

Temporal and spatial variation in cyanogenic glycosides in *Eucalyptus cladocalyx*

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Summary The release of hydrogen cyanide from endogenous cyanide-containing compounds in plants is an effective herbivore deterrent. We investigated temporal and spatial variations in cyanogenic glycoside concentration in greenhouse-grown seedlings and 6-year-old plantation trees of *Eucalyptus cladocalyx* F. Muell., which allocates up to 20% of leaf nitrogen to the cyanogenic glycoside, prunasin. The highest cyanogenic glycoside concentrations were in the young, developing vegetative and reproductive tissues. Both the overall cyanogenic glycoside concentration and the proportion of nitrogen allocated to cyanogenic glycoside decreased as tissues matured. Cyanogenic glycoside and nitrogen concentrations were similar at all positions on the leaf blade. There was no change in concentration of cyanogenic glycosides either diurnally or following wounding of the tissue, suggesting that these compounds are constitutive. Cyanogenic glycoside concentration varied seasonally in young leaf tips of field-grown *E. cladocalyx*, but not in mature, fully expanded leaves. Although some of the changes in cyanogenic glycoside concentration in young leaf tips may have been driven by changes in leaf nitrogen, there was a significant decrease in the proportion of nitrogen allocated to cyanogenic glycosides in young leaves during the summer, coinciding with the peak flowering period. Mobilization of cyanogenic glycosides may have occurred to provide nitrogen for reproduction. Most of the observed temporal and spatial variations in cyanogenic glycosides are consistent with the optimal use of resources, particularly nitrogen.

Keywords: cyanide, herbivore defense, prunasin, resource allocation.

Introduction

Cyanogenesis is the process by which plants and other living organisms release hydrogen cyanide (HCN) from endogenous cyanide-containing compounds (Seigler 1991). It is estimated that 11% of all plant species are cyanogenic, with representatives from all major taxa (Jones 1998). The most common endogenous cyanogens are cyanogenic glycosides, which comprise an α -hydroxynitrile (or cyanohydrin) stabilized by a glycosidically linked sugar moiety (Møller and Poulton 1993). Cyanogenesis is generally initiated by tissue disruption and involves cleavage of the sugar moiety from the cyanogenic gly-

coside followed by degradation of the cyanohydrin to produce HCN and an aldehyde or ketone (Conn 1981). Because of the toxicity of these products, especially HCN, cyanogenesis is highly effective in reducing tissue loss to herbivores in many species (Nahrstedt 1985, Jones 1998). Autotoxicity in intact tissue is prevented by spatial separation—either at the sub-cellular or tissue level—of the degradative enzymes and the cyanogenic glycoside (Conn 1981, Hughes 1993).

Although significant advances have recently been made in understanding the molecular, genetic and biochemical basis of cyanogenic glycoside synthesis in some species (e.g., Hughes 1993, Kahn et al. 1997), several important physiological questions remain unanswered. For example, it is not known whether cyanogenic glycosides are constitutive or whether they can be induced following tissue damage—an important point in terms of resource-use efficiency. In one of the few studies to examine this topic, Kojima et al. (1983) found that the cyanogenic glycoside concentration of root tissue excised from *Manihot esculenta* Crantz. increased over time; however, it could not be determined whether the observed changes occurred in response to wounding or to dehydration of the plant tissue.

Longer-term regulation of cyanogenic glycoside concentration is also not well understood (see Okolie and Obasi 1993). Turnover rates of cyanogenic glycosides are sufficient to accommodate significant diurnal changes in cyanogenic glycoside concentration in *Sorghum* (Bough and Gander 1971, Adewusi 1990) and *M. esculenta* (Kojima et al. 1979). However, no clear relationship between time of day and overall concentration has been documented. On the other hand, seasonal variations in cyanogenic glycoside concentrations have been studied in several species, but these changes have never been unequivocally linked with any single set of environmental variables.

Because a relatively large proportion of leaf nitrogen is allocated to cyanogenic glycosides in most cyanogenic species, modulation of cyanogenic glycoside concentration over time could have important consequences not only for herbivore defense, but also for other processes that depend on energy and nitrogen allocation. We investigated temporal variation in cyanogenic glycoside concentration in the tree species, *Eucalyptus cladocalyx* F. Muell. Specifically, we examined (1) whether the concentration of cyanogenic glycosides varies di-

urnally and seasonally, and (2) whether these compounds are constitutive or inducible. We also present a description of the spatial distribution of cyanogenic glycosides in seedlings and trees. *Eucalyptus cladocalyx* was chosen because it is highly cyanogenic, allocating up to 20% of leaf nitrogen to the cyanogenic glycoside, prunasin (Gleadow et al. 1998, Gleadow 1999).

Materials and methods

Plant material and greenhouse experiments

Seeds of *E. cladocalyx* were collected from a small group of trees at Wilmington, South Australia (32°41' S, 138°06' E, Seedlot 19348, Australian Tree Seed Centre), and germinated in seed trays in a greenhouse. After one month, seedlings were transplanted to 12-l pots containing a 1:1:1 (v/v) mixture of sterilized sand, vermiculite and perlite. Pots were flushed twice each day with one-quarter strength Hoagland's solution containing 6 mol m⁻³ nitrogen, supplied as nitrate, with sodium as the balancing cation (see Gleadow et al. 1998). The air temperature and humidity of the greenhouse were measured with a Vaisala (Helsinki, Finland) HMP 35A sensor every 15 min. Temperature and humidity (day/night ± 1 SD) were 23.2 ± 1.4/22.3 ± 0.2 °C and 48.3 ± 3.9/54.4 ± 2.3%, respectively. Photosynthetic photon flux density (PPFD) in the greenhouse was measured every 5 min with two visible light sensors (LI 190-SA, Li-Cor, Inc., Lincoln, NE), one at either end of the bench. The photoperiod was approximately 14 h, and mean daytime PPFD (± 1 SE) was 543 ± 28.19 μmol m⁻² s⁻¹.

Cyanogenic glycoside concentration of the seedlings was determined when the seedlings were 2 weeks old and 5–8 cm tall. Thirty seedlings were randomly divided into three groups of 10, separated into leaves (two cotyledons, 1–2 leaf pairs), stem and roots and the material pooled for chemical analysis. In addition, the distribution of cyanogenic glycosides in leaf blades, petioles, and stem and root tissues was mapped based on measurements made on three replicate branches of a single 6-month-old *E. cladocalyx* seedling. Abscised, senescent leaves were collected from the surface of the pots of three other seedlings (8 months old) and analyzed chemically.

Previously, it was shown that leaves from different seedlings, classified according to their age, morphology and node number, had similar cyanogenic glycoside concentrations (see Gleadow et al. 1998). Therefore, to facilitate comparisons between plants from different treatments and ages, all of the following experiments were made on fully expanded, juvenile leaves located 3–5 nodes from the tips. The distribution of cyanogenic glycosides within such leaves was determined by excising 10 discs (area = 1.43 cm²) from the lamina of each of six fully expanded leaves, one leaf from each of six plants, such that almost the whole leaf was sampled. For each chemical analysis, discs from two plants were pooled (i.e., $n = 3$).

The effect of leaf wounding was measured on a set of 24 *E. cladocalyx* 6-month-old seedlings, 1.7 ± 0.3 m tall with 118 ± 13 leaves. Two leaves per plant were damaged *in situ* with a wheel paper-perforator to create rows of perforations

(2 mm long with an interval of 2 mm) similar to that described by Ohnmeiss and Baldwin (1994). Approximately 8–9 rows of damage were made on each side of the midrib, taking care to avoid the main veins. One damaged and one undamaged leaf from the set of plants (eight plants per time interval, $n = 4$) were harvested approximately every 2 h over a 24-hour period (11 harvests). Because damaged and undamaged leaves had been randomly assigned *a priori* to one of the harvest times, over the course of the experiment four leaves were sampled from each plant, but not at the same time. Preliminary experiments showed that excising leaf discs, removing an entire leaf at the petiole or damaging a separate leaf on a plant had no significant, systemic effect on the cyanogenic glycoside concentration of the remaining leaves.

Field studies

The field site at Creswick (37°43' S, 143°90' E) is 130 km west of Melbourne, Victoria, Australia. Mean annual rainfall is 756 mm, but during the study year (1997) only 469 mm was recorded. Summers (December–February) are generally warm and dry. The soil profile typically consists of clay, grading to a light or medium clay, overlying weathered sedimentary rock. Cores of soil (0.1-m diameter) were collected 1 m from the base of trees ($n = 13$) at depths of 0–0.2 m (A) and 0.2–0.4 m (B) in November 1997 and analyzed for total nitrogen. There was no significant difference in total soil nitrogen concentration between sites. Overall, total nitrogen of the A and B horizons was 0.13 ± 0.010% and 0.06 ± 0.01%, respectively.

Seed was collected from a single provenance of *E. cladocalyx* near Dimboola, Victoria (36°34' S, 141°64' E) and sown at Creswick in July 1990 (R. Hatley, School of Forestry Creswick, University of Melbourne, Australia, personal communication). At the time of this study, the trees were, on average, 6 m tall and the leaves, whether newly emerged or fully expanded, were all of the lanceolate, mature form rather than the orbicular, juvenile leaf type borne by seedlings. Newly formed young leaves (leaf tips) were present on most trees throughout the year. Leaf tips and mature leaves were sampled from 13 replicate trees every three months: November 19, 1996, and February 19, May 1, August 19 and November 25, 1997. Surface leaf litter was collected 1 m from the base of each tree in November 1997. When present, flowers, buds and fruits at various stages of maturity were also sampled. Trees were always sampled between 1300 and 1500 h on fine, still days to minimize any diurnal variation. The maximum/minimum temperatures on each of the five sampling days were: 15.0/5.9, 37.8/14.0, 13.7/5.2, 12.1/1.6, and 26.2/6.4 °C.

Chemical analyses

Sampled material was snap-frozen in liquid nitrogen within 30 s of sampling, freeze-dried and ground in a cooled IKA Labortechnik A10 microgrinder (Janke and Kunkel GmbH Co. KG, Staufen, Germany). Cyanogenic glycoside concentration of plant tissue was determined by hydrolysis and trapping of the resultant HCN in an NaOH well (Brinker and Seigler 1989). Hydrolysis was achieved by adding 1 ml of 0.1 mol m⁻³ citrate buffer (pH 5.5) to approximately 0.02 g of freeze-

dried leaf material in a sealed glass vial and incubating for 15 h at 35 °C (Gleadow 1999). Exogenous β -glucosidase from almond (*Prunus amygdalis* (L.) Benth. & Hook.; β -D-glucoside glucohydrolase; EC 3.2.1.21, Sigma, St. Louis, MO) was added to the buffer (1.12 units ml⁻¹) to ensure complete conversion to cyanide (Gleadow et al. 1998). Previous experiments had shown that crude protein extract from *E. cladocalyx* was capable of hydrolyzing prunasin (D-mandelonitrile β -D-glucoside, Sigma) without the addition of exogenous α -hydroxynitrile lyase (Gleadow and Woodrow, unpublished data). Aliquots (100 μ l) of 1 M NaOH were taken from the well inside the sealed vial and assayed for cyanide with a Merck Spectroquant Cyanide Detection Kit (Merck, Darmstadt, Germany). The amount of cyanide (CN) detected by this method is a measure of the cyanogenic component of the cyanogenic glycosides in the tissue, principally prunasin, and is hereafter referred to as the amount of "cyanide." The concentration of free cyanide in undisturbed tissue was assumed to be negligible.

Total nitrogen was determined on 0.02 g of finely ground plant material by a micro-Kjeldahl method with ammonium sulfate as the standard. Total nitrogen of soil samples was measured by a modified Kjeldahl method (Adams and Attwill 1986).

Statistical analysis

Analyses of variance (ANOVA), Pearson's correlation coefficients (i.e., product-moment correlation), linear regression and regression ANOVAS (Campbell 1974) were calculated with Minitab 10extra[®] software. Data were tested for homogeneity and normality and transformed as necessary. The identity of individual plant replicates was used as a covariable in all analyses of variance. Correlation coefficients were determined with Pearson's method and the significance of regression equations tested with Minitab 10extra[®] software.

Results

Spatial variation

Variation in seedlings Overall, cyanide concentration of 1-week-old seedlings was low compared with that of 6-month-old seedlings and 6-year-old trees (see below). Among plant organs, leaves and cotyledons had the highest cyanide concentration (0.18 ± 0.05 mg CN g_{dw}⁻¹), whereas stems had a marginally lower cyanide concentration (0.12 ± 0.04 mg g_{dw}⁻¹). Roots of 1-week-old seedlings contained trace amounts of cyanide (0.03 ± 0.01 mg g_{dw}⁻¹).

A study of branch tissues from a single 6-month-old *E. cladocalyx* seedling yielded mean cyanide concentrations of 7.1 ± 0.8 , 6.1 ± 0.4 and 4.8 ± 0.7 mg CN g_{dw}⁻¹ for young, fully expanded and old juvenile leaves, respectively (Figure 1). Concentrations of cyanide in leaf tips of 6-month-old seedlings were higher than in young leaves (8.6 ± 0.8 versus 7.1 ± 0.8 mg CN g_{dw}⁻¹). Abscised, senescent leaves contained relatively low amounts of cyanide, with a mean of 2.0 ± 0.4 mg CN g_{dw}⁻¹. The cyanide concentration in petioles was about

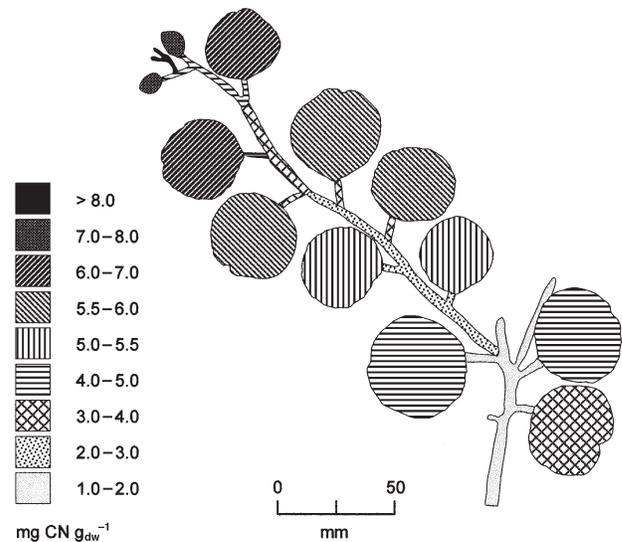


Figure 1. Mean variation in cyanide concentration within a branch, based on measurements on three branches of similar size on a single seedling grown in a greenhouse and supplied with nutrient solution containing 6 mol m⁻³ nitrogen. Means (± 1 SE) were sorted into six classes and coded, with dark shading representing tissue containing high concentrations of cyanide and light shading representing tissues containing low concentrations of cyanide.

50% lower than in the adjacent leaf blade (e.g., 2.8 ± 0.1 mg CN g_{dw}⁻¹ for petioles of fully expanded leaves; Figure 1). The concentration of cyanide in stem tissue was strongly associated with height, decreasing from 4.2 ± 0.4 mg CN g_{dw}⁻¹ in the apical segment to 0.5 ± 0.1 mg g_{dw}⁻¹ at the base of the plant (data not shown). In contrast to the roots of the 1-week-old seedlings, no cyanide was detected in any of the root samples of the 6-month-old seedling.

The inverse relationship between cyanogenic glycoside concentration and leaf age was not simply an effect of dilution as the leaf expanded. The total amount of cyanide per leaf in seedlings increased from 2.75 ± 0.19 mg in young leaves to 10.64 ± 1.20 mg in fully expanded juvenile leaves, decreasing again as the leaves aged (data not shown). On the other hand, the amount of cyanide remained constant in each 0.2-m segment of the main stem (2.5 ± 0.8 mg per 0.2 m segment), suggesting that the observed decrease in cyanogenic glycoside concentration at the stem base may be partially a result of increases in stem biomass.

Analysis of samples from 10 evenly spaced locations on single leaves of similar morphology and position from six replicate 6-month-old seedlings showed that leaf cyanide and nitrogen concentration did not vary significantly across the leaf ($P = 0.21$ and $P = 0.99$, respectively). From this group of seedlings, the pooled mean leaf cyanide concentration was 2.56 ± 0.18 mg CN g_{dw}⁻¹ and mean leaf nitrogen concentration was 22.7 ± 1.0 mg g_{dw}⁻¹.

Variation in adult trees In 6-year-old trees, the newly formed, young adult leaves (leaf tips) always contained more cyanide than fully expanded leaves on the same plant ($P <$

0.0001). On average, the concentration of cyanide in mature leaves was only 25% of that in leaf tips (0.86 versus 3.29 mg g_{dw}^{-1}). In addition, leaf tips had a higher leaf nitrogen concentration than mature leaves (15.6 ± 0.7 versus 11.8 ± 0.3 mg g_{dw}^{-1} ; $P < 0.05$). In terms of resource allocation, the proportion of leaf nitrogen present as cyanide decreased from $11.0 \pm 0.7\%$ in leaf tips to $3.8 \pm 0.3\%$ in mature leaves. The dry weight of single leaves collected in the field was not determined; however, the amount of cyanide per leaf was estimated based on the mean dry weight of adult and newly emergent leaves. Cyanide per leaf was substantially higher in fully expanded adult leaves than in leaf tips (0.6 versus 0.2 mg). Surface leaf litter contained 0.006 ± 0.002 mg CN g_{dw}^{-1} , equivalent to only $0.03 \pm 0.005\%$ of total litter nitrogen.

Reproductive organs at various stages of development were also cyanogenic. Young flower buds were the most cyanogenic, with a mean cyanide concentration of 4.2 mg CN g_{dw}^{-1} (Figure 2), comparable with concentrations found in old juvenile leaves of 6-month-old greenhouse-grown seedlings (Figure 2). Progressively lower concentrations were recorded as the flower bud developed into a fruit. The mean cyanide concentration of flowers was 2.1 mg g_{dw}^{-1} , but most of that cyanide resided in the receptacles (2.6 mg g_{dw}^{-1}) with a concentration of only 0.3 mg g_{dw}^{-1} in the stamens. On the other hand, the total amount of cyanide (estimated based on mean dry weights of the different organs) increased progressively from buds (0.02 mg CN) to flowers (0.05 mg CN) to fruits (0.2 mg CN), concomitant with the increase in biomass. Seeds contained trace amounts of cyanide, representing less than 0.2% of the total seed nitrogen (Figure 2). There was little variation in nitrogen concentration among the different reproductive developmental stages, with approximately 10 mg g_{dw}^{-1} in the buds and 8 mg g_{dw}^{-1} in different parts of the flowers and fruits (data

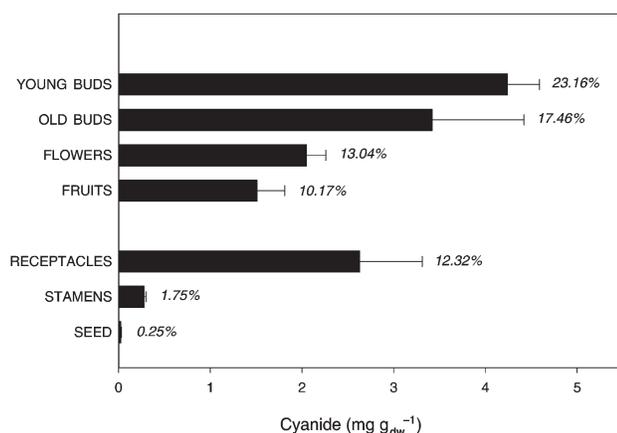


Figure 2. Cyanide concentration (± 1 SE) in reproductive organs of *E. cladocalyx* collected from 6-year-old trees at Creswick during 1997. Organs were classified according to developmental stages: young buds, old buds (both including operculae), flowers and fruits (woody capsules). Flowers were separated into receptacles and stamens for further analysis. Seed included seed and chaff. Numbers beside histograms represent the amount of nitrogen in cyanide calculated as a proportion of the total nitrogen in each organ.

not shown). Thus, there was an overall decline in the proportion of nitrogen allocated to cyanide in the developing reproductive organs from 23% in the young buds to 10% in the woody fruits (Figure 3).

Temporal variation

Diurnal variation and wounding effects To test whether cyanogenic glycosides varied temporally, either naturally or in response to wounding, 88 leaves of comparable age and morphology were compared. Undamaged leaves showed no diurnal variation in either cyanide ($P = 0.63$) or nitrogen ($P = 0.13$) concentration (Figure 3). Furthermore, there was no alteration in either cyanide ($P = 0.64$) or nitrogen ($P = 0.98$) concentration in response to wounding over the same 24-hour period, and there was no significant difference between the wounded and undamaged leaves over time (i.e., the treatment \times time interaction was not significant for any parameter). Overall, combining all leaves analyzed, the mean leaf cyanide was 6.66 ± 0.28 mg g_{dw}^{-1} and mean total leaf nitrogen was 26.5 ± 0.5 mg g_{dw}^{-1} . The relative water content of wounded leaves, calculated as a percentage of dry weight, was on average only 2% less than that of undamaged leaves after 24 h ($62.9 \pm 0.7\%$ compared with $60.9 \pm 0.6\%$). Although significant ($P < 0.05$), this difference represents a small change and suggests that the wounding method employed minimized the effects on leaf water relations that often confound wounding experiments (Ohnmeiss and Baldwin 1994).

Seasonal variation Mature leaves contained roughly the same amount of cyanide throughout the year (Figure 4a), whereas leaf tips showed a marked decline in cyanide concentration during the warmer months, decreasing to values similar

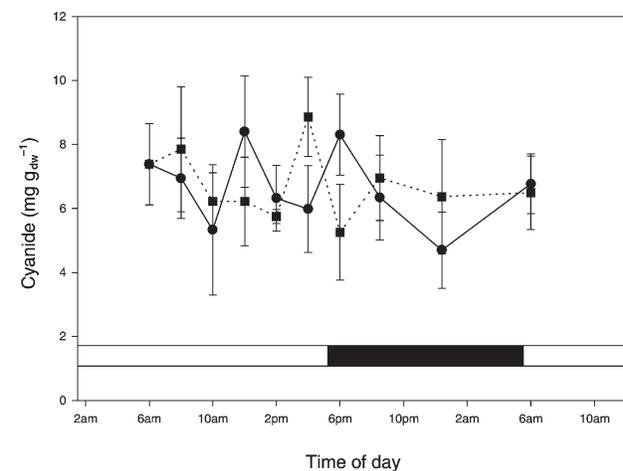


Figure 3. Mean cyanide concentration of entire leaves of *E. cladocalyx* seedlings sampled over a 24-h period. Leaves were either undamaged (\bullet) or wounded with perforations (\blacksquare) and left on the seedling for various lengths of time. Each value is the mean of four leaves. There was no significant difference in cyanide concentration between treatments or over time. Plants were grown in a greenhouse with a natural photoperiod. The bar represents the hours of daylight (open) and darkness (filled).

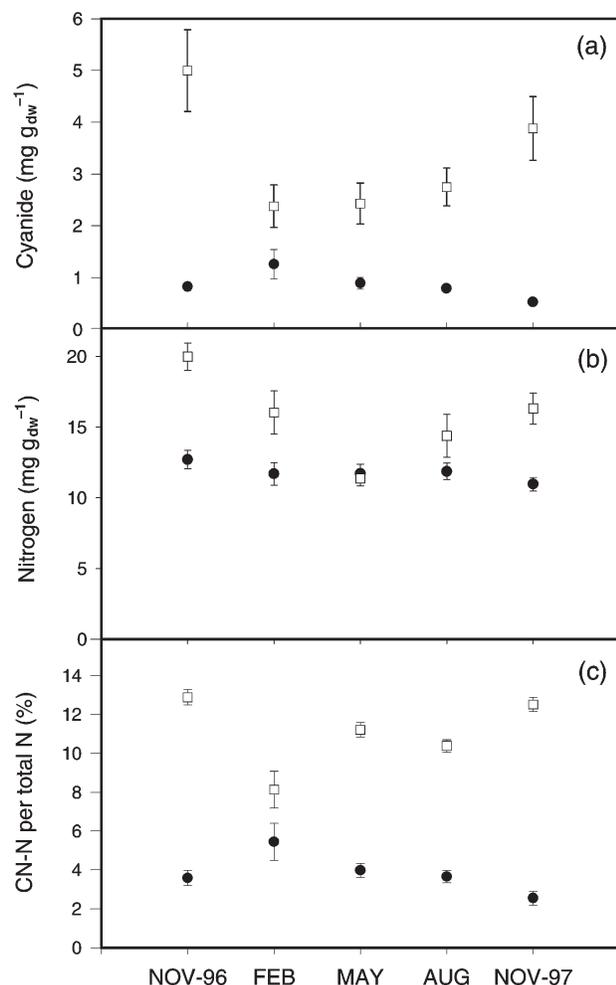


Figure 4. Seasonal variation (± 1 SE) in: (a) cyanide concentration; (b) total leaf nitrogen (% dry weight); and (c) proportion of nitrogen in cyanide as a proportion of total leaf nitrogen (cyanide-nitrogen per nitrogen, %). Both newly produced leaf tips (\square) and mature leaves (\bullet) were sampled from 6-year-old trees at Creswick from November 1996 to November 1997.

to those found in mature leaves. This difference in seasonal response of the different leaf types was highly significant ($P < 0.001$ for the age \times season interaction). The seasonal pattern of changes in leaf nitrogen concentration was similar to the seasonal changes in leaf cyanide concentration (Figure 4b), suggesting that the two may be related. Overall, there was no significant seasonal variation in the proportion of nitrogen occurring as cyanide in mature leaves (Figure 4c), the proportion in leaf tips fluctuated from season to season and was lowest in February (summer). As a result of this variation, the age \times season interaction for cyanide-nitrogen per total nitrogen was significant ($P < 0.05$).

Discussion

Spatial variation

The highest concentrations of cyanide in *E. cladocalyx* were

found in young, developing leaves of both adult plants and seedlings and concentrations decreased with leaf age (Figures 1 and 4). In a study of *E. cladocalyx* seedlings grown at elevated CO_2 concentration, Gleadow et al. (1998) also observed that the concentration of cyanogenic glycoside decreased with leaf age (Table 1). Moreover, the proportion of nitrogen allocated to cyanogenic glycosides decreased with leaf age, from $13.3 \pm 1.1\%$ in young leaves to $7.7 \pm 1.2\%$ in old, but not senescent leaves. As found here, these changes did not correlate with leaf size. Fully expanded leaves contained significantly more cyanide (6.7 mg CN) than either young (1.9 mg CN) or old leaves (3.5 mg CN) (Table 1, Gleadow 1999). Similar allocation patterns have been reported for the leaves (e.g., Lamont 1993, Bennett et al. 1997) and stems (Martin et al. 1938) of several other cyanogenic plants, as well as for plants defended by other nitrogenous allelochemicals, such as alkaloids (e.g., Hartmann et al. 1997).

In some species, cyanogenic glycoside concentration is heterogeneously distributed within leaves, with greater concentrations of defense chemicals in areas more susceptible to herbivory. For example, in *Sorghum vulgare* Pers. and *Pteridium aquilinum* L. Kuhn, the softer leaf apices have a higher concentration of cyanogenic glycosides than the more fibrous leaf bases (Martin et al. 1938, Cooper-Driver et al. 1977). Leaves of *E. cladocalyx* seedlings, however, showed no variation in either cyanide or nitrogen concentration. This finding does not necessarily mean that defense was homogeneous across the leaf surface because other forms of defense, such as terpenoids or leaf toughness, may be unevenly distributed (Voirin and Bayet 1996).

Plants with cyanogenic reproductive organs are not uncommon (e.g., Nahrstedt 1985, Lamont 1993, Thomsen and Brimer 1997), but there have been few detailed studies on cyanogenesis in flowers other than *Trifolium repens* L. (Kakes 1997). Considerable amounts of cyanide were found in the reproductive organs of *E. cladocalyx*. The young buds contained the highest concentration of cyanogenic glycoside (Figure 2), with values similar to those in juvenile leaves (Figure 1). Flowers also contained high concentrations of cyanide, particularly the receptacles that house the ovules. The fruits, by con-

Table 1. Cyanide concentration, cyanide content per leaf and the proportion of total nitrogen allocated to cyanide (CN-N per N) of 6-month-old seedlings of *E. cladocalyx* grown under conditions similar to those described in this study (adapted from Gleadow et al. 1998, Gleadow 1999). Leaves, all of the orbicular, juvenile form, were classified into young, fully expanded and old, based on age, position and morphology. Cyanogenic glycoside concentration (measured as evolved cyanide), amount of cyanide per leaf and CN-N per N differed significantly between leaf classes ($P < 0.05$). Each value is the mean of four replicates (± 1 SE).

Leaf class	Cyanide (mg g^{-1})	Cyanide (mg leaf^{-1})	CN-N/N (%)
Young	8.0 ± 0.6	1.9 ± 0.3	13.3 ± 1.1
Fully-expanded	6.8 ± 0.6	6.7 ± 1.2	11.8 ± 1.2
Old	4.8 ± 0.6	3.5 ± 0.4	7.7 ± 1.2

trast, were much less cyanogenic, possibly because as the fruits develop they become increasingly woody and presumably less accessible to herbivores. Similarly, the cyanogenic glycoside concentration in *Macadamia ternifolia* F. Muell. fruits decreases with increasing woodiness (Vivian Smith 1995). Compared with other plants with cyanogenic glycosides in their seeds (e.g., *Hevea brasiliensis* (Willd. ex A. Juss) Müll. Arg., Selmar 1993), the seeds of *E. cladocalyx* contained very low concentrations of cyanogenic glycosides. Significant concentrations of cyanogenic glycosides were, however, detected in 3-week-old seedlings, suggesting that they are synthesized *de novo*, as in *Trifolium repens* (Hughes 1993).

Most aspects of spatial variation in cyanogenic glycosides were consistent with the optimal use of resources, particularly nitrogen. As formalized in the optimal allocation theory of plant defense (Bloom et al. 1985), we found that chemical defenses against herbivores were concentrated in the young, vulnerable tissues, and these defenses declined with age and photosynthetic capacity. Similarly, reproductive organs were afforded relatively high levels of defense.

Temporal variation

There was no change in cyanogenic glycoside concentration either diurnally or following wounding of the tissue. These findings contradict those of two studies of cassava (*Manihot esculenta*). Okolie and Obasi (1993) reported that field-grown plants had higher cyanogenic glycoside concentrations in the late afternoon, and Kojima et al. (1983) reported that the cyanogenic glycoside concentrations of cubes of cassava root incubated in air on the laboratory bench increased with time. These differing results could be indicative of a species difference, or they could be to the result of confounding variables in the cassava experiments. For example, in the field experiments, plants of different cultivars were sampled from fields in different locations. In the laboratory experiment, atmospheric humidity was not controlled, allowing the cassava tissue to slowly dehydrate. Water stress has been shown to affect cyanogenesis (Bokanga et al. 1994).

The reason for a lack of temporal change in cyanogenic glycoside concentration in *E. cladocalyx* is not known. It could be argued that natural selection would favor increased deployment of defense compounds, such as cyanogenic glycosides, when the density of herbivores is relatively high, especially in view of the considerable amount of nitrogen required for synthesis of these compounds (Gulmon and Mooney 1986). Extensive studies of alkaloids generally support this hypothesis (Ohnmeiss and Baldwin 1994). However, because the proportion of nitrogen in prunasin is low (5.1%) relative to that of nicotine (17.3%), for example, it may be that the benefit of inducing and diurnally modulating the production of prunasin does not outweigh the cost of maintaining the processes required to do this.

The concentration of cyanogenic glycoside varied seasonally in young leaf tips of field-grown *E. cladocalyx*, but not in the mature, fully expanded leaves (Figure 4). Some of the changes in cyanide concentration in leaf tips may have been

driven by changes in leaf nitrogen concentration (see Figure 5), because, in summer, the proportion of leaf nitrogen allocated to cyanogenic glycosides dropped significantly. Seasonal variation in cyanogenic glycoside concentration has been observed in a number of species and several explanations have been proposed. These include availability of nitrogen from the soil (e.g., Dement and Mooney 1974), climate (e.g., Hughes 1993, Frehner et al. 1997), abundance of herbivores (e.g., Cooper-Driver et al. 1977, Auxiliadora et al. 1983), and reallocation of resources to other organs (e.g., Briggs and Schultz 1990).

The latter explanation is supported by our data. The decrease in nitrogen allocation to cyanogenic glycosides in *E. cladocalyx* during summer coincided with the peak flowering period for this species (Boland et al. 1992). Because of the considerable nutrient requirements for flowering and fruiting, mobilization of cyanogenic glycosides may have occurred to provide extra nitrogen for reproduction. A similar conflicting demand for nitrogen occurred during reproduction in *Lotus corniculatus* L. (Briggs and Schulz 1990). Additional support for this hypothesis comes from studies of other *Eucalyptus* species that show a seasonal translocation of nitrogen within the canopy corresponding to an increased demand for nitrogen by new shoots (e.g., Wendler et al. 1995).

Temperature effects cannot be ruled out, however, because the decrease in leaf cyanide in *E. cladocalyx* coincided with the hottest time of year (Figure 4). However, a review of the literature indicates that the effect of temperature on cyanogenic glycoside production is not consistent either within or between species. For example, there is evidence that cyanogenic glycoside production in *Lotus corniculatus* is very sensitive to temperature, but both increases and decreases in cyanogenic glycoside concentration have been correlated with increases in temperature (Jones 1988). By contrast, Stochmal

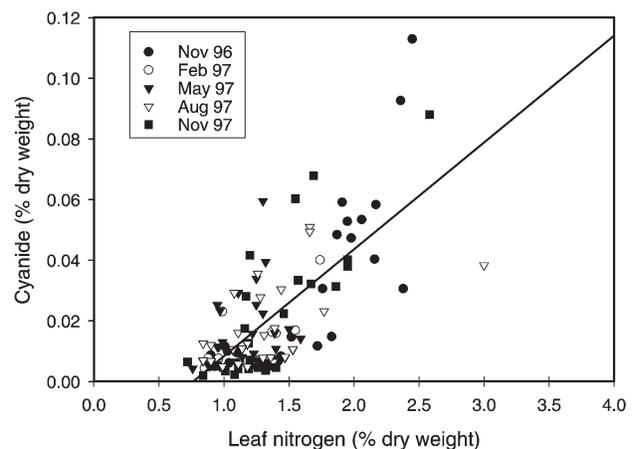


Figure 5. Leaf cyanide as a function of leaf nitrogen in *E. cladocalyx* leaves sampled five times from 6-year-old trees of *E. cladocalyx* over the period November 1996–November 1997 at Creswick ($r^2 = 0.44$ and $P < 0.001$). Leaf tips collected during February are not included because they had a significantly different proportion of nitrogen allocated to cyanide (see Figure 4c).

and Oleszek (1997) found that cyanogenic glycosides accumulated in leaves of *Trifolium repens* when the mean air temperature fell below 15 °C.

At the study site, herbivory of young leaves by insects tended to be highest during autumn (data not shown). In some species, the production of nitrogen-based defense compounds increases in parallel with increasing frequency of herbivores (e.g., Cooper-Driver et al. 1977, Hartmann et al. 1997). In some cases this increase can be attributed to induction (e.g., Baldwin 1994), but in others it appears to be the result of co-evolution (e.g., Auxiliadora et al. 1983). A similar coincidence has been observed in plants employing carbon-based defense systems (e.g., Zou and Cates 1995).

Allocation of resources to defense

Consistent with theories of plant defense based on resource acquisition (e.g., Bryant et al. 1983), it has been found that plants with high leaf nitrogen concentrations also contain high concentrations of cyanogenic glycosides (Kriedemann 1964, Gladow et al. 1998). When field data for all leaves were pooled, a highly significant correlation between the concentrations of cyanide and nitrogen was detected, with an overall Pearson's coefficient of 0.72 ($P < 0.001$; Figure 5), supporting resource-acquisition theories. On the other hand, given a limited set of resources, plants may change their allocation pattern in response to external stimuli or developmental stage, as described by defense theories based on resource allocation (e.g., Bloom et al. 1985, van Dam et al. 1996). In our study, cyanogenic glycosides appeared to be reallocated from leaf tips to the developing flowers during summer, consistent with resource-allocation theory. Furthermore, the proportion of nitrogen allocated to cyanogenic glycosides in senescent juvenile leaves and leaf litter from adult trees was much less than in living leaves, indicating that nitrogen was reallocated from the cyanogenic glycosides during senescence to more actively growing parts of the plant. Mobilization and reallocation of cyanogenic glycosides within a plant has also been observed in *Hevea brasiliensis* (Selmar 1993) and *Trifolium repens* (Hughes 1993). However, whether cyanogenic glycosides are translocated intact in *E. cladocalyx*, as seen in *H. brasiliensis*, or whether the nitrogen is mobilized from the cyanide and transported in some other form, remains unknown.

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