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Essential oils and their combinations with iprodione fungicide as potential antifungal agents against withe rot (*Sclerotium cepivorum* Berk) in garlic (*Allium sativum* L.) crops

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ABSTRACT

This study was conducted to evaluate essential oils (EOs) and their binary combinations with fungicide iprodione (Ip) as a potential fungicide against *Sclerotium cepivorum* Berk in order to reduce the amount of pesticide applied in garlic crops. Five essential oils (EOs) from different plants grown in Argentina were studied: *Tagetes minuta* L. (Su), *Tagetes filifolia* L. (An), *Origanum vulgare* L. spp. *Vulgare* (OCom), *Origanum x majoricum* (OMen), and *Laurus nobilis* L. (Ba). The chemical composition of EOs was analyzed by GC–MS. The minimum fungicide concentration (MFC) and minimum inhibitory concentration (MIC) were determined for each compound. A total of 84 combinations of EOs and Ip were assessed looking for synergistic interactions. A phytotoxicity assay was carried out to identify EOs with a potential negative effect on garlic. The effect of EOs, Ip and mixtures on white rot was evaluated in a field study. Fungicide Ip had the best antifungal activity in the *in vitro* test. OMen and OCom showed high antifungal activity, but also had a strong phytotoxic effect on garlic plants. Su had a moderate antifungal effect with distinguished synergism with Ip, but none can be used due to its phytotoxic effect. An and Ba both had moderate antifungal activity. These EOs had no negative effect on garlic germination and production, being non-phytotoxic for this crop. In this study, the combinations of An and Ba them with Ip have synergistic interactions. Sclerotia density and disease incidence were reduced by these mixtures in a field study. In conclusion, An and Su could be used as natural fungicides to control white rot in garlic, reducing the fungicide dose required. However, future studies are needed to adjust final concentrations to be applied in field crops.

1. Introduction

In many countries, garlic (*Allium sativum* L.) is widely cultivated and used as a flavoring condiment in foods (Zewde et al., 2007). Argentina represents the second largest country in production of this crop in the world; the export markets generate 100 million US dollars per year. In addition to the economic value, garlic has a social impact because it is grown by small farmers. Production areas of garlic in Argentina include Mendoza, San Juan, Córdoba, and Buenos Aires provinces. In total, 85% of the garlic crop is exported in bulk to over 30 countries, while a minor portion is kept as "seed" (Burba, 2003). Garlic production is highly affected by biotic and abiotic factors. Among the biotic factors, fungal diseases are the major problems affecting yield and productivity, as well as the quality of this high value crop. The white rot caused by *Sclerotium cepivorum* Berk is one of the most important diseases which produces garlic rot and plant death. This pathogen also produces a large amount of small size sclerotia which can survive long periods of time and are sources of inoculums. During germination, mycelium penetrates the root epidermis causing tissue degradation, and plants die pre-

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maturely. This disease constitutes a global limiting factor in garlic production. In Argentina and Brazil, losses up to 100% have been reported due to white rot (Crowe, 2008; Pinto et al., 2000).

Chemical control using fungicides is the most common method for white rot management. Several synthetic fungicides which belong to benzimidazoles and triazoles groups have been reported to be effective against this pathogen (Zewide et al., 2007). Iprodione (3-(3.5-dichlorophenyl)-*N*-isopropyl-2.4-dioxo-imidazolidine-1-carboxamide) is commonly used in agricultural production to reduce the negative impact of crop diseases, including white rot in garlic and onion (Miñambres et al., 2010). Treatments with this fungicide applied to the soil achieve infection reduction up to 75–95% (Utkhede and Rahe, 1979; Zewide et al., 2007). Fungicide Iprodione can alter microbial communities and reduce the fungal biomass in soil, including beneficial fungi (Miñambres et al., 2010). Also, chemical residues from fungicides have high persistence in soil and water because of their slow degradability. These are the reasons why the use of synthetic fungicides is recommended as part of an integrated fungal control program (Sosa-Gomez et al., 2003).

Essential oils (EOs) are complex volatile compounds synthesized by aromatic plants in order to protect themselves against diseases and are considered safe for humans and animals (Isman, 2000). Their compounds like terpenes and terpenoids have demonstrated antifungal activity and are easily degraded (Camiletti et al., 2014; Isman, 2000). As a result, EOs are presented as a natural alternative to reduce the negative impact of synthetic fungicides. EOs from oregano (*Origanum* spp.), suico (*Tagetes minuta* L.) and peppermint (*Mentha x piperita* L.) have been reported to have antifungal activity against *Aspergillus* and *Penicillium* species (Camiletti et al., 2014). However, the efficacy of EOs against fungi pathogens is lower than synthetic compounds. Synergic, additive or antagonistic interactions are expected when two antimicrobial substances are mixed. Consequently, the antifungal activity of EOs is evaluated in combination with synthetic fungicides in search of synergic interactions (Pavela, 2014; Pyun and Shin, 2006; Shin and Kang, 2003). According to the Additive and Abbot method, synergism is declared when the efficacy of the mixture is higher than the expected efficacy (Kosman and Cohen, 1996).

On the other hand, phytotoxic effects of EOs have been studied as possible natural herbicides (Batish et al., 2012; Rolli et al., 2014). This biological activity on seed germination is a result of accumulative effects of the whole complex such as membrane damage and chlorophyll degradation (Poonpaiboonpipat et al., 2013). Because of this scenario, phytotoxic effects on garlic seeds have to be studied to identify the appropriate EOs to control fungal diseases.

The objectives of this work were to determine the presence of synergistic, antagonistic or additive effect of five EOs from aromatics plants grown in Argentina with iprodione against the pathogen *S. cepivorum* Berk by two different methods *in vitro*, and in a field study, and to evaluate the phytotoxic effects of these EOs on garlic seeds.

2. Materials and methods

2.1. Fungal strain

S. cepivorum Berk was provided by Laboratorio de Manejo Integrado de Plagas y Fitopatología (Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba).

2.2. Plant material and essential oil extraction

EOs were obtained from aromatic plants harvested at the end of the 2012 growing season in Argentina. Leaves of *T. minuta* L. "suico" (Su) and *Tagetes filifolia* L. "anisillo" (An) were collected from Characato, Cordoba province (31°5′30.12″S, 64°55′59.88″W). Aerial parts of other four aromatic plants were harvested from the Experimental Station of the "Facultad de Ciencias Agropecuarias" (National University of Cordoba), Córdoba province (30°30′0″S, 64°30′0″W): *Origanum vulgare* L. spp. *Vulgare* "oregano compacto" (OCom), *Origanum x majoricum* "oregano mendocino" (OMen), and *Laurus nobilis* L. "bay" (Ba). Plant material was dried under the shade and the extraction was carried out by steam distillation during 2 h using a (Clevenger-type) apparatus coupled with an extraction chamber. EOs were kept in dark flasks at −20 °C in freezer (Asensio et al., 2015).

2.3. Essential oil chemical analysis

EOs were analyzed using a gas chromatograph (PerkinElmer® Clarus 600, Waltham, MA, USA) coupled to a Flame Ionization Detector (FID) and a Mass Spectrometry Detector (MSD). A capillary column DB-5 (30 m, 0.25 mm i.d., and 0.25-mm coating thickness) was used to separate the components. Helium was the carrier gas with 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 35–450. The identification and quantification of the different peaks was performed according to Asensio et al. (2015).

2.4. Antifungal activity

A broth method was used to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of EOs and iprodione according to Camiletti et al. (2014). Growing medium was prepared (20% potato lixiviated; 2% glucose; pH 4.5) and transferred (20 mL) into Petri plates of 9-cm diameter. EOs previously diluted in ethanol and iprodione solutions were added to the culture medium in the desired concentrations (varying each 100 μL/L). The final concentration of ethanol never exceeded 1% v/v. A fungal colony was grown for 7 days at 21 °C in PDA medium before screening. Five-millimeter diameter agar discs of these Petri dishes containing fungal colonies were taken and used to inoculate the tested treatments. A control treatment (C) was also prepared without the addition of an antifungal agent. Treatments were incubated for 7 days at 21 °C in a culture chamber. After incubation, the diameter of the mycelium was measured and the results were expressed as a percentage of growth inhibition (PGI) using the equation described by Camiletti et al. (2014). MFCs were defined as the lowest concentration of EO required to achieve a PGI of 100%. MICs (PGI = 50%) were calculated from the curve obtained after linear regression (Abbaszadeh et al., 2014; Camiletti et al., 2014).

2.5. Mixture effects

The method described previously was also used to study the interaction of different concentrations of EOs with iprodione. All possible combinations between EOs and iprodione were prepared as follows: 20, 40, 60 or 80% of the previously determined MFCs of EOs were mixed with 20, 40, 60 and 80% of the previously determined MFC of iprodione. The mixtures were prepared in Eppendorf tubes, volumes were measured with micropipettes and mixed in a vortex mixer. A total of 80 combinations were tested. Data expressed as PGI were obtained and assessed after incubation (Gadban, 2011; Kordali et al., 2008). Synergism analysis was performed using two methods: the additive method and the Abbot method. In the additive method, the expected PGI of the mixture was obtained by adding the individual PGI of the EO concentration and the individual PGI of iprodione. The calculated values were

compared with the observed PGI. Synergism was determined when the observed PGI was higher than the expected PGI of the mixture. When the observed PGI value was equal to or lower than the expected PGI, an additive or antagonistic effect was considered, respectively (Gadban, 2011).

In the Abbott method, the expected PGI (EPGI) for each mixture was calculated following the equation:

$$EPGI = PGI_{EO} + PGI_{IP} - \left(\frac{PGI_{EO} \times PGI_{IP}}{100}\right)$$

where PGI_{EO} and PGI_{IP} are individual values of PGIs for EO and iprodione at the given concentrations.

The effect of the mixtures was designated assessing the Abbot Index (AI):

$$AI = \frac{OPGI}{EPGI}$$

where OPGI is the observed PGI and EPGI is the expected PGI.

A synergistic effect was assigned for AI ≥ 1.5, an additive effect when it was ≥0.5–1.5 and antagonistic when it was ≤0.5 (Gadban, 2011; Kosman and Cohen, 1996)

2.6. Phytotoxicity assays

2.6.1. Garlic germination study

A seed germination study was carried out to identify EOs with phytotoxic effects. Garlic cloves with a diameter of 1.5-cm were purchased in a local market and kept at 20 °C in paper bags. A stock solution of liquid culture medium was prepared according to Díaz Báez et al. (2004). The medium was diluted 10 times and the pH was adjusted to 7.0. Test tubes (10 × 1.5 cm) were filled with culture medium (9 mL) and EOs were added to achieve their MFCs. The epidermis of the seeds was removed before the test. Garlic cloves were put on top of the tube with the root zone submerged in the culture medium. Tubes were incubated at 20 °C for 72 h in darkness. A solution of Cu^{2+} was used as a positive control. The negative control was growth medium without EO. After incubation, the lengths of the roots and leaves were measured. The results were expressed as roots growth inhibition (%) and leaves growth inhibition (%), using the equation $[(C - T)/C] \times 100$, where C is the mean value of the positive control and T is the mean value of the treatment. Non-phytotoxic essential oils (NPEOs) were identified in this study.

2.6.2. Garlic yield

Previously identified NPEOs were tested in a field assay in order to determine their effect on garlic production. MFC of NPEOs and MFC plus an extra 50% (MFC50+) of this dose (to supplement the losses by volatilization and light degradation) of NPEOs were tested. Bins (16 liters) were filled with soil collected from the Experimental Station of "Facultad de Ciencias Agropecuarias" where garlic had never been cultivated, and buried at the same place. Twenty eight garlic seeds were planted in four rows of seven seeds each. After 30, 50 and 70 days of seeding, broths with the EOs were applied to the soil. After the growing season (120 days), the dry weight of garlic cloves was obtained and the yield was expressed as kg/ha.

2.7. Field study

A field study was carried out to determine whether the MFCs (of NPEOs) obtained in the *in vitro* tests had the same effect in soil inoculated with *S. cepivorum* Berk. MFC and MFC50+ doses of NPEOs were tested. Ip treatment, the combinations of Ip + MFCs, Ip + MFC50+, and a control (C) treatment were also tested. In total, 10 treatments were evaluated. Sclerotia were collected from fungi colonies previously grown at 21 °C for 7 days in PDA medium and then mixed with the soil. Bins (16 liters) containing the inoculated soil were buried at the same location as where the soil was collected. Twenty eight garlic seeds (distanced 7 cm) were planted in four lines. Broths with EOs, Ip or a combination were prepared in an Erlenmeyer flask and applied to the soil with a pipette along each line (20 mL), next to the plant neck. Three applications were made: after 30, 50 and 70 days of the seeding date. The incidence (% of diseased plants) was recorded every 15 days from the first day that a diseased plant was detected, in order to describe the progress of white rot (Zewide et al., 2007). Data were used to calculate the area below the disease progress curve (ABDPC) for each treatment using SigmaPlot version 13.0 (Systat software, San Jose, USA).

Samples were harvested at the end of the growing season (120 days). Soil sclerotia density (SSD) (number of viable sclerotia/100 g) was measured 30 days post-harvest according to Zewide et al. (2007).

2.8. Statistical analysis

Data analysis was carried out using Infostat software version 2014 (Di Rienzo et al., 2014). Treatments were replicated three times. A randomized complete block design was used in the field study. Simple regression equations were used to calculate the MICs and to compare sclerotia density according to their slopes. Analysis of variance (ANOVA, $\alpha = 0.05$) and DCG's multiple range test were performed to determine significant differences between means.

3. Results and discussion

3.1. Chemical analysis

The compositions of An, Su, OCom, OMen, and Ba are presented in Table 1. An was characterized by a great predominance of phenylpropanoids (87.1%). Anethole was the major component representing 63.80% of the essential oil composition, followed by estragole (23.81%). Sphatulenol (3.26%) was the third main component. Gadban (2011) reported a great prevalence of anethole and estragole as principal component in An extracted from plants grown in Argentina (77.7% and 22.5%, respectively). These results agree with other authors (De Feo et al., 1998), who reported a similar composition of An extracted from plants collected in Peru. Previous studies indicated that the chemical profile of this EO is not influenced by the location of the plants (Maestri et al., 1991). A total of 9 compounds were identified in Su. The main component was verbenone (28.31%), followed by E-tagetone (18.73%) and β-ocimene (13.40%). Dihydrotagetone and β-ocimene were reported as major compounds in other samples from Argentina (Camiletti et al., 2014; Gil et al., 2000). Different chemotypes of this EO have been described to be influenced by seasonality and environmental conditions (Gil et al., 2000). Bay was composed mainly of oxygenated monoterpenes. The major compounds in this EO were eucalyptol (42.23%), linalool (15.19%), terpinene 4–acetate (8.91%) and methyl eugenol (5.46%). A similar composition was reported by Di Leo Lira et al. (2009), who analyzed the chemical profile of this EO extracted from a crop from Buenos Aires province (Argentina). These results are in accordance with other authors (Da Silveira et al., 2014) who found eucalyptol and linalool in similar proportions as the main components in Ba extracted in Brazil. The chemical compositions of OCom and OMen were rich in components. The terpenes *o*-cymene (14.25%), terpinen-4-ol (12.48%), E-β-terpineol (10.4%) and thymol (10.13%) were the main components in OCom. Thymol (27.51%) was the major compound in OMen, followed by α-terpinyl acetate (21.89%), γ-terpinene (13.92%) and terpinen-4-ol (10.86%). These results are in accordance with previously published data (Asensio et al., 2015). Theses EOs extracted from plants grown

Table 1
Chemical profile and relative percentage of main compounds present in the five essential oils according to the GC–MS analysis.

RI	Compounds	Relative percentages (%)[a]				
		An[b]	Su[b]	Ba[b]	OCom[b]	OMen[b]
930	α-Thujene	0.00	0.00	0.00	0.66 ± 0.00	0.59 ± 0.00
937	1-R-α-pinene	0.00	0.00	0.37 ± 0.04	0.51 ± 0.00	3.14 ± 0.01
939	α-Pinene	0.00	0.00	3.59 ± 0.11	0.00	0.39 ± 0.00
954	Camphene	0.00	0.00	0.31 ± 0.03	0.00	0.00
973	Sabineno	0.00	0.00	0.00	1.65 ± 0.01	0.00
978	Morillol	0.00	0.00	0.00	1.45 ± 0.00	0.00
979	B-Pinene	0.00	0.00	3.29 ± 0.52	1.14 ± 0.00	0.99 ± 0.00
991	B-Myrcene	0.00	0.00	0.90 ± 0.01	0.00	0.00
1005	α-Phellandrene	0.00	0.00	0.00	0.00	0.23 ± 0,00
1011	3-Carene	0.00	0.00	0.47 ± 0.01	0.00	0.00
1018	α-Terpinene	0.00	0.00	0.55 ± 0.02	3.08 ± 0.01	0.00
1020	o-Cymene	0.00	0.00	0.00	**14.25 ± 0.04**	0.00
1023	m-Cymene	0.00	0.00	0.27 ± 0.01	0.00	2.76 ± 0.02
1025	p-Cymene	0.00	0.00	0.00	0.00	0.00
1031	Limonene	0.00	4.06 ± 0.07	0.00	3.72 ± 0.01	0.71 ± 0.02
1033	1,8Cineole	0.00	0.00	**42.23 ± 0.15**	0.00	0.00
1050	(Z)-β-Ocimene	0.00	**13.40 ± 0.03**	0.00	0.00	0.00
1062	γ-Terpinene	0.00	0.00	0.80 ± 0.00	9.07 ± 0.02	**13.92 ± 0.03**
1068	(Z)-Sabinene hydrate	0.00	0.00	2.04 ± 0.02	1.80 ± 0.00	1.24 ± 0.01
1071	β-terpinene	0.00	0.00	0.00	0.00	2.61 ± 0.03
1084	Terpinolene	0.00	0.00	0.39 ± 0.03	1.44 ± 0.00	0.69 ± 0.01
1095	α-Pinene oxide	0.00	1.56 ± 0.02	0.00	0.00	0.00
1098	Linalool	0.00	0.93 ± 0.02	**15.19 ± 0.11**	0.00	0.00
1108	α-Terpinyl acetate	0.00	0.00	0.00	0.00	**21.89 ± 0.07**
1144	(E)-β-Terpineol	0.00	0.00	0.00	**10.40 ± 0.03**	0.00
1146	(E)-Tagetone	0.00	**18.73 ± 0.05**	0.00	0.00	0.00
1153	(Z)-Tagetone	0.00	**3.99 ± 0.05**	0.00	0.00	0.00
1182	4-ol-Terpinen	0.00	0.00	0.00	**12.48 ± 0.03**	**10.86 ± 0.04**
1189	α-Terpineol	0.00	0.00	3.72 ± 0.03	3.33 ± 0.01	1.71 ± 0.01
1195	Estragole	**23.81 ± 0.11**	0.00	0.00	0.00	0.00
1204	Verbenone	0.00	**28.31 ± 0.07**	0.00	0.00	0.00
1235	Thymol methyl ether	0.00	0.00	0.00	0.69 ± 0.00	1.74 ± 0.01
1244	Anisole	0.00	0.00	0.00	2.12 ± 0.01	0.00
1252	p-Anisaldehyde	**2.49 ± 0.05**	0.00	0.00	0.00	0.00
1289	Anethole	**63.80 ± 0.35**	0.00	0.00	0.00	0.00
1290	Tymol	0.00	0.00	0.00	**10.13 ± 0.03**	**27.51 ± 0.05**
1298	Carvacrol	0.00	0.00	0.00	5.58 ± 0.01	1.58 ± 0.01
1340	Terpinene 4-acetate	0.00	0.00	**8.91 ± 0.09**	0.00	0.00
1342	Piperitenone	0.00	3.09 ± 0.04	0.00	0.00	0.00
1348	β-Terpinyl acetate	0.00	0.00	0.68 ± 0.02	0.00	0.00
1356	Eugenol	0.00	0.00	0.70 ± 0.02	0.00	0.00
1375	β-Elemene	0.00	0.00	0.45 ± 0.01	0.00	0.00
1380	β-maaliene	1.37 ± 0.04	0.00	0.00	0.00	0.00
1388	β-Bourbonene	0.00	0.00	0.00	1.00 ± 0.00	0.00
1401	Methyl eugenol	1.65 ± 0.06	0.00	**5.46 ± 0.06**	0.00	0.00
1418	β-Carophyllene	0.00	0.00	0.00	1.98 ± 0.01	0.47 ± 0.00
1485	Germacrene D	0.00	0.00	0.00	0.55	0.82 ± 0.01
1509	β-Bisabolene	1.69 ± 0.04	0.00	0.00	1.20 ± 0.01	0.00
1524	δ-Cadinene	0.00	0.00	0.00	0.69 ± 0.00	0.00
1573	Caryophyllene oxide	0.00	0.00	0.00	1.76 ± 0.01	0.00
1576	Sphatulenol	**3.26 ± 0.07**	3.06 ± 0.03	0.00	1.91 ± 0.00	0.00
1949	(E)-Phytol	1.93 ± 0.05	0.00	0.00	0.00	0.00

[a] Bold numbers denote major compounds in chemical profiles.
[b] An (*Tagetes filifolia* L.), Su (*Tagetes minuta* L.), Ba (*Laurus nobilis* L.), OCom (*Origanum vulgare* L. sspp. *Vulgare)* and OMen (*Origanum x majoricum*).

in Argentina were described to have a great proportion of thymol and trans-sabinene hydrate in previous studies (Dambolena et al., 2010).

3.2. Antifungal activity

The results of the antifungal activity screening are shown in Table 2. All of the studied compounds (An, Su, Ba, OCom, OMen and Ip) showed a positive effect against *S. cepivorum* (Berk). The synthetic fungicide (Ip) showed the best antifungal activity with an MFC and MIC of 1 and 0.65 μL/L, respectively. All studied EOs were able to inhibit the growth of the fungus. The strongest antifungal was shown by OMen with an MFC of 300 μL/L and an MIC of 150 μL/L. Oregano "Compacto" had an MFC and MIC of 800 and 525 μL/L, respectively. This EOs showed a substantially lower activity than OMen. Despite the similar composition between both EO, the difference is related to a higher content of thymol in OMen.

Table 2
Antifungal activities of the essential oil and iprodione against *Sclerotium cepivorum* Berk.

	Essential Oils					Fungicide
	An[a]	Su[a]	Ba[a]	OCom[a]	OMen[a]	Ip[a]
MFC[b]	600	700	600	800	300	1.00
MIC[b]	310	480	350	490	150	0.58
R^2	0.96	0.72	0.91	0.93	0.92	0.97

[a] An (*Tagetes filifolia* L.), Su (*Tagetes minuta* L.), Ba (*Laurus nobilis* L.), OCom (*Origanum vulgare* L. sspp. *Vulgare)*, OMen (*Origanum x majoricum)*and Ip (iprodione).
[b] Mean values ($n = 3$) of MFC (Minimum Fungicidal Concentration) and MIC (Minimum Inhibitory Concentration) for each compound. Values expressed in μL/L.

Table 3
Effects of essential oils combined with iprodione against *Sclerotium cepivorum* Berk.

Compound A		Compound B	Percentages of Growth Inhibition						Additive method	Abbott method	
Essential oil[a]	Concentration[b]	Ip Concentration[b]	Pure compounds		Mixture				Effect	AI	Effect
			Observed A	Observed B	Observed[c]		Expected[c]				
OCom	20	20	9.62	22.41	9.44	B	31.85	a	Antagonistic	0.32	Antagonistic
OCom	40	20	28.96	22.41	24.63	B	47.04	a	Antagonistic	0.55	Additive
OCom	60	20	37.04	22.41	38.33	B	60.74	a	Antagonistic	0.75	Additive
OCom	80	20	61.85	22.41	57.04	B	79.44	a	Antagonistic	0.81	Additive
OCom	20	40	9.62	33.33	25.56	B	58.89	a	Antagonistic	0.64	Additive
OCom	40	40	28.96	33.33	32.96	B	66.30	a	Antagonistic	0.63	Additive
OCom	60	40	37.04	33.33	43.33	B	76.67	a	Antagonistic	0.75	Additive
OCom	80	40	61.85	33.33	64.81	B	98.15	a	Antagonistic	0.87	Additive
OCom	20	60	9.62	46.85	35.37	B	82.22	a	Antagonistic	0.68	Additive
OCom	40	60	28.96	46.85	51.67	B	98.52	a	Antagonistic	0.83	Additive
OCom	60	60	37.04	46.85	67.22	B	100.00	a	Antagonistic	1.01	Additive
OCom	80	60	61.85	46.85	75.56	B	100.00	a	Antagonistic	0.95	Additive
OCom	20	80	9.62	64.63	66.11	B	100.00	a	Antagonistic	0.97	Additive
OCom	40	80	28.96	64.63	79.26	B	100.00	a	Antagonistic	1.06	Additive
OCom	60	80	37.04	64.63	98.15	A	100.00	a	Additive	1.26	Additive
OCom	80	80	61.85	64.63	100.00	A	100.00	a	Additive	1.16	Additive
OMen	20	20	11.48	22.41	29.81	B	33.89	a	Antagonistic	0.95	Additive
OMen	40	20	27.04	22.41	45.83	A	49.45	a	Additive	1.06	Additive
OMen	60	20	49.44	22.41	78.61	A	78.85	b	Additive	1.29	Additive
OMen	80	20	77.04	22.41	100.00	A	99.45	a	Additive	1.22	Additive
OMen	20	40	11.48	33.33	33.89	B	44.81	a	Antagonistic	0.83	Additive
OMen	40	40	27.04	33.33	55.56	B	60.37	a	Antagonistic	1.08	Additive
OMen	60	40	49.44	33.33	78.33	A	82.77	a	Antagonistic	1.18	Additive
Omen	80	40	77.04	33.33	100.00	A	100.00	a	Additive	1.18	Additive
OMen	20	60	11.48	46.85	54.44	A	58.33	a	Additive	1.03	Additive
OMen	40	60	27.04	46.85	77.50	A	73.89	a	Additive	1.27	Additive
OMen	60	60	49.44	46.85	100.00	A	96.29	a	Additive	1.37	Additive
OMen	80	60	77.04	46.85	100.00	A	100.00	a	Additive	1.14	Additive
OMen	20	80	11.48	64.63	69.17	B	76.11	a	Antagonistic	1.01	Additive
OMen	40	80	27.04	64.63	82.50	B	91.67	a	Antagonistic	1.11	Additive
OMen	60	80	49.44	64.63	100.00	A	100.00	a	Additive	1.22	Additive
OMen	80	80	77.04	64.63	100.00	A	100.00	a	Additive	1.09	Additive
An	20	20	3.33	22.41	8.70	B	25.70	a	Antagonistic	0.35	Antagonistic
An	40	20	25.93	22.41	12.59	B	48.3	a	Antagonistic	0.3	Antagonistic
An	60	20	59.44	22.41	22.96	B	81.8	a	Antagonistic	0.34	Antagonistic
An	80	20	69.81	22.41	32.59	B	92.2	a	Antagonistic	0.43	Antagonistic
An	20	40	3.33	33.33	43.70	A	36.7	b	Synergistic	1.23	Additive
An	40	40	25.93	33.33	53.15	A	59.3	a	Additive	1.05	Additive
An	60	40	59.44	33.33	58.52	B	92.8	a	Antagonistic	0.80	Additive
An	80	40	69.81	33.33	68.33	B	100.00	a	Antagonistic	0.86	Additive
An	20	60	3.33	46.85	58.70	A	50.2	b	Synergistic	1.21	Additive
An	40	60	25.93	46.85	81.00	A	72.8	b	Synergistic	1.10	Additive
An	60	60	59.44	46.85	100.00	A	100.00	a	Additive	0.97	Additive
An	80	60	69.81	46.85	100.00	A	100.00	a	Additive	0.96	Additive
An	20	80	3.33	64.63	66.11	A	68.00	a	Additive	1.00	Additive
An	40	80	25.93	64.63	78.70	B	90.60	a	Antagonistic	1.07	Additive
An	60	80	59.44	64.63	92.78	B	100.00	a	Antagonistic	1.08	Additive
An	80	80	69.81	64.63	100.00	A	100.00	a	Additive	1.12	Additive
Su	20	20	2.59	22.41	63.89	A	25.00	b	Synergistic	2.62	synergistic
Su	40	20	7.04	22.41	76.94	A	29.45	b	Synergistic	2.76	synergistic
Su	60	20	23.33	22.41	97.41	A	45.74	b	Synergistic	2.40	synergistic
Su	80	20	55.56	22.41	100.00	A	77.97	b	Synergistic	1.53	synergistic
Su	20	40	2.59	33.33	76.94	A	35.92	b	Synergistic	2.19	synergistic
Su	40	40	7.04	33.33	78.89	A	40.37	b	Synergistic	2.07	synergistic
Su	60	40	23.33	33.33	99.07	A	56.66	b	Synergistic	2.03	synergistic
Su	80	40	55.56	33.33	100.00	A	88.89	b	Synergistic	1.42	Additive
Su	20	60	2.59	46.85	93.89	A	49.44	b	Synergistic	1.95	synergistic
Su	40	60	7.04	46.85	99.26	A	53.89	b	Synergistic	1.96	synergistic
Su	60	60	23.33	46.85	100.00	A	70.18	b	Synergistic	1.69	synergistic
Su	80	60	55.56	46.85	100.00	A	100.00	a	Additive	1.31	Additive
Su	20	80	2.59	64.63	97.67	A	67.22	b	Synergistic	1.49	Additive
Su	40	80	7.04	64.63	98.89	A	71.67	b	Synergistic	1.47	Additive
Su	60	80	23.33	64.63	100.00	A	87.96	b	Synergistic	1.37	Additive
Su	80	80	55.56	64.63	100.00	A	100.00	a	Additive	1.19	Additive
Ba	20	20	2.96	22.41	11.85	B	25.37	a	Antagonistic	0.48	Antagonistic
Ba	40	20	11.3	22.41	35.56	A	33.71	a	Additive	1.14	Additive
Ba	60	20	30.74	22.41	36.67	B	53.15	a	Antagonistic	0.79	Additive
Ba	80	20	62.59	22.41	38.52	B	85.00	a	Antagonistic	0.54	Additive
Ba	20	40	2.96	33.33	35.56	A	36.29	a	Additive	1.01	Additive
Ba	40	40	11.3	33.33	52.78	A	44.63	b	Synergistic	1.29	Additive
Ba	60	40	30.74	33.33	63.52	A	64.07	a	Additive	1.18	Additive
Ba	80	40	62.59	33.33	76.85	B	95.92	a	Antagonistic	1.02	Additive

Table 3 (*Continued*)

Compound A		Compound B	Percentages of Growth Inhibition						Additive method	Abbott method	
Essential oil[a]	Concentration[b]	Ip Concentration[b]	Pure compounds		Mixture				Effect	AI	Effect
			Observed A	Observed B	Observed[c]		Expected[c]				
Ba	20	60	2.96	46.85	63.33	A	49.81	b	Synergistic	1.31	Additive
Ba	40	60	11.3	46.85	72.04	A	58.15	b	Synergistic	1.36	Additive
Ba	60	60	30.74	46.85	75.37	A	77.59	a	Additive	1.19	Additive
Ba	80	60	62.59	46.85	92.78	B	100.00	a	Antagonistic	1.16	Additive
Ba	20	80	2.96	64.63	77.59	A	67.59	b	Synergistic	1.18	Additive
Ba	40	80	11.3	64.63	94.63	A	75.93	b	Synergistic	1.38	Additive
Ba	60	80	30.74	64.63	100.00	A	95.37	a	Additive	1.32	Additive
Ba	80	80	62.59	64.63	100.00	A	100.00	a	Additive	1.15	Additive

[a] An (*Tagetes filifolia* L.), Su (*Tagetes minuta* L.), Ba (*Laurus nobilis* L), OCom (*Origanum vulgare* L. sspp. *Vulgare*), OMen (*Origanum x majoricum*)and Ip (iprodione).
[b] Concentration expressed as percentages of their minimum fungicidal concentrations.
[c] The same letter in the row means that there are not significant differences at $\alpha = 0.05$ ($n = 3$, DGC test).

This monoterpene has been tested against other fungi showing a strong activity (Marei et al., 2012). Several studies indicate that thymol is an antifungal agent and is the main component associated with the bioactivityof these EOs (Asensio et al., 2015; Sokovic et al., 2002). An and Ba had a similar activity against *S. cepivorum* and their MFCs was 600 μL/L. However, their MICs were 350 and 450 μL/L, respectively. The antifungal activity of An can be associated with the presence of anethole as the major constituent. Several studies mentioned that this phenylpropanoid has inhibitory effects on the growth of yeast and bacteria (Fujita et al., 2007; Fujita and Kubo, 2004). The monoterpene 1.8-cineole is a component with antifungal activity present in great proportion in Ba. Previous studies reported an MFC of 1200 μL/L of this monoterpene against *S. cepivorum* (Lucini et al., 2006). This fact evidences the synergistic action taking place between the EO compounds in comparison with one compound tested alone. Su had a high MFC of 700 μL/L and an MIC of 450 μL/L. A sample of this EO with a similar chemical profile was tested against *Aspergillus flavus* and *Penicillium* sp. and showed antifungal activity because of a strong oxidation effect on membrane lipids (Camiletti et al., 2014).

3.3. Essential oils and Iprodione combined effects study

To explore the possibility of reducing fungicides doses, a synergism study was performed. The objective was to find an effective combination of EOs and Ip against *S. cepivorum*. A total of 80 combinations were tested: 20, 40, 60 or 80% of the MFC of each EO was combined with 20, 40, 60 or 80% of the MFC of Ip. Synergism, antagonism or additive effect was determined using two different methods: the Additive method and the Abbott method (Table 3). Three EOs (An, Su and Ba) showed synergistic effects with Ip. Su EO showed a remarkable synergistic effect; 14 combinations of different concentrations of this EO with Ip showed this effect. The mixture of 80% Su MFC and 20% Ip MFC showed 100% inhibition. Synergistic effects were also observed when Ba or An were combined with Ip (3 and 5 combinations, respectively). Moreover, when 40% of An MFC was mixed with 60% of Ip MFC, the inhibition percentage reached 81%. The combination of 80% Ip MFC and 40% Ba MFC showed even greater inhibition (94.63%). On the contrary, only antagonistic or additive effects were observed for combinations of OCom or OMen with the synthetic fungicide iprodione. Synergistic interactions between natural products and chemical fungicides remarkably reduce the MFCs of each compound. These combinations can be used as a part of modern integrated pest management strategies in order to reduce ecological impacts of pesticides (Thompson and Kreutrweiser, 2007). Previous works have shown the interactions between essential oils and antifungal drugs when evaluating alternative therapies in medicine (Amber et al., 2010; Pereira et al., 2014). Other authors (Khan and Ahmad, 2011) reported that the EOs of *Cinnamomum verum* and *Syzygium aromaticum* had synergistic interactions with medicinal fungicides against *Aspergillus fumigatus*. However, essential oils in combinations with agricultural fungicides have not yet been investigated.

Table 4
Effect of the five essential oils on root and leaf elongation of *Allium sativum* L.

Essential Oils	Inhibition[b]	
	Roots length (%)	Leaves length (%)
An[a]	15.19 ± 0.72 a	30.90 ± 0.47 a
Su[a]	47.25 ± 0.40 b	53.73 ± 0.32 b
Ba[a]	22.78 ± 0.36 a	29.68 ± 0.79 a
OCom[a]	100.00 ± 0.00 c	100.00 ± 0.00 c
OMen[a]	99.16 ± 0.12 c	100.00 ± 0.00 c

[a] An (*Tagetes filifolia* L.), Su (*Tagetes minuta* L.), Ba (*Laurus nobilis* L), OCom (*Origanum vulgare* L. sspp. *Vulgare)* and OMen (*Origanum x majoricum*).
[b] Data is expressed as mean ± standard deviation ($n = 3$). The same letter in the column means that there are not significant differences between treatments at $\alpha = 0.05$ ($n = 3$; DGC test).

3.4. Phytotoxicity assays

3.4.1. Garlic germination study

Phytotoxic effects on roots and leaves of EOs are shown in Table 4. NPEOs were considered those that inhibit the growth of roots and leaves by less than 50%. Significant differences were found in the length of roots and leaves when they were exposed to EOs. The lowest inhibitions in radicle and leaves elongation were observed when garlic cloves were treated with An and Ba (15.19% and 30.0% and 22.78% and 29.68%, respectively). The growth of roots and leaves was partially inhibited in treatments with Su. On the other hand, OCom and OMen inhibited completely roots and foliar growth, presuming toxic effects on garlic. Some terpenes usually show a suppressing effect on germination and seedling growth. The toxic effect of OCom and OMen can be attributed to their main components, thymol and carvacrol, which have been documented as potential herbicides against various plant species (Kordali et al., 2008; Pérez-Vásquez et al., 2008).

3.4.2. Garlic yield

The effect of NPEOs on the dry weight of garlic cloves was analyzed. Control sample weighted an average of 3.88 g whereas An 600 (μL/L), An 900 (μL/L), Ba 600 (μL/L) and Ba 900 (μL/L) cloves weighted 3.87 g, 3.66 g, 3.75 g and 3.41 g, respectively. No significant differences were found between NPEOs treatments and the control sample. Similar results were observed when garlic yield was calculated. Control treatment had a yield of 7833.39 (±195.81) (kg/ha). There were no significant differences in garlic yield (kg/ha) between control and EOs treatments. These results confirm that

Table 5
Effect of the essential oils and their combinations with iprodione on *Sclerotium cepivorum* Berk sclerotia density and in white rot incidence.

Compound A		Compound B	Sclerotia density[c]			Disease Incidence
Essential Oil[a]	Concentration[b]	Ip concentration[a,b]	Initial	Final	Slope[e]	ABDPC[d,e]
Control	–	–	20 ± 1.00	656.80 ± 44.63	4.25 a	3427.69 ± 473.51 a
Ip	–	0.6	14.67± 1.55	732.00 ± 39.65	4.78 a	2835.73 ± 79.12 a
An	600	0	8.0 ± 0.50	425.47 ± 35.70	2.78 b	2552.01 ± 176.72 b
An	900	0	23.0 ± 2.08	460.13 ± 54.60	2.92 b	2838.04 ± 204.16 b
Ba	600	0	17.0 ± 1.00	441.33 ± 35.90	2.83 b	2469.15 ± 39.55 b
Ba	900	0	24.00 ± 2.83	286.5 ± 10.19	1.95 c	2578.06 ± 243.24 b
An	600	0.6	35.0 ± 5.00	366.6 ± 33.50	2.21 c	2388.37 ± 84.61b
An	900	0.6	30.67 ± 7.02	295.0 ± 15.00	1.76d	1882.99 ± 79.05 c
Ba	600	0.6	31.0 ± 1.00	372.4 ± 22.60	2.28 c	3377.23 ± 72.63 b
Ba	900	0.6	30.0 ± 6.00	298.4 ± 8.61	1.79d	1695.93 ± 98.55 c

[a] Control (without essential oil), An (*Tagetes filifolia* L.), Ba (*Laurus nobilis* L) and Ip (iprodione).
[b] 600 = MFC; 900 = MFC + 50% of this dose; 0.6 = 60% of iprodione MFC. Concentrations expressed as μL/L.
[c] Means and standard deviations($n = 3$) of sclerotia density per 100 g of soil sample.
[d] Means and standard deviations ($n = 3$) of areas below the disease progress curve (ABDPC).
[e] The same letter in the column means that there are not significant differences between treatments at $\alpha = 0.05$ ($n = 3$; DGC test).

there was no phytotoxic effect influenced by An and Ba Eos on plants and garlic production.

3.5. Field study

Differences between the initial and final sclerotia density in each treatment were calculated. Significant differences within the slopes (β1) of the lineal regression of each treatment equation were analyzed (Table 5). The treatment Ip (0.6 μL/L Ip) was no different from the control treatment, showing the highest slopes. This result confirmed that there was no effect of the synthetic fungicide on the production of sclerotia by the fungi. Miñambres et al. (2010) found that Ip had no effect on soil sclerotia density. Similar effects were observed for An (600 and 900 μL/L) and Ba (600 μL/L); no significant differences were found between these samples. According to their slopes, moderate sclerotia inhibition was shown by An and Ba (600 μL/L) when combined with Ip (0.6 μL/L). However, the best sclerotia inhibition was observed in the combinations of An or Ba (900 μL/L) with Ip 0.6 μL/L. These combinations not only showed the lowest slopes of sclerotia production but also confirm the synergistic effect of these EOs with Ip.

In addition, the antifungal effect of each treatment on white rot progress was evaluated by calculating area below the disease progress curve (ABDPC) (Table 5). Significant differences were found in ABDPC for all treatments ($p < 0.05$). The highest values observed were for the control and Ip samples. The combinations Ip with An (0.6 μL/L + 900 μL/L) and Ip with Ba (0.6 μL/L + 900 μL/L) had the lowest ABDPC values, being significantly different to the rest of the samples. All other treatments showed intermediate ABDPC values. Our results are in accordance with those observed by Zewide et al. (2007), who reported lower ABCDPC and sclerotia density in garlic crops treated when a synthetic fungicide was applied to the soil.

4. Conclusion

The management of plant diseases with natural compounds is highly needed nowadays. The results of this study evidence that the antifungal power of non-phytotoxic EOs can be used to reduce doses of synthetic compounds that are applied to garlic crop. Anisillo and bay EOs have both moderate antifungal activity but no phytotoxic effect. Moreover, some combinations of these EOs with Ip can reduce the fungicide dose by up to 40% due to the synergistic interactions taking place. Therefore, these EOs are a novel alternative to control "white rot" caused by *S. cepivorum* and can be applied with Ip not only to reduce the amount of chemical fungicide in the soil, but also to reduce the disease incidence and the sclerotia density after white rot disease. Moreover, the persistence of fungicides and the amount of money spent on them can be reduced. Future studies are needed to adjust the applied concentrations.

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