The effect of UV light intensity on anthocyanin content of *Richea continentis* leaves

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Abstract

The aim of this study was to determine the effect of UV light intensity on anthocyanin content in *Richea continentis* leaves. Anthocyanins play an important role in protecting plants from environmental stresses such as high UV exposure and freezing temperatures. This study was conducted because in the context of climate change it is important to understand how increased UV exposure will affect alpine plants. It was hypothesised that anthocyanin content in *R. continentis* leaves would increase as UV light intensity increases. Three samples of *R. continentis* were collected from each of four different elevations at Charlotte Pass, NSW, and UV light intensity at these sites was recorded. The leaves' anthocyanin content was determined using thin layer chromatography and chlorophyll fluorescence (F_v/F_m) was measured as an indicator of leaf stress using a Plant Efficiency Analyser. There was a significant positive correlation between UV light intensity and anthocyanin content, and a significant negative correlation between F_v/F_m and anthocyanin content. No significant correlation was found between light intensity and F_v/F_m, suggesting that anthocyanins act to reduce UV damage in *R. continentis* leaves.

Key Words

Alpine regions, chlorophyll fluorescence

Introduction

Flavonoids are a class of secondary metabolites found in all higher plant and bryophyte tissues (Hernández *et al.* 2009). Flavonoids perform a variety of actions, including protection from pathogens, light screening, antioxidative processes, and pollination (Hernández *et al.* 2009). Flavonoids are most often induced by environmental stresses such as drought, high UV levels, and wounding (Agati *et al.* 2009; Hernández *et al.* 2009). There are more than 9,000 molecules in the flavonoid class, divided into several subgroups.

Anthocyanins are one such subgroup. These compounds are red or purple pigments primarily found in vacuoles of plant epidermal cells (Chalker-Scott 1999). The synthesis of anthocyanins in leaves may be induced by a variety of environmental stressors, such as freezing temperatures, nutrient deficiencies and light exposure (Chalker-Scott 1999). In particular, studies have found that UV-B radiation correlates with high levels of anthocyanins in epidermal cells (Barnes *et al.* 2000).

The presence of anthocyanins reduces UV light absorption by chlorophylls, as anthocyanins reflect the UV light before it can reach the chloroplasts. This reduces light damage (Hernández *et al.* 2009) and is known as light or UV screening (Bilger *et al.* 2007). To protect leaves from overexposure to UV, anthocyanin concentration is higher on the leaf side facing the sun (Nicotra *et al.* 2003).

Anthocyanins (and other flavonoids) are also used by plants to dissipate excess energy under conditions of high light and low temperatures, when the photosystem produces large amounts of adenosine triphosphate (ATP) and reduction equivalents, but these cannot be used by the slower reactions of carbon fixation (Hernández and Van Breusegem 2010). Under these conditions, synthesis of flavonoids uses ATP and reduction equivalents as an alternative endpoint to carbon fixation, allowing the photosystem to remain active (Hernández and Van Breusegem 2010). In addition, the produced anthocyanins have anti-stress functions in the plant, as described above, so the energy is not 'wasted'.

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Much research has been done into the functions of anthocyanins within plants. However, while most of the work on the relationship between UV light and anthocyanins has been done on lowland plants, it can be argued that examining this relationship is even more important in alpine plants.

Solar UV radiation increases with altitude more than the average increase with altitude across all wavelengths (Blumthaler 1996). Thus, alpine plants may be exposed to higher UV levels than lowland plants (Blumthaler 1996). Overall, the harmful effect of UV overexposure on leaf health is reduced because alpine plants spend the winter months under snow cover, where they are protected from solar radiation.

However, climate change, which is likely to affect alpine regions more than other regions, means that snow cover will continue to decrease, with less snowfall and earlier thaw times (Thompson 2016). Thus, alpine plants will soon be exposed to more UV over the course of a year than what they are used to (Worboys and Good 2011). The likelihood of UV damage may increase dramatically unless plants can adapt to the changing climate (Korner 2003).

In order to conserve the alpine flora, it is vital to understand the challenges these plants are, or will, be facing as time goes on. The current study aimed to investigate the effect of UV light intensity on anthocyanin content in the leaves of *Richea continentis*. *Richea continentis* is a branched shrub endemic to Australia (Wahren and Williams 1999). It is commonly found in alpine and subalpine bogs, on peat soils (McDougall 2007).

To better understand the relationship between anthocyanin and UV light intensity, leaf stress was also considered in this study. Leaf stress measurements can be used to assess UV damage to a plant. The technique used in this study is chlorophyll fluorescence (Murchie 2003). This technique measures the light re-emitted by a leaf after exposure to a known amount of light (Murchie 2003). Any light energy absorbed by a leaf can be either used for photosynthesis, dissipated as heat, or re-emitted as light, resulting in chlorophyll fluorescence (Murchie 2003).

When a leaf is dark-adapted and then exposed to a flash of light, most of the absorbed energy is discarded through chlorophyll fluorescence (Murchie 2003). Thus, a leaf's chlorophyll fluorescence indicates the efficiency of photosynthetic components within the leaf, or its photosynthetic potential (Murchie 2003). The ratio of re-emitted light to absorbed light (F_v/F_m) is used to quantify leaf photosynthetic potential (Tian-Shan 2017).

A lower F_v/F_m ratio indicates a more light-damaged leaf (Tian-Shan 2017). In healthy plants of most species, F_v/F_m is about 0.77–0.81 (Tian-Shan 2017). F_v/F_m can drop when a plant is exposed to stress and has been found to be negatively correlated with light intensity (Tian-Shan 2017).

Based on previous research, it was hypothesised that anthocyanin content of *R. continentis* leaves would increase with UV light intensity and that this would provide some protection from the incidence of UV light.

Methods

Four sites of increasing elevations (1,752, 1,785, 1,823 and 1,960 m above sea level) were selected at Charlotte Pass based on the availability of *R. continentis*. Three samples of *R. continentis* were collected from each site, from three different plants growing within several metres of each other. For each sample, the tips of non-flowering shoots were collected. Additionally, UV light intensity (μmol m^{-2} s^{-1}) was measured using a light probe.

In the lab, 12 Eppendorf tubes were labelled 1A-C, 2A-C, 3A-C or 4A-C to correspond with samples A, B and C from each of the four sites. Then, the top 2 cm were excised from each shoot and ground using a mortar and pestle. About 250 mg of the samples were placed into the corresponding Eppendorf tubes and 0.5 mL of ethanol was pipetted into the tubes. The samples were placed into a centrifuge for 1 minute at 13,000 rpm. Then, equal volumes of each of the resulting solutions were blotted onto silica gel thin layer chromatography (TLC) plates (one plate per site, with samples A–C on each plate). The plates were run to separate anthocyanins from other leaf pigments (the mobile phase was an 18:18:3:1 solution of ethyl acetate, water, acetone and formic acid), then left to dry.

After 30 minutes, the plates were photographed and the relative concentration of each anthocyanin spot was determined using the ImageJ software. The image was converted to black and white and the colours were inverted. Then, each anthocyanin spot (originally red in colour) was selected and the 'integrated density' function was used to determine the relative concentration of anthocyanin on the plate.

Finally, three mature leaves of a similar developmental stage from each site were placed into Plant Efficiency Analyser (PEA) clips and left to dark-adapt for 30 minutes. Then, they were placed into the PEA one by one, and each leaf's F_v/F_m was determined.

Results

There was a clear positive correlation between anthocyanin content and UV light intensity, which means anthocyanin content in *R. continentis* leaves increased as UV light intensity increased (Figure 1). There was some variation between the samples from the same site. This was to be expected, as it was partly cloudy on the day the measurements were taken, and the cloud cover shifted constantly. Since deleting the two potential outliers did not affect the trendline, it was decided that the data points would not be removed from the dataset.

The data were analysed using the JMP software package using a linear regression. The obtained *p*-value was 0.0018. Since 0.0018 is much lower than 0.05, the relationship between anthocyanin content and UV light intensity is highly statistically significant. This means that it is unlikely that the observed trend is due to random variation in the data.

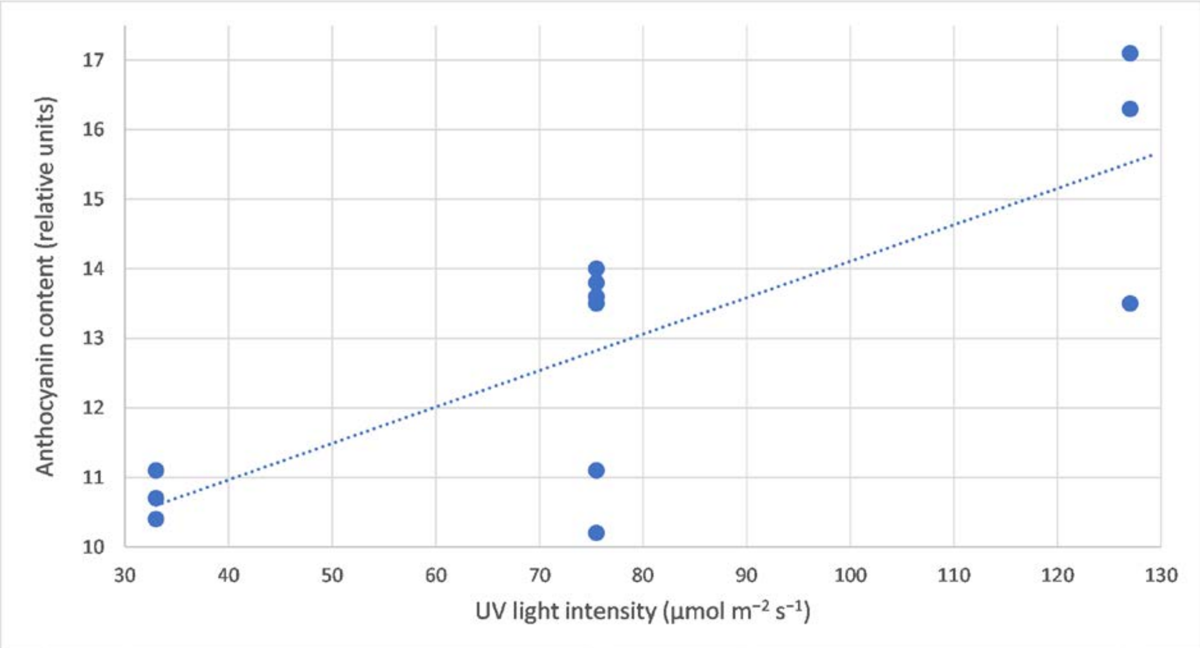

Figure 1: The relationship between UV light intensity and anthocyanin content of *R. continentis* leaves across four different sites at Charlotte Pass, NSW, Australia. Two sites had the same UV intensities. Each dot represents a single sample's anthocyanin content.

There was a negative correlation between UV light intensity and F_v/F_m (Figure 2). Chlorophyll fluorescence of *R. continentis* leaves decreased as UV light intensity increased. Three data points were much lower than the rest of the data. These were not considered outliers because, while the samples in question came from different sites, all three follow the same general trend as the rest of the data. This suggests that they are a result of natural variation in *R. continentis* leaves rather than an experimental error, in which case the outliers would likely be more random.

When a linear regression was performed on the data (using JMP), a *p*-value of 0.0752 was obtained, which showed the observed relationship was not statistically significant.

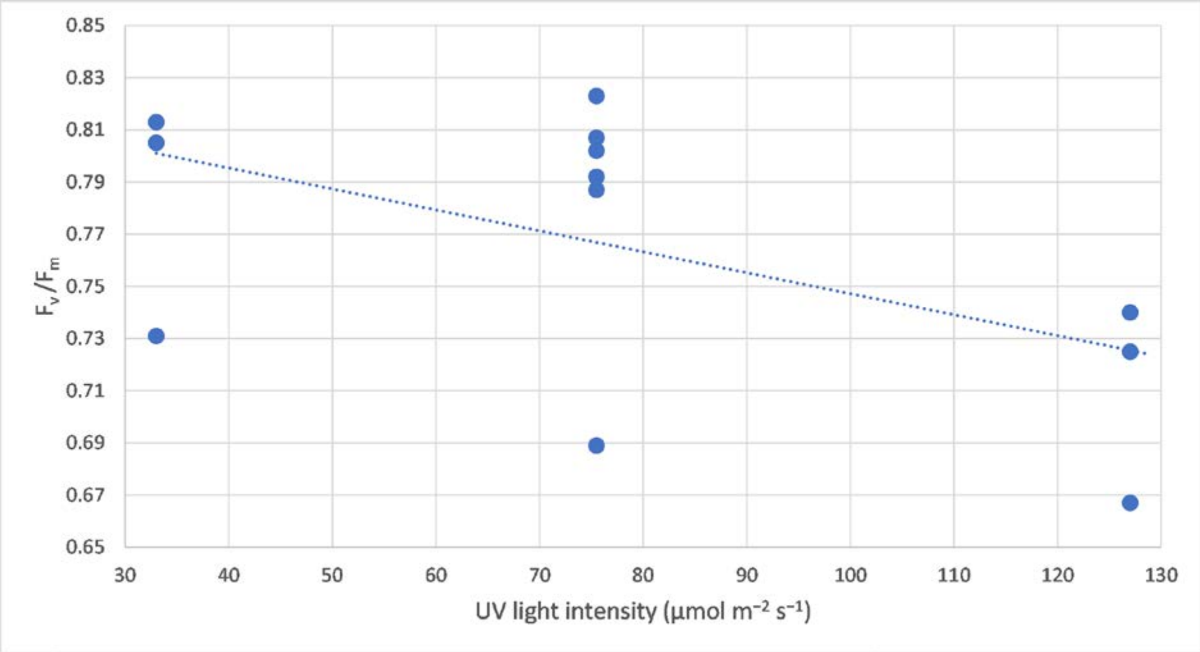

Figure 2: The relationship between UV light intensity and F_v/F_m of *R. continentis* leaves across the four different sites. Each dot represents a single sample's F_v/F_m value.

We also found a negative correlation between anthocyanin content and F_v/F_m, which means that F_v/F_m of *R. continentis* leaves increased as anthocyanin content of the leaves decreased (Figure 3). A linear regression was performed on the data using JMP, resulting in a *p*-value of 0.0262. The relationship between anthocyanin content and F_v/F_m of *R. continentis* leaves is statistically significant, as $0.0262 < 0.05$.

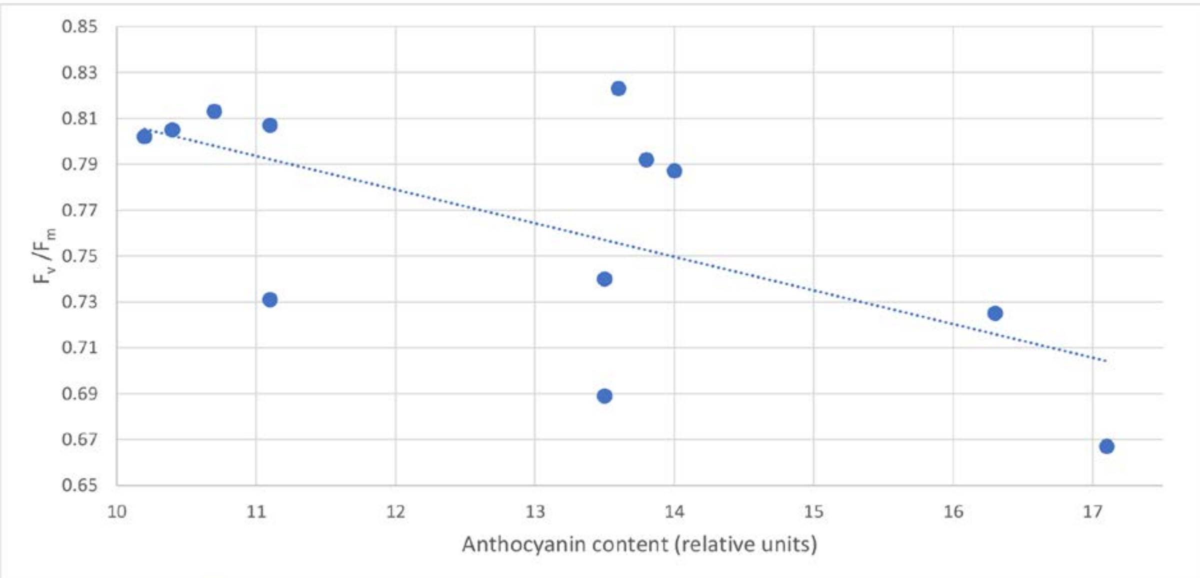

Figure 3: The relationship between anthocyanin content and F_v/F_m of *R. continentis* leaves. Each dot represents a single sample's anthocyanin content.

Discussion

The results of this study supported the first half of the hypothesis – there was a positive correlation between anthocyanin content in *R. continentis* leaves and UV light intensity. This relationship was found to be significant. This finding is consistent with previous research. Past studies have found that anthocyanin synthesis is photoinducible – stimulated by the presence of light (Chalker-Scott 1999). Barnes *et al.* (2000) concluded that UV-B radiation in particular significantly increases the amount of anthocyanin present in plant leaves.

Analysis of F_v/F_m of *R. continentis* leaves showed no significant relationship between UV light intensity and F_v/F_m. Thus, it cannot be concluded that UV light causes damage to *R. continentis* leaves. There was, however, a negative correlation between the two factors, with leaf health (represented by F_v/F_m) decreasing as UV light intensity increases. This finding is supported by previous research. UV light has been shown to contribute to plant stress (Korner 2003), and Tian-Shan (2017) found that F_v/F_m tends to decrease as a plant is exposed to higher levels of stress. It is possible that with a larger sample size the correlation would become significant.

There was a significant correlation between F_v/F_m and anthocyanin content of *R. continentis* leaves. Anthocyanin content was higher in more damaged leaves (those with lower F_v/F_m). This could mean, as Bilger *et al.* (2007) suggested, that anthocyanins are not very efficient at protecting leaves from UV exposure, or that increased stress triggers the production of anthocyanins. However, it must be noted that even the lowest F_v/F_m value in the data set (0.67) is not far outside the healthy range of 0.77–0.81 (Tian-Shan 2017). This, together with the fact that the correlation between UV light intensity and F_v/F_m was not significant, suggests that anthocyanin does play a role in photoprotection. It is possible that its increased concentration in leaves exposed to higher UV levels prevents some but not all UV damage to leaves.

As a result of increases in temperature due to climate change, snow depth and coverage in the alpine regions are expected to be reduced dramatically (Thompson 2016; Worboys and Good 2011). The snow is also likely to thaw earlier (Worboys and Good 2011). As a result, alpine plants such as *R. continentis* that are usually protected from light damage during the winter months by snow will be exposed to more UV light per year. One of the roles of anthocyanins in leaves is photoprotection (Hernández *et al.* 2009). If anthocyanin content is positively correlated with UV light intensity, it is not unreasonable to predict that more sun exposure (due to lack of snow cover) will lead to increased anthocyanin production as global warming progresses.

Increased anthocyanin production could be beneficial for alpine plants. Anthocyanins are antibacterial agents, help prevent leaf freezing and have antioxidant properties (Agati *et al.* 2013; Agati *et al.* 2009; Barnes *et al.* 2000). Therefore, effects of increased anthocyanin production could be increased pathogen resistance and overall plant resilience. Higher anthocyanin content could also help alpine plants survive extremely low temperatures that are likely to result from lack of snow cover (Korner 2003; Worboys and Good 2011). Their role in releasing excess energy could increase plant heat tolerance, a trait that would be vital as temperatures rise (Hernández and Van Breusegem 2010). However, it is also possible that increased synthesis of anthocyanins could compete with other biosynthesis reactions, so future studies should test whether increased anthocyanin response is correlated with loss of biomass.

The findings of this study are important because alpine regions are expected to be more quickly and more severely affected by climate change than lowland regions (Thompson 2016). Thus, it is vital to understand how alpine flora will respond to changes in the environment if these plant species are to be protected. This study suggests that at least some plants may be able to adapt to the changing conditions.

However, more research is required in this area. This was a short-term study that investigated one plant species in one general location (Charlotte Pass). Only 12 samples were used, and the method of quantifying leaf anthocyanin content was not very accurate. From these data, it is difficult to predict how other species may react to increasing UV levels, or even if *R. continentis* in other regions will show the same correlations. This study could be improved by using high-pressure liquid chromatography to determine the amount of anthocyanins in leaves. A larger sample size with more locations across the Australian Alps could improve the generalisability of the data. Additionally, a longer study could be conducted, monitoring UV light intensity for several years, to determine whether anthocyanin production changes in response to rapid changes in UV levels or to longer-term patterns.

Further studies could look at the relationship between UV light and anthocyanins in other alpine species or other plant functional types (trees vs shrubs, for example). This would give a better indication of how alpine plants may cope with the challenges of climate change more generally. The relationship between UV light and anthocyanins in alpine and lowland plants could be compared. It is possible that alpine plants, which are adapted to rapid weather changes, may be better equipped to deal with the changing climate than lowland plants.

Research into the relationship between F_v/F_m and anthocyanin content could also be done. It may also be beneficial to compare plant samples exposed to the same amounts of UV light but with different anthocyanin content when grown under the same conditions (e.g. in a glasshouse). This could determine whether leaves with higher concentrations of anthocyanins suffer less light damage than those with low anthocyanin concentrations when exposed to UV light of the same intensity.

Conclusions

In conclusion, the hypothesis was supported by the results of the experiment. Anthocyanin content in *R. continentis* leaves increased as UV light intensity increased. At the same time, the negative correlation between UV light intensity and F_v/F_m was not significant. This suggests that anthocyanins protect chlorophyll within leaves from light damage and that alpine plants may be able to adapt to increased UV light exposure due to climate change by increasing their anthocyanin production.

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