CSIRO Publishing

Functional Plant Biology

Continuing *Australian Journal of Plant Physiology*

VOLUME 29, 2002

All enquiries and manuscripts should be directed to:

Functional Plant Biology
CSIRO Publishing
PO Box 1139 (150 Oxford St)
Collingwood, Vic. 3066, Australia

Telephone: +61 3 9662 7625
Fax: +61 3 9662 7611
Email: publishing.fpb@csiro.au

Published by CSIRO Publishing
for CSIRO and the Australian Academy of Science

www.publish.csiro.au/journals/fpb

Funct. Plant Biol., 2002, **29**, 103–110

Influence of water stress on cyanogenic capacity in *Eucalyptus cladocalyx*

Ian E. Woodrow[A], *Damian J. Slocum and Roslyn M. Gleadow*

School of Botany, The University of Melbourne, Vic. 3010, Australia.
[A]Corresponding author; email: iewood@unimelb.edu.au

Abstract. Cyanogenesis in many plant species is an effective herbivore deterrent, which appears to be influenced by a range of environmental variables. There is evidence that one such variable, soil water availability, increases cyanogenic capacity (i.e. leaf cyanogenic glycoside concentration), but it is not clear whether this is a relatively direct or indirect effect. To shed light on this issue, we compared the cyanogenic capacity of individuals from two populations of *Eucalyptus cladocalyx* F.Muell. from areas of South Australia that differ markedly in rainfall. Stable carbon isotope analysis confirmed that trees at the drier site were more water-stressed. We found a large range in leaf cyanogenic capacities, from 0 to 1.01 mg cyanide g^{-1} dry weight. Importantly, this is the first record of acyanogenic *E. cladocalyx*. Mean cyanogenic capacity was 30% higher in trees from the drier site, and they suffered less damage from herbivores. However, these trees also contained higher concentrations of leaf nitrogen (N). Correlative analysis of data for individual plants from both sites showed that leaf N was able to account for a significant amount of the variation in cyanogenic glycoside concentration (28%). Water availability on its own, however, was not able to account significantly for any such variation. We conclude that most of the variation in cyanogenic capacity is due to genetic differences between individuals, while the remaining variation is due to differences in leaf N.

Keywords: cyanide, cyanogenesis, eucalypt, herbivore defence, water stress.

Introduction

Cyanogenesis is the process by which plants liberate hydrogen cyanide (HCN) on tissue disruption (Poulton 1990). In most species cyanogenesis is due to the catalysed hydrolysis of cyanogenic glycosides, which is generally initiated by herbivore damage (Møller and Seigler 1999). Cyanide is a respiratory toxin and can cause acute poisoning or act as a feeding deterrent to many herbivores (Nahrstedt 1985; Jones 1998).

Despite the apparent effectiveness of cyanogenesis in herbivore defence, natural populations of most cyanogenic species contain a significant number of individuals that lack the biochemical capacity to synthesise cyanogenic glycosides (Aikman *et al.* 1996; Lechtenberg and Nahrstedt 1999). Moreover, the cyanogenic individuals in these populations typically display a considerable range of cyanogenic glycoside concentrations, which apparently reflects variation in their biosynthetic capacity (Hughes 1991). It is thought that one of the main factors maintaining such variation in cyanogenesis in natural populations is the significant resource and energetic 'cost' of cyanogenesis (Kakes 1989, 1997; Briggs and Schultz 1990; Skogsmyr and Fagerström 1992). Thus, in the absence of herbivory, acyanogenic plants have higher growth rates and reproductive fitness. It is also thought that the 'cost' of cyanogenesis can be moderated by environmental factors such as water and nutrient availability (Foulds and Grime 1972*b*; Gershenzon 1984; Herms and Mattson 1992).

Our interest in this paper is in the effect of water availability on the cyanogenic capacity of individuals in natural populations. Water availability is perhaps the best-studied environmental effector of cyanogenesis, but despite the focus of research on just a few species, the results have been sometimes contradictory (see Pederson *et al.* 1996). For example, Caradus *et al.* (1990) found that the frequency of cyanogenic *Trifolium repens* was higher in areas of low rainfall in New Zealand, while Foulds and Grime (1972*a*), in a study of *T. repens* in England, found the converse to be true. The results of surveys of cyanogenesis in *Lotus corniculatus* are, however, more consistent. For example, on Mainland, Orkney, a clear negative correlation between soil moisture and the frequency of the cyanogenic form was found (Abbott 1977). Similar results have been recorded for *L. corniculatus* in numerous other European populations (Foulds and Grime 1972*b*; Blaise *et al.* 1991). In some species not only was the frequency of cyanogenic forms higher in drier areas, but the mean capacity for

Abbreviations used: CN, cyanide; DBH, diameter at breast height; dw, dry weight; HCN, hydrogen cyanide; N, nitrogen; SLW, specific leaf weight; WUE, water use efficiency; $\delta^{13}C$, natural abundance of ^{13}C.

 10.1071/PP01116 1445-4408/02/010103

cyanogenesis was also increased (e.g. *Sorghum*, Nelson 1953; *Manihot esculenta*, Calatayud *et al.* 1994; Bokanga *et al.* 1994; *Turnera ulmifolia*, Schappert and Shore 1999). In such cases, it is possible that both selection of individuals with a higher cyanogenic capacity and phenotypic enhancement of cyanogenesis contributed to the overall increase in cyanogenesis in dry areas.

In order to verify this latter conclusion, however, it is necessary to be sure that water stress is the only significant variable that can be associated with the increase in cyanogenic capacity. This has not been the case in the aforementioned studies. Moreover, it has generally been assumed that a degree of water stress is correlated with low rainfall, but this is not always a valid assumption. In some species, morphology can be modified under conditions of low rainfall, thereby minimising the effects of soil moisture deficit (e.g. Hsiao 1973; Arndt *et al.* 2000). Furthermore, variation in rainfall can affect soil N availability (Fisher 1987), which in turn can regulate cyanogenic capacity (Gleadow *et al.* 1998). For example, seasonal studies of the chaparral shrub *Heteromeles arbutifolia* actually showed an increase in cyanogenic capacity during the wetter spring months, which the authors attributed to the associated increase in soil N availability rather than to changes in soil moisture *per se* (Dement and Mooney 1974).

There is a clear need for a study of cyanogenesis in which water stress is quantified and all of the other important environmental variables are either constant or controlled. This is the aim of the present study. We will focus on two populations of cyanogenic eucalypts (*Eucalyptus cladocalyx* F.Muell.) that grow under markedly different rainfall regimes. This species is ideal for the project because it is highly variable in its genetically based cyanogenic capacity, allocating up to 20% of leaf N to the cyanogenic glycoside prunasin (Gleadow *et al.* 1998; Gleadow and Woodrow 2000*a*, *b*). Moreover, other than rainfall, the only significant environmental variable that apparently differs between the sites is soil N.

Materials and methods

Species and site selection

Eucalyptus cladocalyx F.Muell. is endemic to South Australia where it occurs in four disjunct areas: one in the southern Flinders Ranges in close proximity of Spencer Gulf; one on Kangaroo Island; and two on the eastern side of the Eyre Peninsula (see Fig. 1; Gleadow and Woodrow 2000*b*). In high rainfall areas, *E. cladocalyx* grows in open-forest, but occurs in woodlands in drier areas. Individuals growing in the Flinders Ranges and on Kangaroo Island tend to be taller and straighter. The variety *Eucalyptus cladocalyx* var. *nana* is found on the Eyre Peninsula, and is shorter with a branching, mallee habit.

To measure plants subject to varying degrees of water stress, two sites were chosen that were known to differ markedly in annual rainfall but not in temperature: Rocky River on Kangaroo Island (35°80′ S, 137°33′ E, elevation 196 m, slope 2°) and Cowell, approximately 105 km SW of Whyalla on South Australia's Eyre Peninsula (33°38′00″ S, 136°40′45″ E; elevation 380 m; slope 3°). Long-term climate records from meteorological stations near the sites show that the rainfall at Kangaroo Island is nearly three times higher than on the Eyre Peninsula (626.7 and 284.9 mm, respectively, recorded at Cape Border, Kangaroo Island and Cowell, Eyre Peninsula). Trees at both sites were growing in a woodland of even age. At the Kangaroo Island site trees were taller (up to 25 m) and straighter, with a shrubby understorey. By contrast, the open woodland at the Eyre Peninsula site was dominated by the bushy form (8–12 m) with very little undergrowth.

Soils from Kangaroo Island were uniform calcareous sands that consisted largely of comminuted shell fragments. There was little pedologic organization in the soils, which were weakly coherent in a moderately moist state. There was some accumulation of organic matter in the top 20 cm of the profiles. Value/chroma ratings for the soils were typically 2 or 3, and the soils were brownish-grey to dull yellow-brown in colour. In contrast to the uniform calcareous soils of Kangaroo Island, soils from the Eyre Peninsula site were duplex and had surface textures ranging from loamy sands to sandy clay loams in their profiles. Few peds were evident in the clayey B-horizon and value and/or chroma ratings on the Eyre Peninsula were generally 4 or 5. Soils were red to orange in colour indicating the presence of iron oxide. Up to ten soil samples were collected randomly from three depths at each site and analysed for total N using a CHNS analyser (see Chemical analyses).

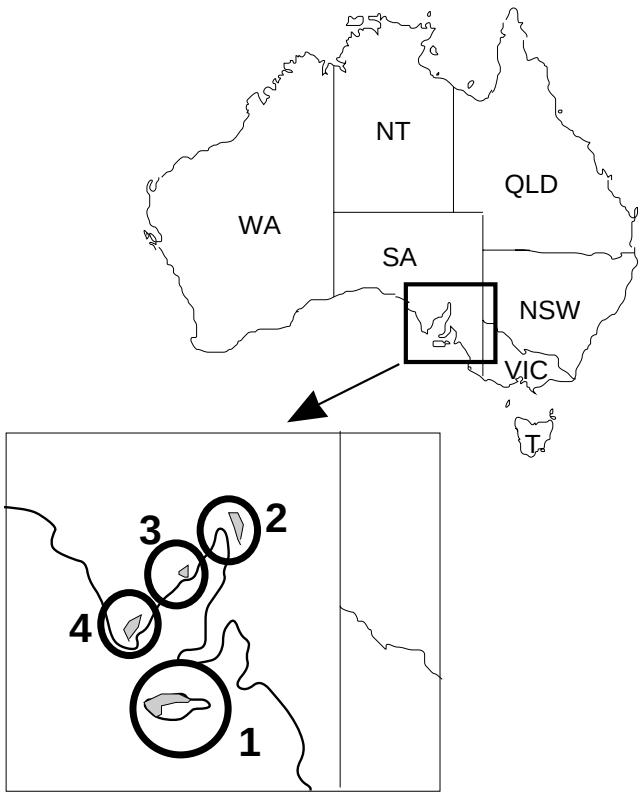

Fig. 1. Natural distribution of *Eucalyptus cladocalyx* in Australia. The species is endemic to South Australia where it occurs in four disjunct areas: on Kangaroo Island (1); in the southern Flinders Ranges (2) and on the eastern side of the Eyre Peninsula (3 and 4). Locations (1) and (3) represent the populations that were sampled in this study.

Field sampling

A total of 52 trees were sampled in April 1999 in dry weather: 31 from the Eyre Peninsula and 21 from Kangaroo Island. Trees of relatively uniform size were chosen at random from within a large stand trees. Ten fully expanded mature leaves were sampled from the northerly aspect of each tree. Leaf discs were excised from the middle of the blade, snap-frozen and stored in liquid N. After freeze-drying, leaves were ground using a ball mill (Ultramat 2 dental grinder; Southern Dental Industries Ltd, Melbourne, Australia). A further 20 leaves from the same branch on each tree were sampled at random to estimate the amount of damage by herbivores. Leaves were scanned and the area missing was calculated using digital imaging (Gleadow 1999; Schappert and Shore 1999). Voucher specimens of *E. cladocalyx* from each site have been lodged with the herbarium at the School of Botany, The University of Melbourne.

Chemical analyses

The concentration of cyanogenic glycosides (i.e. cyanogenic capacity) in plant tissue was determined by hydrolysing all of the cyanogenic glycoside and trapping the resultant HCN in a well containing 1 M NaOH (Brinker and Seigler 1989). Hydrolysis was achieved by adding 1 mL 0.1 M sodium citrate–HCl buffer (pH 5.5) containing 1.12 units mL^{-1} exogenous β-glucosidase emulsin (β*D*-glucoside glucohydrolase; EC 3.2.1.21; Sigma, Sydney) from almond (*Prunus amygdalis* (L.) Benth. & Hook.) to approximately 0.02 g of freeze-dried leaf material in a sealed glass vial and incubating at 37°C for 15 h (Gleadow *et al.* 1998). Cyanide in the NaOH well was neutralised with 1 M acetic acid and assayed using the König reactions (Lambert *et al.* 1975; Brinker and Seigler 1989). The method was miniaturised for microtitre analysis (50-μL sample) and the absorbance was measured at 595 nm using a Labsystems Multiskan Ascent spectrophotometer (Pathtech Pty Ltd, Melbourne, Australia). The amount of cyanide detected by this method is directly proportional to the amount of cyanogenic glycoside in the tissue and in this paper will be referred to as the amount of 'cyanide'.

The total amount of N and carbon in approximately 10 mg of freeze-dried leaf material was measured using a Perkin-Elmer 2400 Series II CHNS/O Analyser (Perkin-Elmer Corporation, Norwalk, Connecticut, USA) with high-purity acetanilide as the standard. The same process was used to determine the amount of N in soil, except that 40-mg samples were used.

$\delta^{13}C$ determination

Carbon isotope ratios in finely ground samples of freeze-dried leaves were measured by an on-line VG Isochrom mass spectrometer (VG Microtech, Uckfield, East Sussex, UK; precision 0.1‰) after combustion in a Carlo Erba 1110 elemental analyser (ThermoQuest Australia Pty Ltd, Rydalmere, Australia). Carbon isotope ratios were expressed as $\delta^{13}C$ using the PeeDee Belemnite standard (Farquhar *et al.* 1989). In interpreting the data, the carbon isotope ratio of source air was assumed to be similar at the two sites, given the open nature of the woodlands and the large degree of air circulation.

Statistical analysis

Statistical analyses were carried out using Minitab release 10 Xtra for Windows (Minitab Inc., State College, PA, USA). Statistical comparisons were made using analysis of variance. Pearson's correlation coefficients and linear regression were used to compare cyanide against independent variables such as leaf N, $\delta^{13}C$ values and herbivory. Most data were normally distributed. Cyanide data, however, required a square root transformation to satisfy the assumptions of normality required by the analysis of variance.

Results

Cyanogenic capacity and growth characteristics of E. cladocalyx *at two sites*

There was a large range in the cyanogenic capacity of trees both within and between sites, from 0.03 to 0.92 mg cyanide (CN) g^{-1} dw in trees from Kangaroo Island and from 0 to 1.01 mg CN g^{-1} dw in those from the Eyre Peninsula (Fig. 2). One milligram of CN measured in this way is equivalent to 11.81 mg of the cyanogenic glycoside prunasin. Despite this variation, there was a significant difference ($P < 0.05$) in the mean cyanogenic capacity of *E. cladocalyx* between the two sites. Overall, the average cyanide concentration (± s.e.) of foliage from the Eyre Peninsula was 0.45 ± 0.05 mg CN g^{-1} dw, compared with 0.29 ± 0.04 mg CN g^{-1} dw in foliage from Kangaroo Island (Table 1). It is noteworthy that one plant from the Eyre Peninsula was found to be lacking in cyanogenic glycosides and, as such, is the first record of acyanogenic *E. cladocalyx*. This finding was confirmed by analysing up to five times the tissue quantity used in the routine cyanogenic glycoside assays.

It is not possible to attribute the differences in mean cyanide content at the two sites to rainfall alone. Soil from the Kangaroo Island site contained significantly less N than soil from the Eyre Peninsula site at all depths ($P < 0.001$; Table 2). For example, the total N concentration in surface soil (0–5-cm depth, ± s.e.) was 3.8 mg g^{-1} (± 0.5) at the Eyre Peninsula but only 2.6 mg g^{-1} (± 0.2) at Kangaroo Island (Table 2). This, in turn, was reflected in the significantly higher total leaf N ($P < 0.0001$) in trees from the Eyre Peninsula (10.3 ± 0.3 mg g^{-1} compared with 8.6 ± 0.2 mg g^{-1} at Kangaroo Island; Table 1). The carbon to N ratio was also

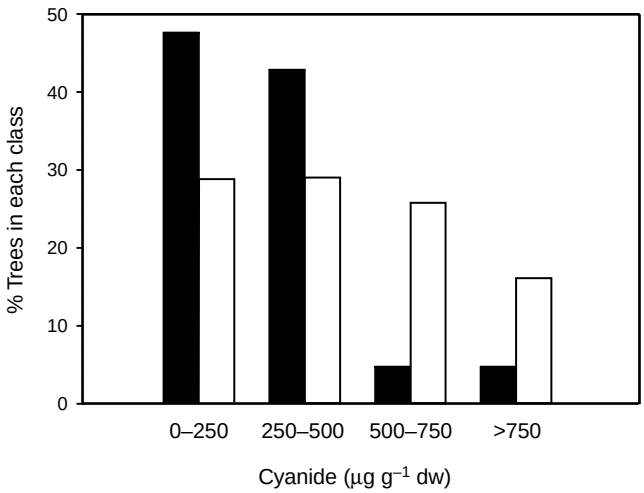

Fig. 2. Variation in cyanogenic capacity of *Eucalyptus cladocalyx* from Kangaroo Island (black bars) and Eyre Peninsula (open bars). Trees were allocated to four classes based on the amount of cyanide released from macerated tissue. One milligram of cyanogenic nitrogen is equivalent to 11.81 mg of the cyanogenic glycoside prunasin.

Table 1. Comparison of *Eucalyptus cladocalyx* from two sites in South Australia for a range of chemical and physical parameters

Twenty-one plants were sampled on Kangaroo Island and 31 on the Eyre Peninsula. SLW, specific leaf weight; DBH, diameter at breast height; C:N, carbon to nitrogen ratio; $\delta^{13}C$, the proportion of stable carbon isotopes. Herbivory was based on the proportion of leaf area missing from the interpolated area of an entire leaf. *P*-values indicate the degree of significance of the difference between the two sites from the analysis of variance. Data are the mean ± s.e. measured on fully expanded mature leaves ($n = 52$)

Analysis	Kangaroo Island	Eyre Peninsula	*P*-value
Total leaf cyanide (mg g^{-1})	0.28 ± 0.04	0.45 ± 0.05	0.049
Totals leaf nitrogen (mg g^{-1})	8.66 ± 0.22	10.30 ± 0.31	<0.0001
$\delta^{13}C$	–28.61 ± 0.22	–26.80 ± 0.12	0.0001
C:N ratio	62.18 ± 1.89	53.66 ± 1.86	0.0013
SLW (g m^{-2})	267 ± 5	305 ± 5	<0.0001
Height (m)	15.8 ± 1.6	8.6 ± 0.4	<0.0001
DBH (m)	1.06 ± 0.18	0.33 ± 0.04	<0.0001
Herbivory (% loss)	9.7 ± 1.6	3.4 ± 0.20	<0.0001

significantly lower ($P < 0.01$) in the foliage of the plants from the Eyre Peninsula compared with those from Kangaroo Island, with mean C:N ratios (± s.e.) of 53.7 ± 1.8 and 62.2 ± 1.9, respectively (Table 1).

In addition to higher leaf cyanide, leaves from the drier Eyre Peninsula site tended to have a higher mass per leaf area, which can be indicative of water stress. The average specific leaf weight (SLW ± s.e.) of leaves from the Eyre Peninsula site was 305 ± 5 g m^{-2}, significantly higher than the 267 ± 5 g m^{-2} recorded for leaves from Kangaroo Island ($P < 0.001$; Table 1). Trees at the Kangaroo Island site were significantly taller ($P < 0.0001$) and had a higher diameter at breast height (DBH) ($P < 0.0001$) than those at the Eyre Peninsula site (Table 1). Correlative analysis of the data for each site, however, indicated that there was no relationship between leaf cyanogenic capacity and either DBH or height (data not shown). We assume, therefore, that the difference in tree size between the two sites did not contribute to differences in cyanogenic capacity.

Water availability and cyanogenesis in the field

In order to gauge the degree of water availability at each site, foliage samples were analysed for stable carbon isotope content. In general, more negative $\delta^{13}C$ values indicate greater water availability (Farquhar *et al.* 1989). Consistent with this, plants from the Eyre Peninsula site, which had the lower rainfall, had on average a significantly higher $\delta^{13}C$ value than plants from the Kangaroo Island site, with mean values (± s.e.) of –26.8 ± 0.12 and –28.61 ± 0.48, respectively, ($P < 0.001$; Table 1).

As there appears to be two significant differences between the sites that could influence cyanogenic capacity (i.e. water and N availability), individual plants were compared using correlative analysis. We first examined the relationship between leaf N and cyanide content. Pooling the data from both sites, leaf cyanide concentration was positively correlated with leaf N ($r^2 = 0.28$; Pearson's = 0.533; $P < 0.0001$; Fig. 3). This correlation was significant for the Eyre Peninsula data on their own ($P = 0.008$), but not for the Kangaroo Island data ($P = 0.22$), which was possibly due to the smaller sample size and the relative lack of variability in leaf N at this site.

Table 2. Total nitrogen content (% dry weight) of soil collected at three depths from ten sites at Kangaroo Island and the Eyre Peninsula

Site	Total nitrogen content (%) 0–5 cm	5–10 cm	>10 cm
Kangaroo Island	0.262 ± 0.019	0.033 ± 0.004	0.013 ± 0.002
Eyre Peninsula	0.380 ± 0.051	0.103 ± 0.033	0.063 ± 0.009

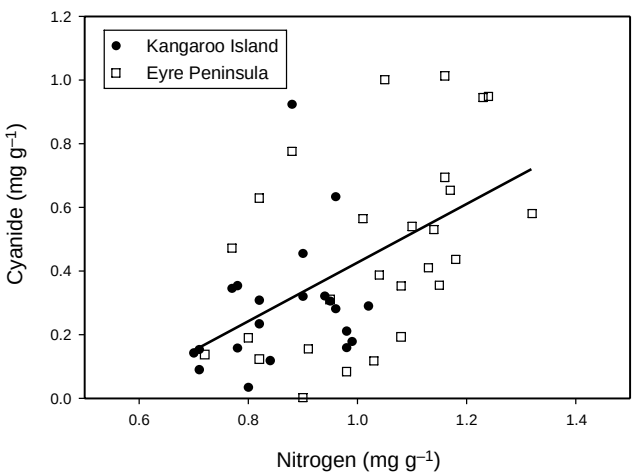

Fig. 3. Leaf cyanogenic glycoside concentration as a function of leaf nitrogen for *Eucalyptus cladocalyx* trees from Kangaroo Island (closed circles) and the Eyre Peninsula (open squares). One milligram of cyanogenic nitrogen is equivalent to 11.81 mg of the cyanogenic glycoside prunasin. The linear regression is for all data were significant ($r^2 = 0.28$, $y = –0.488 + 0.914x$, $P < 0.0001$).

We next tested the hypothesis that some of the variation evident in the cyanide versus N plot (Fig. 3) could be accounted for by water availability ($\delta^{13}C$). First, the leaf cyanide-N to total N ratio (i.e. the proportion of leaf N allocated to cyanide) was calculated in order to effectively eliminate any N effect on cyanide concentration, and this was then plotted against $\delta^{13}C$ (Fig. 4). Regression analysis showed a weak correlation, with a Pearson's correlation coefficient of 0.251 ($r^2 = 0.09$). While this is not significant at the 95% level ($P = 0.086$), it is suggestive of a dependent relationship given the high degree of variability of the data.

Herbivory between two sites

The degree of damage by herbivores to *E. cladocalyx* leaves was next compared by estimating the missing area of leaves from trees growing on the Eyre Peninsula ($n = 31$) and Kangaroo Island ($n = 21$) using digital analysis (20 leaves per tree). Nearly 10% of the leaf area was missing from leaves sampled at Kangaroo Island, compared with 3% from leaves sampled at the Eyre Peninsula ($P < 0.0001$). This is consistent with the higher mean cyanide concentration and SLW measured on leaves from the Eyre Peninsula (Table 1). Interestingly, a small but significant positive correlation was detected across both sites between the amount of leaf area missing and the degree of water stress, as measured by $\delta^{13}C$ ($r^2 = 0.13$; Pearson's = –0.355, $P < 0.05$; Fig. 5). On the other hand, the rate of herbivory did not correlate with leaf N (Pearson's = –0.159, $P = 0.20$), leaf cyanide concentration (Pearson's = –0.206, $P = 0.10$) or SLW (Pearson's = –0.192; $P = 0.12$).

Discussion

The use of stable isotopes is becoming increasingly common in ecological research as a means of measuring water stress. A strong correlation exists between ^{13}C abundance ($\delta^{13}C$) and water use efficiency (WUE), which is determined by the carbon assimilation to transpiration ratio (Farquhar *et al.* 1989). A relatively high WUE is generally characteristic of water-stressed plants because stomatal conductance is low relative to photosynthetic capacity. This seems to have been the case with the *E. cladocalyx* in this study where leaf $\delta^{13}C$ values from the drier Eyre Peninsula were, on average, significantly higher than those from the Kangaroo Island sites (Table 1). The range of the $\delta^{13}C$ values is, moreover, consistent with those previously published for eucalypts and other species from similar habitats (e.g. Ehleringer 1993; Stewart *et al.* 1995; Schulze *et al.* 1998).

It is possible, however, that variation in photosynthetic capacity on its own could account for the difference in $\delta^{13}C$ between the two sites. To test this, we measured total leaf N — which, under some circumstances, is highly correlated with photosynthetic capacity (Field and Mooney 1986) — for leaves from both sites (Table 1). If the higher mean level of foliar N at the Eyre Peninsula site was translated into a proportionally higher photosynthetic capacity, then given no difference in stomatal conductance at all, this could account for the difference in $\delta^{13}C$ between the two sites. In other words, the different $\delta^{13}C$ values may not reflect differences in average stomatal conductance, and thus water availability, between the sites. We suggest, however, that it is unlikely that differences in photosynthetic capacity were solely responsible for differences in $\delta^{13}C$ because we did not find a significant correlation between foliar N and $\delta^{13}C$ at either

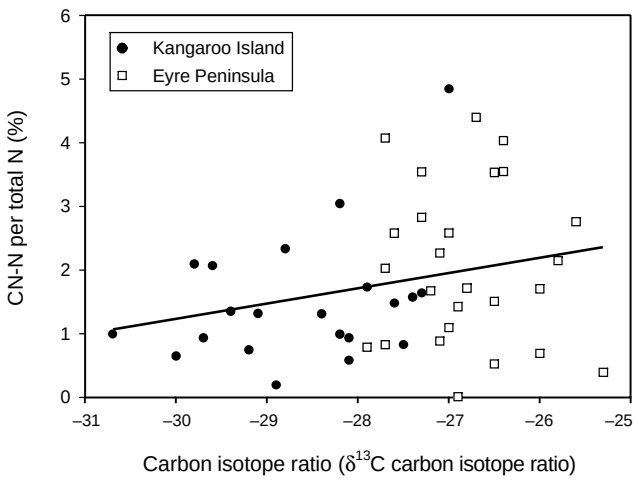

Fig. 4. Proportion of nitrogen allocated to cyanogenic glycosides (i.e. nitrogen in cyanide per total nitrogen) in leaves as a function of water availability ($\delta^{13}C$ ratio) for *Eucalyptus cladocalyx* trees from Kangaroo Island (closed circles) and the Eyre Peninsula (open squares). One milligram of cyanogenic nitrogen is equivalent to 11.81 mg of the cyanogenic glycoside prunasin. The correlation was not significant ($r^2 = 0.12$, $P = 0.086$).

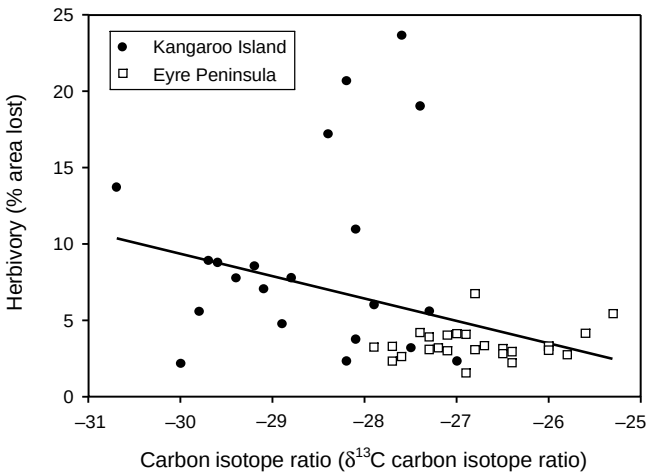

Fig. 5. Degree of herbivory on *Eucalyptus cladocalyx* (measured by interpolating the area missing from entire leaves) as a function of water stress (measured as $\delta^{13}C$) from trees growing at Kangaroo Island (closed circles) and the Eyre Peninsula (open squares). A significant inverse correlation was detected ($r^2 = –0.13$, $P < 0.05$).

site (Fig. 6). This is especially noteworthy at the Eyre Peninsula site where there was a very wide range of leaf N levels (Fig. 6). On this basis, and in view of the morphological differences between the foliage and whole plants (Table 1), we conclude that *E. cladocalyx* plants on the Eyre Peninsula had a lower mean stomatal conductance and a higher WUE as a result of the lower soil water availability at this site.

As has been found before for a range of cyanogenic species, including *E. cladocalyx* (Gleadow and Woodrow 2000*b*), there was considerable variation within sites in both the absolute foliar cyanogenic glycoside concentration and the proportion of foliar N allocated to cyanogenic glycosides (Fig. 2). It is highly likely that most of this variation is due to inherent differences between plants, a conclusion we base on two lines of evidence. First, heritability studies of cyanogenesis in red box (*Eucalyptus polyanthemos*) by J. Goodger (pers. comm.) have demonstrated genetically based variation in cyanogenic glycoside content similar to that found in this study. Similarly, a study of *Turnera ulmifolia* on Jamaica found a wide range of cyanogenic capacities in natural populations, with approximately 80% of the variation attributable to genetic differences between individuals (Schappert and Shore 1999). Second, other than N, which accounted for some 28% of the variation across both sites (Fig. 3), we could simply identify no other environmental or developmental variable that could account for the remainder of the variation in foliar cyanogenic glycoside content.

Despite this variation in foliar cyanogenic capacity within the sites, we found that plants at the drier Eyre Peninsula site were on average significantly more cyanogenic than their counterparts at Kangaroo Island (Table 1). Correlative analysis, however, indicates that most, if not all, of this difference between sites can be accounted for by differences in foliar N (Fig. 3). We found a significant correlation between foliar N and cyanogenic glycoside concentration at the Eyre Peninsula site (the slope of the regression line was 0.094 and the *x*-intercept was 0.53% N) but not at the Kangaroo Island site, possibly due to the relatively small range of N in these leaves. Nevertheless, when we combined these latter data with those of Gleadow and Woodrow (2000*b*) — which were obtained from the same Kangaroo Island site — we did find a significant correlation between leaf N and cyanogenic glycoside concentration. In this case, the slope of the regression line was 0.083 and the *x*-intercept was 0.51% N. Importantly, analysis of variance indicated that the slopes and intercepts of the correlations for each site were not significantly different. In view of this consistent relationship between foliar N and cyanogenic glycoside concentration, we next plotted the proportion of N allocated to cyanogenic glycosides against $\delta^{13}C$ (Fig. 4). We did not find a significant correlation; therefore, we cannot attribute any of the variation in cyanogenic glycoside concentration to variation in $\delta^{13}C$.

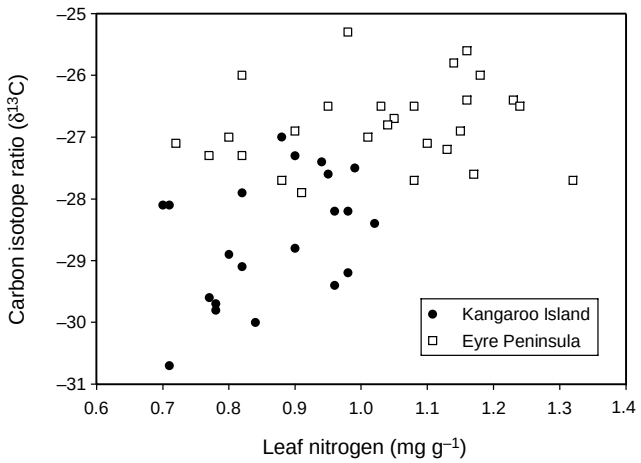

Fig. 6. Total nitrogen concentration and carbon isotope discrimination ($\delta^{13}C$) of leaves from *Eucalyptus cladocalyx* from two regions in South Australia differing in rainfall. No significant correlation was detected for trees at each site (Eyre Peninsula: Pearson's = 0.178; $P = 0.37$, $r^2 = 0.03$; Kangaroo Island Pearson's = 0.409, $P = 0.07$; $r^2 = 0.17$).

Because low water availability is often associated with higher foliar N (e.g. Cunningham *et al.* 1999), our results raise the possibility that previous measurements of enhanced cyanogenic glycoside concentration under water stress (e.g. Majak *et al.* 1980; Bokanga *et al.* 1994; Calatayud *et al.* 1994; Schappert and Shore 1999) reflected variation in leaf N, rather than a direct physiological response by the plant. Controlled environment experiments are clearly required to finally resolve the role of water relations in regulating the allocation of N to cyanogenic glycosides.

Measurements of a decrease in the frequency of acyanogenic forms in relatively low-rainfall areas (e.g. Caradus *et al.* 1990) do, however, suggest that these areas contain plants with a higher genetically based cyanogenic capacity. It seems unlikely that such a difference exists between the two sites examined here because a phenotypic response to N availability alone could account for the higher mean cyanogenic capacity at the Eyre Peninsula site. It has been shown for several species (e.g. Forslund and Jonsson 1997), including *E. cladocalyx* (Gleadow *et al.* 1998), that increasing N supply and foliar N effect an increase in both cyanogenic glycoside concentration and cyanide N to total N ratio not unlike that observed here across both sites. In *E. cladocalyx* seedlings grown in a glasshouse, for example, increasing mean foliar N from 0.76 to 1.11% (by increasing soil N) resulted in a rise in mean cyanogenic glycoside concentration from 1.03 to 2.25 mg g^{-1} dw (R. M. Gleadow, unpublished results). Nevertheless, such an effect may not necessarily be sustained in adult trees and further tests

involving either clones or the progeny of selected trees would be required to resolve whether the frequency distribution of genetically based cyanogenic capacities is the same at both sites.

Finally, we present evidence that *E. cladocalyx* trees from the drier Eyre Peninsula site suffered less leaf damage than those from Kangaroo Island (Table 1). While it is tempting to attribute this to the higher level of cyanogenic glycosides, it may also be the result of lower leaf water content (as indicated by the $\delta^{13}C$ ratios), differences in the concentrations of carbon-based defence chemicals, or merely of differences in the density of herbivores at the two sites.

Acknowledgments

We thank Mr Barry Smith of the Glenville Stud for access to trees on private land and Mr Bruce Webber for assistance in the field. Field work was carried out under a permit supplied by South Australian Department of Natural Resources (Permit no. S23857-02). This research was supported by funds from an Australian Research Council Grant to IEW.

References

Abbott RJ (1977) A quantitative association between soil moisture content and the frequency of cyanogenic form of *Lotus corniculatus* L. at Birsay, Orkney. *Heredity* **38**, 397–400.

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**, 637–645.

Arndt SK, Wanek W, Clifford SC, Popp M (2000) Contrasting adaptations to drought stress in field-grown *Ziziphus mauritiana* and *Prunus persica* trees: water relations, osmotic adjustment and carbon isotope composition. *Australian Journal of Plant Physiology* **27**, 985–996.

Blaise S, Cartier D, Reynaud J (1991) Evolution and differentiation of *Lotus corniculatus/Lotus alpinus* populations from French south-western alps. I. Morphologic and cyanogenic variations. *Evolutionary Trends in Plants* **5**, 137–148.

Bokanga M, Ekanayake IJ, Dixon AGO (1994) Genotype-environment interactions for cyanogenic potential in cassava. *Acta Horticulturae* **375**, 131–139.

Briggs MA, Schultz JC (1990) Chemical defense production in *Lotus corniculatus* L. II. Trade-offs among growth, reproduction and defense. *Oecologia* **83**, 32–37.

Brinker AM, Seigler DS (1989) Methods for the detection and quantitative determination of cyanide in plant materials. *Phytochemical Bulletin* **21**, 24–31.

Calatayud PA, Tertuliano M, Le Ru B (1994) Seasonal variation in secondary compounds in the phloem sap of cassava in relation to plant genotype and infestation by *Phenacoccue manihoti* (Homoptera: Pseudococcidae). *Bulletin of Entomological Research* **84**, 453–459.

Caradus JR, MacKay AC, Charlton JFL, Chapman DF (1990) Genecology of white clover (*Trifolium repens* L.) from wet and dry hill country pastures. *New Zealand Journal of Agricultural Research* **40**, 1–7.

Cunningham SA, Summerhayes B, Westoby M (1999) Evolutionary divergences in leaf structure and chemistry, comparing rainfall and soil nutrient gradients. *Ecological Monographs* **69**, 569–588.

Dement WA, Mooney HA (1974) Seasonal variation in the production of tannins and cyanogenic glucosides in the Chaparral shrub, *Heteromeles arbutifolia*. *Oecologia* **15**, 65–76.

Ehleringer JR (1993) Carbon and water relations in desert plants: an isotopic perspective. In 'Stable isotopes and plant carbon–water relations'. (Eds JR Ehleringer, AE Hall and GD Farquhar) pp. 155–172. (Academic Press: San Diego, CA)

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**, 503–537.

Field C, Mooney HA (1986) The photosynthesis–nitrogen relationship in wild plants. In 'On the economy of plant form and function'. (Ed. TA Givinish) pp. 25–55. (Cambridge University Press: London)

Fisher RF (1987) 'Properties and management of forest soils. 2nd Edn.' (John Wiley & Sons: New York, NY)

Forslund K, Jonsson L (1997) Cyanogenic glycosides and their metabolic enzymes in barley, in relation to nitrogen levels. *Physiologia Plantarum* **101**, 367–372.

Foulds W, Grime JP (1972*a*) The influence of soil moisture on the frequency of cyanogenic plants on populations of *Trifolium repens* and *Lotus corniculatus*. *Heredity* **28**, 143–146.

Foulds W, Grime JP (1972*b*) The response of cyanogenic and acyanogenic phenotypes of *Trifolium repens* to soil moisture supply. *Heredity* **28**, 181–187.

Gershenzon J (1984) Changes in the levels of plant secondary metabolites under water and nutrient stress. *Recent Advances in Phytochemistry* **8**, 273–320.

Gleadow RM (1999) Resource allocation in cyanogenic *Eucalyptus cladocalyx*. PhD thesis, The University of Melbourne.

Gleadow RM, Woodrow IE (2000*a*) Temporal and spatial variation in cyanogenic glycosides in *Eucalyptus cladocalyx*. *Tree Physiology* **20**, 591–598.

Gleadow RM, Woodrow IE (2000*b*) Polymorphism in cyanogenic glycoside content and cyanogenic β-glucosidase activity in natural populations of *Eucalyptus cladocalyx*. *Australian Journal of Plant Physiology* **27**, 693–699.

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**, 12–22.

Herms DA, Mattson WJ (1992) The dilemma of plants: to grow or to defend. *The Quarterly Review of Biology* **67**, 283–335.

Hsiao TC (1973) Plant responses to water stress. *Annual Review of Plant Physiology* **24**, 519–570.

Hughes MA (1991) The cyanogenic polymorphism in *Trifolium repens* L. (white clover). *Heredity* **66**, 105–115.

Jones DA (1998) Why are so many food plants cyanogenic? *Phytochemistry* **47**, 155–162.

Kakes P (1989) An analysis of the costs and benefits of the cyanogenic system in *Trifolium repens* L. *Theory of Applied Genetics* **77**, 111–118.

Kakes P (1997) Difference between the male and female components of fitness associated with the gene *Ac* in *Trifolium repens*. *Acta Botanica Neerlandica* **46**, 219–223.

Lambert JFS, Ramasamy J, Paukstelis JV (1975) Stable reagents for the colorimetric determination of cyanide by modified Konig reactions. *Analytical Chemistry* **47**, 916–918.

Lechtenberg M, Nahrstedt A (1999) Cyanogenic glycosides. In 'Naturally occurring glycosides'. (Ed. R Ikan) pp. 147–191. (John Wiley & Sons: Chichester, UK)

Majak W, McDiarmid RE, Hall JW, Van Ryswyk AL (1980) Seasonal variation in cyanide potential of arrow grass (*Triglochin maritima*). *Canadian Journal of Plant Science* **60**, 1235–1241.

Møller BL, Seigler DS (1999) Biosynthesis of cyanogenic glycosides, cyanolipids, and related compounds. In 'Plant amino acids: biochemistry and biotechnology'. (Ed. BK Singh) pp. 563–609. (Marcel Dekker Inc.: New York, NY)

Nahrstedt A (1985) Cyanogenic compounds as protecting agents for organisms. *Plant Systematics and Evolution* **150**, 35–47.

Nelson CE (1953) HCN content of certain sorghums under irrigation as affected by nitrogen fertilizer and soil moisture stress. *Agronomy Journal* **45**, 615–617.

Pederson G, Fairbrother TE, Greene SL (1996) Cyanogenesis and climatic relationships in US white clover germplasm collection and core subset. *Crop Science* **36**, 427–433.

Poulton JE (1990) Cyanogenesis in plants. *Plant Physiology* **94**, 401–405.

Schappert PJ, Shore JS (1999) Cyanogenesis in *Turnera ulmifolia* L. (Turneraceae). I. Phenotypic distribution and genetic variation for cyanogenesis on Jamaica. *Heredity* **74**, 392–404.

Schulze E-D, Williams RJ, Farquhar GD, Schultze W, Langridge J, Miller JM, Walker BH (1998) Carbon and nitrogen isotope discrimination and nitrogen nutrition of trees along a rainfall gradient in northern Australia. *Australian Journal of Plant Physiology* **25**, 413–425.

Skogsmyr I, Fagerström T (1992) The cost of anti-herbivory defence: an evaluation of some ecological and physiological factors. *OIKOS* **64**, 451–457.

Stewart GR, Turnbull MH, Schmidt S, Erskine PD (1995) ^{13}C natural abundance in plant communities along a rainfall gradient: a biological integrator of water availability. *Australian Journal of Plant Physiology* **22**, 51–55.

Manuscript received 26 April 2001, accepted 16 July 2001