FLAVONOIDS AND COUMARINS IN *FRAXINUS* AND THEIR POTENTIAL UTILITY IN HYBRID VERIFICATION

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ABSTRACT. -- In contradiction of previous reports, it was not possible to distinguish between F. *americana* and F. *pennsylvanica* on the basis of flavonoids. Leaves of both species contained the same two flavone glycosides and no coumarins. Species in other botanical Subsections produced flavonol glycosides as well as a number of coumarin compounds. Chemical verification of interspecific hybridity in many combinations, especially those involving white or green ash, should be readily accomplished using simple paper chromatographic analyses.

SPECIES OF THE GENUS *FRAXINUS,* the ashes, are important timber and landscape trees in North America and Europe. The Asiatic ash species are not widely utilized, but they represent a potential new source of diversity for future testing and selection. Of the approximately 65 ash species known, the most recent monograph on *Fraxinus* (Scheller 1977) listed 25 species as being cultivated in the West.

The most widely used classification scheme in *Fraxinus* has been that of Rehder (1940), but Scheller (1977) proposed a few minor extensions of that scheme. Most notable was the creation of two series within the Subsection *Bumelioides.* In this paper I have followed Scheller's classification.

Recently, there has been increased interest in the ashes, both from forestry and landscape viewpoint. Provenance tests of native species re underway and further introduction of exotic species is being attempted. Selection of superior trees, some at the cultivar level, will follow. Presumably this new research interest will also stimulate activity in controlled hybridization. The present study was undertaken as an adjunct to a proposed breeding program.

CONTROLLED HYBRIDIZATION -- A HISTORY

Perhaps the first reference to controlled interspecific hybridization in Fraxinus was Henry (1914-15), who reported on attempts by himself and others during the period 1909 to 1914. The cross of Fraxinus excelsior L. 'Pendula' x F. angustifolia Vahl. was made in Cambridge, England in 1909, resulting in 11 seedlings. In 1912, a female tree of F. oregona Nutt. (=F. latifolia Benth.) at Kew Gardens was used in crosses with F. pennsylvanica Marsh. (12 seedlings), F. americana (2 seedlings), and F. excelsior (fruit but no seed). Other apparently successful crosses of 1912 were F. excelsior x americana (5 seedlings) and F. excelsior x pennsylvanica (8 seedlings No fruit was produced to crosses of F. ornus L. with F. americana or F. pennsylvanica.

In 1913, seed was set to crosses of F. excelsior 'Heterophylla' with F. angustifolia and F. excelsior, but again the cross F. oregona x excelsior failed to produce viable seed.

In 1914, the following failures were recorded from Kew: F. oregona x americana, F. pennsylvanica x excelsior, and F. pennsylvanica x americana. There was seed set to the following crosses: F. oregona x excelsior, F. oregona x pennsylvanica, F. pennsylvanica x oregona, F. pennsylvanica x pennsylvanica, F. excelsior x oregona, and F. excelsior x pennsylvanica.

Little is known of the fate of the putative hybrid seedlings or hybrid seed reported by Henry. Two of the hybrids (from 1914 seed?) were apparently seen by Edgar Anderson in 1934 (Anderson and Whelden 1936) and were apparently accepted as true hybrids by this distinguished botanist. The plants of F. excelsior x oregona were superior in growth rate to those of F. excelsior x pennsylvanica.

Johnson and Heimburger (1946) reported high seed sets and good seed germination from all their attempted crosses in Fraxinus: F. excelsior 'Aureo-variegata' pollinated by F. americana, F. pennsylvanica, and F. quadrangulata Michx. and F. richardi Bosc. (=F. pennsylvanica) crossed with F. americana and F. pennsylvanica. It is interesting to note that the supposed intraspecific cross F. richardi x pennsylvanica gave the poorest seed sets of all combinations.

According to Rohmeder (1963), a number of hybrids of F. excelsior x americana were raised from natural crossing in Bavaria in 1948. The putative hybrids were reported to grow faster and show more frost resistance than either parent.

Wright (1953) reported on hybridization attempts carried out by the USFS Northeastern Forest Experiment Station from 1947 to 1950. He was able to obtain true hybrids only from reciprocal crosses between F. pennsylvanica and F. velutina Torr. Failures were recorded in the following combinations: F. americana x pennsylvanica (and reciprocal), F. americana x velutina, F. americana x excelsior, F. pennsylvanica x excelsior (and reciprocal), and F. excelsior x quadrangulata.

Lazarescu (1956), in Rumania, obtained hybrids by artificial crossing of F. pennsylvanica with F. excelsior and further (Lazarescu 1967) recommended the production of such hybrids by natural pollination in seed orchards. Benea et al (1963) reported that the hybrid fruit developing m on F. pennsylvanica following controlled pollination were slightly smaller than normal but that the fruit produced on the Fl hybrid were significantly longer than either parent. Benea (1960) mentioned also the successful combination F. pennsylvanica x holotricha Koehne.

This history of controlled interspecific hybridization in Fraxinus has one serious omission. None of the authors were able to offer uncontestable proof of hybridity of any of the species combinations that they reported. Certainly, the results of some of the studies cast doubts on the validity of others. What is needed are techniques, preferably non-morphological, to verify hybridity.

CHEMICAL STUDIES

The earliest chemotaxonomic data on ash species was provided by Lingelsheim (1916) who showed that water extracts of the bark of various species gave a blue or blue-green fluroescence under ultraviolet light. This fluorescence was caused by coumarins (and coumarin gluycosides) and followed the Subsectional classification scheme. Of special interest was the fact that species of Subsection Melioides such as F. americana and F. pennsylvanica did not exhibit fluorescence. Species of Subsection Bumelioides, including the European F. excelsior and F. holotricha and the American F. nigra Marsh. and F. quadrangulata did fluoresce.

These observations were later confirmed and extended by Plouvier (1954) and a number of other authors cited by Hegnauer (1969) in his "Chemotaxonomie der Pflanzen". Although most of these references pertain to bark analyses, Paris and Stambouli (1960) did analyze leaf tissue of F. excelsior, F. nigra, and F. ornus.

As for flavonoids, Paris and Stambouli (1960) reported rutin (the 3-rhamnosylglucoside of the flavonol quercetin) in the leaves of F. excelsior, F. nigra, and F. ornus. Bate-Smith (1962) found both querceti and kaempferol in the acid-hydrolyzed leaves of F. excelsior. The work of Fitzgerald and Reines (1969) was the first to show that the leaves of F. americana contained flavone glycosides rather than flavonol glycosides Furthermore, Fitzgerald and Reines reported two flavone glycosides, luteolin 7-glucoside and luteolin 3-glucoside, in F. americana but did no find either of these compounds in F. pennsylvanica -- although they did not report what they did find in green ash.

The chemical data given above suggested that there was sufficient chemical variation among important landscape species to allow for biochemical verification of interspecific hybridity. The inheritance of flavonoids and coumarins that occur in substantial quantities is usually additive, and any true hybrid should contain the major compounds found in the parental species.

MATERIALS AND METHODS

The trees used in this study were mainly those contained in the specimen and test plantings of the National Arboretum. For biochemical analyses, fresh foliage was extracted by boiling for 1 hour in 70% ethanol to a final volume of 1 ml. for each gram of leaf tissue. The extracts were cleared by centrifugation before being stored in a refrigerator at 5°C. In the typical sequence of analysis, the extracts were first spotted on Whatman No. 30MM filter paper (20x20 cm) and subjected to two-dimensional ascending chromatography in BAW (n-buranol-glacial acetic acid-water, 4:1:5 v/v upper phase) and water.

These preliminary chromatograms, after being fumed with ammonia and viewed under ultraviolet light, provided preliminary information on the number and behavior of flavonoid and coumarin compounds likely to be found in the extract. The compounds were purified by repeated banding and elution of one-dimensional runs in BAW, water, or 15% acetic acid, in whatever sequence was most appropriate for complete separation from other substances. Purified materials were then tested against known compounds (quercetin 3-glucoside, quercetin 3-rutinoside, esculin, chlorogenic acid, etc.) in all three solvents, and occasionally in TBA (tertiary butanol-glacial acetic acid-water, 3:1:1 upper phase). Hydrolysis of purified substances to remove sugars was accomplished by boiling in 2N HC1 for one hour. Aglycones were chromatographed, with quercetin or esculetin controls, in BAW and Forestral solvent (acetic-acid-HC1-water, 30:3:10).

Sugars were analyzed by ascending paper chromatography in iso-propanol- water (4:1, v/v), with glucose control. Ultraviolet absorbtion spectra in 95% ethanol were determined before and after hydrolysis. Spectral shifts with a variety of additives (sodium acetate, boric acid, aluminum chloride) were recorded. The methods of analyses and standards for comparison are contained in Harborne (1967) and Mabry et al (1970).

A modest number of interspecific crossings were attempted using the techniques reported by Wright (1953) or by merely pollinating without bagging of female flowers on isolated trees. Most of the attempted crosses involved F. pennsylvanica. We especially wanted to determine if we could achieve true hybrids between this species and F. excelsior or F. rhynchophylla Hance.

RESULTS AND DISCUSSION

In Table 1 are listed the various flavonoid and coumarin compounds found in the species studied. Table 2 gives the Rf values, the movement of the compounds relative to the front, in several solvent systems.

It was found that leaf extracts of F. americana and F. pennsylvanica gave virtually identical flavonoid profiles. The major flavonoids were the flavone glycosides luteolin 7-glucoside and luteolin 7-diglucoside. Although the latter compound could have been mistaken for luteolin 3'-glucoside, the lack of a spectral shift with added sodium acetate indicated that the sugars were attached to the 7-position. We also found a trace of the flavonol glucoside rutin (quercetin 3-rutinoside) in both species and also appreciable amounts of chlorosenic acid. As shown in Table 1, the other species of Subsection Melioides were similar to white and green ash. A single specimen of F. velutina var. glabra 'Modesto' was analyzed and found not to contain the typical luteolin glycosides. Improper labeling and documentation of this particular plant was likely but the possibility remains that there may be some major variations within taxa. None of the Melioides species contained fluorescent coumarin compounds.

Coumarins were, however, found in all other species examined. Esculetin (6,7-dihydroxy coumarin) and its glucosides esculin and chicoriin were the most common coumarins. Esculin and esculetin tended to exhibit a blue-white fluorescence and chicoriin showed a rose-colored fluorescence. Fraxin (8-gluco-6-methoxy-7-hydroxy coumarin) fluoresced green under ultraviolet light. The presence of chlorogenic acid was noted in a member of species, but it did not mask the appearance of those coumarins with similar Rf values. A pink fluorescent spot (see Table 2) with distinctive Rf values was found in F. ornus, F. sieboldiana Blume, and F. rhynchophylla but it was not investigated further.

No viable seed was produced to crosses of green and white ash on F. excelsior, F. holotricha, or F. quadrangulata. We did, however, grow a number of seedlings from semi-controlled (no bagging) crosses of F. excelsior, F. holotricha, and F. rhynchophylla on F. pennsylvanica. Chromatographic analyses of the leaves of these seedlings showed that none were true interspecific hybrids.

Table 1. Flavonoids and coumarins found in leaves of *Fraxinus* species. [a/]

Species	Q-3-G [b/]	Q-3-R	L-7-G	L-7-GG	Esc	Esct	Chic	Frax	Chl
Section *Ornus*									
Subsection *Ornus*									
ornus	X	X	0	0	tr	X	X	0	0
sieboldiana	X	X	0	0	X	X	X	0	0
Subsection *Ornaster*									
rhynchophylla	X	X	0	0	X	X	0	0	0
Section *Fraxinus*									
Subsection *Sciadanthus*									
xanthoxyloides	0	X	0	0	0	X	X	X	?
Subsection *Melioides*									
americana	0	tr	X	X	0	0	0	0	X
biltmoreana	0	tr	X	X	0	0	0	0	X
pennsylvanica	0	tr	X	X	0	0	0	0	X
tomentosa	0	tr	X	X	0	0	0	0	X
velutina	0	tr	X	X	0	0	0	0	X
Subsection *Bumelioides*									
Series *Paniculatae*									
excelsior	X	X	0	0	X	X	0	0	X
quadrangulata	X	X	0	0	X	X	X	0	X
Series *Racemosae*									
holotricha	0	X	0	0	tr	X	0	0	X

a/ "X" denotes presence and "0" absence of a particular compound. "tr" denotes trace amounts.

b/ Q-3-G=quercetin 3-glucoside; Q-3-R=quercetin 3-rutinoside; L-7-G=luteolin 7-glucoside; L-7-GG=luteolin 7-diglucoside; Esc= esculin; Esct=esculetin; Chic=chicorin; Frax=fraxin; Chl=chlorogenic acid.

Table 2. R_f values (X 100) of flavonoids, coumarins, and related substances in ash species.

Compound	BAW	15% AC	H_2O
Quercetin 3-glucoside	58	37	08
Quercetin 3-rutinoside	45	51	23
Luteolin 7-glucoside	44	15	01
Luteolin 7-diglucoside	40	29	05
Esculetin	80	58	34
Esculin	53	76	56
Chicoriin	47	--	64
Fraxin	25	74	74
Chlorogenic acid	51	--	72
Pink spot	28	07	00

The variation in leaf flavonoids and coumarins among the ash species investigated in this study suggests that simple biochemical analyses could be used to verify interspecific hybridity in many combinations. Such analyses would be especially useful in controlled intersectional crosses where luteolin-containing species of Subsection Melioides were the male parents. True hybrid seedlings would then contain significant amounts of both quercetin and luteolin glycosides.

If species of Subsection Melioides are used as female parents, the presence of trace amounts of quercetin glucosides in these species could lead to erroneous conclusions as to hybridity with quercetin-containing species. If, however, the male parent species were strong coumarin producers, any true hybrids should contain these coumarins.

At this point, we have not been able to analyze any of the putative hybrids involving green ash or white ash that were developed by other research projects. It may be that none of these plants are, in fact, true hybrids. Until such time as ash hybridization activity is increased, the utility of the present study is rather speculative. However, flavonoid analyses have been used to verify hybridity in other genera and should also be of value in Fraxinus.

ACKNOWLEDGMENT

I would like to thank Harold E. Vettel for technical assistance on this project.

LITERATURE CITED

Anderson, Edgar and C.M. Whelden, Jr.
1936. STUDIES IN THE GENUS FRAXINUS II. DATA ON THE FLOWERING AND FRUITING HABITS OF THREE AMERICAN SPECIES OF ASH -- OF POSSIBLE ECONOMIC IMPORTANCE IN THE PRODUCTION OF RAPID-GROWING FOREST TREES. J. Hered. 27: 473-474.

Bate-Smith, E.C.
1962. THE PHENOLIC CONSTITUENTS OF PLANTS AND THEIR TAXONOMIC SIGNIFICANCE. Jour. Linn. Soc. (Bot.) 58: 95-173.

Benea, Vasile
1960. RECHERCHES SUR LES HYBRIDIZATIONS SEXUEES AUX ESPECES DU GENRE QUERCUS, FRAXINUS, ET PILAUS, EFFECTUEES DANS LA R.P.R. Proc. Fifth World Forestry Cong. vol. 2, p. 825-826.

Benea, V., C. Lazarescu, and L. Leandru
1963. RECHERCHES BIOMETRIQUES SUR LES FRUITS ET GRAINES DANS LES TRAVAUX DE SELECTION ET D'AMELIORATION DU FRENE. Proc. World Consult. Forest Genetics and Tree Impr. Document FAO/FORGEN 63-2b/11, 3 p.

Fitzgerald, Charles H. and Mervin Reines
1969. A COMPARATIVE STUDY OF THE FLAVONOID CONTENT OF FRAXINUS AMERICANA AND FRAXINUS PENNSYLVANICA. Castanea 34: 192-194.

Hegnauer, R.
1969. CHEMOTAXONOMIE DER PFLANZEN. Vol. 5, Birkhauser Verlag.

Henry, Augustine
1914-15 THE ARTIFICIAL PRODUCTION OF VIGOROUS TREES. Jour. Dept. Agr. and Tech. Instr. for Ireland 15: 34-52.

Johnson, L.P.V. and C. Heimburger
1946. PRELIMINARY REPORT ON INTERSPECIFIC HYBRIDIZATION IN FMK TREES. Can. J. Res. 24: 308-312.

'Lazarescu, C.
1956. CONTRIBUTION TO THE METHODS OF OBTAINING AND BREEDING ASH-TREE HYBRIDS. Rev. Biol. (Bucarest) 1: 117-126.

Lazarescu, C.
1967. (THE POSSIBILITY OF OBTAINING ASH HYBRIDS IN SEED ORCHARDS) Rev. Padurilor 82: 449-459. (In Rumanian) For. Abstr. 29: 34

Lingelsheim, A.
1916. DIE FLUORESZENZ WASSRIGER RINDENAUSZUGE VON ESCHEN IN HEE BEZIEHUNG ZUR VERWANDTSCHAFT DER ARTEN. Ber. Deut. Botan. Ges, 34: 665-675.

Paris, R. and A. Stambouli
1960. LES HETEROSIDES DU FRENE OFFICINAL (FRAXINUS EXCELSIOR) ET DE QUELQUES ESPECES VOISINES NOTAMMENT FRAXINUS ORNUS. Ann. Pharm. France 18: 873-887.

Plouvier, M. Victor
1954. SUR LA COMPOSITION HETEROSIDIQUE DE QUELQUES FRAXINUS (OLEACEES). Comot. Rend. Acad. Sci. Paris 238: 1835-1837.

Rehder, Alfred
1940. MANUAL OF CULTIVATED TREES AND SHRUBS. Ed. 2 Macmillan.

Rohmeder, E.
1963. EXPERIMENTS ON FOREST TREE HYBRIDS IN BAVARIA FROM 1936 to 1962. Proc. World Consult. Forest Genetics and Tree Impr. Document FAO/FORGEN 63-2b/1, 6 p.

Scheller, H.
1977. KRITISCHE STUDIEN UBER DIE KULTIVIERTEN FRAXINUS-ARTEN. Mitt. Dtsch. Dendrol. Ges. 69: 49-162.

Wright, Jonathan W.
1953. SUMMARY OF TREE-BREEDING EXPERIMENTS BY THE NORTHEASTERN FOREST EXPERIMENT STATION 1947-50. Northeast. For. Expt. Sta., Sta. Paper No. 56, 46 p.