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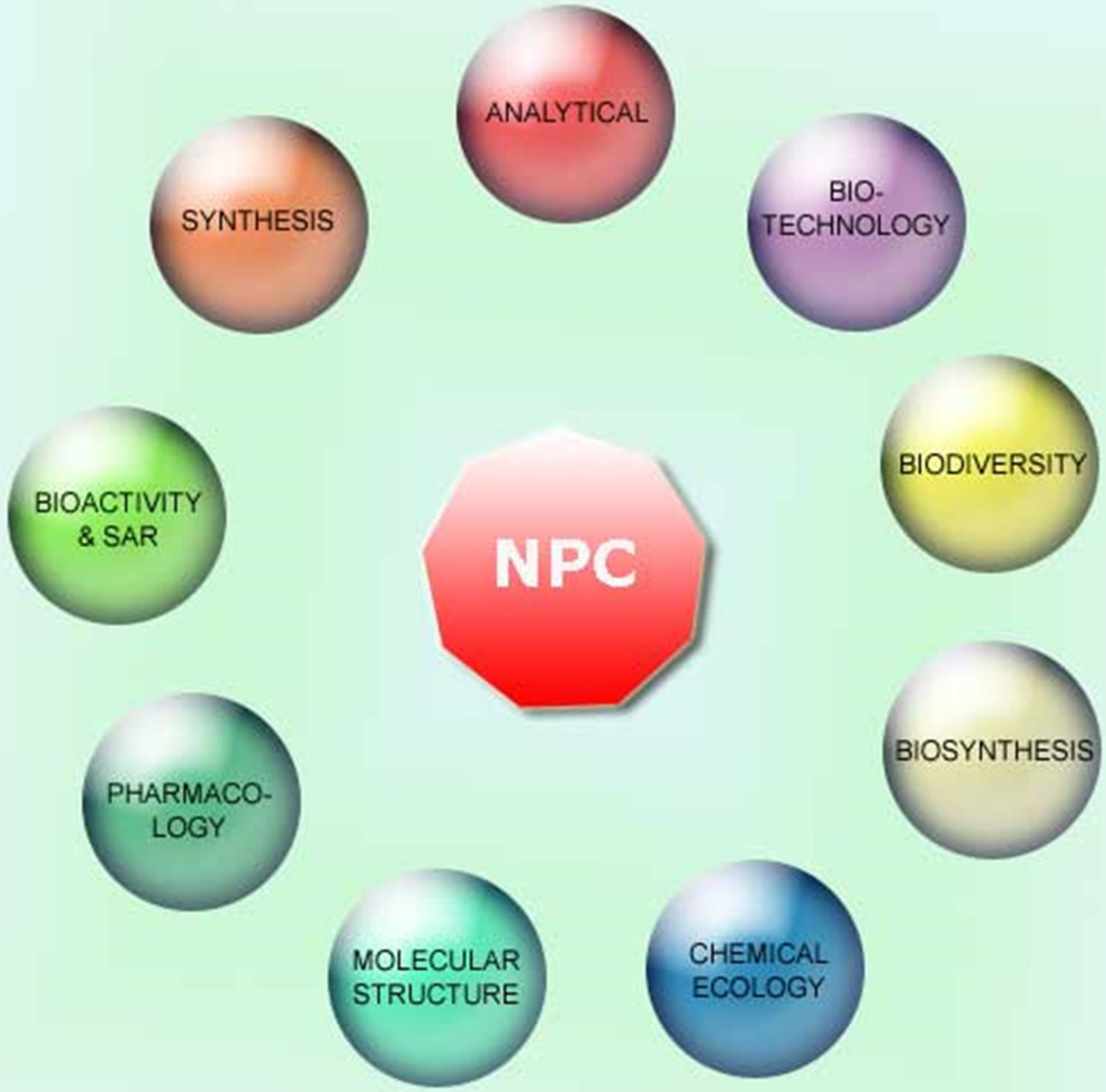

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Essential Oils from *Schinus* Species of Northwest Argentina: Composition and Antifungal Activity

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The composition of the essential oils from leaves (Sal) and fruits of *S. areira* (Saf), and fruits of *S. fasciculatus* (Sff) and *S. gracilipes* (Sgf) were analyzed by GC/MS. The major compounds identified were sabinene (26.0±0.5%), bicyclogermacrene (14.5±0.4%), and E-citral (6.7±0.2%) in Sal oil, limonene (27.7±0.7%), sabinene (16.0±0.5%), β-phellandrene (14.6±0.8%) and bicyclogermacrene (8.1±0.2%) in Saf oil, sabinene (22.7±0.6%), α-phellandrene (18.7±0.3%), β-phellandrene (15.7±0.4%), and bicyclogermacrene (8.1±0.2%) in Sff oil and β-pinene (25.4±0.8%), α-pinene (24.7±0.7%), and sabinene (13.6±0.4%) in Sgf oil.The antifungal activity of the four oils was evaluated on strains of *Fusarium verticillioides* and *F. graminearum*, and the results compared with the effect of epoxyconazole, pyraclostrobin and thyme oil. The Sff oil had the highest antifungal activity among the *Schinus* oils tested, with MIC_{100} (*F. graminearum*) = 6 ‰ and MIC_{100} (*F. verticillioides*) = 12 ‰. A principal component analysis suggests that 9 constituents (α-thujene, α-terpinene, *p*-cymene, γ-terpinene, terpinolene, 1-terpineol, α-calacorene, α-phellandrene, and terpinen-4-ol) explain the higher antifungal effect of Sff. The MIC_{100}s of *Schinus* oils were on average 30-60 and 8.5-17 fold lower than those obtained for thyme oil on *F. verticillioides* and *F. graminearum*, respectively. In the case of commercial fungicides, their MIC_{100}s were three orders of magnitude lower than those of *Schinus* oils. The last ones showed an additive interaction when assayed in mixtures with the commecial fungicides and thyme oil. The results suggest that the doses of fungicides required for control of the *Fusarium* species can be reduced when they are assayed in mixtures with the *Schinus* oils.

Keywords: *Schinus fasciculatus*, *Schinus gracilipes*, *Schinus areira*, Essential oils, *Fusarium*, Antifungal activity.

The genus *Schinus* (Anacardiaceae) comprises 30 species native to South America, including *S. areira* L., *S. fasciculatus* (Griseb.) I.M. Johnst and *S. gracilipes* I.M.Johnst. endemic for northwest Argentina [1]. These plants are known as false peppers because their berries have been used to spice food in South American countries for centuries. The fruits are used in the preparation of a beverage named "Chicha de molle". In the case of *S. areira*, the stems and leaves are used in folk medicine as an antiseptic, antimicrobial and as insect repellents [2]. The fruit oil showed antimutagenic activity and is used as a substitute for black pepper in formulation of spices [3]. The essential oil of leaves and fruits of *S. areira* displayed several biological activities including inhibition of acetylcholinesterase, and insecticidal, allelopathic, antibacterial and antioxidant activities in laboratory tests [4a-4c].

Fusarium graminearum (teleomorph *Gibberella zeae*) and *F. verticillioides* (teleomorph *G. moniliformis*) cause ear rot diseases in cereals from the subtropical and temperate regions of Argentina [5]. They not only reduce cereal yields but also contaminate infected grains with mycotoxins noxious for human and animal health. Exposure to fumonisins produced by *F. verticillioides* has been associated with several diseases in animals including leucoencephalomalacia in equines, pulmonary edema in swine, liver cancer in rats, and immunosuppression in poultry. Epidemiological studies suggest that fumonisins increase the incidence of human esophageal cancer in Africa, Brazil, China, and Italy [6]. Application of fungicides at cereal flowering is a strategy performed to reduce the mycotoxigenic risk. However, the intensive use of these biocides has also increased the resistance to xenobiotics of the *Fusarium* species [7]. New fungicides, or additives to commercial ones, able to control fungal progress are needed in order to solve this problem. Essential oils might be used to either replace or reduce the doses of fungicides applied for control of the toxigenic *Fusarium* species. As part of a systematic study of the chemical composition of essential oils and the antifungal potential of aromatic plants that occur in the northwest of Argentina, the aim of the present study was to describe the chemical composition of the essential oils obtained from fruits and leaves of *S. areira*, *S. fasciculatus*, and *S. gracilipes* and their antifungal activity on *F. verticillioides* and *F. graminearum*.

The hydrodistillation of leaves from *S. areira* (Sal) and fruits from *S. areira* (Saf), *S. fasciculatus* (Sff) and *S. gracilipes* (Sgf) yielded pale yellow oils with markedly different aromas. Fruits yielded a high percentage (w/w) of essential oils (Saf = 4.5%±0.2%, Sff = 3.4±0.1%, and Sgf 3.4±0.1%), while Sal had the lowest yield (1.7±0.1%) and no essential oil was extracted from leaves of the remaining *Schinus* species. GC-MS analysis of the oils revealed a total of 38, 41, 29 and 30 constituents for Sal, Saf, Sff, and Sgf, respectively (Table 1). These analyses accounted for more than 89% of the chemical composition of the oils. Hydrocarbon monoterpenes (Saf = 63.4±0.2, Sff = 74.2±0.2, and Sgf = 81.0±0.4) dominate the terpene composition of the fruit oils, a feature also observed in fruit oils of *S. molle* and *S. terebinthifolius* [8a-8b]. In contrast, hydrocarbon monoterpenes and sesquiterpenes were found in similar levels in Sal. Regarding the major constituents, Sal contained sabinene (26.0±0.5%), bicyclogermacrene (14.5±0.4%), and E-citral (6.7±0.2%), while Saf contained limonene (27.7±0.7%), sabinene (16.0±0.5%), β-phellandrene (14.6±0.8%) and bicyclogermacrene (8.1±0.2%). Sff had high contents of

Table 1: Constituents of the essential oils obtained from leaves of *S. areira* and from fruits of *S. areira*, *S. fasciculatus* and *S. gracilipes*.

Compounds	KI[1]	Area %[2]			
		Sal	Saf	Sff	Sgf
α-Thujene	930	0.4±0.1	0.2±0.0	0.9±0.1	0.4±0.1
α-Pinene	939	0.7±0.1	1.3±0.2	5.5±0.2	24.7±0.7
Camphene	953	0.5±0.0	0.2±0.0	-	0.5±0.1
Sabinene	975	26.9±0.5	16.0±0.5	22.7±0.6	13.6±0.4
β–Pinene	979	0.3±0.1	0.1±0.0	0.8±0.1	25.4±0.8
β-Myrcene	990	0.4±0.1	1.1±0.2	2.1±0.1	6.3±0.3
α-Phellandrene	1002	tr	0.8±0.3	18.7±0.3	tr
α-Terpinene	1017	-	-	1.0±0.1	-
p-Cymene	1026	1.9±0.2	1.0±0.1-	4.4±0.2	0.6±0.1
Limonene	1029	1.2±0.1	27.7±0.7	tr	6.9±0.4
β-Phellandrene	1031	3.7±0.3	14.6±0.8	15.7±0.4	2.6±0.3
γ-Terpinene	1059	0.7±0.1	0.2±0.0	1.7±0.2	-
cis-Sabinene Hydrated	1070	0.2±0.1	0.2±0.0	0.1±0.0	-
Terpinolene	1088	0.4±0.1	0.2±0.1	0.7±0.1	-
1-Terpineol	1120	-	0.1±0.0	0.2±0.0	-
Menth-2-en-1-ol	1140	0.2±0.1	0.2±0.1	0.3±0.1	-
Terpinen-4-ol	1177	2.8±0.2	1.2±0.3	5.2±0.1	0.3±0.0
Isopinocarveol	1180	-	-	-	0.3±0.0
4-Isopropyl-2-Cyclohexen-1-one	1188	-	1.6±0.2	-	0.3±0.3
E-citral	1271	6.7±0.2	-	-	-
Bornyl acetate	1283	1.0±0.1	-	-	0.6±0.2
α –Copaene	1337	0.3±0.0	0.1±0.0	0.4±0.1	-
Linalyl propionate	1338	-	-	0,5±0.2	-
β –Cubebene	1387	-	0.3±0.0	-	-
β-Elemene	1391	1.1±0.2	0.4±0.1	0.2±0.0	0.4±0.1
Junipene	1408	-	0.9±0.1	-	-
α-Gurjunene	1410	2.0±0.1	0.6±0.2	0.3±0.0	0.5±0.3
β-Caryophyllene	1418	1.7±0.1	0.4±0.1	1.2±0.2	0.9±0.2
Aromadendrene	1447	0.6±0.2	0.4±0.2	0.2±0.1	-
α-Humulene	1455	1.7±0.1	0.7±0.1	0.3±0.2	0.4±0.0
(Z,E)-α-Farnesene	1480	-	-	-	1.1±0.2
Germacrene	1480	0.5±0.1	0.1±0.0	-	3.2±0.1
β-Selinene	1489	-	-	-	0.3±0.2
Valencene	1496	-	0.2±0.0	-	-
Bicyclogermacrene	1499	14.5±0.4	8.1±0.2	-	-
α –Muurolene	1500	1.3±0.2	0.5±0.1	0.9±0.2	0.3±0.2
α-Amorphene	1506	0.4±0.1	0.5±0.1	0.4±0.1	0.3±0.0
δ-Cadinene	1522	5.1±0.2	1.3±0.2	2.9±0.3	0.7±0.1
1S,*cis*-Calamenene	1526	0.3±0.1	-	-	-
α–Cadinene	1537	0.3±0.1	-	-	-
α-Calacorene	1544	-	-	0.2±0.0	-
Elemol	1548	0.3±0.1	0.3±0.0	-	1.5±0.4
Ledol	1565	-	0.2±0.1	-	-
Palustrol	1567	0.4±0.1	0.2±0.0	-	-
Spathulenol	1577	5.5±0.2	3.8±0.2	0.9±0.2	1.0±0.2
Caryophyllene oxide	1582	-	-	0.5±0.0	0.3±0.0
Viridiflorol	1597	1.0±0.2	0.5±0.2	0.2±0.1	-
Guaiol	1600	2.0±0.1	1.8±0.3	-	-
10-epi-γ-Eudesmol	1622	-	-	-	0.4±0.1
Isospathulenol	1639	0.2±0.1	0.2±0.0	-	-
T-Muurolol	1640	1.1±0.2	1.0±0.2	-	-
α-Eudesmol	1652	-	-	-	0.9±0.2
α-Cadinol	1660	0.8±0.1	0.3±0.1	-	-
Vulgarol	1701	1.9±0.2	1.3±0.3	-	0.9±0.2
Patchulane	2095	-	-	-	0.4±0.0
monoterpene hydrocarbons		**37.1±0.2**	**63.4±0.2**	**74.2±0.2**	**81.0±0.4**
oxygenated sesquiterpenes		**4.2±0.1**	**3.3±0.1**	**6.3±0.1**	**1.5±0.1**
monoterpene hydrocarbons		**31.7±0.2**	**15.8±0.1**	**7.0±0.1**	**9.4±0.1**
oxygenated sesquiterpenes		**18.0±0.1**	**11.3±0.1**	**1.6±0.1**	**4.1±0.2**
TOTAL		**91.0±0.2**	**93.8±0.1**	**89.1±0.1**	**96.0±0.2**

[a]Compounds listed based on elution from a non-polar DB-5 column; [1]Kovats Index calculated from retention times in relation to those of a series of *n*-alkanes on a 30 m DB-5 capillary column; [2]percentage of total area. SD = Standard deviation; Sal = leaf oil of *Schinus areira*; Saf = fruit oil of *S. areira*; Sff = fruit oil of *S. fasciculatus*; Sgf = fruit oil of *S. gracilipes*; tr = traces; - not detected.

sabinene (22.7±0.6%), α-phellandrene (18.7±0.3%), β-phellandrene (15.7±0.4%), and bicyclogermacrene (8.1±0.2%). Sgf contained β-pinene (25.4±0.8%), α-pinene (24.7±0.7%), and sabinene (13.6±0.4%). The remaining components were found in the oils at levels below 6%. Thyme oil, included as a positive control of antifungal activity, contained *p*-cymene (45.1±1.2%) and thymol (21.5±1.5%) as main constituents (not shown).

Table 2: Concentrations of the *Schinus* oils needed to inhibit 100% (MIC_{100}) of the growth of *F. verticillioides* (strains LABI6 and LABI28) and *F. graminearum sensu stricto* (LABI25 and LABI26) in microdilution assays. Fungi grew in YES semiliquid medium. Commercial fungicides and thyme oil (from leaves of *Thymus vulgaris*) were assayed as positive controls.

	MIC_{100} (‰)			
Strains	Sal	Saf	Sff	Sgf
LABI6	24	24	12	24
LABI25	12	12	6	12
LABI26	12	12	6	12
LABI28	24	24	12	24

	MIC_{100} (ppm)			MIC_{100} (‰)
	Epoxyconazole	Pyraclostrobin	Ketoconazole	*Thymus vulgaris*
LABI6	2	50	16	0.4
LABI25	8	25	32	0.7
LABI26	8	25	32	0.7
LABI28	2	50	16	0.4

Essential oils from the same plant source and organ often show qualitative and quantitative variations in chemical composition related to their geographical origin. In Buenos Aires province, leaf oils of *S. areira* contained α-phellandrene (28.5%), 3-carene (20.8%), and camphene (10.9%) as major constituents while fruit oils contained α-phellandrene (24.8%), 3-carene (21.3%) and β-myrcene (19.7%) [4a]. In Jujuy province (north end of northwest Argentina), α- and β-phellandrene were found as the major constituents of the fruit oils [9]. To the best of our knowledge this work reports for the first time the composition of fruit oils from *S. fasciculatus* and *S. gracilipes*.

The essential oils of *Schinus* spp. inhibited the growth of *F. graminearum* more than that of *F. verticillioides* (Table 2). However, the MIC_{100}s of *Schinus* oils were on average 30-60 and 8.5-17 fold lower than those obtained for thyme oil on *F. verticillioides* and *F. graminearum*, respectively. Thyme oil also had biocidal activity at the MIC_{100} while *Schinus* oils were only fungistatic in the range of concentrations assayed. In the case of commercial fungicides, their MIC_{100}s were three orders of magnitude lower than those of *Schinus* oils. The complex composition of the tested essential oils makes it difficult to elucidate which constituents contribute to the antifungal activity. The expected order of contribution of the constituents, in order of more to less antifungal activity, is phenols > alcohols > aldehydes > ketones > ethers > hydrocarbon compounds [10]. In agreement with this explanation, thyme oil was the only one containing phenolic compounds and showed the highest antifungal activity. The fruit oil of *S. fasciculatus* showed the highest contents of alcohols and was the *Schinus* oil with the highest inhibitory activity on fungal growth. The biological activity of essential oils is often explained as the joint action of several of their minor and/or major constituents [11]. Principal component analysis (PCA) based on relative participation of constituents and MIC_{100} of *Schinus* oils suggests this situation. Figure 1 shows the bidimensional graph of components 1 and 2 explaining 76.1% of the total variance, with an important divergence of Saf from the other *Schinus* oils. The contents of 9 constituents (α-thujene, α-terpinene, *p*-cymene, γ-terpinene, terpinolene, 1-terpineol, α-calacorene, α-phellandrene, and terpinen-4-ol) were directly correlated (r2>0.8, $p = 0.05$) to component 1 and seem to explain the inhibitory effect of Sff on both *F. verticillioides* and *F. graminearum*. Most of the 9 constituents were found in the Sff at contents below 5%, except α-phellandrene (18.7±0.3%) and terpinen-4-ol (5.2±0.1%). The variation explained by component 2 and constituents correlated to it were not associated with the antifungal activity.

Several essential oils with low or no biological activity have synergistic antifungal effects in mixture with biocides [11]. To check this possibility, the *Schinus* oils were assayed in mixtures

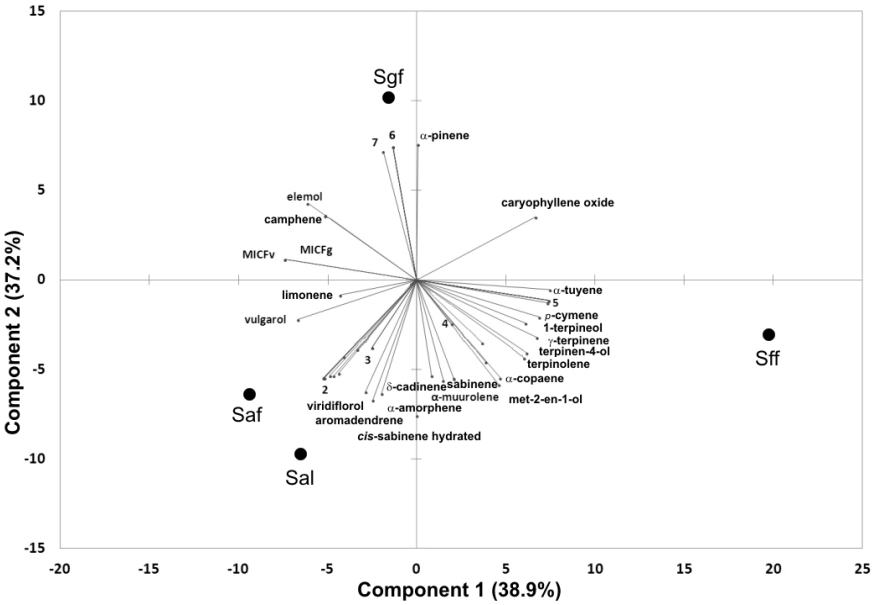

Figure 1: Please Bi-plot of the two first principal components computed from means of the contents of the essential oil constituents, and of the MIC_{100} for *F. verticillioides* (MICFv) and *F. graminearum sensu stricto* (MICFg). 1: ledol, valencene, m-cresol, jupinene, β-elemene, β-cubenene; 2: T-muurolol, isospathulenol, palustrol, 5-guaiol, spathulenol, bicyclogermacrene, α-gurjunene, α-humulene; 3: E-citral, α-cadinene, 1-S-*cis*-calamenene; 4: β-caryophyllene; 5: α-phellandrene, α-calacorene, α-terpinene; 6: isopinocarveol, patchulane, (Z,E)-α-farnesene, β-selinene, 10-*epi*-γ-eudesmol; 7: α-eudesmol, germacrene.

Table 3: Fractional inhibitory concentration index (FICI) of combinations of commercial fungicides and the essential oils from *Schinus* species, determined by the checkerboard technique.

	Fusarium verticillioides			
	Pyraclostrobin	Epoxyconazole	Thymol	Interpretation of the joint effect
Sal	2.5-3.1	2.1-3.2	1.9-3.7	Additivism
Saf	1.5-2.5	2.9-4.0	2.0-3.2	Additivism
Sff	2.5-3.1	0.9-2.5	2.9-4.0	Additivism
Sgf	3.1-3.9	1.0-4.0	2.8-3.9	Additivism
	Fusarium graminearum			
	Pyraclostrobin	Epoxyconazole	Thymol	Interpretation of the joint effect
Sal	2.1-3.0	1.5-3.0	1.5-2.9	Additivism
Saf	1.7-2.6	2.5-4.0	1.8-3.2	Additivism
Sff	2.5-4.0	1.5-4.0	2.0-3.0	Additivism
Sgf	1.6-2.8	2.5-3.2	2.0-3.2	Additivism

with epoxyconazole, pyraclostrobin and thymol oil. The selected commercial fungicides are used in the control of the ear rot disease and their action mechanisms are notably different [12]. Epoxyconazol is a triazol derivative that inhibits ergosterol biosynthesis, while pyraclostrobin is a strobilurin that inhibits mitochondrial electron transport. In the case of thyme oil, thymol is believed to cause structural and functional damage to the cytoplasmic membrane, while *p*-cymene is able to modify the structure and permeability of cell membranes [13]. The fractional concentration indexes were higher than 0.5 and lower than 4 in all the mixes assayed. This situation indicates an additive joint action.

The fungitoxicity of the *Schinus* oils was lower than that of synthetic fungicides and thyme oil. However, lower doses of fungicides were required for control of the *Fusarium* species when these xenobiotics were assayed in mixtures with the *Schinus* oils. Further *in vivo* assays are required in order to check the antifungal potential of the mixtures.

Experimental

Plant material: Fruits and leaves of *Schinus gracilipes* were collected in Tafi del Valle while those of *S. fasciculatus* and *S. areira* were collected in Ampimpa. These locations are in the northwest of Tucuman province (northwest Argentina). Identity of the plant materials was confirmed by comparison of voucher specimens with those stored in the Herbarium of the Miguel Lillo Foundation. Voucher specimens were deposited in the LABIFITO (Laboratory of Biology and Bioactive Agents and Phytopathogens, FBQF, UNT). Mature fruits and leaves were placed in paper bags and carried to the laboratory for extraction of essential oils. Then, fruits were manually detached from leaves.

Assayed microorganisms: Strains of *F. graminearum sensu stricto* (LABI25 and LABI26) and *F. verticillioides* (LABI6 and LABI28) belong to the collection of the Laboratory of Biology of Bioactive Agents and Phytopathogens (LABIFITO – FBQF - UNT). 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 g of fresh ground fruits or leaves were placed in the metal baskets of the apparatus. The oily phase was collected and dehydrated over anhydrous sodium sulfate, stored in hermetically sealed glass containers and kept under refrigeration at + 5ºC until analysis and assay of antifungal activity. Total oil yields were expressed as a percentage of the weight of the essential oil divided by the weight of fresh plant material.

Chemical characterization of the essential oils: Analysis of the volatile oils was achieved using a Hewlett Packard (6890) GC-MS system coupled to a quadruple mass spectrometer (model HP 5973) with a capillary column {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 280ºC, 230ºC, and 150ºC, respectively. Carrier gas used was helium with a flow rate of 1.0 mL min^{-1}. The oven temperature was programmed as follows: 60ºC for 1 min then increased from 60 to 220ºC at a rate of 3ºC min^{-1} and then held at 300ºC for10 min.

Identification of components: Most of the components were identified on the basis of comparison of their retention indices and

mass spectra with published data [14], and computer matching was made with the Wiley 275 and National Institute of Standards Technology libraries provided with 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.

Microdilution assays: Mycelial growth was evaluated by the microdilution method in semiliquid YES medium (20 g/L yeast extract, 150 g/L sucrose, 20 g/L agar) in 96 flat bottom well microplates. Protocols were developed according to M38-A and M38-P documents from the National Committee for Clinical Laboratory Standards with some modifications [15]. 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 with 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 either microconidia (*F. verticillioides*) or macroconidia (*F. graminearum*) suspensions. 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 7 serial two fold dilutions comprised between 96 and 1.5 µL/mL, in semiliquid YES medium (20 g/L yeast extract, 150 g/L sucrose, 0.5 g/L magnesium sulfate). The final volume in each well was 200 µL, which corresponded to 100 µL of 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 3 repetitions per microplate. Each microplate was prepared in duplicate. The microplates were incubated for 72 h at 25-26°C. The minimum concentration of the essential oil required to inhibit 100% of the fungal growth was determined after 72 h of incubation. Fungal survival at oil concentrations above the MIC_{100} was checked by transferring 5 µL aliquots from the wells to Petri dishes containing PDA which were then incubated at 25-26°C for 7 days.

Joint effect of essential oil and xenobiotics on fungal growth: The joint effect of essential oils was evaluated in microdilution assays by the chessboard technique. Based on the obtained MIC_{100}, the inhibitory fractionated concentration (FICI) was calculated as: FICI = (Concentration of A in MIC_{A+B}/Concentration of A in MIC_A) + (Concentration of B in MIC_{A+B}/Concentration of B). The FICI is used to interpret the results of the test in the following manner: FICI $\leq$ 0.5, synergy; FICI 0.5-4.0, no interaction; FICI > 4.0, antagonism [16].

Statistical analysis: Data obtained were subjected to one way ANOVA. Differences among means were calculated by the test of the minimum significant difference (LSD test).

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