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Volume 39

Biotechnology and Genetic Engineering II



J N GOVIL

Foreword to the Series
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Biotechnological Utilization of the Indigenous Biosynthetic Capacity of Medicinal and Aromatic Plants. Experience in the Genera *Hypericum*, *Pulsatilla* and Essential Oil Bearing *Artemisia alba* Characteristic for the Balkan Region

KALINA DANOVA^{1*}

ABSTRACT

*It is widely accepted that plant metabolic profile is genetically pre-determined. However, its qualitative and quantitative characteristics are quite dynamic and can vary strikingly depending on the complex interrelations of the plant organism with its surrounding environment. Therefore, the controlled modification of the parameters of the plant's environment, as it is in the in vitro system of tissue culture conditions, makes it possible to target the production of plant biomass with desired properties. This review summarizes 20 years of scientific research, as well as personal experience of the author in the yield of phytopharmaceuticals by tissue culture development of representatives of the genera *Hypericum*, *Pulsatilla* and essential oil bearing *Artemisia alba*.*

Key words: *In vitro*, phyto-therapeutics, *Hypericum*, *Pulsatilla*, *Artemisia alba*

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INTRODUCTION

Today, a wide range of industries rely on the supply of raw material of plant origin for the production of prescription medicinal products, over-the-counter phyto-therapeutics and additives, functional foods, cosmetics ingredients, dyes, natural rubber etc. However, collection of extractable plant biomass from the *in situ* accessions of the respective species sets the consequences of over-exploitation and depletion of the genotype natural accession. Field cultivation, though a reliable alternative to wild collection, is largely dependent on the concrete geographic, climatic or other environmental uncertainties of the respective plantation location and could only offer a seasonal supply of the target plant material.

Given nowadays advances of fundamental and applied plant sciences, plant biotechnology has grown into a prospective supplementary tool to assist existing approaches for raw plant material supply.

PLANT CELL AND TISSUE CULTURE OF MEDICINAL PLANT SPECIES

Micropropagation, has nowadays become a reliable technology commercially applied worldwide for large-scale plant multiplication, germplasm conservation, pathogen elimination, genetic manipulations and supply of selected plants (Lambardi *et al.*, 2013). In addition, research efforts have led to the possibility to optimize biotechnological production of phytopharmaceuticals in the conditions of *in vitro* environment, aside from the field. A number of technologies have been patented for the biotechnological delivery of pharmaceutically relevant constituents (Eibl and Eibl, 2002; Petersen and Amstutz, 2008 and literature cited within).

Till this moment, only a small part of the developed techniques have reached commercial utilization. Such are the industrial production of berberin, purpurin and shikonin by Mitsui Petrochemical Industries, ginsenosides by Nitto Denko Corporation, Japan (Fujita, 2007; Arora, 2010). It is noteworthy to mention that while shikonin accumulation by the roots of *Lithospermum erythrorhizon* reaches 2% in 3–7 years of field cultivation (4500 USD/kg), the developed process of large-scale cell culture in bioreactor yields 23% in 23 days (total of 65 kg per year which is 43% of the Japanese market demands (Renneberg, 2008). Berberine production by extraction from root biomass of *Coptis japonica* takes 5–6 years to accumulate, while cell culture of the plant yields 1.4 g/l for two weeks (Joshi, 2006).

The still low commercial applicability of the successfully laboratory established processes is due to the peculiarities of secondary metabolite biosynthesis in the plant organism. It is well known that plant secondary metabolites are amongst the major factors for interaction of the plant organism with its surrounding environment. Thus the success of their sustainable biotechnological production requires an in-depth investigation and understanding of the complex biochemical processes underlying the biosynthesis of each specific compound. The search of specific genotypes with indigenously high biosynthetic capacity could also provide useful tools for their biotechnological utilization.

HYPERICUM TISSUE CULTURE EXPERIMENTS

The *Hypericum* genus, *Guttiferae* Juss family (Melchior, 1964) includes over 486 species, distributed in 36 sections (Crockett and Robson, 2011). Its representatives are widely utilized in ethnopharmacological, as well as official medicinal practices worldwide. Traditionally *Hypericum* preparations are utilized for the treatment of bacterial infections, respiratory diseases, skin wounds, peptic ulcers and inflammatory conditions (Di Carlo *et al.*, 2001). Amongst the numerous biological activities, attributed to preparations of the *Herba Hyperici* (the utilized aerial parts of the plant), are also their astringent, capillary strengthening, antiviral and antidepressant effects (Anker *et al.*, 1995; Diwu, 1995; Schempp *et al.*, 1999). All contemporary phytotherapeutical reference books provide data on the beneficial applications of St. John's Wort (*H. perforatum*). Phytochemical investigations have led to the isolation and characterization of the main active constituents responsible for the biological activities of *H. perforatum* preparations. Thus the broad secondary metabolite spectrum of the plant includes the naphthodianthrone derivatives hypericin and pseudohypericin, the phloroglucinols hyperforin and adhyperforin, flavonoids (hyperoside, quercitrin, isoquercitrin, rutin, quercetin), biflavones (amentoflavone, biapigenin), phenylpropanes (chlorogenic acid), as well as terpenoid constituents present in the essential oil of the plant (Bruni and Sacchetti, 2009). A survey of 153 origins and breeding lines of *H. perforatum* has led to the identification of glutamin, asparagin, arginin (amino acids) and neurotransmitters like GABA in this plant extract (Franke *et al.*, 1999). In addition, a melatonin-rich germplasm-line has been selected *in vitro* (Murch and Saxena, 2006).

Hypericin and pseudohypericin were initially isolated from *H. perforatum* 70 years ago (Brockmann *et al.*, 1939; Brockmann and Sanne, 1957). The antidepressant properties of hypericin are attributed to its mild inhibitory effect on monoamine oxidase activity leading to the raise

of serotonin levels without side effects characteristic for the conventional MAO-inhibitory synthetic preparations (Suzuki *et al.*, 1984; Muldner and Zoller, 1984). An emerging scientific interest has been recently observed in the photodynamic properties of hypericin and its potential as a selective photosensitizer in the photodynamic therapy of tumors (PDT) (Karioti and Bilia, 2010). Hypericin and to a lesser extent pseudohypericin are also being investigated as antiviral agents. Hypericin was shown to exhibit *in vitro* virucidal activity against a large number of viruses. This activity was shown to be prominently dependent on light and molecular oxygen presence (Kubin *et al.*, 2005). Hypericins levels are considered as an important phytochemical marker for the quality of *Herba hyperici* and due to its high price, the selection and cultivation of hypericin-rich crops is a major factor for raw materials producers (Becker, 2000). Therefore agricultural approaches for optimization of biomass gain and standardization of active constituent content in the plant raw *H. perforatum* material is an issue of major efforts (Buter *et al.*, 1998). Hypericin levels may vary between 0.05–0.3% in the aerals of *H. perforatum* (European Pharmacopoeia, 2008). Generally, levels of pseudohypericin exceed the ones of hypericin 2–4 times, depending on the species (Mauri and Pietta, 2000).

The interest of various therapeutic properties of *H. perforatum* has led to scientific studies of many representatives of the genus. Literature data provide phytochemical information on more than 70 species, most of them studied for their phenolic constituents and essential oil (Kitanov and Blinova, 1987; Bertoli *et al.*, 2011).

One of the problems, however, associated with medicinal plant preparations including St. John's wort is the extreme variability in the phytochemical content, mostly due to environmental factors, and biotic and abiotic contamination during cropping (Zobayed and Saxena, 2004). One of the possible approaches to overcome this drawback is the search of strictly controlled biotechnological systems for the standardized delivery of *Hypericum* phytopharmaceuticals. Thus, led by the scientific interest towards tissue culture secondary metabolites yield, a piling number of works have dedicated efforts to study the biosynthetic capacity in *Hypericum in vitro*.

Firstly, tissue culture initiation and characterization of *H. perforatum* shoots was reported in 1992 by two different working groups (Čellárová *et al.*, 1992; Zdunek and Alfermann, 1992). Then hypericin and pseudohypericin have been quantified by thin layer chromatography and high performance liquid chromatography (Zdunek and Alfermann, 1992). In the following years continued interest toward the exploitation of numerous *in vitro* systems of the genus has been recorded (Table 1).

Table 1: Literature data of the secondary metabolite productivity of plant cell tissue and organ culture of different *Hypericum* species

<i>Hypericum</i> species	Type of the <i>in vitro</i> culture	Secondary metabolites identified <i>in vitro</i>	Reference(s)
<i>H. perforatum</i>	Aerial parts	Hypericin, pseudo-hypericin	Zdunek and Alfermann, 1992
<i>H. perforatum</i> , <i>H. maculatum</i> , <i>H. tomentosum</i> , <i>H. bithynicum</i> , <i>H. glandulosum</i> , <i>H. balearicum</i> , <i>H. olympicum</i>	Cell suspension	Hypericin, pseudo-hypericin, flavonoids	Kartning and Göbel, 1992, Kartning <i>et al.</i> , 1996
<i>H. patulum</i>	Cell suspension	Xanthones	Ishiguro <i>et al.</i> , 1993
<i>H. erectum</i>	Callus, aerial parts	Six procyanidins	Yazaki and Okuda, 1994
<i>H. perforatum</i>	Aerial parts	Total hypericins	Cellarova <i>et al.</i> , 1994; Cellárová <i>et al.</i> , 1995
<i>H. patulum</i>	Suspension	Xanthones,	Ishiguro <i>et al.</i> , 1995a, b; 1996; 1997; 1999a, b
<i>H. androsaemum</i>	Cell culture	Xanthones	Schmidt and Beerhues, 1997
<i>H. perforatum</i>	Callus	Unusual flavonoids–luteolin derivatives	Dias <i>et al.</i> , 1998
<i>H. patulum</i>	Suspension	Phloroglucinol derivatives	Ishiguro K <i>et al.</i> , 1998
<i>H. perforatum</i> <i>H. androsaemum</i>	Callus, suspension	Xanthones	Dias <i>et al.</i> , 1998, 1999, 2000
<i>H. perforatum</i>	Aerial parts	Tryptophan, melatonin	Murch <i>et al.</i> , 2000
<i>H. perforatum</i>	Callus	Hypericin	Rani <i>et al.</i> , 2001
<i>H. perforatum</i>	Aerial parts, suspension	Hypericin, pseudo-hypericin, hyperforin	Kirakosyan <i>et al.</i> , 2000a, b, 2003, 2004
<i>H. perforatum</i>	Aerial parts	hypericin	Smith <i>et al.</i> , 2002
<i>H. perforatum</i>	Aerial parts	Hypericin, pseudo-hypericin, hyperforin	Sirvent and Gibson, 2002
<i>H. perforatum</i>	Suspension	Hypericin	Walker <i>et al.</i> , 2002
<i>H. perforatum</i>	<i>In vitro</i> grown early seedlings	Hypericins, phloroglucinols	Košuth <i>et al.</i> , 2003
<i>H. perforatum</i>	Aerial parts	Hypericin	Santarém and Astarita, 2003
<i>H. perforatum</i>	Callus, suspension, aerial parts	Hypericin	Bais <i>et al.</i> , 2002, 2003
<i>H. perforatum</i>	Aerial parts re-generated through hairy roots	Total hypericins	Di Guardo <i>et al.</i> , 2003

Table 1: (Contd...)

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<i>Hypericum</i> species	Type of the <i>in vitro</i> culture	Secondary metabolites identified <i>in vitro</i>	Reference(s)
<i>H. perforatum</i> cv. <i>Topas</i>	Suspension, callus, <i>in vitro</i> regenerated organs	Hypericin, pseudo-hypericin, hyperforin, adhyperforin, xanthones	Pasqua <i>et al.</i> , 2003
<i>H. perforatum</i>	Aerial parts in bioreactor	Hypericin, pseudo-hypericin, hyperforin-hyperforin	Zobayed and Saxena 2004; Zobayed <i>et al.</i> , 2004
<i>H. calycinum</i>	Cell suspension	Hyperforin, adhyperforin	Boubakir <i>et al.</i> , 2005; Klingauf <i>et al.</i> , 2005;
<i>H. perforatum</i>	Aerial parts, callus	Hypericin, pseudo-hypericin	Gadzovska <i>et al.</i> , 2005
<i>H. perforatum</i>	Suspension	hypericin	Xu <i>et al.</i> , 2005
<i>H. perforatum</i>	Aerial parts	Hypericin, pseudo-hypericin, hyperforin, adhyperforin	Karppinen <i>et al.</i> , 2006, 2007
<i>H. perforatum</i>	Aerial parts	Hyperforin, secohyperforin	Charchoglyan <i>et al.</i> , 2007
<i>H. perforatum</i>	Aerial parts	Hypericins	Kornfeld <i>et al.</i> , 2007
<i>H. perforatum</i>	Callus	Hypericins	Song <i>et al.</i> , 2007
<i>H. perforatum</i>	Aerial parts, regenerated through hairy roots	Hypericin, hyperosid, chlorogenic acid	Bertoli <i>et al.</i> , 2008
<i>H. perforatum</i> var. <i>angustifolium</i>	Callus, regenerated aerial parts	Anthocyanidins, xanthones	Mulinacci <i>et al.</i> , 2008
<i>H. polyanthemum</i>	Aerial parts	Total phenolics, benzopyranes	Nunes <i>et al.</i> , 2009

Surprisingly, except in the recent work comparing the secondary metabolites in *H. hirsutum* (*Taeniocarpium* Jaub. et Spach section) and *H. maculatum* (*Hypericum* section) (Coste *et al.*, 2011) hypericin productivity *in vitro* has not yet been explored for representatives of the evolutionarily most developed sections of the genus. Throughout the last decade, intensive research effort has been given towards the optimization of the productive capacity of *Hypericum in vitro* systems through biotic and abiotic elicitation experiments. Along with biotic and abiotic elicitation models, also media formulations have been broadly investigated regarding their effect on *Hypericum* growth and secondary metabolites productivity *in vitro*. Modifications comprise growth regulators, inorganic salts variations, based on the individual empirical experience of the tissue culture laboratory (Table 2). Noteworthy is that

Table 2: Secondary metabolite production stimulation experiments of *Hypericum* plant cell tissue and organ culture

<i>Hypericum</i> species	Type of the <i>in vitro</i> culture	Approach utilized	Secondary metabolites elicited <i>in vitro</i>	Reference(s)
<i>H. perforatum</i>	Shoot cultures	Mannan, β -1,3-glucan, pectin	Hypericins	Kirakosyan <i>et al.</i> , 2000
<i>H. perforatum</i> cv. Anthos and NewStem	Meristem cultures	Salicylic acid, methyljasmonate	Hypericins, hyperforin	Sirvent and Gibson, 2002
<i>H. perforatum</i>	Cell culture	Jasmonic acid (Salicylic acid and fungal cell wall-unsuccessful in the studied work)	Hypericin	Walker <i>et al.</i> , 2002
<i>H. perforatum</i>	Shoot culture	Modification of carbon dioxide and/or sucrose concentration in bioreactor system	Hypericin, pseudohypericin, hyperforin	Zobayed <i>et al.</i> , 2003
<i>H. perforatum</i>	Cell culture	Methyl jasmonate, salicylic acid, <i>Colletotrichum gloeosporioides</i> treatment	Xanthone, quercetin apigenin, and luteolin derivatives	Conceição <i>et al.</i> , 2006
<i>H. perforatum</i>	<i>In vitro</i> seedlings	Chromium treatment	Hypericin, pseudohypericin	Tirillini <i>et al.</i> , 2006
<i>H. perforatum</i>	Shoot culture	Saccharose, polyethylene glycol, methyl jasmonate, inactivated bacterial culture of <i>Agrobacterium tumefaciens</i>	Hypericin, hyperforin	Pavlik <i>et al.</i> , 2007
<i>H. perforatum</i> , <i>H. sampsonii</i>	Shoot cultures	Plant growth regulators, nitrogen source, Methyl jasmonate, 2, 3-dihydroxypropyl jasmonate	Hypericin, pseudohypericin, hyperforin	Liu <i>et al.</i> , 2007a
<i>H. perforatum</i>	Liquid shoot cultures	L-phenylalanine, L-tryptophan, cinnamic acid and emodin, methyl jasmonate	Hypericin, pseudohypericin, hyperforin	Liu <i>et al.</i> , 2007b
<i>H. perforatum</i>	Cell culture	Jasmonic acid	Phenylpropenoids, naphthodianthrones	Gadzovska <i>et al.</i> , 2007

Table 2: (Contd...)

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<i>Hypericum species</i>	Type of the <i>in vitro</i> culture	Approach utilized	Secondary metabolites elicited <i>in vitro</i>	Reference(s)
<i>H. perforatum</i>	Cell suspension	Heat shock	hypericin	Xu <i>et al.</i> , 2008
<i>H. perforatum</i>	Adventitious roots, bio-reactor system	Plant growth regulators treatments and macrosalts and inoculum density modifications; sucrose supplementation	Total phenolics, flavonoids hypericins, chlorogenic acid	Cui <i>et al.</i> , 2010a,b
<i>H. hirsutum</i> , <i>H. maculatum</i>	Shoot cultures	Plant growth regulators, jasmonic acid, salicylic acid	Hypericin, pseudohypericin, hyperforin	Coste <i>et al.</i> , 2011
<i>H. perforatum</i>	Cell culture	Novel protein elicitor (PB90) isolated from <i>Phytophthora boehmeriae</i>	hypericin	Xu <i>et al.</i> , 2011
<i>H. perforatum</i>	Nodal stem segments of <i>in vitro</i> shoots	Modification of total immersion (TI), partial immersion (PI), and paper bridge support (PB) liquid culture conditions	Phenolic compounds, hypericin	Savio <i>et al.</i> , 2012
<i>H. perforatum</i> ssp. <i>angustifolium</i>	<i>In vitro</i> roots	Three stage tissue culture: (i) benzyl adenine; (ii) 2, 4-dichlorophenoxyacetic acid, kinetin and 1-naphthalenacetic acid; (iii) chitosan	Xanthones	Tocci <i>et al.</i> , 2012
<i>H. perforatum</i>	Shoots, callus, and cell suspension cultures	Salicylic acid	Hypericin, pseudohypericin, flavonols, flavanols, anthocyanins	Gadzovska <i>et al.</i> , 2013
<i>H. adenotrichum</i>	<i>In vitro</i> seedlings	Mannan, pectin	Hypericins	Yamaner <i>et al.</i> , 2013

is spite of the intensive development of this field of biotechnological research, except for the work of Yamaner *et al.* (2013) elicitation research

has been exclusively dedicated to *in vitro* culture system of *H. perforatum*.

A number of infra-genetic investigations of representatives of the genus have shown that the species of the *Drosocarpium* section are characterized by the highest content of hypericins as compared with other representatives of the genus (Kitanov, 2001; Smelcerovic *et al.*, 2006; Bruni and Sacchetti, 2009). Nevertheless, this section of the genus has been yet neglected in the search of new genotypes for the biotechnological delivery of *Hypericum* secondary metabolites. The biotechnological development of certain *Hypericum* species, characterized by a higher biosynthetic capacity indigenous to the wild genotype could be a promising approach for the development of highly productive *in vitro* culture system with targeted metabolic profile. In addition the comparison of tissue culture model systems of different *Hypericum* species, is an experimental approach to reveal the biochemical and physiological basis of the different secondary metabolites production characteristic for the representatives of this genus.

In Bulgaria the *Hypericum* genus comprises 22 species, divided into 8 subspecies, 35 var. and 11 forms. There are 5 Balkan and 1 Bulgarian (extinct) endemic species (Iordanov and Kojuharov, 1970; Robson, 1977; Kitanov, 2001; Petrova and Vladimirov, 2009). Studies, dedicated on the Bulgarian ethopharmacological traditions have shown that also the other indigenous Balkan *Hypericum* representatives of the genus possess curative properties commensurable to the widely studied *H. perforatum* (Kitanov and Blinova, 1987). Therefore research work in our Laboratory has been dedicated to elucidating the biosynthetic capacity of *Hypericum* species, from the more developed sections of the genus, and indigenous to the Balkan region. Experimental work on the optimization of secondary metabolite productivity of shoot cultures has been performed in our Laboratory on *H. rumeliacum* and *H. richeri* (*Drosocarpium* section). As a comparative model system *H. calycinum* (hypericin non-producing, *Ascyrea* section) and *H. tetrapterum* were also investigated.

- ***H. rumeliacum* Boiss. (*Drosocarpium*)** - this species is Balkan endemic (Fig. 1a). Literature survey has led to the isolation and identification of flavonols kaempferol, quercetin and myricetin, the anthocyanidins leucocyanidin and delphinidin (Kitanov and Blinova, 1987); flavonol glycosides hyperoside, quercitrin, naphthodianthrone hypericin and pseudohypericin and phloroglucinol hyperforin (Kitanov, 1979; Kitanov, 2001; Smelcerovic and Spiteller, 2006; Smelcerovic *et al.*, 2006). Essential oil studies have shown the presence of α - and β -pinene,

dehydroaromadendrene, α -copaene and limonene and established its antimicrobial activity (Couladis *et al.*, 2003; Saroglou *et al.*, 2007). Recent reports have also demonstrated the antimicrobial, anti-inflammatory and antiradical activities of the methanolic extract of *H. rumeliacum* (Radulovi  *et al.*, 2007; Galati *et al.*, 2008). The volatile profile of the species has been shown to be dependent on the stage of its phenological development (Radulovi  and Blagojevi , 2012)

- ***H. richeri* Vill (*Drosocarpium*)** (Fig. 1b) is distributed in Europe, the Mediterranean region and southwestern Asia. In Bulgaria it is found in mountainous regions up to 2600 m asl (Iordanov and Kojuharov 1970). Recently quercetin and I3, II8-biapigenin were found to be dominant in the sunflower extracts of *H. richeri* and were shown to be related to the anti-inflammatory and gastroprotective properties of these preparations (Zdunic *et al.*, 2010). The ethyl acetate extract of the plant was shown to possess potent antioxidant activity in a test system of superoxide radical and ABTS scavenging assays and inhibition of lipid peroxidation (Zdunic *et al.*, 2011). Essential oil of Croatian accessions of *H. rumeliacum* was shown to be dominated by germacrene D, bicyclogermacrene, alpha-pinene, beta-pinene, decanoic acid, beta-caryophyllene, delta-cadinene, spathulenol and tetracosane (Jerkovic *et al.*, 2013). Strong antimicrobial activity of the essential oil of Italian accessions of the species was established in a test system against five different microbial strains by the broth-microdilution method (Maggi *et al.*, 2010).
- ***H. tetrapterum* Fries (*Hypericum*)** is distributed throughout Europe with the exception of its north- and east most parts of the continent (Fig. 1c). It also occurs in the Mediterranean region, northwestern Africa, southwestern Asia, the Caucasus and Siberia. The presence of leucocyanidin, leucodelphinidin, kaempferol, quercetin, hyperoside, isoquercitrin, quercetin-3-O-rhamnoside, rutin, quercitrin, hyperforin, hypericin were reported (Kitanov and Blinova, 1987; Smelcerovic and Spitteller, 2006). Studies have shown that in comparative studies with other Balkan *Hypericum* representatives, *H. tetrapterum* possessed the highest levels of hyperforin and quercitrin (Smelcerovic *et al.*, 2006). In a comparative study of nine Balkan *Hypericum* species the methanolic extracts of *H. rumeliacum* and *H. tetrapterum* were shown to possess a broad spectrum of antimicrobial activity *in vitro* (Radulovi  *et al.*, 2007). Noteworthy, the authors established that actually the widely studied *H. perforatum* even displayed lowest activity.

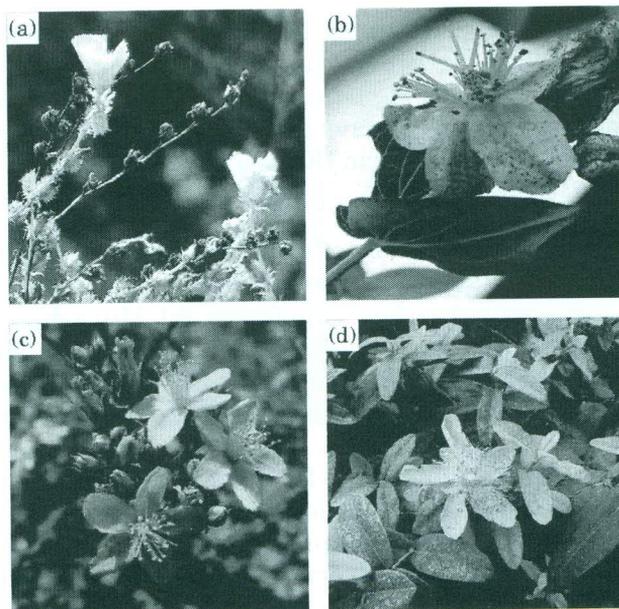


Fig. 1: Photographs of the wild growing *H. rumeliacum* (A), *H. richeri* (B), *H. tetrapterum* (C) and *H. calycinum* (D)

- ***H. calycinum* (Ascyrea)** (Fig. 1d) is a hypericin non-producing species. It was shown to produce leucocyanidin, quercetin, hyperoside, rutin, luteolin (Kitanov and Blinova, 1987). Decosterd *et al.* (1991) report on the isolation of a phloroglucinol derivative with certain fungicide and antimalarial activities. In addition, as compared with *H. perforatum* the species demonstrated commensurable antidepressant activity (Öztürk *et al.*, 1996). Newer report has established the presence of caffeic acid derivatives, as well as several flavonoid and flavanol compounds (Kirmizibekmez *et al.*, 2009). The compounds showed strong DPPH and moderate NO scavenging activities in a concentration dependent manner.

When screening samples of the wild habitats of the investigated species, it was established that while the wild growing *H. tetrapterum* and *H. rumeliacum* had commensurable amounts of total phenolic and flavonoid compounds, the hypericin non-producing *H. calycinum* showed significantly higher quantities of these metabolites on a total assay level. It's worth mentioning, that while hypericin was significantly higher in *H. rumeliacum* as compared with *H. tetrapterum*, pseudohypericin in the latter species exceeded the values for *H. rumeliacum*. Thus total hypericins were commensurable for the two species (Danova *et al.*, 2012a)

As far as the author's knowledge is concerned, there are no literature reports of other working groups on the biotechnological delivery of secondary metabolites of *H. rumeliacum*, *H. richeri* and *H. tetrapterum*. Cell cultures of *H. calycinum* have been investigated for elucidation of the biogenetic steps of hyperforin (Klingauf *et al.*, 2005; Boubakir *et al.*, 2005).

For tissue culture initiation, *ex situ* sterilized stem segments of *H. rumeliacum*, *H. richeri*, *H. tetrapterum* and *H. calycinum* were placed in 0.3–0.5 mg/l benzyl adenine supplemented Murashige and Skoog (1962) basal medium (MS). Then the induced shoots were further maintained in plant growth regulators-free (PGR-free) culture medium for slow growth stock shoot culture (Figs. 2a, d and e) (Danova, 2010; Danova *et al.*, 2010; Danova *et al.*, 2012a, b). However, long-term *in vitro* culture was not possible in the PGR-free MS medium for *H. richeri*. After 3–4 weeks in this formulation the species did not exhibit any multiplication and explants displayed gradual necrosis (Fig. 2a). Therefore this species was long-term maintained in a medium supplementation consisting of 0.2 mg/l BA + 0.1 mg/l indole-3-butyric acid (IBA) to MS medium formulation (Fig. 2b).

Shoots of *H. rumeliacum* in PGR-free medium were characterized by a low multiplication rate (3–4 axillary shoots per explant). When investigation effect of exogenous BA (0.1–1.0 mg/l) supplementation to *H. rumeliacum* shoot culture it was established that intensive biomass formation (>35 axillary shoots per explant) was observed, accompanied however by a significant drop (almost double) of the polyphenolics observed. Therefore a study of the regenerative capacity of different explants of the species led to the development of a double stage culture for polyphenolics production. The first stage consisted of inoculation of nodal explants onto 0.2 mg/l BA supplemented medium. Then induced axillary shoots were transferred to PGR-lacking MS. The polyphenolics assays showed that this protocol led to restoration of the polyphenolic biosynthetic capacity of *H. rumeliacum* shoots in the latter medium (Danova *et al.*, 2010). The morphometric study showed that in BA-supplemented MS, index of compactness (number of leaf pairs per cm of shoot length) significantly dropped as compared with PGR-lacking medium formulation. Thus plant biomass with prevalence of leaf biomass turned out to be more productive for phenolic and flavonoid compounds. Further investigations on secondary metabolites productivity in shoot cultures of Balkan *Hypericum* species consisted of modification of vitamin supplementation in the comparative model of *H. rumeliacum* (high hypericin producing), *H. tetrapterum* (moderate hypericin producing) and *H. calycinum* (hypericin non-producing). It was established that for all three species the MS vitamin supplementation

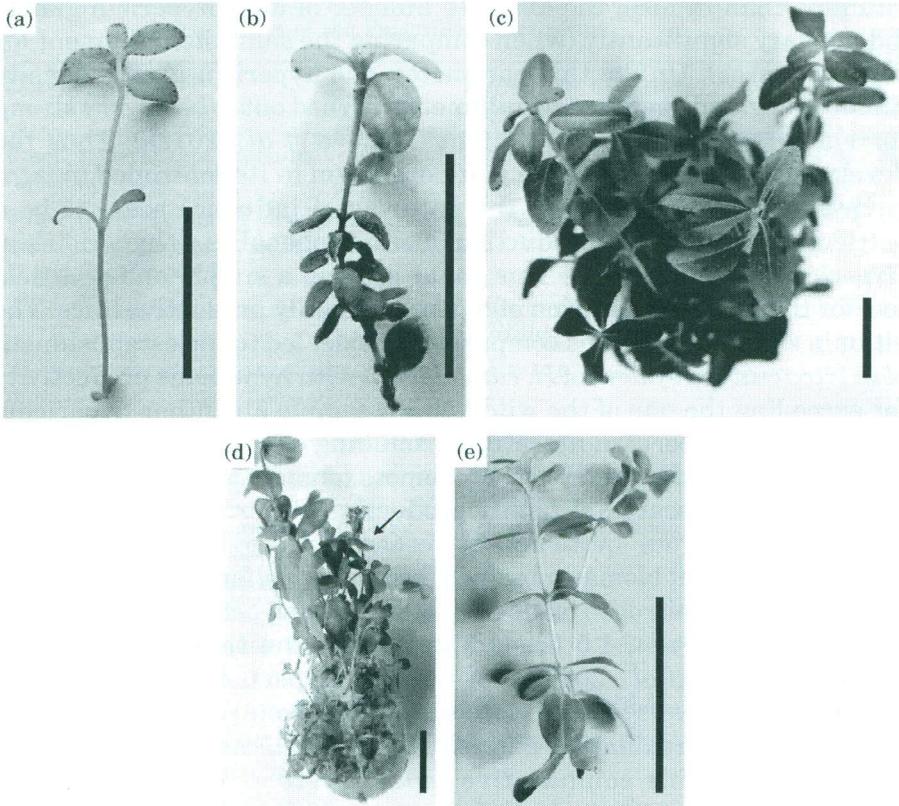


Fig. 2: Photographs of slow growing shoot cultures of the four *Hypericum* species. 12 weeks grown *H. rumeliacum* in PGR-free MS medium (a) Arrow pointing at necrotic leaves in 3–4 weeks grown shoot culture of *H. richeri* in PGR-free MS medium, (b) Multiple shoot formation in 0.2 mg/l BA + 0.1 mg/l IBA supplemented MS medium, (c) *H. tetrapterum* in PGR-free MS after 12 weeks of culture, arrow pointing at *in vitro* formed blossoms, (d) *H. calycinum* in PGR-free MS medium after 12 weeks of culture. Space bar = 1 cm.

was connected to lower growth rates in hypericin producing species and significantly elevated phenolic and flavonoid content (for all three species) as compared with the Gamborg vitamins supplemented to the MS macro- and micro-salts formulation (Danova *et al.*, 2012a). Likewise to the previous experiment it was established that higher polyphenolics were related to a higher compactness and prevalence of leaf tissue in the obtained plant biomass. Unlike polyphenolics levels, hypericin and pseudo-hypericin were shown to be reversely influenced by vitamin supplementation. Thus the lower leaf compactness led to a raise in hypericin levels. Further investigations revealed that irrespective of the species (strikingly lower hypericins in *H. tetrapterum* than *H. rumeliacum*) and the medium (much higher hypericins in Gamborg

vitamins than in MS), the absolute number of dark hypericin glands did not vary significantly (when comparing the same stem segment for different cases). Unlike this parameter, the hypericin glands density (number of glands per square millimetre) turned out to be in very strong correlation with hypericins content (Danova *et al.*, 2012a). Thus the developmental patterns of the plant organism in its controlled *in vitro* environment, irrespective of the pathway of influence seem to be a bottleneck to target the production of either phenolics or hypericins in *Hypericum* model system. This could provide a simple and practical tool for the targeted selection of hypericin-highly productive lines. The vitamin supplementations comparative model led to the establishment of *in vitro* culture system of *H. rumeliacum* with hypericins productivity far exceeding the one of the wild habitat sample and other hypericum tissue culture reports in literature (excluding elicitation models) up to now (Danova *et al.*, 2012a). Thus chemotaxonomic approach proves to be useful in the search of highly productive *Hypericum* genotypes *in vitro*. Moreover, our quite recent research showed that methanolic extract of the plant biomass obtained from *H. rumeliacum* and *H. richeri* possessed potent nitrogen oxide radical scavenging activity, as compared with *H. tetrapterum*, 1.5 times higher than the referent vitamin C (Mehandzhiyski *et al.*, 2013, in print). It is important to mention that high hypericin levels *in vitro* were shown to be connected with elevated levels of lipid peroxidation and oxidative stress markers in the MS/Gamborg vitamins comparative model system. When comparing physiological status of *H. rumeliacum* shoots, regenerated after cryostorage, however, elevated hypericins were not so strongly related to elevated levels of lipid peroxidation and oxidative stress of the respective regenerated lines (Danova *et al.*, 2012b). Hypericins are characteristic for the evolutionary more developed sections of the genus. Hence question might arise whether their production could actually have evolved not only as deterrent phototoxic agent but also as a chemical adaptive factor mediating the overcoming stress impacts of the environment

PULSATILLA SPECIES TISSUE CULTURE EXPERIMENTS

Asian *Pulsatilla* species are traditionally utilized as anti-tumor and spasmolytic remedies, and for enteritis treatment. Recent studies also reveal the cognitive-enhancing and neuroprotective properties of *P. koreana* preparations. A number of investigations have revealed the phytochemical basis of the pharmacological properties of several Asian species (*P. koreana*, *P. cernua*). The triterpenoid saponins are the active ingredients, determining the antitumor activities of *P. koreana*, *P. cernua* and *P. chinensis* (Table 3).

Table 3: Triterpenoid saponins and other triterpenoids reported on the Asian representatives of the *Pulsatilla* Miller genus.

<i>Compound</i>	<i>Pulsatilla species</i>	<i>Plant part</i>	<i>Reference(s)</i>
4 new hederagenin saponins (oleanane type)	<i>P. cernua</i>	Roots	Shimizu <i>et al.</i> , 1978
3 new hederagenins of oleanane type; pulsatilosides A, B, D; caulosides D and F; calcoside D	<i>P. campanella</i>	Roots	Li <i>et al.</i> , 1990
5 oleanane saponins	<i>P. patens</i> var. <i>multifida</i>	Roots	Ye <i>et al.</i> , 1995
23-OH-betulinic acid; pulsatillic acid; pulsatiloside A; pulsatiloside B	<i>P. chinensis</i>	Roots	Ye <i>et al.</i> , 1996
Pulsatiloside C (lupine type)	<i>P. chinensis</i>	Roots	Ye <i>et al.</i> , 1998
5 new oleanane saponins	<i>P. patens</i> var. <i>multifida</i>	Roots	Ye <i>et al.</i> , 1999
Saponins (1–7) of oleanolic acid or of hederagenin; two glucosides of oleanolic acid; (+) pinoresinol, β -peltatin	<i>P. chinensis</i>	Roots	Mimaki <i>et al.</i> , 1999
Two oligoglycosides of oleanane type, cernuosides A and B	<i>P. cernua</i>	Roots	Zhang <i>et al.</i> , 2000
4 lupane saponins	<i>P. chinensis</i>	Roots	Mimaki <i>et al.</i> , 2001
Chinensioside A, chinensioside B, hederasaponin C, glycoside III	<i>P. chinensis</i>	Roots	Glebko <i>et al.</i> , 2002
5 lupane, 1 oleanane saponin	<i>P. koreana</i>	Roots	Bang <i>et al.</i> , 2005a
8 lupane, 9 oleanane saponins	<i>P. koreana</i>	Roots	Bang <i>et al.</i> , 2005b
Radedanoside R ₁₉	<i>A. raddeana</i>	Roots	Li <i>et al.</i> , 2008
Type I, II oleanane and type III, IV and V lupine saponins	<i>P. chinensis</i>	Roots	Sun <i>et al.</i> , 2010

According to newer studies oleanolic acid has been shown to possess *in vitro* inhibitory activity on the development of HIV-1 virus, which is being explained by inhibition of HIV-1 protease activity (Mengoni *et al.*, 2002). Extract of *Pulsatilla pratensis*, together with *Chimaphila umbellata*, *Populus tremula* and *Equisetum arvense* extracts, as well as the oil of *Triticum aestivum* formulate the active ingredient of Eviprostat®, a phytotherapeutic agent for benign prostatic hyperplasia (Oka *et al.*, 2007). The study demonstrated the radical scavenging activity of the active ingredients of Eviprostat® which were supposed to contribute to the anti-inflammatory effect of this medical preparation. The increased scientific interest towards the pharmacological activities of active principles isolated from the roots of the Asian *Pulsatilla* species has led to the isolation and characterization of constituents with beneficial properties and lower toxicity – as the oleanane and lupine

type saponins isolated from roots of *P. chinensis* which do not exhibit the acute haemolytic activity characteristic for this class of compounds but exert defined immune adjuvant activities (Sun *et al.*, 2010). A recent study on the mechanism of action of *Pulsatilla* decoction for treatment of inflammatory bowel disease showed that the effect could be due to inhibiting the expression of pro-inflammatory cytokines (Yu *et al.*, 2011). *Pulsatilla koreana* extract (PKE) widely utilized in Korean and Chinese Traditional medicine as an anti-inflammatory agent and for treating dysentery, was quite recently shown to also exert apoptosis, as well as to inhibiting cell growth and angiogenesis in anaplastic thyroid cancer cells. PKE has been suggested as a potent anticancer drug candidate (Park *et al.*, 2013).

Surprisingly, in spite of the great interest towards the phytochemistry and pharmacology of the Asian *Pulsatilla* species little attention has been given to plant cell tissue and organ cultures of this genus. Research has been orientated towards micropropagation, *ex vitro* adaptation and re-introduction of rare and valuable representatives of the genus. Literature survey has led to a few works dedicated on this subject, which are summarized in Table 4.

Noteworthy in these works is the complexity of the rooting process established by the authors. Addition of plant growth regulators, longer culture period and/or multistep procedures for root formation were reported (Table 4). This might explain the reason why secondary metabolites productivity yet has not been explored for this genus *in vitro*. Since the main bio-active principles of *Pulsatilla* are produced by the roots, given the complicated rooting *in vitro* (Table 4) and possible difficulties of the maintenance of root culture could be a reasonable ground to neglect research into this direction.

However, the recently intensified research on the phenolic constituents of the plant led to isolation and identification of additional pharmacologically relevant molecules of also other parts of the plant, which have been shown to also contribute to the biological activity of Asian *Pulsatilla* preparations (Table 5)

Thus cinnamic acid derivatives isolated from roots of *P. cernua* were shown to inhibit growth of pathogenic bacteria such as *Clostridium perfringens* and *Escherichia coli*, while not having any adverse effects towards beneficial bacteria such as *Bifidobacteria* and *Lactobacillus acidophilus* (Lee *et al.*, 2001). The anti-enteritis application of the roots in Asian traditional medicinal practices was attributed to the activity of these active constituents. Further Lee (2002) also established a marked tyrosinase-inhibitory activity of the isolated cinnamic acid

Table 4: Tissue culture studies on representatives of the *Pulsatilla* genus.

Species	Explant utilized	Tissue culture technique applied	Medium composition	Reference(s)
<i>P. chinensis</i>	Stem apex, root cutting, axillary bud	i) Stock shoot culture	i) MS+ (1-3) mg/l BA + (0-0.5) mg/l NAA + 30 g/l sucrose	Zhang <i>et al.</i> , 2004
		ii) Differentiation and subculture medium	ii) MS + 0.2 mg/l BA + 0.2 mg/l NAA + 0.1 × 10 ⁻³ BR + 30 g/l sucrose	
		iii) Rooting	iii) ½ MS + 0.4 mg/l NAA + 20 g/l sucrose	
<i>P. patens</i>	Sterile seed germination -62% in 96 days	i) Sterile multiplication	i) 0.1-1.5 mg/l BA, 0.1-0.1 mg/l Kin. 0.1-0.5 IAA	Klavina <i>et al.</i> , 2004
		ii) Rooting (poor)	ii) Plant growth regulators-free medium	
<i>P. koreana</i> Nakai	i) Leaf explant ii) Callus	i) Callus induction	i) MS + 2,4-D + BA	Jung <i>et al.</i> , 2007
		ii) Indirect morphogenesis of aerial parts	ii) PVP + CK	
	iii) Leaf explant	iii) Adventitious root formation	iii) ½ MS + IBA	
	iv) Adventitious root segments	iv) Direct morphogenesis of aerial parts	iv) 2-ip treatment	
	v) Induced aerial parts	v) Rooting	v) ½ MS + IAA	
<i>P. cernua</i> var. <i>koreana</i>	i) Apical meristems	i) Embryogenic and organogenic callus induction	i) MS + NAA + BA	Ko and Kim, 2008
		ii) Shoot and shoot buds induction	ii) MS + 0.5 mg/l NAA + 1.0 mg/l BA	
		iii) Callus proliferation, shoot multiplication and elongation	iii) 10% coconut water on MS + 0.5 mg/l NAA + 1.0 mg/l BA	
		iv) Rooting	iv) MS + 2.0 mg/l NAA or 0.5 mg/l NAA + 1.0 mg/l BA	
<i>P. pratensis</i> (L.) Miller ssp. <i>nigricans</i> (Störck) Zamelis	i) Sterilized seeds	i) 58.3 % germination	i) pH 5.8, MS + 2.9 µMGA ₃ , GA ₃ + BA; highest number of shoots - MS and ½ MS + GA ₃ + BA	Naumovski <i>et al.</i> , 2009
		ii) Sterile shoots	ii) MS + 2.9 µMGA ₃ + 0.5 µMBA	
		iii) Shoots excised from multiple culture	iii) MS + 8.5 µM IAA	

Table 4: (Contd...)

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Species	Explant utilized	Tissue culture technique applied	Medium composition	Reference(s)
<i>P. vulgaris</i> <i>P. grandis</i>	i) Sterilized seeds	i) Germination within 20 days	i) MS + BA + IAA (1:1 ratio); 16 h photoperiod	Daulienė and Brinkytė, 2009
	ii) Shoots of sterile seedlings	ii) Multiplication	ii) MS + BA + IAA (1:1; 1:2; 1:5); MS	
<i>P. patens</i>	i) Sterilized seeds	i) Germination	i) ½ MS 16 h photoperiod	Priede and Kiviõja, 2011
	ii) Sterile shoots	ii) Multiplication	ii) 0.2–0.75 mg/l BA; 0.1–0.5 mg/l topolin; 0.1–5 mg/l Kin	
	iii) Sterile shoots	iii) Multistage rooting experiment	iii) ½ MS darkness + 5 mg/l NAA + riboflavin + auxin photooxidation	
	iv) Rooted sterile plantlets	iv) <i>ex vitro</i> adaptation	iv) Peat substrate mixed with sand; transplantation to semi-natural conditions; flowering after one year	
<i>P. koreana</i> Nakai	i) <i>ex situ</i> leaf, petiole, pedicel	i) Multiple shoots formation	i) MS + 0.5 mg/l IAA + (Zn, Kin or BA)	Lin <i>et al.</i> , 2011
	ii) Explants and generated sterile shoots	ii) Somatic embryogenesis	ii) MS + 0.25 mg/l Zn + 0.5 mg/l IAA	
	iii) Regenerated shoots from organogenic or embryogenic calluses	iii) <i>in vitro</i> multiplication	iii) 1.5 mg/l Zn + 0.05 mg/l IAA or 1.0 mg/l BA + 0.05 mg/l IAA	
	iv) Shoots initiated by organogenesis and somatic embryogenesis	iv) Rooting	iv) ½ MS + 1.5 mg/l NAA	
	v) Rooted plantlets	v) <i>ex vitro</i> adaptation	v) Mixture of mountain soil and perlite	

Abbreviations: BA—Benzyl adenine, NAA—1-Naphthaleneacetic acid, BR—Brassinolide, MS—basal Murashige and Skoog culture medium, 2,4-D—2,4-dichlorophenoxyacetic acid, IBA—Indole-3-butyric acid, Kin—Kinetin, IAA—Indole-3-acetic acid, PVP—Polyvinylpyrrolidone, CK—Cytokinins, 2-ip—2-isopentenyladenine, GA₃—Gibberellic acid, Zn—Zinc.

Table 5: Secondary metabolites with phenolic structure isolated and identified in the *Pulsatilla* genus.

<i>Species</i>	<i>Plant part</i>	<i>Compound</i>	<i>Reference(s)</i>
<i>P. cernua</i>	Sepals	Pelargonidin 3-[2''-(2''-trans-caffeoyl- β -D-glucopyranosyl)- β -D-galactopyranoside]	Yoshitama <i>et al.</i> , 1998
<i>P. slaviankae</i>	Aerial parts	Astragalin, isoquercitrin, quercetin, kaempferol, caffeic acid	Nikolova <i>et al.</i> , 1998
<i>P. montana</i>	Aerial parts	Isorhamnetin	Nikolova and Asenov, 2006
<i>P. multifida</i>	Aerial parts	Quercetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-rhamnoside, kaempferol-3-O-glucoside, kaempferol-3-O-rhamnoside	Kolesnikov and Gins, 2001
<i>P. cernua</i>	Roots	3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid	Lee <i>et al.</i> , 2001; Lee, 2002
<i>P. koreana</i>	Aerial parts	Three new phenolic compounds -pulsatillosides A (1), B (2) and C (3); two known - trans-tiliroside (4) and kaempferol-3-O- β -glycoside (5)	Liu <i>et al.</i> , 2012

derivatives. Recently Liu *et al.* (2012) isolated and identified three novel, together with two known phenolic compounds in the aerial parts of *P. koreana*. The research also established the defined anti-adipogenic activities of these constituents

In Bulgaria, there are five species of the genus *Pulsatilla* Miller, divided into five subspecies and two varieties (Iordanov and Kojuharov, 1970). Among them, *P. halleri* ssp. *rhodopaea* and *P. slaviankae* are included in the red data book of the country (Sopotlieva, 2009; Bancheva, 2009). Unlike the widely studied wild Asian species, quite scarce information is available on the Balkan representatives of the genus. In a chemotaxonomic study Nikolova *et al.* (1998) reported on the presence of astragalin, isoquercitrin, quercetin, kaempferol and caffeic acid in the aerals of *P. slaviankae*. In a further study, surface aglycon isorhamnetin was found in the aerals of *P. montana* (Table 5). Therefore research in our Laboratory was targeted towards tissue culture development and polyphenolics stimulation of *Pulsatilla* species characteristic for the Balkan flora. Shoot cultures of Balkan endemic *P. montana* ssp. *balcana* (Fig. 3), Balkan endemic *P. halleri* ssp. *rhodopea* and local endemic *P. slaviankae* were initiated and explored for modification of polyphenolics production *in vitro* (Danova *et al.*, 2007, 2009). Shoot cultures were

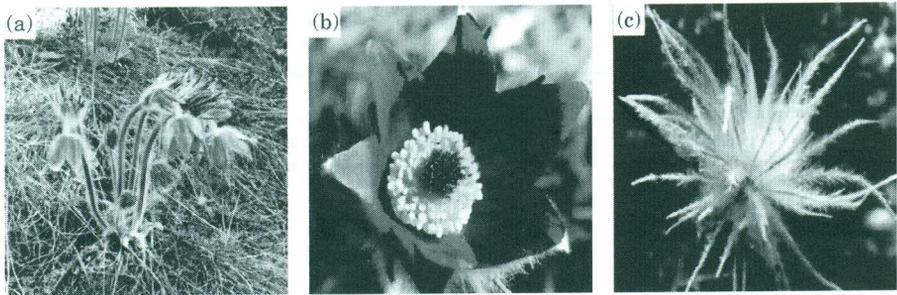


Fig. 3: *In situ* Balkan endemic *Pulsatilla montana* ssp. *balcana* in its wild habitat in the western Balkan mountain in Bulgaria. Typical clumps of the plant (a), Plant's blossom (b) and ripening seeds prior of being dispersed by the wind (c).

initiated by sterile germination of surface sterilized seeds collected from the wild growing species (Fig. 3c, Fig. 4a). The effects of BA and IBA treatments on polyphenolic compounds and photosynthetic pigments content were studied (Danova *et al.*, 2009). It was established that prolonged sub-culture, as well as stimulation of *in vitro* multiplication

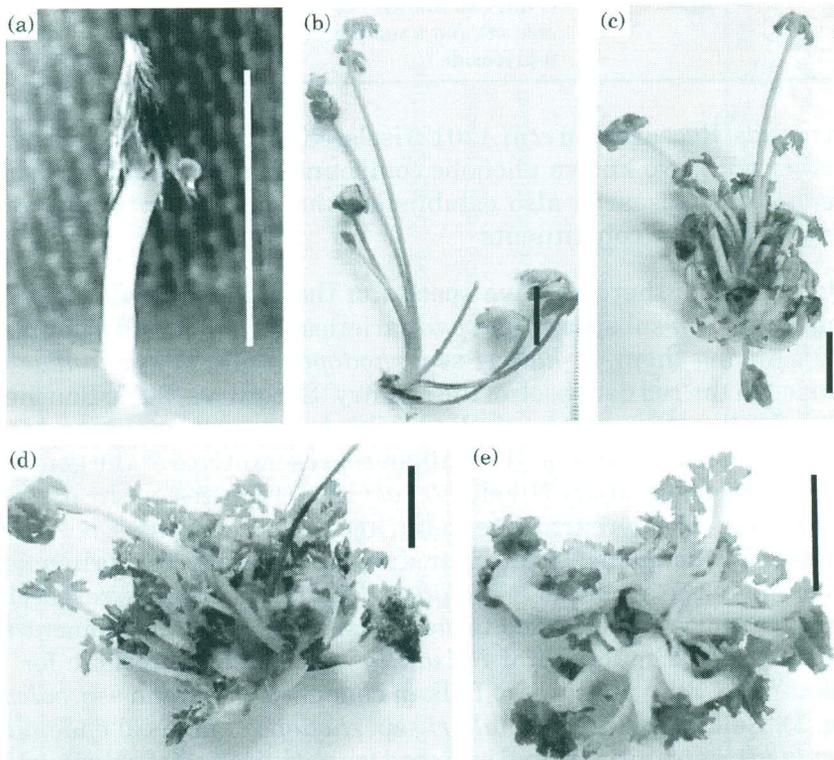


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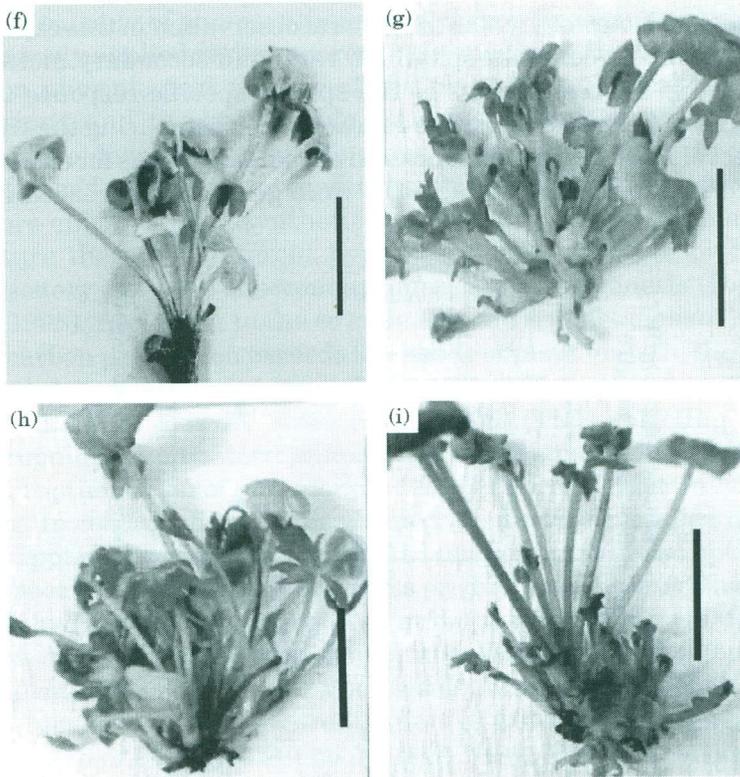


Fig. 4: Tissue culture of *Pulsatilla* species. Sterile germination in plant growth regulators (PGR)-free MS medium (a) Media supplementations for stimulation of polyphenolics production of *Pulsatilla* sp. *in vitro*: *P. slaviankae* in PGR-free, (b) 0.7 mg/l BA + 0.1 mg/l NAA, (c) 0.7 mg/l BA (d) and 0.7 mg/l BA + 0.5 mg/l NAA, (e) supplemented MS media; *P. halleri* in PGR-free, (f) 0.2 mg/l BA + 0.1 mg/l IBA, (g) 0.2 mg/l BA + 0.5 mg/l NAA, (h) and 0.7 mg/l BA + 0.1 mg/l NAA, (i) Supplemented MS media. Space bar = 1 cm.

through growth regulators treatments, increase flavonoid content at the expense of a decrease of the lower molecular phenolic compounds in the studied *in vitro* systems. It was further established that PGR treatments showed species-specific dependence of these parameters. Thus in PGR-free MS medium the total phenolic, flavonoid and anthocyanin levels in shoot cultures of *P. montana* and *P. halleri* were significantly higher as compared with *P. slaviankae.*, *P. montana* was shown to be superior in polyphenolics production as compared with the other two species. After the supplementation of 0.2 mg/l BA the same ratio was observed, as for each of the three species quantities were higher than in PGR-free MS medium (Danova *et al.*, 2009). Combination of 0.2

mg/l BA + 0.1 mg/l IBA further raised the polyphenolics up to levels, commensurable for the three species with only a drop of anthocyanidins for *P. halleri*. Noteworthy was the general observation in this experiment on relations between chlorophyll a/b ratio and secondary metabolites studied (Fig. 5). Irrespective of the species-specific response to PGR treatments, there was a positive relation when comparing the trend for the effect of PGR on polyphenolics and chl a/b ratio. Thus for each species the levels of secondary metabolites were generally increased in PGR

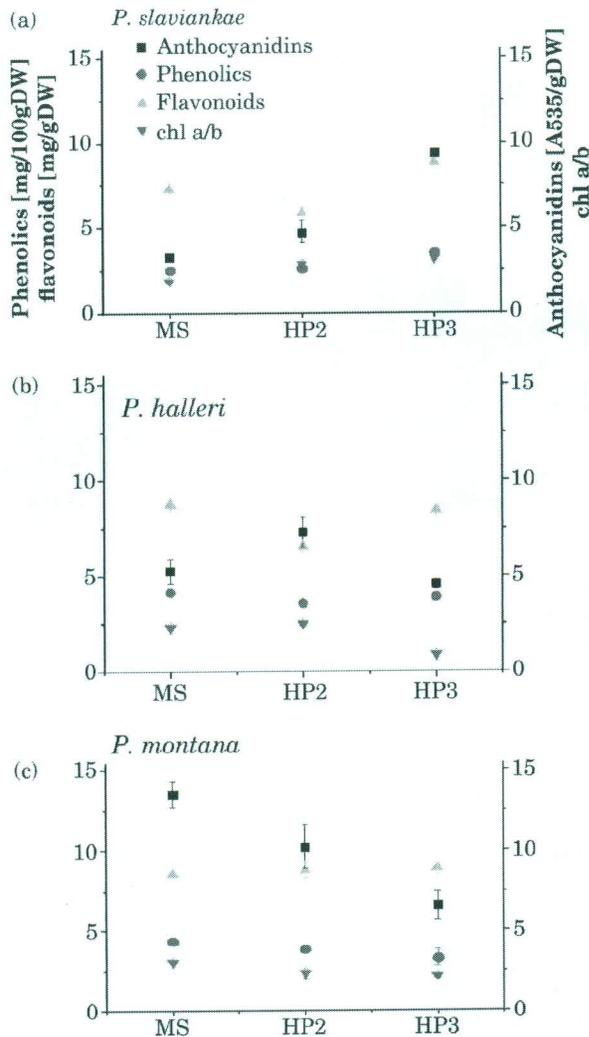


Fig. 5: Impact of PGR-treatment on the content of polyphenolic compounds and chlorophyll a/b (chl a/b) Ratio in *P. slaviankae* (a), *P. halleri* (b) and *P. montana* (c) MS, HP2 (MS + 0.2 mg/l BA) and HP3 (0.2 mg/l BA + 0.1 mg/l IBA media).

treatments with an increased chl a/b ratio. According to Lucchesini *et al.* (2009) in optimal mixotrophic conditions, *in vitro* cultures aerals could be capable of photosynthesis and carbon fixation, aside from the exogenously applied carbon source in the medium. The high ratio of chl a/b is a physiological marker of the plant adaptiveness to *in vitro* conditions and to the possible capacity for mixotrophic or even autotrophic development of the plant organism *in vitro*. It could be hypothesized that in these conditions there might exist prerequisites for more effective photosynthetic process and carbon assimilation. In literature there are different hypotheses explaining phenotypic and evolutionary patterns of secondary metabolites biogenesis (Heyworth *et al.*, 1998). According to the so called “over-flow metabolism” concept when carbon production exceeds the needs of plant growth, the surplus carbon is being channeled towards biogenesis of secondary metabolites (Matsuki, 1996; Mosaleeyanon *et al.*, 2005). Thus selecting optimal mixotrophic or even autotrophic *in vitro* conditions might be utilized as a tool in optimization of secondary metabolites productivity in *Pulsatilla* species. In continuation of this research we further modified different PGR supplementation to the basic MS medium in order to optimize *in vitro* shoot biomass and polyphenolics production (Table 6). Thus it was established that *P. halleri* required a combination of low BA (0.2 mg/l) and low to medium IBA supplementation (0.1 mg/l and 0.5 mg/l) for optimal multiplication (Fig. 6). Successful multiplication of *P. slaviankae* was achieved upon 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 (Fig. 7). Though increased auxin concentration reduced this hyperhydricity effect, explants were still characterized by depressed growth or reduced number

Table 6: Plant growth supplementations for the optimization of *in vitro* multiplication and polyphenolics productivity in *P. hallei* ssp. *rhodopaea* and *P. slaviankae* shoot cultures

Abbreviation	PGR supplementation to MS medium
1	PGR-free control
2	0.2 mg/l BA
3	0.7 mg/l BA
4	0.2 mg/l BA + 0.1 mg/l IBA
5	0.2 mg/l BA + 0.5 mg/l IBA
6	0.2 mg/l BA + 0.1 mg/l NAA
7	0.2 mg/l BA + 0.5 mg/l NAA
8	0.7 mg/l BA + 0.1 mg/l IBA
9	0.7 mg/l BA + 0.5 mg/l IBA
10	0.7 mg/l BA + 0.1 mg/l NAA
11	0.7 mg/l BA + 0.5 mg/l NAA

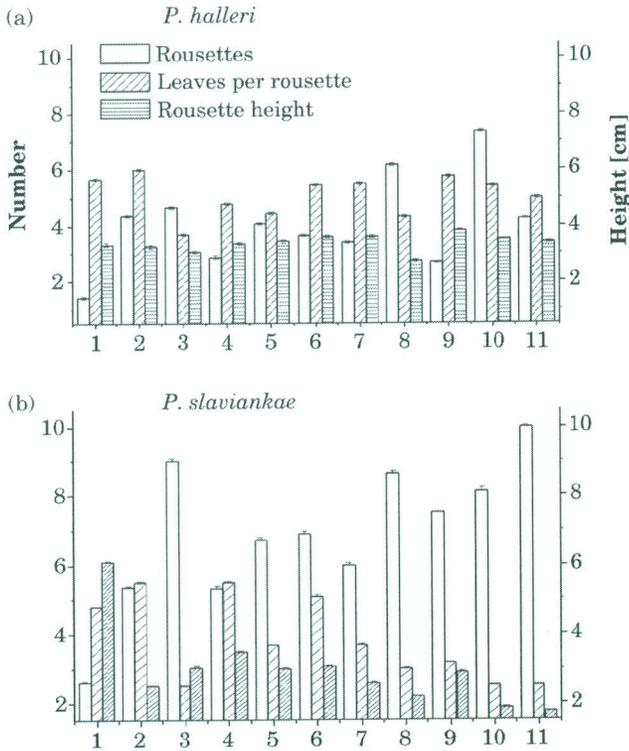


Fig. 6: Influence of BA and IBA on the main morphometric parameters of *P. halleri* (a) and *P. Slaviankae* (b) *in vitro*

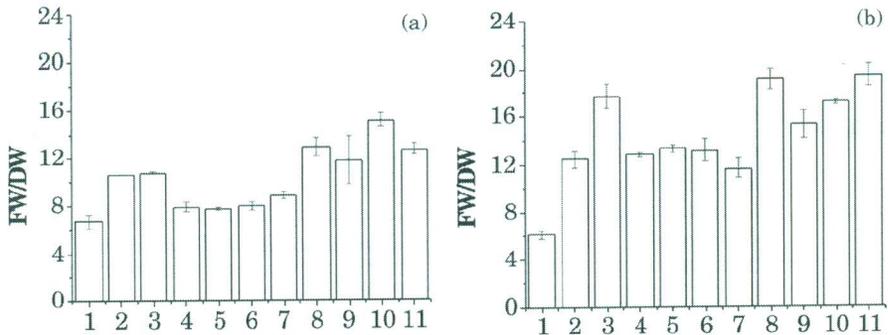


Fig. 7: Effect of PGR treatments on the FW/DW (fresh weight/dry weight) ratio of *P. halleri* ssp. *Rhodopaea* (a) and *P. Slaviankae* (b) shoot cultures

of leaves per rosette, worsening their quality for micropropagation purposes. *In vitro* phenolics/flavonoids accumulation in *P. halleri* however (Fig. 8), was stimulated in media with a combination of low BA

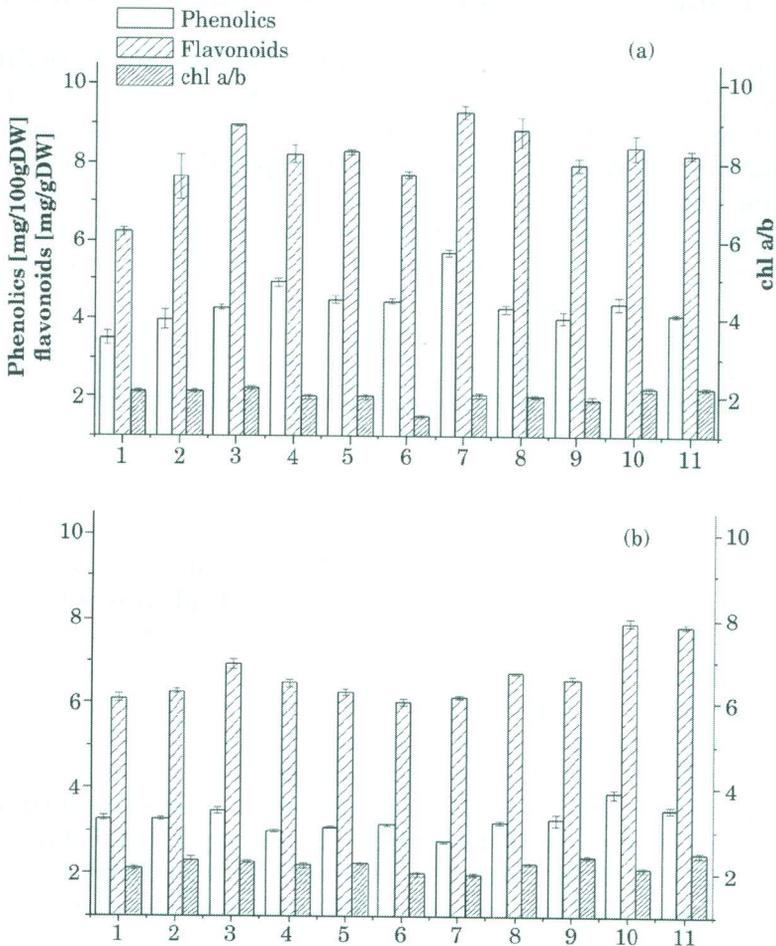


Fig. 8: Effect of PGR on the polyphenolic contents of *P. halleri* ssp. *Rhodopaea* (a) and *P. slaviankae* (b) shoot cultures.

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. Noteworthy is the higher sensitivity of *P. slaviankae* to the PGR-supplementations expressed as the higher hydration of tissues as result of the PGR-supplementations. As a result media supplementations with optimal growth and polyphenolics production were selected for *P. halleri* (Figs. 4g, h and i) and *P. slaviankae* (Figs. 4 c, d and e)

ARTEMISIA ALBA TURRA TISSUE CULTURE EXPERIMENTS

Artemisia alba Turra is a fragrant shrub distributed in Southern Europe. Although not as prominent as *A. annua* for producing anti-malarian artemisinin, the species has been reported to exert spasmolytic, antimicrobial and anti-diabetes activity, especially from its essential oil. The scientific studies of its essential oil have shown its strong spasmolytic and antimicrobial properties (Ronse and De Pooter, 1990; Stojanovic *et al.*, 2000). However, surveys have revealed a great variability of its terpenoid profile, attributed by different authors to environmental conditions, geographic distribution and/or to genetic factors (Radulovic and Blagojevic, 2010 and ref. cited within). As far as the author's knowledge is concerned there is scarce information on tissue culture development for this species. Available data concern comparison of the essential oils obtained from *ex vitro* and *in situ* samples of *A. alba* (Ronse and De Pooter, 1990) and tissue culture initiation with conservational purposes (Holobiuc and Blindu, 2006–2007). In order to investigate the peculiarities of essential oils production in a controlled tissue culture environment we established shoot cultures of the species from surface sterilized stem segments of its field grown commercial cultivar (Danova *et al.*, 2012c). Shoots were firstly induced on 0.5 mg/l MS basal salts with Gamborg (1968) vitamins supplemented medium. Then stock shoot were maintained on PGR-free Gamborg vitamins medium with a period of regular subculture of three months. For experimenting on the effect of PGR-supplementations five media were compared: GAIP_0 – PGR-free, GAIP_1 – 0.5 mg/l IBA, GAIP_2 – 1.0 mg/l IBA, GAIP_3 – 0.2 mg/l BA + 0.5 mg/l IBA and GAIP_4 – 0.2 mg/l BA + 1.0 mg/l IBA supplemented MS salts and Gamborg vitamins medium (Fig. 9). Plants were maintained for 2 months then the basic growth and development parameters (Fig. 10), as well as essential oil content were investigated (Danova *et al.*, 2012 c). Supplementation with 0.5 mg/l IBA caused only slight increase in primary shoot length in comparison with the control (Fig. 10a). Further raise in IBA concentration to 1.0 mg/l resulted in increased shoot number and compactness (IC = number of leaves per cm of shoot length). Addition of 0.2 mg/l BA increased primary and secondary axillary shoots formation, which were however characterized by compressed length (Figs. 10 a and b). Secondary shoots were absent in the control and their length and number were higher in the BA supplemented media, while their compactness was highest in the 1.0 mg/l IBA supplemented GAIP_2. IBA stimulated root formation in GAIP_1 and GAIP_2, as induced roots were characterized by higher number and thickness, but lower length in comparison with the control (Fig. 10c). Increased IBA concentration also stimulated the formation of root branches, thus markedly enhancing

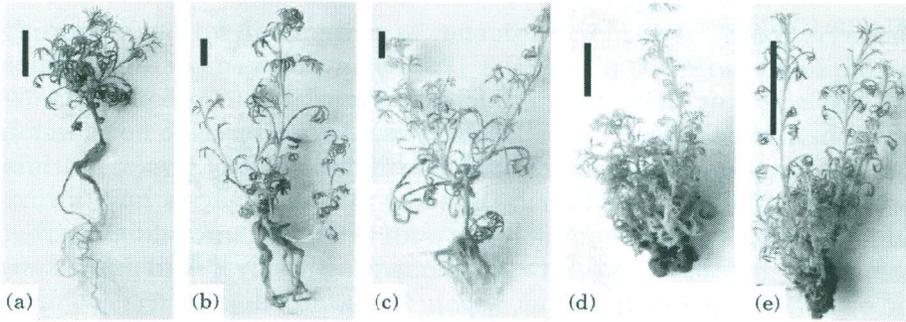


Fig. 9: *A. alba* in PGR-treatments experiment *in vitro*. PGR-free control GAIP_0 (a), 0.5 mg/l IBA supplemented GAIP_1 (b), 1.0 mg/l IBA supplemented GAIP_2 (c), 0.2 mg/l BA + 0.5 mg/l IBA supplemented GAIP_3 (d) and 0.2 mg/l BA + 1.0 mg/l IBA supplemented GAIP_4 (e). Space bar = 1 cm.

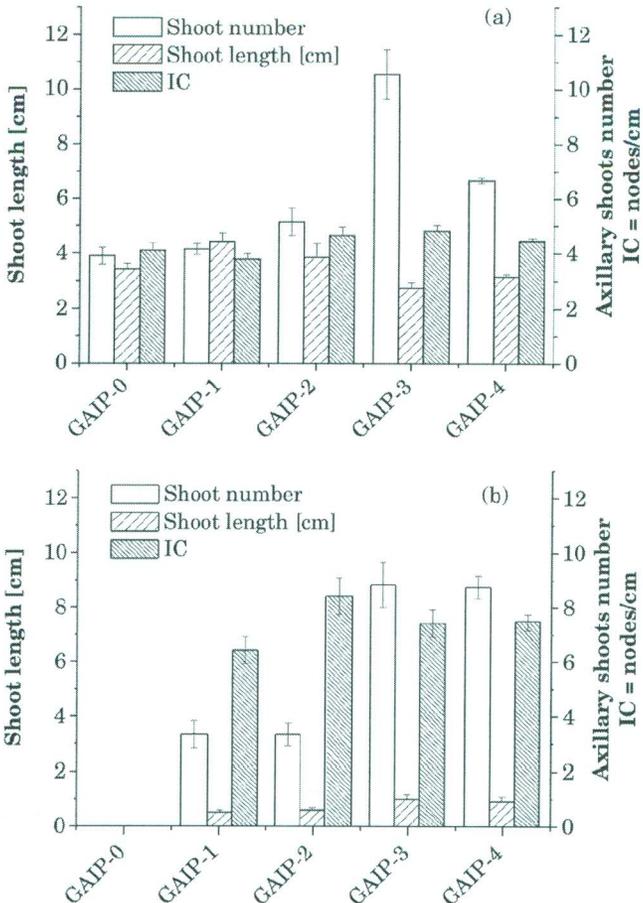


Fig. 10: (Contd...)

Fig. 10: (Contd...)

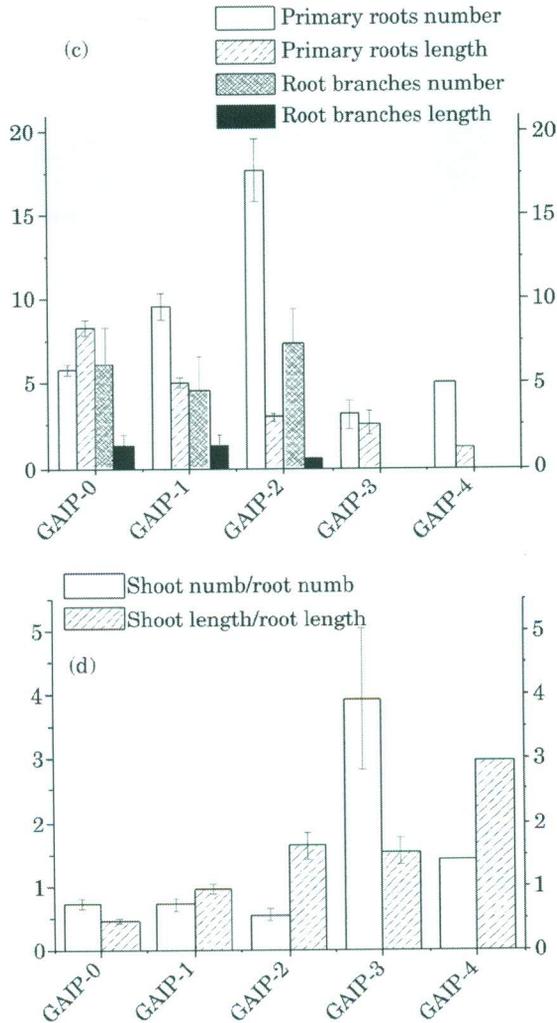


Fig. 10: Impact of PGR on primary (a) and secondary (b) axillary shoots formation (IC is the Index of compactness = number of leaves per cm of shoot length), as well as on root (c) and ratios of shoot/root development (d). It must be kept in mind that in GAIP_3 and GAIP_4 root formation is very scarce and occurs at the end of the collection period (only about 15–20% of the individual plants form roots).

the overall area of the root system. Furthermore, 1.0 mg/l IBA also stimulated aerial parts development, thus raising the shoot/root length when compared with GAIP_0 and GAIP_1 (Fig. 10d). Noteworthy, disregarding the different PGR-treatments in GAIP_0, GAIP_1 and GAIP_3, the essential oils of the aerial parts of these plants were

characterized by domination of monoterpenoid over sesquiterpenoid components in them (Danova *et al.*, 2012c). On the other hand plants which lacked (or very rarely developed) roots were characterized by domination of sesquiterpenoids in the oils. In a recent work Radulovic and Blagojevic (2010) established that the essential oil from *A. alba* growing on serpentine soils (characterized by water deficiency and indispensable mineral nutrients) was dominated by sesquiterpenoids, containing high levels of germacrene D, while on the contrary, samples from calcareous soils contained mostly monoterpenoids. These two oil types obtained in tissue culture experiments in our Laboratory, correlate to types from the wild accessions of the species. Having in mind the established influence of the experimented PGR-treatments on root and callus development, our findings indicate that a root-to-shoot signaling factor might play a key role for the determination of the pathways of terpenoid biosynthesis in *A. alba*. Active research is in progress to identify the biochemical and physiological parameters involved in terpenoid biogenesis of *A. alba* tissue culture. The obtained practical tools for modification of the essential oil profile will further be utilized for the targeted delivery of essential oil with determined terpenoid profile.

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