ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. XLV, nr 4
1976

The chemotaxonomic investigations on the flavonoid compounds in the leaves of *Saxifraga aizoon* Jacq.

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(Received: January 22, 1976)

Abstract

On the basis of the investigations on flavonoid compounds a division of *Saxifraga aizoon* species into two races is proposed one containing cyanidin 3-glucoside and the other devoid of this compound.

INTRODUCTION

The investigations on the variability of *Saxifraga aizoon* Jacq. done by Staszkiewicz and Wójcicki (1977) showed considerable morphological differences in these species but they were insufficient for phylogenetic relationships between specific local populations, and therefore had to be followed by chemotaxonomic investigations.

The investigations were made on flavonoid pigments which constitute an appropriate group of compounds for this purpose and they may be useful as genetic markers. They vary to a great extent, which is explained in terms of different places of substitution, the kind of substitute (hydroxyl group, methoxyl group, sugar or acyl groups) and the number of substitutions. All these compounds have characteristic physicochemical properties. Each of the reactions in pathway of biosynthesis of these compounds is catalyzed by a different specific enzyme (Harborne 1962, Grisebach 1965, Zaprometov 1971).

The basic composition of flavonol and leucocyanidin aglycons in the species of the *Saxifragaceae* family was established by Jay (1969). She found kaempferol, quercetin, leucocyanidin, leucodelphinidin and chlorogenic acid in *Saxifraga aizoon*. A similar compositions of flavonoids for the species *Saxifraga* was established by Hegnauer (1973).

EXPERIMENTAL

Material

The speciments of *Saxifraga aizoon* were collected in 1970 from the following localities:

A. Hala Gąsienicowa	Tatra Mts
B. Dolina Kościeliska	Tatra Mts
C. Dolina Chochołowska	Tatra Mts
D. Świstówka	Tatra Mts
E. Giewont	Tatra Mts
F. Ociemne (St. Kinga's Chapel)	Pieniny Mts
G. Czerwone Skałki	Pieniny Mts
H. Biała Woda	Pieniny Mts
J. Sokolica	Babia Góra Mt

Then they were vegetatively multiplied and cultivated in the same conditions on the plot near the Institute of Botany in Cracow. The investigations were performed in 1971 and 1972 on the fresh leaves of rosettes in the early autumn.

Methods

A) Extraction of flavonoid compounds

2 g of fresh leaves were collected from each clone and were subsequently ground with pure sand and degreased with petroleum ether. The remainder was soaked in 25 ml 1% HCl in methanol and kept for 24 hours, and after it was filtered. All above mentioned preparative operations were made, performed in the approximate temperature 0°C.

B). Purification and isolation of flavonols and anthocyanins

The methanolic extracts were fractionated on the columns (1,5× 15 cm) with Koch-Light's polyamide 120/500. The columns were successively eluted with 20 ml portions of: water, 20%, 50%, 80% and 100% methanol in water. 20 fractions 5 ml each (4 fractions for each solvent) were thus obtained.

C). Methods of identification

1). Thin layer chromatography.

The degreased plates 20 × 20 cm were covered with about 2 mm thin layer of cellulose MN 300 powder. The chromatography was performed in following systems:

BAW — n-butanol : acetic acid : water (4:1:5 v/v)
15% AcOH — acetic acid : water (3:17 v/v)
HCl · AcOH — water : hydrochloric acid : acetic acid (5:1:5 v/v)
Forestal acetic acid : water : hydrochloric acid (30:10:3 v/v)

The methanolic extract (0.5 ml of each sample) was put on the plates and was developed in twodimensional chromatography. BAW was used in one dimensional, and 15% AcOH in the other. The fractions which were obtained from the columns were examined in unidimensional chromatography on the plates 11×18 cm and were developed in all of the above mentioned systems.

2). Development

All chromatograms were developed by means of ammonia vapour. Basides, a test with 3% iron chloride in ethanol was used for anthocyanins, and the following tests were applied for flavonols: a) with 2% solution of zirconium oxychloride in methanol and next citric acid (Hörhammer, Müller 1954a), b) saturated solution of aluminium chloride in methanol, c) reduction test with the application of a magnesium strip and 2 n HCl (Hörhammer, Müller 1954b).

3). Spectral measurements

The fractions obtained from the columns were examined by twodimensional chromatography, and then the flavonols and anthocyanins were eluted from chromatograms by methanol for the next twenty four hours in the approximate temperature of 0°C. The spectral measurements were made on the spectrophotometer Unicam SP 800.

4). Hydrolysis

After the chromatography on polyamid gel the fractions obtained were examined with twodimensional chromatography and eluted for the next 24 hours with 1% HCl in methanol. The eluates were warmed with 2 n HCl in boiling water for 20 minutes.

Analysis of aglycons. Hydrolyzates (0.2 ml from each sample) were put on the plates parallel with the standards and they were developed in the systems BAW and HCl · AcOH.

Analysis of sugars. 0,5 ml of each hydrolyzate was subjected to chromatographic separation on paper Whatman No 1, with standard

sugars. They were developed in BAW and BBPW system (benzene : n-butanol : pyridine : water 1:5:3:3 v/v) and treated by ammonia solution of silver nitrate and aniline phtalane in butanol.

RESULTS

On the basis of occurrence of the studied compounds on chromatograms which were obtained as a result of twodimensional chromatography of methanolic extracts the examined *Saxifraga aizoon* clones may be divided into two groups. Chromatograms of the first group (Fig. 1),

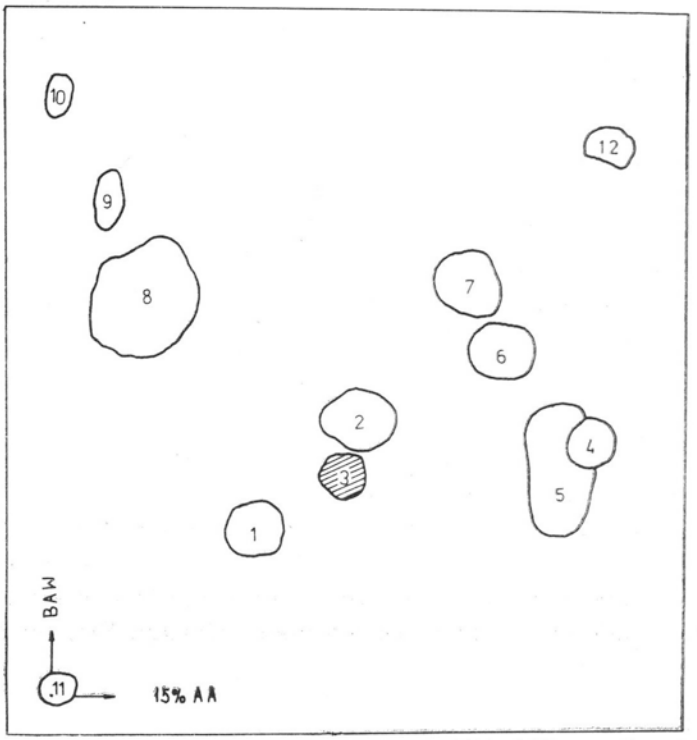

Fig. 1. Characteristic chromatograms of *Saxifraga aizoon* clones from A, B, C, D and E localities

characteristic for clones of the localities A, B, C, D, E, showed twelve spots: three pink (marked on the chromatograms as numbers 1, 2, 3), five yellow (4, 6, 7, 9 and 10), two colourless spots which turn violet in UV (5 and 12), one green (11), and one colourless, turning fluorescent blueish in UV light (8). The chromatograms of the second group (Fig. 2.) yield a similar arrangement of spots but they lack spot No. 3.

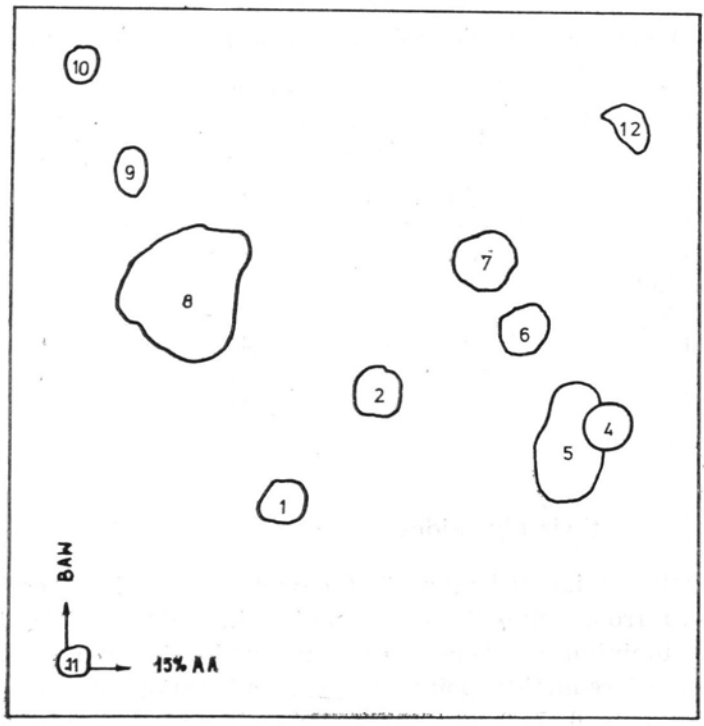

Fig. 2. Characteristic chromatograms of *Saxifraga aizoon* clones from F, G, H and J localities

Identification

1. Anthocyanins

Table 1 shows the results of chromatographic identification of anthocyanins which were obtained after separation on columns with polyamid gel (fractions 16 — 20). All R_f values given for these compounds, and the test reactions with iron chloride (the colour changes into blue) show that they are cyanidin glycosides. Hydrolyzate of spot No. 2 indicated the presence of rhamnose (Tab. 3). The identity of this compound with the product of reduction of quercitrin shows that it is cyanidine 3-rhamnoside. Hydrolyzate of spot No. 3 proves the presence of glucose (Tab. 3), and of spot No. 1 — the presence of xylose (Tab. 3). R_f values for the compound marked 3, agree with those established by Hayshi (1962) and Fahselt, Ownbey (1968) for cyanidine 3-glucose — chrysanthemin. Attempts at the final identification of the compound appearing as spot 1 have not succeeded.

Table 1

Characteristics of anthocyanins from the leaves of *Saxifraga aizoon*

Compound (spot number)	Solvent system				Absorption spectra* (nm)
	BAW	15% AcOH	HCl·AcOH	Forestal	
1	0.24	0.24	0.46	0.38	521
2	0.47	0.40	0.68	0.54	522
3	0.40	0.36	0.62	0.54	
Cyanidin standard	0.68		0.40		
After hydrolysis					
1	0.67		0.40		
2	0.68		0.41		
3	0.68		0.38		

* in methanolic 1% HCl.

2. Flavonols and their glycosides

The results of identification of flavonols and their glycosides (fractions 10—19 from column) are presented in Table 2. The tests with ammonia, aluminium chloride, zirconium oxychloride and citric acid and the reaction of reduction point to the classification of the compounds occuring in spots 6, 7, 9 and 10 as flavonols and their glycosides. The maximum absorption of these compounds in UV, compared with the values given by M a b r y and others (1970), prove so. The compounds 9 and 10 proved to be aglycons: 9 — quercetin and 10 — kaempferol (which was found in all examined clones in vestige amounts). Hydrolyzate of compound marked 6 indicated the presence of rhamnose and glucose (Tab. 3), and the hydrolyzate of compound 7 the presence of rhamnose (Tab. 3). All the values characterizing compound 6, and the comparison with the standard of rutin indicate that it is quercetin 3-glucorhamnoside. The comparison of all characteristic data for the compound marked No. 7 with the standard of quercitrin suggests that it is quercetin 3-rhamnoside.

3. Phenolic acids

The results of identification of phenolic acids are shown in Table 4. The comparison of R_f values of the compound marked on the chromatograms with number 11 with the standard of ellagic acid, and compound 12 with the standard of chlorogenic acid allowed to suggest the identity of these acids. After hydrolysis compound 5 resembles chlorogenic acid and compound 8 — the presence of ferulic acid. Therefore it is probable that compounds located in these spots are the esters of these acids.

Spot 4 has been not identified.

Table 2

Characteristic of the flavonols and their glicosides from the leaves of *Saxifraga aizoon*

Compound (spot number)	+ $ZrOCl_2$	+ citric acid	Solvent system				Absorption spectra* (nm)
			BAW	15% AcOH	HCl·AcOH	Forestal	
6	ż	disappears	0.51	0.70	0.87	0.85	259, 266, 299, 360
7	ż	disappears	0.54	0.66	0.46	0.76	256, 265, 301, 350
9	ż	does not disappear	0.75	0.15	0.34	0.32	255, 269, 301, 368
10	żz/b	does not disappear	0.89	0.05	0.49	0.39	253, 266, 294, 322 368
Standards							
quercetin	ż	does not disappear	0.75		0.34		255, 269, 301, 370
kaempferol	żz	does not disappear	0.88		0.50		253, 266, 294, 322, 367
rutin	ż	disappears	0.50		0.87		258, 266, 299, 359
quercitrin	ż	disappears	0.56		0.43		256, 265, 300, 350
After reduction							
6			0.39	0.66	0.71		
7			0.47	0.40	0.68		
9			0.68		0.40		
10			0.81		0.55		
After hydrolysis							
6			0.75		0.34		
7			0.76		0.35		

z — yellow, żz — yellowgreen
* in methanol

Table 3

R_f measurements of the sugars in solvent systems BAW and BBPW

Compound after hydrolysis	BAW	BBPW
1	0.26	0.55
2	0.35	0.71
3	0.10	0.36
6	0.10	0.35
	0.33	0.70
7	0.33	0.70
Standard		
rhamnose	0.32	0.69
glucose	0.10	0.35
xylose	0.26	0.54

Table 4

R_f measurements of phenolic acids in solvent systems: BAW, 15% AcOH and HCl·AcOH

Compound	BAW	15% AcOH	HCl·AcOH
11	0.93	0.06	0.25
12	0.90	0.75	0.85
5 After hydrolysis	0.90	0.77	0.84
8 After hydrolysis	0.85	0.59	0.87
Standard			
Ellagic acid	0.93	0.06	0.25
Chlorogenic acid	0.90	0.75	0.84
Ferulic acid	0.84	0.59	0.87

DISCUSSION

The studied clones of *Saxifraga aizoon* constitute two groups. To first group belong the specimens from Hala Gąsienicowa, Dolina Kościeliska, Dolina Chochołowska, Świstówka and Giewont, all from the Tatra Mts region. They contain (Tab. 5): kaempferol, quercetin, rutin, quercitrin, cyanidin 3-rhamnoside, chrysanthemin, cyanidin 3-xyloside (?) and phenolic acids, and their esters. The clones of the second group from the Pieniny region: Ociemne (St. Kinga's Chapel), Czerwone Skałki, Biała Woda and Sokolica — Babia Góra Mt., contain the same flavonoid compounds but they do not contain cyanidin 3-glucoside (chrysanthemin).

Considering the fact that all clones were cultivated in identical conditions after they had been collected, it is plausible to assume that the populations of the first group contain an extra enzyme (glycosyl--transferase) catalysing the synthesis of cyanidin 3-glucoside from cyanidin and UDPG. It should be emphasized that vestige amounts of chrysanthemin, with high amounts of the remaining cyanidin glycosides detected in the Giewont Mt. clones, presumably reflect very low activity of glycosyl-transferase of cyanidin 3-glucoside. This, as other enzymes is determined by a corresponding cistron (structure gene). It may therefore be inferred from the tests that the populations of the first group contain this cistron, while those of the second do not. Thus the genotypical composition of the two populations groups of *Saxifraga aizoon* is different, and the species may consequently be treated in terms of two races. The extra cistron can be assumed to have originated as the results of genetic change of structure gene determining glycosyl--transferase of cyanidin 3-rhamnoside or cyanidin 3-xylose.

Table 5

Flavonoid compounds found in studied clones of *Saxifraga aizoon*

Spot number	1	2	3	4	5	6	7	8	9	10	11	12
Compound / Locality	cyanidin 3-xyloside (?)	caynidin 3-rhamnoside	cyanidin 3-glucoside	not identified	chlorogenic acid ester	quercetin 3-rhamnoglucoside	quercetin 3-rhamnoside	ferulic acid ester	quercetin	kaempferol	ellagic acid	chlorogenic acid
Gąsiennicowa Valley	++	+++	+++	++	++	+++	++	++	+	śl.	+++	+++
Kościeliska Valley	+	+++	++	++	++	+++	++	++	+	śl.	++	++
Chochołowska Valley	+	++	+	++	++	++	++	+	+	śl.	++	++
Świstówka Mt.	+	++	+	+	+	++	++	+++	++	śl.	++	++
Giewont Mt.	+	+++	śl.	+	+	++	++	+	++	śl.	++	++
Ociemne (St. Kinga chapel)	+	++	—	+	+	++	++	+	++	śl.	++	++
Czerwone Skałki Mt.	+	+++	—	+	+	++	++	+	++	śl.	++	++
Sokolica (Babia Góra) Mt.	++	+++	—	+	+	+++	++	++	++	śl.	+++	++
Biała Woda	+	+	—	+	+	++	+	+	+	śl.	++	++

Number of plus signs corresponds with the intensity of spot (śl. — vestigial).

Hybridization of this mutant (containing chrysanthemin) with normal specimens (which biosyntheses cyanidin 3-rhamnoside and cyanidin 3-xyloside), yielded phenotypes which biosyntheses three cyanidin glycosides. Because this phenotypic type (which biosyntheses chrysanthemin) was not found in the Tatra Mts Region (i.e. it was not submitted to any other transfering), it may be considered that this process took place after the post-glacial period.

Before mentioned studies of J a y (1969) stated the presence of leucocyanidins in *Saxifraga aizoon* leaves, nevertheless she did not find anthocyanins whose presence in this species has been proved recently. Probably this difference results from J a y's making her experiments on herbarium material whereas the present research has been made on fresh leaves.

Acknowledgements

I would like to thank Doc. dr hab. J. S t r a s z k i e w i c z who encouraged me to undertake this work. I particulary appreciate Doc. dr hab. J. W i l s k a - J e s z k e's valuable advice and methodical instructions during the examinations, and Prof. dr hab. St. L e w a k for his helpful criticism and suggestions.

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Badania chemotaksonomiczne związków flawonoidowych Saxifraga aizoon Jacq.

Streszczenie

Przebadano związki flawonoidowe występujące w świeżych liściach rozetkowych klonów *Saxifraga aizoon* pochodzących z dziewięciu górskich stanowisk (Tatry, Pieniny, Babia Góra), a następnie hodowanych w jednakowych warunkach. W badanym materiale stwierdzono obecność: kempferolu, kwercetyny, kwercetryny (3-ramnozyd kwercetyny), rutyny (3-rutynozyd kwercetyny), 3-ramnozydu cyjanidyny, chryzanteminu (3-glukozyd cyjanidyny), 3-ksylozydu cyjanidyny, kwasu chlorogenowego, kwasu elagowego oraz estrów kwasu ferulowego i kwasu chlorogenowego. Klony pochodzące ze stanowisk tatrzańskich posiadały wszystkie wyżej wymienione związki, natomiast w liściach klonów z pozostałych stanowisk było brak chryzanteminu. Na podstawie tej mikrocechy (przez analogię do makrocech anatomicznych lub morfologicznych) można postulować podział tego gatunku na dwie rasy czy odmiany.