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In Vitro and *in Situ* Antifungal Potential of Essential Oils and Nanoemulsions from Two Cameroonian Aromatic Plants against Pathogens Responsible for Post-Harvest Fruit Rot of *Solanum lycopersicum* L. (Solanaceae)

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Abstract

Introduction: Post-harvest tomato storage faces several phytosanitary challenges, particularly damage caused by pathogenic microorganisms. The objective of this study is to evaluate the *in vitro* and *in situ* antifungal potential of essential oils and nanoemulsions from two Cameroonian aromatic plants against pathogens responsible for post-harvest tomato fruit rot, with the aim of extending the shelf life of this food product. **Methodology:** This study was conducted on healthy and infected tomato (*Solanum lycopersicum* L.) fruits of the (Rio) variety, grown in Dschang, in the West Region of Cameroon. Samples were brought to the laboratory between July and October 2024. The two Cameroonian aromatic plants used for essential oil extraction were fresh thyme (*Thymus vulgaris*) leaves and fennel (*Foeniculum vulgare*) seeds. Extraction was performed using a Clevenger hydro-distiller. Nanoemulsions were obtained using an emulsification method with some modifications. Extraction yields were calculated and the oils were characterized by gas chromatography-mass spectroscopy (GC/MS). The medium used for fungal culture was pre-

prepared potato dextrose agar. Antifungal activities were evaluated *in vitro* for the *Foeniculum vulgare* oil and *in situ* for the essential oil and the *Thymus vulgaris* nanoemulsion on the mycelial growth of the isolates. These evaluations were performed using the solid-state dilution method, and subsequently, the antimicrobial activity parameters and rot diameters were determined. The data were subsequently analyzed using IBM SPSS Statistics Version 20.0. Duncan's multiple ranges test was used for pairwise comparisons if the ANOVA result was significant. **Results:** This study shows that all essential oils (EOs) have a liquid consistency and a density lower than that of water. EO yields varied from one plant species to another. Chemical analysis by GC/MS revealed 29 and 23 compounds, respectively, identified in the EOs of *Fragmentus vulgare* and *Thymus vulgaris*, with anethole (57.20%) and thymol (40.8%) being the major products. Essential oils and their nanoemulsions exhibited varying degrees of antifungal activity against the mycelial growth of *Rhizopus stolonifer* (MB4) and *Colletotrichum cococodes* (MB5) isolates. These effects were dose-dependent and pathogen-specific. **Conclusion**: These essential oils are rich in bioactive compounds and possess innovative properties. They can serve as a basis for post-harvest tomato preservation against microorganisms responsible for rot.

Keywords

Antifungal Potential, *In Vitro* and *in Situ*, Essential Oils, *Solanum lycopersicum* L., *Foeniculum vulgare*, *Thymus vulgaris*

1. Introduction

Food self-sufficiency is a priority objective worldwide. It reflects the desire of states to better control the evolution of a food system that is showing a trend toward rapid and uncontrolled change [1]. The tomato (*Solanum lycopersicum* L.), from the Solanaceae family, is one of the most important vegetable crops in the world, particularly in Central Africa and Cameroon. It is considered an essential ingredient in many dishes. In fact, after the potato, the tomato is the second most consumed fresh or processed vegetable in the world [2]. Lycopene, a natural pigment belonging to the carotenoid family, synthesized by plants and microorganisms, is the most abundant carotenoid terpene in the tomato fruit [3]. It owes its name to the Latin name for tomato: *Solanum lycopersicum* L. It also has antioxidant properties; that is, it reduces cell damage and lowers the risk of developing cancer or certain diseases [4]. Tomato production is very significant, but due to a lack of processing and preservation facilities, losses are enormous, and this product becomes very scarce during the low-yielding season. These losses are reported globally at around 42% [5] and also between 25 and 45% in developing countries in development [6]. The main reference study, conducted by the FAO on global food loss and waste [7], indicates that globally, one-third of agricultural produc-

tion intended for human consumption is lost or wasted (between the production and consumption stages), and more than 50% in the case of fruits and vegetables in sub-Saharan Africa. Furthermore, tomatoes are highly perishable, and their supply chain remains poorly organized and structured. This alarming observation is based on the spoilage of unsold produce, which represents enormous losses for producers and traders. Thus, the problem of finding alternative solutions arises. The lack of modern production preservation techniques causes damage and production losses [8]. One alternative aimed at better controlling this crop would be the use of essential oils (EOs).

Essential oils are beginning to attract considerable interest as a potential source of bioactive natural molecules, as they provide various functions for plants. It is with this in mind that we are interested in the essential oils of two Cameroonian aromatic plants: *Foeniculum vulgare* and *Thymus vulgaris*. These plants were chosen because of their diverse uses in the pharmaceutical, cosmetic, and agri-food sectors; they are commonly used in traditional medicine for the treatment of nervous and gastrointestinal disorders, as antispasmodics, analgesics, anti-inflammatories, antipyretics, diuretics, and sedatives [9] and [10].

2. Methodology

2.1. Materials

Plant Material

The study was conducted on healthy and infected tomatoes and Cameroonian aromatic plants.

1) Tomato

The tomato (*Solanum lycopersicum* L.) of the (Rio) variety was harvested in Dschang, in the West Region of Cameroon. Samples were brought to the laboratory between July and October 2024.

2) Aromatic Plants

The aromatic plants used for essential oil extraction were fresh leaves of *Thymus vulgaris* and seeds of *Foeniculum vulgare*, both harvested in two cities in Cameroon during specific periods, and identified in the National Herbarium in Yaoundé. Table 1 presents the various details relating to each of the plants.

Table 1. Summary of information on the aromatic plants used.

Plante	Part of the plant used	Origin	Dates of harvest	Identification number	Family
Thymus vulgaris	leaves	Santchou	15th/06/2024	25746/SRF/Cam	Lamiaceae
Foeniculum vulgare	seeds	Dschang	15th/06/2024	25580/SRF/Cam	Apiaceae

2.2. Methods

2.2.1. Culture Medium, Preparation, and Isolation of Microscopic Fungi

The medium used for culturing the fungi was called PDA (Potato Dextrose Agar) supplemented with an antibiotic (Gentamicin) and prepared under the following

conditions:

Boil 200 g of peeled potatoes with 1 liter of distilled water. Once the potatoes are thoroughly cooked, filter the mixture to collect the juice in a 1 L Erlenmeyer flask. Add 20 g of glucose and 15 g of agar. Top up the mixture to 1 L with distilled water and shake. Once the medium has been thoroughly shaken, autoclav at 120˚C for 30 minutes. Mold growth was ensured by incubation at 28°C for approximately 7 days.

2.2.2. Purification of Fungal Isolates

The isolates obtained were purified by successive subculturing, which consists of aseptically removing a mycelial explant from a Petri dish containing an old strain using a loop and transferring it to a Petri dish containing sterile medium. Subculturing was performed in this way until pure culture was obtained [11].

The frequency of occurrence (Fr) of each morphotype was determined according to the formula:

$$\mathrm{Fr}(\%) = \frac{\text{Similary morpphotic number x 100}}{\text{Total isolated number}}$$

2.2.3. Pathogenicity Test

The pathogenicity test was performed based on Koch's postulate. It was carried out in the laboratory on detached tomato fruits according to the method used by [12], with some modifications.

2.2.4. Identification of Fungal Isolates

The identification of the different isolates was based on macroscopic and microscopic characteristics using the identification keys of Chabasse [13] and [14].

1) Macroscopic Identification

Macroscopic examination of pure cultures on PDA was performed after 7 days of incubation at 28˚C. This allowed for the determination of the following cultural characteristics: growth rate, shape, texture, and color of the colonies.

2) Microscopic Identification

Microscopic observations were carried out by examining a slide of mycelial filaments collected with tape and mounted under a light microscope. Observations focused on the type of thallus (septate or not), the structure and color of the hyphae (dark or light), the shape and color of the conidia (fusiform, oval, round, etc.), and the type of conidiogenic cell and conidiospores (aspergillar head, acervuli, pycnidia, etc.).

2.2.5. Essential Oil Extraction

Essential oils are extracted from fresh or dried leaves using a Clevenger-type hydro-distiller consisting of a round-necked heating mantle and a steam condenser (cooler) column.

After weighing, the plant material is placed in the mantle and immersed in water.

The entire system is brought to a boil for 3 hours. The essential oils are carried by the steam, collect in the Florentine flask, and are then gathered using the tap at

the base of the flask. They are then stored in hermetically sealed bottles and kept protected from light at 4˚C until use.

1) Calculation of Essential Oil Yield

Essential oil yield is defined as the ratio between the mass of essential oil obtained after extraction and the mass of the plant material used.

According to the **AFNOR standard** [15], it is given by the following formula**:**

$$Rd = Mh/Mv \times 100$$

where:

Rd: Essential oil yield expressed as a percentage (%)

Mh: Mass of essential oil obtained in grams (g)

Mv: Mass of dry plant material used in grams (g), which is 200 g

2) Calculation of Essential Oil Density

The density of an essential oil is the ratio of the mass of oil extracted to its volume.

2.2.6. Chemical Composition Analysis of Essential Oils by GC/MS

The chemical constituents of the essential oils were identified by gas chromatography (Agilent Technologies 7890A) coupled to mass spectrometry (Agilent Technologies 5975C inert MSD) using electron impact. The chromatograph is equipped with a divider injector programmed at 250˚C and a column HP5MS type capillary (30 m × 0.25 µm film thickness). The carrier gas is helium, with a constant flow rate of 0.8 ml/min in the column. The sample is injected in split mode (50:1). A volume of 1 µl of essential oil diluted in 50% hexane is injected through the injector septum.

2.2.7. Formulation of Essential Oils by Emulsion

The formulation was carried out following the emulsification method used by [16], with some modifications. The emulsion (oil-in-water type) was prepared at 5% by mixing an oil phase (a mixture of the emulsifier and the essential oil) and an aqueous phase (a mixture of distilled water and the first phase). The emulsifier used was Tween 80.

The oily phase (nonpolar phase) was prepared by mixing Tween 80 (non-ionic surfactant) and the essential oil in a 1:1 ratio using a magnetic stirrer (7000 rpm) for 30 minutes. The aqueous phase (polar phase) was prepared by adding the surfactant Tween 80 to distilled water. Tween 80 improves the stability and dispersion of the product (dispersion of the organic phase within the continuous phase).

The emulsion was formed by adding the aqueous phase at a rate of 2 mL/min to the oily phase while it was still being stirred. The mixture was then blended under the same conditions for 2 hours at room temperature (28 ± 2˚C).

The emulsion was subsequently subjected to physicochemical characterization, and its stability was tested.

1) Physicochemical Characterization and Emulsion Stability Tests

The physicochemical characterization and stability tests of the emulsion were

also performed using the same solvent as the emulsification method used by [16].

- Determination of the hydrogen potential pH

The pH was measured using a pH meter initially calibrated. The pH meter electrodes were placed in a 1% aqueous solution of the prepared formula.

- Determination of Foam Persistence

Foam persistence is a measure of the amount of foam likely to be present in a spray tank after dilution of the product with water. The formulated product (2 mL) was placed in a graduated cylinder (100 mL) containing hard water, and the volume was adjusted to the 100 mL mark. The cylinder was sealed, inverted 30 times at a 180˚ angle, and then held in this position for one minute. The volume of foam formed at the top was measured by reading the graduation on the graduated cylinder.

2.2.8. Evaluation of the *in Vitro* Antifungal Activity of Essential Oils

The *in vitro* antifungal activity was evaluated based on the mycelial growth of the isolates. This was performed using the solid medium dilution method (incorporation into agar) as described in [17]. Subsequently, the antimicrobial activity parameters (MIC and MMC) were determined.

1) Principle of the method

This method is based on the ability of a microorganism to grow on an agar medium supplemented with a substance to be tested (essential oil) at specific concentrations.

2) Procedure

- Preparation of Essential Oil Stock Solutions

The stock solution for each essential oil was prepared in a 1:9 (v/v) ratio, solubilizing 1 mL of essential oil in 9 mL of DMSO (dimethyl sulfoxide) to a final volume of 10mL. The DMSO acts as a surfactant, reconciling the aqueous phase (which consists of the PDA culture medium) with the oily phase of the essential oil. The concentration of the stock solution was calculated based on the mass of one milliliter of each essential oil. Preparation was carried out under a laminar flow hood for optimal sterility. Each essential oil obtained is mixing by nanoemulsion and isolate according to Table 2 below.

Table 2. Concentration ranges used for each essential oil EO, nanoemulsion (NE), and isolate.

Oil and emulsion	MB4 isolate	MB5 isolate
EO of *T. vulgaris*	100 - 2000 ppm	100 - 2000 ppm
NE *T. vulgaris*	10 - 700 ppm	100 - 500 ppm
EO *F. vulgare*	1000 - 10,000 ppm	1000 - 10,000 ppm
NE *F. vulgare*	1000 - 8000 ppm	1000 - 10,000 ppm
Reference antifungal	100 - 1000 ppm	1000 - 10,000 ppm

Legend: EO: essential oil, NE: nanoemulsion.

- **Preparation of the stock solution of the reference antifungal**

The stock solution of Plantineb (reference antifungal) was also prepared under a laminar flow hood to avoid any contamination. The preparation involved dissolving 1 g of Plantineb powder in 10 mL of sterile distilled water. The range of Concentrations ranging from 32.5 ppm to 300 ppm were used for all isolates according to the following procedure:

The <<control>> consisted of 10 mL of culture medium only, which was not inoculated with pathogenic isolates;

The <<negative>> control consisted of 10 mL of culture medium only, which was inoculated with the pathogenic isolates;

The <<test>> consisted of 10 mL of culture medium supplemented with the substance to be tested, which is the essential oil (EO);

The <<positive control>> consisted of 10 mL of culture medium supplemented with the reference antifungal (Plantineb).

The media thus prepared were homogenized and poured into 90 mm diameter Petri dishes and left under a fume hood until the agar solidified.

2.2.9. Inoculation of Isolates

After the culture medium solidified in the Petri dishes, a 5 mm diameter mycelial disc, cut with a punch, was removed from the periphery of the pathogenic cultures, which were 4 days old for MB4 and 7 days old for MB5, using a flame-sterilized scalpel. This disc was placed in the center of the previously prepared Petri dishes, except for the control dishes, which did not receive a mycelial disc. All dishes were sealed with Parafilm and incubated upside down (to prevent water vapor from the cooling of the culture medium from contaminating it) at 28 ± 2˚C for 4 and 7 days for isolates MB4 and MB5, respectively. Each of these isolates reached the size of a 90 mm Petri dish after these days. Each trial was repeated 3 times and the entire experiment was repeated 2 times. In each experiment, the tests were carried out under a laminar flow hood near the flame of a Bunsen burner to ensure sterility.

1) Determination of Antifungal Activity Parameters (MIC and MFC) and Nature of Inhibition of Essential Oils and Nanoemulsions *in Vitro* and *in Situ*

Following *in vitro* antifungal tests performed with the essential oils of the two plants studied, the essential oil that showed the best activity was selected for the characterization and formulation of the biopesticide (nanoemulsion) according to the emulsification method used by Purkait [16], and for *in situ* testing.

After 4 and 7 days of incubation, the minimum inhibitory concentration (MIC) was determined. This corresponded to the lowest concentration at which no visible growth of the microorganism was observed. Subsequently, the minimum fungicidal concentration (MFC) was determined. Its determination was made by transferring explants from Petri dishes that showed no visible growth during the entire incubation period into new dishes containing culture medium that was not supplemented with either essential oils or a reference antifungal. After 4 and 7

days of incubation for each isolate, the lowest concentration at which no explant growth resumed was considered the MFC.

The MFC/MIC ratio was calculated to determine the nature of the inhibition exerted by the essential oils on the different isolates. According to Voukeng [18], fungicidal activity is defined as MFC/MIC < 4 and fungistatic activity as MFC/MIC > 4.

Evaluation of Rot Diameter

After 6 days of incubation at 28 ± 2˚C for MB4 and 7 days for MB5, the average rot diameter was measured in mm. From this measurement, the percentage of protection or inhibition was calculated using the following formula:

$$\%I = ((\text{Control diameter} - \text{Treated diameter})/\text{Control diameter}) \times 100.$$

Control diameter: represents fungal growth without treatment.

Treated diameter: represents fungal growth on treated fruit.

3. Results

3.1. Physical Characteristics and Essential Oil Extraction Yield

The physical characteristics and yield obtained after extraction from the two investigated plants are presented in Table 3.

Table 3. Physical characteristics and essential oil extraction yield.

	Aspect	Odeur	Colour	Yield (%)	Density (g/ml)
Thymus vulgaris	Liquid	Soft/citronella	Yellow amber	0.55	0.92
Foeniculum vulgare	Liquid	Anisee	pale yellow	0.56	0.91

Analysis of this table shows that all the essential oils have a liquid consistency and a density lower than that of water. Essential oil yields varied from one plant species to another, ranging between 0.55% and 0.56%.

3.2. Chemical Composition of Essential Oils

GC-MS chemical analysis of pure essential oils allowed for the determination of their quantification and chemical composition from the obtained chromatograms and mass spectra. The results of the pure essential oil analyses are presented in Table 4.

From this table, we can conclude that 29 compounds were identified in the essential oil of *F. vulgare*, representing 96.8% of the total chemical composition, with anethole (57.20%) and limonene (25.05%) being the major compounds. 23 compounds were identified in the essential oil of *Thymus vulgaris*, representing 91.57% of the total chemical composition, the most abundant of which are, respectively: thymol (40.8%), pcymene (12.07%), and β-terpinene (10.25%).

Table 4. Chemical composition of pure essential oils from the two aromatic plants analyzed by GC-MS.

Order	Components	Nber (%)	Nber (%)
		EO of Fv (29)	EO of Tv (23)
•	*α*-pinene	1.3	3.48
•	Anethole	**57.20**	-
•	*α*-thujene	-	1.76
•	Camphene (Bicyclic monoterpene)	2.34	-
•	Ocimene	-	-
•	*β*-pinene	3.42	7.73
•	Fenchone	3.5	-
•	Sabinene	0.87	-
•	Limonene	**25.05**	**-**
•	*β*-myrcene	1.63	1.07
•	*α*-phellandrene	0.31	0.26
•	*α*-terpinene	0.42	0.77
•	D-limonene	**3.70**	**-**
•	1.8-cineole	**-**	-
•	Cis-*β*-ocimene	-	-
•	*γ*-terpinene	1.06	**10.02**
•	Trans-*β*-ocimene	-	-
•	p-cymene	-	**12.07**
•	6-methylhept-5-en-2-one	-	-
•	1-octenyl-3-acetate	0.98	-
•	3-octanol	0.35	-
•	Menthone	-	-
•	*α*-fenchyle acetate	0.22	-
•	Citronellal	-	-
•	Menthofurane	-	-
•	Isomenthone	-	-
•	*β*-bourbonène	-	0.38
•	Linalool	1.13	-
•	Hydrated (E)-sabinène	0.18	0.19
•	methyl acetate	-	-
•	Isoneral	-	-
•	Isopulegol	-	-
•	bornyle acetate	**2.24**	-
•	2-undecanone	-	-
•	terpinen-4-ol	1.33	1.65
•	Menthol	-	-
•	Pulegone	-	-
•	(E)-*β*-farnesene	0.42	-
•	*β*-humulene	0.28	0.49

Continued

• δ-terpineol	0.67	-
• Terpinolene	-	0.16
• Linalool	0.6	5.2
• Borneol	-	0.65
• *α*-zingiberene.	-	-
• Sesquiphellandrene	-	-
• *β*-bisabolene.	-	-
• *α*-farnesene.	-	-
• Curcumene	-	-
• Zingiberol	-	-
• Neral	-	-
• z-citral	-	-
• myrtenyle acetate	0.58	-
• Viridiflorene	-	-
• *α-terpineol*	**3.85**	-
• Endo-borneol	0.30	-
• *α*-pinene	-	0.85
• Camphene	0.61	2.63
• *β*-pinene	-	1.63
• Camphre	-	2.04
• p-menth-2-en-1-ol	-	-
• Estragol	2.95	-
• Piperitone	-	-
• Geranial	-	-
• Carvone	-	-
• Genanyle acetate	-	-
• Citronellol	-	-
• Nerol	-	-
• 2-tridecanone	-	-
• Geraniol	-	-
• *β*-phellandrene.		-
• Methyl-6-isopropyl-3-phenol (thymol)	-	**40.8**
• Methyl-5-isopropyl-2-phenol (carvacrol)	-	**3.6**
• Eugenol	-	-
• Curcumene	-	-
• e-Citral	-	-
• Terpineol	-	1.15
• Leborneol	-	-
• Caryophyllene oxyde	-	0.34
Total	96.8	91.57

Legend: Fv: *F. vulgare*; Tv: *T. vulgaris*; nber: number in percentage.

3.3. Isolation, Purification, Pathogenicity Testing, and Frequency of Occurrence of Fungal Isolates

Isolation and purification yielded a total of 13 fungal isolates. The frequency of occurrence was calculated, grouping them into 5 distinct morphotypes as follows:

A: Morphotype "Light white filamentous": 5 isolates, representing a frequency of 38.46%

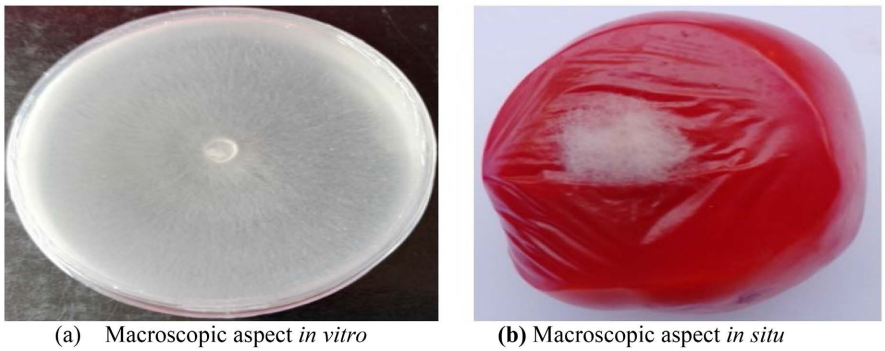
B: Morphotype "Greenish white": 1 isolate, representing a frequency of 7.70%.

C: "White filamentous" morphotype: 5 isolates, representing a frequency of 38.46%

D: "White" morphotype: 1 isolate, representing a frequency of 7.70%

E: "White serrated" morphotype: 1 isolate, representing a frequency of 7.70%

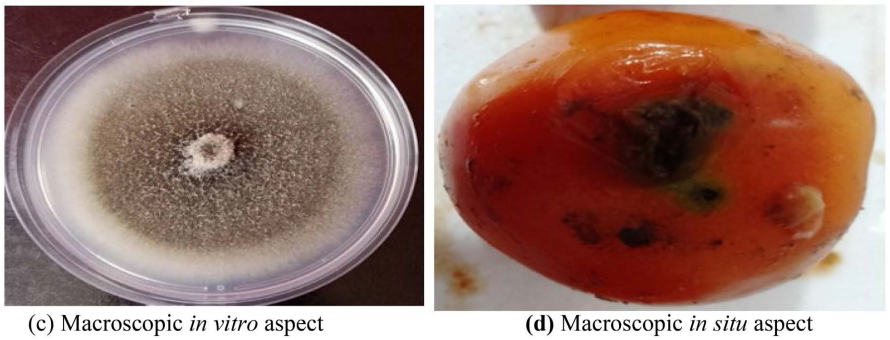
- The isolates that proved pathogenic in the pathogenicity test are morphotypes A and B mentioned above "Light white filamentous" morphotype (designated MB4) was showed in Figure 1 and "Greenish white" morphotype (denoted MB5) in Figure 2.



(a) Macroscopic aspect *in vitro* **(b)** Macroscopic aspect *in situ*

Figure 1. *In vitro* and *in situ* aspects of molds MB4.

- «Greenish white» morphotype (denoted MB5)



(c) Macroscopic *in vitro* aspect **(d)** Macroscopic *in situ* aspect

Figure 2. *In vitro* and *in situ* appearance of the MB5 fungus.

3.4. Macroscopic and Microscopic Identification of Infected Isolates

This step involves determining the macroscopic and microscopic characteristics in order to name the probable species of the pathogens studied in Table 5 and

illustrated in Figure 3.

Table 5. Macroscopic and microscopic characteristics of morphotypes: genera, probable species, and apparent morphotypes.

Characteristics		MB4	MB5
Macroscopic characteristics	Texture	Filamenteos	Duveteos Bombed up at center and flat at the peripheries
	Colour	white, airy appearance, whitish colour	pointed of whitish filaments at the center, greenish at the zone of growth and whitish at the periphery
	Colony size	Invasive	
Caractéristiques microscopiques	Type de thalle	siphonnated thallus siphonated filaments, presence of stolons and rhizoïds, presences of conidies	Septed thallus (segmented)
	Form of dissemination		Conidies unicellulares ovos and small
	Fructification organ		acervule
Genius, probableb species and parental morphotypes	Genius	*Rhizopus*	*Colletotrichum sp.*
	Probable species	*Rhizopus stolonifer*	*Colletotrichun cococodes*

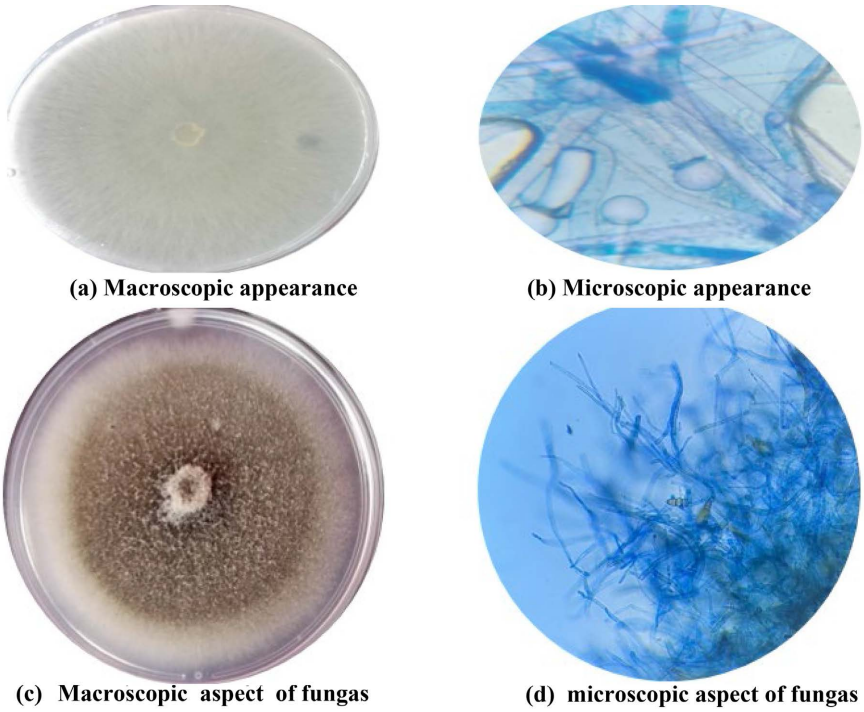


Figure 3. Aspect macroscopic and microscopic of fungus.

3.5. *In Vitro* Antifungal Test of the Different Essential Oils and Their Nanoemulsions with the Pathogenic Isolates

Based on the *in vitro* results obtained on the antifungal potential of Plantineb (the reference antifungal), it appears that the essential oils studied exerted an antifun-

gal effect to varying degrees on the mycelial growth of isolates MB4 and MB5, and these effects were dose-dependent and pathogen-dependent. High concentrations showed no significant effects. It is worth noting that Plantineb and *T. vulgaris* essential oil were the most effective on mycelial growth, with MICs of 0.40 and 0.70 mg/mL, respectively. The MICs obtained with the essential oil nanoemulsions varied not only according to the pathogens but also according to the specific nanoemulsions of *T. vulgaris* and Plantineb essential oils.

3.6. Results of the Formulation of Different Essential Oils by Cold Emulsification

Table 6 below presents the characteristics of the different oils formulated by emulsification.

Table 6. Summary of the physicochemical characteristics of the different nanoemulsions.

Physicochemical characteristics	Thym	Fenouil
Appearance of the formulation	Belge whitish	Translucent white
pH	6.83	6.03
Persistence of the foam	No foam	No faom
Stability of the emulsion	+	+
Storage test:		
4˚C	+	+
27˚C	+	+
55˚C	+	+
Heating and cooling at 4˚C and 40˚C	+	+
freeze-defroze stress: 25˚C et - 18˚C	+	+

Legend: (+): positive test; (−): negative test (phase formation or color change).

The table above shows that:

The different nanoemulsions (NE) are all whitish and translucent, respectively. The essential oils have a pH between 6.83 and 6.03; these values are acceptable because the majority of essential oils are acidic. Regarding the foam test, all the NEs were positive.

3.7. Evaluation of the *in Vitro* Antifungal Activity of Essential Oils and the Reference Antifungal on the Mycelial Growth of Pathogens

The essential oils extracted from the different plants in the study and the reference antifungal (Plantineb) exerted varying inhibitory effects on the mycelial growth of the isolated pathogens. The mycelial growth diameters illustrated in Table 7 and Table 8 represent the inhibition of mycelial growth of isolates MB4 and MB5 at different concentrations of *T. vulgaris* essential oil and Plantineb.

Table 9 below also shows the different values of CMI, CMF and CMF/CMI ratios obtained.

Table 7. Inhibition of mycelial growth of pathogenic isolates (MB4 and MB5) as a function of essential oil concentrations.

Essential oils	Concentrations (mg/mL)	Isolats	
		MB4	MB5
Thym (*Thymus vulgaris*)	0.12	0.0 ± 0.0[a]	77.6 ± 0.4[a]
	0.25	28.2 ± 1.1[b]	81.3 ± 0.8[b]
	0.50	100.0 ± 0.0[c]	89.0 ± 0.6[c]
	1.00	100.0 ± 0.0[c]	100.0 ± 0.0[d]
	2.00	100.0 ± 0.0[c]	100.0 ± 0.0[d]
Fenouil (*Foeniculum vulgare*)	0.12	0.0 ± 0.0[a]	0.0 ± 0.0[a]
	0.25	27.1 ± 0.0[b]	0.0 ± 0.0[a]
	0.50	29.4 ± 0.0[b, c]	1.0 ± 1.0[a]
	1.00	30.0 ± 0.0[c]	9.1 ± 0.6[b]
	2.00	60.4 ± 2.0[d]	16.5 ± 1.0[c]

Legend: For each essential oil tested against a pathogenic isolate, the values marked with different letters in each column are significantly different, according to Duncan's post hoc test at the 5% threshold (a < b < c < d < e). The test was performed in triplicate.

Table 8. Inhibition of the growth of mycelium of the MB4 and MB5 isolates with respect to the concentrations of reference antifungals.

Reference antifungal	Concentrations (mg/mL)	Isolate	Reference antifungal	Concentrations (mg/mL)	Isolate
		MB4			MB5
Plantineb	0.10	51.0 ± 2.5[a]	Plantineb	1.50	45.1 ± 1.3[a]
	0.20	63.9 ± 1.8[b]		2.00	60.0 ± 1.6[b]
	0.30	72.2 ± 2.8[c]		2.50	71.2 ± 0.8[c]
	0.40	83.9 ± 0.5[d]		3.00	85.5 ± 2.0[d]
	0.50	100.0 ± 0.0[e]		3.50	100.0 ± 0.0[e]

Legend: For the reference antifungal tested against a pathogenic isolate, the values indicated by different letters in each column are significantly different, according to Duncan's post-hoc test at the 5% significance level (a < b < c < d < e). The test was performed in triplicate.

Table 7 and Table 8 above show that the pathogen MB5 is the most resistant isolate to the essential oils and Plantineb used. Complete inhibition was observed with Plantineb at a concentration of 3.50 mg/mL, compared to the pathogenic isolate MB4, which was completely inhibited at 0.50 mg/mL. Of the two (02) essential oils in the study, the pathogenic isolates MB4 and MB5 showed a greater sensitivity to the essential oil of *T. vulgaris*.

Table 9. Values of the different MICs, MFCs and MFC/MIC ratios of the essential oils and Plantineb against the pathogenic isolates MB4 and MB5.

	Isolate MB4			Isolate MB5		
	MIC (mg/mL)	CMF (mg/mL)	CMF/MIC	MIC (mg/mL)	CMF (mg/mL)	CMF/MIC
T. vulgaris	0.40	0.90	2.25	0.70	0.90	1.28
F. vulgare	>8.00	ND	/	>8.00	ND	/
Reference antifungal	0.50	0.50	1.00	3.50	3.50	1.00

Table 9 shows that both essential oils and Plantineb inhibited the mycelial growth of pathogenic isolates with varying MICs. The MIC of *T. vulgaris* essential oil against pathogenic isolate MB4 was lower than that of Plantineb (0.50 mg/mL). Both *T. vulgaris* essential oil and Plantineb showed a fungicidal effect against pathogen MB4, in contrast to the fungistatic effect of *T. vulgaris* essential oil.

However, for pathogenic isolate MB5, the MIC of *T. vulgaris* essential oil (0.70 mg/mL) was lower than that of Plantineb (3.50 mg/mL). The fungicidal effect was observed with Plantineb and *T. vulgaris* essential oil. Analysis of these data showed a positive correlation between concentrations and percentages of inhibition. An overall comparison of the antifungal activity of the essential oils and Plantineb using Pearson's bivariate correlation test revealed a significant positive correlation between the activity of the essential oils and that of Plantineb at the 0.01 level.

3.8. Evaluation of the *in Vitro* Antifungal Activity of the Emulsions and Plantineb

In view of the different concentration ranges of the emulsions used on the Mycelial growth values (MIC, CMF, and CMF/MIC) were obtained for isolates MB4 and MB5. These values, along with those obtained with the reference antifungal, are shown in Table 10.

Table 10. MIC, CMF, and CMF/MIC values for essential oil emulsions and the reference antifungal on mycelial growth of MB4 and MB5.

	Isolate MB4			**Isolate MB5**		
	MIC (mg/mL)	CMF (mg/mL)	CMF/MIC	MIC (mg/mL)	CMF (mg/mL)	CMF/MIC
T. vulgaris	0.15	0.30	2.0	0.15	0.15	1.00
F. vulgare	>4.00	ND	/	>4.00	ND	/
Plantineb	0.50	0.50	1.00	3.50	3.50	1.00

Legend: ND: Not Determined.

Table 10 shows that the MICs varied not only according to the pathogens but

also according to the emulsions of *T. vulgaris* and Plantineb essential oils.

The MICs of the emulsions based on *T. vulgaris* and *F. vulgare* essential oils did not change for the two pathogens tested (MB4 and MB5). The *T. vulgaris* and *F. vulgare* emulsions showed identical MICs of 0.15 mg/mL and greater than 4.00 mg/mL, respectively, for both pathogens. As for the reference antifungal (Plantineb), the MICs were 0.50 mg/mL for pathogen MB4 and 3.50 mg/mL for pathogen MB5. The CMF/CMI ratios obtained were less than 4.00 for the emulsions (except for the *F. vulgare* essential oil emulsion) and for Plantineb on the two pathogens tested. These ratios therefore demonstrate a fungicidal effect of these emulsions and of Plantineb on MB4 and MB5.

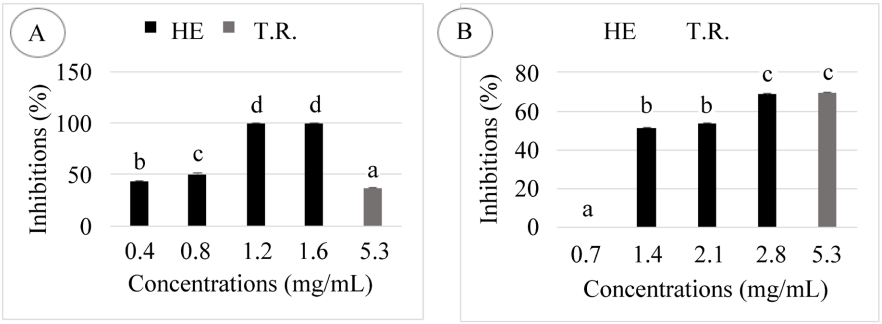
However, compared to the MICs obtained with the essential oils alone, the *T. vulgaris*-based emulsion showed better activity, as the MIC obtained was lower than that obtained with pure *T. vulgaris* essential oil for both isolates. *T. vulgaris* essential oil and its emulsion showed the best antifungal potential against the two pathogenic morphotypes MB4 and MB5. They were selected for *in situ* testing.

3.9. Evaluation of the *in Situ* Antifungal Activity of Essential Oils, Emulsions, and Plantineb

3.9.1. Evaluation of the *in Situ* Antifungal Activity of *T. vulgaris* Essential Oil and Plantineb

The ability of *T. vulgaris* essential oil and Plantineb (reference antifungal) to prevent the proliferation of pathogens MB4 (A) and MB5 (B) in tomato fruits was determined, as shown in Figure 4 and Figure 5 below.

Histograms sharing the same letters indicate no significant difference between the tested concentrations, according to Duncan's post-hoc test at a significance level of $P < 5\%$ ($a < b < c < d$). The test was performed in triplicate. EO: Essential oil; RC: Reference control.



Key: T. MB4: Control infected with MB4; T. MB5: Control infected with MB5; RC: Reference control; mg/mL: Unit of measurement.

Figure 4. *In situ* antifungal effect of T. vulgaris essential oil and the reference antifungal on tomato rots caused by MB4 (A) and MB5 (B).

The *in situ* antifungal effect of *Thymus vulgaris* essential oil and Plantineb against the pathogenic isolates MB4 and MB5 on *Solanum lycopersicum* fruit showed in Figure 5.

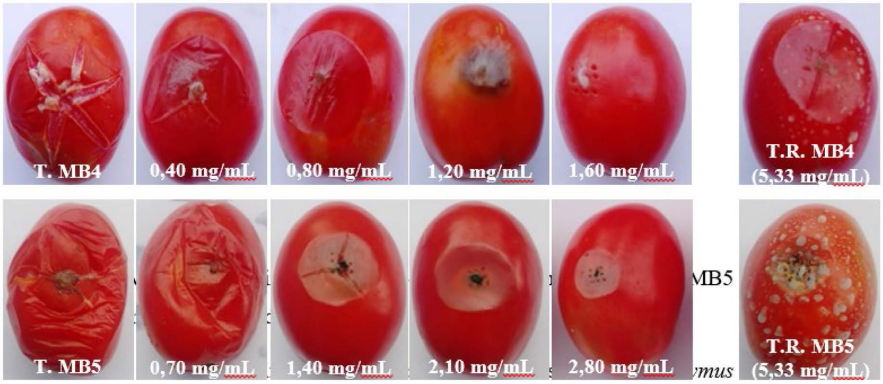


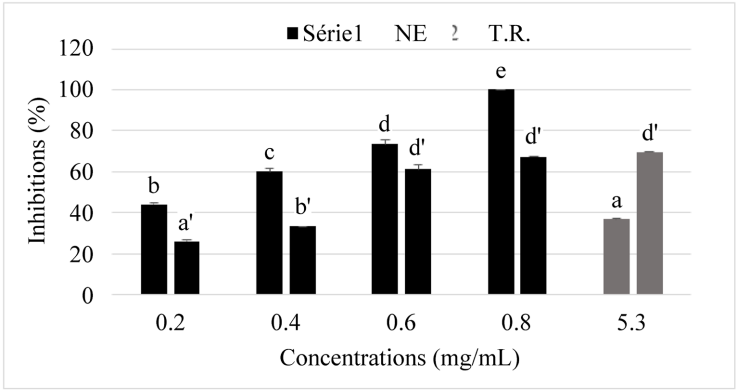
Figure 5. *In situ* antifungal effect of *Thymus vulgaris* essential oil and Plantineb against the pathogenic isolates MB4 and MB5 on *Solanum lycopersicum* fruit.

Figure 4 and **Figure 5** above show that *T. vulgaris* essential oil proved to be more protective of tomatoes against rots caused by the two pathogenic isolates, compared to Plantineb at the manufacturer's concentration (5.33 mg/mL).

The essential oil of *T. vulgaris* completely inhibited the proliferation of the MB4 pathogen at 1.60 mg/mL and partially inhibited that of the MB5 pathogen (68.7 ± 0.3%) at 2.80 mg/mL on tomato fruits.

3.9.2. *In Situ* Antifungal Activity of the *T. vulgaris* Nanoemulsion and the Reference Antifungal

The *in situ* antifungal activity was evaluated by first measuring the average diameters of the observed necrotic lesions and then calculating the percentages of protection. **Figure 6** and **Figure 7** show the antifungal activity of Plantineb and the *T. vulgaris* essential oil emulsion for isolates MB4 and MB5.

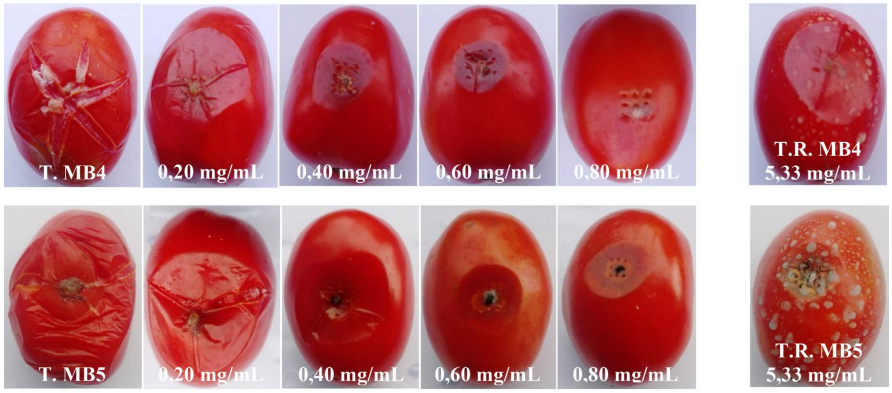


Legend: Histograms sharing the same letters indicate no significant difference between the tested concentrations, according to Duncan's post-hoc test at a significance level of $P < 5\%$ (a < b < c < d < e for pathogen MB4 and a' < b' < c' < d' for pathogen MB5). The test was performed in triplicate. NE: Emulsions; RC: Reference control.

Figure 6. *In situ* antifungal activity of the *T. vulgaris* nanoemulsion and the reference antifungal.

Inhibition of tomato rot caused by MB4 (A) and MB5 (B) as a function of the

concentrations of *T. vulgaris* essential oil emulsion and the reference antifungal was showed in Figure 7.



Legend: T. MB4: Control infected with MB4; T. MB5: Control infected with MB5; mg/mL: Unit of measurement; T. R.: Reference control.

Figure 7. Effect of the *T. vulgaris* essential oil emulsion at different concentrations and of the reference antifungal on tomato fruits infected with MB5 and MB4.

This figure and photograph show that the protection percentages increase with emulsion concentrations for both (2) tested isolates. Comparison of the means using a one-way ANOVA (at a significance level of 0.05) confirmed this observation.

The *T. vulgaris* emulsion showed maximum protection of 100 ± 0.0% and 67.2 ± 0.0% at a concentration of 0.80 mg/mL for isolates MB4 and MB5, respectively. Plantineb, on the other hand, showed protection of 36.8 ± 0.2% and 69.6 ± 0.1% at a concentration of 5.33 mg/mL respectively for the pathogenic isolates tested.

4. Discussion

4.1. Extraction and Yield

4.1.1. Physical Characteristics, Yield, and Chemical Composition of Essential Oils

The evaluation of organoleptic properties is generally part of studies aimed at analyzing the factors that affect the quality of essential oils. The color of essential oils is a very important parameter for certain applications, as it can be determined directly by sight. Each extract is characterized by its organoleptic properties, such as odor, appearance, and color. Essential oils are composed of aromatic molecules with very low molecular weights [19]. They are highly flammable and very fragrant, and are liquid at temperature Essential oils are liquid at room temperature. When exposed to air, they evaporate. They are very rarely colored.

The essential oils studied have a strong and persistent odor, except for *T. vulgaris* essential oil, which appears to be mild. The odor of each oil is linked to its chemical composition. Essential oils are mixtures of numerous compounds, which are relatively simple molecules such as terpenes, phenols, methyl ethers, oxides, esters, and ketones [20]. According to AFNOR [15], essential oils are gen-

erally fragrant products obtained either by steam distillation or by other extraction methods. This definition excludes essences obtained by other extraction processes. Essential oils are liquid at room temperature but also volatile due to their relatively low molecular weight, which gives them the olfactory property that distinguishes them from so-called fixed oils [21]. They are liposoluble and soluble in organic solvents and vegetable oils, steam-transferable but very slightly soluble in water (they are hydrophobic) [22].

The color of an essential oil depends on its constituents. Some solvents have the ability to extract a large amount of pigment, thus intensifying the color of a given oil. Essential oils are perishable and susceptible to oxidation; therefore, their storage requires darkness and humidity; consequently, the use of opaque glass bottles is recommended [23].

Density, or specific gravity, is a physical quantity that characterizes mass: the densities of our essential oils are 0.92 for Thymus vulgaris and 0.91 for Foeniculum vulgare. Their density is generally lower than that of water. They are fragrant and mostly colored (their color varies depending on the aromatic plant used) [24].

Analysis shows that essential oil yields vary from one plant species to another. These yields range from 0.55% to 0.56%. The essential oils from the seeds of *Foeniculum vulgare* are the richest in essential oil, with a yield of 0.56%. This high yield is explained by the extraction method, which does not always produce very good results. This can be due to various factors, including soil type, harvest time, drying time, and the extraction method [25]. These results show that essential oil yield depends on the location and season of harvest, the plant's stage of development, and the soil type.

The quantity of essential oils contained in plants is always small. Several tons of plants are needed to obtain one liter of essential oil, which explains their high cost. However, essential oils are generally diluted before use because of their toxicity at very high concentrations [23]. The essential oil extraction technique plays a crucial role in the oil yield and its chemical composition. Furthermore, numerous factors influence the yield, content, physicochemical characteristics, and chemical composition of essential oils: these include the species of aromatic plant, environmental conditions, drying, harvesting time and environment, cultivation practices, and the age of the plant material. In the essential oil of *Thymus vulgaris*, 23 compounds have been identified, representing 91.57% of the total chemical composition, the most abundant of which are: thymol (40.8%), pcymene (12.07%), and β-terpinene (10.25%).

Numerous studies have revealed that the aerial parts of *Thymus vulgaris* are very rich in several constituents, the content of which varies according to the variability of geographical, climatic, drying, storage, and study methods. The characteristic of *Thymus vulgaris* essential oil is its high thymol content. In studies conducted by [26], 30 compounds were identified and characterized; the most abundant are, respectively: thymol (44.4% - 58.1%), pcymene (9.1% - 18.5%), β-terpinene (6.9% - 18.0%), carvacrol (2.4% - 4.2%), and linalool (4.0% - 6.2%). These

results corroborate those obtained by [26], which show that the essential oil of *T. vulgaris* contained 46.91% oxygenated monoterpenes, the main ones being thymol (35.12%), inalool (4.72%), camphor (2.38%), and carvacrol (2.01%), compared to 45.00% monoterpene hydrocarbons. Sesquiterpene hydrocarbons represented 6.68%, while oxygenated sesquiterpenes were only 1.25%. As for the monoterpene hydrocarbons, the main constituents were p-cymene (25.36%), γ-terpinene (12.48%), myrcene (1.38%), and α-terpinene (1.09%).

A pure essential oil contains several complex chemical substances. Essential oils contain over a thousand chemical components. The number of chemically distinct molecules that make up an essential oil varies. Several essential oils have similar compositions, differing in just a few molecules. According to Bachelot [27], essential oils contain a large number of biochemical elements. The most common are alcohols, ketones, terpene aldehydes, esters, ethers, and terpenes. In addition to the major compounds, there are minor compounds and a number of other components.

The constituents are present in trace amounts and have a relatively low molecular weight, which gives them their volatile character and is the basis of their olfactory property [28]. Essential oils are composed solely of aromatic and volatile molecules and contain no fatty substances, despite their name [23].

Based on the *in vitro* results obtained on the antifungal potential of Plantineb and the essential oils of *Thymus vulgaris* and *Foeniculum vulgare*, it appears that these exerted an antifungal effect to varying degrees on the mycelial development of isolates MB4 and MB5, and the effects are dose-dependent.

It is worth noting that of Plantineb and the two essential oils in the study, the essential oils of *T. vulgaris* and *Foeniculum vulgare*, the most effective on mycelial growth were those with MICs of 0.40 and 0.70 mg/mL, respectively. This potential is explained by the nature of their chemical compounds and their mechanisms of action involved, which can lead to severe membrane damage [29]-[31].

Indeed, the essential oils of *T. vulgaris* and *C. citratus* are composed mainly of terpene molecules such as p-cymene, 2,3-epoxy-geranyl acetate, and phenolic compounds like thymol and citronellol. Terpene molecules are known for their ability to interact with the phospholipids of fungal cell membranes, thus causing a loss of structural integrity of this membrane and an increase in ionic permeability [32]. Furthermore, hydrogen bonds between the polar groups of fungal enzymes and the hydroxyl groups of phenolic or terpenic compounds on the one hand, and the ability of terpenes through their functional groups (phenols and aldehydes) to react on the SH group of the active site of membrane enzymes on the other hand, lead to a decrease in energy production and the inactivation of the synthesis of essential enzymes such as chitin and β (1,3)-glucan, responsible for the formation of the fungal cell wall [33].

Furthermore, the emulsion based on *T. vulgaris* essential oil showed a lower MIC than that of crude *T. vulgaris* essential oil. This was also observed in the work of [34], which showed that the nano-emulsion based on Thymus vulgaris essential

oil was better against several fungal isolates compared to the crude essential oil tested, with a decreased MIC90 for almost all the pathogens tested. Indeed, emulsions allow for homogeneous dispersion of bioactive compounds, thus facilitating their rapid and widespread absorption by target cells [34]. These Bioactive molecules contained in essential oils are protected from UV rays, which cause oxidation or changes in their chemical structure. The presence of the surfactant (Tween 80) in the emulsion facilitates its absorption by the PDA culture medium by giving the final solution a hydrophilic character. This maximizes the antifungal potential of the bioactive molecules in the crude essential oil and thus provides the emulsion with sustained efficacy against pathogens. Furthermore, the formulation, by increasing the stability and solubility of the essential oil, allows for its prolonged release. The dispersed phase of the essential oil acts as a nano-reservoir, releasing an active ingredient over time [32].

4.1.2. *In Situ* Antifungal Potential of *T. vulgaris* Essential Oil and Its Emulsion

The results obtained *in situ* on tomato fruits revealed that the protective percentages of Plantineb and *T. vulgaris* essential oil differed from one isolate to another. The *in vitro* inhibitory concentrations of Plantineb and *T. vulgaris* essential oil on mycelial growth were lower than those obtained *in situ* on tomato fruits. According to the work of [35] [36], the concentrations of essential oils and their compounds necessary to inhibit microbial growth are higher in food than in culture media. This is due, in part, to the interactions between phenolic compounds and the food matrix (the presence of organic acids and sugars can weaken the effects of the essential oil). Since essential oils are made up of many different volatile compounds, residual levels would be low after storage [37], attributed these results to the existence of certain factors such as the effects of pH on stability and antifungal activity, uneven distribution in the food matrix and low solubility of essential oils.

Furthermore, the rot observed on tomato fruits *in situ* is thought to be due to the fact that the phytopathogenic fungi MB4 and MB5 secreted extracellular enzymes capable of degrading the cell walls of tomato fruits, thus facilitating their entry. These enzymes also generated simple molecules that could be assimilated by these fungi, thereby enabling their growth. In the presence of the reference antigen or the essential oil of *T. vulgaris*, the enzymatic activity induced by the phytopathogens was reduced. [38] [39] have shown that essential oils are capable of inducing resistance and/or activity direct antimicrobial action on plants. This phenomenon is characterized by the rapid production of reactive molecules (H_2O_2).

In situ, the *T. vulgaris*-based emulsion exhibited maximum protection of 100 ± 0.0% and 67.2 ± 0.0% at a concentration of 0.8 mg/mL for isolates MB4 and MB5, respectively, which were higher than those obtained with Plantineb. These results can be explained not only by the fact that essential oils have a broad spectrum of activity compared to synthetic fungicides, but also by the improved bioavailability

of the *T. vulgaris* essential oil emulsion in tomato fruits. Indeed, factors such as the size, polarity, and stability of antimicrobial molecules influence their bioavailability *in situ* or in vivo. Small, stable, and amphiphilic molecules have better diffusibility and activity in plant tissues [40].

5. Conclusion

The objective of this section was to evaluate the *in vitro* and *in situ* antifungal potential of essential oils and their emulsions against pathogens responsible for post-harvest tomato fruit rot. During this work, we first determined the chemical composition of the essential oils and their organoleptic characteristics. The yield of *Foeniculum vulgare* was 0.56. Two fungal species were observed, the most prevalent being *Rhizopus stolonifer* (MB4) and *Colletotrichum cococode* (MB5).

The results of the study of the activity of the two essential oils on MB4 and MB5 show that they have an effect on these fungi. Complete inhibition of MB4 and MB5 is achieved with a minimum concentration of 10 µl/20 ml of PDA.

Based on these results, we can conclude that the essential oil of *Foeniculum vulgare* possesses very good antifungal activity against MB4 and MB5 using the direct contact method.

In view of the results obtained *in vitro*, it is interesting to highlight that Plantineb and the essential oil of *Foeniculum vulgare* were the most effective on mycelial growth with MICs of 0.40 and 0.70 mg/mL.

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Conflicts of Interest

The authors declare no conflict of interest.

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