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Short communication

Methylated flavones of the hairy root culture *Scutellaria baicalensis*

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ABSTRACT

Perennial plants in northern Dauria (Zabaikalsky region) grow in low temperatures in winter and in a dry hot summer. The prairies of northern Dauria are rich in a variety of medicinal herbs, including *S. baicalensis*, which has roots that are in demand for traditional Chinese medicine. In addition to two monomethylated flavones (wogonin and oroxylin A), determining the pharmacological significance of the root, there is also a minority of their polymethylated congeners. Little is known about their role in the plant or their connection with the conditions of growth and cultivation of their hairy root culture (HRC). Therefore, the purpose of this study was to determine whether and to what extent the biosynthesis of the latter is retained in the hairy root culture established from wild plants of Dauria. The composition of the main methylated flavones of HRC was established using LC–MS and a previously unknown pentamethylated flavone was found in the roots. This study showed a more significant accumulation of polymethylated flavones in the root of the wild plant than in HRC.

1. Introduction

The important herbaceous plant *Scutellaria baicalensis* Georgi is geographically widespread. The roots of *S. baicalensis*is are among the most popular herbal medicines worldwide, especially in China, Korea and Japan. The roots of this plant are known to produce the root-specific flavones baicalein **3**,wogonin **4**, oroxylin A **5**. A high level of their accumulation led to a proposal for the evolutionary specialization of the appropriate pathway of flavone biosynthesis, responsible for the production of these flavones (Zhao et al., 2016a, 2016b).The occurrence of methylated flavones in both modern plants and ancestral lineages suggests that their original physiological roles might be universal and linked to the challenges of their terrestrial existence (Berim and Gang, 2016).

A metabolimics study of substances in the roots of *S. bacalensis* based on the DAD LC-HRMS/MS method reported 65 O-methylated flavonoids, including glycosides, for a total of 132 substances (Qiao et al., 2016). These methylated flavones include species that carry the 2, 3 and 4 OMe groups alongside the most abundant monomethylated wogonin **4** and oroxylin A **5**. In recent years, the demand for this plant has continued to increase due to the constant expansion of the *S. baicalensis* application leading to the depletion of its wild resources. However, there is a concern about the stability of the quality of the cultivated plant needed to fulfil the market demand. Therefore, it is important to study the wild roots of *S. baicalensis*, especially from the region that gave the species its name.

Interest in the conservation and sustainable use the plant led to an introduction of the hairy root culture (HRC) of *S.* baicalensis (Kuzovkina et al., 2001; Nishikawa and Ishimaru, 1997; Stojakowska and Malarz, 2000; Zhou et al., 1997). Manipulations with genes, media, and nutrition of the HRC have allowed researchers to identify a remarkable content of main flavones (Kim et al., 2012, 2014; Park et al., 2011, 2012, 2016). Apart from the main methylated flavones wogonin **4** and oroxylin A **5**, the plant root contains dozens of various methylated flavones (Qiao et al., 2016). Very few facts have been reliably established regarding the actual physiological role of the methylated flavones in the plant. An environment of HRC sharply differs from the soil naturally supporting the growth of plant roots. Biosynthesis of the flavones in HRC is largely controlled genetically, but it also can be regulated by nutritional and environmental factors (Park et al., 2012; Stojakowska and Malarz, 2000). Therefore, the question arises whether HRC has the same rich repertoire of methylated flavones as the wild root of *S. baicalensis*. This communication provides a comparative pattern of polymethylated flavones in wild roots of *S. baicalensis* from Dauria and the corresponding HRC.

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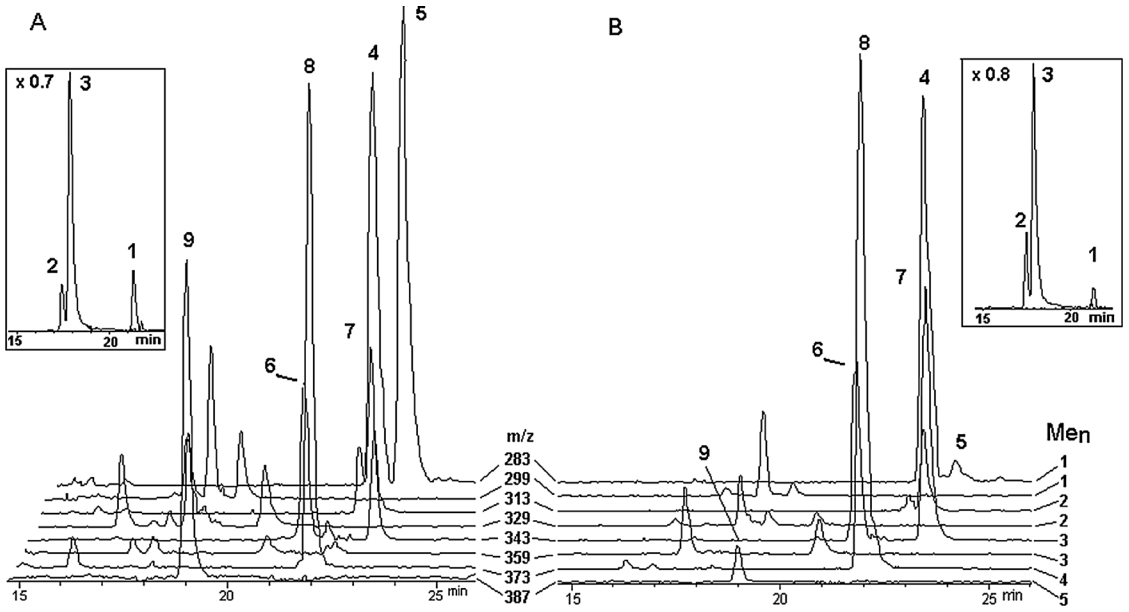
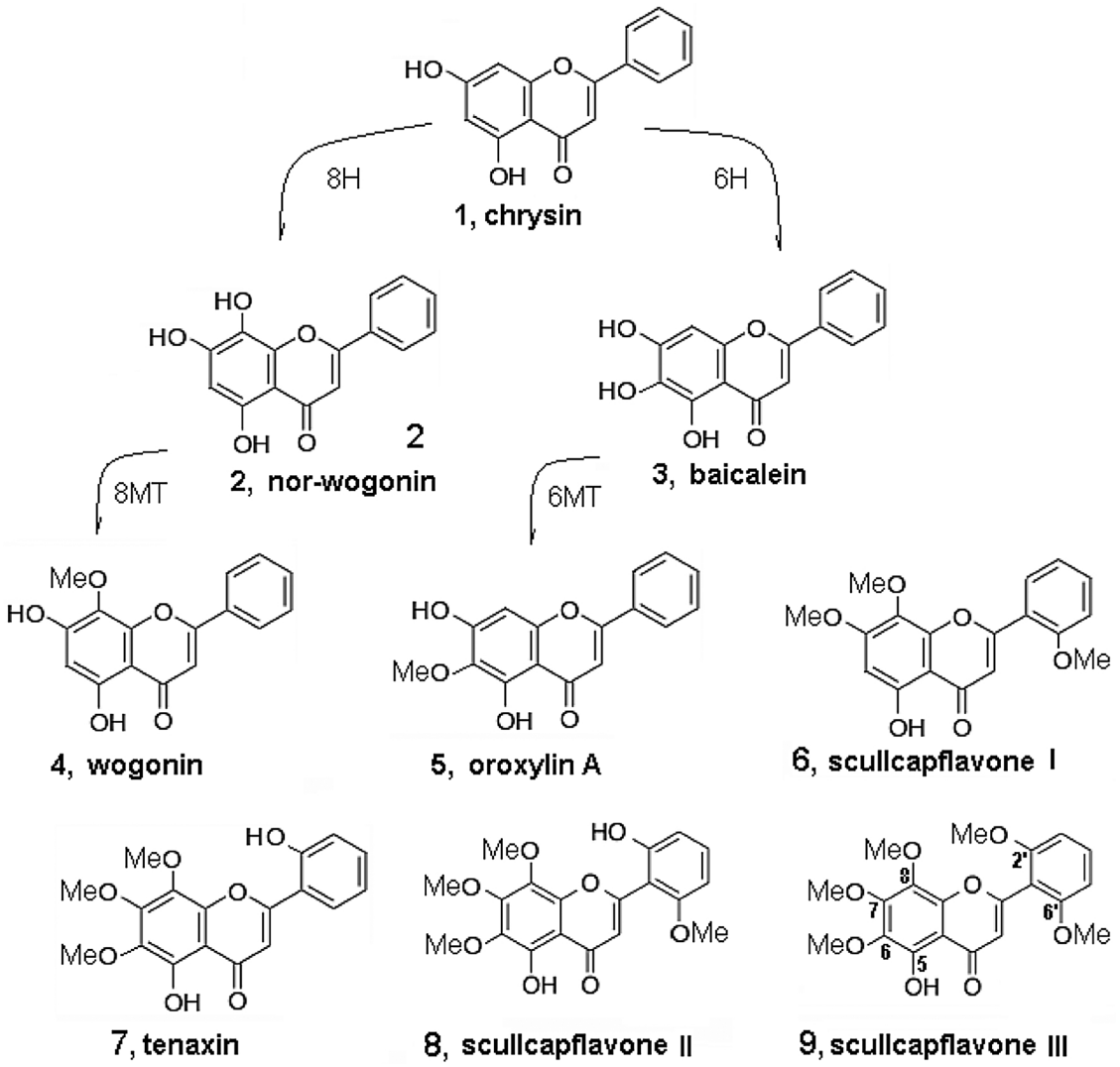


Fig. 1. The ion chromatograms of main methylated flavones: (A) roots of the *S. baicalensis* from Dauria; (B) its hairy root culture. Right number column (Me_n) means the number of OMe groups in a molecule. Inserts: ion chromatograms for the flavones of begin steps biosynthesis.



Scheme 1. The beginning of the path of biosynthesis of flavones in roots and HRC.

2. Materials and methods

The *S. baicalensis* roots were excavated in the Buryat-Aginsky district of Zabaikalsky region in 2017. The hairy root culture of the Baikal skullcap that was used in this investigation is a deposit of the collection of the Institute of Plant Physiology of Russian Academy of Sciences. The culture was established by Prof. Kuzovkina in 1997 using the *A. rhizogenes* A4 wild strain (Kuzovkina et al., 2001). Transformation was conducted by co-cultivation of explants (hypocotyls) and *Agrobacterium rhizogenes* (strain A4). *Agrobacterium*-mediated transformation was shown using the opine test (Petit et al., 1983). The HRC was grown in Gamborg B5 medium without hormones for 5 weeks according to the following scheme: 1 g inoculates were each placed into 100 ml flasks containing 40 ml medium and cultured for 14 days, after which the grown roots were transferred into the 300 ml flasks with 80 ml medium and grown until the culture has reached the age of 35 days.

Dried powder roots (0.5 g) and the lyophilized hairy root culture (2.5 g) of the *S. baicalensis* were twice sonic energize extracted with 96% ethanol at 50 °C. Aliquots of the jointed extracts were centrifuged before injecting in a column of the LC-UV-MS\MS instrument Agilent Technologies 1260 Infinity analytical HPLC system - Bruker HCT ion trap mass spectrometer. The ESI MS analyses were run in negative ion mode. An analytical reverse phase column Zorbax C18, 150 mm, 2.1-mm i.d., 3.5-μm particle size was applied for separation. Separation was carried out using the following conditions: the column temperature was 40 °C, and the mobile phase consisted of 0.1% aqueous acetic acid (A) and acetonitrile (B). The following elution gradient with a flow rate of 0.2 ml/min was used: 0 min 20% B; 3 min 20% B; 25 min 80% B, 30 min 100% B, and then eluent B until 40 min.

3. Results and discussion

The phenoxide-ion [M − H] of ESI mass spectra (Xia, Attygalle, 2016) was used for a comparison of relative abundances of methylated flavones in the both samples, following the protocol that Chinese colleagues used for a metabolomics study of the roots of *S. baicalensis* (Qiao et al., 2016). The ion reflects the relative content of these flavones with greater certainty than UV absorption, even allowing some variations in dissociation of the OH groups on different positions on the carbon skeleton of molecules in the ESI ion source. This study of the obtained MS data by the mass of phenoxide-ions of methylated flavones revealed eight types of abundant flavone carrying from one to five OMe groups. Their ion chromatograms are collected with a time shift in three-dimensional form along the descending mass and the number of OMe groups, as shown in Fig. 1. Since the peaks of chromatograms of phenoxide-ions for chrysin **1**, the flavone from which the diversification of the flavone biosynthetic pathway begins, and it first derivatives norwogonin **2** and baicalein **3** do not overlap like their methylated congeners, they are assembled into a 2D form, as shown in the inserts in Fig. 1. The ion chromatograms of these three flavones show marked differences in their relative content at the beginning of the biosynthetic pathway in the roots and HRC. The flavones **2** and **3** are a result of the action of 8H- and 6H-flavon hydroxylases, respectively (Berim and Gang, 2013). The subsequent combinations of enzymatic methylation and hydroxylation (Berim and Gang, 2016) led to the accumulation of a wide variety of the methyl ester species from mono- (tracks *m/z* 283 and 299) to penta-methylated flavone **9** (track *m/z* 387), as shown in Fig.1. It should be noted that flavone **9** has not previously been found in plants, including the genus Scutellaria.). Indeed, flavone **9** was previously synthesized from tetra-methylated flavone **8** by methylation of the 2′ OH group (Takido et al., 1975). This 6,7,8,2',6'-penta-methyl flavone, denoted as the scullcapflavone III, completes the palette of methylated flavones in the root and HRC of *S. baicalensis* (Scheme 1). It is remarkable that, among the methylated flavones in the roots of this plant in the southern geological provinces, the methylated flavones with OMe group at the C5 carbon atom are not found (Qiao et al., 2016). Apparently, an intermolecular hydrogen bond is also an obstacle for methyltransferases, as for diazomethane in the chemical synthesis of flavone **9** (Takido et al., 1975).

Comparison of the chromatographic profiles of the eight most abundant methylated flavones in both samples demonstrates the difference in the abundances of flavones with one and two OMe groups in the root and HRC relative to the wogonin **4**, as shown in Fig. 1A and B. The constancy of the culture medium likely allows methyltransferases to deplete, to some extent, a part of these intermediates in HRC. It should be noted that the expression of flavone-6-hydroxylase and 6-methyltransferase is much less active in HRC than in wild roots, in which the content of oroxilin A **5** and wogonin **4** is comparable. Together with this, the accumulation of the polymethylated flavones scullcapflavone I **6**, *m/z* 343; tenaxin **7**, *m/z* 343 and scullcapflavone II **8**, *m/z* 373 in both roots and HRC was comparable. We did not aim to measure the content of methylated flavones in weight units, mg. The quantitative production of the wogonin **4** in both the root and HRC of *S. baicalensis* has been measured by many researchers (see Introduction) who have studied this culture, including Kuzovkina. Therefore, taking the ion current of the phenoxide-ion *m/z* 283 for wogonin **4** per unit of measurement, it is possible to see the relative quantitative differences in the production of the polymethylated flavones, as shown in Fig. 1. It should be noted that the quantitative measurement of flavones by UV absorption in LC is a very difficult task for extracts containing several dozen substances due to multiple overlapping chromatogram peaks (Qiao et al., 2016). In addition, the profiles showed that the genes responsible for the final of biosynthetic pathway of flavones in the root of the plant are fully in demand in its HRC. This was demonstrated by the expression of 2′-methyltransferase in the hairy root culture, which caused the production of the new flavone **9** in the wild roots.

4. Conclusion

The results show that geological and climate factors, very cool and dry, of the Dauria eco-geographic system established the conservative genes in the *S. baicalensis* for biosynthesis of polymethoxylated flavones. Their biosynthesis in the hairy root culture obtained from the plant of Dauria is saved almost in the same repertoire. Some polymethylated flavones are found in *S. baicalensis* growing in the Southern geological provinces, but their content in the roots is less relative to monomethylated wogonin **4** and oroxylin A **5** (Liu et al., 2009). These latter constitute the bulk of all methylated flavones in the roots of *S. baicalensis* from Southern areas. Our results show that HRC of *S. baicalensis* from Dauria can be a valuable platform to study influence of media factors on a biosynthesis of the methylated flavonoids. Despite the differences in conditions of existence, the culture of the root hairs preserves the biosynthesis of polymethylated flavones in a ratio close to that observed in the root of the wild plant from which it was obtained. Any relationship between the harsh climate conditions or the mineral environment of the plant roots and enhanced expression of methyltransferases in the root hairs involving genes in the hair root culture requires further investigation.

Author statement

Authors have not conflict interests.

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