Partitioning the Relative Contributions of Inorganic Plant Composition and Soil Characteristics to the Quality of *Helichrysum italicum* subsp. *italicum* (ROTH) G. DON FIL. Essential Oil

by **Ange Bianchini**[a][b], **François Santoni**[c], **Julien Paolini***[a], **Antoine-François Bernardini**[a], **David Mouillot**[d], and **Jean Costa**[a]

[a]) CNRS UMR-6134 SPE, Université de Corse, Laboratoire Chimie des Produits Naturels, F-20250 Corti (phone: (33) 04 95 45 01 93; fax: (33) 04 95 45 01 62; e-mail: paolini@univ-corse.fr)

[b]) Office du Développement Agricole et Rural de Corse, Avenue Paul Giacobbi, F-20600 Bastia

[c]) Office Equipement Hydraulique de Corse, Avenue Paul Giacobbi, F-20600 Bastia

[d]) UMR CNRS-IFREMER-UM2 5119 Écosystèmes Lagunaires, Université Montpellier 2 cc 093, F-34 095 Montpellier

Composition of *Helichrysum italicum* subsp. *italicum* essential oil showed chemical variability according to vegetation cycle, environment, and geographic origins. In the present work, 48 individuals of this plant at different development stages and the corresponding root soils were sampled: *i*) 28 volatile components were identified and measured in essential oil by using GC and GC/MS; *ii*) ten elements from plants and soils have been estimated using colorimetry in continuous flux, flame atomic absorption spectrometry, or emission spectrometry (FAAS/FAES); *iii*) texture and acidity (real and potential) of soil samples were also reported. Relationships between the essential-oil composition, the inorganic plant composition, and the soil characteristics (inorganic composition, texture, and acidity) have been established using multivariate analysis such as Principal Component Analysis (PCA) and partial Redundancy Analysis (RDA). This study demonstrates a high level of intraspecific differences in oil composition due to environmental factors and, more particularly, soil characteristics.

Introduction. – The genus *Helichrysum* (family Asteraceae) from the tribe Inuleae comprises more than 400 species widespread throughout the world [1]. An overview of the numerous works on the genus *Helichrysum* indicates a high degree of polymorphism [2] and suggests some pharmacological applications [3]. Several studies have been reported on the chemical composition of essential oils [4][5] and solvent extracts [6] from *Helichrysum* species of various origins.

Helichrysum italicum (ROTH) G. DON FIL., a typically Mediterranean species, is an aromatic shrub (50–70-cm high) with yellow flowers (blossoming time, May–June) growing on dry cliffs and sandy soils [7]. *H. italicum* oil is widely used in perfume industry and aromatherapy due to their flavouring properties and biological activities (anti-inflammatory, antibacterial, antioxidant). The essential oils of three *H. italicum* subspecies have been studied: subsp. *serotinum* [8], subsp. *microphyllum* [9][10], and subsp. *italicum* [10][11]. Several analyses of essential oils (subspecies not specified) have been also reported in literature [12]. The chemical composition of *H. italicum* essential oil showed chemical variability among geographic origins such as Greece [11–13], Croatia [14], France [15][16], and Italy [16][17].

The essential oil of *H. italicum* has an economic importance in Mediterranean areas, particularly for Corsica Island, due to high content of neryl acetate. Environmental factors (climatic, geographical) and seasonal differences are known to influence essential-oil composition of aromatic plants [18]. Moreover, the chemical polymorphism of this species is outstanding, and its relation with both genetics and environmental factors is not yet well defined. As part of our previous investigations on the characterisation of *H. italicum* oil [15–17], we have observed two chemical compositions (high content of β-diketones or high content of neryl acetate) according to the vegetation cycle. These two chemical compositions appeared also to be dependent on the sampling locations. For instance, Corsican and Sardinian essential oils were characterized by the predominance of oxygenated compounds (neryl esters, eudesm-5-en-11-ol, β-diketones) and the Tuscany oil by the predominance of hydrocarbon compounds (α-pinene, γ-curcumene) [15–17].

Some studies have been also reported on the influence of soil characteristics on the yield and composition of essentials oils from various aromatic plants [19–21]. For instance, *Dialtoff* [21] has studied the effect of nitrogen fertilizers on the yield of essential oil of *Leptospermum* species, while *Dethier et al.* [22] have reported the influence of cultural treatment and harvest time on Vetiver oil quality. More recently, *Razic et al.* [23] have studied the relations between mineral content (eleven metals) of seven herbal drugs and corresponding soils. Hence, the question is no longer whether soil characteristics, vegetation cycles, or inorganic plant composition influence essential-oil composition, but to quantify the relative contribution of each of these factors.

To investigate this question, we have determined the essential-oil composition of *H. italicum* and the element concentrations of plant growing wild in 48 localities of Corsica at different development stages. Principal Component Analysis (PCA) and cluster analyses were carried out on the data of essential-oil composition to identify the various groups gathering plants according to their chemotypes. Furthermore, the soils where plants were harvested were characterized by major elements and physicochemical parameters (texture, and real and potential acidity). Analysis of essential-oil compositions were carried out using GC and GC/MS, and the element concentrations were estimated both in aromatic plants and soil samples by using common techniques (colorimetric analysis and FAAS/FAES). Then, a step further, Redundancy Analysis and its partial form (partial RDA) were used to extract and partition the relative influence of inorganic plant composition and soil characteristics on the composition of *H. italicum* essential oil. Finally, associations between the components of *H. italicum* essential oil, and plant and soil characteristics will be identified, and an ecological interpretation will be provided. To the best of our knowledge, this study is the first to report the effect of environmental variables on the chemical composition of *H. italicum* essential oil. In turn, the study of possible sources of influence on plant inorganic composition and soil parameters on essential-oil composition could be used by commercial producers to select the most appropriate plant samples and to control agricultural conditions. Our results may contribute to a better production of this oil using natural cultural process. Moreover, to our knowledge, little attention has been paid to assay the variability of essential oil among natural populations of *H. italicum*. Such an information is important for the development of conservation programs and for the selection of parental strains for cultivation.

Results and Discussion. – 1. *Chemical Variability of Essential-Oil Composition. H. italicum* subsp. *italicum* were collected on 48 localities (S1–S48) of Corsica Island (*Fig. 1*). The stages of vegetation of each sample were: flowering stage (S1–S13), beginning flowering (S14–S32), and after flowering (S33–S48). *H. italicum* subsp. *italicum* essential oil from each locality was analyzed by GC and GC/MS. The chemical compositions have been characterized for 28 constituents accounting for 67.8–94.9% of the total oil. The identified compounds and the essential-oil compositions (averages of relative percentages of each component from 48 samples S1–S48) were reported in

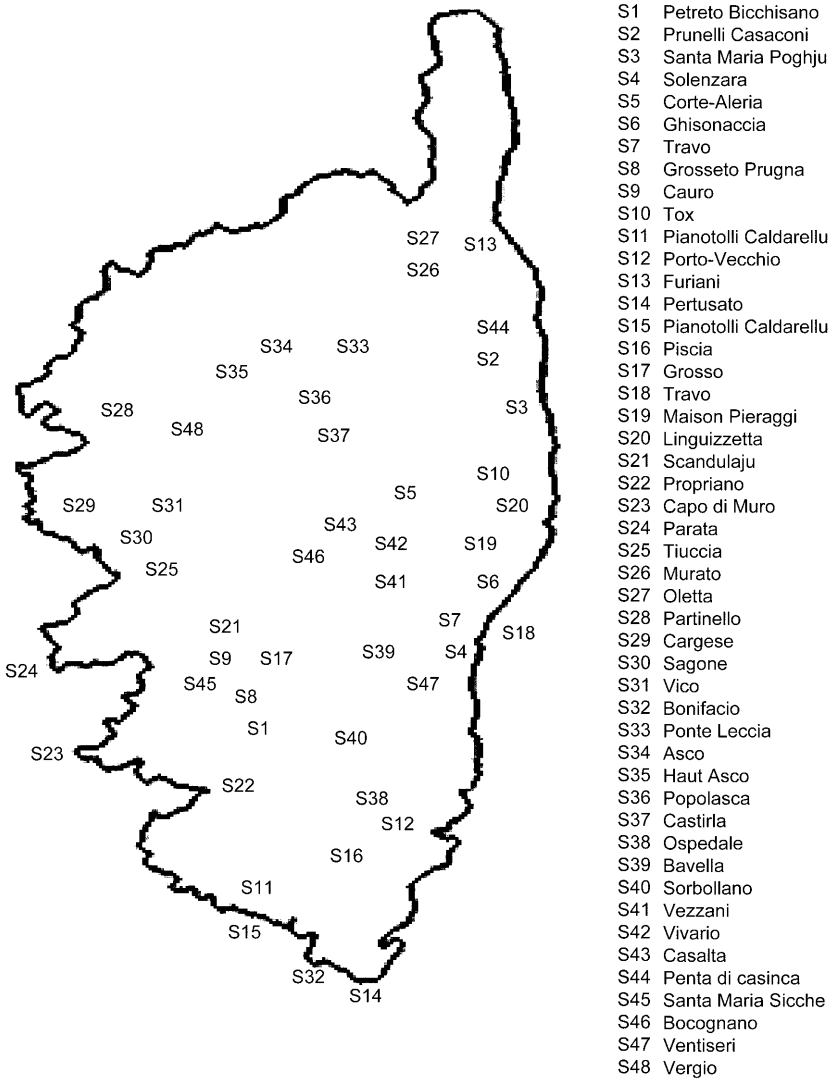


Fig. 1. *Localities of harvesting of* H. italicum *subsp.* italicum *on Corsica*

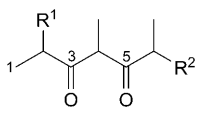
Table 1. The chemical structures of β-diketones (**C9**, **C12**, **C18**, **C20**, and **C21**), unusual compounds in essential oil, were displayed in *Fig. 2*.

These compounds have been classified into eight groups according to their structure: monoterpene hydrocarbons, sesquiterpene hydrocarbons, terpene oxides,

Table 1. *Identified Components and Composition of* H. italicum *Essential Oils*

No.[a])	Components	I_l[b])	I_a[c])	I_p[d])	Essential-oil composition	Identification[f])
C1	Pentan-3-one	678	679	972	1.6±1.4	*I*, MS
C2	2-Methylpentan-3-one	722	732	997	1.8±1.5	*I*, MS
C3	4-Methylhexan-3-one	843	834	1073	2.7±2.1	*I*, MS
C4	α-Pinene	936	929	1013	1.8±1.2	*I*, MS
C5	α-Fenchene	941	940	1046	0.5±0.5	*I*, MS
C6	β-Pinene	978	969	1100	0.5±0.4	*I*, MS
C7	Limonene	1025	1020	1183	5.9±5.2	*I*, MS
C8	1,8-Cineol	1024	1020	1200	1.2±2.0	*I*, MS
C9	2,4-Dimethylheptane-3,5-dione	1068	1071	1507	1.5±1.1	*I*, MS
C10	Linalool	1086	1083	1528	1.4±1.0	*I*, MS
C11	Caryophyllene oxide	1578	1575	1960	1.0±0.6	*I*, MS
C12	4,6-Dimethyloctane-3,5-dione	1158	1162	1585	5.0±3.1	*I*, MS
C13	α-Terpineol	1176	1173	1672	1.7±0.6	*I*, MS
C14	Nerol	1210	1209	1773	4.3±2.7	*I*, MS
C15	Neryl acetate	1342	1344	1703	30.7±12.8	*I*, MS
C16	Isoitalicene	1384	1373	1475	0.4±0.3	*I*, MS
C17	Italicene	1408	1400	1517	1.5±0.8	*I*, MS
C18	4,6,9-Trimethyldec-8-ene-3,5-dione	1417	1415	1865	2.0±2.2	*I*, MS
C19	Neryl propionate	1428	1429	1764	3.5±1.7	*I*, MS
C20	2,4,6,9-Tetramethyldec-8-ene-3,5-dione[g])	1461	1465	1867	1.1±1.0	*I*, MS
C21	2,4,6,9-Tetramethyldec-8-ene-3,5-dione[g])	1467	1468	1869	0.8±1.0	*I*, MS
C22	*ar*-Curcumene	1473	1471	1742	1.5±0.6	*I*, MS
C23	γ-Curcumene	1475	1473	1664	4.4±2.6	*I*, MS
C24	Guiaol	1587	1582	2043	1.1±0.8	*I*, MS
C25	Eudesm-5-en-11-ol	1600	1594	2081	3.8±2.7	*I*, MS
C26	β-Eudesmol	1641	1646	2233	1.6±1.4	*I*, MS
C27	α-Eudesmol	1653	1641	2188	1.3±1.0	*I*, MS
C28	Bulnesol	1665	1651	2171	0.5±0.4	*I*, MS

[a]) Order of elution is given on apolar column (*Rtx-1*). [b]) Retention indices from literature on the apolar column (I_l). [c]) Retention indices on the apolar *Rtx-1* column (I_a). [d]) Retention indices on the polar *Rtx-Wax* column (I_p). [e]) Essential-oil composition: means of 48 samples S1–S48 ($n=48$), relative percentage composition. [f]) *I* = Retention indices; MS: Mass spectrometry in electron-impact (EI) mode. [g]) Mixture of diastereoisomers.



	R^1	R^2
C9	H	Me
C12	H	Et
C18	H	$CH_2CH=CMe_2$
C20/21	Me	$CH_2CH=CMe_2$

Fig. 2. *Structures of* β*-diketones from* H. italicum *essential oil*

monoterpene alcohols, sesquiterpene alcohols, linear ketones, linear diketones, and monoterpene esters (*Table 2*). The oils were generally dominated by oxygenated monoterpenes (11.7–70%), neryl acetate being a major compound of all samples except for localities S17, S27, S33, S41, and S44. However, chemical composition was highly variable, the amount of neryl acetate varying from 2.4 to 58.3% between samples. For a successful commercialization, it should be noted that a high content of neryl acetate is highly desirable in *H. italicum* oil [15].

PCA was used to examine the relative distribution of samples according to their production of different volatile compounds. *Fig. 3* was obtained from the correlation matrix calculated with the standardized matrix. The two first principal axes account for 42% of the whole variability, the two PCA axes indicating 22.44% and 19.61% of the variability, respectively.

The distribution of variables is shown in *Fig. 3*, *a*. Two opposite groups of variables are very well-represented on axis 1: the monoterpene hydrocarbons, **C4**–**C7**, and linear ketones, **C1**–**C3**, **C9**, and **C12**, on the one hand, and monoterpene alcohols, **C10**, **C13**, and **C14**, and sesquiterpene alcohols, **C24**–**C28**, on the other hand. The second PCA axis was positively correlated with sesquiterpene alcohols and linear ketones, and negatively correlated with monoterpenes (alcohols and hydrocarbons). Moreover, it appears that monoterpene esters **C15** and **C19** were very well represented but negatively correlated on axis 2.

As shown in *Fig. 3*, *a*, the plot of the factorial scores relating to each sample on the axis of the first two principal components identified underlines that, in the family components, high percentages of linear and monoterpene hydrocarbons are accompanied by a decrease of alcohols. Moreover, high ester content was accompanied by a decrease of sesquiterpene alcohols and ketones. These results could be explained by their different biosynthesis pathways and suggests a reciprocal regulation.

The plot established according to the first two axes suggests the existence of two groups of essential oils (*Fig. 3*, *b*):

Group I: 24 samples belonged to this group. It was defined by two variables (linear ketones and monoterpene hydrocarbons). The group I was separated into two subgroups: the subgroup Ia contains ten populations, *i.e.*, S18, S19, S20, S24, S25, S33, S35, S39, S41, S42. These individuals were characterized by the production of a high amount of linear ketones, **C1**–**C3**, **C9**, and **C12**, and with low content of monoterpene alcohols, **C10**, **C13**, and **C14**, and esters, **C15** and **C19**; the subgroup Ib with 14 populations, *i.e.*, S12, S14, S15, S17, S21, S22, S23, S26, S27, S32, S34, S38, S40, and S44, was characterized by a large content of monoterpene hydrocarbons, **C4**–**C7**, and low percentages of sesquiterpenes alcohols, **C24**–**C28**, and β-diketones, **C18** and **C20**, **C21**.

Group II: 24 samples belonged to this group. It was defined by three variables (esters, monoterpene alcohols, and sesquiterpene alcohols). This group II was divided into two subgroups: the subgroup IIa includes 13 samples, *i.e.*, S3, S6, S7, S8, S10, S13, S36, S37, S43, S45, S46, S47, and S48. It was defined by sesquiterpene alcohols and by weak content of monoterpene hydrocarbons; the subgroup IIb contained eleven populations, *i.e.*, S1, S2, S4, S5, S9, S11, S16, S28, S29, S30, and S31, whose oils have higher percentages of monoterpenes (alcohols and esters) and lower amount of linear ketones.

Table 2. *Component Families of* H. italicum *Essential Oil from 48 Localities of Corsica*[a])

Locality	Yield of oil [%]	Monoterpene hydrocarbons	Sesquiterpene hydrocarbons	Oxides	Monoterpene alcohols	Sesquiterpene alcohols	Linear ketones	Linear diketones	Monoterpene esters
		C4, **C5**, **C6**, **C7**	**C16**, **C17**, **C22**, **C23**	**C8**, **C11**	**C10**, **C13**, **C14**	**C24**, **C25**, **C26**, **C27**, **C28**	**C1**, **C2**, **C3**	**C9**, **C12**, **C18**, **C20**, **C21**	**C15**, **C19**
S1	0.05	7.3	7.7	8.8	14.2	15.0	1.0	4.1	26.1
S2	0.09	9.5	3.4	8.0	9.7	10.6	4.0	7.7	34.3
S3	0.33	7.8	9.5	2.0	5.7	12.0	2.2	15.2	28.7
S4	0.19	4.7	7.6	0.6	8.0	6.2	1.7	19.5	36.5
S5	0.13	15.0	12.7	0.8	7.5	10.2	2.4	5.8	26.8
S6	0.09	4.8	5.23	0.7	8.3	12.2	1.9	4.1	47.7
S7	0.16	10.0	7.7	0.9	9.9	14.7	2.8	8.9	34.4
S8	0.44	8.6	7.6	0.4	9.6	12.7	1.3	10.2	32.9
S9	0.14	0.5	6.4	3.7	10.0	3.9	1.8	14.2	42.9
S10	0.11	4.3	9.4	0.3	5.5	20.0	3.3	21.3	13.8
S11	0.19	8.8	7.9	7.0	11.1	0.0	1.7	5.0	49.9
S12	0.41	12.3	2.9	2.0	5.8	7.4	5.9	11.1	39.3
S13	0.19	10.3	4.8	0.8	4.2	15.0	3.0	16.9	25.3
S14	0.06	21.7	2.0	1.2	5.5	3.7	2.3	4.1	41.7
S15	0.14	9.1	11.4	2.9	7.4	3.6	3.8	5.1	49.8
S16	0.14	6.4	6.3	4.8	10.0	10.7	2.6	8.1	45.1
S17	0.10	18.8	11.3	1.2	21.1	6.0	3.5	7.6	10.8
S18	0.07	1.8	10.6	1.2	3.6	14.5	13.0	13.7	21.8
S19	0.08	2.3	11.3	1.0	6.7	4.7	12.3	11.8	40.3
S20	0.06	2.0	11.3	3.8	3.6	4.8	16.3	15.6	22.4
S21	0.09	24.6	4.6	2.3	5.1	5.9	11.9	13.0	24.0
S22	0.09	18.1	10.1	0.6	6.2	5.7	5.0	4.3	43.9
S23	0.05	2.3	4.4	1.8	9.3	3.8	5.4	4.9	60.0
S24	0.13	3.6	6.7	1.5	7.5	5.2	12.5	8.5	35.5
S25	0.05	10.8	5.4	1.2	5.5	2.0	13.2	16.4	32.8
S26	0.04	3.2	5.2	1.8	6.2	6.1	8.7	7.0	49.2
S27	0.11	31.3	8.8	1.0	4.4	5.2	6.8	8.6	22.4
S28	0.02	4.5	5.8	0.5	5.7	6.5	1.4	10.6	50.9

Table 2 (cont.)

Locality	Yield of oil [%]	Monoterpene hydrocarbons	Sesquiterpene hydrocarbons	Oxides	Monoterpene alcohols	Sesquiterpene alcohols	Linear ketones	Linear diketones	Monoterpene esters
		C4, **C5**, **C6**, **C7**	**C16**, **C17**, **C22**, **C23**	**C8**, **C11**	**C10**, **C13**, **C14**	**C24**, **C25**, **C26**, **C27**, **C28**	**C1**, **C2**, **C3**	**C9**, **C12**, **C18**, **C20**, **C21**	**C15**, **C19**
S29	0.30	2.0	9.0	1.1	9.7	0.2	2.7	7.8	59.1
S30	0.16	5.5	7.9	0.7	6.6	5.7	2.8	5.5	58.2
S31	0.14	11.1	18.3	0.8	7.8	1.6	1.9	5.8	34.1
S32	0.24	23.1	2.6	1.2	9.7	2.8	2.6	4.4	45.0
S33	0.14	13.6	12.5	0.8	3.3	13.7	13.0	17.6	8.3
S34	0.07	19.5	8.1	2.3	7.3	2.2	8.2	8.4	32.0
S35	0.08	0.0	5.4	1.5	3.3	20.1	5.6	16.4	19.4
S36	0.09	7.1	9.9	0.9	5.8	13.1	6.0	13.1	34.3
S37	0.04	0.9	3.8	1.7	6.9	13.4	1.8	10.7	46.4
S38	0.06	11.2	5.8	2.4	7.7	6.8	10.1	8.1	32.3
S39	0.08	7.4	2.5	3.0	5.5	3.3	20.6	20.0	24.6
S40	0.09	8.2	5.9	1.8	11.7	3.6	5.5	3.2	55.0
S41	0.05	3.8	8.1	1.9	3.5	18.5	14.0	15.5	14.6
S42	0.02	4.4	3.8	3.6	3.7	8.6	9.9	22.7	22.1
S43	0.03	9.6	8.2	1.2	7.0	13.7	5.3	7.8	30.1
S44	0.09	13.8	7.8	2.2	13.7	9.3	12.0	14.0	10.7
S45	0.02	1.8	14.9	7.7	7.4	6.9	6.2	8.9	30.0
S46	0.07	1.5	7.8	0.4	8.8	12.8	0.9	4.4	41.0
S47	0.07	0.8	14.1	2.5	6.8	10.6	4.0	13.7	30.6
S48	0.09	7.5	9.3	0.8	4.9	5.4	3.6	9.1	27.2

[a]) **C1**–**C28**: Identified components; see *Table 1*. S1–S48: Localities of harvesting, see *Fig. 1*.

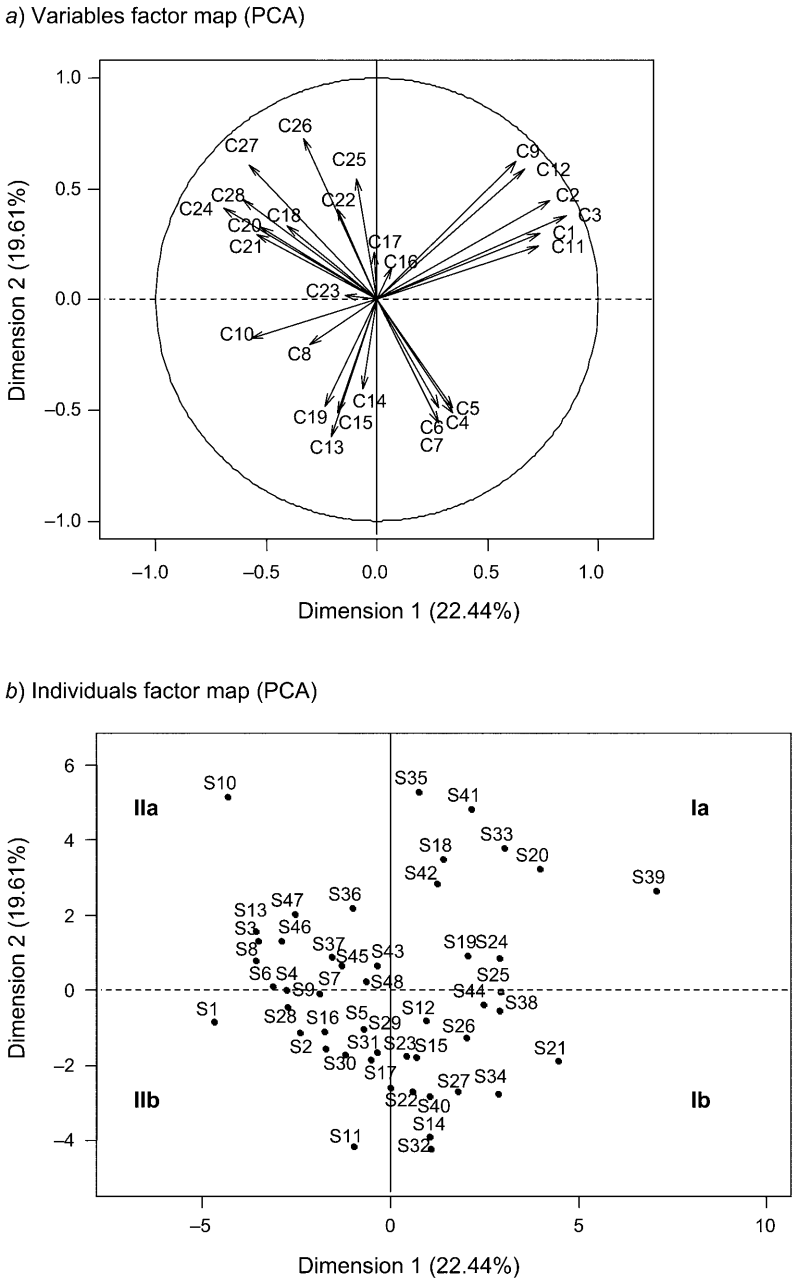


Fig. 3. *Principal Component Analysis* (PCA) *of chemical composition of* H. italicum *essential oil*

The general structure of the dendrogram produced by the *Ward*'s method was consistent with the one obtained with PCA, grouping the 48 populations into four main

clusters (*Fig. 4*). The cluster analysis suggested the existence of two groups based on the amount of components from five compounds families: linear ketones, monoterpene hydrocarbons, sesquiterpene alcohols, and monoterpenes alcohols and esters. This dendrogram reinforces the clustering observed using PCA; two groups were reported (group I: 20 samples and group II: 28 samples) each of which exhibited two subgroups with 10 (subgroup Ia), 10 (subgroup Ib), 12 (subgroup IIa), and 16 (subgroup IIb) samples, respectively. Few differences were reported between PCA and dendrogram results: four populations, S15, S23, S26, and S40, integrated in subgroup Ib with PCA were observed in the subgroup IIb with the dendrogram; two populations, S37 and S45, present in subgroup IIa with PCA belonged to subgroup IIb using the dendrogram; and one population, S5, reported in subgroup IIb with PCA was observed in subgroup IIa by dendrogram.

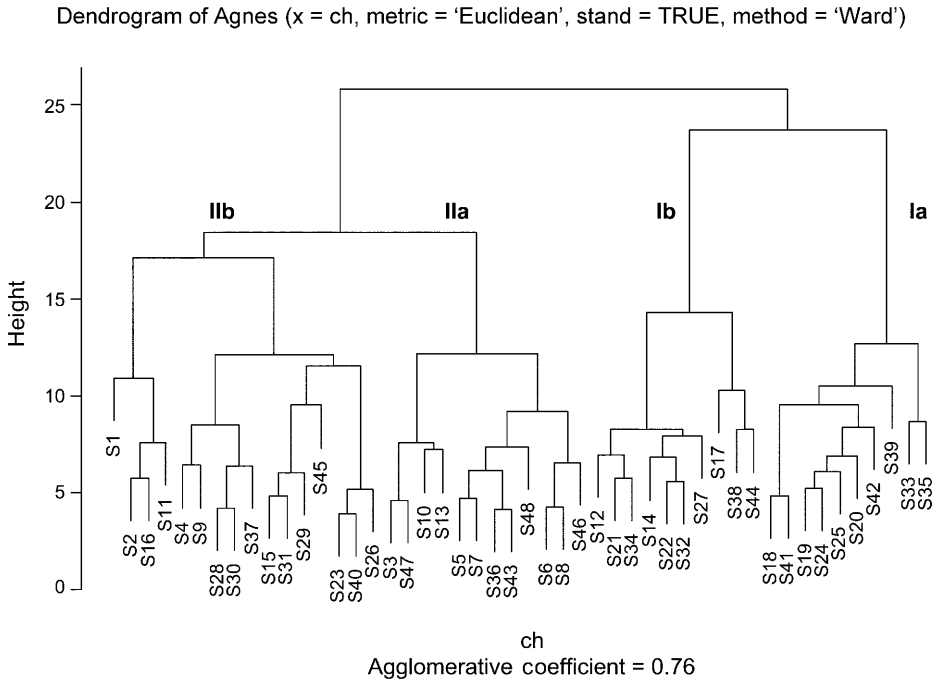


Fig. 4. *Canonical Analysis* (CA) *of chemical composition of* H. italicum *essential oil*

Ours results were in accordance with those reported for *H. italicum* essential oil from various origins and development stage. Indeed, various chemical compositions have been reported depending upon sample locations [10–17].

2. *Influence of the Different Factors on Essential-Oil Variability.* Four quantitative matrices were used to partition the variability in essential-oil composition (family compounds) into four sources of variance: *i*) the inorganic plant composition (*Table 3*); *ii*) the inorganic soil composition (*Table 3*); *iii*) the physico-chemical parameters of soils (texture and acidity) (*Table 4*), and *iv*) the plant stage of vegetation. A series of

Table 3. *Inorganic Compositions of Plant and Soil of* H. italicum *from 48 Localities*[a]) of Corsica

	Inorganic composition of the plant[b])										Inorganic composition of the soil[b])									
	Na	Fe	Mn	Zn	Cu	Ca	N	P	Mg	K	Fe(s)	Mn(s)	Zn(s)	Cu(s)	P(s)	N(s)	Ca(s)	Na(s)	Mg(s)	K(s)
	[mg·kg^{-1}]					[%]					[mg·kg^{-1}]					[%]	milli-equiv. [%]			
S1[d])	10515	339.5	263.5	64.3	13.3	0.92	1.92	0.30	0.27	0.70	110.5	139.50	28.35	1.28	42.3	0.25	4.62	0.27	1.82	0.26
S2	4780	161.5	157.0	27.8	8.7	0.69	0.87	0.16	0.13	1.25	130.5	209.50	4.34	5.21	35.0	0.20	12.00	0.22	0.77	0.17
S3	1240	138.5	200.0	50.4	12.7	0.67	1.39	0.34	0.24	1.85	93.5	318.50	6.06	14.19	99.7	0.24	11.62	0.25	2.00	0.59
S4	4530	179.0	295.5	55.0	11.8	0.48	0.99	0.22	0.19	1.93	44.5	72.00	3.36	2.80	65.3	0.10	8.48	0.54	5.72	0.86
S5	3745	136.5	182.0	33.1	7.1	0.70	0.73	0.27	0.17	1.31	18.0	46.10	2.85	2.09	23.2	0.21	21.52	0.20	0.65	0.31
S6	4315	274.0	118.5	30.2	7.1	0.47	0.77	0.19	0.31	1.09	29.2	41.60	2.04	5.60	21.0	0.10	6.63	0.19	3.29	0.20
S7	2515	146.5	98.5	20.1	5.1	0.62	0.75	0.18	0.13	1.46	34.5	8.25	1.84	0.58	17.2	0.12	4.64	0.19	0.62	0.29
S8	5685	114.0	328.0	40.0	5.9	0.45	0.96	0.29	0.24	1.18	39.5	40.40	0.95	0.58	51.5	0.06	6.52	0.31	3.05	0.20
S9	4330	189.0	337.5	37.7	9.6	0.58	0.96	0.18	0.16	1.83	47.0	98.00	3.72	1.24	15.7	0.12	6.31	0.34	3.52	0.55
S10	5925	152.0	234.5	36.6	11.2	0.82	1.03	0.18	0.41	0.82	34.3	45.75	0.86	3.72	21.7	0.10	9.53	0.23	7.34	0.30
S11	5700	123.5	250.5	55.8	11.3	0.46	0.97	0.18	0.17	1.46	76.5	116.40	4.43	7.30	17.7	0.16	4.15	0.48	1.26	0.31
S12	6400	189.0	182.5	53.8	5.9	0.64	0.95	0.08	0.20	1.02	43.1	17.35	2.92	0.83	15.9	0.11	2.72	0.30	0.91	0.12
S13	3465	142.5	158.0	46.5	10.8	0.76	0.86	0.27	0.15	1.51	49.5	94.20	5.20	5.33	32.3	0.36	15.18	0.30	2.15	0.35
S14	178300	195.0	74.0	37.0	12.9	0.70	2.12	0.28	0.20	1.48	221.5	39.45	5.52	2.42	131.7	0.22	7.35	0.49	3.43	0.27
S15	6125	233.5	161.0	72.3	15.9	0.95	1.72	0.23	0.23	1.87	148.5	53.60	8.64	4.83	24.6	0.24	6.80	0.27	2.33	0.37
S16	8370	138.0	263.0	103.7	35.5	0.61	1.65	0.20	0.15	1.49	157.0	36.50	2.98	1.06	17.4	0.21	4.52	0.36	1.64	0.40
S17	5645	88.5	113.0	48.0	7.8	0.72	1.44	0.26	0.10	1.80	54.5	32.80	3.37	1.78	31.9	0.20	12.55	0.23	0.64	0.35
S18	7745	145.0	125.5	29.3	5.6	0.88	1.62	0.21	0.13	1.43	150.0	30.30	3.99	0.95	26.5	0.19	5.24	0.35	1.42	0.26
S19	4920	102.5	391.5	87.2	15.9	1.01	1.93	0.37	0.16	1.48	22.5	47.60	1.54	1.77	22.5	0.13	18.42	0.19	0.44	0.22
S20	5615	107.5	315.0	60.7	27.8	0.65	1.51	0.28	0.24	1.81	80.5	53.65	2.23	2.25	27.6	0.22	14.23	0.67	7.86	0.56
S21	7440	250.5	260.0	64.4	16.6	0.71	2.16	0.31	0.16	1.91	11.1	70.45	38.05	3.17	74.6	0.46	35.13	0.99	2.93	1.77
S22	8785	169.5	197.0	44.0	7.2	0.87	2.09	0.24	0.21	1.01	60.0	22.45	6.47	1.15	24.3	0.18	2.86	0.21	0.90	0.16
S23	7175	112.0	740.0	68.9	12.4	0.70	1.74	0.43	0.17	2.36	164.5	142.00	4.08	0.67	22.7	0.16	3.89	0.34	1.91	0.34
S24	239500	314.0	457.5	240.4	27.1	0.55	1.26	0.21	0.28	0.90	193.0	85.00	402.20	3.07	25.7	0.32	11.73	4.10	5.72	1.43
S25	168500	257.0	751.0	84.7	15.4	0.46	1.56	0.16	0.24	1.33	144.5	98.00	46.40	1.24	27.8	0.28	3.63	4.16	3.08	0.83
S26	5265	161.0	185.5	42.2	10.8	0.80	1.25	0.28	0.14	1.11	133.0	94.50	6.03	5.49	39.8	0.32	12.21	0.24	3.73	0.37
S27	6405	191.0	146.0	37.0	7.9	0.54	1.70	0.19	0.44	1.57	99.0	121.50	6.06	5.54	47.3	0.27	5.56	0.24	6.87	0.24
S28	9115	147.5	210.5	62.1	9.8	0.70	1.89	0.41	0.21	2.18	141.5	46.80	3.03	1.76	67.9	0.17	4.64	0.25	2.08	0.19
S29	8450	93.5	240.5	34.8	10.9	0.48	1.81	0.31	0.18	1.13	64.0	96.00	2.73	1.37	53.1	0.15	6.28	1.54	5.08	0.18
S30	6685	135.5	351.5	112.3	13.5	0.57	1.38	0.27	0.18	1.96	207.0	40.50	7.67	1.47	47.2	0.16	2.90	0.22	1.36	0.16
S31	5940	950.0	436.0	64.0	8.6	0.70	2.13	0.43	0.20	2.83	242.0	23.50	2.48	1.19	94.5	0.18	2.80	0.22	0.83	0.44

Table 3 (cont.)

	Inorganic composition of the plant[b])										Inorganic composition of the soil[b])									
	Na	Fe	Mn	Zn	Cu	Ca	N	P	Mg	K	Fe(s)	Mn(s)	Zn(s)	Cu(s)	P(s)	N(s)	Ca(s)	Na(s)	Mg(s)	K(s)
	[mg·kg^{-1}]					[%]					[mg·kg^{-1}]					[%]	milli-equiv. [%]			
S32	9835	202.5	82.5	86.7	8.3	0.99	1.22	0.14	0.15	1.38	14.7.0	28.55	7.30	1.63	65.7	0.34	33.66	0.73	2.41	1.15
S33	6745	156.0	82.5	99.8	9.0	0.66	2.11	0.21	0.16	1.33	267.0	47.75	19.07	8.40	157.0	0.31	7.13	0.24	1.17	0.44
S34	3320	167.5	121.0	28.0	7.4	0.74	2.13	0.32	0.16	1.41	149.0	26.85	2.80	1.55	17.5	0.23	4.82	0.23	0.71	0.31
S35	3795	160.0	293.5	49.5	9.0	0.57	1.19	0.15	0.16	0.52	54.5	47.35	2.28	3.32	31.0	0.15	8.16	0.17	1.26	0.28
S36	4995	126.5	422.5	42.4	3.8	0.59	1.55	0.19	0.12	1.39	10.2	30.65	0.31	0.22	23.8	0.03	3.27	0.19	2.14	0.08
S37	3740	199.0	170.5	31.0	9.3	0.60	1.24	0.11	0.10	0.43	57.0	52.60	0.54	0.70	13.2	0.11	2.63	0.18	0.90	0.22
S38	5380	228.0	778.5	60.0	10.4	0.61	1.37	0.13	0.13	0.53	113.5	66.00	1.36	0.69	14.3	0.08	1.79	0.34	1.10	0.24
S39	1830	126.0	431.5	101.8	14.5	0.71	1.13	0.12	0.12	1.01	265.5	49.25	4.00	1.11	17.2	0.46	6.45	0.33	1.82	0.79
S40	7340	143.0	487.5	34.2	3.9	0.94	2.19	0.28	0.12	1.21	84.5	12.15	0.96	0.66	38.4	0.16	2.38	0.38	0.37	0.17
S41	3070	260.0	213.5	35.3	11.2	0.68	1.23	0.20	0.12	1.18	69.0	90.50	0.99	2.29	18.4	0.18	3.57	0.19	0.90	0.28
S42	4165	107.5	174.5	77.6	15.5	0.91	1.83	0.36	0.22	1.17	98.5	28.45	1.11	0.69	16.8	0.24	8.53	0.25	1.65	0.63
S43	140800	357.5	305.0	37.0	12.8	0.92	2.22	0.27	0.19	1.23	93.0	209.50	0	3.68	37.7	0.29	11.48	0.23	1.48	0.23
S44	3065	143.0	76.5	48.0	14.9	0.76	1.72	0.27	0.20	1.03	120.0	77.50	4.91	8.26	39.5	0.26	10.28	0.20	2.77	0.41
S45	5795	269.0	170.0	73.5	12.2	0.92	1.68	0.34	0.18	0.76	75.0	40.40	4.87	2.43	42.0	0.09	5.21	0.29	2.60	0.36
S46	6795	268.0	358.0	44.3	14.1	0.69	1.17	0.21	0.18	1.00	27.9	20.95	5.07	0.70	27.4	0.15	0.71	0.27	0.29	0.26
S47	3970	146.5	202.0	47.2	10.7	0.55	1.49	0.35	0.13	1.37	379.5	88.00	10.74	2.60	44.6	0.40	10.11	0.26	2.00	0.77
S48	6765	285.0	213.5	45.3	15.6	0.81	2.90	0.27	0.16	1.01	69.5	25.30	2.22	1.80	52.8	0.21	2.00	0.19	0.53	0.21

[a]) For localities of harvesting, see *Fig. 1*. [b]) K and Na contents were determined by FAES. Ca, Cu, Fe, Mg, Mn, and Zn contents were determined by FAAS. N and P contents were determined by colorimetric analysis.

Table 4. *Texture* ([%]) *and Acidity of Soils in 48 Localities*[a]) *of* H. italicum *Samples*

Locality	Clay	Fine silt	Coarse silt	Fine sand	Coarse sand	pH_{H_2O}	pH_{KCl}
S1	2.9	9.8	3.2	21.6	62.6	5.62	4.45
S2	2.0	11.1	2.8	23.1	61.0	6.95	6.30
S3	8.2	34.1	8.8	17.0	31.9	6.94	6.27
S4	4.9	7.0	3.6	29.7	54.8	7.00	5.22
S5	9.0	16.6	5.7	37.0	31.7	7.69	7.22
S6	11.3	10.7	3.1	17.9	56.9	7.63	6.79
S7	1.8	2.1	0.4	3.9	91.9	6.45	5.75
S8	4.4	5.3	2.0	14.9	73.5	6.36	4.29
S9	6.4	9.0	1.1	8.1	75.4	5.89	4.84
S10	13.5	12.4	4.5	22.0	47.7	7.57	6.68
S11	7.1	11.1	1.8	13.4	66.6	5.56	4.78
S12	1.7	1.6	0.2	2.1	94.5	6.35	5.71
S13	10.0	39.8	1.5	18.3	30.3	6.20	5.40
S14	4.9	11.2	2.0	17.4	64.5	5.20	4.71
S15	5.1	13.7	1.5	16.7	63.0	5.48	4.92
S16	2.4	16.0	2.9	17.9	60.8	5.15	4.64
S17	0.4	5.4	2.8	16.7	74.8	7.86	7.38
S18	4.4	9.7	2.5	17.7	65.8	5.77	5.02
S19	4.9	10.0	2.6	16.7	65.8	8.22	7.47
S20	11.8	17.9	3.4	18.4	48.4	6.19	4.45
S21	1.1	18.1	2.4	22.6	55.8	7.39	7.15
S22	1.8	0.7	0.7	4.8	92.1	6.24	5.38
S23	7.1	8.1	2.7	14.9	67.2	5.94	4.86
S24	9.7	19.3	5.6	28.3	37.2	6.73	6.27
S25	10.7	14.6	1.5	12.0	61.1	5.94	5.07
S26	10.9	25.3	4.4	19.4	40.0	6.10	5.60
S27	8.5	25.9	4.3	16.0	45.3	6.56	6.10
S28	5.3	8.2	4.0	26.8	55.8	6.00	4.77
S29	6.7	7.1	3.9	15.6	66.8	6.76	4.67
S30	7.1	10.7	5.0	24.1	53.1	4.84	4.06
S31	8.4	10.5	2.8	20.5	57.9	5.07	4.18
S32	6.6	13.0	1.7	21.2	57.5	7.74	7.36
S33	6.7	17.9	4.1	21.9	49.4	5.14	4.59
S34	5.9	30.0	4.1	13.4	46.6	5.20	4.39
S35	9.3	17.0	5.7	22.5	45.5	7.73	7.18
S36	1.5	4.6	1.7	15.9	76.3	7.15	5.15
S37	10.6	16.2	3.8	14.8	54.7	5.59	4.35
S38	8.6	9.7	1.9	14.3	65.5	5.02	4.17
S39	2.9	26.9	0.5	16.6	53.1	4.83	4.23
S40	4.8	10.4	3.3	16.4	65.0	5.05	4.17
S41	24.8	24.6	3.4	12.6	34.6	5.89	4.50
S42	10.2	38.6	2.4	12.2	36.5	5.91	4.97
S43	2.6	18.1	2.8	21.8	54.7	7.00	6.34
S44	6.6	32.9	4.4	20.4	35.8	6.18	5.41
S45	6.0	6.7	2.0	14.3	71.0	6.41	4.48
S46	2.7	13.3	3.1	15.5	65.3	5.35	4.51
S47	10.8	22.4	3.5	18.8	44.5	4.59	4.24
S48	6.3	10.5	2.5	17.6	63.1	5.35	4.44

[a]) For localities of harvesting, see *Fig. 1.*

Redundancy Analysis (RDA) were used to determine the influence of each matrix on oil composition.

Percentages of explained variation for each component estimated from all RDA and partial RDA are displayed in the *Venn* diagram (*Fig. 5*). The percentage of total variation explained by the explanatory matrices was $\Omega = 51\%$, consequently the total unexplained variation was $U = 49\%$.

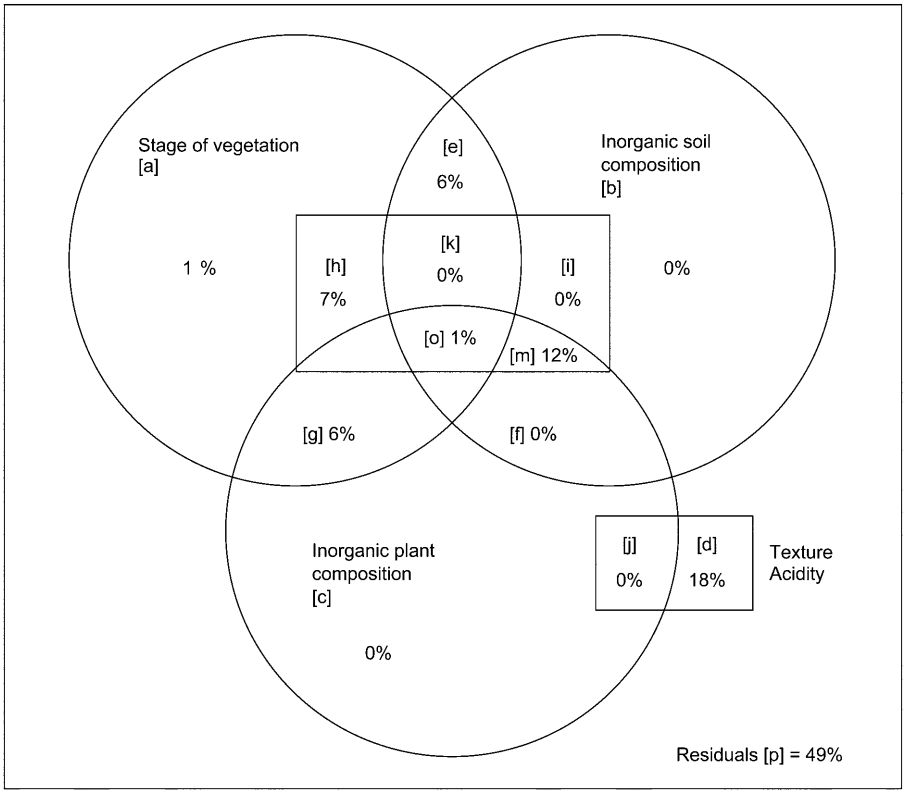


Fig. 5. *Analysis of* H. italicum *essential oil by partition of variance*

The main effect on essential-oil composition was due the physicochemical parameters of soils (texture and acidity) which accounted for 38% of the whole variability (18% of pure effect). At the second rank, the plant stage explained 21% of variation in essential-oil composition, while inorganic soil and plant composition explained a small part of variation in essential-oil composition.

3. *Inorganic Plant Composition and Correlation with Essential-Oil Composition.* According to the present knowledge, numerous elements were recognized as essential for plants [24]. The contents of ten elements, *i.e.*, Ca, Na, Fe, Mn, Zn, Cu, N, P, Mg, and K were determined in plant samples by means of common spectroscopic techniques (FAAS/FAES). The inorganic chemical composition of *H. italicum* subsp. *italicum* was characterized by high content of Ca, Mg, K, N, P, and Na (*Table 3*). All results in *Table 3* are expressed as the average of three sample measurements. The correlations

between the essential-oil composition and inorganic plant composition were determined using a partial RDA where the inorganic plant composition matrix was the explanatory matrix, while the three other matrices were set as co-variables in the analysis. Thus, we could extract the 'pure' relationships between essential-oil composition and inorganic plant composition independently from the inorganic soil composition, the physicochemical parameters of soils (texture and acidity), and the plant stage. As shown in *Fig. 6*, the first RDA axis (eigenvalue 53.1%) positively related Na, P, and Fe to monoterpenes (hydrocarbons, oxides, alcohols, and esters) and opposed N, Mn, Zn, Ca, and Cu to ketones, diketones, and sesquiterpenes. The second RDA axis (eigenvalue 18.9%) positively related Na, Zn, Ca, and Cu to alcohols (mono- and sesquiterpenes), esters, ketones, and diketones, while this axis negatively related Mn, P, N, and Fe to hydrocarbons (mono- and sesquiterpenes).

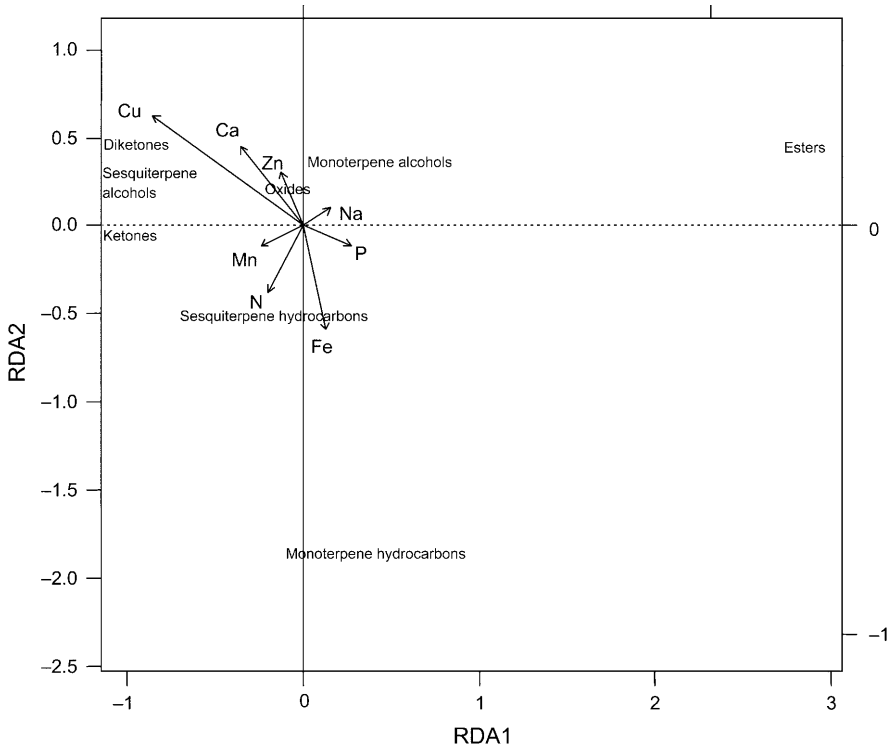


Fig. 6. *Correlations between the inorganic plant composition and* H. italicum *essential-oil composition*

4. *Inorganic Soil Composition and Correlation with Essential-Oil Composition.* Ten elements, *i.e.*, Ca, Na, Fe, Mn, Zn, Cu, N, P, Mg, and K, were used for the characterization of inorganic chemical composition of soils. The major elements of soils were Ca, Na, K, Mg, and N (*Table 3*). All data obtained were subjected to chemometric analyses. The complex relationships between essential oil, plant, and soil have required additional approaches, and chemometry has been used to provide the possible sources

of influence of inorganic soil composition (*Table 3*) on essential-oil composition (*Table 2*).

The correlations between the essential-oil composition and inorganic soil composition were determined using a partial RDA, where the inorganic soil composition matrix was the explanatory matrix, while the three other matrices were set as co-variables in the analysis. Thus, we could extract the 'pure' relationships between oil composition and inorganic soil composition. As shown in *Fig. 7*, the first RDA axis (eigenvalue 64.7%) positively related Zn and Mn to monoterpene hydrocarbons, alcohols, and esters, while this axis opposed Cu, Mg, Na, N, K, and Fe to sesquiterpenes hydrocarbons and alcohols, ketones, and diketones. The second RDA axis (eigenvalue 13.5%) positively related K to sesquiterpene alcohols and monoterpene hydrocarbons, and negatively related Zn, Mn, Cu, Mg, Na, N, and Fe to sesquiterpene hydrocarbons, ketones, diketones, and monoterpene alcohols, oxides, and esters.

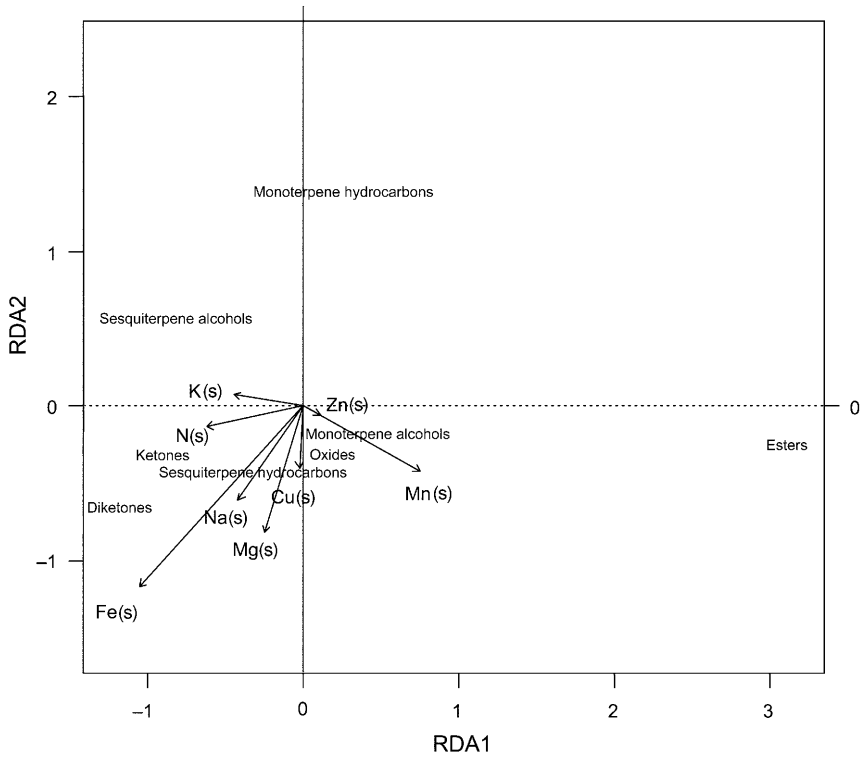


Fig. 7. *Correlations between the inorganic soil composition and* H. italicum *essential-oil composition*

5. *Soil Texture, Soil Acidity, and Correlation with Essential-Oil Composition.* The soil texture (percentages of clay, fine and coarse silt, and fine and coarse sand) has been also reported, and the characteristics were drastically different from the 48 localities (*Table 4*). For instance, the percentages in coarse sand were high for the locality S12 (94.5%) and weak for S44 (35.8%). Keeping in mind the importance of soil acidity for the uptake of metals by plants, measurement was performed in both aqueous and KCl

solutions in order to determine real (measured in aqueous extracts) and potential acidity, as a measure of buffer capacity of the soil. The latter one comprises also the concentration of H^+ ions adsorbed on colloidal particles. The pH values of real and potential acidity (pH_{H_2O} and pH_{KCl}) varies from 4.83 (S39) to 8.22 (S19), and from 4.06 (S30) to 7.47 (S19), respectively (*Table 4*). Weak acidity to weak alkaline reaction of soil favors the strong binding of toxic elements in soil and on the other hand optimal bioavailability of essential elements [25][26].

The correlations between the essential-oil composition and physicochemical parameters of soils were determined using data analysis (*Fig. 8*). To synthesize the effect of the different variables, the canonical RDA were used. The first RDA axis was positively (coarse and fine sand) correlated with monoterpene alcohols, oxides, and esters, and opposed (coarse silt, fine silt, clay, pH_{KCl} and pH_{H_2O}) with monoterpene hydrocarbons, sesquiterpenes (hydrocarbons and alcohols), ketones, and diketones. The second RDA axis was positively correlated (coarse sand and fine silt) with monoterpene alcohols, hydrocarbons, and oxides, and negatively correlated (coarse silt, fine sand, clay, pH_{KCl} and pH_{H_2O}) with sesquiterpenes (hydrocarbons and alcohols) and esters.

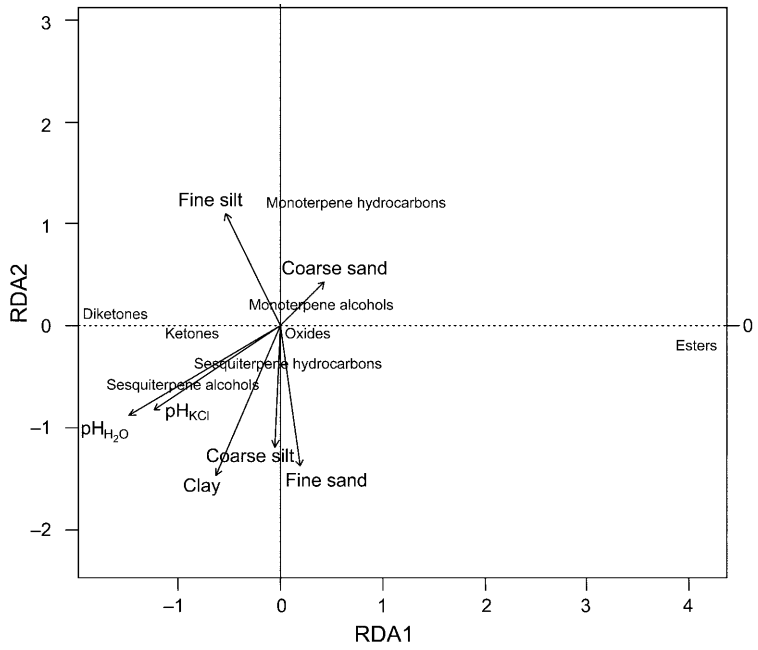


Fig. 8. *Correlations between the texture acidity of soils and* H. italicum *essential-oil composition*

Conclusions. – Our study highlights the need to use statistical methods able to disentangle various sources of variability on plant essential-oil composition. Here, partial RDA allowed us to isolate the pure effect of each factor, independently of the

others. One step further, such methods may also provide predictive models to estimate essential-oil composition according to environmental and biotic factors.

The chemical composition of *H. italicum* essential oil from Corsica exhibited a high chemical variability. Our results showed the correlations between the essential-oil composition, and the texture and acidity of soils. The inorganic composition of plant and soil, as well as the vegetative stage of development intervene lower in a discriminative way on the chemical composition of the essential oils.

For the culture of *H. italicum*, these results were particularly interesting; in effect, the soil parameters could be easily controlled by the producers.

This study demonstrates also a high degree of intraspecific differences in the essential oil due to the environmental factors, particularly the soil composition. The selection of cultural practices of *H. italicum* for desired phytochemical traits can be achieved relatively easy and could be used to improve the quality of *H. italicum* essential oil. Thus, it appears that a high interest in the component neryl acetate essentially correlates, on the one hand, with relatively weak acidity and low percentages of clay, fine sand, and coarse silt, and, on the other hand, with high percentages of coarse sand and fine silt. However, the chemical variability of essential oils may be due to other environmental parameters such irradiance, climate, or water availability.

Experimental Part

1. *Sample Preparation.* Fresh aerial parts of *H. italicum* subsp. *italicum* were hydro-distilled during 5 h using a *Clevenger*-type apparatus in accordance with the method recommended in the *European Pharmacopoeia* [27]. The yields of essential oils were in the range of 0.02–0.46%. The essential-oil yields of each population were reported in *Table 2*. The aerial parts of each plant were also cleaned, air-dried, and crushed; the powder was then mineralized according to the *Kjeldahl* method to measure N and, by dry way, with taking back ashes with HNO_3 to measure other elements. The soil samples were air-dried, N was measured according to the *Kjeldahl* method; P was extracted using H_2SO_4 at 0.002N (pH 3); Ca, Mg, K, and Na with a 1N NH_4OAc soln. and Fe, Mn, Zn, and Cu with a soln. of NH_4OAc (N)/EDTA.

2. *Determination of Essential-Oil Composition.* GC Analyses were carried out with a *Perkin-Elmer Autosystem XL* GC apparatus equipped with dual flame ionization detection (FID) system and fused-silica cap. columns (60 m × 0.22 mm i.d., film thickness 0.25 μm), *Rtx-1* (polydimethylsiloxane) and *Rtx-wax* (polyethyleneglycol). The oven temp. was programmed from 60° to 230° at 2°/min and then held isothermally at 230° for 35 min. Injector and detector temp. was maintained at 280°. Samples were injected in the split mode (1/50), using He as carrier gas (1 ml/min); the injection volume was 0.2 μl of pure oil. Retention indices (RI) of compounds were determined relative to the retention times of series of alkanes (C_5–C_{30}) with linear interpolation, using the *Van den Dool* and *Kratz* equation [28], and software from *Perkin-Elmer*. Component relative concentrations were calculated based on GC peak areas without using correction factors. Samples were also analyzed using a *Perkin-Elmer Turbo* mass detector (quadrupole), coupled to a *Perkin-Elmer Autosystem XL*, equipped with fused-silica cap. columns *Rtx-1* and *Rtx-Wax*. Carrier gas: He (1 ml/min), ion source temp.: 150°, oven temp. programmed from 60° to 230° at 2°/min and then held isothermally at 230° (35 min), injector temp.: 280°, energy ionization: 70 eV, electron-ionization (EI) mass spectra were acquired over the mass range 35–350 Da, split: 1/80, injection volume: 0.2 μl of pure oil. Identification of individual components was based on (*Table 1*): *i*) comparison of calculated RI values, on polar and apolar columns, with those of authentic compounds or literature data [29]; *ii*) computer matching with commercial MS libraries [30] and comparison of mass spectra with those of our own library of authentic compounds or literature data [29][31].

The majority of identified compounds was commercial standard components, and the few others were previously identified at large amount in essential oils or fractions obtained by CC, by comparison with literature spectral data and RIs, and ensured by ^{13}C-NMR [15][16].

3. *Determination of Inorganic Composition of Plant and Soil Samples.* The colorimetric analysis (continuous flux) was carried out using a *Technicon AAII* apparatus equipped with optical filters at 660 nm for N and at 420 nm for P. Samples were analyzed for K and Na by flame atomic emission spectrometry (FAES), and for Ca, Cu, Fe, Mg, Mn, and Zn by flame atomic absorption spectrometry (FAAS), using a *Varian Spectra 300* coupled equipped with an automated passor/diluor *Gilson 222*. The signals were measured at maximum intensity using the background correction (deuterium lamp). Dilution soln.: 5 g l^{-1} La for Ca ($\lambda = 422.6$ nm), K ($\lambda = 766.5$ nm), and Mg ($\lambda = 285.2$ nm), and 5% HNO_3 for Fe ($\lambda = 248.3$ nm), Mn ($\lambda = 279.5$ nm), Na ($\lambda = 590.0$ nm), Zn ($\lambda = 213.9$ nm), and Cu ($\lambda = 324.7$ nm). Other operating parameters were: flame type: air/acetylene; integration time: 3 s.

4. *Texture and Acidity of Soil Samples.* The particle-size analysis, percentages of clays, silt, and sand, was conducted according to the *Aubert* method [32]. The soil acidity was measured in both aq. and KCl solns. in order to determine real and potential acidity. To 10 g of each soil sample, 25 ml of dist. H_2O and KCl were added, respectively. pH Measurements were performed on a pH-*Meter-Hanna Instruments*, model *HI 9017*. To *ca.* 10 g of each soil sample, 25 ml of double dist. H_2O and 1M KCl, respectively, were added. Suspensions obtained were shaked periodically during 30 min, and pH was measured afterwards.

5. *Data Analysis.* Several studies have previously made extensive use of statistical methods to interpret different aspects of the metabolism of aromatic plants, demonstrating the usefulness of Principal Component Analysis (PCA). For instance, PCA has been recently used for determining the chemical variability of essential-oil compositions from *Hypericum* species [33].

PCA and cluster analysis were applied on the matrix linking essential-oil composition and locations in order to *i*) identify possible relationships between compound families and *ii*) gather sample locations in groups according to their oil composition (*Figs. 3* and *4*). Both methods aim at reducing the multivariate space in which objects (sample stations) are distributed but are complementary in their way to present results [34]. Indeed, PCA performs plans where both objects (locations) and variables (oil components) are plotted, while cluster analysis performs a classification tree where objects (station) are gathered.

PCA was carried out using function 'PCA' from the FactoMineR Package of R statistical software. The cluster analysis produced a dendrogram (tree) using the *Ward*'s method of hierarchical clustering, based on the Euclidean distance between pairs of stations. The function 'agnes' from the cluster package (R software) was used.

In a second step, we partitioned the variation in *H. italicum* essential-oil components (Y) into four potential influences (see the *Tables*): the inorganic soil composition, the inorganic plant composition, the physicochemical parameters of soils (texture and acidity), and the plant stage of vegetation.

To this aim, we used a combination of RDA and partial RDA. RDA is an ordination method that can be considered as a multivariate extension of multiple linear regressions. RDA is PCA modified to constrain the ordination axes to be linear combinations of a set of explanatory variables given in a separate matrix [33]. Basically, in a RDA of a matrix Y, where a matrix X of explanatory variables is used to constrain the analysis, the sum of canonical eigenvalues divided by the total trace (or sum of all eigenvalues) corresponds to the amount of variation in the Y data explained by the variables contained in the explanatory data X [34][35]. If the analysis includes a matrix of co-variables W, then these variables may be partialled out of the analysis to determine the 'pure' effect of X [35]. This method, proposed first by *Borcard et al.* [36] for two groups of explanatory variables, was extended to four groups of explanatory variables and is implemented in the function 'varpart' from the R package [37].

With four sets of explanatory variables, the fractions explained uniquely by each of the four sets are [a] to [d], joint fractions between two sets are [e] to [j], joint fractions between three sets are [k] to [n], and the joint fraction between all four set is [o]. Their different fractions were presented on a *Venn* diagram (*Fig. 5*). [p] represents the part of unexplained variation. For instance, [a], which represents the pure effect of the inorganic soil composition on essential oil composition, was estimated using X as a matrix containing inorganic soil components and W containing all the variables belonging to the other

sets (inorganic plant composition, soil texture and acidity, and stage of vegetation). Once each fraction has been estimated ([a] to [o]), then [p] is deduced and the *Venn* diagram is built.

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