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Chemical composition of *Tagetes* hydrolates and *in vitro* and *in vivo* evaluation against disease associated fungi in strawberry (*Fragaria x ananassa*)

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ABSTRACT

Background / Objective. Aromatic plants contain chemical compounds with potential to formulate antifungal products. The objective of this study was to characterize the chemical composition in hydrolates of *Tagetes* species and to evaluate their effect *in vitro* and *in vivo* against disease-associated fungi in strawberry.

Materials and Methods. The hydrolates of *T. coronopifolia*, *T. minuta*, *T. parryi* and *T. terniflora* were analyzed by gas chromatography coupled to a mass spectrometry. Hydrolates at 100, 75, 50 and 25 % and Promyl commercial fungicides were evaluated *in vitro* against *Botrytis cinerea*, *Fusarium oxysporum*, *Rhizoctonia solani* and Ridomil Gold against *Phytophthora capsici*. In the *in vivo* evaluation, strawberry plants sprayed with the hydrolates and 24 h later the plants were inoculated with 1 x 10^6 spore suspension. Data were analyzed by analysis of variance and Turkey's means test ($p \leq 0.05$).

Results. Monoterpenes were the major compounds in the four *Tagetes* species. *T. parryi* hydrolate *in vitro* totally inhibited the growth of *B. cinerea* being effective as a preventive treatment in the *in vivo* evaluation. *F. oxysporum*, *P. capsici* and *R. solani* were less susceptible to all the hydrolats.

Conclusion. *T. parryi* hydrolate can be applied as a preventative against *B. cinerea* on strawberry plants.

Keywords: Fungal diseases, *Fragaria*, inhibition, antifungal.

Introduction

In Mexico, the surface planted with strawberries (*Fragaria x ananassa*) in 2018 was 13,562 ha and in 2022 it decreased to 7,872 ha (SIAP, 2022). This reduction was due to phytosanitary problems that affect their production, associated with different species of fungi, including *Fusarium oxysporum*, *Rhizoctonia* spp. (Golzar *et al*., 2007), *Phytophthora* spp. (Serret-López *et al*., 2016) and *Botrytis cinerea* (Boddy, 2016). This situation results in losses of up to 50% of the production (Bárcenas-Santana *et al*., 2019). To control these fungi, agrochemicals have mainly been used; however, their improper use and dosage has had negative effects on the environment and on humans. Moreover, fungi have developed resistance to fungicides, although their use has intensified and they have become crucial for global food security (Zubrod *et al*., 2019). This situation has led researchers and producers to explore alternatives, such as plant extracts or compounds derived from them, which have been successfully used to control phytopathogenic fungi. Plant extracts have the advantage of containing more than one antifungal compound, making pathogen resistance less likely if the different compounds affect a different metabolic process (Shuping and Eloff, 2017). It is important to consider that in the extracts, the yield and chemical composition are variable and depend on weather conditions, cultivation site and time of harvest (Santos-Gomes *et al*., 2001; Bhat *et al.*, 2016). In addition, due to the volatile nature and thermolability of their components, they are very susceptible to degradation (Odak *et al*., 2018). Therefore, prior scientific knowledge of natural resources or management conditions, both in the field and in storage, is essential for the sustainable local use of plants and their transformation into biocontrol inputs for phytopathogenic fungi as a way to replace pesticides in agricultural production systems.

Tagetes (Asteraceae) is a plant resource, represented in Mexico by approximately 35 species, several of which have the potential to control fungi (Serrato, 2014). The aqueous extracts of some *Tagetes* species have displayed promising results, such as *T. erecta* or the Mexican marigold, is effective in the control of *Fusarium oxysporum* (Wang *et al*., 2022) and *Rhizoctonia solani* (Espejo *et al*., 2010), whereas *in vitro*, the *T. lemmoni* or Lemmon's marigold essential oil completely inhibits the mycelial growth of *B. cinerea* (Larios-Palacios *et al*., 2020). During the process of hydrodistillation of aromatic plants, an aqueous extract known as hydrosol, aromatic water, hydroflorate or hydrolate. Its extraction is quick, inexpensive and characteristically contains low concentrations of essential oil (< 1 mL L^{-1}), as well as some polar, oxygenated and hydrophyllic oil components that for hydrogen bonds with water (Tisserand and Young 2014; Labadie *et al*., 2016; Taglienti *et al*., 2022). Hydrolates are used in perfumes, cosmetics, as food flavorings, aromatherapy and traditional therapies (Rajeswara, 2013). Studies

on the biological effect of hydrolates are still limited in comparison with those performed on the biological effect of essential oils (Yann-Olivier *et al*., 2018). In the scope of agriculture, *Ocimum basilicum*, *Cuminum cyminum*, *Echinophora tenuifolia*, *Daucus carota* subsp. *sativus*, *Rosmarinus officinalis* and *Satureja hortensis* have been successfully used to control some phytopathogenic fungi such as *Alternaria citri*, *Alternaria mali*, *Botrytis cinerea*, *Fusarium oxysporum* f. sp tulipae, *Penicillium expansum* and *Rhizoctonia solani* (Boyraz and Özcan, 2005; Boyraz and Özcan, 2006; Zatla *et al*., 2017). For a long time, hydrolates have been defined as waste products of hydrodistillation, but currently, they are beginning to be used as an alternative to control phytopathogenic fungi. Therefore, the exploration and evaluation of other aromatic species in the control of fungi with a high incidence in crops such as strawberry is essential. Considering that *Tagetes* is an abundant natural resource in Mexico, and thus a potential source of hydrolate as an input for the biocontrol of phytopathogenic fungi, the aim of this investigation was to characterize the chemical composition in hydrolates of *Tagetes* species and evaluate their effect *in vitro* and *in vivo* against diseases associated fungi in strawberry.

Materials and Methods

Plant sampling. In the experimental field of the Chapingo Autonomous University (19.491733, -98.873179), in May of 2022, under greenhouse conditions, *Tagetes* plants were sown: *T. coronopifolia*, *T. minuta*, *T. parryi* and *T. terniflora*, which, in flowering stage, were gathered in the month of October and, using pruning shears, were cut into segments measuring approximately 2 cm. The amount of 4 kg of a mixture of flowers (25%), leaves (35%) and stems (40 %) was introduced in a round-bottomed flask and 2 L of drinkable water were then added, the thermostat was turned on (135±5 °C) and for 40 minutes, the hydrodistillation was carried out (Uddin *et al*., 2023). A total of 400 mL kg^{-1} of fresh hydrolate were obtained, placed in plastic jars and stored at a temperature of 4 °C until use. Three specimens of each species were taken to the Jorge Espinoza Salas herbarium of the Agricultural High School, Chapingo Autonomous University, with registration numbers 36282, 36280, 33299 and 36281, respectively.

Identification of chemical compounds. The hydrolates were analyzed using an Agilent 7890B Gas Chromatograph (GC) coupled with an Agilent 5977ª Mass Selective Detector (MSD) (CRO-E-003) and equipped with a DB-WAX Ultra Inert column (60 m x 250 µm x 0.25 µm). The temperature ramp was 40 °C for 1 min up to 240 °C at a speed of 9 °C min^{-1}. Helium was used as the carrier gas at a

flow rate of 1 mL min^{-1} for the duration of the 24 min run. The hydrolates were filtered in a 45 µm PTFE microfilter, then 100 µL of the sample were dissolved in 400 µL of HPLC grade dichloromethane. The extract was concentrated to a volume of 1 mL and injected into the gas chromatograph in splitless mode. The compounds were identified based on the fragmentation patterns of the mass spectra, which were compared with the mass spectra in the NIST 14 (National Institute of Standards and Technology) database and the Flavor fragrance database (Agilent Technologies). The determination of Kovats retention indices was performed with the C7-C40 alkanes (Sigma-Aldrich). The experimental Kovats retention indices were compared with the Kovats indices from the literature (NIST:webbok.nist.gov/chemistry/), considering that the experimental Kovats index matched (± 50 units) with the literature for the correct identification of the compound. The relative abundance of each compound was estimated based on the area of the peaks.

Origin of the fungi. *Fusarium oxysporum*, *Phytophthora capsici* and *Rhizoctonia solani* were isolated from samples of *manzano* pepper (*Capsicum pubescens*) gathered in a 'manzano pepper' orchard located in the Chapingo experimental field, and *Botrytis cinerea* was isolated from samples of wild rosebushes (*Rosa* sp.) gathered in the municipal area of Acaxochitlán, Hidalgo. The plant tissue was disinfested with sodium hypochlorite at 1% and washed three times with biodistilled water. Later, in a Potato-Dextrose-Agar (PDA) culture medium (BD Bioxon), 5 mm fragments of the plant tissue were placed in order to isolate the fungi and the oomycete was isolated in a culture medium containing 20 g of corn flour, 18 g of agar-agar, 0.8 mL of pimaricin, 0.02 g de rifamycin and 0.25 g L^{-1} of ampicillin diluted in distilled water. The Petri dishes were kept at room temperature and were monitored every 24 hours until mycelial growth was observed. At 72 h, growth was observed and inoculum was transferred to a new culture medium to obtain pure cultures. Subsequently, *F. oxysporum*, *R. solani* and the oomycete *P. capsici* were identified based on their morphology (Nelson *et al*., 1983; Gutiérrez *et al*., 2006; Leslie and Summerell, 2006; Plancarte *et al*., 2017). *B. cinerea* was also identified based on its morphology and pathogenicity tests on strawberry fruits (Barnett and Hunter, 1986; Terrones-Salgado *et al*., 2019). Additionally, DNA extraction was performed using the SDS (Sodium Dodecyl Sulfate) method, followed by PCR amplification using NL4 and ITS5HP primers (Toju *et al*., 2012; Hudagula *et al*., 2022). The thermal program was set to 94 °C for 5 min, followed by 35 cycles at 94 – 56 – 72 °C for 1 – 1 – 2.3 s, respectively, and a final extension at 72 °C for 10 min. The amplified products of the PCR reactions were separated by electrophoresis using 1.2% agarose gels. The amplified and purified fragments were sent to Macrogen for sequencing. The sequenced in FASTA format were the BLAST (Basic Local Alignment Search Tool) database, confirming the identity

of the fungus, and the GenBank accession number for *B. cinerea* is PP401673.1. For the molecular identification of the other organisms, the DNA extraction was carried out using the CTAB method (Weising *et al*., 2005) with modifications. The mycelium was obtained from Petri dishes with *F. oxysporum*, *P. capsici* and *R. solani* growth and placed in mortars. Liquid nitrogen was added and the mycelium was macerated with a sterile pestle. In 2 mL Eppendorf tubes, 1 mL of 2% CTAB + 0.2% β-mercaptoethanol was added, followed by the macerated mycelia, it was shaken by immersion and incubated at 55 °C in a thermoblock for 20 min, stirring every 5 min. Subsequently, the tubes were centrifuged for 5 min at 12,000 x g, the supernatant was transferred to a new tube using micropipettes and 1.2 volumes of 100% isopropanol were added. It was shaken by immersion and centrifuged for 10 min at 12,000 x g, the supernatant was discarded, and washed with 70% ethanol. The tubes with precipitated DNA were centrifuged for 10 min at 12,000 x g and the ethanol was decanted. The tubes were placed upside down on absorbent paper until the pellet dried. Finally, 50 µL of TE were added to each tube, and they were kept at -20 for 24 h. The DNA concentration was determined using a spectrophotometer at 260 nm in a Nanodrop V3.5.2 (Coleman Technologies Inc. For Nanodrop Technologies). The PCR reactions were carried out in a final volume of 25 µL using primers NL4-ITS5SHP, ITS4-ITS5HP, ITS1-NL4, ITS1-ITS4B to amplify the ITS (Toju *et al.*, 2012; Hudagula *et al.*, 2022), primers ITS NMSI-NMS2 to amplify the small subunit of the ribosome, and primers EF1-EF2 to amplify the TEF gene (Raja *et al.*, 2017; Deng *et al*., 2022). The PCR conditions for the ITS region and small ribosomal unit were as follows: a first pre-denaturation cycle at 95 °C for 5 min, followed by 35 denaturation cycles at 95 °C for 1 min, aligning at 58 °C for 45 seconds and an extension at 72 °C for 1 min; finally, an extension at 72 °C for 10 min. The PCR conditions for TEF were as follows: a first pre-denaturation cycle at 95 °C for 5 min, followed by 35 denaturation cycles at 95 °C for 1 min, aligning at 55 °C for 45 s and an extension at 72 °C for 1.5 min; finally, an extension at 72 °C for 10 min. The PCR amplification product was visualized by electrophoresis, 1.2% agarose gel was prepared with 1x TAE buffer, run at 100 volts for 1 h. For scrambling the bands, the gel was stained with ethidium bromide for 15 min, then viewed with the Qwanty One software (BioRad). The size of the bands was estimated using the 1 kb molecular weight markers (Invitrogen) placed at the ends. The amplified products were stored at 4 °C for analysis. The amplified and purified fragments were sent to Macrogen for sequencing. The sequences in FASTA format were compared with the BLAST database (Basic Local Alignment Search Tool), to confirm the identity of the fungi, and then registered in the GenBank database. The accession number for each fungus is *F. oxysporum* (PP729638.1), *R. solani* (PP713022.1) and *P. capsici* (PP922286.1).

***In vitro* evaluation.** PDA medium was mixed with hydrolate in four concentrations (100, 75, 50 and 25% v/v) for every species. The concentrations of the hydrolate were prepared by diluting with bidistilled water and 16 hydrolate treatments were obtained, along with the control with PDA. As reference controls. Commercial fungicides from Distribuidora Técnica Industrial, S. A. de C. V. and Syngenta Agro, S.A de C.V., distributed by Artículos Entomológicos S. A. de C. V. Texcoco, were used. Promyl (Benomyl: Methyl 1-(butylcarbamoyl) bencimidazol-2-il-carbamate) was used for *Botrytis cinerea*, *Rhizoctonia solani* and *Fusarium oxysporum* (Vega-López and Granados-Montero, 2023) and Ridomil Gold (N-(metoxiacetil)-N-(2,6-xilil)-D-alaninato de metilo) for *Phytophthora capsici* (Pons-Hernández *et al*., 2020). Each fungicide was used at 1.5 g L^{-1}. After preparing the culture medium, a disk (3.5 mm) with mycelium from each fungus (10-days-old colonies) was placed in the center of each Petri dish containing the culture medium combined with hydrolate, then incubated in dark conditions with the following temperatures: *B. cinerea* at 20 °C, *P. capsici* at 27 °C, *F. oxysporum* and *R. solani* at 30 °C. The radial growth was measured every 24 h using a digital caliper.

***In vivo* evaluation.** Only the treatments with the highest inhibition effect *in vitro* were retaken for their evaluation *in vivo*. The evaluation was performed in a greenhouse and five-month-old "FESTIVAL" variety strawberry plants were used, from Zamora, Michoacán in the flowering phase kept in pots with red tezontle; fertilization was carried out using 100% Steiner solution, irrigating on a weekly basis and with a weekly application of foliar fertilizerPEKA® (1 mL L^{-1}) (Product manufactured by Química Sagal and distributed by Agroquímicos Texcoco S.A. de C. V.). When the plants began bearing fruits, four mature fruits were selected, and a 0.2 cm-deep perforation was made using a dissection needle sterilized with alcohol, and each plant was sprayed with 20 mL de hydrolate; 24 h after the application of hydrolates, the plants were inoculated with a 1 x 10^6 *F. oxysporum* and *B. cinerea* spore suspension prepared with bidistilled water and 1 mL L^{-1} of Tween 20. The spores were applied by spraying and *P. capsici* and *R. solani* were inoculated via mycelia. The inoculated plants were covered with a polyethylene bag for 48 h to withhold moisture, after which the leaves, mature fruits, immature fruits and flowers were monitored every 24 h until 72 h passed in order to detect symptoms caused by the pathogens (*B. cinerea*, *F. oxysporum*, *P. capsici* and *R. solani*).

Experimental design and analysis of data. The *in vitro* experiment was analyzed under a Completely Randomized Design (CRD) with five repetitions each; a Petri dish was an experimental unit. Using the radial growth data, the percentage of inhibition was calculated (Kagezi *et al*., 2015). The *in vivo* experiment was calculated with a CRD. Four pots were used per treatment and each pot was

considered an experimental unit. The incidence of the disease was calculated using the data recorded on the diseased fruits and flowers (Macías *et al*., 2016). The data underwent an analysis of variance and Tukey's means test ($p \leq 0.05$) using SAS academic Software.

Results and Discussion

Chemical compounds. In the *T. parryi* hydrolate, 14 compounds were identified, 13 in *T. minuta*, 11 in *T. coronopifolia* and a total of 9 in *T. terniflora* (Figure 1). In the *T. parryi* hydrolate, the most abundant compounds were 3-Hexen-1-ol (25.5%), isopiperitenone (14.9 %), 1,3-Di-tert-butylbenzene (6.1%) and alpha-terpineol (5.9%) (Table 1). When comparing with compounds found in this essential oil of the same species, Díaz-Cedillo and Serrato-Cruz (2011) identified seven main compounds: camphene, 3, 6, 6-trimethyl-2-norpinanol, anisole, 4-isopropyl-1-methyl-2-cyclohexenol, cineole, eugenol and alpha-terpineol and González-Velasco *et al*. (2022) recorded 21 compounds, including verbenone, dihydrotagetone, tagetone, eugenol and alpha-terpineol, most of which are monoterpenes and sesquiterpenes. In the *T. parryi* hydrolate, only alpha-terpineol and dihydrotagetone were similar to reports in essential oil, therefore most of the compounds were different to those listed for the essential oil.

In *T. coronopifolia* hydrolate, dihydrotagetone (53.9%), cis-tagetone (11.9%), (Z)-tagetenone (9.7%) and trans-tagetone (5.9%) were found (Table 1); no compounds found in the hydrolate sample coincided with the report for the essential oil by Díaz-Cedillo *et al*. (2013): (1S) -6,6-dimethyl-2-methylene-bicyclo [3.1.1] heptan-3-one, verbenone, methyl 2-oxo-decanoate and crysantenone.

In *T. terniflora* hydrolate, dihydrotagetone (45 %), eucalyptol (13.2 %), trans-3-Hexen-1-ol (10.8 %) and cis-tagetone (4.5 %) (Table 1) were the most abundant. Only the cis-tagetone was common in the analyzed essential oil and hydrolate (Lizarraga *et al*., 2017).

In *T. minuta* hydrolate, the most abundant compounds were 3-Hexen-1-ol (16.4 %), benzene, 1,3-bis(1,1-dimethylethyl)- (14.6 %), cis-tagetone (10.1 %) and dihydrotagetone (5.6 %) (Table 1). Only dihydrotagetone was common with what Karimian *et al*. (2014) reported for this species, which has an essential oil that contains compounds such as: dihydrotagetone, E-ocimene, tagetone, cis-β-ocimene, Z-ocimene, limonene and epoxyocimene.

The compounds 3-Hexen-1-ol, (E)-, dihydrotagetone and cis-tagetone were common in the hydrolates of the species of *Tagetes* under study (Cuadro 1), whereas a comparison between the chemical composition of the hydrolates and of the essential oils shows that these do not coincide in compounds or in abundance,

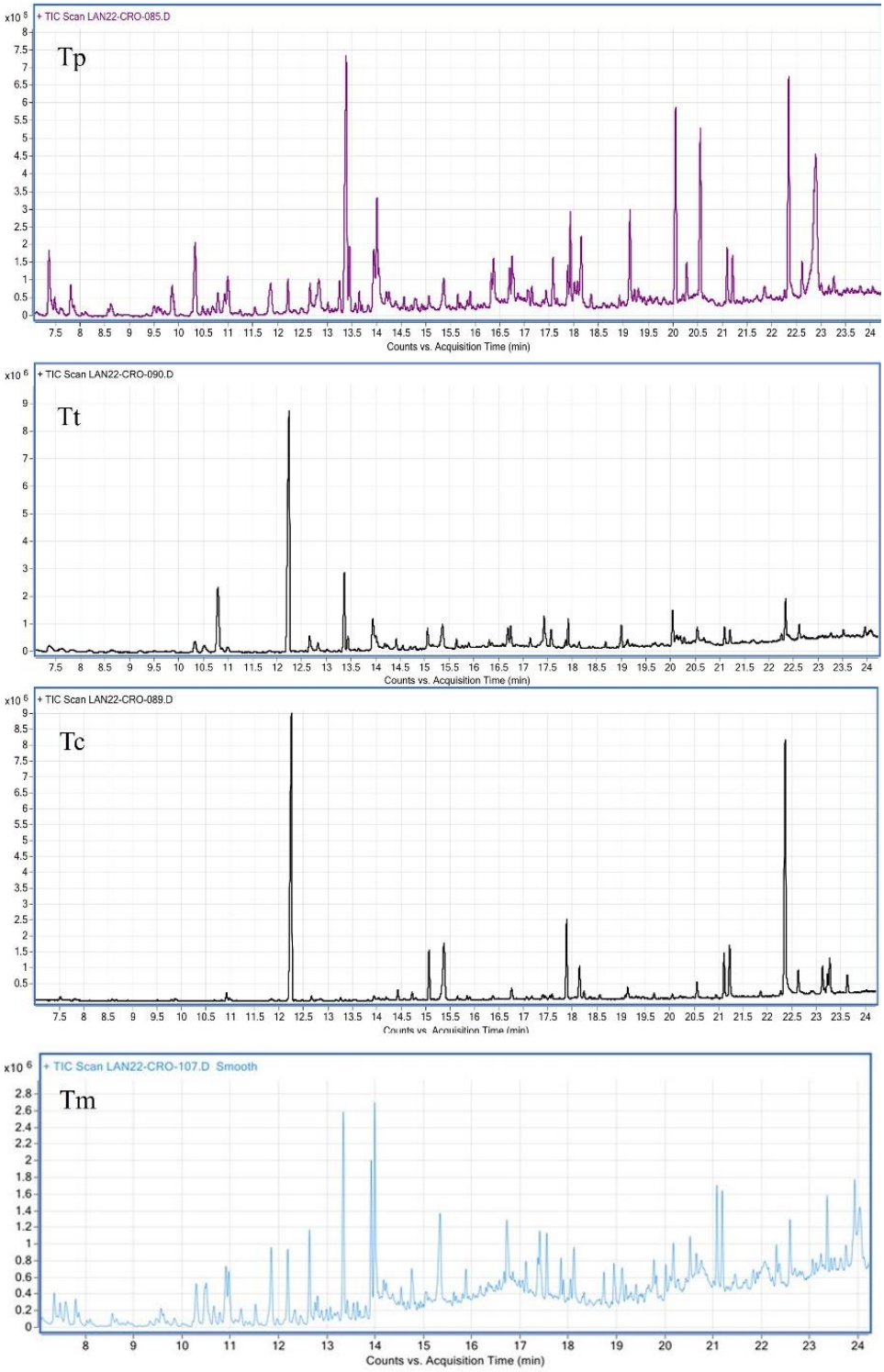

Figure 1. *Tagetes parryi* (Tp), *T. terniflora* (Tt), *T. coronopifolia*, (Tc) and *T. minuta* (Tm) hydrosol chromatograms, showing the peaks of the compounds identified.

Table 1. Relative abundance (%) and Kovats indices (KI) ± standard deviation of chemical compounds found in *Tagetes coronopifolia*, *T. minuta*, *T. parryi* and *T. terniflora* hydrolates analyzed using GC/MSD.

	T. parryi	%	KI		*T. coronopifolia*	%	KI
°	3-Hexen-1-ol, (E)-	25.5	1370 ± 18.5	+	Dihydrotagetone	53.9	1319 ± 3.3
+	Isopiperitenone	14.9	1833 ± 41.9	+	cis-Tagetone	11.9	1500 ± 23.1
-	1,3-Di-terc-butylbenzene	6.1	1426 ± 9.5	+	(Z)-Tagetenone	9.7	1704 ± 9.5
+	α-Terpineol	5.9	1700 ± 14.8	+	trans-Tagetone	5.9	1522 ± 8.2
+	(Z)-Tagetenone	4.5	1704 ± 24.4	*	Methyl 10,11-tetradecadienoate	1.6	1663 ± 19.8
+	cis-Tagetone	3.2	1517 ± 10.7	*	Caryophylene	1.2	1585 ± 33.5
*	Methyl 10,11-tetradecadienoate	2.3	1663 ± 19.8	+	2-(3-methyl-2-cyclopenten-1-yl) -2-methylpropionaldehyde	0.8	1442 ± 31.5
+	Dihydrotagetone	2.2	1319 ± 1.3	*	β-bisabolene	0.7	1715 ± 21.7
+	Terpinen-4-ol	1.9	1619 ± 6.4	+	Estragole	0.6	1676 ± 12.3
´	Benzene, 1-ethyl-2-methyl-	1.7	1254 ± 9.1	+	Isopiperitenone	0.6	1833 ± 41.5
+	Eucalyptol	1.5	1199 ± 23.5	+	Ipsenone	0.3	1444 ± 5.4
	3-Hexen-1-ol, propanoate, (Z)-	1.1	1380 ± 24.3				
°	2-cyclohexene-1-one	1	1424 ± 36.8				
*	Isospathulenol	0.4	2186 ± 0				

	T. terniflora	%	KI		*T. minuta*	%	KI
+	Dihydrotagetone	45	1319 ± 3.7	°	3-Hexen-1-ol, (E)-	16.4	1394 ± 0.28
+	Eucalyptol	13.2	1212 ± 14.6	´	Benzene, 1,3-bis(1,1-dimethylethyl)-	14.6	1436 ± 2.26
°	3-Hexen-1-ol, (E)-	10.8	1394 ± 0.5	+	cis-Tagetone	10.1	1517 ± 10.81
+	cis-Tagetone	4.5	1517 ± 10.6	+	Dihydrotagetone	5.6	1319 ± 1.2
+	α-Terpineol	3.1	1680 ± 28.7	°	2 - Clorocyclohexanol	4.7	1659 ± 24.25
+	trans-Tagetone	2.2	1501 ± 6.2	+	trans-Tagetenone	4.3	1726 ± 8.2
+	Terpinen-4-ol	2.2	1606 ± 15.4	+	Isopiperitenone	2.8	1833 ± 41
°	Phenylethyl Alcohol	0.5	1923 ± 15.8	*	Methyl 10,11-tetradecadienoate	2.5	1663 ± 19.8
°	2-cyclohexene-1-one	0.5	1412 ± 45.4		1-Pentanone, 1-(2-furanyl)-	1.3	1747 ± 10.5
				-	p-Xylene	1.2	1142 ± 11.1
				+	α-terpineol	1	1697 ± 16.4
				+	Terpinen-4-ol	1	1635 ± 4.8
				´	Ethylbenzene	0.3	1146 ± 2.9

+: monoterpenes, *sesquiterpenes, -: phenolic compounds, °: alcohols, ´: benzene compound

therefore the chemical composition of hydrolates and essential oils is always different, both quantitatively and qualitatively (D'Amato *et al*., 2018). Other studies have found that in both hydrolates and essential oils, monoterpenes and oxygenated monoterpenes are abundant, but the same compounds are not found (Vuko *et al*., 2021). Inouye *et al*. (2008) compared 43 hydrolates with essential oils obtained from the same steam distillation process and found that 18 of the 43 hydrolates analyzed displayed a different more abundant compound than the oil.

The *Tagetes* essential oils are monoterpene-rich, and contain low amounts of sesquiterpenes and oxygenated compounds (Salehi *et al*., 2018). In this study, the

more abundant compounds in the four species belong to monoterpenes and, to a lesser degree, to sesquiterpenes, phenolic compounds and some alcohols (Table 1). Despite already having information on the composition of hydrolates of two species from South America (Rajeswara *et al*., 2006; Lima *et al*., 2009), the identification of the compounds in hydrolates of the four *Tagetes* species in Mexico constitutes another important step in advancing the phytochemical knowledge of the genus.

***In vitro* evaluation.** The evaluated treatments displayed significant inhibiting effects on *B. cinerea* ($p < 0.0001$), *F. oxysporum* ($p < 0.0053$), *P. capsici* ($p < 0.0001$) and *R. solani* ($p < 0.0001$) (Table 2). The 100% *T. parryi* hydrolate inhibited the mycelial growth of *B. cinerea* by 71.3%, with no statistical difference with the effect produced by the commercial fungicide. Even dilutions of this hydrolate at 50 and 75% displayed statistical differences with the control, whereas with hydrolate at 100% or dilutions from the other *Tagetes* species, the inhibition of the mycelium was mostly lower than 40%, with no statistical difference with the control (Table

Table 2. Percentage of *in vitro* inhibition of fungus mycelia after applying *Tagetes* species hydrolates.

Treatments	Inhibition (%) of fungal mycelia			
	B. cinerea	*P. capsici*	*F. oxysporum*	*R. solani*
TO	0 ± 0 e	0 ± 0 f	0± 0 c	0 ± 0 c
FC	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a
H100C	40 ± 28.8 bcde	100 ± 0 a	25 ± 15.5 b	34.4 ± 22.2 b
H100M	35 ± 21.6 bcde	54.3 ± 2.5 b	21 ± 4.5 bc	20 ± 11.7 bc
H100P	71.3 ± 9.7 ab	55.3 ± 3 b	23 ± 4 b	26.6 ± 6.8 bc
H100T	25.3 ± 15 cde	57 ± 13.1 b	16.6 ± 2 bc	20 ± 2.6 bc
H75C	22.6 ± 23.5 cde	100 ± 0 a	16.6 ± 3.5 bc	32.6 ± 13.2 bc
H75M	34 ± 7.9 bcde	43.3 ± 9 bc	18.6 ± 7.6 bc	15.6 ± 6 bc
H75P	54.6 ± 18.9 bc	38.3 ± 10.4 bcd	12.3 ± 7 bc	17 ± 13.5 bc
H75T	14 ± 17.8 cde	38.3 ± 3 bcd	11.3 ± 10.5 bc	20 ± 11.5 bc
H50C	22.6 ± 2.3 cde	61.6 ± 20.4 b	9.1 ± 9.4 bc	19.6 ± 10.4 bc
H50M	25 ± 4.5 cde	27 ± 3.6 cde	16 ± 7.9 bc	10.6 ± 8.8 bc
H50P	54 ± 10.5 bcd	28.3 ± 8.5 cde	10.6 ± 6.5 bc	14.3 ± 7.5 bc
H50T	10.3 ± 24.2 de	17 ± 11.7 def	6.3 ± 7.57 bc	10 ± 12.6 bc
H25C	4.6 ± 5.6 e	10.3 ± 11.6 ef	1 ± 1 c	11.6 ± 18 bc
H25M	3.6 ± 6.3 e	16 ± 5 def	10.6 ± 7.5 bc	10 ± 10.7 bc
H25P	33.3 ± 7 bcde	13 ± 3 ef	9.3 ± 3 bc	13 ± 4.5 bc
H25T	0 ± 0 e	4.6 ± 8 ef	4 ± 4 bc	8.4 ± 0 bc
Valor p	0.0001	0.0001	0.0053	0.0001
CV	33	14	21	30
LSD	41	23	39	30

TO: Control, FC: Commercial fungicide, H: Hydrosol, 100, 75, 50 and 25: Concentration (%) of hydrosol used, C: *T. coronopifolia*, M: *T. minuta*, P: *T. parryi*, T: *T. terniflora*, CV: Coefficient of variation, LSD: Least significant difference, means with different letters between columns are statistically different (Tukey, 0.05). Average values ± standard deviation.

2). In *P. capsici*, the *T. coronopifolia* hydrolate at 75 and 100% effectively inhibited mycelial growth, similar to the fungicide. With *T. minuta*, *T. parryi* and *T. terniflora* hydrolates in 50 and 75% dilutions, *P. capsici* inhibition was significant ($p < 0.0001$) compared to the control, but not compared to the fungicide. No concentration of the evaluated hydrolates displayed a fungistatic effect against *F. oxysporum* and *R. solani*. The *T. minuta*, *T. parryi* and *T. terniflora* hydrolates inhibited these fungi by 26.6%. In the case of *T. coronopifolia* at 100%, *F. oxysporum* and *R. solani* were inhibited by 25 and 34.4%, respectively (Table 2). The inhibiting effect of the essential oils and hydrolates is due to the monoterpenes, sesquiterpenes and phenolic compounds (Hu *et al*., 2019; Hill, 2022). Monoterpenes are small and fit between the fatty molecules that make up the cell membrane and affect functions inside the cell, while sesquiterpenes are not small enough to fit through the cell membrane, but they have unique shapes that allow them to adhere to the spaces of three-dimensional protein structures and affect the activity of the protein (Hill, 2022). Hu *et al*. (2019) mention that the phenolic compounds can interfere with the membranes, the cell walls and enzyme action. In *T. parryi* hydrolate, a larger diversity of groups of compounds was found, including the phenolic compound 1,3-Di-terc-butiylbenzene (Table 1). In this regard, Zatla *et al*. (2017) mention that the phenolic compounds of *Daucus carota* subsp. *sativus* have antifungal activity against *B. cinerea*. Belabbés *et al*. (2017) attribute the antifungal effect against *Penicillium expansum* to the oxygenated sesquiterpenes found in the *Calendula arvensis* hydrolate. In *T. parryi*, *T. coronopifolia* and in *T. minuta*, sesquiterpene methyl 11-tetradecadianoate was found, with a relative abundance between 1.6 and 2.5% (Table 1) and the inhibiting effect of these hydrolates was greater (Table 2) to those reported for *T. terniflora*, which did not contain this compound. In addition, the oils with an abundance of monoterpenes inhibit the growth of fungi (Stević *et al*., 2014), which helps highlight that the analyzed *Tagetes* hydrolates contain abundant monoterpenes (Table 1). Although the antifungal effect is attributed to the most predominant compounds, it is the synergic effect of all the compounds that cause the antifungal effects, and also, some compounds are more active than others (Dhifi *et al*., 2016).

***In vivo* evaluation.** The treatments with 100% hydrolates from the four *Tagetes* species displayed the greatest *in vitro* inhibition (Table 2). Therefore, they were picked up for their evaluation *in vivo*. The strawberry plants displayed no phytotoxic effects due to the application of *Tagetes* hydrolates, which gives good expectations for future evaluations in other plant species. The control plants inoculated with *B. cinerea* presented symptoms of the disease, both in fruits and flowers. On the other hand, the plants inoculated with *F. oxysporum*, *P. capsici* and *R. solani* displayed no symptoms of disease in flowers, yet they did in the perforated fruits (Figure 2). Strawberry fruits are highly perishable; in addition, if the fruit presents any

mechanical damage or lesion, it facilitates infections by pathogens (Ángel-García *et al*., 2018). The lack of incidence of the disease in flowers may be related to the lack of lesions and to the fact that these fungi mainly affect roots, the crown and fruits (Koike and Gordon, 2015; Awad, 2016; Barboza *et al*., 2016).

The *T. parryi* hydrolate was effective as a preventive treatment against *B. cinerea*, since the perforated fruits (Figure 2A) and the flowers (Figure 2B) displayed no

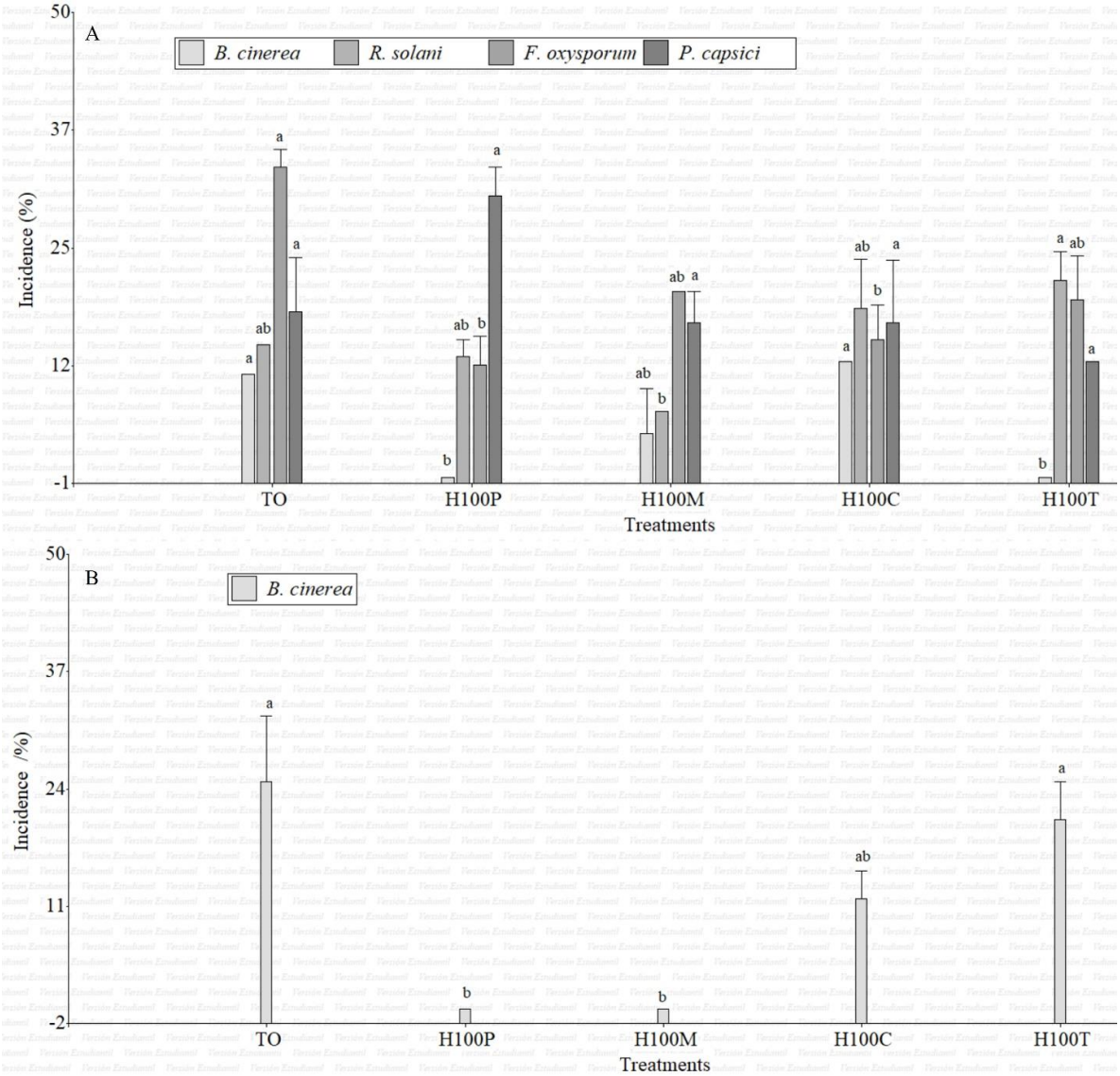

Figure 2. Incidence of *B. cinerea, R. solani, F. oxysporum* and *P. capsici* in strawberry fruits (A) and flowers (B) three days after their inoculation in control (TO) plants and treated with 100% *Tagetes coronopifolia* (H100C), *T. minuta* (H100M), *T. parryi* (H100P) *T. terniflora* hydrolates (H100T).

incidence of the fungus. Therefore, this hydrolate represents an alternative input to chemical fungicides that have negative effects on the environment and have induced resistance in this fungus (Leroux, 2004). Zatla *et al*. (2017) evaluated the effect of the *Daucus carota subsp. sativus* hydrolate in strawberry fruit and it was effective as a preventive treatment, since it inhibited *B. cinerea* entirely until the fifth day. According to these authors, the effect was due to the abundant of phenolic compounds. Due to this, the effect observed with the *T. parryi* hydrolate can also be attributed to the phenolic compound 1,3-Di-terc-butylbenzene, along with the other more abundant monoterpenes and sesquiterpenes found in the hydrolate (Table 1). On the other hand, with the application of the *T. terniflora* hydrolate in the fruits (Figure 2A), no symptoms of the disease were observed, although the flowers were infected and with the *T. minuta* hydrolate, an opposite effect was observed (Figure 2B); this result shows that a joint application of these hydrolates may be effective against this fungus. The *T. coronopifolia* hydrolate did not work as a preventive treatment.

Conclusions

The hydrolates of the tested *Tagetes* species contain between 46 and 72% of monoterpenes, and to a lesser extent, some alcohols (0-33 %), sesquiterpenes (0-27%) and phenolic compounds (0-8 %). Although a fungistatic effect was observed in the evaluation *in vitro* with some hydrolates, this effect did not withstand in the *in vivo* application; only the application of the *T. parryi* hydrolate is effective as a preventive treatment against *B. cinerea* in strawberry plants during flowering and fruiting.

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