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ANTHOCYANINS OF THE PODOCARPACEAE

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Abstract—In the Podocarpaceae anthocyanins occur in some vegetative organs, and in seed-bearing structures where they have a comparable role to angiosperm fruit pigments. Pelargonidin and peonidin are recorded for the first time from gymosperms and several species contain pigments with an unusual substituent that confers highly characteristic chromatographic behaviour. Distribution of anthocyanins does not as yet aid substantially in the taxonomy of the principal genera.

INTRODUCTION

THE PODOCARPACEAE (order: Coniferales) is a large family of gymnosperms found mainly in southern temperate regions and mountains of the Indo-Pacific tropics. Although most species are found in the latter habitats, this probably reflects the high degree of endemism in such areas (where many Podocarp species are of very restricted distribution) and it is in cooler regions such as New Zealand where the family may occupy a dominant role in the flora and be economically important. The Podocarpaceae is the principal southern family of conifers, the order being strongly divided on a geographical basis.[1] The present distribution of conifers is of considerable evolutionary interest, and provides an additional reason for investigating the phylogeny and taxonomy of the Podocarpaceae. Within the Coniferales this group forms a distinct family, based particularly on the morphology of the reproductive structures. The most conspicuous feature (quite unlike northern conifers) is the reduction of the female cone to a structure in which one or two ovules develop, these being often mounted on a fleshy aril-like receptacle or rendered attractive for animal dispersal by development of the surrounding epimatium.

Three principal genera are usually recognised in the Podocarpaceae. *Phyllocladus* is clearly set apart by the morphology of the photosynthetic organs (phylloclades rather than leaves), but *Dacrydium* and *Podocarpus* show several overlapping characteristics, especially in the section *Dacrycarpus* of *Podocarpus*. As there are *ca.* 115 species of *Podocarpus* there have been attempts to subdivide this genus.[2,3] The most recent revision by de Laubenfels[4] creates five new genera from the previous eight sections of *Podocarpus*, and also separates three species of *Dacrydium* with generic status. Pending general acceptance of this revision and for convenience in referring to earlier work, the previous names will be used in this paper.

Chemical investigation of the Podocarpaceae was for some time concerned mainly with the diterpene hydrocarbons from leaf oils, and took place at a few centres suitably located within the range of this family, particularly that under Briggs at the University of Auckland.

[1] H. LI, *Evolution* **7**, 245 (1953).
[2] J. T. BUCHHOLZ and N. E. GRAY, *J. Arn. Arb.* **29**, 49 (1948); **29**, 64 (1948); **32**, 82 (1951); **32**, 93 (1951).
[3] N. E. GRAY, *J. Arn. Arb.* **33**, 67 (1953).
[4] D. DE LAUBENFELS, *J. Arn. Arb.* **50**, 274 (1969).

Since then heartwood constituents of most New Zealand species have been studied intensively, and chemical investigation of species in other areas has increased. Compounds studied include biflavonyls,[5] oxygenated diterpenes,[6] dimeric diterpenoids,[7] a *p*-diphenol oxidase,[8] and most recently ecdysones.[9] Attempts at correlating chemistry and taxonomy in the Podocarpaceae have been limited by the small number of species that have been studied intensively. Rapid survey methods using gas chromatography have indicated that distribution of the diterpene hydrocarbons does not correlate with taxonomy,[10] but results with alkanes of leaf wax have been more positive.[11] Chemotaxonomy of 14 New Zealand species has been reviewed by Cambie and Weston.[12] In *Podocarpus*, the 4 species of section Podocarpus have a similar pattern of heartwood phenolics, different from the rest of the genus. *Dacrydium* is distinguished by the presence of bicyclic non-phenolic diterpenoids instead of the phenolic tricyclic compounds characteristic of *Podocarpus*, and anomalous results from *D. cupressinium* suggest that distribution of heartwood constituents may follow Florin's grouping.[13]

Although some Podocarpaceae have conspicuously coloured seed-bearing structures,* there appears to have been no previous attempt to study the pigments. Anthocyanins, the typical red to blue flower and fruit pigments of the angiosperms, are of rather rare occurrence in gymnosperms. Until recently there was some doubt as to whether they occurred at all, because earlier reports had been based on colour reactions prior to the advent of chromatographic methods.[14] Definitive studies by Santamour have shown the inconspicuous presence of anthocyanins in conelets of many species of Pinaceae,[15] of *Chamaecyparis* (Cupressaceae)[16] and transiently in the spring foliage of certain *Picea* hybrids.[17] Free anthocyanidins have also been found in zones of wound tissue in some North American conifers.[18] Following the observation of strong anthocyanin pigmentation in young leaves of *P. polystachyus*[19] these pigments have been investigated in other accessible members of the Podocarpaceae.

RESULTS AND DISCUSSION

Anthocyanins were identified in 21 species and the results are summarized in Table 1. Most species of the Malayan Peninsula, Borneo and New Zealand were examined, together with some others in cultivation, but not all could be obtained in 'fruit'. Leaf anthocyanin is a transient feature in the tropics and negative results have not been recorded because they may have been due to lack of continuous observation.

* Hereafter referred to as 'fruit'. Although technically incorrect, this term is convenient and serves to emphasize that often there is considerable resemblance to angiosperm fruit.

[5] R. C. CAMBIE and M. A. JAMES, *N.Z. J. Sci.* **10,** 918 (1967).
[6] C. W. BRANDT and B. R. THOMAS, *Nature, Lond.* **170,** 1018 (1952).
[7] S. M. BOCKS, R. C. CAMBIE and T. TAKAHASHI, *Tetrahedron* **19,** 1109 (1963).
[8] R. C. CAMBIE and S. M. BOCKS, *Phytochem.* **5,** 391 (1966).
[9] C. E. BERKOFF, *Quart. Rev.* **23,** 372 (1969).
[10] R. T. APLIN, R. C. CAMBIE and P. S. RUTLEDGE, *Phytochem.* **2,** 205 (1963).
[11] J. BORGES DEL CASTILLO, C. J. W. BROOKS, R. C. CAMBIE, G. EGLINTON, R. J. HAMILTON and W. PELLITT, *Phytochem.* **6,** 391 (1967).
[12] R. C. CAMBIE and R. J. WESTON, *Chemistry in New Zealand* **32,** 105 (1968).
[13] R. FLORIN, Sv. Vet-akad. Hand. III **10,** 1 (1931); as cited e.g. by DE LAUBENFELS, *loc. cit.*
[14] J. B. HARBORNE in *Comparative Phytochemistry* (edited by T. SWAIN), p. 274, Academic Press, London (1966).
[15] F. S. SANTAMOUR, *For. Sci.* **12,** 429 (1966).
[16] F. S. SANTAMOUR, *Morris Arb. Bull.* **17,** 50 (1960).
[17] F. S. SANTAMOUR, *Morris Arb. Bull.* **18,** 41 (1967).
[18] D. B. MULLICK, *Can. J. Botany* (in press).
[19] J. B. LOWRY, *Phytochem.* **7,** 1897 (1968).

TABLE 1. ANTHOCYANINS OF THE PODOCARPACEAE

Species	Collection localities	Location of pigment	Anthocyanins present*
Phyllocladus			
P. alpinus Hook. f.	New Zealand	seed coat	Cy-3-glc
P. hypophyllus Hook. f.	Borneo	seed, receptacle, young leaf	Cy-3-glc
P. trichomanoides D. Don	New Zealand	seed coat	Cy-3-glc
Dacrydium			
Group A			
D. falciforme (Parl.) Pilger	Malaya, Sabah	receptacle, young leaf	Cy-3-glc^1, Podocarpin A^3 Cy-3-glc^2, Podocarpin A^3
Group B			
D. beccarii Parl.	Malaya	receptacle	Podocarpin A^2
D. beccarii var *subelatum*	Sarawak	receptacle	Cy-3-glc^3, Podocarpin A^1
D. cupressinium Lamb.	New Zealand	receptacle	Podocarpin A
D. gibbsiae Stapf	Sabah	receptacle	Cy-3-glc^1, Podocarpin A^2
D. xanthandrum Pilger	Sabah	seed	Pg-3-glc^2, Podocarpin B^2, Cy-3-glc^3
Group C			
D. bidwillii Hook. f.	New Zealand	seed	Cy-3-glc
D. laxifolium Hook. f.	New Zealand	seed	Cy-3-glc^3
		receptacle	Cy-3-glc^2, Pn-3-glc^2
		stem	Cy-3-glc^3
Podocarpus section *Dacrycarpus*			
P. dacrydioides A. Rich	New Zealand	seed	Pg-3-glc^1, Cy-3-glc^3, Dp-3-glc^1
		receptacle	Pg-3-glc^2, Podocarpin B^3, Cy-3-glc^2
P. imbricatus Blume	Malaya	receptacle	Podocarpin B
P. imbricatus var *kinabaluense* Wasscher	Sabah	seed	Cy-3-glc^3, Dp-3-glc^1
		young shoots	Pg-3-glc^1, Cy-3-glc^3, Pn-3-glc^3, Dp-3-glc^1
section *Stachycarpus*			
P. ferrugineus D. Don	New Zealand	epimatium	Cy-3-glc
section *Eupodocarpus*			
P. brevifolius Foxworthy	Sabah	receptacle	Pg-3-glc^1, Pn-3-glc^1, Cy-3-glc^3, Pg-3,5-diglc^1, Pn-3,5-diglc^1, Dp-3,5-diglc^1
		young leaf	Cy-3-glc^3, Dp-3-glc^1
P. hallii Kirk	New Zealand	receptacle	Podocarpin A^3, Cy-3-glc^1
P. neriifolius D. Don	Malaya	receptacle	Cy-3-glc^2, Dp-3-glc^2
		young leaf	Cy-3-glc^3, Dp-3-glc^1
P. nivalis Hook. f.	New Zealand	receptacle	Podocarpin A
P. polystachyus R. Br.	Malaya	receptacle	Cy-3-glc^3, Dp-3-glc^1
		young leaf	Dp-3-glc^1, Dp-3,5-diglc^2
P. totara D. Don	New Zealand	receptacle	Podocarpin A

* Where more than one present an estimate of the relative amounts is indicated by use of superscript number.

Abbreviations: Pg, pelargonidin; Cy, cyanidin; Pn, peonidin; Dp, delphinidin; glc, glucose.

Anthocyanins Present

(a) *Known compounds.* Pigments occurred as the 3-glucosides, rarely as 3,5-diglucosides, and often as a new type of acylated glucoside (see below). The aglycones present were pelargonidin, cyanidin, peonidin and delphinidin. Cyanidin and delphinidin were found by Santamour in the Pinaceae. Outside the angiosperms, pelargonidin glycosides were pre-

viously only known in a single fern species; other ferns have 3-deoxyanthocyanins.[20] The occurrence of a methylated anthocyanin, peonidin, outside the angiosperms also represents a first record and is quite unexpected. This may be of phylogenetic interest, as production of this compound could be regarded as an 'advanced' character.[21]

(b) *New compounds.* During this study a number of extracts yielded pigments that were immediately recognisable as novel compounds because of their high chromatographic mobility. Until identification is complete they are referred to here as podocarpin A and B.

Podocarpin A, first isolated from young leaves of *Dacrydium falciforme*, yielded cyanidin and glucose on hydrolysis. Cyanidin 3-glucoside was the only product of partial hydrolysis carried out under a variety of conditions. This showed that podocarpin A was a substituted cyanidin 3-glucoside but comparative chromatographic data show that it must constitute an anthocyanin of a new class. Thus, the parent compound has higher R_f than the corresponding 3-glucoside in *all* solvent systems commonly used in chromatography of anthocyanins (Table 2). Additional glycosylation has the effect of increasing mobility in aqueous

TABLE 2. COMPARATIVE R_fS FOR NEW PODOCARPACEAE PIGMENTS AND THE CORRESPONDING 3-GLUCOSIDES

Solvent	Cyanidin-3-glucoside	Podocarpin A	Pelargonidin-3-glucoside	Podocarpin B
(a) conc. HCl–H_2O (3:97)	09	51	13	61
(b) H_2O–HOAc–HCl (82:15:3)	28	65	32	69
(c) *n*-BuOH–HOAc–H_2O(4:1:5)	30	43	36	55
(d) *n*-BuOH–2 N HCl (1:1)	25	52	32	61

solvents and decreasing it in the butanolic solvents, relative to the 3-glucoside. The only other easily hydrolysed substituents known in anthocyanins are caffeic, ferulic or *p*-coumaric acid, and these have the reverse effect on chromatographic mobility from that of additional glycosylation. Presence of aromatic acids was also ruled out by the UV spectrum, which was identical to the 3-glucoside, and by resistance to basic hydrolysis at room temp. (under which conditions loss of aromatic acids occurs rapidly).

Podocarpin B, first isolated from fruit of *Podocarpus dacrydioides*, gave pelargonidin on hydrolysis but in all other respects behaved similarly to podocarpin A.

In examining the products of total hydrolysis, in addition to the anthocyanidin and glucose, there was detected a polyhydroxy compound that appeared from chromatographic behaviour to be a low molecular weight aliphatic carboxylic acid, in equilibrium with the corresponding lactone. Unfortunately insufficient of either pigment could be isolated for this compound to be separately identified. Although widespread in the Podocarpaceae (Table 1), there was no reliable and large-scale source of these pigments, and successive chromatographic purifications resulted in considerable losses because of their lability.

Unequivocal results were obtained from the IR spectrum of podocarpin B, which showed strong absorption at 1720 cm^{-1}, completely absent from the spectrum of pelargonidin 3-glucoside which was isolated and measured under the same conditions. These results indicate that Podocarpin A and B are 3-(acylglucosides), in which the acyl group is a simple

[20] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 115, Academic Press, London (1968).
[21] J. B. HARBORNE, *loc. cit.*, p. 311.

polyhydroxy carboxylic acid. Deacylation occurs under very mildly acidic conditions and may be due to intramolecular lactonization of the substituent. This would explain why samples of Podocarpin A and B gave the 3-glucosides on storing as dried films under anhydrous conditions.

While it is regrettable that the complete identity of these compounds has not been established there is no doubt about their chemotaxonomic value for they must be rare in nature, if not unique to the Podocarpaceae.

Location of Anthocyanins

(a) *Young leaves.* This mode of occurrence was found only for the tropical species with rather large leaves, and is in accord both with the rain forest and montane habitats. Many tropical trees have 'flushes' of leaf growth, during which the young leaves are often coloured pink or blue.[22] Production of anthocyanin under these conditions is probably adventitious and the result of physiological factors, such as build-up of free sugar. In lowland species the coloration is seen for only a few days and may occur two or three times in 1 yr. It seems probable that large-leaved tropical *Podocarpus* species such as *P. rumphii*, *P. palembanica* and *P. usambarensis* which so far have given negative results have simply not been observed at the right time. In microphyllous species with reduced scale or needle leaves no anthocyanin could be detected. The pigments, if present, presumably would be located within the foliage bud and be less conspicuous than in those where the lamina is large.

In montane species the coloration is more lasting, but is again probably the result of response to physiological conditions, because many montane species of angiosperms also show this effect.[23]

(a) *Fruit.* Although some of the Podocarpaceae have inconspicuous seeds in most there are definite adaptations to make the 'fruit' attractive to birds or mammals, either by development of epimatium so that the seed is surrounded by a sweet fleshy layer, or by development of the supporting stem into a succulent receptacle. In some *Podocarpus* species this may resemble a small plum. As well as being sweet, juicy and scented these structures are often coloured red or blue. Rarely the blue colour is conferred by a waxy bloom (e.g. *P. rumphii*) and usually it is due to anthocyanin. The presence of anthocyanin in the 'fruiting' structures may be regarded as of different significance from that in the young leaves, or in conelets of the Pinaceae. In the latter a facultative property is revealed by imposed physiological conditions. The 'fruit' pigments however are fulfilling a definite function, rendering the structure attractive for animal dispersal of the seed. This is supported also by the tendency for pigments other than cyanidin 3-glucoside, regarded as the most 'primitive' pigment,[21] to occur in these specialized structures. It has long been recognized that adaptations for seed dispersal by animals existed in this family and it is now evident that the pigments that have evolved for this purpose are typical angiosperm pigments (at least regarding the flavonoid residues). This need not necessarily be so, as other red pigments are found in gymnosperms. The fruit of *Gnetum gnemon* (Gnetaceae) has been found here to be pigmented by carotenoids, and in the Coniferales some colours are due to quinonoid compounds. The frequent appearance of anthocyanins as angiosperm fruit pigments perhaps indicates additional functions; as water-soluble polyphenols they may delay fungal and bacterial attack until the dispersal functions can be achieved. The indication here of a tendency to chemical

[22] P. W. RICHARDS, *The Tropical Rain Forest*, p. 78, University Press, Cambridge (1964).
[23] R. J. WAGNER, A. B. WAGNER and R. A. HOWARD, *J. Arn. Arb.* **50**, 556 (1969).

parallelism with the angiosperms is, interestingly, supported by the lignin chemistry. Podocarpaceae lignin contains syringyl derivatives not found in that of other conifers but typically present in angiosperm lignin.[24]

(c) *Permanent pigmentation.* The only case of this was encountered in *Dacrydium laxifolium*, a dwarf prostrate shrub found in alpine habitats in New Zealand. All exposed vegetative parts, i.e. the wiry stems with scale-leaves, were bronze coloured due to the presence of cyanidin 3-glucoside. This pigment was however absent from well-shaded parts of the plant and is evidently light-induced. Permanent anthocyanin coloration of vegetative parts is a frequent feature of alpine plants, but no adequate proof of physiological function has been obtained. This appears to be the first case of such an effect among the gymnosperms, and is in the case of 'fruit' colour, may have some evolutionary significance.

Systematic Distribution

The principal conclusion from anthocyanin results reported here is that the Podocarpaceae constitute a natural family but that the principal genera are not clearly distinguishable. Thus prodocarpin A and B, both rare pigments and possibly unique to the family, are found in both *Podocarpus* and *Dacrydium*. They are sufficiently widespread to constitute a chemical character of the Podocarpaceae but add little to problems of delimiting these two genera. Similarly, peonidin although found three times, occurs in both *Dacrydium* and *Podocarpus*.

(a) *Phyllocladus.* Three of the seven species were studied and all contained cyanidin 3-glucoside in seeds and arils. Although it has a completely disjunct distribution, *P. hypophyllus* was no different from the other two in this respect. In contrast to other members of the family the young phylloclades of this species *always* showed some anthocyanin coloration, and in high light intensity (11,000 ft altitude near the equator, on Mt. Kinabalu, Sabah) were very deeply coloured.

(b) *Dacrydium.* The results suggest that Group C of Florin[19] may lack the acylated anthocyanins, but the number of species studied was too small. As more elaborate pigments tend to occur in receptacles rather than seed coats their absence from e.g. *D. bidwillii* may simply be because this species has a white aril. *D. falciforme* is one of three uncommon species distinguished by rather unusual doubly falcate leaves. In the recent revision by De Laubenfels[4] these comprise the new genus *Falcatifolium*. As *D. falciforme* has both podocarpin A and cyanidin 3-glucoside in receptacles and young leaves, the anthocyanins are clearly those of *Podocarpus* and *Dacrydium*. The grounds for creating a new genus appear to be largely leaf form, and it may be appropriate to note that the leaves of this species are very similar in shape (although much bigger) to those of the bilaterally flattened juvenile twigs of some other podocarpus (e.g. *P. imbricatus*, *P. dacrydioides*). Leaf polymorphism is common in the Podocarpaceae, and the juvenile forms are often larger than the adult. It may be that the leaf form of *D. falciforme* is a neotenous condition, one means of attaining large leaf size in a habitat (understory of lower montane rainforest) where microphyllous species are not favoured.

(c) *Podocarpus.* At least two sections, Dacrycarpus and Podocarpus, possess the acylated pigments but not enough results are available to show if they are absent from any other groups. At the specific level the results support separation of *P. imbricatus* var. *kinabaluensis* as a new species, *Dacrycarpus kinabaluensis* de Laubenfels. The 'fruit' pigments were quite different, and foliar pigmentation, while uniformly present (independent of altitude) in this species, was never observed in *P. imbricatus*.

[24] R. H. J. CREIGHTON, R. D. GIBBS and H. HIBBERT, *J. Am. Chem. Soc.*, **66**, 32 (1944).

EXPERIMENTAL

Field collections. Plant material collected under expedition conditions was extracted in the field with 1% MeOH–HCl and the solution decanted, concentrated by evaporation, and applied to Whatman 3MM paper. After drying the paper was formed into a roll which was half-filled with silica gel beads, sealed in polythene and protected from light (in this way adequate samples for subsequent identification of pigments could be brought back more easily than herbarium specimens of the same plant. It has been shown that almost no loss of anthocyanin results from this method).[25] On returning to the laboratory these sheets were developed in the usual way.

Identification of anthocyanins. The pigments in extracts of plant material were separated by preparative chromatography on Whatman 3MM paper, the colour bands cut out, eluted, and the purified pigments were co-chromatographed with authentic samples of known compounds in all 4 solvent systems commonly used in the chromatography of anthocyanins (see Table 2). After hydrolysis of the pigment in 2 N HCl at 100° the anthocyanidin was identified by co-chromatography with authentic samples in Forestal solution (HOAc–conc. HCl–H_2O, 30:3:10).

Podocarpin A. Pink young leaves of *D. falciforme* (200 g) were steeped in 0·2% HCl–MeOH overnight at 10° (maceration of tissue gave extracts too gummy to handle conveniently), the solution was decanted and concentrated *in vacuo* at 30°. The extract was applied as a band to 3MM paper and developed with solvent b. The high-R_f band was cut out, eluted, and rechromatographed with solvent d; then with solvent c on paper that had been pre-washed with 15% HOAc. The solution obtained from elution of the coloured band was filtered through sintered glass and evaporated *in vacuo* (15 mg).

Podocarpin B. Receptacles of *P. dacrydioides* were steeped in 0·2% HCl–MeOH at 10° and the solvent changed several times. Combined extracts were evaporated *in vacuo* and worked up as for podocarpin A.

Partial hydrolysis of podocarpin A and B. A purified sample of the pigment was dissolved in 2 N HCl and kept at 100° for 1·5 min then applied as a strong spot on sheets of the corresponding anthocyanidin and 3-glucoside as reference compounds for the second development.

Total hydrolysis of podocarpin A and B. After treatment at 110° in 2 N HCl for 30 min the hydrolysate was shaken with sufficient amyl alcohol to form a second phase. Anthocyanidin separated in the organic layer and was identified as before. The aqueous phase was evaporated *in vacuo* over NaOH and glucose was identified; (1) by co-chromatography with an authentic sample in pyridine–ethyl acetate–H_2O (8:2:1) and solvent c; (2) by glucose oxidase-peroxidase reagent strips ('Clinistix' from Ames Co.).

Base treatment of podocarpin A. A dried sample of pigment was dissolved in 0·2 N NaOH under N_2 at 28°. After 10 min 2 N HCl was added until red colour reappeared, and the resulting solution applied directly to diagnostic chromatograms, and co-chromatographed with cyanidin 3-glucoside and free cyanidin in all 4 solvents.

Anthocyanins for reference samples were isolated from plant material for which the anthocyanins have been completely identified, as listed by Harborne.[20]

Chromatography was carried out on 56 cm sheets of Whatman No. 1 paper with descending flow, at 28°. UV spectra were measured in 1% HCl–MeOH and IR spectra were measured as KBr discs. Specimens of tropical species collected in the field were deposited in the University of Malaya Herbarium (KLU). The New Zealand species were too well-known to require voucher specimens.

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[25] J. B. LOWRY, *Malaysian J. Sci.*, to be published.

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