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Approaches for study of polyphenolics biosynthesis in the *Hypericum* genus *in vitro*

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Hypericins, characteristic for the developed sections of the *Hypericum* genus, are widely studied for their antiviral, antitumor and antidepressant properties. Other phenolics, as well as the volatiles contribute to the wide area of medical applications of *Hypericum* preparations. A great number of works study secondary metabolites in *Hypericum* species *in vitro*. However, research is mainly focused on *H. perforatum* or other species of section *Hypericum*. We have investigated shoot cultures of the Balkan endemic *H. rumeliacum*, belonging to the more developed *Drosocarpium* section. The established amounts of 0.29 mg/gDW and 1.47 mg/gDW for hypericin and pseudohypericin, respectively, are higher than other *Hypericum* shoot cultures, reported in literature so far. In order to better understand their *in vitro* „behavior“, we also studied shoot cultures of *H. tetrapterum* (*Hypericum* section), hypericin non-producing *H. calycinum* (*Ascyreia* section), as well as three *H. rumeliacum* lines, regenerated and cultivated over one and a half year after cryopreservation. We have studied growth parameters, dark glands density, antioxidant enzymes and radical scavenging activity in relation to the physiological status and polyphenolics content. According to our results, high hypericin levels *in vitro* are connected with elevated levels of malondialdehyde and hydrogen peroxide implying of a possible auto-toxic effect of hypericin *in vitro*. This might explain the recalcitrance of *H. rumeliacum* to cryopreservation and the lower recovery rates, than *H. perforatum*, which are reported in literature. Hypericin production is also strongly related to the morphology of shoots, which is an important parameter to be monitored for the targeted selection of hypericin-highly productive lines.

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Phytochemical study of *Anthemis rumelica*

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The genus *Anthemis* (Asteraceae) is represented by 62 species in Europe, which are divided into 3 subgenera. Each of them involves different number of sections. One of the main classes of secondary metabolites characteristic for Asteraceae family, respectively for the genus *Anthemis* are sesquiterpene lactones. This type of natural compounds is important because of their biological properties as well as their use as chemotaxonomical markers. In continuation of our interest in sesquiterpene lactones in the genus *Anthemis*, we studied *Anthemis rumelica*, an endemic species for Bulgaria. Thus, four lactones were isolated, of which two guaianolides (4,9-diacetoxyguaia-2,1(10),11(13)-triene-12,6-olide and 1,4-dihydroxyguaia-2,10(14)-dien-12,6-olide) and two germacranolides (hanphyllin and 1 α ,10 β -epoxy-desacetyl-angeloyllaurenobiolide). In addition, two flavonoids were identified (santin and centaureidin). The structures of the isolated components were determined on the basis of their spectroscopic characteristics. Noteworthy, described above lactones have not been detected previously in the genus *Anthemis*. It should be also noted, that no common feature was observed in their structures-carbon skeleton, substituents, position of the substituents, etc. The importance of sesquiterpene lactones in chemosystematics of the genus *Anthemis* was discussed in our previous article. *A. rumelica* is classified in sect. *Hiorthia* of subgenus *Anthemis*. A chemotaxonomical scheme of genus *Anthemis* based on sesquiterpene lactones as chemotaxonomic markers was discussed in our recent review (Staneva et al., 2008). The obtained results are in accordance with its place in the botanical taxonomical scheme.

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Xanthone content and free radical scavenging activity of hairy root cultures from *Gentiana dinarica* Beck

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Plant species *Gentiana dinarica* was transformed with *Agrobacterium rhizogenes*, strain A4M70GUS. Hairy root cultures were established on solid and liquid MS media. Nontransformed roots cultured on the same medium supplemented with IBA 0.5 mg/l were used as control. Lyophilized roots were extracted with methanol and analysed using HPLC-DAD method. The presence of 1,3,7,8-tetraoxygenated xanthones, which are typical for this species, was confirmed and their content was determined. The concentration of xanthone aglycone norswertianin was 15 times higher in hairy roots than in nontransformed roots. Roots cultured on liquid medium contained higher amount of xanthones than those on solid medium. Relative antioxidative activities of methanolic extracts were tested by measuring of their ability to scavenge the stable free radical – DPPH. Our results showed that hairy roots exhibited free radical scavenging potential which is in accordance with their higher xanthone production.

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In vitro* polyphenolics production of Balkan endemic *Pulsatilla montana* ssp. *balcana

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Although *Pulsatilla* species are widely utilized in Asian traditional medicine, secondary metabolites of the Balkan representatives of the genus are scarcely studied so far. Wild growing Balkan endemic *P. montana* ssp. *balcana* was collected in Bulgaria. Total assays of phenolics and flavonoids in the aerial parts showed levels of 113.1 mg/gDW and 19.8 mg/gDW respectively (flavonoid content 17.5% of the total phenolics). Chicoric, ferulic and rosmarinic acids and their methylated and glycosyl caffeoyl derivatives, as well as 7-O-glucosyl apigenine were isolated and identified from the methanolic extract of the aerial parts in a previous study of the same authors (submitted). Shoot cultures of *P. montana* ssp. *balcana* were initiated from surface sterilized seeds and maintained in Murashige and Skoog medium with a 45 days period of sub-culture. Polyphenolics *in vitro* were monitored during a prolonged period of shoot maintenance. Six months after culture initiation, phenolics were 66.5 mg/gDW and flavonoids – 6.3 mg/gDW (9.5% of the total phenolic content). After eighteen months phenolics levels dropped to 43.7 mg/gDW, while flavonoids reached 8.5 mg/gDW (19.5% of the phenolics). Moreover, addition of benzyl adenine increased flavonoids content to 21.9% of the total phenolics and supplementation of both benzyl adenine and indole-3-butyric acid raised flavonoid content up to 27.05% of the total phenolics. This effect was accompanied by increase in multiplication index and fresh weight accumulation in the shoots. Our results indicate that prolonged sub-culture, as well as stimulation of *in vitro* multiplication through growth regulators supplementation, increase flavonoid content at the expense of a decrease of the lower molecular phenolic compounds in *P. montana* shoot cultures. We are thankful to European Project: EMAP (FP7-PEOPLE-2009-IRSES) N°247548.

Total phenolic and flavonoid contents of *Alnus glutinosa* (L.) Gaertn., *A. incana* (L.) Moench and *A. viridis* (Chaix) DC. extracts

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Methanolic extract obtained from the leaves and bark of *Alnus glutinosa*, *A. incana* and *A. viridis* were estimated for total phenols and flavonoid content. Analysis of total phenolics based on Folin-Ciocalteu assay, gave the following results: 333 mgCAT/g for *A. glutinosa* to 780 mgCAT/g for *A. viridis* dry material. Total flavonoid content, determined by aluminium chloride colorimetric assay, varied from 10.26 to 30.01 mgRUT/g dry material. The highest concentration of total phenolics and flavonoids were found in *A. viridis* samples. The high content of these compounds was correlated with a significant antioxidant activity previously found for the investigated *Alnus* species that could be considered as promising natural antioxidants.

Phenolics accumulation and physiological characteristics of Balkan *Pulsatilla* species *in vitro*

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Pulsatilla species (Ranunculaceae) are utilized in Asian medicine for the treatment of a wide array of ailments. Nevertheless, secondary metabolites in tissue culture have not been studied for them so far. As a part of a broader phytochemical and biotechnological study of the potential of the Balkan and Bulgarian *Pulsatilla* species we have developed tissue culture of rare and endemic representatives of the genus. In our previous research we studied the relations between phenolics accumulation *in vitro* and supplementation of benzyl adenine (BA) and indole-3-butyric (IBA) in *P. montana* ssp. *balcana*, *P. halleri* ssp. *rhodopea* and *P. slaviankae*. In continuation of this experiment, here we present the study of morphometric parameters, and phenolics/flavonoids in *P. halleri* ssp. *rhodopea* and *P. slaviankae* tissue culture. According to our results *P. halleri* required a combination of low BA (0.2 mg/l) and low to medium IBA supplementation (0.1 and 0.5 mg/l) for optimal multiplication. Successful multiplication of *P. slaviankae* was achieved on media supplemented with low concentration of BA alone (0.2 mg/l) or in combination of 0.2 mg/l BA and 0.5 mg/l NAA. High BA concentration (0.7 mg/l) increased the multiplication index, but also led to reduced explant quality due to excessive water accumulation. Though increased auxin concentration reduced this hydropyricity effect, explants were still characterized by depressed growth or reduced number of leaves per rosette, worsening their quality for micropropagation purposes. *In vitro* phenolics/flavonoids accumulation in *P. halleri*, however, was stimulated in media with a combination of low BA supplementation and low IBA or medium NAA supplementation or high BA in combination with low NAA supplementation. These variants were characterized with increased dry/fresh weight ratio. Surprisingly increased phenolics were accumulated in *P. slaviankae* in media with increased FW/DW ratio, i.e. high BA alone or in combination with low to medium NAA concentrations.

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Introduction of virus resistance gene into *Impatiens* plants

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Impatiens walleriana L. and *Impatiens hawkerii* Bull. are important horticultural plants grown in Serbia. Vegetative propagation in commercial production is very risky due to the probability of virus transmission to progeny plants. Viral infections affect the quality and costs of ornamental plant production. To obtain healthy plant material, genetic transformations were applied in addition to *in vitro* culture. *In vitro* culture of *Impatiens* plants on MS medium and the procedure for healthy plant regeneration on cytokinins (CPPU and TDZ) containing media were successfully established. *Pac1* gene, originating from *Schizosaccharomyces pombe*, was used for *Agrobacterium tumefaciens* mediated *Impatiens* transformation in order to enhance virus tolerance/resistance. Selection treatment was done on kanamycin containing media; after 5th subculture no *I. hawkerii* Bull. clones have survived the selection. The presence of the transferred *Pac1* gene was confirmed by PCR analysis in four transformed *I. walleriana* L. clones and its expression was shown by RT-PCR in two clones. Phenotypic differences between transformed and nontransformed plants (shoot and leaf length, number of nodes, number of axillary buds and leaves) were statistically insignificant except for the leaf length. Thus, the overall phenotype of the *I. walleriana* L. transformants was similar to the control plants.

Bud regeneration from recalcitrant to regenerate hairy roots of *Saintpaulia ionantha*

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Hairy roots of african violet were obtained by inoculation of leaves by using *Agrobacterium rhizogenes* strain A4M70GUS. Acquired transgenic line was highly recalcitrant to *in vitro* regeneration. Application of cytokinins Kin, BA or TDZ in wide range of concentration (5–50 μM) did not provoke any type of regeneration for almost a year. In order to induce callus formation, root clumps, consisting of 3–4 root tips, were cultured on MS medium with 5, 10, 30 or 50 μM BA or TDZ for 2 subcultures. TDZ at all concentration induced calli in 100% explants, while BA was less efficient and only at 30 μM and 50 μM BA induced calli in nearly 100% explants. The highest mean number of calli per root clump was attained in 5 μM TDZ (6.3). The resulting calli were isolated and one half was transferred to the same medium, while the other half was transferred to 5 μM BA. Transfer from TDZ to BA was quite efficient and up to 81.7% calli regenerated buds. The highest bud number (25.6 ± 0.2) was achieved in calli induced on 30 μM TDZ. Transgenic nature of hairy roots was confirmed by PCR specific to the *rolA*, *rolB* and *rolC* genes, whereas the absence of residual agrobacteria was confirmed by PCR specific to the *virD1* gene. Regenerated shoots exhibited some symptoms typical for hairy root syndrome, such as wrinkled and rolled leaves. Changed plant morphology may be interesting horticultural trait.

Exploration of the potential of *Artemisia alba* Turra shoot cultures as a source of secondary metabolites

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The medicinal plant: *Artemisia alba* Turra is utilized for various purposes – decoction of the aerial part is utilized as a digestive in traditional medicine; a study on the ethanol extract revealed its anti-inflammatory activity and the essential oils were established to exert strong spasmolytic and antimicrobial properties. However, great variability of the essential oil profiles has been reported in literature by many authors, attributed to the factors of the environment or to the genotype. We have established shoot cultures from surface sterilized stem segments of selected commercial cultivar of the plant. Auxillary shoots induction was achieved on the basic Murashige and Skoog culture medium, supplemented with Gamborg vitamins, 15 g/l sucrose and 0.5 mg/l benzyl adenine. The induced shoots were further maintained on growth regulators-free medium with 45 days of regular subculture. For the study of essential oils composition media variants were experimented, based on the variation of indole-3-butyric acid and benzyl adenine to the basic Murashige and Skoog formula. The essential oils were analyzed by GC-MS. The identified components consisted of mono- and sesquiterpenoids. According to our results strong influence was established by the culture medium variant and the type of terpenoids identified in the shoot cultures. The antioxidant activity in relation to the phenolics content was also studied.

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