Natural Control of Corn Postharvest Fungi *Aspergillus flavus* and *Penicillium* sp. Using Essential Oils from Plants Grown in Argentina

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Abstract: The objective in this study was to evaluate the antifungal activity of essential oils from native and commercial aromatic plants grown in Argentina against corn postharvest fungi and to link the essential oil bioactivity with lipid oxidation and morphological changes in fungus cell membrane. Essential oil (EO) of oregano variety Mendocino (OMen), Cordobes (OCor), and Compacto (OCom), mint variety Inglesa (Mi), and Pehaujo (Mp), Suico (Sui); rosemary (Ro), and Aguaribay (Ag) were tested *in vitro* against 4 corn fungi: *A. flavus* (CCC116–83 and BXC01), *P. oxalicum* (083296), and *P. minioluteum* (BXC03). The minimum fungicidal concentration (MFC) and the minimum inhibitory concentration (MIC) were determined. The chemical profiles of the EOs were analyzed by GC-MS. Lipid oxidation in cell membrane of fungi was determined by hydroperoxides and related with essential oil antifungal activity. The major compounds were Thymol in OCor (18.66%), Omen (12.18%), and OCom (9.44%); menthol in Mi and Mp; verbenone in Sui; dehydroxy-isocalamendiol in Ag; and eucaliptol in Ro. OCor, Omen, and OCom showed the best antifungal activity. No antifungal activity was observed in Ag and Ro EO. The hydroperoxide value depended on the fungi ($P < 0.001$) and the antimicrobial agent ($P < 0.001$).Membrane lipids were oxidized by Sui EO in *A. flavus* BXC01 and *A. flavus* CCC116–83 (0.021 and 0.027 meqO_2/kg, respectively). The results suggest that the EOs of OCor, OMen, OCom, Mi, Mp, and Sui grown in Argentina can be used as natural alternatives to control fungi that produce mycotoxin in maize.

Keywords: antifungal activity, corn essential oils

Practical Application: The use of natural fungicides instead of synthetic ones covers is a present trend in corn postharvest disease control. The quality and food safety of corn could be preserved by the addition of natural antifungal agents as essential oils. This research shows that the essential oils inhibit the growth of fungi and therefore, the formation of mycotoxins. Because of this, the use of these essential oils should be considered for the industry as a natural biocide agent for preserving quality properties and food safety in postharvest cereals, such as corn.

Introduction

Argentina is the 2nd country in corn planting area in the world, producing 22 million metric tons per year. This commodity is used to feed cattle for meat and milk production, to produce human food, and as an ingredient for many different products. Slow drying processes in corn kernels due to wet weather conditions result in consequent pre- and postharvest fungi infections (Pitt and Hocking 2009). In addition, fungal species cause crop quality loss by the presence of mycotoxins. These toxic secondary metabolites can produce adverse effects in human and animal health. The European Commission (2010) has established a maximum regulatory limit of 5 μg/kg aflatoxin B for corn. Aflatoxin B1 (AFB1) has been demonstrated to be the most potent liver carcinogen agent in a variety of animal species. *Penicillium* species produce a diverse range of mycotoxins like ochratoxin and citrinin. Secalonic acid d synthesized by *P. oxalicum* is a mycotoxin that affects animals (Pitt and Hocking 2009).

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Chemical control is the common way used to control fungi worldwide. Synthetic fungicides cause resistant strains of pathogens, thus requiring the increase of fungicide dosages (Gullino and others 2000). Chemical residues from fungicides have a slow degradability rate with high persistence in soil, ground, and water. Also, the multiplication of beneficial fungi as affected by these products. Chemical fungicides are recommended if there are no other management strategies for disease treatment or as part of an integrated fungal control program (Sosa-Gomez and others 2003).

Essentials oils (EOs) are presented as an alternative strategy of control that has nonnegative impact. EOs are characterized by low toxicity to mammals; the quantities needed in a treatment are in the order of milligrams; the compounds are easily degraded by exposure to air, light, and by microorganisms; and they do not remain in water and soil for a long time (Isman 2000). Aromatic plants synthesize volatile compounds like terpenes and terpenoids that have demonstrated to have antifungal activity acting in the cellular membrane level (Lucini and others 2006). EOs have shown a strong control against various species of fungi. Particularly, the antifungal activity of rosemary and oreganos against *Botrytis cinarea* has been studied (Soylu and others 2010).

Oregano EO is considered as a food preservative agent that prevents antifungal infections and mycotoxin production (Kocić-Tanackov and others 2012). Previous studies have shown

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that EOs of oregano (*Origanum sp*.) and suico (*Tagetes minuta* L.) can reduce or inhibit the growth of *Aspergillus* and *Penicillium* species (Kocić-Tanackov and others 2012). Other authors (Beyki and others 2014) have reported that peppermint EO is effective to control *Aspergillus flavus.* Extracts of Aguaribay (*Schinus molle* L.) from Argentina were tested successfully against *Aspergillus* species (Martins and others 2014). Also, extracts of other aromatic plants as *Cicuta virosa* L. and *Satureja hortensis* L. were used to control *Aspergillus flavus* (Dikbas and others 2008; Tian and others 2011).

The objectives of this work were to evaluate the antifungal activity of EOs obtained from native and commercial aromatic plants grown in Argentina against postharvest fungi *Aspergillus flavus* and *Penicillium* spp., and to link this bioactivity with lipid oxidation and morphological changes in the fungus cell membranes.

Materials and Methods

Fungal isolates

Four fungi were studied: 2 *A. flavus* (CCC116–83 and BXC01), *P. oxalicum* (083296), and *P. minioluteum* (BXC03). *A. flavus* (CCC116–83) and *P. oxalicum* (083296) were identified and provided by Facultad de Ciencias Bioquímicas y Farmacéuticas (Univ. Nacional de Rosario, Argentina) and Instituto Nacional de Enfermedades Infecciosas (Buenos Aires, Argentina), respectively. *A. flavus* (BXC01) and *P. minioluteum* (BXC03) were isolated from corn seed samples obtained from Córdoba province and identified according to morphological characteristics. Isolates were kept at 4 °C on potato dextrose agar (PDA) (Pitt and Hocking 2009).

Plant material

EOs from 3 oregano species from Villa Dolores (Córdoba, Argentina) were obtained: *Origanum vulgare* sspp *hirtum* "Cordobes" (OCor), *Origanum vulgare* L. sspp. *Vulgare* "Compacto" (OCom), and *Origanum* x *majoricum* "Mendocino" (OMen). Other 5 aromatic species were collected (crop 2013) from the experimental station of Facultad de Ciencias Agropecuarias (Univ. Nacional de Córdoba, Argentina): *Tagetes minuta* L. "Suico" (Tm), *Mentha* x *piperita* L. var. *Vulgaris Sole* "Inglesa" (Mi), *Mentha* x *piperita* L. "Pehuajo" (Mp), *Rosmarinus officinalis* L. "Romero" (Ro), and *Schinus molle* L. "Aguaribay" (Ag).

Extractions and yields of essential oils

Dried leaves were hydrodistilled for 2 h in a Clevenger-type apparatus with a separated extraction chamber. The EOs were kept in dark flasks at −18 °C in a freezer. Yield was determined measuring the EO volume obtained per plant weight.

Essential oil chemical compositions

EOs were analyzed by CG-MS using a Perkin Elmer Clarus 600 chromatograph coupled with an ion trap mass detector equipped with a capillary column DB-5 (30 m, 0.25 mm i.d., and 0.25-mm coating thickness). The carrier gas was Helium and had a flow rate of 0.9 mL/min. Ionization was performed by electron impact at 70 eV. Mass spectral data were acquired in the scan mode in the m/z range 35 to 450. The compounds were identified by comparing their retention time and mass spectra with published data (Adams 1995) and NIST libraries (version 3.0). The quantitative analysis was performed by peak area normalization (Asensio and others 2011).

Experimental design

Nine essential oils were tested as growth inhibitors × 4 fungi isolates × EO concentrations from 100 to 5500. The concentration was increased for each EO in 100 ppm until minimum fungicidal concentration was reach. Each treatment was replicated 3 times.

Antifungal activity assays

Petri plates of 9-cm dia were prepared with 20 mL of liquid culture medium (20% potato lixiviated; 2% glucose; pH 4.5). EOs were diluted in ethanol and added to culture medium according to desired final concentrations (Lucini and others 2006; De Corato and others 2010; Amini and others 2012). Ethanol concentration was less than 1% v/v. Five-millimeter diameter agar discs were collected from the periphery of 10-d old fungi and placed on Petri dishes (Lucini and others 2006). Treatments were incubated at 25 °C for 10 d in a culture chamber. Diameter mycelium was measured. Antifungal activity was expressed as percentage of growth inhibition (PGI) estimated using the equation (Kordali and others 2008):

$$PGI = [(C - T)/C] \times 100$$

where C is the mean value ($n = 3$) of control hyphal extension (mm), and T is the mean value ($n = 3$) of hyphal extension (mm) from plates treated with essential oil.

The minimum inhibitory concentration (MIC) values were defined as the lowest concentration in which the PGIs were equal to 50%. The minimum fungicidal concentration (MFC) values were defined as the lowest concentrations in which the PGIs were equal to 100% (Abbaszadeh and others 2014)

Hydroperoxides

Petri plates were prepared according to the method explained above (Lucini and others 2006), but EOs were only added at MFCs and incubated at 25 °C for 72 h. Then, the obtained mycelium was dispersed in 5 mL of demineralized water and mixed by homogenizer for 45 s. Lipid hydroperoxides were extracted into a 10 mL methanol:chloroform (1:1, v/v) mixture. Samples were mixed and centrifuged at 10000 × g for 10 min. After that, 1 mL of the chloroform phase was transferred into test tubes and mixed with 1 mL iron (II) thiocyanate solution. Absorbance was measured at 500 nm in UV-V Diode Array, Spectrophotometer Hewlett Packard™ HP 8452 A (Palo Alto, Calif., U.S.A.). Hydroperoxide values (HPV) were based on a calibration curve made with the concentration 0.5, 0.15, 0.25, 0.35, 0.45, 0.55, 0.65, 0.75, 0.85, and 0.95 μg/mL of iron (III) (Dalsgaard and others 2012).

Reflection confocal microscopy

Petri plates were prepared according to the explained in the point antifungal activity assays. Mycelium of *A. flavus* BXC01 were located on Petri plates and were incubated at 25 °C for 10 d in a culture chamber (Lucini and others 2006). Then, the EOs were added to the culture medium at CFM. Membrane damage was observed on an Olympus OLS 4000 (Tokyo, Japan) reflection confocal microscope after 0, 24, and 72 h.

Statistical analysis

The data obtained were analyzed by InfoStat software version 2013 (Facultad de Ciencias Agropecuarias, Univ. Nacional de Córdoba). Treatments were replicated 3 times. Mean values were obtained by Analysis of Variance ($P < 0.05$). LSD Fisher's tests

($\alpha = 0.05$) was utilized to find significant differences among means. The concentration of EOs that inhibited the fungi mycelia growth by 50% (MICs) were determined by a linear regression method (Marei and others 2012). Principal component analysis (PCA) (Souza and others 2011) was performed on the correlation matrix of the standardized (normalized) data from antifungal assays. Associations between different treatments and minimum inhibitory concentrations were explored by PCA. Cluster analysis (CA) was carried out to obtain groups of essential oil treatments with similar behavior. Sample similarities were calculated on the basis of Euclidean distance, and the groups of treatments with similar characteristics were obtained using the average linkage method.

Results and Discussion

Essential oil yields

The EO yields are presented in Table 1 and are expressed as V/W. Between the species of oreganos, the major EO yield was OCor (3.30%). A similar percentage of oregano EO content was reported by Asensio (2013). The yield of Mp (3.22%) was higher than Mi (2.38%). Other authors (Zheljazkov and Astatkie 2012) reported 3.0% EO in mint. Ro had 2.7% EO. Ojeda-Sana and others (2013) reported 2.58% for Ro. Ag showed similar EO yield (2.60%) to Ro. The lowest EO yield was detected in Sui (1.70%). Gil and others (2000) reported 1.75% EO content in Sui from Argentina samples.

Chemical composition

The EO composition of OCor, OMen, OCom, Mi, Mp, Tm, Ag, and Ro are shown in Table 1.The major compounds in OCor and OMen EOs were trans-sabinene hydrate (23.03% and 28.33%, respectively), thymol (18.66% and 12.18%, respectively), γ-terpinene (7.12% and 7.59%, respectively), and 4-ol-terpinen (6.20% and 6.66%). These results are consistent with previous data published by Asensio (2013). In OCom EO the major monoterpenes were o-cymene (14.33%), 4-ol-terpinen (12.55%), (E)-β-terpineol (10.46%), and thymol (9.44%). Olmedo and others (2014) reported γ-terpinene, terpineol-4-ol, and carvacrol as the 3 major compounds. Oregano EO from Argentina was characterized by a great proportion of trans-sabinene hydrate and thymol (Dambolena and others 2010). In Mi and Mp EOs, the major compounds were menthol (43.85% and 36.68%, respectively), menthone (23.22% and 31.68%, respectively), and isomenthone (8.08% and 5.15%, respectively). Zheljazkov and Astatkie (2012) reported menthol and menthone as the major compounds of peppermint EOs. Menthol has been characterized as an important antifungal agent against *A. flavus* and *Penicillium* sp (Abbaszadeh and others 2014). The 4 main components in Sui EO were verbenone (32.78%), (Z)-β-ocimene (11.55%), (E)-tagetone (11.7%), and (Z)-tagetone (2.06%). Other authors (Tankeu and others 2013) reported (Z)-β-ocimene (38.5%) as the major compound, followed by (E)-ocimenone (21.7%). In samples from Argentina, the main compounds found by other authors were β-ocimene, dihydrotagetone, (Z)-tagetone, (E)- and (Z)-tagetenone, and limonene (Gil and others 2000). Ag EO had dehydroxy-isocalamendiol, α-phellandrene, limonene, and elemol (18.66%, 14.20%, 13.30%, and 10.76%, respectively) as main components. Samples of Ag EO from Patagonia (Argentina) described by other researchers showed α-phellandrene (25.9%), limonene (11.7%), and β-myrcene (11.1%) (Martins and others 2014). Compounds like α-phellandrene and limonene are cyclic terpenes (Olmedo and others 2012). A total of 16 compounds were identified in Ro EO. Eucaliptol, β-myrcene, and camphor (24.34%, 20.18%, and 13.99%, respectively) were the main components. Olmedo and others (2013) reported camphor, verbenone, and β-caryophyllene (35.7%, 26.20%, and 15.80%, respectively) as major compounds. On the other hand, Ojeda-Sana and others (2013) found β-myrcene (31.7%) as major component.

Antifungal activity

Antifungal activity of EOs tested against *A. flavus* and *Penicillium* is presented in Table 2. Oregano EOs (OCor, Omen, and OCom) showed the strongest antifungal activity. *Penicillium* species offered less resistance than *Aspergillus* species. OCor EO showed the most effective antifungal activity against *A. flavus* CCC 116–83 and *P. oxalicum* with 600 and 500 ppm MFCs, respectively. On the other hand, OCom and OMen EOs were the most effective compounds against *A. flavus* BXC01 with 700 ppm MFCs and 550 ppm MICs, respectively. OCom EO had the same MFC as that shown by OCor EO and 500 ppm MIC when this EO was tested against *P. minioluteum*. OMen EO was the most effective compound to control *P. minioluteum* with the lowest MIC and MFC (200 and 300 ppm, respectively). Similar results were obtained by Kocić-Tanackov (2012), who investigated the effect of oregano EO on the growth of *Aspergillus* species. Oregano EO caused alterations in the cell membrane of the pathogen resulting in reduced hyphal diameters and lyses of hyphal wall (Kordali and others 2008). Several studies mentioned thymol as the main compound associated with oregano EOs antifungal activity of (Sokovic and others 2002). This monoterpene was tested against *A. niger*, *A. flavus*, and *Penicillium spp.* exhibiting an important antifungal activity (Kocić-Tanackov and others 2012; Marei and others 2012).

Mi EO had better antifungal activity than Mp EO in all tested fungus samples. Mi EO at concentration of 1300 and 1450 ppm was efficient to inhibit the growth (MICs) of *P. oxalicum* and *A. flavus* 116–83, respectively. A concentration of 1800 ppm was enough to fully control (MFC) in both fungi samples. When Mi EO was tested against the obtained fungi from maize, the MFCs were 2300 and 3000 ppm. Mp EO had the same performance against both *Penicillium* species. Concentrations of Mp EO at 2800 and 3000 ppm were needed to totally inhibit (MFC) the growth of *A. flavus* 116–83. *A. flavus* BXC01 showed a higher resistance to Mp EO with 2850 ppm MIC and 3200 ppm MFC. Beyki and others (2014) reported 2100 ppm MFC for Mp EO against *A. flavus.* Also, Tyagi and Malik (2011) tested *Mentha* x *piperita* EO against *Penicillium* sp. and the MIC and MFC were 2.25 and 4,5 μg/mL, respectively.

Sui EO needed concentrations higher than 1500 ppm to affect the growth of fungi. The reference fungi were less resistant than the ones isolates from maize. The CFMs were 1700 and 2300 ppm in *P. oxalicum* and *A. flavus* 116–83, respectively. On the other hand, Sui EO could partially inhibit MIC and totally inhibit MFC, the growth of *P. minioluteum* at concentration of 2400 and 2500 ppm, respectively. The MIC and MFC against *A. flavus* BXC01 were observed at 3700 and 3800 ppm, respectively. Bii and others (2000) studied the effect of Sui EO against *A. niger* and *Penicillium* species and observed that this EO was effective controlling mycelia growth and sporulation.

Although, some authors reported that Ag EO inhibited the growth of *Aspergillus* spp. at 1000 ppm (MFC) (Martins and others 2014), the Ag EO of this study was not effective to control *A. flavus*, *P. minioluteum* or *P. oxalicum*. The maximum concentration tested was 5500 ppm. Limonene was tested against *A. flavus* and completely inhibited the growth at 500 ppm (Singh and others

Table 1–Relative percentages of main terpenoid components in the essential oils of Argentinean aromatic species according to their elution order in the GC-MS analysis.

		Relative percentages (%)[a]							
Compounds	**RI**	**OCor[b]**	**Omen[b]**	**OCom[b]**	**Mi[b]**	**Mp[b]**	**Tm[b]**	**Ag[b]**	**Ro[b]**
Triciclene	926	0.00	0.00	0.00	0.00	0.00	0.00	1.35	0.00
1-Rα- Pinene	937	0.00	0.00	0.51	0.00	0.56	0.00	4.99	0.00
α-Pinene	939	0.75	0.63	0.00	0.00	0.00	0.00	0.00	5.59
Camphene	954	0.41	0.12	0.00	0.00	0.00	0.00	8.10	2.53
Sabinene	977	3.91	4.52	1.66	0.00	0.48	0.00	1.27	0.00
Morillol	978	0.00	0.00	1.40	0.00	0.00	0.00	0.00	0.00
β-Pinene	980	1.36	1.03	1.14	0.81	0.83	0.00	3.79	0.79
β-Myrcene	992	1.80	1.67	0.00	0.00	0.19	0.00	1.01	**20.18**
α-Phellandrene	1006	0.18	0.19	0.00	0.00	0.00	0.00	**14.20**	0.00
α-Terpinene	1018	2.63	3.03	3.10	0.00	0.00	0.00	0.00	2.40
o-Cymene	1020	5.15	7.84	**14.33**	0.00	0.00	0.00	1.20	4.43
Limonene	1029	0.00	0.00	0.00	0.86	3.19	1.92	**13.30**	0.00
β-Phellandrene	1031	1.86	1.96	3.74	0.00	0.00	0.00	0.00	0.00
Eucaliptol	1033	0.00	0.00	0.00	6.21	4.36	0.00	0.00	**24.34**
(E)-β-Ocimene	1040	2.77	1.63	0.00	0.00	0.00	0.00	0.00	0.00
(Z)-β-Ocimene	1051	0.33	0.19	0.00	0.00	0.00	**11.55**	0.00	0.00
γ-Terpinene	1063	**7.12**	**7.59**	9.12	0.00	0.00	0.00	0.00	1.81
(E)-Sabinene hydrate	1071	3.31	3.47	0.00	0.00	0.00	0.00	0.00	0.00
(Z)-Sabinene hydrate	1068	**23.03**	**28.33**	1.81	0.00	0.00	0.00	0.00	0.00
Terpinolene	1084	0.82	0.98	1.45	0.00	0.00	0.00	0.00	0.00
Linalool	1098	0.00	0.00	0.00	0.00	0.20	0.00	0.00	1.93
Camphor	1143	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**13.99**
E-β-Terpineol	1144	0.40	0.44	**10.46**	1.78	0.00	0.00	0.00	0.00
Z-Tagetone	1146	0.00	0.00	0.00	0.00	0.00	**2.06**	0.00	0.00
E-Tagetone	1153	0.00	0.00	0.00	0.00	0.00	**11.47**	0.00	0.00
Menthone	1154	0.00	0.00	0.00	**23.22**	**31.68**	0.00	0.00	0.00
Isoborneol	1156	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.77
Isomenthone	1164	0.00	0.00	0.00	**8.08**	4.71	0.00	0.00	0.00
Borneol	1170	1.47	0.42	0.00	0.00	0.00	0.00	0.00	0.00
Menthol	1173	0.00	0.00	0.00	**43.85**	**36.88**	0.00	0.00	0.00
4-ol-Terpinen	1181	**6.20**	**6.66**	**12.55**	0.00	0.00	0.00	0.00	2.15
Isomenthol	1182	0.00	0.00	0.00	3.58	2.51	0.00	0.00	0.00
α-Terpineol	1192	2.34	2.01	3.35	0.00	0.00	0.00	0.00	3.36
Verbenone	1204	0.00	0.00	0.00	0.00	0.00	32.78	0.00	3.31
Thymol methyl ether	1235	0.27	1.09	0.69	0.00	0.00	0.00	0.00	0.00
Pulegone	1237	0.00	0.00	0.00	1.46	**5.15**	0.00	0.00	0.00
Anisole	1244	0.00	0.00	2.13	0.00	0.00	0.00	0.00	0.00
Carvacrol methyl ether	1247	1.30	1.66	0.00	0.00	0.00	0.00	0.00	0.00
Linalool acetate	1258	1.07	1.82	0.00	0.00	0.00	0.00	0.00	0.00
Piperitone	1282	0.00	0.00	0.00	0.00	1.91	0.00	0.00	0.00
Anethole	1289	0.00	0.00	0.00	0.00	0.00	0.00	2.38	0.00
Thymol	1290	**18.66**	**12.18**	**9.44**	0.00	0.00	0.00	0.00	0.00
Isomenthol acetate	1298	0.00	0.00	0.00	4.56	4.91	0.00	0.00	0.00
Carvacrol	1301	0.21	1.82	5.61	0.00	0.00	0.00	0.00	3.15
Piperitenone	1342	0.00	0.00	0.00	0.00	0.00	1.16	0.00	0.00
β-Cariophillene	1425	2.82	2.18	1.99	1.48	0.00	0.00	0.77	3.85
D Germacrene	1486	1.23	0.30	0.00	1.16	0.16	0.00	2.25	0.00
Bicyclogermacrene	1500	1.72	1.55	0.00	0.00	0.00	0.00	0.00	0.00
β-Bisabolene	1511	0.73	0.23	1.21	0.00	0.00	0.00	0.00	0.00
Elixene	1514	0.00	0.00	0.00	0.00	0.00	0.00	1.63	0.00
δ-Cadinene	1527	0.06	0.03	0.69	0.00	0.00	0.00	3.10	0.00
Elemol	1547	0.00	0.00	0.00	0.00	0.00	0.00	**10.76**	0.00
D-Germancrene 4ol	1574	0.00	0.00	0.00	0.00	0.00	0.00	2.36	0.00
Espatulenol	1576	0.00	0.00	1.92	0.00	0.00	1.16	0.00	0.00
Cariophylene oxide	1588	0.37	0.30	1.77	0.00	0.00	0.00	0.00	0.00
dehydroxy-isocalamendiol	1593	0.00	0.00	0.00	0.00	0.00	0.00	**18.66**	0.00
γ-Eudesmol	1630	0.00	0.00	0.00	0.00	0.00	0.00	2.56	0.00
β-Eudesmol	1649	0.00	0.00	0.00	0.00	0.00	0.00	2.17	0.00
Yield (%, v/w) [c]		3.30	3.15	2.20	2.38	3.22	1.70	2.60	2.75

[a]Bold numbers denote major compound in essential oil compositions.
[b]OCor (*Origanum vulgare* sspp *hirtum*), OCom (*Origanum vulgare* L. sspp. *Vulgare*), OMen (*Origanum* x *majoricum*), Mi (*Mentha* x *piperita* L. var. *Vulgaris Sole*), Mp (*Mentha* x *piperita*), Tm (*Tagetes minuta* L.), Ro (*Rosamarinus officinalis*), and Ag (*Schinus molle*),
[c]Percentages expressed as mL/100 g.

2010). This compound is a major component in Ag EO; however this essential oil did not show antimicrobial activity for *A. flavus*.

Also, Ro EO did not show antifungal activity against *Aspergillus* and *Penicillium*. Rasooli and others (2008) studied the effect of this EO at concentration until 3000 against *Aspegillus* sp. obtaining negative results. β-myrcene and camphor have shown antifungal activity (MIC) against *Aspergillus* sp. (98.5 and 231 ppm, respectively) and *Penicillium* sp. (95.46 and 367 ppm, respectively) (Marei and others 2012). Both these compounds are major compounds in Ro EO but this essential oil had no effect against the studied fungi.

Table 2–Comparative antifungal activity of essential oil against fungi growth.

EO[a]	Aspergillus flavus (CCC116–83)			Penicillium oxalicum (083296)			Aspergillus flavus (BXC01)			Penicillium minioluteum (BXC03)		
	MFC[b]	MIC[b]	R^2	MFC[b]	MIC[b]	R^2	MFC[b]	MIC[b]	R^2	MFC[b]	MIC[b]	R^2
OCor[a]	600	450	0.91	500	350	0.72	800	650	0.90	600	400	0.76
OMen[a]	800	450	0.94	600	450	0.89	700	550	0.83	300	200	0.89
OCom[a]	1100	550	0.62	700	400	0.89	700	550	0.78	600	500	0.73
Tm[a]	2300	2150	0.77	1700	1600	0.92	3800	3700	0.60	2500	2400	0.70
Mi[a]	1800	1450	0.61	1800	1300	0.7	3000	2800	0.73	2300	2200	0.60
Mp[a]	3000	2800	0.92	2300	2050	0.68	3200	2850	0.89	2300	2050	0.92
Ag[a]	>5500	–	–	>5500	–	–	>5500	–	–	>5500	–	–
Ro[a]	>5000	–	–	>5000	–	–	>5000	–	–	>5000	–	–

[a]OCor (*Origanum vulgare* sspp *hirtum*), OCom (*Origanum vulgare* L. sspp. *Vulgare*), OMen (*Origanum* x *majoricum*), Mi (*Mentha* x *piperita* L. var. *Vulgaris Sole*), Mp (*Mentha* x *piperita*), Tm (*Tagetes minuta* L.), Ro (*Rosamarinus officinalis*), and Ag (*Shinus molle*).
[b]MFC (Minimum Fungicidal Concentration), MIC (Minimum Inhibitory Concentration),values expressed in part per million (ppm).

Although these others authors reported that the major compounds of Ro and Ag EOs could inhibit the growth of *Aspergillus sp.* and *Penicillium sp.*, these EOs had negatively antimicrobial effect against fungi control (Singh and others 2010; Marei and others 2012).

Principal components analysis (PCA)

The biplot obtained from the 2 principal components (PC) in the PCA is presented in Figure 1. The 2 principal components explained 99.9% variability of MFC in fungi species. PC 1 represented 99.4% variability. *A. flavus* BXC01, *A. flavus* CCC116–83, *P. Oxalicum* 083296, and *P. minioluteum* BXC03 were placed on the right side of the PC1 in the biplot. EOs that showed higher antifungal activity (lower MFC value) for the studied fungi were also placed on the right side of the biplot (OCor, OMen, OCom, Sui, Mp, and Mi). These EOs were positively related among them. None of the evaluated microorganisms were placed on left side in the biplot. However, Ag and Ro were placed in the opposite side with respect to the others EOs (left side of the biplot) showing no relation among them and their bioactivity. Further, these EOs did not show CFM for these fungi.

A strong association between *A. flavus* CCC116–83 and *P. oxalicum* 083296 was observed. *P. minioluteum* was also related to them (isolated from corn). OCor, Omen, and OCom were the closest treatments to those fungi, presenting higher fungi toxicity (lower CFM values). Sui, Mp, and Mi were highly related among themselves, and were also placed close to OCor, Omen, and OCom. The dispersion of the points showed variability among samples.

Lipid hydroperoxides

The hydroperoxide values (HPV) of fungi tissue samples after 72 h of incubation with EOs addition are shown in Figure 2. Membrane lipid oxidation and its destabilization is one of the antifungal mechanisms that essential oils have. Some essential oils penetrate through the cell wall and cytoplasmic membrane disrupting them, specifically damaging mitochondrial membranes. The mitochondria by changes in electron flow produces free radicals which oxidize and damage lipids, proteins, and DNA (Bakkali and others 2008). The hydroperoxide value depended on the fungi ($P < 0.001$) and the antimicrobial agent ($P < 0.001$). Significant differences were found among EOs. Sui EO had the highest HPV in fungi samples. Membrane lipids were severely oxidized by Sui EO in *A. flavus* BXC01 and *A. flavus* CCC116–83 (0.021 and 0.027 meqO_2/kg, respectively), followed by Mp (0.0098 and 0.0094 meqO_2/kg in *A. flavus* BXC01 and *A. flavus* CCC116–83, respectively), being statistically different from the other EOs. *A. flavus* strains were also affected by Mi (0.0040 and 0.0065 meqO_2/kg in *A. flavus* BXC01 and *A. flavus* CCC116–83, respectively). Menthol is a cyclic terpene alcohol and it was present in high proportion in Mi and Mp (43.85% and 36.88%, respectively); it was found to be the 3rd most active EO component in terms of being fungi-toxic and aflatoxin B1 inhibitory (Mirsha and others 2013). Membrane lipids of *P oxalicum* were also highly oxidized by Sui (0.0079 meqO_2/kg) followed by Mi (0.0065 meqO_2/kg) and Mp (0.0061 meqO_2/kg). Oregano EO prevented membrane lipid oxidation in all treatments. This effect could be attributed to the strong antioxidant activity of the oil and its compounds (Asensio and others 2012; Asensio and others 2013). Chao and others (2005) reported that the thymol present in Oregano EOs composition had strong efficacy as fungal growth inhibitor and aflatoxin suppressor. Moreover, the lipophilic nature of thymol would damage membrane integrity, and has also been reported to inhibit the H^+ ATPase system, leading to intracellular acidification and

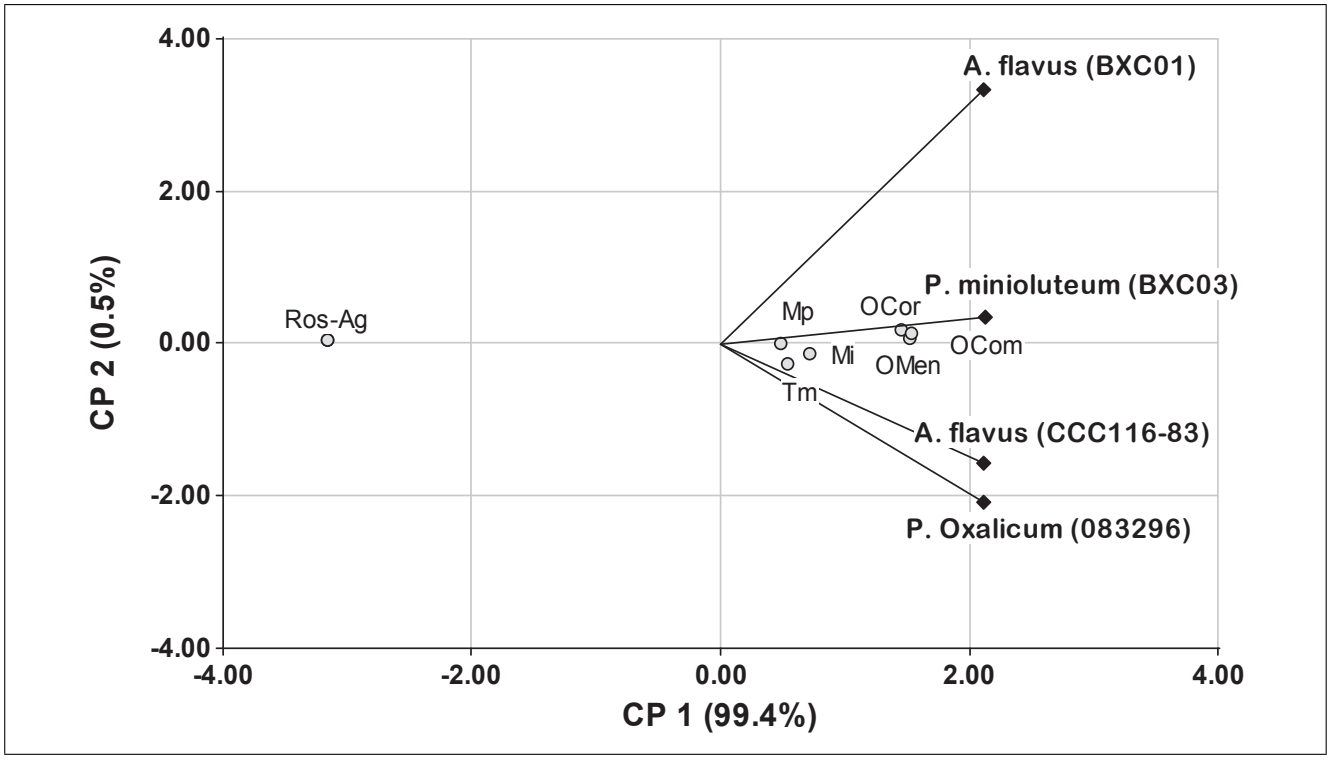

Figure 1–Biplots from the 1st and 2nd principal components of PCA. Variables: minimum fungicidal concentration for each fungi tested. Treatments: essential oil of Tm (*Tagetes minuta* L.), Mi (*Mentha* x *piperita* L. var. *Vulgaris Sole*), Mp (*Mentha* x *piperita*), OCor (*Origanum vulgare* sspp *hirtum*), OCom (*Origanum vulgare* L. sspp. *Vulgare*), OMen (*Origanum* x *majoricum*), Ro (*Rosamarinus officinalis*), and Ag (*Schinus molle*).

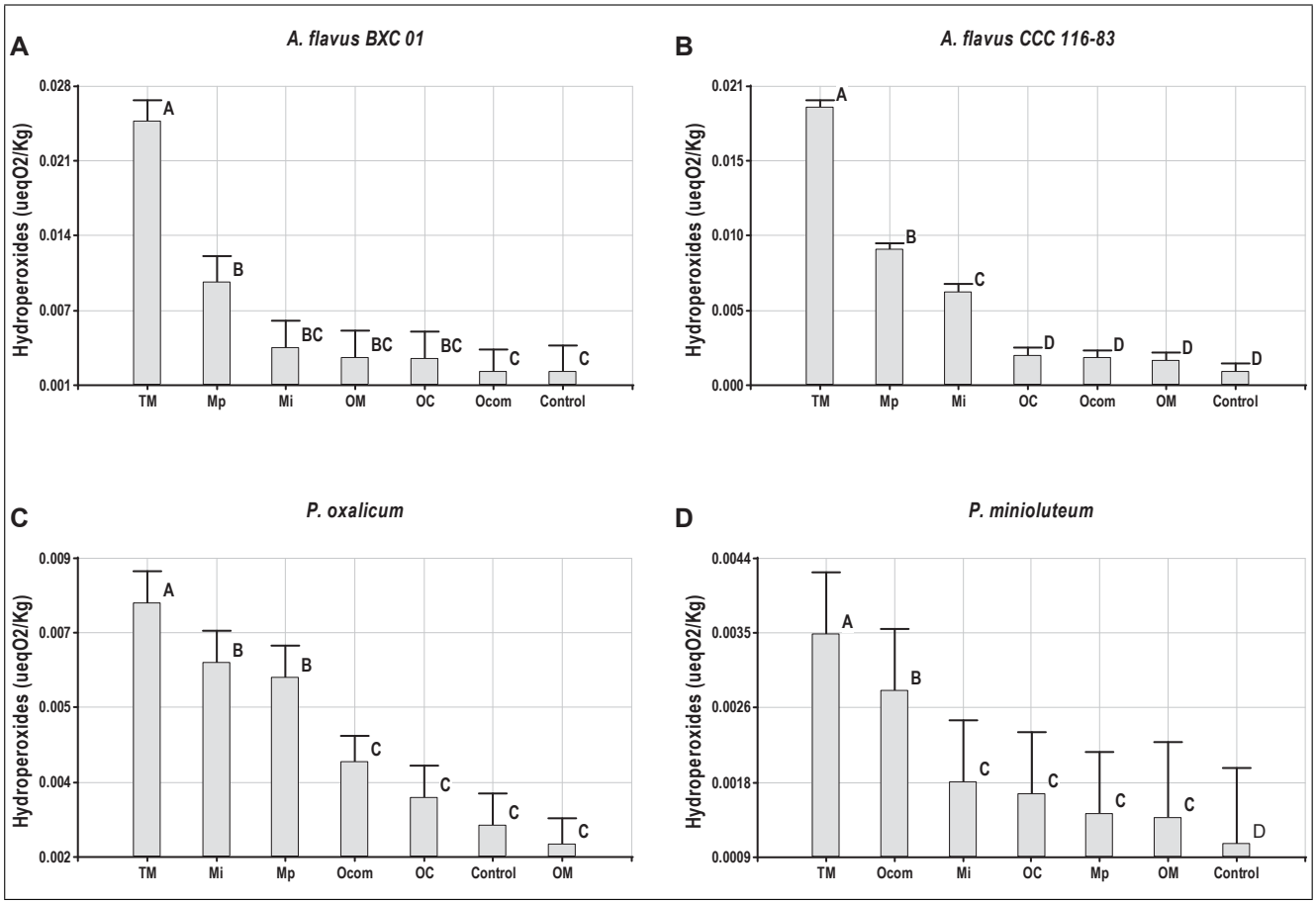

Figure 2–Hydroperoxide values (meqO_2 / kg) of isolated fungi after 72 h of incubation with essential oils: Tm (*Tagetes minuta* L.), Mi (*Mentha* x *piperita* L. var. *Vulgaris Sole*), Mp (*Mentha* x *piperita*), OCor (*Origanum vulgare* sspp *hirtum)*, OCom (*Origanum vulgare* L. sspp. *Vulgare*), OMen (*Origanum* x *majoricum*), Ro (*Rosamarinus officinalis*), and Ag (*Schinus molle*). (A) *A. flavus* BXC 01; (B) *A. flavus* CCC 116–83; (C) *P. oxalicum*; (D) *P. minioluteum*.

cell death (Ahmad and others 2010). The phenol nature of thymol may be responsible for their strong free radical scavenging activity. HPV in *A. flavus* BXC01, *A. flavus* CCC116–83, *P. oxalicum*, and *P. minioluteum* with OCor, OCom, OMen EOs treatments did not show significant differences between them and also, these essential oils showed a behavior similar to the control sample.

Cluster analysis

The results of the CA of fungi samples controlled with different EOs considering the dependent variables (CFM and HPV) is presented as a dendogram in Figure 3. Four groups were obtained: Group 1 was formed only by Sui EO; Group 2 was formed by all oregano EOs (OCom, OMen, and OCor); Group 3 was formed by Mi and Mp EOs; and the Control sample was placed alone in Group 4. The results indicate that oregano EOs had similar fungi toxicity and membrane lipid oxidation damage. Similar results were obtained for Mp and Mi. Sui EO was placed alone in a group, which indicates that it behaved differently, with antifungal activity causing high oxidation in membrane lipids.

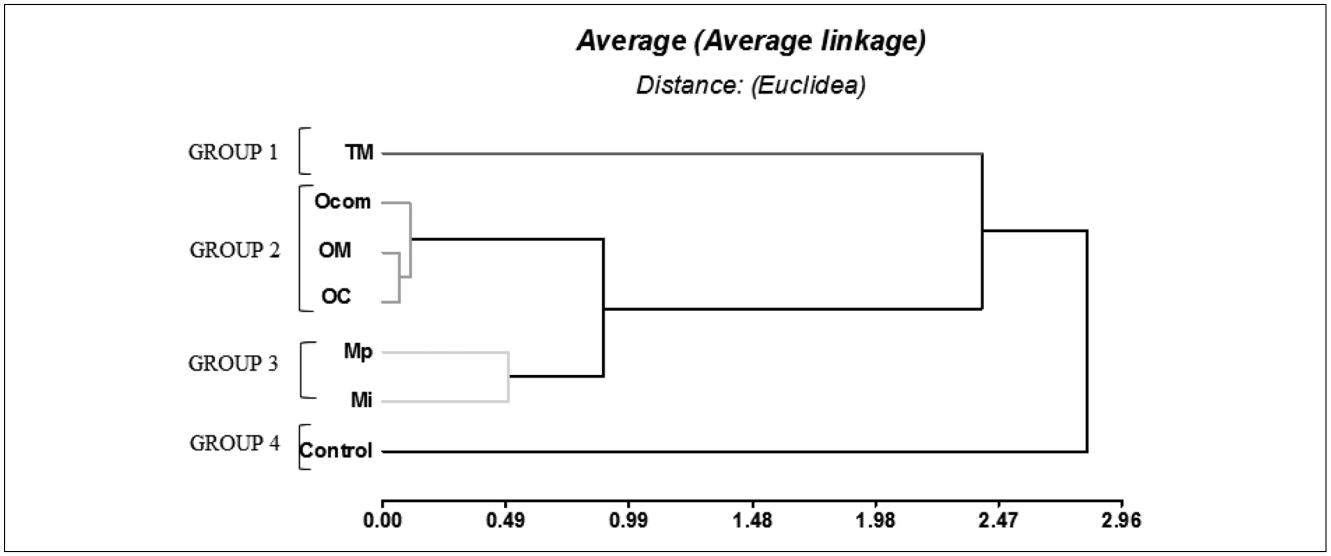

Figure 3–Dendogram from cluster analysis of essential oils grouped according to control effect against fungi samples considering as dependent variables (CFM and HPV).

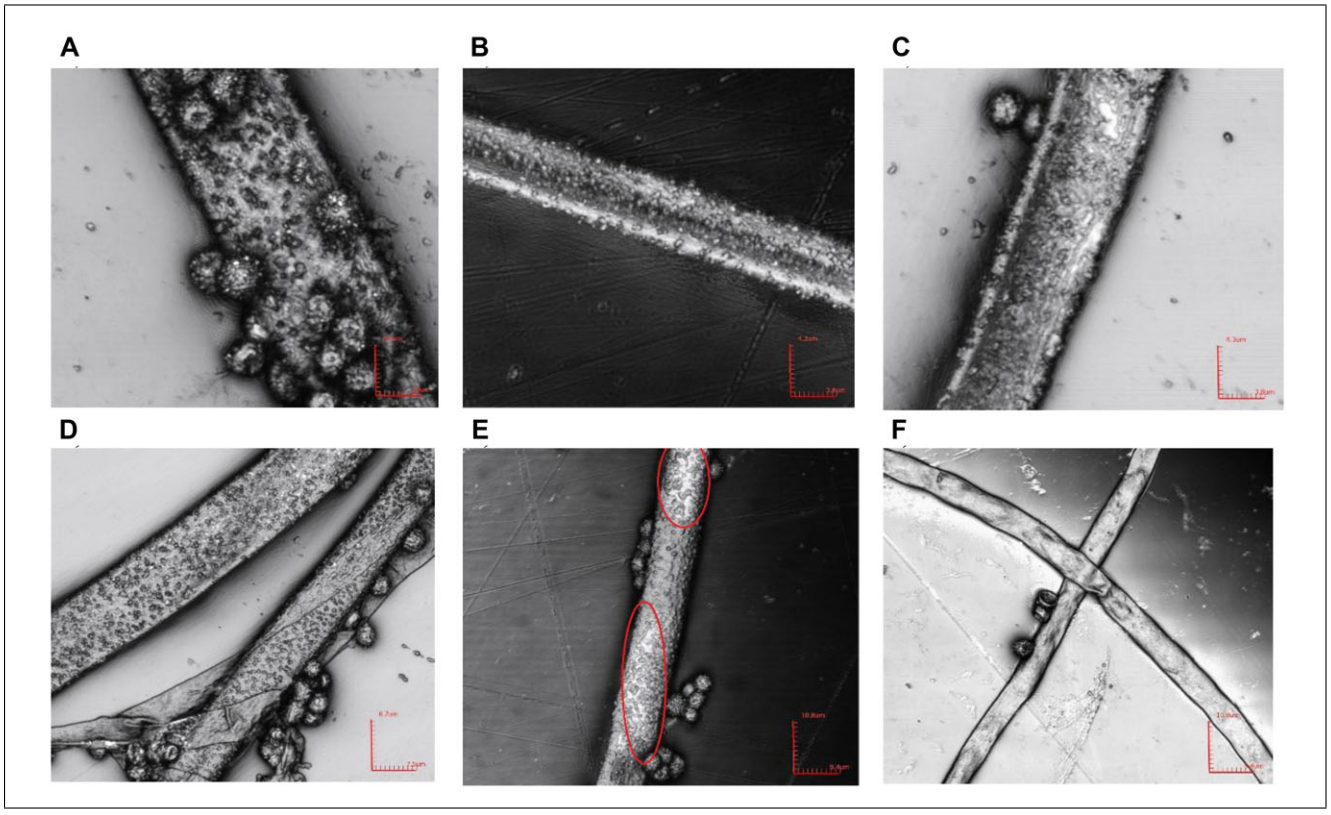

Figure 4–Reflection confocal microscopy images of *A. flavus* BXC01 hyphae. Sample control without addition of esential oil after (A) 0, (B) 24, and (C) 72 h; and samples with the addition of essential oil after (D) 0, (E) 24, and (F) 72 h.

Reflection confocal microscopy

Changes in cell membrane of *A. flavus* hyphae were observed (Figure 4). The control did not show any changes after 0, 24, and 72 h. The action of EO was not instantaneous and treatments did not show changes with EO addition at 0 h. Cell membrane modifications were evidenced after 24 h. After 72 h, the changes in cell membrane were pronounced and the hyphae lost turgidity. The results on membrane damage are in accordance with Lucini and others (2006), who observed morphological changes in mycelium of *Sclerotium* spp. due to the action of EO. Also, Lambert and others (2001) demonstrated changes in membrane integrity by the action of EO.

Conclusion

The results of this research evidence that the chemical profiles of the EOs, the antifungal capacities, and the actions on lipid membrane are related. Oreganos EO have the best antifungal activity due to the presence of thymol as a major component in its composition. The relative percentages of this compound can vary depending on the genotype, but this oscillation does not have a direct effect on the antifungal activity. The oregano EOs do not cause lipid oxidation in cell membrane, probably having another action mechanism. Mint EO has good antifungal activity against *A. flavus* (CCC116–83) and *P. oxalicum* (083296) but Mp and Mi are similar against *A. flavus* (BXC01) and *P. Minioluteum* (BXC03) isolated from maize. The lipid oxidation performed by the essential oils could be the mechanism of control of these fungi. Suico essential oil shows an antifungal activity similar to peppermint essential oil. This last EO has a strong oxidation effect on membrane lipids and seems to be the main action for fungi control.

The results of this study allow us to conclude that the essential oils of oregano, peppermints, and suico grown in Argentina can be used as a natural alternative to control the presence of postharvest fungi in maize. The cereal and food industries may consider the spraying of essential oils as a sustainable and environmentally friendly mechanism to control fungi offering better product quality.

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