Plastic responses to environmental stressors: Biosynthesis of anthocyanins increases in *Eucalyptus pauciflora* and *Richea continentis* with elevation

Gregory Gauthier-Coles, Cynthia Turnbull, Ray Zhang, Tanja Cobden

Abstract

As a subclass of the flavonoid family of secondary metabolites, anthocyanins have been studied closely in recent years for their multitudinous protective properties in plants. Described as 'nature's Swiss army knife', anthocyanins have putative antioxidant, anti-inflammatory, anti-microbial, anti-cancer, photo-protective and colligative characteristics, which have made them a subject of much scientific interest. Anthocyanins are the primarily expressed group of flavonoids in angiosperms such as *Eucalyptus pauciflora* and *Richea continentis*. These species of plants are widely found in the Australian alpine environment of the Kosciuszko National Park, a region that is particularly susceptible to climate change. In studying anthocyanin concentration in the leaves of *E. pauciflora* and *R. continentis* in parts of the Kosciuszko National Park, anthocyanin concentration was found to positively correlate with elevation. In accordance with previous scientific research, anthocyanin accumulation greatly increased in leaves showing evidence of pathogen attack. In spite of methodological limitations, results of this study support the notion of plasticity in the expression of enzymes involved in anthocyanin biosynthesis. Furthermore, these findings may have implications in climate change modelling in relation to plant species distribution of the Australian alpine region and in conservation ecology.

Introduction

The Australian alpine and subalpine environment is characterised by several species of plants, most notably *Eucalyptus pauciflora*, otherwise known as the snow gum (Figure 1A). This robust species of the Australian flora has a large altitudinal range (Boland and McDonald 2006)—they can be found at sea level and make up the alpine tree line in the Australian Alps (William and Potts 1996). *E. pauciflora*'s ability to grow and even thrive in relatively inhospitable alpine and subalpine environments is not only due to its cold hardiness but its tolerance for drought and other stressors (Close et al. 2010). Indeed, the same can be said for many plant species in these environments, such as the much less studied *Richea continentis* (Figure 1B).

The properties that allow such species of plants to exist at high elevation can be explained by important morphological and physiological differences. For example, the seedlings of *E. pauciflora* have been observed to predominantly grow and regenerate on the southern side of the tree. This protects the seedlings from high levels of sunlight and frost damage (Ball et al. 1991). *E. pauciflora* also has a significant capacity for acclimation in its stomatal and photosynthetic responses to changes in temperature and illumination (Körner and Cochrane 1985). The production of biomolecules that have the necessary protective qualities for the survival of plants in alpine and subalpine regions is another example of a set of adaptations that are found in plants like *E. pauciflora* and *R. continentis.* One such group of biomolecules is the flavonoid family of secondary metabolites.

Flavonoids comprise more than 9,000 metabolites, and were an essential biochemical innovation in plants that, well over 500 million years ago, had left the marine environment and needed to adapt to, and endure, severe terrestrial stressors such as higher UV irradiation and greater temperature extremes (Williams and Grayer 2004; Mouradov and Spangenberg 2014). These biomolecules share a common metabolic pathway. The first step in flavonoid synthesis involves the production of aromatic amino acids, such as phenylalanine, using the shikimate pathway. The resulting products of this pathway are then deaminated, hydroxylated and decarboxylated, and a reaction involving three molecules of malonyl-coenzyme A results in the biosynthesis of the first flavonoid, producing either a chalcone or a stilbene (Grotewold 2006). The next step for the majority of flavonoids involves the formation of a C ring, and many subsequent reactions lead to the large diversity of these biomolecules (Hernández and Van Breusegem 2010).

Figure 1: Plant species examined in this study growing in the Kosciuszko National Park: A) *Eucalyptus pauciflora*, B) *Richea continentis*.

Source: Authors' photographs.

A feature of flavonoids is that, unlike proteins, they do not contain nitrogen. Furthermore, due to the many reaction pathways necessary, their synthesis consumes significant amounts of energy. This can be useful under situations, for example, cold temperatures and high light, in which the light reactions of photosynthesis produce a lot of ATP and NADPH; however, the Calvin cycle operates slowly, leading to a buildup of this ATP and NADPH. The flavonoid biosynthesis pathway requires some of the same precursors as the Calvin cycle and uses large amounts of ATP and NADPH. Therefore, it has been hypothesised that flavonoids act as 'energy escape valves' when the Calvin cycle operates slowly (Hernández and Van Breusegem 2010).

This mechanism is considered useful in instances where the photosynthetic machinery is operational to a disproportionate degree to that of the Calvin cycle reactions. The bifurcation found in the glycolysis pathway allows for glyceraldehyde-3-phosphate and dihydroxyacetone phosphate metabolites to be diverted to the shikimate pathway; the photosynthesis reactions can therefore be uncoupled from the Calvin cycle. This prevents the accumulation of the photosynthetic products ATP and NADPH, and in a way that does not exploit nitrogen reserves, which can be strained in certain conditions (Shirley 1996): the upregulation of enzymes involved in flavonoid biosynthesis requires nitrogen in the form of amino acids. This phenomenon may occur in situations where the leaves of a plant are exposed to certain abiotic stressors, such as high illumination and cold temperatures.

One of the most important subclasses of metabolites belonging to the flavonoid family in angiosperms is that of the anthocyanins. Comprising some 500 different structures, anthocyanins are multifunctional chemicals in plants characterised by a host of qualities (Guo et al. 2008). One of these is the ability to pigment the leaves and flowers of plants. Anthocyanins also reflect blue-green and ultraviolet light and therefore act as sunscreens in plants, specifically in palisade and spongy mesophyll cells (Tattini et al. 2005). This is very important in allowing plants to avoid photo-oxidative stress, specifically to photosynthetic machinery when it is exposed to high solar radiation. Stratmann et al. (2007) have partially elucidated the mechanisms by which ultraviolet radiation activates signals that recruit the transcription apparatus for certain structural genes (i.e. those coding for enzymes involved in anthocyanin biosynthesis). This appears to be

an example of physiological plasticity in plants. The sunscreen effect, however, is not the only photo-protective feature of anthocyanins, as they are also potent antioxidants (Rozema et al. 1997).

Reactive oxygen species (ROS) are the products of many biological impacts, such as photo-oxidation. Hydrogen peroxide and superoxide radicals are ROS that are readily created as a result of exposure to high solar radiation, and can react together to form hydroxyl radicals and anions as part of the Haber-Weiss reaction (Haber and Weiss 1932). These products cause serious damage to cellular components including DNA. This same reaction can be catalysed by transition metal ions, namely ferric ions—this is known as the Fenton reaction (Fenton 1894). Since ferric ions naturally occur in various parts of plant cells (including the nucleus), serving a variety of physiological roles, the production of ROS via the Fenton reaction is a serious problem. Flavonoids, including anthocyanins located in the nucleus, exert an anti-oxidative effect indirectly by chelating transition metals, thereby preventing their involvement in Fenton reactions (Melidou et al. 2005). Although this may not be an example of direct anti-oxidative action, vacuolar flavonoids (mostly anthocyanins and proanthocyanidins) have true antioxidant capacities (Hernandez et al. 2009). When the vacuole that contains these flavonoids is breached due to some form of mechanical injury (such as feeding by insect or animal herbivores), anthocyanins and proanthocyanidins are released and neutralise ROS by donating their electrons (Gould et al. 2002).

There is also some evidence that anthocyanins promote cold hardiness in plants that accumulate them; however, this remains an area of contention (Chalker-Scott 1999). Although not fully understood, Christie et al. (1994) found that the anthocyanin production pathway involves cold regulation, or *cor*, genes. It therefore seems that anthocyanin biosynthesis may be induced by cold temperature, but it is not understood if this has an explicit function (i.e. conferring frost resistance in plant tissues). One hypothesis states that anthocyanins, acting as solutes, have an osmotic effect that lowers the freezing point of water in vacuoles (Chalker-Scott 1999). Another hypothesis, not necessarily mutually exclusive with the former, suggests that anthocyanin production is induced by cold temperatures due to the fact that ROS are longer lived in such circumstances (Kramer et al. 1991). This proposal is further supported by evidence that anthocyanin biosynthesis is prevented in cold conditions where visible or UV-B light is absent (Janda et al. 1996; Oren-Shamir and Levi-Nissim 1997).

Anthocyanins are involved in osmotic stress conditions, not only occurring in plants subjected to cold temperatures but also drought. Many plants that are capable of withstanding drought express high levels of anthocyanins. Bahler et al. (1991) found that cultivars of pepper that expressed far greater quantities of anthocyanins (and were purple in colour as a result) had a greater tolerance for drought conditions. *Craterostigma wilmsii* and *Xerophyta viscosa* were also observed to have far higher concentrations of anthocyanins in their roots under drought conditions as opposed to when the plants were fully hydrated.

In summarising the well-established and speculated functions and 'cross-resistances' of anthocyanins, the term 'Swiss army knife' assigned by Kevin S. Gould seems appropriate for such a group of biomolecules (Gould et al. 2002). Furthermore, many of the properties of anthocyanins clearly show a capacity for plasticity. From the biosynthetic pathways that are photo-inductive to those that involve *cor* genes, expression of gene encoding anthocyanin biosynthesis enzymes is determined by numerous genetic and environmental factors. Following on from previous research reported in the literature, the aim of this study was to investigate changes in the anthocyanin concentration in the leaves of *E. pauciflora* and *R. continentis* at different elevation. Given the evidence for plasticity in anthocyanin biosynthesis in response to colder temperatures and increased UV light intensities, it was hypothesised that the anthocyanin concentrations in both these species would increase with elevation in the Australian Alps.

Materials and methods

Sampling of *E. pauciflora* and *R. continentis*

Five samples of *E. pauciflora* and of *R. continentis* were collected at different elevations (range: 1,600 to 2,000 m) in the Charlotte Pass and Mount Stillwell area. Each sample consisted of three replicates (i.e. three branches were selected from the same plant sample). Another *E. pauciflora* sample was taken to analyse anthocyanin expression in leaves that showed evidence of previous damage from herbivory and/or pathogen(s) (Figure 2). Samples were picked, placed in bags and labelled with time of day, elevation and temperature. Elevation was estimated based on information from topographic maps, and temperature determined using an alcohol thermometer. Samples were not selected based on aspect

(exposure to sunlight); however, leaves damaged due to herbivory or the presence of obvious pathogens were excluded. Two samples of *R. continentis* were selected at the same elevation; however, one was found growing adjacent to a snow patch whereas the other was found growing without surrounding snow.

Preparation for thin layer chromatography

One leaf of each *E. pauciflora* and two leaves of each *R. continentis* were weighed and then ground with approximately 0.5 g of glass powder in a mortar and pestle. Because anthocyanin content was clearly different in leaves of different developmental stages (Figure 2), especially for *E. pauciflora*, we always collected the third youngest leaf from each branch. For the leaf showing signs of damage from herbivores and/or pathogens (Figure 2C), which was of a mature stage, we used a mature *E. pauciflora* leaf as a control. The pulverised leaves were then deposited in Eppendorf tubes and an ethanol–water mixture (70:30 v/v) was added. Each solution was then centrifuged for 2.5 minutes. The amount of solvent added related to the mass of the leaves used in each replicate and is shown in the following equation (where V is the volume of solvent and m is the mass of leaf extract):

$$V = m \times 2$$

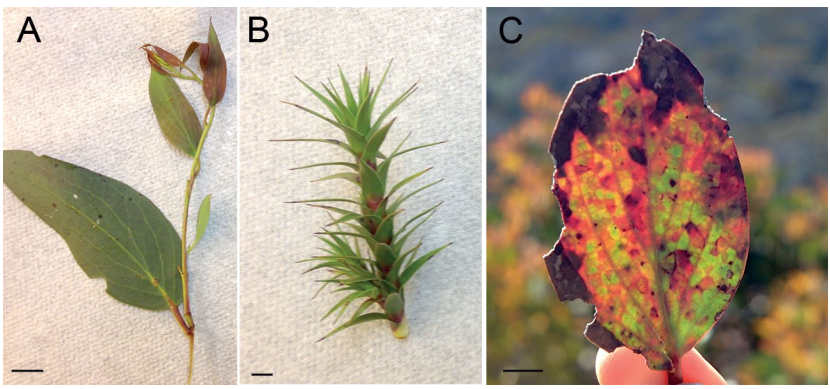

Figure 2: Leaves of *Eucalyptus pauciflora* and *Richea continentis* analysed for anthocyanin content: A) *E. pauciflora*, B) *R. continentis*, C) *E. pauciflora* leaf with obvious damage and anthocyanin accumulation from herbivore and/or pathogen damage.

Note: Magnification bar represents 2 cm in panels A and B and 1.2 cm in C.

Source: Authors' photographs.

Thin layer chromatography

The supernatant of each tube was spotted onto silica-coated thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany) in a way that all three replicates of each sample were allocated to a given plate. A consistent volume of solution was added to each spot; a content equivalent to two 5 cm lengths of a glass capillary tube, 1 mm diameter, was deposited. These spots were placed along a line that was 1 cm above the edge of the plate. After around 5 minutes of drying time, plates were placed in a running buffer solution prepared with ethyl acetate, distilled water, acetone and formic acid (18:18:3:1) for approximately 10 minutes. Plates were then removed and allowed to dry for a further 10 minutes.

Determination of anthocyanin density

One photograph was taken for all the *E. pauciflora* plates and one for all the *R. continentis* plates, under the same illumination, using an iPhone 6 camera. The images were then analysed using ImageJ software. For each photograph, colours were inverted to allow the anthocyanin band to be demarcated. The anthocyanin band was clearly visible as a pink band at the bottom of each plate. A standardised surface area was then used for each photograph and the bands of anthocyanin highlighted. The selected bands were then analysed and an integrated density value obtained for each band. A background integrated density value was also determined (based on a selection of an area of a TLC plate that was blank and unstained) and a net integrated value was determined by subtracting the background integrated density from the anthocyanin band integrated density.

Data analysis

The net integrated densities were averaged for each sample and standard deviations were obtained. Linear regressions tests were then used for the *E. pauciflora* data set and the *R. continentis* data set. A t-test was used for the *E. pauciflora* pathogenic vs non-pathogenic data set, and to compare the *R. continentis* samples from the same elevation differing by proximity to snow. All data analysis was conducted using Microsoft Excel.

Results

Separation of plant pigments from *Eucalyptus pauciflora* and *Richea continentis* leaves using thin layer chromatography showed a clear separation of anthocyanins from other pigments, including chlorophylls and carotenoids. Quantification of the relative band intensities using ImageJ showed that there was a significant positive correlation between elevation and anthocyanin content in both *E. pauciflorus* (Figure 3A) and *R. continentis* (Figure 3B).

An additional observation made during the collection of leaves was that some leaves that showed signs of attack by herbivores and/or pathogens showed intense red pigmentation (Figure 2C). We therefore compared the relative anthocyanin content of a diseased and a healthy leaf, both of a mature developmental stage. As shown in Figure 4, there was a significant increase in anthocyanin content in the diseased leaf.

When collecting samples of *R. continentis*, we observed strong red pigmentation in leaves of specimens growing close to a patch of snow, as opposed to specimens growing in areas without snow at the same elevation (2,000 m). A quantitative comparison of relative anthocyanin content of leaves from each plant showed a higher anthocyanin content in the leaves of the plant growing close to the snow patch compared to the plant growing in a snow-free area. However, this difference was not statistically significant (P>0.05; Figure 5).

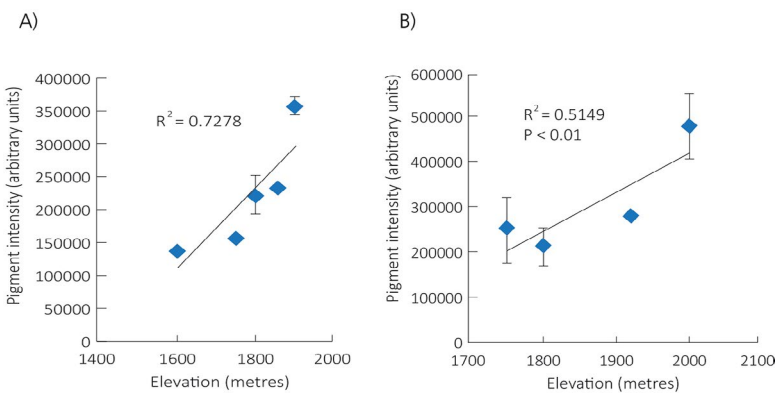

Figure 3: Relationship between elevation and relative anthocyanin concentration in leaves of A) *Eucalyptus pauciflora* and B) *Richea continentis*, analysed by linear regression.

Note: Data points indicate means and standard errors, n=3.

Source: Authors' data.

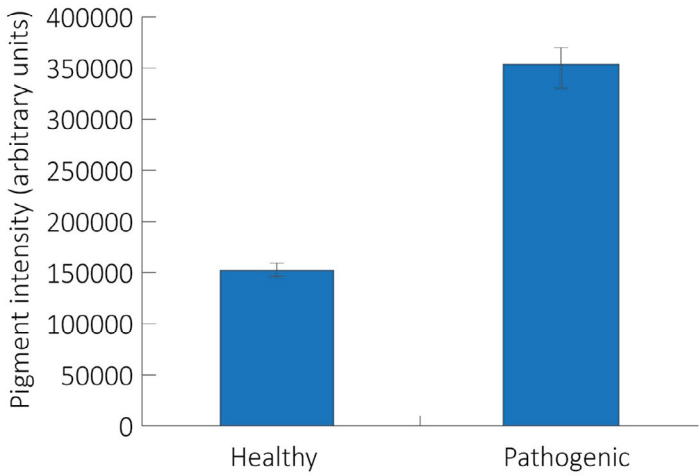

Figure 4: Relative anthocyanin concentrations of healthy vs pathogen-infected *Eucalyptus pauciflora* leaves, $P<0.001$ (tailed, two sample homoscedastic t-test). Data show means and standard errors of three individual leaves.

Source: Authors' data.

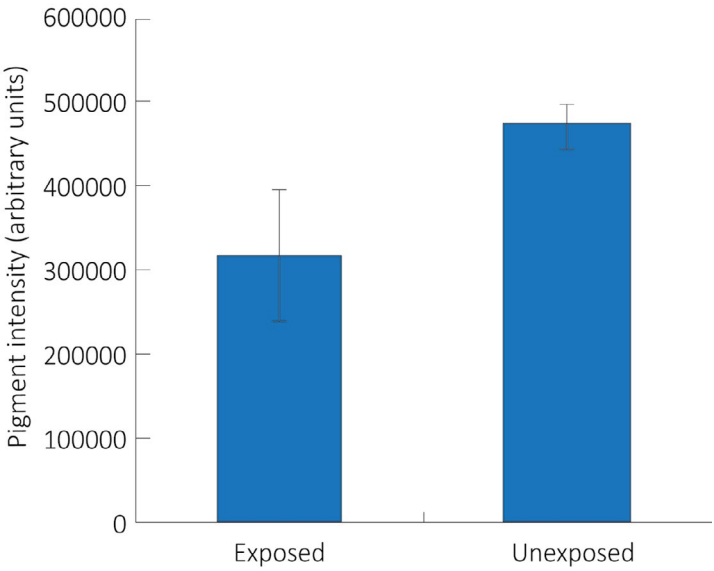

Figure 5: Relative anthocyanin concentrations of *Richea continentis* leaves growing in an exposed area without snow or in an unexposed area close to a snow patch, $P>0.05$ (tailed, two sample homoscedastic t-test). Data show means and standard errors of three individual leaves.

Source: Authors' data.

Discussion

In investigating anthocyanin expression in the leaves of *E. pauciflora* and *R. continentis* at different elevation, the hypothesis that a positive correlation exists between these two factors was supported. The prediction that anthocyanin concentration would be greater in a pathogen-infected *E. pauciflora* compared to a healthy leaf was also supported. The prediction that *R. continentis* leaves growing in an unexposed area compared to those growing in an exposed area at the same elevation could not be supported. These results are largely in keeping with the literature, which finds that plants do produce higher concentrations of anthocyanins at lower temperatures (and, therefore, as elevation increases) (Kramer et al. 1991).

Despite the fact that the R^2 values of the linear regressions performed for each plant against elevation were not very high, the low *P*-values suggest that a relationship between these two variables exists and that further studies should be carried out. Apart from increasing the sample sizes for each experiment (anthocyanin production vs elevation; anthocyanin concentration in pathogen vs healthy *E. pauciflora* leaves; and anthocyanin concentration in exposed vs unexposed *R. continentis* leaves), this experiment could have been improved in several ways. For instance, we did not control for the level of exposure to sunlight in plants. A thermometer was used, but the data was discarded as only one measurement was taken for each sample site at different times of the day. The use of elevation as the independent variable, and as a substitution for temperature changes, may not have been reliable since it did not control for discrepancies in microclimates. For example, the Charlotte Pass area lies in a valley that acts as a cold sink. Temperatures in this valley are frequently below those at higher elevation, which may have affected the way the sampled plants expressed anthocyanins in their leaves. Although the anthocyanin band on the TLC plates was fairly easily identifiable, no standards were used and it is possible that other phytochemicals with similar polarity aggregated at the same level as the anthocyanins. This problem, combined with the use of the ImageJ program, was not the ideal method of determining anthocyanin content in the leaves.

The methodology employed could have therefore been improved in several ways. Sampling could have been selected over a large vertical range and in different areas of the Kosciuszko National Park. This would help control for microclimatic changes at different elevations. Temperature data could

have been used if multiple recordings were taken at each sample site and over several days (preferably over a year-long period). Likewise, the use of an illuminometer may have helped control for sun exposure; however, readings would have needed to be taken at different times of day and over several weeks. Anthocyanin standards could have been used in the TLC assays but an even better analytical technique would comprise the use of high pressure liquid chromatography coupled with mass spectrometry. This method would allow a more precise concentration to be determined, and would elucidate on the ratio of individual anthocyanin constituents.

Although this study had some serious methodological limitations, some important implications can be drawn. The data showed significant plasticity in how these plants express anthocyanins in response to altitudinal gradients. Plasticity was also observed in plant leaves that were infected, whereby anthocyanin concentration is vastly increased in the presence of pathogens. There was also some evidence (albeit statistically insignificant) that suggested that *R. continentis* produces higher levels of anthocyanins close to snow, which may have enhanced light exposure due to reflectance from the snow, and this should be investigated further. The plastic nature of anthocyanin expression in these plant species has implications in climate change research, especially modelling involved in plant distributions in the Australian Alps. These findings and, more broadly, the area of research concerning the production of anthocyanins and other flavonoids may be of interest to conservationists involved in restoration efforts. For instance, plants that are capable of expressing high levels of anthocyanins in response to a variety of stressors (i.e. drought, frost, physical damage) may be good candidates for environmental rehabilitation projects.

Future research should primarily consist of further investigating the aim of this study but in a way that controls for the aforementioned variables and with much greater sample sizes. A study with a high level of ecological validity would share a similar design to the experiment outlined in this report, but would be conducted over a year to investigate seasonal differences in anthocyanin concentration of leaves. Alternatively, many variables could be more easily controlled for in a laboratory setting. A multifactorial laboratory experiment could assess anthocyanin concentrations in *E. pauciflora* and *R. continentis* by individually manipulating temperature and luminescence. Although the idea of anthocyanins acting as super-coolant molecules is a controversial one, and despite the fact that this was not observed in the 'exposed vs unexposed' *R. continentis*, a future

experiment could test for frost avoidance by using anthocyanin structural gene knockouts and wild-type plants at sub-zero temperatures and search for frost damage. These gene knockout experiments could also target the *cor* genes that are suspected to be involved in the expression of enzymes that participate in anthocyanin biosynthesis. Field trials could also be designed to test how well plants that produce high levels of anthocyanins (like *E. pauciflora* and *R. continentis*) help rehabilitate areas damaged by or susceptible to climate change and other destructive human activities.

Acknowledgements

We would like to thank Professor Ulrike Mathesius from the Research School of Biology at The Australian National University for her invaluable contributions to this study.

References

Bahler B, Steffen K, Orzolek M (1991) Morphological and biochemical comparison of a purple-leafed and green-leafed pepper cultivar. *HortScience* 26: 736.

Ball M, Hodges V, Laughlin G (1991) Cold-induced photoinhibition limits regeneration of snow gum at tree-line. *Functional Ecology* 5: 663–8. doi.org/10.2307/2389486

Boland D, McDonald M (2006) *Forest trees of Australia*. CSIRO Publishing, Collingwood.

Chalker-Scott L (1999) Environmental significance of anthocyanins in plant stress responses. *Photochemistry and Photobiology* 70: 1–9. doi.org/10.1111/j.1751-1097.1999.tb01944.x

Christie P, Alfenito M, Walbot V (1994) Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: Enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta* 194: 541–9. doi.org/10.1007/BF00714468

Close D, Davidson N, Churchill K, Corkrey R (2010) Establishment of native *Eucalyptus pauciflora* and exotic *Eucalyptus nitens* on former grazing land. *New Forests* 40: 143–52. doi.org/10.1007/s11056-010-9189-9

Fenton H (1894) LXXIII. Oxidation of tartaric acid in presence of iron. *Journal of the Chemical Society, Transactions* 65: 899–910. doi.org/10.1039/CT8946500899

Gould K, McKelvie J, Markham K (2002) Do anthocyanins function as antioxidants in leaves? Imaging of H_2O_2 in red and green leaves after mechanical injury. *Plant, Cell and Environment* 25: 1261–9. doi.org/10.1046/j.1365-3040.2002.00905.x

Grotewold E (2006) *The science of flavonoids*. Springer Publishing, New York. doi.org/10.1007/978-0-387-28822-2

Guo J, Han W, Wang M (2008) Ultraviolet and environmental stresses involved in the induction and regulation of anthocyanin biosynthesis: A review. *African Journal of Biotechnology* 7: 4966–72.

Haber F, Weiss J (1932) Uber die Katalyse des Hydroperoxydes. *Die Naturwissenschaften* 20: 948–50. doi.org/10.1007/BF01504715

Hernández I, Alegre L, Van Breusegem F, Munné-Bosch S (2009) How relevant are flavonoids as antioxidants in plants? *Trends in Plant Science* 14: 125–32. doi.org/10.1016/j.tplants.2008.12.003

Hernández I, Van Breusegem F (2010) Opinion on the possible role of flavonoids as energy escape valves: Novel tools for nature's Swiss army knife? *Plant Science* 179: 297–301. doi.org/10.1016/j.plantsci.2010.06.001

Janda T, Szalai G, Páldi E (1996) Chlorophyll fluorescence and anthocyanin content in chilled maize plants after return to a non-chilling temperature under various irradiances. *Biologia Plantarum* 38: 625–7. doi.org/10.1007/BF02890623

Körner C, Cochrane P (1985) Stomatal responses and water relations of *Eucalyptus pauciflora* in summer along an elevational gradient. *Oecologia* 66: 443–55. doi.org/10.1007/BF00378313

Kramer G, Norman H, Krizek D, Mirecki R (1991) Influence of UV-B radiation on polyamines, lipid peroxidation and membrane lipids in cucumber. *Phytochemistry* 30: 2101–8. doi.org/10.1016/0031-9422(91)83595-C

Melidou M, Rignakos K, Galaris D (2005) Protection against nuclear DNA damage offered by flavonoids in cells exposed to hydrogen peroxide: The role of iron chelation. *Free Radical Biology and Medicine* 39: 1591–600. doi.org/10.1016/j.freeradbiomed.2005.08.009

Mouradov A, Spangenberg G (2014) Flavonoids: A metabolic network mediating plants adaptation to their real estate. *Frontiers in Plant Science* 5: 620. doi.org/10.3389/fpls.2014.00620

Oren-Shamir M, Levi-Nissim A (1997) UV-light effect on the leaf pigmentation of *Cotinus coggygria* 'Royal Purple'. *Scientia Horticulturae* 71: 59–66. doi.org/10.1016/S0304-4238(97)00073-3

Rozema J, van de Staaij J, Björn L, Caldwell M (1997) UV-B as an environmental factor in plant life: Stress and regulation. *Trends in Ecology and Evolution* 12: 22–8. doi.org/10.1016/S0169-5347(96)10062-8

Shirley B (1996) Flavonoid biosynthesis: 'New' functions for an 'old' pathway. *Trends in Plant Science* 1: 377–82.

Stratmann, J, Stelmach B, Weiler E, Ryan C (2007) UVB/UVA radiation activates a 48 kDa myelin basic protein kinase and potentiates wound signaling in tomato leaves. *Photochemistry and Photobiology* 71: 116–23. doi.org/10.1562/0031-8655(2000)0710116SIPUUR2.0.CO2

Tattini M, Guidi L, Morassi-Bonzi L, Pinelli P, Remorini D, Degl'Innocenti E, Giordano C, Massai R, Agati G (2005) On the role of flavonoids in the integrated mechanisms of response of *Ligustrum vulgare* and *Phillyrea latifolia* to high solar radiation. *New Phytologist* 167: 457–70. doi.org/10.1111/j.1469-8137.2005.01442.x

Williams CA, Grayer R (2004) Anthocyanins and other flavonoids. *ChemInform* 35: 539–73. doi.org/10.1002/chin.200447250

Williams K, Potts B (1996) The natural distribution of *Eucalyptus* species in Tasmania. *Tasforests* 8: 39–165.

This text is taken from *Researching functional ecology in Kosciuszko National Park*, edited by Hannah Zurcher, Chia Ming-Dao, Michael Whitehead and Adrienne Nicotra, published 2017 by ANU eView, The Australian National University, Canberra, Australia.

dx.doi.org/10.22459/RFEKNP.11.2017.03