Identification and Biosynthesis of Glucosylated and Sulfated Flavonols in *Flaveria bidentis*

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Flaveria bidentis, Compositae, Flavonol Glucosides, Flavonol Sulfate Esters, Identification, Biosynthesis

1. The flavonoid constituents of *Flaveria bidentis* have been identified as the 3-O-glucosides of kaempferol, 6-methoxykaempferol and 6-methoxyquercetin (patuletin), as well as six flavonol sulfate esters belonging to quercetin and isorhamnetin. The two latter glucosides are reported here for the first time in this species; whereas quercetin-3,7-disulfate, is a novel compound from nature.

2. The amounts of both glucosides and sulfate esters in young seedlings were highest in buds > stems > leaves on fresh weight basis, whereas they were undetectable in the root. There were striking quantitative differences in the flavonoid pattern of young seedlings, as compared with mature shoots, especially the flavonol sulfate esters which were predominant in seedlings.

3. The biosynthesis of both groups of flavonoids from [^{3}H]cinnamate and [^{35}S]sulfate showed that the former was predominantly incorporated into glucosides whereas the latter, into sulfate esters; thus indicating that sulfation is a later step in the biosynthesis of flavonol sulfate esters.

4. The significance of the glucosylation and sulfation reactions was discussed in relation to the regulation of biosynthesis and compartmentation of both groups of compounds.

Introduction

Organic sulfur compounds are known for their ubiquitous occurrence in plants as amino acids, vitamins, coenzymes, polysaccharides, aliphatic sulphides, glucosinolates and thiophene derivatives [1], to mention a few. Only recently, a new class of sulfur compounds, the flavonoid sulfates has been reported to be of widespread occurrence in a number of plant families [2–4] especially the Compositae [5], among which *Flaveria* is well documented ([6, 7] and refs. cited therein). Although *F. bidentis* is known to contain a number of partially sulfated flavonols [6], however, no complete study of its flavonoid pattern has yet been reported. Furthermore, except for two preliminary reports [8, 9] on the incorporation of $^{35}SO_4^{2-}$ into sulfated flavonoids, very little is known of their biosynthesis in plants.

Preliminary phytochemical investigation of *F. bidentis* indicated the co-occurrence of both flavonol glucosides and flavonol sulfate esters in leaf tissues. The dual nature of these flavonoids prompted us to conduct a systematic identification of these constituents and to study their biosynthesis from labelled precursors.

Materials and Methods

Chemicals

L-[2,3,4,5,6-^{3}H]Phenylalanine (4.44×10^3 GBq/mmol) was purchased from Amersham, Oakville, Ontario, and [$^{35}SO_4^{2-}$]sulfuric acid (1.59×10^3 GBq/mg), from New England Nuclear, Boston, MA. L-Phenylalanine ammonia-lyase (1–3 U/mg) and aryl sulfatase (5–10 U/mg) were obtained from Sigma Chemical Co., St. Louis, MO. [^{3}H]cinnamic acid was prepared enzymatically from labelled phenylalanine [10]. It was purified by TLC and its specific activity was estimated to be 4.03×10^2 GBq/mmol. Quercetin-3-sulfate, quercetin-3-acetyl-7,3′,4′-trisulfate and quercetin-3,7,3′,4′-tetrasulfate were generous gifts from Prof. H. R. Juliani, Cordoba, Argentina. Other flavonoid compounds were from our laboratory collection.

Plant material

Seeds of *Flaveria bidentis* var. *angustifolia* O.K. were obtained from Prof. H. R. Juliani and were germinated in one-cm layer of vermiculite on top of potting soil, under greenhouse conditions.

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Extraction and identification of flavonoids

Fresh leaves, taken from 3–4 month-old plants, were frozen in liquid N_2, ground to a fine powder, then extracted three times with 50% aqueous MeOH. The combined extracts were concentrated under reduced pressure at 30 °C. Partial purification of sulfated flavonoids was carried out on a Sephadex G-10 column using water as eluent. Eluted fractions were analyzed by chromatography on cellulose TLC plates using *n*-BuOH–HOAc–H_2O (6:2:2, v/v/v) as a solvent system, followed by visualization in UV light. The flavonoid-containing fractions were combined, concentrated and chromatographed on semi-preparative cellulose TLC plates using the same solvent system. Bands of individual flavonoids were eluted in 50% aqueous MeOH, concentrated and further purified by HPLC using a Microbondapak C_{18} column (particle size, 10 μm). Best separation of the component flavonoids was carried out according to Harborne [11] after some modification. An isocratic gradient of 60% solvent A (10 mM aqueous tetrabutylammonium dihydrogen phosphate) and 40% solvent B (MeOH–HOAc–H_2O, 18:1:1, v/v/v) was used for 10 min; proceeding to 50% A and 50% B in 40 min and finally to 40% A and 60% B in 10 min; at a flow rate of one ml/min.

Identification of individual flavonoids was carried out by determining their UV absorption spectra, using spectral shift reagents [12]; co-chromatography with reference compounds (when available) on cellulose and polyamide TLC plates, using *n*-BuOH–HOAc–H_2O (6:2:2), 15% aqueous HOAc and MeOH–H_2O (3:2) as solvent systems and visualization in UV light (366 nm). Identification of the aglycone moieties of different flavonoids was carried out after hydrolysis with 2 N HCl at 95 °C for 10 min followed by UV-spectral analysis and co-chromatography with reference compounds. Hydrolysis with aryl sulfatase was used to detect the presence of sulfate groups esterified with flavonoid compounds. Acid or enzyme hydrolysates were co-chromatographed with reference compounds on Polyamide DC 6.6 TLC plates, using benzene–methyl ethyl ketone–MeOH (8:1:1) as solvent. Estimation of the electrophoretic mobility of sulfated flavonoids was performed on Whatman No. 3 chromatographic paper in a formic acid–acetic acid–water (43:147:1820) mixture, pH 2.2 at 250 v and 8 mA for 3 h.

Flavonoid content of different organs

Aqueous methanolic extracts (1:1, v/v) of buds, stems or leaves were analyzed for their total flavonoid content by measuring their absorbance at 340 nm, and for their flavonoid composition by HPLC. Individual peaks were integrated and the quantity of each flavonoid compound was calculated from total absorbance values, using an average molar extinction of 20000.

Isotopic experiments

Excised organs, or leaf disks taken from the first pair of expanded leaves of 3 week-old seedlings, were incubated with the labelled compounds in presence or absence of non-labelled precursors, in the light (ca. 250 f.c.). At the end of the metabolic period, the tissue was thoroughly rinsed with water and homogenized in 50% aqueous MeOH. Aliquots of the methanolic extracts were counted for total

Table I. Characteristics of *Flaveria* flavonoids.

Flavonoid compound	R_f value		R_t [min]	Electro-phoretic mobility[a]	Relative amount [%][b]
	BAW[c]	H_2O			
6-Methoxy-kaempferol-3-glucoside	0.70	–	27	0.8	17
Kaempferol-3-glucoside	0.67	–	26	0.8	13
Patuletin-3-glucoside	0.52	–	16	0.7	19
Isorhamnetin-3-sulfate	0.45	0.45	49	1.0	7.5
Quercetin-3-sulfate	0.36	0.40	44	1.0	19
Isorhamnetin-3,7-disulfate	0.27	0.82	56	1.8	0.5
Quercetin-3,7-disulfate	0.20	0.84	53	1.9	9
Quercetin-3-acetyl-3,7,3′-trisulfate	0.11	0.88	59	2.7	3
Quercetin-3,7,3′,4′-tetrasulfate	0.05	0.94	62	3.1	12

[a] Relative to quercetin-3-sulfate.

[b] Based on integrated peak areas by HPLC analysis of 3–4 month-old shoots and TLC separation of kaempferol-3-glucoside and 6-methoxykaempferol-3-glucoside.

[c] BuOH–HOAc–H_2O (6:2:2, v/v/v).

radioactivity by liquid scintillation. The remainder of the extracts were chromatographed on cellulose TLC plates, then autoradiographed. Individual flavonoids were scraped off the plate, mixed with Cab-O-Sil (colloidal silica) and counted for radioactivity.

Results

Characterization of flavonoid constituents

The chromatographic, electrophoretic and UV-spectral characteristics of the flavonoids isolated from *F. bidentis* are summarized in Fig. 1, Tables I and II. Flavonol sulfate esters could be distinguished from flavonol glucosides by their: (a) low R_f values in organic as compared with aqueous solvents, especially those of highly sulfated compounds; (b) electrophoretic mobility towards the anode (Table I); (c) susceptibility for hydrolysis with aryl sulfatase, except for the 3-sulfates [7]; (d) prominent peak at 1050 cm^{-1} in IR spectra (not shown).

Flavonoid aglycones were identified, after acid or enzymatic hydrolysis of the parent flavonol derivative, by their UV spectra using spectral shift reagents [12], fluorescence in UV-light (366 nm) and co-chromatography with reference compounds. The number of sulfate groups in sulfated flavonols was determined by their electrophoretic mobilities relative to quercetin-3-sulfate (Table I) and the R_t on HPLC of their tetrabutylammonium dihydrogen phosphate [11] derivatives (Fig. 2). The position of sulfation was determined from the UV spectral shifts observed after the addition of specific reagents (Table II), and considering the resistance of flavonol-3-sulfates to hydrolysis with aryl sulfatase [7].

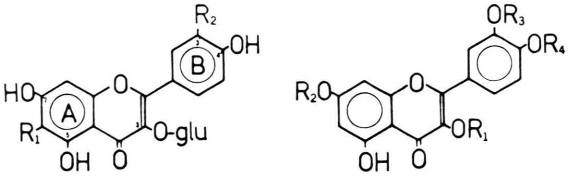

Fig. 1. Structural formulae of the glucosylated (left) and sulfated (right) flavonol constituents of *F. bidentis*.

R_1	R_2	
H	H	Kaempferol-3-glu
OMe	H	6-OMe-kaempferol-3-glu
OMe	OH	Patuletin-3-glu

R_1	R_2	R_3	R_4	
H	H	H	H	Quercetin
H	H	Me	H	Isorhamnetin
		R_1 to R_4		sulfate groups

Table II. UV spectral characteristics of *Flaveria* flavonoids.

Flavonoid compound	MeOH	$AlCl_3$	$AlCl_3$ + HCl	NaOAc+	NaOAc boric acid	MeOH+ HCl
6-Methoxy-kaempferol-3-glucoside	335 270	385 277 300 sh	350 277 295 sh	360 270	335 270	335 270
Kaempferol-3-glucoside	345 265	395 sh 300 sh	395 sh 300 sh	360 270	347 265	345 265
Patuletin-3-glucoside	353 257	430 275	370 265	382 270	380 265	353 257
Isorhamnetin-3-sulfate	347 252	395 365 sh 272 sh	360 390 sh 272 sh	357 267	350 265 sh	365 252
Quercetin-3-sulfate	350 257	395 275	365 268	407 267	388 267	367 257
Isorhamnetin-3,7-disulfate	350 253	397 365 sh 267	392 355 sh 265	355 253	353 253	365 253
Quercetin-3,7-disulfate	355 255	405 272	395 365 sh 268	355 255	375 257	365 255
Quercetin-3-acetyl-7,3′,4′-trisulfate	345 267 245 sh	392 340 272	392 340 272	395 267	350 267	370 267

sh, shoulder.

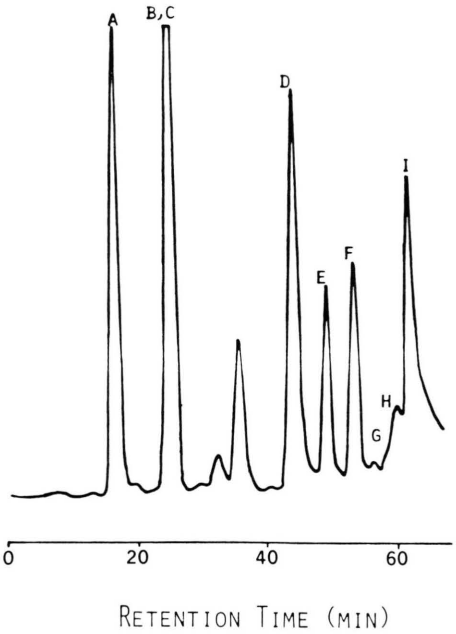

Fig. 2. HPLC separation of the flavonol glucosides and flavonol sulfate esters of *F. bidentis* shoots on a Microbondapak C_{18} column as described in the Methods section. A, patuletin-3-glucoside; B, kaempferol-3-glucoside; C, 6-methoxykaempferol-3-glucoside; D, quercetin-3-sulfate; E, isorhamnetin-3-sulfate; F, quercetin-3,7-disulfate; G, isorhamnetin-3,7-disulfate; H, quercetin-3-acetyl-7,3′,4′-trisulfate; I, quercetin-3,7,3′,4′-tetrasulfate.

Among the different flavonoids characterized in *F. bidentis* (Table I) patuletin-3-glucoside, 6-methoxykaempferol-3-glucoside and quercetin-3,7-disulfate are reported for the first time in this tissue. Due to the less common occurrence of the two latter compounds, further evidence of their identity is given below.

6-Methoxykaempferol-3-glucoside: This compound exhibited 15 nm shift of band I in the presence of $AlCl_3 + HCl$ (Table II) indicating substitution at position 3. However, the low magnitude of that shift suggested a 6-methoxy substitution [13]. Furthermore, there was 25 nm shift in presence of NaOAc, but none with $NaOAc + H_3BO_3$, indicating a free 4′-OH and the absence of an o-dihydroxy-system, respectively. The presence of a C-8 proton (6.25 ppm), as well as a methoxyl group (3.7 ppm) in the ^1H NMR spectrum (DMSO-d_6), confirmed the presence of a 6-methoxy substitution. Furthermore, ring B protons appeared as two groups, of two equivalent protons each (δ + 6.84 and 7.97 ppm), coupled together with an *ortho* coupling ($\delta = 8.8$ Hz) which is characteristic of a disubstitution in 1′,4′, indicating a kaempferol derivative. A 7.7 Hz coupling constant was observed between H-1″ ($\delta = 5.37$ ppm) and H-2″ of the sugar moiety, indicating a β-linked sugar. Acid hydrolysis yielded glucose and 6-methoxy-kaempferol. The fact that the latter aglycone did not co-chromatograph with 8-methoxy-kaempferol [14], provided further, though indirect, evidence of being the 6-substituted analog.

Quercetin-3,7-disulfate: The electrophoretic mobility of this compound (Table I) suggested a disulfate ester which on hydrolysis with sulfatase gave quercetin-3-sulfate [7], and on acid hydrolysis gave quercetin. The spectral shift of the disulfate ester in presence of HCl (Table II) indicated 3-sulfation, whereas the absence of a NaOAc shift in band II suggested 7-sulfation. Furthermore, a 22 nm shift in presence of $NaOAc + H_3BO_3$ demonstrated the presence of o-diOH function on ring B. This compound was tentatively identified as quercetin-3,7-disulfate since it did not co-chromatograph with either the 3,3′- or 3,4′-analogs. To our knowledge, the natural occurrence of quercetin-3,7-disulfate has not previously been reported [2–5]. Further identification and chemical synthesis of this compound are in progress.

Flavonoid content of different organs

Quantitative analysis of various flavonoids in different organs of 3–4 week-old seedlings (Table III) indicated that the highest amounts of individual, as well as total, flavonoids were found in the buds > shoots > leaves. On the other hand, the root system of seedlings was conspicuous by the absence of both glucosylated and sulfated flavonols. The fact that the leaf tissue constitutes the major part (both weight and surface area) of the shoot system, indicates that the leaf is one of the principal sites of flavonoid synthesis and accumulation.

It is interesting to note that the flavonol glucosides in the seedlings amounted to < 10% of total flavonoids (Table III), as compared with approximately 50% in mature shoots (Table I). Quercetin-3,7-disulfate represented the major constituent in the seedlings and amounted to 50–70% of total flavonoids. However, in mature shoots quercetin-3-sulfate, -3,7-disulfate and -3,7,3′,4′-tetrasulfate were the major products among the sulfate esters, even though they formed only 40% of total flavonoids (Table I).

Table III. Flavonoid content of different organs of *Flaveria* seedlings[a].

Flavonoid compound[b]	nmol per gram fresh weight Bud	Stem	Leaf
K-3-glucoside + 6-OMeK-3-glucoside[c]	111	10.4	3.64
P-3-glucoside	162	93.5	7.81
Q-3-sulfate	147	24.7	2.60
IsoR-3-sulfate	38.1	33.6	4.17
Q-3,7-disulfate	1540	686	354
IsoR-3,7-disulfate	19	13.8	6.51
Q-3,7,3′,4′-tetrasulfate	43.3	13.9	2.60

[a] Aqueous (50%) methanolic extracts of 3 week-old seedlings were analyzed by HPLC as described in the Methods section. The amounts of individual compounds were calculated from the integrated peak areas and the absorbance of total extracts using an average extinction coefficient of 20,000.

[b] IsoR, isorhamnetin; K, kaempferol; P, patuletin; Q, quercetin. Q-3-acetyl-7,3′,4′-trisulfate was absent in seedlings.

[c] K-3-glucoside and 6-OMe-K-3-glucoside could not be resolved on HPLC.

Biosynthesis of flavonoids from labelled precursors

Preliminary labelling experiments using three week-old intact seedlings indicated that the highest label incorporation was observed in the terminal bud and the adjacent pair of young leaves (data not shown). Therefore, only the youngest leaf pairs were used for isotopic experiments.

Fig. 3 shows the time course for incorporation of [^{3}H]cinnamate (Fig. 3A) and [^{35}S]sulfate (Fig. 3B) into individual flavonoids, as well as the total methanolic extracts (insert) of leaf disks. Whereas the rate of label incorporation of either precursor was almost linear with time up to 60 min, the order of labelling of the various flavonoid derivatives was different. Comparison of the profiles of glucosylated and sulfated flavonols shows that the cinnamate label was found mostly in flavonol glucosides, as compared with the sulfate esters, with a ratio of 13 to one after 3-h incubation with the labelled precursor (Fig. 3A). The incorporation of label from [^{35}S]sulfate was, as expected, restricted to the flavonol sulfates, and was proportional to the number of sulfate ester linkages of individual compounds (Fig. 3B).

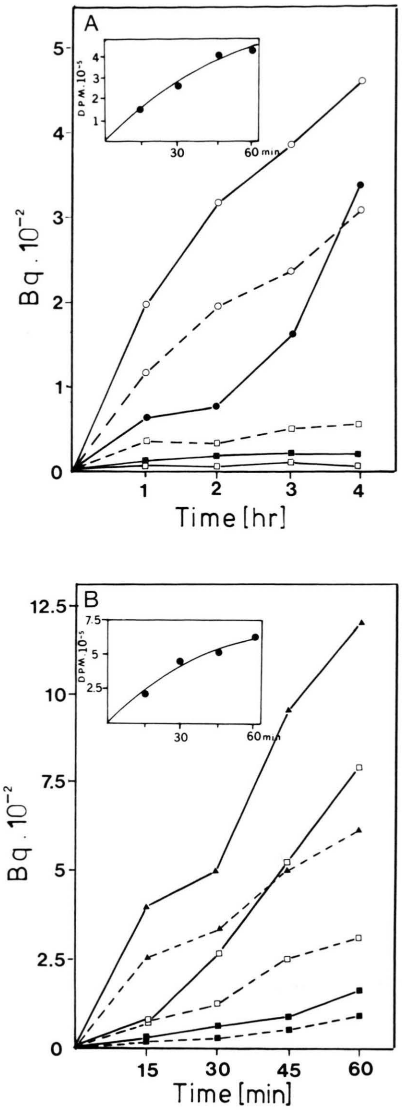

Fig. 3. Incorporation of label of [^{3}H]cinnamate (A) or [^{35}S]sulfate (B) into total MeOH extracts (inserts) and the individual flavonoid constituents of the first pair of seedling leaves of *F. bidentis* at the times indicated. Leaf disks were administered 3.7 × 10^4 Bq cinnamate (4.03 × 10^2 GBq/mmol) or sulfate (1.59 × 10^3 GBq/mg). isoR, isorhamnetin; K, kaempferol; Q, quercetin; P, patuletin. (– – ○ – –), K-3-glu; (—— ○ ——), 6-MeO-K-3-glu; (—— ● ——), P-3-glu; (– – ■ – –), isoR-3-sulfate; (—— ■ ——), isoR-3,7-disulfate; (– – □ – –), Q-3-sulfate; (—— □ ——), Q-3,7-disulfate; (– – ▲ – –), Q-3-acetyl-7,3′,4′-trisulfate; (—— ▲ ——), Q-3,7,3′,4′-tetrasulfate.

When leaf disks were pulsed for 30 min in either precursors, followed by a 3-h chase in water, similar results were obtained (Figs. 4A, B); except for a drop in the radioactivity of quercetin-3-monosulfate and quercetin-3,7-disulfate. The latter compounds seem to have been metabolized to a higher order of sulfated flavonols (Fig. 4B).

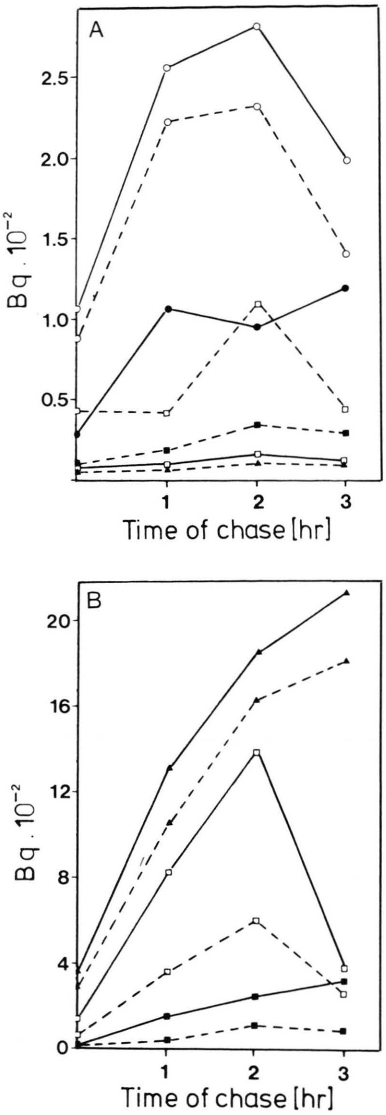

Fig. 4. Incorporation of label of [^{3}H]cinnamate (A) or [^{35}S]sulfate (B) into individual flavonoid constituents of the first pair of seedling leaves, after 30-min pulse with cinnamate or sulfate followed by 3-h chase in water, at the indicated times. Amount of label administered and symbols for individual compounds are as in Fig. 3.

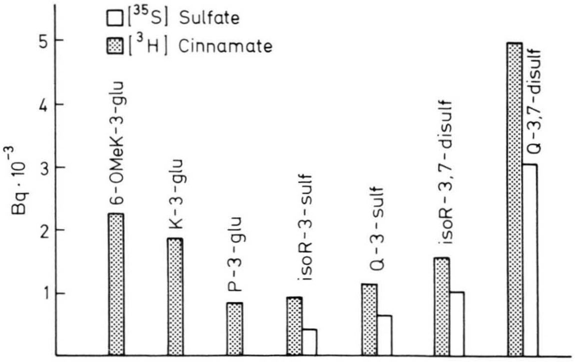

Fig. 5. Incorporation of [^{3}H]cinnamate and [^{35}S]sulfate, after 3-h incubation with both label, into individual flavonoids. Leaf disks were administered 3.7×10^4 Bq cinnamate (4.03×10^2 GBq/mmol) and sulfate (1.59×10^3 GBq/mg). Abbreviations for compounds as in Fig. 3.

Double feeding experiments, using both labelled precursors further indicated that flavonol glucosides were only labelled from cinnamate, whereas the sulfate esters contained the label of both precursors (Fig. 5). The high label incorporation into kaempferol as compared with patuletin glucosides is consistent with the fact that formation of the latter aglycone requires further biosynthetic steps involving modification of both rings A and B (Fig. 1). Similar to the time course experiment (Fig. 3B), [^{35}S]sulfate incorporation increased with increasing level of sulfation of both quercetin and isorhamnetin, with the [^{3}H] to [^{35}S] ratio in sulfated flavonols remaining nearly constant (Fig. 5).

Discussion

We have demonstrated the presence, in *F. bidentis,* of the 3-O-glucosides of kaempferol, 6-methoxykaempferol and 6-methoxyquercetin (patuletin), as well as flavonol sulfate esters belonging to quercetin and isorhamnetin (Fig. 1, Table I). Except for quercetin-3,7-disulfate, all other sulfated flavonols have previously been described ([6] and refs. cited therein). On the other hand, 6-methoxykaempferol-3-glucoside has been reported as a novel compound in *F. brownii* [15]; whereas patuletin-3-glucoside in *F. linearis* and *F. trinervia* [16] as well as other Compositae [17]. These two glucosides together with quercetin-3,7-disulfate are being reported here, for the first time, in *F. bidentis.* To our knowledge, the latter compound represents a hitherto unreported disulfate ester of quercetin [2–4], which complements the two other analogs, quercetin-3,3′- (unpublished results) and quercetin-3,4′-disulfate esters [6].

The co-occurrence of both groups of compounds as the 3-glucosides and the sulfate esters of kaempferol and quercetin derivatives, respectively represents a striking feature of the flavonoid pattern of *F.*

bidentis, as compared with other *Flaveria spp.* [7, 15, 16]. Furthermore, the quantitative differences observed in the different organs of seedlings (Table III) as compared with mature shoots (Table I) represent another interesting feature of this plant. The predominance of the flavonoid sulfate esters in the seedling, especially quercetin-3,7-disulfate which represented 50–70% of total flavonoids as compared with 9% in mature shoots, raises the question as to the significance of sulfated flavonols in the growth and development of this species.

The biosynthesis of *Flaveria* flavonoids from [^{3}H]cinnamate and [^{35}S]sulfate showed that the cinnamate label was incorporated almost predominantly into flavonol glucosides (Figs. 3A, 4A), whereas that of sulfate, into flavonol sulfate esters (Figs. 3B, 4B) which increased with increasing level of sulfation. The lack of incorporation of [^{3}H]cinnamate into sulfated flavonols (Figs. 3A, 4A) may be due to a low endogenous level of 3-phosphoadenosine-5′-phosphosulfate (PAPS), the sulfate donor. However, when both precursors were available (Fig. 5) flavonol sulfate esters were found to contain both [^{3}H] and [^{35}S] labels in almost constant ratio. These results indicate that sulfation is a later step in the biosynthesis of flavonol sulfate esters, and is regulated by PAPS. The low incorporation of the [^{35}S] into isorhamnetin sulfate esters (Figs. 3B, 4B) may have been due to their low concentration in seedling leaves, as compared with that of quercetin analogs (Table III). However, further experiments will be required to demonstrate whether sulfation precedes or succeeds O-methylation in the biosynthesis of the sulfate esters of isorhamnetin, as well as other partially methylated flavonols.

Very recently, we have demonstrated the enzymatic sulfation of quercetin to its mono-, di-, tri- and tetrasulfate esters by cell-free extracts of *F. bidentis* [18]. It will be interesting to find out whether the latter reaction proceeds in a stepwise manner as in the sequential O-methylation of polymethylated flavonols [19]. Furthermore, it is not known whether the glucosylation and sulfation reactions play similar roles in flavonoid metabolism of *F. bidentis, i.e.* the inactivation of reactive hydroxyl groups and/or increasing the solubility of these metabolites. However, the involvement of different aglycone residues in the biosynthesis of both glucosides and sulfate esters suggests possible differences in the regulation of their biosynthesis and their compartmentation within the cells and tissues of this plant. These challenging problems deserve further study.

Acknowledgements

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