SUPPLEMENTARY MATERIAL

Chemical composition and antifungal activity of essential oils from *Senecio nutans, Senecio viridis, Tagetes terniflora* and *Aloysia gratissima* against toxigenic *Aspergillus* and *Fusarium* species

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ABSTRACT

Essential oils from aerial parts of *Senecio nutans, Senecio viridis, Tagetes terniflora and Aloysia gratissima* were analysed by GC-MS and their antifungal activities were assayed on toxigenic *Fusarium* and *Aspergillus* species. Sabinene (27.6±0.1%), α-phellandrene (15.7±0.3%), *o*-cymene (9.6±0.2%) and β-pinene (6.1±0.2%) in *S. nutans*, 9,10-dehydrofukinone (92.7±0.2%) in *S. viridis*, β-thujone (36.1±0.1%), α-thujone (32.2±0.2%), 1,8-cineol (10.7±0.1%) and sabinene (6.2±0.2%) in *A. gratissima*, and *cis*-tagetone (33.6±0.2%), *cis*-β-ocimene (17.1±0.2%), *trans*-tagetone (17.0±0.1%), *cis*-ocimenone (8.0±0.2%) and *trans*-ocimenone (8.2±0.1%) in *T. terniflora*. The oils showed moderate antifungal activity (1.2 mg/mL > MIC > 0.6 mg/mL) on the *Fusarium* species and a weak effect on *Aspergillus* species. The antifungal activity was associated on *F. verticillioides* to the high content of *cis*-tagetone, *trans*-tagetone, *cis*-β-ocimene, *cis*-ocimenone, *trans*-ocimenone and on *F. graminearum* due to the total content of oxygenated sesquiterpenes and 9,10-dehydrofukinone. The oil of *S. viridis* synergized the effect of fungicides and food preservatives on *F. verticillioides*.

Keywords: *Aspergillus*, essential oils, *Fusarium.*

Experimental

Plant material

Aerial parts (stems plus leaves) of *Senecio nutans* (14 kg), *Aloysia gratissima* (9 kg), and *Tagetes terniflora* (10 kg) were collected on February of 2014 in the Tucumán province (Argentina) at Cerro Muñoz, El Mollar and Km 43 of provincial road 307, respectively. Aerial parts of *Senecio viridis* (7 kg) were collected in Antofagasta de la Sierra (Catamarca province, Argentina) on January of 2014. They were identified by Dr. Nora Muruaga and voucher specimens of *Senecio nutans* (LIL 607919), *Aloysia gratissima* (LIL 607922), *Tagetes terniflora* (LIL 607924) and *Senecio viridis* (LIL 607931) are stored at the Herbarium of Miguel Lillo Foundation (Tucuman – Argentina). The plant materials were placed in paper bags and carried to the laboratory for extraction of the essential oils.

Microorganisms

Strains of *Fusarium graminearum* (LABI11) and *F. verticillioides* (LABI7) belong to the collection of the Laboratory of Biology of Bioactive Agents and Phytopathogens (LABIFITO – FBQF - UNT). Strains of *Aspergillus carbonarius* (FRR 5690) and of *A. niger* (FRR 5695) were also assayed. The microbial strains were preserved in SNA medium (Spezieller Nahrstoffarmer agar: 0.1% de K_2HPO_4, 0.1% $NaNO_3$, 0.05% $MgSO_4$. $7H_2O$, 0.05% KCl, 0.02% glucose, 0.2% sucrose and 2% agar) at 4ºC.

Extraction of Essential Oils

The essential oils were extracted from the fresh plant materials by hydrodistillation for 2 h in a Clevenger-type apparatus. Three hundred grams of plant material were placed in the metal baskets of the apparatus. The oily phase was collected and dehydrated over anhydrous sodium sulphate, stored in hermetically sealed glass containers and kept under refrigeration at 5 ºC until analysis and assay of antifungal activity. Total oil yields were expressed based on dry weight of the plant material.

Gas chromatography mass spectrometry of the essential oils

Analysis of the volatile oils was run on a Hewlett Packard (6890) GC-MS system coupled to a quadrupole mass spectrometer (model HP 5973) with a capillary column of HP-5MS (5% phenyl methylsiloxane; length = 30 m, inner diameter = 0.25 mm,

and film thickness = 0.25 μm). GC-MS interphase, ion source, and selective mass detector temperatures were maintained at 280 ºC, 230 ºC, and 150 ºC, respectively. Carrier gas used was helium with a flow rate of 1.0 ml min^{-1}. The oven temperature was programmed as follows: 60 ºC for 1 min then increased from 60 to 220 ºC at a rate of 3 ºC min^{-1} and held at the rate of 300 ºC min^{-1} (10 min). Most of the components were identified on the basis of comparison of their retention indices and mass spectra with published data (Adams, 2007), and computer matching was done with the Wiley 275 and National Institute of Standards Technology libraries provided with the computer controlling GC-MS systems. The retention indices were calculated using a homologous series of n-alkanes C8–C18 and C8–C22 for essential oils, respectively, which are reported in Table 1. The normalization method was used for the chromatographic peaks to calculate the percentage of oil composition. Each oil was injected three times and percentage of relative area are presented as mean±standard deviation. Compounds with percentage of relative area higher than 6% are considered major constituents.

Microdilution assays

The impact of the essential oils on fungal growth was evaluated by the microdilution method in 96 flat well microplates. Fungi were grown in the semiliquid YES medium (20 g/L yeast extract, 150 g/L sucrose, 20 g/L agar. Protocols were developed according to M38-A and M38-P documents from the National Committee for Clinical Laboratory Standards with some modifications (NCCLS, 2002). Fungal colonies were grown in Petri dishes for 7 to 15 days in solid SNA medium. In the case of *F. graminearum*, the Petri dishes were placed under black light at a photoperiod of 12 h light/12 h darkness to stimulate sporulation. Then, the fungal colonies were washed with 2 ml of physiological solution (0.9% of NaCl in distilled water) to obtain suspensions of microconidia (*F. verticillioides* and *Aspergillus* species) or macroconidia (*F. graminearum*). The asexual spores were counted in a neubauer chamber, and the growth medium was diluted to obtain a density of 0.4-5 10^4 spores/ml. The essential oils were assayed in two fold dilution series comprised between 85 and 1.3 mg/ml, in semiliquid YES medium (20 g/L yeast extract, 150 g/L sucrose, 0.5 g/L magnesium sulphate). The final volume in each well was 200 µl, which corresponded to 100 µl of fungal spore suspension and 100 µl of a dilution of the essential oil. Controls of growth and sterility were also performed. Each treatment

(oils or controls) had three repetitions per microplate. Each microplate was prepared twiced. The microplates were incubated 72 h at 25-26 °C for the fungal species. Then, the minimum concentration of the essential oil required to inhibit 100% of the fungal growth (MIC) was visually determined. The absorbance of the microplate wells was read at 630 nm and these data were subjected to probit analysis to calculate the concentration required to inhibit 50% microbial growth (IC_{50}). The MIC and IC_{50} values presented in tables are means of three replicates obtained from two experiments. Potassium sorbate, Calcium propionate, leaf oils of *Thymus vulgaris* and *Origanum vulgare* and the fungicides Vendaval thi-Carb fV (35% thiram-15% carbendazim) and tebuconazole were also included as positive standards of antifungal activity.

Joint effect of essential oils with fungicides and food preservatives

The essential oils *S. viridis* and *T. terniflora* were also assayed on *F. verticillioides* and *F. graminearum*, respectively, in mixtures with fungicides (vendaval or tebuconazole) and food preservatives (potassium sorbate and calcium propionate) in 96-well microplates by the chessboard technique. The values of fractional inhibitory concentration were calculated as FIC = (Concentration of A in MICA+B/Concentration of A in MICA) + (Concentration of B in MICA+B/Concentration of B). The lowest FIC value recorded at each microplate was recorded as the fractional inhibitory concentration index (FICI). The FICI was interpreted as follows: FICI ≤ 0.5, synergy; FICI 0.5-4.0, no interaction; FICI > 4.0, antagonism (Vitale et al. 2005).

Principal component analysis

A principal component analysis based on relative contents of the main constituents and MIC values of the essential oils was performed with XLSTAT v. 2009.3.02.

Figure S1. GC-MS chromatogram representative of the composition of the essential oil of *Senesio nutans*. 1 = β-pinene; 2 = *o*-cymene; 3 = α-phellandrene; 4 = sabinene.

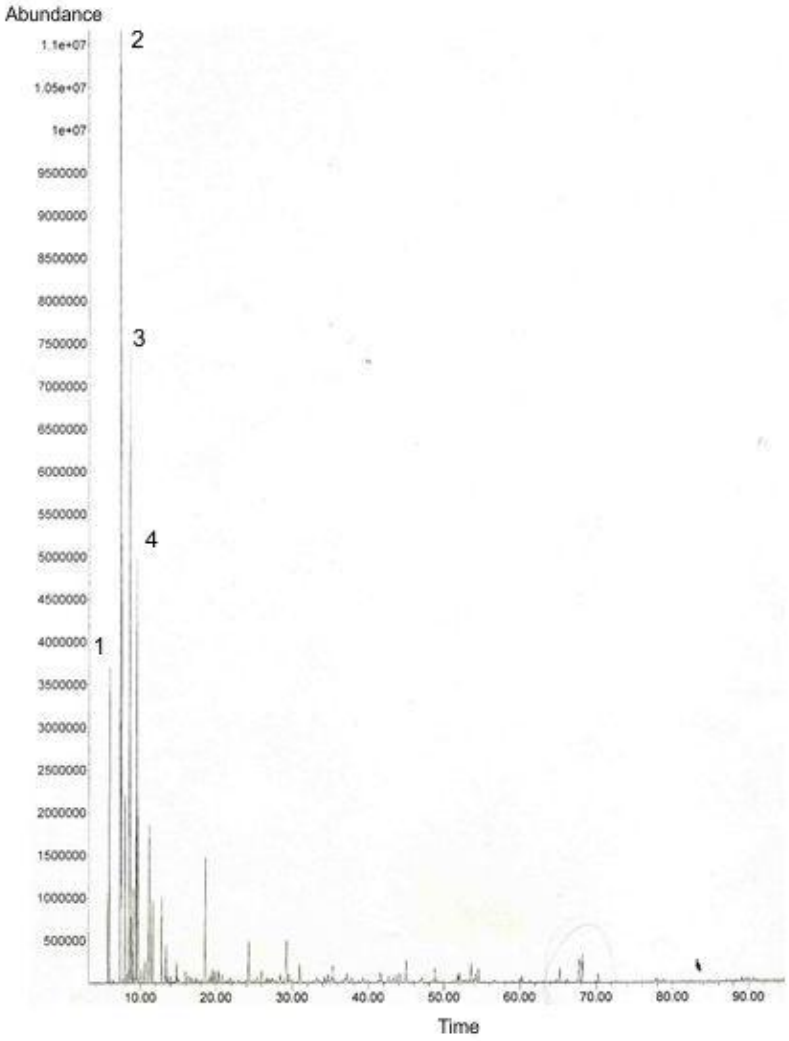

Figure S2. GC-MS chromatogram representative of the composition of the essential oil of *Senesio viridis*. 1 = 9,10-dehydrofukinone.

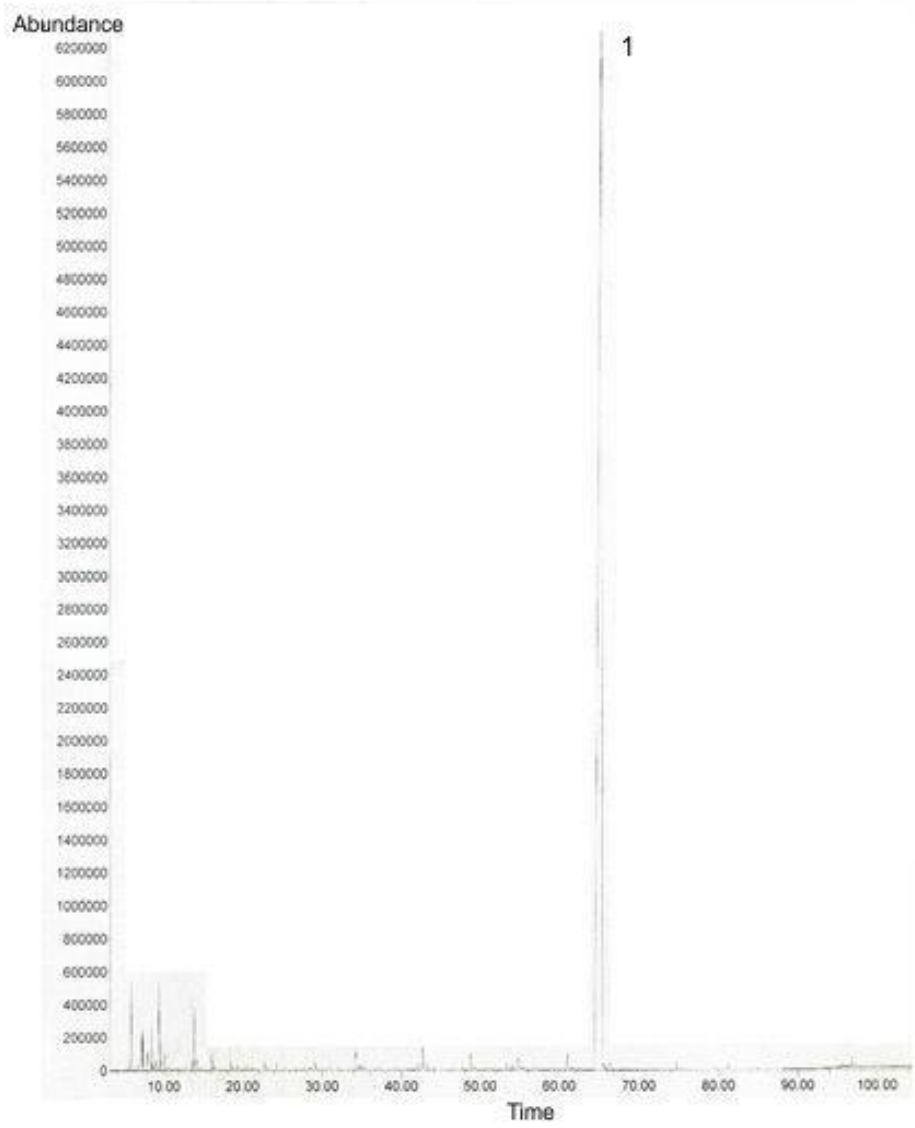

Figure S3. GC-MS chromatogram representative of the composition of the essential oil of *Aloysia gratissima*. 1 =sabinene; 2 = 1,8-cineol; 3 = α-thujone; 4 = β-thujone.

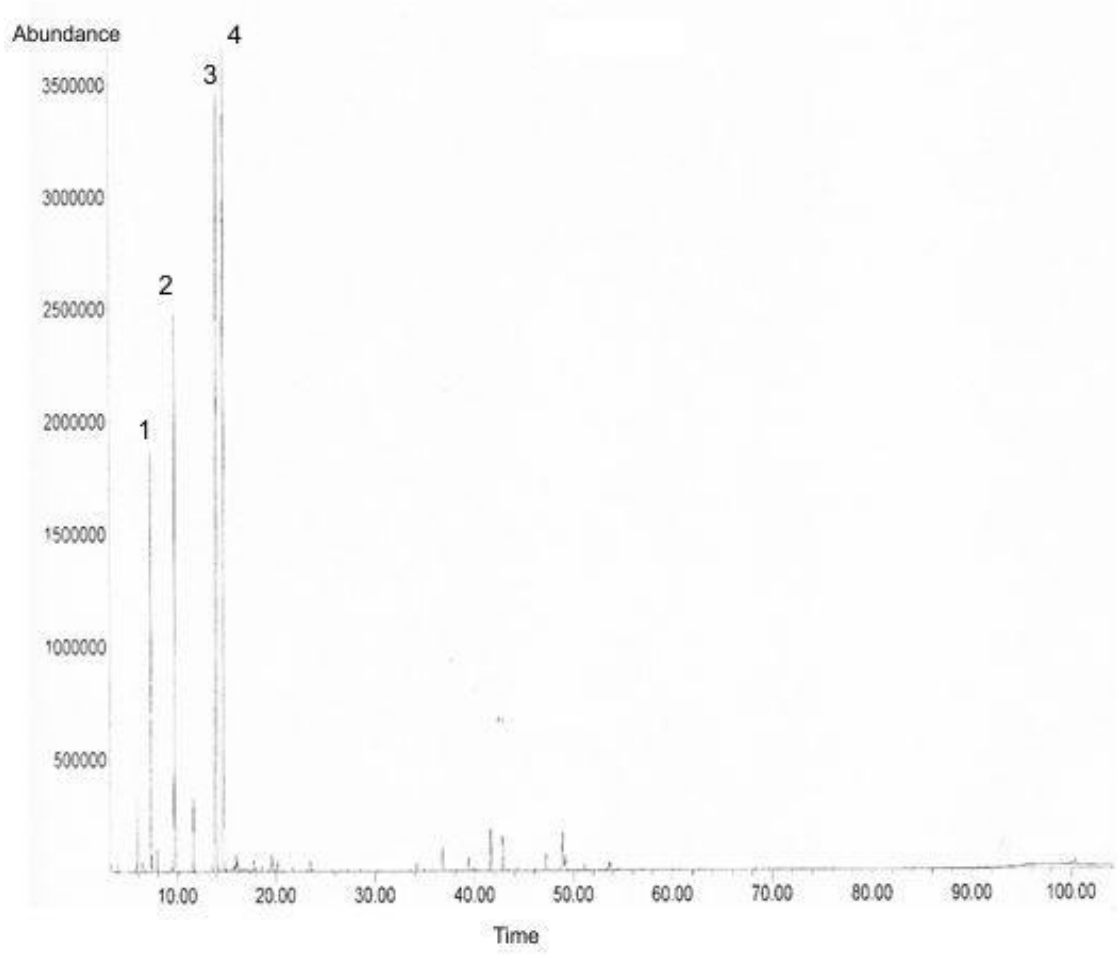

Figure S4. GC-MS chromatogram representative of the composition of the essential oil of *Tagetes terniflora*. 1 = *cis*-β-ocimene; 2 = *trans*-tagetone; 3 = *cis*-tagetone; 4 = *cis*-ocimenone; 5 = *trans*-ocimenone.

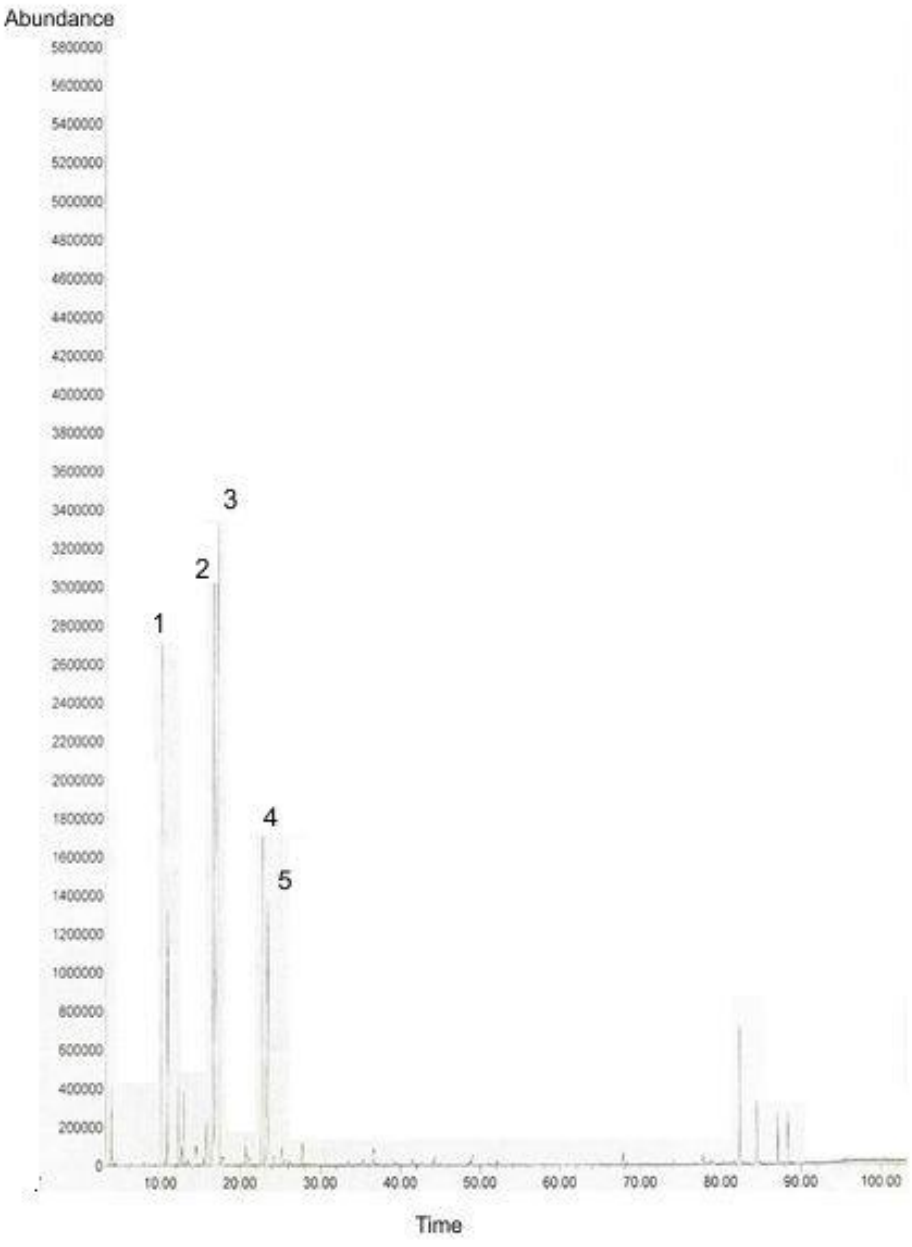

Table S1. Composition of the essential oils from aerial parts of *Senecio nutans*, *Senecio viridis*, *Aloysia gratissima* and *Tagetes terniflora*.

Compounds[a]	RIcalc[1]	RI[2]	Area (%)[3]			
			Senecio nutans	*Senecio viridis*	*Aloysia gratissima*	*Tagetes terniflora*
cis-salvene	845	847	-	-	0.2±0.1	-
α-thuyene	924	924	1.2±0.2	0.1±0.0	-	-
α-pinene	931	932	4.2±0.1	0.7±0.2	0.9±0.1	-
Camphene	946	946	tr	-	0.1±0.0	-
Sabinene	970	969	27.6±0.1	0.3±0.1	6.2±0.2	-
β-pinene	973	974	6.1±0.2	0.4±0.1	0.3±0.1	-
Myrcene	989	988	3.2±0.1	0.2±0.1	0.3±0.1	-
α-phellandrene	1025	1026	15.7±0.3	0.6±0.1	-	-
δ-3-carene	1008	1005	1.0±0.1	tr	-	-
α-terpinene	1014	1011	1.6±0.1	-	-	-
p-cymene	1020	1018	tr	0.9	-	-
o-cymene	1022	1022	9.6±0.2	-	-	-
Limonene	1024	1023	3.3±0.2	-	2.1±0.1	-
β-phellandrene	1025	1028	-	0.4	-	-
1,8-cineole	1028	1026	-	-	10.7±0.1	-
cis-β-ocimene	1032	1031|	0.3±0.1	0.2±0.1	-	17.1±0.2
trans-β-ocimene	1044	1047	0.7±0.1	-	-	0.1±0.0
Dihydrotagetone	1046	1049	-	-	-	2.8±0.2
γ-terpinene	1054	1052	3.5±0.2	-	-	-
cis-sabinene hydrate	1065	1060	1.8±0.1	-	1.7±0.1	-
Terpinolene	1086	1081	0.9±0.1	-	-	-
trans-sabinene hydrate	1094	1094	0.8±0.1	-	0.2±0.1	-
α-thujone	1101	1100	-	-	32.2±0.2	-
β-thujone	1112	1110	-	-	36.1±0.1	-
cis-*p*-menth-2-en-1-ol	1118	1114	0.5±0.1	-	-	-
Allocymene	1128	1122	-	-	-	0.2±0.1
trans-*p*-menth-2-en-1-ol	1136	1130	tr	-	-	-
trans-sabinol	1137	1132	-	-	0.3±0.1	-
trans-verbenol	1138	1137	tr	-	-	-
trans-tagetone	1139	1136	-	-	-	17.1±0.1
cis-tagetone	1148	1150	-	-	-	33.6±0.2
Borneol	1165	1153	-	-	0.3±0.1	-
Terpinen-4-ol	1174	1168	4.1±0.1	-	-	-
p-cymen-8-ol	1179	1174	tr	-	-	-
α-terpineol	1186	1179	0.4±0.1	-	0.5±0.1	-
cis-ocimenone	1226	1221	-	-	-	8.0±0.2
trans-ocimenone	1235	1233	-	-	-	8.2±0.1
Carvone	1239	1231	-	-	0.3±0.1	-
β-bourbonene	1387	1366	-	-	0.3±0.1	-
cis-caryophyllene	1408	1396	-	-	-	0.4±0.1
β-caryophyllene	1417	1406	-	-	0.8±0.1	-
α-humulene	1452	1446	-	-	0.5±0.1	0.1±0.0
α-amorphene	1483	1475	0.3±0.1	-	-	-
Germacrene D	1484	1480	tr	-	1.4±0.2	0.1±0.0
α-muurolene	1500	1489	tr	-	-	-
Bicyclogermacrene	1500	1491	-	0.6±0.1	1.1±0.2	-
γ-cadinene	1513	1508	0.4±0.2	-	-	-
δ-cadinene	1522	1514	0.5±0.1	-	-	-
Spathulenol	1577	1566	0.5±0.1	0.4±0.1	1.5±0.1	-
Caryophyllene oxide	1582	1570	-	-	-	0.2±0.1
epi-α-cadinol	1638	1624	0.8±0.1	-	-	-
α-cadinol	1652	1642	0.5±0.1	-	-	-
9,10-dehydrofukinone	1795	1789	-	92.7±0.2	-	-
Hydrocarbonated monorterpenes			78.9±0.1	3.8±0.1	9.9±0.2	17.2±0.1
Oxygenated monoterpenes			7.6±0.1	-	82.3±0.2	69.7±0.1
Hydrocarbonated sesquiterpenes			1.2±0.1	0.6±0.1	4.|1±0.1	0.6±0.1
Oxygenated sesquiterpenes			1.8±0.1	93.1±0.2	1.5±0.1	0.2±0.1
Total			89.5±0.1	97.5±0.2	97.8±0.2	87.7±0.2

[a]Compounds listed based on elution from a non-polar DB-5 column; [1]Retention Index calculated from retention times in relation to those of a series of n-alkanes on a 30 m DB-5 capillary column; [2]Retention Index taken from Adams (2007); [3]percentage of total área; tr = traces; - not detected.

Table S2. Antifungal activity of essential oils from aerial parts of *Aloysia gratissima*, *Senecio nutans*, *Senecio viridis* and *Tagetes terniflora*. Leaf oils of *Thymus vulgaris* and *Origanum vulgare*, food preservatives (potassium sorbate and calcium propionate) and commercial fungicides (vendaval and tebuconazol) were assayed for comparative purposes.

Strains	*Senecio nutans*		*Senecio viridis*		*Aloysia gratissima*		*Tagetes terniflora*	
	IC_{50}[1] (mg/ml)[5]	MIC (mg/ml)	IC_{50} (mg/ml)	MIC (mg/ml)	IC_{50} (mg/ml)	MIC (mg/ml)	IC_{50} (mg/ml)	MIC (mg/ml)
LABI11 (*Fusarium graminearum*)	1.10 (1.00-1.20)	1.20	0.50 (0.40-0.60)	1.20	0.80 (0.70-0.90)	1.20	0.90 (0.80-1.00)	1.20
LABI7 (*Fusarium verticillioides*)	0.60 (0.50-0.70)	1.20	0.70 (0.60-0.80)	1.20	0.60 (0.50-0.70)	1.20	0.30 (0.20-0.40)	0.60
FRR5690 (*Aspergillus carbonarius*)	> 1.20	>1.20	1.00 (0.90-1.10)	>1.20	>1.20	>1.20	>1.20	>1.20
FRR5695 (*Aspergillus niger*)	>1.20	>1.20	>1.20	>1.20	>1.20	>1.20	>1.20	>1.20

	Potassium sorbate		Calcium propionate		Vendaval		Tebuconazol		*Thymus vulgaris*		*Origanum vulgare*	
	IC_{50} (mg/mL)	MIC (mg/ml)	IC_{50} (mg/mL)	MIC (mg/ml)	IC_{50} (µg/mL)	MIC (µg/mL)	IC_{50} (µg/mL)	MIC (µg/mL)	IC_{50} (mg/ml)	MIC (mg/ml)	IC_{50} (mg/ml)	MIC (mg/ml)
LABI11 (*Fusarium graminearum*)	1.40 (1.2-1.6)	2.4	1.4 (1.2-1.6)	2.4	10.80 (9.30-12.60)	25.0	0.90 (0.80-1.10)	1.50	0.20 (0.10-0.30)	0.30	0.05 (0.08-0.20)	0.15
LABI7 (*Fusarium verticillioides*)	1.30 (1.0-1.5)	2.4	1.3 (1.0-1.6)	2.4	14.10 (12.70–16.10)	25.0	0.30 (0.20-0.40)	0.70	0.10 (0.08-0.20)	0.30	0.05 (0.08-0.20)	0.15
FRR5690 (*Aspergillus carbonarius*)	1.08 (0.8-1.2)	4.8	2.5 (2.2-2.6)	4.8	19.10 (14.50-28.90)	25.0	1.10 (0.90-1.60)	1.50	0.20 (0.10-0.30)	0.60	0.10 (0.10-0.30)	0.30
FRR5695 (*Aspergillus niger*)	1.08 (0.8-1.2)	4.8	2.5 (2.2-2.7)	4.8	10.80 (8.70-15.20)	12.5	0.70 (0.50-1.20)	1.50	0.30 (0.20-0.40)	0.60	0.20 (0.10-0.20)	0.60

[1] Lower and upper limits of the 95% confidence interval are stated into brackets.

Figure S5. Bi-plot of the two first principal components (PC1 and PC2) computed from means of the contents of the main essential oil constituents, and of the MIC for *Fusarium verticillioides* and *F. graminearum*. 1 = 1,8-cineol, α-thujone, β-thujone; 2 = *trans*-ocimenone, *cis*-ocimenone, *trans*-tagetone, *cis*-tagetone, *cis*-β-ocimene; 3 = *o*-cymene, α-phellandrene, hydrocarbonated monoterpenes; 4 = oxygenated sesquiterpenes, 9,10-dehydrofukinone.

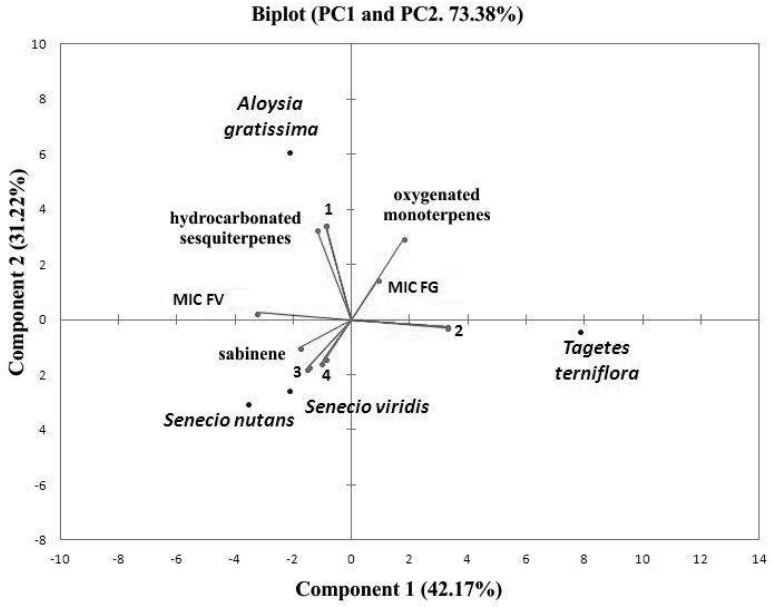

Table S3. Joint action of some essential oils in mixtures with commercial fungicides and food preservants determined by the chessboard technique on growth of LABI 11 (*F. graminearum*) and LABI 7 (*F. verticillioides*).

Compound assayed	FICI		Interpretation of the joint effect[1]
	LABI 11 *(F. graminearum)*	LABI 7 *(F. verticillioides)*	
Oil of *Senecio viridis*			
+ Vendaval	0.31	NA[2]	Synergism
+ Tebuconazole	0.28	NA	Synergism
+ Sorbate potassium	0.35	NA	Synergism
+ Calcium propionate	0.39	NA	Synergism
Oil of *Tagetes terniflora*			
+ Vendaval	NA	1.41	Additivism
+ Tebuconazole	NA	1.25	Additivism
+ Sorbate potassium	NA	1.15	Additivism
+ Calcium propionate	NA	1.25	Additivism

[1]Interpretation of FICI: ≤ 0.5, synergy; 0.5-4.0, no interaction; > 4.0, antagonism. [2]NA: not assayed.