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EFFECTS OF HYBRIDIZATION ON LEAF CONSTITUENTS IN THE GENUS *SALIX*

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Abstract—The distribution of leucoanthocyanidins, phenolic glycosides and piperidine-based imino-acids has been studied in the leaves of *Salix* species and hybrids. When both parents contained either leucocyanidin or leucocyanidin and leucodelphinidin, the hybrid contained the same compounds. When, however, species containing leucocyanidin were crossed with species containing leucocyanidin and leucodelphinidin, the hybrids did not give a uniform result. Phenolic glycosides were detected in all species and hybrids tested, but no obvious taxonomic pattern was noticed. Pipecolic acid was found in many of the leaves tested, but often the yields were low.

INTRODUCTION

IN A RECENT publication Binns, Blunden and Woods[1] reported the distribution of leucoanthocyanidins, phenolic glycosides and piperidine-based imino-acids in the leaves of several *Salix* species. Differences in leucoanthocyanidin content between species were apparent, but the phenolic glycosides were concluded to be of limited chemotaxonomic value. Piperidine imino-acids were found in several species and differences were detected. The work reported in this paper is an extention of the earlier work, including studies on a number of *Salix* hybrids. The studies have been made to determine whether chemical differences between species are of value in the classification of the genus and to obtain information which may aid in the identification of the parents of a hybrid. The present position regarding the classification of the genus *Salix* is complex, because identification of plants is frequently difficult. Plants in the genus hybridise freely, the sexes occur as different trees and there is great variability in many of the species in features such as leaf shape. Difficulty in identification of hybrids is accentuated by the frequent planting of some hybrids away from their parents and by the ease of reproduction from cuttings.[2]

Of the three groups of compounds reported in this communication, phenolic glycosides have been the most widely examined in *Salix*, their structure and distribution having been reviewed by Thieme.[3–6] In his studies, Thieme detected the phenolic glycosides by paper chromatography,[7] but since then a more sensitive TLC procedure has been reported by Audette, Blunden, Steele and Wong[8] and recently, a method has been published by Bolan and Steele[9] for the determination of phenolic glycosides by gas–liquid chromatography.

[1] W. W. BINNS, G. BLUNDEN and D. L. WOODS, *Phytochem.* 7, 1577 (1968).
[2] A. R. CLAPHAM, J. C. TUTIN and E. F. WARBURG, *Flora of the British Isles*, 2nd edition, pp. 581–582, Cambridge University Press, Cambridge (1962).
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[6] H. THIEME, *Planta Medica* **13**, 431 (1965).
[7] H. THIEME, *Pharmazie* **19**, 471 (1964).
[8] R. C. S. AUDETTE, G. BLUNDEN, J. W. STEELE and C. S. C. WONG, *J. Chromatogr.* **25**, 367 (1966).
[9] M. BOLAN and J. W. STEELE, *J. Chromatogr.* **36**, 22 (1968).

Leucoanthocyanidins have been reported in *Salix* by Bate-Smith[10,11] and imino-acids by Virtanen and Kari[12] and Blunden, Challen and Jaques.[13]

RESULTS AND DISCUSSION

Leucoanthocyanidins present in *Salix* leaves were converted to anthocyanidins by heating with HCl, and the compounds so formed were examined by paper chromatography. All the samples tested gave a positive reaction (Table 1). When both parent species of a hybrid

TABLE 1. DISTRIBUTION OF ANTHOCYANIDINS DERIVED FROM LEUCOANTHOCYANIDINS IN *Salix* LEAVES

	Hybrid	Cyanidin*	Delphinidin
S. purpurea†	× *S. viminalis* (*S.* × *rubra*)	+	−
+/−	++/+++		
S. triandra	× *S. viminalis* (*S. hippophaefolia*)	++	++++
+/+	++/+++		
S. purpurea	× *S. triandra* (*S.* × *leiophylla*)	++	−
+/−	+/+		
S. alba	× *S. fragilis* (*S.* × *russelliana*)	++	−
++++/−	++++/−		
S. alba	× *S. pentandra* (*S.* × *ehrhartiana*)	+	−
++++/−	+/−		
S. alba	× *S. babylonica* (*S.* × *sepulcralis*)	++	++
++++/−	++/++++		
S. babylonia	× *S. fragilis* (*S.* × *blanda*)	+++	++
++/++++	++++/−		
S. fragilis	× *S. pentandra* (*S.* × *meyerana*)	++	−
++++/−	+/−		
S. fragilis	× ? *S. triandra* (*S.* × *decipiens*)	++	−
++++/−	+/+		
S. cinerea	× *S. viminalis* (*S.* × *geminata*)	++	+++
++/+++	++/+++		
S. caprea	× *S. lanata* (*S.* × *balfourii*)	+++	+++
++/+	+++/++		
S. caprea	× *S. viminalis* (*S.* × *sericans*)	++	+++
++/+	++/+++		
S. viminalis	× *S.* ? (*S.* × *stipularis*)	++	+++
++/+++			
S. herbacea	× *S. phylicifolia* (*S.* × *moorei*)	+++	++
unavailable	+/+++		
S. lapponum	× *S. phylicifolia* (*S.* × *gillotii*)	+++	−
++++/−	+/+++		

* ++++ Very strong, +++ strong, ++ moderate, + weak.
† Cyanidin/delphinidin yield of parent, e.g. *S. purpurea* +/−.

produced either cyanidin or cyanidin and delphinidin, the hybrid gave the same result. When species yielding cyanidin were crossed with species yielding both cyanidin and delphinidin, the hybrids did not give a uniform result. The cyanidin and delphinidin-producing *S. babylonica*, when crossed with the cyanidin producing species *S. alba* and *S. fragilis*, gave hybrids which yielded both cyanidin and delphinidin. However, the cyanidin-yielding

[10] E. C. BATE-SMITH and N. H. LERNER, *Biochem. J.* **58**, 126 (1954).
[11] E. C. BATE-SMITH and C. R. METCALFE, *J. Linn. Soc. (Botany)* **55**, 669 (1957).
[12] A. I. VIRTANEN and S. KARI, *Acta Chem. Scand.* **9**, 1548 (1955).
[13] G. BLUNDEN, S. B. CHALLEN and B. JAQUES, *Nature* **212**, 514 (1966).

S. purpurea, when crossed with the cyanidin and delphinidin-yielding species *S. viminalis* and *S. triandra*, gave hybrids which produced only cyanidin. A similar result was obtained when *S. lapponum*, which gave cyanidin, was crossed with *S. phylicifolia*, which gave both cyanidin and delphinidin.

In our earlier paper,[1] *S. decipiens* was listed as yielding both delphinidin and cyanidin. The identification of the plant material used in that study was in error and the correct identification should have been *S.* × *sericans*. Leaves of authentic *S. decipiens* were found to contain only cyanidin. This plant is variously regarded as a variety of *S. fragilis* and as a form of the hybrid *S. fragilis* × *S. triandra* or possibly *S. fragilis* × some other species.[2] The presence of cyanidin is consistent with any of these ideas. If the plant be a hybrid of *S. fragilis* and *S. triandra*, it would mean that cyanidin production due to *S. fragilis* was dominant over cyanidin and delphinidin production of *S. triandra*.

Phenolic glycosides were detected in all samples tested (Table 2). Identification of an individual compound was based on its chromatographic position and the colour it produced on thin-layer chromatograms after spraying with 4 per cent H_2SO_4 in EtOH and heating. In certain extracts some glycosides were present in trace quantities and identification was difficult. Although care was taken in interpretation of the chromatograms, there is always an element of doubt when evidence is based on chromatographic data only, particularly when some compounds are present in small quantities. It was with this in mind that the presence of certain phenolic glycosides in willow leaf extracts was marked as uncertain (Table 2). Many of the extracts examined produced unidentified sulphuric acid-positive spots on thin-layer chromatograms in the region of the known phenolic glycosides.

The presence/absence of individual phenolic glycosides did not seem to show any clear cut pattern and the value of these compounds in the determination of the parentage of a hybrid seems to be restricted. There may be some correlation between the major glycoside of a hybrid and its parents. For example, salidrosid was a major component in *S. triandra* and also in the *S. triandra* crosses with *S. viminalis* and *S. purpurea*. However, the presence of salidrosid was uncertain in *S. decipiens*, which may be *S. fragilis* crossed with *S. triandra*. Salidrosid was only a minor component of *S. fragilis*[1] and was not detected in the *S. fragilis* cross with *S. babylonica*.

Pearl and Darling[14] reported that the use of lead subacetate solution at elevated temperatures during the preparation of phenolic glycoside extracts resulted in the migration of the benzoyl group from the 2 position of tremuloidin to the 6 position of populin. That this did not occur in the preparation of our extracts, which employed lead subacetate at room temperature, was shown by the results obtained from a number of different extracts. In these, tremuloidin was present without detectable quantities of populin.

Piperidine imino-acids were detected in the majority of the leaf samples tested. Pipecolic acid was found in *S.* × *hippophaefolia*, *S.* × *leiophylla*, *S.* × *ehrhartiana*, *S. decipiens*, *S.* × *geminata*, *S.* × *balfourii*, *S.* × *sericans*, *S.* × *stipularis*, *S.* × *moorei*, *S. lanata* and *S. lapponum* and an unknown imino-acid, probably based on piperidine, was detected in *S.* × *blanda*. Insufficient leaf material was available to test *S.* × *gillotii*. As reported earlier,[1] the yields of the imino-acids were often low, only trace quantities having been detected in a number of plants. These small yields make it dangerous to draw taxonomic conclusions from negative results. For example, from the sample of *S.* × *geminata* leaf used in our earlier studies, no piperidine imino-acids were detected, but from a second sample used in this investigation a positive reaction was obtained for pipecolic acid. Negative results may only mean that the

[14] I. A. Pearl and S. F. Darling, *Archs Biochem. Biophys.* **102**, 33 (1963).

compounds are present in quantities too small for detection. Both *S. alba*[1] and *S. fragilis*[13] contain 5-hydroxypipecolic acid and another unknown imino-acid, probably based on

TABLE 2. DISTRIBUTION OF PHENOLIC GLYCOSIDES IN *Salix* LEAVES

Hybrid or species	Salicin	Salicortin	Salidrosid	Fragilin	Tremuloidin	Vimalin	Triandrin	Grandidentatin	Populin
S. purpurea × *S. viminalis* (*S.* × *rubra*)	+	+	+	+	+	−	−	+	+
S. purpurea	+	+	+	+	+	+	+	+	+
S. viminalis	+	+	+	+	−	±	+	±	−
S. triandra × *S. viminalis* (*S.* × *hippophaefolia*)	+	+	+	±	±	±	+	+	−
S. triandra	+	+	+	+	+	±	+	±	−
S. viminalis	+	+	+	+	−	±	+	±	−
S. purpurea × *S. triandra* (*S.* × *leiophylla*)	+	+	+	−	−	+	−	±	−
S. purpurea	+	+	+	+	+	+	+	+	+
S. triandra	+	+	+	+	+	±	+	±	−
S. alba × *S. fragilis* (*S.* × *russelliana*)	+	+	+	+	+	+	−	+	−
S. alba	+	+	+	+	±	+	±	+	−
S. fragilis	+	+	+	+	+	±	±	±	+
S. alba × *S. pentandra* (*S.* × *ehrhartiana*)	+	+	±	+	+	+	−	+	+
S. alba	+	+	+	+	±	+	±	+	−
S. pentandra	+	+	+	+	+	+	±	+	±
S. alba × *S. babylonica* (*S.* × *sepulcralis*)	+	+	+	−	−	+	±	±	−
S. alba	+	+	+	+	±	+	±	+	−
S. babylonica	+	+	+	+	+	+	+	±	−
S. babylonica × *S. fragilis* (*S.* × *blanda*)	+	+	−	+	+	+	+	+	−
S. babylonica	+	+	+	+	+	+	+	±	−
S. fragilis	+	+	+	+	+	±	±	±	+
S. fragilis × *S. pentandra* (*S.* × *meyerana*)	+	+	+	+	+	+	+	−	+
S. fragilis	+	+	+	+	+	±	±	±	+
S. pentandra	+	+	+	+	+	+	±	+	±
S. fragilis × ? *S. triandra* (*S.* × *decipiens*)	+	+	±	+	+	+	±	+	−
S. fragilis	+	+	+	+	+	±	±	±	+
S. triandra	+	+	+	+	+	±	+	±	−
S. cinerea × *S. viminalis* (*S.* × *geminata*)	+	+	+	−	+	±	+	+	±
S. cinerea	+	+	+	−	−	+	±	−	−
S. viminalis	+	+	+	+	−	±	+	±	−
S. caprea × *S. lanata* (*S.* × *balfourii*)	+	+	+	−	−	−	−	−	−
S. caprea	+	+	+	−	−	±	−	−	−
S. lanata	Insufficient material available								
S. caprea × *S. viminalis* (*S.* × *sericans*)	+	+	+	+	−	+	+	+	+
S. caprea	+	+	+	−	−	±	−	−	−
S. viminalis	+	+	+	+	−	±	+	±	−
S. viminalis × *S.* ? (*S.* × *stipularis*)	+	+	+	−	−	+	±	+	−
S. viminalis	+	+	+	+	−	±	+	±	−
S. herbacea × *S. phylicifolia* (*S.* × *moorei*)	+	−	+	±	+	±	−	+	−
S. herbacea	Insufficient material available								
S. phylicifolia	+	+	+	+	−	−	−	+	−
S. lapponum × *S. phylicifolia* (*S.* × *gillotii*)	Insufficient material available								
S. lapponum	+	+	+	−	−	+	+	−	−
S. phylicifolia	+	+	+	+	−	−	−	+	−

piperidine. However, no imino-acids were detected in the *S. alba* × *S. fragilis* hybrid, which may have been due to the compounds being present in quantities too minute for identification.

Another surprising result was the detection of pipecolic acid in the *S. alba* × *S. pentandra* hybrid. *S. alba* contains 5-hydroxypipecolic acid and the unknown piperidine imino-acid and *S. pentandra* was recorded as lacking piperidine imino-acids. The unusual result from the hybrid may be due to *S. pentandra* containing pipecolic acid, but not in a sufficient amount for detection.

The result obtained from *S. decipiens* was interesting as pipecolic acid was detected in the leaves. Samples of *S. fragilis* leaf that have been tested for imino-acids have always shown the presence of 5-hydroxypipecolic acid and the unknown piperidine imino-acid. The presence of pipecolic acid in the *S. decipiens* leaves would appear to be inconsistent with it being a variety of *S. fragilis*. However, *S. triandra* contains pipecolic acid and it is quite feasible that a *S. fragilis* × *S. triandra* cross would yield pipecolic acid.

EXPERIMENTAL

The leaf samples of *S.* × *ehrhartiana*, *S.* × *sepulcralis*, *S.* × *blanda*, *S.* × *geminata*, *S.* × *meyerana*, *S.* × *stipularis*, *S.* × *balfourii*, *S.* × *lanata*, *S.* × *moorei*, *S.* × *gillotii*, *S. lapponum* and *S. decipiens* were collected in September 1968 from the nurseries of Hillier and Sons of Winchester. All the remaining leaf samples were collected in September 1967 at the Long Ashton Research Station in Bristol. All the leaf samples were carefully dried in a circulating air oven at a temperature not exceeding 50° and then stored in sealed tins until use.

Examination of Leucoanthocyanidins

The leucoanthocyanidins present in *Salix* leaves were converted into anthocyanidins by the method of Bate-Smith[15] and examined by paper chromatography.[15,16]

Examination for Phenolic Glycosides

The dry, powdered leaves (10 g) were extracted in a Soxhlet with 95 per cent ethanol for 10 hr, the ethanol recovered, and the residue extracted with water (4 × 20 ml). This aqueous solution was extracted with ethyl acetate by continuous liquid–liquid extraction for 6 hr, the ethyl acetate was recovered under reduced pressure and the residue re-dissolved in water (4 × 20 ml). The aqueous solution was treated with 10 ml aqu. lead subacetate and the resultant precipitate was removed by centrifugation. The clear, decanted liquid was treated with H_2S and the precipitate formed was filtered off. The filtrate was concentrated to about 20 ml and extracted with ethyl acetate (5 × 10 ml). The ethyl acetate solution was concentrated to dryness and the residue redissolved in water for chromatographic examination. The phenolic glycoside extracts were separated by two-way TLC, using the technique of Audette, Blunden, Steele and Wong[8] and located by spraying with 4 per cent H_2SO_4 in EtOH, and heating at 110° for 10–15 min.

Examination for Imino-Acids

Extracts of *Salix* leaves for imino-acid evaluation were prepared by boiling 5 g powdered leaf with 3 × 50 ml water and filtering. This extract was passed through a column of Amberlite i.r. 120 as described by Virtanen and Kari.[17] The purified extract was concentrated to about 5 ml, 2–3 drops of conc. HCl were added and the precipitated material was removed by filtration. The filtrate was studied by two-way TLC, using the method of Blunden and Challen,[18] the imino-acids being located with ninhydrin.

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