Bio-morphological and Chemical Characterization of Rosemary (*Rosmarinus officinalis* L.) Biotypes

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Abstract

The use of herbs in the food industry has recently increased significantly because of their antioxidant action on lipid degradation, besides their traditional role in food aroma. In particular, rosemary (*Rosmarinus officinalis* L.) extracts have proved to possess such useful antioxidant properties. The present research that reports on a study on the morphological and chemical characteristics, conducted on rosemary clones obtained from wild material collected in different environments of southern Italy, was aimed at better characterizing and evaluating their potential for present and possible new food technological uses. In addition to morphological characterizations aimed to detect differences among clones, a technological qualitative oil component "fingerprint" was conducted by means of GC-MS, comparing two different extraction techniques of the essential oil fraction (traditional steam distillation and dichloromethane solvent extraction) for the quantitative and qualitative differences.

INTRODUCTION

The genus *Rosmarinus* Linnaeus is by now represented by a single species, *Rosmarinus officinalis* L. (rosemary). However, the systematics of varieties, subspecies and biotypes appears uncertain and confused, as there are numerous cultivars that grow wild in the Mediterranean countries (Giugnolinini, 1985). Even though there are subspecies and varieties of *R. officinalis* L., some infraspecific differences are likely to occur within the species, in that morphologically identical plants may have a different essential oil composition. *R. officinalis* L., traditionally used fresh, dried, or as essential oil, actually contains a great quantity of essential oil (more than 1 %) that is largely used in medicine.

The chemical composition of rosemary oil has been the subject of an extensive study, reviewed by Lawrence (1976–1977,1979–1980,1981–1987,1988–1991,1992–1994, 1995). Two major types of rosemary oil can be distinguished with respect to their main constituents: oils with over 40 % of 1,8-cineole (oils from Morocco, Tunisia, Turkey, Greece, Yugoslavia, Italy, France) and oils with approximately equal ratios (20–30 %) of 1,8-cineole, α-pinene and camphor (oils from France, Spain, Italy, Greece, Bulgaria) (Mastelic, 1997, Tomei et al., 1995). The literature also revealed some unusual chemical compositions for rosemary oils. The more recent interest in this species concerns the biological (antioxidant, antimicrobial and insecticidal) action of specific components of the essential oil of rosemary.

The objective of this research was to characterise the morphological and chemical traits of some rosemary clones, obtained from wild material collected in different environments of southern Italy, to better assess their potentials for present and possible new food technological uses.

Recently, essential oils deriving from medicinal plants such as *Rosmarinus* gained importance as to their quantitative and qualitative composition (Adams, 1995.). In addition to this, oil composition differences proved to be helpful for integrated ecotype-chemiotype characterization approaches, helpful for practical issues such as definition of

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optimum harvesting time or the evaluation of field-plant interactions (European Union Commission 2000, Falchi Delitali et al., 1980).

This research is aimed to characterise the morphological and chemical traits of some clones obtained from wild material collected in different environments of southern Italy, with a view to better assess their potential for present and possible new food technological uses.

MATERIALS AND METHODS

Plant Material

Seven biotypes belonging to the genus *Rosmarinus*, selected from wild populations in different environments of southern Italy (Apulia, Basilicata and Calabria), that were collected since 1999.

Experimental Design

The collection of rosemary clones obtained from wild mother plants was maintained in a collection field at the Agricultural Faculty of Bari (Italy). The field had been fertilized with 120 kg ha^{-1} N, 100 kg ha^{-1} P_2O_5 and 100 K_2O kg ha^{-1}. The experimental design was a randomised complete-block design with 3 replications. The planting distance was 0.75 m between rows and 0.35 m within rows.

Harvest and Processing

Spring shoots used for the determination of morphological parameters were collected for each biotype from three-year-old plants, during the period of slow growth (15th July, 2002). Measurements were made on the total fresh weight, number and weight of early shoots; their weight, length, stem diameter and the number of internodes were measured on defoliated shoots; the number and total weight of leaves of detectable size (>0.5 mm length) occurring on the whole shoot and on side branches was determined. The length, width and surface area of 30 leaves per biotype were measured, using an image analysis software (Scion image program), after acquisition by the videocamera.
Keeping stems and leaves separate, samples were oven-dried at 38°C and re-weighed for the dry matter determination. Entire shoots, once oven-dried, were used for the distillation of essential oils, by dispersing 10 g of dry matter in 400 ml of water, by a 3-hour treatment at a temperature of 90°C.

Content of Essential Oil

For hydrodistillation extraction of oil, vegetal leaf material was air-dried on wooden benches away from direct sunlight for a fifteen days period. Dried leaves were then finely minced with an electric mill and placed in a 3-liter Clevenger-type assembly distillation apparatus. The plant material 150 g in 1500 ml of deionised H_2O; was distilled for 3 hours. The essential oil was collected about 1 h after heating and dehydrated with the addition of anhydrous sodium sulphate (Merck).

For the solvent extraction of oil**,** five grams of oven-dried (35°C for 72 hours) leaf-stem deriving from each single plant were submitted to solvent extraction in the plant material, 0.2 g was placed in 1.5 ml of dichloromethane for 15 min. in ultrasound bath. The obtained essential oil was collected in 2.5 ml borosilicate glass vials sealed by PTFE film, and stored at –18°C up to analysis.

Composition of Essential Oil

Essential oil samples were analyzed by means of gas chromatography mass spectrometry (GC-MS), by a Hewlett Packard 5973 mass selective detector connected with a 6890 Hewlett Packard gas chromatograph. Separation was achieved by a Hewlett Packard HP-5MS fused-silica capillary column (30 m x 0.2 mm I.D., film thickness 0.33 micron).The GC used helium as a carrier gas at a flow rate of 1 ml/min. The temperature ramp was from 50°C (5 min) to 270°C (20 min) at 4°C/min. The injection temperature

was 250°C, and a split ratio of 20:1 was used. The MS used a mass quadrupole detector temperature at 150°C and had an ionization voltage of 70 eV and a . ion source temperature of 230°C

Components were finally identified with the HP Enhanced ChemStation G1701BA Version B.00.00 (Hewlett Packard) by comparing their mass spectra with those of published data (John Wiley & Sons), and confirmed by their gas chromatography retention indices (4).

Statistics

Data were processed for ANOVA and mean separation was performed by Duncan's Multiple Range Test. The percent concentrations of the components in the different oils were used as matrix elements to perform the hierarchical cluster analysis. All PC analyses were carried out using SAS software (SAS Institute Inc., Cary, NC) procedures.

RESULTS AND DISCUSSION

Morphological Traits

Spring shoots show, within the tested biotypes, some variability in the length growth. In particular, the longest shoots were those of biotypes 4, 5 and 7 with values over 185 mm, the others were shorter, with values ranged between 115 and 127 mm (tab.1). The higher shoot length is function of a high number of nodes (5 and 7) or of a high length of internodes (4) (fig. 1). The higher number of nodes induces a relatively high number of leaves on the shoot (biotypes 5 and 7), whereas this character is not differentiated among the biotypes with the change in length of the internodes.

However, it is evident (fig. 2) that the more numerous the leaves, the smaller the leaf size. Based on the leaf size, three different types may be distinguished: biotypes with many (250-500) and small-sized leaves (<0.3 cm^2) (5 and 7); biotypes with a low number (100-200) of medium-sized leaves (0.3-0.5 cm^2) (2 and 6); biotypes with a low number (100-200) of big-sized leaves (>0.5 cm^2) (1, 3 and 4).

In terms of weight, the highest shoots were those of biotypes 7 (4.08 g) and 5 (3.62 g), which were also among the longest, followed by the 3 (3.47 g) that was shorter (fig. 3). These differences are easily attributable both to the leaf type and to the number of axillary shoots, which are correlated with each other. Actually, both type 1 and, secondarily type 3, are shown to be associated with a higher number of axillary shoots (fig. 4). Consequently, the ratio of the leaf weight to the total weight of shoot is higher in the less branched biotypes, belonging to the second type (2 and 6) (fig. 5). The mean dry matter content was 31.35 % with significant fluctuations between 30 and about 33 %.

No notable differences were observed in the stem diameter, which was, on average, 2.7 mm.

Content and Composition of Essential Oils

Analyses conducted for the characterization of the seven ecotypes under study revealed substantial differences in the content of essential oils. In particular, type 1 was characterised by a sharply higher yield (fig. 6), contrary to type 2, which showed the lowest yields. The above analyses revealed the "leaf" types, with medium-sized leaves, poorly branched and with a low essential oil content, as distinguished from the types for "distillation", with small-sized leaves, largely branched and with a high distillation yield, which compensates more than proportionally the lower leaf yield.

As to the quality of essential oil, the gas chromatography of rosemary accessions' essential oils identified 48 components with the predominance of α-pinene, camphor, 1,8 cineole, and verbenone.

Using hierarchical cluster analysis, four main groups of samples were observed (fig. 7). It is thus possible to distinguish clearly the "camphor" chemotype (tab. 2), in the group including biotypes 5 and 7 that are quite similar, since both are mainly constituted

by camphor (43.2 % on average) and 1,8–cineole (9.4 % on average), from the "α-pinene/verbenone" type, with biotypes 2 and 6 in which α-pinene (22.5 % on average) and verbenone (16.7 % on average) are prevailing.

The other groups are less defined: in the second, including only biotype 4, the prevailing components are α-pinene (>20 %), verbenone, 1,8 cineole and bornyl-acetate; in the fourth, with biotypes 1 and 3, α-pinene prevailed (>20 %), followed by verbenone, 1,8 cineole and camphor.

With regard to the different extraction methodologies, data obtained show homogeneous results, both in terms of biotypes considered as a whole and for single biotype identified, except for a single biotype and some compounds (tab. 3). It seems that solvent extraction, much more fast and less requiring in terms of raw material, can be a helpful tool for a preliminary characterisation of the materials under test. However, for a more accurate characterisation on a limited number of biotypes, it seems necessary to conduct a separate control hydrodistillation-based analysis to confirm preliminary screening results and to identify compounds not eventually discriminated by the first characterisation.

CONCLUSIONS

Results obtained showed a clear morphological and chemical characterisation of the seven rosemary biotypes under study. In particular three types of rosemary plants have been identified.

The first (biotypes 5 and 7) is characterised by long shoots with a high number of axillary shoots, small-sized leaves and a high yield of camphor-rich (>40%) essential oils.

The second type (biotypes 2 and 6), instead, exhibits medium-sized shoots and leaves, a low number of small-sized axillary shoots, a low essential oil yield with the predominance of α-pinene/verbenone among its constituents.

Lastly, the third type (biotypes 1, 3 and 4) is featured by a low number of large-sized leaves, a fair number of axillary shoots and quite small shoots in all biotypes except number 4, which showed the longest shoots, in absolute terms, due to the presence of somewhat elongated internodes. The relative essential oil yield was medium and ranged from 1.1 to 1.8, with the predominance of α-pinene (>20 %), verbenone, 1,8 cineole in all the three biotypes.

The most interesting types, under equal shoot weight and number of shoots per plant, are the first and the third, as they are characterised by high foliage that, in turn, is function of the leaf number and size. However, the best leaf weight/shoot weight ratio was observed in the biotypes belonging to the second type, on account of their lower tendency to issue side shoots.

However, these considerations cannot be kept separate from the occurrence of different chemotypes inducing different combinations, which may be more or less desirable according to the final intended use of the product.

Thus, a panel sensory assessment test and an evaluation of the biological activity of a wider range of wild rosemary populations could provide more accurate information on their potential for present and possible new food technological uses of the species.

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Tables

Table 1. Bio-morphological and chemical characters of 7 rosemary biotypes.

Characters	**Type 1 (biotypes 5-7)**			**Type 2 (biotypes 1-3-4)**			**Type 3 (biotypes 2-6)**		
	Range		Mean ± E.S.	Range		Mean ± E.S.	Range		Mean ± E.S.
	Max	Min		Max	Min		Max	Min	
Shoot length (mm)	208.00	163.00	186.17 ± 7.83	145.00	106.00	123.33 ± 5.54	195.00	108.00	144.56 ± 12.09
Shoot fresh weight (g)	6.68	1.91	3.85 ± 0.74	3.45	1.47	2.32 ± 0.28	3.84	2.54	3.19 ± 0.15
Shoot (% dry matter)	33.38	29.32	31.11 ± 0.74	32.46	29.84	30.98 ± 0.38	33.62	29.54	31.77 ± 0.49
Stem diameter (mm)	3.00	2.00	2.75 ± 0.17	2.50	2.00	2.33 ± 0.11	3.00	2.50	2.83 ± 0.08
Internodes (number)	13.00	10.00	11.67 ± 0.42	10.00	7.00	8.33 ± 0.42	9.00	6.00	8.00 ± 0.33
Internodes length(mm)	17.33	13.58	15.99 ± 0.56	15.88	14.25	14.83 ± 0.25	21.67	15.43	17.90 ± 0.89
Leaves (number)	514.00	240.00	352.67 ± 9.11	180.00	78.00	125.00 ± 4.39	184.00	96.00	141.78 ± 8.47
Leaf area (cm^2)	0.27	0.25	0.26 ± 0.01	0.44	0.37	0.40 ± 0.01	0.62	0.50	0.56 ± 0.01
Axillar shoots (number)	15.00	6.00	10.83 ± 1.40	6.00	0.00	3.50 ± 1.15	10.00	5.00	7.67 ± 0.47
Leaves-shoot ratio (%)	0.71	0.68	0.70 ± 0.01	0.80	0.74	0.77 ± 0.01	0.76	0.66	0.71 ± 0.01
Essential oil yield (% of v/w)	4.56	4.12	4.29 ± 0.06	1.04	0.51	0.79 ± 0.08	1.82	0.00	1.21 ± 0.18
Major volatile oil constituents	camphor			α - pinene/verbenone			α - pinene - verbenone - 1,8 cineole		

Table. 2. Essential oil quali-quantitative components (% v/v) in 7 rosemary biotypes.

Compound	RI	Biotypes						
		1	2	3	4	5	6	7
tricyclene	924	0.34	0.25	0.28	0.22	0.20	0.21	0.10
α - thujene	931	0.00	0.23	0.00	0.32	0.27	0.17	0.19
α - pinene	936	23.45	26.54	23.74	23.72	8.65	18.43	0.24
camphene	951	6.07	6.71	5.21	5.17	5.50	4.13	8.85
verbenene	958	0.23	0.00	0.21	0.27	0.00	0.18	5.38
β - pinene	978	3.28	4.27	2.70	3.11	3.74	6.57	3.48
myrcene	993	3.40	4.71	3.95	1.08	0.55	1.21	0.59
α - phellandrene	1005	0.00	0.24	0.00	0.26	0.15	0.00	0.16
δ - 3 - carene	1011	0.00	0.00	0.00	0.00	0.00	0.00	0.00
α - terpinene	1019	0.31	0.69	0.29	0.61	0.32	0.34	0.29
p - cimene	1029	0.20	0.42	0.20	0.54	0.19	0.00	0.19
limonene	1032	2.76	2.43	2.75	2.46	2.02	2.12	2.17
1,8 - cineole	1034	9.15	16.49	8.96	11.48	9.40	16.91	9.43
trans - β - ocimene	1044	0.00	0.00	0.48	0.13	0.00	0.32	0.00
γ - terpinene	1064	0.55	2.05	0.32	2.06	1.28	0.71	1.27
cis - sabinene hydrate	1074	0.37	0.55	0.38	0.86	0.59	0.45	0.58
terpinolene	1091	0.75	0.71	0.61	0.87	0.80	0.47	0.88
trans - sabinene hydrate	1103	0.26	0.26	0.22	0.38	0.30	0.21	0.31
linalool	1105	1.40	1.00	1.74	1.68	0.43	1.68	0.44
crysanthenone	1131	0.67	0.12	0.84	0.41	0.23	0.16	0.21
neo - allo - ocimene	1135	0.00	0.00	0.00	0.00	0.00	0.00	0.00
trans - pinocarveol	1145	0.00	0.00	0.00	0.16	0.00	0.65	0.00
camphor	1150	8.30	6.20	7.57	2.29	42.72	0.43	43.61
verbenol	1153	0.00	0.00	0.00	0.26	0.00	0.00	0.00
pinocamphone	1167	0.19	0.00	0.00	0.06	0.00	0.13	0.22
pinocarvone	1169	0.00	0.00	0.00	0.30	0.19	0.16	0.21
borneol	1173	4.02	3.06	3.66	1.74	1.26	4.24	1.11
cis-3-pinanone	1180	1.45	0.11	1.51	0.53	0.29	1.60	0.25
terpinen-4-ol	1183	0.60	0.65	0.60	0.67	0.37	0.57	0.34
p - cymen-8-ol	1195	0.00	0.00	0.00	0.00	0.00	0.00	0.00
α - terpineol	1197	1.12	1.51	1.20	1.38	1.20	1.36	1.25
myrtenol	1205	0.00	0.00	0.00	0.34	0.00	0.00	0.00
verbenone	1215	9.80	2.84	11.74	19.30	0.71	3.22	0.85
bornyl acetate	1290	7.45	4.65	5.86	11.81	0.61	10.94	0.59
cis - isoeugenol	1370	0.22	0.00	0.19	0.00	0.00	0.19	0.00
α - copaene	1380	0.00	0.00	0.00	0.00	0.00	0.84	0.00
anisyl acetate	1414	0.00	0.00	0.00	0.15	0.00	0.29	0.00
trans - caryophillene	1424	6.34	1.89	6.69	0.00	0.00	7.16	0.00
α - humulene	1459	0.97	0.26	1.01	0.00	0.53	1.17	0.49
γ - curcumene	1483	0.00	0.12	0.00	0.00	0.10	0.75	0.07
α - muurolene	1505	0.00	0.00	0.00	0.00	0.00	0.32	0.00
β - bisabolene	1512	0.00	0.24	0.00	0.00	0.63	0.19	0.57
γ - cadinene	1515	0.00	0.00	0.00	0.00	0.14	0.00	0.13
δ - cadinene	1529	0.00	0.00	0.00	0.00	0.18	1.73	0.16
cadina-1,4-diene	1539	0.00	0.00	0.00	0.00	0.00	0.17	0.00
caryophyllene oxide	1591	1.53	0.84	1.61	0.00	0.00	1.01	0.00
methyl jasmonate	1659	0.00	0.08	0.19	0.00	0.00	0.61	0.00
α - bisabolol	1693	0.00	0.00	0.00	0.00	11.80	0.00	11.14

RI= retention time

Table 3. Comparative average essential oil composition analysed by the two extraction methodologies (only compounds obtained from both techniques are shown).

N.	Compound	RT	Observation [1]	Stem distillation mean	Solvent extraction mean	Differences
		(min)	(%)			
1	tricyclene	7.860	100	0.31	0.23	-0.08
2	α-thujene	8.130	71	0.06	0.17	0.10
3	α-pinene	8.470	100	19.42	17.82	-1.60
4	camphene	8.970	100	6.57	5.95	-0.62
5	verbenene	9.170	71	0.66	0.90	0.23
6	β-pinene[2]	10.100	100	1.45	3.88	2.43
	sabinene[2]	10.105	- -	- -	- -	- -
7	β-mircene	10.790	100	2.57	2.21	-0.35
8	α-phellandrene	11.220	57	0.59	0.12	-0.47
9	α-terpinene	11.730	100	0.81	0.41	-0.40
10	p-cymene	12.100	86	2.64	0.25	-2.39
11	limonene	12.320	100	2.88	2.39	-0.50
12	1,8-cineole	12.380	100	14.10	11.69	-2.42
13	*trans*-β-ocimene	12.690	43	0.20	0.13	-0.06
14	γ-terpinene	13.450	100	1.02	1.18	0.16
15	*cis*-sabinene-hydrate	13.770	100	0.06	0.54	0.49
16	α-terpinolene	14.610	100	1.21	0.73	-0.48
17	*trans*-sabinene-hydrate	15.910	100	0.06	0.28	0.21
18	*trans*-pinocarveol	16.570	29	0.19	0.12	-0.08
19	camphor	16.870	100	17.23	15.87	-1.36
20	borneol	17.650	100	5.38	2.73	-2.65
21	terpin-4-ol	18.040	100	1.30	0.54	-0.75
22	α-terpineol	18.590	100	2.33	1.29	-1.05
23	myrtenol[2]	18.900	14	0.27	0.05	-0.22
	cis-piperitol[2]	18.903	- -	- -	- -	- -
24	verbenone	19.260	100	4.75	6.92	2.17
25	bornyl acetate	21.940	100	1.38	5.99	4.61
26	α-copaene	24.900	14	0.07	0.12	0.05
27	*trans*-cariophyllene	26.330	57	0.80	3.15	2.35
28	α-umulene	27.390	86	0.26	0.63	0.38
29	β-bisabolene	29.090	57	0.07	0.23	0.16
30	γ-cadinene	29.270	29	0.08	0.04	-0.04
31	δ-cadinene	29.540	43	0.11	0.30	0.19

RT = retention time in minutes.

(1) – percent of biotypes in which the compound has been identified in extracted oils by both extraction methodologies.

(2) – in solvent-extracted oils the first of the two compounds only was identified; in the oils extracted by hydrodistillation the two compounds were identified separately, but their peaks were overlapping. As to the quantity the first prevailing compound was considered.

Figures

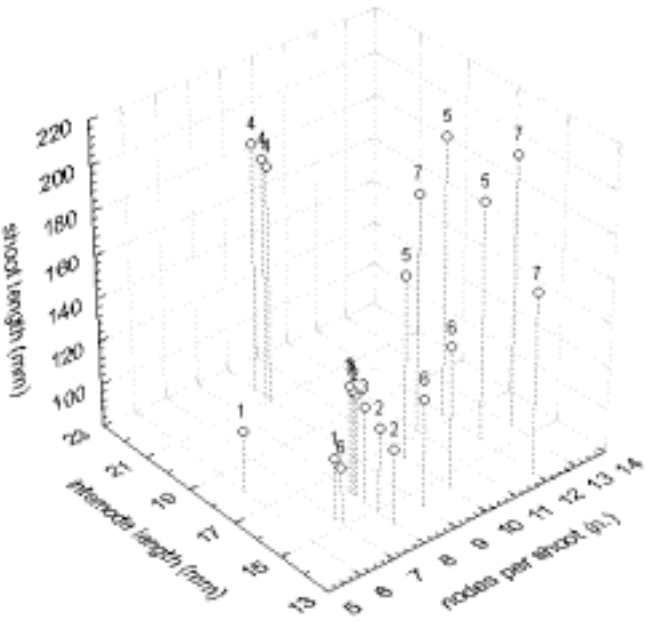

Fig. 1. Shoot length as influenced by internodes length and number of nodes per shoot in 7 rosemary biotypes.

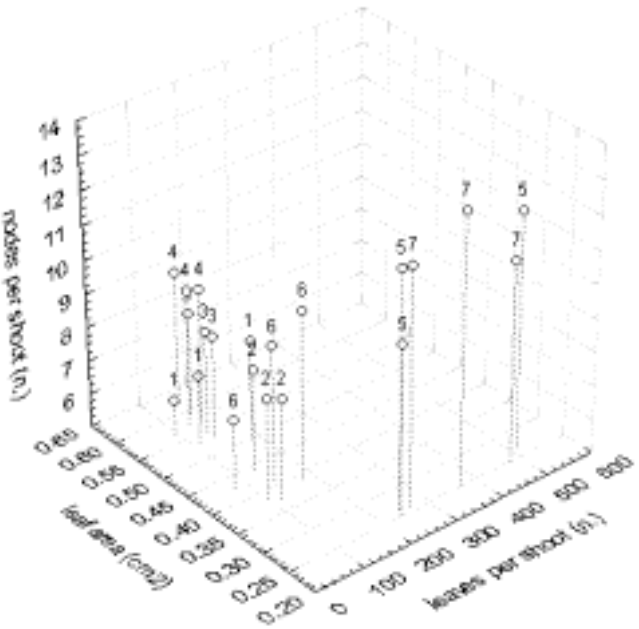

Fig. 2. Relations among leaf area and leaves-nodes number per shoot in 7 rosemary biotypes.

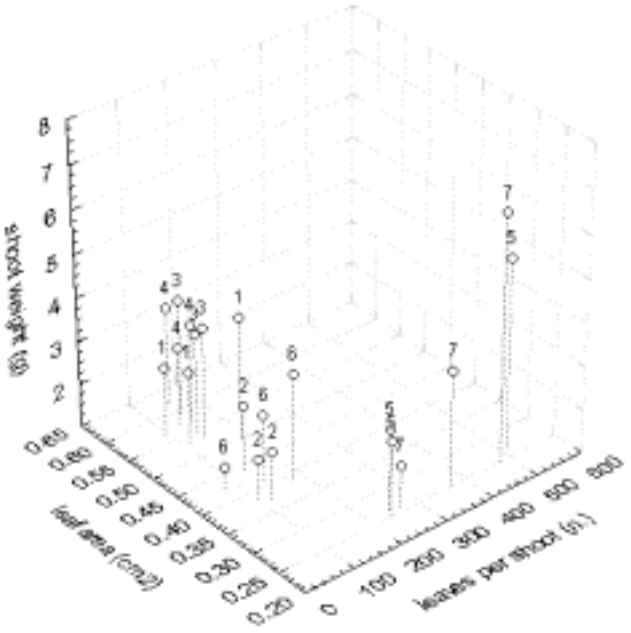

Fig. 3. Shoot weight as influenced by leaf area and number of leaves in 7 rosemary biotypes.

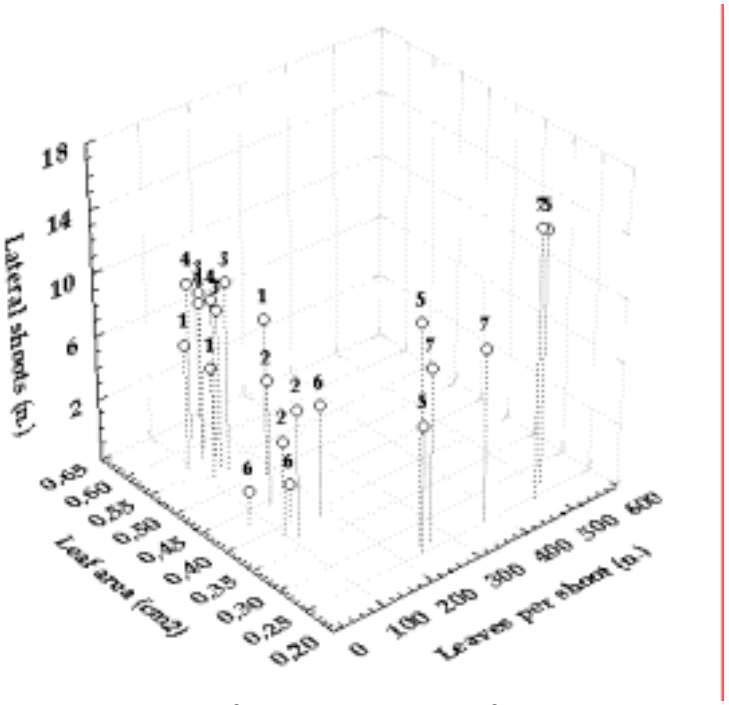

Fig. 4. Relations among number of lateral shoots, leaf area and number of leaves per shoot in 7 rosemary biotypes.

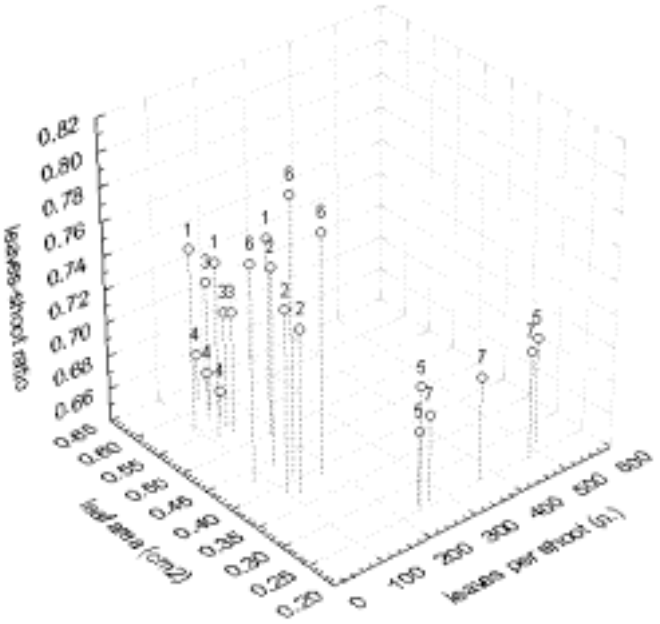

Fig. 5. Leaves-shoot ratio as influenced by leaf area and number of leaves per shoot in 7 rosemary biotypes.

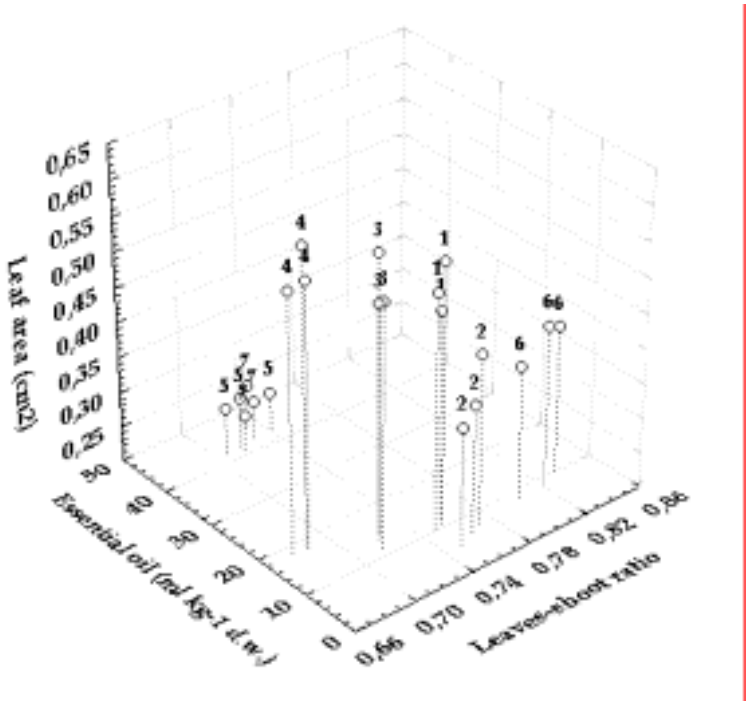

Fig. 6. Relations among essential oil content, leaf area and leaves-shoot ratio in 7 rosemary biotypes.

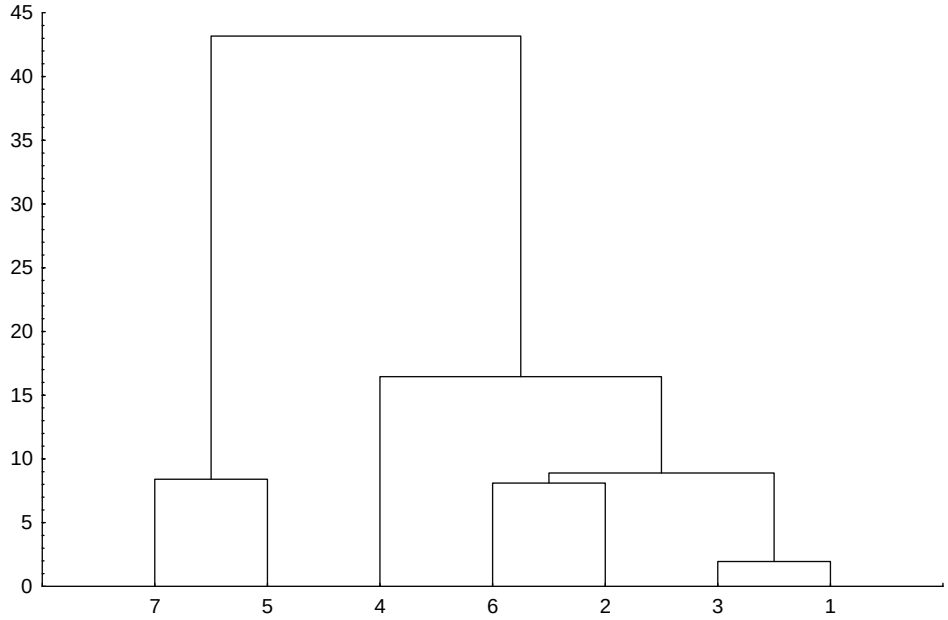

Fig. 7. Cluster analyses of GC essential oils data of 7 rosemary biotypes.