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Cyanogenic *Eucalyptus nobilis* is polymorphic for both prunasin and specific β-glucosidases

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Abstract

Cyanogenesis (i.e. the evolution of HCN from damaged plant tissue) requires the presence of two biochemical pathways, one controlling synthesis of the cyanogenic glycoside and the other controlling the production of a specific degradative β-glucosidase. The sole cyanogenic glycoside in *Eucalyptus nobilis* was identified as prunasin (D-mandelonitrile β-D-glucoside) using HPLC and GC–MS. Seedlings from three populations of *E. nobilis* were grown under controlled conditions and 38% were found to be acyanogenic, a proportion far greater than reported for any other cyanogenic eucalypt. A detailed study of the acyanogenic progeny from a single open-pollinated parent found that 23% lacked a cyanogenic β-glucosidase, 32% lacked prunasin and 9% lacked both. Of the remaining seedlings initially identified as acyanogenics, 27% contained either trace amounts of β-glucosidase or prunasin, while 9% contained trace amounts of both. Results support the hypothesis that the two components necessary for cyanogenesis are inherited independently. Trace amounts are likely to result from the presence of non-specific β-glucosidases or the glycosylation of the cyanohydrin intermediate by non-specific UDP glycosyl transferases.

Keywords: *Eucalyptus nobilis* (Myrtaceae); White gum; Chemical ecology; Chemotaxonomy; Polymorphism; Cyanogenic glycoside; Prunasin; β-glucosidase; Cyanogenesis; Defence

1. Introduction

Cyanogenesis is the process by which plants and other living organisms liberate hydrogen cyanide (HCN) upon tissue disruption (Møller and Seigler, 1999). When cyanogenic plants are chewed by herbivores, an endogenous cyanogen (usually a cyanogenic glycoside) is brought into in contact with a β-glucosidase, hydrolysing the molecule and ultimately liberating toxic HCN (Møller and Seigler, 1999). Cyanide, a respiratory toxin, can cause acute poisoning, but in cyanogenic plants it functions primarily as a feeding deterrent against generalist herbivores (Gleadow and Woodrow, 2002a).

In the important Australian genus *Eucalyptus*, there is evidence that up to 30 of the 600 species are cyanogenic (Finnemore et al., 1935; Gleadow and Woodrow, 2000b; Goodger et al., 2002; Goodger and Woodrow, 2002; E.E. Conn, personal communication), although only three species have been studied in detail (*E. cladocalyx*, *E. polyantemos* and *E. yarraensis*). Two important findings emerged from these latter studies. First, prunasin [(*R*)-mandelonitrile β-D-glucoside] was the only cyanogen found in the three species, and in mature trees it was almost entirely located in the foliage. Second, similar to many other cyanogenic species (e.g. Aikman et al., 1996; Schappert and Shore, 2000), the concentration of cyanogen and the capacity for cyanogenesis was highly variable between individual trees, even within single populations (Gleadow and Woodrow, 2000b; Goodger et al., 2002; Goodger and Woodrow, 2002). What is different between these eucalypts and many other cyanogenic species, however, is the rarity of acyanogenic individuals. This may indicate a difference in the genetic mechanism controlling cyanogenic capacity or, under field conditions, strong selection against acyanogenic individuals, or a combination of both.

Here we describe cyanogenesis in *Eucalyptus nobilis* L. Johnson and K. Hill and show that it is unique amongst cyanogenic eucalypts in having a relatively high inci-

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dence of acyanogenesis. More specifically, we identify the cyanogen in this species and quantify the degree to which acyanogenesis is due to the lack of a cyanogen and a lack of the enzymatic capacity to hydrolyse the cyanogen. Understanding cyanogenesis in this species is important because *E. nobilis* is planted extensively for timber and paper production throughout the world (Swan, 2001) and cyanogenic capacity is most likely a significant effector of productivity. Moreover, *E. nobilis* is classified as endangered in its native habitat in the New England area of NSW, Australia (Anon., 2002) and understanding the polymorphic nature of the species will be important for its conservation.

2. Results and discussion

2.1. Identification of the cyanogenic glycoside

The cyanogen in *E. nobilis* foliage was found to be prunasin [(*R*)-mandelonitrile β-D-glucoside). This conclusion is based on the observation that (1) the cyanogenic fraction from the foliage co-eluted with authentic prunasin using reversed-phase HPLC and, following derivatisation, GC, and (2) the mass spectrum of the derivatised cyanogenic fraction was identical to that of the derivatised standard. There was no evidence for the presence of the related diglycoside, amygdalin [(*R*)-mandelonitrile 6-*O*-β-D-glucosido-β-D-glucoside], which can be separated from prunasin using the HPLC protocol described here. GC–MS analysis did, however, detect minute traces of the epimer sambunigrin [(*S*)-mandelonitrile β-D-glucoside] in both the authentic prunasin supplied by Sigma (98% pure) and the leaf extract. It is possible, however, that this was an artefact of the extraction protocol (E.E. Conn, personal communication).

Prunasin has also been identified in *E. cladocalyx*, *E. polyanthemos* and *E. yarraensis* and it is, therefore, the only cyanogenic glycoside found in *Eucalyptus* so far (Finnemore et al., 1935; Goodger et al., 2002; Goodger and Woodrow, 2002; E.E. Conn, personal communication). All cyanogenic eucalypts belong to the subgenus *Symphyomrytus*. This provides evidence of the relatedness of species within this group.

2.2. Cyanogenic polymorphism

Eucalyptus nobilis is polymorphic with a relatively high proportion of completely acyanogenic plants (Table 1). A total of 135 *E. nobilis* seedlings from three populations were screened for cyanogenesis. Cyanide concentration varied widely, from zero to 153 μg CN g^{-1} dry weight (dw), and there was no significant difference in the mean cyanide concentration between the three progeny groups with an overall mean of 57 ± 7 μg CN g^{-1} ($P = 0.158$, Table 1). Plants returning negative or extremely weak positive tests (< 10 μg CN g^{-1} dw) were classified as acyanogenic (Shore and Obrist, 1992). Considering the cyanogenic plants on their own, the frequency distribution of the cyanogenic glycoside concentration was not significantly different from normal (Kolmogorov–Smirnov test $P = 0.09$; Skewness = 0.83; Fig. 1). Moreover, total leaf nitrogen, a known determinant of leaf cyanogenic capacity (Gleadow and Woodrow, 2000a), was similar in all seedlings with an average (±1SE) of 1.8% ± 0.2.

Table 1
Proportion of acyanogenic trees, and average concentration of prunasin (measured as evolved cyanide) in cyanogenic seedlings of *Eucalyptus nobilis* raised from seed from three different areas of the northern tablelands on NSW

Population	*N*	Cyanide (±1 SE) (μg g^{-1})	Acyanogenic (%)
Nundle	75	55 (11)	35
Kapatar	25	35 (12)	56
Styx	35	67 (8)	20
Overall	135	57 (7)	36

Plants were classified as acyanogenic if < 10 μg CN g^{-1} dry weight was evolved from leaf tissue.

In total, 36% of seedlings were acyanogenic with some variation between the three progeny groups, from 20% in the Styx River group to 56% in the Mt. Kapatar group (Table 1). This proportion of acyanogenics is between 10- and 30-fold higher than found for any naturally growing population of eucalypts (Gleadow and Woodrow, 2000b; Goodger et al., 2002; Goodger and Woodrow, 2002; Woodrow et al., 2002). No progeny trials have yet been undertaken using these other species. The high incidence of acyanogenic plants is

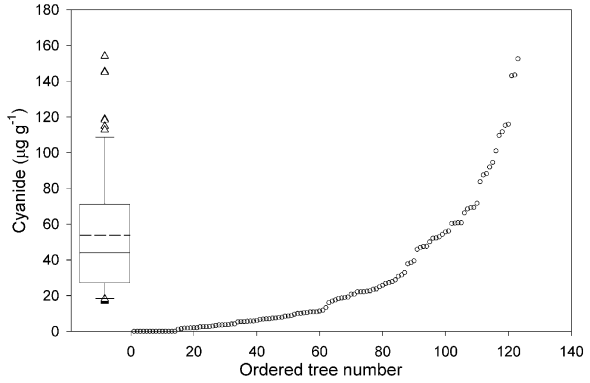

Fig. 1. Cyanogenic status of *Eucalyptus nobilis* seedlings raised from mixed seed from three different areas of the northern tablelands on NSW. Values were ordered according to concentration. The box plot shows the median (solid line) and mean (dashed line) cyanide concentration of all cyanogenic individuals (i.e. > 10 μg CN g^{-1} dw). The boundaries above and below the box mark the 25 and 75th percentiles, respectively. Error bars indicate the 90 and 10th percentiles, respectively.

unlikely to be a developmental effect, although at least one eucalypt changes markedly with development (Goodger et al., 2002).

Until recently *E. nobilis* was considered to be a seven-budded form of *E. viminalis*, which was thought to be polymorphic with respect to cyanogenic capacity. All the cyanogenic *E. viminalis* tested by Finnemore et al. (1935), however, were collected in an area dominated by what is now called *E. nobilis*. It is possible, therefore, that references to cyanogenic forms of *E. viminalis* may have involved what is now known to be *E. nobilis*. Interestingly, "seven budded *E. viminalis*" has been noted to be more resistant to snout beetle in plantations in South Africa than the typical three-budded form (Darrow, 1994). If this is true, it may be that cyanogenic *E. nobilis* would be a better choice for some forestry applications than *E. viminalis*.

2.3. Detailed analysis of acyanogenic seedlings

Detailed analysis was made of seedlings raised from seed collected from a single, open-pollinated tree at Nundle. Of the 60 seedlings tested, 22 were initially found to be acyanogenic (Table 2). Of these, five had cyanogenic glycosides, but lacked a specific cyanogenic β-glucosidase and seven had the β-glucosidase, but lacked the cyanogenic glycoside. Only two lacked both the glycoside and the β-glucosidase. Further testing of the eight remaining acyanogenic plants using very large tissue samples (1 g) revealed that they contained trace amounts of prunasin and/or β-glucosidase, which yielded the positive result (Table 2). As a negative control for these experiments, we used acyanogenic *Eucalyptus fasciculosa* leaves, which always returned completely negative results with the addition of prunasin or β-glucosidase, even when 1 g of tissue was used.

Table 2
Cyanogenic status of *Eucalyptus nobilis* seedlings

Cyanogenic status	*N*	β-glucosidase	Prunasin	Alleles
Cyanogenic	38	+	+	*Ac–Li–*
Acyanogenic	22			
Genotype	5	–	+	*Ac–lili*
	7	+	–	*AcacLi–*
	2	–	–	*Acaclili*
Other[b]	3	Trace	–	
	3	–	Trace	
	2	Trace	Trace	

Seedlings were the progeny of a single, open pollinated parent from Nundle, NSW. Plants were classified as acyanogenic if <10 μg CN g^{-1} dry weight was evolved from leaf tissue. The proposed allelic composition is based on the mendelian model developed for *Trifolium repens* (Corkhill 1942), where Ac and Li control the presence of cyanogenic glycosides and the the degradative β-glucosidase, respectively.[a] Acyanogenic plants would be expected to be homozygous recessive at one or both loci.

[a] The assigning of these alleles to *E. nobilis* is based on the assumption that the principles of biosynthesis and degradation of cyanogenic glycosides are similar in all cyanogenic plants, while recognising that different glycosides are involved.

[b] These plants returned a weak positive result (ca. 5 μg g^{-1}) when the amount of tissue tested was increased 10 fold (0.2 g) above that used in a typical analysis (0.02 g).

The trace amounts of prunasin in some of the samples may be the result of non-specific enzyme activity. Research on *Sorghum bicolor* has shown that dhurrin synthesis from tyrosine involves (1) cyanohydrin production by two membrane-bound cytochrome P450 dependent monooxygenases, followed by (2) stabilisation of the cyanohydrin by glucose addition, which is catalysed by a soluble UDP–glucose glucosyl transferase (Kahn et al., 1997; Jones et al., 1999). Assuming that prunasin synthesis from phenylalanine is similar, it is possible that the acyanogenic seedlings with trace amount of prunasin express functional P450s, but lack the specific glycosyl transferase required to stabilised the cyanohydrin. Low amounts of glucose addition to the cyanohydrin could be catalysed by other non-specific glycosyl transferases (Jones et al., 1999).

Non-specific catalysis may also account for the trace levels of β-glucosidase activity found in some samples. While cyanogenic β-glucosidases are often extremely specific (e.g. Hösel and Nahrstedt, 1975), most cyanogenic glycosides can be broken down to some extent by other, non-specific β-glucosidases. In *Hevea brasiliensis*, for example, the β-glucosidase essential for cyanogenesis is also important in lignin synthesis (Selmar et al., 1987).

3. Concluding remarks

Our results show that the ability to synthesise cyanogenic glycosides and β-glucosidases in *Eucalyptus* is inherited independently. However, the range of phenotypes observed for the cyanogenic plants requires a more complex explanation. Several have been put forward. First, there may be incomplete dominance (e.g. Gorz et al., 1986). Second, the pattern of inheritance may be quantitative with multiple copies of each gene adding together to express the phenotype in proportion to the number of dominant alleles; either in an additive manner, by different forms of the functional alleles or by modifier genes (e.g. Shore and Obrist, 1992; Hu and Poulton, 1997). Third, the expression of the phenotype may be modified by the environment (Hughes, 1991; Gleadow and Woodrow, 2002b). The latter can be ruled out in this study as plants were grown under identical conditions and did not differ significantly in leaf nitrogen concentration.

Conserving allelic diversity is becoming an important consideration (Ryder, 2002). The presence of a stable

polymorphism in *E. nobilis* suggests that the different phenotypes have both advantages and disadvantages—if there were no cost to the production of cyanogenic glycosides, then the defensive traits would become fixed in the population. Cyanogenic plants have slower growth rates and reduced reproductive output in several species (e.g. Briggs and Schulz, 1990; Kakes, 1997; Schappert and Shore, 2000), indicating that there is significant cost in the production of the biosynthetic machinery and deployment of the cyanogenic glycosides. The endangered status of *Eucalyptus nobilis* in its natural habitat makes the conservation of the different genotypes all the more important.

4. Experimental

4.1. Species description

Eucalyptus nobilis L. Johnson and K. Hill (white gum) has recently been classified as a distinct species (Brooker et al., 1997). It is smooth barked, except for the base, and is restricted in its distribution to the northern tablelands of New South Wales, in eastern Australia (Brooker et al., 1997), where it is classed as endangered. Trees are tall (generally 70 m), and they were recently found to be the tallest in that State (79.22 m; Ponder, 1997).

4.2. Plant material and growing conditions

Seed of *Eucalyptus nobilis* was obtained from one open pollinated cyanogenic tree growing near Nundle, NSW (31° 27′ S 151° 15′ E; elevation 1000 m; Australian Tree Seed Centre No. 19806/AS 283). For comparison, mixed seed was obtained from a further two populations from Mount Kapatar National Park (Australian Tree Seed Centre, No. 19805; 30° 17′ S 150° 08′ E; elevation 1250 m) and the Styx River State Forest (Australian Tree Seed Centre No. 15099; 30° 29′ S 152° 18′ E; elevation 1200 m). Seedlings were raised in 2-l pots containing perlite:vermiculite:sand:pine bark (1:1:1:1), with 4 g complete slow-release fertilizer (Osmoscote®) and watered daily. Plants were grown under natural light in a glasshouse with an average photoperiod of 10 h (February–June). The air temperature and relative humidity of the glasshouse (measured every 15 min) were 23.65±1.48 °C and 52.0±2.6% (± 1SD), respectively. Mean daytime PPFD (measured every 10 min with a Li-Cor 190 SA visible light sensor; 8 am–5 pm; ±1SD) was 157±104 μmol m^{-2} s^{-1}, with a maximum of 583 μmol m^{-2} s^{-1}.

Plants were harvested when they were 15-20 cm tall and had 10 fully expanded leaves (ca. 4 months old). All fully-expanded leaves on one side of each plant were removed, snap-frozen in liquid nitrogen and freeze-dried. Leaves were ground in a cooled IKA Labortechnic A10 microgrinder (Janke and Kunkel GmbH Co, KG, Germany) and stored in a desiccator at −20 °C. Remaining leaves were used for identification of the cyanogenic glycoside (see later).

4.3. Testing for cyanogenesis

Cyanogenic glycoside concentration of leaf tissue and fractions was determined by measuring the amount of HCN evolved after hydrolysis. Freeze-dried leaf tissue (0.02 g) was incubated in 1 ml 0.1 mM citrate buffer (pH 5.5) in sealed glass vials for 20 h at 37 °C (Gleadow and Woodrow, 2000a,b). Evolved cyanide was trapped in a NaOH well and assayed using a miniaturised version of the method of Brinker and Seigler (1989; Woodrow et al., 2002). The amount of cyanide (CN) detected by this method is a measure of the amount of cyanogenic glycoside in the tissue and in this paper is referred to as "cyanide".

If no cyanide was detected then samples of leaf tissue (0.02 g) were retested with either exogenous β-glucosidase [2.5 units ml^{-1}; *Prunus amygdalis* (L.) Benth. and Hook.; β-D-glucoside glucohydrolase; EC 3.2.1.21, Sigma-Aldrich], prunasin [10 μl 50 mM (*R*)-Mandelonitrile β-D-glucoside, Sigma-Aldrich], or both, added to the incubation vials. Previous experiments have shown that almond emulsin is capable of hydrolysing the cyanogenic glycosides from *Eucalyptus cladocalyx* F. Muell. and a variety of other cyanogenic mono- and di-glycosides (Fox, 1997). If material still appeared acyanogenic, the amount of leaf tissue was increased 10-fold (0.2 g) and the process repeated. As a final check, 1.0 g of freeze-dried tissue from putative acyanogenic plants was incubated with 2 ml of buffer. Acyanogenic *Eucalyptus fasciculosa* F. Muell. leaf tissue was included as a control and always gave negative results when incubated with buffer alone, buffer plus β-glucosidase or buffer plus prunasin, but yielded a positive result when both β-glucosidase and prunasin were added. The amount of cyanide produced as the result of ethylene biosynthesis was, therefore, assumed to be negligible.

4.4. Identification of cyanogenic glycosides

4.4.1. Extraction

Leaves from twenty 6-month-old cyanogenic *E. nobilis* seedlings were pooled, frozen in liquid nitrogen, freeze-dried, finely ground and the cyanogenic glycosides extracted (see Goodger et al., 2002). Leaf tissue (35 g dw) was defatted with petroleum spirit, and filtered (Whatman® 541). The lipid-free extract was treated with cold methanol (2 ml g^{-1} dw). After filtering, water and chloroform was added (2:4:1, v/v/v, MeOH:-H_2O:$CHCl_3$). The methanol phase was collected, evaporated and resuspended in 20 ml water. The

concentrated extract was fractionated by elution through solid-phase C-18 extraction cartridges (Alltech Maxi-Clean™, 900 mg) using a 0–100% MeOH–H_2O gradient (1 ml min^{-1}). Fractions were collected, concentrated in vacuo and freeze-dried. Complete hydrolysis of cyanogenic glycosides in fractions was achieved by resuspending each freeze-dried fraction in 1 ml 0.1 mM citrate buffer (pH 5.5) containing 2.5 units ml^{-1} almond emulsin and incubated in vials, as earlier. Fractions containing cyanogenic glycosides were further purified by HPLC.

4.4.2. Purification

The cyanogenic glycoside extract was fractionated isocratically using a Phenomenex Luna RP-18 column (250 mm × 4.6 mm × 5 μm particle size) with 20% MeCN–H_2O (1 ml min^{-1}) as the eluent and monitored at 262 nm. Fractions were concentrated in vacuo and tested for the presence of cyanogenic glycosides. The fraction containing cyanogenic glycosides was associated with a single peak (17–18 min).

4.4.3. GC–MS

Cyanogenic glycosides (0.2 mg) purified by HPLC were dissolved in 200 μl Tri-Sil® Reagent (HMDS:TMCS:Pyridine, 2:1:10) (Pierce), incubated in a closed vial for 2 h at room temperature and analysed using GC–MS (Lechtenberg et al., 1999; Buhrmester et al., 2000). Aliquots (1 μl) were injected at 315° C onto a column (HP5MS Hewlett-Packard; 30 m × 0.25 mm × 0.25 μm) and separated using a temperature program increasing from 200 to 315 °C, with a gradient of 5° min^{-1} and a flow rate of 1.3 ml min^{-1}. The spectrum was analysed and compared with 0.5 mg authentic prunasin derivatised in 400 μl Tri-Sil reagent, as earlier.

4.5. Leaf nitrogen

Total concentrations of nitrogen and carbon in 5-mg samples of freeze-dried leaves were measured with a Perkin-Elmer 2400 Series II CHNS/O Analyser (Perkin-Elmer Corporation, Norwalk, CT, USA) with high-purity acetanilide as the standard.

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