Drought, thermal response and climate-patterning in cuticular conductance of the widespread C4 grass, *Themeda triandra*

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Cuticle responses to drought and temperature in *Themeda triandra.*

Highlight

Variation in cuticular conductance under drought and its thermal response reveals diverse drought adaptation strategies among Australian accessions of *Themeda triandra*, a widespread C4 grass.

Abstract

Grasslands are vital ecosystems for biodiversity, carbon cycling and climate resilience, yet their persistence is increasingly challenged by rising temperatures and water scarcity. This study examined intraspecific variation in leaf cuticular traits—minimum cuticular conductance following stomatal closure (g_{min}), and its temperature dependency—across six Australian accessions of *Themeda triandra* from contrasting climates. Plants were grown under well-watered conditions and exposed to experimental drought. Trait variation and drought responses were quantified in relation to climate-at-origin. For well-watered plants there was no relationship between g_{min} and accession climate-at-origin. However, under drought, g_{min} showed clear climate signals, being lower in plants from wetter and cooler regions—in the opposite direction to that predicted. g_{min} also varied clearly (positively) with leaf mass per area, LMA. The six accessions varied substantially in g_{min} responses to drought, from negative to positive. Thermal responses in g_{min} also varied among accessions although, in common, none showed a clear threshold (phase transition) at which g_{min} increased rapidly. That is, g_{min} was more thermally stable than expected. Taken together, these findings highlight substantial intraspecific variation in cuticular traits related to water loss, providing initial steps towards a pathway for selecting climate-resilient genotypes for use in grassland restoration and conservation.

Keywords: drought, leaf cuticle, minimum cuticular conductance, phase transition temperature, *Themeda triandra*, water loss.

Abbreviations

g_{min}: Minimum Cuticular Conductance

LMA: Leaf Mass per Area

AP: Annual Precipitation

AT: Annual Temperature

NSW: New South Wales

NT: Northern Territory

QLD: Queensland

SA: South Australia

T_p: Phase Transition Temperature

WA: Western Australia

Introduction

Grasslands occupy 31% of earth's land area (Petri et al. 2010) and are essential for preserving biodiversity, sequestering carbon and maintaining ecosystem balance (Blair et al. 2014). Grassland ecosystems support a diverse range of plant and animal species, contribute to global food security through livestock grazing, and provide essential ecosystem services such as water infiltration and soil stabilization. However, these ecosystems are especially susceptible to climate change effects, such as alterations in temperature and rainfall and increases in the frequency and severity of extreme weather events (Sala et al. 2000). Indeed, extreme temperatures and droughts are expected to become more intense and frequent worldwide throughout the 21st century (Teuling 2018). These changes pose a risk to grassland resilience, potentially decreasing species diversity, shifting productivity, and causing major alterations to nutrient cycling and soil structure (Overpeck 2013, Edenhofer et al. 2014). Understanding these changes is critical for developing effective conservation and

management strategies for safeguarding grassland ecosystems in a rapidly changing environment.

Under well-watered daytime conditions, stomata remain open to facilitate gas exchange for photosynthesis while simultaneously losing water via transpiration. During drought, plants reduce water loss primarily by closing their stomata. However, even after full stomatal closure, plants continue to lose water through the leaf epidermis ("residual transpiration"; Billon et al. (2020)). This residual transpiration can be significant, particularly under prolonged drought or high temperatures, and is primarily attributed to the permeability of the leaf cuticle, although leaky stomata may also contribute (Muchow and Sinclair 1989, Machado et al. 2021). The rate of this residual water loss from the leaf surface when stomata are fully closed is often referred to as "minimum cuticular conductance" or "g_{min}". g_{min} is increasingly recognized as being ecologically important, indexing the effectiveness of the cuticle for slowing water loss during drought (Schuster et al. 2016). In dryland and savanna species, for example, small differences in g_{min} can significantly affect drought survival (Burghardt and Riederer 2003). Recent work has highlighted that g_{min} is not constant but dynamic throughout dehydration (Burlett et al. 2025). This variability in g_{min} can potentially alter predictions of time to hydraulic failure (Martin-StPaul et al. 2017), thus highlighting the need to understand the response of g_{min} to drought stress, especially against the background of projected increasingly prolonged and severe droughts.

At moderate temperatures (e.g. 20–25 °C), g_{min} of healthy intact leaves is relatively low, typically averaging around 10% of the transpiration through fully open stomata, depending on the species (Holmgren et al. 1965, Boyer et al. 1997). This is because cuticular waxes serve as an efficient barrier against water movement (Boyer et al. 1997, Burghardt and Riederer 2003, Riederer 2006). However, g_{min} may vary with ambient temperature, displaying non-linear dynamics such that it remains relatively stable across these moderate temperatures, then increases sharply beyond some threshold temperature (Schreiber and Schönherr 1990). This threshold temperature is commonly known as the "phase transition temperature" or (T_p). At T_p, the structural integrity of the cuticle begins to shift due to thermal disruption of wax and polymer organization, resulting in a dramatic increase in g_{min} and hence, higher water loss (Schreiber and Schönherr 1990). T_p can vary among species and populations and is considered a marker of thermal stability of the cuticle (Schuster et al. 2016, Bueno et al. 2019, Billon et al. 2020). For example, Schuster et al. (2016) reported that the desert shrub *Rhazya stricta* had a markedly high T_p, suggesting an adaptation to hot, arid

conditions. In the context of climate change and heatwaves, understanding T_p is critical because it determines the threshold temperature at which the primary barrier to plant water loss is compromised during drought. The extent to which these changes are reversible is also unknown. For instance, Schönherr et al. (1979) observed that heating cuticular membranes above their transition temperature irreversibly altered the distribution of soluble cuticular lipids, leading to increased water permeability.

Substantial interspecific variation in g_{min} has been documented; e.g. Duursma et al. (2019) compiled data for 221 species from 40 original studies, reporting values from ~ 0.1 to 50 mmol m^{-2} s^{-1}. By taxonomic order, Poales on average exhibited the highest values, and conifers (Pinales, Araucariales) the lowest, but there was wide variation within any given group. There is evidence showing that g_{min} variation reflects differences in the chemical composition of cuticles; e.g. the richness of cuticular wax components (such as long-chain alkanes) is known to reduce cuticle permeability (Huang et al. 2017, Cheng et al. 2019).

There is little evidence for g_{min} varying interspecifically with site climate (Duursma et al. 2019); however, variation with respect to leaf habit (phenology) is known. For example, research on 24 tropical tree species across varying rainfall conditions found that while g_{min} did not consistently vary along the gradient, it tended to be higher in deciduous species compared to evergreens, suggesting that evergreen species, which retain their leaves throughout the dry season, require more effective water conservation mechanisms (Slot et al. 2021). Similarly, Machado et al. (2021) examined tropical savanna species with contrasting growth strategies and found that deciduous species tended to exhibit higher minimum conductance than their evergreen counterparts, highlighting a trade-off between rapid resource acquisition and water conservation.

Climate-related *intraspecific* variation in g_{min} and cuticle traits has also been observed. For example, some studies have reported g_{min} as lower in plants from arid compared to mesic environments, presumably to minimize residual water loss during drought; this was the case for one of two *Hakea* (Proteaceae) species studied by López et al. (2021). Furthermore, Smith et al. (2006) compared *Digitaria californica* populations from Texas (arid) and Arizona (less arid), as well as the invasive species *Eragrostis lehmanniana*, in a common garden setting. They found that the arid-zone population and *E. lehmanniana* both exhibited significantly lower g_{min} values. In *Nicotiana benthamiana*, two accessions exhibited contrasting drought strategies: plants from arid central Australia relied on rapid

growth and early flowering to escape drought, with minimal changes in cuticle properties or g_{min} under experimental drought (Asadyar et al. 2024). In contrast, plants from tropical northern Australia reduced g_{min} in response to water limitation by altering wax composition, specifically increased very-long-chain alkanes, to minimize water loss under drought conditions (Asadyar et al. 2024). Other studies have also shown acclimation of g_{min} to drought and elevated temperature conditions (Duursma et al. 2019). However, other studies have shown a lack of response in g_{min} to changing growth conditions (Bueno et al. 2020).

Most research on cuticle conductance has focused on woody species and a limited subset of herbaceous plants, primarily high-value annual crops such as wheat and sorghum (Muchow and Sinclair 1989, Araus et al. 1991, Qariani et al. 2000, Lin et al. 2020). In contrast, native and perennial grass species—despite their ecological significance—remain understudied. As a result, our understanding of how perennial grasses regulate cuticular water loss in response to drought remains limited, highlighting the need for broader investigation across diverse grass lineages. Consequently, here we focus on *Themeda triandra*, a perennial C4 tussock grass endemic to Africa, Australia and Asia (Snyman et al. 2013). It has great ecological significance due to its broad distribution across various ecosystems and geological substrates. It is a valuable source of food for both native and introduced herbivores, and plays a crucial role in supporting rural livelihoods, wildlife and livestock production (Snyman et al. 2013). *T. triandra* is widely distributed across Australia and is believed to have been the primary dominant species in many Australian grasslands prior to European settlement (Mitchell and Miller 1990). It grows in habitats as diverse as the hot, semiarid interior, the wet tropics, and cool temperate sub-alpine regions (Mitchell and Miller 1990). This makes it an ideal model for exploring physiological responses to climate-induced stresses in grasses. A reduction in the occurrence of *T. triandra* within grasslands is often associated with a drop in grazing potential, a loss of biodiversity, reduced vegetation cover and a decline in overall health of the ecosystem (Snyman et al. 2013). Despite its huge ecological and economic significance, to this point there has been no attempt to study its cuticle properties.

In this study, we measured g_{min} (both on well-watered and droughted plants) and explored its response to increasing temperature across six distinct accessions of *T. triandra* originating from diverse regions across Australia, spanning a wide range of temperature and rainfall regimes. We had two key aims. First, we sought to elucidate the relationships between climate conditions at the seed source ("climate-at-origin") and two aspects of cuticle permeability: g_{min} and its temperature response (T_p). Second, we sought to characterise the

different accessions in terms of their variation in g_{min} and T_p under control and water-limiting conditions. We also sought to clarify the relationship between g_{min} and the widely-used leaf "economic" trait, leaf mass per area, LMA.

We tested the following hypotheses:

1. *T. triandra* accessions from hotter and drier environments would exhibit generally lower g_{min}, reflecting selection for reduced cuticular water loss under high evaporative demand and limited water availability.

2. Cuticle conductance would show a bi-phasic response to increasing temperature, remaining relatively steady at moderate temperatures, then rising sharply once temperatures exceeded a threshold, T_p, which itself would vary with climate-at-origin. Specifically, we expected plants from hotter environments to have higher T_p; i.e., to maintain cuticular stability up to higher temperatures.

3. Experimental drought would reduce g_{min} in all accessions. This expectation assumes that the lower gmin would reduce plant water loss when savings are needed most. We also expected the proportional change in g_{min} (from control to drought conditions) to be greater in accessions originating from arid or high-temperature regions. This is based on the assumption that plants from such environments have evolved greater plasticity or regulation of cuticle properties to limit water loss during droughts (Bueno et al. 2019).

Materials and methods

Plant material and growth conditions

Six accessions of *T. triandra* (Dalby, QLD; Forbes, NSW; Mt. Fox National Park, QLD, Pannawonica, WA; Rainbow Valley, NT; and Virginia Gardens, SA) (Fig. 1 (a)) were grown from seeds sourced from the Australian Pastures Genebank, Native Seeds Pty Ltd and Nindethana Seed Service Pty Ltd (Albany, Western Australia). The selected accessions represent a range of climate-at-origin conditions (climate data from CHELSA (Karger et al. 2017); Table S1), with mean summer temperatures ranging from 21.0 °C to 32.0 °C, summer rainfall from 75.1 to 435.9 mm, annual temperatures from 16.2 °C and 22.1 °C and annual precipitation from 281 to 712 mm. Plants were propagated from seeds in a climate-controlled growth cabinet. Germinated seedlings were moved into 8 litre round plastic pots containing field soil and placed in climate-controlled glasshouses with 14 h light at a 30/20 °C day/night

temperature regime and 60% relative humidity. The plants were fertilized using a combination of slow-release native-plant fertiliser (10 g Osmocote Native slow release) along with fortnightly application of a commercial water-soluble fertiliser (AQUASOL, Yates, Australia), applied at half regular strength.

Water deficit treatment

Each accession was represented by four replicates, all maintained under well-watered conditions for two months. Plants were irrigated to full field capacity using a drip system. Following this period, all four replicates were first sampled under well-watered conditions. The plants were then subjected to an acute water-deficit treatment by withholding irrigation for three days to simulate a rapid decline in soil moisture and assess plant responses under acute stress conditions. Soil water content was monitored using a soil moisture probe, and leaf water potential was measured with a pressure chamber to assess plant water status (Figs S1 and S2). At the end of the drying period, the plants were re-sampled under water-deficit-treated conditions. All measurements were conducted on four biological replicates per accession per treatment, with each replicate representing a distinct plant individual.

Leaf drying methods used for g_{min} determination

Initially, g_{min} measurements were conducted using the benchtop drying method (Sack and Scoffoni 2010) at a single temperature (30 °C), which was well-suited for assessing the steady state cuticular conductance under controlled conditions. However, as the study progressed, the need to measure g_{min} across multiple temperatures became evident, requiring a more efficient and automated approach. The benchtop method, though reliable, was time- and labour-intensive and limited in its throughput. To overcome these limitations and facilitate rapid measurements across a range of temperatures, we incorporated a "DroughtBox" (Billon et al. 2020) into our workflow, which provided an automated, high-throughput system for assessing g_{min} under precisely controlled environmental conditions.

Description of the DroughtBox

The DroughtBox, originally described by Billon et al. (2020) consists of a 70 cm cubic box with two compartments for semi-automatically determining g_{min} under controlled environmental conditions, specifically temperature and humidity. The system includes strain gauges connected to a data acquisition board (for measuring the mass loss from leaves as they dehydrate), heating resistors for temperature control, and electronically controlled valves for adjusting relative humidity. The original design also incorporates a Raspberry Pi for overall control and monitoring.

In this study, we used a modified version of the DroughtBox to measure g_{min} and T_p. Our version consisted of a 60 cm × 140 cm × 70 cm cuboidal box made of 40 mm thick polystyrene boards with a front door and two compartments (Fig. S3). The upper compartment housed 8 strain gauges (Phidgets single point load cell 780g), fixed in an aluminium frame and directly connected to a Campbell Scientific CR300 datalogger for data acquisition. Leaves for g_{min} measurements were placed in the main lower compartment and attached to the strain gauges through holes in the roof board. An air temperature and relative humidity sensor (Vaisala HMP155) was suspended in the lower compartment to monitor environmental conditions, along with a fine-wire type T thermocouple for rapid response temperature control. Temperature regulation was achieved using two PTC heaters (Joyzan, 220 V, 300 W) mounted in galvanized steel conduits, with a 50 mm 12 V fan to push heated air into the box. A thermostat switch (MULTICOMP T23A150BSR2-15) was installed for safety, shutting off the heaters if they overheat. A solid-state relay (Sparkfun electronics, 40A, 3–32 V DC) was used to allow logger to regulate the heaters via pulse width modulation. Relative humidity in the lower compartment was adjusted by a water-filled plastic container with ultrasonic mist maker (Vaguelly mist maker, 350 mL, 24 V). A 12 V pump (DANXQ, 12 V DC) circulated air through the mist container to introduce fine mist into the box when needed. All environmental variables were monitored and controlled using a Campbell Scientific CR3000 datalogger with program written and compiled in Visual Basic.

Cuticle conductance measurement (benchtop drying)

To assess leaf water loss, 4–5 healthy tillers with fully developed, mature leaves were collected in the morning on each sampling day. The cut ends of the tillers were immediately wrapped in damp paper towels, placed in plastic zip lock bags, and transported to the lab. To relieve xylem tension, the tiller ends were recut under water and allowed to rehydrate in falcon tubes for about 30 minutes. The cut ends were then sealed with parafilm. The tillers were weighed using a balance to record their fully hydrated weight. The leaves were then dehydrated in open plastic trays placed in a controlled growth cabinet set at 30 °C and 60% relative humidity, in the dark, for 2–3 hours, with leaf weight (w) measured every 20 minutes until the rate of water loss stabilized. We considered changes in w to represent the number of water molecules (n_w, mmol) transpiring from the branches. Based on this assumption, transpiration rate, E (mmol m^{-2} s^{-1}) was calculated as

$$E = -\frac{slope\ (n_w;\ t)}{LA} \tag{1}$$

where, t is the time in seconds, and LA represents two-sided leaf area. LA was estimated using the dry leaf mass of each tiller set and empirical relationships between leaf dry mass and fresh leaf area (i.e., LMA). Subsequently, g_{min} was determined from the linear segment of the water loss curve, that is, after stomatal closure, as following

$$g_{min} = -\frac{E}{VPD} \times 101.6 \tag{2}$$

where, E is transpiration in mmol m^{-2} s^{-1}, VPD refers to the vapour pressure deficit of the air in kPa, and 101.6 denotes the atmospheric pressure within the cabinet (described below) in kPa.

At the end of the experiment, the leaves were oven-dried at 70°C for 72 hours, and the final dry weight was recorded.

The percentage change in g_{min} in response to drought (%Δg_{min}) was determined using the g_{min} data from control and drought-treated plants. It was calculated using the formula

$$\%\Delta gmin = \frac{gmin(drought) - gmin(control)}{gmin(control)} \times 100 \quad (3)$$

293

294 **g_{min} measurement using the DroughtBox**

295 The tillers were harvested and prepared for drying in a manner consistent with benchtop
296 drying procedure. Multiple leaf-drying runs were conducted in the DroughtBox at target
297 temperatures of 30, 35, 40, 45, 50 and 55 °C (using a distinct cohort of samples at each
298 temperature), with corresponding increases in vapour pressure deficit (VPD) to maintain a
299 constant absolute humidity of 18 $g(H_2O)m^{-3}$. This standardization ensured consistent
300 conditions during g_{min} measurements. The mass data from the DroughtBox were similarly
301 used to calculate g_{min} from the linear portion of the mass loss curve, as was done following
302 benchtop drying procedure. After the DroughtBox measurements were complete, samples
303 were oven-dried at 70 °C for 72 hours to determine their dry mass.

304

305 **Leaf mass per area (LMA)**

306 LMA was calculated as the ratio of leaf dry mass to leaf area. For each replicate plant, fully
307 expanded leaves were harvested and scanned using a LICOR leaf area meter (model LI-
308 3100C, LI-COR Biosciences, USA) to determine the total leaf area. The scanned leaves were
309 then oven-dried at 60 °C for 72 hours to reach dry mass. LMA ($g\ m^{-2}$) was then calculated
310 by dividing dry mass by leaf area.

311

312 **Statistical analyses**

313 The g_{min} data, including those obtained from both benchtop drying and from multiple
314 temperature runs in the DroughtBox, were visually examined for normality before analysis
315 using histograms and density plots, and log-transformed wherever the data were right-
316 skewed. Significant differences in g_{min} values between accessions and treatments were
317 assessed using a two-way analysis of variance (ANOVA), followed by Tukey's Honestly
318 Significant Difference (HSD) test and Student's t-test for pairwise comparisons. The
319 relationships of g_{min} and $\%\Delta g_{min}$ with climate variables were evaluated through multiple
320 linear regression models using mixed effects models with accession treated as a random

effect. By including both temperature and rainfall in these models, we were able to identify the rainfall-independent effect of temperature and the temperature-independent effect of rainfall. Climate variables were treated both on a yearly basis (annual temperature, AT; annual precipitation, AP), and on a seasonal basis (mean summer temperature; summer rainfall). The relationship between g_{min} and LMA was quantified with ordinary least squares linear regression.

To determine T_p, we used segmented regression applied to Arrhenius-transformed data. Specifically, g_{min} values were natural log-transformed (ln g_{min}) and plotted against the inverse of absolute temperature (1/T, in Kelvin). For each replicate, a segmented linear regression model was fitted using the segmented package in R to estimate a potential breakpoint along the temperature axis, corresponding to a putative T_p. A significance threshold of 0.05 was applied for all statistical tests.

All analyses were conducted using R v.4.4.0 (R Core Team 2025).

Results

Drought-induced changes in cuticular conductance across accessions

Pooling data across the six accessions, g_{min} did not differ between control and droughted plants (Fig. 2). However, substantial variation among accessions was evident, both in mean g_{min} values and in their response of g_{min} to drought (Fig. 3). g_{min} of control plants varied among accessions (ANOVA, $p < 0.001$), being significantly higher in plants from Pannawonica and Mt. Fox than those from Dalby (Tukey HSD, both $p < 0.001$), Forbes (Tukey HSD, $p = 0.003$ and 0.015, respectively), and Virginia Gardens (Tukey HSD, $p = 0.004$ and 0.019, respectively).

For droughted plants g_{min} also varied among accessions (ANOVA, $p = 0.041$), albeit sufficiently weakly that no pairwise differences were statistically significant after post hoc correction (Tukey HSD, all $p > 0.05$). One comparison was very nearly statistically significant, with plants from Rainbow Valley tending to have higher g_{min} than those from Mt. Fox (Tukey HSD, $p = 0.054$).

Although there was no overall difference in g_{min} between control and droughted plants when pooled across all accessions, individual accessions differed in their g_{min} response to

351 drought (accession × treatment interaction: ANOVA, $p < 0.001$), with two exhibiting
352 pronounced reductions in g_{min} (Mt. Fox and Pannawonica; paired t-test, $p = 0.0162$ and $p =$
353 0.0104, respectively), three showing no significant difference in g_{min} with treatment, and one
354 (Dalby) exhibiting increased g_{min} (paired t-test, $p < 0.001$). The asterisks in Figure 3 identify
355 these accessions and the degree of significance of these shifts.

356

357 **Influence of climate-at-origin on g_{min} and its response to drought**

358 To evaluate how climate-at-origin influences g_{min}, we assessed relationships between g_{min}
359 and both seasonal and annual climate variables. Our primary focus was on summer
360 temperature and summer rainfall (Fig. 4), with relationships involving mean annual
361 precipitation and mean annual temperature reported as supplementary results (Fig. S4).

362 Under control conditions, g_{min} showed no relationship with either summer rainfall (p
363 $= 0.484$; Fig. 4a) or summer temperature ($p = 0.286$; Fig. 4b). Similarly, g_{min} showed no
364 relationship to either AP or AT (both $p > 0.12$; Figs S4a,b). By contrast, clear climate-linked
365 patterns emerged under drought conditions, g_{min} being on average higher in plants from drier
366 sites (Figs 4c, S4c; $p = 0.014$ and 0.002 respectively), such that a reduction in summer rainfall
367 from 436 mm to 75 mm corresponded to a 16.5% increase in g_{min}. On average, g_{min} of
368 droughted plants was also higher in plants from sites with warmer summers ($p = 0.003$; Fig.
369 4d), such that an increase in summer temperature from 21 °C to 32 °C corresponded to a
370 23.5% increase in g_{min}. There was no relationship between g_{min} and MAT ($p = 0.961$; Fig.
371 S4d).

372 To assess plasticity in cuticle conductance, we quantified the percentage change in
373 g_{min} from control to drought ($\%\Delta g_{min}$). Contrary to our expectation that accessions from
374 hotter or drier environments would show greater reductions in g_{min}, $\%\Delta g_{min}$ was not
375 statistically related to any of summer rainfall, summer temperature, AP or AT (Figs. 5a, b and
376 S5a, b; all $p > 0.05$).

377

378 **Relationship between g_{min} and LMA**

379 Across accessions, LMA and g_{min} were positively correlated (all $p < 0.001$) for control plants,
380 droughted plants, and for all plants considered together (Figs 6a–c). The relationship was

noticeably tighter under drought conditions ($R^2 = 0.641$) than under control conditions ($R^2 = 0.192$) or in the pooled data ($R^2 = 0.297$).

Temperature dependence of g_{min} across accessions

The "textbook" response of g_{min} to temperature was not observed in our study (i.e., we did not observe a consistent pattern of near-constant g_{min}, rapidly increasing at some threshold temperature, T_p). Overall, the response of g_{min} to increasing temperature was broadly similar across accessions (Fig. 7, a–f). While most accessions (Mt. Fox, Pannawonica, Rainbow Valley and Virginia Gardens) showed a relatively flat response with slightly decreasing g_{min} as temperature increased, plants from Dalby and Forbes displayed a modest rise in g_{min} around 35–40 °C before eventually declining at higher temperatures, like the other accessions. A segmented regression model applied to individual replicates revealed no consistent evidence of a distinct breakpoint across accessions. This statistical outcome aligns with the visual inspection of the plots, where g_{min} appeared largely stable or declined gradually across the experimental temperature range.

Discussion

g_{min} in response to drought

Our study highlights the impact of drought stress on g_{min} across six accessions of *T. triandra,* revealing substantial within-species variability in responses. Although the overall effect of drought on g_{min} was not statistically significant at the species level (Fig. 2), the direction and magnitude of responses differed substantially among accessions (Fig. 3), pointing to accession-specific strategies in cuticular water regulation.

In our experiment, all plants were grown under well-watered conditions and only subjected to a single drought cycle over three days. Such rapid stress treatments can trigger short-term physiological and biochemical shifts in the cuticle. For instance, Macková et al. (2013) showed that *Lepidium sativum* plants subjected to a 7-day exogenous ABA treatment—mimicking drought stress—exhibited a marked shift in cuticular wax composition. Specifically, the proportion of long-chain aliphatic waxes increased, enhancing the hydrophobicity of the cuticle and potentially reducing water permeability, despite no

change in total wax load. Similarly, Cameron et al. (2006) found that short-term periodic dehydration in *Nicotiana glauca* resulted in a 1.5–2.5-fold increase in wax deposition, reducing cuticular transpiration within days.

The significant reductions in g_{min} in response to drought observed in accessions from Mt. Fox (48%, $p = 0.0162$) and Pannawonica (36%, $p = 0.0104$) align closely with the classical drought-adaptive responses. While the drought response of g_{min} was not directly correlated with the accessions' climate-at-origin, these two regions share ecological features—seasonally intense or prolonged drought, high evaporative demand, and unpredictable rainfall. These climate features may favour physiological strategies that reinforce the cuticle under water deficit. In these cases, the decrease in g_{min} may therefore reflect inducible cuticular responses, such as increased wax deposition or structural adjustments that strengthen the water barrier.

However, these patterns were not universal. The Dalby accession exhibited a 31% increase in g_{min} ($p < 0.001$) under drought stress—a response that diverges from the typical drought-induced reduction. This increase may reflect a plastic, rather than adaptive, response to drought—potentially involving alterations in cuticular wax composition that do not enhance water barrier function—as observed in grape berries under water deficit (Dimopoulos et al. 2020). Notably, Dalby had lower g_{min} than the other accessions under control conditions and, even with this increase, its absolute g_{min} remained relatively low. This suggests that plants from Dalby may possess inherently lower cuticular permeability overall, and that the observed increase may still fall within a functionally conservative range.

In contrast, in plants from Forbes, Rainbow Valley and Virginia Gardens, g_{min} did not differ between control and drought treatments. This suggests a high degree of drought tolerance or a mechanism of homeostasis that stabilizes physiological performance under fluctuating water availability. Such accessions might possess robust cuticular barriers that maintain consistent conductance levels regardless of external stressors. Alternatively, these accessions may rely on non-cuticular mechanisms—such as deep or extensive roots systems or osmotic adjustment—to cope with water stress. For instance, efficient root systems can enhance water uptake (Wasson et al. 2012), and osmotic adjustment can maintain cell turgor (Turner 2018) and help plants tolerate water limitation (DaCosta and Huang 2006). Osmotic adjustment involves the accumulation of solutes in plant cells (Hsiao et al. 1976), which aids

in retaining water and sustaining physiological processes during periods of limited water availability. Further research in *Themeda* is needed to investigate these processes.

Climatic correlations with g_{min} and $\%\Delta g_{min}$

Our analysis of *T. triandra* accessions revealed nuanced climate relationships with g_{min}, underscoring the complexity of adaptive trait variation across environmental gradients. Under control conditions, g_{min} showed no association with either annual (AP, AT) or seasonal (summer temperature, summer rainfall) climate variables. This finding contrasts with our hypothesis that accessions from hotter and drier regions would exhibit inherently lower g_{min} to limit water loss. Instead, it suggests that cuticular permeability in this species under control conditions is not tightly linked to climate-at-origin, and that other traits may buffer its expression under transient droughts.

Under drought conditions, however, g_{min} displayed clear climate-linked trends, being typically higher in plants from drier and hotter sites (Figs 4c,d). This runs counter to classical expectations of drought adaptation, which predict reduced g_{min} as a water saving mechanism (Smith et al. 2006). Instead, it suggests that in hot, dry climates, maintaining moderate levels of cuticle permeability may serve alternative functions—such as leaf surface cooling via residual transpiration. However, it is important to note that the magnitude of these trends was relatively modest—across the range of observed summer rainfall (75–436 mm), g_{min} increased by only 16.5%, and across the summer temperature range (21–32 °C), by 23.5%. Notably, MAT was not a significant predictor of g_{min} under either control or drought conditions, emphasizing that seasonal climate variables may be more informative than annual averages for understanding trait-environment relationships in this species.

The climate-linked pattern of g_{min} under drought—specifically, its positive relationship with summer rainfall in fig. 4c—may reflect an evolved alignment of trait means with long-term climate. However, within individual accessions, the short-term drought responses were not always consistent with this trend. For example, Pannawonica—an arid origin accession—showed a reduction in g_{min} under drought. In contrast, Dalby—from a wetter site—showed an increase in g_{min} under drought. This suggests that trait means (shaped by climate adaptation), and trait plasticity (plastic responses to acute stress) are not always aligned. Trait means likely reflect long-term selective pressures at population level, whereas

plastic responses to short-term drought may be shaped by local adaptive strategies, trade-offs, or even stress-related responses. Recognizing that trait means and plasticity are distinct and potentially uncorrelated axes of variation is important for interpreting plant responses to environmental change. Future work should therefore aim to disentangle the genetic, physiological and ecological bases of both axes, ideally by pairing analyses of traits in relation to long-term climate with experiments designed to capture short-term plasticity under controlled-stress scenarios.

We also assessed the plasticity of g_{min} by examining the percentage change from control to drought (%g_{min}), under the expectation that accessions from harsher environments might show greater reductions to conserve water. However, %Δg_{min} was not related to any climatic variable (Fig. S5), suggesting that responsiveness of cuticular conductance to drought is not predictable by climate in this species. This could imply that g_{min} plasticity is under weak environmental selection, for example, or perhaps that traits—such as osmotic adjustment or rooting depth—buffer water loss during drought more effectively.

Taken together, these findings highlight that drought-expressed g_{min} is more strongly shaped by climate-at-origin than baseline g_{min}, particularly in relation to summer climate extremes. Yet, the direction of this relationship is opposite to classical water conservation models, pointing to the multifaceted roles of cuticle conductance in plant adaptation.

Relationship with LMA

LMA is a pivotal trait in plant ecology (Poorter et al. 2009), often linked to various aspects of plant function, including photosynthetic capacity, water-use efficiency, and resilience to environmental stressors. Higher LMA, generally associated with thicker, denser leaf lamina—often resulting from smaller, densely packed cells with thicker walls and reduced air spaces—can limit internal water vapour diffusion, thereby reducing water loss and increasing drought tolerance (De La Riva et al. 2016).

However, our data challenge this expectation. We found a consistent and statistically strong positive correlation between LMA and g_{min} under both control and drought conditions as well as when the data were pooled. However, this may not indicate a direct mechanistic link between these two traits. g_{min} is governed primarily by the composition and organization of the cuticle, with the intracuticular wax layer forming the principal barrier to water loss,

while the contribution of the epicuticular wax layer varies across species depending on the specific wax chemistry (Jetter and Riederer 2016). Thus, any structural features that increase LMA—such as thicker palisade layers, denser mesophyll or more robust cell walls—may not directly affect the barrier properties of the cuticle.

Instead, we suggest that the observed positive correlation between LMA and g_{min} reflects independent responses to shared environmental selection pressures rather than functional integration. Both high LMA and elevated g_{min} may be favoured in hot, dry environments—but for different reasons. For example, high LMA may evolve in response to selection for leaf longevity, structural stability, and carbon-use efficiency in resource-poor systems (Wright et al. 2002, Onoda et al. 2012), while higher g_{min} may be favoured in lineages that benefit from enhanced cuticular flexibility or thermoregulation under high-temperature stress. These are functionally distinct adaptations that may co-occur without influencing one another directly.

Moreover, we speculate that increasing structural investment in high-LMA leaves—in the form of thicker cell walls or denser mesophyll—may interfere with optimal wax deposition or orientation by limiting the available surface area, inadvertently leading to greater cuticular permeability. Alternatively, high g_{min} in these accessions may reflect a relaxed selection pressure to minimize cuticular water loss, especially if other traits—such as deeper rooting systems, efficient stomatal control, or rapid post-drought recovery—are compensating for higher cuticular permeability.

Our findings caution against interpreting trait correlations, however statistically strong, as evidence of causation or functional coordination. The LMA–g_{min} relationship exemplifies how trait co-variation can emerge through ecological convergence rather than physiological integration (James et al. 2023). This supports a broader view in plant functional ecology: that trait syndromes arise from multiple—sometimes competing—selective forces acting on different parts of the phenotype (Murren 2002, Pigliucci 2003).

To better understand the drivers of cuticular conductance and its apparent links to structural traits, future research should move beyond coarse structural proxies like LMA and incorporate detailed chemical and microstructural analyses of the cuticle. Techniques such as gas chromatography–mass spectrometry (GC–MS), Fourier-transform infrared spectroscopy (FTIR), and high resolution microscopy (e.g., SEM/TEM/confocal) could be critical in uncovering how variation in wax composition, thickness, and organization underlie

g_{min} differences across species and environments (Chamel and Maréchal 1992, Urban et al. 2018, Lavergne et al. 2018).

Temperature dependent behaviour of g_{min}

Our investigation into the temperature-dependent behaviour of g_{min} in *T. triandra* accessions revealed a general trend of gradual decline in g_{min} with increasing temperature across most accessions. This pattern suggests that cuticular water loss may be moderately reduced at higher temperatures, possibly due to heat-induced stiffening or reorganization of the wax components. However, the Dalby and Forbes accessions exhibited an initial increase in g_{min} with rising temperatures, followed by a subsequent decrease—a pattern not observed in other accessions. This atypical pattern suggests the involvement of temperature-sensitive physiological pathways or distinct differences in cuticular lipid composition, which is known to play a crucial role in regulating water permeability (Schönherr et al. 1979).

Arrhenius plots of g_{min} did not reveal a clear biphasic pattern, indicating the absence of a distinct phase transition across accessions and suggesting that cuticle remains relatively stable across the temperature range tested. In our study, fresh cohorts from well-watered plants were used for each temperature treatment and leaves from the higher temperature runs (50 °C and 55 °C) did not appear extremely dry or noticeably different from those from lower temperatures. This suggests that markedly reduced water status was unlikely to have prevented an increase in g_{min}, although relative water content (RWC) was not measured and subtle differences cannot be excluded. Such differences could potentially contribute to the absence of a clear T_p. However, these findings imply that *T. triandra* may lack a distinct thermal threshold for cuticle conductance, and that any structural adjustments in the cuticle occur progressively or are buffered by other physiological or biochemical mechanisms. The absence of a common temperature response pattern across accessions further highlights the complexity of thermal strategies within the species.

Implications and future directions

Our findings provide new insights into the drought and thermal responses of *T. triandra* leaf cuticles and their relationship with environmental conditions, highlighting potential mechanisms driving these patterns. The observed variability in g_{min} could inform the

selection of resilient genotypes for ecological restoration or breeding programs aimed at mitigating the impacts of climate change. Future work should focus on the biochemical composition of cuticular waxes and their role in modulating g_{min} under stress conditions. Comparative transcriptomics or metabolomic analyses may also elucidate the genetic underpinnings of these diverse responses. Additionally, field studies assessing the interplay between cuticular properties, drought, and temperature in natural populations would provide valuable context for our controlled experiment findings. While this study provides important insights, the controlled conditions of the experiments of do not fully replicate the complexity of field environments. Further research is needed to validate these findings across broader spatial and temporal scales. Incorporating long-term monitoring of *T. triandra* populations under varying climatic conditions will help refine our understanding of the evolutionary and ecological significance of cuticular adaptations in response to environmental change.

As climate change drives more frequent droughts and heatwaves, understanding how different species/accessions regulate water loss through their cuticles and withstand high temperatures is key to strengthening ecosystem resilience. Additionally, examining how cuticular permeability interacts with other drought responses—such as osmotic regulation and root structure—could improve predictions of plant survival under future climatic conditions. To turn these insights into practical conservation strategies, future studies should expand to include other grassland species and assess their responses in long-term field trials.

Supplementary information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Table S1. Geographic origin and historical climate (1981–2010) for six *T. triandra* accessions, based on CHELSA high-resolution downscaled climate data.

Figure S1. Soil moisture under control and drought conditions.

Figure S2. Midday leaf water potential under control and drought conditions.

Figure S3. Photograph of the DroughtBox with samples set up, prepared to initiate a leaf drying run.

Figure S4. Relationships between g_{min} and annual climate variables (AP and AT) under control and drought conditions across *T. triandra* accessions, based on mixed-effects model outputs.

Figure S5. Relationship between percent change in g_{min} and annual climate variables (AP and AT) across *T. triandra* accessions, based on mixed-effects model outputs.

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Author contributions

AM, VJ and IJW designed and conducted the experiment, analysed the data, and drafted the initial manuscript. IJW, BC, VJ and CJB assisted with data interpretation and manuscript revisions. All authors reviewed and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Data availability

The raw data supporting this study have been uploaded to Zenodo (doi: 10.5281/zenodo.16884594) in restricted mode and will be made publicly accessible upon acceptance.

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Figures

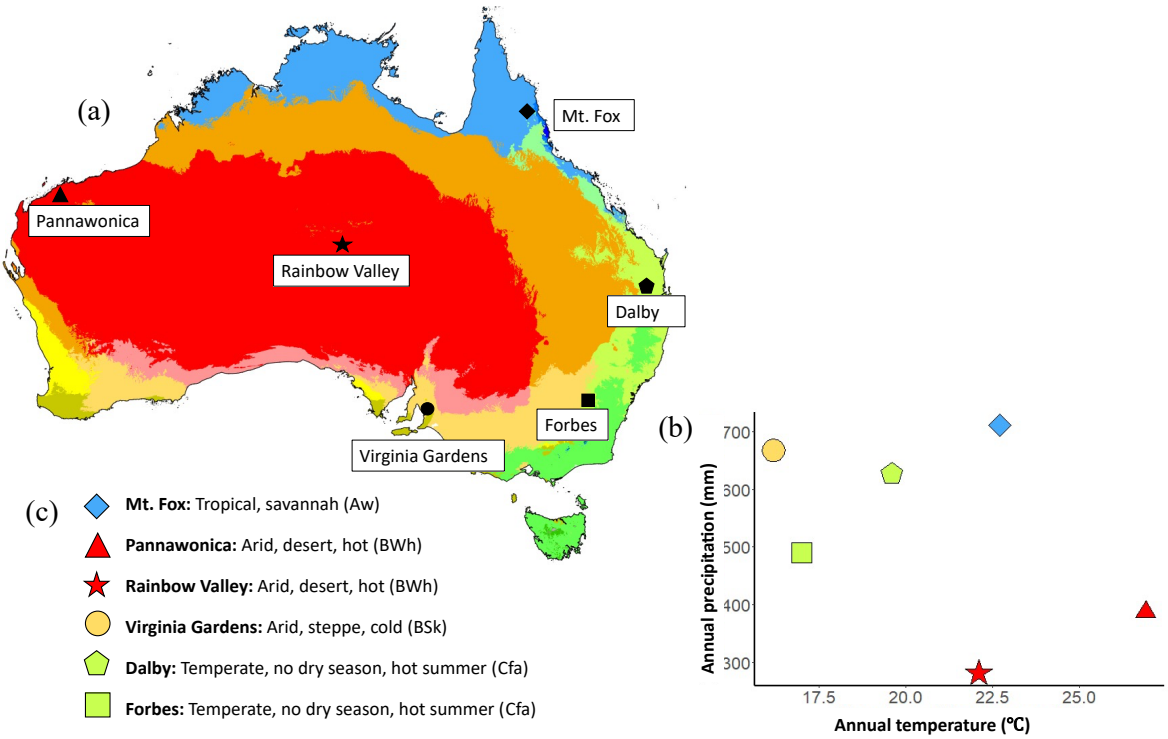

Figure 1. (a) Köppen-Geiger climate classification map of Australia adapted from Peel et al. (2007) showing the distribution of various climate zones. The locations of six *Themeda triandra* accessions included in this study are marked on the map. (b) Scatterplot illustrating the annual temperature (AT) and annual precipitation (AP) of the selected accessions, highlighting the climatic differences across their native habitats. (c) Köppen-Geiger climate classifications represented by distinct colours corresponding to specific climate types based on temperature and precipitation patterns. (The Köppen-Geiger classification system utilizes a combination of letters and colours to denote different climate types. For instance, 'BWh' in red denotes a hot desert climate with high temperatures and minimal precipitation. These classifications are visually represented through distinct colour codes to facilitate easy identification on the map)

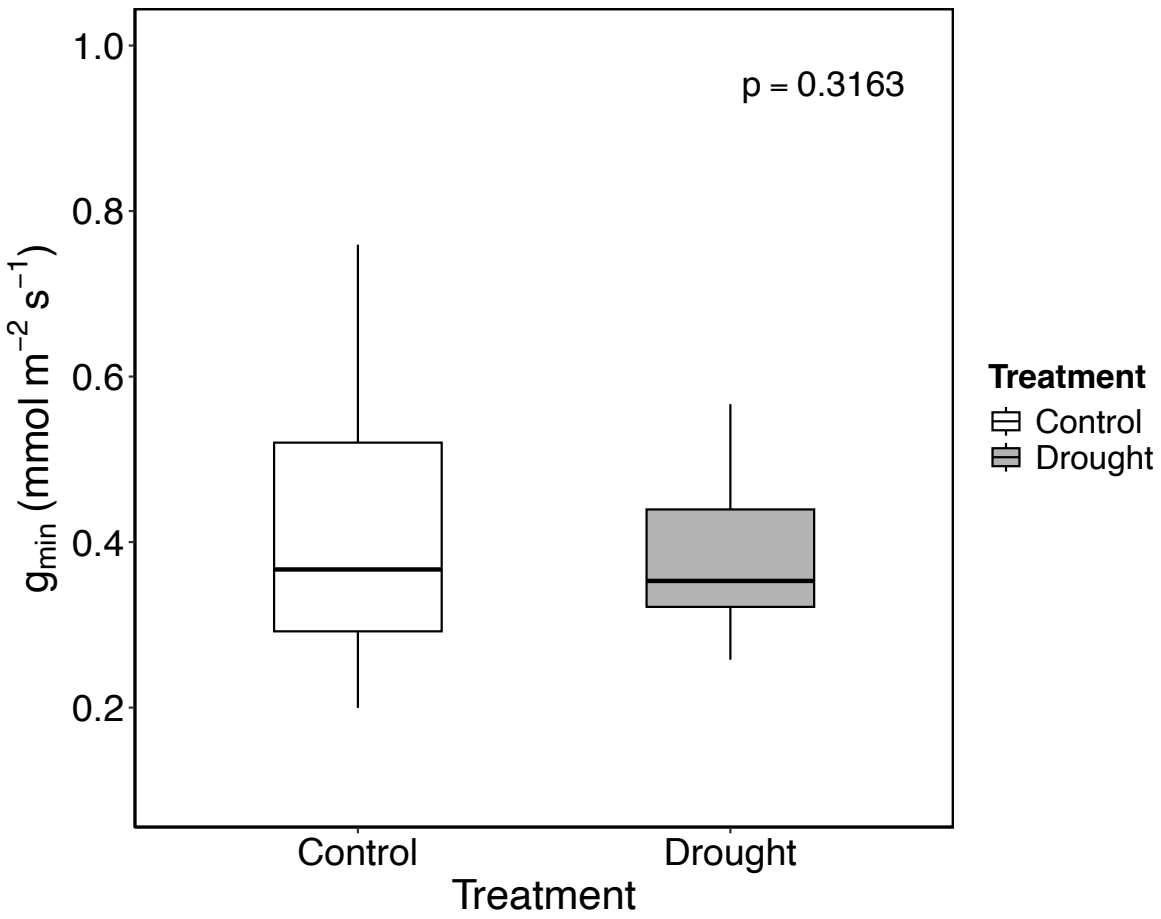

Figure 2. Box plot showing the distribution of g_{min} under control and drought treatments. The boxes represent the interquartile range with median indicated by the central line, and the whiskers extend to the minimum and maximum values. The p-value (0.3163; paired t-test) reflects the statistical comparison between treatments.

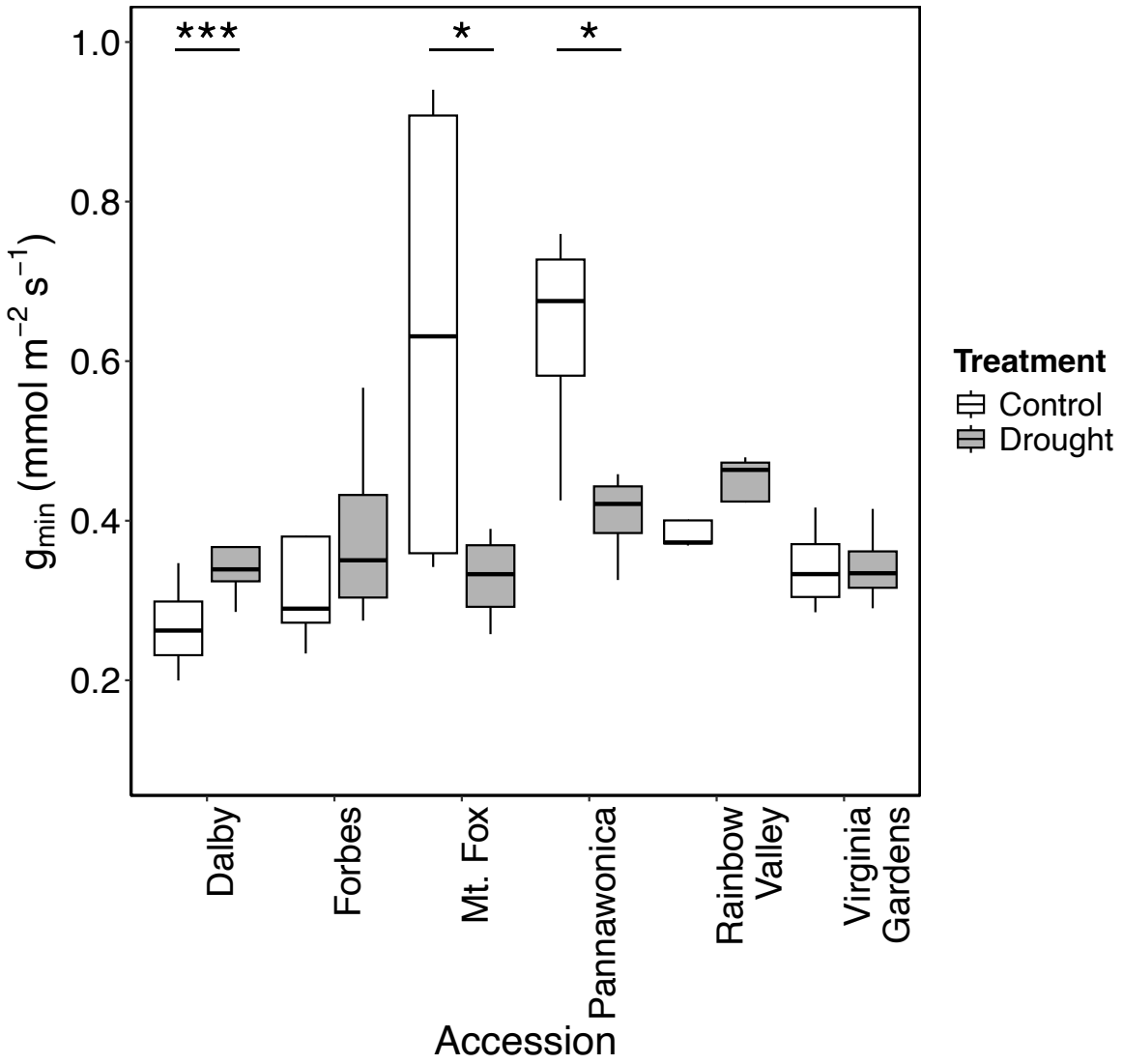

Figure 3. Box plots showing the variation in g_{min} across six accessions of *Themeda triandra* under control and drought treatments. Significant differences between treatments within accessions are denoted by asterisks (*$p < 0.05$, ***$p < 0.001$). The boxes represent the interquartile range (IQR), the horizontal line within each box represents the median, and whiskers extend to 1.5 × IQR.

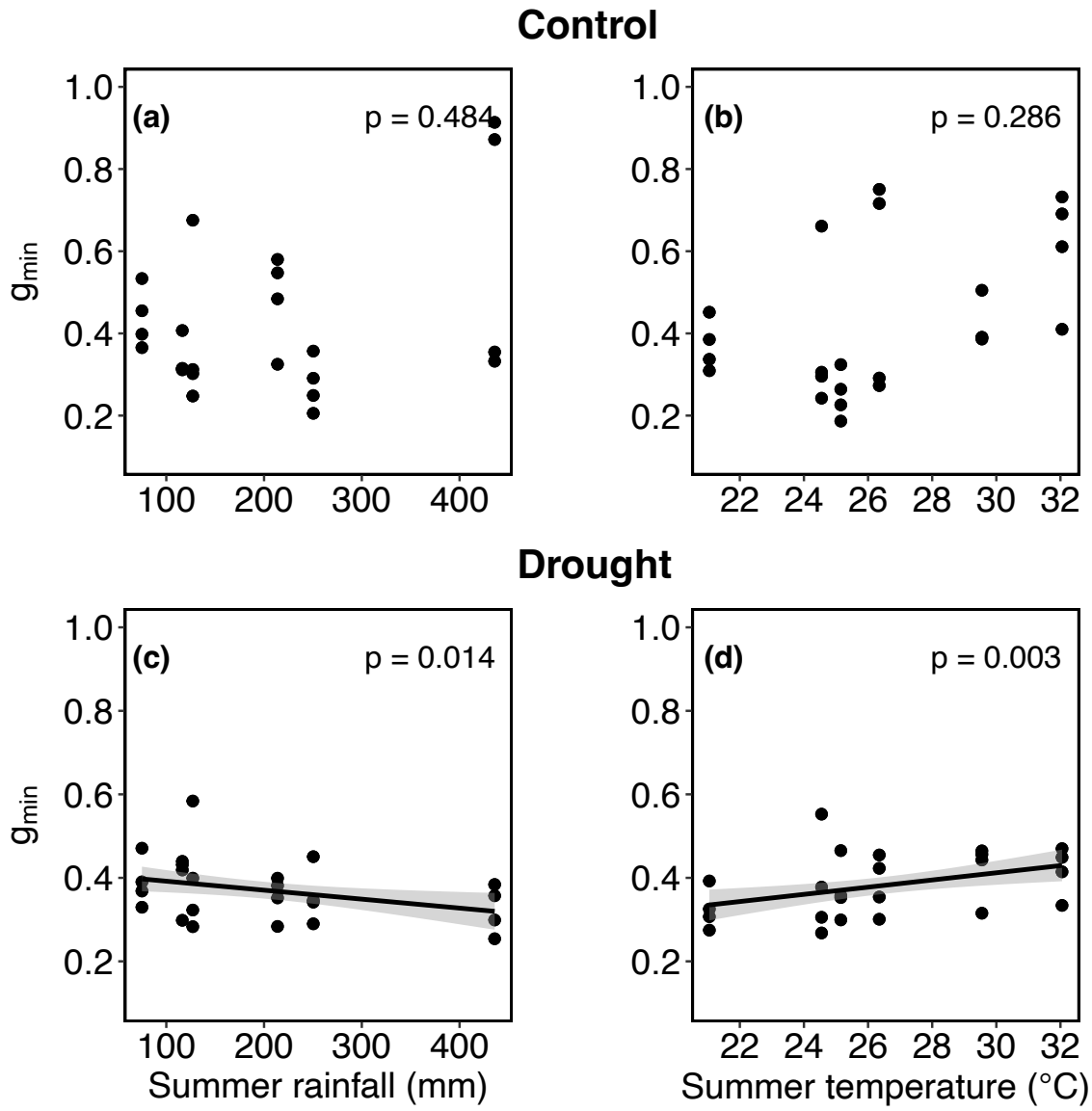

Figure 4. Relationship of g_{min} (mmol m^{-2} s^{-1}) with climate-at-origin variables under control (a–b) and drought (c–d) conditions across *Themeda triandra* accessions. g_{min} is plotted against summer rainfall (a, c) and summer temperature (b, d). Data points represent individual measurements. Black lines and shaded areas depict fitted values and 95% confidence intervals from linear mixed effects models, with accession included as a random effect.

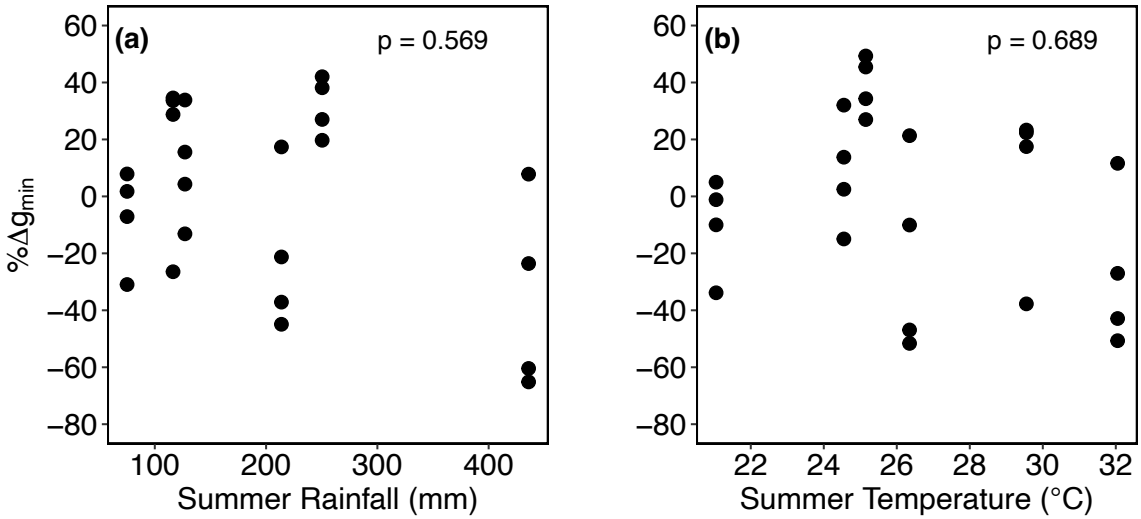

Figure 5. Relationship between $\%\Delta g_{min}$ (mmol m^{-2} s^{-1}) and climate-at-origin variables across *Themeda triandra* accessions (where $\%\Delta gmin = \frac{gmin(drought) - gmin(control)}{gmin(control)} \times 100$). (a) Summer rainfall (mm) and (b) summer temperature (°C) are shown as predictors. Each point represents an individual observation. Relationships were evaluated using linear mixed-effects models, with accession included as a random effect.

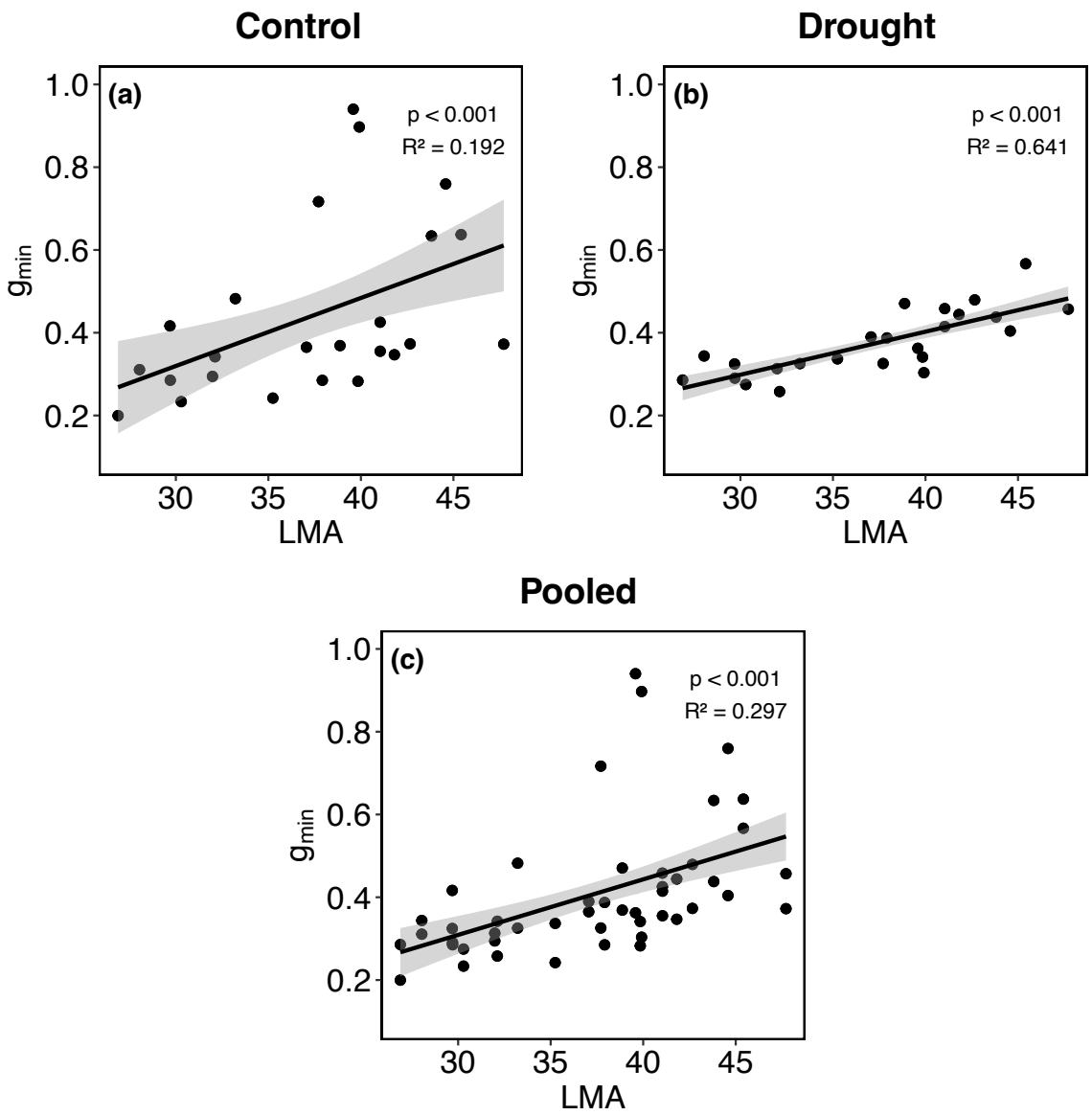

Figure 6. Relationship between g_{min} (mmol m^{-2} s^{-1}) and LMA, g m^{-2} in *Themeda triandra* accessions. (a) g_{min} under control conditions, (b) g_{min} under drought conditions, and (c) pooled g_{min} across both treatments. Each point represents an individual observation. Solid black lines represent fitted values from linear mixed-effects models with accession included as a random effect and shaded areas denote 95% confidence intervals.

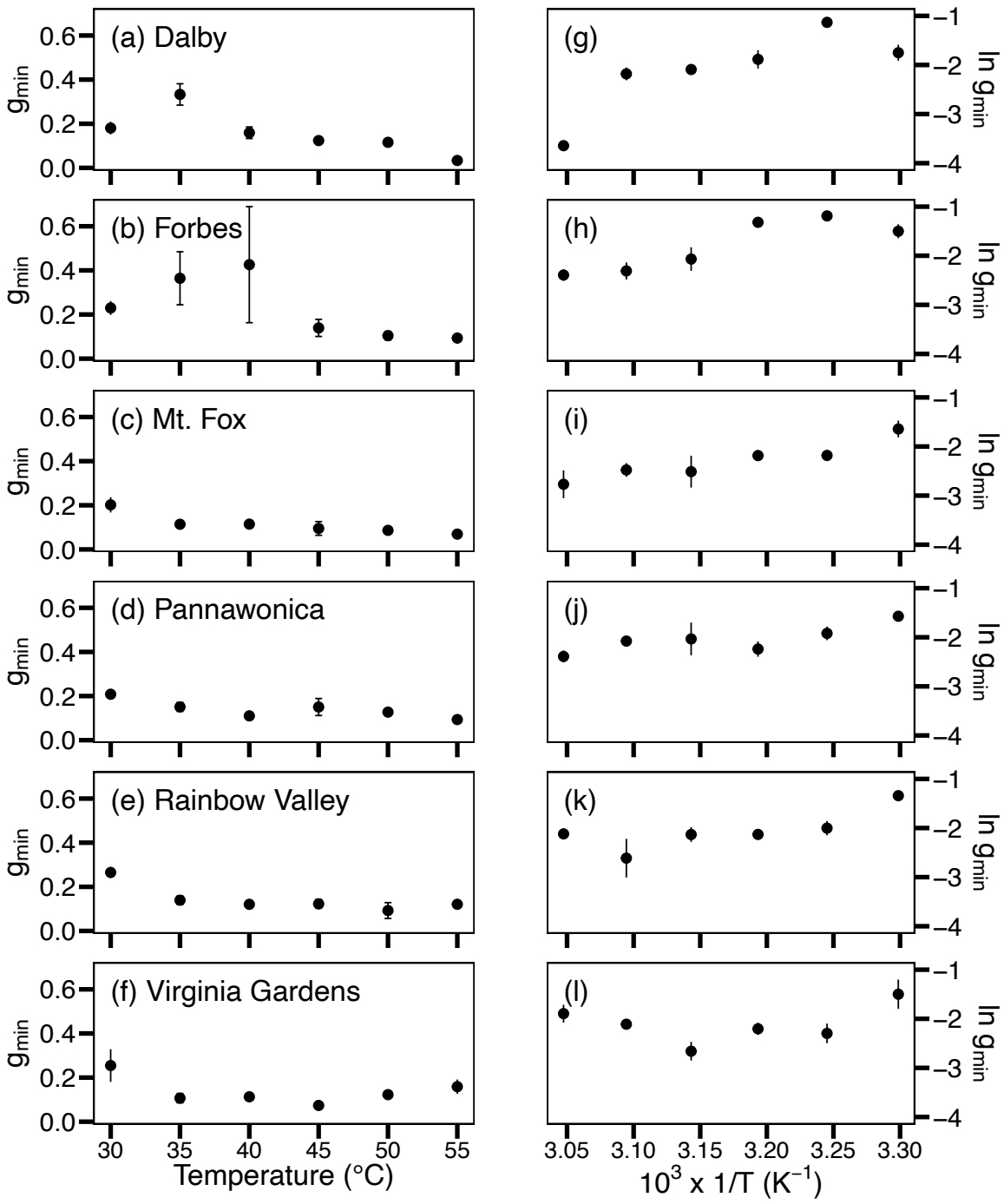

Figure 7. Temperature dependence of g_{min} (a–f) and corresponding Arrhenius plots (g–l) across a temperature range of 30–55°C at a constant absolute humidity of 18 g(H_2O)m^{-3}