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ANTHOCYANINS OF HOLLY FRUITS*

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Abstract—Anthocyanin distribution in the fruits of *Ilex* closely followed an accepted taxonomic classification. Within the evergreen subgenus Aquifolium, species belonging to section Lioprinus produced only cyanidin pigments while in section Aquifolium, pelargonidin was the major anthocyanin. Likewise, in the deciduous subgenus Prinos, species of section Prinoides contained cyanidin pigments and those of section Euprinus had pelargonidin compounds. Exceptions to this pattern and the bearing of pigment studies on breeding are discussed.

THE FRUITS or berries of holly (*Ilex*) species may be red, orange, yellow, white, or black. These fruit colors are found in both evergreen and deciduous species. Red-fruited species predominate among the native and cultivated species in temperate climates, and the major pigments are anthocyanins. Yellow-fruited variants of these species lack anthocyanins and are pigmented by carotenoids. The few orange-fruited cultivars or variants may contain both carotenoid and anthocyanin pigments. Only two black-fruited species are commonly cultivated in the United States: *I. glabra* (L.) Gray, a native species; and *I. crenata* Thunb. from Japan. Mutants lacking fruit anthocyanins have been found in the wild. Fruits of these are white in *I. glabra*, f. *leucocarpa* F. W. Woods (e.g. 'Ivory Queen') and greenish-yellow in *I. crenata* f. *watanabeana* Makino.

Up to the time of the present study, only a few reports on the identification of anthocyanin pigments in holly fruits had been noted in the literature. Robinson and Robinson,[1] using color and distribution tests, determined that the anthocyanin of red berries of *I. aquifolium* L. (English holly) was a pelargonidin 3-bioside. Hayashi[2] crystallized and characterized a cyanidin 3-xyloxylglucoside, which he termed 'ilicicyanin', from the black fruit of *I. crenata*. Hegnauer[3] considered that this disaccharide was primeverose, but Harborne[4] believed it to be sambubiose. Hayashi and Abe[5] reported a pelargonidin 3-hexopentoside and 'chrysanthemin' (cyanidin 3-glucoside), in the ratio of 9:1, in the red fruit of the deciduous species *I. geniculata* Maxim.

An exploratory study of holly fruit anthocyanins was begun at the U.S. National Arboretum in September 1971. The main purpose was an attempt to explain some seemingly unusual inheritance patterns of fruit color that resulted from interspecific hybridization.

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[1] G. M. ROBINSON and R. ROBINSON, *Biochem. J.* **25**, 1687 (1931).
[2] K. HAYASHI, *Acta Phytochim. Japan* **13**, 25 (1942).
[3] R. HEGNAUER, *Chemotaxonomie der Pflanzen*, Band 3, p. 169, Birkhauser Verlag, Basel (1964).
[4] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 131, Academic Press, New York (1967).
[5] K. HAYASHI and Y. ABE, *Misc. Rep. Res. Inst. Nat. Resour. Tokyo* **29**, 1 (1953).

The early results were so interesting that the investigation was extended to include a wide range of species, hybrids, and cultivars.

When this work was complete, two papers by Ishikura[6,7] were brought to my attention. In the first, Ishikura reported the presence of the xylosylglucosides of both cyanidin and pelargonidin in *I. aquifolium*. The second paper noted the same two pigments in two other species of Section Aquifolium (*I. integra* Thunb. and *I. latifolia* Thunb.) as well as in the deciduous species *I. serrata* Thunb. of Section Euprinus. Several species of Section Lioprinus, including *I. crenata*, were reported to contain only cyanidin compounds (3-glucoside and 3-xylosylglucoside).

TABLE 1. ANTHOCYANIN PIGMENTS IN FRUITS OF *Ilex* (HOLLY) SPECIES

Subgenus Section species	Pelargonidin 3-Glucoside	Pelargonidin 3-Xylosylglucoside	Cyanidin 3-Glucoside	Cyanidin 3-Xylosylglucoside
Aquifolium				
Aquifolium	+	+	0	0
aquifolium	+	+	0	0
ciliospinosa	0	+	0	0
corallina	+	0	0	0
cornuta	tr	+	0	0
georgei	0	++	0	+
integra	0	+	0	0
latifolia	0	+	0	0
leucocladia	tr	+	0	0
perado	+	+++	0	0
pernyi	0	+	0	0
vomitoria	0	0	0	+
Lioprinus				
cassine	0	0	0	+
chinensis	0	0	0	+
coriacea	0	0	++	+
crenata	0	0	+	+
glabra	0	0	+(4)	+
myrtifolia	0	0	0	+
opaca	0	0	0	+
pedunculosa	0	0	+	+
sugeroki	0	0	0	+
Prinos				
Euprinus				
serrata	+	+(9)	0	0
verticillata	+	+++	0	0
Prinoides				
amelanchier	0	0	0	+
decidua	0	0	0	+

Key. + indicates definite presence and 0 indicates absence of a particular pigment; number of + signs including numbers in parentheses, denote relative proportions of pigments in a given species; tr indicates trace amounts of pigment.

Inasmuch as Ishikura's results on the Sections Aquifolium and Euprinus differed so substantially from our own, new fruit collections and new analyses were made of several

[6] N. ISHIKURA, *Bot. Mag. Tokyo* **84**, 113 (1971).
[7] N. ISHIKURA, *Phytochem.* **10**, 2513 (1971).

species: *I. serrata*, *I. aquifolium*, *I. latifolia*, and *I. cornuta* Lindl. The results of these analyses did not differ from our earlier determinations.

Holly fruit used in this study were all grown in the grounds or greenhouses of the U.S. National Arboretum in Washington, D.C. There are probably between 450 and 500 species of *Ilex* in the world, but relatively few are in cultivation. The species available for analysis included the majority of those presently cultivated in the United States and used in the Arboretum's extensive holly breeding project between 1956 and 1971.[8] Although we attempted to include at least two different cultivars or individuals of each species in our analyses, this was not possible with some of the rarer species such as *I. georgei* Comber and *I. mitis* (L.) Radlk.

RESULTS

The anthocyanin pigments found in the majority of the species examined are given in Table 1. The species are listed according to their subgeneric and sectional classification following Rehder,[9] with additional information on Asiatic species from Hu.[10] The sectional classification of holly species is by no means settled, but the scheme in Table 1 is currently the most widely used.

It can be seen that at least one accepted classification of cultivated holly species is closely correlated with the presence of cyanidin or pelargonidin as the major pigment type. The only exceptions to this close agreement were *I. vomitoria* Ait. and *I. georgei*. *I. vomitoria* was separated from Section Aquifolium by Loesener[11] and may well have closer affinities to other species in the genus that contain cyanidin pigments. Both cyanidin and pelargonidin pigments were found in the fruit of *I. georgei*. R_fs for the major pigments are given in Table 2. Absorbtion spectra followed data given by Harborne.[4]

TABLE 2. R_fS ($\times$ 100) OF MAJOR ANTHOCYANINS OF *Ilex* FRUITS ON WHATMAN NO. 1 PAPER

Pigment	Solvent BAW	1% HCl	HOAc–HCl	Pigment	Solvent BAW	1% HCl	HOAc–HCl
Cyanidine 3-glucoside	37	07	26	Pelargonidin 3-glucoside	42	11	35
Cyanidin 3-xylosylglucoside	36	25	52	Pelargonidin 3-xylosylglucoside	38	32	60

In addition to the pigments reported in Table 1, *I. crenata* was also found to produce a petunidin 3-bioside (R_f 0·24 in BAW, 0·39 in 1% HCl, 0·69 in HOAc–HCl; λ_{max} 533 nm in MeOH–HCl. The aglycone had an R_f of 0·45 in Forestal and had maximum absorbtion at 543 nm in MeOH–HCl). There also appeared to be a trace of a petunidin 3-monoside in this species.

Two other holly species, of uncertain sectional status, were also investigated. The black-fruited yerba maté of South America (*I. paraguariensis* St. Hil.) contained cyanidin 3-xylosylglucoside and cyanidin 3-glucoside in a 5:1 ratio. The only subequatorial African holly (*I. mitis*), a red-fruited species, produced only cyanidin 3-xylosylglucoside. Of the

[8] G. EISENBEISS and F. S. SANTAMOUR, JR., *Amer. Horticulturist* **51** (2), 32 (1972).
[9] A. REHDER, *Manual of Cultivated Trees and Shrubs*, 2nd edn, Macmillan, New York (1940).
[10] S. Y. HU, *Nat. Hort. Mag.* **36**, 31 (1957).
[11] TH. LOESENER, *Nova Acta (Abh. der Kaiserl, Leop.-Carol. Deutschen Akad. der Naturforscher)* **78**, 1 (1901).

more commonly cultivated species, no fruit of *I. rugosa* Fr. Schmidt (Section Aquifolium) was available for study, but since the hybrid *I. rugosa* × (*cornuta* × *pernyi* Franch.) gave only pelargonidin 3-xylosylglucoside, *I. rugosa* was probably similar to other species of the section in producing only pelargonidin.

Interspecific hybrids between cyanidin- and pelargonidin-containing species were found to produce both pigment types. Such hybrids are usually intersectional crosses and are difficult to achieve, even under conditions of controlled pollination in the greenhouse. Likewise, crosses of *I. vomitoria* (cyanidin producer) with its supposed close relatives in Section Aquifolium (pelargonidin producers) are rarely successful. However, three such hybrids were available and were analyzed: *I. cassine* L. × (*aquifolium* × *perado* Ait.), *I. decidua* Walt. × *aquifolium*, and *I. vomitoria* × (*cornuta* × *pernyi*). All three hybrids produced both cyanidin and pelargonidin pigments.

DISCUSSION

The presence and amount of cyanidin pigments in both the black-fruited and red-fruited species of Section Lioprinus provided some clues for interpretation of breeding results, but the picture is still far from clear. For example, black fruit of *I. glabra* contained about 10 times as much pigment on a fr. wt basis as the red fruit of *I. sugeroki* Maxim. Hybrids between these two species produced fruit with about 50% of the pigment concentration of the black-fruited parent, but the fruits were dark purple. When this first generation hybrid was crossed to a nonpigmented cultivar of *I. glabra*, the fruit of the progeny were black, and pigment concentration was higher than in the original *I. glabra*. Likewise, progeny of a cross between the yellowish-fruited *I. crenata* f. *watanabeana* with red-fruited *I. yunnanensis* Franch. (supposed cyanidin producer) gave black fruit with higher pigment levels than in normal *I. crenata*. Thus, breeding for red-fruited cultivars with the habit, foliar, and cultural characteristics of *I. crenata* and *I. glabra* does not appear feasible when only cyanidin-producing species are used.

It is well known that cyanidin-based anthocyanins may be complexed into bluish pigments by metallic ions or co-pigments.[12] Preliminary investigations of metallic ion concentration of leaf and fruit extracts of a wide range of species and cultivars, using an atomic absorbtion spectrophotometer, did not reveal any significant differences in content of aluminum, magnesium, molybdenum, or several other ions. Likewise, qualitative analyses of PCs of leaf and fruit extracts of black-fruited and red-fruited species showed a similar distribution of flavonoids.

In the absence of more substantial chemical data, we are presently attempting to develop red-fruited plants with the growth habit and leaf characteristics of *I. crenata* and *I. glabra* by crossing white- or yellow-fruited cultivars of these species with species containing pelargonidin pigments as the sole anthocyanins.

At this juncture, it is impossible to reconcile the differences between the results of the present study and those of Ishikura,[6,7] with regard to *I. aquifolium*, *I. integra*, *I. latifolia* and *I. serrata*. In no case did we find cyanidin derivatives in these species as claimed by the Japanese author. The results we report do appear to be consistent with the classification and it does appear that anthocyanins may be extremely valuable chemical characters for delineating taxa in the genus *Ilex*.

[12] J. B. HARBORNE, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), p. 247, Academic Press, New York (1965).

EXPERIMENTAL

Anthocyanin pigments were extracted from mature holly fruits in cold MeOH-1% HCl. In most species, the anthocyanins were only in the skin (epicarp), but in *I. glabra* and other black-fruited species, some internal tissues were also pigmented. Pigments were analyzed and purified by ascending PC, on Whatman No. 1 and No. 3 MM paper, using BAW (*n*-BUOH–HOAC–H_2O, 4:1:5, upper) aq. 1% HCl (97:3) and HOAc–HCl (HOAc–HCl–H_2O, 15:3:82). Extreme care was necessary in purification, since one or more cinnamic acids were frequently eluted with the anthocyanins. When the UV spectra of pigments were determined, these acids gave absorbtion peaks at 316 or 330 nm; and gave the impression that the anthocyanins were acylated.

Purified pigments were hydrolyzed by boiling in 4 N HCl for 20 min. The products of hydrolysis were analyzed by standard PC techniques: aglycones in Forestal solvent (HOAc–HCl–H_2O, 30:3:10) and simple sugars in *iso*PROH–H_2O (4:1). We did not attempt to isolate or identify disaccharides. Absorbtion spectra of the purified anthocyanins and anthocyanidins in the visible and UV range were determined with a recording spectrophotometer.

Note added in proof. Cyanidin 3-sophoroside was the only anthocyanin found in the fruit of *I. pubescens* Hook. & Arn., which is placed in Sect. Pseudoaquifolium Hu by Hu.[10] This disaccharide appears to be unusual in *Ilex*, and may provide a chemical clue to the classification of species belonging to this little-known Section.