Chapter 9
Light environment (sun/shade) effects on lipid peroxidation

9.1 Introduction

The chronic photoinhibition experienced by waratah bracts (Chapter 6) makes them susceptible to photooxidative damage, causing pigment degradation (Chapter 7) and potentially, oxidative damage to other cell components, resulting in browning (Chapter 5). Many studies of photoinhibition induced by high light measure effects on pigment degradation, but other consequences such as oxidative damage or visible discoloration are often not quantified (He *et al.,* 1998; Olsen *et al.,* 2002; Pastenes *et al.,* 2003). The aim of this chapter is to quantify the oxidative damage resulting from chronic photoinhibition in waratah bracts by measuring lipid peroxidation. Membrane lipid peroxidation has been used as a measure of photooxidation in rice leaves under chilling stress (Ji and Jiao, 2001) and during senescence (Jiao *et al.*, 2003), and in pea leaves subject to UV-B damage (van Hasselt *et al.*, 1996). Lipid peroxidation also occurs during senescence of floral tissue (for example, Daylily petals - Panavas and Rubenstein, 1998), which may be an important process increasing susceptibility to photoinhibition in waratah bracts.

9.1.1 How does photooxidative damage occur?

During photosynthesis, photosystem II (PSII) generates strong oxidants in order to oxidise water, which is an extremely stable molecule (Chow, 2001; Anderson and Chow, 2002). Paradoxically, PSII is susceptible to damage from these oxidants, particularly at saturating light levels (Chow, 2001) or when other stresses are present, for example chilling (Wise, 1995).

If electron flow through PSII is blocked (that is, all reaction centres are closed), the electrons of the reaction centre chlorophyll P680 become highly excited, forming triplet state $P680^+$. $P680^+$ is the strongest oxidant in photosynthesis, and is thought by some researchers to be the most likely candidate responsible for photoinactivation of PSII (Chow, 2001). $P680^+$ has a long lifetime and cannot be accumulated in PSII (Powles, 1984; Horton *et al.*, 1996). Triplet $P680^+$ may return directly to the ground state or react directly with oxygen to yield singlet oxygen (Equation 9.1, Anderson and Chow, 2002).

Equation 9.1

$$^3P680 + {}^3O_2 \rightarrow P680 + {}^1O_2$$

Singlet oxygen ($^1O_2$) is highly reactive and needs to be deactivated quickly to prevent damage to photosystem II (Chow, 2001). Singlet oxygen may cause oxidative damage to pigments and proteins, especially the D1 protein of PSII, as well as lipid peroxidation, leading to degradation of whole reaction centres (Asada, 1994; Niyogi, 1999). Damaged reaction centres may then generate oxidised chlorophyll and free radicals, causing further damage to the thylakoid (Horton *et al.*, 1996). Lipid peroxidation occurs when singlet oxygen reacts with unsaturated fatty acids and leads to membrane disruption (Knox and Dodge, 1985).

Singlet oxygen and $P680^+$ are not the only damaging free radicals generated during chronic photoinhibition. Hydroxyl free radicals can also be formed, as described by Chow (2001). Initially, superoxide anions are formed, during electron transfers in an atmosphere containing oxygen (Equation 9.2). Superoxide anions (O_2^-) are no more reactive than oxygen, but superoxide dismutase (SOD) can catalyse the reaction of superoxide to form

the much more reactive hydrogen peroxide (Equation 9.3). Hydrogen peroxide (H_2O_2) can cause DNA breakage and enzyme inactivation (Alscher *et al.*, 1997). It can also react with a reduced iron centre or another superoxide anion to form the hydroxyl radical (Equation 9.4a, Foyer *et al.*, 1994; Chow, 2001). However, the reaction between hydrogen peroxide and superoxide occurs more frequently (Equation 9.4b), producing an hydroxyl radical. The hydroxyl radical ($HO^{\bullet}$) is highly reactive and can modify proteins to make them susceptible to proteolytic attack (Casano *et al.*, 1994). Once damaged, proteins can then be broken down further, leading to cell membrane degradation.

Equation 9.2 $O_2 + e^- \rightarrow O_2^-$

Equation 9.3 $2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$

Equation 9.4a $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$

Equation 9.4b $O_2^- + H_2O_2 \rightarrow O_2 + OH^- + HO^{\bullet}$

9.1.2 Assessing oxidative damage using the malionaldehyde assay

Lipid peroxidation products such as malionaldehyde (MDA) are considered useful and reliable indicators of oxidative damage, due to the susceptibility of membranes to attack by reactive oxygen species (Wise, 1995; Hodges *et al.*, 1999). Malionaldehyde is a secondary end-product of the oxidation of polyunsaturated fatty acids, and reacts with thiobarbituric acid (TBA) to yield a pinkish-red chromagen with maximal absorbance at 532nm (Hodges *et al.*, 1999). Measurements of these thiobarbituric acid reactive substances (TBARS) have been made since the 1950's, often using the method of Heath and Packer (1968). However, many plant tissues contain interfering compounds, including sugars, anthocyanins and other phenolics, that absorb at 532 nm, leading to overestimations of MDA by up to 96.5% (Du

and Bramlage, 1992; Merzlyak *et al.*, 1993; Hodges *et al.*, 1999). The method of Hodges *et al.* (1999) corrects for interfering substances by "subtracting the absorbance at 532 nm of a solution containing plant extract without TBA from an identical solution containing TBA". Since waratah leaves and bracts are known to contain anthocyanins and other phenolics (Chapter 8), the method of Hodges *et al.* (1999) was chosen for assessment of oxidative damage in waratah bracts. Heath and Packer's (1968) calculations of MDA, without correction for interfering substances, can also be made from these results.

The aim of this experiment was to estimate the oxidative damage resulting from chronic photoinhibition by measuring lipid peroxidation in waratah leaves, and inner and outer bracts.

9.2 Methods

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.

Leaf and bract discs (10 mm diameter) were collected, with red and brown bracts analysed separately if present on the same inflorescence. Leaf and bract tissue was stored at -80ºC prior to assay, as described by van Hasselt *et al.* (1996). Leaf or bract discs (two discs x three replicates per sample) were ground in 2 mL of 80% ethanol using a mortar and pestle. The supernatant was transferred to a 2 mL Eppendorf tube and centrifuged at 5000 rpm for 10 min. Solutions with or without thiobarbituric acid (+TBA or –TBA, respectively) were

added to 1 mL of supernatant in a glass centrifuge tube. The –TBA solution comprised 20% trichloroacetic acid (TCA; Sigma, USA) and 0.01% butylated hydroxytoluene (2, 6-Di-*tert*-butyl-4-methylphenol; Sigma Aldrich, WI, USA). The +TBA solution contained the same chemicals as –TBA solution plus 0.65% thiobarbituric acid (TBA; Sigma, MO, USA). Samples were shaken, then heated in a water bath at 95°C for 25 min and then cooled on ice water. Solutions were transferred to Eppendorf tubes containing 1 mL of 80% ethanol (for dilution). The solution was centrifuged at 5000 rpm for 10 min. Absorbance was scanned from 400-600 nm on a Cary 50 Bio UV-Visible scanning spectrophotometer (Varian, Mulgrave, Victoria) with Cary Win-UV scanning kinetics software version 2.0 for data collection. Absorbance at 440, 532 and 600 nm were used for calculation of MDA equivalents by the method of Heath and Packer (1968) (Equation 9.5) and Hodges *et al.* (1999) (Equation 9.6), expressed per 1 cm^2 of tissue.

Equation 9.5 from Heath and Packer (1968)

$$\text{MDA equivalents (nmol.cm}^{-1}\text{)} = 1000[(\text{Abs } 523 - \text{Abs } 600\text{nm})/155]$$

Equation 9.6 from Hodges *et al.* (1999)

(1) $[(\text{Abs } 532\text{nm}_{+\text{TBA}})-(\text{Abs } 600\text{nm}_{+\text{TBA}})-(\text{Abs } 532\text{nm}_{-\text{TBA}}-\text{Abs } 600\text{nm}_{-\text{TBA}})]=A$

(2) $[(\text{Abs } 440\text{nm}_{+\text{TBA}}-\text{Abs } 600\text{nm}_{+\text{TBA}})0.0571]=B$

(3) $\text{MDA equivalents (nmol.mL}^{-1}\text{)} = (A-B/157000)10^6$

Statistical analysis of results was conducted in Genstat (7^{th} edition, Lawes Agricultural Trust, 2003) using the linear mixed model option of the restricted maximum likelihood (REML) procedure. The fixed model included *Treatment*, *Stage*, *Tissue* and *Cultivar* interactions, with *Tissue* nested in *Plant* as the random model. The *Tissue* term was expected to have a higher variance than other terms, so a diagonally correlated error structure for *Tissue* was included in the model. *Anthocyanin* concentration (Chapter 8) was

added as an additional fixed factor in the REML model, since anthocyanins are known to cause overestimation of MDA values. A chi-squared probability of $P \leq 0.05$ was used to assess the significance of interactions and/or main effects. Lipid peroxidation results from red and brown bracts from the same inflorescence are presented using the box plot graphics command in Genstat (Lawes Agricultural Trust, 2003).

9.3 Results

The MDA calculations of Hodges *et al.* (1999) gave a high proportion of negative results for waratah tissues (Appendix Table A5.1). For this reason, statistical analysis was based on the MDA calculations of Heath and Packer (1968). Analysis of this data revealed significant interactions between *Tissue, Stage* and *Treatment* ($P = 0.048$) and *Stage, Cultivar* and *Treatment* ($P = 0.045$). However, the latter results are not presented due to a large number of missing treatment combinations. Anthocyanin concentration was not a significant predictor of MDA value ($P = 0.073$), although correlations do exist between MDA and anthocyanins or UV-B absorbance (Figure 9.3).

Results for the *Tissue, Stage* and *Treatment* interaction are shown in Figure 9.1 for bracts and Figure 9.2 for leaves. At the the intermediate stage of floral development (JO), there was no significant difference between tissues and treatments. At flower maturity (MF stage), late shaded inner bracts had significantly higher MDA values than late shaded leaves and outer bracts (Figure 9.1). Leaf and outer bract values of MDA decreased during flower development, while inner bract values increased (Figures 9.1 and 9.2).

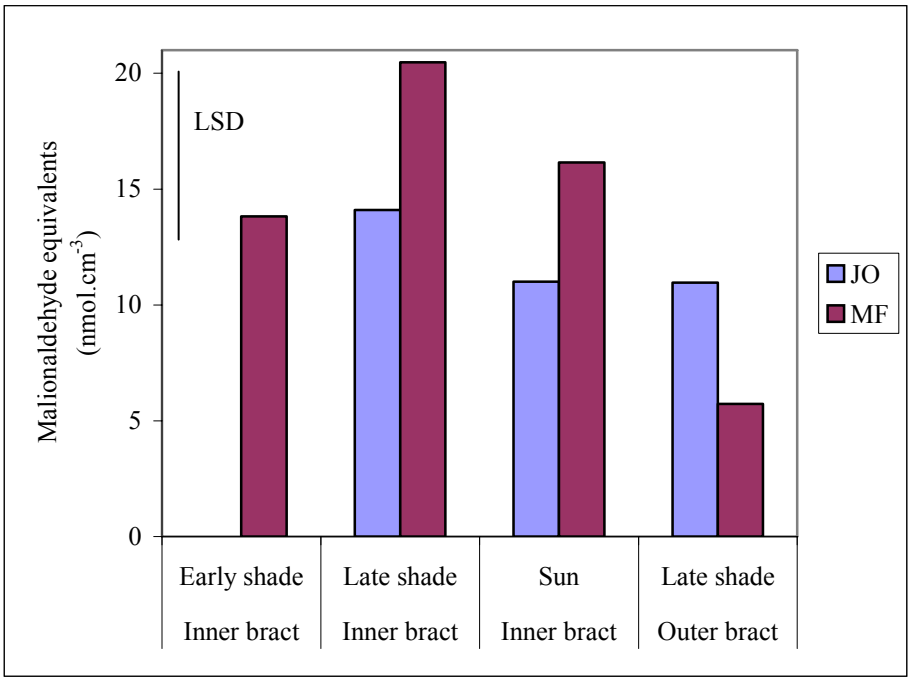

Figure 9.1: Lipid peroxidation (malionaldehyde equivalents) of waratah inner and outer bract tissue pooled from 'Fire and Brimstone' and 'Olympic Flame' cultivars, exposed to full sun or shaded early (from bud initiation) or late (from bud opening). Malionaldehyde (MDA) equivalents were calculated according to Heath and Packer (1968). n = 2 plants for all cultivar and light treatment combinations, except 'Fire and Brimstone' waratahs in the early shade treatment (n=1) and 'Olympic Flame' waratahs in the late shade treatment (n=3 for JO stage only) or early shade treatment (not tested at the JO stage). LSD = 8.762.

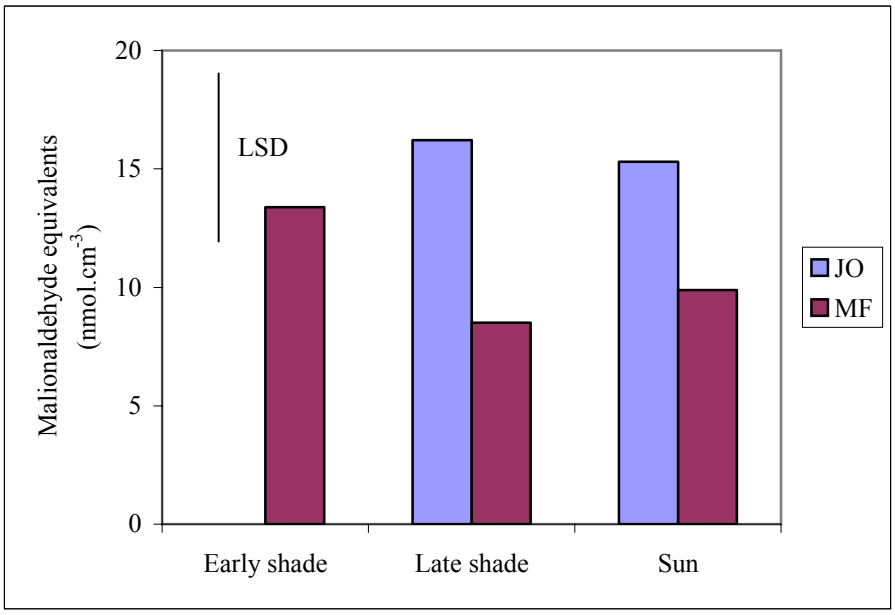

Figure 9.2: Lipid peroxidation (malionaldehyde equivalents) of waratah leaf tissue pooled from 'Fire and Brimstone' and 'Olympic Flame' cultivars, exposed to full sun or shaded early (from bud initiation) or late (from bud opening). Malionaldehyde (MDA) equivalents were calculated according to Heath and Packer (1968). n = 2 plants for all cultivar and light treatment combinations, except 'Fire and Brimstone' waratahs in the early shade treatment (n=1) and 'Olympic Flame' waratahs in the late shade treatment (n=3 for JO stage only) or early shade treatment (not tested at the JO stage). LSD = 8.762.

The measurements of lipid peroxidation appear to be confounded by high concentrations of anthocyanins and flavonoids. Indeed, malionaldehyde equivalents are positively correlated with anthocyanin content and UV absorbance (Figure 9.3). Lipid peroxidation calculations by the method of Hodges *et al.* (1999) result in a lower correlation coefficient for anthocyanins (0.271) and higher coefficient for UV-B absorbance (0.601) than the method of Heath and Packer (1968), indicating that changes to the assay may have reduced the impact of anthocyanins but do not reduce interference by flavonoids. Unexpectedly, quantum yield (Fv/Fm) showed a positive correlation with malionaldehyde (used as an indicator of lipid peroxidation), indicating increasing lipid peroxidation with increasing quantum yield (Figure 9.3).

No significant differences in lipid peroxidation were apparent between brown and red bracts taken from the same inflorescence, calculated by the method of Heath and Packer or Hodges *et al.* (Figure 9.4).

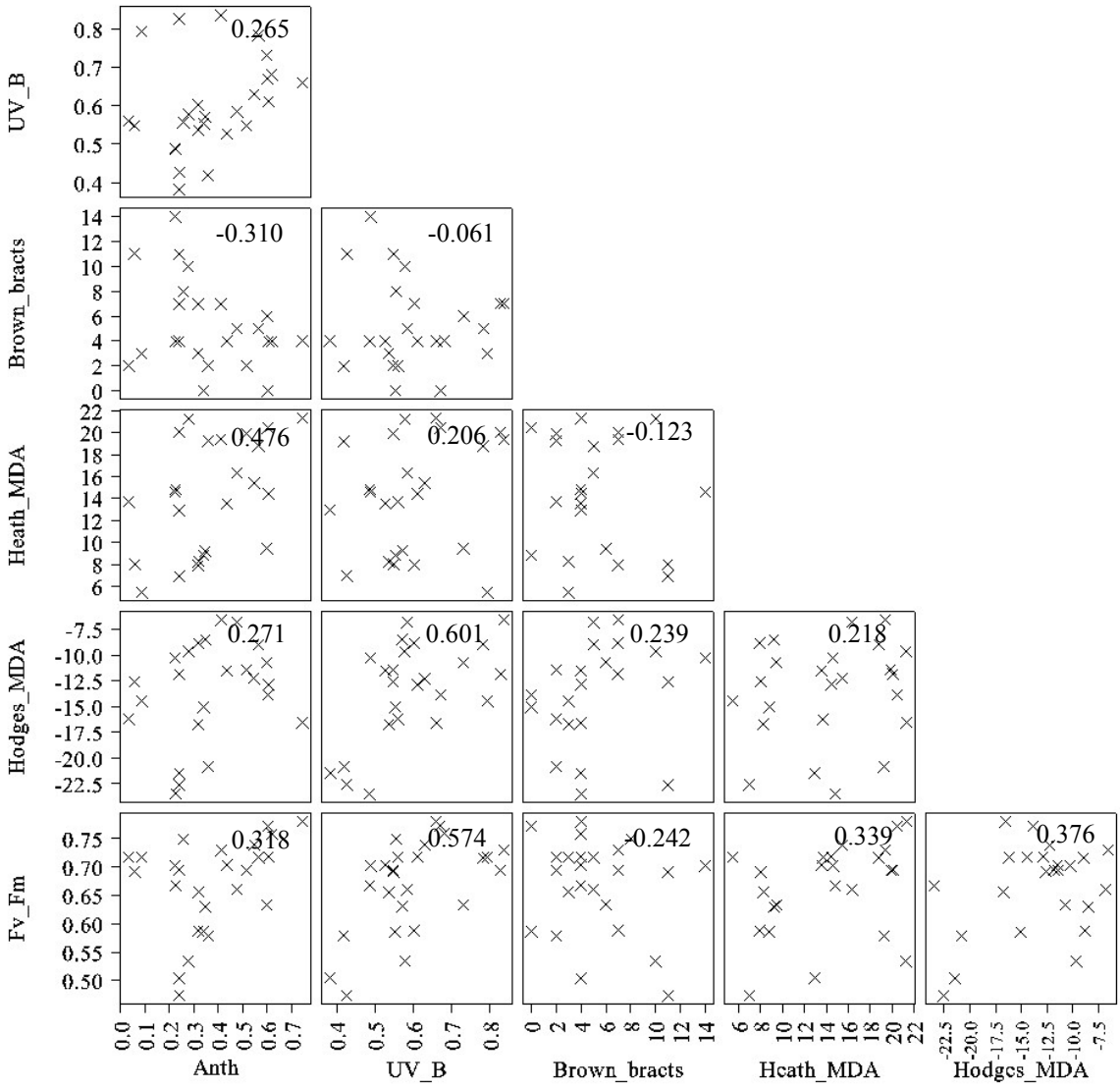

Figure 9.3: Correlation matrix for anthocyanins and UV-B absorbance (abs/cm^2), quantum yield (Fv/Fm), browning of floral bracts (including bracts browned and senesced) and malionaldehyde (MDA) equivalents calculated by the method of Heath and Packer (1968) (Heath_MDA) or Hodges *et al.* (1999) (Hodges_MDA) for 'Fire and Brimstone' and 'Olympic Flame' waratahs at Mount Annan in 2003. Correlation coefficients describing the strength of the relationship between parameters are given in the top right hand corner of each plot.

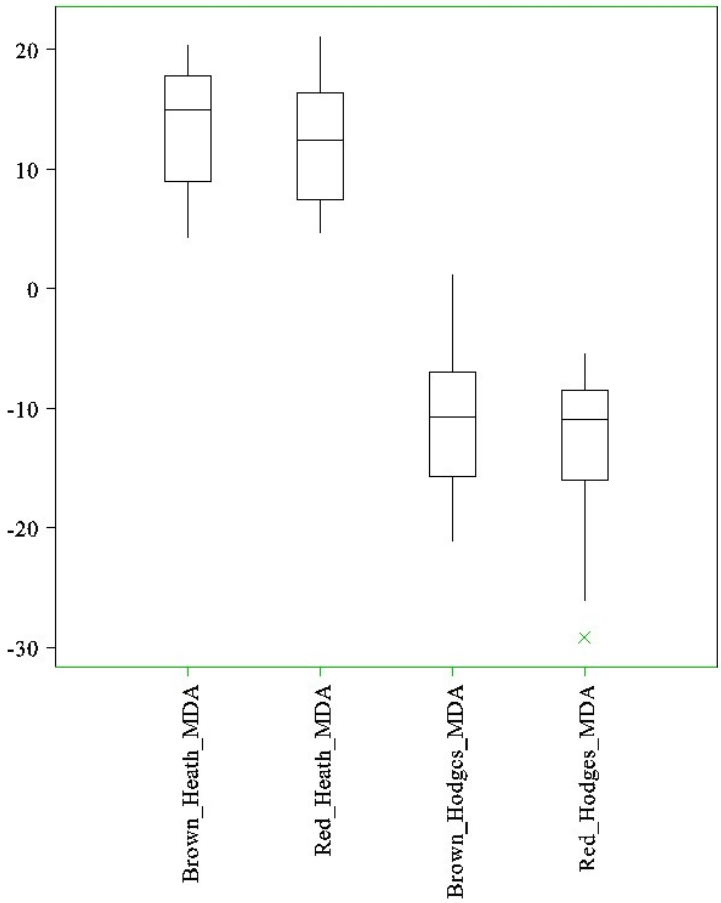

Figure 9.4: Box plot showing lipid peroxidation (MDA equivalents) for brown and red bracts calculated by the method of Heath and Packer (1968) or Hodges *et al.* (1999). Data were pooled for inner and outer bracts of 'Fire and Brimstone' and 'Olympic Flame' waratahs at Mount Annan in 2003, where more than one bract colour was present within an inflorescence. n = 9 plants in total.

9.4 Discussion

9.4.1 Results based on MDA calculations

The unexpected negative results using the MDA calculations of Hodges *et al.* (1999) can be partially explained by examining the raw data and the absorption spectra of a range of samples (for example, Appendix Table A5.1 and Figure A5.1). Two main issues are apparent: (1) higher absorbance was recorded at 532 nm for –TBA extracts compared to +TBA extracts for some samples, so the calculation of component A (Equation 9.6) was negative and (2) absorbance of some samples, particularly outer bract samples, was very high around 440-450 nm for +TBA extracts, leading to negative calculations for MDA equivalents. These issues have been reported previously by other authors, but perhaps not affecting such a wide range of data as reported here.

Hodges *et al.* (1999) reported that occasionally –TBA solutions showed slightly higher absorbance than +TBA solutions, leading to an overall negative value for MDA. They attributed this to TBA binding to specific non-MDA interfering compounds that result in a shift of absorbance away from 532 nm. However, negative results for waratah tissues do not appear to result from a similar shift in absorbance (Figure 9A.1). Hodges *et al.* also suggest that variability between replicates tends to increase, as the concentration of interfering compounds to MDA equivalents increases, as observed here for some samples (for example, outer bracts on plant 40 (40 OB), Appendix Figure A5.1).

The subtraction of absorbance at 440 nm in the assay of Hodges *et al.* (1999) serves to reduce the interference of sucrose in the lipid peroxidation assay, as sugar absorbance is

maximal at this wavelength (Du and Bramlage, 1992). The increased absorbance in waratah tissue at 440 nm therefore suggests interference from a sugary compound. It is possible that some samples were contaminated by nectar from the waratah inflorescence prior to freezing, or that waratah bract tissue contains high concentrations of sugars other than sucrose. Waratah leaves have a total sugar content of 21.6 mg/ g FW, composed primarily of sucrose (46%) and inositol (28%), with smaller amounts of fructose, glucose and bornesitol (9.8, 7.7 and 7.8%, respectively) (Bieleski and Briggs, in prep.). The presence of polyols such as bornesitol in Proteaceae leaves, and potentially, bracts, is thought to confer resistance to drought stress (Bieleski and Briggs, in prep.).

Increased absorbance at 450-460 nm was noted by Merzlyak *et al.* (1993) in a lipid peroxidation assay for senescing autumn leaves, that is, a slight increase in the wavelength of maximal absorption around 440 nm. They attributed the increased absorbance to an increase in compounds such as phenolics, which may accumulate during senescence. In the waratah tissue studied here, the tissues with highest absorbance were often outer bracts, which have decreasing pigment concentrations (Chapter 7) and often senesce towards flower maturity.

Therefore, lipid peroxidation measurements, using malionaldehyde (MDA) equivalents, were confounded by the presence of anthocyanins and flavonoids in waratah bracts and leaves. Both Heath and Packer's (1968) methods and those of Hodges *et al.* (1999) for measuring MDA were positively correlated with the concentration of anthocyanins and UV-B absorbing compounds (possibly flavonoids) in waratah bracts (Figure 9.3).

The results obtained using calculations of Heath and Packer (1968) are not likely to be reliable, since it has been shown previously that compounds such as sugars, anthocyanins and phenolics (all present in waratah tissue) interfere with absorbance at 532 nm (Merzlyak *et al.*, 1993; Hodges *et al.*, 1999; Taulavuori *et al.*, 2001). However, since all waratah tissues tested had low levels of such interfering compounds (Appendix Figure A5.1), comparisons of MDA values can be made in relative terms. In this experiment, MDA values for waratah bracts were correlated with the concentration of anthocyanin and UV-B absorbing compounds (Figure 9.3), although anthocyanin concentration was not a significant predictor of MDA in waratah tissue.

Lipid peroxidation in waratahs (based on the calculations of Heath and Packer, 1968) does not increase with photoinhibition (Figure 9.3) or bract browning (Figure 9.4). Waratah leaves also had similar lipid peroxidation to bracts at the JO stage (Figures 9.1 and 9.2), despite having significantly less photoinhibition than bracts at all stages of development (Chapter 7). These results are in contrast to those of other authors who used a similar method for assessing lipid peroxidation. For example, Ji and Jiao (2001) and Jiao *et al.* (2003) who showed increasing lipid peroxidation with photoinhibition in rice leaves. Van Hasselt *et al.* (1996) and Costa *et al.* (2002) also showed increased lipid peroxidation with increasing UV-B exposure in pea leaves and sunflower cotyledons, respectively. However, a decrease in lipid peroxidation was not evident in shaded waratah tissues compared to those in full sun.

There was also no evidence in waratahs of increasing lipid peroxidation with tissue age, as the inner bracts (youngest tissue) have increasing MDA over time, while leaves and outer

bracts (older tissue) have decreasing lipid peroxidation over time. Merzlyak *et al.* (1993) obtained similar results for senescing autumn leaves of maple, horse chestnut and birch, where lipid peroxidation did not appear to be associated with leaf senescence, in spite of pigment degradation. The lack of association found by Merzlyak *et al.* (1993) may also be attributed to the presence of interfering compounds, which had a greater effect in maple and horse chestnut than in birch leaves. Berger *et al.* (2001) found that large amounts of lipid peroxidation products were found only in the late stages of tissue senescence. These results contrast those of Panavas and Rubenstein (1998) who found increasing lipid peroxidation prior to and during flower opening in daylily petals. Berger *et al.* (2001) noted that lipid peroxidation during seasonal senescence is predominantly non-enzymatic. They suggested that measurement of lipoxygenases (LOX) may allow differentiation of enzymatic and non-enzymatic lipid peroxidation, relating lipid peroxidation to tissue age.

9.4.2 Lipid peroxidation measurements in waratah tissue

The lipid peroxidation method described above may yield more meaningful results if waratah tissue (particularly bract tissue) was rinsed of nectar prior to storage. Polyvinylpyrrolidone (e.g. Polyclar-AT) may also be added at the grinding step, to bind phenolics that interfere with absorbance at 532nm (Blokhina *et al.*, 1999). The addition of insoluble polyvinylpolypyrrolidone (PVPP) has been effectively used to remove phenols and permit quantification of the photosynthetic enzyme rubisco in a range of Australian natives, including species in the family Proteaceae (Warren *et al.*, 2000). These changes were not made in the current project due to the limited availability of inner and outer bract tissue, as well as time constraints. Until further investigation has been made into the nature of interfering compounds in waratah tissue, application of the lipid peroxidation

calculations of Heath and Packer (1968) or Hodges (1999) is not recommended for waratahs.

9.4.3 Other methods of assessing oxidative damage

Although time constraints did not allow their application during this project, other methods of assessing oxidative damage may be applied more readily to waratah tissue. The ferrous oxidation-xylenol orange (FOX) assay has been recommended as a good indicator of early membrane-associated stress events in plant tissue, as it measures initial fatty acid oxidation (Griffiths *et al.*, 2000; DeLong *et al.*, 2001). The FOX assay measures the formation of lipid hydroperoxides resulting from oxidative damage to the polyunsaturated fatty acids in cell membranes (DeLong *et al.*, 2001), rather than the secondary by-products measured using the thiobarbituric acid reaction. The FOX assay has advantages over other methods for detecting lipid hydroperoxides, in that it is sensitive, inexpensive and not affected by ambient oxygen concentrations (DeLong *et al.*, 2001). It has also been successfully applied to floral tissue (petals and sepals) of *Alstroemeria* (Griffiths *et al.*, 2000). However, the method is relatively new and is still being refined (M. Hodges, personal communication). It is also possible that interfering compounds in waratah tissue may yield unreliable results, as they did in the TBA assay.

Measurement of parameters such as those studied by Jiao *et al.* (2003) in senescing rice leaves may further elucidate the chain of damaging reactions leading to browning. Under field conditions, Jiao *et al.* (2003) found that photosynthetic efficiency and chlorophyll content decreased, while singlet oxygen, hydrogen peroxide and lipid peroxidation (MDA)

increased during senescence. The concentration of scavenging enzymes superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) also decreased during senescence.

In laboratory experiments, the production of reactive oxygen species may be studied *in planta*, rather than relying on indirect measurements of oxidative stress (Hideg *et al.*, 2002; Fryer *et al.*, 2002). High resolution chlorophyll fluorescence imaging of *Arabidopsis* leaves showed that superoxide and hydrogen peroxide accumulated in the leaf tip exposed to high light (650 μmol m^{-2} s^{-1}, compared to growth irradiance of 200 μmol m^{-2} s^{-1}) to a much greater degree than basal sections not exposed to photoinhibitory light (Fryer *et al.*, 2002). Singlet oxygen was found to originate in mesophyll tissue, where the photosynthetic apparatus are contained in mesophyll chloroplasts, while hydrogen peroxide accumulated in vascular tissue (Fryer *et al.*, 2002).

9.5 Conclusions

Measurement of lipid peroxidation using the reaction of malionaldehyde and thiobarbituric acid was confounded by the presence of interfering compounds in waratah tissue. Modifications suggested by Hodges *et al.* (1999) to reduce the influence of these interfering compounds (likely to be sugars, anthocyanins and other flavonoids) did not overcome the problem in waratah tissues, particularly due to high absorbance around 440 nm in some samples.

Malionaldehyde was therefore calculated using the method of Heath and Packer (1968) and corrections for interfering compounds were not made. These results do not support the hypothesis of increased lipid peroxidation in browned sun-exposed tissues over undamaged shaded tissue or bract tissue (photoinhibited) over leaf tissue (not photoinhibited). Lipid peroxidation did not appear to be associated with tissue age. Alternative methods for quantification of oxidative damage and investigation of reactions linking chronic photoinhibition and visible browning are given.

Although the lipid peroxidation results do not suggest that oxidative damage has occurred in waratah bracts, a chain of events linking high light, photoinhibition, pigment degradation and bract browning has been established through experiments in Chapters 5 to 9. The following chapter (Chapter 10) brings these results together for discussion.