

# Enhanced CO<sub>2</sub> alters the relationship between photosynthesis and defence in cyanogenic *Eucalyptus cladocalyx* F. Muell.\*

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## ABSTRACT

The effect of elevated CO<sub>2</sub> and different levels of nitrogen on the partitioning of nitrogen between photosynthesis and a constitutive nitrogen-based secondary metabolite (the cyanogenic glycoside prunasin) was examined in *Eucalyptus cladocalyx*. Our hypothesis was that the expected increase in photosynthetic nitrogen-use efficiency of plants grown at elevated CO<sub>2</sub> concentrations would lead to an effective reallocation of available nitrogen from photosynthesis to prunasin. Seedlings were grown at two concentrations of CO<sub>2</sub> and nitrogen, and the proportion of leaf nitrogen allocated to photosynthesis, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), protein and prunasin compared. Up to 20% of leaf nitrogen was allocated to the cyanogenic glycoside, although this proportion varied with leaf age, position and growth conditions. Leaf prunasin concentration was strongly affected by nitrogen supply, but did not increase, on a dry weight basis, in the leaves from the elevated CO<sub>2</sub> treatments. However, the proportion of nitrogen allocated to prunasin increased significantly, in spite of a decreasing pool of leaf nitrogen, in the plants grown at elevated concentrations of CO<sub>2</sub>. There was less protein in leaves of plants grown at elevated CO<sub>2</sub> in both nitrogen treatments, while the concentration of active sites of Rubisco only decreased in plants from the low-nitrogen treatment. These changes in leaf chemistry may have significant implications in terms of the palatability of foliage and defence against herbivores.

*Key-words:* *Eucalyptus cladocalyx*, cyanogenic glycosides, defence, enhanced CO<sub>2</sub>, herbivory, nitrogen, photosynthesis, Rubisco.

## INTRODUCTION

In predicting the outcomes of global atmospheric change, much attention has been paid to the effects of enhanced CO<sub>2</sub> on plant growth and development and the interaction between CO<sub>2</sub> and nutrient supply (e.g. Luo, Field &

Mooney 1994; Drake, González-Meler & Long 1997). Generally, photosynthesis is stimulated when plants are exposed to elevated CO<sub>2</sub>: the carboxylation function of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is stimulated and the oxygenation function inhibited (e.g. Woodrow 1994a). These changes generally result in an increase in biomass. Concomitant with enhanced photosynthesis is an increase in total non-structural carbohydrates and a decrease in leaf nitrogen content (Ceulemans & Mousseau 1994; Poorter *et al.* 1997). There are several secondary effects arising from this decrease in leaf nitrogen, including changes in the nitrogen economy of individual plants, nutrient cycling through ecosystems, rates of turnover of leaf litter and plant–animal interactions (Lambers 1993; Lincoln, Fajer & Johnson 1993; Luo *et al.* 1994; Thompson & Drake 1994; Hughes & Bazzaz 1997). Our interest is in the impact of decreasing leaf nitrogen on folivores. Nitrogen content of plants plays a large part in determining feeding patterns of herbivores (Braithwaite 1996; Hughes & Bazzaz 1997) and any change in leaf nitrogen as a result of CO<sub>2</sub> enhancement could be expected to have an impact on herbivores, particularly folivores, worldwide.

Under enhanced-CO<sub>2</sub> growing conditions, reductions in leaf nitrogen are not entirely attributable to the increase in total nonstructural carbohydrates but occur, to some degree, independently of the carbohydrate changes, particularly when plants are nitrogen limited (Conroy & Hocking 1993; Poorter *et al.* 1997). Moreover, Rubisco, which comprises a large part of the nitrogen in leaves, has also been shown to decrease in a number of species grown at elevated CO<sub>2</sub> (e.g. Sage, Sharkey & Seeman 1989; Majeau & Coleman 1996), again particularly when nitrogen supply is limiting. When nitrogen is saturating, however, some elevated-CO<sub>2</sub> grown plants maintain the concentration of Rubisco at ambient levels, but more of the enzyme exists in its catalytically inactive, decarbamylated form (e.g. Woodrow 1994b). By contrast, there is evidence that when plants grown at enhanced CO<sub>2</sub> are nitrogen limited, transcription of nuclear-encoding genes for the small subunit of Rubisco is reduced, resulting in an overall decrease in concentration of Rubisco and consequently leaf protein (van Oosten & Besford 1995; Majeau & Coleman 1996). This is consistent with the observation that photosynthesis is generally only ‘down regulated’ at enhanced CO<sub>2</sub> when nutrient supply is limiting (Curtis

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\*This paper is dedicated to Professor Bruce Knox who died suddenly in August 1997.

1996; Drake *et al.* 1997). Thus, under conditions of replete nitrogen, excess Rubisco can effectively serve as a storage protein (Woodrow 1994a), but when nitrogen is limiting, it may be redeployed to enhance other processes involved in growth, reproduction or defence.

Whether or not the increase in nitrogen-use efficiency of plants grown at elevated CO<sub>2</sub> allows nitrogen in excess of photosynthetic requirements to be reallocated to other functions, such as constitutive nitrogen-based defence compounds, has not been resolved. We are aware of only one study examining the effect of enhanced CO<sub>2</sub> on nitrogen-based secondary metabolites; it demonstrated no increase in nicotine in cured leaves from field-grown tobacco exposed to twice-ambient CO<sub>2</sub> concentration (Ruffy *et al.* 1989). Bearing in mind that nicotine concentration is highly dependent on nitrogen supply (Baldwin & Ohnmeiss 1994), it is not possible to draw conclusions about the relative allocation of nitrogen to defence from the results of Ruffy *et al.* as they did not measure total leaf nitrogen. Moreover, nicotine is an inducible defence compound (Ohnmeiss & Baldwin 1994), which adds another dimension to the complexity of interpreting their results.

For the experiments presented here, our hypothesis was that at high CO<sub>2</sub>, nitrogen is effectively reallocated away from photosynthetic enzymes to certain constitutive nitrogen-based secondary metabolites. To test our hypothesis, we chose *Eucalyptus cladocalyx*, which invests comparable amounts of nitrogen in photosynthesis and the cyanogenic glycoside, prunasin (Finnemore, Reichard & Large 1935). Plants containing prunasin produce free cyanide when the glycoside is brought into contact with the catabolic enzyme  $\beta$ -glucosidase, usually when the plant tissue is damaged (Poulton 1988). To test our hypothesis, seedlings of *E. cladocalyx* were grown in approximately ambient and double-ambient CO<sub>2</sub> at two levels of nitrogen and the proportion of nitrogen allocated to Rubisco, protein and cyanogenic glycosides compared.

## MATERIALS AND METHODS

### Plant material and growth conditions

Seeds of *E. cladocalyx* F. Muell., collected from a small group of trees at Wilmington, South Australia (32°41'S, 138°06'E; Seedlot 19348, Australian Tree Seed Centre), were germinated in seed trays in ambient and elevated CO<sub>2</sub> (see below). After 1 month, seedlings were transplanted into 12 dm<sup>3</sup> pots containing a mixture of sterilized sand, vermiculite and perlite (1:1:1). Pots were flushed twice each day with one-quarter strength Hoagland's solution containing either 2 mol m<sup>-3</sup> or 6 mol m<sup>-3</sup> nitrogen, supplied as sodium nitrate. Previous work had shown that growth rates saturate at slightly above 2 mol m<sup>-3</sup> nitrogen under similar conditions (Woodrow, unpublished results). The plants were grown in a glasshouse comprising two compartments that were controlled for temperature, humidity and CO<sub>2</sub> (see Woodrow 1994b; Lawler *et al.* 1997).

Air temperature and humidity were measured with HMP 35 A sensors (Vaisala, Helsinki, Finland) and recorded every 15 min. Temperature (day/night  $\pm$  SD) was 23.2 ( $\pm$  1.57)/21.5 ( $\pm$  0.26) °C and 22.5 ( $\pm$  1.01)/20.9 ( $\pm$  0.88) °C in the ambient and elevated CO<sub>2</sub> compartments, respectively. Relative humidity (day/night  $\pm$  SD) was 47.6 ( $\pm$  3.05)/48.5 ( $\pm$  1.27)% and 49.18 ( $\pm$  2.68)/51.5 ( $\pm$  2.04)% for the ambient and elevated CO<sub>2</sub> compartments, respectively. Rapid mixing of air meant that there was little variation in air temperature or humidity between different locations within the glasshouse at the height of the plants.

Before the experiment was started, photosynthetically active photon-flux density (PPFD) was measured every 20 mm down transects along the glasshouse benches using a Li-Cor 190 SA visible light sensor (Li-Cor, Lincoln, Nebraska, USA) Peak PPFD on one sunny day was 1900  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> in each compartment. Average PPFD along each transect ( $\pm$  1 SE) for the ambient and elevated CO<sub>2</sub> compartments was 1240.0  $\pm$  66.5 and 1292.4  $\pm$  47.7, respectively, on a typical sunny day and 46.5  $\pm$  7.2 and 43.0  $\pm$  3.2, respectively, on a heavily overcast day. The variation in PPFD along a bench was caused by structural components of the glasshouse. The effects of this heterogeneity were minimized by rotating plants on each bench each week. Plants were subject to a natural photoperiod of about 10 h. To test further whether there were significant differences in PPFD, temperature and relative humidity between the two glasshouse compartments, we conducted a separate experiment in which conditions were identical to this experiment, except that the CO<sub>2</sub> concentration was the same in both compartments. We found no significant difference in growth rates, photosynthesis or chemical composition between plants from the two compartments. Differences reported here are thus attributable to CO<sub>2</sub> concentration alone.

The throughput of air in each compartment was  $\approx$  15 dm<sup>3</sup> s<sup>-1</sup>. The CO<sub>2</sub> level was enhanced in one glasshouse compartment by injecting pure CO<sub>2</sub> into the return air. The CO<sub>2</sub> level was measured four times each day with an infrared gas analyser (Series 225 Gas Analyser, Analytical Development Co., Hoddesdon, UK). Average CO<sub>2</sub> for the elevated glasshouse was 804  $\mu$ mol mol<sup>-1</sup> (SD = 41.8) and for the ambient was 400.65  $\mu$ mol mol<sup>-1</sup> (SD = 18.7). There was a depression of about 10% in mole fraction of CO<sub>2</sub> in both compartments at noon to  $\approx$  720 and 360  $\mu$ mol mol<sup>-1</sup>, respectively.

### Growth and gas exchange measurements

After 6 months of growth, eight plants from each treatment were measured for photosynthesis and then harvested. Harvests were between 1000 h and 1500 h to minimize any diurnal effects on chemical composition. After the height was recorded, plants were defoliated and leaves sorted into four subclasses based on age, position and morphology. Classifying the leaves ensured that leaves at the same ontogenetic stage could be compared

between treatments. Leaf classes were defined as follows: (1) Y, young: 2–3 cm diameter, up to three nodes from the tips and pale green; (2) E-1, expanded-1: fully expanded, darker green and three to five nodes from the tips; (3) E-2, expanded-2: similar to expanded-1 but five to seven nodes from the tips; (4) O, old: more than seven nodes from tips, but not showing signs of senescence. Five leaves were sampled at random from each age class (10 for young leaves) for chemical analysis. The thickness of each subsampled leaf was measured using a micrometer. Discs were excised from each of the five leaves, frozen in liquid nitrogen and stored at  $-70\text{ }^{\circ}\text{C}$  for Rubisco determination. The remaining portion of the subsampled leaves was ground to a fine powder in liquid nitrogen with a mortar and pestle and freeze dried. Remaining leaves, stems and roots were oven dried at  $70\text{ }^{\circ}\text{C}$  for 48 h, cooled in a desiccator and weighed. Multivariate analyses of variance were performed on biomass, root:shoot ratio, specific leaf weight, leaf area ratio, leaf thickness and height data using Minitab Release 10extra (Minitab Inc., Pasadena, USA). Data were tested for normality and heteroscedacity, and transformed where necessary.

The net  $\text{CO}_2$  assimilation rate was determined for E-1 leaves as a function of  $c_i$  and at a saturating PPFD ( $1200\text{ }\mu\text{mol quanta m}^{-2}\text{ s}^{-1}$ ) with a Li-Cor 6400 Portable Photosynthesis System (Li-Cor, Lincoln, Nebraska, USA) in the week before harvesting. Measurements were made on a single leaf at  $22\text{ }^{\circ}\text{C}$  on each of four replicate plants from each treatment.

### Chemical analyses

Cyanide in plant tissue was determined by hydrolysis of prunasin, the only cyanogenic glycoside found in *E. cladocalyx* (Finnemore *et al.* 1935), and trapping the resultant HCN in NaOH (Brinker & Seigler 1989). Hydrolysis of prunasin was achieved by adding  $1\text{ cm}^3\text{ }0.1\text{ mol m}^{-3}$  phosphate buffer (pH 6.8) to  $\approx 0.02\text{ g}$  of freeze-dried leaf material in a sealed glass vial. While *E. cladocalyx* does contain endogenous  $\beta$ -glucosidase (data not shown), exogenous enzyme ( $\beta$ -D-glucoside glucohydrolase; EC 3.2.1.21) was added ( $1.12\text{ units cm}^{-3}$ ) to ensure complete conversion to cyanide. A well containing  $0.5\text{ cm}^3$  fresh  $1\text{ mol m}^{-3}$  NaOH was placed in the vial, which was incubated overnight at  $37.0\text{ }^{\circ}\text{C}$ . Cyanide trapped in the NaOH solution was assayed using a Merck Spectroquant cyanide detection kit (Merck, Darmstadt, Germany). The amount of cyanide ( $\text{CN}^-$ ) detected by this method is a measure of the amount of the cyanogenic component of the prunasin in the tissue, and in the present paper will be referred to as the amount of 'cyanide'. The level of free cyanide in undisturbed tissue was assumed to be negligible.

Carbon:nitrogen ratios were determined on freeze dried samples using a Leco C:N:S analyser (CNS-2000, Leco, St Joseph, Michigan, USA). Total nitrogen content was determined on finely ground oven-dried leaves (see above) by the Kjeldahl method and measured using a Technicon AutoAnalyser II. Total nitrogen was also determined on

freeze-dried samples using a micro-Kjeldahl method and found to be equal to the determinations on oven-dried samples. Data for oven-dried samples are presented here.

Total leaf protein, together with free amino acids, was estimated by digesting samples of freeze-dried leaves to amino acids in  $1\text{ mol m}^{-3}$  sulphuric acid, according the method of Marks, Buchsbaum & Swain (1985). Duplicate digested samples were neutralized with  $1\text{ mol m}^{-3}$  NaOH and buffered with an equal volume of  $1\text{ mol m}^{-3}$  citrate buffer (pH 5.0). Amino nitrogen was determined using ninhydrin (Yemm & Cocking 1955).

Rubisco concentration was determined by measuring the amount of the transition-state analogue 2-carboxyarabinitol-1,5-bisphosphate (CABP) bound to the carbamylated sites on the enzyme (Butz & Sharkey 1989). Five leaf discs were ground in liquid nitrogen in a chilled mortar and pestle. Soluble protein was extracted with a buffer ( $1\text{ cm}^3$  per leaf disc) containing  $20\text{ mol m}^{-3}$  Bicine-KOH (pH 8.0),  $10\text{ mol m}^{-3}$   $\text{MgCl}_2$ ,  $10\text{ mol m}^{-3}$   $\text{NaHCO}_3$ ,  $1\text{ mol m}^{-3}$  EDTA,  $100\text{ mol m}^{-3}$   $\beta$ -mercaptoethanol, 2% polyvinylolpyrrolidone (w/v), 1% Tween 80 (polyoxyethylene-sorbitan monooleate, v/v), and a protease inhibitor cocktail comprising  $0.2\text{ g m}^{-3}$  AESBSF [4-(2-Aminoethyl)-benzenesulphonyl fluoride],  $0.5\text{ mg m}^{-3}$  leupeptin,  $0.5\text{ mg m}^{-3}$  pepstatin A and  $2.5\text{ g m}^{-3}$  EDTA- $\text{Na}_2$  (Delgado *et al.* 1994). All steps following the initial extraction were done at  $25\text{ }^{\circ}\text{C}$  unless otherwise specified. The presence of  $\text{MgCl}_2$  and  $\text{NaHCO}_3$  in the buffer allowed Rubisco to be fully carbamylated. A  $1\text{ cm}^3$  subsample of the extract was centrifuged for 20 s at  $26\text{ }000\text{ g}$  and the supernatant collected and held at  $4\text{ }^{\circ}\text{C}$ . The pellet was extracted two more times by resuspending the pellet in buffer, and the supernatants were combined and made up to  $1.5\text{ cm}^3$  with distilled water. Fractionation of the extracts and pellet using SDS-PAGE confirmed that more than 95% of the Rubisco was extracted in three washes. The combined extract was incubated for 20 min at  $4\text{ }^{\circ}\text{C}$  with  $10\text{ mm}^3\text{ }0.05\text{ mol m}^{-3}$  [ $^{14}\text{C}$ ]-2-carboxyarabinitol 1,5-bisphosphate (specific activity =  $2.146\text{ GBq mol}^{-1}$ , see below). Rubisco bound to  $^{14}\text{C}$ CABP was separated from unbound  $^{14}\text{C}$ CABP by passing a  $200\text{ mm}^3$  sample down a Superdex 75 HR 10/30 column (Pharmacia, Uppsala, Sweden). The column had been equilibrated with a buffer containing  $50\text{ mol m}^{-3}$  Bicine-KOH (pH 8.0),  $10\text{ mol m}^{-3}$   $\text{MgCl}_2$ ,  $150\text{ mol m}^{-3}$  NaCl,  $10\text{ mol m}^{-3}$   $\beta$ -mercaptoethanol and  $0.5\text{ mol m}^{-3}$  EDTA. The radioactivity eluted from the column with the protein fraction was measured using scintillation counting. Counts were converted to  $\text{mol m}^{-3}$  Rubisco using published procedures (Brooks & Portis 1988).  $^{14}\text{C}$ CABP was synthesised according to Pierce, Tobert & Barker (1980) with the modification that the barium  $^{14}\text{C}$ CABP salts were passed through  $70\text{ cm}^3$  Dowex 50( $\text{H}^+$ ) to remove the barium. The  $^{12}\text{C}$ CABP was synthesised according to Butz & Sharkey (1989).

### RESULTS

Our experiments on *E. cladocalyx* seedlings were divided into three parts. First we quantified the effects of  $\text{CO}_2$  and

nitrogen supply on growth and biomass allocation. Second, we measured the distribution of cyanogenic glycosides within the plant. Finally we examined how CO<sub>2</sub> and nitrogen supply affect the relative allocation of nitrogen between photosynthesis and defence (i.e. prunasin). This involved comparing seedlings grown at  $\approx 720$  and  $360 \mu\text{mol mol}^{-1}$  CO<sub>2</sub> and at ample or limiting nitrogen supply.

### Growth and biomass allocation

Consistent with most other studies of plants grown at enhanced CO<sub>2</sub> (e.g. Curtis 1996), we found CO<sub>2</sub>-effected changes in both biomass accumulation and allocation in *E. cladocalyx* seedlings. First, plants grown at elevated CO<sub>2</sub> were taller ( $P < 0.001$ ) and more massive ( $P < 0.001$ , Table 1). After 6 months, the dry weight of seedlings grown at high CO<sub>2</sub> was approximately double that of the controls. Secondly, leaf area ratio (LAR) was significantly reduced at elevated CO<sub>2</sub> ( $P < 0.05$ ), falling 15% in plants grown at ample nitrogen and 27% in plants grown at limiting nitrogen (Table 1). Nitrogen supply within a CO<sub>2</sub> treatment, by contrast, did not affect LAR significantly (Table 1). Thirdly, specific leaf weight (SLW) was significantly greater in plants grown at elevated CO<sub>2</sub> ( $P < 0.05$ ) and was marginally less at the low nitrogen supply (Table 1). SLW also varied with leaf age, ranging from  $81.23 \text{ g m}^{-2}$  for fully expanded E-2 leaves from the elevated-CO<sub>2</sub>, high-nitrogen treatment to  $47.25 \text{ g m}^{-2}$  for older O leaves in the ambient-CO<sub>2</sub>, low-nitrogen treatment (data not shown). Overall, SLW was greatest in leaves from the E-1 and E-2 classes, and was least in O leaves ( $P < 0.001$ ). Statistical analysis on SLW data from each leaf class showed that the magnitude of the response of leaves to elevated CO<sub>2</sub> and nitrogen was the same for all classes (data not shown). Leaf thickness also increased with leaf age ( $P < 0.001$ , data not shown) and leaves from the elevated CO<sub>2</sub> plants tended to be thicker ( $P < 0.05$ , Table 1), consistent with the observed increase in SLW.

Nitrogen supply also affected biomass allocation (Table 1). Root/shoot ratio was significantly higher in plants grown at limiting nitrogen ( $P < 0.01$ ) at both

ambient (0.49 compared with 0.75) and elevated CO<sub>2</sub> (0.51 compared with 0.56). This ratio was, however, not affected significantly by CO<sub>2</sub> concentration. It is noteworthy that the small increase in biomass in response to increased nitrogen supply was not significant at either CO<sub>2</sub> concentration, although plants grown at high nitrogen were taller ( $P < 0.05$ ). In spite of this lack of a significant response of growth rate to nitrogen supply, evidence from a more extensive study of the effect of nitrogen on growth showed that the low nitrogen level used here is marginally limiting for growth, at least at ambient CO<sub>2</sub> (see Materials and Methods).

### Cyanogenic glycoside distribution

Leaves of *E. cladocalyx* seedlings varied in their content of cyanide — almost all of which was derived from prunasin — depending on their age and position on the plant. For example, in leaves of seedlings grown at high nitrogen and ambient CO<sub>2</sub>, cyanide content varied from more than  $8 \text{ mg CN}^{-1} \text{ g}^{-1}$  dry weight in the young Y leaves to less than  $4 \text{ mg CN}^{-1} \text{ g}^{-1}$  dry weight in O leaves (Fig. 1). This pattern of cyanide distribution within the plant was independent of both CO<sub>2</sub> and nitrogen supply ( $P < 0.001$ , Fig. 1). In all treatments, the young leaves had the highest amount of cyanide, and this decreased with tissue age.

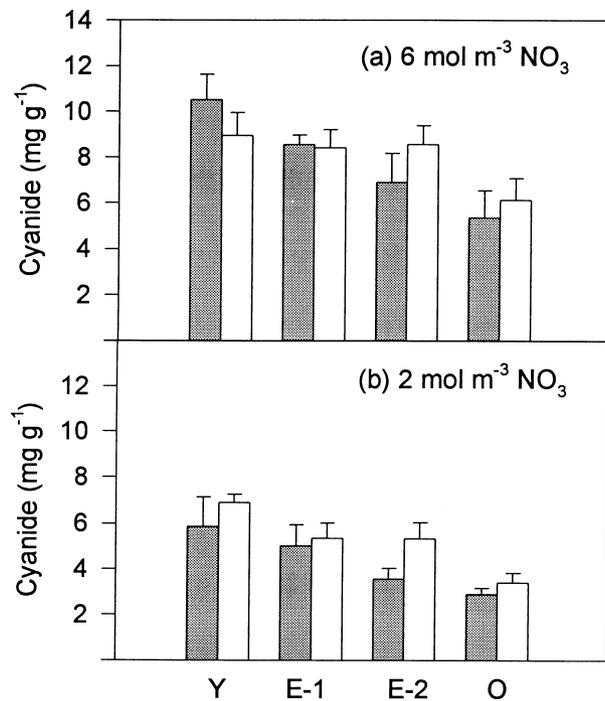
Nitrogen supply alone had a highly significant effect on the absolute amount of cyanide in leaves ( $P < 0.001$ ). For example, for E-1 leaves at ambient CO<sub>2</sub> the amount of cyanide was  $8.4$  and  $5.4 \text{ mg CN}^{-1} \text{ g}^{-1}$  dry weight for the high and low nitrogen treatments, respectively. By contrast, there was no significant difference in cyanide content of leaves from plants grown at elevated and ambient CO<sub>2</sub> (Fig. 1).

### Nitrogen allocation

In the next experiments, we measured the proportion of leaf nitrogen allocated to prunasin. Our hypothesis was that if the amount of nitrogen in leaves were to decrease under elevated CO<sub>2</sub> — as has been found in studies of

	6 mol m <sup>-3</sup> nitrogen		2 mol m <sup>-3</sup> nitrogen		LSD <sub>0.05</sub>
	Elevated	Ambient	Elevated	Ambient	
Height (m)	0.669	0.452	0.493	0.419	0.053
Biomass (g)	56.3	28.5	50.5	21.6	8.2
Root:shoot ratio	0.49	0.51	0.75	0.56	0.07
Specific leaf weight (g m <sup>-2</sup> )	83.6	76.0	82.5	62.5	2.1
Leaf thickness (mm)	0.237	0.222	0.228	0.216	0.052
Leaf area ratio (m <sup>2</sup> g <sup>-1</sup> )	$5.63 \times 10^{-3}$	$6.63 \times 10^{-3}$	$5.31 \times 10^{-3}$	$7.38 \times 10^{-3}$	$0.64 \times 10^{-3}$

**Table 1.** Growth and biomass partitioning in *Eucalyptus cladocalyx* seedlings grown at elevated and ambient CO<sub>2</sub> and 6 mol m<sup>-3</sup> and 2 mol m<sup>-3</sup> nitrogen. Least significant differences (LSD<sub>0.05</sub>) can be used to compare significant differences between means within that row at the 95% probability level. Values are the means of eight replicates per treatment except for data for leaf thickness which represent pooled data from all leaf classes (i.e. 32 replicates). Each specific leaf weight is an overall value for the whole plant but statistical analysis performed on individual leaf age classes showed that each class responded to the environmental treatments in the same way



**Figure 1.** Cyanide content, on a dry-weight basis, of leaves of *Eucalyptus cladocalyx* grown at 6 mol m<sup>-3</sup> or 2 mol m<sup>-3</sup> nitrogen (supplied as nitrate) and at elevated (shaded) or ambient (unshaded) atmospheric CO<sub>2</sub>. Almost all cyanide came from catabolism of the cyanogenic glycoside prunasin. Means ( $\pm$  1 SE) are the average of eight replicate plants. Leaves were sorted into four classes based on morphology, age and position on the plant: young, Y; expanded, E-1 and E-2; old, O (see 'Materials and methods').

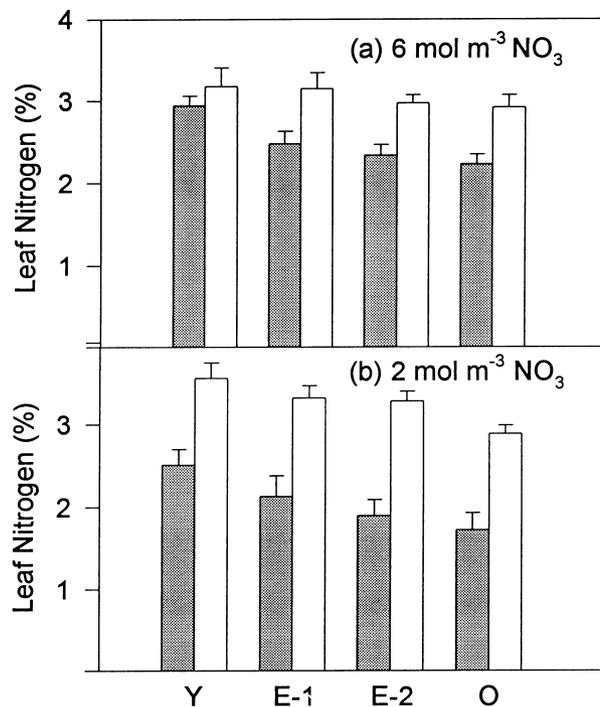
numerous other species, including *Eucalyptus* spp. (e.g. Wong, Kriedeman & Farquhar 1992) — then in view of the cyanide measurements discussed above, the proportion of nitrogen allocated to cyanogenic glycosides must rise.

To test this hypothesis, we first measured total leaf nitrogen for each treatment. Overall, total nitrogen was significantly less, on a dry weight basis, in all seedlings grown at elevated CO<sub>2</sub> ( $P < 0.001$ ; Fig. 2). For the high-nitrogen treatment, average leaf nitrogen concentration on a dry weight basis (combining all age classes) was 18% lower (3.1% compared with 2.5%) under high CO<sub>2</sub>. An even greater reduction in nitrogen of 36% (3.3% compared with 2.1%) was recorded for the low-nitrogen treatment (Fig. 2). Reductions in leaf nitrogen were also observed when nitrogen was expressed on a leaf-area basis ( $P < 0.05$ , Fig. 3), but only in the low-nitrogen treatment. Overall (combining all age classes), nitrogen per unit leaf area was 17% less (1.42 g m<sup>-2</sup> compared with 1.76 g m<sup>-2</sup>) under enhanced CO<sub>2</sub> when nitrogen was limiting. By contrast, when nitrogen supply was high, leaf nitrogen per unit area was similar ( $\approx$  1.80 g m<sup>-2</sup>) at both ambient and elevated CO<sub>2</sub> (Fig. 3). This interaction between CO<sub>2</sub> and nitrogen was highly significant for area-based and dry-weight-based values ( $P < 0.001$ ). Combining both CO<sub>2</sub> treatments, nitrogen content per area was also less in the

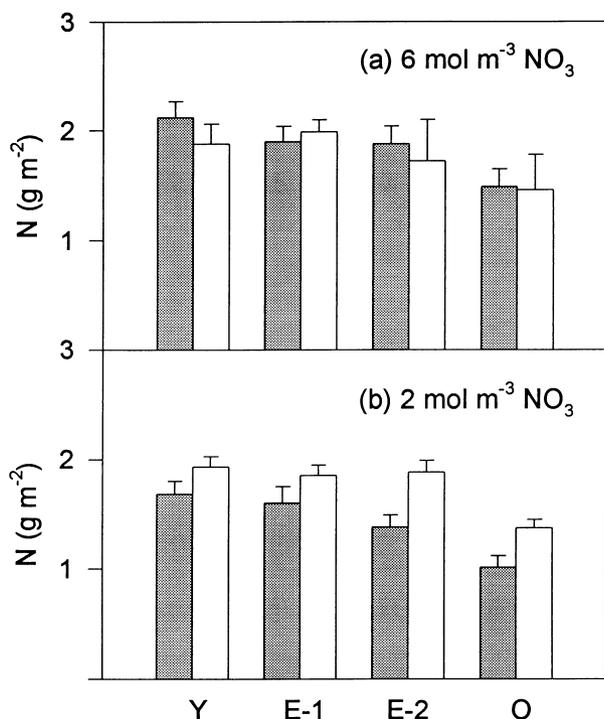
plants grown at low nitrogen ( $P < 0.01$ , Fig. 3). It is noteworthy that the overall nitrogen-age and CO<sub>2</sub>-age interactions were statistically not significantly different, indicating that the effects of nitrogen and CO<sub>2</sub> on leaf nitrogen were independent of leaf age.

Consistent with the reduction in leaf nitrogen at high CO<sub>2</sub>, the carbon:nitrogen ratio was about 20% higher in all leaves from plants grown at elevated compared with ambient CO<sub>2</sub> ( $P < 0.01$ ). For example, this ratio increased from 40 to 55 in E-1 leaves from the high-nitrogen treatment, and from 28 to 40 in E-1 leaves from the low-nitrogen treatment (Fig. 4). Ratios were, however, not significantly different between leaf age classes.

By combining the cyanide and leaf nitrogen data it can be shown that, consistent with our hypothesis, the proportion of nitrogen allocated to cyanide increased in plants grown at elevated CO<sub>2</sub> in both the high- and low-nitrogen treatments ( $P < 0.05$ ; Fig. 5). On average, this increase in cyanide was about 20%, with some variation between leaf age classes. The interaction between CO<sub>2</sub> and leaf age was, however, not statistically significant. There are two other noteworthy aspects of these data. First, plants grown at low nitrogen allocated a smaller proportion of leaf nitrogen to cyanide ( $P < 0.001$ ) at both ambient and elevated CO<sub>2</sub> (Fig. 5). Under ambient CO<sub>2</sub>, for example, the proportion of leaf nitrogen in cyanide was on average 14.6% and 8.5%



**Figure 2.** Total nitrogen as a percentage of leaf dry weight in *Eucalyptus cladocalyx* plants grown at ambient (unshaded) and elevated (shaded) atmospheric CO<sub>2</sub> and 6 mol m<sup>-3</sup> or 2 mol m<sup>-3</sup> nitrogen supplied as nitrate (see 'Materials and methods'). Leaves were sorted into four classes based on morphology, age and position on the plant (see Fig. 1). Means ( $\pm$  1 SE) are the average of eight replicate plants.



**Figure 3.** Total nitrogen as a proportion of leaf area in *Eucalyptus cladocalyx* plants grown at ambient (unshaded) and elevated (shaded) atmospheric CO<sub>2</sub> and 6 mol m<sup>-3</sup> or 2 mol m<sup>-3</sup> nitrogen supplied as nitrate (see 'Materials and methods'). Leaves were sorted into four classes based on morphology, age and position on the plant (see Fig. 1). Means ( $\pm$  1 SE) are the average of eight replicate plants.

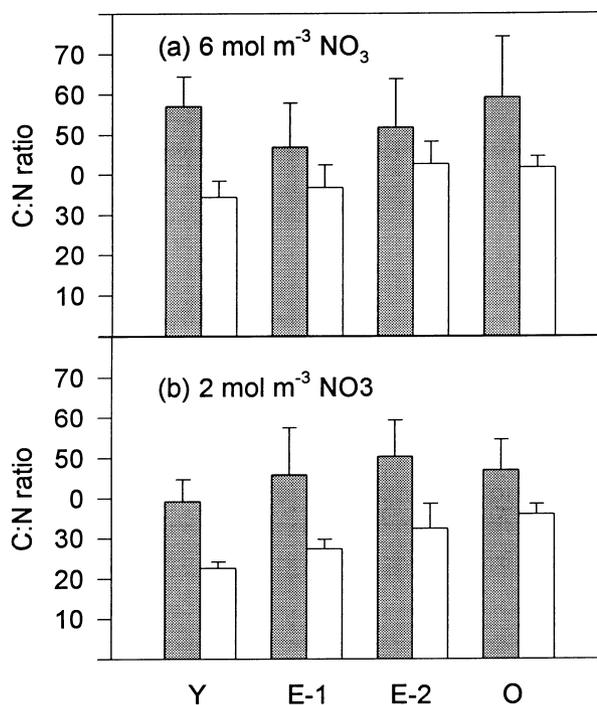
for the high- and low-nitrogen treatments, respectively. There was, moreover, no statistically significant interaction between CO<sub>2</sub> and nitrogen supply. Second, notwithstanding the fact that both nitrogen and cyanide content decreased with leaf age, the proportion of nitrogen present as cyanide decreased significantly with leaf age ( $P < 0.01$ ). For example, at high nitrogen and CO<sub>2</sub>, cyanide nitrogen ranged from almost 20% of the total pool in the young leaves to 14% in the old leaves (Fig. 5).

If the proportion of nitrogen allocated to cyanide increases at elevated CO<sub>2</sub>, one or more other nitrogen pools must decrease. Our hypothesis for this part of the experiment was that at least some of the extra nitrogen allocated to cyanide comes from reductions in leaf Rubisco concentration. Such reductions have been measured in several species (e.g. Sage *et al.* 1989; Besford 1990) and could account for the increase in cyanide. To test this hypothesis, we measured light-saturated photosynthesis as a function of  $c_i$ . We then used the rate equations for carboxylation and oxygenation by Rubisco to calculate the concentration of this enzyme in E1 leaves (see Woodrow & Berry 1988). We measured a significant difference in mean Rubisco concentration (14.7 to 11.6  $\mu\text{mol active sites m}^{-2}$  for the low and high CO<sub>2</sub> treatments, respectively) only when nitrogen was limiting (Table 2,  $P < 0.03$  for overall CO<sub>2</sub> effect). There

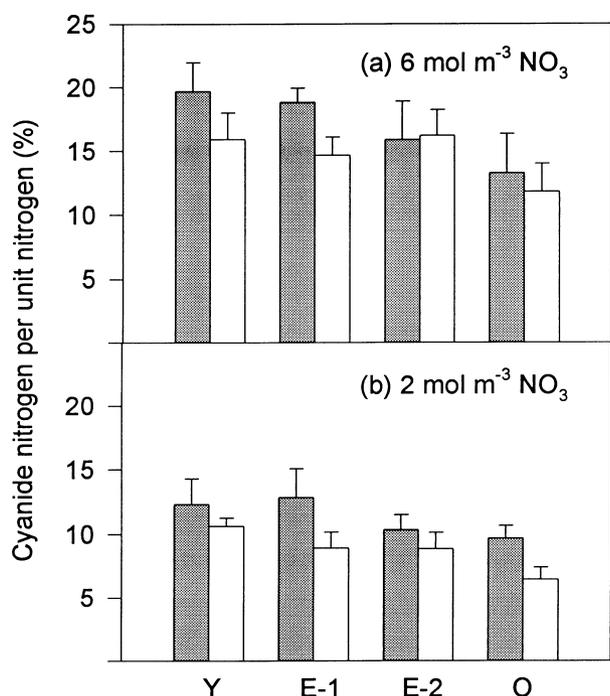
was no change in Rubisco content when plants were grown with sufficient nitrogen, with average values of 15 and 14  $\mu\text{mol active sites m}^{-2}$  from plants grown at ambient and elevated CO<sub>2</sub>, respectively (Table 2).

To verify that changes in the net CO<sub>2</sub> assimilation rate reflected changes in Rubisco concentration and not the proportion of enzyme in the active form, we measured Rubisco concentration in E-1 leaves from the same plants used in the gas-exchange analysis. At low nitrogen, CO<sub>2</sub> enhancement effected a reduction in mean Rubisco concentration of E-1 leaves from 5.8  $\mu\text{mol m}^{-2}$  at ambient CO<sub>2</sub> to 2.8  $\mu\text{mol m}^{-2}$  (Table 2). However, at high nitrogen there was only a small decrease in Rubisco concentration with CO<sub>2</sub> supply, from 4.7 to 4.3  $\mu\text{mol m}^{-2}$ . Importantly, as the CO<sub>2</sub>–nitrogen interaction was significant ( $P < 0.05$ ), consistent with the assimilation measurements, these data show that acclimation of photosynthesis was pronounced only when nitrogen supply was limiting (Table 2). There was no significant change in Rubisco concentration from nitrogen supply alone (Table 2).

There was significantly less protein (measured as total amino nitrogen with ninhydrin) in leaves from plants grown at elevated CO<sub>2</sub> ( $P < 0.01$ , Table 2). E-1 leaves from plants grown at low nitrogen had 36% less amino nitrogen on a dry-weight basis, decreasing from



**Figure 4.** Carbon:nitrogen ratio of *Eucalyptus cladocalyx* seedlings grown at ambient (unshaded) and elevated (shaded) atmospheric CO<sub>2</sub> and either 6 mol m<sup>-3</sup> or 2 mol m<sup>-3</sup> nitrogen supplied as nitrate (see 'Materials and methods'). Leaves were sorted into four classes based on morphology, age and position on the plant (see Fig. 1). Means ( $\pm$  1 SE) are the average of eight replicate plants.



**Figure 5.** Cyanide content of leaves as a proportion of leaf nitrogen of *Eucalyptus cladocalyx* seedlings grown at elevated (shaded) and ambient (unshaded) CO<sub>2</sub> and 6 mol m<sup>-3</sup> or 2 mol m<sup>-3</sup> nitrogen supplied as nitrate (see 'Materials and methods'). Almost all cyanide came from catabolism of the cyanogenic glycoside prunasin. Cyanide and nitrogen were calculated on a dry-weight basis. Leaves were sorted into four classes based on morphology, age and position on the plant (see Fig. 1). Means are averages of eight replicates ( $\pm 1$  SE).

160 mg g<sup>-1</sup> to 100 mg g<sup>-1</sup> dry weight (Table 2). This is equivalent to a reduction from 12 g m<sup>-2</sup> to 8 g m<sup>-2</sup> (see Table 1). Protein concentration in E-1 leaves from plants grown at high nitrogen fell 20%, from 150 mg g<sup>-1</sup> to 100 mg g<sup>-1</sup>, when grown at elevated CO<sub>2</sub> (Table 2), but the CO<sub>2</sub>-nitrogen interaction was not statistically significant.

	6 mol m <sup>-3</sup> nitrogen		2 mol m <sup>-3</sup> nitrogen		LSD <sub>0.05</sub>
	Elevated	Ambient	Elevated	Ambient	
(a) Rubisco active sites (μmol m <sup>-2</sup> )	13.59	14.81	11.64	14.72	0.98
(b) Total Rubisco (μmol m <sup>-2</sup> )	4.35	4.72	2.75	5.80	0.74
(b) Total Rubisco (mg g <sup>-1</sup> )	28	44	20	57	11
(c) Total protein (mg g <sup>-1</sup> )	119	149	102	161	14

**Table 2.** Amount of Rubisco and protein in expanded E-1 leaves of *Eucalyptus cladocalyx* grown at elevated and ambient CO<sub>2</sub>, and 6 mol m<sup>-3</sup> or 2 mol m<sup>-3</sup> nitrogen. The concentration of Rubisco active sites (a) was calculated from the carbon assimilation rate (Woodrow & Berry 1988); total Rubisco was measured using <sup>14</sup>CABP (see Materials and Methods) and expressed on a per-area basis (b) and calculated on a weight-for-weight basis (c) using specific leaf-weight data for E-1 leaves. Total protein (c) was measured as total amino nitrogen and is expressed in bovine serum albumin (BSA) equivalent units. Values are means of six to eight measurements. The least significant difference (LSD<sub>0.05</sub>) can be used to compare means in the same row at the 95% probability level. E-1, see 'Materials and methods'

It is noteworthy that these changes in protein can, to a reasonable degree, be accounted for by the changes in Rubisco concentration (Table 2).

## DISCUSSION

Our results show for the first time that the allocation of nitrogen to photosynthesis and a constitutive nitrogen-based secondary metabolite is altered by the supply of carbon. We showed that the proportion of leaf nitrogen allocated to prunasin increased and that allocated to leaf protein decreased when plants were grown at elevated CO<sub>2</sub> concentrations. Before discussing these results we shall first examine the general effects of CO<sub>2</sub> and nitrogen on biomass partitioning and photosynthesis in *E. cladocalyx*. We shall then discuss the distribution of prunasin, measured as evolved cyanide, within plants from the different treatments and its relationship to total nitrogen. Finally, we shall explore the implications of our findings for theories of plant defence.

### Biomass partitioning

The effects of elevated CO<sub>2</sub> on photosynthesis, growth and biomass allocation in *E. cladocalyx* (Table 1) are consistent with those of numerous other studies of herbaceous and woody plants, including several eucalypts (e.g. Wong *et al.* 1992; Duff, Berryman & Eamus 1994; Roden & Ball 1996). These studies have been reviewed by several workers (e.g. Gunderson & Wullschlegel 1994; Drake *et al.* 1997). Generally, growing plants at elevated CO<sub>2</sub> results in an increased growth rate, a faster photosynthetic rate per unit leaf area under growth conditions, and some alteration to biomass partitioning between leaves, shoots and roots (Ceulemans & Mousseau 1994). We observed higher rates of growth, a higher SLW and a lower LAR in plants grown at elevated CO<sub>2</sub> (Tables 1 & 2). We did not, however, measure a significant change in root:shoot ratio (Table 1), a response noted for its variability between species (Luo *et al.* 1994).

Also consistent with the results of many other studies (Drake *et al.* 1997), we found a reduction in leaf nitrogen under elevated CO<sub>2</sub>. Leaf nitrogen levels in all but the youngest leaves of our *E. cladocalyx* seedlings fell by between 20% and 30% on a dry weight basis under enhanced CO<sub>2</sub> (Figs 2 & 3), a result similar to other published values (Sage 1994). Moreover, as commonly observed in other species, the reduction in nitrogen was more pronounced when nitrogen supply was limiting for growth (Curtis 1996).

Decreased leaf nitrogen in elevated CO<sub>2</sub>-grown plants is often referred to as a 'dilution effect', resulting from greater accumulation of starch and other non-structural carbohydrates (Wong 1990; Luo *et al.* 1994). However, decreases in leaf nitrogen may occur independently of increases in these carbohydrates (Griffin, Winner & Strain 1996; Rogers *et al.* 1996). In many cases it is attributable, at least in part, to a decreased investment in Rubisco and other enzymes involved in leaf carbohydrate metabolism (Stitt 1991). In accord with other studies, we found that overall there was less protein and less Rubisco in leaves from all plants grown at elevated CO<sub>2</sub> concentrations. This effect was even more pronounced when nitrogen supply was limiting (Table 2). Moreover, we measured a reduction in photosynthetic capacity at a  $c_i$  of about 250  $\mu\text{mol mol}^{-1}$  (Table 2), but at the growth  $c_i$ , leaves from plants grown in high CO<sub>2</sub> had higher light-saturated rates of photosynthesis (data not shown). It is noteworthy that the 40% reduction in Rubisco measured here at limiting nitrogen is consistent with Medlyn's (1996) prediction, which is based on a model of allocation of nitrogen at elevated CO<sub>2</sub>.

### Allocation to prunasin

The main difference between the *E. cladocalyx* seedlings used here and almost all other species used in high-CO<sub>2</sub> studies is that in *E. cladocalyx* a large proportion of leaf nitrogen is allocated to a putative defence compound. Up to 20% of leaf nitrogen may be allocated to the cyanogenic glycoside prunasin, although this proportion varies with leaf age, position and growth conditions (Figs 1 & 5). It has been shown for other species that cyanogenic glycosides tend to be concentrated in parts of the plant of high reproductive or photosynthetic potential (Jones 1988; Lamont 1993). In accord with this we showed that the cyanogenic glycoside concentration, measured by the production of cyanide, is highest in young leaves and decreases with leaf age (Fig. 1). Similar results have been found for leaves of variety of other species (e.g. *Sorghum bicolor*, Saunders *et al.* 1977; *Hevea brasiliensis*, Selmar *et al.* 1987).

There has been surprisingly little research on the effect of environmental variables on allocation of resources to cyanogenic glycosides. In one study, *Sorghum alnum* plants were supplied with increased nitrogen, and the leaves were found to contain more cyanide as a proportion of both leaf dry weight and leaf nitrogen (Kriedeman 1964). Similar results for cyanide were obtained for *Heteromeles arbutifolia* (Dement & Mooney 1974) and

*Trifolium repens* (Jones 1972), but in the latter study cyanide as a proportion of leaf nitrogen was not determined. Our data are consistent with these experiments. We showed for *E. cladocalyx* that cyanide, as a proportion of both leaf nitrogen and dry weight, is not constant but varies markedly with nitrogen supply (Figs 1 & 5). Indeed, high nitrogen supply consistently caused a true increase in prunasin concentration in both absolute and relative terms. We also showed, for the first time, that nitrogen allocation to cyanide is affected by carbon supply.

When *E. cladocalyx* seedlings were grown at a high atmospheric CO<sub>2</sub> concentration, there was a significant increase in proportion of total nitrogen allocated to cyanide (Fig. 5). This increase resulted from the absolute amount of leaf cyanide (per dry weight) remaining constant despite a decreasing pool of nitrogen (Figs 2, 3 & 4). By contrast, when leaf nitrogen was reduced by limiting nitrate supply the proportion of nitrogen allocated to prunasin decreased. As far as we are aware, there has been only one other study of the effect of elevated CO<sub>2</sub> on nitrogen-based defence compounds. This study, which examined nicotine in tobacco (Rufty *et al.* 1989), reported a decrease in nicotine as a proportion of leaf dry weight in cured, field-grown tobacco leaves grown at twice-ambient CO<sub>2</sub>. This result, while conflicting with our own, is consistent with the relatively constant relationship between nicotine and leaf nitrogen measured in well fertilized plants (Baldwin & Ohnmeiss 1994), assuming that leaf nitrogen declined under high CO<sub>2</sub>.

### Is allocation to prunasin consistent with defence theories?

The changes in leaf chemistry that we have described for *E. cladocalyx* under high CO<sub>2</sub> may have significant implications in terms of the palatability of foliage and resistance to herbivores. Focussing on nitrogen, we have shown that foliage grown at a high CO<sub>2</sub> concentration is depleted in nitrogen while maintaining the cyanogenic glycoside content; taken together, these qualities present a significantly less palatable combination to herbivores than that presented by the control foliage. A number of studies have shown cyanogenic glycosides to be detrimental to both insect (e.g. Schwarz, Wray & Proksch 1996) and mammalian herbivores (Poulton 1983). While there is some convincing evidence to suggest that they affect feeding behaviour (Fowler 1983), some authors remain ambivalent (e.g. Hruska 1988). Before examining the possible implications of these changes for herbivores, however, we shall first examine whether our results can be interpreted according to the carbon/nutrient balance theory (CNB) of plant defence (Bryant, Chapin & Klein 1983).

The CNB theory argues that growth is given the highest priority in the allocation of a plant's resources; allocation to secondary metabolites increases when resources are in excess of the growth requirement or when there is an imbalance in the supply of resources. For example, when nitrogen availability is low and carbon is in relative excess, the

theory predicts an increase in carbon-based secondary metabolites such as phenolics and terpenoids in plants with the necessary biosynthetic pathways. Although simple, this theory has been able to account for the results of a range of studies in which external factors such as PPFD, water and nutrients have been varied (e.g. Reichardt *et al.* 1991; Cronin & Hay 1996; Rousi *et al.* 1996). The effects of a changing carbon supply have also been quantified by growing plants at high CO<sub>2</sub>. In most of these studies, CO<sub>2</sub> enrichment stimulated growth and increased the concentration of constitutive carbon-based secondary metabolites (Lincoln *et al.* 1993; Lawler *et al.* 1997; Poorter *et al.* 1997).

Relatively few studies, however, have measured the relationship between nitrogen supply and resource allocation to nitrogen-based secondary metabolites, and almost all of these have examined alkaloids. Despite this paucity of research, the findings of most of these studies are broadly consistent with the carbon/nutrient balance theory, showing changes in nitrogen-based defence mirroring changes in nitrogen supply (Kriedeman 1964; Baldwin 1994). It is noteworthy that these changes in nitrogen-based compounds on a dry weight basis have not always been reflected in parallel changes in total leaf nitrogen (Baldwin & Ohnmeiss 1994). Our results for cyanogenic glycosides in seedlings grown at different nitrogen concentrations are clearly consistent with CNB theory. Growth rates changed little in response to increased nitrogen supply, yet cyanogenic glycoside concentration (on a dry-weight and a total-nitrogen basis) increased markedly. Moreover, these increases were of a similar relative magnitude in tissues with quite different cyanogenic glycoside concentrations (e.g. O versus Y leaves).

Interpretation of the effect of CO<sub>2</sub> enhancement on the allocation of resources to cyanogenic glycosides highlights one of the weaknesses of the CNB theory. This involves the difficulty of defining the pools of nutrients that actually reflect or influence the 'availability' of nutrients. In many cases, it has been assumed — either explicitly or implicitly — that the availability of carbon versus nitrogen is reflected by the ratio of these resources in the external environment (Lambers 1993; Kinney *et al.* 1997). It has also been argued that it is the concentration of these resources in the plant itself that determines their availability (Chu, Field & Mooney 1996). Accordingly, Landsberg & Stafford Smith (1992) predicted that the decreased leaf nitrogen content at elevated CO<sub>2</sub> and consequent increase in C:N ratio would lead to a reduction in nitrogen-based defence compounds. It could be argued, however, that the decrease in nitrogen in leaves under high CO<sub>2</sub> simply reflects the increased efficiency of photosynthesis (on a nitrogen basis, Woodrow 1994b), and that given a constant nitrogen availability from the environment and a constant ability to assimilate this nitrogen, there is in fact more nitrogen available for secondary metabolism (Field & Mooney 1986; Conroy & Hocking 1993). In other words, the nitrogen that would otherwise have been used for photosynthesis could be used for secondary metabolism.

Our data are consistent with this latter view. We found that CO<sub>2</sub> enrichment did not deplete leaf cyanogenic

glycoside concentration on a dry-weight basis, and in terms of nitrogen allocation, the proportion of leaf nitrogen allocated to cyanogenic glycosides actually increased (Figs 1 & 5). If, however, we examine the balance between nitrogen- and carbon-based defence, then we find that there is evidence that at least the concentration of total phenolics relative to that of cyanogenic glycosides increases under high CO<sub>2</sub> (Gleadow, unpublished results). This result, it could be argued, is consistent with the rise in leaf C:N ratio and the predictions of the carbon/nutrient balance theory.

Cyanogenic glycoside content, relative to nitrogen, is only part of the defence-potential array deployed by *Eucalyptus* leaves. The inherently low nitrogen content of field grown *Eucalyptus*, together with a variety of leaf waxes, fibre and carbon-based secondary metabolites all contribute to the low digestibility of these leaves (Cork & Foley 1981; Ohmart & Edwards 1991). We have shown that leaves from *E. cladocalyx* grown at elevated CO<sub>2</sub> are not only poorer nutritionally but also have a higher proportion of leaf nitrogen present as prunasin. Several studies have demonstrated that insects raised on plants grown at elevated CO<sub>2</sub> increase consumption to compensate for the low protein content (Salt, Brooks & Whittaker 1995; Lawler *et al.* 1997). If this is the case for *E. cladocalyx* then they would also be ingesting higher amounts of toxins. Alternatively, if the higher proportion of cyanogenic glycosides in leaves grown at elevated CO<sub>2</sub> deters feeding, herbivores may not obtain enough protein for growth and reproduction. Either way, the consequences for both mammalian and insect herbivores feeding on cyanogenic plants in a high-CO<sub>2</sub> world could be serious.

## ACKNOWLEDGMENTS

We thank Jennifer Fox and Arlene McDowell for laboratory assistance, Edward Hammond for preparation of <sup>14</sup>CABP and Michael Kelly for ongoing advice. Thanks are also extended to CSIRO (Wildlife and Ecology) for C:N analyses and Steve Cork and Ivan Lawler for valuable discussions. This research was supported by funds from the Australian Research Council. RMG was a recipient of a Melbourne University Postgraduate Award.

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Received 28 July 1997; received in revised form 3 November 1997; accepted for publication 3 November 1997