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Chemotaxonomic study on *Thymus villosus* from Portugal

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Abstract

The composition of the essential oils of four populations of *Thymus villosus* subsp. *lusitanicus* (Boiss.) Coutinho from Portugal was investigated by GC and GC-MS. To study the chemical polymorphism the results obtained from GC analyses of the volatile oils from individual plants from four populations were submited to Principal Component and Cluster analyses. A comparision with the essential oil of *T. villosus* subsp. *villosus*, previously studied by us was done. Important differences with regard to the major constituents in these two taxa were found. Linalool, geranyl acetate, geraniol and terpinen-4-ol were the main components of the essential oils of *T. villosus* subsp. *lusitanicus*, whereas in the oil of *T. villosus* subsp. *villosus* *p*-cymene, myrcene and α-terpineol were the major ones. Although, both taxa showed chemical polymorphism, different types of essential oils were characterized in each one: linalool; linalool/terpinen-4-ol/*trans*-sabinene hydrate; linalool/1,8-cineole; geranyl acetate/geraniol; geranyl acetate/geraniol/1,8-cineole in *T. villosus* subsp. *lusitanicus* and *p*-cymene/camphor/linalool; *p*-cymene/borneol; linalool/geraniol/geranyl acetate; α-terpineol/camphor/myrcene in *T. villosus* subsp. *villosus*. Thus, the two subspecies of *T. villosus* can be easely differenciated by the composition of their essential oils.

Keywords: *Thymus villosus* subsp. *lusitanicus*; *Thymus villosus* subsp. *villosus*; Lamiaceae; Essential oils; Infraspecific variability; Chemotaxonomy; Multivariate analysis

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1. Introduction

Thymus villosus L. belongs to the section *Pseudothymbra* Bentham of the genus *Thymus* L. Morales (1986) recognized two subspecies, *T. villosus* subsp. *villosus* and *T. villosus* subsp. *lusitanicus* (Boiss.) Coutinho. The former has coloured and dentate bracts, corolla up to 10 mm long and is an endemic plant from central and south-eastern Portugal, while the latter has uncoloured bracts, corolla up to 6.5 mm long and is an Iberian endemic plant (Estremadura and Beira Litoral provinces from Portugal and Cáceres, Ciudad Real and Toledo from Spain). Nevertheless, the latter has a large polymorphism perhaps due to its scattered distribution. In central part of Portugal the two subspecies have similar distribution. So, in that area, sometimes it is difficult to distinguish these two taxa. In previous studies, we already demonstrated the existence of several chemotypes in the essential oil of *T. villosus* subsp. *villosus* (Salgueiro et al., 1997). Thus, the aim of this paper is to investigate the composition and infraspecific variability of the essential oils of *T. villosus* subsp. *lusitanicus* from Portugal, which had not been previously reported in the literature, and compare the results found for both subspecies of *T. villosus* in order to distinguish them through the composition of their essential oils.

In Spain, Pérez-Alonso and Velasco-Negueruela (1984) studied the essential oils of three populations, one of which (Toledo) had camphor and borneol as major compounds whereas the other ones (Cáceres and Ciudad Real) showed high amounts of camphor, linalool and 1,8-cineole. Later, Morales (1986) reported camphor and borneol as the major compounds in a sample from Toledo.

We now report on the results obtained for the essential oil composition and variability of *T. villosus* subsp. *lusitanicus* from Portugal. For that purpose, four different sampling sites were chosen, and the qualitative and quantitative analysis of the volatile oil of a representative population sample of each one was performed by GC and GC-MS. To study the infraspecific variability, the oils of individual plants from each locality were analysed mainly by GC, and the data obtained were submitted to principal component analysis and cluster analysis, in order to detect some pattern distribution of individual plants and to identify which constituents can differentiate the groups of individuals.

2. Material and methods

2.1. Plant material

Aerial parts of the plants were collected at the flowering stage, in June 1992, in the Beira Litoral and Estremadura provinces of Portugal: S. Jorge (A), Azóia (B), Moita Lina-Porto de Mós (C) and Serra do Bouro (D).

Voucher specimens of each population were deposited in the Herbarium of the Instituto Botânico of the University of Coimbra, Portugal (COI).

In order to study the chemical polymorphism, 63 individual plants were also collected. These individuals were collected at random in the same localities at the

same time that the corresponding representative population samples in order to reduce seasonal variability.

2.2. Analysis of the essential oils

The essential oil content of the air-dried plant material was determined according to the European Pharmacopoeia method (Conseil de l' Europe, 1983). Analyses of the essential oils of the representative population samples obtained by water distillation were carried out by GC and GC-MS using fused silica capillary columns with two different stationary phases (Carbowax 20M and Methylsilicone SE-30), as previously described (Adzet et al., 1989; Salgueiro et al., 1995). Components were identified by comparison of their retention indices, relative to a homologous series of fatty acid methyl esters, and mass spectra, with those in our library, literature data and authentic samples.

The quantification of the components was made on the basis of their GC peak areas on the two columns.

2.3. Chemical polymorphism

The essential oil of each individual plant was analyzed by GC and GC-MS on the two stationary phases, using the same analytical conditions indicated above. From all the constituents, those which showed a percentage equal or higher than 2% were selected to be included in the multivariate analysis (20 constituents $\times 63$ individuals $= 1260$ data). Selected constituents are shown in Table 1.

All data were processed by PARVUS (Forina et al., 1988) and ESTATS (Tomàs et al., 1988) chemometric packages.

The whole data set was analyzed by principal component analysis in order to prove if it can be projected in the space defined by the three first principal components and the projection coordinates of each individual in that reduced space ("scores") were evaluated for their posterior graphical presentation.

Cluster analysis was also applied to all individuals data, using the euclidean distance between individuals as an index of their similarity and clustering was performed according to the weighed average linkage method. If clusters are defined, they are also denoted in the graphical presentation of individual scores.

3. Results and discussion

The average yield of essential oil of the air-dried aerial parts of the four populations of *T. villosus* subsp. *lusitanicus* was 1.2% (v/w). More than 95% (86 compounds) of the volatile constituents were identified in each sample. Table 1 shows the tabulated results. Compounds are listed in order of their elution on a carbowax 20M column. Oxygenated monoterpenes were the main group of constituents in all populations (67.2–75.4%). Nevertheless, important differences in the amounts of the major constituents were found, mainly of linalool (24.5–9.0%), geranyl acetate (23.9–11.5%),

Table 1
Composition of the essential oils of four populations of *Thymus villosus* subsp. *lusitanicus* from Portugal

Components[a]	% in sample			
	A	B	C	D
Tricyclene	—	0.1	t	—
α-Thuyene	1.0	0.7	0.9	0.6
α-Pinene	1.1	0.7	1.1	0.6
Camphene	0.3	1.1	0.3	0.1
β-Pinene	1.0	0.5	1.1	0.5
Sabinene	1.1	0.6	0.4	0.8
Myrcene[b]	3.1	4.2	2.1	0.8
α-Phellandrene	0.2	t	0.1	t
α-Terpinene	1.3	0.6	1.3	t
Limonene[b]	1.3	1.1	1.3	2.0
1,8-Cineole[b]	1.1	4.7	1.0	5.0
cis-β-Ocimene	0.1	0.1	0.1	0.1
trans-β-Ocimene	0.3	0.9	0.2	0.8
γ-Terpinene[b]	5.5	1.4	4.8	1.0
Octan-3-one	—	0.3	t	0.2
p-Cymene[b]	5.0	1.6	4.0	1.7
Terpinolene	1.0	0.3	1.0	0.2
1-Octen-3-yl acetate	0.1	t	0.1	0.1
Octan-3-ol	t	0.1	0.1	0.1
Nonanal	t	0.2	0.1	t
Oct-1-en-3-ol	0.1	0.3	0.1	0.2
cis-Linalool oxide	0.3	0.3	0.2	0.1
α-*p*-Dimethylstyrene	0.1	0.2	t	0.2
trans-Sabinene hydrate[b]	2.0	2.0	3.8	1.6
trans-Linalool oxide	0.1	0.1	0.1	t
Campholenal	t	0.1	t	t
β-Bourbonene	0.1	t	t	t
Camphor	0.4	2.0	0.4	0.1
Linalool[b]	12.0	9.0	24.5	24.0
cis-Sabinene hydrate[b]	0.5	2.0	2.0	0.8
α-Gurjunene	t	—	t	0.1
Linalyl acetate[b]	t	1.1	1.1	0.3
Pinocarvone	t	t	t	0.1
Bornyl acetate	0.3	0.1	0.3	0.1
β-Elemene	—	0.3	t	0.4
Terpinen-4-ol[b]	13.0	5.0	15.2	5.5
β-Caryophyllene	0.5	1.1	1.1	1.1
cis-Dihydrocarvone	0.2	—	0.2	—
allo-Aromadendrene	0.2	0.4	0.2	0.4
cis-Verbenol	t	—	t	—
δ-Terpineol	0.3	0.3	0.4	0.3
trans-Verbenol	0.1	0.2	0.1	t
α-Humulene	—	—	t	—
Neral[b]	0.6	0.9	0.2	1.8
α-Terpineol[b]	0.8	2.6	1.2	1.5
α-Terpinyl acetate	0.2	0.2	0.3	0.2
Borneol	0.1	0.8	0.5	0.3
Geranyl formate	t	0.1	—	0.1

Table 1—*continued*

Components[a]	% in sample			
	A	B	C	D
γ-Muurolene	0.1	t	t	0.1
Germacrene D	0.1	0.1	0.1	0.1
Neryl acetate	0.5	0.3	0.2	0.3
Geranial[b]	0.9	1.8	0.3	2.0
Carvone	0.1	t	0.1	—
Geranyl acetate[b]	23.9	19.1	11.5	14.3
Citronellol	t	t	—	0.3
δ-Cadinene	0.4	0.3	0.4	0.5
Nerol	0.4	0.3	0.2	0.9
Geranyl isobutyrate	0.1	t	t	0.2
Geranyl propionate	t	0.1	t	t
trans-Carveol	0.1	t	0.1	t
Cuparene	t	0.4	t	t
Geraniol[b]	8.1	18.0	7.2	14.0
p-Cymene-8-ol	t	0.1	t	0.2
Geranyl butyrate	0.3	0.7	0.1	0.6
Geranyl isovalerate	0.3	0.5	0.1	0.5
β-Caryophyllene oxide[b]	0.2	1.6	0.2	0.3
Ledol	t	0.2	t	0.2
E-Nerolidol	1.2	0.1	0.9	0.1
Cubenol	t	0.5	t	0.1
β-Elemol[b]	0.5	0.5	0.4	2.0
Viridiflorol	0.2	0.2	0.2	0.3
Geranyl caproate	t	0.1	—	0.2
Cuminic alchool	0.1	0.1	t	—
Spathulenol	0.1	0.3	0.1	—
Eugenol	0.2	t	0.1	—
γ-Eudesmol	0.1	t	0.1	0.3
T-Cadinol[b]	0.3	0.6	0.3	0.6
Thymol	0.1	0.1	0.1	—
T-Muurolol	—	—	—	0.3
Carvacrol	—	t	—	—
α-Elemol	—	—	—	t
a-Eudesmol	0.2	t	0.1	—
α-Cadinol	0.2	0.8	0.2	0.8
β-Eudesmol	t	—	t	0.1
Intermedeol[b]	0.2	1.2	0.3	1.3
Eugenyl acetate	—	t	—	—
Monoterpene hydrocarbons	22.4	14.2	18.8	9.5
Oxygenated monoterpenes	67.2	72.9	71.6	75.4
Sesquiterpene hydrocarbons	1.5	2.7	2.0	2.8
Oxygenated sesquiterpenes	3.3	6.1	2.9	6.4
Others	0.6	1.0	0.5	0.7
Total identified	95.0	96.9	95.8	94.8

t: traces ($\leqslant 0.05\%$)

[a]Compounds are listed in order of their elution from a Carbowax 20M column.

[b]Constituents selected for the multivariate analysis.

geraniol (18.0–7.0%) and terpinen-4-ol (15.2–5.0%). The results showed some important differences between the populations, indicating the existence of chemical polymorphism, as it happened in Spain. Meanwhile, the essential oils of the Portuguese plants can be easely distinguished from the Spanish ones. The latter have higher amounts of camphor, borneol and 1,8-cineole than the Portuguese ones, and

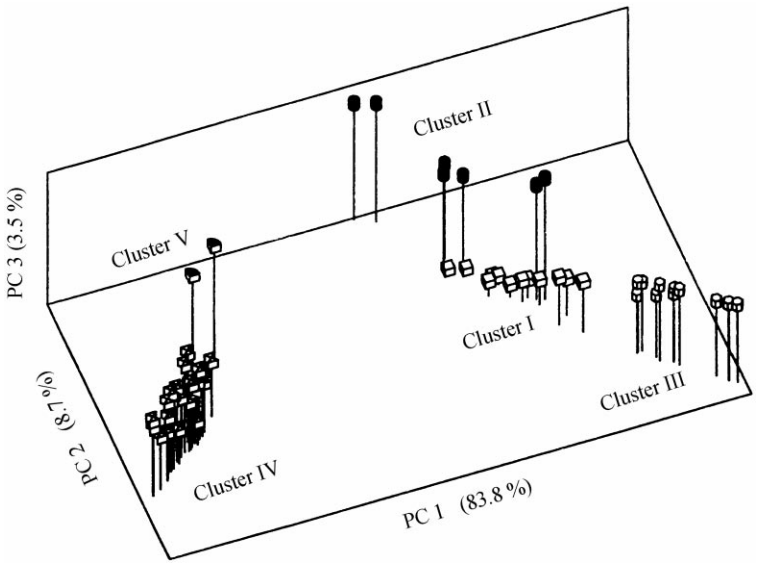

Fig. 1. Relative position of individuals of T. *villosus* subsp. *lusitanicus* in the space defined by the first three Principal components.

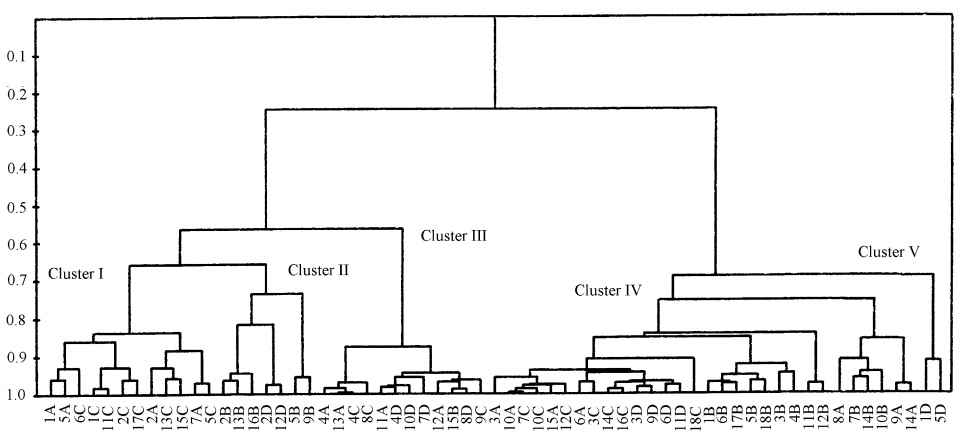

Fig. 2. Two-dimensional dendrogram obtained in the cluster analysis of the essential oils of individual plants of *T. villosus* subsp. *lusitanicus*. Horizontal: samples analysed. Vertical: similarity levels between samples.

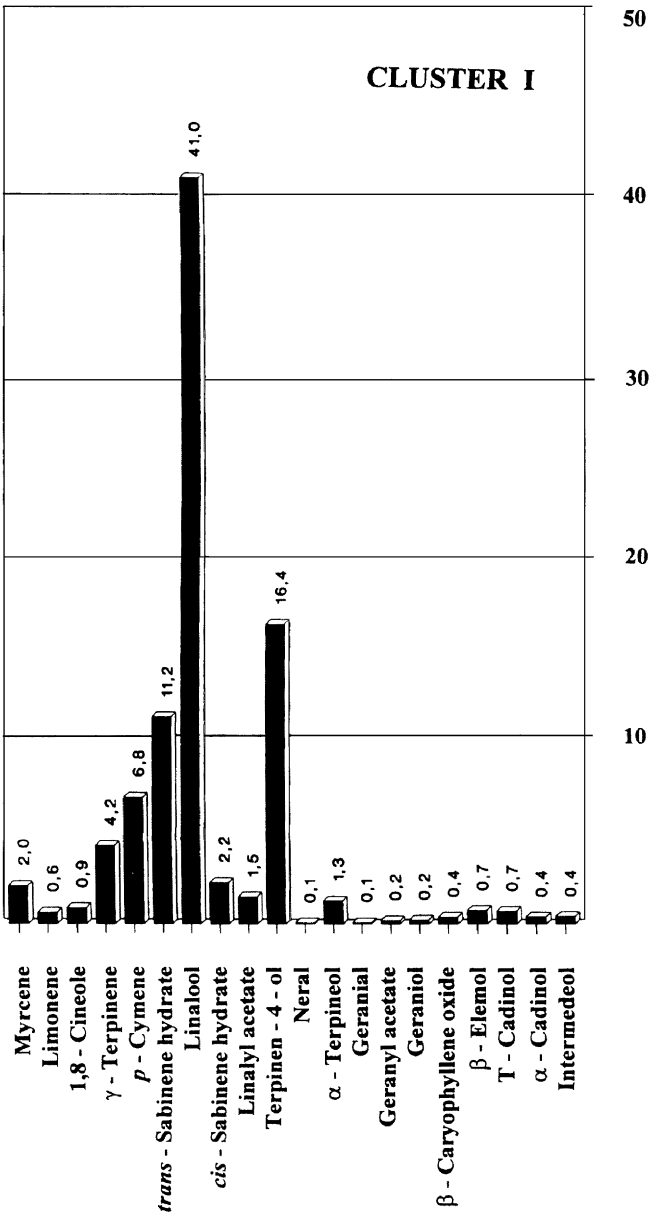

Fig. 3. Mean chemical composition of essential oil of cluster I of *T. villosus* subsp. *lusitanicus*. Vertical: mean percentage in the essential oil.

neither geranyl acetate nor geraniol (Pérez-Alonso and Velasco-Negueruela, 1984), while in the Portuguese essential oils these two compounds have always high percentages.

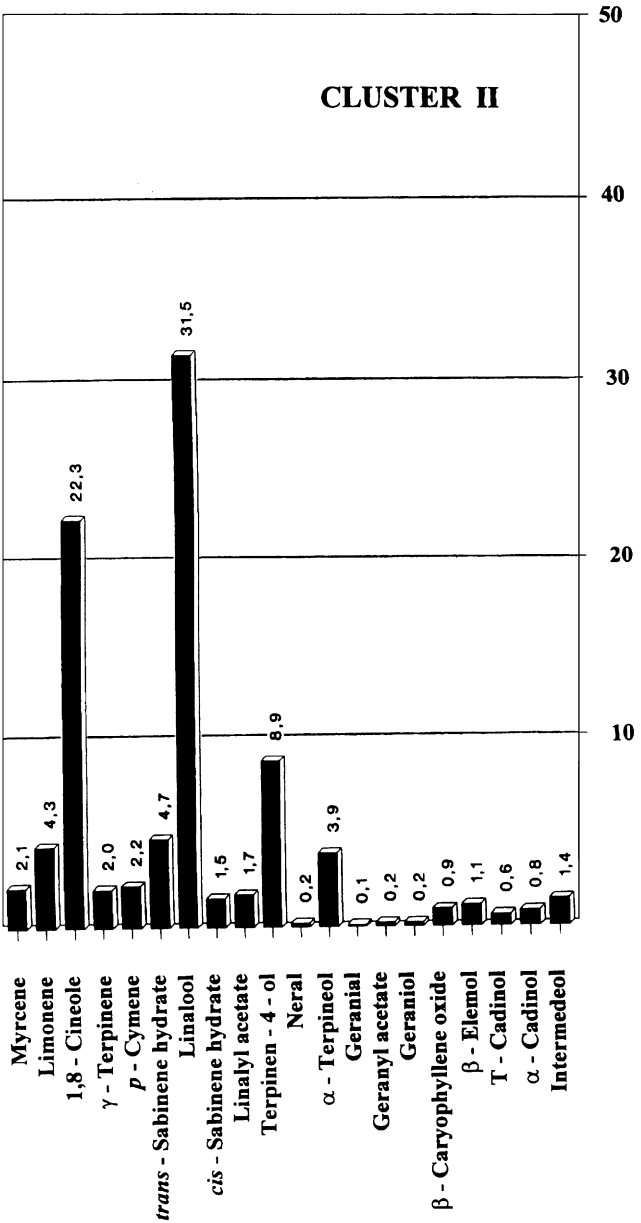

Fig. 4. Mean chemical composition of essential oil of cluster II of *T. villosus* subsp. *lusitanicus*. Vertical: mean percentage in the essential oil.

The application of multivariate analysis techniques to the results obtained in the analysis of the essential oils of individual plants allowed us to establish two main types of essential oils: linalool and geraniol/geranyl acetate. Although in both types some

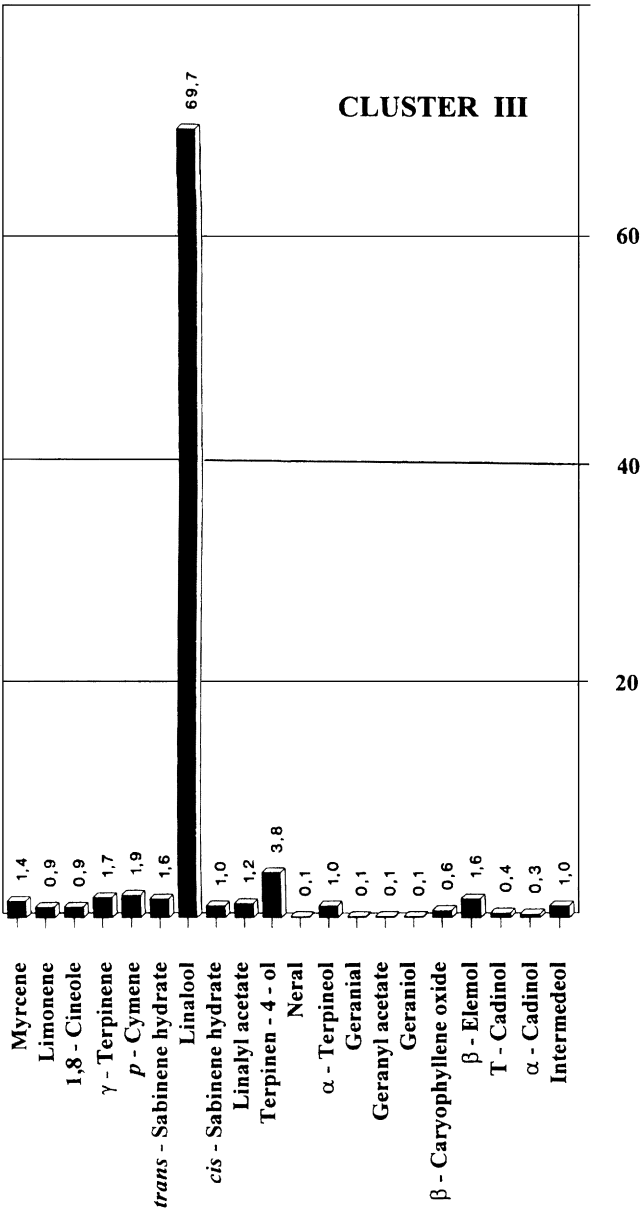

Fig. 5. Mean chemical composition of essential oil of cluster III of *T. villosus* subsp. *lusitanicus*. Vertical: mean percentage in the essential oil.

subgroups could be differentiated by the cluster analysis and principal component analysis (Figs. 1 and 2, respectively), as the first three components from PCA accounted for 83.8, 8.7 and 3.5% of the total variation. Cluster I was characterized by

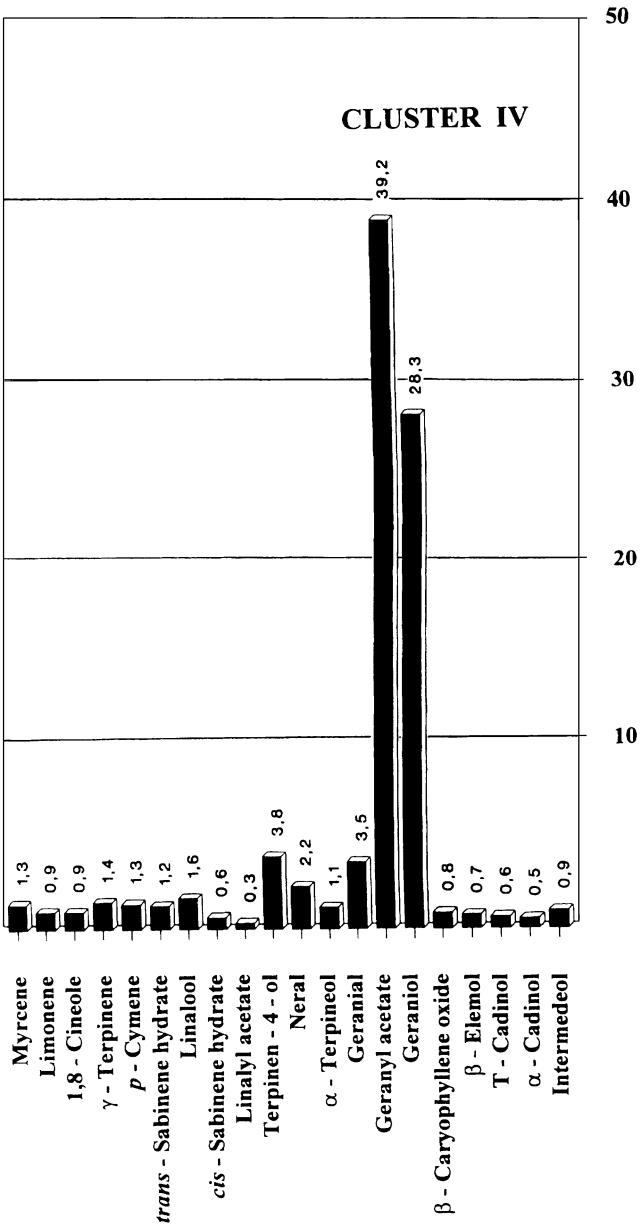

Fig. 6. Mean chemical composition of essential oil of cluster IV of *T. villosus* subsp. *lusitanicus*. Vertical: mean percentage in the essential oil.

a high percentage of linalool, terpinen-4-ol and *trans*-sabinene hydrate (average 41.0, 16.4 and 11.2%, respectively; 19.1% of the samples analyzed); Cluster II had linalool and 1,8-cineole as the major compounds (average 31.5 and 22.3%; 11.1% of the

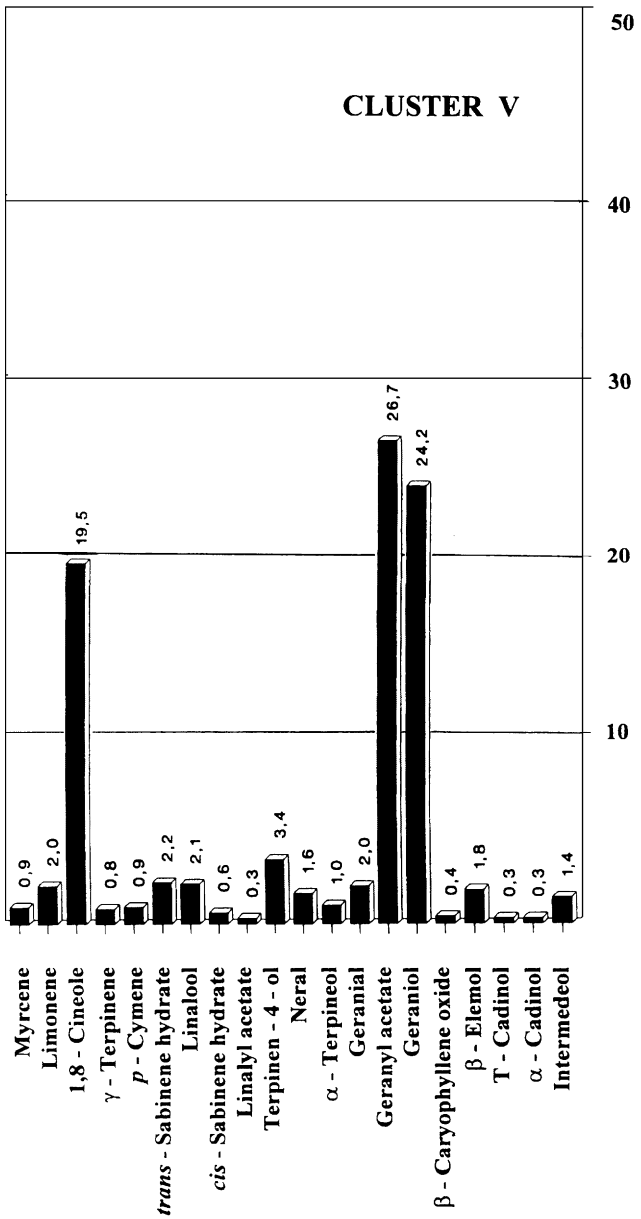

Fig. 7. Mean chemical composition of essential oil of cluster V of *T. villosus* subsp. *lusitanicus*. Vertical: mean percentage in the essential oil.

samples analyzed); in Cluster III linalool was the main constituent (average 69.7%; 19.0% of the samples analyzed); Cluster IV is characterzed by a high rate of geranyl acetate and geraniol (average 39.2 and 28.3%; 47.6% of the samples analyzed); and,

finally, Cluster V showed substantial percentages of geranyl acetate, geraniol and 1,8-cineole (average 26.7, 24.2 and 19.5%; 3.2% of the samples analyzed). The mean chemical composition of the essential oil of each cluster is presented in Figs. 3–7.

In conclusion, the two subspecies of *T. villosus* can be easely differenciated by the composition of their essential oils. *T. villosus* subsp. *lusitanicus* is characterized by a high percentage of oxygenated monoterpenes, whereas *T. villosus* subsp. *villosus* show large proportions of monoterpenes, either hydrocarbons or oxygenated. In this taxon *p*-cymene was always an important constituent (28.0–10.9%), excepting one sample which has a high amount of myrcene (13.0%) and α-terpineol (16.5%). In the essential oils of *T. villosus* subsp. *lusitanicus* *p*-cymene, myrcene and α-terpineol attained only 5.0, 4.2 and 2.6%, respectively. So, important differences with regard to the major constituents in these taxa were observed. Both of them showed chemical polymorphism. Nevertheless, different types of essential oils were characterized in each taxon: linalool; linalool/terpinen-4-ol/*trans*-sabinene hydrate; linalool/1,8-cineole; geranyl acetate/geraniol; geranyl acetate/geraniol/1,8-cineole in *T. villosus* subsp. *lusitanicus* and *p*-cymene/camphor/linalool; *p*-cymene/borneol; linalool/geraniol/geranyl acetate; α-terpineol/camphor/myrcene in *T. villosus* subsp. *villosus*.

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