

Research Article

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Divergent nitrogen regulation of dhuririn biosynthesis in *Sorghum macrospermum*, a crop wild relative of *Sorghum*

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Abstract

In Australia, *Sorghum bicolor* is frequently produced under dryland cropping systems, making these crops particularly susceptible to stress. Sorghum also contains the cyanogenic glucoside dhuririn, a toxic metabolite which is a major concern where sorghum is grown as livestock forage. The wild Australian relatives of *S. bicolor*, including species within the *Chaetosorghum* and *Stiposorghum* subgenera, contain minimal concentrations of dhuririn compared to domesticated sorghum. As the core biosynthetic enzymes are conserved, they provide a valuable comparative system to explore differences in the regulation of dhuririn biosynthesis. This study examined the transcriptional and metabolic response of *Sorghum macrospermum* to nitrogen resupply following nitrogen-limiting growth. Over a 48-hour time-course, changes in nitrate and dhuririn accumulation, and expression of key genes involved in nitrogen metabolism, dhuririn biosynthesis, and candidate transcriptional regulators were measured. In *S. macrospermum*, dhuririn accumulation and upregulation of the biosynthetic gene *SmCYP79A1* occurred in root tissues from 12 to 48 hours post-resupply. Very low dhuririn levels and negligible *SmCYP79A1* expression were observed in leaves and stems. Notably, nitrate levels remained stable over time, suggesting distinct nitrogen management strategies. Candidate regulators previously implicated in dhuririn biosynthesis in *S. bicolor* were not detected in the roots of *S. macrospermum*, indicating that different regulatory mechanisms drive the root-specific response. These findings highlight the evolutionary divergence in nitrogen and dhuririn regulation between domesticated and wild sorghum species. They further highlight *S. macrospermum* as a potential genetic resource for developing low-cyanide and nitrogen-use efficient sorghum with improved resilience to suit dryland cropping systems.

Introduction

Sorghum bicolor L. (Moench) is a multi-purpose crop grown for grain, forage and bioenergy (Rooney *et al.* 2007; Wang *et al.* 2024). In Australia, around 70–80% of sorghum crops are grown under dryland systems, particularly in the North-Eastern regions (Grains Research and Development Corporation (GRDC) 2025). Sorghum is valued for its resilience to stress, primarily drought and heat stress; however, adequate rainfall and nitrogen nutrition are still required to achieve optimum yields (Worland *et al.* 2017; Ostmeyer *et al.* 2022). In Australia, the soils are typically highly weathered and often contain low nitrogen levels that are limiting for crop production. Improving the nitrogen-use-efficiency, as well as improving stress tolerance, are key aims of breeding programs in these regions.

In northern Australia, the use of forage sorghum is complicated by the production of cyanogenic glucosides (CNgls): specialized metabolites that release hydrogen cyanide (HCN) upon tissue damage, a process termed *cyanogenesis* (Gleadow and Møller 2014). Sorghum produces the CNgls *dhuririn* using a simple biosynthesis pathway involving three structural enzymes: CYP79A1, CYP71E1 and UGT85B1 (Halkier and Møller 1989; Gleadow and Møller 2014) (Supplementary Figure S1). Dhuririn provides adaptive benefits, including anti-herbivore defence, abiotic stress tolerance and contributes to nitrogen management (Gleadow and Møller 2014; Gruss *et al.* 2022). However, HCN is a major concern for livestock producers, as high dhuririn levels can cause illness and death in cattle (Giantin *et al.* 2024). This is of particular concern under the environmental conditions in Australia, where events such as drought and nitrogen application can spike dhuririn concentrations in sorghum tissues.

Efforts to reduce HCN toxicity to date have focussed on eliminating CNgls from crops, with success in sorghum and cassava (Blomstedt *et al.* 2012; Tuinstra *et al.* 2016; Juma *et al.* 2022; Gomez *et al.* 2023). However, removing CNgls from crops also removes the benefits they contribute, such as resilience to pests, tolerance to abiotic stress and nitrogen management (Gleadow and Møller 2014; Gruss *et al.* 2022). Rather than eliminating CNgls from crops, altering patterns of CNgls accumulation could enable retention of these benefits while mitigating the risks associated with cyanogenic plants.



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Current knowledge of the molecular regulation of dhurrin accumulation is limited with only one transcription factor identified to date: SbGATA22, a repressor of the dhurrin biosynthesis pathway with maturation (Rosati *et al.* 2024). While other transcriptional regulators of key CNgls genes have been characterized in other cyanogenic species, such as PdbHLH2 in *Prunus dulcis* and LjbHLH7 in *Lotus japonicus* (Sánchez-Pérez *et al.* 2019; Chen *et al.* 2022), application of these findings to regulation of dhurrin in sorghum is complicated by the evolutionary distances between these species and the independent evolution of cyanogenesis in their lineages (Thodberg *et al.* 2020; Hansen *et al.* 2023; Sánchez-Pérez and Neilson 2024). Within *Sorghum*, however, dhurrin biosynthesis appears to be monophyletic and conserved across the 22 undomesticated taxa in the genus (Myrans *et al.* 2020, 2021; Cowan *et al.* 2022), providing a unique opportunity to study regulatory variation.

Sorghum is classified into five subgenera: *Eusorghum* (including *S. bicolor*), *Chaetosorghum*, *Heterosorghum*, *Parasorghum* and *Stiposorghum* (Garber 1950; Dillon *et al.* 2007; Ananda *et al.* 2021). Northern Australia is a hotspot for *Sorghum* diversity, with 17 taxa either native or endemic to the region (Myrans *et al.* 2020). These wild relatives represent valuable genetic resources for breeding resilience traits into domesticated sorghum lines, including reduced dhurrin concentration (Ananda *et al.* 2020; Cowan *et al.* 2020). Among these species, *Sorghum macrospermum* E.D. Garber (subgenus *Chaetosorghum*) is particularly notable. Endemic to a small area in the Northern Territory, it shares key agronomic traits with *S. bicolor*, such as annual growth habit, palatable forage, and large, nutritious grain (Lazarides *et al.* 1991). These characteristics, combined with its superior drought tolerance and lower cyanogenic profile, make *S. macrospermum* a promising candidate for crop improvement (Ananda *et al.* 2020; Cowan *et al.* 2020).

Dhurrin regulation in *S. macrospermum* differs markedly from that in *S. bicolor*. Both species accumulate dhurrin during early seedling growth, followed by a decline with development (Cowan *et al.* 2021). However, *S. macrospermum* has much lower concentrations overall and shows limited responsiveness to nitrogen supply (Myrans *et al.* 2021). Under drought stress, dhurrin accumulates primarily in roots rather than leaves (Cowan *et al.* 2020; Ananda *et al.* 2022). In contrast, *S. bicolor* upregulates foliar dhurrin production under both drought stress and high nitrogen conditions (O'Donnell *et al.* 2013; Blomstedt *et al.* 2018; Rosati *et al.* 2019; Cowan *et al.* 2020; Myrans *et al.* 2021). If varieties of sorghum could be developed with the pattern seen in *S. macrospermum*, the stems and leaves would likely remain safe for grazing under a wide range of conditions.

Because amino acid sequences of the dhurrin biosynthesis genes are highly conserved (Cowan *et al.* 2022), differences in dhurrin accumulate likely reflect divergence in regulatory mechanisms between *S. bicolor* and *S. macrospermum* rather than structural gene variation. Understanding these regulatory factors is critical for developing sorghum suited to dryland and low-input systems, where low toxicity, drought resilience and nitrogen-use efficiency are essential. Furthermore, dhurrin turnover, involving enzymes such as Nitrilase 4B2 (NIT4B2), may contribute to nitrogen recycling (Jenrich *et al.* 2007; Pičmanová *et al.* 2015; Bjarnholt *et al.* 2018). Targeting dhurrin production and turnover may represent a novel target for improving nitrogen-use efficiency in resource-limited cropping systems.

This study investigated short-term response of nitrogen-limited *S. macrospermum* to a high nitrogen supply (12 mM). We measured

changes in nitrate and dhurrin concentrations and expression of key genes in nitrogen assimilation and dhurrin metabolism over 48 hours. We hypothesized that nitrogen application would induce dhurrin biosynthesis in roots, but not in leaves, consistent with previous observations of higher root dhurrin levels in *S. macrospermum*. To test this, dhurrin concentrations and expression of the dhurrin biosynthesis gene *SmCYP79A1* and the turnover enzyme *SbNIT4B2*, was quantified in roots and leaves.

Additionally, we hypothesized that if dhurrin metabolism is activated by nitrogen in *S. macrospermum*, its regulation would involve conserved transcriptional networks shared with *S. bicolor*. Expression of four candidate transcription factors identified in *S. bicolor* (*SmHRS1*, *SmTRFL3*, *SmEIL3* and *SmMYB-related*) were also assessed. Changes in expression of *Glutamine synthetase 2* (*GS2*), involved in primary nitrogen assimilation, was used as positive control for nitrogen response.

Materials and methods

Plant growth conditions

Seeds of *S. macrospermum* (AGG 322277 WSOR3 MHA 716; Australian Grains Genebank) were germinated according to Cowan *et al.* (2020). Germinated seeds were transferred to pots containing washed river sand (Bastion River Sand, Dingo Cement Australia) and left to establish in a greenhouse under ambient light and temperatures between 25°C and 35°C. After two weeks, seedlings were transferred to 10-cm diameter pots with sand and irrigated twice daily with an automated drip irrigation system. Nutrients were supplied with a modified Long Ashton Nutrient Solution containing a final concentration of 0.5 mM nitrogen (Miller *et al.* 2014; Myrans *et al.* 2021), with 200 mL of nutrient solution delivered three times per week (Supplementary Table S1).

Before application of treatment solutions, plants were randomly assigned to one of five time points: baseline (0-hours), 6-, 12-, 24- or 48-hours. Time points were selected based on data from *S. bicolor*, where induction of dhurrin biosynthesis genes occurred within 48-hours of nitrogen treatment (Quinn 2025). Due to limited availability of *S. macrospermum* seeds, each time point included five biological replicates and treatments were compared to the baseline control at the 0-hour time point. A 200 mL of a high-nitrogen treatment solution (12 mM nitrogen) was applied to all plants, except the baseline control (Fig. 1). Measurements were taken for height, leaf number, and chlorophyll content (Apogee Instruments) at each harvest. Tissue was also sampled for chemical and gene expression analysis. Baseline samples for nitrate and biomass measurements were not included.

Chemical analyses

Nitrate (NO_3^-) concentration ($\text{mg}^{-1} \text{g}^{-1} \text{DW}$) was determined spectrophotometrically using $15 \text{ mg} \pm 0.15 \text{ mg}$ of finely ground oven-dried plant material following the method of Cataldo *et al.* (1975) as modified by O'Donnell *et al.* (2013).

Cyanogenic glucoside concentrations were determined as hydrogen cyanide potential (HCNp), which is the maximum amount of HCN capable of being released upon hydrolysis of all CNgls in a sample. HCNp is directly proportional to the dhurrin content in the sample (Gleadow *et al.* 2012). HCNp was measured on fresh tissue from leaf, stem, and root tips collected at the time of harvest according to O'Donnell *et al.* (2013). The assay was modified to account for the lower dhurrin concentrations

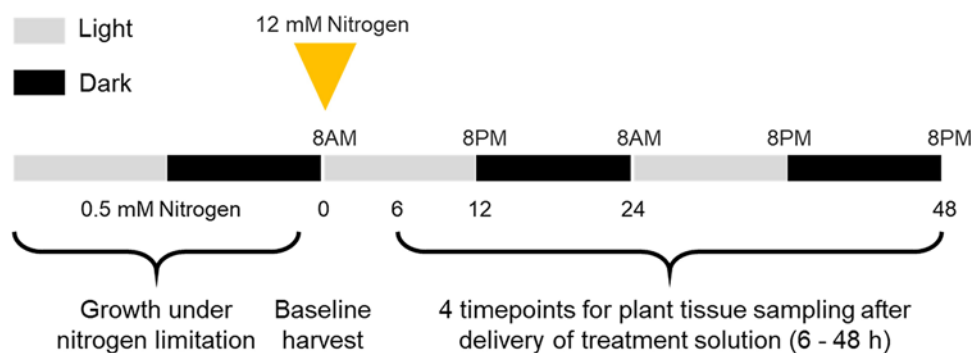


Figure 1. Overview of experiment set up and time points for sampling. *Sorghum macrosperrum* plants were grown under 0.5 mM nitrogen until they reached the 6–7 leaf stage. Samples were collected from leaf, stem and root tips for RNA at 0-hours (baseline), and remaining plant tissue oven-dried ($n = 5$). All remaining plants were treated with a 12 mM nitrogen solution, and harvested in the same way at 6-, 12-, 24- and 48-hours after nitrogen treatment ($n = 5$).

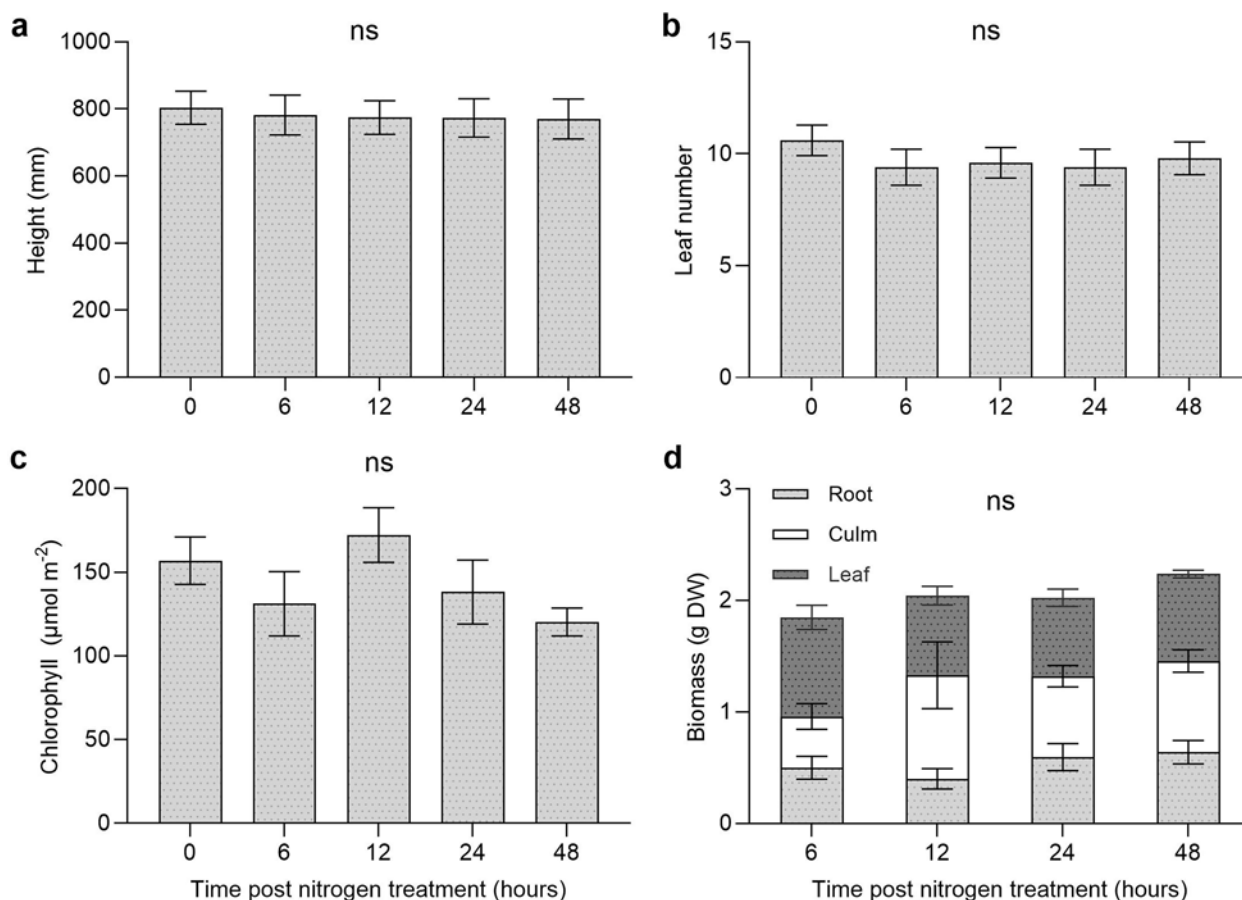


Figure 2. Measurements for (a) height, (b) leaf number and (c) chlorophyll content for nitrogen-limited *S. macrosperrum* in response to a 12 mM nitrogen treatment at four time points up to 48 hours after treatment ($n = 5$). Measurements taken at time of harvest. (d) Total biomass for dried tissue ($n = 5$). Columns represent mean $\pm$ SE. No significant differences between time points were detected using a one-way ANOVA.

produced in *S. macrosperrum* by using 50 μL NaCN samples undiluted and adjusting concentration of acetic acid to 2 M to neutralize the pH of the sample. Remaining plant tissue was oven dried and accurately weighed for calculation of HCNp as μg^{-1} g $^{-1}$ DW.

RNA extraction

Tissues from leaf and root tips were sampled at the time of harvest, frozen immediately in liquid nitrogen and stored at -80°C until

RNA extraction. Plant tissue was ground to a fine powder in a bead mill (FastPrep-24, MP Biomedicals) kept cool with dry ice. RNA was extracted using 1 mL Tri Reagent (Sigma, 92389–100 mL), according to the manufacturer's instructions and resuspended in 100 μL nuclease-free water. RNA integrity was checked with a 2% agarose gel. Quantity and quality were determined using a spectrophotometer (NanoDrop1000, ThermoFisher Scientific). RNA samples where $A_{260}/A_{230} < 1.60$ were re-precipitated and pellets washed with ethanol to remove additional salt contamination and quality control checks repeated.

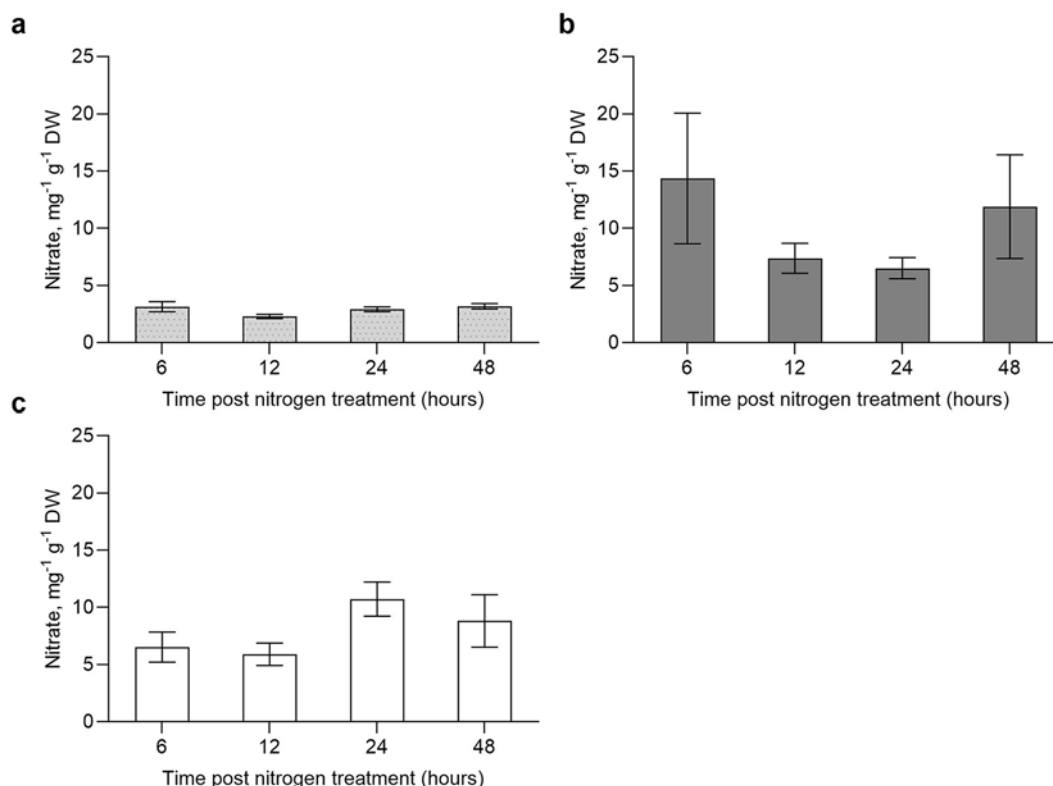


Figure 3. Nitrate concentration of (a) leaf, (b) sheath and (c) root tissue for nitrogen-limited *S. macrospermum* in response to a 12 mM nitrogen treatment ($n = 5$). Columns represent the mean $\pm$ SE. No significant differences between time points were detected using a two-way ANOVA.

Sequence extraction and primer design

No reference sequence for *S. macrospermum* is currently available. However, a reference sequence for closely related *S. bicolor* is available (McCormick *et al.* 2018), and short-read sequence data for *S. macrospermum* is also publicly available (Ananda *et al.* 2021). For design of primers for target genes in *S. macrospermum*, an approach similar to Ananda *et al.* (2021) was used to align the *S. macrospermum* short-read sequencing data (AGG accession 302367, retrieved from NCBI, BioSample SAMN17369313; Ananda *et al.* 2021) to the *S. bicolor* reference assembly (v3.1.1, (McCormick *et al.* 2018)). Reads for *S. macrospermum* were analysed with FastQC to check for and remove low-quality reads before aligning to the *S. bicolor* reference. Reads were aligned using the Map with Bowtie for Illumina tool available in Galaxy Australia, with default parameters for paired-end reads. Alignment was visualized in the Integrative Genomics Viewer (Version 2.17.0) and consensus sequence for target genes in *S. macrospermum* retrieved from alignment to *S. bicolor* genes. A similar approach was used by Ananda *et al.* (2021) in the construction of the nuclear *Sorghum* phylogeny, and sequences for genes involved in cyanogenesis in wild sorghum species, including *S. macrospermum*, have been analysed using this approach (Cowan *et al.* 2022). Sequences retrieved from the alignment by Ananda *et al.* (2021) were used to validate the sequences retrieved in the present study. Consensus sequences for target gene genomic sequences were aligned using MEGA11 (Version 11.0.13) and show similar nucleotide sequences between *S. macrospermum* and *S. bicolor* exons (available at <https://doi.org/10.26180/31200166>). Primers targeting *S. macrospermum* sequences for use in qPCR were designed using PerlPrimer (v1.1.21) (Supplementary Table S2).

cDNA synthesis and reverse-transcription qPCR

One microgram of RNA was DNase-treated (RQ1 DNase, Promega) before cDNA synthesis using First-Strand cDNA synthesis kit (Invitrogen) with oligo(dT) primers. Gene expression was measured using qPCR on a LightCycler 480 instrument (Roche). Reactions were set up in triplicate on 384-well plates using a robotic liquid handling system (Ep-Motion 5070, Eppendorf Research), with each 10 μ L reaction containing 5 μ L $2 \times$ SensiFAST SYBR qPCR mix (Meridian Bioscience, BIO-98050-2000), 2.5 μ L cDNA template (diluted 1:10 with nuclease-free water), and gene-specific forward and reverse qPCR primers to a final concentration of 250 nM each. For each primer pair, the efficiency of the qPCR reaction was determined using a dilution series of a purified PCR product amplified in triplicate alongside the samples. Relative gene expression (mean normalized expression) was calculated using Q-gene software (Simon 2003).

Statistical analyses

Statistical analyses were performed using GraphPad Prism (Version 9.3.1, GraphPad Software, LLC). Data for harvest measurements (height, leaf number and chlorophyll concentration) were analysed using a one-way ANOVA. Data for chemistry and gene expression were analysed using two-way ANOVA. Gene expression data was log-transformed to meet assumptions of normality and homogeneity of variance. Post-hoc analyses for significant ANOVA results were conducted with Dunnett's test for multiple comparisons, where contrasts were made against the baseline time point and subsequent time points.

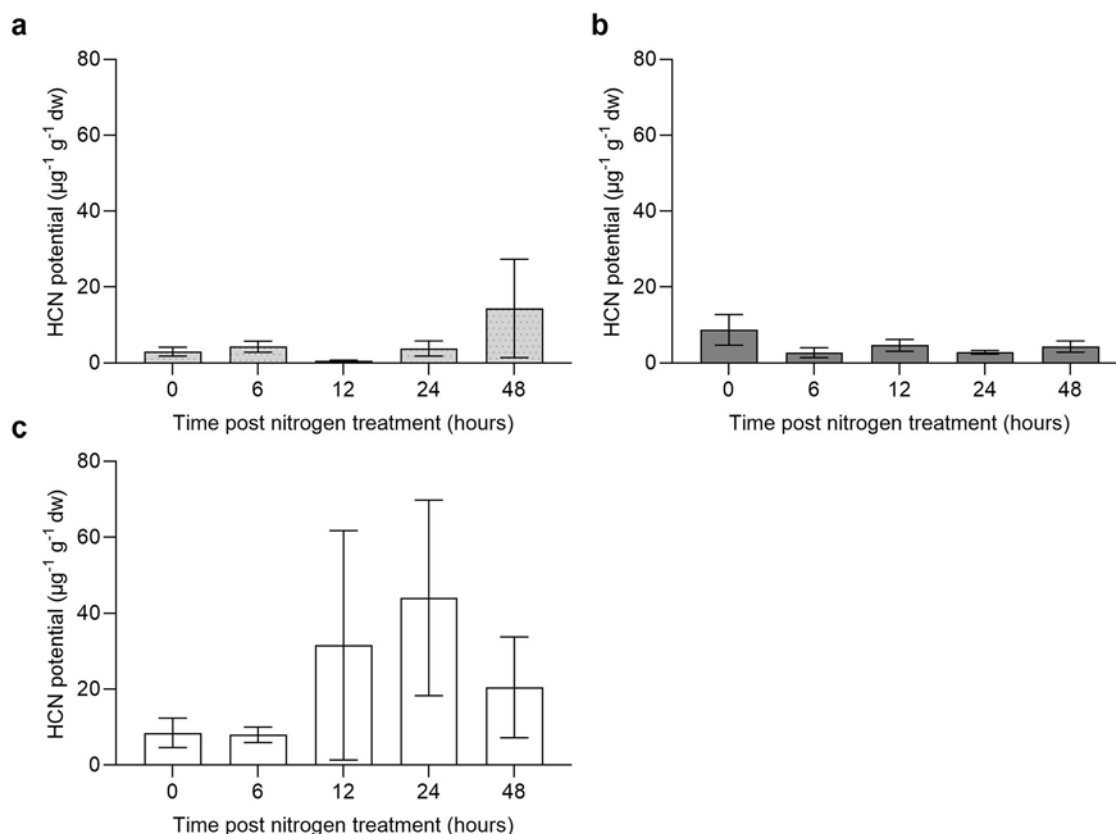


Figure 4. Hydrogen cyanide potential (HCNp) of (a) leaf, (b) stem and (c) root tissue for nitrogen-limited *S. macrospermum* in response to a 12 mM nitrogen treatment ($n = 5$). Columns represent mean $\pm$ SE. No significant differences between time points were detected using a two-way ANOVA.

Results

Harvest measurements

Measurements of growth were taken at the time of harvest for height, leaf number and chlorophyll content (Fig. 2a–c). No significant differences were detected for *S. macrospermum* at any time point in response to the nitrogen surge application. There was no significant increase in total biomass in response to the nitrogen surge treatment during the sampling timeframe (Fig. 2d).

Nitrate concentration

Nitrate concentration was measured on dried and ground tissue for whole samples of leaf, sheath and roots of *S. macrospermum* (Fig. 3). No significant differences in nitrate concentration were detected for any tissue between time points; however, differences between tissue type were identified ($F_{(2, 48)} = 24.25$, $P < 0.0001$), with leaf tissue containing lower amounts of nitrates than sheath ($P < 0.0001$) and root ($P < 0.0001$) (Fig. 3a–c).

Hydrogen cyanide potential

Hydrogen cyanide potential (HCNp) was measured on fresh tissue collected from the last fully emerged leaf, the stem and from root tips of *S. macrospermum*. HCNp was significantly different between tissue types ($F_{(2, 57)} = 4.79$, $P = 0.012$), with roots containing higher HCNp than leaf tissue ($P = 0.008$), which appears to be driven by increased HCNp in a few individuals across the 12- to 48-hour time points in root tissue at the later stages of the sampling period. There was also a slight increase in HCNp in leaf

tissue at the 48-hour time point, although no significant effect of nitrogen treatment on the HCNp was detected in any time point in the tissues tested ($F_{(4, 57)} = 1.02$, $P = 0.40$) (Fig. 4a–c).

Expression of key metabolism genes

Glutamine synthetase 2 (GS2) plays a key role in assimilation of inorganic forms of nitrogen in plants. The nitrogen treatment significantly increased the expression of the *SmGlutamine Synthetase 2* (*SmGS2*) in *S. macrospermum* ($F_{(4, 20)} = 9.70$, $P = 0.0002$), with a similar response seen in both root and leaf tissue (Fig. 5a). Expression of *SmGS2* in leaf tissue was significantly higher than baseline at 6-, 24- and 48-hours after application of the nitrogen treatment. Expression of *SmGS2* in roots also increased from 6-hours after nitrogen treatment, but was only significantly higher than baseline at the 24-hour time point ($P = 0.006$).

Analysis of expression of *SmCYP79A1*, which encodes the gene for the first step in the dhurrin biosynthesis pathway, showed a significant effect of tissue type ($F_{(1, 16)} = 10.43$, $P = 0.005$), but not time point ($F_{(4, 16)} = 2.47$, $P = 0.087$) (Fig. 5b). Differences between the two tissues were largely driven by increased expression of *SmCYP79A1* in the roots from the 12-hour time point onwards, as there is negligible expression of this gene in either root tissue at 0- and 6-hours after nitrogen treatment or at any time point in leaf. Post-hoc analysis comparing all time points to the baseline within tissue types detected significantly higher expression of *SmCYP79A1* from 12-hours after nitrogen application (Fig. 5b).

Like *SmCYP79A1*, there was no significant difference between time points for expression of *SmNitrilase 4B2* (*SmNIT4B2*),

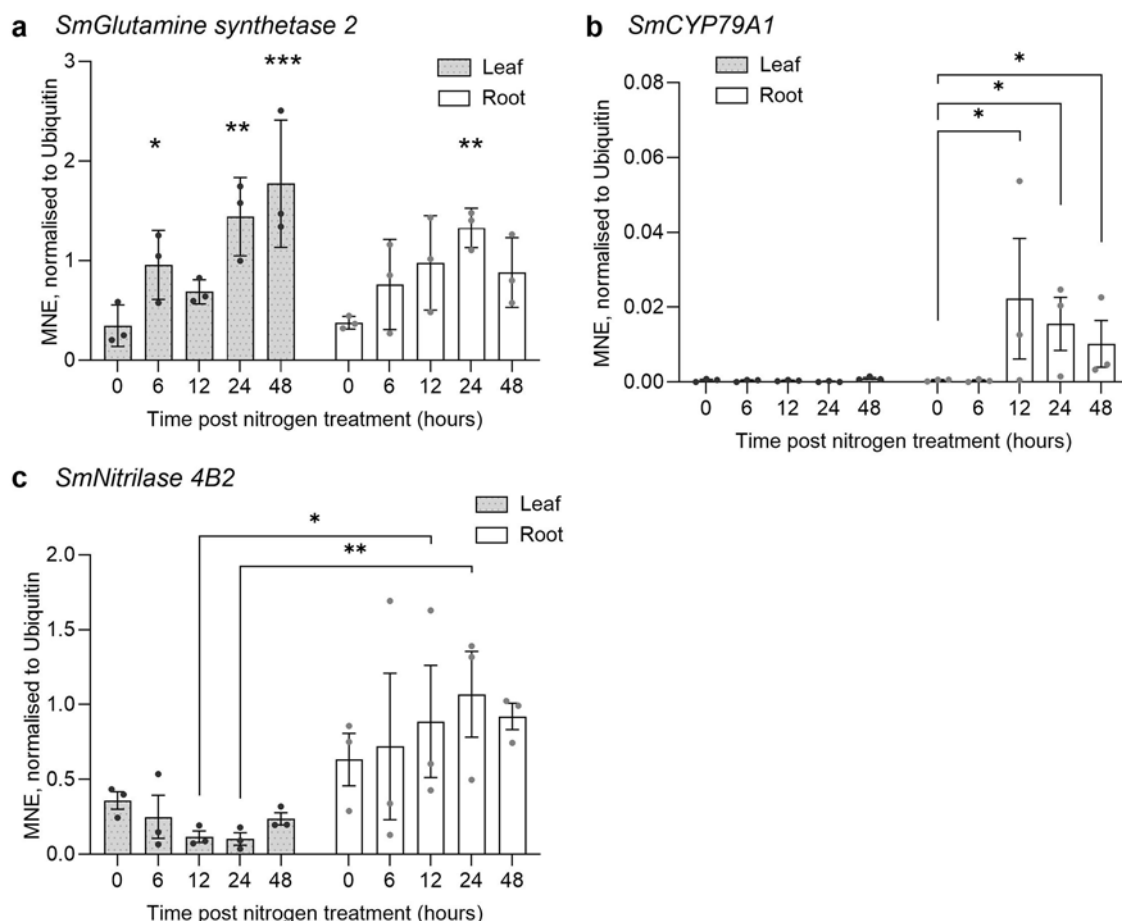


Figure 5. Gene expression normalized to ubiquitin (mean normalized expression) for *Sorghum macrosperrum* leaf and root tissues in response to treatment with a 12 mM nitrogen solution. (a) *SmGlutamine Synthetase 2*, (b) *SmCYP79A1* and (c) *SmNitrilase 4B2*. Columns represent mean ± SE (n = 3). Stars represent significant differences at $P < 0.05$ (*), 0.01 (**) and 0.001 (***) using two-way ANOVA and Sidak's test for multiple comparisons.

although expression was significantly higher in root than leaf tissue ($F_{(1,20)} = 30.04$, $P < 0.0001$) (Fig. 5c). Multiple comparisons between tissue type at each time point showed that expression of *SmNIT4B2* in roots was significantly higher than that in leaf tissue at the 12-hour ($P = 0.016$) and 24-hour ($P = 0.002$) time points. This appears to be the result of both a slight decrease in expression of *SmNIT4B2* in leaf tissue at these time points and a corresponding increase in expression in root tissue.

Expression of candidate transcription factors

Of the genes encoding putative transcription factors, *SmHRS1* was the only candidate with noticeable expression detected in *S. macrosperrum* (Fig. 6). There was essentially no expression of *SmEIL3*, *SmMYB*-like or *SmTRF*-like in either leaf or root at any time point tested (Fig. 6a–c). Expression of *SmHRS1* was detected in both leaf and root tissues, and there was an effect of time point on expression ($F_{(4,20)} = 5.84$, $P = 0.003$), with significantly higher expression of *SmHRS1* in leaf tissue at the 12-hour time point compared to baseline expression levels ($P = 0.004$) (Fig. 6d).

Discussion

Crop wild relatives offer genetic resources for improving crop resilience and productivity, particularly under dryland conditions

where nitrogen is limited and drought is frequent (Ananda *et al.* 2020). *Sorghum macrosperrum* is a promising candidate for breeding due to its low dhurrin content, high productivity with minimal nitrogen and ability to tolerate drought (Cowan *et al.* 2020; Myrans *et al.* 2021). Importantly, *S. macrosperrum* has been successfully crossed with *S. bicolor* making the prospect of introgression much simpler (Price *et al.* 2005; Kuhlman *et al.* 2010). Here, we examined dhurrin regulation in nitrogen-limited *S. macrosperrum* by characterizing the pattern of dhurrin biosynthesis in space and time following a single dose of nitrogen. Findings support the hypothesis that dhurrin biosynthesis occurs primarily in the roots of *S. macrosperrum*. We conclude that this trait could be used to develop low-cyanide, stress-resilient sorghum for low-input dryland systems.

Growth rates of *S. macrosperrum* were delayed under nitrogen limitation, with plants taking 12 weeks to produce 12 leaves. These results are similar to previous reports where *S. macrosperrum* is grown in low-nitrogen conditions (Myrans *et al.* 2021). The high-nitrogen treatment did not significantly affect growth or nitrate concentration over 48 hours, contrasting with *S. bicolor*, which shows rapid nitrate accumulation under similar conditions (Quinn 2025). The lack of increased nitrate concentration suggests *S. macrosperrum* employs a different strategy for nitrogen use and uptake, possibly occurring at a slower rate not detected within the 48-hour sampling timeframe. Alternatively, *S. macrosperrum* may

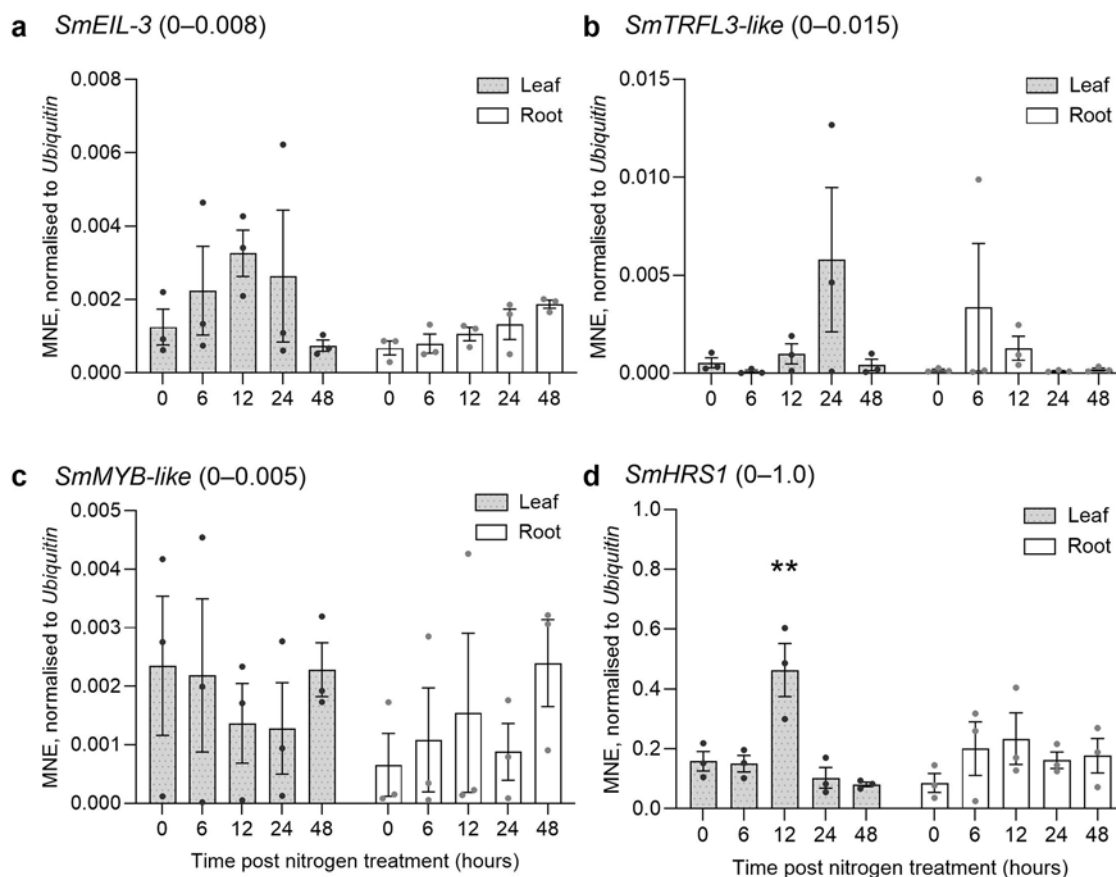


Figure 6. Gene expression normalized to ubiquitin (mean normalized expression) for *Sorghum macrosperrum* leaf and root tissues in response to treatment with a 12 mM nitrogen solution. (a) *SmEthylene Insensitive-like 3*, (b) *SmTRF-like 3*, (c) *SmMYB-like* and (d) *SmHRS1*. Data show mean $\pm$ SE ($n = 3$). Note differences in ranges for Y-axes between a and c are two orders of magnitude lower than for d. Stars represent significant differences at $P < 0.05$ (*), 0.01 (**) and 0.001 (***) using two-way ANOVA and Sidak's test for multiple comparisons.

rely more heavily on nitrate stores, possibly an adaptation to the unpredictable monsoonal region where *S. macrosperrum* grows (Lazarides *et al.* 1991).

Despite the absence of higher nitrate concentrations, elevated expression of *SmGS2* was observed in both roots and leaves. Increased *SmGS2* expression was induced earlier in leaves, likely linked to photosynthesis, while root expression increased gradually, suggesting regulation tied to ongoing nitrogen demand. These findings indicate a transcriptional response to nitrogen application, but further work is needed to clarify additional regulatory mechanisms involved in *S. macrosperrum* nitrogen metabolism, which could provide additional targets for altering NUE in sorghum.

Dhurrin concentrations in *S. macrosperrum* were very low throughout the experiment, both before and after nitrogen application, with no significant changes detected across tissues. These findings align with previous reports showing that Australian sorghum species, including *S. macrosperrum*, typically accumulate less than $50 \mu\text{g g}^{-1}$ HCNp even under high-nitrogen conditions (Cowan *et al.* 2020, 2021; Myrans *et al.* 2021), compared with $> 300 \mu\text{g g}^{-1}$ commonly observed in *S. bicolor* leaves (Blomstedt *et al.* 2018; Myrans *et al.* 2021).

Although overall dhurrin levels remained low, with only a slight increase in root dhurrin after 12 hours, there was a significant upregulation of *SmCYP79A1*, the gene encoding the first and rate-limiting enzyme in the dhurrin biosynthesis pathway (Busk and Møller 2002) (Supplementary Figure S1). Dhurrin concentration

would likely continue to increase, as seen in *S. bicolor*, where an increase in *SmCYP79A1* transcripts has been shown to precede changes in phenotype (Sohail *et al.* 2023). Expression of *SmCYP79A1* was restricted to roots, unlike *S. bicolor*, where nitrogen induces leaf-specific expression of *SbCYP79A1* within 24 hours (Quinn 2025). This pattern, together with previous drought studies, suggests that roots are the primary site of dhurrin production in *S. macrosperrum* in response to environmental cues. The functional significance of root-localized dhurrin is not clear, although CNgls can be effective at managing soil pests, such as nematodes (Abawi and Widmer 2000; Smith *et al.* 2024). However, the dhurrin levels in *S. macrosperrum* roots are so low that they are unlikely to be effective in deterring herbivores (Myrans *et al.* 2021).

Alternatively, dhurrin stored in roots may serve as a nitrogen reserve for regrowth after stress, such as grazing or fire, through a recycling pathway that has been identified in *S. bicolor* and other cyanogenic species (Pičmanová *et al.* 2015; Nielsen *et al.* 2016; Bjarnholt *et al.* 2018). *SbNIT4B2* has been implicated in this pathway (Nielsen *et al.* 2016; Bjarnholt *et al.* 2018), although it also plays a role in the detoxification of HCN after bioactivation (Jenrich *et al.* 2007). In *S. macrosperrum*, *SmNIT4B2* expression was consistently higher in roots than leaves, though unaffected by nitrogen application, leaving its primary role in either defence or nitrogen management uncertain. Examining expression of other bioactivation and detoxification genes, such as dhurrinase or hydroxynitrile

lyase, could help resolve this. Determining whether and how dhuririn contributes to nitrogen recycling in *S. macrospermum*, and elucidating the underlying mechanisms, will be critical for understanding its multifunctional role and for leveraging this trait to develop sorghum varieties with improved nitrogen-use efficiency and resilience, supporting sustainable dryland cropping systems.

The regulatory mechanisms for the induction of dhuririn biosynthesis appear to be different in *S. bicolor* and *S. macrospermum*. To explore whether nitrogen-responsive regulation is conserved, we examined four candidate nitrogen-responsive transcription factors previously identified through a GRN approach in *S. bicolor* that may control expression of genes in the dhuririn biosynthesis pathway (Quinn 2025). Expression of *SmEIL3*, *SmTRF-like*, and *SmMYB-like* were consistently very low across tissues and time points, indicating they are unlikely to play a role in nitrogen response or dhuririn regulation in *S. macrospermum*. A fourth candidate, *SmHRS1*, was expressed in both leaves and roots but showed significant upregulation only in leaves 12 hours after the application of nitrogen. This pattern suggests that it plays a role in the leaf nitrogen response rather than dhuririn regulation *per se*. HRS1 belongs to the NIGT/HRS1 family of GARP-type transcription factors, widely characterized for nitrogen signalling and stress responses (Kiba *et al.* 2018; Li *et al.* 2021). While this group has primarily been characterized in *Arabidopsis*, roles in regulation of nitrogen responses appear to be conserved in monocots, including rice (OsNIGT1) (Yanagisawa 2013; Medici *et al.* 2015), and in networks shared by maize and sorghum (Du *et al.* 2020). Although the HRS1 orthologue has not been functionally characterized in *S. bicolor*, its conservation across monocots suggests a similar role in nitrogen signalling within the Sorghum genus, including *S. macrospermum*.

GARP-type transcription factors could contribute to nitrogen-responsive regulation of dhuririn in *S. macrospermum*, although *SmHRS1* does not appear to control dhuririn biosynthesis in roots. Further interrogation of the differences in regulation of the dhuririn biosynthesis pathway between *S. macrospermum* and *S. bicolor* could enable fine-tuning of dhuririn concentrations in sorghum crops, thereby improving safety of forage varieties. Additionally, understanding the regulatory mechanisms that restrict accumulation of dhuririn to roots could enable development of both safer sorghum crops while retaining the benefits of nitrogen storage in the roots. Completion of the *S. macrospermum* reference genome will allow identification of transcription factor orthologues and CRE differences, providing insight into regulatory evolution and offering genetic resources to improve nitrogen-use efficiency and resilience in sorghum for sustainable dryland cropping systems.

Our study reveals distinct nitrogen-responsive regulation of dhuririn biosynthesis in *S. macrospermum*, with production restricted to roots rather than leaves as in *S. bicolor*. Understanding these species-level differences offers valuable genetic resources for breeding sorghum varieties with improved nitrogen-use efficiency and resilience, supporting sustainable dryland cropping systems.

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Author contributions. The study was conceived by R.M.G., A.A.Q. and C.K.B. A.A.Q. and C.K.B. conducted the greenhouse experiment and harvested plant tissue. A.A.Q. performed experimental analysis, analysed the data and drafted the manuscript. All authors contributed to editing and revising the manuscript.

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Use of artificial intelligence (AI) tools. AI tools were for assistance with editing and restructuring the manuscript to improve clarity, readability and conciseness. All suggestions were reviewed and edited by the authors, who take full responsibility for the accuracy and integrity of the work.

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