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THEME

Study of The Antifungal and Insecticidal Activity of Extracts Essential Oils and Hydrolat of *Mentha rotundifolia*

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Dedication

I dedicate this thesis to the memory of my father,

To my supportive mother,

To my family...

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I would like to express my gratitude to my supervisor, **Mrs.Bouziane Zahira**, for their invaluable advice, expertise, and constant encouragement. Their extensive knowledge and insightful feedback were critical in shaping this thesis. I sincerely appreciate their patience, dedication, and unwavering support.

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List of Abbreviation

A: *Aphis*

B: *Botrytis*

C: *Colletotrichum*

EOs: Essential oils

LD50: Lethal dose 50

M: *Mentha*

MC: Corrected mortality

MGI: Mycelial growth inhibition

mL: milliliter

mm: millimeter

PDA: Potato Dextrose Agar.

PR: Percentage of repellency

sp: species

µL: microliter

Introduction

Lamiaceae species are found all over the world, with varying heights and habitats and greater abundance in the Mediterranean region, including the *Mentha* genus, which contains some of the world's most commonly cultivated spice plants **(Kallunki and Heywood, 1994)**. In the Algerian flora, this genus is represented by five major species: *M. rotundifolia*, *M. longifolia*, *M. spicata*, *M. aquatica*, and *M. pulegium* **(Quezel and Santa, 1962)**. *Mentha rotundifolia L.* is an aromatic plant widely distributed in the north of Algeria. The country's geographical location and climatic diversity have allowed for the development of a very rich and highly diverse aromatic and medicinal plant community, with over 4000 species that have been used to treat a variety of diseases since time immemorial **(Benarba *et al.*, 2015)**. Traditional Algerian medicines use decoction and infusion of the rounded-mint leaves to treat a variety of diseases such as hypertension, diabetes, and digestive and genitourinary system disorders **(Brahmi *et al.*, 2016)**.

The biological applications of this species are primarily related to its essential oils, which have various activities such as antioxidant **(Benabdallah *et al.*, 2018)**, antibacterial, and insecticidal **(Aouadi *et al.*, 2020; Kharoubi *et al.*, 2020);** however, they have been little described in the literature. Despite its importance as a medicinal plant, *M. rotundifolia* has limited industrial potential in Algeria. Only a few studies have been conducted on the biological activities of Algerian *M. rotundifolia*, specifically the antibacterial and antifungal effects **(Boukhebti *et al.*, 2011; Brahmi *et al.*, 2016)**. Yet, this research helps to determine the effectiveness of *M. rotundifolia* essential oils against phytopathogenic agents that infect strawberry crops, which is one of the most economically important crops that is consumed worldwide, in order to isolate the main fungi that cause severe diseases.

Postharvest quality of fresh horticultural crops, on the other hand, is affected by abiotic and biotic factors, including environmental factors such as temperature, relative humidity, air composition, and light. In terms of biotic factors, such as pathogens and pests, physiological changes, physical losses, biochemical changes, and pathological deterioration are all examples of postharvest deterioration. *Botrytis cinerea* postharvest decay is one of the most common fungal pathogens for strawberry infections, causing a destructive grey mould disease. In the field, *B. cinerea* infects various strawberry organs, including fruits, resulting in poor yield quality. As a result, this current infection causes significant economic crop loss in preharvest

stages such as growing seasons, harvesting time, and handling processes, as well as postharvest stages such as transportation and storage conditions **(Rasiukevičiūtė *et al.*, 2018)**.

In addition to the gray mould, we shed light on yet another factor that contributes to strawberry diseases. The causal agents of strawberry anthracnose were previously reported to be *Colletotrichum gloeosporioides* **(Tzean *et al.*, 2019)**, *C. dematium*, *C. fragariae*, and *C. acutatum*, but information about the isolation, pathogenicity, morphology, and sequences of these species is not sufficient for species identification. *Colletotrichum spp*. can infect various strawberry tissues, causing black spots or irregular spots on leaves, sunken black spots or necrosis lesions on petioles, stolons, and fruits, and wilting of the whole plant due to crown rot **(Howard *et al.*, 1992)**.

Furthermore, the essential oils of various mint species have been shown to have high insecticidal activity **(Kumar *et al.* 2011)**. The presence of numerous oxygenated monoterpenes, such as menthone, menthol, pulegone, piperitone oxide, piperitenone, and carvone, has been attributed to this property **(Kasrati *et al.*, 2015)**. Many studies have been published on the insecticidal activity of several Mentha species, including *M. pulegium*, *M. piperita*, *M. suaveolens*, and *M. spicata*, against aphid populations, specifically *Myzus persicae*, *Aphis craccivora*, and *Toxoptera aurantii*. According to WHO data, more than 70% of the population relies primarily on herbal medicines to treat diseases **(Bahmani *et al.*, 2014)**. The growing interest in less industrialized products with functional ingredients has prompted the use of essential oils in a variety of industrial sectors (food, cosmetics, hygiene, and agriculture), where they are used in product preservation and microorganism control **(Sacchitti *et al.*, 2004)**. The advantage of volatile oils over synthetic preservatives is the lower development of toxic byproducts as well as their economic viability **(Kulisic *et al.*, 2004)**. Many parts of Algeria have an important floristic patrimony that can be explored and exploited by using bioactive substances derived from secondary metabolism of these plants in a variety of fields, including the formulation of bio-pesticides.

Therefore, the objective of this study is to characterize the chemical composition of the essential oils of *Mentha rotundifolia* and to improve the medicinal plants of the East Algerian region by identifying and then demonstrating the fungicidal and insecticidal activities of this species essential oils and hydrolat on both strains of *Botrytis cinerea* and *Colletotrichum gloeosporioides;* also *Aphis fabae* pest. In order to minimise the use of synthetic fungicides and

insecticides, this essential oil could be used as an alternative solution in integrated biological control systems against pathogenic agents and insect infestation.

This study is divided into two sections, the first of which deals with the bibliographic synthesis. It consists of chapter one about the medicinal plant *'Mentha rotundifolia'*, a second chapter contains essential oils and the identification of major compounds of *Mentha rotundifolia* with their main biological activities while the third one includes the phytopathogenic fungi and tested pest.

The second section is reserved for detailed experimental tests, the material and techniques used. As for the last axis, it first presents the results obtained from the extraction of *Mentha rotundifolia* essential oils and their analysis by GC-MS, besides the identification of fungal flora isolated from strawberries in the region of Tassoust-Jijel.

In addition, it includes the results and discussion of the evaluation of antifungal and anti-insecticide activities. Thus, a general conclusion, with some perspectives, is presented at the end of the document.

BIBLIOGRAPHY PART

CHAPTER 1

Generalities about Mentha *rotundifolia*

1.1. General Description of *Lamiaceae* Family

The plant family *Lamiaceae*, formerly known as *Labiatae*, is distinguished by bilabiate corollas in its flowers. *Lamiaceae* contains over 7000 species organized into approximately 240 genera, some of these species have aromatic properties, which provide significant economic value to the *Lamiaceae*, as they are used in cosmetics and herbal medicines. *Mentha*, *Ocimum*, *Salvia*, *Clerodendrum*, and *Plectranthus* are examples of genera with such properties.

Lamiaceae species are found all over the world, with varying heights and habitats and greater abundance in the Mediterranean region **(Kallunki and Heywood, 1993)**. They prefer hot climates, but they can also be found in cold climates **(Da Silva *et al.*, 2021).**

According to the same previous author, this family species has a wide range of morphological characteristics and can be herbs, herbaceous plants, shrubs, or trees. Nowadays, this is one of the largest botanical families, with flowers of various sizes and bright colours depending on the species. They are bisexual, with well-defined floral parts, visible sepals and petals, inflorescence, and bilateral symmetry (zygomorphs), and the corolla tube is divided into two distinct parts, resulting in the Labiatae family's distinctive "lip" shape. Their leaves are typically simple, and their fruits are dry and numerous when ripe (schizocarpic fruits).

Many species in the *Limiaceae* family are high in flavonoids and terpenes, with diterpenoids being the most abundant. Thyme, basil, oregano, rosemary, sage, mint, and lemon balm are examples of aromatic spices, the diversity of their bioactive compounds endows *Lamiaceae* with antioxidant, insecticidal, fungicidal and bactericidal properties **(Bekut *et al.*, 2018)**.

1.1.1. Description of *Mentha* Genus

The *Lamiaceae* family includes the genus *Mentha*, whose plants are among the most aromatic and widely distributed around the world **(Harley *et al.*, 2004)**. They have straightforward, distinctive leaves that have a nice perfume. The taxonomy of *Mentha* is extremely complex, with around 42 species, 15 hybrids, hundreds of subspecies, and thousands of cultivars **(Lawrence, 2006)**. From the species *M. arvensis L* and *M. aquatica L*, *M. spicata L*, *M. longifolia L*, and *M. suaveolens*, eleven naturally occurring hybrids have been created. Most of these hybrids are infertile but can spread because of their very invasive rhizome **(Spencer *et al.*, 1993)**. The literature in Algeria reported the presence of six *Mentha* species: *M. rotundifolia*, *M. spicata*, *M. pulegium*, *M. piperita*, *M. longifolia*, and *M. aquatica*, as well as three hybrids of these species: *M. durandoana*, *M.*

niliaca, and *M. schultzii* **(Quezel and Santa, 1963)**. According to the same source, *M. longifolia* and *M. aquatica* were extremely rare at the time and are likely extinct now.

Perennial plants in this genus are used to produce essential oils, mostly in the USA, India, China, and Iran **(Lawrence, 2007)**. *Mentha* species fresh and dried plant materials are frequently utilized in industry as ingredients in confections, flavorings, medicines, cosmetics, and other products. Economically, this genus plants are one of the most important medicinal and aromatic crops in the world. According to various antioxidant activity tests, mint is the richest source of antioxidants; the extract of which has high total phenolic and flavonoid content **(Anwar *et al*., 2019)**.

1.1.2. Chemical Composition of *Mentha*

The mint's various chemical constituents are economically significant. Various derivatives and constituents of mint oil have been used as flavoring agents in the flavoring industry as well as in many different types of foods, herbal products, medicine, and perfumes. Mint oil is soluble in both water and alcohol. The oil contains liquid and solid fractions because it contains hydrocarbon, which prevents menthol crystallization.

Mentha chemical constituents have been studied in depth **(Brahmi et al., 2017)**. Menthol and terpenes, which exist both free and in esters, are the most important chemical compounds discovered in various mint species. Menthol in peppermint oil, for example, has been recognized for its medicinal properties, whereas esters are responsible for its minty flavor and associated sensory fragrance **(Peixoto *et al*., 2009)**. The majority of the literature on mint constituents focuses on their essential oils. Certainly, they are used in various types of food factories. Furthermore, the presence of phenolic compounds has many biological properties.

The mint plant also contains minerals (potassium, iron, sodium, and magnesium, manganese, Zinc, Calcium, Chromium, Copper, Iodine, and Selenium) and vitamins (Vitamin A, C, and carotene activity were found to be higher, while B12, thiamine, folic acid, and riboflavin were also reported) **(Wani *et al*., 2022)**.

1.2. *Mentha rotundifolia*

1.2.1. Botanical Description and Distribution

Mentha rotundifolia according to **Lorenzo *et al.,* (2002)**, it is a hybrid of *Mentha longifolia* and *Mentha suaveolens*, however according to other writers, *Mentha rotundifolia* and *Mentha*

suaveolens are the same species **(Hendriks *et al.*, 1976)**. It thrives in moist areas in Western to Eastern Europe, Northern Africa, and the temperate nations of the Southern Hemisphere **(Lawrence *et al.*, 2007)**. However, **Ouzmil *et al.*, (2002),** reported that this species is native to Southern and Western Europe, extending north to The Netherlands, and has been cultivated as a pot herb and naturalized in Northern and Central Europe. It is commonly found near streams, bogs, and humid areas.

Apple mint, also known as woolly mint or round-leafed mint, is an herbaceous, perennial herb with a sickly-sweet scent that can grow to be 100 cm tall. The stem is erect and quadrangular, with sparse to dense white-tomentose hairs. It has short internodes and is monopodially branched. The foliage is light green, with opposite, wrinkled, sessile or very short petiolate leaves that are ovate oblong to suborbicular in shape and 3 to 4.5 cm long and 2 to 4 cm broad. The leaves are obtuse, cuspidate, or rarely acute, serrate, with 10-20 teeth, hairy above, and usually grey or white-tomentose to lanate. A prostrate branch (creeping sucker) grows from the axil of the leaves at the base of the flowering stem, propagates below ground level, takes root, and then turns upwards to produce a new shoot. Many congested verticillasters form a terminal spike 4 to 9 cm long with several white or pinkish flowers **(El-Kashoury *et al.*, 2013)**. Its flowering season from July to September **(Benbouali, 2006)**.

The foliage of *Mentha rotundifolia* with a medium green color.

The flowering of *Mentha rotundifolia* with tiny white or pinkish melliferous flowers united in spikes.

Figure 1: The species of *Mentha rotundifolia* (**https://www.gammvert.fr/**)

1.2.2. Taxonomy of *Mentha rotundifolia*

Mentha rotundifolia grows wildly in Algeria, is locally known as “timija” **(Brahmi *et al*., 2017)**, and used mostly as a condiment but also for medicinal properties **(Ladjel *et al*., 2011)**. According to **Khadraoui *et al*., (2013)**, it is known as well as “megne essif” and “timarssat”.

The species has numerous nouns depending on the region or the country of its distribution, which is the case for *Mentha rotundifolia.* Its French nouns are Menthe à feuilles rondes, Menthe du Nil, Menthe sauvage **(Benazzouz and Hamdane, 2012)**. Meanwhile, **Umemoto (1998)** affirms that its nouns in the English linguistic are Apple-scented Mint, False Apple-mint**.**

The classification of the species *Mentha rotundifolia* is as follows (**Benoît Bock *et al*., 2021):**

Kingdom: Plantae – plantes, planta, vegetal, plants

Subkingdom: Viridiplantae – green plants

Infrakingdom: Streptophyta – land plants

Super-division: Embryophyta

Division: Tracheophyta – vascular plants, tracheophytes

Subdivision: Spermatophytina – spermatophytes, seed plants, phanérogames

Class: Magnoliopsida

Superorder: Asteranae

Order: Lamiales

Family: Lamiaceae – mints, menthes

Subfamily: Nepetoideae

Genus: *Mentha L.* – mint

Species: *Mentha rotundifolia* (*L.*) Huds – round-leafed mint, mint.

1.2.3. Uses

Mentha species have long been valued for their medicinal and aromatherapeutic properties. Peppermint was used as a flavoring agent for food and as a medicine by the ancient Egyptians,

Romans, and Greeks, while mint essential oils have been used as perfumes, food flavors, deodorants, and pharmaceuticals. Powdered mint leaves were used to whiten teeth during the Middle Ages. Mentha spp. leaves, flowers, and stems are frequently used in herbal teas or as additives in commercial spice mixtures for many foods to add aroma and flavor **(Kumar *et al.*, 2011)**. In fact, *Mentha rotundifolia* is still broadly used in different areas, for instance in Jijel-Algeria, by serving it with couscous which is an Algerian traditional dish.

Aside from its traditional culinary use, it has been used in Mediterranean traditional medicine for a variety of purposes, including tonic, stimulative, stomachic, carminative, analgesic, holeritic, antispasmodic, anti-inflammatory, sedative, hypotensive, and insecticidal **(Moreno *et al.*, 2002)**. For example, decoctions of *Mentha rotundifolia* which are used as condiment in soups in Portuguese cuisine but also as a treatment for cold, influenza and cough were found to have high levels of acetylcholine esterase inhibitory activity **(Ferreira *et al.*, 2006)**.

It has been reported that *Mentha rotundifolia* can be used to cure flatulent dyspepsia and pain in the small and large intestines but also abnormal low blood pressure. Furthermore, boiled upper ground part of *M. rotundifolia* was used to relieve chills and act as a stomachic **(Abbaszadeh *et al.*, 2009).** Whereas **Sutour *et al.,* (2008**) confirms that it acts also as a pain reliever, stimulates appetite, promotes calm, and controls insects.

CHAPTER 2

Essential Oils

2.1. Definition

The term "essential oil" was coined in the 16th century by **Paracelsus Von Hohenheim**, who referred to the active ingredient in a drug as "Quinta essential" **(Guenther *et al*., 1950)**.

Essential oils, also known as aromatic plant essences, are volatile, fragrant substances with an oily consistency produced by plants. They can be more or less fluid, resinous at times, and often range in colour from pale yellow to emerald green and blue to dark brownish red. They are lighter than water, with a density ranging between 0.75 and 0.98 g/cm3. They differ from solid and liquid fatty substances due to their volatility, which increases with increasing temperature **(Balz, 1999)**.

According to the same previous author, essential oils change from a liquid to a gaseous state quickly. One of their physical properties is diathermy, which means that the energy potential of an essential oil in gaseous form is increased by light passing through it because the oil retains the caloric energy of the light.

The mainly types of aromatic plants that produce essential oil in sufficient quantities belong to the *Labiatae* family (lavender, thyme, savory, sage, mint), the *Umbelliferae* family (caraway, anise, fennel), the *Myrtaceae* family (eucalyptus, cajeput, niaouli), the *Conifer* family (pine, cedar, cypress, juniper), the *Rutaceae* family (lemon, orange, bergamot), and the *Laurel* family (cinnamon, borneol, sassafras). Plant essential oils are principally contained in their flowers and leaves, but they are also found in their wood, fruit and peels, bark, seeds and roots. They are soluble in alcohol, ether, and oils, but virtually insoluble in water, where they can only be dispersed with the help of emulsifiers.

2.2. Chemical Composition of Essential Oils

Essential oils are complex combinations of volatile compounds derived from a variety of plants. In general, they are distinguished by the presence of two or three major components at relatively high concentrations (20-70%) in comparison to other components present in trace amounts. The biological properties of which are determined by these major components.

The components are divided into two groups based on their biosynthetic origins. The main group is made up of terpenes and terpenoids, while the other is made up of aromatic and aliphatic constituents, all of which have a low molecular weight **(Bakkali *et al*., 2008)**.

According to the previous author, terpenes form structurally and functionally different classes. The main terpenes are the monoterpenes (C10) and sesquiterpenes (C15), but hemiterpenes (C5), diterpenes (C20), triterpenes (C30) and tetraterpenes (C40) also exist. A terpene containing oxygen is called a terpenoid. They serve a variety of purposes. Some terpenes are effective treatments for cancer **(Ebada *et al*., 2010)**, malaria **(Parshikov and Netrusov, 2012)**, and heart disease **(Liebgott *et al*., 2000)**. Others exhibit insecticidal activity **(Rossi *et al*., 2012)**.

Aromatic compounds are derived from phenylpropane, they compromise Aldehyde, alcohol, phenols, methoxy derivatives and methylene dioxy compounds. This group occurs less frequently than terpenes. Some of the principal plant sources for these compounds are anise, cinnamon, clove and fennel in addition to some botanical families as *Apiaceae*, *Lamiaceae* and *Myrtaceae* **(Bakkali *et al*., 2008)**.

2.3. The Field of Application of Essential Oils

The diverse applications of essential oils account for the intense interest in their research. Such applications can be found in the cosmetic industry as fragrance ingredients, decorative cosmetics, fine fragrances and flavoring, in the food industry as aromas and flavors, in the pharmaceutical industry as active components of medicines and as antibacterials/antimicrobials, and in aromatherapy **(Ester *et al*., 2012)**.

Clove oil and its main compound eugenol, for example, are used in dentistry for their antiseptic and analgesic properties, whereas oils from tea tree, lemongrass, and sandalwood are used in dermatology to treat skin diseases **(Ferrentino *et al.*, 2020)**. Moreover, Mint oils have potent antibacterial properties against Gram+ and Gram- bacteria. Their antimicrobial activity is primarily determined by their chemical composition, specifically the nature of their main volatile compounds **(Hudaib *et al*., 2002)**.

2.4. Methods of Extracting Essential Oils

Essential oils could be extracted using various methods because of their hydrophobic nature and lower density than water but, according to **Balz, (1999)**, the most essential oils are extracted from the plants by the oldest method which is steam distillation water or hydrodistillation, and also through simple hydrodistillation, saturated steam distillation, and hydrodiffusion**.**

For any of the previous methods, the following factors must be considered:

- The optimal time to harvest the plant.
- Plant preparation prior to distillation.
- The best distillation time to obtain the highest yield.
- In the distilling kettle, the correct pressure/temperature ratio.
- Special knowledge is required because it is sometimes advantageous for certain plants to be distilled multiple times with rest periods in between, for example.

2.4.1. Steam Distillation

Steam distillation is one of the oldest methods for extracting essential oils. Water is heated in a pot to produce steam, which flows through the aromatic plant materials from the bottom to the top of the alembic. The steam causes the plant cell structures to rupture, releasing aromatic molecules and removing all volatile components. The essential oils are then transported by steam from the column to a condenser, where the steam is cooled and condensed in a water-oil mixture. After collecting the mixture in a vessel, the oil is separated from the water **(Ferrentino *et al.*, 2020).**

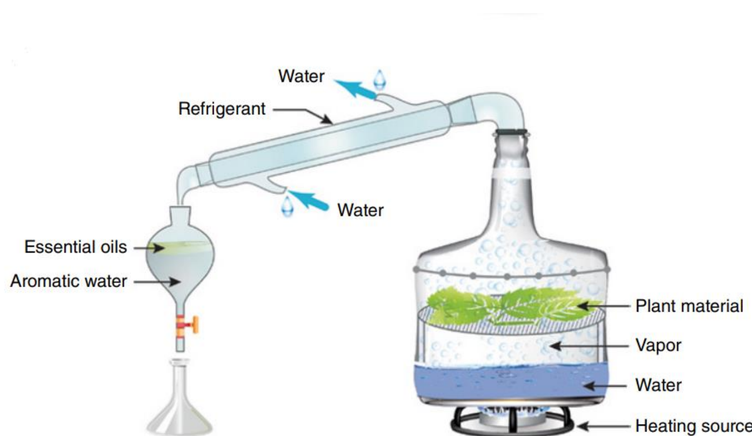

Figure 2: Steam distillation set-up **(Hashemi *et al.*, 2017)**

2.4.2. Hydrodistillation

According to **Meyer-Warnod (1984)**, it is the most basic and traditional method for extracting essential oils. Historically, **Avicenna (980-1037)** was the first to develop alembic extraction. He extracted the first pure essential oil, rose essential oil. The plant material is immersed directly in

the alembic's water, which is then brought to a boil. The extraction device consists of a heating source surrounded by a vessel (alembic) into which we could place plant material and water.

The European Pharmacopoea's third edition recommends hydrodistillation by clevenger system for determining EO yields because it allows for condensate recycling via a cohobage system. This method is suitable for extracting petals and flowers because it prevents plant material from compacting and clumping during extraction **(Asbahani *et al*., 2015)**.

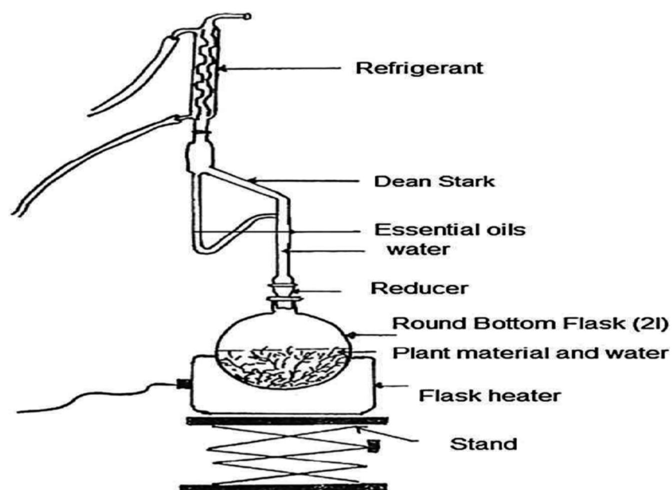

Figure 3: Hydrodistillation of essential oils: Clevenger-type Apparatus materials **(Alaoui *et al*., 2015)**

2.4.3. Solvent Extraction

Solvent extraction requires contacting the plant material containing the essential oils with a solvent for a set amount of time in order to ensure oil dissolution. Following extraction, the liquid mixture goes through filtration and distillation to evaporate the solvent. The majority of essential oils are soluble in hexane. Yet, other solvents such as acetone, petroleum ether, methanol, or ethanol, can also be used for extraction **(Koşar *et al*., 2005).**

Solvent extraction has several drawbacks, including an extended extraction time and a significant amount of heat required during the process **(Ferrentino *et al*., 2020)**.

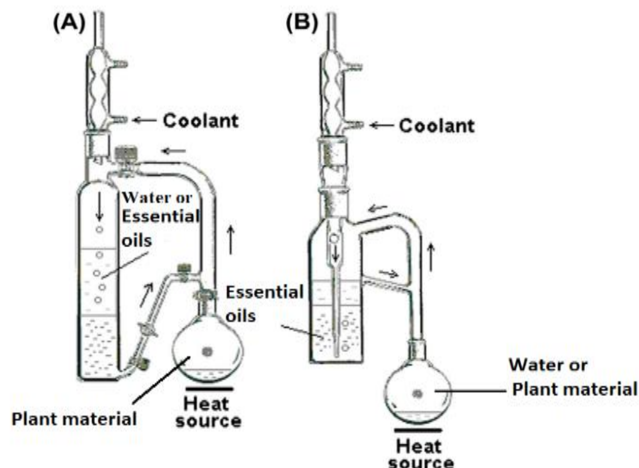

Figure 4: Essential oils solvent extraction **(A)** when the solvent is heavier than the organic phase **(B)** when the solvent is lighter than the organic phase **(Moldoveanu Serban, 2015)**

2.5. Properties of Essential Oils

Essential oils are characterized by a high level of chemical diversity and were found to contain several chemical compounds that were responsible for biological activities.

2.5.1. Antifungal Activity

According to **Karpinski (2020)** and the microbiological data, many significant studies have shown that essential oils have potent antifungal activities including *Lamiaceae* essential oils. Half of them have a good activity against fungi and they could be used alone or in combination with antifungal drugs in the treatment of fungal infections, especially of the skin and mucous membranes.

However, the inhibitory effect depends on the different biochemical composition of the examined essential oils; which is the case with the results represented by **Monforte *et al*., (2011)** about the inhibitory effect of essential oils from Italian *Calamintha nepeta* against *Candida albicans*.

Essential oils have a multidirectional mode of action. They may cause disruption of the cell wall and cell membrane through a permeabilization process but also their lipophilic compounds can pass through the cell wall and damage polysaccharides, fatty acids, and phospholipids, making them to permeable. Changes in the permeability of H+ and K+ cations affect cellular pH and cause organelle damage. Furthermore, essential oils inhibit the synthesis of fungal DNA, RNA, proteins,

and polysaccharides. They can also cause mitochondrial membrane disintegration **(Basak and Guha, 2018)**. In the work of **de Lira Mota *et al*., (2012)**, *Thymus vulgaris* essential oil has also been shown to inhibit the production of aflatoxins by *Aspergillus flavus*, resulting in a decrease in ergosterol production.

2.5.2. Insecticidal Activity

More than 1500 plant species have been reported to have insecticidal properties that are highly dependent on their chemical composition **(Kumar *et al*., 2011)**. Many plants secondary metabolites, such as alkaloids, monoterpenoids, and phenylpropanoids, are toxic to insects; additionally, essential oils extracted from plants have been extensively researched pest control properties, with some providing toxic results **(Aziz and Craker, 2010)**.

According to **Božović *et al*., (2015)**, it is unknown how essential oils, and their constituents work as insecticides, where the fundamental metabolic, biochemical, physiological, and behavioural processes of insects can be disrupted by plant essential oils due to their lipophilic nature. On the report of recent research, essential oils and their constituents affect biochemical processes, specifically disrupting the endocrine balance of insects. They could be neurotoxic or act as insect growth regulators, disrupting the normal morphogenesis process. Furthermore, monoterpenes have been studied for neurotoxicity; they are typically volatile and lipophilic compounds that can quickly penetrate insects and interfere with their physiologic functions by inhibiting acetylcholinesterase activity **(Praveena *et al*., 2011)** and acting on insects' octopaminergic sites **(Price *et al*., 2006)**.

2.5.3. Antibacterial Activity

The Ancient Egyptians used aromatic plants in embalming to prevent bacterial growth and decay, which was attributed in large part to their essential oils. Strong *in vitro* evidence suggests that essential oils can act as antibacterial agents against a diverse range of pathogenic bacteria strains such as *Listeria monocytogenes*, *Listeria innocua*, *Salmonella typhimurium*, *Escherichia coli O157:H7*, *Shigella dysenteria*, *Bacillus cereus*, *Staphylococcus aureus*, and *Salmonella typhimurium* and many others **(Shuaib *et al*., 2016)**.

Many studies have shown that essential oils have antibacterial properties due to their phenolic components carvacrol and thymol, such as oregano, savory, and thyme, which usually have an

inhibitory effect on Gram-positive pathogenic bacteria more than Gram-negative pathogenic bacteria.

The mechanisms by which essential oils inhibit microorganisms involve various modes of action, which may be due in part to their hydrophobicity. As a result, they are partitioned into the cell membrane lipid bilayer, making it more permeable and allowing vital cell contents to leak **(Nazzaro *et al.*, 2013)**. A potential mechanism of action could be the impairment of bacterial enzyme systems **(Wendakoon and Sakaguchi, 1995)**.

2.5.4. Antioxidant Activity

Essential oils, natural antioxidants are becoming more popular due to their safer nature and medicinal benefits when compared to synthetic formulations **(Tafrihi *et al.*, 2021)**. These substances can slow or prevent lipid oxidation caused by high levels of oxygen radicals caused by environmental factors or pathogens. In this regard, extracts and essential oils from a variety of medicinal and food herbs have been studied as a potential source of effective antioxidant agents, with a well-known action against reactive oxygen species and free radicals **(Anwar *et al.*, 2017)**.

Essential oil antioxidant activity may be linked to a variety of systems due to their chemical composition, which includes a variety of functional groups, polarity, and chemical behavior. Several medicinal plants, including the *Mentha* genus, contain high levels of antioxidants such as phenolic compounds, ascorbic acid, and carotenoids, which can delay or inhibit the oxidation of various molecules e.g., phenolic compounds act as free radical scavengers and inhibit lipid peroxidation **(Park et al., 2019)**.

However, **Yadegarinia *et al.*, (2006)**, affirm that oxygenated monoterpenes are the most effective radical scavenging compounds. Whereas **Ruberto and Baratta (2000)** noted that monoterpene hydrocarbons had the highest antioxidant capacity.

In contrast, the antioxidant activity of essential oils may be attributed to minor components rather than major ones **(Mukazayire *et al.*, 2011)**. Furthermore, both minor and major constituents may act synergistically to form a chemical mixture that contributes to the essential oil antioxidant activity **(Wang *et al.*, 2000)**.

One of the effects of which, the acetylcholinesterase inhibitory effect. **Benabdallah *et al.*, (2018)** who studied Algerian wild mint essential oils relating to the acetylcholinesterase inhibitory effect,

observed an important inhibitory effect of oxygenated monoterpenes such as menthofuran, rotundifolone, menthol and pulegone, which are the main components of Algerian mint essential oils. The results obtained by the previous author agree with those of **Miyazawa *et al*., (1998)** from Japan. According to **Aazza *et al*., (2011),** the effect of the essential oils could be explained by their richness in monoterpenes that found to act as competitive or uncompetitive inhibitors due to their hydrophobicity and their ability to interact with the hydrophobic site of this enzyme.

2.5.5. Antiviral Activity

Many viruses, due to their adaptable lifestyle, are resistant to various therapies, making the development of long-term effective antiviral chemotherapeutic agents a very difficult task. However, natural products have always been regarded as the best source for isolating chemically diverse new lead molecules, and they have served as a foundation for the future development of potent and safer antiviral agents **(Tafrihi et al., 2021)**.

It has been reported by **Orhan *et al*., (2012)** that *Mentha spicata* essential oils contain compounds that act as antiviral agents such as phenolic constituents; rosmarinic acid, luteolin, and phytol. However, other preliminary studies suggested that the main component of peppermint oil, menthol, may well act as a natural antiviral agent and protected against *Herpes simplex* but also an inhibitory activity against HIV-reverse transcriptase was observed by its polar substances extract **(Schuhmacher *et al*., 2003)**.

Furthermore, **Yamasaki *et al* (1998)** confirmed that a water-soluble extract of *Mentha piperita* (16 g/mL) had potent anti-human immunodeficiency virus-1 (HIV)-1 activity. Whereas, **Amazazi *et al*., (2003)** reported that *Mentha longlifolia* methanolic and ethyl acetate extracts inhibited human HIV-1 significantly, with the ethyl acetate extract exerting its anti-HIV-1 effects by inhibiting the reverse transcriptase enzyme.

2.6. Essential Oils of *Mentha rotundifolia*

Essential oils and plant extracts have sparked interest as natural product sources. They've been tested for their potential use as alternative treatments for a variety of infectious diseases, as well as for protecting food from the toxic effects of oxidants. Research on plants from various regions has resulted in novel applications of essential oils. The antimicrobial properties of plant oils and extracts, in particular, have served as the foundation for a wide range of applications, including

raw and processed food preservation, pharmaceuticals, alternative medicine, and natural therapies **(Hajlaoui *et al*., 2009)**.

Essential oils contain a variety of volatile molecules, including terpenes and terpenoids, aromatic compounds made from phenol, and aliphatic components, due to the mode of extraction from different parts of aromatic plants **(Bakkali *et al*., 2008)** using classical and advanced techniques **(Mohammadhosseini, 2017)**. Hydrodistillation is the most important traditional approaches to extracting the EOs.

Among the genus *Mentha*, *Mentha rotundifolia* essential oils are one of the widely used around the world besides those of the other species which may vary in their essential oil content and composition. However, the same taxon growing in different areas may have significantly different chemical components and thus biological properties due to environmental factors such as temperature, photoperiod, nutrition, and salinity that have a strong influence on the biosynthesis and metabolism of essential oils **(Aziz and Craker., 2010)**. Variations in oil content and composition are also caused by plant chemotypes, cultivation practices, and extraction methods. Other agronomic and genotype conditions that influence essential oil composition include harvesting time, plant age, and crop density **(Kumar *et al*., 2011)**.

2.6.1. Chemical composition of *Mentha rotundifolia* Essential oil

Research on *Mentha rotundifolia*, in Algeria and around the world, has mainly focused on its chemical composition, which has shown to have a great diversity due to biotic and abiotic factors such as climate specific to plant origin regions, geographical factors (altitude, soil type, and harvest period), and extraction techniques **(Bentoura *et al*, 2021)**.

Table 1: Main chemical compounds isolated from *Mentha rotundifolia* **(Tarfihi *et al.,* 2021)**

Essential Oil Components	Polyphenol Compounds	Reference
Menthol, menthone, menthyl acetate, menthofuran, piperitone oxide, linalyl acetate, neomenthol, piperitone, isomenthone, 1,8-cineole, linalool, geraniol, myrcene, geranyl acetate, germacrene D, carveol, limonene, rotundifolone, p-menthane-1,2,3-triol, D-limonene, piperitol, diosphenol, β-caryophyllene,, germacrene D, calamenene, trans-piperitone epoxide, piperitenone oxide, cis-piperitone oxide, cyclohexanol, trans-sabinene hydrate	Hypericin Apigenin Quercetin Trans-cinamaldehyde acid Rosmarinic acid Quercetin3-O-galactoside Hydroxybenzoic acid Procyanidin B2	**(Satmi and Hossain, 2016)** **(Tsai *et al*., 2013)** **(Politeo *et al*., 2018)** **(Rahmani *at al*., 2018)** **(Ferdjioui *et al*., 2019)**

The analysis of the essential oil of *Mentha rotundifolia* from north, north east, and east Algeria revealed the presence of two chemotypes distinguished by varying levels of piperitone oxide (23,8 %) and piperitenone oxide (30,32 %) **(Derwich *et al*., 2009)**.

Piperitenone oxide has been reported as the major constituent of *Mentha rotundifolia* essential oils in Japan and Uruguay (87.3% and 80.8%, respectively), while piperitone oxide has been reported as the major constituent of essential oils in Greece, Morocco, and Italy (40.5%). According to some research, the most important constituents are oxygenated monoterpenes **(Mailhebiau, 1994)**, such as pulegone (50%) in Morocco and carvone (62.3% and 40.30%, respectively) in Finland and Argentina **(Derwich *et al.,* 2010)**.

Table 2: The percentage of the main components of *Mentha rotundifolia* essential oils according to the literature **(Brahmi *et al*., 2017)**

Species	***Component***	***Origin and the % in the oil***	***Reference***
	Carvone	Argentina (43%), Finland (62%)	**(De la Torre and Torres., 1977; Galambosi *et al*., 1998)**
	Trans-piperitone oxide	Italy (41%), Japan (18-26%)	**(Avato *et al*., 1995; Umemoto *et al*., 1994)**
	Cis-piperitone oxide	Algeria (18-31%)	**(Brada *et al*., 2006)**
	Piperitol	Spain (58%)	**(Perez Raya *et al*., 1990)**
Mentha rotundifolia	Piperitenone oxide	Japan (46%), Japan (8-84%), Morocco (0.9-56%), Algeria (24-39%)	**(Miyazawa *et al*., 1998; Fujita *et al*., 1977 ; Brada *et al*., 2007)**
	2,4(8),6 p-Menthatrien-2,3-diol	Cuba (15%)	**(Pino *et al*., 1999)**
	Lippione	Senegal (80%)	**(Koyalta *et al*., 1993)**
	Pulegone	Morocco (85%), Tunisia (32%)	**(El Arch *et al*., 2003; Riahi *et al*., 2013)**
	Menthol	Morocco (41%)	**(Derwich *et al*., 2010)**
	Piperitenone	Algeria (55%)	**(Brada *et al*., 2007)**
	Trans-piperitone epoxide	Algeria, Bejaia (30%)	**(Brahmi *et al*., 2016)**

2.6.2. Antifungal, Insecticidal and Antibacterial activities of *Mentha rotundifolia* Essential Oils

The biological activity of an essential oil is linked to its chemical composition, to the functional groups of the main compounds and to the possible synergistic effects of these constituents **(Chebli *et al*., 2003)**. While **Durraffourd *et al*., (2002)**, affirm that it is probable that the minority compounds act in synergy; in this way, the value of the essential oil is due to all the components.

According to research, the essential oils of *Mentha rotundifolia* have a strong antibacterial activity against *Staphylococcus aureus* and a moderately strong effect on *Escherichia coli*. The mechanisms by which essential oils inhibit these microorganisms involve different modes of action but may be due in part to their hydrophobicity. As a result, they cause lipid partitioning of bacterial cell membranes and mitochondria, disturbing the cell structures and rendering them more permeable **(Shuaib *et al*., 2016)**.

On the other hand, fungi are resistant and less vulnerable than bacteria where for example, the essential oil of *Mentha rotundifolia* shows a potent effect on the strain of *Candida albicans*, *Saccharomyces cerevisiae* and *Aspergillus niger* but a highly inhibitory effect against *Botrytis cinerea* and *Fusarium solani* by altering the mycelium, inhibiting spore germination but also by inducing morphological changes in the spores causing up to the exuviation of cellular content.

Additionally, essential oils of *Mentha rotundifolia* revealed a strong insecticidal potential against pests like *Tribolium castaneum* and *Anopheles stephensi*. This effective activity respectively could be attributed to its major components as piperitenone oxide, D-Limonene and Cis-piperitone epoxide but also in the mode of application (contact or fumigation) which can be explained by differences in the polarities and volatilities of the individual essential oil components **(Aouadi *et al*., 2021; Cox *et al*., 2001).**

Table 3: Biological activities of the main components of *Mentha rotundifolia* essential oils

Main Component	Biological Activity	Reference
Piperitenone oxide	Antibacterial activity Antifungal Activity Antioxidant Activity	**(Oumzil *et al*., 2002)** **(Brada *et al*., 2007)**
Piperitone oxide and	Insecticidal Activity	**(Tripathi *et al*., 2004)**

piperitenone oxide		
Oxygenated monoterpenes (Pulegone, menthone)	Antifungal Activity	**(Carson and Riley., 1995)**
Phenolic components	Antioxidant Activity	**(Bentoura *et al*., 2021)**
Monoterpenes (limonene, piperitenone, β-pinene, α-pinene and p-cymene)	Insecticidal Activity	**(Samir *et al*., 2009)**

CHAPTER 3

Phytopathogenic Fungi and Tested Pest

3.1. General Description of Phytopathogenic Fungi

Fungi are eukaryotes that digest food and absorb nutrients directly through their cell walls. Most fungi reproduce by spores, and their bodies, known as the thallus, are made of tiny tubular cells called hyphae. They are heterotrophs and rely on other living organisms for their carbon and energy just like animals **(Carris *et al*., 2012)**.

According to **Doehlemann *et al* (2017)**, fungal plant pathogens are traditionally divided into two groups: biotrophic pathogens, which form intimate interactions with plants and can persist in and utilize living tissues (biotrophs), and necrotrophic pathogens, which kill the tissue to extract nutrients (necrotrophs). In addition to these two groups, members of a third, hemibiotrophic pathogens, begin as biotrophs and then shift to necrotrophs which cause necrosis and, in extreme cases, death in infected plants. Whereas biotrophic symptoms may appear mild in many cases, it contains some of the most economically devastating pathogens.

Fungi have developed a variety of strategies for colonizing plants, and these interactions result in a wide range of outcomes, from beneficial interactions to host death. In terms of plant pathogens, fungi are likely the most diverse group of ecologically and economically significant threats. Furthermore, these pathogens are mostly found in the phyla Ascomycota and Basidiomycota. They are classified as Dothideomycetes (e.g., *Cladosporium* spp.), Sordariomycetes (e.g., *Magnaporthe* spp.), or Leotiomycetes (e.g., *Botrytis* spp.) among Ascomycetes. However, the two largest plant pathogen groups represent Basidiomycetes are rusts (Pucciniomycetes) and smuts (distributed throughout the Ustilaginomycotina subphylum).

Table 4: Top 10 fungal plant pathogens **(Dean *et al*., 2012)**

Rank	Fungal pathogen	Author of fungal description
1	*Magnaporthe oryzae*	**Ralph Dean**
2	*Botrytis cinerea*	**Jan A.L., Van Kan**
3	*Puccini spp*	**Zacharias A, Pretorius**
4	*Fusarium graminearum*	**Kim Hammond-kosack**
5	*Fusarium oxysporum*	**Antonio Di Pietro**
6	*Blumeria graminis*	**Pietro Spanu**
7	*Mycosphaerella graminicola*	**Jason J, Rudd**

8	*Colletotrichum spp*	**Marty Dickman**
9	*Ustilago maydis*	**Regine Kahman**
10	*Melampsora lini*	**Jeff Ellis**

3.1.1. *Botrytis cinerea*

3.1.1.1. Definition

The name "*Botrytis cinerea*" is directly related to the morphology of the fungus: "*Botrytis*" is derived from the Greek word for "bunch of grape berries," which describes the grape-like morphology of conidiophores, and "*cinerea*" refers to the grey color of sporulation **(Petrasch *et al*., 2019)**.

Botrytis cinerea (teleomorph: *Botryotinia fuckeliana*) is a necrotrophic airborne plant pathogen that attacks over 200 crop hosts worldwide **(Williamson *et al*., 2007)** and the causal agent of the grey mold disease. It reproduces primarily through asexual conidia or spores, which are easily dispersed by wind, water, or any physical activity. It affects the vegetable and fruit crops, as well as many shrubs, trees, flowers, and weeds **(Amselem *et al*., 2011)**.

This Ascomycete can infect a wide variety of plants at any stage of development and found on all continents. The most notable host is the wine grape, where it not only causes harmful bunch rot but can also cause a beneficial form of berry colonization known as "noble rot" under favourable conditions. For centuries, grapes infected with noble rot have been used to make sweet dessert wines. *Botrytis cinerea* has the potential to cause significant economic losses in a wide range of crops and harvested commodities, including vegetables (lettuce, tomato), fruits (berries, kiwifruit), and ornamentals (rose) **(EnsemblFungi.org)**.

3.1.1.2. Taxonomy

Botrytis is a cosmopolitan genus with 22 recognized species and one hybrid **(Staats *et al*., 2005)**, all of which are plant pathogens. According to the previous author, *Botrytis cinerea* is the only species in the genus with a broad host range, whereas all other *Botrytis* species are thought to be specialized on a single plant species.

The pathogen’s taxonomy is as follows **(Rhouma *et al*., 2022):**

Domain: Eukaryota

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Pezizomycotina

Class: Leotiomycetes

Subclass: Leotiomycetidae

Order: Helotiales

Family: Sclerotiniaceae

Genus: *Botrytis*

Species: *Botrytis cinerea*

3.1.1.3. Morphology

Botrytis cinerea mycelium is branched, septate, and hyaline to brown in colour. Conidiophores, which grow directly from the mycelia or the sclerotia, are tall, slender, and irregularly branched in the terminal portion, with enlarged or rounded apical cells that bear clusters of conidia on short denticles. Conidia are egg-shaped, smooth, hyaline or grey, and have a mean length of 10 µm and a mean width of 5 µm **(Barnett and Hunter, 1998)**. Sclerotia, which are survival structures, are frequently found. They are formed from mycelial branches that fuse together to form a globular mass that is initially hyaline but later turns brown or black due to melanic pigment deposition in the outer rind. Long-term protection from desiccation, UV radiation, and microbial attack is provided by the melanised coat and β-glucans encasing the internal mycelium of sclerotia **(Williamson *et al.*, 2007)**. Sclerotia germinate through the emission of conidiophores or, after a sexual process, the elongation of an apothecium when environmental conditions are favourable for the fungus. However, conidiophore production is the most common mode of germination **(Figure 5) (Jarvis, 1977)**.

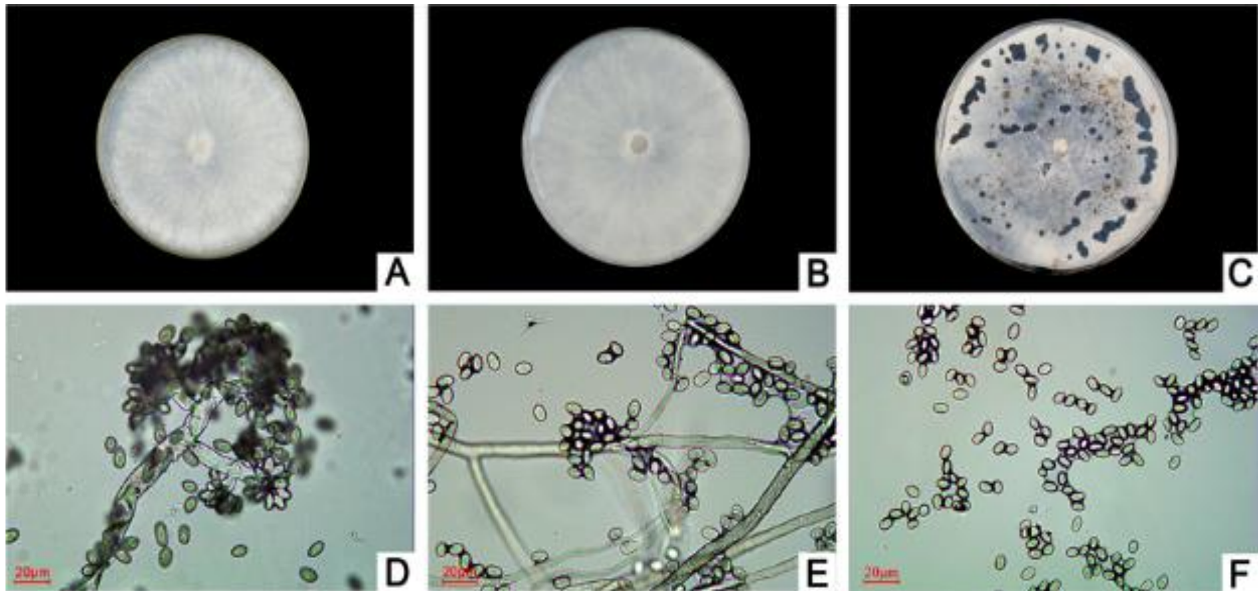

Figure 5: Morphological characteristics of the pathogen *Botrytis cinerea*. **A** and **B**: the front and back of the colony morphology on potato sugar agar (PSA) plate medium. **C**: sclerotia morphology on PSA. **D**, **E** and **F**: conidiophore and conidia morphology on PSA **(Gong *et al*., 2015)**

3.1.1.4. Host Range

Botrytis cinerea has no discernible host preference and can infect over 1000 plant species. The pathogen is found all over the world and causes disease in a variety of fruit, flower, and leafy vegetable crops **(Elad *et al*., 2016).**

Botrytis cinerea is a typical necrotrophic fungus, it kills host plant cells before colonizing the dead tissue **(Amselem *et al*., 2011)**. The minimum temperature for growth is 0°C, the optimal temperature is 20°C, and the maximum temperature is 30°C. As a result, the pathogen thrives during the cold storage of fruits and vegetables **(Romanazzi and Feliziani, 2014)**.

Yet **Droby and Lichter (2004)** affirm that the most severely affected crops are vegetables (such as cabbage, lettuce, broccoli, and beans) and small fruit crops (such as grapes, strawberries, raspberries, and blackberries). With increased international trade in cold-stored produce, *Botrytis cinerea* has grown in importance due to its ability to grow effectively over long periods at temperatures just above freezing in products such as kiwifruit, apples and pears. Additionally, this pathogen has an impact on the important cut flower trade; rose and gerbera flowers are particularly vulnerable. Out-of-season plant culture in heated or unheated greenhouses and under plastic tunnels, which are increasingly being used to supply fruits, vegetables, herbs, and flowers in

northern latitudes, greatly increases the risk of infection, particularly in tomato, cucumber, and sweet pepper plants **(Williamson *et al*., 2007)**.

3.1.1.5. Strawberry *Botrytis cinerea* Infection Process

According to **Bristow *et al*., (1986)**, grey mould in strawberries can develop as a result of *Botrytis cinerea* infections on open flowers as a primary infection or tissue penetration in the fruit receptacle, which is the secondary infection **(Figure 6)**.

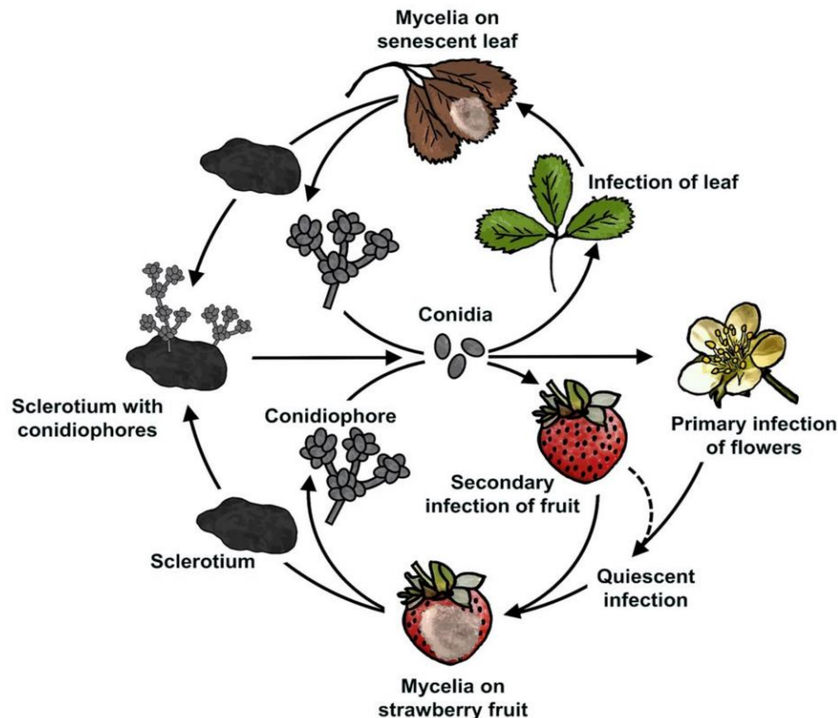

Figure 6: The cycle of *Botrytis cinerea* disease in strawberry including the pathogen inoculum sources, primary infections and secondary infections **(Petrasch *et al*., 2019)**

In primary infections, *Botrytis cinerea* infects flower organs during or shortly after flowering, allowing hyphae to grow into the receptacle where primary inoculum sources include overwintering sclerotia as well as conidia or mycelium from infected neighboring plants **(Jarvis, 1962)**. Whereas, according to **Powelson (1960)**, in fruit, primary infections can be facilitated by infected senescent petals, stamens, and calyxes **(Figure 7)**. However, histological studies, despite the fact that styles are frequently infected, show that fungal growth appears to be severely restricted and never reaches the receptacle. On the contrary belief, fungal growth in colonized stamens can reach the receptacle in some cultivars **(Bristow *et al*., 1986)**.

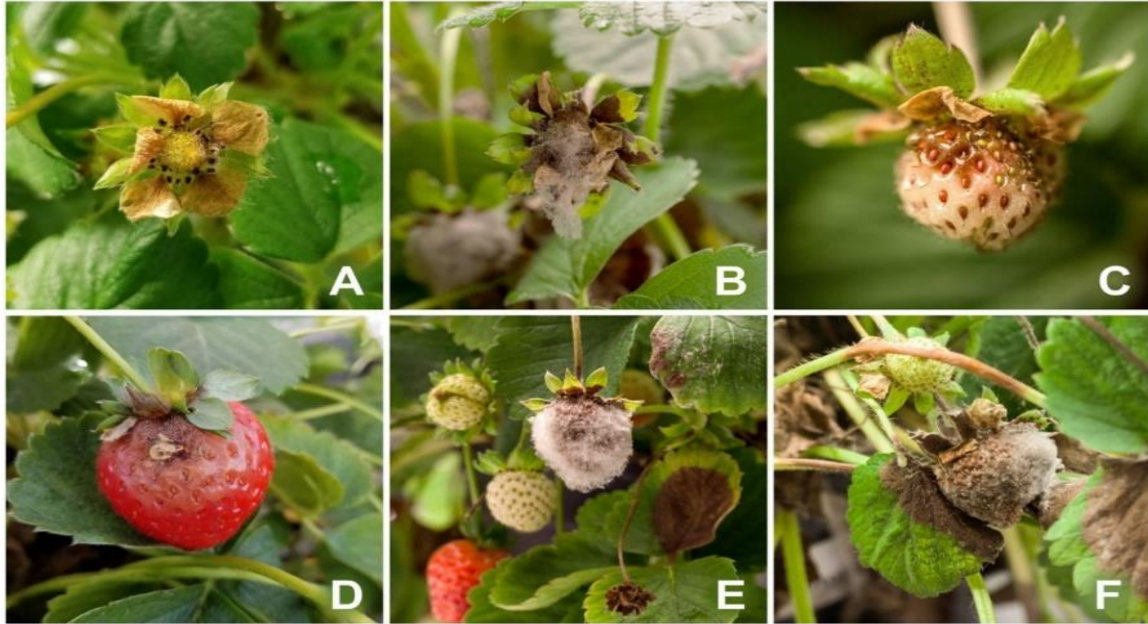

Figure 7: Symptoms of Botrytis cinerea infections in strawberry. **A:** shows a senesced flower with Botrytis cinerea mycelium growth. **B:** shows an advanced floral infection. **C** and **D:** show infections of fruit at different stages, where an infected petal can be seen as the source of fruit infection. **E** and **F:** show browning leaves due to Botrytis cinerea infections **(Petrasch *et al*., 2019)**

Following *Botrytis cinerea* infection of an unripe receptacle, fungal growth usually stops and a symptomless quiescent phase occurs **(Petrasch *et al*., 2019).** It has been proposed that quiescence in unripe fruit is caused by the following factors; a lack of nutrients from the host, the presence of preformed antifungal compounds and an unsuitable environment for fungal virulence factors **(Prusky and Lichter, 2007)**. Unripe strawberries contain factors from all three categories, including a lack of available sugars **(Knee *et al*., 1977)**, preformed antifungal compounds **(Terry *et al*., 2004)**, and high activity of PG-inhibiting proteins (PGIPs) **(Mehli *et al*., 2005)**. According to **Neri *et al*., (2015),** changes in the biochemical composition of the host tissues associated with the ripening process, such as increased sugar content, volatile production, and changes in plant defenses, may trigger the necrotrophic phase in ripe strawberries. All of which promote not only fungal growth but also host susceptibility, for example, through the release of oxalic acid and toxin efflux.

In the other hand, during secondary infections, the fungus enters the necrotrophic phase **(Holz *et al*., 2007)**. Its conidia sources can range from senescent leaves to infected fruit, additionally; conidia from *Botrytis cinerea* infected flower parts are important sources of secondary inoculum **(Figure 6) (Bristow *et al*., 1986).** Furthermore, it has been estimated, according to **Jarvis, (1962),**

that organic fragments in contact with the fruit, such as petals and stamens, contributes to more than 64% of strawberry infections. The same author affirms that senescent flower parts frequently adhere to strawberries, in contrast to other fruits as raspberries, long enough to retain water films for at least 8 hours, which is the time required for *Botrytis cinerea* conidia germination.

Secondary infections can also occur resulting from nesting, which characterized by direct penetration of mycelia growing on neighboring plants organs such as infected leaves and fruit **(Figure 7) (Braun and Sutton, 1988)**. In general, this type of infections, they progress quickly where the pathogen *Botrytis cinerea* can complete germination and infection as quickly as 16h post-inoculation, with a rapid increase in fungal biomass at 48h post-inoculation **(Mehli *et al.*, 2005)**.

3.1.1.6. *Botrytis cinerea* Agronomic and Economic Impact on Strawberry Production

Botrytis cinerea is a lethal airborne pathogen that costs millions of dollars damage to a vast variety of crops worldwide. The pathogen causes pre-harvest and post-harvest diseases resulting in significant economic losses **(Grabke, 2014)**, they can occur not only at pre and post-harvest but at any point in the production process, transportation, selling, and after final sale **(Notte *et al.*, 2021)**. The most economically important host crops are strawberry, grape, lettuce, and cabbage worldwide **(Dianez *et al.*, 2002)**.

According to **Darrow (1966)**, strawberry is an herbaceous perennial plant with short stems (crowns) and densely spaced leaves. These species can produce aggregate and complex accessory fruit made up of achenes and a receptacle. The achenes are small single-seeded fruits, and the receptacle is anatomically equivalent to floral meristem tissue **(Hollender *et al.*, 2012)**.

Strawberries are a good source of macro and micronutrients, vitamins, and antioxidants that promote good health **(Giampieri *et al.*, 2015)**. The most producing countries of this fruit around the world are USA, Spain, Japan, Poland, Korea and Russian Federation where the production was estimated by thousands of tons **(Sharma *et al.*, 2009)**. However, the cultivars are harmed by a variety of pathogens, including fungi, bacteria, viruses, and nematodes. Fungi are the most economically important strawberry pathogens, as they can infect all parts of the plant and cause severe damage or death **(Garrido *et al.*, 2011)**.

Among fungal pathogens, the ascomycete *Botrytis cinerea* is considered the primary pathogen of harvested strawberries worldwide, causing significant economic losses to the strawberry industry. The grey mould pathogen causes fruit and organs senescing, but it can also affect vegetative tissues **(Fig. 5)**. More than 80% of strawberry flowers and fruits can be lost under wet conditions if plants are not sprayed with fungicides **(Ries, 1995)**, while **Petrasch *et al*., (2019)** added that the market value of *Botrytis spp*. fungicide in the Far East and North America was approximately $28.6 million. Strawberry grey mould claimed yield losses of up to 50%, whereas under favorable conditions for the growth and development of this airborne pathogen (100%).

Meanwhile, **Chaves and Wang (2004**) affirm that *Botrytis cinerea* causes significant economic losses in strawberry cultivation, estimated to be around 30% of total production predominately in high humidity conditions where the losses could be about 40-50%. The pathogen is even more aggressive in post-harvest, affecting 95% of the fruits 48 hours after harvest.

3.1.2. *Colletotrichum gloeosporioides*

3.1.2.1. Definition

Colletotrichum is one of the major plant pathogenic genera that causes anthracnose, a plant disease that affects a wide range of hosts, from trees to grasses **(Dean *et al*., 2012)**. The disease is distinguished by sunken spots of varying colors on the leaves, stems, fruits, or flowers. These spots frequently enlarge, causing wilting, withering, and death of infected plant tissues (**Hiremath *et al*., 1993**). To infect different plant hosts, including gymnosperms, angiosperms, ornamental and fruit plants, vegetables, crops, and even grasses, the pathogen requires warm and humid conditions **(Farr *et al*., 2006).**

Gautam (2014) affirms that *Colletotrichum gloeosporioides* **(Penz.) Penz. & Sacc**, is an asexual facultative parasite belonging to the family *Phyllachoraceae* of the division Ascomycota. *Colletotrichum gloeosporioides* is the anamorph imperfect or asexual state of the fungus, while *Glomerella cingulata* is the sexual (perfect) teleomorph state. The fungus prefers a warm, humid environment to uniformly and effectively spread the anthracnose disease.

3.1.2.2. Taxonomy

Primarily based on morphological and microscopic features, *Colletotrichum gloeosporioides* taxonomic description has been thoroughly described and published by various research groups.

According to **Gautam (2014)** the classification is as follow:

Kingdom: Fungi

Division: Ascomycota

Class: Sordariomycetes

Order: Phyllachorales

Family: Phyllachoraceae

Genus: *Colletotrichum*

Species: *Colletotrichum gloeosporioides*

Scientific Name: *Colletotrichum gloeosporioides* **(Penz.) Penz. & Sacc**.

Teleomorph: *Glomerella cingulata* **(Stoneman) Spauld. & H. Schrenk**.

3.1.2.3. Morphology

During the infection process, the fungus primarily invades injured or weakened plant tissues and produces a variety of specialized structures. Conidia, acervulli, setae, and appressoria are specialized structures formed during the host-pathogen interaction. The fungal characteristics on culture media differ between hosts. In general, the fungus forms circular, woolly, or cottony colonies on culture media that are pale brown or grayish white **(Figure 8) (Prabakar *et al*., 2005)**. Growing culture mycelium is hyaline, septate, and branched. Conidiomata are acervular, separate structures made up of hyaline to dark brown septate hyphae. In culture, the fungus produces dark brown, occasionally setose sclerotia. The setae are brown, long, and septate. Conidiogenous cells are enteroblastic, phialidic, and hyaline, with conidia that are hyaline, one celled, straight, cylindrical, and obtuse at the apices **(Gautam, 2014)**.

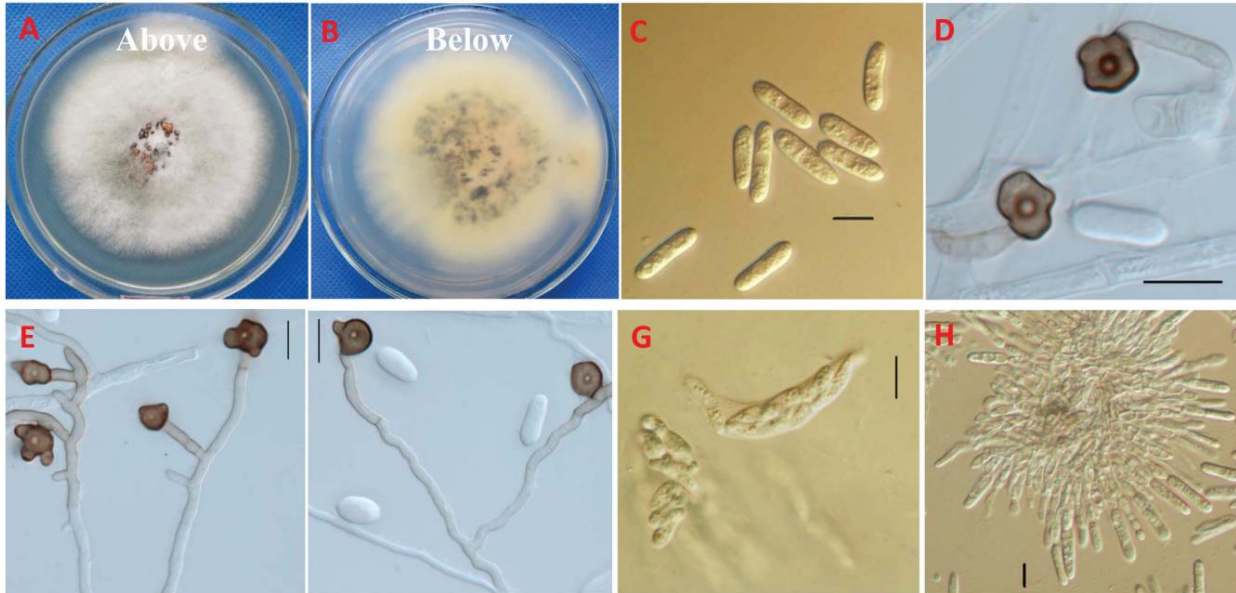

Figure 8: Phenotypical and morphological characteristics of Colletotrichum gloeosporioides. **A** and **B:** colonies in PDA at 7 days post-inoculation. **C:** conidia. **D:** conidial appressoria. **E** and **F:** hyphae appressoria. **D**: asci and ascospores. **H:** conidiophores **(Jian *et al*., 2021)**

3.1.2.4. Infection Process and Symptoms

Interestingly, a single *Colletorichum* species has been found to infect multiple hosts using diverse invasion strategies ranging from intracellular hemibiotrophy to subcuticular intramural necrotrophy. The pathogen develops a series of specific infection structures, including germ tubes, appressoria, intracellular hyphae, and secondary necrotrophic hyphae. *Colletorichum* studies have revealed that the pathogen in nature could be saprophytic, parasitic, and endophytic **(Gautam, 2014)**.

According to the previous author, *Colletotrichum gloeosporioides* colonizes wounds and plant tissues, producing a large number of acervuli and conidia. Conidia can infect other healthy plant tissues and spread over relatively short distances via rain splash or overhead irrigation. The formation of specialized infection structures known as appressoria is generally required for penetration into host tissues. These appressoria allow the fungus to directly penetrate the host cuticle and epidermal cell wall via a narrow penetration peg that emerges from the appressorium's base. Acervli are asexual bodies formed during the infection process in the tissue of the infected host as a small, flask-shaped structure with a small cushion at the bottom, from which short,

crowded conidophores form and can be seen on the surface of diseased plants. Conidia exit through an opening at the top of the acervuli. Setae are typically long brownish-colored filaments that emerge from acervuli **(Vineet *et al*., 2001)**. The entire infection process, including the formation of conidia, acervuli, setae, and appressoria, leads to tissue necrosis. Inocula can also be found in dead wood and plant debris.

Anthracnose disease is defined by sunken, water-soaked spots that rapidly expand on infected plant tissue, becoming soft on full expansion and displaying a range of colors ranging from red-brown to tan to black. However, the symptoms caused by *Colletotrichum gloeosporioides* vary greatly from host to host, some of them are sunken, watersoaked, round to oval, regular to irregular, and brownish red to black spots. It developed irregular reddish brown and sunken necrotic lesions on strawberry leaves, stems, petioles, stolons, crowns, flowers and fruit, which may cause the death of plants **(Figure 9) (Jian *et al*., 2021)**.

Figure 9: Typical symptoms of strawberry anthracnose. **A** and **B:** diseased leaves. **C** to **E:** diseased stolon**. F:** diseased petiole. **G:** diseased crown **(Jian *et al*., 2021)**

It was shown that the nutritional components of culture media, as well as other growth parameters such as temperature, moisture, and pH, could influence *Colletotrichum gloeosporioides* growth and sporulation **(Pandey *et al*., 2012)**. *Colletotrichum gloeosporioides* showed the greatest growth

in the pH range of 6-7 and temperature range of 25-30°C, whereas exposure of the fungus to alternate cycles of 12h light and 12h darkness resulted in the greatest mycelial growth in comparison to 24h exposure to continuous light and 24h exposure to continuous dark **(Manjunath *et al*., 2011)**.

3.1.2.5. *Colletotrichum gloeosporioides* Agronomic and Economic Impact

Most crops grown around the world are susceptible to one or more *Colletotrichum* species. Yet, *Colletotrichum gloeosporioides* causes a serious crown rot of strawberry which is a serious problem in the southeastern United States, because this region is relatively warm all year and has sufficient rainfall to spread the disease but also in India and China **(Mackenzie *et al*., 2007)**.

As a result, the pathogen that cause anthracnose may pose significant economic constraints to crop production around the world, causing significant losses by destroying fruits such as strawberry, mango, citrus, and avocado, bananas, vegetables, and medicinal useful plants. Based on perceived scientific and economic importance, the genus was recently voted the eighth most important group of plant pathogenic fungi in the world **(Gautam, 2014).**

3.2. *Aphis fabae*

3.2.1. Description

Aphids are global pests of numerous agricultural crops, distinguished by their rapid development and high reproductive rate. They not only cause direct damage to their hosts, but they are also important vectors for many plant pathogens. The black bean aphid, *Aphis fabae* Scopoli (Hemiptera: Aphididae) **(Figure 10)**, is a major pest of many cultivated crops, including beans, tomatoes, potatoes, and tobacco, as well as numerous wild and ornamental plant species; its global host range includes over 200 host plant species **(Barnea *et al*., 2005)**. The black bean aphid is one of the most serious pests of the faba bean, *Vicia faba L*., wherever it is grown **(Basedow *et al*., 2006)**.

Figure 10: Black bean aphids *Aphis fabae* on leaves of a broad bean plant (**Paul Maguire, 2022)**

Aphis fabae is found throughout the temperate Northern Hemisphere, as well as South America and Africa. Large colonies of *Aphis fabae* may be very damaging to legumes and beetroot, causing direct phloem feeding damage, resulting in significant impairment of plant growth and yield **(Shannag and Ababneh, 2007)**, leaf deformation, and arrested development, and serving as a vector for approximately 30 plant viruses. Host plants are harmed directly by aphid feeding or indirectly by virus transmission and honeydew excretion **(Neeraj *et al*., 1999)**. Furthermore, individual aphids frequently have highly rapid growth and development rates, allowing aphid populations to rapidly reach levels that are harmful to crop plants under optimal growth conditions. Newborn aphids contain the embryos of their first granddaughters that's why such rapid development is possible. Because of this telescoping of generations, an individual aphid has already completed two-thirds of its development before birth **(Dixon, 1998)**.

3.2.2. Taxonomy

According to **Scopoli (1763),** the classification of *Aphis fabae* is as follows;

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Hemiptera

Family: Aphididae

Sub-family: Aphidinae

Genus: *Aphis* **Linnaeus, 1758**

Species: *Aphis fabae* **Scopoli, 1763**

3.2.3. *Aphis fabae* Biology, Life Cycle and Damage

The apterous adult of *Aphis fabae*, have a squat body that is 2 mm long, dull black to dark greenish, more elongate alatae, shiny black head, and antennae that are not longer than two-thirds of the length of the insect **(Figure 11)**. While the oviparous females are with noticeably enlarged hind tibiae **(HYYP Zoology.org)**.

Figure 11: *Aphis fabae* adult aptera and nymphs on Vicia faba (**InfluentialPoints.com)**

As previously mentioned, this aphid is one of the most polyphagous species which can feed on more than 200 plants **(Barnea *et al*., 2005)**. Their winter eggs, in autumn, are laid on the bark of European spindle (*Euonymus europea*), guelder rose (*Viburnum opulus*), and mock orange (*Philadelphus sp*). In March, Fundatrices give birth to apterae, the progeny of which includes an increasing proportion of winged individuals **(Figure 12)**. These winged virginoparae begin to colonize numerous secondary host plants in May, depositing apterous nymphs on the undersides of leaves or at the tips of stems **(Figure 11)**. However, aphid colonies grow rapidly until mid-June, then gradually decline due to parasites and predators. Winged sexuparae emerge in the autumn and return to their primary hosts; fertilization and egg-laying take place in October **(HYYP Zoology.org)**.

Figure 12: Winged and wingless adult viviparous female *Aphis fabae* **(Department of Agriculture, Western Australia; Rebecca Graham)**

Those species are not only a vector of certain virus diseases but they are also the causal of swollen, rolled beet and common spindle leaves **(Figure 13)** and a non-evenly growth of roots with a lower sugar content. Aphids can significantly spread to seed plant inflorescences and disrupt seed formation. Furthermore, honeydew is formed which spurs sooty molds to grow on it and because of the action of their toxic saliva, growth is hampered and flowers die **(HYPP Zoology.org)**.

Figure 13: Curled leaf pseudogalls by *Aphis fabae* on common spindle **(InfluentialPoints.com)**

EXPERIMENTAL PART

CHAPTER 4

Materials and Methods

4.1. Materials

4.1.1. Plant Material

The aerial parts of *Mentha rotundifolia* were harvested on March 2023 from the region of Taza, 30 km northeast of Jijel on the Mediterranean coast (36°47'34"N 5°39'53"E). The collected samples were air-dried in shadow at room temperature (20–25°C) for two weeks and then were stored in glass boxes for further use **(Figure 14)**. Taxonomic verification was conducted according to the flora of North Africa at Tela botanica (https://www.tela-botanica.org/?in=flore&s=+Mentha+rotundifolia) and the Dr. Sebti Mohamed from the department of environmental and agronomic sciences, University of Mohamed Seddik Benyahia- Jijel.

Figure 14: The aerial parts (leaves and stems) of *Mentha rotundifolia* collected from Taza-Jijel

4.1.2. Extraction of Essential Oils

Essential oils of *Mentha rotundifolia* were extracted from dried leaves and stems (100 g) using Clevenger apparatus during 3h. Essential oils were stored in amber flasks and tightly closed at 4°C until they were used in the below described studies. Essential oils' yields were calculated according to dry weight of the plant materials **(Figure 15)**.

Figure 15: Dried leaves and stems of *Mentha rotundifolia*

4.1.3. Isolation Zone

Fruit samples were taken from the strawberry crop variety “Savana” in two greenhouses in the area of Tassoust-Jijel. The choice of the crop was made based on its economic importance besides its high production and consumption in the region of Jijel and the whole country.

The samples have high incidence of fungal diseases in spring 2022-2023 production cycle. All samples were stored in sterile paper bags, putting them in a cooler until they were transported to the laboratory **(Figure 16)**.

Figure 16: The infected samples of strawberry fruits collected from the two greenhouses located in the region of Tassoust- Jijel

4.1.3.1. Isolation of Fungal Pathogen

Fragments of infected strawberry fruits were cut into small segments (4-5 mm in diameter). The pieces are then disinfected superficially by dipping them in 1% sodium hypochlorite (for 30 seconds to 2 minutes); rinsing it three times with sterile distilled water then drying it with sterile Watman paper and finally planted in Petri dishes containing the culture medium Sabouraud. The four or 5 fragments are placed separately in each box, and then incubated at 25°C for 6 days **(Figure 17) (Choiet *et al*., 1999; Luis Angel Morales-Mora *et al*., 2020**).

Figure 17: The isolation of fungi from strawberry fruits

4.1.3.2. Purification of Fungi

After incubation, the isolates obtained are purified by direct transplanting on the PDA medium until pure strains are obtained **(Botton *et al*., 1991)**.

4.1.3.3. Identification of Isolated Fungi

The identification of isolated fungi is based on two stages:

4.1.3.3.1. Macroscopic identification

The isolates obtained are macroscopically identified by examination of the culture on PDA agar media **(Rapilly, 1968; Botton *et al*., 1991)**. The examination determines the following cropping characteristics: growth rate, thallus texture and colour, culture reverse colour and odour. Mycelial growth patterns, colour, odour and average colour changes of each isolate were daily examined.

4.1.3.3.2. Microscopic identification

This step is carried out by sampling a mycelial fragment and some spores from a young culture of each fungal isolate using a sterile platinum loop, microscopic observation is carried out at magnification (objective 10×40).

This type of identification is based essentially on the morphological study of mycelium (absence or presence of partitions, colour of mycelial filaments,… etc) and spores (shape, colour, size, texture of walls,… etc). However, the identification was achieved by placing a drop of lactophenol on clean slide with the aid of a mounting needle, where a small portion of the colony from the representative fungi cultures was removed and placed in a drop of lactophenol. The mycelium was well spread on the slide with the needle. A cover slip was gently placed with little pressure to eliminate air bubbles. The slide was then mounted and viewed under the light microscope with ×10 and ×40 objective lenses.

The identification of the fungi at a genus level was carried out by comparing the morphological characteristics of the culture (texture, type of mycelia, colour, type of hyphae and measurements of shape and size of anamorphic structures, as well as conidia, conidiophore and sclerotia) which were compared using taxonomic identification keys by **Barnett and Hunter (1998)** in a microculture system using an optical microscope at a magnification of 40×10 **(Ellis, 1971; Samson *et al*., 2014; Zeng *et al*., 2018)**.

4.1.4. Animal Material

The insects used are Black Bean Aphids "*Aphis fabae*". They were harvested from bean crop in the region of Beni kaid-Jijel. This experiment is carried out in the microbiology laboratory at Mohamed Seddik Ben Yahia University, Jijel.

The insects were chosen based on the following criteria:

- The economic importance of host plants.
- The significant damage done to host plants by these insects.
- These insects are widespread and easy to locate.

4.2. Methods

4.2.1. Extraction of Essential Oils

The extraction of *Mentha rotundifolia* essential oils was done at the educational laboratory of the University of Jijel by Hydrodistillation method Clevenger type**.**

4.2.1.1. Experimental Protocol

The hydrodistillation apparatus type combined with Clevenger system is recognized by the European Pharmacopoeia. It has the advantage that the water in which the plant materials are immersed can act as a barrier, protecting the oils from overheating and preventing the deleterious effect of the temperature on the aromatic properties of the essential oils **(Ferrentino *et al.*, 2020)**.

The principle of the process, according to the previous author, is consisted of immersing the plant material in a water bath and heated until it reaches the boiling point. When heated, plant materials release their essential oils, which are then carried through the condensation tube with water vapour. The cooling effect of cold water allows the aromatic water (hydrolat) to separate from the oils by density difference. Despite the fact that the apparatus allows for the recycling of the distillate's aqueous phase in the boiler.

A sample of 100 g was placed in a 2L round bottom flask with 1000 ml of distilled water, subjected to hydrodistillation for 3 hours, using a Clevenger-type apparatus (1928) to produce essential oil **(Figure 17)**. *Mentha rotundifolia* essential oil was preserved in an amber flask at low temperature (4°C) until further analysis.

Figure 18: Hydrodistillation of *Mentha rotundifolia* essential oils Clevenger-type Apparatus

4.2.1.2. Yield Calculation

The essential oil yield is defined as the ratio of the essential oil obtained to the dry mass of the plant material to be treated **(Kaid, 2004).** The following formula calculates the return as a percentage:

$$R = P2/P1 \times 100$$

- **R:** yield of essential oils expressed (percentage).
- **P2:** mass of essential oils in grams.
- **P1:** mass of sample in grams.

4.2.1.3. GC–MS analysis

GC/MS analysis was performed on the isolated volatile compounds using a Shimadzu GC/MS-QP2010, quadripole El 70ev type. The oven temperature was set to 55°C for 4 minutes, then increased to 120°C at a rate of 3°C per minute for 5 minutes later increased to 240°C at a rate of 5°C per minute for 5.33 minutes. The injection port temperature was 250°C (split ratio: 20.0), and

the carrier gas was He with a flow rate of 1.0 mL/min. the sample (1 μL) was injected in the split mode.

The following were the mass spectrometer conditions: the ion source temperature was 200°C, and electron ionization mass spectra were collected over the mass range 40-350 m/z.

The compounds were identified by comparison of their mass spectra with those of NIST02 library data of the GC/MS system, by comparing retention times and mass spectral data with standard compounds injected under the same chromatographic conditions, as well as their retention index relative to a standard mixture of n-alkanes.

4.2.1.4. Preparation of Controls, Essential Oils Concentrations and Hydrolat Dilutions

4.2.1.4.1. Essential Oils Concentrations and Controls

After preliminary tests, in addition to pure essential oils, three concentrations of essential oils were used and prepared by diluting each time in the solvent (ethanol) the successive volumes of 10, 30 and 60 ml of essential oil getting respectively the following concentrations pure EOs (100%), 10%, 30% and 60% **(Figure 19) (Tedonkeng *et al*., 2002)**.

One negative witness was prepared, constituted of ethanol.

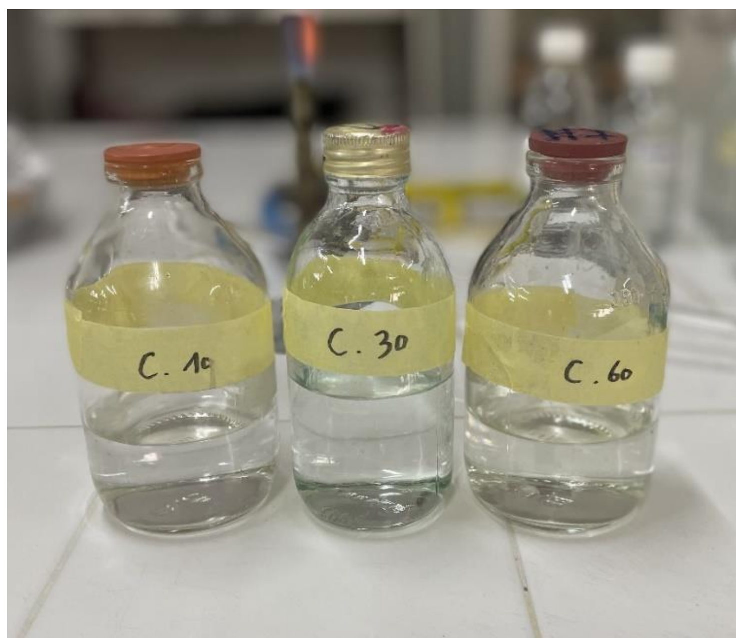

Figure 19: Essential oil concentrations

4.2.1.4.2. Hydrolat Dilutions

A series of dilute hydrolat solutions is prepared starting from a stock solution containing 10 ml of which. Each preparation was made by introducing 9 ml of sterile distilled water into test tubes and the volume is completed to 10 ml by pipetting each time 1 ml of the latter solution in order to have the following dilutions of 10^{-3}, 10^{-6} and 10^{-9} **(Figure 20)**.

Figure 20: Hydrolat dilutions

The effect of the concentrations and dilutions of essential oil and hydrolat (10%, 30%, 60% and 100%), (stock solution, 10^{-3}, 10^{-6} and 10^{-9}) respectively were studied in fungicide and insecticide effects of essential oils.

4.2.2. Biological Activity Tests

4.2.2.1. Evaluation of Antifungal Activity

This test aims to evaluate the antifungal effect of plant extracts of *Mentha rotundifolia* on the development of *Botrytis cinerea* and *Colletotrichum gloeosporioides*. It consists in estimating the inhibition of the growth of these species. The aromatogram test was studied in petri dishes using two techniques: confrontation by direct contact on PDA medium and at a distance **(Appendix n°1)**.

4.2.2.1.1. Direct Contact Method

The antifungal effect of hydrolat of *Mentha rotundifolia* was tested for assessing its contact and effect towards *Botrytis cinerea* and *Colletotrichum gloeosporioides*. For the determination of contact effects, the hydrolat was prepared in solutions (stock solution, 10^{-3}, 10^{-6} and 10^{-9}), after the melting of PDA medium and cooled at (45°C). PDA medium with hydrolat was immediately poured into sterile 85 mm Petri dishes (nearly 15 ml/plate). Then the fungal discs of 6 mm diameter from the young cultures of each species were placed in the middle of Petri dishes and incubated at 25°C during 6 days. When the fungal vegetative growth was covered in control petri dishes, colony diameters of fungus with hydrolat were measured and inhibition percentage of mycelial growth was calculated (MGI) by using the formula of **Mermer Doğu and Zobar, (2014)**;

$$\mathbf{MGI\ (\%) = [(dc - dt) / dc] \times 100}$$

- **dc:** mycelial growth diameter in control.
- **dt:** mycelial growth diameter in treatment.

Three repetitions were performed for each dilution and each control. Then, the negative control consists of PDA mixed with sterile distilled water, while the positive control consists only of the culture medium **(Mermer Doğu and Zobar, 2014)**.

4.2.2.1.2. Volatile activity method

The effect of essential oil vapours against the tested strains (*Botrytis cinerea* and *Colletotrichum gloeospiroides*) was also estimated using the volatile activity technique as described by **Neri *et al.*, (2006)** with slights modifications.

This method consists of transplanting the two disks (mycelial disc and the sterile filter paper disc imbibed with different concentrations) in two separate boxes; subsequently, an assembly is made by superimposing the two boxes, the paper disc imbibed at the bottom and the mycelial disk at the top. The junction between the two boxes is ensured by layers of parafilm to avoid any loss of volatile. The conditions of culture are identical to those of the confrontation by direct contact on culture medium. The positive witness is formed by superimposing two boxes, the top one containing a pellet of the isolate tested, while the bottom contains only PDA medium. However, the negative control (ethanol) was carried out by using the same manipulation but the sterile Watman paper disc was soaked only with ethanol (1ml) and incubated at 25°C during 6 days. Three repetitions were performed for each concentration and each control. Mycelium growth

diameters were noted daily and data were expressed as percentage inhibition of the radial mycelial growth **(Plaza *et al*., 2004; Regnier *et al*., 2008)**. The minimum inhibitory concentration (MIC) was determined for the oil having the broadest antifungal spectrum and is assigned to the lowest concentration able to completely inhibiting fungal growth.

4.2.2.2. Evaluation of Insecticidal Activity

4.2.2.2.1. Repellent Effect of Essential Oils on Filter Paper

The repellent effect of *M. rotundifolia* essential oils against adults of *Aphis fabae* was evaluated using the preferential zone method on filter paper described by **McDonald *et al* (1970)**. Thus, the filter paper discs of 9 cm in diameter used for this purpose were cut into two equal parts each having 31.80 cm of surface. Four concentrations of oil and hydrolat were respectively prepared (10%, 30%, 60% and 100%), (stock solution, 10^{-3}, 10^{-6} and 10^{-9}) by dilution in ethanol and distilled water respectively. After that, 1 ml of each of the solutions then was prepared to be spread evenly over one half of the disc, while the other half received nothing.

Next, the two halves of the disks were resealed using adhesive tape. The filter paper disc that got reconstituted was placed in a Petri dish and a batch of 10 adult aphids randomly selected were placed in the center of each disc. Three repetitions were carried out for each dose. After two hours, the number of insects present on the part of the filter paper treated with essential oil or hydrolat (Nt) and the number of those present on the part non-treated were noted.

4.2.2.2.2. Fumigant Toxicity of Essential Oils and Hydrolat

The test is consisted according to the evaluation of the toxicity by contact of the essential oils and hydrolat by the aphids in the laboratory at room temperature and relative humidity of approximately 75%. Each treatment (concentration) of essential oils and hydrolat (100% and 30%), (stock solution and 10^{-6}) consisted of three repetitions and each repetition consisted of 10 adult aphids "*Aphis fabae*" randomly selected which were carried on a fresh bean leaf and introduced into the petri dish which contained paper discs of 9 cm in diameter cut into two equal parts. However, 1 ml of each of the solutions prepared were spread evenly over one half of the disc, while the other half received nothing.

The controls, the first one, 10 in number and under the same conditions with a fresh uninfected host leaf are placed in each box. The second one, 10 in number and under the same conditions

underwent a pure treatment of ethanol only (1 ml in each box). The Count of dead aphids is done the first 2 hours, 12 hours after treatment and 24 hours until 48 hours **(Figure 21)**.

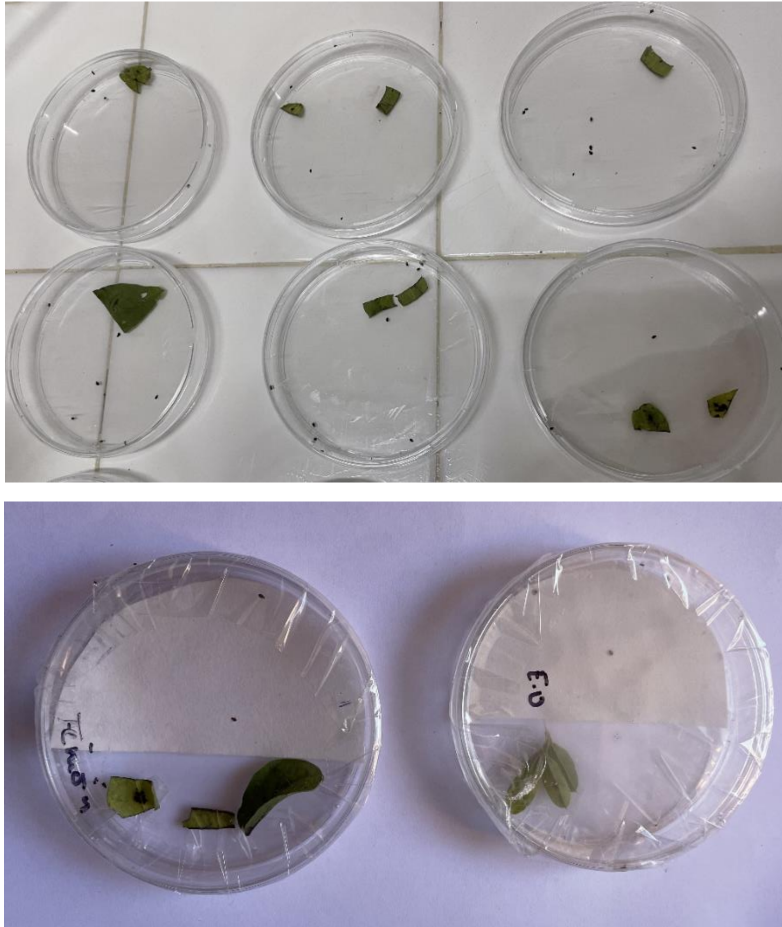

Figure 21: Experimental test of aphids' treatments with essential oils and hydrolat

4.2.2.2.3. Evaluation of Mortality

4.2.2.2.3.1. Percentage of Repellency

After two hours, the number of insects present on the part of the filter paper treated with essential oil or hydrolat **(Nt)** and the number of those present on the part non-treated **(Nc)** were noted. The percentage of repellency for the essential oil and the hydrolat was calculated, according to **(McDonald *et al.*, 1970),** by using the following formula:

$$\mathbf{PR} = \frac{(\mathbf{Nc} - \mathbf{Nt}) \times \mathbf{100}}{(\mathbf{Nc} + \mathbf{Nt})}$$

- **PR:** The percentage of repellency (%).

- **Nt:** The number of insects present on the part of the filter paper treated with essential oil or hydrolat.
- **Nc:** The number of insects present on the part of the filter paper non-treated.

The average percentage of repellency for the essential oil and the hydrolat was calculated and assigned according to the classification of **(McDonald *et al.*, 1970).** The repellency classes were categorized from 0 to V:

- **Class 0** (PR< 0.1%)
- **Class I** (PR= 0.1 - 20%)
- **Class II** (PR= 20.1 - 40%)
- **Class III** (PR= 40.1 - 60%)
- **Class IV** (PR= 60.1 - 80%)
- **Class V** (PR= 80.1 - 100%)

4.2.2.2.3.2.Mortality Correction

The effectiveness of a toxic substance is measured by its LD50. It is determined from the layout of the regression lines. The reason why the corrected mortality percentages are transformed into probits and the doses into log doses.

Therefore, the number of dead individuals in a population treated with a toxic substance is not the real number of individuals killed by this substance. There is a natural mortality in any population which is added to the mortality caused by the substance applied. The mortality percentages must be corrected by **Abbott**'s **(1925)** formula:

$$\mathbf{Mc} = \frac{\mathbf{Mo - Mt}}{\mathbf{100 - Mt}} \times \mathbf{100}$$

- **Mo:** Mortality in the population treated.
- **Mc:** Corrected mortality.
- **Mt:** Natural mortality observed on the control.

4.2.2.2.3.3.LD50 Determination

The median lethal dose LD50 is the quantity of toxic substance resulting in the death of 50% of individuals in the treated population. The method of **Finney (1971)** based on the regression of the

probits of mortalities according to the logarithms of the doses of essential oil made it possible to determine the LD50.

4.2.3. Data Analysis

The statistical processing of the test results of inhibition percentage of mycelial growth (MGI), insecticidal fumigant toxicity and repellency percentage were carried out using XLSTAT 2014, which was used to plot the histograms and curves. As a statistical treatment, an analysis of variance (ANOVA) was used, followed by a Tukey multiple comparison test.

The differences between treatments were considered significant if the p-value was less than "0.05." The lethal doses 50 were calculated using the linear equation of the binary logistic regression with the probit model.

CHAPTER 5

Results and Discussion

5.1. Yield in Essential Oil

The essential oils obtained from *Mentha rotundifolia*'s extraction by hydrodistillation yielded 1.37% **(Figure 22)**. The essential oils obtained were liquid, the colour was light clear yellow and the odour was fresh, characteristic and persistent. The organoleptic characteristics are regrouped in **table 5.**

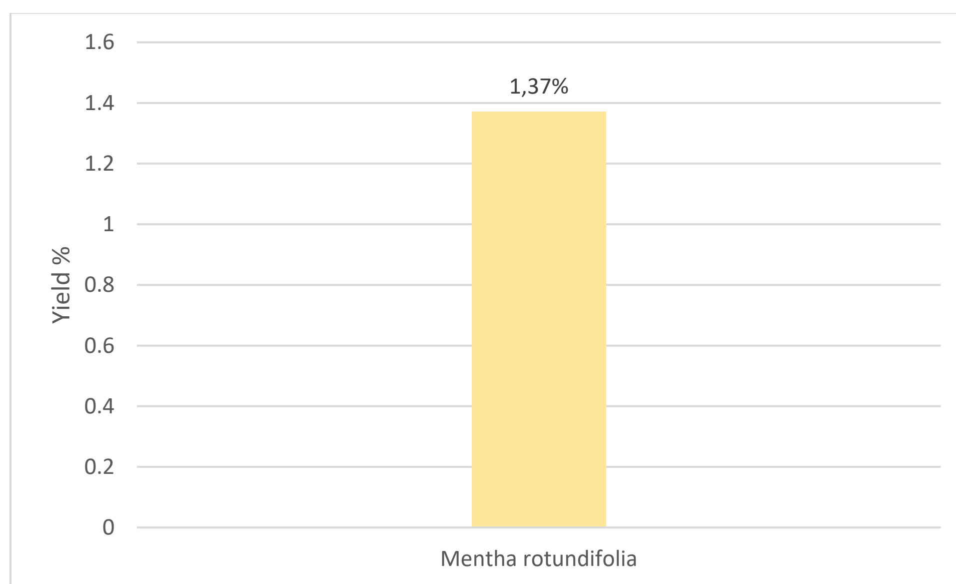

Figure 22: The yield of *Mentha rotundifolia* EOs extracted by hydrodistillation

Table 5: Organoleptic characteristics of *Mentha rotundifolia* extracted EOs

Essential Oils of *M.rotundifolia*	Organoleptic Characteristics			
	Aspect	**Colour**	**Odour**	**Yield**
	Liquide	Light clear yellow	Apple-mint fresh and persistent odour.	1.37%

5.2. Chemical Composition of *Mentha rotundifolia* Essential Oils

Regarding the analysis of *M. rotundifolia* EOs, sixty-seven compounds were identified and the main constituents were Pulegone (39.21%), ß-cubebene (11.72%), caryophyllene (9.18%), D-limonene (4.7%), ß-ocimene (2.39%), myrcene (2.21%), myrtenol (2.1%), ß-farnesene (2.03%) **(Table 6).**

Table 6: Chemical composition of *Mentha rotundifolia* EOs

N°	Rt	Area	Identified Compounds	%	Base m/z
1	4.952	10624717	1R-α-pinene	1.48	104.05
2	6.657	15122041	Myrtenol	2.1	93.10
3	6.798	7653332	β- phellendrene	1.06	93.05
4	7.572	15863440	Myrcene	2.21	93.10
5	8.832	33781311	D-limonene	4.7	68.05
6	9.620	17167452	β- ocimene	2.39	93.05
7	10.244	8960928	α- terpinene	1.25	93.05
8	14.655	15067260	Octen-1-ol, acetate	2.1	43.00
9	26.354	66019651	Caryophyllene	9.18	93.05
10	28.196	8722949	Alpha-caryophyllene	1.21	93.05
11	28.421	14575543	β- farnesene	2.03	69.05
12	29.678	281924721	Pulegone	39.21	67.05
13	30.048	84248841	β- cubebene	11.72	161.10
14	31.261	8455361	Zingergone	1.18	79.05
15	30.896	6831011	α-pinene	0.95	121.10

5.3. Fungi Isolation and Identification

5.3.1. Isolated Fungi

The isolation results obtained showed a marked presence of *Botrytis cinerea* on the strawberry samples of both greenhouses. However, among the other isolates, *Acremonium sp*, *Colletotrichum gloeosporioides*, *Cladosporium sp* and *Fusarium sambucinum* were also isolated from strawberry fruits. By staffing, the spread of other isolates was recorded in both greenhouses.

The identification of the previous fungi was based on their macroscopic characteristics of colonies (appearance, colour, shape, contour, etc) and microscopic characteristics of mycelium and conidia or spores (partition of mycelium, form of spores, form of organs fruiting, etc).

Botrytis cinerea and *Colletotrichum gloeosporioides* were the mainly two strains chosen for the biological test relying to the phenomenon diseases caused by them, which are grey mould and anthracnose.

5.3.2. Description of Identified Fungi

5.3.2.1. *Colletotrichum gloeosporioides*

On PDA medium, the fungal hyphae emerged from the cut ends of the explants within 5-6 days. The culture characteristics are a smooth fungal colony expanded rapidly with typical colours as *C. acutatum*, which usually forming white aerial mycelium during the first few days of growth then becoming orange to pink, or rose with pink conidial masses formation and the cultures were rose to grey on reverse **(Figure 23)**.

C. gloeosporioides isolated from strawberry produced fusiform or tapering, acute end and straight or fusiform conidia. Conidial mass showed salmon pink or orange colour and acervuli were salmon to grey. Setae and sclerotia were absent. However, the presence of hyaline, septate, highly branched mycelia with a large number of more masses, hyaline, cylindrical with rounded ends spores; conidial appressoria were brown to dark black with an irregular shape was revealed by microscopic examination **(Figure 24)**.

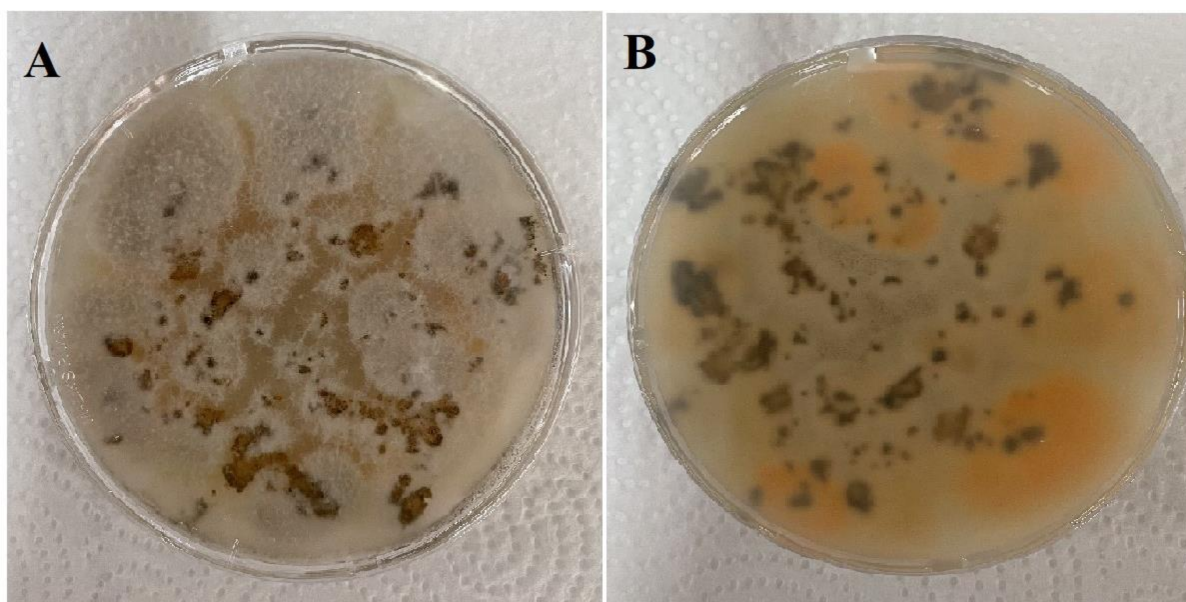

Figure 23: Colony characteristics of *Colletotrichum gloeosporioides* isolated on PDA, from above and below petri dish, after 6 days of incubation

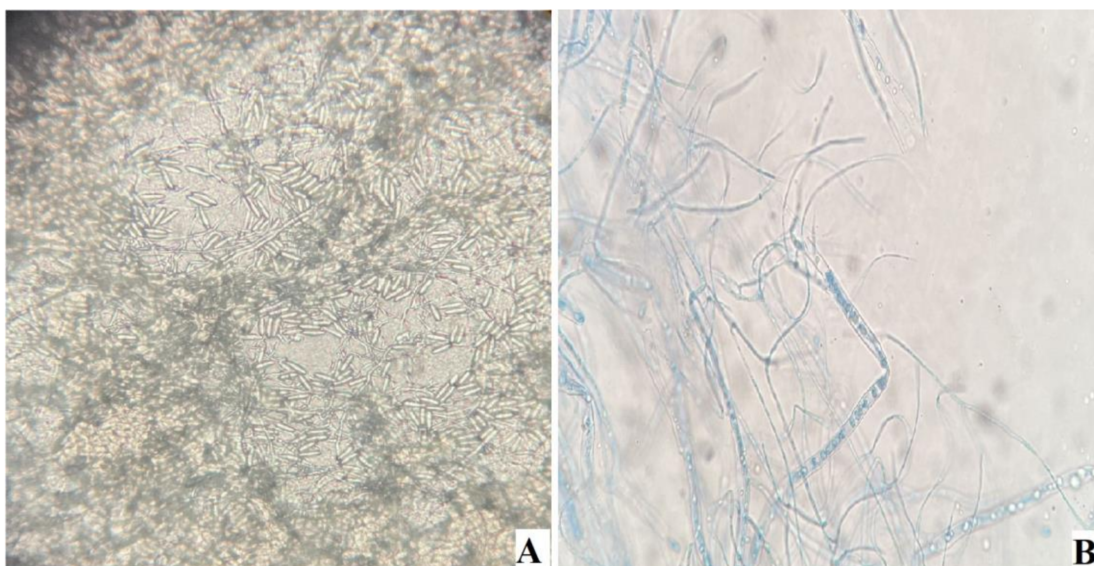

Figure 24: Microscopic aspect of *Colletotrichum gloeosporioides*. **A:** conidia, **B:** mycelium, after 6 days of incubation

5.3.2.2. *Botrytis cinerea*

On PDA medium, the isolates' colonies were warty, fluffy, and appressed, white, dirty white, greyish white to light grey or dark grey, but there were also isolates with hyaline colonies that later became grey. While the reverse was hyaline to greyish **(Figure 25).**

However, microscopic examination revealed that the mycelium was branched, septate, and hyaline to brown in colour. It can produce conidiophore tufts, which is erect and extended, forming an intense grey felting releasing oval conidia. Conidiophores were found growing directly from mycelium or germinated sclerotia which form when conditions become unfavourable to the development of mycelium and conidia. They consist of a whitish aggregate mycelium. As they age, they harden and become blackish **(Figure 26)**.

The conidiophores were mostly straight, septate, monopodial, and branched towards the apex. Conidia were solitary, hyaline or pale brown, but in mass, they appeared grey and darkened with age. The conidia observed in the microscopic fields were ellipsoidal and globose, smooth, often with a slightly protuberant hilum, and unicellular **(Figure 27)**.

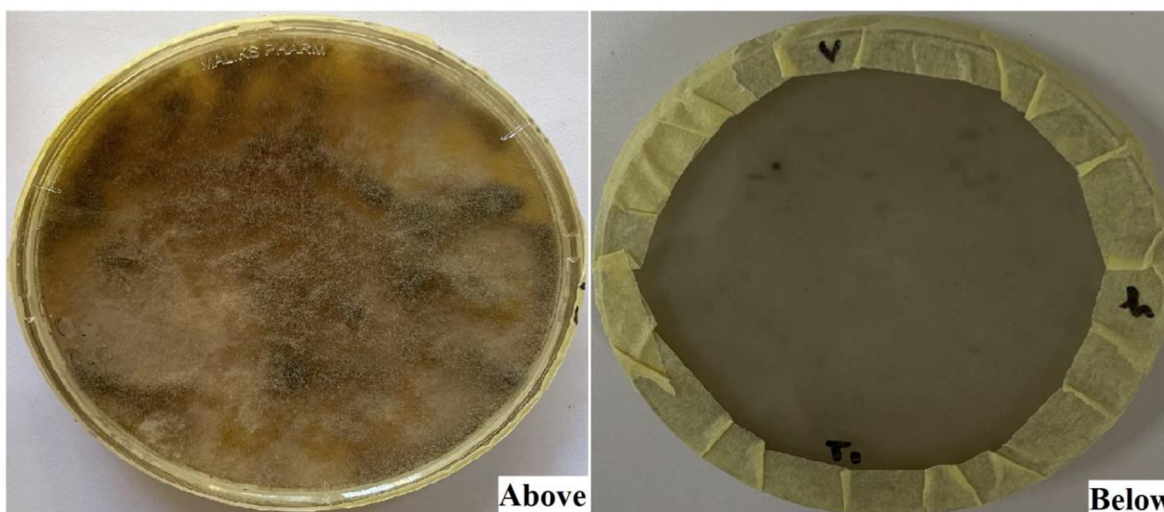

Figure 25: The growth patterns of *Botrytis cinerea* isolates on PDA, from above and below petri dish, after 6 days of incubation

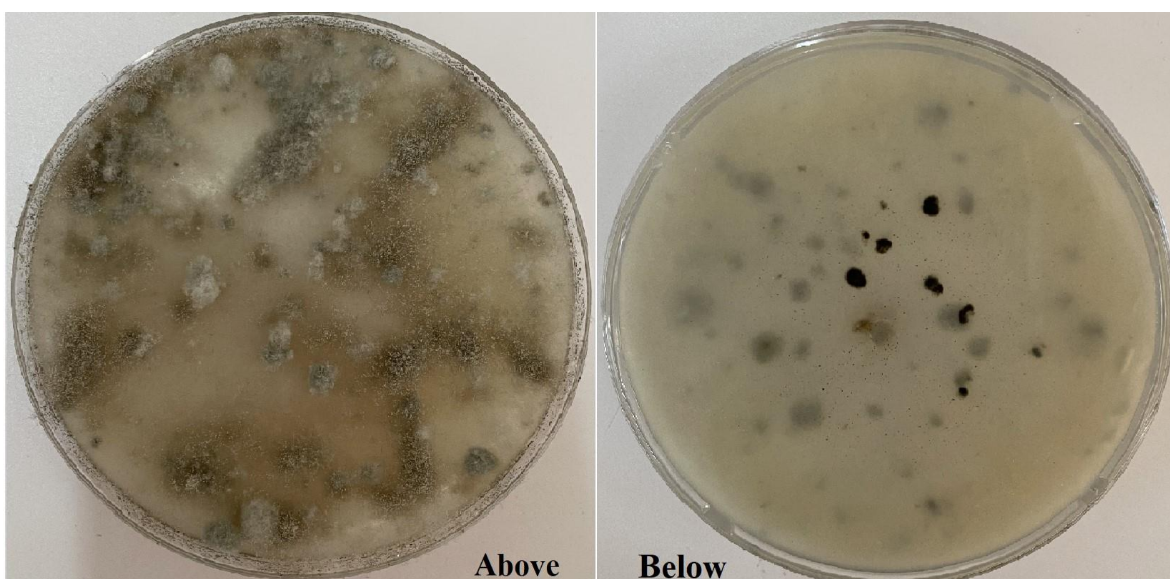

Figure 26: The distribution patterns of the sclerotia of *Botrytis cinerea* isolates on PDA, from above and below petri dish, after 6 days of incubation

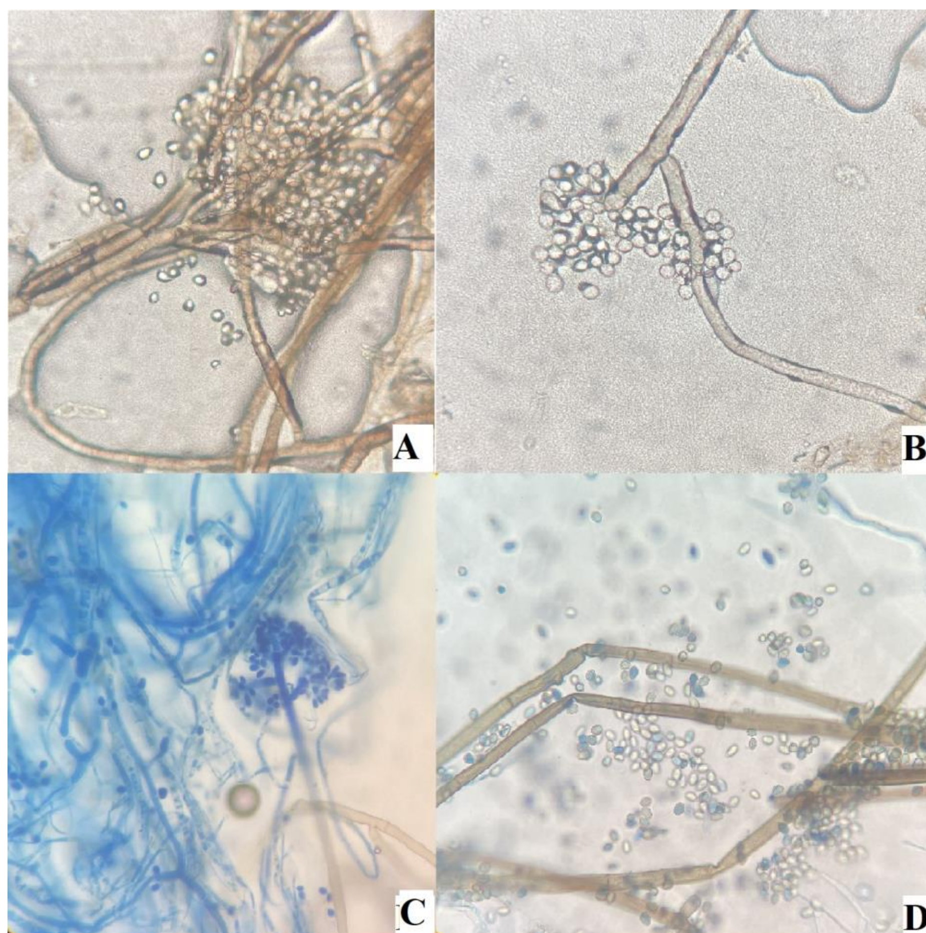

Figure 27: Microscopic aspect of *Botrytis cinerea* isolates on PDA. **A, B and C:** conidiophore morphology. **D:** conidia morphology

5.3.2.3. *Fusarium sambucinum*

These fungi grown on PDA medium are initially white mycelium, getting older of culture age; it will be pale yellow, in certain circumstances pinkish purple with insulated mycelium and form branching **(Figure 28)**. In general, *Fusarium* that observed microscopically usually had micro-conidia and macro-conidia. Micro-conidia is ovoid which generally has 0-1 septum, whereas the macro-conidia form is generally tapered and has 2-6 septums **(Figure 29)**.

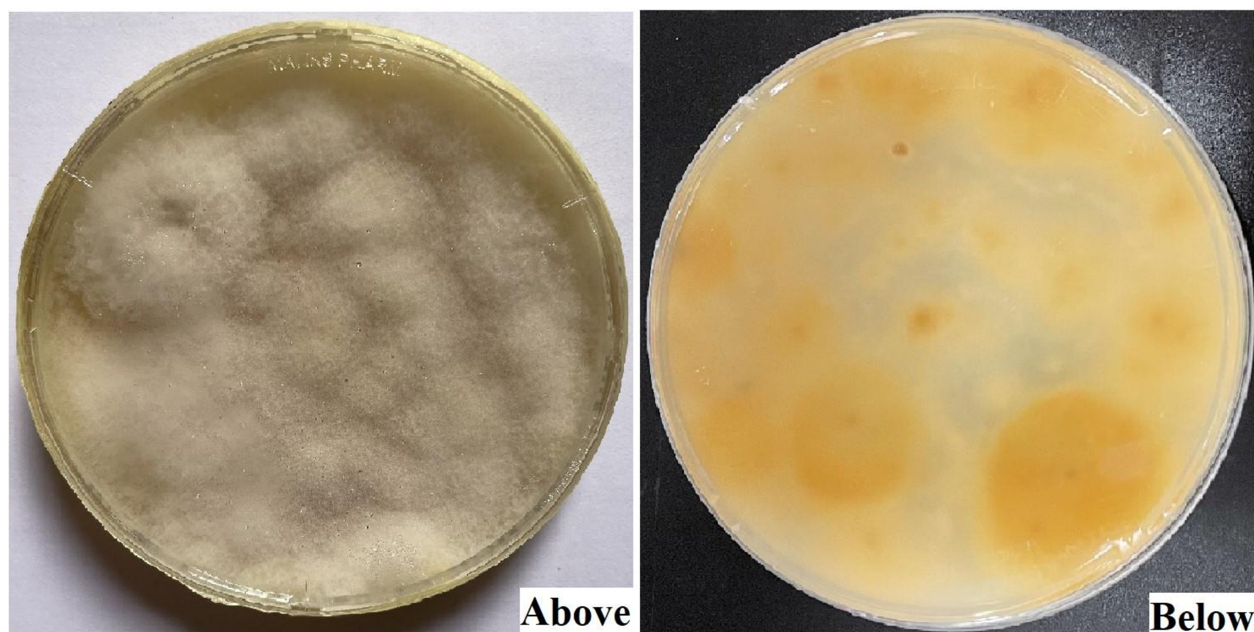

Figure 28: Colony characteristics of *Fusarium sambucinum* isolates on PDA, from above and below petri dish, after 6 days of incubation

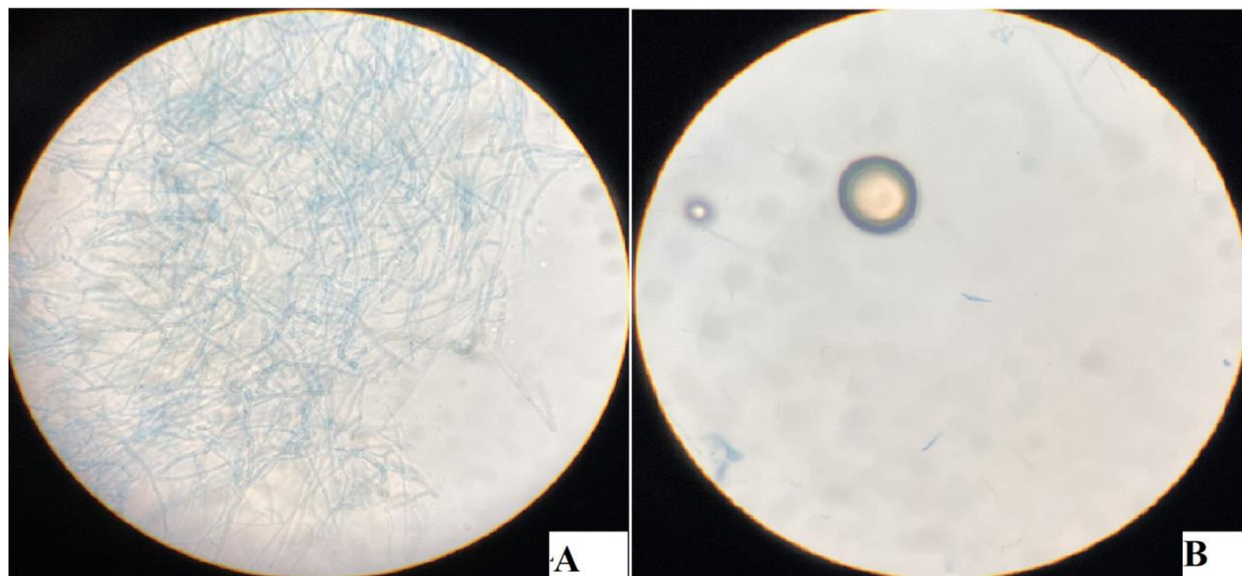

Figure 29: Microscopic aspect of *Fusarium sambusinum* isolates on PDA, A: mycelium, B: conidia, after 6 days of incubation

5.3.2.4. *Cladosporium sp*

The isolate formed on OGA is dark dull green to olivaceous-gray colonie with dark olivaceous reverse; the colonie was velvety with regular margin **(Figure 30)**. The conidiophore was solitary, erect, oblong-cylindrical, nodulose to nodose, sometimes distinctly geniculate, unbranched or

sometimes branched, with swellings, pale to medium brown or olivaceous-brown. Hyphae, straight, sub hyaline and smooth. Conidia were catenate, in branched chains; terminal conidia small, subglobose, obovoid, oval to limoniform and aseptate; intercalary conidia broadly ovoid-ellipsoid and 0–1-septate **(Figure 31)**.

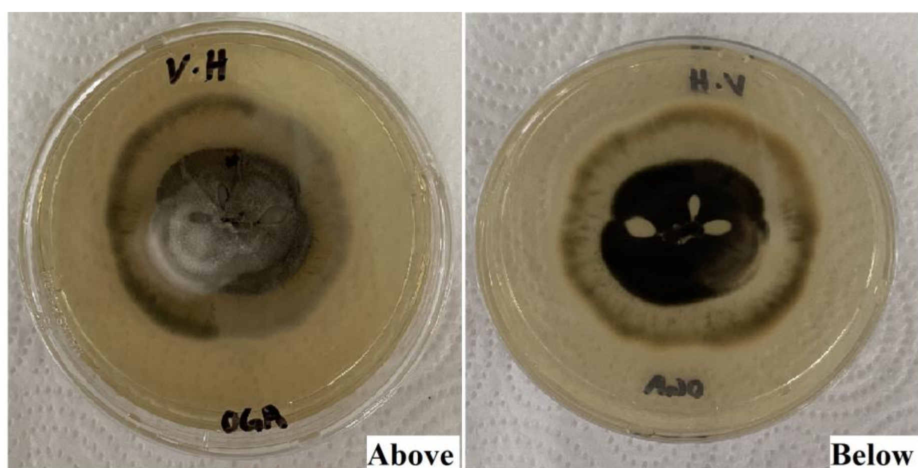

Figure 30: The growth patterns of *Cladosporium sp* isolates on OGA, from above and below petri dish, after 6 days of incubation

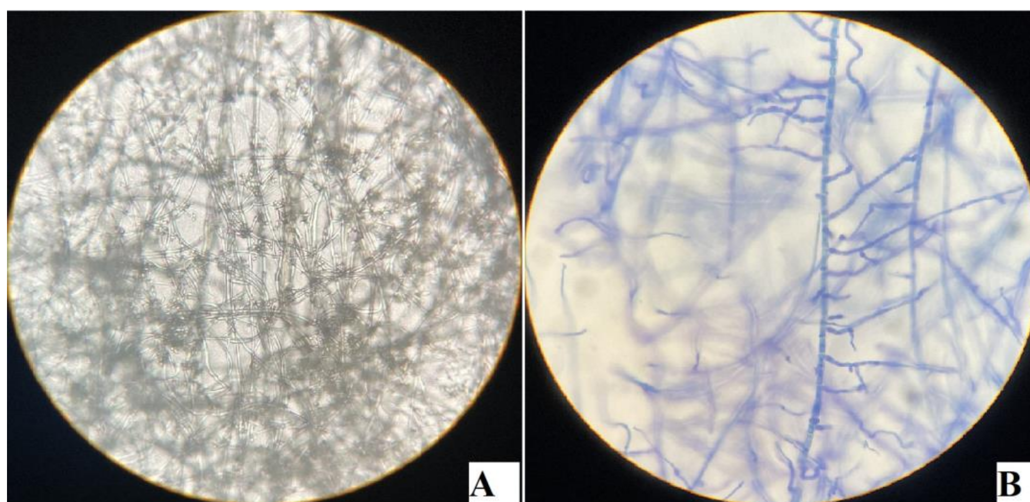

Figure 31: The microscopic aspect of *Cladosporium sp* isolates on PDA, **A:** conidia, **B:** mycelial filaments, after 6 days of incubation

5.3.2.5. *Acremonium sp*

Colony deeply floccose-cottony to tomentosus, whitish to pale pinkish; conidiation abundant, phialides very numerous on hyphal strands, mostly simple and long, distinctly chromophilic near the base **(Figure 32)**. Conidia were aggregated in heads, cylindrical or tapering towards the tips

and slightly fusiform, mostly homopolar, smooth- walled, hyaline; sclerotia formed on nettle stems, scattered in the mycelium, firm, globose, smooth walled, hyaline, consisting of isodiametrical cells of equal size; chlamydospores were absent **(Figure 33)**.

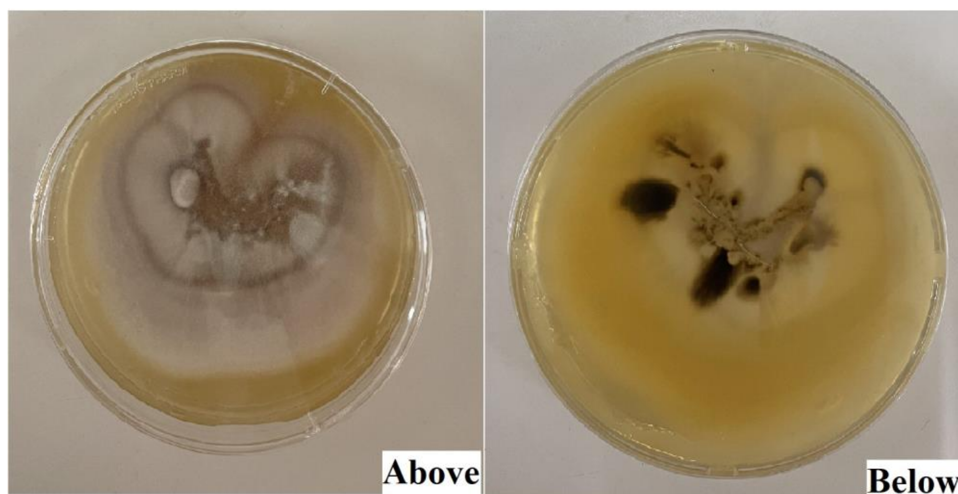

Figure 32: The growth patterns of *Acremonium sp* isolates on OGA, from above and below petri dish, after 6 days of incubation

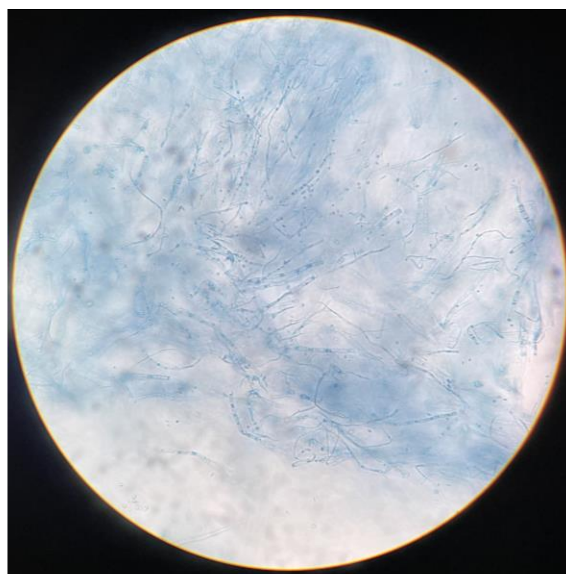

Figure 33: Microscopic aspect of *Acremonium sp* filaments isolates on PDA

5.4. Biological Tests

5.4.1. Antifungal Activity

5.4.1.1. Average Rates of Growth Induced by *Mentha rotundifolia* EOs and Hydrolat

The average growth of *B. cinerea* and *C. gloeosporioides* strains following the treatment with the EOs and hydrolat of *Mentha rotundifolia* in function to various concentrations and various periods of time were treated with statistical analysis.

After incubation of 6 days, statistical analyses revealed that the average growth of strains of *B. cinerea* and *C. gloeosporioides* induced by *M. rotundifolia* essential oils varied significantly according to the essential oils concentration (100%, 60%, 30% and 10%). Screening of antifungal activity by essential oils revealed the efficiency of the concentrations of 100% and 60% against both strains of *B. cinerea* and *C. gloeosporioides* with an average of (26.66, 20.58) and (28.58, 35.45), respectively, compared to both positive and negative controls **(Tables 7 and 8, Appendix n°2 , Figure 34)**.

Table 7: The effect of different concentrations of *M. rotundifolia* EOs against *Botrytis cinerea*, after 6 days

Concentration	Average	Standard Error	Inferior Borne (95%)	Superior Borne (95%)	Groups
Positive Control	56,292	3,373	49,558	63,026	A
Negative Control	52,417	3,373	45,683	59,151	A
10%	44,208	3,373	37,474	50,942	A
30%	43,000	3,373	36,266	49,734	A
100%	26,667	3,373	19,933	33,401	B
60%	20,583	3,373	13,849	27,317	B

Different letters next to the values at each concentration represents significant difference according to Tukey's HSD test ($p \leq 0.05$).

Table 8: The effect of different concentrations of *M. rotundifolia* EOs against *C. gloeosporioides* after 6 days

Concentration	Average	Standard Error	Inferior Borne (95%)	Superior Borne (95%)	Groups
10%	46,375	2,584	41,217	51,533	A
Positive Control	45,625	2,584	40,467	50,783	A
Negative Control	41,292	2,584	36,134	46,450	A
30%	41,100	2,584	35,942	46,258	A

60%	35,458	2,584	30,300	40,616	AB
100%	28,583	2,584	23,425	33,741	A

Different letters next to the values at each concentration represents significant difference according to Tukey's HSD test ($p \leq 0.05$).

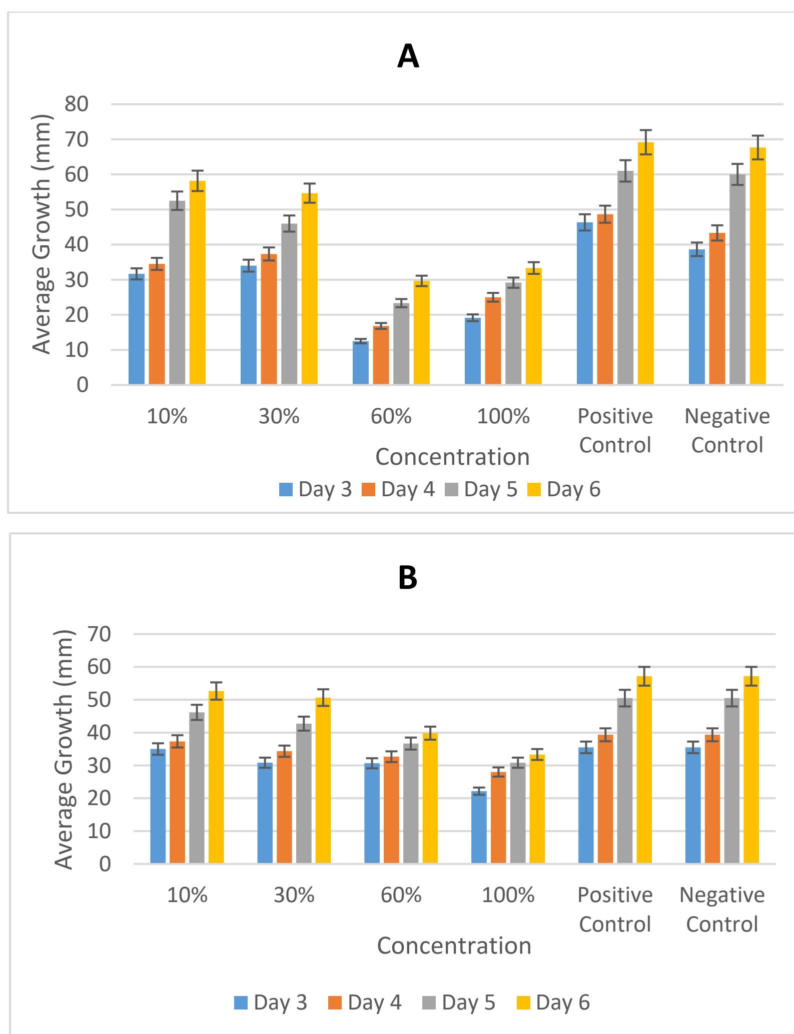

Figure 34: Average growth of *B. cinerea* **(A)** and *C. gloeosporioides* **(B)** strains treated with *M. rotundifolia* EOs, in function to various concentrations and various periods

On the other hand, statistical analyses indicated that the average growth of strains of *B. cinerea* and *C. gloeosporioides* induced by *M. rotundifolia* hydrolat varied significantly according to the various concentrations (stock solution, 10^{-3}, 10^{-6} and 10^{-9}). **Table 9, table 10 and figure 35,** show the efficiency of the hydrolat stock solution and 10^{-3} concentration against both fungi strains of *B. cinerea* and *C. gloeosporioides* with an average of (1.5, 11.37) and (19.95, 32.54), respectively, compared to both positive and negative controls.

Table 9: The effect of different concentrations of *M. rotundifolia* hydrolat against *Botrytis cinerea*, after 6 days

Concentration	Average	Standard Error	Inferior Borne (95%)	Superior Borne (95%)	Groups
Positive Control	52,667	1,775	49,110	56,223	A
10^{-9}	46,208	1,775	42,652	49,765	AB
10^{-6}	42,292	1,775	38,735	45,848	B
10^{-3}	11,375	1,775	7,818	14,932	C
Stock Solution	1,500	1,775	-2,057	5,057	D

Different letters next to the values at each concentration represents significant difference according to Tukey's HSD test ($p \leq 0.05$).

Table 10: The effect of different concentrations of *M. rotundifolia* hydrolat against *C. gloeosporioides*, after 6 days

Concentration	Average	Standard Error	Inferior Borne (95%)	Superior Borne (95%)	Groups
Positive Control	42,125	2,753	36,608	47,642	A
10^{-9}	33,708	2,746	28,205	39,212	A
10^{-6}	32,833	2,746	27,330	38,337	A
10^{-3}	32,542	2,753	27,025	38,059	A
Stock Solution	19,958	2,753	14,441	25,475	B

Different letters next to the values at each concentration represents significant difference according to Tukey's HSD test ($p \leq 0.05$).

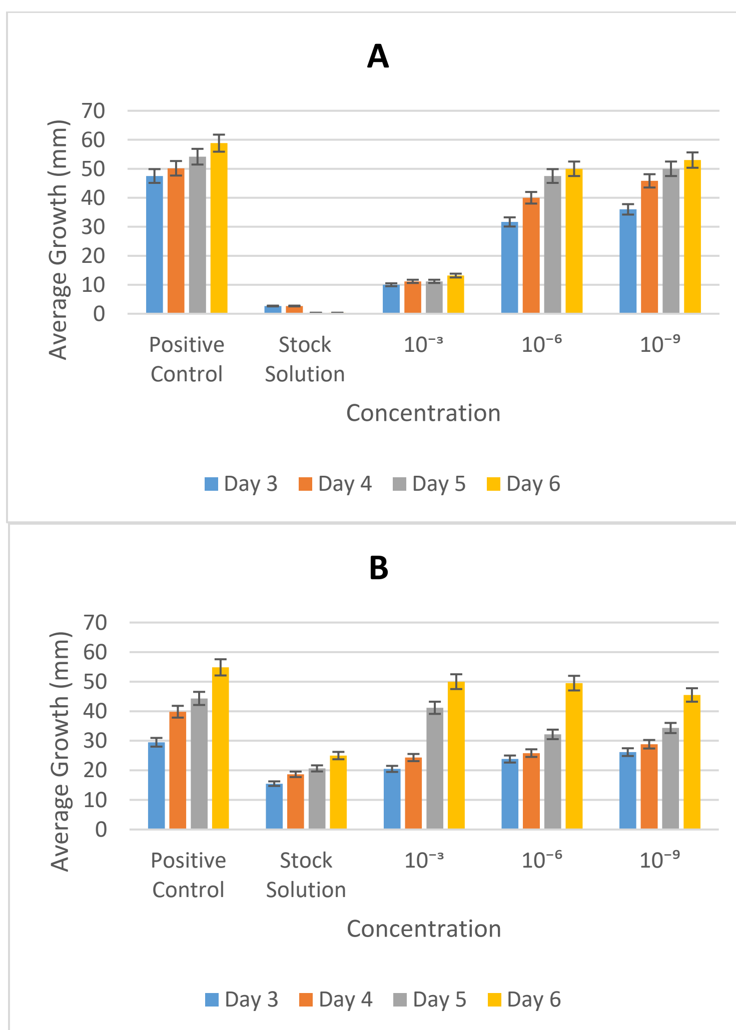

Figure 35: Average growth of *B. cinerea* **(A)** and *C. gloeosporioides* **(B)** strains treated with *M. rotundifolia* hydrolat, in function to various concentrations and various periods

5.4.1.2. Inhibition Percentage

5.4.1.2.1. *Mentha rotundifolia* Essential Oils Inhibition Effect

The concentrations of *Mentha rotundifolia* essential oils were tested at 10%, 30%, 60%, and 100%. Based on the statistical analyses, the growth inhibition effect of *Mentha rotundifolia* essential oils on *Botrytis cinerea* and *Colletotrichum gloeosporioides* strains is concentration-dependent. Higher concentrations (60% and 100%) inhibited growth more than lower concentrations (10% and 30%). The concentrations of 60% and 100% were highly significant against *Botrytis cinerea* strains (F= 109.659, P<0.0001) with inhibition percentages of 62.79% and 51.08%; the inhibition percentage of 60% concentration is higher than the one of 100% referring to the injection of only 0.25 ml of

EOs. These findings rank the both concentrations in the same group C according to Tukey's HSD test witch allowed for the identification of homogeneous groups, indicating significant differences between the negative control and the various essential oils concentrations while, non-significant differences between the two previous concentrations. Furthermore, the other tested concentrations (10% and 30%) according to Tukey's HSD test, were ranked into group A with no significant differences between them but a high significant difference compared to the negative control, with inhibition percentages of 18.79% and 19.5% **(Table 11 and Figure 36, 37)**.

Table 11: The growth inhibition effect of *M. rotundifolia* EOs against *B. cinerea* strains after 6 days

Concentration	Average	Standard Error	Inferior Borne (95%)	Superior Borne (95%)	Homogenous Grroups
Negative Control	6,102	2,464	0,851	11,354	A
10%	18,795	2,620	13,210	24,380	B
30%	19,500	2,620	13,915	25,085	B
100%	51,083	2,620	45,497	56,668	C
60%	62,798	2,620	57,212	68,383	C

Different letters next to the values at each concentration represents significant difference according to Tukey's HSD test ($p \leq 0.05$).

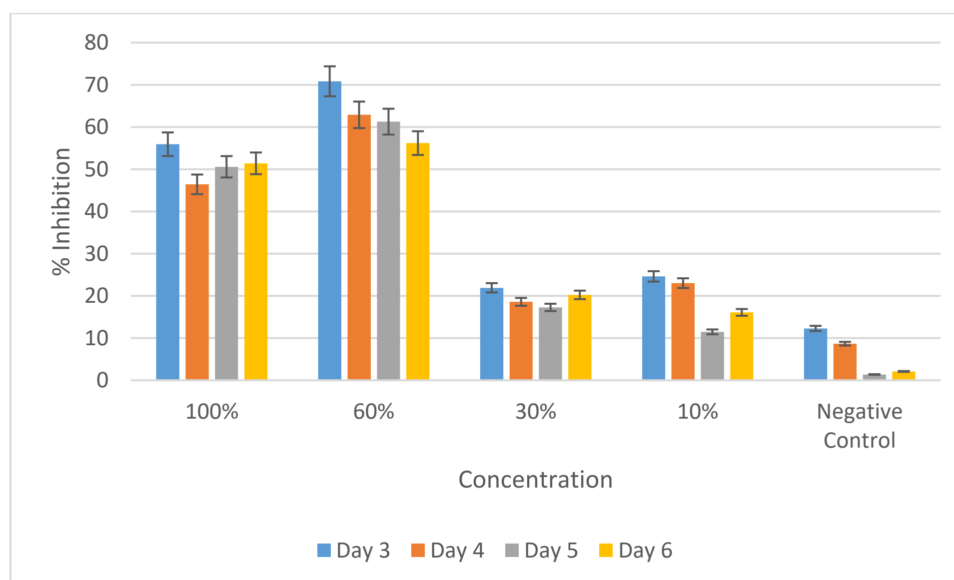

Figure 36: Inhibition percentage induced by various concentrations of *Mentha rotundifolia* essential oils on the growth of *B. cinerea*

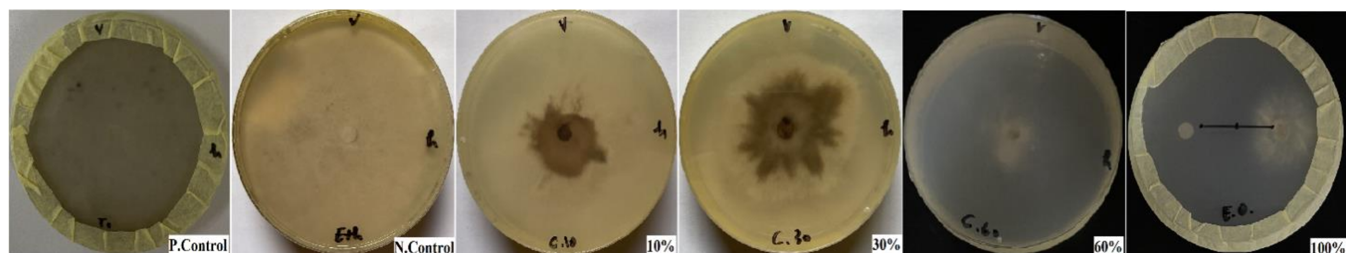

Figure 37: Inhibition effect of different application concentrations of *Mentha rotundifolia* EOs on the mycelial growth of *B. cinerea* after 6 days of incubation

The greatest antifungal activity of EOs concentrations against *Colletotrichum gloeosporioides* strains was observed with 100% dose revealing an inhibition percentage of 36.77%, followed by 60% and 30% concentrations with inhibition percentages of 19.78% and 12.77%, respectively. The 10% dose showed less potential for the control of mycelial growth, providing 5.41% **(Table 12 and Figure 38, 39)**. The results obtained were highly significant (F= 17.371, P<0.0001) compared to the negative control **(Appendix n°2)**.

Table 12: The growth inhibition effect of *M. rotundifolia* EOs against *C. gloeosporioides* strains after 6 days

Concentration	Average	Standard Error	Inferior Borne (95%)	Superior Borne (95%)	Groups
100%	36,779	2,964	30,839	42,720	A
60%	19,980	2,964	14,039	25,921	B
30%	12,777	2,964	6,836	18,717	B
Negative Control	9,348	2,964	3,407	15,288	BC
10%	5,418	2,964	-0,523	11,358	C

Different letters next to the values at each concentration represents significant difference according to Tukey's HSD test (p ≤ 0.05).

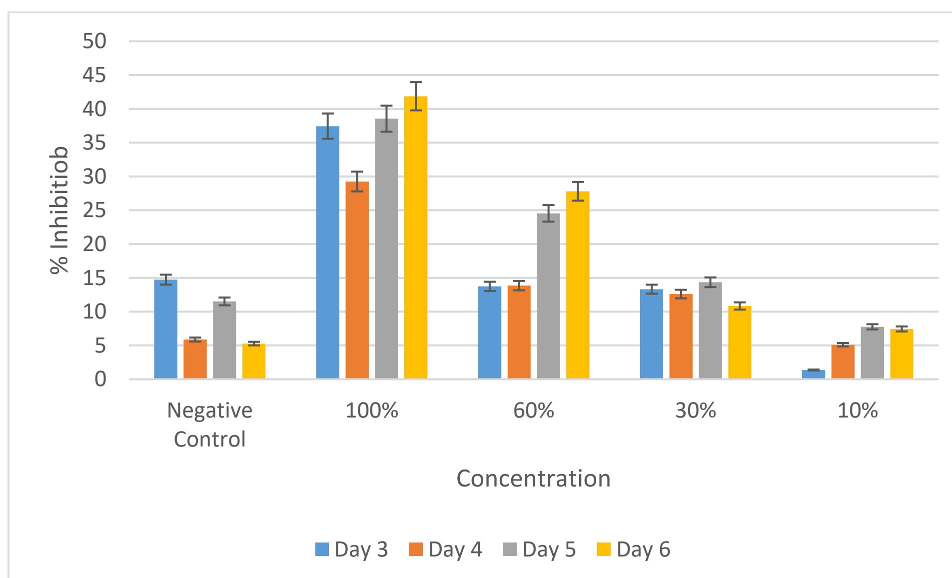

Figure 38: Inhibition percentage induced by various concentrations of *Mentha rotundifolia* EOs on the growth of *C. gloeosporioides*

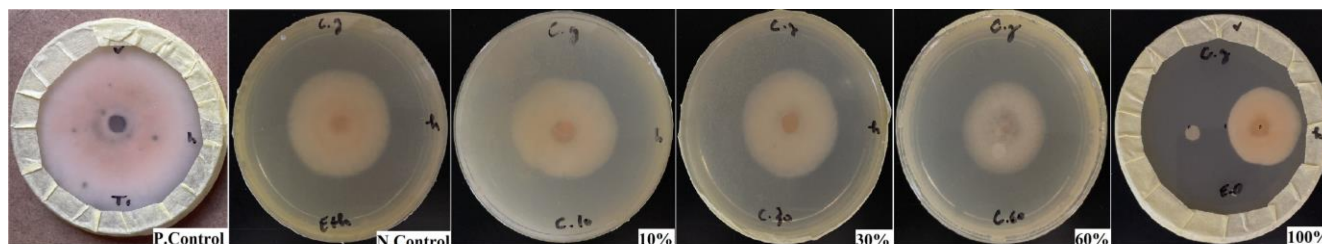

Figure 39: Inhibition effect of different application concentrations of *Mentha rotundifolia* EOs on the mycelial growth of *C. gloeosporioides* after 6 days of incubation

5.4.1.2.2. *Mentha rotundifolia* Hydrolat Inhibition Effect

According to findings mentioned in **Table 13**, **Appendix n°2** and **Figure (n°40** and **41)**, the effect of *M. rotundifolia* hydrolat against *Botrytis cinerea* strains is highly significant (F =227.347, P< 0.0001) which suggest that the hydrolat inhibits the growth of this fungus in a dose-dependent manner. The higher concentrations (stock solution and 10^{-3}) inhibited more effectively than lower concentrations (10^{-6} and 10^{-9}). The stock solution resulted the highest mycelial growth inhibition against B. cinerea strains providing a percentage of 94.28% and assigning into group A. However, the next concentration of 10^{-3} was assigned to group B with a mycelial inhibition percentage of 78.37%. The results assigned the lowest concentrations 10^{-6} and 10^{-9} to group C, which indicates the presence of significant differences in growth inhibition between the treated groups.

Table 13: The growth inhibition effect of *M. rotundifolia* hydrolat against *B. cinerea* strains after 6 days

Concentration	Average	Standard Error	Inferior Borne (95%)	Superior Borne (95%)	Groups
Stock Solution	94,288	2,706	88,835	99,742	A
10^{-3}	78,372	2,706	72,918	83,825	B
10^{-6}	20,385	2,706	14,931	25,839	C
10^{-9}	13,117	2,706	7,663	18,570	C

Different letters next to the values at each concentration represents significant difference according to Tukey's HSD test ($p \leq 0.05$).

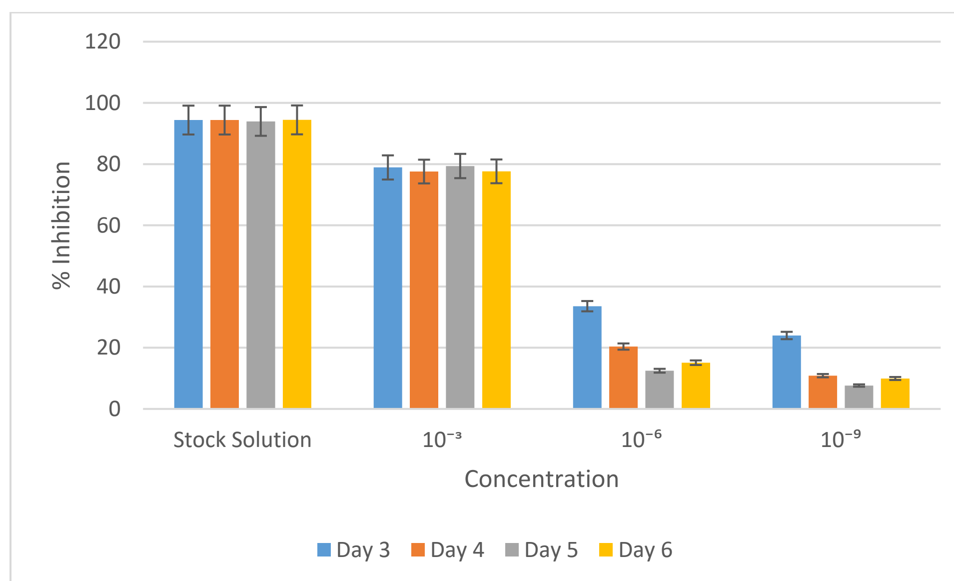

Figure 40: Inhibition percentage induced by various concentrations of *Mentha rotundifolia* hydrolat on the growth of *B. cinerea*

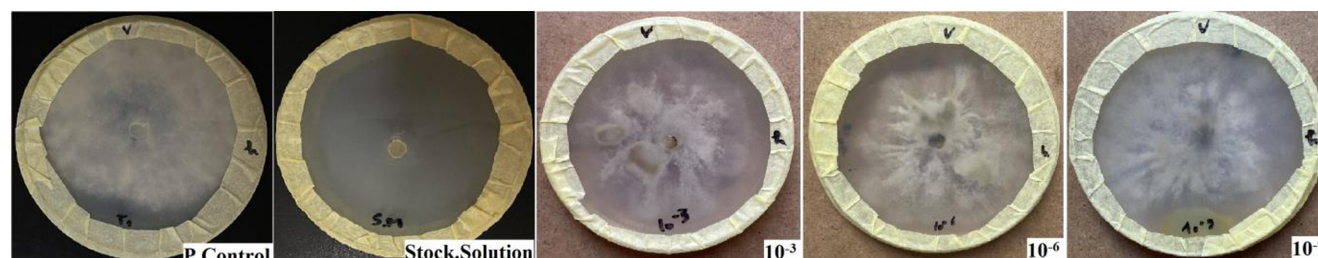

Figure 41: Inhibition effect of different application concentrations of *Mentha rotundifolia* hydrolat on mycelial growth of *B. cinerea* after 6 days of incubation

Moreover, according to statistical analysis mentioned in **Table 14** and **Figure n°42** and **43** about *Mentha rotundifolia* hydrolat inhibition effect against *Colletotrichum gloeosporioides* strains, we can conclude that all three *M. rotundifolia* hydrolat concentrations (10^{-3}, 10^{-6} and10^{-9}) resulted in lower mycelial growth against *Colletotrichum gloeosporioides* when compared to the stock solution. The higher concentration provided higher strain inhibition of 52.12% followed by the previous other concentrations with inhibition percentages of 24.10, 22.75 and 22.46%,

respectively. Furthermore, the concentrations were assigned by Tukey's HSD test to the same group "B," indicating that there is no significant difference in growth inhibition among them while, the stock solution was ranked into group A with significant differences with the other ones. These results are statistically significant with a calculated F of 19.204 and p-value of 0.0001 (**Appendix n°2).**

Table 14: The growth inhibition effect of *M. rotundifolia* hydrolat against *C. gloeosporioides* strains after 6 days

Concentration	Average	Standard Error	Inferior Borne (95%)	Superior Borne (95%)	Groups
Stock Solution	52,120	3,314	45,441	58,799	A
10^{-3}	24,106	3,314	17,427	30,785	B
10^{-6}	22,758	3,314	16,079	29,438	B
10^{-9}	22,461	3,314	15,782	29,140	B

Different letters next to the values at each concentration represents significant difference according to Tukey's HSD test ($p \leq 0.05$).

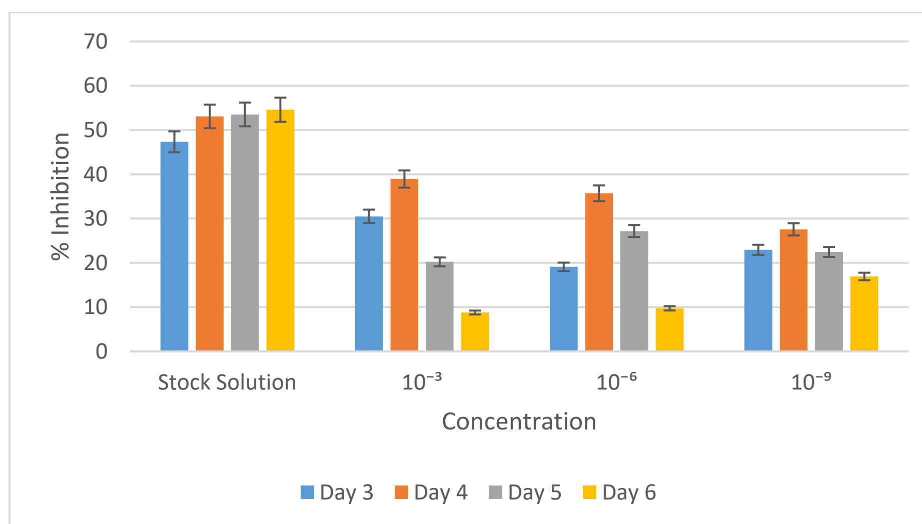

Figure 42: Inhibition percentage induced by various concentrations of *M. rotundifolia* hydrolat on the mycelial growth of *C. gloeosporioides*

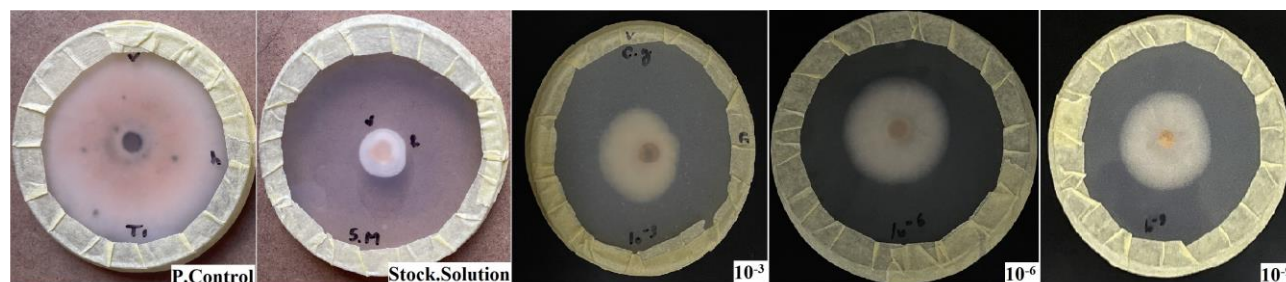

Figure 43: Inhibition effect of different application concentrations of *M. rotundifolia* hydrolat on the mycelial growth of *C. gloeosporioides* after 6 days of incubation

5.4.1.3. Microscopic Observations

The effect of *Mentha rotundifolia* essential oils and hydrolat caused a mycelial growth inhibition of the two pathogens of strawberry, *Botrytis cinerea* and the agent of anthracnose *Colletotrichum gloeosporioides*. In addition, the essential oils concentrations completely suppressed the germination and formation of the appressorium of *Botrytis cinerea* spores, which were found to be involved in the degradation of walls and membranes plant pathogen cells (**Figure 44**). As well as, it causes the lysis of the cell membrane of the mycelium of both pathogens, which appears emptied and siphoned. Partial degradation of the mycelium has been recorded **(Figure 45)**.

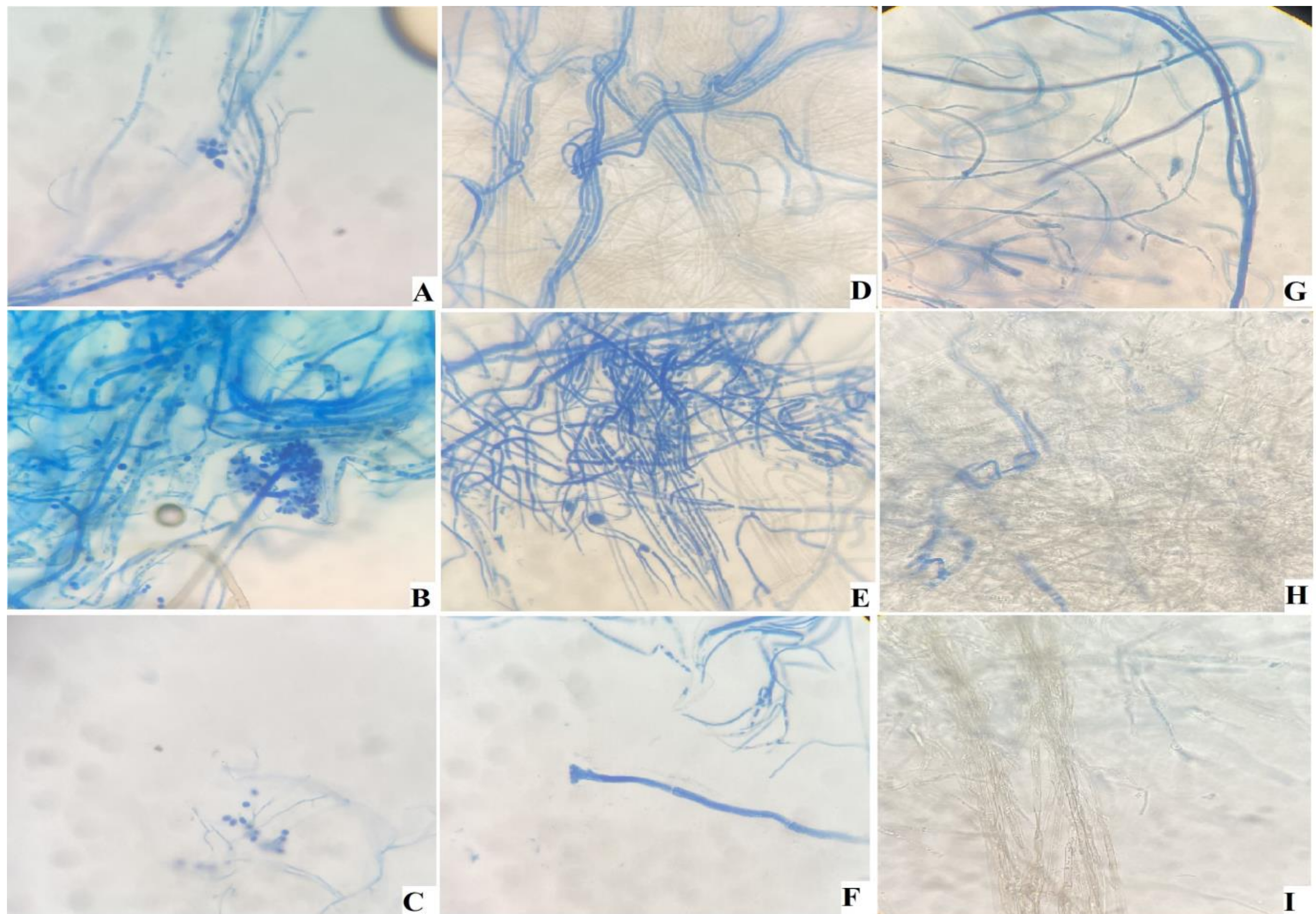

Figure 44: Microscopic aspect of *B. cinerea* treated with *M. rotundifolia* EOs and hydrolat. **A, B, C:** positive control rich of conidial germination and conidiophores. **D, E, F, G, H, I:** treated isolates with EOs and hydrolat showing: degradation of mycelium and membrane lysis, absence of conidial germination

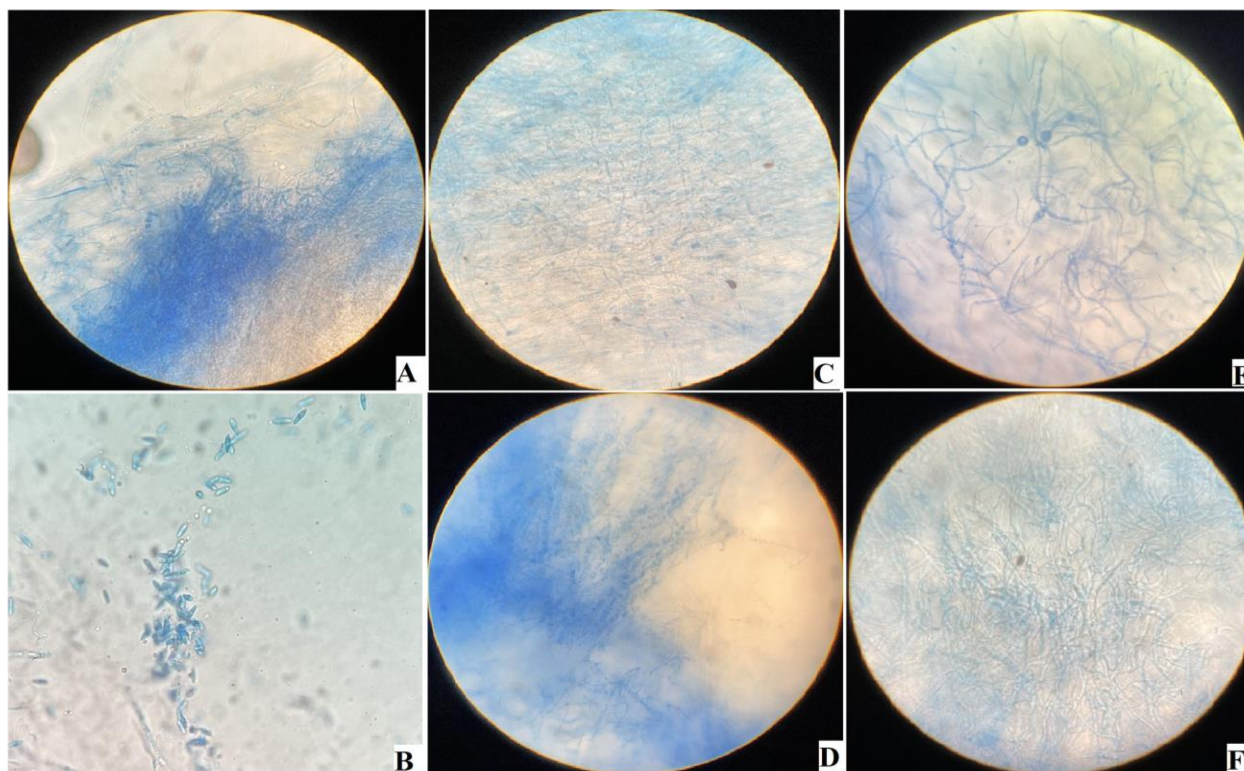

Figure 45: Microscopic aspect of *C. gloeosporioides* treated with *M. rotundifolia* EOs and hydrolat. **A, B, C:** positive control rich of conidial germination**. D, E, F, G, H, I:** treated isolates showing degradation of mycelium and absence of conidial germination

5.4.2. Insecticidal Activity

5.4.2.1. Average Repellency Effect and Percentages Following Treatment with EO and Hydrolat of *Mentha rotundifolia*

5.4.2.1.1. Average Repellency Effect

The repellency effects of different concentrations of *M. rotundifolia* essential oils and hydrolat (aromatic water) on *Aphis fabae* adults, in function to concentration and duration of exposure, are presented in **Table 15 and Table 16.**

Table 15: The repellency effect of *Mentha rotundifolia* EOs on *Aphis fabae* after 2 hours of exposure

Concentration	Average	Standard Error	Inferior Borne (95%)	Superior Borne (95%)	Homogenous Groups
100%	7,667	0,527	6,451	8,882	A
30%	7,000	0,527	5,785	8,215	A

Positive Control	0,000	0,527	-1,215	1,215	B
Negative Control	0,000	0,527	-1,215	1,215	B

Different letters next to the values at each concentration represents significant difference according to Tukey's HSD test ($p \leq 0.05$).

Table 16: The repellency effect of *M. rotundifolia* hydrolat on *Aphis fabae* after 2 hours of exposure

Concentration	Average	Standard Error	Inferior Borne (95%)	Superior Borne (95%)	Homogenous Groups
10^{-6}	8,667	0,471	7,580	9,754	A
Stock Solution	7,667	0,471	6,580	8,754	A
Positive.Control	0,000	0,471	-1,087	1,087	B
Negative.Control	0,000	0,471	-1,087	1,087	B

Different letters next to the values at each concentration represents significant difference according to Tukey's HSD test ($p \leq 0.05$).

After 2 hours, the highest repellency effect on *Aphis fabae* adults was found in both essential oil and hydrolat of *Mentha rotundifolia* compared to negative and positive controls. The effect with concentrations of 100% and 30% were found to be statistically significant ($F=64.800$, $P<0.0001$) with an average of 7.66 and 7, respectively. While with stock solution and 10^{-6} concentration of *M. rotundifolia* hydrolat, it was found that the average of repellency effects to be statisticaly significant ($F = 100.792$, $P<0.0001$) with an average of 7.66 and 8.66, respectively **(Figure 46 and Appendix n°3)**.

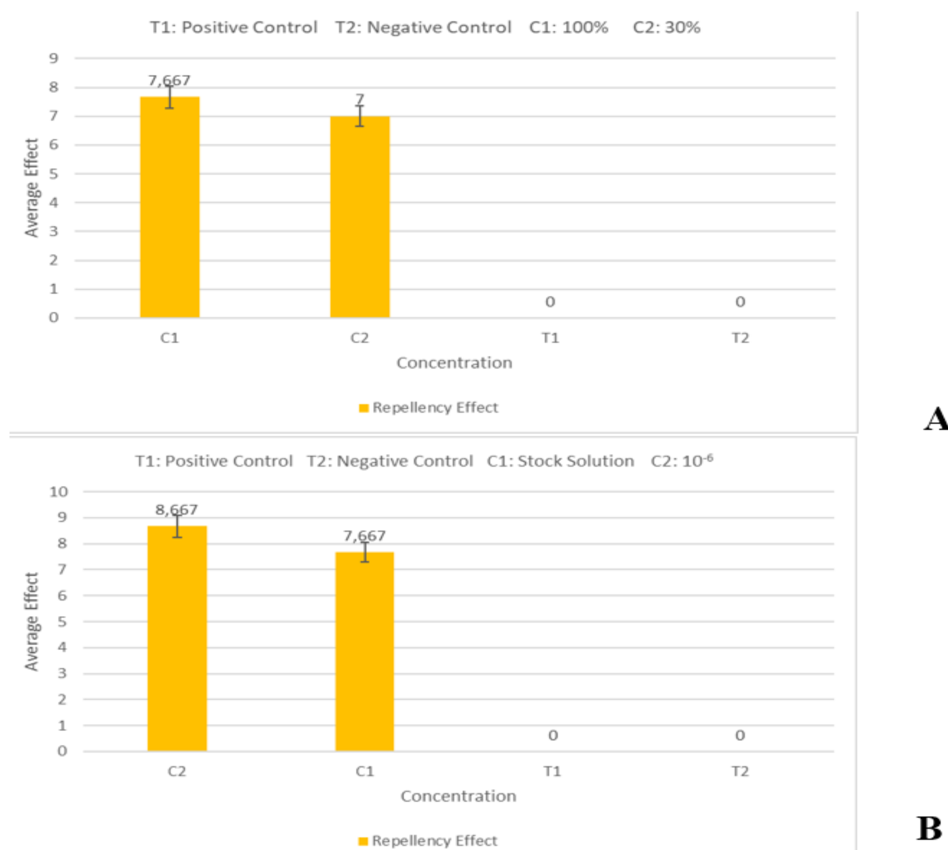

Figure 46: Average rates of repellency effect of *M. rotundifolia* EOs **(A)** and hydrolat **(B)** against *Aphis fabae* adults in function of concentration and exposure time

5.4.2.1.2. Repellency Percentage

According to 50 <R^2 = 72% <100 and p-value (P<0.014) associated with the statistic F calculated (F= 6.800), the information provided by the explanatory variables is significant **(Appendix n°3)**. Additionally, according to **Figure 47**, after 2 hours of exposure on filter paper, the two concentrations of essential oils (100% and 30%) showed nearly the same repellent activity against *Aphis fabae* adults. The repellency percentages, respectively, were 53.33% and 40% ranking them in class **II** and **III** according to the classification of **McDonald et al**. While the stock solution of hydrolat and the 10^{-6} concentration showed a higher repellent effect compared with essential oils, highly statistically significant with 50 < R^2 = 86% < 100 and p-value (0.0001) associated with the statistic F calculated (F = 15.792). Their repellency percentages were, respectively, 53.33% and 73.33% witch rank them in class **III** and **IV (Figure 47 and Appendix n°3)**.

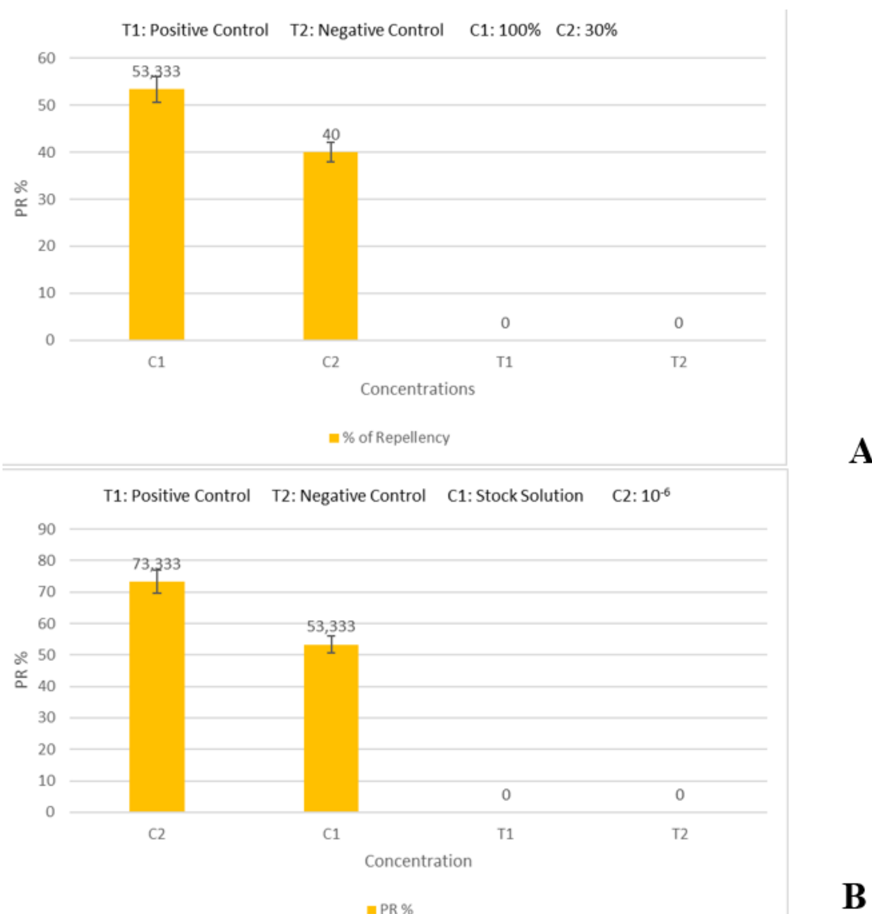

Figure 47: Rates of repellency percentage of *M. rotundifolia* EOs **(A)** and hydrolat **(B)** effect against *Aphis fabae* adults in function to concentration and exposure time

5.4.2.2. Average Mortality Following Treatment with EO and Hydrolat of *Mentha rotundifolia*

The average mortality (effect) and standard deviations following the treatment of the *Aphis fabae* populations with the EOs and hydrolat of *Mentha rotundifolia* in function to concentration and exposure duration are shown in **(Figure 48).**

The different concentrations of EOs and hydrolat, compared to positive and negative control, affected the insects for the first two hours. This mortality effect grew over time, as after twelve hours, almost the total insects died under not only the influence of EOs both concentrations 100% and 30% but also the stock solution of hydrolat. While, the lowest concentration (10^{-6}) killed five insects out of ten in the 12 first hours.

The average effect of EOs and hydrolat of *Mentha rotundifolia*, in function to concentration and exposure duration, relying to R^2 with both EOs and hydrolat is respectively (99% and 94%) in addition to p-value and statistic F calculated (F = 1663.278, P<0.0001; F = 162.142, P<0.0001) is statically significant **(Appendix n°3)**.

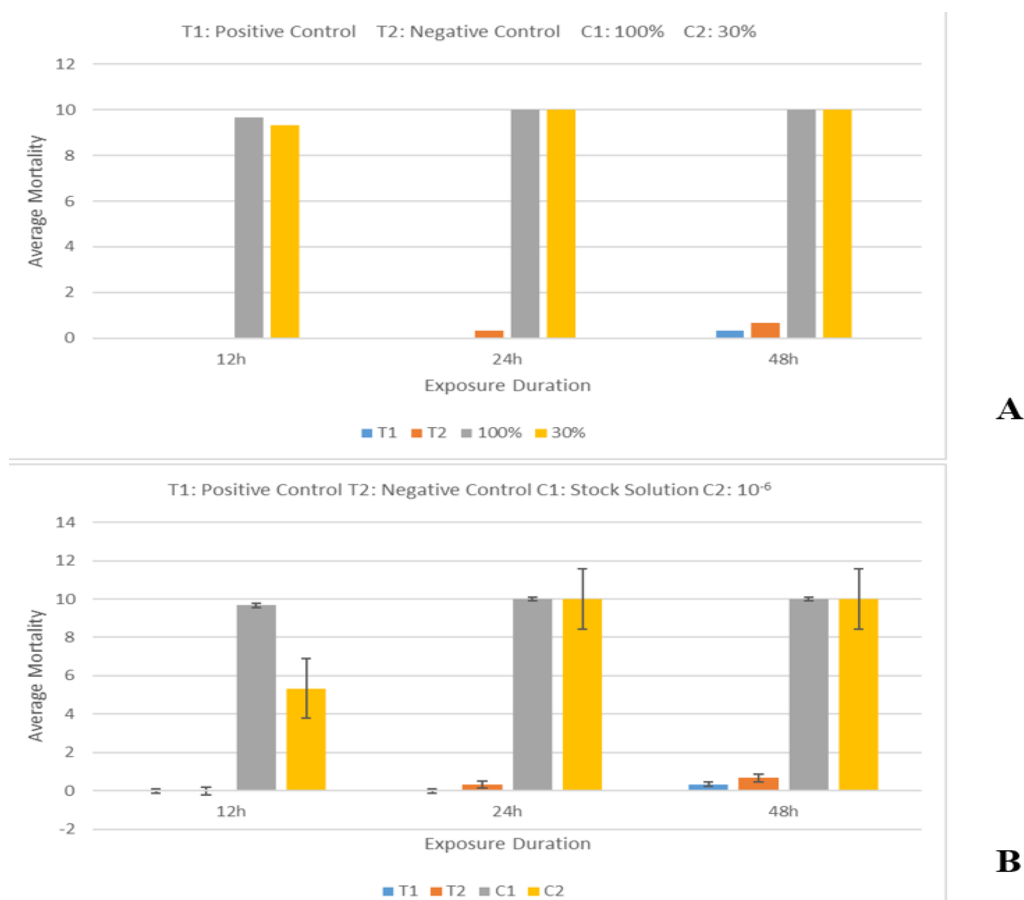

Figure 48: Average mortality of *Aphis fabae* populations' trated with EOs **(A)** and hydrolat **(B)** of *M. rotundifolia* in function to concentration and exposure duration

5.4.2.3. Mortality Correction

5.4.2.3.1. Observed Mortality Percentage

The average percentage of observed mortality and standard deviations following the treatment of *Aphis fabae* populations with the EOs and hydrolat of *Mentha rotundifolia* in function to concentration and exposure duration are shown in **Figure 49**.

After exposure of 12h, the highest concentration of EOs (100%) and hydrolat stock solution marked a mortality percentage of 96.6% while, the lowest concentrations 30% and 10^{-6} registered mortality

percentages of 93.3% and 53.33%, respectively. However, a total mortality of 100% was revealed after 24h of exposure.

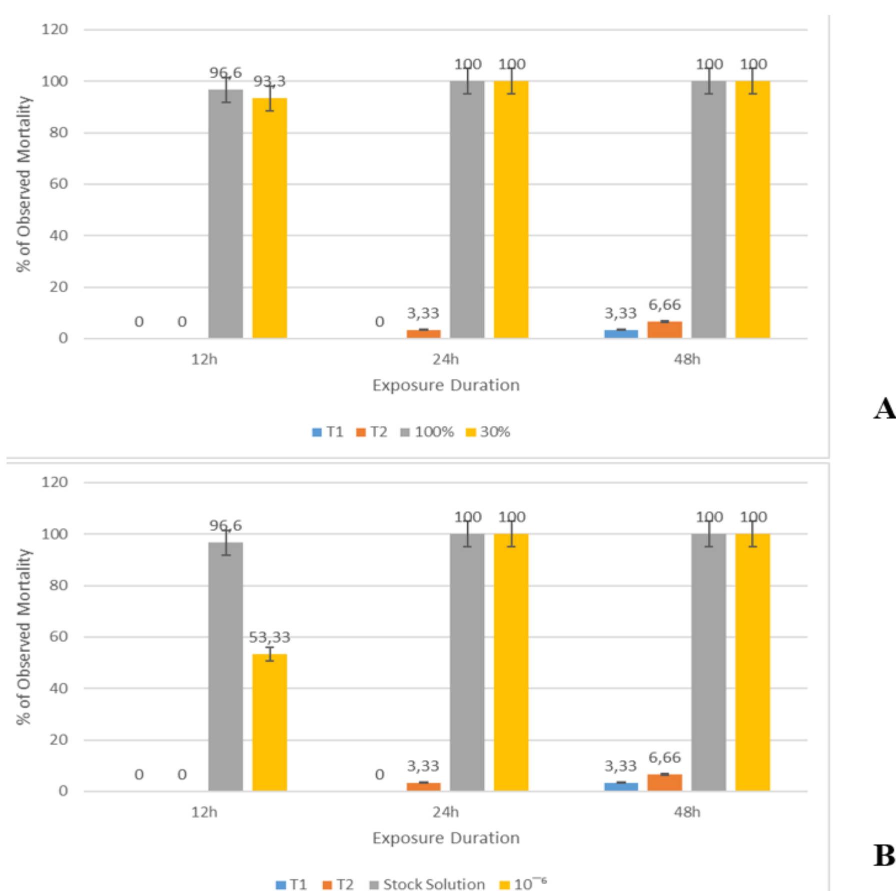

Figure 49: Percentage of observed mortality in *Aphis fabae* populations treated with EOs **(A)** and hydrolat **(B)** in function to concentration and exposure duration

5.4.2.3.2. Corrected Mortality

The tables **(n° 17 and 18)** show the average rates of corrected mortality percentage after treating *Aphis fabae* populations with *Mentha rotundifolia* EOs and hydrolat in function to concentration.

Adult mortality results revealed a dose-response relationship with oils and hydrolat concentrations with a coefficient of correlation R^2 of 100% and 94%, respectively. In fact, as essential oils concentrations increased, mortality increased significantly. As showed in **Appendix n°3**, *M. rotundifolia* essential oils exhibited statistically high significant results with high fumigant toxicity against *Aphis fabae* adults ($F = 1287.865$, $P \leq 0.001$) comparatively to its hydrolat which recorded, also, statistically significant results ($F = 44.126$, $P \leq 0.001$). Indeed, the mortality increased

significantly with increasing essential oils, hydrolat concentrations and exposure time. For *M. rotundifolia* EOs, the highest concentration (100%) induced complete mortality with percentage of 100% after 12 hours of exposure time; however, the lowest concentration (30%) induced complete mortality after 24 hours of exposure time whereas no mortality was registered in the same conditions with positive control (0%).

After exposition of 12 hours, the stock solution of *M. rotundifolia* hydrolat caused 96.6% mortality compared to 53.33% mortality recorded at the concentration of 10^{-6}. Moreover, the mortality of *Aphis fabae* adults attained 100% the previous concentrations after 24 hours of exposure **(Figure 50)**.

Table 17: Mortality percentages of *Aphis fabae* after 48 hours of exposure to the EOs of *M. rotundifolia*

Concentration	Average	Error Standard	Inferior Borne (95%)	Superior Borne (95%)	Homogenous Groups
100%	98,867	1,551	95,289	102,444	A
30%	97,767	1,551	94,189	101,344	A
Positive Control	0,783	1,551	-2,794	4,361	B
Negative Control	3,013	1,551	-0,564	6,591	B

Different letters next to the values at each concentration represents significant difference according to Tukey's HSD test ($p \leq 0.05$).

Table 18: Mortality percentages of *Aphis fabae* after 48 hours of exposure to the hydrolat of *M. rotundifolia*

Concentration	Average	Error Standard	Inferior Borne (95%)	Superior Borne (95%)	Homogenous Groups
Stock Solution	98,867	7,853	80,759	116,975	A
10^{-6}	84,443	7,853	66,335	102,551	A
Positive Control	0,783	7,853	-17,325	18,891	B
Negative Control	3,013	7,853	-15,095	21,121	B

Different letters next to the values at each concentration represents significant difference according to Tukey's HSD test ($p \leq 0.05$).

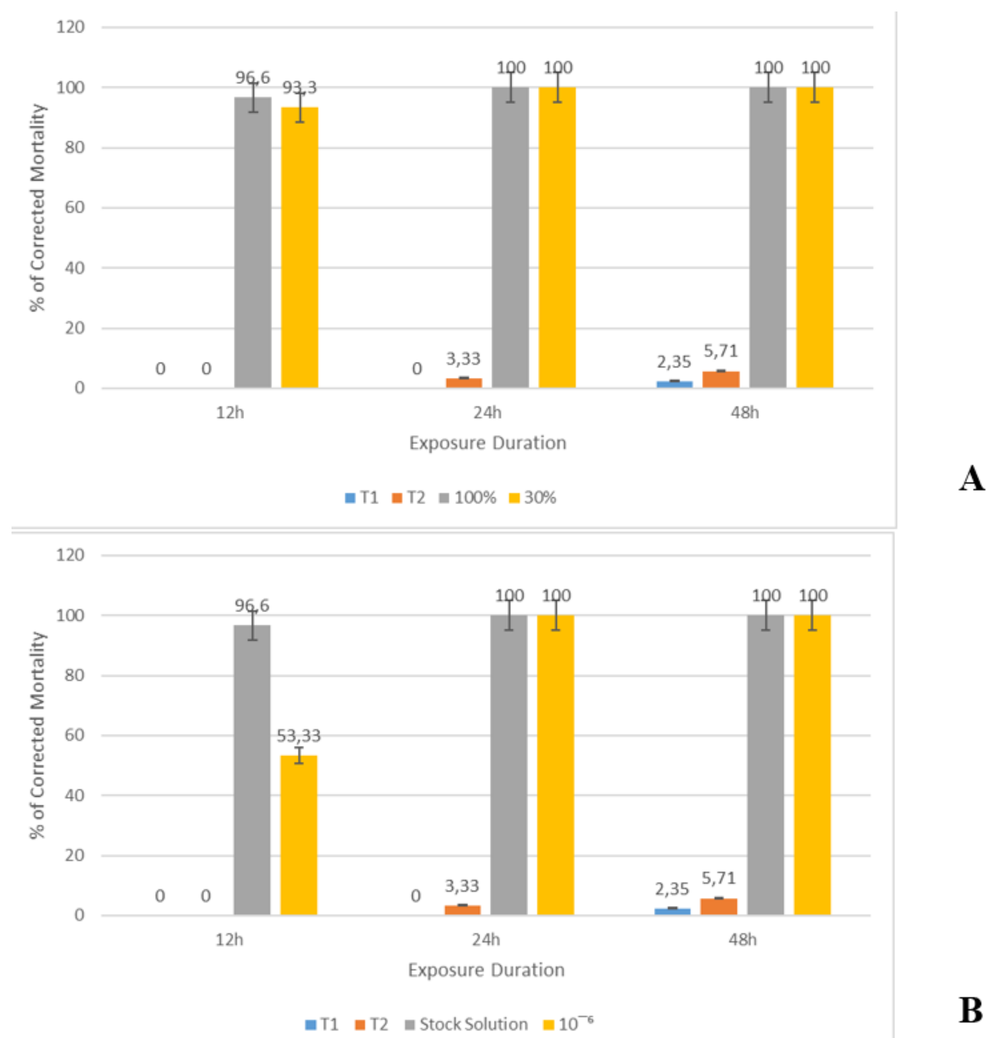

Figure 50: Variation in mortality rate as a function of concentration and duration of exposure. **A:** percentage of mortality in *Aphis fabae* populations treated with EOs. **B:** mortality in *Aphis fabae* populations treated with hydrolat

5.4.2.4. LD50 Determination

The lethal dose at 50% of *M. rotundifolia* essential oil and hydrolat for *Aphis fabae* was calculated using the linear equation of the binary logistic regression with the probit model **(Figure 51 and 52)**. The lethal dose LD50 of essential oils resulting in mortality of 50% of treated aphids population is 3.88×10^{-3} g/ml while, it was measured by 2.882×10^{-13} g/ml in aphids population treated with *Mentha rotundifolia* hydrolat **(Appendix n°3 and 4).**

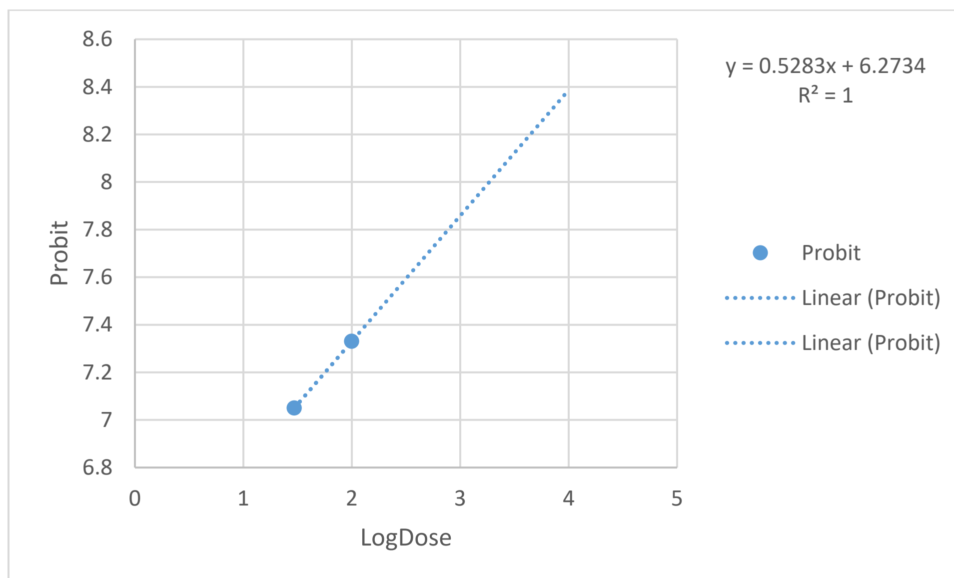

Figure 51: Regression line of LD50 of *M. rotundifolia* EOs for *Aphis fabae* adults' toxicity, in function to doses and mortality percentages

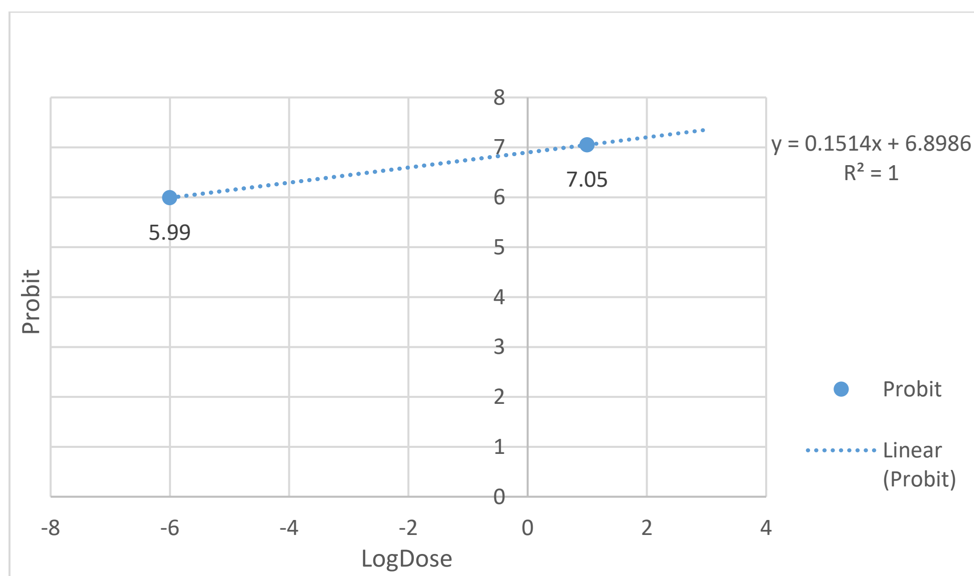

Figure 52: Regression line of LD50 of *M. rotundifolia* hydrolat for *Aphis fabae* adults' toxicity, in function to doses and mortality percentages

5.3. Discussion

Mentha rotundifolia species, which are widely distributed in northern Africa, its essential oils widely recommended in traditional medicine to treat a variety of health problems; they were also screened for chemical, insecticidal, and antifungal properties.

Mentha rotundifolia harvested in our region yielded 1.37%, which is higher than Tunisia's 1.26% **(Riahi *et al.*, 2013)** and Morocco's 1.17% **(Kasrati *et al.*, 2015)**, but lower than Algeria's 1.8% **(Brada *et al.*, 2007)**. The highest yield (4.33%) was obtained from *Mentha rotundifolia* from Morocco **(Derwich *et al.*, 2010)**; however, low levels were obtained by hydrodistillation and microwave from Naciria (60 km east of Algiers) (0.22 and 0.13%, respectively) **(Haddache *et al.*, 2017)**. *Mentha rotundifolia* of Corsica produced very low yields (0.08% -0.10%) **(Sutour *et al.*, 2008)**. The low value obtained can be attributed to several intrinsic (growth stages and plant material age) and extrinsic (plant as the period and middle of harvest, cultural practices, and all soil and climate conditions, drying and extraction methods) parameters **(Benayad, 2008)**. Furthermore, abiotic or physicochemical characteristics have an impact on essential oils yield, such as humidity, temperature, and sunshine time.

The essential oils of *M. rotundifolia* leaves and stems were GC/MS analyzed. Its analysis revealed sixty-seven components; the major component was identified as pulegone (39.21%), a cyclic monoterpene, including other important compounds. The various chemotypes of *M. rotundifolia* have been clearly identified, with an oxygenated menthane derivative being the main component present in almost all chemotypes **(Lawrence, 2007)**. This is consistent with the findings of various studies on *M. rotundifolia* chemical compositions from various parts of the world containing different principal constituents, such as pulegone **(Riahi *et al.*, 2013)**, menthol **(Derwich *et al.*, 2010)**, cis-jasmone **(Riahi *et al.*, 2013),** β-Caryophyllene **(Wang *et al.*, 2013)**, cis-piperitone oxide **(Brada *et al.*, 2007),** trans-piperitone epoxide **(Brahmi *et al.*, 2016)**. These results confirms previous research, which has found different chemotypes of *M. rotundifolia* growing in different parts of the world. The observed differences in yield and composition for the investigated EOs are most likely due to abiotic factors such as the climate specified in the regions of provenance of the samples, geographical factors such as altitude, and the nature of the soil **(Brahmi *et al.*, 2016)**. According to **Riahi *et al.*, (2013)**, genetic factors should not be ruled out when explaining EO chemo-variation. Furthermore, chemical differences in the oil composition of plant species were

reported in relation to harvesting, season, and extraction and analysis conditions **(Brahmi *et al.*, 2016)**.

The findings of our study revealed that essential oils extracted from *M. rotundifolia*, likewise its hydrolat, had potent antifungal activity. They were found to be highly effective against the tested fungal strains with inhibition percentages of 62.79 and 36%, respectively, against *B. cinerea* and *C. gloeosporioides.* Additionally, hydrolat effect was highly significant with mycelial inhibition percentages of 94 and 52% against fungal strains of *B. cinerea* and *C. gloeosporioides.* Regarding minimum inhibitory effect, it should be noted that the sensitivity of microorganisms to essential oils action varied greatly depending on the method of application. Indeed, *M. rotundifolia* essential oils was fungitoxic by contact, in contrast to vapors, which were fungistatic by fumigation **(Aouadi *et al.*, 2021)**. The differences in the polarities and volatilities of the individual essential oil components, according to **Cox *et al.*, (2001)**, can explain the variability in essential oil efficacy related to the mode of application (contact or fumigation). Hydrophilic polar constituents mix and diffuse easily in aqueous media, resulting in greater effects in the direct contact method, in accordance with our findings where the effect of hydrolat by contact application showed higher results compared with volatile application of EOs.

The extracted essential oils, as well as the hydrolat, have also induced morphological changes resulting in mycelium degradation, wall and membrane degradation, cellular content exuviation among the tested fungal strains. Several studies have highlighted the inhibitory action of essential oils on the germination of fungal spores **(Vitoratos *et al.*, 2013; Farzaneh *et al.* 2015),** whereas, the mechanism of action remains ambiguous and misunderstood. Nonetheless, **Pei *et al.*, (2020)** and **Carson *et al.*, (2002)**, affirm that their activity flows from their ability to disrupt the structure of fungi cell membranes; which explains the results obtained about the antifungal activity of *Mentha rotundifolia* against *B. cinerea* and *C. gloeosporioides* that can be probably attributed to the majority oil compounds. The hydrophobic nature of the hydrocarbon constituents of EOs allows them to be inserted into the lipid layers of the microbial cell membrane and mitochondria, disrupting structures and making them more permeable. As a result, leakage of ions and other cell components can occur **(Sikkema et al., 1994; Carson et al., 2002)**.

Plants, particularly aromatic herbs and oils, have a long history of use as insect repellents in herbal folklore **(Kumar *et al.*, 2011)**. In this study, repellent action was dependent upon oils concentration

as well as exposure duration, and there was significant results ($P < 0.0001$) between both EOs and hydrolat (water extract) of *Mentha rotundifolia*. After 2 hours of exposure on filter paper, the two concentrations of essential oil (100% and 30%) showed nearly the same repellent activity against *Aphis fabae* adults (53.33% and 40%) while, the stock solution of hydrolat and the 10^{-6} concentration showed a higher repellent effect compared with essential oils (53.33% and 73.33%). According to **McDonald *et al*., (1970),** these oils belong to repellency class **II** and repellency class **III** (i.e. Moderate Repulsive). However, the hydrolat concentrations belong to repellency classes **III** and **IV**. Our findings showed almost the same repellent activity of *Mentha rotundifolia* against *R. dominica* adults, demonstrated in **Brahmi *et al*., (2016)**, with a percentage of 47.54% (class **III**). In the other hand, they are consistent with the repellent properties of essential oils and extracts of other *Mentha* species on other *Diptera* pests. For example, *M. longifolia* (L.) Huds essential oil was found to be 100% repellent to *Sitophilus zeamais* **(Odeyemi *et al*., 2008)** and 85% repellent to *C. chinensis* **(Kumar *et al*., 2009)**.

The current study's data revealed, afterwards chemical composition determination, that *M. rotundifolia* essential oils and hydrolat had potent fumigant activity against aphids (statistically significant with $p < 0.0001$). Mortality results of *Aphis fabae* adults following the treatment of different concentrations of EOs and hydrolat are, respectively, 98.86% and 98.86%. **Yaka, (2007)** discovered that the toxic or repulsive effects of an essential oil or plant extract depend on a variety of factors, including concentration, chemical composition, and the level of sensitivity of the target insects; this supports the findings. Yet, essential oils, according to **Chiasson and Beloin, (2007)**, act directly on the cuticle of insects and mites, particularly those with soft bodies such as aphids. The essential oil's effectiveness can be attributed to its chemical composition in general, and especially to its major compound and its high level. Since the insecticidal toxicity of oils and their constituents were independent of each other, **Ndomo *et al*., (2009)** proposed that different constituents of essential oils might interact synergistically/antagonistically for its activity. However, recent research has found that essential oils have a variety of effects on insect physiology. Furthermore, oxygenated monoterpenes were tested for neurotoxicity **(Kumar *et al*., 2011)**; they are typically volatile and lipophilic, and can quickly penetrate insects and interfere with their physiological functions **(Lee *et al*., 2002)** by inhibiting acetylcholinesterase activity **(Lopez and Pascual-Villalobos, 2010)** and acting on insect octopaminergic sites. Octopamine, a biogenic structure structurally similar to noradrenaline in vertebrates, functions as a neurotransmitter in

invertebrates. It regulates invertebrate heartbeat, motor function, ventilation, flight, and metabolism **(Roeder, 1999)**. Essential oils would work by binding to octopamine receptors, which are good biopesticide targets **(Taleb-Toudert, 2015)**.

This demonstrates the high toxicity of *M. rotundifolia* essential oils but also its hydrolat, which was reported by **Lablalta *et al*., (2020).** The previous author confirmed the positive effect of *Mentha rotundifolia* EOs against aphids, in agreement with the obtained results of LD50. This parameter value represents the dose required to produce a specific effect, in this case, a 50% mortality rate in the treated population; it is frequently used to assess the toxicity or potency of a substance. More previously demonstrated, *Mentha rotundifolia* hydrolat has significantly higher aphid toxicity than essential oils. It has a much lower concentration required to cause a 50% mortality rate in the aphid population with an LD50 value of 2.882×10^{-13} g/ml when compared to essential oils which have an LD50 of 3.88×10^{-3} g/ml. The extracted water and EOs also caused rapid darkening of the aphids, the colour changing in aphid cadavers is less intense with oils containing phenolic constituents like carvacrol, thymol, γ -terpinene and p-cymene, but oils with these compounds still induced mortality at the very low concentrations **(Sampson *et al*., 2005)**.

The difference in LD50 results may be justified by the minimal quantity of EOs that has been injected; however, the method of application and the duration of exposure can both influence the LD50 value. Various application methods, such as foliar sprays, fumigation, or direct contact, can cause varying degrees of insect mortality. Furthermore, physiological factors such as detoxification mechanisms or specific target sites can influence toxic substance response. Temperature, humidity, and other environmental factors can also have an effect on it **(Salman *et al*., 2015)**.

Sampson *et al*., (2005) studied 23 essential oils, including *Mentha pelgium L*, against turnip aphids, *Lipaphis erysimi* (Kaltenbach) (Hemiptera: Aphididae), and showed that some individuals overcame paralysis induced by pulegone and its analog isomenthone. On the other side, **Miresmailli and Isman**, **(2006)**, discovered in another study that all concentrations of essential oils and EcoTrol pesticide, which contains rosemary, were effective on *T. urticae* and had no phytotoxic effect on the host plant, the tomato. Accordingly, essential oils appear to be suitable sources of active vapours that can be used as alternatives to chemical pesticides in the control of pests.

Conclusion

M. rotundifolia grown in Jijel (Algeria) hydrodistilled essential oil yielded 1.37%, its chemical composition was determined using GC/MS. The main component was identified as pulegone (39.21%).

Our findings revealed that *Mentha rotundifolia* essential oils and hydrolat have potent antifungal and insecticidal activity. The bioassay results show that the essential oils of *M. rotundifolia*, which is rich in bioactive molecules, besides its hydrolat are extremely effective against the phytopathogenic fungus. Indeed, it inhibited completely the mycelial growth of the two tested fungal strains and completely stopped spore germination by inducing deep morphological changes that led to their explosion. On the other hand, fumigation application showed a high effectiveness against the insect pest *Aphis fabae* showing high toxicity and high repellency effect. The mortality rate of the essential oil and hydrolat against the tested pest was high effective with all concentrations, which was emphasized with LD50 values. The in vitro efficiency could be explained by the richness of this plant in aromatic compounds.

The effect/concentration response for both aphids and phytopathogenic fungi was significant. As a result, our findings support the use of *M. rotundifolia* essential oils as a biological control against plant diseases and pest infestations. We recommend using essential oils as a new ideal and innovative strategy with ecological properties to protect plant crops. However, their practical application as safe alternatives of synthetic compounds necessitate further research to develop formulations that improve their effectiveness and stability. In addition, it is recommended that the various biochemical molecules identified in vitro be tested on various biological models with the view of using them for therapeutic and food preservation purposes.

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Appendix

Appendix n°1

Preparation of PDA culture medium:

- Boil 200g sliced, unpeeled potatoes in 1 liter distilled water for 30 minutes to make potato infusion.
- Filter through cheesecloth, saving the effluent (potato infusion).
- Boil 20g glucose, 20g agar, and the 1 L of potato infusion together to dissolve. pH final 6-6.5.
- Autoclave 30 minutes at 110°C.
- Fill sterile petri dishes with 20-25 ml portions.

Appendix n°2

Table 19: Growth diameters of tested strains of *C. gloeosporioides* with *M. rotundifolia* EOs

Growth Diameter of *Colletotrichum gloeosporioides* (mm)												
Repetitions / Concentrations	**Day 3**			**Day 4**			**Day 5**			**Day 6**		
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
Positive Control	36.5	35	35	39	40	39	41.5	60	50	44.5	64	63
Negative Control	36	25	30	39	35	37	41	46	45	44.5	62	55
100%	20	20	26.5	24.5	29.5	30	24.5	32	36	25	35	40
10%	35	35	35	36	39	37	40	50	48.5	43	60	55
30%	36	26.5	30	38	30	35	40	47.5	40.7	42	60	50
60%	34.5	28	29.5	37	30	31	40	30	40	42	32.5	45

Table 20: Growth diameters of tested strains of *B. cinerea* with *M. rotundifolia* EOs

Growth Diameter of *Botrytis cinerea* (mm)												
Repetitions / Concentrations	**Day 3**			**Day 4**			**Day 5**			**Day 6**		
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
B. cinerea (T1)	65	44	30	67.5	46.5	32	77.5	55.5	50	77.5	65	65
Ethanol (T2)	44	42	30	55	43	32	75	55	50	75	64	64
E.O (C1)	20	22.5	15	30	25	20	30	27.5	30	32.5	32.5	35
10% (C2)	30	35	30	32.5	40	31	55	52.5	50	67.5	57	50
30% C3	40	32	30	40	41	31	45	50	43	52.5	56.5	55
60% C4	15	10	12.5	20	14	16.5	27	23.5	19.5	31.5	30	27.5

Table 21: Growth diameters of tested strains of *C. gloeosporioides* with *M. rotundifolia* hydrolat

Growth Diameter (mm)												
Fungi	***Colletotrichum gloeosporioides***											
Repetitions / **Concentrations**	Day 3			Day 4			Day 5			Day 6		
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
Witness (T)	30	28.5	30	40	38.5	41	45.5	42.5	45	56	53.5	55
Stock Solution (C1)	19.5	17	10	22.5	18	15.5	25	17.5	19.5	30	17.5	27.5
10^{-3} (C2)	16.5	20	25	21	24.5	27.5	32.5	35	38.5	50	50	50
10^{-6} (C3)	25	25	21.5	25.5	27	25	30	36.5	30	50	50	48.5
10^{-9} (C4)	26	26.5	26	28	29	29.5	32	35	36	40	46.5	50

Table 22: Growth diameters of tested strains of *B. cinerea* with *M. rotundifolia* hydrolat

Growth Diameter (mm)												
Fungi	***Botrytis cinerea***											
Repetitions / **Concentrations**	Day 3			Day 4			Day 5			Day 6		
	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
Witness (T)	47.5	45	50	47.5	49	54	55	52.5	55	60	57.5	59
Stock solution C1	8	-	-	8	-	-	10	-	-	10	-	-
10^{-3} C2	10	10	10	12.5	11	10	12.5	11	10	14	12.5	13
10^{-6} C3	30	27.5	37.5	42.5	31	46.5	47.5	40	55	51.5	42.5	56
10^{-9} C4	40	35	33	47.5	43	43	55	50	45	59	52.5	47.5

Table 23: Statistical analysis of inhibition effect of *M. rotundifolia* EOs against *B. cinerea* by ANOVA Test, xlstat 2014

Analyse de la variance (% Inhibition) :

Source	DDL	Somme des carrés	Moyenne des carrés	F	Pr > F	Codes de signification des p-valeurs
Modèle	4,000	12047,597	3011,899	109,659	**<0,0001**	***
Erreur	15,000	411,992	27,466			
Total corrigé	19,000	12459,589				

Calculé contre le modèle Y=Moyenne(Y)

*Codes de signification : 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < ° < 1*

Table 24: Statistical analysis of inhibition effect of *M. rotundifolia* EOs against *C. gloeosporioides* by ANOVA Test, xlstat 2014

Analyse de la variance (% Inhibition) :

Source	DDL	Somme des carrés	Moyenne des carrés	F	Pr > F	Codes de signification des p-valeurs
Modèle	4,000	7326,521	1831,630	17,371	**<0,0001**	***
Erreur	55,000	5799,316	105,442			
Total corrigé	59,000	13125,837				

Calculé contre le modèle Y=Moyenne(Y)
*Codes de signification : 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < ° < 1*

Table 25: Statistical analysis of inhibition effect of *M. rotundifolia* hydrolat against *C. gloeosporioides* by ANOVA Test, xlstat 2014

Analyse de la variance (% Inhibition) :

Source	DDL	Somme des carrés	Moyenne des carrés	F	Pr > F	Codes de signification des p-valeurs
Modèle	3,000	7593,532	2531,177	19,204	**<0,0001**	***
Erreur	44,000	5799,328	131,803			
Total corrigé	47,000	13392,861				

Calculé contre le modèle Y=Moyenne(Y)
*Codes de signification : 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < ° < 1*

Table 26: Statistical analysis of inhibition effect of *M. rotundifolia* hydrolat against *B. cinerea* by ANOVA Test, xlstat 2014

Analyse de la variance (% Inhibition) :

Source	DDL	Somme des carrés	Moyenne des carrés	F	Pr > F	Codes de signification des p-valeurs
Modèle	3,000	59932,139	19977,380	227,347	**<0,0001**	***
Erreur	44,000	3866,349	87,872			
Total corrigé	47,000	63798,488				

Calculé contre le modèle Y=Moyenne(Y)
*Codes de signification : 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < ° < 1*

Appendix n°3

Table 27: Repellency rates of *Aphis fabae* populations treated with *M. rotundifolia* EOs after 2 hours of exposure

Repetitions / Concentrations	2h		
	R1	**R2**	**R3**
Positive Control	-	-	-
Negative Control	-	-	-
100%	7	8	8
30%	8	5	8

Table 28: Statistical analysis of repellency effect of *M. rotundifolia* EOs against *Aphis fabae* by ANOVA Test, xlstat 2014

Source	DDL	Somme des carrés	Moyenne des carrés	F	Pr > F	Codes de signification des p-valeurs
Modèle	3,000	831,639	277,213	1663,278	**<0,0001**	***
Erreur	32,000	5,333	0,167			
Total corrigé	35,000	836,972				

Calculé contre le modèle Y=Moyenne(Y)

*Codes de signification : 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < ° < 1*

Table 29: Repellency rates of *Aphis fabae* populations treated with *M. rotundifolia* hydrolat

Repetitions / Concentrations	2h		
	R1	**R2**	**R3**
***Aphis fabae* Control**	-	-	-
Ethanol Control	-	-	-
Stock Solution	7	8	8
10^{-6}	10	9	7

Table 30: Statistical analysis of repellency effect of *M. rotundifolia* hydrolat against *Aphis fabae* by ANOVA Test, xlstat 2014

Source	DDL	Somme des carrés	Moyenne des carrés	F	Pr > F	Codes de signification des p-valeurs
Modèle	3,000	729,639	243,213	162,142	**<0,0001**	***
Erreur	32,000	48,000	1,500			
Total corrigé	35,000	777,639				

Calculé contre le modèle Y=Moyenne(Y)

*Codes de signification : 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < ° < 1*

Table 31: Mortality rates of *Aphis fabae* populations treated with *M. rotundifolia* EOs after 48 hours of exposure

Repetitions / Concentrations	12h			24h			48h		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
***Aphis fabae* Control T1**	-	-	-	-	-	-	-	1	-
Ethanol Control T2	-	-	-	-	1	-	-	1	1
E.O C1	10	10	9	10	10	10	10	10	10
30% C2	9	10	9	10	10	10	10	10	10

Table 32: Statistical Analysis of Mortality Percentage of *Aphis fabae* treated with *M. rotundifolia* EOs by ANOVA Test, xlstat 2014

Analysis of variance (% of Corrected Mortality)

Source	DDL	Somme des carrés	Moyenne des carrés	F	Pr > F	Codes de signification des p-valeurs
Modèle	3,000	27898,759	9299,586	1287,865	**<0,0001**	***
Erreur	8,000	57,767	7,221			
Total corrigé	11,000	27956,527				

Calculé contre le modèle Y=Moyenne(Y)
*Codes de signification : 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < ° < 1*

Table 33: Mortality rates of *Aphis fabae* populations treated with *M. rotundifolia* hydrolat after 48 hours of exposure

Repetitions / Concentrations	12h			24h			48h		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
***Aphis fabae* Control**	-	-	-	-	-	-	-	1	-
Ethanol Control	-	-	-	-	1	-	-	1	1
Stock Solution	9	10	10	10	10	10	10	10	10
10^{-6}	5	5	6	10	10	10	10	10	10

Table 34: Statistical Analysis of Mortality Percentage of *Aphis fabae* treated with *M. rotundifolia* Hydrolat by ANOVA Test, xlstat 2014

Analysis of variance (% of Corrected Mortality) Hydrolat

Source	DDL	Somme des carrés	Moyenne des carrés	F	Pr > F	Codes de signification des p-valeurs
Modèle	3,000	24488,286	8162,762	44,126	**<0,0001**	***
Erreur	8,000	1479,900	184,988			
Total corrigé	11,000	25968,186				

Calculé contre le modèle Y=Moyenne(Y)

*Codes de signification : 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < ° < 1*

Appendix n°4

Table 35: Probit calculation of LD50 mortalities of *Aphis fabae* adults treated with *M. rotundifolia* EOs

Dose	LogDose	Corrected Mortality %	Probit
30	1.47	97.76	6.88
100	2	98.86	7.05

Table 36: Probit calculation of LD50 mortalities of *Aphis fabae* adults treated with *M. rotundifolia* hydrolat

Dose	LogDose	Corrected Mortality %	Probit
10^{-6}	-6	84.44	5.99
10	1	98.86	7.05

Abstract
Essential oils derived from aromatic or medicinal plants have recently proven useful in a variety of fields including the production of medicines, perfumes, and foodstuffs. The purpose of this research is to determine the antifungal and insecticidal activities of essential oils and hydrolat extracted from *Mentha rotundifolia* species against pathogenic strains *Botrytis cinerea*, *Colletotrichum gloeosporioides*, and pests *Aphis fabae*. The hydrodistillation with Clevenger type is used to extract essential oils. The results show that the essential oils yield is in the order of 1.37%, and their chemical composition is determined by the gas chromatography (GC) technique, with pulegone (39,21%) being the main compound obtained.
The essential oils of *Mentha rotundifolia* and its hydrolat have been shown to be effective against *Botrytis cinerea* and *Colletotrichum gloesporioides* in terms of antifungal activity recording significant statistical results ($p<0,0001$). Furthermore, both the liquid and vapour phases of the essential oils inhibit the growth of the fungal strains. The bioassays used to determine essential oils and hydrolat dilution toxicity to pest insects *Aphis fabae* revealed a very high effect that increases significantly with concentration and time of exposure. Similarly, the repellent effect test demonstrates that these extracts are an effective insect repellent. According to the findings, the plant under consideration is promising as a source of natural pesticides and lends itself well to research in the field of fungi and pest control using biochemical alternatives.
Keywords: *Mentha rotundifolia*, essential oils, hydrolat, antifungal activity, insecticidal activity.

Résumé
Les huiles essentielles issues de plantes aromatiques ou médicinales se sont récemment avérées utiles dans plusieursdomaines, telle la production de médicaments, de parfums et de denrées alimentaires. Le but de cette étude est de déterminer les activités antifongiques et insecticides des huiles essentielles et de l'hydrolats extraits de l'espèce *Mentha rotundifolia* contre les souches pathogènes *Botrytis cinerea, Colletotrichumgloeosporioides* et l'isecte nefaste *Aphis fabae*. L'hydrodistillation de type Clevenger a été utilisé pour extraire les huiles essentielles.
Les résultats de notre étude ont montré que le rendement en huiles essentielles est de l'ordre de 1.37%. Leur composition chimique a également été déterminée, et ce en faisant appel àla technique de chromatographie en phase gazeuse (GC). Le principal composé obtenu était la pulégone (39.21%).
En ce qui concerne l'activité antifongique, les huiles essentielles de *Mentha rotundifolia* et son hydrolat étaient efficacies contre les deux espèces *Botrytis cinerea* et *Colletotrichum gloessporioides* avec des résultats statistiques hautement significatifs ($p<0.0001$). Il a aussi été montré, suite à cette étude, que les phases liquide et vapeur des huiles essentielles peuvent inhiber la croissance des souches fongiques étudiées. Les tests biologiques utilisés pour déterminer la toxicité des huiles et de la dilution d'hydrolat pour les insectes nuisibles *Aphis fabae* a révélé un effet très élevé qui varie significativement avec la concentration et le temps d'exposition. De même, le test de l'effet répulsif a montré que ces extraits peuvent avoir d'importants effets insectifuges.
En conclusion, grâce à cette étude nous avons pu montrer que la plante étudiée est prometteuse comme source de pesticides naturels et constitut une bonne piste de recherche dans le domaine de la lutte antifongique et antiparasitaire à l'aide d'alternatives biologique.
Mots clés: ***Mentha rotundifolia*, huiles essentielles, hydrolat, activité antifongique, activité insecticide.**

ملخص

أثبتت الزيوت الأساسية المشتقة من النباتات العطرية أو الطبية مؤخرًا أنها مفيدة في مجموعة متنوعة من المجالات بما في ذلك إنتاج الأدوية
والعطور والمواد الغذائية. الغرض من هذا البحث هو تحديد الأنشطة المضادة للفطريات والمبيدات الحشرية للزيوت العطرية وماء التقطير
المستخرج من أنواع النعناع المستدير ضد السلالات الممرضة *بوتريتيس سينيريا* و*كوليتوتريكوم غلويوسبوريويد* وآفاة حشرات المن. لاستخراج
الزيوت الأساسية تم استخدام التقطير المائي عن طريق جهاز كليفنجر. أظهرت النتائج أن محصول الزيوت العطرية في حدود 1.37%، وتم
تحديد تركيبها الكيميائي بواسطة تقنية كروماتوجرافيا الغاز مع المركب الأساسي بوليغون 39.21%
اثبتت الزيوت الأساسية لعنصر النعناع المستدير وهيدراته فعاليتهما من حيث النشاط المضاد للفطريات مسجلاً
نتائج إحصائية مهمة. علاوة على ذلك، فإن كلاً من المرحلتين السائلة والبخارية للزيوت الأساسية تمنع نمو السلالة الفطرية. أظهرت الاختبارات
الحيوية المستخدمة لتحديد سمية الزيوت العطرية والتخفيف المائي لحشرات المن تأثيرًا عاليًا للغاية يزداد بشكل كبير مع
التركيز ووقت التعرض. وبالمثل، يوضح اختبار التأثير الطاردأن هذه المواد الفعالة هي طارد فعال للحشرات. وفقًا للنتائج، فإن النبات قيد
الدراسة واعد كمصدر للمبيدات الطبيعية ويصلح بشكل جيد للبحث في مجال مكافحة الآفات باستخدام البدائل الكيميائية الحيوية

الكلمات المفتاحية: النعناع المستدير، الزيوت الأساسية، هيدرولات، نشاط مضاد للفطريات، نشاط مبيد حشري