Effect of plant growth regulators on the structure and biosynthetic capacity of *in vitro* roots of *Valeriana officinalis* (*Valerianaceae*)

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Abstract: *In vitro* plantlets of *Valeriana officinalis* L. were regenerated from raceme stalk segments of a selected individual in bloom, then root cultures were grown on basal MS medium and on media supplemented with three commonly used plant-growth regulators (BAP or Kin alone or in combination with NAA), or with double sucrose amount. Root growth and anatomical structure were influenced by the medium composition. GC/MS analyses showed that biosynthetic capacity of *in vitro* roots was lost, or differed significantly from that of the blooming parent valerian plant.

Key words: Auxins, cytology, cytokinins, essential oil, *in vitro* roots, *Valeriana*

Introduction

The genus *Valeriana* comprises over two hundreds species spread mainly in the temperate climate zones of the Northern Hemisphere. Of these, only two are of pharmaceutical importance: *V. officinalis,* used in Europe and North America, and *V. wallichii* DC., native to India, Nepal and China. *Valeriana officinalis* is divided into three subspecies: *V. officinalis* subsp. *officinalis*, *V. officinalis* subsp. *collina* and *V. officinalis* subsp. *sambucifolia*. The first two occur in Bulgaria, both in diploid and tetraploid forms (Evstatieva & al. 1993).

V. officinalis is an important medicinal plant species, valuable mainly for its sedative, anxiolytic and hypotensive action. The used plant parts are roots and rhizomes, with iridoids as main constituents, known as valepotriates, pyridine alkaloids, steroids, essential oil containing monoterpenoids, and cyclopentane sesquiterpenoids, including valerenic acid (Barnes & al. 2007). The distinctive smell of valerian is due to isovaleric acid (Houghton 1997). There are many products on the market such as ethanolic extract, capsules, tablets, and herbal teas and infusions, prescribed for treatment of insomnia, anxiety, muscular tension, spasms or cramps, intestinal colic, asthma, arthritis, hypertension, hysteria, etc. (Thomsen 2009). Besides, in the past, valerian was used for treatment of the plague, and as pain-relieving and anti-venom remedy, often in combination with licorice, raisins and aniseed, to facilitate breathing and expectoration (Breverton 2011).

Market demand for *V. officinalis* products is increasing worldwide, while its natural resources are limited. In Bulgaria, the species is protected by the Medicinal Plants Act (2000), and its gathering is prohibited

on the territory of the whole country. A study into the content of valepotriates had been carried out 25 years ago on 60 natural populations in 18 floristic regions of the country, completed with field-cultivated plants introduced in the Botanical Garden in Sofia and originating from 31 of these populations (Evstatieva & al. 1993). Authors found out cytotypes that differed a lot in their valepotriate pattern and quantity, including some populations with low iridoid concentration. Essential oils were not analyzed, however, because it was valepotriates that were considered then the most valuable compounds in the valerian drug. Once cytotoxicity of valtrate and didrovaltrate had been revealed, the interest was focused on the cultivation of chemoraces free of valepotriates. Moreover, the drug's healing properties were proved to be related mainly to the valerenic acid and valeron, and the requirements for the essential oils content were specified in the *Pharmacopoeia* (2008).

Chemoraces rich in essential oils and with low valepotriate content could be used as a parent plants source for clonal propagation. Plant biotechnologies offer different alternatives for ensuring biomass of the threatened medicinal plants. One of them is enhanced *in vitro* multiplication of high-productive plant individuals, in order to produce genetically identical plants for the establishment of commercial plantation. Another one is *in vitro* bioproduction of the valuable secondary metabolites by root cultures. The present study is aimed at elucidating the effect of three commonly used plant growth regulators (PGRs) on the root structure and biosynthetic capacity of *in vitro* cultured valerian.

Material and methods

Plant material

The *V. officinalis* parent plant was a 2-year old individual, growing in the *ex situ* collection of the Institute of Biodiversity and Ecosystem Research in Sofia, chosen for its vigor and fast growth. Raceme stalks were gathered during flowering and used for *in vitro* culture initiation. Samples of *in vitro* roots were used for anatomic observation. Roots of both parent plant and *in vitro* root cultures were dried at room temperature and used for phytochemical analyses.

In vitro cultivation

Raceme stalks were surface sterilized by treatment with 70 % ethanol for 1 min, followed by soaking in 50 % solution of commercial bleach (chlorine < 5 %) for 7 min, and rinsing with distilled sterile water thrice for 10 min each. *In vitro* plantlets of *V. officinalis* were regenerated from raceme stalk segments on agar-solidified basal MS medium (Murashige & Skoog 1962) supplemented with 30 g/l sucrose, then the roots were removed and subcultured on MS based media containing different plant growth regulators: 6-benzylaminopurine (BAP) or 6-furfurylaminopurine/kinetin (Kin) alone in concentration 0.5 mg/l (media B and K), or in combination with α-naphthalene acetic acid (NAA), with predominance of either cytokinin or auxin (medium BN: 1 mg/l BAP + 0.5 mg/l NAA; medium KN: 1 mg/l Kin + 0.5 mg/l NAA; medium NB: 0.5 mg/l BAP + 1 mg/l NAA; medium NK: 0.5 mg/l Kin + 1 mg/l NAA), as well as on control medium free of PGRs (medium MS), and MS medium with double sucrose concentration (medium MS60). Roots were cultivated in plastic containers for four months in a culture room with diurnal temperature 23±2 °C and light regime 16/8 h light/dark, light intensity 2000 lx.

Anatomical analysis

Samples of *in vitro* valerian roots cultured for three months on the eight tested media were collected, fixed in FAA (formaldehyde-acetic acid-ethanol) for 24 hours and transferred to 75 % ethyl alcohol for storage (Hendry & al. 1993). Free-hand semi-permanent glycerin slides of transverse sections of the roots were prepared. Observations and photographs of the 5 samples were made with a digital light microscope Motic DMBA210 (Motic Incorporation Ltd., China), using the image analysis software Motic Images Plus version 2.0., with a general magnification of ×100.

Phytochemical analyses

The *in vitro* roots cultured for four months on the respective media, and the roots of the parent plant gathered during the flowering season were dried at room temperature and the essential oils were obtained by distillation-extraction with Likens-Nickerson apparatus. The GC/MS analysis was performed with Hewlett-Packard Gas Chromatograph 5890 Series II Plus linked to Hewlett-Packard 5972 mass spectrometer system equipped with HP5-MS capillary column (60 m × 0.25 mm, film thickness 0.25 μm). The temperature was programmed from 40 °C to 280 °C at a rate 6 °C/min. The ion source was set at 250 °C and ionization voltage at 70 eV. Helium was used as carrier

gas. The identification was based on computer searches in HP Mass Spectral Library NIST98 and Wiley, and comparison of MS spectra of identified compounds with those published in literature (Adams 2009).

Results

In vitro root cultures

Root segments excised from *in vitro* plantlets and growing on media with different plant growth regulators, as well as on basal MS medium, turned green, ramified, and formed thichomes. They covered the entire surface of the cultivation vessels. Media B and K, containing cytokinin alone, enhanced root growth, while media BN, KN, NB, and NK, supplemented with combinations of auxin and cytokinin, produced roots and white friable callus (Plate I, Figs 1-5). The exogenous PGRs influenced root morphology, and during the first subcultivation some root cultures were strong and resilient, e.g. roots grown on medium B, while other were fragile and crumbly, as in the case of medium NK. Roots growing on all media were able to regenerate plantlets (Plate I, Fig. 6). Calli formed mainly roots and callus-derived roots remained white, unlike the other roots (Plate I, Fig. 7). Double sucrose concentration in medium MS60 led to delay of the root growth. All root cultures slowed down the growth rate in time, and turned dark-green-brown (Plate I, Fig. 8).

Anatomical structure

Anatomical study of the *in vitro* roots grown on media with different composition revealed significant differences in regard to the cell structure and size, thickness of the cell wall, and especially the presence and quantity of the starch grains into the cortex parenchyma cells (Plate II, Figs 1-8). In media with usual sucrose concentration (all except MS60), the roots developed normal epidermis cells and root hairs, and one layer of exodermis with rounded cells (Plate II, Fig. 1). The cortex, however, was not divided into outer and inner zones. The amount of starch grains in the cortex parenchyma cells differed in line with the medium composition; thus, starch grains were abundant in the cortex cells of roots grown on MS and BN media, and scarce when roots were grown on K medium (Plate II, Figs 2, 4 & 5). Furthermore, in the plants on medium K there were wide intercellular spaces. Most drastic were changes in the anatomical structure of the roots grown on medium MS60, where all cells had thin walls, epidermal cells were bigger, and the base of the root hair was balloon-like deformed (Plate II, Fig. 8).

Phytochemical analyses

The results of GC/MS analysis showed that the basic compounds in the essential oils of root samples from the parent plant were isovaleric acid (55.2 %)[1] and squalene (19.7 %). There were identified some other constituents, typical for *Valeriana* root oils: spatulenol (4.2 %), valerenal (2.3 %), caryopilene oxide (1.4 %). Besides, pentanoic acid-3-methyl, bornyl acetate, viridiflorol, valerenyl acetate, geranyl isovalerate, *trans*-valerenyl isovalerate, patchouli alcohol, and some unidentified sesquiterpenoic alcohols (MW 220) were detected in amounts ≤ 1 %.

Concerning the *in vitro* cultures, secondary metabolites were detected only in the roots cultivated on medium B. The GC/MS analysis of the essential oil of the roots grown on medium B showed that the main identified compounds were monoterpene citronellol (18.1 %) and sesquiterpene ketone valeranon (17.2 %). Lesser amounts of geraniol (6.0 %), viridiflorol (1.4 %) and valeranal (1.3 %) were also found.

Discussion

Application of plant growth regulators is a powerful tool for manipulation of both growth and biosynthetic ability of *in vitro* cultures. They are widely used to enhance secondary metabolites production in many species. However, the genotype features have been proved as the most important factor determining whether or not the desired compounds would be produced and to what degree their biosynthesis could be stimulated. Thus, the wild populations of *V. officinalis* in the Netherlands were proved to comprise three chemical races (Hazelhoff & al. 1979). The variation in essential oil content was ascertained not only between populations, but also on individual level by screening the plants obtained from commercial seeds of *V. officinalis* (Nell & al. 2009). The high genotype variance concerned the chemical content and the composition of sesquiterpenic acids; therefore, researchers used clonal propagation to ensure genetically identical plants for their experiments related to plant-fungus interaction. We also

[1] % of total ion current

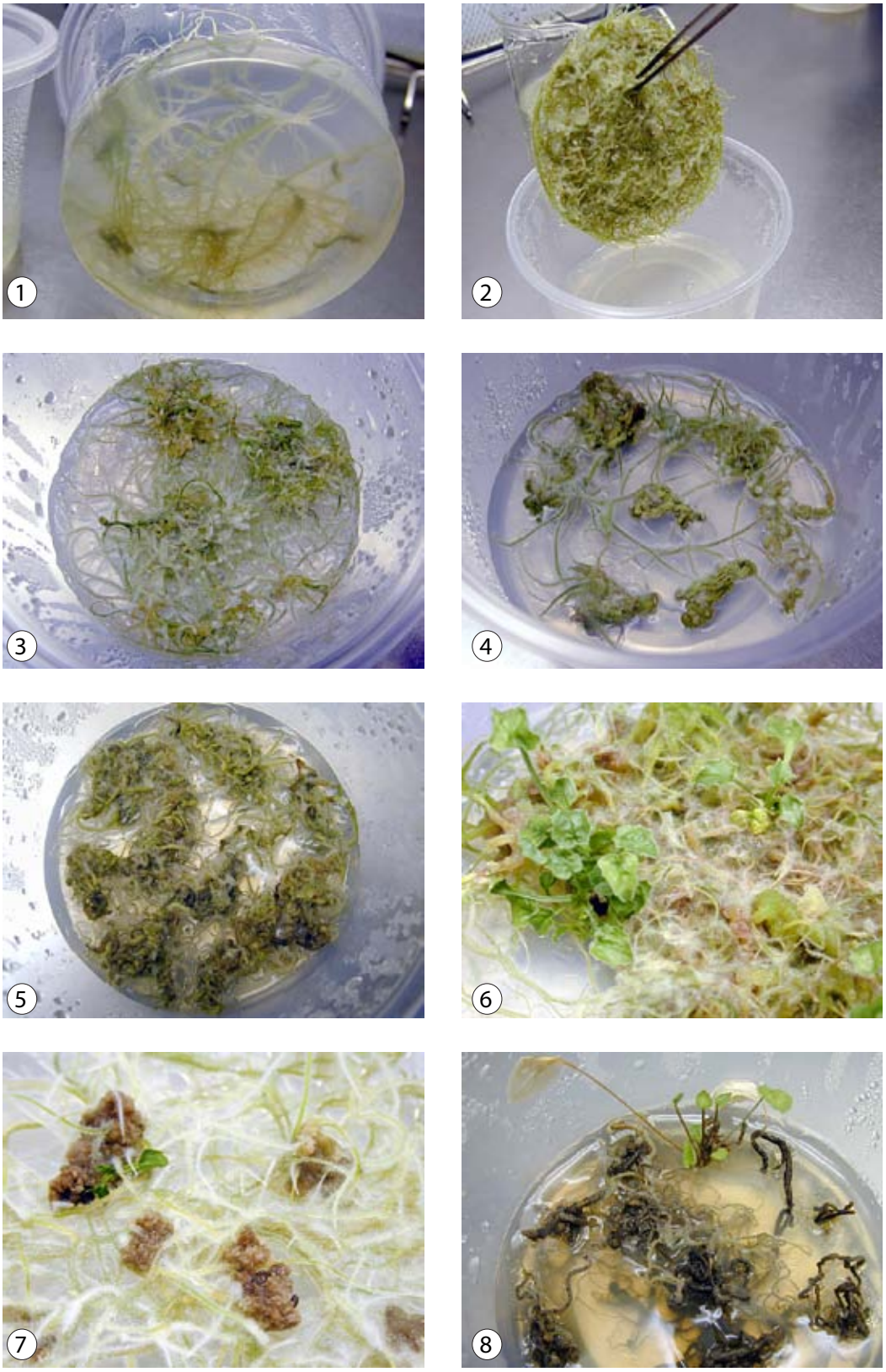

Plate I. *In vitro* roots of *V. officinalis* cultured on different media: Fig. 1. Roots on control MS medium free of PGRs. **Fig. 2.** Roots on medium B. **Fig. 3.** Roots on medium K. **Fig. 4.** Roots on medium BN. **Fig. 5.** Roots on medium NK. **Fig. 6.** Plantlets regenerated on roots. **Fig. 7.** Callus-derived roots on MS medium. **Fig. 8.** Root browning with the time.

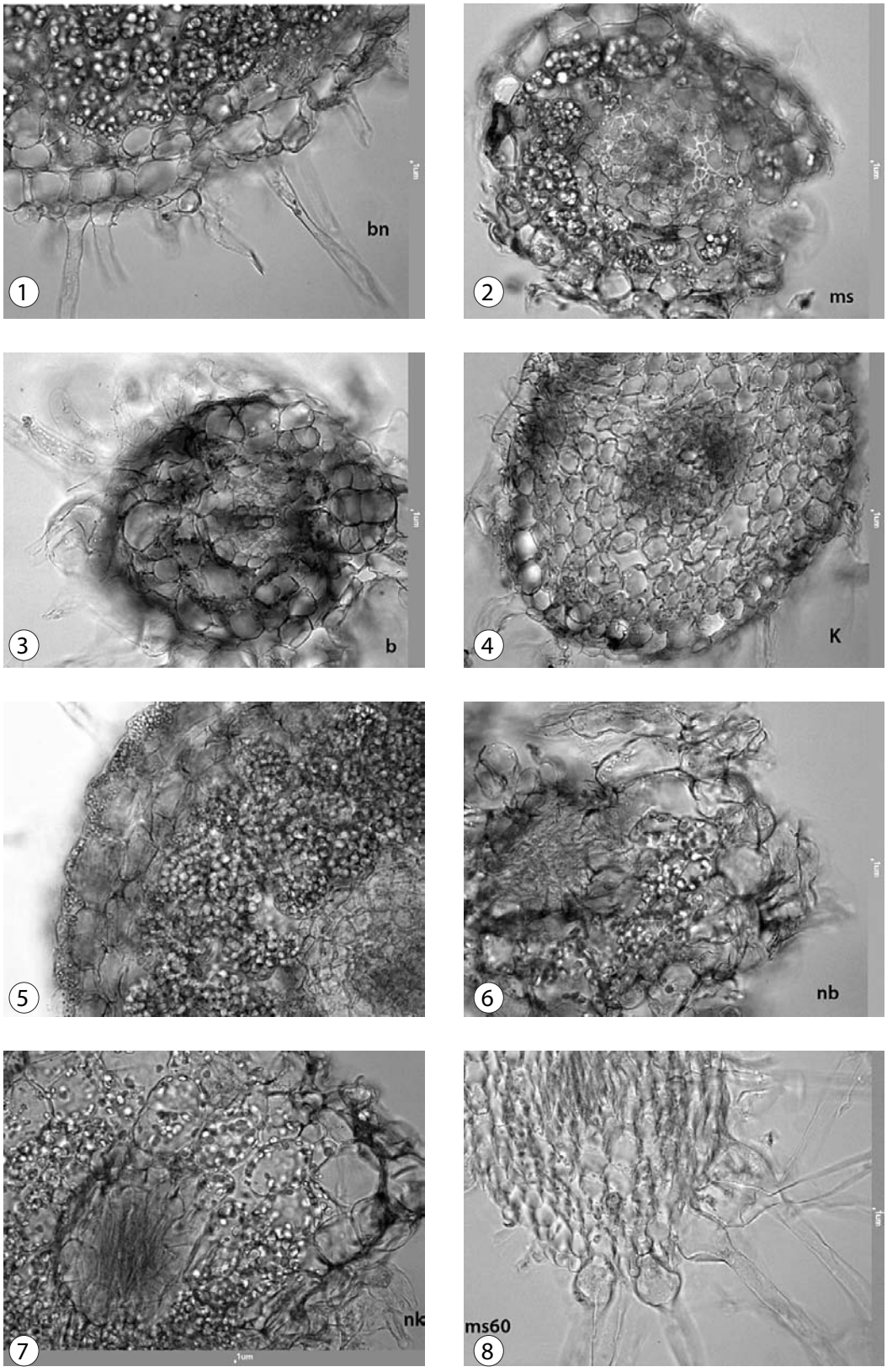

Plate II. Cross-sections of *V. officinalis* roots cultured *in vitro* on different media: **Fig. 1.** Normal exodermis. **Fig. 2.** Control MS medium free of PGRs. **Fig. 3.** Medium B. **Fig. 4.** Medium K. **Fig. 5.** Medium BN: cells overfilled with starch grains. **Fig. 6.** Medium NB. **Fig. 7.** Medium NK. **Fig. 8.** Medium MS60

applied this strategy choosing raceme stalks deriving from a single parent plant as initial material for plantlets multiplication. In this way, the possible differences due to the plant genotype were eliminated and PGRs were the sole factor influencing the biosynthetic activity of the obtained *in vitro* roots.

The essential oil of valerian represents a mixture of mono- and sesquiterpenoids, the latter being of greater importance for both their biological activities and chemotaxonomic features (Houghton 1997). Valerenic acid and its derivatives, acetoxyvalerenic and hydroxyvalerenic acids, cause the main pharmacological effect of *V. officinalis* (Houghton 1997; Trauner & al. 2008). Obviously, the specific smell of the parent plant roots was due to the isovaleric acid, which predominated in the total essential oil, while *in vitro* cultures were unscented. Squalene and other constituents were also identified in the roots of the parent plant, but none of the important sesquiterpenic acids was detected. This corresponds to a recent ethnobotanical study in Bulgaria revealing that *V. officinalis* has been traditionally used for treatment of many and different ailments, such as respiratory, digestive, cardiovascular, and anti-inflammatory disorders, but the plants were not known as a sedative (Nedelcheva & al. 2016). Valerian-based remedies contained leaves or roots and were used either for external application or as herbal teas (Nedelcheva & al. 2015). After HPLC screening of many plants, the authors found out that the root samples originating from wild Bulgarian *V. officinalis* populations contained a relatively low amount of sesquiterpenic acids, without any clearly dominant group, while the roots of the crop cultivated valerian plants had significantly higher content of sesquiterpenic acids, with valerenal, valerenic acid and acetoxyvalerenic acid as dominant components. Earlier studies into the Bulgarian valerian populations referred only to the content of diene and monoene valepotriates (Evstatieva & al. 1993), since the healing effect of valerian was mainly attributed to these compounds during the third period of *V. officinalis* selection starting early in the 1970s (Bernath 1997; Houghton 1997). At that period, the Bulgarian Samokov variety was introduced as crop culture, and an *ex situ* collection was established with plants gathered from different natural populations. Studies into the essential oils of the widest cultivated Shipka cultivar showed an oil yield of 0.42 % (v/m), with valeranone, valerenal, bornyl acetate, and spathulenol (Bos & al. 2000) as main components. Obviously, despite its vigorous growth, our parent plant was not a promising individual for multiplication, because of the lack of valerenic acid, the most important secondary metabolite of valerian, which should be at least 0.17 % in content in the dried roots, according to the *European Pharmacopeia* (2008).

Secondary metabolites are usually in much lower concentrations in *in vitro* cultures, as compared to those in wild and field-cultivated plants. There are many reasons for this, the most evident being the changes occurring in the cell structure, the osmotic stress, and the release of compounds in the culture medium followed by their metabolization. Another important reason, which is usually neglected by the researchers in biotechnology, is the seasonal dynamics of biosynthetic activity of the outside plants. Thus, it was revealed that the best harvesting time for the production of essential oil from *Valeriana jatamansi* growing in western Himalaya was May, while the highest yield of valepotriates was in November and January (Singh & al. 2010). Indeed, the content of many secondary metabolites is increasing during the blooming season, but the appropriate time to harvest medicinal plants depends on the targeted compound. Soni & al. (2015) reviewed the available information on many species from different genera, such as *Thymus, Mentha, Laurus, Eucalyptus, Artemisia, Achillea, Ocimum, Origanum, Melissa, Cistus, Clinopodium, Sclerocarya, Thymbra, Pistacia, Porcelia, Plectranthus,* and *Micromeria*, and concluded that the essential oil yield and composition were significantly influenced not only by the phenological stage, but also by the seasons and, in some cases, by diurnal variations as well. In this relation, mention deserves the fact that we evaluated the essential oil composition of the parent plant only in the blooming stage, while the *in vitro* root cultures were analyzed six months later. Quite probably, variations occur in the essential oil composition of the outdoor plants following the seasons, hence, the differences noticed between the *in vitro* root cultures and the parent plant could be smaller than initially presumed.

According to the *European Pharmacopoeia* (2008), the transverse section of valerian root shows one row of small epidermal cells, some with root hair, one or two layers of large, suberized exodermal cells, often containing droplets of essential oil, outer and inner cortex, the latter consisting of many layers of polygonal to rounded cells filled with simple or compound

starch grains; the pericycle and the parenchyma cells of the rhizome pith are also starch-filled. Indeed, globules of volatile oil occur in the exodermis of the young roots and in the endodermis of the rhizome, both having a large cortex rich in starch (Evans 2009). The four-month old *in vitro* roots developed exodermis on all tested media; however, no essential oil was detected in the root samples except for medium B. These results are consistent with the earlier findings in valerian cultivation revealing that changes in environmental conditions decreased not only essential oil accumulation, but also the number of the essential oil components (Corsi & al. 1984; Babahanjan 1997; Berbec 1965a, b). In the artificial ambiance, cell structure was most influenced in the case of medium MS60, because of the drastic difference in osmotic pressure. Cell and tissue cultures of many other species could not accumulate essential oils, because of lack of special thichomes, receptacles and canals, which resulted in the release of synthesized compounds in the medium and their decomposition in a short time period of about fifteen hours (Evans 2009). In addition, *in vitro* roots were growing under constant ambient conditions of 16 h light and temperature of 23±2 °C, i.e. without seasonal synchronization with the outdoor plants, which most probably affected their biosynthetic capacity. The presence of starch granules in the inner cortex parenchyma cells of the root is typical for *V. officinalis*. Starch grains were observed in almost all *in vitro* roots, thus proving the normal root functioning. Their absence in the cortex cells of roots growing on medium K was an exception and could be caused by the presence of kinetin and related to the phosphorus metabolism, as suggested by Lazarova & al. (1992).

Furthermore, dedifferentiated cells of the callus and cell suspensions often totally lose their biosynthetic capacity, as in the case of hypericin production by *Hypericum*, and α-citral and β-citral by *Cymbopogon* (Wilken & al. 2005). In our case, the presence of auxin caused callus formation, along with the root growth, which further decreased the biosynthetic ability of cultures cultivated on media BN, KN, NB, and NK. Indeed, secondary metabolites were detected only in the root culture grown on medium B. Interestingly, the main compounds in root samples from the parent plant, isovaleric acid and sqalene, were not detected in the *in vitro* roots. Furthermore, monoterpene citronellol and sesquiterpene ketone valeranon, which were identified as main compounds in the *in vitro* root sample, were not produced by the parent plant. Most of the other constituents detected in lower amounts were also different, and only valerenal and viridiflorol were found in both root type samples. This variance was most probably due to the medium composition, especially to the presence of BAP. Modifications in medium composition related to phosphate and nitrate deficiency were found to cause almost parallel ultrastuctural and biochemical changes in the cells of *in vitro* roots of *V. officinalis* too, concerning the valepotriate content and reserve substances (Lazarova & al. 1992).

Decrease of the secondary metabolites in *in vitro* cultures is usual for many medicinal plant species; however, their content is normally recovering during *ex vitro* adaptation of the vegetatively regenerated plants. Therefore, despite the influence of the nutrient medium on the secondary metabolites composition of *V. officinalis*, phytochemical screening of the potential initial plants should be carried out prior to the *in vitro* cultivation. Fast growing root cultures of a single *V. officinalis* plant were successfully obtained and sub-cultured for several months on media containing 0.5 mg/l cytokinins, without callus formation. It is worth continuing the study with some preselected individuals chosen as parent plants for their high content of valerian sesquiterpenoic acids – most valuable from the pharmacological viewpoint.

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