Heritability and plasticity of cyanogenesis in wild and domesticated *Sorghum*

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School of Biological Sciences

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Abstract

Plants produce a diverse array of endogenous compounds that modulate interactions with the biotic and abiotic environment. One such class of defence compounds, or specialised metabolites, is the nitrogen-based cyanogenic glucosides. Cyanogenesis is the process by which hydrogen cyanide (HCN) is released from cyanogenic glucosides upon tissue disruption, providing plants with an immediate defensive response to herbivore attack. Cyanogenic glucosides are widespread in the plant kingdom, yet their occurrence and accumulation appears to be disproportionately high amongst crop species. Previous studies speculated that cyanogenic glucosides were unintentionally selected for during domestication as a natural pesticide. Recent evidence suggests that cyanogenic glucosides possess additional physiological functions that benefit general plant metabolism, potentially offsetting production costs in stable, cultivated environments. This thesis utilises the diversity of the *Sorghum* genus as a model to explore the biological mechanisms driving differences in cyanogenesis between wild and domesticated plants.

Sorghum bicolor is an important food and forage crop that accumulates high concentrations of the cyanogenic glucoside dhurrin in all vegetative tissues, particularly leaves and sheaths. Prior to this thesis, the cyanogenic status of the 19 largely Australian endemic wild *Sorghum* species was unknown. It was hypothesised that wild species would show a reduced cyanogenic capacity based on the variable, generally nutrient-poor environments in which they are naturally distributed. Results of dhurrin content and potential for dhurrin-derived HCN release (HCNp) confirmed this. However, leaves of the 17 wild species tested were essentially phenotypically acyanogenic, much lower than originally expected (Chapter 2). Whole genome sequencing showed that cyanogenic gene structure was largely intact in the wild species, pointing to the likely importance of regulatory mechanisms in controlling the expression of the cyanogenic phenotype.

In *S. bicolor*, dhurrin concentration and HCNp are spatially and temporally regulated *in planta*, with higher concentrations in young and developing shoot tissues, decreasing with plant age. Tissue- and age-dependent regulation patterns studied in two wild Australian species were found to be broadly similar to *S. bicolor*, with comparable HCNp

in sheath and root tissue during seedling development (Chapter 3). However, both wild species had consistently low leaf HCNp throughout the experimental growing period, several orders of magnitude below *S. bicolor*.

Severe abiotic stress such as chronic drought also affects the deployment of dhurrin in *S. bicolor*. This is problematic for farmers relying on *S. bicolor* forage for cattle, as HCN can reach toxic concentrations. This was observed in the experiment that compared general plant performance, HCNp and nitrogen management of *S. bicolor* and seven wild *Sorghum* species grown under water-limited conditions (Chapter 4). *Sorghum bicolor* was generally affected most in terms of biomass, and also developed a very high shoot HCNp under stress, whereas the growth and leaf HCNp of wild species were less affected.

The high tissue-specific polymorphism of HCNp in the undomesticated *Sorghum* species suggests that these plants do not prioritise dhurrin in a defensive role. This implies that there may be additional functions for cyanogenic glucosides that have been selected for in natural ecosystems other than chemical defence.

Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in a peer reviewed journal. The core theme of the thesis is cyanogenesis. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences under the supervision of Prof Roslyn Gleadow and Dr Cecilia Blomstedt.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 4 my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status**	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution**	**Co-author(s), Monash student Y/N**
4	Crop wild relatives as a genetic resource for generating low-cyanide, drought-tolerant *Sorghum*	Published (*Environmental and Experimental Botany*)	85%. Concept, collecting data and writing first draft	1) Roslyn Gleadow: concept, input into manuscript 5% 2) Cecilia Blomstedt: concept, input into manuscript 4% 3) Sally Norton: methods help, input into manuscript 2% 4) Robert Henry: input into manuscript 2% 5) Birger Lindberg Møller: input into manuscript 2%	N

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

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Student signature: **Date:** 01 December 2019

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Roslyn Gleadow

Main Supervisor signature: **Date:** 01 December 2019

Publications during enrolment

1. Heraud, P., **Cowan, M. F.**, Marzec, K. M., Møller, B. L., Blomstedt, C. K. and Gleadow, R. (2018). Label-free Raman hyperspectral imaging analysis localizes the cyanogenic glucoside dhurrin to the cytoplasm in sorghum cells. *Scientific Reports* **8**, Article number: 2691. doi:10.1038/s41598-018-20928-7

Conference presentations

1. **Cowan, M.,** Blomstedt C.K. and Gleadow, R. (2017) Crop wild relatives as a resource for generating low-cyanide, drought tolerant *Sorghum*. Society for Experimental Biology Annual Meeting 2017, Gothenburg, Sweden (oral presentation)

2. **Cowan, M.,** Blomstedt C.K., Norton, S., Henry, R., Møller, B.L. and Gleadow, R. (2019) Crop wild relatives as a resource for generating low-cyanide, drought tolerant *Sorghum*. Australian Society of Plant Scientists Conference 2019, Melbourne, Australia (oral presentation)

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Table of Contents

Abbreviations

ACC	Aminocyclopropanecarboxylate oxidase
CAS	β-cyanoalanine synthase
CM	Chorismate mutase
CWR	Crop wild relative
CYP	Cytochrome P450
DHR	Dhurrinase
GA_3	Gibberellic acid
GST	Glutathione S-transferase
HCN	Hydrogen cyanide
HCNp	Hydrogen cyanide potential
HNL	Hydroxynitrile lyase
LAR	Leaf area ratio
MATE	Multidrug and toxic compound extrusion
NIT4	Nitrilase 4
NO_3^-	Nitrate
POR	Cytochrome P450 reductase
RGR	Relative growth rate
ROS	Reactive oxygen species
RWC	Relative water content
SLA	Specific leaf area
SLN	Specific leaf nitrogen
SNV	Single nucleotide variant
TLA	Total leaf area
UGT	UDP-glucosyltransferase

Chapter 1 – Introduction

1.1 Plant specialised metabolites

Plants have evolved a wide range of dynamic defence systems to withstand biotic and abiotic pressures (Hanley *et al.*, 2007; Pichersky and Lewinsohn, 2011; Neilson *et al.*, 2013). These are very often highly specific to particular plant-herbivore interactions and relevant external environmental factors (Agrawal, 2011). A major form of chemical defence comes in the production of an array of endogenous natural compounds, the specialised metabolites. The anti-herbivore defensive roles of specialised metabolites are well-established (Mithöfer and Boland, 2012). Some classes are directly toxic to organisms (e.g. alkaloids, flavonoids) (Galati and O'Brien, 2004; Matsuura and Fett-Neto, 2017). Others are stored as inactive conjugates that can be activated by specific hydrolases to release toxins (Bones and Rossiter, 1996; Gleadow and Møller, 2014); the cyanogenic glucosides are one such class.

Cyanogenic glucosides are chemically inert compounds, but they can be hydrolysed by specific degradative enzymes to release toxic hydrogen cyanide (HCN). This process is known as cyanogenesis and it provides plants with an immediate response to herbivore attack (Tattersall *et al.*, 2001). Cyanogenic glucosides occur throughout the plant kingdom, with approximately 60 different structures identified in more than 3000 species from vascular taxa (Conn, 1980; Zagrobelny *et al.*, 2008). However, the distribution of these compounds appears to be disproportionately high in cultivated plants (Jones, 1998; Gleadow and Møller, 2014). Many economically important crop species produce cyanogenic glucosides, including sorghum (*Sorghum bicolor*), cassava (*Manihot esculenta*), barley (*Hordeum vulgare*), almond (*Prunus dulcis*), lima bean (*Phaseolus lunatus*), flax (*Linum usitatissimum*) and the rubber tree (*Hevea brasiliensis*). This presents an unusual pattern in the context of other groups of specialised metabolites; numerous studies have found that domesticated plants, including several crop species, exhibit reduced and often negligible concentrations of such compounds relative to their wild progenitors and natural relatives (Mithen *et al.*, 1987; Massei and Hartley, 2000; Lindig-Cisneros *et al.*, 2002; Gols *et al.*, 2008; Mondolot *et al.*, 2008; Kroc *et al.*, 2017; Moreira *et al.*, 2018; Whitehead and Poveda, 2019). This potential aspect of the plant domestication syndrome is yet to be explored in the cyanogenic glucosides.

Twenty years ago, Jones (1998) questioned why so many cultivated plants are cyanogenic. Cyanogenesis is a significant social and economic issue in two staple crop species, sorghum and cassava, as the high potential for HCN release in these plants limits their use as food and animal feed (Wheeler *et al.*, 1990; Burns *et al.*, 2010; Cliff *et al.*, 2011; Finnie *et al.*, 2011). Jones (1998) speculated that perhaps cyanogenic glucosides were selected for during domestication due to their capacity to provide plants with a form of natural pesticide. While this seems likely, knowledge of the biological complexity of these compounds was relatively limited at that time. We now know that the synthesis and expression of cyanogenic glucosides is influenced by a range of biotic and abiotic factors, varying extensively not just across different plant taxa, but also within individual plants (Gleadow *et al.*, 2003; Gleadow and Møller, 2014). In recent years it has become clear that rather than simply acting as defensive compounds, cyanogenic glucosides may possess additional physiological functions that contribute to general plant fitness and potentially offset the resource costs of their production (Neilson *et al.*, 2013). This review outlines our current understanding of the metabolism, function, plasticity and frequency of cyanogenic glucosides in plants. Novel approaches comparing cyanogenic features in closely related domesticated and natural species may help us further understand why so many crop plants, and indeed why so many plants in general, are cyanogenic.

1.2 Metabolism of cyanogenic glucosides

1.2.1 Biosynthesis

The biochemical pathway of cyanogenic glucoside synthesis was first elucidated in *S. bicolor* and its production of the monoglucoside dhurrin [(S)-4-hydroxymandelonitrile-β-D-glucopyranoside] (MacFarlane *et al.*, 1975; Møller and Conn, 1979). In the simplest sense, biosynthesis involves the conversion of the amino acid tyrosine to a stable cyanogenic glucoside through the sequential actions of two cytochrome P450 enzymes (CYP79A1 and CYP71E1) (Koch *et al.*, 1995; Kahn *et al.*, 1999) and a soluble UDP-glucosyltransferase (UGT85B1) (Jones *et al.*, 1999) (**Fig. 1.1**). CYP79A1 catalyses the first step in the pathway, converting L-tyrosine to (*E*)-*p*-hydroxyphenylacetaldoxime (Sibbesen *et al.*, 1995). This is converted to a cyanohydrin (*p*-hydroxymandelonitrile) in a reaction catalysed by CYP71E1 (Bak *et al.*, 1998), before glucosylation of this

intermediate by UGT85B1 generates the final product, dhurrin (Jones *et al.*, 1999). In combination with an NADPH-dependent cytochrome P450 oxidoreductase (POR), these enzymes form a metabolon complex that is thought to improve the channelling efficiency of the toxic intermediates produced during dhurrin biosynthesis (Nielsen *et al.*, 2008; Møller, 2010a; Laursen *et al.*, 2016). The cyanogenic pathways characterised in other species to date (in *Lotus japonicus,* cassava and almond) all involve similar key enzymes encoded by genes from the *CYP* and *UGT* families (Jørgensen *et al.*, 2011; Kannangara *et al.*, 2011; Takos *et al.*, 2011; Thodberg *et al.*, 2018).

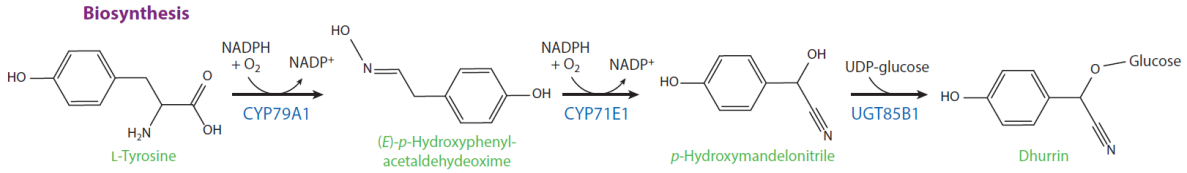


Figure 1.1: Biosynthetic pathway of the cyanogenic glucoside dhurrin in *Sorghum bicolor*. In sorghum, biosynthesis is characterised by the conversion of an L-tyrosine precursor to a stable final product, dhurrin. Reactions are catalysed by sequential actions of two cytochrome P450 enzymes (CYP79A1 and CYP71E1) and a UDP-glucosyltransferase (UGT85B1). Adapted from Gleadow and Møller (2014) with permission from Annual Reviews.

1.2.2 Cyanogenesis

Cyanogenic glucosides are not directly toxic to aerobic organisms; the true defensive function of these compounds resides in their capacity to release hydrogen cyanide when destabilised by specific β-glucosidase enzymes (Morant *et al.*, 2008). In sorghum, dhurrin is hydrolysed by the β-glucosidase dhurrinase (DHR) (Cicek and Esen, 1998), forming an unstable cyanohydrin (*p*-hydroxymandelonitrile) that disassociates and releases HCN if it is not rapidly glucosylated (**Fig. 1.2a**) (Poulton, 1990). HCN binds to and inhibits activity of the mitochondrial cytochrome *c* oxidase in the electron transport

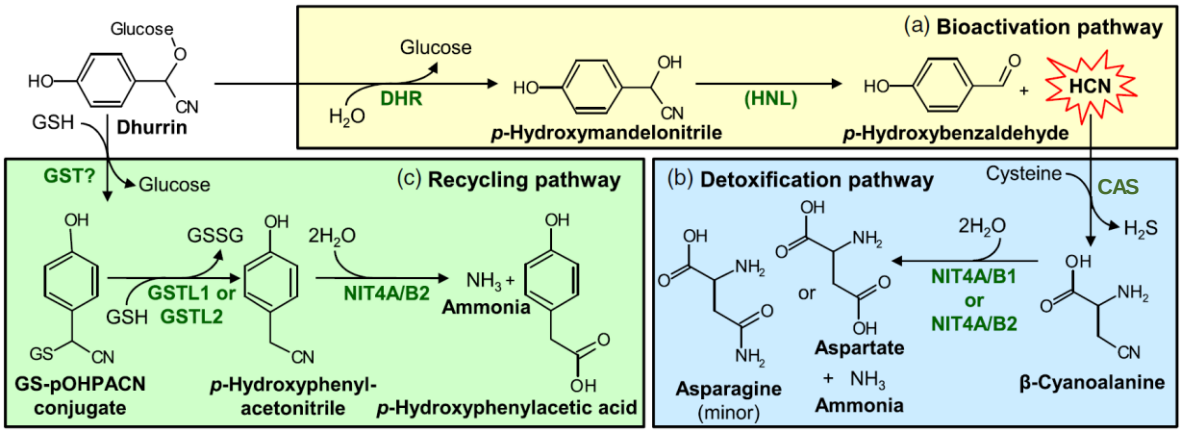


Figure 1.2: Bioactivation, HCN detoxification and nitrogen recycling pathways of the cyanogenic glucoside dhurrin in *S. bicolor*. (a) Illustrates the bioactivation pathway (cyanogenesis), in which the glucose group of dhurrin is cleaved by a specific β-glucosidase enzyme, dhurrinase (DHR), forming an unstable cyanohydrin (*p*-Hydroxymandelonitrile) that releases hydrogen cyanide (HCN). (b) Illustrates the detoxification pathway. Here, HCN is converted to β-cyanoalanine by the enzyme β-cyanoalanine synthase (CAS), which can be converted to nitrogenous compounds including free ammonia by members of the nitrilase 4 (NIT4) enzyme family. (c) Illustrates the proposed nitrogen recycling pathway, in which nitrogen (in the form of ammonia) can be gained *in planta* without the release of HCN. Adapted from Bjarnholt *et al.* (2018).

chain, blocking cellular respiration (Nelson, 2006; Leavesley *et al.*, 2008). Plants avoid self-toxicity through compartmentalisation of the cyanogenic substrate and enzyme in different parts of the cell (Thayer and Conn, 1981; Poulton and Li, 1994; Morant *et al.*, 2008; Heraud *et al.*, 2018). As such, HCN can only be released upon disruption of plant tissue when these components are mixed. This system, cyanogenesis, provides plants with an immediate targeted response to herbivore attack (Nahrstedt, 1985; Tattersall *et al.*, 2001; Zagrobelny *et al.*, 2008; Ballhorn *et al.*, 2009).

1.2.3 Detoxification

As trace amounts of HCN are generated during production of the hormone ethylene (Peiser *et al.*, 1984; Yip and Yang, 1988), all plants possess an endogenous system in which HCN can be detoxified (Blumenthal *et al.*, 1968; Piotrowski and Volmer, 2006). In this pathway, HCN is first converted to the toxic intermediate β-cyanoalanine in a

reaction catalysed by β-cyanoalanine synthase (CAS) (**Fig. 1.2b**) (Castric *et al.*, 1972). A combination of nitrilase 4 (NIT4) family enzymes then catalyse the production of ammonia, asparagine and aspartate, all of which are nitrogenous compounds that could potentially be recovered for use in plant metabolism (Piotrowski and Volmer, 2006; Jenrich *et al.*, 2007). It is thought that plants may also utilise this mechanism to recapture and detoxify any HCN released as a result of cyanogenesis (Swain and Poulton, 1994), although this is yet to be experimentally verified. A second HCN detoxification pathway also occurs in mammals, insects and some plants. In this pathway, HCN is converted to thiocyanate through the catalytic actions of the mitochondrial enzyme rhodanese (Beesley *et al.*, 1985; Zagrobelny *et al.*, 2008). Unlike CAS, rhodanese does not appear to be present in all plants (Kakes and Hakvoort, 1992; Hatzfeld and Saito, 2000). It is currently unclear whether rhodanese can detoxify cyanogenic glucoside-derived HCN *in planta*, although detection of high enzymatic activity in extremely cyanogenic sorghum seedlings (Miller and Conn, 1980) provides supporting evidence for this potential function.

1.3 Functions of cyanogenic glucosides

1.3.1 Defence against herbivory

Cyanogenesis is thought to be the key driver in the evolution of cyanogenic glucosides in plants (Bak *et al.*, 2006). Several controlled feeding trials and observational studies in natural environments have demonstrated the effectiveness of cyanogenesis as a chemical deterrent to generalist herbivores. Feeding experiments have often detected negative correlations between the concentration of cyanogenic glucosides in plant tissue and rate of insect grazing (Patton *et al.*, 1997; Ballhorn *et al.*, 2007; Ballhorn *et al.*, 2009), while field studies note that mammalian and insect herbivores generally avoid cyanogenic plants in natural systems (Cooper-Driver *et al.*, 1977; Schreiner *et al.*, 1984; Kakes, 1989; Crush and Caradus, 1995; Gleadow and Woodrow, 2000b). The negative health impacts of cyanogenic plant consumption are well documented in humans (Nahrstedt, 1985; Cliff *et al.*, 2011) and livestock (Cheeke, 1995; Soto-Blanco *et al.*, 2001; Finnie *et al.*, 2011). Interestingly, a small proportion of arthropods have developed the ability to sequester HCN for their own purposes, and in some cases even synthesise cyanogenic glucosides *de novo* (Zagrobelny *et al.*, 2018). In doing so these

specialist insect herbivores circumvent the release of HCN and are able to safely consume cyanogenic plant tissue.

1.3.2 Nitrogen recycling

The production of cyanogenic glucosides, like other specialised metabolites, has traditionally been considered to come at cost to primary plant metabolism (Herms and Mattson, 1992), tying up vital resources for non-vital functions. However, recent evidence suggests that they have acquired additional physiological functions that might not only offset any production costs, but also improve plant fitness under certain environmental conditions (Neilson *et al.*, 2013; Gleadow and Møller, 2014). For example, while the defensive functions of the nitrogenous cyanogenic glucosides are well established, it has long been suggested that they might also serve as mobilisable nitrogen storage compounds (Lieberei *et al.*, 1985; Selmar *et al.*, 1988; Selmar, 1993). Recent studies have characterised such a recycling pathway in sorghum (**Fig. 1.2c**), demonstrating the capacity for plants to utilise cyanogenic glucosides as a metabolic source of reduced nitrogen without the release of HCN (Pičmanová *et al.*, 2015; Nielsen *et al.*, 2016; Bjarnholt *et al.*, 2018). The initial step of the classic HCN detoxification pathway is catalysed by CAS (**Fig. 1.2b**), whereas the recycling pathway is mediated by enzymes of the glutathione S-transferase (GST) family. It proceeds via replacement of the glucose functional group of the dhurrin compound, possibly through the actions of an unknown GST (Bjarnholt *et al.*, 2018). The resulting conjugate (*p*-hydroxyphenyl(S-glutathione) acetonitrile) is cleaved by lambda class GSTs (GSTL) and releases *p*-hydroxyphenyl acetonitrile, which is hydrolysed by NIT4 enzymes to release free reduced nitrogen in the form of ammonia. The initial step of the pathway might also provide glucose for use in other metabolic functions.

1.3.3 Additional physiological roles

Additional metabolic functions have been proposed for cyanogenic glucosides. In some *Prunus* species the formation and subsequent recycling of cyanogenic glucosides (prunasin and amygdalin) may play a role in the control of germination from dormancy (Barros *et al.*, 2012), flowering time and floral development (Del Cueto *et al.*, 2017). The synthesis of cyanogenic glucosides might also improve plant fitness under adverse

environmental conditions by mitigating oxidative stress caused by excess production of reactive oxygen species (ROS), although evidence for this is limited and based on the biochemistry and not physiological studies (Selmar and Kleinwächter, 2013; Gleadow and Møller, 2014). ROS are important in basic cellular function, but accumulation can result in severe damage to the photosynthetic apparatus. Schmidt *et al.* (2018) suggested that rapid decreases in linamarin content of cassava plants detected after light spikes could be due to the ability of cyanogenic glucosides to scavenge ROS (e.g. hydrogen peroxide, H_2O_2) and be converted into amides (Sendker and Nahrstedt, 2009), compounds with a wide range of biological functions.

1.4 Plasticity of cyanogenic glucosides

1.4.1 Qualitative variation between and within plants

The heritability and accumulation of cyanogenic glucosides varies substantially at multiple scales across different plant taxa, beginning at the species level. In some species, such as cultivated sorghum and cassava, all individual plants are cyanogenic at some level (McBee and Miller, 1980; McMahon *et al.*, 1995). More commonly, species can contain both cyanogenic and phenotypically acyanogenic individuals (e.g. *Trifolium repens, Lotus japonicus*, some species of *Eucalyptus* and *Acacia*) (Jones, 1977; Foulds, 1982; Conn *et al.*, 1989; Hughes, 1991; Schappert and Shore, 1995; Gleadow *et al.*, 2003). A classic model for this polymorphic heritability is observed in *T. repens*, in which two independent genetic loci (*Ac* and *Li*) respectively control the production of the cyanogenic glucoside (linamarin or lotaustralin) and hydrolysing β-glucosidase enzyme (linamarase). As both components are required, natural cyanogenesis cannot occur if a plant lacks functional alleles at either of these loci (Hughes, 1991; Olsen *et al.*, 2008). This polymorphism is thought to be maintained in natural populations as a consequence of opposing biotic and abiotic selection pressures for the trait (Hayden and Parker, 2002; Olsen and Ungerer, 2008). This is also apparent in the large *Eucalyptus* genus, in which only a small number of species restricted to three subgenera are capable of naturally releasing cyanide (Goodger *et al.*, 2002; Gleadow *et al.*, 2008). In the large genus *Acacia*, some species synthesise cyanogenic glucosides but lack the associated β-glucosidases capable of activating them (Conn *et al.*, 1989). However,

plants that produce only the cyanogenic glucoside component can still be toxic to ruminant animals. Livestock such as cattle contain microbes in the rumen capable of degrading cyanogenic glucosides and releasing HCN (Finnie *et al.*, 2011).

Cyanogenic glucosides are also differentially expressed within individual plants. Cassava and white clover accumulate cyanogenic glucosides in all tissue types (McMahon *et al.*, 1995; Stochmal and Oleszek, 1997). All vegetative tissues of sorghum contain dhurrin, but it is absent in the mature reproductive organs (Loyd and Gray, 1970; Nielsen *et al.*, 2016). In some species of *Eucalyptus,* cyanogenic glucosides are present in all aboveground parts of the plant but not in the roots (Hughes, 1991; Gleadow and Woodrow, 2000b). *Lotus japonicus* lacks both linamarin and lotaustralin in the seeds and roots (Forslund *et al.*, 2004). Species that produce multiple structures of cyanogenic glucosides (e.g. cassava, almond, *Trifolium repens, Turnera ulmifolia)* sometimes differentially express these between shoots and roots (Stochmal and Oleszek, 1997) or at different ontogenetic stages (Schappert and Shore, 2000; Sánchez-Pérez *et al.*, 2012), although the factors driving these expression patterns remain unclear.

1.4.2 Developmental and environmental regulation

The regulation of cyanogenic glucosides *in planta* appears to be controlled by a complex interaction of biotic and abiotic factors, largely dependent on developmental stage (Gleadow and Møller, 2014). The concentration of cyanogenic glucosides, and the resulting quantitative potential to release HCN (HCNp), is generally highest in young and developing tissues (Till, 1987; Dahler *et al.*, 1995; Gleadow and Woodrow, 2000b; Forslund *et al.*, 2004; Lieberei, 2007). In young sorghum seedlings, for example, dhurrin is accumulated at very high concentrations and may constitute more than 20% of total dry plant biomass in the tip of the coleoptile (Loyd and Gray, 1970; Halkier and Møller, 1989). This high expression of dhurrin is correlated with increased transcript levels of *CYP79A1* in the first week of growth (Busk and Møller, 2002). Regarding changes in concentration over time, younger leaves typically exhibit higher HCNp than mature leaves in many species including sorghum (O'Donnell *et al.*, 2013; Gleadow *et al.*, 2016b), cassava (Jørgensen *et al.*, 2005), lima bean (Ballhorn *et al.*, 2005) and the rubber tree (Kongsawadworakul *et al.*, 2009). This expression pattern might indicate

that plants accumulate cyanogenic glucosides in their more fragile, developing tissues as a deterrent to herbivores (Morant *et al.*, 2008; Ballhorn *et al.*, 2010). However, clear exceptions to the pattern have been detected in some species of the *Eucalyptus* section *Maidenaria*. These species are essentially acyanogenic in the early seedling stage, before HCNp increases with maturity (Goodger *et al.*, 2006). The typical developmental trend towards lower HCNp is also reversed in lima bean (*P. lunatus*), in which HCNp was found to be higher in older leaf tissue compared to younger developing leaves (Ballhorn *et al.*, 2008).

External environmental factors have some degree of influence over the accumulation of cyanogenic glucosides in plants. An increased concentration and subsequent higher HCNp is generally observed in plants exposed to abiotic stress such as drought (Vickery *et al.*, 1987; Liang, 2003; Ballhorn *et al.*, 2011; Gleadow and Møller, 2014). In studies investigating response to drought in sorghum, leaves of plants grown under prolonged water deficit exhibited higher HCNp than leaves of well-watered plants (Boyd *et al.*, 1938; Duncan, 1996; O'Donnell *et al.*, 2013; Neilson *et al.*, 2015; Gleadow *et al.*, 2016b). Tubers and leaves of drought-stressed cassava plants have been shown to contain much higher concentrations of the cyanogenic glucosides linamarin and lotaustralian in comparison to watered plants (Vandegeer *et al.*, 2013). Field and glasshouse studies of *Eucalyptus cladocalyx* also detected higher HCNp in the leaves of plants growing under water-limited conditions (Gleadow and Woodrow, 2002b; Woodrow *et al.*, 2002). The mechanisms driving these types of increases have proven difficult to determine, however, as the response varies based on length and severity of stress and the availability of soil nutrients. More acute moisture stress appears to have less of an impact on plant HCNp (Wheeler *et al.*, 1990; Hayden and Parker, 2002). The actual biosynthetic rate of cyanogenic glucosides may not be affected by abiotic factors; rather, the reduced biomass of stressed plants could be responsible for the increase in concentration across a smaller area of tissue (i.e. a dilution effect) (Selmar and Kleinwächter, 2013). Nevertheless, there is some evidence to suggest that the biosynthetic genes are upregulated at the transcriptional level under water deficit: in a cell culture study of *Escholtzia californica*, cultures that were osmotically stressed produced higher concentrations of cyanogenic compounds similar to dhurrin (Hösel *et al.*, 1985). Numerous studies have demonstrated that a host of other environmental

factors, such as temperature, CO_2, salinity, frost and light, also affect the capacity to release cyanide in various cyanogenic species (Stochmal and Oleszek, 1997; Burns *et al.*, 2002; Ballhorn *et al.*, 2011; Brown *et al.*, 2016; Gleadow *et al.*, 2016a). It is possible that both potential regulatory processes, a reduction in plant biomass or a transcriptional enhancement of cyanogenic glucoside production, factor into the stress responses seen across these studies.

1.5 Cyanogenesis in wild and cultivated plants

Considering the potential social and economic impacts of cyanogenesis and the release of HCN on human and animal health, research into cyanogenic glucosides has primarily focused on domesticated plant species (Jones, 1998; Gleadow and Møller, 2014). Despite this, a large body of literature documents the occurrence of cyanogenic glucosides and cyanogenesis in natural plant genera. Knowledge of the frequency of cyanogenesis across the plant kingdom has generally been obtained through separate surveys of specific taxonomic groups or of plant communities in particular regions. In regard to particular taxa, many cyanogenic surveys have been undertaken: in Australia, occurrence of cyanogenesis has been extensively studied in different genera of the *Fabaceae* family, particularly in *Acacia* (Conn *et al.*, 1985; Maslin *et al.*, 1988; Conn *et al.*, 1989) and species of the subfamily *Papilionoidae* (Maslin *et al.*, 1990), and in the large *Eucalyptus* genus (Gleadow *et al.*, 2008). The presence of cyanogenic glucosides has also been established in genera of the plant family *Passifloraceae* (Spencer and Seigler, 1985; Olafsdottir *et al.*, 1990), and in chemotaxonomic surveys of *Lotononis* and *Buchenroedera* (family *Fabaceae*) species (Van Wyk, 1989). Although the frequency of cyanogenesis has been comparatively less studied in region-specific plant communities, surveys have been conducted around the world, for example in the Galapagos Islands (Adsersen *et al.*, 1988), in communities across the United States (Seigler, 1976) and in the tropical rainforests of Queensland (Miller *et al.*, 2006).

While the results of these studies provide a fundamental understanding of the frequency and variation of cyanogenesis in nature, they have relied on qualitative measures (presence/absence). This makes it difficult to contextualise findings in the general metabolism of cyanogenic glucosides, particularly as environmental responses may be unique to particular species or genera. Nevertheless, the information obtained

from these studies forms the basis of cyanogenic frequency estimates between wild and cultivated plants (Jones, 1998). Taken together, it also appears that cyanogenic plants are generally clustered in discrete phylogenetic groups (e.g. particular genera or subgenera). It is unclear how domestication might disrupt this clustering pattern, as studies directly comparing cyanogenesis between wild and crop species in a single genus are rare. In fact, only one dataset has quantitatively assessed HCN in the undomesticated wild relatives of any major cyanogenic crop species. Nassar and Fichtner (1978) found that tuber HCN content in six wild relatives of cassava (*M. esculenta*) varied significantly between species, with some species showing lower HCN content than cultivated cassava and some higher (although *M. esculenta* was not tested in this particular study). Today, more robust quantitative methods can assess the potential of plants to release HCN directly from cyanogenic glucosides (Gleadow *et al.*, 2012). The prevalence of cyanogenesis remains completely unknown in the wild relatives of many important crops, including sorghum

1.6 Thesis outline

Nassar (1986) and Jones (1998) raised the idea of harnessing the genetic diversity of cyanogenesis in crop wild relatives (CWRs) as a way to reduce the toxic hydrogen cyanide potential of crop species. Evidence for non-defensive functions of cyanogenic glucosides raises the possibility that their reduction or removal may negatively affect plant growth, as they appear to be intrinsically linked to general metabolism (Neilson *et al.*, 2013; Zidenga *et al.*, 2017). The domesticated and undomesticated species of the *Sorghum* genus provide a unique opportunity to explore how cyanogenic glucosides have adapted to modulate plant plasticity under highly divergent selective pressures.

Sorghum (*S. bicolor*) is a major global cereal crop and a dietary staple for food-insecure people in parts of Africa and Asia. Grown mainly in semi-arid and sub-tropical regions due to its high tolerance of abiotic stress, sorghum is grown for the grain, as forage and feed for livestock and as a source of biofuel (Mathur *et al.*, 2017). Sorghum produces the cyanogenic glucoside dhurrin in all vegetative tissues, and at high enough concentrations the potential released cyanide can cause respiratory poisoning in feeding livestock (Finnie *et al.*, 2011). This issue is more prevalent during times of drought, as accumulation of dhurrin is increased in plants exposed to abiotic stress

(O'Donnell *et al.*, 2013; Neilson *et al.*, 2015). While sorghum was initially domesticated in Africa (Smith *et al.*, 2019), 14 of its 19 undomesticated wild relatives are endemic to Australia (Dillon *et al.*, 2007b). The cyanogenic status of these genetically isolated endemic species is unknown.

This thesis investigates and directly compares cyanogenesis and the synthesis of cyanogenic glucosides between related wild and cultivated species for the first time. Three main research questions are asked: 1) Are the undomesticated species within the *Sorghum* genus cyanogenic, and at what capacity? 2) Does functional expression of cyanogenesis vary in these species, as might be expected in plants adapted to variable natural environments? 3) Do sorghum's wild relatives show differential expression of cyanogenic glucosides depending on plant ontogeny, tissue type or response to drought?

The first step is to understand the diversity and underlying mechanics of cyanogenesis in wild *Sorghum*. **Chapter 2** undertakes the first study of cyanogenesis across the *Sorghum* genus. It examines and compares quantitative cyanogenic potential, cyanogenic glucoside content and underlying genetic structure in the undomesticated and domesticated species of *Sorghum*. It discusses the potential advantages and disadvantages of cyanogenesis in natural and cultivated environments in the context of these results.

The regulation of cyanogenic glucosides and their resulting expression of hydrogen cyanide are controlled by a multitude of factors, including plant ontogeny and specific tissue type. Young sorghum seedlings contain particularly high concentrations of dhurrin (Halkier and Møller, 1989; Busk and Møller, 2002; Blomstedt *et al.*, 2018). **Chapter 3** investigates the differential developmental and tissue-dependent regulation of cyanogenesis between *S. bicolor* and two of its wild relatives during seedling development. It aims to identify potential trade-offs in the allocation of nitrogen resources for growth and defence, and explores how the wild species might balance the potential metabolic functions of cyanogenic glucosides.

Drought is one of the major limiting factors of agriculture. Although *S. bicolor* is relatively drought tolerant, studies have demonstrated that plants grown under water

deficit accumulate high concentrations of dhurrin in vegetative tissues (O'Donnell *et al.*, 2013; Gleadow *et al.*, 2016b). **Chapter 4** aims to identify the potential of wild *Sorghum* to improve tolerance to drought in *S. bicolor* and subsequently reduce the accumulation of dhurrin under adverse environmental conditions. It investigates and compares plant performance of *S. bicolor* with seven of its wild relatives under well-watered and water-limited conditions, with an emphasis on the tissue-specific response of dhurrin to abiotic stress.

Chapter 2 – Cyanogenesis in the *Sorghum* genus: from genotype to phenotype

2.1 Introduction

2.1.1 Cyanogenesis in *Sorghum bicolor*

Sorghum (*Sorghum bicolor* (L.) Moench) is a major cereal crop and the sixth most planted crop worldwide (Zhao *et al.*, 2019). Cultivated predominately in arid and semi-arid regions, the grain is a staple food for more than 500 million food-insecure people in parts of Africa and Asia (Mace *et al.*, 2013). Sorghum is a multi-purpose crop grown for the production of grain, as forage and feed for livestock, and increasingly as a source of biofuel (Reddy *et al.*, 2008; Mathur *et al.*, 2017). As a C_4 plant, sorghum has several advantages over other major cereals such as wheat and rice under harsh growing conditions as a result of increased photosynthetic efficiency and a higher tolerance to drought and elevated temperatures (Stout and Simpson, 1978; Harold Brown, 1999; Paterson *et al.*, 2009; Sage and Zhu, 2011; Tari *et al.*, 2013).

Sorghum produces the cyanogenic glucoside dhurrin in all vegetative tissues. Cyanogenesis describes the process whereby cyanogenic glucosides are hydrolysed by specific degradative β-glucosidase enzymes to release hydrogen cyanide (HCN) (Gleadow and Møller, 2014). Plants avoid autotoxicity by the spatial separation of cyanogenic substrate and enzyme at the cellular or subcellular level (Poulton, 1990; Selmar, 1993; Morant *et al.*, 2008; Heraud *et al.*, 2018). Cyanogenesis can therefore only occur upon plant tissue disruption, for example through herbivore-induced damage or food processing practices. This binary system has been demonstrated to provide plants with an immediate targeted response to herbivore attack (Tattersall *et al.*, 2001; Gleadow and Woodrow, 2002a; Zagrobelny *et al.*, 2008; Ballhorn *et al.*, 2009). However, cyanogenesis also limits the use of sorghum as livestock feed and forage (Wheeler *et al.*, 1990). Studies have shown that sorghum plants subjected to abiotic stress or high application of nitrogen can accumulate high concentrations of dhurrin in aboveground vegetative tissues (Busk and Møller, 2002; O'Donnell *et al.*, 2013; Neilson *et al.*, 2015; Gleadow *et al.*, 2016b), in many cases above the 600 ppm threshold considered unsafe for consumption by cattle (Hunt and Taylor, 1976).

2.1.2 Why are so many crop plants cyanogenic?

Cyanogenic glucosides are widespread throughout the plant kingdom, yet these compounds occur at a disproportionately high frequency in cultivated plants (Jones, 1998). It is currently unclear why so many crop plants, including *S. bicolor,* are cyanogenic. It is generally proposed that an increased production of cyanogenic glucosides may have been indirectly selected for during domestication as a form of natural pesticide (Jones, 1998; McKey *et al.*, 2010; Gleadow and Møller, 2014). However, while cyanogenesis is an effective deterrent against generalist herbivores, some of the most common and damaging insect pests of *S. bicolor* [e.g. cotton bollworm (*Helicoverpa armigera*), sorghum midge (*Stenodiplosis sorghicola*)] feed mainly on the acyanogenic mature grain rather than the cyanogenic vegetative tissues, thereby avoiding any potential HCN toxicity effects (Sharma *et al.*, 2002; Franzmann *et al.*, 2008). In addition, some specialist arthropods sequester or synthesise cyanogenic glucosides *de novo* and are therefore able to safely consume cyanogenic plant tissue (Nahrstedt, 1988; Zagrobelny *et al.*, 2018). The initial reasons for domestication and early cultivation of *S. bicolor* must also be considered – sorghum was likely cultivated primarily for the production of the acyanogenic grain rather than as animal feed (Dillon *et al.*, 2007b; Fuller and Stevens, 2018; Winchell *et al.*, 2018). The presence of dhurrin in all vegetative parts of the plant may therefore have provided benefits in terms of deterring herbivores, without any impact of cyanide toxicity on human health. However, this explanation does not seem to apply to some other domesticated crops, such as cassava (*Manihot esculenta* Cranz), where the highly cyanogenic tubers are directly consumed by humans, often with detrimental effects if not properly processed (Burns *et al.*, 2010).

The prevalence of cyanogenesis in domesticated and undomesticated plants has generally been studied independently (Jones, 1998; Gleadow and Møller, 2014). Direct comparisons of cyanogenic traits between major crop species and their genetically isolated wild relatives have rarely been performed. For example, Nassar and Fichtner (Nassar and Fichtner, 1978; Nassar, 2000) assessed the quantitative HCN content of six undomesticated cassava (*Manihot*) species, but did not examine domesticated varieties (*M. esculenta*) in the same study. Variation in cyanogenic capacity has been investigated more thoroughly in naturalised populations of domesticated species, for example in lima bean (*Phaseolous lunatus*) (Ballhorn *et al.*, 2009), white clover (*Trifolium repens*)

(Blaim and Nowacki, 1979; Blaise *et al.*, 1991), legumes (*Lotus spp.*) (Band *et al.*, 1981), *Macadamia spp.* (Dahler *et al.,* 1995) and the rubber tree (*Hevea spp.*) (Selmar *et al.*, 1991). There are no published studies on the degree and extent of cyanogenesis in the undomesticated species in the genus *Sorghum.*

2.1.3 The *Sorghum* genus

Currently, there are 25 recognised species within the *Sorghum* genus, separated into five morphologically distinct sub-genera: *Eusorghum, Chaetosorghum, Heterosorghum, Parasorghum* and *Stiposorghum* (Garber, 1950; De Wet, 1978; Dillon *et al.*, 2007a). *Sorghum bicolor*, all *S. bicolor* subspecies and landraces, domesticated *Sorghum* varieties and interspecific naturalised domesticated × wild hybrids belong to the *Eusorghum*. The four other subgenera contain 19 species, wild relatives of *S. bicolor*: the *Chaetosorghum* and *Heterosorghum* each contain a single native Australian species, *S. macrospermum* and *S. laxiflorum*, respectively; the *Parasorghum* subgenus contains seven species, five native to Australia (*S. grande, S. leiocladum, S. matarankense, S. nitidum* and *S. timorense*) and two native to Africa and Asia (*S. purpureosericeum* and *S. versicolor*); and the *Stiposorghum* contains ten species all endemic to northern Australia (*S. amplum, S. angustum, S. brachypodum, S. bulbosum, S. ecarinatum, S. exstans, S. interjectum, S. intrans,* and *S. plumosum*) (Lazarides *et al.*, 1991). The genus may also be categorised into three distinct genepools based on relatedness and interspecific compatibility within plant taxa (Harlan and de Wet, 1971). The primary and secondary genepools contain the fully and partially inter-fertile *Eusorghum* species, and the tertiary genepool includes all species from the four other subgenera. All species of *Eusorghum* are thought to produce dhurrin at similar concentrations (Gray *et al.*, 1968; Nicollier *et al.*, 1983). However, the cyanogenic status, both qualitative and quantitative, of the undomesticated species that make up the four other *Sorghum* subgenera is currently unknown.

Sorghum bicolor was originally domesticated in northeastern Africa around 6000 years ago (De Wet and Harlan, 1971; Winchell *et al.*, 2017), and most *Eusorghum* are also native to the African continent. *Sorghum halepense* (Johnson grass) originated in Africa but is now a serious weed found all over the world, while *S. propinquum* is a naturalised species found in southeast Asia (Mace *et al.*, 2013). A large proportion of

undomesticated *Sorghum* species (15 of 19) are distributed exclusively in the remote, relatively undisturbed regions of northern Australia (**Fig. 2.1**) (Lazarides *et al.*, 1991; Norton *et al.*, 2017). Due to the varied geographic distribution and likely extensive period of genetic isolation between species of the *Sorghum* genus (Dillon *et al.*, 2007b), the endemic Australian species provide a unique opportunity to investigate the evolutionary drivers for the deployment of cyanogenic glucosides; differences in composition may have arisen in plants as a result of natural selection rather than anthropogenic artificial selection.

2.1.4 Aims

Resource allocation theories predict that the synthesis and maintenance of the nitrogen-based cyanogenic glucosides must come at a cost to plant growth (McKey, 1974; Herms and Mattson, 1992). Accordingly, plants distributed in natural, resource-limited environments (e.g. undomesticated *Sorghum*) might show a reduced capacity for cyanogenic glucoside production and subsequent release of HCN. In this chapter, a phenotype-genotype approach was employed with the aim of constructing the first profile of cyanogenesis across the undomesticated species of the *Sorghum* genus. Phenotypic variation in cyanogenic status was assessed through analysis of differences in two distinct parameters: firstly, the quantitative potential to release hydrogen cyanide from leaf tissue was measured at different developmental stages in species of the five *Sorghum* subgenera; secondly, the identity and relative content of any cyanogenic glucosides present was determined. Phenotypic analyses were conducted at pre-flowering stages, as dhurrin concentration peaks during seedling development in *S. bicolor* (Busk and Møller, 2002). Genotypic differences were also investigated by analysing structural variation in genes known or thought to be involved in one of several cyanogenic pathways, including dhurrin biosynthesis (Darbani *et al.*, 2016; Nielsen *et al.*, 2016), cyanogenesis (Hayes *et al.*, 2015), HCN detoxification and endogenous turnover of dhurrin (Nielsen *et al.*, 2016; Bjarnholt *et al.*, 2018) (see **Chapter 1 Figs. 1.1-1.2**).

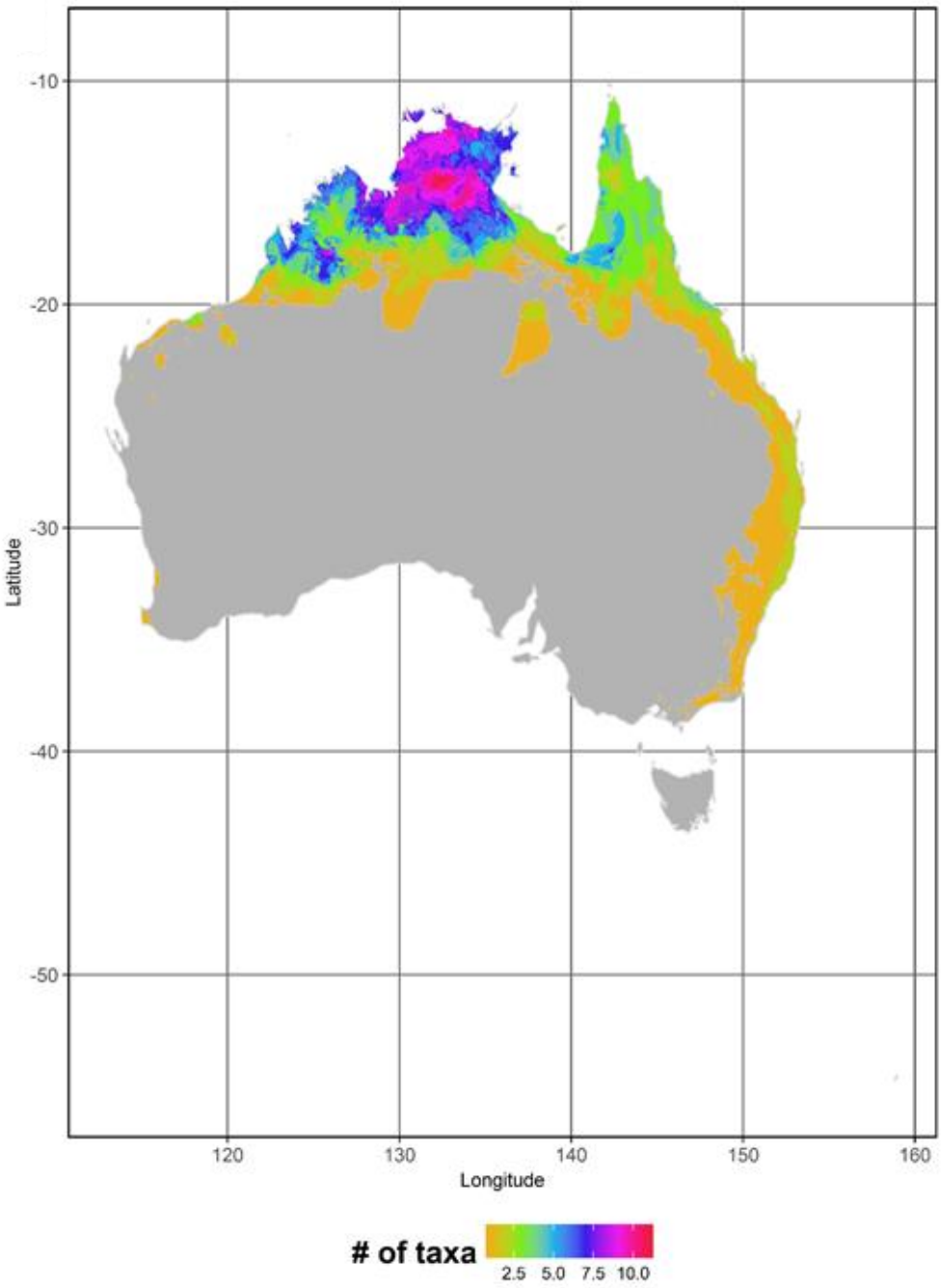


Figure 2.1: Predicted distributions and species richness of the 16 Australian native species of undomesticated *Sorghum.* Potential distribution models of each species were generated based on current known occurrence records and climatic conditions, and overlaid on the map. The blue to red colours indicate areas that potentially containing a higher number of species, while green to orange colours show areas of lower potential species richness. Reproduced from Norton *et al.* (2017) with permission from CSIRO Publishing.

2.2 Methods

2.2.1 Plant material and growing conditions

Seeds from accessions of 15 undomesticated *Sorghum* species from subgenera *Chaetosorghum, Heterosorghum, Parasorghum* and *Stiposorghum* (*Chaetosorghum: S. macrospermum* E.D. Garber; *Heterosorghum: S. laxiflorum* F.M. Bailey; *Parasorghum: S. leiocladum* (Hack.) C.E. Hubb., *S. purpureosericeum* (Hochst. Ex A. Rich.) Asch. & Schweinf. and *S. versicolor* Andersson.); *Stiposorghum: S. amplum* Lazarides, *S. angustum* S.T. Blake, *S. brachypodum* Lazarides, *S. bulbosum* Lazarides, *S. ecarinatum* Lazarides, *S. exstans* Lazarides, *S. interjectum* Lazarides, *S. intrans* F. Muell. Ex Benth., *S. plumosum* (R.Br) P. Beauv., *S. stipoideum* (Ewart & Jean White) C.A. Gardner & C.E. Hubb), and two *Eusorghum* species (*S. halepense* (L.) Pers. and *S.* × *almum* Parodi), were obtained from the Australian Grains Genebank (AGG), Horsham, Victoria (**Table 2.1**). *Sorghum bicolor* (BTx623) seed was supplied by the Queensland Alliance for Agriculture and Food Innovation (QAAFI), University of Queensland (UQ).

The following protocol was developed to achieve an acceptable rate of germination in the wild plants. Each caryopsis was removed from the seed covering using a scalpel and forceps and placed in a 2 mL Eppendorf tube containing 500µL of 3mM Gibberellic Acid (GA_3) (Growth Regulator, G7645, Sigma-Aldrich) and left at room temperature (24°C) for approximately 15 h. The GA_3-treated caryopses were then transferred to Petri dishes on two layers of filter paper moistened with sterile double distilled water and sprayed with 3mM GA_3 solution. The dishes were incubated in a constant temperature cabinet set at 35°C day (9am-5pm) and 25°C overnight, with a 14 h photoperiod from 6am-8pm. Petri dishes were wrapped in foil for the first 24 h in the cabinet. The caryopses were checked daily and kept moist by spraying with sterile double distilled water. Once the caryopses had germinated and formed a small root and shoot (approximately 1 cm), they were transplanted into soil in small trays until the seedlings had established, approximately 4-5 d post-germination. Seedlings were then planted out into larger pots containing the soil mix and perlite (4:1 ratio). All plants (total n = 180) were grown under controlled greenhouse conditions at Monash University (coordinates: 37°54'36"S 145°08'02"E) with a mean temperature of 27.8°C ± 2.6°C and 18.3°C ± 2.1°C day/night, and an average photoperiod of 14 h (average photosynthetic

Table 2.1: Individual accessions of *Sorghum* species (excluding *S. bicolor*) examined in the current study. Seeds were obtained from the Australian Grains Genebank (AGG), Horsham, Victoria (Coordinator: Dr Sally Norton). *denotes species of the *Eusorghum* subgenus.

Species	Accession number	Accession name	Provenance	Latitude	Longitude
S. amplum	302623	JC 2361	Kimberley, WA	-14.5982	125.7928
S. angustum	302596	JC 2280	Central Highlands, QLD	-13.4583	142.9613
S. brachypodum	302670	IDC 7561	Kakadu, NT	-12.5667	-132.8833
S. bulbosum	302645	JC 2320	Kimberley, WA	-16.0965	128.39
S. ecarinatum	302656	JC 2342	Kimberley, WA	-17.1838	124.9158
S. exstans	302577	JC 2247	Melville Island, NT	-11.6403	130.6317
S. interjectum	302428	JC 2067	Kimberley, WA	-15.7695	128.6462
S. intrans	302394	JC 2030	Katherine, NT	-14.4503	132.2432
S. plumosum	302415	JC 2053	Katherine, NT	-14.7922	131.9427
S. stipoideum	302442	JC 2084	Kimberley, WA	-18.0412	127.8037
S. macrospermum	302367	JC 2253	Katherine, NT	-14.4097	132.1977
S. laxiflorum	302525	JC 2196	Katherine, NT	-16.6477	135.8495
S. leiocladum	300170	ATCGRC 0062	New England, NSW	-28.9263	152.3453
S. matarankense	302521	JC 2188	Katherine, NT	-16.0797	136.3077
S. timorense	302660	JC 2340	Kimberley, WA	-17.3657	124.2937
S. purpureosericeum	318068	IS 18945	NA	NA	NA
S. versicolor	321128	IS 18941	Usagara, Tanzania	NA	NA
*S. halepense**	300167	ATCGRC 0056	Western Downs, QLD	-28	152
*S. × almum**	316842	ATCGRC 0014	Central Highlands, QLD	-24.1187	148.0901

photon flux density: 421 ± 71 µmol quanta m^{-2} s^{-1}). Supplementary light from sodium lamps (MK-1 Just-a-shade, Ablite Australia) was used to maintain the irradiation level when the natural photoperiod decreased over autumn. All plants were checked and watered daily as required.

2.2.2 Cyanide assays

Cyanogenic glucoside concentration was determined using the photometric evolved HCN detection method using the König reactions following Gleadow *et al.* (2012). The hydrogen cyanide potential (HCNp) is the total amount of HCN evolved from hydrolysis of the entire content of endogenous cyanogenic glucosides. It is used as a proxy for dhurrin, such that each mg of HCN is equivalent to 11.5 mg of dhurrin in the plant tissue. Freeze-dried tissue of the youngest fully emerged leaf was ground to a fine powder using a MM 300 MixerMill (Retsch, Haan, Germany). Samples (10 mg) were placed into 2mL screw-cap glass vials containing 300µl of 0.2% w/v β-glucosidase almond emulsion (β-$_D$-glucoside glucohydrolase, G-0395, Sigma) in 0.1M citrate buffer (pH 5.6) (trisodium citrate, Sigma). A microcentrifuge tube containing 200µl of 1M NaOH was inserted into the vial. Vials were frozen overnight at -20°C, thawed at room temperature and then incubated at 37°C for approximately 20 hours. This freeze-thaw cycle was performed to disrupt the cells and allow the cyanogenic glucoside to come into contact with the β-glucosidase enzyme and cause the release of HCN. The β-glucosidase was present in excess to ensure complete conversion of dhurrin to HCN during incubation. The HCN gas diffuses into the NaOH in the microcentrifuge tube, forming NaCN. The microcentrifuge tube was removed from the vial and the NaCN-containing solution was diluted 1 in 10 in MilliQ H_2O. 50µl of each diluted sample was pipetted into a 96 well microtitre plate, followed by 50µl of 0.5M acetic acid, 125µl 2 x succinimide reagent and 50µl barbituric acid in pyridine reagent. After incubating the plate for 15 minutes at room temperature, the absorbance was read at 595nm using a spectrophotometer (Fluostar Galaxy, BMG Lab Technologies). An altered method not involving dilution of the 1M NaOH in the inner collecting tube was used where concentrations were very low to improve detection. All assays were performed in triplicate, and NaCN standards were included on each plate to create a standard curve. Data are expressed as cyanide potential (HCNp, mg CN g^{-1} dry weight), that is, the maximum cyanide release per mg cyanogenic glucoside and includes any free cyanide that may be present in the tissue.

2.2.3 LC-MS analysis and identification of cyanogenic glucoside(s)

The youngest fully emerged leaf from plants of individual accessions of *Sorghum* species (*Chaetosorghum*: *S. macrospermum*; *Stiposorghum*: *S. brachypodum*, *S. interjectum*; *Parasorghum*: *S. purpureosericeum*, *S. stipoideum*; *Eusorghum*: *S. halepense, S. propinquum*) (n = 3) was removed at the ligule at six weeks post-germination. The presence and identification of cyanogenic glucosides was analysed using an Advance UHPLC system (Bruker, Bremen, Germany) linked to an EVOQ Elite TripleQuad mass spectrometer (Bruker, Bremen, Germany). The relative quantities of any cyanogenic glucosides were calculated as the ratio of the peak area (normalised with an internal standard) to the sample weight (50 mg). LC-MS analysis was performed by Dr Camilla Knudsen at the Section for Plant Biochemistry, University of Copenhagen, Denmark. Leaf HCNp of the same sampled plants was determined by me at Monash University.

2.2.4 DNA extraction and quantification

Leaf material for the extraction of DNA for genome sequencing was harvested from accessions of six wild *Sorghum* species (*Chaetosorghum*: *S. macrospermum*; *Heterosorghum*: *S. laxiflorum*; *Stiposorghum*: *S. brachypodum*; *Parasorghum*: *S. leiocladum, S. matarankense, S. purpureosericeum*) grown by Dr Sally Norton at the Australian Grain Genebank (AGG), Horsham, Victoria (coordinates: 36°43'S 142°12'E). See Section 2.2.1 for the method of germination. Once germinated, seedlings were transplanted into pots containing Richgro commercial potting mix and transferred to a poly tunnel igloo. Plants were grown under average temperatures of approximately 30°C/17°C day/night and watered 2-3 times per week by an irrigation system. Leaf tissue (4 g total) was harvested from individual plants at multiple time points during growth to ensure plants survived. Material was placed on dry ice and sent to UQ for analysis.

High quality plant DNA was extracted using a modified CTAB protocol following Furtado (2014). Frozen plant material was first coarsely ground in liquid nitrogen using a mortar and pestle, and then finely ground using a QIAGEN Tissue Lyser 400. DNA quality was determined by measuring absorbance using a spectrophotometer, with the extent of shearing visualised on a 0.7% agarose gel in 0.5× TBE buffer.

2.2.5 DNA sequencing and data processing

Extracted DNA was sent to the Ramaciotti Centre for Genomics, University of NSW, Sydney for Next Generation Sequencing. Samples for paired end sequencing were prepared using a Nextera DNA Flex Library Prep Kit. Whole genome shotgun sequencing was performed using an Illumina NovaSeq 6000 Sequencing System (Illumina, San Diego, CA). Sequencing data was analysed in CLC Genomics Workbench 12.0 (CLC Bio, Aarhus, Denmark) at Monash University, accessing servers located at the Queensland Alliance for Agriculture and Food Innovation (QAAFI). Twenty genes were selected based on known and putative roles in cyanogenesis (dhurrin biosynthesis, catabolism, turnover) as well as in the synthesis of tyrosine (the amino acid substrate for the first step in dhurrin biosynthesis) and ethylene (**Table 2.2**). The *S. bicolor* genomic sequences for these genes were downloaded to CLC from Phytozome (https://phytozome.jgi.doe.gov) (Paterson *et al.*, 2009) and used to map the reads generated from the sequencing of the six wild *Sorhgum* species. Single nucleotide variants (SNVs) for each gene were called using the basic variant detection tool in CLC with a minimum coverage of 10, read count of 2 and allele frequency of 10%. Multi-allelic nucleotide variations were not included in this study as these were likely to be false positives, potentially caused by sequencing errors or errors in variant detection. Predicted effects on the protein sequence of all SNVs for all species were investigated in *CYP79A1*, the gene encoding the rate-limiting step of dhurrin biosynthesis (Busk and Møller, 2002), using the online Ensembl Variant Effect Predictor software (McLaren *et al.*, 2016). The impact of these changes on the amino acid sequence of CYP79A1 and protein function was determined using the online software program SNAP2 within PredictProtein (https://www.predictprotein.org/) (Yachdav *et al.*, 2014).

2.2.6 Statistical analysis

All quantitative data were analysed using GraphPad Prism version 7.02 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). Ordinary one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons tests, was used to compare HCNp between and within species across different time points. A 95% confidence level was set for all statistical tests.

Table 2.2: Enzyme family, identification, function, and reference information for the genes examined in this study. *denotes proposed gene function.

Gene	Gene ID	Enzyme	Function	Reference
CYP79A1	Sobic.001G012300	Cytochrome P450	Dhurrin biosynthesis	(Nielsen *et al.*, 2016)
CYP71E1	Sobic.001G012200	Cytochrome P450	Dhurrin biosynthesis	(Nielsen *et al.*, 2016)
UGT85B1	Sobic.001G012400	UDP-glucosyltransferase	Dhurrin biosynthesis	(Nielsen *et al.*, 2016)
POR	Sobic.002G295100	Cytochrome P450 reductase	Dhurrin biosynthesis	(Nielsen *et al.*, 2016)
MATE	Sobic.001G012600	MATE transporter	Dhurrin biosynthesis*	(Darbani *et al.*, 2016)
HNL	Sobic.004G335500	α-hydroxynitrile lyase	Cyanogenesis	(Nielsen *et al.*, 2016)
DHR1	Sobic.008G080400	β-glucosidase	Cyanogenesis	(Hayes *et al.*, 2015)
DHR2	Sobic.008G079800	β-glucosidase	Cyanogenesis	(Hayes *et al.*, 2015)
BCAS	Sobic.006G016900	β-cyanoalanine synthase	HCN detoxification	(Nielsen *et al.*, 2016)
CAS26	Sobic.003G333700	β-cyanoalanine synthase	HCN detoxification	(Akbudak *et al.*, 2019)
NIT4A	Sobic.004G225200	Nitrilase	HCN detoxification, N recycling*	(Nielsen *et al.*, 2016)
NIT4B1	Sobic.004G225000	Nitrilase	HCN detoxification, N recycling*	(Nielsen *et al.*, 2016)
NIT4B2	Sobic.004G225100	Nitrilase	HCN detoxification, N recycling*	(Nielsen *et al.*, 2016)
GST1	Sobic.001G012500	Glutathione S-transferase	N recycling*	(Hayes *et al.*, 2015; Nielsen *et al.*, 2016)
GST1B	Sobic.001G065800	Glutathione S-transferase	N recycling*	(Nielsen *et al.*, 2016)
GST3	Sobic.003G416300	Glutathione S-transferase	N recycling*	NA
ACC	Sobic.003G197200	ACC oxidase	Ethylene synthesis	(Nielsen *et al.*, 2016)
CM2	Sobic.004G065500	Chorismate mutase	Tyrosine synthesis	NA
CM3	Sobic.003G306100	Chorismate mutase	Tyrosine synthesis	NA
CM7	Sobic.007G141500	Chorismate mutase	Tyrosine synthesis	NA

2.3 Results

2.3.1 Hydrogen cyanide potential in the wild relatives of *Sorghum*

Hydrogen cyanide potential (HCNp), as a proxy for cyanogenic glucoside concentration, was determined for leaf tissue harvested from *S. bicolor* and 18 related *Sorghum* species. Hydrogen cyanide was detected in the leaves of all species at three time points during plant development (**Fig. 2.2**). Foliar HCNp was extremely low in all wild species from the *Chaetosorghum, Heterosorghum, Parasorghum* and *Stiposorghum* (tertiary) subgenera compared with the three *Eusorghum* species at 2, 4 and 6 weeks post-germination ($p < 0.001$). Overall average HCNp across the 16 tertiary species was similar at each harvest point with an average of 0.33 ± 0.04 μg g^{-1} at 2 weeks post-germination, 0.37 ± 0.03 μg g^{-1}at 4 weeks, and 0.33 ± 0.03 μg g^{-1} at 6 weeks (**Fig. 2.2**). This was up to three orders of magnitude lower than HCNp in the three *Eusorghum* species, which together had an overall average of 0.88 ± 0.07 mg g^{-1} (880 ± 70 μg g^{-1}) at 2 weeks, 0.66 ± 0.05 mg g^{-1} (660 ± 50 μg g^{-1}) at 4 weeks, and 0.52 ± 0.05 mg g^{-1} (520 ± 0.50 μg g^{-1}) at 6 weeks (**Fig. 2.2**). HCNp did not vary significantly among the *Eusorghum* species at the different time points, except for at 2 weeks when *S. bicolor* had significantly higher HCNp (1.20 ± 0.11 mg g^{-1}) than both *S. halepense* (0.67 ± 0.08 mg g-1) and *S.* × *almum* (0.79 ± 0.09 mg g^{-1}) ($p < 0.05$). There was a general trend towards a decrease in HCNp over time in each *Eusorghum* species, though the differences were not statistically significant in either *S. halepense* or *S.* × *almum* ($p > 0.05$). While HCNp varied significantly among the 16 tertiary species at the μg scale, the differences were minute in terms of final HCN concentration.

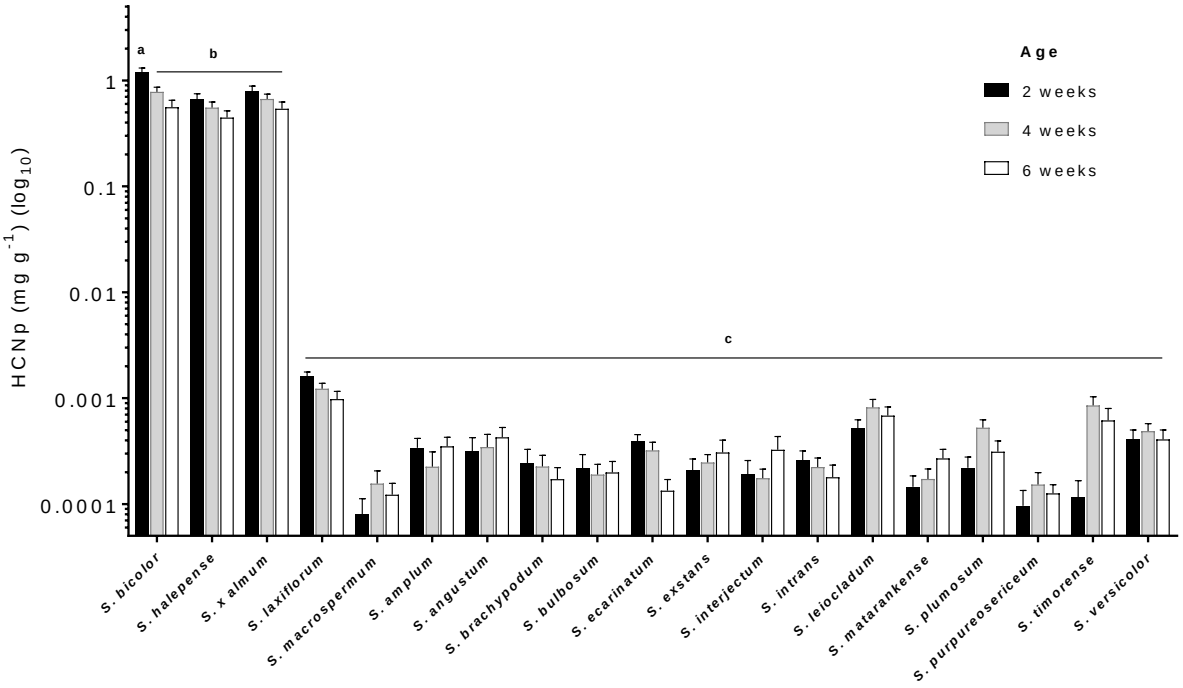


Figure 2.2: Hydrogen cyanide potential (HCNp) in dried, finely ground leaf tissue of 19 *Sorghum* species at three time points during plant development plotted on a logarithmic scale. Data for HCNp at 2 weeks post-germination are represented by black columns, at 4 weeks by grey columns and at 6 weeks by white columns. Graph shows mean ± 1 standard error for three *Eusorghum* species (n = 15), nine *Stiposorghum* (n = 8-10), four *Parasorghum* (n = 8-10) and the individual *Chaetosorghum* (n = 12) and *Heterosorghum* (n = 8) species. Columns with different letters are statistically significant ($p < 0.05$).

2.3.2 Relative cyanogenic glucoside content

LC-MS analysis identified the cyanogenic glucoside as dhurrin, the same cyanogenic glucoside as in *S. bicolor*, in the leaves of all five tested tertiary species (*Chaetosorghum: S. macrospermum; Stiposorghum: S. brachypodum; S. interjectum; Parasorghum: S. purpureosericeum, S. stipoideum*) and two tested *Eusorghum* species, *S. halepense* and *S. propinquum* (**Fig. 2.3**). The two *Eusorghum* species each showed significantly higher relative dhurrin concentrations than any of the five wild species, up to two orders of magnitude in some cases ($p < 0.05$) (**Fig. 2.3A**). *Sorghum propinquum* had the highest relative dhurrin concentration (14893.67 ± 587.48 relative peak area/mg FW) between

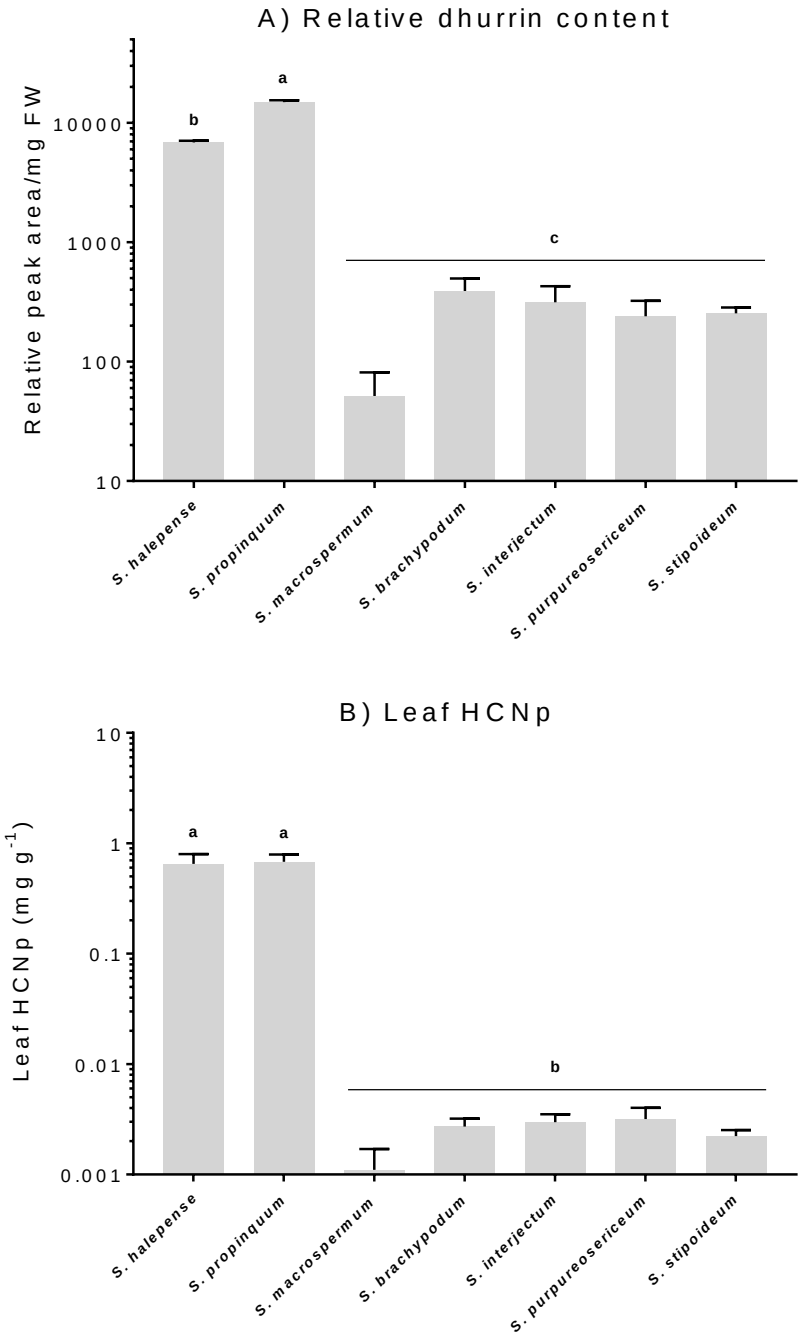


Figure 2.3: Relative dhurrin content and hydrogen cyanide potential (HCNp) in the youngest mature leaf of seven *Sorghum* species at 6 weeks post-germination plotted on a logarithmic scale. A) Relative dhurrin content; B) leaf HCNp. Graphs show mean ±1 SE (n = 3). Columns with different letters are statistically significant ($p < 0.05$).

the two *Eusorghum*, more than twice that of *S. halepense* (6865.49 ± 587.48 relative peak area/mg FW) ($p < 0.05$). Amongst the wild species, *Sorghum brachypodum* had the highest relative dhurrin concentration (390.64 ± 106.18 relative peak area/mg FW) and *S. macrospermum* the lowest (51.53 ± 29.75 relative peak area/mg FW) ($p < 0.05$), although the difference between the two was not statistically significant when all species were considered together ($p > 0.05$) (**Fig. 2.3A**). The pattern seen in the results from the LC-MS was similar to the HCNp detected in leaves from the same individual plants of each species (**Fig. 2.3B**). Both *Eusorghum* species showed significantly higher HCNp than all wild species, although unlike in relative dhurrin content the difference between *S. propinquum* and *S. halepense* was not statistically significant ($p > 0.05$).

2.3.3 Genomic variation of cyanogenesis in *Sorghum*

Focusing initially on a set of selected genes in the cyanogenesis pathway (dhurrin synthesis and catabolism), as well as associated pathways, the trimmed reads were mapped to the genomic sequence of these selected 20 *S. bicolor* genes. The alignment of the reads identified numerous single nucleotide variants (SNVs) in all genes in the six species (**Table 2.3**). In *CYP79A1*, the gene identified as the rate-limiting step of dhurrin biosynthesis, *S. macrospermum* (*Chaetosorghum*) had the fewest SNVs (146) and *S. leiocladum* (*Heterosorghum*) the most (406). This is an interesting result, as these tertiary species are the most closely related and one of the more distantly related, respectively, to *S. bicolor* and the *Eusorghum* according to the current phylogeny (Dillon *et al.*, 2007a). In *UGT85B1,* another essential gene in the formation of dhurrin, *S. brachypodum* had the fewest SNVs (99) and *S. matarankense* the most (177). The genes more directly involved in cyanogenic metabolism, including dhurrin biosynthesis (*CYP79A1, CYP71E1, UGT8B1, POR*) and cyanogenesis (*DHRs, HNL*), generally varied most across all six species. Genes encoding enzymes that function in more fundamental metabolic processes, such as ethylene synthesis (*ACC*) and tyrosine biosynthesis (*CMs*), were generally more highly conserved across all species. Interestingly, the glutathione S-transferase (*GST*) family genes thought to be involved in the endogenous turnover of cyanogenic glucosides (Bjarnholt *et al.*, 2018) (see **Chapter 1, Fig. 1.2B**) were also relatively conserved compared to the biosynthetic and bioactivation genes. The nitrilase 4 class (*NIT4*) genes, likely involved in endogenous turnover and the general HCN

Table 2.3: Number of SNVs called for examined genes in the six wild *Sorghum* species – *S. laxiflorum* (lax), *S. macrospermum* (mac), *S. brachypodum* (bra), *S. leiocladum* (lei), *S. matarankense* (mat) and *S. purpureosericeum* (pur) – when mapped to the genomic *S. bicolor* sequence of the selected genes.

	***Sorghum* species**					
Gene	**lax**	**mac**	**bra**	**lei**	**mat**	**pur**
CYP79A1	285	146	258	406	338	235
CYP71E1	130	306	270	232	232	157
UGT85B1	157	104	99	155	177	154
POR	295	331	322	297	233	234
NIT4A	167	237	248	198	181	149
NIT4B1	321	403	493	187	344	356
NIT4B2	186	330	314	224	174	191
GST1	116	54	150	42	106	109
GST1B	134	138	162	132	138	116
GST3	41	81	69	61	52	58
BCAS	148	98	247	119	123	132
CAS26	125	250	205	156	139	147
MATE	222	188	165	188	177	155
HNL	342	251	528	437	297	386
DHR1	183	332	429	86	100	110
DHR2	216	329	477	130	206	354
ACC	27	63	70	34	33	75
CM2	51	57	49	75	53	55
CM3	131	67	61	131	135	143
CM7	109	265	231	119	119	136

detoxification pathway (see **Chapter 1, Fig. 1.2C**), varied substantially in all species relative to *S. bicolor*.

2.3.4 Variation within key biosynthesis gene, *CYP79A1*

The dhurrin biosynthetic genes were investigated further by examining predicted effects of all SNVs on the protein sequence. A total of 1668 SNVs were detected across all six species in the rate-limiting biosynthetic gene *CYP79A1*. Of these SNVs, 1022 (61%) were predicted to result in a synonymous variant in the amino acid sequence (low impact on protein). Thirty-four percent of SNVs (567) were predicted to have a moderate impact on the protein as a result of missense mutation (**Fig. 2.4**), although the majority of amino acid changes occurred within the hydrophobic class and are likely to have a lower impact on protein function. Overall, 45% of SNVs resulted in positive amino acid changes, that is, changes occurring within the same class. Six variants affecting the CYP79A1 protein sequence were conserved in all six species (**Fig. 2.5**).

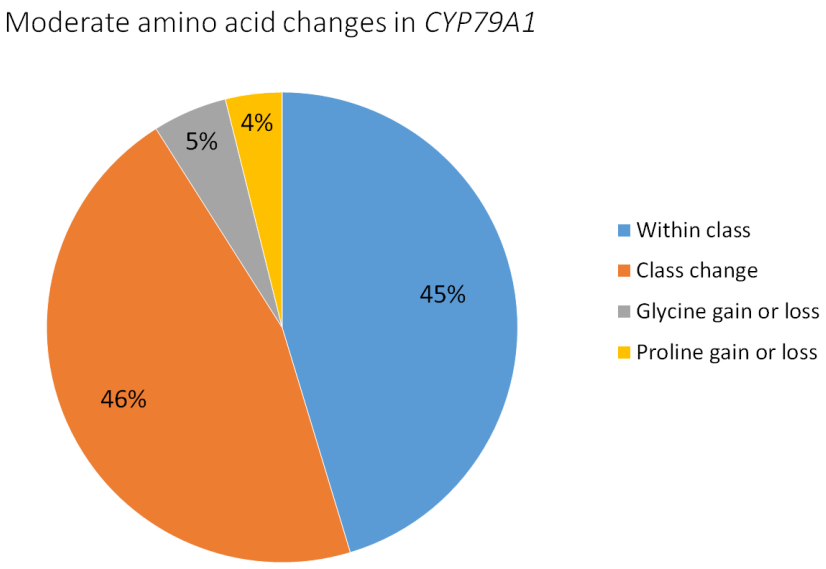
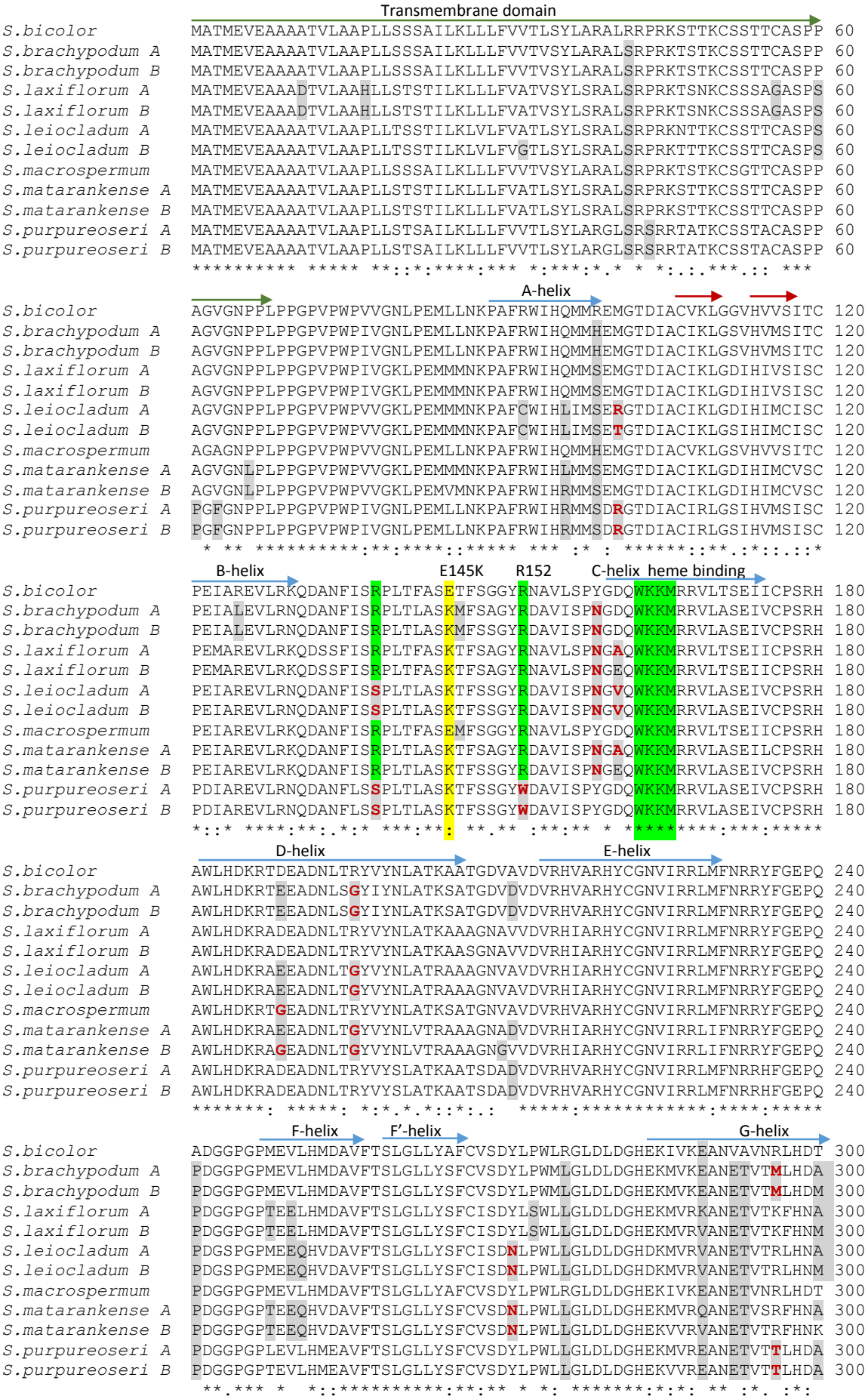


Figure 2.4: Proportions of amino acid property changes predicted to have a moderate impact on CYP79A1 protein function across the six wild *Sorghum* species. Forty-five percent of SNVs are predicted to result in positive amino acid changes, 46% in a class change between hydrophobic, polar or charged amino acids, and 9% in a gain/loss of glycine or proline.

Homology models of CYP79A1 have been made using the solved crystal structures of relevant P450s as a template (Jensen *et al.*, 2011; Vazquez-Albacete *et al.*, 2017). CYP79A1 contains 12 conserved major α-helices and 6 β-strands forming two highly conserved β-sheets. The correct folding of the CYP79A1 protein is important for functionality, ensuring heme-binding and correct docking of the substrate, tyrosine. There are several conserved amino acids that play key roles in these processes. To evaluate the potential effect of the identified SNVs on the function of CYP79A1 in each of the wild species, the SNVs that resulted in amino acid changes were incorporated into protein sequence and aligned with CYP79A1 from *S. bicolor* (**Fig. 2.5**). Of the six variants affecting the CYP79A1 protein sequence that were conserved in all wild species, A293T, V5041 and S555A are considered positive residue changes with less impact on the protein, whilst R42S, A241P and V292E could potentially have an effect, but analysis using the SNAP2 software within the PredictProtein program (https://predict-protein.org/) suggested a minimal impact of these changes. This may be due to the locations within the protein, for example in the N-terminal transmembrane domain anchoring CYP79A1 to the endoplasmic reticulum. However, analysis does indicate that some of the amino acid changes in particular species could have an impact on CYP79A1 function (**Fig. 2.5**). Modelling and mutation studies have identified key amino acids and specific motifs required for CYP79A1 activity, and the majority of these are conserved across the six wild species. However, two arginine residues are not conserved, R152W identified in *S. purpureosericeum* and R411S/C in *S. laxiflorum*, *S. leiocladum* and *S. matarankense* (**Fig. 2.5**). The R152 residue is involved in binding to the tyrosine substrate (Jensen *et al.*, 2011; Vazquez-Albacete *et al.*, 2017) whilst R411 is part of the E-R-R triad formed by the correct folding of the protein and therefore the formation of a salt bridge with the E408 (glutamic acid) and R411 of the XEXXR and R458 in the PERF motif (a P450 signature sequence). This interaction is considered to lock the heme pocket of the active site and to stabilise the overall protein structure (Hasemann *et al.*, 1995). Disruption to this tertiary folding of the protein may reduce the synthesis of dhurrin.

The amino acid change E145K is present in all wild species except *S. macrospermum* (**Fig. 2.5**). This change has previously been identified in *S. bicolor* mutants with elevated


Transmembrane domain
A-helix
B-helix
E145K
R152
C-helix_heme binding
D-helix
E-helix
F-helix
F'-helix
G-helix

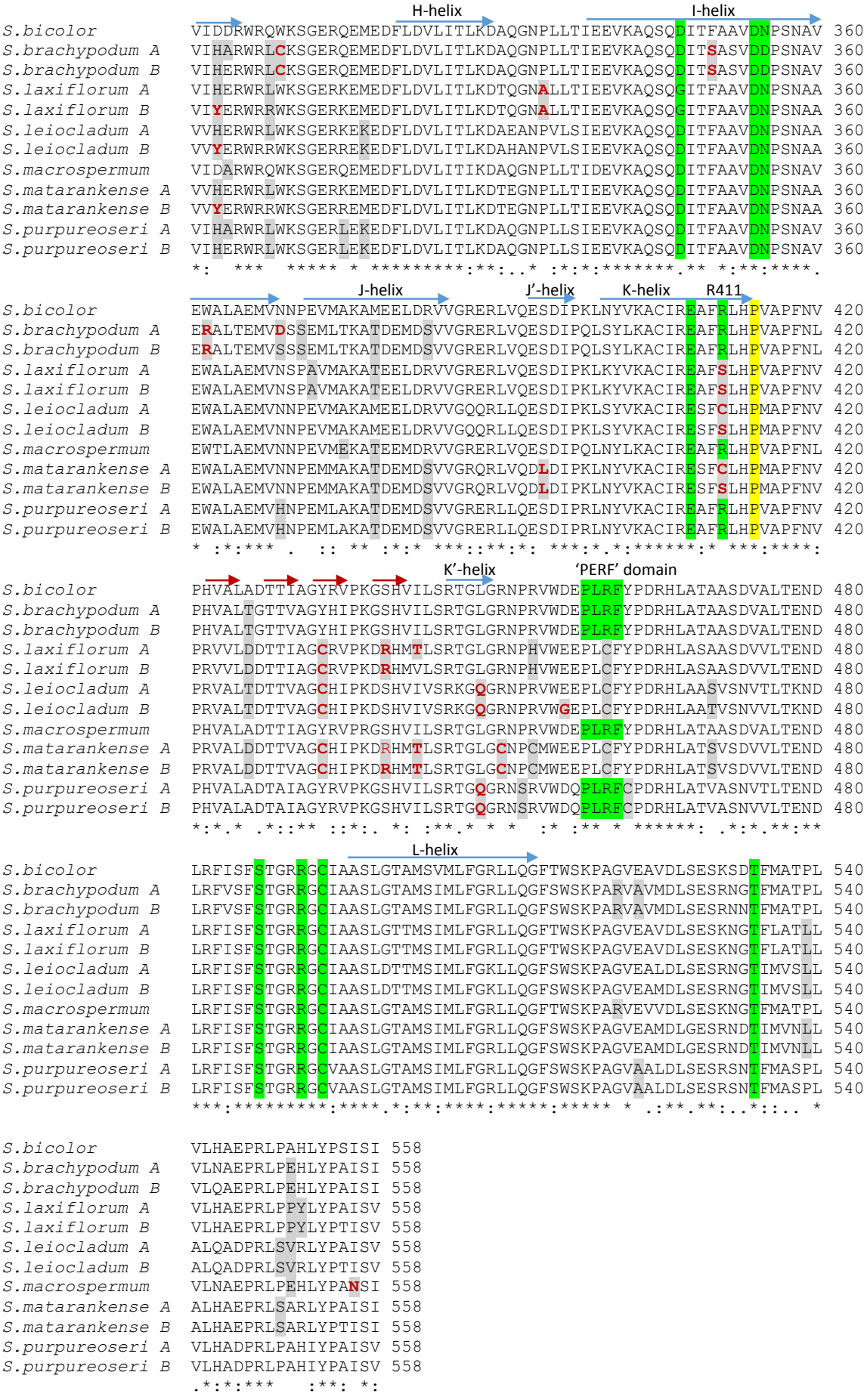

H-helix
I-helix
S.bicolor VIDDRWRQWKSGERQEMEDFLDVLITLKDAQGNPLLTIEEVKAQSQDITFAAVDNPSNAV 360
S.brachypodum A VIHARWRLCKSGERQEMEDFLDVLITLKDAQGNPLLTIEEVKAQSQDITSASVDDPSNAV 360
S.brachypodum B VIHERWRLCKSGERQEMEDFLDVLITLKDAQGNPLLTIEEVKAQSQDITSASVDDPSNAV 360
S.laxiflorum A VIHERWRLWKSGERKEMEDFLDVLITLKDTQGNALLTIEEVKAQSQGITFAAVDNPSNAA 360
S.laxiflorum B VIYERWRRWKSGERKEMEDFLDVLITLKDTQGNALLTIEEVKAQSQGITFAAVDNPSNAA 360
S.leiocladum A VVHERWRLWKSGERKEKEDFLDVLITLKDAEANPVLSIEEVKAQSQDITFAAVDNPSNAA 360
S.leiocladum B VVYERWRRWKSGERREKEDFLDVLITLKDAHANPVLSIEEVKAQSQDITFAAVDNPSNAA 360
S.macrospermum VIDARWRQWKSGERQEMEDFLDVLITIKDAQGNPLLTIDEVKAQSQDITFAAVDNPSNAV 360
S.matarankense A VVHERWRLWKSGERKEMEDFLDVLITLKDTEGNPLLTIEEVKAQSQDITFAAVDNPSNAA 360
S.matarankense B VVYERWRRWKSGERREMEDFLDVLITLKDTEGNPLLTIEEVKAQSQDITFAAVDNPSNAA 360
S.purpureoseri A VIHARWRLWKSGERLEKEDFLDVLITLKDAQGNPLLSIEEVKAQSQDITFAAVDNPSNAV 360
S.purpureoseri B VIHERWRLWKSGERLEKEDFLDVLITLKDAQGNPLLSIEEVKAQSQDITFAAVDNPSNAV 360
J-helix
J'-helix
K-helix
R411
S.bicolor EWALAEMVNNPEVMAKAMEELDRVVGRERLVQESDIPKLNYVKACIREAFRLHPVAPFNV 420
S.brachypodum A ERALTEMVDSSEMLTKATDEMDSVVGRERLVQESDIPQLSYLKACIREAFRLHPVAPFNL 420
S.brachypodum B ERALTEMVSSSEMLTKATDEMDSVVGRERLVQESDIPQLSYLKACIREAFRLHPVAPFNL 420
S.laxiflorum A EWALAEMVNSPAVMAKATEELDRVVGRERLVQESDIPKLKYVKACIREAFSLHPVAPFNV 420
S.laxiflorum B EWALAEMVNSPAVMAKATEELDRVVGRERLVQESDIPKLKYVKACIREAFSLHPVAPFNV 420
S.leiocladum A EWALAEMVNNPEVMAKAMEELDRVVGQQRLLQESDIPKLSYVKACIRESFCLHPMAPFNV 420
S.leiocladum B EWALAEMVNNPEVMAKAMEELDRVVGQQRLLQESDIPKLSYVKACIRESFSLHPMAPFNV 420
S.macrospermum EWTLAEMVNNPEVMEKATEEMDRVVGRERLVQESDIPQLNYLKACIREAFRLHPVAPFNL 420
S.matarankense A EWALAEMVNNPEMMAKATDEMDSVVGRQRLVQDLDIPKLNYVKACIRESFCLHPMAPFNV 420
S.matarankense B EWALAEMVNNPEMMAKATDEMDSVVGRQRLVQDLDIPKLNYVKACIRESFSLHPMAPFNV 420
S.purpureoseri A EWALAEMVHNPEMLAKATDEMDSVVGRERLLQESDIPRLNYVKACIREAFRLHPVAPFNV 420
S.purpureoseri B EWALAEMVHNPEMLAKATDEMDSVVGRERLLQESDIPRLNYVKACIREAFRLHPVAPFNV 420
K'-helix
'PERF' domain
S.bicolor PHVALADTTIAGYRVPKGSHVILSRTGLGRNPRVWDEPLRFYPDRHLATAASDVALTEND 480
S.brachypodum A PHVALTGTTVAGYHIPKGSHVILSRTGLGRNPRVWDEPLRFYPDRHLATAASDVALTEND 480
S.brachypodum B PHVALTGTTVAGYHIPKGSHVILSRTGLGRNPRVWDEPLRFYPDRHLATAASDVALTEND 480
S.laxiflorum A PRVVLDDTTIAGCRVPKDRHMTLSRTGLGRNPHVWEEPLCFYPDRHLASAASDVVLTEND 480
S.laxiflorum B PRVVLDDTTIAGCRVPKDRHMVLSRTGLGRNPHVWEEPLCFYPDRHLASAASDVVLTEND 480
S.leiocladum A PRVALTDTTVAGCHIPKDSHVIVSRKGQGRNPRVWEEPLCFYPDRHLAASVSNVTLTKND 480
S.leiocladum B PRVALTDTTVAGCHIPKDSHVIVSRKGQGRNPRVWGEPLCFYPDRHLAATVSNVVLTKND 480
S.macrospermum PHVALADTTIAGYRVPRGSHVILSRTGLGRNPRVWDEPLRFYPDRHLATAASDVALTEND 480
S.matarankense A PRVALDDTTVAGCHIPKDRHMTLSRTGLGCNPCMWEEPLCFYPDRHLATSVSDVVLTEND 480
S.matarankense B PRVALDDTTVAGCHIPKDRHMTLSRTGLGCNPCMWEEPLCFYPDRHLATSVSDVVLTEND 480
S.purpureoseri A PHVALADTAIAGYRVPKGSHVILSRTGQGRNSRVWDQPLRFCPDRHLATVASNVTLTEND 480
S.purpureoseri B PHVALADTAIAGYRVPKGSHVILSRTGQGRNSRVWDQPLRFCPDRHLATVASNVVLTEND 480
L-helix
S.bicolor LRFISFSTGRRGCIAASLGTAMSVMLFGRLLQGFTWSKPAGVEAVDLSESKSDTFMATPL 540
S.brachypodum A LRFVSFSTGRRGCIAASLGTAMSIMLFGRLLQGFSWSKPARVAVMDLSESRNGTFMATPL 540
S.brachypodum B LRFVSFSTGRRGCIAASLGTAMSIMLFGRLLQGFSWSKPARVAVMDLSESRNNTFMATPL 540
S.laxiflorum A LRFISFSTGRRGCIAASLGTTMSIMLFGRLLQGFTWSKPAGVEAVDLSESKNGTFLATLL 540
S.laxiflorum B LRFISFSTGRRGCIAASLGTTMSIMLFGRLLQGFTWSKPAGVEAVDLSESKNGTFLATLL 540
S.leiocladum A LRFISFSTGRRGCIAASLDTTMSIMLFGKLLQGFSWSKPAGVEALDLSESRNGTIMVSLL 540
S.leiocladum B LRFISFSTGRRGCIAASLDTTMSIMLFGKLLQGFSWSKPAGVEAMDLSESRNGTIMVSLL 540
S.macrospermum LRFISFSTGRRGCIAASLGTAMSIMLFGRLLQGFTWSKPARVEVVDLSESKNGTFMATPL 540
S.matarankense A LRFISFSTGRRGCIAASLGTAMSIMLFGRLLQGFSWSKPAGVEAMDLGESRNDTIMVNLL 540
S.matarankense B LRFISFSTGRRGCIAASLGTAMSIMLFGRLLQGFSWSKPAGVEAMDLGESRNDTIMVNLL 540
S.purpureoseri A LRFISFSTGRRGCVAASLGTAMSIMLFGRLLQGFSWSKPAGVAALDLSESRSNTFMASPL 540
S.purpureoseri B LRFISFSTGRRGCVAASLGTAMSIMLFGRLLQGFSWSKPAGVAALDLSESRSNTFMASPL 540
S.bicolor VLHAEPRLPAHLYPSISI 558
S.brachypodum A VLNAEPRLPEHLYPAISI 558
S.brachypodum B VLQAEPRLPEHLYPAISI 558
S.laxiflorum A VLHAEPRLPPYLYPAISV 558
S.laxiflorum B VLHAEPRLPPYLYPTISV 558
S.leiocladum A ALQADPRLSVRLYPAISV 558
S.leiocladum B ALQADPRLSVRLYPTISV 558
S.macrospermum VLNAEPRLPEHLYPANSI 558
S.matarankense A ALHAEPRLSARLYPAISI 558
S.matarankense B ALHAEPRLSARLYPTISI 558
S.purpureoseri A VLHADPRLPAHIYPAISV 558
S.purpureoseri B VLHADPRLPAHIYPAISV 558

Figure 2.5: The CYP79A1 protein sequence of the six tertiary wild *Sorghum* species, incorporating the amino acid changes predicted from the SNVs, aligned to the *S. bicolor* CYP79A1 reference sequence. For all but *S. macrospermum,* at several residues the SNVs identified generated two alternate amino acids, indicated by A and B after the species name. Grey highlights show the changed residues and those in red bold font are predicted to have an impact on protein function (https://predictprotein.org/); green highlights indicate conserved amino acids involved in heme-binding and tyrosine substrate docking; yellow highlights indicate amino acids shown to be important for correct function of CYP79A1 identified through mutation studies. Alignment was generated using ClustalO (https://www.uniprot.org/align). Information on key amino acids is based on previous studies (Hasemann *et al.*, 1995; Jensen *et al.*, 2011; Blomstedt *et al.*, 2012; Knoch *et al.*, 2016; Vazquez-Albacete *et al.*, 2017; Ehlert *et al.*, 2019).

HCNp (Blomstedt *et al.*, 2012), opposed to the low HCNp detected in the leaves of all wild species in the current study (**Fig. 2.2**). A second study specifically mutated this site in the CYP79A1 protein and saw a reduction in enzyme activity (Vazquez-Albacete *et al.*, 2017). This may suggest that the E145K mutation does result in reduced HCNp and the observed increase in the EMS mutant (Blomstedt *et al.*, 2012) was due to additional unidentified mutations. *Sorghum macrospermum* does not contain the E145K change but does have the D189G downstream of the heme-binding site. This has not been identified previously as an important amino acid but it is in a highly conserved region and the resulting change is from the charged aspartic acid to the very simple amino acid glycine. There are additional amino acid changes that are predicted to have an impact on CYP79A1 function and are located close to the heme-binding site or the regions around the conserved XEXXR and PERF motif.

2.4 Discussion

The current study investigated cyanogenesis in the wild relatives of *Sorghum bicolor* for the first time. The major cyanogenic components, including individual cyanogenic glucoside structure and the genomic machinery driving dhurrin production and bioactivation, were found to be broadly similar in all tested species across the *Sorghum* genus. However, phenotypic expression of hydrogen cyanide potential (HCNp) was substantially reduced in all species from the four undomesticated subgenera compared to *S. bicolor* and the domesticated *Eusorghum*. While a lower cyanogenic capacity in the tertiary *Sorghum* species was not unexpected, the magnitude of the difference between cultivated and wild *Sorghum* was greater than anticipated.

2.4.3 Phenotypic variation of cyanogenesis in *Sorghum*

In the current study, *S. bicolor* and other *Eusorghum* species (*S.* × *almum, S. halepense* and *S. propinquum*) showed a similarly high HCNp and dhurrin content. The production of specialised metabolites such as cyanogenic glucosides has long been thought to come at a metabolic cost to plants, tying up resources that could otherwise be utilised in growth and development (McKey, 1974; Herms and Mattson, 1992). Under the relatively controlled environmental conditions of cultivated systems, these costs may be partially offset by a more stable uptake of essential resources, such as water and soil nutrients. However, production costs are likely to be more keenly felt in highly variable natural environments. This pattern may be reflected in the relatively low dhurrin content and negligible HCNp detected in the undomesticated species of the *Chaetosorghum, Heterosorghum, Parasorghum* and *Stiposorghum* subgenera. A large proportion of species in these groups are endemic to northern Australia, with the main centre of diversity extending from the northerly monsoonal tropics to the arid and semi-arid regions of Central Australia (**Fig. 2.1**). Soils in these regions are typically characterised by low concentrations of available nitrogen (Dillon *et al.*, 2007b), the fundamental building block of cyanogenic glucosides. This could suggest that the endemic wild *Sorghum* species with their low HCNp prioritise allocation of available nitrogen to general metabolic processes in growth and development rather than the production of dhurrin.

Leaf tissue of wild species expressed cyanide potential at a scale lower than 1 μg g^{-1}, translating to dhurrin concentrations of less than 1 ppm. For feeding herbivores, plant tissue containing dhurrin at this scale would be virtually indistinguishable from tissue with no capacity to release HCN (below the concentration threshold considered functionally acyanogenic) (Gleadow *et al.*, 2003). Interestingly, this might suggest that the undomesticated *Sorghum* species are not utilising cyanogenic glucosides as a form of chemical defence, particularly as leaves are considered the plant organ most vulnerable to predation. Production of dhurrin in *S. bicolor* is developmentally regulated, with concentrations often found to be highest in young, developing tissues such as newly formed leaves (Busk and Møller, 2002; O'Donnell *et al.*, 2013; Miller *et al.*, 2014; Gleadow *et al.*, 2016b; Blomstedt *et al.*, 2018). In terms of cyanogenesis this pattern is consistent with optimal resource allocation theories, in that leaves are generally the most heavily defended organ as they house the photosynthetic apparatus (Bloom *et al.*, 1985; Wiedemuth *et al.*, 2005). With the potentially high costs of dhurrin synthesis in nitrogen-poor environments, the undomesticated *Sorghum* species may instead place greater emphasis on other defensive mechanisms to deter herbivores. For example, the production of less costly carbon-based physical structures such as trichomes (Levin, 1973; Johnson, 1975; Tian *et al.*, 2012) could be preferable under such conditions. It is currently unclear which specific herbivores feed on the endemic Australian species, although marsupials have been seen feeding on some species in the field (pers. comm. Dr Sally Norton). Recent evidence indicates that cyanogenic glucosides possess additional physiological functions beyond defence, in particular acting as storage compounds for reduced nitrogen that can be recovered for use in general plant metabolism upon demand (Pičmanová *et al.*, 2015; Nielsen *et al.*, 2016; Zidenga *et al.*, 2017; Bjarnholt *et al.*, 2018; Blomstedt *et al.*, 2018). In the wild *Sorghum* species, dhurrin may be turned over immediately after production to release free nitrogen (which would also point to a reduced defensive role). Future studies could further explore this by measuring the content and composition of any known derivative products of dhurrin turnover and HCN detoxification.

An extremely low concentration of dhurrin and resulting potential for HCN release was consistently observed across all undomesticated *Sorghum* species examined in the current study. In previous studies, high quantitative and qualitative intraspecific

variation of cyanogenesis has been recorded within species of other plant genera (Gleadow and Møller, 2014). Some species of *Eucalyptus* show high quantitative variation for cyanogenic traits both within and between different populations (Gleadow and Woodrow, 2000a; Goodger *et al.*, 2002; Gleadow *et al.*, 2003; Gleadow *et al.*, 2008). In several species of *Lotus,* the potential for HCN release has been observed to vary both quantitatively between individual plants, and qualitatively at the population scale (Armstrong *et al.*, 1913; Jones, 1977; Blaise *et al.*, 1991; Aikman *et al.*, 1996). Within natural populations of the domesticated white clover (*Trifolium repens*), individual plants can be either cyanogenic or completely acyanogenic, representing true polymorphism of the trait (Armstrong *et al.*, 1913; Blaim and Nowacki, 1979; Hughes, 1991; Kakes and Hakvoort, 1994; Olsen *et al.*, 2007). In cassava (*Manihot esculenta*), another major cyanogenic crop, genetically isolated wild relatives (equivalent to the tertiary *Sorghum* species) showed quantitative variation in their potential for HCN release in tubers under stable environmental conditions (Nassar and Fichtner, 1978; Nassar, 2000). Such levels of intraspecific variation were not apparent in the undomesticated *Sorghum* species, although limitations of the current study need to be taken into account. Different populations may vary substantially in the capacity to produce dhurrin, but only a single accession of each species was examined here. This is due primarily to the difficulty in obtaining plant material, as the endemic Australian species are currently not well-represented in genebanks and herbaria (Castañeda-Álvarez *et al.*, 2016; Norton *et al.*, 2017). Many species are also naturally distributed in remote regions, making collection of seeds challenging.

2.4.2 Genomic variation of cyanogenesis in *Sorghum*

The preliminary whole genome sequencing analysis reported here specifically focused on the genetic variation in cyanogenic metabolism within six undomesticated *Sorghum* species. The results suggest that the majority of single nucleotide variants (SNVs) do not have a major effect on the predicted protein function of CYP79A1, the important initial step of dhurrin biosynthesis. However, there are several nonsynonymous changes that are predicted to affect enzyme activity. Of particular note is the E145K change which occurs within the previously defined substrate recognition site 1 (SRS-1)(Vazquez-Albacete *et al.*, 2017). Initially, studies using an EMS (ethyl methanesulfonate)-generated *S. bicolor* mutant suggested that this change may result in increased substrate

binding, as HCNp in these mutants was higher than in the parental line (Blomstedt *et al.*, 2012). In contrast, a subsequent study by Vazquez-Albacete *et al.* (2017) generated recombinant CYP79A1 proteins with substitutions of previously identified key amino acids, including E145K, which showed that CYP79A1 enzyme activity decreased as a result. This agrees with the results reported here, where the wild *Sorghum* species had significantly reduced HCNp and dhurrin concentration in comparison to the domesticated *S. bicolor*. The high HCNp seen in the EMS-generated *S. bicolor* mutant may be due to other unidentified mutations. The E145K change is positioned close to the highly conserved heme-binding motif (WKKM) within the SRS-1 region and may alter the shape and charge distribution within the substrate binding pocket. *Sorghum macrospermum* was the only species that did not have the E145K mutation, with a CYP79A1 protein sequence very similar to *S. bicolor,* but it did have an amino acid change (D189G) which is predicted to affect protein function. The D189G change is located in the highly conserved region downstream of the heme-binding motif and may also impact on the formation of the substrate binding pocket. Blomstedt *et al.* (2012) also identified a P414L change in another *S. bicolor* mutant that caused misfolding of the protein and abolished CYP79A1 activity entirely. In the current study, P414 was found to be conserved in all sequenced wild species.

In this preliminary analysis of the selected cyanogenic genes in wild *Sorghum*, copy number variation and ploidy level must be taken into consideration. For example, there is a *CYP79A1*-like gene on chromosome 10 that shows 75% identity to *CYP79A1* in *S. bicolor,* and more detailed analysis of the whole genome sequence will confirm that the sequence reads have been assigned to the correct gene. The ploidy level of the different *Sorghum* species also varies (2n = 20 in *S. bicolor* and *S. leiocladum*, 2n = 10 in *S. brachypodum*, *S. matarankense* and *S. purpureosericeum*, and 2n = 40 in *S. macrospermum* and *S. laxiflorum*), potentially affecting the synthesis and/or turnover of dhurrin.

The genomic sequencing results analysed to date show that the dhurrin biosynthetic genes are present and largely intact in the geographically and genetically isolated tertiary *Sorghum* species. However, there were substantial differences in HCNp and concentration of dhurrin between undomesticated and undomesticated *Sorghum*. Therefore, expression of the key dhurrin biosynthesis gene *CYP79A1* is likely to be

controlled by regulatory mechanisms. It has previously been suggested that the biosynthesis of dhurrin in *S. bicolor* is regulated at the transcriptional level (Busk and Møller, 2002). Ehlert *et al.* (2019) also found evidence for transcriptional regulation of the cyanogenic glucoside epiheterodendrin in barley. Future studies should include transcriptomic analysis of the tertiary *Sorghum* species at different stages of plant development, in different tissue types, and in plants grown under different environmental conditions to further understand the regulation of dhurrin. The genomic and phenotypic variation apparent in this one functional trait, cyanogenesis, suggests a high degree of genetic diversity in wild *Sorghum* germplasm. These species therefore shape as a valuable genetic resource for the breeding of more climate-resilient *Sorghum* crops in the future (Dillon *et al.*, 2007b; Brozynska *et al.*, 2016).

2.4.3 Conclusion

Cyanogenesis is a highly variable functional trait**,** its expression controlled by a complex interaction of internal and external factors (Gleadow and Møller, 2014). The current study took the fundamental initial step of characterising cyanogenesis in the undomesticated species of the genus *Sorghum*. While impacts of structural genetic variation of the cyanogenic machinery were limited, all tested species of *Chaetosorghum, Heterosorghum, Parasorghum* and *Stiposorghum* showed negligible potential for HCN release in leaf tissue. In simple terms, this low phenotypic expression of cyanogenesis might reflect the conditions in their natural environments, such as a limited access to nutrient resources and/or differences in herbivore pressures (Gleadow and Woodrow, 2000a). The reality is likely to be much more complex. Regulation of dhurrin and HCNp is also dependent on plant ontogeny and specific tissue type in *S. bicolor* (Halkier and Møller, 1989; O'Donnell *et al.*, 2013; Blomstedt *et al.*, 2018). In order to better understand the potential utilisation and growth-defence trade-offs of cyanogenic glucosides in general plant metabolism, detailed tissue- and age-dependent variation of HCNp in wild species in comparison to *S. bicolor* is required (see **Chapter 3**).

Chapter 3 – Variation in cyanide production through early plant development: a comparison of wild and domesticated *Sorghum*

3.1 Introduction

Plant domestication theories hypothesise that crop plants have decreased herbivore resistance compared to their wild relatives due to human-induced (artificial) selection for desirable agronomic traits (Rosenthal and Dirzo, 1997; Gepts, 2004; McCall and Fordyce, 2010; Meyer *et al.*, 2012). One of the proposed explanations for this is that plant breeding may have selected for a reduction in defensive specialised metabolites to improve palatability and reduce toxicity (Mithen *et al.*, 1987; Maag *et al.*, 2015). Interestingly, the frequency and accumulation of cyanogenic glucosides, one such group of specialised metabolites, appears to be disproportionately high in crop plants relative to wild species (Jones, 1998; Gleadow and Møller, 2014). Jones (1998) suggested that a potential factor behind this discrepancy could be the inadvertent selection of cyanogenic plants through domestication, likely due to their increased resistance to pests compared to acyanogenic varieties. Recent evidence points to another possible contributing factor: cyanogenic glucosides are likely not restricted to defensive roles, but rather have additional beneficial functions for plant metabolism (Møller, 2010b; Neilson *et al.*, 2013; Gleadow and Møller, 2014; Schmidt *et al.*, 2018). For example, cyanogenic glucosides may play a role in the storage, transport and endogenous turnover of nitrogen in general metabolism, allowing plants to manage their nitrogen under variable environmental conditions (Selmar *et al.*, 1988; Burke *et al.*, 2013; Neilson *et al.*, 2013; Gleadow and Møller, 2014; Miller *et al.*, 2014; Pičmanová *et al.*, 2015; Bjarnholt *et al.*, 2018; Blomstedt *et al.*, 2018); they might also function to mitigate oxidative stress caused by excess production of reactive oxygen species (ROS) (Møller, 2010b; Selmar and Kleinwächter, 2013; Burke *et al.*, 2015; Schmidt *et al.*, 2018). These proposed functions may partially offset production costs of the nitrogenous cyanogenic glucosides under the relatively stable, controlled conditions of cultivated environments (Neilson *et al.*, 2013).

Several studies have examined variation in cyanogenic traits between cultivated and naturalised populations of domesticated species [e.g. cassava (*Manihot esculenta*): Wang *et al.* (2014), Bradbury *et al.* (2013); lima bean (*Phaseolus lunatus*): Ballhorn *et al.* (2009), Shlichta *et al.* (2018)], but rarely has cyanogenesis been directly compared between cultivated and undomesticated species within the same genus [cassava: Nassar and Fichtner (1978), Nassar (2000)]. In this chapter, I compared growth, hydrogen cyanide potential (HCNp), concentration of nitrate and *in planta* allocation of nitrogen between three related species in the *Sorghum* genus – *Sorghum bicolor* (L.) Moench (subgenus *Eusorghum*), an important crop plant native to Africa, and two undomesticated species endemic to northern Australia, *S. brachypodum* Lazarides (subgenus *Stiposorghum*) and *S. macrospermum* E.D. Garber (subgenus *Chaetosorghum*). *Sorghum bicolor* is a multi-purpose cereal crop grown for the grain, as forage, and more recently as a source for biofuel production. As a C_4 grass, sorghum is predominantly cultivated in the arid- and semi-arid tropics due to its tolerance to drought and high temperatures. *Sorghum bicolor* contains the cyanogenic glucoside dhurrin and can be highly cyanogenic in all vegetative tissues (Loyd and Gray, 1970; Miller *et al.*, 2014). *Sorghum brachypodum* is an annual grass that occurs across the Darwin and Arnhem Land regions of the Northern Territory (Lazarides *et al.*, 1991). Due to pollen-pistil incompatibilities *S. brachypodum* does not successfully hybridise with *S. bicolor* (Hodnett *et al.*, 2005). *Sorghum macrospermum* is an annual species native to the Katherine region of the Northern Territory, with a highly restricted distribution (Lazarides *et al.*, 1991). It is more closely related to *S. bicolor* than is *S. brachypodum* and has been studied in comparatively greater detail (Price *et al.*, 2005; Price *et al.*, 2006; Dillon *et al.*, 2007b; Kuhlman *et al.*, 2008; Kuhlman *et al.*, 2010). *Sorghum macrospermum* also has a relatively high tolerance of several *S. bicolor* pests and diseases, including sorghum downy mildew (*Peronosclerospora sorghi*) and shoot fly (*Atherigona soccata*) (Kamala *et al.*, 2002; Sharma *et al.*, 2005).

Screening of all 14 endemic Australian wild *Sorghum* species, including *S. brachypodum* and *S. macrospermum*, detected HCN at very low concentrations in leaf tissue (see **Chapter 2.3.1**). LC-MS analysis confirmed the presence of dhurrin (see **Chapter 2.3.2**). Due to their isolation from domesticated *Sorghum*, differences in age- and tissue-specific composition of dhurrin may have arisen in the wild species as a result of

distinct selective pressures. In this study, I examined hydrogen cyanide potential (HCNp) in all vegetative tissues (leaves, sheaths and roots) at multiple stages during seedling development. This growth period was selected for two main reasons – firstly, dhurrin concentration peaks in young *S. bicolor* seedlings (constituting more than 20% of dry mass in some cases) before decreasing over the first few weeks of growth (Halkier and Møller, 1989; Busk and Møller, 2002; Emendack *et al.*, 2017); secondly, germination and early development are highly vulnerable stages for plants in terms of herbivore attack (McKey, 1974; McCall and Fordyce, 2010). Newly formed tissues are also generally more cyanogenic than older tissues in *S. bicolor* plants, particularly in the leaves where concentration decreases with leaf maturity (Gleadow and Møller, 2014; Miller *et al.*, 2014). The presence and concentration of dhurrin during this period is unknown in *S. brachypodum* and *S. macrospermum*.

This chapter aimed to determine whether there are potential links between cyanogenic capacity, nitrogen allocation and growth in domesticated and undomesticated *Sorghum* species. Specifically, the following questions were asked. (1) At what concentration, in which tissues and at which growth stages are these species of wild *Sorghum* capable of releasing HCN? (2) Does differential expression of hydrogen cyanide and nitrate support the emerging theory of non-defensive roles for cyanogenic glucosides, and are these compounds potentially utilised differently between wild and cultivated plants?

3.2 Methods

3.2.1 Plant material and growing conditions

Seeds from accessions of two Australian wild *Sorghum* species (*S. brachypodum* Lazarides and *S. macrospermum* E.D.Garber) were obtained from the Australian Grains Genebank (AGG), Horsham, Victoria (**Fig. 3.1**). Seed of *S. bicolor* (BTx623) was sourced from the Queensland Alliance for Agriculture and Food Innovation (QAAFI), University of Queensland (UQ). The wild species were selected based on phylogeny, distribution and cyanogenic potential. The method used to ensure optimal germination in the wild species is outlined in **Chapter 2.2.1**. Once the caryopses had germinated and formed a small root and shoot (approximately 1 cm), they were transplanted into 140 mm plastic pots containing three parts seed raising soil mix to one part perlite.

A) Species	Accession number	Accession name	Provenance	Latitude	Longitude
S. brachypodum	302480	JC 2125	Arnhem Land, NT	-12.7145	132.4167
S. macrospermum	302367	JC 2253	Katherine, NT	-14.4097	132.1977

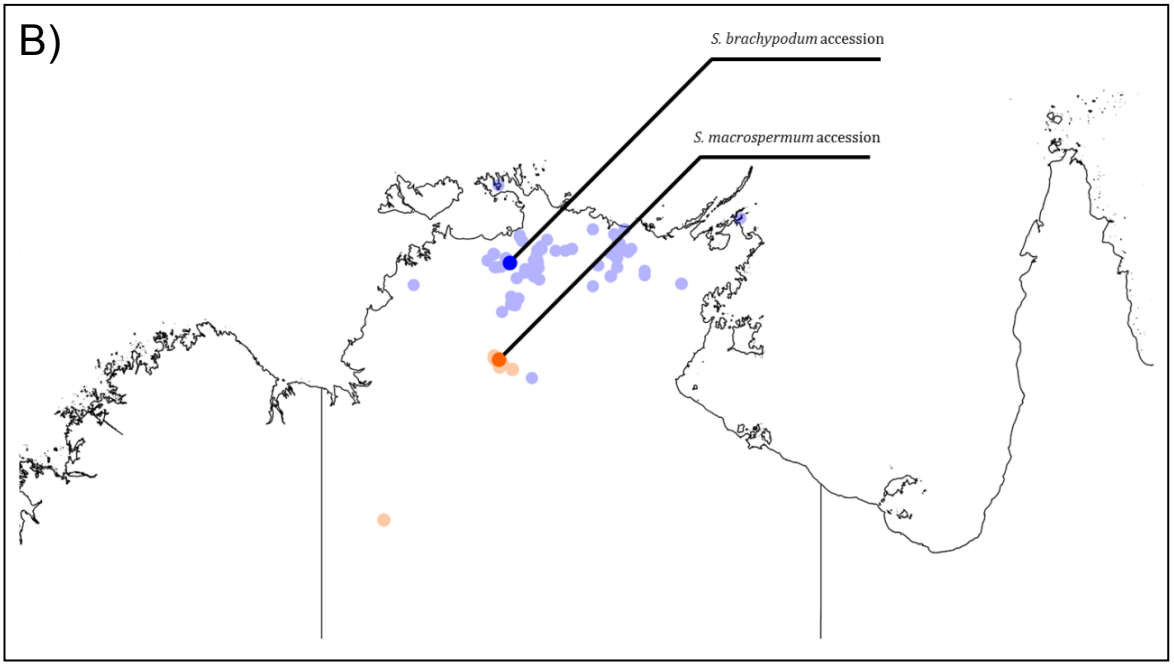


Figure 3.1: A) Details of individual accessions of wild *Sorghum* species *S. brachypodum* and *S. macrospermum* examined in the current study. Seeds were obtained from the Australian Grains Genebank (AGG), Horsham, Victoria. B) Occurrence records of *S. brachypodum* and *S. macrospermum* obtained from the Atlas of Living Australia (ALA), http://www.ala.org.au. Each blue circle represents an occurrence record of *S. brachypodum* and each orange circle represents *S. macrospermum*. Collection localities of individual accessions examined here are marked by darker coloured circles.

All plants (n = 90, 30 for each species) were grown under controlled greenhouse conditions at Monash University (coordinates: 37°54'36"S 145°08'02"E) from July-September 2018 with a mean temperature of 28.4°C ± 2.8°C and 18.3°C ± 2.1°C day/night, and natural light (average photosynthetic photon flux density: 412 ± 76 µmol quanta m^{-2} s^{-1}). Supplementary light from sodium lamps (MK-1 Just-a-shade, Ablite

Australia) was used to extend the photoperiod to 14 hours. All plants were watered once daily.

3.2.2 Harvesting and growth analysis

Plants of each species were destructively harvested at six time points (n = 5) over the first five weeks of development: 3 days, 7 days, 14 days, 21 days, 28 days and 35 days post-germination. Height of plants was measured from the base to the ligule of the most recently unfurled leaf, and the number of leaves, tillers and dead leaves counted. Plants were separated into leaf, sheath and root tissue and weighed to obtain the fresh weight (FW). Leaves were removed from the sheath at the ligule and total leaf area (TLA) measured using a LI-3000 portable area meter and LI-3050A belt conveyor (LI-COR Biosciences, Nebraska, USA). Roots were washed out and the length measured from the base of the seed to the tip of the longest root. All material was then immediately snap-frozen in liquid nitrogen and kept at -80°C before being freeze-dried. Dried material was weighed using a digital balance to obtain dry weight (DW). The root:shoot ratio was calculated by dividing total belowground biomass by total aboveground biomass.

Growth parameters – relative growth rate (RGR), leaf area ratio (LAR), specific leaf area (SLA) and specific leaf nitrogen (SLN) – were derived from the harvest data using the following equations, after Neilson *et al.* (2015) and Gleadow and Rowan (1982):

$$\text{RGR day}^{-1} = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$$

$$\text{LAR } (\text{m}^2\text{g}^{-1}) = \frac{A}{W}$$

$$\text{SLA } (\text{m}^2\text{g}^{-1}) = \frac{A}{W_\text{L}}$$

$$\text{SLN g g}^{-1} = (N)\left(\frac{W_\text{L}}{A}\right)$$

Where W is total biomass, W_L is leaf biomass, A is leaf area, t is time and N is leaf nitrogen concentration.

3.2.3 Chemical assays

Freeze-dried leaf, sheath and root tissue was ground to a fine powder using a MM 300 MixerMill (Retsch, Haan, Germany). Cyanogenic glucoside (dhurrin) concentration was determined using the evolved HCN method following Gleadow *et al.* (2012). The hydrogen cyanide potential (HCNp) is the total amount of HCN evolved from hydrolysis of the entire content of endogenous cyanogenic glucosides. It is used as a proxy for any cyanogenic glucoside, such that each mg of HCN is equivalent to 11.5 mg of cyanogenic glucosides in the plant tissue. The amount of cyanide in the tissue arising from ethylene biosynthesis was assumed to be negligible (Peiser *et al.*, 1984; Yip and Yang, 1988). Data are expressed as cyanide potential (mg CN g^{-1} dry weight), that is, the maximum cyanide release per mg dhurrin. Due to limited tissue, total nitrate (NO_3^-) concentration (mg NO_3^- g^{-1} dry weight) was only measured in each tissue of 35 day old plants via a colorimetric assay in 96 well microtiter plates using 15 mg of tissue per sample following O'Donnell *et al.* (2013).

Total elemental nitrogen and carbon concentration of leaf, sheath and root samples was analysed using a 2400 Series II CHNS/O Elemental Analyser in CHN mode (PerkinElmer, Massachusetts, USA) in 35-day old plants (n = 3). In order to assess how nitrogen was partitioned, the proportion of nitrogen found as cyanogenic glucosides (CN^--N/N %) or nitrate (NO_3^--N/N %) was calculated as a proportion of total elemental nitrogen on both tissue and whole plant bases following Gleadow *et al.* (1998) using the following equations:

$$\mathrm{CN^-} - \mathrm{N/N\%} = [(\mathrm{CN^-}\ (\mathrm{mg\ g^{-1}\ dwt}) \times 14/26)/\mathrm{Total\ nitrogen\ (mg\ g^{-1})}] \times 100$$

$$\mathrm{NO_3^-} - \mathrm{N/N\%} = [(\mathrm{NO_3^-}\ (\mathrm{mg\ g^{-1}\ dwt}) \times 14/62)/\mathrm{Total\ nitrogen\ (mg\ g^{-1})}] \times 100$$

3.2.4 Morphology diagrams

The hydrogen cyanide potential measured in a section of sheath, root and individual leaf tissue was overlaid on a traced image of an *S. bicolor*, *S. brachypodum* and *S. macrospermum* at the six harvest points to summarise and compare morphological and biochemical changes over time. A colour scale was used to represent the concentration

of dhurrin in each tissue type. Images were created using Adobe Illustrator® and Adobe Photoshop® software.

3.2.5 Statistical analysis

All data were analysed using GraphPad Prism version 7.02 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). Standard one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests was used to compare traits between and within each species at each harvest point. Data that were not normally distributed were log transformed to satisfy the assumptions of the ANOVA. A 95% confidence level was set for all statistical tests.

3.3 Results

3.3.1 Growth differences in wild and cultivated *Sorghum*

Differences in plant growth between *S. bicolor* and two of its wild relatives, *S. brachypodum* and *S. macrospermum* were assessed at six harvest points over five weeks post-germination through analysis of biomass, growth indices and morphological traits. Overall, *S. bicolor* was taller, and had a higher biomass than *S. brachypodum* and *S. macrospermum* in the first week of growth; by the final harvest *S. bicolor* was low tillering and had fewer, but larger leaves than the two wild species (**Table 3.1; Figs. 3.2-3.3**). There were several differences between species in the various growth parameters at different times. However, there was no significant difference in RGR among the species ($p < 0.05$).

Plant height and root length followed similar patterns across the growth period with *Sorghum bicolor* being significantly taller and with longer roots than both wild species at 3 and 7 days post-germination ($p < 0.05$) (**Table 3.1**). There was no significant difference in height between *S. bicolor* and either of the wild species at 14 days, although *S. brachypodum* was significantly shorter than *S. macrospermum* ($p < 0.05$). From 21 to 35 days, height was not significantly different between any species ($p > 0.05$). *Sorghum macrospermum* had the longest roots of all species at 28 days (312 ± 12.3 mm; $p < 0.05$). At 35 days post-germination both *S. brachypodum* and

Table 3.1: Summary of morphological measurements (plant height, root length and number of tillers) of *S. bicolor* (*bic*), *S. brachypodum* (*bra*) and *S. macrospermum* (*mac*) plants at six harvest points over 35 days post-germination. Significance of one-way ANOVA is presented for all species at each harvest point. *p < 0.05; **p < 0.01; ***p < 0.001; ns = not significant. Within each morphological measurement, values with different letters at each harvest point are significantly different (p < 0.05).

Age (d)	Plant height (mm)				Root length (mm)				Number of tillers			
	bic	***bra***	***mac***	**p**	***bic***	***bra***	***mac***	**p**	***bic***	***bra***	***mac***	**p**
3	38.8±3.5^{a}	16±1.2^{c}	23.8±0.8^{b}	**	64.2±9.7^{a}	28.4±6.5^{b}	25±7.2^{b}	**	0±0^{a}	0 ± 0^{a}	0±0^{a}	ns
7	56.2±1.7^{a}	37.8±2.4^{b}	44.2±5.8^{b}	*	127±10.1^{a}	62.2±6.9^{b}	39±5.1^{b}	***	0±0^{a}	0 ± 0^{a}	0±0^{a}	ns
14	71.6±0.9^{ab}	63.8±4.1^{b}	80.4±2.6^{a}	*	201.2±21.2^{a}	146.4±6.5^{a}	202.4±6.7^{a}	ns	0±0^{a}	0 ± 0^{a}	0±0^{a}	ns
21	76.6±3.8^{a}	84.8±9.5^{a}	85±4^{a}	ns	276.8±6.7^{a}	172.4±37.5^{b}	249±4^{ab}	ns	0±0^{a}	0±0^{a}	0±0^{a}	ns
28	102.6±2.9^{a}	93.8±6.8^{a}	102.6±4.4^{a}	ns	237.2±23.8^{a}	246.6±23.4^{a}	312±12.3^{a}	ns	0±0^{c}	0.6±0.4^{b}	2.4±0.2^{a}	**
35	151.6±7.9^{a}	137.6±9.5^{a}	139.2±11.6^{a}	ns	396.2±15.6^{a}	262±15.8^{b}	353±13.9^{ab}	*	0.6±0.2^{b}	3.8±0.9^{a}	4.4±0.7^{a}	**

S. macrospermum had significantly more tillers, with approximately four tillers per plant compared to less than one tiller per plant for *S. bicolor* (**Table 3.1**). *Sorghum macrospermum* (2.4 ± 0.2) developed tillers earlier and had significantly more tillers at 28 days post-germination than *S. brachypodum* (0.6 ± 0.4) ($p < 0.05$).

Total biomass differed between species in the early stages of the growth period, though overall relative growth rate (RGR) between 3 and 35 days was not significantly different between the three species ($p > 0.05$) (**Fig. 3.2**). *Sorghum bicolor* had significantly higher total biomass and had fewer and larger leaves than *S. brachypodum* and *S. macrospermum* at 3 and 7 days post-germination ($p < 0.05$). *Sorghum brachypodum* had the lowest total biomass of all species at 3 days (0.01 ± 0.001), and *S. macrospermum* had the lowest biomass at 14 days (0.03 ± 0.004) ($p < 0.05$). After 28 days, total biomass was not significantly different between any species ($p > 0.05$).

Sorghum bicolor had significantly fewer leaves than the wild species at 35 days post-germination (6.8 ± 0.2, $p < 0.05$) (**Fig. 3.3A**). However, these leaves were larger, as *S. bicolor* (102.7 ± 7.5 cm^2) and *S. macrospermum* (93.4 ± 5.2 cm^2) maintained a significantly higher total leaf area (TLA) than *S. brachypodum* (66.7 ± 7.1) at this time point ($p < 0.05$) (**Fig. 3.3B**). *Sorghum bicolor* also had the highest specific leaf area (SLA) of all species at 3 days post-germination (705 ± 78 $cm^2\ g^{-1}$, $p < 0.05$), before gradually decreasing over time, and at 35 days post-germination it was not significantly different to *S. macrospermum* (**Fig. 3.3C**). *Sorghum macrospermum* maintained a stable SLA throughout the growing period, while *S. brachypodum* decreased at 14 days post-germination before stabilising significantly lower than both *S. bicolor* or *S. macrospermum*. Leaf area ratio (LAR) fluctuated within each species across the sampling period, with LAR lower in *S. brachypodum* than the other species from 14 days post-germination onward (14 days: 88.1 ± 7.3 $cm^2\ g^{-1}$, $p < 0.05$; 35 days: 127.3 ± 4.2 $cm^2\ g^{-1}$, $p < 0.05$) (**Fig. 3.3D**).

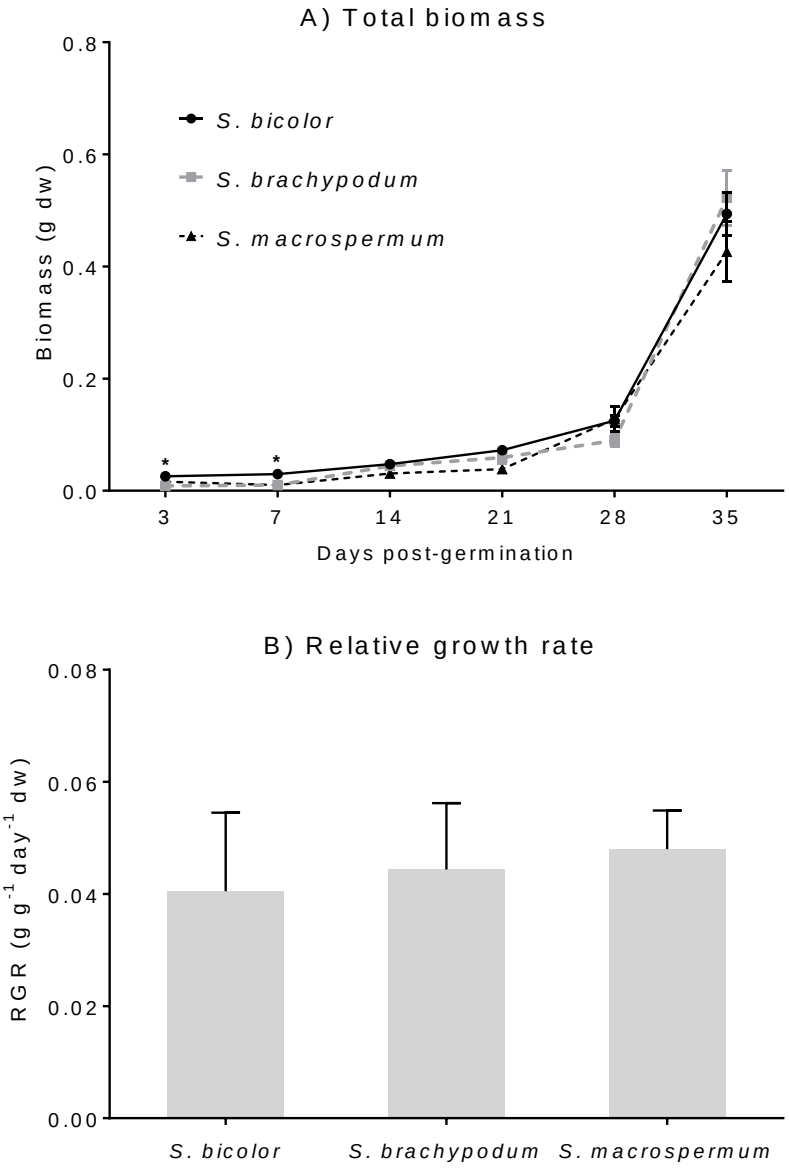


Figure 3.2: Biomass and growth rate of *S. bicolor*, *S. brachypodum* and *S. macrospermum* plants at six harvest points during the first 35 days post-germination. A) Total biomass; B) relative growth rate. Data for *S. bicolor* plants are represented by circles, *S. brachypodum* by squares, and *S. macrospermum* by triangles. Graphs show mean ± 1 standard error (n = 5), with statistically significant differences indicated at each time point: *$p < 0.05$

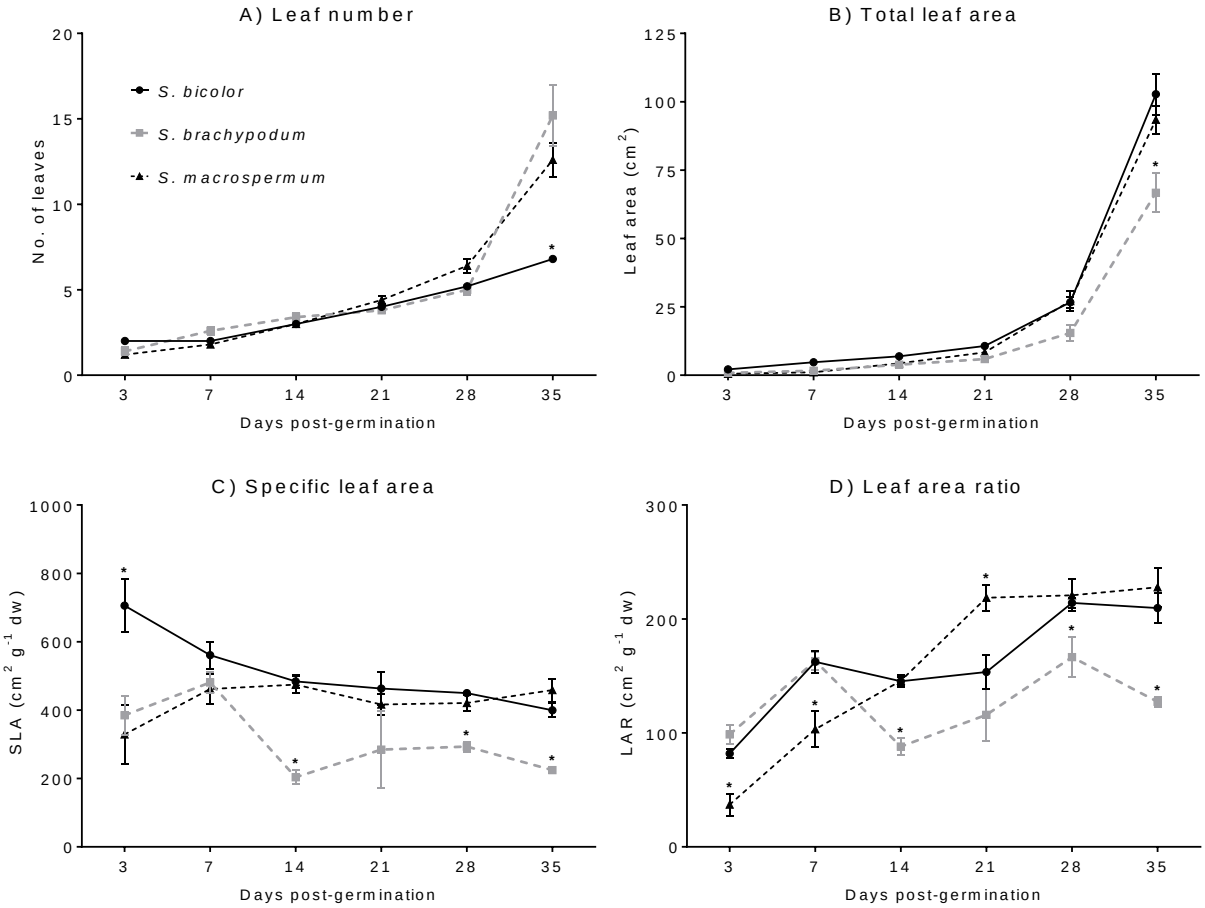


Figure 3.3: Leaf characteristics of *S. bicolor*, *S. brachypodum* and *S. macrospermum* plants at six harvest points during the first 35 days post-germination. A) Total leaf number; B) total leaf area (TLA); C) specific leaf area (SLA); D) leaf area ratio (LAR). Data for *S. bicolor* plants are represented by circles, *S. brachypodum* by squares, and *S. macrospermum* by triangles. Graphs show mean ± 1 standard error (n = 5), with statistically significant differences indicated at each time point: *$p < 0.05$

3.3.2 Cyanide potential and concentration of nitrate in wild and cultivated *Sorghum*

Hydrogen cyanide potential (HCNp), a measure of cyanogenic glucoside concentration, was determined for leaf, sheath and root tissue harvested from *S. bicolor*, *S. brachypodum* and *S. macrospermum* plants at each time point over the 5-week growing period (**Fig. 3.4**). HCNp was detected in the leaves, sheath and roots of all species. There were two major findings regarding variation in HCNp: firstly, it was negligible in the leaves of both wild species at all harvest points, approximately three orders of magnitude lower than in *S. bicolor* (**Fig. 3.4A**); secondly, while the concentration differed at multiple stages, variation in sheath and root HCNp in the wild species broadly followed the same patterns as *S. bicolor*, highest in the first week of growth before gradually decreasing and stabilising after 14 days (**Fig 3.4B,C**). In the wild species, foliar HCNp was extremely low at all harvest points compared with *S. bicolor*, with an overall average of ≤ 0.03 mg g^{-1} across the growing period (**Fig. 3.4A**, $p < 0.05$). *Sorghum bicolor* plants showed the typical age-dependent pattern with the highest HCNp recorded in the cotyledon at 3 days post-germination (2.2 ± 0.3 mg g^{-1}) before decreasing continuously over the remaining growth period to 0.51 ± 0.07 mg g^{-1} in leaf tissue at 35 days. Sheath HCNp followed a similar pattern to the leaves, decreasing over time in all species, before stabilising after 14 days (**Fig. 3.4B**). There were, however, significant differences among species, with significantly higher sheath HCNp in *S. bicolor* than both *S. brachypodum* and *S. macrospermum* at 3, 7, 21, and 28 days post-germination ($p < 0.05$). *Sorghum brachypodum* had lower sheath HCNp than *S. bicolor* at 14 and 35 days post-germination, but the differences were not statistically significant ($p = 0.063$ and $p = 0.059$ respectively). *Sorghum macrospermum* had very low sheath HCNp from 14 days onward (≤ 0.05 mg g^{-1}), between 67% and 98% lower than *S. bicolor* at all harvest points during this period ($p < 0.05$). *Sorghum brachypodum* had higher sheath HCNp than *S. macrospermum* at all harvest points (2× higher at 3 days post-germination, $p < 0.05$), though the differences were not statistically significant after 7 days ($p > 0.05$).

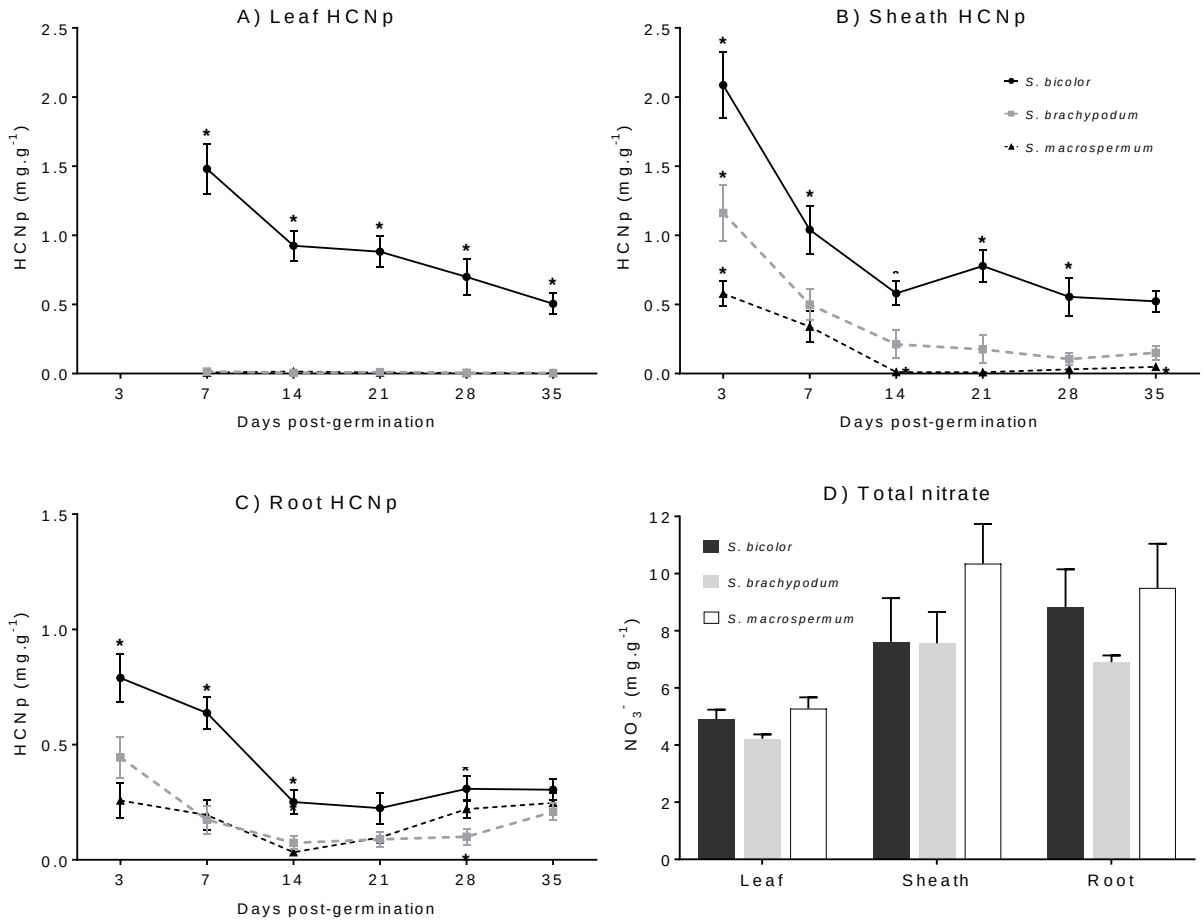


Figure 3.4: Hydrogen cyanide potential (HCNp) and concentration of nitrate (NO_3^-) in dried, finely ground tissues of *S. bicolor*, *S. brachypodum* and *S. macrospermum* plants at six harvest points during the first 35 days post-germination (at 35 days for NO_3^-). A) Leaf HCNp; B) sheath HCNp; C) root HCNp; D) total NO_3^-. Graphs show mean ± 1 standard error (n = 5), with statistically significant differences indicated at each time point: *p < 0.05. Data for leaf HCNp at 3 days post-germination are not shown as a true leaf had not emerged at this stage.

Root HCNp in *S. bicolor* was lower than that detected in the leaf and sheath tissue but followed a similar pattern, being highest at 3 days post-germination before decreasing and stabilising after 14 days (**Fig. 3.4C**). In contrast to the above ground plant parts, root HCNp of *S. bicolor* was only significantly higher than both wild species during the first two weeks of growth, at 3 days (0.78 ± 0.11 mg g^{-1}), 7 days (0.64 ± 0.07 mg g^{-1}) and 14 days post-germination (0.25 ± 0.05 mg g^{-1}) ($p < 0.05$). Within *S. macrospermum* plants, root HCNp gradually increased from 14 days post-germination (0.03 ± 0.01 mg g^{-1}) to similar levels as at 3 days post-germination, though these differences were not statistically significant ($p > 0.05$). HCNp was not significantly different between the roots of all species after 14 days post-germination, save for 28 days when *S. bicolor* (0.31 ± 0.11 mg g^{-1}) was significantly higher than *S. brachypodum* (0.10 ± 0.03 mg g^{-1}, $p < 0.05$)(**Fig. 3.4C**).

The HCNp of individual leaves and sections of the roots and sheath was used to generate a 'heat map' of HCNp (a proxy for dhurrin concentration) in a representative plant of each species at each harvest point over the 35 day duration of the experiment (**Figs. 3.5-3.7**). This illustrates both variation in HCNp distribution and concentration over time and the differences in morphology between the domesticated *S. bicolor* and the two wild species, *S. brachypodum* and *S. macrospermum*. All three species exhibited similar growth habits during the first three weeks (up to 21 days) of plant development, with more pronounced morphological differences apparent between species towards the end of the growing period (**Figs. 3.5-3.7**). At 35 days post-germination, *S. brachypodum* was the most morphologically distinct of the three species (**Fig. 3.6**). *Sorghum bicolor* and *S. macrospermum* were more similar, perhaps reflecting their closer relationship according to the current phylogeny (Dillon *et al.*, 2007a).

Total nitrate concentration was determined for leaf, sheath and root tissue harvested from plants of each species at 35 days post-germination. Total nitrate concentration was similar between all species in all tissues (**Fig. 3.4D**). The highest nitrate concentration was recorded for *S. macrospermum* with an average of 5.28 ± 0.39 mg g^{-1}, 10.35 ± 1.39 mg g^{-1} and 9.50 ± 1.54 mg g^{-1} for the leaves, sheath and roots respectively, compared with *S. brachypodum*, which had the lowest concentrations (leaf: 4.22 ± 0.15 mg g^{-1}, sheath: 7.56 ± 1.09 mg g^{-1}, root: 6.90 ± 0.23 mg g^{-1}), but the differences were not statistically significant between any species or tissue type ($p > 0.05$).

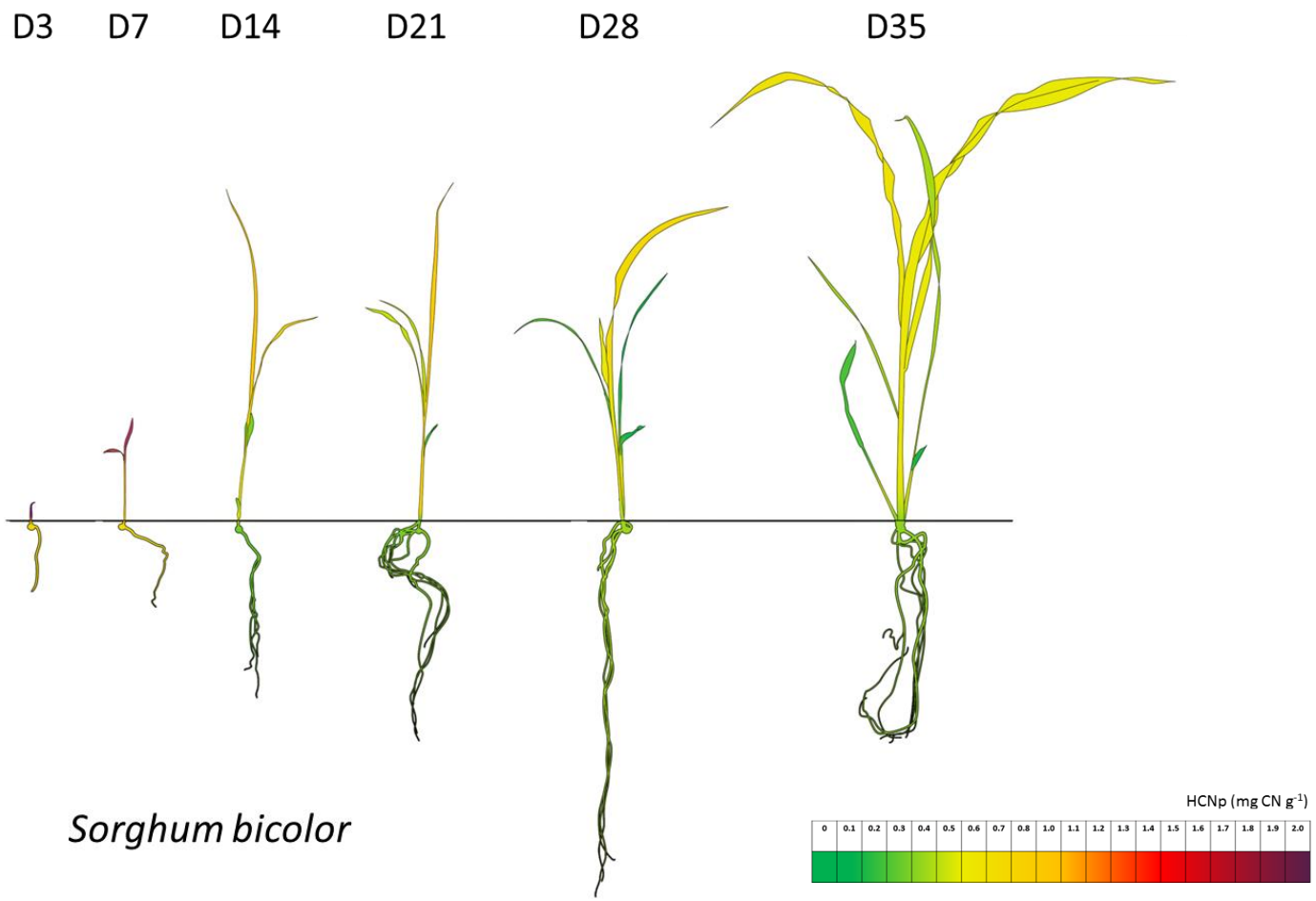


Fig. 3.5: Tissue-specific hydrogen cyanide potential (HCNp) and morphology of an individual *S. bicolor* plant at six time points during seedling development. The HCNp of a section of the sheath, roots, and each individual leaf was measured at 3, 7, 14, 21, 28 and 35 days post-germination. Colour scale indicates HCNp and is used as a proxy for dhurrin concentration (green = low; red = high).

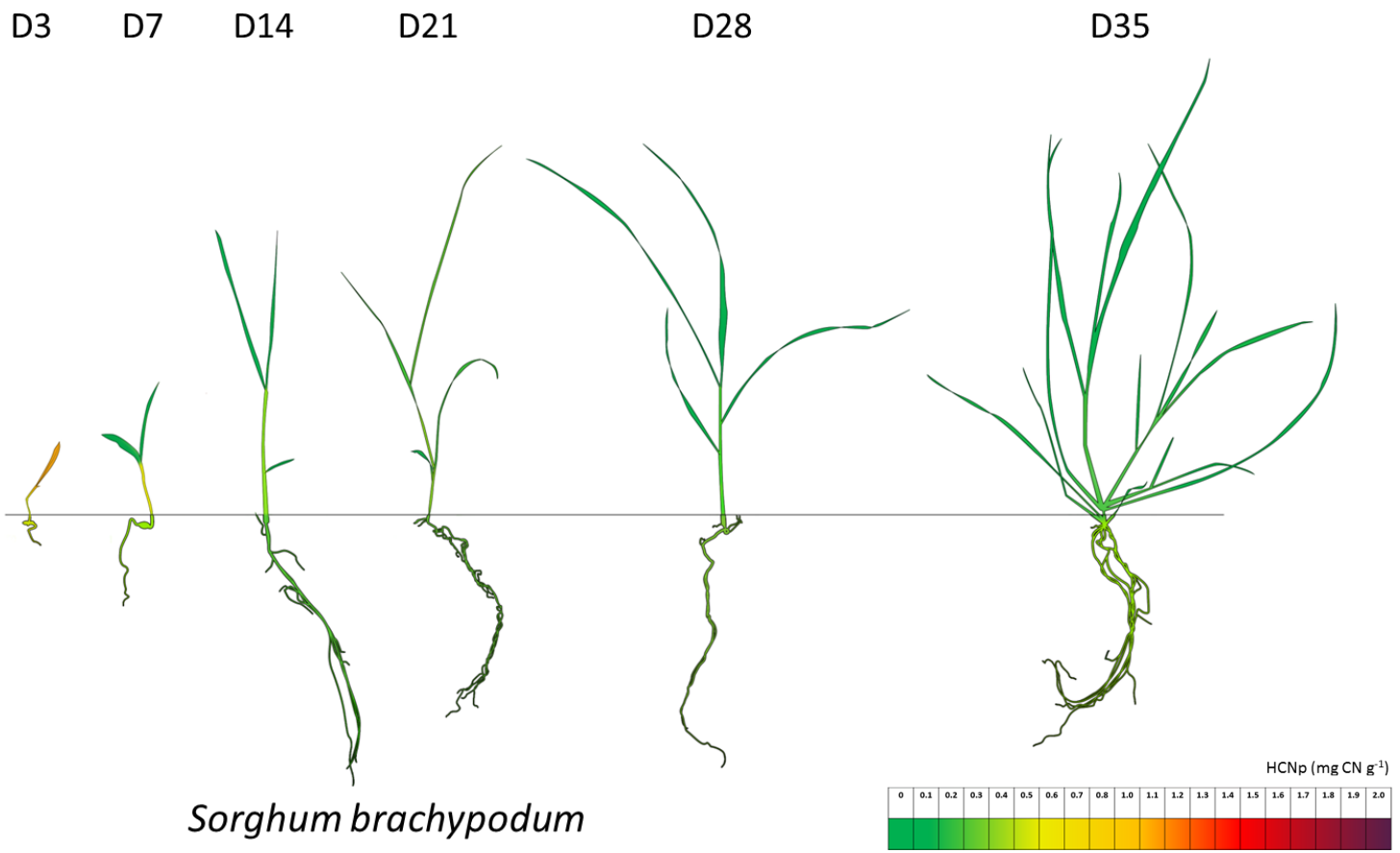


Fig. 3.6: Tissue-specific hydrogen cyanide potential (HCNp) and morphology of an individual *S. brachypodum* plant at six time points during seedling development. The HCNp of a section of the sheath, roots, and each individual leaf was measured at 3, 7, 14, 21, 28 and 35 days post-germination. Colour scale indicates HCNp and is used as a proxy for dhurrin concentration (green = low; red = high).

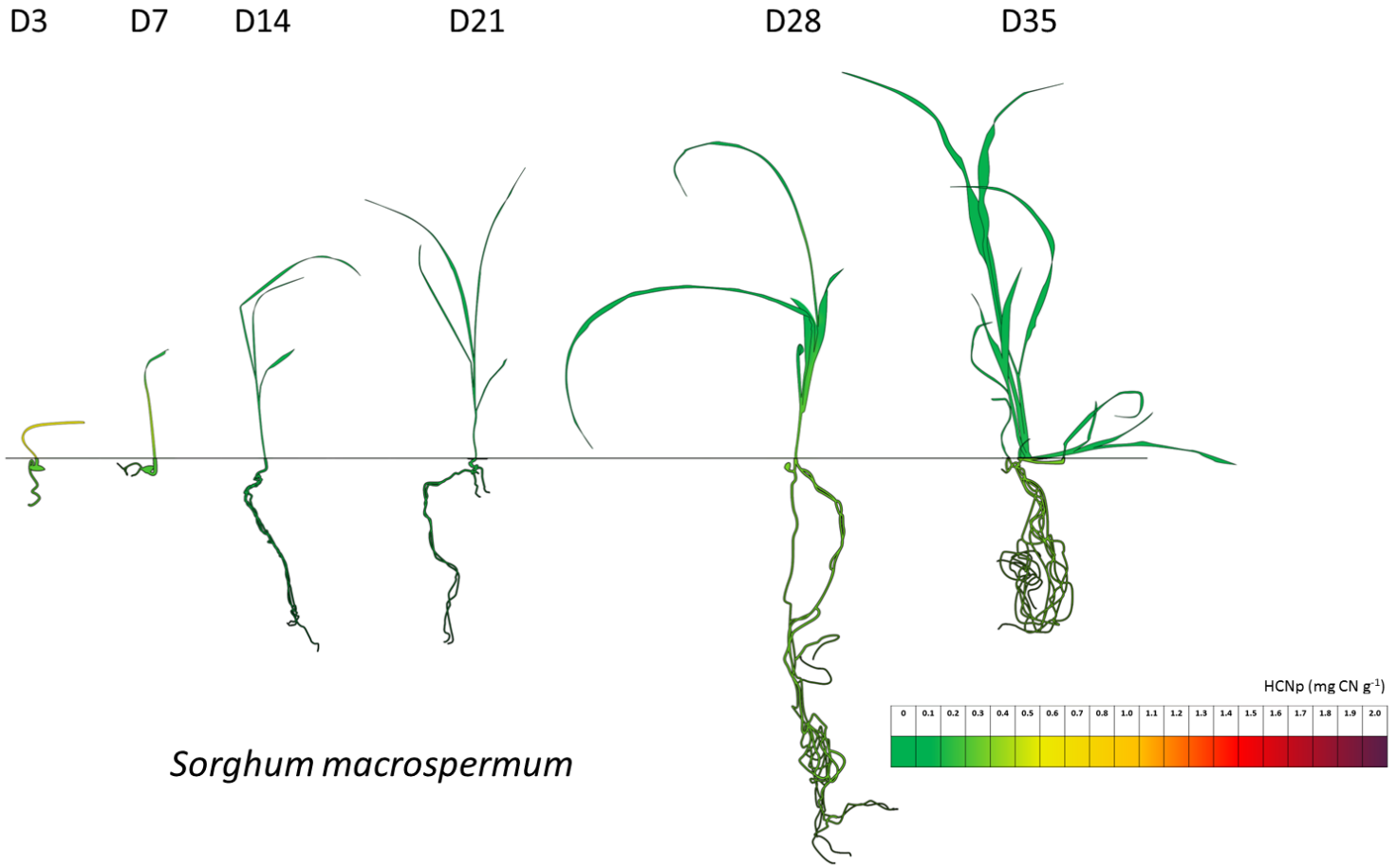


Fig. 3.7: Tissue-specific hydrogen cyanide potential (HCNp) and morphology of an individual *S. macrospermum* plant at six time points during seedling development. The HCNp of a section of the sheath, roots, and each individual leaf was measured at 3, 7, 14, 21, 28 and 35 days post-germination. Colour scale indicates HCNp and is used as a proxy for dhurrin concentration (green = low; red = high).

3.3.3 Tissue allocation of nitrogen to dhurrin and nitrate

Total elemental nitrogen (N%), specific leaf nitrogen, the proportion of nitrogen allocated to dhurrin (using HCNp as a proxy) and nitrate, and the ratio of carbon to nitrogen were determined for leaf, sheath and root tissue harvested from three individual plants of each species at 35 days post-germination (**Table 3.2, Fig. 3.8**). Total nitrogen varied between species in leaf ($p < 0.001$), sheath ($p < 0.01$) and particularly root tissue ($p < 0.001$) (**Table 3.2**). *Sorghum bicolor* had the highest leaf N% (5.2 ± 0.2%, $p < 0.05$) and lowest root N% (2.6 ± 0.1%, $p < 0.05$) of all species, while *S. brachypodum* had the lowest leaf (2.7 ± 0.1%) and sheath N% (2.6 ± 0.2%) ($p < 0.05$). Sheath N% was similar in *Sorghum macrospermum* (4.0 ± 0.2%) and *S. bicolor* (4.1 ± 0.2%) ($p > 0.05$), with N% of the two wild species being similar in the roots (*S. brachypodum*: 3.7 ± 0.1%; *S. macrospermum*: 3.8 ± 0.1%, $p > 0.05$). Specific leaf nitrogen (SLN) was similar in all species ($p > 0.05$) (**Table 3.2**).

Table 3.2: Total elemental nitrogen (%) of leaf, sheath and root tissue and specific leaf nitrogen (SLN) of *S. bicolor*, *S. brachypodum* and *S. macrospermum* plants at 35 days post-germination. Values are mean ± 1 standard error (n = 3). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests were performed for each tissue type: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns = not significant. Values with different letters are significantly different ($p < 0.05$).

	Leaf N%	**Sheath N%**	**Root N%**	**SLN**
S. bicolor	5.2 ± 0.2^{a}	4.1 ± 0.2^{a}	2.6 ± 0.1^{b}	0.013 ± 0.001
S. brachypodum	2.7 ± 0.1^{c}	2.6 ± 0.2^{b}	3.7 ± 0.1^{a}	0.012 ± 0.001
S. macrospermum	3.9 ± 0.1^{b}	4.0 ± 0.2^{a}	3.8 ± 0.1^{a}	0.010 ± 0.001
ANOVA (p)	***	**	***	ns

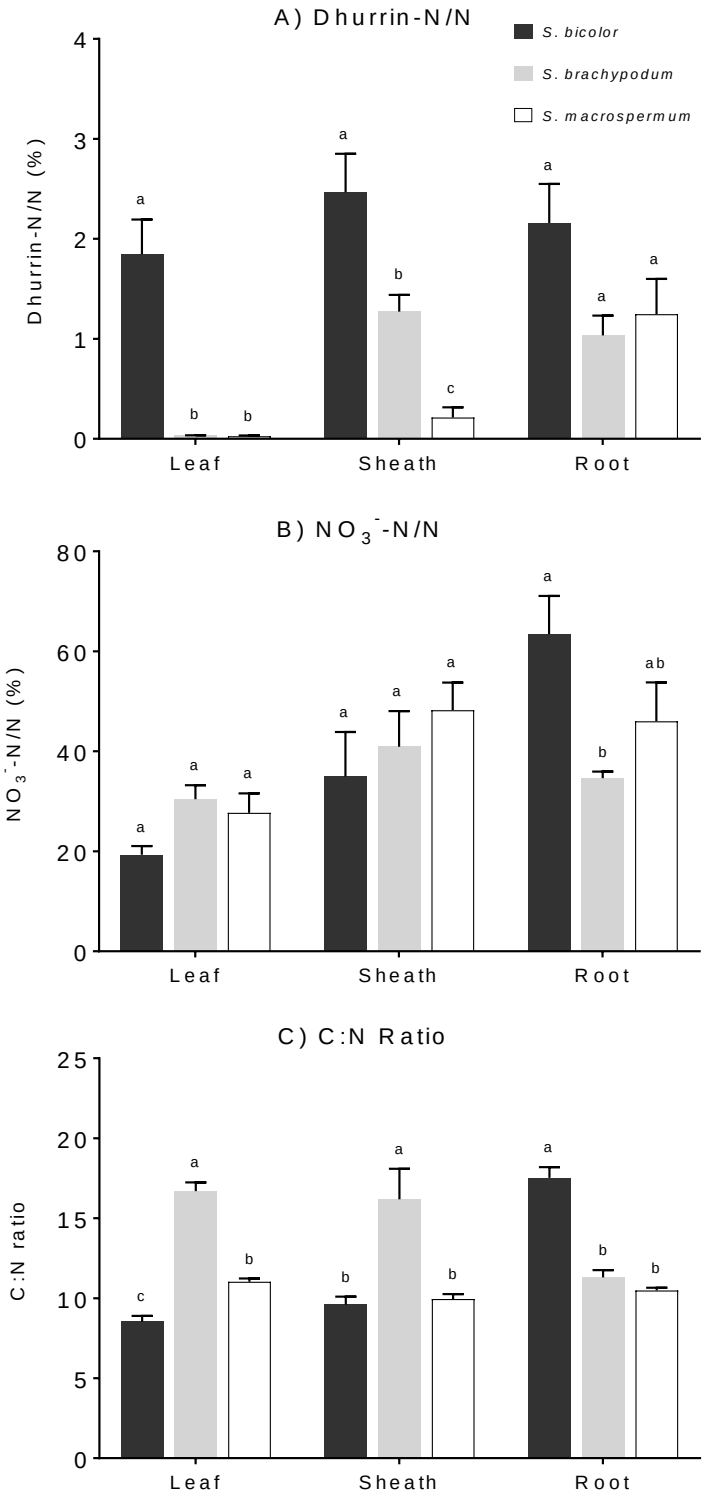


Figure 3.8: Proportion of nitrogen allocated to dhurrin and nitrate (NO_3^-) in dried, finely ground tissues of *S. bicolor*, *S. brachypodum* and *S. macrospermum* plants at 35 days post-germination. A) Dhurrin allocation; B) nitrate allocation; C) C:N ratio. Data for *S. bicolor* plants are in black, *S. brachypodum* in grey, and *S. macrospermum* in white. Graphs show mean ± 1 standard error (n = 3). Columns with different letters within each tissue are significantly different ($p < 0.05$).

The proportion of total nitrogen allocated to dhurrin was much lower than the allocation to nitrate for each tissue type (**Fig. 3.8**). As expected based on the HCNp results, the proportion of nitrogen allocated to dhurrin in leaf tissue was significantly higher in *S. bicolor* (1.8 ± 0.3%) compared to both wild species (< 0.04%, $p < 0.05$) (**Fig. 3.8A**), while allocation to nitrate was similar in both leaf and sheath tissue of all species ($p > 0.05$) (**Fig. 3.8B**). Sheath dhurrin allocation varied among species, being highest in *S. bicolor* (2.5 ± 0.4%) and lowest in *S. macrospermum* (0.2 ± 0.1%, $p < 0.05$). Root dhurrin allocation did not vary significantly between species ($p > 0.05$), though root nitrate allocation did, ranging from 34.6% in *S. brachypodum* to 63.5% in *S. bicolor*. This proportion of total nitrogen as nitrate was approximately 30 times the amount allocated to dhurrin in the roots. *Sorghum brachypodum* had the highest ratio of C:N in the leaf and sheath ($p < 0.05$), with *S. bicolor* significantly lower than both wild species in leaf tissue ($p < 0.05$) (**Fig. 3.8C**). Root C:N ratio was highest in *S. bicolor*, whilst there was no significant difference between the two wild species.

3.4 Discussion

There is growing evidence to suggest that cyanogenic glucosides, a group of specialised metabolites primarily involved in plant chemical defence, play other dynamic roles in plant growth and metabolism (Selmar *et al.*, 1988; Neilson *et al.*, 2013; Pičmanová *et al.*, 2015; Bjarnholt *et al.*, 2018; Schmidt *et al.*, 2018). The nitrogen stored in these compounds can potentially be recovered and recycled for use in primary plant metabolism when needed, without the release of hydrogen cyanide (HCN) (Jørgensen *et al.*, 2005; Møller, 2010b; Pičmanová *et al.*, 2015; Nielsen *et al.*, 2016; Bjarnholt *et al.*, 2018). *Sorghum bicolor* and its wild relatives provide an opportunity to investigate these potential functions, by comparing how the balance of cyanogenic glucoside utilisation in growth and defence differs between domesticated and undomesticated plants. The current study investigated growth, hydrogen cyanide potential (HCNp) and allocation of nitrogen during early plant development in an *S. bicolor* cultivar compared to two of its Australian wild relatives, *S. brachypodum* and *S. macrospermum*. The results indicate that while growth patterns were similar, tissue- and age-dependent regulation of cyanide varied widely between *S. bicolor* and the two wild species under stable

environmental conditions. This may be the result of different evolutionary drivers for plants in natural and cultivated systems.

3.4.1 Wild and cultivated *Sorghum* show similar growth but vary in cyanogenic potential during seedling development

Analysis of morphological features and graphical illustration of the growth habits of the three investigated species show the distinct differences between domesticated and wild *Sorghum* (**Figs. 3.5-3.7**). The wild species, *S. brachypodum* and *S. macrospermum*, generally showed more intraspecific variation in growth form than *S. bicolor* across the growing period, particularly towards the latter stages. Tillering, for example, was much more variable in the wild species after 28 days. Considering the differences between crop plants, cultivated over centuries for increased yield and growth efficiency under controlled conditions, and wild plants that need to retain higher inherent variability to persist in more variable natural systems, this is not unexpected (Vollbrecht and Sigmon, 2005; Meyer *et al.*, 2012; Meyer and Purugganan, 2013).

In contrast to growth rate, hydrogen cyanide potential varied substantially over time and across different plant parts between *S. bicolor* and the two wild species throughout the growing period (**Figs. 3.5-3.7**). Results of the current study show that the leaves of *S. brachypodum* and *S. macrospermum* contain very low concentrations of the cyanogenic glucoside dhurrin during seedling development when grown under stable environmental conditions (**Fig. 3.4A**). At 3 days post-germination the coleoptiles of these species were cyanogenic at a capacity comparable to *S. bicolor*, after which the first-emerged true leaf and all following leaves were much lower. LC-MS analysis (see **Chapter 2.3.2**) showed that this differential expression of HCNp in the leaves of the wild species was not due to any inhibition of the release of HCN or to by-products from ethylene biosynthesis (Peiser *et al.*, 1984; Yip and Yang, 1988). In contrast to the wild species, *S. bicolor* had high leaf HCNp throughout seedling development, particularly in the first week of growth. This is consistent with the findings of previous studies, though the regulatory factors behind this high cyanide expression in early ontogeny are not fully understood (Busk and Møller, 2002; Miller *et al.*, 2014; Blomstedt *et al.*, 2018). A similar pattern has been observed across many plant genera (Till, 1987; Dahler *et al.*, 1995; Gleadow and Woodrow, 2000b; Lieberei, 2007; Webber and Woodrow, 2009),

although exceptions to this have been recorded in lima bean (*Phaseolus lunatus*) (Ballhorn *et al.*, 2008) and some species of *Eucalyptus* sect. *Maidenaria* (Goodger *et al.*, 2006). In addition, younger leaves are hypothesised to be more valuable than older leaves, and therefore more heavily defended, due to being more photosynthetically active (Wiedemuth *et al.*, 2005). Regarding cyanogenic glucosides this was the case in *S. bicolor* but not in the wild species, as both *S. brachypodum* and *S. macrospermum* had very consistent, low HCNp in younger and older leaves during early plant development. Dhurrin is thought to be synthesised in the coleoptile in very young *S. bicolor* seedlings (cotyledon stage) (Halkier and Møller, 1989). This appears to also be the case in both *S. brachypodum* and *S. macrospermum*, albeit the rate of synthesis was lower.

According to the optimal allocation theory, leaf tissue is expected to be one of the most heavily defended parts of a plant, as leaves are vulnerable to predation and most important to plants in terms of photosynthesis and overall carbon assimilation (Wiedemuth *et al.*, 2005; Meldau *et al.*, 2012). While this was consistent with leaf dhurrin concentration results in *S. bicolor*, this was the least cyanogenic tissue type in the wild species, suggesting that *S. brachypodum* and *S. macrospermum* utilise other forms of defence to protect their leaves. It was noted during this study that on the leaves and sheaths of both wild species there was a high density of long epidermal trichomes that were not seen on *S. bicolor* leaves or sheaths. This was particularly apparent on *S. macrospermum* leaves (**Fig. 3.9**). These plant hairs can function as a deterrent to herbivores (Levin, 1973; Satish *et al.*, 2009; Tian *et al.*, 2012; Hauser, 2014), and this type of less costly carbon-based physical defence may be more suitable in the highly variable natural environments in which *S. brachypodum* and *S. macrospermum* are distributed. The presence of such physical defences might also deter common *S. bicolor* crop pests: *S. macrospermum*, for example, has a relatively high tolerance of shoot fly (*Atherigona soccata*) (Sharma *et al.*, 2005), an insect that bores into young *S. bicolor* shoots and damages plants during seedling development. HCNp is very high in young *S. bicolor* shoots, suggesting that another form of defence is deterring this insect in *S. macrospermum*. Two *S. macrospermum* individuals also had small spines on leaf margins at the end of the growing period (**Fig. 3.9A**). These are likely silica deposits, a key physical defence in many grasses (Hartley and DeGabriel, 2016; Simpson *et al.*, 2017). It has been shown that the margins of young leaves of domesticated

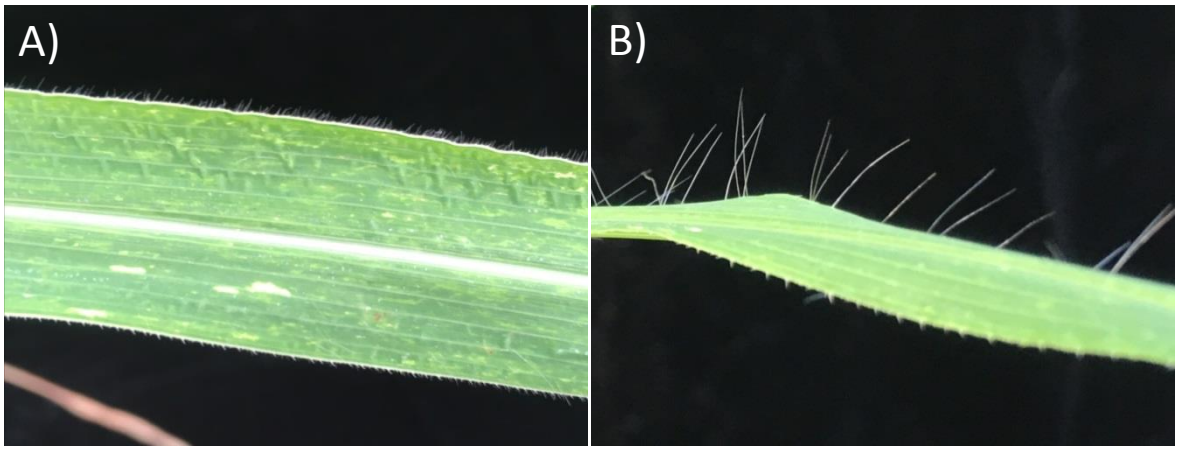


Fig. 3.9: Leaf margin structures of an *S. macrospermum* plant at the end of the growing period. A) Part of a mature leaf blade showing a high density of short trichomes and sharp spines (likely silicon-based) on the leaf margins; B) Section of a younger emerged leaf with sparse, much longer trichomes on leaf margins. *Sorghum bicolor* is not known to produce trichomes, but plants have been observed to produce small spines on their leaves during seedling development (although this was not apparent in the current study).

sorghum are serrated and that these structures contain silica (Lanning *et al.*, 1958), whilst in older leaves it appears that silicon accumulates within leaves in specialised epidermal cells, not external spines or similar structures (Lux *et al.*, 2002; Kumar *et al.*, 2017). This age-dependent shift could be due to human-induced artificial selection for sorghum as livestock forage. Simpson *et al.* (2017) found that within-leaf silicon concentration was similar between *S. bicolor* and its immediate wild progenitor *S. arundinaceum* (interfertile with *S. bicolor*); further examination of silicon accumulation and physical defensive structures between the cultivated and genetically isolated endemic Australian species would be well worth exploring in future studies**.**

Differences in the above- and below-ground distribution of dhurrin reported in the current study raise interesting questions about the utilisation of cyanogenic glucosides for additional physiological functions. Hydrogen cyanide potential in the roots of *S. brachypodum* and *S. macrospermum* plants was much higher than in the leaves, and much more comparable to the cultivated *S. bicolor* (**Fig. 3.4C**). These results support the notion that dhurrin is differentially regulated at the transcriptional level in different plant parts (Busk and Møller, 2002), and the presence of cyanogenic glucosides in roots

may point to their proposed metabolic roles in nitrogen storage and transport (Pičmanová *et al.*, 2015; Bjarnholt *et al.*, 2018). Production and storage of these compounds in the roots, rather than the aboveground tissues, may be advantageous for the wild species in their natural environment. In cassava (*M. esculenta*), another cyanogenic crop species, storage of cyanogenic glucosides (linamarin and lotaustralin) in the roots/tubers likely provides a source of reduced nitrogen that can be mobilised to other parts of the plant upon demand (Siritunga and Sayre, 2003; Zidenga *et al.*, 2017). Studies have also suggested that the presence of cyanogenic glucosides in the roots could provide a system of defence against nematode herbivory (Curto *et al.*, 2012; Djian-Caporalino *et al.*, 2019), or play a role in plant allelopathy (Mahmoodzadeh, 2010). A defensive role seems probable given the importance of roots to plants in water and nutrient uptake (Rasmann and Agrawal, 2008; Moore and Johnson, 2017).

3.4.2 Tissue-dependent allocation of nitrogen varies in wild and cultivated *Sorghum*

Nitrate is a primary metabolite and the preferred source of nitrogen for most plants (Bloom, 2015; Gleadow *et al.*, 2016b; Blomstedt *et al.*, 2018). However, like the nitrogen-containing dhurrin it can also be toxic to grazing animals if accumulated in high concentrations (Finnie *et al.*, 2011). Gleadow *et al.* (2016b) found some evidence of a trade-off in the allocation of plant total nitrogen between dhurrin and nitrate in different tissue types in *S. bicolor*. As such, it was thought that in the current study perhaps the lower cyanide potential across *S. brachypodum* and *S. macrospermum* plants would be correlated with enhanced accumulation of nitrate, particularly in the leaves. The results did not support this hypothesis, with nitrate concentration not significantly different within any tissue between *S. bicolor* and either of its wild relatives at 35 days post-germination (**Fig. 3.4D**). Furthermore, the allocation of total elemental nitrogen (N%) to nitrate was not significantly higher in any tissue of either wild species compared to *S. bicolor* ($p > 0.05$), with the domesticated species showing the highest allocation of nitrogen to nitrate in the roots (**Fig. 3.8B**). Interestingly, while both *S. brachypodum* and *S. macrospermum* maintained higher N% in their roots than *S. bicolor*, this did not translate to a higher allocation of nitrogen to either nitrate or dhurrin (**Table 3.2**). In combination with the HCNp detected in the roots of each species, it

appears that management of nitrogen *in planta* varies between *S. bicolor* and the wild species. This also points to differences in the relative importance of the diverse roles of cyanogenic glucosides in plant growth and defence.

3.4.3 Cyanogenic glucosides in resource allocation and domestication

Resource allocation theories of plant defence predict that the synthesis of defensive compounds, such as cyanogenic glucosides, comes at a cost to growth, in that plants with higher production of such compounds exhibit reduced growth rate and biomass accumulation (McKey, 1974; Herms and Mattson, 1992; Gleadow *et al.*, 1998; McCall and Fordyce, 2010; Endara and Coley, 2011; Meldau *et al.*, 2012). In the current study all species showed similar growth during seedling development, but pronounced differences in age- and tissue-dependent expression of cyanide potential. *Sorghum bicolor* produced far more dhurrin in terms of total mass across the plant, particularly in the leaves, yet it grew at a similar rate to its less cyanogenic wild relatives *S. brachypodum* and *S. macrospermum*, at least during seedling development. As these species have experienced very different evolutionary pressures, it is difficult to directly compare potential trade-offs in dhurrin production between them (Züst and Agrawal, 2017; Whitehead and Poveda, 2019). Within *S. bicolor,* however, analysis of an acyanogenic mutant showed a reduced growth rate during early development, lending support to the proposed nitrogen recycling role of cyanogenic glucosides, especially during seedling emergence (Blomstedt *et al.*, 2012; Bjarnholt *et al.*, 2018; Blomstedt *et al.*, 2018). Based on this and the results of the current study, the production of dhurrin does not necessarily come at a cost to growth and development in sorghum. That is, the potential primary metabolic functions of cyanogenic glucosides may offset the costs of their production under stable, resource-rich conditions (Neilson *et al.*, 2013; Blomstedt *et al.*, 2018).

In the context of optimal allocation and other defence theories, plant domestication theory hypothesises that cultivated plants will generally have reduced chemical defences, as more resources can be diverted to metabolic processes for improved growth and crop yields (Rosenthal and Dirzo, 1997; Gepts, 2004; Meyer *et al.*, 2012; Chen *et al.*, 2015; Simpson *et al.*, 2017). Several studies have detected a general reduction in specialised defence compounds in domesticated plants compared to their

closely related wild progenitors, though understanding of the main drivers behind these differences remains limited (Lindig-Cisneros *et al.*, 2002; Chaudhary, 2013; Chen *et al.*, 2015; Whitehead *et al.*, 2017). Some recent studies and meta-analyses of chemical defences, particularly phenolic compounds, did not find a clear reduction through domestication across a wide range of species (Turcotte *et al.*, 2014; Simpson *et al.*, 2017; Whitehead *et al.*, 2017). This was true of the results of the current study, in which the concentration of dhurrin was higher in domesticated sorghum compared to its two wild relatives. A caveat here is that the two species analysed (*S. brachypodum* and *S. macrospermum*) are not the direct wild progenitors of *S. bicolor*; *S. arundinaceum* (*S. bicolor* subsp. *verticilliflorum*) is considered the immediate wild progenitor (Wiersema and Dahlberg, 2007; Mace *et al.*, 2013), but this species was not available for this study. Further studies will be required but the results presented point to a diverse set of roles for cyanogenic glucosides, and support the growing body of evidence that plant domestication does not necessarily result in a widespread reduction in chemical defence.

3.4.4 Conclusion

This is the first study to evaluate and compare the age- and tissue- dependent regulation of hydrogen cyanide and its potential roles in plant nitrogen metabolism in a *S. bicolor* cultivar and two of its closely related wild relatives from the same genus. We found clear variation in the cyanogenic potential of the wild species *S. brachypodum* and *S. macrospermum* compared to *S. bicolor*, particularly in the leaves. Allocation of nitrogen to dhurrin and nitrate was also found to vary across tissue types and between species. Together, the results of growth, cyanide potential, nitrates and nitrogen allocation point to differences in the utilisation and balance of function of dhurrin production between wild and cultivated sorghum. To further understand the differences in the regulation of cyanogenesis in *S. bicolor* and its wild relatives, future studies could investigate ontogenetic and tissue-specific expression patterns of the major genes involved in dhurrin biosynthesis, the two cytochrome P450s (*CYP79A1* and *CYP71E1*) and a UDP-glucosyltransferase (*UGT85B1*) (Gleadow and Møller, 2014). Results here support the growing body of evidence that cyanogenic glucosides play dynamic roles in general plant metabolism and are not restricted to chemical defence.

Chapter 4 – Crop wild relatives as a genetic resource for generating low-cyanide, drought-tolerant *Sorghum*

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4.1 Abstract

Using a *Sorghum bicolor* cultivar and seven wild *Sorghum* species endemic to Australia as our experimental system, we monitored their different responses to drought by assessing growth and morphological, physiological and biochemical parameters. Drought stress significantly decreased height, biomass, the maximum potential quantum efficiency of photosystem II, photosynthetic rate and relative water content in *S. bicolor*, while several of the wild species were much more tolerant. Drought significantly increased dhurrin concentration in aboveground tissue in *S. bicolor* but not in the wild species. Root dhurrin content was unaffected by drought in *S. bicolor*, in contrast to the varied responses observed in the wild species. *Sorghum macrospermum* and *S. brachypodum* maintained relatively high growth and photosynthetic performance under drought, with negligible aboveground dhurrin content. These wild species are promising candidates for sorghum crop improvement.

4.2 Introduction

Drought is one of the strongest factors limiting global crop production, threatening sustainable agriculture and food security (Araus *et al.*, 2008; Dhankher and Foyer, 2018). *Sorghum bicolor* (L.) Moench (sorghum) is the fifth largest cereal crop in terms of global production, grown mainly in semi-arid and sub-tropical regions with an annual grain production of approximately 60 million metric tonnes (Sanders *et al.*, 2019). Sorghum is cultivated widely for forage and the grain is a staple throughout Africa and Asia (Paterson *et al.*, 2009; Mace *et al.*, 2013; Hariprasanna and Rakshit, 2016; Sanders *et al.*, 2019). Typical of many C_4 plants, *S. bicolor* is fast-growing with a high water use efficiency, and is tolerant of high temperatures and dry conditions (Tuinstra *et al.*, 1997). Nevertheless, it is affected by severe drought especially at the later growth stages, often resulting in a reduction in grain yield and biomass (Prasad *et al.*, 2008;

O'Donnell *et al.*, 2013; Borrell *et al.*, 2014). There is a need to generate varieties that are more resilient to drought, particularly if yields are to be maintained in the face of climate change.

Breeding and crop improvement efforts rely on exploiting the genetic diversity present in different crop biotypes and landraces. A much less explored option exists in the genetic diversity in the species most closely related to crop plants, the crop wild relatives (CWR) (Hajjar and Hodgkin, 2007; Brozynska *et al.*, 2016). These wild species have evolved through natural selection to be highly resilient and productive across a diverse range of habitats (Lazarides *et al.*, 1991; Dillon *et al.*, 2007b). Compared to other major cereal crops, such as wheat and maize, relatively little is known about the diversity in the wild relatives of sorghum (Lazarides *et al.*, 1991; Price *et al.*, 2006; Dillon *et al.*, 2007a; Dillon *et al.*, 2007b; Kuhlman *et al.*, 2008). Of the 25 species in the *Sorghum* genus, 14 are endemic to Australia and a further three are native to Australia and SE Asia (Dillon *et al.*, 2007a). These species are primarily found in the remote areas of northern Australia (Lazarides *et al.*, 1991), making research in the field challenging. Moreover, constraints in inter-species hybridisation and lateral gene transfer between wild and cultivated *Sorghum* have not been addressed (Hodnett *et al.*, 2005; Price *et al.*, 2005; Varshney *et al.*, 2014; Migicovsky and Myles, 2017). The difficulty of gene transfer between domesticated plants and their wild relatives is currently the greatest barrier to utilisation of CWRs in crop improvement strategies in general, although advances in biotechnology, gene sequencing and more recently gene transfer technologies such as the CRISPR/Cas9 system are improving the outlook for crop breeding programs (Bevan *et al.*, 2017; de Wit, 2017; Lemmon *et al.*, 2018; Zsögön *et al.*, 2018).

Abiotic stress not only affects primary growth and development, it also impacts the metabolic pathways responsible for the accumulation of specialised metabolites such as cyanogenic glucosides (Bohnert *et al.*, 1995; Selmar and Kleinwächter, 2013). A range of experiments have demonstrated that many plant species exposed to drought stress accumulate higher concentrations of these products (Gleadow and Woodrow, 2002b; Woodrow *et al.*, 2002; Liang, 2003; O'Donnell *et al.*, 2013; Neilson *et al.*, 2015; Gleadow *et al.*, 2016b). A major problem limiting the use of sorghum as a livestock forage crop is that it is cyanogenic, producing the cyanogenic glucoside dhurrin. Dhurrin is a nitrogen-

containing natural product, which upon tissue damage releases hydrogen cyanide (HCN). HCN is a strong inhibitor of cytochrome *c* oxidase preventing mitochondrial electron transport and blocking oxidative phosphorylation and ATP production in animals, causing respiratory inhibition, and cellular hypoxia or histotoxic anoxia (Leavesley *et al.*, 2008; Nicholson, 2012). Autotoxicity in plants is avoided by spatial separation at the organelle or tissue levels of the cyanogenic glucoside and the specific β-glucosidase required for its hydrolysis (Frehner and Conn, 1987; Heraud *et al.*, 2018; Knudsen *et al.*, 2018). To be safely consumed by cattle, released HCN levels in the ingested plant material should be below 600 ppm (Boyd *et al.*, 1938; Hunt and Taylor, 1976). Prolonged exposure to drought stress may increase the concentration of cyanogenic glucosides (Duncan, 1996; O'Donnell *et al.*, 2013). Drought-stressed forage sorghum, for example, has been reported to contain more than 750 ppm HCN, making it unsuitable as a feed for livestock (O'Donnell *et al.*, 2013; Neilson *et al.*, 2015; Gleadow *et al.*, 2016b). Under drought conditions, farmers in northern Australia have been forced to leave sorghum crops ungrazed or unprocessed due to the increased potential HCN release (pers. comm. Dr Peter Stuart).

Cyanogenic glucosides are typically produced and accumulated in young and developing aboveground parts and provide plants with an immediate chemical defence response against herbivores (Halkier and Møller, 1989; Tattersall *et al.*, 2001; Gleadow and Woodrow, 2002a; Zagrobelny *et al.*, 2008; Neilson *et al.*, 2013; Hansen *et al.*, 2018; Thodberg *et al.*, 2018). Additionally, cyanogenic glucosides appear to play non-defensive roles in plants, functioning as a mobilisable store of reduced nitrogen (Gleadow and Woodrow, 2002a; Miller *et al.*, 2014; Pičmanová *et al.*, 2015; Bjarnholt *et al.*, 2018; Blomstedt *et al.*, 2018). Cyanogenic glucosides may also serve as a system for transporting nitrogen and carbon (Selmar *et al.*, 1988; Kongsawadworakul *et al.*, 2009), and function in mitigating oxidative stress (O'Donnell *et al.*, 2013; Selmar and Kleinwächter, 2013; Gleadow *et al.*, 2016a; Schmidt *et al.*, 2018). In cherry and almond, the formation and turn-over of cyanogenic glucosides have been demonstrated to play a role in the regulation of bud break and flowering time (Del Cueto *et al.*, 2017; Ionescu *et al.*, 2017a; Ionescu *et al.*, 2017b).

Identification of suitable traits in sorghum's wild relatives could be used to develop cultivars that are both more tolerant to drought conditions without compromising yield and biomass, and with lower dhurrin production during stress. On the other hand, the transfer of yield and productivity traits known to be important in domestication from *S. bicolor* back into wild species could provide an alternative path to crop improvement. This latter approach was recently demonstrated in wild tomato, which was essentially 'redomesticated' by inserting known domestication genes (Lemmon *et al.*, 2018; Zsögön *et al.*, 2018).

Currently there are no reports on the cyanogenic potential of wild relatives of *S. bicolor* or on their drought tolerance. It remains unknown whether cyanogenic glucoside formation is regulated differently in the wild species compared to *S. bicolor*, or whether the relative importance of the diverse roles of cyanogenic glucosides is different among the wild species. This is important because improved stress resistance may lead to a lower, more stable HCN potential within the plant, breaking the drought-HCN nexus. Wild plants displaying higher tolerance to drought, and a lower cyanide potential (HCNp) in the aboveground tissues of the plant under stress, would be of particular interest for sorghum crop improvement programs in the future, both as grain and forage.

The aim of this study was to investigate the effects of chronic drought on growth, morphology, and physiological and biochemical characteristics of seven wild *Sorghum* species, in comparison to the cultivated *S. bicolor*. The phylogenetic relationship within the *Sorghum* genus has been resolved by molecular analysis demonstrating five main subgenera, *Eusorghum*, *Chaetosorghum, Heterosorghum*, *Parasorghum* and *Stiposorghum* (Dillon *et al.*, 2007a). Six of the wild species (*S. amplum, S. brachypodum, S. bulbosum, S. ecarinatum, S. intrans* and *S. matarankense*) selected for this study are within the diverse Australian *Stiposorghum* and *Parasorghum* groups (Garber, 1950; De Wet, 1978). *Sorghum macrospermum*, also endemic to Australia, is the single species of the *Chaetosorghum* subgenus, and *S. bicolor* belongs to the domesticated *Eusorghum*. All selected wild species are annuals and are widely distributed across the highly variable environments of northern Australia. These environments are generally characterised by low soil nutrient availability, high day temperatures, high insolation and distinct wet-

dry seasonal shifts (Lazarides *et al.*, 1991; Dillon *et al.*, 2007b). The undomesticated *Sorghum* species might therefore be better adapted to tolerate harsh conditions (*i.e.* low water availability), and accumulate less of the primarily defence-related specialised metabolite dhurrin. Identification of CWR with these characteristics would provide a germplasm resource for the breeding of new and novel 'climate change ready' sorghum varieties.

4.3 Materials and Methods

4.3.1 Plant material and growing conditions

Sorghum bicolor (BTx623) was used as the domesticated germplasm. Seeds from accessions of seven wild *Sorghum* species (*S. amplum* Lazarides, *S. brachypodum* Lazarides, *S. bulbosum* Lazarides, *S. ecarinatum* Lazarides, *S. intrans* F.Muell ex.Benth, *S. macrospermum* E.D.Garber and *S. matarankense* E.D.Garber & L.A.Snyder) were obtained from the Australian Grains Genebank (AGG), Horsham, Victoria. Selection of accessions was based on availability and to represent a range of distributions from across northern Australia (**Table 4.1**). A protocol was developed to achieve an acceptable rate of seed germination. The caryopsis was removed from the seed covering (including the lemma and palea) using a scalpel and forceps and placed in a 2 mL Eppendorf tube containing 500µL 3mM Gibberellic Acid (GA_3) (Growth Regulator, G7645, Sigma-Aldrich) and kept at room temperature (24°C) for approximately 15 h.

The GA_3 treated caryopses were then placed in Petri dishes on two layers of filter paper wetted with sterile double distilled water and sprayed with 3mM GA_3 solution. The dishes were incubated in a constant temperature cabinet set at 35°C day (9am-5pm) and 25°C overnight, with a 14 h photoperiod from 6am-8pm. Petri dishes were wrapped in foil for the first 24 h in the cabinet. The caryopses were checked daily and kept moist with sterile double distilled water. Once the caryopses had germinated and formed a small root and shoot, they were transplanted into soil in small trays until seedlings had established, approximately 4-5 d post-germination. Individual seedlings were then transferred to larger pots (20×14 cm, 3.1 L) containing Debco seed raising soil mix

Table 4.1: Individual accession details and origin of collection for the seven wild *Sorghum* species examined in this study: *S. amplum, S. brachypodum, S. bulbosum, S. ecarinatum, S. intrans, S. macrospermum* and *S. matarankense*. All seeds were obtained from the Australia Grains Genebank, Horsham, Victoria.

Species	Accession number	Accession name	Provenance	Latitude	Longitude
S. amplum	302623	JC 2361	Kimberley, WA	-14.5982	125.7928
S. brachypodum	302480	JC 2125	Arnhem Land, NT	-12.7145	132.4167
S. bulbosum	302645	JC 2320	Kimberley, WA	-16.0965	128.39
S. ecarinatum	302656	JC 2332	Kimberley, WA	-17.2447	122.9102
S. intrans	302668	IDC 7545	Darwin, NT	-12.4333	131.0667
S. macrospermum	302367	JC 2253	Katherine, NT	-14.4097	132.1977
S. matarankense	302521	JC 2188	Katherine, NT	-16.0797	136.3077

(Debco Pty Ltd) and perlite (4:1 v/v ratio). All plants (total n = 200) were grown under controlled greenhouse conditions at Monash University (coordinates: 37°54'36"S 145°08'02"E) from February-April 2017 with a mean temperature of 28°C ± 2°C and 18°C ± 2°C day/night, and an average photoperiod of 14 h (average photosynthetic photon flux density: 430 ± 87 µmol quanta m^{-2} s^{-1}). Supplementary light from sodium lamps (MK-1 Just-a-shade, Ablite Australia) was used to maintain the irradiation level when the natural photoperiod decreased. All plants were watered daily to 100% soil water capacity for the first week and then every second day. A baseline harvest of six to eight individuals of each species was performed at the end of the second week. Remaining plants were then split into two treatment groups: the well-watered group (n = 10 of each species), where plants continued to be watered under the same regime; and the water-limited group (n = 10 of each species), where soil water capacity was gradually reduced to 15% over 10 d and kept at that level by maintaining pot weight every second day. This soil water capacity was selected based on the results of a

previous experiment in which *S. bicolor* plants accumulated higher concentrations of dhurrin when grown at 15% field capacity (Rosati *et al.*, 2019). Pots were weighed in order to account for any potential differences in plant biomass and leaf area. All plants were destructively harvested after six weeks of treatment.

Soil water holding capacity for each individual pot was determined following Hasanuzzaman *et al.* (2017); target soil water content (W_T) was calculated via the equation:

$$W_T = W_P + W_D + \%RSWC \times W_S \qquad \textit{Equation 1}$$

where W_P is the weight of an empty pot, W_D is the dry soil weight (obtained by subtracting the weight of an empty pot from a standard dry soil-filled pot), *%RSWC* is the relative soil water content (15% in this study) and W_S is the soil water content (wet pot weight minus dry pot weight). Soil water holding capacity was calculated using five pots.

4.3.2 Quantum yield and carbon assimilation

The maximum quantum yield (F_V/F_m ratio) of PSII was measured with a PAM-2000 Chlorophyll Fluorometer (WALZ, Effeltrich, Germany) using the youngest fully unfurled leaf from seven-week-old control and drought-stressed plants, *i.e.* one week prior to the final harvest (n = 8 of each species). Measurements were carried out on leaves dark adapted for 30 min. Photosynthetic carbon assimilation (A) was measured using the same leaf on a different day with a LI-6400XT Portable Photosynthesis System (LI-COR Biosciences, Nebraska, USA), avoiding the midrib. Parameters were set at 1000 µmol $m^{-2}s^{-1}$ PAR, 400 ppm CO_2 and 28°C (growth temperature).

4.3.3 Harvesting and growth analysis

Plants were destructively harvested two weeks after transplanting (baseline harvest, all plants well-watered) (n = 6-8 of each species), and six weeks after treatments were initiated (final harvest) (n = 10 of each species). Plant height was measured from the base to the ligule of the uppermost leaf. The number of leaves, tillers and dead leaves was counted. Leaves were then removed at the ligule and total leaf area (TLA) of the

blades was measured using a LI-3000 portable area meter and a LI-3050A belt conveyor (LI-COR Biosciences, Nebraska, USA). Plants were separated into leaf, sheath and root tissue and the fresh weight (FW) of each tissue determined. All tissues were then snap-frozen in liquid nitrogen and kept at -80°C before being freeze-dried and weighed (DW). The root:shoot ratio was calculated by dividing total belowground biomass by total aboveground biomass. Relative water content (%RWC) was determined following Blomstedt *et al.* (1998) using a subsample of leaf tissue (DW included in total biomass). The leaf sample was immersed in water for 24 h to give turgid weight (TW), followed by oven drying at 55°C to give DW. %RWC was calculated using the following equation:

$$\%\mathrm{RWC} = \left(\frac{\mathrm{FW}-\mathrm{DW}}{\mathrm{TW}-\mathrm{DW}}\right) \times 100 \qquad \textit{Equation 2}$$

Relative growth rate (RGR) was calculated using the equation:

$$\mathrm{RGR} = \frac{(\ln W_2 - \ln W_1)}{t_2 - t_1} \qquad \textit{Equation 3}$$

where W_1 and W_2 are the dry biomass at the baseline harvest (t_1) and final harvest (t_2). Specific leaf area (SLA) was calculated as leaf area per gram dry leaf mass.

4.3.4 Chemical assays

Cyanogenic glucoside concentrations were determined spectrophotometrically from the hydrogen cyanide potential (HCNp) using the König reaction (Gleadow *et al.*, 2012). It is used as a proxy for the dhurrin content, such that each mg of HCN is equivalent to 11.5 mg of dhurrin in the plant tissue. Free hydrogen cyanide released as a result of ethylene biosynthesis was assumed to be negligible. Freeze-dried leaf, sheath and root tissue (n = 8 of each species) was ground to a fine powder using a MM 300 MixerMill (Retsch, Haan, Germany). Data are expressed as HCNp (mg HCN g^{-1} dry weight), that is, the maximum amount of HCN release upon hydrolysis of all dhurrin present as ascertained by addition of β-glucosidase (300µl of 2 mg mL^{-1} almond emulsion, Sigma, Missouri, USA). Total nitrate (NO_3^-) concentration (mg NO_3^- g^{-1} dry weight) was measured by a colorimetric assay using 96 well microtiter plates with 15 mg of tissue per sample (n = 8 of each species) following O'Donnell *et al.* (2013). Total elemental nitrogen and carbon

(%) of each tissue type (n = 5) were analysed using a 2400 Series II CHNS/O Elemental Analyser in CHN mode (PerkinElmer, Massachusetts, USA). In order to assess how nitrogen was partitioned, the proportion of nitrogen found as dhurrin or nitrate was calculated as a proportion of the total elemental nitrogen at both the tissue and whole plant level. Specific leaf nitrogen (SLN) was calculated via the following equation:

$$\text{SLN g g}^{-1} = (N)\left(\frac{W_L}{A}\right) \qquad \textit{Equation 4}$$

where W_L is leaf biomass, A is leaf area and N is leaf nitrogen concentration.

4.3.5 Statistical analysis

All data were analysed using GraphPad Prism version 7.02 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) and R version 3.5.1 (R Core Team, 2017). Ordinary two-way ANOVA followed by Tukey's multiple comparisons tests was used to compare the performance of traits between species and treatments, and among species in the same treatment group (n = 5-10, dependent on the measured trait). A principal component analysis (PCA) was performed to assess differences in growth and physiology across all treatment groups using R. Data were tested for normality and homogeneity of variances prior to analysis and transformed where required. A 95% confidence level was set for all statistical tests.

4.4 Results

4.4.1 Changes in plant growth in response to drought

The impact of chronic drought on plant growth was assessed through analysis of biomass, growth indices and morphological traits. In each treatment involving the wild species there was much greater variation between individual plants than observed with the domesticated *S. bicolor*. Above- and below-ground biomass decreased in several, but not all, species under water-limited conditions (**Fig. 4.1A, B**). *Sorghum bicolor* had the highest shoot (15.6 ± 0.9 g DW) and root (4.0 ± 0.2 g DW) biomass of all species among well-watered plants and had the largest decrease under drought in both shoots (2.6 ± 0.3 g DW, 83% decrease; $p < 0.05$) and roots (0.87 ± 0.17 g DW, 78% decrease; $p < 0.05$). In most of the wild species, an overall trend of decreased shoot biomass was observed

under water deficit, but the difference was significant only in *S. ecarinatum* (69% decrease; $p < 0.05$) and *S. macrospermum* (62% decrease; $p < 0.05$), in both species a significantly smaller decrease than observed in *S. bicolor*. Root biomass also decreased significantly in *S. ecarinatum* (82% decrease; $p < 0.05$) and *S. macrospermum* (65% decrease; $p < 0.05$) (**Fig. 4.1B**). Water deficit resulted in a less severe and non-significant reductions of biomass in *S. amplum*, *S. bulbosum*, *S. intrans* and *S. matarankense* ($p > 0.05$), with *S. brachypodum* being the overall least affected species. It is noteworthy that even under well-watered conditions these species had a much smaller biomass than *S. bicolor*.

Relative growth rate (RGR) was significantly lower in drought-stressed *S. bicolor* (66% decrease) and *S. intrans* (76% decrease) ($p < 0.05$) (**Fig. 4.1C**). In the remaining wild *Sorghum* relatives, the RGR was not significantly different between well-watered and water-limited treatments (**Fig. 4.1C**). *Sorghum macrospermum* had the highest RGR of all species under both well-watered (0.08 ± 0.01 g g^{-1} day^{-1} DW) and water-limited conditions (0.06 ± 0.01 g g^{-1} day^{-1} DW), although RGR was not significantly higher than several other species ($p > 0.05$). *Sorghum bicolor* and six of the wild species showed a similar root:shoot ratio under well-watered and water-limited conditions (**Fig. 4.1D**). *Sorghum brachypodum* was the only species in which there was a significant difference in root: shoot ratio (0.12 ± 0.01, 38% decrease) ($p < 0.05$).

Watering treatment had a significant effect on total leaf area (TLA) in *S. bicolor, S. amplum, S. ecarinatum* and *S. macrospermum* plants (**Fig. 4.2A**). *Sorghum bicolor* had the highest TLA among well-watered plants (2260 ± 60 cm^2), followed by *S. macrospermum* with an area of (1650 ± 330 cm^2). TLA decreased by 85% in droughted *S. bicolor* to 310 ± 37 cm^2 ($p < 0.05$).)*Sorghum macrospermum* had the highest TLA among all drought-stressed plants with an average area of 720 ± 110 cm^2 ($p < 0.05$), 2.3× higher than *S. bicolor*.

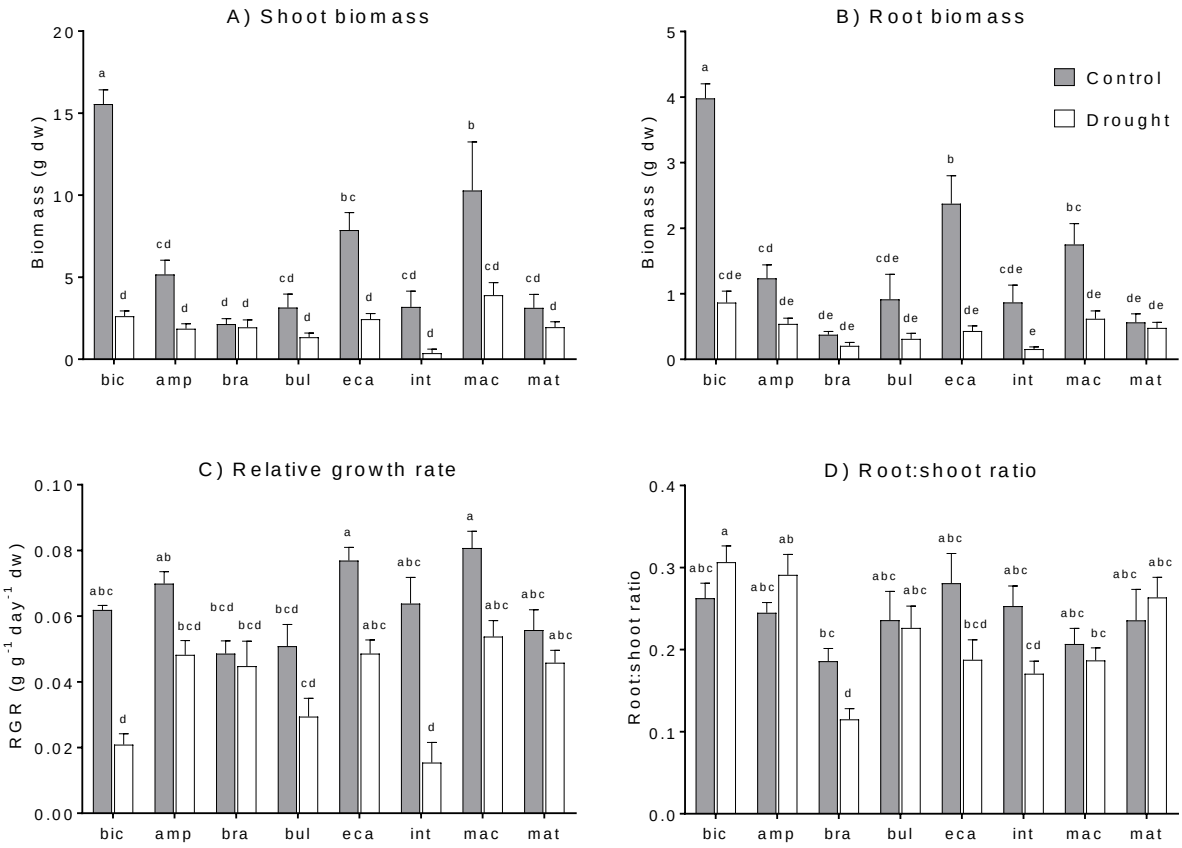

Figure 4.1: Biomass and growth measurements of 8-week-old *S. bicolor* (bic), *S. amplum* (amp), *S. brachypodum* (bra), *S. bulbosum* (bul), *S. ecarinatum* (eca), *S. intrans* (int), *S. macrospermum* (mac) and *S. matarankense* (mat) plants under well-watered (control) and water-limited (drought) conditions. A) shoot biomass; B) root biomass; C) relative growth rate; D) root:shoot ratio. Data for well-watered plants are shown in grey columns, and drought-stressed plants in white. Graphs show mean ± 1 standard error (n = 10). Columns with different letters are significantly different ($p < 0.05$).

Specific leaf area (SLA) was not significantly affected by water limitation in any species (**Fig. 4.2B**). SLA increased in *S. macrospermum* under water deficit (340 ± 30 cm^2 g^{-1} DW; 18% increase), but the difference was not significant ($p > 0.05$). Five wild species (*S. amplum, S. bulbosum, S. intrans, S. macrospermum* and *S. matarankense*) showed similarly high SLA under water deficit. *Sorghum ecarinatum* had the lowest SLA under both well-watered (149 ± 15 cm^2 g^{-1} DW) and water-limited conditions (177 ± 15 cm^2 g^{-1} DW). Among the well-watered plants, mean relative water content (%RWC) of the first

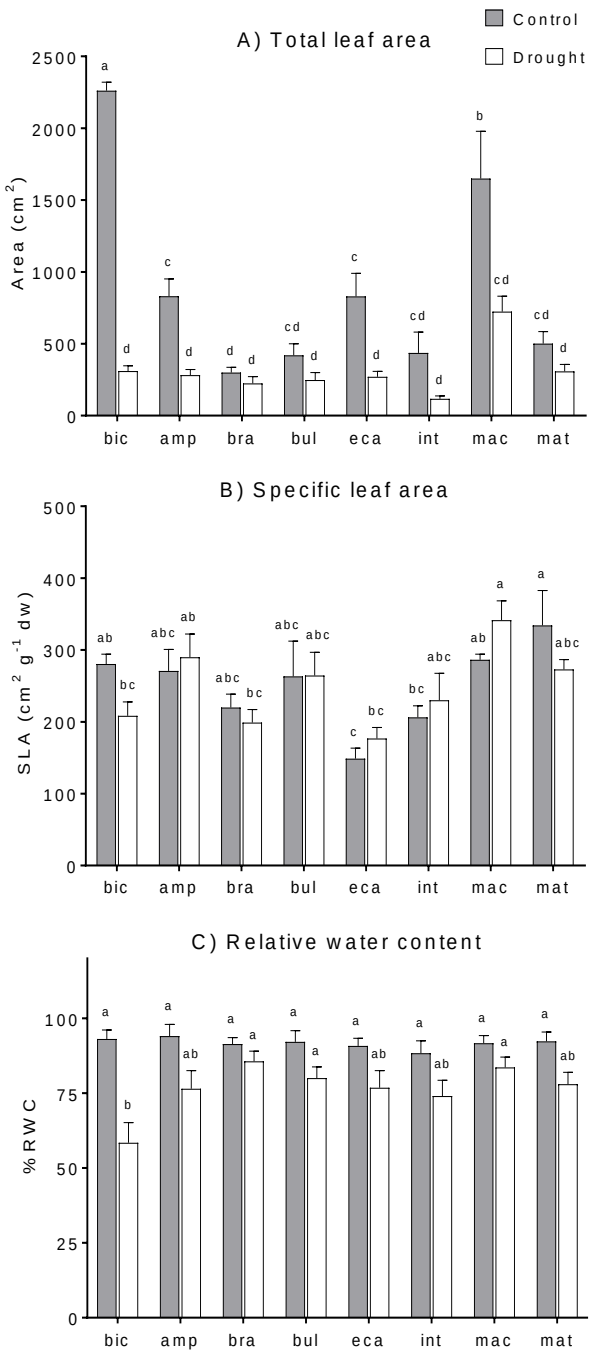

Figure 4.2: Leaf characteristics of 8-week-old *S. bicolor* (bic), *S. amplum* (amp), *S. brachypodum* (bra), *S. bulbosum* (bul), *S. ecarinatum* (eca), *S. intrans* (int), *S. macrospermum* (mac) and *S. matarankense* (mat) plants under well-watered (control) and water-limited (drought) conditions. A) total leaf area (TLA); B) specific leaf area (SLA); C) relative water content (%RWC). Data for well-watered plants are shown in grey columns and drought-stressed plants in white. Graphs show mean ± 1 standard error (n = 10; for %RWC, n = 6). Columns with different letters are significantly different ($p < 0.05$).

fully expanded leaf across all plants was 91%, with no significant difference between species ($p > 0.05$) (**Fig. 4.2C**). Water deficit had no significant impact on the %RWC in any of the wild species, but there was a significant decrease in %RWC in *S. bicolor* (control: 93.1%, drought: 58.5%; 37% decrease; $p < 0.05$).

The wild species differed morphologically and architecturally from *S. bicolor*, generally exhibiting a less upright growth habit and producing a large number of narrower leaves (**Fig. 4.3**). *Sorghum bicolor* had the fewest leaves of all species in both treatment groups (10.0 ± 0.1 under well-watered conditions, 6.5 ± 0.3 under water-limited conditions) (**Table 4.2**). Leaf number was much higher in the wild species due to the presence of side tillers, with only one *S. bicolor* individual growing a side tiller (**Table 4.2**). All wild species showed a similar number of tillers under water-limited conditions. *Sorghum bicolor* was significantly taller than all the wild species under well-watered conditions (47.2 ± 3.0 cm, $p < 0.05$), but subject to the sharpest decrease under drought (21.2 ± 0.7 cm, 55% decrease; $p < 0.05$) (**Table 4.2**). Though the wild species were generally shorter under stress, the difference was significant only in *S. macrospermum* (20.6 ± 1.0 cm, 36% decrease; $p < 0.05$). Height among all drought-stressed plants was similar ($p > 0.05$).

4.4.2 Photosynthetic changes in response to drought

The quantum yield of PSII (chlorophyll fluorescence F_V/F_m ratio), and carbon assimilation rate (photosynthetic rate), were measured in the first fully-developed leaf in all species (**Fig. 4.4**). F_V/F_m values ranged from 0.78 – 0.81 in all species under well-watered conditions. F_V/F_m decreased by 29% in *S. bicolor* (0.57 ± 0.02; $p < 0.05$) and by 14% in *S. brachypodum* (0.69 ± 0.02, $p < 0.05$) under water-limited conditions (**Fig. 4.4A**). F_V/F_m values in the six remaining wild species were not significantly different from each other ($p > 0.05$).

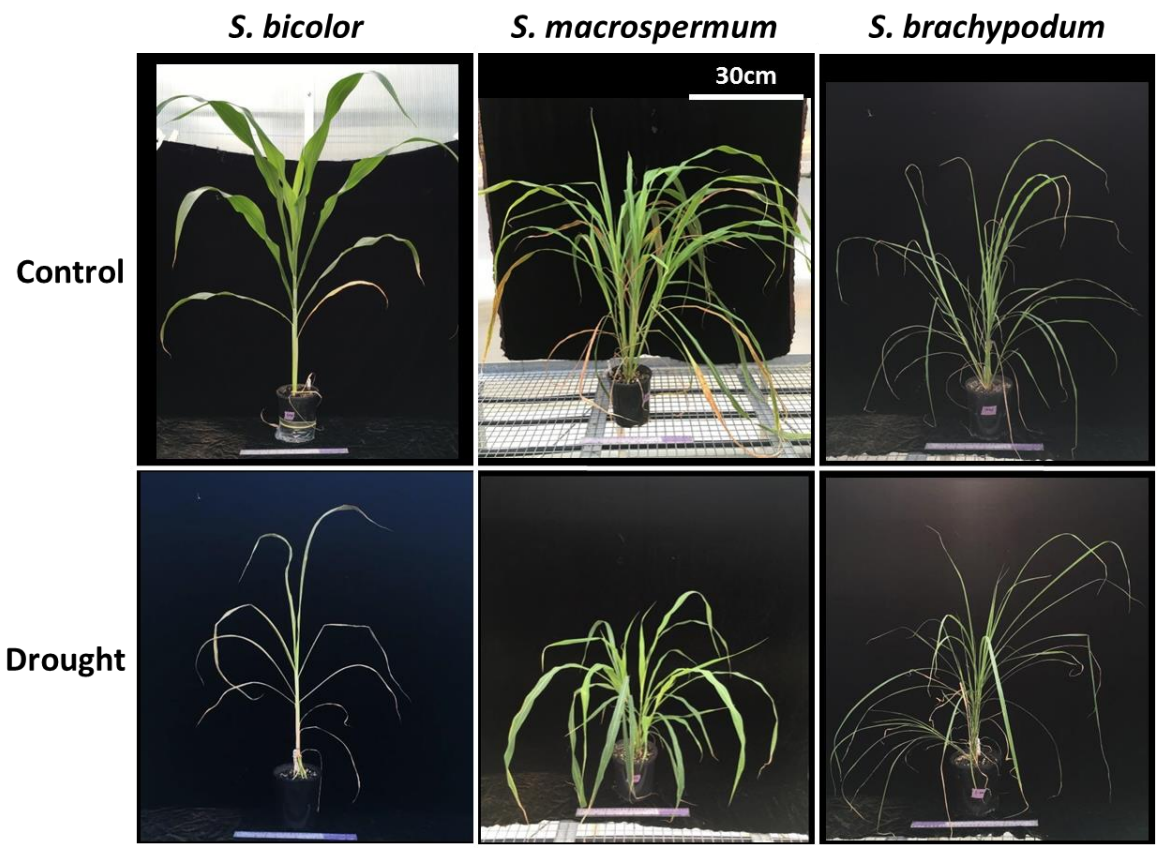

Figure 4.3: Individual 8-week-old *S. bicolor*, *S. macrospermum* and *S. brachypodum* plants grown under well-watered (Control) and water-limited (Drought) conditions (six week duration). Water-limited *S. bicolor* plants were generally much smaller than well-watered plants with severely wilted leaves. *Sorghum macrospermum* and *S. brachypodum* were less affected by drought, showing a smaller reduction in size and biomass, and only slightly wilted leaves.

Under well-watered conditions, photosynthetic rate ranged from 11.2 mmol m^{-2} s^{-1} in *S. intrans* to 14.1 mmol m^{-2} s^{-1} in *S. amplum* (**Fig. 4.4B**). Water deficit significantly decreased photosynthetic rate in *S. bicolor* (6.6 ± 0.6 mmol m^{-2} s^{-1}, 42% decrease, $p < 0.05$) and in two wild species, *S. brachypodum* (7.6 ± 0.6 mmol m^{-2} s^{-1}, 35% decrease; $p < 0.05$) and *S. bulbosum* (7.2 ± 0.5 mmol m^{-2} s^{-1}, 39% decrease; $p < 0.05$). Though photosynthetic rate generally decreased across all remaining species under water-limited conditions, these differences were not statistically significant ($p > 0.05$).

Table 4.2: Summary of morphological measurements of *S. bicolor* and seven wild *Sorghum* species under well-watered (WW) and water-limited (DS) conditions at 8 weeks post-germination. Values are mean ± 1 standard error (n = 10). Two-way ANOVA followed by Tukey's multiple comparisons was performed for each measurement, with the interaction between genotype (G) and treatment (T) presented: *p < 0.05; **p < 0.01; ***p < 0.001; ns = not significant. Values with different letters are significantly different (p < 0.05).

	No. of leaves		**No. of side tillers**		**Height (cm)**		**No. of senescent leaves**	
	WW	**DS**	**WW**	**DS**	**WW**	**DS**	**WW**	**DS**
S. bicolor	10.0 ± 0.1^{de}	6.5 ± 0.3^{e}	0.0	0.1 ± 0.1^{c}	47.2 ± 3.0^{a}	21.2 ± 0.7^{cde}	4.2 ± 0.4^{bc}	3.4 ± 0.2^{c}
S. amplum	46.1 ± 4.2^{ab}	33.9 ± 4.7^{abc}	8.4 ± 0.8^{ab}	7.0 ± 1.6^{ab}	26.2 ± 1.5^{bcd}	19.3 ± 1.1^{cde}	4.1 ± 0.5^{bc}	4.4 ± 0.5^{bc}
S. brachypodum	26.7 ± 3.0^{bcde}	18.6 ± 3.6^{cde}	4.6 ± 0.7^{b}	4.2 ± 1.0^{b}	19.7 ± 0.9^{cde}	16.6 ± 0.5^{de}	3.9 ± 1.6^{bc}	5.5 ± 0.5^{abc}
S. bulbosum	26.0 ± 4.3^{bcde}	27.6 ± 7.1^{bcd}	4.3 ± 1.0^{b}	5.9 ± 1.7^{b}	26.4 ± 2.6^{bc}	18.3 ± 1.3^{cde}	3.4 ± 0.5^{c}	5.7 ± 1.4^{abc}
S. ecarinatum	51.9 ± 11.4^{a}	24.4 ± 2.1^{bcde}	11.3 ± 2.4^{a}	5.0 ± 0.7^{b}	25.9 ± 3.9^{bcd}	22.6 ± 1.5^{cde}	5.8 ± 0.8^{abc}	6.6 ± 1.1^{ab}
S. intrans	37.4 ± 4.5^{abc}	31.0 ± 7.3^{abcd}	7.4 ± 1.2^{ab}	6.5 ± 1.7^{ab}	22.6 ± 4.3^{cde}	15.6 ± 1.3^{e}	4.9 ± 0.4^{bc}	5.8 ± 1.6^{abc}
S. macrospermum	30.4 ± 2.5^{abcd}	27.2 ± 4.1^{bcde}	5.4 ± 0.4^{b}	4.8 ± 1.0^{b}	33.5 ± 3.2^{b}	20.6 ± 1.0^{cde}	5.0 ± 0.7^{bc}	3.9 ± 0.5^{bc}
S. matarankense	38.8 ± 6.8^{abc}	28.1 ± 3.0^{bcd}	8.1 ± 1.4^{ab}	6.6 ± 0.6^{ab}	21.4 ± 1.3^{cde}	17.8 ± 0.8^{cde}	5.3 ± 0.6^{abc}	8.6 ± 1.1^{a}
Genotype (G)	***		***		***		ns	
Treatment (T)	***		*		***		*	
G x T	ns		ns		***		ns	

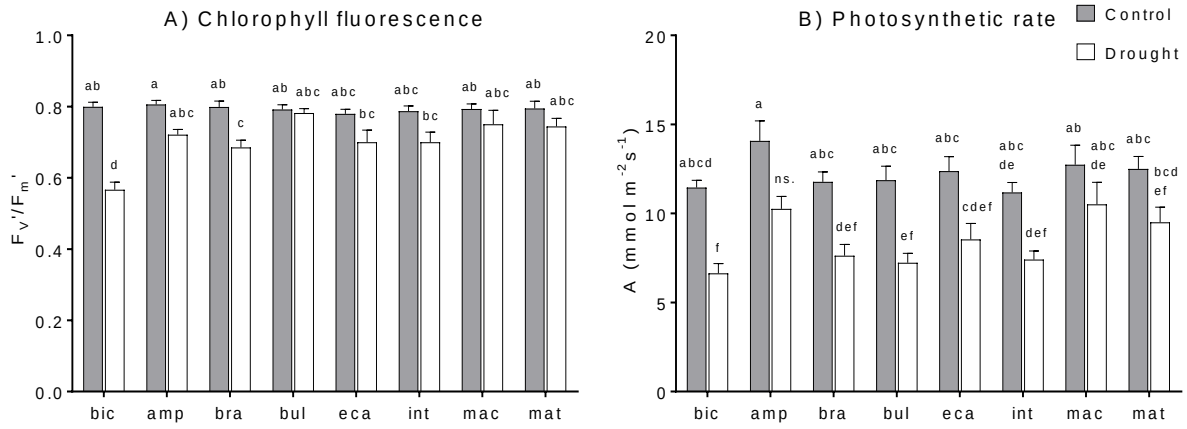

Figure 4.4: Photosynthetic parameters of the youngest fully-unfolded leaf of 8-week-old *S. bicolor* (bic), *S. amplum* (amp), *S. brachypodum* (bra), *S. bulbosum* (bul), *S. ecarinatum* (eca), *S. intrans* (int), *S. macrospermum* (mac) and *S. matarankense* (mat) plants under well-watered (control) and water-limited (drought) conditions. A) dark-adapted chlorophyll fluorescence (FV/Fm); B) carbon assimilation rate. Data for well-watered plants are shown in grey columns and drought-stressed plants in white. Graphs show mean ± 1 standard error (n = 8). Columns with different letters are significantly different ($p < 0.05$), ns. = not significantly different to any other column.

4.4.3 Hydrogen cyanide potential in wild species and changes in response to drought

The dhurrin content in leaf, sheath and root tissue harvested from well-watered and drought-stressed plants was assessed by determination of the hydrogen cyanide potential (HCNp) (**Fig. 4.5**). Among well-watered plants, leaf HCNp was highest in *S. bicolor* (0.52 ± 0.05 mg g^{-1}). Leaf HCNp was several orders of magnitude lower in all wild species, ranging from 0 to 0.002 mg g^{-1} (2 µg g^{-1}) (**Fig. 4.5A, insert**). Water deficit significantly increased leaf HCNp in *S. bicolor* (1.06 ± 0.06 mg g^{-1}, 104% increase; $p < 0.05$), but had no significant effect on HCNp in any of the wild species ($p > 0.05$). Sheath HCNp varied among the wild species under control conditions, with *S. amplum* (0.25 ± 0.05 mg g^{-1}), *S. brachypodum* (0.23 ± 0.04 mg g^{-1}), *S. bulbosum* (0.08 ± 0.02 mg g^{-1}), *S. ecarinatum* (0.12 ± 0.01 mg g^{-1}), *S. intrans* (0.23 ± 0.08 mg g^{-1}) and *S. matarankense* (0.4 ± 0.1 mg g^{-1}) exhibiting comparable levels to *S. bicolor* (0.23 ± 0.02 mg g^{-1}) ($p > 0.05$) (**Fig. 4.5B**). Water deficit significantly increased HCNp in *S. bicolor* sheaths (1.03 ± 0.03 mg g^{-1}, 348% increase; $p < 0.05$). In the wild species, sheath HCNp significantly

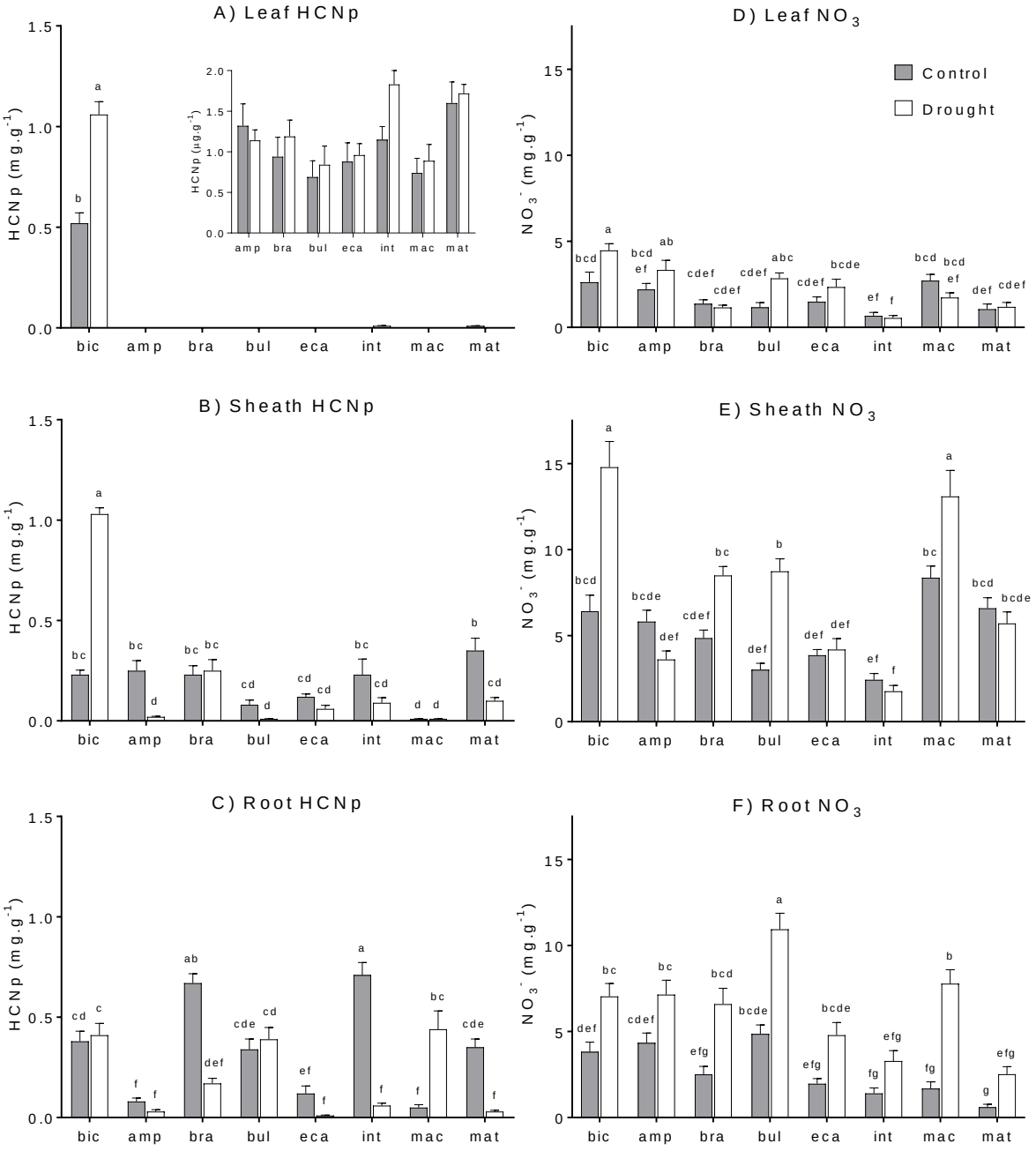

Figure 4.5: Hydrogen cyanide potential (HCNp) and total nitrate (NO3-) in dried, finely ground tissues of 8-week-old *S. bicolor* (bic), *S. amplum* (amp), *S. brachypodum* (bra), *S.* bulbosum (bul), *S. ecarinatum* (eca), *S. intrans* (int), *S. macrospermum* (mac) and *S. matarankense* (mat) plants under well-watered (control) and water-limited (drought) conditions. A) leaf HCNp shown at two different scales; B) sheath HCNp; C) root HCNp; D) leaf NO3-; E) sheath NO3-; F) root NO3-. Data for well-watered plants are shown in grey columns and drought-stressed plants in white. Graphs show mean ± 1 standard error (n = 8). Columns with different letters are significantly different ($p < 0.05$).

decreased in *S. amplum* (92% decrease; $p < 0.05$) and *S. matarankense* (71% decrease; $p < 0.05$) under water-limited conditions. There was no change in the sheath HCNp of drought-stressed *S. macrospermum* and *S. brachypodum* compared to well-watered plants ($p > 0.05$). *Sorghum macrospermum* also had very low sheath HCNp in both treatment groups (control: 0.01 ± 0.01 mg g^{-1}; drought: 0.01 ± 0.01 mg g^{-1}; $p > 0.05$). There was greater variation in the HCNp of roots, both across species and in response to low water availability. Under well-watered conditions, the highest root HCNp was observed in *S. intrans* (0.7 ± 0.1 mg g^{-1}) and *S. brachypodum* (0.7 ± 0.1 mg g^{-1}) (**Fig. 4.5C**). Root HCNp was unchanged between treatments in *S. bicolor*, *S. amplum* and *S. bulbosum*. Three wild species showed a significant decrease in root HCNp under water-limited conditions – *S. brachypodum* (0.17 ± 0.03 mg g^{-1}, 75% decrease; $p < 0.05$), *S. intrans* (0.06 ± 0.01 mg g^{-1}, 92% decrease; $p < 0.05$) and *S. matarankense* (0.03 ± 0.01 mg g^{-1}, 91% decrease; $p < 0.05$) – while *S. macrospermum* increased by more than an order of magnitude, from 0.05 ± 0.01 mg g^{-1} under well-watered conditions to 0.4 ± 0.1 mg g^{-1} under drought ($p < 0.05$).

4.4.4 Nitrate concentration in wild species and changes in response to drought

Total nitrate concentration was determined in leaf, sheath and root tissue harvested from well-watered and drought-stressed plants (**Fig. 4.5D-F**). Leaf NO_3^- concentration did not vary significantly among well-watered plants, ranging from 0.7 ± 0.2 mg g^{-1} in *S. intrans* to 2.7 ± 0.4 in *S. macrospermum* (**Fig. 4.5D**). Water deficit significantly increased leaf NO_3^- in *S. bicolor* (4.5 ± 0.4 mg g^{-1}, 70% increase; $p < 0.05$), but did not have a significant effect on NO_3^- levels in any of the wild species ($p > 0.05$). *Sorghum macrospermum* had the highest sheath NO_3^- concentration under well-watered conditions (8.4 ± 0.7 mg g^{-1}), though it was not significantly higher than *S. bicolor*, *S. amplum* or *S. matarankense* ($p > 0.05$) (**Fig. 4.5E**). Three species showed a significant increase in sheath NO_3^- under drought: *S. bicolor* (14.8 ± 1.5 mg g^{-1}, 130% increase; $p < 0.05$), *S. bulbosum* (8.7 ± 0.7 mg g^{-1}, 189% increase; $p < 0.05$) and *S. macrospermum* (13.1 ± 1.5 mg g^{-1}, 56% increase; $p < 0.05$). There was no significant change in NO_3^- concentration under water limitation in the remaining species ($p > 0.05$). In the roots, water deficit significantly increased NO_3^- in four species, *S. bicolor* (7.0 ± 0.8 mg g^{-1}, 84% increase; $p < 0.05$), *S. brachypodum* (6.6 ± 0.9 mg g^{-1}, 163% increase; $p < 0.05$), *S. bulbosum* (10.9 ± 0.9, 125% increase; $p < 0.05$) and *S. macrospermum* (7.8 ± 0.8 mg g^{-1},

360% increase; $p < 0.05$) (**Fig. 4.5F**). Root NO_3^- concentration in *S. bulbosum* was significantly higher than in all other species under water-limited conditions ($p < 0.05$).

4.4.5 Plant composition: tissue dependent allocation of nitrogen to dhurrin and nitrate

Total elemental nitrogen (N%), the proportion of nitrogen allocated to dhurrin and nitrate, and the ratio of carbon to nitrogen were determined for leaf, sheath and root tissue harvested from well-watered and drought-stressed plants. Water deficit did not have a significant effect on leaf N%, sheath N% or root N% within any species, though the response varied when comparing species (**Table 4.3**). Leaf and root N% differed significantly comparing species within each treatment ($p < 0.05$), with *S. bicolor* (control: 3.4 ± 0.2%, drought: 3.2 ± 0.2%) and *S. macrospermum* (control: 3.1 ± 0.4%, drought: 3.2 ± 0.1%) showing the highest leaf N% under both treatments. Sheath N% and specific leaf nitrogen (SLN) did not differ significantly between any of the treatment groups ($p > 0.05$). The ratio of carbon to nitrogen (C:N) in the leaf and sheath was not significantly affected by water deficit in any species ($p > 0.05$) (**Table 4.4**). The root C:N ratio varied between species but there was only a significant difference (decrease) observed in *S. amplum* in response to drought.

There were significant differences in the allocation of elemental nitrogen (N) to dhurrin and NO_3^- in each tissue type across the different treatment groups (**Fig. 4.6**). N allocation to NO_3^- was generally much higher than to dhurrin in each tissue type, consistent with the observed high nitrate levels. In *S. bicolor* leaves, the proportion of N allocated to dhurrin was significantly increased under water-limited conditions (2.9 – 6.3%, 117% increase; $p < 0.05$), while in the wild species the change to this parameter was negligible (**Fig. 4.6A inset**). Allocation of N to NO_3^- varied between species under water deficit, higher in the leaves of *S. bicolor* and four wild species (**Fig. 4.6D**) and lower in *S. brachypodum* (19% lower) and *S. macrospermum* (35% lower), though these differences were not statistically significant ($p > 0.05$). N allocation to dhurrin in the sheath was significantly increased in drought-stressed *S. bicolor* plants (2.3 – 7.3%, 215% increase; $p < 0.05$), with varied responses but no significant differences between treatments in any of the wild species (**Fig. 4.6B**). Allocation to NO_3^- in the sheath generally increased under water limitation, and was significantly higher in stressed

S. bicolor and *S. bulbosum* plants ($p < 0.05$) (**Fig. 4.6E**). In the roots, N allocation to dhurrin decreased, except for allocation in *S. macrospermum*, but these changes were not statistically significant (**Fig. 4.6C**). Allocation of N to NO_3^- under water-limited conditions did not change significantly ($p > 0.05$) though there was a trend to increased NO_3^- under drought (**Fig. 4.6F**).

4.4.6 Principal component analysis of all traits

A principal component analysis (PCA), incorporating all growth, morphological, physiological and chemical data, clearly differentiated *S. bicolor* from all wild *Sorghum* relatives grown under well-watered and water-limited conditions (**Fig. 4.7**). There was also a distinct treatment difference in *S. bicolor*. By contrast, the effect of watering regime on the wild species was less evident.

Table 4.3: Total elemental nitrogen (%) of leaf, sheath and root tissue and specific leaf nitrogen (SLN) of *S. bicolor* and seven wild *Sorghum* species under well-watered (WW) and water-limited (DS) conditions at 8 weeks post-germination. Values are mean ± 1 standard error (n = 5). Two-way ANOVA followed by Tukey's multiple comparisons was performed for each tissue type, with the interaction between genotype (G) and treatment (T) presented: *p < 0.05; **p < 0.01; ***p < 0.001; ns = not significant. Values with different letters are significantly different (p < 0.05).

	Leaf N (%)		**Sheath N (%)**		**Root N (%)**		**SLN**	
	WW	**DS**	**WW**	**DS**	**WW**	**DS**	**WW**	**DS**
S. bicolor	3.4 ± 0.2^{a}	3.2 ± 0.2^{ab}	2.1 ± 0.2	2.7 ± 0.2	1.7 ± 0.1^{cd}	2.6 ± 0.3^{abc}	0.11 ± 0.01	0.14 ± 0.02
S. amplum	2.0 ± 0.1^{d}	2.5 ± 0.1^{abcd}	2.7 ± 0.2	2.8 ± 0.3	1.4 ± 0.1^{d}	2.5 ± 0.2^{abcd}	0.08 ± 0.01	0.11 ± 0.02
S. brachypodum	2.5 ± 0.2^{bcd}	2.2 ± 0.0^{d}	2.6 ± 0.4	2.2 ± 0.1	3.0 ± 0.1^{ab}	2.7 ± 0.1^{abc}	0.12 ± 0.01	0.11 ± 0.01
S. bulbosum	2.5 ± 0.1^{bcd}	2.4 ± 0.1^{bcd}	2.2 ± 0.3	2.6 ± 0.3	1.8 ± 0.1^{cd}	2.2 ± 0.3^{abcd}	0.12 ± 0.02	0.09 ± 0.01
S. ecarinatum	2.2 ± 0.4^{d}	2.4 ± 0.1^{bcd}	3.6 ± 0.7	2.9 ± 0.2	1.9 ± 0.3^{bcd}	2.5 ± 0.3^{abcd}	0.12 ± 0.02	0.15 ± 0.01
S. intrans	2.5 ± 0.1^{bcd}	2.6 ± 0.04^{abcd}	3.6 ± 0.3	2.3 ± 0.2	3.2 ± 0.1^{a}	2.2 ± 0.1^{abcd}	0.13 ± 0.01	0.15 ± 0.02
S. macrospermum	3.1 ± 0.4^{abc}	3.2 ± 0.1^{abc}	2.8 ± 0.8	3.2 ± 0.1	2.8 ± 0.5^{abc}	3.0 ± 0.3^{ab}	0.11 ± 0.01	0.10 ± 0.01
S. matarankense	2.7 ± 0.1^{abcd}	2.3 ± 0.04^{cd}	3.0 ± 0.2	2.5 ± 0.1	2.5 ± 0.2^{abcd}	2.5 ± 0.2^{abcd}	0.09 ± 0.02	0.08 ± 0.01
Genotype (G)	***		ns		***		**	
Treatment (T)	ns		ns		*		ns	
G x T	ns		ns		**		ns	

Table 4.4: Carbon to nitrogen ratios (C:N) of leaf, sheath and root tissue of *S. bicolor* and seven wild *Sorghum* species under well-watered (WW) and water-limited (DS) conditions at 8 weeks post-germination. Values are mean ± 1 standard error (n = 5). Two-way ANOVA followed by Tukey's multiple comparisons was performed for each tissue type, with the interaction between genotype (G) and treatment (T) presented: *p < 0.05; **p < 0.01; ***p < 0.001; ns = not significant. Values with different letters are significantly different (p < 0.05)

	Leaf C:N		**Sheath C:N**		**Root C:N**	
	WW	**DS**	**WW**	**DS**	**WW**	**DS**
S. bicolor	13.4 ± 0.7bc	13.7 ± 0.8bc	19.7 ± 1.7	15.4 ± 1.1	26.2 ± 1.4abc	17.2 ± 2.2bc
S. amplum	22.2 ± 2a	16.9 ± 0.6abc	15.0 ± 1.4	14.1 ± 1.5	32.6 ± 1.5a	17.5 ± 1.8bc
S. brachypodum	17.8 ± 1.1abc	19.4 ± 0.1ab	16.6 ± 2.6	17.7 ± 0.3	14.2 ± 0.7bc	14.7 ± 0.7bc
S. bulbosum	17.4 ± 0.6abc	18.1 ± 0.3abc	20.1 ± 3.1	16.1 ± 2.2	25.1 ± 2.0abc	20.8 ± 3.9abc
S. ecarinatum	21.4 ± 3.5a	17.4 ± 0.4abc	13.4 ± 3.1	14.0 ± 1.0	27.0 ± 5.6ab	18.0 ± 2.0bc
S. intrans	17.0 ± 0.8abc	16.7 ± 0.2abc	10.9 ± 0.9	17.9 ± 1.5	13.2 ± 0.3c	19.5 ± 0.9abc
S. macrospermum	15.0 ± 1.9bc	13.2 ± 0.5c	19.0 ± 4.0	12.5 ± 0.5	18.1 ± 5.2bc	13.2 ± 2.4c
S. matarankense	16.5 ± 0.5abc	18.6 ± 0.3abc	13.5 ± 1.2	16.0 ± 0.6	17.3 ± 1.5bc	17.4 ± 1.4bc
Genotype (G)	***		ns		***	
Treatment (T)	ns		ns		***	
G x T	*		*		**	

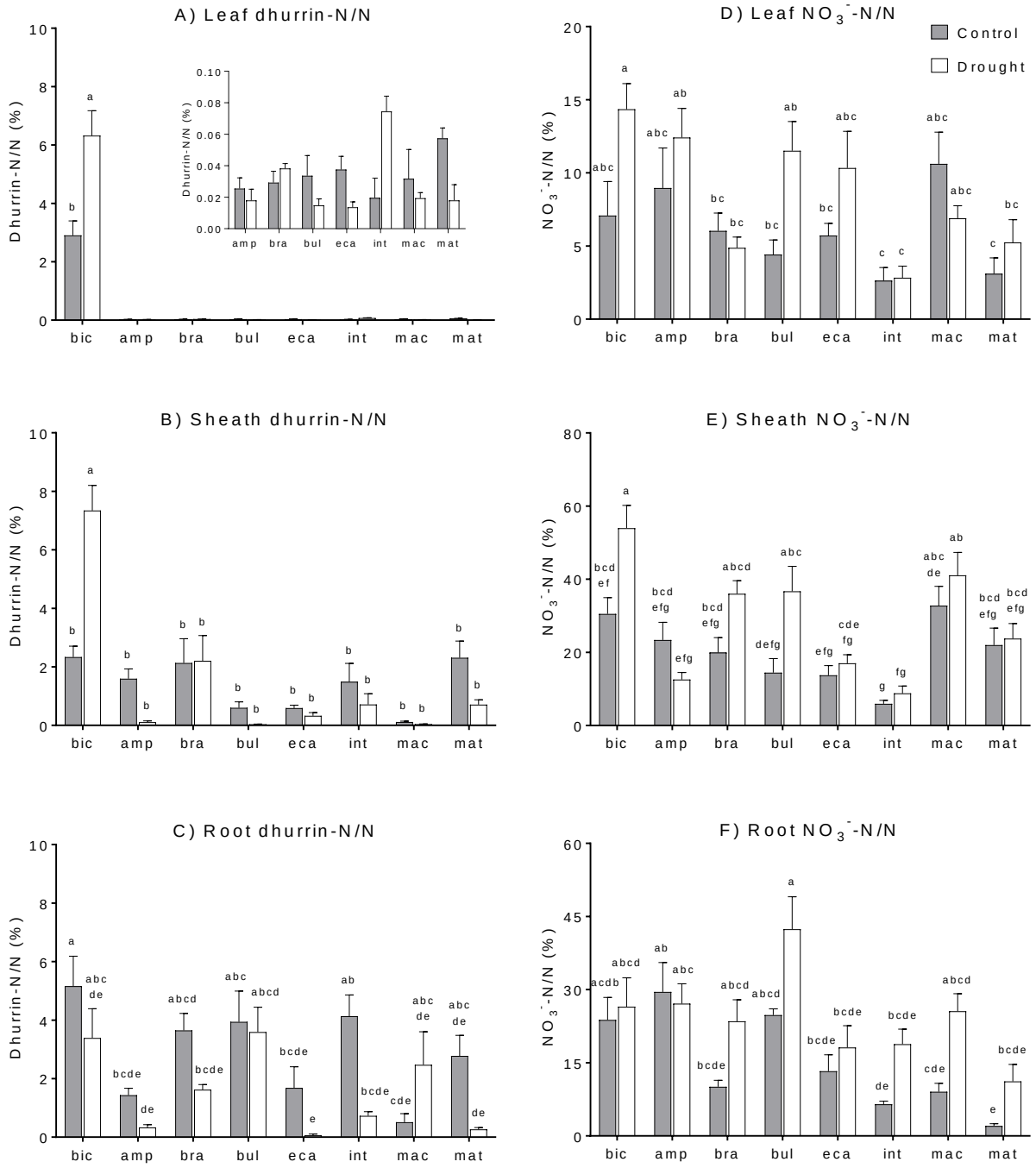

Figure 4.6: Proportion of nitrogen allocated to dhurrin and nitrate in dried, finely ground tissues of 8-week-old *S. bicolor* (bic), *S. amplum* (amp), *S. brachypodum* (bra), *S. bulbosum* (bul), *S. ecarinatum* (eca), *S. intrans* (int), *S. macrospermum* (mac) and *S. matarankense* (mat) plants under well-watered (control) and water-limited (drought) conditions. A) leaf dhurrin allocation; B) sheath dhurrin allocation; C) root dhurrin allocation; D) leaf NO3- allocation; E) sheath NO3- allocation; F) root NO3- allocation. Data for well-watered plants are shown in grey columns and drought-stressed plants in white. Graphs show mean ± 1 standard error (n = 5). Columns with different letters are significantly different ($p < 0.05$).

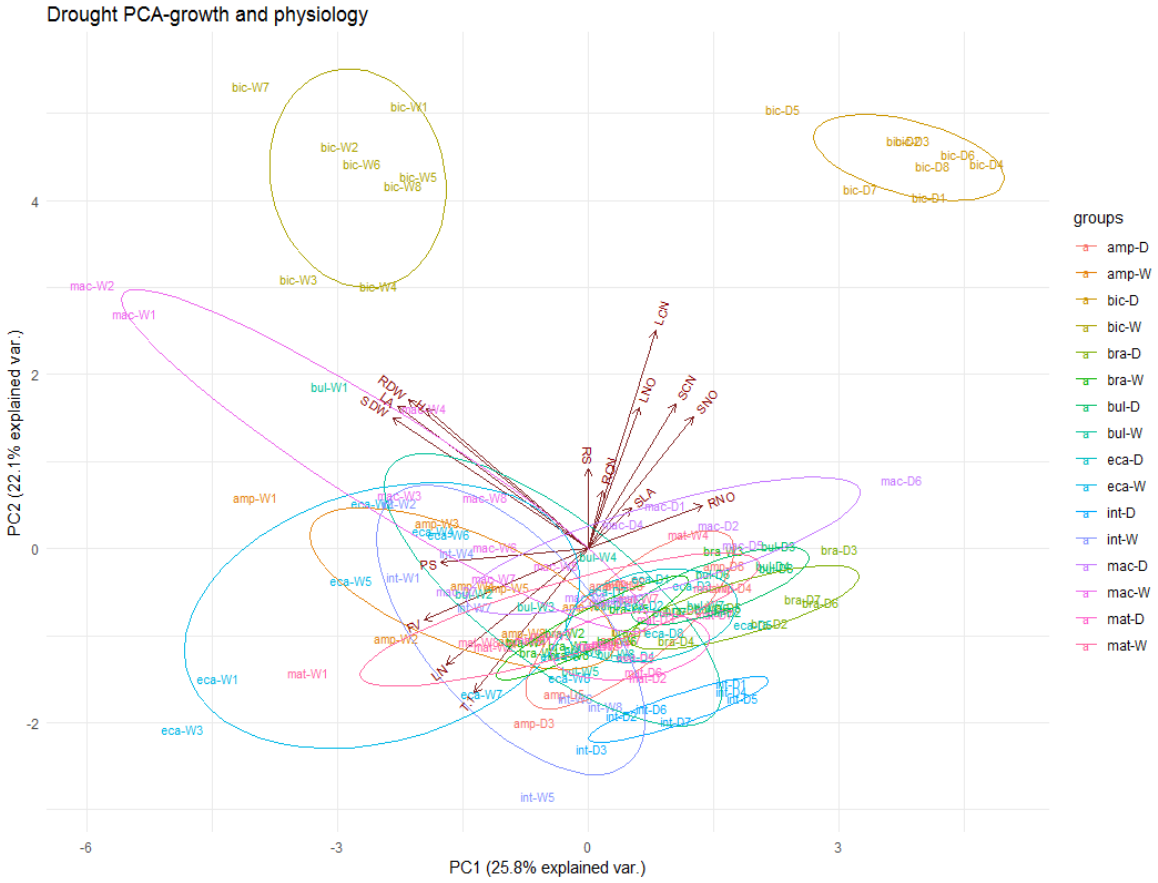

Figure 4.7: Principal component analysis (PCA) of growth and physiological measurements of *S. bicolor* and seven wild *Sorghum* species, *S. amplum* (amp), *S. brachypodum* (bra), *S. bulbosum* (bul), *S. ecarinatum* (eca), *S. intrans* (int), *S. macrospermum* (mac) and *S. matarankense* (mat), under well-watered (W) and water-limited (D) conditions. The first two principal components explain 47.9% of the total variance. Key: RDW=root DW, SDW = shoot DW, LA = total leaf area, PS = assimilation rate, Fv = Fv/Fm, H = plant height, LN = no. of leaves, T.1 = no. of side tillers, RS = root: shoot ratio, SLA = specific leaf area, L/S/RCN = leaf/sheath/root cyanide potential, L/S/RNO = leaf/sheath/root nitrate concentration.

4.5 Discussion

In this study, growth, morphological, physiological and biochemical characteristics of seven wild species of *Sorghum* were assessed and compared to *S. bicolor* under well-watered and severe drought conditions. Several wild species were discovered to perform better in terms of growth, including biomass, leaf area, height, and RGR, than *S. bicolor* under stress. The chemical composition (HCNp, NO_3^- and total N) of the wild relatives was also affected differently under drought, and to different degrees in different species.

4.5.1 Wild Sorghum species show fewer signs of stress and better growth under water-limited conditions

The investigations of growth, biomass and physiological properties demonstrated that several of the wild species were less affected by severe drought than cultivated *S. bicolor*. This is consistent with the northern Australian native range of the seven wild species. In this environment, the species generally germinate during the rainy season and continue to grow after the shift to the dry season, in which soil water availability can become extremely limited (Mott *et al.*, 1976; Lazarides *et al.*, 1991). Two wild species in particular stood out: *S. brachypodum,* which when subjected to drought conditions maintained its biomass, growth rate, leaf area and %RWC; and *S. macrospermum*, which despite having reduced biomass and leaf area under stress maintained its growth rate, photosynthetic rate and %RWC. Additionally, morphological characteristics such as tillering, common in the wild species, may result in improved overall growth and lead to higher yields and biomass in favourable growing seasons.

Sorghum bicolor grew taller and produced more biomass than the wild species under well-watered conditions, but showed the most pronounced decrease in shoot and root biomass and total leaf area when subjected to drought. Under drought, *S. macrospermum* shoot biomass was larger than *S. bicolor*, and had the highest leaf area. Biomass for three of the seven wild species was essentially unaffected by low water availability, with *S. brachypodum* being particularly noteworthy for its ability to continue to grow under water-limited conditions. Roots often experience the initial abiotic stress from water deficit and are an important parameter determining drought

tolerance (Passioura, 2007). Here, root:shoot ratio showed little change in *S. bicolor*, consistent with results reported by O'Donnell *et al.* (2013) for a different variety. *Sorghum macrospermum* was also able to continue growing under water-limited conditions, although RGR was lower than *S. brachypodum* and specific leaf area (SLA) Growth indices such as relative growth rate (RGR) and specific leaf area (SLA) actually increased, suggesting that both species are quite tolerant of this level of drought.

Physiological measurements, including the maximum potential quantum efficiency of PSII (F_V/F_m), photosynthetic rate and relative water content (%RWC) revealed that *S. bicolor* was the least tolerant to severe drought among all species tested. F_V/F_m is an indicator of plant health and stress resistance, with a value of 0.8 indicative of a healthy plant (Kautz *et al.*, 2014). Here, F_V/F_m decreased to a low of 0.56 in drought-stress*ed S. bicolor*, significantly lower than F_V/F_m in the next lowest species under stress, *S. brachypodum*. Relative water content is a measure of the degree of stress caused by withholding water – a plant that can maintain more turgid leaves under stress would be able to maintain growth more efficiently, as growth is turgor-dependent (Smart and Bingham, 1974). Here, %RWC was very similar among all species under well-watered conditions, with the largest reduction in drought-stressed plants again seen in *S. bicolor*. This suggests that the wild species, most notably *S. brachypodum* and *S. macrospermum*, are able to maintain more turgid leaves and therefore more efficient growth (supported by the RGR results) compared to *S. bicolor* under severe water stress.

4.5.2 Hydrogen cyanide, nitrate and total nitrogen vary in concentration among Sorghum species and in response to water limitation

If cyanogenic glucosides and the cyanogenesis pathway evolved as an anti-herbivore defence mechanism, it is reasonable to expect that the leaves - being the most vulnerable part of the plant - would be the most heavily defended (McKey, 1974; Gleadow and Woodrow, 2002a). This is not the case in the wild *Sorghum* accessions tested. Leaf hydrogen cyanide potential (HCNp) was up to 1,000-fold lower in the tested wild *Sorghum* species compared to their cultivated counterpart. Sheath HCNp, by contrast, was comparable between *S. bicolor* and six of the seven wild species under well-watered conditions.

A major and surprising difference between the wild and cultivated species was observed in the response to water deficit. Prior to this study, it was known that *S. bicolor* plants with limited access to water had elevated HCNp, frequently reaching concentrations above the 600 ppm (0.6 mg g^{-1}) and thus considered unsafe for livestock feed (Gray *et al.*, 1968; Wheeler *et al.*, 1990; Blomstedt *et al.*, 2012). The current study confirmed this, with concentrations in the aboveground (leaf and sheath) tissues of more than 1,000 ppm (1.0 mg g-1) in *S. bicolor*. Water limitation, however, had no significant effect on leaf HCNp in any of the wild species. This suggests that either the degree of stress was not enough to induce higher cyanogenic glucoside production in the wild species (supported by the growth and biomass results), or that cyanogenic glucosides are regulated differently in these species.

One wild species, *S. macrospermum*, had very low sheath HCNp in comparison to all other species under both well-watered and water-limited conditions. *Sorghum macrospermum* showed negligible levels of dhurrin in the leaf and sheath above ground tissues. Given its observed high degree of drought tolerance, this makes *S. macrospermum* a promising wild species for further investigation for beneficial agronomic traits. Low leaf and sheath HCNp combined with high drought tolerance would be ideal traits to introduce into cultivated sorghum varieties, and there may even be a link between these characteristics in *S. macrospermum*. Likewise, *S. brachypodum* maintained good growth in response to drought. While its sheath HCNp was comparable to *S. bicolor* under well-watered conditions, the plants did not become more cyanogenic as result of the drought stress. The results obtained in this study are consistent with previous reports where the concentration of nitrate has been shown to increase under drought in *S. bicolor* (Gleadow *et al.*, 2016b). None of the wild species showed a significant increase in leaf nitrate concentration during water stress, and overall concentration of nitrate in shoots of the wild species did not exceed 1.5% of dry weight, a threshold considered dangerous to grazing animals (Stoltenow and Lardy, 2008).

Root HCNp of sorghum has been documented in only a small number of studies (Blomstedt *et al.*, 2012; O'Donnell *et al.*, 2013; Blomstedt *et al.*, 2018). Here, it was found to be highly variable within and between species. Under well-watered conditions, *S. brachypodum* and *S. intrans* both had higher HCNp in their roots compared to *S. bicolor*. Unlike in the leaves and sheath, drought did not have a significant effect on root HCNp

in *S. bicolor*. It did, however, significantly decrease HCNp in *S. brachypodum* and *S. intrans*, with quite low levels under drought. These results again suggest that cyanogenic glucosides possess other roles in addition to herbivore defence. In *S. macrospermum*, water stress significantly increased root HCNp to levels similar to those in *S. bicolor* plants. The roots were the only plant tissue in *S. macrospermum* that contained cyanogenic glucosides at a comparable capacity, and essentially only under water-limited conditions. It was also the only species in which root HCNp increased under water deficit. From a forage crop improvement perspective, HCNp in the roots is not necessarily a problem. In fact, these results raise an interesting question – why are cyanogenic glucosides present in the roots? It may suggest again that cyanogenic glucosides have other roles outside defence, although root herbivory may be a factor (Gange and Brown, 1989; Curto *et al.*, 2012; Djian-Caporalino *et al.*, 2019).

Positive characteristics of *S. macrospermum* and *S. brachypodum*, in relation to increased drought tolerance and a lack of increased production of dhurrin when grown under water-limiting conditions, were recorded in 8-week-old plants. In future studies, this will be extended to assess their post-flowering drought tolerance, altered regulation of dhurrin biosynthesis and grain and forage yield at maturity. The molecular basis for improved characteristics of the two wild *Sorghum* species in comparison to cultivated *S. bicolor* will be investigated with a focus on possible changes in the epicuticular wax load, dhurrin biosynthesis and stay-green characteristics (Hayes *et al.*, 2016; Awika *et al.*, 2017; Emendack *et al.*, 2017).

The HCNp results reported in this study support the notion that cyanogenesis is more prevalent in cultivated crops compared to wild plants (Jones, 1998). This may be due to unintentional selection early in domestication, where cyanogenic species may have had higher pest resistance and lower exposure to herbivory (McKey *et al.*, 2010; Bredeson *et al.*, 2016). In some cyanogenic crops such as cassava, a need for processing to remove the cyanogenic glucosides and liberated hydrogen cyanide would also render species with a high HCNp less subject to theft by trespassers and thus better suited for cultivation (Jones, 1998; Nzwalo and Cliff, 2011). Results presented here support the hypothesis that cyanogenic glucosides may play additional, non-defensive roles in plant metabolism (Møller, 2010a; Gleadow and Møller, 2014; Burke *et al.*, 2015; Pičmanová *et al.*, 2015; Bjarnholt *et al.*, 2018; Blomstedt *et al.*, 2018). A greater understanding of

cyanogenic glucoside regulation by abiotic stress could provide a more detailed interpretation of the results of this study. Currently, the specific mechanism of dhurrin regulation by drought and other abiotic factors is unclear (Gleadow and Møller, 2014). In almond (*Prunus dulcis*), a bHLH transcription factor controls the transcription of the P450 monooxygenase-encoding genes *PdCYP79D16* and *PdCYP71AN24* involved in the biosynthesis of the cyanogenic glucoside amygdalin (Sánchez-Pérez *et al.*, 2019). In cassava (*Manihot esculenta*), the transcripts of the orthologous genes *CYP79D1*, *CYP79D2*, *CYP71E7/E11* involved in the biosynthesis of the cyanogenic glucosides linamarin and lotaustralin show strong diurnal regulation (Schmidt *et al.*, 2018). Studies in young *S. bicolor* plants have shown that biosynthesis of dhurrin correlates well with transcript levels of the two biosynthetic enzymes, CYP79A1 and CYP71E1 (Busk and Møller, 2002). The differences observed in the content and tissue distribution of dhurrin in the wild species reported in the current study are thus likely to reflect regulatory differences at the transcriptional level.

4.5.3 Implications for adaptive plasticity of cyanogenic glucosides

The question remains: why is the pattern of cyanogenic glucoside deployment so distinct in wild and domesticated *Sorghum*. The answer could be in the very different selective pressures of natural and cultivated environments (**Fig. 4.8**). In natural systems, plants need to adapt to variable conditions in order to maximise survival and ensure reproductive output over their lifetime. In more nutrient-poor environments, such as those characterising the native ranges of these wild *Sorghum* species (Lazarides *et al.*, 1991; Dillon *et al.*, 2007b), it would make sense that cyanogenic glucosides are concentrated in the roots; if there is a period of drought, the shoots may die off, but the roots would have a ready store of reduced nitrogen in the form of dhurrin to promote growth when conditions improve. Dhurrin in roots may also protect the roots by serving as a deterrent to belowground herbivores (Curto *et al.*, 2012; Djian-Caporalino *et al.*, 2019). In cultivated environments, by contrast, humans have selected for maximum yield under relatively stable, controlled conditions. There may be less of a requirement for the storage of additional nitrogen in roots of crop plants, as

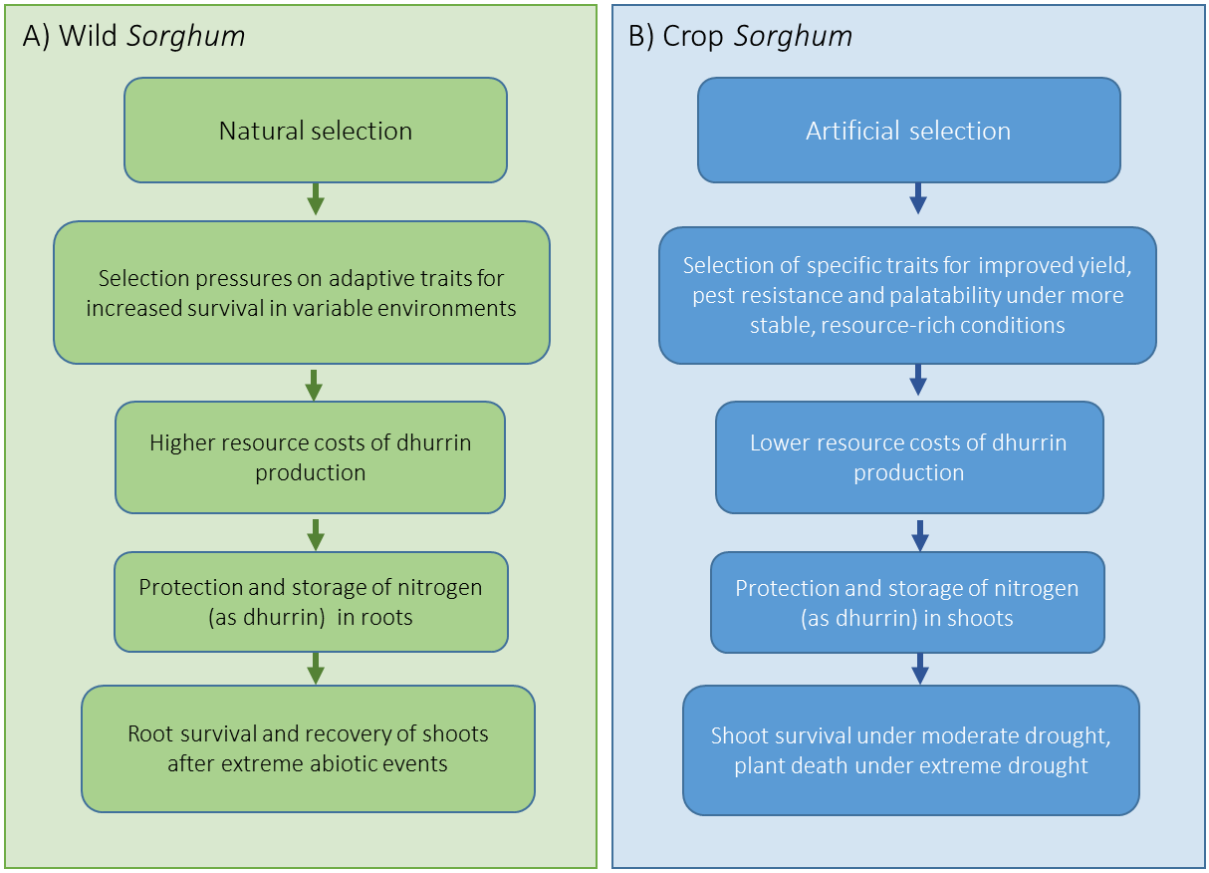

Figure 4.8: Possible underlying mechanisms driving the variable cyanogenic patterns between wild and domesticated *Sorghum* species in response to drought. A) In the wild species, dhurrin may be concentrated in the roots to maximise survival under more variable, harsh environmental conditions. B) In cultivated *S. bicolor*, dhurrin potentially accumulates in shoots as a deterrent to insect pests, and with a more stable available nitrogen pool in soil the costs of dhurrin production are lower. Consequently, plant shoots can survive moderate water limitation to still produce yields, but are vulnerable to more extreme instances of abiotic stress.

there is likely an adaptive advantage for protecting shoots from pests and disease. To help resolve this question, it would be important to investigate in future experiments how undomesticated and domesticated *Sorghum* species respond to different concentrations of available soil nitrogen in terms of growth and cyanogenic traits.

4.5.4 Conclusion

This is the first study to assess the impacts of drought stress on wild *Sorghum* species in comparison to the cultivated *S. bicolor*. We demonstrate that the wild species offer untapped genetic diversity that can be exploited to improve abiotic stress tolerance in *S. bicolor*, although technical constraints still hamper the use of crop wild relatives in breeding and crop improvement efforts. *Sorghum bicolor* × *S. macrospermum* hybrids have been successfully recovered via embryo rescue and *in vitro* culture (Price *et al.*, 2005; Kuhlman *et al.*, 2008; Kuhlman *et al.*, 2010). Fortunately, the outlook is improving through advances in biotechnology, sequencing platforms and newer genetic approaches, such as the transfer of genes from cultivated to natural plants (Lemmon *et al.*, 2018; Zsögön *et al.*, 2018). It is worth noting that this study examined one accession of each species. As was already apparent in this study, the natural variation would be expected to be greater across different populations due to the influence of variation in the environment from which they were sourced. Further experiments assessing multiple populations of individual species under natural drought conditions should be initiated to reveal the variability ranges in different natural environments. Nevertheless, *S. brachypodum* was the species least affected by water deficit according to growth and physiological measures and its HCNp was generally low across all plant tissues, even under stress. *Sorghum macrospermum* also stands out with its higher tolerance to drought compared to *S. bicolor,* its negligible aboveground HCNp and its closer phylogenetic and morphological relation to *S. bicolor*. A complete removal of cyanogenic glucosides may cause unintended side effects in *S. bicolor* due to its likely additional roles outside defence, although a reduction under stress would be ideal. Improved drought tolerance may translate to a lower, more stable hydrogen cyanide potential in *S. bicolor*.

Chapter 5 – Final discussion and conclusions

Cyanogenesis has long been regarded as an effective chemical defence system in plants. However, it is becoming increasingly evident that the nitrogenous cyanogenic glucosides have acquired additional physiological functions in general plant metabolism (Møller, 2010b). Plants are potentially able to utilise cyanogenic glucosides as a source of reduced nitrogen, capable of releasing free ammonia without the release of hydrogen cyanide (Pičmanová *et al.*, 2015; Bjarnholt *et al.*, 2018). This might not only offset the resource costs of their synthesis under stable environmental conditions, but also improve plant fitness in more variable natural systems. The metabolic benefits conferred by such a role may offer another explanation for the high occurrence and accumulation of cyanogenic glucosides in crop plants (Jones, 1998). The core aim of this thesis was to explore the underlying biological mechanisms, both established (defence) and proposed (nitrogen recycling), driving the evolution of cyanogenic glucosides in plants. It used the domesticated and undomesticated species of the *Sorghum* genus as a model.

Prior to this thesis, the cyanogenic status of the 19 undomesticated *Sorghum* species was unknown. Across all experiments beginning with **Chapter 2**, the concentration of the cyanogenic glucoside dhurrin and hydrogen cyanide potential (HCNp) were found to be consistently extremely low in the leaves of all tested wild species. Leaf HCNp was low enough for these plants to be considered essentially acyanogenic using previous definitions (Gleadow *et al.*, 2003). The native Australian species in the four tertiary gene pools (*Chaetosorghum, Heterosorghum, Parasorghum* and *Stiposorghum* species) are generally adapted to low-nutrient soils (Dillon *et al.*, 2007b). It was therefore thought that cyanogenic capacity would be lower in species of wild *Sorghum* due to the predicted nitrogen costs of dhurrin biosynthesis. While this was the case, the magnitude of the observed decrease was unexpected. Analysis of sequenced genomic DNA in species representing each undomesticated subgenera revealed a high number of single nucleotide variants (SNVs) in genes encoding the cyanogenic machinery (e.g. dhurrin biosynthesis, cyanogenesis, endogenous turnover), but most of these were synonymous variants with little predicted effect on protein function. The combined phenotypic and genomic results point to a greater emphasis on regulatory mechanisms in control of dhurrin biosynthesis and cyanogenic expression. Analysis of the transcriptome in the

undomesticated *Sorghum* species would be a useful method to explore such regulation in future studies.

The experiments described in **Chapter 3** found that the two tested Australian endemic species, *S. brachypodum* and *S. macrospermum*, were capable of releasing hydrogen cyanide at a similar capacity to domesticated *Sorghum* in sheath and root tissue. Even during the earliest stages of seedling development, at the stages when dhurrin concentration is highest in *S. bicolor* (Halkier and Møller, 1989), leaf HCNp was once again negligible in these wild species. Interestingly, HCNp in the coleoptile was comparable in all species before leaf and sheath tissue had differentiated.

The high tissue-specific polymorphism of HCNp (expressed in sheaths and roots, but not in leaves) in the examined wild *Sorghum* species strongly suggests that these plants are not utilising dhurrin in its well established defensive role, at least in aboveground parts. The very low production of dhurrin in leaves of the wild species may be compensated for by other defence mechanisms in resource-limited environments. Both *S. brachypodum* and *S. macrospermum*, but not *S. bicolor*, produce long trichomes on leaves and sheaths, suggesting that these species may invest more heavily in less costly carbon-based physical structures to deter potential herbivores (Levin, 1973; Johnson, 1975; Tian *et al.*, 2012). Spines were also observed on leaf margins of *S. macrospermum* plants. *Sorghum bicolor* has been noted to form sharp silicon-based spines on younger leaf margins (Lanning *et al.*, 1958), whereas silicon accumulates in cellular compartments in older leaves (Lux *et al.*, 2002; Kumar *et al.*, 2017). As silicon plays a key physical defensive role in many grasses (Hartley and DeGabriel, 2016; Simpson *et al.*, 2017), future studies should consider comparing the accumulation of leaf silicon in undomesticated and domesticated species of *Sorghum*.

HCNp in the wild species followed the same age-dependent expression pattern as *S. bicolor* in sheaths and roots, i.e. HCNp decreased with increasing leaf number in early seedling development before stabilising after two weeks. The underlying molecular mechanisms for this remain relatively unknown. Dhurrin biosynthesis is thought to be transcriptionally regulated in *S. bicolor* (Busk and Møller, 2002). Therefore, an important next step is to investigate whether transcript levels of the biosynthetic genes (*CYP79A1, CYP71E1* and *UGT85B1*) in the different tissues are correlated with observed

HCNp and dhurrin concentration. This is now feasible given that these genes have been characterised in the undomesticated *Sorghum* species. Expression patterns in leaf tissue across various developmental stages would also be worthy of examination.

Severe environmental effects such as drought stress are thought to impact on the metabolic pathways controlling the accumulation of specialised metabolites in plants (Bohnert *et al.*, 1995; Selmar and Kleinwächter, 2013). Studies investigating the effects of drought on dhurrin accumulation in *S. bicolor* have noted differential responses, although the concentration of dhurrin typically increases under chronic water deficit (Boyd *et al.*, 1938; Duncan, 1996; O'Donnell *et al.*, 2013; Neilson *et al.*, 2015). Amongst the seven undomesticated species of *Sorghum* examined in **Chapter 4**, tissue-specific concentration of dhurrin varied substantially both within and between species in response to drought. The wild species (*S. amplum, S. brachypodum, S. bulbosum, S. ecarinatum, S. intrans, S. macrospermum* and *S. matarankense*) once again exhibited very low leaf HCNp, and drought stress did not induce higher production of dhurrin, unlike *S. bicolor*. HCNp of sheath tissue was similar across all species (including *S. bicolor)* under well-watered conditions, and increased under water deficit in *S. bicolor* but not in the wild species. Root HCNp was most variable between species in terms of drought response. HCNp in roots appeared to be regulated independently of the shoots, something that has been observed previously (Busk and Møller, 2002). Whether or not the concentrations of dhurrin observed here are high enough to be effective in mitigating stress, or act as a nitrogen store remains unknown.

Regarding general plant performance, the domesticated *S. bicolor* was the species most affected by chronic drought stress. This was unlikely to be a consequence of species differences in plant size as this was taken into account in the way the experiment was conducted. Future studies should examine the effects of water deficit on grain yield, an aspect that was beyond the scope of this experiment. In the context of alleviating toxicity in drought-stressed forage sorghum, *S. brachypodum* and *S. macrospermum* both showed a particularly promising response to chronic drought stress. While interspecific hybridisation is currently limited between *S. bicolor* and the undomesticated species (Kuhlman *et al.*, 2008), biotechnological advances will hopefully facilitate more rapid transfer of genetic material in the future.

While this thesis has advanced knowledge of cyanogenesis in wild *Sorghum*, all experiments were limited by low availability of plant material and low germination rates. This meant that multiple populations of a single species were unable to be investigated. It is possible that undomesticated *Sorghum* species will show high intraspecific variation in cyanogenesis, reflecting diverse selective pressures in different environments. Therefore, it is important that these species are both conserved in nature, and collected and maintained in herbaria and genebanks for ongoing research purposes and to provide a rich genetic resource for the future (Brozynska *et al.*, 2016; Castañeda-Álvarez *et al.*, 2016; Norton *et al.*, 2017).

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