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Cyanogenic glucosides in grapevine: polymorphism, identification and developmental patterns

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Abstract

Twelve grapevine (*Vitis vinifera* L.) cultivars were surveyed for 'cyanide potential' (i.e. the total cyanide measured in β-glucosidase-treated crude, boiled tissue extract) in mature leaves. Two related cultivars (Carignan and Ruby Cabernet) had mean cyanide potential (equivalent to 110 mg HCN kg^{-1} fr. wt) ca. 25-fold greater than that of the other 10 cultivars, and so the trait is polymorphic in the species. In boiled leaf extracts of Carignan and Ruby Cabernet, free cyanide constituted a negligible fraction of the total cyanide potential because β-glucosidase treatment was required to liberate the major cyanide fraction – which is therefore bound in glucosylated cyanogenic compound(s) (or cyanogenic glucosides). In addition, cyanide was liberated from ground leaf tissue of Ruby Cabernet but not Sultana (a cultivar with low cyanide potential). Hence, the high cyanide potential in Ruby Cabernet leaves is coupled with endogenous β-glucosidase(s) activity and this cultivar may be considered 'cyanogenic'. A method was developed to detect and identify cyanogenic glucosides using liquid chromatography combined with tandem mass spectrometry (LC–MS/MS). Two putative cyanogenic glucosides were found in extracts from leaves of Carignan and Ruby Cabernet and were identified as the epimers prunasin and sambunigrin. Cyanide potential measured at three times over the growing season in young and mature leaves, petioles, tendrils, flowers, berries, seeds and roots of Ruby Cabernet was substantially higher in the leaves compared with all other tissues. This characterisation of cyanogenic glucoside accumulation in grapevine provides a basis for gauging the involvement of the trait in interactions of the species with its pests and pathogens.

Keywords: *Vitis vinifera* L; Vitaceae; Grapevine; LC–MS/MS; Cyanogenesis; Cyanogenic glucoside

1. Introduction

Release of cyanide by living organisms upon tissue disruption is termed cyanogenesis. This trait is widespread amongst plants and is most often attributed to the coupled occurrence of cyanogenic glycoside(s) with at least one degrading enzyme (β-glycosidase; Seigler, 1998). The capacity for cyanogenesis is modulated by the amount of cyanogenic glycoside available for degradation (i.e. cyanide potential) as well as by the activity of β-glycosidase(s) and these two components of cyanogenesis can be inherited independently (e.g. *Trifolium* spp.: Corkill, 1942; Williams and Williamson, 2001 and *Eucalyptus nobilis*: Gleadow et al., 2003). Cyanogenic glycosides and their degrading enzymes are differentially compartmentalised and thus the plant avoids toxicity (Thayer and Conn, 1981; Gruhnert et al., 1994). But when the compartment boundaries are disrupted and the cyanogenic glycosides and degrading enzyme(s) become co-located, cyanogenesis occurs.

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As a consequence of their implications for taxonomy, population studies, animal toxicity, and pest and pathogen interactions, cyanogenesis and cyanogenic glycosides have been the subject of much review and discussion (Robinson, 1930; Conn, 1981; Nahrstedt, 1985; Poulton, 1990; Jones, 1998; Seigler, 1998; Møller and Seigler, 1999; Jones et al., 2000; Gleadow and Woodrow, 2002). With respect to interactions of plants with pests and pathogens, antagonistic, stimulant and neutral effects have been attributed to cyanogenic glycosides and cyanogenesis (Møller and Seigler, 1999).

A thorough biochemical and genetic characterisation of the synthesis of the cyanogenic glycoside, dhurrin, in *Sorghum bicolor* culminated recently with the transfer of three genes that constitute the entire pathway for dhurrin biosynthesis from that species to *Arabidopsis thaliana*, which does not accumulate cyanogenic glycosides naturally (Tattersall et al., 2001). This engineered accumulation of dhurrin in *Arabidopsis* corresponded with acquired resistance to the flea beetle, *Phyllotreta nemorum*, demonstrating directly that cyanogenic glucosides can provide protection from an insect pest (Tattersall et al., 2001).

At least 60 different cyanogenic glycosides have been identified in plants (Seigler, 1991). They are most often derived from one of five amino acids (L-tyrosine, L-phenylalanine, L-valine, L-isoleucine and L-leucine; Seigler, 1998) and are generally *O*-β-glucosides of α-hydroxynitriles (Fig. 1; Jones et al., 2000). Cyanogenic glycosides have typically been detected and identified by isolating active fractions using chromatography, followed by structure determination using nuclear magnetic resonance spectroscopy and/or mass spectrometry (MS) (e.g. Erb et al., 1979; Goodger and Woodrow, 2002; Seigler et al., 2002). Amongst other alternative approaches, selective isolation of glycosylated compounds followed by GC–MS analysis of trifluoroacetylated derivatives has been used to detect and identify cyanogenic glucosides (in passion fruit; Chassagne et al., 1996). To our knowledge, however, the LC–MS/MS technique for screening for glycosides by monitoring their characteristic and common fragmentation (Qu et al., 2001) has not previously been applied to the detection and identification of cyanogenic glycosides.

In grapevine (*Vitis vinifera* L.), appreciable cyanogenesis from ground leaves has been detected previously in one of four tested cultivars (Deibner, 1967). For the cyanogenic cultivar (Carignan rouge),[1] other tissues (including seeds and berries) were acyanogenic by comparison. Since the absence of cyanogenesis from the different grapevine cultivars and the different grapevine tissues may have been a consequence of either the absence of the cyanogenic compounds (i.e. cyanide potential is low) or the absence of appropriate enzyme(s) for degrading cyanogenic compounds, exogenous β-glucosidase was added to extracts of seed and ripe berry skin of Carignan rouge. However, cyanide release was still negligible and other tissues and cultivars were not investigated (Deibner, 1967).

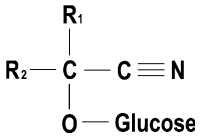

Fig. 1. General structure of cyanogenic glucosides.

Here, we expand on the work of Deibner (1967) by measuring the cyanide potential of various grapevine cultivars and grapevine tissues and by identifying the major cyanogenic glucosides involved. This information may be useful for assigning to the trait function with respect to interactions of *V. vinifera* with its pests and pathogens.

2. Results

2.1. Cyanide potential and cyanogenesis in grapevine leaves

The cyanide potential of mature leaves collected in spring from 12 grapevine (*V. vinifera*) cultivars was determined by measuring the free cyanide in boiled crude extract after treatment with β-glucosidase (Fig. 2). Two cultivars (Carignan and Ruby Cabernet) had appreciable cyanide potentials (mean = 110 mg HCN kg^{-1} fr. wt) that were ca. 25-fold greater than those measured for

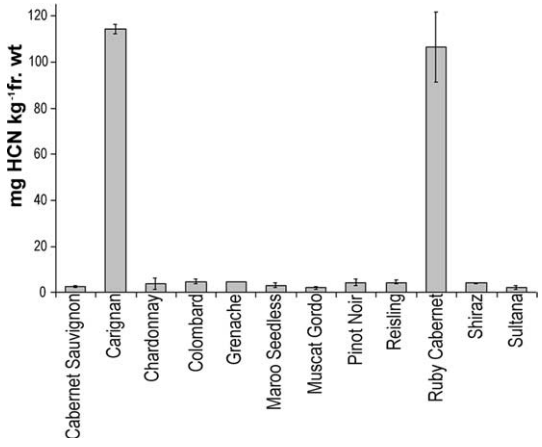

Fig. 2. Cyanide potential (cyanide equivalent to mg HCN kg^{-1} fr. wt) of mature leaf extracts from 12 grapevine cultivars at spring. Values are the mean of three replicates (or two replicates for Grenache) and replicates were assays of extracts prepared from different plants. Bars are ± SD.

[1] While no reference to Carignan rouge appears in authoritative texts (Viala and Vermorel, 1909; Galet, 1990), we assume that it is a synonym for Carignan (*syn* Carignan noire).

the other 10 cultivars (mean = 4 mg HCN kg^{-1} fr. wt) and cyanide was only liberated from boiled Carignan and Ruby Cabernet leaf extracts after treatment with β-glucosidase (Fig. 3(a)). Furthermore, in a comparison of 'cyanogenesis' of Ruby Cabernet with Sultana (which had low cyanide potential), appreciable levels of free cyanide were liberated from Ruby Cabernet but not Sultana leaf extracts (prepared without boiling) that were incubated overnight (without addition of β-glucosidase; Fig. 3(b)).

2.2. A method for screening for cyanogenic glucosides using LC–MS/MS

In a preliminary experiment, dhurrin cyanogenic glucoside was analysed by LC–MS and mass spectra were acquired in full scan mode. A sodium adducted molecular ion $[M + Na]^+$ (*m/z* 334) was the dominant ion observed (data not shown). This ion $[M + Na]^+$ was fragmented to *m/z* 307 and 185 by collision-induced dissociation (CID), which were deduced to be $[M + Na–HCN]^+$and [dehydrated glucose + $Na]^+$, respectively (data not shown). Since the two fragment ions resulting from losses of HCN and aglycone from the $[M + Na]^+$ would be typically derived from the common structure of cyanogenic glucosides, it was possible that these characteristic fragment pathways could be used to screen for cyanogenic glucosides. This possibility was tested by analysing dhurrin by LC–MS/MS, monitoring for the losses of HCN and aglycone by neutral loss scan of 27 Da and precursor ion scan of *m/z* 185, respectively. To do this, the two different scan modes were looped to one scan program that ran repetitively along with the LC run. For both scan modes, one peak each was observed at the same retention time (15.90 min) and each of the peaks gave the same ion (*m/z* 334; Fig. 4), indicating that masses of HCN and aglycone were eliminated from a common ion corresponding to $[M + Na]^+$ of dhurrin. Thus, the LC–MS/MS technique could be used to screen for cyanogenic glucosides in grapevine extracts.

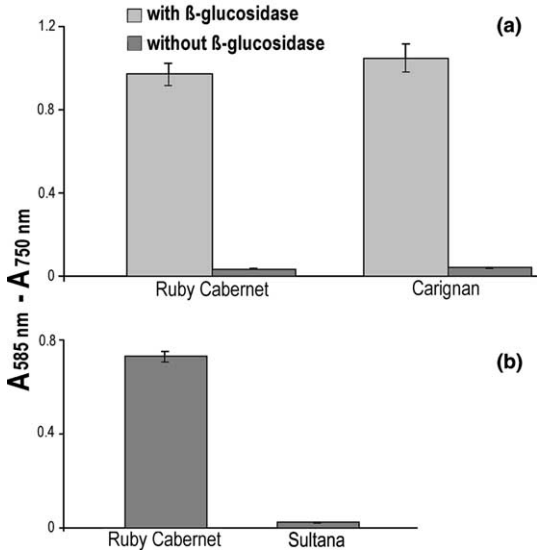

Fig. 3. Liberation of cyanide from grapevine leaf extracts. Absorbance ($A_{585\ nm}$–$A_{750\ nm}$) is proportional to cyanide. (a) boiled leaf extracts with and without β-glucosidase treatment. Values are the mean of two replicates and replicates were assays of extracts prepared from two different plants. Bars are ± SD. Concentration of tissue in the final assay was equivalent to 5 mg fr. wt ml^{-1}. (b) cyanogenesis of (unboiled) leaf extracts after overnight incubation at 37 °C (without β-glucosidase treatment). Values are the mean of two replicates and replicates were assays of independent extracts from two different leaves. Bars are ± SD. Concentration of tissue in the final assay was equivalent to 7 mg fr. wt ml^{-1}.

2.3. Application of the screening method to the detection and identification of the major grapevine cyanogenic glucosides

Ruby Cabernet, Carignan and Sultana leaf extracts were screened for cyanogenic glucosides using the LC–MS/MS described above. For the Ruby Cabernet and Carignan – but not Sultana – extracts, a single significant common precursor ion of *m/z* 318 was detected for both scan modes.

When the Ruby Cabernet and Carignan extracts were analyzed by LC–MS in scan mode, the extracted ion chromatograms of *m/z* 318 showed two peaks that eluted less than 0.4 min apart at ca. 19.5 min. (Fig. 5(a)). The mass spectra of both peaks exhibited ions at *m/z* 318 and 313 (Fig. 5(b)) corresponding to $[M + Na]^+$ and $[M + NH_4]^+$, respectively. Furthermore, a product ion scan of *m/z* 318 from the extracts confirmed that the ions *m/z* 291 (–HCN) and *m/z* 185 (–aglycone) were derived from *m/z* 318 as fragment ions (Fig. 5(c)). The nominal molecular mass (*M*) of the cyanogenic glucosides was therefore deduced to be 295, which is the molecular mass of prunasin and sambunigrin cyanogenic glucosides.

The Ruby Cabernet extract was analyzed with or without the addition of prunasin (20 μg ml^{-1}) by LC–MS in narrow scan mode (*m/z* 300–330). The size of the first eluting peak (19.6–19.8 min) was enhanced in the extract to which prunasin had been added while the late eluting peak (19.9–20.1 min) remained unchanged (results not shown). In the same way, sambunigrin was confirmed to be consistent with the late eluting peak. Furthermore, the two cyanogenic glucoside-like compounds found in the grapevine extracts had mass spectral properties that were consistent with those of the prunasin and sambunigrin reference compounds, confirming their respective identities. By comparing the ion intensity of the corresponding sodium adducts, the ratio of prunasin to sambunigrin in Ruby Cabernet and Carignan was estimated to be 0.22 and 0.33, respectively.

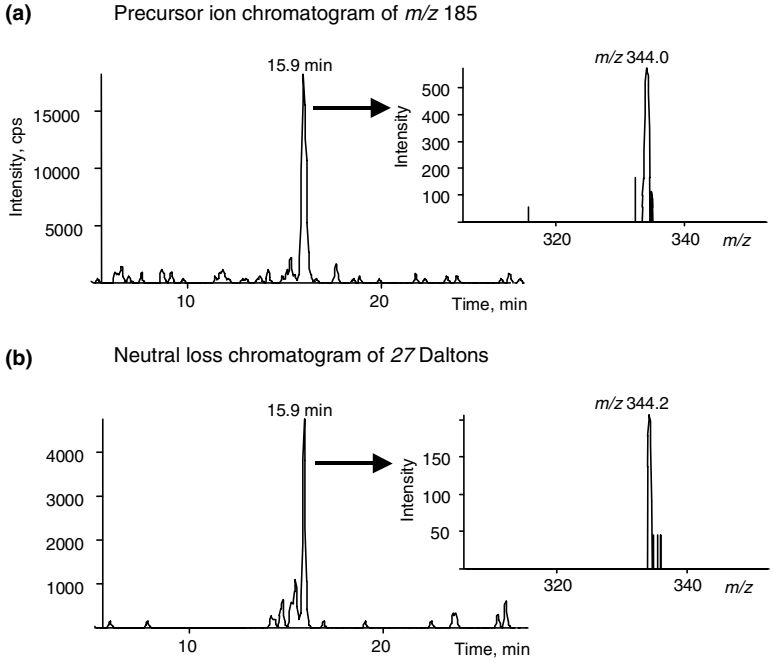

Fig. 4. LC–MS/MS analysis of dhurrin, monitoring for the loss of aglycone as well as HCN. Insets are the mass spectra of the peaks with a retention time of 15.9 min. (a) precursor ion chromatogram of *m/z* 185 showing a single ion with *m/z* 34 (*inset*) eluting at 15.9 min; (b) chromatogram of neutral loss of 27 Da showing a single ion with *m/z* 334 (*inset*) eluting at 15.9 min.

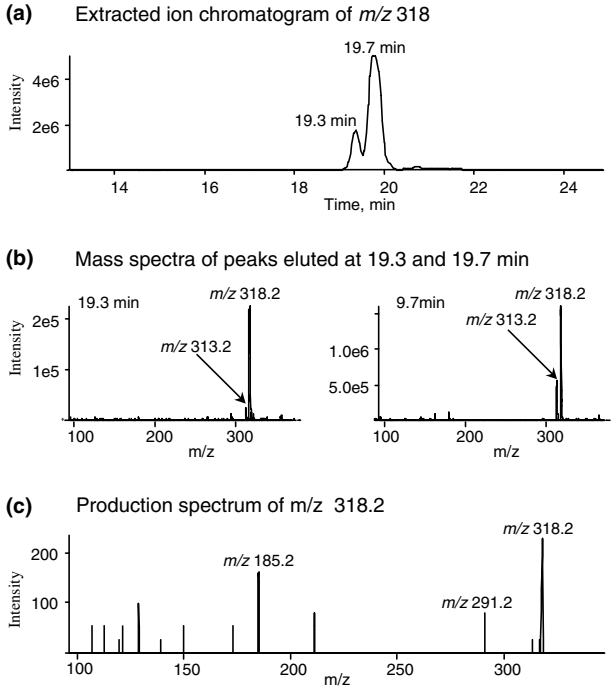

Fig. 5. LC–MS and LC–MS/MS analysis of grapevine Ruby Cabernet leaf extract. (a) LC–MS in scan mode: extracted ion chromatograms of *m/z* 318. (b) Mass spectra of the two peaks in (a). (c) Representative product ion scan of the two peaks in (a).

The presence of prunasin and sambunigrin in diglucosidic forms in the extracts was also investigated by LC–MS in scan mode. An extracted ion chromatogram for *m/z* 480 i.e. [318 + dehydrated glucose (162)]$^+$ also showed a single peak that eluted at 16.9 min in extracts of Ruby Cabernet and Carignan but not Sultana (data not shown). However, the peak with *m/z* 480 could not be characterized by LC–MS/MS due to its insufficient quantity.

2.4. Developmental survey of cyanide potential in grapevine leaves

The cyanide potentials of crude extracts of various Ruby Cabernet tissues (young and mature leaves, roots, tendrils, young and mature petioles, flowers, berries and seeds) were compared for each of three collection times (spring, preveraison and postveraison), except for when data for young petioles at preveraison and tendrils at postveraison was missing (Fig. 6). The mean cyanide potential of leaf extracts (young and mature) ranged from 123 to 329 mg HCN kg^{-1} fr. wt (raw means presented) and was always substantially higher than that of other tissue extracts for which raw mean values ranged from 1 to 14 mg HCN kg^{-1} fr. wt. The cyanide potential of young leaf extracts was significantly higher than that of mature leaves for the spring and postveraison collections, but the cyanide potential of extracts of these two leaf types were not significantly different for the preveraison collection. For each of the respective collection times, extracts from roots (class I), flowers and seeds had cyanide potential significantly higher than that of extracts from tendrils, petioles (young and mature)

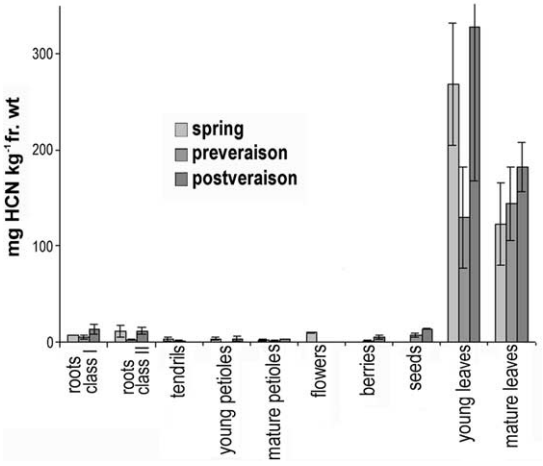

Fig. 6. Cyanide potential (cyanide equivalent to mg HCN kg^{-1} fr. wt) of extracts from various Ruby Cabernet tissues at spring, preveraison and postveraison. Tissue extracts for the three different collection times were assayed in three independent experiments. Values are the mean of three replicates and replicates were assays of extracts prepared from different plants. Bars are ± SD. Values for tendrils at postveraison; flowers at preveraison and postveraison; and berries at spring are absent because the tissues were not available for collection at those times. Data for young petioles at preveraison was not collected. Classification of roots (classes I and II) is described in (Section 4.2).

and berries (i.e. whenever the tissues for comparison were available and collected at that time). Whereas, the cyanide potential of extracts from roots (class II) was only significantly higher than that of extracts from tendrils and petioles (young and mature) at spring and postveraison, mature petioles at preveraison, and berries at postveraison (i.e. when the respective tissues were available and collected). For the preveraison collection, the cyanide potential of root (class II) extracts was not significantly different from that of tendrils, and berries and was significantly less than that of seeds.

Selected extracts were also monitored for prunasin and sambunigrin cyanogenic glucosides using LC–MS/MS in MRM (transition monitored: m/z 318 → m/z 185). While both of these compounds were detected in Carignan and Ruby Cabernet leaf extracts, neither was detected in extracts of Sultana leaf or Ruby Cabernet seed, berry or root (Table 1).

3. Discussion

3.1. Cyanide potential and cyanogenesis in grapevine leaves

Measurement of the cyanide potential (i.e. the total cyanide in β-glucosidase-treated crude, boiled tissue extract) of mature leaves at spring for 12 grapevine (*V. vinifera*) cultivars showed that this species is polymorphic for the trait, with the tested cultivars exhibiting either high cyanide potential (equivalent to ca. 110 mg HCN kg^{-1} fr. wt) or low cyanide potential (ca. 4 mg HCN kg^{-1} fr. wt). The two grapevine cultivars that had relatively high cyanide potential – Carignan and Ruby Cabernet have reasonable international importance in wine grape production and are related, with Ruby Cabernet being the product of a cross between Carignan and Cabernet Sauvignon (which had low cyanide potential).

Release of cyanide from boiled Ruby Cabernet and Carignan leaf extracts was dependent on treatment with an exogenous β-glucosidase and this implies: first, that if any cyanide exists in the tissue in an unbound form, it represents only a minor fraction of the cyanide potential and, second, that cyanogenic glucosides are involved. It is also possible, however, that other types of cyanogenic compounds that are not susceptible to degradation by almond β-glucosidase, also occur in grapevine, but were not detected here.

Our measurement of cyanide potential in grapevine leaves builds on a much earlier report by Deibner (1967) of cyanogenesis from ground leaf material of Carignan but not from three other tested cultivars (Valdiguié rouge, Cinsault rouge and Ternet blanc). Indeed we also observed that ground leaf tissue of Ruby Cabernet is cyanogenic. Hence, the two components of cyanogenesis – cyanide potential plus activity of degrading

Table 1
Detection of cyanogenic glucosides (prunasin and/or sambunigrin) in different grapevine tissues by LC–MS/MS in MRM

Cultivar	Tissue	Season	Prunasin/sambunigrin
Carignan	Mature leaf	Spring	+[a]
Sultana	Mature leaf	Spring	–[a]
Ruby Cabernet	Mature leaf	Spring	+[a]
Ruby Cabernet	Mature leaf	Postveraison	+
Ruby Cabernet	Seeds	Postveraison	–
Ruby Cabernet	Berries	Postveraison	–
Ruby Cabernet	Roots (class I)	Spring	–
Ruby Cabernet	Roots (class II)	Spring	–

Two replicates – which were extracts from different plants were analysed for each tissue type except where indicated otherwise.
Classification of roots (classes I and II) is described in (Section 4.2).
[a] A single replicate was analysed.

enzymes occur coincidently in Carignan and Ruby Cabernet leaves. This capacity for cyanide release from ground leaf tissue over an extended (overnight) period may, however, have limited biological relevance because an immediate cyanogenic response upon tissue disruption is likely to be more applicable to interactions with invasive organisms.

Interestingly, Deibner (1967) found that the amount of cyanide released from ground (but not boiled) Carignan leaves was enhanced with the addition of exogenous β-glucosidase (emulsin from almond). We have also observed this effect for Ruby Cabernet (results not shown). An implication of these observations is that there are at least two different kinds of cyanogenic compounds in leaves of these grapevine cultivars and these compounds are differentially sensitive to degradation by glucosidases. Therefore, in nature, the full cyanide potential of Carignan and Ruby Cabernet would not be realised without the action of (e.g. insect borne) exogenous degrading enzymes. It is also possible that at least one direct function of cyanogenic compounds in grapevine is not related to the release of free and toxic cyanide.

3.2. A novel method for screening for cyanogenic glucosides

A novel method was used to screen for and identify two major cyanogenic glucosides (prunasin and sambunigrin) in minimally purified grapevine leaf extracts. To our knowledge, LC–MS/MS in combination with neutral loss and precursor ion scans has not previously been used to screen for cyanogenic glucosides. Without the need to selectively purify extracts, this method improves on previous approaches through its simplicity. Similar approaches would also be appropriate for detecting non-glucosidic cyanogenic glycosides.

Prunasin and sambunigrin are epimers that are apparently derived from L-phenylalanine (Møller and Seigler, 1999). Their occurrence together – both as major components within a single tissue source has been previously reported (e.g. Seigler et al., 2002; Miller et al., 2004), albeit infrequently. Even so, it needs to be acknowledged that artifacts arising from extensive epimerisation during extraction, storage and/or analysis of samples may affect observations. At least we can partly exclude this possibility with respect to our analysis because when the prunasin standard was analysed, sambunigrin was absent by comparison.

3.3. Cyanide potential in different grapevine tissues

Accumulation of cyanogenic glucosides is evidently developmentally regulated in grapevine because cyanide potential measured at three times over the growing season in crude, boiled extracts of young and mature leaves, petioles, tendrils, flowers, berries, seeds and roots of Ruby Cabernet was substantially higher in the leaves compared with all other tissues. It is possible that the enzyme-based assay for cyanide potential was inhibited by compounds such as tannins (Goldstein and Spencer, 1985), which may occur at different levels in the different tissues. Therefore, selected extracts were screened for prunasin and sambunigrin using LC–MS/MS in MRM. The analysis confirmed that, in comparison with leaves, accumulation of these cyanogenic glucosides is negligible in seeds, berries and roots in Ruby Cabernet, but the possibility that other cyanogenic compounds are present in these tissues remains to be excluded.

The cyanide potential that we have measured in leaves of Carignan and Ruby Cabernet is in the same range as that measured in the edible root pith of some cultivars of cassava (up to ca. 230 mg HCN kg^{-1} fr. wt; Bokanga, 1994) – which, in the tropics, is a human staple sometimes associated with acute and chronic cyanide poisoning. This is noteworthy because grapevine leaves feature in the diet of some cultural groups.

Cyanogenic glucosides and cyanogenesis have been implicated in exacerbation of fungal infections of plants (Lieberei, 1986; Pourmohseni and Ibenthal, 1991) acting, for example, through cyanide-mediated interference with the defense response (Lieberei et al., 1989). Consequently, we speculate that accumulation of cyanogenic glucosides in leaves of Ruby Cabernet and Carignan is related to anecdotal reports that these cultivars are particularly susceptible to fungal diseases compared with other *V. vinifera* cultivars (e.g. Kerridge and Antcliff, 1999).

4. Materials and methods

4.1. Chemicals

The dhurrin standard used to develop the method for screening and for identifying cyanogenic glucosides was obtained from Professor Birger Møller (Royal Veterinary and Agricultural University, Denmark). The prunasin reference compound was obtained from Sigma and a mixture of prunasin and sambunigrin was generated by treating prunasin (0.1 mg ml^{-1}) at pH 11 for 1 h at 25 °C, as described by Chassagne et al. (1996).

4.2. Plant material and sampling

For the survey of different grapevine cultivars and different Ruby Cabernet tissues, plant material was collected during the 2000–2001 growing season from established grapevine plants in the Coombe vineyard at The University of Adelaide, Waite Agricultural Research Institute, Urrbrae, South Australia. Unless otherwise indicated, three extracts for three replicated assays of

each tissue were prepared from three different plants. Definitions of tissue types were as follows: a young leaf was the third fully expanded one, counting from the shoot tip and a mature leaf was the fourth from the shoot base. Young and mature petioles were detached from young and mature leaves, as defined above, and tendrils were opposite young leaves. For extract preparation from young leaves, tendrils and petioles, one entire structure was ground. For mature leaves, only a fraction (1/8–1/2) of the leaf was sampled for extract preparation. Roots designated 'class I' had diameters up to ca. 3 mm and roots designated 'class II' had diameters greater than ca. 5 mm and the extracts were prepared from a number of pieces collected from different roots. All of the flowers from a single bunch were combined for extract preparation and berry extracts were prepared from ca. 30 berries (sampled from a single bunch) after seeds had been removed. Those seeds were pooled to prepare seed extract.

For the cultivar survey, mature leaves were collected in spring ca. 76 days before veraison (when ca. 50% of berries were coloured red) of Ruby Cabernet. For the survey of different Ruby Cabernet tissues, the spring collection was made over a period of a week, 63–70 days before veraison. Flowers were collected – at the end of that period – from bunches from which caps had dropped from ca. 30–40% of flowers. The preveraison collection was made over two days, ca. 18 days before veraison. The postveraison collection of tissues was made at either 22 days postveraison (leaves and petioles) or 32 days postveraison (roots, berries and seeds). At this time, the mean total soluble solid content of berries in the three bunches that were sampled ranged from 19° (SD = 1.8, $n = 8$) to 23° Brix (SD = 0.6, $n = 10$). Bunches of such berries would generally be considered almost ripe for harvest.

4.3. Assays for cyanide potential and cyanide liberation – extract preparation and statistical analysis of data

Fresh plant material was ground to a fine powder in liquid N_2 using a mortar and pestle or – for berries, roots and seeds – using an electric coffee grinder followed by a mortar and pestle. The ground tissue (11–190 mg) was transferred to a precooled microfuge tube and boiled for 10 min in ×10 volume of 85% (v/v) methanol (i.e. 1 ml 100 mg^{-1}). For extraction of tissues for the varietal survey, the microfuge tubes used for boiling extracts were 2 ml capacity Safe-Lock (Eppendorf) with lid clamp, and volume loss during boiling was adjusted afterwards by weight. Otherwise, the microfuge tubes used for boiling extracts were 1.5 ml capacity screw cap tubes with O-ring (Quality Scientific Plastics), which suffered negligible volume loss during boiling. Determination of cyanide potential was by a method similar to that described by Busk and Møller (2002), modified from Halkier and Møller (1989). Extract (100 μl) or extract diluted in 85% (v/v) methanol was added to 200 μg of β-glucosidase from almond (Sigma) in 400 μl of 50 mM MES (pH 6.5) and incubated at 30 °C for 11/2 h. Afterwards, 80 μl of NaOH (6 M) was added and the sample was incubated for a further 30 min at room temperature. Reference KCN solutions (in 50 mM MES, pH 6.5) were treated in the same way as the extracts. Cyanide concentration was determined using the method of Halkier and Møller (1989) except that all reagent volumes were doubled and absorption was measured at 585 and 750 nm using either a Novaspec II (Pharmacia Biotech) or a SmartSpec™ 3000 (Bio-Rad) spectrophotometer.

For the cultivar survey, three sets of replicate extracts were assayed for cyanide potential in three independent experiments. For the survey of different Ruby Cabernet tissues, the sets of replicated extracts for each of the three collection times were assayed for cyanide potential in three independent experiments.

For comparison of cyanide liberation from boiled extracts with or without the addition of β-glucosidase, 100 μl of extract, prepared by boiling in 85% methanol exactly as described above, was mixed with 900 μl of 50 mM MES pH 6.5 (±450 μg β-glucosidase, as appropriate) and incubated at 30 °C for 11/2 h. After incubation, 160 μl of NaOH (6 M) was added and the sample was incubated for a further 30 min at room temperature. Reagents for cyanide-specific colour development were added directly to the mixture, exactly as described above.

For the cyanogenesis assay, leaf tissue was ground to a fine powder in liquid N_2, then ca. 20 mg was mixed immediately with 1 ml of 50 mM MES, pH 6.5, in a microfuge tube and incubated overnight at 37 °C. After incubation, 160 μl of NaOH (6 M) was added to the tube and the sample was incubated for 30 min at room temperature. The tube containing mixture was centrifuged (12,000*g*) for 2 min and 580 μl of the supernatant was assayed for cyanide-specific colour development, by addition of reagents exactly as described above.

Data (transformed as appropriate) was analysed using a General Analysis of Variance (Genstat 6th edition software), testing for significance at the 5% level and assuming that the treatments (i.e. different tissues) were independent.

4.4. Screening and confirmation of cyanogenic glucosides by LC–MS and LC–MS/MS

Sample preparation: for the initial detection and identification of cyanogenic glucosides, leaves (2 g) were ground to a fine powder in liquid nitrogen then boiled for 10 min in ×10 volume of 85% (v/v) methanol. The extract was centrifuged at 3000*g* for 10 min and the supernatant was vacuum dried at 45 °C. The extract was

resuspended in 8 ml water and centrifuged at 3000*g* for 2 min. Supernatant (5 ml for Carignan and 8 ml for Ruby Cabernet and Sultana) was purified by reversed phase extraction using a C18 Sep-Pak® column (1 g bed; Waters), eluting with of 20% methanol (15 ml). The central third of the column eluent was recovered and a portion (0.4–0.5) of this was concentrated by vacuum drying at 60 °C (to ca. 150 μl) before analysis. Otherwise, tissue extracts (i.e. after grinding and boiling in ×10 volume of 85% (v/v) methanol; Section 4.3) were purified as described above, except that an equivalent of ca. 55 mg of tissue was applied to a C18 column (Alltech "Maxi-Clean" or Waters SePak® Plus - 300 mg bed) and the entire eluent (2 ml; 20% methanol) was collected and adjusted to 85 μl, which is the equivalent of 0.6 mg tissue (original fw) μl^{-1}.

LC conditions: a 20 μl aliquot of the extract sample was injected by an autosampler (HP1100, Agilent) and separated by a Synergi 4μ Hydro-RP 80Å (2.1 × 150 mm: Phenomenex) LC column. The method used was a binary gradient with mobile phases containing 50 μM sodium acetate (A) and methanol (B). The elution conditions were as follows; 200 $\mu l\,min^{-1}$, linear gradient from 10%B to 50%B in 30 min, from 50%B to 80%B in 10 min and 80%B for 5 min. The eluent from the LC was split using a T-piece and delivered at 22.5% of the total flow (200 $\mu l\,min^{-1}$) to the mass spectrometer and at 77.5% to an UV detector (HP1100, Agilent), monitoring wavelengths 230 and 280 nm.

MS and MS/MS conditions: mass spectrometric analysis was carried out with a PE-Sciex API-300 triple quadrupole mass spectrometer equipped with an electrospray ion source (MDS-Scies, Concord, ON, Canada). The electrospray needle, orifice and ring potentials were set at 5000, 40 and 250 V, respectively. The curtain gas (nitrogen) and nebulizer (air) gases were set at 8 and 12, respectively. Positive ion mass spectra were recorded in appropriate mass ranges (*m*/*z* 50–1000 or *m*/*z* 300–330 for narrow scan).

For LC–MS/MS, nitrogen gas was used as a collision gas (set at 2 units) and the collision energy was optimized in a range of 30–50 V. LC–MS/MS in neutral loss, precursor ion and product ion scan modes were carried out with a step size of 0.2 Da and a dwell time of 2 ms in appropriate mass ranges. LC–MS/MS in multiple reaction mode (MRM) was carried out with a dwell time of 200 ms.

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