Changes in pigment accumulation as indicators of physical stress in *Eucalyptus pauciflora*

Cynthia Turnbull[1]

Abstract

Investigating whether pigments can be used to reduce the effects of environmental stress and leaf damage is a crucial part of re-evaluating current treatments for herbivory and infection in plants, while also predicting future scenarios with warmer climates. This study investigated whether two pigments (chlorophylls and anthocyanins) could be used as indicators of compromised leaf health in the iconic Australian species *Eucalyptus pauciflora* Sieber ex Spreng, found in Kosciuszko National Park, NSW, Australia. Additionally, leaf age was also assessed as a contributing factor of leaf damage. Leaf age (represented by leaf area) was associated with increased occurrences of leaf damage in *E. pauciflora,* with a significant increase in the degree of wounding and pathogen infection observed in older leaves. Chlorophyll content (relative intensity/area) decreased with wounding and infection, supported by suspected damage to photosystems upon physical leaf stress. Anthocyanin content (relative intensity/area) did not change significantly between healthy and damaged leaves, potentially indicating the presence of anthocyanins with different biological functions in *E. pauciflora* that were not determined by our methods of extraction, but which could be the topic of future studies.

Key Words

Anthocyanin, chlorophyll, pathogen, herbivory

Introduction

In order to survive, plants of all varieties produce pigments to obtain energy from their surroundings but also to adapt to environmental stresses. These stresses often include high UV-light exposure, extreme temperature changes, increased herbivory from insects and risks of pathogen infection (both bacterial and fungal). The pigment chlorophyll does more than providing the characteristic green colouration of plant leaves. It plays a vital role in the capture of light required for the fixation of inorganic carbon (carbon dioxide – CO_2) to organic carbon during the Calvin cycle. This process, known as photosynthesis, generates an accessible supply of carbon which can be used to generate energy (in the form of adenosine triphosphate – ATP), needed to sustain plant survival and growth (Aldea *et al.* 2006). Whilst chlorophyll accumulation is vital for the production of energy in plants, it can be reduced by damage to photosystems through excess UV-light exposure (Aldea *et al.* 2006).

One way plants in different microclimates protect photosystems is through the production of a large group of secondary metabolites called flavonoids. These biomolecules consist of approximately 10,000 structures across the plant kingdom and are characterised largely by different substituents around a basic C6–C3–C6 skeleton (Hichri *et al.* 2011). Flavonoid synthesis requires some of the same carbon precursors as the products of the Calvin cycle, both lack nitrogen in their structures, and their synthesis requires large amounts of energy (Agati *et al.* 2012). As such, these biomolecules have been referred to as energy sinks/escape valves. This means that under conditions where a slow Calvin cycle persists, flavonoid synthesis can be upregulated to limit the excessive build-up of its carbon precursors and ATP (Hernández *et al.* 2009). Energy sinks then help to protect the photosystem from damage under conditions of high incident light at low temperatures, i.e. when the photosystem is active but carbon fixation through the Calvin cycle is slow. This is especially important for alpine and subalpine plants such as *Eucalyptus pauciflora*, an Australian iconic species commonly referred to as snow gum, which is frequently exposed to high light intensity and cold temperatures (Mouradov and Spangenberg 2014).

One common subclass of flavonoids found in plants is anthocyanins. Consisting of over 500 structures, anthocyanins are not only responsible for the pigments associated with the display of brilliant colours

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[1] Corresponding author: u5801218@anu.edu.au, Research School of Biology, ANU, Canberra, ACT, Australia

in angiosperm flowers but also the adaption of plants to stresses in microclimates (Guo *et al.* 2008). These adaptions include actions as energy sinks described above but also the characteristic ability to reflect blue-green light, behaving like 'plant sunscreens' (Guo *et al.* 2008). Studies on alpine plants have also demonstrated a potential use of anthocyanins as antifreeze compounds where the biomolecules act as solutes to lower the freezing point of water in plant cells (Chalker-Scott 1999). By lowering the freezing point anthocyanins prevent the water in cells from expanding when turning into ice, which can cause cell lysis (the rupture of the cell membrane). The ability of anthocyanins to act as energy sinks, plant sunscreens and antifreeze compounds helps healthy leaves adapt to environmental stresses such as high UV-light and temperature extremes characteristic of Kosciuszko National Park, New South Wales (NSW), Australia, where *E. pauciflora* thrives at a range of elevations.

Anthocyanin production is not limited to healthy leaves, but can also be induced under pathogen attack and wounding through herbivory. Leaf-surface flavonoids can act as anti-herbivory agents by mimicking the chemical signals released from certain types of pathogens (Mouradov and Spangenberg 2014). Anthocyanin production in response to pathogen infection has also been demonstrated as a requirement for host cell survival in plants infected with bacterial pathogens such as phytoplasma, which infects potato grapevine and maize. Upregulation of anthocyanins is triggered by significant increases in sucrose levels upon phytoplasma infection and is often observed through a characteristic purple top (reddening) of leaves (Himeno *et al.* 2014). Interestingly, plants with purple top leaves (high levels of anthocyanins) also displayed reduced cell death when compared to infected plants unable to synthesise anthocyanins (Himeno *et al.* 2014).

The mechanisms through which anthocyanins can prevent cell death and resist disease progression are not clearly defined; however, recent studies have implicated the antioxidant properties of flavonoids as a key element in increasing cell survival. This includes flavonoid-facilitated reduction of accumulative reactive oxygen species (ROS), which can be toxic in cells (Agati *et al.* 2013). The reduction of ROS has also been shown to vary with the structure of flavonoids. Glycosylated flavonoids in trichomes tend to donate H^+ ions to reduce pre-existing ROS while flavonoids with hydroxylated B-ring substituents found in the nucleus and chloroplasts of mesophyll cells react with Fe^{2+} and Cu^{2+} ions to prevent the generation of ROS (Agati *et al.* 2013). The effect of anthocyanins on ROS could explain the ability of the biomolecules to alleviate the symptoms of disease and subsequent wounding effects but also provide a strong line of defence against pathogens. Anthocyanin accumulation was shown to double shelf life in inoculated tomatoes by altering the spread of ROS bursts during infection with *Botrytis cinerea*, the bacterium responsible for the over-ripening process (Zhang *et al.* 2013). Research into the effects of anthocyanins on ROS could then benefit developments in food microbiology and perhaps reduce the occurrence of pathogen infection in agricultural crops. Similarly, the antioxidant properties of anthocyanins have also been implicated in stress responses following wounding. Post-wounding responses often include the formation of an anthocyanin band around the wounded site (e.g. insect bite in leaf), which acts to contain the spread of ROS. This was observed after the application of controlled mechanical injury in *Pseudowintera colorata* leaves, where the formation of an anthocyanin band was preceded by a significant decrease in hydrogen peroxide (H_2O_2) levels (Gould *et al.* 2002).

Additionally, the ability to reduce ROS in both conditions of leaf damage (infection and wounding), but also high UV-light exposure, is implicated in the protection and maintenance of efficient photosystems (Aldea *et al.* 2006). For example, the scavenging of free radicals, including DPPH (1,1-diphenyl-2-picrylhydrazyl) and superoxide anions, is one of the active roles anthocyanins play in photoprotection (Gan *et al.* 2014). This action of anthocyanins is particularly relevant in the juvenile stages of leaf development when immature photosystems are more vulnerable to damage through ROS or excess energy (Zhu *et al.* 2016). Consequently, anthocyanin accumulation is often greater in younger leaves where the photosystems are not fully developed (Zhu *et al.* 2016). As leaves mature, anthocyanin content generally decreases while chlorophyll content rises with the maturation of photosystems and the need to generate energy via photosynthesis (Lobato *et al.* 2010; Menzies *et al.* 2015). In the case of wounding and infection the opposite situation occurs, i.e. anthocyanin synthesis is upregulated to protect the plant from excessive leaf damage while chlorophyll content decreases often as a result of associated damage to photosystems through ROS exposure (Aldea et al. 2006; Himeno *et al.* 2014).

This study will first investigate whether leaf age (indicated by leaf area) is a factor in increasing the occurrence of wounding and infection in a native Australian species, *Eucalyptus pauciflora,* at Charlotte Pass, Kosciuszko National Park, NSW, Australia. Due to the predicted decrease in anthocyanin levels in older leaves, it is expected that despite having high chlorophyll content and photosynthetic capacity, older leaves will be more vulnerable to the effects of wounding and infection. Younger leaves are expected to show a smaller degree of leaf damage due to increased anthocyanin accumulation. Pigment (chlorophyll and anthocyanin) accumulation in both wounded and infected leaves, and leaves with combined damage were compared to healthy leaves. Healthy leaves were classified as leaves which display no visible signs of damage but are still exposed to the environmental stresses of the Charlotte Pass region, including cold exposure and high UV-light exposure. Chlorophyll content is expected to be highest in healthy leaves and lowest in wounded or infected leaves, due to photoinhibition. Conversely, anthocyanin content is hypothesised to be lowest in healthy leaves due to the absence of excessive leaf stress in response to physical damage. The study of pigment accumulation will seek to implicate changes in chlorophyll and anthocyanin content as stress responses to both wounding and infection in native Australian flora.

Methods

Collection of leaf samples

Leaves from *E. pauciflora* were collected for analysis of anthocyanin and chlorophyll content with wounding and infection along Mount Stillwell in Charlotte Pass, Kosciuszko National Park, NSW, Australia. Collection sites were of similar elevation (between 1,800 and 1,900 m) with 28 leaves sampled from nine trees in total. Two additional branches (with 46 leaves) were also sampled to determine the degree of infection and wounding with leaf age.

Degree of infection and wounding with leaf age

All 46 leaves from sampled *E. pauciflora* branches were ranked by degree of wounding and infection. Each leaf was given a score of 0–4 for both infection and wounding, where 0 = no damage, 1 = mild damage, 2 = moderate damage, 3 = moderate-acute and 4 = acute damage (Table 1).

Since leaves accumulate biomass and increase in size as they develop from juvenile to adult stages, leaf area (cm^2) was used as a proxy for leaf age (Menzies *et al.* 2015). As such, leaf area for all 46 leaves was measured using ImageJ 1.48 imaging software. For wounded leaves, areas missing from the leaf (e.g. insect bites) were estimated and included in total leaf area in order to allow for a more representative indication of leaf area, had wounding not occurred. This was done to ensure that the age of the leaf was not misrepresented, i.e. large leaves with many bites would not have the same area as a smaller juvenile leaf with no bites. It should be noted that the cause of infection in *E. pauciflora* leaves was not deduced. We instead focused on pigment changes as a potential response to infection.

Extraction of pigments

The *E. pauciflora* leaves from nine sites within the same elevation range where classified into four categories; healthy (no wounds or infection spots), wounded, infected or combined damage (presented both spots and wounds). Pigments were extracted from the leaf by grinding leaves into a fine powder using a mortar and pestle. Ground samples were weighed and extracted in 70 per cent ethanol at a 2:1 solvent to leaf mass ratio. Formic acid was then added to the supernatant of centrifuged diluted sample solutions at a 1:10 acid to supernatant ratio (v/v). Acid was added to hydrolyse sugars from the polar glycosylated anthocyanins whilst in solution, forming less-polar anthocyanin structures referred to as aglycones. The aglycones were used because anthocyanin standards (delphinidin chloride, malvidin chloride, cyanidin chloride, peonidin chloride, all from Extrasynthese) were also in the aglycone form. However, under the conditions used here, we could not definitively identify the extracted anthocyanin spots against the standards as all had very similar retention times.

Table 1: Classification for the degree of leaf damage in *E. pauciflora* leaf samples collected from Charlotte Pass.

	Degree of leaf damage				
	None	Mild	Moderate	Moderate-Acute	Acute
Wounding	No wounds 	One wound 	2 wounds 	2-4 wounds 	Wounds all around leaf exterior 
Infection	No visible infection spots 	Beginnings of infection- one visible zone of infection e.g spot on leaf 	2 zones of infection 	2-4 infections zones with purple discoloration in leaf 	Significant zones of infection dominating leaf surface 

Separation of pigments using thin layer chromatography (TLC)

Anthocyanin aglycones and chlorophyll pigments were separated using TLC with a mobile phase consisting of formic acid, hydrochloric acid, water and ethyl acetate (6:1:3:5). The pigments were separated by their solubility in the mobile phase, forming visible ‘spots’ on the TLC plate at different retention factors.

Quantification of pigments and statistical analyses

Through ImageJ 1.48 software, relative spot intensity per area was then used to quantify chlorophyll and anthocyanin accumulation. This was achieved by first converting the photographed image of the TLC plate to black and white, inverting the image and then using the area tool to calculate the integrated density over a selected pigment spot. This was repeated for all 28 leaves. Statistical analyses included one-way ANOVA and Welch’s *t*-tests using R Studio 3.3.1 software. These were performed to contrast the relationship between leaf area, chlorophyll concentration and anthocyanin concentration with infection and wounding.

Results

Degree of leaf damage increased with leaf age

As leaf area (LA; indicative of leaf age) increased, the degree of wounding and infection in *E. pauciflora* leaves also increased. Significant differences in the degrees of leaf wounding and infection were observed between smaller and larger leaves through one-way ANOVAs ($p = 0.002$ and $p = 2.68 \times 10^{-7}$) (Figures 1 and 2). Smaller leaves (average LA ≈ 2 cm^2) tended to display smaller degrees of wounding while larger leaves (average LA ≈ 5.5 cm^2) had a higher incidence of acute damage, with wounds surrounding the whole exterior leaf (Figure 1).

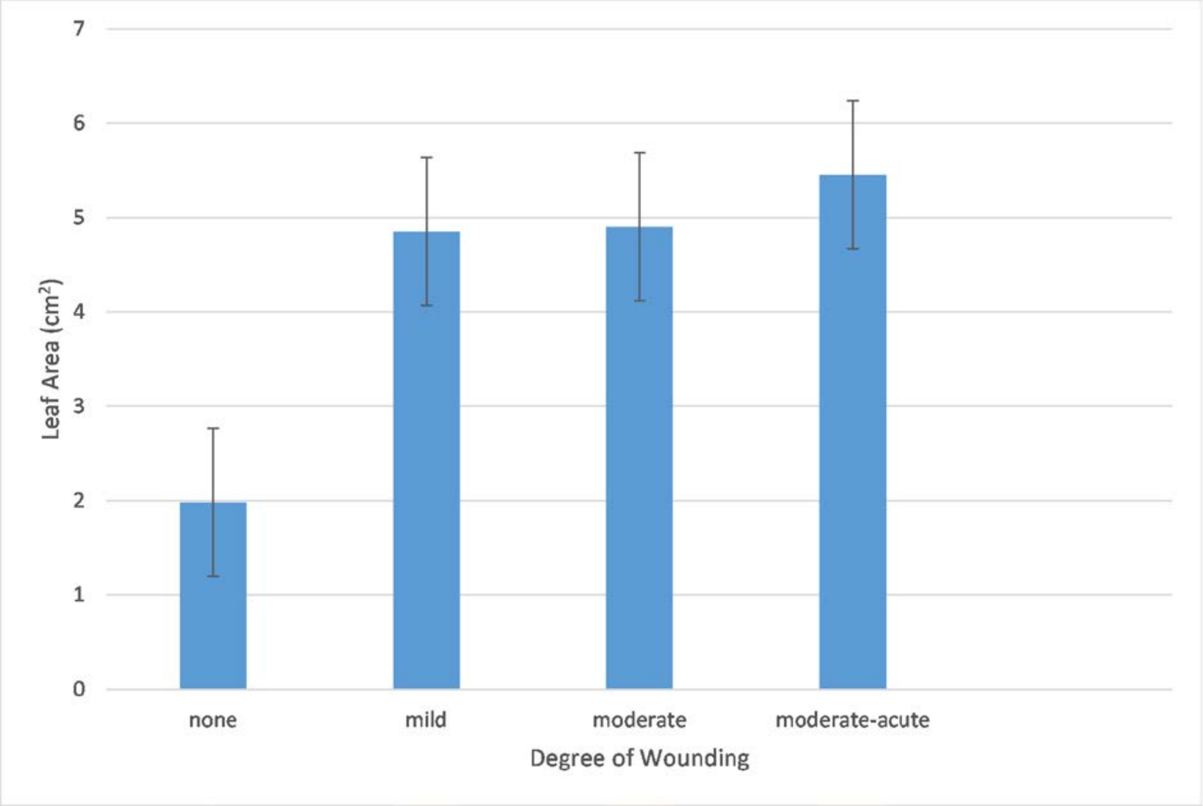

Figure 1: Degree of wounding with leaf area in *Eucalyptus pauciflora*, Kosciuszko National Park. Larger leaf areas were associated with greater degree of wounding ($p = 0.002$, F-value = 13.090, one-way ANOVA). No leaves were classified with acute wounding (Table 1). Standard error bars have been included.

Similarly, smaller leaves displayed lower incidences of severe infection, with leaf areas from 0.5 to 3 cm^2 showing no visible signs of infection while leaves with acute infection had areas from approximately 6 to 8 cm^2 (Figure 2). From these results, leaf area as a proxy for leaf age can be seen as an attributing factor in the severity of leaf damage in the form of infection and wounding.

Anthocyanin concentration with leaf damage

Anthocyanin concentration initially appeared to increase with leaf damage when we compared the relative intensity per area of 104.8 in healthy leaves (Figure 3). However, following an ANOVA test, no significant difference between anthocyanin concentration in healthy, wounded, infected or combined damaged leaves was observed ($p = 0.596$). The greatest increase in anthocyanin content compared to healthy levels was seen in leaves with observable signs of both wounding and infection (combined damage), with a mean relative intensity per area of 117.1 (Figure 3). A Welch's t-test between two leaf types was consistent with the ANOVA, wherein no significant difference between anthocyanin content in healthy and combined damages leaves was observed (p-value = 0.250). This led to the conclusion that anthocyanin content within *E. pauciflora* leaves did not vary with leaf damage in the form of wounding by herbivory and/or pathogen infection.

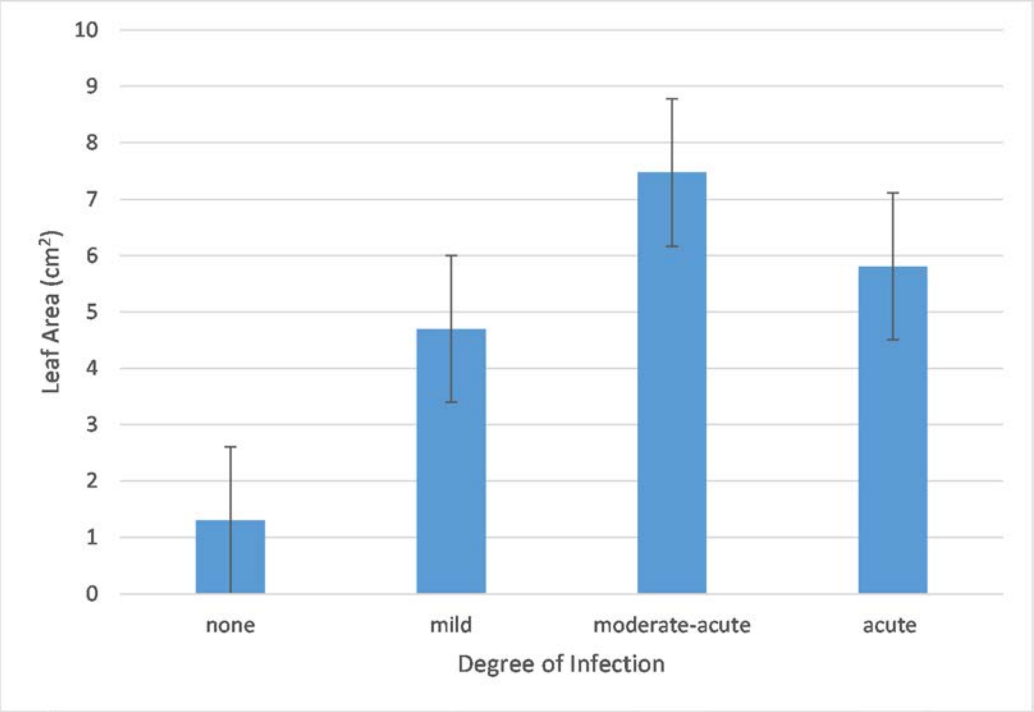

Figure 2: Degree of infection with leaf area (cm^2) in *Eucalyptus pauciflora*. Large areas were associated with large degree of infection from the moderate-acute to acute categories ($p = 2.68 \times 10^{-7}$, *F*-value = 53.120, one-way ANOVA). No leaves were classified with moderate infection (Table 1). Standard error bars have been included.

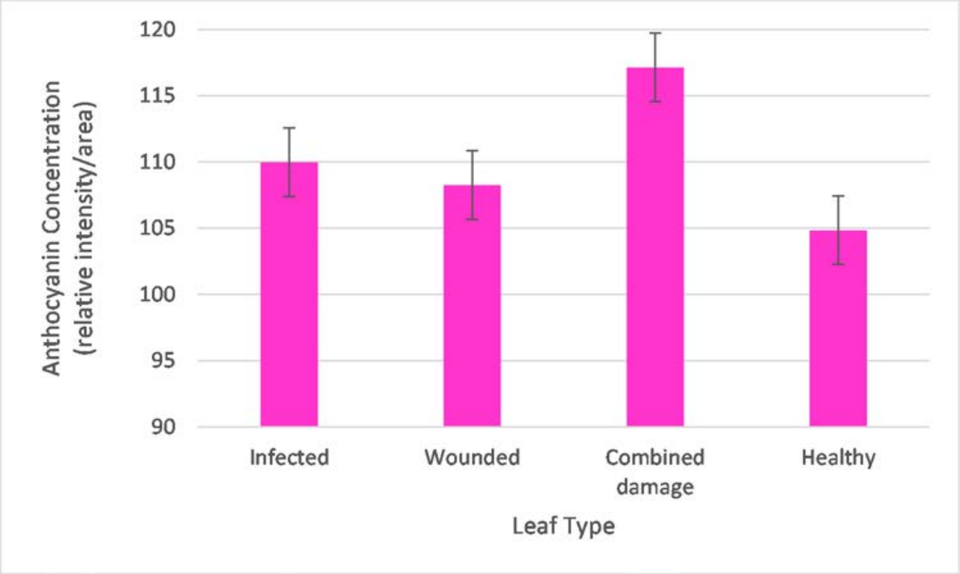

Figure 3: Anthocyanin content (relative intensity/area) with leaf damage through infection and wounding relative to healthy leaves in *Eucalyptus pauciflora*. Anthocyanin levels did not vary significantly with leaf damage ($p = 0.596$, *F*-value = 0.641, one-way ANOVA). The highest accumulation of anthocyanins was seen in leaves with both forms of damage. However no significant difference between combined damaged and healthy leaves was observed ($p = 0.250$, Welch's *t*-test). Standard error bars included.

Chlorophyll concentration decreases with leaf damage

Wounded and pathogen-infected leaves displayed lower average chlorophyll content with mean relative intensities of 95.46 for infected, 108.05 wounded and 106.59 for combined damage, compared to 110.43 (relative intensity/area) in healthy leaves (Figure 4). A significant difference between mean chlorophyll content in wounded, infected and healthy leaves was confirmed ($p = 0.02$), suggesting that changes to leaf health caused variations in chlorophyll content (Figure 4).

In summary, leaf area as an indicator of leaf age is a contributing factor towards the occurrence of leaf damage. Increased leaf area was associated with larger degrees of leaf damage i.e. high incidences of wounding and infection. Anthocyanin content did not vary significantly with leaf damage, through wounding, infection or combined damage when compared to healthy leaves. Conversely, chlorophyll content was higher in healthy leaves when compared to wounded and pathogen-infected leaves, as confirmed by a significant difference between average intensity per area in healthy and damaged leaf groups.

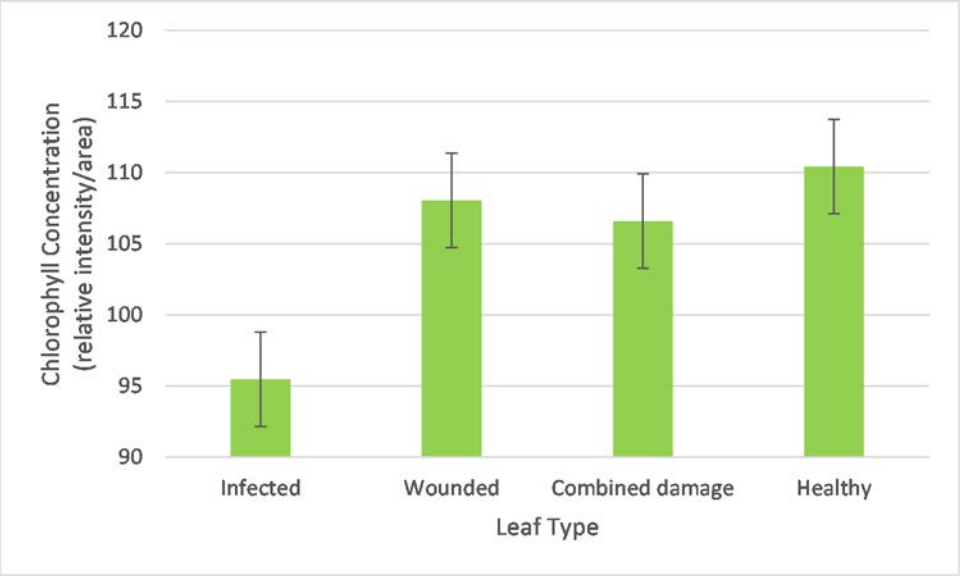

Figure 4: Chlorophyll content (relative intensity/area) with wounded and infected leaves when compared to healthy leaves in *Eucalyptus pauciflora* ($p = 0.0173$, one-way ANOVA). Chlorophyll accumulation was highest in healthy leaves and lowest in infected leaves. Standard error bars have been included.

Discussion

This study investigated the role of leaf age as a determining factor in leaf damage, while also assessing if changes to pigment accumulation could be used as indicators of leaf stress during infection and post wounding. As predicted, older leaves in *E. pauciflora* saw an increase in the degree of infection and wounding. Chlorophyll content (relative intensity/area) decreased with leaf damage, whether through infection or wounding, while no significant difference in anthocyanin content (relative intensity/area) between damaged and healthy leaves was observed.

Leaf age significantly influenced the degree of leaf damage in *E. pauciflora*, with a positive correlation between increasing leaf area and higher degrees of wounding and infection (Figures 1 and 2). Large degrees of leaf damage in older leaves may simply be a consequence of larger probabilities of wounding or infection over time (Zhu *et al.* 2016). However, this could also be influenced by changes to anthocyanin levels throughout leaf development. The presence of larger anthocyanin accumulations in younger leaves has been theorised as a method of photoprotection, wherein anthocyanins act as antioxidants and sunscreens to protect immature photosystems from excess light and ROS exposure (Zhu *et al.* 2016). As leaves grow, the need for anthocyanins decreases with reduced risks of damage to mature photosystems (Lobato *et al.* 2010; Mouradov and Spangenberg 2014). Leaf biochemical pathways are also driven strongly towards the upregulation of chlorophyll over anthocyanins in order to maintain the photosynthetic rates needed to sustain larger leaf mass (Aldea *et al.* 2006; Lobato *et al.* 2010; Menzies *et al.* 2015). Decreasing levels of anthocyanins have the potential to then inadvertently increase plant vulnerability to the effects of wounding and infection. This is because of the crucial role anthocyanins play in reducing the effects of wounding and progression of acute infections (Gould *et al.* 2002; Zhang *et al.* 2013). These include antioxidant activity through the limiting of ROS accumulation which can damage photosystems and lead to cell death during both infection and post wounding (Hernández *et al.* 2009). The possibility of anthocyanin loss with age could then be used to support increases in the degrees of both infection and wounding found during this study (Figures 1 and 2). To confirm this, future studies could build on this experiment by not only measuring changes to the degree of wounding and infection over the course of leaf development but also by comparing the levels of anthocyanin content before damage, during infection and post wounding in leaves of different ages.

As expected, changes to leaf health through wounding and/or infection saw significant variations in chlorophyll levels (Figure 4). This may be explained by the damage caused to photosystems during mechanical and chemical injury (e.g. exposure to toxic ROS) (Aldea *et al.* 2006). Damaged photosystems often result in lowered photosynthetic rates (Menzies *et al.* 2015). This is detrimental to the plant as reduced photosynthetic rates are unable to produce sufficient amounts of energy needed to sustain cell processes, leading to severe leaf damage or death (Aldea *et al.* 2006). Future studies might investigate this further by observing changes in photosynthetic rates between healthy and damaged leaves through the use of a LICOR (Menzies *et al.* 2015). Chlorophyll content could also be obtained more precisely through measurements of chlorophyll fluorescence, rather than the TLC spot intensity/area used in this study (Lobato *et al.* 2010).

It was predicted that upon infection and wounding, rising levels of anthocyanin accumulation would emerge as part of a defence response against ROS damage to leaf cells, associated with the effects of both forms of leaf damage (Gould *et al.* 2002; Zhang *et al.* 2013). Surprisingly, no significant difference in anthocyanin concentration (relative intensity/area) between healthy and damaged cells was observed (Figure 3). This was an unusual result since increased anthocyanin accumulation has often occurred upon the upregulation of particular biochemical pathways during infection (Himeno *et al.* 2014). For example, the upregulation of pathway genes such as chalcone synthase and dihydroflavonol 4-reductase have previously been demonstrated to increase anthocyanin content. In tomato plants infected with phytoplasma, this led to purple colouration of leaves and increased cell survival (Himeno *et al.* 2014). Additionally, anthocyanin accumulation is also recognised as a contributing element of reduced ROS accumulation after mechanical injury and pre-wounding defences (Gould *et al.* 2002). An example of pre-wounding defences includes the role of anthocyanins as herbivory agents. This was recently demonstrated through the reduction of insect host colonisation in *Pseudowintera colorata* as a consequence of increased anthocyanin concentrations (Menzies *et al.* 2015).

The absence of a significant difference between average total anthocyanin concentration (relative intensity/area) in healthy and damaged plants is not a definitive indicator of constant anthocyanin levels despite environmental (high UV light, temperature extremes) and physical (infection and wounding) stresses facing *E. pauciflora* leaves in Charlotte Pass. It may rather be that specific anthocyanins were elevated with different stresses to leaf health. Anthocyanins associated with responses to UV-light and cold exposure may have been increased in the 'healthy' leaves as part of adaptions to the temperature extremes found in the Charlotte Pass region. Conversely, damaged or infected leaves may have accumulated higher levels of other anthocyanins specific to leaf damage. The idea of anthocyanins with selected biological roles is still undefined, but a past study did observe the production of different anthocyanin patterns in *Arabidopsis thaliana* under stress responses to pH, nutritional deficiencies and osmotic stress through high-performance liquid chromatography (HPLC) extraction (Kovinich *et al.* 2014). As the generalised TLC extraction method used in this study only observed total pigment accumulation, it was unable to distinguish between the different structures of anthocyanin types. To test the presence of different types of stress-specific anthocyanins, future experiments could utilise more specific extraction techniques such as HPLC. This study also did not identify the cause of disease in *E. pauciflora* leaves but only classified leaves as 'infected' by the appearance of infection sites/spots. The sole use of visible representations as identifiers of infection may have unintentionally resulted in the misidentification of infected but spot-free leaves as healthy leaves. Future experiments could provide better controls for pathogen infection by using laboratory-grown leaves inoculated with a specific pathogen such as phytoplasma, to ensure all ‘infected’ leaves are suffering from the same disease and will not be confused with healthy plants (Himeno *et al.* 2014). Possible mutant or transgenic plants of *E. pauciflora* could also be used to further determine the roles of anthocyanin upregulation during infection. This could be done through a comparison of cell death and ROS accumulation between mutants or transgenic plants unable to activate anthocyanin pathway genes compared to wild-type plants (Hichri *et al.* 2011).

Conclusions

This study investigated whether changes in pigment accumulation (chlorophylls and anthocyanins) could be attributed to defensive responses to physical environmental stresses such as wounding and infection, whilst also investigating whether incidences of leaf damage would accumulate during leaf ageing.

Leaf age was a significant factor in leaf damage with increases in the degree of infection and wounding in *E. pauciflora* associated with larger leaf areas. Decreased levels of chlorophyll in damaged leaves, when compared to healthy leaves, indicated damage to photosystems, which is often the result of wounding and infection (Aldea *et al.* 2006). There was no significant difference in average anthocyanin levels in healthy leaves when compared with damaged leaves, contradicting current literature (Hernández *et al.* 2009; Mouradov *et al.* 2014). This lead to the suggestion that numerous anthocyanins acting as combatants to different leaf stressors (high UV light, cold exposure, pathogen infection and wounding through herbivory) existed within sampled leaves (Kovinich *et al.* 2014).

Further investigation into the specific roles of anthocyanins could aid the understanding of their dynamic relationship within plants as defences against many stresses to leaf health, both physical and biochemical. Additionally, the response of chlorophyll to leaf damage is imperative to the prediction of future scenarios with warmer climates. Chlorophyll content in relation to photosystem damage is particularly important, as both intense light exposure and CO_2 increase have already been associated with damage to photosystem II (Aldea *et al.* 2006). Since warmer climates could also propagate the spread of pathogenic diseases both in human and plant species, investigations into specific anthocyanins involved in defences against infection could provide insights into new medicinal therapies. It could also allow for advances in the treatment of crop diseases which are crippling agricultural industries (Himeno *et al.* 2014; Zhang *et al.* 2013). The investigation into the action of pigments as indicators of compromised leaf health is therefore a highly beneficial avenue for future research.

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