BIOCHEMICAL VERIFICATION OF HYBRIDITY IN WEEPING WILLOW

by Frank S. Santamour, Jr.

Abstract. The identification of some species of *Salix* (willow) and their interspecific hybrids can be easily accomplished by two-dimensional paper chromatography of alcoholic leaf extracts. Clones of *S. babylonica*, including 'Babylon,' and 'Tortuosa,' and two individuals called *S. matsudana* 'Pendula' contained the flavone luteolin 7-glucoside as the only flavonoid glycoside. Some clones of *S. alba* produced only the flavonol rhamnazin 3-glucoside, but others also contained rhamnazin 3-rutinoside. Both rhamnazin compounds were found in *S. fragilis* and it is possible that some clones purported to be *S. alba* are actually hybrids between *S. alba* and *S. fragilis*. The presence of luteolin 7-glucoside in the leaves of many "weeping" clones was sufficient to indicate the involvement of *S. babylonica* in their parentage. The red anthocyanidins formed during acid hydrolysis of leaf tissue varied among species, but were not helpful in hybrid identification because of inter-clonal differences in *S. alba*.

The botanical origins of most of the weeping willows cultivated in Europe and in North America are largely unknown, but have been subject to various interpretations. Some trees are undoubtedly pure *Salix babylonica* L., represented by the cultivar 'Babylon' and one or two clones sometimes called *S. matsudana* Koidz. 'Pendula' (13). Most of the other weeping willows could be placed in one or the other of two hybrid groups: *S. x sepulcralis* Simonkai (*S. babylonica* x *S. alba* L.) or *S.* x *blanda* Andersson (*S. babylonica* x *S. fragilis* L.). Still, some reputed hybrids are often considered under *S. alba*, with 'Tristis' or 'Vitellina Pendula' as varietal or cultivar designations (13). With regard to the parentage of *S.* x *blanda*, Bean (3) stated that the male parent may have been *S.* x *rubens* Shrank (*S. alba* x *S. fragilis*) or even *S. pentandra* L.

In horticultural and nursery practice, the classification and application of nomenclature in weeping willows has been based largely on opinions of "experts" in various texts and treatises, whether or not such opinions were based on detailed morphological analyses. The high degree of morphological variability within putative parental taxa makes such analyses of doubtful value. It would be desirable to develop simple and reliable non-morphological criteria for the identification of species and hybrids. Analyses of the leaf flavonoids of a few taxa in 1989, performed before a thorough review of the literature, indicated that further studies might yield significant results.

Flavonoid compounds, which include anthocyanins, flavonols, flavones and related substances, have been widely utilized in "chemical taxonomy". Their structural variability, widespread distribution, general stability, and ease of identification make flavonoids ideal as chemical markers in plant classification and phylogeny. Futhermore, most flavonoids are inherited in a codominant fashion and hybrids usually contain all of the major compounds present in both parents.

Of the four *Salix* species noted above as possibly being involved in the gene pool of weeping willows, the leaf flavonols and flavones of only two taxa have been reported. In 1968, Thieme (15) isolated, identified, and fully characterized the sole flavonol in the leaves of *S. alba* as rhamnazin 3-glucoside. Rhamnazin is the 7,3' dimethylether of the most common flavonol (quercetin) in plant tissues and rather sophisticated analytical procedures were necessary for its identification. In 1988 (8), the leaves of *S. babylonica* were reported to contain the flavonol kaempferol 7-glucoside and the flavones apigenin 7-glucoside and luteolin 4'-glucoside. This study was done by a group of Indian scientists on leaves sent to them from Nepal. Many investigations on plant biochemistry are performed by chemists who have little knowledge of plant material and, more often than not, have never seen the plants from which their samples were collected. Inasmuch as one of our first analyses (performed before we were even aware of the Indian work) showed that none of these compounds occurred in *S. babylonica*, we suspect that their plants had not been correctly identified.

The literature on another group of flavonoids, the proanthocyanidins, is more extensive but far

from straightforward. Proanthocyanidins, formerly called leucoanthocyanins, form red pigments (anthocyanidins) when cleaved by acid hydrolysis of condensed tannins in leaf extracts. The most common anthocyanidins are cyanidin and delphinidin, and these compounds are identical to the aglycones of anthocyanins found as flower petal pigments in many plants. Some investigators have reported only the presence or absence of such compounds while others have provided more exact identification. Among the taxa in this study, *S. alba* has been reported as both positive (4,6,7) and negative (1) for these compounds. Two investigations on *S. babylonica* (2,4) gave positive results. For both *S. fragilis* and *S. pentandra*, there have been negative (6) and positive (4,7) reports.

The purposes of the present study were to investigate the distribution of flavonoids in the leaves of those willow taxa that might have been involved in the ancestry of "weeping willow" selections and to use such data to more properly delineate the botanical classification of these trees.

Materials and Methods

All of the willows used in this study were growing in the collections of the U.S. National Arboretum (NA) in Washington, DC. However, most of the clones had been grown from cuttings of parent trees in the permanent collections of other arboreta: the Arnold Arboretum of Harvard University (AA), the Morton Arboretum (MA), and the University of Minnesota Landscape Arboretum (UM). Because of the confused nomenclature and identification of these willows, the original accession numbers of trees at the various arboreta will be given when appropriate, to allow the possibility of future research dealing with these clones as living plants or herbarium specimens. Many of these clones had been used previously in our works on nomenclature (13) and root-knot nematodes (14).

The details of paper chromatographic analyses of flavones, flavonols, and anthocyanidins performed in our laboratory have been given in previous papers (10,11,12). Basically, the flavones and flavonols are identified by their movement (R^f value) in various solvent systems and their ultraviolet absorbtion spectra. These compounds are easily visualized as yellow spots when the chromatography paper is fumed with ammonia vapor. The analyses of anthocyanidins utilizes different solvents and the compounds appear as red spots on the papers. Data pertaining to the identification of most of the common flavonoids are contained in standard reference works (5,9).

Results and Discussion

Data on the identification of purified flavonoids found in this study are provided in Table 1. The only flavonoid glycoside in the leaves of *S. babylonica* 'Babylon' (NA 44011) was luteolin 7-glucoside. This compound was, likewise, the sole flavonoid in trees labelled *S. matsudana* 'Tortuosa' (NA 44014) and *S. matsudana* 'Pendula' (NA 464511, AA 175-61). The identification of luteolin 7-glucoside was confirmed by co-chromatography with authentic material isolated from the foliage of carrot. None of the trees that we consider to represent true *S. babylonica* contained any of the flavonoids previously reported (8) in this species.

We accepted Thieme's (15) identification of the aglycone of the *S. alba* flavonoid as rhamnazin, because of the complex analyses necessary for verification were beyond our capabilities. The ultraviolet spectrum and the R^f of rhamnazin 3-glucoside on two-dimensional chromatograms of crude leaf extracts were very similar to those of quercetin 3-glucoside. However, the movement of the aglycone in Forestal solvent differs markedly from quercetin. The identification of rhamnazin 3-rutinoside was achieved by partial hydrolysis. Since there are no published data on paper chromatography of rhamnazin or its glycosides, the values given in Table 1, adjusted by comparison to authentic controls of queretin compounds, may serve as approximations until absolutely pure materials can be tested.

Some trees of *S. alba* contained only rhamnazin 3-glucoside, as reported by Thieme (15). These included NA 44016, 'Vitellina' (MA 0466-23), 'Rockanje' (MA 0310-65), and 'Hutchinson Yellow-Barked' (NA 57831). On the other hand, trees of 'Calva' (UM 600375) and 'Britzensis' (from Klehm Nurs.) also produced rhamnazin 3-rutinoside.

Unfortunately, we were only able to examine a

Table 1. R^f values (x 100) of willow leaf flavonoids in various solvent systems.

	Solvent [1]		
Flavonoid	*Forestal*	*BAW*	*27% AC*
Luteolin	66	—	—
7-glucoside	—	44	03
Quercetin	41	—	—
3-glucoside	—	58	11
3-rutinoside	—	45	28
Rhamnazin	58	—	—
3-glucoside	—	62	10
3-rutinoside	—	49	21

[1]. Solvents: Forestal (acetic acid: HCL: water, 30:3:10); BAW (n-butanol:acetic acid: water, 4:1:5, upper phase); 2% AC (2% acetic acid).

single clone of S. *fragilis* (*S. fragilis* var. *decipiens*, MA 00059-61), and this tree also contained both the glucoside and the 3-rutinoside of rhamnazin. It is possible that the clones of *S. alba* (noted above) that produced both the rhamnazin glycosides were really hybrids between *S. alba* and *S. fragilia* (*S.* x *rubens*) but extensive studies of wild populations of both species would be necessary before such a conclusion could be made. It is noteworthy, however, that both compounds were found in the leaves of 'Natural Red' (NA 57834, PI 502253), a hybrid between *S. alba* 'Vitellina' and *S. fragilis* var. *decipiens* from the Long Ashton Research Station, Bristol, England. Our single specimen of *S. pentandra* (NA 44015) contained the 3-glucoside and 3-rutinoside of quercetin (no luteolin or rhamnazin compounds).

Leaves of the clones listed below were also analyzed.

Arnold Arboretum

AA 7235	*S. alba* var *tristis*
AA 17950	*S. alba* var *tristis*
AA 2654-5	*S. alba* var *tristis*
AA 13-46	*S.* x *blanda*
AA 386-62	*S.* x *blanda*
AA 19-64	*S. elegantissima*
AA 719-71	*S. elegantissima*
AA 178-63	*S.* x *sepulcralis*

Morton Arboretum

MA 0183-53 (MA 0830-55)	*S. alba* 'Tristis'
MA 0128-44 (MA 0261-59)	*S. alba* 'Tristis'
MA 0463-48	*S. babylonica*
MA 0716-58	*S. babylonica*
MA 0564-23	*S. blanda* 'Niobe'
MA 0055-61	*S.* x *elegantissima*
MA 0068-61	*S.* x *sepulcralis*

National Arboretum

NA 43995	*S. alba* 'Tristis'
NA 46452	*S* x *blanda* 'Niobe'
NA 60127	*S.* x *blanda* 'Niobe'
NA 8929	*S.* x *chrysocoma*

University of Minnesota

UM 580495	*S. alba* 'Tristis'
UM 660586	*S. babylonica*
UM 600376	*S.* x *blanda*
UM 660585	*S.* x *blanda*

All of these clones, except one, contained luteolin 7-glucoside, rhamnazin 3-glucoside, and rhamnazin 3-rutinoside, indicating that they were hybrids between *S. babylonica* and either *S. alba* or *S. fragilis* (or their hybrid, *S.* x *rubens*). Thus, those plants labelled *S. alba* 'Tristis' are not *S. alba* nor are those labelled *S. babylonica* true to name. However, we could not determine which clones could be categorized as *S.* x *sepulcralis* (*babylonica* x *alba*) or *S.* x *blanda* (*babylonica* x *fragilis*). Our failure to find any quercetin glycosides in these weeping willows tends to rule out any involvement of *S. pentandra* in their parentage.

One of the exceptions noted above was MA 0055-61 (*S.* x *elegantissima*) which, because of the synonomy of *S. elegantissima* and *S.* x *blanda*, should be a hybrid between *S. babylonica* and *S. fragilis*. This non-weeping clone did not produce any luteolin 7-glucoside and indicated that *S. babylonica* was not part of its lineage.

The analyses of anthocyanidins in acid-hydrolized leaf extracts did not aid in hybrid verification. None of the clones of *S. babylonica* or *S.*

fragilis produced cyanidin, but *S. pentandra* was weakly positive. Some clones of *S. alba* were positive (NA 44016, 'Vitellina', Rockanje', Britzensis') but others were negative ('Calva', 'Hutchinson Yellow-Barked').

Although the analyses of leaf flavonoids do not answer all of the questions regarding the origin of most weeping willows, we should be able to distinguish trees that are pure *S. babylonica* and those in which *S. babylonica* was parentally involved.

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Research Geneticist
U.S. National Arboretum
ARS-USDA
Washington, D.C. 20002

Résumé. L'identification de certaines espèces de Salix (saule) et de leurs hybrides interspécifiques peut être aisément réalisée par une chromatographie bidimensionnelle sur papier d'un extrait alcoolique de feuilles. Les clones de *S. babylonica*, incluant 'Babylon' et 'Tortuosa', et deux individus nommés *S. matsudana* 'Pendula' contenaient la lutéoline 7-glucoside de flavone comme seule glycoside flavonoïde. Certains clones de *S. alba* produisaient seulement la rhamnazine 3-glucoside de flavonol, mais d'autres renfermaient aussi de la rhamnazine 3-rutinoside. Les deux composés de rhamnazine étaient retrouvés chez *S. fragilis* et il est possible que certains clones, sensés être de *S. alba*, sont actuellement des hybrides entre *S. alba* et *S. fragilis*. La présence de lutéoline 7-glucoside dans les feuilles de plusieurs clones «pleureurs» était suffisante pour indiquer l'implication de *S. babylonica* dans les liens de parenté. Les anthocyanidines rouges, formés durant l'hydrolyse acide du tissu de feuille, variaient parmi les espèces, mais n'étaient pas utiles dans l'identifications de l'hybride en raison des différences interclonales chez *S. alba*.