Biochemical Identification of *Sideritis serrata* × *S. bourgaeana* Hybrids by HPLC Analyses of Flavonoids

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The existence of sympatric interspecific hybrids of *Sideritis serrata* Cav. ex Lag. and *S. bourgaeana* Boiss. Reut. has been shown by HPLC analysis of the flavonoid glycosides accumulated in the cell sap, and by the methylated flavonoid aglycones exudated by the plants and deposited onto the plant surfaces. The hybrids tend to produce in a single plant the compounds characteristic of the parental taxa. This "complementation" effect in the hybrids permitted relatively easy detection of F1 hybrids. Generally, there was a close correlation between the biochemical results and a morphological analysis which used the Anderson's index, although a specimen which morphologically was a hybrid, showed a flavonoid pattern as *S. bourgaeana*. The external flavonoids analysis has revealed that the activity of sideritoflavone O-methyltransferases is much more important in *S. serrata* than in *S. bourgaeana*, while the hybrids show intermediate values. Moreover, *S. bourgaeana* accumulates sideritoflavone 3′-methylether whereas *S. serrata* accumulates the 4′-methylether. The hybrids produce both compounds in similar amounts. In this study, external flavonoids have proved more useful than vacuolar flavonoid glycosides in the biochemical documentation of hybridization. This is probably due to the fact that methylated flavonoid aglycones are physiologically more stable as they are externally located and therefore preserved from the action of enzymes.

Introduction

The section *Sideritis* of genus *Sideritis* L. is taxonomically difficult since it is currently developing. Hybridization plays a very important role in this process. Interspecific hybrids within the section *Sideritis* have been recorded from many localities in Spain, and it seems probable that much of the taxonomic problems is due to the occurrence of these hybrids and possibly hybrid swarms [1]. The identification of those hybrids by morphological studies is often difficult, and flavonoids have been used successfully for biochemical identification of sympatric hybrids [2, 3].

In the present work, the existence of sympatric interspecific hybrids of *S. serrata* Cav. ex Lag. and *S. bourgaeana* Boiss. Reut. is morphologically and biochemically documented, by means of the Anderson's hybrid index and HPLC analyses of vacuolar and external flavonoids.

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Results

Morphological study

This has been achieved on interbreeding populations of *S. serrata* and *S. bourgaeana* and their hybrid, in the hybrid zone of Abenuj (Tobarra, Spain). The distribution of the hybrid index values through the sample follows a sinuous curve. The theoretically pure parent of *S. bourgaeana* has a hybrid index of 0, though in real populations, values between 0 and 6 are observed (group **a** in Fig. 1), this being clearly separated from the next group by a gap. Individuals with values between 6 and 12, are transitional to the typical hybrid (group **b**) and probably correspond with individuals produced by back-crossing between the hybrid and *S. bourgaeana*. The group **c** has values between 12 and 30, showing great variability this being the typical hybrid *S.* × *rodriguezii* Borja [4] including F_1, F_2, etc., individuals. The last group, **d**, includes the individuals belonging to *S. serrata*, with hybrid index values between 36 and 57. None has reached the upper limit of 60 which belongs to the theoretically pure parental *S. serrata*.

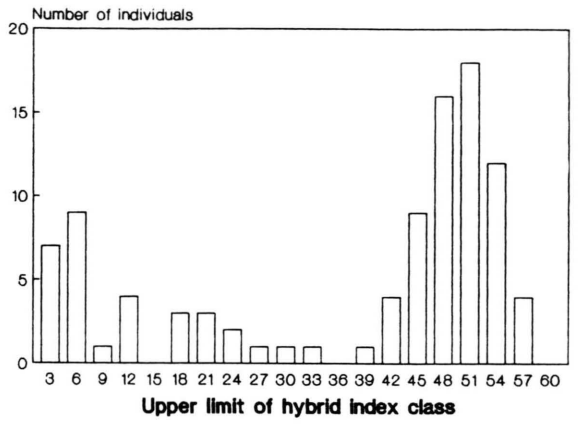

Fig. 1. Frequencies of Hybrid Index (Hi) values and their taxonomic interpretation. Hi = 0–6, *Sideritis bourgaeana* pure (group **a**); Hi = 6–12, the putative back crossing *S. bourgaeana* × *S.* × *rodriguezii* (group **b**); Hi = 12–30, *S.* × *rodriguezii* hybrids, of F_1, F_2, *etc.* (group **c**); Hi = 36–60, *S. serrata* pure (group **d**).

Biochemical study

The external flavonoids where studied with HPLC in 14 selected individuals belonging to the different morphological groups described above, and results are summarized in Table I. Although most of the individuals analyzed grew sympatrically, one individual of both parentals growing allopatrically was analyzed for comparison purposes. Thus sample 1 is a typical *S. bourgaeana* specimen collected 30 km away from the hybrid zone, and sample 14 is a typical *S. serrata* specimen growing up the hills where *S. bourgaeana* does not grow. These results show that both parents produce similar flavonoid patterns, differing in the methyl ethers of sideritoflavone (5,3′,4′-trihydroxy-6,7,8-trimethoxyflavone) since *S. serrata* produces only the 4′-methyl ether, while *S. bourgaeana* produces only the 3′-methyl ether. As could be expected, the hybrids produce both compounds in relative amounts which depend on the degree of hybridization (hybrid index). Thus, the proper hybrids, *S.* × *rodriguezii,* produce both methyl ethers in similar amounts, while individuals which belong to the intermediate morphological groups produce principally one of the methyl ethers. However, the occurrence of sideritoflavone 3′,4′-dimethyl ether (5-desmethylnobiletin), which should be biosynthesized by the action of both methyltransferases which are present in the hybrid, was not detected. In addition, when regarding the relative abundance of sideritoflavone and their methyl ethers, *S. bourgaeana* produces sideritoflavone as the major compound, while *S. serrata* produces mostly the methyl ether (Table I). This is clearly in

Table I. Methylated flavonoids in the different specimens analyzed.

Specimen number	Hybrid index	ciol	sid	cirm	cirn	xant	8ME	8MC	Other
1	4.50	10.0	58.1	0.6	1.1	9.3	–	13.8	7.1
2	4.45	9.6	59.2	0.8	1.9	7.8	–	15.1	5.6
3	3.10	12.5	48.7	1.2	3.0	4.7	sh	22.0	7.9
4	4.25	7.1	46.4	0.4	1.0	14.1	sh	21.4	9.6
5	27.90	16.0	48.8	1.9	2.3	5.5	8.0	12.7	5.7
6	25.66	8.4	40.6	1.0	1.1	21.4	8.9	14.7	3.9
7	16.53	13.8	47.3	1.1	1.8	6.4	10.8	13.6	5.2
8	15.58	12.2	41.1	0.7	2.4	8.6	14.4	16.3	4.3
9	15.45	10.9	37.1	1.7	1.6	18.4	22.6	sh	7.7
10	35.40	4.1	8.5	0.8	4.8	23.5	47.6	–	10.7
11	44.43	12.0	19.0	1.6	10.2	9.7	42.0	sh	5.5
12	40.60	7.0	23.9	1.0	3.7	16.1	44.7	–	3.6
13	42.50	8.0	18.2	1.3	6.4	8.4	45.5	–	12.2
14	52.41	2.1	5.9	–	12.9	5.9	62.6	–	10.6

Numbers are % of the total absorbance of the HPLC chromatogram.
(ciol) cirsiliol = 5,3′,4′-trihydroxy-6,7-dimethoxyflavone; (sid) sideritoflavone = 5,3′,4′-trihydroxy-6,7,8-trimethoxyflavone; (cirm) cirsimaritin = 5,4′-dihydroxy-6,7-dimethoxyflavone; (cirn) cirsilineol = 5,4′-dihydroxy-6,7,3′-trimethoxyflavone; (xant) xanthomicrol = 5,4′-dihydroxy-6,7,8-trimethoxyflavone; (8ME) sideritoflavone 3′-methyl ether; (8MC) sideritoflavone 4′-methyl ether. (–) not detected, (sh) detected as a shoulder of the peak corresponding to the other sideritoflavone methyl ether.

connection with the activity of flavonoid O-methyltransferases, which are much more active, or more abundant in *S. serrata* than in *S. bourgaeana*. In Fig. 2, the percentage of sideritoflavone and their methyl ethers are represented for each specimen analyzed, and different groups are observed which generally correspond with the morphological groups shown in Fig. 1. It is noteworthy that sample number 5, which morphologically is a hybrid, biochemically is very similar to *S. bourgaeana*.

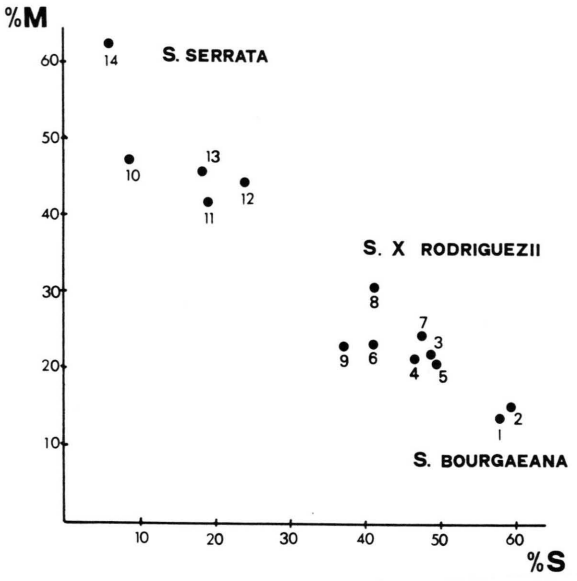

Fig. 2. Precentage of sideritoflavone and their 3′- and 4′-O-methyl ethers in the different specimens analyzed, as an expression of flavonoid O-methyltransferase activity. %S = percentage of sideritoflavone. %M = percentage of sideritoflavone 3′-methyl ether + sideritoflavone 4′-methyl ether. Percentages are obtained from the HPLC analyses.

The vacuolar flavonoid glycosides were analyzed by HPLC and results are summarized in Table II. Both parents accumulate hypolaetin and isoscutellarein glycosides in accordance to previous work [5]. However, *S. serrata* is characterized by accumulation of isoscutellarein derivatives (isoscutellarein 7-allosylglucoside and its 4′-methyl ether), while *S. bourgaeana* produces both hypolaetin and isoscutellarein 7-glycosides in similar amounts. In addition, hypolaetin 8-glucoside which is typical of *S. bourgaeana*, is absent from *S. serrata*. As a general rule, the hybrids show "complementation" patterns with significant relative amounts of isoscutellarein derivatives as for *S. serrata* and with hypolaetin 8-glucoside as for *S. bourgaeana*. However, some aberrant flavonoid patterns have been observed, such as that of sample 5 which accumulated hypolaetin 8-glucoside in a very high relative amount. When regarding the relative amounts of isoscutellarein 7-allosylglucoside and its 4′-methyl ether (Table II), which are in connection with isoscutellarein 4′-methyltransferase activity in the different samples, it seems clear that *S. bourgaeana* individuals produce mostly the isoscutellarein

Table II. Vacuolar flavonoid glycosides in the different specimens analyzed.

Specimen number	Hybrid index	H7G	H8G	8HC7G	I7G	4′MI7G	Other	4′MI7G/I7G
1	4.50	9.7	8.2	6.5	31.0	5.4	39.2	0.17
2	4.45	9.1	6.2	6.1	29.3	4.7	44.6	0.16
3	3.10	15.7	4.5	16.6	17.1	6.0	40.1	0.35
4	4.25	13.0	2.0	7.5	27.3	1.0	49.2	0.04
5	27.90	9.0	12.1	6.1	7.2	5.4	60.2	0.75
6	25.66	21.7	4.6	16.6	7.0	13.3	36.8	1.90
7	16.53	17.9	2.8	22.7	10.8	13.4	32.4	1.24
8	15.58	15.8	4.1	20.5	12.3	15.6	31.7	1.27
9	15.45	8.6	4.2	11.4	7.2	10.6	58.0	1.47
10	35.40	10.1	–	12.7	7.9	17.9	51.4	2.26
11	44.43	12.1	–	12.3	10.2	15.9	49.5	1.56
12	40.60	14.1	–	14.3	5.6	14.3	51.7	2.55
13	42.50	12.2	–	14.0	5.9	12.5	55.4	2.12
14	52.41	2.7	–	2.6	17.6	25.5	51.6	1.45

Numbers are % of the total absorbance of the HPLC chromatogram.
(H7G) Hypolaetin 7-glycoside; (H8G) Hypolaetin 8-glucoside; (8HC7G) 8-hydroxychrysoeriol 7-glycoside; (I7G) isoscutellarein 7-allosylglucoside; (4′MI7G) isoscutellarein 4′-methyl ether 7-allosylglucoside; (Other) the rest of UV absorbing compounds in the extract, which include minor flavonoids and phenolic acids.

glycoside while the *S. serrata* and the hybrid individuals accumulate the 4′-methyl ether derivative. It is remarkable that the hybrid specimens produce even more isoscutellarein 4′-methyl ether than the parental *S. serrata*.

Discussion

The results obtained demonstrate the existence of sympatric hybrids of *S. serrata* × *S. bourgaeana* and show that the complementary analysis of flavonoids, both methylated flavonoid aglycones, located externally, and flavonoid glycosides, located in the vacuole, can be used to study hybridization. The hybrids tend to produce in a single plant the compounds characteristic of the parental taxa, and this "complementation" effect in the hybrids permits relatively easy detection of F1 hybrids. Correlations between the morphological and biochemical data are generally observed, although one specimen (number 5) which morphologically is a hybrid, biochemically is very similar to *S. bourgaeana*. This could probably be explained by an introgression effect [2], but this statement needs to be confirmed. In the present study, external flavonoids analysis has been much more useful for the chemical documentation of hybridization than vacuolar flavonoids analysis. This is probably due to the fact that external flavonoids are physiologically much more stable than internal flavonoids, since the latter are subjected to the action of enzymes.

From the chemosystematic point of view, it is interesting that *S. bourgaeana* produces hypolaetin 8-glucoside, since this compound is a typical marker of *S. angustifolia* group [5] and links *S. bourgaeana* with *S. angustifolia via S. leucantha*. In addition, specimen number 5 produces hypolaetin 8-glucoside as a major compound, this being a characteristic of *S. angustifolia* [5], confirming this close relationship.

In addition, these results deserve some comments on methylated flavonoids biosynthesis. In this process, flavonoid O-methyltransferases play a very important role. It has been suggested that plants produce several position specific O-methyltransferases [6]. Our present results confirm this suggestion, since *S. serrata* produces sideritoflavone 4′-O-methyltransferase and *S. bourgaeana* the 3′-O-methyltransferase instead, while the hybrids produce both enzymes and synthesize both sideritoflavone 3′-methyl ether and the 4′-methyl ether. However, the presence of sideritoflavone 3′,4′-dimethyl ether in the hybrids, as a product of the activity of both enzymes, was not detected. This suggests that while sideritoflavone is a good substrate for both methyltransferases, sideritoflavone 3′-methyl ether and sideritoflavone 4′-methyl ether are not good substrates for methylation by the respective enzymes. This means that the introduction of a methyl group at the 3′- or 4′-position of sideritoflavone must result in a drop in the methyltransferase activity. These results agree with a previous work in which substitution of quercetin at 3′-position to give isorhamnetin, or at 4′-position to give tamarixetin, resulted in a complete loss of methyltransferase activity [7].

Experimental

Plant material

Aerial parts were collected in an area of 900 m^2, situated in the northern slope of a hill at the south border of the "Sierra de Abenuj" (Tobarra, Albacete, Spain). 14 Clumps, composed of 540 individuals, were localized and from these were taken 96 samples, avoiding the destruction of any plant. Another 8 additional specimens were collected from neighbouring places for comparison purposes. Specimens were prepared by drying and pressing. Voucher specimens were deposited in the Department of Botany, Murcia University.

Morphological analysis

Dried specimens were studied under the binocular microscope. The following characters were measured several times and scored to give the hybrid value of every specimen: Number of flowers per whorl, length of the N/2th internode, length of the bract, breadth of the bract, number of teeth per bract, length of the calyx, length of the calyx teeth, length of the leaves, breadth of the leaves, type of leaf margin, hair covering in the base of the twigs, hair covering of the spike axis, presence or absence of glands on the spike axis. The Anderson's index was calculated for each specimen as described previously [8].

Flavonoid analysis

Flavonoids were extracted as reported previously [5]. Secretory flavonoid aglycones were analyzed by TLC and HPLC as described [9] (a brand new C-18

column was used in order to discriminate between sideritoflavone 3′-methyl ether and the 4′-methyl ether). Vacuolar flavonoid glycosides were analyzed by TLC on cellulose with 30% HOAc, and the different spots visualized under UV light (360 nm) before and after spraying with Naturstoffreagenz A. The extracts were chromatographed with authentic markers isolated previously. HPLC analyses were done on a reversed phase column Spherisorb C-8, 5 μm (25 × 0.46 cm), run isocratically with a mixture of THF–ACN–MeOH–H_2O as described previously [10]. The flow rate was 1 ml/min. Samples of 5 μl were injected, and peaks were detected at 340 nm. The UV spectra of the different flavonoids were recorded by a photodiode detector coupled to the HPLC equipment.

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