Tree Physiology 30, 1111–1117
doi:10.1093/treephys/tpq049

PART OF AN INVITED ISSUE ON TREE NUTRITION

Allocation of nitrogen to chemical defence and plant functional traits is constrained by soil N

JUDY SIMON,[1,2,3] ROSLYN M. GLEADOW[1,4] and IAN E. WOODROW[1]

[1] *School of Botany, The University of Melbourne, Parkville, Victoria 3010, Australia*

[2] *Present address: Institute of Forest Botany and Tree Physiology, Chair of Tree Physiology, University of Freiburg, Georges-Koehler-Allee 53/54, 79110 Freiburg, Germany*

[3] *Corresponding author (judy.simon@ctp.uni-freiburg.de)*

[4] *Present address: School of Biological Sciences, Monash University, Clayton, Victoria 3800, Australia*

Received February 16, 2010; accepted April 29, 2010; published online June 23, 2010

Summary Plants have evolved a vast array of defence mechanisms to avoid or minimize damage caused by herbivores and pathogens. The costs and benefits of defences are thought to vary with the availability of resources, herbivore pressure and plant functional traits. We investigated the resource (nitrogen) and growth cost of deploying cyanogenic glycosides in seedlings of *Eucalyptus cladocalyx* (Myrtaceae). To do this, we grew the plants under a range of soil N conditions, from levels that were limiting for growth to those that were saturating for growth, and we measured correlations between foliar chemical and performance attributes. Within each N treatment, we found evidence that, for every N invested in cyanogenic glycosides, additional N is added to the leaf. For the lowest N treatment, the additional N was less than one per cyanogenic glycoside, rising to some two Ns for the other treatments. The interaction between cyanogenic glycosides and both condensed tannins and total phenolic compounds was also examined, but we did not detect correlations between these compounds under constant leaf N concentrations. Finally, we did not detect a correlation between net assimilation rate, relative growth rate and cyanogenic glycoside concentrations under any soil N treatment. We conclude that the growth cost of cyanogenic glycosides was likely too low to detect and that it was offset to some degree by additional N that was allocated alongside the cyanogenic glycosides.

Keywords: chemical defence, cyanogenesis, defence cost, eucalypt, nitrogen allocation, phenolics.

Introduction

Plant growth and reproduction, and thus plant fitness, can be affected by damage caused by herbivores or pathogens (Coley et al. 1985, Westoby et al. 2002). The deployment of defences is, therefore, beneficial to a plant by keeping the loss of resources to a minimum (e.g., Coley et al. 1985, Herms and Mattson 1992). Although chemical defence mechanisms and the interactions between plant functional traits have been studied extensively during past decades, relatively few studies have examined how chemical defence deployment may interact with leaf functional attributes (e.g., leaf structure, function and longevity) with regard to potential negative effects on plant growth/fitness due to the diversion of resources to defence (Coley et al. 1985, Reich et al. 1997, Westoby et al. 2002). For example, with increased investment in N-based chemical defence, photosynthetic capacity may be reduced, as a sizeable proportion of leaf N (up to 15% of leaf N for cyanogenesis; Gleadow et al. 1998, Miller et al. 2004) may be allocated to the synthesis and maintenance of defence compounds (e.g., Herms and Mattson 1992, Gleadow et al. 1998). What is missing from these studies is an appraisal of how different chemical defence types (e.g., C- and N-based defences) and changes in leaf N economy interact and possibly mask their potential influence on plant growth/fitness.

The theoretical basis for a trade-off between resource investment in growth, reproduction or defence has been widely accepted (e.g., Herms and Mattson 1992, Zangerl and Bazzaz 1992, Agrawal and Fishbein 2006). However, in many studies, costs of defence have not been detected. This is possibly because costs vary as a function of the environmental conditions under which the study was undertaken (Agrawal and Fishbein 2006). Defence costs should, theoretically, be easier to detect in resource-limited environments because defence forms a larger part of the overall resource budget of the plant (Craine et al. 2003). Consequently, prior knowledge is required about which resources are limiting and at what levels when designing an experiment for cost detection. However, since this has been done in only few studies, it is not surprising that the correlations between defence mechanisms and plant fitness are not very well understood (but see Mutikainen et al. 2002). Furthermore, many studies examin-

ing the costs of plant defence are only poorly controlled for the underlying genetic polymorphism reflecting genetic variables uncontrolled rather than the actual costs of defence (Strauss et al. 2002, Agrawal and Fishbein 2006).

Plant growth may compete with the biosynthesis of phenolic compounds for a common substrate because phenylalanine is the precursor used for the synthesis of phenolics and it is also an essential amino acid for protein synthesis (Boudet 2007). Some cyanogenic glycosides are derived from phenylalanine as well (Zagrobelny et al. 2004), increasing the likelihood of interactions between defence mechanisms and growth. These relationships between N- and C-based chemical defence and also plant growth may be altered depending on soil N availability. Calculations of the growth and reproductive costs involved in plant defence usually focus on a single defence system such as phenolics (e.g., Herms and Mattson, 1992) or cyanogenic glycosides (e.g., Kakes 1989, Goodger et al. 2004). Given that plants often employ a suite of defence strategies, it may, therefore, be more relevant to examine the trade-offs (or synergisms) between multiple traits and growth (Agrawal and Fishbein 2006, Read et al. 2009). Moreover, tests of plant defence theories have generally been biased towards carbon-based defence compounds, such as phenolics and tannins (e.g., McKey et al. 1979, Coley 1987, 1988).

Goodger et al. (2006) examined the growth cost of N-containing cyanogenic glycosides in cyanogenic eucalypts but were unable to detect a growth cost associated with these compounds. It was suggested that this was due to at least three factors. First, plants were grown under a soil N concentration that was saturating for growth, which would possibly mask a growth cost. Second, and consistent with the first suggestion, higher levels of foliar cyanogenic glycosides were associated with higher levels of N, which may have sustained the biosynthesis and maintenance of these compounds and offset a change in leaf performance. Third, parallel variation in other abundant defence compounds may have masked the effects on growth of cyanogenic glycosides. Our aim in this study is to extend the work of Goodger et al. (2006) by measuring the allocation cost of cyanogenic glycosides under a range of soil N conditions, taking into account variation in other major groups of defence compounds. We chose for the experiments the most cyanogenic of the three species examined by Goodger et al. (2006)—*Eucalyptus cladocalyx*, as it is constitutively cyanogenic with little variation with seedling age (Gleadow et al. 1998; Gleadow and Woodrow 2000a). We included seedlings with genetically different abilities for the synthesis of cyanogenic glycosides (see Woodrow et al. 2002) in this experiment. Multiple regression analysis was used to test the following hypotheses: (i) Increasing foliar cyanogenic glycoside concentration is associated with an increase in foliar N concentration. The magnitude of this relationship is moderated by soil N supply. (ii) Within N treatments, increasing cyanogenic glycoside concentration is associated with reduced concentrations of carbon-based defence compounds (i.e., total phenolics and condensed tannins). (iii) An increase in cyanogenic glycoside concentration is related to a decrease in the productive capacity of leaves [net assimilation rate (NAR)]. The magnitude of this decrease is reduced as N supply to the plant is increased.

Materials and methods

Plant material and growth conditions

Seeds of *E. cladocalyx* var. *nana* were collected from four open-pollinated trees in two populations (two from each population) located ~25 km W of Cowell, South Australia (see Woodrow et al. 2002). One population was located in a valley (33°38′06″ S, 136°40′35″ E; elevation 310 m a.s.l.; slope 3°), and the other on a slope ~400 m away (33°38′00″ S, 136°40′45″ E; elevation 380 m a.s.l.; slope 15°). Seedlings were grown in pots (1.5 l) containing a mixture of sterile sand, vermiculite and perlite (1:1:1). Pots were flushed with modified one-quarter strength Hoagland's solution containing 3 mM N, supplied as ammonium and nitrate (1:5, mole:mole) with sodium as the balancing cation (Gleadow and Woodrow 2000*a*). After 5 months of growth, seedlings were screened for cyanogenic glycosides (determined in the fifth leaf from the top of each seedling). Based on this screening, 51–52 seedlings were chosen for each of four N treatments (1.5, 3, 5 or 8 mM N), taking the cyanogenic glycoside concentration into consideration. For example, four seedlings with similar cyanogenic glycoside concentrations were assigned to each of four treatments to ensure a range of cyanide concentrations in each treatment. For the duration of the N treatments, seedlings were supplied with modified one-quarter strength Hoagland's solution containing either 1.5, 3, 5 or 8 mM N, supplied as ammonium and nitrate (1:5, mole:mole). The total growing period was about 6 months (October–April). Mean air temperature [day and night (±SD)] was 25.4 ± 2.7 and 19.6 ± 1.1 °C, respectively. Mean relative humidity [day and night (±SD)] was 50.2 ± 10.4 and 66.4 ± 16.2%, respectively. Mean daily PPFD ranged from 214.62 to 414.78 μmol m^{-2} s^{-1}. The photoperiod was approximately 11.5 h. Plants were rotated on the bench weekly to minimize any local variation in PPFD.

Harvesting and sample handling

In order to minimize phenological effects on defence (e.g., Goodger et al. 2006), plants were harvested at a common size, when they reached 0.45 m. Keeping size constant had the added benefit of avoiding any potential dilution effects. Seedlings biomass was then divided into leaf tips (width < 1 cm), leaves, stems and roots, weighed and leaf area was measured. The material was snap frozen in liquid nitrogen, and after freeze-drying for 96 h, the dry weight (dw) was determined. Unexpanded and senescent leaves were excluded from the leaf pool for chemical analyses. For the calculation of the relative growth rates, five randomly chosen seedlings

from each family were harvested before the start of the treatments.

Quantitative foliar chemical analyses

Freeze-dried leaf material was ground in a cooled IKA Labortechnic A10 analytical mill (Jahnke & Kunkel GmbH Co., Staufen, Germany) and stored at −20 °C for further analysis. All chemical analyses were undertaken with <20 mg freeze-dried tissue (except for N/C analysis and Rubisco quantification). Cyanogenic glycoside concentration was measured as cyanide (CN), extracted and quantified according to the method by Brinker and Seigler (1989) as modified by Woodrow et al. (2002). For the quantification of cyanogenic glycosides, exogenous β-glucosidase enzyme [from almond emulsin (*Prunus amygdalis* (L.) Benth. & Hook.); β-glucosidase; EC 3.2.1.21; Sigma-Aldrich, Steinheim, Germany] was added to the extraction buffer (1.12 units per millilitre) to ensure complete conversion of the cyanide. The cyanide detected is directly proportional to the concentration of prunasin, the cyanogenic glycoside in *E. cladocalyx* (Gleadow et al. 2008).

Total N and C concentrations were quantified (sample size 5–10 mg) using a Carlos Erba NA 1500 Series 2 NCS Analyser and AS-200 Autosampler (Fisons Instruments, Milan, Italy), using acetanilide as analytical standard (Perkin-Elmer #0204-1121).

Quantification of total phenolics (TP) was determined using the Folin–Ciocalteu assay described in Cork and Krockenberger (1991) with gallic acid as a standard as modified by Burns et al. (2002). Condensed tannins (CT) concentration was quantified according to the method of Julkunen-Tiitto (1985) with (+)-catechin as a standard. Total chlorophyll concentration (Chl) was quantified according to the method of Burns et al. (2002) using the equations of Jeffrey and Humphrey (1975).

Rubisco (Rub) was extracted using the method described in Gleadow et al. (1998) and quantified following the method of Kelly (1998). Rubisco enzyme was extracted from freeze-dried leaf material (~5 mg) at room temperature for 20 min in 1 ml extraction buffer containing 20 mM Bicine–KOH (pH 8.0), 1 mM ethylenediaminetetraacetic acid, 2.5% polyvinylpolypyrrolidone (w/v), 1.5% Tween 20 (polyoxyethylene–sorbitan monolaurate; v/v) and 5 mM 1,4-dithiothreitol (a protease inhibitor). After centrifugation at 15,000*g* for 30 s, the extracts were prepared for SDS–PAGE by adding 5 µl of solubilizing buffer [6% sodium dodecyl sulphate (SDS; electrophoresis grade), 30% glycerol, 0.1% bromophenol blue, 0.1875 mM Tris–HCl (pH 7.5) and 2.5% 2-mercaptoethanol] to a 10-µl sample or standard. The samples were heated at 95 °C for 30 s and then separated using SDS–PAGE using 15-well, 0.1 cm × 10 cm × 8 cm precast 10% Tris–glycine gels (10% acrylamide; Life Therapeutics, French Forest, NSW, Australia), loaded with a 10-µl sample and ran at 100 V for 2 h using NOVEX XCELL II Mini-gel System (Novex, San Diego, CA). Rubisco from *Nicotiana tabacum* was used as a standard. Gels were fixed in a 40% methanol and 10% glacial acetic acid solution for 30 min, washed three times for 3 min each in distilled water, stained with GelCode® Blue Stain Reagent (Pierce #24590, Rockford, IL) for 1 h and finally destained overnight in 6% acetic acid. After scanning the gel using a GeneGenius Bio Imaging System (Syngene Ltd, Frederick, MD), the amounts of Rubisco were calculated using GeneSnap and GeneTools (both Syngene Ltd, Frederick, MD).

Growth and biomass allocation indices

Average relative growth rate (RGR, g g^{-1} dw day^{-1}) was calculated as $RGR = (\ln W_1 - \ln W_2)/t_2 - t_1$ where W_1 is the total plant biomass (g dw) at t_1 calculated from the average biomass of five seedlings harvested at t_1, t_1 (days) is the day the nitrogen treatment commenced, W_2 is the individual total plant biomass (g dw) at t_2 and t_2 (days) is the day of harvesting. It was assumed that average RGR equals RGR at t_2 based on the observation by Goodger et al. (2006) who found that RGR in two related species of *Eucalyptus* was approximately constant during the first few months. The rest of the analysis used biomass indices and chemical data from t_2. Other biomass indices calculated were leaf area per unit leaf weight [specific leaf area (SLA)], leaf mass as a proportion of total plant biomass [leaf mass ratio (LMR)] and total leaf area as a proportion of total plant biomass [leaf area ratio (LAR)]. NAR was calculated by dividing RGR by LAR. Leaf nitrogen productivity (LNP), an index of plant growth relative to leaf N, was determined as follows: $LNP = RGR/(N_a \times LAR)$ where N_a is the foliar nitrogen concentration per unit leaf area.

Statistical analysis

Minitab® Release 15 (Minitab Inc., Pasadena, CA) was used to perform normality tests and Levene's test for homogeneity of variances. Data were transformed where necessary to satisfy the assumptions of normality. To detect differences between nitrogen treatments two-sample *t*-tests and/or one-way ANOVAs were conducted. Single and multiple regression analysis were performed using SigmaPlot 11.0 (SPSS Inc., Chicago, IL) to test the hypotheses of this study.

Results

Impact of N supply on leaf N and N defence

The first hypothesis that we tested was that increasing foliar cyanogenic glycoside concentration is associated with an increase in foliar N concentration and that soil N supply moderates the magnitude of the relationship. To do this, we used the multiple regression approach of Goodger et al. (2006) which involved fitting the data to the following equation: $N_a = N_{a0} + aN_{CNa} + b\text{SLA}$, where N_a is the leaf N per

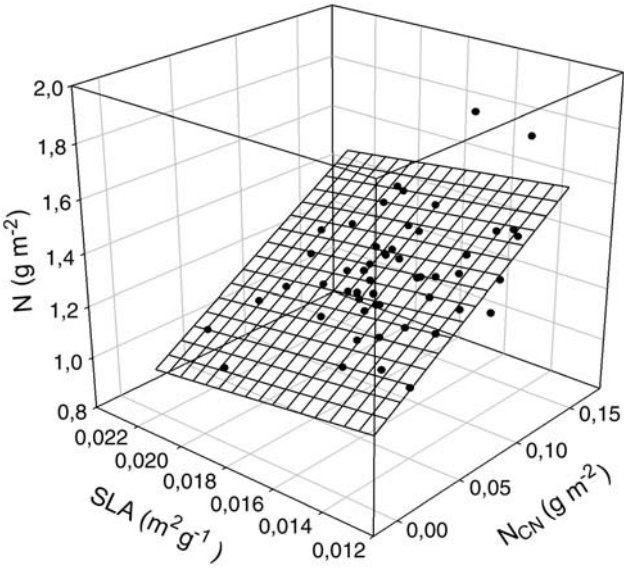

Figure 1. Variation in leaf area-based N (N_a) with SLA ($m^2\ g^{-1}$) and area-based N invested in cyanogenic glycosides (N_{CNa}) in *E. cladocalyx* seedlings. A multiple regression analysis of the data from the 3.0-mM N treatment (black circles) was performed (grid lines) using the following equation: $N_a = N_{a0} + aN_{CNa} + b$SLA where N_{a0} is the N_a intercept when N_{CNa} and SLA are zero and *a* and *b* are the slopes defining the N_a–N_{CNa} and N_a–SLA relationships, respectively (r^2 = 0.509, $P < 0.001$). Estimated values for *a*, *b* and N_{a0} are 3.32 g g^{-1} N, −11.24 g N g^{-1} leaf and 1.17 $m^2\ g^{-1}$, respectively.

unit area, N_{a0} is the axis intercept, N_{CNa} is the area-based N content of cyanogenic glycosides and *a* and *b* are slopes defining the N_a–N_{CNa} and N_a–SLA relationships, respectively. We found slopes (i.e., values) of the N_a–N_{CNa} relationship (i) for the 1.5-mM N treatment of 1.56 ± 0.46 (P = 0.014), (ii) for the 3-mM N treatment of 3.32 ± 0.50 ($P < 0.001$), (iii) for the 5-mM N treatment of 3.22 ± 0.78 (P = 0.001) and (iv) for the 8-mM treatment 2.47 ± 0.69 (P = 0.008). The relationship between the three variables for the 3-mM N treatment is shown in Figure 1. It should be noted that the increase in N supply effected an overall increase in the cyanogenic glycoside content of leaves (Table 1, Figure 2), but within each treatment, it has previously been shown that variation is largely genetically determined (Gleadow and Woodrow 2000b). Interestingly, for the three higher N treatments, there is little variation in the *a* values, showing that for every N effectively added to the leaves as a cyanogenic glycoside, some two Ns are added on top of this investment. In the lowest N treatment, however, less than one N is added alongside every cyanogenic glycoside. Some of this extra investment could be in extra chlorophyll or Rubisco for photosynthesis, and we did detect weak but significant correlations between these variables within the 1.5- and 3-mM N treatments for chlorophyll ($r^2 < 0.203$, $P < 0.001$; see Figure 3) and in the 1.5- and 8-mM N treatments for Rubisco ($r^2 < 0.165$, $P < 0.028$). There was also a strong correlation across treatments for chlorophyll ($r^2 = 0.524$, $P < 0.001$) but not for Rubisco ($r^2 = 0.128$ and $P < 0.001$) (data not shown).

Increasing N-based defence is related to decreases in C-based defence systems

The second hypothesis that we tested was that, within treatments, increases in cyanogenic glycoside concentration are

Table 1. Descriptive statistics for biomass partitioning and leaf chemical parameters for *E. cladocalyx* seedlings subjected to four N treatments. Mean, SE mean and number of replicates (*n*) are presented for each treatment (mM N). Leaf chemical parameters are shown on mass basis. R: S_m, root:shoot ratio; RGR, relative growth rate; LAR, leaf area ratio; SLA, specific leaf area; LMR, leaf mass ratio; NAR, net assimilation rate; LNP, leaf nitrogen productivity; N, nitrogen concentration; C, carbon concentration; CN, cyanide concentration; TP, total phenolics concentration; CT, condensed tannins concentration; Chl, total chlorophyll concentration; Rub, Rubisco concentration. One-way ANOVAs were performed between treatments. The number of replicates varied for all parameters between 51 and 52, except for Rubisco concentration where it was between 33 and 36 depending on the treatment.

	Mean ± SEM				One-way ANOVA	
Variable	1.5	3.0	5.0	8.0	*F*	P-value
Growth allocation indices						
$R:S_m$	0.6339 ± 0.0195	0.6135 ± 0.0176	0.5272 ± 0.0240	0.4671 ± 0.0151	16.07	<0.001
RGR (g g^{-1} d^{-1})	0.0156 ± 0.0004	0.0248 ± 0.0007	0.0297 ± 0.0073	0.0319 ± 0.0007	155.60	<0.001
LAR (m^2 g^{-1})	0.0056 ± 0.0001	0.0069 ± 0.0001	0.0083 ± 0.0002	0.0088 ± 0.0002	88.27	<0.001
SLA (m^2 g^{-1})	0.0131 ± 0.0002	0.0170 ± 0.0003	0.0193 ± 0.0004	0.0198 ± 0.0004	81.90	<0.001
LMR	0.4245 ± 0.0064	0.4077 ± 0.0054	0.4306 ± 0.0062	0.4433 ± 0.0045	6.99	<0.001
NAR (g m^{-2} d^{-1})	2.8544 ± 0.0928	3.6570 ± 0.1120	3.6550 ± 0.1080	3.6999 ± 0.0879	17.30	<0.001
LNP (g $g(N_m)^{-1}$ d^{-1})	2.430 ± 0.101	3.111 ± 0.122	2.727 ± 0.104	2.475 ± 0.067	9.56	<0.001
Foliar chemical composition						
N_m (g g^{-1} dw)	0.0169 ± 0.0004	0.0220 ± 0.0004	0.0287 ± 0.0007	0.0325 ± 0.0006	149.38	<0.001
C_m (g g^{-1} dw)	0.5166 ± 0.0015	0.5101 ± 0.0015	0.5036 ± 0.0018	0.5051 ± 0.0017	13.19	<0.001
CN_m (g g^{-1} dw)	0.0017 ± 0.0001	0.0030 ± 0.0001	0.0044 ± 0.0001	0.0052 ± 0.0002	115.25	<0.001
TP_m (g g^{-1} dw)	0.0100 ± 0.0002	0.0082 ± 0.0002	0.0063 ± 0.0002	0.0057 ± 0.0002	96.09	<0.001
CT_m (g g^{-1} dw)	0.0034 ± 0.0001	0.0026 ± 0.0001	0.0017 ± 0.0001	0.0015 ± 0.0001	76.75	<0.001
Chl_m (g g^{-1} dw)	0.0038 ± 0.0001	0.0057 ± 0.0001	0.0076 ± 0.0002	0.0081 ± 0.0002	189.51	<0.001
Rub_m (g g^{-1} dw)	0.0262 ± 0.0017	0.0367 ± 0.0027	0.0464 ± 0.0035	0.0526 ± 0.0034	13.61	<0.001

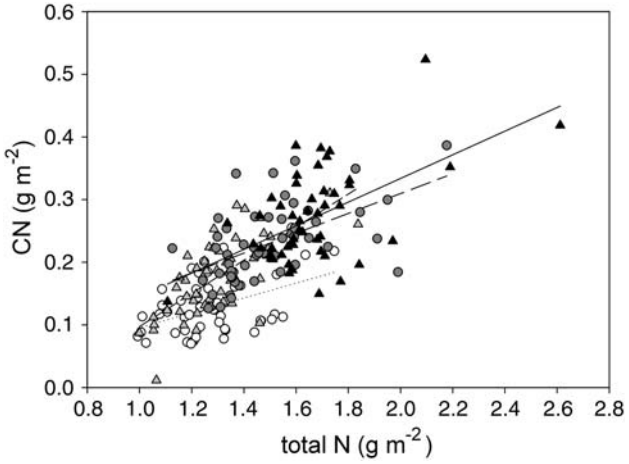

Figure 2. Relationship between foliar cyanide concentration (CN_m, g m^{-2} dw) and total foliar N concentration (g m^{-2} dw) in *E. cladocalyx* seedlings at four different N levels. N treatments are presented as follows: white circles for the 1.5-mM N treatment (r^2 = 0.305, P = 0.001), light grey triangles for the 3.0-mM N treatment (r^2 = 0.332, P < 0.001), dark grey circles for the 5.0-mM N treatment (r^2 = 0.483, P < 0.001) and black triangles for the 8.0-mM N treatment (r^2 = 0.376, P < 0.001).

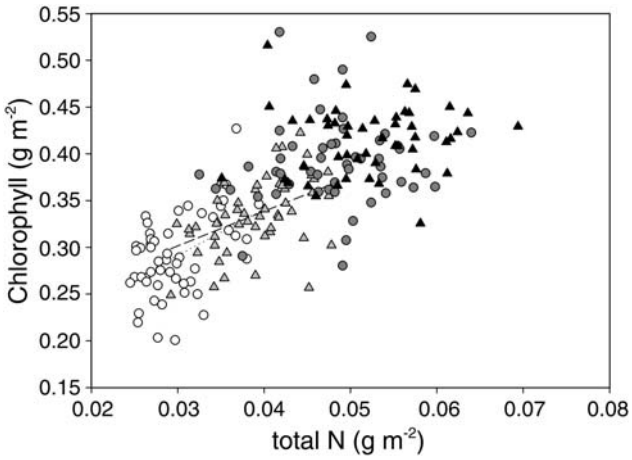

Figure 3. Relationship between foliar N concentration (N_a, g m^{-2} dw) and chlorophyll concentration (Chl, g m^{-2} dw) in *E. cladocalyx* seedlings at four different N levels. N treatments are presented as follows: white circles for the 1.5-mM N treatment (r^2 = 0.187, P = 0.002), light grey triangles for the 3.0-mM N treatment (r^2 = 0.203, P = 0.001), dark grey circles for the 5.0-mM N treatment (r^2 = 0.017, P = 0.356) and black triangles for the 8.0-mM N treatment (r^2 = 0.018, P = 0.353).

associated with decreases in both total phenolics and the important condensed tannins, a component of the total phenolics. To test this, we used multiple regression analysis involving the following equations: (i) $CN = CN_0 + cN + dTP$ where CN_0 is the intercept when N and TP are zero and c and d are the slopes defining the CN–N and CN–TP relationships, respectively, and (ii) $CN = CN_0 + eN + fCT$

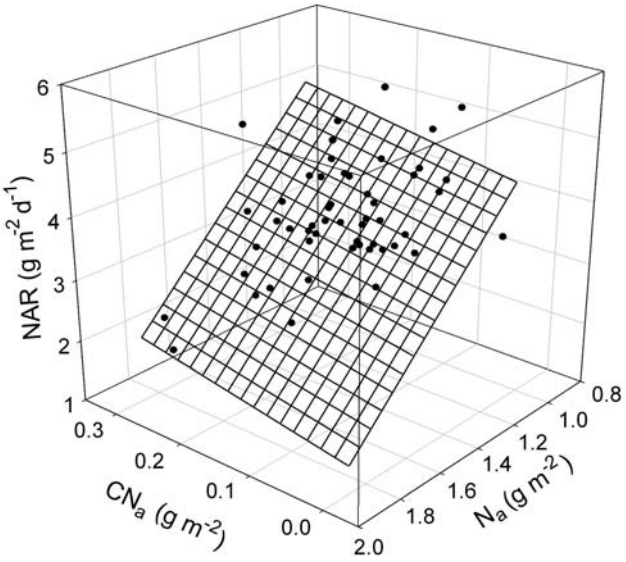

Figure 4. Variation in area-based N (N_a) with NAR (g m^{-2} day^{-1}) and area-based cyanogenic glycosides (CN_a) in *E. cladocalyx* seedlings. A multiple regression analysis of the data from the 3.0-mM N treatment (black circles) was performed (grid lines) using the following equation: $NAR = NAR_0 + mCN_a + nN_a$ where NAR_0 is the NAR intercept when CN_a and N_a are zero and m and n are the slopes defining the NAR–CN_a and NAR–N_a relationships, respectively (r^2 = 0.387, P < 0.001). Estimated values for m and n are 2.21 g g^{-1} CN day^{-1} and −3.68 g g^{-1} N day^{-1}, respectively.

where CN_0 is the intercept when N and CT are zero and e and f are the slopes defining the CN–N and CN–CT relationships, respectively. Despite the overall negative correlations between cyanogenic glycosides and both condensed tannins and total phenolics (r^2 = 0.436, P < 0.001 and r^2 = 0.520, P < 0.001, respectively), we did not detect such a negative correlation within any treatment when foliar N was effectively held constant (i.e., slopes d and f were not significantly different from zero; data not shown).

Are levels of N-based defence inversely correlated with the productive capacity of leaves?

Finally, given the fact that neither total phenolics nor condensed tannins were varying in parallel with cyanogenic glycosides (no clear pattern detected), we used the analysis of Goodger et al. (2006), which involves just total N and cyanogenic glycoside concentration as variables, to test our next hypothesis: (i) an increase in cyanogenic glycoside concentration is associated with a decrease in the productive capacity of leaves (NAR) and (ii) the magnitude of this decrease is reduced as the supply of nitrogen to the plant is increased. The equation used was $NAR = NAR_0 + mCN_a + nN_a$ where NAR_0 is the NAR intercept when CN_a and N_a are zero and m and n are the slope of the NAR–CN_a and NAR–N_a relationships, respectively. Interestingly, we only detected a significant relationship between NAR and CN_a in the 3-mM N treatment: m = −4.180 ± 1.827 (P = 0.027) (Figure 4).

Discussion

Our measurements of the effect of increasing soil N on growth, biomass allocation and foliar chemical composition of *E. cladocalyx* seedlings are broadly consistent with the findings of other studies, including some on the same species (e.g., Gleadow and Woodrow 2002, Goodger et al. 2006). We found an inverse relationship between N defence and C defence compounds in response to N supply, consistent with the carbon/nutrient balance hypothesis (Bryant et al. 1983). When the N supply was low, plants allocated resources to C-based defences (phenolics, tannins and also low SLA) (Table 1). With increasing availability of N, investment in C-based defences decreased, while the concentration of N-rich cyanogenic glycosides and components of the photosynthetic system (chlorophyll, Rubisco) progressively increased (Table 1, Figure 2). This pattern is consistent with other studies of tree species subjected to different N treatments (Gershenzon 1984, Keinänen et al. 1999), including *E. cladocalyx* (Burns et al. 2002, Goodger et al. 2006). The changes in leaf chemistry under increasing nitrogen were paralleled by increases in SLA, shoot:root ratio and RGR, although NAR was largely unaffected (Table 1).

The picture was, however, more complex within each N treatment. We deliberately included plants with considerable genetically based variation in foliar cyanogenic glycoside concentration in this experiment (see Woodrow et al. 2002). This enabled us to effectively titrate cyanogenic glycoside concentration and measure the effects on other factors such as leaf N and NAR against a background of constant N supply. Like Goodger et al. (2006), we found that increasing foliar cyanogenic glycoside levels were associated with higher leaf N when the possibly confounding effects of variation in SLA were taken into account. Interestingly, the slope of the relationship was greater than one for each treatment, indicating that additional N-containing molecules are effectively being added to the leaf together with the cyanogenic glycosides. The amount of these molecules is not relatively high; the slope was less than two for the lowest N treatment, and around three for the other treatments. These latter results are close to the value of 2.5 estimated by Goodger et al. (2006), but in this study, only one N treatment (which was saturating for growth) was employed. The value of about 0.5 additional N per cyanogenic glycoside under the lowest soil N treatment is consistent with likely tight N economy under these conditions. It is likely that the extra N that accompany the cyanogenic glycosides is involved in synthesis and maintenance activities. Mechanisms for both processes are well documented (Jørgensen et al., 2005, Jenrich et al. 2007), and both require an energy input and an array of catalysts. Consistent with this, we found significant correlations between components of the photosynthetic system (Rubisco and chlorophyll) and cyanogenic glycoside concentration.

We did not detect a negative correlation between RGR or NAR and cyanogenic glycoside concentration within any treatment. As suggested by Goodger et al. (2006), this may reflect the fact that increasing cyanogenic glycoside concentrations are to some degree compensated for (in terms of leaf performance) by the accompanying increase in N described above. We used the analysis suggested by Goodger et al. (2006) to take the extra N into account, but we only detected a significant negative effect of NAR in one treatment (i.e., the 3-mM treatment) (Figure 4). We would have preferred to have used a wider spread of leaf cyanogenic glycoside concentrations for these experiments to increase our ability to detect a significant slope. Goodger et al. (2006), who detected a negative correlation between NAR and cyanogenic glycoside content, achieved such a spread by combining data from two species with widely different cyanogenic capacities.

We also measured variation in other leaf defence compounds (condensed tannins and total phenolics) within N treatments to check whether such variation could mask or enhance correlations between cyanogenic glycosides and other variables. In contrast to the strong correlation between the C-based defence compounds and cyanogenic glycoside concentration across treatments (which is apparently driven largely by N), we failed to detect such correlations within any treatment when N was effectively held constant in the analysis. This result is at odds with our hypothesis of opposing change, which was based on the observation that synthesis of both the cyanogenic glycoside in *E. cladocalyx* (prunasin) and phenolic compounds may compete for the common intermediate, phenylalanine (Gleadow and Woodrow 2002, Boudet 2007).

In conclusion, the growth and morphological responses of *E. cladocalyx* to soil N were found here to be typical of most tree seedlings. The increase in RGR was primarily the result of higher SLA with increasing N supply, although there was also a significant increase in LNP (Table 1). This also correlated with the increase in leaf chlorophyll and Rubisco concentration with increasing N supply. Within each N treatment, we found evidence that deployment of cyanogenic glycosides is accompanied by additional deployment of N, which we suggest is involved in biosynthesis and maintenance activities. We did, however, detect a significant cost (in terms of NAR) of cyanogenic glycoside deployment in only one of the four N treatments. It appears that, in this species, there is a genetically determined base rate of N resources allocated to cyanogenic glycosides. The relative decrease in cost of N when N supply is in excess of this amount allows the plant to increase the total concentration of cyanogenic glycosides without any detectable growth sacrifice. Likewise, the relative cost of C defence decreases when growth is limited by low N supply. Changes to photosynthetic efficiency in the future with increasing atmospheric CO_2 could alter this balance, with a consequent impact on herbivores.

Acknowledgments

We would like to thank Ron Teo from the School of Land and Environment, University of Melbourne, for his help with the total nitrogen analyses. We also thank Steve Elefteriadis for the help with the glasshouse set up and Pascal Stroh for harvest assistance.

References

Agrawal, A.A. and M. Fishbein. 2006. Plant defense syndromes. Ecology 87:S132–S149.

Boudet, A. 2007. Evolution and current status of research in phenolic compounds. Phytochemistry 68:2722–2735.

Brinker, A.M. and D.S. Seigler. 1989. Methods for the detection and quantitative determination of cyanide in plant materials. Phytochem. Bull. 21:24–31.

Bryant, J.P., F.S. Chapin III and D.R. Klein. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40:357–368.

Burns, A.E., R.M. Gleadow and I.E. Woodrow. 2002. Light alters the allocation of nitrogen to cyanogenic glycosides in *Eucalyptus cladocalyx*. Oecologia 133:288–294.

Coley, P.D., J.P. Bryant and F.S. Chapin III. 1985. Resource availability and plant antiherbivore defense. Science 230: 895–899.

Coley, P.D. 1987. Interspecific variation in plant anti-herbivore properties: the role of habitat quality and rate of disturbance. New Phytol. 106:251–263.

Coley, P.D. 1988. Effects of plant growth rate and leaf lifetime on the amount and type of anti-herbivore defense. Oecologia 74: 531–536.

Cork, S.J. and A.K. Krockenberger. 1991. Methods and pitfalls of extracting condensed tannins and other phenolics from plants: insights from investigations on *Eucalyptus* leaves. J. Chem. Ecol. 17:123–134.

Craine, J.M., W. Bond, W.G. Lee, P.B. Reich and S. Ollinger. 2003. The resource economics of chemical and structural defenses across nitrogen supply gradients. Oecologia 137: 547–556.

Gershenzon, J. 1984. Changes in the levels of plant secondary metabolites under water and nutrient stress. *In* Recent Advances in Phytochemistry, vol 18. Eds. C. Steelink, B.N. Timmermann and F.A. Loewus. Plenum Press, New York, USA, pp 273–320.

Gleadow, R.M. and I.E. Woodrow. 2000*a*. Temporal and spatial variation in cyanogenic glycosides in *Eucalyptus cladocalyx*. Tree Physiol. 20:591–598.

Gleadow, R.M. and I.E. Woodrow. 2000*b*. Polymorphism in cyanogenic glycoside content and cyanogenic beta-glucosidase activity in natural populations of *Eucalyptus cladocalyx*. Aust. J. Plant Physiol. 27:693–699.

Gleadow, R.M. and I.E. Woodrow. 2002. Defense chemistry of cyanogenic *Eucalyptus cladocalyx* is affected by water supply. Tree Physiol. 22:939–945.

Gleadow, R.M., W.J. Foley and I.E. Woodrow. 1998. Enhanced CO_2 alters the relationship between photosynthesis and defense in cyanogenic *Eucalyptus cladocalyx* F. J. Muell. Plant Cell Environ. 21:12–22.

Gleadow, R.M., J. Haburjak, J.E. Dunn, M.E. Conn and E.E. Conn EE. 2008. Frequency and distribution of cyanogenic glycosides in *Eucalyptus* L'Hérit. Phytochemistry 69:1870–1874.

Goodger, J.Q.D., P.K. Ades and I.E. Woodrow. 2004. Cyanogenesis in *Eucalyptus polyanthemos* seedlings: heritability, ontogeny and effect of soil nitrogen. Tree Physiol. 24:681–688.

Goodger, J.Q.D., R.M. Gleadow and I.E. Woodrow. 2006. Growth cost and ontogenetic expression pattern of defence in cyanogenic *Eucalyptus* ssp. Trees 20:757–765.

Herms, D.A. and W.J. Mattson. 1992. The dilemma of plants: to grow or defend. Q. Rev. Biol. 67:283–335.

Jeffrey, S.W. and G.F. Humphrey. 1975. New spectrophotometric equations for determining chlorophylls a, b, c_1 and c_2 in higher plants, algae and natural phytoplankton. Biochem. Physiol. Pflanzen 167:191–194.

Jenrich, R., I. Trompetter, S. Bak, C.E. Olsen, B.L. Moller and M. Piotrowski. 2007. Evolution of heteromeric nitrolase complexes in Poaceae with new functions in nitrile metabolism. Proc. Natl Acad. Sci. USA 104:18848–18853.

Jørgensen, K., S. Bak, P.K. Busk, C. Sørensen, C.E. Olsen, J. Puonti-Kaerlas and B.L. Møller. 2005. Cassava (*Manihot esculenta* Cranz.) plants with a depleted content of cyanogenic glucosides in leaves and tubers. Distribution of cyanogenic glucosides, their site of synthesis and transport and blockage of the biosynthesis by RNAi technology. Plant Physiol. 139:363–374.

Julkunen-Tiitto, R. 1985. Phenolic constituents in the leaves of northern willows: methods for the analysis of certain phenolics. J. Agric. Food Chem. 33:213–217.

Kakes, P. 1989. An analysis of the costs and benefits of the cyanogenic system in *Trifolium repens* L. Theor. Appl. Genet. 77: 111–118.

Keinänen, M., R. Julkunen-Tiitto, P. Mutikainen, M. Walls, J. Ovaska and E. Vapaavuori. 1999. Trade-offs on phenolic metabolism of silver birch: effects of fertilization, defoliation, and genotype. Ecology 80:1970–1986.

Kelly, M.A. 1998. Role of Rubisco in limiting CO_2 assimilation in understorey environments. MSc Thesis. The University of Melbourne, Melbourne, Australia.

McKey, D. 1979. The distribution of secondary compounds within plants. *In* Herbivores: Their Interactions with Secondary Plant Metabolites. Eds. G.A. Rosenthal and D.H. Janzen. Academic Press, New Jersey, pp 55–133.

Miller, R.E., R.M. Gleadow and I.E. Woodrow. 2004. Cyanogenesis in tropical *Prunus turneriana*: characterisation, variation and response to low light. Funct. Plant Biol. 31:491–503.

Mutikainen, P., M. Walls, J. Ovaska, M. Keinänen, R. Julkunen-Tiitto and E. Vapaavuori. 2002. Costs of herbivore resistance in clonal saplings of *Betula pendula*. Oecologia 133:364–371.

Read, J., G.D. Sanson, E. Caldwell, F.J. Clissold, A. Chatain, P. Peeters, B.B. Lamont, M. De Garine-Wichatitsky, T. Jaffre and S. Kerr. 2009. Correlations between leaf toughness and phenolics among species in contrasting environments of Australia and New Caledonia. Ann. Bot. 103:757–767.

Reich, P.B., M.B. Walters and D.S. Ellsworth DS. 1997. From tropics to tundra: global convergence in plant functioning. Proc. Natl Acad. Sci. USA 94:13730–13734.

Strauss, S.Y., J.A. Rudgers, J.A. Lau and R.E. Irwin. 2002. Direct and ecological costs of resistance to herbivory. Trends Ecol. Evol. 17:278–284.

Westoby, M., D.S. Falster, A.T. Moles, P.A. Vesk and I.J. Wright. 2002. Plant ecological strategies: some leading dimensions of variation between species. Annu. Rev. Ecol. Syst. 33:125–159.

Woodrow, I.E., D.J. Slocum and R.M. Gleadow. 2002. Influence of water stress on cyanogenic capacity in *Eucalyptus cladocalyx*. Funct. Plant Biol. 29:103–110.

Zagrobelny, M., S. Bak, A.V. Rasmussen, B. Jørgensen, C.M. Naumann and B.L. Møller. 2004. Cyanogenic glycosides and plant–insect interactions. Phytochemistry 65:293–306.

Zangerl, A.R. and F.A. Bazzaz. 1992. Theory and pattern in plant defense allocation. *In* Plant Resistance to Herbivores and Pathogens. Eds. R.S. Fritz and E.L. Simms. The University of Chicago Press, Chicago, USA, pp 363–391.