Chapter 7
Light environment (shade/sun) effects on pigmentation

7.1 Introduction

The colour of waratah flowers and their subtending bracts can change rapidly as the flower bud develops and matures (Nixon, 1997). Bracts are initially green due to chlorophyll pigments, with increasing development of red colouration (due to anthocyanins) towards maturity in red cultivars. The white cultivar 'Wirrimbirra White' remains predominantly green-white in colour at maturity with no obvious anthocyanin accumulation. Pigmentation of waratah bracts is likely to be linked to photoinhibition, established as a cause of browning in Chapter 6. Changes in pigment content may significantly influence browning, particularly altering the susceptibility of bracts to photoinhibition. Differences in the pigment content of leaves and bracts may also explain why bracts are susceptible to browning, while leaves are not affected.

7.1.1 Chlorophyll a *and chlorophyll* b

The chlorophyll pigments (chlorophyll *a* and chlorophyll *b*) give plant tissues their characteristic green colour, due to maximum absorption of light at blue (435-450 nm) and red (650-680 nm) wavelengths. Only chlorophyll *a* is present in reaction centers of photosystem I (PSI) and photosystem II (PSII), while both chlorophyll *a* and *b* are present in the PSI and PSII light harvesting antennae (Haldimann, 1998). As chlorophyll *b* is only present in light harvesting antennae, a higher chlorophyll *b* concentration and lower chlorophyll *a*:*b* ratio, indicates a greater proportion of light harvesting antennae. Chlorophyll *a*:*b* ratios vary from 2.6 to 4.2 in sun-adapted species that have many PSII reaction centers and smaller light harvesting antennae. However, the chlorophyll *a*:*b* ratio

can be as low as 2.0 in shade and low-light species that have fewer PSII complexes and larger light harvesting antenna (Anderson *et al.*, 1998; Atwell *et al.*, 1999; Krause *et al.*, 2003). Chlorophyll *a*:*b* ratios are also linearly correlated with electron transport, chlorophyll protein composition, photosynthetic capacity, D1 protein degradation and membrane stacking and inversely correlated with the extent of photoinhibition (Anderson and Aro, 1994; Anderson *et al.*, 1997).

7.1.2 Carotenoids including xanthophylls

Carotenoids (yellow-orange pigments) are essential components of chlorophyll proteins in the PSII reaction centre, protecting chlorophyll from the damaging effects of both UV and visible light (Lovelock *et al.*, 1992). Carotenoids can quench excitation energy from triplet chlorophyll and singlet oxygen (Niyogi, 1999; Anderson and Chow, 2002) and are usually present at about one-third the abundance of total chlorophyll.

7.1.3 Loss of chlorophyll and carotenoids

Chlorophyll loss can reduce the capacity for photosynthesis and can occur at many stages of plant growth (Hendry *et al.*, 1987; Gossauer and Engel, 1996). Loss may coincide with expansion of the flower, possibly followed by the loss of bracts or spathes subtending the flower. For example, chlorophylls are destroyed in the membranous green spathe of daffodils immediately after flower expansion (Hendry *et al.*, 1987). Chlorophyll can also be destroyed by photooxidation following chilling injury, excessive or prolonged heat, UV radiation, high irradiances of visible light combined with low temperatures, or darkness. For example, sunscald development in apples was correlated with loss of chlorophyll and

carotenoids (Merzlyak *et al.*, 2002), while chlorophyll concentrations were reduced in green and black portions of blackleaf-affected grapevine leaves (Lang *et al.*, 1998).

7.1.4 Anthocyanins and other flavonoids — added protection?

Anthocyanins are water-soluble flavonoids common in flowers, fruits and chlorophyllous tissues, including leaves. The colours produced by anthocyanins, from red to blue and purple, are determined by the structure of the anthocyanin present, modifications with sugar and acyl groups, the presence of co-pigments and metal ions, location of the pigmented tissue, cell shape and pH in the vacuole (Lancaster and Dougall, 1992; Mol *et al.*, 1998). If vacuole pH is high, anthocyanins may also be colourless (Gould *et al.*, 2000). Anthocyanin expression can vary seasonally or ontogenetically (Gould *et al.*, 2002). The red pigmentation of anthocyanins in leaves is often masked by the presence of chlorophylls or carotenoids (Manetas *et al.*, 2003).

7.1.5 Function of anthocyanins

Anthocyanins are induced by a wide range of environmental, biotic and anthropogenic stressors (Chalker-Scott, 1999; Gould *et al.*, 2002). They absorb in the visible region of the spectrum (465-550 nm) and, if acylated, in the UV region (around 290 and 310 nm) (Burger and Edwards, 1996). Anthocyanins can also effectively scavenge active oxygen species (Gould *et al.*, 2002).

The function of anthocyanins depends on their location within the plant tissue. In true flowers, anthocyanins are found in the vacuoles of epidermal cells, while in bracts, such as poinsettia and hydrangea, they are located in the vacuoles of internal cells (Asen, 1976). In

leaves, anthocyanins occur most commonly in the vacuole of palisade and spongy mesophyll cells, but in some species they also occur in the epidermis, hypodermis or vascular parenchyma or in leaf hairs (Neill and Gould, 1999; Gould and Quinn, 1999; Lee and Collins, 2001).

Red leaves tend to absorb more radiation of green-yellow wavelengths (500-600 nm) than green leaves. However, it is unlikely that the light energy absorbed by anthocyanins can be transferred to chloroplasts because they are physically separate in the cell (Gould *et al.*, 2002). Anthocyanins are more likely to act as simple light filters, located in the upper epidermis and/or hypodermis to screen UV-B (Gould *et al.*, 2000). Acylated anthocyanins (phenolic acids) are more likely to be effective in UV protection, due to the superimposition of the aromatic acid absorption on the pigment absorption (Woodall and Stewart, 1998; Chalker-Scott, 1999). Anthocyanins located in the mesophyll are more likely to protect chloroplasts from photoinhibition and scavenge free radicals (Beggs and Wellmann, 1985; Gould *et al.* 2000 and 2002; Lee and Collins, 2001). The interception of light energy (photoabatement) by anthocyanins confers protection on the photosynthetic apparatus, reducing the risk of photoinhibition and encouraging recovery from photoinhibition (Gould *et al.*, 2002).

In addition to anthocyanins, other flavonoids may offer protection from oxidative damage and high light, particularly in the ultraviolet region. UV-B damage has been described in species such as kangaroo paw (*Anigozanthus* spp.), resulting in black flecking on more than 80% of leaves (Ben-Tal and King, 1997). The relative contribution of anthocyanins to the pool of antioxidants (which includes other phenolic compounds and antioxidants such as

superoxide dismutase, catalase and ascorbate peroxidase) varies across species (Gould *et al.*, 2002).

In this chapter, the pigment composition of bracts is measured in several cultivars, as the pigmentation of bracts is likely to significantly influence their susceptibility to browning (Chapter 5) and photoinhibition (Chapter 6). Initially, the pigment composition of mature waratah flowers from commercial growers was quantified (section 7.2). In subsequent experiments, the pigmentation of bracts and leaves were compared as the waratah flower developed and matured (section 7.3). These experiments were conducted on red waratahs at Mount Annan in 2002 and 2003, white waratahs at Jervis Bay in 2002 and naturally occurring waratahs in the Royal National Park.

The first experiment aimed to determine whether significant quantities of chlorophylls, carotenoids, anthocyanins and UV absorbing compounds are present in waratah inner and outer bracts at the mature flower stage, across different varieties grown in different environments.

7.2 Pigment concentration of waratah bracts from commercially grown cultivars in 2001

7.2.1 Methods

Three waratahs of the same cultivar were collected at the mature flower (MF) stage from each property during September-October 2001, with 'Fire and Brimstone' waratahs from three growers and 'Cardinal' and 'Wirrimbirra White' from one grower each. 'Fire and Brimstone' waratahs were grown at Mangrove Mountain on the Central Coast of NSW

(Grower 1), Ulmarra on the Far North Coast of NSW (Grower 2) and Wauchope on the North Coast of NSW (Grower 4). Further details of growing locations are given in Table 3.1.

At the laboratory, inner and outer bract tissue was separated, cut into small pieces and 0.2 g samples of tissue weighed out. Tissues were ground in liquid N and stored in Eppendorf tubes at -80ºC. Ground tissue was transferred to a mortar and pestle and ground again with 2 mL of 80% acetone. The sample was centrifuged for 2 min at 12 000 rpm and the supernatant removed. The clear green supernatant contained extracted chlorophyll and carotenoids and was made up to 3 mL with 80% acetone.

Absorbance was measured at 470, 647 and 663 nm, using an Ultrospec II 4050 UV/Visible spectrophotometer (LKB Biochrom, Cambridge, England). The concentration of chlorophylls *a* and *b*, and total carotenoids, in units of μg/ g FW, was estimated using the equations of Lichtenthaler (1988) (Equation 7.1a-c). All results are normalised to 1g FW of tissue.

Equation 7.1a-c: Chlorophylls *a* and *b* and carotenoids

$$\text{Chlorophyll } a = 12.25A_{663} - 2.79A_{646}$$
$$\text{Chlorophyll } b = 21.50A_{646} - 5.10A_{663}$$
$$\text{Carotenoids} = (1000A_{470} - 1.82\text{Chl } a - 85.02\text{Chl } b)/198$$

Sample preparation and storage for anthocyanins and UV-absorbing compound extraction follows the method for chlorophyll and carotenoids above. On removal from the freezer, 2 mL of acidified methanol (3M HCl:H_2O:MeOH 1:3:16 v/v) was added to the ground tissue and tubes were held at 4°C for 24 hours. The solution was filtered with a 0.22 μm Millipore

filter. Absorbance was measured at maximal absorbance (about 530 nm) and at 653 nm for anthocyanins. Maximal absorption was found at 527 nm, from scanning spectrophotometry. Anthocyanin concentrations were estimated according to Murray and Hackett (1991) (Equation 7.2a). For UV-absorbing compounds, the supernatant was diluted 50 fold and absorbance measured at 300 and 350 nm as an estimate of UV-B and UV-A absorbing compounds, respectively (Day, 1993) (Equation 7.2b,c). Results are presented as absorbance/g FW of tissue surface area.

Equation 7.2a-b: Anthocyanins and UV-A and UV-B absorbance

$$\text{Anthocyanins} = A_{530} - 0.24A_{653}$$
$$\text{UV-A absorption approx.} = A_{350}$$
$$\text{UV-B absorption approx.} = A_{300}$$

The results for each cultivar were analysed separately using the ANOVA procedure in Genstat Release 6.1 (Lawes Agricultural Trust, 2002). 'Fire and Brimstone' data required log transformation to reduce variance in the residual analysis, while 'Cardinal' and 'Wirrimbirra White' data did not require transformation.

7.2.2 Results

The concentration of each pigment analysed in inner and outer bracts was compared separately for each cultivar (Tables 7.1 - 7.3). Carotenoid concentrations were significantly higher in outer than inner bracts of 'Cardinal' (Table 7.1), 'Wirrimbirra White' (Table 7.2) and 'Fire and Brimstone' waratahs from two out of three growers (Table 7.3d). Anthocyanin concentrations were higher in inner than outer bracts of 'Cardinal' and 'Fire and Brimstone' waratahs from two out of three growers (Tables 7.1 and 7.3e, respectively).

Table 7.1: Pigment concentration (μg/g FW) of 'Cardinal' bracts at the mature flower (MF) stage. Bold type indicates significant difference between inner and outer bract at $P < 0.05$. n = 3 buds.

Tissue	Chlorophyll *a*	Chlorophyll *b*	Total Chlorophyll	Total carotenoid	Abs Anthocyanin	Abs UV-B	Abs UV-A
Inner bract	4.19	2.78	6.98	**2.98**	**7.68**	0.423	0.148
Outer bract	6.45	3.43	9.88	**6.93**	**1.59**	0.598	0.235

Table 7.2: Pigment concentration (μg/g FW) of 'Wirrimbirra White' bracts at the mature flower (MF) stage. Bold type indicates significant difference between inner and outer bract at $P < 0.05$. n = 3 buds.

Tissue	Chlorophyll *a*	Chlorophyll *b*	Total Chlorophyll	Total carotenoid	Abs Anthocyanin	Abs UV-B	Abs UV-A
Inner bract	4.91	2.15	7.07	**2.24**	0.12	0.31	0.28
Outer bract	7.63	3.79	12.41	**4.42**	0.16	0.26	0.15

As well as significant differences between inner and outer bracts, pigment concentrations varied between 'Fire and Brimstone' waratahs grown in different areas. Chlorophylls *a*, *b* and total chlorophyll concentrations were significantly different at $P < 0.05$ between outer bracts from Growers 1 and 4 (Table 7.3 a-c). Carotenoid concentrations were significantly different for both inner and outer bracts of Growers 2 and 4, while inner bracts were significantly different between Growers 1 and 4 (Table 7.3 d). Anthocyanin concentrations were significantly different for inner and outer bracts of Growers 2 and 3 (Table 7.3e). UV-A and UV-B absorbance were significantly different for outer bracts from Growers 1 and 2 (Table 7.3 f-g). No measurements of UV absorbance were made for Grower 4.

Table 7.3a-g: Pigment concentration (μg/g FW) of 'Fire and Brimstone' bracts from growers at Mangrove Mountain on the Central Coast (1), Ulmarra on the Far North Coast (2) and Wauchope on the North Coast (4). Bold type indicates significant difference between inner and outer bract at $P < 0.05$. n = 3 buds from each of 3 growers.

(a)	Log_{10} transformed chlorophyll *a*			Back transformed chlorophyll *a*		
Tissue	Grower 1	Grower 2	Grower 4	Grower 1	Grower 2	Grower 4
Inner bract	0.41	**0.34**	**0.26**	2.55	2.17	1.81
Outer bract	0.73	**0.82**	**1.09**	5.35	6.58	12.39

(b)	Log_{10} transformed chlorophyll *b*			Back transformed chlorophyll *b*		
Tissue	Grower 1	Grower 2	Grower 4	Grower 1	Grower 2	Grower 4
Inner bract	**0.33**	**0.23**	**0.11**	2.11	1.69	1.29
Outer bract	**0.53**	**0.55**	**0.81**	3.40	3.53	6.52

(c)	Log_{10} transformed total chlorophyll			Back transformed total chlorophyll		
Tissue	Grower 1	Grower 2	Grower 4	Grower 1	Grower 2	Grower 4
Inner bract	**0.67**	**0.59**	**0.50**	4.68	3.90	3.16
Outer bract	**0.95**	**1.01**	**1.28**	7.81	10.14	17.92

(d)	Log_{10} transformed carotenoid			Back transformed carotenoid		
Tissue	Grower 1	Grower 2	Grower 4	Grower 1	Grower 2	Grower 4
Inner bract	0.53	**0.39**	**0.13**	3.38	2.43	1.36
Outer bract	0.67	**0.72**	**0.86**	4.68	5.26	7.28

(e)	Log_{10} transformed anthocyanin			Back transformed anthocyanin		
Tissue	Grower 1	Grower 2	Grower 4	Grower 1	Grower 2	Grower 4
Inner bract	0.66	**0.65**	**0.42**	4.55	4.45	2.61
Outer bract	0.70	**0.14**	**-0.07**	5.00	1.36	0.84

(f)	Log_{10} transformed Abs UV-B		Back transformed Abs UV-B	
Tissue	Grower 1	Grower 2	Grower 1	Grower 2
Inner bract	-0.34	-0.42	0.45	0.38
Outer bract	-0.24	-0.43	0.58	0.38

(g)	Log_{10} transformed Abs UV-A		Back transformed Abs UV-A	
Tissue	Grower 1	Grower 2	Grower 1	Grower 2
Inner bract	-0.69	-0.81	0.21	0.15
Outer bract	-0.69	-0.86	0.21	0.14

Pigment concentrations of different cultivars were not compared statistically, because of different growing practices at each site. However, chlorophylls and carotenoids occur at comparable concentrations for the three cultivars studied (Figures 7.1 and 7.2). 'Wirrimbirra White' waratahs appear to have a similar pigment profile to red cultivars, except for very low concentrations of anthocyanin (Figure 7.3).

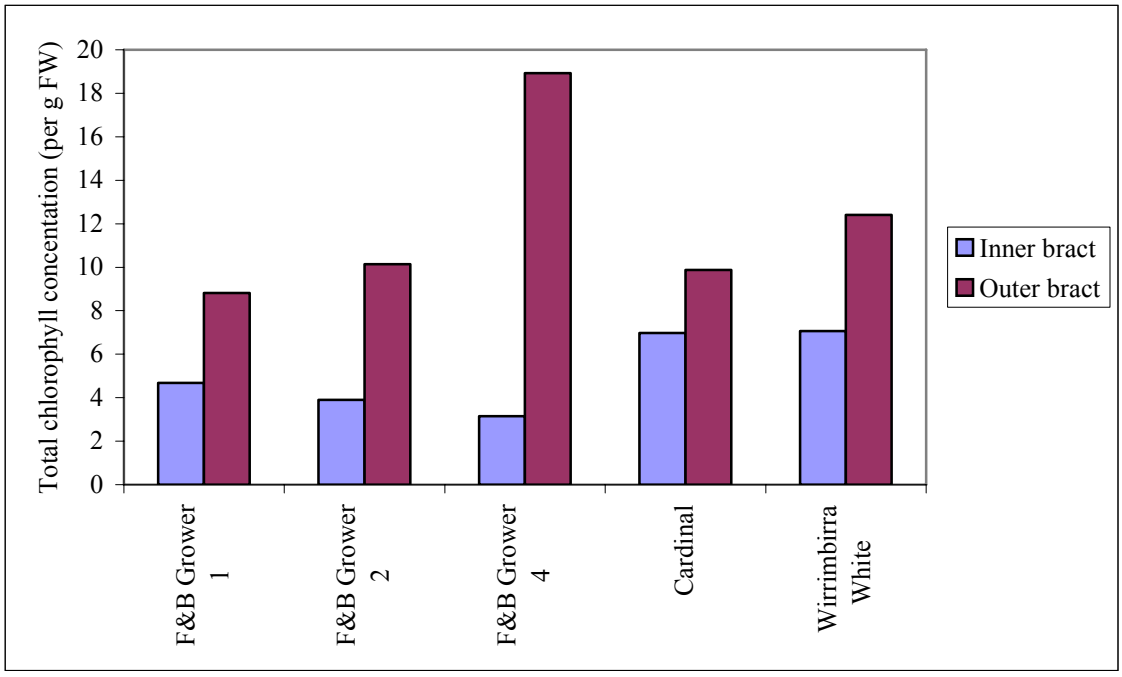

Figure 7.1: Total chlorophyll concentration (µg/g FW) of bracts at the mature flower (MF) stage from 'Fire and Brimstone' (F&B), 'Cardinal' and 'Wirrimbirra White' waratahs grown commercially in NSW. 'Fire and Brimstone' results are growers at Mangrove Mountain on the Central Coast (1), Ulmarra on the Far North Coast (2) and Wauchope on the North Coast (4). n = 3 buds for each grower/ cultivar combination.

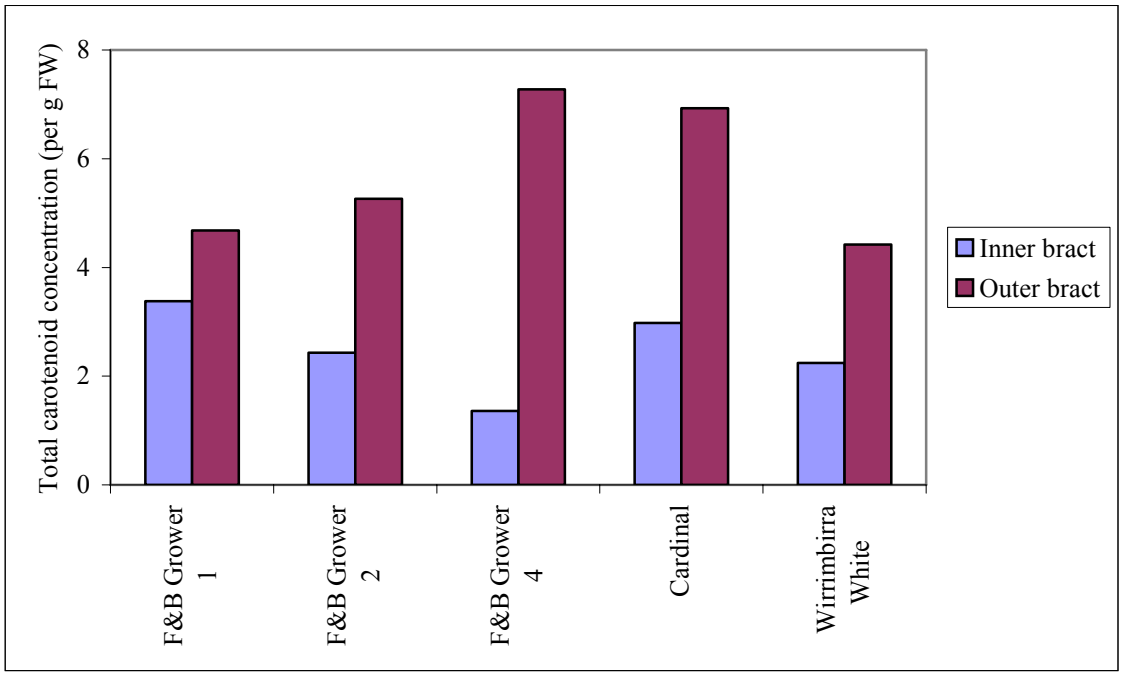

Figure 7.2: Total carotenoid concentration (µg/g FW) of bracts at the mature flower (MF) stage from 'Fire and Brimstone' (F&B), 'Cardinal' and 'Wirrimbirra White' waratahs grown commercially in NSW. 'Fire and Brimstone' results are growers at Mangrove Mountain on the Central Coast (1), Ulmarra on the Far North Coast (2) and Wauchope on the North Coast (4). n = 3 buds for each grower/ cultivar combination.

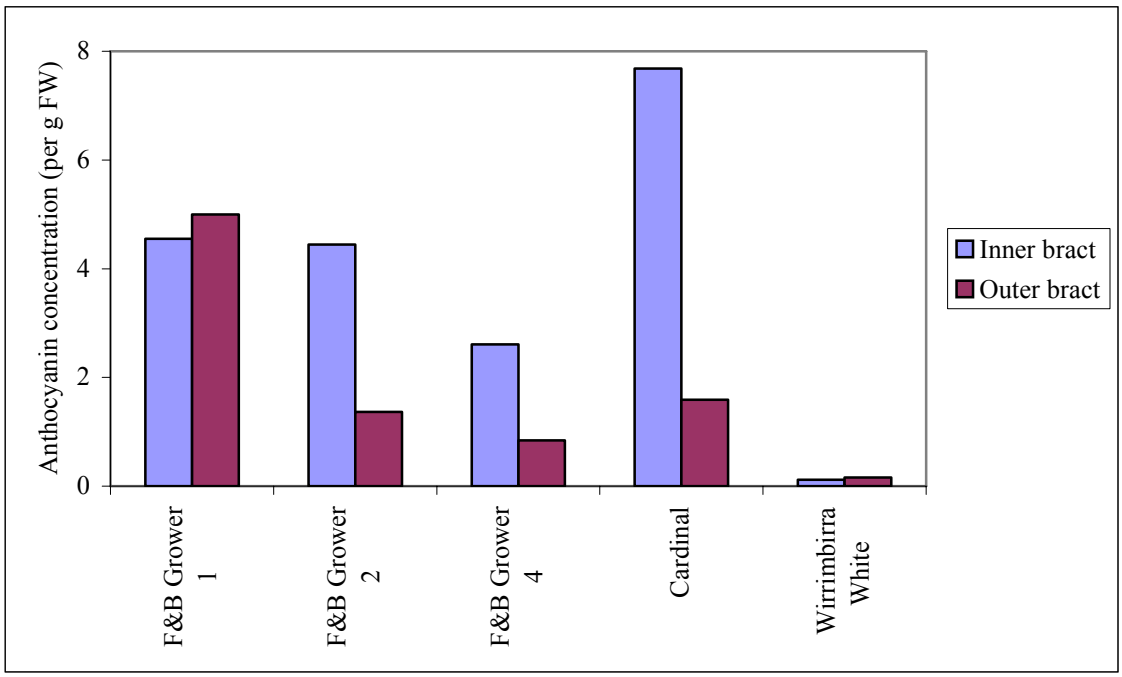

Figure 7.3: Total anthocyanin concentration (absorbance per g FW) of bracts at the mature flower (MF) stage from 'Fire and Brimstone' (F&B), 'Cardinal' and 'Wirrimbirra White' waratahs grown commercially in NSW. 'Fire and Brimstone' results are growers at Mangrove Mountain on the Central Coast (1), Ulmarra on the Far North Coast (2) and Wauchope on the North Coast (4). n = 3 buds for each grower/ cultivar combination.

Since there were significant differences in pigment concentrations between inner and outer bracts at flower maturity, further experiments were designed to investigate whether waratah bract pigments are synthesized or destroyed as flower buds develop. These experiments also investigated the effect of shade on bract pigmentation and were conducted at Mount Annan on 'Fire and Brimstone' waratahs in 2002 and both 'Fire and Brimstone'and 'Olympic Flame' in 2003, as well as 'Wirrimbirra White' waratahs at Jervis Bay in 2002. The pigment concentration of bracts and leaves was compared in the above experiments, as well as in naturally occurring waratahs at flower maturity in 2003.

7.3 Pigment concentration of bracts and leaves of red and white waratah cultivars during flower development and naturally occurring waratahs at flower maturity

7.3.1 Methods

7.3.1.1 Methods in 2002

Leaf and bract samples were collected from 'Fire and Brimstone' waratahs at tight bud (TB), juvenile open bud (JO) and mature flower (MF) stages at Mount Annan. Plants were grown in full sun or shaded early (from bud initiation) or late (from bud opening) with 50% shade cloth.

Similarly, leaf and bract samples were collected from 'Wirrimbirra White' waratahs in 2002 at tight bud (TB), juvenile open bud (JO) and mature flower (MF) stages at Jervis Bay. Plants were grown in full sun or shaded from bud opening with 50% shade cloth.

Leaf and bract discs (10 mm diameter) were collected and stored at -80ºC. Discs were ground in liquid N and transferred to 2 mL Eppendorf tubes. Chlorophyll and carotenoids were extracted by adding 1mL of 80% acetone, and gently agitating on a rocker for 24 hrs at 4ºC in the dark (Gould *et al.*, 2000). Tubes were centrifuged at 13000 rpm for 2 min. The supernatant was removed and diluted with a further 1 mL of 80% acetone, followed by another 2 min centrifugation.

Absorbance was measured at 470, 647 and 663 nm, using an Ultrospec II 4050 UV/Visible spectrophotometer (LKB Biochrom, Cambridge, England). The concentration of chlorophylls *a* and *b*, and total carotenoids was estimated using the equations of Wellburn (1994) for a spectrophotometer with 1-4 nm resolution (Equation 7.3a-e). All results are normalised to 1 mL solution and 1 cm^2 of tissue surface area. Results are expressed in $\mu g/cm^2$ except for chlorophyll *a*:*b* ratio, which is expressed in as a mole ratio, as suggested by Hendry and Price (1993).

Equation 7.3a-e: Chlorophyll and carotenoids

$$\text{Chlorophyll } a = 12.21A_{663} - 2.81A_{646}$$
$$\text{Chlorophyll } b = 20.13A_{646} - 5.03A_{663}$$
$$\text{Carotenoids} = (1000A_{470} - 3.27\text{Chl } a - 104\text{Chl } b)/198$$
$$\text{Chlorophyll } a\ (\mu\text{mol}) = 1.119\ \text{Chlorophyll } a\ (\mu g)$$
$$\text{Chlorophyll } b\ (\mu\text{mol}) = 1.102\ \text{Chlorophyll } b\ (\mu g)$$

Anthocyanins and UV-absorbing compounds were extracted using 1 mL of acidified methanol (3M HCl:H_2O:MeOH 1:3:16 v/v). Sample preparation follows the method for chlorophyll and carotenoids above. Absorbance was measured at 530 and 653 nm for anthocyanins and concentrations were estimated according to Murray and Hackett (1991) (Equation 7.4a). For UV-absorbing compounds, supernatant was diluted 50 fold and absorbance measured at 300 and 350 nm as an estimate of UV-B and UV-A absorbing

compounds, respectively (Day, 1993) (Equation 7.4b,c). Results are presented as absorbance/cm^2 of tissue surface area.

Equation 7.4a-c: Anthocyanins and UV-absorbing compounds

$$\text{Anthocyanins} = A_{530} - 0.24A_{653}$$
$$\text{UV-A absorption approx.} = A_{350}$$
$$\text{UV-B absorption approx.} = A_{300}$$

Data were $\log_{10}$ transformed to reduce variance in the residual analysis. In this chapter, data are presented on a log-transformed scale with least significant differences (LSD's) for comparison, as well as back-transformed values.

The experimental design at Mount Annan was unbalanced, due to different numbers of plants in each treatment being available for measurement at each stage. A restricted maximum likelihood (REML) model was fitted, as described in Chapter 4, with *Treatment*, *Stage* and *Tissue* and their interactions as fixed factors and *Tissue* nested in *Plant* as random factors. Correlated error terms (diagonal) were introduced because *Tissue* variation is not independent, as leaf means are often much higher or lower than bract means. This error structure further reduced variance in the residual analysis. Interactions or individual terms were considered significant at $P \leq 0.05$.

7.3.1.2 Methods in 2003

Leaf and bract samples were collected from 'Fire and Brimstone' and 'Olympic Flame' waratahs at JO and MF stages at Mount Annan. Plants were grown in full sun or shaded early (from bud initiation) or late (from bud opening) with 50% shade cloth.

In the Royal National Park, waratah leaf and bract tissue was collected from mature flowers at the three sites described in Chapter 6 on 06/10/03. Discs (10 mm) were punched from four leaves and five to six inner red floral bracts of each flower.

Leaf and bract discs (19 mm diameter) were collected and stored at -80ºC. Pigment extraction was carried out as described in Experiment 7.3.1.1. Absorbance was measured from 400-700 nm (chlorophylls and carotenoids) and 300-700 nm (anthocyanins and UV absorbance) using a Cary 50 Bio UV-Visible scanning spectrophotometer (Varian, Mulgrave, Victoria) with Cary Win-UV scanning kinetics software version 2.0 for data collection.

Statistical analysis was carried out as described in Experiment 7.3.1.1 for three groups of data from Mount Annan to minimize effects of missing treatment combinations: (1) 'Fire and Brimstone' waratahs comparing late shade and sun treatments, and inner bracts and leaves at JO and MF stages (section 7.3.2.4); (2) 'Olympic Flame' waratahs comparing late shade and sun treatments, and inner bracts, outer bracts and leaves at JO and MF stages (7.3.2.5); (3) Inner bracts only of 'Fire and Brimstone' and 'Olympic Flame' waratahs comparing early shade, late shade and sun treatments at JO and MF stages (7.3.2.6). For Royal National Park data, *Plant* was a fixed term and *Tissue* nested in *Plant* were random terms in the REML analysis.

7.3.2 Results

7.3.2.1 Combined results for all cultivars and sites

The results for pigment experiments at the three sites – Mount Annan, Jervis Bay and Royal National Park – over several cultivars were similar in all years. Therefore, combined results for all sites and cultivars are collated in this section, followed by figures from each cultivar and year of study. Results for total chlorophyll are presented, rather than separate results for chlorophylls *a* and *b*, as cultivar and treatment effects were similar for total chlorophyll or its components in both 2002 and 2003. Similarly, results are presented for UV-A or UV-B absorbance only, as results are similar. Significant interactions between tissue type (leaf, outer or inner bract) and stage of floral development (TB, JO or MF), as well as between treatment (full sun, early or late shading) and stage of floral development, are evident for many of the pigments investigated.

Leaves had a concentration of chlorophylls about ten times higher than inner and outer bracts in all experiments (Tables 7.4, 7.12, 7.20, 7.24 and 7.32). Outer bracts showed pigment degradation from early to mid (JO) or late (MF) floral development, with significant decreases in chlorophylls *a* and *b*, total chlorophyll (Tables 7.4, 7.12 and 7.24) and carotenoids (Tables 7.6 and 7.25). In contrast, inner bracts synthesised pigments over time, with significant increases in chlorophylls *a* and *b*, total chlorophyll (Tables 7.4 and 7.20) and carotenoids (Tables 7.6 and 7.25) For example, total chlorophylls in 'Fire and Brimstone' and 'Olympic Flame' inner bracts increased from 0.273 μg/cm^2 at the JO stage to 0.380 μg/cm^2 at the MF stage ($P = 0.012$). Similarly, total carotenoids in these cultivars increased from 0.100 μg/cm^2 at the JO stage to 0.199 μg/cm^2 at the MF stage ($P < 0.001$).

The exceptions to this trend was inner bract total chlorophyll in 'Wirrimbirra White' waratahs, which decreased significantly from the TB to MF stages of development (Table 7.12). Chlorophylls *a* and *b* showed similar trends to total chlorophyll.

Leaf chlorophyll *a:b* ratios were higher than those of bracts 'Fire and Brimstone' (3.211 in leaves compared to 1.731 in bracts, $P < 0.001$) and 'Olympic Flame' in 2003 (3.607 in leaves compared to 1.914 in inner bracts and 2.854 in outer bracts, $P < 0.001$) as well as naturally occurring waratahs (Table 7.32) but not in 'Fire and Brimstone' in 2002 (Table 7.8). Bracts also had a significantly higher carotenoid:chlorophyll ratio than leaves in most situations (Tables 7.9 and 7.32). For example, in 'Wirrimbirra White', carotenoid:chlorophyll in leaves was 0.221, while in inner bracts it was 0.531 and in outer bracts, 0.414 ($P < 0.001$). In some instances, the difference between bract and leaf carotenoid: chlorophyll was not always significant, although bract levels tended to be higher in all cases (Tables 7.21 and 7.26).

Bracts grown in full sun showed significant decreases in chlorophylls and carotenoids compared to shade grown bracts, at different stages of development. In 2002, 'Fire and Brimstone' tissues grown in full sun showed significant decreases in chlorophylls and carotenoids from TB to JO stages, while early shade prevented this decrease (Tables 7.5 and 7.7). In this experiment in 2002, early and late shading resulted in comparable chlorophyll concentrations. Significantly lower concentrations of chlorophylls and carotenoids were found in mature 'Wirrimbirra White' flowers grown in the sun (Table 7.13 for chlorophylls and Table 7.15 for carotenoids). Similarly, significantly lower concentrations of chlorophylls were present in sun exposed 'Fire and Brimstone' (1.399

μg/cm^2 in sun and 2.030 μg/cm^2 in shade, $P = 0.017$) and sun exposed 'Olympic Flame' in 2003 (1.399 μg/cm^2 in sun and 2.150 μg/cm^2 in shade, $P = 0.014$).

Visually, the high chlorophyll content of waratah leaves masked the presence of anthocyanins. The anthocyanin content of inner bracts was significantly higher than that of leaves in 'Fire and Brimstone' and 'Olympic Flame' in 2003 (Tables 7.22 and 7.27) and in waratahs in the Royal National Park (Table 7.32). Exceptions to this trend include 'Fire and Brimstone' in 2002 (Table 7.10) and 'Wirrimbirra White' (Table 7.18), which had very low anthocyanin concentrations in all tissue. Anthocyanin concentrations in red waratah cultivars tended to increase in bract tissues during development, for example, in early shaded inner bracts of 'Fire and Brimstone' in 2002 (Table 7.10), inner bracts of 'Fire and Brimstone' in 2003 (Table 7.22, JO to MF stages) and in outer bracts of 'Olympic Flame' in 2003 (Table 7.27, JO to MF stages). Shaded inner bracts accumulated significantly higher concentrations of anthocyanins late in development compared to sun exposed inner bracts (Table 7.10 and 7.31 for early shade treatment). Shading also allowed a greater increase in anthocyanins than observed in sun bracts during development (Table 7.10 and 7.31 for late shade treatment).

UV absorbance was approximately three to six times higher in leaves than bracts for most cultivars (Tables 7.11, 7.19, 7.23, 7.28, 7.29 and 7.32). Sun-exposed tissues generally had a higher UV-A and/or UV-B absorbance than shaded tissues. For example, 'Fire and Brimstone' UV-A absorbance in 2002 was 1.620 units/cm^2 in the sun compared to 1.118 units/cm^2 in the shade, while in 2003, UV-A absorbance in the sun was 0.695 units/cm^2

compared to 0.554 units/cm^2 in the shade (P = 0.016). UV-B absorbance in 'Wirrimbirra White' in the sun was 2.116 units/cm^2, compared to 1.672 units/cm^2 in the shade (P =0.047), while in 'Olympic Flame' UV-B absorbance was 0.879 units/cm^2 in the sun compared to 0.753 units/cm^2 in late shaded tissues (P = 0.004).

Pigmentation of cultivars grown at different sites cannot be compared directly, due to differences in growing environment, plant age and cultural practices at each site. However, these results indicate that 'Fire and Brimstone', 'Wirrimbirra White' and 'Olympic Flame' have the potential to produce similar chlorophyll concentrations. Only inner bracts of 'Olympic Flame' and 'Fire and Brimstone' could be directly compared in 2003, due to missing treatment combinations for other tissues. 'Olympic Flame' had higher chlorophyll *a*:*b* concentrations than 'Fire and Brimstone' (ratio of 2.241 compared to 1.658, P = 0.024). Carotenoid:chlorophyll ratios were higher in 'Fire and Brimstone' at the JO stage and in 'Olympic Flame' at the MF stage (Table 7.30).

7.3.2.2 Results for 'Fire and Brimstone' waratahs at Mount Annan in 2002

Table 7.4: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions ($P < 0.001$) in total chlorophyll concentrations (μg /cm^2) of 'Fire and Brimstone' waratahs in 2002, pooled for full sun and early and late shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 4 plants in each light treatment for TB (tight bud) stage, and 8 early shade plants, 5 late shade plants and 7 full sun plants at the MF (mature flower) stage.

	Log_{10} total chlorophyll		Back transformed total chlorophyll	
	Stage			
Tissue	TB	MF	TB	MF
Leaf	1.072 Aa	1.065 Aa	11.814	11.625
Inner bract	-0.616 Ab	0.102 Bb	0.242	1.265
Outer bract	0.383 Ac	-0.133 Bb	2.417	0.737
LSD	0.253			

Table 7.5: Log transformed means (compared by LSD) and back transformed means for significant treatment by stage interactions ($P = 0.003$) in total chlorophyll concentrations (μg /cm^2) of 'Fire and Brimstone' waratahs in 2002, pooled for leaf, inner bract and outer bract tissues. Upper case letters indicate significant differences within the same treatment at different stages of development, while lower case letters indicate significant differences between treatments at the same stage. n = 4 plants in each light treatment for TB (tight bud) and JO (juvenile open) stages, and 8 early shade plants, 5 late shade plants and 7 full sun plants at the MF (mature flower) stage.

	Log_{10} total chlorophyll			Back transformed total chlorophyll		
	Stage					
Treatment	TB	JO	MF	TB	JO	MF
Sun	0.306 Aa	-0.081 Ba	0.354 Aa	2.022	0.831	2.258
Early shade	0.229 Aa	0.092 Aa	0.411 Ba	1.695	1.235	2.575
Late shade	0.305 Aa		0.270 Aa	2.018		1.863
LSD	0.201					

Table 7.6: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions ($P < 0.001$) in total carotenoid concentrations (μg /cm^2) of 'Fire and Brimstone' waratahs in 2002, pooled for full sun and early and late shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 4 plants in each light treatment for TB (tight bud) and JO (juvenile open) stages, and 8 early shade plants, 5 late shade plants and 7 full sun plants at the MF (mature flower) stage.

	Log_{10} total carotenoid		Back transformed total carotenoid	
	Stage			
Tissue	TB	MF	TB	MF
Leaf	0.534 Aa	0.647 Aa	3.422	4.436
Inner bract	-0.021 Ab	0.232 Bb	0.952	1.708
Outer bract	0.522 Aa	0.092 Bc	3.327	1.237
LSD	0.127			

Table 7.7: Log transformed means (compared by LSD) and back transformed means for significant treatment by stage interactions (P = 0.012) in total carotenoid concentrations (μg /cm^2) of 'Fire and Brimstone' waratahs in 2002, pooled for leaf, inner bract and outer bract tissues. Upper case letters indicate significant differences within the same treatment at different stages of development, while lower case letters indicate significant differences between treatments at the same stage. n = 4 plants in each light treatment for TB (tight bud) and JO (juvenile open) stages, and 8 early shade plants, 5 late shade plants and 7 full sun plants at the MF (mature flower) stage.

	Log_{10} total carotenoid			Back transformed total carotenoid		
	Stage					
Treatment	TB	JO	MF	TB	JO	MF
Sun	0.448 Aa	0.210 Ba	0.362 Aa	2.802	1.621	2.304
Early shade	0.232 Ab	0.383 Bb	0.323 ABa	1.705	2.415	2.102
Late shade	0.356 Aab		0.287 Ba	2.269		1.935
LSD	0.138					

Table 7.8: Log transformed means (compared by LSD) and back transformed means for tissue by stage interactions (P = 0.452) in chlorophyll *a*:*b* ratios of 'Fire and Brimstone' waratahs in 2002, pooled for full sun and early and late shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 4 plants in each light treatment for TB (tight bud) and JO (juvenile open) stages, and 8 early shade plants, 5 late shade plants and 7 full sun plants at the MF (mature flower) stage.

	Log_{10} chlorophyll *a*:*b*		Back transformed chlorophyll *a*:*b*	
	Stage			
Tissue	TB	MF	TB	MF
Leaf	0.014 Aa	0.091 Aa	1.032	1.233
Inner bract	0.418 Ab	0.440 Ab	2.618	2.754
Outer bract	0.465 Ab	0.443 Ab	2.920	2.771
LSD	0.100			

Table 7.9: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions (P < 0.001) in total carotenoid:chlorophyll ratios of 'Fire and Brimstone' waratahs in 2002, pooled for full sun and early and late shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 4 plants in each light treatment for TB (tight bud) and JO (juvenile open) stages, and 8 early shade plants, 5 late shade plants and 7 full sun plants at the MF (mature flower) stage.

	Log_{10} car:chlorophyll		Back transformed car:chlorophyll	
	Stage			
Tissue	TB	MF	TB	MF
Leaf	-0.543 Aa	-0.421 Aa	0.286	0.379
Inner bract	0.599 Ab	0.130 Bb	3.974	1.349
Outer bract	0.138 Ac	0.219 Ab	1.374	1.654
LSD	0.185			

Table 7.10: Log transformed means (compared by LSD) and back transformed means for significant tissue by treatment by stage interactions ($P < 0.001$) in anthocyanin concentration (abs/cm^2) of 'Fire and Brimstone' waratahs in 2002. Upper case letters indicate significant differences within the same treatment and tissue combination at different stages of development, while lower case letters indicate significant differences between treatment and tissue combinations at the same stage. n = 4 plants in each light treatment for TB (tight bud) and JO (juvenile open) stages, and 8 early shade plants, 5 late shade plants and 7 full sun plants at the MF (mature flower) stage.

		Log_{10} anthocyanin			Back transformed anthocyanin		
		Stage					
Tissue	Treatment	TB	JO	MF	TB	JO	MF
Leaf	Sun	-1.269 Aab	-1.241 Aa	-1.159 Aa	0.054	0.057	0.069
Leaf	Early shade	-1.460 Aa	-1.193 Aa	-1.408 Aa	0.035	0.064	0.039
Leaf	Late shade	-1.246 Aab		-1.166 Aab	0.057		0.068
Inner bract	Sun	-0.860 Ab	-1.383 Ba	-1.226 ABab	0.138	0.041	0.059
Inner bract	Early shade	-1.203 ABab	-1.482 Ba	-0.842 Ab	0.063	0.033	0.144
Inner bract	Late shade	-0.874 Ab		-1.137 Aa	0.134		0.073
Outer bract	Sun	-1.420 Aa	-1.226 Aa	-0.796 Bbc	0.038	0.059	0.160
Outer bract	Early shade	-1.399 Aa	-1.427 Aa	-0.410 Bc	0.040	0.037	0.389
Outer bract	Late shade	-1.357 Aa		-0.666 Bbc	0.044		0.216
	LSD	0.412					

Table 7.11: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions ($P < 0.001$) in UV-A absorbance (abs/cm^2) of 'Fire and Brimstone' waratahs in 2002, pooled for full sun and early and late shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 4 plants in each light treatment for TB (tight bud) and JO (juvenile open) stages, and 8 early shade plants, 5 late shade plants and 7 full sun plants at the MF (mature flower) stage.

	Log_{10} UV-A abs		Back transformed UV-A abs	
	Stage			
Tissue	TB	MF	TB	MF
Leaf	0.748 Aa	0.612 Aa	5.599	4.090
Inner bract	-0.270 Ab	-0.053 Bb	0.537	0.885
Outer bract	0.244 Ac	-0.092 Ab	1.755	0.808
LSD	0.190			

7.3.2.3 Results for 'Wirrimbirra White' at Jervis Bay in 2002

Table 7.12: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions (P = 0.002) in total chlorophyll concentrations (μg /cm^2) of 'Wirrimbirra White' waratahs in 2002, pooled for sun and shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 5 plants in each light treatment (sun or shade).

	Log_{10} total chlorophyll			Back transformed total chlorophyll		
	Stage					
Tissue	TB	JO	MF	TB	JO	MF
Leaf	0.889 Aa	0.751 Aa	0.795 Aa	7.739	5.635	6.237
Inner bract	-0.607 Ab	-0.750 ABb	-0.828 Bb	0.247	0.178	0.149
Outer bract	0.183 Ac	-0.225 Bc	-0.158 Bc	1.524	0.596	0.695
LSD	0.161					

Table 7.13: Log transformed means (compared by LSD) and back transformed means for significant treatment by stage interactions (P < 0.001) in total chlorophyll concentrations (μg /cm^2) of 'Wirrimbirra White' waratahs in 2002, pooled for leaf, inner and outer bract tissues. Upper case letters indicate significant differences within the same treatment at different stages of development, while lower case letters indicate significant differences between treatments at the same stage. n = 5 plants in each light treatment (sun or shade).

	Log_{10} total chlorophyll			Back transformed total chlorophyll		
	Stage					
Treatment	TB	JO	MF	TB	JO	MF
Sun	0.088 Aa	-0.111 Ba	-0.198 Ba	1.225	0.774	0.634
Shade	0.166 Aa	-0.039 Ba	0.070 ABb	1.465	0.915	1.176
LSD	0.140					

Table 7.14: Log transformed means (compared by LSD) and back transformed means for the significant tissue term (P < 0.001) in total carotenoid concentrations (μg /cm^2) of 'Wirrimbirra White' waratahs in 2002, pooled for sun and shade treatments and stages of flower development. Lower case letters indicate significant differences between tissues. n = 5 plants in each light treatment (sun or shade).

Tissue	Log_{10} carotenoid	Back transformed carotenoid
Leaf	0.156 a	1.433
Inner bract	-1.028 b	0.094
Outer bract	-0.447 c	0.358
LSD	0.115	

Table 7.15: Log transformed means (compared by LSD) and back transformed means for significant treatment by stage interactions ($P < 0.001$) in total carotenoid concentrations (μg /cm^2) of 'Wirrimbirra White' waratahs in 2002, pooled for leaf, inner and outer bract tissues. Upper case letters indicate significant differences within the same treatment at different stages of development, while lower case letters indicate significant differences between treatments at the same stage. n = 5 plants in each light treatment (sun or shade).

	Log_{10} total carotenoid			Back transformed total carotenoid		
	Stage					
Treatment	TB	JO	MF	TB	JO	MF
Sun	-0.403 Aa	-0.438 Aa	-0.606 Ba	0.395	0.365	0.248
Shade	-0.367 Aa	-0.434 Aa	-0.390 Ab	0.430	0.368	0.408
LSD	0.115					

Table 7.16: Log transformed means (compared by LSD) and back transformed means for significant tissue by treatment by stage interactions ($P = 0.003$) in chlorophyll *a*:*b* concentrations of 'Wirrimbirra White' waratahs in 2002. Upper case letters indicate significant differences within the same tissue and treatment combination at different stages of development, while lower case letters indicate significant differences between tissue and treatment combinations at the same stage. n = 5 plants in each light treatment(sun or shade).

		Log_{10} chlorophyll *a*:*b*			Back transformed chlorophyll *a*:*b*		
		Stage					
Tissue	Trt	TB	JO	MF	TB	JO	MF
Leaf	Shade	0.333 Aa	0.445 Aa	0.210 Aa	2.151	2.789	1.621
Leaf	Sun	0.347 Aa	0.440 Aa	0.388 Aac	2.222	2.756	2.446
Inner bract	Shade	0.471 Aab	0.475 Aa	0.735 Bbc	2.960	2.984	5.438
Inner bract	Sun	0.629 Ab	0.419 ABa	0.378 Bac	4.257	2.622	2.386
Outer bract	Shade	0.473 Ab	0.561 Aa	0.475 Ac	2.971	3.636	2.984
Outer bract	Sun	0.534 Ab	0.412 Aa	0.430 Aac	3.423	2.582	2.691
	LSD	0.239					

Table 7.17: Log transformed means (compared by LSD) and back transformed means for the significant stage term ($P = 0.005$) in carotenoid:chlorophyll concentrations of 'Wirrimbirra White' waratahs in 2002, pooled for sun and shade treatments and leaf, inner and outer bract tissues. Lower case letters indicate significant differences between stages. n = 5 plants in each light treatment (sun or shade).

Stage	Log_{10} car:chlorophyll	Back transformed car:chlorophyll
TB	-0.515 ac	0.305
JO	-0.364 b	0.433
MF	-0.434 bc	0.368
LSD	0.092	

Table 7.18: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions ($P < 0.001$) in anthocyanin concentrations (abs/cm^2) of 'Wirrimbirra White' waratahs in 2002, pooled for sun and shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 5 plants in each light treatment (sun or shade).

	Log_{10} anthocyanin			Back transformed anthocyanin		
	Stage					
Tissue	TB	JO	MF	TB	JO	MF
Leaf	-1.293 Aa	-1.444 Ba	-1.362 Aa	0.051	0.036	0.043
Inner bract	-2.256 Ab	-2.332 Ab	-2.429 Bb	0.006	0.005	0.004
Outer bract	-1.853 Ac	-1.833 Ac	-1.882 Ac	0.014	0.015	0.013
LSD	0.079					

Table 7.19: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions ($P < 0.001$) in UV-B absorbance (abs/cm^2) of 'Wirrimbirra White' waratahs in 2002, pooled for sun and shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 5 plants in each light treatment (sun or shade).

	Log_{10} UV-B absorbance			Back transformed UV-B absorbance		
	Stage					
Tissue	TB	JO	MF	TB	JO	MF
Leaf	0.790 Aa	0.677 Aa	0.819 Aa	6.167	4.758	6.595
Inner bract	-0.030 Ab	-0.229 Ab	-0.038 Ab	0.933	0.590	0.917
Outer bract	0.072 ABb	0.473 Aa	-0.064 Bb	1.181	2.969	0.862
LSD	0.432					

7.3.2.4 Results for 'Fire and Brimstone' waratahs at Mount Annan in 2003

Table 7.20: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions ($P = 0.002$) in total chlorophyll concentrations (μg /cm^2) of 'Fire and Brimstone' waratahs in 2003, pooled for sun and late shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 2-3 plants in each cultivar and treatment combination, except for 'Fire and Brimstone' early shade treatment (JO (juvenile open) and MF (mature flower) stages), with n = 1.

	Log_{10} total chlorophyll		Back transformed total chlorophyll	
	Stage			
Tissue	JO	MF	JO	MF
Leaf	1.079 Aa	0.824 Aa	12.006	6.673
Inner bract	-0.638 Ab	-0.359 Bb	0.230	0.438
LSD	0.268			

Table 7.21: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions (P = 0.022) in total carotenoid: chlorophyll concentrations of 'Fire and Brimstone' waratahs in 2003, pooled for sun and late shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 2-3 plants in each cultivar and treatment combination, except for 'Fire and Brimstone' early shade treatment (JO (juvenile open) and MF (mature flower) stages), with n = 1.

	Log_{10} car:chlorophyll		Back transformed car:chlorophyll	
	Stage			
Tissue	JO	MF	JO	MF
Leaf	-0.702 Aa	-0.381 Aa	0.199	0.416
Inner bract	-0.248 Ab	-0.351 Aa	0.565	0.446
LSD	0.356			

Table 7.22: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions (P < 0.001) in anthocyanin concentrations (abs/cm^2) of 'Fire and Brimstone' waratahs in 2003, pooled for sun and late shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 2-3 plants in each cultivar and treatment combination, except for 'Fire and Brimstone' early shade treatment (JO (juvenile open) and MF (mature flower) stages), with n = 1.

	Log_{10} anthocyanin		Back transformed anth	
	Stage			
Tissue	JO	MF	JO	MF
Leaf	-1.292 Aa	-1.313 Aa	0.051	0.049
Inner bract	-0.567 Ab	-0.260 Bb	0.271	0.550
LSD	0.135			

Table 7.23: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions (P = 0.041) in UV-A absorbance (abs/cm^2) of 'Fire and Brimstone' waratahs in 2003, pooled for sun and late shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 2-3 plants in each cultivar and treatment combination, except for 'Fire and Brimstone' early shade treatment (JO (juvenile open) and MF (mature flower) stages), with n = 1.

	Log_{10} UV-A abs		Back transformed UV-A abs	
	Stage			
Tissue	JO	MF	JO	MF
Leaf	0.116 Aa	0.148 Aa	1.306	1.406
Inner bract	-0.650 Ab	-0.443 Ab	0.224	0.360
LSD	0.268			

7.3.2.5 Results for 'Olympic Flame' at Mount Annan in 2003

Table 7.24: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions ($P < 0.001$) in total chlorophyll concentrations (μg /cm^2) of 'Olympic Flame' waratahs in 2003, pooled for sun and late shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 2-3 plants in each treatment combination, except for the MF (mature flower) stage in the sun, with n = 1.

	Log_{10} total chlorophyll		Back transformed total chlorophyll	
	Stage			
Tissue	JO	MF	JO	MF
Leaf	1.073 Aa	1.009 Aa	11.828	10.212
Inner bract	-0.592 Ab	-0.388 Ab	0.256	0.409
Outer bract	0.404 Ac	-0.071 Bb	2.535	0.849
LSD	0.428			

Table 7.25: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions ($P < 0.001$) in total carotenoid concentrations (μg /cm^2) of 'Olympic Flame' waratahs in 2003, pooled for sun and late shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 2-3 plants in each treatment combination, except for the MF (mature flower) stage in the sun, with n = 1.

	Log_{10} total carotenoid		Back transformed total car	
	Stage			
Tissue	JO	MF	JO	MF
Leaf	0.336 Aa	0.598 Aa	2.167	3.961
Inner bract	-1.167 Ab	-0.624 Bb	0.068	0.238
Outer bract	-0.206 Ac	-0.377 Ab	0.622	0.420
LSD	0.384			

Table 7.26: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage by treatment interactions ($P = 0.007$) in total carotenoid: chlorophyll concentrations of 'Olympic Flame' waratahs in 2003. Upper case letters indicate significant differences within the same tissue and treatment combination at different stages of development, while lower case letters indicate significant differences between tissue and treatment combinations at the same stage. n = 2-3 plants in each treatment combination, except for the MF (mature flower) stage in the sun, with n = 1.

		Log_{10} car:chlorophyll		Back transformed car:chlorophyll	
		Stage			
Tissue	Treatment	JO	MF	JO	MF
Leaf	Late shade	-0.760 Aa	-0.433 Ba	0.174	0.369
Leaf	Sun	-0.715 Aa	-0.390 Ba	0.193	0.407
Inner bract	Late shade	-0.575 Aa	-0.291 Bab	0.266	0.511
Inner bract	Sun	-0.596 Aab	-0.150 Bb	0.254	0.708
Outer bract	Late shade	-0.750 Aa	-0.335 Bab	0.178	0.462
Outer bract	Sun	-0.470 Ab	-0.284 Aab	0.339	0.520
	LSD	0.192			

Table 7.27: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions ($P < 0.001$) in anthocyanin concentrations (abs/cm^2) of 'Olympic Flame' waratahs in 2003, pooled for sun and late shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 2-3 plants in each treatment combination, except for the MF (mature flower) stage in the sun, with n = 1.

	Log_{10} anthocyanin		Back transformed anth	
	Stage			
Tissue	JO	MF	JO	MF
Leaf	-1.339 Aa	-1.394 Aa	0.046	0.040
Inner bract	-0.631 Ab	-0.399 Ab	0.234	0.399
Outer bract	-1.380 Aa	-0.705 Bb	0.042	0.197
LSD	0.420			

Table 7.28: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions ($P = 0.038$) in UV-A absorbance (abs/cm^2) of 'Olympic Flame' waratahs in 2003, pooled for sun and late shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 2-3 plants in each treatment combination, except for the MF (mature flower) stage in the sun, with n = 1.

	Log_{10} UV-A abs		Back transformed UV-A	
	Stage			
Tissue	JO	MF	JO	MF
Leaf	0.336 Aa	0.207 Aa	2.167	1.609
Inner bract	-0.562 Ab	-0.351 Ab	0.274	0.446
Outer bract	-0.503 Ab	-0.396 Ab	0.314	0.402
LSD	0.291			

Table 7.29: Log transformed means (compared by LSD) and back transformed means for significant tissue by stage interactions ($P < 0.001$) in UV-B absorbance (abs/cm^2) of 'Olympic Flame' waratahs in 2003, pooled for sun and late shade treatments. Upper case letters indicate significant differences within the same tissue at different stages of development, while lower case letters indicate significant differences between tissues at the same stage. n = 2-3 plants in each treatment combination, except for the MF (mature flower) stage in the sun, with n = 1.

	Log_{10} UV-B abs		Back transformed UV-B	
	Stage			
Tissue	JO	MF	JO	MF
Leaf	0.297 Aa	0.221 Aa	1.983	1.665
Inner bract	-0.374 Ab	-0.208 Ab	0.422	0.619
Outer bract	-0.262 Ab	-0.196 Ab	0.547	0.636
LSD	0.119			

7.3.2.6 Results of cultivar comparisons at Mount Annan in 2003

Table 7.30: Log transformed means (compared by LSD) and back transformed means for significant cultivar by stage interactions (P = 0.018) in total carotenoid: chlorophyll concentrations of inner bracts of 'Fire and Brimstone' and 'Olympic Flame' waratahs in 2003, pooled for sun and late shade treatments. Upper case letters indicate significant differences within the same cultivar at different stages of development, while lower case letters indicate significant differences between cultivars at the same stage. n = 2-3 plants in each cultivar and treatment combination, except for 'Fire and Brimstone' early shade treatment (JO (juvenile open) and MF (mature flower) stages) and 'Olympic Flame' sun treatment (MF stage only), with n = 1.

	Log_{10} car:chlorophyll		Back transformed car:chlorophyll	
	Stage			
Cultivar	JO	MF	JO	MF
Fire & Brimstone	-0.285 Aa	-0.303 Aa	0.519	0.498
Olympic Flame	-0.584 Ab	-0.260 Bb	0.261	0.550
LSD	0.032			

Table 7.31: Log transformed means (compared by LSD) and back transformed means for significant treatment by stage interactions (P = 0.016) in anthocyanin concentrations (abs/cm^2) of inner bracts of 'Fire and Brimstone' and 'Olympic Flame' waratahs in 2003. Upper case letters indicate significant differences within the same treatment at different stages of development, while lower case letters indicate significant differences between treatments at the same stage. n = 2-3 plants in each cultivar and treatment combination, except for 'Fire and Brimstone' early shade treatment (JO (juvenile open) and MF (mature flower) stages) and 'Olympic Flame' sun treatment (MF stage only), with n = 1.

	Log_{10} anthocyanin		Back transformed anth	
	Stage			
Tissue	JO	MF	JO	MF
Sun	-0.594 Aa	-0.402 Aa	0.255	0.396
Early shade	-0.286 Ab	-0.416 Aa	0.518	0.384
Late shade	-0.594 Aa	-0.261 Ba	0.255	0.548
LSD	0.282			

Table 7.32: Back transformed mean pigment concentration of leaves and bracts of waratahs at Royal National Park. Log-transformed leaf and inner bract means were significantly different for each pigment at $P < 0.05$. Lower case letters indicate significant differences between leaves and bracts for each pigment. n = 4 plants with leaves and bracts sampled, and another 4 plants with leaves only sampled.

Pigment	Units	Leaf	Inner bract
Chlorophyll *a*	µg/cm2	11.16 a	0.12 b
Chlorophyll *b*	µg/cm2	3.27 a	0.12 b
Total chlorophyll	µg/cm2	14.45 a	0.24 b
Total carotenoid	µg/cm2	2.77 a	0.09 b
Chlorophyll *a*:*b*		3.49 a	0.96 b
Car: chlorophyll		0.19 a	0.36 b
Anthocyanin	abs/cm2	0.03 a	0.67 b
UV-A absorbance	abs/cm2	1.81 a	0.71 b
UV-B absorbance	abs/cm2	1.96 a	0.88 b

In addition to quantifying pigment concentrations, the location of both anthocyanins and browning compounds was investigated in mature 'Olympic Flame' waratah bracts (section 7.4). The composition of brown compounds was not revealed in pigment analysis but their presence may be related to pigment concentrations in damaged bracts. The location of anthocyanins in waratah bract tissues may be as important as their concentration, particularly if anthocyanins function as a screen for intense light. The location of anthocyanic and brown compounds is therefore investigated in section 7.4.

7.4 Location of anthocyanins and brown compounds using light microscopy

7.4.1 Methods

'Olympic Flame' waratahs grown in full sun or shaded late were harvested from Mount Annan on 9/10/03 and 13/10/03 and kept in water until required. Inner bracts were selected from full sun (with bract browning) or late shade (without bract browning) environments (Figures 7.4 and 7.5). Transverse sections were cut from the center of the bract by hand and mounted in 80% glycerol. Sections were cut from the center of bracts, as bract edges were too difficult to section. Sections were viewed under a light microscope (Orthoplan, Leitz Wetzlar, Germany) fitted with a microscope camera (Orthomat, Leitz Wetzlar, Germany).

Figure 7.4: 'Olympic Flame' bracts from full sun environment, used for sectioning.

Figure 7.5: ‘Olympic Flame’ bracts from late shade treatment (shaded from bud opening) used for sectioning.

7.4.2 Results

Anthocyanins were most frequently located in epidermal and subepidermal layers in shade bracts (Figures 7.6 and 7.7) and in sun bracts (Figure 7.8), and occasionally in mesophyll cells. In brown sections of sun bracts, the anthocyanic pigments of abaxial subepidermal layers were replaced by brown cell contents (Figures 7.9 and 7.10), while the adaxial surface retained the red pigmentation of anthocyanins (Figure 7.9) or remained unpigmented (Figure 7.10). Chloroplasts appear undamaged (Figure 7.8).

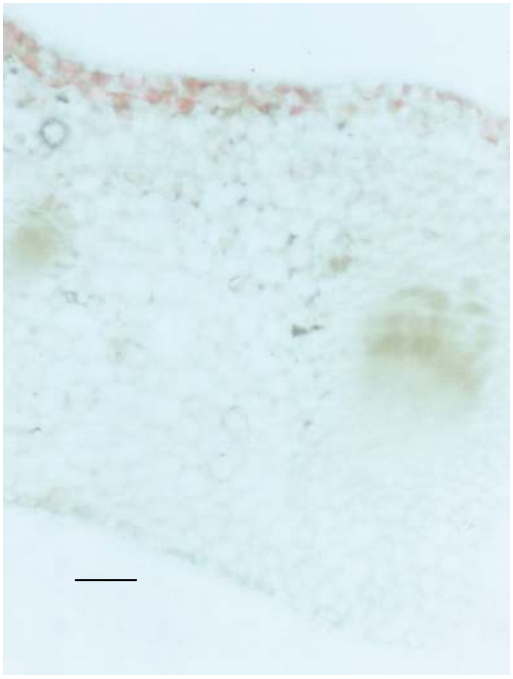

Figure 7.6: Transverse section of ‘Olympic Flame’ shade bract. Scale bar = 0.1 mm.

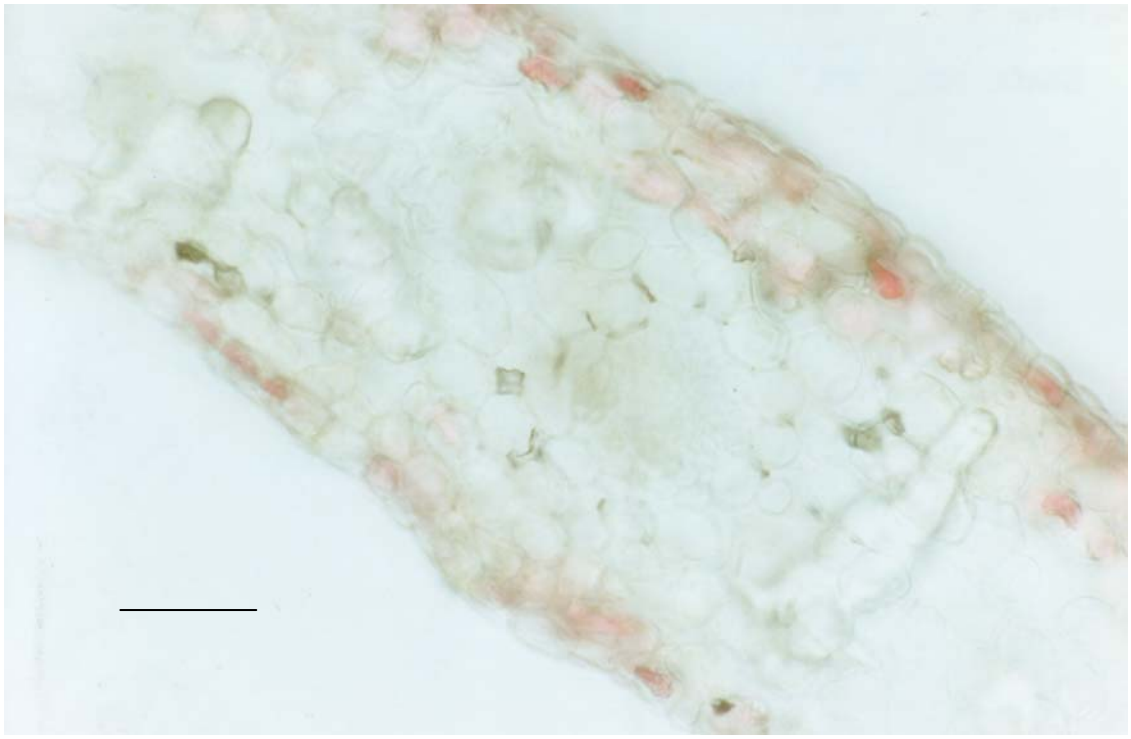

Figure 7.7: Transverse section of ‘Olympic Flame’ shade bract. Scale bar = 0.1 mm.

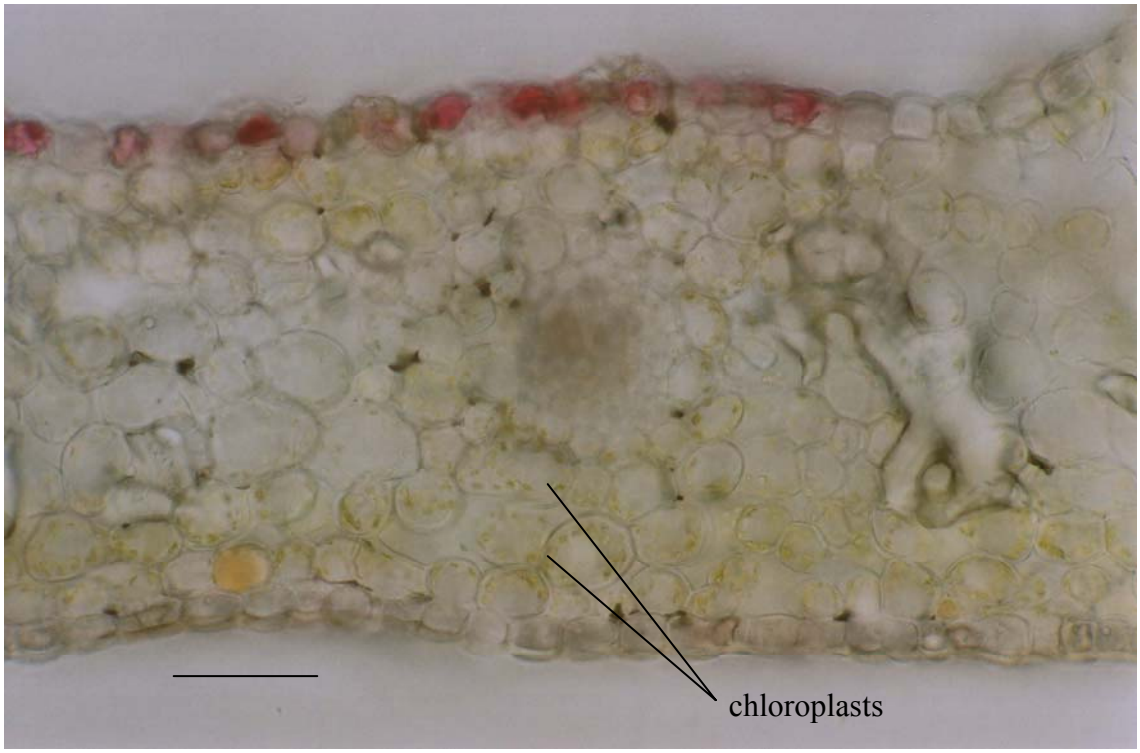

Figure 7.8: Transverse section of ‘Olympic Flame’ sun bract. Scale bar = 0.1 mm.

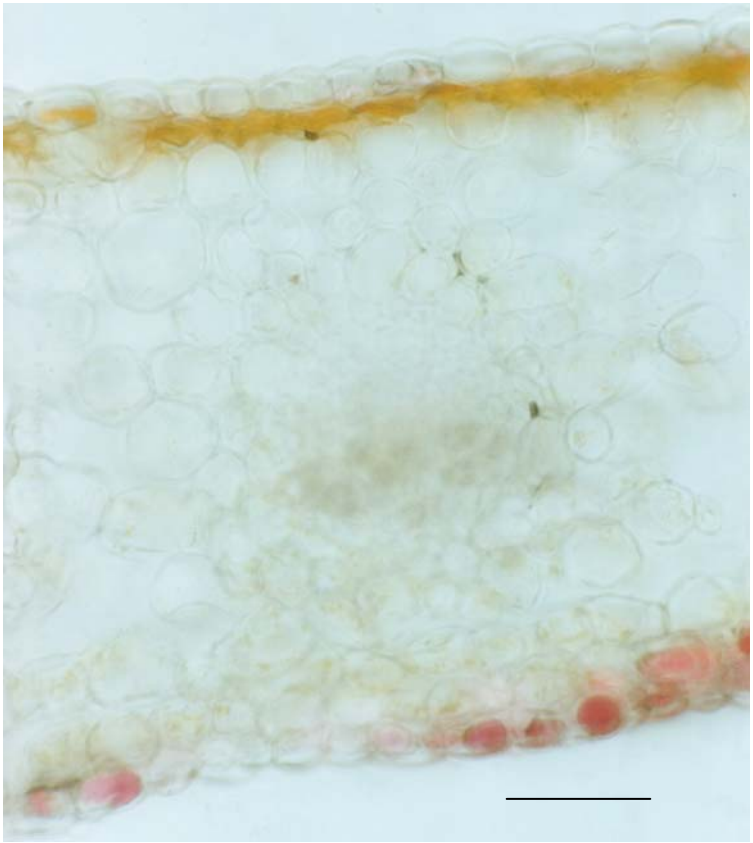

Figure 7.9: Transverse section of ‘Olympic Flame’ sun bract. Scale bar = 0.1 mm.

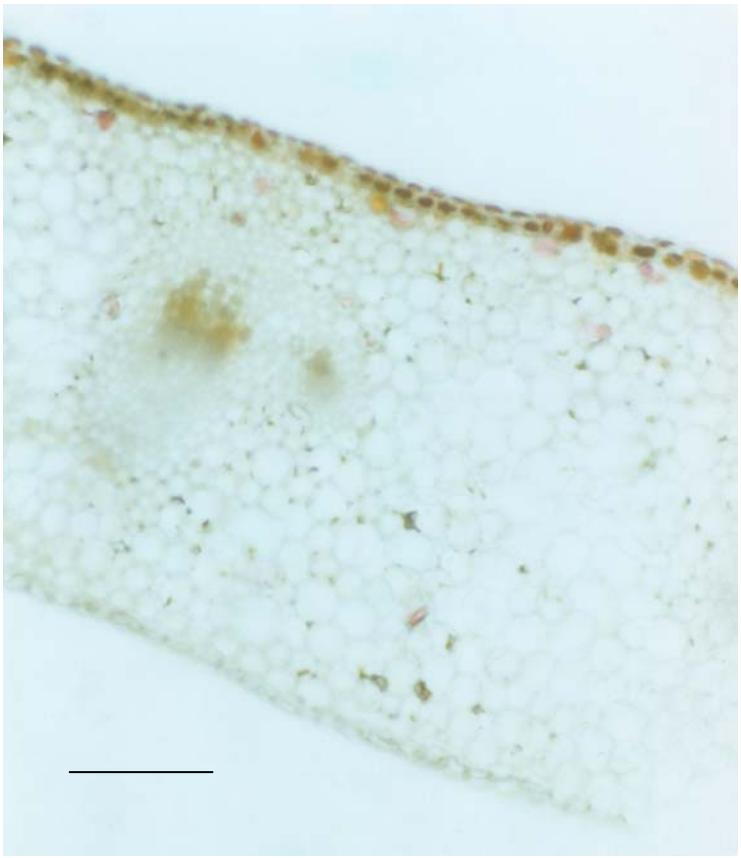

Figure 7.10: Transverse section of ‘Olympic Flame’ sun bract. Scale bar = 0.1 mm.

7.5 Discussion

7.5.1 Differences in pigmentation of sun, early and late shade tissues

Shading prevented significant decreases in chlorophyll, carotenoid and anthocyanin concentrations from TB to JO stages in ‘Fire and Brimstone’ tissues in 2002. Shade tissues also had significantly higher chlorophyll concentrations than sun-exposed tissues of ‘Fire and Brimstone’ and ‘Olympic Flame’ in 2003. ‘Fire and Brimstone’ and ‘Olympic Flame’ shaded early in 2003 had significantly higher anthocyanin concentrations in inner bracts at the JO stage, compared to inner bracts on plants shaded late or exposed to full sun. Chlorophyll concentrations were significantly higher in shaded ‘Wirrimbirra White’ bracts at the MF stage. Shaded ‘Wirrimbirra White’ inner bracts also had a significantly higher

chlorophyll *a*:*b* concentration and higher total carotenoids than sun-exposed inner bracts at flower maturity.

The reduction in pigment concentration observed in waratah bracts under high light has also been observed in leaves and floral tissues of other species. For example, chlorophyll concentration and chlorophyll *a*:*b* was reduced in leaves of coffee plants exposed to full sunlight (Nunes *et al.*, 1993). In coffee, this photodamage was visible after two days of exposure as copper-coloured areas on leaves, which became necrotic after two weeks and were eventually shed (Nunes *et al.*, 1993). In several *Illicium* (star anise) species, photooxidative bleaching and necrosis in high light environments were correlated with decreased total chlorophyll, measured spectrophotometrically or with a SPAD chlorophyll meter (Olsen *et al.*, 2002). Similarly, waratah bract browning occurred in full sun but was significantly reduced by shade (Chapter 5). Plants subjected to other stresses, such as chilling, show similar decreases in pigments resulting in photoinhibition of photosynthesis. For example, maize plants grown at chilling temperatures showed reduced chlorophyll content and chlorophyll *a*:*b* ratio, but an increased carotenoid:chlorophyll ratio (Haldimann, 1998).

The corollary of reduced pigmentation in full sun is the ability to maintain pigment concentrations under shade, as observed in waratah bracts and leaves and floral tissues of other species. For example, shade prevented a decrease in chlorophyll content in *Dendrobium* flower parts, while chlorophyll was reduced in intermediate and full sunlight environments (He *et al.*, 1998). *Schefflera* leaves in the shade had higher total chlorophyll than plants in full sun but lower chlorophyll *a*:*b* ratio (Schiefthaler *et al.*, 1999), as did

shade-grown tropical root crops such as yam, taro, tannia, cassava and sweet potato (Johnston and Onwueme, 1998) and shaded *Acacia mangium* seedlings (Yu and Ong, 2001). Increased chlorophyll concentrations in the shade enable plants to harvest the limited light available, while lower chlorophyll *a*:*b* ratios indicate maintenance of larger light harvesting antennae associated with PSII (Johnston and Onwueme, 1998; Yu and Ong, 2001). A higher chlorophyll *a*:*b*, as observed in 'Wirrimbirra White' inner bracts, suggests adaptation to full sun is occurring, by decreasing PSII antenna size and minimizing the potential for harvesting of excess light and thus, photoinhibition (Pastenes *et al.*, 2003). Red waratah cultivars 'Fire and Brimstone' and 'Olympic Flame' did not show significant differences in chlorophyll *a*:*b* when grown in different light environments, suggesting their light harvesting system may be better adapted to high or low light conditions than 'Wirrimbirra White' waratahs. However, more accurate comparisons could be made between waratah cultivars by growing them in the same location, using the same management practices. The growing environment can have a significant impact on pigment concentrations, as observed in Experiment 7.2 where the cultivar 'Fire and Brimstone' was grown at different locations. The maintenance, or increase, in carotenoid concentrations in shaded waratah bracts indicates that bracts retain the ability to harmlessly dissipate excess light energy via non-photochemical quenching, as observed in Chapter 6. In plants with senescent tissues, for example *Lolium* leaves (Mae *et al.*, 1993) shading can extend the green duration of mature leaves by maintaining total chlorophyll concentration. Similarly, waratahs appeared to retain more floral bracts in the shade than under full sun, possibly as a consequence of increased chlorophyll.

Shaded waratah bracts on red cultivars had higher concentrations of anthocyanins than sun-exposed bracts, indicating a more intense red coloration and hence, better flower quality. The reduction in anthocyanin concentrations in full sun may be explained by the inhibition of anthocyanin synthesis under excessive UV-B radiation (Chalker-Scott, 1999). This phenomenon is observed in red lychee fruit, which have the least red colouration on the side of the tree intercepting the most radiation (Tyas *et al.*, 1998) and red raspberry cultivars prone to 'white drupelet' disorder unless grown under shade cloth (Renquist *et al.*, 1987). Anthocyanins may be bleached by the superoxide radical (Yamasaki *et al.*, 1996), which can be produced during chronic photoinhibition. The anthocyanin concentration of waratah bracts also decreases during flower senescence (Faragher, 1989), which may be a factor in pigmentation of outer waratah bracts.

UV absorbance was often higher in sun tissues of waratah, compared to those in the shade, as observed in other species. Thus, shade tissue is likely to be more susceptible to photoinhibition, as observed in Chapter 6, caused by visible or UV radiation. For example, shade leaves and understorey plants had a lower absorbance at 305 and 375 nm than sun leaves of the same species, suggesting insufficient protection against UV radiation (Krause *et al.*, 2003). Similarly, Lovelock *et al.* (1992) found that shade leaves of mangroves have significantly less phenolics than sun leaves. Shade leaves also have a smaller pool of anti-oxidants than sun leaves (Demmig-Adams *et al.*, 1997). This suggests that shade adapted tissues have insufficient protection against UV radiation and may be damaged by even short exposures to UV radiation (Krause *et al.*, 2003).

The protected inner bracts of waratahs had lower concentrations of UV-A absorbing compounds than outer bracts at the TB stage in 'Fire and Brimstone' and 'Wirrimbirra White' in 2002. Inner bracts of 'Wirrimbirra White' waratahs also had a lower concentration of UV-B absorbing compounds than outer bracts at the TB stage in 2002. Although this early (TB) stage was not measured for any cultivar in 2003, the results suggest that inner bracts may have similar qualities to shade tissues, and be equally susceptible to UV damage. However, the protection of the inner bracts at this early stage of development means that photoinhibition does not occur until later in inner bract development (Chapter 6). Thus, the outer bracts of waratah appear to offer protection to inner bracts, similar to the way that bud scales protect conifer needles from the damaging effects of UV-B radiation in the early stages of needle development, as epidermal screening properties of needles are not well developed at early stages of growth (DeLucia *et al.*, 1992).

7.5.2 Changes in bract pigmentation during development

During 2002, 'Fire and Brimstone' inner bracts had a higher carotenoid:chlorophyll ratio at the TB stage than at flower maturity. Total chlorophylls increased more rapidly than total carotenoids from the TB stage to flower maturity. The higher proportion of carotenoids in inner bracts at the TB stage indicates a greater capacity for photoprotection. Similarly, young grapevine leaves had a higher car:chlorophyll ratio than mature leaves, and a higher proportion of carotenoids as xanthophyll cycle pigments (Bertamini and Nedunchezhian, 2003). Carotenoid:chlorophyll ratios did not change significantly from JO to MF stages in 'Fire and Brimstone' waratahs in 2003, although changes from TB to JO stages were not measured and could be significant. Waratahs at the Royal National Park also had lower

carotenoid:chlorophyll contents in leaves compared to bracts at flower maturity, indicating increased potential for photoprotection in bracts.

In 2003, carotenoid:chlorophyll ratios in 'Olympic Flame' were higher at flower maturity than at the intermediate (JO) stage of development for all tissues. This is due to increased total carotenoids in leaves and inner bracts, and decreased total chlorophylls in outer bracts. The increase in carotenoid content corresponds to a higher capacity for non-photochemical quenching (qN) at flower maturity than at the JO stage of development (Chapter 6). Similarly to waratah bracts, loss of chlorophyll was correlated with an increased carotenoid content in senescing leaves and ripening apple fruit (Merzlyak and Solovchenko, 2002; Merzlyak *et al.*, 2002), indicating a greater capacity for photoprotection. Merzlyak and Solovchenko (2002) have identified two groups of carotenoids: the first group, associated with chloroplast membranes, is destroyed along with chlorophyll; while the second group is more light stable.

7.5.3 Differences in leaf and bract pigmentation

Consistent significant differences in the concentration of pigments in waratah leaves and bracts were revealed during experiments over two years on two red waratah cultivars 'Fire and Brimstone' and 'Olympic Flame' and one white cultivar 'Wirrimbirra White', as well as in one natural population. Leaves had higher UV absorbance, and higher concentrations of chlorophylls *a* and *b*, total chlorophylls and total carotenoids compared to bracts. Khoo *et al.* (1997) observed similar results in *Dendrobium* orchids, with higher chlorophyll and carotenoid concentrations in leaves compared to flower parts. Weiss *et al.* (1988) found that petunia corollas had a maximal chlorophyll concentration about 40% of that of green

leaves. The concentration of waratah leaf pigments tended to show little variation during flower development (for example, Table 7.4), so where all tissue types are grouped together (for example, Table 7.5), changes over time are attributed to bracts.

Significant differences between inner and outer bracts were observed, and the pigment concentration of these tissues changes during flower development and maturation. Inner bract tissue is younger than outer bract tissue and is mostly protected until the juvenile open stage of flower development. The concentration of chlorophylls, carotenoids, UV-absorbing compounds and anthocyanins in inner bracts increases during flower development. The pigment development of inner bracts is similar to that of petunia corollas (Weiss *et al.*, 1988), with increasing chlorophyll and anthocyanin concentrations over time. Accumulation of anthocyanins in petunia corollas (Weiss *et al.*, 1988) and inner waratah bracts did not correspond to a decrease in chlorophyll concentration. Instead, anthocyanins visually masked the presence of chlorophylls.

On a waratah inflorescence, outer bract tissue is older than inner bract tissue, and has been exposed to the environment for a longer period of time at flower maturation. The concentration of chlorophylls, carotenoids and UV-absorbing compounds in outer bracts decreases during flower development. A reduction in chlorophyll and carotenoid concentrations was also observed in *Dendrobium* flower parts during flower development, indicating senescence (Khoo *et al.*, 1997). Waratah outer bracts are particularly susceptible to photoinhibition early in development, as they are not protected like inner bracts (Chapter 6). Halevy and Mayak (1979) reviewed pigment changes in ageing flowers, noting a decreased carotenoid concentration in senescing chrysanthemum flowers. A decrease in

chlorophyll content may result from the transition of chloroplasts to chromoplasts or gerontoplasts (Khoo *et al.*, 1997; Matile *et al.*, 1999). Gerontoplasts control the removal of nitrogen and lipid carbon from the thylakoid membrane, while ensuring inactivation of chlorophyll during senescence (Thomas, 1997; Matile *et al.*, 1999). The breakdown products of chlorophyll are colourless (Matile *et al.*, 1996), and thus, chlorophyll breakdown is not directly responsible for the brown colouration observed in waratah bracts. Bract senescence is also likely to increase susceptibility to photoinhibition, as the concentration of chlorophyll and carotenoids decreases, reducing the capacity of the bracts to dissipate light via photosynthesis or non-photochemical quenching.

Differences between leaves and bracts, and outer and inner bracts, may partly be attributed to tissue age, as a lower chlorophyll content per unit leaf area and lower capacity for O_2 evolution was observed in young leaves of grapevines (Bertamini and Nedunchezhian, 2003) and young leaves of different tropical forest species (Krause *et al.*, 1995). Krause *et al.* (1995) suggested that the low chlorophyll content would result in a higher fraction of excess light and higher average chlorophyll excitation in young leaves at the same light exposure, a proposal that has been supported by Bertamini and Nedunchezhian (2003). However, Khoo *et al.* (1997) suggest that the low chlorophyll content of mature flowers leads to less absorbed radiation and minimization of photoinhibition. Further work is required to determine whether the amount of light absorbed by bracts changes over time, as the light absorbance of waratahs bracts was studied at one stage only (Experiment 6.1).

The high chlorophyll content visibly masked the presence of anthocyanins in leaves of all cultivars. Anthocyanin concentrations of bracts increased during flower development in 'Fire and Brimstone' in 2002 (inner and outer bracts) and in 2003 (inner bracts). The potential for anthocyanins to reduce photoinhibition, as found in reddish-purple *Bauhinia* pods compared to green (Smillie and Hetherington, 1999), red *Quintinia serrata* leaves (Gould *et al.*, 2000) and red *Quercus coccifera* leaves (Manetas *et al.*, 2003) will be examined in detail in Chapter 9. However in waratah bracts, photosynthetic yield was more closely linked with UV absorbing compounds than anthocyanins (Chapter 9). The relative contribution of anthocyanins and UV absorbing compounds (such as the flavonoid group of phenolics) to potential photoprotection differs between species.

UV-B absorbing compounds, measured spectrophotometrically at 300 nm, are strongly correlated with the total phenolic levels of a range of Mediterranean plants (Levizou and Manetas, 2002). Other species in the Proteaceae (*Protea* and *Aulax*) are known to have relatively high concentrations of UV-B absorbing compounds, with the exception of *Leucadendron* (average UV-absorbing capacity) (Wand, 1995). The relationship between UV absorbance and phenolic content may be further investigated in waratah tissues using the current method for UV-B absorbance and the Folin-Ciocalteau assay for phenolic concentrations (Levizou and Manetas, 2002). However, the potential for flavonoids and other phenolics to screen out UV-B radiation depends on the location of these compounds, as well as their concentration (Day, 1993; Wand, 1995) (section 7.5.6).

7.5.4 Differences in pigmentation between cultivars

The white cultivar showed different trends in pigmentation to red cultivars, with 'Wirrimbirra White' inner bracts having very low concentrations of chlorophylls compared to outer bracts and leaves, and a higher carotenoid: chlorophyll ratio. 'Wirrimbirra White' bracts also had much lower anthocyanin concentrations than red cultivars (although leaf anthocyanin concentrations were similar) and higher UV-A and UV-B absorbance, possibly to compensate for lower anthocyanin content. Geraldton wax (*Chamelaucium uncinatum*) flowers of the white cultivar 'Alba' similarly had no anthocyanins and no coloured vacuoles, while 'Purple Pride' and 'CWA Pink' had magenta or pink flowers, respectively and pigmented vacuoles (Klyne *et al.*, 2003). Flavonoid composition can also alter pigmentation, for example, in white carnations, the three nearly pure white cultivars lack flavonoids, while the other white cultivars contain the flavonol kaempferol (Onozaki *et al.*, 1999).

7.5.5 Differences in pigmentation due to environment (site and year effects)

Variation in pigmentation within the same cultivar grown at different sites, as well as between cultivars, was evident from the initial experiments on waratah bracts (section 7.2). Similarly, variation within a cultivar between years at the same site was observed in later experiments ('Fire and Brimstone' grown at Mount Annan in 2002 and 2003). Thus, these differences between sites and years can be attributed to environmental variation.

The proportion of PSII reaction centers and light harvesting antenna of leaves and bracts, indicated by the chlorophyll *a*:*b* ratio, appears to be flexible in waratah leaves and bracts exposed to different environmental conditions each year. As an example, 'Fire and

Brimstone' and 'Wirrimbirra White' bracts had a higher chlorophyll *a*:*b* ratio than leaves in 2002. In contrast, 'Fire and Brimstone' and 'Olympic Flame' leaves had a higher chlorophyll *a*:*b* than bracts in 2003, as did waratahs in the Royal National Park in the same season.

Within the cultivar 'Fire and Brimstone', bracts had a higher carotenoid: chlorophyll ratio than leaves in 2002 while leaves and bracts had a similar carotenoid:chlorophyll ratio in 2003. An increase in carotenoids compared to chlorophylls reduces light absorbance in the antenna and increases capacity for photoprotection (Haldimann 1998), via the xanthophyll cycle. These results suggest that the capacity for photoprotection varies from year to year. Carotenoids have been found to protect against both visible and UV radiation in mangroves and other species (Lovelock *et al.*, 1992). The ratio of chlorophylls to carotenoids is also a sensitive indicator of potential for oxidative damage, as chlorophylls are sensitive to oxidative attack while carotenoids act as anti-oxidants and quenchers of light-induced excitation (Hendry and Grime, 1993). Over all the cultivars examined, these results suggest that waratah bracts have a similar or greater capacity than leaves for photoprotection due to a higher proportion of carotenoids. Measurements of non-photochemical quenching (NPQ) in waratah bracts corroborate these results, as a decrease in photosynthetic yield during the day was attributed to significant increases in dissipation of light via NPQ.

Similarly, environmental factors appear to impact on anthocyanin development within a season, as trends in the anthocyanin response of early shaded bracts are not consistent from year to year. Anthocyanin concentrations increased from JO to MF stages in 2002 and decreased from JO to MF stages in 2003, potentially due to environmental influences.

7.5.6 Location of anthocyanins and brown compounds in bracts

The epidermal and subepidermal location of anthocyanins observed in waratah bracts, is a common feature of floral tissue of many species (Asen, 1976; Mudalige *et al.*, 2003). For example, in *Dendrobium* orchids, anthocyanins were found as a single layer of epidermal or subepidermal cells in pale, pastel and white/purple flowers, while more intensely coloured flowers had anthocyanins in epidermal and subepidermal cells or in many layers of the epidermis and mesophyll (Mudalige *et al.*, 2003). Staining for flavonoids and phenols in waratah bracts was not successful, as insufficient time was available to modify the technique for waratah tissues (data not presented). However, flavonoids in leaves of other species are typically found in the epidermis and are ideally placed to intercept UV radiation, although some species have high concentrations in the mesophyll (Day, 1993). For example, flavonols were located in cuticles and epidermal cells of *Quintinia* leaves, with concentrations in red leaves twice that of green leaves (Neill *et al.*, 2002).

The brown pigments replacing the anthocyanins in waratahs with bract browning were not identified, although Halevy and Mayak (1979) note that aging of petals in some flowers results in browning or blackening. They attribute the browning and blackening to oxidation of flavones, leucoanthocyanins and other phenols, and accumulation of tannins. Gossauer and Engel (1996) note that brown tannins accumulate in the vacuole along with anthocyanins in senescing leaves. Condensed tannins (also known as proanthocyanidins) are produced in a biosynthetic pathway closely linked to flavonoid and anthocyanin production, athough the production of condensed tannins is not well understood (Robbins *et al.*, 2003).

Accumulation of condensed tannins has been linked with poinsettia bract necrosis (McAvoy *et al.*, 1998). Dark occlusions were noticed under the light microscope in epidermal cells as necrotic lesions began to form, while none were observed in healthy bract tissue. These occlusions enlarged, eventually filling the adaxial and abaxial epidermal cells, as well as some subepidermal layers (McAvoy *et al.*, 1998). Transmission electron micrographs suggested these occlusions were condensed tannins, and this was confirmed with a modified vanillin-HCL microassay for condensed tannins (McAvoy *et al.*, 1998). However, the trigger for bract necrosis has not been identified. Accumulation of phenolics, particularly tannins, was also found in leaves of beech trees subjected to environmental stress including wind and intense solar radiation (Bussotti *et al.*, 1998). The tannins initially accumulate in vacuoles of the upper epidermal and palisade mesophyll cells, and eventually solubilize in the cytoplasm and impregnate the outer wall of the epidermal cells (Bussotti *et al.*, 1998). Thus, browning in waratah bracts may result from tannin accumulation in response to stress, an hypothesis which may be tested in the future using the vanillin-HCl microassay (McAvoy *et al.*, 1998).

The brown compounds accumulating in the epidermis of waratah bracts may form a protective screen against further UV damage. For example, in colonies of the alga *Ulva*, the bleached top layer of algae acts as a selective UV-B screen, preventing subcanopy thalli from damage caused by excessive radiation (Bischof *et al.*, 2002). Similarly, the brown epidermal layer in waratah bracts may protect the chloroplasts of the mesophyll from UV damage, although this hypothesis requires further investigation.

Alternatively, browning of waratah bracts may be the result of cell breakdown following lipid peroxidation and oxidative damage to other cell components (Chapter 8). In this case, enzymes may facilitate browning reactions, similar to those observed in lychee pericarp browning. During storage, browning of the lychee pericarp increased while anthocyanin content decreased (Zhang *et al.*, 2001). The product of anthocyanin degradation has a similar structure to catechol and may accelerate browning via the enzymes polyphenol oxidase and anthocyanase (Zhang *et al.*, 2001).

7.6 Conclusions

Shading prevented significant decreases in chlorophyll and carotenoid concentrations of bracts of 'Fire and Brimstone', 'Olympic Flame' and 'Wirrimbirra White' at different stages of flower development. The maintenance of pigment compositions in the shade appears to be linked to a reduction in the photoinhibition experienced by bracts in full sun. Shaded tissue of the red waratah cultivars 'Fire and Brimstone' and 'Olympic Flame' tended to have a higher anthocyanin concentration than sun-exposed tissue. Anthocyanins were located in epidermal and subepidermal layers in waratah bracts. In brown bract tissue, anthocyanic layers were replaced by unidentified brown compounds, possibly tannins, which may function as screens against visible or UV radiation.

Waratah leaves had a higher UV absorbance, and higher concentrations of chlorophylls a and *b*, and total carotenoids compared to bracts. The high chlorophyll content of leaves masked the visible presence of anthocyanins in all cultivars. 'Wirrimbirra White' waratahs had very low concentrations of anthocyanins in both leaf and bract tissues, and inner bracts had very low concentrations of chlorophylls compared to outer bracts and leaves.

The concentration of chlorophylls, carotenoids, UV-absorbing compounds and anthocyanins in inner bracts increased during flower development, while the concentration of these compounds in outer bracts decreased, indicating senescence. The increased exposure of outer bracts at early stages of development and their senescence late in development is likely to increase their susceptibility to photoinhibition.

The relationship between pigmentation, photoinhibition and browning of waratah bracts alluded to in this chapter will be investigated statistically in Chapter 8, using correlation techniques to examine the relationship between browning and changes in bract physiology (namely, photoinhibition and pigmentation).