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Flavonoids in the leaves of polish species of the genus *Betula* L.

III. The flavonoids of *B. oycoviensis* Bess. leaves

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Abstract

B. oycoviensis Bess. leaves were found to contain compounds characteristic of *B. "nova"* i.e. myricitrin, isoquercitrin and probably also kaempferol 3-rhamno-7-glucoside, quercetin 3,7,4′-trimethyl ether, and quercetin 7,3′,4′--trimethyl ether. They also contain compounds which occur in *B. penduia* Roth. (kaempferol 3-glucoside, isorhamnetin 3-glactoside, 6-methoxykaempferide, acacetin 7-glucoside, and probably scutellarein 7-glycoside). These biochemical traits bring out still better the hybrid origin of *B. oycoviensis*.

INTRODUCTION

Among Polish species of the genus *Betula*, *B. oycoviensis* attracted particular attention and was described for the first time in 1809 by Besser, because it was considered to be an endemic Polish plant. However, further studies showed it also grew in Denmark, Sweden, Czechoslovakia, Romania, and the USSR (Korczyk, 1967a). Detailed morphological and systematic studies carried out by Jentys-Szaferowa and co-workers proved *B. oycoviensis* to be a hybrid of *B. pendula* (= *B. verrucosa* Ehrh.) and the so-called *B. "nova"* (Jentys-Szaferowa, 1953, 1967; Korczyk, 1967b, 1967c; Więckowska, 1967; Szwabowicz, 1971, 1972, 1976; Jentys-Szaferowa et al. 1974.) *B. pendula*, *B. "nova"*, and *B. oycoviensis* differ from one another by a number of morphological traits and biological properties such as, e.g. the shape and number of leaves on fructifying short shoots (Fig. 1) the time of first florescence, and the distribution of male catkins. Besides, there is a great variety of transition forms of *B. oycoviensis* between a shrub typical of *B. "nova"* and a tree characteristic of *B. pendula*.

It seemed interesting to study whether and how far the hybrid character of *B. oycoviensis* would be reflected in its flavonoids complex.

MATERIAL AND METHODS

1. Material for comparative-taxonomic studies

Comparative studies were performed on fresh leaves of the long shoots. Those of the plants growing in the Botanical Garden of the Jagiellonian University in Cracow were subjected to morphological examinations. For this reason their symbolic designation was continued in the present work. Chemotaxonomic analysis was carried out on the following plants:

"OB" — grows in the Botanical Garden; it was transplanted from Hamernia in 1920 (Jentys-Szaferowa, 1967); it is a large shrub with small leaves,

"B-81" — grew in the Botanical Garden, bred by the crossing of "A-39" with "OB" (Więckowska, 1967); it was a small 16-year-old tree with very small leaves; in the spring of 1975 it was removed from the garden,

"O-1" — grows in the Kobylańska Valley (KV); it is a mature tree with small leaves,

"O-2a" — grows in KV; it is a young tree with very small leaves,

"O-2b" — grows in KV beside "O-2a"; it is a young tree with small leaves,

"O-3" — grows in KV; it is a mature large under-shrub with small leaves.

The analyses were made on material collected on 8th July and 4th September, 1972, and on 15th July and 27th August, 1973.

Moreover, six 15-year-old synthetic specimens, i.e. three *B. pendula* × *B. "nova"* (the H_6 generation — Jentys-Szaferowa et al., 1974), denoted here by symbols "pxn 1-3", and three plants of *B. "nova"* × *B. pendula* (the H_7 generation — Jentys-Szaferowa et al., l.c.) designated as "nxp 1-3", were the object of comparative studies. Material from them was collected on 27th August, 1978.

2. Material for quantitative studies

In order to obtain additional data on the relationship of predominant flavonoids, examinations were carried out for the content of these com-

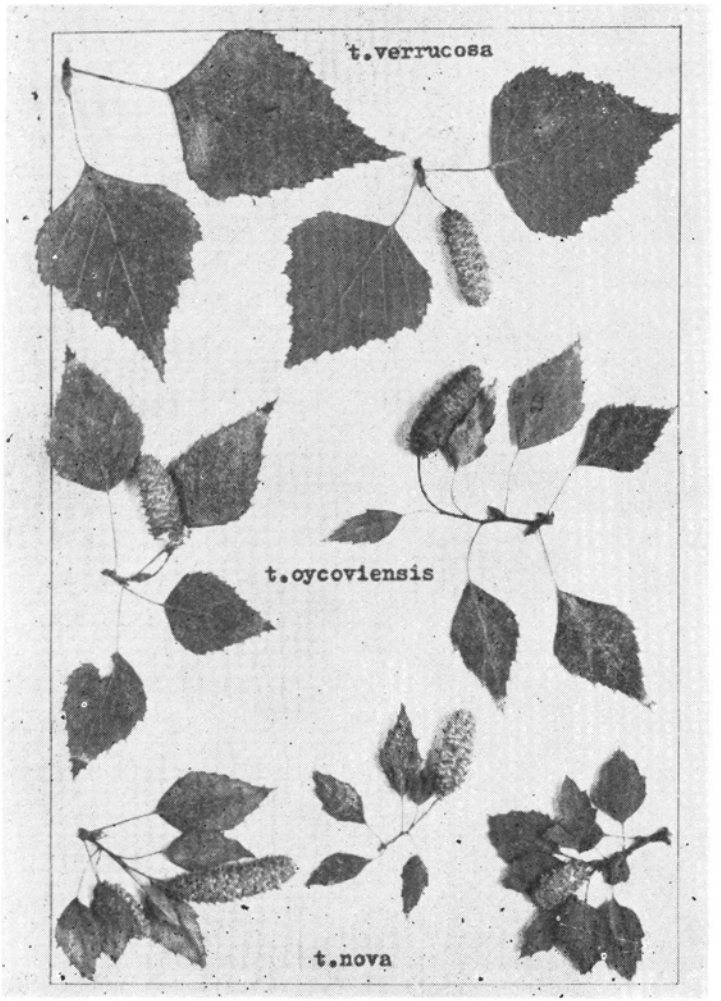

Fig. 1. Fructifying short shoots of *B. pendula*, *B. oycoviensis* and *B. "nova"* according to Jentys-Szaferowa.

pounds. The analysis was made twice on the plants "OB", "B-81", "O-1", "O-2a", "O-2b", and "O-3": first on the material collected on 30th August, 1974, and was repeated on the leaves gathered on 18th August, 1975. No leaves of "B-81" were collected in 1975, as this specimen had been removed from the Botanical Garden in the spring of that year.

3. Material for analysis in initial vegetation period

In order to follow the succession of occurrence of flavonol glycosides, the flavonoids were identified in extracts of the material collected in the initial vegetation period. Every few days 2 g of leaves was gathered from each specimen: in 1972 on 5th, 13th, 17th, and 21st April, and in 1973 on 12th and 18th April and on 10th May. The material from the first gathering in the year (5th April, 1972, and 12th April, 1973) was in the leaf bud phase. In 1972 "OB", "B-81", and "A-39" were investigated. In the subsequent year the material came only from the first two specimens, as the third one perished in the winter of 1972/1973. It was not taken into consideration in taxonomic analysis since no more than 1.5 g of leaves could be collected from the whole plant in July 1972. "A-39", which had been bred from the seeds brought from Karniowice, grew in the Botanical Garden; it was a small shrub with very small leaves.

The material for all examinations was gathered from the most insolated part of the crown of tree or shrub, on sunny days, at the most suitable time, i.e. between 1.00 and 2.00 p.m. (P a w ł o w s k a, 1976).

4. Isolation and identification

Isolation and identification of flavonoids was performed as described in the earlier paper (P a w ł o w s k a, 1980a).

5. Quantitative determination of myricitrin, hyperoside, and quercitrin

Fresh leaves were put on dry ice. The weighed amount of 15.000 g ± 0.009 was homogenized with petroleum ether. After one hour the homogenate was centrifuged. The extract was decanted and petroleum ether was poured over the material again. Washing was repeated until the extract became colourless. The remainder was infused with methanol and left to stand for 24 hours at about 4°C. Next, following centrifugation the extract was poured off and methanol was poured in again. This procedure was repeated until the extract became colourless. Extracts

from single specimen belonging to the same group were mixed together and evaporated to the volume of about 50 ml, this was treated with an equal amount of chloroform. After thorough shaking, the preparation was left to stand for 15 minutes, and subsequently separated. The upper methanol-water phase was subjected to further procedure, whereas the bottom, chloroform phase was discarded.

The methanol-water extract was separated by means of ascending thin-layer chromatography on glass plates measuring 30/24 × 50 cm and covered with a mixture of MN 300 cellulose and kieselguhr G (1:1 w/w), and it was developed in the solvent system amyl alcohol : isoamyl alcohol : 80% formic acid (1 : 1 : 1 v/v). Fractions from the bands: I of Rf 0.29 -0.41, II of Rf 0.42-0.55, and III of Rf 0.70-0.80 were eluted with methanol and rechromatographed on MN 300 cellulose, using water as a moving phase. The fractions were eluted with 50 ml methanol, then filtered, the sediment being repeatedly washed with 10-ml portions of methanol until complete washing was achieved. The eluates of myricitrin and hyperoside were made up to a volume of 100 ml and that of quercitrin to 50 ml, readings of the extinction values were then taken on a Beckman Model 25 spectrophotometer: for hyperoside, myricitrin and quercitrin at 257, 255 and 256 nm respectively.

The amounts of these compounds were read from standard curves. The latter were plotted on the basis of the read-out extinction values for methanol solutions of the standard substances of these compounds, at varying concentrations. Standard quercitrin and hyperoside were Fluka products, while standard myricitrin was obtained by isolation from *Corylus avellana* L. leaves (Pawłowska, 1980b).

RESULTS

1. Composition of flavonoid compounds

Identification of the flavonoid compounds present in the leaves of *B. pendula* and *B. oycoviensis* was reported earlier (Pawłowska, 1980a). The identification of the compounds which were found to occur in the *B. "nova"* leaves as well as in those of *B. oycoviensis* is discussed in another paper (Pawłowska, 1980b).

A compilation of two-dimensional chromatograms of *B. "nova"*, *B. oycoviensis*, and *B. pendula* is presented in Fig. 2. The compounds which occur in *B. "nova"* and *B. oycoviensis* leaves are dotted, while those present in *B. pendula* and *B. oycoviensis* leaves are chequered.

The flavonoids and polyphenol acids detected in the leaves of *B. oycoviensis* specimens under study are shown in Table 1.

Table 1

Composition of flavonoid compounds in *B. oycoviensis* specimens and parental species

No. of compound		1	2	3	4	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	24
Species/° Specimen \ Name		quercitrin	myricitrin	hyperoside	myricetin 3-digalactoside	hesperidin	isorhamnetin 3-galactoside	isoquercitrin	quercetin 3,7,4′-trimethyl ether	kaempferol 3,4′-dimethyl ether	quercetin 3-glycoside-7,4′-dimethyl ether (?)	quercetin 7,3′,4′-trimethyl ether (?)	dihydrohyperoside	kaempferol 3-rhamno-7-glucoside	6-methoxy-kaempferide	scutellarein 7-glycoside (?)	caffeic acid	gallic acid	chlorogenic acid	quercetin 3-arabinoside	acacetin 7-glucoside	kaempferol 3-glucoside
B. "nova"		+	+++	++	++	+	−	+	tr.	−	−	tr.	+	+	−	−	+	+	+	+	−	−
B. oycoviensis	B-81	++	+++	+++++	++	+	−	++	tr.	−	−	−	+	tr.	−	−	+	+	+	+	−	tr.
	0-2a	+++	+++	+++++	++	+	+	++	−	−	−	−	+	+	−	−	+	+	+	++	−	tr.
	0-2b	+	++	++++	++	+	tr.	++	tr.	−	−	tr.	+	+	+	−	+	+	+	+	−	tr.
	n×p1	+++	+++	+++++	++	+	−	++	tr.	−	−	−	+	+	−	−	+	+	+	++	−	+
	n×p2	+++	+++	+++++	++	+	+	+	−	tr.	−	−	+	tr.	tr.	−	+	+	+	++	tr.	tr.
	n×p3	++	+++	+++++	++	+	−	+	−	−	−	−	+	−	−	−	+	+	+	+	+	tr.
	p×n1	++	++	+++++	+	+	tr.	+	−	−	−	−	+	+	+	−	+	+	+	+	+	tr.
	p×n3	++	++	+++++	++	+	+	+	tr.	−	−	tr.	+	−	+	tr.	+	+	+	tr.	+	tr.
	p×n2	+++	+++	+++++	++	+	+	−	−	−	−	−	+	++	+	tr.	+	+	+	+	+	tr.
	0-1	+	+++	+++	+	tr.	+	+	−	tr.	tr.	−	+	−	+	tr.	+	+	+	+	−	+
	0-3	++	+++	+++	++	+	tr.	−	−	tr.	−	−	+	−	+	−	+	+	+	+	tr.	tr.
	OB	+	+	+++	+	tr.	+	−	−	−	−	−	+	−	++	tr.	+	+	+	tr.	+	+
B. pendula		++	−	++++	+	+	+	−	−	tr.	tr.	−	+	−	+	tr.	+	+	+	tr.	tr.	tr.

I° — B. "nova" and *B. pendula* an earlier study (Pawłowska, 1980a, 1980b); tr. — trace amount; "+" — observable in visible light after development; "+" to "+++++" — correspondingly more amount

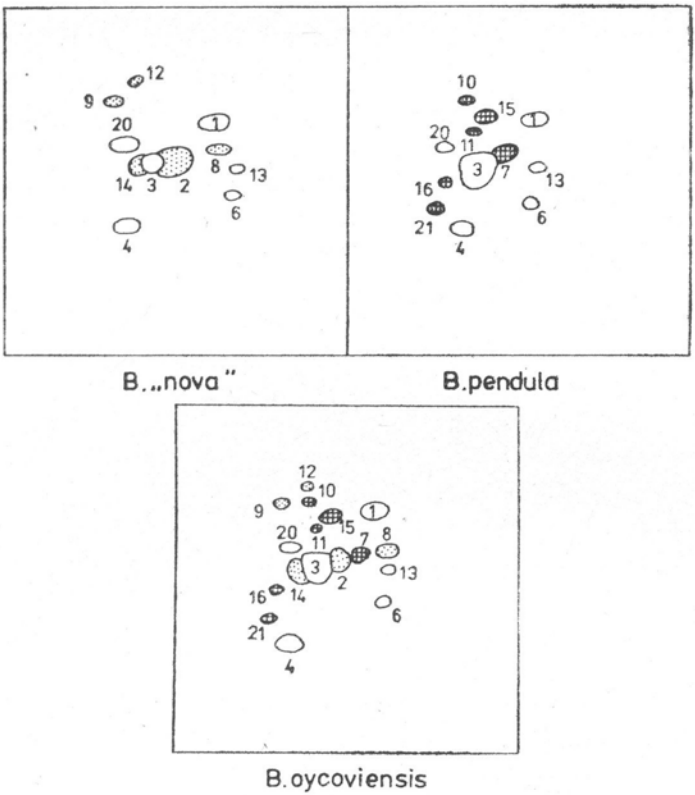

Fig. 2. Schemes of two-dimensional chromatograms for *B. oycoviensis* and its parental forms in the systems: 15% acetic acid in first dimension — abscissa; BAW (n-butanol : acetic acid : water 12 : 3 : 5 v/v) in the other dimension — ordinate.

2. Myricitrin, hyperoside and quercitrin content

The amounts of myricitrin and hyperoside in three specimens ("O-2a", "O-2b", and "O-3") were equivalent, in two ("B-81" and "O-1") the myricitrin content exceeded that of hyperoside, whereas in "OB" the proportions of the two compounds were reversed (Table 2). The amount of quercitrin in all samples examined was lower than of myricitrin and hyperoside, but it was most stable. In four specimens ("OB", "B-81", "O-1", and "O-2a") the ratio of quercitrin to hyperoside content was constant and was equal to 1 : 3.

3. Flavonoids of the initial vegetation period

Samples gathered in the leaf bud phase from "B-81", "A-39", and "OB" (5th April, 1972) and from "B-81" and "OB" (12th April, 1973)

Table 2

Amounts of principal flavonol glycosides in *B. oycoviensis* specimens

Specimen	Compound						Mean values			Content ratios	
	1		2		3		1	2	3	3:1	3:2
	a	b	a	b	a	b					
"OB"	0.098	0.110	0.099	0.115	0.300	0.350	0.104	0.103	0.325	3.13	3.15
"B-81"	0.137	—	0.220	—	0.370	—				2.70	1.68
"0-1"	0.070	0.080	0.210	0.225	0.220	0.200	0.075	0.218	0.210	2.80	0.96
"0-2a"	0.126	0.141	0.153	0.160	0.350	0.328	0.133	0.157	0.336	2.53	2.14
"0-2b"	0.068	0.066	0.130	0.145	0.305	0.240	0.067	0.138	0.272	4.06	1.97
"0-3"	0.130	0.132	0.165	0.195	0.170	0.250	0.131	0.180	0.210	1.60	1.17

The values are expressed in % per fresh leaf mass. Material collected: a — August 30, 1974; b — August 18, 1975

revealed the presence of negligible amounts of free flavonols. Quercetin occurred in all these samples, whereas vestigial amounts of myricetin were found in "A-39" and "B-81" and trace amounts of kaempferol in "OB" (Table 3). The appearance of quercitrin (in all specimens under study) was noted in the material collected some days later (13th April, 1972, and 18th April, 1973) as well as vestigial amounts of myricitrin (in "A-39" and "B-81") or hyperoside (in "OB"), but flavonols were detected no more. In further samples gathered on 17th and 21st April, 1972, and on 10th May, 1973 the observed quercitrin content did not undergo any significant change, and the amounts of myricitrin and hyperoside increased.

Table 3

Occurrence of flavonoids in *B. oycoviensis* leaves in the initial period of vegetation

Specimen	Date of collection						
	1972				1973		
	5.04	13.04	17.04	21.04	12.04	18.04	10.05
"A-39"	Q, tr. M	M, 1	tr. 1, 2 tr. 3	1, 2, 3	—	—	—
"B-81"	Q, tr. M	1, tr. 2	1, 2, 3	1, 2, 3	Q, tr. M	1, tr. 2	1, 2, 3
"OB"	Q, tr. K	1, tr. 3	1, 3	1, tr. 2, 3	Q, tr. M tr. K	1, 3	1, tr. 2, 3

K — kaempferol; M — myricetin; Q — quercetin; 1 — quercitrin; 2 — myricitrin; 3 — hyperoside

DISCUSSION

By free pollination of *B. oycoviensis* Więckowska (1967) obtained two types of birches: *B. oycoviensis* and *B. pendula* in the ratio 1:1. Next, by crossing *B. oycoviensis* "A-39" with *B. oycoviensis* "OB" three types of segregants were obtained: *B. "nova", B. oycoviensis,* and *B. pendula* in the proportion 1:2:1 (Jentys-Szaferowa et al., 1974). Crossing of *B. "nova"* with *B. pendula* yielded a uniform progeny bearing the traits of *B. oycoviensis* (Jentys-Szaferowa, 1967). These investigations have proved *B. oycoviensis* to be a hybrid, despite the regular progress of microsporogenesis and normally developed pollen in it (Szwabowicz, 1971, 1976). It should be emphasized that all three types of segregants are equipped with the same chromosome set 2n = 28 (*B. pendula* — Woodworth, 1929, 1930; *B. oycoviensis* — Skalińska et al., 1959; *B. "nova"* — Szwabowicz, 1972).

The present investigations show the hybrid character of *B. oycoviensis* in its biochemical aspect as well. The specimens of this species were

found to contain compound specific for *B. "nova"*, i.e. myricitrin, quercetin 3-glucoside (isoquercitrin), kaempferol 3-rhamno-7-glucoside, quercetin 3,7,4′-trimethyl ether, and quercetin 7,3′,4′-trimethyl ether. At the same time some compounds were detected which point to the descendance of *B. oycoviensis* from *B. pendula;* these were isorhamnetin 3-galactoside, 6-methoxykaempferide, kaempferol 3,4′-dimethyl ether, kaempferol 3-glucoside, acacetin 7-glucoside, scutellarein 7-glycoside, and quercetin 3-glycoside-7,4′-dimethyl ether.

The quantitative analyses of the main flavonol monoglycosides revealed the hyperoside content (in 5 specimens out of 6 studied) to be nearly twice as high as that of myricitrin, and even three times the amount of it, as observed in "OB", and about three times higher than the quercitrin content (Table 2). It was solely specimen "0-1" which contained more myricitrin than hyperoside. On the basis of assessment with the naked eye of the intensity and size of the spot on two-dimensional chromatograms, the synthetic specimens were found to be characterized by similar proportions of the mentioned monoglycosides present therein, independently of the direction of the cross (i.e. which of the two species was mother and which father).

On the basis of the occurrence of some definite compounds — biochemical properties inherited from parental forms — a differees can be observed within the group of *B. oycoviensis* samples studied; some of them are more like *B. "nova"* ("B-81", "O-2b", and "O-2a", and only one synthetic "nxp 1") and others resemble more *B. pendula* ("OB", "O-1", "O-3" and one synthetic "pxn 2"), this being illustrated in Table 1. Likewise "OB" and "O-1" are similar in shape to *B. pendula*. Jentys-Szaferowa (1967) also revealed differentiation among the cultivated *B. oycoviensis* plants from the B generation (*B. oycoviensis* × *B. oycoviensis*) into those which in their morphology are more like *B. "nova"* and others resembling *B. pendula*. However, the composition of the leaf flavonoids in the majority of synthetic specimens (4 out of 6 studied) does not exhibit a predominance of the compounds characteristic of any of the parental species (Table 1 — middle group). The studies by Jentys-Szaferowa et al. (1974) have shown that, among the *B. oycoviensis* specimens investigated, synthetic birches are the most uniform as regards their morphological properties as well.

Similarly as in *B. pendula*, a certain correlation is noted between the size of leaf-blades of *B. oycoviensis* and the presence in them of betuletol (6-methoxykaempferide). The specimens with larger leaves contain this compound, whereas it was not found in the plants with smaller leaf-blades (Pawłowska, 1980a). Nevertheless, this correlation is less conspicuous in *B. oycoviensis* than in *B. pendula*.

During growth of the leaves of not only *B. oycoviensis* (Table 3) but also *B. pendula* and *B. pubescens* (Pawłowska, unpublished) quercitrin appears as the first flavonoid glycoside. Besides, this compound occurs in all hitherto investigated species of the family *Betulaceae* except. *B. humilis* (Hänsel, Hörhammer, 1954, Hörhammer et al., 1956; Pawłowska, 1976, 1980a, 1980b). Moreover, the leaves of *B. oycoviensis* under study contain an amount quercitrin which is very stable as compared with that of the flavonoid monoglycosides whose content was determined and which, as has already been said, are the main representatives of flavonoids in this species (Table 2). The here presented data encourage the assumption that in the family *Betulaceae* quercitrin is evolutionally an earlier flavonol glycoside than hyperoside, myricitrin or myricetin 3-digalactoside.

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Flawonoidy liści polskich gatunków z rodzaju Betula L. *III. Flawonoidy liści B. oycoviensis* Bess.

Streszczenie

Analiza składu jakościowego flawonoidów ze świeżych liści pochodzących z dwunastu osobników *B. oycoviensis* potwierdziła charakter mieszańcowy tego gatunku. Osobniki *B. oycoviensis* posiadały zarówno cechy biochemiczne charakterystyczne dla *B. "nova"*, a mianowicie myrycytrynę, izokwercytrynę, 3-ramno-7--glukozyd kempferolu oraz 3,7,4'- i być może 7,3',4'-trójpochodne kwercetyny, jak i dla *B. pendula* tj. 3-galaktozyd izoramnetyny, 3-glukozyd kempferolu, betuletol (6-metoksy-kempferyd), 7-glukozyd akacetyny oraz być może 3-glikozyd 7-4'-dwumetylo-kwercetyny i 7-glikozyd skutelaryny.