buffer and held on ice for the duration of the experiments. All the purification steps were undertaken at 4 ^{o}C under low light.

Anthocyanin purification

Anthocyanins, previously identified as cyanidin-3-malonylglucoside (Ferreres *et al.*, 1997), were purified from leaves grown under the high light treatment. Leaves were ground in 2 % HCl in MeOH, filtered, centrifuged at 7,800 g for 5 mins, and concentrated under vacuum. They were further purified by paper chromatography using t-BuOH:H_2O:MeOH (3:1:1) for 24 hrs. The anthocyanic band was eluted in 2 % HCl in MeOH, freeze dried and resuspended in 50 mM MOPS buffer at pH 7.0 for the $HO_2^{\cdot}$-scavenging assay. The purified cyanidin-3-malonylglucoside was also resuspended in 20 mM potassium acetate buffer, pH 4.0, to produce flavylium forms typical of those in the vacuole.

Effects of antioxidants on extrachloroplastic superoxide concentrations

Precisely 0.5 ml of the chloroplast suspension from leaves grown under the low light treatment was inserted into a quartz cuvette containing 0.1 mL of 3 mM nitroblue tetrazolium (NBT) in chloroplast resuspension buffer and 2.4 mL of 50 mM MOPS (pH 7.0). The chloroplast suspensions were constantly agitated at 23 ^{o}C and irradiated white light ranging from 75–850 μmol m^{-2} s^{-1} (Chapter 4 - Fig. 4.5 A) from a Schott KL 1500 halogen collimated light source (Mainz, Germany). Superoxide generation was quantified by recording the formation of the dark blue diformazan pigment (A_{560} values) over 20 min at 2-min intervals using a Hitachi U-2001 dual beam spectrophotometer. Absorbance values after 8 min were found to be linearly correlated to the applied irradiance and were subsequently used as a measure of superoxide production across the treatments (Chapter 4 – Fig. 4.5 B).

Irradiance was adjusted to 300 μmol m^{-2} s^{-1} and superoxide scavenging was quantified after the addition of the following antioxidants: 2, 8, 17, and 26 μM purified anthocyanin; 26 μM quercetin; 26 μM quercetin 3β-D rutinoside (rutin; Fig. 7.1); 100 units $mL^{-1}$of superoxide dismutase (SOD); and 100 units $mL^{-1}$of catalase (CAT). The addition of the anthocyanin in 50 mM MOPS buffer (pH 7.0) resulted in a significant increase in PAR absorption relative to the controls because of the presence of blue quinoidal forms. In order to standardise the light flux incident on the suspended chloroplasts, the irradiance was increased in proportion to the enhanced absorption, so that for each treatment the chloroplast suspension received exactly 300 μmol m^{-2} s^{-1}. MOPS was used in preference to HEPES, (which was used in the extraction of chloroplasts), because HEPES formed strong coloured complexes with the extracted

anthocyanins at pH 7.0 and altered the absorption properties of NBT (as described by Beyer and Fridovich, 1987).

Photobleaching of anthocyanin

The light activation of riboflavin in the presence of EDTA provides a mechanism for generation of $O_2^{\cdot-}$ *in vitro* (Fig. 7.2) (Takahama, 1987; Yamasaki *et al*., 1996). A total of 1 mL of a 9 μM anthocyanin solution was added to 2.0 mL of a solution containing 5 μM riboflavin, 3 mM EDTA, and 20 mM potassium acetate buffer (pH 4.0). The reaction mixture was irradiated with 300 μmol m^{-2} s^{-1} provided by a Schott KL 1500 halogen collimated light source, and the decrease in absorbance at 525 nm (λ_{max} of cyanidin-3-malonylglucoside at pH 4.0) was followed for 15 mins at 1 min intervals. The individual effects of 50 units of SOD mL^{-1}, 50 units of CAT mL^{-1}, and 20 mM mannitol (scavenger of $OH^{\cdot}$), on the rate of anthocyanin bleaching were also analysed.

The bleaching of anthocyanin by singlet oxygen ($^1O_2$) generated by Rose Bengal was also tested. However, the overlapping absorbance peaks of anthocyanin and rose bengal at 515-549 nm masked any bleaching effects caused by $^1O_2$.

Cyclic voltammetry of anthocyanins

Cyclic voltammetry was used to characterise the antioxidant capacity of cyanidin-3-malonylglucoside before and after bleaching by $O_2^{\cdot-}$ in the EDTA-riboflavin system. Cyclic voltammetry measurements were taken using a 100A electrochemical analyser (Bioanalytical systems, West Lafayette, USA), with a BAS C2 cell stand Kilmartin (2001). A glassy carbon disk electrode (BAS M-2012) used for the measurements was cleaned with 3 μm alumina powder (PK-4) between runs. All measurements were recorded at a scan rate of 100 mVs^{-1} with a maximum potential before the reverse scan of 500 mV, approximately 30 mV past the first anodic peak. These measurements were simultaneously compared with a silver/ silver chloride reference electrode and a platinum counter electrode. Cyclic voltammograms were obtained by subtracting the current from blank scans made with 1 mL MeOH and 2 mL the EDTA-riboflavin solution.

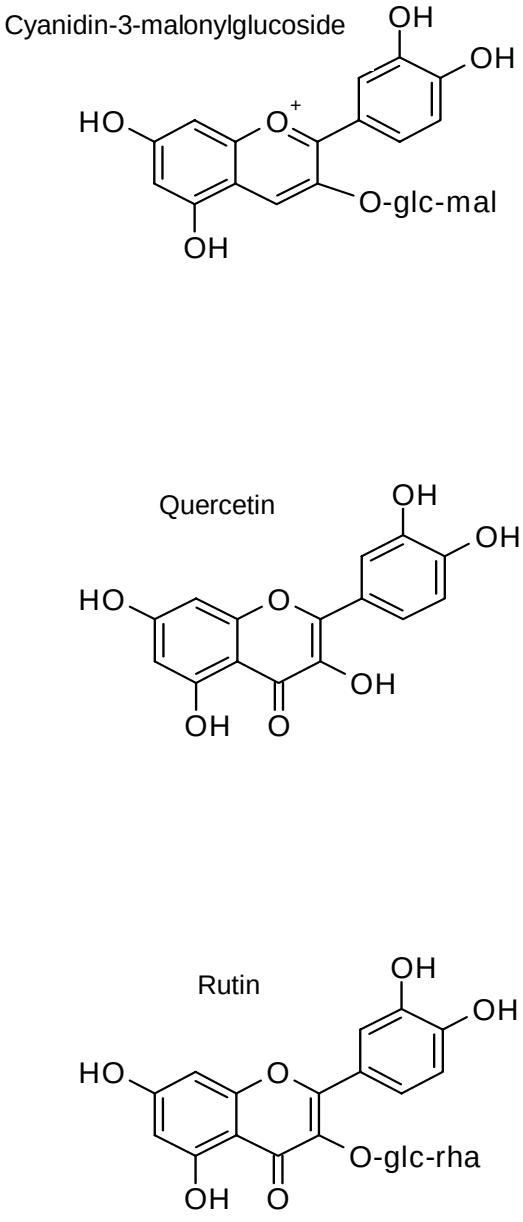


Fig. 7.1 Structural features of cyanidin-3-malonylglucoside extracted from *Lactuca sativa* and of the related flavonoid compounds used in the $O_2^{\cdot -}$-scavenging assay (see Fig. 7.3). Glc = glucose; mal = malonyl; and rha = rhamnose.

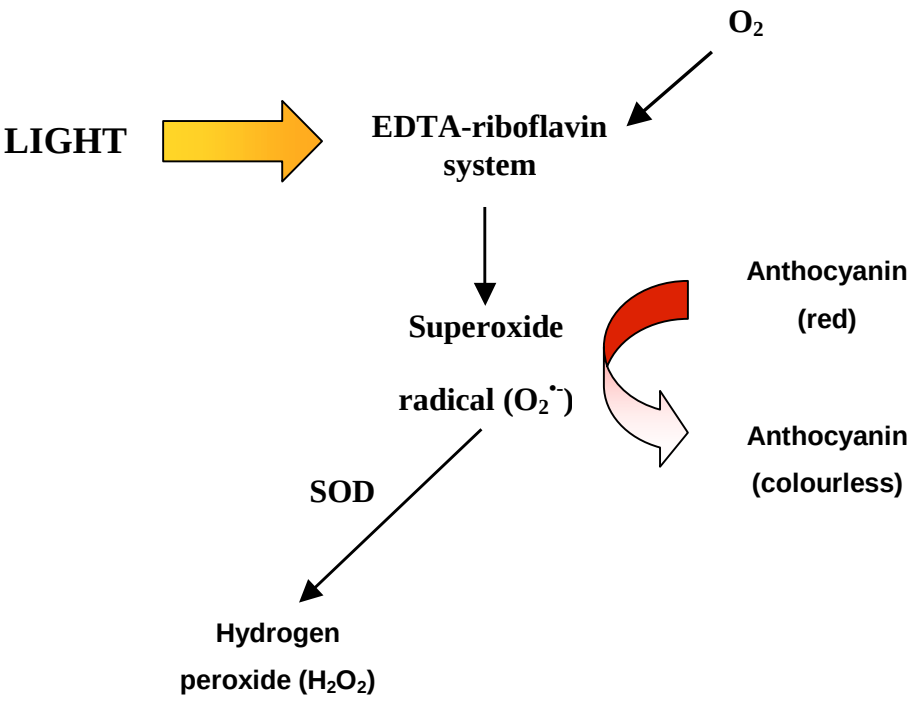


Fig. 7.2 Superoxide generation through the EDTA-riboflavin system, and subsequent quenching by; i) anthocyanin; and ii) superoxide dismutase (SOD)

Results

Extrachloroplastic superoxide concentrations

Conversion of nitroblue tetrazolium (NBT) to the dark blue insoluble diformazan pigment is proportional to the number of $O_2^{\cdot-}$ radicals present (Halliwell and Gutteridge, 1998). NBT conversion in the chloroplast solution increased exponentially over time (Chapter 4 - Fig. 4.5 A), and was linearly correlated to the applied irradiance (Chapter 4 - Fig. 4.5 B) demonstrating the photo-inducible production of $O_2^{\cdot-}$. There was no evidence of NBT conversion in the MOPS buffer in the absence of light, nor in the presence of light without chloroplasts. The specificity of NBT for $O_2^{\cdot-}$ was established by the addition of SOD to the assay solution, which resulted in a significant 43 % decrease in $HO_2^{\cdot}$ levels compared to that of the control (Fig. 7.3 A). The addition of 100 units of CAT had little effect (3 % reduction) on NBT reduction.

The detection of $O_2^{\cdot-}$ from the isolated chloroplasts was significantly reduced (17 %) when cyanidin-3-malonylglucoside was added to the suspension (Fig. 7.3 A). The effects of the anthocyanin on $O_2^{\cdot-}$ levels were concentration dependant (Fig. 7.3 B). Other flavonoids also resulted in lower $O_2^{\cdot-}$ concentration. The addition of 26 μM effected a similar reduction (16 %) in NBT conversion as that seen with anthocyanin. However, the structurally related flavonoid rutin only caused a 7 % reduction.

Photobleaching of anthocyanin

Illumination of the EDTA-riboflavin system with 300 μmol m^{-2} s^{-1} white light resulted in a significantly (t-test; P=<0.001) more rapid loss of absorbance of cyanidin-3-malonylglucoside at 525 nm relative to that observed in the dark (Fig. 7.4). This bleaching of anthocyanins was not associated with changes in pH or temperature, which remained constant throughout the experiment. Negligible bleaching occurred when cyanidin was illuminated in the absence of the superoxide generating system. SOD significantly reduced the rate of colour loss at 525 nm (t-test; P<0.001) relative to that of cyanidin-3-malonylglucoside alone, demonstrating the involvement of $O_2^{\cdot-}$ in anthocyanin bleaching. CAT and 20 mM mannitol both failed to reduce the rate of cyanidin bleaching when illuminated in separate experiments.

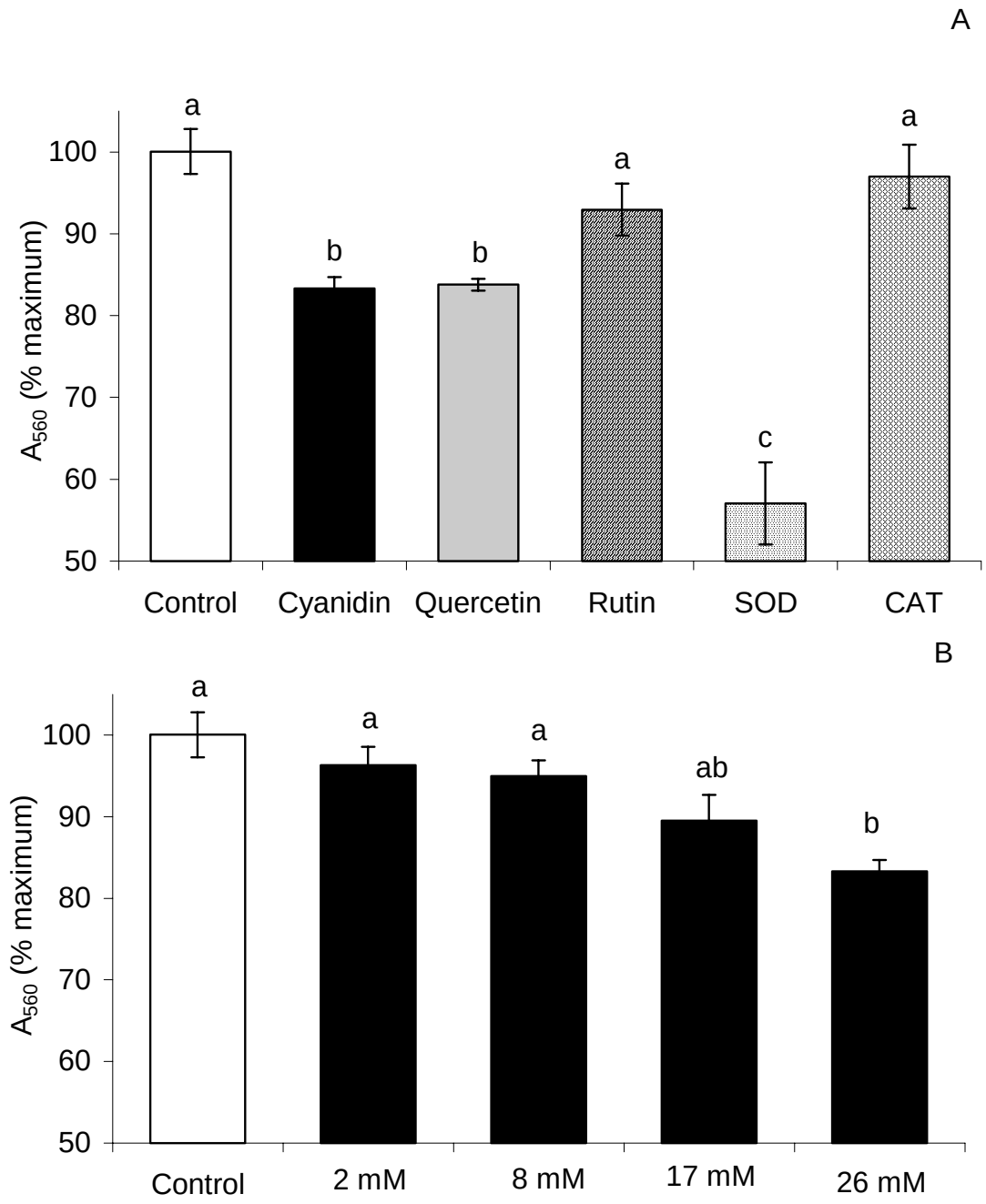


Fig. 7.3 Superoxide production relative to that of a control in irradiated chloroplasts from *Lactuca sativa*. The suspensions were supplemented with (A) 26 μM cyanidin-3-malonylglucoside, quercetin, rutin, 100 units of SOD, and CAT in 50 mM MOPS buffer at pH 7.0, or (B) a range of cyanidin-3-malonylglucoside concentrations. Values that are significantly different ($P<0.05$) are indicated by different letters (Tukey-Kramer HSD). N = 3 for each treatment. Bars show ± s.e.

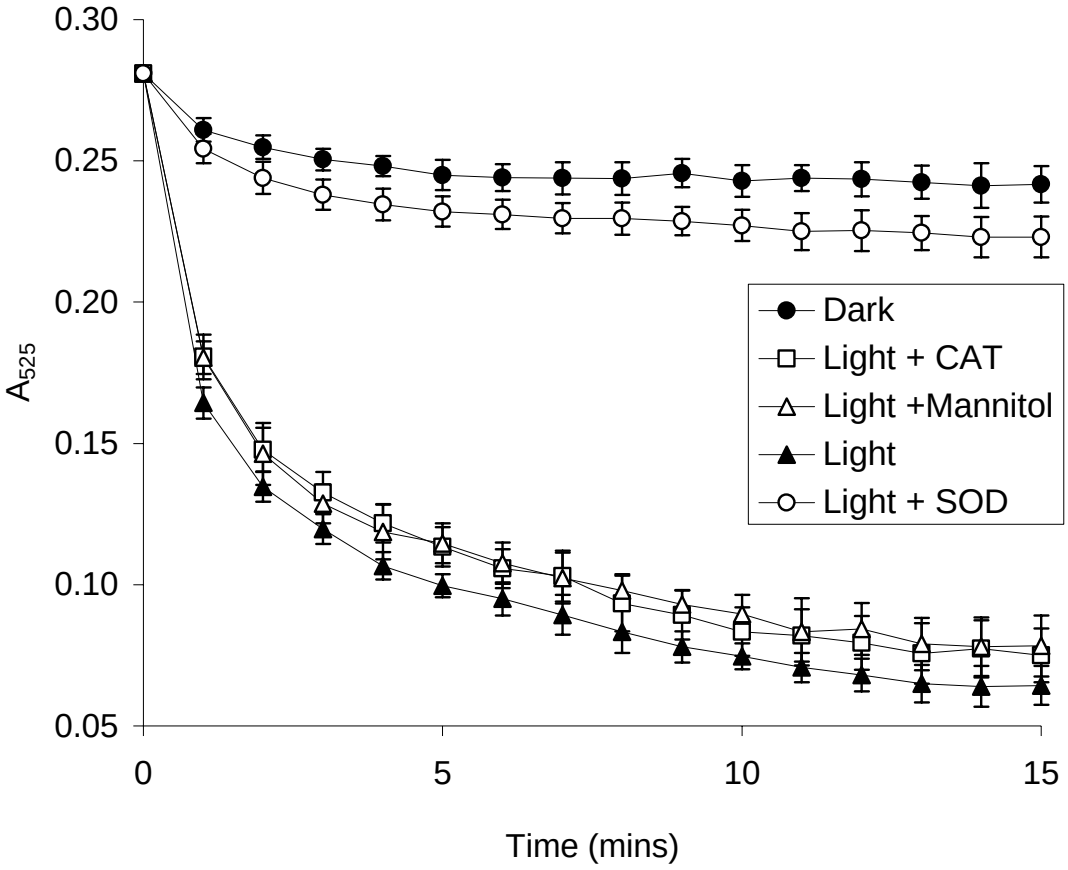


Fig. 7.4 Time-course of cyanidin-3-malonylglucoside photobleaching by superoxide generated through the EDTA-riboflavin system. The effects of SOD and CAT (50 units mL^{-1} each) and 20 mM mannitol are shown. Anthocyanin absorbance (A_{525}) are given as the means (± s.e.) of 3 replicates.

The antioxidant activity of cyanidin-3-malonylglucoside was measured as the ability to donate electrons during cyclic voltammetry, both before and after photobleaching by the EDTA-riboflavin system. A single, distinct voltammetric peak was evident at 471 mV for both treatments (Fig. 7.5), indicative of the presence of an ortho-diphenol compound. Both the natural and photobleached anthocyanin forms demonstrated a high degree of voltammetric reversibility, as shown by the negative current peak at 405 mV on the reverse scan. However the relative levels of natural anthocyanin were reduced by 40 % after the 15 min light treatment, as shown by the significantly lower positive and negative peak currents.

Discussion

Our data indicate that anthocyanins present within photosynthetic cells exposed to photooxidative stress can confer significant antioxidant protection at pH values representative of the cytoplasm. We postulate that anthocyanins act as important low molecular weight antioxidants, which supplements the overall antioxidant pool of the plant cell.

Protection of the cytoplasm by colourless anthocyanins

The chloroplast suspension system, which we consider to be representative of the cytoplasmic environment, demonstrates that colourless carbinol pseudobase and chalcone pseudobase forms (as well as a small proportion of blue quinoidal forms) (Brouillard, 1982) can directly scavenge $O_2^{\cdot -}$ generated by photooxidative processes in the chloroplast (Fig. 7.3). Although anthocyanins have been implicated in scavenging $O_2^{\cdot -}$ generated from artificial systems previously (Yamasaki *et al.*, 1996), this is the first report to demonstrate their importance as antioxidants from ROS produced from chloroplasts.

Colourless cyanidin-3-malonylglucoside and quercetin both reduced the amount of $O_2^{\cdot -}$ detectable extrachloroplastically in solution at pH 7.0. An equivalent concentration of rutin, however, had little effect. These data are consistent with the structural requirements of phenolic antioxidants, as described by Bors *et al*. (1990), Rice-Evans *et al*. (1995 b), and Rice-Evans *et al*. (1997). The slightly greater scavenging capacities of cyanidin-3-malonylglucoside as compared to quercetin can be explained by the acylation of the anthocyanin. As organic acids display moderate antioxidant activities themselves

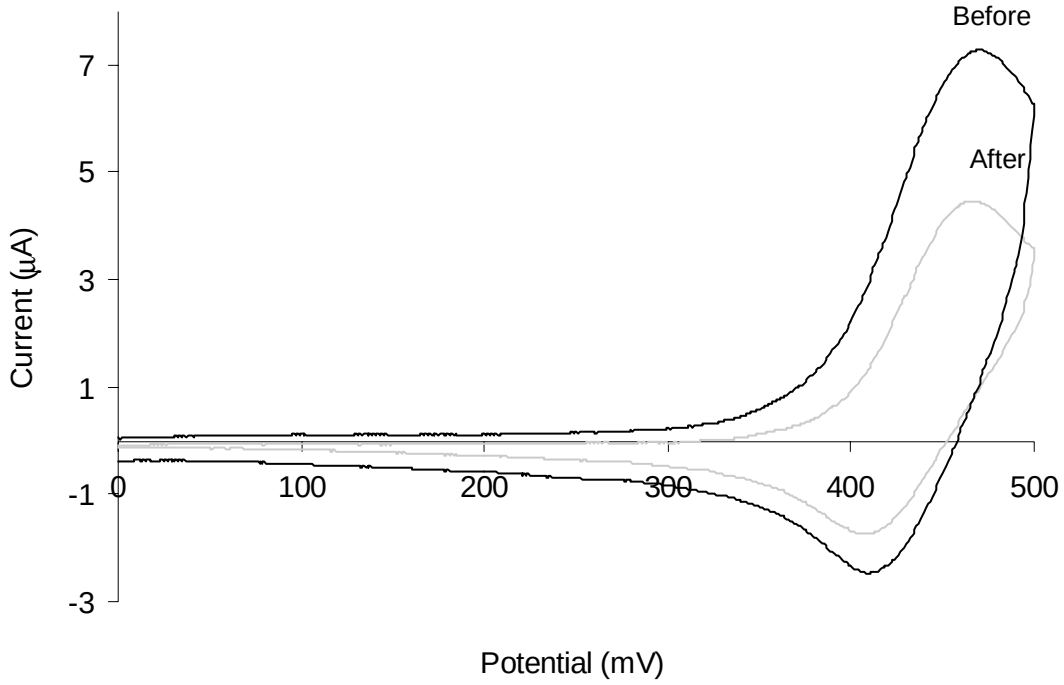


Fig. 7.5 Cyclic voltammograms of cyanidin-3-malonylglucoside before (black line) and after (grey line) bleaching by $O_2^{\cdot -}$ in the EDTA-riboflavin system. Scans were measured at 100 mVs^{-1} at a 3 mm glassy carbon electrode, and taken to 500 mV.

(Ribereau-Gayon, 1972; Markham, 1982) it can be expected that acylation increases the overall scavenging potential.

We postulate that the accumulation of colourless anthocyanins represents a flexible mechanism to scavenge membrane permeable radicals and ROS generated through photooxidative processes. Anthocyanins can directly scavenge H_2O_2 and $O_2^{\cdot -}$. They are also likely to i) act as reducing agents for general peroxidases (Takahama, 1989; Takahama and Oniki, 1997; Yamasaki *et al.*, 1997; Yamasaki and Grace, 1998), which are extremely abundant in the cytoplasm (Polle, 1997); and ii) inhibit the formation of $OH^{\cdot}$ radicals through Haber-Weiss-Fenton reactions by chelating transition metals (Van Acker *et al.*, 1996). Recent work using flavonoid-deficient petunia has indirectly demonstrated the importance of flavonoids in cellular antioxidant protection (Repka and Fischerova, 2000). Chalcone synthase-deficient petunia displayed higher rates of peroxidase activity, possibly to compensate for the lack of flavonoids and the associated accumulation of ROS, relative to individuals with flavonoids.

One key antioxidant feature of anthocyanins lies with their ability to retard further radical reactions through the delocalisation of unpaired electrons following a scavenging event (Van Acker *et al.*, 1996). It seems likely that in the cell, oxidised forms of anthocyanins would depend on interactions with other antioxidant molecules to restore their reducing capability. Indeed, recent work suggested that flavonoid radicals could be regenerated to their fully reduced state through an ascorbic acid/peroxidase system (Takahama and Oniki, 2000), or may be reduced directly by the enzyme monodehydroascorbate (MDHAR) reductase (Sakihama *et al.*, 2000).

The capacity of anthocyanins to scavenge ROS offers an explanation as to why they are most commonly associated with the chlorophyllous tissues in leaves (Lee and Collins, 2001), which are especially susceptible to oxidative damage (Halliwell and Gutteridge, 1998; Foyer *et al.*, 1994; Tsuda *et al.*, 1996 b). It also explains why anthocyanins often accumulate under stressful conditions (Chalker-Scott, 1999). Furthermore, as colourless anthocyanins are synthesised in close proximity to other metabolically active organelles, such as the mitochondria, peroxisomes, as well as chloroplasts (Hrazdina *et al.*, 1978), they possess the potential to swiftly scavenge membrane permeable ROS and thus prevent the oxidation of

biologically important compounds. Interestingly, recent work also indicates that anthocyanins might play a more direct role in preventing ROS generation in photosynthetic tissues. The shikimate pathway, which generates aromatic precursors for anthocyanin biosynthesis, is localised within plastids (Herrmann, 1995) and could provide an alternative electron sink for excess reducing power under conditions of stress. This would sustain the turnover of the photosynthetic apparatus and reduce photooxidative generation of ROS (Grace and Logan, 2000).

A build up of oxidised anthocyanins and other flavonoids in the cytoplasm is toxic to cellular processes (Debeaujon *et al.*, 2001), and usually leads to the formation of polymers, such as tannins (Larson, 1997). Glycosylation, which marks the last step in anthocyanin biosynthesis, both increases the water solubility of flavonoids in the cytoplasm (Markham, 1982; Rice-Evans *et al.*, 1997), and generally renders anthocyanins less reactive towards ROS (Tsuda *et al.*, 1996 b; Rice-Evans *et al.*, 1997). Therefore, subcellular transport to the vacuole via specific flavonoid-binding proteins (Mueller *et al.*, 2000) is likely to represent a mechanism to store potentially toxic or reactive radical intermediates (Marrs and Walbot, 1997; Debeaujon *et al.*, 2001).

Antioxidative protection of the vacuole

The presence of $O_2^{\cdot-}$ results in a marked loss of colour of flavylium forms of cyanidin-3-malonylglucoside (Fig. 7.4), in accordance with the findings of Yamasaki *et al.* (1996) for cyanidin at more neutral pH values. This 'bleaching' represents a significant reduction in the amounts of active anthocyanin available to scavenge $O_2^{\cdot-}$, as shown by the lower voltammetric currents in Fig. 7.5. Therefore, the bleaching of the characteristic red flavylium forms of anthocyanins, which occupy the low pH environment of the vacuole, is likely to corresponds to the scavenging of $O_2^{\cdot-}$.

The scavenging of ROS may not be the only function of anthocyanins located in the vacuole. Anthocyanins do not usually exist in isolation within the vacuole, but are instead stabilised by interactions with metals, other flavonoids, and/or acyl groups, which protects against nucleophilic attack of the central chromophore. These interactions effectively maintain the coloured flavylium form over a larger range of pH values (Brouillard, 1982; Figueiredo *et al.*, 1999). Consequently, aswell as providing antioxidant protection, this colour stability is

expected to be of importance in lessening the extent of photoinhibition and photooxidation in underlying tissues through the shielding of excess quanta (Chapter 4).

In conclusion, anthocyanins have the potential to serve as potent $O_2^{\cdot -}$ scavengers in both the cytoplasmic and vacuolar compartments. Sequestration of anthocyanins to the vacuole may represent a mechanism to store oxidised and condensed anthocyanins, which are known to be toxic to the cytoplasm (Martinoia *et al.*, 1993; Marrs *et al.*, 1995; Coleman *et al.*, 1997; Lu *et al.*, 1997; Marrs and Walbot, 1997; Debeaujon et al., 2001). Stabilisation of these products as flavylium forms is likely to confer photoprotection by shielding underlying chloroplasts from excess irradiance.

Numerous *in vitro* studies have suggested a role of anthocyanins *in vivo*. These include: i) diminishing the extent of oxidation of ascorbate by chelating metal ions (Sarma *et al.*, 1997); ii) inhibiting the formation of pyrimidine dimers by UV irradiation (Takahashi *et al.*, 1991); iii) form complexes with DNA to protect against $OH^{\cdot}$ induced damage (Sarma and Sharma, 1999); and iv) reducing the formation of malondialdehyde by UV irradiation in liposomal systems (Tsuda *et al.*, 1996 b). Certainly, *in vivo* localization studies suggest that flavonoids may be associated with a variety of other subcellular organelles, including the apoplast, chloroplast, and nucleus (Saunders and McClure, 1976; Ibrahim, 1992; Grandmaison and Ibrahim, 1996; Hutzler *et al.*, 1998). Anthocyanins therefore may serve a number of protective roles and could present widespread antioxidant protection throughout the plant cell.

CHAPTER 8 – General discussion

Anthocyanins are a group of highly modified flavonoid compounds, which constitute one of the most abundant classes of plant secondary metabolites. However, despite extensive knowledge of their biosynthesis and molecular control, their physiological function within leaves remains obscure. This study has presented evidence that anthocyanins provide multifaceted phytoprotection in leaves through; i) the absorption of visible radiation; and ii) the scavenging of free radicals and ROS. The implications of such versatile protection mechanisms, which are expressed in the leaves of certain species, at specific stages of leaf development, and/or under specific environmental conditions, are discussed further.

Anthocyanins absorb both UV and visible radiation. However, the fact that they are often located beneath the epidermis in most species, and that non-acylated anthocyanins absorb poorly in the 310-320 nm range indicate that UV protection is not a principle function. By contrast, the absorption of visible light by red-pigmented vacuoles has important implications for leaf physiology. Although we found no evidence of enhanced light capture through the back-scattering of red light by anthocyanins located in abaxial tissue, as suggested by Lee *et al*. (1979) and Lee (1986), we established that anthocyanins increase the absorbance of total PAR (400-700nm) by up to 17 %, depending on concentration (Chapter 3). This extra absorbance occurs primarily within the green-yellow waveband (500-600 nm) and is not subsequently available to chloroplasts (Gould, unpublished data). Thus, the accumulation of anthocyanins within leaves provides an effective mechanism to attenuate incident irradiance.

Anthocyanins are commonly associated with the photosynthetic tissue of leaves (McClure, 1975; Gould and Quinn, 1999; Lee and Collins, 2001) and are often accumulated during perturbations in mineral, nutrient, or water availability, or during conditions of sub- or above optimal, temperature, light, or UV. These same stresses are known to exacerbate the susceptibility of plants to photoinhibition (Powles, 1984) and photooxidation (Alscher *et al.*, 1997). In Chapter 4, we show that the photoabatement of high-energy quanta by anthocyanins serves two main functions (summarized in Fig. 8.1). First, an anthocyanic layer reduces the probability of light saturation in underlying chloroplasts, and effectively reduces the requirement for non-photochemical quenching through the xanthophyll cycle. This would

minimize wastages associated with photoinhibition and photorespiration. Secondly, the presence of a red-pigmented shield in adaxial tissue significantly reduces the generation of $O_2^{\cdot-}$ through the Mehler reaction. This diminishes the extent of $O_2^{\cdot-}$ associated damage, such as the degradation of the PSII D1 reaction centre proteins and the bleaching of chlorophyll. The specific attenuation of predominantly green light may represent an evolutionary compromise between the beneficial elimination of free radicals and ROS from photooxidative processes, and the effective maintenance of CO_2 fixation. However, under light-limiting conditions, the attenuation of green light would reduce photosynthetic yield, which has implications especially for the growth of shade plants. Under certain situations, the reduced incidence of photoinhibition and the enhanced protection from free radical damage could outweigh losses associated with a diminished photosynthetic capacity. This would especially benefit plants that are venerable to high intensity sun flecks, or plants experiencing constantly elevated irradiances, such those inhabiting alpine and tundra areas. In addition, the synthesis of anthocyanins at critical stages of plant development, such as during leaf expansion, leaf senescence, is likely to provide protection from excess quanta during the assembly or dismantling of photosynthetic apparatus.

In addition to their shielding effects, anthocyanins are also effective electron donors and serve as potent scavengers of free radicals and ROS (Bors *et al.*, 1990; Rice-Evans *et al.*, 1995 a; Rice-Evans *et al.*, 1997). Our evidence indicates that this potential of the anthocyanins can be realised both at the sites of origin (at cytosolic pH values), as well as site of storage (at acidic pH) (Chapter 7). The colourless forms of anthocyanins provide an effective mechanism to directly scavenge potentially damaging radicals. Purified cyanidin-3-malonylglucoside can effectively scavenge superoxide radicals (probably as the protonated form, $HO_2^{\cdot}$) generated by photooxidative processes by chloroplast suspensions at pH 7.0 (indicative of the cytoplasm).

It has been postulated that once an anthocyanin molecule has scavenged a radical, the unpaired electron is delocalised throughout the molecule, thereby retarding further radical reactions (Van Acker *et al.*, 1996). Anthocyanins might then be regenerated by either i) reduction through an ascorbic acid/peroxidase system (Takahama and Oniki, 2000); ii) the action of monodehydroascorbate reductase (Sakihama *et al.*, 2000); or iii) polymerisation with other radicals, e.g. to form tannins (Larson, 1997). The build-up of such oxidised phenolic compounds is known to be toxic to the cytoplasm (Debeaujon *et al.*, 2001). Glycosylation,

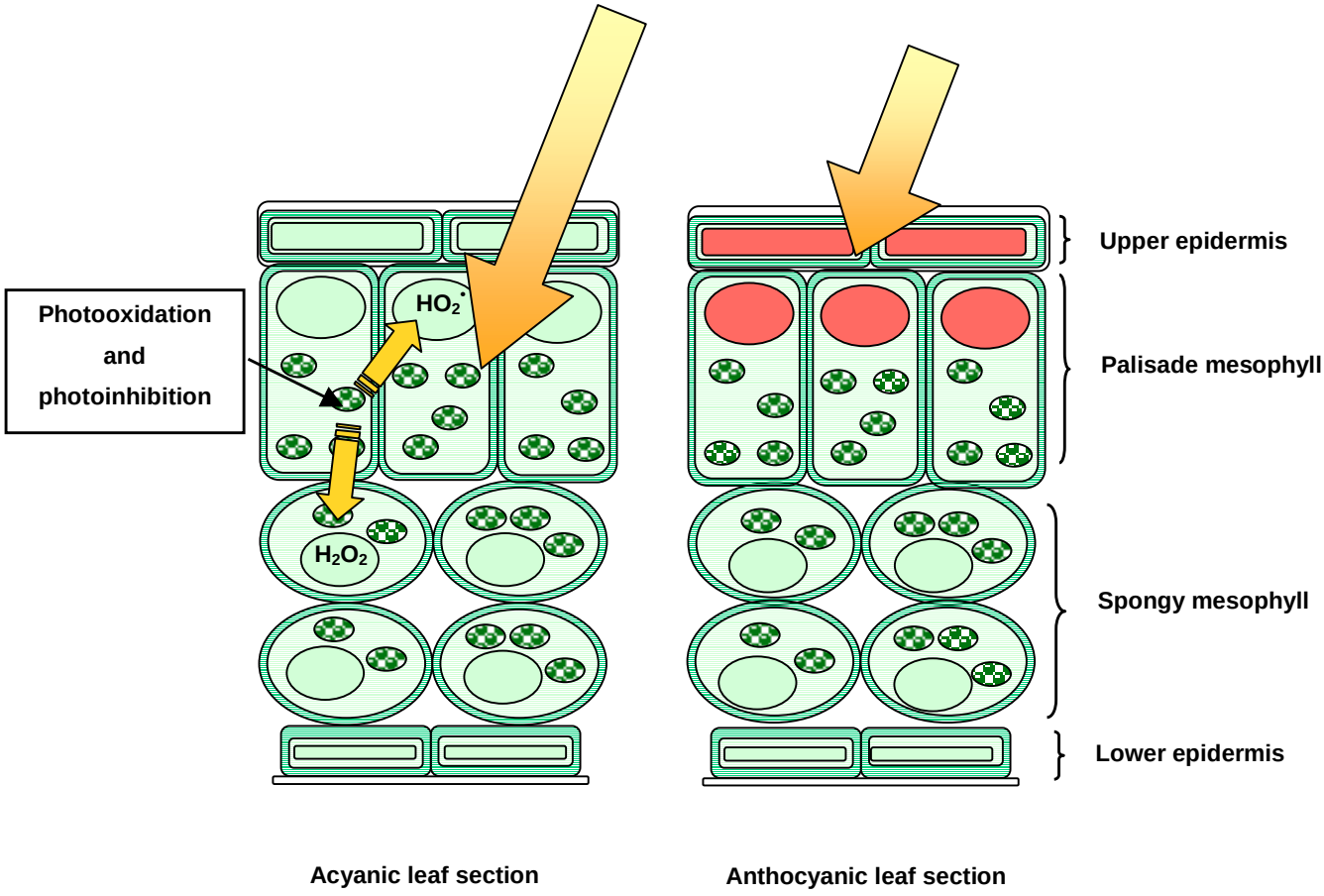


Fig. 8.1 A simplified diagram illustrating the reduction of quanta incident on underlying chloroplasts in an anthocyanic leaf relative to an acyanic leaf. In the anthocyanic leaf, the internal light gradients are more marked as a result of anthocyanic light-filtering, predominantly in the green wavebands, as shown by the large arrows. Such a reduction in total PAR absorptance represents an effective mechanism to reduce the occurrence of photoinhibition and ROS generation through photooxidative processes within the chloroplasts.

which marks the last step of anthocyanin biosynthesis, increases the water solubility of the anthocyanins and permits them to be translocated to the vacuole, and therefore may represent a method of storing anthocyanins polymers and/or reactive radical intermediates.

Anthocyanins, extracted from two native New Zealand plant species displayed strong antioxidant potentials at both neutral and acidic pH values (Chapter 6). Indeed, generation of $O_2^{\cdot -}$ resulted in the rapid oxidation and loss of the characteristic red colour of cyanidin-3-malonylglucoside at low pH values (Chapter 7). Together, these findings indicate that anthocyanins in their flavylium forms are likely to serve an antioxidant role in the vacuole. As anthocyanins have also been suggested to inhibit lipid peroxidation (Chauhan *et al.*, 1992; Jovanovic *et al.*, 1994), they may help to prevent the rupture of the tonoplast membrane, thus safeguarding against the release of highly acidic hydrolytic enzymes and toxic compounds into the cytoplasm.

As well as the ability of anthocyanins to directly scavenge radical species (as shown here for $O_2^{\cdot -}$), they may also serve as the substrate for the scavenging of H_20_2 by general peroxidases (Takahama, 1989; Takahama and Oniki, 1997; Yamasaki *et al.*, 1997; Yamasaki and Grace, 1998). High concentrations of general peroxidases, such as guaiacol peroxidase occur within both the cytoplasm and the vacuole (Polle, 1997). A final antioxidant feature of the anthocyanins lies within their ability to chelate transition metal ions, thereby reducing the formation of potentially damaging hydroxyl radicals ($OH^{\cdot}$) through Fenton-Haber-Weiss reactions (Van Acker *et al.*, 1996; Brown *et al.*, 1998; Noda *et al.*, 1998). Thus, anthocyanins have the potential to regulate oxidative damage by interrupting radical-driven chain reactions, such as localised lipid peroxidation events, and under severe conditions of stress, by scavenging radicals and/or ROS leaked from organelles such as the chloroplast, mitochondria, and peroxisomes in the cytoplasm (see Fig. 8.2). They are likely to constitute a significant component of the low molecular weight antioxidant (LMWA) pool and would be expected to act in concert with other established antioxidant systems within the cell to provide complementary protection.

Examination of anthocyanin accumulation in polymorphic native New Zealand plant species has yielded evidence for both the photoprotective and antioxidant hypotheses. In *Elatostema rugosum*, juvenile leaves of anthocyanic morphs were better equipped to scavenge ROS than

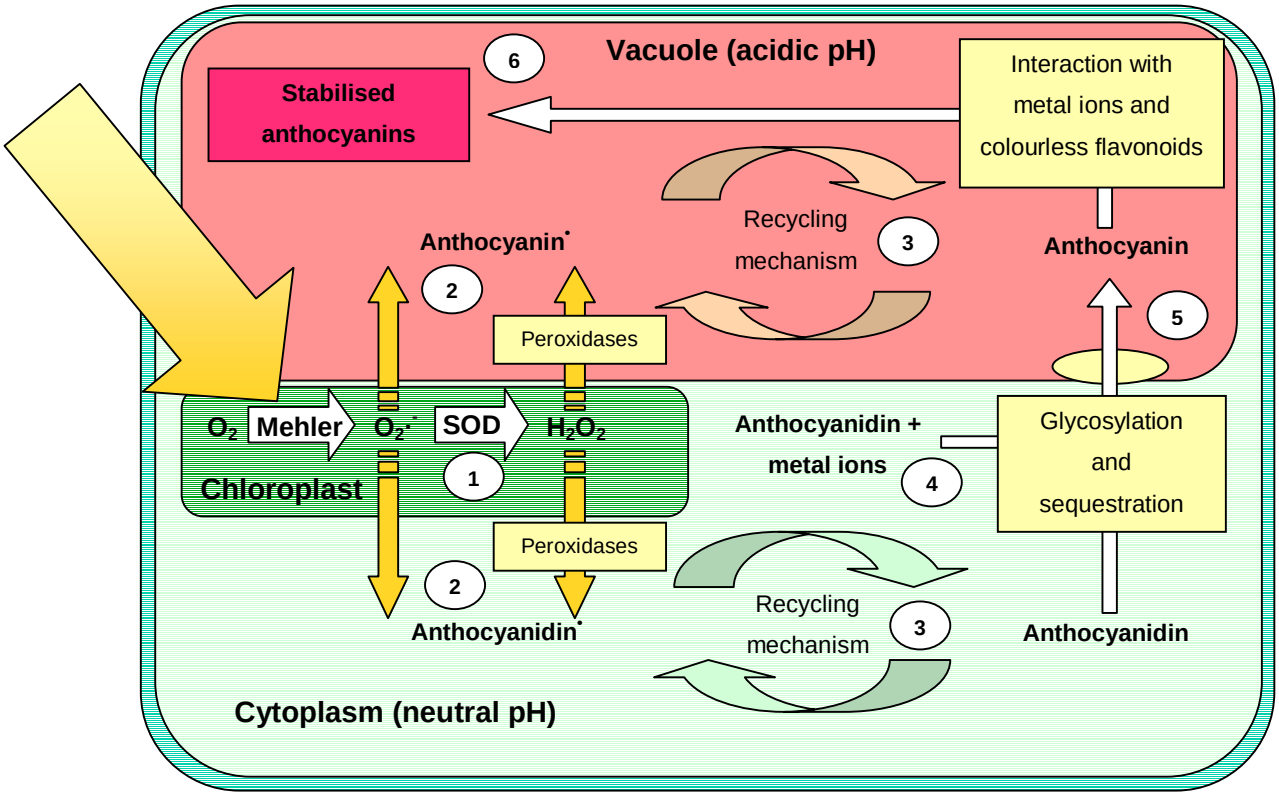


Fig. 8.2 Summary diagram illustrating the potential intracellular antioxidative roles of anthocyanins within a plant cell. 1) Excessive excitation of the chloroplast can lead to the formation of damaging ROS such as superoxide ($O_2^{\bullet-}$) through the Mehler reaction. Conversion of $O_2^{\bullet-}$ by superoxide dismutase (SOD) leads to the production of hydrogen peroxide (H_2O_2). Both H_2O_2 and $O_2^{\bullet-}$ (in its protonated form $HO_2^{\bullet}$) can cross membranes and present the cytoplasm and vacuole with an increased oxidative burden. 2) Colourless anthocyanidins/anthocyanins and red forms of anthocyanins are able to directly scavenge $O_2^{\bullet-}$, and act as a substrate for H_2O_2 scavenging by peroxidases. 3) Anthocyanin radicals can be regenerated through reduction with an ascorbate/peroxidase system, through the action of monodehydroascorbate reductase, or stabilised by the polymerisation of another radical intermediate. 4) Chelation of transition metal ions by anthocyanins reduces the generation of ROS through Haber-Weiss-Fenton reactions. 5) Glycosylation and sequestration of anthocyanins into the vacuole is a mechanism for removing oxidised and/or polymers of anthocyanin which are toxic to the cytoplasm. 6) Stabilisation of these anthocyanins with other flavonoids and/or metals maintains strong coloration, which indirectly reduces the formation of ROS in underlying chloroplasts through the shielding of excess quanta.

were those from acyanic morphs (Chapter 6). Furthermore, anthocyanins contributed a greater percentage to the overall LMWA pool than all other constituent phenolics, as shown by the higher IC_{50} values, and thus indicating their primary role of anthocyanin as an antioxidant. The presence of anthocyanins in shade-adapted leaves appears to enhance ROS scavenging and shields subjacent chloroplasts, enabling *E. rugosum* to occupy more exposed areas of the forest floor.

In contrast, red and acyanic leaves of the canopy tree *Quintinia serrata* are likely to experience comparative levels of photooxidative stress (Gould *et al.*, 2000) and as shown in Chapter 6, exhibit comparable antioxidant activities. In this case, anthocyanins do not appear to be a prerequisite for antioxidant protection, but certainly enhanced the overall capacity of the LMWA pool. As anthocyanins are not translocated by the vascular system (McClure, 1975), and only accumulate in the cells in which they are synthesised, the specific accumulation of anthocyanins in discrete cells or groups of cells may represent a mechanism to reduce the number of photons incident on a cell's photosynthetic apparatus. Hence, the primary role of anthocyanins may be to shield chloroplasts, which are as a result of herbivore damage, or mineral deficiency predisposed to photoinhibition and the generation of ROS.

One may question why the leaves of most terrestrial plants are green, when the accumulation of anthocyanic pigments apparently offers such clear physiological benefits. It seems likely that in the course of evolution, plants have developed disparate mechanisms in order to accommodate environmental stressors. Indeed, it may be argued that the ability of anthocyanins to attenuate light is not a unique function, as the presence of carotenoids, and betacyanins all effectively reduce PAR transmittance to underlying tissues. Likewise, the antioxidant function of anthocyanins is not a specialized role, but is instead a general feature of many plant phenolics (Takahama, 1988; Castelluccio *et al.*, 1995). Most phenolic compounds are colourless, which explains why a large number of leaves appear green.

Apart from the light shielding effects, our findings for anthocyanins are consistent with the postulated functions of other flavonoids which are also up-regulated as a general response to biotic and abiotic stressors (see reviews by Stafford (1990); Koes *et al.* (1994); Dixon and Paiva (1995); and Harborne and Williams (2000). Recent evidence suggests that phenolic precursors generated within the chloroplast via the shikimate pathway (Herrmann, 1995) and

which are used later in the assembly of anthocyanins, may provide an alternative electron sink for metabolic reducing power. This could have the effect of sustaining turnover of the photosynthetic apparatus under conditions of stress (Grace and Logan, 2000). It is known that levels of carbohydrates can become excessive during environmental stress, which can impair phosphate recycling in the chloroplasts, and thus reducing the availability of acceptors for photosynthetic electron transport (Grace and Logan, 2000). The possibility exists that anthocyanins, which are also triggered by high carbohydrate levels (Cormier *et al.*, 1989; Murray and Hackett, 1991; Suzuki, 1995; Russain *et al.*, 1996), can alleviate increased rates of ROS produced by the chloroplasts as a result of insufficient electron sinks (Osmond and Grace, 1995) by providing an 'overflow' pathway for excess captured light energy. As anthocyanins represent an energetically expensive compound to synthesise, they may well be favoured over other flavonoid compounds to utilise a greater amount of reducing power. Furthermore, as anthocyanins are responsible for reducing photoinhibition, photorespiration, photooxidation, possess the ability to scavenge radicals and/or ROS, and to

Whilst the postulated roles of anthocyanins within leaves presented here seem to explain their occurrence in the leaves of at least two naturally occurring species, there are almost certainly exceptions. Such cases would warrant further investigation. Experiments exploring the phytoprotective hypotheses of anthocyanins *in vivo* would be of significant merit. Such experiments might include:

i) The use of probes and/or spin traps specific for ROS.

As plant cells contain a suite of enzymatic and LMWAs, it is uncertain how anthocyanins interact with ROS, whether they are depleted under conditions of stress, and what biomolecules they actually protect. In order to investigate the antioxidant activity of anthocyanins *in vivo* a number of compounds are available, that in the presence of various ROS cause detectible changes in the compound's fluorescence or absorbance characteristics in proportion to the amount of ROS present. Measuring ROS and quantifying scavenging is technically very challenging. Delicate infiltration of specific ROS probes, such as Amplex Red, dichlorofluorescein, and scopoletin may aid the detection of transient ROS and minimise their creation through mechanical stress (Allan and Fluhr, 1997). However, the presence of naturally occurring fluorescent and coloured compounds, such as flavonoids and anthocyanins respectively, often interfere and even mask probe signals. A more feasible method of

monitoring the antioxidant function of anthocyanins may involve the use of *in vivo* NMR and chromotropic nitrone spin traps recently developed at Florida International University, USA (Becker, 1996). Such a method would be of particular use to compare the competitive scavenging activities of anthocyanins within intact red and green areas of the same leaf. Interfering coloured compounds, such as the anthocyanins, would not interfere with the NMR measurements. In addition, stable spin adducts formed through interactions with radicals can be extracted and analysed using electron paramagnetic resonance to make distinctions regarding the specific radical species involved.

ii) The use of transgenic plants.

The role of anthocyanins in reducing photooxidation and photoinhibition might be further investigated through the use of transgenic plants. Flavonoid biosynthesis is well studied and a large number of the genes encoding the biosynthetic enzymes have been determined. Various plant species have been transformed to produce anthocyanic leaves, for example the gene for maize leaf colour (Lc) has been successfully introduced into Petunia (cv. Mitchell). However, these transformants also exhibit marked up-regulation of several other flavonoid specific biosynthetic genes (Marie Bradley *et al.*, 1998). A superior system would involve the silencing of a relatively late occurring biosynthetic gene (e.g anthocyanidin synthase) in a naturally anthocyanic plant, then comparing the photosynthetic parameters, and the competitive advantages of acyanic mutants and wild-type plants. Even in this idealised system, care must be taken to ensure downstream flavonoid compounds such as the flavonols are not up regulated as a consequence of a 'blocked' pathway, as an increase in such compounds could complicate any differences.

REFERENCES

Adachi, M., and Shimokawa, K. 1995. Evidence for the involvement of superoxide anion in the ethylene-induced chlorophyll *a* catabolism of *Raphanus sativus* cotyledons. *Phytochemistry* 39: 527-530.

Ahmed, M. S., Ainley, K., Parish, J. H., and Hadi, S. M. 1994. Free radical-induced fragmentation of proteins by quercetin. *Carcinogenesis* 15: 1627-1630.

Alfenito, M. R., Souer, E., Goodman, C. D., Buell, R., Mol, J., Koes, R., and Walbot, V. 1998. Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione S-transferases. *Plant Cell* 10: 1135-1149.

Allan, A. C., and Fluhr, R. 1997. Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. *Plant Cell* 9: 1559-1572.

Allen, H. H. 1961. *Flora of New Zealand*, Volume 1. Government Printer, Wellington. Pp 1085.

Alscher, R. G., Donahue, J. L., and Cramer, C. L. 1997. Reactive oxygen species and antioxidants: relationships in green cells. *Physiologia Plantarum* 100: 224-233.

Anderson, J. M. 1986. Photoregulation of the composition, function and structure of thylakoid membranes. *Annual Review of Plant Physiology* 37: 93-136.

Anderson, J. M., Chow, W. S., and Park, Y. 1995. The grand design of photosynthesis: acclimation of the photosynthetic apparatus to environmental cues. *Photosynthesis Research* 46: 129-139.

Anderson, J. M., and Osmond, C. B. 1987. Shade-sun responses: compromises between acclimation and photoinhibition. In Kyle, D. J., Osmond, C. B., and Arntzen, C. J., eds. *Photoinhibition*. pp 1-38. Elsevier, Amsterdam.

Asada, K. 1994. Production and action of active oxygen in photosynthetic tissue. In Foyer, C. H. and Mullineaux, P., eds. *Causes of photooxidative stress and amelioration of defence systems in plants*. pp 77-104. CRC Press, Boca Raton.

Asada, K. 1999. The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. *Annual Review of Plant Physiology and Plant Molecular Biology* 50: 601-639.

Asada, K., and Takahashi, M. 1987. Production and scavenging of active oxygen in photosynthesis. In Kyle, D. J., Osmond, C. B., and Artntzen, C. J., eds. *Photoinhibition*. pp 227-288. Elsevier, Amsterdam.

Asen, S., Stewart, R. N., and Norris, K. H. 1972. Co-pigmentation of anthocyanins in plant tissues and its effect on colour. *Phytochemistry* 11: 1139-1144.

Asen, S., Stewart, R. N., and Norris, K. H. 1976. Anthocyanin, flavonol copigments, and pH responsible for larkspur flower colour. *Phytochemistry* 14: 2677-2682.

Auclair, C., and Voisin, E., 1985. Nitroblue tetrazolium reduction. In Greenwald, R. A., ed. *CRC Handbook of methods of oxygen radical research*. pp. 123-132. CRC Press, Boca Raton.

Babior, B.M., Kipnes, R. S., and Curnette, J. T. 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *Journal of Clinical Investigation* 52: 741-744.

Balakumar, T., Hani Babu Vincent, V., and Paliwal, K. 1993. On the interaction of UV-B radiation (280-315 nm) with water stress in crop plants. *Physiologia Plantarum* 87: 217-222.

Barabas, K. N., Szegletes, Z., Pestenacz, A., Fulop, K., and Erdei, L. 1998. Effects of excess UV-B irradiation on the antioxidant defence mechanisms in wheat (*Triticum aestivum* L.) seedlings. *Journal of Plant Physiology* 153: 146-153.

Barker, D. H., Seaton, G. G. R., and Robinson, S. A. 1997. Internal and external photoprotection in developing leaves of the CAM plant *Cotyledon orbiculata*. *Plant, Cell and Environment* 20: 617-624.

Beauchamp, C., and Fridovich, I. 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry* 44: 276-287.

Becker, D. A. 1996. Highly sensitive colorimetric detection and facile isolation of diamagnetic free radical adducts of novel chromotrophic nitrone spin trapping agents readily derived from guaiazulene. *Journal of the American Chemical Society* 118: 905-906.

Beggs, C. J., and Wellmann, E. 1985. Analysis of light-controlled anthocyanin formation in coleoptiles of *Zea mays* L: the role of UV-B, blue, red, and far red light. *Photochemistry and Photobiology* 41: 481486.

Beggs, C. J., and Wellmann, E. 1994. Photocontrol of flavonoid biosynthesis. In Kendrick, R. E. and Kronenberg, G. H. M., eds. *Photomorphogenesis in Plants*. pp 733-750. Kluwer, Dordrect, The Netherlands.

Beyer, W. F., and Fridovich, I. 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Analytical Biochemistry* 161: 559-566.

Biran, I., and Halevy, A. H. 1974. Effect of varying light intensities and temperature treatments applied to whole plants, or locally to leaves or flower buds, on growth and pigmentation of 'Braccara' roses. *Physiologia Plantarum* 31: 175-179.

Blois, M. S. 1958. Antioxidant determination by the use of a stable free radical. *Nature* 181: 1199-1200.

Bolhar-Nordenkampf, H. R., and Oquist, G. 1993. Chlorophyll fluorescence as a research tool in photosynthesis research. In Hall, E. D., Scurlock, J. M. O., Bolhar-Nordenkampf, H. R., Leegood, R. C., and Long, S. P., eds. *Photosynthesis and production in a changing environment - a field and laboratory guide*. pp 193-206. Chapman and Hall, London.

Bongue-Bartelsman, M., and Phillips, D. A. 1995. Nitrogen stress regulates gene expression of enzymes in the flavonoid biosynthetic pathway of tomato. *Plant Physiology and Biochemistry* 33: 539-546.

Bors, W., Heller, W., Michel, C., and Saran, M. 1990. Flavonoids as antioxidants: determination of radical-scavenging efficiencies. *Methods in Enzymology* 186: 343-355.

Bors, W., Michel, C., and Saran, M. 1994. Flavonoid antioxidants: rate constants for reactions with oxygen radicals. *Methods in Enzymology* 234: 420-437.

Botha, C. E. J., Cross, R. H. M., Van Bel, A. J. E., and Peter, C. I. 2000. Phloem loading in the sucrose-export-defective (*SXD-1*) mutant maize is limited by callulose deposition at plasmodesmata in bundle sheath-vascular parenchyma interface. *Protoplasma* 214: 65-72.

Bowler, C., Van Camp, W., Van Montagu, M., and Inzé, D. 1994. Superoxide dismutases in plants. *Critical Reviews in Plant Science* 13: 199-218.

Boyer, M., Miller, J., Belanger, M., Hare, E., and Wu, J. 1988. Senescence and spectral reflectance in leaves of Northern Pine Oak (*Quercus palustris* Muenchh). *Remote Sensing of Environment* 25: 71-87.

Brandt, K., Giannini, A., and Lercari, B. 1995. Photomorphogenic responses to UV radiation III: a comparative study of UV-B effects on anthocyanin and flavonoid accumulation in wild-type and *aurea* mutant of tomato (*Lycopersicon esculentum* Mill.). *Photochemistry and Photobiology* 62: 1081-1087.

Brouillard, R. 1982. Chemical structure of anthocyanins. In Markakis, P., ed. *Anthocyanins as food colours*. pp 1-40. Academic Press, New York.

Brouillard, R. 1988. Flavonoids and flower colour. In Harborne, J. B., ed. *The Flavonoids: advances in research since 1980*. pp 525-538. Chapman and Hall, London.

Brouillard, R., and Dangles, O. 1993. Flavonoids and flower colour. In Harborne, J. B., ed. *The Flavonoids: Advances in Research since 1986*. pp 565-588 Chapman and Hall, London.

Brouillard, R., and Dangles, O. 1994. Anthocyanin molecular interactions - the first step in the formation of new pigments during wine aging. *Food Chemistry* 51: 365-371.

Brouillard, R., and Delaporte, B. 1977. Chemistry of anthocyanin pigments. 2. Kinetic and thermodynamic study of proton transfer, hydration, and tautomeric reactions of malvidin 3-glucoside. *Journal of American Chemical Society* 99: 8461-8468.

Brown, J. E., Khodr, H., Hider, R. C., and Rice-Evans, C. A. 1998. Structural dependence of flavonoid interactions with Cu^{2+} ions: Implications for their antioxidant properties. *Biochemical Journal* 330: 1173-1178.

Bueno, P., Varela, J., Gimeenez-Gallego, G., and Del Rio, L. A. 1995. Peroxisomal copper, zinc superoxide dismutase. Characterization of the isoenzyme from watermelon cotyledons. *Plant Physiology* 108: 1151-1160.

Burchard, P., Bilger, W., and Weissenböck, G. 2000. Contribution of hydroxycinnamates and flavonoids to epidermal shielding of UV-A and UV-B radiation in developing rye primary leaves as assessed by ultraviolet-induced chlorophyll fluorescence measurements. *Plant, Cell and Environment* 23: 1373-1380.

Burger, J., and Edwards, G. 1996. Photosynthetic efficiency, and photodamage by UV and visible radiation, in red versus green leaf *Coleus* varieties. *Plant Cell Physiology* 37: 395-399.

Caldwell, M. M., Robbertecht, R., and Flint, S. D. 1983. Internal filters: prospects for UV-acclimation in higher plants. *Physiologia Plantarum* 58: 445-450.

Castelluccio, C., Paganga, G., Melikian, N., Bolwell, G. P., Pridham, J., Sampson, J., and Rice-Evans, C. 1995. Antioxidant potential of intermediates in phenylpropanoid metabolism in higher plants. *Federation of European Biochemical Societies Letters* 368: 188-192.

Chalker-Scott, L. 1999. Environmental significance of anthocyanins in plant stress responses. *Photochemistry and Photobiology* 70: 1-9.

Chauhan, N. P., Fatma, T., and Mishra, R. K. 1992. Protection of wheat chloroplasts from lipid peroxidation and loss of photosynthetic pigments by quercetin under strong illumination. *Journal of Plant Physiology* 140: 409-413.

Chevion, S., Chevion, M., Boon Chock, P., and Beecher, G. R. 1999. Antioxidant capacity of edible plants: extraction protocol and direct evaluation by cyclic voltammetry. *Journal of Medicinal Food* 2: 1-10.

Choinski, J. S., and Johnson, J. M. 1993. Changes in photosynthesis and water status of developing leaves of *Brachystegia spiciformis* Benth. *Tree Physiology* 13: 17-27.

Christie, P. J., Alfenito, M. R., and Walbot, V. 1994. Impact of low temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta* 194: 541-549.

Coleman, J. O. D., Randall, R., and Blake Kalff, M. M. A. 1997. Detoxification of xenobiotics in plant cells by glutathione conjugation and vascular compartmentalization: a fluorescent assay using monochlorobimane. *Plant Cell & Environment* 20: 449-460.

Coley, P. D., and Aide, M. T. 1989. Red coloration of tropical young leaves: a possible antifungal defence? *Journal of Tropical Ecology* 5: 293-300.

Cooper-Driver, G. A., and Bhattacharya, M. 1998. Role of phenolics in plant evolution. *Phytochemistry* 49: 1165-1174.

Cormier, F. 1997. Food colourants from plant cell cultures. In Romeo, T., ed. *Functionality of food phytochemicals - recent advances in phytochemistry*. pp 201-218. Plenum Press, London.

Cormier, F., Crevier, H. A., and Do, C. B. 1989. Effects of sucrose concentration on the accumulation of anthocyanins in grape (*Vitis vinifera*) cell suspension. *Canadian Journal of Botany* 68: 1822-1825.

Costa-Arbulú, C., Gianoli, E., Gonzáles, W. L., and Niemeyer, H. M. 2001. Feeding by the aphid *Sipha flava* produces a reddish spot on leaves of *Sorghum halepense*: an induced defence? *Journal of Chemical Ecology* 27: 273-283.

Creelman, R. A., Tierney, M. L., and Mullet, J. E. 1992. Jasmonic acid/methyl jasmonate accumulation in wounded soybean hypocotyls and modulate wound gene expression. *Proceedings of the National Academy of Science* 89: 4938-4941.

Czichi, U., and Kindl, H. 1975. Formation of *p*-coumaric acid and *o*-coumaric acid from L-phenylalanine by microsomal membrane fractions from potato: evidence of membrane-bound enzyme complexes. *Planta* 125: 115-125.

Dangles, O., Elhabiri, M., and Brouillard, R. 1994. Kinetic and thermodynamic investigation of the aluminium-anthocyanin complexation in aqueous solution. *Journal of American Chemical Society - Perkin Transactions II* 12: 2587-2596.

Dangles, O., Saito, N., and Brouillard, R. 1993. Anthocyanin intramolecular copigment effect. *Phytochemistry* 34: 119-124.

Das, D. K. 1994. Naturally occurring flavonoids: structure, chemistry, and high-performance liquid chromatography methods for separation and characterization. *Methods in Enzymology* 234: 410-420.

Davies, J. M. 1997. The bioenergetics of vacuolar H^+ pumps. In Leigh, R. A. and Sanders, D., eds. *The plant vacuole*. Academic Press, New York.

Davies, K. M. 2000. Plant colour and fragrance in Verpoorte, R. and Alfermann, A. W., eds. *Metabolic engineering of plant secondary metabolism*. pp. 127-163. Kluwer Academic, Dordrecht.

Day, T. A. 1993. Relating UV-B radiation screening effectiveness of foliage to absorbing-compound concentration and anatomical characteristics in a diverse group of plants. *Oecologia* 95: 542-550.

De Majnik, J., Weinman, J., Djordjevic, M., Rolfe, B., Tanner, G., Joeseph, R., and Larkin, P. 2000. Anthocyanin regulatory gene expression in transgenic white clover can result in an altered pattern of pigmentation. *Australian Journal of Plant Physiology* 27: 659-667.

Debeaujon, I., Peeters, A. J. M., Léon-Kloosteriel, K. M., and Koorneef, M. 2001. The TRANSPARENT TESTA 12 gene of *Arabidopsis* encodes a multidrug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. *Plant Cell* 13: 853-871.

Dedaldechamp, F., Uhel, C., and Macheix, J.-J. 1995. Enhancement of anthocyanin synthesis and dihydroflavonol reductase (DFR) activity in response to phosphate deprivation in grape cell suspensions. *Phytochemistry* 40: 1357-1360.

Deikman, J., and Hammer, P. E. 1995. Induction of anthocyanins by cytokinins in *Arabidopsis thaliana*. *Plant Physiology* 108: 47-57.

Del Rio, L. A., Ortega, M. G., Lopez, A. L., and Gorge, J. L. 1977. A more sensitive modification of the catalase assay with the clark oxygen electrode. *Annals of Biochemistry* 80: 409-415.

Demmig-Adams, B. 1990. Carotenoids and photoprotection in plants: a role for the xanthophyll zeaxanthin. *Biochimica et Biophysica Acta* 1020: 1-24.

Demmig-Adams, B., and Adams III, W. W. 1991. Light, photosynthesis, and the xanthophyll cycle. In Pell, E. and Steffen, K., eds. *Active oxygen/oxidative stress and plant metabolism*. pp 171-179. American Society of Plant Physiologists, Pennsylvania.

Demmig-Adams, B., Adams III, W. W., and Grace, S. C. 1997. Physiology of light tolerance in plants. In Janick, J., ed. *Horticulture reviews*. pp 215-246. John Wiley & Sons, Inc., New York.

Demmig-Adams, B., and Adams III, W. W. 2000. Harvesting sunlight safely. *Nature* 403: 371-374.

Deng, H., and Berkel, V. 1998. Electrospray mass spectrometry and UV/visible spectrophotometry studies of aluminium (III)-flavonoid complexes. *Journal of Mass Spectrometry* 33: 1080-1087.

Dixon, R. A., Harrison, M. J., and Lamb, C. J. 1994. Early events in the activation of plant defence responses. *Annual Review of Phytopathology* 32: 479-501.

Dixon, R. A., and Paiva, N. L. 1995. Stress-induced phenylpropanoid metabolism. *Plant Cell* 7: 1085-1097.

Dodd, I. C., Critchley, C., Woodall, G. S., and Stewart, G. R. 1998. Photoinhibition in differently coloured juvenile leaves of *Syzygium* species. *Journal of Experimental Botany* 49: 1437-1445.

Doke, N., Miura, Y., Leandro, M. S., and Kawakita, K. 1994. Involvement of superoxide in signal transduction: responses to attack by pathogens, physical and chemical shocks and UV-irradiation. In Foyer, C. H. and Mullineaux, P. eds. *Causes of photooxidative stress and amelioration of defence systems in plants*. pp. 177-197..CRC Press Inc, Boca Raton.

Dominy, N. J., and Lucas, P. W. 2001. Ecological importance of trichromatic vision to primates. *Nature* 410: 363-366.

Dooner, H. K., and Robbins, T. P. 1991. Genetic and developmental control of anthocyanin biosynthesis. *Annual Review of Genetics* 25: 173-199.

Ehleringer, J. R., and Björkmann, O. 1978. Pubescence and leaf spectral characteristics in a desert shrub, *Encelia farinosa*. *Oecologia* 36: 151-162.

Eller, B. M., Glättli, R., and Flach, B. 1981. Optical properties and pigments of sun and shade leaves of the Beech (*Fagus silvatica* L.) and the Copper Beech (*Fagus silvatica* cv. *Atropunicea*). *Flora* 171: 170-185.

Englemann, T. W. 1882. Uber sauerstoffausscheidung von pflanzenzellen im mikrospecrum. *Botanische Zeitung* 40: 419-426.

Evans, J. R., Jakobsen, I., and Ogren, E. 1993. Photosynthetic light-response curves. 2. Gradients of light absorption and photosynthetic capacity. *Planta* 189: 191-200.

Feder, N., and O'Brien, T. P. 1968. Plant microtechnique: some principles and new methods. *American Journal of Botany* 55: 123-142.

Feild, T. S., Lee, D. W., and Michele Holbrook, N. 2001. Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. *Plant Physiology* 127: 566-574.

Fergusson, L. R. 2001. Role of plant polyphenols in genomic stability. *Mutation Research* 475: 89-111.

Ferreres, F., Gil, M. I., Castaner, M., and Thomas-Barberan, F. A. 1997. Phenolic metabolites in red pigmented lettuce (*Lactuca sativa*). Changes with minimal processing and cold storage. *Journal of Agriculture & Food Chemistry* 45: 4249-4254.

Figueiredo, P., George, F., Tatsuzawa, F., Toki, K., Saito, N., and Brouillard, R. 1999. New features of intramolecular copigmentation by acylated anthocyanins. *Phytochemistry* 51: 125-132.

Forkmann, G. 1994. Genetics of flavonoids. In Harborne, J. B., ed. *The Flavonoids: Advances in research since 1986*. pp 537-564. Chapman and Hall, London.

Foyer, C. H. 1993. Ascorbic acid. In Alscher, R. G. and Hess, J. L., eds. *Antioxidants in Higher Plants*. pp 31-58. CRC Press Inc., Boca Raton.

Foyer, C. H. 1997. Oxygen metabolism and electron transport in photosynthesis. In Scandalios, J., ed. *Molecular biology of free radical scavenging systems*. pp 587-621. Cold Spring Harbor Laboratory Press, New York.

Foyer, C. H., Lelandais, M., and Kunnert, K. J. 1994. Photoxidative stress in plants. *Physiologia Plantarum* 92: 696-717.

Foyer, C. H., Lopez-Delgado, H., Dat, J. F., and Scott, I. M. 1997. Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. *Physiologia Plantarum* 100: 241-254.

Foyer, C. H., and Noctor, G. 2000. Oxygen processing in photosynthesis: regulation and signalling. *New Phytologist* 146: 359-388.

Franceschi, V. R., and Grimes, H. D. 1991. Induction of soybean vegetative storage proteins and anthocyanins by low-level atmospheric methyl jasmonate. *Proceedings of the National Academy of Sciences* 88: 6745-6749.

Frankel, E. N., Waterhouse, A. L., and Teissedra, P. L. 1995. Principal phenolic phytochemicals in selected California wine and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins. *Journal of Agricultural & Food Chemistry* 43: 890-894.

Fridovich, I. 1995. Superoxide radical and superoxide dismutases. *Annual Review of Biochemistry* 64: 97-112.

Gabrielska, J., Oszmianski, J., Komorowska, M., and Langner, M. 1999. Anthocyanin extracts with antioxidant radical scavenging effect. *Zeitschrift für Naturforschung* 54 c: 314-324.

Garcia-Plazaola, J. I., Artetxe, U., and Becerril, J. M. 1999. Diurnal changes in antioxidant and carotenoid composition in the Mediterranean sclerophyll tree *Quercus ilex* (L) during winter. *Plant Science* 143: 125-133.

Gausman, H. W. 1982. Visible light reflectance, transmittance, and absorptance of differently pigmented cotton leaves. *Remote Sensing of Environment* 13: 233-238.

Genty, B., Briantais, J.-M., and Baker, N. R. 1989. The relationship between quantun yield of photosynthetic electron transport and the quenching of chlorophyll fluorescence. *Biochemica et Biophysica Acta* 990: 87-92.

George, F., Figueiredo, P., and Brouillard, R. 1999. Malvin Z-chalcone: An unexpected new open cavity for the ferric cation. *Phytochemistry* 50: 1391-1394.

Ghirardi, M. L., and Melis, A. 1984. Photosystem electron-transport capacity and light-harvesting antenna size in maize chloroplasts. *Plant Physiology* 21: 84-99.

Ghiselli, A., Nardini, M., Baldi, A., and Scaccini, C. 1998. Antioxidant activity of different phenolic fractions separated from an Italian red wine. *Journal of Agricultural & Food Chemistry* 46: 361-367.

Givnish, T. J. 1990. Leaf mottling: relation to growth form and leaf phenology and possible role as camouflage. *Functional Ecology* 4: 463-474.

Gorton, H., and Vogelmann, T. C. 1996. Effects of epidermal cell shape and pigmentation on optical properties of *Antirrhinum* petals at visible and ultraviolet wavelengths. *Plant Physiology* 112: 879-888.

Goto, T., and Kondo, T. 1991. Structure and molecular stacking of anthocyanins- flower colour variation. *Angewandte Chemie International Edition* 30: 17-33.

Gould, K. S., Kuhn, D. N., Lee, D. W., and Oberbauer, S. F. 1995. Why leaves are sometimes red. *Nature* 378: 241-242.

Gould, K. S., Markham, K. R., Smith, R. H., and Goris, J. J. 2000. Functional role of anthocyanins in leaves of *Quintinia serrata* A. Cunn. *Journal of Experimental Botany* 51: 1107-1115.

Gould, K. S., and Quinn, B. D. 1999. Do anthocyanins protect leaves of New Zealand native species from UV-B? *New Zealand Journal of Botany* 37: 175-178.

Grace, S. C., and Logan, B. A. 1996. Acclimation of foliar antioxidant systems to growth irradiance in three broad-leaved evergreen species. *Plant Physiology* 112: 1631-1640.

Grace, S. C., and Logan, B. A. 2000. Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. *Philosophical Transactions of the Royal Society of London. B* 355: 1499-1510.

Grace, S. C., Logan, B. A., and Adams, W. W., Iii. 1998 a. Seasonal differences in foliar content of chlorogenic acid, a phenylpropanoid antioxidant, in *Mahonia repens*. *Plant Cell & Environment* 21: 513-521.

Grace, S. C., Salgo, M. G., and Pryor, W. A. 1998 b. Scavenging of peroxynitrite by a phenolic/peroxidase system prevents oxidative damage to DNA. *Federation of European Biochemical Societies Letters* 426: 24-28.

Grandmaison, J., and Ibrahim, R. K. 1996. Evidence for nuclear protein binding of flavonol sulphate esters in *Flaveria chloraefolia*. *Journal of Plant Physiology* 147: 653-660.

Grayer, R. J., and Harborne, J. B. 1994. A survey of antifungal compounds from higher plants. *Phytochemistry* 37: 19-42.

Greenberg, B. M., Wilson, M. I., Huang, X.-D., Duxbury, C. L., Gerhardt, K. E., and Gensemer, R. W. 1997. The effects of ultraviolet-B radiation on higher plants. In Wang, W.,

Grisebach, H. 1982. Biosynthesis of anthocyanins. In Markakis, P., ed. *Anthocyanins as food colours*. pp 69-92. Academic Press, New York.

Groden, D., and Beck, E. 1979. H_20_2 destruction by ascorbate dependent systems from chloroplasts. *Biochimica et Biophysica Acta* 546: 426-435.

Haenen, G. R. M. M., and Bast, A. 1999. Nitric oxide radical scavenging of flavonoids. *Methods in Enzymology* 301: 490-503.

Hahlbrock, K., and Scheel, D. 1989. Physiology and molecular biology of phenylpropanoid metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology* 40: 347-369.

Hall, D. O., and Rao, K. K. 1994. *Photosynthesis*. Fifth edition. Cambridge University Press, Cambridge. Pp 211.

Halliwell, B. 1984. Chloroplast metabolism: The structure and function of chloroplasts in green leaf cells. Claredon Press, New York. Pp. 206.

Halliwell, B. 1990. How to characterize a biological antioxidant. *Free Radical Research Communication* 9: 1-32.

Halliwell, B., and Gutteridge, J. M. C. 1998. *Free radicals in biology and medicine*. Volume 3. Clarendon Press, Oxford. Pp 897.

Hamilton, W. D., and Brown, S. P. 2001. Autumn tree colours as a handicap signal. *Proceedings of the Royal Society of London B* 268: 1489-1493.

Harborne, J. B. 1967. *Comparative biochemistry of the flavonoids*. Academic Press, New York. Pp 383.

Harborne, J. B. 1988. The flavonoids: recent advances. In Goodwin, T. W., ed. *Plant pigments*. pp. 299-343. Academic Press, New York.

Harborne, J. B., and Grayer, R. J. 1988. The anthocyanins. In Harborne, J. B., ed. *The flavonoids: advances in research since 1980*. pp. 1-20. Chapman and Hall, London.

Harborne, J. B., Mabry, T. J., and Mabry, H. 1975. *The flavonoids*. Chapman and Hall, London. Pp 1204.

Harborne, J. B., and Williams, C. A. 2000. Advances in flavonoid research since 1992. *Phytochemistry* 55: 481-504.

Hasegawa, H., Fukasawa-Akada, T., Okuno, T., Niizeki, M., and Suzuki, M. 2001. Anthocyanin accumulation and related gene expression in Japanese parsley (*Oenanthe stolonifera*) induced by low temperature. *Journal of Plant Physiology* 158: 71-78.

Haupt, W., and Scheuerlein, R. 1990. Chloroplast movements. *Plant, Cell and Environment* 13: 595-614.

Heber, U., and Krause, G. H. 1980. What is the physiological role of photorespiration? *Trends in Biochemical Science* 5: 32-34.

Heller, W., and Forkmann, G. 1993. Biosynthesis of flavonoids. In Harborne, J. B., ed. *The flavonoids: advances in research since 1986*. pp. 499-535. Chapman and Hall, London.

Henmi, T. 1997. Oxygen is required for the degradation of the D1 protein in Photosystem II during the donor-side photoinhibition. *Plant Physiology Supplement* 114: 206.

Herrmann, K. M. 1995. The shikimate pathway: early steps in the biosynthesis of aromatic compounds. *Plant Cell* 7: 907-919.

Hess, J. L. 1993. Vitamin E, alpha-tocopherol. In Alscher, R. G. and Hess, J. L., eds. *Antioxidants in higher plants*. pp 111-134. CRC Press, Boca Raton.

Hoch, W. A., Zeldin, E. L., and Mccown, B. H. 2001. Physiological significance of anthocyanins during autumnal leaf senescence. *Tree Physiology* 21: 1-8.

Holton, T. A., and Cornish, E. C. 1995. Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell* 7: 1071-1083.

Hopp, W., and Seitz, H. U. 1987. The uptake of acylated anthocyanins into isolated vacuoles from cell suspension culture of *Daucus carota*. *Planta* 170: 74-85.

Hossain, M., Nakano, K., and Asada, K. 1984. Monodehydroascorbate reductase in spinach chloroplasts and its participation in the regeneration of ascorbate for scavenging hydrogen peroxide. *Plant and Cell Physiology* 61: 385-395.

Hrazdina, G. 1992. Compartmentation in aromatic metabolism. In Stafford, H. A. and Ibrahim, R. K., eds. *Phenolic metabolism in plants.* pp 1-23. Plenum Press, New York.

Hrazdina, G., Wagner, G. J., and Siegelman, H. W. 1978. Subcellular localization of enzymes of anthocyanin biosynthesis in protoplasts. *Phytochemistry* 17: 53-56.

Hsiao, T. C. 1973. Plant responses to water stress. *Annual Review of Plant Physiology* 24: 519-570.

Hutzler, P., Fischbach, R., Heller, W., Jungblut, T. P., Reuber, S., Schmitz, R., Veit, M., Weissenbock, G., and Schnitzler, J.-P. 1998. Tissue location of phenolic compounds in plants by confocal laser microscopy. *Journal of Experimental Botany* 49: 953-956.

Ibrahim, R. K. 1992. Immunolocalization of flavonoid conjugates and their enzymes. In Stafford, H. A. and Ibrahim, R. K., eds. *Phenolic metabolism in plants.* pp 25-61. Plenum Press, New York.

Inada, K. 1976. Action spectrum for photosynthesis in higher plants. *Plant and Cell Physiology* 17: 355-365.

Jackman, R. L., and Smith, J. L. 1996. Anthocyanins and betalins. In Hendry, G. A. F. and Houghton, J. D., eds. *Natural Food Colourants.* pp. 244-309. Chapman and Hall, London.

Janda, T., Szalai, G., and Páldi, E. 1996. Chlorophyll fluorescence and anthocyanin content in chilled maize plants after return to a non-chilling temperature under various irradiances. *Biologia Plantarum* 38: 625-627.

Jang, J.-C., and Sheen, J. 1997. Sugar sensing in higher plants. *Trends in Plant Science* 2: 208-213.

Janzen, D. 1979. New horizons in the biology of plant defenses. In Rosenthal, G. A., Janzen, D. H., and Applebaum, S. W. eds. *Herbivores: their interactions with secondary plant metabolites*. pp 331-350. Academic Press, New York.

Jeje-A, and Zimmermann, M. 1983. The anisotropy of the mesophyll and CO_2 capture sites in *Vicia faba* L. leaves at low light intensities. *Journal of Experimental Botany* 34: 1676-1694.

Jovanovic, S. V., Steenken, S., Tosic, M., Marjanovic, B., and Simic, M. G. 1994. Flavonoids as antioxidants. *Journal of the American Chemical Society* 116: 4846-4851.

Kaiser, W. M. 1979. Reversible inhibition of the Calvin cycle and activation of oxidative pentose phosphate cycle in isolated chloroplasts by hydrogen peroxide. *Planta* 145: 377-382.

Kautsky, H., Appel, W., and Amann, H. 1960. Chlorophyllfluorescenz und kohlensaureassimilation. *Biochemische Zeitschrift* 322: 277-292.

Keli, S. O., Hertog, M. G. L., Feskens, E. J. M., and Kromhout, D. 1996. Dietary flavonoids, antioxidant vitamins and incidence of stroke: the Zutphen study. *Archives of Internal Medicine* 156: 637-642.

Kendrick, R. E., and Kronenberg, G. H. M. 1994. *Photomorphogenesis in plants*. Kluwer Academic Publishers, Dordrecht. Pp 828.

Kilmartin, P. A. 2001. Electrochemical detection of natural antioxidants: principles and protocols. *Antioxidants and Redox Signalling* 3: 941-956.

Kilmartin, P. A., Zou, H., and Waterhouse, A. L. 2001. A cyclic voltammetry method for characterizing antioxidant properties of wine and wine phenolics. *Journal of Agricultural and Food Chemistry* 49: 1957-1965.

Klaper, R., Frankel, S., and Berenbaum, M. R. 1996. Anthocyanin content and UV-B sensitivity in *Brassica napa*. *Photochemistry and Photobiology* 63: 811-813.

Klein, M., Martinoia, E., Hoffmann-Thoma, G., and Weissenbock, G. 2000. A membrane-potential dependent ABC-like transporter mediates the vacuolar uptake of rye flavone

glucuronides: Regulation of glucuronide uptake by glutathione and its conjugates. *The Plant Journal* 21: 289-304.

Klein, M., Weissenbock, G., Dufaud, A., Gaillard, C., Kreuz, K., and Martinoia, E. 1996. Different engergization mechanisms drive the vacuolar uptake of a flavonoid glucoside and a herbicide glucoside. *Journal of Biological Chemistry* 271: 29666-29671.

Knox, J. P., and Dodge, A. D. 1985. Singlet oxygen and plants. *Phytochemistry* 24: 889-896.

Koes, R. E., Quattrocchio, F., and Mol, J. N. M. 1994. The flavonoid biosynthetic pathway in plants: function and evolution. *BioEssays* 16: 123-132.

Koostra, A. 1994. Protection from UV-B induced DNA damage by flavonoids. *Plant Molecular Biology* 26: 771-774.

Krause, G. H., and Weis, E. 1991. Chlorophyll fluorescence and photosynthesis: the basics. *Annual Review of Plant Physiology and Plant Molecular Biology* 42: 331-349.

Krauss, W., Schiebel, G., Eberl, D., and Hager, A. 1987. Blue light induced, reversible in-activation of the tonoplast-type H^+-ATPase from corn coleoptiles in the presence of flavins. *Photochemical and Photobiology* 45: 837-844.

Krizek, D. T., Semeniuk, P., Moline, H. E., Mirecki, R. M., and Abbott, J. A. 1995. Chilling injury in coleus as influenced by photosynthetically active radiation, temperature and abscisic acid pretreatment. I. Morphological and physiological responses. *Plant, Cell and Environment* 8: 135-142.

Krol, M., Gray, G. R., Hurray, V. M., Oquist, G., Malek, L., and Huner, N. P. 1995. Low-temperature stress and photoperiod affect an increased tolerance to photoinhibition in *Pinus banksiana* seedlings. *Canadian Journal of Botany* 73: 1119-1127.

Kroller, D. 1990. Light-driven leaf movements. *Plant, Cell and Environment* 13: 615-632.

Kubo, H., Peeters, A. J. M., Aarts, M. G. M., Pereira, A., and Koorneef, M. 1999. ANTHOCYANINLESS2, a homeobox gene affecting anthocyanin distribution and root development in *Aradibopsis. Plant Cell* 11: 1217-1226.

Kunz, S., Burkhardt, G., and Becker, H. 1994. Ricciondins A and B, anthocyanidins from the cell walls of the liverwort *Ricciocarpus natans*. *Phytochemistry* 35: 233-235.

Lambers, H., Chapin, F. S., and Pons, T. L. 1998. *Plant physiological ecology*. Springer, New York. Pp 540.

Landry, L. G., Chapple, C. C. S., and Last, R. L. 1995. *Arabidopsis* mutants lacking phenolic sunscreens exhibit enhanced ultraviolet-B injury and oxidative damage. *Plant Physiology* 109: 1159-1166.

Lapidot, T., Harel, S., Akiri, B., Granit, R., and Kanner, J. 1999. pH-dependent forms of red wine anthocyanins as antioxidants. *Journal of Agricultural & Food Chemistry* 47: 67-70.

Larson, R. 1995. Plant defences against oxidative stress. *Archives of Insect Biochemistry and Physiology* 29: 175-186.

Larson, R. A. 1988. The antioxidants of higher plants. *Phytochemistry* 27: 969-978.

Larson, R. A. 1997. *Naturally occurring antioxidants*. Lewis Publisher, Boca Ranton. Pp 195.

Law, M. Y., Charles, S. A., and Halliwell, B. 1983. Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts. *Biochemical Journal* 210: 899-903.

Lee, D. 1987. The spectral distribution of radiation in two neotropical rainforests. *Biotropica* 19: 161-166.

Lee, D., Brammeier, S., and Smith, A. P. 1987. The selective advantage of anthocyanins in developing leaves of Mango and Cacao. *Biotropica* 19: 40-49.

Lee, D. W. 1986. Unusual strategies of light absorption in rain-forest herbs. In Givnish, T. J., ed. *On the economy of plant form and function*. pp 105-131. Cambridge University Press, Cambridge.

Lee, D. W., and Collins, T. M. 2001. Phylogenetic and ontogenetic influences on the distribution of anthocyanins and betacyanins in leaves of tropical plants. *International Journal of Plant Science* 162: 1141-1153.

Lee, D. W., and Graham, R. 1986. Leaf optical properties of rainforest sun and shade extreme shade plants. *American Journal of Botany* 73: 1100-1108.

Lee, D. W., and Lowry, J. B. 1980. Young leaf anthocyanin and solar ultraviolet. *Biotropica* 12: 75-76.

Lee, D. W., Lowry, J. B., and Stone, B. C. 1979. Abaxial anthocyanin layer in leaves of tropical rain forest plants: Enhancer of light capture in deep shade. *Biotropica* 11: 70-77.

Leegood, R. C., and Walker, D. A. 1993. Chloroplasts and protoplasts. In Hall, E. D., Scurlock, J. M. O., Bolhar-Nordenkampf, H. R., Leegood, R. C., and Long, S. P., eds. *Photosynthesis and production in a changing environment - a field and laboratory manual*. pp 268-282. Chapman and Hall, London.

Legendre, L., Yeuh, Y. G., Crain, R., Haddock, N., Heinstein, P. F., and Low, P. S. 1993. Phospholipase-C activation during elicitation of oxidative burst in cultured plant cells. *Journal of Biological Chemistry* 268: 24559-24563.

Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79: 583-593.

Lewis, C. E., Walker, J. R. L., Lancaster, J. E., and Conner, A. J. 1998. Light regulation of anthocyanin, flavonoid and phenolic acid biosynthesis in potato minitubers *in vitro*. *Australian Journal of Plant Physiology* 25.

Leyva, A., Jarillo, J. A., Salinas, J., and Martinezzapater, J. M. 1995. Low temperature induces the accumulation of *phenylalanine ammonia-lyase* and *chalcone synthase* mRNAs of *Arabidopsis thaliana* in a light-dependent manner. *Plant Physiology* 108: 39-46.

Li, J., Ou-Lee, T. M., Raba, R., Amundson, R. G., and Last, R. L. 1993. *Arabidopsis* flavonoid mutants are hypersensitive to UV-B irradiation. *Plant Cell* 5: 171-179.

Lichtenthaler, H. K. 1987. Chlorophyll and carotenoids: pigments of photosynthetic biomembranes. *Methods in Enyzmology* 148: 350-385.

Logan, B. A., Demmig-Adams, B., Adams Iii, W. W., and Grace, S. 1998 a. Antioxidants and xanthophyll cycle-dependant energy dissipation in *Cucurbita pepo* L. and *Vinca major* L. acclimated to four growth PPFDs in the field. *Journal of Experimental Botany* 49: 1969-1879.

Logan, B. A., Grace, S. C., Adams Iii, W. W., and Demmig-Adams, B. 1998 b. Seasonal differences in xanthophyll cycle characteristics and antioxidant in *Mahonia repens* growing in different light environments. *Oecologia* 116: 9-17.

Lois, R., and Buchanan, B. B. 1994. Severe sensitivity to ultraviolet radiation in an *Arabidopsis* mutant deficient in flavonoid accumulation. II. Mechanisms of UV - resistance in *Arabidopsis*. *Planta* 194: 504-509.

Løvaas, E. and Olsen, J.E. (1998) No induction of polyamines and radical scavenging antioxidants in *Nicotiana tabaccum* exposed to iron excess, as investigated by the DPPH assay and differential spectroscopy. *Journal of Plant Physiology* 153: 401-408.

Low, P. S., and Merida, J. R. 1996. The oxidative burst in plant defence: function and signal transduction. *Physiologia Plantarum* 96: 533-542.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* 193: 265-275.

Lu, Y. P., Li, Z. S., and Rea, P. A. 1997. AtMRP1 gene of *Arabidopsis* encodes a glutathione S-conjugate pump: isolation and functional definition of a plant ATP-binding cassette transporter gene. *Proceedings of the National Academy USA* 94: 8243-8248.

Mallick, N., and Mohn, F. H. 2000. Reactive oxygen species: response of algal cells. *Journal of Plant Physiology* 157: 183-193.

Mancinelli, A. L. 1985. Light-dependent anthocyanin synthesis: a model system for the study of plant photomorphogenesis. *Botanical Review* 51: 107-157.

Mancinelli, A. L. 1990. Interaction between light quality and light quantity in the photoregulation of anthocyanin production. *Plant Physiology* 92: 1191-1195.

Marie Bradley, J., Davies, K. M., Deroles, S. C., Bloor, S. J., and Lewis, D. J. 1998. The maize *Lc* regulatory gene up-regulates the flavonoid biosynthetic pathway of *petunia*. *The Plant Journal* 13: 381-392.

Markham, K. R. 1982. *Techniques of flavonoid identification*. Academic Press, New York. Pp 113.

Markham, K. R. 1988. Distribution of flavonoids in the lower plants and its evolution significance. In Harborne, J. B., ed. *The flavonoids - advances in research since 1980*. pp 427-468. Chapman and Hall, London.

Markham, K. R., and Bloor, S. J. 1998. Analysis and identification of flavonoids in practice. In Rice-Evans, C. A. and Packer, L., eds. *Flavonoids in health and disease*. pp 1-34. Marcel Dekker, New York.

Markham, K. R., Gould, K. S., Winefield, C. S., Mitchell, K. A., Bloor, S. J., and Boase, M. R. 2000 a. Anthocyanic vacuolar inclusions- their nature and significance in flower colouration. *Phytochemistry* 55: 327-336.

Markham, K. R., Ryan, K. G., Bloor, S. J., and Mitchell, K. A. 1998. An increase in the luteolin:apigenin ratio on *Marchantia polymorpha* on UV-B enhancement. *Phytochemistry* 48: 791-794.

Markham, K. R., Ryan, K. G., Gould, K. S., and Richards, G. K. 2000 b. Cell wall sited flavonoids in lisianthus flower petals. *Phytochemistry* 54: 681-687.

Marrs, K. A., Alfenito, M. R., Lloyd, A. M., and Walbot, V. 1995. A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*. *Nature* 375: 397-400.

Marrs, K. A., and Walbot, V. 1997. Expression and RNA splicing of the maize glutathione S-transferase Bronze2 gene is regulated by cadmium and other stresses. *Plant Physiology* 113: 93-102.

Martinoia, E., Grill, E., Tommasini, R., Kreuz, K., and Amrhein, N. 1993. ATP-dependent glutathione *S*-conjugate 'export' pump in the vacuolar membrane of plants. *Nature* 364: 247-249.

Matile, P. 2000. Biochemistry of Indian summer: physiology of autumnal leaf coloration. *Experimental Gerontology* 35: 145-158.

Maxwell, K., and Johnson, G. N. 2000. Chlorophyll fluorescence - a practical guide. *Journal of Experimental Botany* 51: 659-668.

McClure, J. W. 1975. Physiology and functions of flavonoids. In Harborne, J. B., ed. *The flavonoids*. pp 971-1055. Chapman and Hall, London.

Mehlhorn, H. 1990. Electron spin resonance evidence for the formation of free radicals in plants exposed to ozone. *Physiologia Plantarum* 79: 377-383.

Mendez, M., Jones, D. G., and Manetas, Y. 1999. Enhanced UV-B radiation under field conditions increases anthocyanin and reduces the risk of photoinhibition but does not affect growth in the carnivorous plant *Pinguicula vulgaris*. *New Phytologist* 144: 275-282.

Meneguzzo, S., Navari-Izzo, F., and Izzo, R. 1999. Antioxidative responses of shoots and roots of wheat to increasing NaCl concentrations. *Journal of Plant Physiology* 155: 274-280.

Middleton, E., Jr. 1996. Biological properties of plant flavonoids: an overview. *International Journal of Pharmacognosy* 34: 344-348.

Mol, J., Grotewold, E., and Koes, R. 1998. How genes paint flowers and seeds. *Trends in Plant Science* 3: 212-217.

Mol, J., Jenkins, G. I., Schäfer, E., and Weiss, D. 1996. Signal perception, transduction, and gene expression involved in anthocyanin biosynthesis. *Critical Reviews in Plant Science* 15: 525-557.

Mooney, H. A., Ehleringer, J. R., and Björkmann, O. 1977. The energy balance of leaves of the evergreen desert shrub *Atriplex hymenelytra*. *Oecologia* 29: 301-310.

Moore, K. G. 1965. Senescence in leaves of *Acer pseudoplatanus* L. and *Parthenocissus tricuspidata* Planch.1. Changes in some leaf constituents during maturity and senescence. *Annals of Botany (New Series)* 29: 433-444.

Morel, I., Lescoat, G., Cognel, P., Sergent, O., Pasdeloup, N., Brissot, P., Cillard, P., and Cillard, J. 1993. Antioxidant and iron-chelating activities of the flavonoids catechin, quercetin an diosmetin on iron loaded rat hepatocyte cultures. *Biochemical Pharmacology* 45: 13-19.

Mueller, L. A., Goodman, C. D., Silady, R. A., and Walbot, V. 2000. AN9, a petunia glutathione *S*-transferase required for anthocyanin sequestration, is a flavonoid-binding protein. *Plant Physiology* 123: 1561-1570.

Murray, J. R., and Hackett, W. P. 1991. Dihydroflavonol reductase activity in relation to differential anthocyanin accumulation in juvenile and mature phase *Hedera helix* L. *Plant Physiology* 97: 343-351.

Nakajima, J., Tanaka, Y., Yamazaki, M., and Saito, K. 2001. Reaction mechanism from leucoanthocyanidin to anthocyanidin 3-glucoside, a key reaction for colouring in anthocyanin biosynthesis. *Journal of Biological Chemistry* 276: 25797-25803.

Nakano, Y., and Asada, K. 1987. Purification of ascorbate peroxidase in spinach chloroplasts; its inactivation in ascorbate-depleated medium and reactivation by monodehydroascorbate radical. *Plant Cell Physiology* 28: 131-140.

Neff, M. M., Frankhauser, C., and Chorey, J. 2000. Light: an indicator of time and place. *Genes and Development* 14: 257-271.

Neill, S. O., and Gould, K. S. 1999. Optical properties of leaves in relation to anthocyanin concentration and distribution. *Canadian Journal of Botany* 77: 1777-1782.

Neuhaus, G., Bowler, C., Kern, R., and Chua, N.-H. 1993. Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways. *Cell* 73: 937-952.

Nick, P., Ehmann, B., Furuya, M., and Schäfer, E. 1993. Cell communication, stochastic cell responses, and anthocyanin pattern in mustard cotyledons. *Plant Cell* 5: 541-552.

Niki, E. 1987. Lipid antioxidants: how they may act in biological systems. *British Journal of Cancer* 55: 153-157.

Nishio, J. N. 2000. Why are higher plants green? Evolution of the higher plant photosynthetic pigment complement. *Plant, Cell and Environment* 23: 539-548.

Nishio, J. N., Sun, J., and Vogelmann, T. C. 1993. Carbon fixation gradients across spinach leaves do not follow internal light gradients. *Plant Cell* 5: 953-961.

Niyogi, K. K., Grossman, A. R., and Björkmann, O. 1998. *Arabidopsis* mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. *Plant Cell* 10: 1121-1134.

Noctor, G., and Foyer, C. H. 1998. Ascorbate and Glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology* 49: 249-279.

Noctor, G., Veljovic-Jovanovic, S., and Foyer, C. H. 2000. Peroxide processing in photosynthesis: antioxidant coupling and redox signalling. *Philosophical Transactions of the Royal Society of London. B.* 355: 1465-1475.

Noda, Y., Kaneyuki, T., Igarashi, K., Mori, A., and Packer, L. 1998. Antioxidant activity of nasunin, an anthocyanin in eggplant. *Research Communications in Molecular Pathology and Pharmacology* 102: 175-187.

Nozue, M., Yamanda, K., Nakamura, T., Kubo, H., Kondo, M., and Nishimura, M. 1997. Expression of a vacuolar protein (VP24) in anthocyanin-producing cells of sweet potato in suspension culture. *Plant Physiology* 115: 1065-1072.

Nozzolillo, C. 1994. Anthocyanoplasts: organelles or inclusions? *Polyphenols Actualites* 11: 16-18.

Ntefidou, M., and Manetas, Y. 1996. Optical properties of hairs during the early stages of leaf development in *Platanus orientalis*. *Australian Journal of Plant Physiology* 23: 535-538.

Oberley, L. W., and Spitz, D. R. 1985. Nitroblue tetrazolium. In Greenwald, R. A., ed. *CRC Handbook of methods for oxygen radical research*. pp 217-220. CRC Press, Boca Raton.

Ogawa, K., Kanematsu, S., Takebe, K., and Asada, K. 1995. Attachment of Cu,Zn SOD to thylakoid membranes at the site of superoxide generation (PSI) in spinach chloroplasts: detection by immunogold labelling after rapid freezing. *Plant Cell Physiology* 36: 565-573.

Oren-Shamir, M., and Levi-Nissim, A. 1997. UV-light effect on the leaf pigmentation of *Cortinus coggygria* 'Royal Purple'. *Science and Horticulture* 71: 59-66.

Osmond, C. B. 1981. Photorespiration and photoinhibition: some implications for the energetics of photosynthesis. *Biochimica et Biophysica Acta* 639: 77-98.

Osmond, C. B. 1994. What is photoinhibition? Some insights from comparisons of sun and shade plants. In Baker, N. R. and Boyer, J. R., eds. *Photoinhibition: from molecular mechanisms to the field*. pp 1-24. Bios Scientific Publications, Oxford.

Osmond, C. B., and Grace, S. C. 1995. Perspectives on photoinhibition and photorespiration in the field: quintessential inefficiencies of the light and dark reactions of photosynthesis. *Journal of Experimental Botany* 46: 1351-1362.

Pathak, D. P. K. S. A. K. 1991. Flavonoids as medicinal agents recent advances. *Fitoterapia* 62: 371-390.

Pecket, R. C., and Small, C. A. 1980. Occurrence, location and development of anthocyanoplasts. *Phytochemistry* 19: 2571-2576.

Pietrini, F., and Massacci, A. 1998. Leaf anthocyanin content changes in *Zea mays* L. grown at low temperature: Significance for the relationship between the quantum yield of PSII and the apparent quantum yield of CO_2 assimilation. *Photosynthesis Research* 58: 213-219.

Polle, A. 1997. Defence against photooxidative damage in plants. In Scandalios, J.G. ed. *Oxidative stress and molecular biology of antioxidant defences*. pp 623-666. Cold Spring Harbour Laboratory Press, New York.

Post, A. 1990. Photoprotective pigment as an adaptive strategy in the Antarctic moss *Ceratodon purpureus*. *Polar Biology* 10: 241-246.

Post, A., and Meret, V. 1992. Photosynthesis, pigments, and chloroplast ultrastructure of an Antarctic liverwort from sun-exposed and shaded sites. *Canadian Journal of Botany* 70: 2259-2264.

Powles, S. B. 1984. Photoinhibition of photosynthesis induced by visible light. *Annual Review of Plant Physiology* 35: 15-44.

Pratt, D. F., and Hudson, B. J. F. 1990. Natural oxidants not exploited commercially. In Hudson, B. J. F., ed. *Food antioxidants*. pp. 171-184. Elsevier, Amsterdam.

Prior, R. L., Cao, G., Martin, A., Sofic, E., McEwen, J., O'Brien, C., Lischner, N., Ehlenfeldt, M., Kalt, W., Krewer, G., and Mainland, C. M. 1998. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. *Journal of Agricultural & Food Chemistry* 46: 2686-2693.

Quick, W. P., and Horton, P. 1984. Studies on the induction of chlorophyll fluorescence in barley protoplasts. I. Factors affecting the observation of oscillations in the yield of chlorophyll fluorescence and the rate of oxygen evolution. *Proceedings of the Royal Society of London B* 220: 361-370.

Rao, M. V., Halle, B. A., and Ormrod, D. P. 1995. Amelioration of ozone-induced oxidative damage in wheat plants grown under high carbon dioxide. *Plant Physiology* 109: 421-432.

Reddy, V. S., Goud, K. V., Sharma, R., and Reddy, A. R. 1994. Ultraviolet-B-responsive anthocyanin production in a rice cultivar is associated with a specific phase change of phenylalanine ammonia lyase biosynthesis. *Plant Physiology* 105: 1059-1066.

Repka, V., and Fischerova, I. 2000. Serologically-related anionic peroxidases from petunia and cucumber can substitute flavonoid antioxidants. *Biologia Plantarum* 43: 105-112.

Ribereau-Gayon, P. 1972. *Plant Phenolics*. Oliver and Boyd, Edinburgh. Pp. 254.

Rice-Evans, C., Diplock, A. T., and Symons, M. C. R. 1991. *Techniques in free radical research*. Elsevier, New York. Pp 291.

Rice-Evans, C., Miller, C., Bolwell, G. P., Bramley, P. M., and Pridham, J. B. 1995 a. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Research* 22: 375-383.

Rice-Evans, C. A., Miller, J., and Paganga, G. 1997. Antioxidant properties of phenolic compounds. *Trends in Plant Science* 2: 152-159.

Rice-Evans, C. A., Miller Nicholas, J., and Paganga, G. 1995 b. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology & Medicine* 20: 933-956.

Robinson, J. M. 1988. Does O_2 photoreduction occur within the chloroplast *in vivo*? *Physiologia Plantarum* 72: 666-680.

Robinson, T. 1991. *The organic constituents of higher plants*. Cordus Press, North Amherst. Pp 353.

Rueber, S., Bornman, J. F., and Weissenböck, G. 1996. Phenylpropanoid compounds in primary leaves of rye (*Secale cereale*) - light regulation of their biosynthesis and possible role in UV-B protection. *Physiologia Plantarum* 97: 160-168.

Russain, W. A., Evert, R. F., Vanderveer, P. J., Sharkey, T. D., and Briggs, S. P. 1996. Modification of a specific class of plasmodesmata and loss of sucrose export ability in the *sucrose export defective1* maize mutant. *Plant Cell* 8: 645-658.

Sadka, A., Dewald, D., May, G. D., Park, W. D., and Mullet, J. E. 1994. Phosphate modulates transcription of soybean vspB and other sugar-inducible genes. *Plant Cell* 6: 737-749.

Saito, K., Kobayashi, M., Gong, Z., Tanaka, Y., and Yamazaki, M. 1999. Direct evidence for anthocyanidin synthase as a 2-oxogluatarate-dependent oxygenase: molecular cloning and functional expression of cDNA from a red forma of *Perilla frutescens*. *The Plant Journal* 17: 181-189.

Sakihama, Y., Mano, J., Sano, S., Asada, K., and Yamasaki, H. 2000. Reduction of phenoxyl radicals mediated by monohydroascorbate reductase. *Biochemical & Biophysical Research Communications* 279: 949-954.

Sandermann, H. 1992. Plant metabolism of xenobiotics. *Trends in Biological Science* 17: 82-84.

Sanger, J. E. 1971. Quantitative investigations of leaf pigments from their inception in buds through autumn coloration to decomposition in falling leaves. *Ecology* 52: 1075-1089.

Saran, M., Michel, C., and Bors, W. 1998. Radical functions *in vivo*: a critical review of current concepts and hypotheses. *Zeitschrift für Naturforschung* 53: 210-227.

Sarma, A. D., and Sharma, R. 1999. Anthocyanin-DNA copigmentation complex: mutual protection against oxidative damage. *Phytochemistry* 52: 1313-1318.

Sarma, A. D., Sreelakshmi, Y., and Sharma, R. 1997. Antioxidant ability of anthocyanins against ascorbic acid oxidation. *Phytochemistry* 45: 671-674.

Sato, K., Nakayama, M., and Shigeta, J.-I. 1996. Culturing conditions affecting the production of anthocyanin in suspended cell cultures of strawberry. *Plant Science* 113: 91-98.

Saunders, J. A., and McClure, J. W. 1976. The distribution of flavonoids in chloroplasts of twenty species of vascular plants. *Phytochemisty* 15: 809-810.

Scandalios, J. 1994. Regulation and properties of plant catalases. In Foyer, C. H. and Mullineaux, P., eds. *Causes of photooxidative stress and amelioration of defence systems in plants*. pp 275-316. CRC Press,. Boca Raton.

Schemelzer, E., Jahnen, W., and Hahlbrock, K. 1988. *In situ* localization of light-induced chalcone synthase mRNA, chalcone synthase, and flavonoid end products in epidermal cells of parsley leaves. *Proceedings of the National Academy of Science* USA 85: 2989-2993.

Schnitzler, J.-P., Jungblut, T. P., Heller, W., Köfferlein, M., Hutzler, P., Heinzmann, U., Schmelzer, E., Ernst, D., Langebartels, C., and Sandermann, H. 1996. Tissue localization of UV-B screening pigments and of chalcone synthase mRNA in needles of Scots pine seedlings. *New Phytologist* 132: 247-258.

Schoner, S., and Krause, G. H. 1990. Protective systems against active oxygen species in spinach: response to cold acclimation in excess light. *Planta* 180: 383-389.

Schreiber, U., Bilger, W., Harman, H., and Neubauer, C. 1998. Chlorophyll fluorescence as a diagnostic tool: basics and some aspects of practical relevance. In Raghavendra, A. S., ed. *Photosynthesis: a comprehensive treatise*. pp 320-336. Cambridge University Press, Cambridge.

Schreiber, U., and Neubauer, C. 1990. O_2-dependent electron flow, membrane energization and the mechanism of non-photochemical quenching of chlorophyll fluorescence. *Photosynthesis Research* 25: 279-293.

Shang, W., and Feierabend, J. 1999. Dependence of catalase photoinactivation in rye leaves on light intensity and quality and characterization of a chloroplastic-mediated inactivation in red light. *Photosynthesis Research* 59: 201-213.

Sherwin, H. W., and Farrant, J. M. 1998. Protection mechanisms against excess light in the resurrection plants *Craterostigma wilmsii* and *Xerophyta viscosa*. *Plant Growth Regulation* 24: 203-210.

Shibata, K., Benson, A. A., and Calvin, M. 1954. The absorption spectra of suspensions of living micro-organisms. *Biochemica et Biophysica Acta* 15: 461-470.

Shirley, B. W. 1996. Flavonoid biosynthesis: 'new functions' for an 'old pathway'. *Trends in Plant Science* 1: 301-317.

Smillie, R. M., and Hetherington, S. E. 1999. Photoabatement by anthocyanin shields photosynthetic systems from light stress. *Photosynthetica* 36: 451-463.

Smirnoff, N. 1993. The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytologist* 125: 27-58.

Smirnoff, N. 2000. Ascorbate biosynthesis and function in photoprotection. *Philosophical Transactions of the Royal Society of London. B* 355: 1455-1464.

Smith, A. M. 1909. On internal temperature of leaves in tropical isolation, with special reference to their effect of their colour on the temperature; also observations on the periodicity of the appearance of young coloured leaves of trees growing in Peradeniya gardens. *Annals of the Royal Botanical Gardens* Peradeniya 4: 229-297.

Smith, H. 2000. Phytochromes and light signal perception by plants - an emerging synthesis. *Nature* 407: 585-591.

Smith, I. K., Kendall, A. C., Keys, A. J., Turner, J. C., and Lea, P. 1985. The regulation of the biosynthesis of glutathione in leaves of barley (*Hordeum vulgare*). *Plant Science Letters* 41: 1-18.

Smith, M. W., and Doolittle, R. F. 1992. A comparison of evolutionary rates of the two major kinds of superoxide dismutase. *Journal of Molecular Evolution* 34: 175-184.

Sponga, F., Deitzer, G. F., and Mancinelli, A. L. 1986. Crytochrome, phytochrome, and the photoregulation of anthocyanin production under blue light. *Plant Physiology* 82: 952-955.

Stafford, H. A. 1990. *Flavonoid Metabolism.* CRC Press, Boca Raton. Pp 298.

Stafford, H. A. 1994. Anthocyanins and betalains: evolution of the mutually exclusive pathways. *Plant Science* 101: 91-98.

Stapleton, A. E., and Walbot, V. 1994. Flavonoids can protect maize DNA from the induction of ultraviolet radiation damage. *Plant Physiology* 105: 881-889.

Steffen, K. 1991. Avoidance of photooxidative stress: balancing energy flux within the chloroplast. In Pell, E. and Steffen, K., eds. *Active oxygen-oxidative stress and plant metabolism*. pp 119-130. American Society of Plant Physiologists, Pennsylvania.

Stone, B. C. 1979. Protective coloration of young leaves in certain Malaysian palms. *Biotropica* 11: 126.

Strack, D., and Wray, V. 1989. Anthocyanins. In Dey, P. M. and Harborne, J. B., eds. *Methods in plant biochemistry*. pp 325-356. Academic Press, New York.

Strack, D., and Wray, V. 1993. The anthocyanins. In Harborne, J. B., ed. *The flavonoids: Advances in research since 1986*. pp 1-22. Chapman and Hall, London.

Sturgeon, K. B., and Mitten, J. B. 1980. Cone colour polymorphism associated with elevation in white fir, *Abies concolor*, in southern Colorado. *American Journal of Botany* 67: 1040-1045.

Sun, J., Nishio, J. N., and Vogelmann, T. 1996. High-light effects on CO_2 fixation gradients across leaves. *Plant, Cell and Environment* 19: 1261-1271.

Suzuki, M. 1995. Enhancement of anthocyanin accumulation by high osmotic stress and low pH in grape cells (*Vitis hybrida*). *Journal of Plant Physiology* 147: 152-155.

Swain, T. 1986. The evolution of flavonoids. In Cody, V., Middleton Jr, E., and Harborne, J. B., eds. *Plant flavonoids in biology and medicine, biochemical, pharmacological, and structure-activity relationships*. pp 1-14. Alan R. Liss Inc, New York.

Takahama, U. 1987. Oxidation products of kaempferol by superoxide anion radical. *Plant Cell Physiology* 28: 953-957.

Takahama, U. 1988. Hydrogen peroxide-dependent oxidation of flavonoids and hydroxycinnamic acid derivatives in epidermal and guard cells of *Tradescantia virginiana* L. *Plant Cell Physiology* 29: 475-481.

Takahama, U. 1989. A role of hydrogen peroxide in the metabolism of phenolics in Mesophyll cells of *Vicia faba* L. *Plant Cell Physiology* 30: 295-301.

Takahama, U., and Oniki, T. 1997. A peroxide/phenolics/ascorbate system can scavenge hydrogen peroxide in plant cells. *Physiologia Plantarum* 101: 845-852.

Takahama, U., and Oniki, T. 2000. Flavonoids and some other phenolics as substrates of peroxidase: physiological significance of redox reactions. *Journal of Plant Research* 113: 301-309.

Takahashi, A., Takeda, K., and Ohnishi, T. 1991. Light-induced anthocyanin reduces the extent of damage to DNA in UV-irradiated *Centaurea cyanus*cells in culture. *Plant Cell Physiology* 32: 541-547.

Takenaka, Y., Miki, M., Yasuda, H., and Mino, M. 1991. The effect of α-tocopherol as an antioxidant on the oxidation of membrane protein thiols induced by free radical generation in different sites. *Archives of Biochemistry and Biophysics* 285: 344-351.

Tekeda, J., and Abe, S. 1992. Light-induced synthesis of anthocyanin in carrot cells in suspension. IV. The action spectrum. *Photochemistry and Photobiology* 56: 69-74.

Tevini, M., Braun, J., and Fieser, G. 1991. The protective function of the epidermal layer of rye seedlings against ultraviolet - B radiation. *Photochemistry and Photobiology* 53: 329-333.

Tholakalabavi, A., Zwiazek, J. J., and Thorpe, T. A. 1997. Osmotically-stressed poplar cell cultures: anthocyanin accumulation, deaminase activity, and solute composition. *Journal of Plant Physiology* 151: 489-496.

Timberlake, C. F. 1980. Anthocyanins - occurrence, extraction and chemistry. *Food Chemistry* 5: 69-80.

Timberlake, C. F., and Bridle, P. 1975. The anthocyanins. In Harborne, J. B., Mabry, T. J., and Mabry, H., eds. *The flavonoids*. pp 214-266. Chapman and Hall, London.

Trull, M. C., Guiltinan, M. J., Lynch, J. P., and Deikman, J. 1997. The response of wild-type and ABA mutant *Arabidopsis thaliana* plants to phosphorus starvation. *Plant Cell & Environment* 20: 85-92.

Tsuda, T., Kato, Y., and Osawa, T. 2000. Mechanism for the peroxynitrite scavenging activity by anthocyanins. *Federation of European Biochemical Societies* 484: 207-210.

Tsuda, T., Ohshima, K., Kawakishi, S., and Osawa, T. 1996 a. Oxidation products of cyanidin 3-*O*-β-D-glucoside with a free radical initiator. *Lipids* 31: 1259-1263.

Tsuda, T., Shiga, K., Ohshima, K., Kawakishi, S., and Osawa, T. 1996 b. Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from *Phaseolus vulgaris* L. *Biochemical Pharmacology* 52: 1033-1039.

Tsuda, T., Watanabe, M., Ohshima, K., Norinobu, S., Choi, S.-W., Kawakishi, S., and Osawa, T. 1994. Antioxidative activity of the anthocyanin pigments cyanidin 3-O-β-D-glucoside and cyanidin. *Journal of Agricultural & Food Chemistry* 42: 2407-2410.

Tsukaya, H., Oshima, T., Naito, S., Chino, M., and Komeda, Y. 1991. Sugar-dependant expression of the CHS-A gene for chalcone synthase from petunia in transgenic *Arabidopsis*. *Plant Physiology* 97: 1414-1421.

Van Acker, S. A. B. E., Van Den Berg, D. J., Tromp, M. N. J. L., Griffioen, D. H., Van Bennekom, W. P., Van Der Vijgh, W. J. F., and Bast, A. 1996. Structural aspects of antioxidant activity of flavonoids. *Free Radical Biology & Medicine* 20: 331-342.

Van Camp, W., Bowler, C., Villaroel, R., Tsang, E. W. T., Van Montagu, M., and Inzé, D. 1991. Characterization of iron superoxide dismutase cDNAs from plants obtained by genetic complementation in *Escherichia coli*. *Proceedings of the National Academy of Science USA* 87: 9903-9907.

Van Kooten, O., and Snel, J. F. H. 1990. The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynthesis Research* 25: 147-150.

Wade, H. K., Bibikova, T. N., Valentine, W. J., and Jenkins, G. I. 2001. Interactions within a network of phytochrome, cryptochrome and UV-B phototransduction pathways regulate chalcone synthase gene expression in *Arabidopsis* leaf tissue. *The Plant Journal* 25: 675-685.

Wagner, R. J., Wagner, A. B., and Howard, R. A. 1969. The ecology of an elfin forest in Puerto Rico, 9. Chemical studies of coloured leaves. *Journal of Arnold Arboretum* 50: 556-565.

Walker, A. R., Davidson, P. A., Bolognesi-Winfield, A. C., James, C. M., Srinivasan, N., Blundell, T. L., Esch, J. J., Marks, M. D., and Gray, J. C. 1999. The TRANSPARENT TESTA GLABRA1 locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. *Plant Cell* 11: 1337-1349.

Wardman, P., and Candeias, L. P. 1996. Fenton chemistry: an introduction. *Radiation Research* 145: 523-531.

Weckx, J. E. J., and Clijsters, H. M. M. 1996. Oxidative damage and defence mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amounts of copper. *Physiologia Plantarum* 96: 506-512.

Weiss, D. 2000. Regulation of flower pigmentation and growth: multiple signalling pathways control anthocyanin synthesis in expanding petals. *Physiologia Plantarum* 110: 152-157.

Weiss, D., and Halavely, A. H. 1989. Stamens and gibberellin in the regulation of corolla pigmentation and growth in *Petunia hybrida*. *Planta* 179: 89-96.

Weiss, D., and Halavely, A. H. 1991. The role of light reaction in the regulation of anthocyanin synthesis in petunia corollas. *Physiologia Plantarum* 81: 127-133.

Weisshaar, B., Armstrong, G. A., Block, A., Silva, O. D. C., and Hahlbrock, K. 1991. Light-inducible and constitutively expressed DNA-binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. *The EMBO Journal* 10: 1777-1786.

Wiering, H., and De Vlaming, P. 1984. Genetics of flower and pollen colours. In Sink, K. C., ed. *Petunia*. pp 49-75. Springer- Verlag, Berlin.

Winkler, B. S., Orselli, S. M., and Tonia, S. 1994. The redox couple between glutathione and ascorbic acid: a chemical and physiological perspective. *Free Radical Biology & Medicine* 17: 333-349.

Winston, G. A. 1990. Physiochemical basis for free radical fromation in cells: production and defences. In Alscher, R. G. and Cumming, J. R., eds. *Stress responses in plants: adaptation and acclimation mechanisms*. pp 57-86. Wiley-Liss, New York.

Woodall, G. S., Dodd, I. C., and Stewart, G. R. 1998. Contrasting leaf development within the genus *Syzygium*. *Journal of Experimental Botany* 49: 79-87.

Woodall, G. S., and Stewart, G. R. 1998. Do anthocyanins play a role in UV protection of red juvenile leaves of *Syzygium*? *Journal of Experimental Botany* 49: 1447-1450.

Xin, Z., and Browse, J. 2000. Cold comfort farm: the acclimation of plants to freezing temperatures. *Plant, Cell and Environment* 23: 893-902.

Xu, W., Shioiri, H., Kojima, M., and Nozue, M. 2001. Primary structure and expression of a 24-kD vacuolar protein (VP24) precursor in anthocyanin-producing cells of sweet potato in suspension culture. *Plant Physiology* 125: 447-455.

Yamasaki, H. 1997. A function of colour. *Trends in Plant Science* 2: 7-8.

Yamasaki, H. 2000. Nitrate-dependent nitric oxide production pathway: implications for the involvement of active nitrogen species in photoinhibition *in vivo*. *Philosophical Transactions of the Royal Society of London. B* 355: 1477-1488.

Yamasaki, H., and Grace, S. C. 1998. EPR detection of phytophenoxyl radicals stabilized by zinc ions: evidence for the redox coupling of plant phenolics with ascorbate in the H_2O_2-peroxidase system. *Federation of European Biochemical Societies* 422: 377-380.

Yamasaki, H., Heshiki, R., Yamasu, T., Sakihama, Y., and Ikehara, N. 1995. Physiological significance of the ascorbate regenerating system for the high-light tolerance of chloroplasts. In Mathis, P., ed. *Photosynthesis: from light to biosphere*. pp 291-294. Kluwer, Dordrecht, The Netherlands.

Yamasaki, H., Sakihama, Y., and Ikehara, N. 1997. Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H_2O_2. Plant Physiology: 1405-1412.

Yamasaki, H., Uefuji, H., and Sakihama, Y. 1996. Bleaching of the red anthocyanin induced by superoxide radical. *Archives of Biochemistry & Biophysics* 332: 183-186.