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HYPERICUM MACULATUM CRANTZ SUBSP. *MACULATUM* X *H. PERFORATUM* L. (*HYPERICACEAE*): CORROBORATION OF NATURAL HYBRIDIZATION BY SECONDARY METABOLITE ANALYSIS

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Abstract: The presence of various flavonoids, naphthodianthrones and phloroglucinol derivatives was studied in the natural pentaploid hybrid *H. maculatum* CRANTZ subsp. *maculatum* × *H. perforatum* L. The hybrid taxon was shown to have secondary metabolites in common with both putative parents thus confirming its parentage. Morphological and nomenclatural questions are briefly discussed.

INTRODUCTION

If gathered with sufficient care, morphological data can be very effective in the resolution of problems involving hybridization. But it can also be very misleading, especially when attempting to assess the extent of hybridization (HARBORNE & TURNER 1984). The finding of the rare native hybrid *Hypericum maculatum* CRANTZ subsp. *maculatum* × *H. perforatum* L., which is the hybrid of the diploid species *H. maculatum* subsp. *maculatum* (2n=16) and the tetraploid facultative apomict (NOACK 1939) *H. perforatum* (2n=32), stimulated us to study the presence or absence of certain secondary metabolites in the flower tissue of all three taxa in order to confirm or refute the presumed parentage of the hybrid.

Several groups of secondary metabolites are accummulated by representatives of the genus *Hypericum* and they are synthesised by various biosynthetic pathways. Three groups of secondary metabolites are dealt with in this paper.

Hypericin and pseudohypericin from the group of naphthodianthrones are known in many *Hypericum* taxa and they are present in the flowers of both *H. perforatum* and *H. maculatum* (MATHIS & OURISSON 1963). There are additional data for *H. perforatum* in e.g. FREYTAG 1984, HÖLZL & OSTROWSKI 1987, SOUTHWELL & CAMPBELL 1991, HÄBERLEIN et al. 1992).

The next group are the flavonoids. I3,II8- and I3',II8-biapigenin (BERGHÖFFER & HÖLZL 1987, 1989) and rutin are present in *H. perforatum* and quercetin glycosides (hyperosid [=hyperin], isoquercitrin, quercitrin) in both species. *H. maculatum* differs from *H. perforatum* in the absence of rutin (MICHALUK 1961, LEIFERTOVÁ 1966).

The third group, produced by the polyketide biosynthetic pathway, consists of unstable acylphloroglucinol derivatives. The most recent papers show that they are present in many species; data are given e. g. for *H. revolutum* VAHL (hyperrevolutin A and B, DECOSTERED et al. 1989) and *H. japonicum* THUNB. (sarothralin G, ISHIGURO et al. 1990; sarothralen C and D, ISHIGURO et al. 1994). Hyperforin and adhyperforin are confirmed in *H. perforatum* (BYSTROV 1978, MAISENBACHER & KOVAR 1992, HÖLZL & OSTROWSKI 1987).

H. perforatum is stable from the point of view of the secondary metabolite character. Although the variation of the compounds is rather large and types with low and high content can be distinguished, no differences in the presence/absence of secondary metabolites are known and variation in quantity of each compound is likely to follow normal distribution. At the same time, the amount of individual compounds varies considerably during ontogeny as shown in *H. perforatum* during the blooming period (MÁRTONFI & REPČÁK 1994). In comparison with *H. perforatum*, *H. maculatum* has not been studied for its secondary metabolite chemistry.

MATERIAL AND METHODS

Plant material

Hybrid *H. maculatum* subsp. *maculatum* × *H. perforatum* was collected in Eastern Slovakia [Prakovce, by the side of a forest path, 3. VIII. 1993, leg. MÁRTONFI *1452* (herbarium specimen KO, no. 9063)]. The hybrid population was found growing among the parents which were also documented as herbarium specimens (*H. perforatum* KO, no. 9061, *H. maculatum* subsp. *maculatum* KO, no. 9038).

Flower samples were taken from fully open flowers both from the hybrid plant and the presumed parents for secondary metabolite analyses. The hybrid was marked and left in situ to mature and produce seed. During the next visit to the locality in autumn 1993 seeds were collected and in August 1994 more flower samples were taken for chemical analyses.

Further flower samples of *H. maculatum* subsp. *maculatum* × *H. perforatum* from the following herbarium specimens were used: Slovakia, Valaská Belá, above the challet on Homolka, 17. VII. 1965, leg. SCHIDLAY (SAV); Czech Republic, Lomnice nad Lužnicí, Velká Dubová, 1886, leg. A. WEIDMANN (BRA).

Chromosome numbers were counted in shoot meristems. Meristems were pre-treated with 0.1% colchicine, fixed in a mixture of ethanol and acetic acid (3:1), hydrolysed in 60 °C warm 1N HCl for 5 min., squashed in a drop of 45% acetic acid under cellophane square (MURÍN 1960) and stained in Giemsa stain (10 ml stock solution diluted with 90 ml M/15 SÖRENSEN phosphate buffer, pH 6.8).

Extraction and HPLC estimation of secondary metabolites

Air-dried flowers (9.8-15.5 mg) were extracted in methanol and immediately injected by Rheodyne sample injector (20 µl). A gradient method was used for resolution of flavonoids, naphthodianthrones and acylphloroglucinols (HÖLZL & OSTROWSKI 1987). Equipment: gradient pump (Ecom, Praha), variable UV VIS detector (Hewlett-Packard, Palo Alto, model 1050), column (3×150 mm) SCX C18, 7 µm (Tessek, Praha), integrator Apex (Ecom, Praha). HPLC purity solvents (Fluka) were used. Standard compounds of rutin (Fluka), quercetin

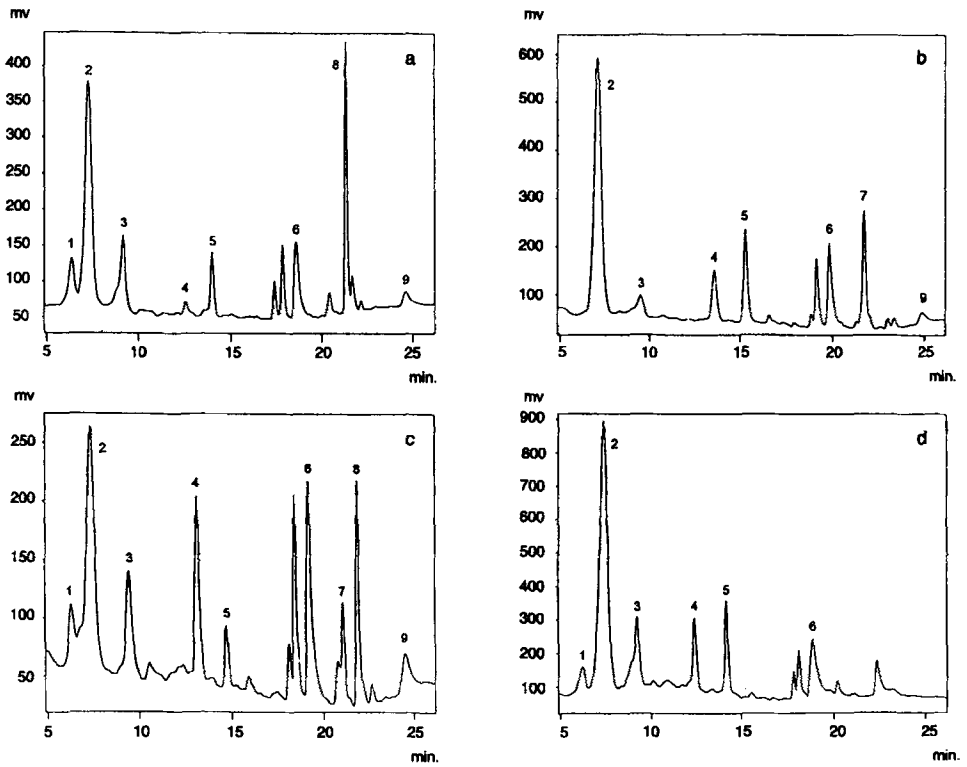

Fig. 1. HPLC chromatograms of flower methanol extracts. a – *Hypericum perforatum*, b – *H. maculatum* subsp. *maculatum*, c – *H. maculatum* subsp. *maculatum* × *H. perforatum*, d – as previous (c), 30 year old herbarium specimen. (1 – rutin, 2 – hyperoside and isoquercitrin, 3 – quercitrin, 4 – quercetin, 5 – biapigenin, 6 – pseudohypericin, 7 – unknown Rt=21.1, 8 – hyperforin, 9 – hypericin).

(Sigma), hyperoside, isoquercitrin, quercitrin, hypericin and amentoflavone [= I3',II8-biapigenin] (Roth) were used for comparative identification and quantitative determination. Hyperforin was isolated from acetone extract of flowers by means of silica gel column chromatography. UV spectral analysis was carried out to confirm compound identification.

RESULTS AND DISCUSSION

Morphology, chromosomes and nomenclature

On the basis of morphological characters hybrid *H. maculatum* subsp. *maculatum* × *H. perforatum* was identified in situ in Prakovce (Slovakia). The habit of the hybrid is closer to *H. perforatum* but the stem is 4-ridged, with two lines more conspicuous than the other two. The sepals are ovate or oblong, acute with an hairy apex and often minute dentations. The leaf venation, petal shape and hypericin gland distribution on the petals are intermediate. The chromosome number, counted in stem meristems of the hybrid, was 2n=40. The same

Table 1. Comparison of secondary metabolites in *Hypericum maculatum* subsp. *maculatum* × *H. perforatum* and their parents.

Taxa	Secondary metabolite 1	2	3	4	5	6	7	8	9
H. perforatum	+	+	+	+	+	+	-	+	+
H. maculatum	-	+	+	+	+	+	+	-	+
hybrid	+	+	+	+	+	+	+	+	+

Key: 1 – rutin, 2 – hyperoside and isoquercitrin, 3 – quercitrin, 4 – quercetin, 5 – biapigenin, 6 – pseudohypericin, 7 – unknown Rt=21.1, 8 – hyperforin, 9 – hypericin.

chromosome number was found in seedlings produced from seeds of the hybrid plant i.e. 2n=40 or 2n=ca. 40.

These chromosome numbers correspond with a pentaploid hybrid, classified by ROBSON (1981) as *H.* ×*desetangsii* nm. *perforatiforme* (A. FRÖHL.) N. ROBSON (basionym: *H.* ×*desetangsii* f. *perforatiforme* A. FRÖHL. Mitt. Naturwiss. Vereines Steiermark 51: 229, 1915). He stated that this hybrid is almost indistinguishable from *H. perforatum*. However, this does not fully correspond with our results given above. ROBSON (1981) attributes intermediate habit to the triploid type (2n=24) *H.* ×*desetangsii* LAMOTTE nm. *carinthiacum* (A. FRÖHL.) N. ROBSON. He attributed both nothomorphs [corresponding to the rank of nothovariety in the sense of the present Code (GREUTER et al. 1994)] to *H. maculatum* subsp. *maculatum* × *H. perforatum*.

In our opinion the hybrid name *H.* ×*desetangsii* LAMOTTE Bull. Soc. Bot. Fr. 21:121, 1874 is most probably not the correct name for the hybrid *H. maculatum* × *H. perforatum*, because of the earlier legitimate name *H.* ×*mixtum* DU MOULIN Oesterr. Bot. Z. 17: 390, 1867. However, *H.* ×*mixtum* DU MOULIN still requires typification, to determine its proper interpretation.

Secondary metabolites

The following secondary metabolites were found in *H. perforatum* (Fig. 1a): flavonoids: rutin, hyperoside and isoquercitrin, quercitrin, quercetin, I3,II8-biapigenin; naphthodianthrones: pseudohypericin and hypericin; and acylphloroglucinol derivatives: hyperforin and adhyperforin. These results correspond with our previous analyses of *H. perforatum* on more than 150 samples (1992-94), in which plants from natural populations and plants cultivated in breeding programmes were studied. In *H. maculatum* subsp. *maculatum* (Fig. 1b) the following secondary metabolites were found: flavonoids: hyperoside and isoquercitrin, quercitrin, quercetin and I3,II8-biapigenin; naphthodianthrones: pseudohypericin and hypericin and a compound with retention time 21.1 min. (in Fig. 1 and Tab. 1 it is marked no. 7), which has not been further identified. From its retention time and hyperforin-like UV spectra (max=272 nm, obtained by HPLC analysis of flower extract) it appears to be an acylphloroglucinol derivative. These findings correspond well with our 21 previous analyses of *H. maculatum* subsp. *maculatum* from various localities in Slovakia.

H. maculatum subsp. *maculatum* × *H. perforatum* shows an additive pattern of secondary metabolites from both parents i. e. rutin, hyperoside, isoquercitrin, quercitrin, quercetin, I3,II8-biapigenin; pseudohypericin, hypericin, hyperforin and adhyperforin, as well as the unidentified compound no. 7.

The presence of further compounds was recorded in all the samples (Rt 18.2 min. and 18.5 min., before compound no. 6), but their quantity varied considerably between the different taxa studied. These compounds were not identified.

The results obtained confirmed previous findings using paper chromatography in which differences in flavonoid constituents were reported between *Hypericum* taxa (LEIFERTOVÁ 1966), as well as additional compounds in *H. perforatum*, recorded by HÖLZL & OSTROWSKI (1987). We could find no report of biapigenin in *H. maculatum* in the literature. The results for *H. maculatum* subsp. *maculatum* × *H. perforatum* reveal that it is another case of F_1 chemical complementation for different compounds in the interspecific hybrid (cf. HARBORNE & TURNER 1984).

It was also interesting to ascertain whether secondary metabolites could be used to identify the hybrid from herbarium specimens (see Material and Methods). A specimen, dated 1886, i.e. more than 100 years old, transpired to be unusable although peaks corresponding with hyperoside + isoquercitrin, quercetin, biapigenin and pseudohypericin were identified. The other compounds could not be identified.

In a 30 year old *H. maculatum* subsp. *maculatum* × *H. perforatum* herbarium specimen (from 1965, Fig. 1d), the following metabolites were identified: rutin, hyperoside and isoquercitrin, quercitrin, quercetin, biapigenin and pseudohypericin. The presence of rutin was a good indicator for the hybrid in question. Acylphloroglucinol derivatives are unstable compounds and could not be identified in material of this age.

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