

SHORT RESEARCH ARTICLE

Regulation of cyanogenic glucosides in wild and domesticated *Eusorghum* taxa

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ABSTRACT

- Domesticated sorghum (*Sorghum bicolor* [L.] Moench subsp. *bicolor*) diverts significant amounts of nitrogen away from primary metabolism to the synthesis of cyanogenic glucosides (CNglc) – specialized metabolites that release toxic hydrogen cyanide (HCN). Our aim was to identify the point in the genus *Sorghum* Moench at which plants gained the ability to maintain hazardous concentrations of cyanogenic glucosides in their leaves into maturity (HCN potential $>0.4 \text{ mg g}^{-1}$). This ability occurs in domesticated sorghum (in the subgenus *Eusorghum*), but not in wild taxa in other *Sorghum* subgenera.
- Eight accessions from the subgenus *Eusorghum* were grown in a common garden: an improved sorghum line, five sorghum landraces, the crop's wild progenitor (*S. bicolor* subsp. *verticilliflorum* [Steud.] de Wet ex Wiersema & J. Dahlb.) and wild *Sorghum propinquum* (Kunth) Hitchc. HCN potential was measured in plants ($n = 80$) at the three-leaf stage and at 6 weeks old.
- All study accessions, including the wild taxa, had hazardous CNglc concentrations in the leaves at both the three-leaf stage (mean HCN potentials $\geq 2.5 \text{ mg g}^{-1}$) and at 6 weeks old (mean HCN potentials $\geq 0.68 \text{ mg g}^{-1}$), greatly contrasting the much lower mature leaf HCN potentials previously found in wild *Sorghum* taxa outside subgenus *Eusorghum* (generally $\leq 0.01 \text{ mg g}^{-1}$).
- Our results suggest that the ability to maintain hazardous leaf HCN potentials into maturity might have arisen during the divergence of *Eusorghum* from other *Sorghum* subgenera, rather than during the speciation or domestication of *S. bicolor*, and highlights the value of utilizing *Sorghum* taxa outside *Eusorghum* in efforts to improve the crop safety of sorghum.

INTRODUCTION

Cyanogenic glucosides (CNglc) are stored in the cells of around 11% of plant species and are thought to have multiple functions, most commonly as a deterrent to generalist herbivores (Gibbs 1974; Gleadow & Møller 2014). CNglc themselves are not necessarily harmful, but, upon physical damage to the plant, are broken down by β -glucosidase enzymes to release toxic hydrogen cyanide (HCN) in a process called cyanogenesis. Stored CNglc concentrations can be quantified as HCN potential (mg g^{-1}): the total amount of HCN produced by hydrolysing all CNglcs present. HCN potentials above 0.6 mg g^{-1} are above the toxicity threshold for cattle primarily fed on forage sorghum (Boyd *et al.* 1938; Hunt & Taylor 1976). Another potential function of CNglc in plants is the storage of reduced nitrogen (Gleadow & Møller 2014), although whether this is positive or negative for plant growth is unknown (Rosati *et al.* 2019). Putative pathways have been identified in multiple species in which CNglc are catabolized, with the nitrogen recycled from them and no HCN produced (Pičmanová *et al.* 2015).

Sorghum Moench is a genus of Poaceae whose members produce the CNglc dhurrin. Every *Sorghum* taxon tested thus far has the ability to produce and store dhurrin, but HCN potential greatly differs between organs and species (Cowan *et al.* 2022).

Domesticated sorghum (*Sorghum bicolor* [L.] Moench subsp. *bicolor*) is a member of the subgenus *Eusorghum* and, when mature, has relatively high HCN potentials in its leaves ($0.4\text{--}1.2 \text{ mg g}^{-1}$) and roots ($0.25\text{--}0.50 \text{ mg g}^{-1}$) (Table 1). This greatly contrasts with *Sorghum* taxa outside *Eusorghum*, all of which are wild and consistently have much lower HCN potentials in their mature leaves ($0\text{--}0.01 \text{ mg g}^{-1}$), across multiple studies and treatments (Table 1). Mature root HCN potential in these wild taxa is more variable ($0\text{--}0.75 \text{ mg g}^{-1}$). The difference in CNglc storage patterns between *Sorghum* taxa poses questions regarding the point in the *Sorghum* phylogeny at which high HCN potentials in mature leaves arose.

It is not uncommon for a crop to have a higher HCN potential than wild plants. Jones (1998) found crops to be greatly overrepresented in a list of cyanogenic plants, compared to what would be expected by chance. One potential explanation for this is that cyanogenesis is agronomically useful as a natural pest deterrent, which might seem counterintuitive given that HCN is also toxic to humans. However, Jones (1998) posits that, while most generalist pests are unable to detoxify cyanogenic crops, humans are able, through processing, to mitigate that risk. It is plausible that high leaf HCN potential could have been artificially selected for in domesticated sorghum, but this hypothesis remains untested.

Table 1. Ranges of HCN potential in the leaves and roots of *Sorghum* taxa.

HCN potential in mature plants	Taxa
High in leaves (0.4–1.2 mg g ⁻¹)	<i>Sorghum bicolor</i> subsp. <i>bicolor</i> (BTx623) ^{a,b,c,d}
High in roots (0.25–0.50 mg g ⁻¹)	
High in leaves (~0.7 mg g ⁻¹)	<i>Sorghum propinquum</i> (Kunth) Hitchc ^a
Unknown in roots	
Low in leaves (0–0.01 mg g ⁻¹)	<i>Sorghum amplum</i> Lazarides ^{a,b} ; <i>Sorghum brachypodium</i> Lazarides ^{a,b,c,d} ;
Low–high in roots (0–0.75 mg g ⁻¹)	<i>Sorghum bulbosum</i> Lazarides ^{a,b} ; <i>Sorghum ecarinatum</i> Lazarides ^{a,b} ; <i>Sorghum intrans</i> F. Muell. ex Benth. ^{a,b} ; <i>Sorghum laxiflorum</i> F. M. Bailey ^{a,d} ; <i>Sorghum leiocladum</i> (Hack.) C. E. Hubb ^{a,d} ; <i>Sorghum macrospermum</i> E. D. Garber ^{a,b,c,d} ; <i>Sorghum matarankense</i> E. D. Garber & Snyder ^{a,b,d} ; <i>Sorghum plumosum</i> (R. Br.) P. Beauv ^{a,e} ; <i>Sorghum stipoides</i> (Ewart & Jean White) C. A. Gardner & C. E. Hubb ^{a,e} ; <i>Sorghum timorensis</i> (Kunth) Büse ^{a,e}
Low in leaves (0–0.001 mg g ⁻¹)	<i>Sorghum angustum</i> S. T. Blake ^a ; <i>Sorghum exstans</i> Lazarides ^a ;
Unknown in roots	<i>Sorghum interjectum</i> Lazarides ^a ; <i>Sorghum purpureosericeum</i> (Hochst. ex A. Rich.) Schweinf. & Asch ^a <i>Sorghum versicolor</i> Andersson ^a
Unknown	<i>Sorghum bicolor</i> subsp. <i>verticilliflorum</i> (Steud.) de Wet ex Wiersma & J. Dahlb.; <i>Sorghum grande</i> Lazarides; <i>Sorghum nitidum</i> (Vahl) Pers.; <i>Sorghum trichocladum</i> (Rupr. ex Hack.) Kuntze. <i>Cleistachne sorghoides</i> Benth

^aCowan *et al.* (2022).^bCowan *et al.* (2020).^cCowan *et al.* (2021).^dMyrans *et al.* (2021), excluding plants under the nitrogen-deficient treatment.^eMyrans (2022).

Uncovering the point in the *Sorghum* phylogeny at which the ability to maintain high leaf HCN potentials arose would enable a better understanding of the selection pressures acting on cyanogenesis and allow a more targeted approach to studying the genetic basis for such changes. We grew eight *Eusorghum* accessions in a common garden in order to test four hypothetical points at which maintenance of high leaf HCN potential could have arisen in *Eusorghum*: (i) during *Eusorghum*'s divergence from other subgenera (Fig. 1A); (ii) during the speciation of *S. bicolor* (Fig. 1B); (iii) during the domestication of *S. bicolor* subsp. *bicolor* (Fig. 1C); or (iv) during the breeding of certain lines within *S. bicolor* subsp. *bicolor*.

MATERIAL AND METHODS

Study taxa

Eight *Eusorghum* accessions were selected for this study, including: wild *S. propinquum*, the crop's wild progenitor (*S. bicolor*

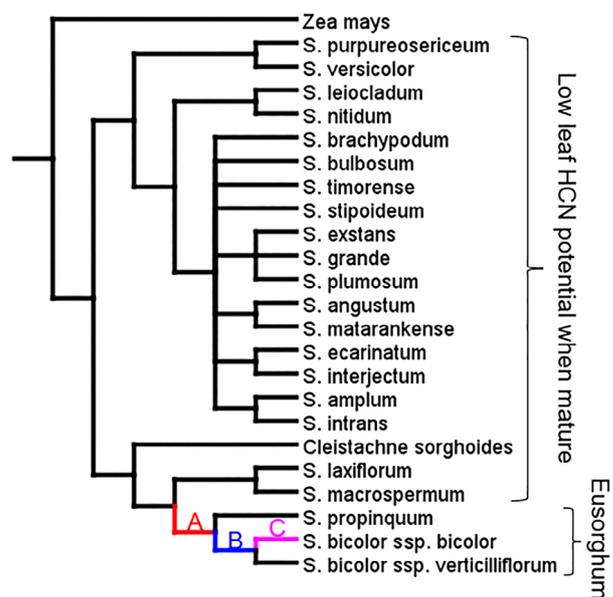


Fig. 1. Phylogeny of the genus *Sorghum* according to Dillon *et al.* (2007). Three potential points at which the ability to maintain high leaf HCN potentials when mature could have arisen in subgenus *Eusorghum* are highlighted: (A) during *Eusorghum*'s divergence from other subgenera; (B) during the speciation of *S. bicolor*; (C) during the domestication of *S. bicolor* subsp. *bicolor*.

subsp. *verticilliflorum*), an improved line of domesticated *S. bicolor* subsp. *bicolor* and five landraces of subsp. *Bicolor* each belonging to a different race of the crop (*caudatum*, *durra*, *kafir*, *guinea* and *margaritifera*) (Table 2). It is thought that *caudatum*, *durra*, *guinea* and *kafir* derive from a single domestication event, while *margaritifera* derives from a separate domestication event (Deu *et al.* 2006; Mace *et al.* 2013; Morris *et al.* 2013). Seeds were supplied by the Australian Grains Genebank (VIC, Australia) and Queensland Department of Agriculture and Fisheries (QLD, Australia).

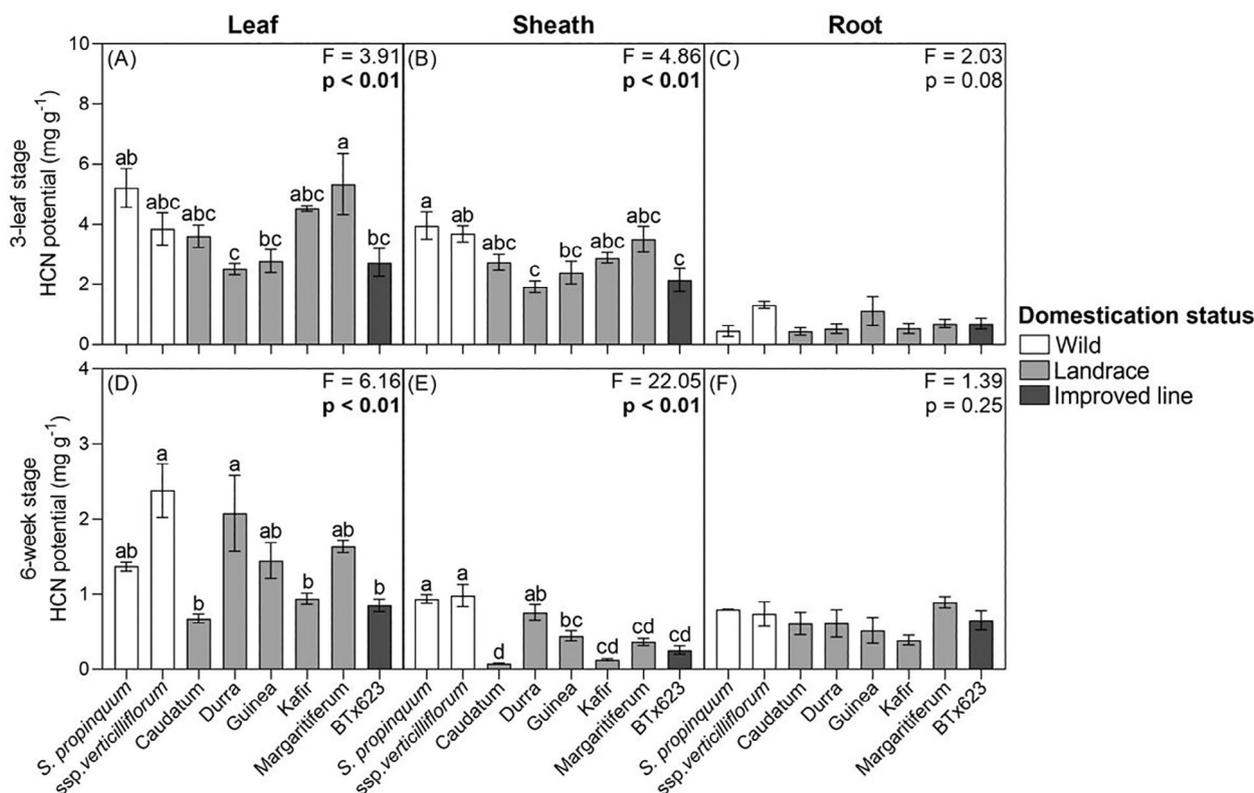
Plant growth conditions

All plants ($n = 80$) were grown under controlled greenhouse conditions at Monash University (-37.91° , 145.14°) between September and November 2021, with mean temperatures of $30.81 \pm 0.07^\circ\text{C}$ and $23.88 \pm 0.02^\circ\text{C}$, day/night, and an average photoperiod of 13.2 h (average daytime illuminance was 4183 ± 49 lx). Supplementary light was provided using sodium lamps for 13 h per day (MK-1 Just-a-shade; Ablite, NSW, Australia).

Seeds were germinated following Cowan *et al.* (2020). Once the coleoptile and radicle had emerged, plants were transferred to the greenhouse and planted in seedling trays containing Debco seed raising substrate (Evergreen Garden Care, NSW, Australia) and perlite (1:1 v/v ratio) for 1 week. Half of the seedlings were then transplanted into 1-l pots, and the other half were transplanted into 1.9-l pots. All pots contained Debco seed raising substrate, perlite and coarse sand (2:2:1 v/v/v) and were watered three times per week to saturation; twice with water, once with Thrive all-purpose soluble fertilizer (Yates Australia, NSW, Australia), with an N:P:K ratio of

Table 2. Details and origins of collection for the eight *Eusorghum* accessions examined in this study: *S. propinquum*, *S. bicolor* subsp. *verticilliflorum*, an improved line of *S. bicolor* subsp. *bicolor* and five landraces of subsp. *bicolor*.

Accession ID	Taxon	Domestication status	Geographic origin
AGG324437	<i>S. propinquum</i>	Wild	Philippines
AusTRCF317961	<i>S. bicolor</i> subsp. <i>verticilliflorum</i>	Wild	Australia (naturalized)
BTx623	<i>S. bicolor</i> subsp. <i>bicolor</i>	Improved line	USA
IS9710	<i>S. bicolor</i> subsp. <i>bicolor</i> (race caudatum)	Landrace	Sudan
IS8525	<i>S. bicolor</i> subsp. <i>bicolor</i> (race kafir)	Landrace	Ethiopia
IS3614-2	<i>S. bicolor</i> subsp. <i>bicolor</i> (race guinea)	Landrace	Nigeria
PI585749	<i>S. bicolor</i> subsp. <i>bicolor</i> (race durra)	Landrace	Mali
PI525695	<i>S. bicolor</i> subsp. <i>bicolor</i> (race margaritifera)	Landrace	Mali

**Fig. 2.** HCN potential in the (A) leaves, (B) sheaths and (C) roots at the three-leaf stage, and the (D) leaves, (E) sheaths and (F) roots at 6 weeks post-germination in eight *Eusorghum* accessions grown in a common garden. Accessions included: *S. propinquum*, *S. bicolor* subsp. *verticilliflorum*, landraces belonging to five races of *S. bicolor* subsp. *bicolor* (caudatum, durra, kafir, guinea and margaritifera), and an improved line of subsp. *bicolor* (BTx623). Data were analysed by one-way ANOVA. F- and P-values are presented, with significant P-values in bold. Columns with different letters are significantly different ($P < 0.05$). Values are means \pm SE ($n = 5$).

25:5:8.8 (v/v/v). Pot positions were randomized and rotated weekly to minimize microclimate effects.

Plants in the 1-l pots were harvested at the three-leaf stage and plants in the 1.9-l pots were harvested at 6 weeks post-germination. Harvested plants were separated into roots, sheaths and leaves, and oven dried at 50 °C for 72 h.

Cyanide analysis

The HCN potential of dried tissue was measured following Gleadow *et al.* (2012) through colorimetric assay in 96-well

microtiter plates, using up to 10 mg tissue per sample. Three-leaf stage samples were chopped into 1-cm sections before analysis, while 6-week-old samples were finely ground before analysis.

Statistical analysis

GraphPad Prism version 9.0.2 (GraphPad Software, CA, USA) was used for statistical analysis with one-way ANOVA. When significant effects were detected, *post-hoc* comparisons were made using Tukey tests. For all tests, P -values < 0.05 were considered significant.

RESULTS

At the three-leaf stage, mean leaf HCN potential ranged from 2.50 mg g⁻¹ in the durra landrace to 5.32 mg g⁻¹ in the margaritifera landrace. Leaf HCN potential significantly varied among the study plants ($F = 5.83$, $P < 0.01$), with no domesticated subsp. *bicolor* accession having a significantly higher mean leaf HCN potential than either wild accession (Fig. 2A). Mean sheath HCN potential ranged from 1.91 mg g⁻¹ in the durra landrace to 3.95 mg g⁻¹ in *S. propinquum*. Sheath HCN potential significantly varied among the study plants ($F = 4.86$, $P < 0.01$), with both wild accessions having significantly higher mean sheath HCN potentials than the improved line (Fig. 2B). Mean root HCN potential ranged from 0.42 mg g⁻¹ in the caudatum landrace to 1.31 mg g⁻¹ in subsp. *verticilliflorum*. Root HCN potential did not significantly vary among study plants ($F = 2.03$, $P = 0.08$; Fig. 2C).

At 6 weeks old, mean leaf HCN potential ranged from 0.68 mg g⁻¹ in the caudatum landrace to 2.38 mg g⁻¹ in subsp. *verticilliflorum*. Leaf HCN potential significantly varied among the study plants ($F = 6.16$, $P < 0.01$), again with no domesticated subsp. *bicolor* accession having a significantly higher mean leaf HCN potential than either wild accession. The improved line had a significantly lower mean leaf HCN potential than subsp. *verticilliflorum* (Fig. 2D). Mean sheath HCN potential ranged from 0.07 mg g⁻¹ in the caudatum landrace to 0.98 mg g⁻¹ in subsp. *verticilliflorum*. Sheath HCN potential significantly varied among the study plants ($F = 22.05$, $P < 0.01$), with both wild accessions again having significantly higher mean sheath HCN potentials than the improved line (Fig. 2E). Mean root HCN potential ranged from 0.39 mg g⁻¹ in the kafir landrace to 0.89 mg g⁻¹ in the margaritifera landrace. Root HCN potential did not significantly vary among study plants ($F = 1.39$, $P = 0.25$; Fig. 2F).

DISCUSSION

While HCN potential did significantly vary in the leaves and sheaths between our study accessions at the three-leaf stage and at 6 weeks old, the two wild taxa (*S. propinquum* and *S. bicolor* subsp. *verticilliflorum*) did not have a significantly lower mean leaf HCN potential than any domesticated *S. bicolor* subsp. *bicolor* accession at either stage. On the contrary, subsp. *verticilliflorum* had the highest mean leaf and sheath HCN potentials of any accession at 6 weeks old (Fig. 2). These results contrast with previous studies in which undomesticated *Sorghum* taxa have consistently much lower HCN potentials than domesticated sorghum, sometimes even differing by degrees of magnitude (Cowan *et al.* 2020; Myrans *et al.* 2021). One of the key traits that separated domesticated and wild *Sorghum* taxa in those studies was domesticated sorghum's ability to maintain much higher leaf HCN potentials into maturity than its wild congeners. In *S. propinquum* and subsp. *verticilliflorum*, we have identified two wild *Sorghum* taxa that break this trend, more closely matching the CNglc regulation patterns of domesticated sorghum. High HCN potential (~0.7 mg g⁻¹) had previously been detected in mature *S. propinquum* leaves by Cowan *et al.* (2022), supporting our result. *Sorghum bicolor* subsp. *verticilliflorum* had not previously had its HCN potential formally quantified.

Our results provide support for the hypothesis that the ability to maintain high HCN potentials in mature leaves arose during *Eusorghum*'s divergence from other *Sorghum* subgenera (Fig. 1A). The two most closely related taxa to *Eusorghum* – *S. macrospermum* E. D. Garber and *S. laxiflorum* F. M. Bailey (Fig. 1) – have both previously been found to have low leaf HCN potentials when mature (Myrans *et al.* 2021). That high leaf HCN potential occurs in subsp. *verticilliflorum*, domesticated sorghum's wild progenitor, offers some explanation as to how both sorghum domestication events produced highly cyanogenic sorghum races (caudatum, durra, guinea and kafir in the first; margaritifera in the second). Rather than supporting the hypothesis that high leaf HCN potential was artificially selected for in sorghum during domestication (Jones 1998), our results more closely resemble the cassava (*Manihot esculenta* Crantz) lineage, in which the crop lines are actually less cyanogenic than their wild progenitors (Wang *et al.* 2014).

In order to gather further evidence for the origin of the ability to maintain high HCN potentials in mature leaves in *Sorghum*, further physiological and genetic research into *Eusorghum* and its sister groups will be needed. In the first instance, it would be informative to test HCN potential in a variety of accessions of *S. propinquum* and subsp. *verticilliflorum*, both of which are widely distributed taxa with high niche variability (Myrans *et al.* 2020). It might also be valuable to test the HCN potential of *Cleistachne sorghoides* Benth., an African taxon within the *Sorghum* clade, closely related to *Eusorghum* (Fig. 1), that is yet to have its HCN potential assessed.

The results of this study have crop improvement implications, with historical sorghum improvement efforts largely focussing on crossbreeding lines within *Eusorghum* (Duncan *et al.* 1991). While this strategy presents fewer reproductive barriers than attempting to cross sorghum with its more distantly related congeners (Kuhlman *et al.* 2010; Ananda *et al.* 2020), it is becoming apparent that introgression of genes from the broader *Sorghum* gene pool might be required in order to lower sorghum's HCN potentials and improve crop safety.

In conclusion, this study provides some evidence that both wild and domesticated taxa within the *Eusorghum* subgenus are able to maintain high aboveground HCN potentials into maturity, unlike wild *Sorghum* taxa of other subgenera. This result is subject to further research but could have significant implications regarding crop improvement and studies into the evolution of cyanogenesis regulation within the *Sorghum* genus.

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