

COULD EVOLUTIONARY FACTORS AFFECT ENDOGENOUS CYTOKININ POOLS IN *HYPERICUM* SPECIES *IN VITRO*?

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Abstract: In the present work, shoot cultures of hypericin non-producing *Hypericum calycinum* L. (of the primitive *Ascyreia* section), hypericin producing *H. perforatum* L. and *H. tetrapterum* Fr. (both section *Hypericum*) and *H. richeri* Vill. (the evolutionary most advanced *Drosocarpium* section in our study) were developed. For the purpose of multiplication stimulation, cytokinin (CK) N⁶-benzyladenine and auxin indole-3-butyric acid were exogenously applied in different combinations. The content of total endogenous CKs were studied and their pools and ratios between *cis*- and *trans*-zeatin types were compared.

INTRODUCTION

The *Hypericum* genus comprises over 450 species distributed worldwide and classified into 36 sections (Danova, 2015). The most widely studied representative of the genus is *H. perforatum*, which has been applied in medicinal practice since the 1st century A.D. up to modern times (Danova 2015, and references cited within). The species has been utilized for a wide array of pharmacological properties such as antimicrobial, antiviral, anti-inflammatory, wound-healing and anticancer, amongst many others (Barnes et al., 2001; Saddiqe et al., 2010; Süntar et al., 2010). These pharmacological properties are due to the wide array of biologically active compounds characteristic for *H. perforatum*, but also to the other representatives of the genus. The main chemical groups of secondary

metabolites characteristic for the *Hypericum* species are polyphenolic compounds, flavonoids, naphthodianthrones and phloroglucinols, and terpenes (Nahrstedt and Butterweck, 1997; Bruni and Sacchetti, 2009; Nahrstedt and Butterweck, 2010).

Research has shown that the production levels of condensed naphthodianthrones hypericin and pseudohypericin are related to the evolutionary development of the species in accordance with their sections' distribution. Thus, while the *Hypericum* species of the most primitive sections lack hypericins production, the levels of these compounds increase with the ascent of the evolutionarily level in the genus (Kitanov, 2001; Smelcerovic et al., 2006; Danova et al., 2014 and references cited within).

The flora of Bulgaria comprises 22 species of the *Hypericum* genus, distributed within 11 sections. Of them, one species is Bulgarian (reported now as extinct), and five are Balkan endemics (Yordanov and Kojuharov, 1970; Robson, 1977; Kitanov, 2001; Anchev et al., 2009).

The aim of the present work was to develop *in vitro* culture of *Hypericum* species from sections of the genus of different evolutionary levels and to study the effects of exogenously applied plant growth regulators on their endogenous cytokinin (CK) pools.

MATERIALS AND METHODS

Plant material

Shoot cultures of the hypericin non-producing *H. calycinum* of the *Ascyreia* section (the most primitive in our study), *H. perforatum* and *H. tetrapterum* of the *Hypericum* section and the representative of the evolutionarily most advanced in the work *Drosocarpium* section were established.

For the purpose of *in vitro* culture initiation plants collected from their natural habitats were used (with the exception of *H. calycinum* for which plants cultivated for ornamental purposes were used).

Tissue culture development

For tissue culture induction, the cuttings of the aerial parts of the plants from natural habitats were surface-sterilized in 0.1 % HgCl₂, triple washed in sterile distilled water and placed in Murashige and Skoog (1962) culture medium, supplemented with 0.2 mg/l CK, N⁶- benzyladenine (BA). After the formation of axillary shoots, stock shoots of the species were maintained in plant growth regulator (PGR)-free medium (with the exception of *H. richeri* which required PGR supplementation for its long-term *in vitro* maintenance). For the purpose of the experiment the following PGR treatments were applied to the PGR-free control (Hyp_M_0): Hyp_M_1 – 0.2 mg/l BA, Hyp_M_2 – 0.2 mg/l BA + 0.1 mg/l indole-3-butyric acid (IBA) and Hyp_M_3 – 0.1 mg/l BA + 0.2 mg/l IBA. For the purposes of endogenous CKs evaluation, plant material of five different cultivation flasks was combined and lyophilized. Two separate aliquot parts of the combined plant material were extracted and subjected to the respective analyses.

Endogenous CK levels analyses

The contents of endogenous phytohormones were quantified using high-performance liquid chromatography electrospray tandem-mass spectrometry (HPLC-ESI-MS/MS) (Danova et al., 2018). Data of the content of endogenous CKs were collected and the total CK pools and ratios between *cis*- and *trans*-zeatin types were compared.

RESULTS AND DISCUSSION

While dark glands, in which hypericins are stored, were clearly visible along the ribs of the hypericin producing *Hypericum* species (illustrated by *H. richeri* in Fig. 1A), in *H. calycinum* only translucent glands, in which essential oils and phloroglucinols are accumulated (Fig. 1B). The close relation between dark glands and hypericin production has been well known and widely discussed in literature (Briskin and Gawienowski, 2001; Onelli et al., 2002; Piovan et al., 2004). The biosynthesis and accumulation of essential oils and phloroglucinols in *Hypericum* species are bound to the translucent glands visible on the leaves of the species (Soelberg et al., 2007).

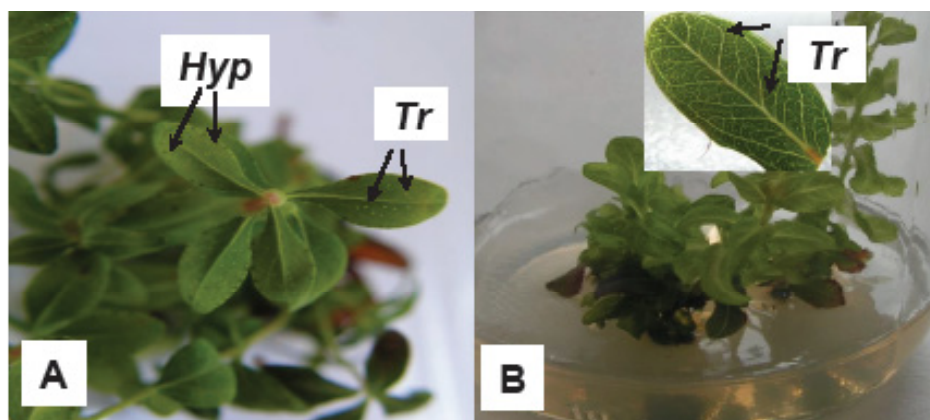


Figure 1: Shoot cultures of the representative of the evolutionary most developed Drosocarpium section - the hypericin-producing *H. richeri* in Hyp_M_1 culture medium (A) and the hypericin non-producing *H. calycinum* in Hyp_M_1 culture medium (B).

Tr = essential oils and hyperforin producing translucent glands, *Hyp* = hypericin producing dark glands, visible on the ribs of leaves

CKs are plant hormones with central role in regulation of plants' vital processes such as development of shoots and roots, leaf senescence, chloroplast development, pathogen defense in higher plants and bud formation in lower plants (Mok and Mok, 2001; von Schwartzenberg et al., 2007).

Analyses of the results of endogenous CK production were indicative for their relations with the evolutionary level of the studied *Hypericum* species. Thus, while the most primitive *H. calycinum* showed the lowest levels of total CKs (Fig. 2), as well as of *cis*- (Fig. 3) and *trans*-zeatins (Fig. 4), the highest

total CK pools were recorded for the most evolutionary developed *H. richeri*. As mentioned above *H. richeri* required PGR supplementation for its long-term *in vitro* maintenance and did not grow on control Hyp_M_0 medium.

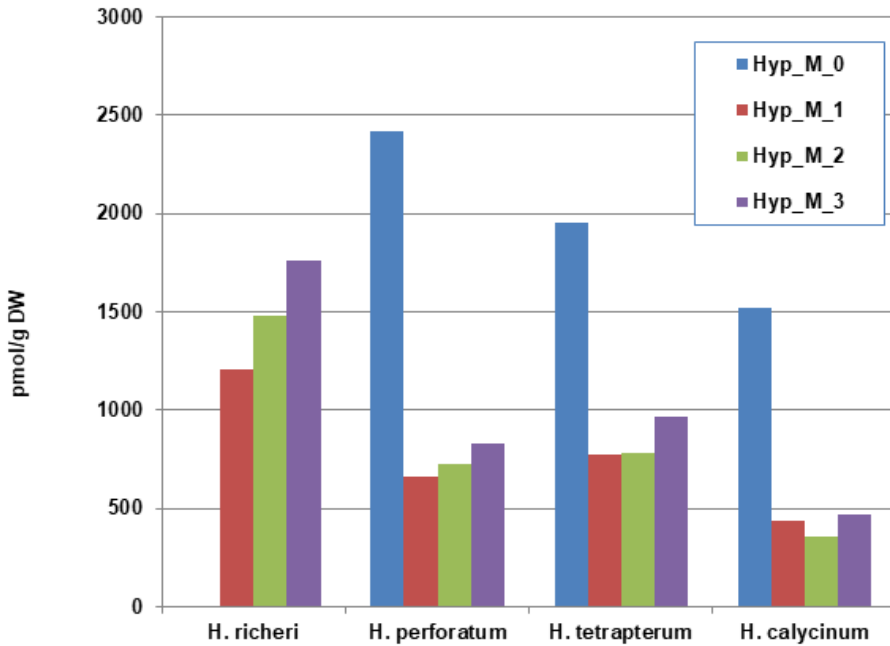


Figure 2: Total CK pools established for the four culture media supplementations for the four *Hypericum* species

The analysis of data regarding the *trans*- and *cis*-zeatin type CKs, however, showed an interesting interplay when comparing amongst the three hypericin producing species. Thus, while the highest levels of *trans*-zeatin types were detected for the most evolutionary developed hypericin producing *H. richeri* (Fig. 3), on the contrary *cis*-zeatins dominated in *H. tetrapterum* (Fig. 4).

The data for different *Hypericum* representatives correspond with our previous results that *trans*-zeatin type CKs prevail generally in evolutionary younger plants whereas *cis*-zeatin types are ubiquitous and predominate in evolutionary older cyanobacteria and algae (Žižková et al. 2017), mosses (Záveská Drábková et al. 2015) as well as in less advanced vascular plants including ferns (Zemanová et al. 2019) and some of seed plants (Gajdošová et al. 2011). Thus, they clearly indicate that the evolutionary factors affect internal CK pool and portion of particular CK forms in *in vitro* grown *Hypericum* species.

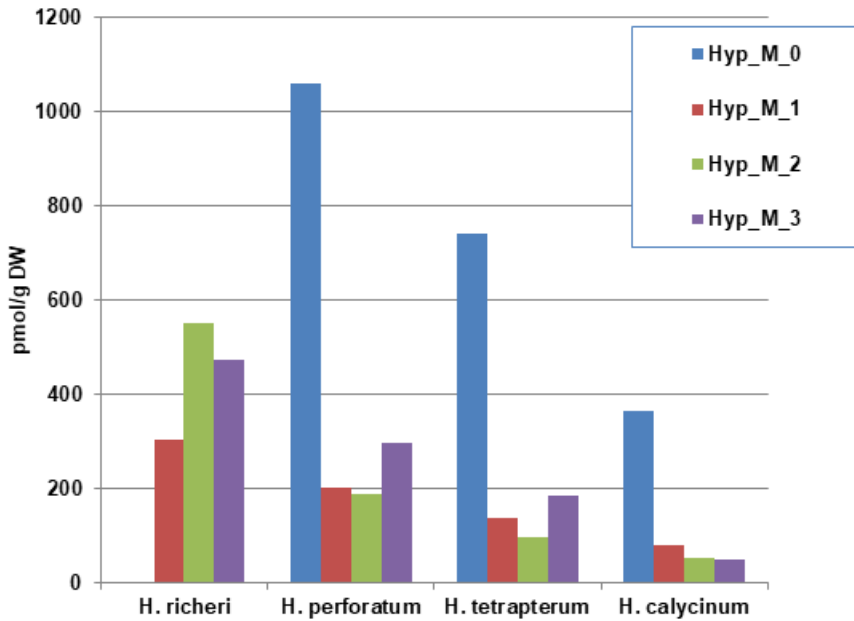


Figure 3: *Trans*-zeatin types CK pools established for the four culture media supplementations for the four *Hypericum* species

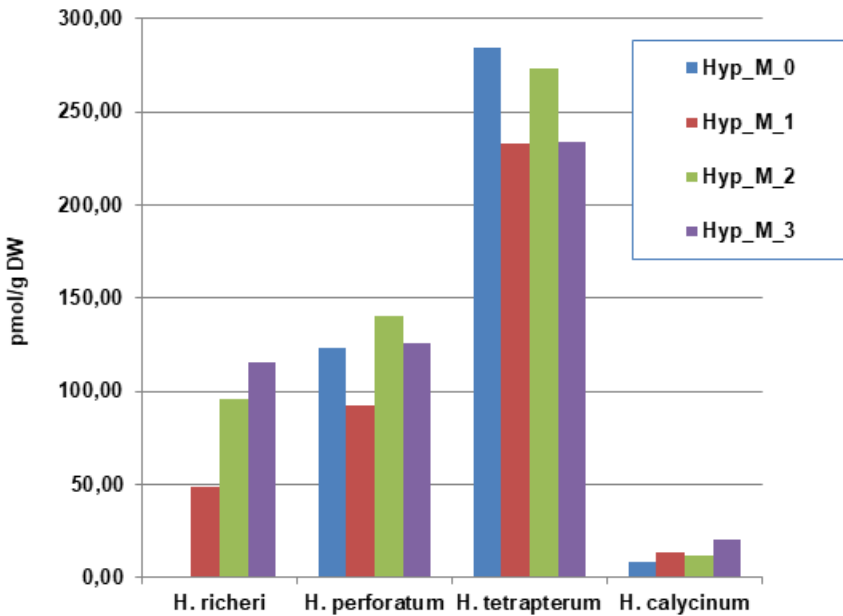


Figure 4: *Cis*-zeatin types CK pools established for the four culture media supplementations for the four *Hypericum* species

CONCLUSION

Our observations indicate that both complexity of evolutionary development, as well as hypericin production capacity, might be in close interplay with parameters of physiological adaptation in the plant organism such as endogenous CKs production..

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Conflicts of Interest: The authors declare no conflict of interest.

Involvement in the presented study: KD developed the shoot cultures of the studied *Hypericum* species and conceived the experimental design and manuscript, VM and PD performed the analyses of endogenous CKs and VM performed the data interpretation of the results. All authors have read and critically reviewed the manuscript.

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