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Chromatographic (GC-MS, HPLC) and virological evaluations of *Salvia sclarea* infected by BBWV-I

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Abstract

Salvia sclarea cultivated at the Herb Garden of Casola–Valsenio (Emilia–Romagna region, Italy) has been found for the first time naturally infected by broad bean wilt fabavirus, serotype I (BBWV-I). Symptomatic plants showed malformed leaves, with chlorotic mosaic followed by yellowing and stunting. BBWV-I was identified by applying virological tests: mechanical inoculations on herbaceous plants, electron microscopy, DAS-ELISA and PAS-ELISA. The essential oil obtained from BBWV-infected material corresponded to 2/3 the quantity of that from healthy material. The GC-MS and HPLC analyses of these oils afforded a comparative analytical profile of the two plant materials attributed to BBWV-I infection. The oils from infected materials showed higher percentages of sesquiterpene hydrocarbons (e.g. germacrene D and β-caryophyllene), monoterpene alcohols (e.g. α-terpineol) and diterpenoids (mainly sclareol). In contrast, lower levels of monoterpene hydrocarbons (e.g. myrcene, limonene and the two ocimene isomers) and the principal components (linalyl acetate and linalool) were observed.

Keywords: *Salvia sclarea*; Broad bean wilt fabavirus; Symptomatology; Essential oil; GC-MS analysis; HPLC analysis

1. Introduction

Many phytopathological problems, such as fungal, viral and bacterial infections, affect the different species of medicinal and aromatic plants cultivated in Italy, reducing the quality and the quantity of fresh and/or dry products [1–5]. In particular, during the past decade, virus spread has become more frequent, causing severe damage to crops located in several geographically distinct areas [6–9]. The symptoms associated with virus presence can affect leaves (vein-clearing, mosaic, necrosis, chlorotic, yellow or brown spots, stripes between the veins, curling, malformation, etc.) and flowers (smalling, necrosis, deformation, breaking patterns on the petals, etc.); in some cases growth can be reduced and the plant does not bloom. All these symptoms are intensified when plants are mixed virus infected.

During an epidemiological survey carried out in 1999 to identify viral infections most frequently occurring in Emilia–Romagna region (northern Italy), some plants of *Salvia sclarea* L. (*Labiatae* family) were found showing virus-like symptoms. *S. sclarea*, also known as clary sage, a plant native to southern Europe, is one of the most important aromatic plants cultivated world-wide as a source of essential oils and many other compounds derived from the different parts of the plant. *S. sclarea* has shown diverse biological activities manifested by the different components (mainly of essential oil) that allowed for the many medicinal and pharmaceutical applications of the plant materials and/or extracts [10]. Recent studies reported analgesic, *anti*-inflammatory and antimicrobial effects of the plant essential oil fractions and their relation to the chemical composition of the oil [11,12]. The effects of the plant extracts on the CNS as well as on skeletal and smooth muscles have also been reported [13,14]. The chemical composition of the essential oil from *S. sclarea* was found to be almost exclusively determined by the geographical habitat

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'source' of the plant growing wild or cultivated [12,15–17] and few (if any) differences were observed from different plant parts (except for leaves) or different harvest and cultivation conditions [15]. In addition, the various bioactivities of the oil were found to correlate with its composition, particularly the major components [11,18].

According to the various pharmacopoeias (USP, BP and European), the quality control of essential oils from medicinally important plants is of great importance and involves evaluation of any modification in the qualities as well as the quantities of its constituents, in particular the principal active ones. In the present communication we report the results of the virological study carried out to identify the virus associated with the disease observed in the open field. Moreover, since comparative research concerning the effect of viral infections on essential oil production by medicinal and aromatic plants has not been reported before, this aspect was also investigated. Therefore, in order to verify possible qualitative and/or quantitative differences in the composition of essential oils from healthy and infected *S. sclarea* plants, GC-MS and liquid chromatography (HPLC) analyses of both oils were performed.

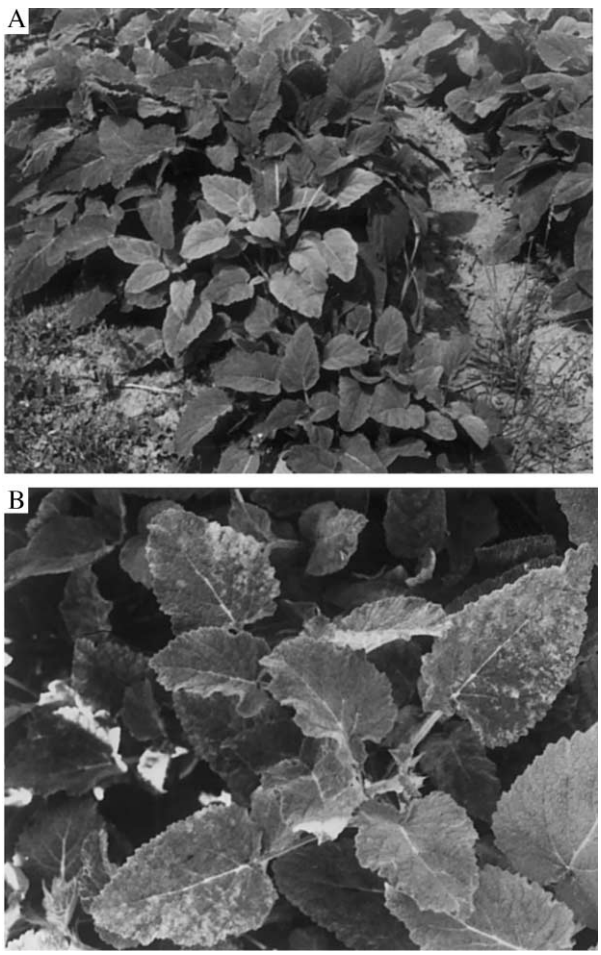

Fig. 1. *S. sclarea* growing at the Herb Garden of Casola–Valsenio. Some plants show virus-like symptoms consisting of (A) stunting and leaf yellowing, and (B) chlorotic mottle on leaves.

2. Materials and methods

2.1. Sample collection

During spring–summer 1999, a severe virus-like disease was observed on 30% of *S. sclarea* plants, obtained by seed, grown in the open at the Herb Garden of Casola–Valsenio (Italy). Leaf symptoms consisted of chlorotic mosaic and malformations, followed by stunting and yellowing of the entire plant (Fig. 1A and B). Several leaf samples from both symptomatic and asymptomatic plants (control) were collected and used in virological tests.

2.2. Virological tests

The identification of the virus was performed by mechanical inoculation on herbaceous hosts belonging to seven botanical families. The infections thus obtained in the test plants were the object of further investigations to identify the isolated virus, such as electron microscopy and serology.

The preparations for electron microscope (Philips CM 10), leaf-dip method, were negatively stained with 2% (w/v) aqueous uranyl acetate (UA) or 1% phosphotungstic acid (PTA) neutralized to pH 7.0. Serological techniques applied were double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) [19] and protein A sandwich (PAS)-ELISA [20]. The commercial kit DAS-ELISA, containing polyclonal antibodies to broad bean wilt fabavirus (BBWV), was obtained from Loewe Biochemica GmbH (Germany). In PAS-ELISA, sera to the following isometric or bacilliform viruses were tested: cucumber mosaic cucumovirus (CMV); alfalfa mosaic alfamovirus (AMV); arabis mosaic nepovirus (ArMV); strawberry latent ring spot nepovirus (SLRSV); tobacco ring spot nepovirus (TRSV); tomato ring spot nepovirus (ToRSV); tomato black ring nepovirus (TBRV); tobacco streak ilarvirus (TSV); BBWV serotype I (BBWV-I) and II (BBWV-II). The sera to CMV, AMV, ArMV, TRSV, ToRSV, TSV were obtained from American Type Culture Collection, ATCC, Rockville, MD; the Istituto di Fitovirologia Applicata, Turin, Italy, provided the sera to BBWV-I and II; anti-SLRSV and anti-TBRV were available at our Institute.

2.3. Plant materials and oils

In June, about 2 kg of samples (leaves) from infected and healthy *S. sclarea* plants were collected and investi-

gated by DAS-ELISA to verify BBWV presence before the oil extraction. The distillation was performed by the method of 'water and steam' (or direct steam distillation) at the Herb Garden. For comparative purposes, a commercial oil sample from *S. sclarea* of a different source (Russia) was obtained from Moellhausen s.p.a (Cologno Monzese (MI), Italy).

2.4. GC-MS analysis

About 0.1 μl volumes of the tested oils without further modifications were injected into a TRACE GC 2000 SERIES (ThermoQuest CE Instruments) gas chromatograph equipped with a split–splitless injector (split ratios of 50:1 and 75:1). The column was an Rtx®-5MS fused silica capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness) consisting of Crossbond® (5% phenyl 95% dimethyl polysiloxane). Helium (He) was the carrier gas at a flow rate of 1.0 ml/min. The GC was interfaced with a GCQ plus (ThermoQuest, Finnigan) mass detector operating in the EI mode (70 eV) using an autotune file. The mass spectra were recorded within 40–650 m/z full scan mode that revealed the total ion current (TIC) chromatograms. A linear temperature program was adopted to separate the different oil components as follows: initial temperature: 70°C (hold time: 10 min), then ramped by 4°C/min to 210°C (hold time: 10 min). The temperatures of the injector base, transfer line, and ionization source were maintained at 250, 250 and 200°C, respectively.

The chemical identities of the separated components were determined by matching their recorded mass spectra with the data bank of mass spectra (General Purpose, Terpene 'ThermoQuest' and NIST libraries) provided by the instrument software, and by comparing their retention indices with literature values measured on columns with identical [21,22] and/or similar [23] polarity. Some structures were further confirmed by authentic standards analyzed under the conditions mentioned above (Table 1). An *n*-alkane hydrocarbon mixture (C_8–C_{24} series) was injected under the above temperature program to calculate the retention indices using the generalized equation by Van Del Dool et al. [24]. Concentrations (% content) were calculated by integrating peak areas assuming a unity response by all.

2.5. Direct MS analysis

Using the direct insertion probe (DIP) technique, some of the fractions collected from the HPLC analyses were pooled, concentrated and directly introduced into the mass spectrometer through the optional MS valve. The samples were rapidly ramped by 50°C/min (initial temperature: 50°C, hold time: 30 s. — final temperature: 350°C, hold time: 30 s.). The mass spectra and the TIC chromatograms were recorded under the same MS conditions mentioned previously.

2.6. Liquid chromatography (HPLC) analysis

All solvents used were of analytical chromatographic grade (Carlo Erba reagents). HPLC was performed with a HP Ti series 1050 liquid chromatograph, equipped with a photodiode array detector (DAD, HP series 1050). Solutions of the tested oils (5% v/v in *n*-hexane or ethanol) were subjected to normal phase HPLC analysis carried out on a Phenomenex Hypersil 5 CN (5 μm, 25 × 4.6 mm) column using a mobile phase of *n*-hexane at a flow rate of 1.0 ml/min. The injector was a Rheodyne model valve with a 20-μl loop. UV detection (DAD) at two wavelengths (245 and 265 nm) was recorded.

Eluate fractions obtained from HPLC analyses (*n*-hexane solutions) were further subjected to the above GC-MS analysis directly or after concentration under vacuum.

3. Results and discussion

3.1. Virological study

Mechanical inoculations made it possible to infect several herbaceous plants, including *Chenopodium quinoa* Willd., *C. murale* L. and *C. amaranticolor* Coste et Reyn., which showed local and systemic symptoms in 3–4 days. *Spinacia oleracea* L., *Gomphrena globosa* L., *Nicotiana tabacum* L., *N. clevelandii* Gray., *Petunia hybrida* Hort., *Vicia fava* L. (broad bean) were also infected. No species belonging to *Labiatae* (*Ocimum basilicum* L., *Origanum majorana* L., *Thymus vulgaris* L., *Salvia officinalis* L.), *Cucurbitaceae* (*Cucumis sativus* L., *C. melo* L., *Cucurbita pepo* L.), and *Umbelliferae* (*Apium graveolens* L., *Petroselinum sativum* Hoffm.) families showed symptoms or was latently infected.

In leaf-dip preparations from field-collected *S. sclarea* (symptomatic and asymptomatic) and inoculated herbaceous plants, no virus particles were observed. DAS-ELISA identified the virus infecting symptomatic *S. sclarea* as BBWV, which was also detected in inoculated host plants. PAS-ELISA provided a better characterization of this virus, which was identified as BBWV-I. No reaction was obtained with the sera to BBWV-II, CMV, AMV, ArMV, SLRSV, TRSV, ToRSV, TBRV, TSV.

The virological investigation carried out at the Herb Garden of Casola–Valsenio shows that BBWV naturally infects *S. sclarea*, causing severe symptoms (stunting and leaf yellowing) which can reduce the yield of the crop. The finding of BBWV here described is the first report of its natural infection in *S. sclarea*.

BBWV has been identified by DAS-ELISA technique, but PAS-ELISA confirmed its characterization as BBWV-I. This serotype, transmitted by aphids (in a

Table 1
Composition of essential oils from *S. sclarea* of different disease states and sources.

No.	R_t	R_I	Comp.	% Content of the oil		
				Healthy	infected	Russian [a]
1	4.70	931	α-pinene	0.02	0.02	0.12
2	5.16	946	camphene	0.01	0.01	0.02
3	5.95	971	sabinene	0.02	0.01	0.01
4	6.10	976	β-pinene	0.03	0.03	0.05
5	6.61	993	myrcene	3.29	2.08	0.68
6	7.24	1007	α-phellanderene	0.05	0.03	0.12
7	7.79	1016	α-terpinene	0.11	0.07	0.06
8	8.22	1024	*p*-cymene	0.05	0.05	0.07
9	8.42	1027	limonene	1.03	0.54	0.42
10	8.97	1036	*Z*-ocimene	1.71	1.05	0.37
11	9.61	1047	*E*-ocimene	2.96	1.89	0.78
12	10.28	1059	γ-terpinene	0.09	0.06	0.04
13	11.20	1075	myrtenol [b]	tr	tr	tr
14	12.08	1089	terpinolene	0.33	0.24	0.33
15	12.86	1103	linalool	10.06	9.01	11.97
16	14.40	1134	α-pyronene [b]	0.09	0.06	0.02
17	15.72	1160	*cis* D.H. carvone	tr	0.01	0.01
18	16.24	1170	borneol	0.03	0.05	0.01
19	16.74	1180	4-terpineol	0.01	0.02	0.04
20	17.41	1193	α-terpineol	1.64	2.30	3.5
21	18.56	1220	β-citronellol	0.16	0.06	0.36
22	19.13	1233	4-terpinyl acetate	0.13	0.21	0.35
23	20.26	1261	linalyl acetate	55.72	54.40	42.75
24	21.40	1288	*trans*-anethol	NF	NF	2.04
25	21.43	1289	isobornyl acetate (α-terpinyl acetate in Russian)	0.06	0.08	0.05
26	21.76	1297	thymol	0.36	0.62	NF
27	22.10	1306	carvacrol	0.07	0.06	NF
28	23.78	1353	geranyl acetate	0.18	0.17	0.07
29	24.35	1369	neryl acetate	1.11	1.27	0.98
30	24.61	1377	α-copaene	2.06	2.33	1.00
31	24.91	1385	β-bourbonene	0.34	0.49	0.13
32	25.03	1388	sabinene hydrate acetate	2.24	2.33	1.75
33	25.14	1392	β-cubebene	0.50	0.55	0.29
34	25.21	1394	α-gurjinene	0.28	0.37	0.18
35	26.11	1421	β-caryophyllene	3.84	4.24	3.00
36	26.44	1431	germacrene D isomer # 1 [b]	0.17	0.23	0.06
37	26.76	1441	longifolene	0.06	0.14	NF
38	27.01	1449	α-humulene	0.04	0.05	0.02
39	27.25	1456	α-guaiene	0.16	0.20	0.11
40	28.15	1484	germacrene D	7.58	9.47	4.35
41	28.37	1491	γ-muurolene	0.91	0.49	0.12
42	28.65	1500	valencene	0.48	0.55	0.84
43	28.80	1504	γ-cadinene	0.10	0.13	0.06
44	29.01	1511	α-*trans*-farnesene	0.19	0.18	0.19
45	29.20	1518	unk.	0.07	0.08	0.10
46	29.30	1521	cedrane diol(8*S*,14) or longiborneol [b]	0.06	0.08	0.10
47	29.49	1528	δ-cadinene	0.58	0.73	0.30
48	29.90	1541	unk.	0.02	0.03	NF
49	30.01	1545	unk.	0.02	0.06	0.02
50	30.11	1548	α-calacorene	0.01	0.04	tr
51	31.14	1583	spathulenol	0.06	0.17	0.19
52	31.31	1589	caryophyllene oxide	0.26	0.60	0.35
53	32.57	1633	*Z*-α-santalol (or patchoulene, ionone)	0.13	0.20	0.05
54	33.25	1657	α-eudesmol	0.05	0.19	0.12
55	33.33	1660	γ-eudesmol	0.06	0.21	0.10
56	39.35	1889	unk. diterpenoid [b]	0.24	1.00	0.61
57	40.75	1947	unk. diterpenoid [b]	0.11	0.48	0.42
58	41.10	1962	cuparene [b]	0.01	0.07	0.15
59	41.43	1976	lanceol acetate or *trans*-*cis*-nuciferol [b]	0.03	0.07	0.10
60	43.51	2065	manool [b]	0.03	0.11	0.07
61	43.72	2080	manool derivative (epimer 13) [b]	0.02	0.05	0.03
62	47.50	2227	sclareol	0.23	1.05	1.22

[a] A plant of a different source (see Section 2).
[b] Most probable compound structure by library or R_I matching. R_t, retention time; R_I, retention index; NF, not found; unk., unknown; tr, traces (less than 0.01%).

non-persistent manner), is very wide spreading in medicinal and aromatic crops in Italy. It has been found infecting *Phytolacca decandra* L. in the Piedmont region [25], *Valeriana officinalis* L. in Trentino–Alto Adige region [7] and also several species cultivated at the Herb Garden of Casola–Valsenio, such as: *Borago officinalis* L., *Digitalis lanata* L., *D. purpurea* L., *Leonorus cardiaca* L., *P. decandra* L. and *Polygonum fagopyrum* L. [26]. BBWV-I has been probably transmitted to *S. sclarea* by aphids from weeds or some other medicinal species growing in the same area.

The damage caused by this virus not only in *S. sclarea* but also in other species of medicinal and aromatic plants, appears serious enough to require control measures, such as: removal of infected plants and elimination of weeds and aphids, natural vectors of BBWV. To prevent virus infections the crops should be located in exposed mountain areas of high rainfall, where aphid infestations are unlikely to occur.

With regards to differences in quantity and quality of the oils produced by healthy and virus-infected *S. sclarea* plants, no data have been reported before in the literature. Similar research has been carried out on medicinal and aromatic plants infected by fungal pathogens. For example, in Egypt, *Sclerotinia sclerotiorum* decreased the volatile oil percentage of anise (*Pimpinella anisum* L.), caraway (*Carum carvi* L.) and fennel (*Foeniculum vulgare* Miller), but the main components were not analyzed [27]. From our results it is evident that BBWV infection decreases the volatile oil fraction which, in infected *S. sclarea* plants, was 2/3 of that obtained from healthy plants. The composition of both oils was then comparatively evaluated by chromatographic analyses.

3.2. Essential oils analysis

The GC-MS analyses of the tested oil samples (healthy, infected and the commercial one) revealed 62 components (Fig. 2A–C), of which 57 compounds were identified using their mass spectra and retention indices (R_I). These compounds are summarized in order of their retention times in Table 1. The essential oil composition of both infected and healthy plants, as indicated by the general GC-MS profile (Table 1), was characterized by high percentages of monoterpene esters (ca. 60%) of which linalyl acetate, the principal component of the oil, corresponded to ca. 90% of the total esters detected. The oil was found to contain also sesquiterpene hydrocarbons (ca. 20%, mainly α-co-

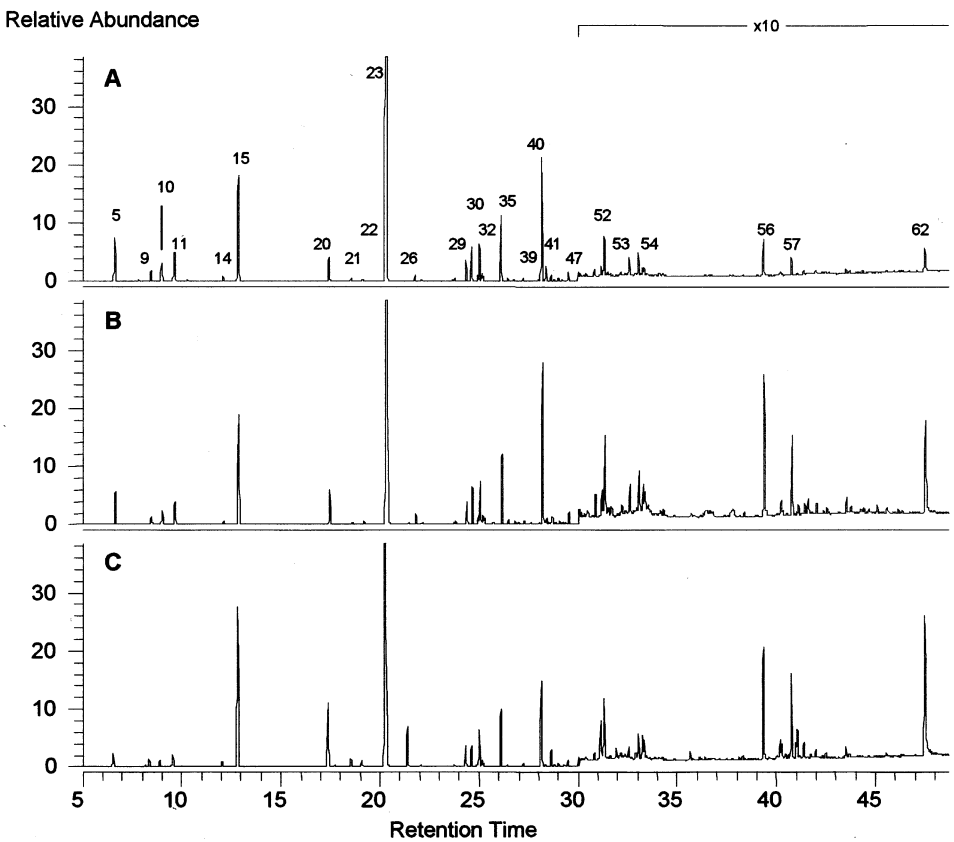

Fig. 2. GC-MS chromatograms of *S. sclarea* essential oils from: (A) healthy plant, (B) BBWV-I infected plant, and (C) commercial source (Russia). For chromatographic conditions see experimental section. Peaks after (RT: 30 min) are ten times amplified. Peaks are numbered according to Table 1.

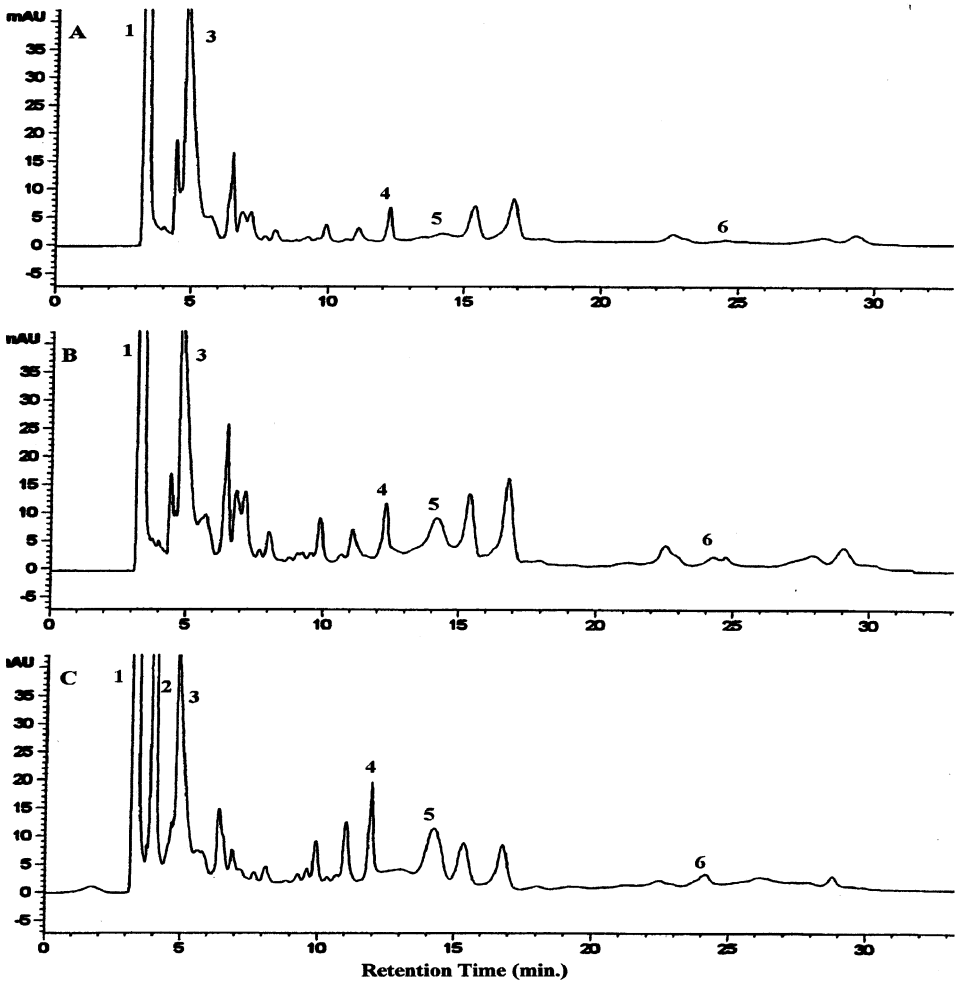

Fig. 3. HPLC chromatograms of *S. sclarea* essential oil solutions (5% v/v in *n*-hexane) from: (A) healthy plant, (B) BBWV-I infected plant, and (C) commercial source (Russia). For chromatographic conditions see experimental section. For chemical identities of numbered peaks refer to text.

paene, germacrene D and β-caryophyllene), alcohols (ca. 12%, mainly linalool and α-terpineol) and monoterpene hydrocarbons (ca. 7.5%, mainly myrcene, limonene and the two ocimene isomers), in addition to minor fractions of oxides, phenolics and other compounds of diverse and/or unidentified structures. The well known diterpenoids, sclareol and manool, were also detected and quantified (Fig. 2 and Table 1). The latter components have rarely been detected in the volatile fractions obtained from *S. sclarea* by steam distillation [17,28,29]. In all of the tested samples, linalyl acetate was the main component that corresponded to about 50% of the oil content with minor differences between the different oils (Table 1). This ester and interchangeably with linalool and α-terpineol, has long been reported as the principal volatile oil constituent of clary sage [12,15–17].

In order to complete and support the data obtained by the GC-MS analyses, liquid chromatography (HPLC) was also applied. The HPLC analyses under normal phase conditions (CN-column and n-hexane as mobile phase) allowed the oil components to be separated into groups of different chemical classes of increasing polarity (mainly hydrocarbons, ethers, esters and alcohols). The composition of the HPLC-fractions was subsequently confirmed by GC-MS analysis. Referring to the HPLC chromatograms (Fig. 3A–C), the first intense peak at R_t: 3.26(1) was found by the GC-MS runs to include almost exclusively all monoterpene and sesquiterpene hydrocarbons of the oil. The peak-fraction at R_t: 4.78(3) proved to be linalyl acetate; however, this ester was detected in trace levels in the subsequent more retained peaks. Fractions at R_t values: 12.24(4) and 24.12(6) were monoterpene alcohols identified as linalool and α-terpineol, respectively. The peak at R_t: 14.15(5) (Fig. 3B) was of particular interest mainly due to its incidence almost only in the infected plant oil (not in the healthy). In addition this peak fraction afforded no components (clear field) by the GC-MS run (except traces of linalyl acetate and linalool). This indicated either very low concentrations (traces) of its UV-absorbing constituents that were

masked by the background of the TIC, or could contain components with high polarity and low volatility that could not be separated and detected by the GC analysis. A larger volume of this fraction was further collected by multiple HPLC runs, concentrated under vacuum and the final residue was subjected to direct MS analysis using the DIP technique. These analyses suggested a mixture-of hydrocarbons (mainly oxygenated) in this fraction. However, after investigating the different mass spectra, particularly observing the masses of the base and molecular ions, and the best library matching compounds, this mixture fraction was found to be almost corresponding to diterpenoids (mainly sclareol and the unknown component at R_t: 39.35, Table 1) and other oxygenated components (possibly spathulenol and caryophyllene oxide) that occurred late in the GC-MS profile. The higher quantities of the latter substances in the infected plant oil as well as in the commercial one (Fig. 2B and C) further supported the correspondence of this peak to these components.

Although the general chemical profile was quite similar, the HPLC and GC-MS analyses of the commercial oil and that of the infected and healthy plants provided some different compositions (Table 1). For instance, the two phenolic compounds thymol (R_t: 21.76) and carvacrol (R_t: 22.1), were only found in the oils from Casola–Valsenio (healthy and infected oils, Table 1). Conversely, in the HPLC analysis, an additional intense peak (R_t: 3.97, 2, Fig. 3C) was observed only in the commercial oil chromatogram and was absent in that from Casola–Valsenio (Fig. 3). This fraction, subjected to GC-MS analysis, was found to contain *trans*-anethole based on its mass spectrum and retention index data. This component (R_t: 21.40, Table 1) was found overlapping with α-terpinyl acetate in the TIC chromatogram of the Russian oil. The two components were then separated into two distinguishable peaks in the single ion current (SIC) chromatograms plotted at the base ion mass corresponding to each of the two compounds. It should be noted that this peak (R_t: 21.43) corresponded to isobornyl acetate in the oils from Casola–Valsenio (Table 1). In all of these examples, liquid chromatography (HPLC), which to our knowledge has not been reported before to evaluate the oil from *S. sclarea*, proved to be a useful subsidiary technique for GC-MS, providing additional data for better characterization of the oil.

In considering the composition variation between the oils from healthy and infected plants, some significant (Student's *t*-test) differences were observed for the major components of both oils as indicated in Table 2. Referring to the GC-MS analyses and by scanning out the individual percentages of the different components of the tested oils, a comparative-quantitative figure can be highlighted (Tables 1 and 2). While higher percentages of monoterpene hydrocarbons were observed in the healthy plant oil (e.g. myrcene, limonene and the two ocimene isomers), the infected plant oil was characterized by higher percentages of sesquiterpene hydrocarbons (mainly germacrene D and β-caryophyllene)

Table 2
Major components of essential oils from *S. sclarea* of different disease states (significance analysis of % content-variations due to viral infection) [a]

R_t	R_I	Comp.	% Content (SD) [b]		P [c]
			Healthy	infected	
6.61	993	myrcene [d]	3.29 (0.21)	2.08 (0.22)	<0.01
8.42	1027	limonene [d]	1.03 (0.18)	0.54 (0.02)	<0.01
8.97	1036	*Z*-ocimene [d]	1.71 (0.14)	1.05 (0.09)	<0.01
9.61	1047	*E*-ocimene [d]	2.96 (0.22)	1.89 (0.17)	<0.01
12.86	1103	linalool [d]	10.06 (0.20)	9.01 (0.27)	<0.01
17.41	1193	α-terpineol [d]	1.64 (0.02)	2.30 (0.02)	<0.01
20.26	1261	linalyl acetate [e]	55.72 (0.79)	54.40 (0.86)	>0.05
24.61	1377	α-copaene [d]	2.06 (0.06)	2.33 (0.05)	<0.01
25.03	1388	sabinene hydrate acetate [e]	2.24 (0.07)	2.33 (0.07)	>0.05
26.11	1421	β-caryophyllene [d]	3.84 (0.19)	4.24 (0.05)	<0.01
28.15	1484	germacrene D [d]	7.58 (0.47)	9.47 (0.87)	<0.05
28.37	1491	γ-muurolene [d]	0.91 (0.04)	0.49 (0.05)	<0.01
29.49	1528	δ-cadinene [d]	0.58 (0.02)	0.73 (0.05)	<0.01
31.14	1583	spathulenol [d]	0.06 (0.01)	0.17 (0.03)	<0.01
31.31	1589	caryophyllene oxide [d]	0.26 (0.02)	0.60 (0.07)	<0.01
39.35	1889	unk. diterpenoid [d]	0.24 (0.01)	1.00 (0.03)	<0.01
47.50	2227	sclareol [d]	0.23 (0.01)	1.05 (0.04)	<0.01

[a] R_t, retention time; R_I, retention index (calculated on Rtx-5MS column).
[b] Mean % content of four samples injected under the same conditions with the standard deviation between brackets.
[c] Probability that the difference is due to chance (according to the Student's *t*-test).
[d] Significant difference.
[e] Non-significant difference.

and oxygenated sesquiterpenes (caryophyllene oxide and spathulenol). With the exception of linalool, higher levels of alcohols (e.g. α-terpineol, borneol and sclareol) were observed in the infected plant oil. Monoterpene esters (e.g. linalyl, neryl, isobornyl and terpinyl acetates) showed comparable figures in both oils, but with minor non-significant differences. In addition, many other components showed variable % contents in the different oils as indicated in Table 1. These data obtained for oils derived from healthy and infected plants cultivated in the same geographical habitat and under the same conditions, collectively supported that viral infection could be responsible for significant modifications of the essential oil composition. Therefore, as the oil activities (e.g. antimicrobial, anti-inflammatory and analgesic) were reported to be depending upon its chemical composition (mainly alcoholic and acetate fractions) [11,12], it is likely that the virus-induced modifications in the oil composition result in an altered pharmacological profile. It should be pointed out, in particular, that altered levels of the principal (linalyl acetate and linalool) and some minor (myrcene, limonene and α-terpineol) components, reported to play an important role in the anti-microbial activity of *S. sclarea* [12,30], were observed in the infected plant oil.

4. Conclusions

Virological tests were carried out on *S. sclarea* plants to identify BBWV-I, a virus found for the first time infecting this plant species. Essential oils hydrodistilled from both healthy and BBWV-I infected *S. sclarea* plants were then analyzed by GC-MS and HPLC systems. As a result qualitative and quantitative differences exhibited by the two plants with different disease states were observed. These can be summarized through two different virus-induced signs: (a) the reduction in the essential oil yield of the infected plant, as well as its crop production (mainly physical sign) and (b) the different oil compositions as indicated by the GC-MS and HPLC analyses as the second, mainly chemical, sign of BBWV-I infection. Although not marked, the chemical differences in the oil compositions were well established and useful to characterize the product, whose pharmacological activity is frequently reported to correlate with its composition. These analytical data, that helped to differentiate infected from healthy plant materials, were also observed for several commercial oils from *S. sclarea*, suggesting a non-selective plant collection by their providers. Therefore, in addition to the routine growth-stage control during the plant life cycle, attention should be also paid to the plant disease-status upon their collection. Having this study as the first report of the infection of clary sage by BBWV-I, further investigations on the biogenetic and phytochemical influences of the virus on this plant and other plant species should be undertaken.

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