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Article

Distillates and homeopathic oil of *Tagetes remotiflora*: effect on *Botrytis cinerea*

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Abstract

A phytogenetic resource in Mexico is *Tagetes remotiflora* Kunze, with potential for the control of fungal diseases. The objective was to describe the composition of *T. remotiflora* essential oil and to evaluate distillates (oil and hydrolate) and homeopathic essential oil on the control of *Botrytis cinerea in vitro* in 2021. Essential oil and hydrolate were obtained by hydrodistillation of flowering plants, the essential oil was also prepared in its homeopathic form, and the oil was analyzed using the GC-MS technique. The treatments evaluated were PDA medium (39 g L^{-1}), Cabrio C fungicide (1 g L^{-1}), Tween 20 (0.1 ml L^{-1}), essential oil (0.0001, 0.001, 0.01, 0.1, 0.5, 1 and 2%), hydrolate (25, 50, 75, and 100%), and homeopathic essential oil (1, 2, 3, 4, 5, 6, 60 and 200 CH). The data on the percentage of inhibition and mycelial growth rate were subjected to an analysis of variance and Tukey's test ($p \leq 0.05$) and minimum inhibitory concentration 50 (MIC_{50}) and 90% (MIC_{90}) were determined by probit analysis. Thirty-seven chemical compounds were identified, four of which were the majority: trans-β-Ocimene, 2-Carene, Cyclohexene, 1-methyl-4-(1-methylethylidene)- and 5,7-Octadien-4-one, 2,6-dimethyl-, (E)-. Mycelial growth was inhibited by 92% with the 0.5% essential oil, and by 100% with 1 and 2%, as with the fungicide; with the homeopathic oil 6 CH, the inhibition was 19.2% and with 75 or 100% hydrolate, the inhibition was from 74 to 85%. The probit analysis confirms the biocidal effect of the essential oil and the fungistatic effect of the hydrolate against *B. cinerea*.

Keywords:

Tagetes remotiflora, fungal disease, hydrolate, inhibition.

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Introduction

One of the ways to the use of aromatic plants in agriculture is to subject them to distillation processes to obtain compounds; most of them are secondary metabolites with biological activity. During the extraction of essential oils by hydrodistillation, three substances are generated (essential oil, hydrolate, and wastewater or infusion), which have the potential to be used as biocontrollers of pests and diseases (Proto *et al*., 2022); however, essential oil is what is most used.

In Mexico, aromatic genetic resources are numerous, especially those related to the botanical families Asteraceae, Burseraceae, Lauraceae, Fabaceae, Rubiaceae, Euphorbiaceae, Solanaceae, Lamiaceae, and Verbenaceae; it is estimated that there are around 450 to 600 aromatic species (Calvo-Irabien, 2018). Asteraceae in Mexico is made up of 3 113 species (Villaseñor, 2018). *Tagetes* (Asteraceae) is a genus with a wide distribution in Mexico and is considered an alternative natural resource for pest and disease control (Serrato, 2014).

The antimicrobial and antioxidant activity of *Tagetes* essential oil has already been studied (Salehi *et al*., 2018). Nonetheless, research on its antifungal activity is still limited (Mutlu-Ingok *et al*., 2020). *T. remotiflora* is an annual herbaceous plant with regional distribution in Mexico, of ruderal condition (Serrato, 2014), with antibacterial activity (Rincón *et al*., 2012), and currently, its chemical composition or its properties against phytopathogenic fungi have not been explored.

Hydrolate usually contains 0.05 to 0.2 g L^{-1} of essential oil and other compounds (Jakubczyk *et al*., 2021), a characteristic that gives it antioxidant (Jakubczyk *et al*., 2021), organoleptic, antimicrobial, antifungal, medicinal, and food industry properties (Aćimović *et al*., 2020); there are few studies on its use in the control of fungal diseases, among which the application of hydrolates of garlic (*Allium sativum* L.) and onion (*Allium fistulosum* L.) stand out, which inhibit the development of *Botrytis allii* Munn and *Sclerotium cepivorum* Berk (Lozano *et al*., 2000); nevertheless, the exploration of *Tagetes* hydrolates against fungi has not been considered so far.

There are aromatic plants with fungicidal or fungistatic properties, but their low yield of essential oil limits their economic viability as an active ingredient for the control of phytopathogenic fungi. For this reason, the exploration of homeopathic essential oils is considered, which, in addition to representing a cheap input, of personal preparation, and without polluting effect on the environment or humans (Toledo *et al*., 2011), is a way that contributes to obtaining healthy foods; hence, it is strategic to verify their biological effect.

Homeopathy experimentation includes a few studies on homeopathic essential oils against phytopathogenic fungi (Teixeira *et al*., 2017; Larios-Palacios *et al*., 2020). The essential oil in homeopathic form of *Eucalyptus citriodora* Hook. and *Cymbopogon citratus* (DC.) Stapf control *Alternaria solani* Sorauer and *Corynespora cassiicola* (Berk. & M.A. Curtis) (Oliveira *et al*., 2017) and the nosode of *B. cinerea* (homeopathic preparation from the fungus that causes the disease) in dilution 7 CH and arsenic 6 CH partially inhibit the mycelial growth of *B. cinerea in vitro* (Larios-Palacios *et al*., 2020).

Although the essential oils of *T. lemmonii* A. Gray. (Larios-Palacios *et al*., 2020) and *T. patula* L. (Romagnoli *et al*., 2005) have an inhibitory effect against *B. cinerea*, there is still no background on homeopathic essential oils of *Tagetes* in the *B. cinerea* model, hence the importance of generating basic information on *T. remotiflora* for its use as an organic input against *B. cinerea*, which causes estimated economic losses between 10 and 100 billion dollars, is difficult to control and is adaptive in nature to changing environments; unfortunately, the application of chemical fungicides for its control leads to problems of pollution to the environment and humans (Romanazzi and Feliziani, 2014; Boddy, 2016).

The study aimed to describe the composition of *T. remotiflora* essential oil and to evaluate distillates (oil and hydrolate) and homeopathic essential oil on the control of *B. cinerea in vitro*. It is expected that some compounds in *T. remotiflora* essential oil will match other species of *Tagetes*, but others may be specific and that, both distilled and homeopathic, will have an effect on the mycelial growth of *B. cinerea*.

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Materials and methods

Sampling of plant material

During October 2021, specimens of *T. remotiflora* (3.25 kg in dry weight) in the flowering stage were sampled in the municipality of San Martín Itunyoso, Oaxaca (17° 11' 58.37" north latitude, 97° 52' 40.13" west longitude); this species is mainly distributed in climatic transition areas, not towards cold or warm temperate climates. The sampled sites were georeferenced with a UTM Geo Map 3.1.7 geopositioner. The botanical characterization and deposit of specimens with registration number 35880 were carried out at the Jorge Espinosa Salas JES Herbarium-Hortorio of the Department of Agricultural High School of the Chapingo Autonomous University (UACH, for its acronym in Spanish) in the State of Mexico.

Essential oil and hydrolate extraction

Whole plants (root, stem, leaves, and flowers) were collected and crushed with 8" pruning shears; the extraction of essential oil and hydrolate was done by hydrodistillation (Rodríguez *et al*., 2012) for 45 min in an Italian-type glass distiller with a capacity of 6 kg. The hydrolate (600 ml) and essential oil (1.6 ml) were kept for five months in glass bottles until use.

Identification of chemical compounds in the essential oil

In an amber bottle, 1 ml of essential oil was added and a solid-phase micro-extraction fiber (PALsystem Ingenious Simple Handling) was placed for 1 min. The sample was then injected using the injector of the gas chromatograph coupled to a mass spectrometer (GC-MS) for 45 min (Bicchi, 2000) and two replications were performed. For the chemical characterization of the sample, a gas chromatograph coupled to a mass spectrometer (GC-MS Agilent Technologies 7890 A GC System) was used.

A VF-5ms GC capillary column, 30 x 0.25 (0.25), was used. The temperature of the column furnace was started at 60 °C for 2 min, then at 120 °C for 15 min, and a final temperature of 250 °C; helium was used as a carrier gas at a constant flow of 0.5 ml min^{-1}. The compounds were identified by comparing the retention time and mass spectrum with the mass spectral library of the National Institute of Standards and Technology (NIST).

Isolation of *Botrytis cinerea*

Samples of *Rosa* sp. flowers with symptoms of *Botrytis cinerea* were collected during January 2022 in the municipality of Acaxochitlán, Hidalgo. The samples were labeled and placed in polyethylene bags. In Petri dishes with PDA culture medium (BD Bioxon), the fungus was isolated by passing fragments of 3-4 mm of rose petals previously disinfested with 1% sodium hypochlorite, then washed three times with double-distilled water, and dried on filter paper.

The dishes were left at room temperature and the growth of the fungus was recorded every 24 h; after three days, a PDA disc with mycelium was obtained with a sterile punch of 3.5 mm in diameter and was transferred to a new PDA culture medium to purify the fungus. To corroborate the identity of the fungus, pathogenicity tests were carried out on strawberry fruits of the 'CP Zamorana' variety obtained from a greenhouse of the Chapingo experimental field.

Ripe and unripe fruits were selected; the samples were immersed for 1 min in 1% sodium hypochlorite, then they were washed three times with double-distilled water and dried on filter paper; subsequently, the ripe and unripe fruits were placed separately in a sterile Petri dish and three replications were made. The fruits were inoculated by contact with a sterile swab containing seven-day-old mycelium. Only disinfested samples were considered as controls. The samples were left at room temperature until fungal growth was observed (three days).

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Identification of *B. cinerea*

A drop of lactophenol was added to a slide, and a scrape was made in the Petri dishes with growth of the fungus (colony) with a dissection needle to obtain mycelium and it was placed in the drop of lactophenol, then it was covered with the coverslip to observe under the Olympus CX31 microscope (40x). For the morphological identification of the fungus, the following structures were observed: gray mycelium and long and branched conidiophores, whose rounded apical cells produce clusters of ovoid, unicellular, colorless, or gray conidia (Brandhoff *et al*., 2017); identification keys were used to identify the color of the colony and the shape of the conidia, mycelium, and sclerotia formation (Abata *et al*., 2016). Likewise, the document Taxonomy and Identification in Illustrated Genera of Imperfect Fungi (Barnett and Hunter, 1986) was consulted.

Preparation of treatments

The bioassay used the poisoned agar method, which consisted of integrating the treatment substances into the culture medium (Balouiri *et al*., 2016). One hundred milliliters of PDA medium (39 g L^{-1}) were prepared for each treatment in 250 ml Erlenmeyer flasks. Essential oil of *T. remotiflora*, double-distilled water, and Tween 20 (0.1%) were used to prepare an emulsion to obtain seven concentrations (0.0001, 0.001, 0.01, 0.1, 0.5, 1, and 2%) of essential oil and hydrolate in four concentrations (100, 75, 50 and 25%) prepared with double-distilled water.

The homeopathic substances (1, 2, 3, 4, 5, 6, 60, and 200 CH) were prepared by Dr. Felipe de Jesús Ruíz Espinoza from the Anahuac Regional University Center of the UACH in accordance with Larios-Palacios *et al*. (2020) protocol. The fungicide was Cabrio® C (BASF, 2023) (Boscalid 25.20% + Pyraclostrobin 12.8%) (1 g L^{-1}). Cabrio® C and Tween® 20 (Thermo Fisher Scientific, 2023) (0.1 L^{-1}) were used as reference controls; in the case of Tween 20 (Tw20), a biological effect was observed in previous trials and we wanted to confirm it. PDA 39 g L^{-1} was the absolute control.

One drop of the homeopathic oils was added to each flask and succussion was made for 2 min. The flasks with the treatment substances were sterilized for 20 min in an autoclave (AESA model CV 300) at 120 °C and poured into sterile 90 mm glass Petri dishes. The culture medium was left to gel at room temperature for 24 h. Subsequently, a sterile punch of 3.5 mm in diameter was used to obtain a PDA disc with the inoculum of the mycelium of the seven-day-old fungus and a dissection needle was used to place the inverted disc in the center of the Petri dish. The Petri dishes with the treatment substances and inoculum were incubated in darkness at 18 ±2 °C in an oven as it is an optimal temperature for the growth of the fungus.

Experimental design

The experiment was established in a completely randomized design with eight homeopathic treatments and 14 more, result of the essential oil, hydrolate, control, Tween 20, and commercial fungicide, each with five replications and an absolute control; the experimental unit was a Petri dish with PDA culture medium with the treatments to be evaluated.

Data recording

With a digital vernier, the radial growth of the fungus was measured every 24 h until the control completely covered the Petri dish with mycelium (five days). The growth rate (GR) and inhibition percentage (%I) were calculated with the formulas shown in (Table 1).

Table 1. Formulas for calculating the variables evaluated.

Growth rate (GR)	Percentage of inhibition (%I)
$GR = \frac{Df - Di}{Tf - Ti}$	$\%I = \frac{D1 - D2}{D1}(100)$

Df= final growth diameter (mm); Di= initial growth diameter (mm); Ti= initial growth time (days); Tf= final growth time (days); D1= diameter of the mycelial growth of the control (mm); and D2= diameter of the mycelial growth of the influenced (mm).

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Data analysis

The recorded data were subjected to an analysis of variance and Tukey's mean comparison test ($p \leq 0.05$) using the SAS OnDemand for Academics software (SAS Institute Inc, 2024). The analysis was carried out by groups of treatments: oil, hydrolate, by considering the variable of percentage of inhibition and homeopathic substances with the variables of percentage of inhibition and growth rate.

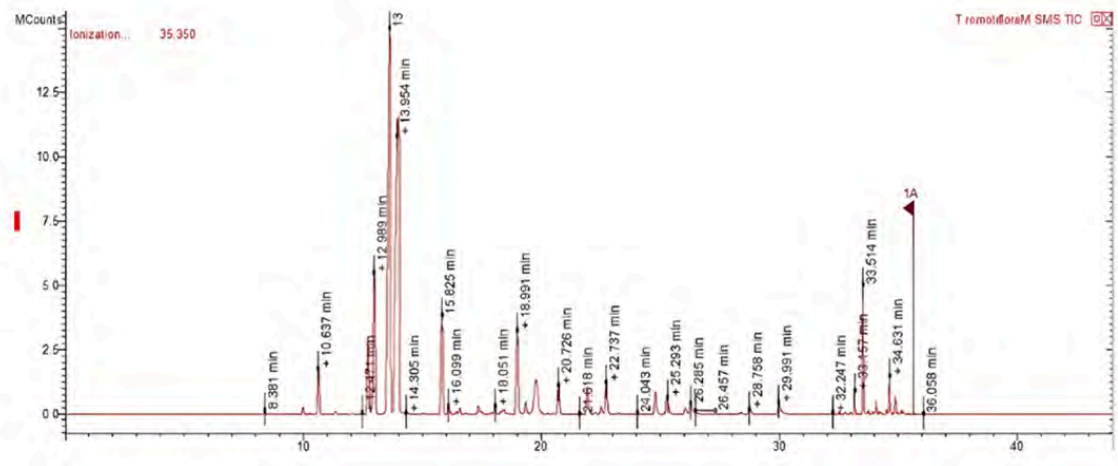
Probit analysis

Regression estimates and the concentration that produced inhibition (minimum inhibition dose) of 50 (MIC_{50}) and 90% (MIC_{90}) were generated by probit analysis in SAS OnDemand for Academics (SAS Institute Inc, 2023). The MIC allowed us to know the concentration of the treatment substance with which it is possible to inhibit, in percentage, the growth of mycelium.

Results and discussion

The retention time of compounds with the CG-EM equipment fluctuated between 8.38 and 36.05 min, a period in which 37 chemical compounds were detected, four of which were the majority: trans-β-Ocimene (6.98%), 2-Carene (4.98%), Cyclohexene, 1-methyl-4-(1-methylethylidene)- (4.87%), and 5,7-Octadien-4-one, 2,6-dimethyl-, (E)- (4.22%) (Figure 1). The compounds found in *T. remotiflora* oil, which are first identified, especially the major trans-β-Ocimene, have been found in other species of *Tagetes* (Xu *et al*., 2012) whereas 2-Carene appears to be specific to this species (Figure 1).

Figure 1. Chromatogram showing the peaks and retention times of compounds identified in samples of essen tial oil of *T. remotiflora*.



The morphology of reproductive structures confirmed the identity of *B. cinerea*, which was verified by pathogenicity tests that showed growth of the fungus that causes gray mold in strawberry fruits (Brandhoff *et al*., 2017). The isolates produced black sclerotia, the colonies were dark gray, aerial mycelium, and oval-shaped conidia.

In the toxicological evaluation, it was observed that, when the concentration of the oil was reduced, the inhibition decreased (100 to 3.45%) and the growth rate increased (0 to 10.13 mm day^{-1}) (Figure2).

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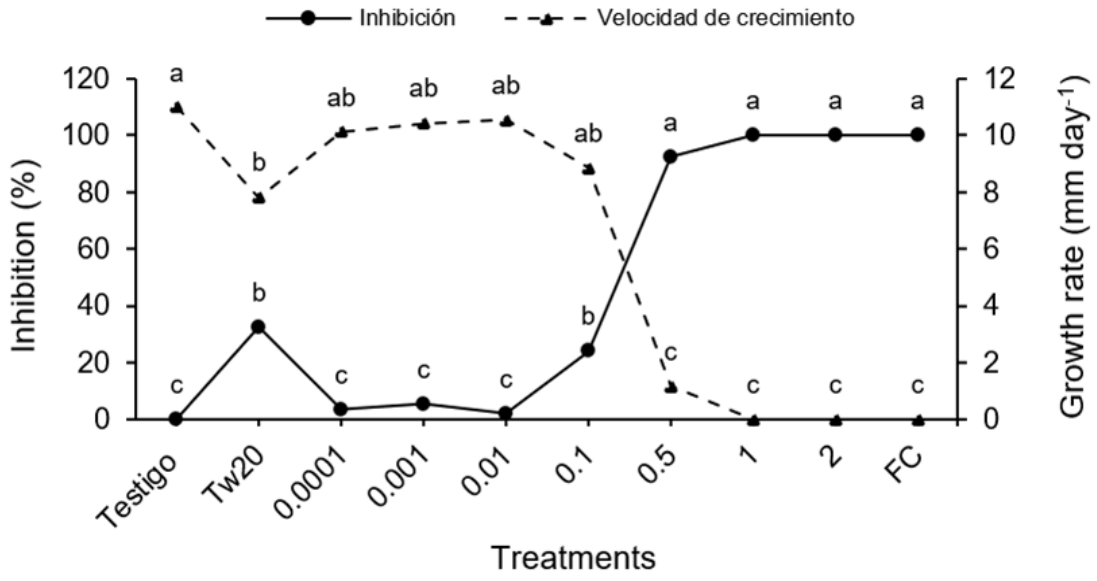
Essential oil at 1 and 2% and commercial fungicide (CF) totally inhibited mycelial growth (Figure 2), with statistically significant differences ($p< 0.0001$). The variability of *B. cinerea* sporulation can be explained by the complex interactions between climatic conditions and inoculum load, spore germination, and fungal growth in the culture medium (Fedele *et al*., 2019), a result that agrees with others obtained for the same fungus when applying *T. lemmonii* oil at 1% (Larios-Palacios *et al*., 2020) and *T. patula* at 1% and 0.1% (Romagnoli *et al*., 2005).

Figure 2. Effect of the essential oil of *T. remotiflora* on the mycelial growth of *B. cinerea* after five days. FC= commercial fungicide; Tw20= Tween 20 0.1%; 2= essential oil 2%; 1= essential oil 1%; 0.5= essential oil 0.5%; 0.1= essential oil 0.1%; 0.01= essential oil 0.01%; 0.001= essential oil 0.001% and 0.0001= essential oil 0.0001%. Means with the same letters in each variable are not statistically different (Tukey, $p \leq 0.05$).



At concentrations of 0.1, 0.01, 0.001 and 0.0001%, the inhibition of mycelium was greater (24.05 to 3.45%) than that recorded in the control (0%); with 0.1% oil, the growth rate was 8.84 mm day^{-1}, lower than the control (11 mm day^{-1}). *T. remotiflora* essential oil also reports inhibitory activity in the growth of bacteria, such as *Dikeya dadantii* and *Pseudomonas syringae* pv. *phaseolicola* (Rincón *et al*., 2012).

Essential oils and their components act on the membrane and cytoplasm and in certain situations, completely alter the morphology of cells; their activity could be caused by the properties of terpenes/ terpenoids (highly lipophilic and with low molecular weight) that are capable of altering the cell membrane (inhibiting ergosterol synthesis), thinning, distorting the hyphal wall, causing cell death, or inhibiting the sporulation and germination of fungi (Nazzaro *et al*., 2017).

The concentrations of 0.5, 1, and 2% did not show statistical differences in mycelial growth (Figure 2); therefore, the concentration of 0.5% (5 ml L^{-1}) was promising from an economic point of view since, being a lower concentration, a higher volume can be prepared as only 5 ml L^{-1} is required; for its part, with the concentration of 1 and 2%, 10 and 20 ml L^{-1} are required, respectively, so it would be advisable to implement *in vivo* evaluations.

Undoubtedly, isolating each major compound and evaluating their individual biological effect will be the next task, as will the experimental evaluation of different concentrations of the surfactant, since their inhibitory effect on mycelial growth was evident and their interaction with the essential oil will have to be discriminated. With 75 (H75) and 100% (H100) hydrolate, the inhibition percentage was 74 and 85% and the growth rate was 5 and 3.54 mm dia-1, respectively.

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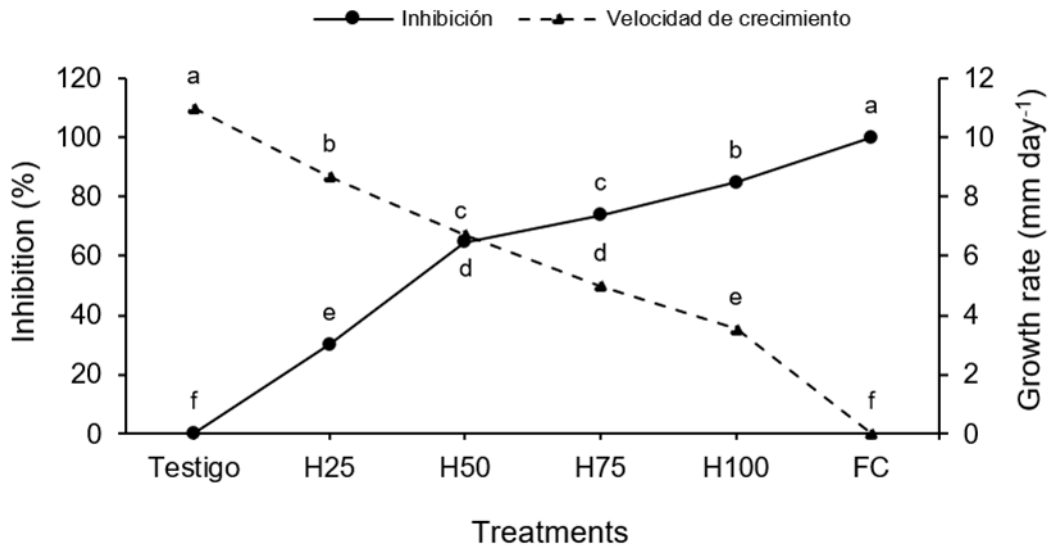
By reducing the concentration, inhibition in mycelial growth was also reduced, as in the case of 25% hydrolate (H25), which inhibited by 30%; this same trend was followed by the mycelial growth rate (Figure 3). Some studies on hydrolates of several plant species in *in vitro* and *in situ* antifungal activity highlight biological effects attributable to their chemical composition and the very small amounts of essential oils that remain dispersed (Jakubczyk *et al*., 2021).

Figure 3. Effect of the hydrolate of *T. remotiflora* on the mycelial growth of *B. cinerea* after five days. FC= commercial fungicide; H25= hydrolate 25%; H50= hydrolate 50%; H75= hydrolate 75%; and H100= 100% hydrolate. Means with the same letters in each variable are not statistically different (Tukey, $p \leq 0.05$).



The active components of the hydrolate can diffuse better into the aqueous medium around the microorganisms and their activity increases compared to essential oils that need initial solubilization in an organic solvent before their introduction into the aqueous medium (Karampoula *et al*., 2016); this situation reinforces the need to explore the role of surfactant in bioassays with essential oil, which, to dissolve in water, requires this input.

The inhibitory effect of *T. remotiflora* hydrolate on the growth of *B. cinerea* mycelium coincides with other studies that have used hydrolates of different species, such as *Origanum vulgare* L., *Tradescantia spathacea* Sw., and *Zingiber officinale* Roscoe, which inhibit the *in vitro* development of *Moniliophthora roreri* H.C. by 50%, Ramírez *et al*. (2011), and *Allium sativum* and *Allium fistulosum* that inhibit *Botrytis allii* and *Sclerotium cepivorum* (Lozano *et al*., 2000).

The inhibitory effect of 74 to 85% on *B. cinerea* (Figure 3) is the first evidence of *Tagetes* hydrolates as a fungistatic substance; therefore, in the perspective of its *in vivo* evaluation, the description of the chemical profile by liquid chromatography is necessary. In general, hydrolates have been explored little in their composition (Jakubczyk *et al*., 2021).

The control treatment with the surfactant Tween 20 (Tw20), used only for the preparation of essential oil treatments, led to inhibition of mycelial growth (32%) compared to the control (0%) and had an effect similar to that produced with essential oil at 0.0001 to 0.5% (Figure 2) and hydrolate at 25% (Figure 3).

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In the probit analysis (Table 2), low MIC values corresponded to the essential oil (0.16 and 0.51%) while high values to the hydrolate (41 and 127%); that is, trends of partial or complete inhibition of mycelial growth were detected when applying distillates of *T. remotiflora*; it is highlighted that doses of 0.51% of oil can inhibit the growth of the mycelium of *B. cinerea* by 90%, a promising result for establishing *in vivo* evaluations.

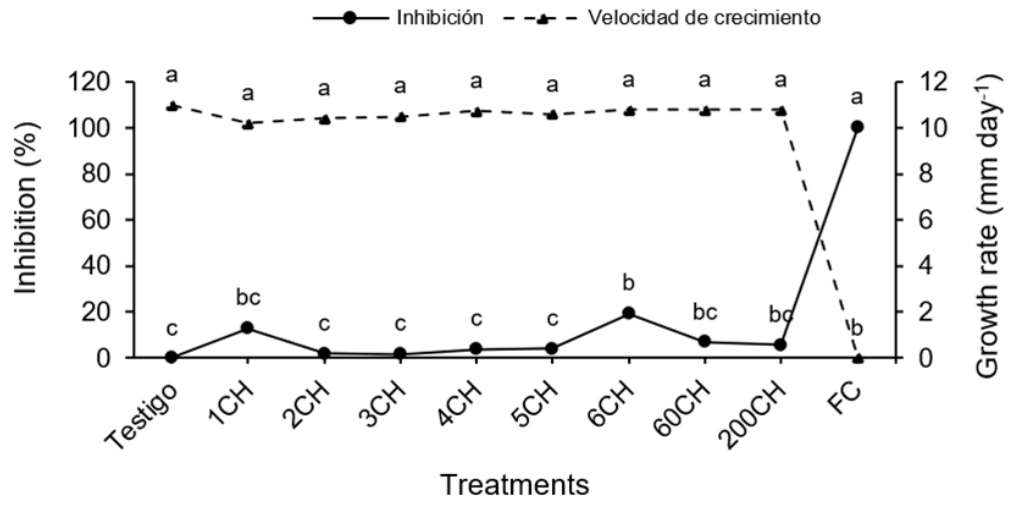
Table 2. Minimum inhibition doses for *B. cinerea*, estimated by probit analysis at five days of growth.

Distillates	Doses	
	MIC 50 (%)	MIC 90 (%)
Essential oil	0.16	0.51
Hydrolate	41.15	127.51

MIC= minimum inhibitory dose.

With homeopathic essential oil 6 CH, it was possible to inhibit the mycelial growth of *B. cinerea* with statistical significance ($p< 0.0001$). As dilution increased, no definite behavior was observed in mycelium growth; for example, 1 CH (low dilution), 4 CH (low dilution), 60 CH (medium dilution), and 200 CH (high dilution) inhibited 12.93, 3.8, 6.94, and 5.72%, respectively (Figure 4).

Figure 4. Effect of the homeopathic essential oil of *T. remotiflora* on the mycelial growth of *B. cinerea* after five days. CH= Hahnemannian centesimal dilution scale. Means with the same letters in each variable are not statistically different (Tukey, $p\leq 0.05$).



The biological effect of homeopathic substances has been observed with homeopathic sulfur 100 CH (Vargas *et al*., 2016), *Arnica montana* Hook and *Thuja occidentalis* L. 30 CH (Hanif and Dawar, 2017), calcarea carbonica 1 000 CH (Dos Reis and Ottoni, 2021), and *Sclerotinia sclerotiorum* (Lib.) de Bary 24 CH (Rissato *et al*., 2016), which inhibit from 40 to 90% mycelial growth and sclerotia production in *S. sclerotiorum*, *Phytophthora colocasiae* Racib., *Alternaria solani*, *Aspergillus niger* Tiegh., *Fusarium* spp., *Rhizoctonia solani* Kühn, and *Macrophomina phaseolina* (Tassi) Goid.

In *B. cinerea* , only nosode of *B. cinerea* 7 CH and arsenic 6 CH have been evaluated, inhibitory by 31.2 and 11.8%, respectively (Larios-Palacios *et al*., 2020), within these inhibition percentages was the inhibition response (19.2%) derived from the use of homeopathic oil 6 CH of *T. remotiflora* (Figure 4). The effect of homeopathic substances is due to the fact that the process of dilution and succussion generates nanoparticles (from the source material, in this case, the essential oil) with greater biological activity than with macromolecules (Rajendran, 2017).

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That is, during this process (dilution-succussion), the solvent can be modified in its physical structure by the presence, dilution, and succussion of the different solutes, even when they have disappeared in the progressive dilutions (Montfort, 2019) and this transfer and application of information that is linked to the solvent molecules is what maintains the effect or mechanism of action (Liu *et al*., 1996).

The evaluation of the homeopathic essential oil of *T. remotiflora* on the *B. cinerea* model is the first with *Tagetes* plants and adds to the scarce works on homeopathic essential oils of aromatic plant species against phytopathogenic fungi. The need to reduce the use of agrochemicals in Mexico's agriculture has increased interest in the possible application of plant extracts, essential oils, and homeopathic substances in the control of phytopathogens, hence attention to the study and use of the country's aromatic species, as in the case of *Tagetes*, could be a source of promising solutions; therefore, the *in vivo* evaluation of the results of this work is necessary, which is in process.

Conclusions

T. remotiflora oil contains 37 chemical compounds, four of which are the majority. Depending on the concentration, both of the taxon essential oil and its hydrolat, it is possible to partially or completely inhibit the mycelial growth and sporulation of *B. cinerea*; the probit analysis confirms the biocidal effect of the essential oil and the fungistatic effect of the hydrolate against *B. cinerea*. Homeopathic essential oil has minor fungistatic effects. The *in vivo* evaluation of *T. remotiflora* distillates and homeopathic essential oil is important to confirm their effectiveness as biocontrol inputs against *B. cinerea* and the mechanism of action of these substances requires research to understand their effect at the molecular level.

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