Influence of arbuscular mycorrhizal fungi on water relations in C_3/C_4 grasses under extreme heat and water stress

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Word Counts: Introduction – 1 263, Methods – 2 106, Results - 914, Discussion – 1 798

Abstract

The increasing occurrence of heat waves compounded with water stress is placing native grassland communities under growing pressure. In particular, combined heat and water stress can be particularly damaging for plant physiological performance. Arbuscular mycorrhizal (AM) fungi may improve grass physiological stress tolerance, with responses likely to be different between C_3 vs C_4 photosynthetic pathways. The role of AM fungi in mitigating physiological responses to heat and water stress, and their combination, was investigated in six Australian grass species under a simulated heatwave event. Grasses exposed to combined heat and drought stress had additively deleterious effects compared to single stressors. AM fungi did not improve host grass performance, resulting in decreased transpiration rate of native grasses under heat stress and water stress. As expected, C_4 grasses outperformed C_3 grasses across all physiological measures, but both groups showed seasonally acclimated responses, with greater resilience in spring than in winter. The limited effects of AM fungi on native grass performance under heat and water stress underscores the importance of using native species to understand natural community responses to climate stressors. Further, while C_4 grasses showed greater resilience than C_3 grasses to the applied stressors, both groups showed considerable damage under combined heat and water stress, highlighting the risk to native grasslands from

increasing compound stress events.

Key Words: Heatwaves, grasslands, water use efficiency, photosynthesis, compound events

Author Contributions

SJ wrote the manuscript, designed and conducted the experiment, undertook data collection and performed data analysis. CG assisted in data collection. KF, SR, MM and AL supervised the experimental design and execution and provided revisions to the manuscript. All authors read and approved the final version of the manuscript.

Funding

This work was supported by grants from Friends of Grasslands, the Australian Wildlife Society student award, the Ecological Society of Australia's Holsworth Research Endowment and CSES enhancement grant and Environmental Futures enable grant. SLJ was supported by an Australian Government RTP stipend. SAR is supported by an Australian Research Council Australian Laureate Fellowship (FL240100032) and this work was possible because of the ARC Flora and Fauna Research Facility grant (LE0775666).

Acknowledgements

Thank you to Connor Bland, Zahra Spitznagel-Reeves, Eva Watts, Gilbert Coombes and Georgia Watson for assisting in experiment set up and execution.

1. Introduction

Globally, grasslands are one of the most threatened ecological communities, largely being placed under threat from urbanisation and agricultural use (Scholtz & Twidwell 2022). Increases in extreme climate events also place global grassland communities at elevated risk (Li *et al.* 2023). Given that native grasslands are one of the most important ecological communities for their role in carbon sequestration and biodiversity (Bai & Cotrufo 2022; Lyons

et al. 2023), it is becoming increasingly important to understand their responses to increased climatic stress. Their patchy spatial distribution coupled with high exposure to irradiation due to sparse tree coverage, places grasslands at particularly high risk from extreme heat events (Li *et al.* 2023).

Heatwaves are characterised by extreme temperatures, well above a region's average for three or more days (Trancoso *et al.* 2020). Recently, there has been an increase in the frequency and severity of extreme temperatures occurring concurrently with water deficits or drought, known as compound events (Zscheischler *et al.* 2018; Mukherjee & Mishra 2021; Lee *et al.* 2023). Heat stress compounded by water stress can present additional challenges to plants than either stressor in isolation. While plants can produce protective compounds, such as heat shock proteins (Wang *et al.* 2004; Davies *et al.* 2018) and antioxidants (Demidchik 2015), the ability to cool via thermoregulation during high heat can also help to maintain leaf temperatures within functional limits (Michaletz *et al.* 2015). By increasing stomatal aperture, plants may mitigate damage to thermo-labile cellular components via evapotranspiration which evaporatively cools plants at the leaf's surface following stomatal transpiration (Leigh *et al.* 2017; Arnold *et al.* 2025) although this response can be species-dependent (Marchin *et al.* 2022). Further, this process generally requires water availability to be relatively high (Cook *et al.* 2021). As such, when plants are simultaneously water stressed and heat stressed, the capacity for plants to reduce thermal damage may be limited. Water stress induces water-saving mechanisms in plants. A chronic response can be the diversion of energy from shoot growth to root growth to increase water scavenging; a more immediate response is to partially or fully close stomata to reduce water loss via transpiration, although this can ultimately limit production of protective compounds (Zia *et al.* 2021). As a result, the key physiological mechanisms that plants require to limit thermal damage can be disrupted when plants are simultaneously heat and water stressed.

Arbuscular mycorrhizal (AM) fungi are known to facilitate plant performance during periods of abiotic stress (Wang *et al.* 2023). In response to AM fungal colonisation, several physiological adjustments and adaptive mechanisms have been demonstrated on plant responses to drought, which may also benefit plants during a compound stress event. For example, AM fungi can improve host plant nutrition, which can boost plant responses to environmental stressors and increase the production of protective free radical scavenging compounds (Begum *et al.* 2020). Also, association with AM fungi can alleviate plant stress during low water availability or high-water demand by mediating the plant's water saving mechanisms and improving the plant's water relations in several ways. AM fungi can modify host gene expression to improved water regulation and nutrient uptake via aquaporins (Sharma *et al.* 2021; Wan *et al.* 2024), increase solute concentrations in plant cells (Xiong & Zhu 2002; Zhu *et al.* 2010; Ma *et al.* 2015), and modify edaphic properties to increase water and solute uptake (Rillig & Mummey 2006; Zhang *et al.* 2019; Cheng *et al.* 2022; Pauwels *et al.* 2023). Under a range of conditions, AM fungi have been found to increase stomatal conductance and transpiration of colonised plants by as much as 20%, with this rate being species-dependent (Khalvati *et al.* 2005; Ruth *et al.* 2011). Another way that AM fungi benefit plant-water relations is via improved soil water retention, largely facilitated by the secretion of glomalin, a glycoprotein that enhances soil structure by promoting the formation of soil aggregates (Rillig & Mummey 2006; Zhang *et al.* 2019). Additionally, AM fungi improve water movement through the soil. The proliferation of fungal mycelium through the soil works to effectively extend the host plant's zone of root depletion, increasing the water scavenging potential of the plant via fungal hyphal cell membranes (Smith & Read 2010; Kakouridis *et al.* 2022). Moreover, in some substrates, AM fungi can improve soil hydraulic conductivity, which alleviates resistance to water movement through the soil (Bitterlich *et al.* 2018a; Bitterlich *et al.* 2018b; Pauwels *et al.* 2023). However, reported benefits conferred to host species vary,

with AM fungal colonisation showing no effect on transpiration and stomatal conductance under droughted conditions (Cheng *et al.* 2021).

While the majority of examples presented above come from studies investigating AM fungal benefits to plant drought responses alone, they suggest, that since AM fungi have the ability to improve water relations in host plants, they might also mitigate plant stress during a combined heat and water stress event. Such examples also demonstrate context-specific responses to AM fungi under stress, highlighting the need to explore effects on host plants with contrasting water use strategies. For grasses, one key difference in this regard is photosynthetic pathway. As water use and energy expenditure differ greatly between C_3 species and C_4 species, the influence of AM fungi has unsurprisingly been found to be disparate between the two groups (Frew 2019). Under ambient conditions, C_4 grasses are considered to be more mycorrhizal than C_3 grasses (Hetrick *et al.* 1988; Hetrick *et al.* 1990), with studies finding greater increases in plant nutrition in C_4 species than C_3 species (Frew 2019). Previous work, however, that when native grasses are placed under stress, photosynthetic pathway is not the main driver of AM fungi colonisation (Jones 2025).

The mycorrhizal dependency continuum suggests that the functional benefits of AM fungal colonisation vary in accordance with the level of 'need' of the host plant (Johnson, 2010). C_4 grasses are more resilient to both heat and water stress, owing to their morpho-physiological adaptations for improved water use efficiency (WUE) in hotter and drier climates (Bräutigam & Gowik 2016). As these species can maintain high functioning water relations during stress, they have been found to have decreased damage under water- and heat-stressed conditions (Davies *et al.* 2018). C_3 grasses, on the other hand, are highly sensitive to heat stress and water stress. Their photosynthetic pathway favours temperate climates, where they have greater energy efficiency over C_4 plants, but they have poor responses in high heat and low water

conditions (Opoku *et al.* 2024). Therefore, during compound stress events, C_3 grasses may become more dependent on AM fungi as they are more susceptible to damage during stressful periods in comparison to C_4 grasses. To our knowledge, this question has not previously been explored but is key to unravelling variation in responses of the two functional groups when predicting the persistence of co-occurring grassland species.

Therefore, we sought to determine the role of AM fungi in facilitating plant physiological responses during compound stress events. Six native grasses, including both C_3 and C_4 species, were exposed to either water stress, heat stress or combined heat and water stress. For each species-treatment combination, half of the plants were inoculated with AM fungi and half were not. The first aim was to determine whether AM fungi improved water relations in native grass species when subjected to water stress, heat stress, and the combination of both. The second aim was to explore whether C_3 or C_4 photosynthetic pathways of the host plant modified the facilitatory role of AM fungi under these stress conditions. Together, these questions address how plant–fungi interactions may influence grassland resilience in the face of increasing climate extremes.

2. Methods

2.1 Study Species

Six grass species were used in the heat and water stress experiments. Three species were C_3 grasses: *Poa labillardierei* Stued., *Austrostipa scabra* subsp. *scabra* (Lindl.) S. W. L Jacobs & J. Everett and *Microlaena stipoides* (Labil.) R.Br. The three remaining species were C_4 grasses: *Themeda triandra* Forssk., *Chloris truncata* R.Br and *Cymbopogon refractus* (R.Br) A. Camus. All six grass species are co-occurring within their natural distributions, with several species, *T. triandra*, *P. labillardierei*, *C. truncata* and *A. scabra*, being dominant species in temperate grassland communities along the south-eastern coast of Australia (Mott & Groves 1994; Keith

2004). All grasses were grown from seed purchased from Nindethana Seed Service Pty Ltd (Albany, WA, Australia).

Pre-treatment growing conditions

Seeds were germinated in separate seedling trays per species in a sterile soil mix (1:3 river sand: topsoil) and transferred into individual replicate pots (20 cm diameter) after seedlings had 4-5 true leaves. The number of seeds per tray was dependent on the species' variation in seed size and viability. Viability was tested for each species separately to determine the number of seeds needed for each species, prior to germinating seeds for the experiment. Given the variable germination success of individual species and the scale of this experiment, germination and transferring of the seedlings occurred over 4 months (November 2021 – May 2022). All soil was autoclaved prior to use for 90 minutes at 120 °C and all pots and other equipment was surface sterilised using 70% ethanol solution. Each pot was filled with 5 kg of sterilised soil (1:3 river sand: topsoil) and was watered fully every 2-3 days using Reverse Osmosis (RO) water. Seedlings were sprayed with a micronutrient fertiliser (Yates Health Tonic Trace Element Chelates) according to the manufacturer's instructions following transplanting. All seedlings were grown inside temperature-controlled greenhouses at the Ecological Research Centre, University of Wollongong. The temperature inside the greenhouse was maintained between 18 °C and 25 °C for the duration of the experiment.

Mycorrhizal inoculation preparation

To prepare a natural mycorrhizal inoculant, soil was collected at the Australian Botanic Garden Mount Annan, NSW. All six study species occur naturally within remnant patches of native vegetation at this site; therefore, it was considered suitable for co-adapted soil microbes and AM fungi of the six study species. Soil was collected on four different occasions, to align with the staggered planting of the seedlings. At each collection time, 8 kg of soil was collected from

four to five locations, with three to four subsamples taken from within a location. Each location was selected based on low disturbance and the presence of at least one of the study species. Soil was collected at least 50 m away from tracks and other disturbances such as clearings and roads and at least 50 m from other sample locations to minimise the chance of sampling similar communities and to maximise genetic diversity of fungal species (Brundrett 2009; Hazard et al. 2013; Rayment et al. 2020). Soil was collected from the top 10 cm of soil surrounding the base of plants, including soil aggregates and roots in the sample. The top 0.5 cm was scraped off to limit moss and algae growth.

All the samples from a given collection time were combined to form a homogenous mixture. RO water was added to the soil (1:1, soil:water v/v) to form the inoculant which was then stirred and agitated by hand to break apart soil aggregates then left to rest and settle for 10 minutes. The soil mix was then strained through a course mesh (2 mm) to remove large debris which formed the mycorrhizal (AM) inoculant (containing soil microbes, mycorrhizal spores, and hyphal fragments). To form the non-mycorrhizal (NM) inoculant, half of the above mixture (M) was strained through a 50 μm mesh to remove mycorrhizal spores or hyphal fragments (Jones & French 2021). The spores and hyphal fragments collected on the mesh were subsequently added to the mycorrhizal inoculant to increase spore density and colonisation potential.

All pots were inoculated twice during the growth stage with 100 mL of either AM inoculant (50% pots) or NM inoculant (other 50% of the pots). The first inoculation was 2-4 weeks after seedlings were transplanted into the individual pots, the second inoculation was 2-4 weeks before entering the stress treatments. Therefore, replicate pots received a mixture of different AM inoculants made from different soil collections.

Microscopic analysis confirmed the non-mycorrhizal status of NM treated plants, showing an

average hyphal colonisation of 0.53 % (± 0.18 SE) with no arbuscule, coil, or vesicle formation, verifying the effectiveness of the non-mycorrhizal inoculant.

2.2 Experimental Design

Once matured, all plants were subjected to one of four stress treatments: control (C), heat stress (HS), water stress (WS) or heat + water stress (HWS). The experimental design included 480 plants: 10 replicates x 6 species x 2 mycorrhizal treatments x 4 stress treatments. The control treatment received maximum daytime (8 h) temperatures of 25 °C, minimum night-time (8 h) temperatures of 18 °C (ramp rate 1.75 °C/h) and deionised water each afternoon, maintaining soil moisture levels at or above 30% (VWC). The HS treatments had 7 days at these control temperatures and on day 8 (d 8), temperatures were increased incrementally to simulate a heatwave; day-time maximum temperature was increased to 35 °C and minimum night-time temperature to 25 °C. Temperature was ramped up 1 °C every day for the following 4 d, peaking at 39 °C on d 12. On d 13, temperature was decreased back to 35 °C and on d 14 the temperature was further decreased to 30 °C; the night temperature was maintained at 25 °C, with daytime temperatures returned to control temperatures on d 15. HS plants were watered fully in accordance with the control treatment. The WS plants received no water from d 1 until they reached a soil moisture level below 5 %. If the soil moisture dropped below 5% plants received a small amount of water to maintain volumetric water content (VWC) at 5%. On days 13 and 14, WS plants received up to 600 ml of deionised water to increase VWC back to 30%. Temperatures for the WS plants were the same as the control treatment. The HWS plants received a combination of both the heat and water stress treatments in full, as described above.

The stress treatments were completed in six rounds of separate replicate plants. Approximately, 90 plants were placed into the stress treatments each round. Rounds 1, 2 and 3 were carried out in the austral winter, beginning in May 2022 and finishing in early August 2022. The final

rounds, 4, 5 and 6, were carried out in austral spring, beginning in late August 2022 and finishing in October 2022. As such, season was used as a factor to account for timing differences over the separate rounds. Additionally, plants were staggered during the germination and growth stage to minimise differences in the age of replicates in each round.

2.3 Physiological response measurements

Plant gas exchange (carbon assimilation (A), transpiration (E) and stomatal conductance, (g_{sw}) was measured using a LI-6800 Portable Photosynthesis System (Li-Cor, Lincoln, Nebraska, USA), using consistent cuvette conditions (Supplementary Table 1). Measurements were taken at midday on d 12, peak stress day (from approximately 11:00 to 14:00 to capture peak heat) on one healthy, fully expanded leaf per plant. The surface area of the portion of leaf in the cuvette was calculated for every measurement using photos taken alongside a ruler reference. Surface area of each measured leaf was then calculated using Image J (Schneider *et al.* 2012). The total number of replicates per species per stress treatment was n = 6.

To assess the ability of photosystem II (PSII) to recover from stress, maximum quantum yield (F_V/F_M) was measured pre-dawn on every third day on two healthy, dark-adapted leaves per plant using a fluorpen (FluorPen FP 100-MAX-D). For each plant, the average of both measurements was calculated (n = 10 per species per stress treatment).

To determine the extent of water stress, leaf water potential (ψ) was measured every third day. Healthy, inner leaves were cut as close to the base as possible and placed into a sealable plastic bag for 5-10 mins before measuring ψ using a pressure chamber (PMS Instruments, Model 1505D) with an almond gasket. Water potential was measured every three days on alternating plants to reduce the amount of stress on the plants caused by defoliation. The total number of replicates was n = 5 per species per stress treatment. Fluorescence and leaf water potential measurements were taken on days 0, 3, 6, 9, 12 and 15 to capture changes over time.

Stable carbon isotope composition ($\delta^{13}C_p$) was measured to estimate intrinsic water use efficiency (iWUE) of grasses under stress. Inner, fully expanded healthy leaves were collected on the final day of the stress experiment (d 15) for each experiment round (1-6). After collection, samples were dried at 70 °C and stored at room temperature until processing (n = 5 per species per stress treatment). Samples were processed at the Australian National University (ANU), Canberra, Australia to determine $\delta^{13}C$ values, using the following equation (Farquhar *et al.* 1989):

$$\delta^{13}C_p = (R_{sample} / R_{standard} - 1) * 1000$$

where $\delta^{13}C_p$ represents the $\delta^{13}C$ of the sample plant. These same samples were also used to measure changes in plant carbon to nitrogen ratio (C:N = C / N) using the Dumas combustion analysis coupled with isotopic radio mass spectrometry (Dumas 1831).

2.4 Statistical Analysis

A full factorial generalised linear model (GLMM) was used to determine the role of AM fungi in influencing F_V/F_M, water potential (ψ), C%, N% and C:N ratio. Mycorrhizal treatment (AM = with AM fungi, NM = no AM fungi), stress treatment (C = control, HS = heat stress only, WS = water stress only, HWS = heat + water stress), photosynthetic pathway (C_3 and C_4) and season (winter and spring) were all considered fixed effects. F_V/F_M was modelled using a beta distribution (link = log); all others were fitted using a gamma distribution (link = log). Statistical analysis was not conducted on changes in F_V/F_M and leaf water potential (ψ) across time points due to reduced statistical power from repeated measures; however, the data is presented in Supplementary material (Supplementary Table 1).

Differences in $\delta^{13}C_p$ amongst treatments were identified via two separate GLMMs for C_3 and C_4 species. Due to the bimodal distribution of $\delta^{13}C_p$ with both photosynthetic pathways

included, two separate analyses were performed. For both C_3 and C_4 groups, a full factorial model was employed with mycorrhizae, stress treatment, species, and season as fixed factors. Both models were fitted using normal distributions (link = identity).

For the gas exchange data, a multivariate analysis of variance (MANOVA) was used to determine the joint response of E and A to the stress treatments, AM fungi and photosynthetic pathway. To fit the assumption of normality and homogeneity of variance, E was log transformed. Due to the occurrence of negative A values as well as E being 0 for some samples, particularly in the combined heat and water stress treatment, instantaneous water use efficiency (WUE =A/E) could not be calculated. Instead, a MANOVA was employed to gain insight into carbon-water exchange dynamics and serve as a proxy for WUE (pWUE). As the negative and zero values occurred predominately in the combined heat and water stress treatment, removing these samples was not an option as it would cause a very unbalanced design. Subsequently, univariate analyses (GLMM) were then used to assess the separate responses of A and E. The response of g_{sw} was also analysed using a GLMM in response to the stress treatments. Due to high correlation of photosynthetic pathway and species in the gas exchange data, species was not included as a fixed factor in the model to facilitate model convergence. To confirm whether experimental round explained a significant proportion of the data variability, plotted residuals were assessed and a Levene's test was performed; this confirmed that timing did not explain a significant proportion of variation in the data and therefore was not included as a random factor in the model. Similarly, season was not included as a main effect as there were no gas exchange measurements taken during round 1. For the GLMMs, E and g_{sw} were fitted with gamma distributions (link = log) and A was fitted using a normal distribution (link = identity).

The distributions of dependent variables were determined by visual inspection of histograms and QQ plots where required. To understand effects driving significant interactions, post-hoc

Tukey HSD tests were performed. All analyses were performed using SAS 3.81 (SAS Institute Inc., Cary, NC, USA).

3. Results

Influence of AM fungi and stress on gas exchange and plant water use

AM inoculation did not affect parameters associated with carbon assimilation (A and pWUE) (Figure 1a; Figure 1b; Table 1). Instead, the rate of carbon assimilation (A) was influenced by photosynthetic pathway and stress treatment (Table 1). Irrespective of mycorrhizal treatment, pWUE varied significantly between C_3 and C_4 photosynthetic groups (Figure 1a; Figure 1b; Table 1). C_3 grasses showed the greatest variability in responses across stress treatments, having no change in the relationship between A and E in response to the HS treatment but having an increased transpiration rate with lower assimilation rates in the WS and HWS treatment (Figure 1a). C_4 species showed minimal response to the stress treatments in relation to pWUE (Figure 1b).

The presence of arbuscular mycorrhizal fungi did affect gas exchange of grasses, with these effects varying, depending on the response parameter. Inoculation treatment (AM: with AM fungi; NM: non-mycorrhizal) significantly influenced parameters associated with water vapour loss, namely transpiration rate (E) and stomatal conductance (g_{SW}) (Table 1), with the effect depending on stress treatment (Figure 1). Heat stress alone (HS) increased transpiration rate and stomatal conductance overall, however, this effect was not significant for either AM or NM treatments (Figure 1f, Figure 1h). For the non-mycorrhizal (NM) grasses under the water stress (WS) treatment, both transpiration rate and stomatal conductance were also significantly lower than control NM grasses, with an 80% decrease in transpiration rate (Figure 1f, Figure 1h). However, for the AM fungi inoculated grasses, exposure to water stress alone did not significantly lower transpiration rate or stomatal conductance in comparison to the control.

Under combined heat and water stress (HWS), both AM and non-AM treated grasses had significantly decreased stomatal conductance and transpiration rates when compared to the control (Figure 1f, Figure 1h). Differences in stomatal conductance between C_3 and C_4 grasses were also evident. C_3 grasses had significantly reduced stomatal conductance in the WS and HWS treatments compared to the control and HS (Figure 1g). C_4 grasses, however, only exhibited a significant difference in conductance between the WS and HS treatments, with HS alone showing significantly greater stomatal conductance than the WS treatment (Figure 1g), and transpiration rates showing mostly similar trends across both groups (Figure 1e).

Influence of photosynthetic pathway and stress on gas exchange and plant water use

Overall, C_4 grasses had greater assimilation rates than C_3 grasses (Figure 1c). While grass responses were not significantly different in the HS or WS treatments in comparison to the control group, assimilation rates of WS treated grasses were lower than the HS treated grasses (Figure 1d). Grass assimilation rates were significantly lower in the HWS treatment compared to both the control and HS treatments, being more than 30% lower in grasses exposed to HWS than grasses exposed to HS (Figure 1d).

Changes in water potential were also not dependent on AM fungi but were influenced by photosynthetic pathway and stress treatment (Figure 2a; Table 1) and season (Figure 2b; Table 1). C_3 grasses showed large drops in leaf water potential when exposed to the WS and even more to HWS treatments, dropping from ~ -0.5 MPa (control) to -2.5 MPa and -3.5 MPa, respectively (Figure 2a). C_4 grasses did not show a significant drop in water potential in response to WS or HS alone, but water potential did decrease significantly in response to HWS, from -0.29 MPa in the control group to -1.3 MPa in the HWS treatment (Figure 2a). Water potential was also found to be significantly lower in winter than in spring (Figure 2b). For $\delta^{13}C$, responses to the stress treatments varied significantly for both C_3 and C_4 grasses (Table 2).

However, post-hoc Tukey's tests found no significant differences among stress treatments for each photosynthetic pathway group (Figure 2c). For C_3 grasses, changes in $\delta^{13}C$ were seasonal and dependent on species: *A. scabra* and *P. labillardierei* increased $\delta^{13}C$ in winter, while *M. stipodies* had no seasonal differences in $\delta^{13}C$ (Figure 2d). C_4 grasses had no seasonal responses, but did vary across species, with all three C_4 species having small but significant differences in $\delta^{13}C$ (Figure 2e).

Table 1 Results of a 3-way MANOVA testing the effects of mycorrhizal treatment (mycorrhizal, non-mycorrhizal), stress treatment (control, heat stress, water stress and heat + water stress), photosynthetic pathways (C_3 and C_4) on pWUE (A and E); a 3-way generalised linear mixed model testing the effects of mycorrhizal treatment, stress treatment, C_3/C_4 photosynthetic pathways on assimilation rate (A), transpiration rate (E) and stomatal conductance (g_{sw}); and a 4-way generalised linear mixed model testing the effects of mycorrhizae, stress treatment, photosynthetic pathway and season on water potential (Ψ). Wilks' lambda values are reported for MANOVA results.

	df	F	p
pWUE *(A and E)* -			
Mycorrhizae	2, 288	0.17	0.842
Stress treatment	6, 576	**57.02**	**<.0001**
Photosynthetic pathway	2, 288	**19.38**	**<.0001**
Mycorrhizae*stress treatment	6, 576	1.79	0.0987
Mycorrhizae*photosynthetic pathway	2, 288	0.81	0.8371
Stress treatment*photosynthetic pathway	6, 576	**9.12**	**<.0001**
Mycorrhizae*stress treatment*photosynthetic pathway	6, 576	0.29	0.9419
Carbon assimilation rate (A)			
Mycorrhizae	1, 289	0.25	0.6197
Stress treatment	3, 289	**6.17**	**0.0004**
Photosynthetic pathway	1, 289	**35.3**	**<.0001**
Mycorrhizae*stress treatment	3, 289	1.56	0.1993
Mycorrhizae*photosynthetic pathway	1, 289	0.02	0.8845
Stress treatment*photosynthetic pathway	3, 289	1.17	0.3203
Mycorrhizae*stress treatment*photosynthetic pathway	3, 289	0.23	0.8768
Transpiration rate (E)			
Mycorrhizae	1, 289	1.27	0.261
Stress treatment	3, 289	**29.4**	**<.0001**
Photosynthetic pathway	1, 289	**13.77**	**0.0002**
Mycorrhizae*stress treatment	3, 289	**2.91**	**0.0347**
Mycorrhizae*photosynthetic pathway	1, 289	0.67	0.4128
Stress treatment*photosynthetic pathway	3, 289	**15.73**	**<.0001**
Mycorrhizae*stress treatment*photosynthetic pathway	3, 289	1.07	0.3606
Stomatal conductance (g_{sw})			
Mycorrhizae	1, 289	1.37	0.2429
Stress treatment	3, 289	**58.12**	**<.0001**
Photosynthetic pathway	1, 289	**19.8**	**<.0001**

Mycorrhizae*stress treatment	3,	289	**4.57**	**0.0039**
Mycorrhizae*photosynthetic pathway	1,	289	1.86	0.1018
Stress treatment*photosynthetic pathway	3,	289	**10.52**	**<.0001**
Mycorrhizae*stress treatment*photosynthetic pathway	3,	289	0.94	0.5161
Water potential (Ψ)				
Mycorrhizae	1,	438	0.01	0.9131
Stress treatment	3,	438	**20.13**	**<.0001**
Photosynthetic pathway	1,	438	**119.02**	**<.0001**
Season	1,	438	**20.33**	**<.0001**
Mycorrhizae*stress treatment	3,	438	0.23	0.8722
Mycorrhizae*photosynthetic pathway	1,	438	2.42	0.1208
Photosynthetic pathway*stress treatment	3,	438	**3.23**	**0.0222**
Season*mycorrhizae	1,	438	0.33	0.5662
Season*stress treatment	3,	438	0.46	0.7072
Season*photosynthetic pathway	1,	438	3.5	0.062
Photosynthetic pathway*mycorrhizae*stress treatment	3,	438	0.64	0.5877
Season*mycorrhizae*stress treatment	3,	438	0.07	0.9752
Season*photosynthetic pathway*mycorrhizae	1,	438	0.07	0.7935
Season*photosynthetic pathway*stress treatment	3,	438	0.45	0.7188
Season*photosynthetic pathway*mycorrhizae*stress treatment	3,	438	0.56	0.640

Seasonal variation in photosystem recovery and C:N

Optimal efficiency (F_V/F_M) of photosynthesis also varied significantly by photosynthetic pathway in response to the stress treatments (Figure 3a; Table 3). Again, C_3 grasses had more pronounced responses than C_4 grasses following exposure to WS and HWS. In C_3 grasses F_V/F_M dropped more than 10% and 30% for the WS and HWS treated grasses respectively, in comparison to the control group (Figure 3a). On the other hand, F_V/F_M did not significantly decrease for the WS or HWS C_4 plants in contrast with the control treatments but did show a small but still significant difference of 6% when comparing the responses of HWS exposure to the HS treatment. Stress responses to F_V/F_M were also shown to be seasonal (Figure 3b; Table 3), with winter grown grasses in the HWS treatment showing less F_V/F_M recovery than spring grown grasses. Differences between treatment groups were greatest on d 12 (peak stress day), showing a more rapid decrease in F_V/F_M and leaf water potential for C_3 grasses in comparison to C_4 grasses (Supplementary Figure 1).

Table 2 Results of 4-way generalised linear mixed model testing the effects of mycorrhizae, stress treatment, species and season on intrinsic water use efficiency ($\delta^{13}C_p$) for both photosynthetic groups, C_3 and C_4. Values in bold are significantly different.

	df	F	p
C_3			
Mycorrhizae	1, 95	0.26	0.6102
Stress treatment	3, 95	**3.23**	**0.0257**
Species	2, 95	**8.09**	**0.0006**
Season	1, 95	**19.61**	**<.0001**
Mycorrhizae*stress treatment	3, 95	0.1	0.9602
Species*mycorrhizae	2, 95	0.09	0.9141
Season*mycorrhizae	1, 95	0.15	0.7005
Species*stress treatment	6, 95	1.07	0.3859
Season*stress treatment	3, 95	1.24	0.2981
Season*species	2, 95	**3.17**	**0.0464**
Species*mycorrhizae*stress treatment	6, 95	1	0.4274
Season*mycorrhizae*stress treatment	3, 95	0.43	0.7315
Season*species*mycorrhizae	2, 95	1.31	0.2753
Season*species*stress treatment	6, 95	0.53	0.7825
Season*species*mycorrhizae*stress treatment	6, 95	0.86	0.526
C_4			
Mycorrhizae	1, 93	3.14	0.0797
Stress treatment	3, 93	**2.72**	**0.0489**
Species	2, 93	**51.72**	**<.0001**
Season	1, 93	0.45	0.5055
Mycorrhizae*stress treatment	3 93	0.03	0.9942
Species*mycorrhizae	2, 93	1.04	0.3563
Season*mycorrhizae	6, 93	0.42	0.8672
Species*stress treatment	1, 93	0.03	0.871
Season*stress treatment	3 93	2.06	0.1114
Season*species	2, 93	0.74	0.4795
Species*mycorrhizae*stress treatment	6, 93	0.27	0.9479
Season*mycorrhizae*stress treatment	3, 93	0.95	0.4176
Season*species*mycorrhizae	2, 93	1.11	0.3333
Season*species*stress treatment	6, 93	1.11	0.3624
Season*species*mycorrhizae*stress treatment	6, 93	1.03	0.4123

Table 3 4-way generalised linear mixed model testing the effects of mycorrhizae, stress treatment, C_3/C_4 photosynthetic pathways and season on F_V/F_M. Values in bold are significantly different.

	df	F	p
F_V/F_M			
Mycorrhizae	1, 458	0.12	0.7272
Stress treatment	3, 458	**67.63**	**<.0001**
Photosynthetic pathway	1, 4	**37.51**	**0.0036**
Season	1, 458	0.03	0.8572
Mycorrhizae*stress treatment	3, 458	0.82	0.4839

Mycorrhizae*photosynthetic pathway	1, 458	0.35	0.5518
Photosynthetic pathway*stress treatment	3, 458	**26.92**	**<.0001**
Season*mycorrhizae	1, 458	0.16	0.6872
Season*stress treatment	3, 458	**3.43**	**0.017**
Season*photosynthetic pathway	1, 458	0.83	0.3626
Photosynthetic pathway *mycorrhizae*stress treatment	3, 458	0.06	0.9787
Season*mycorrhizae*stress treatment	3, 458	0.22	0.8814
Season*photosynthetic pathway*mycorrhizae	1, 458	0.44	0.5098
Season*photosynthetic pathway*stress treatment	3, 458	0.93	0.4237
Season*photosynthetic pathway*mycorrhizae*stress treatment	3, 458	0.86	0.4618

Differences in % C were not detected, however, changes in % N and C:N ratio was found to be dependent on photosynthetic pathway and season (Figure 4; Table 4). % N was found to be significantly higher in spring grown C_3 grasses, which is reflected in the C:N ratio (Figure 4a; Figure 4b). C_4 grasses had no seasonal responses. Neither % N nor % C was influenced by AM fungal colonisation (Table 4).

Table Error! No text of specified style in document. Results of 4-way generalised linear mixed model testing the effects of mycorrhizae, stress treatment, C_3/C_4 photosynthetic pathways and season on % C, % N and C:N ratio. Values in bold are significantly different.

	df	F	p
% C			
Mycorrhizae	1, 248	0.54	0.4616
Stress treatment	3, 248	1.01	0.3881
Photosynthetic pathway	1, 4	2.63	0.1801
Season	1, 248	0.47	0.493
Mycorrhizae*Stress treatment	3, 248	0.98	0.404
Mycorrhizae*photosynthetic pathway	1, 248	2.02	0.157
Photosynthetic pathway*stress treatment	3, 248	0.32	0.8116
Season*mycorrhizae	1, 248	0.67	0.4126
Season*stress treatment	3, 248	0.24	0.8655
Season*photosynthetic pathway	1, 248	0.56	0.4547
Photosynthetic pathway*mycorrhizae*stress treatment	3, 248	0.66	0.5743
Season*mycorrhizae*stress treatment	3, 248	1.32	0.2671
Season*photosynthetic pathway*mycorrhizae	1, 248	0.1	0.7516
Season*photosynthetic pathway*stress treatment	3, 248	1.44	0.2318
Season*photosynthetic pathway*mycorrhizae*stress treatment	3, 248	1.14	0.3334
% N			
Mycorrhizae	1, 248	2.59	0.1089
Stress treatment	3, 248	0.82	0.4858
Photosynthetic pathway	1, 4	0.15	0.719

Season	1,	248	78.95	**<.0001**
Mycorrhizae*stress treatment	3,	248	1.03	0.3811
Mycorrhizae*photosynthetic pathway	1,	248	0.16	0.6939
Photosynthetic pathway*stress treatment	3,	248	0.13	0.9428
Season*mycorrhizae	1,	248	1.15	0.2844
Season*stress treatment	3,	248	1.35	0.2581
Season*photosynthetic pathway	1,	248	47.43	**<.0001**
Photosynthetic pathway*mycorrhizae*stress treatment	3,	248	1.13	0.3388
Season*mycorrhizae*stress treatment	3,	248	0.4	0.7497
Season*photosynthetic pathway*mycorrhizae	1,	248	0.11	0.742
Season*photosynthetic pathway*stress treatment	3,	248	2.11	0.1
Season*photosynthetic pathway*mycorrhizae*stress treatment	3,	248	0.29	0.8358
C:N				
Mycorrhizae	1,	248	1.31	0.2541
Stress treatment	3,	248	0.67	0.5712
Photosynthetic pathway	1,	4	0.71	0.4455
Season	1,	248	91.4	**<.0001**
Mycorrhizae*stress treatment	3,	248	0.4	0.7547
Mycorrhizae*photosynthetic pathway	1,	248	0	0.9913
Photosynthetic pathway*stress treatment	3,	248	0.1	0.9593
Season*mycorrhizae	1,	248	1.24	0.2659
Season*stress treatment	3,	248	1.54	0.2052
Season*photosynthetic pathway	1,	248	60.34	**<.0001**
Photosynthetic pathway*mycorrhizae*stress treatment	3,	248	0.79	0.5021
Season*mycorrhizae*stress treatment	3,	248	0.43	0.7352
Season*photosynthetic pathway*mycorrhizae	1,	248	0.35	0.5544
Season*photosynthetic pathway*stress treatment	3,	248	2.18	0.0909
Season*photosynthetic pathway*mycorrhizae*stress treatment	3,	248	0.11	0.9547

4. Discussion

Contrary to expectations, this study found that arbuscular mycorrhizal fungi had no influence on most ecophysiological response parameters of the six native Australian grass species under compound stress events. With the exception of a significant effect on transpiration and stomatal conductance, the presence of AM fungi did not change carbon assimilation, water use efficiency, photosystem function, or plant nutrition. This study does, however, highlight the disparate responses of C_3 and C_4 grasses under climate stress and suggests that the differing capacities of each functional group may have adaptive consequences in their responses to climate stress events.

Influence of AM fungi on gas exchange, water use efficiency and plant nutrition under stress

There was some evidence for the potential role of AM fungi in mitigating plant responses to stress exposure. Differences among stress treatments highlighted a significant trend for grasses inoculated with AM fungi to have small but significant increases in transpiration rates and stomatal conductance. These results suggest that native grasses grown without AM fungi have increased stomatal closure and thus have a greater capacity to reduce transpirational water loss. These results also suggest that by improving plant water uptake, AM fungi should support greater gas exchange and therefore carbon fixation when exposed to water stress. However, increased stomatal conductance in AM grasses did not influence assimilation, plant water status, photosystem, or measures of plant nutrition. Further, AM fungi did not significantly affect either measure of water use efficiency. Also, total plant biomass was reduced by AM fungal colonisation in these grasses (Jones *et al.* in review). These results indicate that the influence of AM fungi on stomatal conductance to water vapour did not ultimately have a beneficial effect on carbon gain or plant growth.

The role of AM fungi in improving plant performance under water limitation is well documented (Sun *et al.* 2017; Tuo *et al.* 2017; Bitterlich *et al.* 2018b; Duc *et al.* 2018; Li *et al.* 2019; Mathur *et al.* 2019; Leventis *et al.* 2021; Liu *et al.* 2022; Xiao *et al.* 2023) and more recently, their role in mitigating heat stress has also been recognised (Haddidi *et al.* 2021; Reva *et al.* 2021; Ndeko *et al.* 2022). Notably, all the above studies are on agricultural cultivars, and are evidently not reflective of native species outcomes as reflected in this study. Another notable difference between past studies and this study is in the use of single AM fungal isolates compared to using field collected inoculum containing multiple AM fungal taxa. Inoculation with a single AM fungal species can often result in favourable outcomes for the host (Wu *et al.* 2024) as the push and pull between the host and symbiont is disrupted, and the mutualistic

– parasitic continuum (Johnson *et al.* 1997) favours the host if they are competitively dominant (Jansa *et al.* 2008; Knegt *et al.* 2016; Püschel *et al.* 2016). Duc *et al.* (2023) presents one of the only other studies on the effect of AM fungi on plant performance under combined heat and water stress. Their findings demonstrate the interplay between host outcomes and symbiont identity, with different fungal species showing large variations in tomato host performance, both positive and negative, in response to heat and water stress (Duc *et al.* 2023).

As this study used field collected inoculum, with as many as 25 separate AM fungal taxa present and up to nine individual AM fungal taxa colonising a single plant (Jones *et al.* in prep), the results presented here may be more reflective of plant responses under natural conditions. In field conditions, with a diverse range of competing symbionts, host responses may be limited as balanced mutualisms favour neutral leaning symbioses in facultative hosts (Bennett & Groten 2022). Therefore, in grassland communities, where continual AM fungi recruitment is ensured by a steady rate of colonised neighbours and fungal spores in the soil (Mony *et al.* 2021), grassland species may have neutral outcomes when colonised by multiple AM fungal taxa. However, changes in AM fungal community composition in response to climate stressors may occur (Millar & Bennett 2016). While the impact of these potential community shifts remain poorly understood, we do know that AM fungal identity influences symbioses form and function, and thus changes in community composition will see direct influences on plant community outcomes (Frew 2023).

Impact of ecologically relevant compound events on stress responses in native grasses

In response to the combined heat and water stress, overall physiological function of grasses was lower than experienced during single stressors, heat or water stress, alone. For both C_3 and C_4 grasses, leaf water potential, photosystem function and CO_2 assimilation were lowered when grasses were exposed to combined heat and water stress. When looking at responses to heat

stress alone, grasses showed little sign of physiological stress, with all measured parameters, aside from transpiration rate, being maintained at control levels. Conversely, grass responses to water deficit alone were variable, but did show signs of lessened performance, largely driven by C_3 grasses. These results make emphasise that that grasses can tolerate prolonged heat stress when water availability is not limiting (Marchin *et al.* 2022).

Water stress, therefore, was demonstrated as a key driver of physiological stress in native grasses. When water stress is compounded with heat stress, plants begin to reach their physiological thresholds and have a lower capacity to mitigate stress (Cook *et al.* 2021). The current study underscores a key physiological strategy and potential limitation of some plant species under ecologically relevant compound stressors: that when co-stressed by high heat and low water, they are unable to upregulate stomatal conductance and their ability to cool leaves through transpiration is compromised, limiting the plants ability to reduce thermal damage (Marchin *et al.* 2022). Even when plants can maintain transpiration, the increased evaporative demand placed on plants during compound events has been shown to exacerbate plant tissue damage (Carins-Murphy *et al.* 2023). In this study, impaired function of photosynthetic apparatus of grasses was suggested by the reduced photosystem efficiency and lowered assimilation rates, particularly for C_3 grasses.

Greater resilience of C_4 grasses to stress events in comparison to C_3 grasses

Across all physiological parameters, C_4 grasses were found to outperform C_3 grasses in response to stress exposure. C_4 grasses had greater resilience to heat and water stress, as well as their combination, showing minimal decreases in performance. The impact of combined heat and water stress was shown to be additive, being greater than both the responses to single heat or water stressors combined, contrasting with the results of past findings (Hoover *et al.* 2014). The improved stress performance of C_4 grasses compared to C_3 grasses is likely

attributed to their adaptive morphophysiological structure which increases photosynthetic water use efficiency (Bräutigam & Gowik 2016), demonstrated in the significantly greater $\delta^{13}C$ of C_4 grasses found here. The conservative, but stable gas exchange rates of the C_4 grasses, imparted by their ability to concentrate CO_2 and reduce the rate of water lost per carbon gained, allows for a steady rate of leaf cooling via transpiration and the water reserves to facilitate this (Sage & McKown 2005). This study adds to the growing body of evidence of C_4 persistence in response to climate stressors and also highlights the existing concerns of further shifts in grassland community composition (Havrilla *et al.* 2023) as compound stress events increase in frequency and severity (Mukherjee & Mishra 2021); and; for example, where hardier C_4 grasses exclude the less resilient C_3 grasses (Xie *et al.* 2022).

While C_4 grasses in this study were consistently more resilient to climate stressors, C_3 grasses demonstrated more dynamic responses as well as seasonally dependent responses. C_4 species largely showed the same pattern across stress treatments for most parameters; aside from small differences in $\delta^{13}C$ among C_4 species and differing transpiration responses for one C_4 species, likely owing to their limited range of plasticity (Sage & McKown 2005). C_3 species, however, showed notable variation in responses across most parameters. This variability limits the ability to make reliable predictions about C_3 grass outcomes under stress exposure. This variability of C_3 species is corroborated by past studies (Davies *et al.* 2018). Further, these results suggest that within the less resilient C_3 group there will be winners and losers across grassland communities as climate extremes increase. Understanding which C_3 species show adaptive responses to compound heat and water stress, such as seasonal acclimation, would support predictions about grassland community shifts under climate change.

There was some evidence of grasses having an improved response in spring. For example, $F_V/F_{M,}$ both C_3 and C_4 grasses was higher in spring than in winter when exposed to combined

heat and water stress. There were additional positive responses for C_3 species, such as *A. sachne* and *P. labillardierei*, which had lowered $\delta^{13}C$ values, signalling increased photosynthetic activity. There was also an increased C:N ratio for C_3 grasses, reflective of increased investment into biomass growth; supporting the responses of improved growth responses to heat and water stress in spring compared to winter for C_3 grasses (Jones *et al.* in review). Improved plant performance during climate stress events in spring suggests that seasonal primed resilience and recovery is important for plants to respond adaptively to increasing stressors (Milner *et al.* 2023) the responses of grasses will depend on season. As high heat and low water stress events continue to increase during cooler winter periods (Trancoso *et al.* 2020; Adnan *et al.* 2022), we can expect to see grassland species and grassland communities having poorer outcomes and reduced ecosystem functioning.

Conclusions

This study provides valuable insights into the complex interactions between arbuscular mycorrhizal fungi under ecologically relevant climate stressors, along with the roles of plant functional types and seasonal acclimation. While AM fungi are often lauded for their stress-mitigating roles in agricultural contexts, their influence on native grass species under compound stress events in this study was minimal. More broadly, this research highlights the differential stress responses of C_3 and C_4 grasses, with C_4 species exhibiting greater resilience and stability under heat and water stress, and C_3 species demonstrating more plastic, albeit less predictable, responses. These findings emphasise the risk of further shifts in grassland community composition as climate extremes intensify, favouring hardier C_4 species. While the findings suggest that further reductions in overall C_3 species' abundance can be expected under increasing compound events, they also indicate species variation among C_3 species, meaning some persistence in this group. Additionally, this study demonstrates the important role of

seasonal acclimation in plant stress responses, with greater spring resilience, suggesting that seasonal context is essential to predicting plant responses and highlighting the risks associated with increasing compound stress events in cooler seasons. Grasslands dominated by C_3 species are likely at higher risk of being negatively impacted by heatwaves during droughts and as such the resulting ecosystem resilience under these conditions will be impacted by the suite of co-occurring C_3 and C_4 grasses.

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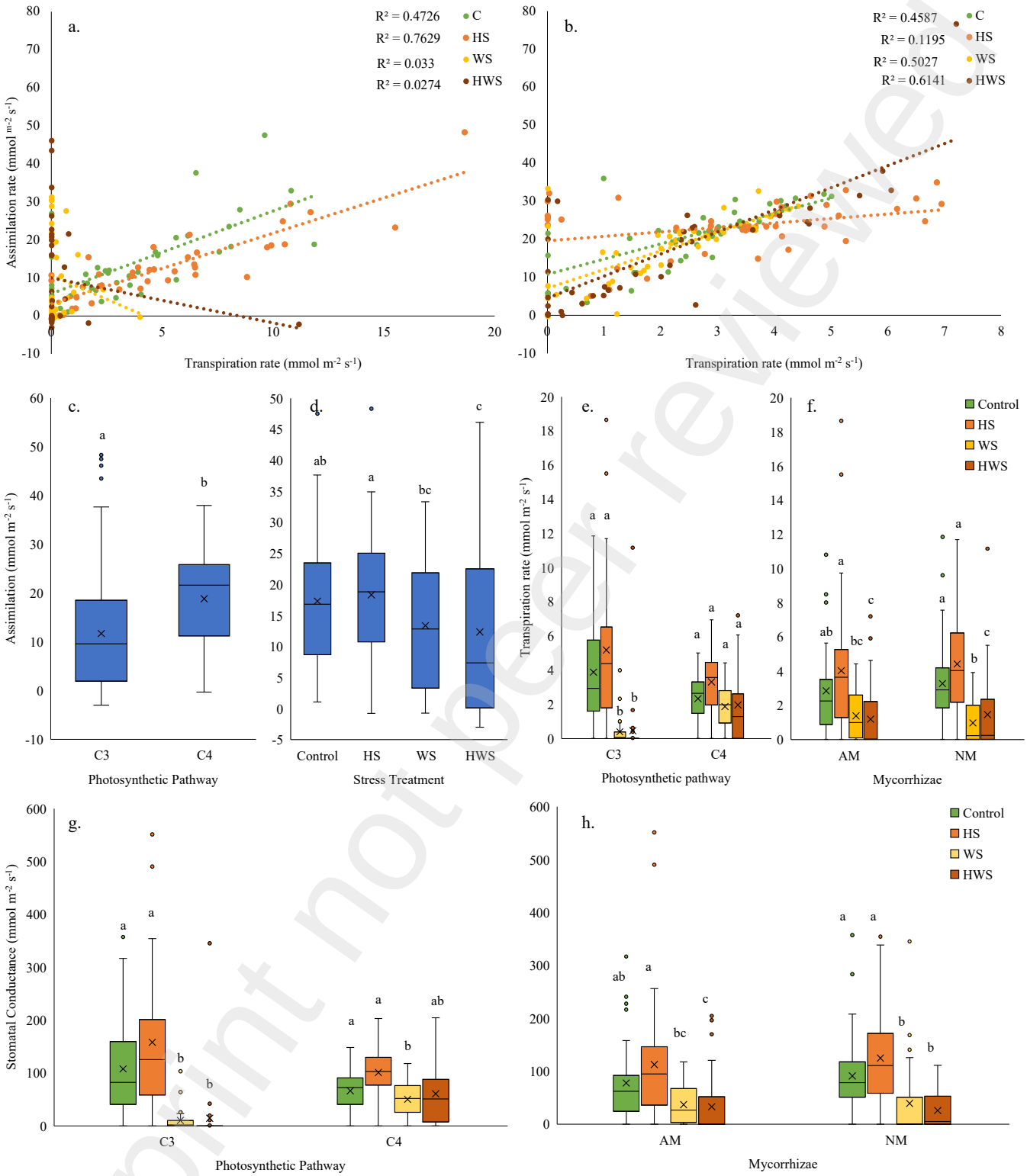

Figure 1 Results of a 3-way MANOVA showing the effects of stress treatment on the relationship between A and E for **a.** C_3 species and **b.** C_4 species. Results of general linear mixed models (GLMM) to determine the effects of assimilation rates by **c.** photosynthetic pathway (C_3/C_4) and **d.** stress treatment (control (C), heat stress (HS), water stress (WS) and heat + water stress (HWS); transpiration rate by **e.** photosynthetic pathway by stress treatment and **f.** mycorrhizal treatment (AM / NM) by stress treatment; and stomatal conductance by **g.** photosynthetic pathway by stress treatment and **h.** mycorrhizal treatment by stress treatment. Letters denote the results of post-hoc Tukey's tests within groups. Shared letters indicate NS. Boxplots indicate mean (×), interquartile range (box), minimum and maximum bounds (whiskers).

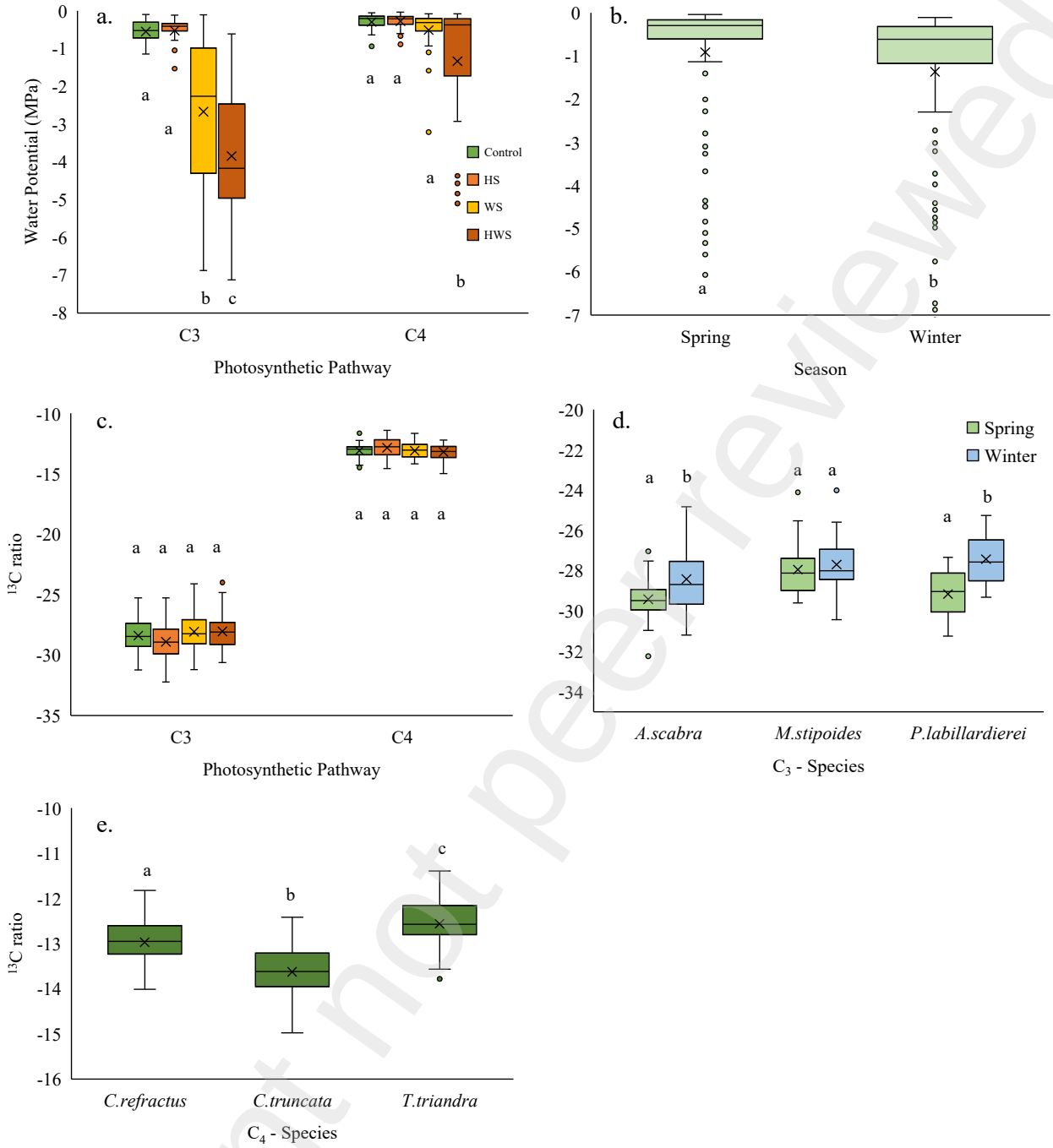

Figure 2 Results of general linear mixed models to determine the effects of **a.** water potential by photosynthetic pathway (C_3 / C_4) and stress treatment (control, HS = heat stress, WS = water stress and HWS = heat + water stress), letters denote results of Tukey's tests performed within photosynthetic pathway, among stress treatments; **b.** water potential by season (spring / winter); letters denote results of Tukey's test; **c.** $\delta^{13}C_p$ ratio by photosynthetic pathway and stress treatment, Tukey's tests were performed within photosynthetic pathway, among stress treatments; **d.** $\delta^{13}C_p$ ratio by C_3 species (*Austrostipa scabra, Microleana stipoides* and *Poa labillardierei*) and season; Tukey's tests were performed between seasons within species; **e.** $\delta^{13}C_p$ ratio by C_4 species (*Cymbopogon refractus, Themeda triandra* and *Chloris truncata*); letters denote results of post-hoc Tukey's test. Boxplots indicate mean (×), interquartile range (box), minimum and maximum bounds (whiskers).

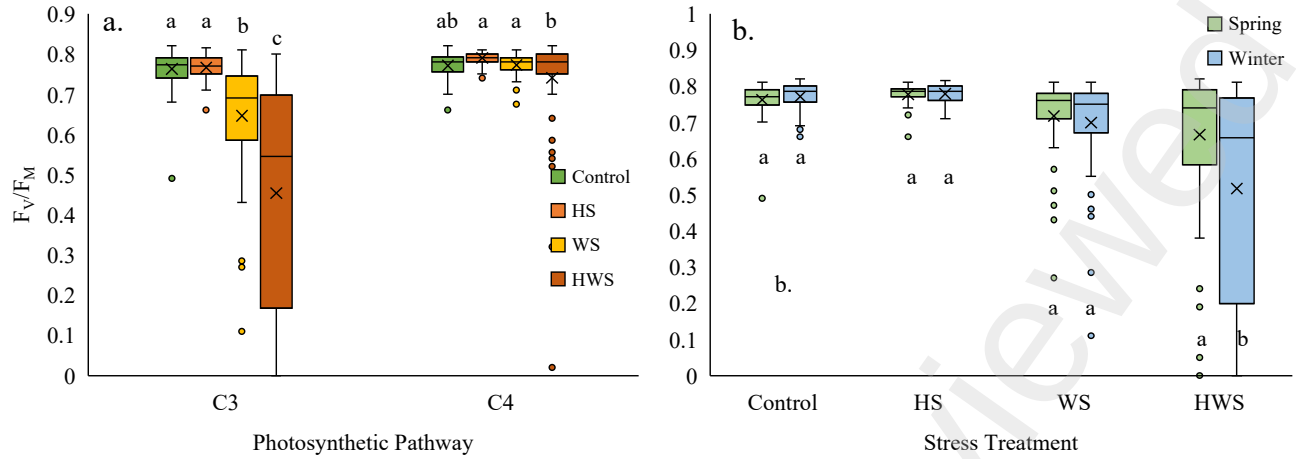

Figure 3 Results of general linear mixed models to determine the effects of **a.** F_V/F_M by photosynthetic pathway (C_3 / C_4) and stress treatment (control, HS = heat stress, WS = water stress and HWS = heat + water stress), letters denote results of Tukey's tests performed within photosynthetic pathway, among stress treatments; **b.** F_V/F_M by stress treatment and season (spring / winter), Tukey's tests were performed between seasons within stress treatment. Boxplots indicate mean (×), interquartile range (box), min. and max. bounds (whiskers).

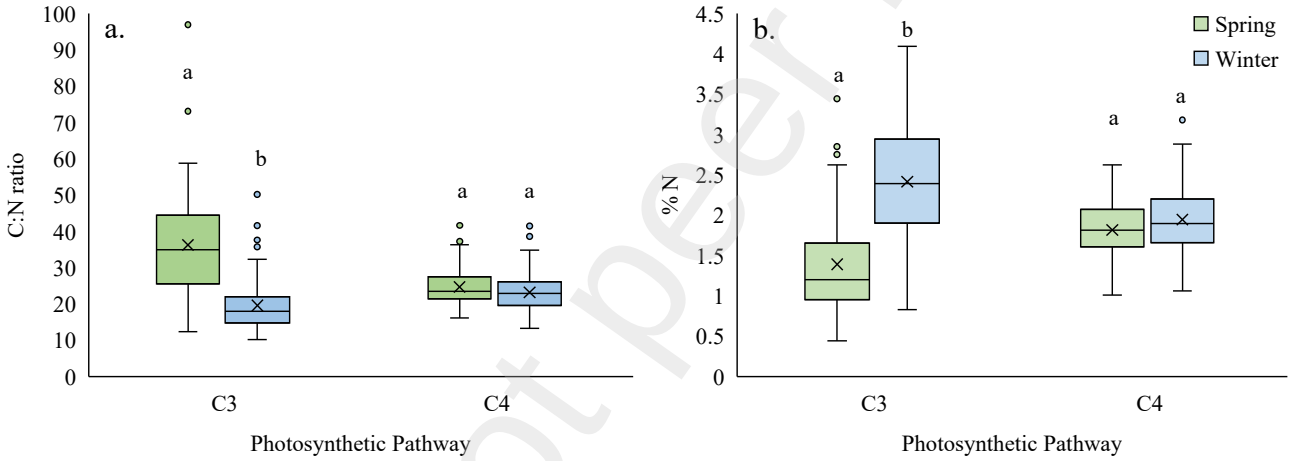

Figure 4 Results of general linear mixed models to determine the effects of **a.** C:N ratio by photosynthetic pathway and season (spring / winter); Tukey's tests were performed within photosynthetic pathway between season and **b.** %N by photosynthetic pathway and season; Tukey's tests were performed within photosynthetic pathway between seasons. Boxplots indicate mean (×), interquartile range (box), minimum and maximum bounds (whiskers).