# Variable responses of alpine grassland plant species to drought in Kosciuszko National Park

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## Abstract

Understanding how plant species in alpine Australia will respond to climate change is vital for the future management of these ecosystems. Plant and leaf traits can provide key insights into how an individual is responding to environmental stresses such as reduced water availability. By assessing traits across species and functional groups, a picture can begin to develop about whole community responses to drought stress. This study recorded a variety of traits for six alpine plant species across three functional groups to understand how plant stress responses may influence shifts in community composition in the Australian Alps. The results of this study varied greatly between species and functional groups, highlighting the need for further longer-term studies to provide a more comprehensive understanding of species-specific responses.

## Introduction

Attempting to predict the effects of climate change on global ecosystems is of great interest within the worldwide scientific community. The coming decades are expected to bring more extreme weather events and changes in weather regimes that have been somewhat reliable until recently (Hennessy et al., 2007). In higher alpine areas such as the Mt Kosciuszko region, higher temperatures and shifts in precipitation regimes are expected, resulting in changes in water availability (Björk and Molau, 2007; Morrison and Pickering, 2013; Mondoni et al., 2022; Ji et al., 2022). Kosciuszko National Park has been a location of focus for much of Australia's research into the effects of climate change on alpine ecosystems. With high levels of endemism, many alpine plant species are highly specialised, creating uncertainty around their ability to survive in and adapt to an environment that is rapidly changing (Björk and Molau, 2007). Research on alpine plant responses to decreased water availability and water stress can provide key insights into the capacity of these plants to tolerate predicted future climates. This knowledge is essential to understand and predict shifts in community composition and function in the future (Raddi et al., 2022).

There is great interest in determining not only how different species respond to these climatic changes, but also how different growth forms respond. As different growth forms (i.e., shrubs, herbs, graminoids) play different functional roles within their communities, it is important to understand how changes in community assemblage could shift the functionality of that community (Ackerman et al., 2017; Duchicela et al., 2021; Steinbauer et al., 2022; Wang et al., 2023). A study by Scherrer and Pickering (2005) in Kosciuszko National Park found that graminoids recovered from drought quickly, in comparison to herbs which were much slower and showed decreased abundance after a drought event. In summary, studying growth form responses to drought within the same community will improve models predicting future changes in community composition.

Plants have a range of ways of responding to water stress. These responses can either be a result of trying to cope with water stress, or a plastic response to building resistance to water stress (e.g. producing smaller leaves) (Hsiao, 1973; Wang et al., 2004). Producing smaller and thicker leaves which are more robust and have lower surface areas reduces the amount of radiation received, and therefore the amount of water lost to transpiration (Fang and Xiong, 2014; Zargar et al., 2017). Palisade tissues and vascular bundles are involved in water transportation and retention, and so increasing the thickness of these tissues can provide higher capacities for water retention (Wright et al., 2004; Fang and Xiong, 2014). The presence of smaller and denser stomata is also an indicator of drought tolerance (Fang and Xiong, 2014). These are all traits that can be measured with minimal equipment and can provide key insights into plant stress responses, but we can also look deeper at how biological processes are responding.

When a plant experiences drought, responses such as the closure of stomata, thereby limiting stomatal conductance (limitation of gas exchange) will result in a reduction in photosynthetic efficiency (through less access to atmospheric $CO_2$) (Chaves et al., 2008; Zargar et al., 2017). When drought stressed plants limit their open stomata, they prevent water loss via transpiration, which can often lead to a reduction in cooling and increase in leaf temperature to potentially damaging levels (Mehri et al., 2008; Buchner et al., 2017). Measuring stomatal conductance and transpiration can provide a better understanding of how plants are responding to their environment.

Reflectance spectroscopy can provide estimations of photosynthetic pigment content in leaves, which can then be used to make postulations about whether a particular plant is experiencing drought stress, and if they have built up any resistance to this stress (Ballester et al., 2018; Burnett et al., 2021; Raddi et al., 2022). Under drought stress, the membrane system in plant cells becomes damaged or destroyed, which is thought to either directly or indirectly impact chlorophyll content in the plant (Fang and Xiong, 2014). As a result of this, chlorophyll content in a leaf can be used as a bio-indicator of whether the plant is experiencing stress (Esteban et al., 2014). Similarly, the upregulation of pigments such as anthocyanins and carotenoids can indicate a stress response, and so reflectance spectroscopy can provide insights into these changes in a non-destructive way, before other signs of stress may be visible to the naked eye (Andersen et al., 1984; Chalker-Scott, 1999; Burnett et al., 2021).

### *Specific aims*

This project aims to assess a variety of plant and leaf traits in alpine species to determine if and how these plants are responding to drought. Additionally, it aims to understand how plant responses can be used to make meaningful insights into community composition under future climate change.

It is hypothesised that when exposed to drought, plants will be affected in a variety of ways:

- Internal processes will be affected (decreased rates of transpiration, decreased stomatal conductance and decreased photosynthetic efficiency - which will lead to an inability to regulate leaf temperature, and so higher leaf temperature will be observed).
- Chemical responses will occur (upregulation of carotenoids and anthocyanins, downregulation of chlorophyll).
- Plastic responses will better equip plants for future stresses (increased leaf mass per unit area, increased stomatal density and thicker epidermis).

Additionally, it is hypothesised that shrubs will be more tolerant of drought than forbs because of their deeper roots, allowing for water acquisition at lower depths.

## Methods

### *Site and Drought Treatment*

Data was collected from six plots (three treatment and three control) previously established by the Australian Mountain Research Facility (AMRF) in Kosciuszko National Park (148.430, -36.372, 1600m a.s.l), over a period of 7 days in late November 2023. Maximum daytime temperatures over the week fluctuated from 14.0°C to 16.6°C, and 58mm of rainfall was received over the week (Bureau of Meteorology, 2023, Station 071075).

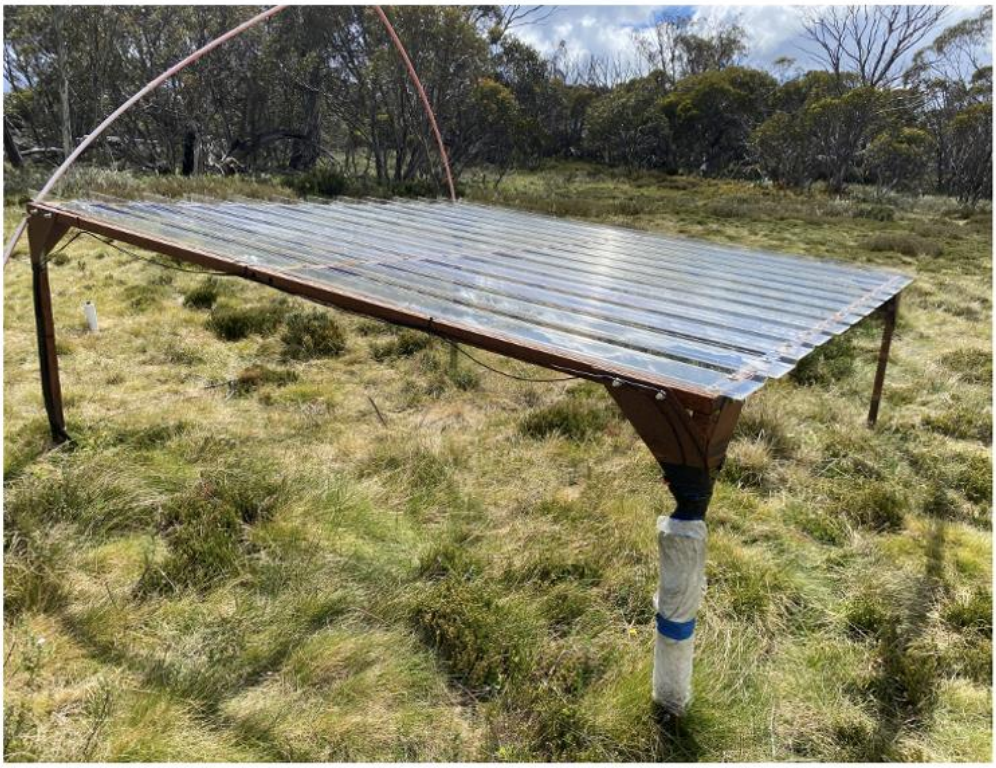

Figure 1. Drought shelters established by the Australian Mountain Research Facility, which divert 60% of incident rainfall.

The drought treatment was created by overhead rainfall interception shelters, diverting 60% of incident rainfall. Rainfall is intercepted by polycarbonate plastic panels fixed over the plots on a steel frame (Figure 1). To ensure sufficient panel water draining, shelters were designed to slope downhill (parallel to the slope they were positioned on). Each shelter covers 3 m x 3 m; research plots occupy the central 2 m x 2 m with a 50cm rainfall buffer along each edge under the shelter (Figure 2). Sampling occurred primarily within the inner 20 cm of the buffer zone to both minimise impacts on other long-term experiments being conducted within the research plots, while still ensuring drought treatments. Open-air ‘control’ plots were also established by AMRF within 10 m directly uphill of each drought shelter.  Due to study species not being present in the control plots, some sampling was conducted outside of these plots. In these cases, sampling occurred on the individual of the desired species that was the closest distance to the plots.

Soil moisture was measured with a soil moisture probe (ML3 ThetaProbe Soil Moisture Sensor, Delta-T Devices) for each plot. Measurements were taken along each edge of the central 2 m x 2 m research plots as well as in the centre of the plot (Figure 2). The five measurements were used to create an average soil moisture measurement for each plot.

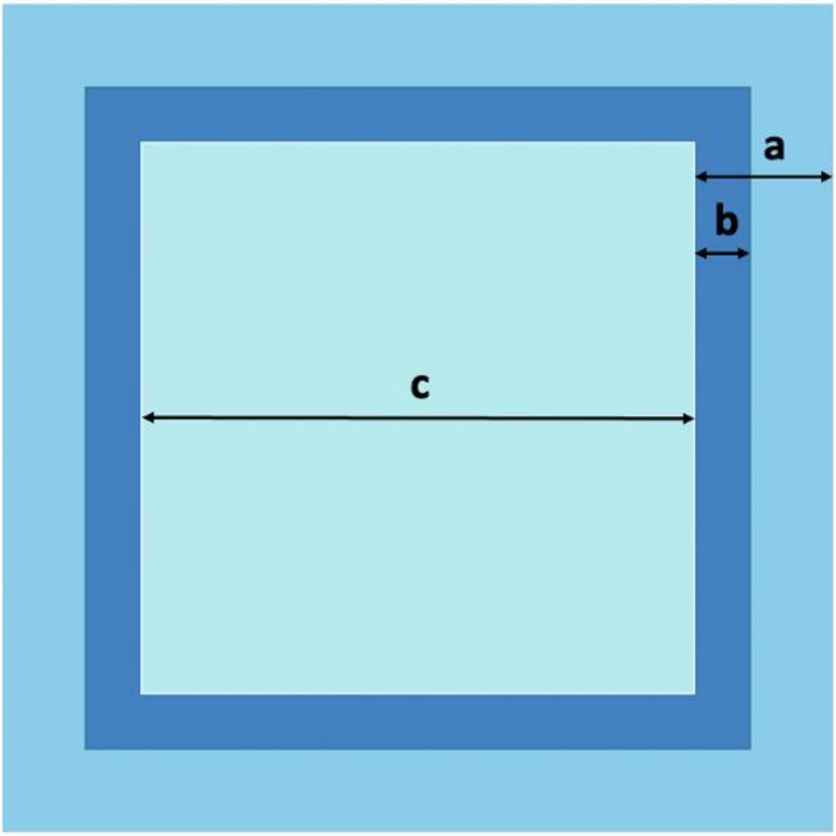

Figure 2. 3 m x 3 m drought shelter, a) 50 cm rainfall buffer zone, b) the inner 20c m of the buffer zone in which data collection primarily took place, c) inner 2 m x 2 m research plot.

## *Study Species*

Six species were selected for sampling, based primarily on their availability in the drought plots; secondarily on their presence in and around the control plots; and thirdly on their representation of different functional groups. Some species were only present in two of three drought shelters but were still included to cover a larger range of species. Species list included 1 shrub species, *Melicytus dentatus* ((DC.) Molloy and Mabb.); 1 graminoid species, *Luzula novae-cambriae* (Gand.); and 4 forb species, *Ranunculus graniticola* (Melville), *Geranium antrorsum* (Carolin), *Brachyscome decipiens* (Hook.f.), and *Craspedia sp.* (J. Everett and Joy Thomps.). Species were identified using Kosciuszko Alpine Flora (Costin et al. 2000) in the field with supplemental identification keys from PlantNet (PlantNet, 2023) as required to further classify species. *Craspedia sp.* could not be identified to a species level due to hybridisation and high plasticity within this site.

## *Leaf Traits*

A variety of leaf traits were collected from the study species, with two replications for each species within each plot, using either the two newest fully expanded leaves on one single plant, or each newest fully expanded leaf on two plants. Leaf thickness was measured as the thickest part of the leaf using precision callipers and was measured before the leaf was removed from the plant. Shortly after removing the leaf from the plant, leaf area was measured using the LeafByte app for iPhone (version 1.3.0)

(Getman-Pickering et al., 2020). Leaves were dried in a drying oven for 10 hours at 60°C, then weighed individually. Leaf dry weight (DW, g) could then be used in conjunction with leaf area (A, $m^2$) to produce leaf mass per area (LMA, $g/m^2$). LMA was calculated using the below equation:

$$LMA = \frac{DW}{A}$$

Plant height and width were recorded for each plant sampled. Plant height was measured from the base of the plant to the tallest point on the plant, excluding any temporary reproductive structures. Plant width was measured as the maximum diameter of the plant.

### *Spectroscopy*

A SpectraVue Leaf Spectrometer (CID Bio-Science Inc.) was used to measure leaf reflectance. Measurements of reflectance were taken on the upper side of the newest fully expanded leaf, avoiding measurements over the mid-rib where possible. Between three and five measurements were recorded for each species in each plot, either from the most recently expanded leaves on one plant, or across multiple plants. Reflectance data was then used to generate values for various reflectance indices (Supplementary Material: Table S1).

### *Porometry/Fluorometry*

A LI-600 Porometer/Fluorometer (LI-COR Inc.) was used to measure stomatal conductance (gsw), transpiration ($E$), leaf temperature (LT) and quantum yield of fluorescence ($\Phi PSII$) [also referred to as 'photosynthetic efficiency']. Measurements were taken on the upper side of the newest fully expanded leaf, avoiding measurements over the mid-rib where possible. Between three and five measurements were recorded for each species in each plot, either from the most recently expanded leaves on one plant, or across multiple plants. Measurements were only taken in full sun, and between 12:30 pm - 2:00 pm on a single day.

### *Stomatal Density*

Stomatal peels were collected for each leaf sample by applying a thin layer of clear nail varnish to the underside of each leaf. Once the varnish had dried, cellotape was placed over the varnish to allow for removal from the sample without damaging the peel. This tape was then applied to a microscope slide, and each slide was viewed under 400x magnification using a compound microscope. After setting the eyepiece scale, the area of the eyepiece could be determined. The number of stomata within that area were then counted, and these values used to calculate a stomatal density (number of stomata/$mm^2$) for each sample.

### *Leaf Cross Sections*

A small sample was cut from each leaf (approximately 1 cm x 1 cm) and placed onto a damp sponge inside a pillbox compartment to prevent dehydration, samples were stored in a refrigerator between collection and processing. A cross section for each leaf sample was collected using the carrot method (adapted from Destario Metusala (2017) using a carrot in place of cassava cork) and placed into a solution of methylene blue diluted with distilled water for approximately 30 seconds to allow for the stain to take effect. Cross sections were mounted onto a microscope slide and covered with a cover slip, then viewed under 400x magnification using a compound microscope. A camera mount was attached to the eyepiece and an iPhone 11 used to take between five and twenty images along the cross section of each leaf. Images were uploaded into ImageJ (Schneider et al., 2012) and the scale of each leaf thickness was set to 1 to allow for ratios of cell layers to be calculated as a proportion of the total leaf thickness.

### *Statistical Analysis*

For all traits, measurements were condensed into average values for each species in the two treatments. Data analysis was conducted in Microsoft Excel (Microsoft Corporation, 2018), where two-sample t-tests were used to test for significant differences between the drought and control treatments. The significance threshold used was $P < 0.05$. Mean values, standard deviations and sample sizes for results can be found in the supplementary material (Table S2 and Table S3).

When the data collected via fluorometry/porometry (leaf temperature, transpiration rate, stomatal conductance, and photosynthetic efficiency) was analysed, *Luzula atrata* was excluded from this portion of the analysis as most of the measurements taken for this species returned values that were deemed erroneous.

## Results

### *Soil Moisture*

Soil moisture in drought plots was significantly lower than in control plots ($P < 0.01$), confirming the efficacy of the drought treatment.

### *LMA*

*Ranunculus graniticola* was the only species to show higher LMA in the control treatment ($\bar{x} = 0.0082$, $\sigma = 0.0002$, $n = 3$) than drought treatment ($\bar{x} = 0.0076$, $\sigma = 0.0013$, $n = 3$). All other species showed higher LMA in the drought than control, however none of the results were significantly different.

### *Stomatal Density*

*Craspedia sp.* had significantly higher stomatal density in the drought treatment than the control treatment ($P = 0.0086$) (Figure 3). *Melicytus dentatus, Luzula atrata,* and *Ranunculus graniticola* all followed the same trend, but were not significantly different. *Geranium antrorsum* showed the opposite, with higher stomatal density in the control treatment ($\bar{x} = 15.951$) than in the drought treatment ($\bar{x} = 30.675$), but results were not statistically significant (Supplementary Material, Figure S1). *Brachyscome decipiens* only had a sample size of one for the drought treatment, so a test of significance could not be run.

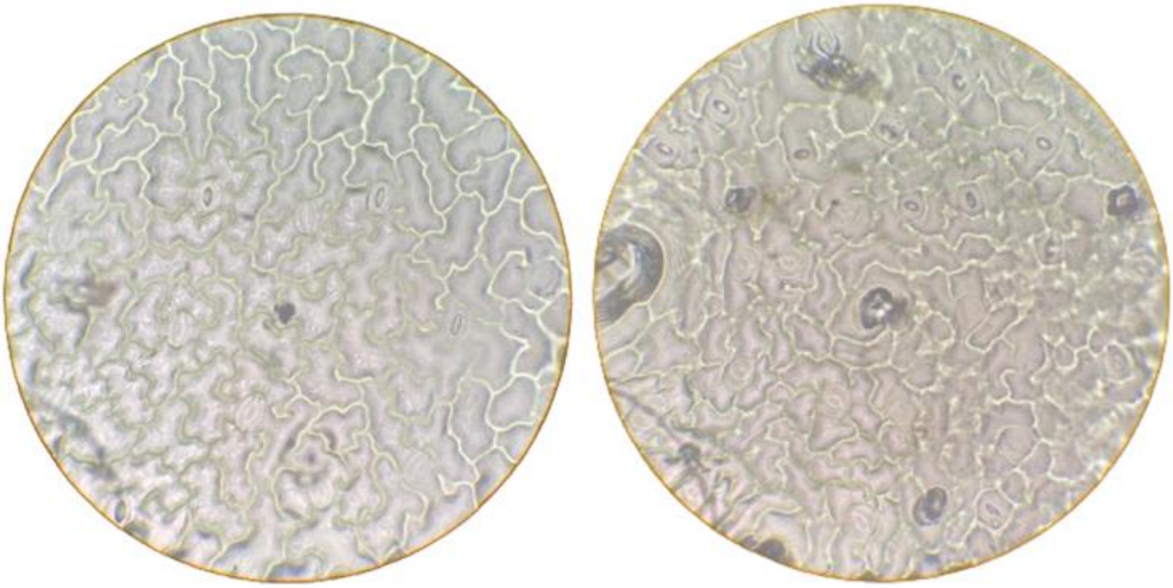

Figure 3. Microscope images of stomatal density on leaves of *Craspedia sp.* not exposed to drought (left) and exposed to drought (right). Images by T. Walker.

## *Epidermal Ratio*

The ratio of epidermal cells to mesophyll cells (epidermal ratio) was significantly higher in leaves of droughted plants than in non-droughted plants of *R. graniticola* ($P < 0.001$) (*Figure 4*). *Melicytus dentatus* also followed this trend, however it was not significant. All other species trended in the opposite direction. Of these, *G. antrorsum* was the only species to show statistical significance for higher values under the control treatment than drought treatment ($P = 0.0056$).

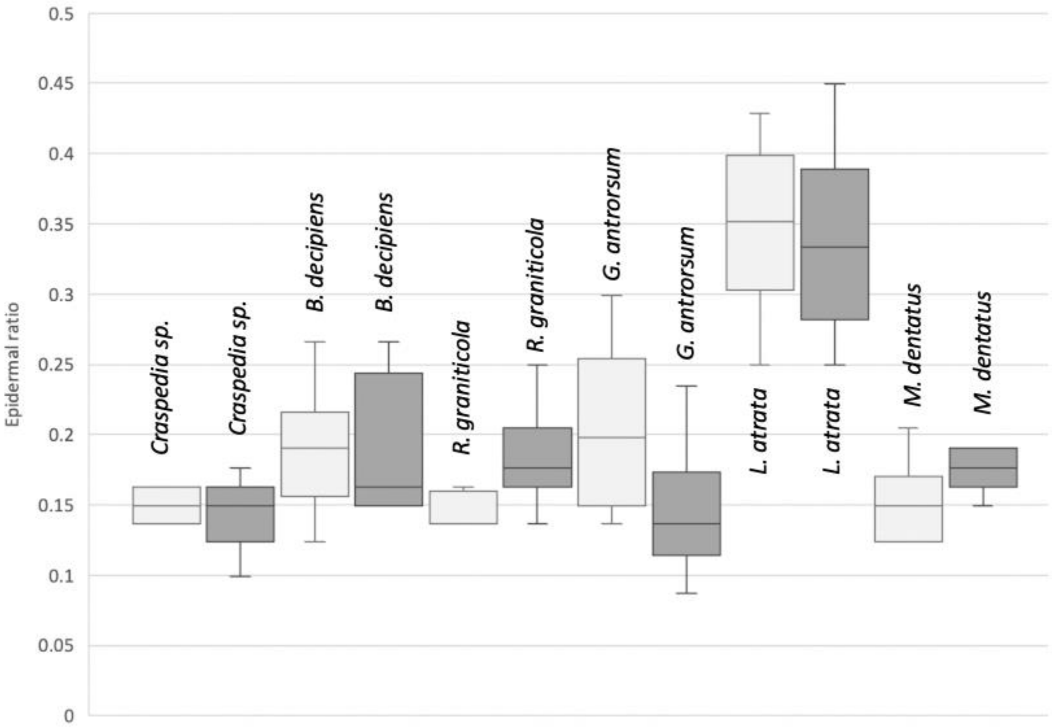

Figure 4. Ratio of epidermis to mesophyll thickness in leaves of alpine plant species. Unshaded boxes indicate control treatment and shaded boxes indicate drought treatment.

## *Leaf Temperature*

Leaf temperature of *B. decipiens* was significantly higher in control than drought treatments (P = 0.0059) (Figure 5). This was also the case for *M. dentatus* and *Craspedia sp.* however, they were not statistically significant. Both *R. graniticola* and *G. antrorsum* had higher temperatures under drought conditions than control conditions but were also not statistically significant.

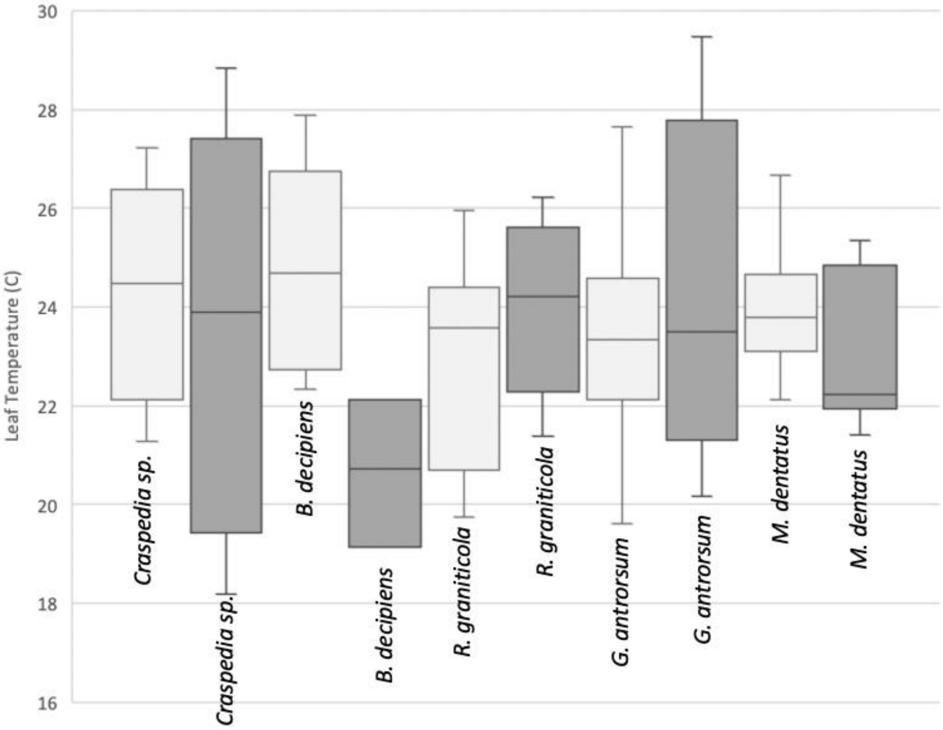

Figure 5. Temperature (degrees Celsius) of leaves of alpine plant species. Unshaded boxes indicate control treatment and shaded boxes indicate drought treatment.

## *Transpiration*

*Brachyscome decipiens* had significantly higher rates of transpiration under control conditions than drought conditions (P = 0.0293) (Figure 6). *Craspedia sp.*, *R. graniticola*, and *G. antrorsum* all followed the same trend, but were not statistically significant. *Melicytus dentatus* was also not significant but trended in the opposite direction: with transpiration rates higher under drought conditions than control conditions.

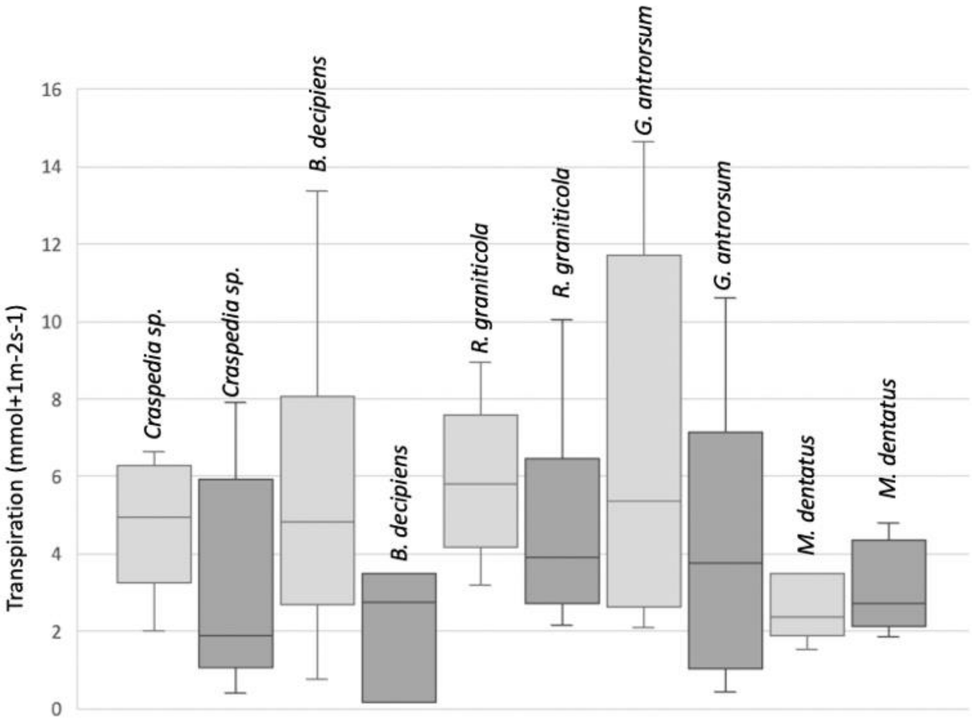

Figure 6. Rate of transpiration of alpine plant species. Unshaded boxes indicate control treatment and shaded boxes indicate drought treatment.

## *Stomatal Conductance*

Results for all species showed average rates of stomatal conductance were higher under control conditions than drought conditions, however none of the differences were statistically significant.

## *PhiPSII*

*Brachyscome decipiens* had significantly higher photosystem II efficiency in droughted plants than in control plants (P = 0.0039) (Figure 7). *Ranunculus graniticola*, *G. antrorsum*, and *M. dentatus* also showed higher photosystem II efficiency in drought compared to control treatments but were not statistically significant. *Craspedia sp*. was the opposite, with higher photosystem II efficiency in the control treatment than in the drought treatment, however the difference was not statistically significant.

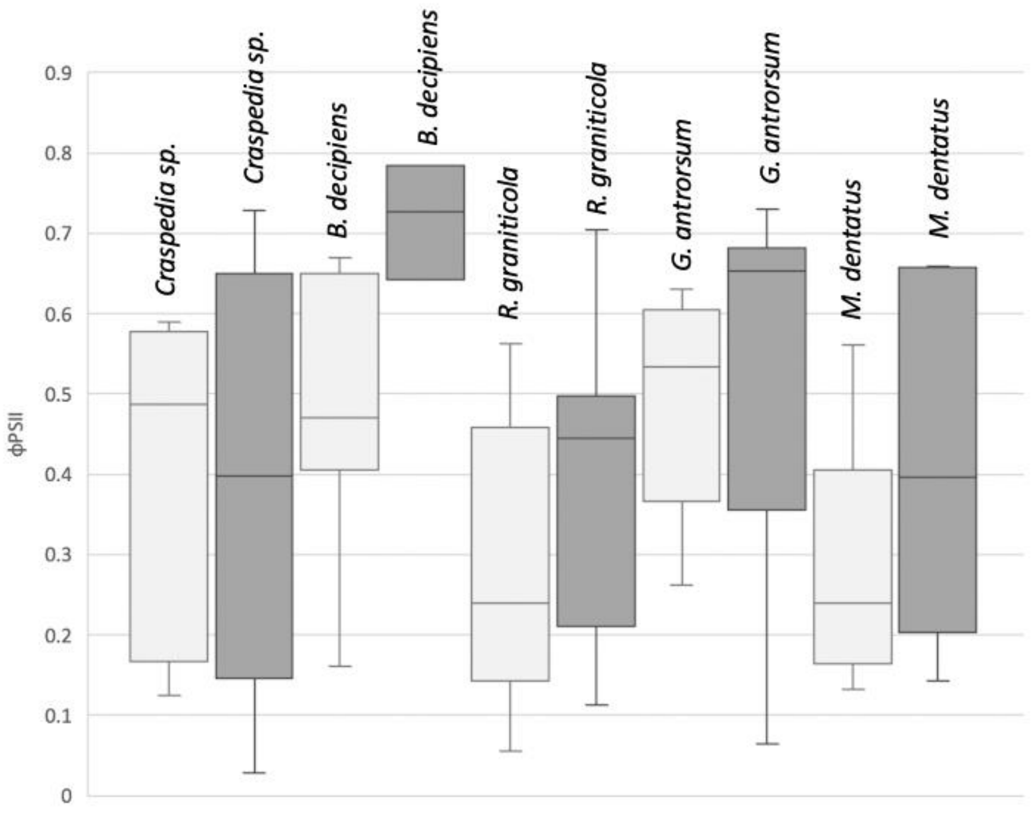

Figure 7. Photosystem II efficiency of alpine plant species. Unshaded boxes indicate control treatment and shaded boxes indicate drought treatment.

## *Spectroscopy*

*Craspedia sp.* only showed significant results for the Plant Senescence Reflectance Index (PSRI), indicating higher levels of plant senescence in drought treatments than control treatments ($P < 0.001$) (Table 1).

*Brachyscome decipiens* had significantly higher levels of senescence and anthocyanins in drought treatments than in control treatments (PSRI: $P < 0.001$, Anthocyanin Reflectance Index 1 (ARI1): $P = 0.0059$, Anthocyanin Reflectance Index 2 (ARI2): $P = 0.0059$). Higher values on the Water Band Index (WBI) ($P = 0.01$), Greenness Index (G) ($P = 0.017$) and Photochemical Reflectance Index (PRI) ($P = 0.0061$) in control plots indicate higher water content and chlorophyll concentrations. In contrast to this, higher Chlorophyll Normalised Difference Vegetation Index (CNDVI) ($P = 0.0032$) values in drought plots indicate healthier vegetation and higher chlorophyll concentrations in these plants (Table 1).

*Melicytus dentatus* indicated healthier vegetation and higher chlorophyll contents in plants under drought conditions (Normalised Difference Vegetation Index (NDVI): $P = 0.051$, G: $P = 0.017$). It also showed higher levels of anthocyanins and carotenoids in control treatments; however, these results were not statistically significant (Table 1).

*Luzula atrata* had significantly higher levels of anthocyanins present in leaves under control treatments than drought treatments (ARI1: $P = 0.0377$, ARI2: $P = 0.0203$) (Table 1).

*Geranium antrorsum* indicated healthier vegetation and higher chlorophyll levels in control plots (NDVI: P = 0.0044, CNDVI: P = 0.0199, G: P = 0.0428), however also had higher concentrations of anthocyanins (ARI1: P = 0.0041, ARI2: P = 0.0037), carotenoids (Carotenoid Reflectance Index 1 (CRI1): P = 0.0169, Carotenoid Reflectance Index 2 (CRI2): P = 0.0104), and higher levels of senescence (PSRI: P = 0.014) (Table 1).

*Ranunculus graniticola* had higher NDVI (P = 0.01) and G values (P = 0.0137) in control plots indicating healthier vegetation and higher chlorophyll levels (Table 1).

Table 1. Spectroscopy results for alpine plants species, using vegetation indices. D represents leaves exposed to drought; C represents leaves in control treatment. Indices in green are expected to be higher in control plots and indices in orange are expected to be higher in drought plots. Statistically significant results are bolded, and asterisks indicate significance levels (* 0.05 > 0.01, ** 0.01 > 0.001, *** < 0.001).

| | *Craspedia sp.* | *Brachyscome decipiens* | *Melicytus dentatus* | *Luzula atrata* | *Geranium antrorsum* | *Ranunculus graniticola* |
|---|---|---|---|---|---|---|
| NDVI | D > C | D > C | **D > C *** | C > D | **C > D **** | **C > D *** |
| CNDVI | D > C | **D > C **** | D > C | C > D | **C > D *** | C > D |
| WBI | C > D | **C > D *** | D > C | D > C | D > C | C > D |
| G | D > C | **C > D *** | **D > C *** | D > C | **C > D *** | **C > D *** |
| PRI | C > D | **C > D **** | D > C | D > C | D > C | C > D |
| PSRI | **D > C ***** | **D > C ***** | D > C | D > C | **C > D *** | C > D |
| ARI1 | D > C | **D > C **** | C > D | **C > D *** | **C > D **** | D > C |
| ARI2 | D > C | **D > C **** | C > D | **C > D *** | **C > D **** | C > D |
| CRI1 | D > C | C > D | C > D | C > D | **C > D *** | D > C |
| CRI2 | D > C | D > C | C > D | C > D | **C > D *** | D > C |

## Discussion

This study first hypothesised that drought would influence internal processes by decreasing transpiration rates, stomatal conductance, and photosynthetic efficiency - which would in turn result in higher leaf temperatures. The only part of this hypothesis that was supported was decreased transpiration rates in *Brachyscome decipiens* under drought conditions.

This study secondly hypothesised that plants exposed to drought would respond with the upregulation of carotenoids and anthocyanins, and downregulation of chlorophyll.

*Craspedia sp., B. decipiens* and *M. dentatus* all provided evidence to support this hypothesis; with higher levels of carotenoids, anthocyanins, or both, under drought conditions. Additionally, *B. decipiens, M. dentatus, G. antrorsum* and *R. graniticola* all supported the downregulation of chlorophyll, with indicators of decreased chlorophyll concentrations in plants undergoing drought treatment.

Finally, this study hypothesised that plants exposed to drought conditions would have plastic responses that would better equip them under future drought by increasing LMA, stomatal density, and the thickness of the epidermis. *Ranunculus graniticola* provided evidence to support thickening of epidermal cells, and *Craspedia sp.* supported the increase in stomatal density, however no results supported an increase in LMA.

## *Process changes*

A decrease in transpiration, as seen in *Brachyscome decipiens*, suggests a plant response to the imposed drought by attempting to regulate water loss. This has also been reported in alpine grasslands (Tello-García et al., 2020), wheat (Li et al., 2017) and various forests worldwide (Granier et al., 2007). Results from Tello-García et al. (2020) also support differing responses between growth forms. All other species in this study did not show a decrease in transpiration under drought conditions. Granier et al. (2007) found that in a period of drought, transpiration rates would spike after a precipitation event. During the sampling period of the present study, 58 mm of rain that fell at the site. Despite the shelters aiming to reduce incident rainfall by 60%, the presence of the remaining rainfall may have led to an increase in transpiration - resulting in no observable difference between the droughted and non-droughted plants. Similarly, Irvine et al. (1998) found that transpiration only decreased in Scots Pine once water content in the topsoil decreased to a threshold value. The present study only measured soil moisture as an average between the plots to ensure a drought treatment had been imposed, but if instead measurements were taken from below each plant as data was collected, this could provide a better understanding of the conditions each plant is experiencing.

Stomatal conductance and transpiration are linked processes, which supports the results of only one species showing changes in both processes under drought conditions.  studies have suggested that responses to decreased water availability such as decreased stomatal conductance could have negative effects on photosynthetic efficiency (Damour et al., 2008; Pirasteh-Anosheh et al., 2016; Zou et al., 2022). Seeing as the results in this study showed no trend supporting decreased stomatal conductance, this supports the lack of effects on photosynthetic efficiency also observed.

Siddique et al., (2000) found that plants with higher leaf temperatures also had higher photosynthetic efficiency, and so it is interesting that while *B. decipiens* had a higher temperature in control plants, photosynthetic efficiency was higher in droughted plants. Higher temperatures in water stressed leaves is well supported in literature, suggested to be due to an inability to cool via transpiration (Siddique et al., 2000; Buchner et al., 2017; Gräf et al., 2021). The higher leaf temperatures and decreased photosynthetic efficiency of *B. decipiens* under control conditions indicates that there may be a confounding factor in play. During the sampling period, *B. decipiens* was flowering (though sampling was not completed on actively flowering individuals) it is possible that recent or upcoming flowering may have been an additional stress on this species.

### *Chemical responses & indicators*

Increases in anthocyanin synthesis can indicate a stress response, as seen in *Brachyscome decipiens* (Andersen et al., 1984; Chalker-Scott 1999; 2002). *Brachyscome decipiens* also showed lower photosystem II efficiency in control plots, which could be the result of photoinhibition. Anthocyanins can help a plant to prevent photoinhibition, which could explain why *B. decipiens* has higher photosynthetic efficiency in drought plots - where anthocyanins are in higher concentrations (Gould, 2004). The photosynthetic reflectance index (PRI) can also be used as an indicator of photosynthetic efficiency, and so with the high values on the PRI in *B. decipiens* in droughted plants, this supports higher levels of photosynthetic efficiency in this species under drought conditions (Garbulsky et al., 2011). Along with drought response, increased anthocyanin concentrations can also be a result of cold stress in plants (Chalker-Scott, 2008). Previous studies have shown that low temperatures can induce the production of anthocyanins (Kroll et al., 1995; Oren-Shamir and Levi-Nissim, 1997). It is possible that the rain-out shelters act as an additional layer of protection to the droughted plants on colder mornings, leaving the control plants more exposed to frost and cold stress by comparison (Chalker-Scott, 2008). I speculate this may explain why some species show higher concentrations of anthocyanins in the control plots.

Increases in carotenoid concentration in response to drought is believed to be linked to the role carotenoids play as antioxidants, which have been shown to be a drought stress response (Shafiq et al., 2015; Zhang et al., 2021). This was supported by the increased concentrations of carotenoids observed in droughted *B. decipiens* individuals. While most research suggests an increase in carotenoid concentrations during periods of water stress, there are also exceptions to this, which may explain why we see a decrease in carotenoids in droughted *Geranium antrorsum* and *Luzula atrata*. Mibei et al. (2017) found that carotenoid concentrations would increase with plant growth in African eggplant. While our study aimed to maintain consistency by using the newest fully expanded leaf for measurements, the life stage of the plant itself may influence these results. Additionally, Terzi & Kadioglu (2006) found that carotenoid concentrations in *Ctenanthe setosa* decreased in the early stages of drought, before then increasing in the later stages. Without observing the plants over a prolonged period, it cannot be said for sure at what life stage these individual plants are at, or how this may influence results.

Several studies have provided support for chlorophyll parameters being used as metrics to measure both responses to environmental stress, and whole-plant mortality (Figueroa et al. 1997; Hakam et al., 2000; Baker & Rosenqvist, 2004; Valladares, 2005; Li et al. 2006). Decreased chlorophyll concentrations in *B. decipiens*, *G. antrorsum* and *R. graniticola* support this, as plants that have reduced their photosynthetic activity will require less chlorophyll. The spectroscopy portion of this study yielded some conflicting results under different reflectance indices. There could be great benefit in using chemical analysis to ground-truth these results, to determine if these indices vary in their application across species.

### *Plasticity*

The degree to which a plant will experience drought under reduced water availability is a result of the water requirements of the plant. For this reason, smaller leaf surface area (smaller transpiring surface) can be beneficial in dry environments (Poorter et al., 2009; Zou et al., 2022). This, in combination with smaller, more tightly packed cells produces a higher leaf mass per area (LMA). Although LMA is considered a good indicator of whether a plant is experiencing water stress, there are also other factors that can affect this and could be confounding why the changes seen in this study were not significant. Thomas and Winner (2002), and Niinemets (2006) both found that LMA increased with plant age, and other studies have also supported an increase in LMA over time in herbaceous species (Poorter & Pothmann, 1992; Villar *et al*., 2005). Niinemets (2006) also found that low-light plants have lower LMA than high-light plants, and while the material of the drought shelters was designed to not impact light penetration, it is possible that this additional barrier could be causing a 'shade' effect.

Most water loss in plants occurs via transpiration through the stomata, and so by altering the density of these stomata on the leaf epidermis, plants can minimise water loss. Environmental factors influence this change by recognition of water limitation in mature leaves, which then alter the stomatal frequency in developing leaves (Casson and Gray, 2008; Casson and Hetherington, 2010; Pillitteri and Torii, 2012). In this study, *Craspedia sp.* demonstrates increases in stomatal density under water limitations. This result has also been observed in rice crops and olive cultivars exposed to water stress (Kawamitsu et al., 1996; Bosabalidis and Kofidis, 2002). However, there are also many examples within the literature that support leaves exposed to water stress having lower stomatal densities in order to regulate water loss via transpiration (Chen et al., 2001; Kusvuran et al., 2010; Pirasteh-Anosheh et al., 2016; Wang et al., 2016).

Higher ratios of epidermal: mesophyll cells under water limitation - as seen in *Ranunculus graniticola* - is the result of decreased cell size in the mesophyll which thereby increases the ratio between the two cell types (Lees, 1984; Makbul et al., 2011). This decrease in cell size is suggested to be significantly more resistant to cell collapse in arid conditions (Oertli et al., 1990). Several studies that have seen this increase in epidermis thickness also supported increases in stomatal density in these leaves (Bosabiladis and Kofidis, 2002; Ennajeh et al., 2010; Taratima et al., 2019).

## Conclusions

The results of this study have shown a wide range of variability in species' responses to drought in the Australian Alps. With no clear trend shown across either species or growth form, this study has identified a gap in knowledge to be filled for other species that make up these alpine grassland communities. While this study has provided an important snapshot of the state of the plants at the time of measurement, to really understand the underpinnings of how these plants are responding to these changes in water regimes, similar studies need to be completed over a larger time scale. By doing this, we can collect essential information for the management of alpine grasslands under projected climate change and make clear predictions of how species' responses may lead to shifts in community composition in Kosciuszko National Park.

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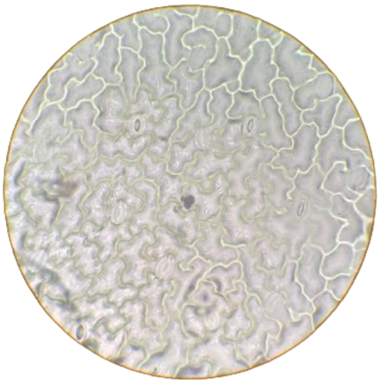
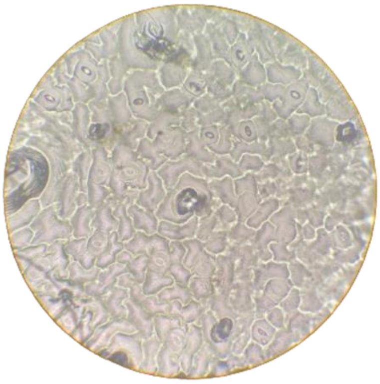
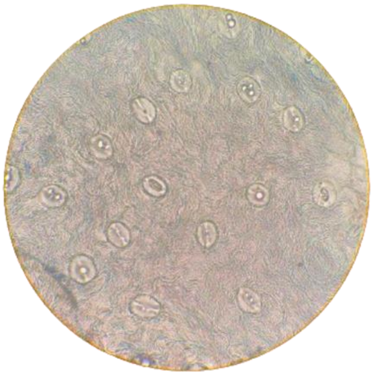
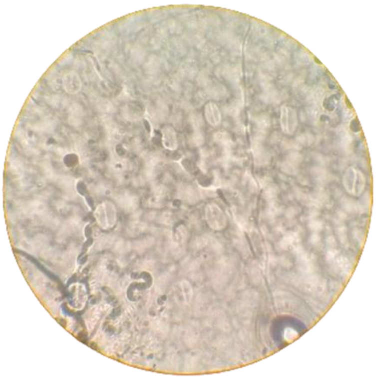
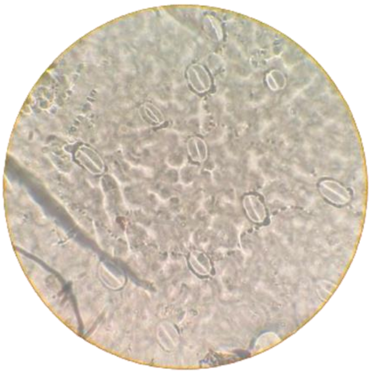
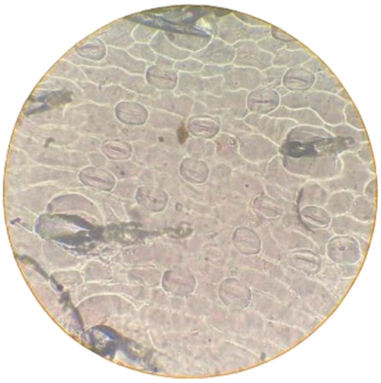
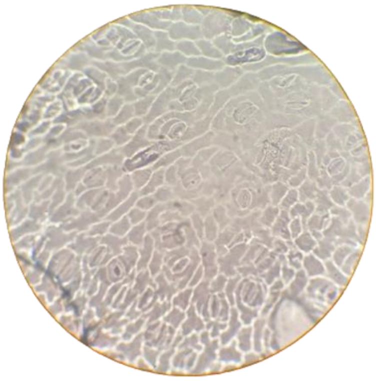
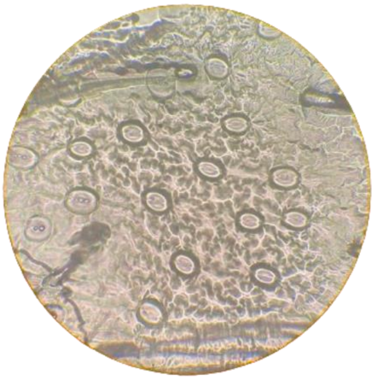
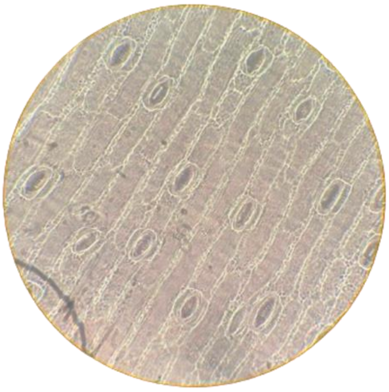
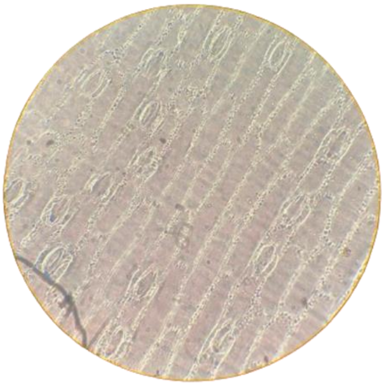
# Supplementary Material

| | Control | Drought |
|---|---|---|
| *Craspedia sp.* |  |  |

| | | |
|---|---|---|
| *Brachyscome decipiens* |  |  |
| *Ranunculus graniticola* |  |  |
| *Geranium antrorsum* |  |  |
| *Luzula atrata* |  |  |

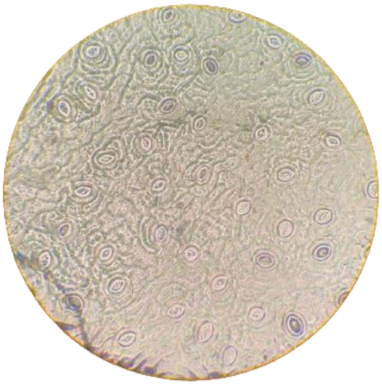
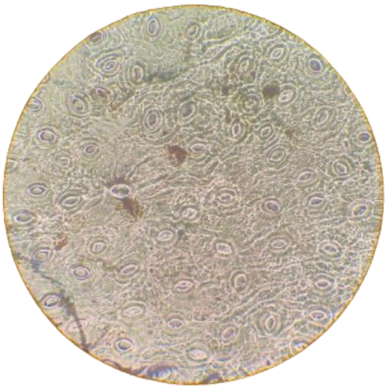
| | | |
|---|---|---|
| *Melicytus dentatus* |  |  |

**Figure S1**. Microscope images of stomatal density of six alpine plant species exposed to control (left) treatments and drought (right) treatments. Images by T. Walker.

Table S1. Spectral vegetation indices equations and descriptions, adapted from Lowe et al. (2017).

| Index Name | Index Abbreviation | Equation | Description |
|---|---|---|---|
| Normalised Difference Vegetation Index | NDVI | (R800 - R680) / (R800 + R680) | An area with nothing growing in it will have an NDVI of zero. NDVI will increase in proportion to vegetation growth. An area with dense, healthy vegetation will have an NDVI of one. |
| Chlorophyll Normalised Difference Vegetation Index | CNDVI | (R750 - R705) / (R750 + R705) | Similar to Normalised Difference Vegetation Index (NDVI) but uses the ratio of Near-Infrared and the edge of Red, CNDVI is more affected by chlorophyll content when compared to the NDVI. |
| Carotenoid Reflectance Index 1 | CRI1 | (1/R510) - (1/R550) | Weakening vegetation contains higher concentrations of carotenoids, so this index is one measure of stressed vegetation. Higher CRI1 values mean greater carotenoid concentration relative to chlorophyll. |
| Carotenoid Reflectance Index 2 | CRI2 | (1/R510) - (1/R700) | This index is a modification to CRI1 that provides better results in areas of high carotenoid concentration. Higher CRI2 values mean greater carotenoid concentration relative to chlorophyll |

| | | | |
|---|---|---|---|
| Photochemical Reflectance Index | PRI | (R531 - R570) / (R531 + R570) | The PRI is sensitive to changes in carotenoid pigments in live foliage, it can also serve as an index of relative chlorophyll: carotenoid levels |
| Plant Senescence Reflectance Index | PSRI | (R680 - R500) / R750 | Based on the chlorophyll/carotenoid ratio which undergoes major changes as a consequence of differential breakdown rates of these pigments during early senescence. |
| Water Band Index | WBI | (R900 / R970) | Correlated with the plant water content. |
| Anthocyanin Reflectance Index 1 | ARI1 | (1/R550) - (1/R700) | Weakening vegetation contains higher concentrations of anthocyanins, so this index is one measure of stressed vegetation. Increases in ARI1 indicate canopy changes in foliage via new growth or death. |
| Anthocyanin Reflectance Index 2 | ARI2 | R800 * ((1/R550) - (1/R700)) | This index is a modification of ARI1 that provides corrections based on leaf density and thickness. |
| Greenness Index | G | R554 / R677 | Higher greenness values correspond to increased presence of chlorophyll in the vegetation being monitored |

Table S2. Mean, standard deviation and sample size for soil moisture measurements between drought and control plots.

| | | Mean (x̄) | Standard deviation ($\sigma$) | Sample size (n) |
|---|---|---|---|---|
| SOIL MOISTURE | Control | 4.5933 | 2.0899 | 15 |
| | Drought | 2.5267 | 1.2937 | 15 |

Table S3. Mean (x̄), standard deviation ($\sigma$) and sample size (n) for measurements between drought and control plots for each species.

| | | Control | | | Drought | | |
|---|---|---|---|---|---|---|---|
| | | x̄ | $\sigma$ | n | x̄ | $\sigma$ | n |
| **Stomatal Density** | *Brachyscome decipiens* | 17.178 | 4.424 | 3 | 13.497 | N/A | 1 |
| | *Craspedia sp.* | 10.225 | 2.834 | 3 | 22.904 | 0.708 | 3 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Geranium antrorsum* | 30.675 | N/A | 2 | 15.951 | N/A | 2 |
| | *Luzula atrata* | 15.951 | 3.246 | 3 | 18.814 | 5.108 | 3 |
| | *Melycitus dentatus* | 42.127 | 13.005 | 3 | 33.129 | 3.246 | 3 |
| | *Ranunculus graniticola* | 29.039 | 15.342 | 3 | 22.699 | N/A | 2 |
| **Epidermal Ratio** | *Brachyscome decipiens* | 0.191 | 0.425 | 12 | 0.185 | 0.0552 | 4 |
| | *Craspedia sp.* | 0.149 | 0.015 | 4 | 0.146 | 0.027 | 7 |
| | *Geranium antrorsum* | 0.203 | 0.053 | 14 | 0.147 | 0.04 | 12 |
| | *Luzula atrata* | 0.347 | 0.058 | 8 | 0.339 | 0.066 | 15 |
| | *Melycitus dentatus* | 0.154 | 0.038 | 13 | 0.172 | 0.024 | 15 |
| | *Ranunculus graniticola* | 0.14 | 0.02 | 8 | 0.184 | 0.028 | 16 |
| **Leaf Temperature** | *Brachyscome decipiens* | 24.836 | 2.064 | 10 | 20.66 | 1.491 | 3 |
| | *Craspedia sp.* | 24.284 | 2.2 | 9 | 23.595 | 4.074 | 6 |
| | *Geranium antrorsum* | 23.451 | 2.221 | 9 | 24.354 | 3.379 | 9 |
| | *Melycitus dentatus* | 23.989 | 1.316 | 9 | 22.996 | 1.526 | 7 |
| | *Ranunculus graniticola* | 22.926 | 2.089 | 9 | 23.924 | 1.754 | 9 |
| **Transpiration** | *Brachyscome decipiens* | 5.675 | 3.942 | 10 | 2.139 | 1.747 | 3 |
| | *Craspedia sp.* | 4.74 | 1.65 | 9 | 3.106 | 2.877 | 6 |
| | *Geranium antrorsum* | 7.109 | 4.869 | 9 | 4.533 | 3.491 | 9 |
| | *Melycitus dentatus* | 2.516 | 2.782 | 9 | 1.443 | 3.109 | 7 |
| | *Ranunculus graniticola* | 5.783 | 1.954 | 9 | 4.663 | 2.552 | 9 |
| **Stomatal Conductance** | *Brachyscome decipiens* | 0.305 | 0.184 | 10 | 0.183 | 0.151 | 3 |
| | *Craspedia sp.* | 0.281 | 0.139 | 9 | 0.182 | 0.159 | 6 |
| | *Geranium antrorsum* | 0.594 | 0.567 | 9 | 0.328 | 0.268 | 9 |
| | *Melycitus dentatus* | 0.137 | 0.133 | 9 | 0.854 | 0.156 | 7 |
| | *Ranunculus graniticola* | 0.374 | 0.113 | 9 | 0.273 | 0.183 | 9 |
| **PhiPSII** | *Brachyscome decipiens* | 0.486 | 0.162 | 10 | 0.718 | 0.072 | 3 |
| | *Craspedia sp.* | 0.413 | 0.201 | 9 | 0.394 | 0.284 | 6 |
| | *Geranium antrorsum* | 0.481 | 0.132 | 9 | 0.529 | 0.225 | 9 |
| | *Melycitus dentatus* | 0.289 | 0.146 | 9 | 0.410 | 0.225 | 7 |
| | *Ranunculus graniticola* | 0.279 | 0.179 | 9 | 0.387 | 0.189 | 9 |