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Research Article

Volatile metabolic profiles of cell suspension cultures of *Lavandula vera*, *Nicotiana tabacum* and *Helianthus annuus*, cultivated under different regimes

Cell suspension cultures of *Lavandula vera* (Lamiaceae), *Nicotiana tabacum* (Solanaceae), and *Helianthus annuus* (Asteraceae) were cultivated in three different ways: in shake flasks both as free suspensions and in two-phase systems (in the presence of Amberlite XAD-4 resin as a second phase), as well as in 3-L stirred tank reactor, and their volatile metabolic profiles were studied using GC-MS. A number of compounds, some of them having allelochemical and biological activities, were identified in all the three cell suspension cultures under study. Also the presence of some compounds, unusual for the intact plants, was observed. It was found that the cultivation mode strongly influences the production and the transport (secretion into the culture medium) of the low-molecular-mass volatile metabolites. Principal component analyses of 12 common hydrocarbons showed discrimination between the different cultivation modes (shake flasks and two-phase systems cultivation) by first principal component (PC1) and second principal component (PC2).

Keywords: Bioreactor / GC-MS / Plant cell suspensions / Principal component analysis / Volatiles

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1 Introduction

Plant *in vitro* cultures were recognized as an attractive alternative of the classical technologies for mass production of important plant-derived metabolites with various biological activities [1]. The progress in this field leads to the mass production of several important metabolites, such as taxol (paclitaxel), shikonin, and berberin by different companies [1]. However, for their production several problems have to be overcome, mainly connected with their cultivation and high sensitivity to different stress factors/conditions [2, 3]. The influence of the cultivation conditions in plant cell suspensions was mainly investigated in the frame of single compound production and the influence on the whole metabolism was more or less neglected. Recent metabolic profiling attempts seem to be able both to provide more information on plant metabolic pathways and to give us the opportunity for better understanding and exploitation of plant biochemical machinery for production of desired compounds [1, 4, 5]. GC-MS is an essentially nonbiased technique, which allowed identification of the number of compounds [6] and was successfully applied for quantitative comparisons between different groups of metabolites and different cultivation modes [4, 5].

Plant volatile compounds act as a language that plants use for their communication and interaction with the surrounding environment [7]. They defend the plants against a wide spectrum of enemies [7] and some of them possess valuable biological activity [8].

In our previous study, a large GC-MS survey of cell suspension culture from *Rosa damascena* was performed [4], which revealed the significant differences in metabolic profiles of low mass molecular compounds between different cultivation modes applied. Thus, in this study the volatile metabolic profiles of three cell suspension cultures of different

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Abbreviation: PC, principal component

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families – *Lavandula vera* (Lamiaceae), *Nicotiana tabacum* (Solanaceae), and *Helianthus annuus* (Asteraceae) – were presented and compared with their metabolic profiles, when they were cultivated under different regimes (in two-phase systems in the presence of resin and in stirred tank bioreactors).

2 Materials and methods

2.1 Cell cultures and experimental scheme

The callus cultures of *L. vera* MM, *N. tabacum*, and *H. annuus* used in this work were obtained between 1985 and 1990 [9]. All of them were maintained on a solid Linsmayer–Skoog (LS) nutrient medium [10], supplemented with 30 g/L sucrose, 0.2 mg/L 2,4-dichlorophenoxyacetic acid and 5.5 g/L plant agar and were grown for 3 wk at 26°C, in the dark. The cell suspension cultures were cultivated in liquid LS medium of the same composition (without agar) under the following conditions:

Mode I: in 500 mL conical flasks with 1/5 net volume, on an orbital shaker (11.6 rad/s), in the dark, at 26°C;

Mode II: under the above-mentioned conditions, but in the presence of 10 g/L Amberlite XAD-4 (Sigma-Aldrich, St. Louis, MO, USA) as a second phase;

Mode III: in a 3-L bioreactor (2.25-L working volume) BioFlo 110 (New Brunswick, NJ, USA), equipped with propeller impeller (at 100 rpm; tip speed 0.318 m/s), dissolved oxygen at 30% of air saturation (polarizable DO electrode, InPro 6000, type T-96, Mettler Toledo, Switzerland), at 26°C.

The inoculation was always performed with 20% v/v cell suspensions cultivated in the flasks for 7 days under the above-mentioned conditions.

After 9 days of cultivation, the cell suspension cultures reached their maximum growth and accumulated about 10–12 g dry biomass/L in all cultivation modes applied.

2.2 Sample preparation procedures for plant cell biomass, culture media, and second phase

Samples of biomasses (~20 g fresh weight each) were subjected to a 4-h distillation in a Lickens–Nickerson apparatus [11] and the volatile compounds were extracted from the distillate with diethyl ether. For the investigation of the volatile compounds, adsorbed on the second phase, the resin (10 g Amberlite XAD-4) was extracted with boiling acetone (3 × 400 mL). The acetone extracts were combined, evaporated under reduced pressure and the dry residue (~100 mg) was subjected to distillation as described above. The culture media (~13 mL each) were extracted consecutively with chloroform (3 × 200 mL) and then the chloroform fraction was subjected to distillation as described above.

2.3 Chromatographic conditions

The extracted volatiles were dissolved in diethyl ether and investigated by GC-MS on Hewlett Packard 6890+MS 5973 instrument (Hewlett Packard, Palo Alto, CA, USA) equipped with HP5-MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness, Agilent Technologies, Wilmington, Delaware, USA). The oven temperature was programmed from 40 to 280°C at a rate of 6°C/min. Helium was used as a carrier gas (0.8 mL/min). Split–splitless injector was used at 240°C.

2.4 Interpretation of the results

The GC-MS investigation was based on the interpretation of the mass spectral fragmentation followed by comparisons of the spectra obtained with those in a HP Mass Spectral Library NIST98 (Hewlett Packard). When the spectra of some isomers were very similar and these compounds could not be identified unambiguously, comparisons of the GC retention times, obtained under the same conditions, were used. When there were no suitable authentic samples and/or spectra for comparison, no identification was proposed.

The presented percentages were from the total ion current of the GC-MS of the volatile fractions, accepted as 100%. Different compounds have different ion currents, depending on their quantities and fragmentation. The sum of the percentages of the compounds is always $<100\%$ since part of them cannot be identified. The ion current generated depends on the characteristics of the investigated compounds and is not a true quantification. For this reason, the GC-MS analyses do not give the exact quantitative data. The data obtained from GC-MS were used only for comparison between the same groups of compounds biosynthesized under different modes of cultivation.

The presented results are the means of two independent experiments.

2.5 Principal component analysis

Principal component (PC) analysis was performed using CHEMDA software (http://www.kosnos.com/chemometrics/pca/), working both under the correlation and covariance methods. The output of the PC analysis consisted of the relative amounts (biomass and culture medium for the free cell suspension and bioreactor cultivation, while for the two-phase systems cultivation the yield consisted of biomass, culture medium, and the amounts from the resin) of 12 common hydrocarbons for all the cultures under study.

3 Results and discussion

Plant cell culture technologies are an attractive alternative for overcoming the production of useful metabolites by extraction from intact plants. However the plant cells, cultivated *in vitro* (on agar or submerged in liquid medium) have a very different physiological and biochemical environment, compared with the respective intact plants. Hence, detailed intra- and extra-metabolic analyses have to be performed on every plant cell culture system for obtaining the needed information, concerning its biochemical potential. Many factors can affect the production of metabolites by a plant cell culture as they are

Table 1. Volatile compounds from *L. vera* MM cell suspension cultures cultivated under different regimes (% from the total volatiles)[a)]

Compounds	Regime of cultivation						
	As free suspension		In two-phase system			In bioreactor	
	Biomass (1)	Culture medium (2)	Biomass (3)	Culture medium (4)	Amberlite XAD-4 (5)	Biomass (6)	Culture medium (7)
Hydrocarbons	**8.2**	**5.7**	**18.2**	**8.9**	**6.1**	**5.4**	**18.3**
Tetradecane	–	–	–	0.1	0.1	–	–
Hexadecane	–	–	–	–	–	–	0.4
Heptadecane	–	0.7	–	–	0.3	0.4	0.8
Octadecane	0.1	0.8	0.5	0.9	0.4	0.2	0.7
Nonadecane	0.4	0.6	0.7	0.9	0.3	0.8	1.6
Eicosane	0.5	0.8	0.8	1.1	0.3	0.4	2.0
Heneicosane	–	0.6	1.1	1.1	0.5	0.3	1.9
Docosane	0.7	–	0.7	1.1	0.4	0.4	2.4
Tricosane	0.8	–	0.7	0.9	0.4	0.3	1.9
Tetracosane	1.2	–	1.4	1.1	0.7	–	1.4
Pentacosane	1.4	–	1.5	0.3	0.7	–	1.4
Hexacosane	1.0	–	1.6	–	–	–	0.7
Heptacosane	0.8	–	1.8	–	–	–	0.6
Octacosane	0.2	–	2.2	–	–	–	0.2
Nonacosane	0.1	–	2.3	–	–	–	0.1
Triacontane	–	–	2.1	–	–	–	–
1-Tetradecene	–	–	–	–	0.4	0.2	–
Biphenyl	–	–	–	0.1	–	0.1	–
2,6,10,14-Tetramethyl-hexadecane	–	–	–	–	–	0.3	–
Trimethyl naphthalene	–	–	–	–	0.1	–	–
Squalene	0.8	1.2	0.8	1.0	0.5	0.9	0.5
Phenanthrene	0.2	0.9	–	0.3	0.1	0.7	1.7
Anthracene	–	–	–	–	0.1	0.4	–
Methylphenanthrene	–	–	–	–	0.4	–	–
Methylphenanthrene (isomer)	–	–	–	–	0.4	–	–
Methylfluorene	–	0.1	–	–	–	–	–
Fatty acids	**1.4**	**0.6**	**1.8**	**2.4**	**4.3**	**0.5**	**2.2**
Heptanoic acid	–	–	–	0.1	0.1	0.1	0.1
Octanoic acid	–	–	0.1	0.1	0.1	0.1	0.1
Nonanoic acid	0.1	–	0.2	0.3	0.1	0.2	0.2
Decanoic acid	0.1	–	0.1	0.1	0.1	0.1	0.2
Tetradecanoic acid	0.1	–	–	0.9	0.5	–	1.4
Hexadecanoic acid	1.1	0.6	1.4	0.9	3.4	–	0.2
Esters	**10.0**	**0.7**	**3.2**	**4.8**	**1.9**	**3.6**	**0.1**
Methyl dihydrojasmonate	0.1	–	0.1	–	–	–	–
Methyl hexadecanoic acid	0.7	–	–	–	0.5	–	–
Isopropyl myristate	0.7	0.2	0.5	3.7	1.4	–	–
Methyl ester of octadienoic acid	0.5	–	–	–	–	0.3	–
Methyl ester of octatrienoic acid	4.6	–	1.6	–	–	2.1	–

Table 1. Continued

Compounds	Regime of cultivation						
	As free suspension		In two-phase system			In bioreactor	
	Biomass (1)	Culture medium (2)	Biomass (3)	Culture medium (4)	Amberlite XAD-4 (5)	Biomass (6)	Culture medium (7)
Ethyl ester of octadienoic acid	3.2	–	–	–	–	–	–
Ethyl ester of octatrienoic acid	–	–	0.9	–	–	1.2	–
Glycerol tricaprylate	0.2	–	0.1	0.5	–	–	0.1
Methyl ester of pentadecanoicacid	–	0.5	–	0.6	–	–	–
N-containing compounds	**0.3**	**19.5**	**0.2**	**0.9**	**0.3**	**0.5**	**1.8**
Aniline	0.1	0.1	–	0.3	0.2	0.1	0.1
N-Methyl aniline	–	–	–	–	–	–	0.1
N,N-Dibuthyl formamide	–	0.1	–	–	–	–	0.1
Methyl isoquinoline	–	–	–	–	–	–	0.1
Carbazole	–	–	–	–	–	–	0.6
Methyl carbazole	–	–	–	–	–	–	0.8
Cyclohexane isothiocyanate	–	7.8	0.1	–	0.1	0.1	–
Propane nitrile, 3-(phenyl amino)	0.2	–	0.1	0.3	–	0.1	–
Diphenyl amine	–	–	–	0.3	–	0.2	–
Cyclohexamine	–	2.9	–	–	–	–	–
1,3-Dicyclohexyl urea	–	8.6	–	–	–	–	–
Aldehydes	**0.2**	**–**	**–**	**0.1**	**0.2**	**0.3**	**–**
Benzaldehyde	0.1	–	–	0.1	0.1	0.1	–
Vanilline	0.1	–	–	–	0.1	0.2	–
Phenols	**0.2**	**0.1**	**–**	**0.2**	**0.1**	**0.2**	**0.3**
Phenol	0.2	0.1	–	0.2	0.1	0.1	0.2
2-*O*-Methyl-4-vinyl phenol	–	–	–	–	–	0.1	–
4-Chloro-3-methyl phenol	–	–	–	–	–	–	0.1
Other compounds							
Monoterpene, $M^+ = 164$	0.1	–	–	0.1	0.7	–	–
$M^+ = 160$	23.2	10.6	22.3	23.1	11.0	20.0	8.2
$M^+ = 162$	18.5	0.9	0.9	22.5	11.5	21.0	0.5
Dichloro compound ($M^+ = 110$)	–	–	–	0.1	–	–	–
Dichloro compound ($M^+ = 188$)	–	–	–	0.5	–	–	–
Trichloro compound ($M^+ = 242$)	–	–	–	–	0.1	–	–
Tetrachloro compound ($M^+ = 204$)	–	–	–	0.1	–	–	–
Benzoic acid	–	–	–	–	0.1	–	–
Benzothiazole	–	–	–	0.1	–	–	–
Methyl dibenzothiophene	–	–	–	–	–	–	0.1

[a] The ion current generated depends on the characteristics of the compound and is not a true quantification, but can be used for quantitative comparisons.

Table 2. Volatile compounds from *N. tabacum* cell suspension cultures cultivated under different regimes (% from the total volatiles)[a)]

Compounds	Regime of cultivation						
	As free suspension		In two-phase system			In bioreactor	
	Biomass (1)	Culture medium (2)	Biomass (3)	Culture medium (4)	Amberlite XAD-4 (5)	Biomass (6)	Culture medium (7)
Hydrocarbons	**11.6**	**5.1**	**4.3**	**14.2**	**12.8**	**0.9**	**11.5**
Tetradecane	–	–	–	–	0.1	–	–
Pentadecane	–	–	–	–	0.4	–	–
Hexadecane	1.1	–	–	–	–	–	–
Heptadecane	1.3	–	0.1	1.2	1.4	0.1	0.1
Octadecane	1.5	–	0.3	1.6	1.1	0.2	–
Nonadecane	1.0	0.1	0.6	0.1	1.4	0.3	1.1
Eicosane	0.4	0.3	0.6	1.3	1.4	0.2	1.3
Heneicosane	–	0.7	–	1.1	–	0.1	1.5
Docosane	4.1	1.1	0.1	2.1	2.5	–	1.8
Tetracosane	–	1.5	0.7	2.3	1.7	–	2.1
Pentacosane	–	1.4	0.6	2.2	–	–	2.2
Hexacosane	–	–	0.1	0.9	0.2	–	–
Heptacosane	2.2	–	1.2	1.4	1.2	–	1.4
Octacosane	–	–	–	–	0.1	–	–
Nonacosane	–	–	–	–	1.1	–	–
Hentriacontane	–	–	–	–	0.2	–	–
Fatty acids	**5.9**	**–**	**6.4**	**0.5**	**0.8**	**0.3**	**0.5**
Heptanoic acid	–	–	–	–	0.1	–	–
Octanoic acid	0.1	–	–	0.1	0.1	0.1	0.1
Nonanoic acid	0.4	–	–	0.2	0.2	0.1	0.2
Decanoic acid	0.2	–	–	0.2	0.4	0.1	0.2
Tetradecanoic acid	0.2	–	0.1	–	–	–	–
Hexadecanoic acid	–	–	6.3	–	–	–	–
Linoleic acid	5.0	–	–	–	–	–	–
Esters	**6.9**	**–**	**1.1**	**6.1**	**11.0**	**–**	**1.6**
Octyl benzoate	–	–	–	–	–	–	0.1
Linoleic acid ethylester	4.2	–	–	2.4	–	–	–
Hexadecanoic acid ethylester	1.3	–	–	1.1	–	–	–
Glycine, *N*-phenyl-, ethylester	0.2	–	1.0	1.2	1.5	–	1.5
Isopropyl myristate	1.2	–	0.1	1.4	–	–	–
Decyl oleate	–	–	–	–	9.4	–	–
Isopropyl palmitate	–	–	–	–	0.1	–	–
Others compounds							
Aniline	5.6	2.4	7.5	4.2	8.5	7.5	7.6
Vanilline	0.1	–	1.1	0.2	1.1	0.1	0.3
o-Hydroxy biphenyl	0.1	–	–	–	–	–	1.1
1-Hexadecanol	–	–	6.3	–	–	–	1.2
Phenol	–	–	–	0.2	0.1	0.1	0.1
Benzoic acid	–	–	–	–	0.1	–	–
Formamide-*N,N*-dibutyl	–	–	–	–	–	–	0.1

[a)] The ion current generated depends on the characteristics of the compound and is not a true quantification, but can be used for quantitative comparisons.

Table 3. Volatile compounds from *H. annuus* cell suspension cultures, cultivated under different regimes (% from the total volatiles)[a)]

Compounds	Regime of cultivation						
	As free suspension		In two-phase system			In bioreactor	
	Biomass (1)	Culture medium (2)	Biomass (3)	Culture medium (4)	Amberlite XAD-4 (5)	Biomass (6)	Culture medium (7)
Hydrocarbons	**14.0**	**3.1**	**3.5**	**9.2**	**11.2**	**13.9**	**15.9**
Tetradecane	–	–	–	–	–	0.2	3.9
Octadecane	1.1	–	–	–	–	0.7	–
Nonadecane	0.7	–	–	–	2.1	–	–
Eicosane	1.2	–	–	1.3	3.1	–	0.1
Heneicosane	–	–	–	3.9	2.0	–	–
Docosane	1.5	1.6	2.0	–	2.0	0.9	2.5
Tricosane	0.8	–	–	–	–	1.1	0.1
Tetracosane	–	1.5	1.5	2.5	–	–	4.5
Pentacosane	3.2	–	–	1.5	2.0	3.8	4.8
Hexacosane	–	–	–	–	–	1.5	–
Heptacosane	1.3	–	–	–	–	2.8	–
Octacosane	1.8	–	–	–	–	–	–
Nonacosane	2.4	–	–	–	–	–	–
Triacontane	–	–	–	–	–	1.3	–
Hentriacontane	–	–	–	–	–	1.6	–
Fatty acids	**14.1**	**7.0**	**3.5**	**11.2**	**3.3**	**3.6**	**5.4**
Octanoic acid	–	–	0.1	–	0.5	–	–
Nonanoic acid	–	–	0.1	–	1.2	–	–
Decanoic acid	–	–	–	–	0.1	–	–
Tetradecanoic acid	–	–	–	–	–	1.1	–
Pentadecanoic acid	–	0.1	–	–	–	1.2	–
Hexadecanoic acid	–	–	–	–	–	1.3	–
Linoleic acid	14.1	6.9	3.3	11.2	–	–	5.4
Octadecenoic acid	–	–	–	–	1.5	–	–
Esters	**–**	**–**	**0.2**	**–**	**1.0**	**9.3**	**–**
Glycine, N-phenyl-, ethyl ester	–	–	0.1	–	0.6	1.1	–
Glycine, N-phenyl-, methyl ester	–	–	0.1	–	0.4	1.8	–
Methylphenyl-carbonic acid ethyl ester	–	–	–	–	–	6.4	–
Aromatic compounds and derivates	**–**	**–**	**1.2**	**1.3**	**7.0**	**–**	**0.4**
2-Metoxy-benzoic acid, methyl ester	–	–	1.2	1.1	4.3	–	–
Benzothiazole	–	–	–	0.2	–	–	0.4
Ethylbenzoic acid	–	–	–	–	1.8	–	–
Vinylbenzoic acid	–	–	–	–	0.9	–	–
Other compounds							
Triphenyl phosphate	1.8	–	–	–	–	–	–
Aniline	10.1	2.3	6.7	4.6	6.7	2.7	0.9
Phenol	0.3	0.2	–	–	0.2	0.1	0.3
Isoquinoline, 1-methyl-	–	–	–	–	0.6	–	–
Cyclohexanone	0.2	–	–	–	–	0.1	–
Cyclohexane isocyanate	–	–	–	0.1	–	0.1	–
2-Ethyl-1-hexanol	–	0.1	–	–	–	–	–
1-Hexadecanol	–	–	–	–	4.2	4.5	–

[a)] The ion current generated depends on the characteristics of the compound and is not a true quantification, but can be used for quantitative comparisons.

sensitive to various external stresses, which can alter both the overall metabolite production and the composition of the metabolite profile. Because of that the production of secondary metabolites by plant cell cultures required the monitoring and maintaining of their biosynthetic status and checking their metabolite profiles during their cultivation under different regimes and even at every step of the up-scaling processes.

It is known, that the plant cell volatiles are an important group of compounds, which fulfill the potentialities of the cell to interact with surrounding environmental situations [7] and their biosynthesis can be altered under different cultivation conditions. The metabolic profiles of volatiles were studied in three different cell suspension cultures, obtained from plants, belonging to different families (*N. tabacum* (Solanaceae), *H. annuus* (Asteraceae) and *L. vera* (Lamiaceae)). Further, the investigation was extended to the checking of these profiles during cultivation of the cell suspension cultures under study in bioreactor and in the presence of the second phase (Tables 1–3). The volatile compounds in biomasses, culture media, and second phases of the three cell suspensions under study were analyzed by GC-MS. Previously it was established that the main constituents of the volatiles of *R. damascena* cell suspension were hydrocarbons, free acids, and esters [4]. The same groups of compounds formed the volatile fractions of the three cell suspensions under study, but their profiles were specific for each culture (Tables 1–3). Their amounts and distribution between biomasses, culture media, and second phases depended on the regimes of cultivation as well, as much as under different regimes adaptation to the different environmental conditions had to be performed by the cell.

3.1 Volatiles from cell suspension culture of *L. vera*

The results from GC-MS analysis of the *L. vera* biomass, culture medium, and Amberlite XAD-4 resin from different culture conditions (Table 1) showed that the main constituents of the volatiles fraction were hydrocarbons as well as some monoterpenes (molecular masses 160 and 162; Table 1).

Most of the hydrocarbons (26 hydrocarbons were detected) contained saturated straight chains with 14–30 atoms. Only one unsaturated (squalene) and one-branched (2,6,10,14-tetramethylhexadecane) hydrocarbons were identified. Squalene, which is a 30-carbon isoprenoid, possesses valuable antioxidant and chemopreventive activities [12, 13]. A few polycyclic hydrocarbons and their methylated derivates were found in relatively low concentrations, mainly in the resin and in the culture media. Highest content of the straight chain saturated hydrocarbons was found in the biomass with or without resin and in the culture medium from bioreactor cultivation (Table 1).

Another important group of metabolites appeared to be the free fatty acids. Fatty acids containing 7–18 carbon atoms were identified; the hexadecanoic acid was the main fatty acid.

A significant number of esters were identified, some of them with known allelochemical activities. The main group of esters was methyl esters of fatty acids, which are common for many plants [14]. The presence of ethyl esters is believed to be artifacts, produced through extractions and chromatographic separations by ethanolysis of lipids by the ethanol used. However, the presence of only two ethyl esters did not support this opinion (if artifacts they should be much more) and more importantly there was no ethanol used in the extraction procedure.

The unusually high number of nitrogen-containing compounds was detected mainly in the culture medium of the *L. vera* cell suspension (Table 1). The low presence of alkaloidal components is aberrant for a representative of family Lamiaceae, in which very rarely nitrogen-containing metabolites are found. Among other *N*-containing compounds carbazole was detected, which is known to possess anticancer activities [15].

Aldehydes are important allelochemicals, responsible for the insect–plant interactions [16, 17]. However, we found them only in traces in the *L. vera* cell cultures. The phenols, other important defensive metabolites in plants, were also found in small amounts in the investigated culture (Table 1).

L. vera plants contain significant amounts of terpenoids [18, 19], but in the *in vitro* culture from this plant species we found only limited number of terpenoids (Table 1). Two of them are often found in the higher plants (2,6,10,14-tetramethylhexadecane and squalene), but only the latter is a known allelochemical. The GC-MS study of the total volatile fraction showed that its main components (usually both of them were responsible for about the half of the volatile fractions) were two compounds, whose molecular masses were 160 and 162. This suggests that they may be monoterpenes, containing an aromatic ring (intensive molecular ion peaks and *m/z* 77 and *m/z* 51 peaks). Weak peak for the elimination of oxygen atoms from the molecular ion peaks is an indication for the presence of one of the oxygen atoms as epoxy group. Elimination of the CHO fragments from the two compounds is an indication for the phenolic hydroxyl group, while the elimination of 42 mass units from the molecular ion peak at *m/z* 162 is an indication for the presence of conjugated carbonyl group. Eliminations of methyl groups are a further indication that both the discussed compounds are terpenoids. Such compounds have not been found in intact *Lavandula* sp. so far, and therefore we were not able to identify them only by mass spectra.

Furthermore, three unidentified chlorine-containing compounds were found in low amounts in the culture medium from two-phase cultivation and one was found adsorbed on the resin (Table 1). Representatives of the same group of compounds were found earlier in cell suspension cultures of *R. damascena* [4].

3.2 Volatiles from cell suspension culture of *N. tabacum*

The results from GC-MS analysis of the *N. tabacum* biomass, culture medium, and Amberlite XAD-4 resin from different culture conditions (Table 2) showed that the main constituents of the volatiles fraction were also hydrocarbons, but their hydrocarbon pool was presented only from saturated straight chains with 14–30 atoms. The composition and the amounts of the hydrocarbons biosynthesized were also influenced by the culture conditions. Some of them (tetradecane, pentadecane, octacosane, nonacosane, and hentriacontane) were detected only at the second phase (Amberlite XAD-4).

From the free fatty acids pool, hexadecanoic and linoleic acids were the main ones (Table 2) detected. Hexadecanoic acid is a common saturated fatty acid, which was only found in the *Nicotiana* biomass samples from two-phase cultivation mode. Linoleic acid, also found in relatively high amounts, was present only in the *Nicotiana* biomass from cultivation as a free suspension, indicating that the cultivation mode strongly influences the cell's metabolism.

The esters produced from *N. tabacum* cell suspension culture were mainly those of fatty acids (Table 2) and the highest amounts were achieved during two-phase cultivation. The main ester appeared to be decyl oleate, widely used in many cosmetic products due to its emollient properties [20]. Among the other compounds produced by *Nicotiana* cells, 1-hexadecanol was found mainly in the biomass from the two-phase cultivation mode (Table 2). Ratajczak *et al.* [21] recently found that hexadecanol competitively displaces cholesterol from the membrane lipids.

Note that benzoic acid, a compound with antibacterial and antifungal activities, was detected in traces in the second phase (Table 2). This compound which plays defensive functions in the plant cells was also detected on the second phase during the cultivation of *L. vera* cell suspension culture (Table 1) and earlier during the cultivation of *R. damascena* cell suspension culture [4]. Probably because of its biological activities, mentioned above, benzoic acid has a negative effect on cells and is secreted in the culture medium only in small amounts, needed for fulfillment of its defensive function and it could be detected only when adsorbed on the second phase.

3.3 Volatiles from cell suspension culture of *H. annuus*

The results from the GC-MS survey of the *H. annuus* cell suspension culture, grown under different modes showed that again the main groups of volatile compounds identified were hydrocarbons and fatty acids (Table 3). The hydrocarbon fraction was presented by compounds with saturated straight chains with 14–31 atoms and its content was almost identical with that of *N. tabacum* cell suspension culture. As it has been observed for the other two cultures under study, the hydrocarbons content was strongly influenced from the cultivation conditions as the main amounts of them were found in the biomasses from free suspension and bioreactor cultivations as well as in the culture medium from the bioreactor culture (Table 3).

In opposite of the *L. vera* and *N. tabacum* cell cultures, the cell suspensions from sunflower produced higher amounts of fatty acids (Table 3). Among the saturated fatty acids (8–16 carbon chain), one monounsaturated (18:1) and a polyunsaturated linoleic acid (18-carbon chain) were detected. Linoleic acid, which is the main fatty acid produced by *Helianthus* cells (Table 3), belongs to the essential fatty acids group and is essential for the dietary requirement of humans [22].

The esters were detected in relatively high amounts only in the biomass of *Helianthus* and some of them (ethyl and methyl esters of glycine-*N*-phenyl) adsorbed on the second phase (Table 3). Benzothiazole, a compound with antitumor properties [23] was detected in the culture media from two-phase and bioreactor cultivation modes. No amount of benzoic acid was detected, however two of its derivates (ethylbenzoic acid and vinylbenzoic acid) were found also adsorbed on the second phase. Aniline, an aromatic amine, was detected in all three cultures under study (at relatively high amounts in *N. tabacum* and *H. annuus* cell suspension cultures). However, no information is available in the literature on the functions of this compound in plant cells.

The results presented showed that the volatiles, produced by all the three plant cell suspensions under study, their amounts and distribution were changed, depending on cultivation conditions used (Tables 1–3). Placing the plant cells in bioreactor imposed another environmental situation (caused by agitation and aeration of the cell suspension) very different from that when the culture was cultivated in flasks on a shaker (*e.g.* different engineering parameters, in particular oxygen transfer efficiency, fluid flow conditions, power input and thus shear stress would result in different cell growth and product formation). For example, the hydrocarbons were found mainly in the biomasses from cultivation in shake flasks but those from bioreactor cultivation were distributed between biomass and culture medium and in the case of *L. vera* and *N. tabacum* cell suspension cultures they were distributed mainly in culture medium. The two-phase cultivation (in the presence of Amberlite XAD-4 as a second phase) changed even more radically the metabolic profiles of volatiles, compared with the regimes of cultivations as free suspension in flasks and in bioreactor. In this case, the volatiles were distributed between all phases (biomass, culture medium, and second phase). In the cell suspension cultures, cultivated as free suspension (in flasks or in bioreactor) the volatiles were found distributed between biomass and culture medium. Some of them, which possess defensive functions, usually had negative effect on the cells and were secreted in small amounts in the culture medium. Because of that they could not be identified or were found in very low amounts. When the cell suspensions were cultivated in the presence of second phase, these compounds were accumulated on the resin and could be identified and because of that in the group of volatiles new compounds appeared.

3.4 PC analysis

PC analysis is an unsupervised clustering method, which is a powerful tool for analysis of multivariate data, without requiring any knowledge of the dataset [24]. For the dataset, 12 common hydrocarbons for all *in vitro* cultures under study were used (tetradecane, octadecane, nonadecane, eicosane, heneicosane, docosane, tetracosane, pentacosane, hexacosane, heptacosane, octacosane, and nonacosane). For better comparison, the relative amounts (as described in Section 2) of the selected hydrocarbons were used. Both correlation and covariance methods were applied; however, better separation was reached using covariance method. An advantage of the covariance method is that the eigenvectors retain the scale of the original data [25]. A good separation between samples in score plots was achieved by combining the first principal component (PC1) with the second principal component (PC2), which cumulatively accounted for 68.1% of the variance. As evident in Fig. 1 the combination of

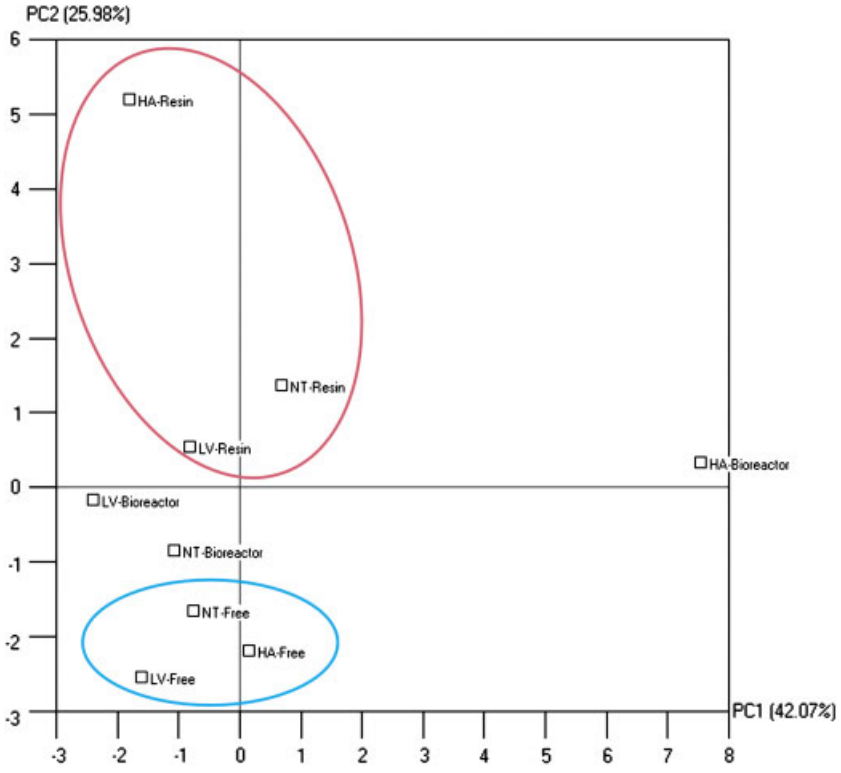

Figure 1. Score plot of PC analysis of the hydrocarbons (tetradecane, octadecane, nonadecane, eicosane, heneicosane, docosane, tetracosane, pentacosane, hexacosane, heptacosane, octacosane, and nonacosane) of *Lavandula vera* (LV), *Nicotiana tabacum* (NT), and *Helianthus annuus* (HA) cell suspensions, cultivated in shake-flasks (Free), two-phase systems (Resin) and in stirred tank reactors (Bioreactor). The ovals are used merely with the aim to draw attention to the classes without any statistical meaning.

PC1 and PC2 allowed a clearly separate trajectory between all the three suspension cultures under study, cultivated in shake flasks as free suspensions (down side) and in two-phase systems (upper side). This is probably indicating that the cultivation regime is having common influence on the production of hydrocarbons from plant *in vitro* cultures from three different families. Concerning the bioreactors cultivation the closeness between cell suspensions of *L.vera* and *N. tabacum* was distinguishable, while the cell suspension of *H. annuus* was distant. This is due to the cultivation in stirred tank reactors, where the environment created influences the cell metabolism and therefore different responses appeared. In fact, these differences impose the necessity of the optimization of bioreactor culture environment for each *in vitro* culture [26]. The closeness between shake-flasks cultivation and bioreactor cultivation (except for *H.annuus* cell suspension culture) should also be noted, which further reveals the differences between cultivation as free suspensions (shake-flasks or stirred tank reactors) and cultivation in two-phase systems (in the presence of Amberlite XAD-4 resin).

Conflict of interest

The authors have declared no conflict of interest.

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