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Studies on the influence of fertilization on the quality of *Rozmarinus offficinalis* L. essential oil for the purpose of superior capitalization

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Abstract

In this paper, we studied the physiological and biochemical behavior of rosemary under the influence of differentiated fertilizations. The plants were grown in vegetation pots with the help of different variants of the Hoagland nutrient solution, namely: control variant (Vo - Hoagland), variant 1 - Hoagland with addition of K X 2 and variant 2 - Hoagland with addition of P X 2. During the experiment we carried out the measuring of the intensity of photosynthesis by gas exchange method and the quantitative determination of chlorophyll pigments. From a biochemical point of view, we performed the extraction and analysis of volatile compounds. The main compounds identified on the basis of gas chromatographic and mass spectrophotometry analyzes were monoterpenoids and in lower concentrations sesquiterpenoids. The researches were carried out in the Laboratory of Plant Physiology at the Faculty of Horticulture and Forestry, as well as in the Laboratory of Plant Extracts at the Faculty of Food Engineering. The experiment revealed that K fertilization had a positive influence on both the amount of dry matter and photosynthesis. Regarding the biochemical analysis of the volatile oil, analyzed in the case of variant V1, with positive results in the case of physiological determinations, over 16 compounds were identified, and the best represented were: terpinen and cymen.

Keyword: fertilization, bioactive compounds, chlorophyll content, photosynthetic intensity

1. Introduction

Rosemary (*Rosmarinus officinalis* Linn.) is a common houseplant, cultivated in many parts of the world. It is used for flavoring food, beverages, and for certain cosmetics. In folk medicine, it is used as an antispasmodic in renal colic and dysmenorrhea, in relieving respiratory disorders and stimulating hair growth. The rosemary extract relaxes the smooth muscles of the trachea and intestine, and has a choleretic, hepatoprotective and anti-tumorigenic effect. The most important components of rosemary are caffeic acid and its derivatives, such as rosmarinic acid, compounds with antioxidant effect. The rosmarinic acid is well absorbed from the gastrointestinal tract and through the skin. It influences the increase of prostaglandin E2 production and reduces the production of B4 leukotrienes from human polymorphonuclear leukocytes, and inhibits the complement system.

Rosemary acid derivatives and its constituent elements, especially caffeic acid, have a therapeutic potential in the treatment or prevention of asthma, spasmogenic disorders, peptic ulcer, inflammatory diseases, hepatotoxicity, athe[1]; rosclerosis, ischemic heart disease, cataracts, cancer and sperm motility (AL-SEREITIA M.R., ABU-AMERB K.M., SENA P., 1999[1]; KOSAKA and YOKOI, 2003[2]; Petersen and Simmonds, 2003[3]; BOZIN et al., 2007[4]). Various nutrition preparations and diets have been made based on rosemary in various treatments (BOTSOGLOU et al., 2007[5]; MOÑINO et al., 2008[6]; NIETO et al., 2010[7]).

Due to the importance of the active principles contained in rosemary, (Wellwood and Cole, 2004[8]) developed methods to identify and select accessions of rosemary, *Rosmarinus officinali*s (L.), producing optimum antioxidant activity.

NABAVI et al.,2015 [9] considers that rosmarinic acid is one of the most important and well known natural antioxidant compounds, which possesses neuroprotective effects in different models of neuroinflammation, neurodegeneration, as well as chemicalinduced neurotoxicity and oxidative stress.

Due to the importance of rosemary compounds, some research has studied the enzymatic control of rosmarinic acid biosynthesis (PETERSEN et al., 2009[10]).

Some studies have highlighted the influence of vegetation and nutrition factors on the content of active ingredients (PEÑUELAS and LLUSIÀ, 1997[11]), and others have studied the seasonal variation of rosemary active compounds (LUIS and JOHNSON, 2005[12]). HIDALGO et al.,1998, [13] observed an excellent correlation (r = 0.93) between the carnosic acid concentration and photoperiod in *Rosmarinus officinalis*. The results presented can be used to improve the selection of raw materials for the extraction of carnosic acid from rosemary.

Given the importance of rosemary through bioactive compounds and the essential oils it contains, the present study looked at how differentiated fertilization with P and K-added nutrient solutions influences the main physiological processes as well as the quantitative and qualitative analysis of rosemary volatile oil.

The oil is extracted by steam distillation of the leaves and flowers. The oil can be used to treat pain from arthritis, asthma, bronchitis, mental fatigue, memory loss and muscle pain. It can be beneficial for skin conditions, dandruff, candida and supports the proper functioning of the immune system and is also a carminative. The main constituents of the oil are alpha-pinene and beta-pinene, camphene, camphor, sabinene, limonene, beta-caryophyllene), phenolic acids (caffeic, gentisic, vanillic, syringic), caffeic acid derivatives (rosmarinic acid), diterpenes (rosmanol, carnosol, carnosic acid), triterpenes (alpha-amirenol and beta-amirenol, ursolic acid, oleanolic acid), flavonoids (diosmin, diosmetin, hesperidin), tannins, waxes, etc. Rosemary oil and its main constituents, ether 1 and cineole-8, act as a good remedy against exhaustion. The locomotor activity of the tested animals was significantly increased by inhaling this oil.

Another common name for rosemary is garden rosemary. The parts used are the leaves and flowers, and the medicinal properties: stimulant, antispasmodic, emenagogue, astringent, diaphoretic, carminative, nervine, aromatic, cephalic.

Rosemary is a plant of Mediterranean origin, and the limiting ecological factor is temperature, rosemary requiring a mild climate, without large temperature variations and not tolerating wintering except in particularly favorable conditions, without temperatures below -2 °… -3°C, protected of snow and a layer of mulch. It requires direct light. It does not have special demands on humidity, due to the thick hairs on the back of the leaf, and the mature plant tolerates relatively well the dry periods, as well. It requires deep, calcium-rich, light soils that are easily heated and permeable, with southwest exposure.

2. Material and method

Rosmarinus officinalis plants were grown in vegetation pots, under controlled conditions of temperature, light and humidity. The nutrient substrate was composed of a mixture of perlite and peat in a ratio of 1: 3. The plants were fed with the help of a Hoagland nutrient solution in the following variants:

Table 1. Nutritive solution Hoagland – control variant (Vo)

Components	**Supply solutions**	**Ml Supply solutions/1L**		
2M KN_3	202g/L	2.5	5	2.5
2M $Ca(NO_3)_2$ x $4H_2O$	236g/0.5L	2.5	2.5	2.5
Fier	15 g/L	1.5	1.5	1.5
2M $MgSO_4$ x $7H_2O$	493 g/L	1	1	1
1M NH_4NO_3	80 g/L	1	1	1
Microelements				
H_3BO_3	2.86 g/L	0.5	0.5	0.5
$MnCl_2$ x $4H_2O$	1.81 g/L	0.5	0.5	0.5
$ZnSO_4$ x $7H_2O$	0.22 g/L	0.5	0.5	0.5
$CuSO_4$	0.051 g/L	0.5	0.5	0.5
H_3MoO_4 x H_2O	0.09 g/L	0.5	0.5	0.5
1M KH_2PO_4	136 g/L	0.5	0.5	1

Research Methods of physiological processes

Determination of the amount of accumulated dry matter – Fragments of leaves were used as plant material, using the Kern MLS 50 thermobalance

Determination of perspiration intensity – by the ratio obtained after the successive weighing of some organs of the plant and the relation to their fresh mass

Quantitative determination of chlorophyll pigments – using the chlorofimeter SPAD 502 Konika Minolta

Measurement of photosynthesis intensity by gas exchange method – with the help of the CO_2 ANALYSIS PACKAGE, Qubit Systems (Canada), which measures the change in CO2 concentration following the photosynthesis process

Analysis Method of volatile oils

For the study of the composition of volatile oils of mint, rosemary and lavender, hexane was used for sample dilution (GC purity, Sigma), and for the determination of Kovats indices for volatile compounds in oils, a standard solution of linear alkanes C_8-C_{20}, obtained from Fluka Chemie AG, was used.

GC-MS analysis. A chromatographic gas analysis system coupled with a mass spectrometry detection system was used to analyze the volatile oils of mint, rosemary and lavender. We used a GC Hewlett Packard HP 6890 Series coupled with a mass spectrometre Hewlett Packard 5973 Mass Selective Detector. The GC analysis conditions were:

- column: HP-5 MS, length 30 m, inner diameter 0.25 mm, film width 0.25 μm;
- temperature program: 50°C - 250°C with a speed of 6°C/min;
- injector temperature: 280°C;
- detector temperature: 280°C;
- injection volume: 2 μl;
- carrier gas: He.

For the MS detector we worked with an EI energy of 70eV, at a source temperature of 150°C, scanning range 50-300 amu, scanning speed of 1 s^{-1} for mass spectrometry, and the obtained spectra were compared with the database NIST/EPA/NIH Mass Spectral Library 2.0 (2002). The data acquisition was done with the help of the software package Hewlett Packard Enhanced ChemStation G1701BA ver. B.01.00/1998, and the processing of gas chromatography and mass spectrometry data was performed using the program Hewlett Packard Enhanced Data Analysis from the software package above.

ORIGINAL PAPER

Table 2. Kovats retention index (KI) and retention time (RT) values for linear alkanes C_8-C_{20}

Nr.	Linear alkane	KI	RT (min)	Nr.	Linear alkane	KI	RT (min)
1	octane, C_8	800	3.07	8	pentadecane, C_{15}	1500	17.39
2	nonane, C_9	900	4.37	9	hexadecane, C_{16}	1600	19.39
3	decane, C_{10}	1000	6.23	10	heptadecane, C_{17}	1700	21.3
4	undecane, C_{11}	1100	8.44	11	octadecane, C_{18}	1800	23.11
5	dodecane, C_{12}	1200	10.76	12	nonadecane, C_{19}	1900	24.83
6	tridecane, C_{13}	1300	13.06	13	eicosane, C_{20}	2000	26.48
7	tetradecane, C_{14}	1400	15.28				

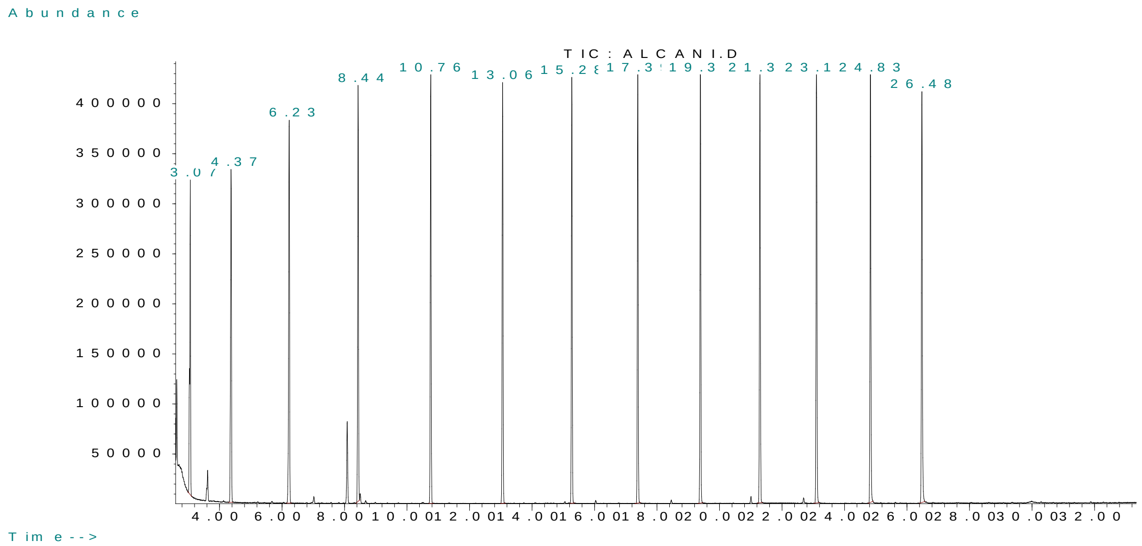

Figure 1. Gas chromatogram for the standard mixture of linear alkanes C_8-C_{20} used to determine Kovats retention indices

Determination of Kovats retention indices (KI). The identification of the compounds following the GC-MS analysis was performed, in parallel with the identification based on the MS spectra, based on the Kovats retention indices (KI), too, for the cases where retention index data were available for the corresponding standards. Important mono- and sesquiterpenoids could be identified in this way. In addition to the possibility of identifying compounds, for many of the components of the analyzed samples, which could be clearly identified based on the MS spectra, the corresponding Kovats indices were determined for the GC column used (HP-5 MS).

The values of these indices depend only on the type of column used. The temperature program, injector and detector temperatures or carrier gas flow have no influence on this parameter.

To determine the Kovats retention indices, we proceeded as follows: a sample of 2 μl of a mixture of standard solution of linear alkanes C_8-C_{20} in chromatograph gas was injected into the same chromatographic column and the same conditions of analysis as in the case of basil volatile oil. After confirmation (based on MS spectra) of the linear alkane structures separated by GC analysis (Figure 1), we determined the retention time (RT) for each component of the mixture. The correlation of the conventional Kovats retention indices (Table 4) with the retention times obtained from GC analysis leads to a polynomial calibration curve that was used to determine Kovats index values for the compounds studied by interpolating the corresponding retention times (determined from GC analysis) on the KI *vs* RT chart.

3. Results and discussions

Regarding the experimental results obtained regarding the total chlorophyll content, we may observe that there are differences between the experimental fertilization variants as follows:

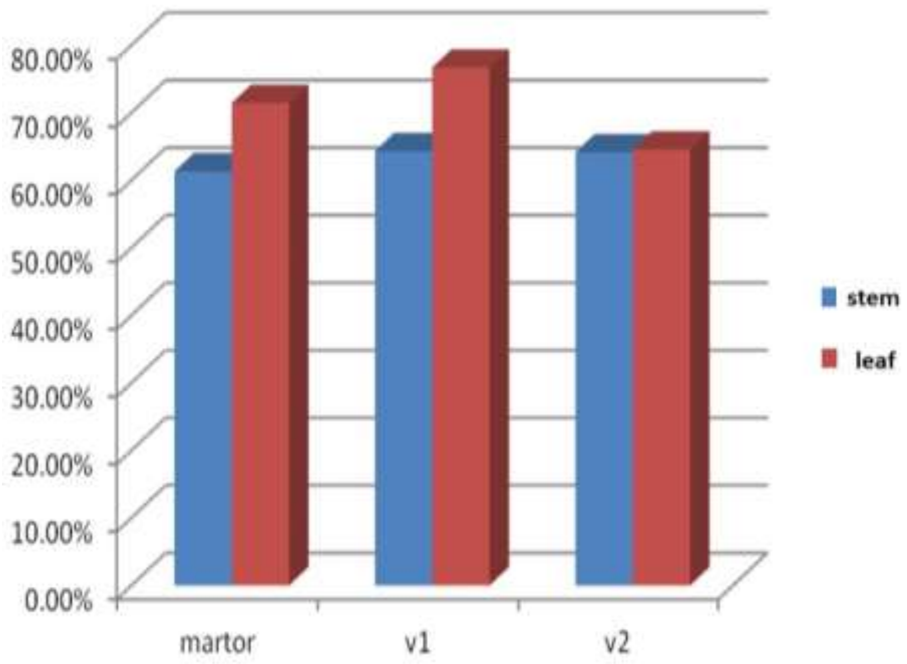

Figure 2. Dry matter percentage - rosemary

Regarding the experimental results obtained, we may notice that a high percentage of dry matter is recorded in the case of samples from the leaf compared to those from the strain. It can also be observed in the leaf fragments that there are significant differences between the studied experimental variants, higher values being registered in variant V1, followed by the control variant and V2.

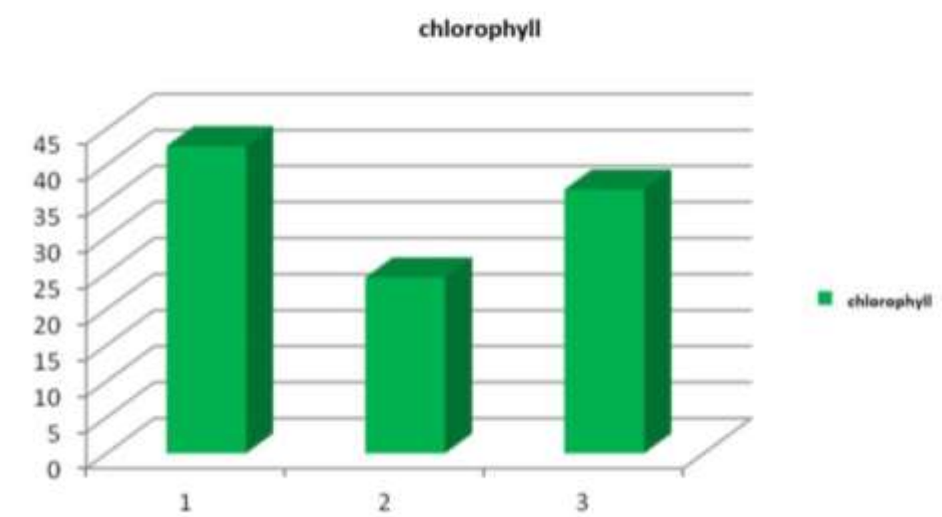

Figure 3. Determination of total chlorophyll content (SPAD)

Regarding the total chlorophyll content expressed in SPAD units, it can be seen that a higher value was found in the control variant (45 SPAD), followed by variant V3, while a lower value was recorded in variant V2 (27.5 SPAD).

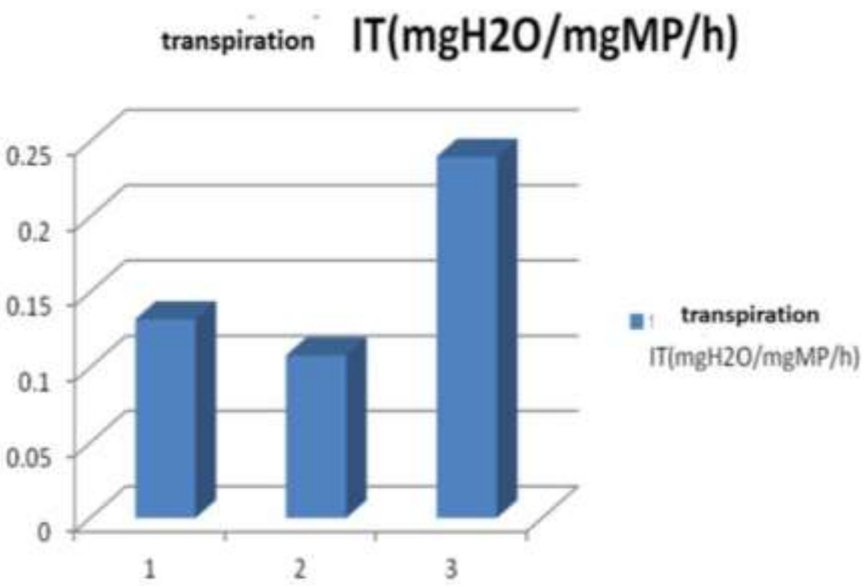

Figure 4. Intensity of perspiration in the case of the studied experimental variants

Another physiological process studied was transpiration expressed by the amount of water removed relative to the fresh mass of plant material per unit time (sweat rate). In this case it can be seen that there are differences between the studied variants, so a higher rate of perspiration was observed in the case of variant V3 compared to the control variant and V2, which had a lower value.

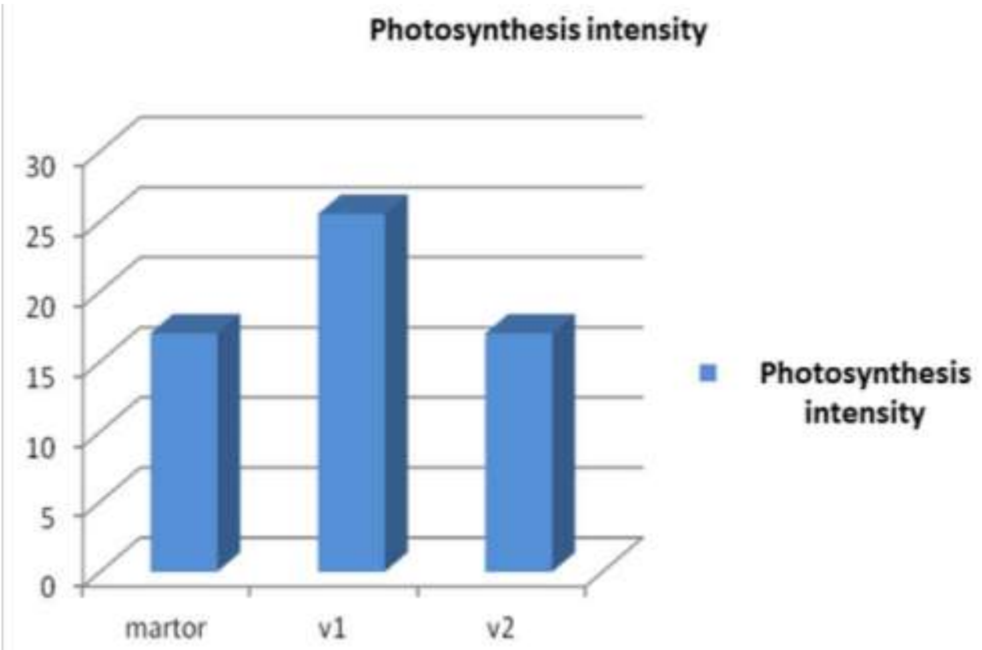

Figure 5. Results on the determining of photosynthesis intensity

In the case of determinations made in order to establish the photosynthetic intensity by the gas exchange method (CO_2 uptake and O_2 release), we observed the existence of differences between the three variants studied, so a higher intensity was recorded in the case of variant V1 (added with phosphorus), followed by variant V2 (added with K) and control variant.

Analysis of volatile rosemary oil

The main compounds identified and quantified in rosemary volatile oil based on gas chromatographic analyzes coupled with mass spectrometry, GC-MS, were monoterpenoids and, to a lesser extent, sesquiterpenoids.

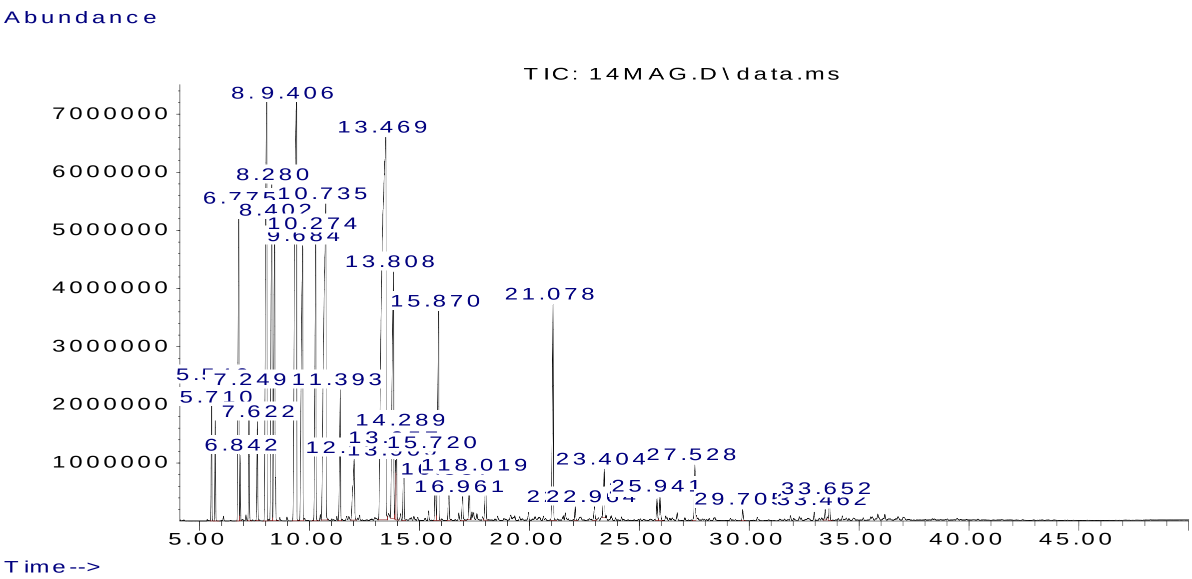

Figure 6. Gas chromatogram from GC-MS analysis of rosemary volatile oil samples

Table 6. Results of GC-MS analyzes (identification of MS and relative percentage concentrations) for rosemary volatile oil

Nr.	RT (min)	Kovats Index	Identification MS	Area (rosemary) (%)
1	5.71	936	α-Pinene	0.900
2	6.775	976	β-Phellandrene	3.732
3	6.842	979	β-Pinene	0.465
4	7.249	993	β-Myrcene	1.236
5	7.622	1006	α-Phellandrene	0.981
6	8.05	1021	α-Terpinen	7.839
7	8.28	1028	*p*-Cymene	4.600
8	8.402	1032	Limonene	4.225
9	9.406	1064	γ-Terpinen	11.025
10	9.684	1073	Terpineol	5.339
11	10.274	1091	Terpinolen	3.698
12	10.735	1105	Terpineol, cis-beta-	8.932
13	13.469	1187	Terpinen-4-ol	19.276
14	13.808	1197	α-Terpineol	4.811
15	15.87	1259	Linalyl acetate	2.962
16	21.078	1421	β-Caryophyllene	2.987
			Other compounds	*16.99*

Of the monoterpenoids, the most concentrated was terpinen-4-ol (19%), γ-terpinene (11%), cis-β-terpineol (9%) şi α-terpinene (8%), followed by other mono- and sesquiterpenoides in concentrations below 6% (β-phellandrene – 3.7%, limonene – 4.2%, terpineol – 5.3%, terpinolene – 3.7%, α-terpineol – 4.8%, linalyl acetate – 3% and β-caryphyllene – 3%). The experimental mass spectra and from the NIST database are shown in the figures 6. This is also confirmed by the literature. PRABODH SATYAL et al.,2017[14], in a similar experiment, identified that oils were dominated by (+)-α-pinene (13.5%–37.7%), 1,8-cineole (16.1%–29.3%), (+)-verbenone (0.8%–16.9%), (−)-borneol (2.1%–6.9%), (−)-camphor (0.7%–7.0%), and racemic limonene (1.6%–4.4%). (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5368539/)

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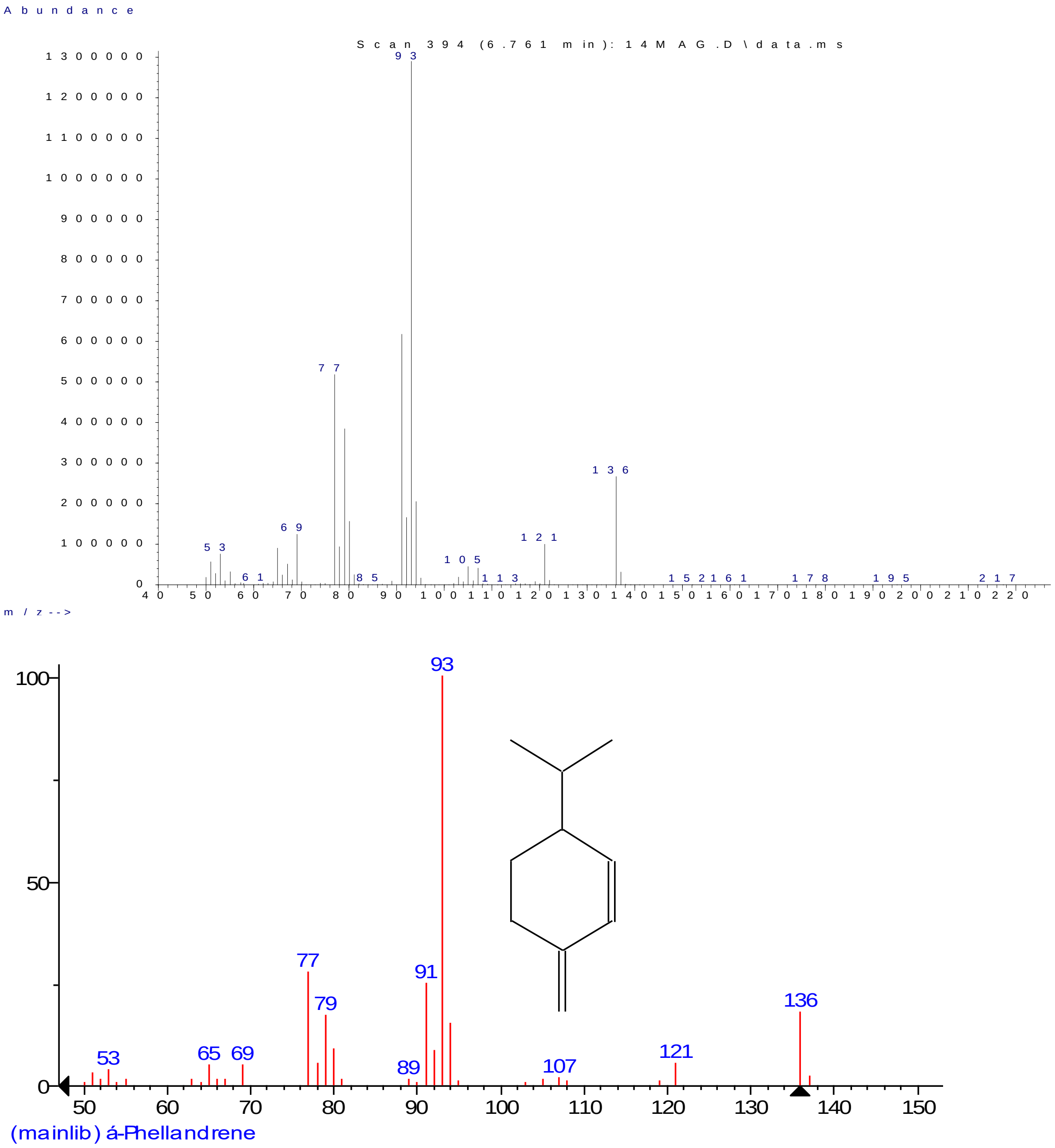

Figure 7. Experimental mass spectra (top) and NIST database (bottom) for β-phellandren

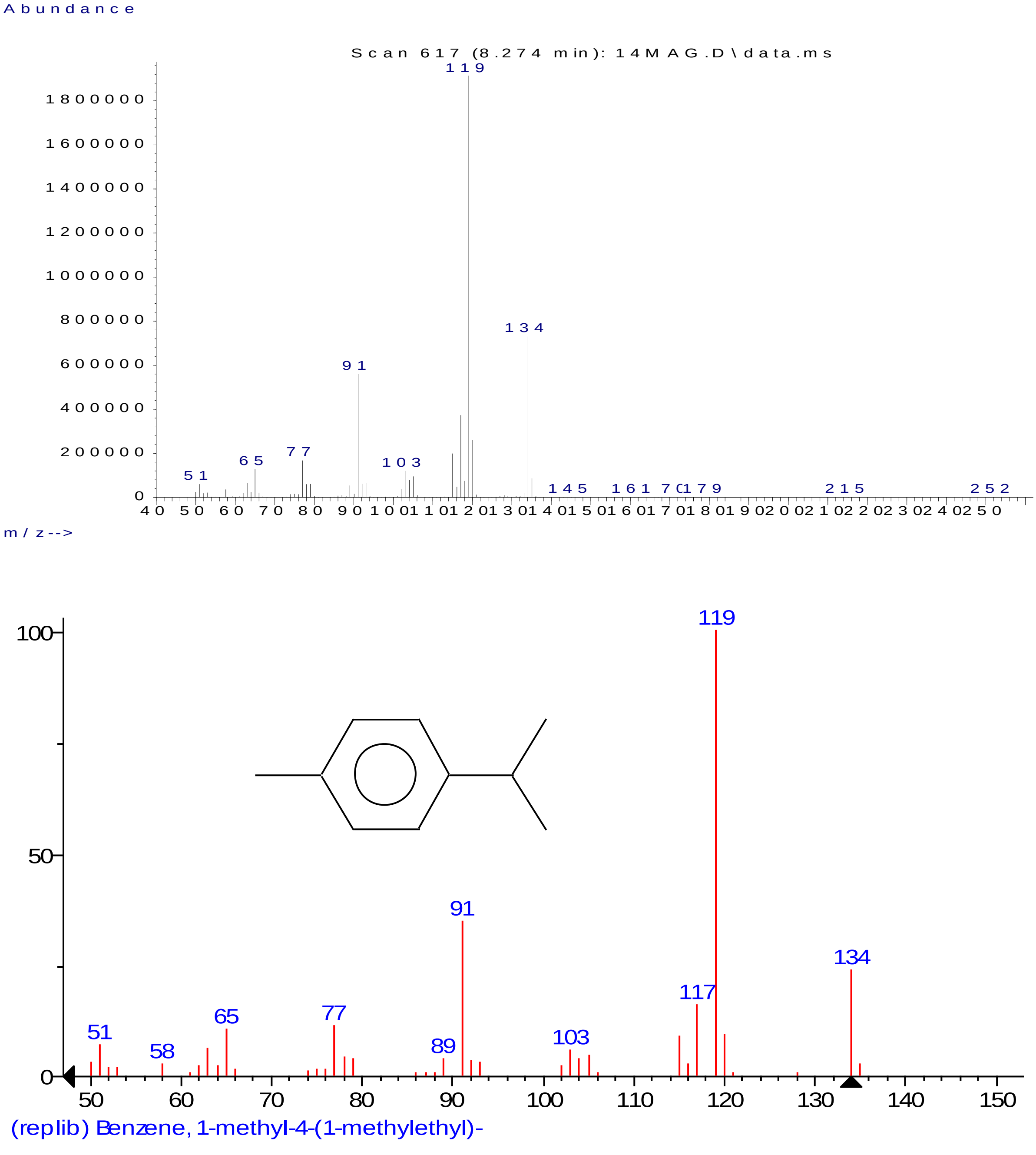

Figure 8. Experimental mass spectra (top) and from the NIST database (bottom) for *p*-cymene

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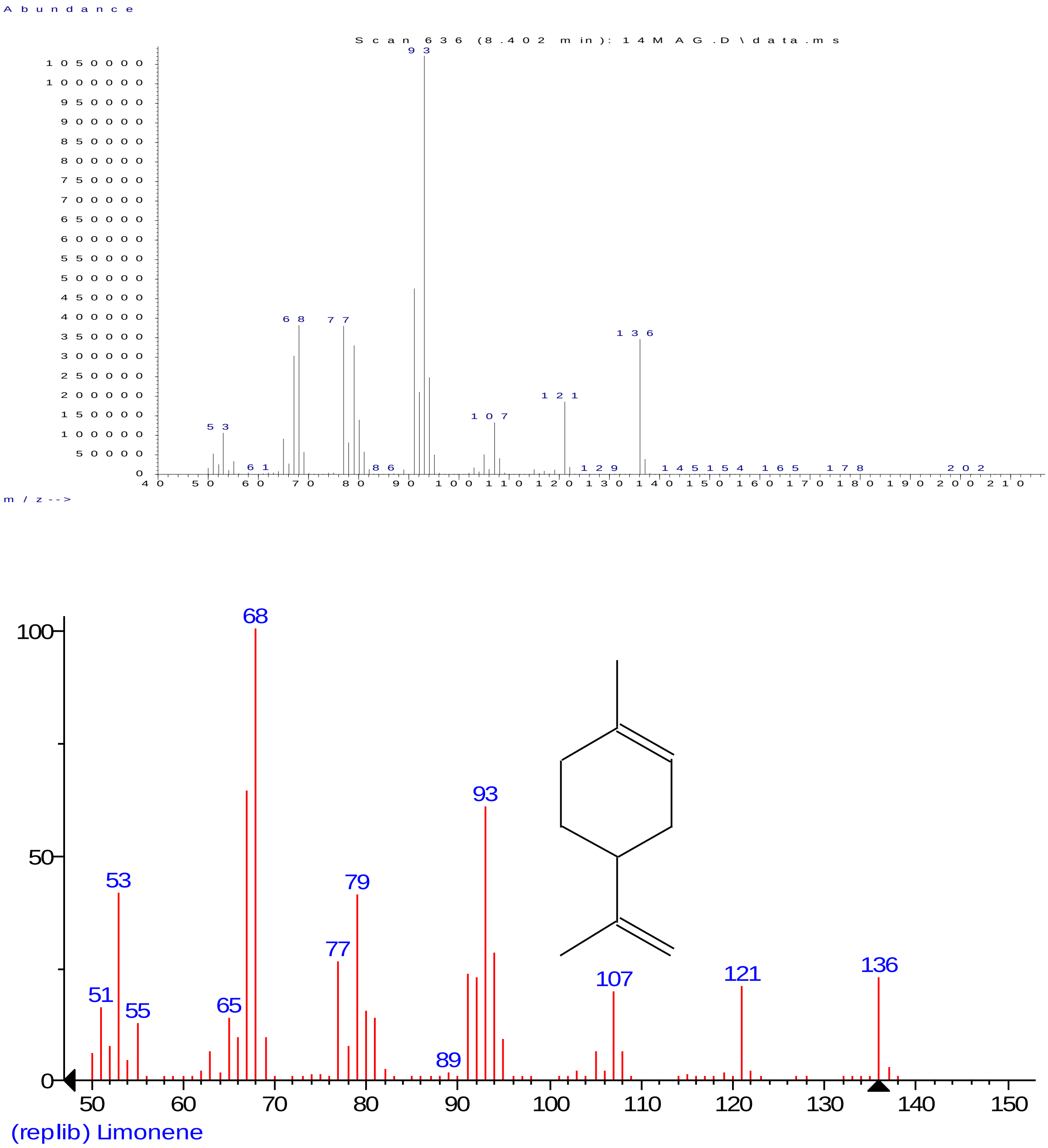

Figure 9. Experimental mass spectra (top) and from the NIST database (bottom) for limonene

C. BOUTEKEDJIRET et all (2003) [15]), in an experiment in which he analyzed volatile oil from 5 samples from different areas of Algeria, found that the main compounds identified were pinene and camphene. Also Iram Ayoob1 et al. conclude, analyzing samples of rosemary from Kashmir province, India, that the essential oil analysis of *R. officinalis* lead to the identification of 38 constituents. The essential oil was dominated by α-pinene (16.33), 1, 8-cineole (14.33), camphor (22.01), camphene (9.28), β-pinene (5.97), β-phellandrene (5.19), bornyl acetate (4.59), myrcene (4.31) and borneol (3.35).

The differentiated distribution of the active principles (phenolic diterpenes, flavonoids and rosmarinic acid) during the vegetative cycle in different parts of the plant (stems, leaves, flowers) was highlighted (DEL BAÑO et al., 2003[16]). The nutritional factors have played a role in directing rosemary production and nutritional principles. MORETTI et al.,1998 [17 studied the influence of iron administered foliar to rosemary under irrigated and non-irrigated cultivation conditions and found a significant increase in the concentration of verbenone in the oil of irrigated plants. This was attributed to more intense oxidative processes under the influence of iron, which converted α-pinene into verbenyl derivatives. Higher concentrations of verbenone in *R. officinalis* oil after iron-based treatment increase its value for the perfume industry. DEEF, 2007[18] highlighted the influence of copper on plant growth and the content of minerals, carbohydrates and essential oils of rosemary in protected conditions. The obtained results revealed that the applied copper treatments determined a significant increase of the content of essential oils and the increase of the concentration of verbenone and 1,8-cineole in oil, at the same time with the decrease of α-pinene content. The influence of copper on physiological parameters (growth, leaf relative water content - LRWC, cell membrane permeability) and on some processes and compounds in rosemary plants (lipid peroxidation, and total phenolic content - TPC) were studied by HEJAZI-MEHRIZI et al.,2012[19] in a hydroponic condition.

4. Conclusions

From a physiological point of view, in terms of dry matter percentage, we may observe that the highest percentage was registered in the case of samples from leaves, in the experimental variant V1. In the case of determination of total chlorophyll content, the highest value was found in the case of the control variant. The intensity of perspiration was found to have the highest rate in the case of variant V3. The intensity of photosynthesis was found to be higher in the case of variant V1.

In terms of biochemical characterization, the volatile oils analyzed showed a significant number of volatile compounds (over 16 volatile compounds identified), most belonging to the class of monoterpenoids and sesquiterpenoids. Rosemary volatile oil had a more uniform distribution of the constituent compounds, the most concentrated being the tertiary alcohol terpinen-4-ol (19%) and monoterpene γ-terpinen which is most likely one of the main sources in the biosynthesis process of terpinene-4-ol.

Following the studies carried out, it is recommended the differentiated application of fertilizers with phosphorus and potassium in order to make a superior use of rosemary essential oil on the market of profile products.

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