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THESIS:

Biocidal Effect of Plant Extracts Based on Nanoparticles

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ABSTRACT

Biocidal Effect of Plant Extracts Based on Nanoparticles

This study fits into the framework of fighting fungal phytopathogens by focusing on the antifungal potential of natural substances and silver nanoparticles (AgNPs). It is divided into two main parts: the synthesis and characterization of AgNPs, and the evaluation of antifungal activity of AgNPs biosynthesized using *Aloysia citrodora* essential oil, as well as essential oils from *A. citrodora* (verbena) and *Salvia sclarea* (sage), against *Fusarium oxysporum* and *Alternaria sp.*

The synthesis of AgNPs was confirmed by visual observation (color change to yellowish-brown) and UV-Visible spectroscopy. The antifungal tests revealed an overall significant reduction in mycelial growth for both fungi. For *F. oxysporum*, the *A. citrodora* essential oil showed the highest inhibition at all doses: **75.55%** at 100%, **68.88%** at 50%, and **57.77%** at 25%. Sage EO showed **71.11%** inhibition at 100%, while AgNPs had moderate but stable effects, peaking at **62.96%**.

For *Alternaria sp.*, *A. citrodora* EO again exhibited strong efficacy: **84.44%** at 100%, **74.81%** at 50%, and **64.44%** at 25%. Sage EO had **77.03%** inhibition at 25%, while AgNPs recorded **78.51%** inhibition at 100%.

These results confirm the high antifungal potential of *A. citrodora* EO at all concentrations against both fungi, followed by AgNPs and *S. sclarea* EO. The observed differences in fungal response suggest potential escape or adaptation mechanisms, especially for *F. oxysporum* when treated with nanoparticles. The findings support the use of nanoformulated plant extracts as promising, eco-friendly alternatives to chemical fungicides in integrated plant protection strategies.

Keywords: Silver nanoparticles (AgNPs), Essential oils, Aloysia citrodora (Verbena), Salvia sclarea (Sage), Fusarium oxysporum, Alternaria sp., Antifungal activity

RESUME

Effet de biocide d'extraits végétal à base de nanoparticules

Cette étude s'inscrit dans le cadre de la lutte contre les phytopathogènes fongiques en mettant l'accent sur le potentiel antifongique des substances naturelles et des nanoparticules d'argent (AgNPs). Elle est structurée en deux parties principales : la synthèse et la caractérisation des AgNPs, suivies de l'évaluation de leur activité antifongique lorsqu'elles sont biosynthétisées à partir de l'huile essentielle d'**Aloysia citrodora**, ainsi que des huiles essentielles de **A. citrodora** (verveine) et de **Salvia sclarea** (sauge) contre **Fusarium oxysporum** et **Alternaria sp.**

La synthèse des AgNPs a été confirmée par un changement de couleur (brun jaunâtre) et une analyse spectroscopique UV-Visible. Les tests antifongiques ont révélé une inhibition significative de la croissance mycélienne pour les deux champignons. Contre *Fusarium oxysporum*, l'huile essentielle d'*A. citrodora* a montré une inhibition de **75,55 %** à 100 %, **68,88 %** à 50 %, et **57,77 %** à 25 %. L'huile essentielle de sauge a atteint **71,11 %** à 100 %, tandis que les AgNPs ont présenté une efficacité plus modérée, avec un maximum de **62,96 %**.

Concernant *Alternaria sp.*, *A. citrodora* a également présenté une forte activité antifongique avec **84,44 %** à 100 %, **74,81 %** à 50 %, et **64,44 %** à 25 %. L'huile de *S. sclarea* a atteint **77,03 %** à 25 %, tandis que les AgNPs ont montré une inhibition de **78,51 %** à 100 %.

Ces résultats confirment l'efficacité élevée des huiles essentielles d'*A. citrodora* à toutes les concentrations testées, suivies des AgNPs et de l'huile de sauge. Les différences observées entre les réponses fongiques pourraient refléter des mécanismes d'adaptation ou de contournement, notamment chez *Fusarium*. Ces données soutiennent le potentiel des extraits végétaux nanoformulés comme alternatives écologiques et prometteuses aux fongicides chimiques.

Mots-clés : Nanoparticules d'argent (AgNPs), Huiles essentielles, Aloysia citrodora (Verveine), Salvia sclarea (Sauge), Fusarium oxysporum, Alternaria sp., Activité antifongique.

ملخص

التأثيرات المبيدة للكائنات الحية للمستخلصات النباتية القائمة على الجسيمات النانوية

تندرج هذه الدراسة ضمن إطار مكافحة الفطريات الممرضة للنباتات، مع التركيز على الإمكانيات المضادة للفطريات للمواد الطبيعية والجسيمات النانوية الفضية .(AgNPs) تنقسم الدراسة إلى قسمين رئيسيين: تخليق وتوصيف الجسيمات النانوية، ثم تقييم النشاط المضاد للفطريات للجسيمات النانوية المُخلقة باستخدام الزيت العطري لنبات **Aloysia citrodora**، إلى جانب الزيوت الأساسية المستخلصة من **Aloysia citrodora**(الفرفينة) و **Salvia sclarea**(المريمية) ضد كل من **Fusarium oxysporum**و**Alternaria sp.**

تم تأكيد تخليق الجسيمات النانوية من خلال تغير اللون إلى البني المصفر وتحليل الطيف المرئي-فوق البنفسجي. أظهرت اختبارات الفعالية المضادة للفطريات انخفاضًا كبيرًا في نمو الميسيليوم لكلا الفطرين. بالنسبة إلى *Fusarium oxysporum*، سجل زيت *A. citrodora* أعلى نسب تثبيط عند **75.55%**بتركيز 100%، **68.88%**بتركيز 50%، و**57.77%** بتركيز 25%. أما زيت المريمية فقد بلغ **71.11%**عند تركيز 100%، في حين أظهرت الجسيمات النانوية تأثيرًا متوسطًا بلغ حده الأقصى .**62.96%**

بالنسبة إلى *Alternaria sp.*، فقد سجل زيت *A. citrodora* مرة أخرى فعالية قوية بنسب تثبيط بلغت **84.44%** عند 100%، **74.81%**عند 50%، و**64.44% عند 25%. أظهر زيت *S. sclarea* فعالية جيدة خاصة عند تركيز 25% بنسبة **77.03%**، بينما سجلت الجسيمات النانوية **78.51%**عند تركيز 100%.

تؤكد هذه النتائج الكفاءة العالية لزيت *A. citrodora* الأساسي بجميع التراكيز المختبرة، تليه الجسيمات النانوية وزيت *S. sclarea*. ويُحتمل أن تكون الفروقات في الاستجابة الفطرية ناتجة عن آليات هروب أو تكيف، خصوصًا لدى *Fusarium*. وتدعم هذه النتائج استخدام المستخلصات النباتية المُحملة على جسيمات نانوية كبدائل واعدة وصديقة للبيئة للفطريات الكيميائية.

الكلمات المفتاحية: الجسيمات النانوية الفضية (AgNPs)، الزيوت الأساسية، Aloysia citrodora (الفرفينة)، Salvia sclarea (المريمية)، Fusarium oxysporum، Alternaria sp.، النشاط المضاد للفطريات.

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DEDICATION

In the name of Allah,

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To my parents*, whose quiet sacrifices built the ground beneath my feet,*
whose strength taught me resilience, and whose love,
even in silence, lit the way forward.
You never asked for perfection—only that I try,
and in that space, I found the courage to grow.

To my siblings,
thank you for the stubborn hope, the tough love,
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You reminded me, time and again, that I was capable—
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who became more than just names on lab benches and shared deadlines.
Thank you for the chaos we laughed through,
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for choosing persistence over perfection,
and for showing up again and again,
even when the path felt uncertain.
This is for the version of me that kept going.
You made it.
And you made it matter.

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LISTE OF ABBRIVIATION

- **AGNPs**: silver Nanoparticles
- **Eos**: essential oil
- **D.W:** distilled water
- **RO. W**: Reverse osmosis water
- **PDA**: Potato Dextrose Agar
- **ml:** millilitre
- **nm:** Nanometre
- **µl** : microliter
- **rpm**: Revolution Per Minute
- **Mm**: millimetre
- **Cm**: centimetre
- **CSEO** : Clary sage essential oil

INTRODUCTION

➢ INTRODUCTION

Phytopathogenic fungi from the genera ***Alternaria*** and ***Fusarium*** pose a major threat to global food security, including in Algeria, causing significant yield losses across a wide range of food and ornamental crops (agrios, 2005; FAO, 2022). These pathogens, known for their ability to produce toxins (mycotoxins) dangerous for human and animal consumption, are particularly challenging to control due to their complex life cycle, genetic diversity, and capacity to develop fungicide resistance (Leslie and Summerell, 2006)**.** Fungal diseases (e.g., Powdery mildew, Rust, Fusarium wilt, Take-all, Septoria blotch, Ergot) are estimated to be responsible for approximately 80% of diseases affecting cereals, impacting not only nutritional value but also the technological and sanitary quality of harvests, leading to economic problems nationally and internationally (HAMANI, 2020 and REZINE & BOUKEZIOUA, 2021). In Algeria, like other countries, fungal diseases, especially fusarioses, are widespread and cause significant damage to various crops such as lentils, chickpeas, tomatoes, and date palms (abdelhadi and bachir, 2023; benlabiod and menakh, 2019; rezine and boukezioua, 2021)

Controlling these phytopathogenic fungi proves particularly difficult due to their rapid adaptability and wide distribution in the environment Historically, chemical pesticides have been the primary control method. However, this approach is increasingly being questioned. Not only do less than 0.3% of pesticides actually reach the targeted pests, resulting in over 99.7% of the product dispersing into the environment (ABGI, 2025), but phytopathogenic fungi also rapidly develop resistance, rendering fungicides less and less effective (INRAE, 2020; Yarou et al., 2017) Furthermore, the massive use of these chemical products poses proven dangers to human health (acute and chronic poisoning, neurological diseases like Parkinson's, cancers) and the environment (contamination of soil, water, and atmosphere, disruption of ecosystems, especially for pollinators) (ABGI, 2025; DPCE, Centre Léon Bérard, 2025; Eurofins Biomnis, 2024; pimentel, 1995; US EPA, 2022; Yarou et al., 2017)

Facing these challenges, **biological control** emerges as a promising, more environmentally friendly and health-conscious alternative. Among biological approaches, the use of **essential oils (EOs)** from aromatic and medicinal plants is gaining increasing

interest due to their proven antifungal properties against various phytopathogenic fungi, including those affecting crops like *Fusarium sp.* and *Aspergillus niger* **.** Their effectiveness is attributed to the rich array of their bioactive compounds (terpenes, phenols, isoprenoids, phenylpropanoids) which can disrupt fungal membrane integrity, inhibit enzyme production, or interfere with their metabolic pathways (Bakkali et al., 2008)

However, the instability, volatility, and low water solubility of EOs limit their field efficacy. This is why their **integration with nanoparticles (nanoencapsulation)** is an innovative and optimized strategy (seghiri and haci, 2023)**.** Nanoparticles protect essential oils from environmental degradation, improve their stability, increase their solubility and bioavailability, and enable controlled and targeted release of active compounds (Bakkali et al., 2008) This nanobiotechnological approach thus optimizes the antifungal efficacy of essential oils at lower doses, reducing risks to the environment and human health, while offering a more sustainable control solution against pathogens like ***Alternaria*** and ***Fusarium*****.**

We hypothesize that the integration of plant extracts with nanoparticles will significantly enhance their biocidal properties compared to unformulated plant extracts. This improvement is attributed to several synergistic factors:

- Increased stability of the bioactive compounds from plant extracts within the nanostructure.

- Better bioavailability and penetration of biocidal agents into the target cells of microorganisms due to nanometric size.

- Controlled and prolonged release of active principles, allowing for a more durable biocidal action and potentially at lower concentrations.

Our primary objective is to evaluate the biocidal (antifungal) effect of these plant extracts formulated in nanoparticle form. More specifically, this study aims to:

- Synthesize silver nanoparticles using essential oils extracted from Aloysia citrodora.
- Quantify and compare the biocidal (antifungal) activity of nano-essential oils and/or pure essential oils against the tested pathogens, namely Alternaria sp. and Fusarium sp.

CHAPITRE I

➢ CHAPTER I: Nanoparticles Incorporated with Essential Oils

1. General Overview

Nanotechnology is defined by the International Organization for Standardization (ISO) as the study and manipulation of matter at the nanoscale, specifically within the range of 1 to 100 nanometers (nm) (Singh et al., 2021). At this scale **(Figure 1),** nanoparticles exhibit unique properties that differ significantly from their bulk counterparts, enabling enhanced interactions with plants, animals, and microbes. These nanoparticles, made from various metallic materials such as copper, zinc, titanium, magnesium, gold, alginate, and silver, act as integrated systems with active components that enhance beneficial effects within experimental organisms (Hasan, 2015). Furthermore, the size and shape of nanoparticles significantly influence their biological activity; although many particles are spherical, variations in size and composition complicate comparative evaluations of their (Slavin and Bach, 2022)**.** These factors together emphasize the critical role of nanoparticle characteristics in their application to biological systems **(Figure 2).**

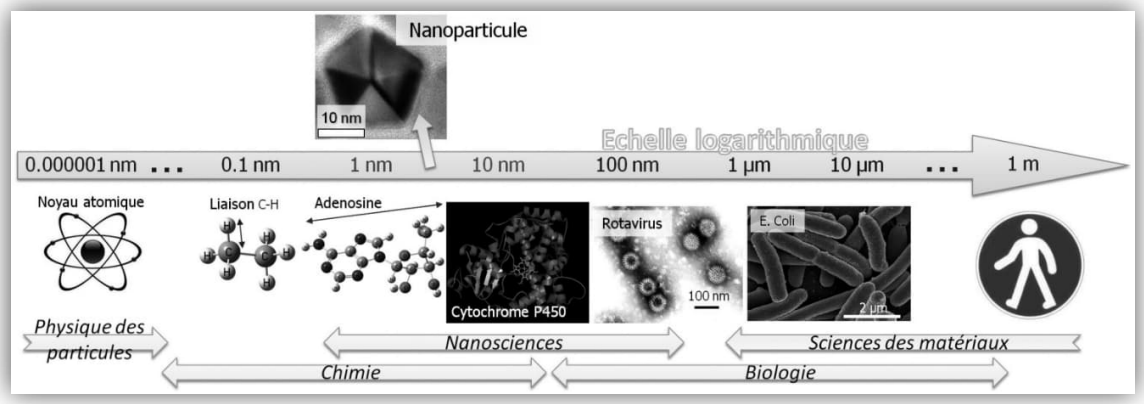


Figure 1: Spatial scales with the different aspects related to this technological revolution

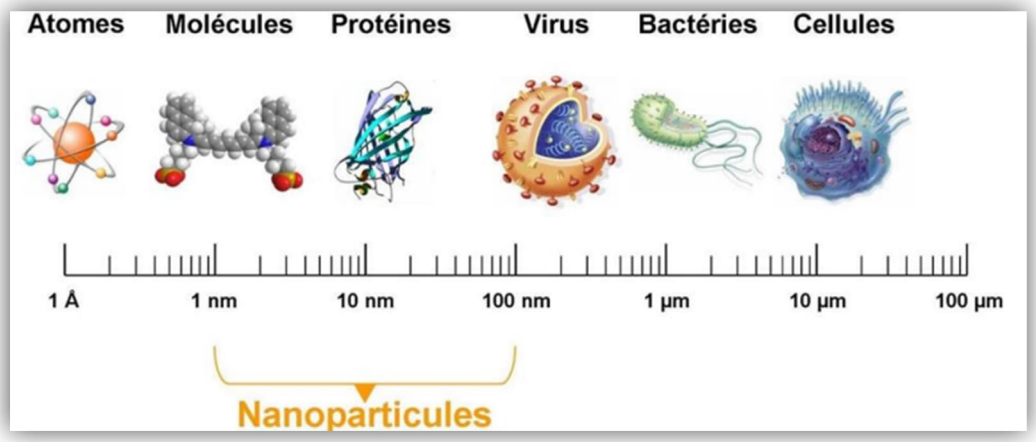


Figure 2: Size range of nanoparticles compared to major chemical and biological structures. (Mathieu Goutayer, 2008)

2. Silver Nanoparticles

Silver nanoparticles (Ag NPs) are among the most widely used nanomaterials in agricultural practices, typically ranging in size from 10 to 20 nanometers. Composed primarily of silver ions, these nanoparticles demonstrate high activity and efficacy within living systems, making them valuable for combating harmful microbes. Applications of Ag NPs include breaking seed dormancy, improving seed vigor index, and increasing the fresh weight of seedlings (Singh et al., 2021).

Due to their smaller size compared to microorganisms, Ag NPs can diffuse into cells, often disrupting cell walls—an effect observed through scanning electron microscopy (SEM) and transmission electron microscopy (TEM) imaging of nanoparticle suspensions interacting with pathogens (Siddiqi et al., 2018) Their significant antimicrobial efficacy spans bacteria, viruses, and other eukaryotic microorganisms, leading to their widespread use in agriculture, textiles, water treatment, and cosmetics such as sunscreens (Hasan, 2015)**.** Moreover, successful biosynthesis of Ag NPs has been reported using various plant species such as *Azadirachta indica*, *Capsicum annuum*, and *Carica papaya*.

2.1. Synthesis Methods

Nanoparticle synthesis can be approached through two main strategies: the "bottom-up" and "top-down" approaches (Figure 3). (**Yael N. Slavin and Horacio Bach, 2022 and Hongda Lu et al., 2020).**

The" bottom-up" method is generally preferred for nanoparticle production due to its ability to yield more homogeneous products with fewer defects. This approach utilizes catalysts such as reducing agents and enzymes to build nanostructures **.**Conversely, the "top-down" method starts with bulk materials, reducing their size to the nanoscale through techniques like thermal decomposition, mechanical milling, and spraying. However, this method often results in surface defects that can impair the physical properties and surface chemistry of the nanoparticles. (**Yael N. Slavin and Horacio Bach, 2022 and Hongda Lu et al., 2020).**

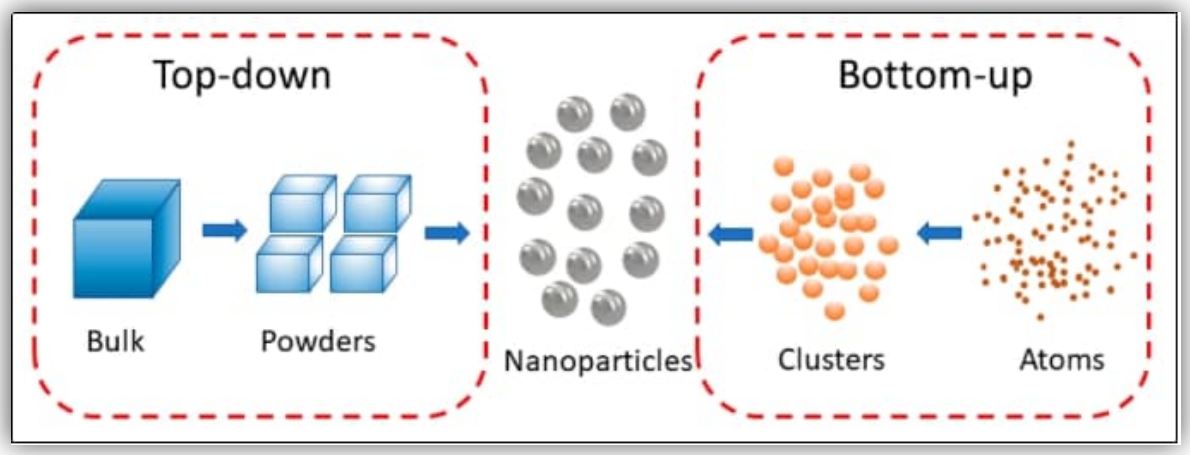


Figure 3 : the bottom-up and top-down approaches for the fabrication of nanoparticles. (Hongda Lu et al., 2020)

Various techniques exist for synthesizing silver nanoparticles (AGNPs), including chemical, physical, and biological methods (Siddiqi et al., 2018; Verma et al., 2023)**.**

2.1.1. ***Chemical synthesis;*** involves techniques such as chemical reduction, electrochemical methods, and pyrolysis, requiring metallic precursors.

2.1.2. ***Physical methods;*** including arc discharge and physical vapor condensation, tend to produce Ag NPs with a narrow size distribution but are often energy-intensive.

2.1.3. ***Biological synthesis;*** using plant extracts or microorganisms has emerged as a promising approach due to its simplicity, cost-effectiveness, and environmental friendliness. This method relies on natural plant components containing various biomolecules that act as reducing agents, producing nanoparticles without toxic byproducts and with generally lower toxicity than those generated by non-green methods.

2.1. Mechanism of Green Synthesis of Metallic Nanoparticles from Plant Extracts

Green synthesis of metallic nanoparticles (NPs) from plant extracts involves a highly coordinated, three-step mechanism: activation, growth, and termination. Each phase reflects specific chemical interactions between metal ions and plant-derived biomolecules, ultimately resulting in the formation of stable, shape-controlled nanoparticles (Khandel et al., 2018; Nasrollahzadeh et al., 2019)

2.2.1. Activation Phase: Ion Reduction and Nucleation

The process begins with the introduction of metal ions (e.g., Ag^{+}, Au^{3+}, Zn^{2+}) into a solution containing plant extracts. These ions, derived from metal salts, are highly reactive in their oxidized ionic form. Plant extracts are rich in diverse bio-reductants such as terpenoids (e.g., eugenol), flavonoids, polyphenols, alkaloids, sugars, phenolic acids, and proteins. These metabolites donate electrons or hydrogen atoms to the metal ions, effectively reducing them from their ionic state to their zero-valent metallic form (M^{0}). This chemical reduction step initiates nucleation, where the reduced metal atoms cluster together to form nanometric seeds—the initial cores of nanoparticles. (Figure 4) (Khandel et al., 2018; Nasrollahzadeh et al., 2019).

2.2.2. Growth Phase: Nanoparticle Formation and Morphological Evolution

Following nucleation, the process enters the growth phase. Additional reduced metal atoms deposit onto the existing seeds, and the nanoparticles begin to grow and evolve into distinct shapes (e.g., spheres, cubes, triangles, rods). Continued biological reduction of metal ions occurs as more biomolecules interact with remaining metal ions. The type and structure of the plant compounds heavily influence growth rate, shape, and size of the nanoparticles. This phase allows for size- and shape-controlled development, although uncontrolled nucleation may lead to aggregation or irregular morphologies. (Figure 4) (Khandel et al., 2018; Nasrollahzadeh et al., 2019)

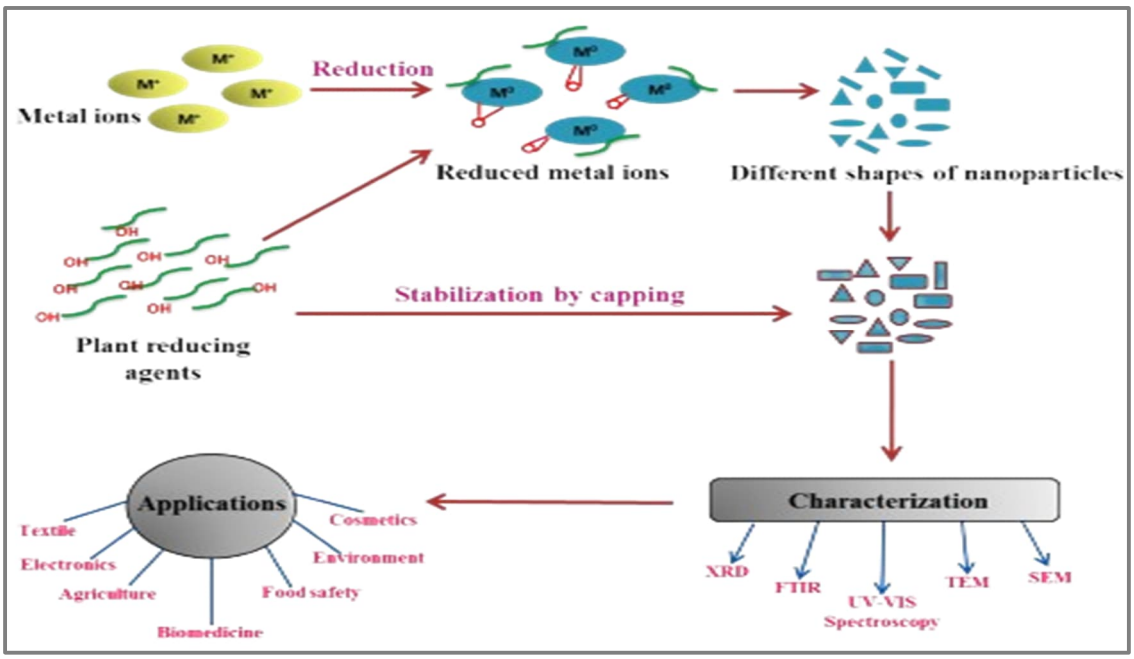


Figure 4: Schematic representation of the green synthesis of metal nanoparticles by plants: from reduction to application.. (Khandel et al., 2018)

2.2.3. Termination Phase: Capping and Stabilization

When nanoparticles reach their optimal size and shape, the termination phase begins. Here, plant metabolites act as capping and stabilizing agents. The nanoparticle surface binds with hydroxyl (–OH), carboxyl (–COOH), amine ($–NH_2$), and carbonyl (=O) groups from plant compounds. These biomolecules adsorb onto the nanoparticle surface, forming a protective organic shell that prevents aggregation and enhances

colloidal stability. The result is a stable suspension of plant-metabolite-capped metallic nanoparticles, ready for various applications. (Figure 4) (Khandel et al., 2018; Nasrollahzadeh et al., 2019)

2.2. Mechanism of Action

2.1.1. Antifungal Activity:

Silver nanoparticles (Ag NPs) exhibit antifungal properties through multiple mechanisms targeting fungal cells. A key aspect of their activity is their ability to disrupt fungal membrane integrity: Ag NPs adhere to and penetrate membranes, compromising membrane potential and causing cell death. This disruption impairs essential cellular functions such as budding and respiration, ultimately halting cell division. The increased intracellular release of silver ions enhances these antifungal effects (Figure 5) (Nasrollahi et al., n.d.)

Size is a critical factor affecting Ag NP efficacy; smaller nanoparticles generally display stronger antifungal activity due to their higher surface-to-volume ratio, facilitating better binding and diffusion to target sites. Exposure to Ag NPs causes significant alterations in fungal cells, including membrane damage, surface deformation, and loss of structural integrity, evidenced by increased intracellular vesicles and changes in organelle distribution. Additionally, smaller nanoparticles can enter fungal cells via fluid-phase endocytosis, causing intracellular damage such as mitochondrial fragmentation and chromatin disruption. Ag NPs also deform hyphal structures, leading to clumping and altered growth patterns (Slavin and Bach, 2022)

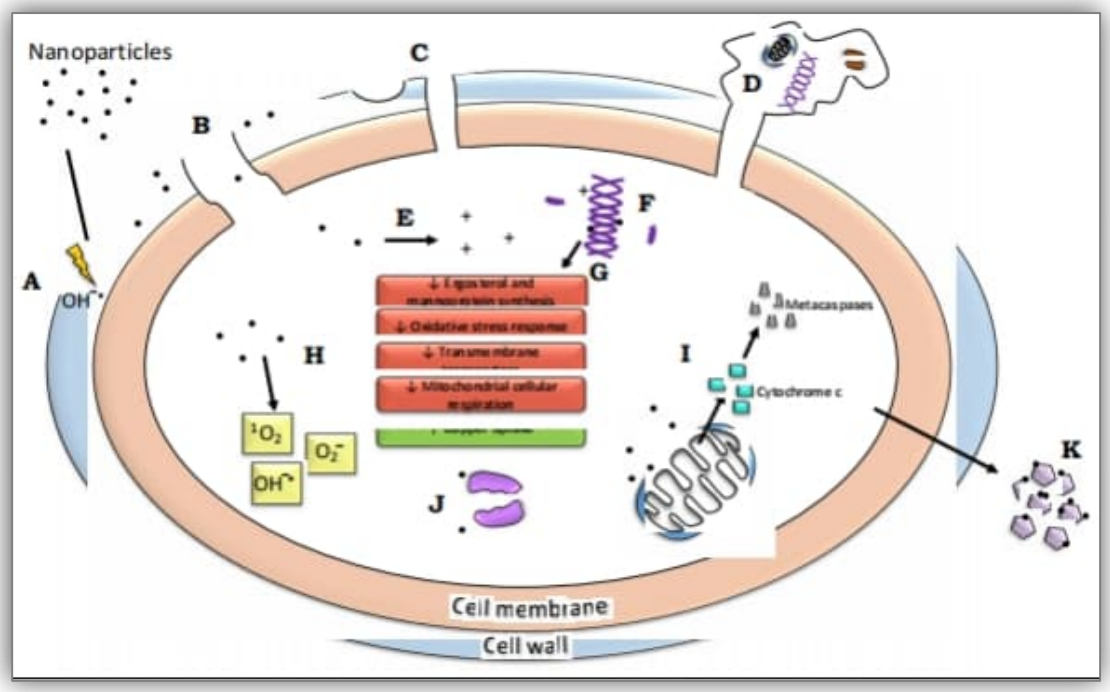


Figure 5. mecanisme of the anti-fungal acitivty of the nanoparticles (Slavin and Bach, 2022)

2.1.2. Cytotoxicity:

Ag NPs demonstrate a tendency to envelope and penetrate microbial cells, leading to the inhibition of vital functions. Cytotoxic effects are largely attributed to the generation of reactive oxygen species (ROS), which deplete glutathione levels and impair antioxidant defenses. Ag NPs also interfere with enzymatic activities, such as lactate dehydrogenase (LDH), and can cause DNA damage. Their small size allows adherence to bacterial cell walls, restricting nutrient flow and ultimately leading to microbial death. The greater surface area of smaller nanoparticles enhances their antibacterial efficacy by enabling better contact with microbial surfaces, penetrating cell membranes, disrupting cell walls, denaturing proteins, and causing cell death (Siddiqi et al., 2018)

2.1.3. Antibacterial Effects:

Metallic nanoparticles exert antibacterial effects through multiple mechanisms, including the induction of oxidative stress, interaction and permeabilization of cellular membranes, release of metal ions, and interaction with proteins and/or DNA. The release of metal ions from nanoparticles contributes to bacterial cell death by depleting

ATP and destabilizing membranes—a process driven by reactive oxygen species (ROS) generated during nanoparticle dissolution (Vernain, n.d.)

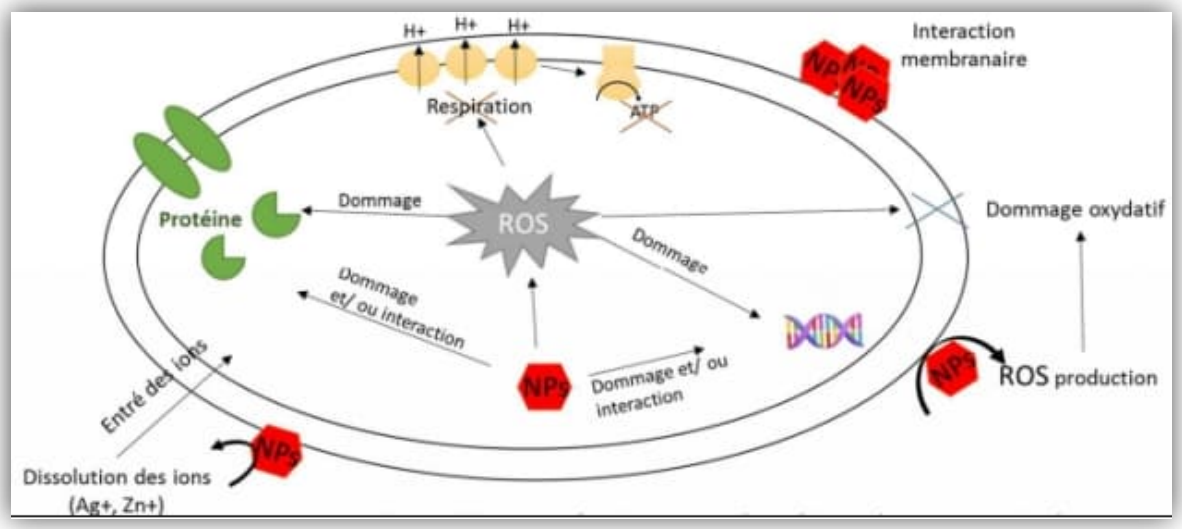


Figure 6. Nanoparticles anti-bacterial activity mechanism (Mijnendonckx et al., 2013)

3. Nanoparticles Incorporated with Essential Oils Used as Control Agents

The integration of essential oils (EOs) into nano-carriers represents a significant advancement in the development of more effective and sustainable control solutions across various sectors. While EOs are known for their antimicrobial, insecticidal, and antioxidant properties, they are also susceptible to degradation, volatility, and low water solubility (Scarlette Lizeth Recio-Cázares et al., 2023)

Incorporating EOs into nanoparticles (NPs) helps protect active compounds, enhances their stability and bioavailability, and allows for targeted delivery, thereby amplifying their potential as control agents.

3.1. Control of Phytopathogens

One of the most promising applications of EO-loaded NPs is the control of bacteria, fungi, and viruses. Encapsulating EOs in nanoemulsions, polymeric nanocapsules, or lipid nanoparticles (such as SLNs and NLCs) significantly enhances their antimicrobial activity compared to free EOs (AMMAR et al., 2023; DIMA et al., 2022; HAMED et al., 2022**).** These nano-carriers facilitate the penetration of active compounds through microbial cell membranes, thereby increasing their efficacy (AL-NEMARI et al., 2024)

For example, silver or gold nanoparticles synthesized via green methods using EOs combine the antimicrobial properties of metallic nanoparticles with the phytochemical compounds of the EOs, providing enhanced antimicrobial effects against a wide range of pathogenic strains (ALI et al., 2023) In agriculture, encapsulating EOs in NPs improves the control of plant diseases caused by fungi, bacteria, or viruses by increasing the persistence and effectiveness of EOs on crops (Javanmardi et al., 2023). This results in long-lasting protection against phytopathogens while minimizing environmental impact.

3.2. Pest Control

EO-loaded NPs are increasingly being explored as eco-friendly alternatives to synthetic chemical pesticides and insecticides. Nano-encapsulated EOs have proven effective as insecticides, larvicides, and repellents. They extend the action of EOs, reduce their volatility, and increase their lethality against pests such as mosquitoes (e.g., *Aedes albopictus*) and other parasites, offering safer solutions for public health and the environment (Parichanon et al., 2025; RABY et al., 2023)

3.3. Control of Food Spoilage

Incorporating EOs into NPs is a promising strategy for food preservation and spoilage prevention. EO-loaded NPs can be incorporated into active packaging films or directly added to food products. Their antioxidant and antimicrobial actions inhibit the growth of spoilage-causing microorganisms (bacteria and fungi) and prevent lipid oxidation, thereby extending the shelf life of food products (GHASEMI et al., 2023; RABY et al., 2023). This application helps reduce food waste and enhances food safety.

➢ CHAPTER II: Aloysia citrodora (Lemon Verbena): A Versatile Plant with Ancient and Modern Uses

1. General Overview

Aloysia citrodora Palau, more commonly known by its vernacular names such as Lemon Verbena, Sweet Verbena, Louisa Herb, or Tea Verbena, is a perennial shrub that is deciduous or semi-evergreen, belonging to the family *Verbenaceae* (which also includes *Verbena officinalis*, although they are distinct genera). Native to the temperate regions of South America—particularly Argentina, Chile, and Peru—it was introduced to Europe in the 18th century, where it quickly gained popularity for its distinctive fragrance (Polumackanycz et al., 2022). In Peru, the plant is used in the production of the national soft drink *Inca Kola*, which is the top-selling carbonated beverage in the country (DORE, et al., 2020)**.**

The leaves of *Aloysia citrodora* are its most valued part. Emitting a strong lemon scent, they are widely used in herbal teas, cooking, and medicinal applications. These leaves are rich in a wide array of phytochemicals responsible for both their aromatic and therapeutic properties. The essential oil, extracted primarily from the leaves, is dominated by monoterpenes and sesquiterpenes, notably citral (including both neral and geranial, which give it the lemony scent), limonene, and caryophyllene. Beyond essential oils, the plant contains a wealth of phenolic compounds, including flavonoids (such as luteolin and apigenin), phenylpropanoids (especially verbascoside, also known as acteoside), iridoids, triterpenes, and mucilages (AMMAR et al., 2023; Scarlette Lizeth Recio-Cázares et al., 2023) These bioactive constituents provide *Aloysia citrodora* with a broad spectrum of pharmacological properties supported by modern scientific research. It is especially noted for its potent antioxidant effects, attributed to its high content of phenolic compounds, which help neutralize free radicals and reduce oxidative stress. Studies have also demonstrated its anti-inflammatory, anxiolytic, and sedative properties, making it useful for alleviating stress, insomnia, and anxiety. Furthermore, it is both traditionally and scientifically recognized for its digestive benefits, aiding in the relief of bloating, gastrointestinal spasms, and indigestion (AMMAR et al., 2023; Polumackanycz et al., 2022)

2. Distribution and Habitat of Aloysia citrodora

3.1. Global Distribution

Aloysia citrodora, commonly referred to as Lemon Verbena, is a perennial shrub whose natural range is centered in the Andean regions of South America, primarily including Argentina (especially the northwest), Bolivia, Paraguay, Chile, and Peru (Todorović et al., 2023) **(Figure 7).**

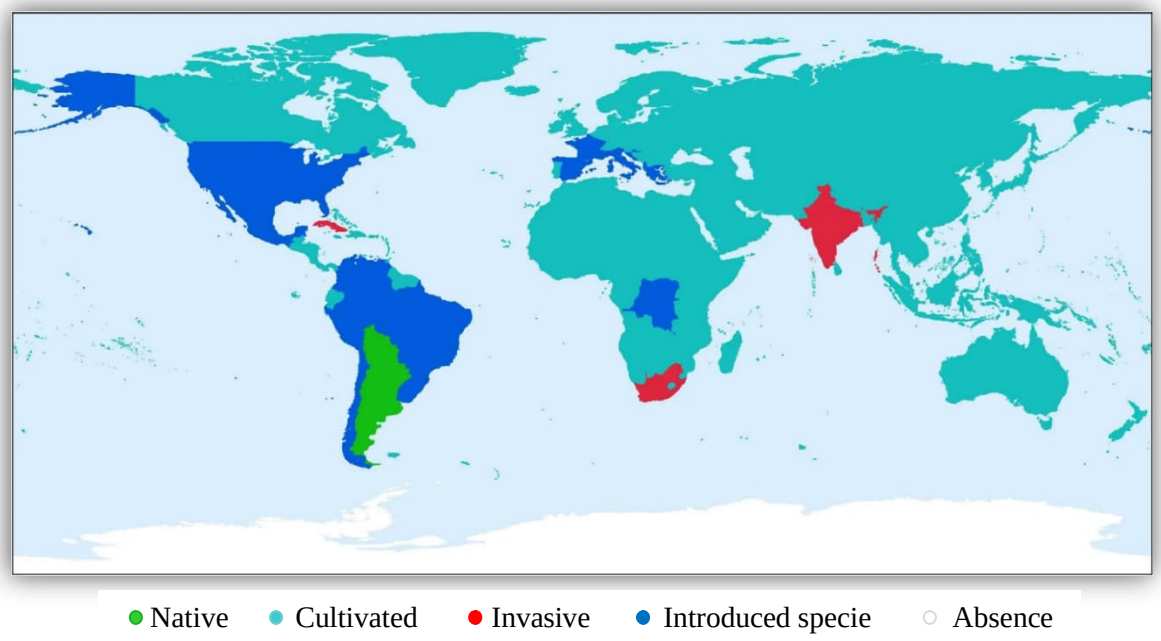


Figure 7 . global Geographical distribiution of *Aloysia citrodora.* (Anonyme 01, 2025)

Due to its highly prized aromatic and medicinal qualities, *Aloysia citrodora* has been widely introduced and cultivated in many temperate and subtropical regions around the world.It is now found cultivated—or occasionally naturalized—in Europe (particularly the Mediterranean Basin, including France, Spain, and Greece), North Africa (notably Algeria and Morocco), Asia (China, India), various parts of the Americas, and even Australia (Anonyme 02, 2025; Molla, A et al., 2024)**.** This global expansion is largely driven by increasing consumer demand for its fragrant leaves used in herbal infusions and aromatherapy **(Figure 7).**

As for its preferred habitat, *Aloysia citrodora* generally thrives in sunny, warm environments. It adapts to a variety of soils, though it prefers well-drained, light, fertile, and moist soils with a neutral to slightly acidic or alkaline pH. While it can grow in

semi-arid habitats in its native Andean range, it also tolerates more humid environments provided proper drainage is ensured (Anonyme 03, 2025; Rojas et al., 2010)**.** Being sensitive to frost, it is often cultivated as an annual or brought indoors during winter in climates where temperatures fall below 0°C (Anonyme 04, 2025). It is commonly found in open fields, dry scrublands, and along roadsides, indicating a certain capacity to establish in disturbed environments.

2.3. In Algeria

Although native to South America, Lemon Verbena (*Aloysia citrodora*) is a cultivated and naturalized plant in several regions, particularly due to its adaptability to Mediterranean climates and its high aromatic and medicinal value (Aouadi, S. et al., 2017)**.** It is commonly encountered in private gardens, herbalist shops, and occasionally even cultivated fields, reflecting its successful establishment driven by local demand.

The climatic and soil conditions in Algeria—characterized by hot, dry summers and mild winters in coastal and Tell regions—are favorable for its growth. *Aloysia citrodora* thrives particularly in well-drained, sunlit soils typical of Mediterranean zones. Its cultivation is observed in areas such as the Mitidja plain, the Algiers coast, and even in certain parts of the High Plateaus such as tellian atlas where conditions are suitable and irrigation is available. Although the plant is tolerant to heat, it is sensitive to prolonged drought (Benchaâbane, A. et al., 2018). Its presence in Algeria is usually the result of intentional introduction for its varied uses rather than spontaneous, widespread distribution as a native species.(figure 8)

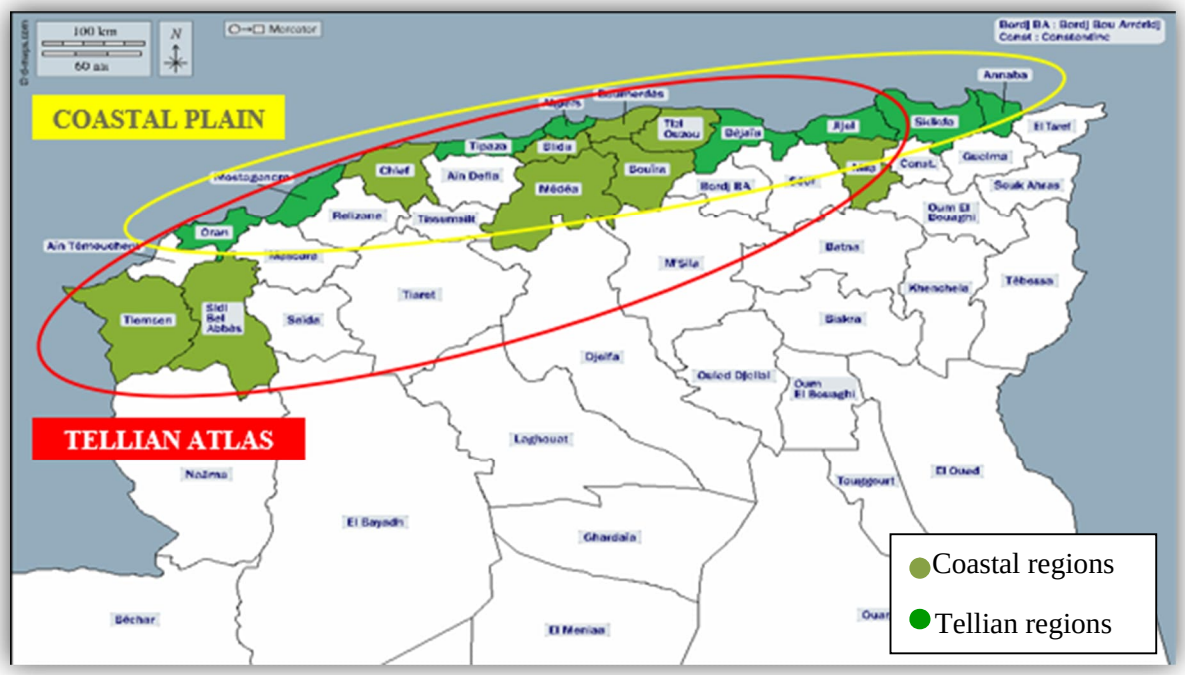


Figure 8. distripution of aloysia cintrodora in north algeria ("North Algeria," 2025)

3. Botanical Description

Aloysia citrodora **(Figure 9),** often described as a ruderal species due to its distribution across various regions of the world, is known by many names that reflect its "magical" reputation **(Rojas-Sandoval, 2019).**

▸ Taxonomic Classification

- **Domain** : Eukaryota
- **Kingdom** : Plantae
- **Phylum** : Spermatophyta
- **Subphylum** : Angiosperms
- **Class** : Dicotyledons
- **Order** : Lamiales
- **Family** : Verbenaceae
- **Genus** : *Aloysia*
- **Species** : *Aloysia citrodora*

Other Scientific Names (Synonyms):

1. *Aloysia citridora* Paláu.
2. *Aloysia sleumeri* Moldenke.
3. *Aloysia triphylla* Britton.
4. *Aloysia triphylla* f. *serrulata* Moldenke.
5. *Cordia microcephala* Willd. ex Roem &Schult.
6. *Cordia microcephala* Willd., 1819.
7. *Lippia citriodora* (Lam.) Kunth.
8. *Lippia citrodora* (Palau) Kunth.
9. *Lippia triphylla* Kuntze
10. *Lithocardium microcephalum* Kuntze
11. *Verbena citriodora* (Lam.) Cav.
12. *Verbena citrodora* (Palau) Cav.
13. *Verbena fragrans* Salisb.
14. *Verbena triphylla* L'Hér.
15. *Zappania citriodora* Lam.
16. *Zappania citrodora* (Palau) Lam

Common Name: Lemon Verbena

Common Names in Other Languages:

- **English**: Lemon bush, lemon-scented verbena.
- **Spanish**: Cedrón, cidrón, hierba Luisa, verbena.
- **French**: Verveine citronnelle.
- **Portuguese**: Cidreira, salva-limão.

Local Names:

- **Cuba**: Yerba Luisa.
- **Algeria** : Luisa.
- **Morocco**: Lwiza roumya.



Figure 9. Leaves and flowers of Aloysia citrodora in the region of Mitidja (original picture taken by the farm owner 25/10/2025)

Aloysia citrodora is a branched, perennial shrub that can reach a height of 1 to 3 meters when cultivated. Its bright green leaves are lanceolate to oblong-lanceolate, typically measuring 3 to 10 cm in length. They are arranged in whorls of three (rarely four) around the stem. When crushed, the leaves release a strong and pleasant lemon aroma—a key identifying trait of the plant. Flowering occurs in summer, producing small white to pale lilac flowers **(Figure 9)** clustered in loose, elongated terminal spikes **(**(Ríos-Chauca, M. R et al., 2024)**).**

4. Phytochemical Properties and Therapeutic Effects of Aloysia citrodora

2.4. Biological Properties of Lemon Verbena

The aromatic and medicinal properties of *Aloysia citrodora* are intrinsically linked to its rich composition of phytochemical compounds with therapeutic effects. These bioactive molecules, identified particularly in the leaves and essential oil, can be grouped into several chemical families, each with specific structures that confer distinct biological functions (Figure 10).

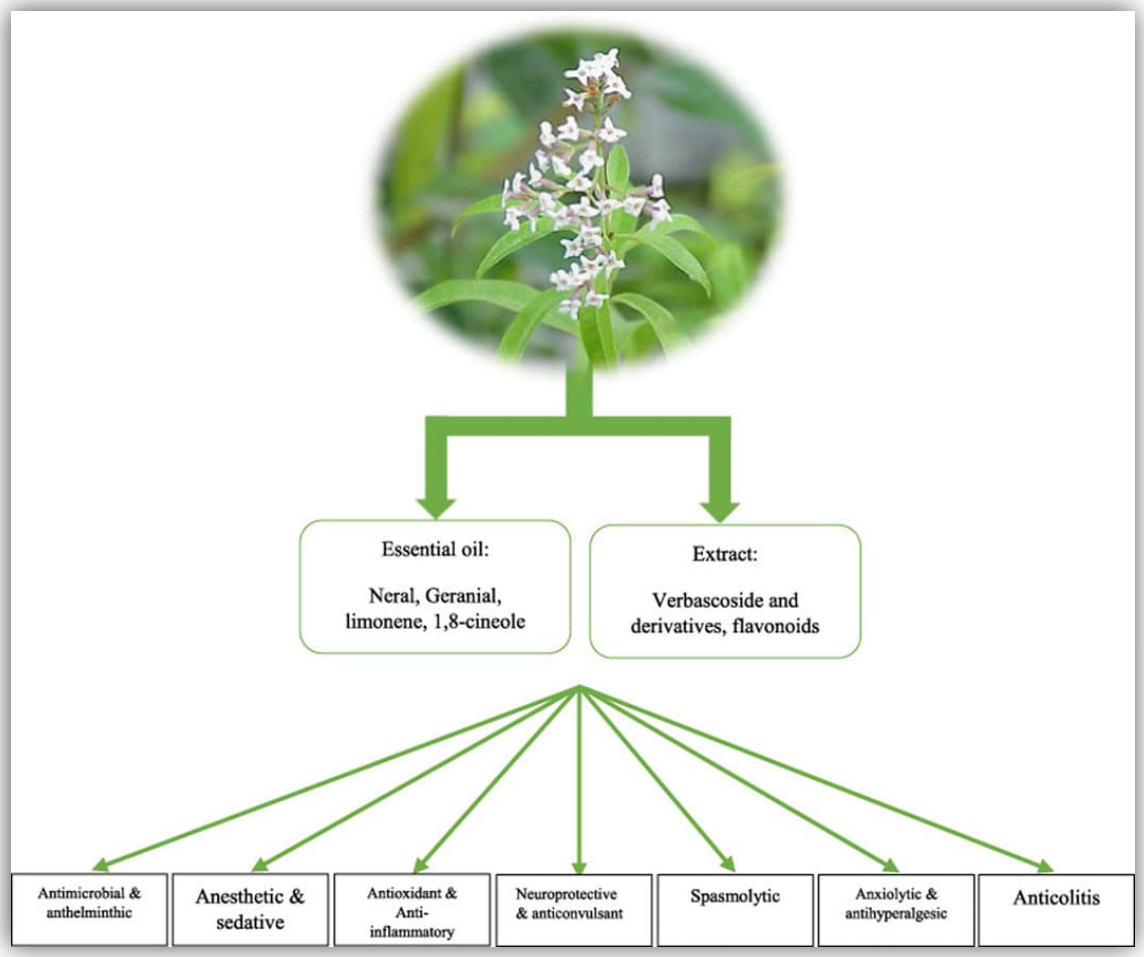


Figure 10. Propriétés biologique et Effets Thérapeutiques d'*Aloysia citrodora* (Bahramsoltani, R. et al., 2018)

4.1.1. Terpenes (Essential Oil)

The characteristic lemon scent of *Aloysia citrodora* essential oil (EO) is primarily due to monoterpenes, volatile molecules composed of 10 carbon atoms. These compounds are largely responsible for the plant's properties. The EO is mainly made up of volatile monoterpenes, with citral—a mixture of aldehyde isomers (geranial or trans-citral and neral or cis-citral)—as the dominant component (AMMAR et al., 2023; Gil, A. et al., 2007; Ríos-Chauca, M. R et al., 2024). Other terpenes include limonene (a cyclic monoterpene with six carbons), as well as sesquiterpenes (with fifteen carbon atoms) such as spathulenol (a bicyclic oxygenated sesquiterpene), caryophyllene and its oxide (bicyclic sesquiterpenes), and 1,8-cineole (a bicyclic monoterpene oxide), though their

proportions can vary depending on environmental and genetic factors **(Polumackanycz et al., 2022; Ali et al., 2008; Sprea et al., 2023).**

❖ Therapeutic Properties and Effects:

- Antimicrobial and Antifungal**:** Citral and other terpenes give the EO significant antibacterial (Dos Santos et al., 2017; Sprea, RM et al., 2023) and antifungal activity against a broad range of microorganisms, including gastrointestinal and genitourinary pathogens (AMMAR et al., 2023; Rojas et al., 2010), and have also been effective against *Aeromonas hydrophila* in fish (Dos Santos et al., 2017) .
- Anti-inflammatory**:** Certain EO terpenes contribute to anti-inflammatory potential **(**(AMMAR et al., 2023; Sprea, RM et al., 2023)
- Anxiolytic and Sedative**:** Traditionally used for its calming effects, the EO has demonstrated pharmacological and neuroprotective profiles, helping alleviate agitation and **insomnia** (Abuhamdah, S. et al., 2015; Rashid, Hasan M. et al., 2022; Sprea, RM et al., 2023) Ethanolic extracts have also shown effects on neuropathic pain (Amin, B. et al., 2018).
- Insecticidal/Larvicidal**:** The EO exhibits insecticidal and larvicidal properties (Ali et al., 2008; Parichanon et al., 2025)**.**

4.1.2. Phenylpropanoids

These compounds, derived from aromatic amino acids, are essential for the antioxidant and anti-inflammatory activities of lemon verbena. Verbascoside (also called acteoside, a glycosylated phenylethanoid heteroside) is the most abundant and well-studied phenylpropanoid in *Aloysia citrodora* extracts. Other related compounds like isoverbascoside, eukovoside, and martinoside share similar structures with variations in their sugar or phenolic groups (Sánchez-Marzo et al., 2019; Tammar et al., 2021; Ammar et al., 2023).

❖ Therapeutic Properties and Effects:

- Powerful Antioxidants**:** Verbascoside is a strong antioxidant, vital in scavenging free radicals and reducing oxidative stress (Polumackanycz et al.,

2022; Rashid, Hasan M. et al., 2022; Sánchez-Marzo, N et al., 2019)**.** Its activity is especially evident in infusions **(**(Peixoto, J. A. B et al., 2023).

- Anti-inflammatory**:** Verbascoside contributes to the plant's anti-inflammatory properties (AMMAR et al., 2023; Sprea, RM et al., 2023)**.**
- Neuroprotective**:** Studies have highlighted a neuroprotective profile potentially linked to these compounds (Abuhamdah, S. et al., 2015).
- Effects on Colonic Inflammation**:** The bioavailability of lemon verbena polyphenols, including phenylpropanoids, has been studied for their impact on colonic inflammation (Felgines, et al., 2014)**.**

4.1.3. Flavonoids

- Flavonoids, another class of polyphenols in *Aloysia citrodora*, primarily include flavones and flavonols, often as glycosides (sugar-bound forms). Compounds like luteolin, apigenin, and chrysoeriol, typically present as glycosides (e.g., diglucuronides), significantly contribute to the plant's antioxidant activity (Bilia, A. R. et al., 2008; Peixoto, J. A. B et al., 2023; Quirantes-Piné et al., 2009).

❖ Therapeutic Properties and Effects:

- Antioxidants: These flavonoids strongly enhance the plant’s antioxidant capacity (Aldeen et al., 2015; Peixoto, J. A. B et al., 2023)**.**
- Anti-inflammatory: They also exhibit notable anti-inflammatory properties (Rashid, Hasan M. et al., 2022; Sprea, RM et al., 2023)

- Anxiolytic and Sedative: Some flavonoids may support the plant’s calming effects (Maliki, I. et al., 2021)**.** This is one of the EO's most renowned features—widely used to ease nervous tension, anxiety, mental fatigue, and sleep disorders like insomnia (Abuhamdah, S. et al., 2015)**.** Its soft lemony scent creates a relaxing atmosphere conducive to sleep. Studies suggest a neuroprotective profile and emotional regulation potential (Abuhamdah, S. et al., 2015; Bahramsoltani, R. et al., 2018)

4.1.4. Other Compounds

In addition to the major classes above, *Aloysia citrodora* contains other phytochemicals in smaller amounts, including lignans, tannins, mucilages, and iridoids. The synergy and interaction among these compounds contribute to the plant's extensive therapeutic profile and distinct aroma (Rashid, Hasan M. et al., 2022).

❖ General Therapeutic Effects:

- Digestive Aid**:** Lemon verbena infusions are traditionally used to relieve indigestion, bloating, cramps, and diarrhea (Bahramsoltani, R. et al., 2018; Felgines, et al., 2014; Rashid, Hasan M. et al., 2022; Rezig, L. et al., 2019)**.**
- Diuretic and Antispasmodic**:** Widely used in the pharmaceutical industry for its diuretic and antispasmodic properties (Bahramsoltani, R. et al., 2018; Felgines, et al., 2014; Rashid, Hasan M. et al., 2022).
- Anticancer and Antiproliferative**:** *Aloysia citrodora* has shown anticancer and antiproliferative activity in vitro and in vivo, including effects on gene expression in cancer cells and reduced cell viability **(**(AMMAR et al., 2023; Peixoto, J. A. B et al., 2023)**,** attributed to its EO and active constituents (Rashid, Hasan M. et al., 2022; Sprea, RM et al., 2023)**.**
- Immunomodulatory**:** Immunomodulatory properties have also been suggested (Gil, A. et al., 2007)

5. Physicochemical Properties of Lemon Verbena Essential Oils

The essential oils (EOs) of lemon verbena, primarily extracted from *Aloysia citrodora* (or *Lippia citriodora*), are defined by their physicochemical characteristics, directly tied to their volatile chemical composition. These EOs are clear, pale to dark yellow liquids with a fresh, sweet, and intensely lemony scent. They typically have a density ranging from 0.870 to 0.920 g/cm³ and a refractive index between 1.470 and 1.500 at 20°C. They are lipophilic—insoluble in water but soluble in organic solvents and vegetable oils. Since they are sensitive to oxidation from air and light, proper storage is essential to preserve their integrity and therapeutic qualities.

Lemon verbena EO contains around 40 different compounds, giving it a diverse range of properties. Its composition can vary significantly due to genetic, geographical, and

environmental factors, which must be considered during identification and quality control (Gil, A. et al., 2007; Parodi, T. V. et al., 2020).

➢ CHAPTER III :Salvia Sclarea (Sage)

1. Generality :

Salvia sclarea L., commonly known as *clary sage*, is a biennial or short-lived perennial herbaceous species native to Southern Europe. Taxonomically, it belongs to the *Aethiopis* section of the *Lamiaceae* family ((DANAE PITAROKILI et al., 2002)). Due to its extensive cultivation in countries such as France, Russia, and the United States, *S. sclarea* has gained considerable commercial significance worldwide, primarily for the high-value essential oil it produces. This oil is widely used in the perfume, cosmetics, and food industries (Kacˇániová, M et al., 2023; Pitarokili, D. et al., 2002).

The plant is especially prized for its highly aromatic essential oil, mainly extracted from its inflorescences. This oil is distinguished by its sweet, herbaceous fragrance, reminiscent of ambergris (Sharifi-Rad et al., 2018). The history of *Salvia sclarea* as a cultivated herb dates back centuries, with its medicinal uses documented as early as the 4th century BCE by Theophrastus and in the 1st century CE by Dioscorides and Pliny the Elder. The generic name *Salvia* is derived from the Latin word *salveo,* meaning “to save or to heal,” reflecting the traditional medicinal attributes of plants in this genus. It was historically used to flavor muscat wines and beers, earning it the nickname "muscatel sage." Its seeds were also used in traditional medicine to remove foreign particles from the eye (Huxley et al., 1992). Today, *Salvia sclarea* is widely cultivated for its essential oil, with significant production in regions such as Bertie County in northeastern North Carolina, USA.

From a phytotherapeutic perspective, *S. sclarea* has a broad range of traditional medicinal uses. Infusions made from its leaves and flowers have been traditionally used to treat ailments such as sore throats, gynecological disorders, ulcers, digestive issues, kidney diseases, coughs, colds, blood purification, wounds, irritated eyes, dysentery, and bloody diarrhea ((Sharifi-Rad et al., 2018; Teresa Tuttolomondo et al., 2020). The essential oil is especially valued for its anticonvulsant, anxiolytic, and anti-

inflammatory properties (Abasi et al., 2022; Abdel-Massih and Beyrouthy, 2022; Sharifi-Rad et al., 2018). It is also used to relieve menstrual discomfort, nervous fatigue, insomnia, and stress-related conditions, often through aromatherapy applications (Sharifi-Rad et al., 2018; Abdel-Massih & Beyrouthy, 2022). Modern pharmacological studies have also highlighted its potential antibacterial, antifungal, antiviral, antioxidant, anticancer, and antidiabetic activities (E.Yuce et al., 2014; Kacˇániová, M et al., 2023) Chemically, the therapeutic and aromatic qualities of clary sage essential oil are primarily attributed to constituents such as linalyl acetate, linalool, and sclareol. These compounds contribute not only to its physiological effects but also to its appeal in perfume and cosmetic formulations due to their fragrance profiles and fixative properties ((Sharifi-Rad et al., 2018)). The oil is also found in a variety of products, including soaps, lotions, and even tobacco (Sharifi-Rad et al., 2018). In culinary traditions, the leaves and flowers are used to prepare herbal teas and sometimes serve as flavoring agents in liqueurs and food products ((Aćimović, M. et al., 2018; Huxley et al., 1992).

Recent research has expanded the scope of *S. sclarea* to agricultural and environmental sectors. Its essential oil has been studied as a natural preservative and bioinsecticide, while the plant's ability to grow in contaminated soils suggests potential usefulness in phytoremediation efforts (Sharifi-Rad et al., 2018). With a rich history rooted in traditional medicine and a growing array of industrial applications, *Salvia sclarea* remains a significant subject of pharmacological, ecological, and commercial interest.

2. Distribution and Habitat of Salvia sclarea

2.1. Global Distribution

Its primary geographic origin lies in the northern Mediterranean basin, with its natural range extending into adjacent regions of North Africa and Western Asia. *Salvia sclarea* is native to a broad range of countries, including Afghanistan, Albania, Algeria, Bulgaria, Corsica, Cyprus, France, Greece, Iran, Italy, Kazakhstan, Kyrgyzstan, Crimea, the Levant (Lebanon-Syria), the North Caucasus, Palestine, Portugal, Romania, Sardinia, the Sinai Peninsula, Spain, Tajikistan, Transcaucasia, Tunisia, Turkmenistan, Turkey, Ukraine, Uzbekistan, and the western Himalayas (Govaerts, R., 2025)*(Figure 11)*

Ecologically, clary sage exhibits remarkable ecological plasticity. It thrives in dry, well-drained soils typical of sunny coastal, submontane, and mountainous environments, often on loose rocky or calcareous substrates. It is commonly found on dry slopes, hilly terrains, and in anthropogenic habitats such as meadows, fields, roadsides, and pastures (Govaerts, R., 2025)). Its adaptability to various climates allows it to spread across temperate and subtropical regions, especially in the Mediterranean and Central Europe.

Globally, *S. sclarea* has been widely cultivated and naturalized beyond its native range, particularly in the United States, where it spans from the northeast to the west coast, and in regions like Australia and parts of Central Europe. In some countries, particularly in North America, it is considered invasive and classified as a noxious weed due to its potential to displace native flora (Govaerts, 2025).

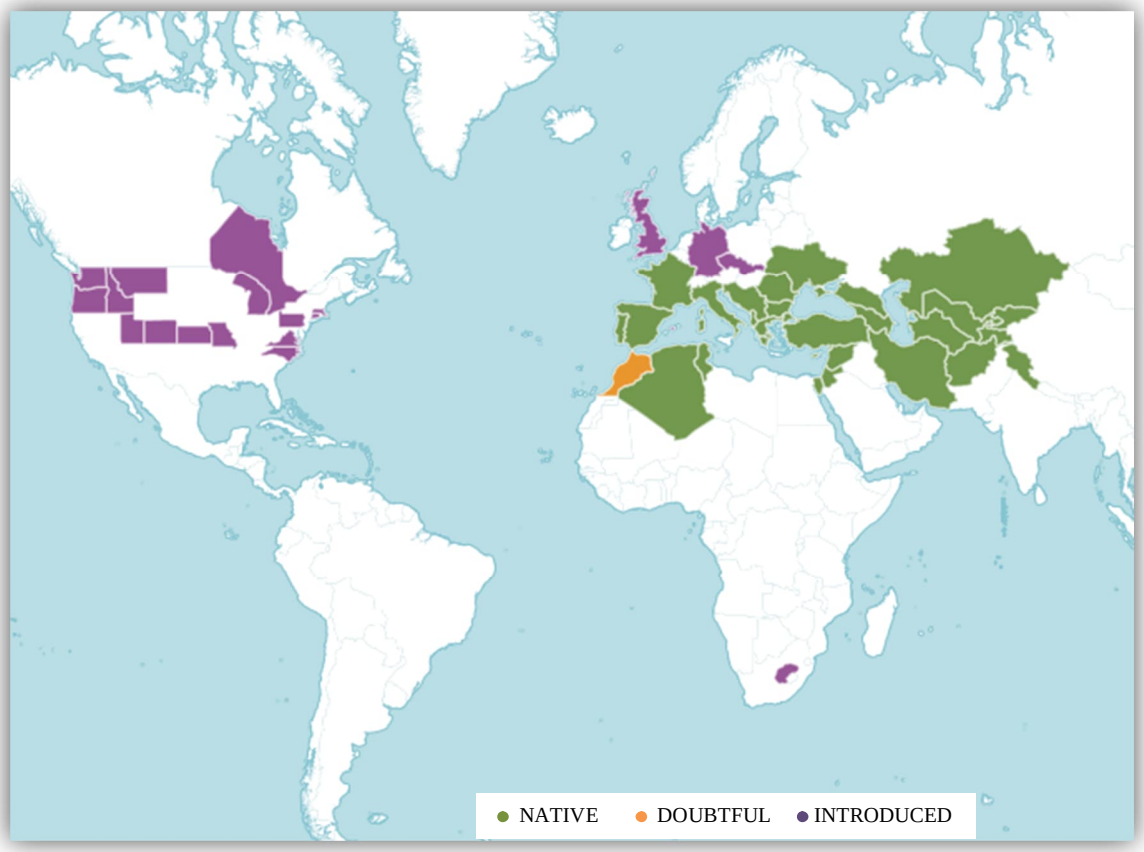


Figure 11. worldwide distribution of Salvia sclarea L. (Govaerts, R., 2025)

2.2. In Algeria

In Algeria, *Salvia sclarea* is part of both the wild and cultivated flora, benefiting from the Mediterranean climate that favors aromatic and medicinal plants. Its distribution in

Algeria falls within its natural range, which includes North Africa (Govaerts, 2025). Algeria, with its varied climates and terrains (Mediterranean to Saharan), hosts many *Salvia* species. It is noted that the *Salvia* genus includes 23 recorded species in Algeria (Quezel, P. and Santa, S., 1963). Specifically, clary sage adapts to sunny coastal, submontane, and mountainous environments, often on rocky or calcareous soils. It is also present in arid regions with dry, well-drained soils. While a detailed distribution map of *Salvia sclarea* in Algeria was not available in our search, its presence as a native species is confirmed (Govaerts, R., 2025), and it is cultivated and found growing wild in areas such as Tlemcen and other mountainous or semi-arid zones.

3. Botanical Description

Salvia sclarea is a biennial or short-lived perennial species. It is known by several names (Govaerts, R., 2025):

▸ Taxonomic Classification (Govaerts, R., 2025)

- **Domain :** Eukaryota
- **Kingdom :** Plantae
- **Phylum :** Streptophyta
- **Class :** Equisetopsida
- **Sub-class :** Magnoliidae
- **Order :** Lamiales
- **Family :** Lamiaceae
- **Genus :** *Salvia*
- **Species :** *Salvia sclarea*

▸ Synonyms:

According to botanical databases such as Plants of the World Online ((Govaerts, R., 2025)) from Kew Science, which is a key reference, the synonyms of Salvia sclarea L. include:

➢ Homotypic synonyms:

1. *Aethiopis sclarea* (L.) Opiz
2. *Sclarea vulgaris* Mill.

➢ Heterotypic synonyms:

1. *Salvia altilabrosa* Pau.
2. *Salvia calostachya* Gand.
3. *Salvia coarctata* Vahl.
4. *Salvia haematodes* Scop. (Nom. illeg.)
5. *Salvia lucana* Cavara & Grande.
6. *Salvia pamirica* Gand.
7. *Salvia sclarea var. calostachya* (Gand.) Nyman
8. *Salvia sclarea var. hispanica* Gavioli
9. *Salvia sclarea var. turkestaniana* Mottet
10. *Salvia simsiana* Schult.
11. *Salvia turkestanica* Noter

▸ Common Names:

- **French** : Sauge Sclarée, Sclarée, Sauge Muscat, Orvale.
- **English** : Clary Sage, Muscatel Sage.
- **Spanish** : Esclarea, Salvia Romana, Hierba de los Ojos (“eye herb”).
- **Portuguese** : Esclareia, Salva-esclareia.

▸ Local Name (Algeria):

- **Algeria (Arabic):** *Maramia* (مريمية) – although this more frequently refers to *Salvia officinalis*, it can also describe other cultivated or wild *Salvia* species, including *clary sage*, due to their similar appearance and uses. In Kabyle, it is sometimes referred to as *Timijja* or *Tizeggilt*, though further research is needed to confirm usage for *S. sclarea*.
- **Morocco:** *Salmia* (سالمية)

Salvia sclarea (family *Lamiaceae*) is an herbaceous biennial or short-lived perennial, reaching 0.6–1.2 m in height, and occasionally over 1.5 m under optimal conditions. Its root system consists of a tapered taproot that extends 15–70 cm into the soil, providing anchorage and nutrient uptake (Özdemir, F. and Şenel, S., 1999). During the first year, it develops a basal rosette of large, velvety, aromatic leaves; in the second year it sends up a flowering stem.

The stems (figure 12.,B) are upright, quadrangular, and typically 50–120 cm tall. They are densely hairy, especially toward the base, and often bear glandular hairs that secrete essential oils, lending the plant its characteristic balsamic scent (Aćimović, M. et al., 2018; Peana, A. T. and Moretti, M. D., 2002)

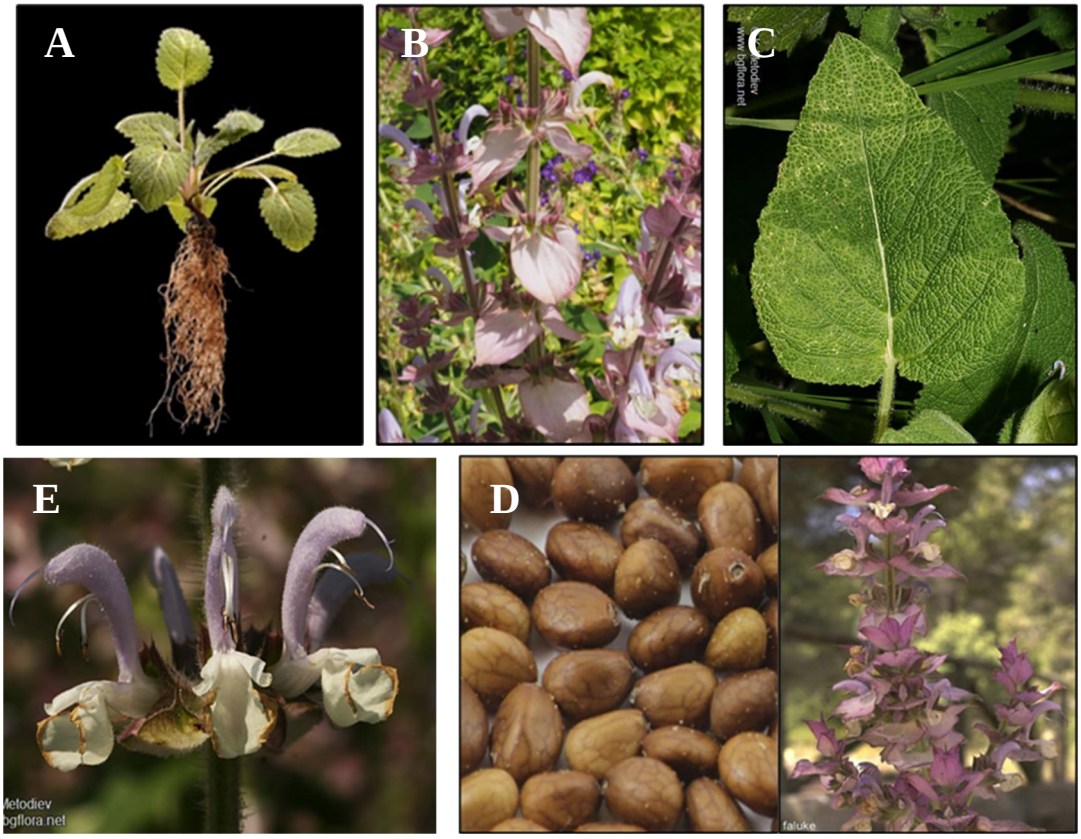


Figure 12. the different parts of S. sclarea ((Özdemir, F. and Şenel, S., 1999; "Salvia sclarea. (Informations générales sur la fiche plante).," n.d.))
Legend: A: root; B: stem; C: leaves; D: flowers; E: seed and fruits (schizocarp)

The leaves (Figure 12,C) are large, simple, oval to oblong, with a wrinkled, velvety (tomentose) surface due to abundant glandular trichomes. Their color ranges from gray-green to glaucous, featuring crenate-erosive margins and prominent pinnate venation. Basal leaves may reach 20 cm long with petioles of 3–9 cm, releasing a strong musky fragrance when crushed (Missouri Botanical Garden; Özdemir & Şenel, 1999).

The inflorescence (Figure 12.,D) is a terminal panicle composed of verticillasters of 2–6 zygomorphic (bilaterally symmetrical) flowers. The calyx is ovate-campanulate and hairy. The corolla, measuring 20–30 mm, features a distinctive falcate lilac upper lip and a cream-colored lower lip. The flowers are protandrous and bloom from mid-May

to August, attracting pollinators such as bees, butterflies, and hummingbirds with their nectar and pollen ((Peana, A. T. and Moretti, M. D., 2002)).

After flowering (Figure 12,E), the plant produces small nutlet-type fruits. Each brown, rounded, three-angled nutlet is about 2–3 mm long and seated within membranous mauve bracts. These seeds germinate readily, facilitating effective propagation of the species (Huxley et al., 1992)

4. Phytochemical Constituents, Properties, and Therapeutic Effects of Salvia sclarea

4.1. Biological Properties of S. sclarea

Species of the genus *Salvia*, including *clary sage*, are abundant in valuable secondary metabolites. These bioactive compounds, located in the plant's roots, leaves, and flowers, are responsible for many of its pharmacological and therapeutic effects. They encompass a range of chemical classes, notably terpenoids, flavonoids, and phenolic acids ((Ma et al., 2024; Quradha et al., 2024)). The distribution of these metabolites is organ-specific: monoterpenes and triterpenes are concentrated in the aerial parts, while diterpenes and phenolic acids are found mainly in the roots (Behnaz RAHMANIFAR et al., 2023)

4.1.1. Terpenoids of Clary Sage

Terpenoids are a major component of the phytochemical profile of *S. sclarea*, especially in its essential oils. These oils are rich in mono- and sesquiterpenoids. Oxygenated monoterpenes, notably linalyl acetate and linalool, can together account for up to approximately 82% of the total oil content, underscoring their dominant role in the plant's characteristic aroma and therapeutic applications (Kacˇániová, M et al., 2023). The essential oil also contains diverse sesquiterpenes, such as (E)-β-caryophyllene. Additionally, diterpenoid alcohols like sclareol, though non-volatile and present in smaller quantities in the essential oil, are major components of the plant’s resin; they are widely recognized for their anti-inflammatory and anti-cancer bioactivities (Ma et al., 2024; Zhou et al., 2022).

The rich and complex mixture of terpenoids contributes directly to the numerous reported bioactivities of *S. sclarea* essential oil.

Table 1:Main chemical components of *Salvia sclarea* essential oil

Compound Class	Major Compounds	Content Range / percentage	Notes & References
Oxygenated Monoterpenes	Linalyl acetate, Linalool	Linalyl acetate: 45–87%; Linalool: ~15–25%; combined: ~82.4%	Dominant in EO; major contributors to aroma and bioactivity **(Kačániová et al., 2023)**
Monoterpene Esters	Geranyl acetate, Neryl acetate	Geranyl acetate: 4–5%; Neryl acetate: minor	Contribute to EO complexity **(Kačániová et al., 2023)**
Monoterpene Alcohols	Geraniol, Nerol, Terpineol	Significant amounts, specific percentages unspecified	Contribute to fragrance and bioactivity **(Zhumaliyeva et al., 2023)**
Monoterpene Hydrocarbons	p-Cymene, α- and β-pinene, Limonene	~4.9–5% each; minor in overall composition	Contribute to aroma profile **(Kačániová et al., 2023)**
Sesquiterpenes	(E)-β-caryophyllene, α-copaene	~5% for (E)-β-caryophyllene	Minor yet significant constituents **(Kačániová et al., 2023)**
Diterpenes	Sclareol	Present in small amounts; contributes to resin components	Known for bioactivities **(Callan et al., 1999; Zhou et al., 2022)**
Other Components	Borneol, Camphor, Myrcene, 1,8-Cineole	Small quantities	Minor constituents with sensory and bioactive roles **(Callan et al., 1999)**

4.1.2. Phenolic and Flavonoid Compounds: Potent Antioxidants

The phenolic profile of *S. sclarea* is rich in hydroxycinnamic acids—namely rosmarinic and caffeic acids—and hydroxybenzoic acids, all predominantly present in the aerial parts. These compounds play a major role in the plant's antioxidant activity (Ma et al., 2024; Quradha et al., 2024)). Clary sage also contains several flavonoids such as rutin, quercetin, luteolin, apigenin, and myricetin, which enhance its

antioxidant and bioactive potential (Ma et al., 2024; Quradha et al., 2024). Other phenolic constituents—gallic acid, syringic acid, coumarins, and cinnamates—add further complexity to its phytochemical profile.

4.1.3. Other Bioactive Compounds

Besides these primary secondary metabolites, *S. sclarea* produces minor amounts of coumarins and glycosides, characteristic of the *Salvia* genus. The roots and leaves also contain bitter principles, such as daucosterol derivatives and triterpenes, historically used in herbal medicine (Mahboubi, M., 2020).

- **Antioxidant activity:** The oil has modest radical-scavenging activity, neutralizing about ~12% of DPPH and ~30% of ABTS radicals at test concentrations—indicating weak antioxidant power relative to stronger standards. By contrast, phenol-rich extracts (e.g., methanolic) show substantially higher antioxidant activity thanks to rosmarinic and caffeic acids ((Sharifi-Rad et al., 2018)).
- **Anti-inflammatory and analgesic effects:** Both the oil and extracts have demonstrated anti-inflammatory effects. In animal models, sclareol significantly reduced inflammation and pain (Zhou et al., 2022). Studies report analgesic activity of the whole oil, and ethnobotanical records emphasize clary sage's use for muscular pain and menstrual cramps (Sharifi-Rad et al., 2018). These effects are partly due to sclareol and other terpenoids that modulate inflammatory pathways (Sharifi-Rad et al., 2018; Zhou et al., 2022).

4.2. Physicochemical Properties of *Salvia sclarea* Essential Oil

Clary sage essential oil is a clear, mobile liquid, with physicochemical properties essential for identification and quality assurance. Its color ranges from colorless to pale yellow or yellow-brown. The scent is distinctive—often described as herbaceous, sweet, ethereal with amber, musky, lightly woody, hay-like, or even tobacco-like notes ((Aćimović, M. et al., 2018; Tasheva et al., 2021)).

Physically, at 20 °C, the oil's density typically ranges from 0.890 to 0.908 g/mL. The refractive index at 20 °C usually lies between 1.455 and 1.5. Its optical rotation at 20 °C is levorotatory, ranging approximately from –30° to –10°. The flash point

generally falls between 57 °C and 79 °C, up to 85 °C under certain measurement conditions**.(Tasheva et al.,2021 , Aćimović et al.,2018)**

Regarding solubility, clary sage oil is soluble in alcohol, fats (vegetable oils and butters), and emulsions (creams), but insoluble in water. This necessitates use of a dispersant (e.g., Solubol) for aqueous preparations. These physicochemical and compositional parameters—dominated by linalyl acetate and linalool—are key indicators of essential oil purity and quality, ensuring its efficacy and safe use.(Dhifi, W., et al., 2016; EFSA (FEEDAP) et al., 2024; NAHA, 2025)**)**

5. Effects of Salvia sclarea on Plant Bioaggressors

5.1. Insecticidal Activity

Clary sage essential oil (CSEO) and its extracts exhibit significant insecticidal potential (Šućur, J., et al., 2015). Studies (table 2) have demonstrated its effectiveness against pests such as the lime bug (*Oxycarenus lavaterae*), where the highest tested concentration of CSEO achieved 66% insecticidal activity (Yemets, O. Y. et al., 2021). Aqueous extracts of *S. sclarea* have also shown toxicity against greenhouse whitefly (*Trialeurodes vaporariorum*), with a 0.1% concentration causing 56.66% mortality (Mansour, S. A., et al., 2013). The broader *Salvia* genus is known for toxicity to various insects, suggesting a wider insecticidal capacity across the species (Kacˇániová, M et al., 2023; Sharaby, A. and El-Nujiban, A., 2015)

Table 2:Insecticidal Activity of Salvia sclarea Extracts Against Linden Bug and Greenhouse Whitefly (Sharaby & El-Nujiban, 2015; Kačániová et al., 2023).

Pests	Extract/oil type	Concentration	Mortality Rate (%)	Time (if specified)
Linden bug ***(Oxycarenus lavatera)***	Essential oil	Highest concentration tested	66,66	*Not specified*
Greenhouse whitefly ***(Trialeurodes vaporariorum)***	Aqueous Extract	0,1%	56,66	*Not specified*

Although the specific insecticidal mechanisms of *Salvia sclarea* are not as extensively elucidated as its antimicrobial effects in the referenced literature, the observed

mortality rates—such as 66.66% against *Oxycarenus lavatera*—indicate a potent effect (Šućur et al., 2015). By analogy with other *Salvia* species, particularly *Salvia officinalis*, the potential mechanisms may include; Disruption of insect hormonal balance, Anti-feeding (repellent or feeding-reduction) activity and Effects on larval development and reproduction, such as malformed pupae, reduced egg-laying, and sterility ((Sharaby, A. and El-Nujiban, A., 2015))

Fungicidal Activity

Clary sage essential oil (CSEO) exhibits inhibitory effects on the mycelial growth of key phytopathogenic fungi. Singh et al. (2019) evaluated the antifungal, anti-aflatoxigenic, and antioxidant activities of *Salvia sclarea* essential oil and its combination with linalyl acetate as a protective agent against contamination.

The tested pathogens included *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *Verticillium dahliae*, two soil-borne wilt diseases of major economic significance in agriculture. The antifungal activity was dose-dependent (Table 3); for instance, at a concentration of 8 µL per Petri dish, *Verticillium dahliae* mycelial growth was reduced by 46.49%, while *Fusarium oxysporum* growth decreased by 39.06%. *F. oxysporum* appeared more sensitive to CSEO than *V. dahliae*. CSEO also shows broad-spectrum fungitoxicity against several common mold species known to degrade pharmaceutical products, including *Aspergillus flavus*, *Botrytis cinerea*, and *Penicillium citrinum*. Notably, the combination of CSEO with linalyl acetate (1:1) demonstrated superior antifungal and anti-aflatoxigenic activity compared to either component alone.

Table 3: Antifungal activity of *Salvia sclarea* essential oil against phytopathogenic fungi (Singh et al., 2019)

Phytopathogenic Fungi	CSEO Concentration (µl/Petri dish)	Inhibition of Mycelial Growth (%)
Verticillium dahliae	0,5	0
	1	0
	2	26,16
	4	37,50
	8	46,49
Fusarium oxysporum f. sp. radicis-lycopersici	0,5	1,81
	8	39,06

Mechanistically, the combination of CSEO and linalyl acetate targets the fungal cell membrane—a critical site of action. It significantly reduces ergosterol levels, a key structural component of fungal membranes essential for integrity and function. This disruption leads to increased leakage of vital ions and UV-absorbing materials from the cells, confirming the plasma membrane as the primary target (Singh, V. K., et al., 2019)).

Moreover, CSEO and its constituents inhibit intracellular methylglyoxal (MG), a cytotoxic alpha-oxo-aldehyde implicated in oxidative damage and aflatoxin B1 secretion in molds. By neutralizing MG, CSEO helps protect host cells and reduce fungal toxin production (Singh, V. K., et al., 2019)

Importantly, the antimicrobial activity of CSEO's vapor phase is significantly stronger than that of direct contact application—particularly for microorganisms growing on surfaces such as those found on potatoes. This highlights the crucial role of the oil's volatile compounds in its antimicrobial efficacy ((Cui, H., et al., 2015; Xiao, R., et al., 2017)).

CSEO's primary antimicrobial mechanism involves broad-spectrum physical disruption of microbial cell membranes, causing leakage of essential intracellular components such as ATP and DNA. This non-specific mode of action makes it more difficult for microbes to develop resistance compared to synthetic chemical agents that target specific metabolic pathways or molecular sites (Cui, H. et al., 2017; Xiao, R., et al., 2017) This characteristic positions CSEO as a valuable tool in addressing the growing issue of pesticide resistance in agriculture—offering a sustainable and effective alternative.

Allelopathic Effects

Allelopathy refers to the direct or indirect, beneficial or harmful effects of one plant on another, typically mediated by biomolecules known as allelochemicals. Aqueous extracts of *S. sclarea* have demonstrated allelopathic effects that vary depending on the receiving plant species.

For example, on black nightshade (*Solanum nigrum L.*) seedlings, a low concentration (0.1%) of *S. sclarea* aqueous extract induced lipid peroxidation (LP) in roots, indicating oxidative stress and resulting in increased production of malondialdehyde (MDA). In contrast, the same extract did not significantly increase LP in bell pepper (*Capsicum annuum L.*) seedlings, and MDA accumulation remained low—suggesting that the bell pepper's defense systems were effective against the allelochemicals.This points to species-specific phytotoxic effects, highlighting the potential for selective weed management (Singh, V. K., et al., 2019; Šućur, J., et al., 2015).

The body of evidence regarding *S. sclarea*'s effectiveness against specific pests (*whiteflies*, *Oxycarenus lavatera*) ((Sharaby, A. and El-Nujiban, A., 2015; Šućur, J., et al., 2015)), major phytopathogenic fungi (*Fusarium*, *Verticillium*, *Aspergillus*), and its selective allelopathic effects (toxic to *S. nigrum*, less so to *C. annuum*) underscores its great potential as a targeted biopesticide (Džamić, A., et al., 2008; El-Baky, N. A. and Amara, A. A. A. F., 2021; Singh, V. K., et al., 2019). This specificity represents a major advantage over broad-spectrum synthetic chemicals, allowing for more ecologically precise interventions that minimize harm to non-target organisms and beneficial crops. Such properties are particularly relevant within the framework of modern sustainable agriculture, where reducing environmental impact is a key priority.

➢ CHAPITRE V: Phyto-Fungi exemple Fusarium et Alternaria

1. Phytopathogenic Fungus: Fusarium sp.

1.1. Overview :

The genus *Fusarium*, a cosmopolitan fungus belonging to the phylum Ascomycota, is commonly found in agricultural soils and plant debris. Certain species are powerful plant pathogens, responsible for significant crop losses through diseases such as vascular wilt, root and crown rot, seedling blight, and Fusarium head blight in cereals, legumes, vegetables, and other crops. Moreover, *Fusarium* species pose a risk to human health through the production of various mycotoxins, which represent a serious threat to food safety (**Todorović et al., 2023).**Although many filamentous *Fusarium* species exhibit perfect (teleomorphic) forms, the sexual stage remains unknown for several species (GEISER et al., 2013; O'DONNELL et al., 2000)**.**

The classification of *Fusarium* (Figure 13) is particularly complex and has undergone significant evolution. Initially based on morphological characteristics, the taxonomy has been refined with the advent of increasingly sophisticated molecular techniques (GEISER et al., 2013)**.** This advancement in classification methods is critical for the accurate identification of species, which is essential for understanding host specificity, pathogenicity, and ultimately for the effective management of the diseases they cause.

Taxonomy (O'Donnell et al., 2015)

- **Kingdom**: Fungi
- **Phylum**: Ascomycota
- **Class**: Sordariomycetes
- **Subclass**: Hypocreomycetidae
- **Order**: Hypocreales
- **Family**: Nectriaceae
- **Genus**: *Fusarium (Link, 1809)*



Figure 13.Fusarium sp. (Halewy et Chevalier., 2025)

a) Classification Traditionnelle : Morphologie et Complexes d'Espèces

Historiquement, l'identification des espèces de *Fusarium* reposait principalement sur des critères morphologiques et culturaux (Nelson et al., 1983). Les chercheurs examinaient l'aspect des colonies (forme, texture, couleur), leur vitesse de croissance, ainsi que la production de pigments diffusibles sur milieu PDA (Jeunot, 2005 ; Heit, 2015) (Figures 14&15).

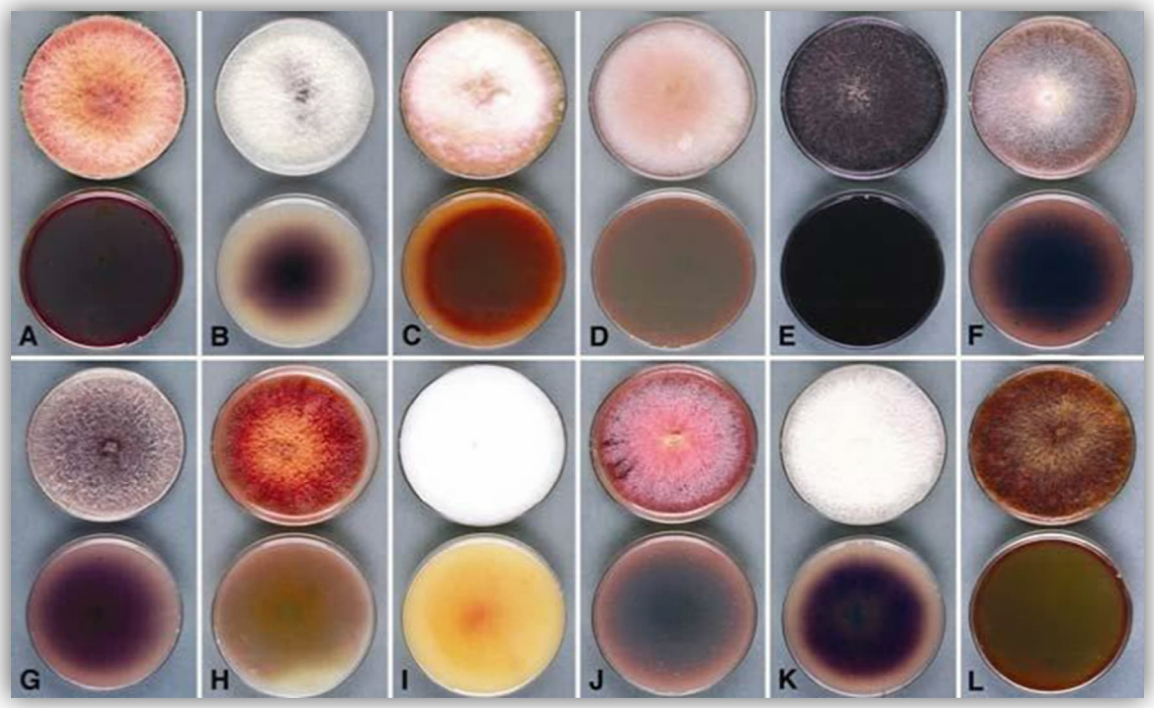


Figure 14. Morphologie de différentes espèces de *Fusarium* sur PDA (Summerell et al., 2003).
Légende : Chaque paire de boîtes montre le recto (mycélium aérien, en haut) et le verso (vue inférieure, en bas). A : *F. poae*, B : *F. oxysporum*, C : *F. acuminatum*, D : *F. nelsonii*, E : *F. subglutinans*, F : *F. nygamai*, G : *F. pseudonygamai*, H : *F. lateritium*, I : *F. thapsinum*, J : *F. decemcellulare*, K : *F. verticillioides*, L : *F. culmorum*.

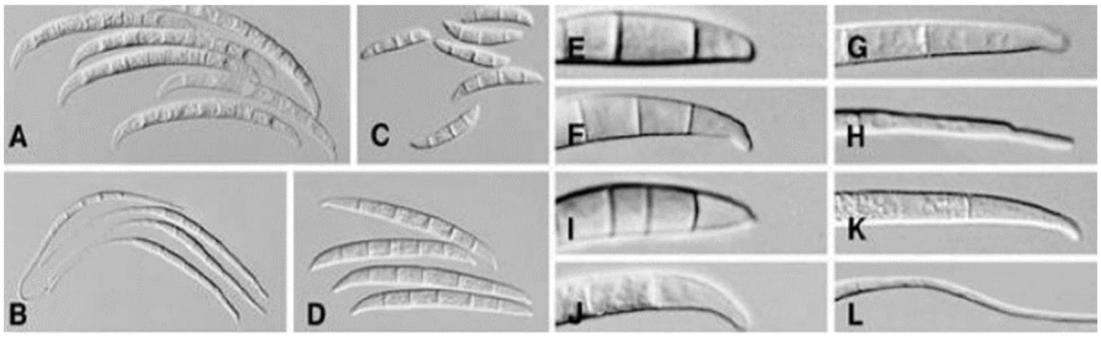


Figure 15. Morphologie des macroconidies observée au microscope (Summerell et al., 2003).

Légende : A–D : variation de la taille et de la forme générale ; E–H : variations de la base des macroconidies ; I–L : variation de la partie apicale. A et J : *F. decemcellulare*, B, H et L : *F. longipes*, C, E et I : *F. culmorum*, D : *F. chlamydosporum*, F : *F.*

Under the microscope, identification was based on specific spore characteristics (Figure 15):

- Presence or absence of microconidia, their shape and arrangement.
- Presence or absence of chlamydospores, their location (intercalary or terminal), and abundance.
- Shape and size of macroconidia, as well as the form of the basal cell.

This method enabled the definition of many species and sections within the genus. However, morphological classification soon revealed its limitations. The high variability in colony characteristics, phenotypic plasticity (the ability of a species to change appearance based on conditions), and especially the presence of cryptic species (genetically distinct but morphologically indistinguishable) made identification difficult and often inconsistent. This led to genetically distinct species being grouped under the same name, forming species complexes (Leslie and Summerell, 2006)

Formae speciales (ff. spp.) are a significant sub-classification used for phytopathogens. They designate populations within the same morphological species that are specific to one or more plant hosts. For example, *Fusarium oxysporum* is a species complex that includes many *formae speciales* (e.g., *F. oxysporum* f. sp. *lycopersici* causing tomato wilt), each pathogenic to a specific host but with no significant morphological divergence **(MICHIELSE and REP, 2009).** This classification is functional and useful for disease management, but it does not always reflect actual phylogenetic relationships.

b) Molecular Classification: The Era of Phylogeny

The emergence of molecular biology techniques revolutionized *Fusarium* classification by uncovering evolutionary (phylogenetic) relationships between lineages. DNA sequence analysis of conserved genes—such as rDNA (especially ITS), the translation elongation factor 1-alpha (TEF1-α), and the second largest subunit of RNA polymerase (RPB2)—has become standard (GEISER et al., 2013; O'DONNELL et al., 2000) (Figure 16).

These molecular studies confirmed the existence of Cryptic Species Complexes (CSCs), where multiple genetically distinct species share similar morphological traits. This led to a major revision of *Fusarium* taxonomy, including proposals for new genera and redefinition of many groups (GEISER et al., 2013). For example, the genus *Fusarium* sensu stricto was redefined, and many species previously classified under *Fusarium* were reassigned to other genera such as *Neocosmospora* (e.g., *Fusarium solani* complex) or the *Nectriaceae* family (for species producing red perithecia) (LOMBARD et al., 2015)

Today, a **polyphasic approach** is preferred, integrating morphological, molecular (multi-gene phylogeny), and ecological data (host specificity, geographic distribution) for a more robust and precise classification of phytopathogenic *Fusarium* (CROUS et al., 2021; Leslie and Summerell, 2006) This modern classification is crucial for research in plant pathology, disease surveillance, and the development of effective control strategies.

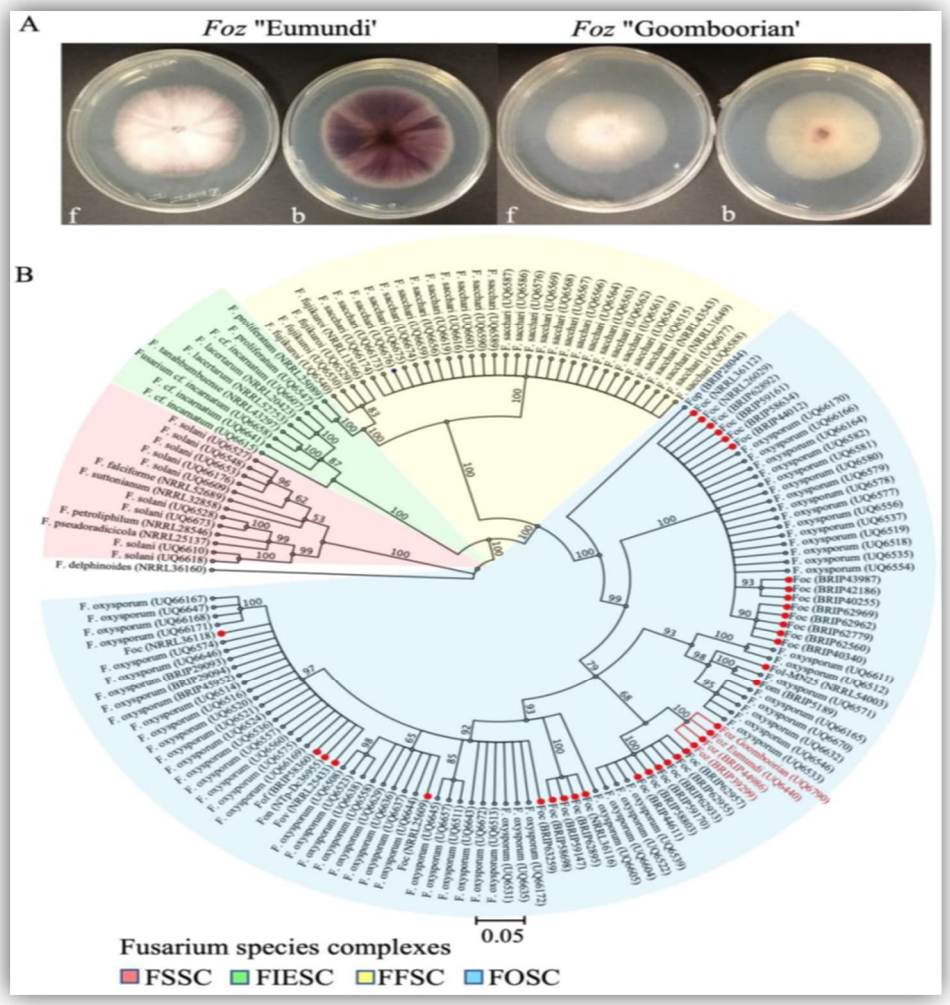


Figure 16. Morphology of Foz 'Eumundi' and Foz 'Goomboorian' and their phylogenetic classification (Prasath et al., 2023).
***Caption*: (A) Colony morphology of two monoconidial isolates of Foz, Foz 'Eumundi' and Foz 'Goomboorian', cultured on full-strength potato dextrose agar for five days. f = front view, b = back view. (B) Bayesian inference phylogeny based on TEF-1*α* sequences, representing four *Fusarium* species complexes: FIESC (*F. incarnatum-equiseti* complex), FFSC (*F. fujikuroi* complex), FOSC (*F. oxysporum* complex), and FSSC (*F. solani* complex). Pathogenic *formae speciales* within FOSC are indicated with red circles. Branch labels show posterior probabilities in percentages. The Foz clade within FOSC is highlighted in red, including members Foz 'Goomboorian', Foz 'Eumundi', Foz BRIP44986, and Foz BRIP39299. A scale bar of 0.05 is shown below the tree.**

1.2.Life Cycle

Fusarium species are filamentous Ascomycetes that grow as haploid mycelium and primarily reproduce via asexual spores. The fungus produces mitotic conidia—multicellular macroconidia and smaller microconidia—on sporulating hyphae; these asexual spores disperse to colonize new host plants. Thick-walled chlamydospores or hyphal fragments can also form and survive in soil and plant debris, acting as overwintering structures that restart infection cycles when conditions become favorable (Figure 17).

A minority of *Fusarium* species (≈20%) also undergo sexual reproduction, producing perithecia (ascocarps) in which karyogamy and meiosis generate ascospores. In some homothallic species (e.g., *F. graminearum*), self-fertilization yields recombinant or clonal ascospores. Overall, *Fusarium* life cycles alternate between vegetative growth with prolific asexual sporulation and occasional sexual reproduction (Ajmal et al., 2022; Buttar et al., 2024) (Figure 17).

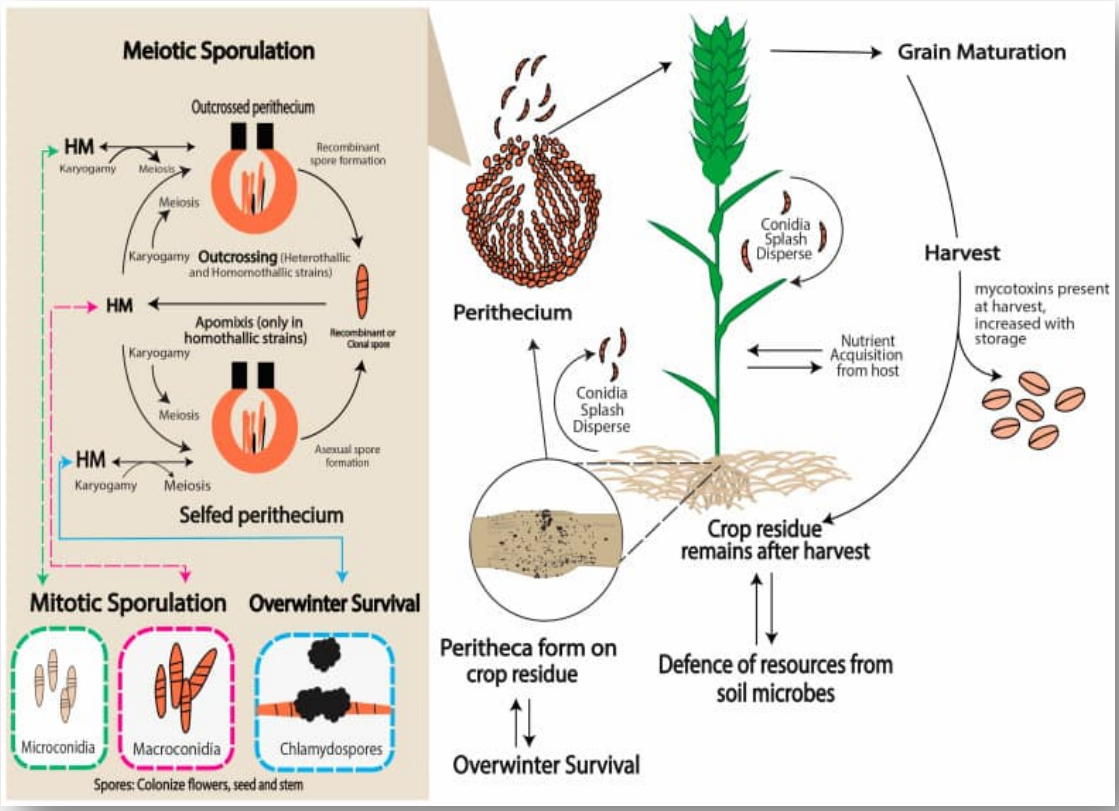


Figure 17. Generalized life cycle of *Fusarium* spp. (Buttar et al., 2024)

1.3. Virulence Factors

Fusarium species deploy a variety of secreted molecules and enzymes to invade hosts. These include secondary metabolites (mycotoxins), cell wall-degrading enzymes, and protein effectors (including small RNAs).

4.1.3. Mycotoxins

Fusarium species synthesize many toxic secondary metabolites (Figure 18), known as **mycotoxins**. The most studied include fumonisins, trichothecenes, and zearalenone. Each class is biosynthesized by dedicated gene clusters (polyketide- or terpene-based enzymes) and functions as a virulence factor. For example:

- **Fumonisin B_1 (FB_1)**: A long-chain polyketide (sphinganine analogue) produced by *F. verticillioides* and *F. proliferatum*. Its biosynthesis is governed by the **FUM gene cluster (**(ELLIS, 1971; SIMMONS, 1992)FB_1 inhibits ceramide synthase, disrupting sphingolipid metabolism and triggering programmed cell death (PCD) in host cells. It is considered a **necrotrophic**

effector: its activity promotes colonization of maize plants, and deletion of the FUM cluster eliminates virulence in seedlings **(Perochon et al., 2024; Chen et al., 2020).**

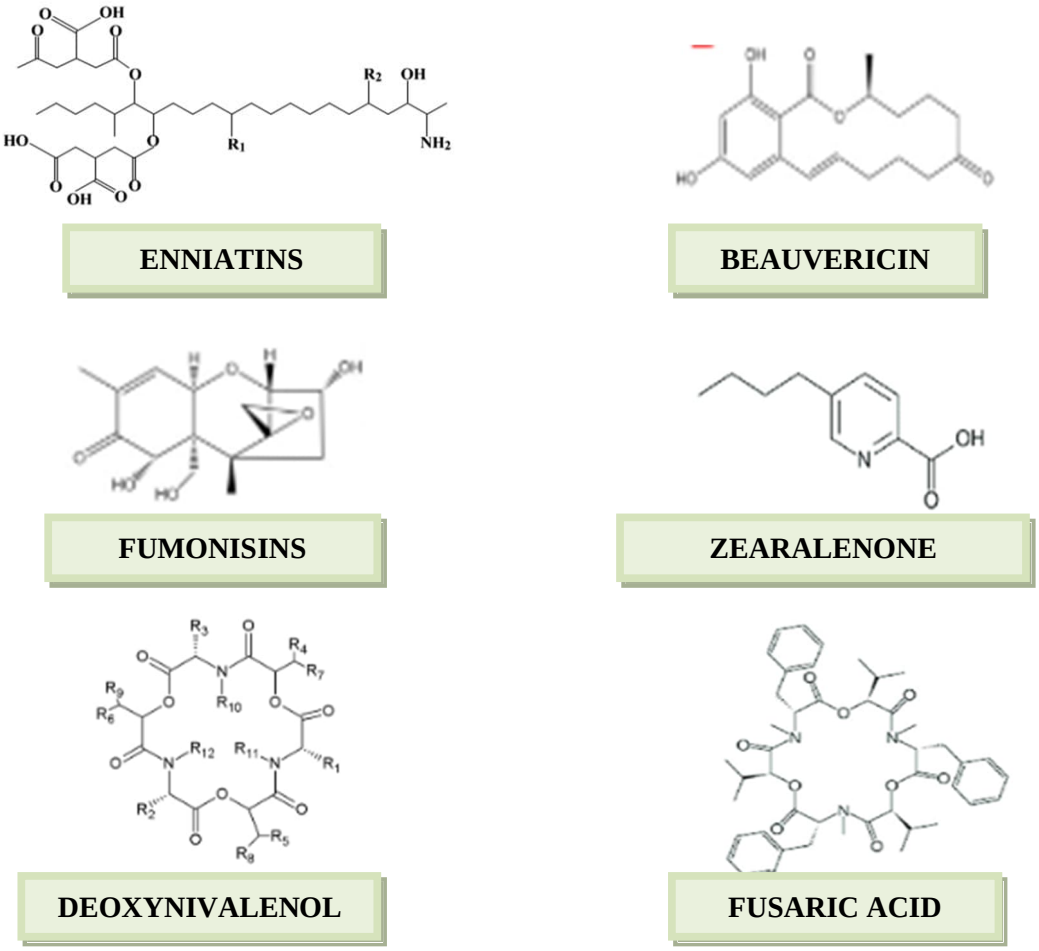


Figure 18. Chemical structures of *Fusarium* spp. mycotoxins (Urbaniak, M. et al., 2020; Ekwomadu et al., 2021; Lázaro et al., 2024; Deyu Huang et al., 2019)

- **Trichothecenes** (e.g., deoxynivalenol, DON): A family of sesquiterpenoid epoxide toxins produced by *F. graminearum*, *F. culmorum*, etc. The *Tri* gene cluster encodes enzymes (e.g., *Tri5*, *Tri6*) involved in trichothecene biosynthesis. DON inhibits eukaryotic ribosomes, halting protein synthesis and leading to cell death. It also promotes the spread of the fungus in cereal grains

(wheat, barley, maize). Its biosynthesis and regulation are tightly linked to the fungus's virulence **(Chen et al., 2020; Perochon et al., 2024).**

- **Zearalenone (ZEA):** A non-steroidal estrogenic mycotoxin synthesized primarily by *F. graminearum* and *F. culmorum*. It is produced through a type I polyketide synthase pathway (encoded by the *ZEA* gene cluster). ZEA interferes with hormonal balance in animals and can weaken plant defenses, although its precise role in virulence remains less defined **(Urbaniak et al., 2020; Huang et al., 2019).**

4.1.4. . Cell Wall-Degrading Enzymes (CWDEs):

Fusarium species secrete numerous hydrolytic enzymes to penetrate and colonize plant tissues. These include:

- **Pectinases** (e.g., polygalacturonases, pectin lyases),
- **Cellulases**,
- **Xylanases**, and
- **Proteases**.

These enzymes degrade structural components of plant cell walls, facilitating fungal invasion and nutrient acquisition. The expression of CWDEs is often induced in response to host cues and is essential for successful infection.

4.1.5. Effectors and Small RNAs:

Fusarium also deploys **effector proteins**—small secreted molecules that manipulate host immunity, suppress defense responses, or trigger necrosis. Some effectors act inside plant cells, while others target the apoplast.

Recent studies have also shown that **small RNAs (sRNAs)** produced by Fusarium can interfere with host gene expression by hijacking the plant's RNA silencing machinery. This form of cross-kingdom RNA interference (ck-RNAi) has been documented in *F. graminearum* and represents a novel strategy for host manipulation **(Kim et al., 2023).**

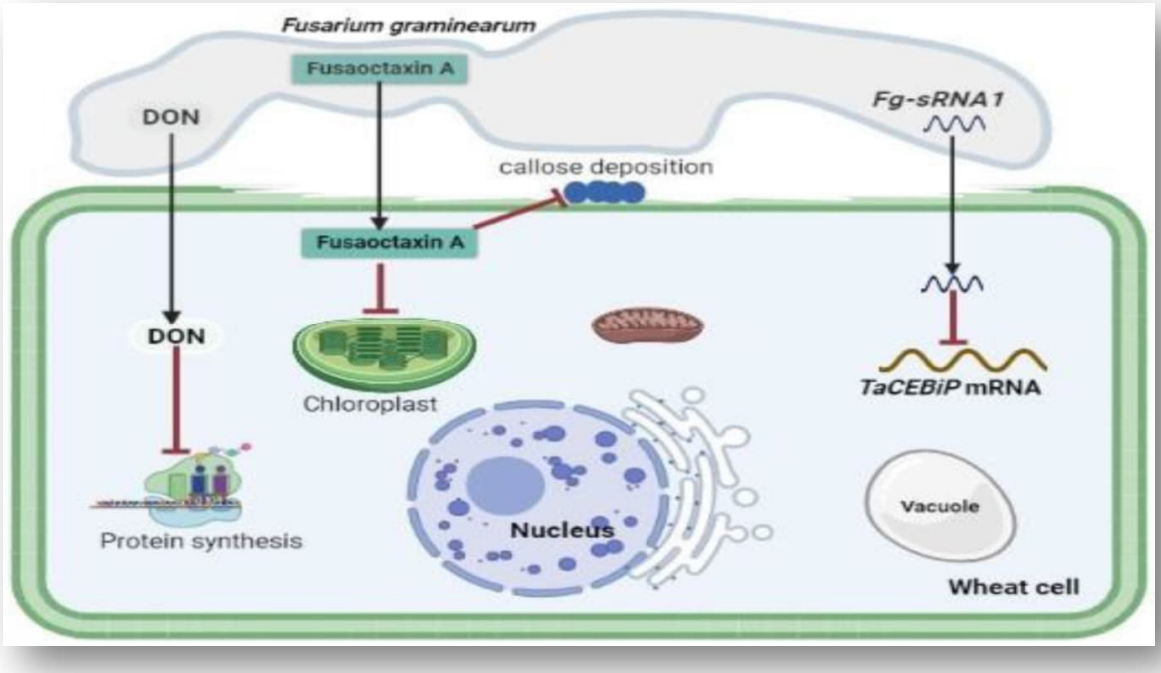


Figure 19. Mode of action of *F. graminearum*-**secreted pathogenicity factors during** fungi-plant **interaction. (Mapuranga, J Et al.,2023)**

2. Pathogenic Fungus: Alternaria sp.

2.1. General Overview:

The genus *Alternaria* (Figure 20), a group of ubiquitous filamentous fungi belonging to the phylum *Ascomycota*, is recognized as one of the most formidable fungal phytopathogens worldwide. These fungi are known to cause a wide array of diseases across a broad range of crops—including fruits, vegetables, cereals, and ornamental plants—both pre- and post-harvest (Thomma, 2003; Woudenberg et al., 2013; Lou et al., 2013). They result in significant global economic losses and are ranked among the most damaging pathogens to agricultural yields (Saleh et al., 2024).

Beyond their agricultural impact, the roughly 100 ubiquitous *Alternaria* species (Simmons, 2007) are characterized by dark, multicellular conidia. Most *Alternaria* species are necrotrophs, destroying host tissues, while others are common saprophytes that act as widespread decomposers (Rotem, 1994). They cause a variety of symptoms including leaf spots, blight, and fruit rot (Schmey et al., 2024). Their metabolites can be toxic, producing phytotoxins that harm plants and mycotoxins that affect animals (Duke and Dayan, 2011).

The taxonomy of this complex genus—particularly its phytopathogenic species—has long posed a challenge. However, molecular tools have brought significant and necessary advances in its classification.

Taxonomy (Nasraoui, 2015)

- Kingdom: Fungi
- Phylum: Ascomycota
- Subphylum: Pezizomycotina
- Class: Dothideomycetes
- Order: Pleosporales
- Family: Pleosporaceae
- Teleomorph genus: *Lewia*
- ***Anamorph genus: Alternaria***



Figure 20. Alternaria spp. (Halewy et Chevalier., 2025)

a) Traditional Classification: Based on Conidial Morphology

Historically, classification of *Alternaria* species was primarily based on morphological traits of their conidia (asexual spores) and the formation of conidial chains **(Ellis, 1971; Simmons, 1992)** (Figure 21). Key criteria included:

- Shape and size of conidia, which vary from pyriform to obclavate, ellipsoidal, or ovoid.
- Number and arrangement of septa (transverse and longitudinal partitions) within conidia.
- Length and shape of the beak (rostrum) in species such as *Alternaria alternata* and *A. solani*.
- Mode of conidial chain formation—short or long chains.
- Cultural characteristics of colonies (color, texture, growth rate).

This morphological approach led to the identification of numerous species and "species groups" based on phenotypic similarities. However, the phenotypic plasticity of *Alternaria* (the ability of the same species to exhibit morphological variation under different conditions), high intra-species variability, and the existence of cryptic species (genetically distinct but morphologically similar) made identification and classification difficult and often confusing (LAWRENCE et al., 2013; Woudenberg et al., 2015)This has led to the formation of species complexes, such as the *Alternaria muriformis* complex (Figure 21), which includes many pathogenic species with different host specificities but nearly identical morphologies under certain culture conditions (Iturrieta-González and Gené, 2023**).**

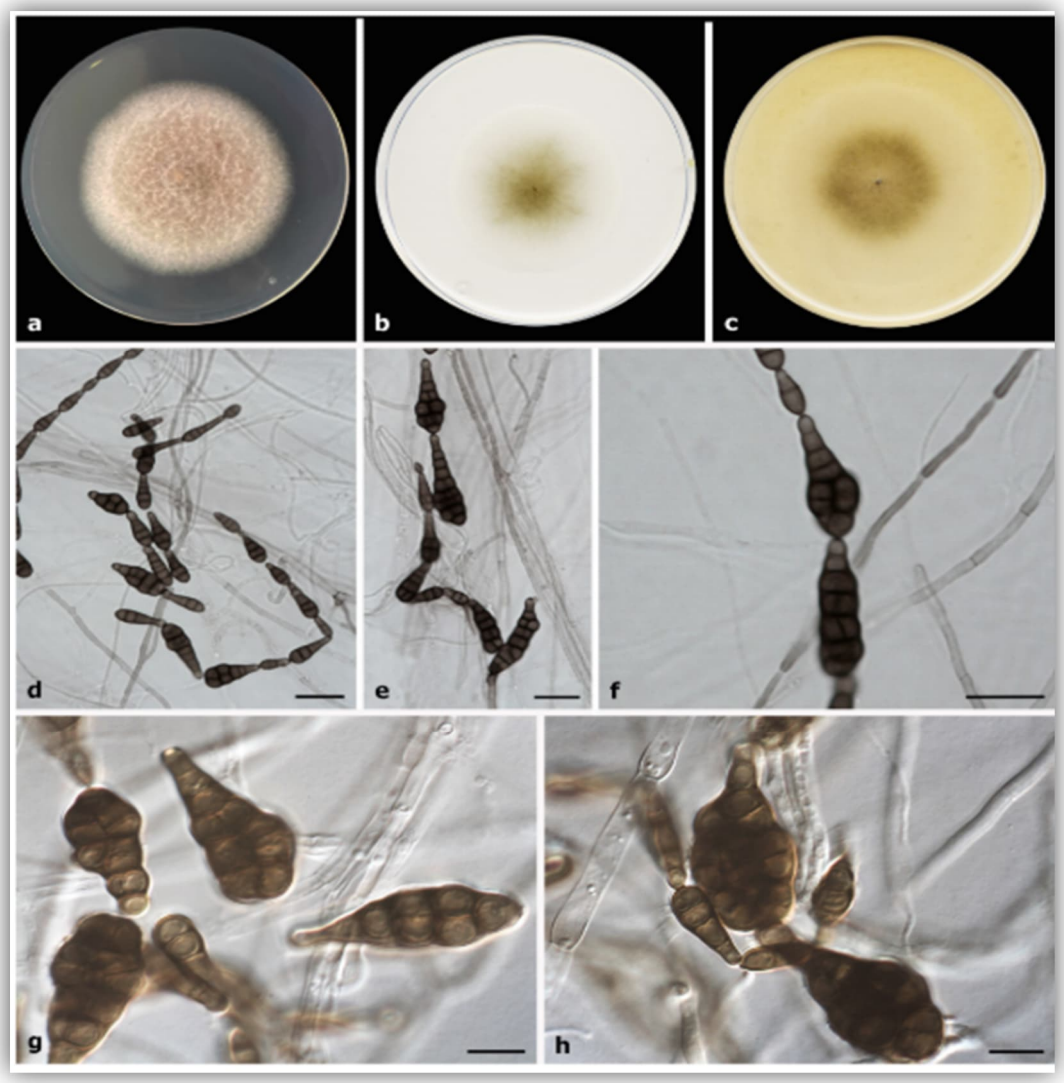


Figure 21. ***Alternaria muriformis*** **(ex-type FMR 17518) (Iturrieta-González & Gené, 2023).**
Caption: (a) Colonies on PDA; (b) colonies on PCA; (c) colonies on OA; (d–h) conidia. Scale bars: (d–f) = 20 µm and (g, h) = 10 µm.

b) Molecular Classification and Polyphasic Approach

The introduction of molecular biology techniques has revolutionized *Alternaria* taxonomy, resolving many morphological ambiguities and revealing the true phylogenetic relationships among species (Figure 22). DNA sequence analysis of genetic markers such as the internal transcribed spacer (ITS) region of rDNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the second-largest subunit of

RNA polymerase (RPB2), and P-type ATPase has become essential for species discrimination (PRYOR and MICHAILIDES, 2002; Woudenberg et al., 2015)

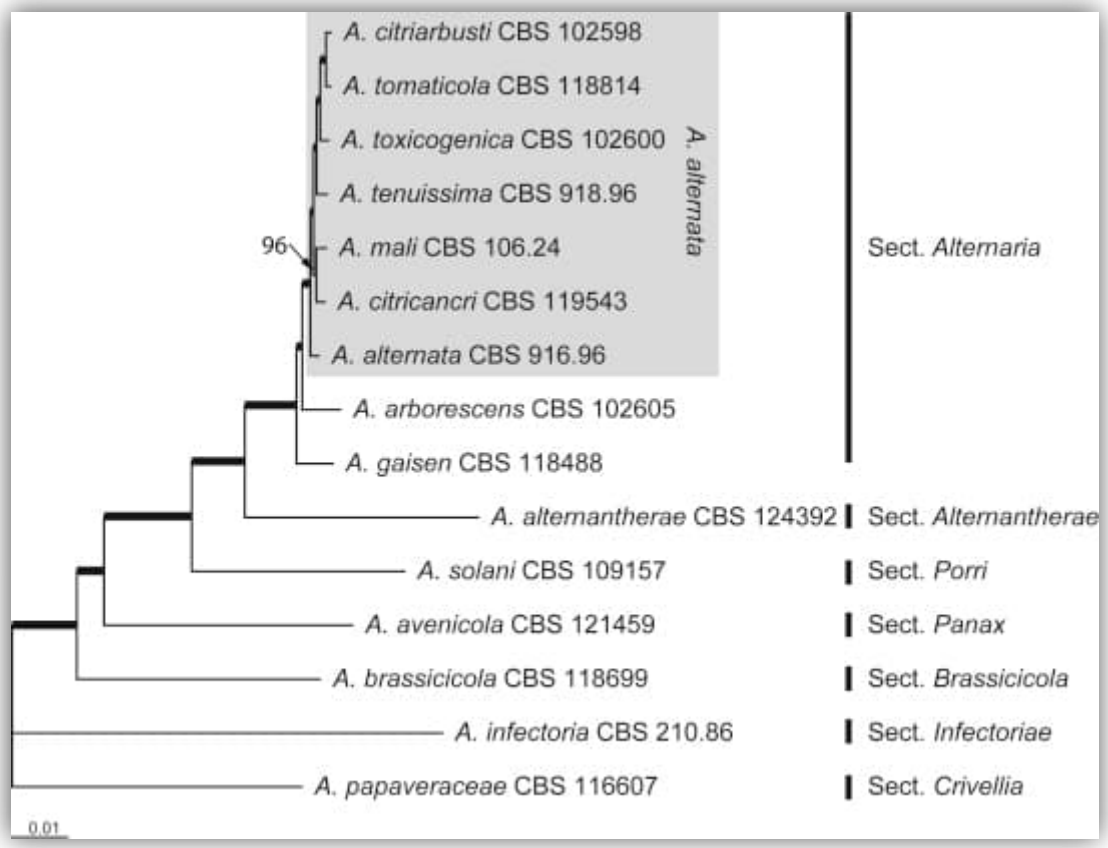


Figure 22: PhyML tree based on whole-genome and transcriptome reads from 15 *Alternaria* species using REALPHY (Woudenberg et al., 2015).
Legend: Bootstrap support values are shown at the nodes; thick branches indicate full support. The gray box shows species now synonymized under *A. alternata*. The tree is rooted with *A. papaveraceae* (CBS 116607).

Molecular phylogenetic studies have made it possible to:

- Delimit species complexes with greater precision, revealing many cryptic species within previously lumped groups (e.g., the *A. alternata* complex has been subdivided into over 20 distinct species based on molecular markers and host specificity, despite similar morphologies) (Lawrence et al., 2013). The same is true for *Alternaria burnsii* (Figure 23).
- Clarify evolutionary relationships within the genus and with related genera.
- Update nomenclature, leading to reclassification and the description of new species.

Today, the classification of phytopathogenic *Alternaria* is based on a **polyphasic approach**, integrating morphological data (though no longer used exclusively), molecular data (multigene phylogeny), and ecological information (host specificity, disease symptoms, geographic distribution, and toxin production) (Woudenberg et al., 2013; Zaccardelli et al., 2015). This integrated method is essential for accurate identification of *Alternaria* phytopathogens, which is crucial for early disease detection, reliable diagnostic development, and effective control strategies.



Figure 23. Conidia and conidiophores of *Alternaria burnsii* (Woudenberg et al., 2015) Legend: (A–B): CBS 108.27; (C–D): CBS 879.95; (E–F): CBS 118816; (G–H): CBS 118817. Scale bars = 10 µm.

2.2.Life Cycle of the Alternaria Genus

Alternaria species are also filamentous Ascomycetes (Dothideomycetes) but reproduce almost exclusively asexually. No sexual stage (teleomorph) is known for the small-spored *Alternaria* pathogens (Schmey et al., 2024) (Figure 24). Instead, they produce multicellular conidia on specialized conidiophores. These conidia typically form long chains of thick-walled, dark spores that detach easily. The spores are widely dispersed by wind and rain splashes (Schmey et al., 2024). Under warm and humid conditions (~25°C optimal, with dew periods), the conidia germinate within hours and infect plants. Germ tubes penetrate plant tissues through wounds, via appressoria, or by

secreting cell wall-degrading enzymes (pectinases, cutinases, etc.) to breach the epidermis (Schmey et al., 2024).

After initial infection, the lesions quickly produce new conidia for secondary spread (Schmey et al., 2024). Infected tissues often show expanding necrotic spots surrounded by yellow halos (caused by fungal toxins diffusing into adjacent tissue) (Schmey et al., 2024). At the end of the season or under unfavorable conditions, *Alternaria* forms survival structures: intercalary hyphal cells convert into chlamydospores that aggregate into melanized microsclerotia. These survive in soil or plant debris (often with higher virulence than conidia) (Figure 24) and germinate to infect new crops (Schmey et al., 2024). If no host is available, *Alternaria* can adopt a saprophytic lifestyle on dead organic matter (Schmey et al., 2024).

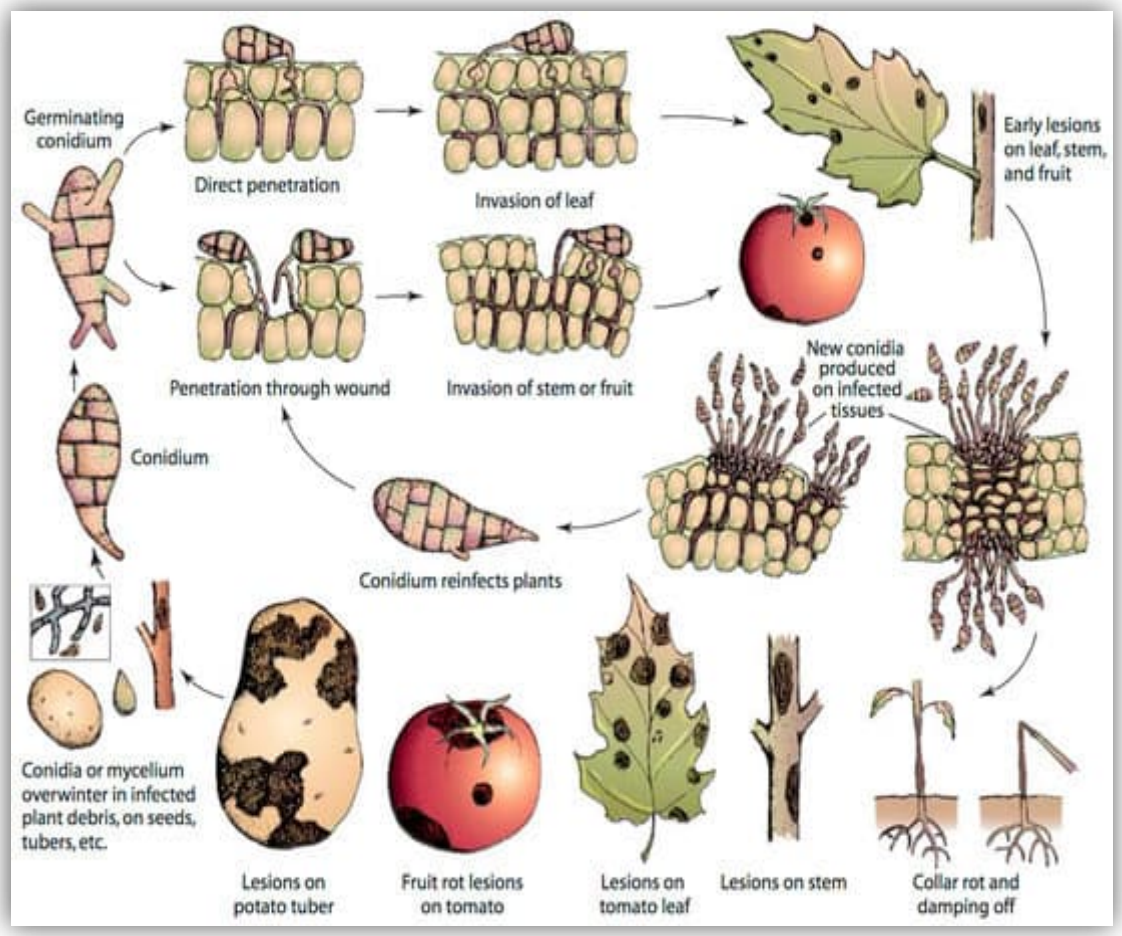


Figure 24. Life cycle of *Alternaria* sp. (Adhikari et al., 2017)

2.3. Virulence Factors

Alternaria pathogens deploy similar strategies using their own repertoires of toxins and effectors.

2.3.1. Phytotoxins and Mycotoxins

a) Host-Selective Toxins (HSTs) (Figures 25 and 26):

- **AAL toxins**: These are sphinganine-analog mycotoxins produced by *Alternaria alternata* f. sp. *lycopersici* (the tomato pathotype). Structurally similar to *Fusarium* fumonisins, AAL toxins interfere with sphingolipid metabolism by inhibiting ceramide synthase. This disruption leads to the accumulation of toxic intermediates, inducing programmed cell death (apoptosis) in susceptible host plant cells (Brandwagt et al., 2000).
- **ACT toxins**: Synthesized by *A. alternata* f. sp. *citri* (the tangerine pathotype), ACT toxins target citrus species. They act by altering plasma membrane permeability and disrupting mitochondrial function, thereby triggering oxidative stress and cell death in sensitive citrus cultivars (Tsuge et al., 2013).
- **AK-toxins**: These are specific to Japanese pear (*Pyrus pyrifolia*), produced by *A. alternata* f. sp. *kikuchiana*. AK-toxins inhibit photosynthesis and disrupt chloroplasts in susceptible cultivars, leading to necrotic lesions (Tanaka et al., 1999).
- **AF-toxins**: Produced by *A. alternata* f. sp. *fragariae*, these toxins affect strawberry plants, particularly during fruit maturation stages.
- **AT-toxins and AC-toxins**: These are secreted by pathotypes infecting apple and rough lemon, respectively. These compounds show host specificity and vary structurally but generally function by inducing reactive oxygen species (ROS) accumulation, mitochondrial dysfunction, and cellular leakage (Tsuge et al., 2013).

Each pathotype of *A. alternata* produces a unique HST required for pathogenicity, meaning that deletion or inactivation of the HST biosynthetic genes renders the fungus non-pathogenic toward its specific host (Tsuge et al., 2013). The biosynthesis of these toxins is often governed by genes located on conditionally dispensable chromosomes (CDC), which are not essential for saprophytic growth but crucial for virulence (Akamatsu et al., 1999).

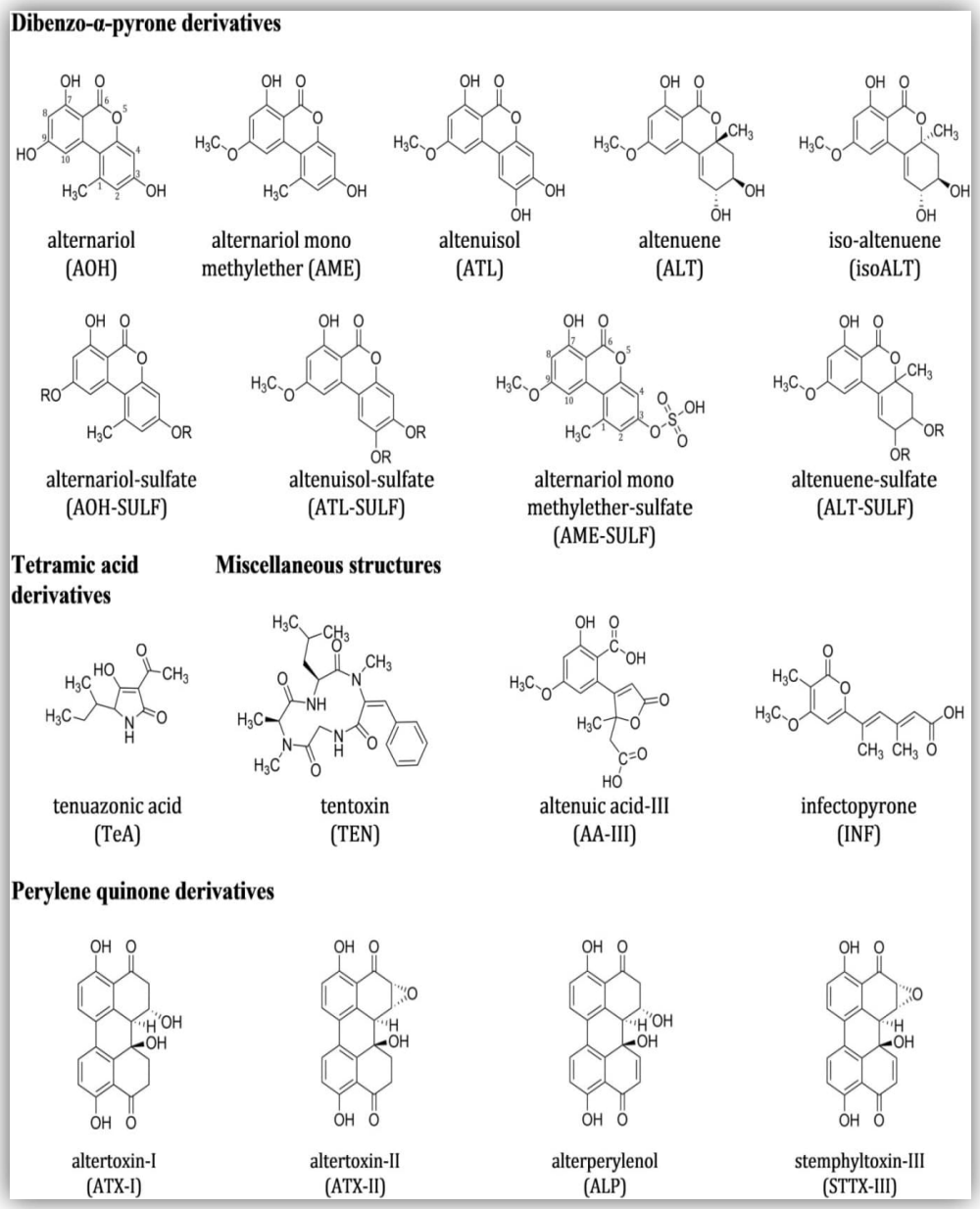


Figure 25. Chemical structures of the Non specific toxins produced by *Alternaria spp* (Zwickel et al., 2018)

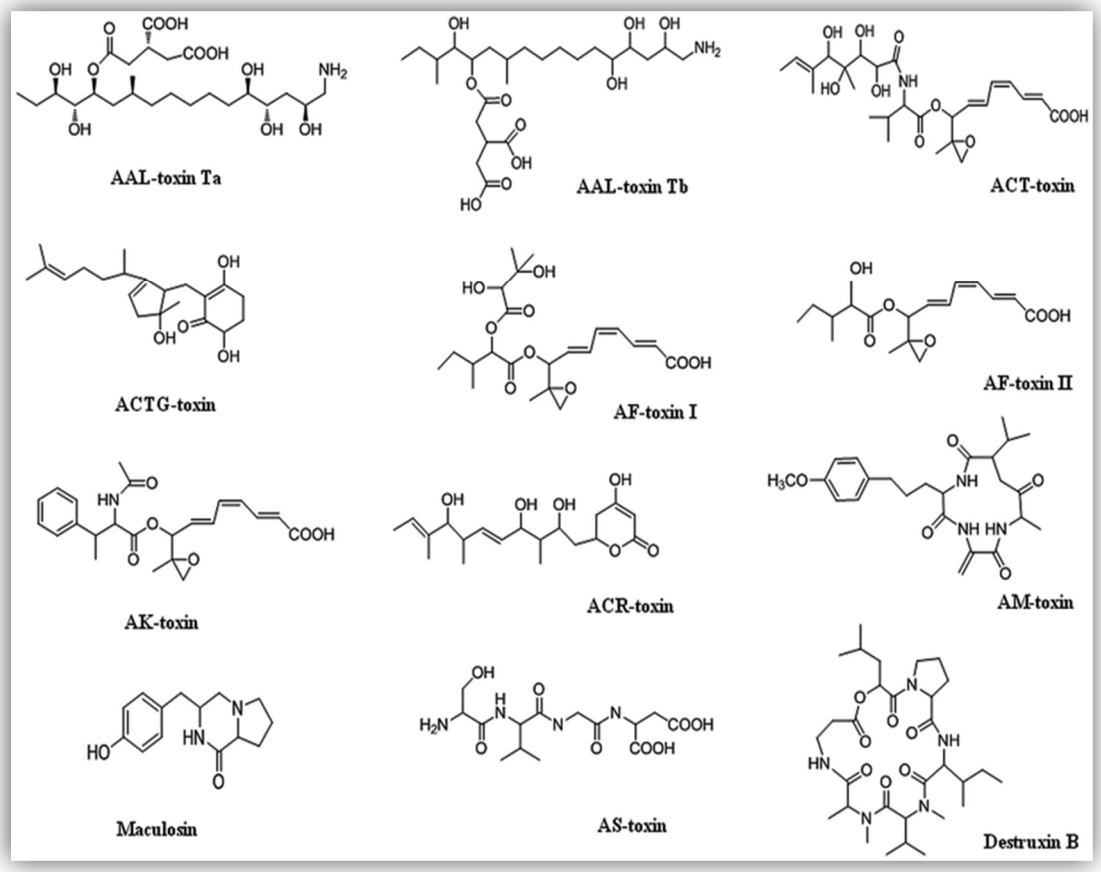


Figure 26. Chemical structures of HSTs produced by *Alternaria spp*

- **ACT Toxin:** A cyclic tetrapeptide host-selective toxin (with a 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety) produced by the tangerine (citrus) pathotype of *Alternaria alternata*. ACT toxin induces rapid electrolyte leakage and necrosis in citrus tissues of susceptible cultivars **(Ma et al., 2019).**
- **AM Toxin:** A cyclic peptide produced by the apple pathotype of *A. alternata* (*Alternaria mali*). The AM toxin is essential for Alternaria leaf spot in apple – *A. alternata* "produces the host-specific AM toxin, a key pathogenicity factor" **(Cao et al., 2024).**
- **Others:** Other host-selective toxins include AK toxin (Japanese pear pathotype), AF toxin (rough lemon pathotype), and various AK and AF analogs. All HSTs target specific hosts (often by disrupting membranes or mitochondria) and are indispensable for disease development on those plants **(Wang et al., 2022).**

b) Non-Host Selective Toxins (NHSTs): *(Figures 24 and 25)*

- **Alternariol (AOH) and Alternariol Monomethyl Ether (AME):** Dibenzo-α-pyrone polyketides produced by many *Alternaria* spp. They exhibit genotoxic and phytotoxic activity by inhibiting DNA and protein synthesis. The biosynthesis of AOH and AME requires the *pksJ* gene and a transcriptional regulator, *altR* **(Saha et al., 2012).**
- **Tenuazonic Acid (TeA):** A simple tetramic acid first identified in *A. tenuis*, produced by numerous *Alternaria* species. TeA inhibits protein synthesis and photosynthesis, exhibiting strong herbicidal activity against a variety of plants **(Wang et al., 2022).**
- **Tentoxin:** A cyclic tetrapeptide produced by several *Alternaria* species (*A. alternata*, *A. citri*, *A. longipes*, etc.) that specifically inhibits chloroplast F_1-ATPase (photophosphorylation). Tentoxin causes chlorosis (bleaching of leaves) in sensitive plants by targeting a subunit of the chloroplast ATPase **(Wang et al., 2022).**
- **Other NHSTs:** These include altenuene, altertoxins, zinniol, brefeldin A, and other polyketides or cyclic peptides. For instance, various cyclic peptides and macrolides (e.g., altertoxin I) and melanin precursors also contribute to host damage or interfere with host metabolism **(Wang et al., 2022).**

Each toxin class has a defined biosynthetic origin, often involving polyketide synthases (PKS) or non-ribosomal peptide synthetases (NRPS). For example, the AOH/AME biosynthetic cluster in *A. alternata* encodes a type I iterative PKS (PksJ) and associated tailoring enzymes **(Saha et al., 2012).** Many *Alternaria* toxins (including tentoxins and altertoxins) are derived from PKS-NRPS hybrid pathways or cyclic peptide synthetases. Similar to *Fusarium*, *Alternaria* toxins function as necrotrophic effectors: HSTs are required for host invasion, while NHSTs typically intensify disease symptoms and promote pathogen growth **(Wang et al., 2022).**

2.3.2. Cell Wall-Degrading Enzymes (CWDEs):

Alternaria secretes a variety of CWDEs to penetrate plant tissues. The genomes of *A. alternata*, *A. brassicicola*, and others encode numerous carbohydrate-active enzymes. Experimental evidence (e.g., in the citrus pathotype) shows that cutinases, cellulases, polygalacturonases, and other CWDEs are necessary alongside toxin production for full virulence (Ma H et al., 2019). For instance, deletion of *cutinase* genes (*AaCut3*, *AaCut7*) in the mandarin pathotype of *A. alternata* reduced cutinase activity and led to smaller lesions (Ma H et al., 2019). Key CWDEs include:

- **Cutinases** – degrade the plant cuticle (cutin), facilitating fungal penetration.
- **Cellulases** – break down cellulose microfibrils.
- **Pectinases** (e.g., polygalacturonases, pectate lyases) – degrade pectic polysaccharides in the middle lamella and cell wall.
- **Xylanases and other hemicellulases** – target hemicellulose components.
- **Proteases and lipases** – degrade structural wall proteins and cuticular waxes.

Studies have shown that in *A. alternata*, both host-specific toxins and CWDEs "are necessary for full virulence" (Ma H et al., 2019). Generally, CWDEs act synergistically with toxins: toxins kill or weaken host cells, and CWDEs macerate tissues to enable fungal spread.

2.3.3. Protein Effectors:

Alternaria secretes a variety of small proteins that modulate host responses. For example, *A. brassicae* (a necrotroph of Brassicaceae) encodes two Nep1-like proteins (NLPs), AbrNLP1 and AbrNLP2 (Duhan et al., 2021). AbrNLP2 is secreted into plant tissues and strongly induces necrotic cell death in both host (mustard) and non-host plants (Duhan et al., 2021). In the apple pathotype of *A. alternata*, exosome proteomics identified several secreted proteins associated with pathogenicity. Three of these proteins (AltABC, AltAO, and AltPDE) were confirmed to suppress apple defenses: when each was overexpressed in apple plants, transgenic leaves displayed increased sensitivity (Cao et al., 2024). These and other similar effectors (often small, uncharacterized secreted proteins) likely help the fungus overcome plant immunity, for example by interfering with signaling or degrading host defense proteins.

CHAPITRE II

1. Introduction:

This study is based on the assumption that the integration of silver nanoparticles (AgNPs), synthesized from the essential oils of *Aloysia citrodora*, will significantly enhance their biocidal properties.

Our primary objective is to evaluate the biocidal (antifungal) effect of these plant extracts formulated in nanoparticle form. More specifically, this study aims to:

- Synthesize silver nanoparticles using essential oils extracted from *Aloysia citrodora*.
- Quantify and compare the biocidal (antifungal) activity of nano-essential oils and/or pure essential oils against the tested pathogens, namely *Alternaria* sp. and *Fusarium* sp.

2. Biological Material

2.1. Origin of the Essential Oils

Samples of lemon verbena (*Aloysia citrodora*) leaves and flowers were harvested in October 2024 from a private farm located in Hattatba, Tipaza. Simultaneously, sage leaf samples were collected from various locations in the Mitidja region of Blida, also in October 2024.

Immediately after harvesting, the fresh leaves were cleaned and sorted, retaining only healthy leaves for extraction. The essential oils were then extracted through steam distillation (hydrodistillation) using a stainless-steel (INOX) still. This cylindrical still measured 80 cm in height and 30 cm in diameter and was equipped with a double jacket and a perforated plate (figure 27). The extraction lasted 3 hours.

The hydrodistillation process relies on the volatility and water-insolubility of essential oils. As the steam passes through the plant material, it ruptures the cells and carries the essential oil molecules. The resulting steam-oil mixture is then directed to a condenser, where it comes into contact with cold water and liquefies. The condensate obtained—a mixture of water and essential oil—is collected in a Florentine flask. The essential oil, being less

dense and immiscible with water, floats on the surface and is separated from the remaining water, referred to as hydrosol or floral water.

Following this step, an additional decantation was performed using a separatory funnel to separate the two immiscible phases: the organic phase (essential oil) and the aqueous phase (floral water). The essential oils obtained were stored in a refrigerator at 4°C.

These essential oils were then used to prepare solutions of varying concentrations (100%, 50%, and 25%) using distilled and sterilized osmosed water. The same preparation procedure was applied to the solutions synthesized with silver nanoparticles intended for antifungal testing against phytopathogenic fungi.



Figure 27. Device used for steam distillation extraction of verbena and sage

2.2. Phytopathogenic Fungal Strains

The fungal strains used in this study, namely *Alternaria* sp. and *Fusarium* sp., were obtained in May 2025 from the Boufarik Plant Protection Station (S.I.N.P.V) in the Blida province.

These strains were replicated in the laboratory to generate sufficient biomass for evaluating the biocidal effects of the solutions we prepared. This replication step is crucial to ensure the reliability of our experiments on antifungal activity.

3. Plant-Based Synthesis of Silver Nanoparticles from Essential Oil:

3.1. Preparation of Silver Nitrate [$AgNO_3$]

Silver nitrate [$AgNO_3$] is a white crystalline compound with a molecular weight of 169.87 g/mol and is highly soluble in water. It serves as the primary source of silver ions (Ag^+), which are essential for synthesizing silver nanoparticles (AgNPs). When reduced, Ag^+ is converted into elemental silver (Ag), facilitating nanoparticle formation.

The use of $AgNO_3$ allows for better control over the size and morphology of the nanoparticles, as its concentration directly influences their growth and aggregation. Furthermore, the stability of silver nitrate solutions makes it an ideal precursor for green synthesis of AgNPs (silver nanoparticles) particularly when using natural reducing agents such as plant extracts or essential oils.

3.2. Green Synthesis of Silver Nanoparticles Using Essential Oils

The green synthesis of silver nanoparticles using essential oils was carried out according to the methodology described by **Matheus Vinicius de Oliveira, Brisola Maciel, et al. (2019).**

4.1.1. Preparation of the Silver Nitrate Solution (Figure 28)

a. Preparation of $AgNO_3$ Stock Solutions

The required amount of silver nitrate ($AgNO_3$) was calculated based on its molar mass (approximately 169.87 g/mol). To prepare a 0.31 mmol/L solution, 5.23 mg of silver nitrate powder were weighed.

b. Dissolution

The measured silver nitrate was added to approximately 50 mL of distilled water and another 50 mL of reverse osmosis (RO) water in the same clean beaker. The mixture was stirred thoroughly until the silver nitrate was fully dissolved.

4.1.2. Procedure for AgNP Synthesis (Figure 28)

a. Preparation of Working Essential Oil Solutions

One milliliter of *Aloysia citrodora* essential oil was measured and diluted with 40 µL of Tween-20, 5 mL of ethanol, and a combined total of 8 mL of distilled and reverse osmosis (RO) water. This mixture was formulated to improve essential oil dispersion and promote its integration into the aqueous medium. The same procedure was followed for sage oil.

b. Mixing the Solutions

Silver nanoparticle synthesis was performed by slowly adding 4 mL of the diluted essential oil solution dropwise to 60 mL of preheated silver nitrate solution. The mixture was kept under vigorous stirring and heating until it reached boiling point, and then the stirring continued for an additional 30 minutes. The successful formation of silver nanoparticles was indicated by the appearance of a yellow-brown color in the solution, confirming the reduction of silver ions and nanoparticle formation.

c. Reaction Monitoring

The absorbance of each solution was measured at regular intervals (e.g., hourly) using a UV-Vis spectrophotometer in wavelength-scan mode. The presence of absorption peaks around 420–450 nm was checked to confirm nanoparticle formation.

d. Centrifugation

Reaction mixtures were centrifuged at 5000 rpm for 10 minutes to separate the formed nanoparticles from the solution. The supernatant was carefully discarded without disturbing the nanoparticle pellet at the bottom.

e. Washing the Nanoparticles

The nanoparticle pellets were resuspended in 10 mL of RO water to remove any unreacted materials. The mixture was centrifuged again under the same conditions for another 10 minutes. The supernatant was removed using a micropipette. This washing step was repeated twice more, including a final resuspension in 10 mL of distilled water for pellets derived from the distilled water batch, to ensure optimal purification of the nanoparticles. A vortex mixer was used during each resuspension step to prevent pellet aggregation.

f. Final Suspension

The purified nanoparticle pellets were resuspended in a small volume (e.g., 1 to 5 mL) of reverse osmosis water or buffer solution for further characterization or storage, using an ultrasonicator set to 30% amplitude for 2 minutes.

g. Storage

The synthesized silver nanoparticles were stored in a cool, dark environment (preferably at 4°C) in sterile, light-resistant containers to prevent photodegradation.

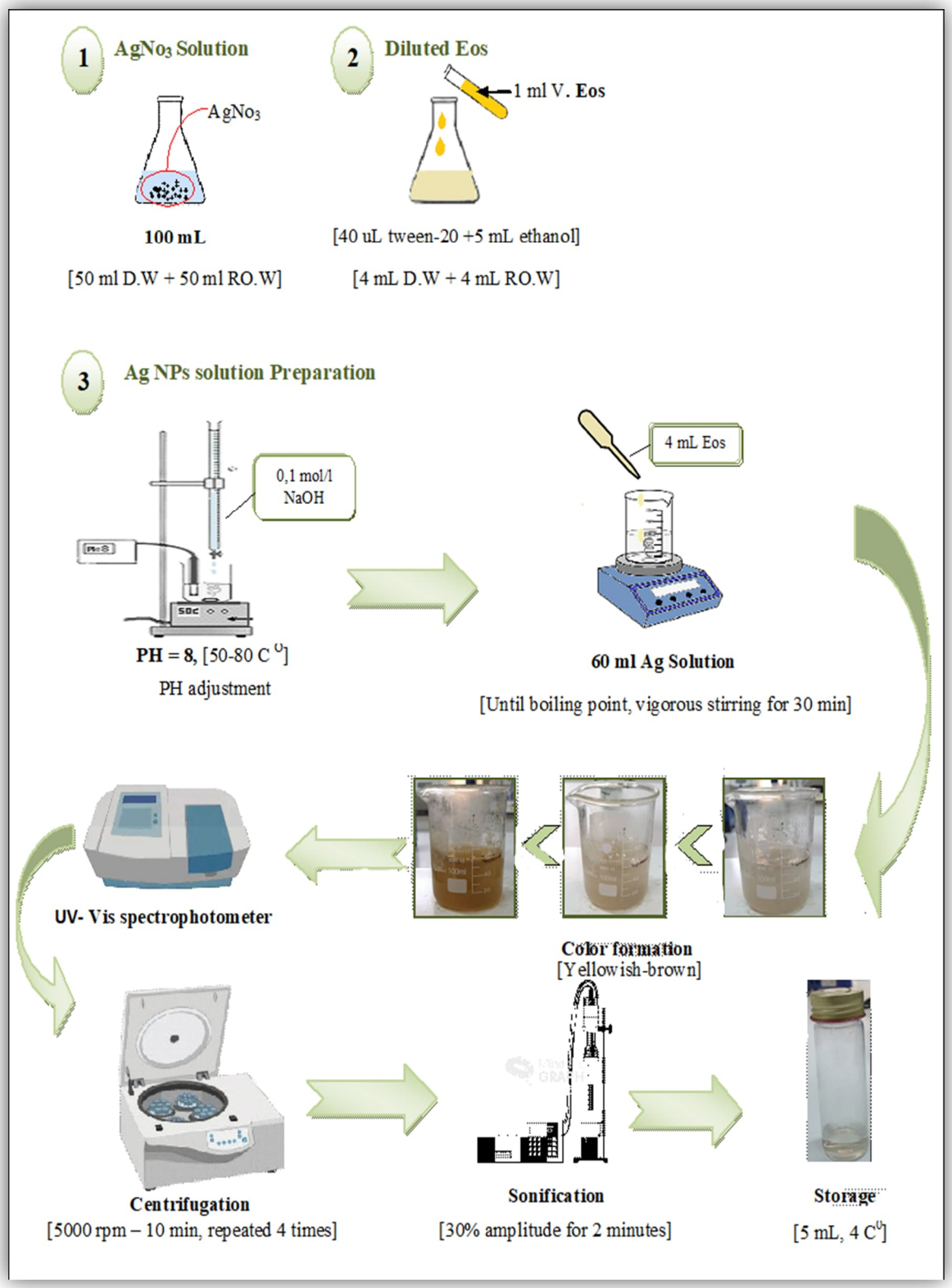


Figure 28 . Green Synthesis of Silver Nanoparticles Using Essential Oils

4. Testing anti-fungus activity of silver Nanoparticles :

4.1. Isolation and identification of phytopathogens

The biocidal effect experiments were conducted in the teaching laboratory of the Ouezra Campus at Yahia Fares University, Médéa.

Before proceeding with the tests, macroscopic and microscopic observations were conducted on the replicated fungal strains. This step was crucial to confirm the purity and identification of the species used as biological models in our study.

4.2. Preparation of Solutions and Implementation of Antifungal Tests (Figure 29)

This section outlines the methodology used to evaluate the antifungal activity of the essential oils (EOs) of lemon verbena (*Aloysia citrodora*) and clary sage (*Salvia sclarea*), as well as the solutions prepared with silver nanoparticles (AgNPs), using both negative and positive controls.

4.2.1. Negative Control

The negative control was conducted using standard PDA (Potato Dextrose Agar) medium, without the addition of any antifungal agents, including the two essential oils or their AgNP-formulated solutions. This medium allowed for the observation of normal, unimpeded growth of *Fusarium* sp. and *Alternaria* sp. fungal strains, serving as a reference point to assess the effectiveness of antifungal treatments.

4.1.3. Positive Control

Positive controls involved the preparation and testing of essential oil solutions and silver nanoparticles according to a defined protocol.

a) Preparation of Essential Oil Solutions (EOs of Verbena and Sage)

The EOs of *Aloysia citrodora* (lemon verbena) and *Salvia sclarea* (sage) were tested at three concentrations: 100 µL/mL, 50 µL/mL, and 25 µL/mL.
For the 50% and 25% concentrations, the EOs were first emulsified with ethanol (5 mL), then diluted with distilled water (D.W.) to reach the desired final concentrations (e.g., 2

mL of EO emulsified with 2 mL D.W. for 50%, and 2 mL with 6 mL D.W. for 25%). All emulsions were vortexed to ensure perfect homogeneity.

For application, sterile PDA medium (20 mL/plate) was poured into Petri dishes. Once solidified, three wells of 6 mm in diameter were created in each plate. Each well was filled with 50 µL of the corresponding EO emulsion. Plates were left at room temperature for 5 minutes to allow pre-diffusion of the compounds into the medium before fungal inoculation.

b) Preparation of Silver Nanoparticle (AgNP) Solutions

AgNPs were also prepared and tested at three concentrations: 100 µL/mL, 50 µL/mL, and 25 µL/mL. Dilutions were made by mixing the concentrated AgNP solution with distilled water (e.g., 2 mL of concentrated solution with 2 mL D.W. for 50%, and 2 mL with 6 mL D.W. for 25%). All dilutions were vortexed for uniform dispersion.

The application protocol was similar to that of the EOs: sterile PDA medium was poured into Petri dishes, and after solidification, three 6 mm wells were punched. Each well received 50 µL of the respective AgNP solution. Plates were left at room temperature for 5 minutes to allow initial diffusion of the nanoparticles.

4.3. Inoculation and Evaluation

After the test solutions (EOs or AgNPs) were added and allowed time for pre-diffusion, all plates (negative and positive controls) were inoculated with a 5 mm diameter disc of an active fungal culture, placed at the center. Plates were incubated at 25–28 °C. Antifungal activity was assessed by measuring fungal growth. Each test was repeated five times to ensure reproducibility and reliability of results. The interpretation of the results was based primarily on measuring the radial growth diameter of the fungal mycelium over time in order to calculate the inhibition rate.

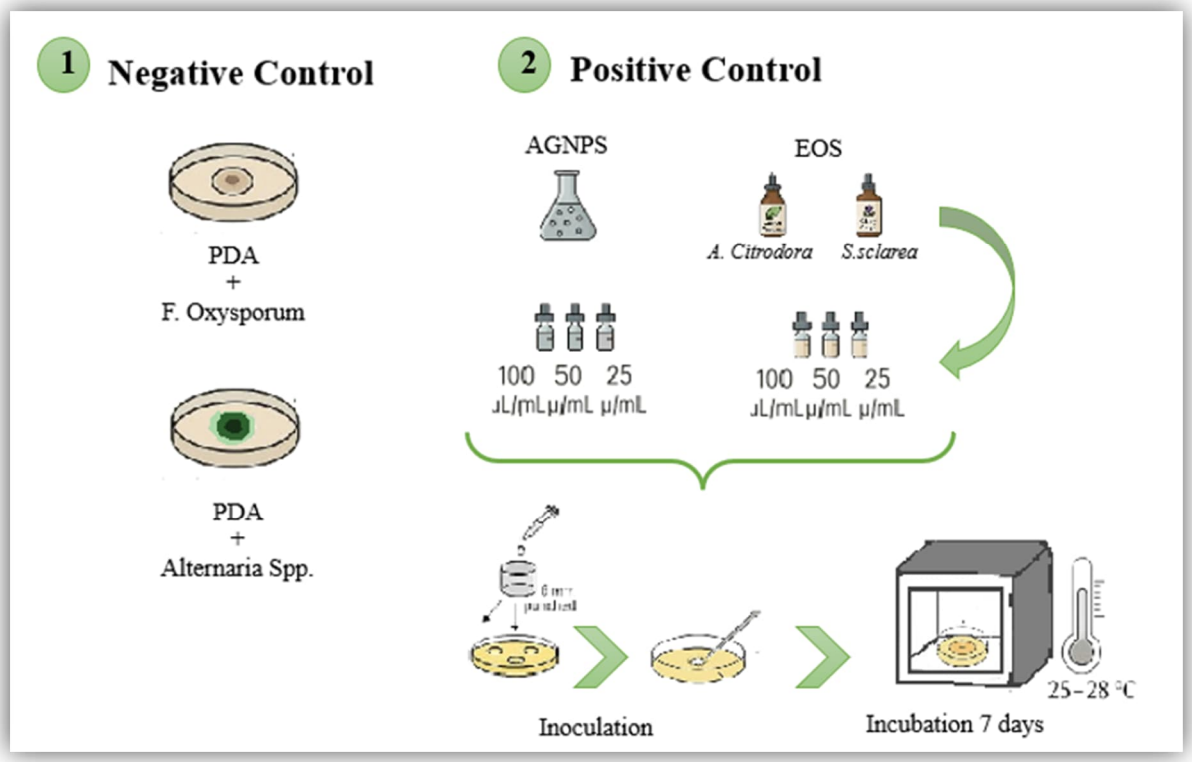


Figure 29. in-vitro antifungal activity testing of A. citrodora & S. sclarea Eos and AGNPS.

4.3.1. Evaluation of Mycelial Growth Inhibition Percentage

The antifungal effect of the EOs on mycelial growth was expressed as the percentage of mycelial growth inhibition (%) and calculated using the following formula (Hu et al., 2019):

$$\text{Inhibition \%} = [(C-T)/(T)] \times 100$$

Where:

C = Average colony diameter in the control group (cm).

T = Average colony diameter in the treated group (cm).

4.3.2. Evaluation of Mycelial Growth Percentage

This provides a standardized comparison of fungal vigor in treated vs. untreated samples. The calculation was based on the maximum possible radial growth corresponding to the full diameter of the Petri dish (8.5 cm), and expressed using the following formula:

Growth % = (measured diameter / diameter of the petri dish) ×100

5. Statistical Analysis

We evaluated the biocidal effectiveness of essential oils from lemon verbena *(Aloysia citrodora*) and clary sage *(Salvia sclarea*), as well as silver nanoparticles (AgNP) biosynthesized from *Aloysia citrodora* essential oil. The impact of these substances on the mycelial growth of Fusarium oxysporum and Alternaria sp. was quantified by calculating the inhibition percentage, allowing for the determination of the Minimum Inhibitory Dose (MID).

The raw results, including graphs and tables, were generated using Excel software.

For the statistical analysis of significant differences between treatment groups, we used **PAST software (Hammer, 2023).** The calculations involved the **Kruskal-Wallis** test, a nonparametric test suitable for comparing more than two independent groups when the data do not follow a normal distribution. When the **Kruskal-Wallis** test indicated a significant overall difference, **a post-hoc Dunn** test was applied. This nonparametric test is specifically used for pairwise comparisons between groups, enabling precise identification of which treatments differ significantly from each other in terms of biocidal effectiveness.

CHAPITRE III

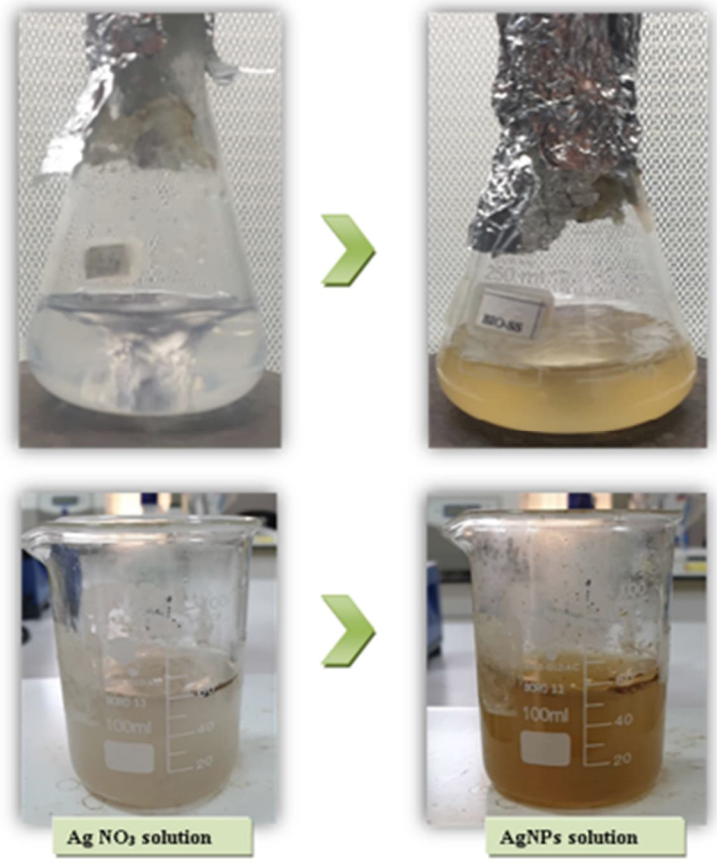
This section of the study aims to present our experimental findings, which highlight significant advances in the fight against fungal phytopathogens. Through analyses and graphical presentations, the antifungal potential of these natural substances and silver nanoparticles (AgNPs) is clearly demonstrated. The study is divided into two main parts.

First, we present the synthesis of silver nanoparticles (AgNPs) and their stability, which influences the preparation of various biocidal test formulations derived from lemon verbena essential oil and ensures their effectiveness in different applications.

Second, we delve into the antifungal activity of two strains, Fusarium sp. and Alternaria sp., which were used as model phytopathogens for these evaluations. We will present the results of tests conducted with essential oils extracted from Lemon Verbena (Aloysia citrodora) and Clary Sage (Salvia sclarea), both known for their bioactive properties. In parallel, we will evaluate the antifungal efficacy of the biosynthesized silver nanoparticles.

1. Characterization of Silver Nanoparticles (AgNPs) by UV-Visible Spectroscopy

The characterization of nanoparticles is a crucial step in understanding their physical properties and predicting their behavior. Among the available techniques, UV-Visible (UV-Vis) spectroscopy is a rapid and effective method for confirming the formation of metallic nanoparticles and obtaining preliminary information regarding their size and morphology.



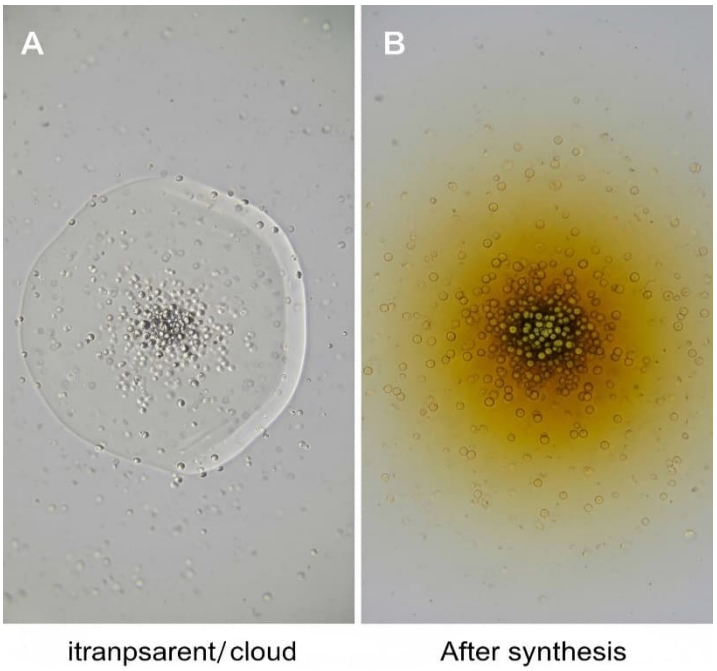


Figure 30. Illustration of the color change in the substance containing Aloysia citrodora essential oil and silver nanoparticles (AgNPs) before (A) and after (B) synthesis (image generated by artificial intelligence).

The synthesis process of silver nanoparticles (AgNPs) is initially indicated by a visual change in the color of the solution (Figure 30), shifting from a transparent or cloudy state to a yellowish-brown hue. This color shift, illustrated in Figure 30, is a key indicator of AgNP formation. It is attributed to the excitation of surface plasmon resonance (SPR) (Kumari et al., 2022), an optical phenomenon in which a collective oscillation of conduction electrons occurs on the surface of the nanoparticles following the bioreduction of silver ions (Ag^{+}) in aqueous suspension (Nagajyothi et al., 2011). This phenomenon has also been reported in several other studies that observed a color change in the reaction mixture during the biosynthesis of silver nanoparticles from species such as Oscillatoria, Allium sativum, Zingiber officinale, Capsicum frutescens, and Azadirachta indica (Otunola et al., 2017; Adebayo-Tayo et al., 2019; Kumari et al., 2022).

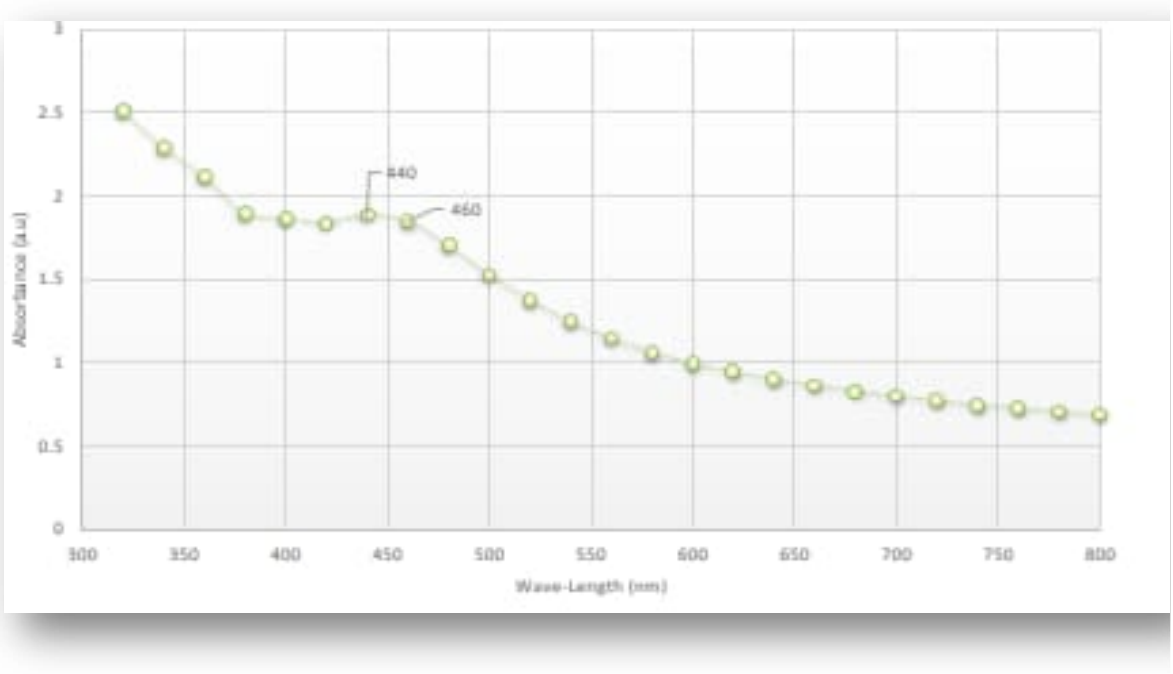


Figure 31 .UV Spectrum (2019) of silver nanoparticles (AgNPs) biosynthesized using Aloysia citrodora essential oil (EO).

This visual observation was confirmed through a detailed analysis using a UV-Vis Optima spectrophotometer (SP-3000 Nano). The spectra obtained revealed the presence of a single, relatively symmetrical SPR (Surface Plasmon Resonance) peak within the range of 440 to 460 nm (Figure 31). The position of this peak is indicative of nanoparticle size: a peak around 440 nm suggests the presence of smaller nanoparticles, while a shift toward

460 nm is generally associated with larger particles and potentially a broader size distribution (Sarkar et al., 2010).

Furthermore, analysis of the full width at half maximum (FWHM) of the SPR peak showed relatively broad bands. This characteristic indicates a polydisperse population of AgNPs, meaning the nanoparticles exhibit a diversity of shapes and sizes (Maciel et al., 2019). This wide distribution was particularly pronounced in the AgNPs synthesized using Aloysia citrodora essential oil, suggesting that the synthesis method or the specific reducing agents in this essential oil may influence the uniformity of the nanoparticles produced.

2. Identification of Fungal Phytopathogenic Strains

Before evaluating the effectiveness of our antifungal treatments, a fundamental step in our study involved identifying and confirming the purity of the fungal phytopathogenic strains used. Accurate identification is crucial to ensure the reliability of our results and to confirm that the observed effects can be directly attributed to interactions with the target species, namely Fusarium sp. and Alternaria sp. This section presents the methods and observations used to authenticate these microorganisms, which serve as a solid foundation for all subsequent experiments on their growth and sensitivity to biocidal agents.

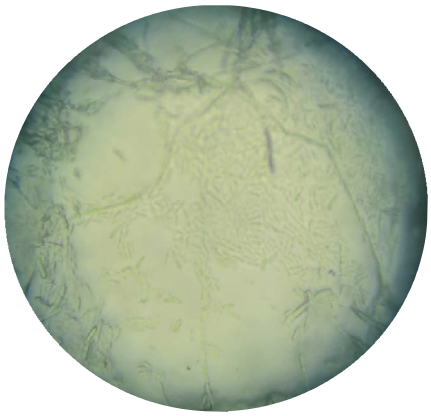
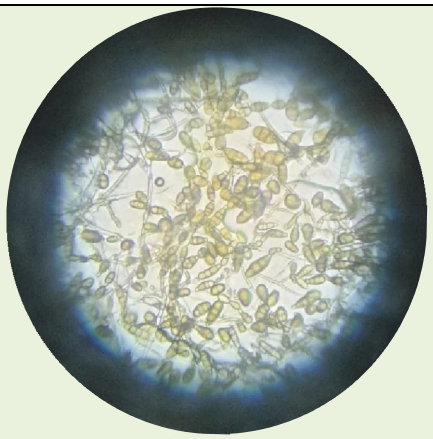
Macroscopic and microscopic observations of the two strains studied are summarized in Table 5.

For the Fusarium sp. strain, microscopic examination revealed a septate, hyaline (colorless) mycelium. We also observed sickle-shaped, multicellular macroconidia (with 3 to 5 septa), as well as oval to elliptical, unicellular microconidia. The presence of chlamydospores was also noted. With the support of specialists from the Plant Protection Station of Boufarik (S.I.N.P.V) in Blida Province, the species was formally identified as Fusarium oxysporum.

However, despite detailed microscopic observations of the Alternaria sp. strain—which revealed a dark, septate mycelium and large muriform conidia (with both transverse and

longitudinal septa), formed in chains or singly, and typically club-shaped or obclavate with a beak-like tip—the specialist was unable to confirm the exact Alternaria species.

Table 4: Observations macroscopiques et microscopiques de *Fusarium oxysporum* et *Alternaria sp.*

Genus	Macroscopic Identification	Microscopic Identification
	Macroscopic Observation	**Microscopic Observation (G × 40)**
Fusarium oxysporum	 - Cottony texture -White to pinkish or violet pigmentation -No water droplets (no exudate	 - Septate, hyaline (colorless) mycelium - **Macroconidia**: sickle-shaped, multicellular with 3–5 septa - **Microconidia**: oval to elliptical, unicellular - **Chlamydospores** present
Alternaria spp.	 - Cottony to velvety - Dark green to blackish gray - Concentric rings and irregular margins	 - Dark, septate mycelium - **Conidia**: large, muriform (both transverse and longitudinal septa) - Formed in chains or singly - Club-shaped or obclavate with a beak-like tip

Accurate identification of fungal phytopathogen species is a crucial step in plant pathology for understanding disease epidemiology, implementing effective control strategies, and ensuring food security. However, this task is often challenging and may encounter significant difficulties, especially with ubiquitous and morphologically variable genera such as Fusarium and Alternaria (Dupont, 2023).

Traditionally, identification relied mainly on morphological and cultural characteristics observed under microscopy and on culture media (Leslie & Summerell, 2006). For Fusarium, for instance, discriminating features include the shape and size of macroconidia and microconidia, the presence of chlamydospores, mycelium coloration, and pigment production. However, these traits can vary depending on culture conditions (e.g., temperature, nutrient media) and exhibit high phenotypic plasticity, making accurate identification difficult for non-specialists or in the absence of reference strains (Nelson et al., 1983; Jeunot, 2005; Heit, 2015). Moreover, many Fusarium species form cryptic species complexes (e.g., the F. oxysporum or F. solani complexes; F. oxysporum f. sp. lycopersici), in which genetically distinct species share nearly identical morphologies but differ in pathogenic profiles and fungicide sensitivities (Michielse & Rep, 2009; O'Donnell et al., 2000). For this reason, even for specialists, species-level identification may require supplementary tools.

The genus Alternaria presents similar, if not greater, challenges. Although certain characteristics—such as the shape and size of muriform conidia, their arrangement in chains or singly, and the presence of long conidial beaks—are considered diagnostic, there is considerable intraspecific morphological variability (Lawrence et al., 2013). Sporulation can be difficult to induce in vitro for some species, and morphological descriptions often fail to clearly delineate species boundaries. As with Fusarium, Alternaria includes many cryptic species complexes—for example, the Alternaria alternata complex, which comprises morphologically indistinguishable but genetically distinct species with varying host specificities (Woudenberg et al., 2015). Furthermore, toxins produced by certain Alternaria species, which are critical to their pathogenicity, are not reliably correlated with morphological traits.

In response to these challenges, the identification of fungal phytopathogen species has gradually shifted toward molecular approaches. Sequencing of marker genes such as

the ITS (Internal Transcribed Spacer) region of ribosomal DNA, the elongation factor 1-alpha (TEF1-α) for Fusarium, or the RNA polymerase II largest subunit (RPB2) region for Alternaria has become standard practice (Leslie & Summerell, 2006; Woudenberg et al., 2015). These markers offer improved taxonomic resolution and enable differentiation among species within cryptic complexes. However, even with molecular tools, the absence of comprehensive reference databases or the presence of intraspecific variation can make species-level identification uncertain, often requiring multilocus approaches or support from expert reference centers.

While morphological observations remain a useful and rapid preliminary step for guiding identification, they are frequently insufficient for accurate species determination, particularly in complex genera like Fusarium and Alternaria. A combined approach using morphology, molecular tools, and expert validation is essential to overcome these challenges and ensure reliable identification of fungal phytopathogens.

3. Evaluation of the Antifungal Biocidal Effects on Fusarium sp. and Alternaria sp.

In this section, we explore the impact of various prepared solutions—including essential oils of verbena and sage, as well as silver nanoparticles—on the mycelial growth of two important fungal strains: Fusarium oxysporum and Alternaria sp. These fungi are well-known as plant pathogens and agents of crop spoilage. The goal is to quantify the antifungal efficacy of our treatments by precisely measuring their capacity to inhibit the development of these microorganisms. The results presented here will help assess the potential of these solutions as biocidal agents, offering prospects for more eco-friendly and effective crop protection strategies.

3.4. Evaluation of the Influence of the Tested Solutions on the Mycelial Growth of Fusarium sp. and Alternaria sp.

To assess the influence of our solutions on the mycelial growth of Fusarium oxysporum and Alternaria sp., we tested sterilized solutions based on essential oils extracted from

Verbena (Aloysia citrodora) and Sage (Salvia sclarea). We also included silver nanoparticles (AgNPs) biosynthesized using Aloysia citrodora essential oil.
Each solution was tested at three different concentrations to determine the minimum inhibitory dose (MID):

- **100% Dose (D100%):** Corresponds to the raw, undiluted solution.

- **50% Dose (D50%):** Prepared by mixing equal volumes of the raw solution and sterile distilled water (V_{raw solution} + V_{sterile distilled water}).

- **25% Dose (D25%):** Prepared by mixing one quarter of the raw solution with three quarters of sterile distilled water (1/4 V_{raw solution} + 3/4 V_{sterile distilled water}).

Results were expressed as the average of four replicates for each dose. Mycelial growth was monitored on the 3rd, 5th, and 7th days after application, with growth measured in centimeters.

Figures 32 and 33 show the macroscopic observations of mycelial growth development in the control strains of Fusarium oxysporum and Alternaria sp. These controls served as references for evaluating the effectiveness of the various treatments.

We then compared the effects of the silver nanoparticles (AgNPs) biosynthesized from Aloysia citrodora essential oil, as well as the effects of essential oils extracted from Verbena (Aloysia citrodora) and Sage (Salvia sclarea). These treatments were applied at varying concentrations (D100%, D50%, and D25%), and mycelial growth was quantified on the 3rd, 5th, and 7th days following application.

Mycelial growth was observed in all Petri dishes, including those treated, throughout the entire experimental period (Figures 34 to 39). To determine significant differences between the mycelial growth observed on the 3rd, 5th, and 7th days, a Kruskal–Wallis test was conducted. This was followed by a post-hoc Dunn's test.

3ed DAY 5thDAY 7th DAY



Figure 32. Macroscopic observation of mycelial growth development of *Fusarium oxysporum* in the control group.

3ed DAY 5thDAY 7th DAY

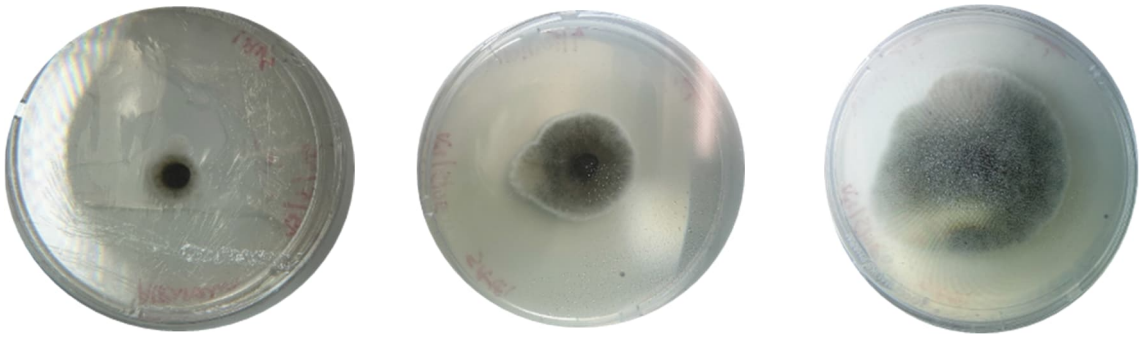


Figure 33 .Macroscopic observation of mycelial growth development of *Alternaria sp.* in the control group

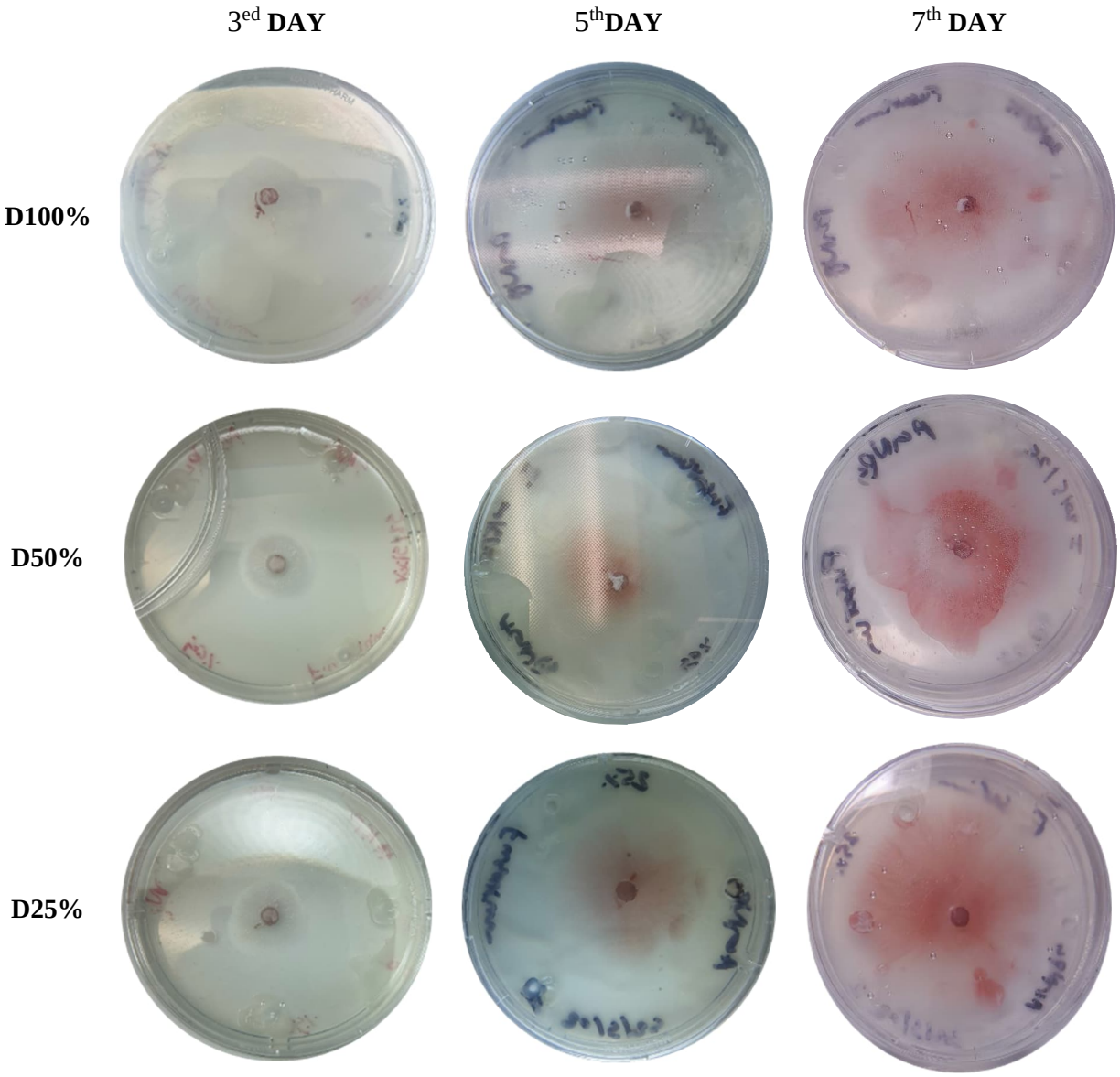


Figure 34. Macroscopic observation of the antifungal activity of *Fusarium oxysporum* under different concentrations (D100%, D50%, and D25%) of AgNP solutions biosynthesized using *Aloysia citrodora*.

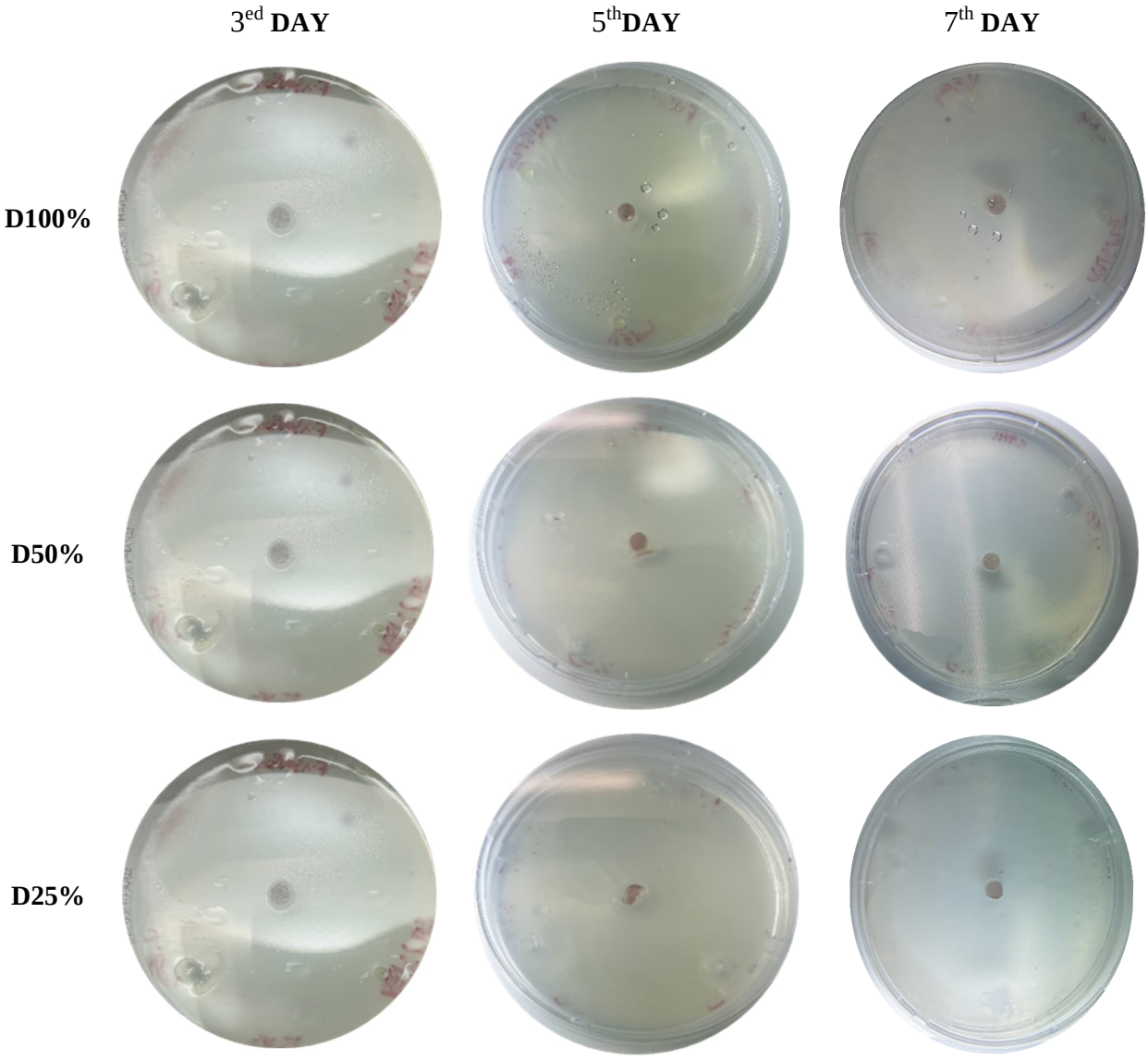


Figure 35 Macroscopic observation of the antifungal activity of *Fusarium oxysporum* under different concentrations (D100%, D50%, and D25%) of *Aloysia citrodora* essential oil.

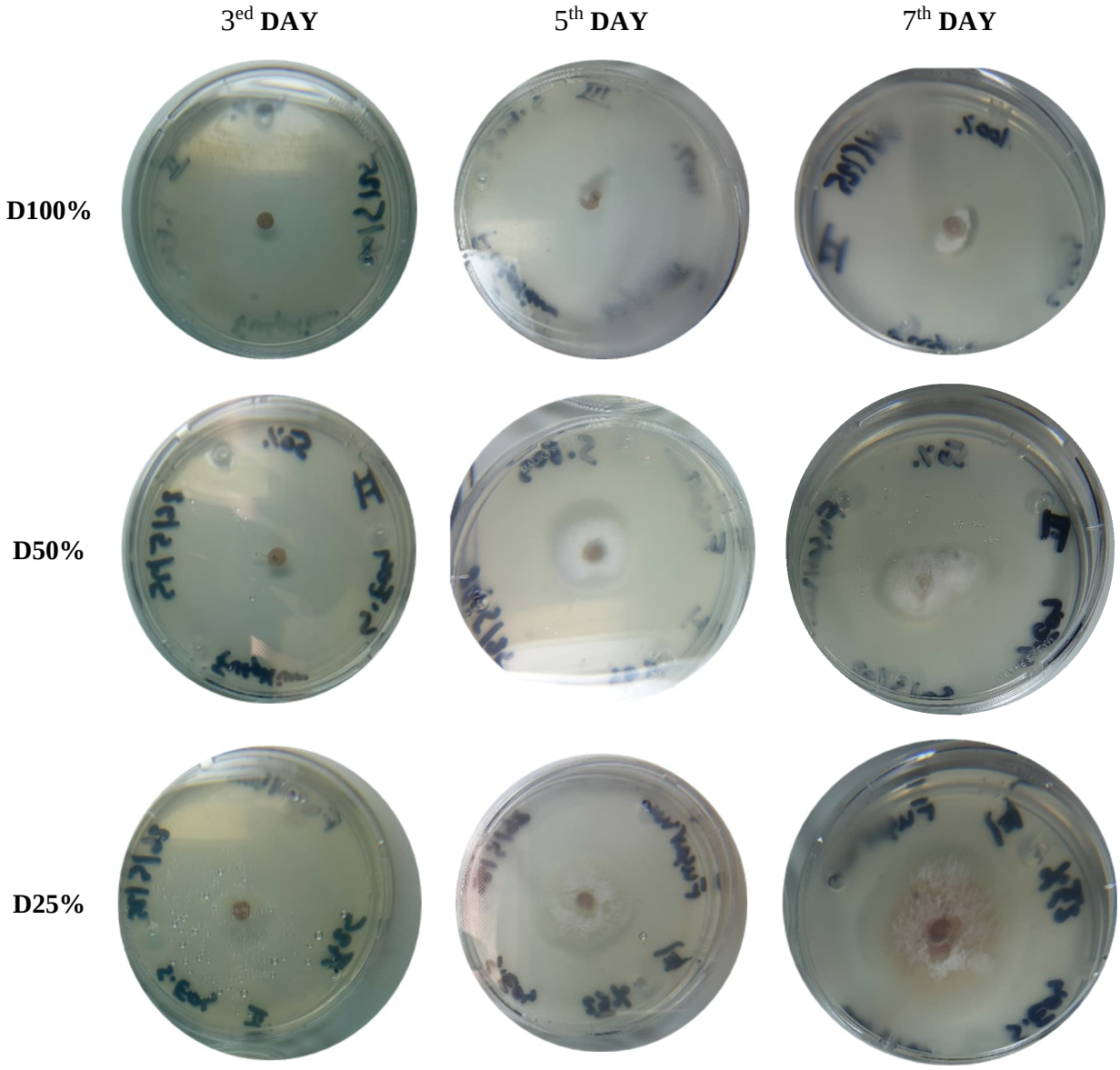


Figure 36: Macroscopic observation of the antifungal activity of *Fusarium oxysporum* under different concentrations (D100%, D50%, and D25%) of *Salvia sclarea* essential oil.

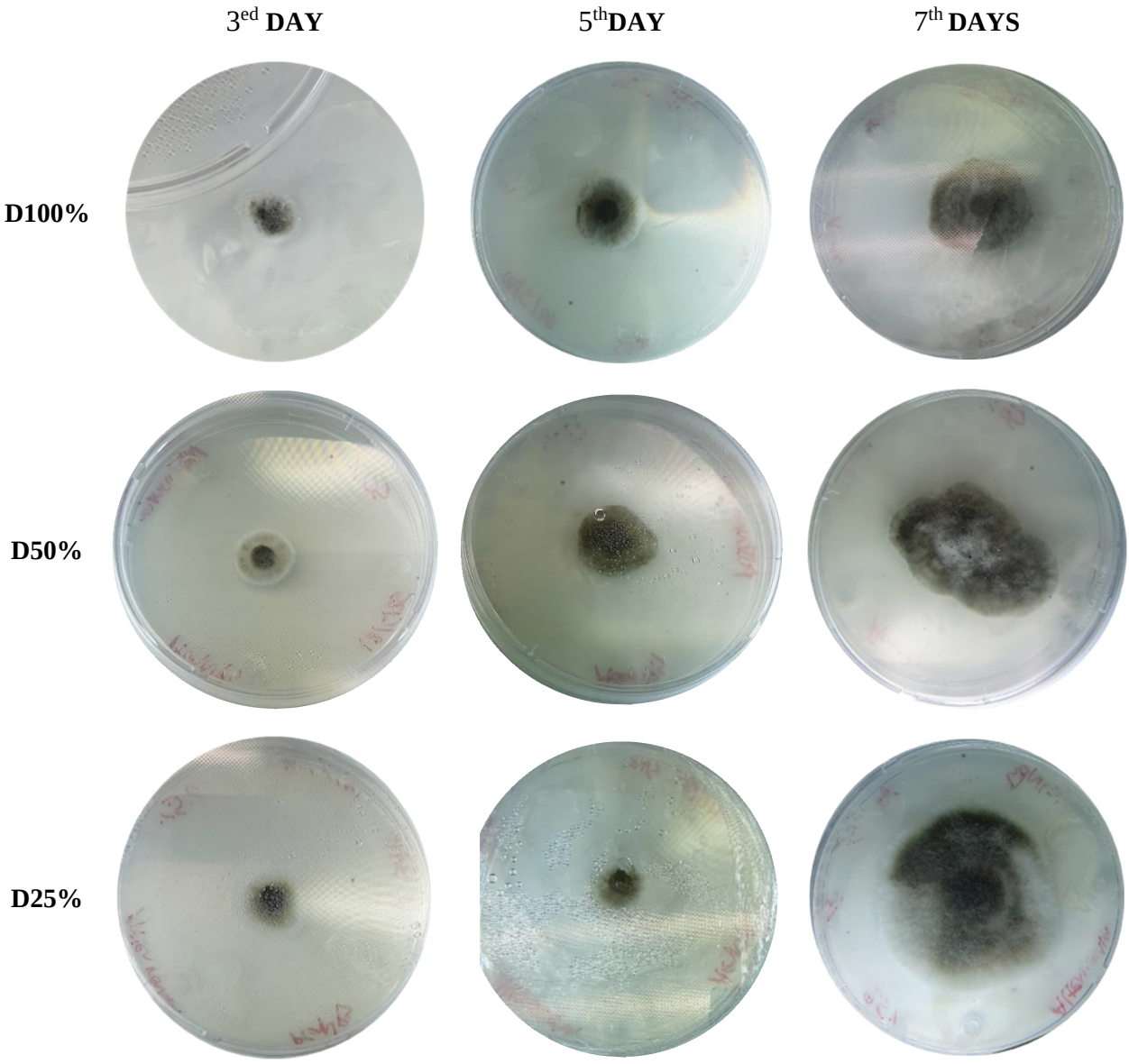


Figure 37. Macroscopic observation of the antifungal activity of *Alternaria sp.* under different concentrations (D100%, D50%, and D25%) of AgNP solutions biosynthesized using *Aloysia citrodora*.

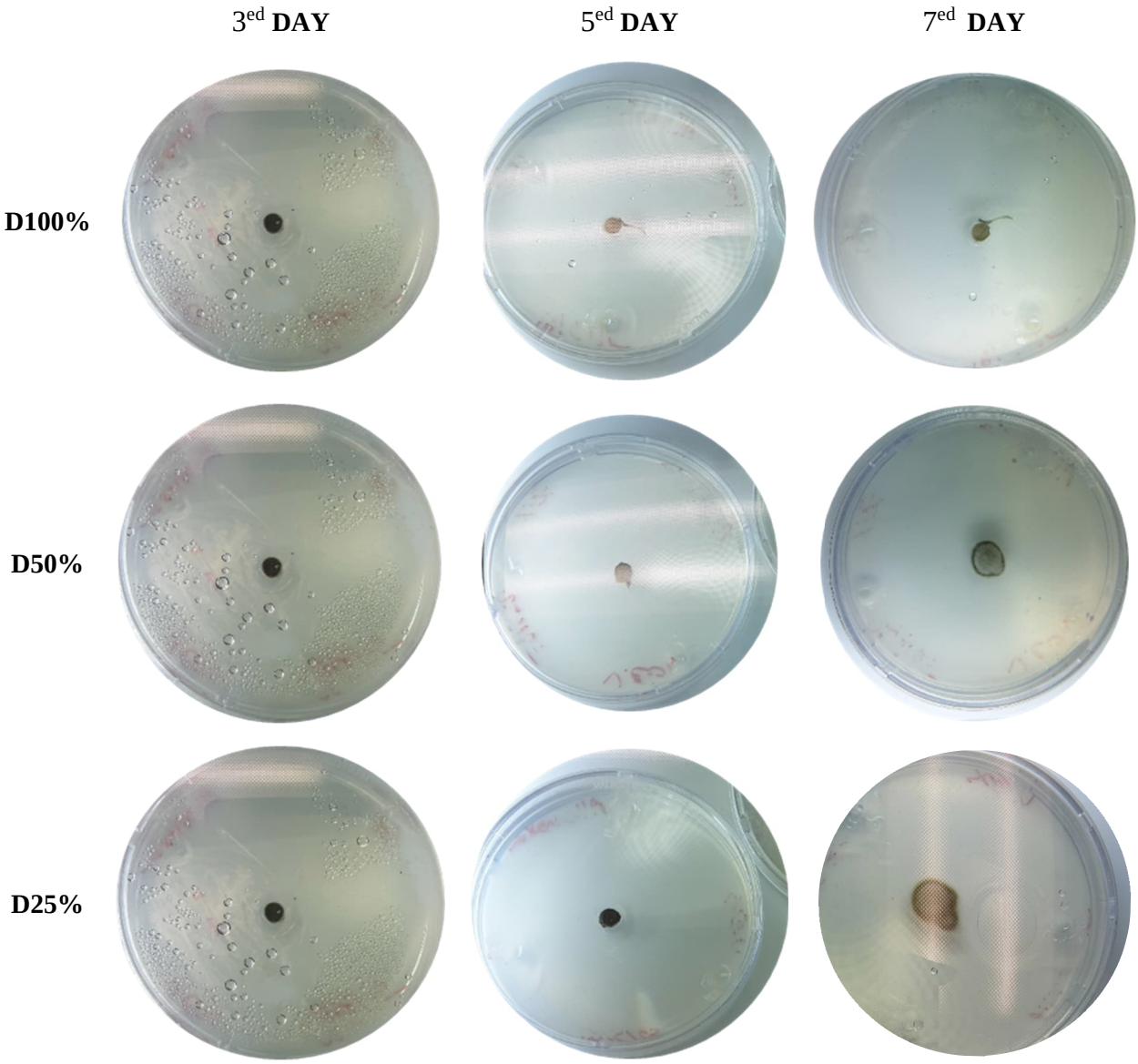


Figure 38 Macroscopic observation of the antifungal activity of *Alternaria sp.* under different concentrations (D100%, D50%, and D25%) of *Aloysia citrodora* essential oil.

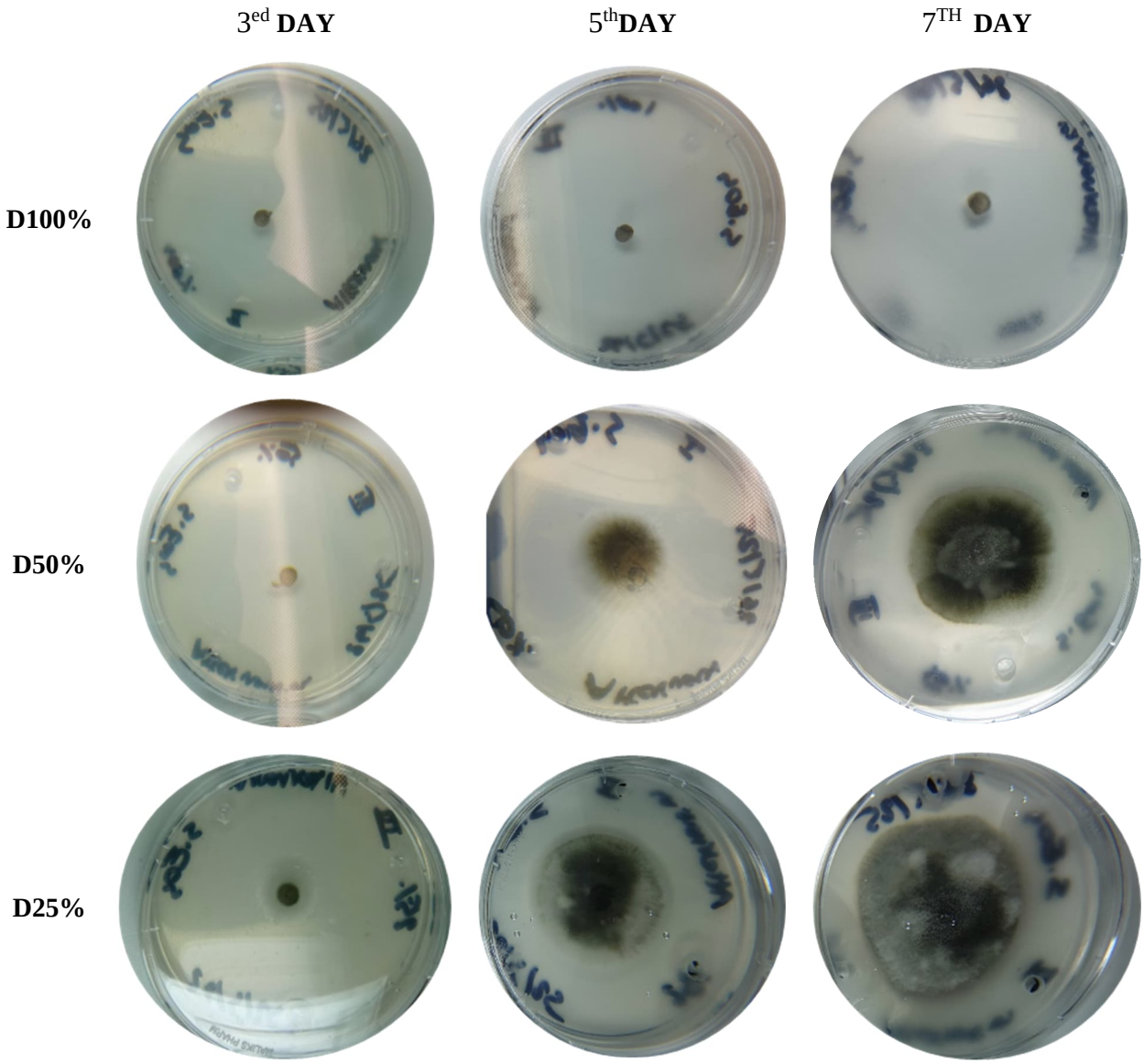


Figure 39 Macroscopic observation of the antifungal activity of *Alternaria sp.* under different concentrations (D100%, D50%, and D25%) of *Salvia sclarea* essential oil.

Identify the pairs of groups that are significantly different. The detailed results of these statistical analyses are presented in Table 6, accompanied by Figure 40, which highlights these findings.

Table 5: Variation in mycelial growth of treatments over time (by day).

	Kruskal-Wallis Test	Dunn's post-hoc test		
		3edDay	5thDay	7th Day
3ed Day	1.78 X 10^{-3}**	/	4.54 X 10^{-2}**	3.89 X 10^{-3}**
5th Day		4.54 X 10^{-2}**	/	1.81 X 10^{-3}**
7th Day		3.89 X 10^{-3}**	1.81 X 10^{-3}**	/

Asterisks indicate statistically significant differences at the 5% level (*) or highly significant differences at the 0.1% level (**).

The results of the statistical analysis using the Kruskal–Wallis test revealed a highly significant difference in mycelial growth between the various treatments applied in the Petri dishes, including the controls. This observation applies to both *Fusarium oxysporum* and *Alternaria sp.* across all time points (3rd, 5th, and 7th days), with a p-value of $P = 1.78 \times 10^{-3}$. Such a result indicates that, overall, the treatments had a significant impact on fungal growth compared to the controls, and that there were also significant differences among the treatments themselves.

To further investigate these findings, a Dunn's post-hoc test was conducted to compare treatment pairs and assess the progression of growth over time. This test revealed significant differences between the observation days, as follows:

- Between day 3 and day 5: $P = 4.54 \times 10^{-2}$
- Between day 3 and day 7: $P = 3.89 \times 10^{-3}$
- Between day 5 and day 7: $P = 1.81 \times 10^{-3}$

All these p-values fall below the common significance threshold of 0.05, confirming statistically distinct changes in mycelial growth over the experimental period. Figure 40 visually illustrates these growth dynamics.

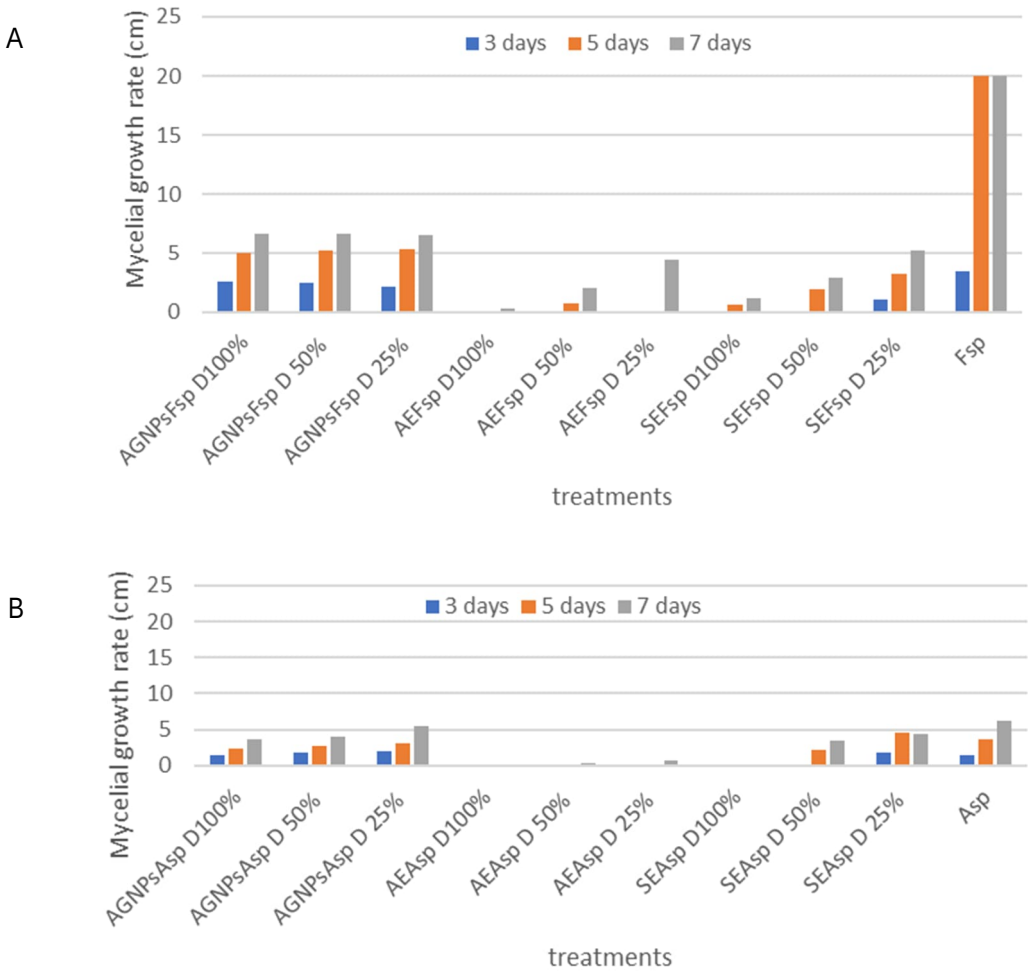


Figure 40. Variation in mycelial growth of treatments over time (A: *Fusarium oxysporum*, B: *Alternaria sp.*)

Legend:AGNPsFsp D100%: Treatment with 100% concentration of silver nanoparticles biosynthesized from *A. citrodora* essential oil on *F. oxysporum*.**AGNPsFsp D50%**: Treatment with 50% concentration of silver nanoparticles biosynthesized from *A. citrodora* essential oil on *F. oxysporum*.**AGNPsFsp D25%**: Treatment with 25% concentration of silver nanoparticles biosynthesized from *A. citrodora* essential oil on *F. oxysporum*.**AGNPsAsp D100%**: Treatment with 100% concentration of silver nanoparticles biosynthesized from *A. citrodora* essential oil on *Alternaria sp*.**AGNPsAsp D50%**: Treatment with 50% concentration of silver nanoparticles biosynthesized from *A. citrodora* essential oil on *Alternaria sp*.**AGNPsAsp D25%**: Treatment with 25% concentration of silver nanoparticles biosynthesized from *A. citrodora* essential oil on *Alternaria sp*..**AEFsp D100%**: Treatment with 100% concentration of *A. citrodora* essential oil on *F. oxysporum*.**AEFsp D50%**: Treatment with 50% concentration of *A. citrodora* essential oil on *F. oxysporum*.**AEFsp D25%**: Treatment with 25% concentration of *A. citrodora* essential oil on *F. oxysporum*.**SEFsp D100%**: Treatment with 100% concentration of *S. sclarea* essential oil on *F. oxysporum*.**SEFsp D50%**: Treatment with 50% concentration of *S. sclarea* essential oil on *F. oxysporum*.**SEFsp D25%**: Treatment with 25% concentration of *S. sclarea* essential oil on *F. oxysporum*.**AEAsp D100%**: Treatment with 100% concentration of *A. citrodora* essential oil on *Alternaria sp*.**AEAsp D50%**: Treatment with 50% concentration of *A. citrodora* essential oil on *Alternaria sp*.**AEAsp D25%**: Treatment with 25% concentration of *A. citrodora* essential oil on *Alternaria sp*.**SEAsp D100%**: Treatment with 100% concentration of *S. sclarea* essential oil on *Alternaria sp*.**SEAsp D50%**: Treatment with 50% concentration of *S. sclarea* essential oil on *Alternaria sp*.**SEAsp D25%**: Treatment with 25% concentration of *S. sclarea* essential oil on *Alternaria sp.* **Fsp**: Control with *F. oxysporum.* **Asp**: Control with *Alternaria sp.*

Table 6 : Différence de la croissance mycélienne des traitements réalisé en fonction de leur origine et dose

	AGNPsFsp D100%	AGNPsFsp D 50%	AGNPsFsp D 25%	AGNPsAsp D100%	AGNPsAsp D 50%	AGNPsAsp D 25%	AEFsp D100%	AEFsp D 50%	AEFsp D 25%	AEAsp D100%	AEAsp D 50%	AEAsp D 25%	SEFsp D100%	SEFsp D 50%	SEFsp D 25%	SEAsp D100%	SEAsp D 50%	SEAsp D 25%	Fsp	Asp
AGNPsFsp D100%		**0.0435***	0.0745	0.1275	0.1235	0.2026	0.4191	0.1736	0.4191	**1**	0.4191	0.4191	**0.0438***	**0.0362***	**0**.0872	**1**	**0.0074***	0.2768	0.2475	0.1316
AGNPsFsp D 50%	**0.0435***		**0.0310***	0.1711	0.1670	0.2461	0.4627	0.2172	0.4627	**1**	0.4627	0.4627	0.0874	**0.0073***	0.1308	**1**	**0.0510***	0.2333	0.2039	0.1752
AGNPsFsp D 25%	0.0745	**0.0310***		0,3974	0,4752	0,6819	**0,0103***	**0,0604***	0,0622	**0,0048****	**0,0111***	**0,0129***	**0,0355***	**0.0383***	0,5256	**0,0048****	0,1732	0,7211	0,751	0,7112
AGNPsAsp D100%	0.1275	0.1711	0,3974		**0.0040****	0,6626	0,0856	**0.0460***	0,3086	**0,0488***	0,0905	0,1011	0,2091	0,4834	**0.0403***	**0,0488***	0,6061	0,6247	0,2446	**0.0041****
AGNPsAsp D 50%	0.1235	0.1670	0,4752	**0.0040****		0,761	0,0641	**0.0501***	0,25	**0,0355***	**0,0680***	0,0764	0,165	0,4048	**0.0362***	**0,0355***	0,5171	0,7211	0,3024	**0.0081****
AGNPsAsp D 25%	0.2026	0.2461	0,6819	0.0750	0,761		**0,0311***	**0.0289***	0,1458	**0,0161***	**0,0332***	**0,0379***	0,0905	0,2555	0,8222	**0,0161***	0,3411	0,9578	0,4671	**0.0709**
AEFsp D100%	0.41916	0.4627	**0,0103***	0,0856	**0,064*1**	**0,0311***		0,4917	**0.0032****	0,8016	**0.0032****	**0.0032****	0,6435	0,3086	**0,0535***	0,8016	0,2289	**0,0272***	**0,0039****	0,0281*
AEFsp D 50%	0.1736	0.2172	**0,0604***	**0.0460**	**0.0501***	**0.0289***	0,4917		0,9895	0,3478	0,5085	0,543	0,8222	0,741	0,2139	0,3478	0,6061	0,1284	**0,0281***	0,1317
AEFsp D 25%	0.41916	0.4627	**0,0622***	0,3086	0,25	0,1458	**0.0032****	0,9895		0,3411	**0.0032****	**0.0032****	0,8119	0,751	0,2188	0,3411	0,6154	0,1317	**0,0291***	0,1351
AEAsp D100%	**1**	**1**	**0,0048****	**0,0488**	**0,0355***	**0,0161***	0,8016	0,3478	0,3411		**1**	**1**	0,4752	0,2043	**0,0291***	**1**	0,1458	**0,0139***	0,0017**	**0,0144***
AEAsp D 50%	0.4191	0.4627	**0,0111***	**0,0905**	0,0680	**0,0332***	**0.0032****	0,5085	**0.0032****	0,7813		**0.0032****	0,6626	0,3214	**0,0569***	0,7813	0,2393	**0,0291***	0,0042**	**0,0301***
AEAsp D 25%	0.4191	0.4627	**0,0129***	0,1011	0,0764	**0,0379***	**0.0032****	0,543	**0.0032****	0,741	0,9578		0,7014	0,3478	0,0641	0,741	0,2611	**0,0332***	0,0050**	**0,0343***
SEFsp D100%	0.0438	0.0874	**0,0355***	0,2091	0,165	0,0905	0,6435	0,8222	0,8119	0,4752	0,6626	0,7014		**0.0801**	**0.0433***	0,4752	**0.0364***	0,0809	**0,0155***	0,0832
SEFsp D 50%	**0.0362***	**0.0073****	**0.0383***	0,4834	0,4048	0,2555	0,3086	0,741	0,751	0,2043	0,3214	0,3478	0,5787		0,3616	0,2043	**0.0436***	0,234	**0,0622***	0,2393
SEFsp D 25%	0.0872	0.1308	0,5256	**0.0403***	**0.0362***	0,8222	**0,0535***	0.0864	0,2188	**0,0291***	**0,0569***	0,0641	**0.0433***	0,3616		**0,0291***	0,4671	0,7813	0,3411	0,7914
SEAsp D100%	**1**	**1**	**0,0048****	**0,0488***	**0,0355***	**0,0161***	0,8016	0,3478	0,3411	**1**	0,7813	**1**	**1**	0,2043	**0,0291***		0,1458	**0,0139***	0,0017**	**0,0144***
SEAsp D 50%	**0.0074****	**0.0510***	0,1732	0,6061	0,5171	0,3411	**0,0022****	0,6061	0,6154	0,1458	0,2393	0,2611	**0.0364***	**0.0436***	0.0797	0,1458		0,3149	0,0931	0,3214
SEAsp D 25%	0.2768	0.2333	0,7211	0,6247	0,7211	0,9578	**0,0272***	0,1284	0,1317	**0,0139***	**0,0291***	**0,0332***	0,0809	0,234	0,7813	**0,0139***	0,3149		0.0293*	0,9895
Fsp	0.2475	0.2039	0,751	0,2446	0,3024	0,4671	**0,0039****	0,0281	0,0291	0,0017**	0,0042**	0,0050**	**0,0155***	0,0622	0,3411	0,0017**	0,0931	0.0293*		0,4917
Asp	0.1316	0.1752	0,7112	**0.0041****	**0,0473***	0.0709	0,0281*	0.0419*	0,1351	**0,0144***	**0,0301***	**0,0343***	0,0832	0,2393	0.0444	**0,0144**	0,3214	0,9895	0,4917	

Asterisks indicate statistically significant differences at the 5% level (*) or highly significant differences at the 0.1% level (**).

The legend is provided below Figure 40.

The detection of significant mycelial growth even under treatment conditions (as suggested by the Kruskal–Wallis test results and previous observations), along with persistent growth in the control samples, reflects the quality of the culture medium (PDA). These findings confirm that the medium was conducive to the development of fungal phytopathogens, which is essential for validating the reliability of inhibition tests (Leslie & Summerell, 2006). A medium favorable to pathogen growth ensures that any observed reduction is truly due to the biocidal effect of the tested substances, and not to nutrient or environmental limitations.

Figure 40 shows a difference in growth between *Fusarium oxysporum* and *Alternaria sp.* The growth rate of fungal phytopathogens such as *Fusarium* and *Alternaria* plays a crucial role in their impact on crops. Although both are major plant pathogens, their growth rates and development can differ significantly due to distinct life cycles and specific environmental requirements, particularly in terms of temperature and humidity.

The genus *Fusarium* is characterized by great life cycle diversity. Many *Fusarium* species can produce a variety of spores, including conidia (macro- and microconidia) and chlamydospores. Chlamydospores are thick-walled survival structures that allow the fungus to persist in soil or plant debris for extended periods, even in the absence of a host or under unfavorable conditions (Agrios, 2005; Munkvold, 2003). Some *Fusarium* species also have a sexual stage (teleomorph), often classified in the genus *Gibberella*, which enables genetic recombination and the production of wind-dispersed ascospores, facilitating rapid long-distance spread (Tudzynski & Marasas, 2004). Most *Fusarium* plant pathogens (e.g., *F. graminearum*, *F. verticillioides*) have optimal growth temperatures typically ranging from 20°C to 30°C, with good growth possible over a broader range (Munkvold, 2003). Cooler temperatures may slow growth, while extremely high temperatures can be inhibitory. Although some *Fusarium* species can tolerate relatively dry conditions once established (due to chlamydospores), most require high humidity or free water for spore germination and initial infection (Munkvold, 2003).

Alternaria species reproduce mainly asexually through multicellular conidia of various shapes and sizes. These conidia are generally wind-dispersed and highly efficient at initiating new infections, especially on the aerial parts of plants (leaves, stems, fruits) (Agrios, 2005). Their life cycles are often shorter and directly linked to the presence of

susceptible plant tissues. Their rapid development on the host can give the impression of faster epidemic growth, but their long-term survival in the absence of a host or infected debris is more limited compared to *Fusarium*, which forms durable survival structures. *Alternaria* species (e.g., *A. solani*, *A. alternata*) also have specific temperature requirements, often similar to or slightly lower than those of *Fusarium*, with optimal growth typically between 20°C and 25°C, though some species grow well up to 30°C. They are especially problematic in warm, humid temperate climates (Agrios, 2005). *Alternaria* species rely heavily on the presence of free water on plant tissues or very high relative humidity (above 90%) for several hours for conidial germination and infection. Prolonged morning dew, rain, or overhead irrigation creates ideal conditions for their development (Agrios, 2005).

The differences in life cycles and environmental requirements explain why *Fusarium* and *Alternaria* may dominate under different conditions.

To evaluate the influence of dose and the 18 different treatments on the mycelial growth of the two fungal phytopathogen strains (*Fusarium oxysporum* and *Alternaria sp.*), we used a non-parametric statistical approach, including a Kruskal–Wallis test followed by Dunn's post-hoc test. This methodology is particularly appropriate when growth data do not follow a normal distribution or when variances are not homogeneous—common conditions in biology (Sokal & Rohlf, 1995).

The Kruskal–Wallis test revealed a highly significant overall difference among the treatment groups, with a p-value of $P = 2.148 \times 10^{-3}$. This result indicates that at least one of the treatments differs significantly from the others in terms of impact on mycelial growth. To precisely identify which specific comparisons were significant, Dunn's post-hoc test was performed (Zar, 2010).

The detailed results of the Dunn test are presented in Table 6. This table highlights numerous statistically significant—and in some cases, highly significant—probabilities for cross-comparisons between treatments and doses, for both fungal strains.

It is important to note that Table 6 reveals a consistent and pronounced effect of treatments based on essential oils of Verbena (*Aloysia citrodora*), Sage (*Salvia sclarea*), and silver nanoparticles (AgNPs) biosynthesized from *Aloysia citrodora* essential oil. At

concentrations of 100% (D100%) and 50% (D50%), these treatments exerted a significant inhibitory effect on the mycelial growth of both phytopathogenic fungal strains. In several cases, this effect led to near-complete or total inhibition of mycelial growth, thus demonstrating a strong biocidal effect at these concentrations. This high antifungal activity of essential oils is often attributed to the synergistic action of their terpenic and phenolic compounds, which disrupt fungal cell membrane integrity, inhibit respiration, and alter enzymatic mechanisms (Bakkali et al., 2008). As for silver nanoparticles, their antifungal mechanism of action is related to the release of Ag^+ ions that interfere with fungal proteins and DNA, as well as the generation of reactive oxygen species (ROS), which induce oxidative stress and cellular damage (Sharma et al., 2019). These findings underscore the remarkable antifungal potential of these natural substances and their associated nanomaterials, paving the way for the development of new strategies to combat these pathogens (Hammer et al., 2012; Sharma et al., 2019).

3.2. Evaluation of the Inhibitory Effect of the Tested Treatments

After characterizing our fungal strains and preparing the various solutions, we now present the crucial section of the results: the evaluation of the inhibitory effect of our treatments on mycelial growth. This section provides quantitative data demonstrating the ability of verbena (*Aloysia citrodora*) and sage (*Salvia sclarea*) essential oils, as well as biosynthesized silver nanoparticles, to slow or halt the growth of the fungal phytopathogens *Fusarium oxysporum* and *Alternaria sp.* We analyze the different concentrations (D100%, D50%, and D25%) and the inhibition dynamics observed over time (3rd, 5th, and 7th days) for each tested agent.

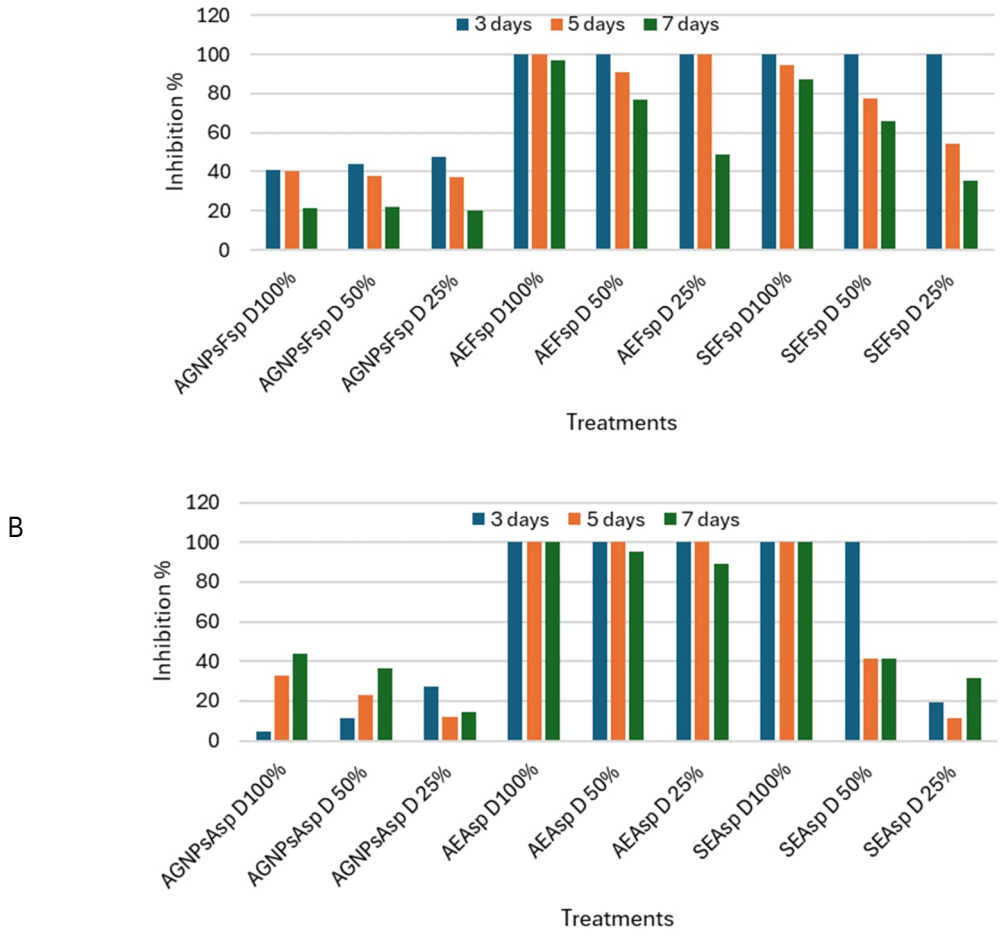


Figure 41 Inhibitory Effect of the Tested Treatments on *Fusarium oxysporum* (A) and *Alternaria sp.* (B)

Legend:AGNPsFsp D100%: Treatment with 100% concentration of silver nanoparticles biosynthesized from *A. citrodora* essential oil on *F. oxysporum.***AGNPsFsp D50%**: Treatment with 50% concentration of silver nanoparticles biosynthesized from *A. citrodora* essential oil on *F. oxysporum.***AGNPsFsp D25%**: Treatment with 25% concentration of silver nanoparticles biosynthesized from *A. citrodora* essential oil on *F. oxysporum.***AGNPsAsp D100%**: Treatment with 100% concentration of silver nanoparticles biosynthesized from *A. citrodora* essential oil on *Alternaria sp.***AGNPsAsp D50%**: Treatment with 50% concentration of silver nanoparticles biosynthesized from *A. citrodora* essential oil on *Alternaria sp.*.**AGNPsAsp D25%**: Treatment with 25% concentration of silver nanoparticles biosynthesized from *A. citrodora* essential oil on *Alternaria sp.*.**AEFsp D100%**: Treatment with 100% concentration of *A. citrodora* essential oil on *F. oxysporum.***AEFsp D50%**: Treatment with 50% concentration of *A. citrodora* essential oil on *F. oxysporum.***AEFsp D25%**: Treatment with 25% concentration of *A. citrodora* essential oil on *F. oxysporum.***SEFsp D100%**: Treatment with 100% concentration of *S. sclarea* essential oil on *F. oxysporum.***SEFsp D50%**: Treatment with 50% concentration of *S. sclarea* essential oil on *F. oxysporum.***SEFsp D25%**: Treatment with 25% concentration of *S. sclarea* essential oil on *F. oxysporum.***AEAsp D100%**: Treatment with 100% concentration of *A. citrodora* essential oil on *Alternaria sp.***AEAsp D50%**: Treatment with 50% concentration of *A. citrodora* essential oil on *Alternaria sp.***AEAsp D25%**: Treatment with 25% concentration of *A. citrodora* essential oil on *Alternaria sp.***SEAsp D100%**: Treatment with 100% concentration of *S. sclarea* essential oil on *Alternaria sp.***SEAsp D50%**: Treatment with 50% concentration of *S. sclarea* essential oil on *Alternaria sp.***SEAsp D25%**: Treatment with 25% concentration of *S. sclarea* essential oil on *Alternaria sp.***Fsp**: Control with *F. oxysporum.***Asp**: Control with *Alternaria sp.*

Antifungal activity was evaluated by measuring the growth of fungal mycelium in Petri dishes containing PDA medium. Eighteen different treatments, varying by dose and

targeted fungal species, were applied. The results, expressed as the average of four replicates per dose, are presented in Figure 41.

The antifungal performance of silver nanoparticles (AgNPs), *Salvia sclarea* essential oil (EO), and *Aloysia citrodora* EO was assessed against *Fusarium oxysporum* and *Alternaria* spp. over three observation points (3, 5, and 7 days).

Against Fusarium, AgNPs demonstrated a clear dose- and time-dependent inhibition pattern, with moderate early effectiveness that declined over time. At the highest dose (100), inhibition decreased from 40.98% (day 3) to 21.52% (day 7). Lower doses followed similar trajectories, indicating a short-term impact that diminishes rapidly.

In contrast, *Aloysia citrodora* EO exhibited robust and immediate inhibition, with all doses achieving 100% inhibition by day 3. Though its efficacy slightly declined by day 7—particularly at lower concentrations—it remained significantly potent, especially at doses 100 (96.82%) and 50 (76.82%).

Salvia sclarea EO also achieved full inhibition initially (day 3), but its persistence was more limited compared to *Aloysia*. While the highest dose retained strong activity (87.29% by day 7), lower doses saw a more pronounced decline (e.g., Dose 25 dropped to 35.17% by day 7), suggesting moderate durability.

Against Alternaria, AgNPs showed moderate cumulative effects over time at higher doses. For instance, inhibition at Dose 100 increased steadily from 4.51% (day 3) to 43.95% (day 7), while lower doses were less consistent and less durable.

Aloysia citrodora EO again proved highly effective, maintaining 100% inhibition at Doses 100 and 50 throughout the entire period. Even at Dose 25, inhibition remained high, only slightly decreasing from 100% to 88.85% by day 7. This underscores its sustained antifungal activity across all concentrations.

Salvia sclarea EO showed excellent efficacy at full concentration (100% across all days at Dose 100), but its activity dropped sharply at lower doses after day 3. Dose 50 declined to ~41%, and Dose 25 showed weak and inconsistent inhibition, ranging between 11% and 31.55%.

To analyze the statistical significance of the differences observed between treatments, a Kruskal–Wallis test was performed based on the means of the 18 treatments, followed by Dunn’s post-hoc test. The results of these analyses are presented in Table 7. Figure 41 and

Tables 7, 8, and 9 highlight variations in the inhibitory response depending on the treatment and the phytopathogenic fungal strain.

Table 7: Inhibitory Effect of the Tested Treatments on *Fusarium oxysporum* and *Alternaria sp.*

	Fusarium oxysporum	*Alternaria sp*
Kruskal-Wallis Test	2.632×10^{-2}*	6.197×10^{-3}**

Asterisks indicate statistically significant differences at the 5% level (*) or highly significant differences at the 0.1% level (**).

For the fungus *Fusarium oxysporum* (Figure 41.A; Tables 7 and 8), the tested treatments showed a positive impact on the inhibition of mycelial growth, with a p-value of $P = 2.632 \times 10^{-2}$. Greater effectiveness was observed with essential oils of verbena (*Aloysia citrodora*) at all tested doses (100%, 50%, 25%). The essential oil of sage also demonstrated notable efficacy at the 100% dose, showing a strong initial inhibitory effect that tended to decrease over time. In contrast, the silver nanoparticles biosynthesized from *Aloysia citrodora* essential oil exhibited a weaker effect on *F. oxysporum*, but this effect proved more stable over time. This relative stability could be attributed to escape mechanisms or adaptive responses developed by the fungus against the AgNPs (Sharma et al., 2019).

Regarding the fungus *Alternaria sp.* (Figure 41.B; Tables 7 and 9), the treatments also had a positive impact ($P = 6.197 \times 10^{-3}$). The greatest efficacy was demonstrated by the verbena essential oil at all concentrations. The sage essential oil also showed good efficacy, especially at the 25% dose, with a more stable inhibitory effect over time compared to *Fusarium*. However, the silver nanoparticles biosynthesized from *Aloysia citrodora* essential oil had a weaker effect on *Alternaria sp.*, with an efficacy that appeared to increase slightly over time.

Table 8: Inhibitory Effect of the Tested Treatments on *Fusarium oxysporum*

	AGNPsFsp D100%	AGNPsFsp D 50%	AGNPsFsp D 25%	AEFsp D100%	AEFsp D 50%	AEFsp D 25%	SEFsp D100%	SEFsp D 50%	SEFsp D 25%
AGNPsFsp D100%		0.9584	0.9584	0.0106*	0.0507*	0.0326*	0.0348*	0.0722	0.241
AGNPsFsp D 50%	0.9584		0.917	0.0123*	0.0571*	0.0371*	0.0395*	0.0808	0.2626
AGNPsFsp D 25%	0.9584	0.917		0.0091**	0.0448*	0.0286*	0.0305*	0.0643	0.2208
AEFsp D100%	0.0106*	0.0123*	0.0091**		0.549	0.6768	0.6578	0.4499	0.1673
AEFsp D 50%	0.0507*	0.0571*	0.0448*	0.549		0.8553	0.8758	0.8758	0.4344
AEFsp D 25%	0.0326*	0.0371*	0.0286*	0.6768	0.8553		0.9792	0.7348	0.3351
SEFsp D100%	0.0348*	0.0395*	0.0305*	0.6578	0.8758	0.9792		0.7546	0.3483
SEFsp D 50%	0.0722	0.0808	0.0643	0.4499	0.8758	0.7348	0.7546		0.5318
SEFsp D 25%	0.241	0.2626	0.2208	0.1673	0.4344	0.3351	0.3483	0.5318	

Asterisks indicate statistically significant differences at the 5% level (*) or highly significant differences at the 0.1% level (**).
The legend is provided below Figure 41.

Table 9: Inhibitory Effect of the Tested Treatments on *Alternaria sp.*

	AGNPsAsp D100%	AGNPsAsp D 50%	AGNPsAsp D 25%	AEAsp D100%	AEAsp D 50%	AEAsp D 25%	SEAsp D100%	SEAsp D 50%	SEAsp D 25%
AGNPsAsp D100%		0.8313	0.6701	0.0290*	0.06234	0.0702	0.029**	0.2414	0.6701
AGNPsAsp D 50%	0.8313		0.8313	0.0165*	0.0378*	0.043*	0.0165*	0.1662	0.8313
AGNPsAsp D 25%	0.6701	0.8313		0.009**	0.022*	0.0253*	0.009**	0.1101	1
AEAsp D100%	0.0290*	0.0165*	0.009**		0.7493	0.7093	1	0.3116	0.009**
AEAsp D 50%	0.06234	0.0378*	0.0220*	0.7493		0.9575	0.7493	0.4888	0.022*
AEAsp D 25%	0.0702	0.0430*	0.0253*	0.7093	0.9575		0.7093	0.5228	0.0253*
SEAsp D100%	0.0290*	0.0165*	0.009**	1	0.7493	0.7093		0.3116	0.009**
SEAsp D 50%	0.2414	0.1662	0.1101	0.3116	0.4888	0.5228	0.3116		0.1101
SEAsp D 25%	0.6701	0.8313	1	0.009**	0.022*	0.0253*	0.009**	0.1101	

Asterisks indicate statistically significant differences at the 5% level (*) or highly significant differences at the 0.1% level (**).
The legend is provided below Figure 41.

For F. oxysporum, this may suggest escape or adaptation mechanisms on the part of Alternaria sp. (Ma & Michailides, 2007). It is possible that the slow diffusion of nanoparticles or their larger size at certain concentrations limited their rapid interaction with mycelial growth *(*Maciel et al., 2019).

The essential oil of Aloysia citrodora demonstrated exceptionally high inhibition at all tested doses and against both phytopathogenic fungal strains throughout the experiment. This suggests that verbena oil exerts powerful immediate effects and sustained activity over time. Chemically, the superior efficacy of A. citrodora essential oil can be explained by its richness in citral (geranial and neral isomers) and related terpenes—compounds well known for their strong antifungal activity. Citral, in particular, is known to rapidly disrupt fungal cell membrane integrity, leading to leakage of cell contents and cell death (Leite et al., 2014). Studies have shown that citral alone can strongly inhibit molds and yeasts, including Candida species, within a few hours (Leite et al., 2014). This observation is consistent with reports that A. citrodora essential oil inhibits Alternaria at low concentrations (Mariotto et al., 2023).

The essential oil of Salvia sclarea also showed a significant inhibitory effect on the growth of Fusarium oxysporum and Alternaria sp. This antifungal activity is strongly influenced by concentration, demonstrating a dose-dependent relationship: fungal inhibition becomes more pronounced as the concentration increases. Notably, the highest concentration tested produced the greatest reduction in fungal growth compared to lower doses. In comparison, clary sage oil is primarily composed of linalyl acetate (which has weaker antifungal activity) and smaller amounts of linalool (moderately active). Experimental studies have shown that linalool is very effective against fungi, while linalyl acetate is considered "almost ineffective" (D'Auria et al., 2005). Thus, the chemical profile of S. sclarea oil—rich in linalyl acetate—may explain its comparatively lower activity, requiring higher doses to achieve fungicidal effects (Dzamic et al., 2008).

Silver nanoparticles also demonstrated strong antifungal activity. AgNPs inhibit fungi through multiple mechanisms: they can adhere to the cell surface and slowly release Ag^+ ions, which then enter cells and interfere with vital processes (Mussin & Giusiano, 2022). Inside the cell, Ag^+ ions induce the accumulation of reactive oxygen species (ROS), disrupt ionic balance (e.g., causing potassium efflux), and impair mitochondrial respiration (Mussin & Giusiano, 2022). These multifaceted attacks damage cellular membranes and organelles, leading to fungal cell death. Since AgNPs act on several targets simultaneously, they tend to exhibit sustained and broad-spectrum inhibition. Our experiments confirmed

that AgNP treatment clearly inhibited growth in a dose- and time-dependent manner: higher doses provided more nanoparticles (and Ag^+ ions), and prolonged exposure allowed greater accumulation, consistent with previous findings (Mussin & Giusiano, 2022).

In all treatments, the observed increase in inhibition from day 3 to day 7 suggests that a certain period is required for full biocidal action. For essential oils (like citral), continuous exposure allows for slow volatilization and progressive penetration of oil components into fungal cells, ultimately leading to membrane collapse (Leite et al., 2014). For AgNPs, longer incubation allows gradual Ag^+ release and cellular uptake, intensifying oxidative stress over time (Mussin & Giusiano, 2022). Thus, the time-dependent patterns likely reflect the kinetic mechanisms of antifungal agents. Initial growth may occur during a latency phase, but by day 7, most spores or hyphae had been compromised. The strongest effects observed on day 7 suggest that treatments did not act instantaneously, but continued to inhibit growth even after fungal development had begun. These findings align with other reports of slow-acting antimicrobial effects (Leite et al., 2014; Begum et al., 2022).

The variability in response of phytopathogenic fungi from the genera Fusarium and Alternaria to the tested treatments is often linked to mechanisms of escape and adaptation. These fungi have developed sophisticated strategies to survive and thrive despite pressure from control agents (Agrios, 2005). These mechanisms include:

1. **Mycotoxin Production**: Fusarium and Alternaria are major producers of mycotoxins, secondary metabolites that play a crucial role in their virulence (Frisvad et al., 2007). In the presence of toxic compounds, some strains may increase or alter their mycotoxin production profiles. These toxins can weaken the host plant, facilitating infection, or act directly or indirectly to neutralize antifungal agents. For example, Alternaria alternata produces toxins such as alternariol and tenuazonic acid, essential to its pathogenicity (Frisvad et al., 2007).

2. **Development of Resistance and Genetic Adaptation**: This is the most documented mechanism. Populations of Fusarium and Alternaria can rapidly develop resistance to fungicides through various genetic and physiological pathways (Gisi & Cohen, 2015). These include targeted point mutations in genes encoding fungicide target proteins, which alter the target's structure and reduce fungicide binding. This phenomenon is widely observed, for instance, in resistance to benzimidazoles (Gisi & Cohen, 2015). Such evolution is often driven by genetic

variation from sexual recombination (though rare) and asexual mechanisms such as mutations, parasexual recombination, horizontal gene or chromosome transfer, and chromosomal structural changes (Tudzynski & Marasas, 2004). These changes can confer fungicide resistance or enhanced ability to overcome plant defenses.

3. **Efflux Pumps**: Fungal membrane systems can actively expel fungicide molecules from cells, reducing their intracellular concentration to non-lethal levels (Gisi & Cohen, 2015).

4. **Metabolic Detoxification**: Fungi can acquire the ability to degrade or chemically modify fungicides, rendering them harmless before they reach their target site (Gisi & Cohen, 2015).

5. **Intrinsic Variability and Evolution**: The genera Fusarium and Alternaria exhibit high natural genetic variability within their populations. This diversity provides fertile ground for the rapid emergence of resistant strains under fungicide selection pressure (Munkvold, 2003). Sexual reproduction in certain Fusarium species (e.g., teleomorph Gibberella) enhances genetic recombination, accelerating the emergence of new combinations with adaptive advantages (Tudzynski & Marasas, 2004). Specific cases of resistance, such as Alternaria alternata to QoI fungicides, have been well documented (Ma & Michailides, 2007).

The results of our study can be explained by the intrinsic properties of the treatments. The superior performance of A. citrodora oil likely stems from its high citral content and the strongly membrane-disruptive nature of monoterpenes (Leite et al., 2014). S. sclarea oil, which contains weaker components like linalyl acetate, proved effective but to a lesser extent (D'Auria et al., 2005). Silver nanoparticles provided a complementary mode of action via sustained silver ion release and intracellular attacks (Mussin & Giusiano, 2022). The clear dose- and time-dependent trends observed across all samples are consistent with cumulative antimicrobial action and support the conclusion that the effectiveness of each agent depends on its concentration and duration of exposure. These tests align with previous studies on essential oil-based antifungals and nanoparticle-based antimicrobials, reinforcing the conclusion that plant-derived compounds and nanomaterials can

significantly inhibit phytopathogens (Mussin & Giusiano, 2022; Dzamic et al., 2008; Mariotto et al., 2023).

CHAPITRE V

1. Business Model Overview

This project proposes a natural, eco-friendly biocide based on *Aloysia citrodora* essential oil and green-synthesized silver nanoparticles (AgNPs), designed to combat fungal phytopathogens in line with organic agriculture standards. The concept aims to address Algeria's growing need for sustainable plant protection alternatives amid increasing awareness of the harmful effects of chemical pesticides.

The product targets Algerian **organic farmers**, **nurseries**, **agro-cooperatives**, and **agricultural research institutions**, particularly in regions like **Médéa, Blida, Mitidja, and Tell Atlas**, where crop losses due to *Fusarium* and *Alternaria* infections are significant.

Our Business Model Canvas is structured around the following key components:

- **Key Partners**: Algerian universities (e.g., Yahia Fares University of Médéa), INPV Boufarik, certified organic farms, nanotech researchers, and agricultural support programs such as **ANSEJ**, **ANADE**, and the **Ministry of Agriculture**.
- **Key Activities**: Cultivation of *Aloysia citrodora*, extraction of essential oil, biosynthesis of AgNPs, antifungal testing, R&D, product formulation, and preparation for phytosanitary certification.
- **Value Proposition**: An effective, residue-free antifungal product that respects the principles of organic farming and offers an Algerian-made alternative to imported synthetic fungicides.
- **Customer Segments**: Farmers engaged in organic or sustainable agriculture, greenhouse operators, plant nurseries, local cooperatives, and academic institutions.
- **Customer Relationship**: Field demonstrations, technical assistance, loyalty programs, and post-sale support.
- **Distribution Channels**: Direct sales at agricultural fairs (e.g., *SIMA–SIPSA*), partnerships with *Chambres Agricoles*, local cooperatives, and online platforms such as *Ouedkniss* or *Jumia DZ*.

- **Key Resources**: Small-scale EO extraction units, university laboratories, expert researchers, access to medicinal plant growers, and certification bodies like **ECOCERT Algeria**.
- **Cost Structure**: Raw material cultivation (verveine), nanoparticle synthesis, lab research, eco-certifications, transport, packaging, and marketing.
- **Revenue Streams**: Sales to farmers, seasonal subscription plans for plant protection, consulting for greenhouse installations, and potential licensing to agro-pharmaceutical companies.

2. Challenges and Limitations in the Algerian Context

Despite the potential of this innovation, several **practical limitations** were encountered during development, many specific to the Algerian ecosystem:

a. Financial Limitations

The most critical barrier was financial. Due to a lack of dedicated project funding or startup capital, several development phases were delayed or not completed. Key processes such as advanced formulation, encapsulation, and packaging were halted because of equipment and material shortages.

Algeria's national funding programs (like **ANSEJ**, now **ANADE**) are often difficult to access for early-stage university-based projects, particularly in the phytosanitary product sector, which requires specialized validation and regulatory approval.

b. Unavailability of Key Materials

Local unavailability of high-purity **silver nitrate**, specialized **emulsifiers**, and standardized essential oils posed significant logistical problems. Imports of chemical precursors and lab-grade tools are subject to customs delays and high taxes, making it hard to sustain consistent R&D.

c. Incomplete Prototyping

Due to these resource constraints, the project could not advance to the industrial prototyping stage. The spray formulation was not finalized, and the final packaging and

shelf-life stability tests were not performed. The product remains at the **laboratory validation stage**, with proof of concept but lacking industrial scalability.

d. Regulatory and Administrative Delays

In Algeria, obtaining approval for phytosanitary or biocidal products is a lengthy and expensive process. The need for toxicological tests, environmental impact assessments, and certification through INPV or other bodies posed a major administrative hurdle.

e. Lack of Industrial Partners

While university support was present, **industrial partnerships** (for bottling, marketing, or mass production) were not yet established. Most agrochemical companies in Algeria focus on imports rather than development of local organic solutions, creating a gap in the value chain.

3. Future Perspectives

Despite these limitations, the business model remains **feasible and aligned with Algeria's strategic goals** in sustainable agriculture. With proper funding and institutional support, the product could be further developed, certified, and commercialized both nationally and eventually regionally (e.g., in the Maghreb or Sahel zones).

Recommendations for the next phase include:

- Applying for funding through **start-up incubators** or university-industry collaboration programs (e.g., *FABLAB, StartUp Algeria*).
- Partnering with **local distilleries** or cooperatives that already extract medicinal plant oils.
- Securing **pilot testing fields** in collaboration with INPV or CRAs (Centres de Recherche Agronomique).
- Exploring **international cooperation** (France, Tunisia, etc.) for access to formulation equipment and technical mentorship

CHAPITRE IV

➢ Conclusion

This study explores significant advances in the fight against fungal phytopathogens, focusing on the antifungal potential of natural substances and silver nanoparticles (AgNPs). The study is divided into two main parts: the synthesis and characterization of AgNPs, then the evaluation of the antifungal activity of biosynthesized AgNPs and essential oils (EOs) of verbena (Aloysia citrodora) and sage (Salvia sclarea) against Fusarium oxysporum and Alternaria sp.

The synthesis of AgNPs was confirmed by visual observation (color change to yellowish-brown) and UV-Visible spectroscopy analysis. The spectra revealed a surface plasmon resonance (SPR) peak between 440 and 460 nm, indicating the formation of nanoparticles. The full width at half maximum (FWHM) of the peak suggests a polydisperse AgNP population, with a diversity of sizes and shapes, particularly pronounced for AgNPs synthesized with Aloysia citrodora essential oil.

Before evaluating antifungal activity, rigorous identification of the strains was carried out. Macroscopic and microscopic observations confirmed the purity and identified the Fusarium sp. strain as Fusarium oxysporum, based on the presence of hyaline mycelium, sickle-shaped macroconidia, and chlamydospores, confirmed by specialists. For Alternaria sp., despite detailed morphological characteristics (dark mycelium, muriform conidia in chains or isolated), the exact species could not be formally identified.

The biocidal efficacy of the treatments was evaluated by measuring the inhibition of mycelial growth of the two fungal strains. Treatments included essential oils of verbena and sage, as well as AgNPs, applied at different concentrations (D 100%, D 50%, D 25%). Growth was monitored on the 3rd, 5th, and 7th days. Non-parametric statistical tests (Kruskal-Wallis followed by Dunn’s post-hoc test) were used to analyze the significant differences.

The results showed an overall highly significant difference in mycelial growth between the treatments and the controls for both strains ($P = 1.78\times10^{-3}$ and $P = 2.148\times10^{-3}$ for the general tests). Temporal analyses also revealed significant differences between observation days ($P < 0.05$ between D3, D5, and D7).

• For Fusarium oxysporum, verbena essential oil showed the highest efficacy at all doses, followed by sage essential oil at 100%. AgNPs had a weaker but more stable effect over time.

• For Alternaria sp., verbena essential oil was also the most effective at all doses, followed by sage essential oil at 25%, with a more stable inhibitory effect. AgNPs had a weak effect, although slightly increasing over time.

In general, Aloysia citrodora essential oil demonstrated exceptionally high and persistent inhibition on both strains and at all concentrations, probably due to its richness in citral, a powerful antifungal agent. Salvia sclarea essential oil showed significant antifungal activity, especially at high concentrations, highlighting a dose-dependent relationship consistent with its chemical composition. AgNPs exerted robust antifungal activity through multiple mechanisms, depending on dose and exposure time.

The variability in the response of fungi is attributed to escape and adaptation mechanisms, including mycotoxin production, genetic resistance development (mutations, efflux pumps, metabolic detoxification), and the high intrinsic variability of these fungal genera.

In conclusion, the study confirms that the efficacy of the tested agents depends on concentration and exposure duration. Aloysia citrodora EO stands out for its strong and consistent antifungal activity, followed by AgNPs and Salvia sclarea EO. These results confirm the hypothesis that nanoformulation improves the activity of essential oils by increasing their stability, bioavailability, and duration of action. The synergy between plant extracts and nanotechnology opens promising prospects for the development of sustainable biopesticide solutions, in response to the growing limitations of chemical fungicides in terms of resistance, toxicity, and environmental impact.

However, this study presents certain limitations. The tests were conducted in vitro, under controlled conditions that do not accurately reflect the complexity of real agricultural environments. In addition, the pathogenic spectrum assessed was limited to two fungal genera, and the ecological safety of the formulations was not examined. The environmental fate of nanoparticles and their long-term stability also remain unknown.

To go further, additional research is needed, including trials under real cultivation conditions, toxicity evaluation on non-target organisms, as well as optimization of nanometric delivery systems. Expanding testing to other pathogens and crops would strengthen the validity and applicability of the results. Ultimately, integrating these formulations into integrated pest management programs would constitute a promising strategy for sustainable plant protection.

By combining traditional plant knowledge with advances in nanotechnology, this work contributes significantly to the development of greener agriculture. It highlights the potential of bio-nanotechnological formulations as viable and effective alternatives to conventional plant protection products, in an Algerian and international context concerned with reconciling agricultural productivity and environmental sustainability.

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