Correlation between the Chemical and Genetic Relationships among Commercial Thyme Cultivars

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The essential oil composition and genetic variability of six commercial cultivars of thyme (*Thymus vulgaris* L.), a Mediterranean medicinal and aromatic plant, were analyzed by GC-MS and randomly amplified polymorphic DNA (RAPD), respectively. All evaluated cultivars belong to the thymol chemotype, with differences in the concentrations of thymol, γ-terpinene, *p*-cymene, and other minor components. The comparison of the oil components concentration by multivariate analysis allowed separation of the cultivars into two groups. All of the cultivars exhibited characteristic RAPD patterns that allowed their identification. On the basis of the RAPD patterns, the cultivars could be divided into two clusters, which coincides with results obtained by oil GS-MS analysis, with a correlation coefficient of −0.779.

Keywords: *Thymus vulgaris L.; thyme; oil composition; RAPD; molecular markers; genetic distances*

INTRODUCTION

Thymus vulgaris L. (Labiatae), or common thyme, is an aromatic perennial herb of the Mediterranean flora. Thyme is a medicinal plant used from ancient times in Europe. Its essential oil, which represents 0.7−6.5% of the dry weight of leaves and flowers, has antitussive, expectorant, spasmolytic, antiseptic, antifungal, antiphlogistic, rubefacient, and anthelmintic properties. At present, thyme is important in the food and cosmetic industries (*1*, *2*) and is cultivated all over the world.

Genetic constitution and environmental conditions influence the yield and composition of volatile oil produced by thyme plants. Granger and Passet (*3*) described six chemotypes of *T. vulgaris* growing in southern France. The chemotypes could be classified by their components as geraniol, linalool, α-terpineol, thujanol-4 and terpinen-4-ol, thymol, and carvacrol. A seventh type, 1,8-cineole, is known to grow in Spain (*4*). The main components represent 60−95% of the essential oil, depending on the type. Genetic control of the chemical characteristics of thyme plants has been proven, and the monoterpenes accumulated by the plant are controlled by a series of loci with epistatic relationships between them (*5*).

Thyme is propagated by seeds or by stem cuttings. Pollination is mainly effected by bees (*6*). Male sterile and male fertile plants have been identified in several populations (*7*), leading to high levels of polymorphism. Natural populations are usually heterogeneous, composed by plants of different chemotypes. No correlation has been observed between the morphological and anatomical features of a plant and its chemical composition (*3*). However, correlations between chemotype polymorphism, sexual polymorphism, and the environment have been detected (*8*).

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At present, seeds of thyme are commercialized by many companies. Most of the cultivars are not chemically, or genetically, characterized, and the similarity of their morphological and anatomical features poses a problem for adequate identification. The objective of the present work was to evaluate the genetic and chemical variation among commercial thyme cultivars to obtain information about their potential for the extraction of essential oils and their inclusion in breeding programs.

MATERIALS AND METHODS

Plant Material. Commercial seeds form six companies were used in the present study. The cultivars were as follows: Burpee, from the United States; Batlle, from Spain; Tropical, from France; Blumen, from Italy; SEM, from the European Union; and Isla, from Brazil. Seeds were germinated in a mixture of sand, vermiculite, and soil (1:1:2), and the plantlets were grown under greenhouse conditions for 2 months. Thirty plants of each cultivar were transferred to the experimental area of the Institute of Biotechnology of the University of Caxias do Sul. The field experiment was designed in complete randomized blocks of 10 plants with three replications.

Essential Oil Extraction. Plant materials was collected in March 1999. Ten adult flowering plants (2 years old) of each cultivar were used to form a composite sample that represented the six commercial cultivars. Three bulked samples per cultivar were analyzed. Extracts were obtained by 1 h of steam distillation in a Clevenger apparatus by using 150 g of dried plant material. The oil volume was measured directly in the extraction buret. All of the extractions were made and analyzed in duplicate.

Chemical Composition of the Essential Oil. The identification of the chemical components of the essential oil samples was carried out by using an HP 6890 gas chromatograph coupled with a mass selective detector HP 5973 and equipped with ChemStation software and Wiley 275 spectra data. An HP-5 fused silica capillary column (30 × 0.25 μm i.d., 0.25 μm film thickness) was used. The chromatographic conditions were as follows: column temperature, 60 °C (8 min), raised from 60 to 180 °C (3 °C/min) and from 180 to 230 °C (20 °C/min), 230 °C (20 min); interface, 180 °C; split ratio,

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Table 1. Chemical Composition of Essential Oil Extracts from Six Commercial Thyme Cultivars Obtained by GC-MS[a]

	Burpee	Batlle	Tropical	Blumen	SEM	Isla	mean[b]
α-thujene	1.87	1.73^{bc}	1.60^{c}	1.65^{bc}	1.97^{a}	1.35^{c}	1.70
α-pinene	0.82^{b}	0.92^{ab}	0.79^{b}	0.79^{b}	1.07^{a}	0.78^{b}	0.86
camphene	0.45^{c}	0.59^{b}	0.36^{d}	0.41^{cd}	0.74^{a}	0.56^{b}	0.52
β-pinene	0.23^{a}	0.23^{a}	0.21^{a}	0.22^{a}	0.26^{a}	0.20^{a}	0.23^{ns}
myrcene	1.92^{b}	1.96^{b}	1.83^{b}	1.92^{b}	2.12^{a}	1.63^{c}	1.90**
α-phellandrene	0.24^{a}	0.25^{a}	0.22^{a}	0.25^{a}	0.26^{a}	0.20^{a}	0.24^{ns}
α-terpinene	2.45^{a}	2.49^{a}	2.06^{b}	2.45^{a}	2.58^{a}	2.12^{b}	2.36*
p-cymene	13.17^{b}	13.06^{b}	11.69d	12.34^{c}	13.99^{a}	11.68^{d}	12.66**
1,8-cineole	0.34^{a}	0.23^{a}	0.35^{a}	0.34^{a}	0.34a	0.41^{aa}	0.34^{ns}
γ-terpinene	19.21^{b}	22.26^{a}	11.47^{c}	22.28^{a}	22.20 "	19.02^{b}	19.41
cis-sabinene hydrate	1.21^{a}	1.08^{ab}	0.96^{b}	1.06^{ab}	1.09^{ab}	0.68^{c}	1.01
linalool	1.86^{c}	2.16^{b}	2.55^{a}	1.61d	1.80^{c}	2.51	2.08**
borneol	0.90^{b}	1.29^{a}	0.68^{b}	0.75^{b}	1.188	1.208	1.00
terpinen-4-ol	0.49^{b}	0.54^{ab}	0.47^{b}	0.52^{b}	0.48^{b}	0.73^{aa}	0.54**
thymol	46.47^{c}	43.51^{d}	50.47^{a}	44.11^{d}	41.96^{e}	48.64^{b}	45.86*
carvacrol	2.64^{a}	0.06^{b}	0.00^{b}	2.13^{a}	2.30^{a}	0.00^{b}	1.16**
thymyl acetate	0.07^{a}	0.00^{a}	0.07^{a}	0.00^{a}	0.00^{a}	0.07^{a}	0.04^{ns}
β-caryophyllene	0.63^{ab}	0.66^{ab}	0.87^{a}	0.68^{ab}	0.59^{b}	0.77^{ab}	0.70*
germacrene D	0.17^{c}	0.36^{a}	0.26^{bc}	0.31^{ab}	0.17^{c}	0.20^{c}	0.25*
nonoxygenated terpenes	41.16	44.51	31.34	43.30	45.95	38.53	40.80
monoterpenes	40.36	43.49	30.23	42.31	45.19	37.56	39.86
sesquiterpenes	0.80	1.02	1.13	0.99	0.76	0.97	0.95
oxygenated terpenes	53.98	48.87	55.55	50.52	49.15	54.24	52.05*
esters	0.07	0.00	0.07	0.00	0.00	0.07	0.04^{ns}
alcohols	4.46	5.07	4.66	3.94	4.55	5.12	4.63**
ethers	0.34	0.23	0.35	0.34	0.34	0.41	0.34^{ns}
phenols	49.11	43.57	50.47	46.24	44.26	48.64	47.05**

[a] Percentual values. Means followed by different letters (within a line) are significantly different according to the Tukey test ($p = 0.05$). [b] General mean considering all of the cultivars and *F* test results for each component: ns, nonsignificant; ***,** significant ($p = 0.05$); **, significant ($p = 0.01$).

1:100; carrier gas, He (55.4 kPa); flow rate, 1.0 mL/min; ionization energy, 70 eV; mass range, 40−350; volume injected, 0.5 gL; solvent cut, 3.5 min.

Statistical Analysis of Oil Composition. Average Euclidian distances between each pair of cultivars were calculated using patronized data [$xj = Xj/\sigma(Xj)$] of their oil composition (*9*). The average Euclidian distances matrix was used to evaluate the relationship between cultivars by single-linkage and Tocher cluster analysis. The two cluster methods offer different advantages: the single-linkage method shows gradual differences among accessions in the dendrograms, whereas Tocher's method indicates components of the groups according to a progressive comparison from the smallest to the highest distances (*10*). The data were also submitted to principal components analysis to identify the most important variables. All of these analyses were made using the GENES program (*11*).

DNA Extraction and Randomly Amplified Polymorphic DNA (RAPD) Analysis. Equal amounts (0.5 g) of leaf tissue from each of 10 plants of each cultivar were combined to make composite leaf samples of each cultivar. The composite samples were frozen in liquid nitrogen, ground and mixed with a mortar and pestle, and stored at −20 °C or immediately used for DNA extraction.

DNA was extracted from 1.5 g of leaf powder using a CTAB protocol (*12*), and triplicate extractions were made for each entry. DNA pellets were dissolved in 200 μL of TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and the DNA content of all samples was measured using a spectrophotometer at 260 nm.

Fifteen decamer oligonucleotides of kits A, B, and W, obtained from Operon DNA Technologies (Alameda, CA), showing consistent banding patterns and amplification, were chosen for use in this study on the basis of the number of amplification products and the level of polymorphism. The Polymerase Chain Reaction DNA amplification (*13*) was performed in a 25 μL volume containing 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 3 mM $MgCl_2$; 0.25% Triton X-100; 1.25 mM each dATP, dCTP, dTTP, and dGTP (Pharmacia LKB Biotechnology, Inc.); 30 ng of a single decamer primer; 60−80 ng of genomic DNA; and 1.5 units of Taq DNA polymerase (Pharmacia LKB Biotechnology). DNA amplification was performed using a thermal cycler (model PTC100, MJ Research, Watertown, MA). The thermal cycle used was 94 °C for 1 min followed by 45 cycles of 94 °C (1 min), 35 °C (1 min), and 72 °C (2 min), and finally 72 °C for 3 min. A negative control including all components except genomic DNA was included in all thermal cycle runs.

Following amplification, the RAPD products (20 μL) were loaded in 1.5% agarose gels in 1× TBE buffer and separated by electrophoresis. After electophoresis, the amplification products were stained with ethidium bromide and photographed under UV light. The size of amplification products was determined by comparison to Lambda DNA digested with *Eco*RI and *Hin*dIII restriction enzymes.

Bands were scored as present (1) or absent (0). Only bands that were between 100 and 2500 bp and that appeared consistently among three independent runs were rated. Bands that were not well-defined were not included in the data set, as these were assumed to be unreliable markers. Band-sharing analysis (Jaccard's coefficient) was conducted using an NTSYS-pc (*14*).

RESULTS AND DISCUSSION

Oil yields ranged from 0.47 to 0.64%. The highest yields were obtained from the cultivars Tropical and SEM (0.64 and 0.63%, respectively), and the lowest yields were obtained form the cultivars Burpee and Isla (0.47%). Oil yields were relatively low when compared with other reports (*15*). This can be attributed to the environmental conditions in which the plants were grown and the genetic constitution of the cultivars, as these two factors greatly influence both oil yield and composition of thyme (*16*).

Analysis of the oil composition (Table 1) showed that all of the commercial cultivars assayed belong to the thymol chemotype, with a high level of thymol (41.96−50.47%) compared with the other components, especially those that characterize the other chemotypes: geraniol, linalool, terpineol, thujonol-4, terpinen-4-ol, carvacrol, or 1,8-cineole (*2*−*4*). Other important compounds de-

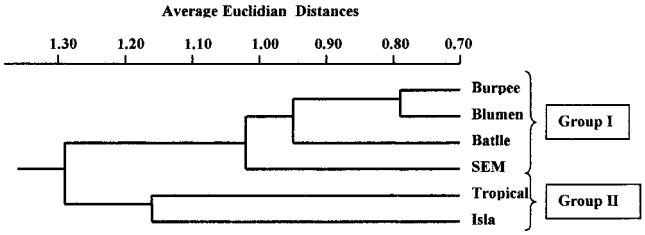

Figure 1. Dendrogaram obtained by single-cluster analysis of six commercial cultivars of thyme based on the chemical composition of their essential oils.

tected were γ-terpinene and *p*-cymene. The highest variation among the main components of the essential oil was observed for γ-terpinene concentration, which varied between 11.47% for the Tropical cultivar and 22.28% for the Blumen cultivar.

Multivariate analysis of the chemical composition of the essential oils allowed separation of the cultivars into two different groups (Figure 1). Group I was formed by the cultivars Burpee, Blumen, Batlle, and SEM and group II by the cultivars Tropical and Isla. The most important variables (components) for discrimination were α-terpinene, borneol, germacrene D, γ-terpinene, and thymol. The cultivars that form group II are characterized by low levels of α- and γ-terpinene and high concentration of thymol.

To evaluate the genetic relationships, 15 decamer oligonucleotide primers were used to amplify aleatory segments of DNA of the genome of the six commercial thyme cultivars. These primers were initially selected from kits A, B, and W on the basis of the number of amplification products and the stability of the patterns. The number of bands obtained by primer varied from 5 (OPA-04) to 18 (OPA-01). An example of the banding patterns obtained is shown in Figure 2.

From the total of 163 fragments amplified and scored, 104 were polymorphic (63.8%). With this many bands analyzed it can be considered there is a good representation of the genome of the cultivars. The percentage of polymorphic bands varied from 18.2% (OPA-03) to 90.9% (OPA-05) depending on the primer. The level of polymorphism obtained is within the range expected for allogamous plants (*17*).

The ability to differentiate all tested cultivars by RAPD bands suggests that this technique may provide a rapid and inexpensive method for the identification of thyme cultivars, even between phenotypically similar materials, as is the case for identification of other plant species (*17*). Moreover, RAPD fingerprinting will help in the identification of hybrids and in the evaluation of genetic purity of thyme cultivars.

The similarity of the cultivars, estimated by the Jaccard's coefficient, based on RAPD data, is depicted in the unweighted pair-group method with arithmetic averages (UPGMA) dendrogram (Figure 3). The six cultivars of thyme fell into two clusters at a 0.70 level of similarity. Group I was formed by four cultivars, Burpee, Blumen, Batlle, and SEM, and group II by two cultivars, Tropical and Isla.

Comparing the groups formed by the cluster analysis based on the chemical composition (Figure 1) and that obtained from RAPD data (Figure 3), we can observe that the groups formed in both cases have the same cultivars, in the same order. The correlation (Pearson coefficient) between the distances obtained by both methods was -0.779 ($p = 0.01$). These results suggest that oil composition is controlled by a high number of genes widely distributed over the plant genome. High correlation between genetic and chemical distance matrices was obtained in other aromatic and medicinal plants such as *Ocimum gratissimum* (*18*) and *Tanacetum vulgare* (*19*).

The results show that both oil composition and RAPD analysis can be used to identify thyme cultivars and to evaluate their relationships, especially when associated with multivariate analysis. The correlation between the oil composition and the genetic data indicates that RAPD analysis, a rapid and inexpensive method, can be a useful tool for marker-assisted selection in thyme

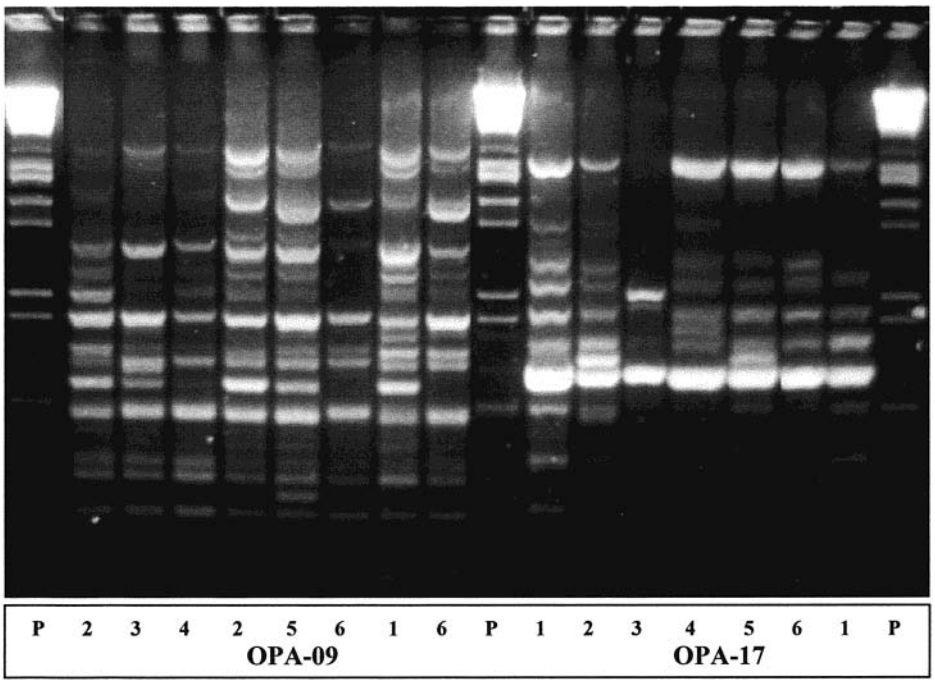

Figure 2. RAPD patterns of six commercial thyme cultivars obtained with primers OPA-09 and OPA-17: 1, Burpee; 2, Batlle; 3, Tropical; 4, Blumen; 5, SEM; 6, Isla; P, Lambda *Eco*RI/*Hin*dIII.

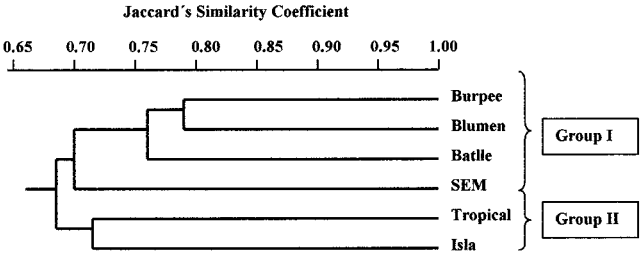

Figure 3. Unweighted pair-group method with arithmetic average (UPGMA) dendrogram showing the genetic similarity among six commercial thyme cultivars as determined by the analysis of 163 RAPD markers.

breeding programs directed to the improvement of essential oil composition.

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