Regulation of cyanogenesis in forage sorghum

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Abstract

The C_4 plant Sorghum combines high water use efficiency with heat tolerance making it suitable as a fodder crop in the dry tropics. However, it also produces dhurrin (Conn 1981) which leads to the release of hydrogen cyanide (HCN) when plants are damaged. The HCN potential (HCNp) of sorghum is subject to spatial and temporal regulation and levels can vary greatly between varieties (Benson *et al.* 1969; Haskins *et al.* 1987). Sorghum HCNp is also affected by environmental factors including drought, frost and soil fertility, and levels within a given variety can differ from one season to the next (Gray *et al.* 1968). Experienced farmers know that young sorghum plants, or older plants that have been subjected to drought, are often toxic to grazing cattle. Three key genes catalyse the biosynthesis of dhurrin in sorghum, two cytochrome P450s (CYP79A1 and CYP71E1) and a (UDP)-glycosyltransferase (UGT85B1). The CYP79A1, CYP71E1 and UGT85B1 enzymes, along with the redox partner NADPH-dependant cytochrome P450 reductase (CPR) (Ellis *et al.* 2009; Jensen & Møller 2010), are thought to form a metabolon to enable channelling of toxic intermediates (Nielsen *et al*. 2008). CYP79A1 is thought to be the rate limiting step in the pathway, but little more is known about the regulation of these genes.

It has been hypothesised in numerous studies that stress, in particular osmotic and nutrient related stress, play a large role in the regulation of HCNp in sorghum. However, it is not known how these factors influence the cyanogenesis pathway. The overall aim of this project was to generate new varieties of sorghum with lower cyanogenic glucoside content for safer consumption by cattle, particularly after drought. Within this larger project the specific aims of this PhD project were to (1) to determine the ontogenetic changes in the HCNp of forage sorghum, (2) to determine the effect of environmental stress on dhurrin concentration, and (3) to identify a viable low dhurrin producing sorghum line.

The variation in HCNp due to ontogenic changes was investigated which confirmed that the HCNp of sorghum is highest in seedlings and decreases with plant age. However, in sorghum the total dhurrin content increases with age. Numerous abiotic and biotic stresses (osmotic, high/low nitrogen, wounding and different hormone treatments)

were applied to the plants to monitor the effect on accumulation of dhurrin in various parts of sorghum plants. Results showed that the dhurrin concentration increased in the shoots under osmotic stress, high nitrogen (plants >6 leaves), and when treated with the hormones Methyl jasmonate, Kinetin and Abscisic acid. In general the different treatments did not induce HCNp in the roots.

The transcript level of *CYP79A1*, the rate limiting step in dhurrin biosynthesis, was analysed by quantitative RT-PCR (qPCR) and an inverse relationship was found between transcript levels and HCNp in leaf sheath tissue. The relationship between *CYP79A1* transcript levels and HCNp in the leaf blade and roots was not as defined and will require further detailed investigation. Evidence presented in this thesis suggests that the change in dhurrin concentration in sorghum may be due to a reduction in plant growth caused by environmental stresses, leading to a feedback loop that inhibits the turnover of dhurrin, rather than an increase in activity of key biosynthetic genes *per se*.

Targetted Induced Local Lesions in Genomes (TILLING) and cyanide assays identified several mutant lines with altered HCNp. Detailed characterisation of an acyanogenic line, termed *total cyanide deficient* (*tcd2*), identified the mutation as being in the *UGT85B1* gene resulting in a stop codon. The *tcd2* line is novel because it was previously thought that a mutation the *UGT85B1* gene would result in the accumulation of toxic intermediates and therefore a non viable plant. However growth studies found other than a stunted phenotype and a small delay in development, the *tcd2* line is fully functional and fertile. The *tcd2* line is now being used to further characterise the putative dhurrin turnover pathway.

The findings detailed in this thesis have wide implications for further characterisation of the molecular mechanisms that regulate dhurrin synthesis in sorghum as well as practical applications for farmers in to agricultural regions experiencing environmental stress due to climate change. The cattle industry that use forage sorghum as fodder will benefit from an understanding of the regulation of cyanogenesis in sorghum. Additionally, the information gained on the regulation of cyanogenesis will have worldwide implications as it may be transferable to other cyanogenic crops which are important food resources, such as cassava.

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Declaration and statement of authorship

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(i) The thesis comprises only my original work, except where due acknowledgement has been made in the Preface to the thesis and in the text to all other material used.

(ii) The thesis is less than 100,000 words in length, exclusive of figures, tables, references and appendices.

(iii) The thesis reflects work done during the period of candidature but may include related preliminary material provided that has not contributed to an award of a previous degree.

(iv) Research data and records collected, used and maintained in the conduct of my research will be retained and accessible for five years from the point of thesis submission unless publication, or public release of the work of research subsequently occurs, in which case the research data and records will then be retained for five years after publication, or public release, of the work of research.

Natalie O'Donnell

15 – 05 – 2012

Preface

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In chapter 3, the format of Figure 3.6 was first created by Catherine Adams in her Honours project. The figure shown in this thesis was created by original data obtained by Natalie O'Donnell.

In chapter 6 of this thesis, the generation and selection of cyanogenesis mutants was done in collaboration with Dr. Cecilia Blomstedt.

Publications:

1. Blomstedt CK, Gleadow RM**, O'Donnell N**, Naur P, Jensen K, Laursen T, Olsen CE, Stuart P, Hamill JD, Møller BL and Neale AD (2012) A combined biochemical screen and TILLING approach identifies mutations in Sorghum bicolor L. Moench resulting in acyanogenic forage production. *Plant Biotechnology Journal* **10**, 54-66.
2. Gleadow RM, Møldrup ME, **O'Donnell NH**, Stuart PN (2012) Drying and processing protocols affect the quantification of cyanogenic glucosides in forage sorghum. *Journal of the Science of Food and Agriculture.* In press
3. Fox G, Stuart P, **O'Donnell N** and Gleadow RM (2012) Estimating hydrogen cyanide in forage sorghum (*Sorghum bicolor*) by near infrared spectroscopy. In press

Conference presentations:

1. **O'Donnell N**., Blomstedt C., Møldrup M., Adams C., Stuart P., Neale, A., Hamill J., Møller B. and Gleadow R. (2008) Accurate and biological meaningful

measurements of HCN potential in sorghum. Poster Presentation, Combio, September 22nd-26th 2008, Canberra, Australia. (poster presentation)

2. **O'Donnell N.**, Blomstedt C., Stuart P., Neale, A., Hamill J., Møller B. and Gleadow R. (2009) Hormonal effects on cyanogenic glycosides in forage sorghum", Oral Presentation, Combio, 6th-10th December, Christchurch, New Zealand 10/12/09 (**oral presentation**)
3. **O'Donnell N.H.**, Blomstedt C., Stuart P., Neale, A., Hamill J., Møller B. and Gleadow R. (2010) "Environmental regulation of the cyanogenic glycoside, dhurrin, in sorghum." Ozbio, September 26th – October 1st 2010, Melbourne, Australia. (poster presentation)
4. **O'Donnell N.H.**, Blomstedt C., Stuart P., Neale, A., Hamill J., Møller B. and Gleadow R. (2011) Environmental Stress and Cyanogenesis In Forage Sorghum. Second International Conference on Plant Metabolism. June 30th – July 3rd 2011, Qingdao, China. (poster presentation)
5. **O'Donnell N.**, Blomstedt C., Stuart P., Neale, A., Hamill J., Møller B. and Gleadow R. (2011) Environmental Influences on the Cyanogenesis Pathway in Forage Sorghum. International Botanical Congress. July 24th – 30th 2011, Melbourne, Australia 25/07/11 (**oral presentation**)

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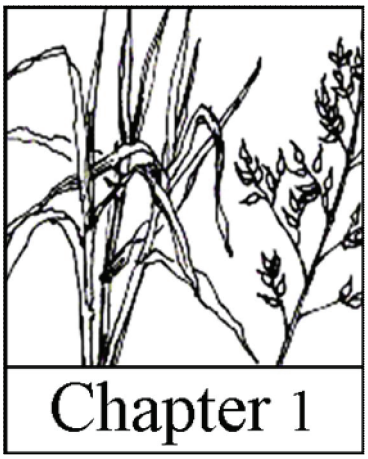
Abbreviations

aa	amino acid
ABA	abscisic acid
acdc	adult cyanide deficent
ANOVA	analysis of variance
bp	base pair
CTAB	cetyl trimethylammonium bromide
cDNA	complementary DNA
CFP	cyan fluorescent protein
CN	cyanide
CN-N/N%	cyanide-nitrogen as a proportion of total nitrogen
C:N	carbon to nitrogen ratio
CPR	cytochrome P450 reductase
d	day
DNA	deoxyribonucleic acid
dwt	dry weight
ecp	elevated cyanide potential
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methanesulfonate
EtOH	ethanol
GA	gibberellic acid
h	hour
HCN	hydrogen cyanide, hydrocyanic acid, prussic acid
HCNp	hydrogen cyanide potential
IAA	indole-3-acetic acid
K	kinetin
LA	leaf area
LAR	leaf area ratio
MeJa	methyl jasmonate
MOPS	4-Morpholinepropanesulfonic acid
MQ	Milli-Q ultrapure water

NAR	net assimilation rate
n	number of replicates
N	nitrogen (total number of nitrogen atoms measured in the plant)
N_2	nitrogen (molecule)
NO_3^-	nitrate
NO_3-N/N%	nitrate-nitrogen as a proportion of total nitrogen
p	statistical probability
PCR	polymerase chain reaction
PEG	polyethylene glycol
qPCR	quantitative (real-time) polymerase chain reaction
RGR	relative growth rate
RNA	ribonucleic acid
RNAi	RNA intereference
rpm	revolutions per minute
rtPCR	reverse transcription polymerase chain reaction
R:S	root to shoot ratio
RWC	relative water content
SA	salicylic acid
SE	standard error
SDS	sodium dodecyl sulphate
SLA	specific leaf area
TAE	Tris-acetate EDTA buffer
tcd	totally cyanide deficient
v/v	volume per volume
wt	wildtype
w/v	weight per volume
YFP	yellow fluorescent protein



Literature Review & Project Aims

1.1 Introduction

The ability to produce enough food to meet the needs of a growing world population is becoming a prominent issue (Burns *et al.* 2010). In order to meet this growth, it has been estimated that food production will need to be doubled, using half the current resources, by the year 2050 (Balcombe *et al.* 2009). There is a call for the improvement of cultural practices and crop genotypes suitable for drought-prone areas and other marginal lands. The understanding of the mechanisms behind drought resistance and the efficient use of water by the plants is fundamental for the achievement of those goals (Xin *et al.* 2008).

Sorghum [*Sorghum bicolor* (L.) Moench] is a food crop grown worldwide for grain, forage and more recently as source of biomass for biofuel. Grain sorghum is the fifth most widely-grown cereal crop in the world (www.fao.org). Sorghum grows well in arid and semi-arid regions and therefore is a desirable forage choice for farmers (Price *et al.* 2005). The high drought tolerance traits of sorghum are also a driving factor for the recent increase in sorghum research (Xin *et al.* 2008). Forage sorghum is used for grazing or to make hay and cattle feed from all parts of sorghum plants, including the grain, leaves and stem (Stuart 2002).

Many plants produce defence compounds for protection against herbivory. Cyanogenic glycosides, produced in sorghum, are such compounds. Hydrogen cyanide (HCN, otherwise known as prussic acid) is released from cyanogenic glycosides in a process known as cyanogenesis. The plants do not contain free or soluble cyanide, but have the potential to release HCN from the cyanogenic glycosides stored within certain tissues. The hydrogen cyanide potential (HCNp) of the plants is defined as the cyanide concentration of any specific tissue if all the cyanogenic glycosides present were completely hydrolysed to release all possible HCN (Gorashi *et al.* 1980). HCN can be released from both cyanogenic glycosides and cyanogenic lipids (Robinson 1930; Mikolajczak 1977). Cyanogenic lipids are less common and will not be reviewed here (see Mikolajczak 1977; Selmar *et al.* 1990; Selmar 1991).

Many studies have examined cyanogenic glycosides, however, most studies have focused on either the metabolic processes or the resulting HCNp. This review summarises the current knowledge on regulation, synthesis, mobilization and turnover of cyanogenic glycosides, in the context of using sorghum as a forage crop.

1.2 Cyanogenic glycosides

1.2.1 Occurrence of cyanogenic glycosides

Cyanogenic glucosides are an important and widespread class of defense chemicals, which have been recorded in over 2650 plant species from all major vascular plant taxa (Aikman *et al.* 1996), including *Sorghum* (*S. bicolor*), *Eucalyptus*, *Lotus*, *Cassava* (*Manihot esculenta*), flax (*Linum usitatissimum*), barley (*Hordeum vulgare*), giant bamboo (*Cathariostachys madagascariensis*), lima bean (*Phaseolus lunatus*), white clover (*Trifolium repens*), rubber tree (*Hevea brasiliensis*) and almond (*Prunus dulcis*) (Robinson 1930; Conn 1981; Cutler *et al.* 1985; Lechtenberg & Nahrstedt 1999; Møller & Seigler 1999; Pourmohseni & Ibenthal 1991; Pourmohseni *et al.* 1993 Gleadow *et al.* 2003). Cyanogenic glycosides are not only confined to the plant kingdom. A number of cyanogenic insect species; including species of butterflies and moths, and *Zygaena* (burnet moth) larvae, can not only sequester cyanide from the plants they eat, but also synthesise it *de novo* (Nahrstedt 1988; Zagrobelny *et al.* 2008). It is thought that insects

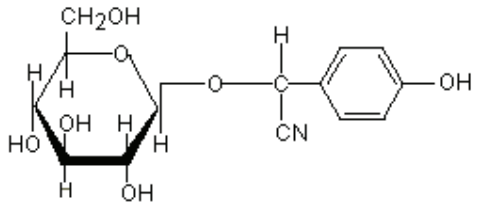
use the cyanogenic glycosides as a defence mechanism but this is not fully understood yet (Nahrstedt, 1985; Zagrobelny *et al.* 2011).

1.2.2 Biosynthesis of cyanogenic glycosides

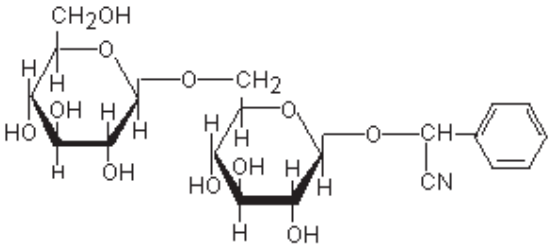
Many different cyanogenic glycosides have been identified (Conn 1969; Seigler 1975; Chassagne *et al.* 1996; Seigler *et al.* 2002). All of which are synthesised from either one of five protein amino acids (L-valine, L-isoleucine, L-leucine, L-phenylalanine, L-tyrosine) or the non-protein amino acid L-2-(20-cyclopentenyl) glycine (Zagrobelny *et al.* 2008). The diversity in chemical structures may be limited in part by the channeled nature of the biosynthetic pathway (Figure 1.1; Møller & Conn 1980). Most cyanogenic species contain a single cyanogenic glycoside. However, both, linamarin and lotaustralin, are produced within the same plant, such as *Lotus*, cassava, white clover, lima beans as well as within insect species, such as *Zygaena* (Robinson 1930; Zagrobelny *et al.* 2007). It is not understood why the two cyanogenic glycosides are coexpressed. Linamarin is the predominant cyanogenic glycoside compared to lotaustralin in cassava (Andersen *et al.* 2000), but in *L. japonicus*, lotaustralin is predominant (Forslund *et al.* 2004).

Dunstan *et al*. (1906) first identified the presence of dhurrin, the cyanogenic glycoside found in *S. bicolor* in 1901, but it was Conn (1981) that identified the structure of dhurrin (Figure 1.1a). Dhurrin was the first cyanogenic glycoside to have the entire biosynthetic pathway defined. Dhurrin is synthesised by the conversion of L-tyrosine to the stable cyanogenic glycoside (Conn 1981). The reaction is catalysed by two cytochrome P450s (CYP79A1 and CYP71E1), a soluble UDP-glucosyltransferase (UGT85B1) (Jones *et al,* 1999; Bak *et al.* 2006) is also required in conjunction with the redox partner NADPH-dependant cytochrome P450 reductase (CPR) (Ellis *et al.* 2009; Jensen & Møller 2010; Figure 1.2). It is postulated that the enzymes involved in dhurrin synthesis form a metabolon to promote channelling of toxic intermediates and to prevent metabolic cross-talk (Jørgensen *et al.* 2005b; Kristensen *et al.* 2005; Nielsen & Møller 2005; Nielsen *et al.* 2008). The metabolon is proposed to be anchored to the cytosolic surface of the endoplasmic reticulum (ER) membrane (Koch *et al.* 1995; Bak *et al.* 1998) via the N-terminal sequences of CYP79A1 and CYP71E1 (Figure 1.3; Tattersall *et al.* 2001). Single O2 molecules are required for use in the dhurrin synthesis and an

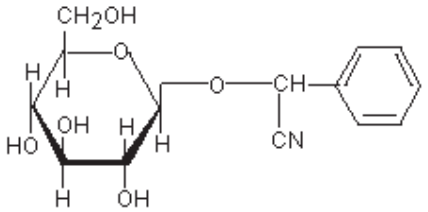
a) dhurrin



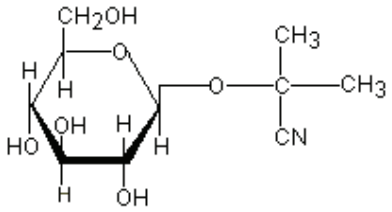
b) amygdalin



c) prunasin



d) linamarin



e) lotaustralin

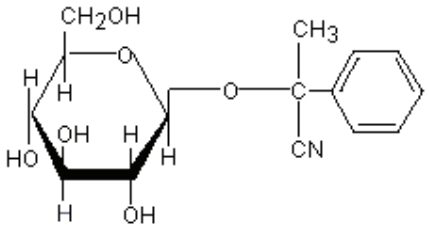


Figure 1.1 Chemical structures of five common cyanogenic glycosides. (a) dhurrin, found in sorghum, (b) amygdalin, found in almonds, (c) prunasin, found in eucalyptus, and (d & e) linamarin and lotaustralin, the two cyanogenic glycoside found in cassava, lotus, white clover, lima beans and *Zygaena*.

NADPH dependent membrane bound NADPH-cytochrome P450 oxidoreductase supplies electrons for the reducing power (Nielsen *et al.* 2008).

The CYP79A1 shows high substrate specificity to tyrosine whereas CYP71E1 has lower substrate specificity to (*Z*)-*p*-hydroxyphenyl-acetaldehyde oxime, hence CYP79A1 is considered as the rate limiting step of the pathway (Kahn *et al.* 1999). The regulation of *CYP79A1* and *CYP71E1* genes have been shown to be at the transcriptional level (Busk & Møller 2002), but little is known about the factors affecting regulation of these genes involved in dhurrin synthesis. The CYP79A1 enzyme catalyses the first step in dhurrin biosynthesis, whereby L-tyrosine is converted to (Z)-*p*-hydroxyphenylacetaldoxime (Figure 1.2; Sibbesen 1994; Halkier et al. 1995). The oxime is then converted to cyanohydrin *p*-hydroxymandelonitrile, via a dehydration and a C-hydroxylation reaction catalysed by CYP71E1 (Halkier *et al.* 1989; Kahn *et al.* 1997; Bak *et al.* 1998) and the product is stabilised by glycosylation via a soluble UDP-glucosyltransferase (UGT85B1) to produce dhurrin (Jones *et al.* 1999).

Much of the understanding of the dhurrin synthesis pathway has come from the ectopic expression of the key enzymes, CYP79A1, CYP71E1 and UGT85B1, in the usually noncyanogenic plants, *Arabidopsis thaliana* and *Nicotiana tabacum* (tobacco), to study the activity of these enzymes (Bak *et al.* 1998; Bak *et al.* 2000; Tattersall *et al.* 2001; Kristensen *et al.* 2005). The expression of CYP79A1 in *A. thaliana* alone, resulted in the accumulation of a high amount of the tyrosine-derived glucosinolate, *p*-hydroxybenzylglucosinolate. *P*-hydroxybenzylglucosinolate does not occur naturally in *A. thaliana* (Bak *et al.* 1998; Petersen *et al.* 2001; Tattersall *et al.* 2001). This diversion of the product of the CYP79A1 reaction indicates that the glucosinolate pathway enzymes have low substrate specificity (Bak *et al.* 1999). In addition, it has been suggested that the glucosinolate pathway arose from alterations in the cyanogenesis pathway (Halkier *et al.* 2002; Bak *et al.* 2006). When both CYP79A1 and CYP71E1 were expressed in tobacco and *Arabidopsis* plants, the activity of CYP79A1 was higher than that of CYP71E1. This resulted in the accumulation of several *p*-hydroxyphenylacetaldoxime-derived products as well as those derived from *p*-hydroxymandelonitrile (Bak *et al.* 2000). However, when the *CYP79A1*, *CYP71E1* and

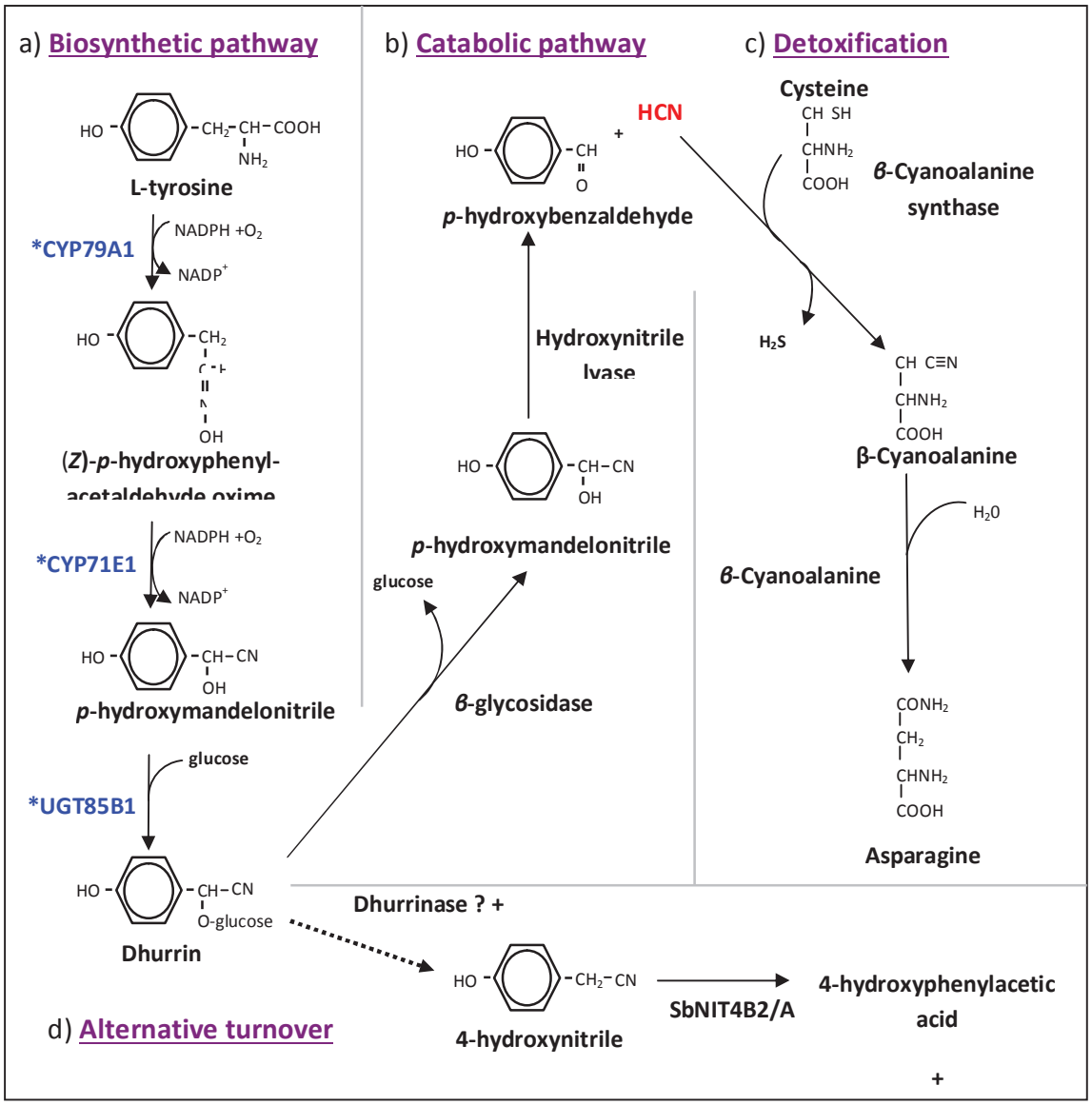


Figure 1.2 The cyanogenesis pathway in Sorghum. (a) The dhurin biosynthesis pathway, (b) the dhurrin catabolic pathway, (c) the HCN detoxification pathway, and (d) the alternative dhurrin turnover pathway, that avoids the release of HCN (Figure altered from Blomstedt, O'Donnell, *et al*. (2012).

UGT85B1 genes were all inserted there was an accumulation of 4% dry-weight of dhurrin (Kristensen *et al.* 2005).

In *S. bicolor*, the intermediates produced in the dhurrin synthesis pathway are difficult to detect and it is thought that the intermediates are toxic to the plant (Tattersall *et al.* 2001), supporting the theory that the enzymes involved in dhurrin biosynthesis are organised into a metabolon (Figure 1.3; Jørgensen *et al.* 2005b; Kristensen *et al.* 2005; Nielsen *et al.* 2008). Studies by Nielsen *et al.* (2008) on the metabolon formation following insertion of the pathway into *A. thaliana* found that when yellow or cyan fluorescent proteins (YFP or CFP, respectively) were fused to either CYP79A1, CYP71E1 or UGT875B1, dhurrin synthesis was detected *in planta.* There was no effect on the catalytic activity of the individual enzymes due to the protein fusion. However, when YFP and CFP were fused to both the cytochrome P450 enzymes, but not to UGT85B1 and co expressed in Arabidopsis, no dhurrin was produced. (Neilsen *et al*. 2008). When YFP and CFP were fused to the CYP71E1 and UGT85B1 proteins, but not to CYP79A1, dhurrin syntheses was detected at low levels (Nielsen *et al.* 2008). This suggests that the fusion of the YFP and CFP to both the cytochrome P450 proteins interfered with the enzyme interaction and substrate channeling (Nielsen *et al.* 2008).

1.2.3 Cyanogenesis

Cyanogenesis is the process by which HCN is released when cyanogenic glycosides are hydrolysis by a β-glucosidase (Figure 1.2b). The most common cause of cyanogenesis is tissue disruption by chewing, freezing and trampling (Lloyd *et al.* 1970; Conn 1991; Møller & Siegler, 1999; Goodger *et al.* 2006; Gleadow, O'Donnell *et al.* 2012). The cyanogenic glycoside, dhurrin found in sorghum, is hydrolysed when it comes into contact with the β-glucosidase, dhurrinase and an α-hydroxynitrile and a glucose are released. At pH ≥ 6 the α-hydroxynitrile spontaneously dissociates into a sugar, a keto compound and HCN, but at pH ≤ 6 a hydroxynitrile lyase converts the α-hydroxynitrile to *p*-hydroxybenzaldehyde and HCN (Figure 1.2; Conn 1981). The α-hydroxynitrile lyases appear to be located in the same tissues as the β-glucosidases specific to the degradation of cyanogenic glycosides (Jørgensen *et al.* 2005). However, the α-hydroxynitrile lyase activity appears to be confined to protein bodies (Swain *et al*. 1992)

rather than the chloroplasts or the apoplastic space as usually found for β-glucosidases (Hickel *et al.* 1996; Zagrobelny *et al.* 2008).

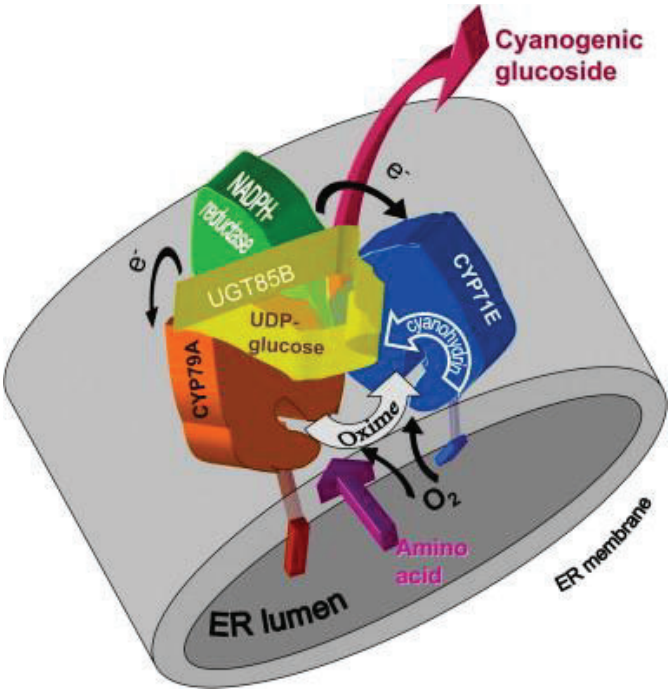


Figure 1.3 Proposed metabolon formed betweeen CYP79A1, CYP71E1 and UGT85B1 for the channeling of intermediates formed during dhurrin synthesis. The figure is from Nielsen *et al*. (2008) and represents the structure of the metabolon formed during dhurrin synthesis. Two O_2 molecules are used in the conversion of L-tyrosine to (Z)-*p*-hydroxyphenylacetaldoxime via the CYP79A1 (Nielsen *et al.* 2008). CYP71E1 then converts the (Z)-*p*-hydroxyphenylacetaldoxime to cyanohydrin *p*-hydroxymandelonitrile consuming a single O_2 molecule (Nielsen *et al.* 2008). This is then glycosylated by UGT85B1, to form dhurrin.

Plants avoid self toxicity by storing the cyanogenic glycosides and β-glucosidases separately. In the monocotyledonous sorghum plants the dhurrin is stored in the vacuole of the leaf epidermis (Saunders *et al.* 1977; Saunders & Conn 1978) whereas the enzymes involved in its degradation (dhurrinase and hydroxynitrile lyase) are localised in the mesophyll tissue (Kojima et al. 1979; Thayer & Conn 1981). The hydroxynitrile lyase is thought to be cytoplasmic and the dhurrinase mostly chloroplast-associated (Thayer & Conn 1981). In dicotyledonous plants, such as cassava, the β-glucosidases appear to be stored in the apoplast or cell walls (Kojima *et al.* 1979). β-glucosidases generally have an acidic pH optima of 5-6 and a high specificity towards a precise β-glucoside (Esen 1993). Sorghum produces two β-glucosidases, dhurrinase-1 and dhurrinase-2 (Cicek & Esen 1998). Dhurrinase-1 accumulates in the mesocotyl and the root tip, whereas dhurrinase-2 accumulates in the leaves (Thayer & Conn, 1981). However, it is currently unknown if there is any functional difference between these two enzymes.

Cyanide (CN) toxicity inhibits cytochrome C oxidase activity (Leavesley *et al.* 2008). Plants have an alternative respiratory pathway not involving cytochrome C oxidase (Lambers 1982; Millenaar & Lambers 2003). This alternative respiratory pathway is thought to be important when the cytochrome C pathway is restricted (Millenaar & Lambers 2003) and therefore the release of HCN is thought less toxic to plants than animals. HCN can be detoxified by two reactions (Conn, 1980). The first HCN detoxification pathway is present in plants and insects (Castric *et al.* 1972). This pathway involves cysteine synthase an enzyme that catalyses the reaction between cysteine and HCN, to produce β-cyanoalanine and H_2S. In plants, the β-cyanoalanine synthase activity is primarily located in mitochondria, the organelle that is most vulnerable to HCN toxicity (Meyers & Ahmad 1991; Watanabe *et al.* 2008). β-cyanoalanine is a potent neurotoxin and its accumulation in some plants may serve to deter predators (Ressler *et al.* 1969). The β-cyanoalanine is then converted to asparagine by β-cyanoalanine hydrolase (Figure 1.3c; Møller & Siegler, 1999). This pathway may also be present in insects (Zagrobelny *et al.* 2004). The second CN detoxification pathway is present in plants, insects and higher animals (Zagrobelny *et al.* 2004). This pathway involves the conversion HCN to thiocyanate by a reaction catalysed by rhodanese (Lang 1933; Bordo & Bork 2002). Unlike β-cyanoalanine synthase,

rhodanese is not universally present in plants and its *in vivo* function is not well understood (Zagrobelny *et al.* 2008). It appears that few, if any studies examining the efficiency of the two detoxification pathways in plants have been conducted.

It has been proposed that an alternative dhurrin turnover pathway exists that does not result in the release of HCN (Jenrich *et al.* 2007). This alternative turnover pathway avoids the risk of self-toxicity by converting dhurrin to asparagine and aspartic acid without releasing HCN (Figure 1.2d; Piotrowski & Volmer 2006; Jenrich *et al.* 2007; Piotrowski 2008). The first step in this pathway, the conversion of dhurrin to 4-hydroxynitrile, is yet to be defined (Jenrich *et al.* 2007; Piotrowski 2008). However, it is known that *Sb*NIT4A/B2, a nitrilase heterodimer, catalyses the conversion of 4-hydroxynitrile to ammonia and 4-hydroxyphenylacetic acid (Jenrich *et al.* 2007). Additionally, *p*-hydroxyphenyl acetic glucoside was also shown to actively degrade dhurrin in etiolated sorghum seedlings (Halkier & Møller 1989; Jenrich *et al.* 2007). This alternative pathway is of particular interest as it avoids the release of HCN and supports the theory that dhurrin serves as a nitrogen store in the plant (see section 1.2.4).

1.2.4 Function of cyanogenic glycosides

Plants are sessile organisms that require defence systems to protect themselves from attack from pathogens and herbivory (Berenbaum 1995). Cyanogenic glycosides are thought to be a defence mechanism against generalist herbivores (Poulton 1990; Gleadow & Woodrow 2002b; Zagrobelny *et al.* 2011). Despite this, there are some specialist insect species that favour cyanogenic plants. For example, the moth species *Zygaena filipendulae* sequester cyanogenic glycosides from the *Lotus* plants they ingest. However, *Zygaena* maintain a cyanogenic status no matter if the plants ingested contain cyanogenic glycosides or not (Zagrobelny *et al.* 2007). This indicates that *de novo* synthesis of cyanogenic glycosides occur in *Zygaena.* Additionally, *Zygaena* appear to use the cyanogenic glycosides in their own defence systems (Zagrobelny *et al.* 2007). The males also give a nuptial gift of cyanogenic glycosides to the female during mating and females resist mating with males with low cyanogenic glycoside contents. Hence, males containing a higher cyanogenic glycoside concentration have a reproductive fitness advantage (Zagrobelny *et al.* 2008). It is not known whether the cyanogenic glycoside

gift received by the female is used for her own defence or purely to pass onto the offspring, as the pupae are known to have a high cyanogenic glycoside content (Zagrobelny *et al.* 2011).

Bell (1972) proposed the dual functions of defense and nitrogen storage for canavanine, a secondary metabolite that accumulates in high concentration in the seeds of several legumes, and disappears during germination. Since then, it has also been suggested that cyanogenic glycosides are not only for defence but also serve as storage compounds of sugar and reduced nitrogen (Selmar *et al.* 1988; Sánchez- Pérez *et al.* 2008). This theory is supported by the presence of an alternative dhurrin turnover pathway that does not result in the release of HCN and allows the nitrogen to be remobilised (Jenrich *et al.* 2007) and further supported by the transport of cyanogenic glycosides around the plant (Selmar *et al.* 1988; Frehner *et al.* 1990; Selmar 1993; Neilson *et al.* 2011). In cassava the cyanogenic glucosides, linamarin and lotaustralin, are primarily synthesised in the shoot apex (Andersen *et al.* 2000) and transported to the tubers (Bokanga 1994; Jørgensen *et al.* 2005a). Linamarin and lotaustralin are glycosylated to produce the diglucosides linustatin and neolinustatin for transport around the plant (Lykkesfeldt & Møller 1994). The cyanogenic glycosides are transported as diglucosides to prevent cyanogenesis and the release of HCN. The β-glucosidases have a high specificity towards a precise β-glucoside (Esen 1993) and therefore do not hydrolyse the diglucosides (Selmar *et al.* 1988; Selmar 1993). Similar studies on the production of the cyanogenic glucoside, prunasin, and the cyanogenic diglucoside, amygdalin, in almonds support this transport theory (Sánchez-Pérez et al. 2008). The diglucoside dhurrin-6-glucoside has been identified in *S. bicolor* (Selmar *et al.* 1996) and is thought to have a similar function in transport as the diglucosides present in cassava and almond (Møller 2010b). Additionally, a high concentration of dhurrin-6-glucoside was detected in the mature seed of sorghum (Selmar *et al.* 1996). Mature sorghum seeds are known to have no HCNp but a high HCNp is present in young seedlings post-germination (Halkier & Møller 1989; Frehner *et al.* 1990 Ahmed *et al.* 1996).

1.2.5 Regulation of cyanogenic glycosides

The concentration of cyanogenic glycosides within a plant is, at least to some degree, under environmental control. In order to understand why sorghum can produce excessive levels of dhurrin, especially when exposed to water deficit, it is necessary to identify the regulatory mechanisms affecting the cyanogenesis pathway. It has not been established whether the response is primary, the synthesis of additional cyanogenic glycosides via stress-related up-regulation of the biosynthetic pathway, or secondary, an accumulation of dhurrin due to a reduced growth rate (Gleadow *et al*. 1998) or if these two processes are linked. Nevertheless, there has been a general consensus that the cyanogenesis defence system is constitutively regulated (Hartmann 1996; Cipollini & Gruner 2007).

1.2.5.1 Indirect regulation of cyanogenic glycosides

There appears to be three factors that regulate the HCNp of a plant; plant maturity, soil nutrients and the surrounding environment. There are age related effects associated with plant cyanogenic glycoside content, yet there is no definite relationship that covers all cyanogenic species. Plants such as sorghum, rubber, cassava and some *Eucalyptus* species have a high HCNp in young plants which decreases with age (Wheeler *et al.* 1984; Haskins *et al.* 1987; Gleadow & Woodrow 1998; Jørgensen *et al.* 2005a; Kongsawadworakul *et al.* 2009). The youngest petiole on a cassava plant has the highest HCNp per gram fresh weight (Jørgensen *et al.* 2005a). Similarly, studies on rubber plants have found the younger the leaf, the higher the HCNp (Kongsawadworakul *et al.* 2009). In sorghum the HCNp is highest approximately one week after soil emergence (Loyd & Gray 1970). Contradictorily, one study on sorghum reported a significant decline in HCNp only when the plants were fully mature, ~100 days after planting (Eck 1976). There are very different age effects related to the HCNp of *Eucalyptus* species. *Eucalyptus cladocalyx* has a high cyanogenic glycoside content in young plants (Gleadow *et al.* 1998), whereas *E. yarraensis* and *E. camphora* produce a low amount or no cyanogenic glycosides until they reach a defined maturity stage and then there is an increase in HCNp with plant development (Goodger *et al.* 2006;Goodger *et al.* 2007; Neilson *et al.* 2011). There are conflicting accounts of the HCNp in *E. polyanthemos*. Goodger *et al*. (2004) found the foliar cyanogenic glycoside concentrations in *E. polyanthemos* trees were on average about 70% lower in seedlings than in mature

trees, whereas Gras *et al.* (2005) reported an absence of cyanogenic glycosides in the mature leaves of *E. polyanthemos,* but cyanogenic glycosides were present in the young leaves.

A strong association between nitrogen supply and cyanogenic glycoside content has been identified in many cyanogenic species, but not all. The leaf prunasin concentration in *E. cladocalyx*, was strongly affected by nitrogen supply (Gleadow *et al*. 1998) and further studies on *E. cladocalyx* that applied increased nitrogen treatments, found that for every N invested in cyanogenic glycosides, additional N was added to the leaf (Simon *et al.* 2010). A number of sorghum studies have found an increase in HCNp after nitrogen fertilisation (Kriedeman 1964; Eck 1976; Wheeler *et al.* 1980, 1990; Busk & Møller 2002). Moreover, Busk and Møller (2002) found an age and nitrogen related association with HCNp in sorghum plants. Nitrogen fertilisation did not have an effect on the dhurrin concentration of sorghum seedlings, however, when the plants were older (five week old) there was a positive correlation between nitrogen supply and dhurrin concentration (Busk & Møller 2002). This result is in agreement with dhurrin acting as a nitrogen store (Zagrobelny *et al*. 2008). Conversely, nitrogen supply was found to have no effect on cyanogenic glycoside concentration in cassava plants (Gleadow *et al.* 2009b). Similarly, the cyanogenic glycoside concentration in the rainforest tree, *Beilschmiedia collina* B. Hyland, was not different in soils containing higher nutrients, including nitrogen, although an increase in cyanogenic glycoside concentrations was associated higher rainfall (Simon *et al.* 2007).

There have also been numerous studies that have found a relationship between drought and the HCNp of cyanogenic plants. A survey on the HCNp of cassava flour from African communities over the 1998 - 2000 growing seasons showed varying HCNp, but all were high, ranging from 26 to 186 ppm. The four samples with the highest HCNp from each site five week old correlated with low rainfall for the growing season (Ernesto *et al.* 2002). A study on *E. cladocalyx* showed that HCNp was higher in plants subject to prolonged drought stress in the field (as determined by $\partial^{13}C$ values). However, there was a significant correlation between increased leaf nitrogen content and HCNp in the same study (Woodrow *et al.* 2002 and the authors therefore concluded that not all variation was due to water stress. However, further glasshouse experiments found that

water stressed *E. cladocalyx* plants had higher HCNp compared to well watered controls under conditions of both low and high nitrogen (Gleadow & Woodrow 2002a). When white clover plants were allowed to wilt (reducing plant matter yield by up to 13%) prior to harvest the HCNp increased up 24% compared to unwilted control plants (Vickery *et al.* 1987). Similarly, drought studies using sorghum likewise found an increase in HCNp compared to non-stressed sorghum plants (Kriedeman 1964; Wheeler *et al.* 1980). One study by Wheeler et al. (1990) found the opposite, with HCNp was lower after drought treatments, but in this case it was not possible to separate the decrease in HCNp with the normal decline in HCNp associated with increasing plant age (Wheeler *et al.* 1990). Additional environmental stress factors such as salinity, temperature, frost and light have also been shown to have an effect the HCNp of cyanogenic plants (Dunstan 1906; Robinson 1930; Kriedeman 1964; Foulds & Grime 1972; Gleadow & Woodrow 2002b; Liang 2003; Ballhorn *et al.* 2011). For example, a sorghum study by Wheeler *et al.* (1990), found the increase in HCNp caused by nitrogen supply was more pronounced in normal light than in shade.

1.2.5.2 Biochemical regulation of cyanogenic glycosides

There is little knowledge of the regulation of the enzymes involved in the cyanogenic glycoside biosynthesis. In sorghum, the first enzyme in the dhurrin biosynthetic pathway, CYP79A1, has been shown to be the rate limiting enzyme (Figure1.2). Furthermore, the CYP79A1 and CYP71E1 enzymes in sorghum have been shown to be transcriptionally regulated (Busk & Møller 2002). The transcript level of the CYP79A1 and CYP71E1 enzymes are not inducible in young plants, however, in five week old plants the transcript level correlates to the relative HCNp (Busk & Møller 2002). A similar result has been found in cassava, with the first enzymes in the linamarin/lotaustralin pathway CYP79D1/D2 found to be inducible in the roots but the HCNp was not measured in these plants (Du *et al.* 1995). The biosynthesis of cyanogenic glycosides primarily occurs in young and developing tissues (Halkier & Møller 1989). It has been suggested that the decrease in the cyanogenic glycoside concentration of older plant tissues is due to the biosynthesis of cyanogenic glycosides occurring at a lower rate than catabolic turnover pathway, or biosynthesis cannot keep up with the net gain in plant biomass (Busk & Møller 2002).

Moreover, microarray studies have revealed transcriptional changes in the sorghum transcriptome in response to hormone application or wounding (Zhu-Salzman *et al.* 2004; Buchanan et al. 2005; Salzman *et al.* 2005). Specifically, the application of Methyl Jasmonate (MeJa), Salicylic acid (SA) and aminocyclopropane carboxylic acid (ACC) treatments found that MeJa induced dhurrinase and hydroxynitrile lyase transcripts in the shoots, whereas SA suppressed dhurrinase transcripts in roots but not in the shoots (Salzman *et al.* 2005). Both dhurrinase and hydroxynitrile lyase are involved in the catabolism of dhurrin (Figure 1.2). Studies in other species that produce different nitrogen containing secondary metabolites have found many of the genes involved in the synthesis of the secondary metabolites are hormonally regulated (Baldwin *et al.* 1997; Baldwin 1998; DeBoer 2009). For example, in the alkaloid producing species *Nicotiana tabacum*, alkaloid production is increased following the application of MeJa (Baldwin *et al.* 1997; Sinclair *et al.* 2004). The age, nutrient and environmental effects outlined above suggest the synthesis of cyanogenic glycosides may also be regulated at the hormone level.

1.3 Sorghum

Sorghum is a member of the Poaceae family (Clark *et al.* 1995). The *Sorghum* genus is currently comprised of 25 species that have been taxonomically classified into five subgenera: *Chaetosorghum, Eusorghum, Heterosorghum, Parasorghum and Stiposorghum* (Garber 1950). Species within the sorghum genus have $2n$ = 10, 20, 30 or 40 chromosome numbers (Garber 1950; Lazarides *et al.* 1991; Price *et al.* 2005). *Sorghum bicolor* and *Sorghum propinquum* are classified as *Eusorghum* and are both diploid species with $2n$ = 20 chromosomes (Doggett 1988; Price *et al.* 2005). Whereas *Sorghum halpense* commonly known as Johnson grass, is a polyploid with $2n$ = 40 chromosomes (Duara & Stebbins 1952; Tang & Liang 1988). A recent survey of wild sorghum species native to Australia has identified a number non-cyanogenic sorghum species (pers. comm. A/Prof. Ros Gleadow), but none of the wild species tested were *S. bicolor*.

Sorghum is most closely related to the non-cyanogenic species, maize (*Zea mays* L.) and rice (*Oryza sativa* L.) (Doebley *et al.* 1990). The appearance of maize and sorghum are most similar, and both species have been cultivated for grain and forage production.

Grain sorghum has been bred to reach ~1.5 meters at maturity, maximising the nutrient availability for grain production and shortening the time required for growth (Williams & Rao 1981). Forage sorghum is generally a hybrid cross of *S. bicolor* subspecies bicolor and *S. bicolor* subspecies *drummondii* (Stuart 2002). Sorghum hybrids are more desirable for forage because of the hybrid vigor that allows the plants to grow better in nutrient poor regions (Gibson & Schertz 1977). Like grain sorghum, forage sorghum is fast growing and at maturity can reach over three meters (Stuart 2002). Although, good forage is associated with a high leaf to stem ratio and therefore is usually consumed at younger and more palatable growth stage (Hoveland & Monson 1980).

The qualities that make for a good forage crop, such as sorghum, include: good nutritional value, high biomass yield, good digestibility, physiological-chemical characteristics that allow rapid outflow, high nonstructural carbohydrate content, and low lipid and condensed tannin content (Gourley & Lusk 1978; Haveland & Monson 1980; Wheeler & Corbett 1989; Kalton 1988; Duncan 1996). A comparison of crops found sorghum was more nutritional to cattle in forages grown in summer compared to autumn (Fulkerson *et al.* 2008). Additionally, research in regions lacking good water supply have shown that when sorghum was grown over summer, there was no adverse effect on the growth (e.g. lack of water) of subsequent winter crops (Postlethwaite & Coventry 2003). Drought is one of the greatest limitations to crop expansion from present-day agricultural areas (Falkenmark *et al.* 1998; Challinor *et al.* 2007). Sorghum is a C_4 plant which is advantageous under high temperatures, low rainfall and historically low atmospheric CO_2 (Osbourne & Beerling 2006). Sorghum also grows well on marginal land, leaving the fertile land for the production of other food crops that require the better growing conditions (Xin *et al* 2009).

All known *S. bicolor* varieties produce dhurrin, and the HCNp of sorghum crops are highly variable and the HCNp can vary from one season to the next (Kriedeman 1964; Gray *et al.* 1968). It is known that very young plants are toxic, as are older plants subjected to drought (Nelson 1953, Gray *et al.* 1968; Wheeler *et al.* 1990). The majority of farmers do not have their fields tested for toxicity before allowing their cattle to feed on it, relying on knowledge of how the growing conditions relate to plant HCNp alone (pers. comm. Peter Stuart). Testing for HCNp is expensive and may be impractical as the

HCN concentrations may change before the results are received. In Australia there is an estimated loss of over $20 million a year from fields of sorghum that cannot be grazed due to fears of cyanide poisoning (Blomstedt, O'Donnell *et al.* 2012). In addition, there is likely to be a reduced productivity of grazing animals due to low-level toxicity. Thus, sorghum lines of low HCNp are desirable for use as fodder.

Farmers use forage sorghum because it has good nutritional benefits for the cattle and grows well under dry conditions (Stuart *et al.* 2002). However, there is frequently not enough sulphur present in the forage to be used in the HCN detoxification pathway and for other required metabolic functions (Wheeler *et al.* 1980). It is important to know the HCNp of the plants, because 200 ppm dwt cyanide is considered safe for grazing, whilst 600-800 ppm dry weight is potentially toxic and anything above 1000 ppm dwt is considered toxic to grazing animals (Stuart, 2002). Although, the size of the animal consuming the cyanogenic material and the rate of ingestion, are important factors when deciding whether the HCNp of a plant is toxic. A lethal dose of HCN has been calculated to be 0.5 - 3.5 mg CN kg^{-1} of body weight (Stochmal & Oleszek 1997).

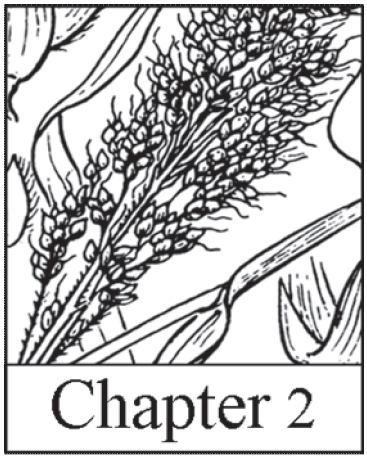
1.4 PhD aims and hypotheses

For the efficient use of sorghum as forage it is important to gain knowledge about factors affecting production, storage and turnover of the cyanogenic glycosides. Little is known about the molecular mechanisms regulating cyanogenesis, but dhurrin production is known to be strongly influenced by environmental factors that also affect the growth of the plant, such as water availability and soil nutrients. How the aforementioned factors influence the HCNp of plants remains unknown.

This research project focused on *S. bicolor* subspecies *bicolor*, *S. bicolor* subspecies *drummondii* and hybrids of the two. Based on the literature, it was hypothesised that stress, in particular osmotic and nutrient related stress, play a large role in the regulation of sorghum HCNp. It is not know how these factors influence the cyanogenesis pathway. In order to answer this question, more information was needed on the response of sorghum to various growth conditions. The overall aim of this project was to generate new varieties of sorghum with lower cyanogenic glucoside content for

safer consumption for cattle, particularly after drought. The increase in dhurrin in stressed plants could be due to the reduction of nitrogen allocation to growth and therefore the reallocation of nitrogen to dhurrin production via the up-regulation of the biosynthetic enzymes. Alternatively, the observed increase in dhurrin concentration in stressed plants could be due to the constant expression of the genes involved in dhurrin biosynthesis and subsequent accumulation of dhurrin due to reduced growth, or a combination of the two. It is hypothesised that a combination of drought, nitrogen and other environmental factors impact on the cyanogenesis pathway. The specific aims of this PhD project were: (1) to determine the ontogenetic changes in HCNp forage sorghum, (2) to determine what environmental factors influence dhurrin concentration, and (3) to identify a viable low dhurrin producing sorghum line.

In order to answer these questions, sorghum plants were grown under controlled environment conditions and subjected to different degrees of osmotic stress, nitrogenous fertilisers, wounding, and hormonal applications. The HCNp was measured for all experiments. The *CYP79A1* transcript level was used as a measure of gene expression and compared to the dhurrin concentration and other nitrogenous metabolites (total N, nitrate, and growth parameters). In addition to the dhurrin characterisation studies, a population of sorghum mutants was created. A number of 'cyanogenesis' mutant lines were identified, including the *totally cyanide deficient 2* (*tcd2*) line. The growth characteristics of the *tcd2* line was investigated further.



General Methods

2.0 Introduction

This chapter outlines the methods that are common to many of the experiments described in subsequent chapters. Later chapters will include methods that are specific to those experiments. There are five sections to this chapter; (1) species description, (2) growth conditions, (3) growth analysis, (4) chemical analytical methods and (5) molecular biology.

2.1 Species description

Sorghum grasses are monocotyledons and part of the Poaceae family. The sorghum genus is comprised of many grass species, many of which are wild and uncharacterised (Dillon *et al.* 2004). *Sorghum bicolor* L. Moench has been cultivated for grain, animal fodder and recently as a source for biofuels (Simmons *et al.* 2008; Xin *et al.* 2009). Modern *S. bicolor* cultivars used for their grain have been bred to reach 1.5 meters at maturity. However, the *S. bicolor* cultivars grown for forage can reach more than three meters at maturity. Many forage sorghum varieties are formed by crossing *S. bicolor* subspecies *bicolor* and *S. bicolor* subspecies *drummondii* to form hybrids, as sorghum hybrids are often more hardy and grow better in difficult regions. *S. bicolor* subspecies *drummondii* was previously classified as *S. sudanense* (Zhan *et al.* 2008) and often still referred to in this form in the literature. Thus, species names quoted in this thesis will be as they were found in the primary research article. The sorghum architecture is much

like other grasses, comprising numerous tillers coming off a central tiller. The tillers are made up of leaves, comprising the leaf blade and the leaf sheath, and roots, and growth of new leaves is from the central basal meristematic tissue. The reproductive tillers also arise from the meristematic tissue at the base of the plant forming the central stem with a flag leaf and head containing the flowers/seed (Figure 2.1).

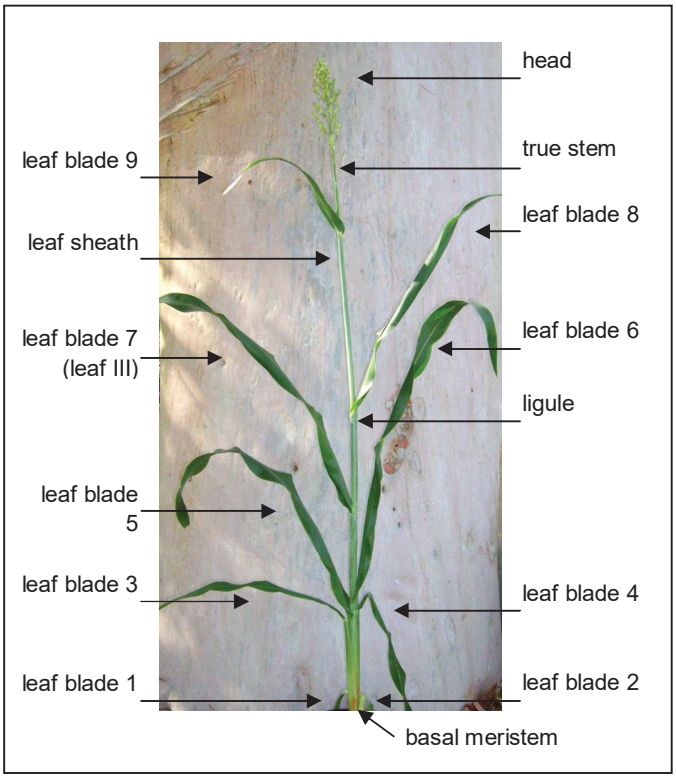


Figure 2.1. Architecture of a sorghum plant. The oldest tissue of a sorghum plant is at the base. Each leaf is numbered chronologically from the bottom to the top. The roots are directly under the basal meristem.

All seed used in this project were supplied by Pacific Seeds (PTY LTD, Toowoomba, Australia). The SB-b1 seed line (*Sorghum bicolor* L. Moench *subspecies bicolor*), otherwise referred to as the Elite line, has been selectively bred for several years and is thought to be near isogenic. The Elite line is the maternal parent to the Hybrid 1 and Hybrid 2 lines which are commercial forage sorghum hybrids available from Pacific Seeds PTY LTD. The paternal parents to the Hybrid 1 and Hybrid 2 lines are two different lines of *Sorghum bicolor subspecies drummondii,* previously referred to as

Sorghum sudanense (Table 2.1). The Elite, Hybrid 1 and Hybrid 2 lines are the main seed lines used in this project, however, other seed lines were also used and are listed in Table 2.1.

Table 2.1. The sorghum seed line and species used in this research project.

Seed line	Species	Sub-species
SB-b1 (Elite)	*S. bicolor* (SJ & SJ-LPA parent)	*bicolor*
SB-b2	*S. bicolor*	*bicolor*
SB-b3	*S. bicolor*	*bicolor*
SB-d1	*S. bicolor* (SJ parent)	*drummondii*
SB-d2	*S. bicolor* (SJ-LPA parent)	*drummondii*
SB-d3	*S. bicolor*	*drummondii*
SB-d4	*S. bicolor*	*drummondii*
Hybrid 1	*S. bicolor*	*bicolor x drummondii*
Hybrid 2	*S. bicolor*	*bicolor x drummondii*

The experiments conducted in this thesis were primarily on sorghum plants with single vegetative tillers and the developmental stage is indicated by the leaf number, rather than the number of days, because leaf number is indicative of the developmental stage of the plant and different sorghum cultivars develop at different rates (chapter 3). The leaf number of a plant refers to the number of fully unfurled leaves and does not include the two or three immature leaves often present. The numbering system, adapted from Vanderlip *et al.* (1993) was used to identify individual leaves of a plant, numbered consecutively from basal to apical leaf of the plant (Figure 2.1). Analysis of the leaves was done on the leaf blade and leaf sheath separately. The leaf blade is defined as the section of leaf above the ligule and leaf sheath as the section of leaf below the ligule (Figure 2.1). The true stem of a plant (containing basal meristematic tissue) is not present in experiments described in this thesis unless otherwise stated. The first and third fully unfurled leaves, at any stage of a plants development, are referred to as leaves I and III, respectively, (Figure 2.1), to avoid any confusion with the Vanderlip *et al.* (1993) numbering system. These two top-most leaves were sampled for physiological data and biochemical assays to provide a consistent point of reference between experiments. The plant height was measured from the base of the plant to the ligule of leaf I.

2.2 Plant growth conditions

Plants were grown in a greenhouse, in Melbourne, Australia (37°50'S, 145°0'E), kept at mean temperature of 26.2 °C ± 7.2 (SE) day and 19.5 °C ± 4.7 (SE) night, with a relative humidity of 51.7% ± 13.6 (SE) / 59.11% ± 11.9 (SE) day/night. The temperature and humidity was measured using a HOBO® data logger (Onset). Plants were grown over the summer between the months of September – April, with an average photoperiod of 14:44 hours. When lower than the average, the photoperiod was extended to at least 14 hrs with supplementary incandescent lights yielding an intensity of 100-150 mE m^{-2} s^{-1}.

Seeds were germinated in punnets containing Debaco seed raising mix and perlite (3:1 ratio) supplemented with slow a release fertiliser (Growcoat, 5 g per 12 L soil mix) and sprayed with water daily. At the three leaf stage seedlings were transferred into either individual pots (125 mm diam. x 120 mm deep) containing the same soil mix as above or into hydroponic containers (see below). Plants growing in soil were watered once per week with a modified Hoagland's nutrient solution containing 5 mM NO_3^- and flushed 1-2 times per week with water to prevent build up of salts in the pots. Pots were allowed to drain freely between watering. The Hoagland's nutrient solution was modified to contain 5 mM NO_3, and 90 µM Fe^+ all other nutrients were not changed.

The hydroponic system was based on the model described in Mason (1990). Plants were grown in opaque pots (140 mm x 105 mm x 50 mm deep) with 200 mL modified Hoagland's nutrient solution, containing 5mM NO_3^-. A rock wool (Grow wool Horticulture Systems, NSW, Australia) plug was inserted into the lid of the pot suspending the base of the stem above the nutrient solution, preventing the stem from becoming wet, but allowing the roots to protrude into the nutrient solution (Figure 2.2). The nutrient solution was changed once a week for the first three weeks and twice a week after that and pots were rinsed each time the nutrient solution was changed to prevent the build up of salts. Pots were rotated regularly to reduce any subtle variations in microenvironment within the glasshouse.

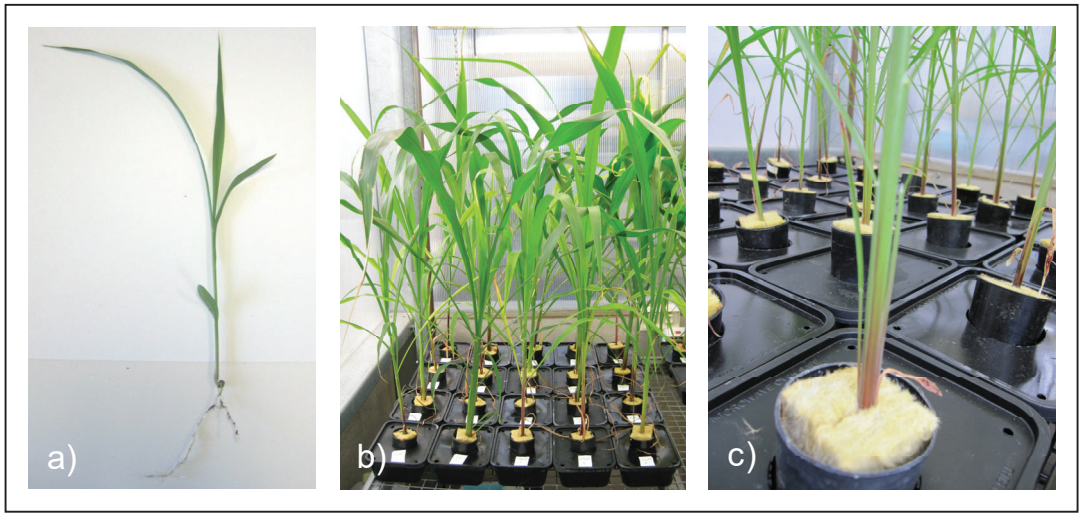


Figure 2.2 The hydroponic system established for sorghum. (a) a plant at the 3-leaf stage, ready to be transferred into hydroponics, (b) plants at the 7-8 leaf stage growing in hydroponics in the glasshouse and (c) the base of a sorghum plant growing in hydroponic system, demonstrating how the rockwool supports the plant.

2.3 Harvesting protocols and growth analysis

Prior to harvesting the height of the plants was measured and the number of leaves counted. Leaf blades were then removed from the sheath at the ligule and total leaf surface area (cm^2) measured using a Li-Cor portable area meter (LI-3000, Lambda Instruments Corporation). The roots of soil grown plants were cleaned of soil and blotted dry, while roots of hydroponically grown plants were rinsed of nutrient solution and blotted dry. Leaf blade, leaf sheath and root tissue was dried in an oven at 50°C for 5 days and weighed.

Plant water status was estimated by determining the relative water content (% RWC) of leaf segments (Slatyer 1967). A section of leaf blade (~10 cm) was taken ~5 cm from the ligule of leaf I and the fresh weight taken. The turgid weight was obtained by immersing the tissue in MQH_2O for 15-20 hours and blotting dry. The tissue was then oven dried and dry weight determined. The %RWC was calculated using the following equation.

$$\% \text{ RWC} = \frac{(\text{ fresh weight} - \text{dry weight})}{(\text{ turgid weight} - \text{dry weight})} \times 100$$

The potential maximum quantum yield (F_v/F_m) of photosystem II (PSII) was measured using a PAM- 210 Chlorophyll Fluorometer (WALZ). The F_v/F_m ratio is an indicator of the degree of potential photosynthetic ability and nutrient stress. Healthy plants usually have an F_v/F_m ratio of around 0.8, while an F_v/F_m ratios of < 0.5 is indicative of a plant under stress (Atwell *et al.* 1999). The F_o, F_m and F_t values were measured after 20–30 min of dark adaptation. Measurements were taken from the middle ten cm of leaf I in triplicate and averaged. The midrib of the leaf was avoided when taking measurements.

Growth parameters were calculated using the equations described in Atwell *et al.* 1999. Specific leaf area (SLA cm^2 g^{-1} dwt), which is the ratio of total leaf area to leaf dry weight, is related to leaf thickness and density. Leaf area ratio (LAR cm^2 g^{-1} dwt) is a measure of the leaf area relative to total plant biomass. Leaf weight ratio (LWR dwt) is a measure of biomass allocation to the leaves. Relative growth rate (RGR d^{-1} dwt) was calculated using formula:

$$RGR = (\ln W_2 - \ln W_1) / (t_2 - t_1)$$

where, W_1 is the initial dry mass of the total plant, W_2 is the final dry mass, t_1 is number of days of growth when the treatment commenced, and t_2 the final number of days of growth. RGR is also the product of LAR, SLA and LWR. Net assimilation rate (NAR g m^{-2} d^{-1} dwt) is a measure of the photosynthetic efficiency of the plants. NAR was calculated

$$NAR = \frac{(W_2 - W_1)(\ln A_2 - \ln A_1)}{(A_2 - A_1)(t_2 - t_1)}$$

where, W_1 is the initial dry mass of the plant, W_2 is the final dry mass of the plant, t_1 is number of days of growth when the treatment commenced, t_2 the final number of days of growth, and A is the total area of the leaves.

Root length (RL) was measured using the method described by Tennant (1975). The roots were cut into 1 cm long sections and sub-sampled. The subsample was weighed and then spread across a grid. The number of times the root sections intersect with the grid was recorded and the following equation used to calculate RL:

$$RL = \frac{L \times G \times (11/14)}{W_S} \times W_R$$

where, L is the length of the side of the squares in the grid, G the number of times the roots intersect with the grid, W_S is the subsample weight and W_R is the total root weight. 11/14 is a constant. Specific root length (SRL cm g^{-1} dwt) is the ratio of root length to root weight and is an indicator of the relative thickness or spread of the roots.

2.4 Chemical analysis

2.4.1 Cyanogenic glycoside, dhurrin.

Cyanogenic glycosides, such as dhurrin, can be measured directly or indirectly. LC-MS analysis directly measures the dhurrin concentration in plant extracts, while indirect methods are a measure of evolved HCN (Feigl-Anger and Colorimetric assays) from the hydrolysed dhurrin. The amount of evolved HCN is directly proportional to the amount cyanogenic glycosides in the tissue (Gleadow & Woodrow, 2000).

2.4.1.1 Feigl-Anger assay

Feigl-Anger (FA) paper screening is a qualitative assay to identify the presence/absence of HCN (Feigl & Anger, 1966; Miller *et al.* 2006). A concentration dependant blue coloured precipitate forms on the FA paper when HCN is present. Three leaf discs (~0.5cm diameter) were taken from the youngest fully expanded leaf of test plants and placed in a 96-well micro-titre plate. The wells were covered with a sheet of FA paper, which is filter paper imbibed with tetrabase and copper ethylacetoacetate (Feigl & Anger, 1966; Takos *et al*. 2010), and sealed with a lid. The plate was frozen and thawed twice and then incubated at 25 °C for ~30 mins. The freezing and thawing of the plant tissue disrupts the cells and allows the dhurrin and dhurrinase to come into contact,

resulting in the formation of volatile HCN and FA paper above wells turned blue (Miller *et al.* 2006; Takos *et al.* 2010). The FA papers were photographed to provide a permanent record of the staining intensity, as the colour fades over time. Each plate included leaf disc samples from the Elite (parent line) plants as a baseline for cyanide comparison.

2.4.1.2 Colorimetric cyanide assay

A colorimetric assay was used to quantify evolved HCN for sorghum tissue (Brinker & Seigler 1989; Woodrow *et al.* 2002). Plant tissue was dried and ground to a fine powder using a Mixer Mill 301 (Retsch). A 10 mg sample was weighed into a 4 mL scintillation vial containing 300 µL of 2 mg mL^{-1} β-glucosidase (almond emulsion, G-0395 Sigma, Melbourne), in 0.1 M citrate buffer (trisodium citrate, Sigma), pH 5.6. Before sealing the vial a 0.2 mL microcentrifuge tube containing 200 µL 1 M NaOH was inserted (Figure 2.3). Vials were frozen in liquid N_2 and allowed to thaw at room temperature (approx. 22 °C) or at 37 °C, to ensure complete disruption of all the cells. Once thawed the vials were left at room temperature for 1-2 hrs, for the β-glucosidase to hydrolyse the dhurrin and allow complete release of HCN. Vials were then incubated at 37°C for 15-20 hrs, to allow the HCN to diffuse into the NaOH, forming NaCN and H_2O (Gleadow *et al.* 1999). The microcentrifuge tube containing the 200 µL NaCN/NaOH was removed from the scintillation vial and the concentration of the NaCN in the tube was determined using a colourimetric assay as described by Lambert *et al*. (1975).

Aliquots (20 µL) taken from the 1 M NaCN/NaOH were diluted to 1/10 with MilliQ H_2O and mixed. 50 µL aliquots of the 0.1M NaOH mixture were pipetted in triplicate into a 96-well microtitre plate and the following reagents were added with shaking in between, 50 µL 0.5 M acetic acid, to neutralise the solutions, then 125 µL 2× succinimide reagent and 50 µL barbituric acid (in pyridine reagent). Once the barbituric acid was added, the plate was incubated at room temperature (approx. 22 °C) for 15-20 mins to allow colour to develop, previous study has found that the reaction is stable within this time frame (Gleadow 1999). The absorbance was measured at 595 nm using a FLUOstar *Galaxy* plate reader (BMG, Melbourne). The HCN concentration of the samples were calculated using a NaCN standard curve (0, 5, 10, 25, 50, 60, 75, 90, 100, 115, 125 or 150 µM NaCN), in units of mg CN g^{-1} dry weight of tissue.

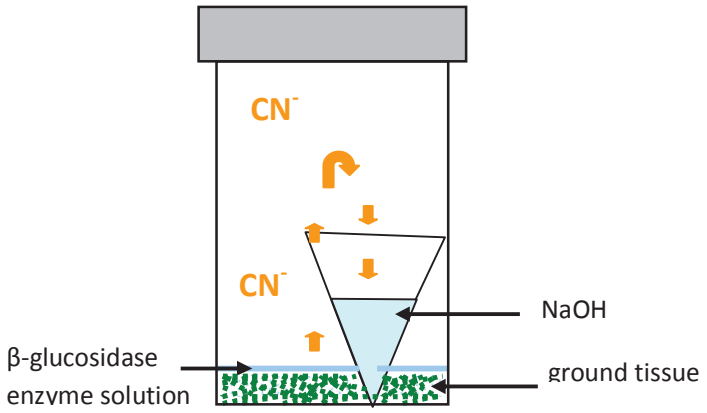


Figure 2.3 Extraction and quantification of HCN from sorghum tissue. A known amount of ground tissue is incubated at 37 °C in the presence of 300 µL β-glucosidase in citrate buffer, with a tube containing 200 µL NaOH within the sealed vial. The β-glucosidase releases the volatile HCN, which diffuses into the NaOH, forming NaCN and H_2O.

2.4.1.3 Direct measure of dhurrin using LC-MS

Dhurrin was measured directly using the method of Bjarnholt *et al.* (2008) as modified by Gleadow *et al.* 2011. Plant tissue was ground in liquid N_2 and 100 mg - 200 mg of tissue boiled in 500 µL of 85% methanol for 3 mins before cooling on ice. Samples were then centrifuged for 3 mins at 10,000 rpm. The supernatant was removed and stored at -20°C in 1 ml glass scintillation vials. Before the LC-MS analysis, the methanol extracts were thawed and diluted 1/5 in water, to achieve a final methanol concentration of 20%. Aliquots (50 µL) of the diluted sample extracts were filtered via centrifugation using 0.45 µm PVDF spin filter plate (Milipore) for 5 minutes at 3000 rpm. Samples of the filtered extract (30 µL) was transferred to the glass insert inside 1 mL scintillation vials. Dhurrin standards (1, 2.5, 5, 10, 25, 50, 100, 200, 300, 500 µM dhurrin) were run with the samples of interest, with an additional dhurrin reference sample run every 30 samples as quality control. The standards were also filtered before adding to the scintillation vials.

Analytical LC-MS was performed using an Agilent 1100 Series LC (Agilent Technologies, Germany) coupled to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) by Prof. C. E. Olsen Copenhagen University, Denmark. A Zorbax SB-C18 column (Agilent; 1.8 μM, 2.1 × 50mm), at a flow rate of 0.2 mL min^{-1}, and an oven temperature of 35 °C was used. The mobile phases were: A, water with 0.1% (v/v) HCOOH and 50μM NaCl; B, acetonitrile with 0.1% (v/v) HCOOH. The gradient program was: 0 to 0.5 min, isocratic 2% B; 0.5 to 7.5 min, linear gradient 2 to 40% B; 7.5 to 8.5 min, linear gradient 40% to 90% B; 8.5 to 11.5 isocratic 90% B; 11.60 to 15 min, isocratic 2% B. The flow rate was increased to 0.3 mL min^{-1} in the interval 11.2 to 13.5 min. The mass spectrometer was run in positive electrospray mode. The dhurrin concentrations were determined by comparison to the standards and data analysis was carried out using the Bruker Daltonics DataAnalysis software (Tableau software, USA).

2.4.2 Nitrate analysis

Total nitrate concentration was measured in dried sorghum material using the method described in Cataldo *et al.* (1975), modified to fit 96-well microtitre plate format. Approximately 15 mg tissue was weighed into 1 mL tubes in a 96-well format, 500 μL of MilliQ H_2O and a tungsten bead (Qiagen, Austalia) was added followed by shaking in a Mixer Mill 301 (Retsch) at 25 oscillations per second for two minutes. The samples were then incubated at 45 °C for one hour with inversion every 20 mins. The tubes were centrifuged at 15,000 rpm in a plate centrifuge for 10 minutes and 200 μL of the supernatant collected for analysis. Samples were stored at 4 °C until analysis, within 48 hours.

The nitrate concentration was determined using a colorimetric assay. Five microliters of each sample was added to a 96 well microtitre plate, in triplicate. In the fumehood, 20 μL 5% salicylic acid (SA) in concentrated H_2SO_4 was added to each well and incubated for 20 min at room temperature. 300 μL 3 M NaOH (to bring pH >12) was then added and allowed to cool to room temperature for three to five minutes before reading the absorbance at 410 nm in the FLUOstar *Galaxy* plate reader (BMG, Australia). The nitrate concentrations were determined by comparison to the standard curve (0, 0.025, 0.0625,

0.1, 0.125, 0.15, 0.2 and 0.25 g/L NO_3^-) included in each microtitre plate and converted to the unit mg g^{-1} dwt.

2.4.3 Total nitrogen

The plant material was dried in a 50°C oven before grinding to a fine power. A 5 mg subsample was then analysed for total carbon, nitrogen and hydrogen content using an Elementar Vario Micro Cube, CHNS anlayser. Acetanilide (Merck, Australia) was used as an internal standard.

2.4.4 Total chlorophyll and carotenoids

Chlorophyll *a* and *b*, and carotenoids were measured following the method of Burns *et al.* (2002), using extracts from frozen tissue (Warren & Adams 2004). Leaf discs (~1.5 cm diameter) were taken from the centre of the youngest fully unfurled leaf, avoiding the leaf midrib and fresh weight was recorded before freezing the tissue in liquid N_2 for later analysis. Pigments were extracted by grinding the tissue in 1 mL cold 80% acetone in a mortar and pestle. The suspension was added to a 1.5 mL micro-centrifuge tube and the volume adjusted to 1.5 ml and held on ice. The mortar and pestle was rinsed with another 1 mL cold 80% (v/v) acetone and added to a second micro-centrifuge tube with the volume adjusted with 80% acetone to 1.5 ml. After centrifuging for 3 minutes at 10,000 rpm, the supernatant from both tubes was pooled. The absorbance of each sample was read at 450 nm, 647 nm and 664 nm. Chlorophyll *a* and chlorophyll *b* concentrations were calculated using the equations of Jeffrey and Humphrey (1975) (see below) and an estimate of carotenoid concentration was made using absorbance at 450 nm. Leaf Chlorophyll (i.e. chlorophyll a + b) and carotenoids were expressed on weight (mg g^{-1} fwt) and leaf area (g cm^{-1}) basis.

$$\text{Chlorophyll } a = 11.93\ A_{664} - 1.93\ A_{647} \text{ mg/L}$$

$$\text{Chlorophyll } b = 20.36\ A_{647} - 5.50\ A_{664} \text{ mg/L}$$

$$\text{Carotenoids} = 4.1\ A_{450} - 0.6435 \text{ Chlorophyll } a - 0.367 \text{ Chlorophyll } b \text{ mg/L}$$

2.5 Molecular biology

2.5.1 DNA techniques

2.5.1.1 Isolation of genomic DNA

Genomic DNA was isolated from sorghum plants using a hot phenol CTAB method as described in Allen *et al*. (2006). Approximately 0.5 g fresh tissue was ground to a fine powder in liquid nitrogen. A 1.2 mL aliquot of preheated extraction buffer (1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 2% (w/v) CTAB, 1% (v/v) β-mercaptoethanol) was added to the sample, vortexed for ~30 secs and incubated at 65 °C for 30 min, mixing the tube every 5–10 minutes. Samples were centrifuged at 13,000 rpm for 10 min at RT to pellet the debris and the supernatant transferred to 2 mL microcentrifuge tube containing 800 mL phenol:chloroform:isoamyl alcohol and mixed for 20 min at RT. The phases were separated by centrifugation for 10 mins at 13,000 rpm at RT, with the upper aqueous layer transferred to a fresh 2-ml microcentrifuge tube containing 800 mL cold isopropanol and mixed before incubation at RT for 10 min to precipitate the DNA. The DNA pellet was collected by centrifugation for 10 minutes at 13,000 rpm, the supernatant removed and the pellet re-suspended in 250 µL 1 TE (pH 8.0). The DNA was RNase treated with 2.5 µL of RQ1 DNase-free RNase (Promega, Australia) for 30 min at 37 °C. The DNA was re-precipitated with 25 µL 3 M NaAc and 600 µL cold ethanol, incubated at RT for 20 mins and collected via centrifugation for 10 minutes at 13,000 rpm. The DNA pellet was washed with 250 µL of cold 70% (v/v) EtOH by centrifugation for 10 minutes at 13,000 rpm at RT, the supernatant removed and the pellet allowed to dry for 5-10 minutes to remove residual ethanol. The pellet was then re-suspended in 25 µL MilliQ H_2O. The DNA concentration was quantified by using a Nanodrop® ND-1000 spectrophotometer (Biolab Australia) at 260 nm. The readings were also taken at 230 and 260 nm and the 230:260 and 280:260 ratios give an indication of protein and carbohydrate contamination, respectively. DNA purity was visualized on a 1% agarose / 1x TAE (40 mM TRIS-acetate and 1 mM EDTA by running 5 µL of each DNA preparation. DNA was stored at - 20 °C.

2.5.1.2 Polymerase chain reaction

Polymerase chain reactions (PCR) were run on a Mastercycler EpGradient S (Eppendorf) PCR machine. Gene specific primers were designed based on the *S. bicolor* genes

published on GeneBank; CYP79A1 based on gene accession SBU32624, CYP71E1 based on gene accession AF029858, and UGT85B1 based on gene accession AF199453. Using 100 ng of genomic DNA per reaction gave the most consistent results. The two CYP genes were amplified in two sections with two sets of forward and reverse primers. CYP79A1 was amplified using the primers: 5' for - CTCATCGGGTGATCGATCAG, 5' rev – CTAACGTGGCGGTGAACAG, 3' for – GCCTCATGTTCAACAGGC and 3' rev – AATGCAAGTGT GACCAGCAG and CYP71E1 was amplified using the primers: 5' for – TAGAAGCAGCTCAC ACTCCAC, 5' rev - ACGGAGCTGGTGTCGATG, 3' for – CGAGGACTTCTTCCCCAAC and 3' rev - CTTTGCGGCACTAAAACA. UGT85B1 was amplified using primers for – GGGGTCGGGGA TATTGTATT and rev – CAGAACCACTTATTGCAAACTC. PCR conditions were as follows. CYP79A1 contains a 97 bp intron which needed to be taken into consideration for amplification and sequencing.

CYP79A1: PCRs (total volume, 50 µL) performed in 1× PCR buffer (Invitrogen), 200 µM of each dNTP, 1.5 mM MgCl2, 100 ng of forward and reverse primers, and 0.5 µL of Platinum *Taq* polymerase (Invitrogen). Thermal cycling parameters were as follows: 94°C for 2 mins, followed by 30 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 2 mins. CYP71E1: PCRs (total volume, 50 µL) performed in 1× PCR buffer (Invitrogen), 200 µM of each dNTP, 1 mM MgCl2, 100 ng of forward and reverse primers, and 0.5 µL of Platinum *Taq* polymerase (Invitrogen). Thermal cycling parameters were as follows: 94°C for 2 mins, followed by 35 cycles of 94°C for 30 s, 54°C for 1 min, and 72°C for 2 mins. UGT85B1: PCRs (total volume, 50 µL) performed in 1 × PCR buffer (Invitrogen), 200 µM of each dNTP, 1.5 mM MgCl2, 100 ng of forward and reverse primers, and 0.5 µL of Platinum *Taq* polymerase (Invitrogen). Thermal cycling parameters were as follows: 94°C for 2 mins, followed by 30 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 2 mins. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide staining under UV light. The correct sized PCR products were excised from the gel and purified using a Wizard SV gel/clean-up kit according to manufacturer's instructions (Promega), with an extra ethanol precipitation step at the end to get remove any residual contaminations.

2.5.1.3 Sequencing of genomic DNA

Purified PCR products were sequenced using gene specific primers. Sequencing reactions were set up with 1 µL of 2.5× Applied Biosystems PRISM BigDye Terminator Mix (Applied biosystems), 2 µL of 1 × Reaction Buffer (Promega, Australia), 50 ng primer, ~100 ng purified DNA made up to 20 µL with MilliQ H_2O. Cycle sequencing was performed with 30 cycles of 30 s at 96 ºC, 15 s at 52 ºC and 4 mins 60 ºC. The products were purified by a standard sodium acetate clean up. Three µL 3 M NaAc (pH 5), 62.5µL ethanol (96%) and 14.5µL MilliQ H_2O were added to the sequencing reaction and incubated for 45 – 90 minutes at RT. Samples were centrifuged at 13,000 rpm for 25 minutes, the supernatant removed and the pellet washed with 150 µL of 70% EtOH, centrifuged for 5 minutes and the supernatant removed again. The pellet was then left to dry at 37ºC for 30 minutes. Electrophoresis of the sequencing reaction was performed at Micromon services (Monash University, Clayton).

2.5.2 RNA techniques

2.5.2.1 Isolation of total RNA

Total RNA was extracted from sorghum using a Hot Phenol:Chloroform:IAA method modified from Verwoerd *et al.* (1989). The cryogenic tube holder of the FastPrep -24 (MP biomedicals) was cooled using liquid N_2. Approximately 100 mg plant tissue was placed in 1.7 mL screw-cap microcentrifuge tube with two small tungsten beads. The tissue was ground for two cycles (with settings at 40 seconds, speed 6, CRY) in the FastPrep. Samples were placed in liquid N_2 for 5 mins between cycles (FastPrep has a 5 min recovery time between runs). The FastPrep tube holder was then allowed to thaw. 750 µL preheated phenol (pH 4.5):TLES (100mM Tris, 100mM LiCl, 1mM EDTA, 1% SDS) solution was added to each screw-cap tube and tubes were shaken in the FastPrep under the same conditions as above but at RT. The contents of each tube was then poured into a 15 mL plastic tube containing an additional 750 µL hot Phenol/TLES solution, and vortexed, before adding 750 µL chloroform/IAA (24:1) and vortexing again. The solution was then decanted into two 1.7 mL microcentrifuge tubes and centrifuged for 5 minutes at 10,000 rpm. The supernatant was transferred to a fresh microcentrifuge tube and an equal volume of 4M LiCl added. Tubes were inverted and placed at 0 – 4°C for approximately 15 hrs to precipitate the RNA. The samples were

centrifuged for 30 mins at 10,000 rpm at 4°C. The supernatant was removed and the pellet resuspended in 150 µL H_2O. Duplicates of the same sample were transferred to the one tube and the RNA was re-precipitated by adding 30 µL of 2.5 M sodium acetate (pH 6.0) and 600 µL of absolute ethanol and stored at -20 °C > 15 hrs. The samples were then centrifuged for 20 minutes at 10,000 rpm at 4°C. The supernatant was discarded and the pellet washed with 250 µL 70% ethanol and centrifuged for 5 mins at 10,000 rpm. The ethanol was discarded and pellet allowed to air dry until almost dry. The pellet was resuspended in 50 µL of H_2O and stored at -80 °C. For tougher samples (leaf sheath and basal meristem) a mortar and pestle was used to grind the tissue.

2.5.2.2 Total RNA quantification

The total RNA concentration was quantified using a Nanodrop® ND-1000 spectrophotometer (Biolab Australia) and the readings at the wavelengths 230, 260 and 280 were taken. The reading at 260nm is used to calculate the RNA concentration (O.D.260 of 1.0 = 40 µg ml^{-1} RNA) whilst the 230:260 and the 280:260 ratios give an indication of protein and carbohydrate contamination, respectively. One µg of each RNA extraction was also run on an agarose formaldehyde gel (1x MOPS, 2.2 M formaldehyde, 3% agarose, 4 µL ethidium bromide and run in 1x MOPS buffer, as described in Maniatis *et al.* (1982)) to determine if the RNA was of good quality.

2.5.2.3 cDNA synthesis

Three µg RNA was DNase treated (Promega RNase-free DNase) in a total volume of 10 µL. The DNase was inactivated by incubation at 65°C for 10 minutes. cDNA was synthesised using oligo-dT primers and the SuperScriptIII First-Strand Synthesis System for RT-PCR kit (Invitrogen), following the manufacturers instructions. In short, 2 µg DNase treated RNA was combined with 1 µL of 50 uM oligo-dT, 1 µL of 10 mm dNTPs, made up to 10 µL with H_2O and pre-heated to 65°C for 5 minutes. The samples were then cooled on ice and a master mix containing 2 µL 10X RT buffer, 4 µL 25 mm $MgCl_2$, 2 µL 0.1 M DTT, 1 µL RNaseOUT, 1 µL SuperScriptIII RT was added. The cDNA synthesis was the carried out at 50°C for 50 min, with an additional 5 min at 85°C. RNase H (1 µL) was then added and the tube incubated at 37°C for 20 mins. The cDNA was stored at -20°C until use.

2.5.2.4 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed on a Rotor-gene 6000 light cycler (Roche) using SensiMix SYBR No ROX (Bioline). The reactions were performed in a volume of 25 µL (half volume stated in the Bioline SensiMix SYBR protocol). Amplifications were performed using gene specific primers for the gene of interest (CYP79A1) and β-actin as the reference gene. The *CYP79A1* gene was amplified using the primers: CYP79A1for - ACCTCGCCACCGCCGCGTCC and CYP79A1rev – AGCAAGCCCG CCGGGGTGGA, with an efficiency of 1.06. β-actin was amplified using the primers: actin5' - ACCCCCGCCATGTACGTCGCCA and actinrev – GACCTCGCTGGCCGCGACCT, with an efficiency of 1.11. The resulting amplicons were 172 bp and 170 bp, respectively. Reactions for both the gene of interest and the reference gene were performed in triplicate. A positive control containing sorghum cDNA from actively growing young plants high in dhurrin, and negative controls containing no template were included in every run.

A 6× master mix was made up separately for each cDNA sample and contained 75 µL Sensi-mix SYBR, 6 µL cDNA (100 ng per qPCR reaction) and 57 µL H_2O. This ensured that each reaction had the same cDNA concentration, thus making reactions more uniform. 23 µL of the master mix was added to each tube already containing 2 µL of either 5µM actin primers or 5µM CYP79A1 primers. The qPCR reactions were run using the following reaction times: 95°C for 10 minutes and then 50 cycles of 95°C for 15 seconds, 58°C for 20 seconds and 72°C for 10 seconds

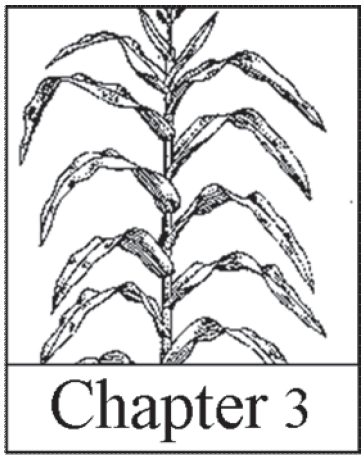
Analysis of the data was done using Rotor-Gene Q Series Software 1.7. The quanitification cycle (C_q) is the cycle at which the transcript level is calculated. The $\Delta\Delta C_q$ method was used to calculate the amount of CYP79A1 transcript relative to β-actin in each sample and standardised to the positive control run in each cycle.

$$\text{Relative transcript level} = 2^{(\Delta\Delta Cq)}$$

where, ΔC_q is the C_q level of the gene of interest minus the C_q level of the reference gene per sample and $\Delta\Delta C_q$ is the sample ΔC_q minus the positive control ΔC_q.

2.6 Statistical analysis

Results were analysed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) and Minitab 15 (Minitab Inc.). Data was tested for normality and homogeneity of variances before analysis and data that was not normally distributed was log transformed. Experiments with two treatments were compared using a *Student's t-test*. A one-way analysis of variance (*ANOVA*) was performed on groups of plants that had more than two treatment groups. If the results did not have a normal distribution, a Kruskal-Wallis one-way *ANOVA* was used. When there was more than one variable to consider a two-way ANOVA was performed. Means that were significantly different were compared *post hoc* using Tukey's t-tests when one treatment was compared and Bonferroni post tests when two treatments compared. A 95% confidence level was set for all statistical tests.



The spatial and temporal distribution of the cyanogenic glycoside, dhurrin, in sorghum

3.1 Introduction

Sorghum is an important crop grown throughout the world, particularly in the dry tropics and on marginal lands, for both grain and forage consumption. Sorghum has a high growth rate and can produce large quantities of feed in a short time. Sorghum produces the stable cyanogenic glycoside, dhurrin (Conn 1981), which breaks down to release toxic hydrogen cyanide (HCN) when plant tissue is damaged, for example by chewing. Cattle are able to metabolise a small amount of cyanide (Cheek 1995), but concentrations above 0.5 mg CN g^{-1} dwt can cause respiratory poisoning, possibly leading to death (Nelson 1953; Poulton 1993; Lechtenberg & Nahrstedt 1999). Several factors can cause the dhurrin concentration to increase which can pose major problems for farmers as fields of apparently nutritious and healthy sorghum plants, which may be the only fodder available, may be unfit for animal consumption.

Dhurrin synthesis (Figure 1.2) is catalysed by two cytochrome P450s (CYP79A1 and CYP71E1), a soluble UDP-glucosyltransferase (UGT85B1) (Jones *et al,* 1999; Bak *et al.* 2006), and the redox partner NADPH-dependant cytochrome P450 reductase (CPR) (Ellis *et al.* 2009; Jensen & Møller 2010). CYP79A1 has been reported as the rate limiting step (Kahn *et al.* 1999) and with regulation occurring primarily at the transcriptional level (Busk & Møller 2002). Although dhurrin is generally thought to act as defence

compounds against herbivory, as are other cyanogenic glycosides, (Tattersall *et al.* 2001; Gleadow & Woodrow, 2002; Møller, 2010a), it may also play a role in nitrogen storage *in planta* (Selmar 1988; Adewusi, 1990; Gleadow & Woodrow, 2000a, 2002a; Jørgensen *et al.* 2005a; Kongsawadworakul *et al.* 2009; Møller 2010b).

Although cyanogenic glycosides are present in over 2500 species and have been studied extensively in *Eucalyptus* (Gleadow & Woodrow 2002a, 2002b), *Lotus* (Forslund *et al.* 2004; Takos *et al.* 2010; Zagrobelny *&* Møller 2011), cassava (Gleadow *et al.* 2009b; Jørgensen *et al.* 2011) and white clover (Collinge & Hughes 1982; Hughes 1991; Olsen *et al*. 2007, 2008; Gleadow *et al.* 2009a), relatively little is known about the regulatory mechanisms controlling their synthesis. The cyanogenic glycoside concentration often varies depending on the growth conditions as well as plant age and is often highest in young plants grown with an abundant nitrogen supply (Muldoon 1985; Brunnich 1903; Robinson 1930; Harrinton 1966; Gorashi *et al.* 1980; Panasiuk & Bills 1984; Wheeler *et al.* 1984; Haskins *et al.* 1987; Ikediobi & Olugboji 1988; Selmar *et al*, 1991; Cipollini & Gruner 2007). Following germination, the HCNp of sorghum seedlings typically peaks one week post-soil emergence and then declines with age (Loyd & Gray 1970). The HCNp also varies within the plant, with a decline in HCNp in the leaf tissues harvested from the top (younger) to the bottom (older) of the plant (Gray *et al.* 1968). Whilst these older studies of Gray *et al*. (1968) and Loyd and Gray (1970) remain useful as general indicators of likely HCNp in sorghum, the plant age was presented chronologically without any indication of the actual developmental stage of the plant at the time of analysis. In addition, it is possible that figures quoted may be well below the true HCNp as exogenous β-glucosidase was not used to release the HCN, which ensures that the endogenous enzyme is not limiting. Also the quantification method used by these authors required repeated re-opening of vessels containing the sample over the course of the quantification process, thereby potentially resulting in loss of volatile HCN.

There has been some discussion in the literature as to whether roots of cyanogenic plants contain cyanogenic glucosides. Sorghum and cassava roots have been reported to contain cyanogenic glycosides (Akazawa *et al.* 1960; Lloyd & Gray 1970; Ikediobi & Olugboji 1988; Du *et al.* 1995 Magalhaes *et al.* 2000; Morant *et al.* 2008) whilst roots of eucalyptus and white clover lack cyanogenic glycosides (Gleadow & Woodrow 2000b;

Hughes 1991). It is likely, however, that there has been a misrepresentation of the conclusion of Akazawa *et al*. (1960) that free HCN is not found in the seed or root, to mean there is no HCN (i.e. dhurrin) present in the seed or roots. Hence, relatively few sorghum HCNp studies have included roots in their analyses, e.g. (Gorashi *et al*, 1980).

Cultivated forage sorghum is usually an interspecific hybrid between *S. bicolor* subspecies *bicolor* and *Sorghum bicolor* subspecies *drummondii* (Sudan grass) (Doggett 1967; Haussmann *et al*, 1998). Apart from the general enhanced tillering and vigour of such hybrids, it is also generally assumed that the *Sorghum bicolor* subspecies *drummondii* parent imparts lower HCNp to the plants which renders them less toxic to grazing ruminents, particularly cattle (Gray *et al.* 1968). The cyanide concentration in forage sorghum cultivars has been reported to vary considerably (Barnett & Caviness 1968; Benson *et al.* 1969; Haskins *et al.* 1987). However, it is difficult to compare results from various studies as many where not conducted under the same conditions and used different sampling methods. Additionally, research related to uncovering the dhurrin biosynthetic pathway (Jones *et al*. 1999; Ellis *et al*. 2009; Jensen & Møller 2010; Jørgensen et al. 2005b; Kristensen et al. 2005; Nielsen *et al*. 2008) and dhurrin turnover pathways (Jenrich *et al*. 2007) have focused on *S. bicolor* subspecies *bicolor* with few studies on Sudan grass included.

In the current study, a series of experiments were designed to investigate the spatial and temporal distribution of cyanide in forage sorghum. It was hypothesised that a reduction in cyanide concentration with plant maturity in sorghum might be due to a redistribution of the dhurrin that is synthesised (i.e. a dilution effect) as has been reported in eucalypts (Gleadow *et al.* 1998). If this is the case, differences in cyanide concentrations between varieties may not be due to variations in the regulation of expression of the biosynthetic genes at the translational and transcriptional level.

Specifically the main aims of experiments described in this chapter were: (1) to examine HCNp within individual sorghum plants at a number of ontological stages to account for changes in plant HCNp in relation to plant maturity; (2) to determine if a range of vegetative plant tissues express CYP79A1 transcripts at detectable (and similar) levels and if so, is there a correlation between the HCNp concentration and CYP79A1 transcript

level in these tissues? and (3) to determine whether any noteworthy differences exist in the coding region of the dhurrin biosynthetic gene sequences encoding the CYP79A1, CYP71E1 or UGT85B1 enzymes of the different cultivars. Such differences, if present, could possibly be useful for future long term studies to determine whether they lead to altered kinetics of the dhurrin-synthesising metabolon that might account, at least in part, for differences observed in the cyanide concentrations between cultivars. The coding sequences of key dhurrin biosynthetic genes (*CYP79A1*, *CYP71E1* and *UGT85B1*) were therefore sequenced for five cultivars; Elite, SB-d1, SB-d2, Hybrid 1 and Hybrid 2.

In order to determine the degree of variation across lines, the shoot cyanide concentrations and shoot CYP79A1 transcript levels were compared among nine forage sorghum cultivars (Table 2.1). Detailed studies were carried out on the Elite, Hybrid 1 and Hybrid 2 plants to separate the effects of age and development on HCNp within the plants. An additional experiment was designed, using the developmental growth stages defined by Vanderlip *et al*. (1993), to examine the spatial and temporal distribution of dhurrin and to ascertain whether variations in the concentrations correlated with alterations in CYP79A1 transcript levels. Surprisingly perhaps, the question of whether a reduction in the cyanide concentration of the plant as it grows is due to the increase in plant mass (a dilution effect) or whether the rate of dhurrin reduction with age has not previously been explicitly investigated in the literature. The experiments conducted in this chapter were primarily conducted on Elite, Hybrid 1 and Hybrid 2 sorghum cultivars. The characterisation of these sorghum lines are of particular interest to a larger, industry-funded mutagenesis project to identify sorghum lines with altered HCNp, which is ongoing in the laboratory and which is described in more detail in chapter 6.

3.2 Methods

3.2.1 Comparison of sorghum cultivars

Seeds were supplied by Pacific Seeds PTY LTD, Toowoomba, Queensland, Australia. The shoot cyanide concentration and CYP79A1 transcript levels of nine sorghum varieties (Table 2.1) were compared. Plant dhurrin content was measured using an evolved cyanide method and expressed as mg CN g^{-1} dwt (section 2.4.1). Seeds were sown in

punnets containing a soil/perlite mix and grown in glasshouse conditions, as described in section 2.2. Thirty plants from each variety were grown to the 5-leaf stage and 15 plants, at the most uniform developmental stage were randomly assigned to either cyanide or RNA analysis. The remaining 15 plants were discarded. Plants were harvested by cutting at the soil line and ten plants from each line were dried at 50°C for cyanide analysis (section 2.4.1) whilst the remaining five were stored at -80°C for subsequent RNA extraction and transcript analysis (section 2.5.2).

3.2.2 Sequencing of the genes in the dhurrin biosynthesis pathway

Genomic DNA was extracted (Allen *et al.* 2006), and the target sequences PCR amplified using gene specific primers (section 2.5.1). The resulting DNA fragments were purified using the Promega Wizard Kit (Promega) and sequenced using the Applied Biosystems PRISM BigDye Terminator Mix (Applied biosystems), as per section 2.5.1. Full length coding sequences were amplified from each line and analysed in triplicate from separate DNA preparations from individual plants. The coding regions for *CYP79A1*, *CYP71E1* and *UGT85B1*, were sequenced from the Elite, SB-d1, SB-d2, Hybrid 1 and Hybrid 2 sorghum lines and compared to the respective gene sequence from *S. bicolor* subspecies *bicolor* sequences available in Genebank (SBU32624, AF029858 and AF199453, respectively). Dhurrin biosynthesis gene sequences, *CYP79A1*, *CYP71E1* and *UGT85B1*, have not yet been published for *S. bicolor* subspecies *drummondii*. Sequence comparison was conducted to determine if there were any differences in the known functional regions of the enzymes which could account for the differences seen in cyanide concentrations across cultivars.

3.2.3 Spatio-temporal dhurrin analysis

3.2.3.1 Spatial analysis of dhurrin

There were two parts to this aspect of the study. First, the variation in cyanide concentration within plants was measured, and second a more detailed study into the cyanide concentration of the leaf blades and roots was conducted.

Elite and Hybrid 1 plants were grown in pots containing soil:perlite mix under glasshouse conditions, as described in section 2.2. The plants were grown to the 3-leaf,

5-leaf, 10-leaf growth stages (n = 5) and harvested. The cyanide variation was measured for individual leaf blades, cross-sections of the 'stem' (see below) and the roots. The shoots were separated from the roots at the soil surface. Each leaf blade was detached at the ligule and dried for cyanide analysis. The leaf sheath was then sectioned into segments (3 cm long, basal to apical). The tissues were dried at 50 °C and ground separately for cyanide analysis. In order to ensure sufficient material for analysis, plants at the 3-leaf stage were first pooled into groups of three (n = 5).

Field grown (as described in section 6.2.1) Hybrid 1 plants at approximately the 12-leaf stage were used for this study. The cyanide concentration along the leaf lamina was measured (n = 3), by taking leaf discs approximately 10 cm apart, from ligule to the leaf tip. The cyanide concentration was also measured on roots that were dug out of the ground. The fibrous roots were removed and 1 cm sections approximately 5 cm apart were tested (n = 3). Cyanide was also measured on the pooled fibrous roots.

3.2.3.2 Temporal analysis of dhurrin

In order to separate the effect of plant age from the age of the specific tissue, Elite plants were grown in soil (as described in section 2.2) and harvested at the 3-leaf, 5-leaf, 10-leaf stages. The cyanide concentration (n = 6) and CYP79A1 transcripts (n = 3) were measured on the plant tissues as outlined in section 3.2.3 (the leaf blade, leaf sheath, shoot base and the roots). Although, in this experiment 'shoot base' was defined as the bottom 2 cm of stalk and consists of leaf sheath and the anatomical true stem. The shoot base was sampled separately because it contains the basal meristem, where new tissue is generated. At each growth stage the plants were harvested as follows. Firstly, the roots and shoot base were separated then each leaf was removed, including the sheath. The leaves were then cut at the ligule to separate the leaf blade from the leaf sheath. As both cyanide and transcript analysis was required on the same tissue, larger quantities of plant material were required. At each harvest the leaves (blade and sheath separately) from the same plant were pooled and classified into age classes as follows; α - leaves 1 to 3 (basal), β - leaves 4 to 6, γ - leaves 7 to 10, and δ leaves 11 to 12 (apical), this last group contained unfurled leaves (Figure 3.1). Each of the four tissues (leaf blade, leaf sheath, shoot base and roots) were sliced down the centre, with one half stored at -80°C for RNA extraction and transcript analysis and the other half dried at

50°C for cyanide analysis. The α leaves at the 5-leaf and 10-leaf stages were not separated for cyanide and RNA analysis as there was not enough leaf mass. Hybrid 1 and Hybrid 2 plants were also grown and harvested in the same manner as above, but only the cyanide concentrations were measured (n = 6).

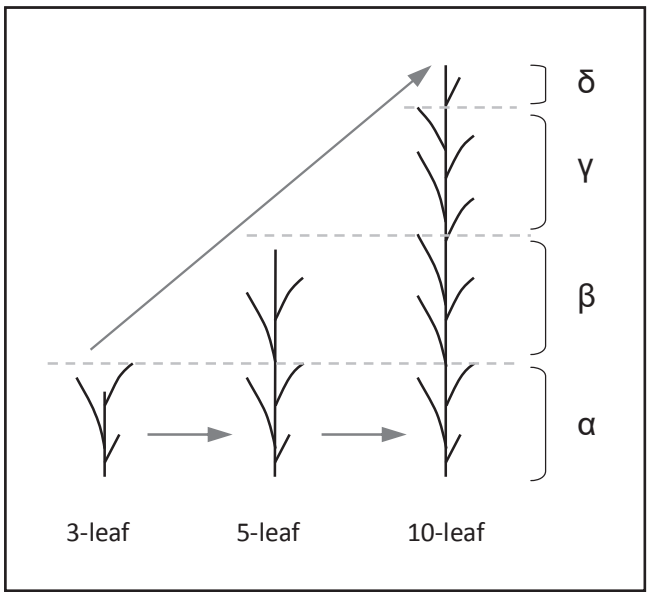


Figure 3.1 Classification of pooled leaf blade and leaf sheath tissue for temporal analysis of cyanide concentration and CYP79A1 transcript levels. α - leaves 1 to 3, β - leaves 4 to 6, γ - leaves 7 to 10, and δ leaves 11 to 12 (immature leaves). The leaves were separated into age classes before analysis.

3.2.4 Sample analysis

Dhurrin analysis

Dhurrin concentration was measured as evolved cyanide. Approximately 10 mg (weighed accurately to 4 decimal places for each sample) of dried ground tissue was analysed for HCNp using a colorimetric cyanide assay as described in Gleadow, O'Donnell, *et al*. (2012), section 2.4.2.

CYP79A1 transcript analysis

Total RNA was extracted from frozen tissue using a method modified from Verwoerd *et al*. (1989). cDNA was first synthesised using a commercial kit (SuperScriptIII First-Strand Synthesis System for RT-PCR (Invitrogen)) using oligo-dT primers. Quantitative real-time PCR (qPCR) was conducted using CYP79A1 gene specific primers with actin as the reference gene. SensiMix SYBR No ROX (Bioline) was used in a total reaction volume of 25 µL (half volume stated in the Bioline SensiMix SYBR protocol), and amplified on a Rotor-gene 6000 light cycler (Roche). Reactions for both the gene of interest and the reference gene were performed on three individual (biological replicates) and in triplicate (technical replicates). See section 2.5.2.4 for details.

Statistical analysis

Results were analysed using GraphPad prism 5 (GraphPad Software Inc., USA). Normality and homogeneity of variances tests were performed prior to analysis. Data that was not normally distributed was log transformed, to satisfy the assumptions of the *ANOVA*. Experiments with two treatments were compared using a *Student's t-test*. A one-way analysis of variance (*ANOVA*) was performed on groups of plants that had more than two treatment groups. Means that were significantly different were compared *post hoc* using Tukey's t-tests, where the same letter superscript indicates significant differences between means. Correlation coefficients and regression equations were calculated using Minitab 15 (Minitab Inc.). Mean values are followed by one standard error of the mean (± 1 SE).

3.3 Results

3.3.1. Comparison of sorghum cultivars

The HCN potential of nine sorghum cultivars harvested at the 5-leaf stage were compared by measuring the shoot cyanide concentration. Levels of CYP79A1 mRNA were also measured in RNA extracted from comparable samples (Figure 3.2). The cyanide concentration of all sorghum lines was observed to be less than 0.5 mg g^{-1} dwt (toxic threshold is ~0.6 mg g^{-1} dwt). Elite and SB-d1 had the highest cyanide concentrations of 0.47 ± 0.02 and 0.49 ± 0.03 mg CN g^{-1} dwt, respectively. SB-d3 had the

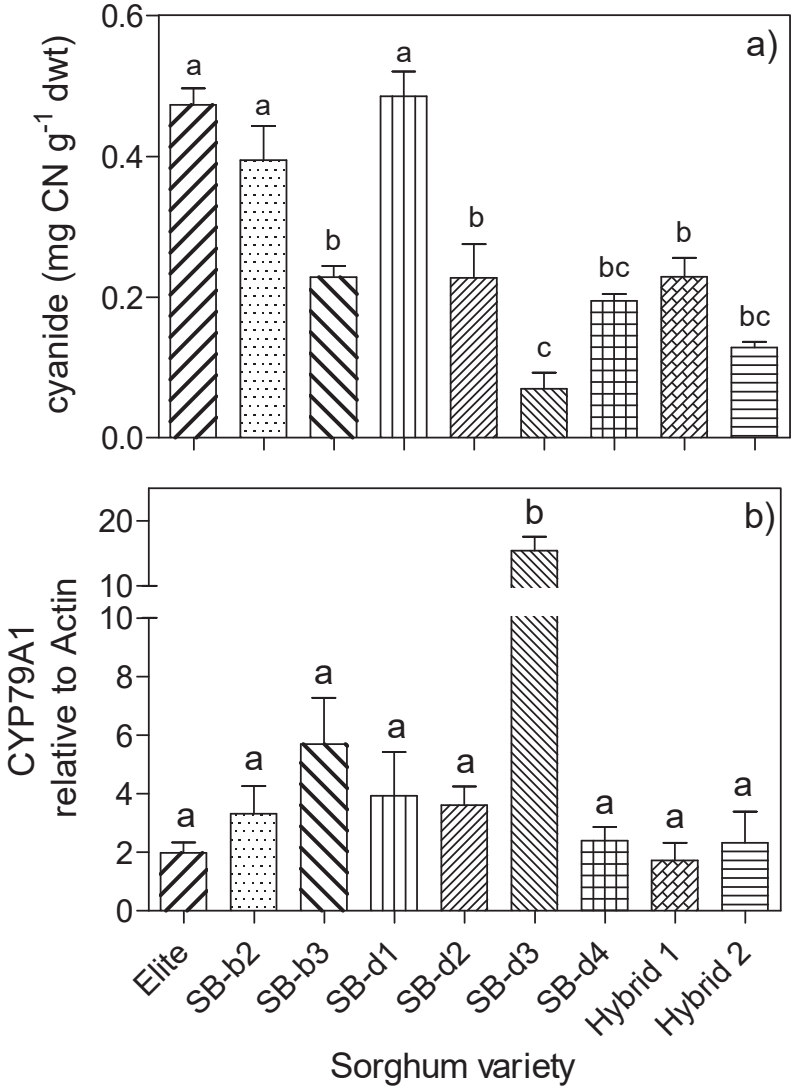


Figure 3.2 (a) cyanide concentration and (b) CYP79A1 transcripts ($\Delta\Delta C_q$ values relative to actin) in the total shoot of nine different sorghum varieties analysed at the 5-leaf growth stage. Plants were grown in soil under uniform glasshouse conditions. Values are mean (± 1 SE) data, cyanide n = 10 and transcripts n = 3. Significant variation was found between cyanide concentrations ($p < 0.0001$) and CYP79A1 transcripts ($p < 0.0001$). Tukey's tests were performed and letters indicate significant difference at $p < 0.05$.

lowest cyanide concentration (0.07 ± 0.02 mg CN g^{-1} dwt), 85% less than Elite plants. The cyanide concentrations of the remaining sorghum lines were from highest to lowest: SB-b2, Hybrid 1, SB-b3, SB-d2, SB-d4 and Hybrid 2 (Figure 3.2a). Interestingly, the cyanide concentrations of commercial Hybrid 1 and Hybrid 2 cultivars did not reflect the cyanide concentrations of their parents (paternal = SB-d1 and SB-d2 respectively, maternal = Elite for both; Table 2.1), with both Hybrid 1 and Hybrid 2 cyanide exhibiting concentrations that were less than half of that of their parents.

The Elite and Hybrid 1 lines showed the lowest relative CYP79A1 values at 1.97± 0.4 and 1.71± 0.6, respectively (Figure 3.2b). Conversely, SB-d3 had the highest relative CYP79A1 at 15.42 ± 2.1, between 60% and 89% higher than all other cultivars. This is the opposite of that observed for the relative cyanide concentrations between the different sorghum varieties. The cyanide concentration and relative CYP79A1 transcript levels were regressed and no statistical correlation was found (p = 0.1965). However, this general trend is similar to the results presented below (section 3.3.3; Figure 3.12b), where the cyanide concentration and CYP79A1 transcript levels are negatively correlated.

3.3.2 Sequencing of the genes in the Dhurrin biosynthesis pathway

CYP79A1, *CYP71E1* and *UGT85B1* are key genes in dhurrin biosynthesis. The coding region of each of the biosynthetic genes was sequenced from the Elite, SB-d1 and SB-d2 sorghum lines. The sequences were compared to the published Genebank sequences from *S. bicolor* subspecies bicolor, SBU32624, AF029858 and AF199453 for the three genes, respectively, to determine if there are any allelic differences between lines which could be linked with variations in the observed cyanide concentration of lines. As expected, the Hybrid 1 and Hybrid 2 sequences reflected the differences observed in the parent lines (Elite and Sb-d1 or Elite and SB-d2). Therefore sequence differences are not discussed for the Hybrid 1 and Hybrid 2 lines specifically. Two sequences are shown for both the Hybrid 1 and Hybrid 2 lines to show the multiple nucleotides when there was a nucleotide difference between the parent lines ("a" or "b" is in the title to indicate the different the sequences).

Compared to Genebank accession SBU32624 (*S. bicolor CYP79A1*), the *CYP79A1* gene sequences of the sorghum varieties under examination had two nucleotide changes that resulted in altered amino acid sequence in the deduced proteins (Figure 3.3; Appendix 2) with an additional five silent nucleotide changes (Appendix 2). Thus Elite and SB-d2 showed changes of V207A and A60G, respectively, while SB-d1 was identical to Genebank accession SBU32624.

The Elite CYP71E1 nucleotide sequences had one silent nucleotide change within the coding region (Appendix 4), two nucleotide changes that resulted in altered amino acid sequence in the deduced proteins (S40N and N42S) and a two amino acid insertion/deletion at positions 45 and 46 of the deduced amino acid sequence when compared to Genebank accession AF029858 (*S. bicolor* CYP71E1; Figure 3.4; Appendix 4). The CYP71E1 sequences for SB-d1 and SB-d2 were identical to Genebank accession AF029858. Notably, the S*. bicolor* subspecies *drummondii* lines had the same sequence as the *S. bicolor* subspecies *bicolor* line in database.

Figure 3.3 Elite, SB-d1 and SB-d2, Hybrid 1 and Hybrid 2 CYP79A1 amino acid sequences compared to Genebank gene accession SBU32624. Hybrid 1 and Hybrid 2 have two variations in the sequence, shown here as sequence “a” or “b”.

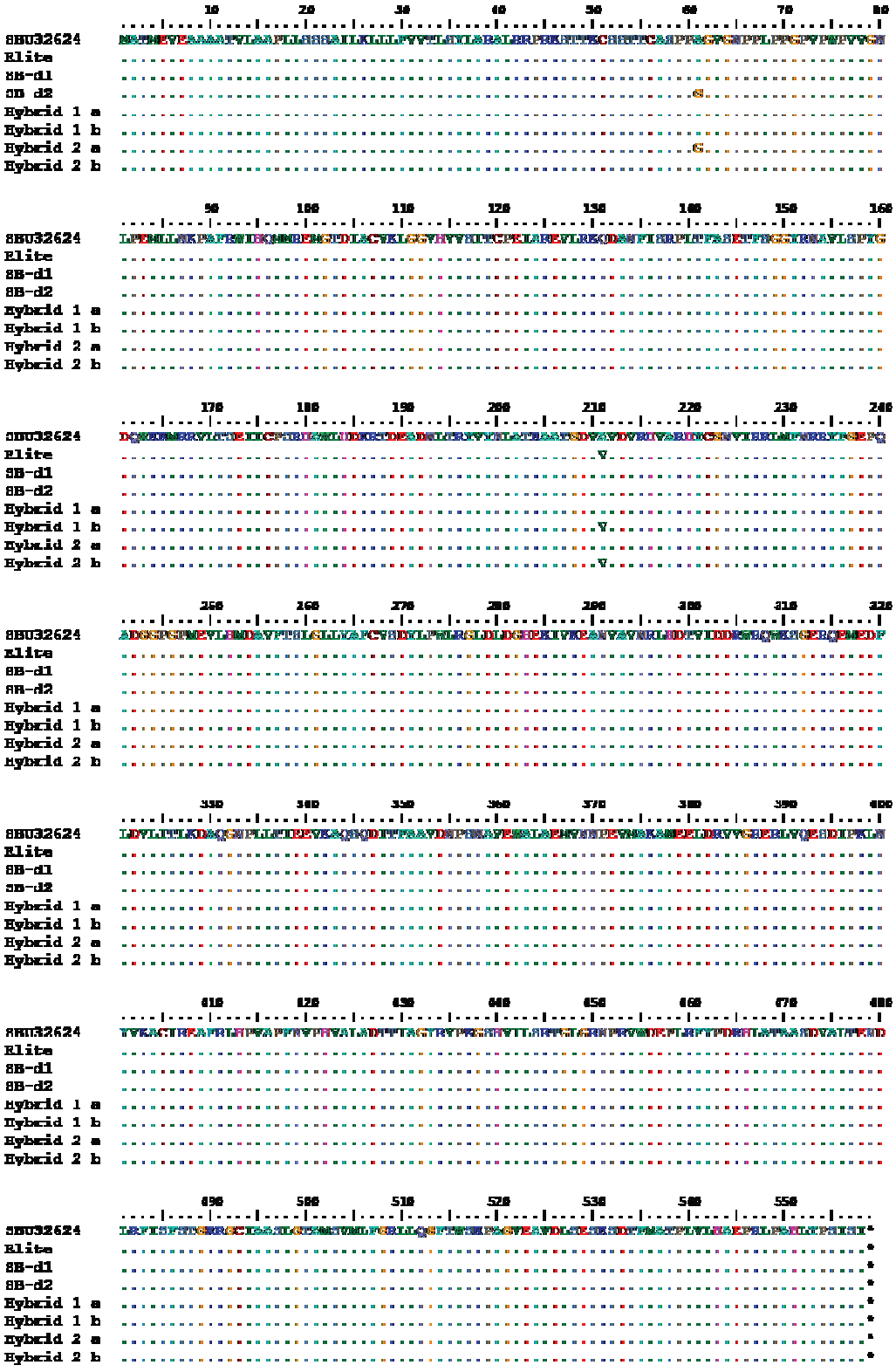
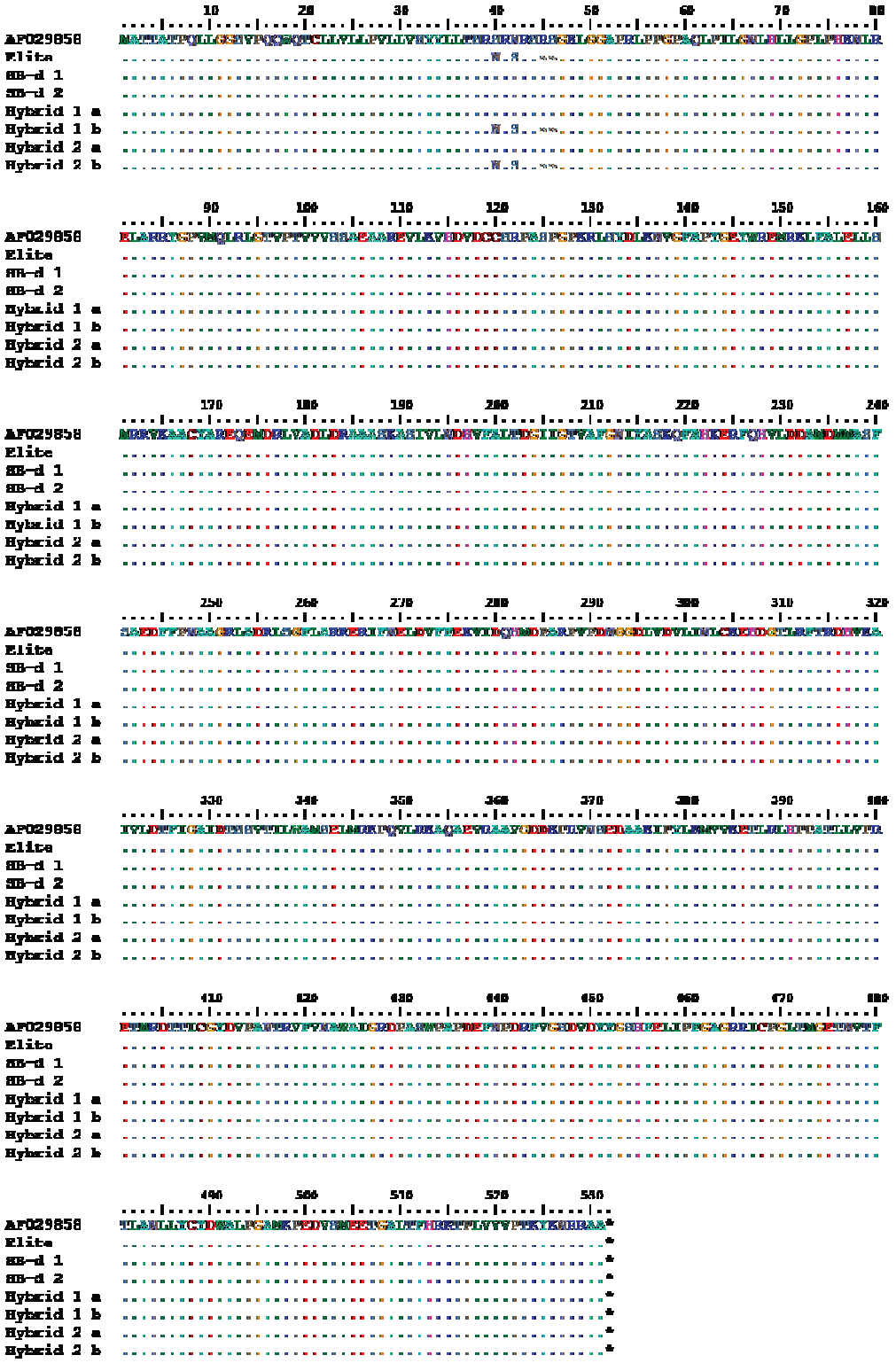


Figure 3.4 Elite, SB-d1 and SB-d2, Hybrid 1 and Hybrid 2 CYP71E1 amino acid sequences compared to Genebank gene accession AF029858. Hybrid 1 and Hybrid 2 have two variations in the sequence, shown here as sequence “a” or “b”.



The UGT85B1 gene sequences from the Elite, SB-d1 and SB-d2 showed nine silent nucleotide changes within the coding region (Appendix 6) and five nucleotide changes which resulted in amino acid changes of the deduced protein compared to the sequences of the Genebank accession AF199453 (*S. bicolor* UGT85B1; Figure 3.5; Appendix 6). Elite and SB-d2 UGT85B1 gene sequences both had changes at G118E, A182T, A274D and Q287K. The SB-d2 line had an additional nucleotide change of F254V, while SB-d1 again had the identical sequence as Genebank accession AF199453.

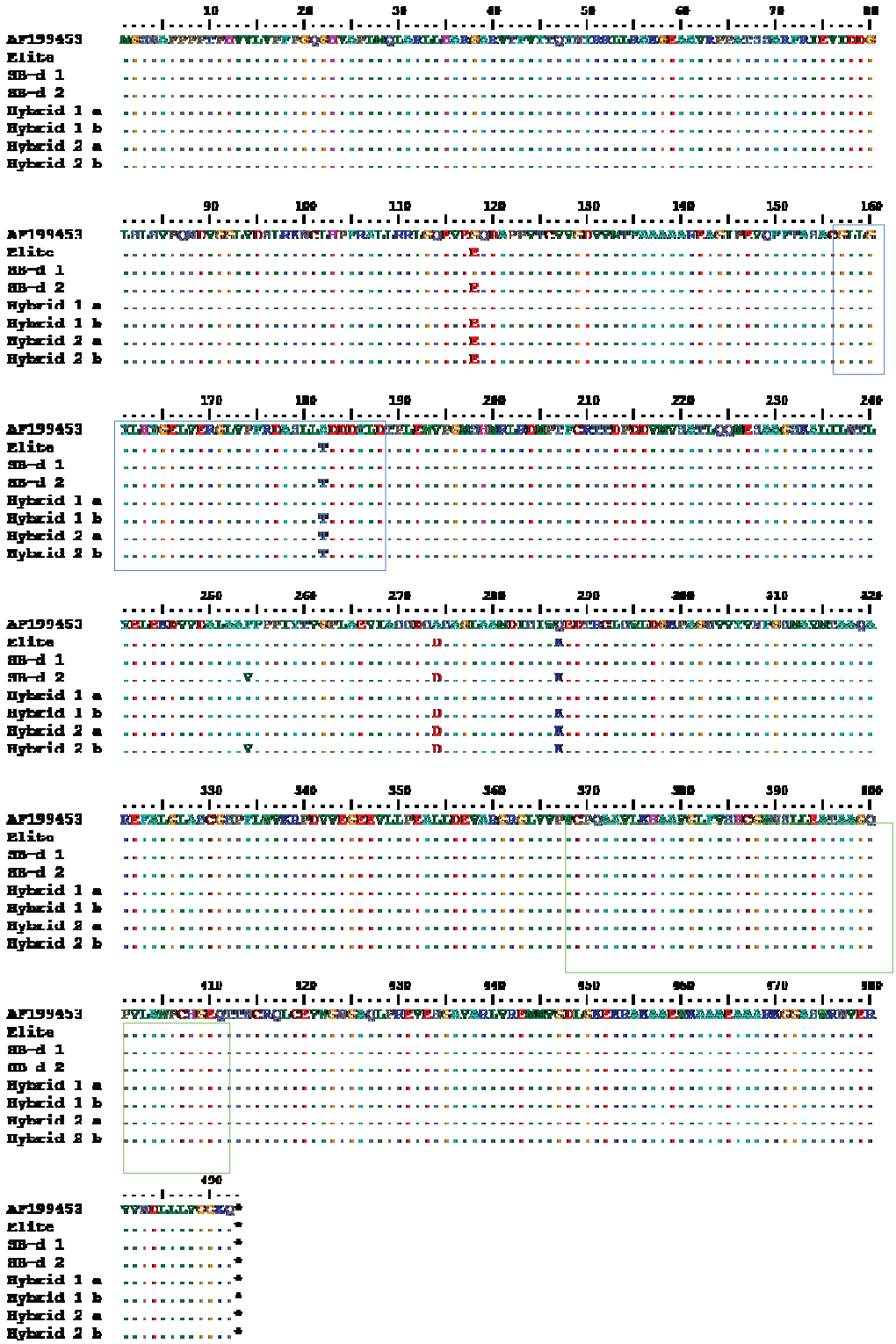
3.3.3 Spatio-temporal dhurrin analysis

3.3.3.1 Spatial analysis of dhurrin

The cyanide concentration of the plants analysed in this experiment varied significantly during the life cycle of the plant (Figure 3.6). The overall trend showed that young plants had a high cyanide concentration which decreased with age. Cyanide was detected in all plants and all plant tissues analysed, except the mature seed, imbibed seed and newly emerged base (Table 3.1 & Figure 3.7). Elite plants had between 75% - 100% higher cyanide concentrations in the leaf blades and sheath cross sections, respectively, compared to Hybrid 1 plants (Figure 3.7). Further analysis of the cyanide concentration of each leaf blade, cross-sections of the sheath and the roots were measured in the Elite and Hybrid 1 lines at the 3-leaf, 5-leaf and 10-leaf growth stages (Figure 3.7).

The cotyledon (leaf blade 1) at the 3-leaf stage for both Elite and Hybrid 1 lines contained the highest cyanide concentration compared to all tissues measured at all time points (Elite 7.64 ± 1.1 mg CN g^{-1} dwt and Hybrid 1 7.94 ± 0.64 mg CN g^{-1} dwt; Figure 3.7a). The cyanide concentrations in the leaf blade of the plants measured at the 3-leaf stage decreased by 85% from leaf 1 to leaf 4 (Elite: p = 0.0003; Hybrid 1: p = 0.0007). At the 5-leaf stage there was no significant difference in the cyanide concentration of all the leaf blades present on the Elite plants (p = 0.1109), but there was a general decrease in cyanide concentration, compared to the leaf blades at the 3-leaf stage. Hybrid 1 plants at the 5-leaf stage showed a different trend in leaf blade cyanide concentration than the Elite plants at the 5-leaf stage. The leaf blades 1-3 of Hybrid 1 , harvested at the 5-leaf stage, showed very low cyanide concentrations (0.06 ± 0.012 to 0.08 ± 0.01 mg CN g^{-1} dwt), while leaf blades 4-7 showed considerably higher

Figure 3.5 Elite, SB-d1 and SB-d2, Hybrid 1 and Hybrid 2 UGT85B1 amino acid sequences compared to Genebank gene accession AF199453. Hybrid 1 and Hybrid 2 have two variations in the sequence, shown here as sequence “a” or “b”. The blue box indicates Loop B which is involved in metabolon formation and dhurrin synthesis. The green box indicates the nucleotide diphosphate sugar- binding domain (Thorsøe *et al.* 2005).



cyanide concentrations (0.73 ± 0.07 to 2.08 ± 0.72 mg CN g^{-1} dwt; Figure 3.7a). At the 10-leaf stage, the leaf blades showed a gradient of cyanide concentrations in both varieties. The cyanide concentration increased from the basal (leaf blade 1) to apical (leaf blade 12) leaves for the Elite (0.02 ± 0.002 to 1.28 ± 0.13 mg CN g^{-1} dwt, $p < 0.0001$) and Hybrid 1 lines (0.03 ± 0.004 to 0.73 ± 0.07 mg cng^{-1} dwt, $p < 0.0001$; Figure 3.7a).

The leaf sheaths for each growth stage showed similar trends to that observed in leaf blades (Figure 3.7b). Overall, the cyanide concentration was higher in the sheath at the 3-leaf stage, but the pattern was not consistent across the age classes. The distribution was also different between the Elite and Hybrid 1 lines. The sheath segments at 3-leaf stage of Elite plants showed a gradient of cyanide concentrations, decreasing 55% from cross-section A (basal) to D (apical) ($p < 0.0001$). A different trend was observed for Hybrid 1: at the 3-leaf stage, cyanide concentration of sheath cross-section A (basal; 1.38 ± 0.19 mg CN g^{-1} dwt) was not significantly different to sheath cross-section D (apical; 1.21 ± 0.09 mg CN g^{-1} dwt), while sheath cross-section B spiked to 3.11 ± 0.18 mg CN g^{-1} dwt ($p < 0.0001$). At the 5-leaf stage, sheath cross-sections A (containing basal meristem tissue; Figure 3.7b) for both Elite and Hybrid 1 lines had cyanide concentrations of > 1 mg CN g^{-1} dwt and had between 25% and 50% higher cyanide concentration than sheath cross-sections B-F at the same growth stage ($p < 0.0001$). Additionally, the cyanide concentrations of the sheath cross-sections B-F in Hybrid 1 plants at the 5-leaf stage were not significantly different to each other, but the cyanide concentration in the sheath cross-sections of the Elite plants doubled from sheath cross-section B to F ($p < 0.0001$). At the 10 leaf stage, the most basally located leaf sheath cross-section (cross-sections A, containing basal meristem tissue; Figure 3.7b) for both the Elite and Hybrid 1 lines had up to 80% higher cyanide concentration than the remaining sheath segments (cross sections B-I; $p < 0.0001$). The sheath cross sections B-I in Hybrid 1 showed less variation than sheath cross-sections B-I in the Elite plants.

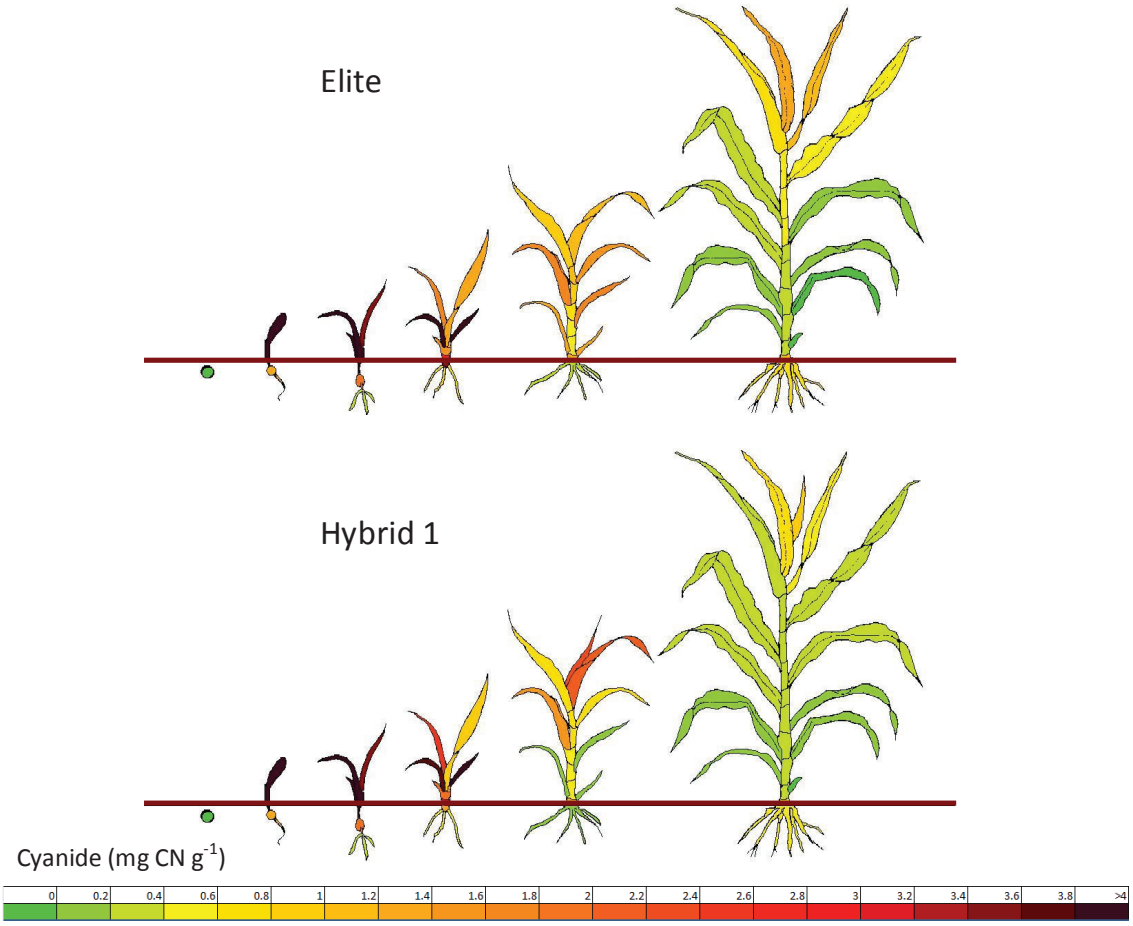


Figure 3.6 Cyanide concentration in individual plant sections from the seed to the 10-leaf stage of Elite and Hybrid 1 sorghum varieties. Each leaf blade, sheath section and the roots were measured separately. The diagram is a summary of Table 3.1 and Figure 3.7. Values are mean (± 1 SE) data, n = 5.

Table 3.1 Cyanide concentration in dry seed and plant tissue up to the 2-leaf stage of Elite and Hybrid 1 plants. Imbibed seed were placed in MQ H_2O for 48 hrs before the cyanide concentration was measured. Newly emerged seedlings were assayed prior to the cotyledon unfurling. Just germinated, 1-leaf and 2-leaf stage seedlings were measured as total shoot, plant base (including the remaining seed) and roots separately. Values are mean (± 1 SE) data, *n* = 5 with each replicate a pooled sample from 3 plants.

		Elite	**Hybrid 1**
Dry Seed		0.02 ± 0.001	0.02 ± 0.001
Imbibed seed		0.02 ± 0.001	0.02 ± 0.001
Newly emerged seedling	shoot	22.85 ± 0.67	26.84 ± 0.63
	base	0.04 ± 0.004	0.03 ± 0.002
	root	1.13 ± 0.12	1.13 ± 0.21
1-leaf	shoot	6.71 ± 0.20	6.60 ± 0.18
	base	1.54 ± 0.07	1.46 ± 0.08
	root	0.73 ± 0.03	0.67 ± 0.03
2-leaf	shoot	3.84 ± 0.17	3.65 ± 0.20
	base	1.91 ± 0.16	1.83 ± 0.15
	root	0.31 ± 0.04	0.28 ± 0.03

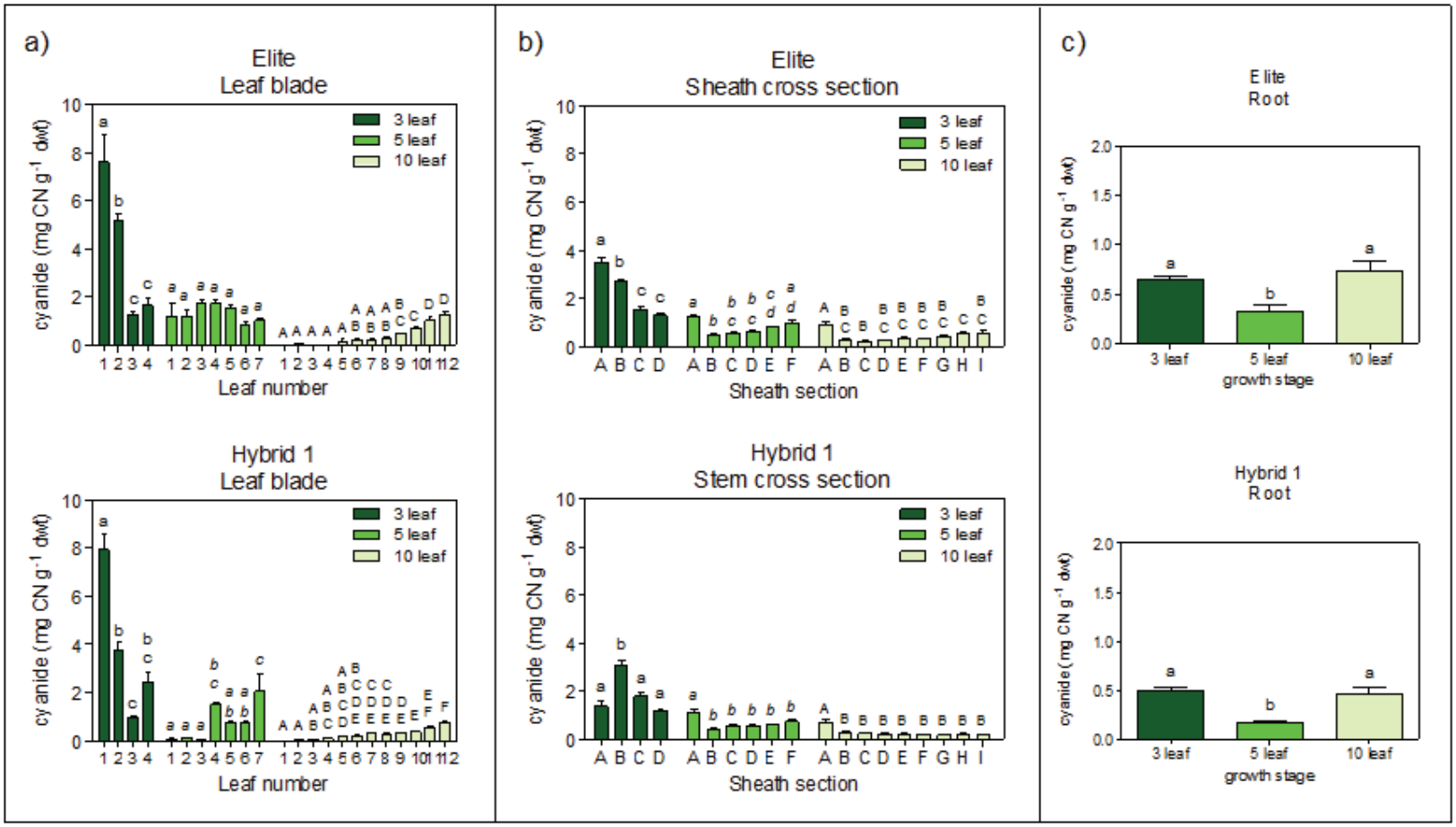


Figure 3.7 Cyanide concentration of individual plant tissues at three different growth stages: (a) leaf blade – 1(cotyledon) to 12 (youngest developing leaf), (b) leaf sheath cross sections – A (basal) to I (apical), and (c) whole root system, from Elite and Hybrid 1 plants. Section A of the leaf sheath contained basal meristematic tissue. Values are mean (± 1 SE) data, n = 5. Significant variation was found in the cyanogenic glycoside concentration of individual tissues at separate growth stages. Post-hoc Tukey's tests were performed and the letters indicate significant difference at $p < 0.05$.

The root cyanide concentration was lower in plant at the 5-leaf stage for both Elite (p = 0.0051) and Hybrid 1 (p = 0.0004) plants compared to the root cyanide concentrations at the 3-leaf and 10-leaf stages (Figure 3.7c), but all samples remained below the toxic threshold.

The field grown Hybrid 1 plants that had the cyanide concentration measured at increments along the leaf blade, showed the tissue next to the ligule contained the highest cyanide concentration (1.17 ± 0.01 mg CN g^{-1} dwt), and decreased to 0.57 ± 0.07 mg CN g^{-1} dwt along the leaf to the apex (p = 0.0027; Figure 3.8). In the roots, the highest cyanide concentration was found at the base of the plant, the root/shoot junction (2.31 ± 0.01 mg CN g^{-1} dwt; Figure 3.9), this tissue was green. The cyanide concentration decreased by more than half in first 10 cm of the root and then maintained a constant cyanide concentration to the root tips (p < 0.0001). This trend was also seen in the root of the Hybrid 2 cultivar (data not shown).

3.3.3.2 Temporal dhurrin analysis

This experiment was designed to allow the cyanide concentration and relative CYP79A1 transcript levels to be determined for equivalent tissue of glasshouse grown plants, with either leaves of the same age from different developmental stages (i.e. lower three leaves from plants at the 3 leaf, 5 leaf or 10 leaf stage) or the same leaves measured over time (Figure 3.10). The Elite line was used for this experiment and overall trends in cyanide concentration observed here are similar to those seen in the spatial experiment (section 3.3.3.2), but a clear distinction in age and position was observed. Taking the leaves from the same position on the plant and following them over time, several patterns emerged. First, the leaves at the base of the plant (α) decreased in cyanide. The cyanide concentration of the α- leaf blades and leaf sheath decreased by 98% and 97%, respectively, from the 3-leaf to the 10-leaf stage (Figure 3.10 and Table 3.2). A similar decrease in cyanide concentration with age was also observed for the β age class. Second, the youngest leaves (groups α, β and δ) present on the plant were found to decrease in cyanide concentration as the plant aged (Figure 3.10). Overall, the cyanide concentration of the youngest leaf blades decreased from 3.18 ± 0.37 mg CN g^{-1} dwt to

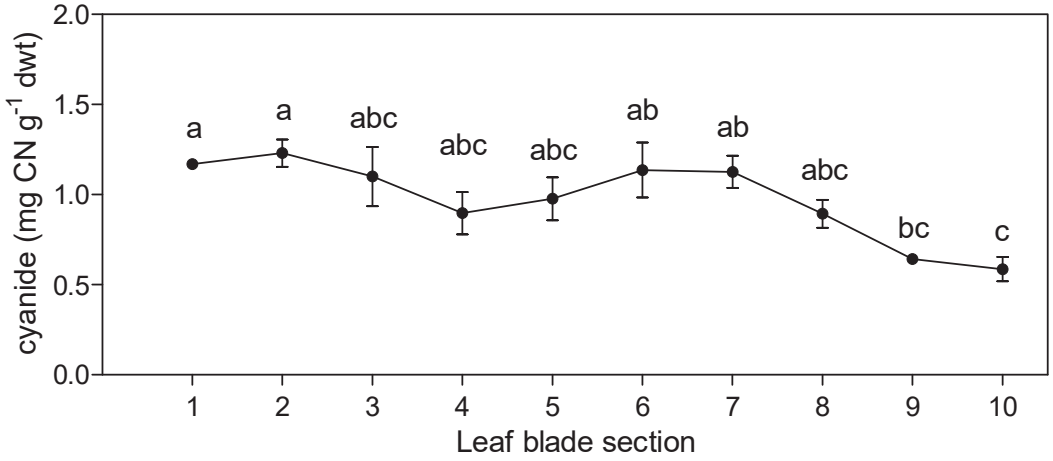


Figure 3.8 Cyanide concentration along the leaf blade of mature field grown Hybrid 1 sorghum plants. Leaf sections were taken from next to the ligule (section 1) and approximately 10 cm apart to the tip (section 10). Values are mean (± 1 SE) data, $n = 3$. Significant variation was found in the cyanide concentration along the leaf blade ($p = 0.0027$). A posthoc Tukey's test was performed and the letters indicate significant difference at $p < 0.05$.

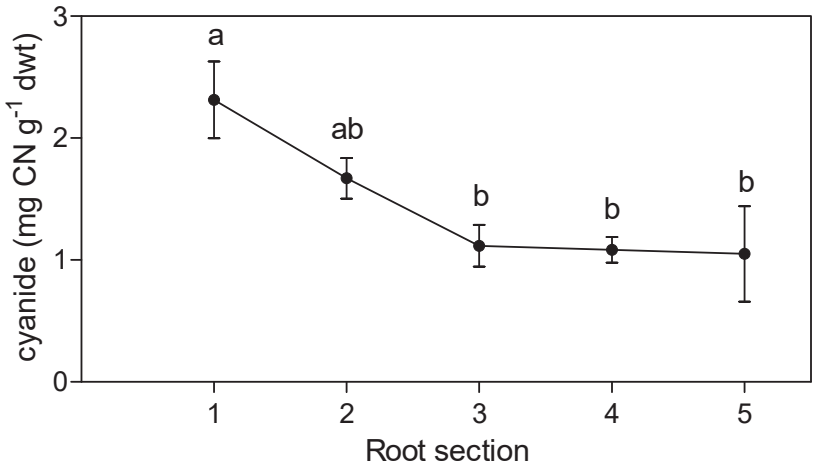


Figure 3.9 Cyanide concentrations along the root of mature field grown Hybrid 1 plants. Measurements were taken approximately 5 cm apart (plant base to root tip). Values are mean (± 1 SE) data, $n = 3$. Significant variation was found in cyanide concentration along the root ($p < 0.0001$). Post hoc Tukey's tests were performed and the letters indicate significant difference at $p < 0.05$.

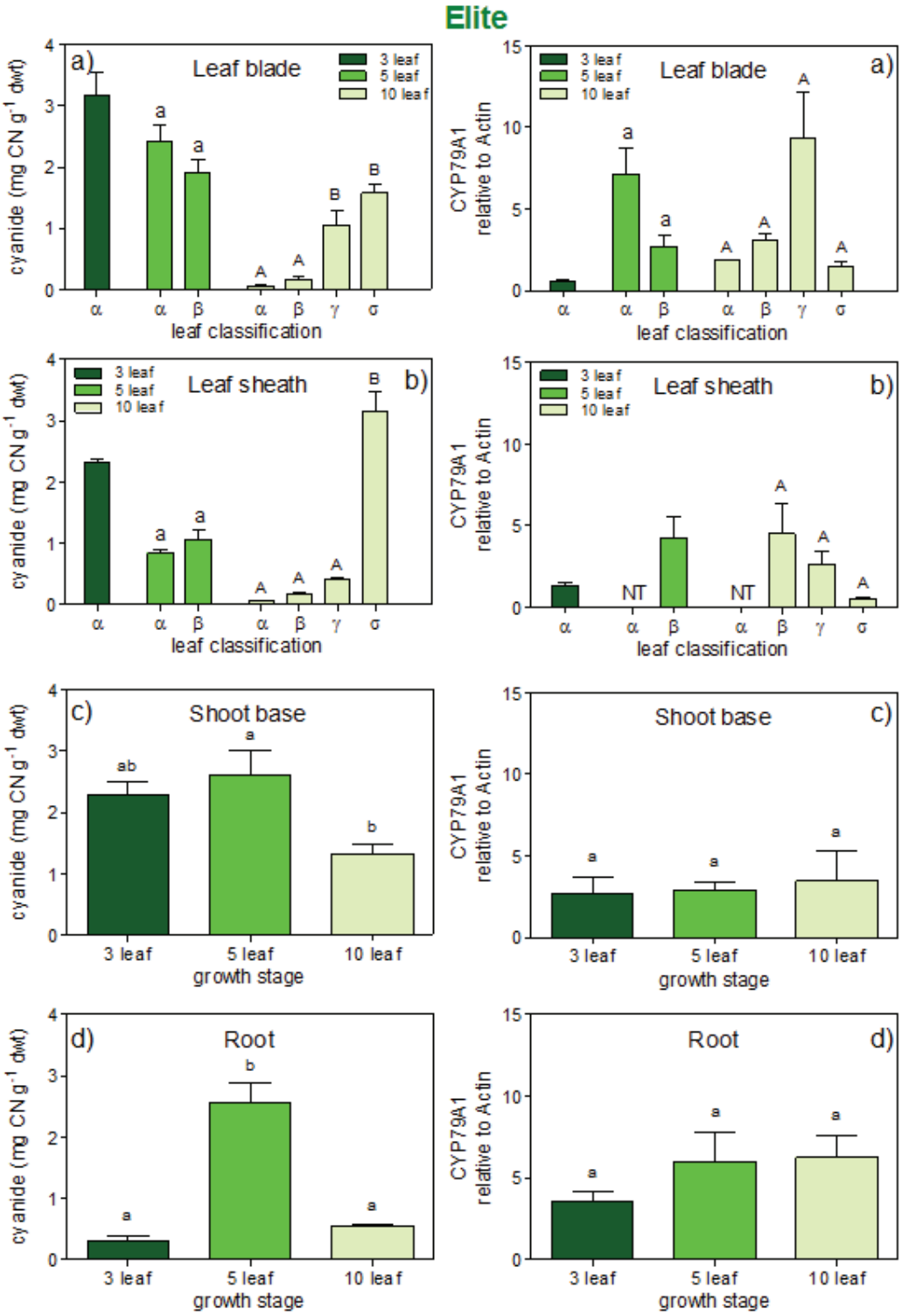


Figure 3.10 Cyanide concentrations and CYP79A1 transcripts of pooled comparative plant tissues at three growth stages in the Elite sorghum line. The tissues measured were (a) leaf blades, (b) leaf sheaths, (c) meristematic tissue and (d) roots. The leaf blades and leaf sheaths were pooled three-fold for the analysis. Values are mean (± 1 SE) data, n = 6 for cyanide analysis and n = 3 for transcript analysis. NT = not tested. Significant variation in the cyanogenic glycoside concentration and CYP79A1 transcript levels was found within plants and between growth stages (Tables 3.2 & 3.3). Posthoc Tukey's tests were performed and different letters indicate significant difference at $p < 0.05$.

1.90 ± 0.23 mg CN g^{-1} dwt between the 3-leaf and 5-leaf stages (α and β leaf groups) respectively. The cyanide concentration observed decreased further to 1.58 ± 0.14 mg g^{-1} dwt in tissues harvested at the 10-leaf stage (δ leaf group; $p < 0.0001$).

The cyanide concentration in the shoot base (containing basal meristem) of the Elite plants varied over time, with a cyanide concentration of 2.3 ± 0.21 mg CN g^{-1} dwt at the 3-leaf stage, increasing to 2.6 ± 0.40 mg CN g^{-1} dwt at the 5-leaf stage and again decreasing to 1.3 ± 1.7 mg CN g^{-1} dwt at the 10-leaf stage (Figure 3.10 & Table 3.3). The root cyanide concentration at 3-leaf stage was 0.32 ± 0.07 mg CN g^{-1} dwt and increased to 2.57 ± 0.32 mg CN g^{-1} dwt at the 5-leaf stage and then decreased again to 0.54 ± 0.02 mg CN g^{-1} dwt at the 10-leaf stages (Figure 3.10 & Table 3.3).

Cyanide concentrations of commercial Hybrid 1 and Hybrid 2 plants showed the same trends for all tissue types analysed as observed in the Elite line (Figure 3.11). However, the Elite line cyanide concentrations were higher than the Hybrid 1 and Hybrid 2 in all plant tissues and the Hybrid cyanide concentrations decreased more rapidly over the time interval than in the Elite line. The total cyanide content in the shoots and roots of Elite, Hybrid 1 and Hybrid 2 lines were also determined. Data showed that the total cyanide content of plants increased with age (Elite $p < 0.0001$, Hybrid 1 $p < 0.0001$ and Hybrid 2 $p = 0.0002$; Figure 3.13). Total shoot cyanide content at 10-leaf stage was compared across cultivars and Elite shoot cyanide content was found to be 37% higher than Hybrid 1 and 51% higher than Hybrid 2 ($p = 0.0031$), but no difference was found in total root cyanide content ($p = 0.2650$).

The relative concentration of CYP79A1 transcripts were determined for the same leaf tissue described for cyanide concentrations for the Elite line. There was no clear relationship between cyanide concentration and relative CYP79A1 transcript abundance in the leaf blades, but in the leaf sheath an inverse correlation was found between the cyanide concentration and CYP79A1 transcript level (Figure 3.12; sheath $r^2 = 0.3124$; p = 0.030). The CYP79A1 transcript level in the α leaf blades were significantly higher at the 5-leaf stage compared to 3-leaf and 10-leaf stages ($p = 0.0126$; Figures 3.10a & 3.10b). Similarly, the CYP79A1 transcript level in the youngest leaves (groups α, β and δ) was significantly higher at 5-leaf stage for leaf blades ($p = 0.0369$; Figure 3.10a) and leaf

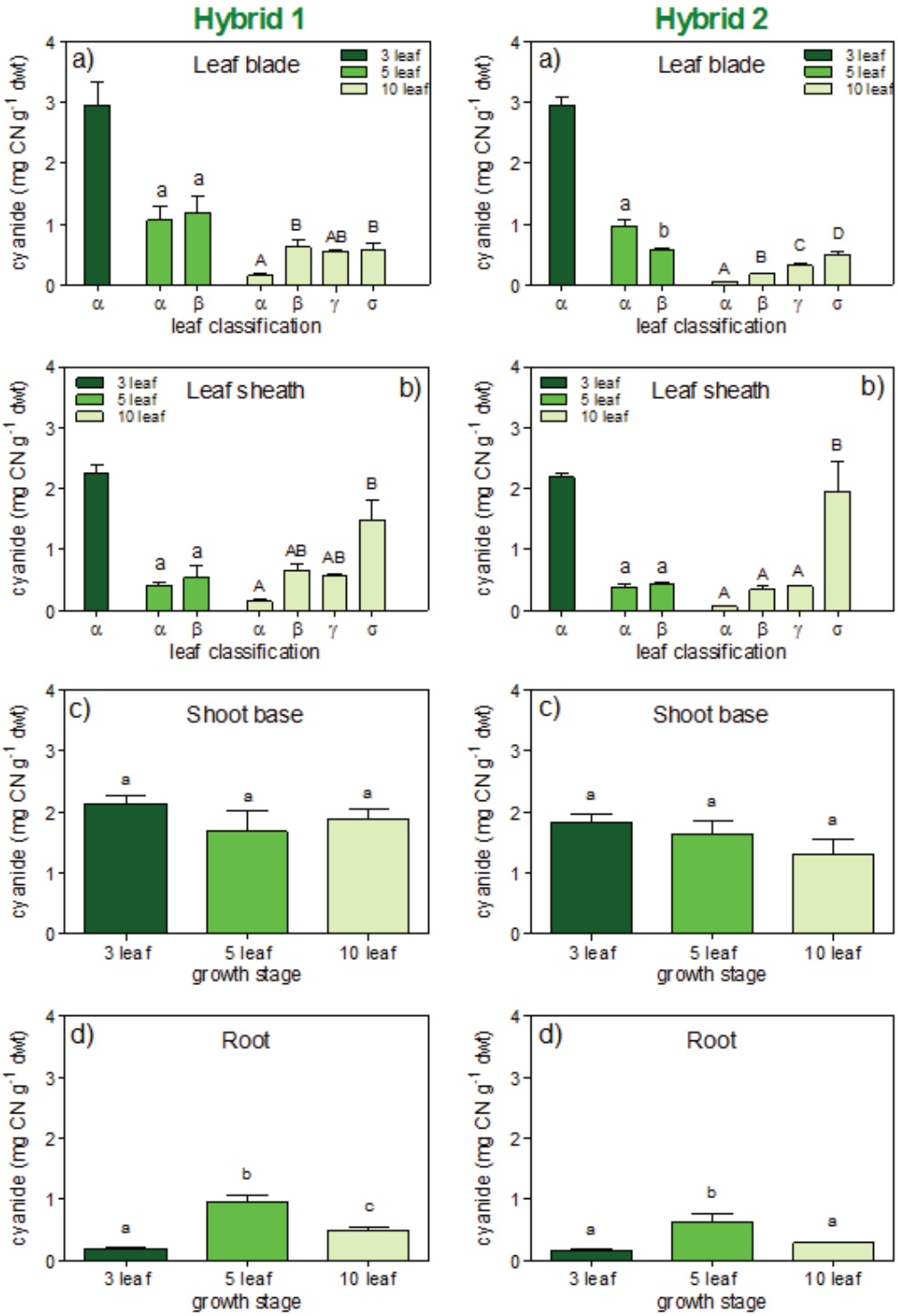


Figure 3.11 Cyanide concentrations of pooled tissues of Hybrid 1 and Hybrid 2 sorghum lines at three growth stages. The tissues measured were (a) leaf blades, (b) leaf sheaths, (c) meristematic tissue and (d) roots. The leaf blades and leaf sheaths were pooled three-fold for the analysis. Values are mean (± 1 SE) data, n = 5. Significant variation was found in the cyanogenic glycoside concentration of samples at separate growth stages and within a growth stage (Tables 3.2 & 3.3). Posthoc Tukey's tests were performed and a different letters indicate significant difference at $p < 0.05$.

sheaths (p = 0.0298; Figure 3.10b) compared to levels observed in tissues harvested at the 3-leaf and 10-leaf stages. When relative levels of CYP79A1 transcripts were regressed against the cyanide concentration a statistically significant negative correlation was found for leaf sheath (cyanide = 2.09-0.266CYP79A1, r^2=0.3124, p = 0.0303; Figure 3.12b). However, relative levels of leaf blade CYP79A1 transcripts were not correlated with cyanide concentrations (p = 0.4654; Figure 3.12a). The shoot base CYP79A1 transcripts did not significantly vary over time (p = 0.9042; Figure 3.10c). Similarly, there was no significant difference in the CYP79A1 transcripts in the roots over time (p = 0.2686; Figure 3.10d).

Table 3.2 Statistical analysis of the data resulting from the cyanide and transcript analysis of Elite, Hybrid 1 and Hybrid 2 lines. One way ANOVA's were conducted and significant p-values are in bold. Four comparisons were made for cyanide concentration and CYP79A1 transcript levels: within plants at 5-leaf and 10-leaf growth stages, α leaves at 3-leaf, 5-leaf and 10-leaf growth stages, and youngest leaves (α, β and δ) over 3-leaf, 5-leaf and 10-leaf growth stages. Cyanide concentration and relative CYP79A1 transcript levels were measured in the Elite line. Only the cyanide concentrations were determined for the Hybrid 1 and Hybrid 2 commercial lines.

	Elite				**Hybrid 1**		**Hybrid 2**	
	CN conc.		*CYP79A1 trans.*		*CN conc.*		*CN conc.*	
	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath	Leaf blade	Leaf sheath
5-leaf stage	0.1818	0.1800	0.0608	NT	0.5787	0.7632	**0.0051**	0.5508
10-leaf stage	**<0.0001**	**<0.0001**	0.0526	0.1332	**0.0062**	**0.0004**	**<0.0001**	**0.0004**
α leaves	**<0.0001**	**<0.0001**	**0.0126**	NT	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
young leaves (α, β and δ)	**0.0025**	**<0.0001**	**0.0369**	**0.0298**	**<0.0001**	**0.0027**	**<0.0001**	**0.0027**

Table 3.3 Statistical analysis for cyanide and transcript analysis of Elite, Hybrid 1 and Hybrid 2 lines. One way ANOVA's were conducted and significant p-values are in bold. Comparisons were made for shoot base and root tissue across 3-leaf, 5-leaf and 10-leaf growth stages. Cyanide concentration and relative CYP79A1 transcript levels were measured in the Elite line. Only the cyanide concentrations were determined for the Hybrid 1 and Hybrid 2 commercial lines.

	Elite		**Hybrid 1**	**Hybrid 2**
	CN conc.	*CYP79A1 trans.*	*CN conc.*	*CN conc.*
Shoot base	**0.0180**	0.9042	0.3754	0.1843
Root	**<0.0001**	0.2686	**<0.0001**	**0.0014**

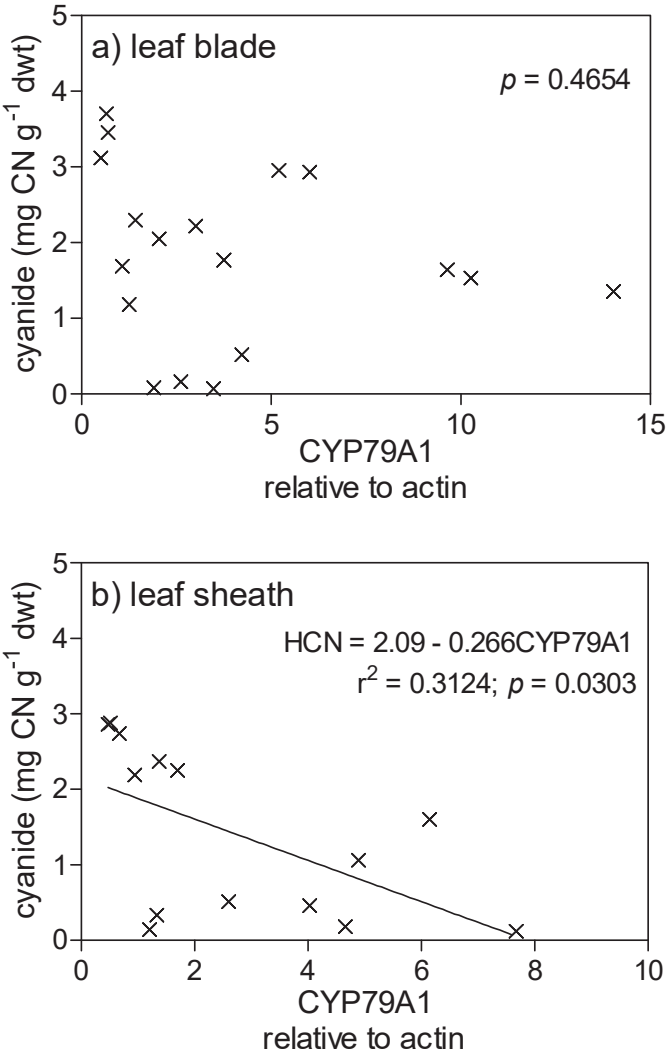


Figure 3.12 The relationship between (a) leaf blade and (b) leaf sheath cyanide concentration and CYP79A1 transcript level of Elite plants at 3-leaf, 5 leaf and 10 leaf stages. Leaf sheath shows a significant negative correlation between cyanide concentration and relative CYP79A1 transcript levels (p = 0.0303), but leaf blade does not show a significant correlation (p = 0.4654).

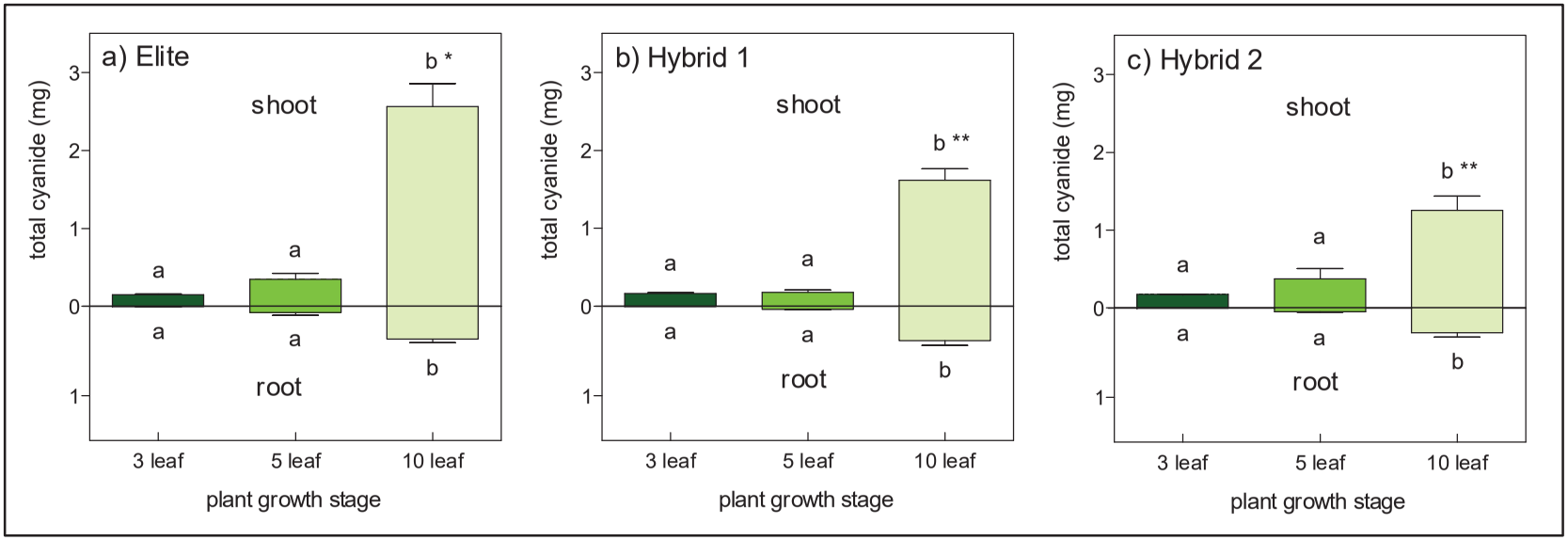


Figure 3.13 Total amount of cyanide in the shoots and roots of the Elite, Hybrid 1 and Hybrid 2 lines at three growth stages. Values are mean (± 1 SE) data, $n = 6$. One-way ANOVA's showed significant variation in the total cyanide contents within a line ($p < 0.0001$). Post-hoc Tukey's tests were performed, the letters indicate significant difference at $p < 0.05$. Shoot cyanide content of Elite lines is significantly higher than Hybrid 1 and Hybrid 2 plants at the 10 leaf stage (* indicates significant difference).

3.4 Discussion

The current study set out to determine, whether variation in cyanide levels, CYP79A1 transcript abundance relative to actin and also coding sequences of the dhurrin biosynthetic genes *CYP79A1*, *CYP71E1* and *UGT85B1* exists in different cultivars of forage sorghum. Results obtained showed that high levels of variation in cyanide concentration between sorghum cultivars were observed. Cyanide concentrations of individual plants were also found to vary spatially, and the spatial pattern observed was age dependant. CYP79A1 transcripts were detected in all plant tissues analysed, with the general relationship between cyanide concentration and CYP79A1 transcript levels showing a negative correlation. Some variation was observed in the coding sequences of genes encoding dhurrin biosynthetic enzymes in samples sourced from different cultivars.

3.4.1 Spatio-temporal dhurrin analysis

As previously established, the cyanide concentration in sorghum decreases with plant maturity (Robinson 1930; Loyd & Grey 1970; Haskins *et al.* 1987). The results presented here show that there are two contributing factors: tissue age and plant age. When leaf blades and leaf sheaths of plants were compared as they aged, a statistically significant decrease in cyanide concentration in older plants was observed. Similarly, when leaf blades of comparable maturity, but from plants of increasing age, were compared there also was a decrease in cyanide concentration. However, leaf sheath of comparable maturity from plants of increasing age (same tissue age from different plants of increasing age), showed cyanide concentrations initially decreased but then returned to a concentration similar to that measured at the first harvest (Figure 3.10). Busk and Møller (2002) reported a marked shift in dhurrin synthesis from the leaf to the stem at 5 weeks of growth. However, due to the young age of the plants in their study it is likely that leaf sheath was measured (Busk & Møller 2002; Møller personal communication), rather than true stem as defined in the current study.

The present study showed that CYP79A1 transcripts were detected in leaf blades and roots at all developmental stages tested, unlike the results reported by Busk and Møller (2002) who showed that CYP79A1 transcripts were only detected in the stem tissue in

the five week old plants and that the dhurrin concentration was positively correlated with the CYP79A1 transcript level. Surprisingly, in the current study the relative CYP79A1 transcript abundance and cyanide concentration in the leaf sheath were negatively correlated. However, the plants in the current study were older than the plants in Busk and Møller's study (2002) and the cyanide concentration of shoot bases containing the basal meristematic tissue was consistently high across all cultivars and growth stages, even though CYP79A1 transcript levels were uniformly comparatively low. It is quite possible that observed HCNp and CYP79A1 transcript levels would have been markedly altered if harvested after the shoot changed to the reproductive phase. Vanderlip *et al*. (1993) found that plants changed from vegetative (leaf producing) to reproductive (head producing) at 30 days post germination, however this was not found in these studies. It is not known whether this is due to the difference in cultivar or growing conditions.

Consistent with earlier studies (Panasiuk & Bill 1984; Selmar *et al.* 1988; Ikediobi & Olugboji 1988; Busk & Møller 2002), the cyanide concentration of seedlings increases rapidly in the first week after germination (Table 3.1). Surprisingly, young sorghum plants (3-leaf; Figure 3.7) had the highest cyanide concentration in the oldest leaves and cyanide concentration declined in the younger uppermost leaves. Around the 5-leaf stage there was a switch, where all leaves contained relatively similar cyanide concentrations. In the plants at the last stage tested (10-leaf) there was a gradient in cyanide concentration where the youngest and most apical leaves contained the highest cyanide concentration and there was a linear decline in cyanide concentration with age, to basally located leaves. Consistent with the literature, the reduction in cyanide concentration with age, resulted in an increase in the total HCNp of the plant with age (Loyd & Gray, 1970). This outcome is also consistent with studies on alkaloid chemical defence compounds, where the total alkaloid content steadily increased over periods of active growth, reaching maximum alkaloid content at or about the time of flowering (James 1950; Gupta *et al.* 1973; Schaffner *et al.* 2003). This indicates that the plant continues to synthesise dhurrin throughout the plant life cycle and the decrease in cyanide concentration is due to a dilution effect, and not necessarily due to reduced synthesis, consistent with the hypothesis made by Gleadow *et al*. (1998).

Previous spatial studies on HCNp of sorghum have mainly concentrated on the shoots (Wheeler *et al.* 1984; Haskins *et al.* 1987) with few studies also looking at the HCNp in the roots of the plants being analysed (Akazawa *et al.* 1960; Lloyd & Gray 1970; Starr *et al.* 1983; Ikediobi & Olugboji 1988; Du *et al.* 1995). Ikediobi and Olugboji (1988) found the root HCNp of six day old sorghum seedlings was approximately 1/6 of shoot HCNp. The results of the current experiments and another study reported in the literature found that roots of sorghum plants contain cyanide concentrations high enough to provide defence against soil borne pests (Starr *et al.* 1983). Furthermore, CYP79A1 transcripts were detected, which may indicate dhurrin is also synthesised in the roots, like alkaloids in *Nicotiana* species (DeBoer 2009; Sinclair *et al.* 2004).

3.4.2 Dhurrin comparison across sorghum varieties

Consistent with the literature (Barnett & Caviness 1968; Benson *et al.* 1969, Haskins *et al.* 1987), variation in HCN potential has been found between sorghum cultivars. In the current study plants grown under controlled growth conditions and tested at the same developmental stage (Figure 3.2) confirmed inherent differences between sorghum lines. Such high degrees of variation in HCNp in sorghum may be due to the comparatively recent domestication, compared to the noncyanogenic crops such as wheat or maize (de Alencar Figueiredo *et al.* 2008). Classically it was believed that cyanide concentrations of *S. bicolor* subspecies *bicolor* plants were higher than *S. bicolor* subspecies *drummondii* plants (Gray *et al.* 1968). Contradictorily to the published literature, both *S. bicolor* subspecies *bicolor* and *S. bicolor* subspecies *drummondii* plants were found to have representative varieties with cyanide concentrations safe for grazing and as well as varieties that were potentially toxic (Figure 3.2; Poulton 1993; Lechtenberg & Nahrstedt 1999). To date no natural occurring populations of *S. bicolor* have been found that are acyanogenic (Blomstedt, O'Donnell *et al.* 2012).

As found in the spatio-temporal dhurrin analysis (section 3.4.1), the relative level of CYP79A1 transcripts for each sorghum cultivar did not positively relate to the shoot cyanide concentration, although there appears to be a negative trend between the relative CYP79A1 transcript levels and the shoot HCN concentration. The Elite, SB-d1, SB-d2 showed little variation in the gene sequences for CYP79A1, CYP71E1 and UGT85B1, but a high amount of variation for down stream HCNp (Figure 3.2).

Additionally, the HCNp of commercial Hybrid 1 and Hybrid 2 did not relate to the HCNp of the respective parents (Elite and Sb-1, and Elite and SB-d2). There was little variation in the CYP79A1, CYP71E1 or UGT85B1 gene sequences and high variation in the HCNp of the plants. The transcript level of the CYP79A1 mRNA was not measured for the Hybrid 1 and Hybrid 2 plants in this experiment. However, the results of the Elite line showing an inverse correlation between CYP79A1 transcript level and HCNp may suggest the variation in the Hybrid lines could be due to regulatory differences, but transport or stability / turnover of the transcript may also be contributing factors.

Dhurrin regulation may not necessarily be at a transcriptional level in older plants, it is important to analyse the dhurrin biosynthetic genes (CYP79A1, CYP71E1 and UGT85B1) further. To our knowledge, none of the genes in the dhurrin biosynthetic pathway in *S. bicolor* subspecies *drummondii* have previously been sequenced. A number of polymorphisms were detected between the Genebank, Elite, SB-d1, SB-d2, Hybrid 1 and Hybrid 2 lines, but most were silent mutations (Figures 3.3, 3.4 and 3.5). As the genetic make up of commercial Hybrid 1 and Hybrid 2 come from the crossing of Elite and SB-1, and Elite and SB-d2, respectively, and the sequences reflect this, only Elite, SB-d1 and SB-d2 are discussed. In most cases the Elite line and the SB-d2 line have the same sequences. The SB-d1 sequence was generally the same as the Genebank sequences.

The alanine to glycine amino acid change (A60G) found in CYP79A1 of SB-d2 (Figure 3.3), is outside any known functional areas in the protein (Werk-Reichhart & Feyereisen, 2000; Jensen *et al.* 2011). The alanine to valine change at position 211 of the deduced protein (A211V) is located between regions known to be associated with heme anchoring (Jensen *et al.* 2011). However, due to the similar properties displayed by each of alanine and valine (same polarity and charge), it may be unlikely that they would impact markedly upon the structure of the protein and/or the resulting dhurrin-synthesising metabolon. The three translational changes found in CYP71E1, a two amino acid deletion (R and S), S119N and N125S in Elite line, are clustered close to the membrane-spanning region (pers. comm. Birger Møller) hence may have an effect on the anchoring of the CYP71E1 to the endoplasmic reticulum, therefore may weaken and/or strength the interaction between the CYP79A1 and CYP71E1, and alter the efficiency of the dhurrin biosynthesis.

In the sorghum lines examined in the present study, a total of five amino acid changes were observed in deduced UGT85B1 proteins. Four of these alterations are in the Elite and SB-d2 cultivars (G118E, A182T, A274D and Q287K) and SB-d2 also contains F254V. The green box in Figure 3.5 highlights the sugar phosphate binding domain of UGT85B1 (Osmani *et al.* 2009), and therefore would not affect substrate binding. The blue box in Figure 3.5 highlights loop B, this region is thought to have a role in the interaction with the two cytochrome P450s (CYP79A1 and CYP71E1) (Thorsøe et al. 2005). The amino acid difference A182T is localised in the hydrophobic cluster of the Loop B. Alanine is neutral and non-polar and threonine is neutral and polar, but it is predicted that this change would have little impact on protein structure Several other domains have been characterised (Thorsoe *et al.* 2005; Kannangara *et al*. 2011), but it is difficult to determine where these domains are conserved in the sequences under examination, as UGT's have generally low sequence conservation, but highly conserved secondary and tertiary structures (Thorsoe et al. 2005; Osmani *et al.* 2009). From studying the structural domains described in Osmani *et al*. (2009) and comparing the amino acid changes found for UGT85B1, it is not thought that the A182T, F254V or A274D amino acid changes would not affect the structure of the protein, however, G118E and Q287K cannot be ruled out. The glycine to glutamine amino acid change, G118E, is a non-polar to polar amino acid substitution, which may be important as the position of this residue lies close to Nβ4 and loopN4 in the N-terminal domain (Osman *et al.* 2009), and therefore may influence the structure of the protein. Similarly, the glutamine to lysine amino acid change, Q287K, changes the pH and is possibly in the Cα0 in the C-terminal domain (Osman *et al.* 2009), hence this alteration may also impact upon the protein. Structural modeling of each of the CYP79A1, CYP71E1 and UGT85B1 proteins and incorporating the amino acid changes found in this study would enable us to gain a better understanding of the protein structures and how together they influence the function of the proposed dhurrin synthesising metabolon as a whole.

3.4.3 Why does sorghum change the allocation of dhurrin?

A good defence system is fundamental to plant survival. A range of hypotheses and theories exist to explain how and why plants allocate valuable resources such as nitrogen to chemical defence systems (Ohnmeiss & Baldwin 1994; Lou & Baldwin 2004;

Simon *et al.* 2010). It has been suggested, however, that most theories suit the specific systems for which they were proposed and often do not hold up when applied elsewhere (Berenbaum 1995). The results of the current study examining the differences in HCNp within sorghum at different stages during over plant maturation, fit well into the optimal allocation of defence hypothesis described in detail by McKey (1974) whereby chemical defense properties of individual plant tissues are determined by two factors: (1) "value," or cost to the plant if the tissue is damaged from, or lost completely to, herbivory; and (2) "vulnerability," the probability of the particular plant tissue being successfully attacked by herbivores in the absence of chemical defenses. It should be noted, however, that this theory is based on the assumption that natural selection has influenced within-plant distribution of defensive compounds (McKey 1974). Goodger *et al*. (2007) pointed out this theory neglects to consider that the level of chemical defence may not be directly proportional to the total cost of defence because other factors may be impacting on plant growth and metabolism (Purrington 2000; Stowe *et al.* 2000; Strauss *et al.* 2002). In experiments described here, the plants were grown under nutrient rich conditions, to endeavor to study effects of age without the possible confounding data stemming from nutritional limitations.

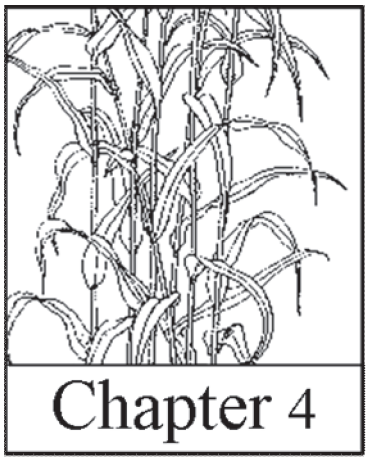
The large range in HCNp observed across the plant and at different stages of maturity is consistent with a defensive cost being associated with a capacity to produce dhurrin in sorghum. If there were no cost associated with defence then presumably all parts of the plant would be equally defended (Webber & Woodrow, 2009). It is thought that young shoots are more highly defended because young tissue is valuable and vulnerable to attack because of the concentrated nutrients (Palo 1984; Schardl & Chen 2001), thus herbivores often prefer them to older, less nutrient-rich tissues.

3.4.4 Conclusion

The experiments detailed in this chapter have furthered the understanding of the distribution of dhurrin within sorghum plants. This not only improves the current understanding of HCN potential of sorghum, but is also of benefit to farmers by helping to determine when to use sorghum fodder for their animals.

It is not known whether the changes detected in the dhurrin biosynthesis gene sequences would affect enzyme activity, but it is unlikely because the number of sequence variations does not account for larger variation observed in HCNp. It is more likely that the genes are under regulatory control and the regulation is the subject of continuing investigation. The current research also found that the dhurrin allocation within the plant shifts with time. This result shows that consistent harvesting methods should be used in order for results to be meaningful. The ontogenetic and phenological stages are both more important drivers of HCNp, than chronological age. Moreover, leaf number is a good indicator of ontogenetic stages.

It is already widely known that young sorghum should not be fed to cattle due to toxicity. Rotational grazing of cattle in separated fields allows for the production of more forage on an area basis, owing to the increased time given for the plants to grow, while also minimising the risk of cattle grazing on the highly toxic re-growth. Such practices should be continued until a simple field-based testing kit is readily available.



Effects on hormones, wounding and altered nitrogen nutrition on dhurrin in sorghum

4.1 Introduction

Cyanogenic glycosides, such as dhurrin, are generally thought to act as defence compounds against herbivory (Tattersall *et al.* 2001; Gleadow & Woodrow 2002; Møller 2010a) but have also been reported to play a role in nitrogen turnover and storage (Selmar *et al.* 1988; Adewusi 1990; Gleadow & Woodrow 2000a, 2002b; Jørgensen *et al.* 2005; Kongsawadworakul *et al.* 2009; Møller 2010b). Cyanogenic glycosides are synthesised from aromatic or aliphatic amino acids (Conn 1981) that are first converted to an oxime and then to a nitrile that is finally stabilized by glycosylation. The three enzymes involved in this process (CYP79A1, CYP71E1 and UGT85B1), and also a NADPH-dependant cytochrome P450 reductase (CPR) (Møller & Conn 1979; Jones *et al.* 1999; Bak *et al.* 2006; Ellis *et al.* 2009; Jensen & Møller 2010), have been shown to function as components of a dhurrin synthesising metabolon in sorghum (Jørgensen *et al.* 2005b; Kristensen *et al.* 2005; Nielsen *et al.* 2008). Relatively little is known about how these pathways are regulated.

4.1.1 Phenotypic expression of HCNp

Cyanogenic glycoside concentration is typically higher in young plants in most species (Panasiuk & Bills 1984, Ikediobi & Olugboji 1988; Gleadow & Woodrow 1998, 2000a), and also in plants where growth is limited by some other environmental variable such as

low phosphorous concentrations (Kriedeman 1964; Wheeler 1990), or drought (Aikman *et al.* 1996; Gleadow & Woodrow 2002b; Woodrow *et al.* 2002), although it is not clear how this is mediated. Application of a high amount of fertiliser containing high concentrations of nitrogen results in an increased HCNp in many species (Kriedeman 1964; Muldoon 1985; Wheeler 1990; Jorgensen *et al.* 2005a). However, a correlation has not been detected between nitrogen supply and HCNp in several tropical plants including cassava (*Manihot esculenta* Cranz. Gleadow *et al.* 2009b), *Rhyparosa kurrangii* (Webber & Woodrow 2009) and *Prunus turneriana* (FM Bailey) Kalkman (Miller *et al*. 2004). Indeed, multiple studies have found that the cyanogenic glycoside concentration is more highly associated with genetic traits rather than with environmental factors, such as nitrogen supply (Briggs 1990; Gleadow & Woodrow 2000a; Goodger & Woodrow 2002; Goodger *et al.* 2004).

In young sorghum seedling (<2 weeks post germination), there is no apparent correlation between nitrogen supply and dhurrin. Yet in older plants, where the constitutive levels of dhurrin are lower, a correlation between nitrogen supply and dhurrin has been repeatedly detected in both glasshouse (Busk & Møller, 2002a) and field studies (Kriedeman 1964; Eck, 1976; Wheeler *et al.* 1980, 1990). Kriedeman's (1964) studies indicated that there was a threshold where an increase in soil nitrogen did not further increase the dhurrin concentration. The Wheeler *et al.* (1980) study also found an increase in HCNp with nitrogen fertilisation, however, there was also a reduction in dry matter yield in plants grown under higher nitrogen when there was not an adequate supply of phosphorous. The true significance of these results are difficult to interpret as there was an inconsistent establishment of plants in the different field sites, and plants may not have received the same nutrients across sites due to differences in planting density. A further study by Wheeler *et al.* (1990) included two nitrogen treatments and a considerable increase in dhurrin was observed in the high nitrogen treatment group. As found for Wheeler *et al.* (1990), it is often assumed that higher nitrogen in the soil will result in higher foliar dhurrin concentrations, however, the results in this case are confounded by plant age, the possibility of a sensitivity threshold level and genotype.

4.1.2 Phytohormones and cyanogenic glycoside production

Phytohormones are important signaling molecules that positively and/or negatively regulate physiological processes within plants, primarily growth, differentiation and development (Davies 2010). Although little is known about the link between cyanogenic glycosides and nitrogen, it is possible that alterations in endogenous hormones may play a critical role. Simplistically, it is often proposed in text books that each hormone controls specific metabolic processes, although increasingly it is realized that a complex interplay between levels and types of hormones is likely to be involved in the control of most, if not all aspects of plant growth and metabolism (Jaillais & Chory 2010). This section focuses on effects of Gibberellic acid (GA), Kinetin (K), Abscisic acid (ABA), Methyl jasmonate (MeJa) and Salycilic acid (SA), as these hormones are known to regulate processes that are also known to influence cyanogenic glycoside concentrations. GA is primarily involved in plant development, such as seed germination, elongation growth, flowering time and floral development (Archard *et al.* 2007; Jiang *et al.* 2007). Cytokinins are also involved in promoting plant development (Mok & Mok 2001; Howell *et al.* 2003) by increasing cell division and altering patterns of cellular differentiation (Shumaker & Gizinski 1993; Gan & Amasino 1995; Zhang *et al.* 1996; Riou-Khamlichi *et al.* 1999). Among other things, ABA is a hormone which regulates stomatal apertures (Cornish & Zeevart 1985; Radin & Hendrix 1987) and is therefore thought to be associated with responses to drought. There have also been conflicting reports that ABA may have direct effects on photosynthesis (Fischer *et al.* 1986; Ward & Bunce 1987; Rascke & Patzke 1988). The jasmonates (jasmonic acid, JA) and salicylic acid (SA) are known to be associated with wounding response within sessile organisms (Baldwin 1998; Lee *et al*, 2004; Hildreth *et al.* 2012). JA, and its volatile methylated derivatives methyl jasmonate (MeJa) (Baldwin *et al.* 2006) has a multiplicity of roles affecting seed germination, root growth, fertility, fruit ripening, senescence and chemical defence (Wasternack & Parthier 1997; Creelman & Rao 2002; Wasternack & Haus 2002). SA also influences seed germination, seedling establishment, cell growth, respiration, stomatal closure, senescence-associated gene expression, responses to abiotic stress and fruit yield (Rate *et al.* 1999; Morris *et al.* 2000; Metwally *et al.* 2003; Clarke *et al.* 2004; Norman *et al.* 2004; Rajou *et al.* 2006).

JA and SA-regulated genes have been found to be upregulated in sorghum (*S. bicolor*) plants treated with MeJa, SA or attacked by phloem-feeding greenbug aphid (*Schizaphis graminum*) (Zhu-Salzman *et al.* 2004). An additional study of sorghum investigating the transcriptional changes following MeJa, SA and aminocyclopropane carboxylic acid (ACC) treatments found MeJa induced dhurrinase and hydroxynitrile lyase transcripts in the shoots, these enzymes are involved in the catabolism of dhurrin (Figure 1.2). In contrast, SA suppressed dhurrinase transcripts in roots but not in the shoots (Salzman *et al.* 2005). It appears that MeJa and SA may have opposite effects on the genes involved with the breakdown of dhurrin. Whether or not the genes in the dhurrin biosynthetic pathway were included in these studies is not clear.

4.1.3 Wounding effects in plants

Different methods of wounding plants have also been shown to have different effects on plant metabolism, for example, in *Nicotiana tabacum* alkaloid synthesis is up-regulated by mechanical wounding and JA treatment (Baldwin *et al.* 1994; Baldwin 1998; Park *et al.* 2002; Cane *et al.* 2005). Unlike sorghum and the dhurrin biosynthesis pathway, nicotine synthesis takes place in the roots and the alkaloids are transported to the shoots (Baldwin *et al.* 1994; Hildreth *et al.* 2012). Extensive studies into the relationship between wounding, JA and alkaloid synthesis has shown a relatively rapid increase in JA in roots after wounding of the leaves (1-2 hours) (Baldwin *et al.* 1997) and a much more gradual increase in leaf alkaloids, peaking about 1 week after wounding (Baldwin *et al.* 1997; Sinclair *et al.* 2004). It was concluded that wound-induced increases in leaf JA are an important component of the long-distance signal-transduction pathway (Baldwin *et al.* 1997). Wounding and herbivory studies conducted on other cyanogenic species have found no induction of HCNp (Bennet & Wallsgrove 1994; Gleadow & Woodrow 2000b; Ballhorn *et al.* 2007; Hayden *et al.* 2010) or a reduction in the HCNp of the plants post herbivory (Lieberei 1986; Cipollini & Gruner's 2007). Furthermore, there are no known studies in sorghum that look at wounding and the resulting dhurrin concentration.

4.1.4 Aim and hypothesis

To date there have been few in depth studies into the regulation of dhurrin biosynthesis. It is known that certain conditions lead to an increase in HCNp but the causes are not yet

understood. It is hypothesised here that sorghum HCNp increases when the plants are experiencing abiotic and biotic stress with certain phytohormones playing key roles in mediating this increase in dhurrin content. The aims of the experiments described in this chapter were thus (1) to determine whether the application of different classes of phytohormones affect the dhurrin concentration of sorghum plants; (2) to determine if mechanical wounding has an effect on HCNp in sorghum plants and if so, whether different forms of wounding provoke similar or different effects on dhurrin and (3) to examine the difference in the effect of nitrogen supply on HCNp in 3 week and 6 week old plants.

Specific experiments were designed to test each of the research questions. A hydroponic system for sorghum was established which enabled plants to be grown uniformly and to ensure they all received adequate nutrients (section 2.2). Experiments were designed to determine whether wounding, specific hormones and nitrogen had an effect on dhurrin concentration. The hormones used in the following experiments were chosen based on the results of preliminary studies (data not shown). The wounding study was designed not only to gain a better understanding of the regulation of the HCNp of sorghum plants, but also to clarify whether sampling methods used for other experiments in this research program can adversely affect deduced cyanide concentrations of plant tissues.

4.2 Methods

All experiments conducted in this chapter were performed on Hybrid 1 sorghum plants grown in a glasshouse in the hydroponic system, described in section 2.2. All seeds were germinated in soil and seedlings were transferred to the hydroponic system at the 3-leaf stage. Plants received altered Hoagland's nutrient solution which was changed once a week for the first four weeks in hydroponic system and replaced twice a week after that, due to the increased nutrient requirements of larger plants. The nutrient solution was never allowed to be fully depleted and the hydroponic pots were rinsed when the nutrient solution was changed to remove residual nutrient solution. See section 2.2 for full details.

4.2.1 Wounding of sorghum plants

One hundred and twenty nine plants were grown to the 9-10 leaf stage (see Figure 2.1) with altered Hoagland's nutrient solution containing 5mM NO_3^-. The plants were randomly assigned to one of six wounding treatment groups (Figure 4.1): (i) control (21 plants) – no wounding; (ii) hole punch (21 plants) – a single hole (0.9 cm diameter) was excised from the top four fully developed leaves using a hand held hole punch, leaf punches were positioned in the middle of the leaf lamella, avoiding the leaf midrib; (iii) pin board (21 plants) – a 10×10 cm board covered in 0.5 mm point pins 0.5 cm apart was pressed into each leaf blade and the stem (leaf sheath); (iv) leaf removal (21 plants) – the youngest fully expanded leaf was removed at the ligule; (v) perforator (21plants) – a perforating wheel was rolled across each leaf in a criss-cross pattern; and (vi) root removal (21 plants) – one quarter of the root mass was removed. The cyanide concentration of the leaf blades, leaf sheath and roots from all treatments (n = 3) was measured at time (T) = 0, 12 h, 24 h, 2 d, 4 d, 6 d and 8 d. Plant height was also measured for all plants at time 0 h, 2 d, 4 d, 6 d and 8 d post-wounding.

4.2.2 Hormone effects on dhurrin concentration

Eighty four plants were grown in the hydroponic system described in section 2.3, with the modified Hoagland's nutrient solution containing 5mM NO_3^-. When the plants reached the 9-10 leaf stage, they were randomly assigned to one of five treatment groups: Abscisic acid (ABA), Gibberellic acid (GA), Kinetin (K), Methyl jasmonate (MeJa), Salicylic acid (SA), and control (C). The hormones were dissolved in the appropriate solvent before adding to the hydroponic nutrient solution (Table 4.1), resulting in the following concentrations; 150 µM ABA, 100 µM GA, 20 µM K, 100 µM MeJa, and 1 mM SA, along with the unaltered nutrient solution control. Due to the volatile nature of MeJa, plants within this treatment were sealed within plastic bags. A preliminary study was conducted which included a control for the bagging of plants and the bags were shown to have no effect on the HCNp of the plants (Adams 2008).

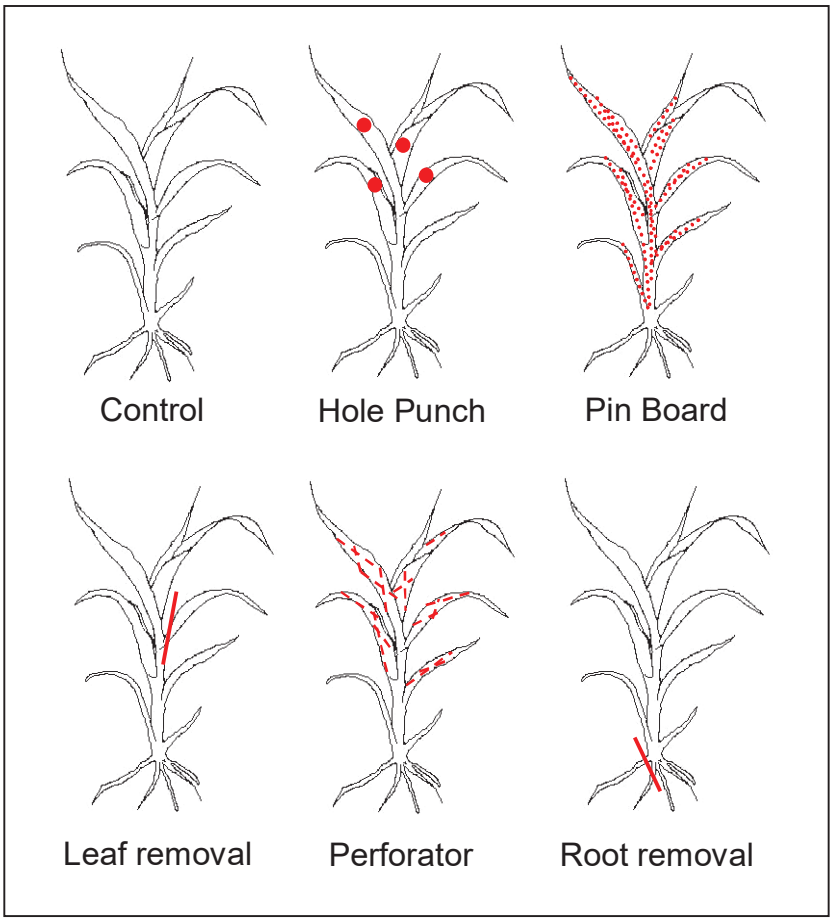


Figure 4.1 Wounding treatments. Control – no treatment. Hole punch – avoiding the leaf midrib, a single hole (0.9 cm diameter) punched into the middle of the top four fully developed leaves using a single hand held hole puncher. Pin board – a board pins 0.5 cm apart pressed into each leaf blade and the stem (leaf sheath). Leaf removal – the youngest fully expanded leaf cut at the ligule of the leaf. Perforator – a perforating wheel rolled across each leaf in a criss-cross pattern. Root removal – one quarter of the roots removed.

Table 4.1 Hormone treatments.

Treatment name	Chemical used	Solvent	Final concentration
Control	no treatment		-
ABA	Abscisic acid	1 M NaOH	150 µM
GA	Gibberellic acid	100% EtOH	100 µM
K	Kinetin	1 M NaOH	20 µM
MeJa	Methyl Jasmonate	100% EtOH	100 µM
SA	Salicylic acid	100% EtOH	1 mM

The plant height, leaf number and the cyanide concentration was measured using the leaf disc method (section 2.5; Gleadow, O'Donnell *et al.* 2012) before treatments were imposed (T0) and 2, 5 and 10 days following treatment onset. The height of the MeJa treated plants was not measured due to difficulties associated with being contained within a bag.

4.2.3 Nitrogen effects on sorghum

At the 3-leaf stage of plant growth, twenty Hybrid 1 sorghum seedlings were transferred into the hydroponic system, described in section 2.3. The plants were separated into five treatment groups (n = 4) and each group was given Hoagland's nutrient solution containing either, 1.25, 2.5, 5, 7.5 or 15 mM NO_3^-. The KNO_3 and $Ca(NO_3)_2$ were adjusted with KCl and CaCl, respectively, to balance the compounds. This experiment was conducted twice with both sets of plants grown under the same conditions, however, one experiment was terminated after 3-weeks and the other after 6-weeks. The nutrient solutions were replaced each week for the plants grown in 3-week trial. During the 6-week trial, nutrient solutions were changed once a week for the first four weeks, then twice a week thereafter. At the time of harvest, the number of mature leaves and plant height was recorded and shoot and root material was oven dried, weighed and assayed for cyanide concentration, and total nitrogen and carbon content. Due to the small root size after the 3-week treatment, root material was not collected and only the shoot tissue was harvested. Both the shoot and root tissue was harvested for the 6-week treated plants. The harvested tissue was dried and weighed before the cyanide concentration and the total carbon and nitrogen were determined.

4.2.4 Sample analysis

Dhurrin analysis

The concentration of dhurrin was measured as evolved cyanide from 0.01 g of dried ground tissue. The cyanide was extracted with the addition of exogenous β-glucosidase and a colorimetric cyanide assay was used, as described in Gleadow, O'Donnell *et al.* (2012), section 2.4.2.

Carbon-nitrogen analysis

Total carbon-nitrogen concentrations were determined on 5 mg dried plant material using an Elementar Vario Micro Cube, CHNS anlayser and acetanilide (Merck, Australia) was used as an internal standard.

Statistical analysis

Results were analysed using GraphPad prism 5 (GraphPad Software Inc., USA). Normality and homogeneity of variances tests were performed prior to analysis. Data that was not normally distributed was log transformed. Experiments with two treatments were compared using a *Student's t-test*. A one-way analysis of variance (*ANOVA*) was performed on groups of plants that had more than two treatment groups. Means that were significantly different were compared *post hoc* using Tukey's t-tests and different letters indicate significant differences. Two-way analysis of variance (*ANOVA*) was performed on experimental data with more than two treatments and significantly different means were compared *post hoc* using Bonferroni's post-test. Correlation coefficients and regression equations were calculated using Minitab 15 (Minitab Inc.). Where the y-axis intercept and regression slopes of two test groups were not significantly different, the data was treated as a single population for regression analysis. Mean values are followed by one standard error of the mean (± 1 SE).

4.3 Results

4.3.1 Wounding of sorghum plants

The effect of mechanical wounding on cyanogenic glucoside concentration was tested in sorghum using various wounding techniques (Figure 4.1). Pin board, perforator and root removal wounding methods resulted in significant changes in cyanide concentrations but these changes were not uniform across the wounding types. 'Hole punch' and 'leaf removal' wounding showed no significant difference in cyanide concentration across the plant or a change in height, compared to the controls (Table 4.2). A noteworthy observation was that there was a general decrease in cyanide concentration in all treatments and tissues at six days. The nutrient solution for the plants was changed at four days. It is not believed that this could be the cause.

'Pin board' wounding had the largest effect on cyanide concentration compared to all other wounding treatments. The cyanide concentration of both the leaf blade and leaf sheath peaked at four days. The leaf blade cyanide concentrations of the 'pin board' wounded plants were up to four-fold higher than the control plants at two days, four days, and eight days post wounding (Figure 4.2a; Table 4.2). The cyanide concentration in the leaf sheath also increased by up to four-fold compared to the control plants at two days, four days, six days and eight days post wounding, however, the overall cyanide concentration in the leaf sheath was approximately 45% lower than the leaf blade cyanide concentrations (Figure 4.2b; Table 4.2). There was no effect from the 'pin board' on cyanide concentration in the roots. The leaf blade cyanide concentrations of the 'perforator' wounded plants were up to three-fold higher than the control plants at two days, four days and eight days post wounding (Figure 4.2a; Table 4.2). Wounding with the 'perforator' had no effect on the cyanide concentration in the leaf sheath or the roots.

Interestingly, cyanide concentrations were highly variable, but not significantly different, in the roots across all wounding treatments. For example, the cyanide concentration four day post wounding ranged from 0.56 ± 0.05 mg CN g^{-1} dwt to 0.93 ± 0.18 mg CN g^{-1} dwt (p = 0.3460; Figure 4.2c). The plant in the wound treatment group that had a quarter of their roots removed consistently had the highest cyanide concentration across time-points measured. The cyanide concentration of the 'root removal' plants eight days post wounding was almost double the cyanide concentration

of the control plants and was the only time-point to be significantly higher than the control plants for the roots (Table 4.2).

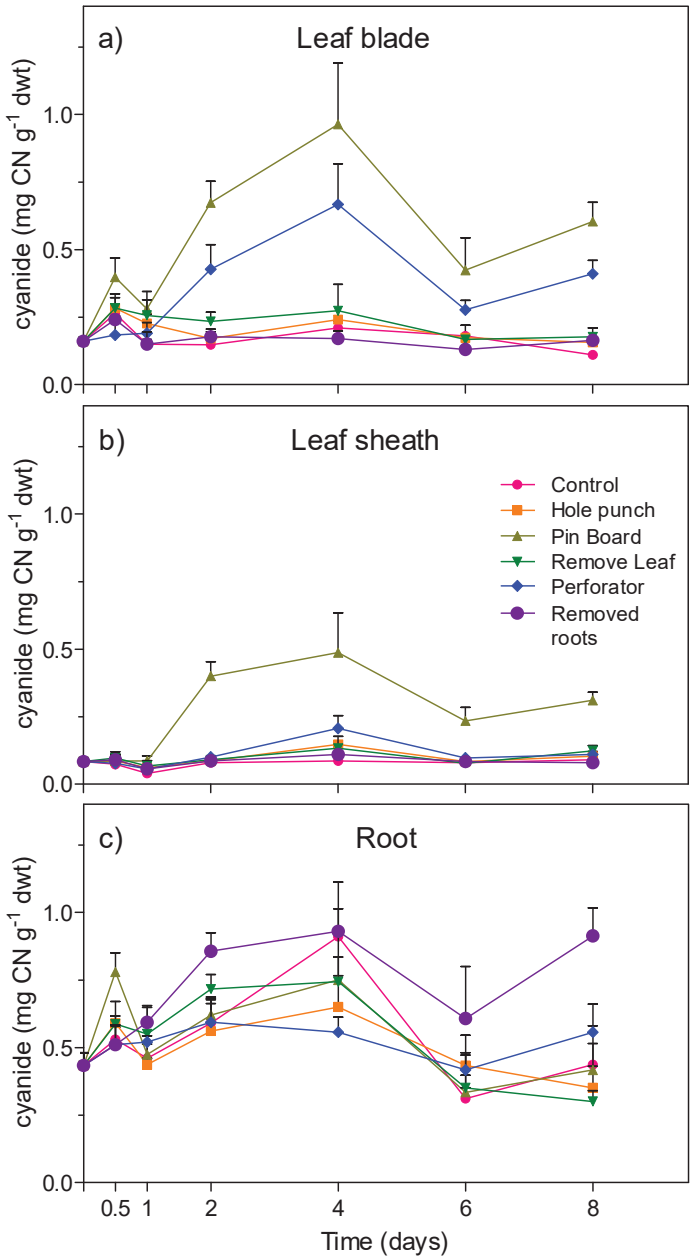


Figure 4.2 The (a) leaf blade, (b) leaf sheath and (c) root cyanide concentrations of hydroponically grown sorghum plants wounded by the 5 methods detailed in section 4.2.2; hole punches to the leaves, pins board punctured the whole plant, a leaf cut off, a perforator run over the leaves and one quarter of the roots cut off (Figure 4.1). Untreated treatment control plants were also tested at each time point. Values are mean (± 1 SE) data from n = 3 plants.

Leaf blade	Time since treatment														
	0			2 days			4 days			6 days			8 days		
	B	*S*	*R*	*B*	*S*	*R*	*B*	*S*	*R*	*B*	*S*	*R*	*B*	*S*	*R*
Control v Hole punch	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Control v Leaf removed	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Control v Root removed	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	**$p < 0.01$**
Control v Pin board	ns	ns	ns	**$p < 0.001$**	**$p < 0.001$**	ns	**$p < 0.001$**	**$p < 0.001$**	ns	ns	**$p < 0.01$**	**$p < 0.001$**	**$p < 0.001$**	ns	ns
Control v Perforator	ns	ns	ns	**$p < 0.05$**	ns	ns	**$p < 0.001$**	ns	ns	ns	ns	ns	**$p < 0.01$**	ns	ns

Table 4.2 Significant *p*-values generated by Bonferroni post tests comparing the cyanide concentration of the leaf blades (*B*), leaf sheathes (*S*) and roots (*R*) of wounded plants to the leaf blades of control plants at the five time points post treatments. $p < 0.05$ was considered significant.

The height of the pin board wounded plants was, 22 ± 0.8 cm tall, 15% shorter than control plants (27 ± 0.9 cm) eight days post wounding ($p < 0.05$ Bonferroni post-test). Wounding by the perforator, root removal, leaf removal and holes punched from the leaves showed no significant difference in plant height compared to the controls ($p = 0.7218$; Figure 4.3).

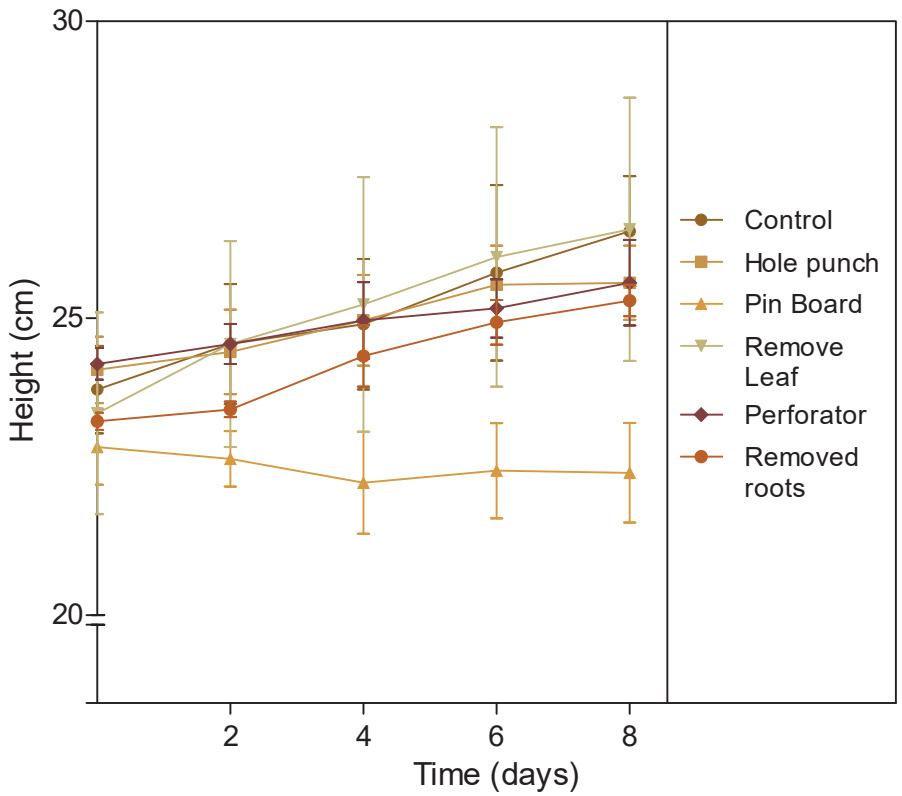


Figure 4.3 The height of wounded and control plants at times 0, 2, 5 and 10 days post wounding. Values are mean (± 1 SE) data from $n = 3$ plants. The plants wounded with the pin board were significantly shorter than the controls at 10 days ($p < 0.05$) post treatment but there was no significant difference in height for any other treatment at any time point ($p > 0.05$).

4.3.2 Hormone effects on dhurrin concentration

Specific hormones were added to the nutrient solution of sorghum plants growing in a hydroponic system and the effect on the cyanogenic glucoside, dhurrin, was measured. Abscisic acid (ABA), Kinetin (K) and Methyl jasmonate (MeJa) induced a significant increase in cyanide concentration compared to the control plants five days after application, with a 300% 200% and 250% increase in dhurrin compared to the control plants, respectively, ($p < 0.001$ for all treatments at T = 5 days; Figure 4.4). By 10 days post treatments, the cyanide concentrations of the ABA, K and MeJa treated plants had returned to control levels. In contrast, Salicylic acid (SA) and Gibberellic acid (GA) were not found to affect cyanide concentration (Figure 4.4, respectively).

The plants treated with GA showed a considerable growth response, with a significant increase in height ($p < 0.001$) and leaf number ($p < 0.001$; Figures 4.5 and 4.6). Specifically, GA treated plants increased in height by 34 ± 1.01 cm in ten days and gained three extra leaves, compared to the control plants which only grew 2.26 ± 0.41 cm and gained one extra leaf. Interestingly, ABA treated plants produced significantly fewer leaves than control plants after five days ($p < 0.05$; Figure 4.6), but this trend was not reflected in plant height (Figure 4.5). Ten days after treatments, the ABA treated plants were statistically the same height and had the same number of mature leaves as the control plants. The height and number of mature leaves for the SA, ABA and Kinetin treated plants were not significantly different to the control ($p > 0.05$), in addition, the MeJa treated plants had the same number of leaves as the controls ($p > 0.05$).

Table 4.3 The *p*-values generated by Bonferroni post test comparing the cyanide concentration of various treatments of sorghum plants to the control plants after 10 days. ns = not significant

Treatment comparison	Time since treatment			
	0	2 days	5 days	10 days
Control v ABA	ns	ns	***p* < 0.001**	ns
Control v GA	ns	ns	ns	ns
Control v K	ns	ns	***p* < 0.001**	ns
Control v MeJa	ns	ns	***p* < 0.001**	ns
Control v SA	ns	ns	ns	ns

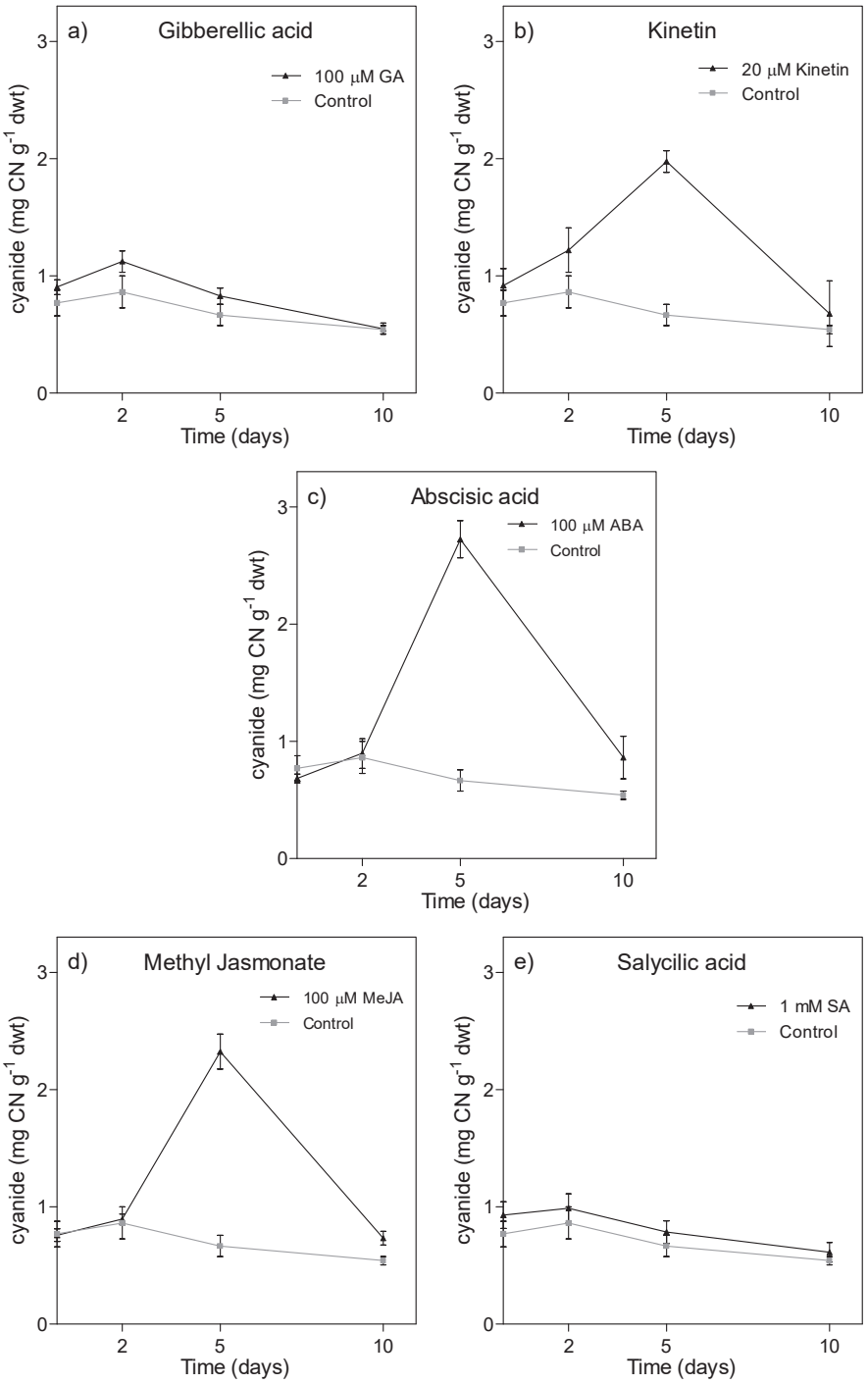


Figure 4.4. Leaf blade cyanide concentrations of hydroponically grown sorghum plants treated with (a) 100 μM Gibberellic acid (GA), (b) 20 μM Kinetin, and (c) 100 μM Abscisic acid (ABA), (d) 100 μM Methyl Jasmonate (MeJa), and (e) 1 mM Salicylic acid (SA). Untreated control plants were also tested at each time point. Values are mean (± 1 SE) data from n = 7 plants.

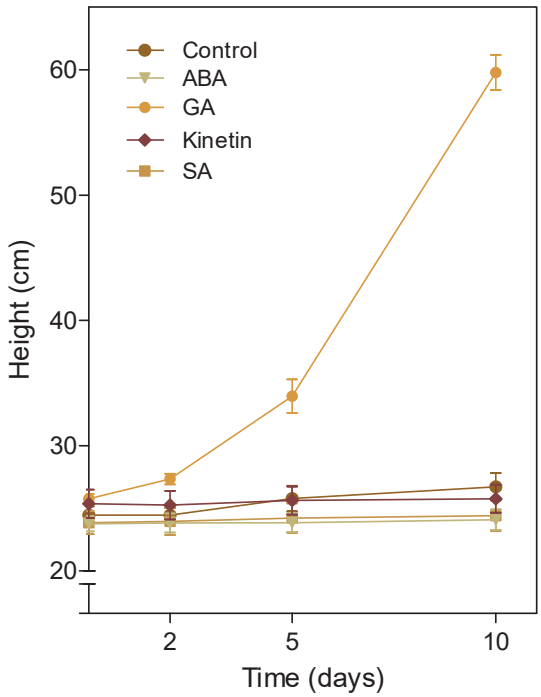


Figure 4.5 The height of hormone treated plants at times 0, 2, 5 and 10 days post hormone treatments. Values are mean (± 1 SE) data from n = 7 plants.

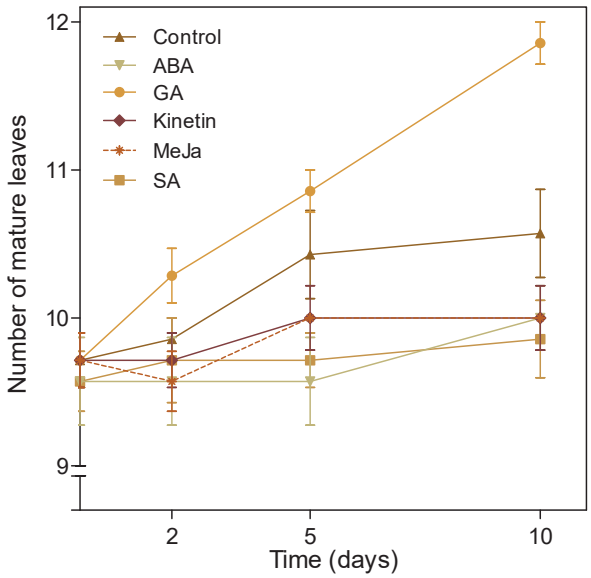


Figure 4.6 Number of mature leaves present for hormone treated plants at times 0, 2, 5 and 10 days post treatments. Values are mean (± 1 SE) data from n = 7 plants. GA treated plants at 10 days ($p < 0.001$) have significantly more mature leaves and ABA treated plants at 5 days ($p < 0.05$) have significantly less mature leaves than the control plants.

4.3.3 Nitrogen effects on sorghum

The height of plants supplied with different nitrate concentrations in the nutrient solution were not significantly different after 3-weeks (p = 0.516) or after 6-weeks treatment (p = 0.294) and no interaction was found between the harvests (p = 0.3573; Figure 4.7a). The dry shoot mass was also not significantly different between plants with different nitrogen supply after 3-weeks (p = 0.806) or 6-weeks (p = 0.153) and no statistical interaction was detected between harvest times (p = 0.1164). Although, there was a non-significant trend showing that shoot mass increased in plants grown with more nitrate (Figure 4.7b). After six weeks there was no significant difference in dry root mass in plants from the different treatments (p = 0.8618; Figure 4.7b). Surprisingly the dry mass of the roots and shoots were statistically the same across treatments (p = 0.609).

The cyanide and nitrogen concentrations in the shoot tissue of nitrate treated plants were determined. Overall, the shoots from 3-week nitrate treated plants showed higher cyanide concentrations than plants treated for 6-weeks and there was no statistical interaction detected between plants treated for different amounts of time (p = 0.0993; Figure 4.8). After 3-weeks of nitrate treatments there was no significant difference in shoot cyanide concentration (p = 0.2241), in spite of this there appeared to be a positive trend, in that the plants treated with the lowest concentration of nitrate (1.25 mM NO_3^-) had the lowest cyanide concentration of (1.23 ± 0.14 mg CN g^{-1} dwt) and the plants in the maximum nitrate treatment of 15 mM NO_3^- had the highest cyanide concentration (1.80 ± 0.21 mg g^{-1} dwt). After 6-weeks nitrate treatments, there was a significant increase in cyanide concentration in the plants treated with increased nitrate application (p < 0.0001; Figure 4.8). However, the 5 mM NO_3^- treatment appeared to be the threshold for maximum cyanide concentration in older plants, with 5 mM, 7.5 mM and 15 mM NO_3^- treated plants having cyanide concentrations that were not significantly different to each other (p = 0.9535; Figure 4.8), whilst the cyanide concentration of the 1.25 and 2.5 mM NO_3^- treated plants was 76% and 66% less than the 5 mM NO_3^- treated plants, respectively. The cyanide concentration of the roots were also measured for plants treated with nitrate for 6-weeks and no significant difference was found between treatments (p = 0.0777).

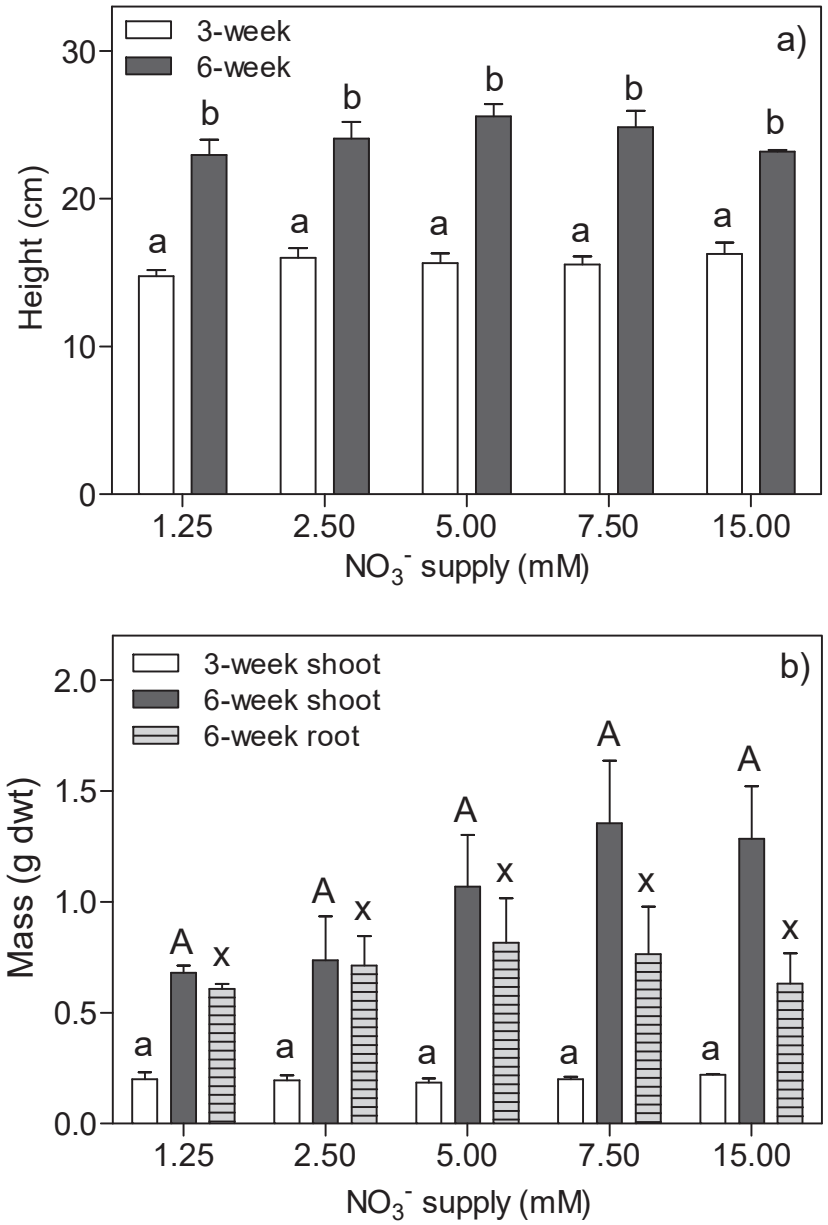


Figure 4.7 The (a) height and (b) dry mass of sorghum plants grown in hydroponics for three or six weeks with either 1.25, 2.5, 5, 7.5 or 15 mM NO_3^{-1} in the nutrient solution. The dry mass of the shoots and roots of the plants was measured separately. Values are mean (± 1 SE) data from *n* = 4 plants.

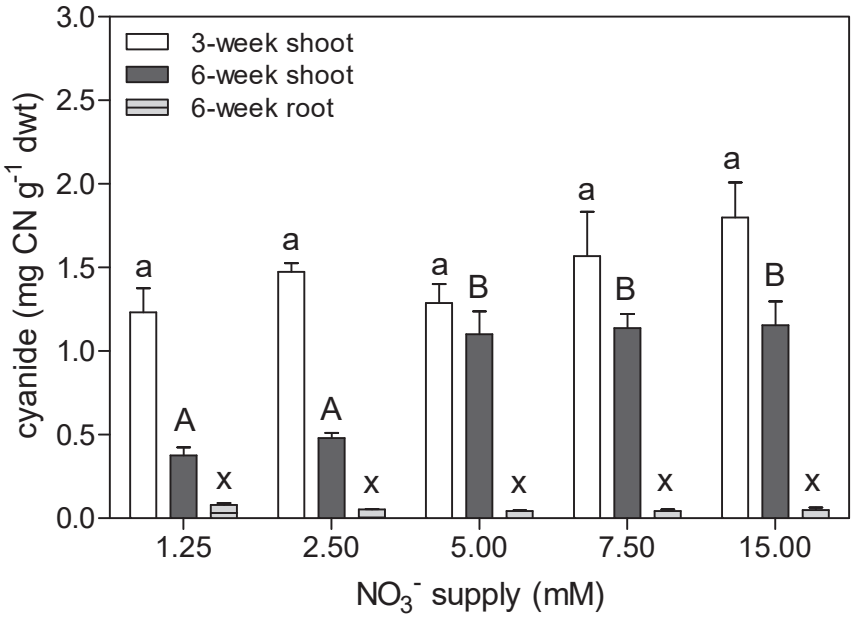


Figure 4.8 Cyanide concentration of sorghum plants grown in hydroponics for 3-weeks or 6-weeks with either 1.25, 2.5, 5, 7.5 or 15 mM NO_3^- in the nutrient solution. Cyanide measurements were taken on the shoots and roots for the 6-week treatment group. Values are mean (± 1 SE) data from n = 4 plants.

The plant nitrogen concentrations were measured and showed similar trends to that observed for the cyanide concentrations. Overall, the shoots from 3-week nitrate treated plants showed higher nitrogen concentrations than plants treated for 6-weeks (Figure 4.9). An interaction was found for the nitrogen concentration of the plants between the 3-week and 6-week treatment groups (p = 0.0123). After 3-weeks of different nitrate supply, the plants grown with the lowest concentration of nitrate (1.25 mM NO_3^-) had a nitrogen concentration of 33 ± 2.8 mg N g^{-1} dwt which was significantly lower than all other treatment groups (p < 0.0001; Figure 4.9). Additionally, the plants treated with 5 mM, 7.5 mM and 15 mM NO_3^- had statistically the same nitrogen concentration. A significant increase in nitrogen concentration was also seen with increased nitrate application after 6-weeks treatment (p < 0.0001; Figure 4.9). The 1.25 mM NO_3^- treated plants contained the lowest nitrogen concentration, at 14.3 ± 0.848 mg g^{-1} dwt, significantly lower than the plants treated with 2.5 mM, 7.5 mM and 15 mM NO_3^-, but not

to the 5 mM NO_3^- treated plants (23.9 ± 3.5 mg N g^{-1} dwt). The nitrogen concentration of the 2.5 and 5 mM NO_3^- treated plants were approximately 40% lower than in the 15 mM NO_3^- treated plants. Unlike the effect on cyanide concentration, increased nitrate application showed no maximum threshold for nitrogen concentration within the shoots.

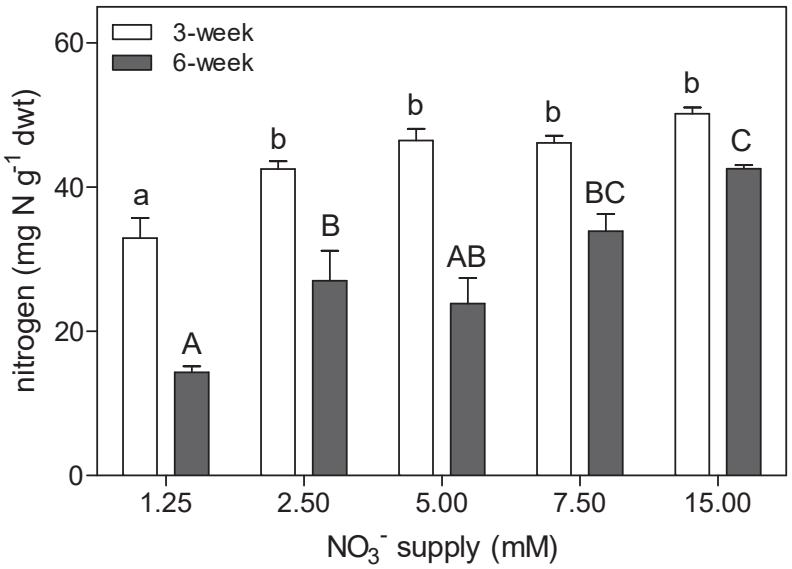


Figure 4.9 Nitrogen concentrations of sorghum plants grown in hydroponics for 3-weeks or 6-weeks with either 1.25, 2.5, 5, 7.5 or 15 mM NO_3^{-1} in the nutrient solution. Nitrogen measurements were taken on the shoots (sht). Values are mean (± 1 SE) data from n = 4 plants.

The carbon-nitrogen ratio (C:N) (Figure 4.10) and the proportion of nitrogen allocated to cyanide in the shoot, represented by CN-N/N% (Figure 4.11), were calculated for plants treated with nitrate for 3-weeks and 6-weeks. A statistical interaction was found for the C:N of the plants between the 3-week and 6-week treatment groups ($p < 0.0001$). After 3-weeks C:N in 1.25 mM NO_3^- treated plants was 20% higher than in the plants treated with between 2.5 – 15 mM NO_3^- ($p = 0.0006$), whilst after 6-weeks of treatment, the C:N of the 1.25 mM NO_3^- treated plants was over double the C:N of the 2.5 – 15 mM NO_3^- treatment groups ($p < 0.0001$). The increase in C:N was driven by the reduced

nitrogen content of the 1.25 mM NO_3^- treated plants. After 3-weeks of nitrate treatments, there was no significant difference in the proportion of nitrogen allocated to cyanide in the shoots for plants treated with different nitrate concentrations (p = 0.7404). After 6-weeks of nitrate treatment, the 2.5 mM NO_3^- treated plants had 65% less nitrogen allocated to cyanide than the 5 mM NO_3^- treated plants (p = 0.0113), but both were not significantly different to 1.25, 7.5 or 15 mM NO_3^- treated plants (Figure 4.11). A statistical interaction was detected for the proportion of nitrogen allocated to cyanide between the 3-week and 6-week treatment groups (p = 0.0271).

The total amount of cyanide and nitrogen in the entire shoot were calculated using the concentrations and known final mass. The total amount of cyanide was also calculated in the roots of the 6-week treated plants. A significant interaction was detected for the total amount of cyanide in the shoots between the 3-week and 6-week treatment groups (p = 0.0008). After 3-weeks no significant difference in total shoot cyanide content was found between nitrate treatments (p = 0.1543; Figure 4.12). After 6-weeks there was a significant overall increase in total shoot cyanide content (p = 0.0025). In the 1.25 mM NO_3^- treated plants the total shoot cyanide content (0.26 ± 0.04 mg) was approximately 80% lower than 7.5 and 15 mM NO_3^- treated plants. The 2.5 mM NO_3^- treated plants (0.47 ± 0.05 mg) had 70% lower total shoot cyanide content than the 15 mM NO_3^- treated plants, while other treatment groups were not significantly different to each other (Figure 4.12). Interestingly, 1.25 mM NO_3^- treated plants had statistically the same total cyanide content after 3- and 6-weeks treatment (p = 0.7014). The total cyanide content of the roots was calculated for plants treated with nitrate for 6-weeks and no significant difference was found between treatments (p = 0.8507).

The differences observed in the total cyanide contents were similar to those of the total nitrogen content. The total nitrogen content was not significantly different between treatments after 3-weeks (p = 0.0758; Figures 4. 13), however, after 6-weeks the total shoot nitrogen content significantly increased with nitrate supply (p = 0.0038; Figures 4.13) and a significant interaction was detected between the 3-week and 6-week treatment groups (p = 0.0013). Total shoot nitrogen content of 1.25 mM NO_3^- (9.64 ± 0.11 mg) was approximately 80% lower than the 7.5 mM and 15 mM NO_3^- treated plants, while 2.5 mM NO_3^- treated plants (19.65 ± 5.09 mg) were 64% lower than the 15 mM

NO_3^- treated plants and all other treatment groups were not significantly different (Figure 4.13). As seen for the total cyanide content, the total nitrogen content of the 1.25 mM NO_3^- treated plants was statistically the same after 3- and 6-weeks of the nitrate treatments (p = 0.1011).

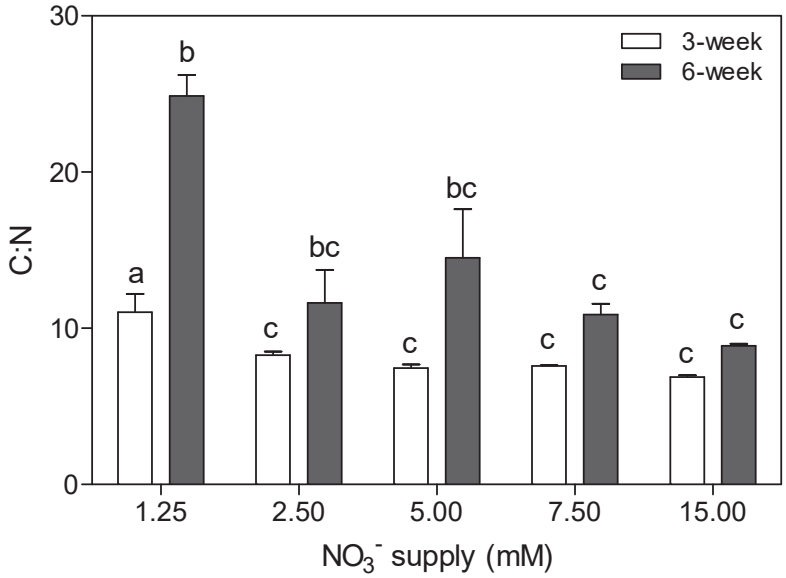


Figure 4.10 The carbon-nitrogen ratio in shoots of sorghum plants grown in hydroponics for 3-weeks or 6-weeks with either 1.25, 2.5, 5, 7.5 or 15 mM NO_3^{-1} in the nutrient solution. Values are mean (± 1 SE) data from n = 4 plants.

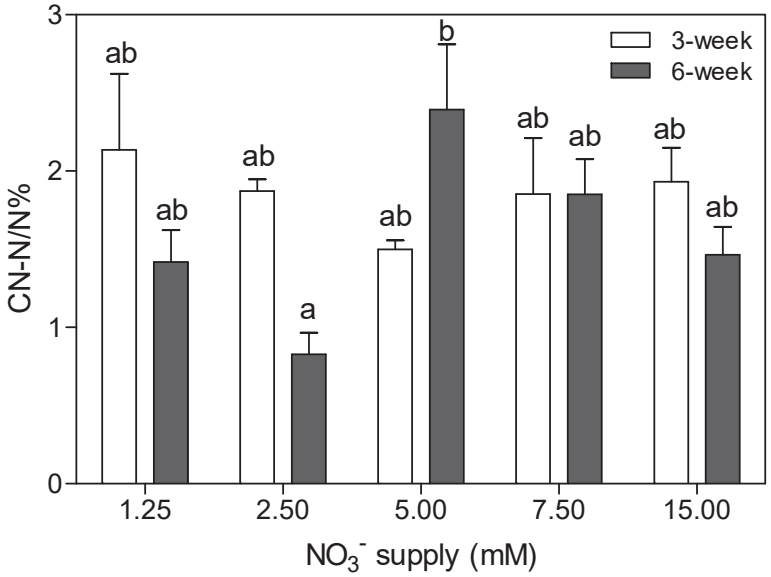


Figure 4.11 The proportion of nitrogen in cyanide in the whole plant (CN-N/N%) in shoots of sorghum plants grown in hydroponics for 3-weeks or 6-weeks with either 1.25, 2.5, 5, 7.5 or 15 mM NO_3^{-1} in the nutrient solution. Values are mean (± 1 SE) data from n = 4 plants.

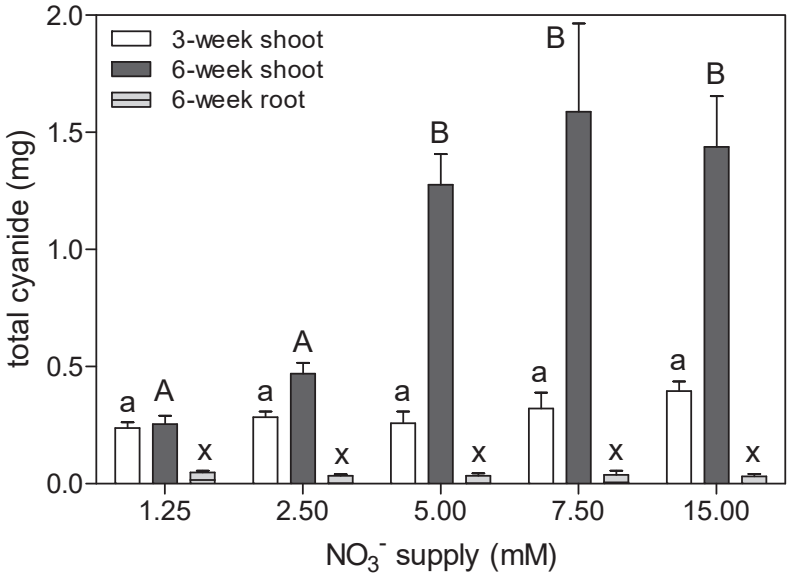


Figure 4.12 The total cyanide content of sorghum plants grown in hydroponics for 3-weeks or 6-weeks with either 1.25, 2.5, 5, 7.5 or 15 mM NO_3^{-1} in the nutrient solution. Cyanide measurements were taken on the shoots and roots for the 6-week treatment group. Values are mean (± 1 SE) data from *n* = 4 plants.

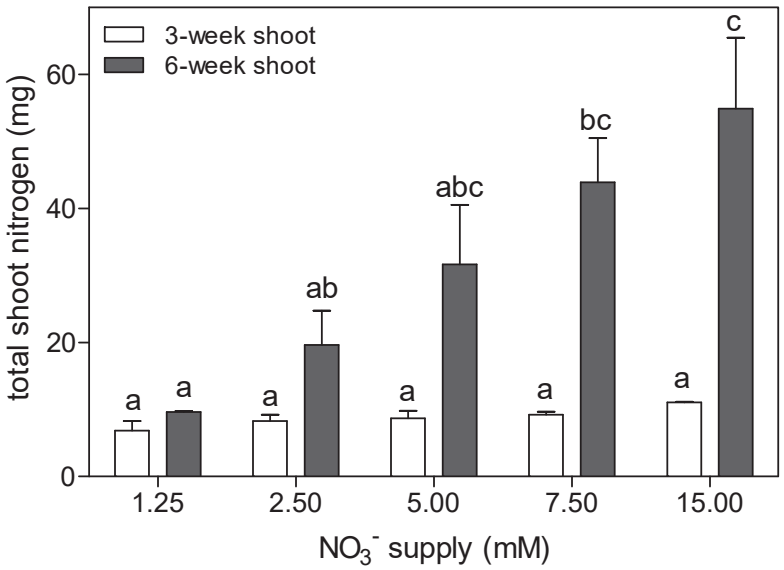


Figure 4.13 The total nitrogen content of sorghum plants grown in hydroponics for 3-weeks or 6-weeks with either 1.25, 2.5, 5, 7.5 or 15 mM NO_3^{-1} in the nutrient solution. Nitrogen measurements were taken on the shoots for both the 3-week and 6-week treatment groups. Values are mean (± 1 SE) data from *n* = 4 plants.

4.4 Discussion

The results from the experiments detailed in this chapter showed that specific hormone treatments and certain wounding methods increased the HCNp of sorghum. Additionally, nitrate supply influenced the HCNp of more mature sorghum plants. The studies resulted in four key findings. First, the HCNp of a plant increases with the application of the hormones, ABA, MeJa and Kinetin. Second, plant growth rapidly increased after GA application but the HCNp remained at control levels. Third, specific wounding methods increased sorghum HCNp. Fourth, the level of nitrogen application correlated with HCNp of more mature sorghum plants, but not with the growth of these plants. These findings are discussed in two distinct sections; (1) the HCNp responses, and (2) nitrogen supply. The HCNp responses section covers the response to growth stimuli, drought simulation and pathogen/herbivory attack, while the nitrogen supply section looks at the flow-on costs and benefits resulting from changes in nitrogen supply.

4.4.1 HCNp responses

The HCNp of sorghum was found to increase with kinetin, MeJa, and ABA application but not SA or GA (Figure 4.4). It is well documented that kinetin and GA are associated with development in both plant systems. Kinetin is known to be involved in cell division (Zhang *et al.* 1996) and has also been said to be associated with anti-aging effects in humans (Kaminek 1992). With this in mind, the increase in cyanide concentration seen in the plants treated with kinetin may be associated with the age-related effects associated with cyanide concentration, as described in chapter 3, where young tissue has demonstrably higher cyanide concentrations. Addition of exogenous kinetin has been shown to be an anti-stress hormone in tobacco plants infected with tobacco mosaic virus (TMV) (Balazc *et al.* 1977; Sziraki *et al.* 1980; Barciszewski *et al.* 1999) and also to slow down development and ageing, to prolong the lifespan of the fruit fly *Zaprionus paravittiger* (Sharma *et al.* 1995). In this instance, the increase in plant cyanide concentration after the application of kinetin, could it be due to the cyanogenesis pathways functioning as it would in a younger plant, an age reversal triggered by the presence of kinetin. Furthermore, 'stay-green' which is a sorghum genotype that has a higher resistance to pre- or post-flowering senescence (Rosenow 1983) and like the

name says, stays green longer than other genotypes. The sorghum stay-green hybrids also contain higher cytokinin levels than senescent genotypes under stressed conditions, such as drought (McBee 1984; Ambler *et al.* 1987). In contrast, the plants treated with GA, a hormone also associated primarily with growth and cell elongation (Jiang *et al.* 2007), did not show an increase in HCNp, but doubled in height in 10 days, which is consistent with numerous studies on GA effects on plants (Morgan *et al.* 1977; Richards *et al.* 2001; Hedden 2003; Zhu *et al.* 2005). At the conclusion of the experiment the GA treated plants had the same cyanide concentration as the control plants. This result suggests that there was an increase in the total cyanide content of the plant but also in total plant mass (not recorded) in order to maintain the same cyanide concentration as the control plants. ABA is associated with stomatal closure and drought (Cornish & Zeevart 1985; Radin & Hendrix 1987). In sorghum, HCNp is higher in drought stressed plants (chapter 5; Dunstaann 1906; Robinson 1930; Kriedeman 1964; Wheeler *et al.* 1990), consistent with the response observed here with ABA. Interestingly, Buchanan *et al.* (2005) reported that twenty of the sorghum genes shown to be either induced or repressed by factors associated with water stress, ABA, NaCl, or PEG treatment, are also involved in plant defence against insects.

Plants possess inducible defense systems to combat pathogen and herbivore attacks. JA and SA are known to regulate the metabolic pathways associated with plant defence (Glazebrook 2005; Lorenzo & Solano 2005; Loake & Grant 2007). Although JA and SA wounding responses are not mutually exclusive, defence pathways are often primarily regulated by either JA or SA (Doares *et al.* 1995; Pieterse & van Loon 1999; Kniskern *et al.* 2007; Zarate *et al.* 2007). Evidence has suggested that MeJa regulated pathways respond to herbivory (Orozco-Cardenas *et al.* 2001), while SA regulated pathways respond to pathogen attacks (Penninckx *et al.* 1996; Loake & Grant 2007). However, there is evidence to suggest that there is a crosstalk between the two pathways (Doars *et al*. 2005; Bari & Jones 2009). For instance, an insect feeding study in sorghum found an increase in transcripts of defense related genes, including SA-dependent pathogenesis-related genes, in addition to a weak induction of MeJA-regulated defense genes (Zhu-Salzman *et al.* 2004). There have been numerous studies on cyanogenic species involving MeJa and/or SA treatments (Creelman & Mullet 1995; Zhu-Salzman *et al.* 2004; Salzman *et al.* 2005; Chen *et al.* 2007), but only one has measured the HCNp

response. Cipollini and Gruner (2007) examined five different varieties of garlic mustard (*Alliaria petiolata*) treated with SA and found a decrease in HCNp in one variety but no change in the other four. In the current study, the HCNp of the plants treated with SA remained at control levels over the 10 days measured.

The application of MeJa to Nicotiana species has been found to induce the synthesis of nitrogen containing alkaloids over seven days (Sinclair *et al.* 2004). Similarly, the HCNp of sorghum plants treated with MeJa was found to increase to more than double the HCNp of the control plants five days post treatments, but then returned to control levels by 10 days. Following the mechanical wounding studies by Baldwin *et al*. (1997) and Sinclair *et al.* (2004) in *Nicotiana*,that showed increased JA in damaged leaves within 90 min and systemically in the roots 180 min after wounding (Baldwin *et al.* 1997). The current study used a similar technique to looked at the effects on dhurrin from mechanical wounding, measured by HCNp. A detailed study covering various wounding techniques was conducted and found that certain methods of wounding had an effect on plant HCNp, but not all. The plants wounded with a pin board (Figure 4.1) showed the largest HCNp effects with an increase in shoot dhurrin concentration peaking at 4 days post wounding and a reduction in growth, compared to the controls. Plants wounded by a perforator also had an increase in dhurrin concentration compared to the controls, but only in the leaf blade. The plants that had one quarter of the roots cut showed a small increase in root HCNp at eight days post wounding, while the plant growth and the shoot HCNp was not effected. No change in plant growth or HCNp was observed for the plants that had discs punched from the leaves (avoiding the midrib) or a whole leaf cut off. These results are consistent with the wounding studies in *Eucalyptus cladocalyx* that found no effect on HCNp in the 24 hours post wounding by hole punch and perforator (Gleadow & Woodrow 2000b). It is hypothesised that no change in HCNp was detected from the perforator because the HCNp was not measured for a long enough time period. Conversely, an aphid feeding study in garlic mustard found a reduction in the cyanogenic glycoside concentration (Cipollini & Gruner's 2007). Whereas the current study found an increased HCNp in the plant that were wounded in a similar way to aphid attack. It is unclear whether the two studies can be compared as aphids can be phloem-feeding and studies suggest that aphids are able to avoid triggering activation of

cyanogenesis, possibly through their particular mode of feeding (Gleadow & Woodrow 2002b; Zhu-Salzman *et al.* 2004).

The results of the hormone and wounding experiments described here contradict the reports in the literature that have found no induction of cyanogenic glycosides with wounding or herbivory (Bennet & Wallsgrove 1994; Hayden *et al.* 2010). For example, Hayden *et al*. (2010) found no effect on cyanogenic glucoside content in white clover (*Trifolium repens*) after wounding or snail herbivory. It was then concluded that cyanogenic glucosides are not inducible and that cyanogenesis is a constitutive defence system. The present study found wounding increased HCNp, but only specific wounding types and only for a finite amount of time. It is supposed that wounding studies in other cyanogenic species were unable to detect a change in HCNp due to the wounding technique and the timing of the treatments (Bennet & Wallsgrove 1994; Gleadow & Woodrow 2000b; Ballhorn *et al.* 2007; Hayden *et al.* 2010). Moreover, a number of cytochrome P450s have been shown to be induced by wounding or pathogens (Donaldson & Luster, 1991; Mizutani *et al.* 1998; Persans *et al.* 2001), therefore it is possible that the P450s (CYP79A1 and CYP71E1) in the dhurrin biosynthesis pathway (Figure 1.2) are also induced by wounding and/or pathogen attack, resulting in the higher HCNp observed in these studies.

The results of the wounding studies have lead to the hypothesis that the increase in HCNp in the sorghum plants wounded by the 'pin board' and the 'perforator' was due to a response by the metabolic pathways that respond to insect attacks, such as aphids. In contrast, it is also hypothesised that the plants that had larger section removed (leaves 'hole punched', 'leaf removal' and 'root removal') did not increase in HCNp because sorghum does not trigger defence to larger events of herbivory. In addition to a wounding response, there was a MeJa response, therefore it is likely that the wounding response was JA mediated.

4.4.2 Nitrogen supply and the associated growth costs and benefits.

The nitrogen supply to sorghum plants in the experiments described had no significant effect on the growth of sorghum plants, with plant height and dry mass as the measure.

Yet, there was an increase in the root to shoot ratios with increased nitrogen supply. The unexpected results for plant growth may be attributed to the fact that the plants were grown in a hydroponic system and it can be difficult to simulate specific growth conditions in a model system (Passioura 2006). However, the plants with the lowest nitrogen supply had an increased C:N ratio, after 3- and 6-weeks treatments. This is indicative of limited nitrogen availability in the plants grown with 1.25 mM NO_3^- supply.

The plants grown with different nitrogen supplies for 3-weeks (up to ~6-leaf stage) did not show a difference in HCNp between treatments. This is consistent with findings that nitrogen supply to young plants has no impact on HCNp (Busk & Møller 2002) and may be due the plants having a threshold HCNp which sorghum seedlings meet and therefore the HCNp in seedlings is not inducible. Whereas the older sorghum plants in the current study, grown at a range of nitrogen concentrations for 6-weeks (up to ~10-leaf stage), showed nitrogen availability was associated with shoot HCNp. This is also consistent with the idea that defence is not likely to be consistent throughout the entire life span of the plant (Weiner 2004). Moreover, when the shoot cyanide concentrations and nitrogen concentrations for plants in 3-weeks and 6-weeks treatments were regressed, a significant positive correlation was found ($CN = 0.0684 - 0.03043N$; $r^2 = 0.5017$; $p<0.0001$; Figure 4.14a). A similar result was found when the total shoot cyanide and total nitrogen contents were regressed ($CN = 0.0834-0.0278N$; $r^2 = 0.7479$; $p<0.0001$; Figure 4.14b). These results are in keeping with the carbon-nitrogen balance (CNB) hypothesis, which predicts that concentrations of nitrogen-based secondary metabolites, such as cyanogenic glycosides, are inversely proportional to the C:N of the plant (Bryant *et al.* 1983). In addition, these correlations also strongly support the hypothesis proposed by Gleadow *et al.* (1998) where nitrogen not required for growth is allocated to defence. However, these results conflict with the HCNp data of other cyanogenic species grown under varying nitrogen supplies (Briggs 1990; Goodger *et al.* 2002; Goodger *et al.* 2004).

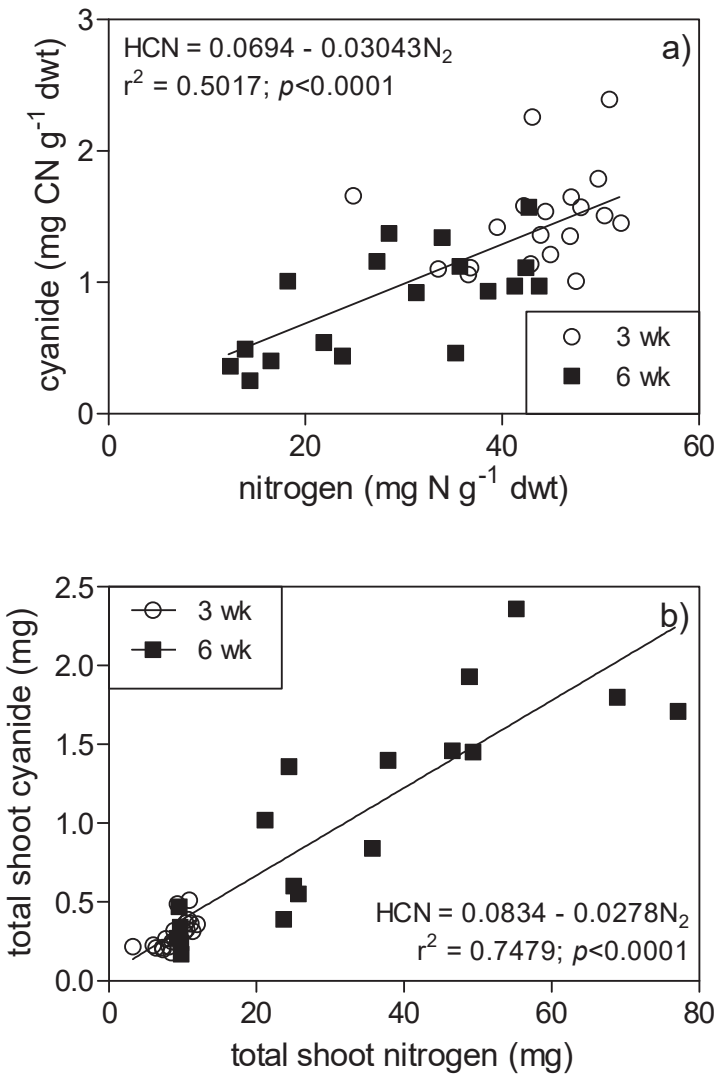


Figure 4.14 The relationships between (a) cyanide concentration and nitrogen concentration, and (b) total cyanide content and total nitrogen content of sorghum plants grown in hydroponics conditions for either 3-weeks or 6-weeks with 1.25, 2.5, 5, 7.5 or 15 mM NO_3^{-1} in the nutrient solution.

Gleadow *et al.* (1998) hypothesis is further supported by an observed nitrogen threshold. Plants grown at 5mM NO_3^- and above had the same shoot HCNp. This idea of a threshold of maximum production of dhurrin was first reported by Kriedeman (1964). Additionally, the shoots of the plants grown at 5mM NO_3^- and above were at a level that would be toxic to cattle (Cheek 1995). Despite the higher HCNp observed in the plants

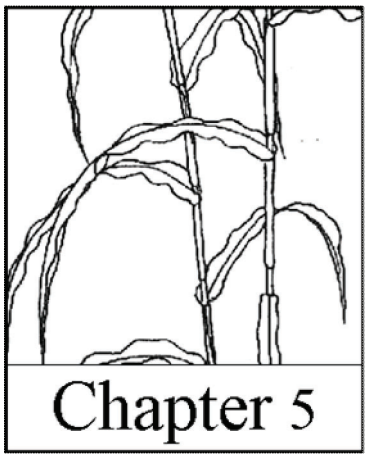
grown in ≥ 5mM NO_3^- for 6-weeks, the HCNp of all nitrogen treatments of these older plants, were lower than the HCNp of the plants after 3-weeks nitrogen treatments. This reduction in HCNp over time is consistent with the results detailed in chapter 3, and important for farmers to know that even with increased nitrogen supply, the age effects on HCNp will outweigh the nitrogen effects. There was no effect on root HCNp due to plants being grown at different nitrogen concentrations. The current study found variable results with nitrogen supply and the proportion of nitrogen allocated to dhurrin. A significant difference in the allocation of nitrogen to dhurrin was found between plants after 6-weeks treatments. However, there was no clear relationship observed.

Previous research on plant nitrogen content and HCNp has not been able to conclude that nitrogen supply alone is the cause of the change in cyanogenic glycosides (Goodger *et al.* 2002, 2004). It has been proposed that HCNp is highly dependant on genotype (Goodger *et al.* 2002, 2004), suggesting that there may be a multifactorial association with HCNp. It has been postulated that if the supply of defence compounds is limited, then the distribution within the plant is dependant on the needs of the different tissues (McKey 1974). What would happen to the distribution of dhurrin within plants with limited nitrogen supply if they were wounded or treated with a hormone associated with increased HCNp (ABA, Kinetin or MeJa)? Would there be the same response, or would there be a shift in the relative needs for chemical defense? Moreover, what would happen in plants grown with either high or low nitrogen supply that were treated with GA?

4.5 Conclusion

The current study found nitrogen supply affects the HCNp of sorghum plants, with no significant reduction in growth, it has been reported that reduced nitrogen supply limits the growth and productivity of the plants (Zhao *et al.* 2005). Hence, these results support the idea raised by Burns *et al*. (2010) that it is important that the link between plant HCNp and soil nutrients are further explored. While it has previously been shown that nitrogen supply effects HCNp in sorghum, the hormone and wounding effects on HCNp observed in this chapter are novel and will require further analysis to determine the primary cause of the observed increase in HCNp. The HCNp in sorghum is inducible

but the hormone applications and mechanical wounding that resulted in increased in HCNp here, had short term effects. The current experiments were not extensive enough to answer the question of the specific cause of the increase in HCNp. The increase in dhurrin could be due to increased synthesis or a reduction in dhurrin turn over, or perhaps both. Further studies into transcription and protein expression of the genes involved in the dhurrin biosynthesis and the multiple turnover pathways (Figure 1.2) would be beneficial to the understanding regulation of cyanogenesis in sorghum.



The effects of osmotic stress on the growth and cyanogenesis of sorghum

5.1 Introduction

Environmental stress factors such as drought, salinity, elevated temperature, and rising CO_2, affect plant growth and pose a growing threat to sustainable agriculture (Ahuja *et al.* 2010). Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Boyer 1982; Bray *et al.* 2000). Sorghum has become a popular choice for forage because of its natural tolerance to adverse conditions, in particular drought. Moreover, it is emerging as a model for studying drought resistance among the grasses (Sanchez *et al.* 2002). Sorghum growth is stunted under drought conditions and senescence of the lower leaves occurs (Rosenow *et at.*, 1983), yet the young leaves of sorghum often maintain a high photosynthetic rate (Carmo-Silva *et al.* 2010), indicating that the available resources are concentrated in younger developing tissues. Studies include: the ability of sorghum to maintain high relative water content (RWC %) under osmotic stress (Haussmann *et al.* 1998; Bhargava & Paranjpe, 2004), response of stomata (Bhargava & Paranjpe, 2004), stay-green traits (Borrell & Hammer, 2000), grain yield under drought stress (Haussmann *et al.* 1998), and gene regulation under osmotic stress (Buchanan *et al.* 2005; Dugas *et al.* 2011).

Sorghum produces the cyanogenic glycoside, dhurrin, which breaks down to release hydrogen cyanide (HCN) when mixed with degradative enzymes, most common during

herbivory (Seigler 1981, Jones 1998). HCN is a respiratory toxin, with an acute lethal dose for mammals as low as 0.5 mg HCN kg^{-1} body weight (Lechtenberg & Nahrstedt 1999). Cyanogenic glycosides occur in approximately 5% of all plant species (Conn *et al.* 1989; Miller *et al.* 2006; Gleadow *et al.* 2008) and have been shown to increase under water deficit (Foulds & Grime, 1972; Gleadow & Woodrow, 2002; Liang 2003; Ballhorn *et al.* 2011). Fields of sorghum experiencing drought have shown increased cyanogenic glycoside concentrations (Dunstaan 1906; Robinson 1930; Kriedeman 1964), making them unsuitable for forage. Although, the studies that specifically investigated HCNp of sorghum were limited in scope and, as these were field experiments, they had a number of confounding variables. For example, one study investigating the impact of drought on sorghum reported reduced HCNp but could not rule out effects associated with plant age (Wheeler *et al.* 1990). It can be difficult to measure the HCNp in sorghum because there are such large ontological effects (chapter 3). Moreover, sorghum drought studies often target grain yield rather than forage quality (Flower *et al.* 1990; Tanpremsri *et al.* 1991a, 1991b). Few drought studies have looked at roots (Hattori *et al.* 2005), and none of those studies have included HCNp. Additionally, few studies have looked at recovery of the plants once the drought stress (osmotic stress) was removed and how this affects HCNp or the rate of plant development under osmotic stress which may also affect HCNp (Jones & Rawson, 1979).

The HCNp has been little studied in detail in any cyanogenic species. A study of the cyanogenic species, *Eucalyptus cladocalyx,* found plants located in a field site with lower average rainfall were water-stressed and had 30% higher cyanogenic glycoside concentration than plants at a field site with a higher average rainfall. However, adding to the complexity of this response, it was also found that there was a significant correlation between increased leaf nitrogen content and HCNp (Woodrow *et al.* 2002). From these findings, it was concluded that some cyanogenic variation is due to differences in nitrogen concentration in the leaves, although the background genetic variation was also very high (Woodrow *et al.* 2002).

Dhurrin is not the only compound in sorghum that can be potentially toxic to cattle. The nitrate-nitrite level can also be toxic to cattle (Bennet *et al.* 1990). Plant nitrate concentrations less than 1000 ug g^{-1} dwt are considered safe for grazing, but nitrate

concentrations greater than 1500 ug g^{-1} dwt are considered toxic to ruminants (Bennet *et al.* 1990; McKechnie *et al.* 2004). A relationship between cyanide and nitrate concentrations within sorghum plants has been found where field grown drought stressed sorghum plants have a sharp decrease in cyanide concentration after rain, while the nitrate concentrations increase to toxic levels (Stuart 2012). Hence, it is important to measure both HCNp and nitrate in stressed plants. Although nitrate poisoning is well known (McKechnie *et al.* 2007), the interaction between HCNp and nitrate concentration has not been extensively studied. It is not known whether the purported switch between HCNp and nitrate is coupled to a change in resource allocation associated with growth, or whether another mechanism is involved. The proposed interaction between HCNp and nitrate concentration further supports the theory that dhurrin may also play a role in nitrogen turnover and storage but it is a complex interaction (Selmar 1988; Adewusi 1990; Gleadow & Woodrow, 2000a, 2002a; Jørgensen *et al.* 2005a; Kongsawadworakul *et al.* 2009; Møller,2010b).

Many of the drought studies on sorghum have been developed to answer specific questions that are not related to cyanogenesis therefore the results cannot be applied to the current question of whether the HCNp increases in sorghum under drought? It is hypothesised that the increase in HCNp of sorghum subjected to drought stress is due to an increase in dhurrin synthesis, as a result of excess nitrogen due to the reduced growth of plants exposed to osmotic stress. It is also hypothesised that if osmotically stressed sorghum plants were allowed to recover there would be a reduction in HCNp. The aims of this chapter are, therefore, to determine: (1) whether dhurrin synthesis is higher in plants under osmotic stress; (2) if the changes observed in HCN, NO_3^- or N_2 concentrations in plants under osmotic (PEG) stress are a consequence of reduced growth; (3) if the shoots and roots respond in the same way to the osmotic (PEG) stress; and (4) whether any increase in dhurrin in osmotically stressed plants is reversible when plants are re-watered and allowed to recover.

Passioura (2007) points out that 'drought' may have different meaning depending on who is measuring it: meteorologists (annual rain fall); agronomists (yield limited by water availability); molecular biologists (sudden severe water deficits); and farmers (management of all afore mentioned issues). All these definitions and the consequent

impact on plants are relevant to farmers and their ability to use crops when water is limiting. Many different experimental systems have been used to simulate drought stress, including bench top drying (Seki *et al.* 2002; Zhou *et al.* 2007), field drought (Semel *et al.* 2007), controlled soil based pot experiments (Rizhsky *et al.* 2004) and polyethylene glycol (PEG)-induced drought (ie osmotic stress) (Lagerwerff *et al.* 1961; Pilon-Smits *et al.* 1999, Zheng *et al.* 2006; Haussmann *et al.* 1998; Foito *et al.* 2009; Dugas *et al.* 2011). Some preliminary studies have shown that it can be difficult to impose a sustained water stress on sorghum plants growing in a controlled system without the plants dying (Passioura, 2006; O'Donnell 2007; pers. comm. Peter Stuart). The addition of polyethylene glycol (PEG) to the nutrient solution of a hydroponic system allows for consistent treatments of plants and the study of the effects of 'drought' on sorghum, in the form of osmotic stress (Anikó & László, 1996; Haussmann *et al.* 1998; Dugas, *et al.* 2011). The present study is broken into three sections: First, the immediate effects on HCN due to osmotic stress (within the first 24 hours). Second, the effects of prolonged osmotic stress (four weeks) on plant growth and resource allocation. Third, the effects of mild and moderate osmotic stress on plant growth and resource allocation and plant growth and resource allocation of plants once the osmotic stress was removed.

5.2 Methods

This chapter outlines the use of polyethylene glycol (PEG-8000) (Sigma-Aldrich) as an osmoticant. Commercial Hybrid 1 sorghum seeds supplied by Pacific Seeds PTY LTD were used for all experiments described in this chapter (Table 2.1). The seeds were germinated in soil: perlite (3:1) mix and seedlings transferred to the hydroponic system at the 3-leaf stage, as described in section 2.2. Plants were supplied with a modified Hoagland's nutrient solution containing 5 mM NO_3^-. PEG-8000 (Sigma-Aldrich) was added to the nutrient solution, at different concentrations, to induce osmotic stress. The concentrations of PEG ranged from 0% to 20% which correspond to osmotic pressures (OP) of 0 to -5.11 (Table 5.1; Michel 1983).

Table 5.1 Conversion of percent of PEG in nutrient solution to osmotic pressure (OP) at 25 °C (Michel 1983).

PEG % (w/v)	PEG-8000 g/100 mL	Osmotic pressure at 25°C
0	0	0.00
2.5	0.025	-0.17
5	0.05	-0.47
10	0.1	-1.48
15	0.15	-3.02
20	0.2	-5.11

5.2.1 PEG-mediated osmotic stress

Plants were acclimatised to the hydroponic system for one week before treatments commenced (section 2.2). The plants were supplied with Hoagland's nutrient solution containing either, 0%, 2.5%, 5%, 10%, 15% or 20% PEG (n = 5 plants). The nutrient solution ± PEG was replaced weekly, with the excess nutrient solution removed and the pots rinsed of residual nutrient solution each time. The plants were grown for a further four weeks and then destructively harvested. Parameters measured at the time of harvest were: sheath height, sheath width, leaf number, leaf blade length, leaf blade width, total leaf surface area, % RWC and F_V/F_M. The leaf length, leaf width, %RWC and F_V/F_M measurements were determined on the youngest fully expanded leaf (leaf I). The plant tissue was divided into leaf blade, leaf sheath and root tissues (section 2.1), dried and weighed. The cyanide concentration was measured on finely ground dried tissue. The biomass data for this experiment expressed on dry weight basis was determined by conversion of fresh weight to dry weight using the RWC.

5.2.2 Osmotic stress followed by rehydration

The plants in this experiment were grown as described above (section 5.2.1). At the start of the experiment six untreated plants at the 3-leaf stage were harvested to provide a baseline (Harvest 0). There were three initial treatment groups; 0% PEG, 10% PEG and 20% PEG treated plants with 12, 18 and 18 plants, respectively, in each group. The plants were grown under these treatments for 2 weeks, then six plants from each treatment were harvested (Harvest 1) as described above (5.2.2; Figure 5.1). The

physiological parameters of %RWC and F_v/F_m were measured on the first and third fully unfurled leaves (leaf I and III; see Figure 2.1). After Harvest 1, six plants from both the 10% and 20% PEG treatment groups were transferred nutrient solution without PEG (0% PEG). All plants were grown for a further two weeks and harvested (Harvest 2) in the same way as detailed for Harvest 1. The harvested material from all time points was divided into the three tissue classes (leaf blade, leaf sheath and roots) with half the tissue dried for cyanide, nitrate and total nitrogen analysis and the other half stored at -80°C for RNA extraction.

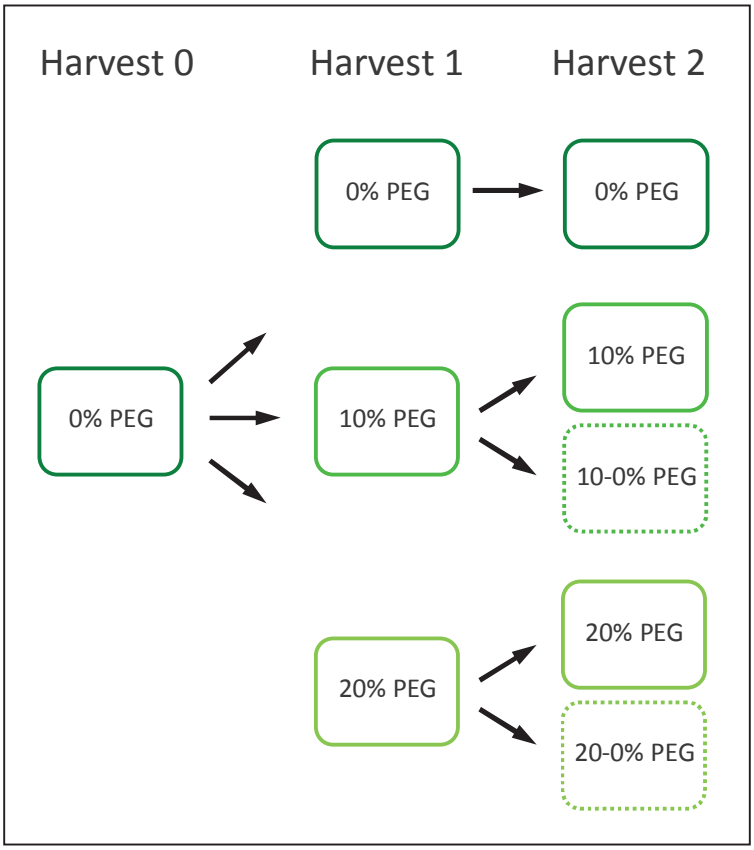


Figure 5.1 Overview of PEG treatments for the osmotic stress and rehydration of sorghum plants (section 5.2.2).

5.2.3 Short term osmotic stress

In this experiment the plants were transferred to the hydroponic system, described in section 5.2.1 at the 3-leaf stage and grown to the 10-leaf stage prior to the osmotic stress being imposed. The plants were allocated to either 0% or 20% PEG treatment groups and were grown for a further 8 days (n = 27 plants for 0% PEG and n = 24 plants for 20% PEG). These treatments were chosen based on the information collected in experiments 5.2.1 and 5.2.2. Three 0% PEG control plants were harvested at time (T) 0. The PEG was added to the nutrient solution of the 20% PEG treatment group and three plants were harvested from each treatment group at time, T = 3 h, 6 h, 12 h, 24 h, 2 d, 4 d, 6 d and 8 d after treatment. The leaf blade cyanide concentration of all remaining plants at each harvest was measured by taking leaf discs (section 2.5.1). The relative water content (RWC %), number of mature leaves and plant height were measured at T 0, 2 d, 4 d, 6 d and 8 d, using the method described in section 2.4. The base of the sheath, which includes the basal meristem tissue, was harvested and stored for RNA extraction and analysis. The rest of the plant was sectioned into leaf blade, leaf sheath and roots and dried for cyanide, nitrate and total carbon/nitrogen analysis (sections 2.4).

5.2.4 Chemical and molecular analysis

Dhurrin analysis

The dhurrin concentration was measured as evolved cyanide, using 0.01 g ground dried tissue for the long term PEG experiments and fresh leaf discs for the short term PEG experiment, using the colorimetric cyanide assay as described in Gleadow, O'Donnell, *et al*. (2012; section 2.4.2). Exogenous β-glucosidase (Amygdalin, Sigma) was added to ensure complete conversion of dhurrin to HCN. Fresh leaf discs were frozen and thawed twice to disrupt the cells allowing contact between the dhurrin and β-glucosidase, and the release of cyanide (Gleadow, O'Donnell, *et al.* 2012)

Nitrate analysis

The soluble nitrate concentration was measured on 0.015 g ground dried tissue, using the method described by Cataldo *et al*. (1975), see section 2.4.2.

Total carbon-nitrogen analysis

The total carbon and nitrogen concentrations was measured on 5 mg ground dried plant material on Elementar Vario Micro Cube, CHNS anlayser, as described in section 2.4.3.

Carbon isotope analysis

The isotopic ratio ($\delta^{13}C$) of dried ground plant material was determined using a Fisons Isochrom Continuous-flow Isotope Ratio Mass Spectrometer, after combustion (at 1050 °C) in a Carlo Erba 1110 CHN-S elemental analyser. Laboratory standards were analysed at the same time to determine the carbon isotope ratios of the samples, *i.e.* C_3 beet sucrose with δ value of -24.62‰ VPDB (relative to the Vienna PeeDee Belemnite scale) and C_4 cane (ANU) sucrose (an international standard), which is accepted as -10.45‰ VPDB; as well as USGS Glutamic Acids 40 and 41, which provide very depleted and very enriched carbon. In interpreting the data, the carbon isotope ratio of source air was necessarily assumed to be similar as plants were growing in the same greenhouse.

CYP79A1 transcript analysis

Total RNA was extracted from frozen tissue using the method described by Verwoerd *et al*. (1989). cDNA was synthesised using the SuperScriptIII First-Strand Synthesis System for RT-PCR kit (Invitrogen) using oligo-dT primers. Quantitative real-time PCR (qPCR) was conducted using gene specific primers for CYP79A1 and β-actin, as the reference gene. SensiMix SYBR No ROX (Bioline) was used in a total reaction volume of 25 µL (half the volume stated in the Bioline SensiMix SYBR protocol), and the target genes amplified using the Rotor-gene 6000 light cycler (Roche). Reactions for both the gene of interest and the reference gene were performed on three individual (biological replicates) and in triplicate (technical replicates). See section 2.5.2.4 for details.

Statistical analysis

Results were analysed using GraphPad prism 5 (GraphPad Software Inc., USA). Data sets were tested for normality and homogeneity of variances prior to analysis. Data that was not normally distributed was log transformed. Comparisons of two treatment groups were performed using a *Student's t-test*. A one-way analysis of variance (*ANOVA*) was performed on groups of plants that had more than two treatment groups. Means that

were significantly different were compared *post hoc* using Tukey's t-tests and different letters indicate significant differences. Mean values are followed by one standard error of the mean (± 1 SE).

5.3 Results

5.3.1 PEG-mediated osmotic stress

The plants in this experiment were grown in a range of PEG concentrations for four weeks. Plant growth was reduced with increasing concentrations of PEG (2.5% - 20% PEG; Table 5.2), with a linear decrease in height with each increase in PEG, from 19.8 ± 1.1 cm for 0% PEG control plants to 9.8 ± 1.3 cm for 20% PEG treated plants (r^2 = 0.7744; $p < 0.0001$; Figure 5.2a). The total leaf surface area of the controls was 296 ± 19 cm with the leaf surface area of 10% and 20% PEG treated plants reduced to 140 ± 11 cm and 46 ± 10 cm, respectively ($p < 0.0001$). A reduction in total biomass was also seen, with the control 1.54 ± 0.22 g dwt and the 20% PEG treated plants 0.58 ± 0.09 g dwt (p = 0.0033). The exception was the number of immature leaves produced per plant, which stayed relatively constant over all PEG treatments (p = 3.699; Table 5.2).

In general, the plants grown in 0 - 15% PEG did not show symptoms of physiological stress, as measured by RWC, F_v/F_m, chlorophyll and carbon isotopes ($\delta^{13}C$). The F_v/F_m of the 0 to 15% PEG treated plants was not significantly different, with an overall mean of 0.72 ± 0.01 (p = 0.8204). Plants treated with 20% PEG showed a significant decline in F_v/F_m to 0.49 ± 0.07 (p = 0.0026; Figure 5.2c), indicating the plants in the 20% PEG group were stressed. The RWC was not significantly different in the 0 to 15% PEG treated plants either, whilst the plants from the 20% PEG treatment group had a RWC of 70% and were significantly lower than the 0% PEG control plants. Yet, the RWC of the 15 and 20% PEG treated plants were not significantly different (Table 5.2). The chlorophyll a:b ratio was not significantly different under any PEG treatment. However, the chlorophyll a+b concentration in the 20% PEG treated plants was significantly lower than that measured in the 2.5 and 5% PEG treated plants, but it was not significantly lower than that determined for the 0% PEG control plants (p = 0.0016). Due to sorghum plants having a C_4 photosynthesis system, it was unexpected to find a significant

increase in C^{13} isotopes in the 15% and 20% PEG treated plants compared 0% PEG control plants (p = 0.0004).

The HCN concentration was significantly higher in plants grown at 20% PEG (p = 0.0011; Figure 5.2b). While there was no significant difference in the HCN concentration of plants treated with 0 - 15% PEG, there was a significant linear correlation in HCN concentration from 5 - 20% PEG (0.2 to 0.58 mg CN g^{-1} dwt), resulting in an increase of 23 µg cyanide per 1% increase in PEG (r^2 = 0.4356; p = 0.0015).

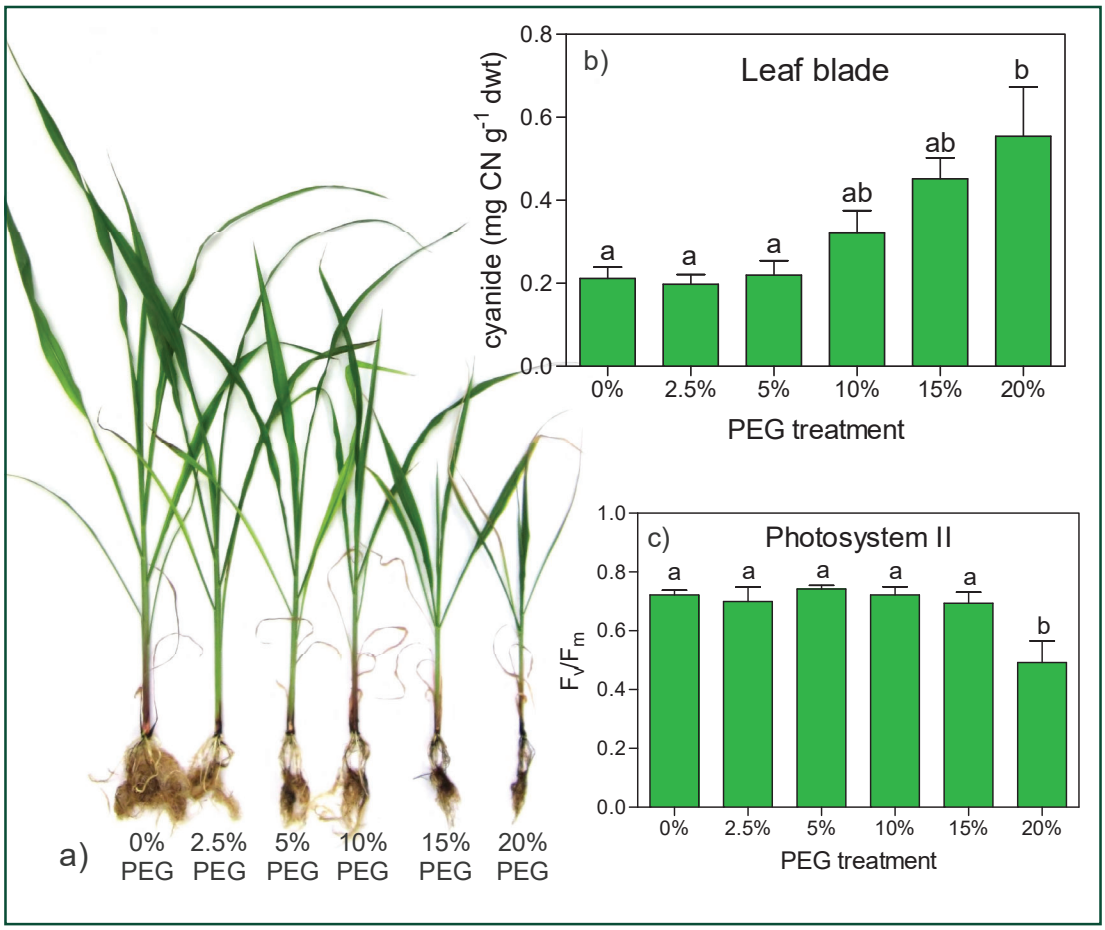


Figure 5.2 (a) The phenotype of 8 weeks old plants, after 4 weeks of PEG treatments, (b) cyanogenic glycoside concentration of the leaf blade and (c) efficiency of photosystem II measured by F_v/F_m ratios of eight week old hydroponically grown PEG treated sorghum plants. Plants were treated for four weeks with either 0%, 2.5%, 5%, 10%, 15% or 20% PEG in the nutrient solution. Values for b and c are mean (± 1 SE) data from n = 5 plants. Letters indicate significant difference at $p < 0.05$, using Tukey's tests.

Table 5.2 Summary of physiological measurements of sorghum plants treated with +/- PEG. Plants were eight weeks old and treated for four weeks with either 0%, 2.5%, 5%, 10%, 15% and 20% PEG in the nutrient solution. Leaf I refers to the first fully unfurled leaf. Values are mean (± 1 SE) data from n = 5 plants. One-way analysis of variance (ANOVA) was performed with $p < 0.05$ confidence interval, bold values are significant.

	Plant section	Control	2.5% PEG	5% PEG	10% PEG	15% PEG	20% PEG	*p*-value
Height (cm)		19.8 ± 1.09^{a}	17.4 ± 1.25^{ab}	18.74 ± 0.70^{a}	13.88 ± 0.77^{bc}	11.92 ± 0.58^{cd}	9.8 ± 0.59^{d}	**< 0.0001**
Leaf surface area (cm^2)		296.2 ± 19.3^{a}	224.0 ± 33.20^{a}	232.1 ± 12.37^{a}	139.5 ± 11.29^{b}	83.83 ± 12.11^{bc}	45.78 ± 9.58^{c}	**< 0.0001**
No. of mature leaves		7.8 ± 0.20^{a}	7.8 ± 0.20^{a}	7.8 ± 0.20^{a}	7.0 ± 0.32^{a}	6.8 ± 0.20^{a}	5.0 ± 0.32^{b}	**< 0.0001**
No. of immature leaves		1.8 ± 0.20	1.8 ± 0.20	1.6 ± 0.40	2 ± 0.32	1.2 ± 0.20	2.0 ± 0.32	0.3699
Leaf length (cm)	leaf I	54.4 ± 2.30^{a}	47.0 ± 3.96^{ab}	50.0 ± 2.29^{a}	37.4 ± 1.84^{bc}	34.3 ± 1.19^{cd}	27.2 ± 1.09^{d}	**< 0.0001**
Leaf width (cm)	leaf I	2.30 ± 0.06^{a}	2.00 ± 0.07^{ab}	1.88 ± 0.08^{b}	1.46 ± 0.09^{c}	1.38 ± 0.08^{c}	0.78 ± 0.04^{d}	**< 0.0001**
Stem width (cm)		0.74 ± 0.04^{a}	0.70 ± 0.10^{ab}	0.72 ± 0.02^{ab}	0.56 ± 0.02^{bc}	0.48 ± 0.04^{cd}	0.38 ± 0.04^{d}	**< 0.0001**
Dry mass (g)	leaf blade	0.50 ± 0.07^{a}	0.37 ± 0.05^{ab}	0.50 ± 0.07^{a}	0.28 ± 0.03^{ab}	0.23 ± 0.05^{b}	0.17 ± 0.04^{b}	**0.0007**
	leaf sheath	0.54 ± 0.08^{a}	0.40 ± 0.06^{ab}	0.55 ± 0.09^{a}	0.29 ± 0.04^{ab}	0.24 ± 0.04^{b}	0.22 ± 0.04^{b}	**0.0009**
	root	0.50 ± 0.09^{a}	0.27 ± 0.05^{ab}	0.48 ± 0.10^{a}	0.35 ± 0.06^{ab}	0.28 ± 0.07^{b}	0.19 ± 0.03^{b}	**0.0301**
	total	1.54 ± 0.22^{a}	1.04 ± 0.56^{ab}	1.52 ± 0.25^{a}	0.92 ± 0.14^{ab}	0.78 ± 0.15^{a}	0.58 ± 0.09^{b}	**0.0033**
Relative water content (%)	leaf I	98.46 ± 2.30^{a}	100.41 ± 2.52^{a}	89.8 ± 3.78^{a}	100.3 ± 3.77^{a}	92.13 ± 3.35^{ab}	69.8 ± 13.89^{b}	**0.0273**
F_v/F_m	leaf I	0.72 ± 0.02^{a}	0.75 ± 0.05^{a}	0.74 ± 0.01^{a}	0.72 ± 0.03^{a}	0.70 ± 0.04^{a}	0.49 ± 0.07^{b}	**0.0026**
Chlorophyll *a*+*b* (mg g^{-1} dwt)		19.7 ± 3.11^{abc}	21.9 ± 4.03^{ab}	28.5 ± 3.80^{a}	18.3 ± 1.20^{abc}	14.3 ± 1.28^{bc}	9.25 ± 1.88^{c}	**0.0016**
Chlorophyll a:b		3.5 ± 0.44	3.1 ± 0.71	3.4 ± 0.19	4.3 ± 0.05	3.0 ± 0.40	4.1 ± 0.31	0.2017
Carotenoids (mg g^{-1} dwt)		3.3 ± 0.43	2.9 ± 0.10	4.0 ± 0.52	3.0 ± 0.15	2.5 ± 0.29	2.1 ± 0.46	0.2482
C isotopes		-12.3 ± 0.10^{a}	-12.9 ± 0.39^{ab}	-12.6 ± 0.04^{a}	-12.9 ± 0.10^{ab}	-13.4 ± 0.16^{b}	-13.6 ± 0.10^{b}	**0.0004**

5.3.2 Osmotic stress followed by rehydration

5.3.2.1 Physiological measurements

In this experiment two concentrations of PEG were used, 10% or 20% PEG, in addition to the 0% PEG control. After 2 weeks in PEG, half the plants were returned to 0% PEG and allowed to recover (Figure 5.1). Plants were first harvested before treatments began (Harvest 0), after 2 weeks (Harvest 1) and again at the end of the four weeks of treatments (Harvest 2; Figure 5.3). The plants at Harvest 0 were at a uniform developmental stage (Table 5.3a). The PEG (10% and 20%) treated plants at Harvest 1 and 2 showed the same trends of stunted growth with increased PEG, as seen in section 5.3.1. The plants at Harvest 2 showed a more pronounced effect of osmotic stress on growth than that observed at Harvest 1 (Tables 5.3b & 5.3c). Trends observed include a significant reduction; in height ($p < 0.0001$), leaf surface area ($p < 0.0001$), total leaf number ($p < 0.0001$), leaf length ($p < 0.0001$), leaf width ($p < 0.0001$), stem width ($p < 0.0001$) and total dry mass of the plant (Harvest 1 $p = 0.0005$; Harvest 2 $p < 0.0001$). At Harvest 2, the recovery plants (10-0% and 20-0% PEG) showed an increase in some but not all physiological measurements compared to the 10% and 20% PEG treated plants, respectively (Table 5.3c). The 10-0% and 20-0% 'recovery' plants showed a significant increase in total number of leaves present per plant ($p = 0.0030$) and a significant increase in the growth the new leaves since the PEG was removed, leaf III leaf length ($p = 0.0026$) and leaf width ($p = 0.0341$) compared to 10% and 20% PEG treatments, respectively. However, the plant height ($p = 0.0711$), total leaf surface area ($p = 0.0785$), and total dry mass did not increase ($p = 0.6499$).

The RWC and F_v/F_m were measured for the first and third fully expanded leaves (leaf I and III, Figure 2.1 in chapter 2). There was a significant decrease in both RWC and F_v/F_m values for 20% PEG treated plants at Harvest 1 and 2 compared to the control plants (0% PEG), indicating that at 20% PEG plants experienced osmotic stress (Figure 5.4; Table 5.3b&c). The RWC and F_v/F_m measurements for leaf III were consistently lower than leaf I for the 20% PEG treated plants. Yet the 0%, 10% and 10-0% PEG treatments showed no significant difference in both leaf I and III for the RWC and F_v/F_m. At Harvest 1, the RWC of the 20% PEG treated plants was reduced by 40% and > 80% for leaves I and III, respectively, compared to the 0% PEG control plants ($p = 0.0086$ and $p < 0.0001$, respectively). At Harvest 2, the RWC of the 20% PEG treated plants was reduced by 60%

and 70% for leaves I and III, respectively, compared to the 0% PEG control plants. The RWC of the 20-0% recovery plants showed a different trend, whereby the RWC of leaf I for the 20-0% PEG plants was not significantly different from 0% PEG controls (p = 0.1262), and leaf III had a RWC only 40% lower (p = 0.0469) than the control. The overall trends seen for the F_v/F_m measurements are the same as outlined for the RWC for both Harvest 1 and 2. At Harvest 1, the F_v/F_m of leaf I was reduced by 40% and leaf III by 60% for the 20% PEG treated plants (p = 0.0086 and p < 0.0001, respectively; Figure 5.4; Table 5.3b). At Harvest 2, the F_v/F_m of the 20% PEG treated plants was 70% and almost 80% lower for leaves I and III, respectively (p < 0.0001), compared to the 0% PEG plants (Figure 5.4b and Table 5.3c). As seen for the RWC, the F_v/F_m for leaf I of the 20-0% recovery plants was not significantly different from 0% PEG controls (p = 0.0572), and leaf III had a F_v/F_m 50% lower than leaf I (p = 0.0457). Both leaves I and III in the 20-0% PEG treatment group showed photosynthetic recovery compared to the 20% PEG plants, with increased RWC and F_v/F_m, hence the 20-0% plants had begun to recover from osmotic stress.

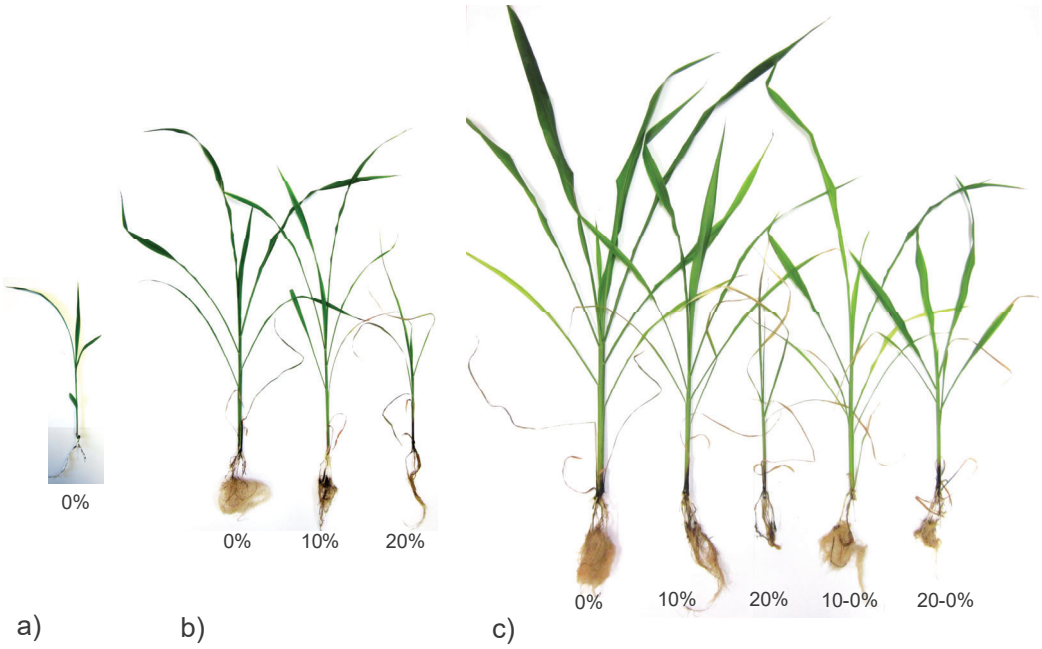


Figure 5.3 The phenotype of PEG treated hydroponically grown sorghum plants from (a) Harvest 0, (b) Harvest 1 and (c) Harvest 2. Untreated, baseline plants were harvested at Harvest 0. Plants were then grown in nutrient solution with three concentrations of PEG. After 2 weeks, a subsample of plants from all PEG treatments were harvested (Harvest 1). Half the remaining plants were returned to nutrient solution with PEG and half to nutrient solution without PEG and allowed to recover (denoted 10-0% and 20-0%, respectively). After a further two weeks growth, all plants were harvested (Harvest 2).

Table 5.3a Physiological measurements at Harvest 0 of the sorghum plants before PEG treatments were imposed, which provides a baseline for growth and development. Plants were 3 weeks old. Leaf I and III a first and third youngest fully expanded leaves respectively. Values are mean (± 1 SE) data from *n* = 10 plants.

Harvest 0	**Plant section**	**Control**
Height (cm)		9.4 ± 0.229
Leaf surface area (cm^2)		24.11 ± 1.622
No. of mature leaves		3 ± 0.000
No. of immature leaves		1.8 ± 0.133
Leaf length (cm)	leaf I	24.25 ± 0.899
	leaf III	2.35 ± 0.143
Leaf width (cm)	leaf I	0.70 ± 0.047
	leaf III	0.61 ± 0.023
Stem width (cm)		0.22 ± 0.015
Dry mass (g)	leaf blade	0.035 ± 0.003
	leaf sheath	0.023 ± 0.002
	root	0.015 ± 0.002
	total	0.073 ± 0.006
F_v/F_m	leaf I	8.13 ± 0.013

Table 5.3b. Physiological measurements for PEG treated sorghum plants at Harvest 1. Plants were six weeks old and treated for two weeks with either 0%, 10% or 20% PEG in the nutrient solution. Leaf I and III refer to the first and third fully expanded leaves respectively. Values are mean (± 1 SE) data from $n = 6$ plants. One-way analysis of variance (ANOVA) was performed and letters indicate significant difference at $p < 0.05$, using Tukey's tests, bold values are significant.

Harvest 1	Plant section	Control	10% PEG	20% PEG	*p*-value
Height (cm)		18.6 ± 0.405[a]	14.4 ± 0.391[b]	11.6 ± 0.227[c]	**< 0.0001**
Leaf surface area (cm^2)		192.0 ± 18.31[a]	149.1 ± 11.51[a]	43.7 ± 4.723[b]	**< 0.0001**
No. of mature leaves		7.07 ± 0.071[a]	6.45 ± 0.114[b]	4.95 ± 0.053[c]	**< 0.0001**
No. of immature leaves		1.71 ± 0.125	1.75 ± 0.123	1.95 ± 0.143	0.4139
Leaf length (cm)	leaf I	50.40 ± 0.830[a]	40.75 ± 1.038[b]	30.32 ± 0.726[c]	**< 0.0001**
	leaf III	30.50 ± 0.971[a]	30.35 ± 0.924[a]	20.42 ± 0.681[b]	**< 0.0001**
Leaf width (cm)	leaf I	1.96 ± 0.037[a]	1.54 ± 0.053[b]	0.79 ± 0.057[c]	**< 0.0001**
	leaf III	1.22 ± 0.048[a]	0.93 ± 0.066[b]	0.38 ± 0.016[c]	**< 0.0001**
Stem width (cm)		0.68 ± 0.015[a]	0.54 ± 0.011[b]	0.38 ± 0.010[c]	**< 0.0001**
Dry mass (g)	leaf blade	0.808 ± 0.070[a]	0.727 ± 0.081[a]	0.360 ± 0.033[b]	**0.0004**
	leaf sheath	0.437 ± 0.054[a]	0.341 ± 0.039[a]	0.179 ± 0.009[b]	**0.0011**
	root	0.398 ± 0.043[ab]	0.480 ± 0.055[a]	0.235 ± 0.044[b]	**0.0078**
	total	1.643 ± 0.164[a]	1.548 ± 0.135[a]	0.773 ± 0.085[b]	**0.0005**
Relative water content	leaf I	97.96 ± 0.415[a]	98.29 ± 0.559[a]	61.11 ± 14.340[b]	**0.0086**
(%)	leaf III	96.056 ± 0.716[a]	95.46 ± 1.157[a]	16.86 ± 2.031[b]	**< 0.0001**
F_v/F_m	leaf I	0.75 ± 0.012[a]	0.68 ± 0.022[a]	0.45 ± 0.050[b]	**< 0.0001**
	leaf III	0.69 ± 0.011[a]	0.72 ± 0.010[a]	0.27 ± 0.034[b]	**< 0.0001**

Table 5.3c. Physiological measurements taken for PEG treated sorghum plants after Harvest 2. Plants were eight weeks old and either treated for four weeks with 0%, 10% or 20% PEG, or first treated for two weeks with either 10% or 20% PEG and then two weeks with 0% PEG in the nutrient solution. Leaf I and III refer to the first and third fully expanded leaves respectively. Values are mean (± 1 SE) data from n = 6 plants. One-way analysis of variance (ANOVA) was performed and letters indicate significant difference at $p < 0.05$, using Tukey's tests, bold values are significant.

Harvest 2	Plant section	Control	10% PEG	20% PEG	10 - 0% PEG	20 - 0% PEG	*p*-value
Height (cm)		22.3 ± 0.368^{a}	16.8 ± 0.341^{b}	11.5 ± 0.475^{c}	19.8 ± 0.610^{a}	13.983 ± 1.258^{c}	**< 0.0001**
Leaf surface area (cm^2)		516.2 ± 39.24^{a}	263.5 ± 22.96^{b}	53.81 ± 13.22^{c}	329.5 ± 41.13^{b}	120.4 ± 34.62^{c}	**< 0.0001**
No. of mature leaves		9.27 ± 0.173^{a}	8.17 ± 0.307^{ab}	5.67 ± 0.333^{c}	8.83 ± 0.167^{a}	7.50 ± 0.428^{b}	**< 0.0001**
No. of immature leaves		2.29 ± 0.173^{a}	2.17 ± 0.167^{a}	2.00 ± 0.258^{ab}	2.00 ± 0.000^{ab}	1.33 ± 0.211^{b}	**0.0073**
Leaf length (cm)	leaf I	67.29 ± 1.113^{a}	47.00 ± 2.887^{bc}	34.00 ± 0.632^{d}	56.33 ± 3.263^{ab}	36.50 ± 5.045^{cd}	**< 0.0001**
	leaf III	50.86 ± 1.587^{a}	39.67 ± 1.926^{bc}	24.00 ± 1.880^{d}	41.33 ± 2.716^{ab}	33.00 ± 1.713^{c}	**< 0.0001**
Leaf width (cm)	leaf I	2.60 ± 0.117^{a}	1.77 ± 0.128^{b}	0.80 ± 0.163^{c}	2.05 ± 0.118^{b}	1.70 ± 0.073^{b}	**< 0.0001**
	leaf III	2.03 ± 0.064^{a}	1.43 ± 0.105^{b}	0.45 ± 0.072^{c}	1.67 ± 0.109^{ab}	0.93 ± 0.214^{d}	**< 0.0001**
Stem width (cm)		1.04 ± 0.028^{a}	0.73 ± 0.033^{b}	0.38 ± 0.031^{c}	0.867 ± 0.049^{b}	0.52 ± 0.065^{c}	**< 0.0001**
Dry mass (g)	leaf blade	2.32 ± 0.177^{a}	1.40 ± 0.122^{b}	0.47 ± 0.060^{c}	1.46 ± 0.200^{b}	0.64 ± 0.135^{c}	**< 0.0001**
	leaf sheath	1.15 ± 0.202^{a}	0.83 ± 0.125^{a}	0.24 ± 0.039^{b}	0.66 ± 0.100^{ab}	0.24 ± 0.065^{b}	**< 0.0001**
	root	1.32 ± 0.199^{a}	1.05 ± 0.123^{a}	0.27 ± 0.018^{bc}	0.80 ± 0.153^{ab}	0.21 ± 0.050^{c}	**< 0.0001**
	total	4.80 ± 0.514^{a}	3.27 ± 0.298^{b}	0.98 ± 0.111^{c}	2.91 ± 0.447^{b}	1.09 ± 0.247^{c}	**< 0.0001**
Relative water content	leaf I	97.79 ± 0.836^{a}	95.46 ± 1.135^{a}	37.42 ± 14.417^{b}	96.52 ± 0.714^{a}	92.81 ± 13.141^{a}	**< 0.0001**
(%)	leaf III	96.71 ± 0.694^{a}	96.72 ± 0.765^{a}	30.35 ± 11.996^{b}	97.32 ± 1.290^{a}	55.72 ± 18.076^{ab}	**< 0.0001**
F_v/F_m	leaf I	0.70 ± 0.004^{a}	0.70 ± 0.004^{a}	0.21 ± 0.123^{b}	0.70 ± 0.009^{a}	0.66 ± 0.012^{a}	**< 0.0001**
	leaf III	0.66 ± 0.018^{a}	0.67 ± 0.022^{a}	0.16 ± 0.085^{bc}	0.60 ± 0.054^{a}	0.34 ± 0.077^{ac}	**< 0.0001**

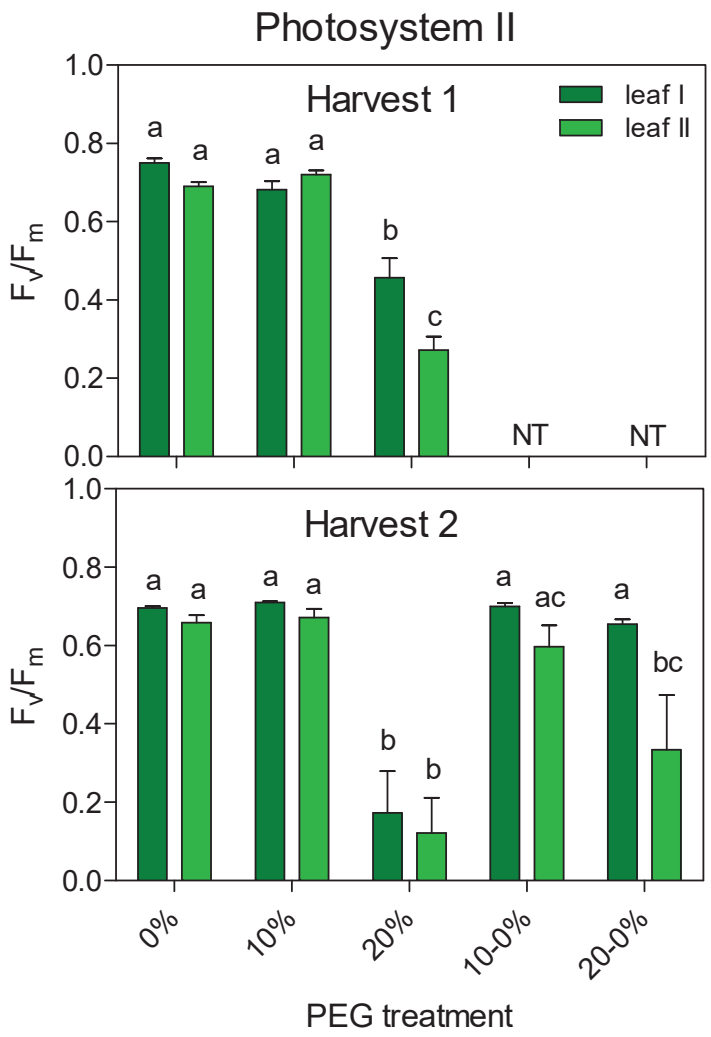


Figure 5.4 Efficiency of photosystem II measured by F_v/F_m ratios in PEG treated hydroponically grown sorghum plants. Harvest 1 plants were six weeks old and treated for two weeks with either 0%, 10% or 20% PEG in the nutrient solution. Harvest 2 plants were eight week old plants, and either treated for four weeks with 0%, 10% or 20% PEG, or treated for two weeks with 10% or 20% PEG and then two weeks with no PEG in the nutrient solution. Values are mean (± 1 SE) data from plants where n = 6 – 20 plants. The, the Fv/Fm ratios varied significantly in Harvests 1 and 2 for both leaf I and leaf II ($p < 0.0001$ for each). Letters indicate significant difference at $p < 0.05$, using Tukey's tests.

5.3.2.2 Growth rate and biomass partitioning

Growth parameters were measured and compared over the three harvests. The specific leaf area (SLA) (Figure 5.5a) and relative growth rates (RGR) (Figure 5.5b) both showed a similar trend at Harvest 1, with no significant difference between 0% and 10% PEG, but the 20% PEG treated plants had a SLA half that of the 0% PEG control plants ($p < 0.0001$) and RGR was reduced to a quarter ($p = 0.0002$) of the RGR of 0% PEG control plants. At Harvest 2, there was no significant difference in the SLA between the 0%, 10%, 10%-0% and 20%-0% PEG treated plants, but the 20% PEG treated plants had half the SLA of the 0% PEG controls ($p = 0.0026$). Between Harvest 1 and 2, the RGRs were reduced by a half for all PEG treatments (Figure 5.5b) and at Harvest 2 the RGR's of the 0%, 10% and 10-0% PEG treated plants were not significantly different. The 20% and 20-0% PEG were ~80% lower than the 0% PEG control plants ($p = 0.0015$), yet there was no significant difference between 10%, 10-0%, 20% and 20-0% treatment groups.

PEG treatment had no effect on net assimilation rate (NAR) at both Harvest 1 ($p = 0.5584$) or Harvest 2 ($p = 0.1291$; (Figure 5.5c). However, NAR decreased by over 50% during the two weeks from Harvest 1 to Harvest 2. The leaf area ratio (LAR) significantly declined with increased PEG treatment for Harvest 1 ($p < 0.0001$) and Harvest 2 ($p = 0.0010$), however, the LAR of the recovery (10-0% and 20-0% PEG) plants at Harvest 2 returned to the LAR of the 0% PEG control plants (Figure 5.5d). Likewise, the total plant biomass decreased with increased PEG concentrations (Figure 5.5e). At Harvest 1, the total biomass of the 20% PEG treated plants was half the biomass of the plants in the 0% PEG control group ($p = 0.0005$). However, there was no significant difference in the total biomass of the plants exposed to 10% PEG compared to the controls. At Harvest 2 there was a significant decrease in total plant biomass for both 10% and 20% PEG treatment groups compared to the control plants, and there was no significant change in the total plant biomass of recovery (10-0% and 20-0% PEG) plants compared to the 10% and 20% PEG treated plants, respectively, (Figure 5.5e), indicating no plant recovery for this parameter. The root to shoot ratio was not significantly different across PEG treatments at Harvest 1 ($p = 0.0621$; Figure 5.5f). At Harvest 2 the 20-0% PEG ($p = 0.0005$) was significantly lower than all PEG treatments except 10%-0% PEG, while 0%, 10%, 20% and 10-0% PEG showed no significant difference in root shoot ratio.

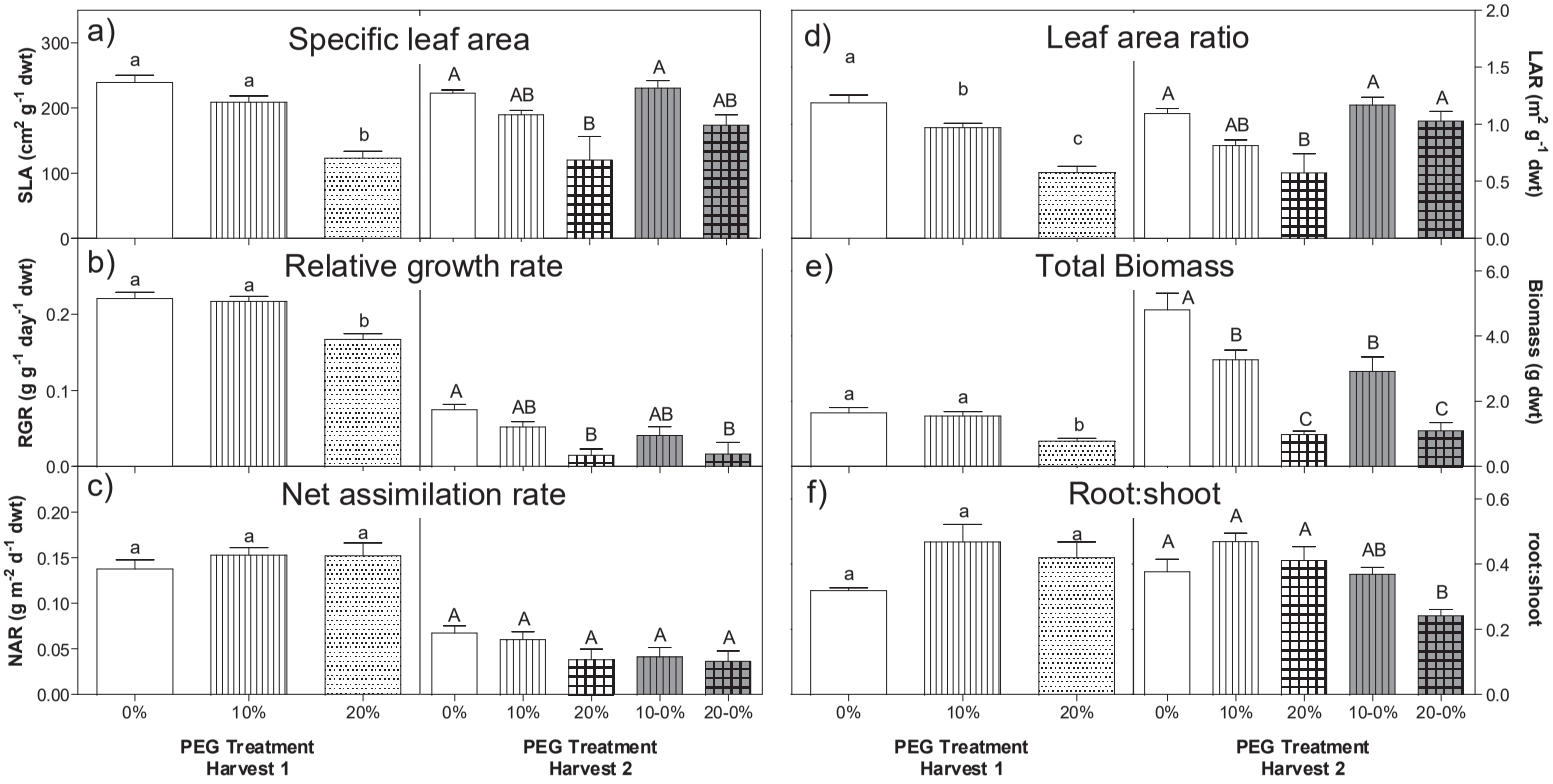


Figure 5.5 Summary of mean growth rate and biomass partitioning for PEG treated sorghum plants at Harvests 1 and 2. Harvest 1 plants were 6 weeks old and treated for two weeks with either 0%, 10% or 20% PEG in the nutrient solution. Harvest 2 plants were eight weeks old and treated for four weeks with either 0%, 10% or 20% PEG, or first treated for two weeks with either 10% or 20% PEG and then two weeks with 0% PEG in the nutrient solution. Values are mean (± 1 SE) data from n = 6 plants. Letters indicate significant difference at $p < 0.05$, using Tukey's tests.

5.3.2.3 Cyanogenic glycosides

The cyanide concentrations were determined for the leaf blade, leaf sheath and roots in the PEG experiment incorporating the recovery treatments (Figure 5.6). The cyanide concentration was highest in young sorghum tissue and decreased with age, consistent with earlier studies (Chapter 3), and this trend was observed for the leaf blade, leaf sheath and roots of the +/- PEG treated plants. For example, at Harvest 0 leaf blade cyanide concentration in 0% PEG plants was 1.88 ± 0.267 mg CN g^{-1} dwt, 80% and 90% higher than at Harvests 1 and 2, respectively. The 0% PEG leaf sheath and roots showed similar declines in cyanide concentrations (Figure 5.6).

Overall, there was no significant difference in the cyanide concentrations of the plants treated with 0%, 10% and 10-0% PEG (p = 0.0642). In general, the 10% and 10-0% PEG treated plants tended to have a higher cyanide concentration than the control (0% PEG) plants, but this was not supported statistically. The 20% PEG treated plants, had a significantly higher leaf blade and leaf sheath cyanide concentration, for example, at Harvest 1 the leaf blade cyanide concentration was 0.98 ± 0.07 mg CN g^{-1} dwt compared with 0.53 ± 0.10 mg CN g^{-1} dwt in the 0% PEG treated control plants (p = 0.0031; Figure 5.6a). Continuing, at Harvest 2, the leaf blade cyanide concentrations for 20% and 20-0% PEG treated plants were over 50% higher than the 0% PEG control plants (p < 0.0001) but there was no significant difference between the 20% and 20-0% PEG treatments, though there is a non-significant decrease in the HCN of the 20-0% PEG treated plants. The cyanide concentration in the leaf sheath of the 20-0% PEG treated plants was 40% lower than the 20% PEG plants at harvest 2 (Figure 5.6b), indicating that these plants may be recovering due to the rehydration treatment.

As found in the leaves, there was no significant difference in root cyanide concentration between 0%, 10% and 10-0% PEG at Harvest 1 or 2 (p = 0.2033 and p = 0.7440; Figure 5.6). However, the cyanide concentrations in the root of the plants treated with 20% PEG did not follow the same trends as observed in the leaf blade and leaf sheath. At Harvest 1, the root cyanide concentration of the 0% PEG control plants was 0.41 ± 0.05 mg CN g^{-1} dry weight, 60% higher than the 20% PEG treated plant (p = 0.0109). While the cyanide concentrations of the 0% PEG control plants at Harvest 2 was 70% and 25%

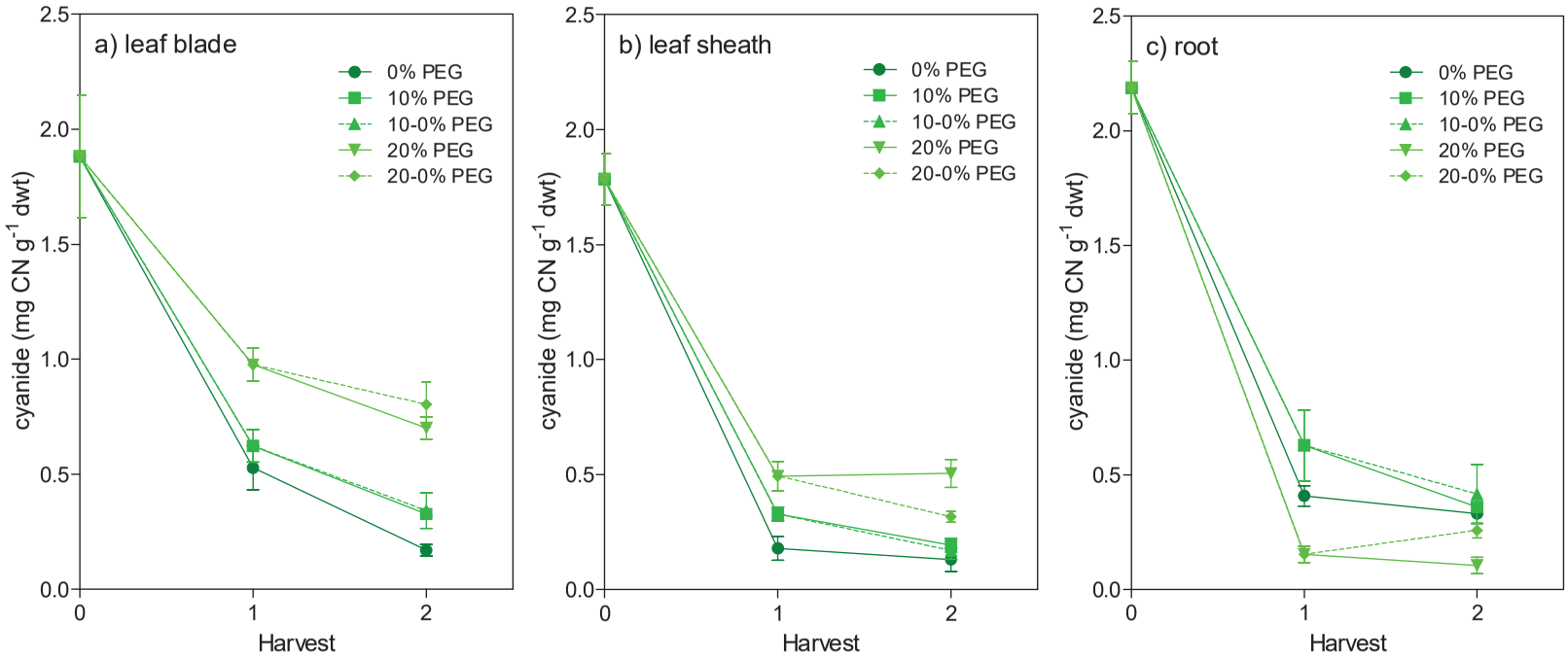


Figure 5.6 Cyanide concentrations of the leaf blade (a), leaf sheath (b) and roots (c) of PEG treated hydroponically grown sorghum plants. The baseline Harvest 0 plants were three weeks old. Harvest 1 plants were 6 weeks old and treated for two weeks with either 0%, 10% or 20% PEG in the nutrient solution. Harvest 2 plants were eight weeks old and treated for four weeks with either 0%, 10% or 20% PEG, or first treated for two weeks with either 10% or 20% PEG and then two weeks with 0% PEG in the nutrient solution. Values are mean (± 1 SE) data from $n = 6$ plants. The cyanide concentration varied significantly between leaf blade (Harvest 1: $p = 0.0031$, Harvest 2: $p < 0.0001$), leaf sheath (Harvest 1: $p = 0.0021$, Harvest 2: $p < 0.0001$) and roots (Harvest 1: $p = 0.0109$, Harvest 2: $p = 0.0222$) for both harvests. Letters indicate significant difference at $p < 0.05$, using Tukey's tests.

higher than the 20% and 20-0% PEG treated plants, respectively (p = 0.0222). This trend is the opposite of what was observed in the leaves.

The total amount of cyanide (mg) in the shoots and roots of PEG treated plants was calculated for the three time points (Harvests 0, 1 and 2). The plants contained the same total plant cyanide content in the shoot for all PEG treatments at Harvest 1 and 2 (Figure 5.7). The total shoot and root cyanide contents of 0% PEG treated plants increased 4-fold from Harvest 0 to Harvest 1. The total shoot cyanide content of the 0% PEG treated plants did not significantly increase further at Harvest 2 (p = 0.5941). In contrast, the total root cyanide content of 0% PEG treated plants at Harvest 2 increased by 11-fold compared to Harvest 0. There was no significant difference in the shoot cyanide content for 0, 10 or 20% PEG treated plants at Harvest 1 (p = 0.2411) or the 0, 10 or 20% PEG treated and recovery (10-0% and 20-0% PEG) plants at Harvest 2 (p = 0.5744; Figure 5.7). The total cyanide content of the roots again differed to the shoots. At Harvest 1 the total root cyanide content of the 20% PEG treated plants was 80% lower than the 0% PEG control plants (p = 0.0036;), whilst the total root cyanide content of 10% PEG treated plants was not significantly different to the 0% PEG control plants (Figure 5.7). At Harvest 2, the total root cyanide content of the 0%, 10% and 10-0% PEG treated plants were not significantly different to each other (p = 0.3957; Figure 5.7), but the total root cyanide content of the 20% and 20-0% PEG treated plants were approximately 90% lower than the 0% PEG control plants (p < 0.0001).

5.3.2.4 Nitrates

Nitrate concentrations for the PEG treated plants were also measured for the leaf blade, leaf sheath and roots (Figure 5.8). At Harvest 0, the leaf sheath nitrate concentration of plants 0% PEG control plants was 70% higher than the leaf blades and roots (Figure 5.8). At Harvest 1, the leaf sheath nitrate concentration of the plants grown in 0% PEG was 20% higher than at Harvest 0. Yet at Harvest 2, the leaf sheath nitrate concentration of the 0% PEG treated plants was 20% lower than at Harvest 0. The leaf sheath nitrate concentrations of the 10% and 20% PEG treated plants did not follow the same trend as the 0% PEG controls. At Harvest 1, 10% and 20% PEG treated plants had 40% and 50% less nitrates than the 0% PEG control plants, respectively (p = 0.0007). At Harvest 2 the decline in nitrate concentration continued, with the 10% and 20% PEG treated plants

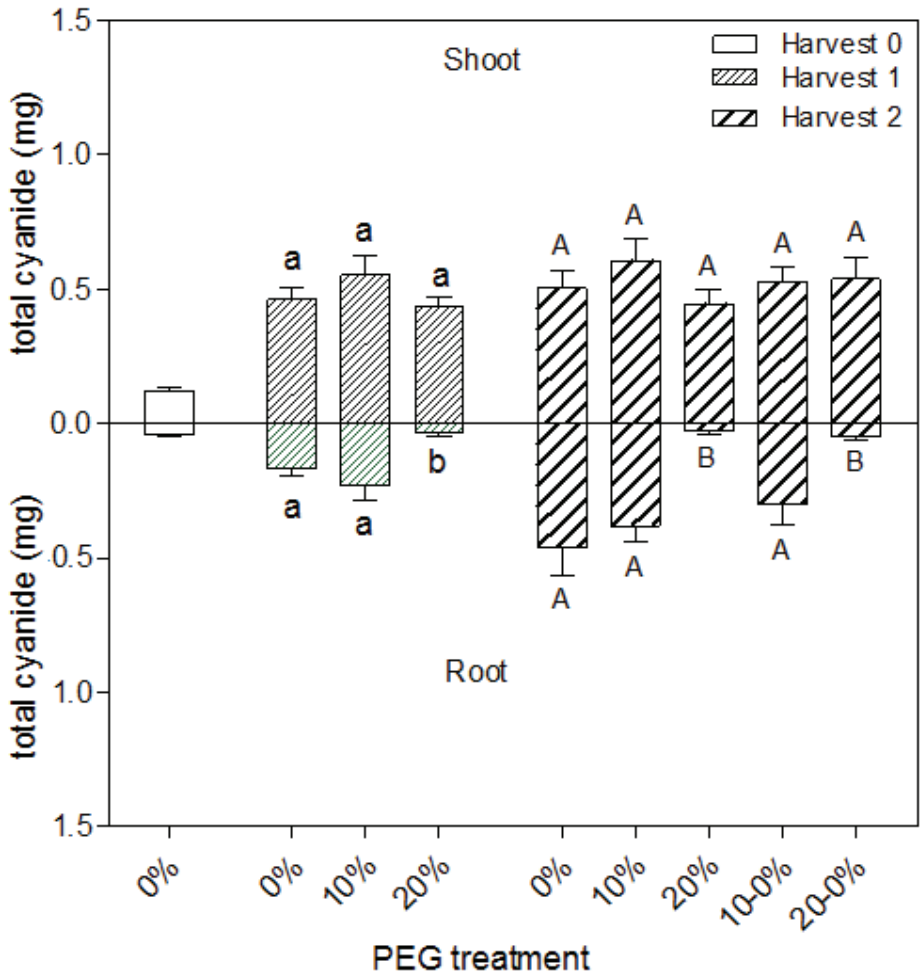


Figure 5.7 The total amount of cyanide in the shoots and roots of PEG treated hydroponically grown sorghum plants from Harvests 0, 1 and 2. The untreated baseline plants were three weeks old at Harvest 0. Harvest 1 plants were 6 weeks old and treated for two weeks with either 0%, 10% or 20% PEG in the nutrient solution. Harvest 2 plants were eight weeks old and treated for four weeks with either 0%, 10% or 20% PEG, or first treated for two weeks with either 10% or 20% PEG and then two weeks with 0% PEG in the nutrient solution. Values are mean (± 1 SE) data from $n = 6$ plants. The total cyanide content varied significantly between roots (Harvest 1: $p = 0.0036$, Harvest 2: $p < 0.0001$), but not shoots (Harvest 1: $p = 0.2411$, Harvest 2: $p = 0.5744$). Letters indicate significant difference at $p < 0.05$, using Tukey's tests.

having approximately half the nitrate concentration of the 0% PEG control plants (p = 0.0041). However, the nitrate concentrations of the recovery (10-0% and 20-0% PEG) plants increased by 45% from Harvest 1 to Harvest 2. However, the nitrate concentrations of the recovery plants, was not significantly different to the 0% PEG control plants at Harvest 2 (p = 0.3566).

The root nitrate concentrations followed the same trends as observed in the leaf sheath. The nitrate concentration of the roots increased by 60% from Harvest 0 to Harvest 1, with the root nitrate concentration at Harvest 2 the same as observed at Harvest 0 (Figure 5.8c). At Harvest 1, the roots of 10% and 20% PEG treated plant had nitrate concentration that were 75% and 80% less than the 0% PEG control plants, respectively (p = 0.0002). At Harvest 2, the root nitrate concentration of the 10% and 20% PEG treated plants root declined further, but had approximately half the nitrate concentration of the 0% PEG control plants. However, the nitrate concentrations of the recovery plants (10-0% and 20-0% PEG) at Harvest 2 increased by 25% and 40%, compared to Harvest 1, and were not significantly different to the Harvest 2 0% PEG control plants (p = 0.1132).

Unlike the nitrate concentrations determined for the sheath and root, the leaf blade nitrate concentration of the 0% PEG treated plants decreased by 40% from Harvest 0 to Harvest 1, and maintained a similar nitrate concentration at Harvest 2. At Harvest 1, the leaf blade nitrate concentrations of the 10% and 20% PEG treated plants decreased to by up to 60% of the 0% PEG control plants (p = 0.0007; Figure 5.8a). At Harvest 2, the 10% PEG treated plants maintained the same leaf blade nitrate concentration as Harvest 1, while the leaf blade nitrate concentration of the 20% PEG treated plants increased by 40%. As seen for the leaf sheath and root tissue, the leaf blade nitrate concentration of the recovery (10-0% and 20-0% PEG) plants increased at Harvest 2. However, the leaf blade nitrate concentrations of the recovery plants and were not significantly different to the 0% PEG control but where 55% and 30% higher than the 10% and 20% PEG plants, respectively (p = 0.0039).

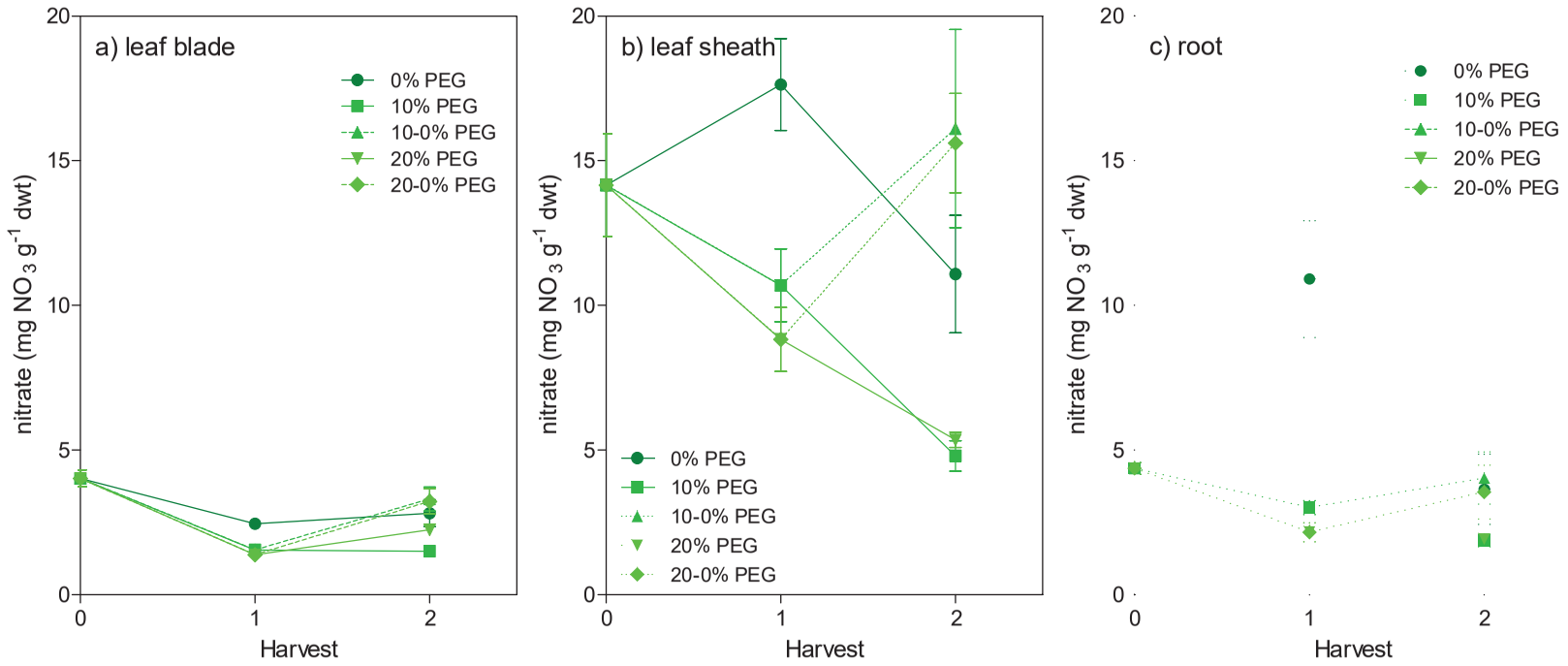


Figure 5.8 Nitrate concentrations in the leaf blade (a), leaf sheath (b) and roots (c) of PEG treated hydroponically grown sorghum plants. The baseline Harvest 0 plants were three weeks old. Harvest 1 plants were 6 weeks old and treated for two weeks with either 0%, 10% or 20% PEG in the nutrient solution. Harvest 2 plants were eight weeks old and treated for four weeks with either 0%, 10% or 20% PEG, or first treated for two weeks with either 10% or 20% PEG and then two weeks with 0% PEG in the nutrient solution. Values are mean (± 1 SE) data from $n = 6$ plants. Based on a one-way ANOVA, the nitrate concentration varied significantly between leaf blade (Harvest 1: $p = 0.0007$, Harvest 2: $p = 0.0039$) and leaf sheath (Harvest 1: $p = 0.0007$, Harvest 2: $p = 0.0004$) at both harvests. However, the roots showed a significant difference at Harvest 1 ($p = 0.0002$) but not Harvest 2 ($p = 0.1132$). Letters indicate significant difference at $p < 0.05$, using Tukey's tests.

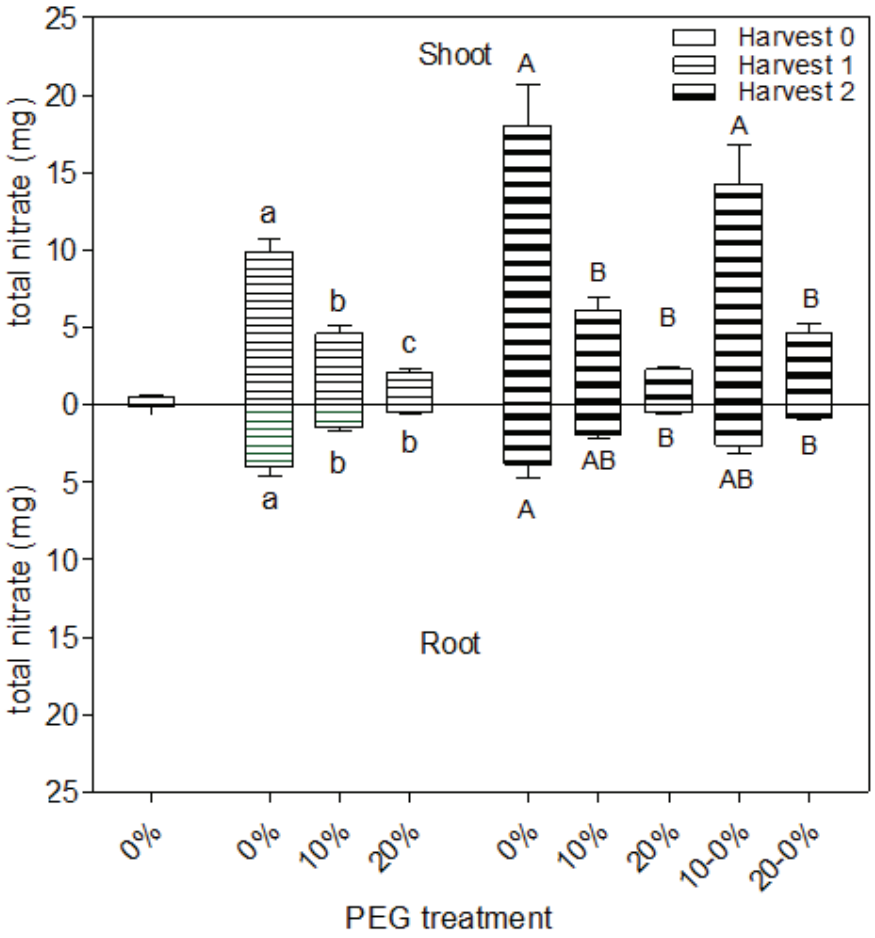


Figure 5.9 The total amount of nitrate in the shoots and roots of PEG treated hydroponically grown sorghum plants from Harvests 0, 1 and 2. The baseline Harvest 0 plants were three weeks old. Harvest 1 plants were 6 weeks old and treated for two weeks with either 0%, 10% or 20% PEG in the nutrient solution. Harvest 2 plants were eight weeks old and treated for four weeks with either 0%, 10% or 20% PEG, or first treated for two weeks with either 10% or 20% PEG and then two weeks with 0% PEG in the nutrient solution. Values are mean (± 1 SE) data from n = 6 plants. The total nitrate content varied significantly between shoots (Harvest 1: $p < 0.0001$, Harvest 2: $p < 0.0001$) and roots (Harvest 1: $p < 0.0001$, Harvest 2: $p = 0.0014$). Letters indicate significant difference at $p < 0.05$, using Tukey's tests.

The total amount of nitrate (mg) in the shoots and roots of PEG treated plants was calculated for the three time points (Harvests 0, 1 and 2). Contrary to the total cyanide content, which stayed constant, the shoots and roots contained less total nitrate with increased PEG treatments (Figure 5.9), with the roots containing approximately one third of the nitrate content of the shoots. Moreover, the total plant nitrate content increased with age ($p < 0.0001$). At Harvest 0 the nitrate content of the shoots was very low and it was almost undetected in the roots, the levels then increased with each harvest. At Harvest 1, the shoots of the 20% PEG treated plants had 80% less total nitrate content than the 0% PEG control plants ($p < 0.0001$) and the same was observed for the 20% PEG treated plants at Harvest 2. The nitrate content of the recovery (10-0% and 20-0% PEG) plants at Harvest 2 varied, with the total shoot nitrate content for the 10-0% PEG not significantly different to 0% PEG control plants, however, the 20-0% PEG treated plants remained low in nitrates ($p < 0.0001$). The total nitrate content of the roots reflected the same trends as observed in the shoots. At Harvest 1 the total nitrate in the roots of 10 and 20% PEG treated plants was significantly lower than the untreated control plants. Whilst at Harvest 2 the total root nitrate content of plants in the 20% and 20-0% treatment groups were significantly lower than that in the 0% PEG treated plants ($p < 0.0001$; Figure 5.9). However, the total nitrates of the roots of plants grown in 0%, 10% and 10-0% PEG were not significantly different ($p = 0.1130$), and the total root nitrate content of the 10%, 10-0%, 20% and 20-0% PEG treated plants were also not significantly different.

5.3.2.5 Total nitrogen

The total carbon and nitrogen concentrations of the leaf blade, leaf sheath and roots were measured for all PEG treatments at the three harvests (Harvests 0, 1 and 2). The leaf blade and leaf sheath nitrogen concentration of the 0% PEG control plants dropped over time, while the root nitrogen concentrations in 0% PEG control plants increased from Harvest 0 to 1 and then declined at Harvest 2 (Figure 5.10). The nitrogen concentration in the recovery (10-0% and 20-0% PEG) plants increased at Harvest 2 for each tissue. The total nitrogen content of the leaf blade at Harvest 0 had the highest nitrogen concentration, 8% and 43% higher than the leaf sheath and roots at Harvest 0, respectively ($p = 0.0001$).

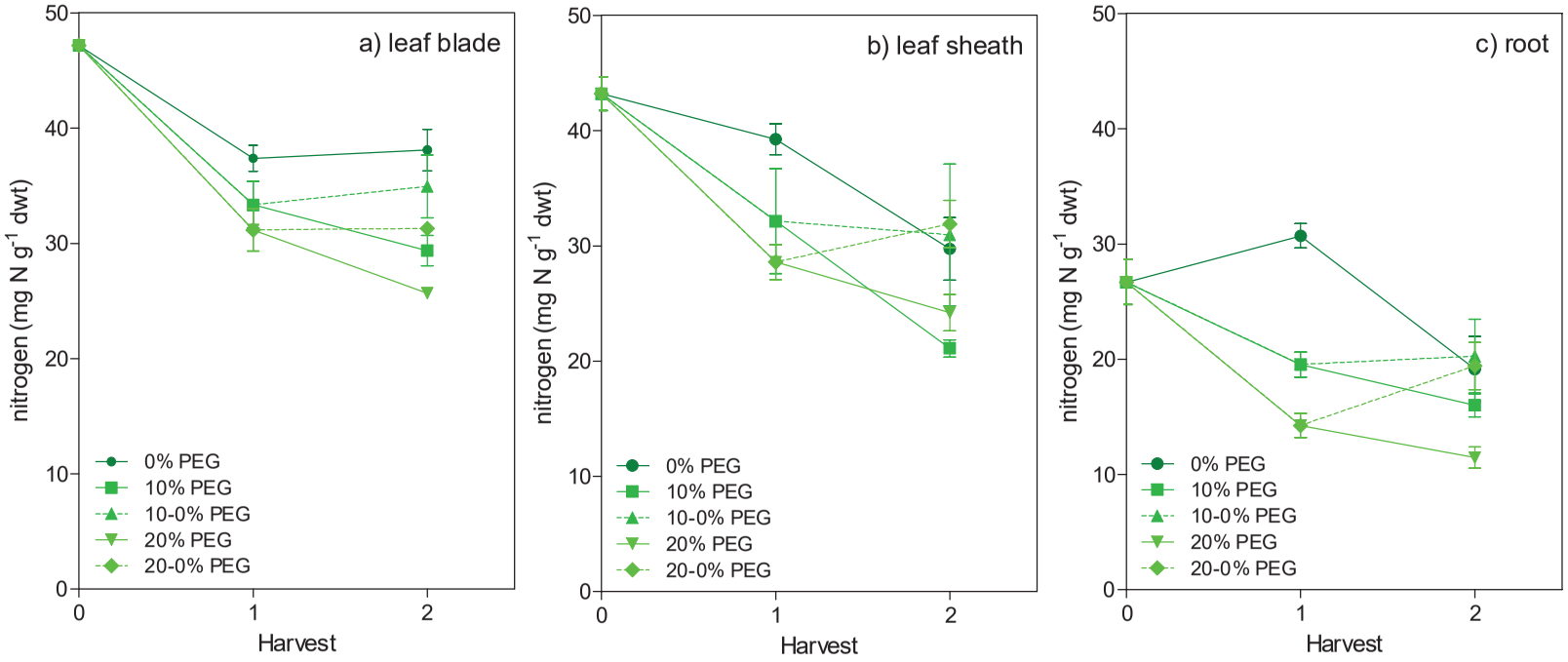


Figure 5.10 Nitrogen concentrations in the leaf blade (a), leaf sheath (b) and roots (c) of PEG treated hydroponically grown sorghum plants. The baseline Harvest 0 plants were three weeks old. Harvest 1 plants were 6 weeks old and treated for two weeks with either 0%, 10% or 20% PEG in the nutrient solution. Harvest 2 plants were eight weeks old and treated for four weeks with either 0%, 10% or 20% PEG, or first treated for two weeks with either 10% or 20% PEG and then two weeks with 0% PEG in the nutrient solution. Values are mean (± 1 SE) data from n = 6 plants. The leaf blade and root nitrogen concentrations vary significantly between PEG treatments at Harvest 2 (blade $p = 0.0021$; root $p < 0.0001$) but not Harvest 1 (blade $p = 0.1025$; root $p = 0.0933$). There is not significant difference in nitrogen concentrations of the leaf sheath (Harvest 1: $p = 0.0950$, Harvest 2: $p = 0.1572$). Letters indicate significant difference at $p < 0.05$, using Tukey's tests.

The leaf blade nitrogen concentrations for 10% and 20% PEG treated plants decreased by 11% and 17% compared to the 0% PEG control plants at Harvest 1 (p = 0.0007; Figure 5.10a). At Harvest 2 the leaf blade nitrogen concentrations of the 10% and 20% treated plants decreased further by 24% and 43% compared to the 0% PEG control plants, respectively. Statistically, the leaf blade nitrogen concentration of the 10-0% and 20-0% PEG treated plants maintained the same nitrogen concentration at Harvest 2 as at Harvest 1, and was not significantly different to the 0% PEG control plants (p = 0.0933).

The leaf sheath nitrogen concentration of 10% and 20% PEG were 20% and 30% lower than the 0% PEG control at Harvest 1 but this was not a significant difference (p = 0.0950; Figure 5.10b). At Harvest 2 leaf sheath nitrogen concentration was reduced by 35% in the 10% PEG treated plants and reduced by 15% in 20% PEG treated plants compared to Harvest 1. The leaf sheath of the 10-0% PEG recovery plants maintained a nitrogen concentration of 30.95 ± 6.16 mg g^{-1} dwt and the 20-0% PEG increased by 11% compared to Harvest 1.

Root nitrogen concentrations followed the same trends as the leaf sheath. The root nitrogen concentration at Harvest 1, for 10% PEG and 20% PEG treated plants had 36% and 54% less nitrogen concentration, respectively, than that found in the 0% PEG control plants (p < 0.0001). At Harvest 2 the nitrogen concentration in the roots of the 10% and 20% PEG treated plants were by 17% and 40%, respectively, lower than the 0% PEG control plants. Again at Harvest 2 10-0% PEG slight increase in nitrogen concentration compared to Harvest 1, and the nitrogen content of the 20-0% PEG treated plants increased by 27% compared to Harvest 1, and was not significantly different to the 0% PEG control (p = 0.1132).

The total amount of nitrogen (mg) in the shoots and roots of PEG treated plants was calculated. The results showed that the total nitrogen content of the plants increased over time but decreases with increased PEG concentration (Figure 5.11), which was the same pattern as that observed for the total nitrate content. The shoots contained approximately three quarters more nitrogen than the roots, but both followed the same trends between PEG treatments. The shoots of the 0% PEG treated plants increased by

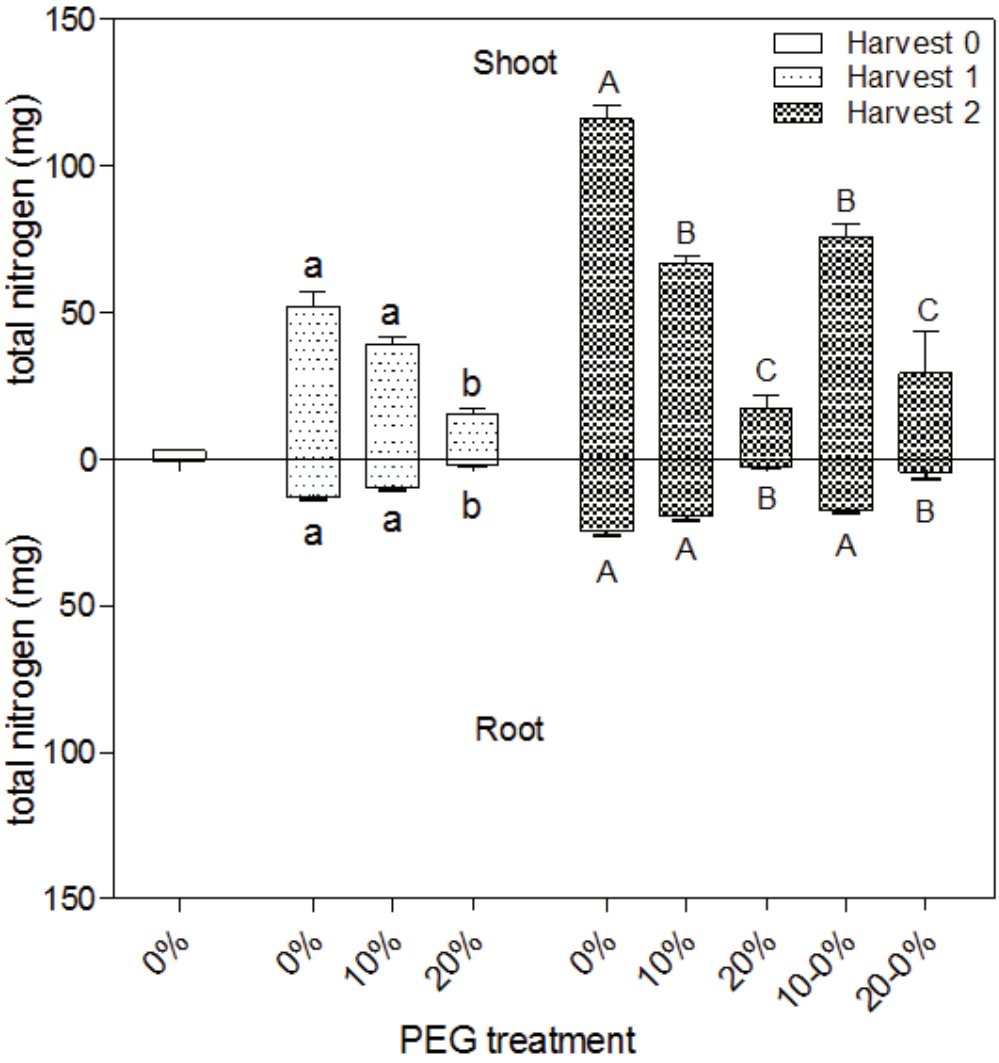


Figure 5.11 The total nitrogen content in the shoots and roots of PEG treated hydroponically grown sorghum plants from Harvests 0, 1 and 2. The baseline Harvest 0 plants were three weeks old. Harvest 1 plants were 6 weeks old and treated for two weeks with either 0%, 10% or 20% PEG in the nutrient solution. Harvest 2 plants were eight weeks old and treated for four weeks with either 0%, 10% or 20% PEG, or first treated for two weeks with either 10% or 20% PEG and then two weeks with 0% PEG in the nutrient solution. Values are mean (± 1 SE) data from n = 6 plants. The total nitrogen content varied significantly between shoots (Harvest 1: p = 0.0006, Harvest 2: p < 0.0001) and roots (Harvest 1: p = 0.0003, Harvest 2: p < 0.0001). Letters indicate significant difference at p < 0.05, using Tukey's tests.

94% between Harvest 0 and Harvest 1 and 98% by Harvest 2. The roots of the 0% PEG treated plants increased 96% between Harvest 0 and Harvest 1 and by a total of 98% by Harvest 2. At Harvest 1 the 20% PEG treated plants had 72% less nitrogen in the shoots (p = 0.0006) and 83% less in the roots (p = 0.0003) compared to the respective 0% PEG controls. The total nitrogen in the shoots and roots of the 10% PEG treated plants also decreased from Harvest 0 to Harvest 1 but it was not significantly different to the controls (p = 0.0663). Determination of the total shoot nitrogen content at Harvest 2 showed that with increased PEG concentration there was a significant decrease in total nitrogen (Figure 5.11). The total shoot and root nitrogen content of the plants in the recovery (10-0% and 20-0% PEG) plant were not significantly different to the 10% or 20% PEG treated plants, respectively. The 20% and 20-0% PEG treated plants had lower total root nitrogen than the control (0% PEG) plants at Harvest 2 (p < 0.0001) and the 0%, 10% and 10-0% PEG were not significantly different (p = 0.0648).

5.3.2.6 Nitrogen allocation

The proportion of nitrogen allocated to cyanide (represented by CN-N/N%) and the proportion if nitrogen allocated to nitrate (represented by NO_3^--N/N%) were calculated for the leaf blade, leaf sheath and roots for all plants from Harvest 0-2 (Figure 5.12). Overall the proportion of nitrogen allocated to cyanide decreased with age regardless of treatment and the 20% PEG treated plants allocated more nitrogen to cyanide in the shoots but not in the roots (Figure 5.12a). The CN-N/N% of the leaf blade and sheath of the 20% PEG treated plants was significantly higher than the CN-N/N% of the 0% PEG control plants (Harvest 1: blade p = 0.0036; sheath p < 0.0001; Harvest 2: blade p < 0.0001; sheath p = 0.0231). While the roots of the 20% PEG treated plants had a significantly lower CN-N/N% than the 0% PEG control plants (Harvest 1 p = 0.0025; Harvest 2 p = 0.0886).

The proportion of nitrogen allocated to nitrate did not show a clear relationship between treatments (Figure 5.12b). Although there was one clear effect, the recovery (10-0% and 20-0% PEG) plants allocated more nitrogen to nitrate in the leaf blade after growing in 0% PEG for 2 weeks (Harvest 2; p = 0.0031). At Harvest 1 the NO_3^--N/N% was not significantly different across PEG treatments for leaf blade (p = 0.3949), or root (p = 0.0699), yet the NO_3^--N/N% in the leaf sheath of the 10% and 20% PEG treated

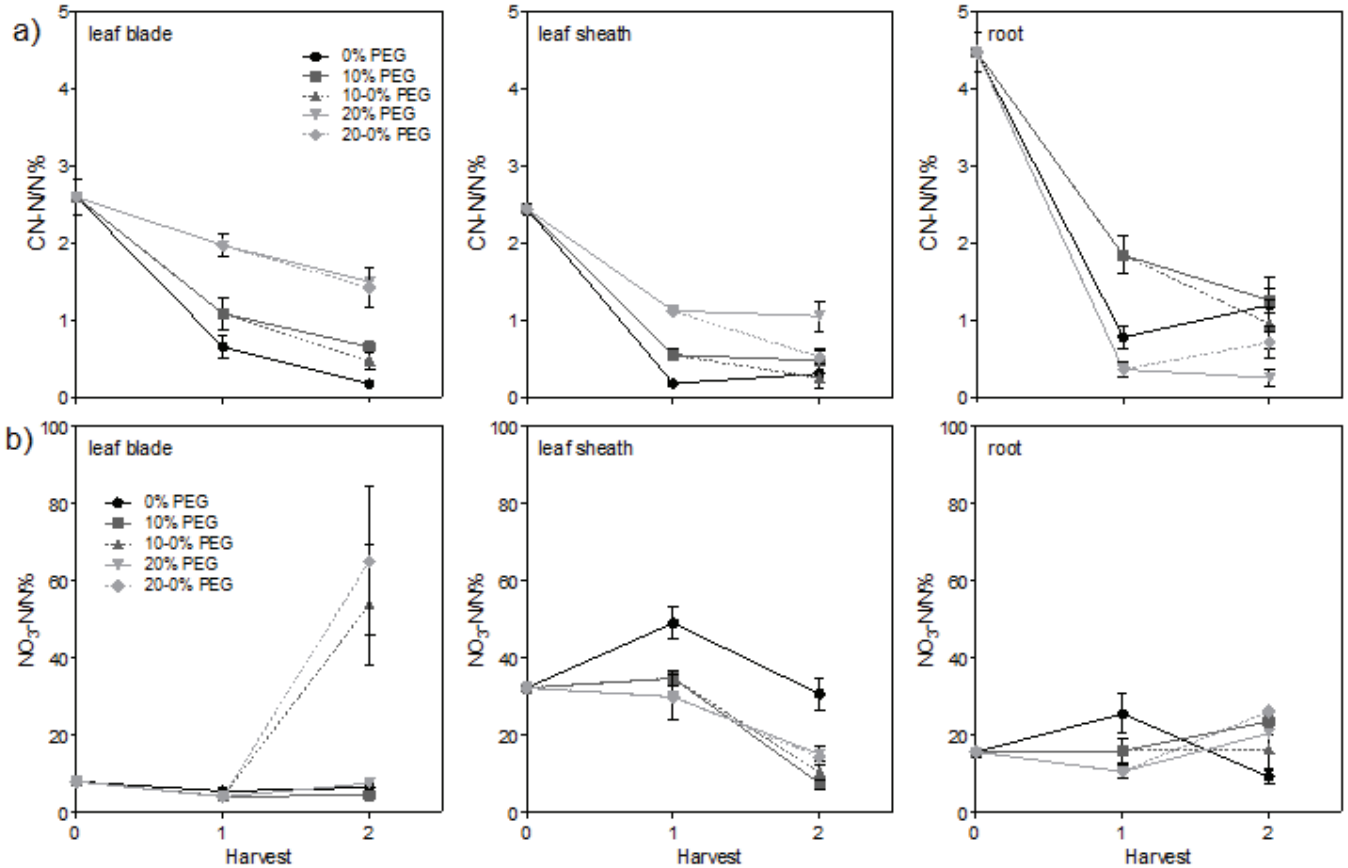


Figure 5.12 Proportion of nitrogen in (a) cyanide (represented by CN-N/N%) and (b) nitrate (represented by NO_3^--N/N%) in whole tissue for PEG treated sorghum plants. Harvest 0: four week old plants – PEG treatments. The baseline Harvest 0 plants were three weeks old. Harvest 1 plants were 6 weeks old and treated for two weeks with either 0%, 10% or 20% PEG in the nutrient solution. Harvest 2 plants were eight weeks old and treated for four weeks with either 0%, 10% or 20% PEG, or first treated for two weeks with either 10% or 20% PEG and then two weeks with 0% PEG in the nutrient solution. Values are mean (± 1 SE) data from *n* = 3 plants.

plants was significantly lower than the 0% PEG control plants (p = 0.0456). The leaf sheath and roots at Harvest 2 also showed significant variation in NO_3^--N/N% (leaf sheath p = 0.0456; root p = 0.0333), but there seems to be no distinct relationship.

5.3.2.7 CYP79A1 transcripts

The bottom of the stalk containing leaf sheath and the basal meristematic tissue was used to isolate total RNA to determine CYP79A1 transcript levels for PEG treated plants at Harvests 1 and 2. A trend was seen for both harvest times where PEG treated plants had lower CYP79A1 transcript levels than in the 0% PEG control plants (Figure 5.13), however, not all PEG treatments were significantly different to the 0% PEG control plants. At Harvest 1, the 20% PEG treated plants had a 2 fold reduction in CYP79A1 transcripts compared to the 0% PEG control plants (p = 0.0348; Figure 5.13). Consistently, the CYP79A1 transcript level of the 10% PEG treated plants at Harvest 1 and the 10% and 10%-0% PEG treated plants at Harvest 2 were not significantly different from the 0% PEG control plants at the respective harvests, whilst the 20% and 20-0% PEG treated plants had less than half the CYP79A1 transcripts of the 0% PEG control plants (p = 0.0068). Moreover, the CYP79A1 transcript levels of 10%, 20%, 10-0% and 20-0% PEG treated plants were not significantly different from each other (p = 0.1042).

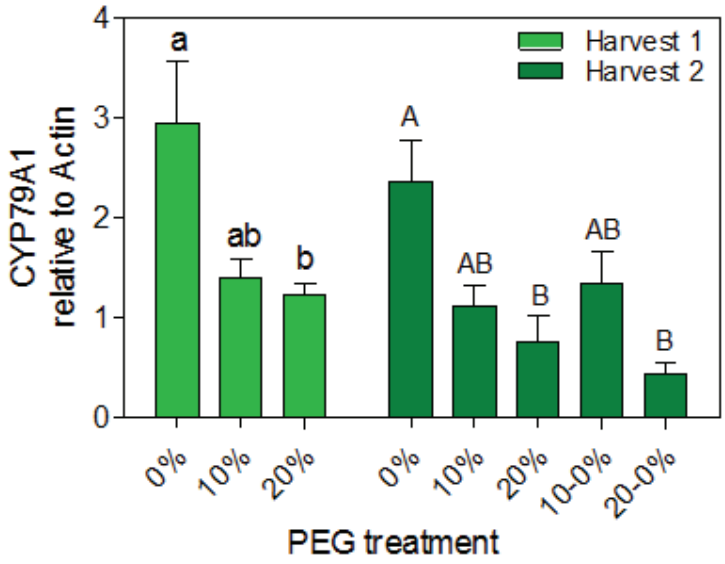


Figure 5.13 CYP79A1 transcript levels, relative to actin, in PEG treated sorghum leaf sheath. Harvest 1 plants were 6 weeks old and treated for two weeks with either 0%, 10% or 20% PEG in the nutrient solution. Harvest 2 plants were eight weeks old and treated for four weeks with either 0%, 10% or 20% PEG, or first treated for two weeks with either 10% or 20% PEG and then two weeks with 0% PEG in the nutrient solution. Values are mean (± 1 SE) data from $n = 3$ plants. Letters indicate significant difference at $p < 0.05$, Tukey's tests.

5.3.3 Short term PEG-mediated osmotic stress

Sorghum plants were treated with +/− PEG nutrient solution (0% or 20% PEG). The RWC (youngest fully unfurled leaf) and leaf cyanide concentrations were measured over eight days and the CYP79A1 mRNA gene transcripts measured over the first 24 hours of treatments. The cyanide concentration of plants treated with 20% PEG decreased up to 50% of the control (0% PEG) plants over the eight days of PEG treatments (Figure 5.14a). It should be noted that, although the plants were randomised for the treatment groups, the cyanide concentration of plants in the 20% PEG treatment group were 25% lower than the 0% PEG control plants when the experiment commenced. The RWC between 0% and 20% PEG plants were not significantly different until eight days of PEG treatments (0% PEG 93.5 ± 0.6, 20% PEG 80.8 ± 5.6, p = 0.0297; Figure 5.14b), indicating the plants were not osmotically stressed for up to eight days of growth in 20% PEG.

The CYP79A1 transcripts were measured in leaf sheath tissue (sheath tissue containing the true meristem was used for RNA isolation) and results showed that the 20% PEG treated plants had lower CYP79A1 transcripts than 0% PEG controls (Figure 5.15). The relative CYP79A1 transcript levels did not vary significantly vary between 0% and 20% PEG treatments at three hours post treatment (p = 0.2827), however, the 20% PEG treated plants were 80% lower at 6 hours (p = 0.0165), 90% lower at 12 hours (p = 0.033) and 90% lower at 24 hours (p = 0.0024), compared to the 0% PEG control plants. The results found here for the short term PEG treatment were consistent with the CYP79A1 transcript results for long term PEG treatments (section 5.3.2.7).

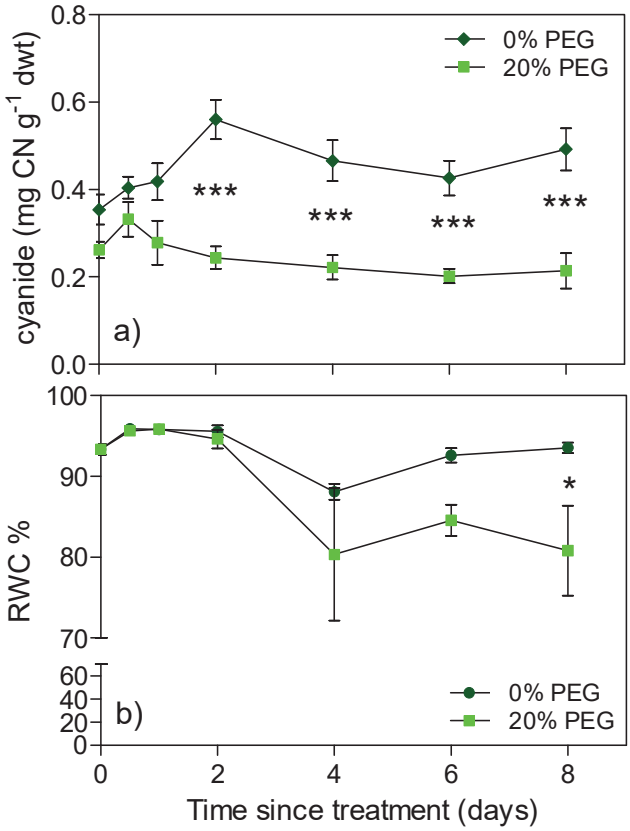


Figure 5.14 The (a) leaf blade cyanide concentration and (b) relative water content (RWC) of hydroponically grown sorghum plants treated with PEG for 8 days. Plants were ten weeks old when PEG treatments began. Values are mean (± 1 SE) data. n = 5 plants for RWC and cyanide. * and *** indicate significant difference of $p < 0.05$ and $p < 0.001$ respectively, determined by Bonferroni's post test.

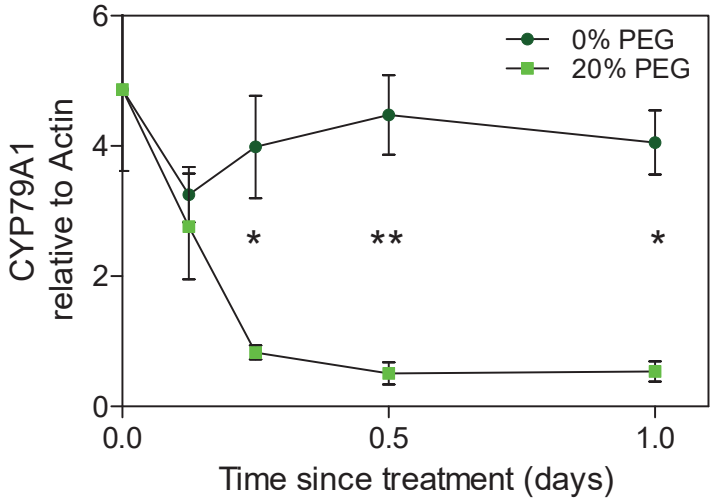


Figure 5.15 The CYP79A1 transcripts in hydroponically grown sorghum plants treated with PEG for 1 day. Plants were ten weeks old when PEG treatments began. Values are mean (± 1 SE) data. n = 3 plants. * and ** indicate significant difference of $p < 0.05$, $p < 0.01$ respectively, determined by Bonferroni's posttest.

5.4 Discussion

The experiments detailed in this chapter resulted in four key findings; the cyanide concentration of sorghum shoots increased under osmotic stress, but the shoots of osmotically stressed and non-stressed plants produced approximately the same total cyanide content; the CYP79A1 transcripts were reduced under osmotic stress; roots showed less ability to recover from osmotic stress and the osmotic stress did not have the same effect on HCNp in the roots as the shoots; and the amount of nitrogen allocated to defence increased in plants under osmotic stress. These findings are discussed in relation to; (1) how osmotic stress was established, (2) the HCNp of the shoots, and CYP79A1 transcript levels, (4) the effect of osmotic stress on roots, and (5) the allocation of resources.

5.4.1 Induction of osmotic stress in shoots

Published research has established that it is difficult to induce osmotic stress in sorghum plants under field conditions (Flower *et al.* 1990; Tangpremsri *et al*, 1991a & 1991b; Haussmann *et al.* 1998; O'Donnell 2007). The current study used a range of PEG

treatments and found that lower PEG concentrations (2.5% - 15%) did not initiate osmotic stress (measured by RWC and Fv/Fm) in sorghum but did result in a linear negative relationship between plant size and PEG treatments across the parameters measured. The plants found to be under osmotic stress, the 20% PEG treated plants, had stunted growth, reduced SLA, reduced PSII, and a decrease in RGR, SLA, LAR and total biomass, consistent with the findings of Jones and Rawson (1979). Moreover, the osmotic stress had no effect on NAR in any of the treatments, conflicting with the trade-off theories outlined by Herms and Mattson (1992).

The present study found that osmotic stress was only measurable, at a phenotypic level by RWC, a minimum of eight days after PEG (20%) treatments began. This observation contradicts Bhargava and Paranjpe (2004), who showed a decrease in relative water content (RWC %) and quantum yield PSII electron transport (F_v/F_m) in sorghum plants treated with 10-25% PEG for five days. Moreover, they found a decrease in stomatal conductance, even before the decrease in RWC or CO_2 fixation was observed. The discrepancy in the level of stress measured for the 10-15% PEG treated plants between the two experiments is believed to be due to a difference in the classification of PEG treatments. The current study classified percent PEG on a w/v PEG basis, while the Bhargava and Paranjpe (2004) study treated the plants with 5% PEG with increased time intervals and used a PEG with a lower molecular weight. Nevertheless, both the present study and the study of Bhargava and Paranjpe (2004) found a decrease in photosynthetic ability and osmotic potential of plants subjected to osmotic stress. A study of C_4 grasses, found that the Rubisco concentration decreased in the leaves for *Cynodon dactylon*, and *Zoysia japonica* plants under drought, suggesting that the lower Rubisco level could contribute to the drought-induced decrease in photosynthetic capacity (Carmo-Silva *et al.* 2010). The observed decrease in chlorophyll *a* + *b* concentrations in the plants under osmotic stress in the current study is consistent with such a decrease in photosynthetic activity. The decrease in chlorophyll *a* + *b* is thought to have been predominantly driven by the imposed osmotic stress. Though, it should be noted that leaf chlorophyll concentration is sensitive to low nitrogen supply (Burns *et al.* 2002) and the plants under osmotic stress in the current study had an increased nitrogen concentration. This indicates that osmotic stress has more of an impact on chlorophyll concentration than the plant nitrogen status.

The older leaves of the plants under osmotic stress showed increasing signs of senescence, a result consistent with research into the phenotypic response of pre-flowing senescence in sorghum (Rosenow 1993). The phenotype of the recovery plants was not consistent with a study that found sorghum plants took three days to recover photosynthetically and 6-11 days to recover osmotically from a period of water stress (Jones & Rawson 1979). The plants in the present study that were transferred out of PEG into nutrient solution and grown for two weeks showed signs of recovery in tissues established during the stress (increased RWC and F_v/F_m), although they did not restore function to control levels and new tissue that developed since the removal of stress, functioned at the same level as the control plants. Additionally, the recovery plants increased in height, but not total biomass, on dry weight basis, suggesting plants had redistributed resources within the plant into growth (Bryant *et al.* 1983; Selmar *et al.* 1988).

5.4.2 Shoot HCN potential

High cyanogenic glycoside concentrations in drought stressed plants have previously been reported (Boyd *et al.* 1938; Franzke *et al.* 1945; Harrington 1966; Gleadow & Woodrow, 2002; Liang 2003; Ballhorn *et al.* 2011). However, the underlying mechanisms causing the increase in cyanogenic glycoside concentrations have not been determined. Here, only the plants showing signs of stress (reduced RWC or F_v/F_m) had a higher cyanide concentration in the shoots. Despite the difference in cyanide concentration, all treatment groups (0%-20% PEG and recovery plants) produced the same total amount of cyanide in the shoots. This result has not been found before but is consistent with the hypothesis that the increase in the HCNp of drought stressed sorghum is due to a concentration effect from stunted growth of the plants (Gleadow & Woodrow, 2002a). Furthermore, the cyanide concentration of plants in the recovery groups (10-0% and 20-0% PEG) did not significantly decrease in the timeframe measured after the osmotic stress was removed, as has been observed in field grown plants (Stuart 2012). The recovery plants did not show the expected reduction in cyanide concentration, possibly because the total plant biomass (dwt) did not increase. This was despite the increase in LAR and marginal increase in SLA.

It was hypothesised that the increase in HCNp was due to an increase in dhurrin synthesis. Therefore the CYP79A1 mRNA was measured in plants under osmotic stress, because CYP79A1 has been shown to be the rate-limiting step in dhurrin synthesis and it has been proposed that CYP79A1 is transcriptionally regulated (Kahn et al. 1999; Busk & Møller 2002). Unexpectedly, the CYP79A1 mRNA transcripts in PEG treated plants were lower than in the control plants. However, it should be noted that transcript levels do not always relate directly to protein activity and the turnover rate of CYP79A1 protein has not been examined in this study. There are only two known published studies that have looked at sorghum genes in plants under osmotic stress (Buchanan *et al* 2005; Dugas *et al.* 2011). Micro-array screening of sorghum seedlings exposed to osmotic stress (20% PEG), NaCl and ABA, showed no change was found in the regulation of the genes involved in dhurrin biosynthesis, CYP79A1, CYP71E1 or UGT85B1 (Buchanan *et al.* 2005). The result of no change in the genes involved in dhurrin biosynthesis was anticipated as these seedlings are quite young and it has been found that dhurrin concentration is not inducible in young plants (chapter 4, Busk & Møller, 2002). Dugas *et al*. (2011) found a strong interplay between genes involved in metabolic pathways associated with Abscisic acid (ABA), Salicylic acid (SA), Jasmonic acid (JA) and other defence pathways. ABA is known to be involved in drought responses (Cornish & Zeevart 1985; Radin & Hendrix 1987; Reddy *et al.* 2004).

A similar study on the defence mechanisms in sorghum measured the effects on gene expression in sorghum treated with methyl jasmonate (MeJa) and SA, and found that in the shoots, MeJa induced dhurrinase and hydroxynitrile lyase activity, which are involved in the catabolism of dhurrin. SA suppressed dhurrinase in the roots but not in the shoots (Salzman *et al.* 2005). The transcript levels of the genes encoding these enzymes increased in the shoots of plants in response to MeJa. Considering that biosynthetic and catabolic pathways have been found to interact and also that the current study found *CYP79A1* was down-regulated when the plant growth was reduced due to osmotic stress, it is possible that the genes involved in the dhurrin degradative pathways were also down regulated under drought conditions as a means of preserving nitrogen resources. The decrease in activity of the degradation pathway may allow an increase in HCNp despite reduced CYP79A1 transcript activity.

The HCNp of the plants from all treatments at Harvest 1 were approaching toxic concentrations (0.5 mg CN g^{-1} dwt; Lechtenberg & Nahrstedt 1999), yet two weeks later at Harvest 2, only the plants that were under osmotic stress, 20% PEG treated plants, were potentially toxic. The reduction of cyanide concentration with age is well documented (chapter 3; Brunnich 1903; Robinson 1930; Harrinton 1966; Panasiuk & Bills 1984; Wheeler *et al.* 1984; Haskins *et al.* 1987; Ikediobi & Olugboji, 1988; Selmar *et al*, 1991; Cipollini & Gruner, 2007). However, the question of whether cyanide concentration is dependant on plant size, rather than maturity, remains unanswered.

5.4.4 Effects of osmotic stress on sorghum roots

The roots of the plants in the current study were found to behave differently to the shoots when exposed to osmotic stress. The root biomass was inversely proportional to the PEG treatment and the plants under osmotic stress showed no increase in biomass between Harvests 1 and 2. Furthermore, the root to shoot ratios were consistent between 0%, 10% and 20% PEG treatments but decreased in the recovery plants. In contrast to Hattori et al. (2005), who found that the root to shoot ratio was reduced in sorghum plants exposed to drought. The current study found that the size of the shoots of the recovery plants (20-0%) increased when PEG was removed but the roots remained the same size, showing no recovery in growth. The decreased ability of roots to regenerate post osmotic stress is consistent with observations of root death when plants were treated with PEG for 27 hours, although in this study the plants were ultimately able to recover (Buchanan *et al.* 2005). Phenology, size and depth of root systems are thought to be constitutive traits allowing for drought tolerance (Blum 1984; Passioura 2002). The phenotype of the roots in the current study may not be reflective of the root systems of field grown plants under stress, because they were in an unnatural hydroponic system under osmotic stress. Increased root growth would not benefit plant in the hydroponic system, as it does in the field (Passioura 2006).

The root HCNp of osmotically stressed sorghum plants was also different to the HCNp of the shoots. The plants under osmotic stress had a lower root cyanide concentration than the control plants. Likewise, the total cyanide content in the roots of osmotically stressed plants was lower than the control plants. This result is consistent with the

finding that the total cyanide content in the roots is lower in smaller plants (chapter 3). The plants under osmotic stress where not only smaller, they were less advanced developmentally, which again raises the question; is HCNp dependent on size rather than age? However, it is not known whether the difference in total cyanide content of the roots was due to the increased root mass of the healthier plants or the reduced root mass of the plants under osmotic stress due to the unusual growing conditions.

5.4.5 Resource allocation: growth, defence, or storage?

There are many theories that attempt to explain the allocation of plant resources to either the growth, defence and/or storage of compounds such as cyanogenic glycosides (Loomis, 1932, 1958; McKey 1974; Bryant *et al.* 1983; Coley *et al.* 1985; Lorio 1986, 1988; Tuomi *et al.* 1990; Herms & Mattson, 1992; Purrington 2000; Stowe *et al.* 2000; Strauss *et al.* 2002). The carbon-nitrogen balance (CNB) hypothesis predicts that concentrations of nitrogen-based secondary metabolites, such as cyanogenic glycosides, are inversely proportional to the C:N of the plant (Bryant *et al.* 1983). The growth-differentiation balance (GDB) hypothesis is based on the existence of a physiological trade-off between growth and differentiation processes, including secondary metabolism (Loomis, 1932, 1958; Lorio, 1986, 1988; Tuomi *et al.* 1990). The GDB hypothesis is different to the CNB hypothesis because it attempts to address the developmental constraints on secondary metabolism, as well as other factors affecting secondary metabolism. The GDB hypothesis encompasses the CNB hypothesis, but expands it to predict that environmental factors that slow plant growth more than the photosynthetic rate can increase the resource pool available for allocation to secondary metabolism (Loomis, 1932, 1958). McKey (1974) proposed that the level of defence for any particular tissue is dependant on two factors; the cost to the plant if the tissue is damaged, and the probability of the tissue being attacked. However, in the current study chemical defence is not directly proportional to the total cost of defence, as outlined in the literature (Purrington 2000; Stowe *et al.* 2000; Strauss *et al.* 2002 Goodger *et al.* 2007). Moreover, the resource availability in the environment appears to be the major determinant of both the amount and type of plant defense (Coley *et al.* 1985).

In the current study, all plants put the same resources into defending the shoots but at different costs. The total shoot cyanide content of the sorghum plants did not vary but the plants under osmotic stress allocated proportionally more nitrogen to cyanide defence than watered controls and stressed plants had less nitrogen to start with. Cyanogenic glycosides may not only have a role in defense (Seigler & Price, 1976; Seigler, 1977), but also as a temporary nitrogen store (Wink & Witte, 1985; Selmar *et al.* 1988; Harborne 1990; Poulton 1990). This has been shown by the study that found an inverse correlation between the cyanide concentration and nitrate concentration in field grown sorghum plants under drought stress, before and after rain (Stuart 2012). The result of the current study was not as apparent as field-based studies, but when the osmotic stress was removed, there was a reallocation of nitrogen to nitrate in the leaf blade, suggesting resources were re-allocated to growth. Furthermore, the differences in the onset and rate of senescence within the stressed plants could be explained by differences in the nitrogen content at leaf and whole-plant levels (Borrell *et al.* 2000; Borrell & Hammer, 2000), as there is a gradient of nitrogen concentrations within sorghum plants (Anten *et al.* 1998). Therefore, the nitrogen from the lower older leaves may have been withdrawn to maintain limited growth in the younger leaves, as the plants under osmotic stress contained less total nitrogen the control plants and the PSII showed leaf three had little photosynthetic ability. This may have been associated with the low nitrogen content of the plant as longevity of a leaf is intimately related to its nitrogen status (Thomas & Rogers, 1990).

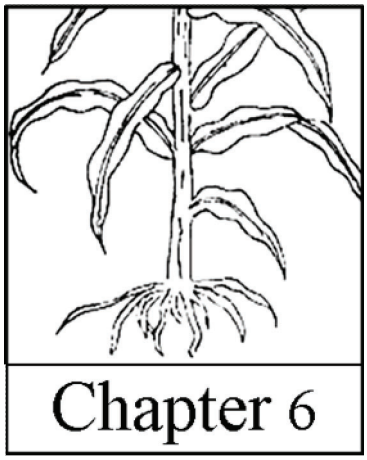
Overall the differences in growth, cyanogenic glycoside and nitrogen concentrations observed between control plants and stressed plants are best explained by the GDB hypothesis (Loomis, 1932, 1958; Lorio, 1986, 1988; Tuomi *et al.* 1990). Nevertheless, the primary driving factor is still unknown. Using this study with plants under osmotic stress as an example, the reduction in the total nitrogen could be due to the smaller leaf surface area and therefore less photosynthesis, leading to reduced shoot and root growth, feeding back into less nitrogen in plant. But which step is the driving factor?

5.5 Conclusion

Sorghum is grown mostly in semi-arid climates where unpredictable rainfall constitutes a major production constraint. It is important to have a good understanding of how the

cyanogenic glycoside and nitrate concentrations vary under drought conditions, because plants exposed to such stress change the proportion of resources that go into defence. Although the resource allocation theories can make quite reliable predictions about how a plant may grow or the level of defence compounds that may be present in a plant grown under specific conditions, they do not address the question at the molecular level. In order to have a more complete understanding of stress, it will be important to look at the molecular level of both the physiological and metabolic effects. The current study shows clearly that with growth limited by increased osmotic stress, more nitrogen is devoted to HCNp (dhurrin). Thus, it is important that farmers are aware of the effects caused by drought and continue to monitor their crops.

The results outlined in this chapter also demonstrated the use of a model system to routinely stress and characterise plants under osmotic stress. This system will be useful in the detailed characterisation of the mutant lines with reduced cyanide generated in the TILLING program (chapter 6), avoiding the possibility of generating a sorghum variety that is low in HCNp but still toxic to cattle due to the high nitrate concentration.



The Generation of a novel acyanogenic sorghum variety using EMS mutation and TILLING

6.1 Introduction

Forage and grain sorghum are grown worldwide, particularly in the dry tropics (Rai *et al.* 1999). The recent interest in sorghum research is driven, in part, by its tolerance to drought, heat stress and its potential as a bioenergy feedstock (Xin *et al.* 2008). Sorghum is also emerging as a model system for the study of C_4 grasses and as a source of genes for the C_4-rice project (Furbank *et al.* 2009). A disadvantage to the use of sorghum as forage is that it produces the cyanogenic glycoside, dhurrin, which releases hydrogen cyanide (HCN) when hydrolysed by the β-glucosidase, dhurrinase. HCN is a respiratory toxin, with an acute lethal dose for mammals as low as 0.5 mg HCN kg^{-1} body weight (Lechtenberg & Nahrstedt 1999). The HCN potential of sorghum varies depending on the growing conditions (chapters 3-5; Gray *et al.* 1968). Dhurrin is produced from the conversion of the amino acid L-tyrosine, in a process catalysed by two cytochrome P450s (CYP79A1 and CYP71E1), a soluble UDP-glucosyltransferase (UGT85B1) (Figure 1.2) (Jones *et al,* 1999; Bak *et al.* 2006), and the redox partner NADPH-dependant cytochrome P450 reductase (CPR) (Ellis *et al.* 2009; Jensen & Møller 2010), with CYP79A1 reported as the rate limiting step (Kahn et al. 1999).

Approximately 5% of all plant species produce cyanogenic glycosides (Conn *et al.* 1989; Miller *et al.* 2006; Gleadow *et al*, 2008). Some species are polymorphic with cyanogenic and acyanogenic genotypes, these include clover (Foulds & Grime 1972; Dritschilo *et al.*

1979; Majumdar *et al.* 2004), lotus (Foulds 1982; Kakes & Hakvoort 1992) and Eucalyptus (Gleadow *et al.* 2002; Gleadow & Woodrow 2002; Woodrow *et al.* 2002; Alonso-Amelot & Oliveros-Bastidas 2005; Simon *et al.* 2007). For example, in natural populations, acyanogenic *Eucalyptus cladocalyx* occurs at a frequency of 1 per 200 (Woodrow *et al.* 2002). Similarly, Goodger *et al*. (2002) found that 6 in 300 *Eucalyptus polyanthemous* were acyanogenic. However, in *Eucalyptus nobilis* about 25% of plants are acyanogenic (Gleadow *et al*. 2003), whilst in *Lotus australis* ~12% are acyanogenic (Foulds 1982). In contrast, other genera are considered to be entirely cyanogenic such as *Hevea*, *Cassava* and *Sorghum,* with no known naturally occurring acyanogenic *S. bicolor* reported to date (Blomstedt, O'Donnell*, et al.* 2012).

The genes involved in the biosynthesis of dhurrin in sorghum have been extensively studied and the genes have been utilized to identify the corresponding genes in other cyanogenic species (Andersen *et al.* 2000; Jørgensen *et al.* 2011). In addition the sorghum genes have been used to manipulate the cyanogenesis pathway in other species, both cyanogenic and acyanogenic, to observe the effects on plant growth and metabolism (Bak *et al.* 1998; Bak *et al.* 2000; Tattersall *et al.* 2001; Jorgensen *et al.* 2005a; Kristensen *et al.* 2005; Franks *et al.* 2006). The genes encoding the enzymes of the dhurrin biosynthetic pathway, *CYP79A1*, *CYP71E1* and *UGT85B1*, from sorghum have been transformed into non-cyanogenic species, such as *Arabidopsis thaliana* and *Nicotiana tabacum* (tobacco) to study the activity of the enzymes (Bak *et al.* 1999, 2000; Tattersall *et al.* 2001; Kristensen *et al.* 2005). The *A. thaliana* plants that expressed all three enzymes accumulated 4% dry-weight dhurrin with minor effects on plant morphology (Kristensen *et al.* 2005). However, transgenic *A. thaliana* plants expressing CYP79A1 and CYP71E1, had a stunted phenotype, changes to the transcriptome and additional glucosides derived from the detoxification of intermediates in the dhurrin pathway were detected (Kristensen *et al.* 2005). The insertion of only CYP79A1 into the *A. thaliana* plants resulted in the accumulation of a novel glucosinolate, *p*-hydroxybenzylglucosinolate (Petersen *et al.* 2001; Kristensen *et al.* 2005). The transgenic tobacco plants expressing CYP79A1 and CYP71E1 showed similar phenotypes to the *A. thaliana* plants (Bak *et al.* 2000). These studies indicate that CYP79A1, CYP71E1 and UGT85B1 are all required for the production of dhurrin.

Additionally, if only part of the pathway is present, there can be adverse effects to the plant metabolism.

Genetic manipulation to eliminate the production of cyanogenic glycosides has been attempted in several cyanogenic species (Jørgensen *et al.* 2005a; Takos *et al.* 2010; Blomstedt, O'Donnell, *et al.* 2012). These studies have focused on the first enzyme in the biosynthesis pathway of cyanogenic glucosides, for example in sorghum CYP79A1 is targeted. It is thought that mutations in *CYP71E1* and *UGT85B1* or their homologues would result in nonviable plants because of the build up of toxic intermediates within the plants (Tattersal *et al.* 2001; Bak *et al.* 1999, 2000; Blomstedt, O'Donnell, *et al.* 2012). Studies on the naturally occurring cyanogenic crop species, cassava (*Manihot esculenta*), have produced plants with acyanogenic leaves and near acyanogenic roots. RNA interference (RNAi) was used to block expression of *CYP79D1* and *CYP79D2*, the two genes encoding the enzymes in the first committed step in the synthesis of the two cyanogenic glycosides, linamarin and lotaustralin (Jørgensen *et al.* 2005a). The RNAi cassava plants with depleted cyanogenic glycoside content grown in vitro did not grow well. These plants were small, few leaves developed and the plants rarely set roots. However, when the plants were transferred to soil the phenotype was almost completely recovered (Jørgensen *et al.* 2005a).

The overall aim of this part of the project was to produce acyanogenic sorghum germplasm for use in a commercial plant breeding program. Transformation systems for sorghum can be problematic (Zhao et al. 2000; Zhensheng *et al.* 2005), although recent work by Liu & Godwin (2012) is returning a 25% success rate. Moreover, GMO sorghum is unlikely to be accepted by the farming community, due to fears of outcrossing into closely related weeds, such as Johnsongrass (*Sorghum halpense*) (Arriola & Ellstrand 1996). An alternative to genetic modification is the reverse genetic technique, Targetted Induced Local Lesions in Genomes (TILLING; Figure 6.1; Till *et al.* 2003). The TILLING process involves chemical mutagenesis of seeds to induce random point mutations. The resulting plants are then genetically screened for mutations in target genes (Till *et al.* 2003). Natural polymorphisms within sequences are also detected in the TILLING process, hence it is important to use a homogenous seed line. The mutant lines selected

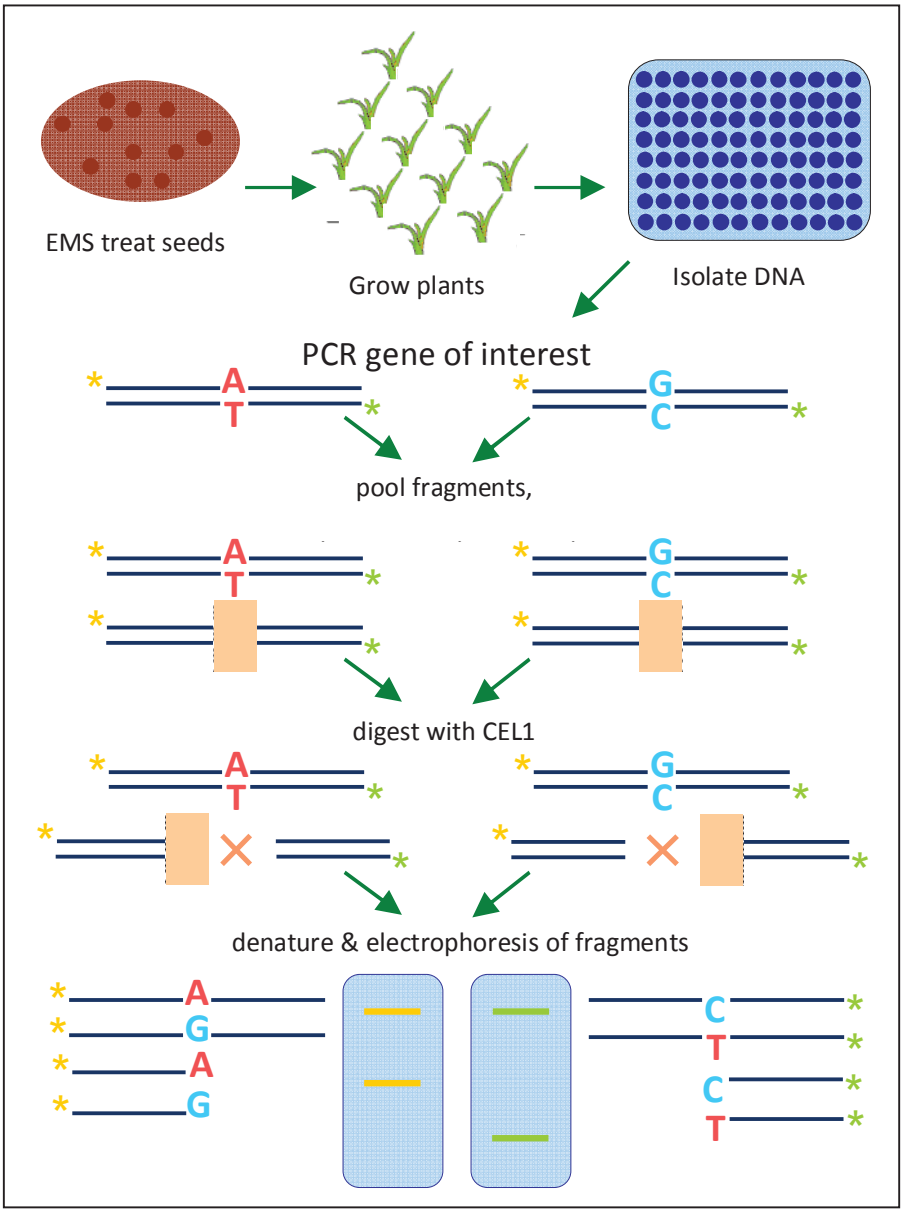


Figure 6.1 The targetted Induced Local Lesions in Genomes (TILLING) process. EMS mutagenised seeds are grown, the plants are allowed to self-pollinate and the second generation is grown. DNA is isolated from the M_2 plant and the gene of interest is amplified by PCR. The primers have fluorescent tags (wavelengths 700 and 800) attached. Fragments are denatured and re-annealed to allow heteroduplexes to form between the PCR products from wild-type and EMS mutated plants. If heteroduplexes are formed these are cleaved at the mismatch by the CEL1 enzyme. Products are run on a Li-cor 4200 DNA Analyser and viewed at 700 and 800 wavelengths. Figure adapted from Jing, 2005.

in the TILLING process were then crossed with conventional breeding lines to produce crops with the desired genotypes without the introduction of foreign DNA. TILLING is, therefore, classified as 'selective' breeding and does not fall into the GMO category. Sorghum plants with the brown midrib (bmr) phenotype, a trait associated with altered lignin content and increased digestibility, have previously been identified using TILLING (Xin *et al.* 2008). Additionally, TILLING was used successfully to identify acyanogenic varieties of the cyanogenic species, lotus (Takos *et al.* 2010).

6.1.2 Aims and hypotheses

Defence hypotheses state that there is a balance in cost to the allocation of resources to herbivore defence compounds (McKey 1974; Herms & Mattson 1992; Goodger *et al.* 2007; Simon *et al.* 2010). Simon *et al*. (2010) calculated the growth and nitrogen cost associated with the synthesis of cyanogenic glycosides in *Eucalyptus cladocalyx.* The plants grown under higher nitrogen concentrations showed increased cyanogenic glycoside content and evidence suggested that this was accompanied by additional nitrogen content (Simon *et al*. 2010). It is, therefore, hypothesised that if sorghum were engineered to be acyanogenic the nitrogen would be diverted to the many other metabolites, potentially resulting in a higher growth rate. It is expected that acyanogenic sorghum lines would have a higher nitrate concentration than cyanogenic plants. It is also hypothesised that only mutations in the first enzyme of the dhurrin synthesis pathway would result in viable plants, as the inactivation of either the second or third enzymes in the dhurrin synthesis pathway (Figure 1.3) would results in the build up of toxic intermediates within the plant.

The work described in this chapter was conducted by the Monash Cyanogenesis group, of which I was part of between 2006 and 2012. Ethylmethyl sulfide (EMS) was used to generate a sorghum mutant population, as described in Blomstedt, O'Donnell, *et al*. (2012). The Blomstedt, O'Donnell *et al*. (2012) study focused on CYP79A1 mutants and are not described in detail here (full paper included as Appendix 10). An acyanogenic sorghum line with a mutation in the coding region of the *UGT85B1* gene was also identified in the mutagenesis study. The specific aim of this chapter was therefore to examine the health and growth of the UGT mutant line named *total cyanide deficient 2*

(*tcd2*). This mutant was of particular interest to see if, in sorghum, there are UGTs that can take over the role of UGT85B1. Control plants and segregating mutant offspring were grown in pots under controlled glasshouse conditions for comparison. This chapter includes: (1) the generation and selection of the original sorghum mutant population and identification of lines with altered levels of HCNp; (2) the identification of *tcd2*; and (3) the analysis of the growth and health of the *tcd2* line.

6.2 Methods

6.2.1 Generation of the mutant sorghum population and selection of specific lines with mutations affecting cyanogenesis

Multiple generations of the sorghum TILLING populations were grown at the Pacific Seeds PTY LTD research station in Gatton, Queensland (27°54'S, 152°34'E). Prior to planting, the soil was plowed with 120 Kg Ha^{-1} of Urea (46% N) and 120 Kg Ha^{-1} of CK-88 (15.1% N; 4.4% P; 11.5% K; 13.6% S). Seeds from each generation were sown in early October and screened for cyanide in the November. The panicles of each plant containing mature seed were harvested in January or February, depending on the exact sowing date, maturity rate of the plant line and growing conditions. Depending on the time of year, later generations were either grown at the field research station or in the ground in a temperature regulated plastic house at Pacific Seeds LTD PTY in Toowoomba, Queensland (27°56'S, 151°88'E). Soil from the research station in Gatton was moved to the plastic houses and fertilizer added in the same way as in the field. The average max/min temperatures during the growing season were 32.5 °C / 16.3 °C day/night, and an average relative humidity of 57% at 9 am and 39% at 3 pm. The plots were irrigated weekly.

A mutant *S. bicolor* population was generated from Elite line seeds (Table 2.1) using TILLING (Figure 6.1), which were treated with 0.15-0.4% ethylmethyl sulfide (EMS) in six batches, totaling 53,000 seeds (Table 6.1). The seeds were treated in Melbourne and freighted to the field site in Gatton, approximately 24 hours transit, were they were grown (as above). Approximately 16,300 M_1 plants germinated in October, 2007. Paper bags were placed over the panicle prior to anthesis to prevent cross-pollination and ensure plants were self-fertilised. Overall 4,185 M_1 plants produced seed, with between

1 and > 200 seeds per plant. Up to five seeds from each M_1 plant were planted in the field to form the M_2 generation of ~6,000 plants.

Each plant in the M_2 population (sown October 2008) was screened at 8 weeks in the field using the Feigl-Anger assay (section 2.4.1; Takos *et al.* 2010). Putative 'cyanogenesis' mutants were identified based on either high or low cyanide concentration compared to parental control plants. The first fully expanded leaf (leaf I) of each putative mutant was removed (at either 8 weeks or ~12 weeks of growth) and dried at 70°C for two days before being sent to Melbourne for quantitative cyanide analysis (section 2.4). The dried leaf tissue was also used to extract genomic DNA. Mutations in the coding regions of the *CYP79A1* or *UGT85B1* gene were identified by amplifying the genes of interest, using fluorescent-labeled primers. PCR products were pooled three fold, then heated and cooled to form heteroduplexes. The heteroduplex fragments were digested with the enzyme CEL1, a restriction enzyme that cleaves mismatched DNA. PCR products were run on a LI-COR 4200 DNA Analyser to detect multiple fragments. Details are included in Blomstedt, O'Donnell *et al*. (2012). Mutations were confirmed by sequencing (section 6.3). The results from the Feigl-Anger assay and the quantitative cyanide assays were used to select mutants of high or low HCNp for further investigation in the M_3 generation. Up to 50 seeds of each M_3 line were sown in the field in October 2009. At approximately 8 weeks of age, phenotypic analysis (height, health and growth stage) and quantitative cyanide analysis was conducted on every plant in the M_3 population. The M_2 sequencing results and the phenotypic analysis of the M_3 plants was used to select lines of scientific and commercial interest to be carried into the M_4 generation.

The plants in the M_4 generation were grown in a plastic house at Pacific Seeds PTY LTD, Toowoomba, QLD (March-June 2010) and at 8 weeks old the HCNp was measured in Leaf I for each plant (see Figure 2.1). The M_5 generation was chosen based on determination of homozygosity (from phenotypic and/or sequence data) of the M_4 generation. Fifty seeds from each line selected for the M_5 generation were sown in the field in Gatton, October 2010. The seed produced by the M_5 generation was pooled per line.

The seed produced from each plant from the M_1 to M_4 generations were removed and stored separately. A plant numbering system designed to allow tracking of individual plants through each generation was devised. For example; plant 4-882-1-3-1, the first number represents the fourth EMS treatment batch of seeds (Table 6.1), the 882 is the M_1 reference number and each subsequent number identifies the plant selected in the M_2, M_3, M_4 etc. generations, in this case plant 1 of the M_2 generation, plant 3 in the M_3 generation and plant 1 in the M_4 generation.

Table 6.1 EMS treatment groups and the number of established plants in the M_2 generation that resulted from each seed treatment group.

EMS treatment group	EMS (%)	Number of seeds treated	Number of plants screened by FA paper in M_2 generation
1	0.25	10000	84
2	0.15	10000	3050
3	0.25 0.40	5000 4000	91
4	0.20 0.30	7000 7000	2130
5	0.15 0.35	4000 2000	117
6	0.35	4000	55
Total		53000	5527

6.2.2 Totally cyanide deficient 2 (tcd2) sorghum line

6.2.2.1 Identification of the totally cyanide deficient 2 (tcd2) sorghum line

The *total cyanide deficient 2* (*tcd2*) line was obtained by monitoring the cyanide concentration of the progeny of the 4-882-1 plant from the M_2 generation to the M_5 generation. TILLING of *CYP79A1* and *UGT85B1* genes in individual plants of the M_2 generation identified the presence of mutations in several lines. The plants showing mutations in the CYP79A1 gene were characterized in Blomstedt, O'Donnell, et al. (2012). Plants which showed markedly reduced HCNp's but did not show mutations in the CYP79A1 gene, were then tested for mutations in the UGT85B1 gene. Multiple mutant UGT85B1 lines were identified however not all of these lines set seed and

therefore were not able to be further characterised. The *tcd2* line was chosen for further analysis as this line had ample viable seed. One line, 4-882-1, was identified as containing a mutation in *UGT85B1* and was selected for further characterisation. Two groups of 4-882-1 M_3 generation offspring were grown. One group was grown along with the other cyanogenesis mutant lines at the Pacific Seeds PTY LTD research station in Gatton (October 2009 – January 2010) and analysed for HCNp and carried through to further homozygous generations. The second group was grown in glasshouse conditions at Copenhagen University, Denmark (55°41'N, 12°32'E). In this second group forty seeds were grown in punnets of soil:perlite mix and at the 3-4 leaf stage, the whole shoot was removed and the dhurrin concentration measured by LC-MS.

6.2.2.2 Growth characterisation of totally cyanide deficient 2 (tcd2) sorghum line

The *tcd2* (4-882-1) line was grown over several generations to produce a homozygous line that contained the mutation. During this selection process a segregating sibling line without the mutation (TCD2) was also selected as a control for subsequent experiments. The growth characteristics of the *tcd2* plants were studied further with two control plant lines also included in the study, the non-mutated parental Elite line and the TCD2 line. The TCD2 line was included as a control for background EMS mutations which may account for phenotype differences observed in *tcd2* plants compared to the Elite parent.

Elite, TCD2 and *tcd2* plants (n = 4 plants per harvest) were grown in individual pots containing soil, as described in section 2.2, and harvested at either 8 weeks (Harvest 1) or 10 weeks (Harvest 2). Parameters measured at the time of harvest were: sheath height, sheath width, leaf number, leaf blade length, leaf blade width, total leaf surface area, and F_V/F_M. The leaf length, leaf width, and F_V/F_M measurements were determined on the youngest fully expanded leaf (leaf I). The plants were divided into leaf blade, leaf sheath and root tissues, see section 2.1. The roots were further cut into ~1 cm sections and a sample taken for root length measurements (section 2.3). All plant tissues were dried and weighed before analysis for cyanide concentration, nitrate concentration and total carbon-nitrogen (section 2.4). Plant tissue was also extracted for LC-MS analysis as described below.

6.2.3 Sample analysis

Dhurrin analysis

Feigl-Anger (FA), semi-quantitative, assays were performed to test for the presence/absence of dhurrin in leaf discs taken from the first fully unfurled leaf (leaf I) of each plant, following the methods outlined in (Miller *et al.* 2006; Takos *et al.* 2010; Blomstedt, O'Donnell, *et al.* 2012) see section 2.5.1. Following the identification of cyanogenic mutant lines of interest the first fully unfurled leaf was harvested, dried and ground and the dhurrin concentration measured by evolved cyanide, using the colorimetric cyanide assay as described in Gleadow, O'Donnell, *et al*. (2012) and section 2.4.2.

Dhurrin quantification by LC-MS

The dhurrin concentration was also quantified using LC-MS. In the M_3 generation, the total shoot was measured and in the M_5 generation the leaf blade, leaf sheath and root tissue were measured separately. The tissue was homogenized in liquid N_2 before boiling a 100 mg - 200 mg tissue in 500 µL 85% MeOH and cooled on ice. Following extraction, the homogenate was centrifuged (10 min, 3,000 g) and 20 µL aliquots were diluted with 40 µL H_2O and filtered through a membrane by centrifugation. The dhurrin content was determined by injection of 0.1 and 2 µL aliquots in the LC-MS (Gleadow, O'Donnell et al. 2012). The analytical LC-MS was carried out using an Agilent 1100 Series LC (Agilent Technologies) hyphenated to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics). An authentic dhurrin standard was also analysed. See section 2.4.1 for full method.

Nitrate analysis

The soluble nitrate concentration was measured on 0.015 g ground dried tissue, using the method described by Cataldo et al. (1975), see section 2.4.2.

Total carbon-nitrogen analysis

The total carbon and nitrogen concentrations was measured on 5 mg ground dried plant material on Elementar Vario Micro Cube, CHNS anlayser, as described in section 2.4.3.

Detection of mutations in target genes

Genomic DNA was extracted from M_2 plant tissue using the MagAttract DNA extraction kit (Qiagen) and mutations in CYP79A1 and UGT85B1 genes detected as described in Blomstedt, O'Donnell *et al*. (2012) and section 2.5.1.

Confirmation of cyanogenesis mutant lines

CYP79A1 and UGT85B1 mutations were confirmed in the M_2, and M_5 generations by sequencing the gene specific PCR products. Genomic DNA was isolated using the MagAttract DNA extraction kit (Qiagen) or the CTAB method (section 2.5.1), amplified using gene specific primers and purified using the Promega Wizard Kit (Promega) with an added final ethanol precipitation step to optimise DNA purity. The PCR products were sequenced using the Applied Biosystems PRISM BigDye Terminator Mix (Applied biosystems), as per section 2.5.1.

Statistical analysis

Results were analysed using GraphPad prism 5 (GraphPad Software Inc., USA). Data sets were tested for normality and homogeneity of variances prior to analysis. Data that was not normally distributed was log transformed. Comparison of the two treatment groups was performed using a *Student's t-test*. A one-way analysis of variance (*ANOVA*) was performed on groups of plants that had more than two treatment groups. Means that were significantly different were compared *post hoc* using Tukey's t-tests. Different letters indicate means of significant difference. Mean values are followed by one standard error of the mean (± 1 SE).

6.3 Results

6.3.1 Generation of the mutant sorghum population and selection of specific lines with mutations affecting cyanogenesis

An EMS mutant sorghum population was generated and screened for desired, biochemical and molecular traits related to cyanogenesis across multiple generations. The M_2 plants were likely to be heterozygous for numerous mutations throughout the

genome and a wide range of phenotypic differences such as height, leaf width and leaf colour, observed in the M_2 mutant population support this (Figure 6.2).

The preliminary FA assay of the M_2 generation plants at 8 weeks growth (see example in Figure 6.5a) identified 264 putative cyanogenesis mutants, showing either high or low HCNp compared to the Elite non-mutated parent plants. The cyanide concentration of each putative cyanogenesis mutant in the M_2 generation was then quantified from plant tissue at either 8 weeks or 12 weeks growth (Figures 6.3a & 6.3b) and the *CYP79A1* and *UGT85B1* genes screened for the presence of mutations. As, expected, the overall cyanide concentration of the plants quantified at 8 weeks growth was higher than the plants at 12 weeks growth (Figures 6.3a & 6.3b). Elite control plants were included at both time points for comparison. It was apparent by the phenotype and cyanide concentrations of the plants in the M_3 generation that the plants were segregating for numerous traits (Figure 6.4 & Appendix 7).

Several classes of mutants were identified on the basis of plant phenotype, HCNp and TILLING from the plants in the M_2 – M_5 generations. These include *totally cyanide deficient* (*tcd*), *adult cyanide deficient* (*acdc*), *elevated cyanide potential* (*ecp*) and putative β-gluciosidase mutants. Six lines have been selected for further analysis and have been or will be put into a breeding program to produce plants homozygous for the mutations causing the alterations in HCNp (Table 6.2; Blomstedt, O'Donnell *et al.* 2012). Two mutant lines, *tcd1* and *ecp1*, with mutations in the *CYP79A1* gene are of particular interest. *tcd1* has a C to T nucleotide change causing the amino acid change proline to leucine (P414L), resulting in the formation of an inactive protein and no dhurrin production (Blomstedt, O'Donnell, *et al*. 2012). The *ecp1* plants had a nucleotide sequence change of G to A in the coding region of *CYP79A1,* which caused the E145K change at the amino acid level which may increase substrate affinity resulting in higher HCNp (Blomstedt, O'Donnell, *et al*. 2012). The three *acdc* lines were found to be acyanogenic in the adult leaf tissue but not in newly germinated seedlings (< 3 weeks), and to date no mutation has been found to account for the change in HCNp. Several lines were found to have a mutation in the *UGT85B1* gene. However, to date only one mutant line has been characterised in detail and it has been shown to result in a translational change that results in a stop codon in *UGT85B1* but a viable plant line is still generated

(see section 6.3.2). Finally, there were thirty-two EMS mutant lines classified as putative β-glucosidase mutants. There was no or little cyanide detected in the putative β-glucosidase mutants by the M_2 FA paper screen, but there was a moderate to high cyanide concentration in the quantified cyanide assay that used exogenous β-glucosidase. β-glucosidase mutants are not of interest in this project, because the bacteria in the gut of ruminants, such as cattle, produce β-glucosidases that are potentially capable of hydrolysing the cyanogenic glycosides (Majak *et al.* 1983).

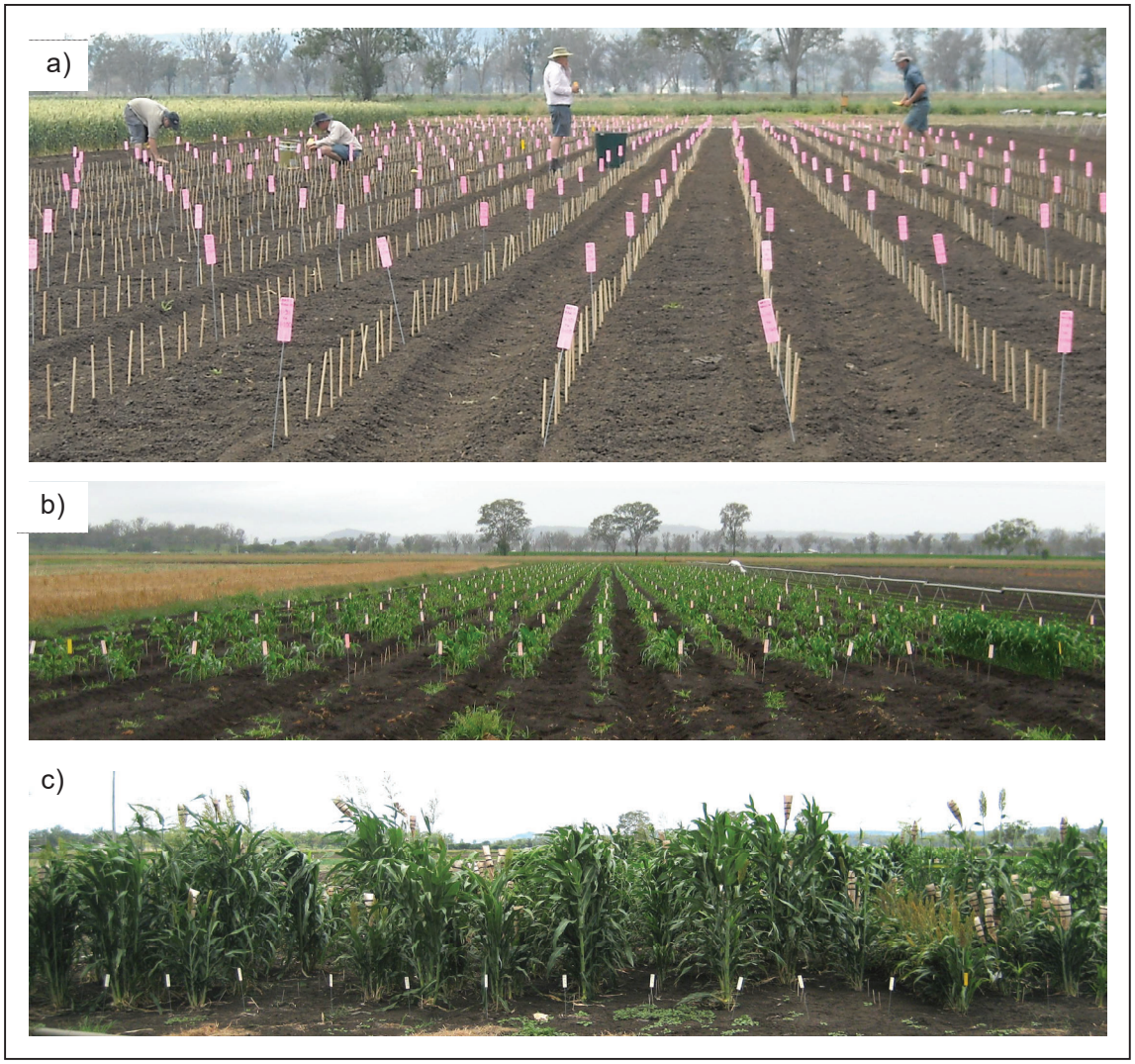


Figure 6.2 The M_2 sorghum mutant population growing over the 2008/2009 season, in the field site in Queensland, Australia. (a) Seed sown in October 2008, (b) 8 weeks of growth and the time at which the plants were assayed for HCNp using the FA paper screen, and (c) 12 weeks growth, clearly showing the range of phenotypic variation caused by the EMS mutations. A row of parental control plants, with panicles showing, can be seen to the right in image c.

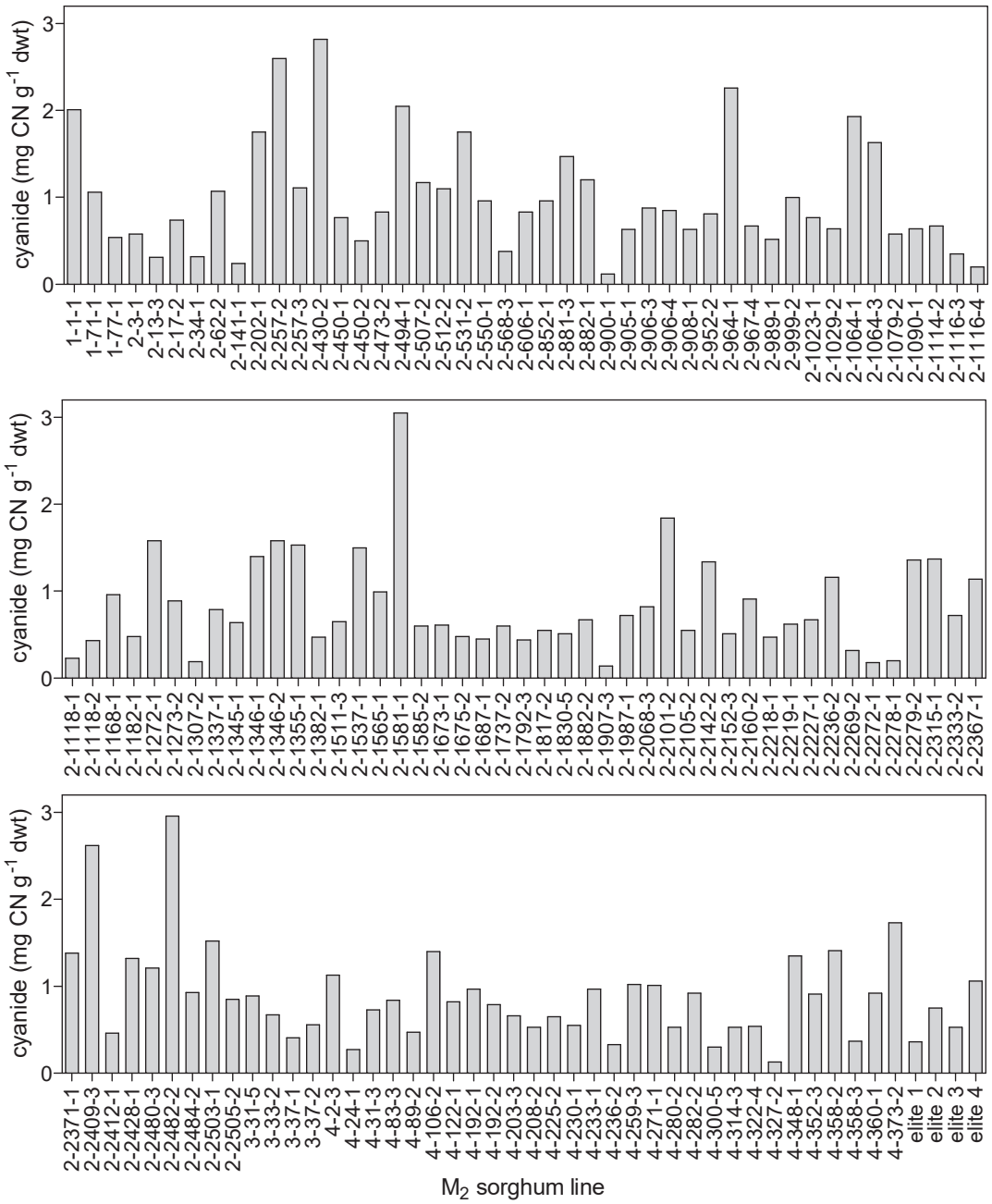


Figure 6.3a The cyanide concentration of the plants in the M_2 generation selected as either high or low HCNp from the Feigl-Anger paper screen. Plants were sampled 8 weeks post planting. The cyanide concentration of Elite plants was included for comparison. Values are the mean of triplicate cyanide assays from n = 1 plant.

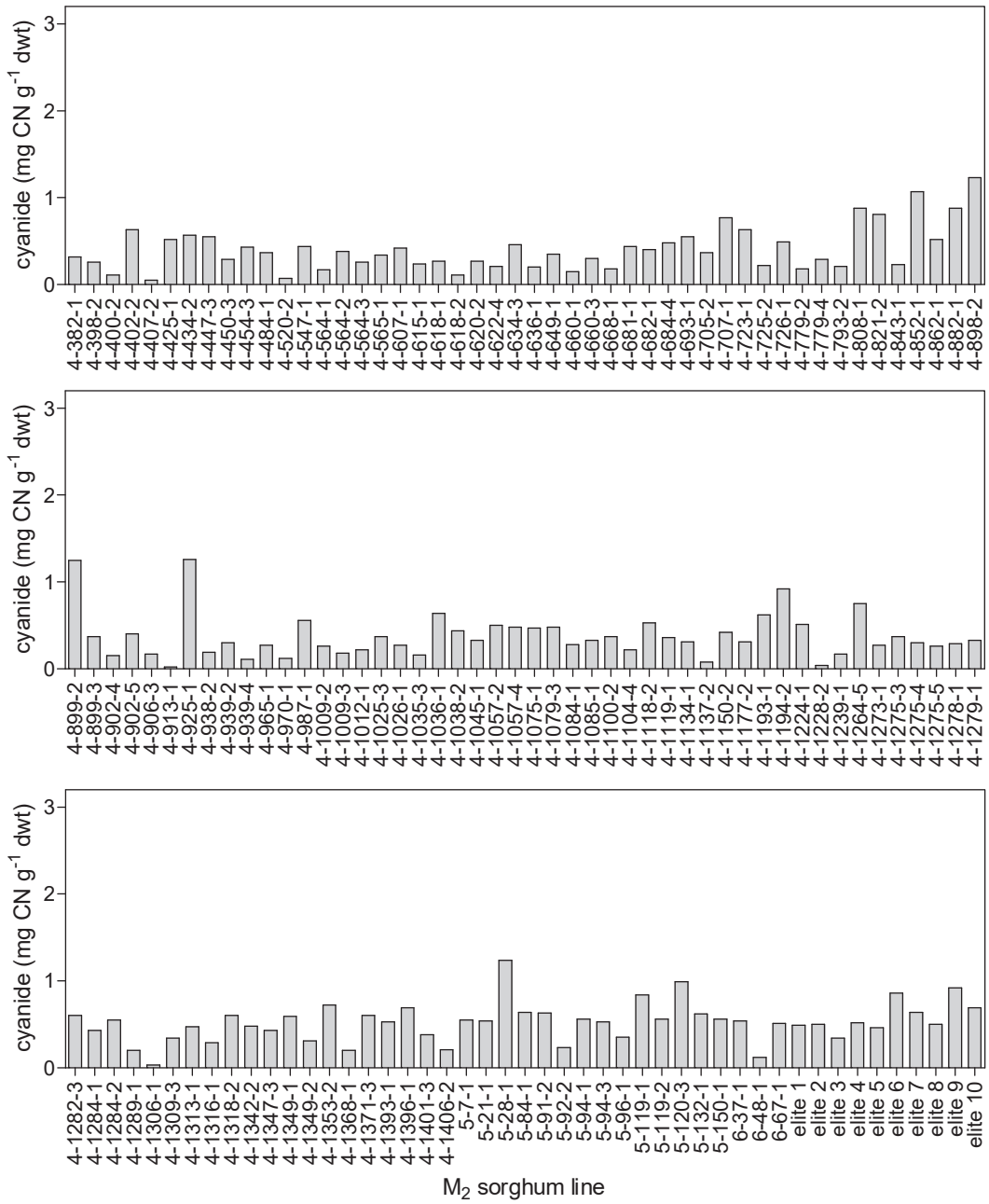


Figure 6.3b Cyanide concentration of the plants in the M_2 generation selected as either high or low HCNp from the Feigl-Anger paper screen. Plants sampled 12 weeks post planting. The cyanide concentration of Elite plants was included for comparison. Values are the mean of triplicate cyanide assays from n = 1 plant.

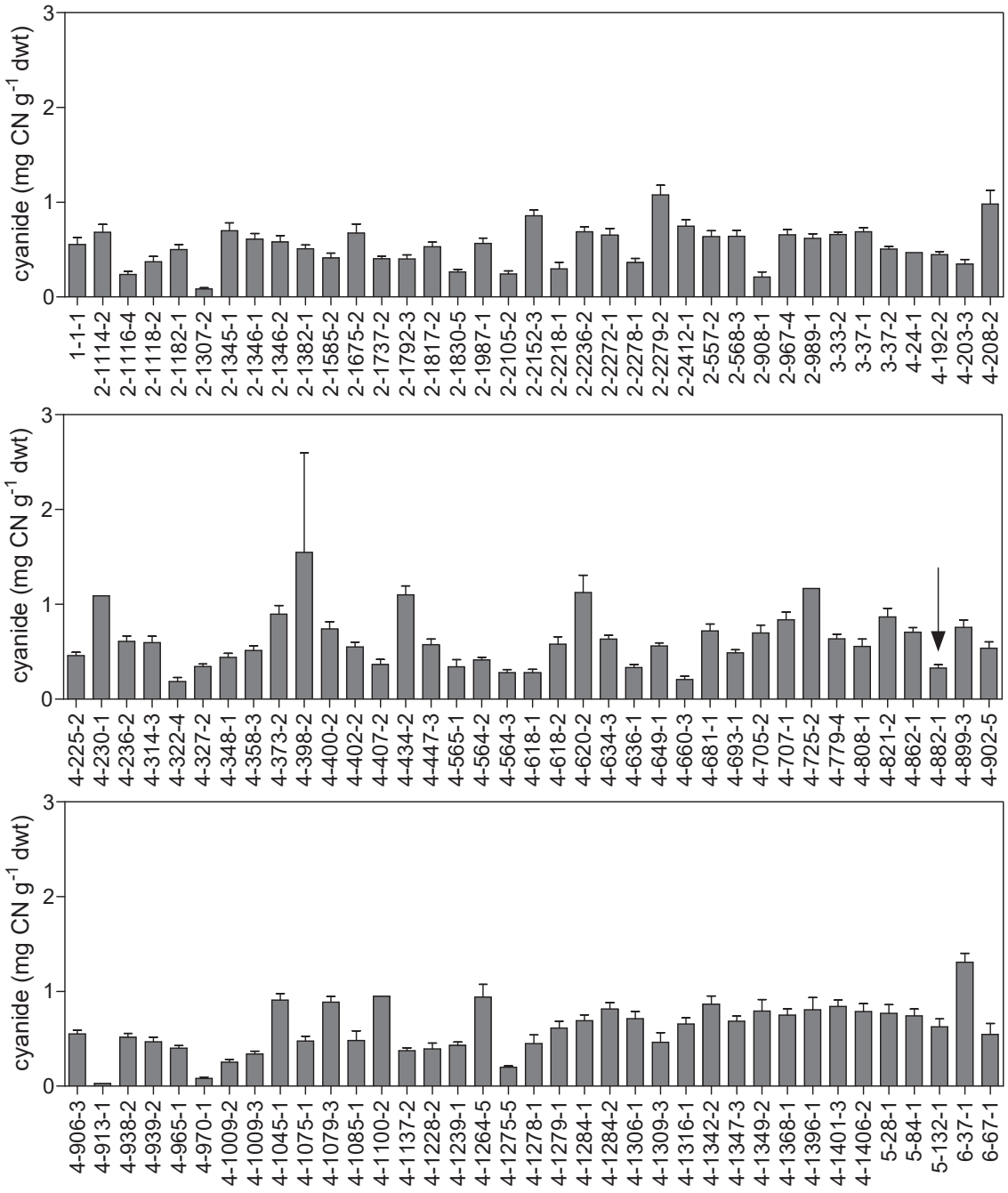


Figure 6.4 Mean cyanide concentration of each line in the M_3 generation. Plants were sampled 6 weeks after emergence. Values are mean (± 1 SE) data from *n* = 1-20 plants (individual plant cyanide concentrations shown in Appendix 7). Arrow highlights the *tcd2* mutant, see section 6.3.2.

Table 6.2 Cyanogenesis mutant lines under further investigation.

M_2 plant ID number	Mutant Name	Mutation	Nucleotide change	Codon change	Viable seed	cyanide class
2-908-1	*tcd1*	CYP79A1	C/T	P414L	yes	acyanogenic
4-882-1-3	*tcd2*	UGT85B1	C/T	Q149*	yes	acyanogenic
2-1307-1	*acdc1*	unknown			yes	adult leaf
4-790-1	*acdc2*	unknown			yes	tissue is
4-565-1	*acdc3*	unknown			yes	acyanogenic
5-84-1	*ecp1*	CYP79A1	G/A	E145K	yes	elevated HCNp

6.3.2 Totally cyanide deficient 2 (tcd2) sorghum line

6.3.2.1 Identification of totally cyanide deficient 2 (tcd2) sorghum line

An acyanogenic sorghum line with a mutation in the coding sequence of the *UGT85B1* gene (Figure 1.3 & Figure 6.7) has been identified. The UGT85B1 mutant line arose from the 4-882-1 plant in the M_2 generation and has been called *totally cyanide deficient 2* (*tcd2*). The HCNp of the 4-882-1 plant was low in the M_2 FA paper screen (Figure 6.5a) but the quantified cyanide concentration on plant tissue one month later was higher than the Elite control plants (Figure 6.5b). Sequencing of total DNA isolated from the 4-882-1 plant from the M_2 generation showed that the mutation was in coding region of the *UGT85B1* gene resulting in the formation of a stop codon, Q149* (Table 6.2; Figure 6.7).

Forty M_3 generation plants (4-882-1 offspring), grown at Copenhagen University, Denmark, had a germination rate of 50% but only a total of 13 plants grew to the 3-4 leaf stage. Metabolomic extracts for each of the mutant plants, as well as Elite control plants, were analysed by LC-MS. Standards showed that dhurrin has a retention time of 5.6 mins. The Elite plants and 12 of the mutant plants showed relatively uniform chromatograms for all metaboiltes (Figures 6.6a & 6.6b). One of the 4-882-1 mutant plants did not have a peak with a retention time of 5.6 mins but had two additional peaks with retention times of 1.4 mins and 3.4 mins (Figure 6.6c), when compared to the control plants. The whole seedling was ground up for the metabolite extraction. This mutant line was considered to be interesting and requiring further detailed analysis.

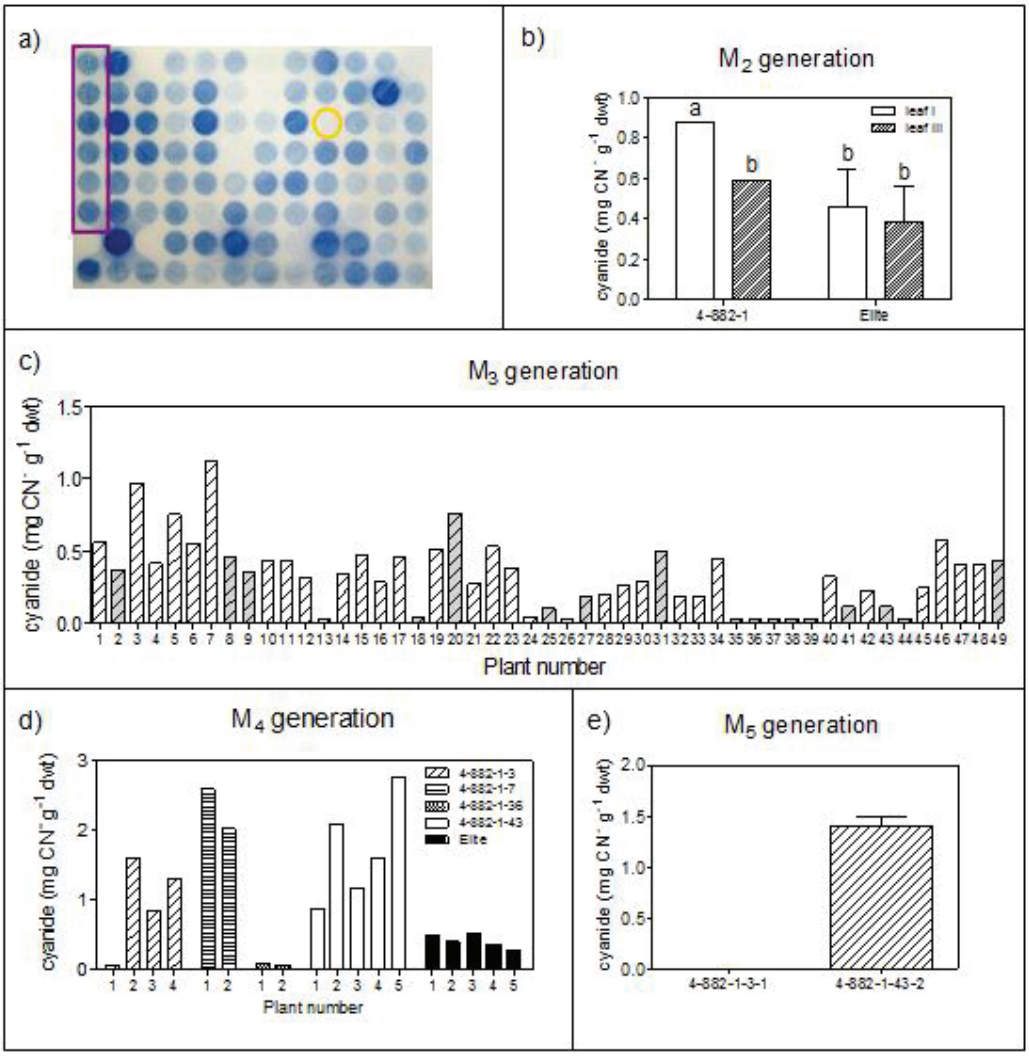


Figure 6.5 HCNp of 4-882-1 mutant line from the M_2 to M_5 generations, resulting in the identification of the *tcd2* mutant line. In the M_2 generation, the cyanide concentration was tested by (a) Feigl-Anger papers at eight weeks, 4-882-1 is highlighted by a yellow circle and controls in purple rectangle; (b) quantitative cyanide assay at 12 weeks for leaves I and III; (c) Variation in cyanide concentration of individual plant in the M_3 generation, the relative heights are indicated by white for 0-25 cm and grey for 26-50 cm; (d) cyanide concentration of the plants in the M_4 generation, showing the segregation of the mutation, for example the, 4-882-1-3 offspring has one individual with low cyanide concentration whilst the other three have high cyanide concentrations; (e) cyanide concentration of the high and low selected sibling lines in the M_5 generation, 4-882-1-3-1 is classified as having no cyanide and was named *tcd2*. Values are from n = 1 plant M_2 – M_4 generations and mean (± 1 SE) data from n = 15 plants for plant in M_5 generation.

Therefore, 100 seeds from the 4-882-1 line were planted in the field in Gatton, Australia. The cyanide concentration was measured and basic phenotypic data recorded for the 49 plants that were present after 6 weeks growth. The offspring of the 4-882-1 line in the M_3 generation were 25 - 50 cm tall and were generally shorter than the control plants (50 – 75 cm tall). The M_3 generation of the 4-882-1 line frequently had multiple tillers, low insect damage and good plant health. The cyanide concentrations of the 4-882-1 offspring plants in the M_3 generation ranged from high (1.1 mg CN g^{-1} dwt) to low (0.03 mg CN g^{-1} dwt; Figure 6.5c). The variation in cyanide concentration was likely to be due to the segregation for the mutation in the *UGT85B1* gene (approximately 1:3 for low:wild type HCNp level). Additionally, the plant heights varied within the 4-882-1 offspring in the M_3 generation (Figure 6.5c; 0-25 cm white and 26-50 cm grey), but plant height did not correlate with the cyanide concentration. Plants with the lowest cyanide concentration in the M_3 generation did not set seed, therefore the plants that showed the lowest cyanide concentration and set seed were selected, along with two sibling lines that had high cyanide concentrations. The four sibling lines were then grown in the M_4 generation.

As found in the M_3 generation, sibling plants in the M_4 generation (with the same M_3 parent) segregated for high and low cyanide concentrations (Figure 6.5d). The 4-882-1-3-1 and 4-882-1-43-5 plants set seed and where selected for their low and high cyanide concentrations, respectively, to be grown in the M_5 generation. Fifty seeds from the 4-882-1-3-1 and 4-882-1-43-5 plants were grown in the field to allow for bulking of the seed. The plants from the respective lines were uniform in growth and health. The cyanide concentrations of 15 randomly selected plants from each M_5 line was measured (Figure 6.5e) and the *UGT85B1* coding gene sequenced to determine whether the mutation had segregated for high or low cyanide potential (Appendix 8). The 4-882-1-43-5 plants had a cyanide concentration of 1.4 ± 0.08 mg CN g dwt and no mutation in the *UGT85B1* coding gene. The 4-882-1-3-1 plants showed no cyanide release and had a C to T nucleotide change in the *UGT85B1* coding gene (Appendix 8), which resulted in the stop codon at Q149 in the amino acid sequence as previously identified in the M2 generation (Figure 6.7). The 4-882-1-3-1 line was classified as *totally cyanide deficient 2* (*tcd2*).

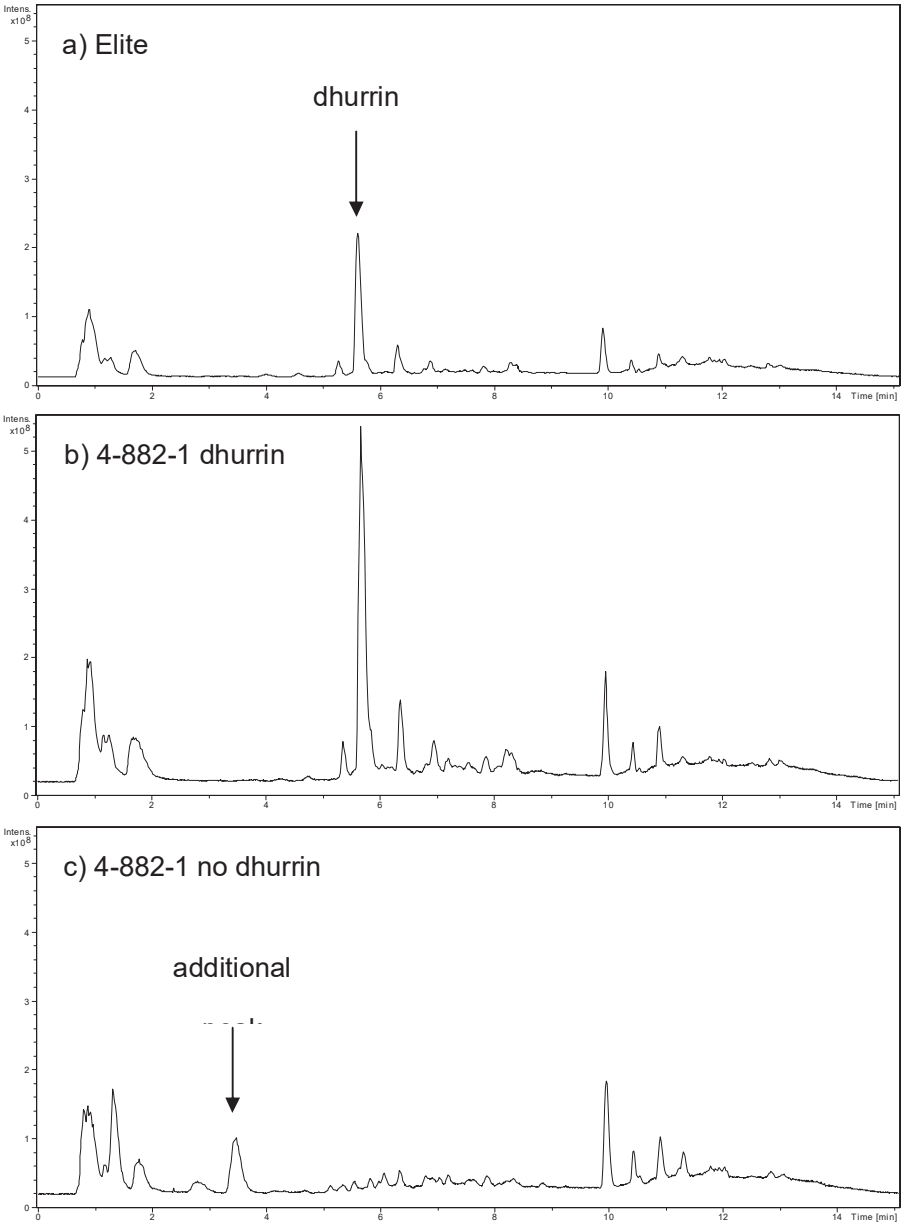
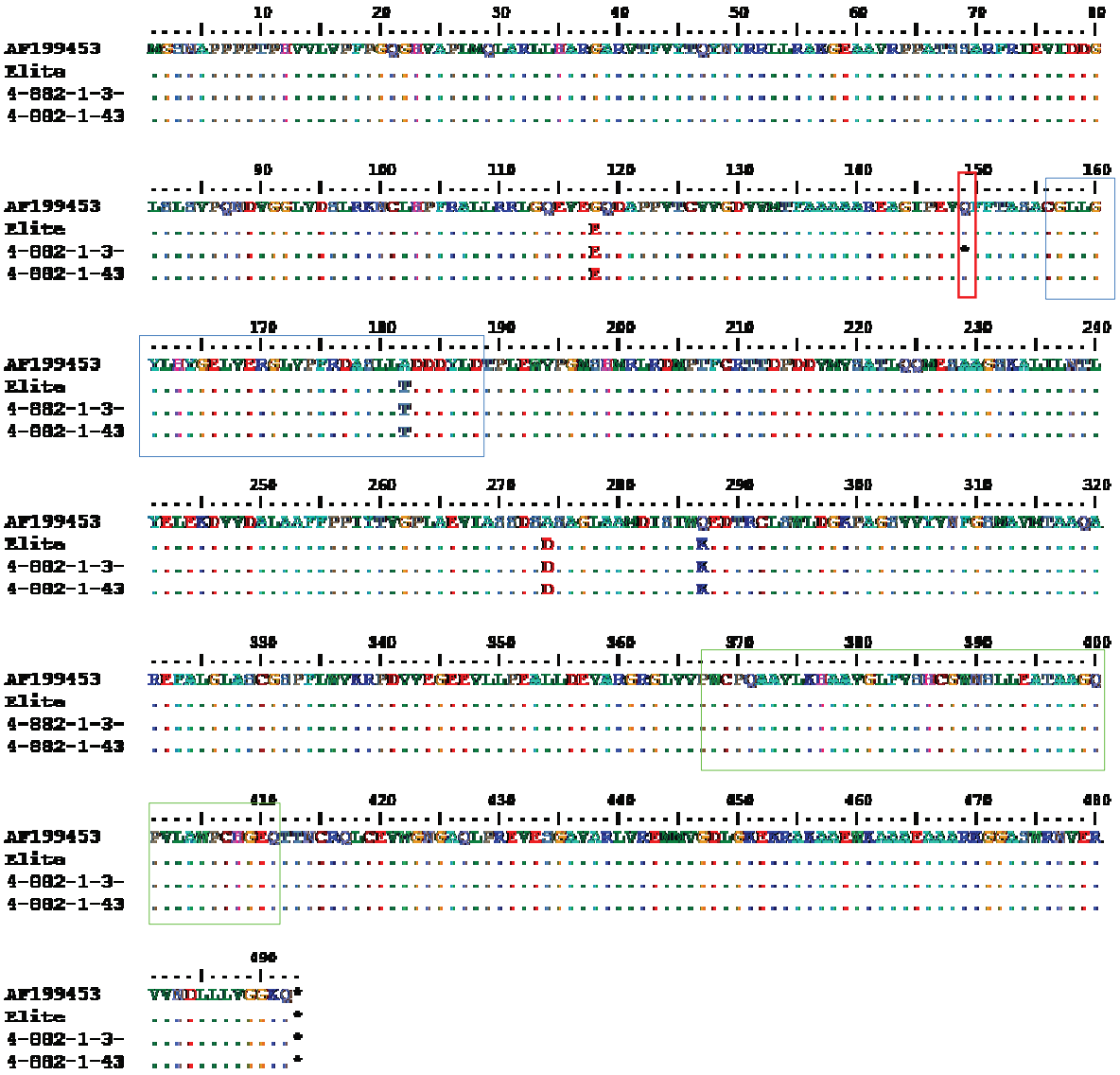


Figure 6.6 LC-MS trace of metabolites extracted from the shoots of Elite and 4-882-1 individuals. Dhurrin has the retention time of 5.6 mins. The additional peak in block c has retention times of 3.4 mins.

Figure 6.7 The UGT85B1 amino acid sequences for Elite and mutant lines 4-882-1-3 and 4-882-1-43, compared to Genebank gene accession AF199453. The red box indicates the amino acid change. The blue box indicates the B loop identified as important for interactions with CYP79A1 and CYP71E1 during metabolon formation (Kannangara *et al*. 2011). The green box indicates the sugar binding domain.



6.3.2.2 Growth characterisation of totally cyanide deficient 2 (tcd2) sorghum line

The tcd2 plants had a low germination rate, therefore there were only four replicates per harvest per plant line. There was no significant difference between the Elite and TCD2 plants for all measurements, except leaf length and the root to shoot ratio (Tables 6.4, 6.5 & 6.6). The *tcd2* plants were significantly smaller at 10 weeks of growth (Figure 6.8; $p < 0.001$). However, at 8 weeks growth there was no significant difference in the total shoot mass ($p = 0.069$; Figure 6.8 b). The same trends were observed for the total root mass at 8 weeks ($p = 0.074$) and ten weeks ($p < 0.001$) growth. The lack of significant difference in the dry mass at 8 weeks growth appears to be due to the large difference in the total dry mass of the Elite plants.

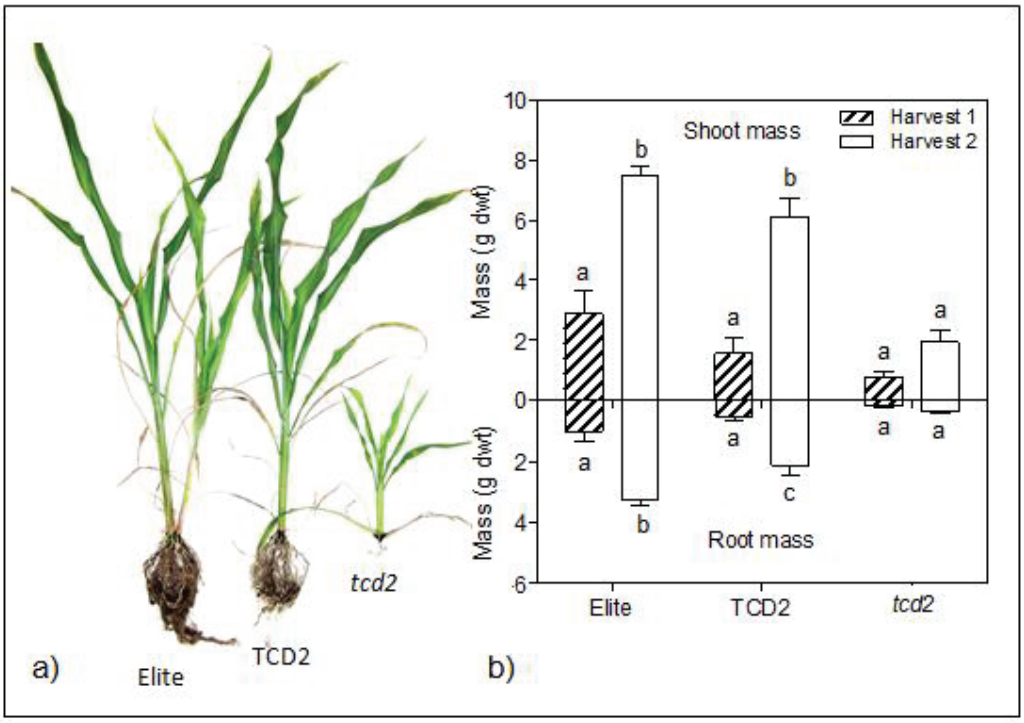


Figure 6.8 (a) Phenotype of Elite, TCD2 and *tcd2* sorghum plants at ten weeks and (b) mass (g dwt) of shoots and roots at 8 and 10 weeks. The Elite line is the parental control line, *tcd1* line is acyanogenic and has mutation the *UGT85B1* gene, TCD2 line are the segregating sibling to *tcd2*, selected for wild-type *UGT85B1*.

Table 6.4 Growth parameters for Elite, TCD2 and *tcd2* plants at Harvest 1 and Harvest 2. Harvest 1 plants were 8 weeks old and Harvest 2 plants were 10 weeks old. Leaf I and III refer to the first and third fully expanded leaves respectively. Values are mean (± 1 SE) data from $n = 4$ plants. One-way analysis of variance (ANOVA) was performed and letters indicate significant difference at $p < 0.05$, bold values are significant.

	Harvest 1				**Harvest 2**			
	Elite	**TCD2**	***tcd2***	***p*-value**	**Elite**	**TCD2**	***tcd2***	***p*-value**
Height (cm)	14.4 ± 0.6[a]	12.5 ± 0.7[a]	9.1 ± 0.5[b]	**<0.001**	22.7 ± 0.7[a]	21.1 ± 0.8[a]	13.1 ± 1.3[b]	**<0.001**
Leaf surface area (cm^2)	425.6 ± 91.4[a]	216.0 ± 48.3[ab]	85.15 ± 23.6[b]	**0.013**	918.2 ± 76.7[a]	794.3 ± 54.9[a]	175.4 ± 24.4[b]	**<0.001**
No. mature leaves	8.5 ± 0.19	7.8 ± 0.31	8.4 ± 0.18	0.123	11.8 ± 0.25[a]	11.0 ± 0.0[ab]	10.5 ± 0.29[b]	**0.016**
No. immature leaves	2.9 ± 0.13[a]	3.3 ± 0.21[b]	2.0 ± 0.0[a]	**<0.001**	3.0 ± 0.0[a]	3.0 ± 0.0[a]	2.3 ± 0.25[b]	**0.014**
Leaf length (cm)								
leaf I	48.1 ± 3.80[a]	31.2 ± 3.98[b]	23.2 ± 1.21[b]	**<0.001**	70.0 ± 0.84[a]	63.8 ± 1.59[a]	26.4 ± 2.01[b]	**<0.001**
leaf III	29.6 ± 4.70[a]	21.0 ± 1.87[ab]	14.9 ± 1.53[b]	**0.012**	59.1 ± 2.05[a]	48.3 ± 2.49[b]	24.9 ± 1.64[c]	**<0.001**
Leaf width (cm)								
leaf I	2.4 ± 0.14[a]	2.0 ± 0.23[ab]	1.6 ± 0.14[b]	**0.007**	4.7 ± 0.19[a]	4.9 ± 0.27[a]	2.5 ± 0.21[b]	**<0.001**
leaf III	1.9 ± 0.12[a]	1.4 ± 0.17[ab]	1.1 ± 0.16[b]	**0.005**	2.6 ± 0.25	3.0 ± 0.067	2.2 ± 0.15	0.054
Stem width (cm)	1.4 ± 0.10[a]	1.1 ± 0.07[ab]	1.0 ± 0.06[b]	**0.005**	1.7 ± 0.08	1.6 ± 0.03	1.7 ± 0.19	0.940
Total root length (cm)	7441 ± 1706	6512 ± 1451	5786 ± 1370	0.739	6998 ± 1706	12722 ± 2400	7067 ± 7067	0.100
Dry mass (g)								
leaf blade	1.80 ± 0.44[a]	0.90 ± 0.21[ab]	0.42 ± 0.11[b]	**0.029**	4.67 ± 0.19[a]	3.96 ± 0.45[a]	0.97 ± 0.17[b]	**<0.001**
leaf sheath	1.08 ± 0.35	0.67 ± 0.31	0.35 ± 0.11	0.195	2.80 ± 0.17[a]	2.15 ± 0.20[a]	0.97 ± 0.23[b]	**<0.001**
root	1.01 ± 0.33	0.50 ± 0.15	0.19 ± 0.05	0.074	3.26 ± 0.15[a]	2.13 ± 0.30[b]	0.34 ± 0.06[c]	**<0.001**
total plant	3.90 ± 1.12	2.08 ± 0.67	0.95 ± 0.23	0.068	10.74 ± 0.40[a]	8.23 ± 0.93[b]	2.30 ± 0.45[c]	**<0.001**

The *tcd2* plants at Harvest 1 were shorter than the Elite control plants ($p < 0.001$). The *tcd2* plants also had a smaller leaf surface area ($p = 0.013$), leaf length (leaf I $p < 0.001$ and leaf III $p = 0.012$) and leaf width (leaf I $p = 0.007$ and leaf III $p = 0.005$) compared to the Elite plants but were not significantly different in size to the TCD2 plants (Table 6.4). At Harvest 2, the *tcd2* plants were smaller than both the Elite and TCD2 plants with regard to height ($p < 0.001$), leaf surface area ($p < 0.001$), leaf length (leaf I $p < 0.001$ and leaf III $p < 0.001$) and leaf width (leaf I $p < 0.001$; Table 6.4). The number of mature leaves on a plant was similar between lines at Harvest 1, but at Harvest 2 the *tcd2* plants had significantly less mature leaves than the Elite plants ($p = 0.016$; Table 6.4). The stem width of the *tcd2* plants at Harvest 1 was 39% smaller than the Elite ($p = 0.005$). However, the stem width of the *tcd2* plants at Harvest 2 was not significantly different to the Elite plants ($p = 0.940$; Table 6.4). The total root length was not significantly different between lines at either harvest (Harvest 1 $p = 0.739$; Harvest 2 $p = 0.100$).

In general, the *tcd2* plants were smaller but other growth parameters within each individual plant were in proportion. The *tcd2* plants had a significantly lower relative growth rate (RGR) from germination to Harvest 1 than the Elite plants ($p = 0.012$), but there was no significant difference in RGR ($p = 0.074$) between Harvests 1 and 2 for all plant lines (Table 6.5). The net assimilation rate (NAR) of all plant lines was not significantly different between harvest 1 and 2 ($p = 0.514$). The specific leaf area (SLA) of the *tcd2* plants was approximately 20% lower than the Elite plants at Harvest 1 ($p = 0.045$). Although, at Harvest 2 there was no significant difference in SLA between plant lines ($p = 0.550$; Table 6.5). The leaf area ratio (LAR) of the *tcd2* plants was 35% lower than the Elite plants at Harvest 1 ($p = 0.036$), yet there was no significant difference in LAR at Harvest 2 ($p = 0.074$; Table 6.5). Interestingly, there was no significant difference in leaf weight ratio (LWR) between *tcd2* and Elite plants (Harvest 1 p = 0.166; Harvest 2 $p = 0.025$; Table 6.5). The root to shoot ratio showed no significant difference between the lines at Harvest 1 ($p = 0.973$; Table 6.5). However, at Harvest 2 the root to shoot ratio for the *tcd2* plants was 60% lower than the Elite plants ($p < 0.001$). Surprisingly there was no statistically significant difference in total root length between Elite, TCD2 and *tcd2* plants at Harvest 1 ($p = 0.739$) or Harvest 2 ($p = 0.100$; Table 6.4).The results had a high standard error rate and from visual inspection of the root systems it appears that the root system of the tcd2 plants are in fact smaller than the control plants.

Additionally, the *tcd2* plants had 90% less specific root length (SRL) than the Elite plants (Harvest 1 $p = 0.046$; Harvest 2 $p = 0.014$; Table 6.5).

At Harvests 1 and 2, the *tcd2* carotenoid concentrations ($p = 0.354$, $p = 0.721$), chlorophyll *a*:*b* ratios ($p = 0.911$, $p = 0.444$), chlorophyll *a*+*b* concentrations (based on weight and area: weight $p = 0.510$, $p = 0.396$ and area $p = 0.425$, $p = 0.790$) were not significantly different to the Elite or TCD2 lines (Tables 6.6a & 6.6b). The efficiency of photosystem II (PSII), measured by F_v/F_m, varied between harvests for the Elite plants but not the *tcd2* plants. There was no significant difference in the F_v/F_m of Leaves I and III at Harvest 1 ($p = 0.8887$, $p = 0.2929$; Figure 6.9). At Harvest 2, there was no significant difference in the F_v/F_m for leaf I ($p = 0.4425$). The leaf III F_v/F_m, however, for the Elite plants was 36% lower than *tcd2* plants. The TCD2 plants also showed a reduced F_v/F_m compared to the *tcd2* plants but this was not significant.

Table 6.5 Summary of mean growth rate and biomass partitioning for Elite, TCD2 and *tcd2* sorghum lines at Harvest 1 (8 weeks) and Harvest 2 (10 weeks). Values are mean (± 1 SE) data from $n = 4$ plants. One-way analysis of variance (ANOVA) was performed with $p < 0.05$ confidence interval, bold values are significant.

	Harvest	Elite	TCD2	*tcd2*	*p*-value
RGR (g g^{-1} day^{-1} dwt)	0-1	0.09 ± 0.005	0.08 ± 0.003	0.07 ± 0.004	**0.012**
	1-2	0.07 ± 0.003	0.10 ± 0.008	0.06 ± 0.013	0.074
NAR (g m^{-2} d^{-1} dwt)	1-2	0.12 ± 0.009	0.17 ± 0.019	0.14 ± 0.035	0.514
SLA (m^2 g^{-1} dwt)	1	243.2 ± 12.26^a	240.6 ± 9.96^a	206.0 ± 6.11^b	**0.045**
	2	197.9 ± 19.21	203.2 ± 13.29	181.1 ± 6.60	0.550
LAR (m^2 g^{-1} dwt)	1	66.82 ± 5.12^a	62.37 ± 5.49^{ab}	49.28 ± 0.88^b	**0.036**
	2	50.51 ± 3.91	56.01 ± 3.54	42.56 ± 2.78	0.074
LWR	1	0.27 ± 0.01	0.259 ± 0.02	0.240 ± 0.01	0.166
	2	0.26 ± 0.01^{ab}	0.276 ± 0.001^a	0.235 ± 0.01^b	**0.025**
Root:shoot	1	0.34 ± 0.02	0.323 ± 0.01	0.307 ± 0.14	0.973
	2	0.44 ± 0.02^a	0.346 ± 0.02^b	0.179 ± 0.01^c	**<0.001**
SRL (cm g^{-1} dwt)	1	3018 ± 1055^a	1079 ± 181^{ab}	258 ± 126^b	**0.046**
	2	9734 ± 245^a	8259 ± 1711^{ab}	932 ± 420^b	**0.014**

Table 6.6a Chemical analysis of Elite, TCD2 and *tcd2* plants at Harvest 1. Plants were 8 weeks old. Leaf I and III refer to the first and third fully expanded leaves respectively. Values are mean (± 1 SE) data from n = 4 plants. One-way analysis of variance (ANOVA) was performed and letters indicate significant difference at $p < 0.05$, bold values are significant.

Harvest 1	Elite	TCD2	*tcd2*	*p*-value
Chlorophyll *a*+*b* (mg g^{-1} dwt)	20.36 ± 4.42	20.96 ± 0.57	15.81 ± 2.69	0.510
Chlorophyll *a*+*b* (mg cm^{-1})	0.041 ± 0.01	0.043 ± 0.002	0.03 ± 0.003	0.425
Chlorophyll a:b	6.28 ± 1.50	5.88 ± 1.09	5.56 ± 0.88	0.911
Carotenoids (mg g^{-1} dwt)	3.41 ± 0.20	3.67 ± 0.46	2.77 ± 0.54	0.354
Dhurrin (M dhurrin g^{-1} dwt)				
leaf blade	21.8 ± 3.4[a]	35.4 ± 9.1[a]	0.0 ± 0[b]	**0.002**
leaf sheath	34.3 ± 2.0[a]	28.0 ± 0.21[b]	0.0 ± 0[c]	**<0.001**
root	32.6 ± 1.4[a]	29.4 ± 7.8[b]	0.0 ± 0[b]	**<0.001**

Table 6.6b Chemical analysis of Elite, TCD2 and *tcd2* plants at Harvest 2. Plants were 10 weeks old. Leaf I and III refer to the first and third fully expanded leaves respectively. Values are mean (± 1 SE) data from n = 4 plants. One-way analysis of variance (ANOVA) was performed and letters indicate significant difference at $p < 0.05$, bold values are significant.

Harvest 2	Elite	TCD2	*tcd2*	*p*-value
Chlorophyll *a*+*b* (mg g^{-1} dwt)	11.81 ± 1.28	10.58 ± 3.84	15.43 ± 2.41	0.396
Chlorophyll *a*+*b* (mg cm^{-1})	0.037 ± 0.003	0.033 ± 0.01	0.040 ± 0.01	0.790
Chlorophyll a:b	4.42 ± 0.17	5.25 ± 0.76	4.51 ± 0.44	0.444
Carotenoids (mg g^{-1} dwt)	2.08 ± 0.19	2.12 ± 0.49	2.43 ± 0.34	0.721
Dhurrin (M dhurrin g^{-1} dwt)				
leaf blade	0.8 ± 0.8	0.0 ± 0	0.0 ± 0	0.463
leaf sheath	1.0 ± 1.0	1.1 ± 1.1	0.0 ± 0	0.583
root	16.5 ± 1.0[a]	11.7 ± 3.1[a]	0.0 ± 0[b]	**<0.001**

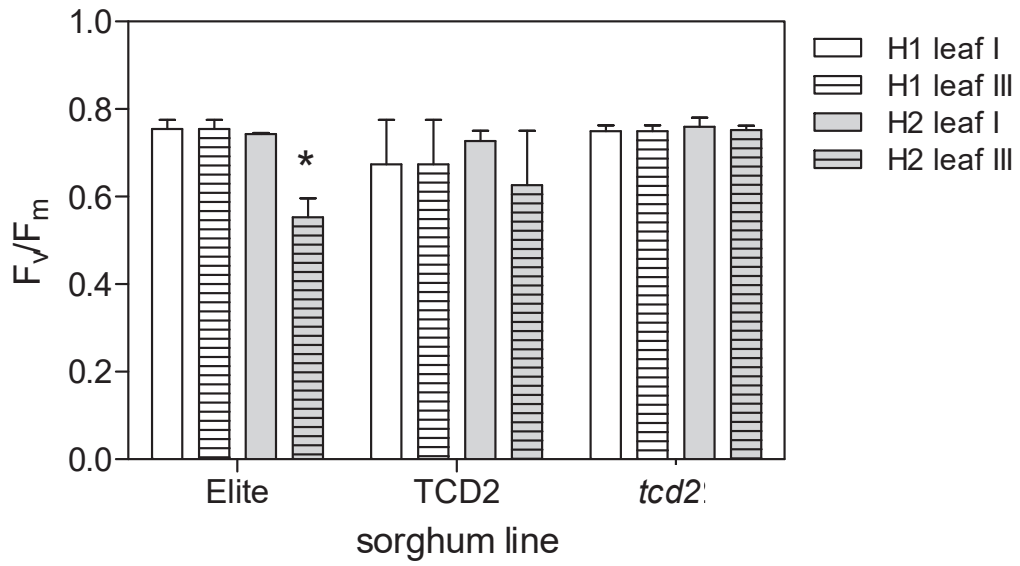


Figure 6.9 Efficiency of photosystem II, measured by F_v/F_m ratios, in leaf I and III from Elite, TCD2 and *tcd2* sorghum lines after eight (H1) and ten (H2) weeks of growth. Values are mean (± 1 SE) data from plants where n = 4-8. * indicates significant difference of p < 0.01.

Cyanogenic glycosides

The dhurrin concentration of the leaf blade, leaf sheath and roots were measured by evolved cyanide (Figure 6.10a) and also directly by LC-MS (Tables 6.6a & 6.6b; Figure 6.10b). The results of the cyanide assays and LC-MS measurements were consistent with one another. The level of evolved cyanide detected in the *tcd2* plants was not significantly different from background (< 0.01 mg g; Gleadow *et al.* 2003), hence indicating that no dhurrin was present in the *tcd2* plants (Figure 6.10a). The cyanide concentration of the Elite and TCD2 plants were not significantly different (p = 0.084). At Harvest 1, the leaf blade cyanide concentration of the Elite control plants was 0.51 ± 0.06 mg CN g^{-1} dwt and 0.01 ± 0.01 mg CN g^{-1} dwt for the *tcd2* plants. The cyanide concentration of the control plant reduced at Harvest 2 to the same level as the *tcd2* plants (p = 0.828; Figure 6.10a). The leaf sheath cyanide concentrations followed the same trends as the leaf blade (Harvest 1 p < 0.001; Harvest 2 p = 0.125). As found in previous chapters, the cyanide concentrations of the roots did not follow the same trends as the cyanide concentration in the leaves. The cyanide concentration in the roots

of the Elite control plants was 0.97 ± 0.11 mg CN g^{-1} dwt at Harvest 1 and decreased by 70% to 0.28 ± 0.01 mg CN g^{-1} dwt at Harvest 2. The roots of the *tcd2* plants contained no cyanide at both harvests (Figure 6.10a).

The total amount of cyanide (mg) in the shoots and roots of Elite, TCD2 and *tcd2* plants was calculated at Harvest 1 and 2 (Figure 6.10b). The small amount of cyanide in the *tcd2* plants was due to limitations in the assay. The LC-MS analysis of confirmed the absence of dhurrin in the *tcd2* plants (Tables 6.6a & 6.6b). There was no significant difference in the total amount of cyanide in the Elite and TCD2 plants ($p = 0.531$). The total shoot cyanide content of the Elite plants decreased 90% from Harvest 1 to Harvest 2. Whereas, the total cyanide content of the roots was not significantly different between the two Harvests for the Elite plants ($p = 0.073$).

LC-MS metabolite analysis on the Elite, TCD2 and *tcd2* lines at both harvest times showed that the metabolites were not expressed equally over time (Figure 6.11; Appendix 9). The LC-MS traces for the *tcd2* plants confirmed that no dhurrin was produced but several new peaks are observed (Figure 6.11a). The LC-MS analysis of the *tcd2* plants also confirmed the presence of the additional peak with a retention time of 3.4 mins, as observed in Figure 6.6. Furthermore, two additional peaks were observed in the *tcd2* plants, these peaks had a retention times of 2.6 and 4.2 mins. These products have mass peaks of m/z 309 at 2.6 min, m/z 323 at 3.4 min and m/z 485 at 4.2 min (Figure 6.11b).

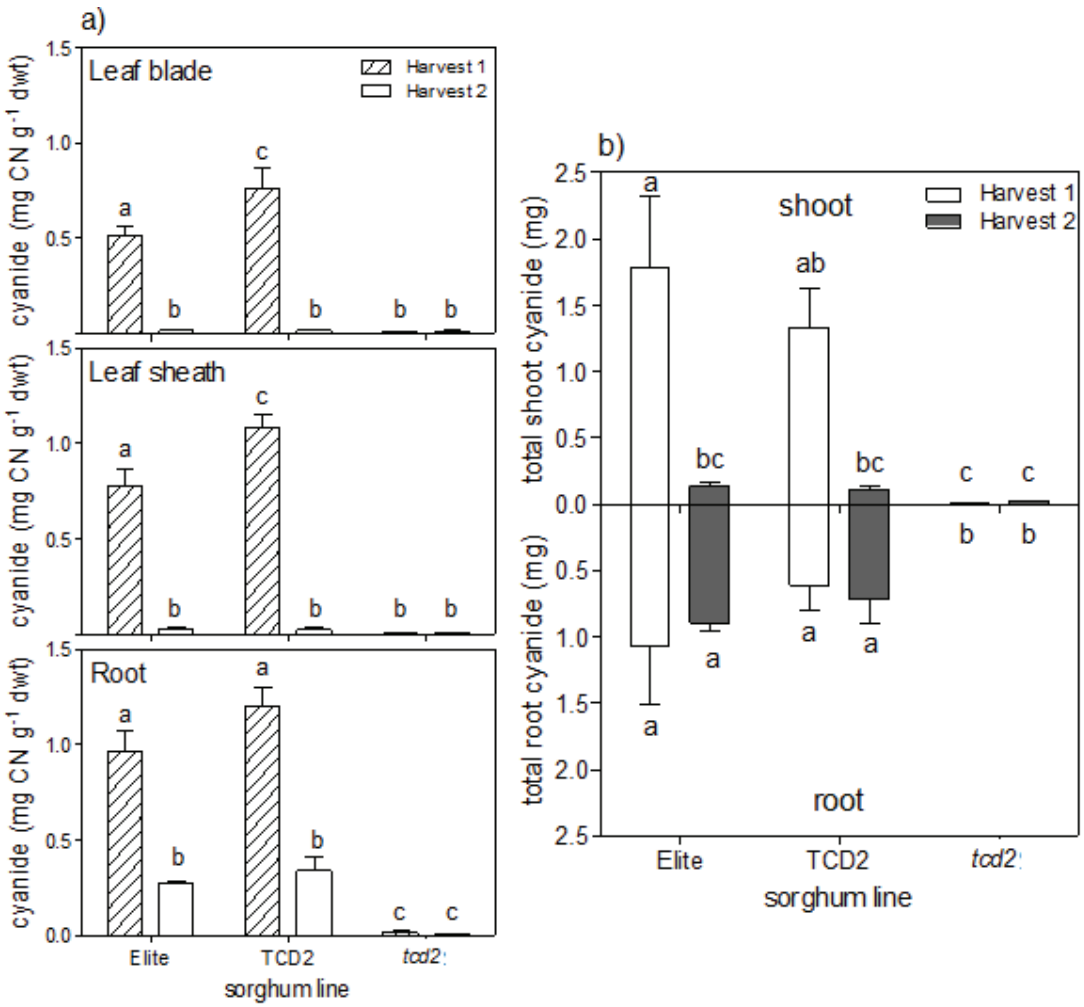
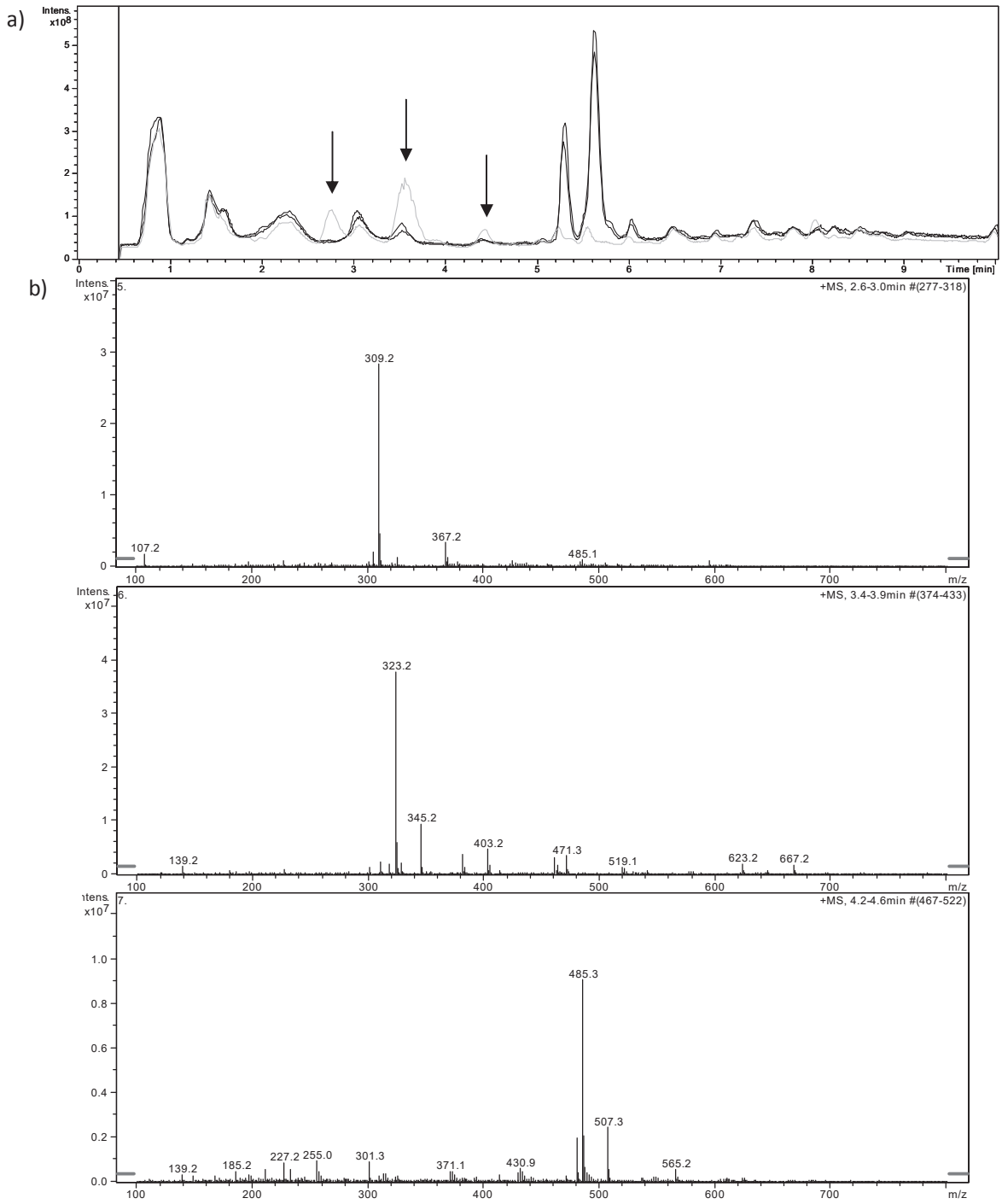


Figure 6.10 (a) Cyanide concentration of the leaf blade, leaf sheath and roots, and (b) total amount of cyanide in the shoots and roots of Elite, TCD2 and *tcd2* sorghum lines at 8 weeks (Harvest 1) and 10 weeks (Harvest 2) growth. Values are mean (± 1 SE) data from $n = 4$ plants. Letters indicate significant difference at $p < 0.05$, using Tukey's tests.

Figure 6.11 LC-Ms analysis of (a) metabolite extracts from Elite (black), TCD2 (black) and *tcd2* (grey) sheath tissue, and (b) mass spectrum of the compounds unique to the tcd2 mutant, indicated by arrows.



Total nitrogen

The total carbon and total nitrogen concentrations of the leaf blade, leaf sheath and roots of the Elite, TCD2 and *tcd2* plants were also measured (Figure 6.12a). The nitrogen concentration of the Elite and TCD2 plants was not significantly different at either harvest. At Harvest 1, the leaf blades of the *tcd2* plants had a nitrogen concentration of 31.9 ± 1.8 mg N g^{-1} dwt which was not significantly different to the Elite or TCD2 plants (p = 0.122; Figure 6.12a). The *tcd2* plants, at Harvest 2, maintained a leaf blade nitrogen concentration statistically the same as at Harvest 1 (p = 0.055), but it was 23% higher than the leaf blade nitrogen concentration of the Elite plants (p = 0.005). The leaf sheath and root nitrogen concentrations showed the same pattern to the leaf blade between the *tcd2* and Elite plants (Figures 6.11a).

The total nitrogen content (mg) of the shoots and roots was calculated per plant for Harvest 1 and 2 (Figure 6.12b). The total nitrogen content in the shoots of the *tcd2* plants was 70% less than the Elite plants at Harvest 1 (p = 0.034) and 60% less than the Elite plants at Harvest 2 (p = 0.003). The total nitrogen content of the TCD2 shoots was not significantly different to the Elite or the *tcd2* plants at either Harvest (Figure 6.12b). The total amount of nitrogen in the root of the Elite, TCD2 and *tcd2* plants reflect the same trends as the shoots, but with one quarter of the nitrogen content (Harvest 1 p = 0.052; Harvest 2 $p < 0.001$; Figure 6.12b).

Nitrates

Nitrate concentrations of the Elite, TCD2 and *tcd2* plants were also measured for the leaf blade, leaf sheath and roots (Figure 6.13a). The leaf blade nitrate concentrations were not significantly different at Harvest 1 (p = 0.708) or Harvest 2 (p = 0.084) for any of the lines. The leaf sheath nitrate concentrations were also not significantly different at Harvest 1 (p = 0.360). However, the *tdc2* leaf sheath nitrate concentration was double the Elite at Harvest 2 (p = 0.007; Figure 6.13a). The root nitrate concentrations follow the same trends as the leaf blades, with no significant difference between the plants at Harvest 1 (p = 0.094) and the *tcd2* root nitrate concentration higher than the Elite plants at Harvest 2 (p = 0.004; Figure 6.13a).

The total amount of nitrate in the shoots and roots of the Elite, TCD2 and *tcd2* plants was calculated (Figure 6.13b). The total nitrate content of the plants followed a similar pattern to the total nitrogen content. The shoots contained at least four-fold more nitrate content than the roots for all plants. At Harvest 1, the total nitrate content of the *tcd2* shoots was 70% less than the Elite shoots (p = 0.013) and the total nitrate content of the TCD2 shoots was not significantly different to the Elite or the *tcd2* plants (Figure 6.13b). Whereas, at Harvest 2, there was no significant difference in the total nitrate content in the shoots of any plants (p = 0.607). The total amount of nitrate in the roots of the *tcd2* plants at Harvest 1 was 75% less than the Elite plants (p = 0.008; Figure 6.13b). At Harvest 2, the Elite plants doubled the total nitrate content of the roots compared to Harvest 1 (p = 0.003). Yet the total root nitrate content of the *tcd2* and TCD2 plants were approximately 80% lower than the Elite plants (p = < 0.001) and the nitrate concentration of the tcd2 roots did not change from Harvest 1 to 2 (p = 0.313; Figure 6.13b).

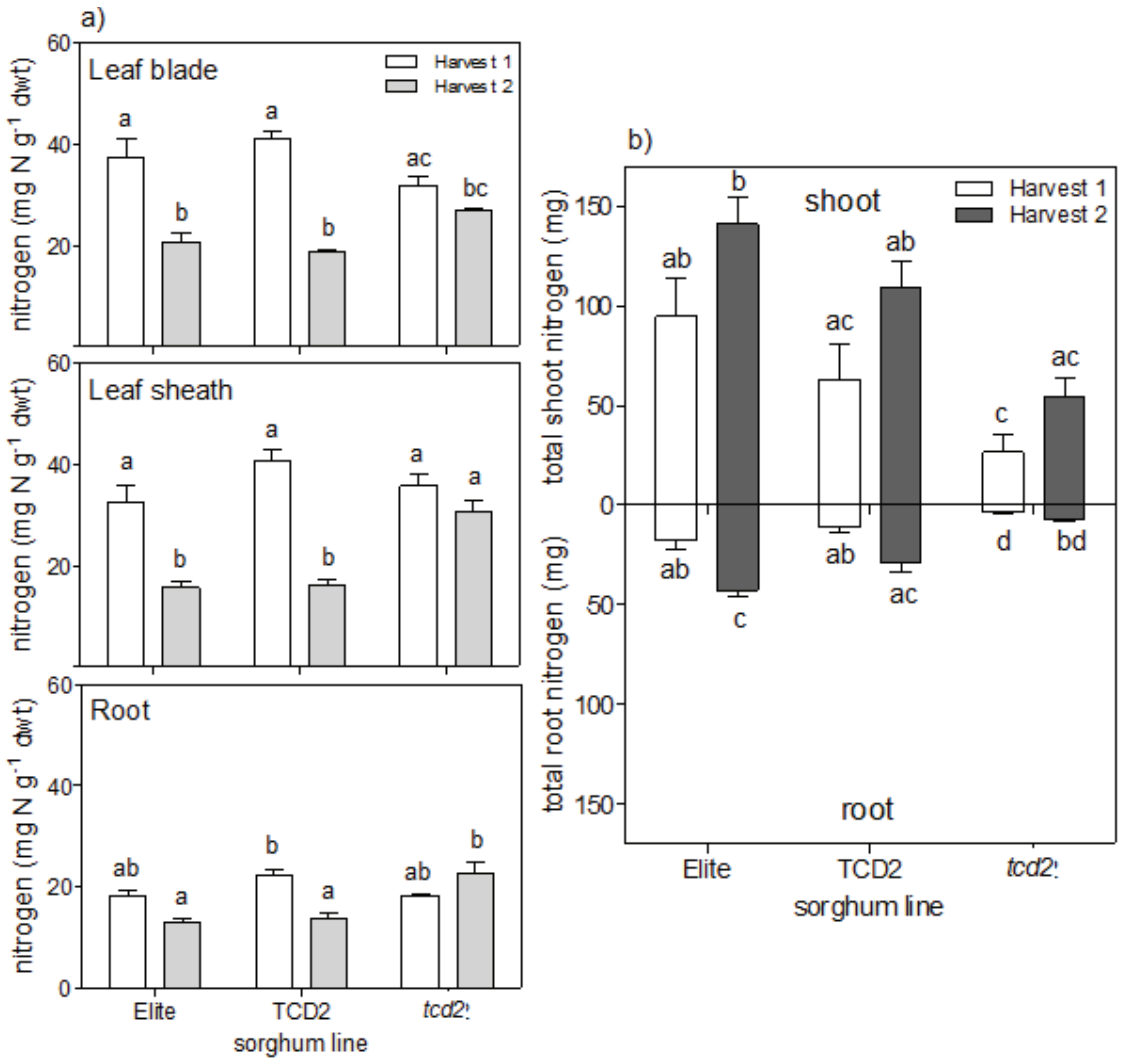


Figure 6.12 (a) Nitrogen concentration of leaf blade, leaf sheath and roots, and (b) total nitrogen content of the shoots and roots of Elite, TCD2 and *tcd2* sorghum lines at eight (Harvest 1) and ten weeks (Harvest 2). Values are mean (± 1 SE) data from n = 4 plants. Tukey's posthoc tests were performed and letters indicate significant difference at p < 0.05.

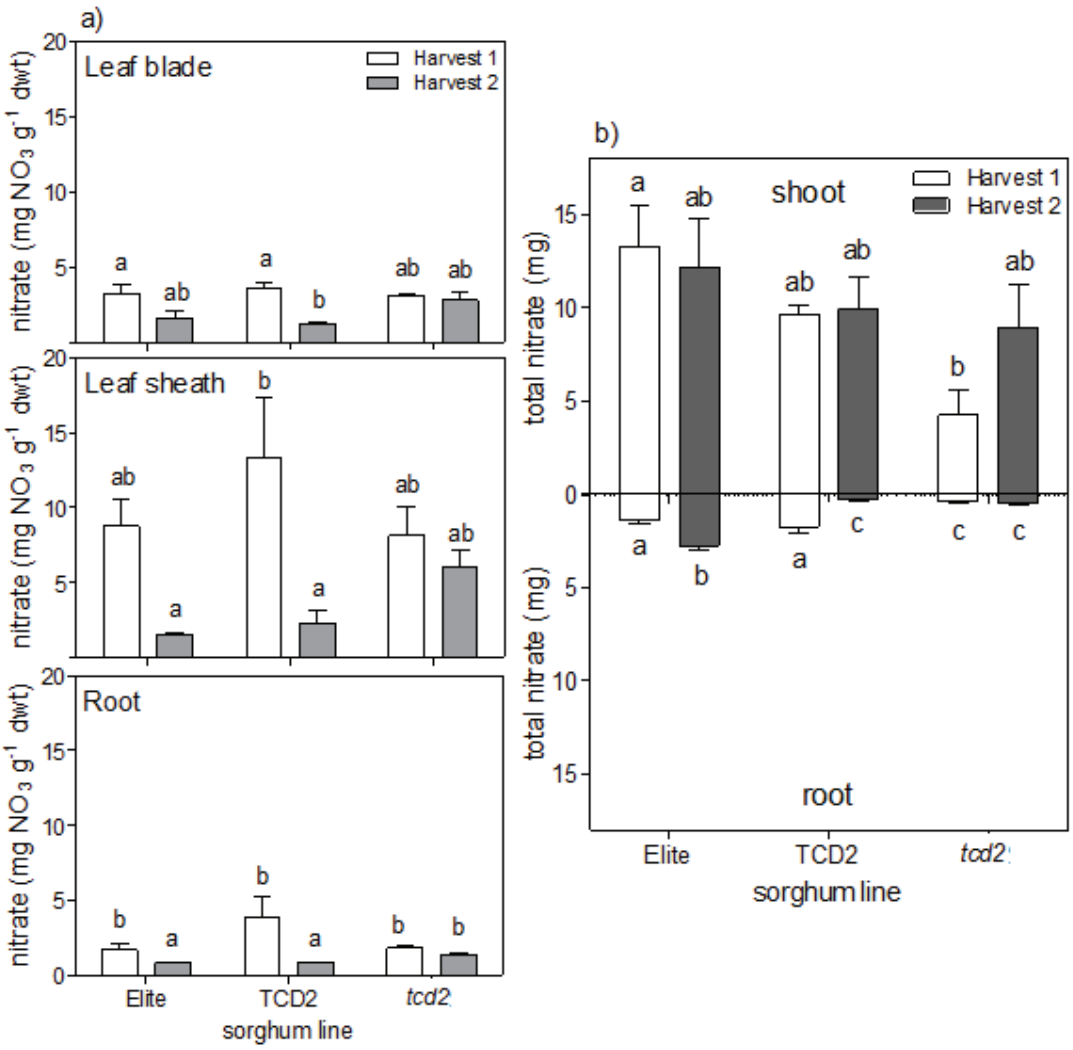


Figure 6.13 (a) Nitrate concentration of the leaf blade, leaf sheath and roots, and (b) total nitrate content of the shoots and roots of Elite, TCD2 and *tcd2* sorghum lines at 8 weeks (Harvest 1) and 10 weeks (Harvest 2) growth. Values are mean (± 1 SE) data from $n = 4$ plants. Letters indicate significant difference at $p < 0.05$, using Tukey's tests.

Nitrogen allocation

The leaf blade, leaf sheath and root carbon-nitrogen ratio (C:N) were calculated (Figure 6.14). The C:N ratio increased between Harvest 1 and 2 for the Elite and TCD2 plants, but remained at a constant level between harvests for the *tcd2* plants. There was no significant difference in the C:N between the lines for the leaf blade (p = 0.133) and leaf sheath (p = 0.291) at Harvest 1. The C:N in the leaf blade and leaf sheath of the *tcd2* plants at Harvest 2 was 30% and 50% lower than the Elite plants, respectively (leaf blade p = 0.014; leaf sheath p < 0.001). The C:N in the roots was different to the C:N in the shoots (Figure 6.14c). At Harvest 1, the roots of the *tcd2* plants had a higher C:N than the Elite plants (p = 0.022), but there was no significant difference in the root C:N between Elite, TCD2 and *tcd2* plants at Harvest 2 (p = 0.032).

The proportion of nitrogen allocated to cyanide (represented by CN-N/N%; Figure 6.15a) and the proportion if nitrogen allocated to nitrate (represented by NO_3^--N/N%; Figure 6.15b) were calculated for the leaf blade, leaf sheath and roots for all plants from Harvest 1 and 2. As observed for the total cyanide content, a low level of CN-N/N% was calculated for the *tcd2* plants and is again due to the limitations in the cyanide assay. The overall proportion of nitrogen allocated to cyanide in the Elite and TCD2 plants was higher in the roots than the shoots (Figure 6.15a). The shoot and root CN-N/N% decreased with time. The shoot CN-N/N% decreased to the same level as in the *tcd2* plants (indicating no cyanide).

The proportion of nitrogen allocated to nitrate in the leaf blade was not significantly different between plant lines at Harvest 1 (p = 0.516) or Harvest 2 (p = 0.239; Figure 6.15b). The leaf sheath followed the same trend as the leaf blade, with no significant difference in the proportion of nitrogen allocated to nitrate at Harvest 1 (p = 0.494) and Harvest 2 (p = 0.143). However, there appeared to be a decline in the NO_3^--N/N% at Harvest 2 for the Elite and TCD2 plants compared to Harvest 1 and this was not seen in the *tcd2* plants (Figure 6.15b). The roots also showed no significant difference in the proportion of nitrogen allocated to nitrate at Harvest 1 (p = 0.117) and Harvest 2 (p = 0.995).

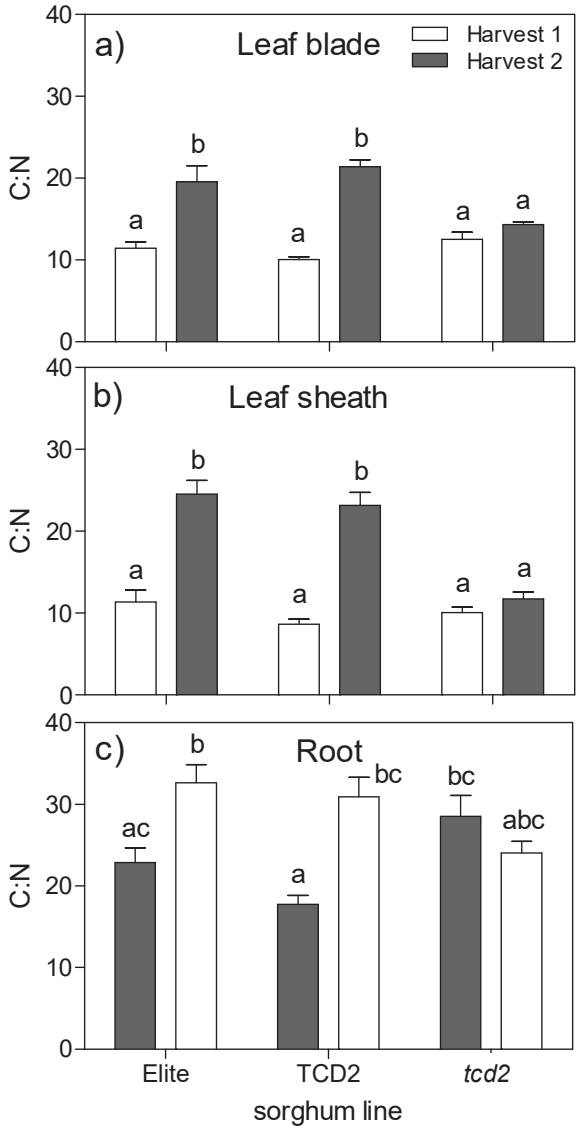


Figure 6.14 Elite, TCD2 and tcd2 carbon-nitrogen ratios (C:N) at eight (Harvest 1) and ten weeks (Harvest 2). Values are mean (± 1 SE) data from n = 4 plants. Tukey's posthoc tests were performed and letters indicate significant difference at $p < 0.05$.

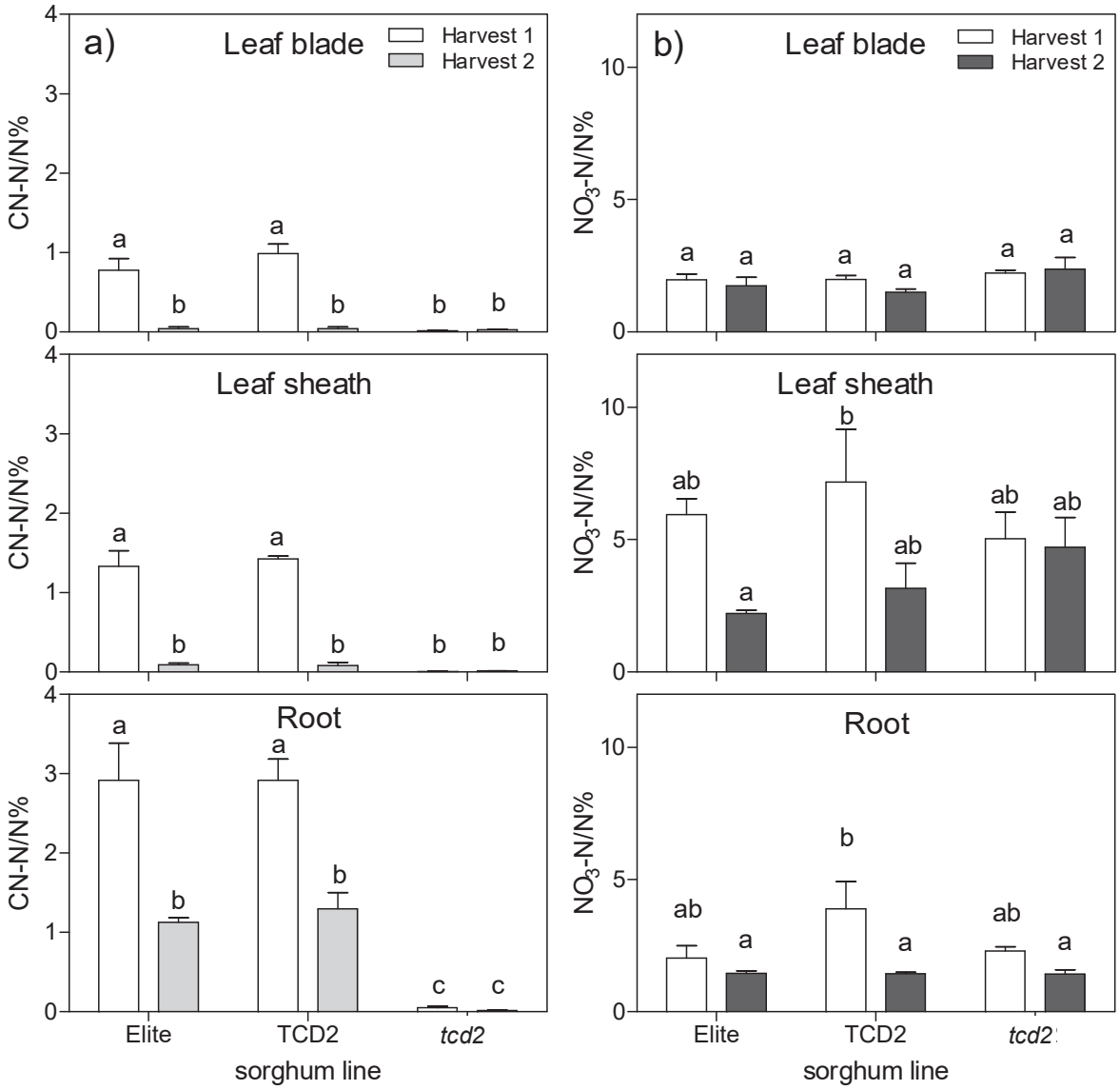


Figure 6.15 Proportion of nitrogen in (a) HCN (represented by CN-N/N%) and (b) nitrate (represented by NO_3^--N/N%) in whole of Elite, TCD2 and *tcd2* sorghum plants at eight (Harvest 1) and ten weeks (Harvest 2). Values are mean (± 1 SE) data from n = 4 plants. Tukey's posthoc tests were performed and letters indicate significant difference at $p < 0.05$.

6.4 Discussion

The sorghum mutagenesis project described in this chapter resulted in the identification of a number of sorghum lines with alterations in dhurrin biosynthesis, but the *tcd2* mutant, with a truncated UGT85B1 protein, was the focus of the chapter. The *tcd2* mutant has a stunted phenotype, it is acyanogenic and the nitrogen content of the plant is altered compared to the control plants. Nevertheless, the photosynthetic capacity of the *tcd2* plants was the same as for the Elite control plants. The identification and subsequent results of the growth and development of the *tcd2* sorghum line has potentially opened up new avenues for future research. This discussion covers: (1) implications of the *tcd2* sorghum line with respect to dhurrin metabolism, (2) nitrogen metabolism and growth of the *tcd2* sorghum line, and (3) the future of the cyanogenesis mutants,

6.4.1 Implications of the *tcd2* sorghum with respect to dhurrin metabolism

The identification of a viable acyanogenic sorghum line with a mutation in the coding sequence of the *UGT85B1* gene will be useful to the further understanding of dhurrin metabolism. The *tcd2* plants had a mutation that caused a premature stop codon (Q149*) in UGT85B1. The *tcd2* plants were not tested to determine the activity of the truncated UGT85B1 enzyme. However, due to the lack of dhurrin production and the *tcd2* UGT85B1 sequence being one third the size of the wild-type protein sequence, it is predicted that if the mutant UGT85B1 protein is made it will be unstable and inactive. This prediction is supported by the results of the Kristensen *et al*. (2005) study that found no dhurrin production in *A. thaliana* when the two sorghum cytochrome P450s (CYP79A1 and CYP71E1) were expressed without UGT85B1. Yet when the all three of the dhurrin biosynthetic genes were inserted and expressed in *A. thaliana*, dhurrin was detected (Kristensen *et al.* 2005). Furthermore, the truncated UGT85B1 protein in the *tcd2* plants does not contain the B loop, which has been identified as important for interactions with CYP79A1 and CYP71E1 during metabolon formation (highlighted by blue box in Figure 6.7; Thorsøe *et al.* 2005; Kannangara *et al*. 2011), therefore the two cytochrome P450s and the UDP-glycosyltransferase would not form the postulated metabolon required for dhurrin production (Jørgensen *et al.* 2005b; Kristensen *et al.* 2005; Nielsen *et al.* 2008).

Based on previous LC-MS data and standards (Kristensen *et al*. 2005) it appears that the dhurrin in leaves of both the Elite and TCD2 plant was converted to glucosylated p-hydroxyphenylacetic acid (retention time of 5.3 min), a product of the alternative turnover pathway in sorghum (Figure 1.2).

The identification of the *tcd2* sorghum mutant line may have contradicted the hypothesis that a sorghum plant containing an inactive UGT85B1 enzyme would not be viable. This hypothesis was based on the studies that characterised the dhurrin biosynthetic pathway (Bak *et al.* 1999, 2000; Petersen *et al.* 2001; Kristensen *et al.* 2005). *A. thaliana* plants that expressed the CYP791A1 and CYP71E1 enzymes did not produce dhurrin, yet had a build up of the toxic intermediate *p*-hydroxymandelonitrile (Figure 1.3; Petersen *et al.* 2001; Kristensen *et al.* 2005). Interestingly, the LC-MS analysis of the *tcd2* line showed that these plants contained metabolites with similar properties to the metabolites observed in the aforementioned *A. thaliana* plants expressing CYP79A1 and CYP71E1, metabolites that resulted from detoxification of products formed from the incomplete dhurrin synthesis pathway (Tattersall *et al*. 2001; Kristensen *et al*. 2005). Hence, the metabiltes with the retention times 2.6 min, 3.4 min and at 4.2 min and are possibly p-glucsyloxy-benzoic acid, p-hydroxybenzoylglucose and p-glucosyloxy-benzylglucose, respectively, but will need to be confirmed by further LC-MS analysis.

As found for the *tcd2* mutants, the A. thaliana plants expressing the two cytochrome P450s were also found to have a stunted phenotype, chlorotic appearance, an accumulation of numerous irregular glucosides (as mentioned above) and an altered transcriptome (Kristensen *et al.* 2005). The *tcd2* plants in the current study also had a stunted phenotype but showed the same photosynthetic potential as the control plants and were ultimately fertile. The inactivation of the UGT85B1 enzyme was predicted to result in the build up of *p*-hydroxymandelonitrile and associated derivates, as found in the studies by Petersen *et al*. (2001) and Kristensen *et al*. (2005), and result in unheathly and infertile plants. This prediction did not take into consideration the detoxification and postulated alternative dhurrin pathways in sorghum (Jenrich *et al.* 2007), which are unlikely to be present in *A. thaliana* and tobacco plants. Instead, the *tcd2* plants are

likely to have metabolized the *p*-hydroxymandelonitrile which would have dissociated into HCN and *p*-hydroxybenzaldehyde (Conn 1981), although the detoxification of HCN is dependant on the availability of cysteine. If the HCN detoxification process was not as efficient as the activity of the CYP79A1 and CYP71E1 enzymes, there would be a build up of HCN within the *tcd2* plants which may account for the stunted phenotype. This indicates there is also only one highly specific UDP-glycosyltransferase in sorghum that will work in the metabolon to produce dhurrin. Despite there being other glycosyltransferases with very similar amino acid sequences to UGT85B1 in the sorghum genome, especially in the B loop region important in metabolon formation.

The *tcd2* mutant is novel and of great interest for the further investigation of the cyanogenesis pathway. In particular it has great implications for the further study of the postulated alternative dhurrin turnover pathway (Jenrich *et al.* 2007).

6.4.2 *tcd2* sorghum growth and nitrogen allocation

It appears that the disruption of the UGT85B1 enzyme in the dhurrin biosynthetic pathway has affected the growth of the *tcd2* plants, but not to the same degree as observed for the *A. thaliana* plants that expressed CYP79A1 and CYP71E1 (Petersen *et al.* 2001; Kristensen *et al.* 2005). The growth characterisation study showed that the RGR of the *tcd2* plants was stunted early in growth, but later in the plant life there was the RGR was the same for all plants measured. The *tcd2* sorghum plants contained the same chlorophyll and carotenoid contents as the control plants, indicating they were healthy. Nevertheless, the *tcd2* plants had a stunted phenotype and the root:shoot was reduced. It should be noted that at Harvest 2 the Elite and TCD2 plants had 'bolted', switched to a reproductive state (Burn *et al.* 1993; Möller-Steinbach *et al.* 2010), but the *tcd2* plants had not. The RGR of the control plants could have slowed for change to reproductive state while the *tcd2* plants were continuing to grow. This may explain the similarity in the RGR for all plant lines between Harvest 1 and Harvest 2. The older leaves of the control plants had also begun to senesce, but both leaves I and III measured for the *tcd2* plants were photosynthetically active. Sorghum shed the older leaves as they mature and when under stress, such as drought (Rosenow & Clark 1995).

Therefore, it is hypothesis that the development of *tcd2* line is delayed and the *tcd2* plants in the current study were developmentally younger than the control plants.

The delay in development may be a consequence of the extra resources required for the metabolism of *p*-hydroxymandelonitrile, or the reduced nitrogen content the *tcd2* plants, or a combination of the two. The growth studies found the *tcd2* plants to initially contain the same nitrogen concentration to the control plants, but had a higher nitrogen concentration at Harvest 2. Nevertheless, there was an overall decrease in total amount of nitrogen in the *tcd2* plants compared to the controls. This reduction in nitrogen supports the theory of dhurrin as a nitrogen storage compound (Selmar *et al.* 1988; Sánchez- Pérez *et al.* 2008; Zagrobelny *et al.* 2008). Furthermore, the control plants decreased in nitrogen concentration over time, but the *tcd2* plants had the same nitrogen concentration for the two time points measured. This result is not consistent with the usual nitrogen status in sorghum plants (chapters 3 & 4; Anten *et al.* 1998; Borrell & Hammer 2000). Moreover, the results of the total nitrogen and nitrate in the *tcd2* plants in the current study do not support the growth-differentiation balance (GDB) hypothesis. The GDB predict that conditions limiting to growth but not photosynthesis can lead to an increase in resources available for secondary metabolism (Loomis, 1932, 1958). The *tcd2* plants showed that acyanogenic sorghum plants do not necessarily allocate more nitrogen to nitrate or have a higher growth rate. If anything, the *tcd2* plants have a lower growth rate.

Interestingly, the HCNp in the Elite and TCD2 plants over the two harvest times was not expected. At Harvest 1 the HCNp of the Elite and TCD2 plants would have been considered potentially toxic. Yet at Harvest 2 the HCNp of the Elite and TCD2 plants had dropped to undetectable. It is well established that the HCNp of sorghum plants declines with maturity (chapter 3; Gray *et al.* 1968; Loyd & Gray 1970). However, no sorghum studies have reported the HCNp to decline in this way (Gray *et al.* 1968; Wheeler *et al.* 1984; Haskins *et al.* 1987; Gleadow & Woodrow 1998; Jørgensen *et al.* 2005a; Kongsawadworakul *et al.* 2009). This rapid decline in HCNp could be due to the change from a vegetative stage of plant growth to the reproductive state and use of CN for nitrogen. Some plant species are known to remove nitrogen stores when they reach maturity to allow prevent loss of nitrogen in senescent tissues. The nitrogen is then

reallocated to the grain (Pommel *et al.* 2006; Jukanti *et al.* 2008). The total nitrogen content of the Elite and TCD2 plants did not decrease with maturity. It is likely that the nitrogen in the dhurrin was remobilised, further still supporting the theory of dhurrin as a nitrogen store and able to be turned over to free the nitrogen when required by the plant (Selmar *et al.* 1988; Jenrich *et al*. 2007; Sánchez- Pérez *et al.* 2008).

TCD2 plants were consistently marginally smaller than the Elite plants for all physiological measurements. Additionally, the TDC2 plants were significantly higher in cyanide concentration than the Elite plants. These results suggest there may have been a background mutation causing part (but not all) of the stunted phenotype in the *tcd2*.

6.4.3 Sorghum cyanogenesis mutant lines of commercial interest

The mutant sorghum population has resulted in three classes of sorghum cyanogenesis mutants (*tcd*, *acdc* and *ecp*). The *tcd1* sorghum plants, with the P414L mutation in CYP79A1 are acyanogenic when homozygous for this mutation and are phenotypically normal, apart from a slightly slower growth rate at an early developmental stage. The *acdc* mutants, capable of producing dhurrin at normal levels in young seedlings but with negligible leaf dhurrin levels in mature plants were also identified, but no mutation has been found in the coding sequence of dhurrin biosynthetic genes in this class of mutants. The *acdc* plants are the same as or taller than the non-mutated parent line, and often leafier than the controls. Further investigation into the cause of the low HCNp of these plants is currently being carried out. Preliminary results have indicated that the *acdc* mutant plants have an insertion/deletion in the putative promoter of the CYP79A1 gene (pers. comm. Samantha Fromhold). This indicates the regulation of the CYP79A1 enzyme could have been altered and be the cause of the low HCNp. The *ecp1* plants had a nucleotide sequence change of G to A in the coding region of *CYP79A1,* which caused the E145K change at the amino acid level. It is postulated that this amino acid change caused an increased affinity to the substrate and therefore an increase in HCNp (Blomstedt, O'Donnell, *et al.* 2012).

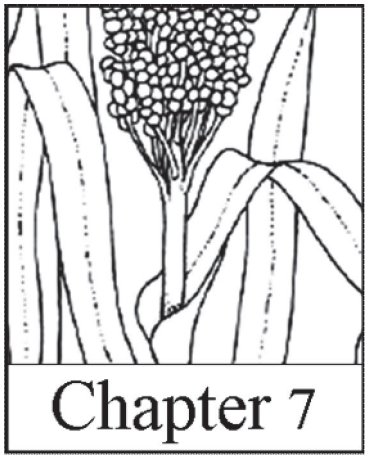
The *tcd*, *acdc* and *ecp* mutant lines are currently undergoing further characterisation and the mutations are being backcrossed in the Elite (parent) line to eliminate any undesired

background EMS mutations. EMS mutagenesis is random and therefore undesired mutations need to be removed (Henikoff *et al*. 2004). Although the phenotypes of the cyanogenesis mutant lines are normal, the background mutations could result in other deleterious traits in future generations, therefore it is crucial to eliminate the background EMS mutations.

6.5 Conclusion

It is predicted that the mutation in the *UGT85B1* gene of the *tcd2* plants causes a truncated UGT85B1 protein to be formed, hence no dhurrin is produced. The change in the plant metabolism also results in a stunted phenotype. However, the outcome of the TCD2 plant growth characteristics, indicates there may be other unidentified mutations that have caused part of the stunted phenotype. Furthermore, the growth study of the *tcd2* sorghum plants showed that acyanogenic sorghum plants do not necessarily allocate more nitrogen to nitrate or have a higher growth rate. Although the *tcd2* plants do appear to have a lower nitrogen content than the control plants. The *tcd2* mutant is novel and of great interest for the further investigation of the cyanogenesis pathway. In particular, for further investigation of the postulated alternative dhurrin turnover pathway.

Further characterisation is required for all the cyanogenesis mutants. The *tcd1* and *acdc* mutant lines look promising for future use as commercial forage sorghum. The *tcd2* plants are phenotypically and developmentally stunted therefore not of use commercially, yet the *tcd2* mutant is of intellectual interest for the study metabolism of dhurrin.



General discussion

This PhD thesis presents original discoveries about the molecular, environmental and developmental regulation of dhurrin and cyanide potential (HCNp) in forage sorghum. The work was organised around three main research questions, or aims. Aim one was to assess the changes in HCNp associated with ontogeny and was primarily addressed in chapter three. The second aim, to determine environmental factors that influence dhurrin concentration, was covered in chapters four and five. The final aim, to produce low dhurrin producing or acyanogenic sorghum germplasm for use in future plant breeding programs, was described in chapter six. The genotype determined the overall cyanogenic status of particular varieties, consistent with studies of other species (e.g. Goodger *et al.* 2002; Gleadow *et al.* 2003). Against this background, there is a large degree of phenotypic plasticity. Four dominant factors were found to drive the HCNp of sorghum: plant age, developmental stage and the availability of nitrogen and water. The results from chapters three, four and five of this thesis clearly show this phenotypic plasticity in the expression of HCNp of sorghum. The implications of these results will be further discussed in this chapter.

7.1 Thesis overview

The ontogenetic studies showed that there was a gradient in HCNp within individuals but that the direction of this gradient was dependent on plant age. In young sorghum plants the HCNp

was highest in the basal leaves, but at approximately the 5-leaf stage there was a switch and by the 10-leaf stage the HCNp gradient was basipetal. Plant age was also a strong predictor or HCNp with the highest HCNp typically found in young plants, consistent with early agronomic studies (e.g. Loyd & Gray 1970). Growing conditions also affected sorghum HCNp. Nitrogen is often considerd an important driver of HCNp in many cyanogenic species (e.g McBee & Miller 1980; Vickery *et al*. 1987; Briggs 1990; Gleadow *et al*. 2009a; Simon *et al*. 2010). However, this is not true of all species, or growing conditions. HCNp in some tropical trees, for example, appears to be completely unaffected by the concentration of nitrogen in the nutrient solution (Miller *et al*. 2004; Simon *et al.* 2007; Webber & Woodrow 2009), Busk and Møller (2002) detected a positive correlation between nitrogen supply and HCNp in older sorghum plants, but no such effect on HCNp of was found in young plants. The results of the nitrogen experiments in the current study were consistent with the results of Busk and Møller (2002). These results emphasise the importance of long term studies. If the majority of the experiments described in this thesis had been conducted in the first 5 weeks after germination, as often reported in the literature (Busk & Møller 2002, Bhargava & Paranjpe 2004; Buchanan *et al.* 2005; Salzman et al. 2005), then the HCNp trends related to nitrogen and osmotic stress would have been missed.

Environmental conditions that limit plant growth, such as drought, have previously been associated with a high HCNp in sorghum (Dunstaan 1906; Robinson 1930; Kriedeman 1964). The results of the current studies showed that this increase in dhurrin concentration may not be due to the increase in dhurrin synthesis, but associated with the stunted growth of the plant. Evidence for this comes from calculating the total amount of dhurrin per plant and quantifying the mRNA transcripts. In non-stressed sorghum plants, the total amount of dhurrin produced per plant was increased with plant size/age. Osmotically stressed sorghum plants produced the same total amount of dhurrin in the shoots as the control plants, despite being stunted resulting in an increase in HCNp on a per mass basis (i.e. a higher concentration). Moreover, the CYP79A1 transcript level decreased in the sheath of the osmotically stressed sorghum plants. On the other hand, HCNp does appear to be under some degree of hormonal control. Of particular relevance here is the higher HCNp in sorghum plants treated with abscisic acid (ABA), a hormone associated with drought responses in plants (Cornish & Zeevart 1985; Radin & Hendrix 1987; Reddy *et al.* 2004), within a week of treatments.

Interestingly, HCNp in the roots did not follow the same pattern observed in the shoots. The roots of the osmotically stressed plants produced less total dhurrin than the roots of the non-stressed plants. It is possible that HCNp in the roots is regulated independently from the shoots.

Additional evidence for this comes from the uncoupling of root and shoot HCNp in the *tcd2* and *acdc* mutants.

Cyanogenic glycosides have long been regarded as a defence against herbivory (Gleadow & Woodrow 2002b; Møller 2010b). Furthermore, until recently they have been almost universally regarded as constitutive, since there is no evidence in the literature that they are inducible or that their concentration increases after wounding (Gleadow & Woodrow 2000b; Ballhorn *et al*. 2011). An important finding of the current studies is the observed increase in HCNp in sorghum plants after certain types of mechanical wounding that mimic insect damage, although no change in HCNp was detected when larger portions of the plant were removed. Additionally, HCNp increased in plants after treatment with Methy jasmonate (MeJa), a hormone known to be associated with a wounding response (e.g. Ohnmeiss & Baldwin 1994). These results indicate that, in contrast to earlier studies, cyanogenic glycosides are indeed inducible. The recent discovery of a pathway for the turnover of dhurrin that does not include a free-HCN step (Møller 2010b) could provide a mechanism to explain how such induction might be regulated.

7.2 Proposed feed-back mechanism in resource allocation

The defence theories outlined in the literature (Loomis, 1932, 1958; McKey 1974Lorio, 1986, 1988; Tuomi *et al.* 1990; Purrington 2000; Stowe *et al.* 2000; Strauss *et al.* 2002 Goodger *et al.* 2007) and discussed in this thesis provide passive explanations to the expression of defence compounds in plants. One argument is that in resource-limited environments there is often a trade-off between investment in growth, reproduction or defence (Herms & Mattson 1992; Zangerl & Bazzaz 1992; Webber & Woodrow 2009). A new model is proposed here (Figure 7.1) to provide a possible explanation as to how the trade-off between growth and defence is mediated. It is proposed that if sorghum plants are grown in a suboptimum environment, such as drought, and growth is limited, then the defence compound, dhurrin, is increased due to a feedback mechanism (Figure 7.1). For example, when growth is limited by resources, such as water supply, there is less demand for nitrogen by the primary metabolism. The alternative dhurrin turnover pathway would then be suppressed to prevent loss of reduced nitrogen, and yet not inhibit nitrogen uptake by the roots. With less dhurrin turnover, there would then be a build up of dhurrin which, in turn, would provide a signal to suppress the production of dhurrin synthesis. This explains both the increase in HCNp in growth-limited plants, and the surprising decrease in CYP79A1 transcripts, observed here, even though CYP79A1 is

reputedly the rate limiting step in dhurrin synthesis (Kahn *et al.* 1999). This hypothesis is further supported by the increase in HCNp of sorghum plants after ABA treatment. ABA could be associated in this proposed feed-back loop and temporarily induce the proposed alternative dhurrin turnover pathway to be down regulated. Once the ABA was metabolised, the alternative dhurrin turnover pathway could then return to the usual level and the HCNp of the plant returned to control levels. Not all steps in the proposed turnover pathway have been characterized (Jenrich *et al*. 2007; Møller 2010b). The model proposed here could provide a framework for testing for the activity of the pathway, and aid in the identification of the missing steps.

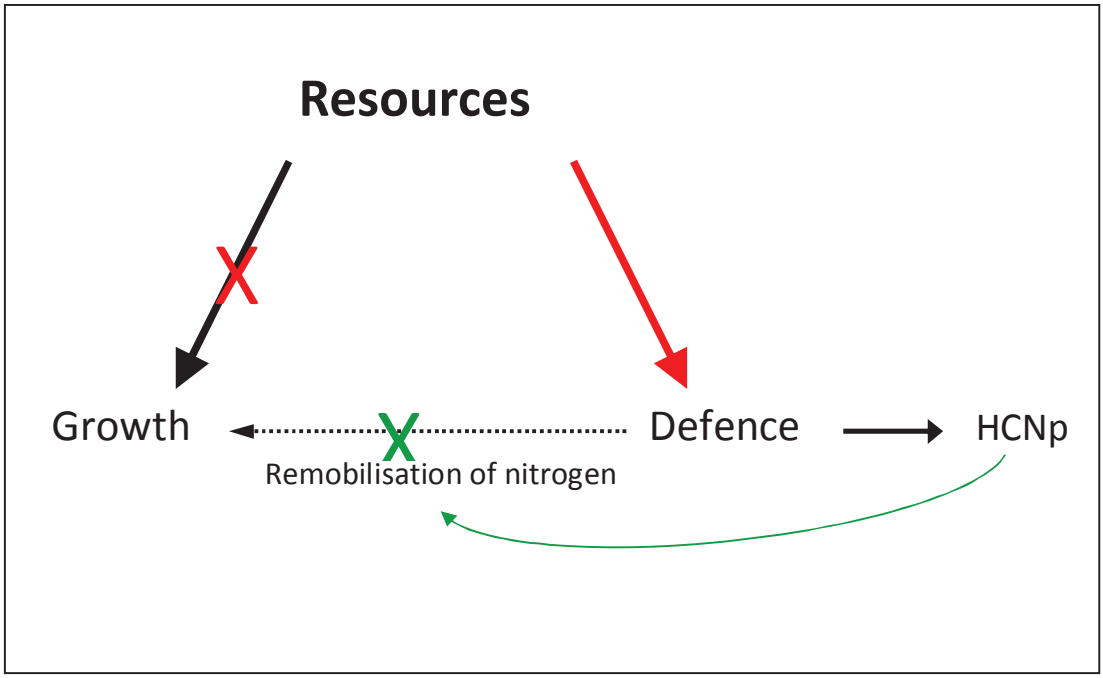


Figure 7.1 Proposed feed-back mechanism associated with resource allocation to growth and defence compounds in stressed sorghum plants. Traditional plant defense theory - trade off between growth and defense- passive. Results presented here suggested the key control point may be at the level of allocation to chemistry downstream thereby addressing underlying regulatory mechanisms for first time.

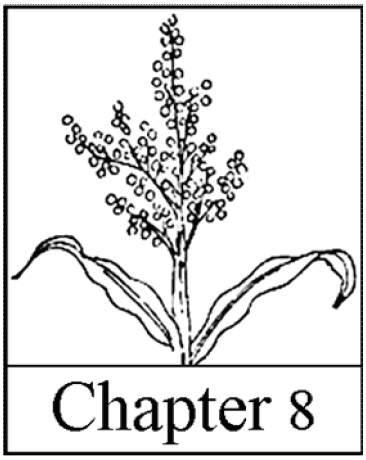
The final part of this thesis deals with the development of varieties of sorghum with altered cyanogenic status (see Chapter 6). An EMS-induced mutant population was created and screened phenotypically and using TILLING. Two mutant lines appear to be totally acyanogenic (*tcd1* & *tcd2*) and several others appear to lose the ability to synthesis dhurrin in the shoots at about the 5-leaf stage when the dhurrin concentration

gradient switching-point within the plants (*acdc* mutants). Moreover, some of these mutants seem to have uncoupled the HCNp in the roots from the shoots. As well as there direct commercial application, these mutants provide an ideal opportunity for testing the diverse roles that dhurrin may play in sorghum. The reduced requirement for nitrogen by totally cyanide deficient mutants opens up unique ways to test the cost of defence compounds in terms of nitrogen use efficiency and growth. In addition, the mutants could be used for testing the regulation of both the known synthesis and detoxification pathways but also the newly proposed turnover pathway. For example, the UGT mutant (*tcd2*) may be using a different UGT to proceed in the pathway and therefore produce a different suite of end products.

The creation of the mutant population also gave rise to additional mutants of interest to sorghum breeders. For example, a striped leaf phenotype and BMR (brown mid rib). As previously stated, the BMR trait is associated with a high lignin content and allows better digestibility for cattle (Cherney 1990). The mutant line with the striped leaf phenotype could be of use to farmers to alleviate any concern of biofumigants being mistaken as general forage. If the striped leaf phenotype were bred into a sorghum line with a high HCNp used as a biofumigant, it would be easily recognised in the field as a biofumigant and not safe as forage. The mutant lines described in this thesis and Blomstedt, O'Donnell et al. (2012), along with additional mutant lines selected by Pacific Seeds, have been placed in a traditional breeding program at Pacific Seeds. The breeding program will backcross the mutant traits into the Elite parent and the appropriate maintainer lines. The backcrossing of the mutant lines is necessary to remove any background mutations that may also have been induced by the EMS treatment and co-selected for with the desired traits. Backcrossing a trait into another line takes several generations to ensure all other genes are the same. This is the same process that plant breeders/farmers used to produce the current cultivars. However, despite the many improvements made to sorghum naturally and through the efforts of traditional plant breeders, as the demand for food increases now and into the future additional changes are necessary to allow sorghum to be utilised on marginal lands. This project is one of the many that shows how modern technology and traditional breeding methods can be used together to help.

7.3 Conclusion

The work done in this PhD thesis used both molecular and physiological tools to address ecological defence theories and has consequently begun to bridge the gap between these diverse fields and yet cover the same area of biology. These studies have been performed in a way that not only furthers scientific knowledge into the cyanogenesis pathway in sorghum, but also allows farmers a better understanding of when they can use their sorghum crops and under which conditions. Work presented here leads to a better understanding of the regulatory mechanisms of cyanogenesis in sorghum. The premise of at the start of this research was that the relationship between transcript levels and phenotype would be simple but they are not. The relationships are complex and there are likely to be several points of active and passive regulation. On a practical level, the results may help to determine under what conditions forage sorghum should be grown. Farmers need to utilise the best growing conditions for the crop in order to produce the best food for their cattle. The development of new acyanogenic sorghum varieties using the germplasm created in the course of this project will provide farmers with options previously unavailable to them. This work shows how bringing together the multidisciplinary approaches of molecular genetics, physiology and agro-ecology can help to develop new climate-change crops ready for a hotter and drier Australia.



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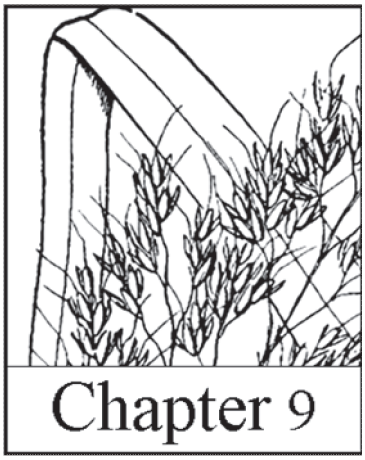
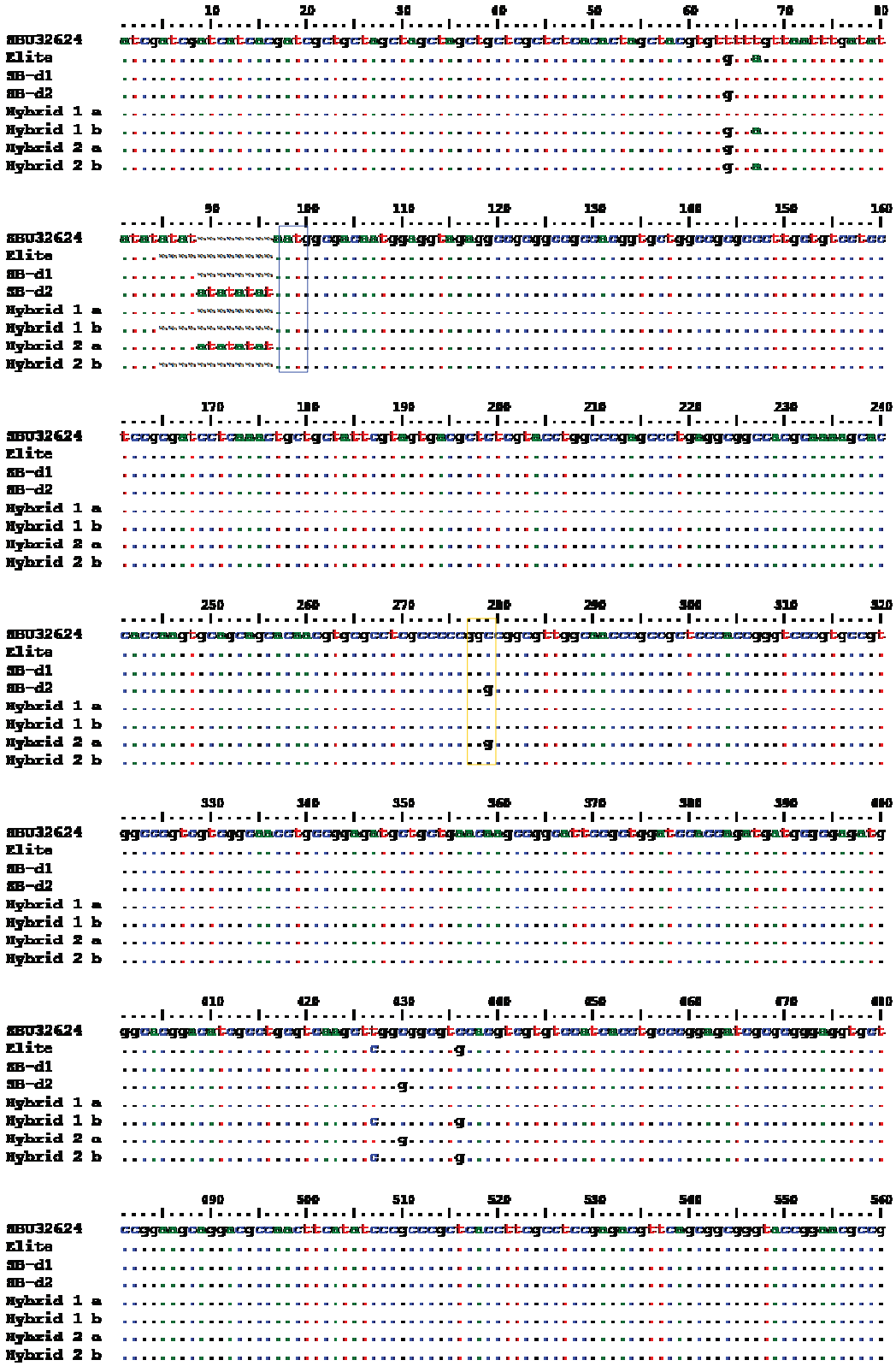


Appendices

Appendix 1 Differences in the CYP79A1 gene coding sequences between sorghum varieties. Bold indicates difference in polarity/charge of amino acid between varieties.

Sorghum line	**Nucleotide sequence**		**Amino acid sequence**			
	position change	codon	position	amino acid	polarity	charge
SBU32624	182 632	g**c**c g**c**c	60 210	Alanine (A) Alanine (A)	nonpolar nonpolar	neutral neutral
SB-b1 (Elite)	182 632	g**c**c g**t**c	60 210	Alanine (A) Valine (V)	nonpolar nonpolar	neutral neutral
SB-d1	182 632	g**c**c g**c**c	60 210	Alanine (A) Alanine (A)	nonpolar nonpolar	neutral neutral
SB-d2	182 632	g**g**c g**c**c	60 210	Glycine (G) Alanine (A)	nonpolar nonpolar	neutral neutral
Hybrid 1	182 632	g**c**c g**c**c/ g**t**c	60 210	Alanine (A) Alanine (A) / Valine (V)	nonpolar nonpolar nonpolar	neutral neutral neutral
Hybrid 2	182 632	g**g**c/ g**c**c g**c**c/ g**t**c	60 210	Glycine (G) / Alanine (A) Alanine (A) / Valine (V)	nonpolar / nonpolar nonpolar / nonpolar	neutral / neutral neutral / neutral

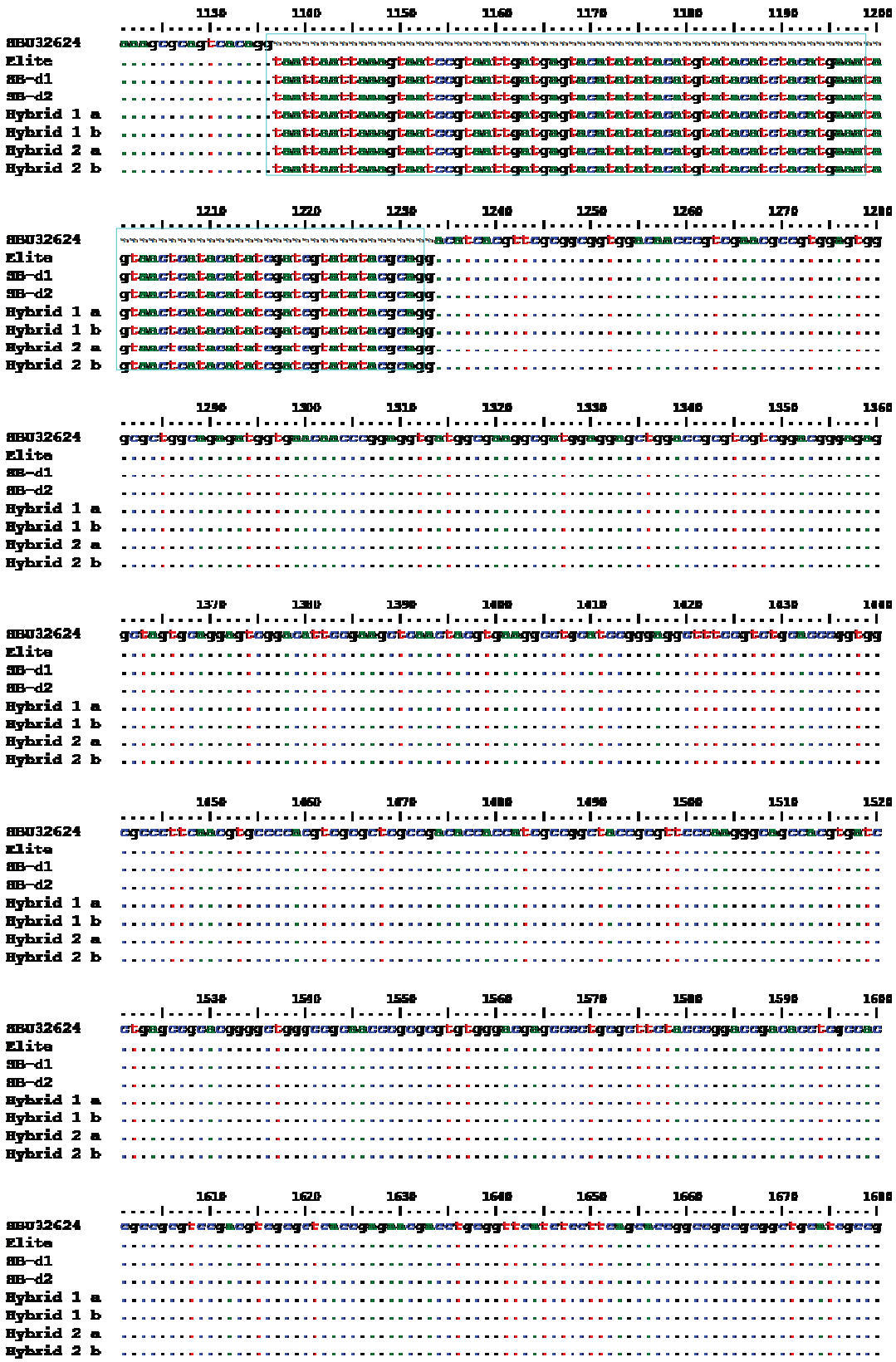
Appendix 2 Elite, SB-d1 and SB-d2, Hybrid 1 and Hybrid 2 CYP79A1 nucleotide sequences compared to Genebank gene accession SBU32624. Hybrid 1 and Hybrid 2 have two variations in the sequence, shown here as sequence “a” or “b”.



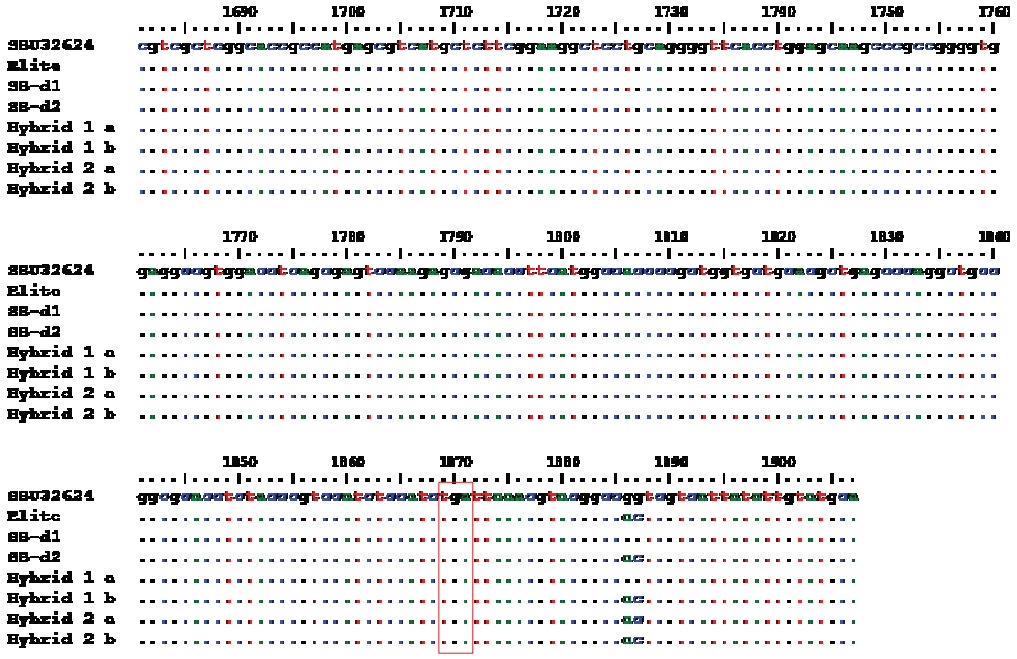
Appendix 2 continued

```
                 570       580       590       600       610       620       630       640
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SBU32624 tgctctcgccctacggcgaccagtggaagaagatgcgccgcgtcctcacctccgagatcatctgcccgtcccgccacgcc
Elite    ........................................................................................
SB-d1    ........................................................................................
SB-d2    ........................................................................................
Hybrid 1 a ......................................................................................
Hybrid 1 b ......................................................................................
Hybrid 2 a ......................................................................................
Hybrid 2 b ......................................................................................

                 650       660       670       680       690       700       710       720
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SBU32624 tggctccacgacaagcgcaccgaagcgaggccgacaacctcaccccgctacgtctcacaacctcgccacccaagccgccaccgg
Elite    ........................................................................................
SB-d1    ........................................................................................
SB-d2    ...........................................g............................................
Hybrid 1 a ......................................................................................
Hybrid 1 b ......................................................................................
Hybrid 2 a ......................................g...............................................
Hybrid 2 b ......................................................................................

                 730       740       750       760       770       780       790       800
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SBU32624 cgacgtcgccgtcgacgtcaggcacgtcgctcgtcactattgcggcaacgttatccgccgcctcatgttcaacaggcgct
Elite    ........t...........................................c...........................
SB-d1    ........................................................................................
SB-d2    ...........................................c............................................
Hybrid 1 a ......................................................................................
Hybrid 1 b ........t..........................................c...........................
Hybrid 2 a ......................................................c...............................
Hybrid 2 b ........t..........................................c...........................

                 810       820       830       840       850       860       870       880
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SBU32624 acttcggcgagccccaggctgacggcggtccggggccgatggaggtgctgcatatggacgccgtgttcacctccctcggc
Elite    ........................................................................................
SB-d1    ........................................................................................
SB-d2    ........................................................................................
Hybrid 1 a ......................................................................................
Hybrid 1 b ......................................................................................
Hybrid 2 a ......................................................................................
Hybrid 2 b ......................................................................................

                 890       900       910       920       930       940       950       960
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SBU32624 ctcctctacgccttctgcgtctccgactacctccccctggctgcggggcctcgacctcgacggccacgagaagatcgtcaa
Elite    ........................................................................................
SB-d1    ........................................................................................
SB-d2    ........................................................................................
Hybrid 1 a ......................................................................................
Hybrid 1 b ......................................................................................
Hybrid 2 a ......................................................................................
Hybrid 2 b ......................................................................................

                 970       980       990      1000      1010      1020      1030      1040
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SBU32624 ggaggctaacgtggcggtgaacaggctccacgacacggtcatcgacgaccggtggaggcagtggaagagcggcgagcggc
Elite    ........................................................................................
SB-d1    ........................................................................................
SB-d2    ........................................................................................
Hybrid 1 a ......................................................................................
Hybrid 1 b ......................................................................................
Hybrid 2 a ......................................................................................
Hybrid 2 b ......................................................................................

                1050      1060      1070      1080      1090      1100      1110      1120
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SBU32624 cggagatggaggacttcctggatgtgctcatcactctcaaggacgcccagggcaacccgctgctgaccatcgaggaggtc
Elite    ........................................................................................
SB-d1    ........................................................................................
SB-d2    ........................................................................................
Hybrid 1 a ......................................................................................
Hybrid 1 b ......................................................................................
Hybrid 2 a ......................................................................................
Hybrid 2 b ......................................................................................
```

Appendix 2 continued



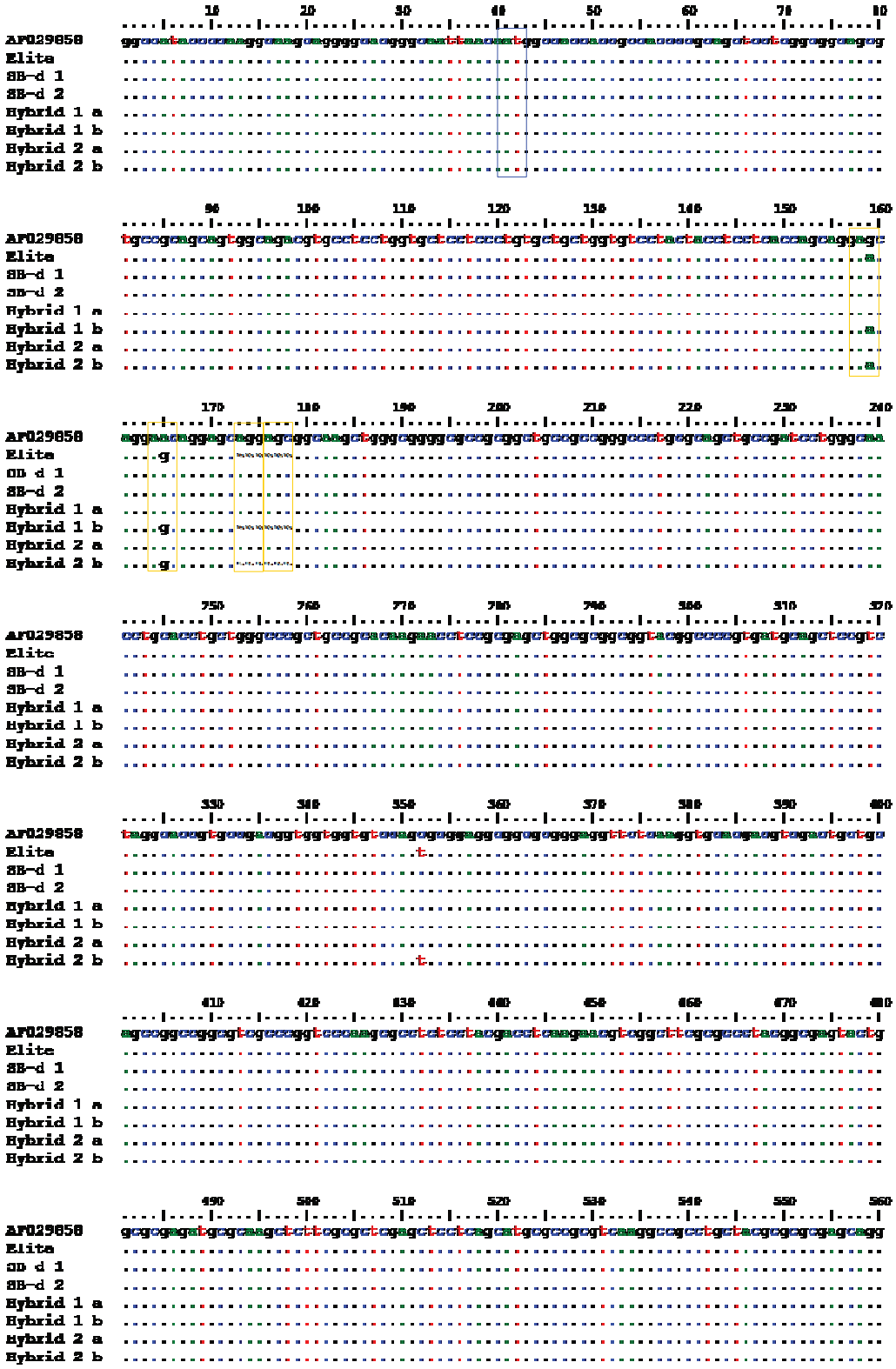
Appendix 2 continued



Appendix 3 Differences in the CYP71E1 gene coding sequences between sorghum varieties. Bold indicates difference in polarity/charge of amino acid between varieties.

Sorghum line	**Nucleotide sequence**		**Amino acid sequence**			
	position change	codon	position	amino acid	polarity	charge
AF029858	119	agc	40	Serine (S)	polar	neutral
	125	aac	42	Asparagine (N)	polar	neutral
	133-135	aag	45	Arginine (R)	polar	neutral
	136-138	agc	46	Serine (S)	polar	neutral
SB-b1 (Elite)	119	aac	40	Asparagine (N)	polar	neutral
	125	agc	42	Serine (S)	polar	neutral
	133-135	-	45	-	-	-
	136-138	-	46	-	-	-
SB-d1	119	agc	40	Serine (S)	polar	neutral
	125	aac	42	Asparagine (N)	polar	neutral
	133-135	aag	45	Arginine (R)	polar	neutral
	136-138	agc	46	Serine (S)	polar	neutral
SB-d2	119	agc	40	Serine (S)	polar	neutral
	125	aac	42	Asparagine (N)	polar	neutral
	133-135	aag	45	Arginine (R)	polar	neutral
	136-138	agc	46	Serine (S)	polar	neutral
Hybrid 1	119	agc/ aac	40	Serine (S) / Asparagine (N)	polar / polar	neutral / neutral
	125	aac/ agc	42	Asparagine (N) /	polar / polar	neutral / neutral
	133-135	aag/ -	45	Serine (S) Arginine (R) /	polar / -	neutral / -
	136-138	agc/ -	46	- Serine (S) / -	polar / -	neutral / -
Hybrid 2	119	agc/ aac	40	Serine (S) / Asparagine (N)	polar / polar	neutral / neutral
	125	aac/ agc	42	Asparagine (N) /	polar / polar	neutral / neutral
	133-135	aag/ -	45	Serine (S) Arginine (R) /	polar / -	neutral / -
	136-138	agc/ -	46	- Serine (S) / -	polar / -	neutral / -

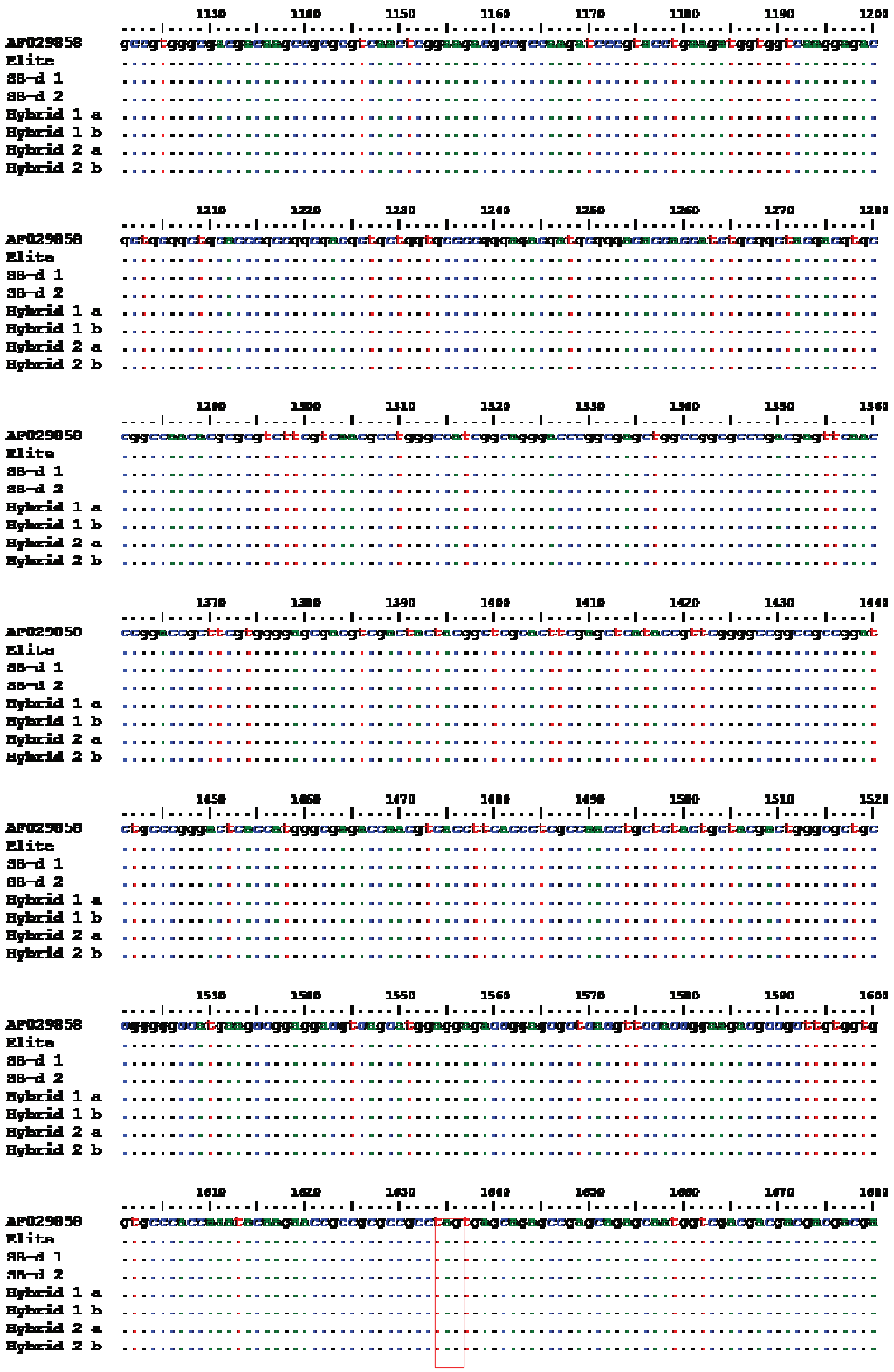
Appendix 4 Elite, SB-d1 and SB-d2, Hybrid 1 and Hybrid 2 CYP71E1 nucleotide sequences compared to Genebank gene accession AF029858. Hybrid 1 and Hybrid 2 have two variations in the sequence, shown here as sequence “a” or “b”.



Appendix 4 continued

```
                    570       580       590       600       610       620       630       640
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AF029858            agatggacaggctcgtcgcgacctcgacccgcgcgccgcgtccaaggcctccatcgtcctcaaagacaacgtcttcgcc
Elite               ........................................................................
SB-d 1              ........................................................................
SB-d 2              ........................................................................
Hybrid 1 a          ........................................................................
Hybrid 1 b          ........................................................................
Hybrid 2 a          ........................................................................
Hybrid 2 b          ........................................................................

                    650       660       670       680       690       700       710       720
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AF029858            ctcaccgacggcatcatcggcaccgtcgcgttcggcaacatctacgcctccaagcagttcgcgcacaaggagcgcttcca
Elite               ........................................................................
SB-d 1              ........................................................................
SB-d 2              ........................................................................
Hybrid 1 a          ........................................................................
Hybrid 1 b          ........................................................................
Hybrid 2 a          ........................................................................
Hybrid 2 b          ........................................................................

                    730       740       750       760       770       780       790       800
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AF029858            gcacgtgctggacgacgccatggacatgatggccagcttctccgcgaggacttcttcccaacgccgccggccgcctcg
Elite               ........................................................................
SB-d 1              ........................................................................
SB-d 2              ........................................................................
Hybrid 1 a          ........................................................................
Hybrid 1 b          ........................................................................
Hybrid 2 a          ........................................................................
Hybrid 2 b          ........................................................................

                    810       820       830       840       850       860       870       880
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AF029858            ccgaccgcctctcgggcttcctcgcccgcgcgagcgcatcttcaacgagctcgacgtcttcttcgagaaaggtcatcgac
Elite               ........................................................................
SB-d 1              ........................................................................
SB-d 2              ........................................................................
Hybrid 1 a          ........................................................................
Hybrid 1 b          ........................................................................
Hybrid 2 a          ........................................................................
Hybrid 2 b          ........................................................................

                    890       900       910       920       930       940       950       960
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AF029858            cagcacatggaccccggcgcgcccgtgcggacaacggcggcgacctcgtcgacgtccctcatcaacctgtgcaaggagca
Elite               ........................................................................
SB-d 1              ........................................................................
SB-d 2              ........................................................................
Hybrid 1 a          ........................................................................
Hybrid 1 b          ........................................................................
Hybrid 2 a          ........................................................................
Hybrid 2 b          ........................................................................

                    970       980       990       1000      1010      1020      1030      1040
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AF029858            cgacggcacgctccgcttcacccgggaccacgtcaaggccatcgtcctcgacacctttcatcggcgccatcgacaccagct
Elite               ........................................................................
SB-d 1              ........................................................................
SB-d 2              ........................................................................
Hybrid 1 a          ........................................................................
Hybrid 1 b          ........................................................................
Hybrid 2 a          ........................................................................
Hybrid 2 b          ........................................................................

                    1050      1060      1070      1080      1090      1100      1110      1120
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AF029858            ccgtcaccatcctgtgggccatgtcggagctgatgcggaagccgcaggtgctgaggaaggcgcaggccgaggtgcgggcc
Elite               ........................................................................
SB-d 1              ........................................................................
SB-d 2              ........................................................................
Hybrid 1 a          ........................................................................
Hybrid 1 b          ........................................................................
Hybrid 2 a          ........................................................................
Hybrid 2 b          ........................................................................
```

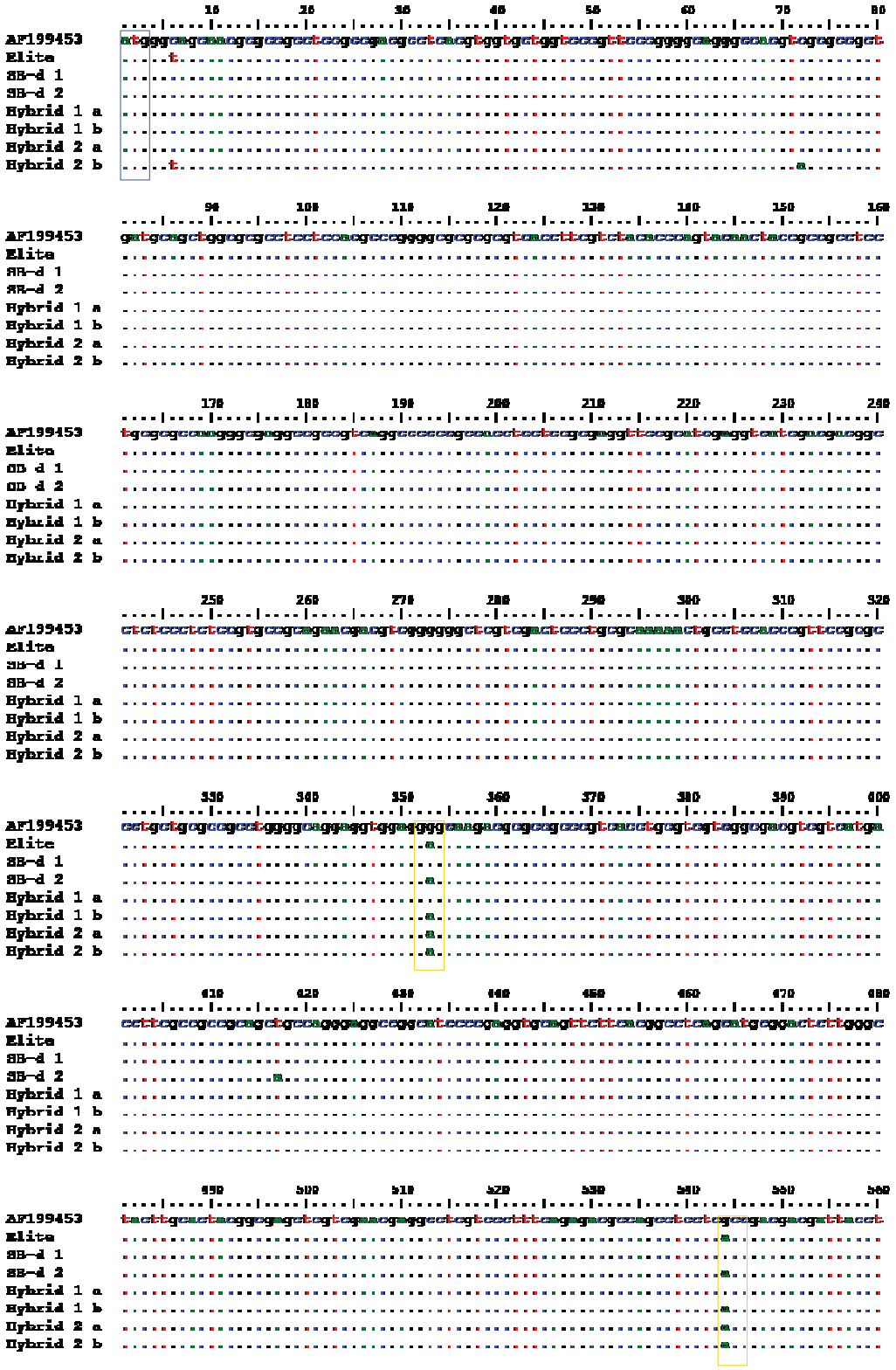
Appendix 4 continued



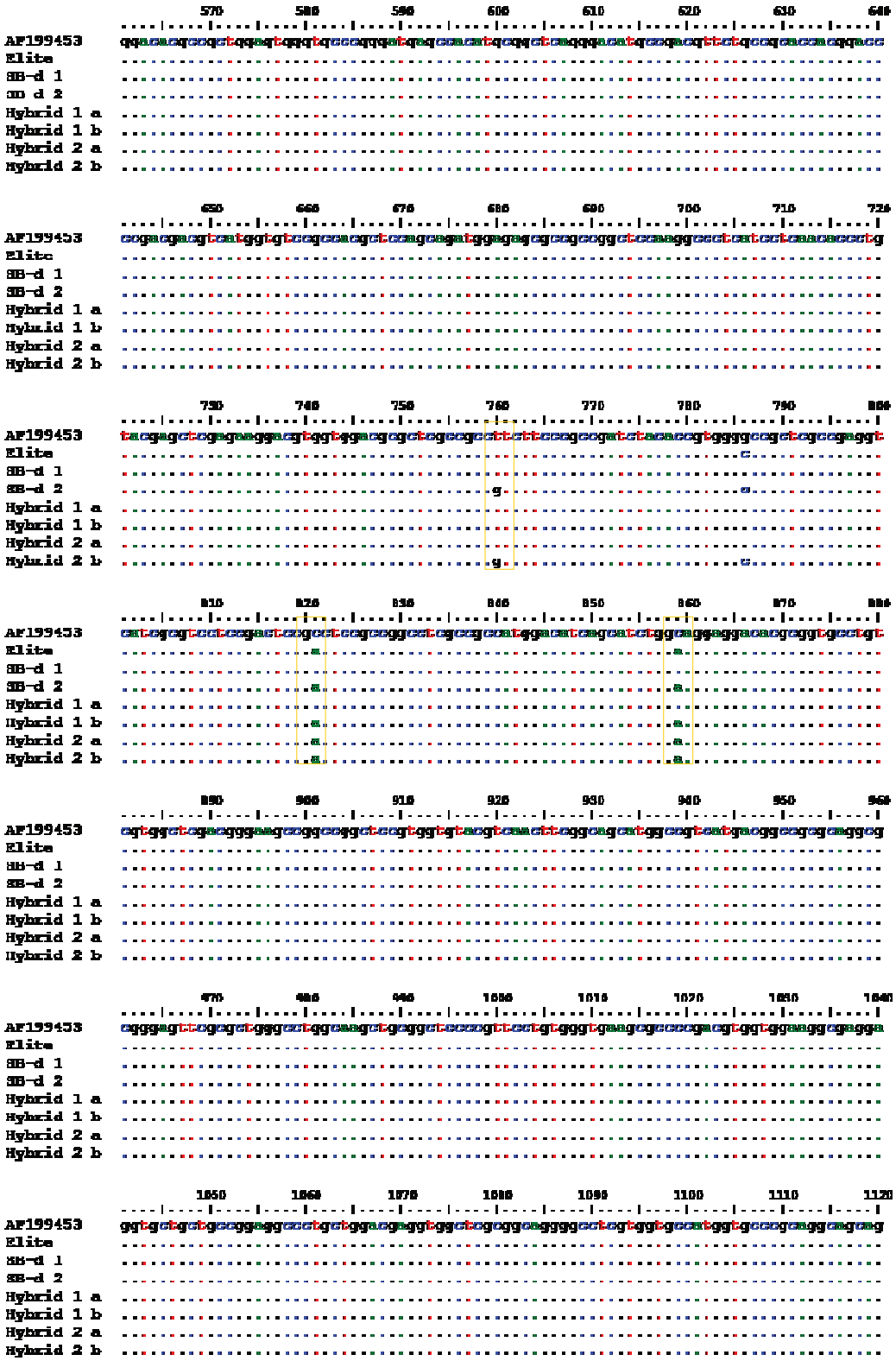
Appendix 5 Differences in the UGT85B1 gene coding sequences between sorghum varieties. Bold indicates difference in polarity/charge of amino acid between varieties.

Sorghum line	Nucleotide sequence		Amino acid sequence		Polarity	Charge
	position change	codon	position	amino acid		
AF199453	354	g**g**g	118	Glycine	**nonpolar**	**neutral**
	546	**g**cc	182	Alanine	**nonpolar**	neutral
	760	**t**tc	254	Phenylalanine	nonpolar	neutral
	821	g**c**c	274	Alanine	**nonpolar**	**neutral**
	859	**c**ag	286	Glutamine	polar	**neutral**
SB-b1 (Elite)	354	g**a**g	118	Glutamic acid	**polar**	**negative**
	546	**a**cc	182	Threonine	**polar**	neutral
	760	**t**tc	254	Phenylalanine	nonpolar	neutral
	821	g**a**c	274	Aspartic acid	**polar**	**negative**
	859	**a**ag	286	Lysine	polar	**positive**
SB-d1	354	g**g**g	118	Glycine	**nonpolar**	**neutral**
	546	**g**cc	182	Alanine	**nonpolar**	neutral
	760	**t**tc	254	Phenylalanine	nonpolar	neutral
	821	g**c**c	274	Alanine	**nonpolar**	**neutral**
	859	**c**ag	286	Glutamine	polar	**neutral**
SB-d2	354	g**a**g	118	Glutamic acid	**polar**	**negative**
	546	**a**cc	182	Threonine	**polar**	neutral
	760	**g**tc	254	Valine	nonpolar	neutral
	821	g**a**c	274	Aspartic acid	polar	**negative**
	859	**a**ag	286	Lysine	polar	**positive**
Hybrid 1	354	g**g**g/ g**a**g	118	Glycine / Glutamic acid	**nonpolar / polar**	**neutral / negative**
	546	**g**cc/ **a**cc	182	Alanine / Threonine	**nonpolar / polar**	neutral / neutral
	760	**t**tc	254	Phenylalanine	nonpolar	neutral
	821	g**c**c/ g**a**c	274	Alanine / Aspartic acid	**nonpolar / polar**	**neutral / negative**
	859	**c**ag/ **a**ag	286	Glumine / Lysine	polar / polar	**neutral / positive**
Hybrid 2	354	g**a**g	118	Glutamic acid	**polar**	**negative**
	546	**a**cc	182	Threonine	**polar**	neutral
	760	**t**tc/ **g**tc	254	Phenylalanine / Valine	nonpolar / nonpolar	neutral / neutral
	821	g**a**c	274	Aspartic acid	**polar**	**negative**
	859	**a**ag	286	Lysine	polar	**positive**

Appendix 6 Elite, SB-d1 and SB-d2, Hybrid 1 and Hybrid 2 UGT85B1 nucleotide sequences compared to Genebank gene accession AF199453. Hybrid 1 and Hybrid 2 have two variations in the sequence, shown here as sequence “a” or “b”.



Appendix 6 continued



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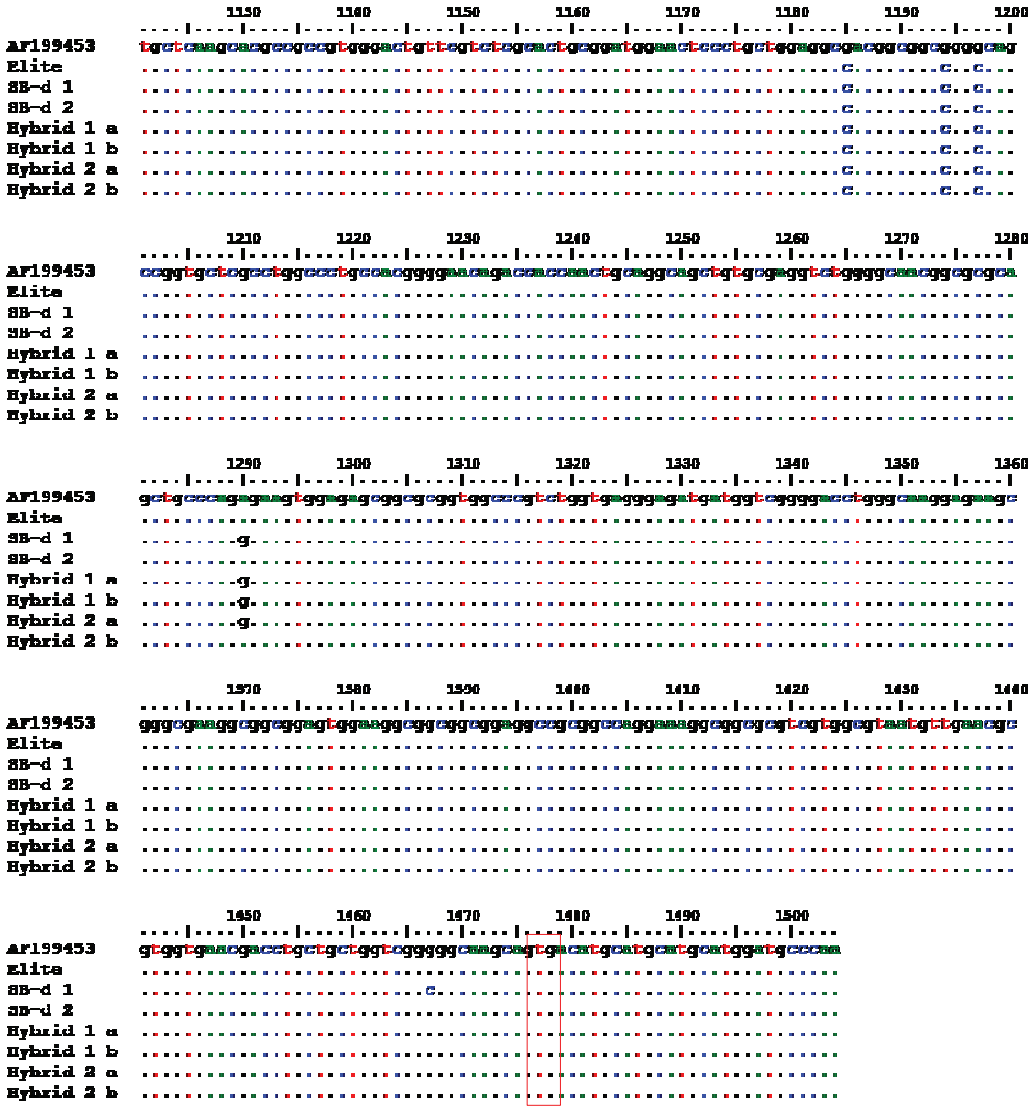
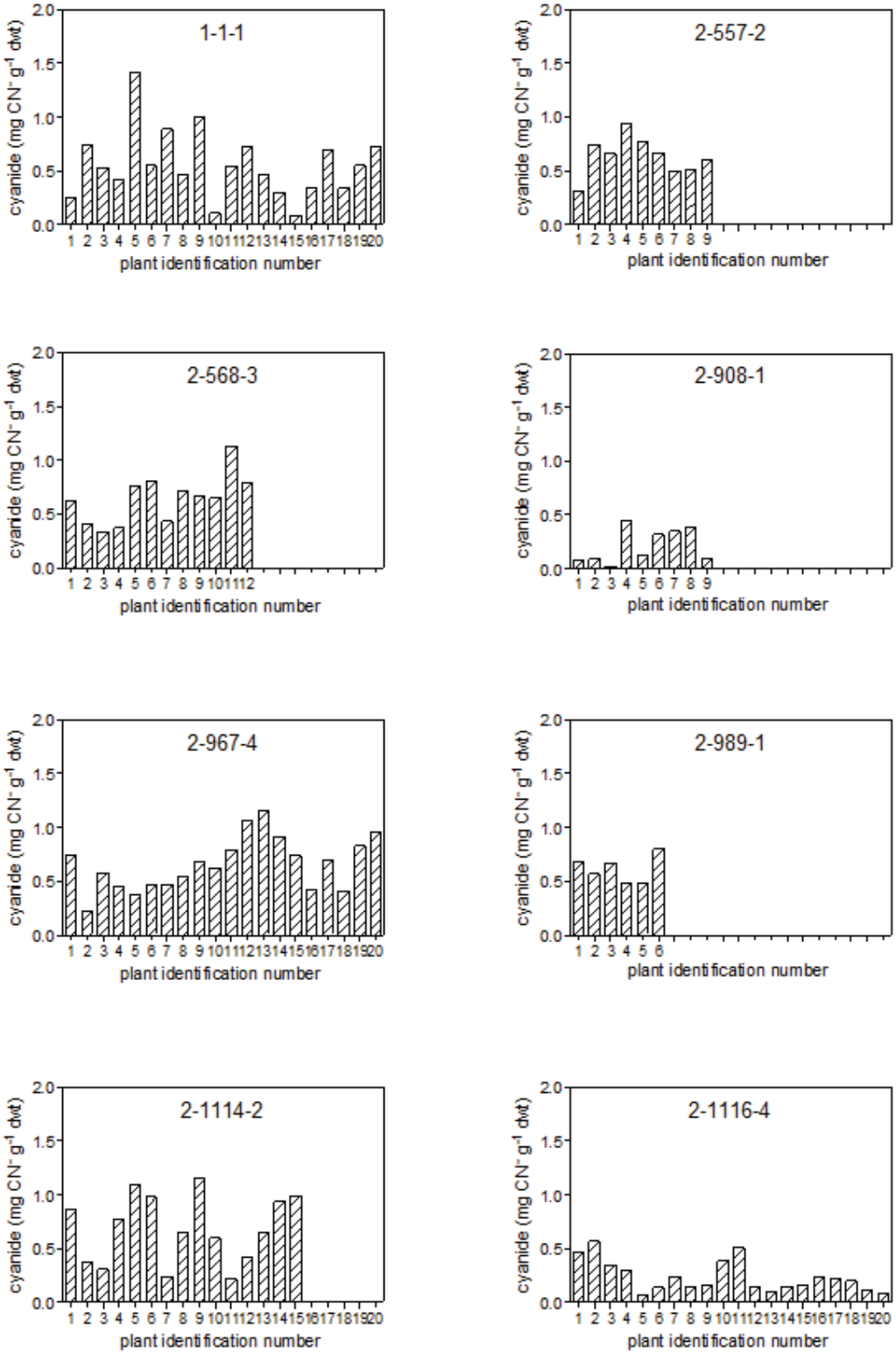
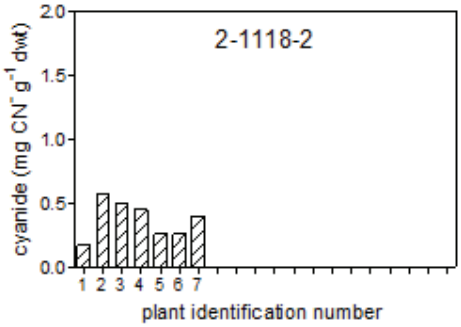


Appendix 7 The HCN concentrations of the first fully expanded leaf of individual plants from the M_3 population. Up to 20 plants from each family was tested. Where there are less than 20 plants, all surviving plants were tested.

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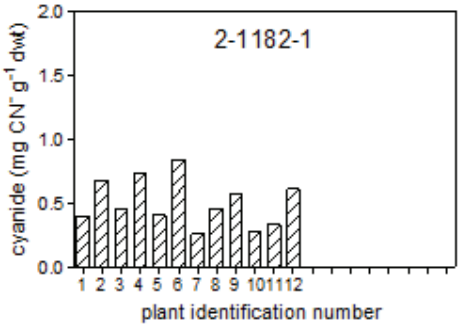
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number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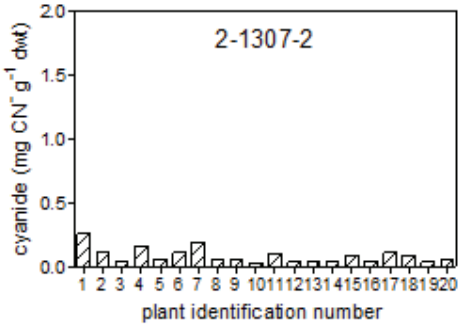
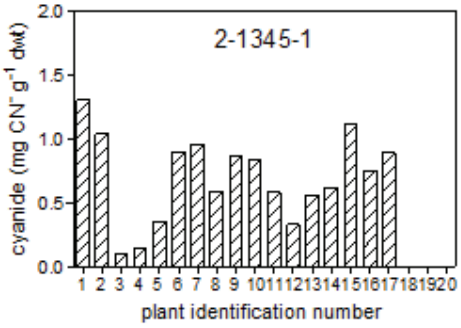
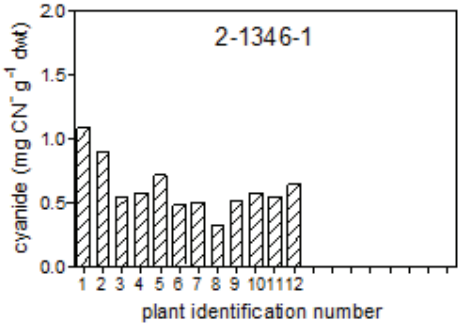
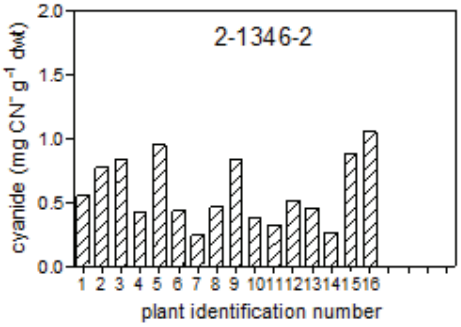
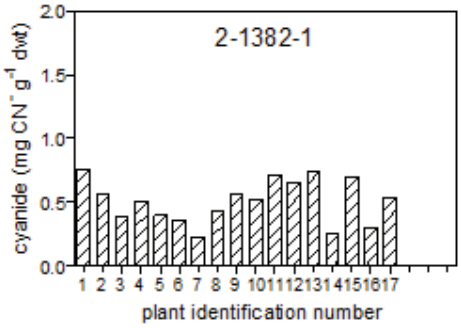
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

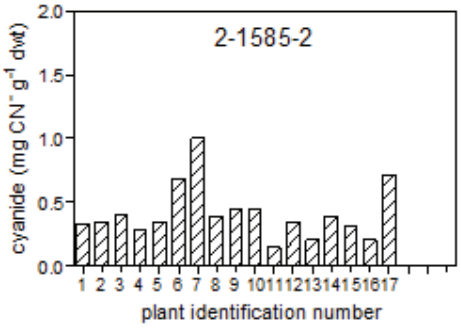

2-1307-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

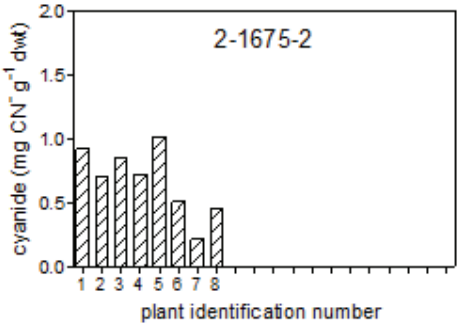

2-1345-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

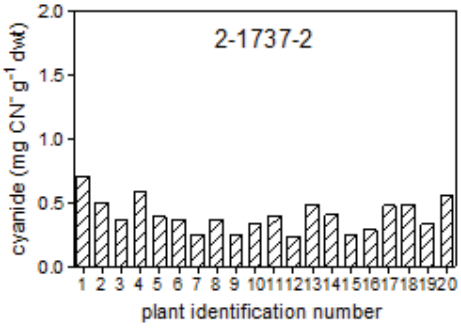

2-1346-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

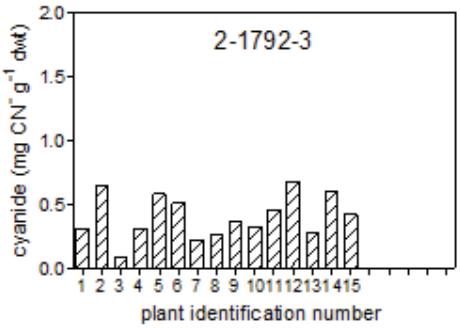

2-1346-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

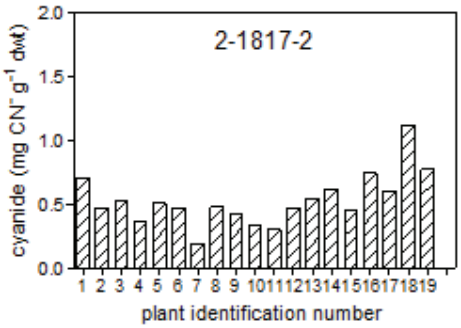

2-1382-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

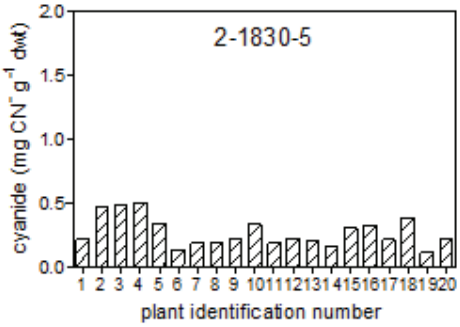

2-1585-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

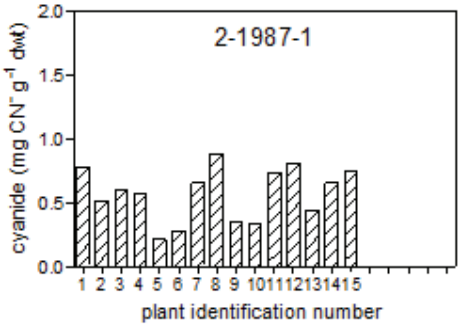

2-1675-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

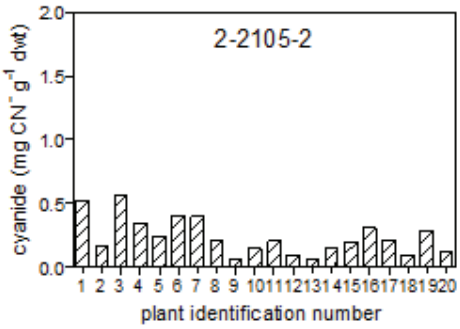

2-1737-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

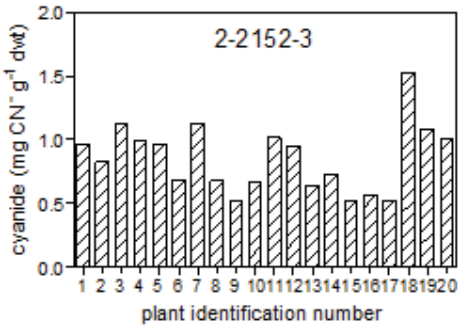

2-1792-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

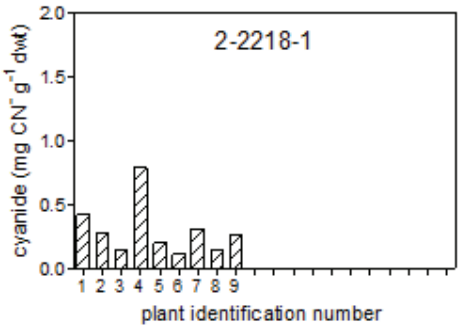

2-1817-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

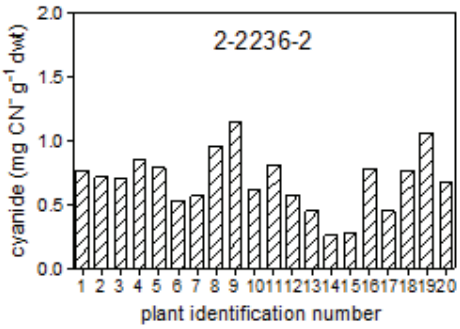

2-1830-5
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

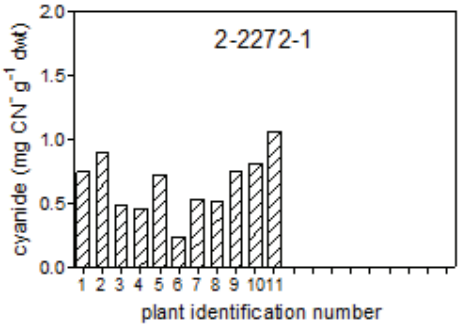

2-1987-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

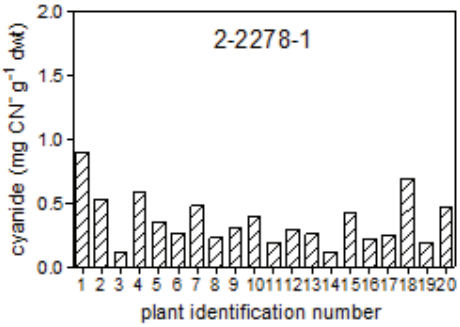

2-2105-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

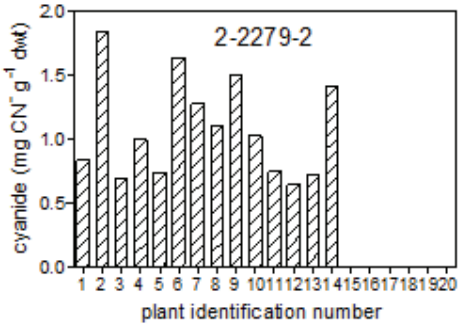

2-2152-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

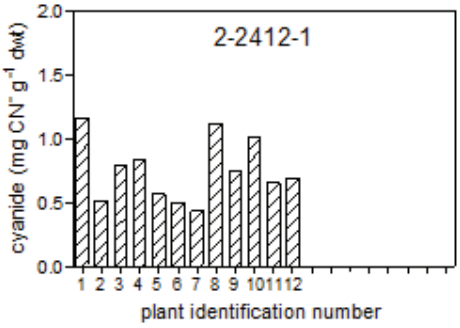

2-2218-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

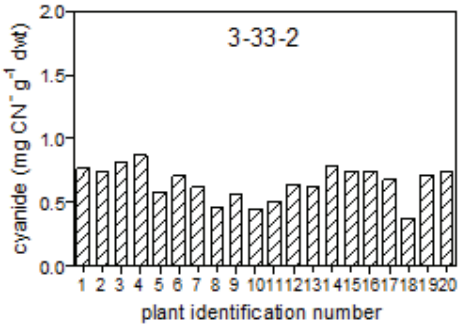

2-2236-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

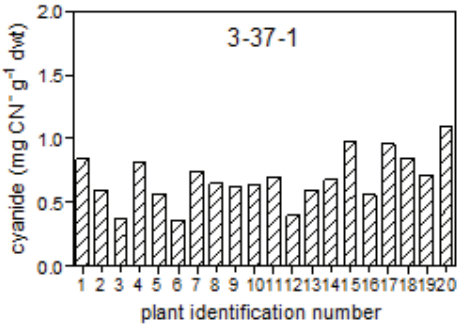

2-2272-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

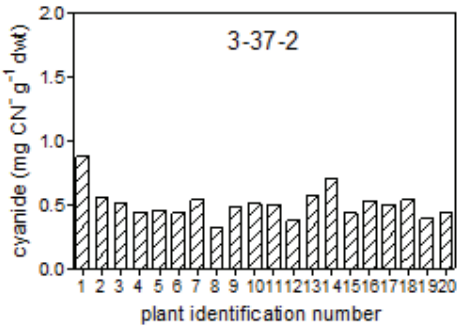

2-2278-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

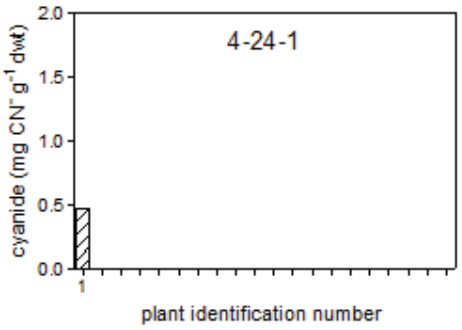

2-2279-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

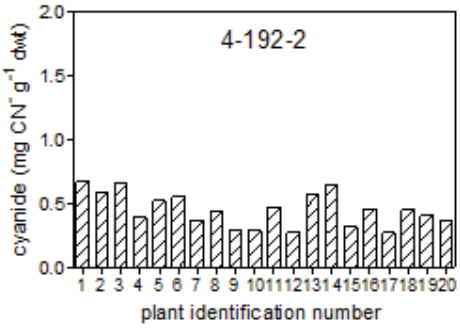

2-2412-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

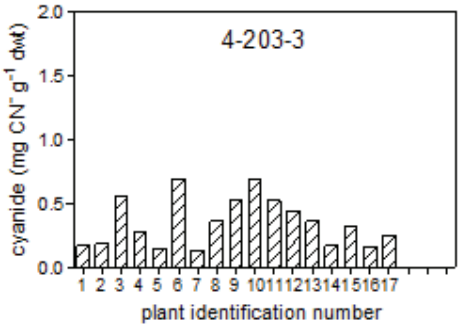

3-33-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

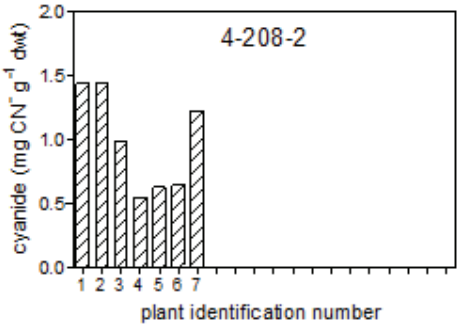

3-37-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

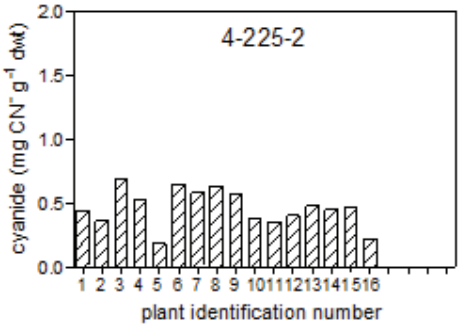

3-37-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

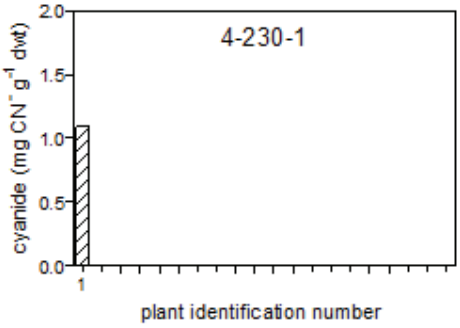

4-24-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

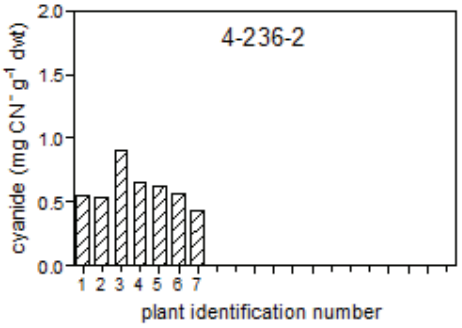

4-192-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

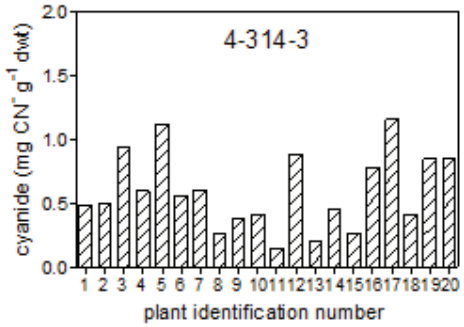

4-203-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

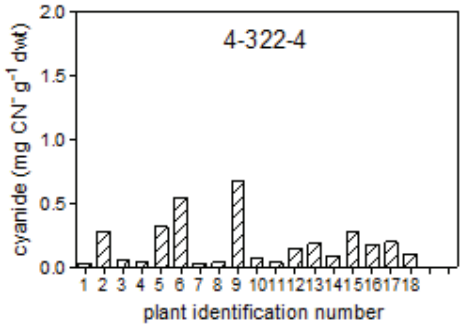

4-208-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

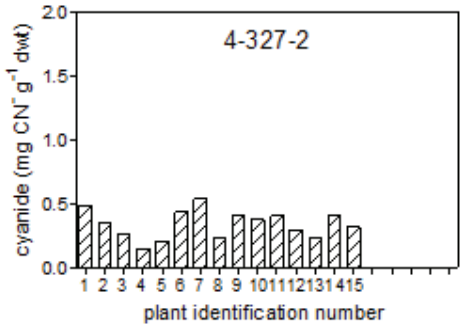

4-225-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

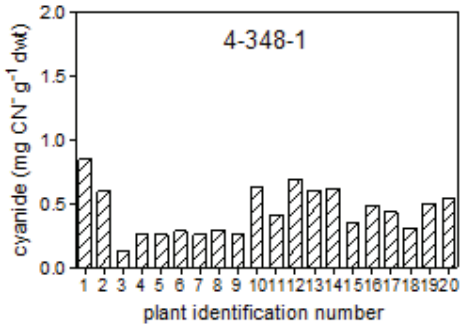

4-230-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

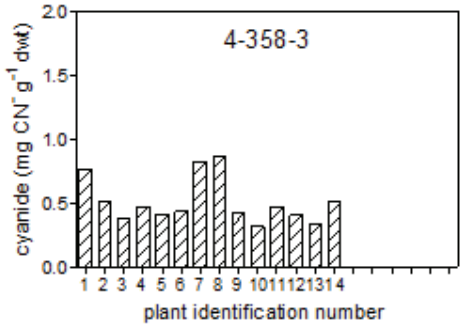

4-236-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

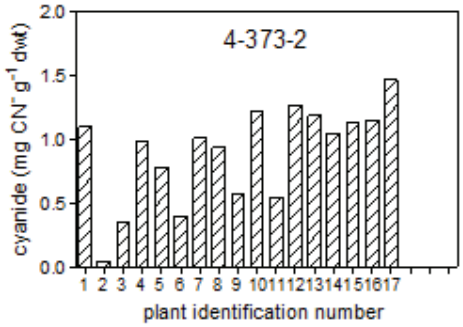

4-314-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

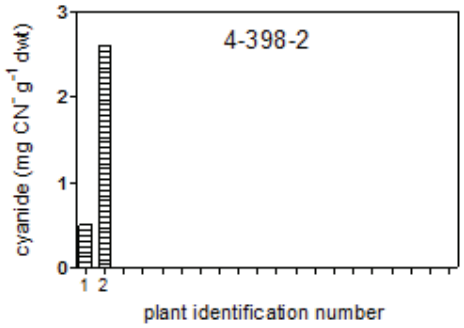

4-322-4
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

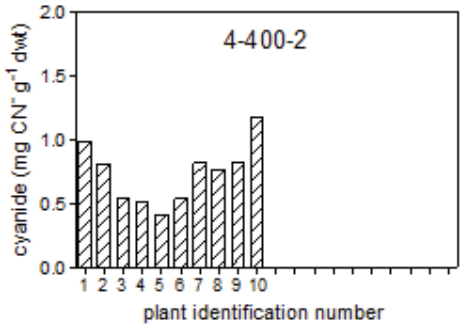

4-327-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

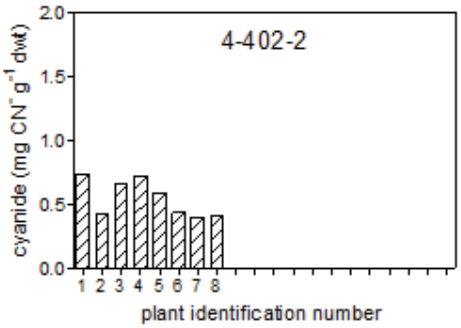

4-348-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

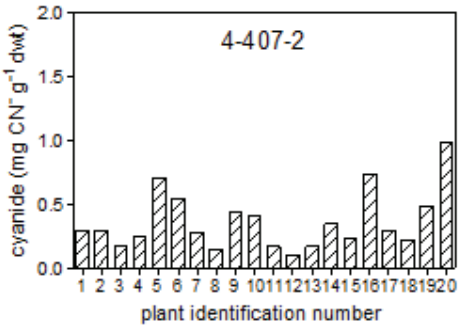

4-358-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

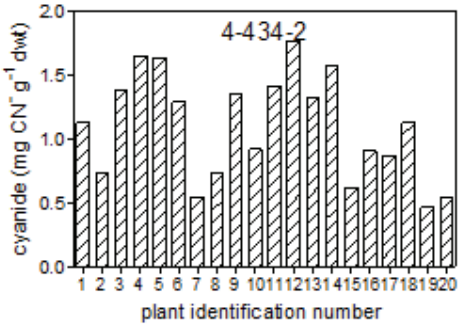

4-373-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

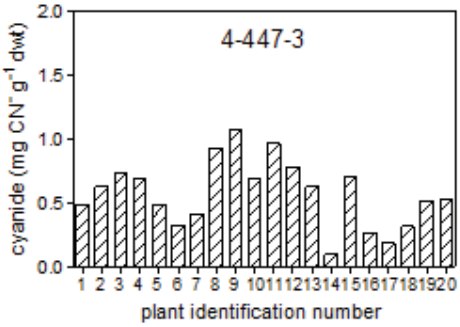

4-398-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

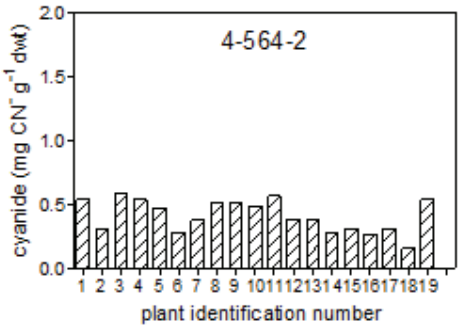

4-400-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

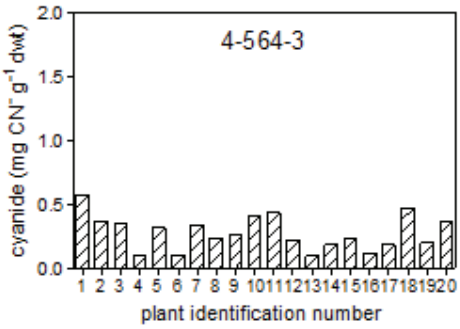

4-402-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

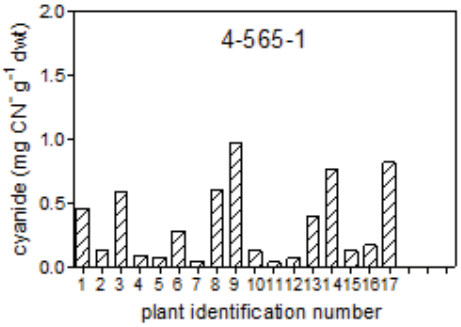

4-407-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

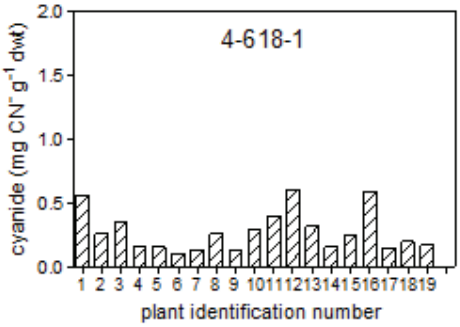

4-434-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

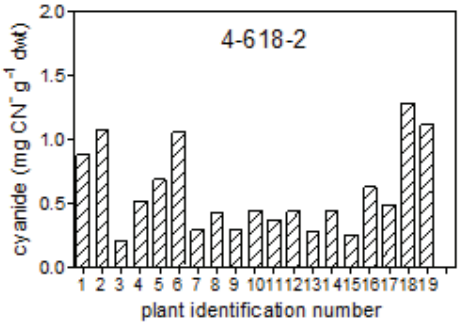

4-447-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

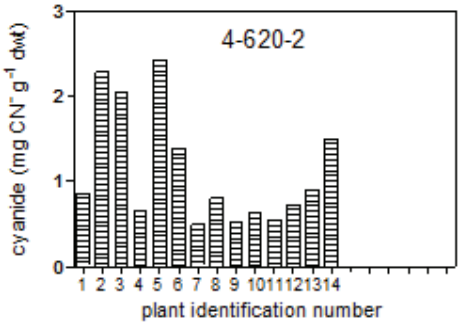

4-564-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

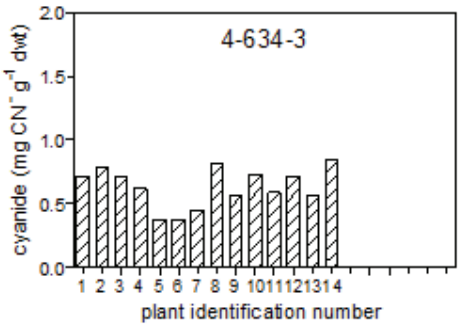

4-564-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

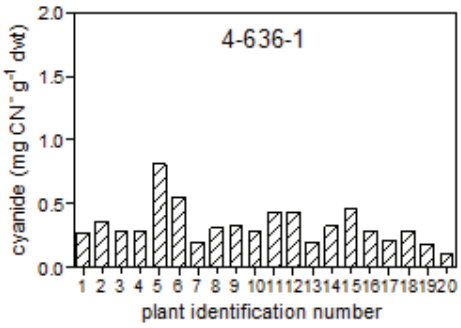

4-565-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

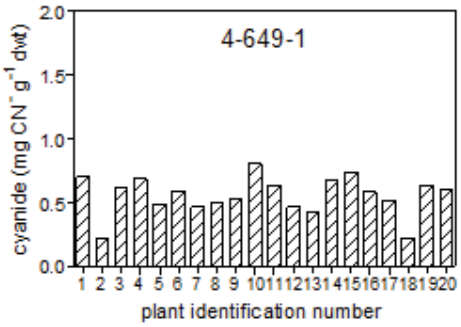

4-618-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

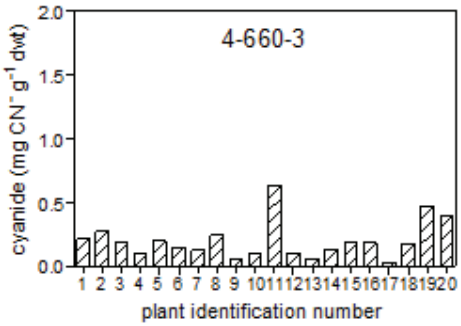

4-618-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

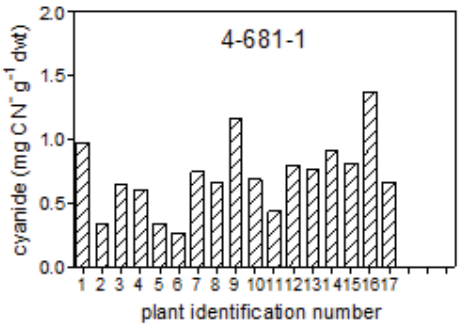

4-620-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

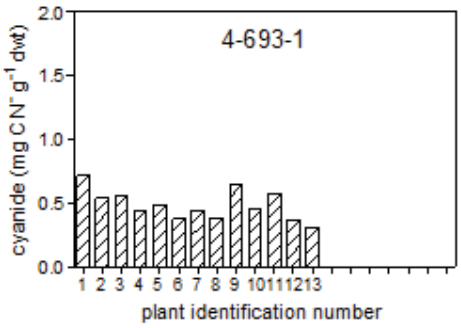

4-634-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

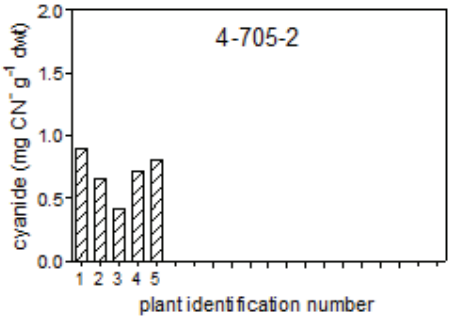

4-636-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

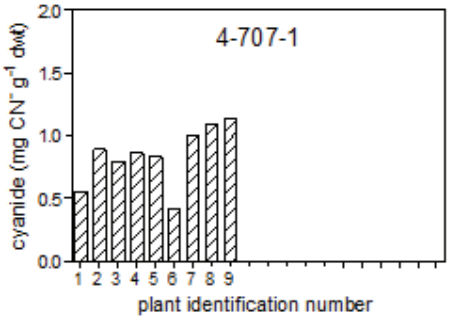

4-649-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

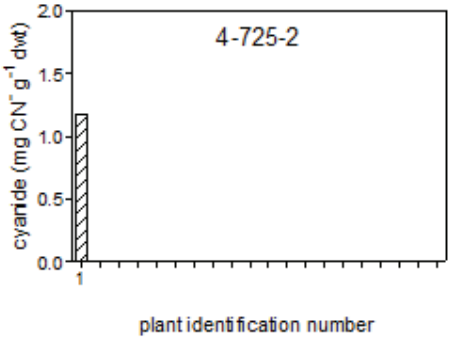

4-660-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

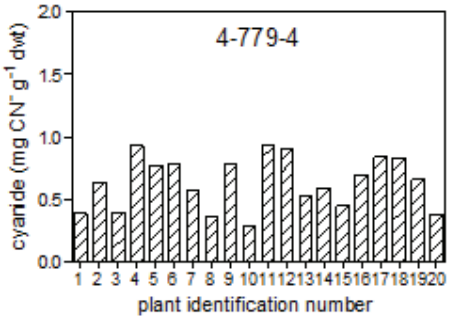

4-681-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

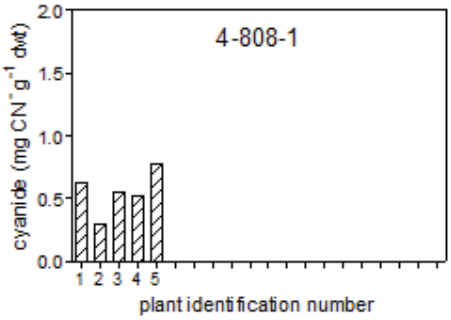

4-693-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

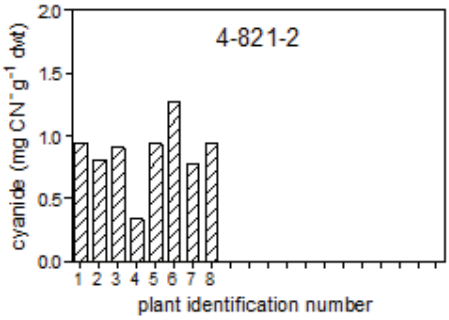

4-705-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

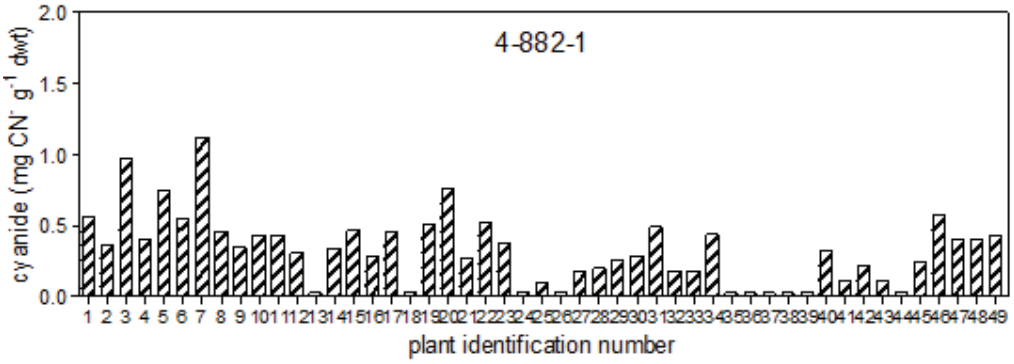

4-707-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

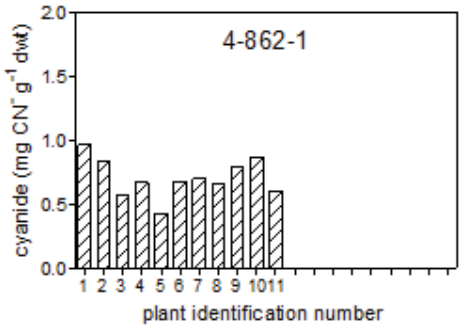

4-725-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

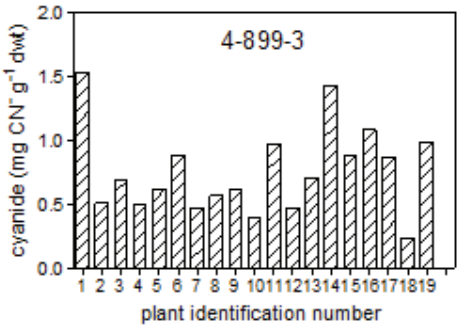

4-779-4
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

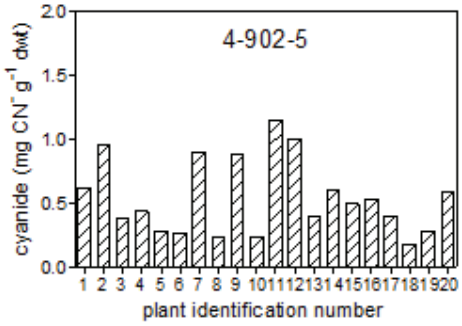

4-808-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

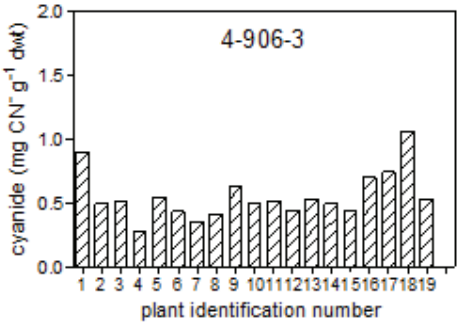

4-821-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

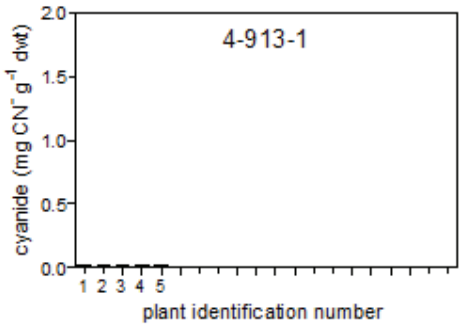

4-882-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

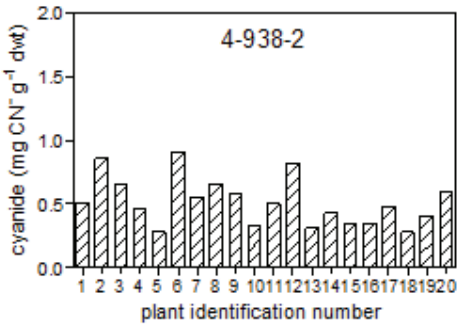

4-862-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

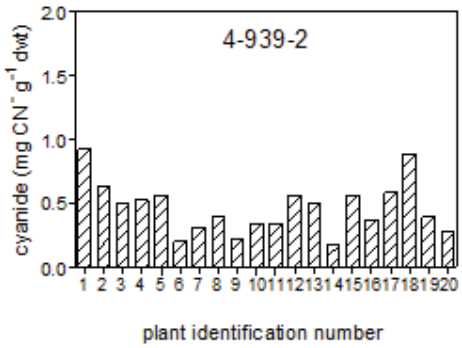

4-899-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

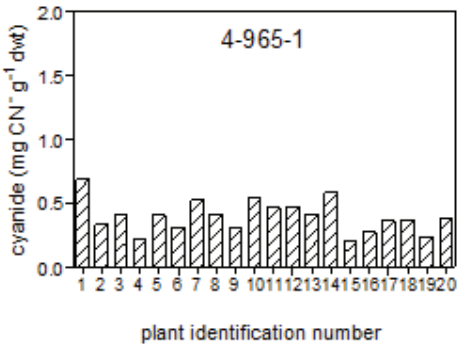

4-902-5
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

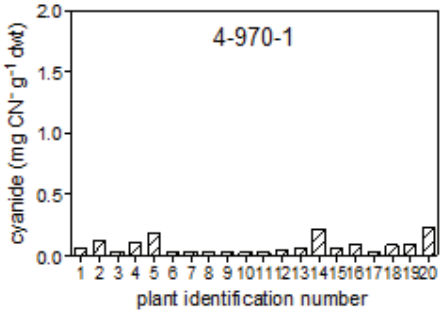

4-906-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

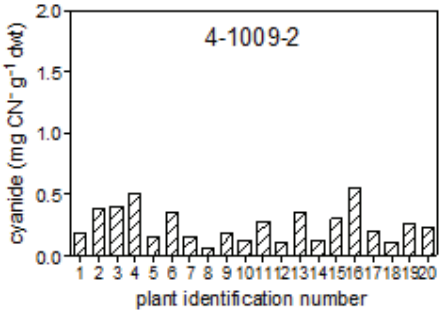

4-913-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

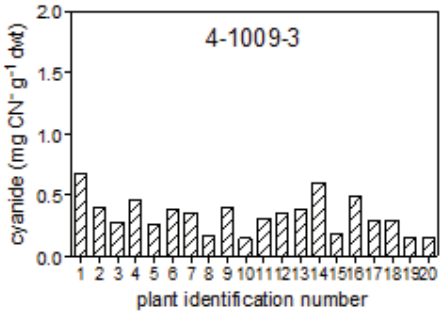

4-938-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

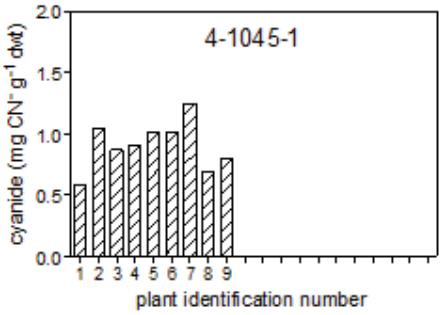

4-939-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

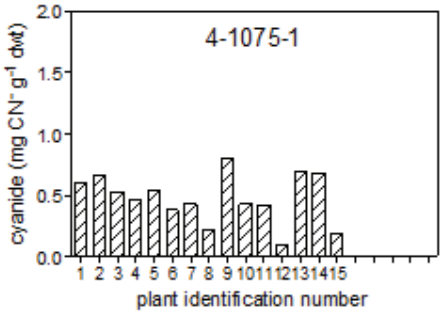

4-965-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

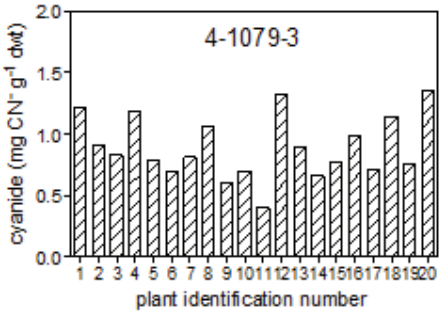

4-970-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

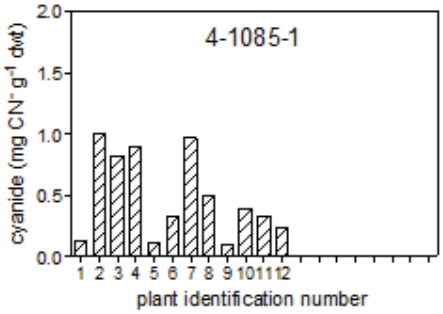

4-1009-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

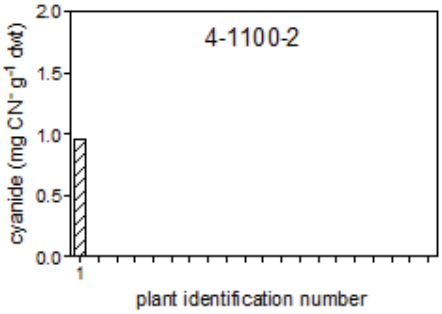

4-1009-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

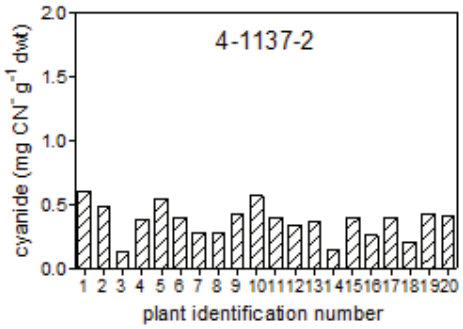

4-1045-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

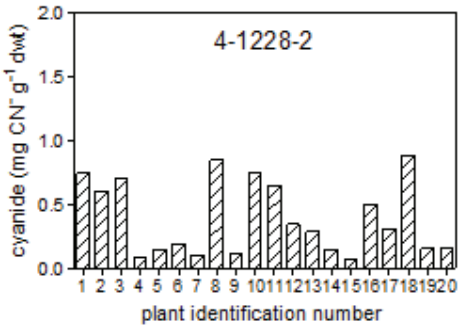

4-1075-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

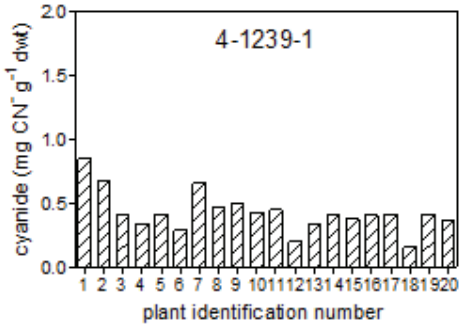

4-1079-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

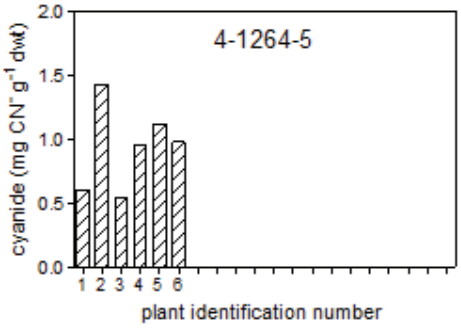

4-1085-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

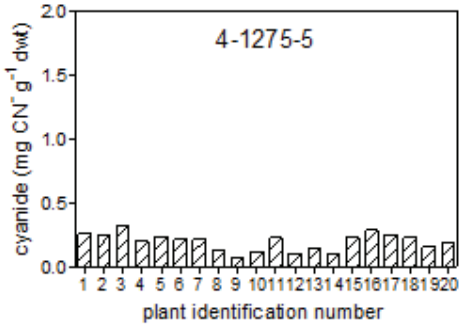

4-1100-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

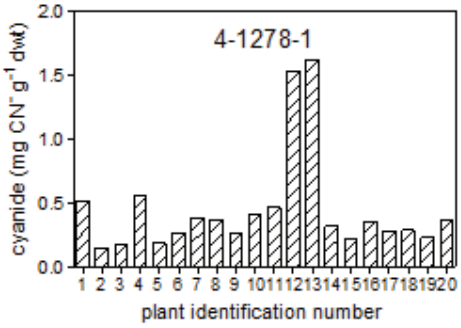

4-1137-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

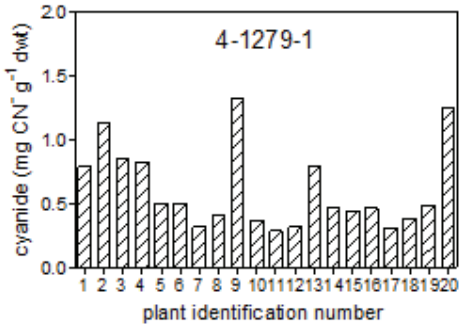

4-1228-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

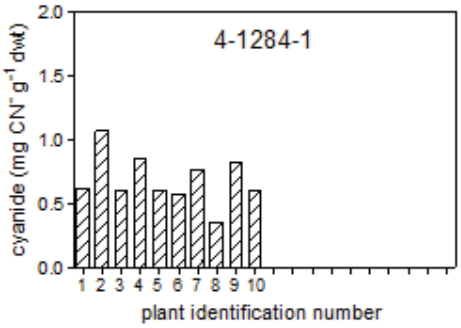

4-1239-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

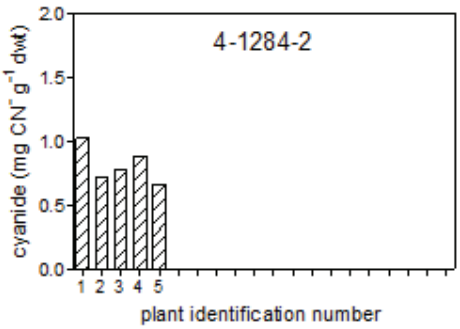

4-1264-5
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

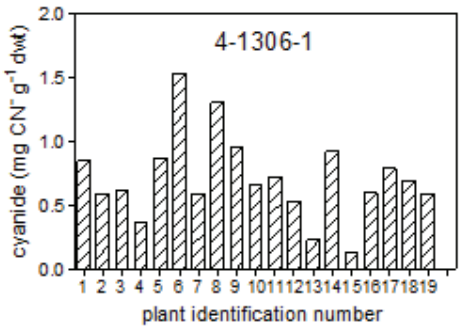

4-1275-5
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

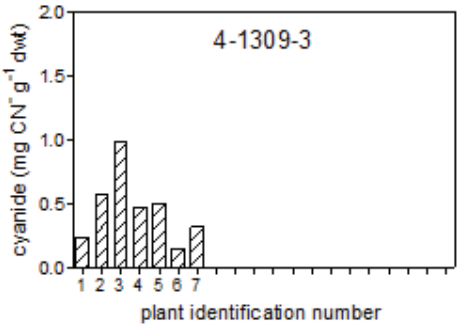

4-1278-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

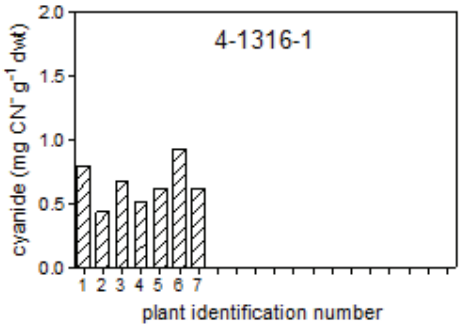

4-1279-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

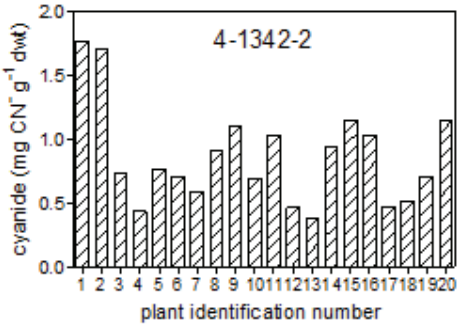

4-1284-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

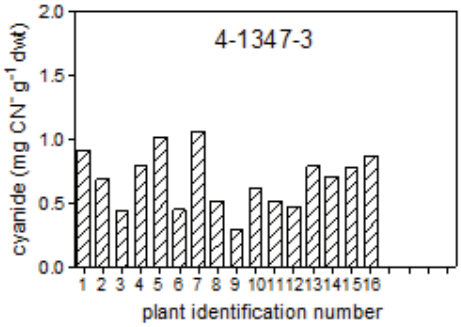

4-1284-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

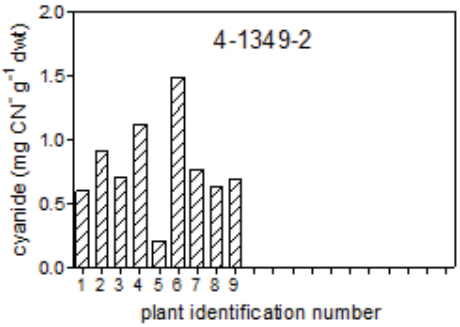

4-1306-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

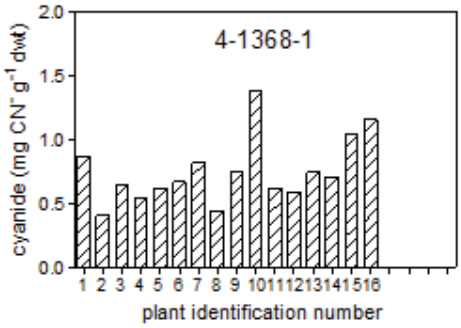

4-1309-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

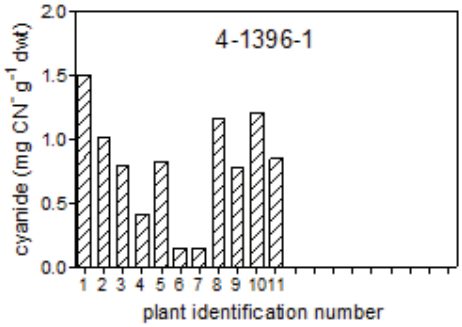

4-1316-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

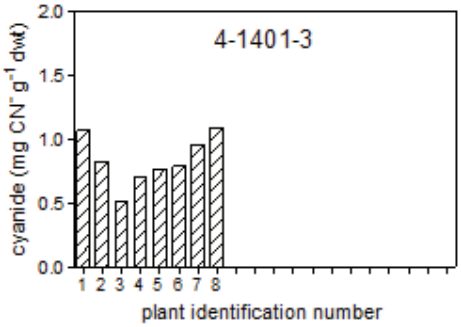

4-1342-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

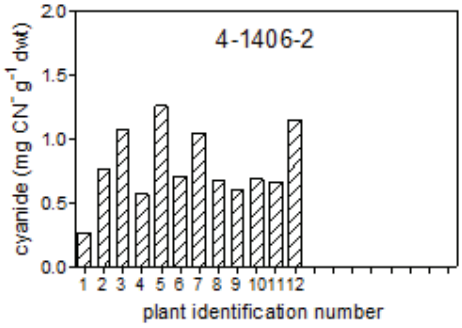

4-1347-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

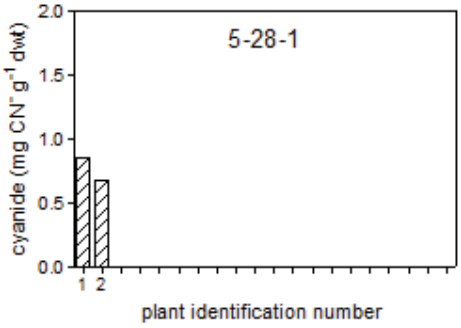

4-1349-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

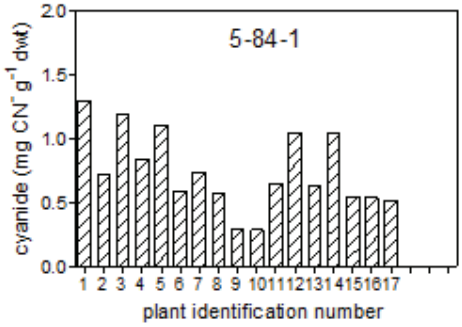

4-1368-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

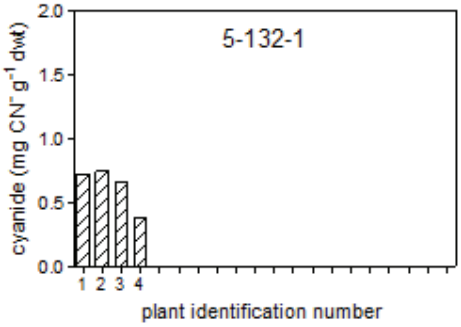

4-1396-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

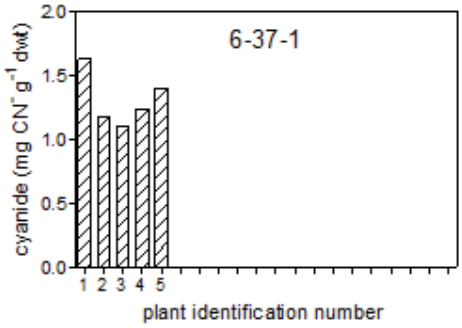

4-1401-3
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

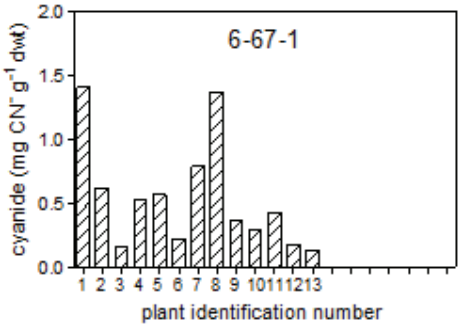

4-1406-2
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number


5-28-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

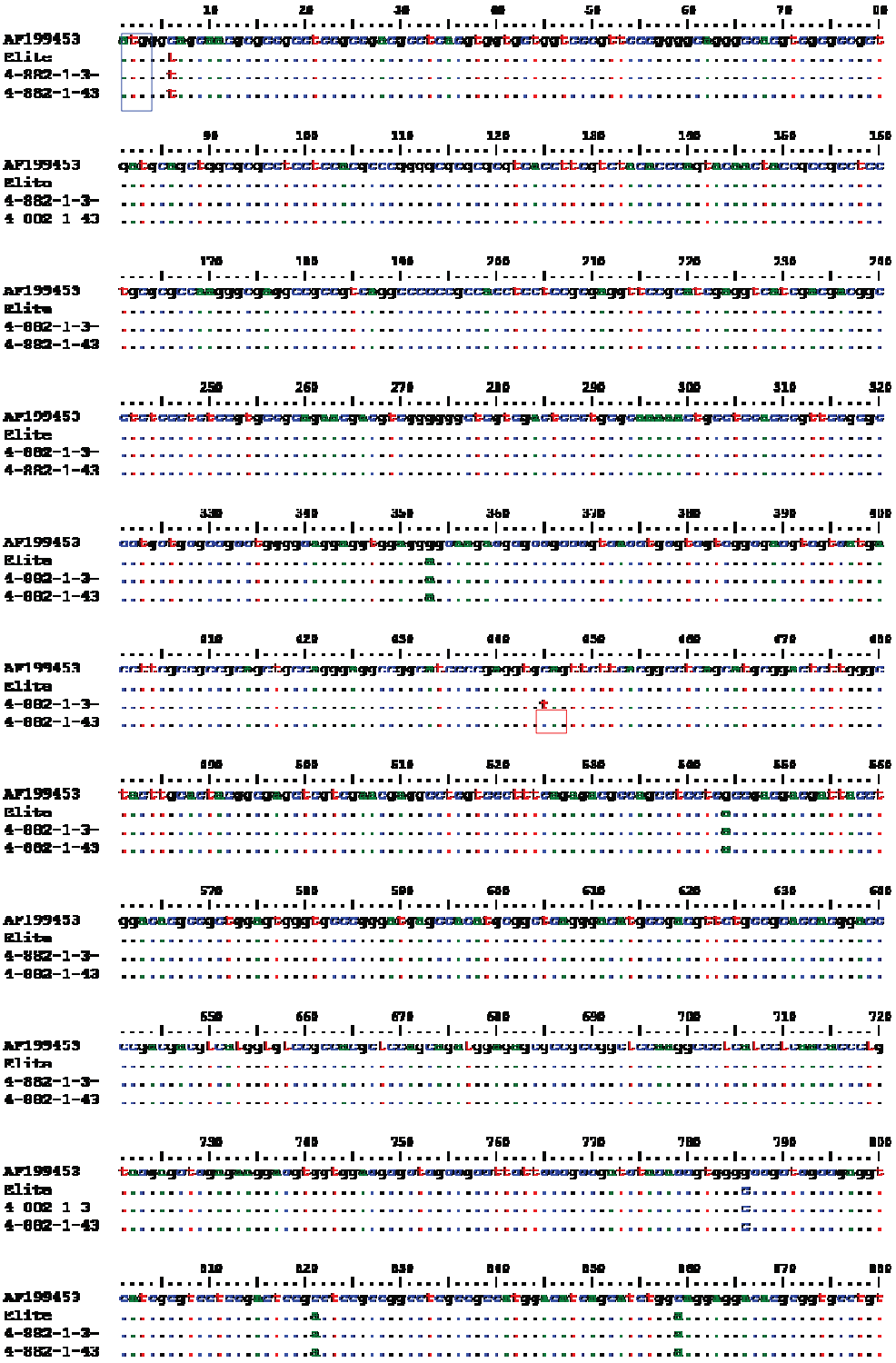

5-84-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

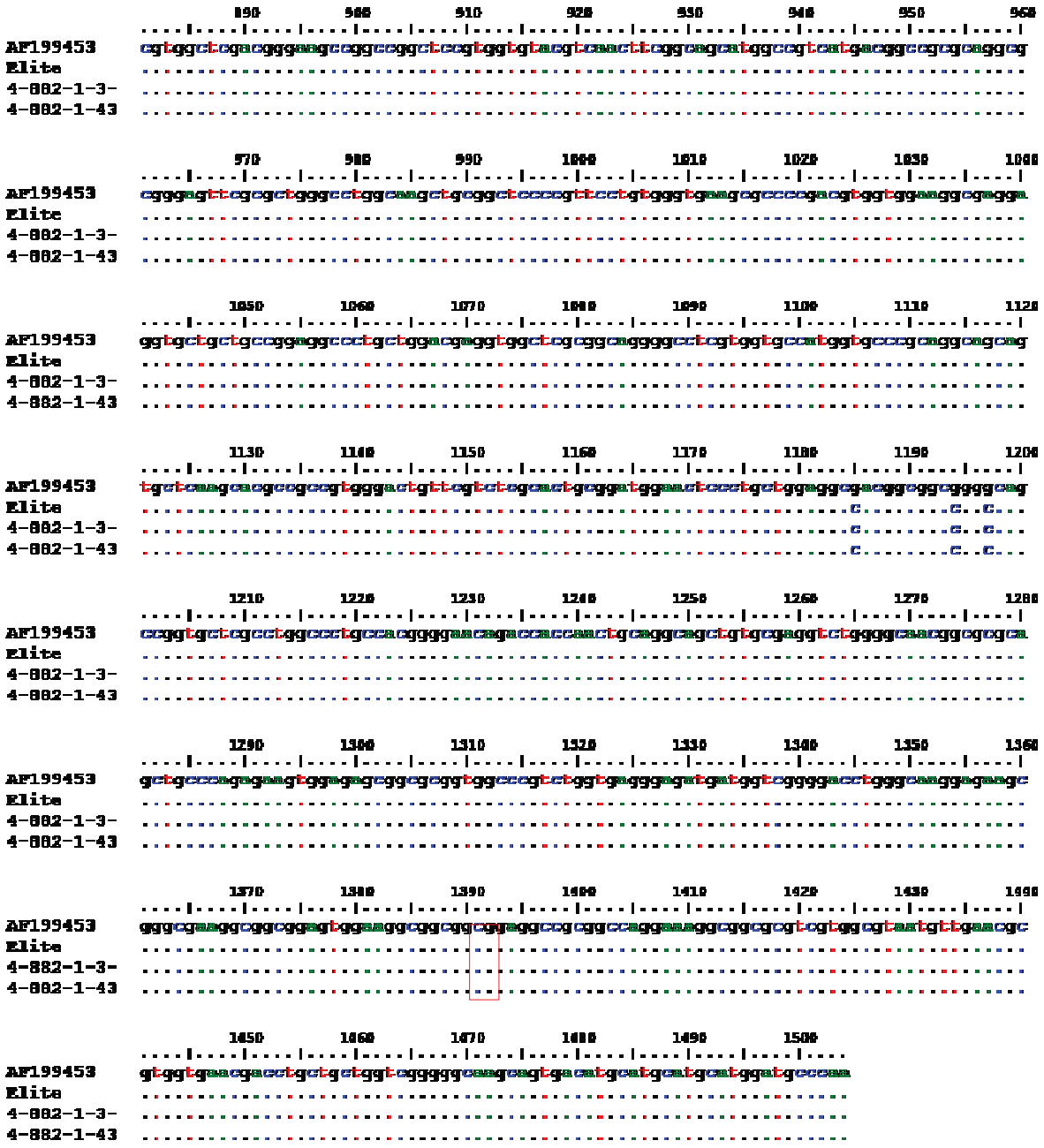

5-132-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number


6-37-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number


6-67-1
cyanide (mg CN⁻ g⁻¹ dwt)
plant identification number

Appendix 8 Nucleotide sequences of the coding region of the UGT85B1 gene for Elite and mutant lines (4-882-1-3 and 4-882-1-43), compared to Genebank gene accession AF199453. Blue box – start codon. Red boxes – stop codon.




AF199453
Elite
4-882-1-3-
4-882-1-43

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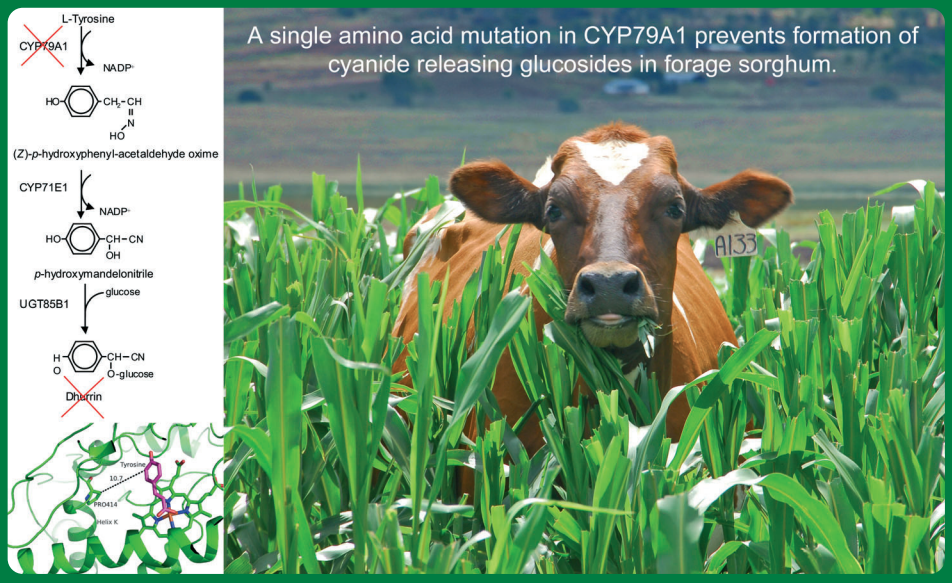

A single amino acid mutation in CYP79A1 prevents formation of cyanide releasing glucosides in forage sorghum.

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A combined biochemical screen and TILLING approach identifies mutations in *Sorghum bicolor* L. Moench resulting in acyanogenic forage production

Cecilia K. Blomstedt[1,*], Roslyn M. Gleadow[1], Natalie O'Donnell[1], Peter Naur[2], Kenneth Jensen[2], Tomas Laursen[2], Carl Erik Olsen[2], Peter Stuart[3,†], John D. Hamill[1], Birger Lindberg Møller[2] and Alan D. Neale[1]

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Summary

Cyanogenic glucosides are present in several crop plants and can pose a significant problem for human and animal consumption, because of their ability to release toxic hydrogen cyanide. *Sorghum bicolor* L. contains the cyanogenic glucoside dhurrin. A qualitative biochemical screen of the M2 population derived from EMS treatment of sorghum seeds, followed by the reverse genetic technique of Targeted Induced Local Lesions in Genomes (TILLING), was employed to identify mutants with altered hydrogen cyanide potential (HCNp). Characterization of these plants identified mutations affecting the function or expression of dhurrin biosynthesis enzymes, and the ability of plants to catabolise dhurrin. The main focus in this study is on acyanogenic or low cyanide releasing lines that contain mutations in CYP79A1, the cytochrome P450 enzyme catalysing the first committed step in dhurrin synthesis. Molecular modelling supports the measured effects on CYP79A1 activity in the mutant lines. Plants harbouring a P414L mutation in CYP79A1 are acyanogenic when homozygous for this mutation and are phenotypically normal, except for slightly slower growth at early seedling stage. Detailed biochemical analyses demonstrate that the enzyme is present in wild-type amounts but is catalytically inactive. Additional mutants capable of producing dhurrin at normal levels in young seedlings but with negligible leaf dhurrin levels in mature plants were also identified. No mutations were detected in the coding sequence of dhurrin biosynthetic genes in this second group of mutants, which are as tall or taller, and leafier than nonmutated lines. These sorghum mutants with reduced or negligible dhurrin content may be ideally suited for forage production.

Introduction

Tolerance to drought, elevated temperatures and high water-use efficiency, make sorghum an ideal cereal crop for arid and semi-arid regions of the world. Over 40 million ha of grain sorghum was harvested globally in 2009 (http://faostat.fao.org/site/567/default.aspx#ancor, accessed 2 December 2010). Forage sorghum is grown widely as a drought-resistant alternative to maize in more arid subtropical to warm temperate regions and has potential as a source for biofuel production on marginal lands not appropriate for food production. Like maize, sorghum is a C4 plant with the attributes of improved photosynthetic efficiency and reduced water loss in hot and dry environments, important traits in the context of current global climate change, limited availability of fertilizer and reduced availability of arable land (Ghannoum, 2009; Gregory *et al.*, 2005).

Several plants, including sorghum produces cyanogenic glucosides (Jones, 1998), which act as defence compounds towards herbivores and pests (Tattersall *et al.*, 2001; Gleadow and Woodrow, 2002a; Møller, 2010a) but may also function as an important antioxidant and play a role in nitrogen turnover and storage (Gleadow and Woodrow, 2000, 2002a,b; Jørgensen *et al.*, 2005a; Kongsawadworakul *et al.*, 2009; Møller, 2010b). In sorghum, the cyanogenic glucoside, dhurrin is derived from tyrosine and is found at higher concentrations in seedlings and young developing leaves compared with mature tissue (Akazawa *et al.*, 1960; Halkier and Møller, 1989; Wheeler *et al.*, 1990; Busk and Møller, 2002). Upon browsing by animals or insects, dhurrin can be hydrolysed by β-glucosidases present in the disrupted leaf tissue, resulting in the release of hydrogen cyanide (HCN), a process termed cyanogenesis (Morant *et al.*, 2008). In response to environmental stresses, which restrict plant growth, such as drought (Wheeler and Mulcahy, 1989), or following nitrogen application (Busk and Møller, 2002) dhurrin concentration typically increases, posing a major threat to livestock. In Australia alone, farmers' reticence to use stressed sorghum as animal fodder decreases the value of the crop by approximately $20 million p.a. (P. Stuart, unpubl. data). Therefore, eliminating the toxicity issues through the development of sorghum varieties with highly reduced or no capacity to produce dhurrin is a key agronomical challenge in sorghum breeding.

Naturally occurring acyanogenic individuals are found in a number of cyanogenic plant species, for example white clover (*Trifolium repens*) (Olsen *et al.*, 2007, 2008), eucalypts (Gleadow *et al.*, 2003) and bird's-foot trefoil (*Lotus corniculatus*; Zagrobelny *et al.*, 2007), demonstrating that cyanogenic glucosides do not play an essential role in primary metabolism but that their involvement in N turnover and storage serves to fine tune primary metabolism under certain circumstances (Kongsawadworakul *et al.*, 2009; Jørgensen *et al.*, 2005a; Møller, 2010b). In white clover, the selective pressures of protection from herbivory in cyanogenic individuals and increased fitness under cold temperatures in acyanogenic individuals are thought to maintain the polymorphism in the *Ac* (CYP79A1 homologue) and *Li* (β-glucosidase) loci, which determines the functional cyanogenic pathway (Olsen *et al.*, 2008). Acyanogenic individuals have not been identified among wild or cultivated populations of sorghum and to date efforts to breed acyanogenic sorghum lines using natural genetic variation have not been successful (Haskins and Gorz, 1986; Duncan, 1996).

Cyanogenesis in sorghum has been studied extensively at the molecular and biochemical levels (Sibbesen *et al.*, 1994; Koch *et al.*, 1995; Kahn *et al.*, 1997; Bak *et al.*, 1998; Jones *et al.*, 1999). Dhurrin synthesis (Figure 1) involves two cytochrome P450s (CYP79A1 and CYP71E1) and one UDP-glucosyltransferase (UGT85B1) (Møller and Conn, 1979; Jones *et al.*, 1999; Bak *et al.*, 2006). These enzymes function co-operatively within a metabolon which allows labile and toxic intermediates to be channelled into dhurrin formation and prevents undesired metabolic cross-talk (Jørgensen *et al.*, 2005b; Kristensen *et al.*, 2005; Nielsen *et al.*, 2008). The identification of all three biosynthetic genes in sorghum has also facilitated attempts to identify the corresponding genes in other species such as cassava (*Manihot esculenta* Cranz) and the model legume *Lotus japonicus* (Forslund *et al.*, 2004; Jørgensen *et al.*, 2005a, 2011). In the present study, knowledge of the gene sequences required for dhurrin synthesis in sorghum was exploited to undertake a TILLING program to generate and identify mutations in the dhurrin biosynthesis enzymes.

TILLING has been used to identify mutations in particular genes of interest in several crop species such as maize, rice and barley (Till *et al.*, 2004, 2007; Talame *et al.*, 2008; Lababidi *et al.*, 2009). Recently, Xin *et al.* (2008) documented the feasibility of TILLING in sorghum by screening a mutagenized population of 768 sorghum plants for alterations in

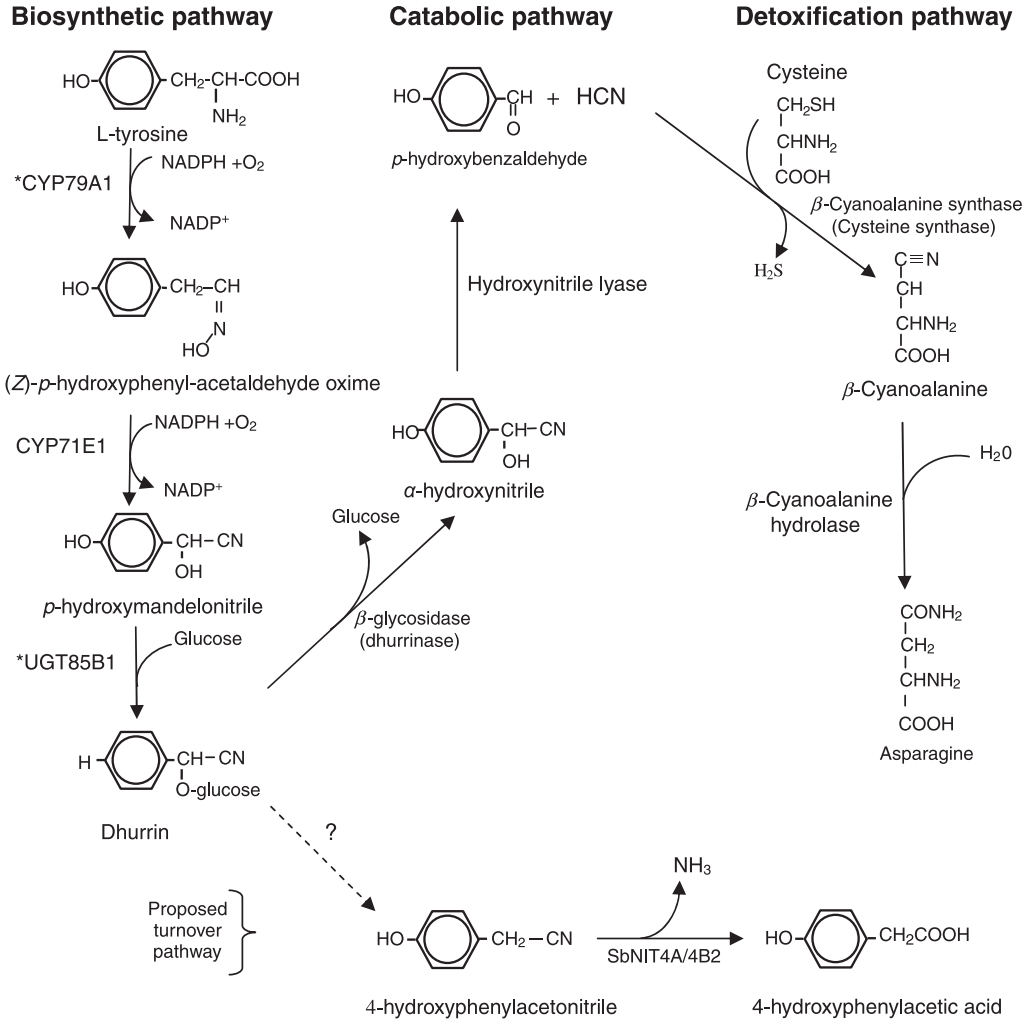

Figure 1 The biosynthetic and detoxification pathways of dhurrin in forage sorghum (from Busk and Møller, 2002; Kristensen *et al.*, 2005; Jenrich *et al.*, 2007).

genes of agronomic value not associated with cyanogenesis. Cyanogenesis-deficient (*cyd*) mutants were obtained in *L. japonicus* using TILLING with mutations in the β-glucosidase gene BGD2 identified and characterized (Takos *et al.*, 2010). In the present study, targeting genes for disrupted β-glucosidase enzyme function was not considered suitable for the production of an acyanogenic forage sorghum crop as cattle contain micro-organisms in their rumen possessing β-glucosidase activity that would release HCN from dhurrin ingested by the animal regardless of the absence of *in planta* β-glucosidase activity (Wheeler and Mulcahy, 1989). Therefore, this study focussed on using a biochemical screen in combination with a TILLING approach to select forage sorghum plants that do not synthesize dhurrin by targetting CYP79A1, the enzyme that catalyses the first committed step in dhurrin synthesis. However, as sorghum lines with a high capacity to release HCN may be of commercial use as biofumigants, mutant lines with elevated HCNp were also targetted. Several mutant lines of sorghum with reduced or no potential for HCN production under field conditions were identified. As well as being of direct benefit to agriculture, such mutants provide an important tool for investigating fundamental questions regarding resource allocation to defence-related secondary metabolites and the associated interactions between primary and secondary metabolism.

Results

Generation and screening for cyanogenesis-deficient mutants in *Sorghum bicolor*

To generate a mutant population, 53 000 seeds of an inbred *S. bicolor* line were treated with varying concentrations of ethyl methanesulfonate (EMS) (0.15%–0.4%), resulting in the germination and growth of approximately 16 300 M1 plants. The higher concentrations of EMS affected the fertility of a large proportion of the plants and only approximately 4200 plants set seed, with some panicles producing <5 seeds. Based on seed availability, up to five M2 seed were planted per line, resulting in a population of 5451 individual M2 plants derived from 2709 independent M2 families. Visual assessment of this M2 population growing in the field showed a wide range of variable phenotypes, suggesting that the EMS treatment had been effective (Figure 2). The initial screening of HCNp of 8-week-old plants was conducted in the field using Feigl–Anger (FA) paper (Feigl and Anger, 1966; Takos *et al.*, 2010) and identified 264 putative mutant lines with substantially differing HCNp, either lower or higher, compared with nonmutated parent plants. Following FA paper screening, samples from the youngest fully unfurled leaf were taken from the 264 putative mutant lines at the time points indicated and the HCNp quantified in the laboratory (Figure S1). In several instances, the HCNp determined by FA paper screening was low, whereas the HCNp determined by the quantitative laboratory-based assay, which included the addition of exogenous β-glucosidase, was high. This suggests that a subset of mutant plants may lack endogenous β-glucosidase activity. In this way, 32 putative β-glucosidase mutants were identified (Table 1).

TILLING of the selected 264 plants resulted in the identification of ten putative *CYP79A1* and 13 putative *UGT85B1* mutant lines (Table 1). DNA sequencing showed that the CYP79A1 mutant lines were independently derived and all mutations identified were G:C to A:T transitions (Figure 3 and Table 2), as expected following EMS treatments (Henikoff *et al.*, 2004). Table 2 shows the HCNp in the M2 and M4 generation of a number of the mutant lines. The full effect of the mutation on HCNp would not necessarily be reflected in the M2 generation as these individuals are likely to be heterozygous. Selected mutant plants were allowed to self-pollinate and subsequent generations assayed for HCNp to identify lines homozygous for the identified mutations. The mutant line carrying the stop codon at amino acid 39 and another carrying an A154T amino acid change in the CYP79A1 protein were lost because of poor growth and infertility in the M2 and M3 generation, respectively. The other eight mutant lines appeared healthy under field conditions. Two lines carried silent CYP79A1 mutations, whereas the remaining six carried alteration to the amino acid sequence of CYP79A1 (Figure 3 and Table 2). Line 2-908-1, with the P414L mutation in CYP79A1 showed only a slight reduction in HCNp in the M2 generation (Table 2). However, subsequent generations, homozygous for the mutation, were found to be completely HCNp deficient in all tissue (Table 3) and grew well under field conditions (Figure 4a,b). Sequence analysis of several M4 2-908-1 lines, which show variation in

Figure 2 Phenotypic variation observed in the M2 mutant population of sorghum growing in the field in Queensland, Australia. In the centre are three rows of M2 mutant plants, note the variation in height from very short plants in the front to quite tall plants further back. Variations in leaf width and colour were also observed. Flanking the M2 plants are nonmutated parent plants on the left and additional buffer rows on the right. Panicles were bagged to prevent cross-pollination.

Table 1 Classification of *Sorghum bicolor* mutant lines obtained in this study

Gene mutated	Phenotype	No. of families
CYP79A1	Absence or reduction in dhurrin content	9
CYP79A1	Increase in dhurrin content	1
Putative *UGT85B1*	Absence or reduced dhurrin content	13
β-glucosidase 1/2	Devoid of or depleted in β-glucosidase activity	32
Unknown	Regulatory mutant	3
Unknown	Brown mid rib (BMR)	7
Unknown	Increased biomass and/or high sugar	2

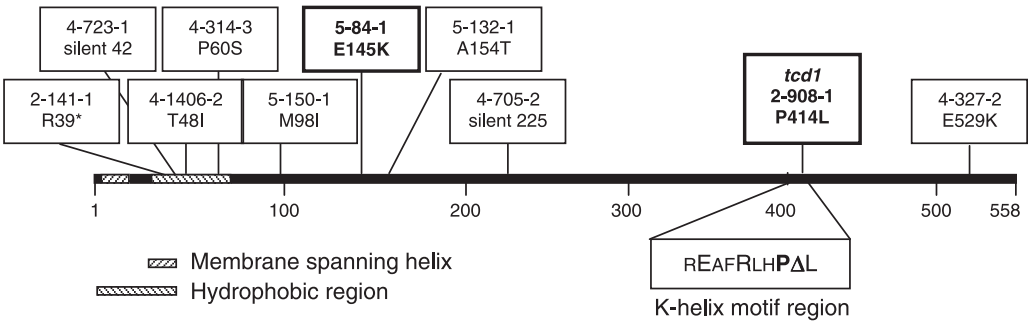

Figure 3 Details and location of the ethyl methanesulfonate-induced mutations identified in CYP79A1. The helix K consensus motif (KETLR, specifically REAFR in sorghum) is located very close to the P414L mutation as shown in the figure.

Table 2 Details of the mutant lines identified to have a mutation in the CYP79A1 gene

			HCNp (mg/g DW)		
			M2 Field grown		M4
Line	nt mutated	Codon change	November 8 ~8 weeks	January 9 ~16 weeks	Greenhouse grown ~5 weeks old
2-141-1	C/T	Stop codon 39	0.24		N/A no seed
4-723-1	C/T	Silent 42		0.43	Not selected
4-1406-2	C/T	T48I		0.11	0.28*
4-314-3	C/T	P60S	0.53		0.18†
5-150-1	G/A	M98I		0.56	Not selected
5-84-1	G/A	E145K		0.97	1.14†
5-132-1	G/A	A154T		0.42	M3 infertile
4-705-2	C/T	Silent 225		0.25	0.31†
2-908-1	C/T	P414L	0.63		<0.03*
4-327-2	G/A	E529K	0.13		0.26†
Control—Av.	–	–	0.71	0.39	0.40

HCNp, hydrogen cyanide potential.

HCNp is higher in younger plants as can be seen from a comparison of the HCNp of the nonmutated parent plants at approximately 8 and 16 weeks. The M4 generation was grown during winter in a greenhouse, which may explain the difference in HCNp observed in the young M2 and M4 control plants.

*Homozygous, confirmed by sequencing.

†Homozygosity not yet confirmed by sequencing.

Table 3 HCNp (mg/g DW) determined in the leaf, sheath and root tissue of adult *tcd1*, *acdc2* and *acdc3* mutants

	Parent line	*tcd1*	*acdc2*	*acdc3*
Leaf	2.09 ± 0.08	0 ± 0.01	0.07 ± 0.03	0.05 ± 0.02
Stem	1.21 ± 0.23	0 ± 0.00	0.36 ± 0.12	0.23 ± 0.03
Root	2.04 ± 0.76	0 ± 0.01	1.04 ± 0.29	1.04 ± 0.64

HCNp, hydrogen cyanide potential.

the HCNp of the different siblings (Figure 5), confirmed that a-cyanogenicity is determined by the presence of the mutation. Lines 2-908-1-1-2, 2-908-1-1-5 and 2-908-1-5-2 are homozygous for the mutation and have negligible HCNp, while the wild-type sibling 2-908-1-3-2 and heterozygous sibling 2-908-1-4-1 have high HCNp (Figure 5). The acyanogenic 2-908-1 mutant line was designated as *tcd1* (totally cyanide deficient 1) and was analysed further.

Three additional independent mutant lines (2-1307-2, 4-565-1 and 4-970-1) identified as having very low HCNp in the leaves of adult plants showed no mutations in the *CYP79A1* and *UGT85B1* structural gene sequences. M5 plants of these three mutant lines grown in the field were healthy with no apparent susceptibility to fungal or insect attack or requirement for additional nitrogen fertilization (Figure 4c–h). The lines grew well, particularly line 2-1307-2, which grew taller and leafier than control plants (Figure 4d,e). While subsequent analysis showed that HCNp in very young leaf tissue (<3-leaf stage; Figure 6a) and etiolated seedlings of these mutants are comparable to the levels in leaves of nonmutated parent lines, the HCNp in these lines drop rapidly to levels below or equivalent to that of the leaves of nonmutated parent plants at the 4-leaf stage (Figure 6a). Quantitative assays in adult M3 and M4 individuals, as well as FA paper assays of adult M5 individuals from each of these lines (Figure 6b), confirm that negligible or very low HCNp is present in mature leaf tissue. However, HCNp of 4-565-1 and 4-970-1 remains high in the sheath and root tissue

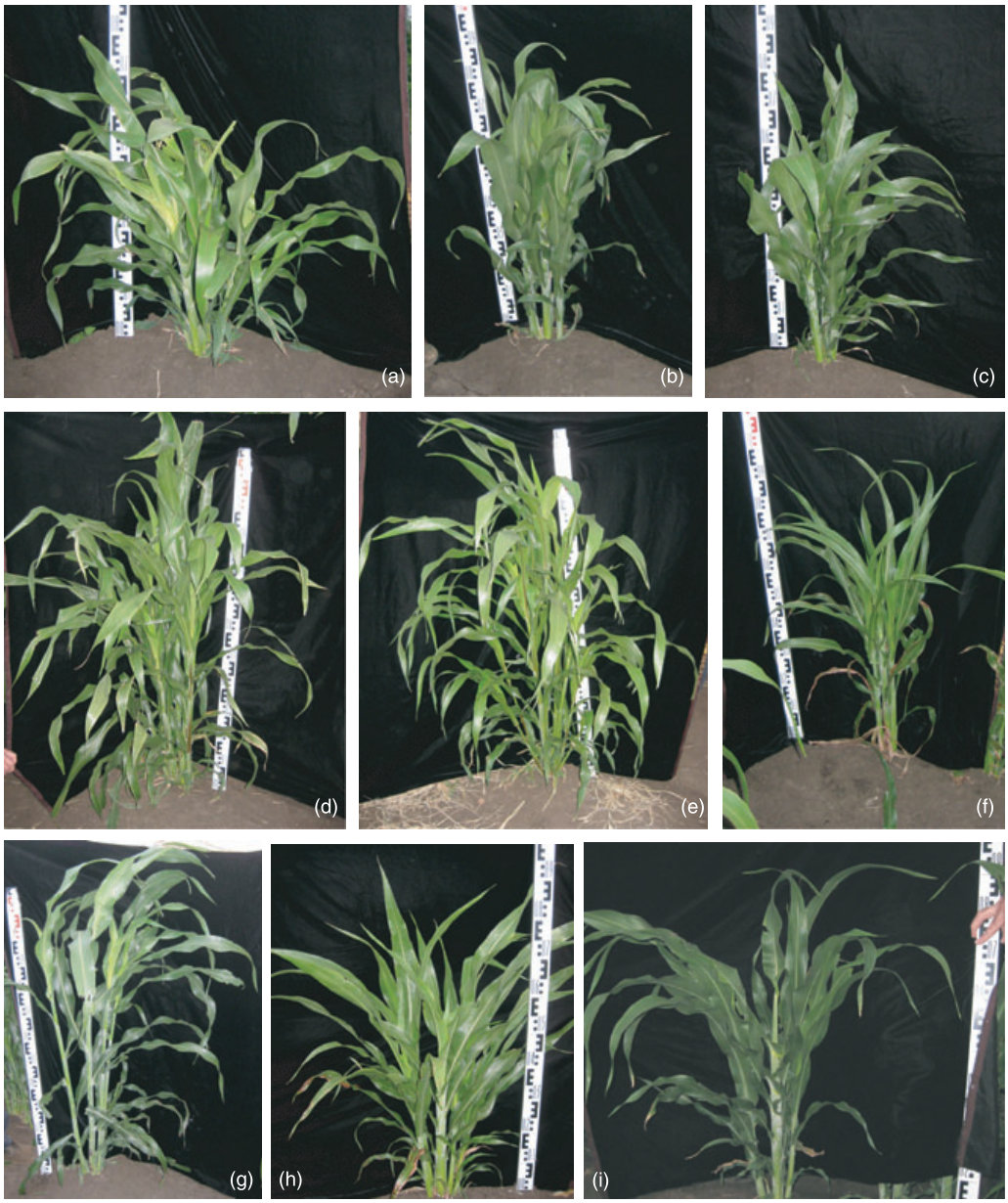

Figure 4 The selected M5 sorghum mutants at approximately 9 weeks of age. (a) acyanogenic *tcd1* individual 2-908-1-1-5 (0.5 m) (b) acyanogenic *tcd1*, 2-908-1-5-2. (0.7 m); (c) *acdc2*, 4-565-1-11-2, (0.6 m); (d) *acdc1*, 2-1307-2-10-1, (0.9 m); (e) *acdc1*, 2-1307-2-10-5, (0.9 m); (f) *acdc3*, 4-970-1-1-2, (0.6 m); (g) *acdc3*, 4-970-1-6-1, (1.0 m); (h) *acdc3*, 4-970-1-10-3, (0.6 m). (i) Nonmutated parent line, (0.6 m). Height stick is marked with 0.1-M increments.

(Table 3). These three lines, 2-1307-2, 4-565-1 and 4-970-1, were designated adult cyanide deficient category (*acdc*) mutants 1-3, respectively.

Analysis of CYP79A1 activity using microsomal preparations

The biochemical activity of CYP79A1 in 4-day-old-etiolated seedlings from the nonmutated parent line and selected *tcd1* and *acdc1-3* mutant lines was measured using microsomal preparations. The germination rate of the seeds from all selected lines was high and the only clear phenotypic effect was observed in the *tcd1* line, which showed slower growth rates at the seedling but not the adult stage. To conserve the valuable seed resource, a procedure was developed to isolate microsomes from as little as approximately 0.5 g etiolated seedling material. Mutant and wild-type seedlings afforded similar yields with approximately 370 μg microsomal protein obtained from 0.5 g of seedling material.

The catalytic activity of CYP79A1 and CYP71E1 requires electron transfer from NADPH-cytochrome P450 oxidoreductase (CPR; Jensen and Møller, 2010). The two P450s as well as CPR are membrane bound and thus recovered in the microsomal preparation. Because the EMS treatment might affect CPR, the CPR activity was measured in wild-type lines and in the individual TILLING lines. The parent line showed an activity of 3.1 nmol cytochrome *c* reduced per min per seedling while the

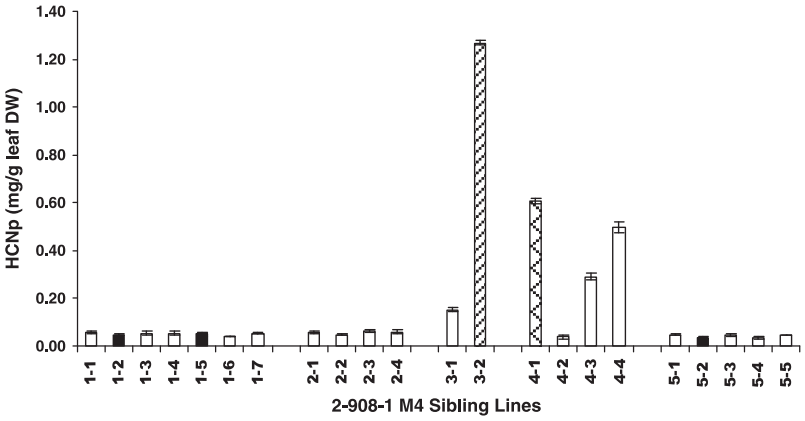

Figure 5 Hydrogen cyanide potential among *tcd1* M4 siblings showing that seedlings homozygous for the P414L mutation are acyanogenic. Solid black, homozygous for the P414L mutation; diagonal grey lines, homozygous for the wild type; hatched pattern, heterozygous for the mutation.

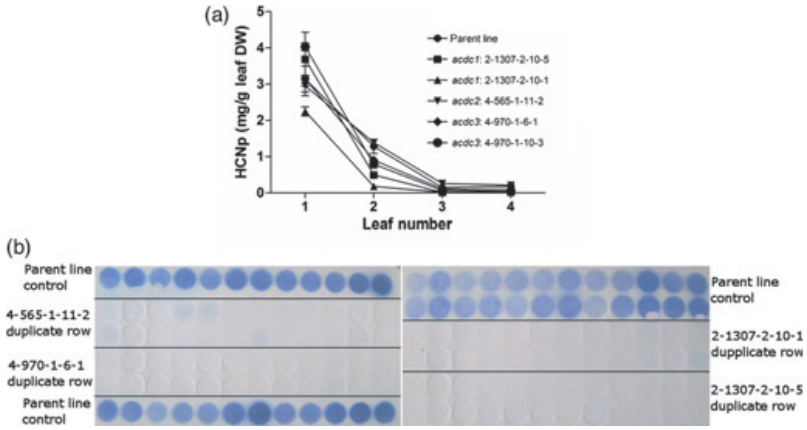

Figure 6 (a) Hydrogen cyanide potential of the leaf tissue of the *acdc1-3* mutant lines during early seedling growth (up to 4-leaf stage). (b) Feigl–Anger test of 15 M5 field-grown adult plants demonstrating that mature leaves of the *acdc* mutant lines are acyanogenic.

activity of the TILLING lines varied in the range from 2.6 to 3.6 nmol cytochrome c reduced per min per seedling (Table 4). Thus, none of the TILLING lines showed a substantial reduction in CPR activity compared with nonmutated parent lines.

The CYP79A1 and CYP71E1 activity in the microsomal membranes was monitored by their ability to metabolize radiolabelled tyrosine in the presence of a saturating amount of NADPH. The analysed *tcd1* M4 line was completely devoid of CYP79A1 activity resulting in no metabolism of the administered radiolabelled tyrosine (Figure 7a). At saturating substrate concentrations, the *acdc* mutants showed little or no reduction compared with the parent line (Figure 7a). The *acdc2* line showed only a 20% reduction of activity, while *acdc1* and the three, *acdc3* siblings showed no reduction in CYP79A1 activity. In none of the TILLING lines did *p*-hydroxyphenylacetaldoxime or *p*-hydroxyphenylacetonitrile accumulate, indicating that the activity of the subsequent enzyme in the pathway, CYP71E1, was not limiting for the conversion of intermediates into *p*-hydroxymandelonitrile (Figure 7a). The latter is the final product of the dhurrin pathway obtained when membrane-bound enzymes of microsomal preparations is used as the source.

Table 4 NADPH-cytochrome P450 oxidoreductase activity in microsomal preparations isolated from different sorghum mutants and from the nonmutated parental line

Sorghum line	cyt c reduced (nmol/min per seedling)
Parent line	3.1
tcd1 (2-908-1-1-5)	3.2
tcd1 (2-908-1-5-2)	3.4
acdc1 (2-1307-2-10-1)	3.5
acdc1 (2-1307-2-10-5	3.1
acdc2 (4-565-1-11-2)	2.9
acdc3 (4-970-1-1-2)	3.6
acdc3 (4-970-1-6-1)	2.6
acdc3 (4-970-1-10-3)	3.5

Analysis of the TILLING lines as 3- to 4-day-old etiolated seedlings showed that the *tcd1* mutant does not contain any dhurrin (Figure 7b). This was consistent with the absence of CYP79A1 activity in the microsomal preparations isolated from the same lines. Dhurrin content of the seedlings from the other mutant lines showed no direct correlation with the measured activity of CYP79A1. In wild-type seedlings, the high dhurrin content of dark grown seedlings decreases rapidly after approximately 4 days and the amount of dhurrin presence reflects the balance between rate of synthesis and turn-over. Hence, a direct relationship between CYP79A1 activity and dhurrin content is not necessarily expected.

CYP79A1 and CYP71E1 content of the sorghum TILLING lines as determined by immunoblotting

Antibodies were raised against different specific surface-exposed peptide sequences of CYP79A1 and CYP71E1. Western

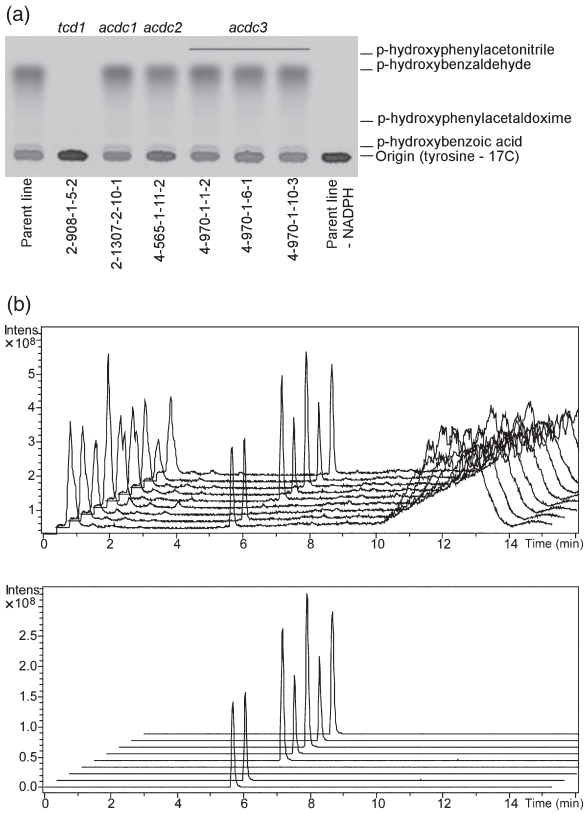

Figure 7 Assessment of CYP79A1 activity in microsomal preparations from nonmutated parent lines and selected TILLING mutants. (a) CYP79A1 and CYP71E1 activity measured by administration of radiolabelled tyrosine to the microsomal system. No accumulation of *p*-hydroxyphenylacetaldoxime is observed demonstrating that all lines possess active CYP71E1. *p*-Hydroxymandelonitrile is labile and is therefore monitored as *p*-hydroxybenzaldehyde. (b) LC-MS analysis of the dhurrin content (RT 5.7 min) shown as total ion traces (top panel) and by specific ion monitoring (lower panel). Traces shown from front to back are two nonmutated parent lines, two *tcd1* siblings, followed by *acdc* mutants.

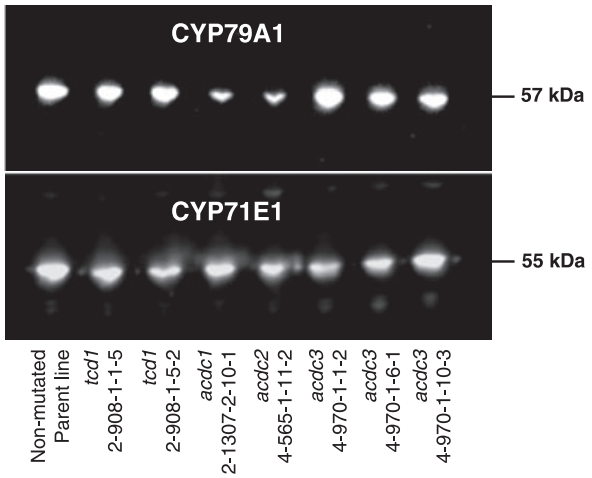

Figure 8 Western blot analysis of CYP79A1 and CYP71E1 in microsomal preparations from the nonmutated parent line and mutant lines.

blot analysis of the CYP79A1 and CYP71E1 content of microsomal preparations isolated from the parent line and from different TILLING lines are shown in Figure 8. CYP79A1 and CYP71E1 proteins were shown to be present in all mutant lines and the content of CYP79A1 and CYP71E1 per seedling did not vary substantially as determined by a semi-quantitative Western blot analysis (Figure 8).

Molecular modelling of mutations in CYP79A1

To gain an understanding of the possible effect of the identified mutations on CYP79A1 activity, a homology model of CYP79A1 was made based on the solved crystal structures of relevant P450s (Figure 9a; Jensen *et al.*, 2011). Two of the mutations (E145K, A154T) are positioned in the proposed substrate-binding site and would directly alter the shape and charge distribution within the tyrosine substrate-binding pocket. The HCNp in both M2 and M4 plants in line 5-84-1 (E145K) was higher than control plants, suggesting that alteration of the negatively charged E residue to a positively charged K residue at position 145 may have the potential to increase substrate affinity. In agreement with this hypothesis, this residue is modelled to interact with the partly negatively charged *p*-hydroxy group of the tyrosine substrate, and the presence of a positively charged K residue in the mutant compared with the negatively charged E residue in the wild-type enzyme may indeed serve to increase substrate affinity. The P414L mutation in *tcd1* is found 10.7 Å away from the substrate-binding site, and consequently, this mutation would not directly interfere with binding of substrate (Figure 9b). However, the P414L mutation is located at the end of helix K (consensus sequence, KETLR), and replacement of this highly conserved P residue (Paquette *et al.*, 2009) with an L so close to this motif is likely to affect enzyme activity. The arginine (R) in the PERF motif (a P450 signature sequence), forms a salt bridge with the glutamic acid (E) and arginine (R) residues in the KETLR motif, and this E–R–R triad is generally thought to lock the haem pocket of the active site into the proper position and to stabilize the overall P450 core structure (Hasemann *et al.*, 1995). The P414L mutation is likely to obstruct this structural organization resulting in overall destabilization and loss of catalytic properties. Two mutations (M98I and E529K) are on the periphery of the enzyme and the enzyme's apparent inability to metabolize tyrosine to *p*-hydroxyphenylacetaldoxime is not easily explained in a structural context. Three additional mutations identified in the screen (R39stop, T48I and P60S) are located in the transmembrane anchor. The transmembrane anchor was not included in the homology model because of the lack of available templates.

Discussion

In this study, a combined biochemical screen and TILLING approach has been used successfully to identify sorghum plants in which cyanogenesis has been substantially altered without using transgenic approaches. We have produced several viable lines, including acyanogenic or low HCNp lines as well as lines that accumulate high HCNp in the adult stages. In addition to being of agronomic value, these lines provide an excellent resource for increasing our understanding of the molecular mechanisms involved in cyanogenesis and factors that affect its regulation. The TILLING population was screened for mutations in two of the three key biosynthetic genes in the cyanogenesis

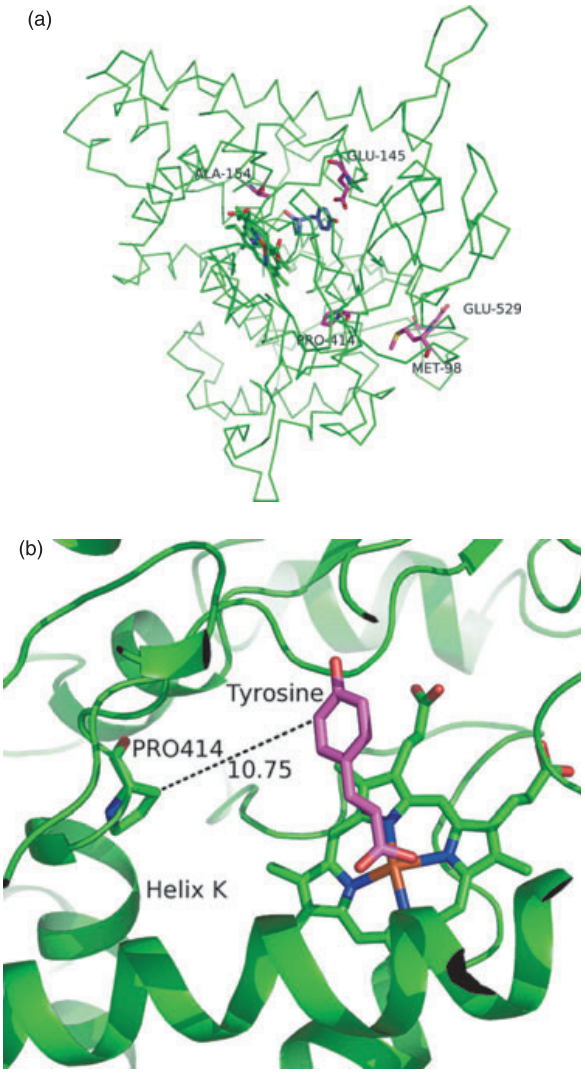

Figure 9 Homology modelling of CYP79A1 based on solved crystal structures of relevant P450s. (a) Molecular model showing the location of five of the identified mutations in CYP79A1. (b) Specific location and details of the P414L mutation in *tcd1*.

pathway, *CYP79A1* and *UGT85B1*. Ten lines that contain mutations in *CYP79A1* were identified. The majority of these lines exhibit lower or negligible HCNp compared with nonmutated parent lines. In contrast, the line 5-84-1 containing the E145K mutation has considerably higher dhurrin content compared with the nonmutant parent lines and will be the subject of further investigations to determine its value as a biofumigant. Analyses of the lines carrying mutations in *UGT85B1* are ongoing. The dhurrin-producing enzymes from sorghum have been expressed in noncyanogenic plants, either individually or in combination (Kristensen *et al.,* 2005), and the results obtained imply that a nonfunctional CYP71E1 protein would result in the accumulation of reactive *p*-hydroxyphenylacetaldoxime (Møller, 2010b). Thus, screening for mutations in *CYP71E1* was not performed as such plants, if viable, would be likely to have reduced fitness (Bak *et al.*, 2006; Morant *et al.*, 2007).

Acyanogenic sorghum has not been detected in natural populations. In some other species, cyanogenic glucosides are synthesized in the shoot and transported to the root and may act as a nitrogen source for root development (Selmar *et al.*, 1988; Jørgensen *et al.*, 2005a). In transgenic cassava plants, created using RNA interference (RNAi) to target CYP79D1 and D2, even a low rate of cyanogenic synthesis in leaves results in transport of substantial amounts of cyanogenic glucoside into tubers. However, in some of the RNAi cassava plants with essentially acyanogenic leaves and a 92% reduction in cyanogenic glucosides in tubers, normal roots and tubers still formed when the plants were grown in soil (Jørgensen *et al.*, 2005a).

The rate of dhurrin synthesis in sorghum is normally high during the first few days post-germination after which rates of degradation exceed those of synthesis (Halkier and Møller, 1989). Catabolism of dhurrin in these early stages of seedling growth leads to the production of ammonia and 4-hydroxyphenylacetic acid (Jenrich *et al.*, 2007). The latter compound, together with other hydroxy- or methoxy-derivatives of phenylacetic acid, was shown to have growth-promoting properties consistent with a role as weak auxins in classical tissue culture experiments (Lee and Skoog, 1965). The observation that *tdc*1 mutants lack dhurrin and show slightly retarded rates of growth when very young may indicate that this cyanogenic glycoside is an important source of growth regulatory metabolites at this critical stage of growth in sorghum. Further experiments to test this hypothesis are underway.

The *tcd1* mutant produces no dhurrin in either the leaves (Figure 7) or the sheath and roots (Table 3). However, growth characteristics of this acyanogenic sorghum *tcd1* mutant, which produces a nonfunctional CYP79A1 protein, is yet to be determined in detail. Under favourable conditions, the growth of the plant is largely unaffected, exhibiting only slightly slower growth in early seedling stages and despite the unusually wet growth conditions experienced during the growing season in 2010 and 2011 in S.E. Queensland, the field grown M5 *tcd1* mutant plants did not show altered susceptibility to insect or fungal attack. Nor do the *tcd1* mutants require additional nitrogen for normal growth and development, as they appear phenotypically similar to parental plants.

The *acdc* mutant lines have a high HCNp in young seedlings and possess a microsomal enzyme system with activity comparable to that of nonmutated parental seedlings yet have a much reduced HCNp in mature leaf tissue compared with control plants. Analysis of the ontogeny of HCNp from seedling stage up to the 4-leaf stage shows that the HCNp drops rapidly in *acdc1* and *acdc3* mutant lines (Figure 6a). The HCNp decrease in *acdc2* is less rapid, but additional experiments demonstrated that the residual HCNp is restricted to the leaf sheath tissue (Table 3) and, like *acdc1* and *acdc3* mutants, is not found in leaf tissue.

Previous studies (Busk and Møller, 2002) indicate that dhurrin biosynthesis in adult plants occurs mainly in the sheath and is upregulated at the transcriptional level (*CYP79A1* and *CYP71E1*) by the addition of exogenous nitrogen. While the *CYP79A1* and *CYP71E1* transcripts in the leaf are easily detectable in the young highly cyanogenic control plants, the transcripts are not detectable by northern analysis in adult leaf tissue in either nonmutated parent plants or any of the mutant lines examined when grown under normal conditions (data not shown). The transcriptional response to increased exogenous nitrogen availability in adult mutant plants is yet to be determined. The Western blot analysis shows the protein levels of CYP79A1 and

CYP71E1 are not altered substantially in the microsomal preparations from young mutant seedlings including the acyanogenic *tcd1* mutant. This suggests the lack of dhurrin production in the *tcd1* mutant has not triggered a compensatory feedback mechanism that is detectable at the protein level.

Dhurrin mobilization and transport in sorghum is not well understood. While the *acdc* mutants produce shoot tissue that is essentially acyanogenic at adult stages, there is no substantial reduction in the dhurrin content in the roots of adult plants (Table 3) compared with levels found in nonmutated parent plants. This may indicate that root accumulation is not dependent on biosynthetic capacity in leaves. While sheath tissue has been identified as the primary site of synthesis in older plants (Busk and Møller, 2002), low levels of *CYP79A1* transcripts have also been observed in sorghum roots (Natalie O' Donnell, unpubl. data), suggesting some synthesis may normally occur in the roots and that this is unaffected in the *acdc* mutants. In cassava, root tissue has also been reported to be able to carry out *de novo* synthesis of cyanogenic glucosides (Du *et al.*, 1995). The low dhurrin levels in the adult leaves of *acdc* mutants could result from mutations in leaf-specific regulatory genes controlling the expression levels of biosynthetic genes and/or degradation pathways and/or alternatively mutations may affect dhurrin transport or storage in adult plants. Because the dhurrin levels are essentially negligible in the leaves and very low in the sheath of these adult plants, a mutation in dhurrin transport seems less likely.

When grown in the field, the *acdc* mutants, as well as the *tcd1* mutant, are as tall or taller than nonmutated parent plants and are leafier. This supports the notion that the lack of dhurrin accumulation does not impede plant growth. The growth/defence nexus assumes that the cost of synthesis of bioactive defence compounds necessitates a trade off between growth and defence (Endara and Coley, 2011). The possible presence of several mutations unrelated to cyanogenesis in the *acdc* mutant plants means that a direct link between low dhurrin accumulation and enhanced growth cannot be made at this stage, but it is noteworthy that the link between the absence of dhurrin in the leaves of adult plants and tall, bushy growth was observed in all three independent *acdc* lines. A similar phenomenon was observed in white clover (Kakes, 1989).

Dhurrin production in sorghum control populations is high during initial germination and seedling growth and then declines as the plant matures (Loyd and Gray, 1970; Wheeler *et al.*, 1990; Busk and Møller, 2002; Møller and Conn, 1980) making it suitable as a highly nutritious forage crop. However, environmental factors, such as drought and high nitrogen, are problematic as they may increase the dhurrin content in adult sorghum plants to toxic levels (Loyd and Gray, 1970; Wheeler *et al.*, 1990). Results from a preliminary drought experiment indicates that dhurrin production is not induced by drought in the shoot tissue of adult *acdc* plants and suggests that dhurrin synthesis or breakdown may be controlled by different developmental and environmental-stress regulatory pathways. The availability of the *acdc* mutants may assist in dissecting the molecular pathways regulating cyanogenesis in response to developmental and environmental signals.

The retention of cyanogenic glucosides throughout young *acdc* mutant seedlings and in the roots of adult plants retains much of the potential protective capacity of cyanogenesis towards crop pests. There are a number of serious fungal diseases in sorghum (Gupta and Paul, 2002). Recent reports suggest that the stoichiometric release of HCN during fungal-induced ethylene production in plants helps retard fungal growth independently of the ethylene-induced responses (Seo *et al.*, 2011). ß-Glucosidase and hydroxynitrile lyase–mediated release of HCN from dhurrin stored in the vacuole following cellular disruption and CYP79A1-catalysed oxime release induced by the oxygen burst associated with the hypersensitive response may potentially inhibit the growth of pathogenic fungi (Møller, 2010a). However, rubber tree (*Hevea brasiliensis*) varieties susceptible to the fungus South American Leaf Blight had a high HCNp, while resistant varieties had low HCNp (Lieberei, 2007). Interestingly, some of the high HCNp mutants identified in the sorghum TILLING population appeared more susceptible to fungal attack than low HCNp mutants. The susceptibility could result from specialised fungal pathogens ability to detoxify HCN (Møller, 2010b). Trials are underway to test the fitness of the selected mutant plants in the field.

To benefit the agricultural industry, the desired characteristic of these forage sorghum plants, lacking the capacity to produce dhurrin, now needs to be introgressed into commercial elite hybrids. Utilization of the mutated *CYP79A1* gene in hybrids will involve a process of crossing the elite mutants with appropriate commercial male and female forage sorghum parent lines. The development of each new parent will involve pedigree crossing and assaying individual plants in the F_2 generation for the absence of HCNp. In addition, selection for broader morphological characteristics will be required.

Experimental procedures

Plant material

The mutant population was generated using a near-isogenic *S. bicolor* (L.) Moench inbred parent line (Pacific Seeds, Toowoomba, Qld, Australia). Seeds were treated with 0.15%–0.4% EMS for 16 h in batches of approximately 1000 seed/500 mL with shaking (approximately 50 r.p.m.), then rinsed thoroughly with water. A total of 53 000 treated seeds were sown in the field at the Pacific Seeds Research Station (Gatton, Queensland). The site was prepared by deep ripping the paddock twice, 3–4 months before planting and spraying with Roundup (Monsanto, http://www.monsanto.com.au). A complete fertilizer [120 kg/ha of Urea (46% N) and 120 kg/ha of CK-88 (15.1% N; 4.4% P; 11.5% K; 13.6% S)] was applied 6 weeks before planting and additional nitrogen (40 kg/ha urea) supplied 5 weeks postplanting. Weeds were controlled with Atrazine (3 L/ha; Syngenta, http://www.atrazine.com) before planting and again after 4 weeks (1 L/ha) and with inter-row cultivation.

To ensure self-pollination, each panicle was bagged prior to anthesis. Panicles were harvested and threshed individually, resulting in 4185 individual M2 selections. Up to five seeds from each M2 line were sown in the field, resulting in 5451 individual M2 plants derived from 2709 independent M2 lines. These plants were screened phenotypically for cyanide levels using Feigl–Anger (FA) paper when approximately 8 weeks old. The main panicle of all M2 plants was bagged and threshed individually. Selected M3 mutant lines were sown in the field, whereas lines of the M4 generation were planted in a greenhouse at Pacific Seeds (Toowoomba) to facilitate growth over the winter. The main panicle for all plants of the

M3 and M4 generations were also bagged to allow generation of homozygous mutants. All seed was stored or used for propagation purposes. Numbering of individual mutant lines allows the identification of the ancestry of each plant and easy tracking through every generation. For example, in 2-908-1-1-5, the first number represents the second EMS seed treatment batch, the 908 is the M1 reference number and each subsequent number identifies the plant selected in the following generations.

Assays of the cyanide content of mutant lines

Feigl–Anger (FA) papers (Feigl and Anger, 1966; Miller *et al.*, 2006) were used to identify mutants with altered HCNp in all M2 plants (approximately 6100) (Gleadow *et al.*, 2010; Takos *et al.*, 2010). Three leaf discs (approximately 0.5 cm diameter) were taken from the youngest fully unfurled leaf of 8-week-old plants and placed in a single well of a 96-well plate, covered with a sheet of FA paper and sealed. Every plate included samples from eight nonmutated parent plants as controls. The sealed plate was frozen and thawed to lyse the cells and incubated at 25 °C for approximately 30 min, allowing HCN to evolve and form a concentration-dependent blue spot above each well (Miller *et al.*, 2006; Takos *et al.*, 2010). FA papers were photographed as a permanent record.

Based on the FA paper screening, 264 individual M2 plants showing substantial differences (either low or high) in HCNp compared with nonmutated parent plants were selected for quantitative HCNp analysis using a colorimetric method (Gleadow *et al.*, 2010). The youngest fully unfurled leaf was sampled from approximately half of the M2 plants at approximately 8 weeks old, while the rest were sampled at approximately 16 weeks. Previous experiments have shown that dhurrin can be quantified accurately from dried material (Haskins *et al.*, 1984). Therefore, dried, ground tissue (approximately 10 mg) was placed in a vial containing 300 μL of 0.1 M citrate buffer (pH 5.5) with 1.12 units/mL β-D-glucoside glucohydrolase (EC 3.2.1.21). A PCR tube containing 200 μL of 1 M NaOH was placed in the vial, which was then sealed and incubated (1 h at RT followed by 15 h, at 37 °C). The NaCN content of the NaOH trap was quantified colorimetrically using NaCN as the standard (Gleadow *et al.*, 2010).

Genomic DNA extraction

Genomic DNA from the selected 264 M2 plants and nonmutated parent plants was isolated using the MagAttract DNA extraction kit (Qiagen, http://www.qiagen.com). Multiple plates, containing 100 ng DNA aliquots in individual wells were prepared to enable the analysis by PCR and TILLING using primers specific to *CYP79A1* and *UGT85B1*.

Detection of mutations in the target genes

Nested PCR with M13 tailed primers (Table S1) was used to amplify the 5′ and 3′ region of *CYP79A1* and *UGT85B1* genes (Genebank accession numbers U32624 and AF199453, respectively). All PCR reaction mixtures (20 μL) contained 0.5 U *Pfu* DNA polymerase (Promega, http://www.promega.com) in buffer containing 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM gene specific forward and reverse primers. Following the first PCR, the products were pooled threefold in a matrix designed to allow identification of individuals, and re-amplified using a combination of 0.2 μM M13 tailed gene specific nested primers and 1 μM of the corresponding forward and reverse M13 primers fluorescently labelled with IR dye 700 and 800 nm, respectively (Eurofins MWG operon, http://www.eurofinsdna.com), to allow visualization following gel separation on the LiCor 4200 (Table S1). The thermocycling conditions are detailed in Table S1.

Following the second PCR, the amplified products were denatured and re-annealed (99 °C—10 min; 70 °C—20 s, −0.3 °C/cycle × 70 cycles; 48 °C—20 s, −0.3 °C/cycle × 50; 4 °C hold) to form heteroduplex DNA. Mutations were detected by digestion of the heteroduplexed DNA using CEL1 enzyme (Oleykowski *et al.*, 1998). Cleaved products were visualized by electrophoresis on the Li-Cor 4200 DNA Analyser (Li-Cor Bioscience, http://biosupport.licor.com). GelBuddy (http://www.gelbuddy.org; Zerr and Henikoff, 2005) was used to analyse the gels and identify the mutations in the M2 individual.

Sequencing of cyanide-deficient mutants

The CYP79A1 gene PCR products were amplified from mutant genomic DNA and purified using the Promega Wizard kit (Promega, http://www.promega.com) with an added final ethanol precipitation step to optimize DNA purity and sequenced using the Applied Biosystems PRISM BigDye Terminator Mix(Applied Biosystems, http://www.appliedbiosystems.com.au/).

Preparation of microsomes

Seventy seeds of *S. bicolor* parent lines and of selected TILLING lines were germinated in fine grade vermiculite in the dark (4 days at 23 °C). Microsomes were isolated from approximately 0.5 g of seedling material. Each class of seedlings was harvested separately, counted to correct for small differences in germination percentage, weighed and ground in a chilled mortar with 600 mg polyvinylpolypyrrolidone in 8 mL isolation buffer [250 mM sucrose, 100 mM Tricine (pH 7.9), 50 mM NaCl, 2 mM DTT]. The homogenate was filtered through a nylon cloth (22 μm mesh) and centrifuged (10 min, 10 000 ***g***). Supernatant was centrifuged (1 h, 100 000 ***g***) and the microsomal pellet resuspended in 8 mL isolation buffer using a tiny marten paintbrush to wash away soluble substrates and cofactors bound to the microsomal membranes. Following re-centrifugation as described earlier, the microsomal pellet was resuspended in 120 μL resuspension buffer [50 mM (pH 7.9), 20 mM NaCl, 2 mM DTT] and final volume was adjusted so that 3.6 μL of the microsomal extract corresponded to the amount of microsomal protein isolated from a single seedling.

Analysis of NADPH-cytochrome P450 oxidoreductase activity

The activity of NADPH-cytochrome P450 oxidoreductase (CPR) in each of the microsomal preparations was measured by its ability to reduce cytochrome *c* (Horseheart cytochrome *c*, Sigma, cat no 7752; http://www.sigmaaldrich.com) as monitored spectrometrically at 550 nm. The sample cuvette (1 mL) contained 50 μL 1 mM cytochrome *c* and 5 μL microsomal membrane preparation in 50 mM KP_i (pH 7.9). Following monitoring of the background reduction rate for 3 min, 10 μL of 62 mM NADPH was added and the initial rate of cytochrome *c* reduction was measured. No NADPH was added to the reference cuvette. In the assay conditions used, 1 nmol of CPR will reduce about 3000 nmol of cytochrome *c* per min enabling quantification of the activity in each sorghum seedling (Guengerich *et al.*, 2009).

Analysis of CYP79A1 activity

The activity of CYP79A1 in the microsomal membrane preparations was assayed in 20.2-μL reaction mixtures containing 2.5 μL L-[UL-^{14}C]-tyrosine (482 mCi/mmol), 2.5 μL 8 mM NADPH and 5 μL resuspension buffer. The reaction was initiated by addition of 7.2 μL microsomes. Following the incubation period (30 min, 30 °C), the entire reaction mixture was applied to silica gel 60F254 TLC plates (Merck, http://www.merck.dk). Following development in ethyl acetate: toluene (1 : 5 v/v), the formation of radiolabelled dhurrin pathway intermediates was monitored and quantified by phosphor imaging (Typhoon; GE Healthcare, http://www.gelifesciences.com). In a parallel set of experiments, the incubation mixtures were fortified with 2.5 μL 1 mM L-tyrosine to saturate the enzyme system with substrate over the entire incubation period. The position of the different intermediates on the TLC plates was determined by co-application of unlabelled reference compounds and monitored by UV absorption.

Quantification of dhurrin content in dark grown seedlings

Ten 4-day-old dark grown seedlings of the nonmutated parent line and each of the four selected mutant TILLING lines were weighed and homogenized in 600 μL 85% MeOH in a microcentrifuge tube using a pointed pestle. Following extraction (4 h, RT, gentle shaking), the homogenate was centrifuged (10 min, 3000 ***g***). Aliquots (20 μL) were diluted with 60 μL H_2O and filtered through a membrane by centrifugation. Dhurrin content was determined by injection of 0.1 and 2 μL aliquots in the LC-MS and analysed as outlined later.

LC-MS experimental data

Analytical LC-MS was carried out using an Agilent 1100 Series LC (Agilent Technologies, http://www.home.agilent.com) hyphenated to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, http://www.bdal.com/). A Zorbax SB-C18 column (Agilent; 1.8 μM, 2.1 × 50 mm) was used at a flow rate of 0.2 mL/min, and the oven temperature was maintained at 35°. The mobile phases were the following: A, water with 0.1% (v/v) HCOOH and 50 μM NaCl; B, acetonitrile with 0.1% (v/v) HCOOH. The following gradient program was used: 0–0.5 min, isocratic 2% B; 0.5–7.5 min, linear gradient 2%–40% B; 7.5–8.5 min, linear gradient 40%–90% B; 8.5–11.5 isocratic 90% B; 11.60–17 min, isocratic 2% B. The flow rate was increased to 0.3 mL/min in the interval 11.2–13.5 min. The mass spectrometer was run in positive electrospray mode. Data was analysed using the Bruker Daltonics Data Analysis software with comparison to dhurrin standards.

Immunoblotting

CYP79A1 specific antibodies were obtained following immunization of rabbits using a 14-residue peptide, AGVEAVDLSESKSD (residues 520–533). In a similar manner, specific antibodies towards CYP71E1 were obtained using the 12-mer peptide, VVPTKYKNRRAA (residues 520–531). Microsomal preparations (2.4 μL) corresponding to the amount of microsomal protein isolated from 0.67 seedlings were electrophoresed on a 12% SDS-PAGE gels in MOPS buffer for 1 h at 200 V. The separated proteins were transferred to nitrocellulose membranes (45 min at 100 V) and the membranes blocked (1 h at room temperature) in 5% skim milk powder solution (PBS-T), washed and the immunoreactions carried out in 5% skim milk powder solution (PBS-T) using either antibodies to CYP79A1 at 1 : 2000 dilution or to CYP71E1 at 1 : 5000 dilution (1 h at room temperature). The blot was incubated with secondary antibody for 1 h at room temperature at a dilution of 1 : 2000.

Homology modelling of mutations affecting CYP79A1

The protein model of CYP79A1 was built using the Orchestra protein modelling component of the Sybyl software (Tripos, http://tripos.com/) using coordinates for the solved crystal structures of relevant CYPs (Jensen *et al.*, 2011). Structural models and position of mutations were visualized using PYMOL (http://www.pymol.org). APBS was used for the calculation and visualization of the electrostatic potential (Baker *et al.*, 2001). The final structural model included 489 residues thus missing the N-terminal residues 1–69.

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Author contributions

The project was initiated by RMG; strategic approach and experimental planning by ADN, RMG, JDH, BLM and CKB. All authors contributed to experimental and/or field work. All authors provided intellectual input into the writing and preparation of this manuscript.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Quantifiable HCNp Assay of the 264 selected M2 plants.

Figure S2 Sequence chromatograms of three 2-908-1 M4 siblings showing a homozygous wild-type line, a heterozygous line and a homozygous P414L line.

Table S1 Sequence of the primers and thermocycling conditions used for the PCR in the TILLING analysis.

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Research Article

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Drying and processing protocols affect the quantification of cyanogenic glucosides in forage sorghum

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Abstract

BACKGROUND: Cyanogenic glucosides are common bioactive products that break down to release toxic hydrogen cyanide (HCN) when combined with specific β-glucosidases. In forage sorghum, high concentrations of the cyanogenic glucoside dhurrin lead to reduced productivity and sometimes death of grazing animals, especially in times of drought, when the dhurrin content of stunted crops is often higher. The aim of this study was to develop harvesting protocols suitable for sampling in remote areas.

RESULTS: Dhurrin concentration in air- and oven-dried leaves was the same as in fresh leaves, with no subsequent losses during storage. Dhurrin concentration was halved when leaves were freeze-dried, although activity of the endogenous dhurrinase was preserved. Direct measurement of dhurrin concentration in methanolic extracts using liquid chromatography/mass spectrometry (LC/MS) gave similar results to methods that captured evolved cyanide. A single freezing event was as effective as fine grinding in facilitating complete conversion of dhurrin to cyanide.

CONCLUSION: Direct measurement of dhurrin using LC/MS is accurate but expensive and not appropriate for fieldwork. Air drying provides an accurate, low-cost method for preparing tissue for dhurrin analysis, so long as the specific β-glucosidase is added. It is recommended that comparative studies like the one presented here be extended to other cyanogenic species.

Keywords: *Sorghum bicolor*; method; cyanogenic glucoside; dhurrin; forage sorghum; freeze-drying; β-glucosidase; cyanide; HCN

INTRODUCTION

Cyanogenic glucosides are bioactive compounds that break down to release toxic hydrogen cyanide (HCN), primarily as a defence against herbivores.[1,2] The targeted delivery of HCN is controlled by the spatial separation within the plant of the glucoside and specific degradative β-glucosidases.[3] The two are only mixed when tissues are macerated by a chewing herbivore[1] or when physically damaged such as by freezing.[4] Many crop plants are cyanogenic, including ones of global importance such as clover, sorghum and cassava.[5,6] Since the ability of herbivores (including humans) to tolerate HCN depends in part on the dose, as well as the rate of consumption, it is important to know whether the concentration is less than any recommended threshold toxicity.[1,7,8]

Forage sorghum (*Sorghum bicolor* subsp. *bicolor* (L.) Moench × *S. bicolor* subsp. *drummondii* Stapf., Poaceae), an important crop for feeding livestock, contains the cyanogenic glucoside dhurrin ((*S*)-4-hydroxymandelnitrile-β-D-glucopyranoside). There is a risk of poisoning of livestock if the effective HCN concentration is above 600 mg kg^{-1} on a dry matter basis.[9] Dhurrin concentration is highest in very young plants, so the crop is only grazed after plants reach the five-leaf stage.[10] The concentration increases again when plants are drought-stressed, leading to uncertainty about the suitability of the crop for grazing during dry weather.[9,10] Farmers can send plant material away for analysis, but doubts have been raised about how much dhurrin is lost in the time between harvesting and testing. There is, therefore, a need for a simple, reliable method for harvesting and processing plant samples that minimises HCN losses prior to analysis.

The effectiveness of the most commonly practised methods for preparing plant tissue for cyanogenic glucoside analysis was evaluated using field- and glasshouse-grown forage sorghum. Dhurrin concentration in fresh, frozen and air-dried leaves was compared with the commonly used laboratory method of freeze-drying.[11–14] Harvesting protocols were validated using other tissues. Concentrations of cyanogenic glucosides can be

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measured by incubating ground leaves with the appropriate β-glucosidase and collecting the evolved cyanide.[15] Here we optimised the use of freezing and thawing as an alternative to grinding to disrupt the cells. Results were then compared with the direct measurement of dhurrin on the same tissues using liquid chromatography/mass spectrometry (LC/MS). To our knowledge, no systematic comparison of these different pre-analysis protocols has been published before.

EXPERIMENTAL

Analysis of cyanogenic glucosides as evolved HCN

Cyanogenic glucosides were determined by measuring evolved HCN following the method of Gleadow and co-workers[15,16] unless specified otherwise. In brief, samples were weighed into vials (300 mm × 10 mm diameter) containing 0.5 mL of 0.1 mol L^{-1} citrate buffer (pH 5.5) alone or citrate buffer plus almond emulsin (0.1 g L^{-1}; β-D-glucoside glucohydrolase, EC 3.2.1.21, Sigma, Sigma-Aldrich, Sydney, Australia). A microcentrifuge tube containing 0.2 mL of 1 mol L^{-1} NaOH was inserted and the apparatus was incubated for 16 h (1 h at ∼22 °C, then 15 h at 37 °C). HCN trapped in the NaOH inner tube was neutralised with 1 mol L^{-1} acetic acid and assayed colorimetrically by using König reactions and reading the absorbance at 595 nm, with NaCN standards.[16] All data are presented on a dry weight (DW) basis. Where whole fresh tissue was used (e.g. in the freezing protocols), it was removed after incubation, rinsed in distilled water and dried overnight at 60 °C and weighed.[17] All assays were done in triplicate unless stated otherwise. Previous experiments have shown that no HCN is evolved from cyanogenic glucosides in the absence of specific degradative β-glucosidases.[3] By testing with and without almond emulsin, we could assess the impact of the various drying methods on the endogenous dhurrinase activity.

Analysis of cyanogenic glucosides using LC/MS

Dried, ground plant material (20 mg) was boiled in 500 µL of 850 mL L^{-1} methanol for 3 min, cooled and centrifuged at 10 000 × *g* for 3 min.[15,18] The supernatant was collected, diluted to 200 mL L^{-1} methanol and filtered through a 0.45 µm filter membrane by centrifuging at 3000 × *g* for 5 min. Analytical LC/MS was carried out using an Agilent 1100 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Extracts (20 µL) were injected onto a Zorbax SB-C18 column (1.8 µm particles, 2.1 mm × 50 mm; Agilent Technologies). The mobile phases were (A) 1 mL L^{-1} HCO_2H and 50 µmol L^{-1} NaCl and (B) CH_3CN with 1 mL L^{-1} HCO_2H. The gradient programme was as follows: 0–0.5 min, isocratic 2% B; 0.5–7.5 min, linear 2–40% B; 7.5–8.5 min, linear 40–90% B; 8.5–11.5, isocratic 90% B; 11.6–15 min, isocratic 2% B. The flow rate was 0.2 mL min^{-1}, increasing to 0.3 mL min^{-1} during the 11.2–13.5 min interval. The mass spectrometer was run in positive electrospray mode, and cyanogenic glucosides were detected as Na^+ adducts. Spectra were compared with dhurrin standards using Bruker Daltonics Data Analysis software, with dhurrin eluting at 5.7 min.[18]

Optimisation of freeze/thaw method

In order to ascertain the optimal number of freeze/thaw cycles required to fully disrupt the tissue, we froze and re-thawed leaf tissue from sorghum up to five times. Leaves were sampled from single 5-week-old plants grown in naturally lit greenhouses supplemented by a combination of Grow-lux T12 fluorescent lamps (Sylvania Company, USA) and cool-white lights at Monash University, Melbourne, Australia (28 °C day/22 °C night and 14 h day/10 h night).[19] Three leaf discs (diameter 9 mm) were excised from the lamina of the middle three leaves (avoiding the midrib and staying to the middle quadrant of the leaf) and immediately placed in a small vial (fresh weight ∼0.017 g). Buffer containing β-glucosidase was added (0.5 mL) to the vial. Vials ($N = 6$) were placed on dry ice until completely frozen (30 min) and then thawed at room temperature (1 h) zero to five times. Vials were incubated overnight and liberated HCN was determined colorimetrically (as above).

Comparison of drying methods

Leaves were collected from plants growing in the ground in a plastic house in Toowoomba, Australia (Pacific Seeds Pty. Ltd). The first two fully expanded leaves were sampled before dawn in March 2007 from adjacent 8-week-old plants. Whole leaves were sealed in plastic ziplock bags, placed on ice and freighted to Melbourne for analysis (total travel time 8 h). The tip and base of each leaf were discarded (∼100 mm) to minimise within-leaf variation.[10] Leaves were then cut into strips (30 mm wide × 50 mm long) and mixed together in a beaker held on ice, but not frozen. This resulted in a large, homogeneous sample of leaves that could be used to compare the different methods. Pooled leaves were subsampled to provide four replicate samples for each of the following seven processing regimes ($N = 4$): F, analysed fresh; FD1, snap frozen in liquid N_2 for 2 min and then lyphopilised by freeze-drying for 48 h; FD2, frozen at −20 °C overnight and then lyphopilised by freeze-drying for 48 h; FD3, frozen at −20 °C overnight, transferred to liquid N_2 for 2 min and then freeze-dried for 48 h; OD1, immediately oven dried at 60 °C for 48 h; OD2, kept at room temperature overnight and then oven dried at 60 °C for 48 h; RT, air dried in paper bags in the dark at room temperature for 7 days.[19] Fresh samples were ground in liquid N_2 and then quickly transferred to preweighed vials and reweighed. Dried samples were ground in an Ultra Dental Mix (Southern Dental Instruments, Melbourne, Australia).[16] Dried, ground leaves (FD1, FD2, FD3, OD1, OD2, RT) were analysed soon after grinding either with or without addition of almond emulsin in the extraction buffer. Material was retested after storage in a desiccator at room temperature for 2 weeks and 12 months.

Comparison of different tissue types

We tested whether the pre-analysis methods described above were equally applicable to different tissues. The three treatments were: Fr1, segments (discs) of tissue disrupted only through freezing and thawing; Fr2, pieces of tissue ground in liquid N_2; OD, pieces of tissue oven dried (50 °C for 48 h) and then ground in liquid N_2. Three leaves from each of four plants at the five-leaf stage ($N = 4$), grown in pots in a greenhouse at Monash University, were sampled.[19] Three leaf discs were removed from the middle of each leaf blade (Fr1). An adjacent segment (∼30 mm long) was divided into blade and midrib segments (∼30 mm long). Each was further divided into two and analysed immediately (fresh, Fr2) or dried prior to analysis (OD, as above). 'Stem' was defined as the compressed tissue between the soil and the apical meristem (∼20 mm high, ∼5 mm diameter), which anatomically contains both true stem and leaf sheaths. Transverse sections of stem (2 mm thick, $N = 4$) were cut from each plant, and the segments were analysed immediately by freezing/thawing (Fr1), snap frozen in liquid N_2 (Fr2) or oven dried (OD). In all cases,

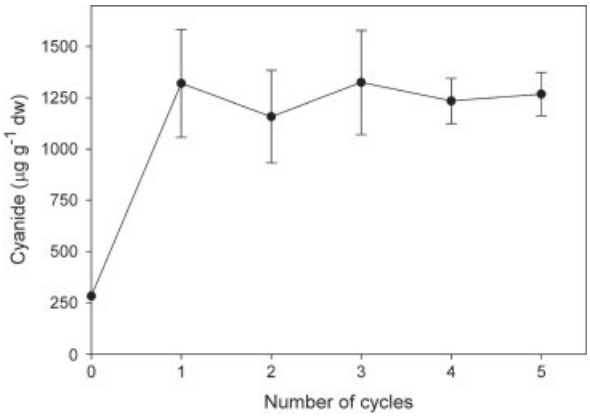

Figure 1. Concentration of HCN in leaf lamina of sorghum measured as evolved HCN using leaf discs ($N = 4$). Discs were frozen and re-thawed between zero (no freezing) and five times in a sealed chamber to disrupt the cells, allowing dhurrin and the endogenous β-glucosidase (dhurrinase) to mix.

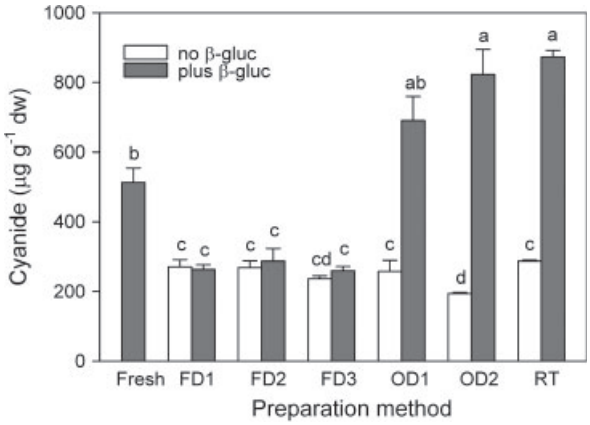

Figure 2. Concentration of HCN released from field-grown leaves of forage sorghum. Leaves ($N = 5$) were collected in the field and transported for analysis. Leaves were either analysed fresh, dried at room temperature (RT), freeze-dried (FD) or oven dried (OD). Bars with the same letter are not significantly different using *post hoc* Tukey's *t* tests ($P < 0.05$).

tissue was incubated together with almond emulsin, and dhurrin concentration was determined by evolved HCN.

Comparison of LC/MS and HCN methods

The absolute concentration of dhurrin in oven-dried sorghum leaves was measured directly using LC/MS and compared with the indirect HCN evolution method. Leaves of *S. bicolor* L. grown in a field trial at USDA, Arizona, USA were collected, dried at 70 °C and stored in paper bags.[20] Data presented here are from plants grown in 1998 and 1999 and harvested at the seven- and 14-leaf stages. Dried, ground tissue was either weighed into sealed vials and frozen/thawed twice to release HCN in the presence of almond emulsin or extracted using hot methanol and measured using LC/MS.

Statistical analysis

Data were tested for normality and homogeneity of variances and then subjected to two-way analysis of variance using the General Linear Models procedure of Minitab 15®, Minitab Ltd, Coventry, U.K. Means that were significantly different were compared *post hoc* using Tukey's *t* tests. Correlation coefficients and regression equations were calculated using SigmaPlot11®, Systat Software Inc. San Jose, CA, USA.

RESULTS AND DISCUSSION

Assessment of freeze/thaw method of tissue disruption

We found freezing and thawing tissue to be a very effective mechanism for disrupting cells and releasing HCN. Takos *et al.*[21] used three cycles of freezing and thawing to macerate *Lotus corniculatus* L. leaves. In the present study a single freezing event was shown to be enough to allow all the dhurrin to be degraded, with no further increase in HCN detected with repeated freeze/thaw cycles (Fig. 1). The small amount of HCN released from the intact tissue (time 0) could be from the cut edges. It seems likely that significant losses of HCN could occur when tissue that is frozen for transporting partially thaws prior to analysis.

Comparison of drying methods using whole leaves

We compared the amount of HCN released from whole sorghum leaves that had been collected in the field and transported to the laboratory for analysis 8 h later using the different processing protocols. The measured concentrations for the methods ranged widely from 194 to 874 µg g^{-1} DW (Fig. 2). HCN concentration in freeze-dried leaves (FD1, FD2, FD3) was only half of that measured in fresh, air-dried (RT) or oven-dried (OD1, OD2) tissue when almond emulsin was added (Fig. 2). It did not make any difference to the measured concentration if samples were stored at −20 or −196 °C (liquid N_2) before freeze-drying (FD1, FD2, FD3). HCN concentration was not significantly different in leaf tissue that was analysed immediately after drying compared with tissue that had been dried and then stored in a desiccator for up to 12 months. HCN concentration in fresh tissue samples was not significantly different from that in OD leaves, but the concentration in RT leaves was higher (Fig. 2).

Less than a third of the amount of HCN was recovered from OD and RT dried tissue when no β-glucosidase was added (Fig. 2), indicating that the enzyme was at least partially denatured during drying. Torres *et al.*[22] reported significant losses of cyanogenic glucosides in leaves of cassava (*Manihot esculenta* Cranz.) that had been dried at 80 °C and then ground, compared with grinding fresh leaves in buffer at room temperature. Since the exogenous β-glucosidase (linamarase) in cassava would have been denatured at this temperature and since the authors did not add any exogenous degradative enzymes, the observed loss of cyanogenic glucosides is likely to be a consequence of the loss of β-glucosidase activity. By contrast, in this study, leaves dried at room temperature retained some β-glucosidase activity, consistent with reports of positive tests from dried herbarium specimens.[23] In a study of *Olinia ventosa* (L.) Cuf., significant amounts of the cyanogenic glucoside prunasin were degraded in leaves when they were dried at 30 °C.[24] A possible explanation for this quite different result for *O. ventosa* could be that, in sorghum, dhurrin and dhurrinase are spatially separated in different tissues in addition to the usual subcellular localisation,[25] reducing the chance of opportunistic mixing.

HCN concentration was consistently lower in tissues that had been freeze-dried compared with other pre-analysis processing methods. This was true whether or not exogenous β-glucosidase had been added (Fig. 2), indicating that activity of the dhurrinase was preserved by freeze-drying. Freeze-drying is considered a safe protocol for the preparation of tissue for a range of secondary metabolites and is widely used in cyanogenic glucoside

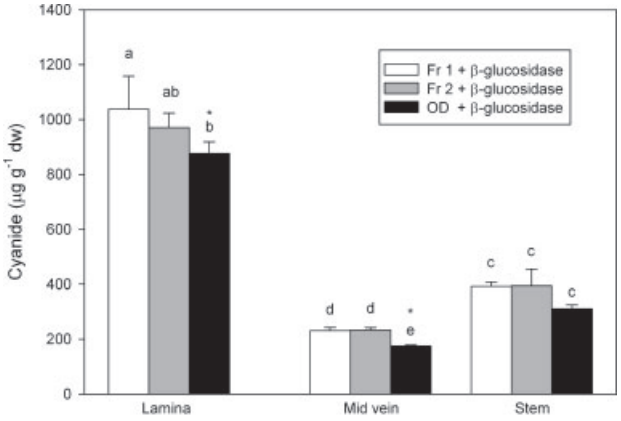

Figure 3. Concentration of HCN released from leaves and stems of sorghum plants ($N = 4$) grown in a glasshouse. Tissue was analysed fresh (Fr 1, discs; Fr 2, segments) or oven-dried (OD, segments only). Bars with the same letter are not significantly different ($P < 0.05$).

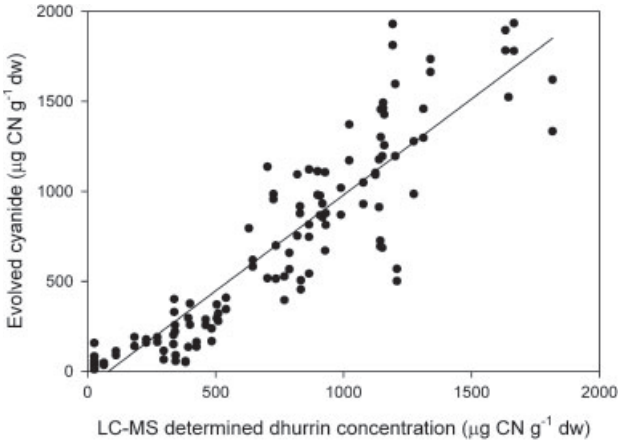

Figure 4. Concentration of dhurrin in oven-dried samples of field-grown *Sorghum bicolor* measured directly using LC/MS and using evolved HCN method ($N = 127$). Exogenous β-glucosidase was added to the latter to ensure conversion of dhurrin to HCN. For comparison, dhurrin is expressed as cyanide equivalents. The regression line is significant and near unity ($y = 1.066x - 84.7, R^2 = 0.85, P < 0.001$).

analysis.[12,17,26–29] Data presented here suggest that caution should be taken when interpreting results for cyanogenic glucosides until sample preparation tests similar to those described here have been done for other species. The reason for the low concentration of dhurrin in freeze-dried material is not known, but the disruption of cells during freezing and the epidermal location of dhurrin in sorghum[25] could be factors. Any thawing of the plant tissue subsequent to freezing would also cause dhurrin to be catabolised to HCN, which would readily dissipate. Thawing could potentially occur in the initial stage of freeze-drying or during the grinding procedure. The catabolic conversion of dhurrin to HCN is very fast[3,30] and it is difficult to completely eliminate losses when weighing out frozen tissue when the thermal mass is low, as is the case with small leaf samples.

Extrapolation to other plant tissues and sampling sizes

In order to test whether freezing/thawing of small samples was representative of larger sampling methods, we compared the recovery of HCN from different tissues (leaf lamina, midrib, stem) that were sliced into discs with the recovery from larger samples that had been ground. There are three points to be made from Fig. 3. First, there was no significant difference in HCN concentration between fresh material that had been analysed using freezing and thawing of discs/slices and the larger samples that had been ground immediately in liquid N_2. Second, there were significant differences in dhurrin concentration in the three plant tissue parts. Third, the effect of oven drying was not the same in all tissues (i.e. the tissue $\times$ treatment interaction was significant, $P < 0.05$). Often only small samples can be taken from living plants, either because they are rare[27] or because the plants themselves are small. Data presented here highlight the importance of sampling in a consistent manner, because whole leaves (e.g. including the midrib) will likely return a lower concentration of cyanogenic glucosides than sampling methods that are restricted to the lamina.

Comparison of LC/MS and evolved HCN methods

In order to test whether the recovery of HCN from lysed cells in OD plants was a true reflection of the dhurrin concentration, we compared the direct determination of dhurrin in methanolic extracts using LC/MS and the amount of HCN released using field-grown *S. bicolor* subsp. *bicolor* (L.) Moench that had been dried and stored for 10 years.[20] There was an almost one-to-one correlation between the two methods, with a regression equation of $y = 1.066x - 84.7$ ($R^2 = 0.85$, $P < 0.001$; Fig 4). The high degree of concordance (Pearson's coefficient of 0.91, $P < 0.001$) strongly supports the contention that methods that rely on evolved HCN can be used to accurately measure cyanogenic glucoside concentration, even where tissue has been stored for long periods.

CONCLUSIONS

Assessment of the impact of environmental variables such as drought, nitrogen and CO_2 on cyanogenic glucosides is contingent on an accurate determination of concentration. Each preparation method tested here has advantages and limitations, thus the choice will ultimately depend on constraints surrounding the collection, transportation and processing of the material. Drying at room temperature or in ovens is an attractive option for preparing sorghum for cyanogenic glucoside analysis and is suitable for sampling in remote locations. Whole leaves, or even entire plants, can be harvested, dried, ground and homogenised. Dried material can then be transported and analysed at a later date.

Direct measurement of cyanogenic glucosides does not depend on intermediate enzymatic steps. Here we show that methods that rely on trapping evolved HCN are highly correlated with the direct measurements. The advantage of using the trapping method is that it is simple, fast, accurate and relatively cheap. Freezing/thawing is an effective way of ensuring that cellular disruption is complete.

In conclusion, based on data presented here for sorghum, analysis of freeze-dried plant tissue leads to a significant underestimation of dhurrin concentration. It is important that tissue preparation methods now be tested for other species. If similar discrepancies are found, then estimates of the effect of drought, nitrogen and elevated CO_2 on HCN toxicity may need to be recalculated where measurements were made on freeze-dried tissue.

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Article

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Estimating Hydrogen Cyanide in Forage Sorghum (*Sorghum bicolor*) by Near-Infrared Spectroscopy

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ABSTRACT: Hydrogen cyanide (HCN) is a toxic chemical that can potentially cause mild to severe reactions in animals when grazing forage sorghum. Developing technologies to monitor the level of HCN in the growing crop would benefit graziers, so that they can move cattle into paddocks with acceptable levels of HCN. In this study, we developed near-infrared spectroscopy (NIRS) calibrations to estimate HCN in forage sorghum and hay. The full spectral NIRS range (400−2498 nm) was used as well as specific spectral ranges within the full spectral range, i.e., visible (400−750 nm), shortwave (800−1100 nm) and near-infrared (NIR) (1100−2498 nm). Using the full spectrum approach and partial least-squares (PLS), the calibration produced a coefficient of determination (R^2) = 0.838 and standard error of cross-validation (SECV) = 0.040%, while the validation set had a R^2 = 0.824 with a low standard error of prediction (SEP = 0.047%). When using a multiple linear regression (MLR) approach, the best model (NIR spectra) produced a R^2 = 0.847 and standard error of calibration (SEC) = 0.050% and a R^2 = 0.829 and SEP = 0.057% for the validation set. The MLR models built from these spectral regions all used nine wavelengths. Two specific wavelengths 2034 and 2458 nm were of interest, with the former associated with C═O carbonyl stretch and the latter associated with C−N−C stretching. The most accurate PLS and MLR models produced a ratio of standard error of prediction to standard deviation of 3.4 and 3.0, respectively, suggesting that the calibrations could be used for screening breeding material. The results indicated that it should be feasible to develop calibrations using PLS or MLR models for a number of users, including breeding programs to screen for genotypes with low HCN, as well as graziers to monitor crop status to help with grazing efficiency.

KEYWORDS: *Dhurrin, feed quality, hydrogen cyanide, NIRS, wavelengths*

INTRODUCTION

Cyanogenesis is the process whereby plants release hydrogen cyanide (HCN) from *in situ* cyanide-containing compounds. While cyanogenic glycosides are nontoxic, in the presence of certain enzymes, these compounds are hydrolyzed to produce HCN, which is highly toxic. The development of HCN may play a role in plant defense against herbivores.[1] In sorghum, the cyanogenic glycoside dhurrin is synthesized from the amino acid tyrosine in a series of steps catalyzed by two P450s and a UGT-transferase. Dhurrin is broken down to HCN when it is mixed with specific β-glucosidases (dhurrinase).[2] Dhurrin and dhurrinase are spatially separated in the living plant, such that HCN is only released when the tissue is damaged, consistent with its putative role in herbivore defense. The amount of cyanide able to be released from dhurrin is known as the cyanide potential (HCNp).

Near-infrared spectroscopy (NIRS) is an analysis tool used routinely in agricultural sciences. Since its development in the 1950s, it has become the main work-horse for cereal-based plant breeding programs,[3] as well as finding applications in forage testing,[4] remote sensing on plants for growth,[5] and crop nutrition status.[6] Specifically, in cereal plant breeding applications, NIRS has been mainly used in crops, such as wheat or barley, where there is a quality specification on commercially delivered crops.[3] For grain sorghum, no target quality specifications exist, although the opportunity to predict feed traits exists using NIRS.[7] In forage sorghum, NIRS has been used to estimate characteristics, such as chemical composition and feed quality.[8] To date, there has been one published report describing the NIRS estimation of the antinutritional factor, namely, HCNp, in forage sorghum[9] using a partial least-squares method.

Recent calibration development strategies use a partial least-squares (PLS) regression approach, i.e., combining all spectral data, of up to 1050 wavelengths. This approach has been used successfully in a number of plant-based agricultural applications, particularly breeding.[3] However, it is possible is to use only a few specific wavelengths [multiple linear regression (MLR)] that are correlated to the trait of interest.[10] The early NIRS instruments used a limited number of specific filters (specific wavelengths) and for very few grain traits, such as moisture, protein, and lipid.[11]

The aim of this study was to determine the suitability of NIRS to estimate HCNp in forage sorghum. A NIRS calibration has been reported, and PLS and MLR models were compared to ascertain if one approach provided a better calibration than

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the other. The development of a NIRS calibration could be used in a number of applications, including breeding to screen lines, because NIRS is a high-throughput, low-cost technology, as well as on the farm for monitoring forage sorghum during the growing season.

MATERIALS AND METHODS

Sorghum Samples. Forage sorghum [*Sorghum bicolor* (L.) Moench × *Sorghum sudanense* Stapf. cv. Superdan] was tested. All samples were collected in 2008 from near Gatton in the Lockyer Valley, Queensland, Australia, except two from South Australia, Australia. A total of 12 additional samples were from an experimental Sorghum × Sudan line. To ensure that the samples represented a wide range of HCN, a further 24 samples were taken from hay made in the same season. Field samples of live plants were harvested, where the uppermost fully unfurled leaf was collected. The hay samples were all whole plant samples. Samples were dried at 70 °C and then ground in a Christie and Norris 200 mm hammer mill, with a 1 mm screen. Initially, 153 samples were combined and split into calibration (109) and validation (44) sets. A further 15 samples were used as an independent test set to test significant differences ($p < 0.05$) between calibration models.

Table 1. Summary of HCN in Forage Sorghum Samples Used

	number of samples	range (%, dry basis)	mean (%, dry basis)	standard deviation
calibration	109	0.021−0.941	0.230	0.156
validation	44	0.036−0.521	0.236	0.129
test set	15	0.022−0.531	0.189	0.160

Measurement of HCNp. Dhurrin in plant tissue was determined as described previously,[12] by hydrolyzing the cyanogenic glycoside and trapping the resultant HCN in a well containing 1 M NaOH. Initially, samples were ground using a Christy and Norris 200 mm cross-arm mill fitted with a 1 mm screen. The dried and ground samples were placed in plastic Ziploc bags and stored in a dehumidified cold room at 4 °C. Hydrolysis was achieved by adding 500 μL of β-glucosidase almond emulsion (0.01%, w/v) (β-D-glucoside glucohydrolase, EC 3.2.1.21, Sigma) in 0.1 M trisodium citrate−HCl buffer (pH 5.5) to approximately 10 mg of ground oven-dried leaf material in a sealed glass vial and incubating at 37 °C for 15 h. Cyanide in the NaOH well was neutralized with acetic acid and assayed. HCN was measured with a FLUOstar OPTIMA UV/vis absorbance spectrophotometer microplate reader (BMG LABTECH, Offenburg, Germany) at 595 nm. A total of 1 g of cyanide (CN) detected by this method is equivalent to 11.6 g of dhurrin and referred to as HCNp. All samples were tested in triplicate.

NIRS. Ground samples were scanned in a small ring cup in the NIRSystem 6500 instrument (Foss NIRSystems, Inc., Silver Spring, MD).

Table 2. Summary of the Best NIR Calibration Model Statistics

region	spectral range (nm)	math treatment[a]	number of factors[b]	R^2 (cal)	SECV (cal)[c]	R^2 (val)	SEP (val)	RPD (test)[d]
			PLS					
full	400−2498	2,4,4,1	5	0.838	0.044	0.824	0.047	3.4
visible	400−800	2,4,4,1	5	0.712	0.079	0.702	0.092	1.7
SWNIR	802−1098	2,4,4,1	9	0.813	0.056	0.798	0.069	2.3
NIR	1100−2498	1,4,4,1	9	0.826	0.049	0.804	0.051	3.1
			MLR					
full	400−2498	1,4,4,1	9	0.836	0.051	0.821	0.054	3.0
visible	400−800	2,4,4,1	3	0.525	0.086	0.467	0.121	1.3
SWNIR	802−1098	1,4,4,1	9	0.798	0.056	0.701	0.078	2.1
NIR	1100−2498	2,4,4,1	9	0.847	0.050	0.829	0.057	2.8

[a]All calibrations produced the best models with SNV detrend scatter correction. [b]Number of factors used in the PLS calibration and number of wavelengths used in the MLR calibration. [c]Standard error of cross-validation for the PLS calibration and standard error of calibration in the MLR calibration. [d]Ratio of SEP (test set) and standard deviation (SD) (test set).

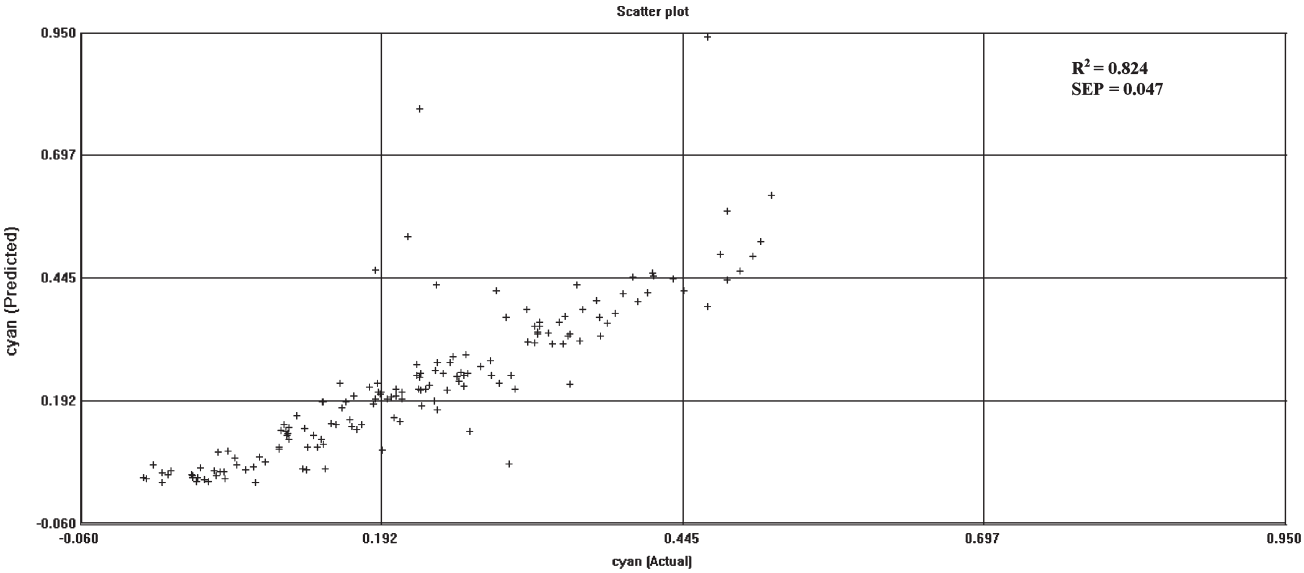

Figure 1. Scatter plot of actual versus predicted values using the best PLS calibration model.

Table 3. Wavelengths Selected in MLR Calibrations

region	spectral range (nm)	wavelength[a] (nm)	chemical assignment[b]
full	400−2498	776	N−H third overtone (OT) NH_2
		628	chlorophyll
		2068	NH_2
		2314	CH_2
		446	unassigned
		546	unassigned
		1704	oil
		2034	C═O second OT
		2316	CH_2
SWNIR	700−1098	736	O−H third OT OH
		888	C−H third OT CH_2
		948	O−H second OT OH
		1056	C−H CB CH_2
		1012	CONH second OT amide
		788	C≡CH third OT
		858	C−H third OT aromatic
		1000	N−H second OT NH_2
		864	C═C third OT
NIR spectra only	1100−2498	2394	no assignment
		1152	C−H second OT CH_3
		1836	C−H CB CH_2
		1398	C−H CB CH_2 CH stretch + CH bend
		1256	no assignment
		2430	no assignment
		2302	C−H bend second OT protein
		1228	C−H second OT CH
		2458	C−N−C stretch first OT protein

[a]Wavelengths are listed in order of selection for the MLR model. [b]Chemical assignments from the WinISI software.

The calibration software (WinISI V1.6, Foss NIRSystems, Inc., Silver Spring, MD) for the NIRS instrument was used for all calibration models using absorbance as a log(1/R) function. Two calibration methods were used in developing a calibration model for HCN. The first was a typical approach using the full spectrum (400−2498 nm at 2 nm intervals) in a modified PLS regression model. A number of scatter corrections were tested, including no treatment, multiplicative scatter correction, and standard normal variate detrend. Math pretreatments tested included none, first, and second derivatives. MLR models were also assessed with the above scatter corrections and premath treatment combinations using a stepwise regression with a maximum number of 10 terms (9 wavelengths) based on the WinISI software. For both calibration approaches, full spectra, visible spectra (400−750 nm), shortwave near-infrared (SWNIR) spectra (800−1100 nm), and near-infrared (NIR) spectra (1100−2500 nm) were tested. In addition, for all calibration models, the spectral outlier (global H) was set at 5.000 GH and cross-validation settings were used, i.e., four groups.

To calculate which model was most accurate from all models developed, the modified Fearn test was used.[13] This method tests the significance of the differences in standard deviation and bias between two models based on the residuals for the references and predicted values for each sample in the tested population. A ratio of standard error of prediction to standard deviation (RPD)[14] was also calculated to ascertain the potential application of the NIRS models for application in breeding or industry screening. RPD values of 1 or less are an indication of an inadequate model. Values greater than 2.5 indicate that the model could be useful for initial screening purposes, whereas a RPD greater than 5 indicates that the model would be good for quality control and prediction.

RESULTS

One of the main objectives in developing NIRS calibrations is to ensure that a suitable range of the trait of interest is sampled and the level of precision in the reference method is acceptable. Table 1 shows the descriptive statistics and number of samples for the calibration, validation, and test sets used in this study. Both sets included samples below and above the HCN range acceptable for cattle (0.6%). However, the average values for each set was below the 0.6% threshold, suggesting the range of values could be expanded to include more samples above the threshold.

PLS Approach. Two calibration methods were used in developing a calibration model for HCN. The first was a typical approach using the full spectrum [400−2498 nm (1050 data points)] with a modified PLS regression model. The best PLS model was using the NIR spectra (400−2500 nm), giving a coefficient of determination (R^2) = 0.837 and standard error of cross-validation (SECV) = 0.044%, using a standard normal variance (SNV) detrend pretreatment with the first derivative, 4 nm gap, and 4 nm smoothing (Table 2). Figure 1 shows the scatter plot for the actual versus predicted values from this model. The use of NIR and SWNIR spectra both produced reasonable calibrations (Table 2). The R^2 for the validation set using the full spectrum was 0.824, with a standard error of prediction (SEP) = 0.047%. The RPD was 3.4, indicating that the predictive model could be used for screening in a breeding program. All other PLS models produced R^2 > 0.66 and RPD above 1.6. As suggested by Williams and Sobbering,[14] these calibration statistics may be useful for screening in a breeding program but not for quality testing applications.

MLR Approach. The second calibration model was developing using a MLR approach, in this case a stepwise wavelength selection process. The NIR spectra produced the most accurate calibration with a 2,4,4,1 math treatment and SNV detrend scatter correction. The R^2 = 0.847, and a standard error of calibration = 0.050%. For the validation set, a R^2 = 0.829, with a SEP = 0.057%. The RPD for this model was 2.8, suggesting that it could also be used for screening in a breeding program. However, the full spectra and NIR spectra regions showed potential for providing useful predictions from a MLR calibration approach with R^2 greater than 0.79, SEPs less than 0.080%, and RPDs greater than 2.0.

Nine wavelengths were selected using the MLR model for all three spectral regions, including the NIR region (Table 3). The visible region produced a very poor calibration model (Table 2), with only four wavelengths selected (not shown). The majority of wavelengths selected were associated with CH bonding (methyl CH_2 or methylene CH_3) (Table 3). However, in the full spectral region and the SWNIR region calibrations, there was one or more wavelengths associated with NH bonding. These included 776, 2034, and 2068 nm in the full spectral region and 1000 and 1012 nm in the SWNIR region. In the "NIR spectra only" calibration, six of the nine wavelengths were associated with CH bonding, while for the remaining three, there was no chemical assignment (Table 3).

As seen in the loading plots (Figure 2), there was a strong influence from the visible region in the full spectrum model (Figure 2a). The first loading explained 60% of the full spectral calibration. However, when using the visible region only, a poor calibration was developed, where the first loading (Figure 2b) explained only 34% of the visible region calibration. The first

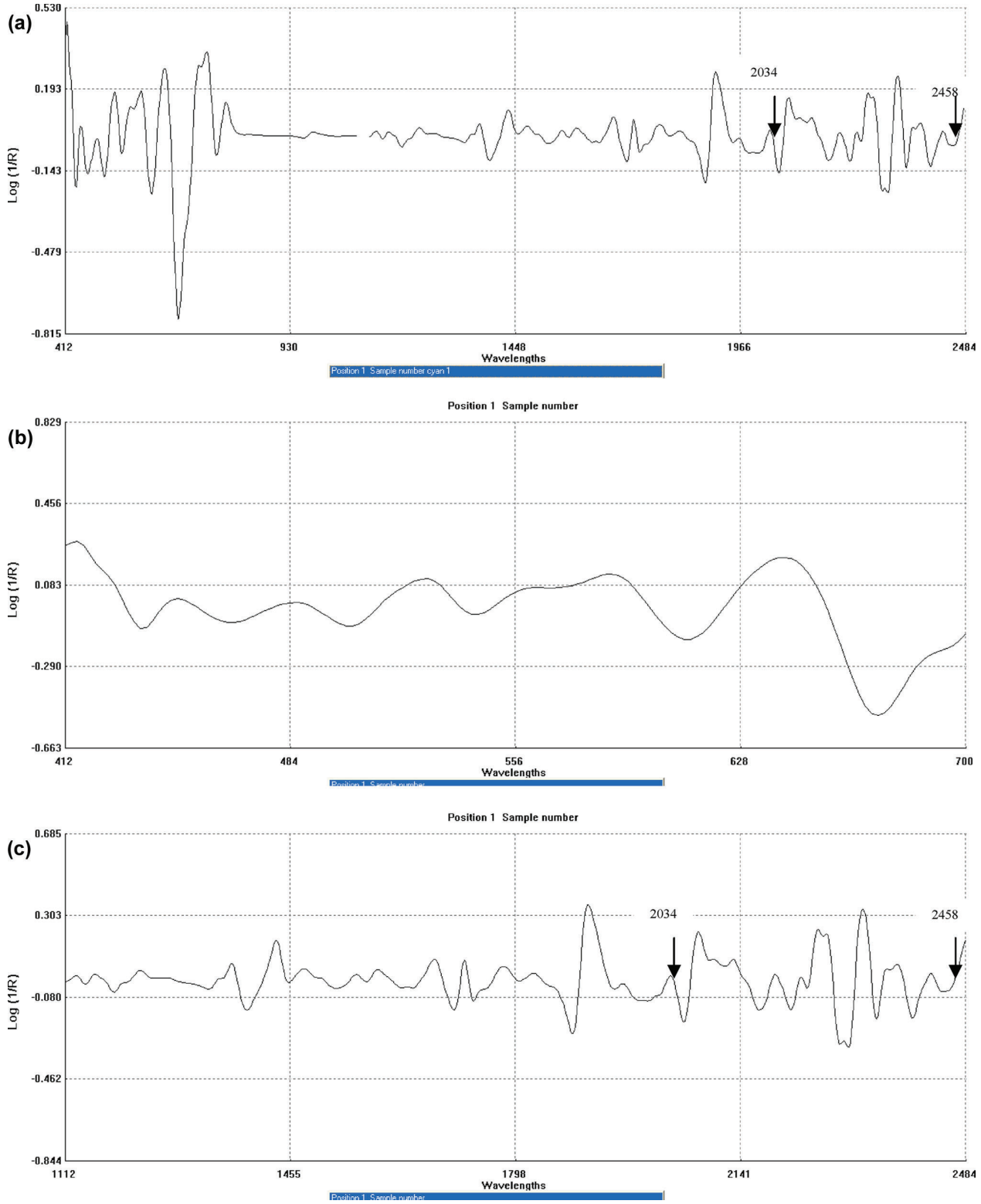

Figure 2. Loading line plot 1 from (a) full spectrum, (b) visible spectrum only, and (c) NIR spectrum only second derivative PLS model.

loading plot for the NIR region is shown in Figure 2c. This loading explained 63% of the NIR spectrum calibration.

While both PLS and MLR calibrations used wavelengths associated with nitrogen bonding, two wavelengths from MLR calibrations were of specific interest. In the full spectrum MLR calibration, the 2034 nm wavelength was selected (Table 3). This wavelength is associated with the C═O bond and assigned as a carbonyl stretch second overtone associated with amide I (urea) (as per the WinISI software). Because the chemical formula for urea is O═C−$(NH_2)_2$, it is possible that the covalent bonding

between the C≡N atoms in HCN could result in a similar chemical stretch to C═O. In the NIR spectrum only calibration, the 2458 nm wavelength was selected (Table 3). This wavelength was assigned as a C−N−C stretch first OT protein in the WinISI software. These two specific regions have been highlighted in panels a and c of Figure 2 for the full spectra and NIR spectra PLS calibrations, respectively.

The modified Fearn test[13] was used to note any significant differences ($p < 0.05$) between calibrations when predicting the test set. The results indicated that there was no significant difference ($p < 0.05$) between the predicted HCN levels for the full spectral, "NIR spectra only", and "SWNIR spectra only" for both PLS and MLR calibrations. There was a significant difference ($p < 0.05$) between the visible spectral region and all of the other regions from both PLS and MLR calibrations. There was also no significant difference ($p < 0.05$) between the "best" PLS (full spectra) and MLR (full spectra) models.

DISCUSSION

In this study, we built NIR calibrations to predicted HCNp. Two calibration approaches were used, with these being the typical full-spectral calibration model and a selected wavelength calibration model. The resultant calibrations showed that both approaches were similar in predicting HCNp in forage sorghum samples. Our results, especially for the PLS model for the NIR spectral region only were comparable to the only other reported NIRS calibration for HCN,[9] in which that study used only the NIR spectral region only.

From our study, when the full, visible spectra, SWNIR spectra, and NIR spectra were compared, the best calibration in terms of R^2, SEP, and RPD values was using the full spectrum. This would suggest that using all wavelengths from the visible, SWNIR, and NIR regions was the best strategy for building a PLS calibration for HCN. The loading plots showed the positive and negative contributions of these regions to the PLS model. However, despite using the 1050 wavelengths available, it was possible to build a predictive calibration with a similar level of accuracy using only nine wavelengths. While for this study, the best MLR calibration was using the NIR spectra, the full spectrum calibration, which included visible spectrum wavelengths, was similar. This would suggest that, for this type of sample, including wavelength from the visible and NIR spectral regions would provide accurate calibrations, regardless of whether choosing MLR or PLS calibration strategies. The use of fewer wavelengths provides an opportunity for the development of specific instruments, which could provide fast estimation in the field-based instrument to predict HCNp levels or potentially using remote sensing technologies.

The approach of using fewer wavelengths was considered specifically for this small chemical molecule. It may not work for some traits, especially where there may be strong interactions between different chemical species in a complex sample matrix, for example, wheat dough, which has starch, protein, and lipid components. However, for chemicals such as HCN, with a simple chemical structure and, hence, associations with specific wavelengths, the MLR approach has merit. This has already been shown where both PLS and MLR models proved suitable.[10]

The possibility to use NIRS to predict HCN in forage sorghum breeding could help in selecting genotypes that inherently produce lower concentrations of dhurrin. Other potential applications include the monitoring of crops to gauge HCNp levels prior to feeding cattle as well as assessing crops to understand how the environment influences the expression of dhurrin. The results of this study have shown the potential to use NIR to predict the HCNp content in forage sorghum, which has good economic potential for graziers and plant breeders alike.

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Notes

The authors declare no competing financial interest.

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