JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Chemical Composition, Plant Genetic Differences, Antimicrobial and Antifungal Activity Investigation of the Essential Oil of *Rosmarinus officinalis* L.

ALBERTO ANGIONI,*,† ANDREA BARRA,† ELISABETTA CERETI,‡ DANIELA BARILE,‡ JEAN DANIEL COÏSSON,‡ MARCO ARLORIO,‡ SANDRO DESSI,† VALENTINA CORONEO,§ AND PAOLO CABRAS†

Dipartimento di Tossicologia, Università di Cagliari, Via Ospedale 72, 09124 Cagliari, Italy, Dipartimento di Scienze Chimiche, Alimentari, Farmaceutiche e Farmacologiche (DISCAFF), Università degli Studi del Piemonte Orientale, via Bovio 6, 28100 Novara, Italy, and Dipartimento di Sanità Pubblica, Laboratorio di Igiene degli Alimenti, Università di Cagliari, Via Porcell 4, 09124 Cagliari, Italy

The chemical composition of the essential oil of the Sardinian *Rosmarinus officinalis* L. obtained by hydro distillation and steam\hydro distillation was studied using GC-FID and MS. Samples were collected at different latitude and longitude of Sardinia (Italy). The yields ranged between 1.75 and 0.48% (v/w, volume/dry-weight). A total of 30 components were identified. The major compounds in the essential oil were α-pinene, borneol, (−) camphene, camphor, verbenone, and bornyl-acetate. Multivariate analysis carried out on chemical molecular markers, with the appraisal of chemical, pedological, and random amplified polymorphic DNA data, allows four different clusters to be distiguished. The antimicrobial and antifungal tests showed a weak activity of Sardinian rosemary. On the other hand, an inductive effect on fungal growth, especially toward *Fusarium graminearum* was observed.

KEYWORDS: ***Rosmarinus officinalis* L.; essential oil; antimicrobial and antifungal activity; RAPD; PCA**

INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) is a spontaneous shrub growing in all Mediterranean countries. In Sardinia, it is spread along the coast and in the inner part, growing from sea level to 1000 meters above sea level. Different species of rosemary exist all over the world: *R. officinalis*, *R. eriocalyx*, *R. laxiflorus*, and *R. lavandulaceus*. *R. officinalis* is the only one that grows naturally in the Mediterranean regions. The variety found is *genuina*, subdivided in three forms: *erectus*, *humilis*, and *albiflorus*. In Sardinia, flowering starts in March and stops in July, with an average yield under 1%, dramatically influenced by the harvest period. The chemo-taxonomy of rosemary was previously studied by many authors; Manunta et al. (*1*) underlined the existence of two oils from two different regions of the north of Sardinia, grown on soils with different pH. In this case, the natural plantations were both at sea level, and the differences between plants and oils were correlated to the difference of the substrate. Falchi Delitala et al. (*2*) studied the taxa occurring in the northwest area of Sardinia, correlating botanical observations to the chemical composition. Tuberoso et al. (*3*) reported that mono-terpenes constitute 50% of the oil, expecially α-pinene (>30%), camphene, and limonene; alcohols ∼7%; and ketones ∼10%. Pintore et al. (*4*) studied the differences between rosemary from Sardinia and Corsica, identifying α-pinene, verbenone, and bornyl acetate chemotypes, respectively.

Elamrani et al. (*5*) studied the chemotaxonomy of Moroccan rosemary essential oils, finding three chemotypes: α-pinene, camphor, and 1,8-cineole. They also studied the influence of the vegetative stage and the species on the oil composition; no differences were found. Rosemary from Lebanon (*6*) is characterized by 1,8 cineole (∼20%), and α-pinene (∼18.8−38.5%), Indian oil (*7*) by 1,8 cineole (∼30%), and camphor (∼30%), while rosemary from Japan (*8*) is characterized by a high content of 1,8-cineole (∼50%) and low levels of α-pinene and camphor (∼10%). Moreover, rosemary harvested in Portugal (*9*) is rich in myrcene (25%) 1,8-cineole, and camphor, and is similar to Argentinean and Brazilian oils (*10*, *11*).

Many researchers studied the antifungal and antimicrobial activity of the essential oil of rosemary. Baratta et al. 1998 (*12*) tested the antibacterial and antifungal activities of a commercial sample (α-pinene, 1,8-cineole, camphor, α-terpineol, chemotype) finding low activity except against *Staphylococcus aureus*.

The Sardinian and Corsican chemotypes (α-pinene/verbenone/bornyl acetate) exhibited a nonsignificant activity, both against Gram (+) (*Staphylococcus aureus*, and *S. epidermidis*) and Gram (−) (*Escherichia coli*, *Pseudomonas aeruginosa*) (*8*). On the other hand, the Argentinean chemotypes (myrcene/1,8-

* To whom correspondence should be addressed. E-mail: aangioni@unica.it.
† Dipartimento di Tossicologia, Università di Cagliari.
‡ Università degli Studi del Piemonte Orientale.
§ Dipartimento di Sanità Pubblica, Università di Cagliari.

10.1021/jf049913t CCC: $27.50
Published on Web 05/06/2004

Table 1. Harvesting Areas, Pedological Characteristics (pH, Altitude) and Essential Oil Yields (% v\w)

	sample locality	region	altitude| m/slm	soil pH	yield[a] (% ± SD) SISS	yield[a] (% ± SD) Clevenger
1	Gutturru Mannu	South	0	7.20	1.18 ± 0.12	1.12 ± 0.11
2	Scaffa	South	0	8.57	0.10 ± 0.01	0.10 ± 0.01
3	S.Antioco	South	0	7.85	0.62 ± 0.08	0.86 ± 0.15
4	Domusnovas (1)	South	300	7.62	0.59 ± 0.01	0.75 ± 0.18
5	Domusnovas (2)	South	300	7.82	0.81 ± 0.03	0.81 ± 0.25
6	Villamassargia	South	0	7.87	0.48 ± 0.00	0.93 ± 0.17
7	Siliqua	South	300	7.7	0.51 ± 0.01	1.23 ± 0.21
8	Sadali (1)	Centre	700	7.98	0.71 ± 0.02	1.32 ± 0.23
9	Sadali (2)	Centre	700	7.96	1.11 ± 0.10	1.21 ± 0.49
10	Seulo	Centre	900	7.63	0.90 ± 0.08	0.76 ± 0.16
11	Capo Caccia	North	200	8.02	0.97 ± 0.07	1.28 ± 0.46
12	Tramariglio	North	200	7.25	1.48 ± 0.13	2.02 ± 0.84
13	Argentiera	North	0	8.16	1.66 ± 0.20	2.13 ± 0.15
14	Talana	East	200	6.09	1.52 ± 0.05	2.02 ± 0.93
15	Baunei	East	700	7.75	1.04 ± 0.12	1.26 ± 0.34
16	Calagonone	East	100	7.96	1.21 ± 0.08	1.90 ± 0.52
17	Quirra	Southeast	300	8.0	1.01 ± 0.09	1.78 ± 0.46

[a] Expressed as 100 g of dried weight.

cineole/camphor) expressed insecticide properties and in vitro antifungal activity against *Ascosphaera apis* (*11*).

In a recent paper, Masatoshi et al. (*8*) pointed out the effectiveness of rosemary against plant parasite; both the essential oil and its major components (α-pinene/1,8-cineole/camphor) had high repellency effect against *Neotoxoptera formosana*, the onion aphid. Daferera et al. (*13*) evaluated the effectiveness of the essential oil (α-pinene/1,8 cineole/borneol, chemotype) from Greece on the growth of *Botritis cinerea*, *Fusarium* sp., and *Clavibacter michiganensis*.

Natural plant species are characterized by different inter- and intra-specific polymorphisms. Flamini et al. (*14*) reported the analysis of two different ecotypes of cultivated *Rosmarinus officinalis:* a α-pinene, and a 1,8-cineole chemotype. The genetic variation of the genus *Rosmarinus* was pointed out only for the species *R. tomentosus* using a PCR-related technique, the random amplified polymorphic DNA (RAPD) (*15*). The RAPD technique involves the amplification of genomic DNA using short primers (*16*, *17*), and it is routinely used to assess the intra-specific polymorphisms of plants and other organisms (*18*−*20*). To organize and explain RAPD complex fingerprints, coupled with a phenotypic (*21*) or chemotypic trait (*22*), the use of PCA (principal component analysis) allows a more objective interpretation. PCA coupled with DNA analysis represents a powerful tool to assess the identity of microorganisms and organisms, as well as to cluster different organisms from different geographic origins. In fact, the use of PCA led to the reducing of descriptor parameters to two or three only (*23*, *24*).

The aims of the present paper were (a) to characterize the chemical composition of the essential oil of wild rosemary from Sardinia, (b) to assess the presence of different chemotypes or ecotypes, and (c) to investigate the antifungal activity against plant pest and the antimicrobial activity.

EXPERIMENTAL PROCEDURES

Harvest: Period and Method. *Plant Material*. Samples of *Rosmarinus officinalis* were collected from 17 different natural stations at different altitude (between sea level and 1000 m above sea level), between February and July, 2003 during the flowering stage (in the same flowering conditions), in Sardinia (Italy) (**Table 1**). The samples were fresh flowering tops and stems and after harvest were carried (in jute-bags at 25 °C) to the laboratory for the analysis. In every location of sampling, three samples SPS (single plant samples) of about 0.2 kg were collected from three single plants and samples RPS (random plant sample) of about 2.5 kg were collected, harvesting the majority of plants of the area. The samples were dried at 100 °C for 1 h to verify their water content. A sample of ground was collected in every location to evaluate the pH. The specimens were identified and deposited in the Herbarium of the Department of Toxicology of the University of Cagliari.

Distillation. The replicate SPS of 130 g were hydro distilled simultaneously for 1 h in a Clevenger-type apparatus, according to the Italian Official Pharmacopoea X (1998) (*25*). The RPSs (2.5 kg) were steam\hydro distilled using a semi-industrial stainless steel apparatus (SISS) of 80 L of capacity. The essential oils were recovered directly, using a micropipet from above the distillate without adding any solvent. The essential oils were stored with anhydrous sodium sulfate in dark vials at 4 °C. Solutions of 1% (v/v) oil were prepared in *n*-hexane before GC\MS analysis.

GC/FID Analysis. A Gas Chromatographer Trace (Thermo Finningan, Rodano, Milan, Italy) equipped with a FID detector, an AS 800 auto sampler and a split-splitless injector, was used.

The capillary column was a fused silica DB5 (30m, 0,25 mm id; 0,25μm film thickness) (J&W Scientific, Folsom, CA). The injector and the detector were operated at 150 °C and 280 °C, respectively. A 1-μL aliquot of each sample was injected in the split mode (1:20). The oven was programmed as follows: 60 °C, raised to 180 °C (3 °C/min), and isothermally held for 15 min. Helium was used as carrier gas, and nitrogen was used for make up at 120 kPA and 80 kPA, respectively.

GC/ITMS Analysis. A Varian CP 3800 gas chromatograph (Varian, Inc., Palo Alto, CA) coupled with a Saturn 2000 ITMS detector, a Varian CP 7800 autosampler, a split-splitless injector, and an MS ChemStation, was used.

The column was a fused silica capillary DB-5MS (5% phenylmethylpolysyloxane, 30-m × 0.25-mm; film thickness 0.25-μm) (J&W Scientific Fisons, Folsom, CA). The injector and interface were at 150 and 280 °C, respectively. The oven temperature was programmed as follows: from 60 to 180 °C (3 °C/min), and isothermally held for 15 min. Helium was used as carrier gas at 1 mL/min; 1 μL of each sample was injected in the split mode (1:20). MS conditions were as follows: ionization mode EI from 50 to 450 amu. The oil components were identified by comparison of their relative retention times with those of standard references or by comparison of their retention index (RI), relative to the series of *n*-hydrocarbons. The computer matching against commercial library (*26*, *27*) and homemade library mass spectra made up from pure substances and component of known oils, as well as MS literature data, were also used for the identification. The KI calculated were in agreement with that reported by Adams (*26*). A quantitative analysis of each oils component (expressed as percentages) was carried out by peak area normalization measurement.

Chemicals. α-Pinene, (−)-camphene, β-pinene, myrcene, α-terpinene, *p*-cimene, 1,8-cineole, γ-terpinene, terpinolene, borneol, terpinen-4-ol, α-terpineol, geraniol, verbenone, carvacrol, thymol, bornyl-acetate, α-cedrene, and β-caryophyllene were obtained from Aldrich, Acros, and Fluka, (Milan, Italy); α-thujene, sabinene, δ-3-carene, limonene, linalool, and α-humulene were obtained from Extrasynthese (Genay, France); and camphor was from Carlo Erba (Milan, Italy). All compounds were analytical standard grade. *n*-Hexane was an analytical grade solvent, and Na_2SO_4 was analytical reagent grade from Carlo Erba (Milan, Italy).

Microbiological Assay. (a) The antimicrobial activity of the rosemary essential oils and their major components (α-pinene, (−)-camphene, verbenone, bornyl-acetate, camphor, and borneol) was studied. The experiments were carried out using a set of microorganisms represented by the following six ATCC strains: *Staphylococcus aureus* (6538), *Staphylococcus epidermidis* (12228), *Escherichia coli* (8739), *Pseudomonas aeruginosa* (9027), and *Candida albicans* (14053). Preliminary tests using agar diffusion assay with disk (ø 6 mm) showed the evident sensibility of *Staphylococcus aureus* (ø 5 cm) to the essential oils extract without dilution.

For each of the above-mentioned strains, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration

(MBC) were determined using a broth microdilution method. Stock standard solutions at 1% in DMSO (Dimethyl sulfoxide) were prepared for each oil. Working solutions were prepared by dilution in microplates at concentrations between 3.5 μL/mL and 900 μL/mL. The bacterial suspensions were added in the microwells at the concentration of 5.105 cfu/mL (colony forming units/mL). The concentration of each inoculum was verified sowing in plates containing agar PDA (potato dextrose agar). The plates were incubated aerobically at 37 °C (*Candida albicans* at 30 °C) for 24h. Bacterial growth was revealed by the presence of turbidity and a "pellet" on the well bottom. MICs were determined as the first well in ascending order that did not produce a pellet. To confirm MIC and establish MBC, 25 μL of broth was removed from each well and inoculated on PDA plates. After aerobic incubation at 37 °C overnight, the number of surviving organisms was determined (MBC was determined when 99.9% of bacteria were dead). Each experiment was repeated three times for each oil or compound and for each test concentration.

(b) The antifungal activity was investigated against *Botrytis cinerea*, *Fusarium oxysporum lycopersici*, *Fusarium graminearum*, *Fusarium culmorum*, and *Rhizoctonia solani*.

The tests were carried out by insemination, with mycelia fragments of 6 mm in diameter (10 days hold), in Petri dishes containing PDA. After the addition of the essential oil (450 and 900 μL/mL), the plates were sealed with Parafilm to avoid the dispersion of the oil and incubated in the dark at 22 °C. Control samples with the mycelia in PDA and distilled water were subjected to the same treatments. The effectiveness of the treatments was evaluated by measuring the average diametric growth of the colonies after 4, 8, and 12 days after the inoculum.

The percentage of inhibition was calculated according to the equation of Zygadlo and Guzman (*28*)

$$I = 100\ (C - T)C - 1$$

where I = inibition, C = average diameter of fungi grown in PDA + water, and T = average diameter of fungi cultivated in PDA + essential oil.

When no mycelium growth was observed, the mycelium was transferred to a plate containing only PDA and incubated for 48 h, to determine if the inhibition was fungistatic or fungicide. All the experiments were replicated three times.

Genetic Analysis. *Plant Material*. DNA samples were obtained from leaves collected in each area from wild plants of *Rosmarinus officinalis.*

DNA Reagents and Instrumentations. Taq-polymerase, $MgCl_2$, and buffer 10× were obtained from Genenco (Milan, Italy) and dNTPs from M-Medical (Milan, Italy). Primers were synthesized by M-Medical (Milan, Italy). Gels were prepared with agarose LE from Euroclone (Pero, Italy). All others chemicals were from Fluka (Buchs, Switzerland).

Amplification was carried out using a PCR system 9700 (PE Applied Biosystem, Monza, Italy). The electrophoretic analysis was made using a Power Pack 300 power supply equipped with a subcell agarose gel electrophoresis system (Bio-Rad, Segrate Milan, Italy).

DNA Extraction. Extraction tubes contained 200 mg of leaf tissue of fresh plants treated with liquid nitrogen.

A 500-μL aliquot of lysis buffer (2% w/v Ctab, 200 mM Tris-HCl pH 8.0, EDTA 20 mM pH 8.9, 1.4 NaCl, 1% w/v PVP) were added and the samples were vortexed and left in thermo mixer at 65 °C for 1 h. After cooling, 100 mL of 2 M ascorbic acid was added. The centrifuged solutions were extracted with 600 mL of cold phenol−chloroform−isoamyl alcohol (25:24:1) solutuion. The supernatants were transferred in new tubes and washed with cold chloroform (about 500 μL). After the centrifugation step (13 000 rpm for 10 min), open tubes were left at 40 °C per 15 min to allow evaporation of chloroform residues and then were treated with RNase A (100 U/mL) for 15 min at 37 °C. Precipitation of DNA was made using 0.7 mL of 2-propanol, and the solutions were mixed by inverting tubes 10−20 times. After a freezing period of 30 min, the solution was centrifuged (3000 rpm for10 min), the supernatant was removed, and the DNA pellet was washed by adding 500 μL of ethanol at 70%. The supernatant was removed, and the washing steps were repeated. The DNA was subsequently air-dried for at least 10 min and finally re-suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Table 2. Details of the Random Primers (10-mer) Used in This Study

primer (operon series)	sequence (5′ to 3′)	melting temp (at 400 nM)	% of G + C
A1	CAGGCCCTTC	31.5	70
A2	TGCCGAGCTG	35.3	70
A4	AATCGGGCTG	32.7	60
A5	AGGGGTCTTG	30.5	60
A8	GTGACGTAGG	15.8	60
C1	TTCGAGCCAG	28.9	60
C4	GATGACCGCC	31.8	70
D1	ACCGCGAAGG	37.7	70
D2	GGACCCAACC	29.9	70
D5	TGAGCGGACA	29.5	60
D8	GTGTGCCCCA	33.3	70

DNA Amplification and Detection. The optimized PCR mixture (50 μL) contained 11.9 μL of ultrapure H_2O, 2.5 μL of 10× buffer [100 mM Tris-HCl, 500 mM KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$, pH 8.7, at 20 °C], 2 μL of dNTPs (dATP, dCTP, dGTP, and dTTP, 2.5 mM cad.), 1 μL of 10 μM oligonucleotide primer, 2.5 μL of 25 μM $MgCl_2$, 0.5 units of Taq-polymerase, and 5 μL of genomic DNA (0.1 ng/μL). Each reaction was overlaid with 50 μL of mineral oil to prevent evaporation during amplification.

The thermal cycler was programmed with the following protocol: initial denaturation at 95 °C for 5 min, 45 amplification cycles at 94 °C for 40 s (denaturation), 32 °C for 40 s (annealing), 72 °C for 90 s (extension), and a final step of extension at 72 °C for 7 min.

In total, 11 (10-mer) random primers (M-Medical, Milan, Italy) were used (**Table 2**).

Aliquots of 12 μL of amplified sample were loaded in agarose gels at 2% and analyzed by electrophoresis for 100 min at 80 V, in TBE buffer 1× (8.8 mM Tris-HCl, 8.8 mM boric acid, and 0.2 mM EDTA). The samples were added to loading buffer 1× stained with 0.5μg/μL ethidium bromide. All RAPD profiles were analyzed using the Fluor-S MultImager detector, equipped with Quantity One software from Bio-Rad (Segrate, Milan, Italy).

Statistical Analysis. Principal component analysis was accomplished by using the software Mathematica (Wolfram Research Inc., Champaign, IL).

RESULTS AND DISCUSSION

Chemical Analysis. Table 1 shows the pedological characteristics (pH, altitude) and the yields in essential oil obtained with the Clevenger apparatus and the semi-industrial apparatus. The majority of samples are from soils with basic pH, except for one sample that was from a soil with acidic pH (number 14). The yields ranged between 0.10 and 2.13 and between 0.10 and 1.66 for the Clevenger and the semi-industrial apparatus, respectively. Because the yields were calculated on dry weight, their variability could not be ascribable to the water content. When the standard deviation of the yields obtained with the two methods was considered, the data could almost all be statically overlapped. Mainly, the yields obtained from the north and east samples were on average 2-fold higher than those from the south and center samples. The oil obtained from the Clevenger apparatus and the semi-industrial apparatus showed the same composition, both qualitative and quantitative. A total of 30 components, listed in **Table 3**, were identified: α-pinene (≈23%), (−) camphene (≈7.6%), borneol (≈16%), camphor (≈4.5%), verbenone (≈9.4%) and bornyl-acetate (≈10.4%) were the most representative. According to Pintore et al. (*4*) the Sardinian rosemary essential oil is an α-pinene\borneol\bornyl-acetate\verbenone chemotype. **Table 4** reports the composition of the essential oils classified considering the chemical group, from these data, it can be seen that the general composition is

Table 3. Constituents (Area % ± SD) of the Oil of Sardinian *Rosmarinus officinalis* L. from Different Harvesting Regions

compound	KI	South (RA[a]% ± SD)	Central (RA[a]% ± SD)	North (RA[a]% ± SD)	East (RA[a]% ± SD)
			Monoterpenes		
α-thujene[b]	930	0.49 ± 0.20	0.52 ± 0.11	0.65 ± 0.83	0.49 ± 0.16
α-pinene[b]	936	19.09 ± 7.19	24.98 ± 5.56	25.82 ± 7.75	21.19 ± 9.27
(–) camphene[b]	952	8.16 ± 2.66	8.39 ± 2.28	6.45 ± 1.5	7.42 ± 1.69
Verbenene[b]	967	0.10 ± 0.12	0.0 ± 0.0	0.06 ± 0.06	0.02 ± 0.02
sabinene[b]	975	3.30 ± 2.08	3.46 ± 1.92	3.72 ± 1.47	2.40 ± 2.38
myrcene[b]	991	2.03 ± 1.95	1.48 ± 0.48	3.37 ± 2.38	1.52 ± 0.77
α-phellandrene[b]	1000	0.91 ± 1.11	0.42 ± 0.51	0.83 ± 0.96	0.77 ± 0.85
α-terpinene[b]	1017	0.56 ± 0.31	0.62 ± 0.19	0.78 ± 0.21	0.65 ± 0.24
p-cymene[b]	1025	4.27 ± 2.12	4.34 ± 1.61	1.69 ± 0.66	3.24 ± 1.90
limonene[b]	1029	4.34 ± 1.27	4.17 ± 0.76	3.91 ± 0.85	4.91 ± 0.96
β-phellandrene[b]	1030	2.73 ± 2.08	3.90 ± 1.35	3.24 ± 1.20	2.36 ± 1.97
δ–3-carene[b]	1031	1.47 ± 1.13	0.44 ± 0.51	0.61 ± 0.42	0.70 ± 0.80
1.8-cineole[b]	1031	1.54 ± 1.52	2.84 ± 1.98	2.42 ± 1.06	0.88 ± 1.62
γ-terpinene[b]	1060	0.69 ± 0.41	0.87 ± 0.36	1.38 ± 0.38	1.02 ± 0.49
terpinolene[b]	1089	0.74 ± 0.33	0.59 ± 0.29	1.18 ± 0.22	1.04 ± 0.45
			Alcohols		
linalool[b]	1097	1.08 ± 0.68	1.07 ± 0.56	1.06 ± 0.32	1.45 ± 0.74
Geijerene	1143	0.22 ± 0.18	0.1 ± 0.11	0.28 ± 0.13	0.27 ± 0.13
Verbenol[b]	1145	3.03 ± 2.14	1.28 ± 1.14	0.70 ± 0.56	1.20 ± 1.96
borneol[b]	1172	16.17 ± 9.08	18.08 ± 3.75	15.08 ± 9.86	14.18 ± 4.65
terpinen-4-ol[b]	1177	0.72 ± 0.23	0.79 ± 0.12	0.80 ± 0.23	0.86 ± 0.20
α-terpineol[b]	1189	1.19 ± 0.46	1.45 ± 0.37	1.44 ± 0.64	1.29 ± 0.57
geraniol[b]	1253				
			Ketones		
camphor[b]	1148	7.33 ± 4.02	4.81 ± 3.09	2.45 ± 1.89	3.35 ± 3.15
cis-pinocanfone	1175	1.01 ± 0.91	0.33 ± 0.29	0.76 ± 0.39	0.80 ± 0.40
verbenone[b]	1209	7.37 ± 3.45	6.84 ± 2.89	11.28 ± 3.06	12.00 ± 4.41
			Phenols		
carvacrol[b]	1299	0.05 ± 0.09	0.01 ± 0.04	0.01 ± 0.03	0.02 ± 0.04
thymol[b]	1287	0.03 ± 0.07			
			Esters		
bornyl-acetate[b]	1292	9.88 ± 4.59	7.55 ± 3.25	9.14 ± 2.90	14.90 ± 7.90
			Sesquiterpenes		
β-caryophyllene[b]	1419	0.51 ± 0.53	0.35 ± 0.33	0.18 ± 0.17	0.05 ± 0.13
α-humulene[b]	1455	0.12 ± 0.17	0.03 ± 0.05	0.02 ± 0.07	0.0 ± 0.0
total identified		99.20	99.64	99.36	99.02

[a] Relative area. [b] Peaks identified by comparison with respective pure standards.

Table 4. Composition (Area % ± SD) of the Oil of Sardinian *Rosmarinus officinalis* L. From Different Harvesting Regions

compound	South (RA[a]% ± SD)	Central (RA[a]% ± SD)	North (RA[a]% ± SD)	East (RA[a]% ± SD)
monoterpenes	50.34 ± 10.58	57.01 ± 5.50	56.14 ± 7.91	48.60 ± 5.86
alcohols	22.40 ± 10.37	22.76 ± 3.70	19.36 ± 9.80	19.26 ± 4.30
ketones	15.87 ± 4.22	11.97 ± 2.27	14.51 ± 3.43	16.19 ± 5.06
phenols	0.08 ± 0.13	0.01 ± 0.04	0.01 ± 0.03	0.02 ± 0.04
esters	9.88 ± 4.59	7.55 ± 3.25	9.14 ± 2.90	14.90 ± 7.90
sesquiterpenes	0.63 ± 0.68	0.34 ± 0.38	0.20 ± 0.2	0.05 ± 0.13

similar in the different areas. The data found are in agreement with Tuberoso et al. (*3*), who reported that monoterpenes constitute 0%, but the average amount of alcohol and ketones is higher (21 vs 7%, and 15 vs 10%). Because the standard deviations were remarkably high, the chemical data were elaborated by PCA.

PCA determines the combination of essential oil constituents that account for the largest amount of variance. Prior to the PCA analysis, all the variables were standardized for a normalized PCA.

Figure 1 represents graphically the relationship between the samples (SPS) using as inputs the essential oils chemical composition. The loadings are the coefficients of the original variables in the principal components vectors, and they can lead

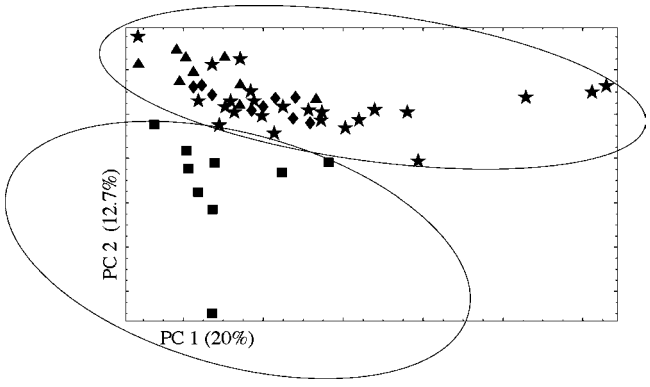

Figure 1. A bi-plot of the samples (SPS) based only on the essential oils chemical composition. South (★); Central (◆); North (▲); East (■).

to determination of the relative contribution of all the variables in constituting each principal component. In this case, all the variables were equally important for each principal component, because they had similar loading, between the values 0.29 and 0.27. The first principal component (PC1) accounted for 20.0% of the variation among the 17 samples, and the second principal component (PC2) accounted for 12.7% of the total variance: these low percentages suggested that the large amount of variation seen among SPS and the area of grown was not referable at a clearly defined pattern; however, two main clusters

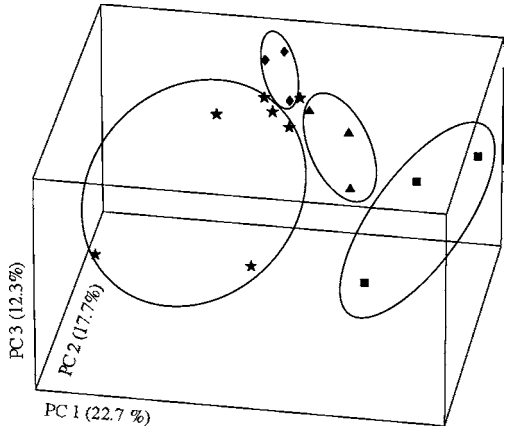

Figure 2. A three-dimensional visualization of the samples (RPS) based only on the essential oils chemical composition. South (★); Central (◆); North (▲); East (■).

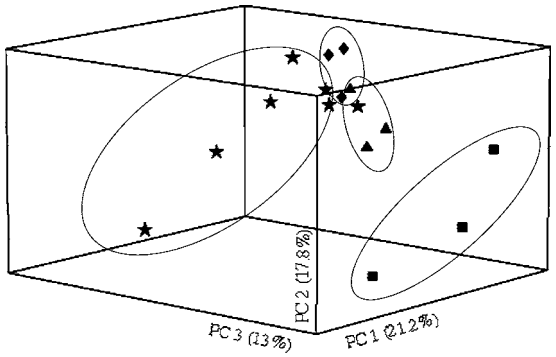

Figure 3. A three-dimensional visualization obtained by coupling chemical composition data from RPS and pedologicals data. South (★); Central (◆); North (▲); East (■).

were differentiated: the first formed by the samples grown in the East area, and the second formed by the samples grown in the other areas of Sardinia (South, Central and North). Inside the second Cluster, two other clusters, formed by the Central and the North samples, were only partially distinguished.

Carrying out a second PCA analysis, considering the data of essential oils from the RPS, one from each area, we obtained a better discrimination among the four clusters, where the three PC accounted for the 52.7% of the total variance (**Figure 2**). The main variables contributing to PC1 were Humulene (loading, 0.327), β-caryophyllene (loading, 0.287), and verbenene (loading, 0.286). The main variables contributing to the PC2 were α-terpineol (loading, 0.422), cis-pinocanfone (loading, 0.408), and bornyl-acetate (loading, 0.310). The main variables contributing to the PC3 were terpinolene (loading, 0.358), γ-terpene (loading, 0.287), and verbenene (loading, 0.282).

In this analysis, the plant individual variation is minimized, and the data are more representative of each harvesting area.

Another PCA was performed coupling chemical data from the RPS with pedological data listed in **Table 1** (pH, altitude, etc); and the total variance of the three principal components was equal to 52% (**Figure 3**). The variables contributing more to PC1 were yield of clevenger (loading, 0.297), soil pH (loading, 0.277), and verbenene (loading, 0.249). The main variables contributing to PC2 were myrcene (loading, 0.339), thujene (loading, 0.335), and α- phellandrene (loading, 0.319). The main variables contributing to PC3 were camphor (loading, 0.342), verbenene (loading, 0.321), and terpinolene (loading, 0.316).

Microbiological Analysis. The essential oils obtained from the plant of Sardinian Rosemary and their main compounds (α-pinene, (−) camphene, verbenone, bornyl-acetate, camphor, and borneol) showed low inhibitory activity against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*, with MIC always over 900 μL/mL.

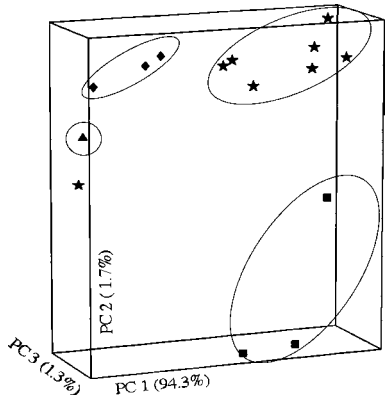

Figure 4. A three-dimensional visualization of the information obtained by using six primers RAPD (98.8% of the total variation). South (★); Central (◆); North (▲); East (■).

No inhibitory effect was observed on the growth of bacteria and fungi; in contrast, it was observed an induction effect of the essential oil, especially toward the growth of *Fusarium graminearum.*

Genetic Analysis. Genomic DNA extracted from *Rosmarinus officinalis* L. samples was amplified using 11 (10-mer) random primer to study the polymorphism. The "classic" approach to establish the polymorphism degree is represented by the count of the bands obtained, considering the number of common bands toward the total number of bands generated by means of RAPD amplification. In fact, this is a nonobjective and nonstandardized approach.

In our work, we have arbitrarily assigned some values to all bands generated, using the same approach described in a previous work (*20*). Briefly, RAPD bands were recorded in a binary form, for example, (1) presence of a band, and (0) absence of a band, and a variable value from 0 to 1 (value based on the total mean bands) indicates the "not amplified" lanes. Bands that were unique to a single sample or did not show reliability within the replicated samples of each taxon were eliminated and not used in the computation of similarities. It should be noted that these bands contain very useful information for the study of genetic variance and individual variation, but are merely "noise" in the present taxonomic study.

Among the 11 random primers used for the amplifications of the rosemary samples are 7 amplified polymorphic products. A total of 42 amplification products were scored, 25 of which were polymorphic (60%). The results obtained by RAPD analysis were studied with PCA (*29*), as reported in **Figure 4**.

Results obtained from PCA showed the existence of a high interpopulation variability among the samples of different Sardinia areas and confirmed the four clusters obtained with chemical data. In this PCA analysis all the variables (bands) are equally important for each principal component, because they assume oscillating loading weights close to 0.27 in the principal components vectors.

Interestingly, the samples coming from the North have the same RAPD fingerprint and then in the three-dimensional plot all are located in a single point. Among the South samples, only one sample was located in an isolated position. In fact, this sample comes from the southeast and is geographically distinct from the other southern samples.

To gain time and to limit the economic weight of the RAPD assays, we have evaluated the reduction of the amplifications to the lowest number of random primers able to show the same information about polymorphisms. Though the choice of primers is somewhat subjective, criteria such as number of bands produced, clarity, and distinctness of bands, as well as reproducibility of RAPD fingerprints were used for the selection process. The primers were carefully screened; testing all the possible combinations, we have found that the minimum number of amplifications providing a constant similarity variance (97.3%) corresponded to six primers (A4, A5, A8, C1, D5, and D8), generating a total of 22 bands.

So, the clustering of genomic data, recorded and processed in the form of binary data, confirmed the clustering obtained by processing all other chemical parameters with PCA. The global evaluation of all molecular markers (genomic plus chemical) was impossible to consider because of the different nature of data.

The essential oils composition obtained with the two distillation apparatus have common characteristics both in quality and in quantity. The multivariate analysis (PCA) carried out on chemical molecular markers, with the appraisal of chemical, pedological and RAPDs data, allows four different clusters corresponding to the four harvesting areas, North, South, East, and West to be distinguished. This suggests the existence of different chemo/eco/genotypes of *Rosmarinus officinalis* L. The study confirmed the weak antimicrobic/fungitoxic activity of Sardinian rosemary as reported by Pintore et al. (4). On the other hand, an inductive effect on fungal growth, especially toward *Fusarium graminearum* was observed.

LITERATURE CITED

(1) Manunta, A. Influences of the pH of the substratum on the composition of the essential oil of *Rosmarinus officinalis* L. *Studi sassaresi sez. III Vol XXXII* **1985**, *86*, 111−118.

(2) Falchi Delitala, L.; Soccolini, F. Investigations on *Rosmarinus officinalis* L. from Sardinia. *Riv. Italiana EPPOS* **1980**, LXII, *4*, 195−201.

(3) Tuberoso, C. I. G.; Satta, M.; Cabras, P.; Garau, V. L. Chemical composition of *Rosmarinus officinalis* of Sardinia. *J. Ess. Oil Res.* **1998**, *10*, 660−664.

(4) Pintore, G.; Usai, M.; Bradesi, P.; Juliano, C.; Boato, G.; Tomi, F.; Chessa, M.; Cerri, R.; Casanova, J. Chemical composition and antimicrobial activity of *Rosmarinus officinalis* L. oils from Sardinia and Corsica. *Flavour Fragr.* **2002**, *17*, 15−19.

(5) Elamrani, A.; Zrira, S.; Benjilali, B. A study of Moroccan rosemary oils. *J. Ess. Oil Res.* **2000**, *12*, 487−495.

(6) Diab, Y.; Auezova, L.; Chebib, H.; Chalchat, J. C.; Figueredo, G. Chemical composition of Lebanese rosemary (*Rosmarinus officinalis* L.) essential oil as a function of the geographical region and the harvest time. *J. Ess. Oil Res.* **2002**, *14*, 449−452.

(7) Jaganmohan R. L.; Meenakshi, S.; Raghavan, D.; Abraham, K. O. Rosemary (*Rosmarinus officinalis* L.): impact of drying on its flavor quality. *J. Food Qual.* **1997**, *21*, 107, 115.

(8) Masatoshi, H.; Hiroaki, K. Repellency of rosemary oil and its components againts the Onion aphid, Neotoxoptera formosana. *Appl. Entomol. Zoot.* **1997**, *32* (2), 3303−310.

(9) Serrano, E.; Palma, J.; Tinocco, T.; Venancio, F.; Martins, A. Evaluation of the essential oils of rosemary (*Rosmarinus officinalis* L.) from different zones of "Alentejo" (Portugal). *J. Ess. Oil Res.* **2002**, *14*, 87−92.

(10) Porte, A.; De, O.; Godoy, R. L.; Lopes, D.; Koketsu, M.; Goncalves, S. L.; Torquilho, H. S. Essential oil of *Rosmarinus officinalis* L. (rosemary) from Rio de Janeiro, Brazil. *J. Ess. Oil Res.* **2000**, *12*, 577−580.

(11) Larràn, S.; Ringuelet, J. A.; Carranza, M. R.; Henning, C. P.; Rè, M. S.; Cerimele, E. L.; Urrutia, M. I. In vitro fungistatic effect of essential oils against Ascosphaera apis. *J. Ess. Oil Res.* **2001**, *13*, 122−124.

(12) Baratta, M. T.; Damien Dorman, H. J.; Deans, S. G.; Biondi, D. M.; Ruperto, G. Chemical Composition, Antimicrobial and Antioxidative Activity of Laurel, Sage, Rosemary, Oregano, and Coriander Essential Oils. *J. Ess. Oil Res.* **1998**, *10*, 618−627.

(13) Daferera, D. J.; Ziogas, B. N.; Polissiou, M. G. The effectivenesss of plant essential oils on the growth of *Botrytis cinerea*, *Fusarium sp*., and *Clavibacter michiganensis* subsp. *michiganensis*. *J. Crop Prot.* **2003**, *22*, 39−44.

(14) Flamini, G.; Cioni, P. L.; Morelli, I.; Macchia, M.; Ceccarini, L. Main agronomic productive characteristics of two ecotypes of *Rosmarinus officinalis* L. and chemical composition of their essential oils. *J. Agric. Food. Chem.* **2002**, *50*, 3512−3517.

(15) Martin, J. P.; Bermejo, J. E. H. Genetic variation in the endemic and endangered Rosmarinus tomentosus Huber-Morath & Maire (Labiate) using RAPD markers. *Heredity* **2000**, *85*, 434−443.

(16) Welsh, J.; McClelland, M. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **1990**, *18*, 7213−7218.

(17) Williams, J. G. K.; Kubelik, A. R.; Livak, K. J.; Rafalski, J. A.; Tingey, S. V. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **1990**, *18*, 6531−6535.

(18) Saunders, G. C.; Parkes, H. C. Random Amplified Polymorphic DNA (RAPD) analysis. In *Analytical Molecular Biology. Quality and Validation*; Royal Society of Chemistry: Cambridge, UK, 1999.

(19) Cobb, B. Optimization of RAPD Fingerprinting. In *Fingerprinting Methods Based on Arbitrarily Primed PCR*; Springer: Berlin, 1997.

(20) Angioni, A.; Barra, A.; Arlorio, M.; Coisson, J. D.; Russo, M. L.; Pirisi, F. M.; Satta, M.; Cabras, P. Chemical composition, plant genetic differences, and antifungal activity of the essential oil of *Helichrysum italicum* G. Don. ssp, *microphyllum* (Willd) Nym. *J. Agric. Food. Chem.* **2003**, *51*, 1030−1034.

(21) Geisen, R.; Cantor, M. D.; Hansen, T. K.; Holzapfel, W. H.; Jakobsen, M. Characterization of Penicillium roqueforti strains used as cheese starter cultures by RAPD typing. *Int. J. Food Microbiol.* **2001**, *65*, 183−191.

(22) Gounaris, Y.; Skoula, M.; Fournaraki, C.; Drakakaki, G.; Makris, A. Comparison of essential oils and genetic relationship of Origanum x intercedens to its parental taxa in the island of Crete. *Biochem. Syst. Ecol.* **2002**, *30*, 249−258.

(23) Munck, L.; Nørgaard, L.; Engelsen, S. B.; Bro, R.; Andersson, C. A. Chemometrics in food science−a demonstration of the feasibility of a highly exploratory, inductive evaluation strategy of fundamental scientific significance. *Chemomet. Intell. Lab. Syst.* **1998**, *44*, 31−60.

(24) Hair, J. F.; Anderson, R. E.; Tatham, R. L.; Black, W. C. *Multivariate Data Analysis*, 5th ed.; Prentice-Hall: Upper Saddle River, NJ, 1998.

(25) Farmacopea Ufficiale Italiana, 10th ed.; droghe vegetali e preparazioni; Istituto Poligrafico e Zecca dello Stato: 1998.

(26) Adams, R. P. Identification of the Essential Oil Components by Gas-Chromatography\Mass Spectroscopy. Allured Publishers: Carol Stream, IL 1995.

(27) The Nist Mass Spectral Search Program for the NIST\EPA\NIM Mass Spectral; Library version 1.7, build 11\05\1999.

(28) Zygadlo, J. A.; Guzman, C. A.; Grosso, N. R. Antifungal properties of the leaf oils of Tagetes minuta L.; T. filifolia Lag. *J. Ess. Oil Res.* **1994**, *6*, 617−621.

(29) Iezzoni, A. F.; Pritts, M. P. Applications of principal component analysis to horticultural research. *HortScience* **1991**, *26*, 334−338.

Received for review January 16, 2004. Revised manuscript received March 25, 2004. Accepted April 4, 2004.

JF049913T