Trees (2006) 20:757–765
DOI 10.1007/s00468-006-0090-2

ORIGINAL ARTICLE

Growth cost and ontogenetic expression patterns of defence in cyanogenic *Eucalyptus* spp.

Jason Q. D. Goodger · Roslyn M. Gleadow · Ian E. Woodrow

Received: 6 March 2006 / Revised: 28 July 2006 / Accepted: 8 August 2006 / Published online: 30 August 2006
© Springer-Verlag 2006

Abstract Plant defences can incur allocation costs and such costs incurred early in ontogeny may result in opportunity costs with effects evident later in life. A unified understanding of the growth cost of defence requires the identification of plants with varying ontogenetic trajectories of preferably resource demanding defences and an appropriate measurement of the growth cost of these defences. To develop such tools, we first compared nitrogen-based chemical defence (cyanogenic glycosides) in juvenile and adult foliage of three species of *Eucalyptus* (Myrtaceae). We found marked differences between the species, with two having much lower concentrations of foliar cyanogenic glycosides in seedlings compared to adults. We next used seedlings of two species to measure the resource (nitrogen) and growth cost of deploying cyanogenic glycosides. We found evidence that for every 1.0 nitrogen invested in cyanogenic glycosides, 1.49 additional nitrogens were effectively added to the leaves. We also found that deployment of cyanogenic glycosides was associated with a reduction in net assimilation rate (NAR) at constant leaf nitrogen. We did not, however, detect an overall growth cost associated with cyanogenic glycoside deployment because the rise in leaf nitrogen associated with this deployment apparently counteracted the reduction in NAR.

Communicated by M. Adams

J. Q. D. Goodger (✉) · R. M. Gleadow · I. E. Woodrow
School of Botany, University of Melbourne,
Victoria 3010, Australia
e-mail: jgoodger@unimelb.edu.au

Present address:
R. M. Gleadow
School of Biological Sciences, Monash University,
Victoria 3800, Australia

Keywords Cost · Cyanogenic glycoside · Eucalyptus · Ontogeny · Plant defence

Abbreviations CN_A: Leaf cyanide per unit leaf area · CN_m: Leaf cyanide per unit leaf mass · CN-N/N: Cyanide–nitrogen as a proportion of total leaf nitrogen · *Ec*: *Eucalyptus cladocalyx* · *Ep*: *E. polyanthemos* · *Ey*: *E. yarraensis* · LAR: Leaf area ratio · LMA: Leaf mass per unit leaf area · LL: Leaf longevity · LNP: Leaf nitrogen productivity · LWR: Leaf weight ratio · N_A: Total leaf nitrogen per unit leaf area · NAR: Net assimilation rate · N_{CN-A}: Nitrogen in cyanide on a leaf area basis · N_M: Total leaf nitrogen per unit leaf mass · RGR: Relative growth rate · SLA: Specific leaf area

Introduction

The response of plants to their environment can be constrained in part by physiological trade-offs between traits involved in defence against herbivory and those that more directly influence growth (Herms and Mattson 1992). The expression of plant defence traits can incur 'allocation costs' due to the energetic demands of synthesis and maintenance and the diversion of limiting nutrients away from growth or reproduction (McKey 1974; Mooney and Gulmon 1982). Allocation costs that are incurred early in ontogeny result in an 'opportunity cost', which involves a loss of growth and competitive status with effects that are evident later in life, even after the defences are diminished or no longer present (Coley et al. 1985; Sagers and Coley 1995).

Allocation and opportunity costs are clearly connected, and a unified understanding of the growth and fitness cost of defence requires measurement not only of the effect on growth and fitness of individual defences, but also of the

lifetime ontogenetic trajectories of these defences (i.e. how they change through time as the plant develops–Boege and Marquis 2005). Considerable progress has been made in detecting the growth and fitness cost of chemical defences especially, and recent meta-analyses by Koricheva (2002) and Bergelson and Purrington (1996) have provided strong evidence for costs associated with various defences across a range of plants and conditions. Nevertheless, many of the reports of an allocation cost for one type of defence may reflect, at least in part, costs or benefits of other co-varying traits. Some recent studies have sought to overcome this problem of confounding variables by genetically modifying plants with regard to a single defence trait (Tian et al. 2003; Zavala et al. 2004). In both cases an appreciable cost was detected, despite the fact that the modified traits did not account for an appreciable amount of plant resources. The authors were thus not convinced that they had indeed resolved the actual cost of the modified trait from that of other co-variables.

Research on the ontogenetic trajectories of individual defences, in contrast, is still in its infancy. In their review of research in this area, Boege and Marquis (2005) noted that while there is indeed evidence for ontogenetic variation in some chemical and physical defence traits in a range of species, no consistent pattern is evident. In fact, some plants show a positive relationship between age and defence (e.g. Goodger et al. 2004) while others show a negative relationship (e.g. Wallace and Eigenbrode 2002). Interestingly, almost all of the studies of ontogenetic variation in defence involved comparing two ontogenetic stages, and as yet, there has been no research on a complete ontogenetic trajectory for any defence type or on whether these trajectories vary qualitatively or quantitatively between plant groups.

The juvenile stages of trees may provide a good opportunity to study the relationship between allocation and opportunity costs. Juvenile trees are often relatively more vulnerable to browsing by herbivores and the resulting damage (or death) may be of greater consequence to maintaining a population than damage to an adult (Bryant et al. 1992; Huntly 1991; Julkunen-Tiitto et al. 1995). Therefore, traits that defend trees from herbivory are likely to be strongly selected for at the seedling and sapling stage. These juvenile stages of trees are also generally the most vulnerable to competition; thus traits that enhance seedling and sapling growth and establishment can also be strongly selected for at the seedling stage (de Jung 1995; Grime and Hunt 1975; Tilman 1990). This potential growth strategy conflict may result in variation in ontogenetic defence trajectories that can be exploited in an appraisal of defence costs.

In order to measure the costs and benefits of varying ontogenetic patterns of resource allocation to growth and chemical defence two tools are required. First, plants with varying ontogenetic trajectories of preferably resource demanding defences must be identified, and second, appropriate measures of the growth cost of the defences must be derived. Our aim in this paper is to define a system involving trees that may provide these tools. More specifically, our aims were to (1) describe contrasting ontogenetic changes in resource (nitrogen) allocation to chemical defence (cyanogenesis, a defence against especially generalist herbivores–Gleadow and Woodrow 2002a) using three closely related species of *Eucalyptus* and (2) develop a measure of the allocation cost of chemical defence in the early stages of the ontogenetic trajectory. We chose cyanogenic *Eucalyptus* trees for this study for two reasons. First, the cyanogenic glycosides in these species can account for up to 15% of total leaf nitrogen (Gleadow et al. 1998; Goodger et al. 2002; Goodger and Woodrow 2002) and thus the allocation costs are likely to be appreciable and measurable. Second, there is apparently a difference in the developmental timing and level of expression of cyanogenic capacity between the species. Fully expanded foliage from seedlings of *E. cladocalyx* is apparently more cyanogenic than is fully expanded foliage from adult trees (Gleadow and Woodrow 2002b), whereas the opposite has been observed in the other two species (Goodger et al. 2004; Goodger and Woodrow 2002).

Materials and methods

Field study

Fully expanded foliage of adult *Eucalyptus polyanthemos* subsp. *vestita* (*Ep*, red box), *E. yarraensis* Maiden &. Cambage (*Ey*, yarra gum), and *E. cladocalyx* var. *nana* F. Muell. (*Ec*, dwarf sugar gum) was sampled from trees growing in natural stands in southeast Australia. The locations of the stands were as follows: *E. polyanthemos*, Sutherland Creek, Brisbane Ranges National Park, Victoria (37°54′S, 144°10′E); *E. yarraensis*, Stony Creek, Brisbane Ranges National Park, Victoria (37°51′S, 144°15′E); and *E. cladocalyx*, 15 km NE of Cowell, South Australia (33°38′S, 136°41′E). Leaves (10 per plant) were snap-frozen in liquid nitrogen within 1 min of removal from the tree, returned to the laboratory and then freeze-dried, ground in a cooled IKA Labortechnic A10 analytical mill (Janke and Kunkel GmbH Co, Staufen, Germany), and analysed chemically as described below. Leaf area and dry weight of an additional 10 randomly sampled leaves per tree were measured and used to calculate LMA (leaf mass per unit area). Twelve random soil samples were collected from within each stand and analysed for total nitrogen (see chemical analyses below). Long-term average rainfall was obtained from nearby meteorological stations (Bureau of Meteorology, Australia, www.bom.gov.au/climate/averages/).

Seedling study

Seedlings deploying juvenile foliage were not present at the sites listed above. Therefore, seeds were collected from a number of open pollinated trees at each site. Seedlings of each species were grown in pots containing a mixture of sand, vermiculite and perlite (1:1:1; v/v) in a glasshouse under the same conditions described in Goodger and Woodrow (2002). Pots were flushed with one-quarter strength Hoagland's solution containing 4 mM nitrogen (supplied as ammonium and nitrate, 1:6 mole:mole) for the first 4 weeks and then 6 mM nitrogen for the remainder of the experiment. This relatively high nutrient regime was planned to approximate the post-fire nutrient replete conditions under which the species generally regenerate (Wellington 1989).

Plants were harvested when 0.5 m tall, at 16–18 weeks after germination. The area of all fully expanded leaves of each seedling was determined, then they were pooled and snap-frozen in liquid nitrogen. Unexpanded and senescent leaves were excluded from the leaf pool for chemical analyses, but their mass was included in biomass determinations. The fully expanded leaf pool from each seedling was freeze-dried, ground and analysed in the same way as leaves from adult trees. Other plant parts were oven-dried at 70°C for 72 h for use in biomass determinations. The final dry weight of each seedling was determined and used to calculate relative growth rate (RGR, g g^{-1} d^{-1}) such that RGR = $(\ln W_2 - \ln W_1)/(t_2 - t_1)$, where t_1 is the day the 6.0 mM nitrogen treatment commenced, W_1 is the plant dry mass at t_1 (calculated from the average mass of 20 seedlings randomly selected from each species' seedling cohort and destructively harvested at t_1), t_2 is the day of harvesting and W_2 is the individual plant dry mass at time t_2. Net assimilation rate (NAR, g m^{-2} d^{-1}) was also calculated as follows: NAR = $[(W_2 - W_1)(\ln A_2 - \ln A_1)]/[(A_2 - A_1)(t_2 - t_1)]$, where A_1 and A_2 are the leaf area at times t_1 and t_2, respectively. Note that seedling mass and leaf area at t_1 was highly uniform within each species.

Chemical analyses

Cyanogenic glycoside concentration was measured by hydrolysing the glycoside in freeze-dried leaf tissue (40–100 mg), and trapping the resultant cyanide in a NaOH well (see Brinker and Seigler 1989; Woodrow et al. 2002). The amount of cyanide detected by this method is directly proportional to the amount of cyanogenic glycoside (i.e. 1 mg CN = 11.35 mg prunasin). The cyanogenic glycoside present in the foliage of the three eucalypts has been identified as prunasin (Gleadow 1999; Goodger et al. 2002; Goodger and Woodrow 2002).

The concentration of total phenolics (including tannins) in the freeze-dried leaves of seedlings and adults was determined by the Folin–Ciocalteu Assay according to the method of Cork and Krockenburger (1991) with modifications described by Burns et al. (2002). Phenolics were extracted from tissue (50 mg) in 50% acetone, and gallic acid was used as the standard.

Total nitrogen was determined on freeze-dried leaf material (5–10 mg) and soil samples (30–40 mg) using a Perkin Elmer 2400 Series II CHNS/O Analyzer (Perkin-Elmer Pty. Ltd., Melbourne, Australia), calibrated with the organic analytical standard, acetanilide (Perkin-Elmer #0204-1121).

Results

Fully expanded juvenile foliage

There were pronounced differences in the foliar chemical and physical properties between seedlings of the three *Eucalyptus* species. The mean cyanogenic glycoside concentration was markedly higher in *E. cladocalyx* seedlings compared to *E. yarraensis* and *E. polyanthemos* (one-way ANOVA $F = 265.1$, $p < 0.001$; Table 2; *Ec*>*Ey*>*Ep*). Mass-based foliar nitrogen concentration paralleled cyanide (*Ec*>*Ey*>*Ep*; $F = 35.1$, $p < 0.001$; Table 2). Phenolic compounds, however, were lowest in the most cyanogenic species (*Ec*<*Ey* = *Ep*; $F = 313.9$, $p < 0.001$; Table 2). Leaf mass per unit leaf area (LMA) showed a similar inverse relationship with cyanogenic glycoside concentration for *E. polyanthemos* and *E. cladocalyx* (*Ep*>*Ec*, $F = 29.6$, $p < 0.001$; Table 2). However, *E. yarraensis* displayed similar LMA to *E. cladocalyx* despite its much lower cyanogenic glycoside concentration (Table 2).

Adult fully expanded foliage

The natural populations of the three eucalypts also showed pronounced species differences in leaf chemistry and LMA, but these did not always mirror those found in the seedlings. In contrast to seedling leaves, adult *E. cladocalyx* leaves had the lowest concentration of cyanogenic glycosides and *E. yarraensis* the highest (*Ey*>*Ep*>*Ec*; $F = 61.8$, $p < 0.001$; Table 3). Nevertheless, as with the seedling leaves, the most cyanogenic foliage was also the highest in nitrogen. Foliar nitrogen in *E. yarraensis* was significantly higher than in both the other species (*Ey*>*Ec*>*Ep*; $F = 528.8$, $p < 0.001$; Table 3).

The phenolic concentration in adult leaves was dissimilar to the generally inverse relationship seen in the seedling leaves. In fact, *E. cladocalyx* foliage remained by far the lowest in phenolics (*Ep*>*Ey*>*Ec*; $F = 121.3$, $p < 0.001$; Table 3) despite also being the lowest in cyanogenic glycosides. The other two species had adult foliar phenolic

Table 1 Annual rainfall (long-term average) and total soil nitrogen (top soil) from field sites for populations of *Eucalyptus polyanthemos*, *E. yarraensis* and *E. cladocalyx*

Species	Site	Average total soil nitrogen (mg g^{-1})			Average annual rainfall (mm)
		0–5 cm	5–10 cm	10–15 cm	
E. polyanthemos	Sutherland Creek, Victoria	0.506	0.217	0.081	690
E. yarraensis	Stony Creek, Victoria	0.290	0.088	0.000	690
E. cladocalyx	Eyre Peninsula, South Australia	0.380	0.103	0.063	285

Twelve randomly chosen soil cores (15 cm) were taken from within each site and divided into three 5 cm depths. The 12 samples at each depth were pooled for each site and analysed for total nitrogen.

concentrations comparable to those of the seedling leaves (Table 3). The LMA of adult foliage, in contrast, was several times higher than the juvenile leaves of seedlings in all three species. Moreover, LMA was not consistent with the rankings for total phenolics, nitrogen or cyanogenic glycosides ($Ec>Ep=Ey$; $F=44.44$; $p<0.001$; Table 3).

The differences in adult foliar cyanogenic glycoside concentration measured here do not appear to be the result of any difference in soil nitrogen at the site of origin (Table 1). Adult *E. yarraensis* leaves contained the highest concentrations of cyanogenic glycosides, and also the highest concentration of leaf nitrogen (Table 3). Soil nitrogen, however, was lower at all depths at Stony Creek where *E. yarraensis* was sampled compared to the other two sites. It should be noted that total soil nitrogen may not accurately reflect the amount of nitrogen available to trees (Kriedemann and Cromer 1996) and moreover, leaf nitrogen does not always co-vary with soil nitrogen in plantation grown eucalypts (e.g. Hooda and Weston 1999).

Resource allocation in juvenile and adult foliage

To examine if the species showed contrasting ontogenetic changes in resource allocation to chemical defence, we made comparisons on the basis of the proportion of foliar N allocated to cyanogenic glycosides (Fig. 1). In *E. polyanthemos* and *E. yarraensis* seedlings, less than 1% leaf nitrogen was allocated to cyanogenic glycosides, with cyanide–nitrogen per total leaf nitrogen ratios (CN-N/N) of 0.09 ± 01 and $0.76 \pm 0.07\%$, respectively. By contrast, in juvenile leaves of *E. cladocalyx* seedlings, a relatively large proportion of an already enhanced leaf nitrogen level was incorporated into cyanogenic glycosides ($4.22 \pm 0.16\%$, CN-N/N). The ranking of N allocation to cyanogenic glycosides was $Ec>Ey>Ep$ ($F=195.32$, $p<0.001$). When adult leaves were compared, however, *E. cladocalyx* allocated the least nitrogen to cyanogenic glycosides ($1.96 \pm 0.23\%$, CN-N/N) compared to 2.62 ± 0.13 and $2.99 \pm 0.14\%$ for *E. yarraensis* and *E. polyanthemos*, respectively. The trend was thus the reverse of that seen in seedlings ($Ep>Ey>Ec$; $F=22.92$, $p<0.001$; Fig. 1).

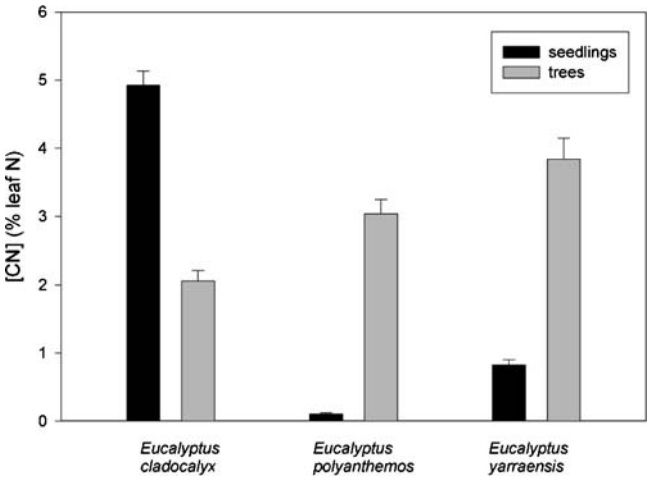

Fig. 1 Proportion of total leaf nitrogen allocated to cyanide (CN-N/N%) in adult *Eucalyptus polyanthemos*, *E. yarraensis* and *E. cladocalyx* growing in the field (*grey bars*) and for seedlings grown from seed collected at the same sites (*black bars*). Data for adults are population means (± 1 SE) estimated using 10 leaves randomly sampled from replicate trees. Values for seedlings are for all fully expanded leaves

Seedling growth and biomass allocation

Seedlings of the three eucalypts also differed significantly in their mean relative growth rates (RGR), with *E. yarraensis* being higher than the other two species ($F=6.52$, $p<0.01$; Table 2). This difference in RGR was a result of differences in both leaf area ratio (LAR; $F=7.91$, $p<0.001$) and net assimilation rate (NAR; $F=6.62$, $p<0.01$; Table 2). Specifically, RGR differences between *E. yarraensis* and *E. cladocalyx* were largely due to differences in NAR, whereas differences between *E. polyanthemos* and *E. yarraensis* were largely due to differences in LAR (Table 2). Focussing on *E. yarraensis* and *E. cladocalyx*, the marked difference in NAR (about 12%) was not due to a higher proportion of non-photosynthetic tissue in the low NAR *E. cladocalyx*, nor was it due to a significantly lower amount of nitrogen per unit leaf area (N_A). In fact, both leaf weight ratio (LWR) and N_A were higher in *E. cladocalyx* compared to that in *E. yarraensis*. The relatively low NAR in *E. cladocalyx* was largely due to the low leaf nitrogen productivity (LNP), which is the efficiency with which leaf nitrogen is used for biomass

Table 2 Growth indices and foliar chemical concentrations for seedlings of *Eucalyptus polyanthemos*, *E. yarraensis* and *E. cladocalyx*

Index	*E. cladocalyx*	*E. polyanthemos*	*E. yarraensis*	*F*	*p*
RGR ($g\ g^{-1}\ d^{-1}$)	0.056 ± 0.001	0.053 ± 0.002	$0.063 \pm 0.002^*$	6.52	<0.01
LAR ($m^2\ g^{-1}$)	0.013 ± 0.000	$0.011 \pm 0.000^*$	0.013 ± 0.000	7.91	<0.001
NAR ($g\ m^{-2}\ d^{-1}$)	$4.485 \pm 0.093^*$	5.006 ± 0.209	5.113 ± 0.170	6.62	<0.01
LWR ($g\ g^{-1}$)	$0.520 \pm 0.005^*$	$0.601 \pm 0.011^*$	$0.499 \pm 0.070^*$	44.34	<0.0001
LMA ($g\ m^{-2}$)	42.27 ± 1.03	$56.76 \pm 1.03^*$	40.93 ± 1.75	29.58	<0.0001
LNP ($g\ m^{-2}\ d^{-1}$)	2.82 ± 0.05	2.97 ± 0.11	$3.75 \pm 0.11^*$	35.80	<0.0001
CN_M ($mg\ g^{-1}$)	$3.556 \pm 0.120^*$	$0.060 \pm 0.009^*$	$0.531 \pm 0.120^*$	265.14	<0.0001
CN_A ($mg\ m^{-2}$)	$0.154 \pm 0.007^*$	0.003 ± 0.001	0.022 ± 0.002	143.87	<0.0001
Ph_M ($g\ g^{-1}$)	$0.024 \pm 0.011^*$	0.184 ± 0.009	0.196 ± 0.022	453.70	<0.0001
N_M ($g\ g^{-1}$)	$0.039 \pm 0.001^*$	$0.030 \pm 0.001^*$	$0.034 \pm 0.001^*$	35.13	<0.0001
N_A ($g\ m^{-2}$)	$1.59 \pm 0.02^*$	$1.70 \pm 0.05^*$	$1.34 \pm 0.04^*$	14.15	<0.0001

Seedlings were grown in a glasshouse under identical conditions. Data are presented as means (± 1 SE) with the results of one-way ANOVA analysis between species (Minitab Release 13). Fisher's least LSD procedure was used to discriminate among the means and statistically significant differences (at $p = 0.05$) are indicated by asterisks. Abbreviations: RGR, relative growth rate; LAR, leaf area ratio; NAR, net assimilation rate; LWR, leaf weight ratio; LMA, leaf mass per unit leaf area; LNP, leaf nitrogen productivity; CN_M, leaf cyanide per unit leaf mass; CN_A, leaf cyanide per unit leaf area; Ph_M, leaf total phenolics per unit leaf mass; N_M, total leaf nitrogen per unit leaf mass; N_A, total leaf nitrogen per unit leaf area.

accumulation. In contrast, no significant difference in NAR was detected between *E. polyanthemos* and *E. yarraensis*, despite a 20% difference in mean LWR. It appears that a relatively high NAR is attained in *E. polyanthemos* by a combination of a significantly higher allocation of mass to leaves (LWR) and enhanced N_A in the thicker (high LMA) leaves.

Measurement of allocation costs

To develop a measure of the allocation cost of chemical defence in the early stages of the ontogenetic trajectory we used the combined data from the seedlings of *E. cladocalyx* and *E. yarraensis*. No difference was detected in LAR between these two species, thus they offered an excellent opportunity to examine the relationship between RGR, NAR and chemical defence. We tested two hypotheses: (1) increasing allocation of foliar nitrogen to cyanogenic glycosides is associated with increasing total foliar nitrogen (with a slope of the relationship greater than one); (2) increasing foliar cyanogenic glycoside concentration is associated with decreasing NAR. In other words, we hypothesised that highly cyanogenic plants deploy more foliar nitrogen to make and maintain the cyanogenic glycosides, and because maintenance has a metabolic 'cost' the productive capacity of leaves is reduced.

To test the first hypothesis, we examined variation in N_A with the area-based amount of nitrogen invested in cyanogenic glycosides (N_{CN-A}) across the two eucalypt species. Because both mass- and area-based nitrogen concentration is generally strongly correlated with LMA, we included all three variables in a multiple regression analysis (SigmaPlot 2004 version 9.01, SPSS Inc., Chicago, USA) as follows:

$$N_A = N_{A0} + aN_{CN-A} + b\mathrm{LMA},$$

where N_{A0} is the N_A intercept when N_{CN-A} and LMA are zero, and a and b are slopes defining the N_A–N_{CN-A} and N_A–LMA relationships, respectively (Fig. 2). We found

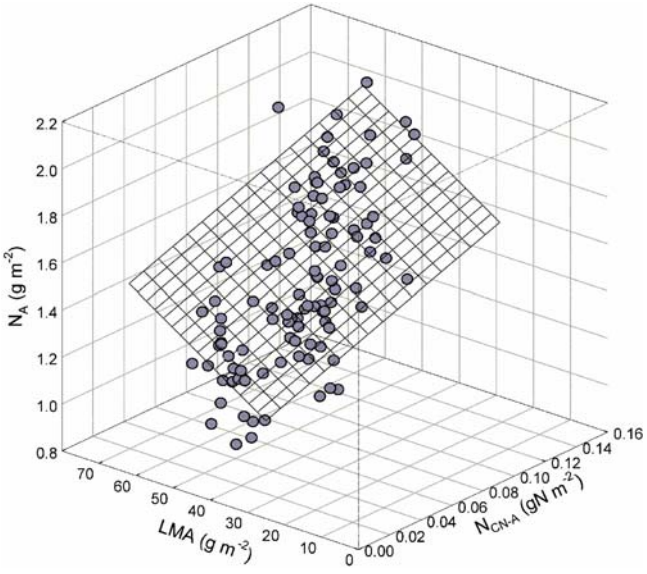

Fig. 2 Examination of the variation in leaf area-based nitrogen (N_A) with leaf mass per unit area (LMA) and area-based nitrogen invested in cyanogenic glycosides (N_{CN-A}). A multiple regression analysis of the combined *Eucalyptus cladocalyx* and *E. yarraensis* data (*grey circles*) was performed (*grid lines*) using the following equation: $N_A = N_{A0} + aN_{CN-A} + b\mathrm{LMA}$, where N_{A0} is the N_A intercept when N_{CN-A} and LMA are zero, and a and b are slopes defining the N_A–N_{CN-A} and N_A–LMA relationships, respectively ($r^2 = 0.54$, $p<0.0001$). Estimated values for a, b and N_{A0} are 2.49 g g^{-1} N, 0.011 g N g^{-1} leaf, and 0.93 g m^{-2}, respectively

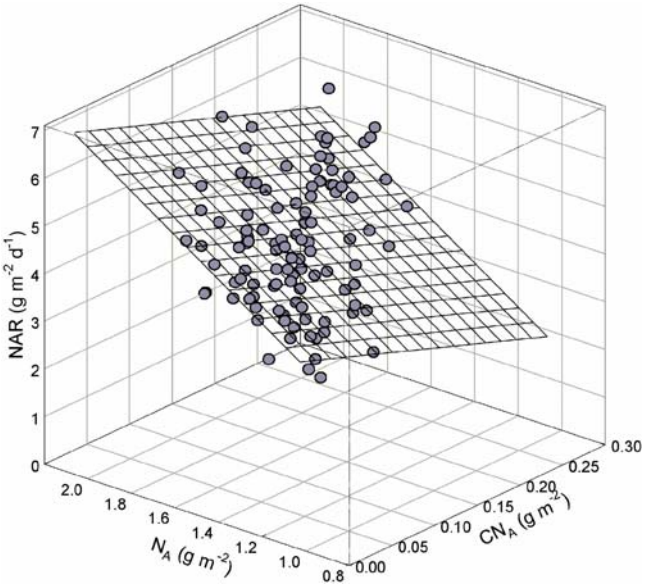

Fig. 3 Examination of the influence of leaf area-based nitrogen (N_A) and cyanide per unit leaf area (CN_A) on net assimilation rate (NAR). A multiple regression analysis of the combined *Eucalyptus cladocalyx* and *E. yarraensis* data (*grey circles*) was performed (*grid lines*) using the following 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 slopes defining the NAR–CN_A and NAR–N_A relationships, respectively ($r^2 = 0.32$, $p<0.0001$). Estimated values for m and n are -6.61 g g^{-1} CN d^{-1} and 3.11 g g^{-1} N d^{-1}, respectively

highly significant relationships between N_A and both variables ($r^2 = 0.54$; $F = 76.40$, $p<0.0001$) and estimated values for a and b of 2.49 ± 0.42 g g^{-1} ($p<0.0001$) and 0.011 ± 0.002 g N g^{-1} leaf ($p<0.0001$), respectively, and an N_{A0} value of 0.93 ± 0.07 g m^{-2} ($p<0.0001$). An a slope of this magnitude indicates that for every nitrogen invested in cyanogenic glycosides, an additional 1.49 nitrogens are effectively added to the leaf. We could not identify any variable other than LMA that affected this correlation between N_A and CN_A.

To test the second hypothesis, we measured the separate effects of N_A and CN_A on NAR using multiple regression analysis. We used 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 slopes defining the NAR–CN_A and NAR–N_A relationships, respectively (Fig. 3). We found highly significant relationships between NAR and both variables ($r^2 = 0.32$, $F = 24.24$, $p<0.0001$) and estimated values for m and n of -6.61 ± 1.25 g g^{-1} CN d^{-1} ($p<0.0001$) and 3.11 ± 0.45 g g^{-1} N d^{-1} ($p<0.0001$), respectively. This result indicates that deployment of cyanogenic glycosides is associated with a productivity 'cost', and that the magnitude of this 'cost' apparently exceeds (on a nitrogen basis) the 'gain' of deploying the nitrogen into productive activities. These results do not appear confounded by parallel variation in the phenolics concentration as we detected a small, but significant negative relationship between area-based phenolics concentration and CN_A ($r^2 = 0.16$, $F = 17.28$, $p<0.001$).

To measure the overall effect of the parallel increases in CN_A and N_A, we plotted CN_A against NAR across both eucalypt species and failed to detect a significant relationship ($r^2 = 0.004$, $F = 0.48$, $p = 0.49$). This indicates that the increase in leaf N accompanying the rise in cyanogenic glycoside concentration maintains NAR. Thus, we have not detected an overall growth 'cost' of cyanogenic glycoside deployment and maintenance.

Discussion

Ontogenetic changes in resource allocation

We have identified contrasting ontogenetic changes in defence strategies in three species of cyanogenic *Eucalyptus*. Amongst the juvenile foliage, *E. cladocalyx* and *E. yarraensis* display high levels of chemical defence (cyanogenesis in *Ec* and phenolics plus cyanogenesis in *Ey*), whereas *E. polyanthemos* appears tougher, as indicated by its relatively high LMA. This pattern changes amongst the adult foliage where it is *E. cladocalyx* that apparently has the toughest leaves and the lowest level of chemical defence. Adult leaves of the other two species have relatively high levels of both cyanogenic glycosides and phenolics (Table 3). Consistent with our first aim, the ontogenetic changes in cyanogenic glycoside concentration were mirrored by changes in the proportional allocation of leaf nitrogen to these compounds. Thus, investment of nitrogen in cyanogenic compounds declines from nearly 5% in juvenile foliage of *E. cladocalyx* to below 2% in adult foliage, but in the other two eucalypts there was an increase from less than 1% to around 3% with leaf ontogeny (Fig. 1).

Measurements of changes in cyanogenic glycoside concentration with age have been made for several other species (e.g. Dahler et al. 1995; Gleadow et al. 1998; Miller et al. 2004; Schappert and Shore 2000; Selmar et al. 1991), and it has been consistently found that 'old' tissue contains lower concentrations of cyanogenic glycosides than does 'young' tissue, no matter whether the definition is based on being developmentally mature (i.e. ontogenetically old) or more persistent (i.e. older in terms of leaf age; see Nahrstedt 1985). Similar patterns have been seen in other nitrogen-based defence systems (e.g. pyrrolizidine alkaloids in *Senecio jacobaea*, de Boer 1999; nicotine in *Nicotiana sylvestris*, Ohnmeiss and Baldwin 2000; glucosinolates in *Brassica napus*, Lambdon et al. 2003). The increase in cyanogenic capacity in *E. yarraensis* and *E. polyanthemos* (and recently

Table 3 Leaf chemical and physical parameters for adult *Eucalyptus cladocalyx*, *E. yarraensis* and *E. polyanthemos* growing in the field and for seedlings grown from seed collected at the same sites

Leaf type	Species	Trees sampled	CN_M (mg g^{-1})	Ph_M (g g^{-1})	N_M (g g^{-1})	N_A (g m^{-2})	LMA (g m^{-2})
Juvenile	*E. polyanthemos*	30	$0.06 \pm 0.01a$	$0.184 \pm 0.009c$	$0.030 \pm 0.001c$	$1.70 \pm 0.05a$	$56.8 \pm 1.8b$
	E. yarraensis	28	$0.53 \pm 0.05bc$	$0.196 \pm 0.022c$	$0.034 \pm 0.001d$	$1.38 \pm 0.05b$	$40.9 \pm 1.8a$
	E. cladocalyx	80	$3.56 \pm 0.12e$	$0.024 \pm 0.011a$	$0.039 \pm 0.001e$	$1.58 \pm 0.02a$	$42.3 \pm 1.0a$
Adult	*E. polyanthemos*	201	$0.63 \pm 0.03c$	$0.294 \pm 0.019e$	$0.010 \pm 0.001a$	$2.45 \pm 0.13c$	$257.3 \pm 7.8c$
	E. yarraensis	100	$1.08 \pm 0.06d$	$0.219 \pm 0.018d$	$0.018 \pm 0.003b$	$4.12 \pm 0.14e$	$224.9 \pm 6.7c$
	E. cladocalyx	31	$0.42 \pm 0.03b$	$0.085 \pm 0.005b$	$0.012 \pm 0.004a$	$3.56 \pm 0.09d$	$308.1 \pm 5.2c$

Data for adults are population means ($\pm$ 1 SE) estimated using 10 leaves randomly sampled fully expanded adult leaves from replicate trees. Values for seedlings are for all fully expanded juvenile leaves. Means with the same superscript letter are not significantly different using Fisher's least significant difference ($LSD_{0.05}$) procedure (Minitab Release 13, Minitab Inc., Pasadena, USA). Field sites: *E. polyanthemos*, Sutherland Creek (Victoria); *E. yarraensis*, Stony Creek (Victoria); *E. cladocalyx*, Eyre Peninsula (South Australia). Abbreviations: CN_M, leaf cyanide per unit leaf mass; Ph_M, leaf total phenolics per unit leaf mass; N_M, total leaf nitrogen per unit leaf mass; N_A, total leaf nitrogen per unit leaf area; LMA, leaf mass per unit leaf area.

observed by our group for *E. camphora* (Neilson et al. 2006) is, to our knowledge, the only recorded example of an increase in cyanogenic glycosides with plant maturation. In some species, a transient increase in cyanogenic compounds in germinating plants has been observed, usually as a result of the transfer of cyanogenic compounds from seed to plant (e.g. *Ungnadia speciosa*, Selmar et al. 1990; *Hevea* spp., Selmar et al. 1991), but there are no examples of seedlings being overall less cyanogenic than are adults of the same species.

Overall, adult leaves had elevated concentrations of phenolics compared to their juvenile counterparts. *Eucalyptus* leaves typically contain 100–200 mg g^{-1} of total phenolics, and these compounds can comprise up to 50% of the leaf dry weight (e.g. Cork and Krockenburger 1991; Lawler et al. 1997). The phenolic concentration in adult and juvenile leaves of *E. polyanthemos* and *E. yarraensis* were within this range. The phenolic values of juvenile and adult *E. cladocalyx* foliage were relatively low and are consistent with previous observations (Burns et al. 2002; Gleadow 1999). With respect to leaf ontogeny, the pattern of deployment of phenolics appears to be species specific: some studies, among tropical tree species in particular, report higher concentrations of phenolics and tannins in young leaves, while others report little change with leaf age (see Coley and Barone 1996). It may be that plants with low phenolics in young leaves are either low in protein or are dependent on other forms of defence, such as cyanogenesis.

Overlying the ontogenetic changes in defence strategy in the three eucalypts was a marked and significant decrease in leaf nitrogen concentration with leaf age. This probably does not reflect a decrease in productive capacity at the leaf level; rather, it reflects the large increase in LMA in adult leaves, which actually results in an apparent increase in nitrogen per unit leaf area in each species (Table 3). Such increases in LMA have been measured for a range of species (e.g. Gleadow et al. 1998; Kouki and Manetas 2002; Lawrence et al. 2003) and in eucalypts, they reflect the well-documented increase in sclerophylly with age (e.g. Lawrence et al. 2003). LMA is the product of leaf thickness and leaf density, and increases in either of these can lead to an increase in physical resistance to herbivory (Coley 1983), especially insect herbivory (e.g. in *Eucalyptus*–Landsberg and Cork 1997). Moreover, the lower mass-based nitrogen concentrations often associated with high LMA can also be a deterrent to herbivores (Berenbaum 1995). Not surprisingly, therefore, it has been found for a wide range of co-existing species, and across species and habitats, that LMA and leaf lifespan (LL) are positively correlated (e.g. Diemer 1998; Reich et al. 1997; Ryser and Urbas 2000).

Finally, it should be noted that there is no ideal experimental system that we could have used to separate genetic and environmental effects on the ontogeny of eucalypt leaf defence traits. We chose to examine juvenile foliage on seedlings raised under glasshouse conditions simply because there were no seedlings growing in the natural populations from which the adult foliage was collected. The environments that support woodland *Eucalyptus* communities are often unfavourable for seedling establishment, and hence stands may become increasingly open with age until the right sequence of conditions (i.e. fire and/or several wet years–Gill 1993; Wellington 1989) enables regeneration to occur (Ashton 2000). Thus, even under natural conditions, seedlings and mature trees would almost certainly be subject to different levels of soil nutrients and water. Previous experimentation by our group has shown that soil water can have a relatively small but significant effect on foliar cyanogenic glycoside concentration (Gleadow and Woodrow 2002b), but manipulation of the amount of nitrogen supplied to cyanogenic *Eucalyptus* seedlings did not modify their cyanogenic capacity (e.g. *E. polyanthemos*–Goodger et al. 2004 and *E. yarraensis*–Goodger 2002). Given this, and the fact that the foliar cyanogenic capacity of seedlings of *E. yarraensis* and *E. polyanthemos* was actually lower than that

of adults, despite being grown under optimal conditions, it is almost certain that the contrasting ontogenetic changes in resource allocation to chemical defence that we observed were not a result of the conditions in which the seedlings were grown.

Costs of defence

The use of seedlings of *E. cladocalyx* and *E. yarraensis*, which did not differ significantly in either SLA or LWR despite a marked difference in foliar cyanogenic glycoside content, enabled us to effectively titrate the influence of relatively sizable changes in cyanogenic capacity on leaf performance against a constant LAR background. We did not detect an overall correlation between leaf performance (i.e. NAR) and cyanogenic glycoside content, but this apparently reflects two counteracting changes. Firstly, we detected a rise in leaf N in parallel with the change in cyanogenic glycosides (Fig. 2). The slope of this rise exceeded the nitrogen invested in cyanide by some 1.49 nitrogens per cyanide nitrogen, and it is possible that these extra nitrogens reflect an increased investment in maintenance to service the extra cyanogenic glycosides. Secondly, consistent with this interpretation, we detected a decline in NAR at constant leaf N (Fig. 3). This leaf cyanide deployment 'cost' was estimated to be 6.61 ± 1.25 g g^{-1} CN d^{-1}, exceeding the estimated 'benefit' of N addition to the leaf of 3.11 ± 0.45 g g^{-1} N d^{-1}.

As far as we are aware, this is the first appropriately quantified 'cost' for a chemical defence in terms of leaf performance. Many studies have demonstrated the existence of measurable costs, in terms of both growth and reproductive fitness, for a variety of chemical defence types (Bergelson and Purrington 1996; Koricheva 2002; Strauss et al. 2002), including cyanogenesis (e.g. Kakes 1989). Nevertheless, associating the measured cost to a change in a single defence chemical has proved to be very difficult indeed due to the presence of unidentified and potentially confounding variables.

The present experiments potentially suffer from such shortcomings. The use of seedlings from two different cyanogenic species, for example, could have introduced species-specific variables into the analysis. We did measure several of these, including SLA, LWR and total phenolics concentration, and we showed that they did not confound our interpretation. Despite this finding, it would certainly have been better to confine the analysis to a more genetically homogeneous group, but we found that there was insufficient variation in the foliar cyanogenic glycoside content, either within each species or half-sib family, to detect significant correlations. A better design may have involved the use of a large number of clones derived from individual seedlings with relatively high and low cyanogenic capacities (with similar SLA and LWR), but a cloning protocol for *E. cladocalyx* and *E. yarraensis* is not yet available. Another noteworthy point regarding our experiments is that the plants were grown at a soil nitrogen concentration that was largely saturating for growth. Under these conditions, it is likely that an overall growth 'cost' for a nitrogen-based defence chemical will be harder to detect because the plants may be able to simply take up 'extra' nitrogen to, at least in part, offset the cost.

In conclusion, we have provided good evidence that deployment of cyanogenic glycosides in the early stages of the ontogenetic trajectory incurs a quantifiable cost in terms of leaf performance, and that this can be overcome by acquisition of extra nitrogen, presumably for both construction and maintenance. The regression analyses, however, were not able to account for all of the variation in NAR or leaf N, and the possibility remains that other variables may have confounded our estimates. Simpler experimental systems, and experiments under limiting soil nitrogen, may enable more refined estimates to be made of costs.

Acknowledgements We thank Mr. Barry Smith of the Glenville Stud, South Australia, for access to trees on private land. The Department of Natural Resources and Environment, Victoria, provided permits to take protected flora (#10001252) and for fieldwork in Brisbane Ranges National Park, Victoria (#10000781). This research was supported by funds from an Australian Research Council Grant to IEW and in part by the Holsworth Wildlife Research Fund (Victorian Community Foundation, ANZ Charitable Trusts). JQDG was a recipient of an Australian Postgraduate Award Scholarship.

References

Ashton DH (2000) Ecology of eucalypt regeneration. In: Keane PJ, Kile GA, Podger FD, Brown BN (eds) Diseases and pathogens of eucalypts. CSIRO, Melbourne, pp 47–60

Berenbaum MR (1995) Turnabout is fair play: secondary roles for primary compounds. J Chem Ecol 21:925–940

Bergelson J, Purrington CB (1996) Surveying patterns in the cost of resistance in plants. Am Nat 148:536–558

Boege K, Marquis RJ (2005) Facing herbivory as you grow up: the ontogeny of resistance in plants. Trends Ecol Evol 20:441–448

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

Bryant JP, Reichart PB, Clausen TP, Provenza FO, Kuropat PJ (1992) Woody plant–mammal interactions. In: Rosenthal GA, Berenbaum MR (eds) Herbivores. Their interactions with secondary plant metabolites. Academic Press, New York, pp 343–370

Burns A, Gleadow RM, Woodrow IE (2002) Light alters the allocation of nitrogen to cyanogenic glycosides in *Eucalyptus cladocalyx*. Oecologia 133:288–294

Coley PD (1983) Herbivory and defensive characteristics of tree species in a lowland tropical forest. Ecol Monogr 53:209–233

Coley PD, Barone JA (1996) Herbivory and plant defenses in tropical forests. Annu Rev Ecol Syst 27:305–335

Coley PD, Bryant JP, Chapin FSI (1985) Resource availability and plant antiherbivore defence. Science 230:895–899

Cork SJ, Krockenburger AK (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

Dahler JM, McConchie CA, Turnbull CGN (1995) Quantification of cyanogenic glycosides in seedlings of three *Macadamia* (Proteaceae) species. Aust J Bot 43:619–628

de Boer NJ (1999) Pyrrolizidine alkaloid distribution in *Senecio jacobaea* rosettes minimises losses to generalist feeding. Entomol Exp Appl 91:169–173

de Jung TJ (1995) Why fast-growing plants do not bother about defense. Oikos 74:545–548

Diemer M (1998) Life span and dynamics of leaves of herbaceous perennials in high-elevation environments: 'news from the elephant's leg'. Funct Ecol 12:413–425

Gill AM (1993) Interplay of Victoria's flora with fire. In: Foreman DB, Walsh NG (eds) Flora of Victoria, vol 1, Introduction. Inkata Press, Melbourne, pp 212–226

Gleadow RM (1999) Resource allocation in Eucalyptus cladocalyx. PhD thesis, School of Botany, University of Melbourne, Melbourne

Gleadow RM, Woodrow IE (2002a) Constraints on effectiveness of cyanogenic glycosides in herbivore defense. J Chem Ecol 28:1297–1309

Gleadow RM, Woodrow IE (2002b) Defence chemistry of cyanogenic *Eucalyptus cladocalyx* seedlings is affected by water supply. Tree Physiol 22:939–945

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 Environ 21:12–22

Goodger JQD (2002) Characterising the polymorphism for cyanogenesis in *Eucalyptus*. PhD thesis, University of Melbourne, Melbourne

Goodger JQD, Woodrow IE (2002) Cyanogenic polymorphism as an indicator of genetic diversity in the rare species *Eucalyptus yarraensis* (Myrtaceae). Funct Plant Biol 29:1445–1452

Goodger JQD, Capon RJ, Woodrow IE (2002) Cyanogenic polymorphism in *Eucalyptus polyanthemos* Schauer subsp. *vestita* L. Johnson and K. Hill (Myrtaceae). Biochem Syst Ecol 30:617–630

Goodger JQD, Ades PK, Woodrow IE (2004) Cyanogenesis in *Eucalyptus polyanthemos* seedlings: heritability, ontogeny and effect of soil nitrogen. Tree Physiol 24:681–688

Grime JP, Hunt R (1975) Relative growth-rate: its range and adaptive significance in a local flora. J Ecol 63:393–422

Herms DA, Mattson WJ (1992) The dilemma of plants: to grow or defend. Q Rev Biol 67:283–335

Hooda N, Weston CJ (1999) The effects of site and fertilizer addition on foliage and litter quality in six-year-old *Eucalyptus globulus* (Labill.) plantations in Gippsland, southeastern Australia. Aust J Bot 47:189–206

Huntly NJ (1991) Herbivores and the dynamics of communities and ecosystems. Annu Rev Ecol Syst 22:477–503

Julkunen-Tiitto R, Bryant JP, Kuropat P, Roininen H (1995) Slight tissue wounding fails to induce consistent chemical defense in three willow (*Salix* spp.) clones. Oecologia 101:467–471

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

Koricheva J (2002) Meta-analysis of sources of variation in fitness costs of plant antiherbivore defenses. Ecology 83:176–190

Kouki M, Manetas Y (2002) Toughness is less important than chemical composition of *Arbutus* leaves in food selection by *Poecilimon* species. New Phytol 154:399–407

Kriedemann PE, Cromer RN (1996) The nutritional physiology of the Eucalypts—nutrition and growth. In: Attiwill PM, Adams MA (eds) Nutrition of the Eucalypts. CSIRO, Melbourne, pp 109–122

Lambdon PW, Hassall M, Boar RR, Mithen R (2003) Asynchrony in the nitrogen and glucosinolate leaf-age profiles of Brassica: is this a defensive strategy against generalist herbivores? Agric Ecosyst Environ 97:205–214

Landsberg JJ, Cork SJ (1997) Herbivory: interactions between eucalypts and the vertebrates and invertebrates that feed on them. In: Williams JE, Woinarski JCZ (eds) Eucalypt ecology. Cambridge University Press, Cambridge, UK, pp 342–372

Lawler IR, Foley WJ, Woodrow IE, Cork SJ (1997) The effects of elevated CO_2 atmospheres on the nutritional quality of *Eucalyptus* foliage and its interaction with soil nutrient and light availability. Oecologia 109:59–68

Lawrence R, Potts BM, Whitham TG (2003) Relative importance of plant ontogeny, host genetic variation, and leaf age for a common herbivore. Ecology 84:1171–1178

McKey D (1974) Adaptive patterns in alkaloid physiology. Am Nat 108:305–320

Miller RE, Gleadow RM, Woodrow IE (2004) Cyanogenesis in tropical *Prunus turneriana*: characterisation, variation and response to low light. Funct Plant Biol 31:491–503

Mooney HA, Gulmon SL (1982) Constraints on leaf structure and function in reference to herbivory. Bioscience 32:198–206

Nahrstedt A (1985) Cyanogenic compounds as protecting agents for organisms. Plant Syst Evol 150:35–47

Neilson EH, Goodger JQD, Woodrow IE (2006) Novel aspects of cyanogenesis in *Eucalyptus camphora* subsp. *humeana*. Funct Plant Biol 33:487–496

Ohnmeiss TE, Baldwin IT (2000) Optimal defense theory predicts the ontogeny of an induced nicotine defense. Ecology 81:1765–1783

Reich PB, Walkers MB, Ellsworth DS (1997) From tropics to tundra: global convergence in plant functioning. Proc Natl Acad Sci USA 94:13730–13734

Ryser P, Urbas P (2000) Ecological significance of leaf life span among Central European grass species. Oikos 91:41–50

Sagers C, Coley PD (1995) Benefits and costs of defense in a neotropical shrub. Ecology 76:1835–1843

Schappert PJ, Shore JS (2000) Cyanogenesis in *Turnera ulmifolia* L (Turneraceae): II. Developmental expression, heritability and cost of cyanogenesis. Evol Ecol Res 2:337–352

Selmar D, Grocholewski S, Seigler DS (1990) Cyanogenic lipids: utilization during seedling development of *Ungnadia speciosa*. Plant Physiol 93:631–636

Selmar D, Lieberei R, Junqueira NTV, Biehl B (1991) Changes in cyanogenic glucoside content in seeds and seedlings of *Hevea* species. Phytochemistry 30:2135–2140

Strauss SY, Rudgers JA, Lau JA, Irwin RE (2002) Direct and ecological costs of resistance to herbivory. Trends Ecol Evol 17:278–284

Tian D, Traw MB, Chen JQ, Kreitman M, Bergelson J (2003) Fitness costs of R-gene-mediated resistance in *Arabidopsis thaliana*. Nature 423:74–77

Tilman D (1990) Constraints and tradeoffs: toward a predictive theory of competition and succession. Oikos 58:3–15

Wallace SK, Eigenbrode SD (2002) Changes in the glucosinolate–myrosinase defense system in *Brassica juncea* cotyledons during seedling development. J Chem Ecol 28:243–256

Wellington AB (1989) Seedling regeneration and population dynamics of eucalypts. In: Noble JC, Bradstock RA (eds) Mediterranean landscapes in Australia. CSIRO, Melbourne, pp 155–167

Woodrow IE, Slocum DJ, Gleadow RM (2002) Influence of water stress on cyanogenic capacity in *Eucalyptus cladocalyx*. Funct Plant Biol 29:103–110

Zavala JA, Patankar AG, Gase K, Baldwin IT (2004) Constitutive and inducible trypsin protease inhibitor production incurs large fitness costs in *Nicotiana attenuata*. Proc Natl Acad Sci USA 101:1607–1612