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Chemotaxonomic Value of Anthocyanins in *Podalyria* and *Virgilia* (Tribe Podalyrieae: Fabaceae)

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Key Word Index—Fabaceae; *Virgilia*; *Podalyria*; anthocyanins; chemotaxonomy; generic relationships.

Abstract—Anthocyanin pigments responsible for purple and pink flower colours in the tribe Podalyrieae have been identified. The considerable variation in flower colour is not reflected in the chemical variation and the flower pigments are surprisingly conservative. *Virgilia* flowers always have the acetic acid esters of cyanidin-3-glucoside and peonidin-3-glucoside as major compounds, together with trace amounts of the coumaroyl ester of cyanidin-3-glucoside. *Podalyria* flowers invariably have the rather more stable coumaroyl ester of cyanidin. The data support a close affinity between the two genera but also show that flower colour is only partially homologous.

Introduction

As part of our continuing studies of taxonomic relationships amongst African Fabaceae, the flower anthocyanins of the tribe Podalyrieae have been investigated. We could find no published information on the chemical basis of flower pigmentation in the two genera *Podalyria* and *Virgilia*. Review papers on flower pigmentation (Kay, 1987; Brouillard, 1988; Harborne, 1988) and on anthocyanins in general (Harborne and Grayer, 1988) have all highlighted the paucity of data for the large family Fabaceae. The main purpose of this study was to investigate the homology of pink and purple flower colours in the two genera and to establish whether anthocyanins can be used for phylogenetic interpretations.

Materials and Methods

Plant materials. Vouchers specimens and locality data of the material used for fresh extraction are listed in Table 1.

Procedures. Fresh petals were extracted in 1% methanolic HCl containing a small amount of BHT (butylated hydroxytoluene). To check for the possible presence of unstable malonyl esters, some samples were extracted in MeOH:acetic acid:H_2O (8:1:1) or MeOH:phosphoric acid:H_2O (8:1:1) and the extracts subjected to paper electrophoresis as described below.

Anthocyanins were studied by analytical HPLC using a diode array detector (Beckman Ultrasphere C_{18} reverse phase column, 5 μm particle size, 250 mm×4.6 mm i.d.; flow rate 1 ml min^{-1}; 20 μl sample loop). The solvent system was the same as described by Akavia and Strack (1980) and Strack *et al.*, (1980) and comprised a 20–100% linear gradient of A in B. A: 1% H_3PO_4, 20% acetic acid and 25% MeCN in H_2O; B: 1% H_3PO_4 in H_2O. The relative concentrations of acetic acid esters remained unchanged when acetic acid in the solvent system was replaced with formic acid. For preparative HPLC we used an isocratic system (varying proportions of A in B), with a wider column (250 × 10 mm i.d.), a 1 ml sample loop and a flow rate of 5 ml min^{-1}. Samples were subjected to partial hydrolysis (usually 0, 15, 30, 45 and 60 min in 2N HCl at 100°C), precleaned by simple filtration (through a fine grade celite), concentrated by C_{18} solid phase extraction and then taken up in a mixture of MeOH and H_2O (at least 50% H_2O). Anthocyanins were identified by their spectral characteristics and by comparison and co-HPLC with known standards (Harborne, 1967). Cyanidin-3-glucoside was isolated from *Acer* leaves, peonidin-3-glucoside from a partially hydrolysed extract of *Fuschia* petals and cyanidin-3-coumaroylglucoside from the bulb scales of *Hyacinthus orientalis*. Further confirmation was obtained by a study of hydrolysis products (Strack *et al.*, 1980) after boiling the samples in 2N HCl for 10, 20, 30, 40 and 60 min. Acetic acid esters were identified according to the methods described by Maccarone *et al.* (1983, 1985), which involve alkaline hydrolysis (yielding free acid) followed by derivitization with *p*-nitrobenzylbromide, using 18-crown-6 as catalyst. The *p*-nitrobenzylacetate was identical to a sample prepared from glacial acetic

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TABLE 1. DISTRIBUTION OF ANTHOCYANINS IN EXTRACTS OF PETALS FROM *PODALYRIA* AND *VIRGILIA*. Anthocyanin distributions are given as percentages of total pigments, as estimated from HPLC results, using a diode array detector (see Materials and Methods)

Genus, species and sample		Anthocyanins present†						Solvent used‡	Date collected	Date analysed
	*Rt**:	**1** 17.2	**2** 22.1	**3** 29.9	**4** 35.3	**5** 35.6	**6** 40.3			
Podalyria biflora (L.) Willd.										
Van Wyk 3212b (Citrusdal)		24	—	—	—	5	71	HCl	13.09.91	25.05.92
P. calyptrata (Retz.) Willd.										
Schutte 697a (Hermanus)		53	—	—	—	4	43	HCl	20.09.91	30.03.92
Betty's Bay		22	—	—	—	8	70	HCl	09.09.92	11.11.92
ex hort., NBI, Pretoria		3	—	—	—	8	89	HCl	10.11.92	11.11.92
P. cuneifolia Vent.										
Van Wyk 3232 (Pearly Beach)		39	—	—	—	7	54	HCl	16.09.91	29.01.93
P. glauca (Thunb.) DC.										
Schutte 728a (Knysna distr.)		59	—	—	—	9	32	HCl	21.09.91	18.11.92
Schutte 728b (Knysna distr.)		27	—	—	—	12	61	HCl	21.09.91	30.03.92
P. hirsuta (Ait.) Willd.										
Van Wyk 3237 (Paarl distr.)		14	—	—	—	6	80	HCl	16.09.91	29.01.93
P. leipoldtii L. Bol.										
Schutte 702 (Citrusdal)		44	—	—	—	9	47	HCl	13.09.91	18.11.92
P. pulcherrima Schinz										
Van Wyk 3224a (Piketberg)		16	—	—	—	6	78	HCl	14.09.91	04.02.93
Virgilia divaricata Adamson										
Schutte 731a (Knysna distr.)		76	24	—	—	—	tr	HCl	21.09.91	30.10.92

Schutte 731a (Knysna distr.)	92	8	—	—	—	tr	HCl	21.09.91	30.10.92
Schutte 731a (Knysna distr.)	83	17	—	—	—	tr	HCl	21.09.91	30.10.92
Van Wyk 3193 (Harkerville)	48	52	tr	tr	—	tr	HCl	10.09.91	07.04.92
ex hort., Cape Town (a)	10	4	32	53	—	1	AcOH	28.12.92	19.01.93
ex hort., Cape Town (a)	10	18	26	45	—	1	H_3PO_4	28.12.92	19.01.93
ex hort., Cape Town (b)	3	2	53	39	—	3	AcOH	28.12.92	19.01.93
ex hort., RAU campus	52	20	18	9	—	1	HCl	01.09.92	26.01.93
Westdene Dam (wing petals)	9	5	56	28	—	2	AcOH	18.11.92	18.11.92
Westdene Dam (keel petals)	8	2	70	18	—	2	AcOH	18.11.92	18.11.92
Johannesburg Botanical Garden	5	4	47	43	—	1	HCl	18.11.92	18.11.92
Johannesburg Botanical Garden	3	2	53	41	—	1	AcOH	18.11.92	18.11.92
Knysna (petals without keels)	22	13	35	29	—	1	HCl	07.09.92	26.10.92
Knysna (keel petals only)	67	13	14	3	—	1	HCl	07.09.92	26.10.92
V. oroboides (Berg.) Salter subsp. *oroboides* (pale pink flowers)									
ex hort., NBI, Pretoria	2	4	28	64	—	2	AcOH	17.11.92	18.11.92
Platteklip, Table Mountain (a)	3	5	23	66	—	3	AcOH	28.12.92	19.01.93
Platteklip, Table Mountain (b)	5	10	19	65	—	1	AcOH	28.12.92	19.01.93
Platteklip, Table Mountain (c)	3	9	14	73	—	1	AcOH	28.12.92	19.01.93
V. oroboides (Berg.) Salter subsp. *ferruginea* B.-E. van Wyk (dark pink flowers)									
Outeniqua Pass, George	3	2	36	59	—	tr	AcOH	04.10.93	20.10.93

*Retention time in minutes, using the HPLC system described under Materials and Methods.

†Anthocyanins are numbered as in Fig. 1: **1** = cyanidin-3-glucoside; **2** = peonidin-3-glucoside; **3** = cyanidin-3-(acetylglucoside); **4** = peonidin-3-(acetylglucoside); **5** = cyanidin-3-glucoside *p*-coumarate (a partially identified minor compound); **6** = cyanidin-3-(*p*-coumaroylglucoside).

‡Solvents used for extraction of anthocyanins: HCl = 1% methanolic HCl; AcOH = MeOH:acetic acid:H_2O (8:1:1); H_3PO_4 = same as previous, but acetic acid replaced by phosphoric acid.

acid (co-TLC and co-HPLC) in the following eluent systems: for TLC—benzene:$CHCl_3$ (1:1) and benzene:ethyl-acetate (17:1); for HPLC—a linear gradient of 50–100% MeOH in H_2O (see Durst *et al.*, 1975). Coumaroyl-glycosides were also subjected to alkaline hydrolysis and the free acids identified by HPLC and TLC using the pure acid as standard. [HPLC system as described by Charpentier and Cowles (1981); the eluent for TLC was $CHCl_3$: formic acid: acetic acid (9:1:1), spray reagent bromochresol green.] Partial hydrolysis of the minor coumaroylglycoside in *Podalyria* yielded cyanidin-3-(*p*-coumaroylglycoside) as intermediate product, but sample limitations have prevented us from a complete characterisation of this compound. Paper electro-phoresis was carried out with Whatman 3MM paper in a 0.1 M (pH 4.5) acetate buffer system, applying current for 5 h at 350 V (*ca* 10 V cm $^{-1}$).

Results

A total of 29 extracts from seven species of *Podalyria* and both species of *Virgilia* were analysed and the results are presented in Table 1. Despite considerable variation in flower colour, only a few anthocyanins were present and the variation within each of the genera was remarkably small. Only six anthocyanins were detected in the two

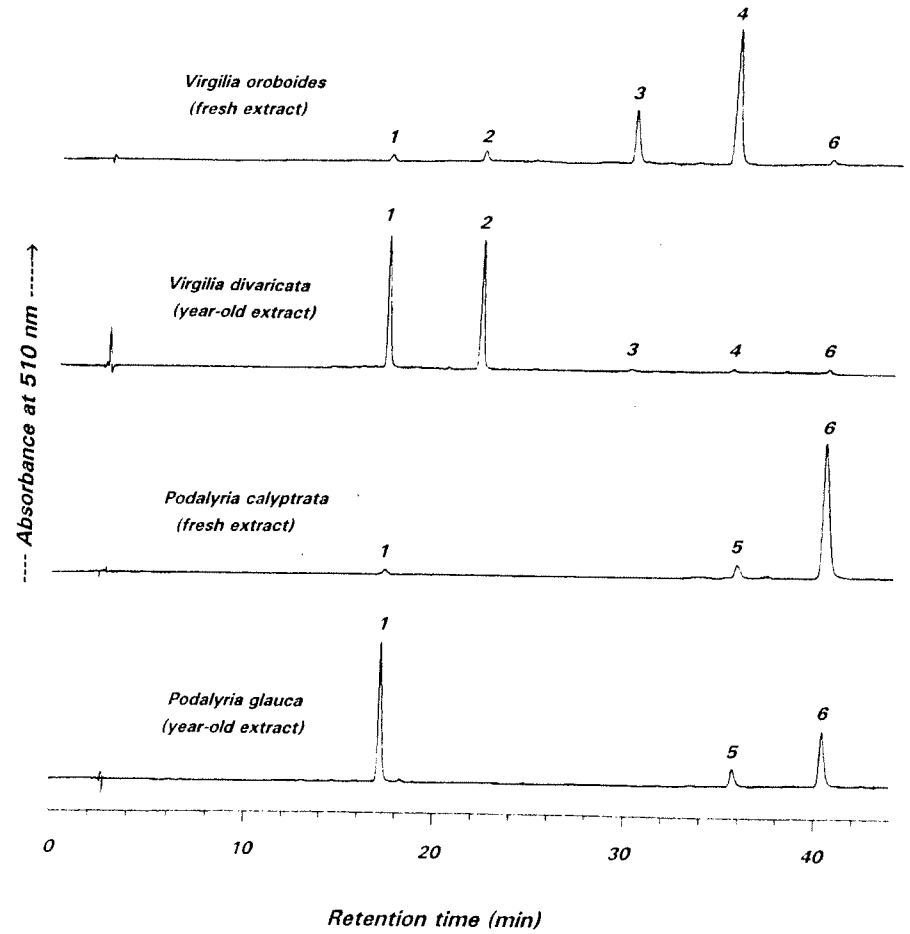

FIG. 1. SUMMARY OF THE DIAGNOSTICALLY DIFFERENT COMPOSITION OF ANTHOCYANIN PIGMENTS IN *VIRGILIA* AND *PODALYRIA* FLOWERS. Extraction and storage of the petals in methanolic HCl result in a gradual hydrolysis of the esters (**3**, **4**, **5** and **6**) to their corresponding glucosides (**1** and **2**). **1** = cyanidin-3-glucoside, **2** = peonidin-3-glucoside, **3** = cyanidin-3-(acetyl-glucoside), **4** = peonidin-3-(acetylglucoside), **5** = cyanidin-3-glucoside *p*-coumarate (a partially identified minor compound), **6** = cyanidin-3-(*p*-coumaroylglucoside).

genera, of which five could be positively identified. These are cyanidin-3-glucoside, peonidin-3-glucoside, cyanidin-3-(acetylglucoside), peonidin-3-(acetylglucoside), cyanidin-3-(*p*-coumaroylglucoside) and a cyanidin-3-glucoside *p*-coumarate (a minor compound).

A large part of the variation is due to different methods of extraction and experiments with different solvent systems and different periods of extraction (see Fig. 1) confirmed that the anthocyanin pigments are present as esters in the fresh flowers and that these are partially or completely hydrolysed during extraction or storage. Table 1 and Fig. 1 show that the two esters of cyanidin-3-glucoside are the most common anthocyanins, occurring in all the samples studied. The identified coumaroyl ester is the only major compound in *Podalyria* flowers (small amounts are present in *Virgilia*), while the acetic acid esters of cyanidin-3-glucoside and peonidin-3-glucoside appear to be restricted to *Virgilia*, where they represent the only major pigments of fresh flowers.

Discussion

The conservative anthocyanin pattern in the Podalyrieae clearly does not explain the considerable variation in flower colour and the different shades of purple and pink. It is well known, however, that the same pigment may give rise to different flower colours. Willstätter's pH theory for explaining flower colour variation has recently been questioned by Goto and Kondo (1991), who showed that colour variation may also result from self-association, copigmentation and intramolecular sandwich-type stacking.

Flower colour is often used in taxonomy without any knowledge of the chemical basis or homology of the character. Our study has shown that flower colour is only partially homologous in *Podalyria* and *Virgilia* and that each of them has a unique and diagnostically different combination of flower anthocyanins. The universal occurrence of cyanidin-3-glucoside and its esters nevertheless supports the idea of an affinity between *Virgilia* and *Podalyria* and provides further support for the transfer of *Virgilia* from the Sophoreae to the Podalyrieae (Polhill, 1981). It may be interesting to extend this study to the closely related tribe Liparieae, where generic circumscriptions have not yet reached stability. The chemical basis of flower colour in the anomalous genus *Hypocalyptus* for example, may give helpful clues about its true affinities within the Fabaceae.

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