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Polymorphism in cyanogenic glycoside content and cyanogenic b-glucosidase activity in natural populations of *Eucalyptus cladocalyx*

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Abstract. Cyanogenesis is the process by which plants release hydrogen cyanide (HCN) from endogenous cyanide-containing compounds and is thought to play a role in plant defence against generalist herbivores. Cyanogenesis is poorly understood in natural populations, and has been little studied in tree species. In this paper we present the first systematic survey of cyanogenesis in the economically and ecologically important genus *Eucalyptus*. We document variability in both the concentration of the cyanogenic glycoside, prunasin, and the accompanying degradative b-glucosidase in a woody plant for the first time. Leaves of 96 *E. cladocalyx* F. Muell. trees growing in natural populations on Kangaroo Island, South Australia were analysed. All trees were cyanogenic, containing both cyanogenic glycosides and active b-glucosidase. Cyanogenic glycoside concentration varied by over two orders of magnitude. The b-glucosidase activity varied widely as well, but plants high in cyanogenic glycosides did not necessarily have higher enzyme activity. A significant proportion of the variation in cyanogenic glycoside concentration can be accounted for by the variation in leaf nitrogen. Most of the variation, however, appears to be the result of genetic polymorphism, which is inherited independently of the level of activity of the degradative b-glucosidase.

Keywords: cyanide, eucalypt, Kangaroo Island, herbivore defence, prunasin.

Introduction

Cyanogenesis has been detected in over 2000 species from over 130 families with representatives from all major vascular plant taxa (Jones 1988, 1998; Hughes 1991). Endogenous cyanogens found in plants are most commonly cyanogenic glycosides, which comprise an a-hydroxynitrile stabilised by a glycosidically linked sugar moiety (Conn 1991). The basic two-step process of cyanogenesis is apparently the same in all plants and is usually initiated by tissue disruption. First, the sugar moiety is cleaved from the cyanogenic glycoside. The cyanohydrin is then degraded to produce the respiratory toxin, hydrogen cyanide (HCN), and an aldehyde or a ketone. Consistent with the toxicity of the latter compounds, there is now substantial evidence that cyanogenic glycosides are primarily involved in defence against generalist herbivores, including mammals, insects and molluscs (Nahrstedt 1985; Conn 1991; Jones 1998). As with all defence systems, however, specialist herbivores have evolved mechanisms to detoxify cyanide and are able to feed on cyanogenic plants, although this may incur a significant metabolic cost (Jones 1988).

There is appreciable evidence, particular for herbaceous species, that cyanogenic potential (i.e. the amount of HCN released from cyanogenic glycosides) varies widely between species and between individuals within a species (e.g. Till 1987; Jones 1998). Cassava (*Manihot esculenta* Crantz), for example, has both low and high cyanogenic forms (McMahon *et al.* 1995). Other species such as white clover (*Trifolium repens* L.) not only have a range of cyanogenic potentials but also have at least some cyanogenic individuals; as such they are truly polymorphic (e.g. Pederson *et al.* 1996; Thomsen and Brimer 1997).

The precise genetic control of cyanogenesis varies between species. Extensive studies on *Trifolium repens* point to a modified dihybrid Mendelian system of inheritance controlled by alleles of two independently segregating loci (Ac, Li) that govern inheritance of the cyanogenic glycoside (i.e. the capacity for biosynthesis) and the degradative b-glucosidase, respectively (Hughes 1991). This accounts for the existence of acyanogenic forms in some species, which are either deficient in the cyanogenic glycoside or the b-glucosidase, or a combination of both. However, the character appears to be inherited quantitatively in *Sorghum* species (Poaceae; Gorz *et al.* 1986).

Just how such polymorphism is maintained in natural populations is not understood, although it is likely that the costs and benefits of cyanogenesis to growth and reproduction are important. In order for different levels of cyanogenesis to be favoured by selection, the advantage of defence in terms of overall increased growth and reproductive fitness must be at least balanced by the metabolic cost of synthesis-

Abbreviations used: DBH, diameter at breast height; SA, South Australia; SLW, specific leaf weight; dw, dry weight.

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ing, storing and maintaining the defensive compounds (Kakes 1997). Environmental conditions are apparently important in affecting this balance. Variation in the frequency of acyanogenic individuals in populations of a single species has been correlated with variation in environmental variables such as temperature (Jones 1988), soil moisture (Foulds 1982), and substrate type (Briggs 1990). It is apparent from these studies that environments with high frequencies of cyanogenic individuals are those where either the costs of synthesis are relatively low (e.g. high soil nitrogen) or the benefits are high (e.g. high herbivore pressure).

Understanding the genetics of cyanogenesis and the factors governing the selection of polymorphic populations will clearly require studies of a range of plant types. So far, studies of cyanogenic polymorphism have largely been confined to pasture and crop species (e.g. Pedersen *et al.* 1996); comparatively little work has been carried out on natural populations of wild plants, especially woody species (e.g. Schappert and Shore 1994; Aikman *et al.* 1996; Thomsen and Brimer 1997). The aim of this paper is to characterise cyanogenic polymorphism in a woody species that shows an exceptionally high degree of cyanogenic potential — *Eucalyptus cladocalyx*, the sugar gum (Gleadow *et al.* 1998; Gleadow and Woodrow 2000). More specifically, we aim to identify acyanogenic forms of *E. cladocalyx* (if any), to estimate the degree of variability in the cyanogenic potentia of naturally occurring *E. cladocalyx*, and to investigate whether, as in white clover, the cyanogenic potential is inde pendent of the activity of the degradative β-glucosidase. Kangaroo Island was chosen for the study because it hac readily accessible, natural population with high morpho logical variability.

Materials and methods

Field description

Kangaroo Island (35°80′S, 137°33′E) is 145 km long and approximately 50 km wide and lies 13 km southwest of Cape Jervis, SA (Fig. 1). Geologically, it is a high plain cut by rivers and streams, with a maximum elevation of 307 m. Soils in the western part of the islan are predominantly deep calcareous sands, with pockets of red-brov sandy soils that are poor in nitrogen and a range of micronutrient (Northcote 1979). The climate is cool and temperate, with a distinc winter rainfall maximum (June–August).

Structurally, the vegetation is predominantly open woodland, interspersed with heath and open forest. There are more than 15 species *Eucalyptus* on the island with a high degree of endemism (Boland *et al.* 1992). *E. cladocalyx* is found chiefly on the wetter, western part of the island. Much of its distribution lies within the Flinders Chase National Park, although there are also a number of populations on the nor coast. *E. cladocalyx* is commonly found along watercourses associatec with *E. fasciculosa* F. Muell., *E. leucoxylon* F. Muell., and less often with *E. ovata* Labill., *E. diversifolia* Bonpl. and *E. cneorifolia* DC. (Boland *et al.* 1992).

Plant material

It was not feasible to sample every tree on the Island. Therefore, it wa decided to sample enough trees so that the probability of sampling tl least common form (i.e. an acyanogenic plant) was approximately 95%. As there were no data on the frequency of the non-functional *ac* and *li* alleles in *Eucalyptus* (assuming a similar genetic system to white clover), the sample size was calculated using an estimate of the degree of rarity of a species (or polymorph) given by McArdle (1990):

$$N = \log (1 - a)/\log (1 - P),$$

where N is the number of plants required, P is the probability of the species appearing in a sampling unit and *a* is the probability (or confidence) that the sample will be detected in a sample of N units. The frequency of cyanogenic plants in this case was assumed to be such that P = 0.03, based on typical probabilities for rare plants (McArdle 1990), while a was set at 0.95. This gave a preferred sample size of 95. A total of 96 trees from Kangaroo Island were tested for cyanogenesis (Fig. 1). Included in the total were adult leaves from 65 trees, young leaf-tips of the adult, lanceolate form from two individuals and one plant with coppice leaves of the juvenile, orbicular leaf form sampled. Samples were taken from a number of different locations on the island in January 1997 (Fig. 1). A further 28 trees were sampled in February 1999. Replicate samples taken in 1997 and 1999 were not significantly different in chemical composition (data not shown).

A single branch from each tree was sampled from the lower part of the canopy with a northerly aspect. Samples were stored on ice for 1–2 h. Leaf discs (area = 1.43 cm^2) were then excised from the middle of the blade of 10 fully expanded, mature leaves, snap-frozen and stored in liquid nitrogen. On returning from the field, samples were freeze-

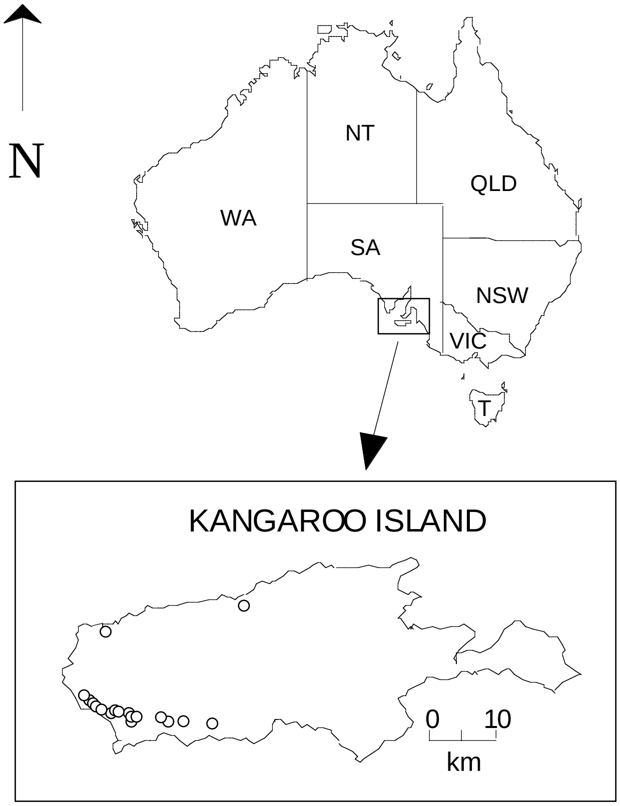


Fig. 1. Map of Australia showing location of Kangaroo Island in South Australia. Inset: map of Kangaroo Island showing localities ( ) where leaves of *E. cladocalyx* plants were sampled. A total of 96 trees were sampled in the western part of the island and tested for cyanogenesis.

dried and stored in a desiccator at 20 °C. Before analysis, samples were ground to a fine powder using a cooled IKA Labortechnic A10 micro-grinder (Janke and Kunkel GmbH Co, KG, Germany). Duplicate samples of leaves were analysed for nitrogen, carbon and cyanogenic potential. The b

The ratio of carbon to nitrogen was, overall, 41±10.896 (± 1 s.e). A significant inverse correlation was detected between leaf cyanide concentration and Carbon: Nitrogen ratio (Fig. 4, Pearson's correlation coefficient = –0.61, $r^2 = 0.37$; $P < 0.001$), reflecting the interdependence between leaf cyanide and the nitrogen component of the ratio.

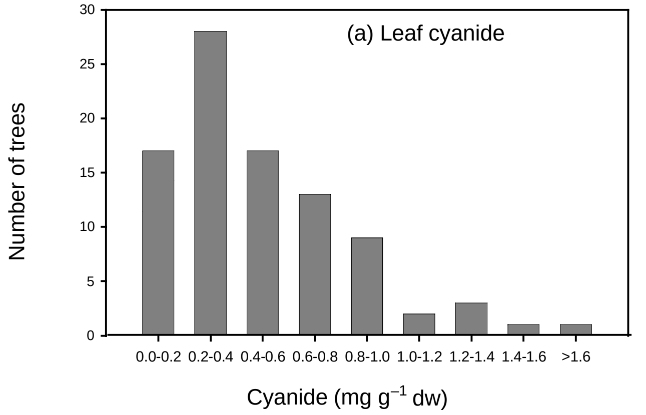
Morphological variation

Trees varied widely in height (2–25 m), but no relationship was detected between the cyanide concentration of fully expanded adult leaves and either tree height or girth. On th other hand, there was a weak, but highly significant, negativ correlation between specific leaf weight (SLW, measure using the dry weight of sampled leaf discs of known are expressed in g m^{-2}) and leaf cyanide concentration (Pearson correlation coefficient = –0.49, $r^2 = 0.24$; $P < 0.001$; Fig. 5). SLW ranged from 126–383 g m^{-2} with a mean (± 1 s.e) of 260 ± 4 g m^{-2}. *E. cladocalyx* trees growing on Kangaroo Island displayed a high degree of polymorphism in fruit morphology (capsule size, shape and degree of ridging) and the colour of the petioles (degree of redness), but this was found to be independent of leaf chemical characteristics (data not shown).

Variation in *b*-glucosidase activity

Enzyme activity was measured on a subsample of 24 trees representing a wide range of cyanogenic potential. *b*-glucosidase activity, when expressed on a dw basis, also ranged widely (0.2–3.8 mg CN^{-1} g dw h^{-1}) with a mean of

Leaf Nitrogen (mg g–1



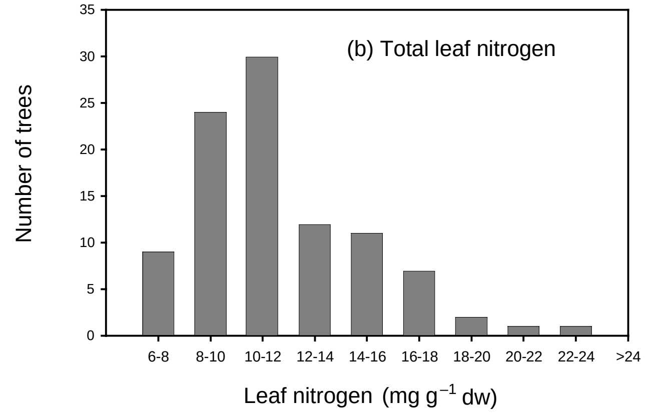
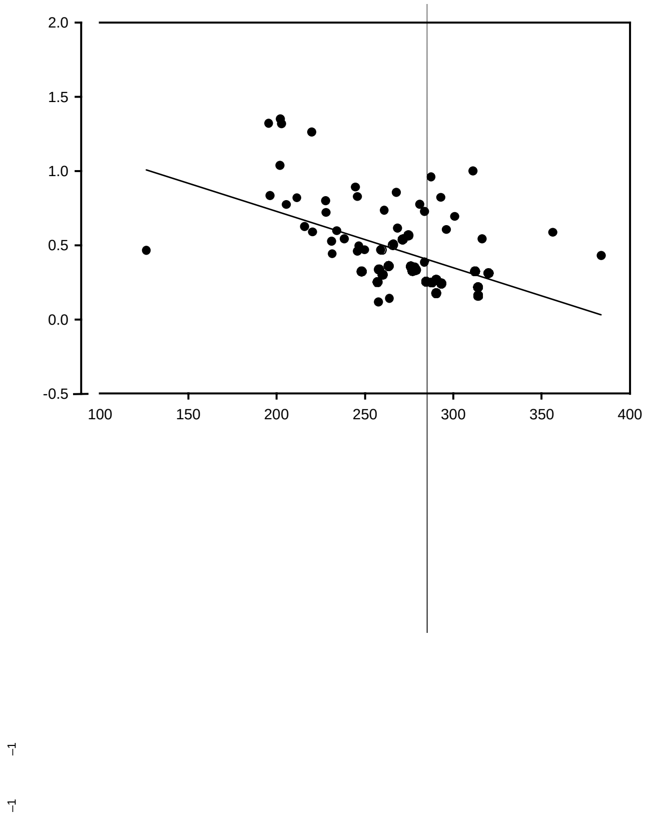


Fig. 2. Frequency of *E. cladocalyx* from Kangaroo Island, occurring in classes based on chemical characteristics of mature, adult leaves: (a) Leaf cyanide concentration, (b) total leaf nitrogen. 96 trees were sampled. Only fully expanded leaves of the adult form are presented in the frequency diagram.

0.90 ± 0.18 mg CN g^{Ð1} dw h^{Ð1} (Fig. 6). Importantly, this variation in b-glucosidase activity was independent of the variation in cyanogenic glycoside concentration (PearsonÕs correlation coefficient = 0.26; r^2 = 0.18; P = 0.25). The differences in observed enzyme activity were not simply due to differences in protein as the concentration of soluble protein was similar in each fraction with a mean (± 1 s.e.) of 82.2 ± 3.2 mg g^{Ð1} dw. Interestingly, the two samples with very high enzyme activities came from sites that had been burnt 8Ð10 years before and therefore, although morphologically similar to adult leaves, may have retained some juvenile features.



Discussion

Our results show the degree of variation in cyanogenic glycosides and the degradative b-glucosidase in a natural population of *Eucalyptus* for the first time. A significant proportion of the variation in cyanogenic glycoside concentration between trees can be accounted for by the variation in leaf nitrogen, and possibly a small proportion by differences in specific leaf weight. Most of the variation, however, appears to be the result of genetic polymorphism, which is inherited independently of the level of activity of the b-glucosidase.

There are a number of possible explanations for the high degree of variation in cyanogenic glycoside content of *E. cladocalyx* foliage observed here. Firstly, there may be incomplete dominance between the alleles, as suggested for *T. repens* by Till (1987). Secondly, 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, similar to that proposed for *Sorghum* (Gorz et al. 1986). It is noteworthy that recent research has shown that biosynthesis of cyanogenic glycosides requires the products of at least three genes: two encoding cytochrome P450 enzymes and one a [UDP]glucose glycosyl transferase (Kahn et al. 1997). Moreover, from early work on white clover, it appears likely that these genes are relatively tightly linked (defined as the Ac locus; Corkhill 1942). Thirdly, because cyanogenic glycoside concentration is a function of not only the rate of biosynthesis but also the rate of degradation (turnover), it is possible that gene products affecting the latter process account for some of the variation in cyanogenic potential. Finally, different patterns of gene expression may be modified further by environmental factors (Hughes 1991). While there is insufficient information on the genetics and metabolism of cyanogenic glycosides in eucalypts to comment on the first three explanations, it is likely that the variation in cyanogenic potential measured here is largely the result of differential gene expression, ameliorated by nitrogen availability.

We suggest that nitrogen is an important factor because it was apparently the only environmental factor that varied significantly at the collection sites. Others factors such as water availability and leaf age may have affected leaf chemistry, as indicated by variation in specific leaf weight (Fig. 5). Nevertheless, the variation in cyanide accounted for by variation in specific leaf weight could almost entirely be attributed to nitrogen. There was an inverse correlation between nitrogen and specific leaf weight (PearsonÕs correlation coefficient = Ð0.39; data not shown). Nitrogen availability has received attention in several other studies of cyanogenic plants, which showed an increase in cyanogenic glycoside concentration with leaf and soil nitrogen (e.g. Kriedemann 1964; Dement and Mooney 1974; Briggs 1990; Frehner et al. 1997). Our study made a similar finding. We found a significant correlation between the concentration of leaf cyanide and total leaf nitrogen (Fig. 3), which is also consistent with the results of a controlled environment study of *E. cladocalyx* (Gleadow *et al.* 1998). In the latter study, there was a large increase in cyanogenic glycoside concentration in the leaves of glasshouse-grown seedlings with increasing leaf nitrogen.

If the range of leaf nitrogen levels measured for the Kangaroo Island plants reflects phenotypic variation (Fig. 3), then the results of the regression analysis highlight two important points about cyanogenesis in *E. cladocalyx*. Firstly, as discussed above, for any leaf nitrogen level there is considerable variability in cyanide concentration. This, we have suggested, is due to genetic variation. The variability in cyanide concentration declines, however, with decreasing leaf nitrogen until, at about 7 mg N g^{Ð1} dw, there is little variability in cyanide concentration, and the cyanide levels are close to zero. There is apparently a threshold level of leaf nitrogen above which leaf nitrogen is allocated increasingly to cyanide. Moreover, the lack of variability in cyanide close to the threshold indicates that the magnitude of the threshold may be similar for all genotypes.

Accepting this argument, the second important value to emerge from the regression analysis is the average apparent cyanide yield. This can be defined as the increase in leaf cyanide-nitrogen associated with a 1 mg g^{Ð1} dw increase in leaf nitrogen. From the slope of the regression line (Fig. 3), this yield is approximately 4.1%. Clearly, genotypes may vary dramatically in their apparent yields, and it would be interesting to test this with a range of isogenic plants.

What is especially interesting about the *E. cladocalyx* populations at Kangaroo Island is that, in contrast to a number of other species, we found no individuals that either lacked the cyanogenic glycoside or the b-glucosidase, or both. This is consistent with studies of *Manihot esculenta*, the only other species to our knowledge that has been tested in this regard, that also failed to detect any correlation between cyanogenic glycoside content (i.e. linamarin) and the activity of the degradative linamerase in high and low cyanogenic varieties (Mkpong *et al.* 1990).

Studies on cyanogenesis in *Trifolium repens* and *Lotus corniculatus* have shown that inheritance of the ability to synthesise cyanogenic glycosides is independent of the ability to produce the cyanogenic b-glucosidase, controlled by the *Ac* and *Li* loci, respectively (Hughes 1991). Our results extend these observations by showing that the differential expression of these two traits is also independent of one another.

Although no acyanogenic plants were found on Kangaroo Island, we did locate several plants with low concentrations of cyanogenic glycosides (< 0.1 mg CN g^{Ð1} dw). Leaf material from these plants consistently tested positive, unlike leaves from neighbouring acyanogenic tree species. Thus, while the biosynthetic machinery was present in these plants, it was poorly expressed. The method we have used here is much more sensitive than the FeiglÐAnger test papers used by many workers (Brinker and Seigler 1989). This highlights one of the problems in many surveys that rely solely on these papers. For example, in this study, preliminary results for several trees using test papers failed to detect cyanogenesis (data not shown). However, further analysis in the laboratory found they were, indeed, cyanogenic, albeit with low concentrations of cyanogenic glycosides. It is noteworthy that a simple kit using picrate papers has recently been developed, and appears to be more sensitive than the FeiglÐAnger method and may, therefore, be more suitable for field use (Bradbury *et al.* 1999).

Finally, the considerable variation in cyanogenic capacity in the populations of *E. cladocalyx* on Kangaroo Island provides an excellent system for investigating not only the cost and benefits of cyanogenesis, but also the factors that select for polymorphism. Certainly, the benefit of cyanogenesis as a defence mechanism against generalist herbivores, particularly mammals, is beyond doubt (Jones 1998). On the other hand, there may be considerable costs in term of reduced growth and reproductive success associated with diverting photosynthate and nitrogen resources away from the primary metabolism (Briggs and Schultz 1990; Kakes 1997; Gleadow *et al.* 1998). The costs of cyanogenesis have, however, not led to the exclusion of *E. cladocalyx* from the island; rather, the distribution appears to be restricted to the more productive sites with adequate soil moisture, and possibly higher soil nitrogen. Selection of high or low cyanogenic individuals is most likely influenced by these balancing costs and benefits, but it may also be influenced by the spatial distribution of trees. Thus, an individual with a less than advantageous balance between the costs and benefits of cyanogenesis may gain an advantage through the protection offered by highly cyanogenic neighbours (Atsatt and OÕDowd 1976).

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References

Aikman K, Bergman D, Ebinger J, Seigler D (1996) Variation of cyanogenesis in some plant species of the midwestern United States. *Biochemical Systematics and Ecology* **24**, 637Ð645.

Atsatt PR, OÕDowd DJ (1976) Plant defense guilds. *Science* **193**, 24Ð29.

Boland DJ, Brooker MIH, Chippendale GM, Hall N, Hyland BPM, Johnston RD, Kleinig DA, Turner JD (1992) ÔForest trees of Australia, 4th edition.Õ (CSIRO: Melbourne)

Bradbury MG, Egan SV, Bradbury JH (1999) Picrate paper kits for determination of total cyanogens in cassava roots and all forms of cyanogens in cassava products. Journal of the Science of Food and Agriculture 76, 39Ð48.

Briggs MA (1990) Chemical defense production in Lotus corniculatus L. I. The effects of nitrogen source on growth, reproduction and defence. Oecologia 83, 27Ð31.

Briggs MA, Schultz JC (1990) Chemical defense production in Lotus corniculatus L. II. Trade-offs among growth, reproduction and defense. Oecologia 83, 32Ð37.

Brinker AM, Seigler DS (1989). Methods for the detection and quantitative determination of cyanide in plant materials. Phytochemical Bulletin 21, 24Ð31.

Conn EE (1991) Metabolic studies on natural products: cyanogenic glycosides and cyanogenesis as possible models. Proceedings of The National Science Council, ROC. Part B. Life Sciences 15, 220Ð225.

Corkhill L (1942) Cyanogenesis in white clover (Trifolium repens L.) V. The inheritance of cyanogenesis. New Zealand Journal of Science and Technology B 23, 178Ð193.

Dement WA, Mooney HA (1974) Seasonal variation in the production of tannins and cyanogenic glucosides in the chaparral shrub Heteromeles arbutifolia. Oecologia 15, 65Ð76.

Foulds W (1982) Polymorphism for cyanogenesis in Lotus australis Andr. populations at Greenough Front Flats, Western Australia. Australian Journal of Botany 30, 211Ð217.

Frehner M, LŸscher A, Hebeisen T, Zanetti S, Schubiger F, Scalet M (1997) Effects of elevated partial pressure of carbon dioxide and season of the year on forage quality and cyanide concentration of Trifolium repens L. from a FACE experiment. Acta Oecologica 18, 297Ð304.

Gleadow RM, Woodrow IE (2000) Temporal and spatial variation of cyanogenic glycosides in Eucalyptus cladocalyx. Tree Physiology 20, 591Ð598.

Gleadow RM, Foley, WJ, Woodrow IE (1998) Enhanced CO_2 alters the relationship between photosynthesis and defence in cyanogenic Eucalyptus cladocalyx F. Muell. Plant, Cell and Environment 21, 12Ð22.

Gorz HJ, Haskins FA, Všgel KP (1986) Inheritance of dhurrin content in mature sorghum leaves. Crop Science 26, 65Ð67.

Hughes MA (1991) The cyanogenic polymorphism in Trifolium repens L. (white clover). Heredity 66, 105Ð115.

Jones DA (1988) Cyanogenesis in animalÐplant interactions. In ÔCyanide compounds in biologyÕ. (Eds D Evered and S Harnett) pp. 151Ð165. (John Wiley and Sons: Chichester)

Jones DA (1998) Why are so many food plants cyanogenic? Phytochemistry 47, 155Ð162.

Kahn RA, Bak S, Svendsen I, Halkier BA M¿ller BL (1997) Isolation and reconstitution of cytochrome P450ox and in vitro reconstitution of the entire biosynthetic pathway of the cyanogenic glucoside dhurrin from sorghum. Plant Physiology 115, 1661Ð1670.

Kakes P (1997) Difference between the male and female components of fitness associated with the gene Ac in Trifolium repens. Acta Botanica Neerlandica 46, 219Ð223.

Kirsten W (1983) ÔOrganic elemental analysis: ultramicro, micro and trace methods.Õ (Academic Press: New York)

Kriedemann PE (1964) Cyanide formation in Sorghum almum in relation to nitrogen and phosphorus nutrition. Australian Journal of Experimental Agriculture and Animal Husbandry 4, 15Ð16.

McArdle BH (1990) When are rare species not there? Oikos 57, 276Ð277.

McMahon JM, White WLB, Sayre RT (1995) Cyanogenesis in cassava (Manihot esculenta Crantz). Journal of Experimental Botany 46, 731Ð741.

Mkpong OE, Yan H, Chism G, Sayre RT (1990) Purification, characterization and localization of linamerase in cassava. Plant Physiology 93, 176Ð181.

Nahrstedt A (1985) Cyanogenic compounds as protecting agents for organisms. Plant Systematics and Evolution 150, 35Ð47.

Northcote KH (1979) Soils. In ÔNatural history of Kangaroo IslandÕ (Eds MJ Tyler, CR Twidale and JK Ling) pp. 39Ð46. (Royal Society of South Australia: Adelaide)

Pederson G, Fairbrother TE, Greene SL (1996) Cyanogenesis and climatic relationships in US white clover germplasm collection and core subset. Crop Science 36, 427Ð433.

Schappert PJ, Shore JS (1994) Cyanogenesis in Turnera ulmifolia L. (Turneraceae). I. Phenotypic distribution and genetic variation for cyanogenesis on Jamaica. Heredity 74, 392Ð404.

Thomsen K, Brimer L (1997) Cyanogenic constituents in woody plants in natural lowland rain forest in Costa Rica. Botanical Journal of the Linnaean Society 124, 273Ð294.

Till I (1987) Variability of expression of cyanogenesis in white clover (Trifolium repens L.). Heredity 59, 265Ð271.

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