Journal of Plant Protection Research ISSN 1427-4345

ORIGINAL ARTICLE

Combinations of *Tagetes filifolia* Lag. essential oil with chemical fungicides to control *Colletotrichum truncatum* and their effects on the biocontrol agent *Trichoderma harzianum*

Laura Carolina Gadban[1], Boris Xavier Camiletti[2,3]*, Ezequiel Darío Bigatton[2], Silvia Graciela Distéfano[1], Enrique Iván Lucini[2]

[1] Estacioń Experimental Agropecuaria Marcos Juárez/Laboratorio de Fitopatologia, Instituto Nacional de Tecnología Agropecuaria (INTA), Córdoba, Argentina

[2] Microbiologia Agricola, Facultad de Ciencias Agropecuarias-Universidad nacional de Cordoba (UNC), Córdoba, Argentina

[3] Instituto Multidisciplinario de Biología Vegetal (IMBIV), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Córdoba, Argentina

Vol. 60, No. 1: 41–50, 2020

DOI: 10.24425/jppr.2020.132202

Received: June 17, 2019
Accepted: August 7, 2019

*Corresponding address:
bcamiletti@agro.unc.edu.ar

Abstract

Soybean [*Glycine max* (L.)], one of the most important crops in Argentina, is commonly infected by *Colletotrichum truncatum*, the causal agent of anthracnose. *Tagetes filifolia* essential oil (EO) is presented as a natural approach to minimize the dose of chemical fungicides applied to the crop. The fungus *Trichoderma harzianum* is used as a biocontrol agent because of its ability to produce secondary metabolites that destroy cell walls of phytopathogenic fungi. However, its performance can be affected when it is exposed to chemical fungicides. The objective of this work was to evaluate the antifungal activity of *T. filifolia* EO both individually and combined with chemical fungicides against *C. truncatum,* and its effect on *T. harzianum*. Fungi were isolated from soybean crops. The following pesticides were assessed: carbendazim (F1), difenoconazole (F2) and trifloxystrobin + cyproconazole (F3). The EO was obtained from native plants and its chemical composition was analyzed by gas chromatography–mass spectrometry (GC–MS). The minimum fungicide concentration (MFC) was determined for each compound. Fungicides were combined with the EO to look for combinations that allowed a reduction of pesticide doses. Among fungicides, F1 showed the strongest antifungal activity against *C. truncatum* (MFC = 0.25 µl · l^{-1}) and *T. harzianum* (MFC = 1.5 µl · l^{-1}). The sensitivity of both fungi to the EO was lower than to fungicides. The EO presented MFCs of 6,000 and 9,000 µl · l^{-1} against *C. truncatum* and *T. harzianum*. The EO and F1 affected the growth of *T. harzianum* at concentrations that controlled *C. truncatum* (31 and 10%). Eight combinations of fungicides and the EO allowed fungicide concentration reductions of up to 80%, although the growth of the biocontrol strain was also affected. The results demonstrated that *T. filifolia* EO can be used to control anthracnose and reduce doses of chemical fungicides applied to soybean crops. Its effect on *T. harzianum* should be considered in the design of integrated pest management strategies.

Keywords: anthracnose, integrated pest management, natural control, soybean

Introduction

Soybean [*Glycine max* (L.)] is widely cultivated in many countries due to its well-developed agronomic performance, characterized by a great protein yield per unit area (Vollmann 2016). In Argentina, 20.5 million hectares of this commodity were sown during the 2016–2017 growing season. Soybean reached an overall production of 70.2 million tons, representing 31% of total exports of the country (Phélinas and

Choumert 2017). Soybean production is highly affected by late season diseases (LS), a combination of several pathogens that may cause yield and productivity losses, as well as seed quality changes (Carmona *et al.* 2015). Anthracnose, mainly caused by *Colletotrichum truncatum,* is one of the most prevalent LS in Argentinean soybean production (Ramos *et al.* 2013). Infections with this fungus are characterized by twisted and aborted pods (Ramos *et al.* 2013; Carmona *et al.* 2015; Dias *et al.* 2016).

The use of chemical fungicides is a common practice employed in the control of soybean diseases on a global scale (Carmona *et al.* 2011; Carmona *et al.* 2015; Dias *et al.* 2016). Several synthetic fungicides that belong to benzimidazoles, strobilurins and triazoles groups have been extensively used in the field to control *C. truncatum* (Gawade *et al.* 2009; Carmona *et al.* 2011; Torres-Calzada *et al.* 2015). However, it is increasingly frequent to find isolates that are less sensitive or even resistant to these fungicides (Fernandez-Ortuño *et al.* 2012; Torres-Calzada *et al.* 2015). Triazole resistance among *Aspergillus* and *Candida* species has been well documented (Goncalves *et al.* 2016). Furthermore, chemical residues from fungicides can exhibit low degradability, remaining in soil and water for years. These are some of the reasons why chemical treatment is recommended as part of an integrated fungal control program (Sosa-Gomez *et al.* 2003).

Essential oils (EOs) are complex mixtures of volatile components synthesized as secondary metabolites by aromatic plants in order to protect themselves against infectious diseases (Isman 2000). These natural extracts may have numerous components, although in general, a few major compounds determine their biological properties (Isman 2000; Bakkali *et al.* 2008). Essential oils are easily degraded by non-target microorganisms, and are considered safe for humans and animals (Isman 2000). Numerous EOs, as well as their individual components, have been characterized by their antifungal activities against several plant pathogens (Abbaszadeh *et al.* 2014; Camiletti *et al.* 2014; Camiletti *et al.* 2016). Thus, the application of EOs has become a very attractive approach for the control of several phytopathogenic fungi (Omidbeygi *et al.* 2007; Bluma *et al.* 2008; Abdel-Kader *et al.* 2011; Camiletti *et al.* 2014; Camiletti *et al.* 2016). Generally, the sensitivity of fungal species to EOs is lower than to chemical fungicides. Therefore, EOs are combined with synthetic compounds in order to improve their performance, as well as to reduce pesticide doses (Pyun and Shin 2006; Lim and Shin 2008; Camiletti *et al.* 2016). To reach this objective, the antifungal activity of EOs is frequently investigated in combination with chemical pesticides in search of synergistic interactions (Shin and Kang 2003; Pyun and Shin 2006; Pavela 2014). However, additive or antagonistic interactions may also appear when two antimicrobial substances are combined (Kosman and Cohen 1996). In previous studies, *Tagetes filifolia* EO was successfully combined with a synthetic fungicide to control *Sclerotium cepivorum* in garlic crops (Camiletti *et al.* 2016).

Species of the fungal genera *Trichoderma*, characterized by antagonistic properties against phytopathogenic fungi, are commonly used to control plant diseases. The success of *Trichoderma* species is mainly due to its ability to colonize different substrates, its effectiveness for nutrient use and strong aggressiveness against phytopathogenic fungi (Benítez *et al.* 2004). The most common strains utilized as biocontrol agents belong to the species *Trichoderma harzianum* (Benítez *et al.* 2004; Sushir *et al.* 2015; Ganuza *et al.* 2017). This fungus destroys cell walls of phytopathogenic fungi by production of lytic enzymes and secondary metabolites (Grondona *et al.* 1997; Sharma *et al.* 2017). *Trichoderma harzianum*, an ubiquitous species present in crops, soil, and debris, may act as a biocontrol agent against ascomycetous, deuteromycetous and basidiomycetous fungi (Benítez *et al.* 2004).

Integrated pest management commonly involves biological, physical and chemical strategies (Abrol and Shankar 2016). Some EOs have been successfully combined with biocontrol agents and constitute an applicable method for controlling plant diseases (Arrebola *et al.* 2010; Abdel-Kader *et al.* 2011). On the other hand, some antifungal compounds inhibit the growth of biocontrol microorganisms and may affect its performance (Sarkar *et al.* 2010). The objective of this work was to evaluate the antifungal activity of *T. filifolia* EO, both individually and combined with chemical fungicides, against *C. truncatum* and their effects on the biocontrol agent *T. harzianum*.

Materials and Methods

Plant material and essential oil extraction

Tagetes filifolia plants were grown in 3-liter pots containing soil collected from the experimental station of the National Institute of Agricultural Technology (INTA) in Marcos Juarez (Córdoba, Argentina). Pots were placed in a greenhouse where the temperature ranged from 25 to 28°C. Developing plants were transferred to field plots at the grown stage of two detectable nodes. Plants were watered every 7 days and harvested when 50% of the plants had at least one corolla open. Aerial parts were dried in a forced air oven at 60°C for 4 days and hydrodistilled for 2 h in a Clevenger-type apparatus coupled to a separated extraction chamber (Asensio *et al.* 2011). The essential oil was kept in dark flasks at –20°C and the yield was determined by measuring the collected volume per plant weight.

Essential oil chemical composition

The essential oil composition was determined by CG-MS in a Perkin Elmer Clarus 600 chromatograph coupled with an ion trap mass detector. Compounds were separated using a capillary column DB-5 (30 m, 0.25 mm internal diameter, and 0.25-mm coating thickness) and helium as the carrier gas with a flow rate of 0.9 ml · min^{-1}. Ionization was performed by electron impact at 70 eV and the mass spectral data were obtained in the scan mode in the m/z range 35 to 450. The temperature program was settled at 60°C for 5 min and from 60 to 250 at a rate of 5°C/min (Asensio *et al*. 2011). The compounds were identified by contrasting their retention time and mass spectra with published data (Adams 1995) and NIST libraries (version 3.0). Quantitative analysis was performed using the area normalization method and the results were expressed as relative percentages (Asensio *et al*. 2011).

Fungal isolates

Seeds, pods and stems that looked infected by *C. truncatum* were collected from a soybean crop cultivated in the Marcos Juarez area at the end of the growing season, immediately before harvest. Samples were surface-disinfected by dipping in ethanol solution (70%) and then washed three times in sterile water. Plant material was placed on Petri plates (9 cm) previously prepared with sterile moistened paper. Samples were placed in a culture chamber at 25 ± 1°C for 4 days, alternating 12 h of light with 12 h of darkness. *Colletotrichum* acervuli were separated and examined under a stereo microscope. Spores of *C. truncatum* were identified according to morphological characteristics (Armostrong-Cho and Banniza 2006), transferred to Petri plates containing potato dextrose agar (Britania, Buenos Aires, Argentina) and incubated as previously described. To confirm identity, Petri plates were prepared with sterile soybean stems and inoculated with mycelium from a 7-day culture (Dhingra and Sinclair 1995). Samples were again incubated as described above. After incubation, acervuli were separated and the morphological characteristics of the spores were compared with those indicated for *C. truncatum* (Manandhar and Hartman 1999; Armostrong-Cho and Banniza 2006). *Trichoderma harzianum* was isolated from soil samples collected from the same soybean crop. Soil samples (10 g) were suspended in physiological saline solution. Serial dilutions were prepared and seeded on Petri plates containing selective medium for *Trichoderma* (Dhingra and Sinclair 1995). Samples were incubated under the same conditions as *C. truncatum.* Isolates characterized as *T. harzianum* were sub-cultured on potato dextrose agar and incubated as previously described. The identity of the isolates was confirmed following the taxonomic schemes described by Rifai (1969). The distinctive features of *T. harzianum* are conidiophores which are compactly branched in a pyramidal shape, the base being highly branched, and the apex usually bearing a solitary phialide. Conidia are less than 3.5 mm long, nearly spherical and smooth-walled (Rifai 1969).

Chemical fungicides

Fungicides included in this study were selected based on their current use to control anthracnose caused by *C. truncatum* in Argentinean soybean crops. Commercial formulations were purchased from local suppliers (Table 1). These products were used instead of active ingredients in an attempt to obtain a more accurate predictive value during the in vitro assay. The response of the pathogens was based on the formulation employed in the field.

Antifungal activity

A broth method was used to determine the antifungal activity of the EO and fungicides against *C. truncatum* and *T. harzianum* according to previous bibliography (Lucini *et al*. 2006; Camiletti *et al*. 2014). Petri plates were prepared with 20 ml of liquid growing medium (20% v/v lixiviated potato; 2% w/v glucose; pH 4.5). The EO and fungicide solutions were added to the liquid medium according to the desired concentration. Prior to addition, the EO and fungicides were diluted in ethanol and sterile water, respectively. The final concentration of ethanol was always less than 1% v/v. A 5-mm diameter agar disc was taken from 7-day-old fungal colonies and used to inoculate selected

Table 1. Fungicides included in the assays

Code	Group	Active ingredient [% w/v]	Formulation	Class*
F1	benzimidazoles	carbendazim (50)	SC	IV
F2	triazoles	difenoconazole (25)	EC	III
F3	strobilurins + triazoles	trifloxystrobin (32) + cyproconazole (14)	SC	IV

EC – emulsifiable concentrate, SC – suspension concentrate

*toxicological categories based on Argentinean standard regulations (CASAFE 2015): IV – product that normally does not represent danger, III – low dangerous product

treatments. Petri plates without the addition of an antifungal agent were included as a control. All treatments were incubated in a culture chamber for 7 days at 25 ± 1°C alternating 12 h of light with 12 h of darkness. After incubation, the diameter of the mycelium was measured to calculate the percentage of growth inhibition (*PGI*) using the following equation (Camiletti *et al.* 2014):

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

where: C and T are the mean values ($n = 3$) of hyphal extension (mm) obtained for the control and treatments with antifungal agents, respectively. The lowest concentration of active ingredient or EO in which the *PGI*s were equal to 100% represented the minimum fungicidal concentration (*MFC*) values (Abbaszadeh *et al.* 2014; Camiletti *et al.* 2014).

Mixture effects

The broth method was also utilized to determine the interaction of different concentrations of fungicides with the EO. The selected treatments were composed of all possible combinations between 20, 40, 60 or 80% of the previously determined *MFC* of EO and 20, 40, 60 or 80% of the previously determined *MFC*s of fungicides (Pyun and Shin 2006; Camiletti *et al.* 2016). Both the EO and the fungicide were diluted as described above and added separately to the culture medium. Petri plates were gently shaken before inoculation to enhance uniformity. All treatments were incubated in a culture chamber under the same conditions. After incubation, data expressed as *PGI* was calculated and assessed. Because of the great number of available methodologies, the interaction of the combination was determined using three methods: the additive method, the Abbott method, and the *FIC* index. The additive and Abbott methods have been traditionally used to determine interactions among agricultural pesticides (Kosman and Cohen 1996). At present, the *FIC* index is the most recommended method to evaluate combinations of antifungal compounds (Odds 2003). In the additive method, the expected *PGI* (*APGI*) for each mixture was calculated by adding the individual *PGI* of the fungicide doses (PGI_F) and the individual *PGI* of the EO doses (PIG_{EO}). Synergism, additive or antagonistic effects were determined when the observed *PGI* (*OPGI*) was statistically higher, equal and lower than the calculated *APGI*, respectively (Camiletti *et al.* 2016). In the Abbott method, the expected *PGI* (*EPGI*) was estimated also using the PGI_F and PGI_{EO} but following the equation:

$$EPGI = PGI_F + PGI_{EO} - [(PGI_F \times PGI_{EO})/100].$$

The effect of the combinations was designated by assessing the Abbot Index (*AI*):

$$AI = OPGI/EPGI.$$

A synergistic interaction was assigned for $AI \geq 1.5$, additive for >0.5–1.4 and antagonistic for <0.5 (Kosman and Cohen 1996).

For each combination, the utilized fungicide (C_{F+EO}) and EO (C_{EO+F}) concentrations served as a basis to calculate a fractional inhibitory concentration (*FIC*) index for the mixture according to the formulas:

$$FIC_{EO} = C_{EO+F}/C_{EO},$$
$$FIC_F = C_{F+EO}/C_F,$$
$$FIC = FIC_{EO} + FIC_F,$$

where: C_{EO} and C_F are the EOs and fungicide concentrations needed to achieve the same *OPGI* when they are applied individually. Predicted values of C_{EO} and C_F were obtained from regression equations. The interaction was interpreted as synergy for $FIC < 0.5$, additive for >0.5–4.0 and antagonistic for >4.0 (Pillai *et al.* 2005; Magi *et al.* 2015; Nikkhah *et al.* 2017).

Statistical analysis

Treatments were replicated three times and data of each experiment were combined after checking for homogeneity of the experimental error variances by the F test. Means were obtained via ANOVA ($p < 0.05$). Significant differences between treatments were determined using Fisher´s protected least significant difference (LSD) test at level $p < 0.05$. The PGI data were regressed against the the log-transformed dose, and the best fit model was selected according to the significance of the regression and coefficient of determination (R^2). Regression equations were used to obtain the predicted values of the minimum inhibitory concentrations (*MIC*s; *PGI* = 50%), C_{EO} and C_F (Marei *et al.* 2012). Data analysis was performed using InfoStat software version 2014.

Results

Essential oil yield and chemical composition

The EO of *T. filifolia* was collected as a 4.0% dry weight yield and its composition was characterized by a great proportion of phenylpropanoids (Table 2). The main components were *cis*-anethole and estragole, representing 97.9% of the oil composition. P-Anisaldehyde, β-bisabolene, methyl eugenol and sphatulenol completed the chemical profile with percentages lower than 1.0%.

Table 2. Chemical profile and relative percentage of main compounds present in the *Tagetes filifolia* essential oil according to GC-MS analysis

RI[a]	*RT*[b]	Compound	Relative percentage [%]
1195	11.88	Estragole	22.11 ± 2.9
1252	12.55	p-Anisaldehyde	0.49 ± 0.3
1289	12.93	Anethole	75.10 ± 5.7
1401	13.89	Methyl eugenol	0.55 ± 0.2
1509	14.85	β-Bisabolene	0.69 ± 0.2
1576	15.59	Sphatulenol	0.26 ± 0.1
Total			99.20

[a]*RI* – retention index, [b]*RT* – retention time

Antifungal activity

The results of the antifungal activity screening are presented in Table 3. All the studied compounds (F1, F2, F3 and the EO) had the capacity to completely inhibit the growth of *C. truncatum* and *T. harzianum*. When the fungi were compared, *T. harzianum* showed a remarkably higher resistance than *C. truncatum* to all four antifungal agents. The fungicide F1 showed the strongest antifungal activity against both fungi, with the lowest being *MIC* and *MFC*, followed by F2 and F3. Surprisingly, the sensitivity of the pathogen to F1 decreased at concentrations higher than the *MFC* (Fig. 1). The chemical fungicide F1 also affected the growth of *T. harzianum*, 10% on average, at the same doses that controlled *C. truncatum* (Fig. 1). The chemical fungicide F2 did not show an antifungal effect on *T. harzianum* when it was evaluated at the same concentration that controlled *C. truncatum*. In fact, the inhibitory effect against the biocontrol fungus started at a concentration of 10 $\mu l \cdot l^{-1}$. The fungicide F3 presented the lowest antifungal activity among the chemical pesticides, showing the highest *MFC* against *C. truncatum*. Furthermore, growth inhibition of *T. harzianum* by this pesticide was not observed up to concentrations higher than 25 $\mu l \cdot l^{-1}$. The sensitivity of *C. truncatum* to the EO was lower than to chemical fungicides. Furthermore, doses of EO that partially inhibited the growth of *C. truncatum* also affected mycelial development of *T. harzianum* (Fig. 2). For example, 4,000 $\mu l \cdot l^{-1}$ of EO showed a *PGI* of 50 and 21% against *C. truncatum* and *T. harzianum*, respectively.

Mixture effects

The synergism assay was carried out to find effective combinations of fungicides and EO that allow a reduction of the pesticide dose required to control *C. truncatum*. The objective was to find combinations

Table 3. Minimum fungicidal concentration of chemical compounds and *Tagetes filifolia* essential oil against *Colletotrichum truncatum* and *Trichoderma harzianum*

Fungi	Essential oil[a]		Fungicides[b]					
	Tagetes filifolia		F1		F2		F3	
	MIC[c]	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*
Colletotrichum truncatum	4,000	6,000	0.13	0.25	0.35	1.0	0.7	1.5
Trichoderma harzianum	7,400	9,000	1.10	1.50	10.00	30.0	75.0	90.0

[a]mean values ($n = 3$) expressed as $\mu l \cdot l^{-1}$ of essential oil
[b]F1 – carbendazim, F2 – difenconazole, F3 – trifloxystrobin + cyproconazole. Mean values ($n = 3$) expressed as $\mu l \cdot l^{-1}$ of active ingredient
[c]*MIC* – minimum inhibitory concentration, *MFC* – minimum fungicidal concentration

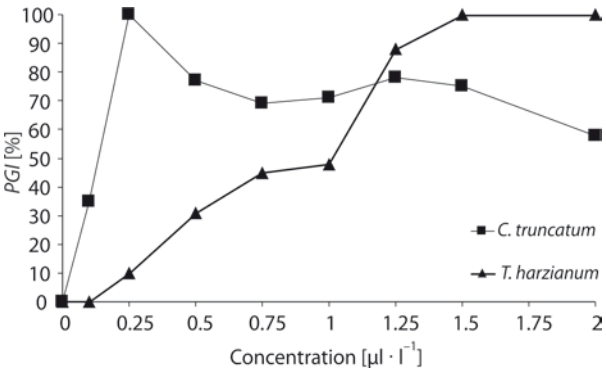

Fig. 1. Percentage of growth inhibition (*PGI*) of *Colletotrichum truncatum* and *Trichoderma harzianum* exposed to different concentrations of carbendazim

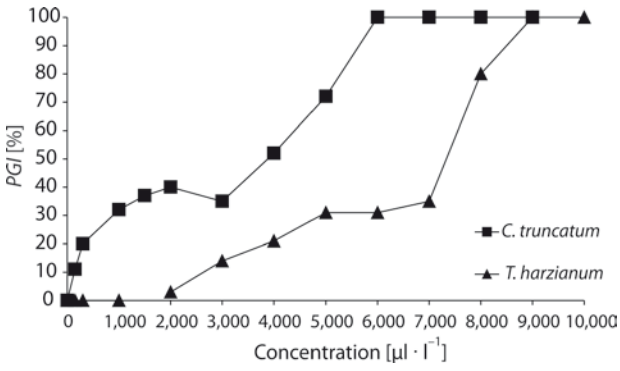

Fig. 2. Percentage of growth inhibition (*PGI*) of *Colletotrichum truncatum* and *Trichoderma harzianum* exposed to different concentrations of *Tagetes filifolia* EO

Table 4. Antifungal activity of chemical fungicides combined with *Tagetes filifolia* essential oil against *Colletotrichum truncatum* and their interactions according to the additive, Abbott and FIC methods

Compound A		Compound B	*PGI*		Additive	Abbott		*FIC*	
fungicide[a]	concentration[b]	EO concentration[b]	observed	expected	effect	index	effect	index	effect
F1	20	20	11 a	4 b	SN	2.89	SN	0,84	AD
F1	20	40	31 a	4 b	SN	8.00	SN	1.03	AD
F1	20	60	59 a	27 b	SN	2.22	SN	1.19	AD
F1	20	80	94 a	85 a	AD	1.11	AD	1.11	AD
F1	40	20	64 a	4 b	SN	16.61	SN	0.91	AD
F1	40	40	73 a	4 b	SN	18.62	SN	1.10	AD
F1	40	60	75 a	27 b	SN	2.80	SN	1.34	AD
F1	40	80	90 a	86 a	AD	1.06	AD	1.35	AD
F1	60	20	78 a	62 a	AD	1.29	AD	0.99	AD
F1	60	40	84 a	62 b	SN	1.39	AD	1.14	AD
F1	60	60	100 a	85 b	SN	1.44	AD	1.20	AD
F1	60	80	100 a	100 b	AD	1.07	AD	1.40	AD
F1	80	20	84 a	62 b	SN	1.40	AD	1.09	AD
F1	80	40	93 a	62 b	SN	1.55	SN	1.26	AD
F1	80	60	96 a	85 b	SN	1.38	AD	1.47	AD
F1	80	80	100 a	100 a	AD	1.07	AD	1.60	AD
F2	20	20	20 b	37 a	AN	0.58	AD	1.79	AD
F2	20	40	25 a	34 a	AD	0.77	AD	1.74	AD
F2	20	60	61 a	57 a	AD	1.25	AD	1.29	AD
F2	20	80	100 a	100 a	AD	1.09	AD	1.08	AD
F2	40	20	1 b	70 a	AN	0.00	AN	22.00	AN
F2	40	40	10 b	67 a	AN	0.16	AN	20.83	AN
F2	40	60	69 b	91 a	AN	0.94	AD	1.60	AD
F2	40	80	100 a	100 a	AD	1.03	AD	1.27	AD
F2	60	20	28 b	77 a	AN	0.40	AN	3.07	AD
F2	60	40	31 b	74 a	AN	0.44	AN	3.17	AD
F2	60	60	53 b	98 a	AN	0.69	AD	2.42	AD
F2	60	80	100 a	100 a	AD	1.05	AD	1.40	AD
F2	80	20	30 b	92 a	AN	0.35	AN	3.81	AD
F2	80	40	33 b	90 a	AN	0.39	AN	3.86	AD
F2	80	60	47 b	100 a	AN	0.53	AD	3.19	AD
F2	80	80	100 a	100 a	AD	1.03	AD	1.60	AD
F3	20	20	35 a	17 b	SN	2.15	SN	0.56	AD
F3	20	40	53 a	10 b	SN	5.43	SN	0.75	AD
F3	20	60	67 a	19 b	SN	3.72	SN	0.96	AD
F3	20	80	88 a	69 b	SN	1.40	AD	1.09	AD
F3	40	20	36 a	11 b	SN	3.31	SN	0.84	AD
F3	40	40	54 a	4 b	SN	14.46	SN	1.01	AD
F3	40	60	77 a	13 b	SN	6.13	SN	1.14	AD
F3	40	80	93 a	62 b	SN	1.54	AD	1.26	AD
F3	60	20	36 a	28 a	AD	1.35	AD	1.12	AD
F3	60	40	73 a	21 b	SN	3.42	SN	1.15	AD
F3	60	60	82 a	30 b	SN	2.87	SN	1.33	AD
F3	60	80	100 a	80 b	SN	1.41	AD	1.45	AD
F3	80	20	78 a	60 b	SN	1.40	AD	1.11	AD
F3	80	40	86 a	52 b	SN	1.64	AD	1.29	AD
F3	80	60	90 a	61b	SN	1.59	AD	1.49	AD
F3	80	80	100 a	100 a	AD	1.24	AD	1.60	AD

PGI – percentage of growth inhibition; *FIC* – fractional inhibitory concentration

[a]F1 – carbendazim, F2 – difenoconazole, F3 – trifloxystrobin (70%) + cyproconazole (30%)

[b]concentration expressed as percentages of their minimum fungicidal concentrations.

The same letter in the row means that there are no significant differences at $p < 0.05$ ($n = 3$; Fisher´s LSD test); SN – synergism, AD – additivity, AN – antagonism

of fungicides with *T. filifolia* EO that improved their performance and to study their effects on the growth of the biocontrol agent *T. harzianum*. A total of 48 combinations were evaluated: 20, 40, 60 or 80% of the *MFC* of each fungicide combined with 20, 40, 60 or 80% of the *MFC* of EO. Synergistic, additive or antagonistic effects were assigned based on three different methods: the additive method, the Abbott method and the *FIC* index (Table 4). Synergistic interactions were not found according to the *FIC* index, being mainly determined as an additive type. The Abbott and additive methods displayed some synergistic interactions although none of them inhibited completely the growth of *C. truncatum*. However, the EO presented a total of eight combinations with different fungicides that allowed reductions of the pesticide doses, conserving its antifungal effect. Combinations of EO with F2 showed the largest number of additive combinations controlling *C. truncatum*. In fact, the mixture of 80% EO *MFC* and 20% F2 *MFC* showed a 100% inhibition which allowed an 80% reduction of the pesticide dose. However, these antifungal agents also presented a greater number of antagonist interactions when they were combined. On the other hand, all combinations that allowed a reduction of the pesticide dose against *C. truncatum* affected the growth of *T. harzianum*. The *PGI*s averaged 31 ± 4%, in accordance with the *PGI*s observed when 60 and 80% of EO *MFC* were evaluated individually (Fig. 2).

Discussion

In Argentina, soybean crops are affected by anthracnose, causing yield and quality losses. Soybean monocropping and conservation tillage have exacerbated the severity of anthracnose, increasing the doses of fungicides applied to the crop (Carmona *et al*. 2015). The *T. filifolia* EO was characterized and investigated as a natural alternative to reduce the amount of chemical pesticides currently applied to soybean crops.

The EO yield obtained from the *T. filifolia* aerial parts was lower than those reported for plants grown in other regions of Argentina (6.3–13.1%), Mexico (6.6–16.8%) and Peru (12.1%) (Maestri *et al*. 1991; Serrato Cruz *et al*. 2003; Marotti *et al*. 2011). Although the obtained EO yield was lower than that reported in earlier literature, the chemical composition showed a similar profile to those previously reported for *T. filifolia* EO. The prevalence of *cis*-anethole and estragole was reported in EO extracted from different cultivars grown in other regions of Argentina, in agreement with our results (Maestri *et al*. 1991; Zygadlo *et al*. 1994; Camiletti *et al*. 2016). In addition, the EO obtained from aerial parts of *T. filifolia* grown in Peru was previously characterized by a similar composition (De Feo *et al*. 1998). Studies by Maestri *et al*. (1991) indicated that EO yield is related to the altitude above sea level of the plant population, acting as an adaptive response mechanism that does not influence the chemical composition of the essence.

All the studied compounds showed antifungal activity against the pathogen *C. truncatum* and the biocontrol agent *T. harzianum*. Among the chemical fungicides, F1 showed the strongest antifungal activity, followed by F2 and F3. This finding is in agreement with other research where members of the benzimidazole group needed lower concentrations to control *C. truncatum* than members of other chemical groups (Zavala León *et al*. 2005). The effectiveness of F1 to control *C. truncatum* isolates was previously demonstrated in studies that included several strains of this pathogen isolated from other crops and regions (Gawade *et al*. 2009; Dias *et al*. 2016). Moreover, Oh and Kang (2002) observed that the antifungal activity of this fungicide decreased at concentrations higher than those that completely controlled *C. truncatum,* which agrees with results obtained in this work. The active ingredients difenoconazole (F2) and cyproconazole (present in F3) were described by a similar antifungal activity against *Colletotrichum* species (Chen *et al*. 2016). Cyproconazole represented only 30% of the active ingredient in F3 and trifloxistrobin (strobilurin), the remaining 70%. In previous studies (Carmona *et al*. 2011; Rampersad *et al*. 2012; Torres-Calzada *et al*. 2015), *C. truncatum* isolates showed intermediate or higher resistance to fungicide members of the chemical group strobilurins, explaining the lower inhibitory effect of F3. In fact, F2 and F3 did not affect the growth of *T. harzianum* at the same concentration that controlled the pathogen as was observed for F1. This biocontrol agent was investigated by its sensitivity to numerous fungicides, indicating that diverse fungicides can inhibit the growth of this fungus (Sarkar *et al*. 2010; Sushir *et al*. 2015).

Tagetes filifolia EO was effective in controlling *C. truncatum*. This EO was previously described by its antifungal activity against several fungi, including species of genera *Alternaria*, *Colletotrichum* and *Sclerotium* (Zygadlo 1994; Camiletti *et al*. 2016). The antimicrobial activity of the major compound in the EO of *T. filifolia* explained the antifungal property of this essence. Anethole demonstrated an inhibitory effect against species of genera *Alternaria*, *Aspergillus*, *Fusarium* and *Rhizoctonia*, among others (Karapinar 1990; Huang *et al*. 2010). The efficacy of the *T. filifolia* EO against *C. truncatum* was lower than that observed for the chemical fungicides, which is in agreement with previous research (Camiletti *et al*. 2016). Furthermore,

this EO affected mycelial development of *T. harzianum*. In fact, some EOs were reported as effective against *T. harzianum* in crops where this fungus is considered to be a plant pathogen (Angelini *et al*. 2008). These results differ from those reported in earlier studies where it was indicated that *T. harzianum* improved the performance of EOs against several phytopathogenic fungi in combined treatments (Abdel-Kader *et al*. 2011). However, the effectiveness of the biocontrol agent by itself was not included in the study carried out by Abdel-Kader *et al*. (2011).

Combinations of fungicides with *T. filifolia* EO showed the prevalence of additive interactions. A similar characterization was previously indicated for several EOs that exhibited this type of interaction when combined with chemical fungicides, such as fludioxonil and iprodione (Camiletti *et al* 2016; Camiletti 2018). Strong synergistic interactions have been mainly documented for mixtures of antifungal agents with different modes of action (Gisi *et al*. 1985; Gisi 1991, 1996; Kosman and Cohen 1996; Camiletti *et al*. 2016). Carbendazim inhibits the polymerization of tubulin, a protein that is essential during cell division (FAO 2005).Triazoles difenoconazole and cyproconazole act by inhibition of ergosterol biosynthesis, affecting the structure and function of cell membranes (Szkolnik 1981). Trifloxystrobin works by interfering with respiration and its site of action is located in the mitochondrial respiratory pathway (FAO 2005). The terpenes commonly present in EOs were previously described as damaging factors of membranous structures, depleting hyphal cytoplasm and organelles (Lucini 2004; Bakkali *et al*. 2008; Plodpai *et al*. 2013). Triazoles and EOs have a similar mode of action and may explain the higher number of interactions with antagonistic effects. Despite different modes of action, combinations of carbendazim and trifloxystrobin with the EO did not display synergistic effects that controlled *C. truncatum*. However, these combinations were characterized by their additive interaction that remarkably reduced the fungicide dose. Thus, these mixtures could be used as a part of integrated management to reduce the ecological impact of chemical pesticides (Thompson and Kreutrweiser 2007). These properties were widely investigated in combinations of EOs with medicinal fungicides (Shin and Kang 2003; Pyun and Shin 2006; Amber *et al*. 2010). Some research has been carried out to investigate combinations of EOs with agricultural fungicides. Some EOs were reported as an alternative to reduce the amount of the chemical fungicide fludioxonil needed to control *A. flavus* and *Penicillium* spp. (Camiletti 2018). The *T. filifolia* EO was reported as a natural antifungal agent against *Sclerotium cepivorum* with a remarkable capacity to reduce the required dose of the fungicide iprodione (Camiletti *et al*. 2016).

The inclusion of natural compounds as part of an integrated management program is critical for controlling plant diseases. This study demonstrates that the antifungal power of the *T. filifolia* EO against *C. truncatum* can be exploited to reduce chemical doses of fungicides commonly applied to soybean crops. *T. filifolia* EO has the potential to be applied individually as well as in combination with chemical fungicides. The antifungal effect of this EO against *T. harzianum* must be considered in the development of new strategies that may include this biocontrol strain. In conclusion, *T. filifolia* EO is a novel alternative to control anthracnose caused by *C. truncatum* and can be used to reduce the amount of chemical fungicide applied to the field. The agrochemical industry should consider *T. filifolia* EO and its main components for the production of environmentally friendly fungicides. Moreover, the persistence of fungicide residues and the amount of money spent on them can be diminished. Further field assays are needed to adjust the applied concentrations.

Acknowledgements

The authors wish to thank the National Institute of Agricultural Technology (INTA), National University of Cordoba, Secretary of Science and Technology (UNC, SECyT) and the Argentinian National Research Council (CONICET) for their financial support. We are also grateful to the College of Agricultural Science (FCA-UNC) for allowing us to use its laboratory equipment.

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