Biological Activity of Essential Oil from Two Aromatic Species on the *in vitro* Control of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.

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ABSTRACT

Objective: Essential oils (EO) from aromatic and medicinal plants (AMP) are considered a viable alternative for controlling phytopathogenic fungi of agronomic importance. This study evaluated the antifungal activity of thyme (*Thymus vulgaris* L.) and rue (*Ruta graveolens* L.) essential oils against *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.

Design/Methodology/Approach: The antifungal activity was assessed using the agar disk diffusion method, with concentrations ranging from 10% to 100% of each EO. The phytochemical composition was analyzed using GC-MS. Morphological observations were conducted with a scanning electron microscope (SEM).

Results: Significant antifungal activity was observed at concentrations of 50-100% of thyme EO. Rue EO exhibited a fungistatic effect for up to six days. Phytochemical analysis identified carvacrol (35.95%) and p-cymene (41.18%) as the major components in thyme EO, and 2-nonanone (24.24%) and 2-undecanone (68.69%) in rue EO.

Findings/conclusions: The evaluated EOs significantly impacted fungal morphology at 60% concentration. The strong antifungal activity of thyme EO against *C. gloeosporioides* suggests its potential as an eco-friendly control alternative.

Keywords: essential oil, antifungal activity, anthracnose, secondary metabolites.

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INTRODUCTION

There are over 8,000 species of phytopathogenic fungi, responsible for more than 70% of plant diseases, causing severe damage and even total loss of agricultural crops (Urbina, 2011). Their control has primarily relied on the use of commercial fungicides (Granados, 2018). However, pathogenic organisms have developed resistance to many fungicidal active ingredients (Gang *et al.*, 2019), in addition to causing harm to the health of producers (Silveira *et al.*, 2018). *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. is a facultative fungus that causes anthracnose in various agriculturally significant crops, leading to severe

economic losses due to its pathogenicity, as it infects more than 1,000 plant species (Pérez *et al.*, 2017; Gang *et al.*, 2019). In search of safer alternatives for producers, more effective disease control methods, and environmentally sustainable solutions, various natural products have been evaluated, including essential oils (EOs), which, depending on the species and their chemical composition, possess properties capable of combating pests and diseases (Granados, 2018; Calvo, 2016).

In this context, the present research aimed to find a sustainable solution to the phytosanitary issues faced in the agricultural sector by evaluating the antifungal activity of *Thymus vulgaris* and *Ruta graveolens* EOs against *C. gloeosporioides*.

MATERIALS AND METHODS

Essential Oil Extraction

Two species of aromatic and medicinal plants (AMP) were used: thyme (*Thymus vulgaris* L.) and rue (*Ruta graveolens* L.), both collected from the greenhouse area at the Colegio de Postgraduados, Montecillo Campus. The essential oils (EO) were extracted from the fresh biomass of leaves and stems. The steam distillation method was employed (Rodríguez-Álvarez *et al.*, 2012), where the plant material (PM) was placed in a 5 kg-capacity distiller and distilled for 3 hours. Table 1 shows the biomass and EO yield obtained from each plant species.

Inoculum Preparation

The COLTOR1 strain of *C. gloeosporioides* (Cruz-Lagunas *et al.*, 2020) was provided by the Faculty of Agricultural and Environmental Sciences at the Autonomous University of Guerrero. The phytopathogen was cultured on PDA (Potato Dextrose Agar) medium, using the dehydrated medium from BD Bioxon®. The PDA was prepared according to the product label instructions (39 g per liter of distilled water, sterilized at 120 °C for 15 minutes). A 5 mm diameter punch was used to cut circular pieces of fungal mycelium, which were then transferred to the Petri dishes with the prepared PDA medium. The Petri dishes were incubated at 28 °C for 15 days until the pathogen sporulated, producing master culture plates (Cruz-Lagunas *et al.*, 2020).

Antifungal Activity Evaluation

The antifungal activity was evaluated using the agar disk diffusion method, with slight modifications (Arce-Araya *et al.*, 2019), to determine the inhibition of mycelial growth. Two 5 mm diameter disks were taken from the fungal master culture using a hole punch and placed on Petri dishes containing PDA as the culture medium. Each disk was positioned at opposite ends of the dish, leaving the central part free, and incubated at 28 °C for 24 hours.

Table 1. Biomass quantity and EO yield of each distilled plant species.

Specie	Biomas (kg)	Yield essential oil (%)
Thymus vulgaris	5	0.2
Ruta graveolens	4	0.16

Subsequently, 10 μL of EO from each treatment was applied to a sterilized Whatman® No. 1 filter paper disk (5 mm diameter). The dishes were incubated at 28 °C for 20 days, and daily observations were made. Dimethyl sulfoxide (DMSO) was used to dilute the EOs. The variable evaluated was the inhibition zone, which was scanned on days 6 and 14 after EO application. The scans were processed using two image analysis programs: GIMP and ImageJ.

A completely randomized experimental design was employed, with each Petri dish as the experimental unit. Thirteen treatments were tested (EO dilutions from 10% to 100%, benomyl fungicide, distilled water, and DMSO solvent), with three repetitions per treatment. An analysis of variance and means comparison using Tukey's test ($p \leq 0.05$) was conducted using the SAS statistical package.

Chemical Composition of EOs

The chemical composition of the EOs was analyzed by gas chromatography-mass spectrometry (GC-MS) using a gas chromatograph (HP-6890) coupled with a mass selective detector (HP-5973) (Ricaldi & Martínez, 2014). A 30 m × 0.25 mm × 0.25 μm HP5-MS column was used for the chromatographic separation. The injection volume for each EO was 1 μL, injected in Split mode (10:1) at a temperature of 280 °C. The oven temperature was initially set at 60 °C and then increased by 5 °C/min until reaching 200 °C, where it was maintained for 1 minute. The column flow was 1 mL/min of ultrapure helium (99.999%). Data acquisition was performed in electron impact mode within a range of m/z 50 to m/z 550 in SCAN mode. The compounds were identified by comparing their mass spectra with the NIST 2011 library and/or with standard compounds.

Scanning Electron Microscopy

The fungi used in the bioassays were observed using a JEOL JSM-6390 scanning electron microscope (SEM) (Gutiérrez-Iparraguirre, 2019). The process began with the fixation of samples by placing thin sections of the fungal mycelium into vials containing 3% glutaraldehyde in 0.1 M Sorensen phosphate buffer at pH 7.2 for 24 hours. The samples were then dehydrated through two rinses with deionized water for 30 minutes, followed by sequential ethanol rinses from 30% to 100% for 20 minutes each. The samples were brought to critical point drying for 2 hours at 1071 psi and 31°C using a Sandri®-780A dryer. The dried samples were mounted on sample holders and coated with gold-palladium.

RESULTS AND DISCUSSION

Biological Activity of *Thymus vulgaris*

The EO of *T. vulgaris* exhibited inhibition at concentrations ranging from 50% to 100% in both recorded evaluations (days 6 and 14), reaching inhibition zones of 1907.4 mm^2 and 1908 mm^2, respectively (Figure 1), relative to the area of the Petri dish (1963.5 mm^2). Similar findings were reported in a study that evaluated the biological activity of thyme EO against *Fusarium* spp. *in vitro*, where complete mycelial growth inhibition was observed at concentrations of 500 and 1000 mg kg^{-1} (Caballero *et al.*, 2018). It has been reported that

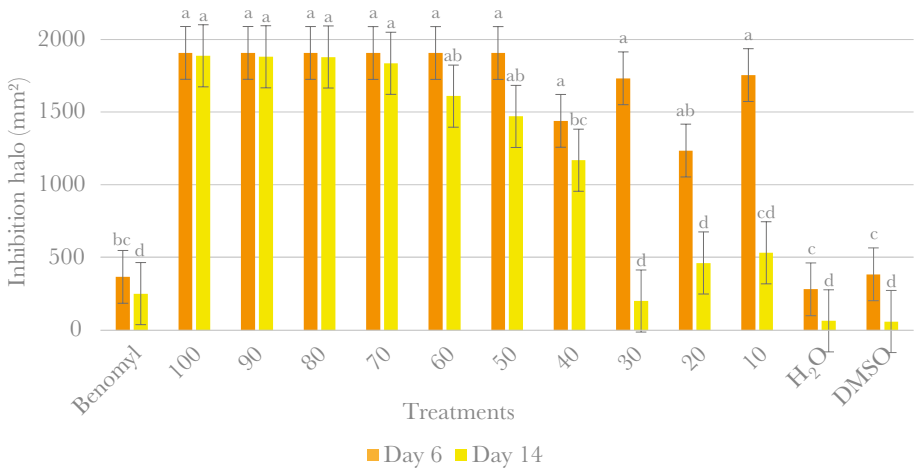

Figure 1. Inhibitory effect of *T. vulgaris* EO on the growth of *C. gloeosporioides*. Each point represents the mean of three replicates taken on days 6 and 14 ± standard error. Different letters in the graphs indicate significance (Tukey, p≤0.05).

thyme EO possesses fungicidal properties primarily due to its terpene content, particularly thymol and carvacrol (Hernández *et al.*, 2018; Morkeliūnė *et al.*, 2021).

Biological Activity of Ruta graveolens

The results of the in vitro test of *R. graveolens* EO showed an inhibitory effect during the first evaluation (day 6) at concentrations of 50%, 60%, 70%, 80%, 90%, and 100%, with inhibition zones of 970.6 mm^2, 924.1 mm^2, 1665.4 mm^2, 1536.4 mm^2, 1572.3 mm^2, and 1649.6 mm^2, respectively. However, by the second evaluation (day 14), the EO's effect diminished, resulting in inhibition zones of 538.3 mm^2, 400.2 mm^2, 1277.8 mm^2, 816.4 mm^2, 158.6 mm^2, and 618.6 mm^2, respectively (Figure 2). This suggests a fungistatic effect where the pathogen's growth was inhibited during the first six days of EO application.

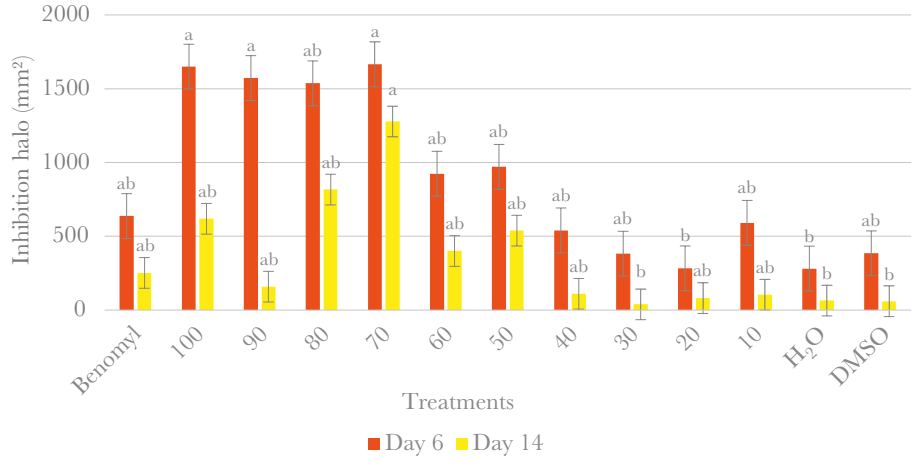

Figure 2. Inhibitory effect of *R. graveolens* EO on the growth of *C. gloeosporioides*. Each point represents the mean of three replicates taken on days 6 and 14 ± standard error. Different letters in the graphs indicate significance (Tukey, p≤0.05).

In contrast, other recent studies have reported fungicidal activity of rue EO against *Coniothyrium phyllachorae*, where the authors observed complete mycelial growth inhibition with the application of 3 μL of EO (Ceja *et al*., 2023). The differences in results between this study and the previous one may be due to the different pathogens tested, as well as the potential higher resistance of *C. gloeosporioides* due to its high pathogenicity.

An important observation is that the commercial fungicide typically used to control *C. gloeosporioides* showed no effect, allowing the pathogen to continue growing. The inhibition zone measurements were 367 mm^2 and 251.8 mm^2 during both evaluations. On the other hand, the DMSO control did not exhibit any inhibitory effect on growth in the evaluations performed, with inhibition zones of 384.7 mm^2 and 59.9 mm^2, respectively. In contrast, pathogen growth in the H_2O control developed successfully, yielding inhibition zones of 281.3 mm^2 and 64.5 mm^2 during both evaluations.

Chemical Composition of Essential Oils

In the phytochemical analysis using GC-MS, 15 volatile compounds were identified in *T. vulgaris*, with carvacrol (35.95%) and p-cymene (41.18%) being the main components, while thymol was present in smaller amounts (0.19%) (Table 2). The primary compounds characterizing *T. vulgaris* EO were the monoterpenes thymol, carvacrol, and p-cymene. Thymol, the most extensively studied component, has demonstrated antimicrobial, antitumor, antiparasitic, antifungal, antioxidant, and anti-inflammatory activities (Vassiliou *et al*., 2023). p-Cymene, found in relatively high concentrations, exhibits strong antiviral, antioxidant, and antitumor properties. Carvacrol, known for its potent antimicrobial and

Table 2. Volatile compounds identified in *Thymus vulgaris* essential oil by gas chromatography-mass spectrometry (GC-MS).

Compounds	Formula	Rt (min)	Score match (%)	Composition (%)
1-hexen-3-ol	$C_6H_{12}O$	5.32	79	0.46
β-myrcene*	$C_{10}H_{16}$	5.60	82	1.20
isoterpinolene	$C_{10}H_{16}$	6.19	87	0.72
p-cymene*	$C_{10}H_{14}$	6.38	88	41.18
eucalyptol	$C_{10}H_{18}O$	6.53	81	0.40
γ-terpinene	$C_{10}H_{16}$	7.17	83	8.72
(*Z*)-sabinene hydrate	$C_{10}H_{18}O$	7.36	79	0.77
linalool*	$C_{10}H_{18}O$	8.13	85	2.76
(+)-2-bornanone	$C_{10}H_{16}O$	9.32	86	0.84
borneol	$C_{10}H_{18}O$	9.87	79	1.37
thymol methyl ether	$C_{11}H_{16}O$	11.89	85	0.89
carvacrol	$C_{10}H_{14}O$	13.18	83	35.95
thymol	$C_{10}H_{14}O$	13.25	80	0.19
6-ethyl-3,4-xylenol	$C_{10}H_{14}O$	13.40	80	1.13
α-farnesene*	$C_{15}H_{24}$	16.48	82	3.40

*Compound compared with a standard.

antifungal effects, is likely the compound responsible for the fungicidal activity observed in thyme EO. However, it is important to note that the antifungal activity of an EO is not dependent solely on a single compound but rather on the synergy among its components (López, 2006; Vassiliou *et al*., 2023).

In *R. graveolens*, 9 chemical compounds were identified, with 2-nonanone (24.24%) and 2-undecanone (68.69%) as the major components, and butyl propyl oxalate ester (0.06%) present in smaller amounts (Table 3). Previous studies have reported aliphatic ketones (2-nonanone and 2-undecanone) as the primary components of *R. graveolens* EO, responsible for its antimicrobial properties (Ceja *et al*., 2023). Although the antifungal activity of rue EO has been attributed to fatty acids such as linoleic, palmitic, and retinoic acids, these compounds were not identified in the phytochemical analysis of this study, which may explain the absence of fungicidal activity (Grande *et al*., 2019; Ceja *et al*., 2023).

Morphological Damage Caused by the Essential Oils

Observations made through SEM indicated that the presence of *T. vulgaris* EO at a 60% concentration significantly affected the morphology of *C. gloeosporioides*. The hyphae exhibited swelling, wrinkling, rupture, and deformations, while the conidia showed deformations, perforations, and collapse.

In contrast, *R. graveolens* EO did not cause morphological damage to the conidia; however, slight damage, such as wrinkling and rupture, was observed in the hyphae (Figure 3). These results suggest that *C. gloeosporioides* conidia are resistant to the phytochemical components of rue EO. The study of morphological alterations caused by EOs in phytopathogenic fungi is limited; however, in the few studies conducted, similar damage has been reported in the hyphae and conidia of *C. gloeosporioides* when treated with chitosan and salicylic acid (Ramos *et al*., 2018).

It has been confirmed that EOs affect the cell membrane, altering its permeability, nutrient uptake, and the mycelial development of the pathogen as part of their fungicidal mechanism (Chávez *et al*., 2020; He *et al*., 2018).

Table 3. Volatile compounds identified in *Ruta graveolens* essential oil by gas chromatography-mass spectrometry (GC-MS).

Compounds	Formula	Rt (min)	Score match (%)	Composition (%)
2-nonanone	$C_9H_{18}O$	7.96	78	24.24
oxalic acid butyl propyl ester	$C_9H_{16}O_4$	8.24	78	0.06
1,2-dimethyl-1,3-cyclopentadiene	C_7H_{10}	9.26	83	0.58
2-decanone	$C_{10}H_{20}O$	10.52	81	1.61
6-methyl-2-heptanol acetate	$C_{10}H_{20}O_2$	11.67	75	0.90
2-undecanone	$C_{11}H_{22}O$	13.25	82	68.69
2-dodecanone	$C_{12}H_{24}O$	15.02	76	1.42
butanimidamide	$C_4H_{10}N_2$	15.77	77	0.78
2-octanone	$C_8H_{16}O$	18.27	78	1.29

*Compound compared with a standard.

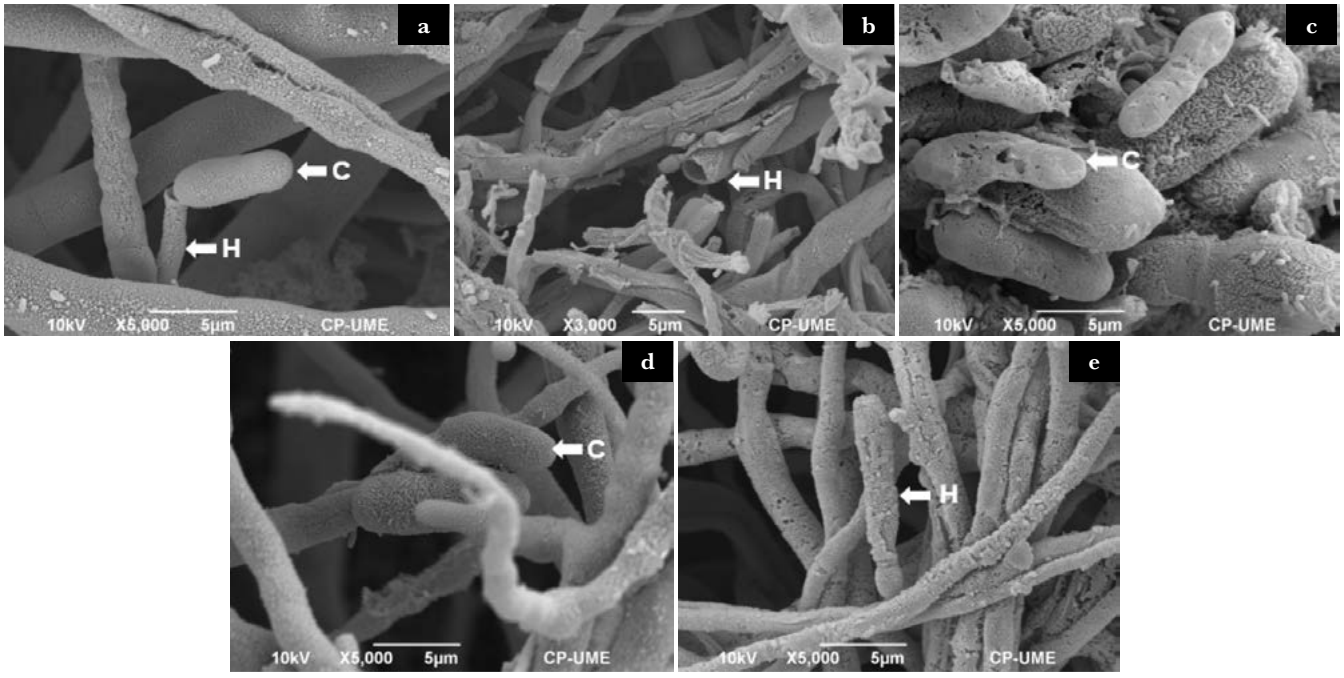

Figure 3. Microphotographs of *C. gloeosporioides* at 5000X, 3000X, 5000X, 5000X, and 5000X magnification, obtained through SEM. a) H_2O control; b) 6 days after applying *T. vulgaris* EO at 60%; c) 14 days after applying *T. vulgaris* EO at 60%; d) 6 days after applying *R. graveolens* EO at 60%; e) 14 days after applying *R. graveolens* EO at 60%. *C: Conidia*; H: Hyphae.

CONCLUSIONS

The main chemical components of *T. vulgaris* EO were carvacrol and p-cymene, while for *R. graveolens* EO, the primary components were 2-nonanone and 2-undecanone. The essential oils of *T. vulgaris* and *R. graveolens* present an alternative for controlling the fungus *C. gloeosporioides*. *T. vulgaris* EO can be used as a fungicidal agent, while *R. graveolens* EO is recommended as a fungistatic agent. For optimal results, periodic applications, at least every six days, are necessary to achieve proper control of *C. gloeosporioides*. The chemical composition of *T. vulgaris* and *R. graveolens* EOs has a morphological impact on *C. gloeosporioides*, even at low concentrations. However, it is recommended to conduct *in vivo* research to compare the results and assess whether the behavior of each EO is consistent under real-world conditions.

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