

Cyanogenesis in tropical *Prunus turneriana*: characterisation, variation and response to low light

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Abstract. This study characterised three aspects of cyanogenesis in the late successional tropical rainforest species *Prunus turneriana* (F.M.Bailey) Kalkman. First, all tissues were found to be highly cyanogenic, containing combinations of the cyanogenic glycosides (*R*)-prunasin, (*S*)-sambunigrin, and amygdalin. Second, the progeny of a single parent tree varied markedly and continuously in their cyanogenic glycoside content, indicating that this variation is genetically based. Third, we investigated resource allocation to cyanogenic glycosides in light treatments representative of rainforest understorey and gap environments. Contrary to our hypothesis that under low light, photosynthetic gain would be maximised by the reallocation of nitrogen from defence to the photosynthetic system, we found no difference in cyanogenic glycoside concentration, or the proportion of nitrogen allocated to cyanogenic glycoside, between high and low light. However, within the plant, shade affected a significant change in distribution of cyanogenic glycosides between young and old leaves. There was an increased allocation of cyanogenic glycosides to old, expanded and photosynthetically productive leaves, a pattern which appears inconsistent with predictions of optimal defence theories, and the results of other studies. We suggest that such a strategy may be advantageous for seedlings of tree species that can only reach a reproductive stage following the creation of a canopy gap.

Keywords: amygdalin, cyanogenic glycoside, defence, light, polymorphism, prunasin, rainforest, regeneration, Rosaceae, sambunigrin.

Introduction

Within the heterogeneous environment of the tropical rainforest, light is the single most variable resource and therefore, the most critical resource limiting growth and survival (Chazdon 1988; Denslow *et al.* 1990). For example, in Costa Rican tropical montane forest, plants in the deep shade of the understorey receive as little as 2% full sunlight, increasing to 3–11% sunlight at gap edges (Denslow *et al.* 1990). Even within large forest gaps, daily photon flux density rarely exceeds 50% full sunlight (Chazdon 1992). Hence, the deeply shaded conditions of the understorey represent a challenge for photosynthesis and the acquisition of sufficient carbon to support growth and survival. Rainforests are characterised by large numbers of shade-tolerant species, including some that tolerate deep shade during part of their development, but require increased irradiance in tree fall gaps for growth and survival (e.g. Denslow 1980; Piñero and Sarukhan 1982; Augspurger 1984; Denslow *et al.* 1990).

The main focus of research on shade-tolerant species and their response to light has been the acclimation of the photosynthetic apparatus (e.g. Langenheim *et al.* 1984;

Chow *et al.* 1988; Reich *et al.* 1991; Chazdon 1992; Turnbull *et al.* 1993). However, this is just one side of the carbon acquisition equation because in the long term, photosynthetic gain depends just as much upon leaf lifespan, especially in the rainforest environment where herbivore densities are relatively high (Coley 1983; Coley *et al.* 1985; Reich *et al.* 1991). It is intriguing, therefore, that many of the attributes that increase leaf lifespan, such as thick leaves and high concentrations of resource-demanding defence chemicals, tend to decrease the capacity for photosynthesis (Mooney and Gulmon 1982; Gulmon and Mooney 1986). As a consequence there has been increasing interest in how leaf defence strategies, particularly chemical defence, respond to environmental variation (e.g. Denslow *et al.* 1990; Höft *et al.* 1996; Ralphs *et al.* 1998; Hägele and Rowell-Rahier 1999).

Most of the research on rainforest plants under varying light conditions has focussed on carbon-based defence chemicals. Underlying much of this research was the hypothesis of Bryant *et al.* (1983), who proposed that under higher light, elevated photosynthesis and carbohydrate

Abbreviations used: A_{max} , maximum carbon assimilation rate; dw, dry weight; LAR, leaf area ratio; PPF, photosynthetic photon flux density; RGR, relative growth rate; SLA, specific leaf area.

accumulation result in an increase in carbon-based defences. Accordingly, sun-grown plants have frequently been reported to have greater foliar tannin concentrations compared with individuals growing in the shaded understorey (e.g. Mole *et al.* 1988; Nichols-Orians 1991). Similarly, Denslow *et al.* (1990) examined seven shrub species, ranging from shade-tolerant to light demanding species, and reported increasing phenolic concentrations in parallel with enhanced growth under higher light. Interestingly, the only species that exhibited a decline in phenolic content was a shade-tolerant species *Miconia gracilis* Triana, known also to contain significant quantities of nitrogen-based alkaloids. However, the nitrogen-based compounds were not quantified in the study by Denslow *et al.* and could have actually confounded the interpretation of the phenolic data.

In comparison with the large body of work on carbon-based defence in tropical rainforests, studies on environmental regulation of nitrogen-based defences (e.g. alkaloids and cyanogenic glycosides) are rare. Höft *et al.* (1996) studied light effects on alkaloid concentration in the tropical tree species *Tabernaemontana pachysiphon* Stapf., but the PFD in the low-light treatment was relatively high, and not comparable with a rainforest understorey. Such nitrogen-based defences are more resource- and energy-demanding, and therefore likely to compromise photosynthesis.

Research into nitrogen-based defence can provide an excellent opportunity for understanding the rules of defence allocation because nitrogen-based defence compounds compete with the proteins of primary metabolism for nitrogen. Therefore, we chose to study resource allocation to nitrogen-based defence (viz. cyanogenesis) in a rainforest plant subjected to a light flux that markedly reduced growth rate. Cyanogenesis is a constitutive and resource-demanding defence (e.g. accounts for up to 15% leaf nitrogen in *Eucalyptus cladocalyx* F.Muell., Gleadow *et al.* 1998), which is known to be effective in reducing tissue loss to herbivores (Seigler 1991; Jones 1998; Gleadow and Woodrow 2002). Cyanogenesis — the evolution of toxic HCN — results from the hydrolysis of cyanogenic glycosides (Conn 1981), typically upon tissue disruption. We studied *Prunus turneriana* ('Almond bark'), a late successional canopy tree species with broad distribution in the lowland and upland rainforests of far north Queensland in Australia. Among shade-tolerant climax species, *P. turneriana* demonstrates dependence on large canopy gaps for survival; seedlings persist with little growth in the understorey, suffer increased mortality with long-term shade, and show rapid growth in gap environments (Osunkoya *et al.* 1993). In such an environment, mechanisms resulting in careful allocation of especially nitrogen to photosynthesis or defence may be under very strong selection pressure. Preliminary investigation indicated *P. turneriana* to be highly cyanogenic, with a significant investment of nitrogen in cyanogenic glycosides (14% leaf

nitrogen) in mature leaves in the field. In addition, cyanogenic glycosides are distributed throughout all tissues of the plant (R.E. Miller, unpublished data).

Our aim was to characterise cyanogenesis in *P. turneriana* by identifying cyanogenic glycosides in different plant tissues, and to document the distribution of cyanogenic glycosides within the plant and compare the cyanogenic capacities of the progeny from a single parent. In addition, we aimed to investigate resource allocation to cyanogenic glycosides in light treatments representative of rainforest understorey (95% shade) and canopy gap (50% shade) environments, and so test the hypothesis that photosynthetic gain under low light would be maximised by the reallocation of nitrogen from cyanogenic glycosides to the photosynthetic system. Given that investment in defence should reflect the cost of losing and replacing the leaf tissue (Givnish 1986; Gulmon and Mooney 1986), we hypothesised that the most valuable parts of plant would be most highly defended.

Materials and methods

Plant material and growth conditions

Seeds of *Prunus turneriana* (F.M.Bailey) Kalkman (Rosaceae) obtained from Department of Primary Industry (DPI) Forest Tree Seed Centre, Queensland, were germinated in a sterile soil:sand (1:1) mix in a glasshouse. Seedlings were transplanted into pots (1.5 L) at the two-leaf stage. Foliar concentration of cyanogenic glycosides at transplanting was determined for two leaf discs (approximately 15 mg dw) excised from the leaves (see below). Plants were assigned to one of three light treatments ($n = 6$) based on the initial leaf cyanogenic glycoside concentration to ensure that each treatment contained plants with a similar range of values. Pots were flushed daily with one quarter strength Hoagland solution containing 1.5 mM nitrogen supplied as nitrate and ammonium (6:1, mol:mol), with sodium the balancing cation (Gleadow *et al.* 1998).

Shade cloths suspended 1.5 m above plants created 50% and 95% shade environments. Photosynthetic photon flux density (PPFD) at plant height was measured every 15 min (as an average) in each treatment for the duration of the experiment using three visible light sensors (LI-190-SA, Li-Cor, Inc., Lincoln, NE). The selection of representative light treatments is important for the relevance of acclimation studies in rainforest species (Chazdon *et al.* 1996). Shade treatments were selected to be representative of the understorey and different gap environments within the rainforest. The light intensity during the experiment conducted in Melbourne (Autumn–Winter; maximum irradiance 1069 $\mu\text{mol m}^{-2} \text{s}^{-1}$) would be significantly less than full sun in the tropics (up to 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, Chazdon 1992) but it would be fairly typical of the mean daily PPFD in a large rainforest canopy gap (see Raich 1989). Average daytime PPFD (± 1 s.d.) was $172 \pm 83 \mu\text{mol m}^{-2} \text{s}^{-1}$ for the full light treatment, and $94 \pm 36 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $12 \pm 6 \mu\text{mol m}^{-2} \text{s}^{-1}$ for the 50% shade and 95% shade treatments, with a natural mean photoperiod of 11 h (March–June). The maximum daily mean PPFD was $332 \mu\text{mol m}^{-2} \text{s}^{-1}$ in the full light treatment, compared to $185 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $24 \mu\text{mol m}^{-2} \text{s}^{-1}$ in the 50% and 95% shade treatments, respectively, with maximum irradiances of 1069, 525 and $56 \mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were rotated fortnightly within treatments to minimise variation in plant growth produced by variation within the glasshouse. Air temperature and relative humidity were measured as 15 min averages using a Spectrum temperature/humidity sensor (Datatronics, Bayswater, Australia.). Mean daytime and nighttime

temperatures (± 1 s.d.) were $27.7 \pm 1.1^\circ\text{C}$ and $27.0 \pm 0.3^\circ\text{C}$; relative humidity was $65.8 \pm 6.6\%$. Initial height and leaf lengths were measured to enable determination of initial biomass for relative growth rate (RGR) calculations.

Plant material for cyanogenic glycoside purification was from a further 10 seedlings, grown as above, but fertilised with Osmocote. Seedlings were harvested for vegetative samples between 6 and 12 months-old. Stem, root and foliage were separated, snap frozen in liquid nitrogen, freeze-dried, ground and homogenised using an IKA Labortechnik A10 analytical mill (Janke and Kunkel, Stanfen, Germany). In addition, immature seed was collected from three trees on the Atherton Tablelands, Queensland and pooled for analysis. Flesh and pericarp were removed and the endosperm was snap frozen in liquid nitrogen, freeze-dried and ground to fine powder. Voucher specimens of *Prunus turneriana* have been lodged at the University of Melbourne Herbarium (MELU) and the Queensland Herbarium (BRI); accession numbers are yet to be assigned.

Plant harvesting and gas exchange of light experiment

Plants were harvested after 60 d when stem, root and leaf tissues were separated, and leaves were further separated into four leaf age categories: young (y; > 2 cm in length, soft, not fully expanded), young 2 (y2; fully expanded, but pale, soft), old (o; fully expanded, dark, toughened leaves), and old 2 (o2; oldest age class, fully expanded, dark, brittle, with some senescence at leaf tip). Leaf area was measured and samples were snap frozen in liquid nitrogen, then freeze-dried. Dry weights were determined, and tissue was ground to a fine powder using a cooled analytical mill (as above).

In order to determine the initial biomass of treatment seedlings for growth analyses, six additional seedlings were harvested before the commencement of the treatments. Biomass was found in the six seedlings to correlate with leaf area, so estimates of leaf area were used to determine biomass at the start of the treatments. Initial leaf area of treatment seedlings was based on the conversion of initial leaf lengths (x), to leaf area (y) using the following relationship: $y = 0.2628 x^{1.9547}$ ($r^2=0.957$; $n = 414$). Relative growth rate (RGR $\text{g g}^{-1} \text{d}^{-1}$) was calculated as follows:

$$\text{RGR} = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$$

where W_1 was the initial biomass of seedlings, W_2 was the final mass of each plant, t_1 was the day where treatment commenced and t_2 the day of harvesting.

In the 2 weeks before harvesting, the relationship between net CO_2 assimilation rate and PPFD was determined at ambient CO_2 concentration ($360 \mu\text{mol mol}^{-1}$). Measurements were made on one mature, fully expanded leaf (class: old) for each seedling at 25°C with a LI-6400 gas exchange system (Li-Cor, Lincoln, NE). Relative humidity in the chamber for each measurement was 50%.

Chemical analyses

Cyanogenic glycosides

Cyanogenic glycoside concentration in plant material and in extracts was measured by hydrolysing the glycoside and trapping the evolved cyanide in a 1 M NaOH well (Gleadow *et al.* 1998; Brinker and Seigler 1989). Freeze-dried, ground plant tissue (15–20 mg) was incubated for 20 h at 37°C with 1 mL of 0.1 M citrate buffer-HCl pH 5.5, conditions which allowed for complete conversion of the cyanogenic glycoside to cyanide (data not shown). Previous experiments had shown that tissue contained sufficient endogenous β -glucosidase for complete hydrolysis of the cyanogenic glycosides (data not shown). To detect and quantify cyanogenic compounds fractions during purification, glycosides were hydrolysed using β -glucosidase enzyme partially purified from the same *P. turneriana*

leaf tissue used for cyanogenic glycoside purification (see below) or by the addition of β -glucosidase from almonds (emulsin from *Prunus amygdalis* (L.) Benth. and Hook.; EC 3.2.1.21, Sigma G-0395) at the rate of 1.12 units mL^{-1} to the buffer (Gleadow *et al.* 1998). Preliminary experiments showed that in all cases, the almond emulsin was as effective as the extracted leaf enzyme. Subsequent assays, therefore, used almond emulsin as it is known to catalyse reactions with both the cyanogenic mono-glycosides and di-glycosides typically found in rosaceous species.

Cyanide in the NaOH well was determined using the method of Gleadow and Woodrow (2002) adapted from Brinker and Seigler (1989) for use with a photometric microplate reader (Labsystems Multiskan[®] Ascent, with incubator, Helsinki, Finland). The cyanide detected by this method is directly proportional to the concentration of cyanogenic glycoside, with, for example, 1 mg CN is equivalent to 11.35 mg glycoside prunasin.

Nitrogen concentration

Total nitrogen concentration of 5–10 mg of freeze-dried leaf, stem and root tissue was determined using a Perkin Elmer 2400 Series II CHNS/O Analyzer (Perkin-Elmer Pty Ltd, Melbourne, Vic.) calibrated using the organic analytical standard acetanilide (Perkin-Elmer no. 0240–1121).

Chlorophyll

Chlorophyll was extracted from ground freeze-dried leaf tissue (0.0020 g) in cold 80% (v/v) acetone (1 mL) according to the method used by Burns *et al.* (2002). On average, 95% chlorophyll was recovered after two extractions (data not shown). The concentrations of chlorophyll *a* and chlorophyll *b* were calculated according to the equations of Jeffrey and Humphrey (1975).

Purification and identification of cyanogenic glycosides

Cyanogenic glycosides were extracted from leaf, root, stem and seeds of *P. turneriana* seedlings using the same procedure as Goodger *et al.* (2002) and Gleadow *et al.* (2003). Homogenised, freeze-dried tissue (10–20 g) was de-fatted by a minimum of three extractions with petroleum ether (solvent:tissue, 10:1 v/w), filtered (Whatman[®] 541 filter paper, Whatman Asia Pacific, San Centre, Singapore), and then twice extracted with cold methanol, and filtered. The filtrate volume was reduced by rotary evaporation (40°C), and an equivalent volume of CHCl_3 was added, with sufficient H_2O to facilitate phase separation. The MeOH phase was collected and concentrated *in vacuo*. Concentrated MeOH extracts were resuspended in H_2O and fractionated by elution through a solid-phase extraction cartridge (Maxi-Clean[™] C₁₈, 900 mg cartridge, Alltech Associates, Baulkham Hills, Australia) at 1 mL min^{-1} , using a methanol gradient (0–100% MeOH in H_2O). Fractions were collected, concentrated *in vacuo*, and tested quantitatively for cyanogenic glycosides by the addition of β -glucosidase enzyme partially purified from the same leaf tissue, or almond emulsin for seed, stem and root extracts. In each tissue extract, cyanogenic glycosides eluted in H_2O and 10% MeOH. The H_2O and 10% MeOH eluent were concentrated *in vacuo* and fractionated isocratically by reverse phase HPLC using either 10% MeCN- H_2O (1 mL min^{-1}) through a Phenomenex Luna C₁₈ column (250 mm \times 4.6 mm \times 5 μm particle size), or 20% MeCN- H_2O (2 mL min^{-1}) through a Phenomenex Luna C₁₈ column (250 mm \times 10 mm \times 5 μm particle size; Phenomenex, Pennant Hills, Australia). Fractions (1 min) were concentrated *in vacuo* and tested for the presence of cyanogenic glycoside. In both the H_2O and 10% MeOH eluants of all tissue extracts, the major cyanogenic glycoside was associated with the same single peak (compound 1; 17–18 min, λ_{max} 208 nm in 20% MeCN). In the seed extract, there was a second cyanogenic peak (compound 2; 10–11 min, λ_{max} 208 nm in

20% MeCN), in addition to the major peak (1) at 17–18 min. These corresponded with the retention times of authentic samples of the mono-glucoside (*R*)-prunasin (Sigma M-0636; 1) and its gentiobiose amygdalin (Sigma A-6005; 2) under the same conditions. Samples of purified cyanogenic glycoside (1) and authentic prunasin (Sigma M-0636) were analysed by ^1H NMR (400 MHz, D_2O and CD_3OD) and LC-ESIMS. Data were compared with reported spectra.

Both ^1H NMR and GC-MS analysis of the TMS-ethers of extracts of compound 1 confirmed the presence of a second compound (compounds 1A and 1B). All extracts (compound 1, 17–18 min HPLC elution time) were derivatised in 100 μL Tri-Sil[®] Reagent (HMMA:TMCS:pyridine, 2:1:10) (Pierce, Rockford, IL), warmed to 70°C, and allowed to stand at room temperature for 10 min. Aliquots (1 μL) were injected at 315°C onto a column (HP5MS Hewlett-Packard, Agilent Technologies, Palo Alto, CA; 30 m \times 0.25 mm \times 0.25 μm) and separated using a temperature programme increasing from 200–315°C, with a gradient of 5°C min⁻¹ and a flow rate of 1.3 mL min⁻¹ (see Buhmester *et al.* 2000; Gleadow *et al.* 2003). The spectrum was analysed and compared with 0.5 mg authentic prunasin (Sigma M-0636) derivatised in 400 μL Tri-Sil[®] reagent, as above. The ratio of the two compounds was determined by ^1H NMR.

The minor cyanogenic glycoside (compound 2) was not present in sufficient concentration to be readily purified for NMR analyses. Compound 2 (HPLC elution time 10–11 min) from the seed tissue, was derivatised in Tri-Sil[®] Reagent as above, and GC-MS spectrum of the derivative was compared with that of the TMS-ether of authentic amygdalin (Sigma A-6005).

Cyanogenic β -glucosidase purification

Cyanogenic β -glucosidase was partially purified from the same *P. turneriana* leaf tissue used for identification of the cyanogenic glycoside (Brinker and Seigler 1989). Freeze-dried tissue (5 g) was extracted at 4°C in a protein extraction buffer [100 mM citrate buffer-HCl, pH 5.5, 10 mM sodium-EDTA, 4% (w/v) polyvinyl-pyrrolidone (PVP) and 1.5% Tween 80 (polyoxyethylene-sorbitan monooleate, v/v); Gleadow *et al.* 1998], filtered, and centrifuged (20 min at 27000 g) to remove remaining tissue. The supernatant was fractionated by adding solid ammonium sulfate and proteins precipitating between 35% and 90% ammonium sulfate saturation were collected and resuspended in a minimum amount of buffer (0.1 M citrate buffer-HCl, pH 5.5) and desalted using a dialysis cassette (Slide-A-Lyzer[®] 3.5K, MWCO 3500, Pierce, Rockford, IL) in 0.1 M citrate buffer-HCl, pH 5.5. Aliquots of the crude enzyme preparation were incubated and tested for cyanide to verify that no cyanogenic glycoside had been extracted in the protein preparation.

Statistical analyses

Statistical analyses were conducted using Minitab Release 13 (Minitab, State College, PA). Data were tested for normality by the Kolmogorov-Smirnov and Ryan-Joiner (Shapiro-Wilks) tests, and transformed where necessary before using one-way and two-way ANOVA. Significant differences between treatments were determined by multiple unplanned comparisons using Dunnett's test (for unequal sample size), or by comparison with the least significant difference (l.s.d.). Regression equations were calculated using Sigmaplot 2000 (SPSS Inc., Chicago, IL).

Results

Cyanogenic glycoside purification and identification

Root, stem and leaf tissue from at least ten seedlings of *P. turneriana* were combined and analysed, and all parts were found to be cyanogenic (Table 1). The highest concentration of cyanogenic glycosides in nine month-old

seedlings was detected in the leaves (4.7 mg CN g⁻¹ dw) while the concentration in stem tissue was the lowest (1.18 mg CN g⁻¹ dw). These tissue samples were then used for purification of the cyanogens.

The extracted compounds were purified by reverse phase HPLC, and analysed using ^1H NMR and liquid chromatography-electrospray ionisation mass spectroscopy (LC-ESIMS). The most abundant cyanogenic compound in all tissues was identical in all respects trimethylsilyl (TMS)-ethers, HPLC, ^1H NMR, LC-ESIMS) to the authentic prunasin standard (Fig. 1a). The LC-ESIMS indicated a molecular weight of 295 amu, isobaric with prunasin [(*R*)-mandelonitrile β -D-glucoside; compound 1A; Fig. 1]. The ^1H NMR spectra in D_2O and CD_3OD revealed resonances consistent with prunasin. In addition, the spectra indicated that the cyanogenic sample purified from leaf, stem and seed tissue also contained varying amounts of a related compound, evident by a second singlet downfield of H-7. This compound was identified as sambunigrin [(*S*)-mandelonitrile β -D-glucoside; compound 1B; Fig. 1]. (*S*)-sambunigrin differs from (*R*)-prunasin in its configuration at a single chiral centre, the carbinol carbon; the ratios of epimers were established by measuring the integrals of each singlet (Table 1). Prunasin and sambunigrin are not resolved by HPLC under these conditions. In addition to ^1H NMR detection, the presence of sambunigrin was confirmed by the analysis of TMS-ethers of the purified cyanogenic samples; the TMS derivatives of prunasin and sambunigrin are resolved by GC-MS (Seigler 1991; Chassagne and Crouzet 1998). The ratios of sambunigrin to prunasin ratio (S:P) varied with tissue type (Table 1). The greatest proportion of sambunigrin was present in leaf (27:73, S:P) and stem (26:74, S:P) samples. No sambunigrin was detected in root tissue.

In addition to prunasin and sambunigrin (19:81, S:P), the extract from immature *P. turneriana* seed contained a further minor cyanogenic peak (compound 2). The retention time by reverse phase HPLC and GC-MS spectrum of the TMS-ether were consistent with the cyanogenic

Table 1. Concentration of cyanogenic glycosides in tissues of *P. turneriana* seedlings

There were two cyanogenic glycosides in all but root tissue: epimers (*R*)-prunasin and (*S*)-sambunigrin. Vegetative samples were homogenised samples from a minimum of 10 glasshouse grown seedlings (9 months old). Seeds (immature), collected from the field, also contained small amounts of the di-glycoside amygdalin. The ratio of sambunigrin to prunasin (S:P) was determined by ^1H -NMR

| Plant tissue | Cyanogenic glycoside concentration (mg CN g ⁻¹ dw) | Ratio of S:P |
|-----------------|---------------------------------------------------------------|--------------|
| Leaf | 4.70 | 27:73 |
| Stem | 1.18 | 26:74 |
| Root | 3.60 | 0:100 |
| Seed (immature) | 2.92 | 19:81 |

di-glycoside amygdalin [(*R*)-mandelonitrile 6-*O*- β -D-glucoside- β -D-glucoside; Fig. 1]. Based on HPLC fraction analysis, amygdalin was estimated to account for less than 5% of the total cyanogen.

Variation in foliar cyanogenic glycoside concentration

The initial foliar concentration of cyanogenic glycosides was measured in 74 young *P. turneriana* seedlings (3 months old) grown from seed sourced from a single parent tree (Fig. 2). Seedlings were sampled at the same (two leaf) stage. All seedlings were cyanogenic but the actual concentration of cyanogenic glycosides varied 4-fold, from 1.05 to 4.01 mg CN g⁻¹ dw. Data were normally distributed around the population mean (\pm 1 s.e.) of 3.1 \pm 0.06 mg g⁻¹ dw (Shapiro–Wilks, $P > 0.1$), not right skewed as has been reported for several other cyanogenic species (e.g. Woodrow *et al.* 2002).

Effect of light on photosynthesis, growth, biomass partitioning and leaf chemistry

Young *P. turneriana* seedlings were allocated to three different light environments based on their cyanogenic glycoside concentration such that initially each treatment contained a similar range of concentrations. After 3 months,

the physiological, morphological and chemical responses of seedlings grown were measured.

Photosynthesis

The light-saturated photosynthetic capacity (A_{\max}) of fully-expanded, old leaves of seedlings grown under 95% shade was about half that of similar leaves of plants grown under both 50% and full light ($P < 0.0001$; Table 2) with a mean (\pm 1 s.e.) of $4.6 \pm 0.5 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$. However, A_{\max} did not differ between seedlings grown in the 50% shade ($9.1 \pm 0.1 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and full light treatments ($9.3 \pm 0.9 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). In addition, the PPFD at which photosynthesis became saturated was significantly lower for plants grown in the 95% shade treatment ($P = 0.002$) compared to both 50% shade and full light grown plants (data not shown).

Growth and biomass analysis

Consistent with the photosynthetic responses, growth and biomass allocation varied markedly between treatments (Fig. 3*a–d*). Seedling growth was strongly reduced in 95% shade, with a mean relative growth rate (RGR) of only $0.015 \pm 0.002 \text{ g g}^{-1} \text{ d}^{-1}$ (mean \pm 1 s.e.) compared with $0.036 \pm 0.002 \text{ g g}^{-1} \text{ d}^{-1}$, and $0.036 \pm 0.004 \text{ g g}^{-1} \text{ d}^{-1}$ (mean \pm 1 s.e.) for 50% and full light grown plants, respectively. It is noteworthy that the RGR of plants grown in 50% shade and full light did not differ. However, there was a significant increase in height with each increase in PPFD with means (\pm 1 s.e.) $36.7 \pm 2.6 \text{ cm}$, $56.5 \pm 6.3 \text{ cm}$ and $76.9 \pm 7.7 \text{ cm}$, respectively ($P < 0.01$).

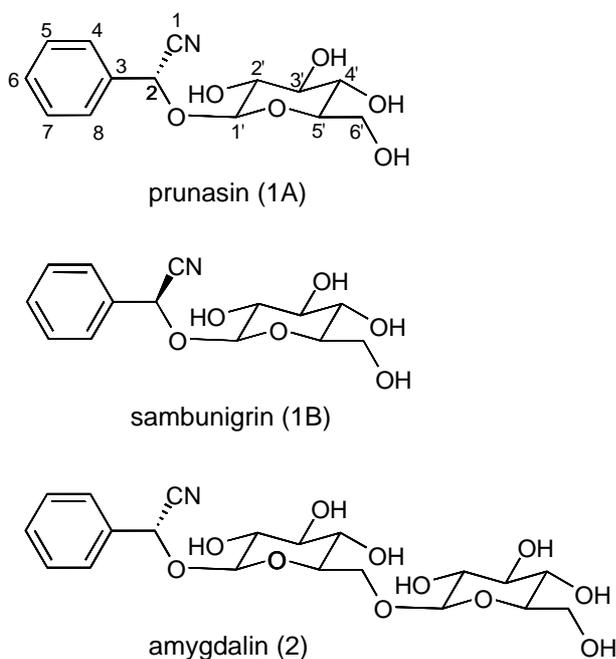


Fig. 1. Chemical structures of mono-glucoside epimers (1A) (*R*)-prunasin [(*R*)-mandelonitrile β -D-glucoside] and (1B) (*S*)-sambunigrin [(*S*)-mandelonitrile β -D-glucoside], and (2) the di-glycoside (*R*)-amygdalin [(*R*)-mandelonitrile 6-*O*- β -D-glucoside- β -D-glucoside]. A mix of epimers (*R*)-prunasin and (*S*)-sambunigrin was detected in leaf, stem and seed tissue; root tissue contained only (*R*)-prunasin. Structures 1A and 1B were elucidated using LC–ESIMS and ¹H NMR. Amygdalin (2) occurred in small amounts in seed tissue only, as determined by GC–MS.

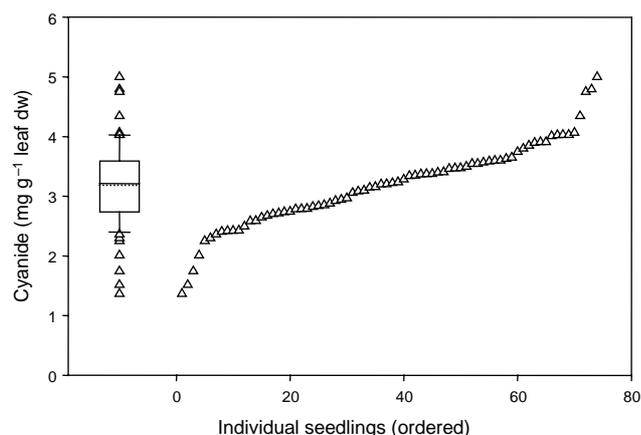


Fig. 2. Foliar cyanogenic glycoside concentrations of *P. turneriana* seedlings, indicating the continuous nature of variation in cyanogenic glycoside concentration. Seed was sourced from single parent tree. The distribution does not differ significantly from a normal distribution (Shapiro–Wilks, $P > 0.1$). Cyanogenic glycoside concentration was determined for all seedlings ($n = 74$) at the two-leaf stage. The box plot represents the entire seedlings population ($n = 74$) delimited by the 25th and 75th percentiles, with error bars denoting the 10th and 90th percentiles. Values outside these ranges are represented by open triangles. Both the mean (dotted line) and median (solid line) concentrations were $3.09 \text{ mg CN g}^{-1} \text{ dw}$.

Consistent with the RGR data, the root-to-shoot ratio was significantly higher in the 95% shade grown plants, compared to the 50% shade grown plants (Fig. 3b). Moreover, seedlings growing in the two shaded environments (50% and 95% shade) had a higher leaf area relative to plant biomass (i.e. leaf area ratio, LAR) than seedlings grown in full light ($P=0.009$; Fig. 3c). In this case, unlike the overall photosynthetic and growth responses, there was no significant difference in LAR between the two shade treatments. Consistent with this greater investment in leaf area relative to total biomass, the area of each leaf relative to its mass (specific leaf area, SLA) was also higher in seedlings grown in deep shade (95%; $P<0.01$; Fig. 3d). Again, plants grown in the two higher light environments (50% and full light) were similar in SLA (Fig. 3d).

Chemical composition

Chlorophyll

Decreased PPFD was associated with a significant increase in the total chlorophyll concentration (chlorophyll *a* + *b*) of leaves of seedlings from the 95% shade treatment with a mean (± 1 s.e.) of 6.99 ± 1.02 mg g^{-1} dw, compared with 3.41 ± 0.48 mg g^{-1} dw in leaves of full light grown plants leaves ($P=0.01$; Fig. 4c). However, total chlorophyll concentrations in the leaves of seedlings grown at 50% and full light were similar ($P>0.05$). The shaded treatments increased the concentration of chlorophyll *b* relative to chlorophyll *a*, again most notably in the 95% shade-grown plants ($P<0.0001$; Fig. 4d).

Nitrogen and cyanogenic glycosides

Mean foliar cyanogenic glycoside concentration did not differ significantly between light treatments, despite an apparent trend towards decreased concentration of cyanogenic glycosides with shading ($P=0.15$; Fig. 4a). Mean concentrations of foliar cyanogenic glycosides ranged from 3.30 mg CN g^{-1} dw in 95% shade-grown plants, to 4.57 mg CN g^{-1} dw in full-light plants (Fig. 4a).

Total leaf nitrogen was quite variable in the 3-month-old *P. turneriana* seedlings, ranging from 8.7 mg N g^{-1} dw to 23.5 mg N g^{-1} dw in plants from the 95% shade treatment, for example. Overall, pooling all leaf age-classes, the concentration of total leaf nitrogen was similar in all treatments ($P=0.679$, Fig. 4b). Of this leaf nitrogen, cyanogenic glycoside nitrogen accounted for 11.46–16.34% of the total leaf nitrogen (CN-N/N %; data not shown). Surprisingly this proportion did not change with the light environment ($P=0.153$).

Further analysis of leaf age classes showed significant differences in cyanogenic glycoside concentration with leaf age. In the full light treatment, for example, young leaves (classes *y* and *y2*) contained 3.39 ± 0.87 mg CN g^{-1} dw while the old leaves (*o* and *o2*) contained 4.87 ± 0.85 mg CN g^{-1} dw. Total leaf nitrogen was not significantly different between the four leaf classes, although the old leaves tended to have a lower mean leaf N (*o*; 16.5 ± 0.25 mg N g^{-1} dw) compared with the young leaves (*y1*; 18.3 ± 0.07 mg N g^{-1} dw) (data not shown).

Importantly, leaves of different ages from seedlings grown at the three different PPFDs responded differently. That is, the interaction between leaf age and light treatment was significant ($P<0.0001$; Fig. 5). Young leaves (*y*) from the plants grown in deep shade had the lowest cyanogenic glycoside concentration with 0.73 ± 0.12 mg CN g^{-1} dw (mean ± 1 s.e.) compared with 4.31 ± 0.86 and 3.39 ± 0.87 mg CN g^{-1} dw in the 50% shade and full light treatments, respectively (Fig. 5). By contrast, the cyanogenic glycoside concentration was not significantly different in old leaves (*o*) of seedlings from the three light treatments with means (± 1 s.e.) of 3.31 ± 0.53 , 4.13 ± 0.39 and 4.87 ± 0.85 , respectively (Fig. 5). This dependence on leaf age in the magnitude of the light response was amplified when the proportion of nitrogen allocated to cyanide in each leaf age class was considered (Fig. 6). In the deep shade treatment, the proportional allocation of nitrogen to cyanide increases by an order of magnitude with leaf age, across all leaf age classes ($P<0.0001$; Fig. 6); from $1.98 \pm 0.27\%$ (mean ± 1 s.e.) nitrogen present as cyanide in

Table 2. Mean light saturated rates of photosynthesis (A_{\max}) and cyanogenic glycoside concentration in different parts of 6-month-old *P. turneriana* seedlings

Seedlings were grown in three light treatments for 60 d: full light, 50% and 95% shade ($n = 5, 6, 6$, respectively). A_{\max} was determined from photosynthetic light response curves, and was measured for at least one old leaf on each plant. A_{\max} in 95% shade-grown plants was significantly lower than in 50%- and full-light-grown plants ($P<0.0001$). Cyanogenic glycoside concentrations for foliage are overall means (± 1 s.e.) incorporating concentrations in all leaf age classes. There were no significant differences between treatments for the same plant part

| Light treatment | A_{\max} ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) (mean ± 1 s.e.) | Foliage (CN mg g^{-1} dw) | Stem (CN mg g^{-1} dw) | Root (CN mg g^{-1} dw) |
|------------------------|------------------------------------------------------------------------------------------|---------------------------------------|------------------------------------|------------------------------------|
| 95% shade ($n = 6$) | 4.62 ± 0.52^a | 3.301 ± 0.287 | 1.138 ± 0.122 | 1.652 ± 0.126 |
| 50% shade ($n = 6$) | 9.11 ± 0.16^b | 4.222 ± 0.358 | 1.202 ± 0.099 | 1.861 ± 0.114 |
| Full light ($n = 5$) | 9.29 ± 0.89^b | 4.565 ± 0.677 | 0.832 ± 0.104 | 2.238 ± 0.379 |

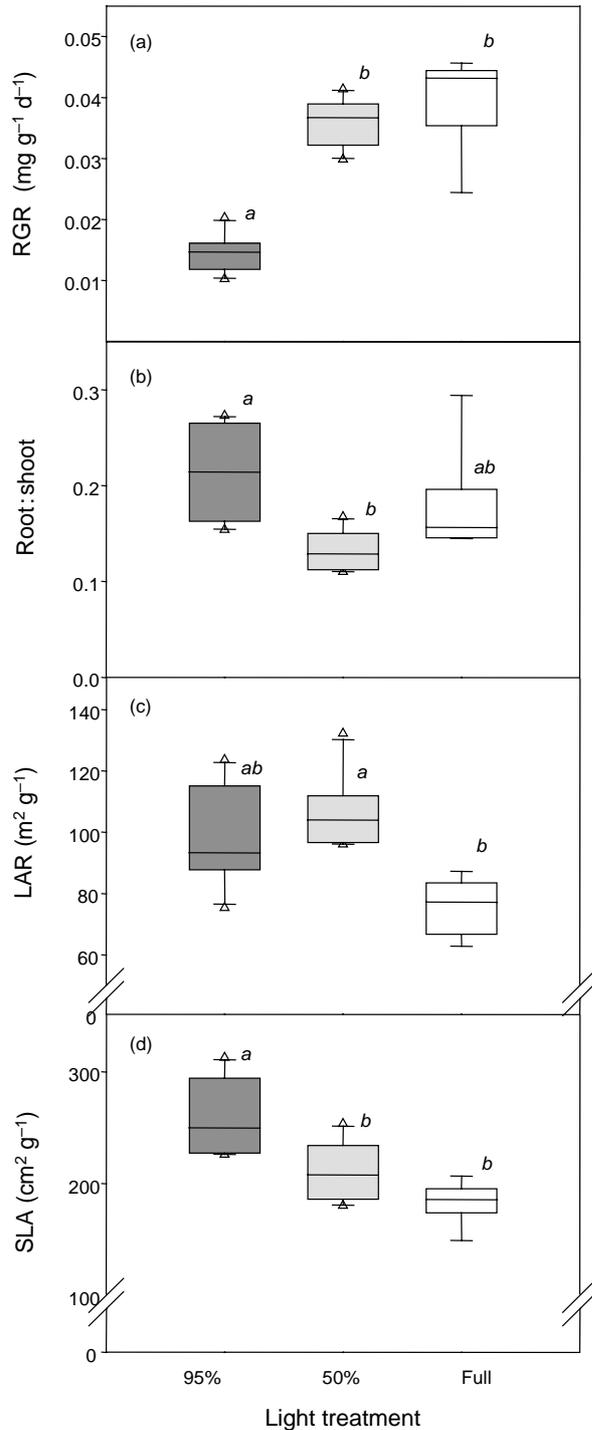


Fig. 3. Growth and biomass partitioning in 6-month-old *P. turneriana* seedlings grown in three light treatments for 60 d: full light (FL) white boxes, 50% shade, light grey boxes and 95% shade, dark grey boxes ($n = 5, 6, 6$, respectively). (a) Relative growth rate (RGR), (b) root:shoot ratio, (c) leaf area ratio (LAR $\text{cm}^2 \text{g}^{-1}$), and (d) specific leaf area (SLA $\text{cm}^2 \text{g}^{-1}$). Box plots show the range of values for six measurements, and are delimited by 25th and 75th quartiles, with error bars indicating the 10th and 90th percentiles. Statistically significant ($P < 0.05$) differences between treatment means (solid lines) are indicated by different letters.

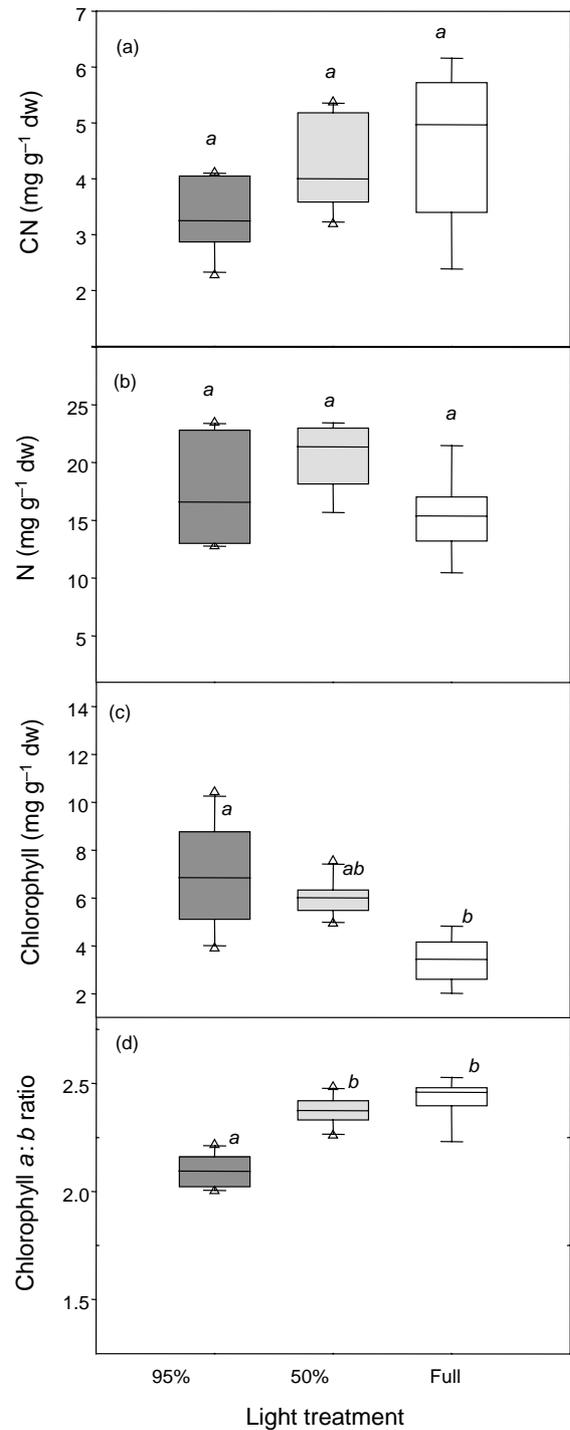


Fig. 4. Chemical composition of leaves of 6-month-old *P. turneriana* seedlings grown in three light treatments for 60 d: full light (FL) white boxes, 50% shade, light grey boxes and 95% shade, dark grey boxes ($n = 5, 6, 6$, respectively). (a) cyanogenic glycoside concentration, (b) total leaf nitrogen, (c) total chlorophyll ($a + b$) concentration, and (d) chlorophyll $a:b$ ratio. Box plots show the range of values for six measurements, and are delimited by 25th and 75th quartiles, with error bars indicating the 10th and 90th percentiles. Statistically significant ($P < 0.05$) differences between treatment means (solid lines) are indicated by different letters.

young, to 3.95 ± 0.8 , 9.64 ± 1.64 , and 15.49 ± 0.47 in young2, old and old2 leaf age classes, respectively (Fig. 6).

Prunus turneriana seedlings allocated a significant proportion of nitrogen to cyanide in the stems and roots as well. Light had no impact on the cyanogenic glycoside concentration of roots and stems (Table 2) or on the allocation of nitrogen to cyanide in root or stem tissue (data not shown). The proportion of nitrogen allocated to cyanide in roots and stems was less than the foliar allocation. In roots, between 5.58 and 10.77% of nitrogen was present as cyanide, whereas in stems this ratio ranged from 4.65 to 6.45% (data not shown).

Discussion

There were three parts to our study of *P. turneriana* seedlings. First we identified the cyanogenic glycosides found in different organs of 9-month-old seedlings. Second, we examined the variability of expression by quantifying the foliar cyanogenic glycoside concentration in progeny from a single parent at the two-leaf stage. Third, we tested the hypothesis that, in addition to the inherent polymorphism, the accumulation of cyanogenic glycosides is opportunistic, decreasing when the resources available for growth, such as light, are limited.

Cyanogenic glycoside purification and identification

Our findings regarding the cyanogens in *P. turneriana* are broadly consistent with other studies of species in the Rosaceae. Predominantly, cyanogenic members of this family have been found to have cyanogens derived from

phenylalanine (typically prunasin and amygdalin; Møller and Seigler 1999). We identified prunasin as the major cyanogen in all tissues of *P. turneriana*, and amygdalin was restricted to the seed. What is unusual about *P. turneriana*, is the presence of significant amounts of the (*R*)-prunasin epimer, (*S*)-sambunigrin. We found a mean ratio of these epimers of 76:24 (P:S) in leaf stem and seed tissue, whereas root tissue contained only prunasin. Such a distribution is quite unique because sambunigrin has only recently been noted in seeds (Kumarasamy *et al.* 2003), and leaves (Ito and Kumazawa 1995) of two *Prunus* species. Moreover, in some cases, sambunigrin been proven to be an artefact of extraction (Nahrstedt 1975).

Racemisation during isolation of prunasin has been reported under alkaline conditions (Nahrstedt, 1975; Chassagne *et al.* 1996). For example, early reported mixes of sambunigrin and prunasin, termed prulaurasin in the cherry laurel (*P. lauracerasus*), were subsequently shown to have resulted from the addition of CaCO_3 during extraction (Conn 1981). In addition, the interconversion of epimers has also been reported during long-term storage in ethanol (Erb *et al.* 1979). That the mixtures of epimers detected in this study actually occur in the plant and are not an artefact of extraction is supported by the fact that the identical extraction protocol across different tissue types yielded different proportions of the epimers, and most notably, by the absence of sambunigrin in root tissue. Moreover, the extraction technique was identical with that employed by both Goodger *et al.* (2002) and Gleadow *et al.* (2003) who reported 100% and 96% prunasin, respectively, in *Eucalyptus* species.

While the presence of a chiral centre in mandelonitrile provides the opportunity for the two β -glucosides, prunasin and sambunigrin, these epimers seldom occur in the same

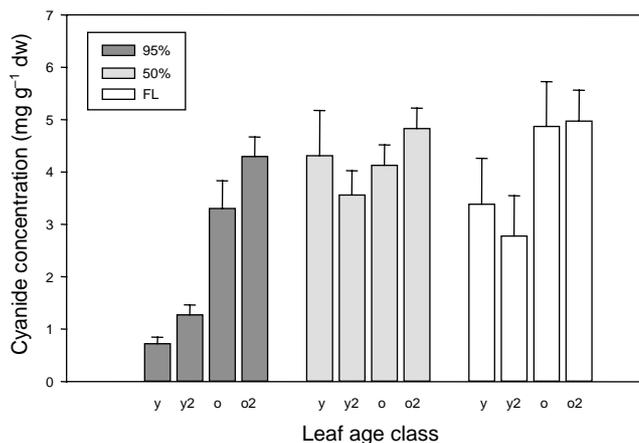


Fig. 5. Concentration of cyanogenic glycosides in leaves of different ages in 6-month-old *P. turneriana* seedlings grown in three light environments: full light (FL), 50% shade and 95% shade for 60 d ($n = 5, 6, 6$, respectively). Leaves were classed according to position on the plant, toughness and colour: yg, newly formed leaves (> 2 cm length); yg2, newly expanded leaves; old, dark, tough, fully expanded leaves; old2, oldest leaves from the lower part of the plant. Values are mean values (± 1 s.e.) and can be compared at the 95% level using $\text{l.s.d.}_{0.05}=1.2$.

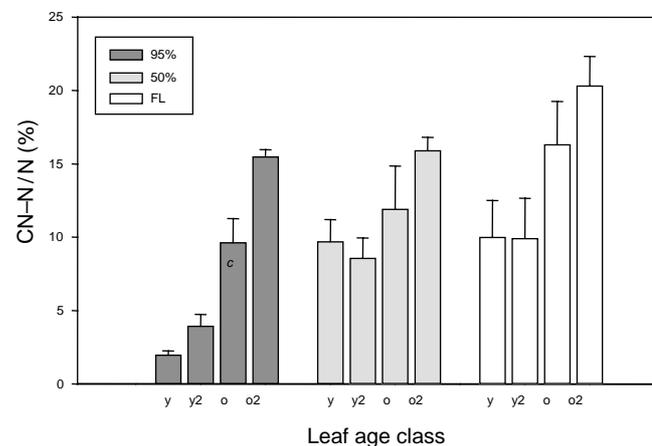


Fig. 6. The proportion of total nitrogen found as cyanide in 6-month-old *P. turneriana* seedlings grown in three light environments: full light (FL), 50% shade and 95% shade for 60 d ($n = 5, 6, 6$, respectively). Leaves classes are defined in legend to Fig. 5. Values are means (± 1 s.e.) and can be compared at the 95% level using $\text{l.s.d.}_{0.05}=1.5$.

plant or even related species (Conn 1973; Lechtenberg and Nahrstedt 1999). One notable exception to this is found in Australian *Acacia* spp. (subgenus Phylloideae), where species contain both epimers, in a spectrum ranging from pure sambunigrin, to a 50:50 mix, to pure prunasin across species (Conn *et al.* 1985; Maslin *et al.* 1988). Similarly, the epimeric pair, dhurrin and taxiphyllin, occur in distinct taxa (Conn 1981). However, the co-occurrence of epimers within a species has previously been reported for epiheterodendrin/heterodendrin in barley (Gramineae; Erb *et al.* 1979, Hübel *et al.* 1980; Pourmohseni *et al.* 1993), tetraphyllin B and an epimer in *Adenia volkensii* (Passifloraceae; Gondwe *et al.* 1978), and prunasin/sambunigrin in non-rosaceous *Sambucus nigra* (Caprifoliaceae; Jensen and Nielsen 1973; DellaGreca *et al.* 2000).

Given the rare co-occurrence of epimers in the same species or even within the same genus, it has been suggested that there must be differences in biosynthetic pathways for the two compounds (Conn 1991). Interestingly, biosynthetic studies in *P. serotina* (black cherry) demonstrate enzyme stereo-specificity in the biosynthesis of prunasin; *in vitro*, strict substrate stereo-specificity of the enzyme *O*-glucosyl-transferase for the (*R*)-enantiomer mandelonitrile was found, which is consistent with the absence of sambunigrin in *P. serotina in vivo* (Poulton and Shin 1983). Similar stereo-specificity in biosynthesis of tyrosine-derived cyanogenic glycosides has been found in *Sorghum* (Reay and Conn 1970), and in *Triglochin maritima* (Hösel and Nahrstedt 1980). The detection of substantial proportions of sambunigrin in *P. turneriana* in this study shows that, within one genus, with the same amino acid precursor, there may be diversity in biosynthetic pathway.

In contrast to the co-occurrence of epimers, the co-occurrence of mono-glucoside and related di-glucoside in the same species is common. In this study, amygdalin was restricted to seeds, while prunasin was found in all plant parts. This distribution pattern is typical of other Rosaceae (e.g. *Prunus dulcis*, Dicenta *et al.* 2002; *Cotoneaster* spp., Nahrstedt 1973). Other di-glycosides are also restricted to seeds of other taxa (e.g. linustation/neolinustatin in linseed; Smith *et al.* 1980). The amount of amygdalin generally increases relative to prunasin during seed maturation in *Prunus* spp. (Frehner *et al.* 1990; Ohtsubo and Ikeda 1994; Zhou *et al.* 2002), and there is evidence that cyanogenic di-glycosides in seeds are metabolised upon germination, and used for synthesis of non-cyanogenic compounds during seedling development (e.g. *Hevea brasiliensis* and *Ungnadia speciosa*; Selmar *et al.* 1988). The isolation of small amounts of amygdalin from immature *P. turneriana* seed (< 5%) in this study is consistent with the pattern of accumulation of the mono-glucoside in immature fruits, and of the di-glucoside amygdalin in mature fruits.

Genetic and environmental variation in cyanogenic glycoside content

Cyanogenic polymorphism

The progeny of a single *P. turneriana* tree were all cyanogenic, but ranged more or less continuously from approximately 1 to 4 mg CN g⁻¹ dw (Fig. 2). Because plants were grown under constant light conditions to the same developmental stage, it is highly likely that this variation in cyanogenesis is genetically based. Similar findings have been made for a range of other species (Conn *et al.* 1985; Gleadow and Woodrow 2000a; Goodger and Woodrow 2002; Goodger *et al.* 2002), including naturally occurring populations of three *Prunus* species (Aikman *et al.* 1996). It is noteworthy that unlike many other cyanogenic species, we did not detect any acyanogenic individuals. This may have been due to the fact that we used only one half-sib family for the analysis, or it may be that in the high herbivory conditions of the rainforest, acyanogenic individuals may not be viable, as there is strong selective pressure for high defence (Coley 1983; Marquis 1984; Coley *et al.* 1985).

Light effects on growth and photosynthesis

The physiological and morphological responses of seedlings grown in the 95% shade treatment were typical of most woody plants adapted to deep shade (e.g. Chow *et al.* 1988; Chazdon 1992; Mooney *et al.* 1995; Burns *et al.* 2002; Figs 2–4, Table 2). While the RGR, A_{\max} and PPFD required to approach A_{\max} were all reduced in shade, there was a marked increase in total chlorophyll concentration and the concentration of chlorophyll *b* relative to chlorophyll *a* (Figs 3, 4, Table 2). Moreover, consistent with other studies of rainforest plants (e.g. Popma and Bongers 1988; Denslow *et al.* 1990), shade-grown plants had a greater investment in leaf surface area relative to plant biomass (LAR; Fig. 3), and a significantly higher SLA (Fig. 3), although root-to-shoot ratio did not differ consistently across light treatments. It is noteworthy that there was little difference in the physiological and morphological responses between the 50% and full sunlight treatments. This is typical of shade-tolerant species, which exhibit reduced plasticity across a range of PFDs (Bazzaz and Pickett 1980; Denslow *et al.* 1990; Chazdon 1992), and probably reflects the fact that even when rainforest gaps appear, plants are exposed to full sunlight for a relatively short period each day.

Light effects on resource allocation and cyanogenic glycosides

Whole-plant level

Based upon the morphological and physiological changes observed in seedlings under deep shade, which are all apparently geared to increasing light capture and the efficiency of net CO₂ assimilation, we hypothesised that there should be a parallel decrease in cyanogenic glycoside

concentration. Our reasoning was that it may be too 'expensive' to maintain a high level of these compounds, which have high rates of turnover (Adewusi 1990) and require a sizeable amount of nitrogen. However, this was not supported by our data at the whole-plant level. We found no reduction in cyanogenic glycoside concentration (in leaves, stems or roots), or in the proportion of nitrogen in cyanogenic glycosides, in seedlings of *P. turneriana*, in low light. Therefore, in comparison with the significant plasticity in photosynthesis, growth response, biomass allocation and allocation to light harvesting (i.e. chlorophyll), *P. turneriana* seedlings exhibit reduced plasticity in the expression of cyanogenic glycoside defence.

This is the first study of light effects on resource allocation to cyanogenic glycosides, and indeed to any nitrogen-based defence chemical, under photon flux densities representative of rainforest understorey and gap environments. There have been studies of plants from other habitats, such as those on the fern *Pteridium aquilinum* (Cooper-Driver *et al.* 1977; Schreiner *et al.* 1984) and white clover (*Trifolium repens*, Vickery *et al.* 1987), in which it was found that cyanogenic glycoside concentration was higher in plants growing in shady compared to sunny areas. In contrast, the cyanogenic glycoside concentration of *Linum usitatissimum* (flax) decreased in shade (Niedzwiedz-Siegi n and Gierasimiul 2001). Similarly, conflicting data were reported for alkaloid-containing species in contrasting light environments, with both increases (H gele and Rowell-Rahier 1999) and decreases (Ralphs *et al.* 1998) in alkaloid concentration reported with shading. Nevertheless, it is difficult to interpret these findings in relation to a trade-off in resource allocation because leaf nitrogen was not measured.

In two studies where nitrogen was measured, results contradicted. In the in the tropical tree *Tabernaemontana pachysiphon* alkaloid concentration, and the proportion of nitrogen in alkaloids, increased under lower light (H ft *et al.* 1996), consistent with the prediction that increased nitrogen in shade-grown plants results in a greater investment in nitrogen-based defence (Bryant *et al.* 1983, 1985). In contrast, in a study in which both light and nitrogen supply were controlled, Burns *et al.* (2002) found both the concentration of cyanogenic glycosides and the proportion of nitrogen in cyanogenic glycosides decreased in shade-grown *Eucalyptus cladocalyx*.

Light effects on within-plant variation in allocation to defence

In contrast to the whole-plant experiments, we identified significant changes in the allocation of resources within the plant. A highly significant interaction between PFD and leaf age was found in relation to both the concentration of cyanogenic glycosides, and the proportion of nitrogen in cyanogenic glycosides (Figs 5, 6). That is, we found different allocation patterns in young and old leaves with

contrasting irradiance. Most significantly, in the 95% shade treatment, cyanogenic glycoside concentration increased by approximately 600% from young to old leaves (Fig. 5). Furthermore, this pattern was amplified when the relative investment of nitrogen in cyanogenic glycosides was analysed (Fig. 6). In the 95% shade treatment, the allocation of nitrogen to cyanogenic glycosides increased from approximately 2% of nitrogen invested in cyanogenic glycosides in young leaves, to just less than 20% in old leaves (Fig. 6). This greater relative investment of nitrogen to defence in old leaves was common to all light treatments (Fig. 6), although the variation with leaf age was less significant in the two treatments where light was not limiting.

To our knowledge, this is the first time that such a pattern of chemical defence has been reported. In almost all cases, young leaves have been found to be the most defended (Coley and Barone 1996). This greater commitment to defence in young leaves has been found for terpenes (Crankshaw and Langenheim 1981), phenolics and tannins (e.g. Coley and Aide 1991), as well as for alkaloids (McKey 1974; H ft *et al.* 1998). Similarly, studies report a decrease in foliar cyanogenic capacity with leaf age across a range of species including species of tropical *Macadamia* (Dahler *et al.* 1995), as well as *Eucalyptus* (Gleadow and Woodrow 2000b), and *Prunus* spp. (Aikman *et al.* 1996).

This pattern is consistent with the optimal allocation theory (McKey 1974, 1979), which predicts that the most vulnerable (most likely to be attacked) and valuable and parts of the plant, in terms of fitness value, will be most highly defended. The allocation of cyanogenic glycosides found here in *P. turneriana* seedlings is inconsistent with the predictions of the optimal allocation theory. Under this theory, young expanding leaves, which suffer high herbivory and which have yet to provide return on investment in terms of carbon gain for the plant, are well defended. More specifically, in young expanding leaves, the deployment of mobile, low molecular weight compounds (e.g. cyanogenic glycosides), which can be re-metabolised, is assumed to be of greater advantage (McKey 1979). By contrast, the cost associated with the ongoing turnover of these compounds, is predicted to be prohibitive for the maintenance of high levels in old leaves (Coley *et al.* 1985). Why then, does resource allocation *P. turneriana* differ from most of these other plants? It may be because this is the first study to look at the effect of deep shade on nitrogen-based defence, previous studies having used relatively high light (H ft *et al.* 1996). It may also be that part of the answer is reflected by the pattern of nitrogen allocation within the seedlings. We found no significant decrease in foliar nitrogen with leaf age, indicating that older leaves in this species may remain highly productive for relatively long periods of time. Moreover, we found that these productive older leaves were very well defended by their relative toughness, and high concentration of cyanogenic glycosides. In the deep shade, it

may be a more productive strategy to prolong the lifespan and maintain the productivity of older leaves (at the expense of new leaves), and in effect, wait for an influx of light from a gap. For *P. turneriana*, it is only under these conditions that there is any likelihood of growth to a reproductive size (see Osunkoya *et al.* 1993).

Acknowledgments

The authors thank Prof Rob Capon and Dr Mike Stewart, Centre for Molecular Biodiversity, University of Queensland for NMR analysis and interpretation, Drew King and Dr Jianping Lin for technical assistance with GC–MS, Rigel Jensen for seed collection and field assistance, and Bill and Wendy Cooper for access to the rainforest on their property. This project was supported, in part, by an Individual Award from The Queen's Trust for Young Australians to REM and an ARC grant to IEW. REM was a recipient of an Australian Postgraduate Award.

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Manuscript received 5 November 2003, accepted 9 January 2004