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Constitutive polymorphic cyanogenesis in the Australian rainforest tree, *Ryparosa kurrangii* (Achariaceae)

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Abstract

Cyanogenesis, the liberation of volatile hydrogen cyanide from endogenous cyanide-containing compounds, is a proven plant defence mechanism and the particular cyanogens involved have taxonomic utility. The cyclopentenoncyanhydrin glycoside gynocardin was the only cyanogen isolated from foliar tissue of the rare Australian rainforest tree, *Ryparosa kurrangii* (Achariaceae). Mechanical damage simulating foliar herbivory did not induce a significant increase in the expression of cyanogenesis over a 24 h period, indicating cyanogenic herbivore defence in *R. kurrangii* is constitutive. The cyanogenic potential of mature leaves was quantitatively polymorphic between trees in a natural population, ranging from 0.54 to 4.77 mg CN g^{-1} dry wt leaf tissue.

Keywords: *Ryparosa kurrangii*; Achariaceae; Cyanogenesis; Cyanogenic glycoside; Gynocardin; Polymorphism; Constitutive defence; Tropical rainforest

1. Introduction

Cyanogenesis involves the liberation of volatile hydrogen cyanide (HCN) from endogenous cyanide-containing compounds, and is widespread in the plant kingdom, recognised in over 3000 species representing more than 550 genera and 130 families (Poulton, 1990; Lechtenberg and Nahrstedt, 1999; Møller and Seigler, 1999; Vetter, 2000). Cyanogenic plants are particularly common in certain families (Seigler, 1976, 1991; Thomsen and Brimer, 1997) and the identification of cyanogenic constituents has been used in the past as an informative taxonomic marker (e.g. Spencer and Seigler, 1985a; Spencer et al., 1985). Relative to the documentation of their presence within plant species, the structural elucidation of cyanogenic glycosides between species remains limited, with around 60 different compounds identified from approximately 18% of plants known to be cyanogenic (Seigler, 1991; Lechtenberg and Nahrstedt, 1999).

In its role as a plant defence mechanism cyanogenesis has been widely studied, and there is now a general consensus that evolved cyanide following tissue disruption functions as a feeding deterrent for generalist herbivores (Nahrstedt, 1985; Hruska, 1988; Jones, 1998; Gleadow and Woodrow, 2002a). What is less clear is the response, if any, of plant cyanogenic potential to herbivore damage, as well as the quantitative variation in cyanogenic concentration that a browsing herbivore may encounter. Both of these factors are critical in determining the potential toxicity of cyanogenic plant taxa. Recent work on a range of tropical rainforest plant species suggests that cyanogenic glycoside concentrations may be considerably higher than in plants from temperate regions (Webber and Woodrow, 2004; Miller et al., 2006b) but there is a distinct paucity of knowledge on the extent of cyanogenic polymorphism in tropical rainforest species.

The Austro-Malesian genus *Ryparosa*, was one of 30 genera assigned to the predominantly cyanogenic Achariaceae,

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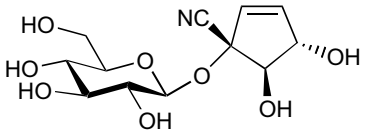
during a recent revision of Flacourtiaceae *sens. lat.* (Chase et al., 2002). Cyanogenesis was first reported in *Ryparosa* in 1907 after an examination of plants in the Bogor Botanic Gardens identified *R. javanica sens. str.* (Blume) Kurz ex. Koord. & Valeton and *R. caesia* Blume as cyanogenic (Greshoff, 1898, 1906a,b; Treub, 1907). A subsequent study of cyanogenesis and systematic relationships in Flacourtiaceae *sens. lat.* confirmed cyanogenesis in *R. hirsuta* J.J.Sm. and *R. hullettii* King, and also identified the presence of the cyclopentenoid glycoside, gynocardin, in *R. acuminata* Merr. (Spencer and Seigler, 1985a). The rare endemic *R. kurrangii* B.L. Webber is the only known *Ryparosa* species in Australia, and forms the south-easterly limits of the generic distribution. In this paper we explore cyanogenesis in *R. kurrangii*. More specifically, our objectives were to (1) identify the cyanogenic compound in foliar material; (2) investigate the possibility of cyanogenic inducibility after tissue damage; and (3) examine inter-plant cyanogenic polymorphism in a natural population.

2. Results and discussion

2.1. Identification of the cyanogen

The cyanogenic compound was extracted from field-collected leaf tissue and purified using solid-phase extraction cartridges followed by RP-HPLC. Fractionation of the leaf extract by preparative C_{18} HPLC identified a single peak (approx RT 9.85 min) of cyanogenic activity. Active fractions were collected and further purified using analytical C_{18} HPLC, yielding a single symmetrical peak (RT 7.3 min) with cyanogenic activity.

Positive mode LC-ESIMS identified the ions *m/z* 326.1 $[M+Na]^+$, and *m/z* 342.1 $[M+K]^+$, indicating that the M_r of the compound was 303 amu. With respect to cyanogenic compounds, this M_r is consistent with the known cyanogenic glycoside gynocardin (**1**) and its theoretically possible epimer, epigynocardin, which has not yet been found to naturally occur ($C_{12}H_{17}NO_8$). The ^1H NMR spectrum (400 MHz, D_2O) revealed resonances that were consistent with these structures (δ 6.1 and 5.9 ppm, consistent with the olefinic protons; δ 3.2 and 5.0 ppm, indicative of deshielded methines and methylenes). A COSY experiment indicated ^1H–^1H interactions that compared favourably with the original characterisation of gynocardin from *Gynocardia odorata* R. Br. (Coburn and Long, 1966), confirming the gross structure as one of gynocardin or epigynocardin. The ^{13}C NMR spectrum (75 MHz, D_2O) was virtually identical to that reported for **1** (Hübel et al., 1981). Chiroptical measurements based on an assumed analysis weight of 0.5 mg, gave an $[\alpha]_D$ of +64.1° ($c = 0.05$, water) which compares favourably with the value obtained by Coburn and Long (1966) for **1** of +72.9° ($c = 0.96$, water). Thus, the only cyanogen detected in *R. kurrangii* foliar tissue was the cyclopentenoncyanhydrin glycoside gynocardin (**1**).



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This result is in concordance with an examination of *Ryparosa acuminata* seed tissue, in which **1** was the only documented cyanogen (Spencer and Seigler, 1985a). Gynocardin, the first cyclopentenoid glycoside to be discovered (Power and Gornall, 1904; Power and Barrowcliff, 1905; Power and Lees, 1905) and have its chemical structure determined (Coburn and Long, 1966; Kim et al., 1970), is the most common glycoside found in Achariaceae taxa, and has been isolated from leaf, stem, root, pericarp and seed tissue (e.g. Spencer and Seigler, 1985a; Jensen and Nielsen, 1986). An initial report on the purification of its theoretical epimer, epigynocardin, from *Pangium edule* Reinw. (Spencer and Seigler, 1985a) was never confirmed and its existence remains highly unlikely (D.S. Seigler, pers. comm.; Jaroszewski and Olafsdottir, 1987).

Cyclopentenoncyanhydrin glycosides, derived from the non-protein amino acid 2-(2-cyclopentenyl)glycine, are restricted to five families within the Violales – Achariaceae, Malesherbiaceae, Passifloraceae, Salicaceae and Turneraceae (Cabalion et al., 1980; Spencer and Seigler, 1985a; Lechtenberg and Nahrstedt, 1999; Chase et al., 2002; Thorne, 2002). These distinctive cyclopentenyl glycosides have been proposed as phylogenetic markers in chemotaxonomic studies (e.g. Saupe, 1981; Spencer and Seigler, 1985b; Spencer et al., 1985) and the structure of 15 distinct cyclopentenoid cyanogens has been documented (Lechtenberg and Nahrstedt, 1999). The recent revision of Flacourtiaceae *sensu lato* (Chase et al., 2002) saw the majority of cyanogenic species reassigned to Achariaceae.

In addition to gynocardin, seven other 2-(2′-cyclopentenyl)glycine-derived glycosides (e.g. epivolkenin, taraktophyllin, deidaclin) have been identified in Achariaceae (e.g. Jaroszewski et al., 1987, 2004), and it appears that there may be spatial separation at an organ level between different cyanogenic compounds. For example, gynocardin and epivolkenin were detected in *Lindackeria dentata* (Oliv.) Gilg seeds (Spencer and Seigler, 1985a; Seigler and Spencer, 1989), while epivolkenin and taraktophyllin were isolated from leaf material of the same species (Jaroszewski et al., 2004). Further chemical purification of cyanogens from different tissue types and taxa within *Ryparosa* would be required to test if gynocardin is the only cyanogenic glycoside in the genus. Previous work has revealed that all tested tissue types, including fruits, flowers and stems, of *R. kurrangii* are cyanogenic (Webber and Woodrow, 2004; Webber, 2005).

2.2. Cyanide potential inducibility

Across sets of three adjacent leaves of a similar age we studied the effect of mechanical damage to leaf tissue on

Table 1
Cyanogenic potential of mature *Ryparosa kurrangii* leaves measured over a 24 h period

Leaf class	Day 1 CN	Day 2 CN	*P*
'Control'	n/a	1.87 ± 0.29	n/a
'Wounded'	1.65 ± 0.19	1.62 ± 0.19	0.905
'Punched'	1.88 ± 0.25	1.77 ± 0.24	0.357

Three adjacent leaves were either wounded (simulated herbivory as well as leaf sampling; 'wounded'), punched (sampled only; 'punched') or left untouched ('control'). After 24 h, leaves were re-sampled and cyanide concentrations compared. Mean cyanide potential (mg CN g^{-1} per dry wt plant tissue ± s.e.) was not significantly different between leaves, or over time.

cyanogenic potential over a 24 h period. Leaves that were damaged to simulate the mechanical aspects of chewing herbivory, in addition to the damage caused by taking samples from the leaf ('wounded'), showed no significant change ($P = 0.905$) in mean cyanide concentration 24 h after mechanical damage (Table 1). Likewise, there was no significant change ($P = 0.357$) after 24 h in leaves that were only damaged by removing leaf samples ('punched'; Table 1). Finally, adjacent leaves that had not been damaged initially ('control') were sampled after 24 h and their cyanide concentration did not differ significantly ($P = 0.588$) from either 'wounded' or 'punched' leaves.

The effect of physical leaf damage on cyanogenic potential is largely unknown, despite the many correlations drawn between plant cyanogenesis and foliar herbivory. Other forms of nitrogen-based defence (e.g. alkaloids) have been reported to be highly inducible following leaf damage (Baldwin et al., 1994; Baldwin and Ohnmeiss, 1994) and cyanogenic glycosides are known to be turned over reasonably rapidly (Jones, 1988; Okolie and Obasi, 1993). However, cyanogenic plant defence, from the perspective of cyanogenic potential, has been shown to be a non-inducible trait (i.e. a 'phytoanticipin'*sensu* VanEtten et al., 1994) in other studies using mechanical damage to simulate herbivory over periods of 24 h (Gleadow and Woodrow, 2000) and 4 weeks (Hayden and Parker, 2002). It is important to recognise that aspects of herbivory other than mechanical damage, such as herbivore oral secretions (e.g. Alborn et al., 1997; Bede et al., 2006) and specific feeding strategies (e.g. Leitner et al., 2005), may induce or inhibit the synthesis of plant chemical defences, although to date there is no evidence of such an effect in cyanogenic species. In the case of *Trifolium repens* L., for example, Hayden and Parker (2002) found no effect of *Helix aspersa* Müller chewing herbivory on plant cyanogenic potential over 5 days. Gleadow and Woodrow (2000) suggest that the considerable cost of maintaining the processes required to enable a rapidly inducible cyanogenic defence, outweighs any potential benefits from such a system. This seems to be the case for *R. kurrangii*.

2.3. Population cyanogenesis studies

Variation in cyanogenic potential between *R. kurrangii* trees in a natural population was quantified using samples from mature leaves. While we detected no qualitative polymorphism for cyanogenesis (i.e. there were no acyanogenic morphs) in *R. kurrangii*, there was considerable quantitative polymorphism at a population level (Fig. 1). Cyanide concentrations ranged between 0.54 and 4.77 mg CN g^{-1} dry wt leaf tissue (mean ± s.e. 1.97 ± 0.09). These upper values compare to some of the highest concentrations of cyanogenic potential recorded in other plant species (Miller et al., 2006a) and reports of cyanide concentrations in excess of 4 mg CN g^{-1} dry wt are rare. 'Dangerous' or 'toxic' concentrations of cyanide, often referred to in relation to poisoning of humans or domestic animals, fall in the region of 0.5–1.0 mg g^{-1} dry wt (e.g. Seigler, 1976; Montgomery, 1980; Hill and Henry, 1996; Jones, 1998).

Tropical rainforests are known to support the greatest diversity of potential herbivores found in any natural ecosystem (Clark and Clark, 1991; Coley and Aide, 1991; Coley and Barone, 1996). In these ecosystems, it may be that the benefits gained from a higher level of plant defence, to counter these threats to plant fitness, may not only select against the maintenance of an acyanogenic phenotype, but also select for higher cyanogenic potential. In support of this notion, Miller et al. (2004, 2006a) found that the tropical rainforest species *Elaeocarpus sericopetalus* F. Muell. (5.2 mg g^{-1} dry wt), *Clerodendrum grayi* Munir. (4.9 mg g^{-1} dry wt) and *Prunus turneriana* (F.M. Bailey) Kalkman (4.8 mg g^{-1} dry wt) all had relatively high levels of cyanogenic glycosides in mature leaves, and no acyanogenic individuals were detected. It seems the number of individuals required to detect qualitative polymorphism in a natural population, if it in fact exists, varies considerably from species to species. On the whole, however, the documentation of uniform population cyanogenesis (i.e. a lack of acyanogenic individuals) is uncommon. The majority of plant species where reasonable numbers of individuals have been tested have reported a certain percentage of functionally

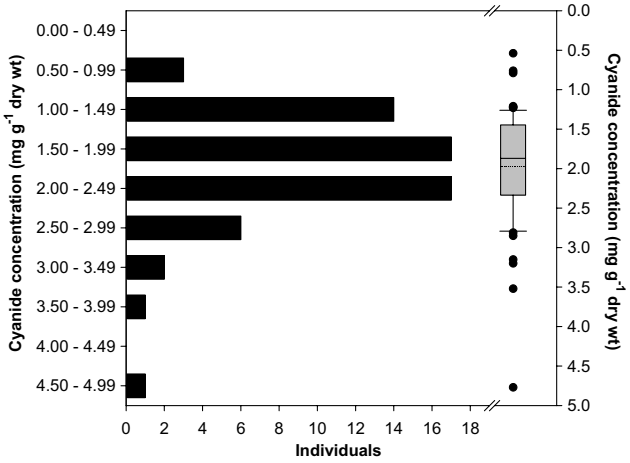

Fig. 1. Population level quantitative polymorphism for foliar cyanogenic potential (mg CN g^{-1} dry wt plant tissue) in a population of *Ryparosa kurrangii* ($n = 61$). The histogram depicts individual cyanogenic potential while the boxplot indicates median (solid) and mean (dotted) cyanide concentrations.

acyanogenic individuals (e.g. Cooper-Driver and Swain, 1976; Ellis et al., 1977; Dirzo and Harper, 1982; Till, 1987; Hughes, 1991; Schappert and Shore, 1995; Aikman et al., 1996; Pederson et al., 1996; Bazin et al., 1997; Goodger et al., 2002; Gleadow et al., 2003; but see Kakes, 1994; McMahon et al., 1995; Solís Neffa et al., 2003). Locating and testing larger populations of the rare *R. kurrangii* will be required to confirm if this species has qualitative as well as quantitative polymorphism for cyanogenesis.

3. Experimental

3.1. Plant material

Ryparosa kurrangii B.L. Webber (Achariaceae; Flacourtiaceae *pro parte*) is endemic to tropical lowland rainforest of the Daintree region in northern Queensland, Australia (Webber and Woodrow, 2006). The understorey tree commonly maintains large leaves (240–280 mm long) at all levels along the main trunk, regardless of light availability. All leaf material was collected from mature leaves of a similar age (fully expanded and toughened but without significant damage or epiphyll colonisation). Trees were generally sampled from a similar height (0.5–2.0 m) and pole pruning shears were used to sample material from greater heights where this was not possible. No direct relationship between sampling height and cyanogenic capacity was detected (data not shown). Given that the rainforest canopy is the main determinant of light availability, absolute aspect was considered to be of negligible influence, and as such, was not kept constant. Samples were collected from field populations of *R. kurrangii* in the Mount Sorrow valley (ca. 16°07′S, 145°28′E; 50 m a.s.l.). Voucher specimens (*BW017, BW018*) have been lodged at The University of Melbourne (MELU; *D*102277, *D*102278), Kew (K) and Leiden (L) herbaria.

To identify the cyanogen in *R. kurrangii*, entire leaves were collected from approximately 15 trees in February and July 1999. These leaves were combined to produce a bulk tissue sample for cyanogenic β-glucosidase and cyanogenic glycoside purification. To study cyanogenic inducibility, three adjacent leaves were chosen on five randomly selected, mature trees. Leaves were designated as 'control' (C), 'wounded' (W) or 'punched' (P) and sampled at 13:00 on two consecutive days. Leaf samples taken from the 'wounded' leaves were punctured in 15 places immediately after sampling on the first day, to simulate chewing herbivory. This was done with the saw blade of a pocket knife forming numerous small holes in the leaf lamina. Care was taken to avoid damaging the main veins of the leaf. 'Punched' leaves were sampled on both days with no additional damage, while 'control' leaves were only sampled on the second day. Four leaf discs (15 mm diameter) were punched from each leaf at evenly spaced points on the leaf lamina, avoiding main veins, at each sampling time. Samples for population cyanogenesis determination were collected during the wet season (February) from a permanent study population on a tributary of Myall Creek. For all individuals in the population ($n = 61$), two leaf discs (15 mm diameter) were punched from either side of the midrib of five spatially proximate leaves, avoiding main veins. Foliar samples were snap-frozen in liquid N_2, temporarily stored on dry ice for transport, freeze dried, ground to a fine homogeneous powder using a ball mill (Southern Dental Industries Ltd., Melbourne, Australia) for small samples or an analytical mill (IKA Labortechnik, Janke and Kunkel GmbH Co., Staufen, Germany) for bulk material, and stored on desiccant at −20 °C.

3.2. Cyanogenic β-glucosidase purification

Partially purified cyanogenic β-glucosidase was extracted from the same bulk tissue sample as that used for cyanogenic constituent identification. Freeze–dried leaf tissue (10 g) was extracted on ice (1 h) in buffer A (50 ml; 100 mM citrate–HCl (pH 5.5), 10 mM Na_2-EDTA, 4% PVPP (w/v), 1.5% Tween 80 (v/v)) then filtered and centrifuged to remove leaf tissue. A 60–90% $(NH_4)_2SO_4$ cut fractionated the supernatant and the resultant precipitate was re-suspended in buffer B (100 mM citrate–HCl (pH 5.5), 1 mM Na_2-EDTA, 200 mM NaCl) and desalted using a G-25 Superfine Hi-Trap column (Pharmacia, Uppsala, Sweden) in buffer B. All eluted protein was pooled to produce the partially purified enzyme extract for *R. kurrangii*, which was tested for CN to confirm that no cyanogen had been extracted in the protein preparation. It was found that the addition of partially purified protein extract (50 μL, equivalent to the quantity of enzyme contained in 70 mg of leaf tissue) from *R. kurrangii* had no significant influence on the amount of HCN liberated in 15 h from leaf tissue ($n = 5$, $P = 0.161$) indicating that the foliage contains sufficient endogenous enzyme to catabolise all cyanogenic glycosides.

Two enzymes with non-specific β-glycosidase activity – almond β-glucosidase from *Prunus amygdalus* Batsch. (E.C. 3.2.1.21, 49290; Sigma–Aldrich Pty. Ltd., Castle Hill, NSW, Australia; added at 2.7 mU ml^{-1}) and pectinase from *Rhizopus* spp. (Macerase® Pectinase, E.C. 3.2.1.14; 441201 Calbiochem®, Calbiochem-Novabiochem Corp., California, USA; added at 1.3 mU ml^{-1}) – were also tested for activity but failed to hydrolyse the cyanogenic glycosides in *R. kurrangii*, which is similar to findings for the activity of these enzymes on other cyclopentenyl cyanogenic glycosides (Seigler, 1975; Seigler et al., 1982). Therefore, the *R. kurrangii* β-glucosidase was used for all subsequent assays of leaf glycoside extracts.

3.3. Detection and quantification of cyanogenic potential

Cyanogenic potential in leaf material and tissue extracts was quantified by measuring the amount of HCN released from glycoside hydrolysis (Lambert et al., 1975; Brinker and Seigler, 1989; Gleadow et al., 1998). Evolved cyanide was trapped in a 1 M NaOH well suspended above 0.1 M

citrate buffer–HCl (pH 5.5) in a sealed glass vial. Either (1) leaf glycoside extracts and partially purified *R. kurrangii* β-glucosidase enzyme (Section 3.2), or (2) leaf tissue (10–15 mg) was added to the buffer. Leaf tissue and extracts were incubated for 15 h at 37 °C, conditions which allowed for the complete hydrolysis of cyanogens (data not shown), but precluded the possibility of microbial cyanogenesis which can occur with incubation times greater than 24 h (Saupe et al., 1982; Brinker and Seigler, 1989). Trapped cyanide was assayed using a miniaturised version of the method of Brinker and Seigler (Brinker and Seigler, 1989; Gleadow and Woodrow, 2002b) with NaCN as the standard. The optical density of the resulting colour reaction was measured at 590 nm, using an incubated photometric microplate reader (Multiskan® Ascent; Labsystem, Helsinki, Finland). The amount of cyanide detected with this method is directly proportional to the total concentration of cyanogens in the tissue (i.e. cyanogenic potential) and is referred to as the amount of 'cyanide' (mg CN g^{-1} dry wt plant tissue).

3.4. Purification of cyanogenic glycosides

Approximately 30 g of freeze–dried homogenised tissue was mixed in cold MeOH and repeatedly stirred for 24 h before being filtered (Whatman® 541 filter paper, Whatman Asia Pacific, San Centre, Singapore) and reduced in volume by rotary evaporation (45 °C). The MeOH extract was then de-fatted with petroleum spirit, before being combined with $CHCl_3$. Partitioning of the phases was achieved with H_2O and the MeOH fraction was retained and concentrated *in vacuo*. Solid-phase extraction cartridges (Maxi-Clean™ C_{18} 900 mg cartridge, Alltech Associates, Baulkham Hills, Australia) were used to further fractionate the MeOH extract across a stepped MeOH–H_2O gradient (1.5 ml min^{-1}; 0, 10, 20, 50, 100% MeOH–H_2O). Eluted fractions were concentrated *in vacuo* and tested for cyanogenic activity using the β-glucosidase partially purified from *R. kurrangii*. All cyanogens eluted in the 100% H_2O fraction.

The H_2O eluant was concentrated *in vacuo* and further purified using RP-HPLC. Initially, a preparative column (3 ml min^{-1}; 250 × 10 mm × 5 μm particle size Phenomenex Luna C18 (2) column; UV detection at 192 nm) with a mobile phase of A:B 80% MeCN + 0.1% H_3PO_4:H_2O + 0.1% H_3PO_4 (A:B 2:98 for 15 min, ramped to 95:5 over 2 min and held constant for 8 min) was used and eluted fractions were concentrated *in vacuo* and tested for cyanogenic activity. The active cyanogenic fractions were associated with a peak at RT 9.85 min, eluting at a similar rate to other unidentified, but non-cyanogenic compounds. Further purification of active fractions was achieved using an analytical column (0.8 ml min^{-1}; 250 × 4.6 mm × 5 μm particle size Phenomenex Luna C18 (2) column; UV detection at 192 nm) with a mobile phase of A:B 100% MeCN: 100% H_2O (A:B 2:98 for 10 min, ramped to 95:5 over 2 min and held constant for 8 min). A single cyanogenic peak with RT 7.3 min was collected and concentrated *in vacuo* for identification.

^1H NMR and COSY (400 MHz) spectra were recorded on a Varian Inova 400 spectrometer, in D_2O and referenced to residual ^1H signals in the deuterated solvent. ^{13}C NMR (75 MHz) spectra were recorded on a Varian Unity 400 plus spectrometer, in D_2O with no internal standard. ESIMS were acquired using a Waters 2790 separations module equipped with a Micromass ZMD mass detector. Chiroptical measurements ($[\alpha]_D$) were obtained on a Jasco Dip-1000 digital polarimeter in a 100 × 3 mm cell. All data were compared to published values for gynocardin (Hübel et al., 1981).

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References

Aikman, K., Bergman, D., Ebinger, J., Seigler, D., 1996. Variation of cyanogenesis in some plant species of the Midwestern United States. Biochem. Syst. Ecol. 24, 637–645.

Alborn, H.T., Turlings, T.C.J., Jones, T.H., Stenhagen, G., Loughrin, J.H., Tumlinson, J.H., 1997. An elicitor of plant volatiles from beet armyworm oral secretion. Science 276, 945–949.

Baldwin, I.T., Karb, M.J., Ohnmeiss, T.E., 1994. Allocation of N-15 from nitrate to nicotine – production and turnover of a damage-induced mobile defense. Ecology 75, 1703–1713.

Baldwin, I.T., Ohnmeiss, T.E., 1994. Swords into plowshares? *Nicotiana sylvestris* does not use nicotine as a nitrogen source under nitrogen-limited growth. Oecologia 98, 385–392.

Bazin, A., Blaise, S., Cartier, D., 1997. Polymorphisme de deux mécanismes de défense ches *Lotus corniculatus* L. (Fabaceae); mise en relation avec des facteurs abiotiques. Acta Botanica Gallica 144, 433–442.

Bede, J.C., Musser, R.O., Felton, G.W., Korth, K.L., 2006. Caterpillar herbivory and salivary enzymes decrease transcript levels of *Medicago truncatula* genes encoding early enzymes in terpenoid biosynthesis. Plant Mol. Biol. 60, 519–531.

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

Cabalion, P., Fournet, A., Mangeney, P., Bouquet, A., 1980. Recherches chimiques préliminaires sur leas plantes de la république populaire du Congo. Fitoterapia 51, 89–111.

Chase, M.W., Zmarzty, S., Lledo, M.D., Wurdack, K.J., Swensen, S.M., Fay, M.F., 2002. When in doubt, put it in *Flacourtiaceae*: a molecular phylogenetic analysis based on plastid*rbc*L DNA sequences. Kew Bull. 57, 141–181.

Clark, D.B., Clark, D.A., 1991. Herbivores, herbivory, and plant phenology: patterns and consequences in a tropical rain-forest cycad. In: Price, P.W., Lewinsohn, T.M., Wilson Fernandes, G.W., Benson, W.W. (Eds.), Plant–Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions. John Wiley and Sons, New York, pp. 209–225.

Coburn, R.A., Long Jr., L., 1966. Gynocardin. J. Org. Chem. 31, 4312–4315.

Coley, P.D., Aide, T.M., 1991. Comparison of herbivory and plant defenses in temperate and tropical broad-leaved forests. In: Price, P.W., Lewinsohn, T.M., Fernandes, G.W., Benson, W.W. (Eds.), Plant–Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions. John Wiley and Sons, New York, pp. 25–49.

Coley, P.D., Barone, J.A., 1996. Herbivory and plant defenses in tropical forests. Ann. Rev. Ecol. Syst. 27, 305–335.

Cooper-Driver, G., Swain, T., 1976. Cyanogenic polymorphism in bracken in relation to herbivore predation. Nature 260, 604.

Dirzo, R., Harper, J.L., 1982. Experimental studies on slug-plant interactions. III. Differences in the acceptability of individual plants of *Trifolium repens* to slugs and snails. J. Ecol. 70, 101–117.

Ellis, W.D., Keymer, R.J., Jones, D.A., 1977. The effect of temperature on the polymorphism of cyanogenesis in *Lotus corniculatus* L. Heredity 38, 339–347.

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

Gleadow, R.M., Vecchies, A.C., Woodrow, I.E., 2003. Cyanogenic *Eucalyptus nobilis* is polymorphic for both prunasin and specific β-glucosidases. Phytochemistry 63, 699–704.

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

Gleadow, R.M., Woodrow, I.E., 2002a. Constraints on effectiveness of cyanogenic glycosides in herbivore defense. J. Chem. Ecol. 28, 1297–1309.

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

Goodger, J.Q.D., Capon, R.J., Woodrow, I.E., 2002. Cyanogenic polymorphism in *Eucalyptus polyanthemos* Schauer subsp. *vestita* L. Johnson and K. Hill (Myrtaceae). Biochem. Syst. Ecol. 30, 617–630.

Greshoff, M., 1898. Tweede verslag van het onderzoek naar de plantenstoffen van Nederlandsch-Indië. Meded. Lands Plantent. XXV, 1–194.

Greshoff, M., 1906a. Sur la distribution de l'acide cyanhydrique dans le régne végétal. Bull. Sci. Pharm. 8, 589–602.

Greshoff, M., 1906b. Ueber die verteilung der blausäure in dem pflanzenreiche. Arch. Pharm. 244, 665–672.

Hayden, K.J., Parker, I.M., 2002. Plasticity in cyanogenesis of *Trifolium repens* L.: inducibility, fitness costs and variable expresson. Evol. Ecol. Res. 4, 155–168.

Hill, E.F., Henry, P.F.P., 1996. Cyanide. In: Fairbrother, A., Locke, L.N., Hoff, G.L. (Eds.), Noninfectious Diseases of Wildlife. Iowa State University Press, Ames, p. 219.

Hruska, A.J., 1988. Cyanogenic glucosides as defence compounds. A review of the evidence. J. Chem. Ecol. 14, 2213–2217.

Hübel, W., Nahrstedt, A., Wray, V., 1981. A structural investigation by ^{13}C-NMR of the cyanogenic glycosides. Arch. Pharm. 314, 609–617.

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

Jaroszewski, J.W., Andersen, J.V., Billeskov, I., 1987. Plants as a source of chiral cyclopentenes – taraktophyllin and epivolkenin, new cyclopentenoid cyanohydrin glucosides from Flacourtiaceae. Tetrahedron 43, 2349–2354.

Jaroszewski, J.W., Ekpe, P., Witt, M., 2004. Cyclopentanoid cyanohydrin glucosides and amides of *Lindackeria dentata*. Planta Med. 70, 1001–1003.

Jaroszewski, J.W., Olafsdottir, E.S., 1987. Monohydroxylated cyclopentenone cyanohydrin glucosides of Flacourtiaceae. Phytochemistry 26, 3348–3349.

Jensen, S.R., Nielsen, B.J., 1986. Gynocardin in *Ceratiosicyos laevis* (Achariaceae). Phytochemistry 25, 2349–2350.

Jones, D.A., 1988. Cyanogenesis in animal–plant interactions. In: Evered, D., Harnett, S. (Eds.), Cyanide Compounds in Biology. John Wiley & Sons, Chichester, pp. 151–170.

Jones, D.A., 1998. Why are so many food plants cyanogenic? Phytochemistry 47, 155–162.

Kakes, P., 1994. The function of cyanogenesis in Cassava. Acta Hort. 375, 79–85.

Kim, H.S., Jeffery, G.A., Panke, D., Clapp, R.C., Coburn, R.A., Long, L.J., 1970. The X-ray crystallographic determination of the structure of gynocardin. J. Chem. Soc. D: Chem. Comm. 6, 381b–382b.

Lambert, J.L., Ramasamy, J., Paukstelis, J.V., 1975. Stable reagents for the colourimetric determination of cyanide by modified König reactions. Anal. Chem. 47, 916–918.

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

Leitner, M., Boland, W., Mithöfer, A., 2005. Direct and indirect defences induced by piercing–sucking and chewing herbivores in *Medicago truncatula*. New Phytol. 167, 597–606.

McMahon, J.M., White, W.L.B., Sayre, R.T., 1995. Cyanogenesis in cassava (*Manihot esculenta Crantz*). J. Exp. Bot. 46, 731–741.

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

Miller, R.E., Jensen, R., Woodrow, I.E., 2006a. Frequency of cyanogenesis in tropical rainforests of far north Queensland, Australia. Ann. Bot. 97, 1017–1044.

Miller, R.E., McConville, M.J., Woodrow, I.E., 2006b. Cyanogenic glycosides from the rare Australian endemic rainforest tree *Clerodendrum grayi* (Lamiaceae). Phytochemistry 67, 43–51.

Møller, B.L., Seigler, D.S., 1999. Biosynthesis of cyanogenic glycosides, cyanolipids, and related compounds. In: Singh, B.K. (Ed.), Plant Amino Acids: Biochemistry and Biotechnology. Marcel Dekker, New York, pp. 563–609.

Montgomery, R.D., 1980. Cyanogens. In: Liener, I.E. (Ed.), Toxic Constituents of Plant Foodstuffs. Academic Press, London, pp. 143–160.

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

Okolie, P.N., Obasi, B.N., 1993. Diurnal variation of cyanogenic glucosides, thiocyanate and rhodanese in Cassava. Phytochemistry 33, 775–778.

Pederson, G.A., Fairbrother, T.E., Greene, S.L., 1996. Cyanogenesis and climatic relationships in U.S. white clover germplasm collection and core subset. Crop Sci. 36, 427–433.

Poulton, J.E., 1990. Cyanogenesis in plants. Plant Physiol. 94, 401–405.

Power, F.B., Barrowcliff, M., 1905. The constituents of the seeds of *Gynocardia odorata*. J. Chem. Soc. 87, 896–900.

Power, F.B., Gornall, F.H., 1904. Gynocardin, a new cyanogenetic glucoside. Proc. Chem. Soc. 19, 137.

Power, F.B., Lees, F.H., 1905. Gynocardin, a new cyanogenetic glucoside. J. Chem. Soc. 87, 349–357.

Saupe, S.G., 1981. Cyanogenic compounds and angiosperm phylogeny. In: Young, D.A., Seigler, D.S. (Eds.), Phytochemistry and Angiosperm Phylogeny. Praeger, New York, pp. 80–116.

Saupe, S.G., Seigler, D.S., Escalante-Semerena, J.C., 1982. Bacterial contamination as a cause of spurious cyanide tests. Phytochemistry 21, 2111–2112.

Schappert, P.J., Shore, J.S., 1995. Cyanogenesis in *Turnera ulmifolia* L. (Turneraceae). I. Phenotypic distribution and genetic variation for cyanogenesis on Jamaica. Heredity 74, 392–404.

Seigler, D.S., 1975. Isolation and characterization of naturally occurring cyanogenic compounds. Phytochemistry 14, 9–29.

Seigler, D.S., 1976. Plants of the northeastern United States that produce cyanogenic compounds. Econ. Bot. 30, 395–407.

Seigler, D.S., 1991. Cyanide and cyanogenic glycosides. In: Rosenthal, G.A., Berenbaum, M.R. (Eds.), Herbivores: Their Interactions with Secondary Plant Metabolites, The Chemical Participants, vol.1. Academic Press, San Diego, CA, pp. 35–77.

Seigler, D.S., Spencer, K.C., 1989. Corrected structures of passicoriacin, epicoriacin and epitetraphyllin B and their distribution in the Flacourtiaceae and Passifloraceae. Phytochemistry 28, 931–932.

Seigler, D.S., Spencer, K.C., Statler, W.S., Conn, E.E., Dunn, J.E., 1982. Tetraphyllin B and Epitetraphyllin B sulphates: novel cyanogenic glucosides from *Passiflora caerulea* and *P. alato-caerulea*. Phytochemistry 21, 2277–2285.

Solís Neffa, V.G., Faloci, M.M., Seijo, J.G., 2003. Cyanogenesis variation in the *Turnera sidoides* L. polyploid complex (Turneraceae). Bot. J. Linn. Soc. 141, 85–94.

Spencer, K.C., Seigler, D.S., 1985a. Cyanogenic glycosides and the systematics of the Flacourtiaceae. Biochem. Syst. Ecol. 13, 421–431.

Spencer, K.C., Seigler, D.S., 1985b. Cyanogenic glycosides of Malesherbia. Biochem. Syst. Ecol. 13, 23–24.

Spencer, K.C., Seigler, D.S., Fraley, S.W., 1985. Cyanogenic glycosides of the Turneraceae. Biochem. Syst. Ecol. 13, 433–435.

Thomsen, K., Brimer, L., 1997. Cyanogenic constituents in woody plants in natural lowland rainforest in Costa Rica. Bot. J. Linn. Soc. 124, 273–294.

Thorne, R.F., 2002. The classification and geography of the flowering plants: dicotyledons of the class Angiospermae (subclasses Magnoliidae, Ranunculidae, Caryophyllidae, Dilleniidae, Rosidae, Asteridae, and Lamiidae). Bot. Rev. 66, 441–647.

Till, I., 1987. Variability of expression of cyanogenesis in white clover (*Trifolium repens* L.). Heredity 59, 265–271.

Treub, M., 1907. Nouvelles recherches sur le rôle de l'acide cyanhydrique dans les plantes vertes II. Ann. Bot. Buitenzorg XXI VI, 79–106.

VanEtten, H.D., Mansfield, J.W., Bailey, J.A., Farmer, E.E., 1994. Two classes of plant antibiotics: phytoalexins versus ''phytoanticipins''. Plant Cell 6, 1191–1192.

Vetter, J., 2000. Plant cyanogenic glycosides. Toxicon 38, 11–36.

Webber, B.L., 2005. Plant–animal interactions and plant defence in the rainforest tree, *Ryparosa*. PhD Thesis. School of Botany, The University of Melbourne, Australia.

Webber, B.L., Woodrow, I.E., 2004. Cassowary frugivory, seed defleshing and fruit fly infestation influence the transition from seed to seedling in the rare Australian rainforest tree, *Ryparosa* sp. nov. 1 (Achariaceae). Funct. Plant Biol. 31, 505–516.

Webber, B.L., Woodrow, I.E., 2006. Morphological analysis and a resolution of the *Ryparosa javanica* species complex (Achariaceae) from Malesian and Australian tropical rainforests. Aust. Syst. Bot. 19, 541–569.