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191. Mitiiti Fujita,[*1] Shuji Hisamichi,[*2] Toshio Ando,[*2] and Nobuyoshi Murakami[*3]: Investigations on Flavonoid Component in the Pollen of Some *Forsythia* Species.

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The dried capsules of *Forsythia suspensa* and others of this species, known as the Chinese drug "Lien k'iao" (連翹), are used for tumor or abscess, and in some cases for scabies, tuberculous cervical lymphadenitis, etc. Pharmacognostical studies of this crude drug were reported by one of the present authors (N. M.) in an earlier paper.[1]

The original plant of this drug is a heterostylous shrub and each stock only bears either short-styled (with long-stalked anther) or long-styled (with short-stalked anther) flowers. Therefore, fertilization is possible only when the former pollens reach the latter stigma or the latter pollens reach the former stigma. Kuhn and Löw[2] assumed that this habit is due to biogenetic distinction between the two types of pollens. They analyzed the petals and pollens from the flowers of *F. intermedia* Zabel and stated that, just because rutin is present in all the petals of every type of flowers, it does not follow that their pollens contain the same glycosides of quercetin. They isolated rutin (10%) and lactose from the pollens (3.8 g.) of short-styled flower, and quercitrin (9%) from the pollens (4.2 g.) of long-styled flower, from which lactose was not detected. Thus, Kuhn and Löw found, as mentioned in the paper of Moewus,[3] that these flavonoid compounds may be responsible for the self-directed sterility in the fertilization of forsythia.

The present paper concerns experiment on pollen flavonoids of four species of forsythia cultivated in Japan; *F. koreans* Nakai, *F. suspensa* Vahl., *F. viridissima* Lindl., and *F. japonica* Makino. Greatest caution was taken to gather the flowers, together with branches before bloom, little by little, in order to avoid contamination of any traces of different kinds of pollens. Almost all the flowers were allowed in the room until in full bloom.

Thus, 200 mg. each of dried pollens were obtained from each type of flowers in every species, from 1957 to 1959. The pollen material was extracted with methanol, after removal of lipids and carotenoids, by the procedure described in the Experimental part and the concentrated fluid obtained therefrom showed positive reaction for flavonoids. Although

Table I. Rf Values for Flavonoid Pigments of Pollens and Authentic Samples

Compound \ Solvent[a)]	1	2	3	4	5	6
Pollen flavonoid[b)]	0.58	0.09	0.36	0.34	0.71	0.64
Pollen flavonoid[c)]	0.58	0.09	0.36	0.34	0.71	0.64
Rutin	0.58	0.09	0.36	0.34	0.71	0.64
Quercitrin	0.82	0.38	0.48	0.64	0.90	0.86

Color reagents used are listed in Table II.

a) Solvent system: 1. BuOH:AcOH:H_2O (4:1:5). 4. Cresol saturated with H_2O.
2. AcOEt saturated with H_2O. 5. iso-PrOH:H_2O (6:4).
3. PhOH saturated with H_2O. 6. BuOH:pyridine:H_2O (45:25:40).

b), *c*) Pollen from long-styled (b) and short-styled (c) flowers.

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1) N. Murakami: Yakugaku Zasshi, **77**, 403, 437(1957).
2) R. Kuhn, I. Löw: Chem. Ber., **82**, 474, 479(1947).
3) F. Moewus: Biol. Zentr., **69**, 181(1950).

Table II. Flavonoid Pigments and Color of their Spots Before and After Treatment with Coloring Reagents

Compound	Original color		Color reagent[a] 1		2		3		4	
	VL	UV	VL	UV	VL	UV	VL	UV	VL	UV
Pollen flavonoid[b]	Y	O B	R B	B	Y	Y	Y	O B	Y	O
Pollen flavonoid[c]	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃
Rutin	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃
Quercitrin	〃	B	Y B	Y	〃	〃	Y B	O Y	〃	〃
Quercetin	〃	Y	〃	O B	〃	G Y	B	O B	B	〃
Kaempferol	〃	〃	Y	Y	〃	〃	Y	B Y	Y	Y

Color of spot in visible light (VL) and ultraviolet ray (UV) :
B, brown; G, green; O, orange; R, red; Y, yellow

a) Color reagent : 1. 1% Na_2CO_3 soln 3. 1% $(AcO)_2Pb$.
2. 1% EtOH solution of $AlCl_3$ 4. 1% basic lead acetate solution

b) and *c*) same as in Table I

the material in hand was not sufficient to complete the work planned, this pollen extract was used for the following series of experiments.

Rf values obtained by chromatography were used for the detection and identification of pollen flavonoids and sugars. Color and fluorescence of the spots of flavonoids developed on the chromatogram were observed under ordinary and ultraviolet ray before and after spraying color reagents. These results are summarised in Tables I, II, and V. The area separated as a wide zone in many of the chromatograms was cut out and extracted with methanol, from which a concentrated fluid was obtained. This fluid was submitted to hydrolysis and a yellowish precipitate was obtained as a hydrolysate. The precipitate and the mother liquor were submitted to paper chromatography to examine the aglycone and combined sugar. Results obtained are listed in Tables III, IV, and V. The area of the paper chromatogram corresponding to the pollen flavonoid and the chromatogram obtained from methanolic solution of the precipitated hydrolysate were cut out and separately extracted with ethanol. Ultraviolet absorption spectra of the ethanolic solution were measured with reference to standards.

Table III. Rf Values of Aglycones from Pollen Flavonoids and Standard Samples

Compound \ Solvent[a]	1	2	3	4	5
Aglycone[b]	0.72	0.77	0.01	0.05	0.36
Aglycone[c]	0.72	0.77	0.01	0.05	0.36
Quercetin	0.72	0.77	0.01	0.05	0.36
Kaempferol	0.88	0.85	0.02	0.31	0.47

a) Solvent system : 1. BuOH:AcOH:H_2O (4:1:5). 4. $CHCl_3$:AcOH:H_2O (2:1:1).
2. BuOH:AcOH:H_2O (4:1:2). 5. AcOH:H_2O (60:40).
3. AcOH:H_2O (15:85).

b) and *c*) same as in Table I.

Table IV. Color of Aglycone from Pollen Flavonoid and of Test Samples

Compound	Original color		Color reagent[a] 1		2		3		4	
	VL	UV	VL	UV	VL	UV	VL	UV	VL	UV
Aglycone[b]	Y	Y	Y B	O B	Y	G Y	B	O B	B	O
Aglycone[c]	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃
Quercetin	〃	〃	〃	〃	〃	〃	〃	〃	〃	〃
Kaempferol	〃	〃	Y	Y	〃	〃	Y	B Y	Y	Y

All notations are the same as those in Tables I and II.

TABLE V. Rf Values of Sugar in the Pollen and Standard Samples

Compound \ Solvent[a]	1		2		3	
Sugar[b]	0.08	0.24	0.35	0.63	0.18	0.42
Sugar[c]	0.08	0.24	0.35	0.63	0.18	0.42
Sugar[d]	0.08	0.21	0.35	0.49	0.18	0.27
Glucose	0.08		0.35		0.18	
Rhamnose	0.24		0.63		0.42	
Fructose	0.21		0.49		0.27	

Color reagent : 1. Aniline hydrogenphthalate (1 g. of aniline and 1.8 g. of phthalic acid in 1000 cc. of BuOH saturated with H_2O)
2. 2% Hippuric acid in MeOH

a) Solvent system : 1. BuOH:AcOH:H_2O (4:1:5) 3. Cresol saturated with H_2O
2. PhOH saturated with H_2O

b) and *c*) : Combined sugars produced by hydrolysis of pollen flavonoid in long-styled (b) and short-styled (c) flowers

d) Free sugar in pollen grains

It was found that, in all the four species of forsythia used as material in the present series of experimets, all the pollens from flowers with short-stalked as well as long-stalked anthers contain nothing but rutin as flavonoid component, since its aglycone and combined sugars were identical with quercetin, glucose, and rhamnose.

After removal of flavonoid from pollen material by extraction with methanol, residual pollens were extracted with hot water to detect free sugar and aqueous extract was concentrated under a reduced pressure. Chromatography of this aqueous extract revealed spots which might be considered to be glucose and fructose, and a spot identical with lactose was not detected. Thus, the result obtained from the present series of experiment did not agree with that of the experiment of Kuhn and Löw mentioned above.

It should be pointed out that *F. intermedia* used by the German workers is a hybrid of *F. suspensa* and *F. viridissima*, and the result obtained by them might have been due to this fact. If, however, this is not the case, it seems that there is no relationship between the pollen flavonoid in forsythia and its rôle in self-sterility.

On the other hand, following studies were made in connection with Kuhn's work. Yamada, *et al.*[4] recently reported that rutin is contained as a flavonoid component in the perianth of *F. koreana* but they did not refer to the morphology of the flowers used as their material. Esser and Straub[5] made comparative studies on germination of pollen grains obtained from the two morphologically different types of flower from *F. intermedia*, *F. viridissima*, and *F. suspensa*, they denied Moewus' hypothesis that there is an enzyme which probably plays an important rôle in fertilization, and stated that self-sterility of forsythia is not dependent on the presence of rutin in the pollens from long-stalked anther and quercitrin in those of another type. It has also been reported from chromatographic assay of flavonoid pigments in the pollens from two varieties of *F. intermedia* VAR. *spectabilis* (a short-styled shrub) and VAR. *densiflora* (a long-styled shrub), that all the pollens contain two flavonol glycosides, rutin being the major component and the other, a kaempferol derivative, but not quercitrin.[6] This worker also gave the same opinion as that of Esser and Straub about the flavonol component of forsythia.

4) S. Yamada, T. Takano, G. Suzushino, K. Hayashi : Botan. Mag. (Tokyo), **73**, 265(1960).
5) K. Esser, J. Straub : Biol. Zentr., **73**, 449(1954).
6) H. Reznik : *Ibid.*, **76**, 352(1957).

Experimental*4

Extraction of Flavonoid from the Pollens—Two hundred milligrams of dried pollens was extracted several times with petr. ether, followed by 30 cc. of Et_2O, on a water bath. After removal of the solvent, residual pollens (I) were extracted three times with 30 cc. each of 95% MeOH for several hours. Removal of the solvent from the MeOH extract left 15 cc. of concentrated yellowish fluid (II). It gives a greenish brown color with $FeCl_3$, yellow color with $Pb(OAc)_2$ or alkaline solutions, and a pink color with Mg and conc. HCl.

Paper Chromatography of Pollen Flavonoid and Aglycone—(II) was submitted to paper chromatography by the usual procedure and a single spot was detected on the paper chromatogram (at 20°). From the Rf value of this spot and its colors in daylight and fluorescent illumination of the spot treated and untreated with color reagents, it was identified with rutin (Tables I and II).

(II) was also placed as a wide band on a line 6 cm. from the shorter edge of a sheet (10×40 cm.) of Toyo Roshi No. 51 filter paper. This was developed for 16 hr. by the ascending technique, using BuOH:AcOH:H_2O (4:1:5) as a solvent (with color reagents listed in Table II) and the area (III) of chromatogram corresponding to rutin (Rf 0.58), in comparison with the guide strip containing the known flavonoid, was cut off. The combined strips obtained from 10 sheets of filter papers treated at the same time by this procedure were eluted with 50 cc. of 95% MeOH. Removal of the solvent left a fluid which was subjected to hydrolysis by heating with 5% H_2SO_4 for 1 hr., the solution was cooled, and the resulting yellowish precipitate (IV) was collected. The precipitate was washed thoroughly with water to remove the acid and dissolved in 20 cc. of hot MeOH. The color reaction of this solution was examined and showed dark greenish brown with $FeCl_3$ in EtOH, orange with $Pb(OAc)_2$, and deep red with Mg and conc. HCl. (IV) was identical with quercetin, as shown in Tables III and IV, by paper chromatogram developed from its MeOH solution.

Paper Chromatography of Sugar in the Pollens——After removal of (IV), the filtrate was treated with an ion-exchange resin, Amberlite IRA-411, to remove the acid and concentrated under a reduced pressure. Paper chromatography of this residual solution (at 20°) revealed the presence of glucose and rhamnose as the combined sugar of the pollen flavonoid.

(I) was extracted with 30 cc. of water on a water bath after removal of flavonoid component with MeOH and the aqueous extract was submitted to paper chromatography by the same procedure as described above. Spots obtained on the chromatogram were identical with those of glucose and fructose, as free sugars in the pollens (Table V).

Spectrophotometric Observations—(III) and the area on the chromatogram obtained from MeOH solution of (IV) were cut out, eluted with EtOH, and each solution was submitted to ultraviolet spectrophotometry, using Cary Model 11 Recording Spectrophotometer. The absorption curves and spectral data of the pollen flavonoid (λ_{max}^{EtOH} mμ ($E_{1cm}^{1\%}$) : 258 (233), 361 (189)) and of its aglycone (λ_{max}^{EtOH} mμ ($E_{1cm}^{1\%}$) : 256 (798), 372 (828)) were identified with those of authentic samples of rutin and quercetin.

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Summary

Examinations were made on the pollens of *Forsythia koreana* and other species by paper chromatography in an attempt to confirm the pollen flavonoid component of forsythia which has two types of flower, one being short-styled and the other, long-styled. It had been reported that rutin is present in the pollens from the former and quercitrin in the pollens of the latter, and that a satisfactory fertilization would be prevented without the action of these different glycosides of quercetin. However, as far as the present series of experiments were concerned, it was found that rutin is solely present as the flavonoid pigment, and glucose and fructose as the free sugars. Lactose was not detected from any of the pollens.

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*4 Most of the flowers for pollen material were gathered in the Botanical Garden, Faculty of Science, University of Tokyo, and all the materials were treated by the same procedure.