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Full Length Research Paper

Essential oil content and composition of three sage varieties grown in Central Italy

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The present study was carried out in order to investigate the differences of three sage varieties: Extrakta (EXT), Regula (REG), and Syn 1 (SYN). The essential oil yields, expressed in ml/kg dry weight varied from one accession to another and in different seasonal collection ranging from 0.22 to 1.6%. The lowest values were observed in EXT variety, at fall collection, while the highest result was observed in REG, at summer collection during the second year. In all samples 1,8 cineol is present in constant range from 11.12 to 12.78%. Larger differences are in α-pinene content that differs from 7.45% in SYN to 2.46% in REG. α-thujone is always the main component (from 20.88, 22.46, and 23.76 to 24.32, 21.55, and 22.89%, respectively in EXT, REG and SYN). Also, at the highest thujone content, none of the selected varieties reach 50%, that appear not in accordance with European Pharmacopeia specification of a thujone rich oil. Flower morphology, analysis of pollen viability, and differentiation were also determined.

Key words: *Salvia officinalis* L., var. Extrakta, var. Regula, var. Syn 1, essential oil.

INTRODUCTION

Salvia officinalis L. (Lamiaceae), is a perennial hardy subshrub native to Mediterranean regions. The herb is abundant and widely distributed, but some authors suggest that many wild populations are derived from old crops (Pignatti, 2003; Tutin et al., 1993).

Long times ago, sage is one of the most popular medicinal and culinary herbs. It is appreciated mainly for the presence of an aromatic essential oil with different composition related to different origins. The phyto-complex of essential oil results from the most interesting plant part, and is related to the use of plant for medicinal purpose, as culinary herb or for essential oil extraction. Several papers have reported on the variation in essential oil composition induced by environmental, physiological and morphological factors (Bernath et al., 1991; Grella and Picci, 1988; Puteievsaki et al., 1986a; Puteievsaki et al., 1986b; Telekova et al., 1994). Several papers report the essential oil composition of sage (Bernotiene et al., 2007; Perry et al., 1996) that is as a result of too much variable depending on genotypes (Chalchat et al., 1998), age (Länger et al., 1993), and environmental influences, such as fertilization (Piccaglia et al., 1989), light intensity (Li et al., 1996), climatic conditions (Máthé et al., 1992), season (Grella and Picci, 1988; Puteievsaki et al., 1986a), salinity and culture site (Perry et al., 1996; Santos-Gomes and Fernandes-Ferreira, 2001).

The International Organization for Standardization defines the following specifications for dried sage: ash (12% w/w) max, acid insoluble ash (2% w/w) max, maximum water content (12% w/w), minimum (1.5% w/w) of volatile oil (V/O) (ISO, 1995). ISO 9909 regulates the amounts of some constituents in sage essential oils: cis-thujone (18.0 to 43.0%), camphor (4.5 to 24.5%), 1,8-cineole (5.5 to 13.0%), trans-thujone (3.0 to 8.5%), α-humulene (≤12.0%), α-pinene (1.0 to 6.5%), camphene (1.5 to 7.0%), limonene (0.5 to 3.0%), bornyl acetate

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(≤2.5%) and linalool + linalyl acetate (≤1.0%) (ISO, 1997). Different chemotypes of sage have been identified in relation to major constituents (Couladis et al., 2002; Marie et al., 2006), such as α-thujone (≤65%), 1,8-cineole (≤59.0%), camphor (≤45.7%), β-thujone (≤40.1%), α-humulene (≤33.7%), linalool (≤35.0%), germacrene D (32.9%), viridiflorol (≤24.0%), α-pinene (≤24.6%), limonene (≤20.3%) and borneol (≤15.0%) (Bernotiene et al., 2007; Couladis et al., 2002; Marie et al., 2006; Mockute et al., 2003; Zawislak and Dyduch, 2006).

Perry et al. (1996) found out that oils from flowering and nonflowering accessions had different compositions, with significantly higher levels of thujone, β-caryophyllene and viridiflorol in oils from flowering accessions. Some authors reported that, to obtain a higher quality sage product, the harvest should be summer or early fall (Grella and Picci, 1988). Actually, it seems not possible to define a standard composition for sage essential oil that show great intrinsic variability, also improved by agricultural practices, chemotypes and high yield selections.

The present study was carried out in order to investigate the differences of three sage varieties: Extrakta (EXT), Regula (REG), and Syn 1 (SYN). Extrakta is a selected strain of commercial interest due to high essential oil production (De Mastro et al., 2006; Scartezzini et al., 2006); Regula is an hybrid variety selected in Switzerland for high essential oil production (De Mastro et al., 2006; Scartezzini et al., 2006), and Syn 1 is a synthetic variety selected by ISAFA from poly-cross between five clones (Aiello et al., 2001). In order to reduce the environmental effects, plants were grown in same conditions.

MATERIALS AND METHODS

Plant

Accessions of *S.officinalis* varieties were kindly furnished by ISAFA (Villazzano, Tn, Italy). A voucher specimen from each plant variety was kept at the Herbarium of Giardino dei Semplici, Dipartimento di Farmacia, Universitàà “G. d’Annunzio”, Chieti, Italy.

Three varieties of *S. officinalis* were used: EXT, SYN, and REG. Accessions were grown in fields for four years, in Valtiberina, a large valley with flat bottom (400 m asl), in Central Italy. In order to limit breeding, fields were selected at not less than 3 km. In each field, 150 plants were planted on rows (0.5 m) apart with 35 cm between plants. Irrigation was applied regularly during the growing season. Fertilizer was applied annually according to soil test results, to ensure that soil fertility was not a limiting factor for growth and development. Plants were trimmed once a year during flowering.

Extraction of essential oils

Fresh plants were hydrodistilled in a Clevenger-type apparatus for 3 h. The essential oils were dried over anhydrous sodium sulphate, and then, was stored in sealed vials protected from the light at -20°C before gas chromatographic analysis.

Three oils were obtained from each sample by hydrodistillation and subsequently analyzed by gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS).

Chemicals

α-Pinene, camphene, β-pinene, myrcene, γ-terpinene, terpinolene, camphor, terpinen-4-ol, and (E)-caryophyllene were purchased from Sigma-Aldrich (Milan, Italy). All compounds were of analytical standard grade. Analytical grade hexane solvent was purchased from Carlo Erba (Milan, Italy); it was successively distilled by a Vigreux column before use.

GC-FID and GC-MS analysis

For GC separations, an Agilent 4890D instrument coupled to a FID was used. Volatile components were separated on a HP-5 capillary column (5% phenylmethylpolysiloxane, 25 m, 0.32 mm i.d.; 0.17 µm film thickness) (J & W Scientific, Folsom, CA), with the following temperature program: 5 min at 60°C, subsequently 4°C min^{-1} up to 220°C, then 11°C min^{-1} up to 280°C, held for 15 min, for a total run of about 65 min. Injector and transfer line temperatures were 280°C. Helium was used as the carrier gas, at a flow rate of 1.2 ml/min; split ratio: 1:20. A mixture of aliphatic hydrocarbons (C8-C30) (Sigma, Milan, Italy) in hexane was directly injected into the GC injector under the aforementioned temperature program, in order to calculate the retention index (as Kovats index) of each compound. Data were collected by using HP3398A GC Chemstation software (Hewlett Packard, Rev. A.01.01).

GC-MS analysis was performed on an Agilent 6890N gas chromatograph coupled to a 5973N mass spectrometer using a HP-5MS (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 µm film thickness) (J & W Scientific, Folsom). The temperature program was the same with that reported earlier. Injector and transfer line temperatures were 280°C. Helium was used as the carrier gas, at a flow rate of 1 ml/min. Split ratio: 1:50; acquisition mass range: 29 to 400 m/z. All mass spectra were acquired in electron-impact (EI) mode with a ionization voltage of 70 eV (Tirillini et al., 2009).

Identification and quantification of volatiles

The volatile components detected in essential oils were identified by comparing the retention index and mass spectrum of the chromatographic peaks with that of standards analyzed under the same conditions. The peak assignments of the other volatile components was based on computer matching of the mass spectra obtained with the WILEY275, NIST 08, and ADAMS libraries, taking into account the coherence of the retention indices of the analyzed compounds with those reported by Adams and NIST libraries. Relative percentages of the components were calculated from peak areas without the use of a correction factor (Adams, 2001; Heller and Milne, 1983; Jennings and Shibamoto, 1980; McLafferty and Staufer, 1989; Stenhagen et al., 1974; Tirillini et al., 2009) .

Productivity

Three sampling were obtained on each field, on August 2009 (1°), on October 2009 (2°) and the last on August 2010 (3°). At every sampling, fresh weight/plant, width of branches/plant, number of leafs/plant, yield as fresh weight/field, yield as dry weight/field, water content in fresh plant, yield of essential oil, and composition of essential oil were determined. Data were expressed as mean values of at least three determinations, otherwise indicated.
Samples of chopped herb were dried to constant weight at 80°C to determine dry matter (DM) content.

Flower morphology and pollen viability

Flower morphology and pollen viability were characterized for each plant varieties. Flowers morphology were determined as mean length of upright spike-like cymes, mean number of flowers on each verticillaster and number of ovary/flower.

Florets, from of at least three flowers from different plants from each variety, were collected and immediately used for pollen viability determination following enzymatic test (TTC-test) and colorimetric test (Alexander, 1969). The TTC test result is one of the most widely used assays for pollen viability estimation. It is a staining technique for an enzymatic viability used to reveal the activity of respiration in living cells (Comtois and Schemenauer, 1991; Sarvella, 1964). Pollen, from fresh collected anthers, was dusted onto a microscope slide with a brush, and leave to incubate in TTC solution (2,3,5-triphenyl-tetrazonium chloride, 1% by weight in 50% sucrose) after being covered with a coverslip and the edges sealed with nail varnish, at 40°C for 30 min. Pollen viability was estimated under light microscope at 100× magnification, by discriminating viable pollen (stained red) from dead grains (yellow/white). Pollen viability is expressed as mean percentage from three replicates developed on pollens from three different plants for each variety. Control experiments were performed using heat-killed pollen (80°C for 2 h) (Dafni and Firmage, 2000).

A colorimetric determination of aborted/non aborted pollen were developed immediately after flower collection (Alexander, 1980). A drop of Alexander's stain was put on microscope slide, pollen from dehisced anthers was added with a platinum needle, gently heated, and mounted on a microscope slide. Alexander's dye is a mixture of malachite green staining the cellulose of pollen walls green, and acid fuchsin staining the pollen protoplast red (Singh, 2003). Under the light microscope (400× magnifications), 100 pollen grains per slide were scored as aborted or non-aborted. A pollen grain was considered aborted if it reveals pale turquoise blue stain and appear empty or had highly degenerated protoplasm. Non-aborted pollen is stained dark blue or purple and reveals a thick walls (ca.4.5 to 6.5 µm) that appears thinner in aborted pollen grains (ca. 2.5 to 3.5 µm) and refractive. At least three flowers from one to six plants were scored per cross combination.

Representative samples of flowers were collected and after recovery of pollen grains, were stored at -20°C.

Statistical analysis

Data reported are expressed as mean values of triplicate experiments. Analysis of variance, significances, correlations and other statistical analysis were performed by GraphPad Prism version 5.00 (GraphPad Software, San Diego, California, USA).

RESULTS AND DISCUSSION

Productivity

Immediately after collection, aerial parts of each plant were weighted. Data of yields and productivity were reported as shown in Table1.

The fresh weight/plant results were much different, both in varieties comparison and in different seasonal collections. The highest productivity in aerial parts results in REG at the early fall collection, while the lowest yield is from EXT collected in summer. During development, the plant productivity seems to increase, with highest value on late summer collection.

The dry matter yield results in higher fall sampling, with values of water amounts lower than 60%. The mean branch width calculated on different varieties is as shown in Table 2. Growing capacity of all varieties seems similar, all of them showed an increase of branch length during summer. Only for variety SYN is an elongation evident, that start earlier during late spring and continue up to fall, until it gets to the maximum value, that are close to those of other varieties.

Number of leafs/plant give variable values from 105.50 in EXT at fall collection, up to 395.75 in SYN during summer. Summing the total number of leaf of all sampling: SYN result is the variety with the highest leaf production in a complete season, while REG result is the most productive in a single sampling (fall).

Yield of fresh plant materials (Figure 1) in terms of mean aerial parts weight/plant result in 21.9, 30.8 and 81.2 g respectively for EXT, REG and SYN. The production strongly increases in late summer collection, when values are two to three times higher.

Similar is also the variation of dry matter. This data result is useful in the determination of optimal time to collect the aerial parts of plants, which represent the crude drug used in medicinal and food industries.

Essential oil

The essential oil yields, expressed in ml/kg DW, varied from one accession to another and in different seasonal collection ranging from 0.22 to 1.6%. The lowest values were observed in EXT variety, at fall collection, while the highest result was in REG, at summer collection, during the second year. In literature some sages with hyper production of essential oil up to 3% were described, but most of the plant cultured gives a yields oscillating around 1.5% (Bernotiene et al., 2007; Mockute et al., 2003; Zawislak and Dyduch, 2006).

All varieties result in medium-high productive essential oil and are useful for industrial applications as well as for medicinal use, but only if collected in summer (the European Pharmacopoeia prescribe a minimum of 10% oil yield from cut dried sage). REG result in the best in the quantity of essential oil production. Unexpected was the weak result for EXT, selected as high productive variety. Data confirm that the preferred time to collect this plant for oil extraction is summer, as traditionally indicate by balsamic time that correspond to full bloom. Yield of essential oil in fall collection results is low, so not advantageous for commercial purpose.

Qualitative and quantitative compositions of essential oils from bloom collection in subsequent year are reported in Table 3. A comparison of main component composition of oil from summer and fall collection is reported in Figure 2. The qualitative analysis shows strong similarities between samples that, in opposition, result markedly different quantity. Statistical analysis showed high correlation value for the yields obtained

Table 1. Data of yield mean values.

Variety	Sampling	A	B	C	D	E	F	G	H	I	J	K	L
	Aug 09	36.78 ±8.5	11.39		30.25	33.31	-	8.45±4.6	22.19	-	189.00	10.28	-
EXT	Oct 09	107.34 ±13.2	-	65.73	40.93	-	26.09	7.11±8.7	-	-18.84	105.50	-	-79.14
	Aug 10	41.51 ±9.6	-	-	45.36	-	-	10.86±4.6	-	-	210.66	-	-
	Aug 09	41.57 ±17.1	8.75	-	34.64	16.65	-	7.58±4.2	28.01	-	270.66	11.93	-
REG	Oct 09	145.98 ±8.9	-	71.52	35.52	-	2.47	7.51±3.2	-	-0.93	207.33	-	-30.54
	Aug 10	45.56 ±16.3	-	-	41.56	-	-	10.53±5.0	-	-	307.33	-	-
	Aug 09	62.01 ±10.7	9.54	-	31.16	36.64	-	7.86±3.6	36.35	-	333.16	15.81	-
SYN	Oct 09	124.93 ±8.55	-	50.36	34.11	-	8.64	10.14±5.2	-	22.48	145.50		-128.97
	Aug 10	68.55 ±11.5	-	-	49.18	-	-	12.35±6.3	-	-	395.75	-	-

A: Fresh weight/plant (g); B : % variation Summer 09/10; C: % variation Summer/Fall 09; D: dry matter content /plant (%); E: % variation Summer 09/10; F: % variation Summer/Fall 09; G: branch length (cm); H: % variation Summer 09/10; I: % variation Summer/Fall 09; J: mean number of leaf/plant; K: % variation Summer 09/10; L: % variation Summer/Fall 09.

Table 2. Yield of essential oil from aerial parts of sage (ml/kg).

Yield	EXT	REG	SYN
AUG 09	10±1.8	14±1.7	13.8±1.6
OCT 09	2.2±0.7	3.6±0.9	6.5±1.2
AUG 10	11.2±1.7	16.0±1.9	12.3±1.1

from different collection (EXT and REG; P<0.01), except for SYN that reveals no significant R squared value.In all samples, 1,8 cineol is present in constant range from 11.12 to 12.78%. The quantity remains constant and seems not be affected by seasonal changes. Larger difference are in α-pinene content that differs from 7.45% in SYN to 2.46 in REG 2°, with strong decrease from summer to fall collections. Similar is the distribution of camphene, which is present in lower percentage. In all tested samples, α-thujone is always the most abundant compound, and the content is statistically equivalent from summer to fall (from 20.88, 22.46, and 23.76 to 24.32, 21.55 and 22.89%, respectively in EXT, REG and SYN). Also at the highest thujone content, none of the selected varieties reach 50%, and probably not indicated for medicinal uses, and the European Pharmacopeia reported that sage has to be rich in thujone (European Pharmacopoeia Commission, 2008). None of studied varieties are related to thujone-chemotypes, confirming the original selection target that was for application in industrial practices, such as food and drink preparations. The actual European law on food (European Parliament and Council, 2008) allows the use of sage and other thujone-containing flavoring plants (except for *Artemisia*) in foods without restrictions, deregulating the previous limit for thujone of 25 mg/kg recommended by the Scientific Committee on Food of the European Commission (SCFEC, 2003).Camphor is present in quantities from 13.88 to 19.46%, and decrease from summer to winter in SYN. Great variation was found in the proportions of the major compounds between the populations examined. Correlation analysis was done to explore the trend of association between individual components and more abundant phytochemicals (Table 4). Analysis using combined data from all samples revealed characteristic significant correlation within single components.

The highest correlation values were α-pinene/α-thujone, borneol/1,8-cineole, bornyl acetate/ camphor and camphor/(E)-caryophyllene (R>0.8). Each of the most abundant components is significantly correlated with at least one other main component, except for camphene that is significantly related only to minor component. Borneol and α-pinene have the highest number

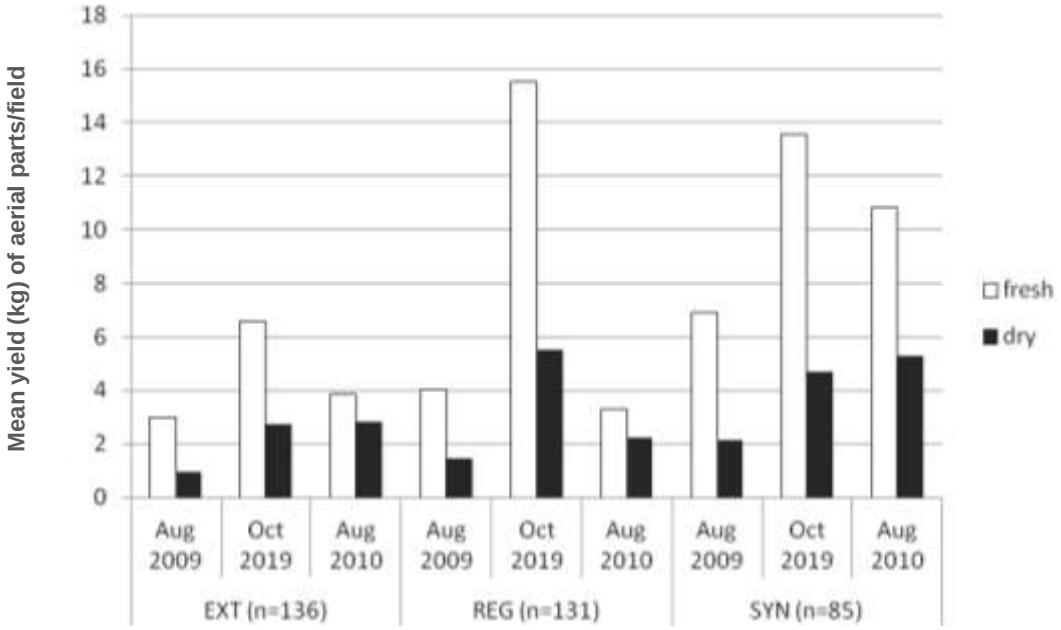

Figure 1. Seasonal aerial part production of sage varieties. Extimated mean yield of fresh plant material from different sampling of all aerial parts of at least three plant/variety.

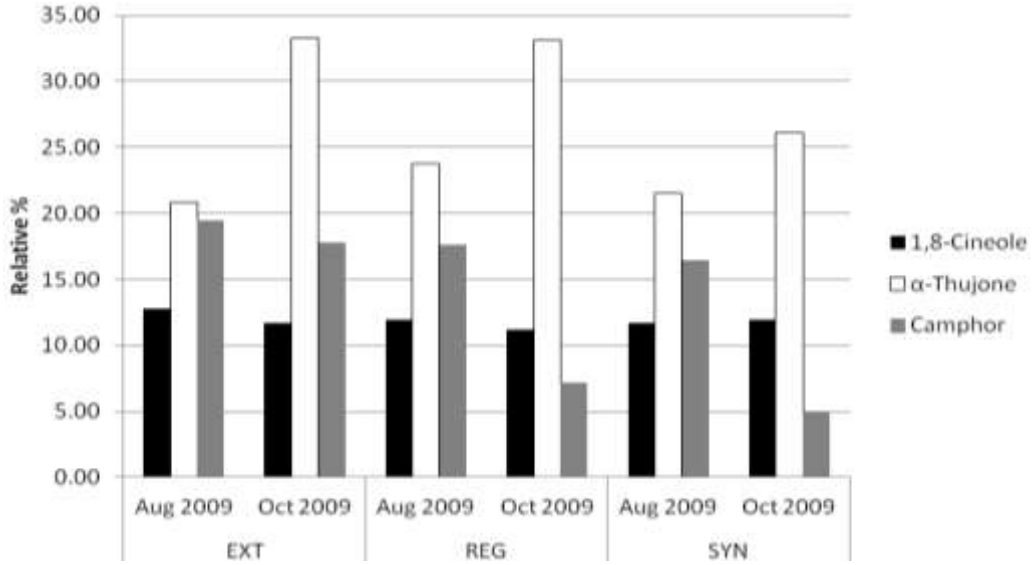

Figure 2. Main component quantity in essential oils .
Comparison of mean relative percentage of most aboundant metabolites in essential oils at summer and fall collection.

(three) of statistically significant correlations.

Flower morphology

Flower morphology was determined on at least three inflorescences from three different plants on each variety. Data of mean length of representative number of upright spike-like cymes, mean number of flowers on each verticillaster and number of ovary on each flower, observed for each plant, are shown in Table 5.

Pollen

Pollen grains collection was difficult. During sampling it

Table 3. Composition, as percentages, of the essential oils of three varieties of *Salvia officinalis* L.

Compound	RI	Var. *extrakta*				Var. *regula*				Var. *syn 1*			
		Aug 09		Aug 10		Aug 09		Aug 10		Aug 09		Aug 10	
		Mean (%)	± S.D.	Mean (%)	± S.D.	Mean (%)	± S.D.	Mean (%)	± S.D.	Mean (%)	± S.D.	Mean (%)	± S.D.
α-Thujene	925	0.44^{a}	0.04	0.53^{bc}	0.05	0.51^{ab}	0.05	0.48^{ab}	0.05	0.63^{c}	0.06	0.57^{bc}	0.05
α-Pinene	938	7.04^{cd}	0.61	6.1^{c}	0.52	3.95^{b}	0.34	2.46^{a}	0.21	7.45^{d}	0.65	3.59^{b}	0.31
Camphene	952	6.99^{abc}	0.48	6.24^{a}	0.43	6.34^{a}	0.44	7.45^{bc}	0.52	6.47^{ab}	0.45	7.58^{c}	0.52
Sabinene	971	0.42^{ab}	0.04	0.38^{ab}	0.03	0.41^{ab}	0.04	0.35^{a}	0.03	0.53^{c}	0.05	0.44^{b}	0.04
β-Pinene	981	4.3^{ab}	0.36	5.19^{c}	0.44	3.88^{a}	0.33	4.23^{ab}	0.36	5.35^{c}	0.45	4.89^{bc}	0.41
Myrcene	992	1.38^{b}	0.12	1.04^{a}	0.09	1.46^{b}	0.12	0.85^{a}	0.07	1.06^{a}	0.09	0.95^{a}	0.08
α-Terpinene	1016	0.24^{c}	0.02	0.18^{ab}	0.01	0.21^{bc}	0.02	0.15^{a}	0.01	0.16^{a}	0.01	0.22^{c}	0.02
p-Cymene	1023	0.13^{bc}	0.01	0.11^{ab}	0.01	0.1^{a}	0.01	0.12^{ab}	0.01	0.15^{c}	0.02	0.19^{d}	0.02
Limonene	1025	0.15^{a}	0.02	0.17^{a}	0.02	0.22^{bc}	0.02	0.18^{ab}	0.02	0.24^{c}	0.02	0.19^{ab}	0.02
d-3-Carene	1030	0.35^{ab}	0.02	0.38^{ab}	0.03	0.45^{c}	0.03	0.41^{bc}	0.03	0.33^{a}	0.02	0.37^{ab}	0.03
1.8-Cineole	1033	12.78^{a}	1.15	12.6^{a}	1.14	11.98^{a}	1.08	11.45^{a}	1.03	11.67^{a}	1.05	11.12^{a}	1
(*Z*)-β-Ocimene	1034	0.09^{c}	0.01	0.05^{a}	0.01	0.1^{c}	0.01	0.05^{a}	0.01	0.07^{b}	0.01	0.09^{c}	0.01
(*E*)-β-Ocimene	1045	0.02^{b}	0.01	0.02^{b}	0.01	0.02^{b}	0.01	0.02^{b}	0.01	0.01^{a}	0.01	0.01^{a}	0.01
γ-Terpinene	1060	0.8^{bc}	0.08	0.56^{a}	0.06	0.71^{ab}	0.07	0.76^{bc}	0.08	0.82^{bc}	0.08	0.88^{c}	0.09
cis-Linalool oxide	1072	0.14^{d}	0.01	0.12^{c}	0.01	0.1^{b}	0.01	0.09^{ab}	0.01	0.12^{c}	0.01	0.08^{a}	0.01
Terpinolene	1088	0.78^{c}	0.07	0.26^{a}	0.02	0.69^{bc}	0.06	0.35^{a}	0.03	0.62^{b}	0.05	0.27^{a}	0.02
α-Thujone	1102	20.88^{a}	2	22.46^{a}	2.15	23.76^{a}	2.28	24.32^{a}	2.33	21.55^{a}	2.07	22.89^{a}	2.2
β-Thujone	1114	3.67^{a}	0.29	4.04^{a}	0.31	3.93^{a}	0.31	4.89^{b}	0.38	3.67^{a}	0.29	4.13^{a}	0.32
Camphor	1143	19.46^{b}	1.67	18.34^{b}	1.57	17.64^{b}	1.51	19.34^{b}	1.65	16.45^{ab}	1.41	13.88^{a}	1.19
Borneol	1164	1.54^{a}	0.11	1.89^{ab}	0.13	2.43^{c}	0.16	2.76^{cd}	0.19	2.01^{b}	0.14	3.12^{d}	0.21
Terpinen-4-ol	1177	0.4^{ab}	0.04	0.46^{bc}	0.04	0.55^{c}	0.05	0.34^{a}	0.03	0.37^{ab}	0.03	0.43^{ab}	0.04
α-Terpineol	1189	0.77^{b}	0.07	0.56^{a}	0.05	0.64^{ab}	0.06	0.59^{a}	0.05	0.65^{ab}	0.06	0.57^{a}	0.05
Bornyl acetate	1283	2.56^{b}	0.23	1.98^{ab}	0.17	2.35^{ab}	0.2	1.77^{a}	0.15	3.23^{c}	0.28	4.89^{d}	0.43
cis-Sabinyl acetate	1286	0.55^{b}	0.05	0.37^{a}	0.03	0.48^{b}	0.05	0.51^{b}	0.05	0.35^{a}	0.03	0.37^{a}	0.03
δ-Elemene	1334	0.05^{d}	0.01	0.02^{b}	0.01	0.02^{b}	0.01	0.01^{a}	0.01	0.02^{b}	0.01	0.01^{a}	0.01
trans-Carvyl acetate	1336	0.02^{b}	0.01	0.02^{b}	0.01	0.02^{b}	0.01	0.01^{a}	0.01	0.01^{a}	0.01	0.02^{b}	0.01
cis-Carvyl acetate	1362	0.01^{a}	0.01	0.01^{a}	0.01	0.01^{a}	0.01	0.01^{a}	0.01	0.01^{a}	0.01	0.01^{a}	0.01
Neryl acetate	1364	0.02^{b}	0.01	0.01^{a}	0.01	0.02^{b}	0.01	0.01^{a}	0.01	0.02^{b}	0.01	0.02^{b}	0.01
α-Copaene	1377	0.12^{a}	0.01	0.28^{b}	0.02	0.14^{a}	0.02	0.32^{bc}	0.03	0.15^{a}	0.02	0.34^{c}	0.03
(*Z*)-Caryophyllene	1409	2.12^{a}	0.2	3.35^{c}	0.31	2.56^{ab}	0.24	4.01^{d}	0.37	2.98^{bc}	0.27	3.45^{cd}	0.32
(*E*)-Caryophyllene	1419	3.77^{a}	0.34	4.89^{b}	0.45	4.67^{ab}	0.42	4.03^{ab}	0.37	4.78^{b}	0.43	5.89^{c}	0.54
α-Humulene	1447	1.05^{bc}	0.09	0.89^{ab}	0.07	1.1^{c}	0.09	1.14^{c}	0.1	0.79^{a}	0.07	1.14^{c}	0.1
Allo-aromadendrene	1457	0.06^{c}	0.01	0.02^{a}	0.01	0.06^{c}	0.01	0.04^{b}	0.01	0.02^{a}	0.01	0.02^{a}	0.01
Germacrene D	1477	0.11^{ab}	0.01	0.09^{a}	0.01	0.12^{bc}	0.01	0.13^{bc}	0.01	0.09^{a}	0.01	0.14^{c}	0.01

Table 3. Contd.

α-Selinene	1491	0.03^{c}	0.01	0.01^{a}	0.01	0.01^{a}	0.01	0.02^{b}	0.01	0.01^{a}	0.01	0.01^{a}	0.01
δ-Cadinene	1520	0.12^{d}	0.01	0.08^{a}	0.01	0.08^{a}	0.01	0.09^{ab}	0.01	0.11^{cd}	0.01	0.1^{bc}	0.01
Ledol	1564	2.55^{ab}	0.18	2.35^{a}	0.16	2.96^{bc}	0.2	2.32^{a}	0.16	2.77^{bc}	0.19	3.11^{c}	0.21
Caryophyllene oxide	1584	0.21^{a}	0.02	0.25^{bc}	0.02	0.23^{ab}	0.02	0.28^{bc}	0.02	0.31^{cd}	0.03	0.35^{d}	0.03
Viridiflorol	1593	2.56^{bc}	0.23	2.12^{ab}	0.2	2.67^{c}	0.24	2.34^{abc}	0.21	2.45^{abc}	0.23	2.03^{a}	0.19
Sclareol	2223	1.07^{c}	0.08	0.88^{ab}	0.07	1.79^{d}	0.13	0.83^{a}	0.06	0.78^{a}	0.06	1.02^{bc}	0.07
Total		99.7^{5}	-	99.3	-	99.37	-	99.51	-	99.26	-	99.38	-

Values are means of three determinations ± standard deviation. RI: Retention indices as determined on HP-5 column. Values within a row for each compound having different letters are significantly different from each other using Tukey's and LSD test (P>0.05).

Table 4. Correlation coefficients within main components of the essential oil.

Component	α-Pinene	Camphene	1.8-Cineole	α-Thujone	α-Thujone	Camphor	Borneol	Bornyl acetate	(Z)-Caryophyllene	(E)-Caryophyllene
Camphene	0.3150									
1.8-Cineole	0.3863	0.3340								
α-Thujone	0.8516**	0.0563	0.2904							
β-Thujone	0.6966*	0.3032	0.2301	0.6606*						
Camphor	0.0149	0.0561	0.4405	0.0001	0.0348					
Borneol	0.7401*	0.3859	0.8069**	0.5555*	0.4015	0.3442				
Bornyl acetate	0.0003	0.1876	0.3124	0.0469	0.0754	0.8547**	0.2191			
(Z)-Caryophyllene	0.3792	0.1801	0.3938	0.4138	0.7016*	0.0310	0.4366	>0.0001		
(E)-Caryophyllene	0.0349	0.0090	0.2990	0.0183	0.0072	0.8661**	0.3356	0.5979*	0.0937	
Ledol	0.0140	0.0121	0.2379	<0.0001	0.1498	0.6746*	0.2040	0.6386*	0.0724	0.4492

*,**Significant at P<0.05 and P<0.01, respectively.

was evident that flowers did not fully develop male component. Externally, anthers were full developed, but most of them result to be empty inside. Pollen collection resulted poor in all samples and only variety EXT results was more consistent.

Analysis of pollen viability and differentiation did not show large differences, as shown in Table 6. Only three plants (EXT (2), SYN (2), and SYN (3)) showed a weak pollen differentiation, while only pollen from sample EXT (2) showed very low viability. All other samples contain less than 1% of viable pollen.

All samples analyzed reveal very poor pollen stainability. The average pollen stainability was lower than 5%, and only few samples reveal higher mean values, deriving large viability differences between pollen of plants from the same population and also between different florets from single plant.

These data suggest the hybrid sterile nature of selected varieties, and is rationally correlated with agricultural practice of asexual propagation for large scale cultivations.

DISCUSSION

All varieties showed good adaptation performance to cultivation in Valtiberina's area. For productive purpose, SYN results in the best variety due to

Table 5. Flowers morphology in *Salvia* varieties.

Salvia variety	Plant sample[1]	Lenght of upright spike-like cymes (cm)	No. florets/ verticillaster	No. ovary/ florets	Number of flowers with same number of ovary (No. of ovary)			
					< 25%	26-50%	51- 75%	>76%
EXT	1[a]	4.5	6	5	4 or 6	-	5	-
	1[b]	3.5	7	4.3	5	-	4	-
	1[c]	4.3	5	4.2	5	-	4	-
	1[d]	6.0	7	4	-	-	-	4
	2[a]	3.2	5	4	-	-	-	4
	2[b]	4.1	5	4.2	5	-	4	-
	2[c]	3.5	5	4	-	-		4
	2[d]	6.0	5	4.1	5	-	4	-
	3[a]	2.5	5	4	-	-	-	4
	3[b]	2.7	5	4	-	-	-	4
	3[c]	5.5	6	4.1	-	-	-	4
SYN	4[a]	3.5	7	4	-	-	-	4
	4[b]	4.3	5	4	-	-	-	4
	4[c]	8.2	6	4	-	-	-	4
	5[a]	3.2	5	4.1	5	-	4	-
	5[b]	3.4	3	4	-	-	-	4
	5[c]	5.5	4	4	-	-	-	4
	6[a]	4.5	5	4.3	5 or 6	-	4	-
	6[b]	7.8	5	4.4	6	-	4	-
	6[c]	6.5	5	4	-	-	-	4
REG	1[a]	6.1	7	4	-	-	-	4
	1[b]	6.8	3	4	-	-	-	4
	1[c]	12.0	6	4	-	-	-	4
	2[a]	5.1	5	4	-	-	-	4
	2[b]	7.5	5	4.2	6	-	4	-
	2[c]	11.0	6	4.3	5	-	4	-
	3[a]	4.5	5	4.2	5	-	4	-
	3[b]	4.3	5	4	-	-	4	-
	3[c]	8.0	5	4	-	-	4	-

[1]Numbers identify the plant and letters different flowers from the same plant.

bigger dimensions, high number of leaves production and low water content. Also concerning the essential oil, SYN is preferable, due to lower quantitative variation as yield of extraction and to the larger number of compounds present, which is over 5%. The resulting essential oil has organoleptic quality more pleasant and equilibrate. The quality of cultivated REG is good as well with productive yield much more abundant than EXT. This variety, also with lower productive yield, is interesting in alimentary industry due to the quality of essential oil with lowest thujone content. The actual European law on food (European Parliament and Council, 2008) allows the use of sage and other thujone-containing flavoring plants (except for Artemisia) in foods without restrictions, deregulating the previous limit of 25 mg/kg of thujone in foodstuffs containing pre-parations based on sage (European Council, 1988) that was considered adequate by the Scientific Committee on Food of the European Commission (SCFEC, 2003). Standard quality for plants intended for medicinal use considers only the essential oil yield (15 ml/kg for whole drug and minimum 10 ml/kg for the cut drug), with no quantitative reference to the thujone content (European Pharmacopoeia Commission, 2008). On the other hand, the European Medicines Agency, in sage monograph indicates 5.0 mg of thujone/ person as acceptable daily intake (ADI) for at least two weeks treatment (EMA, 2009). The limit is re-considered in the 2011 public statement (EMA, 2011) where the increase of ADI limit to 6 mg/person was suggested to be considered for the monograph's revision. Thujone is still

Table 6. Pollen viability and differentiation.

Variety and plant	Percent of differentiated pollen (Alexander test)	Percent of pollen viability (TTC test)
REG 1	<1	<1
REG 2	<1	<1
REG 3	<1	<1
EXT 1	<1	<1
EXT 2	39.9	20.4
EXT 3	<1	<1
SYN 1	<1	<1
SYN 2	9.6	<1
SYN 3	6.2	<1

not allowed as food additive and also if it can be introduced into food using plant containing thujone, the potential risk for the consumer is practically guaranteed by different factors, such as the weak solubility in water that reduce the amount in teas and other water-based formulation (Lachenmeier and Uebelacker, 2010). An extended investi-gation on chemical composition and phytochemical concentration of commercial sage samples from German market do not evidenced health risk associated to the occasional and rational use of sage derived products as food as well as medicines. In fact, in condition of use of up to 6 cup a day of sage tea, the ADI level of thujone is not reached (Walch et al. 2011).

Flowers morphology results are different, most of the flowers has abnormal number of ovary lobes, also with constant number of stamens. These produce a very small amount of pollen grains with weak percentage of differ-rentiation and with no respiratory activity as marker of viability. In these conditions, reproductive process are not regularly guaranteed due to high risk of male sterility rather than ovary anomalies. Such conditions are fre-quent in plants that are hybrids of different species.

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