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Targeted metabolite profiling provides a functional link among eucalypt taxonomy, physiology and evolution

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Abstract

Adaptation to aridity is considered a major factor in the evolution of the genus *Eucalyptus*. For the first time, targeted metabolite profiling has uncovered a quantitative yet discrete phytochemical link with eucalypt taxonomy. The distribution of cyclitols among *Eucalyptus* species, and a range of other Australian tree genera, support their proposed functions in plant tissues and provide putative links with the acclimation of trees to arid environments.

Keywords: Aridity; Eucalypts; Taxonomy; Evolution; Cyclitols; D-Quercitol

1. Introduction

Changing climatic conditions over geological time scales has played a major role in the evolution of Australian plant genera, including *Eucalyptus*. At the beginning of the tertiary period, the Australian land mass began to move northwards to drier latitudes. The resultant general increase in aridity associated with this shift is widely attributed as a defining factor in the evolution of many Australian plant species. For example, even among the large group of 'multistemmed' eucalypts that are generally drought-tolerant, speciation has been attributed to between-habitat variation in water availability of a few percent (Parsons, 1969a,b). At a larger scale, there are many seemingly clear instances of speciation among Australian tree genera that are attributed to isolation of gene pools within environments that differed mainly in the availability of water (Davidson and Reid, 1980; Noble, 1989; Adams, 1996).

Consideration of plant evolution has classically encompassed morphological descriptions of species and their contribution to reproductive fitness. Morphological features remain the primary basis of taxonomy that is, in turn, frequently related to evolution ('morphometric' analysis, sensu Dunlop et al., 1998). In the last few decades, advances in genomic analysis have enabled researchers to observe patterns based upon a universal unit of inheritance and better quantify the evolutionarily crucial interaction of genes with their environment. In part, these interactions are reconciled through changes in metabolism.

The relatively new approach of 'metabolomics' (sensu (Weckwerth, 2003)) offers considerable promise to those interested in the relationships between plant function and their genotype. Analysis of primary and secondary metabolites provides a means of assessing how and to what degree a plant responds to its environment. The analysis of these compounds, collectively termed the 'metabolome' (Tweeddale et al., 1998), helps develop a process-based understanding of plant adaptation to changing environments.

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Table 1
Quantification of cyclitols among a range of subgeneric groups from the genus *Eucalyptus* (X = <1 mg g^{-1}, XX = 1–10 mg g^{-1}, XXX = >10 mg g^{-1} leaf dry weight)

Species	Subgenus	Section	Series	D-Quercitol	D-1-OMMI	D-Pinitol	Viburnitol	L-Quebrachitol	*muco*-Inositol	Leucanthemitol	*chiro*-Inositol	*scyllo*-Inositol	*myo*-Inositol	Growth habit	Annual rainfall	Climatic zone	Altitude	Soil type
Angophora florida	*Angophora*		Floribundinas									X	XX	Medium sized tree 12–20 m height	500–1000 mm	Warm humid to warm subhumid	0–1100 m	…On alluvial soils and deep sandy loams along flats and watercourses
Eucalyptus citriodora	*Corymbia*	Septentrionales	Naviculares									X	XX	Medium to tall up to 50 m height	650–1600 mm	Warm humid to warm subhumid	450–1000 m	…Tolerant of a variety of soils…podsola and residual podsols…
Eucalyptus maculate	*Corymbia*	Septentrionales	Naviculares									X	XX	Tall tree 35–45 m	750–1750 mm	Warm humid to warm subhumid	0–950 m	…Variety of soils…moist but well drained with a moderately heavy texture…
Eucalyptus alpina	*Eucalyptus*	Renantheria	Capitellatae								X	XX	XX	Small…of multistemmed habit 2–6 m[a]			1000–1250 m[a]	…Sandstone peaks of Grampians NP…[a]
Eucalyptus obliqua	*Eucalyptus*	Eucalyptus	Regnantes								X	XX	XX	Tall to very tall tree 45–90 m height	500–2400 mm	Cool-subhumid to humid	0–1000 m	…Wide range of soil with best development on good quality loams
Eucalyptus seiberi	*Eucalyptus*	Cineraceae	Consideration								X	XX	XX	25–35 m	700–1400 mm	Cool to warm, humid to subhumid	0–1100 m	…Wide range of soil…usually sandy type over a well drained clay subsoil
Eucalyptus radiate	*Eucalyptus*	Aromatica	Insulance								X	XX	XX	Medium tree 20–30 m height	650–1100 mm	Warm to cool, humid to subhumid	50–1200 m	Wide range of soil types including sands, skeletel soils and volcanic loams
Eucalyptus elata	*Eucalyptus*	Renantheria	Radiatae								X	XX	XX	Medium tree 20–30 m height	650–1700 mm	Cool to warm, humid to subhumid	0–750 m	Moderately fertile alluvial loams
Eucalyptus dives	*Eucalyptus*	Renantheria	Radiatae								X	XX	XX	medium tree 12–25 m height	600–1100 mm	Cool to warm, subhumid to humid	150–1400 m	Rather poor shallow and stony soils
Eucalyptus richolii[a]	*Eucalyptus*	Renantheria	Piperitae								X	XX	XX	Small to medium sized tree[b]		Northern tablelands of NSW[b]		
Eucalyptus saligna	*Symphyomyrtus*	Latoangulae	Transversae								tr	X	XX	Tall to very tall tree 25–55 m height	900–1800 mm	Warm humid	0–1100 m	Good quality alluvial sandy loams
Eucalyptus botryoides	*Symphyomyrtus*	Latoangulae	Annulares								X	X	XX	30–40 m height	700–1300 mm	Warm humid	0–300 m	Poors sandy soils of coastal locations
Eucalyptus longifolia	*Symphyomyrtus*	Similares								X	X	X	XX	20–35 m	800–1250 mm	Warm humid	0–300 m	Heavy soils derived from shales that do not dry out
Eucalyptus cosmophylia	*Symphyomyrtus*	Incognitae									tr	X	XX	Bushy shrub or small tree 1–10 m		Associate with *E. longifolia*		
Eucalyptus camaldulensis	*Symphyomyrtus*	Exsertaria	Rostratae								X	X	XX	Medium sized to tall tree 25–40 m height	250–1250 mm	Warm to hot, subhumid to semi-arid	20–700 m	Sandy alluvial
Eucalyptus aromaphloia	*Symphyomyrtus*	Maidenaria	Acaciformes								X	X	XX	Moderate size 12–20 m[a]		Associated with E. *viminalis* and *E. obliqua*[a]		
Eucalyptus ovata	*Symphyomyrtus*	Maidenaria	Foveolate								X	X	XX	Medium sized to tall tree up to 30 m	600–1400 mm	Warm to cool subhumid to humid	0–1100 m	Soils are generally sands and clays frequently with poor drainage
Eucalyptus camphora	*Symphyomyrtus*	Maidenaria	Foveolate								X	X	XX	Small to medium sized tree 8–20 m	600–1400 mm	Associated with *E. ovata*		
Eucalyptus globulus	*Symphyomyrtus*	Maidenaria	Globulares								X	X	XX	Tall tree 30–40 m height	700–1200 mm	Warm subhumid to humid	0–1050 m	Heavy soil with good quality loam
Eucalyptus crenulata	*Symphyomyrtus*	Maidenaria	Viminales								X	X	XX	Small tree 4–12 m[a]	600–1400 mm	Associated with *E. ovata*		
Eucalyptus viminalis	*Symphyomyrtus*	Maidenaria	Viminales								X	X	XX	Tall tree 30–50 m height	500–2000 mm	Warm to cool, subhumid to humid	0–1400 m	Moist but well drained alluvial or sandy podsolic soils with clay subsoils
Eucalyptus cladocalyx	*Symphyomyrtus*	Sejunctae		XXX			X			X	tr		X	Small to medium tree	380–650 mm	Warm subhumid	0–600 m	Mainly skeletal or podsolic frequently shallow
Eucalyptus leptophyila	*Symphyomyrtus*	Bisectae	Porantherae	XXX			X			X	X		X	Multistemmed or small tree 2–8 m[a]	250–500 mm	Associated with *E. dumosa*		
Eucalyptus calycogna	*Symphyomyrtus*	Bisectae	Heterostemones	XXX			X			X	X		X	Multistemmed or small tree 3–9 m[a]	250–500 mm	Associated with *E. dumosa*		
Eucalyptus gracillis	*Symphyomyrtus*	Bisectae	Heterostemones	XXX			X			X				Multistemmed or small tree 3–10 m[a]	251–500 mm	Associated with *E. dumosa*		
Eucalyptus astringens	*Symphyomyrtus*	Bisectae	Erectae	XXX			X			X	X		X	Medium sized tree 10–25 m	350–750 mm	Warm subhumid to semi-arid	200–350 m	…On sand or clay loams…on lateritic flats…adaptable to a wide range of soils
Eucalyptus dumosa	*Symphyomyrtus*	Dumaria	Rufispermae	XXX			X			X	X		X	Multistemmed2–10 m	250–500 mm	Warm, semi-arid	0–300 m	Common on solonized brown soils, red-brown earths, desertloams
Eucalyptus behriana	*Symphyomyrtus*	Adnataria	Buxeales	XXX			X			X	tr	tr	X	Multistemmed3–10 m[a]	250–500 mm	Associated with *E. dumosa*		
Eucalyptus largiflorens	*Symphyomyrtus*	Adnataria	Buxeales	XXX			X			X				Medium sized tree10–20 m	200–380 mm	Warm semi-arid to arid	30–300 m	Grey clay loams…self mulching clays…less commonly on fine red brown sands
Eucalyptus viridis	*Symphyomyrtus*	Adnataria	Buxeales	XXX			X			X	X	tr	X	Multistemmed or small tree to 10 m[a]	around 470 mm	Associated with *E. polybractea*		
Eucalyptus polybractea	*Symphyomyrtus*	Adnataria	Buxeales	XXX			X			X	X		X	Multistemmed 5–10 m	Around 470 mm	Warm subhumid to semi-arid	250–350 m	…Red brown loams often with quartz…
Eucalyptus frogattii	*Symphyomyrtus*	Adnataria	Buxeales	XXX			X			X				Multistemmed4–10 m[a]	Around 470 mm	Associated with *E. polybractea*		
Eucalyptus microcarpa	*Symphyomyrtus*	Adnataria	Buxeales	XXX			X			X	X		X	12–25 m height	400–700 mm	Warm subhumid to semi-arid	80–400 m	…Heavy alluvial soils clay loams better quality sandy loams
Eucalyptus polyanthemos	*Symphyomyrtus*	Adnataria	Heterophloiae	XXX			X			X	X		X	Medium sized tree 15–25 m	500–800 mm	Warm subhumid	120–800 m	…Dry stoney or gravelly soils and rather heavy poor soils of sedimentary origin
Eucalyptus leucoxylon	*Symphyomyrtus*	Adnataria	Melliodorae	XXX			X			X	X		X	10–16 m height	400–800 mm	Warm subhumid	0–800 m	…Mainly soils of…shales, granites and quartzites, basalts and limestones.
Eucalyptus sideroxylon	*Symphyomyrtus*	Adnataria	Melliodorae	XXX			X			X				Medium sized…10–25 m	450–1000 mm	Warm subhumid	0–1000 m	…Poor, shallow soils including sands, gravels ironstones and clays
Eucalyptus melliodora	*Symphyomyrtus*	Adnataria	Melliodorae	XXX			X			X				Medium sized to tall tree 15–30 m	500–900 mm	Warm subhumid	150–600 m	…Light to somewhat heavy alluvial soils, loams and sandy loams
Eucalyptus paniculata	*Symphyomyrtus*	Adnataria	Rhodoxylon	XXX			X			X	X	X	X	Medium sized tree up to 30 m	750–1500 m	Warm humid to subhumid	0–500 m	…Prefers good soils especially fertile sandy loams…ability to grow on poor soils…

Species are arranged in a conceptually phylogenetic order from top to bottom as per (Brooker, 2000). Ecological data is compiled from Boland (1992), and footnotes a and b.

[a] See Costermans (1992).

[b] See Brooker and Kleinig (1996).

For eucalypts, semi-quantitative analysis of essential oils has been the only major non-morphological approach used by researchers to link taxonomy to physiology and metabolism e.g. (Dunlop et al., 1998, 1999). This approach ('chemometric' sensu Dunlop et al., 1998) has provided a means of supporting taxonomic separation of species. Essential oil data has been used as supporting evidence in several revisions of series within the genus. Apart from the essential oils, the only classes of metabolites to have been assessed within even a moderate number of *Eucalyptus* spp. are amino compounds (Adams et al., 1995) and a range of cyanogenic glycosides (Gleadow and Woodrow, 2002; Goodger and Woodrow, 2002) and then the analysis has been conducted largely in relation to herbivory.

More recently, studies of a few eucalypts suggested clear taxonomic differences between species in their capacity to synthesise and accumulate a range of sugar alcohols or cyclitols (Adams et al., 2005). These studies have shown that some mallee eucalypts contain D-quercitol up to 30 mg g^{-1} leaf dry weight in contrast to more mesic species that contain no, or very low amounts. Significantly, cyclitols have been clearly identified as key osmotica in higher plants (e.g., Hasegawa et al., 2000) and may thus provide a putative link between adaptation of eucalypts to aridity and their taxonomy. As Bieleski and Briggs (2005) recently concluded from their study of the presence of cyclitols (polyols) in some 80 members of another southern hemisphere genus, the *Proteaceae*: "...persistence of the polyol pathways in the family is the end product of repeated challenges on the family to accommodate drought-stress conditions".

We report here an analysis of some 61 Australian tree species, collected from their native habitat, with representative samples from *Eucalyptus* (Myrtaceae), *Leptospermum* (Myrtaceae), *Melaleuca* (Myrtaceae), *Acacia* (Mimosaceae) *Callitris* (Cupressaceae) and *Heterodendrum* (Sapindaceae) for low molecular weight carbohydrate and polyol (including cyclitol) content. The genus *Eucalyptus* contains 15 subgenera and more than 700 species (Brooker, 2000), with the majority lying within *Corymbia* (~70 spp.), *Eucalyptus* (~110 spp.) and *Symphyomyrtus* (~500 spp.). *Corymbia* spp. dominate the savannas of northern Australia whilst *Eucalyptus* spp. dominate most of the coastal and upland regions of southeast and southwest Australia (Gill et al., 1985). *Symphyomyrtus* spp. are widely distributed across the continent, but are particularly common in more arid regions. We also provide ecological data compiled from various authoritative sources on the ecology of the analysed species. The species selected here for study reflect, and to a first approximation represent, the known distribution of species among eucalypt subgenera. A mixture of GC–MS and GC techniques were used for a targeted metabolic analysis along the lines suggested by Trethewey (2004) and based on our preliminary knowledge of putative taxonomic differences in cyclitol accumulation. In addition, we adopted one of the more consistent extraction techniques (methanol/chloroform/water) to maximise both reproducibility and cross-study comparability (e.g., Weckwerth, 2003).

2. Results and discussion

Apart from common plant sugars such as fructose glucose, sucrose and raffinose the major water-soluble carbohydrates identified using GC–MS in extracts of the range of studied species included: the cyclohexanepentols D-quercitol (Fig. 1a) and L-viburnitol; the cyclohexenetetrol L-leucanthemitol; the *O*-methylated cyclohexanehexols D-pinitol (Fig. 1b), L-quebrachitol (Fig. 1c) and D-1-*O*-methyl-*muco*-insitol (Fig. 1d) and the cyclohexanehexols *muco*-, *chiro*-, *myo*- and *scyllo*-inositol. Qualitatively, the relatively large abundances of some of these cyclitols were immediately obvious from chromatographic output. Using known standards, we quantified the abundance of the dominant cyclitols.

For eucalypts, all species in all subgenera contained one or more forms of inositol (Table 1). The ubiquitous *myo*-inositol was the most widespread form and, in cases with a noted absence, is assumed to be present at concentrations below detection limits.

A most striking result was the complete and consistent absence of L-leucanthemitol, L-viburnitol and most especially D-quercitol, from the subgenus *Eucalyptus*. Equally striking was the abundance of these compounds in some sections of the *Symphyomyrtus* but absence in others. For example, D-quercitol was present in high concentrations (up to 40 mg g^{-1} dry weight) in *Adnataria*, *Dumaria*, *Bisectae* and *Sejunctae* but absent in species from the other five represented sections of this subgenus (Table 1). The relative abundances of cyclitols in the two representatives of the subgenus *Corymbia* most resembled patterns established for *Eucalyptus* although *chiro*-inositol was absent (see Table 2).

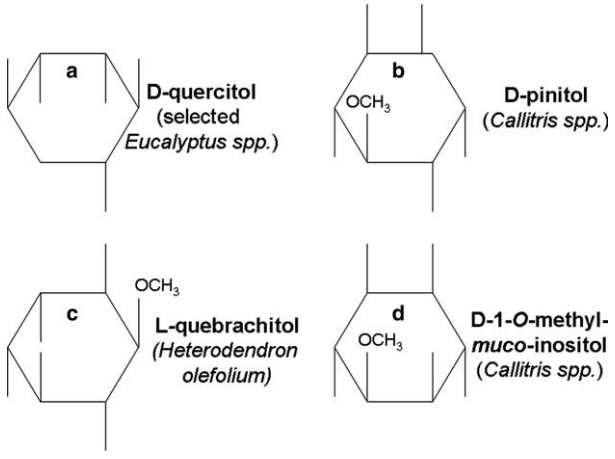

Fig. 1. Cyclitols isolated in major concentrations from *Eucalyptus* (a), *Acacia* (b), *Heterodenrum* (c) and *Callitris* (b and d) originating from contrasting rainfall regions of Australia. Stereoisomeric conformations are adopted based upon previous suggestions among related tree species.

Table 2
Quantification of cyclitols among a selection of Australian tree species from low rainfall regions (X = <1 mg g^{-1}, XX = 1–10 mg g^{-1}, XXX = >10 mg g^{-1} leaf dry weight)

Species	Family	D1-OMMI	D-Pinitol	L-Quebrachitol	*muco*-Inositol	*chiro*-Inositol	*scyllo*-Inositol	*myo*-Inositol	Growth habit	Ecological distribution	Soil type
Acacia baileyana	Mimosaceae		XXX		X	X	X	X	Shrub or tree to 10 m	Open woodland stoney undulating country	On granites and porphyries
Acacia elata	Mimosaceae		XXX			X		X	Tree 7–20 m	Coast and tablelands	Deep sandy soils
Acacia implexa	Mimosaceae		XXX			X	X	X	Tree 3–15 m	Variety of growing conditions	Shallow soils on hills
Acacia mearnsii	Mimosaceae		XXX			X		X	Erect tree 10–16 m	Open forest,woodland or tussock grassland in gullies or on hillsides	Sandy or gravelly clay soils
Acacia melanoxylon	Mimosaceae		XXX			X		X	Tree 6–45 m	Wet sclerophyll forests and cooler rainforest	Diversity however prefers fertile gullies
Acacia pycnantha	Mimosaceae		XXX			X		X	Shrub or tree 3–8 m	Widespread inland,open scrub and health	Sand or loan
Acacia williamsonii	Mimosaceae		XXX			X		X	Bushy shrub up to 2 m	Open forest and open scrub	Stoney gravel or clay loam
Leptospermum juniperinum	Myrtaceae							X	Shrubs or small trees, 1–4 m[b]	Lowland heaths scrubs and forests[b]	On poorly drained soils[b]
Leptospermum laevigatum	Myrtaceae							X	Shrubs to small tree, 2–8 m[b]	Coastal scrub[b]	Coastal sands[b]
Leptospermum myrsinoides	Myrtaceae							X	Wiry shrub 0.5–2.5 m[b]	Heath and heath understories[b]	On poor, sandy soils[b]
Melaleuca halmaturorum	Myrtaceae							X	Shrub or small tree, 3–8 m[b]	Coastal and inland salt lakes[b]	Brackish or muddy saline sites[b]
Melaleuca lanceolata	Myrtaceae							X	Bushy shrub or small tree, 1–8 m[b]	Closed, coastal scrubs[b]	Sandy, calcareous soils[b]
Melaleuca uncinata	Myrtaceae							X	Shrub, sometimes tall and multistemmed 1–5 m[b]	Common in scrublands[b]	Sands and sandy loams[b]
Heterodendrum oleifolium	Sapindaceae			XXX	X	X		X	Small tree 3–6 m[b]	Widespread on inland plains[b]	
Callitris canescens	Cupressaceae	XXX	XXX		X	X		X	Small tree or shrub to 6 m		Variety of soils loamy and calcareous
Callitris columellaris	Cupressaceae	XXX	XXX		X	X		X	Tree to 30 m	Coastal	Deep sands
Callitris drummondii	Cupressaceae	XXX	XXX		X	X		X	Shrub to 10 m	Coastal	Sand over laterite or subcoastal dunes
Callitris endicheri	Cupressaceae	XXX	XXX		X	X		X	Tree to 10 m	Drier sites and rocky outcrops[b]	Shallow soils and rocky sites
Callitris glaucophylla	Cupressaceae	XXX	XXX		X	X		X	Tree to 20 m	Widespread across continent	Various substrates, deep sand
Callitris macleayana	Cupressaceae	XXX	XXX		X	X		X	Tree to 30 m	Subcoastal rainforest and rainforest margins	Poor soils…sandy loams to sandy clay loams[a]
Callitris oblonga	Cupressaceae	XXX	XXX		X	X		X	Tree or shrub to 5 m	Low wet sites	Sand
Callitris presii	Cupressaceae	XXX	XXX		X	X		X	Tree or shrub to 20 m	Coastal	Calcareous sand deposits
Callitris rhomboidea	Cupressaceae	XXX	XXX		X	X		X	Tree to 15 m	Coastal	Variety of substrates

Ecological data is compiled from Boland (1992), and from footnotes a and b.

[a] See Costermans (1992).

[b] See Brooker and Kleinig (1996).

This is the first time, to our knowledge, that a chemical or biochemical analysis has been able to simply and clearly identify discrete yet taxonomically related groups of eucalypts on a quantitative basis. Past research using essential oils has relied on semi-quantitative analysis of many separate compounds and then has only been able to separate groups of eucalypts on the basis of broad patterns in the collective presence/absence and abundance of the many constituent oils (Li et al., 1995, 1996). In contrast, our evidence shows that the species from the subgenus *Eucalyptus Monocalyptus* (Pryor and Johnson, 1971) and within sections *Maidenaria*, *Exsertaria*, *Incognitae*, *Similares* and *Latoangulatae* differ definitively from those in sections *Adnataria*, *Dumaria* and *Bisectaria* in the presence/absence and abundance of a single compound – D-quercitol. Likewise, within the *Symphyomyrtus*, species in the section *Maidenaria* differ definitively in the abundance of cyclitols from those in the sections *Bisectae, Dumaria, Adnataria* and *Sejunctae*. These results, whilst still representing less than 10% of the total number of eucalypt species, offer a putative but highly significant link among: (1) the evolutionary response of Australian native trees, especially eucalypts, to aridity; (2) the role of metabolites such as D-quercitol as adaptations to aridity; and, (3) taxonomy.

Alternative attempts to delineate taxonomic groups of the *Eucalyptus* genus based upon chemical and biochemical physiology have encompassed initial growth rates (Duff et al., 1983), foliar nutrient concentrations (Lambert and Turner, 1983), volatile leaf oils (Li et al., 1995, 1996), respiratory metabolism (Anekonda et al., 1999) and combinations of these parameters (Noble, 1989). Despite extensive efforts (see, for example, the more than 20 papers on links between essential oils and eucalypt taxonomy by Dunlop et al. (1999), Li et al. (1995, 1996) and co-workers), these studies have failed to identify chemical or biochemical characteristics of eucalypt tissue that adequately explain species adaptation to stressful environments. While the distribution of *Eucalyptus* spp. depends on a variety of factors, the availability of water remains the most likely general predictor for species and specific adaptive traits (e.g., Adams, 1996).

In addition to increased aridity, the genus *Eucalyptus* has co-evolved with a general decrease in soil fertility due to leaching and laterisation and, in places, increased salinity (Eldridge et al., 1993). The diversity of *Eucalyptus* is partly maintained by restrictions on gene exchange caused by geographic isolation. Intense debate followed the latest classification of the eucalypt genus, particularly with regard to eucalypt phylogeny. Brooker (2000), based largely on morphological characters, presents a 'conceptually phylogenetic'' classification of eucalypt species recognising seven polyphyletic (including the largest subgenera *Angophora*, *Corymbia* and *Eucalyptus*) and five monophyletic subgenera. On the other hand, molecular analysis (Udovicic et al., 1995) based upon nuclear DNA (5s rDNA, spacer region ITS1, ITS2) and chloroplastic DNA (RFLPs, *trn*L intron, *trn*L-F spacer and *psb*A-*trn*H spacer) sequence homologies support the 'monophyly of eucalypt clades' (Ladiges and Udovicic, 2000) specifically those of *Angophora, Corymbia* and *Eucalyptus*. Our chemometric/metabolite analysis neither accepts nor rejects either classification but strongly supports the existence of monophyletic groups of eucalypts. Our data agrees with the monophyletic clade proposed by Ladiges (1997) within the subgenus *Symphyomyrtus*. Two mechanistic explanations of the observed patterns are worth mentioning. First, the accumulation of specific cyclitols in arid-adapted but geographically isolated species may be a result of common inheritance from a distant ancestor that gave rise to a monophyletic clade with the capacity to radiate into more arid regions of the continent. Secondly, a viable alternative is that the adaptation (quercitol synthesis) arose independently a number of times. Bieleski and Briggs (2005) pondered the question as to why cyclitols, that are present at rather large concentrations (second only to cellulose in some Proteaceae and, as shown here, in some eucalypts), ''have not been discarded during evolution''. We suggest the data presented here lend strong support to suggestions made by Bieleski and Briggs (2005) and Adams et al. (2005) about the importance of cyclitols to the ability of native trees to cope with drought and salinity. The data also support the contention that the presence and abundance of cyclitols in some eucalypt families and sections suggests repeated periods of aridity had much to do with their evolution.

Of the other genera examined, the myrtaceous *Melaleuca* and *Leptospermum*, contained only trace concentrations of one cyclitol – *myo*-inositol. In contrast, *Acacia* species accumulated D-pinitol up to 25 mg g^{-1} dry weight along with detectable concentrations of *chiro*-inositol. Equally, all *Callitris* species contained the *O*-methylated cyclitols D-pinitol (20 mg g^{-1}dw) and D-1-*O*-methyl-*muco*-inositol (15 mg g^{-1} dry weight). Finally, *Heterodendrum oelifolium* (*Sapindaceae*) contained significant concentrations of the methylated cyclitol L-quebrachitol at concentrations up to 35 mg g^{-1} dry weight and *chiro*-, and *myo*-inositol at trace concentrations. In each of the seven species of *Acacia*, nine species of *Callitris* and *Heterodendrum oleifolium* the cyclitols constituted the major portion of extracted water-soluble, carbon based osmolytes.

Cyclitol accumulation, and the general abundance in arid environments of species of *Acacia*, *Callitris* and *Heterodendrum*, further support the putative link between cyclitol accumulation and evolutionary adaptation to aridity in Australian tree genera. Unlike D-quercitol, cyclitols found in these species (D-pinitol, D-1-*O*-methyl-*muco*-inositol and L-quebrachitol) are methylated. Methylation of cyclitols may further increase osmoprotectant capacity by (a) increasing demand for photorespiration products or (b) increasing hydrophobicity and improving plant ability to stabilise tertiary protein structures (for review see Hare and Cress, 1997). As noted above, concentrations of cyclitols recorded here have a large influence over cellular osmolarity. Further, D-pinitol and related cyclitols are inert (Paul and Cockburn, 1989; Sheveleva et al., 1997) and do

not fluctuate greatly in the short-term and the primary role of cyclitols in these *Acacia*, *Callitris* and *Heterodendrum* species seems again likely to be that of a stable osmolyte.

Cyclitols have several other demonstrated roles in higher plants apart from being stable osmotica (Nguyen and Lamant, 1988; Paul and Cockburn, 1989; Vernon et al., 1993; Popp et al., 1997; Sheveleva et al., 1997; Vera-Estrella et al., 1999). Cyclitols function in the sequestration of excess photochemical energy, in the stabilisation of cellular components (Nguyen and Lamant, 1988; Adams et al., 1998; Klages et al., 1999) and in signalling of stress (Koch, 1996; Klages et al., 1999; Nelson et al., 1999). Certainly, the concentrations of cyclitols found in eucalypts from arid environments are sufficient to account for a significant proportion of osmotic potential recorded to date in studies of the genus (e.g., Clayton-Greene, 1983; Myers and Neales, 1986; White et al., 2000).

Unlike previous chemo-taxonomic studies of eucalypts, here we have shown a clear distinction between xeric and mesic eucalypts on the basis of cyclitol concentrations in leaf tissues. The clarity of this distinction is particularly striking given the background of temporal and environmental variation in metabolic processes.

All of the cyclitols identified here have been previously identified in trees. (Plouvier, 1963) first isolated D-quercitol from several species including *E. obliqua*. More recently, Popp et al. (1997) noted accumulation of D-quercitol in *Quercus robur* and quebrachitol in *Acer pseudoplatinus* up to 33 mg g^{-1} dry weight. Crowe et al. (1984) found that D-pinitol accumulated to up to 30 mg g^{-1} dry weight in needles of *Pinus sylvestris* and L-quebrachitol has been detected in the family *Sapindaceae* as well as *Hippocastanaceae*, *Myrtaceae*, *Tiliaceae*, *Proteaceae* and *Rutaceae* (Plouvier, 1963; Kindl and Hoffmann-Ostenhof, 1966). There are suggestions in our data that the distribution of L-quebrachitol within *Eucalyptus* provides another link to evolution in response to aridity.

Plouviers work suggested common patterns of cyclitol accumulation in many higher plant species and genera. These are likely related to the presence/absence of specific enzyme systems. The concurrent accumulation of D-quercitol, viburnitol and leucanthemitol is thought to result from the direct cyclisation of glucose-6-phosphate. With one known exception in a zannichelliacean seagrass (Drew, 1984), the remainder of the thus far identified plant cyclitols arise via the cyclisation of glucose-6-phosphate to *myo*-inositol – a process ubiquitous to plant tissues. Present knowledge of cyclitol biosynthetic pathways in higher plants are largely derived from radioactive labelling studies (Kindl, 1969; Drew, 1984) and have been comprehensively reviewed (Anderson and Wolter, 1966; Loewus and Dickinson, 1982; Drew, 1984; Popp et al., 1997). Some cyclitols can be synthesised via multiple pathways (e.g., L-quebrachitol in *Acer pseudoplatanus* and *Artemisia vulgaris* (Schilling et al., 1972)) and the biosynthetic pathways have been suggested as a basis for taxonomic division (e.g., *Artemisia vulgaris* and *Artemisia dranunculus* Drew, 1984).

Irrespective of the mechanisms by which cyclitols confer an adaptation to aridity in *Eucalyptus*, restricted metabolic profiling has uncovered a putative link between the acclimation of trees to arid environments and plant biochemistry. Elucidation of the physiological roles of cyclitols may place them alongside leaf thickness and the regulation of stomatal aperture as congruent responses of eucalypts to arid environments. In this investigation, we adopted techniques that produce accurate, repeatable measurements. This is particularly important in cross-study comparisons of plant metabolites, given the time- and environment-dependant variability of metabolic processes. Further studies of Australian tree genera using similar approaches will help test some of the hypotheses that have been generated as a result of the present work.

3. Experimental

We selected and sampled a range of Australian tree species in their natural habitats. In the case of *Eucalyptus*, we selected species from the differing taxonomic groups defined by Brooker (2000) hence we sampled seven species from the subgenus *Eucalyptus* (or *Monocalyptus*), two *Corymbia* spp. one *Angophora* spp. and 28 *Symphyomyrtus* spp. We also sampled four *Leptospermum* spp., two *Melaleuca* spp. both of which also belong to the *Myrtaceae* family. In addition, we sampled seven *Acacia* spp. and two other species of two genera common to arid areas – *Callitris* and *Heterodendrum*. At least five replicate trees from separate sites were sampled for all sampled species.

3.1. Sample collection

Samples consisted of the first fully expanded (FFE) leaf on a terminal branchlet. Due to the intermittent growth spurts that are characteristic of many Australian tree species, occasionally the samples collected were of growth up to 3 months old. Due to the diversity of growth habit in Australian tree species, the location within the canopy of the collected foliage varied considerably. Similarly, samples were collected at different times of the year although predominantly during the spring.

Samples were placed in 15 ml Falconer tubes and transferred immediately to liquid nitrogen. The date and location of each sample was recorded. Based upon methods described by Popp et al. (1996) samples were microwaved (30 s, 650 W conventional microwave oven) and then oven dried at 85 °C. Samples were then ground to a powder.

3.2. Extraction procedure

Approximately 40 mg of dried leaf material was weighed into a 2 ml screw-cap micro-tube. One milliliter of methanol/chloroform/water (12:5:3) was added and incubated

at 80 °C for 30 min. The water fraction of the extraction mixture consisted of a 0.1% solution of internal standard. The internal standard used was 0.1% β-glucopysranosyl for GC–MS analysis and a mixture of 0.1% penta-erythritol and 0.1% xylitol for GC analysis.

After cooling, samples were centrifuged (11,400*g*) and 800 μl of the supernatant removed and placed into a clean 2 ml round bottomed micro-tube. A further 200 μl chloroform and 500 μl of deionised water was added to facilitate the separation of phases. Samples were centrifuged and left to stand for 15 min to allow phase separation.

Samples were then centrifuged at 11,400*g* for 3 min and 700 μl of the upper phase (the water–methanol soluble fraction) transferred to a clean 1.5 ml micro-tube to which 300 μl of mixed bed resin (MBR) had already been added. MBR consisted of 1 part Dowex 1 × 8 (50–100 mesh anion exchange resin in the formate form) and 1 part Dowex 50 W × 8 (50–100 mesh cation exchange H+ form). Samples were agitated for a period of 2 h at room temperature. Following pulse centrifugation, 400 μl of the supernatant was transferred to a clean eppendorf tube and stored at −80 °C.

Ion exchange was intensified for GC–MS analysis by the use of two vertical columns packed with either of the resins described above. Samples were suspended in approximately 50 ml of deionised water and passed through the columns at a rate of approximately 15 ml per minute. Due to the subsequent increase in volume, samples were dried and re-suspended in 800 μl of water. The resultant neutral fraction was then stored at −86 °C.

3.3. GC and GC–MS analysis

To facilitate phase transition, samples were derivatised using a 1:10 mixture of trimethylchlorosilane (TMCS) and bis-trimethylsilyl-triflouroacetamide (BSTFA). Sixty microliters of sample solution was dried and resuspended in 400 μl anhydrous pyridine to which 50 μl of the TMCS/BSTFA (Pierce Chemicals) solution was added. Samples were incubated for 1 h at 75 °C and analysed by gas chromatography within 24 h. To facilitate full peak separation (hence identification) subsamples were taken from original extracts and oxime derivatised with hydroxylamine hydrochloride/anhydrous pyridine solution (0.25%). Samples were incubated at 75 °C for 1 h then derivatised with 50 μl of TMCS/BSTFA as outlined above. Detection limits for GC analysis were consistently below 40 ng which equated to 95 μg in the original extract solution.

GC–MS analysis was performed using a Varian Saturn 3 GC–MS using a DB1 column (0.2 mm id, 50 m, 0.33 μm film thickness). Injection was made with an injection port temperature ramping from 85 to 325 °C in 5 min. Initial oven temperature was at 130 °C for 1.5 min then ramping to 190 °C at 15 °C/min then to 325 °C at 6 °C/min and maintained for 2 min. MS spectra were compared to known standards. Non-methylated cyclitol standards were made from commercially available sources (Sigma). Standards for methylated cyclitols and for D-quercitol, viburnitol and leucanthemitol were isolated and purified as previously described by Wanek and Richter (1995) and Peterbauer et al. (1998). GC analysis was performed using a Shimadzu 17A Series Gas Chromatograph with a DB1 column (0.25 mm id, 30 m, 0.25 μm film thickness). Split injection was made at 300 °C with an initial oven temperature program of 60 °C for 2 min ramping to 300 °C at 10 °C/min and maintained for 10 min. Column flow rate was maintained at 1.5 ml per minute. Peak integration was made using Class VP analysis software.

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