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Essential Oils Produced by in Vitro Shoots of Sage (*Salvia officinalis* L.)

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In vitro shoots of sage (*Salvia officinalis* L.) were established under eight different hormonal supplementations and proliferated by subculture of nodal shoot segments. The respective essential oils, obtained by hydrodistillation, were composed of more than 75 compounds, 65 of which were identified. The 10 major compounds were, by order of retention time, α-pinene (4.1−5.4%), camphene (6−7.1%), β-pinene (9.3−14.5%), limonene (2−2.3%), 1,8-cineole (3.6−5.6%), (−)-thujone (13.2−16.1%), (+)-isothujone (6.6−7.4%), camphor (19.8−24%), α-humulene (5.1−6.8%), and manool (4.2−7.7%). Notwithstanding the eight different hormonal supplementations tested, the percentage composition of the shoot essential oils were kept in a narrow range of variation. However, the type and concentration of growth regulators apparently influenced the accumulation of essential oils. The highest accumulation of essential oils and the highest shoot biomass growth were obtained with 2.0 mg/L kinetin and 0.05 mg/L 2,4-dichlorophenoxyacetic acid.

KEYWORDS: ***Salvia officinalis*; sage; essential oils; in vitro shoots**

INTRODUCTION

Salvia officinalis L. (Lamiaceae) is a perennial woody subshrub native to the Mediterranean countries. Like other sage species, namely, *S. lavandulifolia* Vahl. and *S. sclarea* L., *S. officinalis* L. (sage) is cultivated in several countries mainly to obtain dried leaves to be used as raw material in medicine, perfumery, and the food industry (*1*). *S. officinalis* was included in a group of aromatic and medicinal plant species that are being investigated in Portugal to meet the needs of the essential oil and extract industries and the need of finding new agrarian crops as alternatives to the surplus traditional ones. The research program includes the chemical characterization and the definition of micropropagation protocols of the targeted crops. The chemical characterization of the in vivo cultivated plants of this species was accomplished in determining what constitutes the essential oils (*2*) and phenolic acids (*3*). The data show that the composition of the sage essential oil varies significantly depending on soil mineral fertilization (*4*), light intensity (*5*), organ age (*6*), climate conditions (*7*), season (*8*, *9*), and organ, season, and culture site (*2*, *10*). Because of such variation, the sage essential oil composition sometimes does not match the profile defined by standard ISO 9909 (*1*) for official sage essential oil, which, according to Bruneton (*1*), is *cis*-thujone [= (−)-thujone] (18−43%), *trans*-thujone [= (+)-isothujone] (3−8.5%), camphor (4.5−24.5%), cineole (5.5−13%), humulene (0−12%), α-pinene (1−6.5%), camphene (1.5−7%), limonene (0.5−3%), linalool [free and esterified (1% maximum)], and bornyl acetate (2.5% maximum). In December, the levels of *cis*-thujone in essential oils from sage plants cultivated at two different sites from the northern region of Portugal are over the maximum limits of the standard ISO 9909 (*2*), making the respective use unsuitable, by that time, due to the toxicity of thujones, especially on the central nervous system (*11*).

Environment and nutritional factors, as well as growth regulators, can be easily controlled in in vitro cultures. Besides their importance in facilitating plant propagation, in vitro techniques can afford system models to study, often advantageously, the production, accumulation, and metabolism of important metabolites, including those from *Salvia* species (*12*−*14*). Micropropagation of *Salvia* species, through bud proliferation, has been reported, namely, *S. canariensis* (*15*, *16*), *S. valentina* and *S. blancoana* (*17*), and *S. officinalis* (*18*). Molina et al. (*15*) isolated carnosol and some related phenolic diterpene compounds from 25-day-old in vitro plantlets of *S. canariensis* L. Rosmarinic acid was isolated from leaf callus cultures (*19*) and suspended cells (*20*) of *S. officinalis* L. From suspension cultures of this species, ursolic acid, a triterpenoid compound with potent antiinflammatory, anti-hyperlipidemic, and hepatoprotective activity, was also isolated (*21*). In vitro cell suspensions of *S. officinalis* were also used as system models in studies of metabolism (*22*) and catabolism (*23*) of monoterpenoids being demonstrated that the lack of the respective accumulation in these types of undifferentiated sage cultures could be attributed to a low level of biosynthetic activity coupled with a pronounced capacity for monoterpene catabolism.

To our knowledge, the capacity of in vitro sage shoots in the production and accumulation of essential oil compounds was not yet sufficiently studied. However, these in vitro system

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Table 1. Effect of the Hormonal Supplementation of the MS Basal Medium on Shoot Proliferation, Linear, and Biomass Growth of Shoot Cultures Using Nodal Shoot Segments as Explants

hormonal supplementation	N° of shoots per explant[a]	length of shoots (mm)[a]	biomass fresh wt per shoot (mg)[a]	biomass dry wt per shoot (mg)[a]
0.10 mg/L 2,4-D + 1.5 mg/L BA	2.3 ± 0.98	32 ± 13.8	276 ± 109.7	9 ± 3.6
0.10 mg/L 2,4-D + 1.5 mg/L ZEA	1.2 ± 0.35	34 ± 20.2	433 ± 65.5	16 ± 2.4
0.10 mg/L 2,4-D + 1.5 mg/L KIN	1.9 ± 0.75	37 ± 13.8	264 ± 39.6	12 ± 1.8
0.05 mg/L 2,4-D + 1.5 mg/L BA	3.2 ± 1.62	41 ± 9.7	159 ± 29.7	4 ± 0.8
0.05 mg/L 2,4-D + 1.5 mg/L ZEA	1.4 ± 0.44	42 ± 15.0	280 ± 112.1	15 ± 5.9
0.05 mg/L 2,4-D + 1.5 mg/L KIN	1.6 ± 0.71	35 ± 14.8	310 ± 95.5	24 ± 9.6
0.05 mg/L 2,4-D + 2.0 mg/L KIN	1.9 ± 0.80	36 ± 15.2	374 ± 116.6	26 ± 7.7
0.05 mg/L 2,4-D + 4.0 mg/L KIN	2.1 ± 0.91	36 ± 13.9	273 ± 85.9	19 ± 10.2

[a] Each value represents the mean ± standard error of the data from the respective parameter. BA – benzyladenine; ZEA – zeatin; KIN – kinetin; 2,4-D – 2,4 dichlorophenoxyacetic acid. Wt (wt) – weight.

models resemble more closely the respective in vivo plants, and recently in vitro sage shoots were reported to have higher capacity of accumulation of some types of antioxidant phenolic compounds, namely, rosmarinic acid, than the in vivo sage plants (*24*).

The present paper reports on the results of studies on the production and accumulation of essential oils by in vitro shoots of *Salvia officinalis* developed under different hormonal supplementation.

MATERIALS AND METHODS

Plant Material and Sterilization. Aseptic plantlets of *S. officinalis* were obtained by in vitro germination of open pollinated plant seeds supplied by Direcção Regional de Agricultura de Entre Douro e Minho (DRAEM). Seed surface sterilization as well as medium and environment conditions of germination were as previously described (*24*).

Establishment of in Vitro Shoot Cultures. Sage shoot cultures were induced from nodal segments (~10 mm), obtained from aseptic 6-week-old sage seedlings on Murashige and Skoog (*25*) basal medium (MS) supplemented with 20 g/L sucrose, and 0.1 or 0.05 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) combined with benzyladenine (BA) or zeatin (ZEA) or kinetin (KIN) at 1.5 mg/L or with KIN at 2.0 or 4.0 mg/L, for a total of eight hormonal supplementations tested. Each 175-mL plant tissue culture vessel (Sigma) containing 20 mL MS medium solidified with 8 g/L agar and covered with Magenta B-cap was inoculated with three primary explants. A total of about 70 primary explants were used for each MS hormonal medium variant. Cultures were maintained in a growth room at 25 ± 2 °C with a photoperiod of 16 h light/8 h dark. Illumination was supplied by cool white fluorescent tubes with a light intensity of 52.2 μmol m^{-2} s^{-1}. Shoots from the eight hormonal MS medium variants were subcultured to the same medium conditions with intervals of about five weeks. The efficacy of each medium variant in shoot proliferation and growth was determined over the subsequent subcultures by recording the number of induced shoots *per* explant, the length of the shoots, and the mean biomass fresh weight and dry weight. Dry weight was determined after freeze-drying at 0.05 mbar for 72 h. At the end of 7th subculture period, shoots from about 10 flasks of each hormonal medium variant were withdrawn and weighed, and the respective fresh biomass was gathered and submitted to hydrodistillation for essential oil recovery. The fresh weight of the gathered shoots from each hormonal medium variant ranged from 8 to 21 g (0.5–0.8 g of dry weight).

Hydrodistillation and Analysis of the Essential Oils. Hydrodistillation was performed in a Clevenger type apparatus over 1 h, using volumes of 1.0 mL of *n*-hexane, containing 5α-cholestane (1 mg/mL), for retention of the hydrodistillate components. After that, the mixtures constituted by the solvent, *n*-hexane, the shoot essential oil constituents, and the internal standard, 5α-cholestane, were analyzed by gas chromatography (GC) and gas chromatography coupled to mass spectrometry (GC-MS).

GC analyses were performed using a Perkin-Elmer Autosystem gas chromatograph equipped with a fused silica DB-5 column (30 m long × 0.25 i.d., 0.25 μm film thickness) composed by 5% phenyl methylpolysiloxane, J & W Scientific. Temperature program was 60–285 °C at 3 °C min^{-1} for the column, 300 °C for the injector and 320 °C for the flame ionization detector (FID). H_2 was used as carrier gas at flow rate of 1.49 mL/min under a column head pressure of 12.5 psi. Injections were performed in a split/splitless injector with the splitter opened at the 1:13 split ratio. Three GC injections of each sample were made, and the percentage averages of the respective compounds were determined.

The estimation of the specific content of each essential oil compound was made by using 5α-cholestane, as an internal standard, according to the procedure reported before (*2*). The quantitative GC response factors used for each main essential oil compound group were 0.741 (monoterpene hydrocarbons), 1.014 (oxygen-containing monoterpenes), 1.071 (monoterpenyl esters), 0.747 (sesquiterpene hydrocarbons), 1.018 (oxygen-containing sesquiterpenes), 1.263 (sesquiterpenyl esters), and 0.794 (oxygen-containing diterpenes). A GC response factor of 1 was assumed for compounds that did not belong to any of these groups, as they are the cases of *n*-alkanes. The sum of the specific contents of all individual essential oil compounds was assumed as a parameter for the determination of the total specific essential oil yield.

GC-MS analyses were performed with a Perkin-Elmer 8500 gas chromatograph equipped with a fused silica DB-5 column as that of GC, connected with a Finnigan MAT Ion Trap detector (ITD; software version 4.1) operating in electron impact (EI) mode at 70 eV. Injector, interface, and ion-source temperatures were 300, 260, and 220 °C, respectively. The oven temperature program and injection conditions were as described above for GC. Helium was used as carrier gas with a column head pressure of 12.5 psi. The identification of the compounds was performed according to recommendations of the International Organization of the Flavor Industry (*26*), following the methodology used before in the identification of the compounds from essential oils of this species (*2*). Mass spectra libraries, namely, a terpene library containing mass spectra and retention times on a DB-5 column, were used in the identification of all compounds as well as the comparison with published data, namely, retention times and retention indexes of essential oil compounds on DB-5 column (*27*, *28*). The identification of compounds available in the market was confirmed by comparison of their GC retention times and mass spectra with those of reference compounds.

RESULTS AND DISCUSSION

In Vitro Shoot Cultures and Micropropagation of Sage. The induction and development of new in vitro sage shoots from nodal segments of aseptic plantlets, or from routinely cultivated aseptic shoots, occurred with all eight hormonal variants of the Murashige & Skoog (*25*) medium tested (**Table 1**). The combination of 0.05 mg/L 2,4-D with 1.5 mg/L BA afforded the highest number of shoots per explant allowing us to consider it the best hormonal supplementation for multiplication of the culture. The respective linear growth was also among the highest ones recorded. However, shoots maintained with this hormonal

Table 2. Composition (%) of the Essential Oils from in Vitro Shoots of *Salvia officinalis* Maintained and Grown on MS Basal Medium Supplemented with Eight Different Combinations of a Cytokinin (BA or ZEA or KIN) with an Auxin (2,4-D)[a]

	BA [1.5]		ZEA [1.5]		KIN [1.5]		KIN [2.0]	KIN [4.0]
compounds	2,4-D [0.1]	2,4-D [0.05]	2,4-D [0.1]	2,4-D [0.05]	2,4-D [0.1]	2,4-D [0.05]	2,4-D [0.05]	2,4-D [0.05]
1-butyl acetate	0.2	0.1	0.1	0.1	0.1	tr	tr	tr
cis-2-methyl-3-methylene-hep-5-ene	0.1	0.4	0.1	0.1	0.2	0.1	0.1	0.1
trans-2-methyl-3-methylene-hep-5-ene	tr	0.1			tr	tr		
tricyclene	0.3	0.3	0.2	0.3	0.2	0.3	0.3	0.3
α-thujene	0.3	0.3	0.2	0.2	0.2	0.3	0.3	0.3
α-pinene	5.4	5.2	4.4	4.9	4.1	5.2	4.9	5.1
camphene	6.9	6.7	6.0	7.1	6.0	7.1	7.0	7.1
sabinene	0.2	0.3	0.2	0.2	0.1	0.3	0.2	0.3
β-pinene	13.6	14.5	9.3	11.7	11.7	13.1	12.1	12.6
myrcene	0.9	1.0	0.9	1.0	1.0	1.1	1.1	1.1
n-decane	tr	tr	tr		tr	tr	tr	
α-phellandrene	0.1	0.1	0.1	0.1	tr	0.1	0.1	0.1
α-terpinene	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.2
p-cymene	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
limonene	2.1	2.1	2.0	2.3	2.0	2.3	2.2	2.2
1,8-cineole	3.6	4.1	4.2	4.6	4.8	5.6	5.6	4.9
Z-β-ocimene	0.1	0.1	0.1	tr	tr	tr	tr	0.1
E-β-ocimene	tr	tr	tr	tr	tr	tr	tr	tr
γ-terpinene	0.4	0.4	0.3	0.4	0.3	0.4	0.3	0.4
cis-linalool oxide	0.2	0.2	0.1	0.1	0.2	0.2	0.3	0.2
terpinolene	0.4	0.4	0.4	0.5	0.4	0.5	0.5	0.5
n-undecane	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.2
linalool	0.4	0.3	0.4	0.4	0.4	0.3	0.4	0.4
cis-thujone [= (–)-thujone]	13.2	13.4	14.7	14.8	16.0	15.6	16.1	14.8
trans-thujone [= (+)-isothujone]	7.4	7.1	6.6	6.8	6.7	6.6	6.9	6.9
α-campholenal	0.1	0.1	0.1	tr	tr	0.1	0.1	0.1
camphor	19.8	20.4	22.3	21.0	21.4	22.0	24.0	22.3
cis-pinocamphone [= (*cis*-3-)-pinanone]	1.0	1.2	0.7	0.8	0.9	0.7	0.7	0.8
borneol	1.6	0.9	2.2	1.8	1.5	1.4	1.1	1.2
pinocamphone isomer (T)	0.5	0.7	0.4	0.4	0.5	0.4	0.3	0.4
4-terpineol	0.5	0.4	0.5	0.4	0.4	0.4	0.4	0.3
α-terpineol	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1
bornyl acetate	0.6	0.7	0.9	1.1	1.0	0.9	0.9	0.7
cis-sabinyl acetate	0.1	0.1	0.1	0.1	tr	tr	0.1	0.1
δ-elemene	tr	tr	tr	tr	tr	tr	tr	
trans-carvyl acetate		tr		tr	tr	tr	tr	
cis-carvyl acetate	tr	tr		tr	tr	tr	tr	tr
neryl acetate		tr	tr					tr
β-bourbonene + geranyl acetate	tr			0.0	0.1			
E-caryophyllene	0.6	0.6	0.6	0.6	0.6	0.5	0.5	0.5
aromadendrene or α-guaiene (?)	0.3	0.2	0.4	0.4	0.3	0.3	0.3	0.3
α-humulene	6.6	6.5	6.4	6.0	6.8	5.2	5.1	5.8
allo-aromadendrene			tr		tr	tr	tr	
germacrene D isomer #3	tr	tr	0.1	tr	tr	tr	tr	tr
germacrene D	tr	tr	0.1	tr	tr	tr	tr	tr
α-selinene	0.2	0.2	0.3	0.3	0.4	0.2	0.3	0.2
δ-cadinene	0.1	0.1	0.1	tr	tr	0.1	tr	0.1
caryophyllene oxide	0.1	0.1	0.3	0.2	0.1	0.1	0.1	0.1
viridiflorol	0.1	0.1	0.1	0.1	0.1	tr	tr	tr
widdrol (?)	0.5	0.3	0.6	0.4	0.3	0.2	0.2	0.2
n-hexadecane	0.1	0.1	0.1	0.1	tr	tr	tr	tr
n-heptadecane	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.2
n-octadecane	0.2	0.5	0.2	0.2	0.5	0.1	0.1	0.4
(*Z*)-α-*trans*-bergamotol acetate	0.3	0.2	0.3	0.3	0.3	0.2	0.2	0.2
n-nonadecane	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
n-eicosane	0.1	0.1	0.1	0.1	0.1	tr	tr	tr
manool	6.0	5.5	7.7	6.4	6.0	5.1	4.2	5.1
n-heneicosane	tr			tr		tr	0.1	tr
n-docosane	0.3	0.3	0.4	0.3	0.2	0.2	0.2	0.1
n-tricosane	0.8	0.2	0.6	0.3	0.3	0.1	0.1	0.2

Table 2. (Continued)

	BA [1.5]		ZEA [1.5]		KIN [1.5]		KIN [2.0]	KIN [4.0]
compounds	2,4-D [0.1]	2,4-D [0.05]	2,4-D [0.1]	2,4-D [0.05]	2,4-D [0.1]	2,4-D [0.05]	2,4-D [0.05]	2,4-D [0.05]
n-tetracosane	0.1	tr	0.1	tr	tr			
n-hexacosane	0.1	0.1	0.1	0.1	0.1	0.1	tr	0.1
n-heptacosane	0.1	0.1	0.1	0.1	0.1	tr		0.1
n-octacosane	0.3	0.3	0.4	0.2	0.1	0.1	0.1	0.2
Grouped Components								
monoterpene hydrocarbons	31.0	32.2	24.5	29.1	26.7	31.0	29.6	30.2
oxygen-containing monoterpenes	49.2	50.1	53.3	52.0	53.9	54.2	56.6	54.1
monoterpenyl esters	0.9	1.0	1.2	1.4	1.5	1.1	1.3	0.9
sesquiterpene hydrocarbons	7.8	7.6	7.9	7.3	8.3	6.3	6.2	6.9
oxygen-containing sesquiterpenes	0.6	0.4	0.9	0.6	0.4	0.3	0.3	0.3
sesquiterpenyl esters	0.3	0.2	0.3	0.3	0.3	0.2	0.2	0.2
oxygen-containing diterpenes	6.0	5.5	7.7	6.4	6.0	5.1	4.2	5.1
n-alkanes	2.7	2.2	2.6	1.9	1.9	1.2	1.0	1.5
others	1.5	0.9	1.9	1.1	1.0	0.7	0.5	0.7

[a] The identification of the compounds was performed according to the recommendations of the International Organization of the Flavour Industry (*26*). Both mass spectra and retention indexes of the compounds match those of the corresponding compounds from the terpene library of the computer database and those published before (*2*), and the respective retention times and retention indexes are coherent with the corresponding compounds described by other authors (*27*, *28*). BA – benzyladenine; ZEA – zeatin; KIN – kinetin; 2,4-D – 2,4 dichlorophenoxyacetic acid. Concentration units of the growth regulators, in brackets, are mg/L. Tr (tr) – trace amounts (<0.05%).

supplementation were thinner, with fewer and smaller leaves, and with lower weights than the shoots maintained with the other hormonal supplementations tested (**Table 1**). BA was considered the most suitable cytokinin in the shoot proliferation of *S. canariensis* (*15*, *16*) and *S. sclarea* (*29*). The increase in KIN concentration from 1.5 to 2.0 and to 4.0 mg/L, in the presence of 0.05 mg/L 2.4-D tended to increase the shoot proliferation and allowed us to find the combination of 0.05 mg/L 2,4-D with 2.0 mg/L KIN as the most suitable hormonal supplementation, from those tested, for biomass growth, in terms of dry matter reached per shoot (**Table 1**). Similar effects of KIN concentration in Murashige & Skoog (*25*) medium on the shoot proliferation of *S. valentina* was reported (*17*). The results here described on the in vitro shoot proliferation of *S. officinalis* are much better than those reported by Olszowska and Furmanowa (*18*) for a male sterile genotype of the same species. These authors obtained, from that genotype, one shoot per nodal segment with a length of about 1 cm, reached at the end of 5 weeks of culture in a modified Nitsch and Nitsch (*30*) medium containing 0.1 or 10.0 mg/L of indol-3-acetic acid and KIN (0−1.2 mg/L).

Shoot rooting was induced after separation of the multiple shoots followed by their transference to a MS medium devoid of growth regulators. Four weeks after, multiple adventitious roots per each shoot had been formed. The same strategy had already been successful in the micropropagation of *S. valentina* Vahl and *S. blancoana* Webb & Heldr subsp. Figuerola (*17*).

Essential Oils from Shoot Cultures. The essential oils obtained by hydrodistillation of in vitro growing sage shoots were composed of more than 75 compounds, 65 of which were identified, with three of them still doubtful (**Table 2**). The list of total compounds from the essential oil of in vitro sage shoots (**Table 2**) included and exceeded, in number, all the constituents from the essential oils of aerial vegetative organs from the corresponding in vivo cultivated plants (*2*). Comparing the data on the essential oils from in vivo cultivated plants previously reported (*2*), it was found that, in in vitro shoots, the relative amounts of the total monoterpene hydrocarbons (24.5−32.2%), total *n*-alkanes (1.0−2.7%), and that of the only diterpene compound, manool (4.2−7.7%), were higher than those of the corresponding groups from essential oils of in vivo plants. In contrast, the total oxygenated sesquiterpenes (0.3−0.9%) were much less represented in the essential oils from in vitro sage shoots than in those from the corresponding in vivo plants. The percentages of the total oxygen-containing monoterpenes (49.2−56.6%) from in vitro shoots were within the range of percentage variation of this compound group recorded in the respective in vivo plants, while those of sesquiterpene hydrocarbons (6.2−8.3%) were the majority among the lowest ones recorded (*2*). Comparing the eight percentage essential oil profiles from the shoots grown under the effect of the eight different hormonal supplementations tested, it can be seen that variation ranges are much narrower than the corresponding ones previously reported for samples of in vivo plants harvested over the year with intervals of two months (*2*).

The new compounds from the essential oils of in vitro sage shoots, not found in the corresponding ones from in vivo plants, consisted basically of a series of *n*-alkanes, from *n*-hexadecane to *n*-octacosane, *n*-pentacosane being the only lacking alkane. Linalool is another constituent from essential oils of in vitro sage shoots, not found in the essential oils from the respective in vivo plants. However, this compound is a common constituent from the sage essential oil (*1*).

Apparently, the type and concentration of the growth regulators did not influence the composition of the essential oils from the in vitro sage shoots. The sum of the 10 most representative compounds in the essential oils from in vitro sage shoots ranged from 83.4 to 88.2% in shoots grown with 0.1 mg/L 2,4-D + 1.5 mg/L ZEA and in shoots grown with 0.05 mg/L 2.4-D + 2.0 mg/L KIN, respectively. With the exception for shoots grown with BA, the first, second, and the third major compounds were camphor, *cis*-thujone, and β-pinene, respectively. In shoots grown with BA, camphor was the first one, but β-pinene and *cis*-thujone were the second and third ones, respectively (**Table 2**). These compounds are among those whose percentages in the essential oils from in vivo sage plants most vary over the

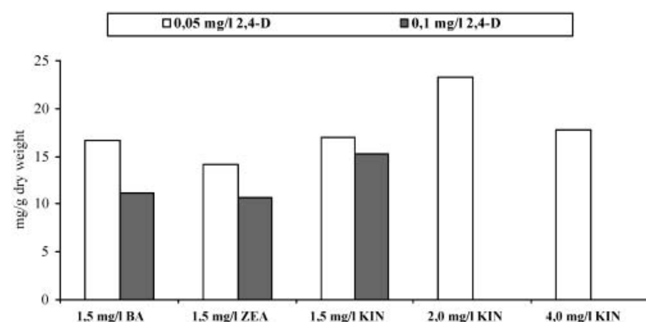

Figure 1. Total essential oil specific contents in the in vitro sage shoots maintained on MS medium under the effect of eight different hormonal supplementations. BA – benzyladenine; ZEA – zeatin; KIN – kinetin; 2,4-D – 2,4 dichlorophenoxyacetic acid.

year (*2*, *8*, *10*). According to some authors, monoterpene compounds from sage essential oils are produced and accumulated in two types of glandular trichomes differentiated on the respective leaves (*31*) and as the leaves expand the camphor content increases (*32*). However, as the plant approaches maturity the content of camphor declines by roughly half, in fully expanded leaves, being metabolized to a water-soluble metabolite via its lactonization to 1,2-campholide followed by conversion to β-D-glucoside-6-*O*-glucose ester of the corresponding hydroxy acid [1-carboxymethyl-3-hydroxy-2,2,3-trimethylcyclopentane] (*33*) which is translocated from leaves to roots where it is subsequently metabolized (*34*). In view of these data, the high levels of camphor in in vitro sage shoots are easily rationalized because, at the time of hydrodistillation, the respective leaves were young and in expanding phase. On the other hand, rhizogenesis had not been induced preventing the metabolism of the bis-glucose derivative of camphor.

An alternative degradative pathway of the camphor described in cell suspension cultures and leaf disks of sage involves the conversion of camphor, in sequence, to 6-hydroxycamphor, 6-oxocamphor, α-campholonic acid, and 2-hydroxy-α-campholonic acid (*22*, *23*). In vitro shoots or in vitro plantlets of sage are system models apparently more suitable for studies of metabolism of terpene compounds than the respective suspension cultures or leaf disks because they resemble more closely the in vivo plants. To our knowledge, however, up to now, no study of metabolism and/or accumulation of terpene compounds in sage shoots was made.

Chemotypes of *S. officinalis* with either *cis*-thujone, *trans*-thujone, camphor, or 1,8-cineole, as the main constituents, or based on their *cis/trans*-thujone ratios 10:1 *cis/trans*, 1.5:1 *cis/trans*, and 1:10 *cis/trans*, have been proposed by some authors (*10*). However, as the percentages of each of these compounds vary greatly in flowers, leaves, and stems, and vary with the season and site where plants grow (*2*), it is not surprising that the composition of the essential oils from the aerial parts of *S. officinalis* plants taken as a whole vary significantly depending on the ratio of leaves/stems/flowers of the biomass used as essential oil source and depending on environmental and soil factors. Such variability would explain the difficulty in ascribing a plant to one or another chemotype. In our view, in vitro shoot cultures could be advantageously used as a tool in determination of chemotypes because such sources of variability would be kept under control.

Besides the utility as tools, in vitro shoot cultures of sage afforded essential oils whose composition, independent of the

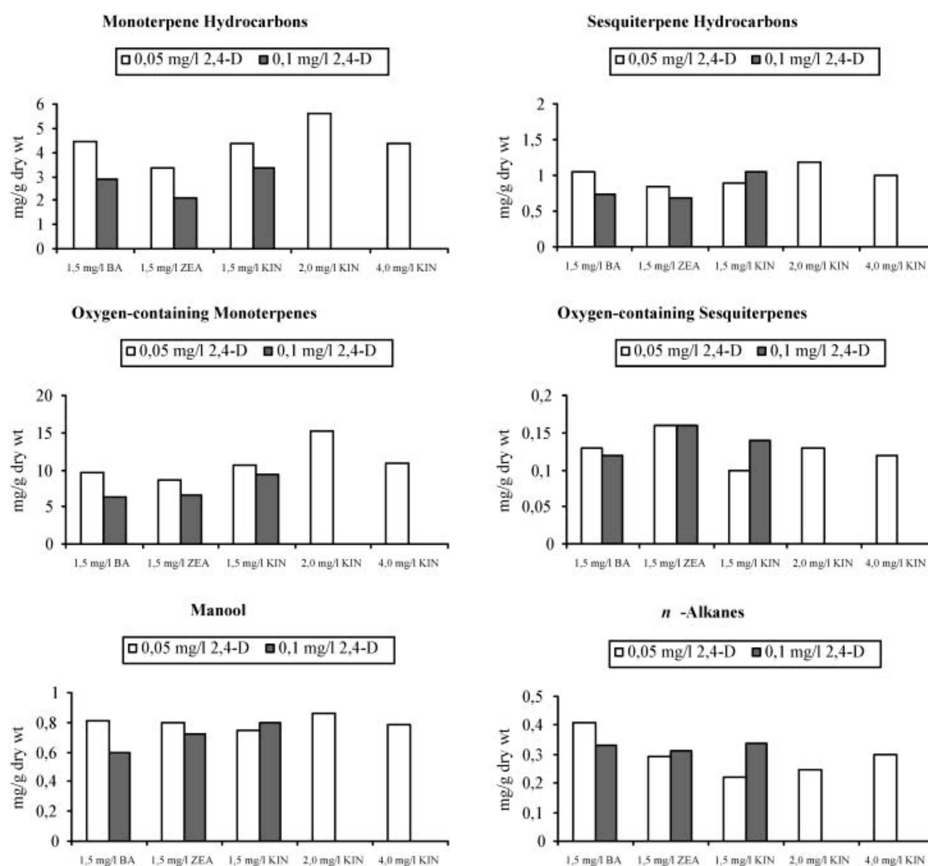

Figure 2. Specific contents of each one of the main compound groups from the essential oils of in vitro sage shoots maintained on MS medium under the effect of eight different hormonal supplementations. BA – benzyladenine; ZEA – zeatin; KIN – kinetin; 2,4-D – 2,4 dichlorophenoxyacetic acid.

growth regulators used, for the majority matches the profile defined by the standard ISO 9909 (*1*). The exception was for *cis*-thujone whose percentage, independent of the hormonal supplementation used, stood lower than the minimum (18%) fixed in the ISO 9909 (*1*).

Figure 1 shows the contents of the essential oils in in vitro shoots grown with each one of hormonal supplementations tested. The influence of the type and concentration of cytokinin and concentration of 2,4-D seems to be more evident for accumulation than for the composition of the essential oils from in vitro sage shoots. According to some authors, the density of glands and the specific essential oil content from in vitro shoots of some species, namely, *Pelargonium* sp., varies with the auxin/cytokinin ratio (*35*). As the essential oil of sage accumulates in leaf glands, an eventual lower density of glands on the leaves induced by the higher auxin/cytokinin ratio would explain the lower specific essential oil accumulation in the in vitro shoots grown with 0.1 mg/L 2,4-D. However, as **Figure 2** shows, the hormonal supplementation seems to influence differentially the accumulation of each one of the main groups of essential oil compounds. For instance, in the presence of 1.5 mg/L KIN, the contents of the total monoterpene hydrocarbons and total oxygen-containing monoterpenes in shoots grown under the effect of 0.1 mg/L 2,4-D were lower than the corresponding contents accumulated in shoots grown with 0.05 mg/L 2,4-D. The contrary occurred, however, with the contents of the total sesquiterpene hydrocarbons, oxygen-containing sesquiterpenes, alkanes, and manool (**Figure 2**). On the other hand, from the three KIN concentrations tested, 4.0 mg/L KIN was the most favorable in *n*-alkanes accumulation while 2.0 mg/L KIN was the most suitable in the accumulation of all the other main compound groups (**Figure 2**). These results hardly can be explained by the effect of growth regulators on the glandular trichomes density as it was observed in *Pelargonium* sp. shoots (*35*).

The three concentrations of KIN (1.5, 2.0, and 4.0 mg/L), in combination with 0.05 mg/L 2,4-D, had already been tested in the production of phenolic antioxidant compounds by in vitro shoots of sage. The results showed that sage shoots developed in the presence of 2.0 mg/L KIN had contents of total phenolics 35% and 23% lower than shoots maintained with 1.5 and 4.0 mg/L KIN, respectively, in inverse correlation with the biomass growth (*24*). In this case, however, shoots developed in the presence of 2.0 mg/L KIN had a content of total essential oil 37% and 32% higher than shoots maintained with 1.5 and 4.0 mg/L KIN, respectively, in positive correlation with the biomass growth. Therefore, the results here reported on the specific accumulation of the total essential oils and on the biomass growth show that from all eight hormonal supplementations tested in MS medium, KIN at 2.0 mg/L with 2,4-D at 0.05 mg/L is indubitably the most suitable for essential oil production by in vitro shoots of *Salvia officinalis* L.

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