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TITLE

BIOCONTROL OF PHYTOPATHOGENIC BACTERIA AFFECTING DATE PALM (*PHOENIX DACTYLIFERA* L.)

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Fares Douis

Biocontrol of phytopathogenic bacteria affecting date palm (*Phoenix dactylifera* L.)

ABSTRACT

The date palm (*Phoenix dactylifera* L.) is a vital crop in arid regions but is increasingly affected by bacterial diseases, particularly *Dickeya chrysanthemi* the causal agent of Sudden Decline (SD) disease. This study explores eco-friendly biocontrol strategies using bacteriophages and rosemary essential oil (REO). A presumptive *Dickeya* isolate was obtained from symptomatic date palms in Ouargla (Algeria) and characterized phenotypically and biochemically (VITEK®, API 20E). Although sequencing issues limited molecular confirmation, the isolate exhibited pathogenic traits consistent with *Dickeya*. Five lytic phages were isolated from environmental samples and formulated into a cocktail that significantly reduced bacterial growth. GC-MS analysis of REO identified Eucalyptol (1,8-cineole) as the major antimicrobial compound. At 10% (v/v), REO showed strong antibacterial activity. Combined application of the phage cocktail and REO produced a synergistic effect, reducing bacterial counts by over 3 $\log_{10}$ CFU/mL *in vitro* and decreasing the Disease Severity Index (DSI) by 55.56% *in planta*. These results suggest that integrating phage therapy with plant-derived antimicrobials represents a promising and sustainable strategy for controlling SD disease in date palms.

Keywords: *Phoenix dactylifera*, Sudden decline, *Dickeya sp*, Bacteriophage, Rosemary, Biocontrol.

Biocontrôle des bactéries phytopathogènes affectant le palmier dattier (*Phoenix dactylifera* L.)

RÉSUMÉ

Le palmier dattier (*Phoenix dactylifera* L.) constitue une culture essentielle dans les régions arides, mais il est de plus en plus menacé par des maladies bactériennes, en particulier *Dickeya chrysanthemi* agent causal du dépérissement soudain. Cette étude évalue des stratégies de biocontrôle écologiques utilisant des bactériophages et l'huile essentielle de romarin (REO). Un isolat présumé de *Dickeya* a été obtenu à partir de palmiers symptomatiques à Ouargla (Algérie), puis caractérisé sur les plans phénotypique et biochimique (VITEK®, API 20E). Bien que des problèmes de séquençage aient limité la confirmation moléculaire, l'isolat présentait des caractéristiques pathogènes compatibles avec le genre *Dickeya*. Cinq phages lytiques ont été isolés à partir d'échantillons environnementaux et formulés en un cocktail ayant significativement inhibé la croissance bactérienne. L'analyse GC-MS de l'huile essentielle de romarin a identifié l'eucalyptol (1,8-cinéole) comme composé antimicrobien majoritaire. À une concentration de 10 % (v/v), l'REO a montré une forte activité antibactérienne. L'application combinée du cocktail de phages et de l'huile essentielle a produit un effet synergique, réduisant les charges bactériennes de plus de 3 $\log_{10}$ UFC/mL *in vitro*, et diminuant l'indice de sévérité de la maladie (DSI) de 55,56 % *in planta*. Ces résultats suggèrent que l'intégration de la phagothérapie et des antimicrobiens d'origine végétale constitue une stratégie prometteuse et durable pour la lutte contre le dépérissement soudain chez le palmier dattier.

Mots-clés : *Phoenix dactylifera*, Dépérissement soudain, *Dickeya* sp., Bactériophage, Romarin, Biocontrôle.

المكافحة البيولوجية للبكتيريا الممرضة للنباتات التي تصيب نخيل التمر(*Phoenix dactylifera* L.)

الملخص

يُعدّ نخيل التمر (*Phoenix dactylifera* L.) من المحاصيل الحيوية في المناطق الجافة، غير أنه يتعرض بشكل متزايد لأمراض بكتيرية، وعلى رأسها *Dickeya chrysanthemi* ، المُسببة لمرض الذبول المفاجئ. تهدف هذه الدراسة إلى استكشاف استراتيجيات مكافحة بيولوجية صديقة للبيئة باستخدام العاثيات البكتيرية وزيت إكليل الجبل العطري .(REO) تم عزل سلالة مشتبه بها من بكتيريا *Dickeya*من نخيل تمر تظهر عليه أعراض المرض في منطقة ورقلة (الجزائر)، وتم توصيفها ظاهريًا وكيميائيًا وحيويًا باستخدام نظامي VITEK® و .API 20Eوعلى الرغم من صعوبات التأكيد الجزيئي بسبب مشاكل في التسلسل، أظهرت السلالة خصائص مرضية متوافقة مع بكتيريا *Dickeya*. تم عزل خمسة عاثيات بكتيرية مذيبة من عينات بيئية، وتم تركيبها في خليط أظهر فعالية كبيرة في تثبيط النمو البكتيري. كشفت تحاليل الكروماتوغرافيا الغازية – مطياف الكتلة (GC-MS) لزيت إكليل الجبل أن المركب المضاد للميكروبات الرئيسي هو الأوكاليبتول (1,8-سينول). وقد أظهر الزيت بتركيز 10٪ (حجم/حجم) فعالية قوية ضد البكتيريا. كما أدى الاستخدام المشترك بين خليط العاثيات والزيت العطري إلى تأثير تآزري، حيث تم تقليص عدد البكتيريا بأكثر من 3 لوغ$_{10}$ وحدة تكوين مستعمرات/مل في المختبر، مع انخفاض في مؤشر شدة المرض بنسبة 55.56٪ داخل النبات. تشير هذه النتائج إلى أن دمج المعالجة بالعاثيات مع المركبات المضادة للميكروبات ذات الأصل النباتي يُمثل استراتيجية واعدة ومستدامة لمكافحة مرض الذبول المفاجئ في نخيل التمر.

الكلمات المفتاحية: *Phoenix dactylifera*، الذبول المفاجئ، *Dickeya sp*.، العاثيات ، إكليل الجبل، المكافحة البيولوجية

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LIST OF ABBREVIATIONS

- **5Kg** – 5-keto-D-gluconate
- **ABLap** – β-alanine arylamidase
- **ADO** – Adonitol fermentation
- **ADH** – Arginine dihydrolase
- **AGAL** – α-galactosidase
- **AGLTp** – Glutamyl arylamidase
- **AGLU** – α-glucosidase
- **AMY** – Amygdalin fermentation
- **API** – Analytical Profile Index
- **APPA** – Ala–Phe–Pro arylamidase
- **ARA** – Arabinose fermentation
- **BALap** – β-alanine arylamidase
- **BGAL** – β-galactosidase
- **BGLU** – β-glucosidase
- **BNAG** – N-acetyl-β-D-glucosaminidase
- **BXYL** – β-xylosidase
- **CFU** – Colony Forming Unit
- **CIT** – Citrate / Citrate utilization
- **CVP** – Crystal Violet Pectate
- **dCEL** – D-cellobiose fermentation
- **dGLU** – D-glucose fermentation
- **dMAL** – D-maltose
- **dMAN** – D-mannitol
- **dMNE** – D-mannose
- **dSOR** – D-sorbitol
- **dTAG** – D-tagatose
- **dTRE** – D-trehalose
- **DNA** – Deoxyribonucleic Acid
- **DSI** – Disease Severity Index
- **GC-MS** – Gas Chromatography–Mass Spectrometry
- **GEL** – Gelatin hydrolysis
- **GGT** – γ-glutamyl transferase
- **GLU** – Glucose fermentation
- **GlyA** – Glycine arylamidase
- **H_2S** – Hydrogen sulfide production
- **ILATK / ILATa** – L-lactate alkalinization
- **IND** – Indole production
- **INO** – Inositol fermentation
- **IARL** – L-arabitol fermentation
- **LDC** – Lysine decarboxylase
- **LIP** – Lipase
- **MAN** – Mannitol fermentation
- **MEL** – Melibiose fermentation
- **MH** – Mueller Hinton (medium)
- **MNT** – Malonate
- **MOI** – Multiplicity of Infection
- **NA** – Nutrient Agar
- **NAGA** – N-acetylgalactosaminidase
- **NGS** – Next-Generation Sequencing
- **OD** – Ornithine decarboxylase
- **OFF** – Glucose oxidation
- **ONPG** – β-galactosidase activity
- **PBS** – Phosphate Buffered Saline
- **PFU** – Plaque Forming Unit
- **PHOS** – Phosphatase
- **PLE** – Palatinose
- **ProA** – Proline arylamidase
- **PyrA** – L-Pyrrolidonyl arylamidase
- **REO** – Rosemary Essential Oil
- **RHA** – Rhamnose fermentation
- **rRNA** – Ribosomal Ribonucleic Acid
- **SAC** – Sucrose / Sucrose fermentation
- **SD** – Sudden Decline
- **SDW** – Sterile Distilled Water
- **SM buffer** – Sodium-Magnesium Buffer
- **SOR** – Sorbitol fermentation
- **SUCT** – Succinic acid alkalinization
- **TDA** – Tryptophan deaminase
- **TyrA** – Tyrosine arylamidase
- **URE** – Urease
- **VP** – Acetoin production (Voges–Proskauer)

INTRODUCTION

INTRODUCTION

The date palm (*Phoenix dactylifera* L.) is a monocotyledonous species belonging to the Arecaceae family, has been cultivated for over 6,000 years in arid and semi-arid regions, particularly across the Middle East and North Africa (Gros-Balthazard *et al*., 2021; Jalal *et al*., 2024) (Figure 01). Beyond its historical significance, the date palm is a keystone species in desert ecosystems. It creates microclimates favorable for intercropping, enhances water and soil conservation, and fosters biodiversity by providing habitats for various faunal and microbial communities. The date palm also contributes to climate regulation through carbon sequestration (Ibrahim, 2018; Ibrahim, 2022). Agronomically, it improves soil fertility in sandy and nutrient-poor environments by adding organic matter and promoting nutrient cycling (Alotaibi *et al.,* 2023; Kavvadias *et al.,* 2024). The date palm industry is economically significant, supporting millions of livelihoods worldwide. Global date production has reached approximately 9.82 million tonnes, with Egypt, Saudi Arabia, and Algeria being the top producers (Sporchia *et al.,* 2023; FAO, 2023). In addition to its economic importance, the date palm holds socio-cultural value, featuring prominently in religious texts like the Quran and serving traditional uses in crafts, construction, and animal husbandry. The extensive genetic diversity of the date palm, with thousands of cultivars adapted to diverse local conditions, enhances its resilience and utility (Mahmoud *et al.,* 2022). Nutritionally, date fruits are functional foods, rich in phytochemicals such as phenolic acids, flavonoids, and carotenoids. These compounds contribute to antioxidant, antimicrobial, and anti-inflammatory properties, offering potential for nutritional and pharmacological applications (Al-Talaqany *et al.,* 2023).

Despite its resilience and multifaceted importance, the date palm remains highly susceptible to a broad range of diseases that impact various organs and significantly diminish both yield quality and quantity. These diseases vary depending on the region, cultivar, and environmental conditions and can be classified symptomatically (e.g., spots, blights, rots, wilts), anatomically (based on the affected organ), or etiologically (according to the causal agents, including fungi, bacteria, viruses, nematodes, insects, and physiological disorders). Among these, bacterial pathogens are increasingly recognized as major threats. For instance, in Iran, offshoot decline and bud rot have been associated with *Klebsiella oxytoca*, *Kosakonia radicincitans*, and *Citrobacter koseri*, which induce symptoms such as foliar yellowing, tissue rot, and foul odors (Abedinzadeh *et al.,* 2024). *Stenotrophomonas* spp. have also been implicated in offshoot wilting and desiccation (Abedinzadeh *et al., 2023*). In Kuwait, *Serratia*

marcescens is responsible for pink rot of inflorescences, producing dark lesions and distinctive pink mucilage (Riaz *et al.,* 2009).

Erwinia chrysanthemi, now reclassified under the genus *Dickeya*, is a significant Gram-negative phytopathogenic bacterium known for its broad host range, affecting a wide variety of plant species worldwide (Hugouvieux *et al.,* 2023). This pathogen is associated with several economically important plant diseases. In Saudi Arabia, *E. chrysanthemi* has been implicated in the sudden decline syndrome of date palms, a condition marked by internal tissue necrosis, frond discoloration, and rapid apical meristem death (Abdalla, 2001). Bacterial infections can also predispose the host to secondary pathogens, compounding yield losses

The increasing impact of bacterial diseases highlights the need for enhanced surveillance and targeted management strategies. Integrated disease management, combining preventive, curative, and sustainable practices, is essential. Cultural control methods like sanitation, pruning, and crop rotation are foundational but their effectiveness depends on local knowledge and resources (Al-Dosary *et al.,* 2016; Benzohra, 2017). Chemical treatments are widespread but raise concerns about environmental persistence, pathogen resistance, and human health risks (El-Shafie, 2019; Matrood *et al.,* 2021). In response, biological control strategies are gaining traction, particularly those employing antagonistic microorganisms such as *Bacillus* spp. and *Trichoderma* spp., which have demonstrated promising results against a variety of phytopathogens (Boulahouat *et al.,* 2022; Zer-Aviv *et al.,* 2024). Furthermore, Integrated Pest Management (IPM) frameworks seek to integrate these biological approaches with advanced technologies, including remote sensing, predictive modeling, and decision-support systems, thereby facilitating precision agriculture (Reddy *et al.,* 2024). However, the large-scale implementation of IPM in date palm cultivation remains limited, primarily due to infrastructural, financial, and educational constraints. This highlights the necessity for coordinated policy support, comprehensive farmer training programs, and interdisciplinary research initiatives aimed at overcoming these barriers (Khoury & Makkouk, 2010).

Figure 1: Photographs of the Date Palm (*Phoenix dactylifera*) in Ouargla (Algeria):(**A**) Morphological view of date palm trees. (**B**) Fruits of the Algerian cultivar *Deglet Nour*. (**C**) Bundles of natural and dyed date palm strips used in traditional handicrafts.

Bacteriophages, or phages, are viruses that specifically infect bacteria and are recognized as the most abundant biological entities on Earth, with an estimated global population of approximately 10^{31}, surpassing the number of their bacterial hosts (Clokie *et al.,* 2011) (Figure 02). Structurally, they are obligate intracellular parasites composed of either DNA or RNA enclosed within a protein capsid, exhibiting notable morphological diversity, including tailed and filamentous forms (Ackermann, 2007). Their remarkable host specificity and potentially bacteriolytic capabilities have fueled a resurgence of interest in phage therapy as a viable alternative to antibiotics, particularly in response to the escalating crisis of antibiotic resistance (Abedon *et al.,* 2011). Since their discovery by F.W. Twort and subsequent characterization by Félix d'Hérelle in the early twentieth century, phages have been extensively explored across diverse sectors such as medicine, biotechnology, and agriculture, where they have shown potential in therapeutic interventions, food safety enhancement, and environmental sanitation (Kutter & Sulakvelidze, 2005). In agricultural contexts, bacteriophages have demonstrated significant efficacy in controlling bacterial phytopathogens offering a highly specific and ecologically sustainable approach with minimal disruption to non-target microbiota (Buttimer *et al.,* 2017).

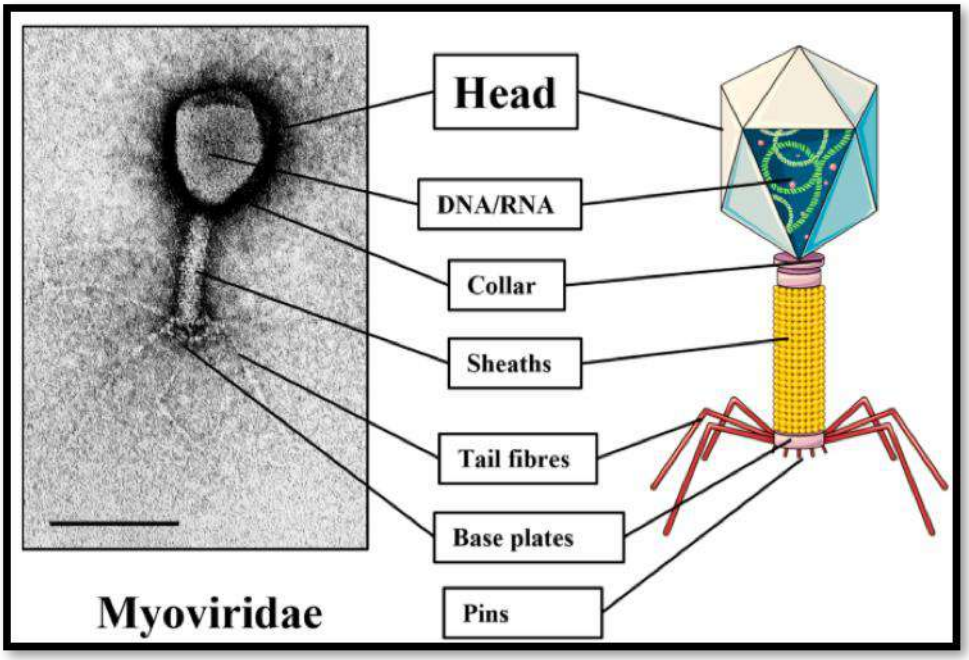

Figure 2*:* Microscopic and schematic structure of a bacteriophage (Waghet *et al.,* 2023)

Essential oils (EOs) are natural, volatile, and aromatic hydrophobic extracts from plants, composed of various secondary metabolites. (Bakkali *et al.,* 2008; Zukowska & Durczyńska, 2024). Their chemical composition varies depending on botanical origin, geographical conditions, climate, soil type, and extraction techniques (Tuberoso & Kowalczyk, 2009; Tripathi *et al.,* 2024). EOs typically include monoterpenes, sesquiterpenes, and phenolic compounds, which contribute to their biological activities, particular antimicrobial properties (Chraibi *et al*., 2020). Due to their low toxicity to non-target organisms, EOs are increasingly used as environmentally friendly alternatives to synthetic pesticides in organic agriculture (El-Mohamedy, 2017).

Among the various essential oils, rosemary essential oil (REO), derived from *Rosmarinus officinalis*, is notable for its strong antimicrobial activity. Its efficacy is mainly attributed to major constituents such as 1,8-cineole, camphor, and α-pinene (Figure 03) along with minor compounds like borneol and bornanone (Walid *et al*., 2022; Arfa *et al*., 2022; Becer *et al*., 2023; Hcini *et al.,* 2023; Hassan & Khalil, 2024; Kabotso *et al.,* 2024). REO has shown effectiveness against a range of pathogens, including Gram-negative plant pathogens like *Erwinia* species (Doukkali *et al*., 2018). Its antimicrobial mechanisms involve disrupting bacterial membranes, inhibiting DNA and protein synthesis, and preventing biofilm formation, making it effective against resistant strains (Kanth *et al*., 2018; Hidayat *et al*., 2024).

The combination of essential oils and bacteriophages is being explored as a strategy to enhance antimicrobial effectiveness. Studies indicate that co-application can lead to greater reductions in bacterial populations compared to individual treatments, suggesting a synergistic effect. This combined approach is a promising alternative to conventional antibiotics, particularly in the context of rising antimicrobial resistance (Elafify *et al*., 2025).

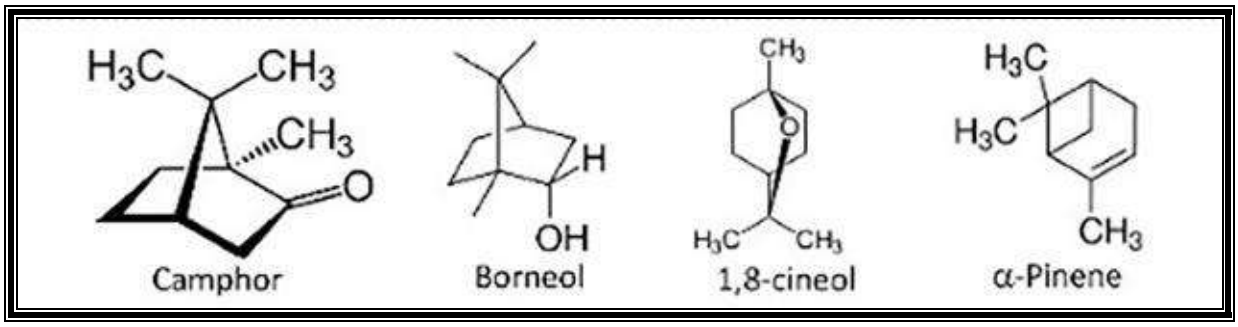

Figure 3 Major bioactive compounds of rosemary (*Rosmarinus officinalis*) essential oil (Chang *et al.,* 2021)

Sudden decline disease in date palms (*Phoenix dactylifera*) constitutes a serious phytosanitary and economic challenge in date-producing regions. It is primarily associated with phytopathogenic bacteria such as *Dickeya chrysanthemi*, which can induce severe tissue maceration and plant mortality. Conventional control methods, including chemical treatments and cultural practices, have demonstrated limited effectiveness and are often accompanied by environmental and health concerns. These limitations underscore the urgent need for alternative, sustainable disease management strategies.

In response to this challenge, the present thesis explores eco-friendly biocontrol strategies that combine lytic bacteriophages with plant-derived antimicrobial compounds. The research is structured around four main objectives:

1. To isolate and identify the bacterial agent responsible for sudden decline in date palm.
2. To isolate and characterize lytic bacteriophages active against the identified pathogen.
3. To evaluate the antibacterial activity of rosemary (*Rosmarinus officinalis*) essential oil.
4. To assess the biocontrol efficacy of bacteriophages and essential oil, both individually and in combination.

MATERIALS AND METHODS

II.1 Isolation of the suspected bacterial pathogen associated with sudden decline in date palm

A field sampling campaign was conducted on January 21, 2025, in the Rouissat region of Ouargla, Algeria (31°55'52.7"N, 5°21'38.8"E), with the objective of isolating the causal agent responsible for Sudden Decline (SD) disease in date palm. A total of seven symptomatic date palm specimens was selected based on characteristic disease symptoms, including rapid canopy wilting, pronounced chlorosis, and leaf desiccation, collectively resulting in a withered and dry crown. Further examination of the root systems revealed extensive tissue degradation, characterized by soft rot and decay. Longitudinal sections of the vascular tissues exhibited distinct internal browning and discoloration. These symptoms, as shown in Figure 04 were consistent with those previously described by Abdalla (2001) in similar cases reported in Saudi Arabia.

Figure 4: Symptoms of Sudden Decline in date palm (*Phoenix dactylifera* L.): (**A**) Canopy wilting and frond necrosis; (**B**) Desiccated frond; (**C**) Vascular browning at the frond base; (**D**) Root discoloration and rot.

Samples from the affected plant tissues were collected and transported under sterile conditions to the laboratory for microbial isolation and identification. The isolation procedure was adopted with minor modifications from the protocol established by Abdalla (2001), and is summarized in Figure 05. Fronds from recently symptomatic palms were selected for

analysis. The samples were initially washed under a fine mist of tap water to eliminate superficial contaminants. Leaf rachis tissues were excised into approximately 5-mm sections using sterile scalpels. Surface sterilization was performed by immersing the tissue pieces in a 0.5% sodium hypochlorite solution for two minutes, followed by two rinses in sterile distilled water (SDW) for two minutes each. Following sterilization, the tissue sections were macerated in 5 mL of SDW supplemented with 0.1 M magnesium sulfate ($MgSO_4$) using a sterile mortar and pestle. The resulting suspensions were streaked onto nutrient agar (NA) plates and incubated at 30°C for 24 hours. Discrete bacterial colonies that emerged were subsequently subcultured onto crystal violet pectate (CVP) medium for preliminary identification and further pathogenicity assessments.

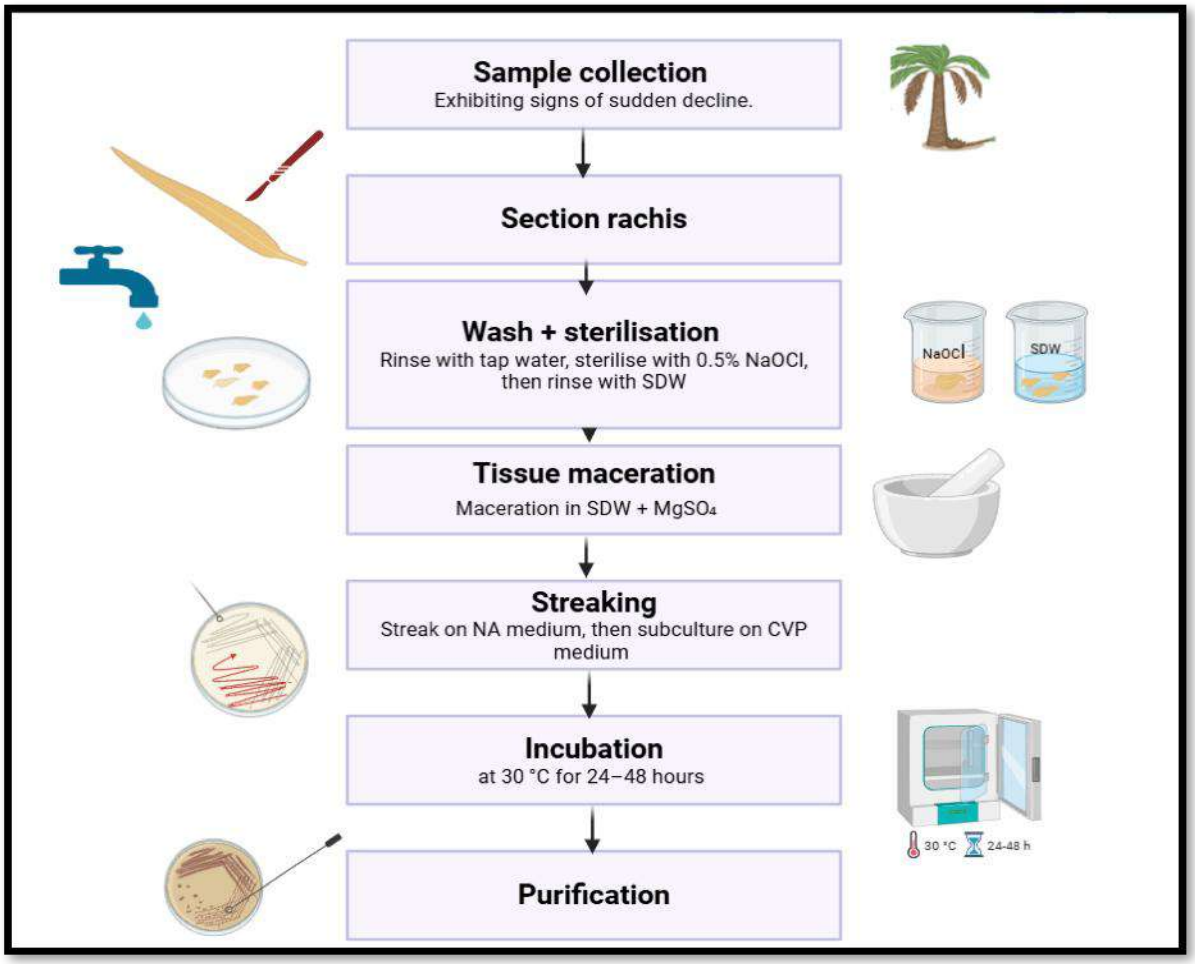

Figure 5: Flowchart of the bacterial isolation procedure for identifying the causal agent of sudden decline in date palm. Created with BioRender.

II.2. Identification of the bacterial isolate

The bacterial isolate was identified through a comprehensive, multi-tiered approach, encompassing phenotypic characterization, biochemical profiling with antimicrobial susceptibility testing, and molecular identification.

II.2.1. Phenotypic characterization

Initial phenotypic characterization was conducted at the Microbiology Laboratory of Kasdi Merbah University, Ouargla (Algeria). This phase involved preliminary identification based on morphological and biochemical traits. Observations included colony morphology on Crystal Violet Pectate (CVP) and Nutrient Agar (NA) media after 24 hours of incubation at 30°C. Gram staining was performed according to the standard protocol of the American Society for Microbiology (ASM), which includes sequential staining and decolourization steps to differentiate bacteria based on cell wall composition (Smith& Hussey,.2005). Catalase test was assessed using the slide (drop) method, whereby a drop of hydrogen peroxide is added to a bacterial smear on a slide; immediate bubble formation indicates a positive result (Reiner 2010). Oxidase test was evaluated using the filter paper method, which detects cytochrome c oxidase based on a reagent-induced colour change (Shields & Cathcart.,2010). Salt tolerance was tested by culturing the isolates on nutrient agar supplemented with 5% NaCl; growth indicated halotolerance (Cappuccino & Sherman.,2013). Gelatin liquefaction was assessed using the nutrient gelatin stab method where a heavy inoculum of a 24-hour-old bacterial culture was stabbed into nutrient gelatin tubes and incubated at 30°C for up to 7 days. To confirm gelatin hydrolysis, the tubes were refrigerated at 4°C for 30 minutes liquefaction of the medium while the inoculated control remained solid, indicated the presence of gelatinase enzymes (Dela & Torres., 2012). The composition of all media used is detailed in Annex 1.

II.2.2. Biochemical profiling and antibiotic susceptibility testing

Biochemical profiling and antimicrobial susceptibility testing were conducted at El Amel Medical Diagnostic Laboratory in Ouargla (Algeria) using the API 20E system and the VITEK® 2 Compact system (BioMérieux), following the manufacturers' instructions.

For the API 20E system, a fresh, pure bacterial colony (24 hours old) was suspended in sterile saline (0.85% NaCl) to achieve a turbidity equivalent to the 0.5 McFarland standard. This suspension was used to inoculate each microtube of the API 20E strip, which contains dehydrated substrates for 20 distinct biochemical reactions. Certain wells, such as ADH, LDC, ODC, H_2S, and URE, were overlaid with mineral oil to maintain anaerobic conditions. The strips were incubated at 37°C for 24 hours, after which color changes were interpreted visually or following the addition of specific reagents (e.g., Kovac's reagent for indole), and results were analyzed using the API Web™.

In parallel, the VITEK® 2 Compact system was employed for automated identification and antimicrobial susceptibility testing (AST), utilizing colorimetric and fluorometric detection of bacterial growth and metabolic activity. A bacterial suspension was prepared from a fresh culture and adjusted to a 0.5 McFarland turbidity, then transferred into a GN identification card (Ref. 21341) along with the corresponding Gram-negative antibiogram card. The loaded cassettes were processed by the VITEK® 2 Compact system, which incubated the samples and performed kinetic optical readings over 6 to 18 hours to assess biochemical activity and determine both the identity of the organism and its antimicrobial susceptibility profile.

The interpretation of the results was based on comparisons with standard references, comprising *Bergey's Manual of Systematic Bacteriology* and Abdalla (2001).

II.2.3. Molecular identification

For accurate species-level identification, molecular analysis of the bacterial isolate was performed by Macrogen Europe B.V. (Amsterdam, Netherlands). Genomic DNA (gDNA) was extracted using either a resin-based protocol or the PrepMan™ (Thermo Fisher) boiling method, wherein 20 µL of resin or PrepMan was mixed with 20 µL of distilled water and a bacterial colony or pellet, followed by centrifugation at 13,000 rpm for 1 minute. The mixture was then incubated at 56°C for 15 minutes, denatured at 99°C for 15 minutes, and held at 12 °C. After an additional centrifugation at 4000 rpm for 5 minutes, 1.5 µL of the supernatant was used as the PCR template. The 16S rRNA gene was amplified using universal primers 27F and 1492R, while sequencing primers 785F and 907R were employed for downstream analysis. PCR reactions were prepared in a 20 µL volume comprising 10 µL of Axen™ H Taq PCR Master Mix (2X), 1 µL each of forward and reverse primers (10 pmol/µL), 1–2 µL of DNA template (20 ng/µL), and 7 µL of HPLC-grade distilled water. The thermocycling conditions included an initial denaturation at 95°C for 5 minutes; 31 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 1.4 minutes; followed by a final extension at 72°C for 10 minutes and a hold at 4 °C. Sequencing was performed using the BigDye Terminator v3.1 cycle sequencing kit and the 3730XL automated DNA sequencing system. Resulting sequences were analyzed for homology using the EzBioCloud 16S database (Annex 4)

II.3. Pathogenicity testing of the isolated strain

To evaluate the pathogenicity of the bacterial isolate associated with sudden decline disease in date palm (*Phoenix dactylifera* L.), both *in vitro* and *in planta* assays were conducted, following the methodology described by Abdalla (2001) with minor

modifications. As illustrated in the accompanying Figure 6, the isolate was first cultured in nutrient broth at 30°C for 24 hours, and the resulting suspension was adjusted to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL).

For the *in vitro* assay, healthy leaflets collected from date palm trees at our university were surface-sterilized and mechanically wounded before being placed in sterile Petri dishes lined with moistened cotton. Each wound site was inoculated with 2 mL of either a bacterial suspension (treatment group) or sterile distilled water (SDW; control group).

In the *in-planta* assay, *Phoenix dactylifera* seedlings, approximately one year old and obtained from wild palms in Khafdji (Ouargla region), were inoculated by injecting 2 mL of the same bacterial suspension (treatment group) or SDW (control group) into the crown region. All samples were maintained under controlled environmental conditions and monitored daily for the appearance of disease symptoms, including necrosis, tissue softening, and wilting. Visual changes were documented photographically.

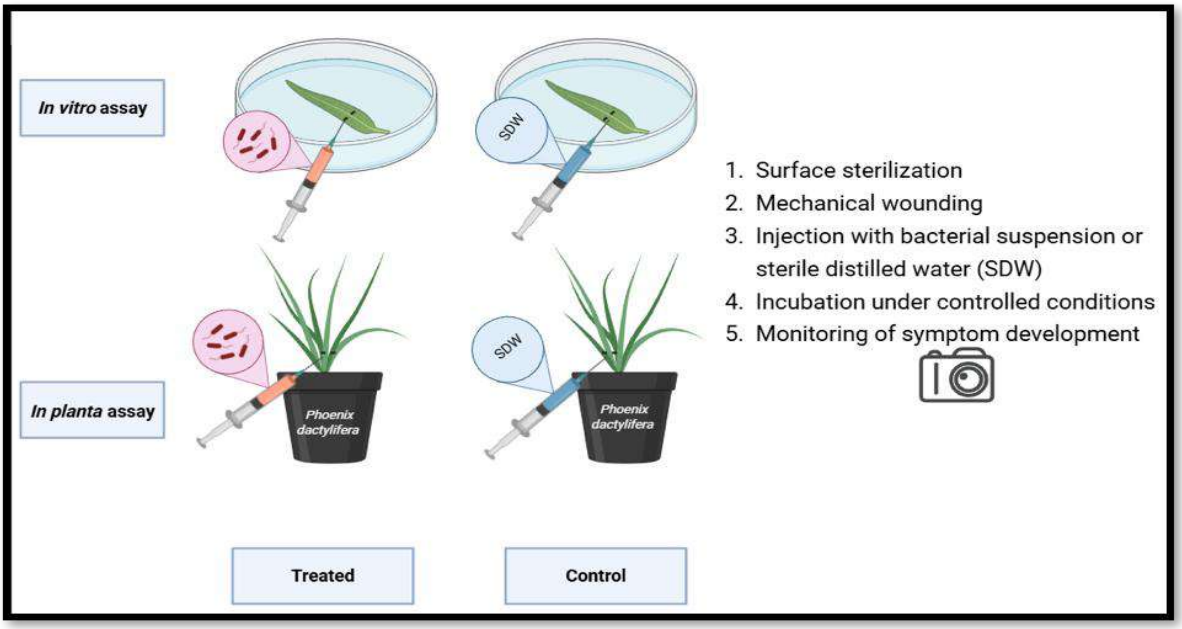

Figure 6: Schematic illustration of the *in vitro* and *in planta* pathogenicity assays used to assess the virulence of the bacterial isolate associated with sudden decline disease in date palm (*Phoenix dactylifera* L.). Created using BioRender.

II.4. Isolation of bacteriophages

The protocol for bacteriophage isolation and characterization was primarily adapted from (Azeredo *et al.*, 2014), with certain modifications tailored to the specific experimental conditions of this study. A simplified schematic of the workflow is presented in Figure 07.

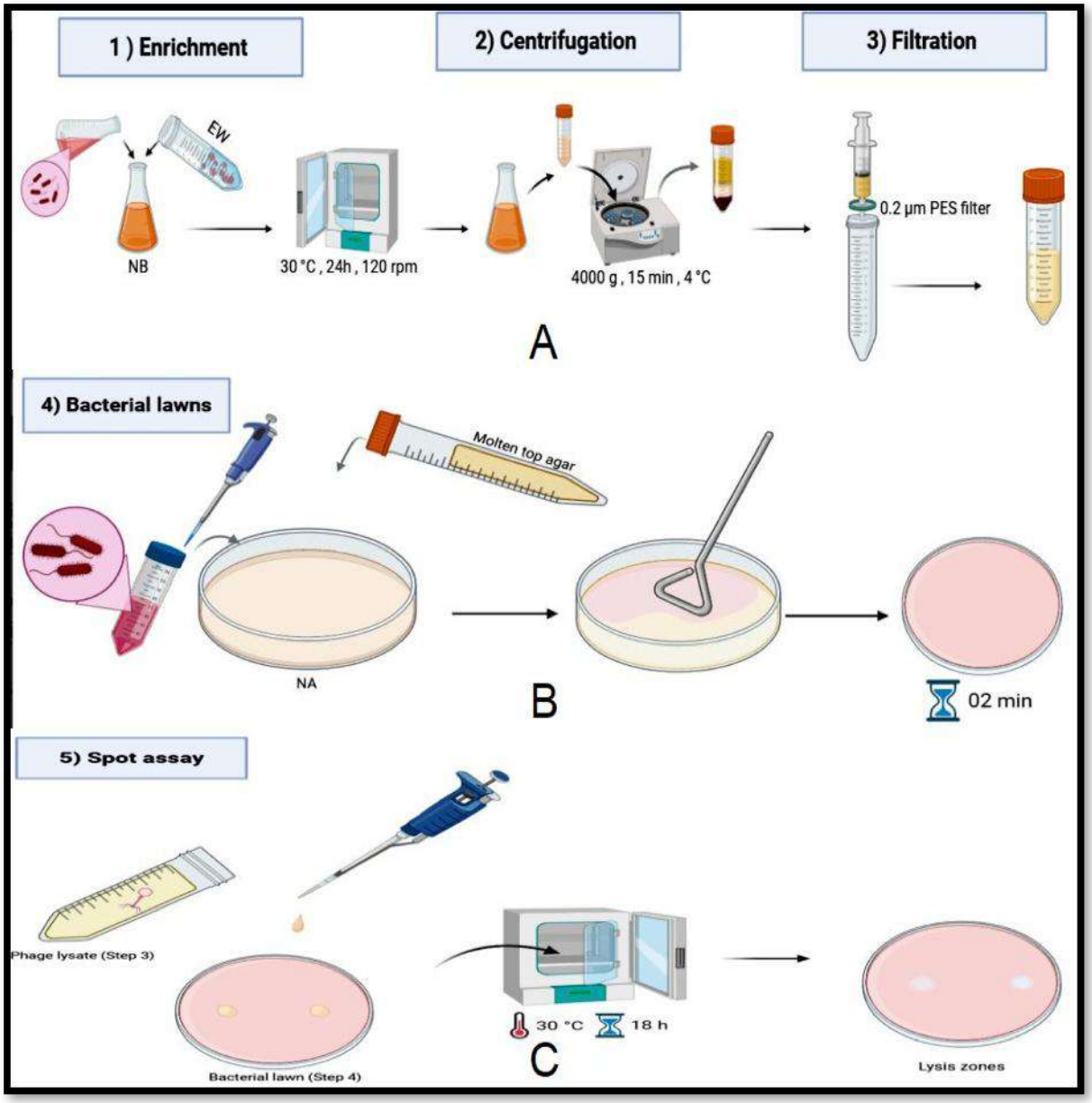

Figure 7: Step-by-step workflow for the isolation of bacteriophages targeting *Dickeya* species associated with sudden decline in date palm (**A**) Preparation of phage lysates through enrichment, centrifugation, and filtration. (**B**) Preparation of bacterial lawns using the pour plate technique with molten top agar.(**C**) Spot assay performed by applying filtered lysates onto the bacterial lawn to detect lytic activity. Figure created with BioRender.

II.4.1. Environmental sample collection and enrichment

Lytic bacteriophages specific to the target bacterial host were isolated from five distinct environmental water samples (designated EW1 to EW5) obtained from irrigation canals in proximity to *Phoenix dactylifera* plantations. These sites are located near the university's experimental agricultural fields. To maintain phage viability, samples were either processed immediately or stored at 4°C for a maximum of 24 hours when immediate processing was not feasible, as cold storage has been shown to stabilize phage particles.

For phage enrichment, 50 mL of each environmental sample was transferred aseptically into a sterile 250 mL Erlenmeyer flask. To each flask, 50 µL of an overnight culture of the target bacterial strain and 50 mL of nutrient broth were added. The mixture was incubated at 30°C for 24 hours with continuous agitation at 120 rpm to promote phage replication. After incubation, the cultures were centrifuged at 4,000 × g for 15 minutes at 4°C to remove bacterial cells and particulate matter. The resulting supernatants were then filtered through 0.2 µm pore-size polyethersulfone (PES) membrane filters (Millipore) to eliminate residual bacterial contamination, thereby yielding sterile phage-enriched lysates suitable for downstream analyses.

II.4.2. Preparation of bacterial lawns for phage detection

To detect lytic activity, bacterial lawns were prepared on nutrient agar plates. An aliquot of 100 µL of an overnight bacterial culture was added to 3–5 mL of molten top agar (nutrient broth supplemented with 0.6% agar) maintained at approximately 47°C to avoid thermal inactivation of the bacteria. This mixture was homogenized and uniformly poured onto solidified nutrient agar base plates (containing 1.5% agar). Plates were left undisturbed at room temperature for 1–2 minutes to allow the overlay to solidify prior to phage application.

II.4.3. Preliminary screening via spot assay

A qualitative spot assay was performed to assess the presence of lytic bacteriophages in the enriched lysates. A 20 µL aliquot of each filtered lysate was spotted onto the surface of the pre-prepared bacterial lawns. Plates were incubated at 30°C for 16–18 hours. The appearance of clear or partially clear zones of lysis indicated phage activity against the host bacterium. Plaque morphology clarity, diameter, and edge definition was visually evaluated to identify isolates with pronounced lytic activity.

II.4.4. Formulation of a broad-spectrum phage cocktail

To enhance the potential antimicrobial spectrum and reduce the likelihood of bacterial resistance development, a composite phage cocktail was formulated. Equal volumes of the five enriched lysates (EW1–EW5) were pooled to create a broad-spectrum preparation. This approach is commonly adopted in phage therapy to increase host range and mitigate resistance evolution. The final cocktail was stored at 4°C for use in further titration and characterization experiments.

II.4.5. Phage titer determination using the plaque assay

Phage concentration, expressed as plaque-forming units per millilitre (PFU/mL), was determined using a small-drop plaque assay (Figure 8). Serial tenfold dilutions of the phage cocktail were prepared in SM buffer. For each dilution, 100 µL was mixed with 100 µL of an overnight culture of the host bacterium and 3 mL of molten top agar (0.6% agar at 47 °C). This mixture was overlaid onto base nutrient agar plates (1.5% agar) and allowed to solidify for 10 minutes. Additionally, a 10 µL drop of each dilution was spotted onto bacterial lawns prepared as described above. Plates were tilted gently to facilitate even absorption of the droplets and incubated overnight at 30 °C. Plaques displaying well-defined morphology were counted. Only dilutions yielding 3 to 30 plaques per spot were considered statistically reliable. The phage titer was calculated using the following formula:

$$\mathrm{PFU/mL} = \frac{\text{Number of plaques} \times \text{dilution factor}}{\text{Volume plated (ml)}}$$

Where ;

Number of Plaques: Count the clear zones (plaques) formed on the cell monolayer.

Dilution Factor: The reciprocal of the dilution used (e.g., for a 10^{-6} dilution, the dilution factor is 10^{6}).

Volume Plated: The volume of the diluted virus sample plated, in milliliters

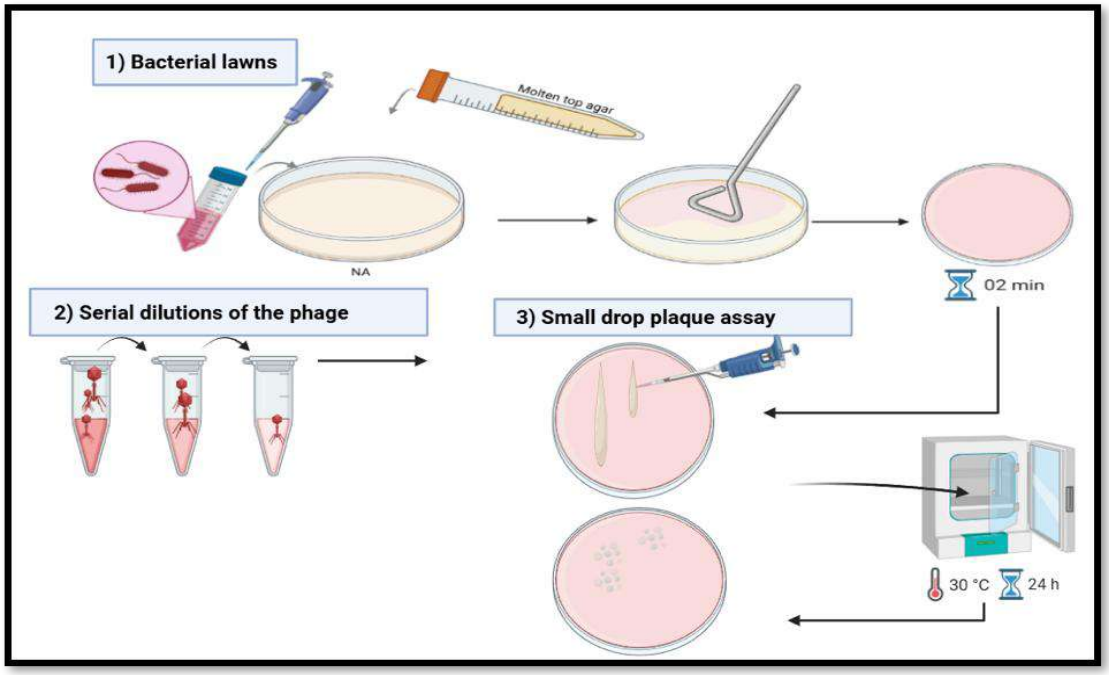

Figure 8: Enumeration of bacteriophages using the small drop plaque assay. Figure created with BioRender.

II.5. Evaluation of the antibacterial activity of rosemary (*Rosmarinus officinalis*) essential oil

The antibacterial activity of rosemary essential oil (REO), as extracted and characterized by Prof. Hammoudi's research team (2024), was systematically evaluated using standardized microbiological methodologies as summarized in Figure 9. To facilitate the solubilization of the hydrophobic REO in aqueous media, emulsions were prepared at concentrations of 0%, 1.5%, 2.5%, 5%, and 10% v/v, utilizing distilled water and Tween 20 a non-ionic surfactant known for its ability to stabilize essential oil dispersions (Malik & Upadhyay, 2023). The antimicrobial activity of these formulations was assessed using the agar disc diffusion method, a widely accepted technique for evaluating plant-based antimicrobials (Hussain *et al.*, 2013; Khalil & Hassan, 2024). Mueller-Hinton agar plates were uniformly inoculated with bacterial suspensions standardized to a 0.5 McFarland turbidity. Sterile 7 mm filter paper discs, each impregnated with 10 µL of the respective REO emulsion, were aseptically placed onto the inoculated agar surfaces. Control discs containing only distilled water and Tween 20 were included to account for any antimicrobial effects attributable to the solvent system. Following incubation at 30°C for 24 hours, the diameters of the zones of inhibition surrounding each disc were meticulously measured in millimeters, providing qualitative data on the antibacterial activity of each REO concentration.

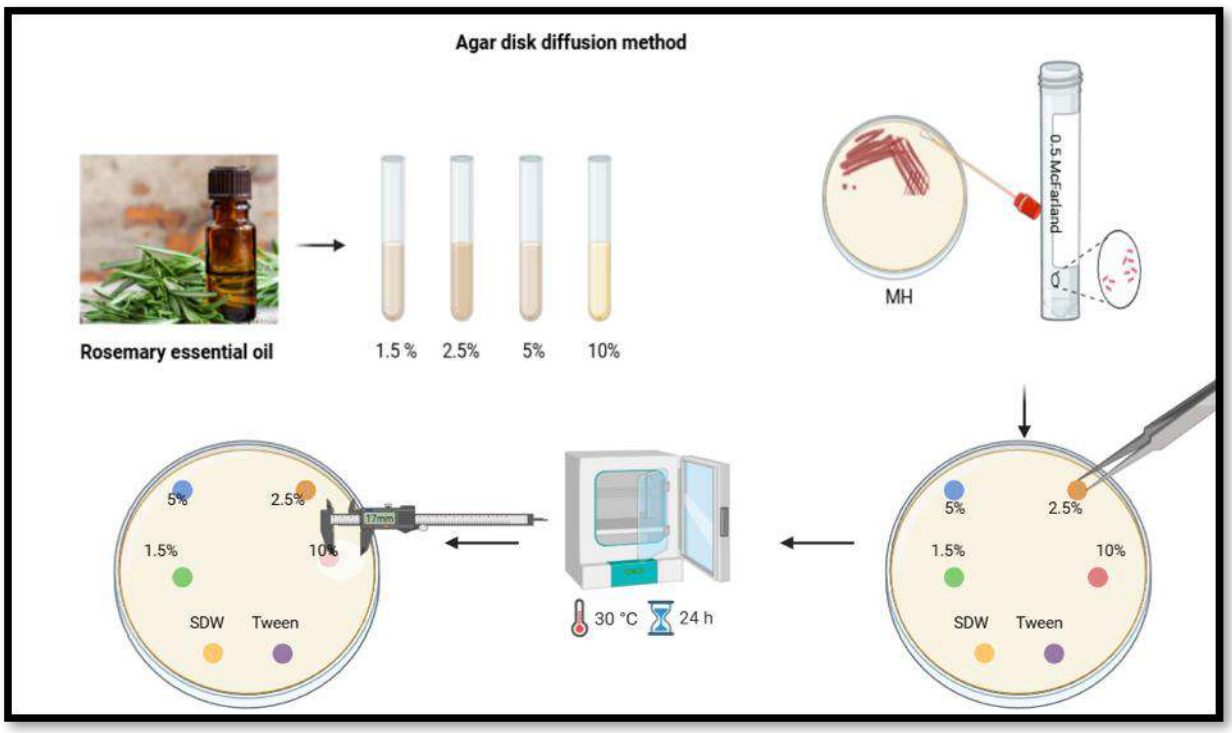

Figure 9: Schematic representation of the agar disc diffusion method used to evaluate the antibacterial activity of rosemary (*Rosmarinus officinalis*) essential oil against *Dickeya sp*., causative agents of sudden decline in date palm. Figure created with BioRender.

II.6. Assessment of biocontrol efficacy

The experimental protocol implemented in this study was adapted from (Sillankorva and Azeredo, 2014) who originally investigated the efficacy of bacteriophage treatments in disrupting microbial biofilms under *in vitro* conditions. While their work was primarily biofilm-focused, the present study diverged from this framework to examine the antibacterial potential of bacteriophages and rosemary essential oil (REO) against *Dickeya* spp., phytopathogenic bacteria known to infect *Phoenix dactylifera* L. The aim was to evaluate the antimicrobial efficacy of these agents both individually and in combination at a 1:1 ratio through a two-phase experimental approach involving *in vitro* and *in planta* assays. Particular emphasis was placed on assessing the synergistic effects of the combined treatment using colony-forming unit (CFU) enumeration *in vitro* and disease severity index (DSI%) as an *in-planta* validation metric. As summarized in Figure 10.

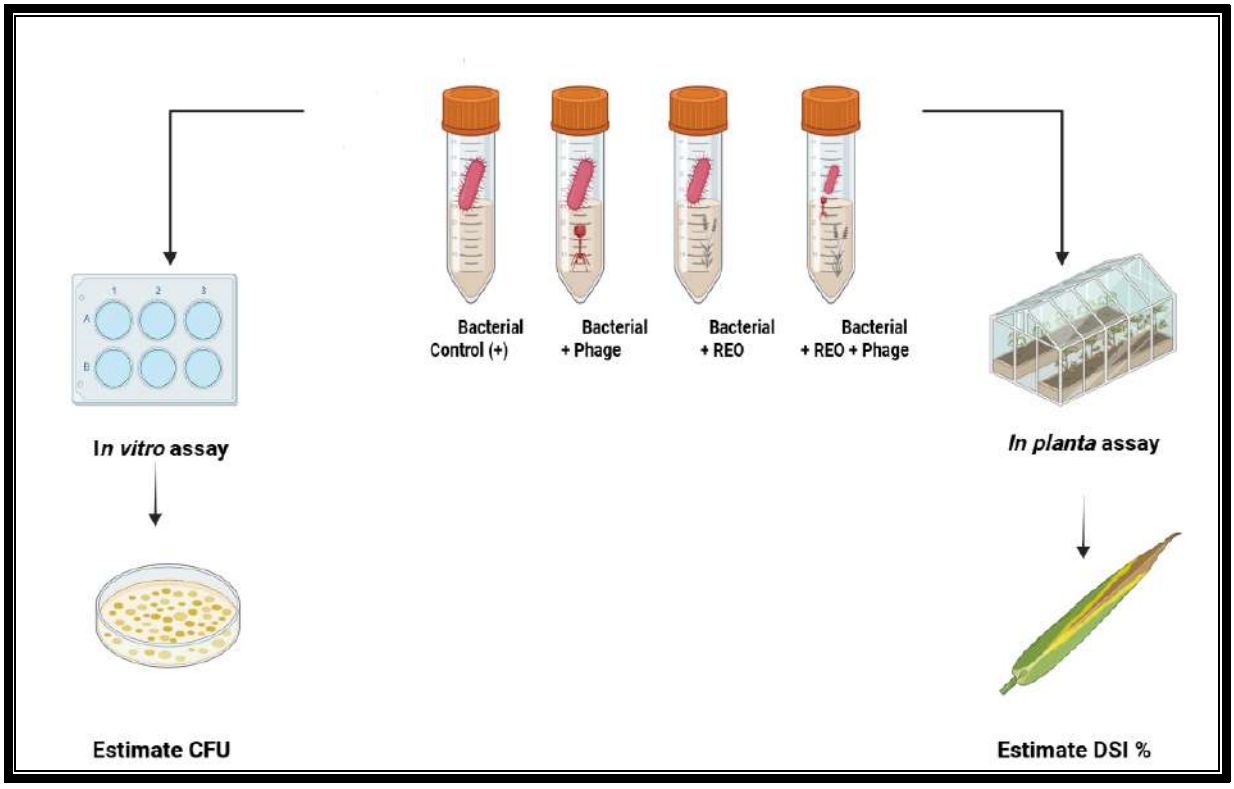

Figure 10:Schematic representation of *in vitro* and *in planta* biocontrol assays using bacteriophages and rosemary essential oil against *Dickeya sp*. causing sudden decline in date palm (*Phoenix dactylifera* L.). Figure created with BioRender.

II.6.1. I*n vitro* assay

The *in vitro* phase consisted of a bacterial inhibition assay performed in sterile 12-well microplates under five treatment conditions: (1) a negative control with 1 mL of sterile Nutrient Broth (NB) without bacterial inoculation; (2) a positive control with 900 µL of NB and 100 µL of bacterial suspension adjusted to ~10^6 CFU/mL, matching the density used in

treated wells; (3) a phage treatment containing 800 µL of NB, 100 µL of bacterial suspension, and 100 µL of a bacteriophage cocktail at 10^6 PFU/mL, achieving a multiplicity of infection (MOI) of 1; (4) a REO treatment composed of 800 µL of NB, 100 µL of bacterial suspension, and 100 µL of 10% (v/v) rosemary essential oil (REO); and (5) a combined treatment with 700 µL of NB, 100 µL of bacterial suspension, 100 µL of the phage cocktail (MOI = 1), and 100 µL of REO emulsion. After 24 hours of incubation at 30 °C under static conditions, bacterial viability was evaluated via serial dilution and plating on Nutrient Agar, and CFU/mL was calculated using standard microbiological methods.

$$\text{CFU/mL} = \frac{\text{Number of colonies} \times \text{dilution factor}}{\text{Volume plated (ml)}}$$

Where;
Number of Colonies: Count the colonies on a plate that has between 30–300 colonies to ensure accuracy.
Dilution Factor: The reciprocal of the dilution
Volume Plated: The volume of the diluted sample plated, in milliliters
The $\log_{10}$ reduction in bacterial counts was calculated as;

$$\text{Log}_{10}\text{ Reduction} = \log_{10}(\text{CFU/mL of positive control}) - \log_{10}(\text{CFU/mL of treatment})$$

Each treatment was conducted in triplicate, and data were statistically analyzed using one-way analysis of variance (ANOVA).

II.6.2. *In planta* assay

The *in-planta* phase aimed to validate these findings under more natural conditions. Healthy *P. dactylifera* seedlings estimated to be three months old, were obtained from our university and cultivated in sterile pot and each plant was mechanically wounded at the leaf axil using a sterile needle, followed by inoculation with 1 mL of the bacterial suspension. After 24 h, treatments were administered at the wound site via sterile syringe. Disease progression was monitored daily for 30 days, and symptoms were recorded photographically. Disease severity was evaluated using a visual 0–5 scale, where 0 indicated no symptoms and 5 denoted complete wilting and necrosis.

$$\text{DSI (\%)} = \left(\frac{\sum(\text{Class frequency} \times \text{Severity score})}{\text{Total number of plants} \times \text{Maximum severity score}}\right) \times 100$$

Where;
Class Frequency: The number of plants observed in each severity class.
Severity Score: A numerical value assigned to each severity class (e.g., 0 for no symptoms, up to 5 for severe symptoms).
Total Number of Plants: The total number of plants assessed.
Maximum Severity Score: The highest possible score in the severity scale used.

RESULTS AND DISCUSSION

III.1. Characterization of the isolated bacterium

III.1.1 Phenotypic profile and initial identification

Following the isolation procedure, a dominant bacterial morphotype was consistently observed on Nutrient Agar (NA) plates incubated at 30°C for 24 hours (Figure 11A). These colonies were typically circular, smooth, and opaque with entire margins. On Crystal Violet Pectate (CVP) medium, the isolate exhibited the formation of pitting surrounding the colonies (Figure 11B), indicative of pectinolytic activity. Growth was also observed at 36°C (Figure 11C), suggesting a degree of thermotolerance. However, no growth was evident on NA supplemented with 5% NaCl after 24 hours of incubation (Figure 11D), indicating a lack of halotolerance under the tested conditions. Microscopic examination following Gram staining revealed the bacterial isolate to be Gram-negative, characterized by a pink coloration, and rod-shaped morphology (Figure 11E). The isolate tested negative for oxidase and positive for catalase activities (Figure 11F and 11G, respectively), as evidenced by the presence of bubble formation upon the addition of hydrogen peroxide and the lack of color change on the oxidase reagent-impregnated filter paper. Furthermore, the isolate demonstrated proteolytic activity, as indicated by the liquefaction of the nutrient gelatin medium after 7 days of incubation at 30°C followed by refrigeration.

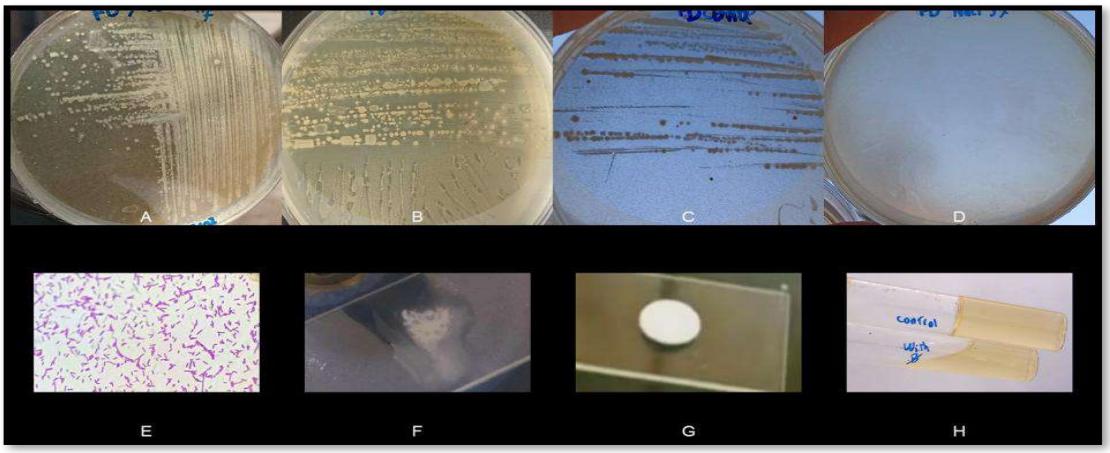

Figure 11:Phenotypic characterization of the bacterial isolate**:** (**A**) Growth of the purified isolate on nutrient agar after 24 hours of incubation at 30 °C. (**B**) Colony development on crystal violet pectate (CVP) medium at 30 °C. (**C**) Growth response at elevated temperature (36 °C) after 24 hours. (**D**) Growth tolerance in the presence of 5% NaCl following 24 hours of incubation at 30 °C. (**E**) Microscopic observation of Gram-stained cells. (**F**) Catalase activity test. (**G**) Oxidase activity test. (**H**) Gelatin liquefaction assay.

The phenotypic characterization of the bacterial isolate obtained from symptomatic date palm tissues in the Rouissat region of Ouargla (Algeria), provides preliminary insights into its identity and potential role in Sudden Decline (SD) disease. The consistent isolation of a dominant bacterial morphotype from multiple symptomatic specimens strengthens the

hypothesis that this isolate may be associated with the observed disease symptoms. The Gram-negative, rod-shaped morphology of the isolate aligns with the characteristics of several bacterial genera known to cause plant diseases, including vascular wilts and soft rots (Agrios, 2005). The observed pectinolytic activity on CVP medium is particularly noteworthy. Pectate lyases and other pectin-degrading enzymes are key virulence factors in many phytopathogenic bacteria, enabling them to break down the plant cell wall middle lamella, leading to tissue maceration and soft rot symptoms, as observed in the root systems of the affected date palms (Collmer & Keen, 1986). The ability of the isolate to grow at 36°C suggests a degree of adaptation to the ambient temperatures prevalent in the date palm-growing regions of Algeria. However, the lack of growth in the presence of 5% NaCl indicates that the isolate is likely not halotolerant under these specific laboratory conditions. This observation could be relevant to understanding the bacterium's ecological niche and its potential interaction with the saline conditions that can sometimes affect date palm cultivation. The results for oxidase and catalase activities provide crucial biochemical markers for the identification process. The oxidase test detects the presence of cytochrome c oxidase, a key enzyme in the electron transport chain, while the catalase test identifies the presence of catalase, an enzyme that detoxifies hydrogen peroxide. The absence of oxidase and the presence of catalase can help narrow down the possible bacterial genera. The positive gelatin liquefaction test signifies the production of gelatinase, an extracellular protease capable of hydrolyzing gelatin. The ability to degrade proteins further suggests the potential of this isolate to break down plant tissues and access nutrients, contributing to the observed tissue degradation in the infected date palms (Dela & Torres, 2012).

Synthesizing the observed phenotypic characteristics including colony morphology, Gram reaction, the presence of pectinolytic and proteolytic enzymatic activities, the ability to grow at a moderately high temperature, and the negative oxidase and catalase reactions reveals a profile that shares similarities with members of the genus *Dickeya*. Notably, Abdalla (2001) previously reported the isolation of a Gram-negative, rod-shaped bacterium exhibiting both pectinolytic and proteolytic activities as the causal agent of a Sudden Decline-like syndrome in date palms in Saudi Arabia, highlighting the potential relevance of these traits in the context of this disease. Furthermore, when considering the taxonomic criteria outlined in *Bergey's Manual of Systematic Bacteriology*, the collective phenotypic attributes of the present isolate align with the general characteristics described for species within the genus *Dickeya*, a well-known group of plant-pathogenic bacteria often associated with soft rot and vascular wilt diseases.

III.1.2 Metabolic and AST characterization of the bacterial isolate

The metabolic profile of the bacterial isolate was assessed using both the API 20E test strip and the VITEK® 2 Compact system. Based on the API 20E results (Table 1), the isolate exhibited positive reactions for arginine dihydrolase (ADH), lysine (LDC) and ornithine decarboxylase (ODC), citrate utilization (CIT), acetoin production (VP), gelatin hydrolysis (GEL), and the fermentation of multiple carbohydrates including glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), saccharose (SAC), melibiose (MEL), and amygdalin (AMY). The isolate tested negative for hydrogen sulfide production (H_2S), indole production (IND), urease (URE), tryptophan deaminase (TDA), and the assimilation of arabinose (ARA) and rhamnose (RHA).

Table 1: Biochemical profile of the bacterial isolate as determined by the API 20E test strip. For detailed descriptions of the biochemical tests and the interpretation of the results, refer to Annexes 2.1 and 2.2.

ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
-	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	+	+	+	-

The symbol "+" denotes a positive biochemical reaction, whereas "–" denotes a negative reaction, as interpreted according to standard criteria.

In parallel, the automated VITEK® 2 system using the GN identification card yielded a partially overlapping metabolic profile (Table 2).

Table 2: Biochemical identification profile of the bacterial isolate obtained using the VITEK® 2 system with GN cards. A detailed description of the biochemical tests and the interpretation of the results is provided in Annex 3.

Well	Test	Result	Well	Test	Result	Well	Test	Result	Well	Test	Result	Well	Test	Result	Well	Test	Result
2	APPA	-	3	ADO	-	4	PyrA	+	5	IARL	-	7	dCEL	+	9	BGAL	+
10	H2S	+	11	BNAG	+	12	AGTLp	+	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	+	18	dMAL	-	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	-
33	SAC	+	34	dTAG	+	35	dTRE	-	36	CIT	+	37	MNT	-	39	5Kg	+
40	ILATK	-	41	AGLU	-	42	SUCT	-	43	NANGA	-	44	AGAL	-	45	PHOS	+
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	-	57	BGUR	-
58	O129R	-	59	GGAA	-	61	IMLTa	-	62	ELLEM	+	64	ILATa	-			

The symbol "+" indicates a positive result, while "–" indicates a negative result.

Antibiotic susceptibility testing (AST) (Table 03), conducted using the VITEK® 2 Compact system (Table 4), showed that the isolate was susceptible to piperacillin/tazobactam (≤ 4 µg/mL), imipenem (≤ 0.25 µg/mL), amikacin (≤ 2 µg/mL), colistin (≤ 0.5 µg/mL), and

chloramphenicol (≤ 2 µg/mL). Intermediate susceptibility was observed for ceftazidime (16 µg/mL), while resistance was noted to gentamicin (≥ 16 µg/mL), cefazolin (≥ 64 µg/mL), cefotaxime (≥ 64 µg/mL), ciprofloxacin (≥ 4 µg/mL), and trimethoprim/sulfamethoxazole (≥ 320 µg/mL).

Table 3: Antibiotic susceptibility profile of the bacterial isolate as determined by the VITEK® 2 system using Gram-negative antibiogram cards.

Antibiotic	MIC (mg/L)	Interpretation	Antibiotic	MIC (mg/L)	Interpretation
Ampicillin	nd	nd	Amikacin	≤ 2	S
Amoxicillin/clavulanic acid	nd	nd	Gentamicin	≥ 16	R
Piperacillin/tazobactam	≤ 4	S	Ciprofloxacin	≥ 4	R
Cefazolin	≥ 64	R	Fosfomycin	-	-
Cefoxitin	-	-	Nitrofurantoin	≤ 2	S
Cefotaxime	≥ 64	R	Chloramphenicol	-	-
Ceftazidime	16	I	Colistin	≤ 0.5	S
Ertapenem	nd	nd	Trimethoprim/sulfamethoxazole	≥ 320	R
Imipenem	≤ 0.25	S			

ND = Not determined, S = Susceptible, I = Intermediate, R = Resistant.

The biochemical and physiological data obtained from both the API 20E and VITEK® 2 Compact systems collectively highlight the metabolic versatility of the isolate, particularly its capacity to ferment a wide spectrum of carbohydrates. This broad saccharolytic capability is a hallmark of soft rot phytopathogens, especially those historically classified under the genus *Erwinia* (Krieg & Holt, 1984). Nonetheless, the consistency of key metabolic traits such as gelatinase and pectinase activity supports the phenotypic association with *Erwinia chrysanthemi*, a species now reclassified within the genus *Dickeya* (Samson et al., 2005). It is essential, however, to recognize the inherent limitations of both identification systems, particularly when applied to plant-pathogenic bacteria, as their databases are primarily optimized for clinical isolates. Furthermore, the antimicrobial susceptibility testing (AST) profile revealed multidrug resistance to several antibiotic classes, including aminoglycosides, fluoroquinolones, and cephalosporins. While such resistance is not typically relevant in the context of plant pathology, it may pose biosafety concerns in the event of accidental human exposure, particularly among immunocompromised individuals. Altogether, the phenotypic and resistance characteristics observed are consistent with a member of the genus *Dickeya*, most likely *Dickeya chrysanthemi*, exhibiting notable metabolic adaptability and antimicrobial resistance.

Comparative analysis with references from Abdalla (2001) and *Bergey's Manual of Systematic Bacteriology* (Table 4) further supported the consistency of these profiles with characteristics described for species within the genus *Dickeya.*

Table 4: Comparative biochemical and physiological characterization of the bacterial isolate, with reference to Abdalla (2001) and Bergey's Manual of Systematic Bacteriology

Characteristic	Isolate tested	Abdalla (2001) *Erwinia chrysanthemi*	Bergey's Manual *Pectobacterium chrysanthemi*
Gram	-	-	-
Catalase	+	+	+
Oxidase	-	-	-
Growth in 5%NaCl	-	-	-
Growth at 36 °C	+	+	+
Pectinase	+	+	+
Gelatinase	+	+	+
Production of acetoin	+	+	+
Phosphatase	+	+	+
Malonate	+	nd	+
Maltose	-	-	-
Cellobiose	-	nd	-
Melibiose	+	nd	+
Citrate	+	nd	+
Urease	-	-	-
Adonitol	-	nd	-
Amygdalin	+	nd	+
l-Arabitol	-	nd	-
D-Mannose	+	nd	+
Palatinose	-	nd	-
Ornithine decarboxylase	-	nd	-
Tryptophan deaminase	-	nd	-

nd = not determined

III.1.3 Overcoming challenges in molecular identification via sanger sequencing

Initial efforts to identify the bacterial isolate through Sanger sequencing of a target gene encountered technical limitations that compromised the reliability and completeness of the generated data (data available at[1]). Analysis via EZBioCloud revealed that the forward and reverse reads achieved only 46.5% and 59.8% alignment coverage, respectively, relative to the full-length 16S rRNA gene. The corresponding chromatograms exhibited overlapping peaks, weak signal intensities, and ambiguous base calls particularly in the middle and late regions of the sequences suggesting sequencing anomalies such as compression, base-calling errors, and polymerase slippage. According to standard Sanger sequencing troubleshooting guidelines (Macrogen, 2020), these patterns are frequently attributed to DNA secondary structures, regions of high GC or AT content, or homopolymeric stretches that promote slippage during amplification. Additionally, the presence of insertion/deletion (indel)

[1] https://dna2.macrogen.com/admin/download_zipLims3.jsp?oN=HC01192485&fn=HC01192485.zip&rgsnDttm=250226&fake=20250307101136

mutations and mixed signal peaks indicated potential template heterogeneity. One plausible explanation is that the DNA sample did not originate from a pure bacterial culture. Contamination by host DNA or co-existing microbial species possibly introduced during transport from Ouargla (Algeria) to Amsterdam (Netherlands) may have led to the co-amplification of multiple templates, thereby producing overlapping chromatogram signals characteristic of mixed-template sequencing (Loman *et al.,* 2012).

Moreover, genomic features intrinsic to the isolate, such as high genetic variability, repetitive sequences, or homopolymeric tracts, may have contributed to the sequencing difficulties observed. These features are well known to interfere with DNA polymerase fidelity, especially in regions susceptible to indel formation (Acinas *et al.,* 2005; Sipos *et al.,* 2007). These anomalies are clearly illustrated in figure 12, which displays the Sanger chromatograms and sequence alignment summaries, highlighting signal ambiguity, sequence dropouts, and reduced gene coverage. Taken together, these observations underscore the limitations of Sanger sequencing despite its foundational contributions to molecular genetics (Sanger *et al.,* 1977; Smith *et al.,* 1995) when applied to complex or low-quality DNA samples, and highlight the critical importance of using high-purity, well-characterized templates for accurate microbial identification.

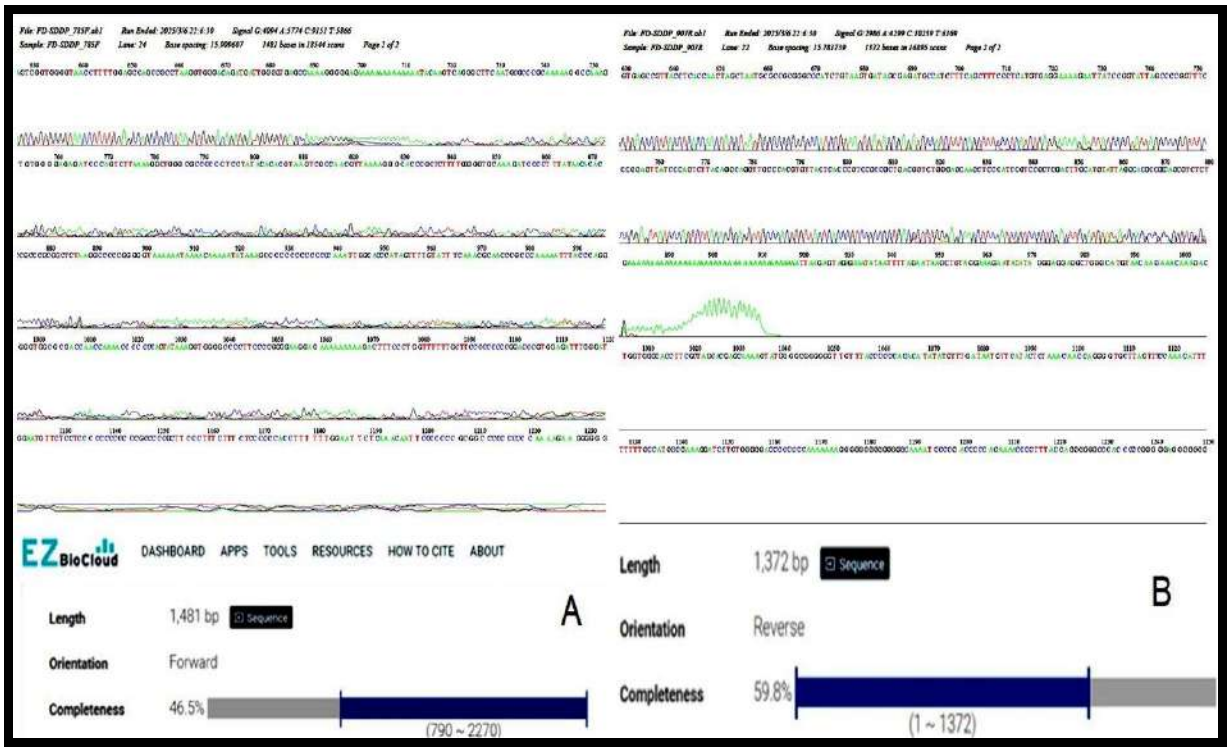

Figure 12: Chromatogram profiles and alignment completeness of partial 16S rRNA gene sequences obtained by Sanger sequencing of bacterial isolate (**A**) Forward strand sequence (1,481 bp) with 46.5% completeness (**B)**Reverse strand sequence (1,372 bp) with 59.8% completeness.

III.2. Assessment of the isolated bacterium's pathogenicity

In vitro inoculation of mechanically wounded date palm leaflets with the isolated bacterial strain resulted in visible necrosis and tissue maceration within 72 hours post-inoculation, with lesions expanding progressively over time (Figure 13A). In contrast, control leaflets treated with sterile distilled water (SDW) remained asymptomatic throughout the observation period (Figure 13B).

Figure 13: *In vitro* assay for evaluating the pathogenicity of the isolated bacterium on date palm leaflets. (**A**) Mechanically wounded leaflets inoculated with the bacterial suspension after 72 hours of incubation at 30 °C.(**B**) Control leaflets treated with sterile distilled water (SDW) under identical conditions.

Similarly, *in planta* experiments confirmed the pathogenic potential of the isolate, as one year old date palm seedlings inoculated in the crown region began to display wilting symptoms by 10 days post-inoculation (dpi), which advanced to chlorosis, necrosis, and frond collapse by 24 dpi. Control seedlings injected with SDW showed no signs of disease and remained healthy over the same period (Figure 14).

Figure 14 *In planta* assay for assessing the pathogenic potential of the isolated bacterium on date palm seedlings. (**Top row**) one year old date palm seedlings inoculated with the bacterial suspension and monitored at 0-, 10-, and 24-days post-inoculation (dpi). (**Bottom row**) Control seedlings injected with sterile distilled water (SDW) and observed over the same time intervals.

The appearance of characteristic decline symptoms in both *in vitro* and *in planta* assays provides compelling evidence for the pathogenicity of the isolated bacterium. These findings align with those reported by Abdalla (2001), who documented similar symptoms following artificial inoculation of date palms with phytopathogenic bacteria. The observed progression from localized necrosis to systemic wilt indicates the presence of virulence factors, likely including cell wall-degrading enzymes and toxins that impair vascular function (Agrios, 2005; Strange, 2003). The complete absence of symptoms in the control treatments further supports that the observed effects were due to the inoculated organism and not to mechanical damage or environmental conditions (Schaad *et al.,* 2001). Moreover, the consistent reproduction of disease symptoms across multiple biological replicates satisfies Koch's postulates, thereby confirming a causal relationship between the isolated bacterium and the sudden decline disease observed in date palms (Lucas *et al.,* 2012).

III.3. Bacteriophage characterization

All five enriched bacteriophage lysates (Ew1 to Ew5) exhibited lytic activity against the target bacterial strain, as evidenced by the formation of turbid lysis zones on bacterial lawns (Figure 15A). These lysis zones were generally turbid and of relatively small diameter, indicating low phage titers or modest lytic efficiency when used individually. In contrast, the phage cocktail, consisting of the five individual isolates, produced a significantly larger and more transparent lytic halo (Figure 15B), suggesting enhanced antibacterial efficacy.

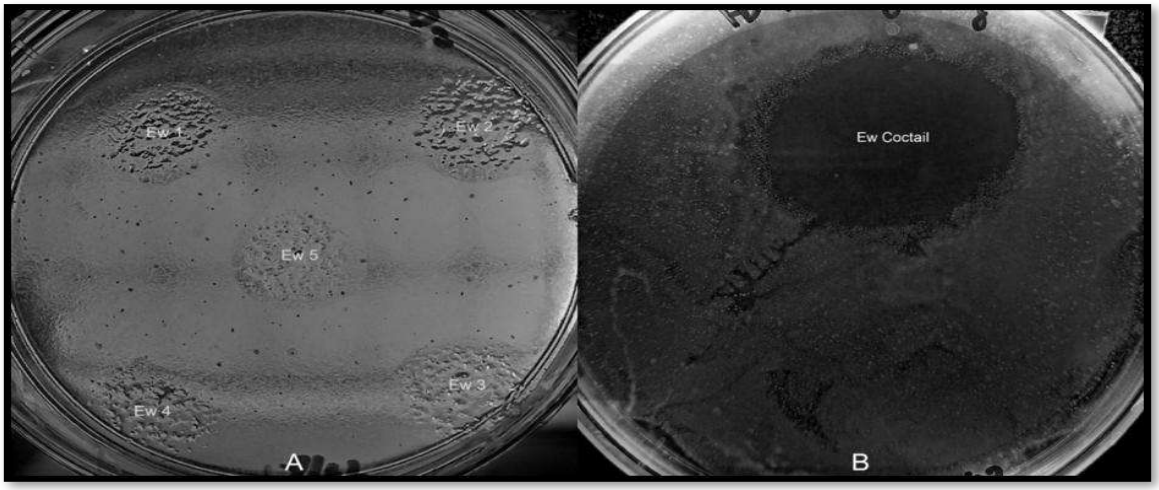

Figure 15:Spot test assay of individual bacteriophages (Ew1–Ew5) and a phage cocktail on the target bacterial isolate following 24 hours of incubation at 30 °C.(**A**) Spot inoculation of individual bacteriophage isolates Ew1 to Ew5 onto a lawn of the target bacterial isolate. (**B**) Spot inoculation of a phage cocktail comprising all five isolates under the same incubation conditions.

Quantification via small-drop plaque assay revealed a titer of 4.0×10^6 plaque-forming units per milliliter (PFU/mL) (Figure 16), confirming a high concentration of infective particles suitable for downstream applications.

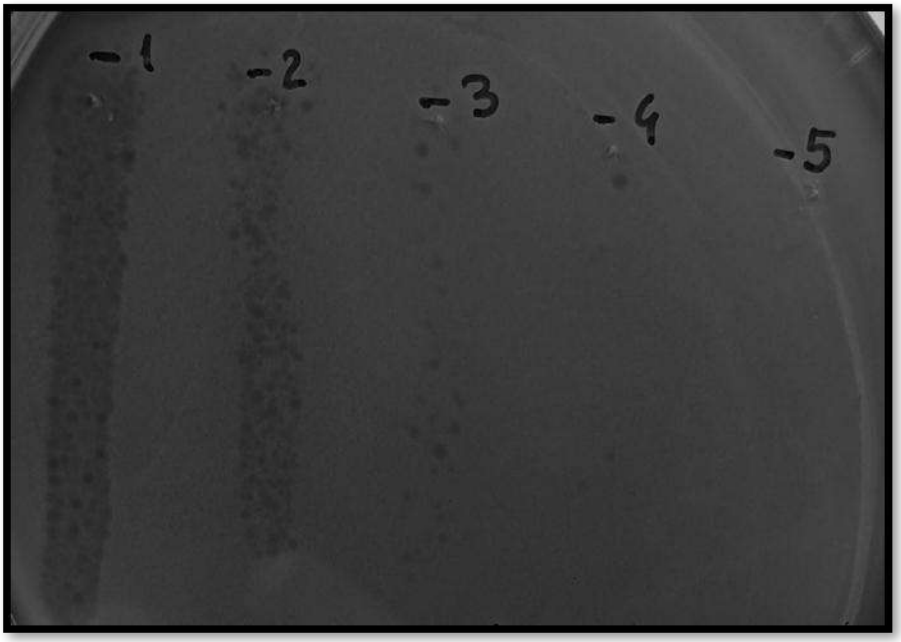

Figure 16:Titration of a phage cocktail using the small-drop plaque assay on a lawn of the target bacterial isolate, followed by incubation at 30°C for 24 hours. Serial tenfold dilutions of the phage suspension, ranging from 10^1 to 10^5, were applied from **left** to **right.**

The observed differences in plaque clarity and size among individual isolates reflect potential variability in infectivity, replication efficiency, and phage-host interaction dynamics. The superior lytic activity exhibited by the phage cocktail is consistent with previous studies demonstrating the benefits of using polyphage formulations to enhance host range, reduce bacterial resistance, and improve lytic potential (Chan *et al.,* 2017). The obtained titer falls within the optimal range reported for experimental biocontrol and therapeutic use, particularly in agricultural settings (Loc-Carrillo & Abedon, 2011). Furthermore, the morphological diversity of plaques ranging from turbid to clear may indicate the presence of both temperate and strictly lytic phages, as turbid plaques are typically associated with incomplete lysis or lysogeny, while clear plaques are indicative of strong lytic cycles (Clokie *et al.,* 2011). Collectively, these findings suggest that the phage cocktail outperforms individual phage isolates and holds promise as an effective candidate for controlling phytopathogenic bacteria involved in the sudden decline disease of date palms.

III.4. Antibacterial activity of rosemary essential oil

A significant antibacterial effect was observed following treatment with rosemary essential oil (REO) at 10% (v/v) against *Dickeya* sp., as demonstrated by the prominent inhibition zone measuring 17 mm in Petri dish assays (Figures 17B). The treated area displayed a clear and defined halo of bacterial growth suppression, contrasting with the absence of any inhibition in control discs containing only sterile distilled water (SDW) or Tween 20 (Figure17 A) This highlights the potent inhibitory capacity of REO at this concentration and confirms that the effect is attributable solely to the essential oil's active constituents.

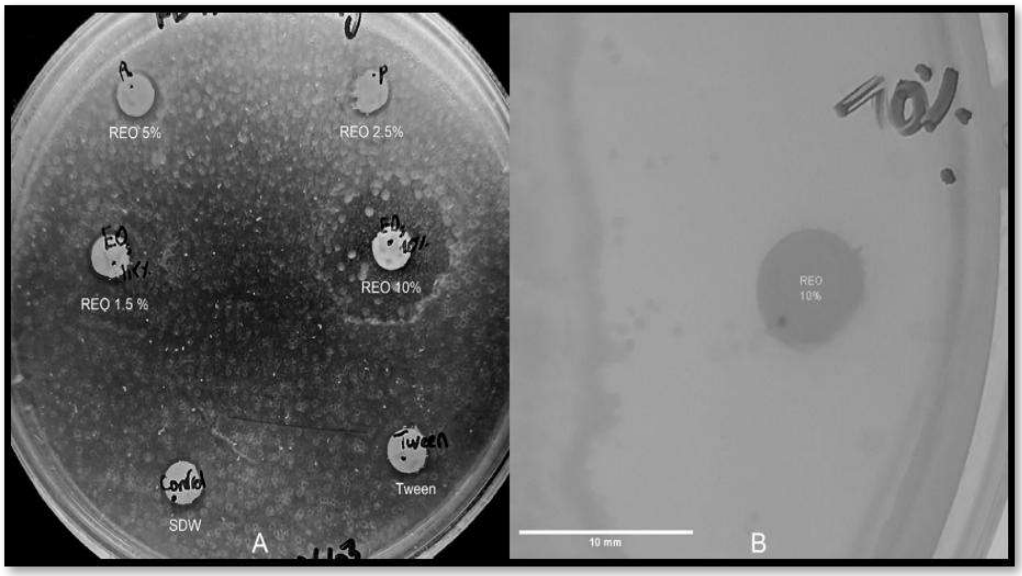

Figure 17: Antibacterial activity of Rosemary Essential Oil (REO) against *Dickeya sp*. Using the agar disc diffusion method. (**A**) REO tested at different concentrations (1.5%, 2.5%, 5%, and 10%). (**B**) Enlarged view of the antibacterial activity at 10% REO.

Complementary analysis using gas chromatography–mass spectrometry (GC-MS) (Figure 18) revealed the chemical composition of the REO. Eucalyptol (1,8-cineole) was identified as the

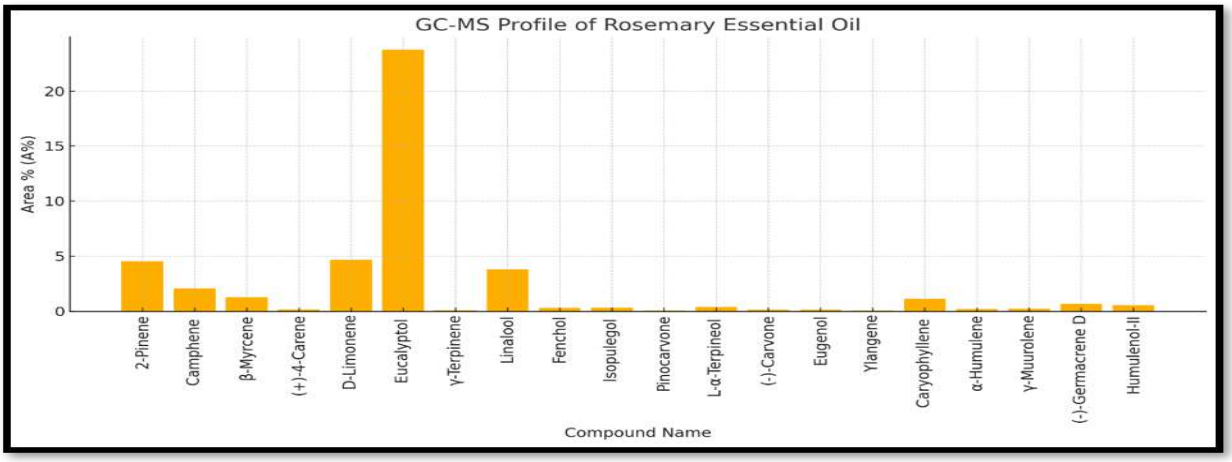

Figure 18:: Gas Chromatography–Mass Spectrometry (GC-MS) analysis of the chemical composition of Rosemary (*Rosmarinus officinalis*) essential oil.

major constituent (23.76%), followed by D-limonene (4.66%), α-pinene (4.53%), linalool (3.78%), and camphene (2.07%). These compounds are widely recognized for their antimicrobial properties and are likely responsible for the observed antibacterial activity.

The strong inhibition of *Dickeya* strains at 10% REO confirms the essential oil's potential as a natural antimicrobial agent. The effectiveness observed aligns with previous research indicating that high concentrations of essential oils enhance their bactericidal activity due to increased availability of active compounds (Košćak *et al.,* 2023). Notably, the absence of inhibition around control discs eliminates the possibility of confounding effects from solvents or surfactants. The predominance of eucalyptol in the GC-MS profile is particularly relevant, as this monoterpene is known to disrupt bacterial membranes, increase permeability, and induce oxidative damage through the generation of reactive oxygen species (ROS) (Merghni *et al.,* 2023). D-limonene similarly disrupts bacterial membranes due to its hydrophobic interaction with lipid bilayers, causing leakage and cell death (Stojiljkovic *et al.,* 2018). α-Pinene contributes by promoting oxidative stress and altering membrane surface characteristics (Shahina *et al.,* 2022), while linalool interferes with enzymatic pathways critical for bacterial metabolism (Hui *et al.,* 2017). Although camphene's precise mode of action is less characterized, its contribution to membrane destabilization and ROS generation is plausible (Rashid, 2010). Together, these compounds likely act synergistically to enhance the antibacterial effect observed with 10% REO. The findings underscore the potential application of REO in managing bacterial phytopathogens and support its use in environmentally friendly plant protection strategies (Talbaoui *et al.,* 2012; Kabotso *et al.,* 2024).

III.5. Biocontrol efficacy in *in vitro* and *in planta* conditions

The antimicrobial efficacy of bacteriophage and rosemary essential oil (REO) treatments against *Dickeya* spp., the causative agent of sudden decline disease in *Phoenix dactylifera* L., was assessed under both *in vitro* and *in planta* conditions (Figure 19 and Figure 20). *In vitro* assays demonstrated statistically significant reductions in bacterial counts across treatment groups. The untreated positive control (bacterial suspension only) showed the highest bacterial density at 1.5×10^{9} CFU/mL. Application of a phage cocktail (MOI = 1) targeting multiple *Dickeya* strains reduced bacterial load to 4.3×10^{5} CFU/mL, indicating a 2.54 $\log_{10}$ reduction. Treatment with 10% (v/v) REO alone decreased bacterial counts to 3.7×10^{6} CFU/mL (1.61 $\log_{10}$ reduction). Notably, the combined treatment of phage cocktail and REO

yielded the most pronounced reduction, lowering CFU to 5.4×10^4 CFU/mL a 3.44 $\log_{10}$ decrease, suggesting a synergistic effect.

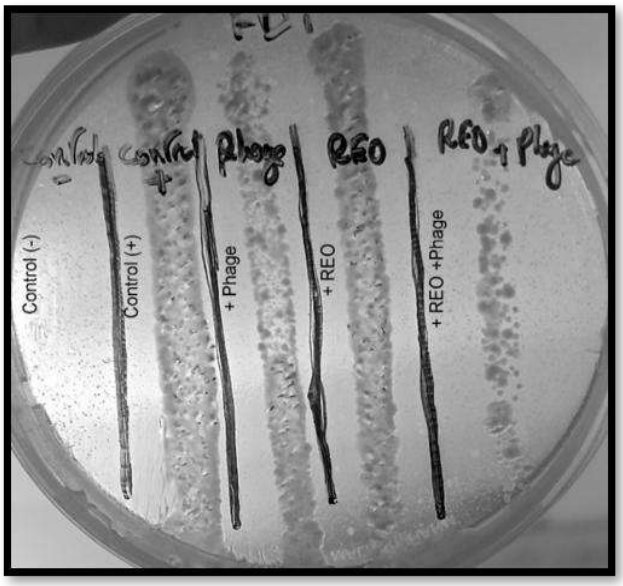

Figure 19: Qualitative streak-line assay of *Dickeya sp*. growth on nutrient agar after 24 hours of incubation at 30°C under different treatment conditions. From **left** to **right**: Negative control (nutrient broth only); Positive control (bacterial suspension); treatment with a lytic phage cocktail (MOI = 1); treatment with rosemary essential oil (REO, 10% v/v); combined treatment with the phage cocktail and REO. Visual differences in bacterial growth density indicate the inhibitory effects of the treatments.

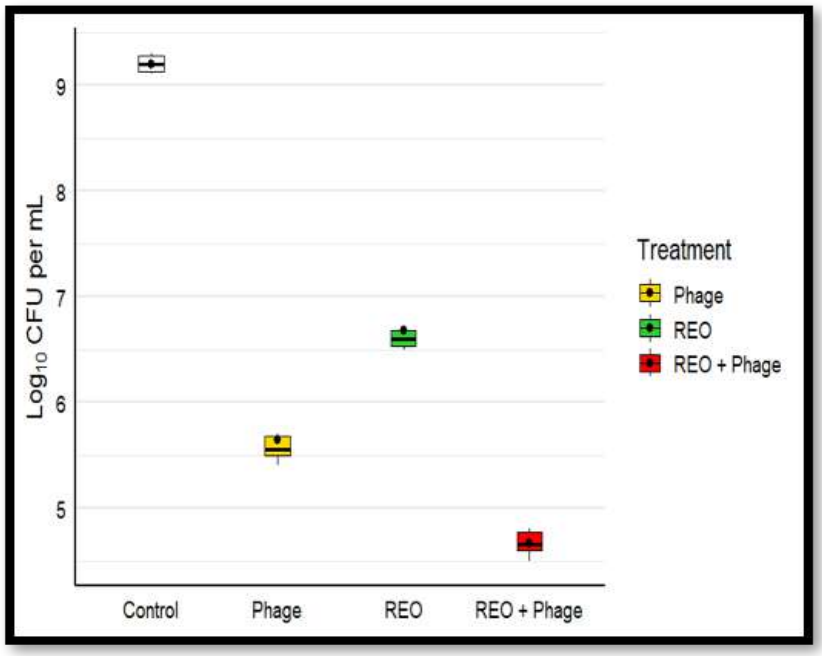

Figure 20: Box Plot Showing reduction of CFU of *Dickeya sp*. on Nutrient agar after 24 hours of incubation at 30°C under different treatment conditions: Positive control (Bacterial suspension only), Treatment with a lytic phage cocktail (MOI = 1), Treatment with Rosemary Essential Oil (REO, 10% v/v), and Combined treatment with phage cocktail and REO. Analysis Performed in RStudio (v4.5.0) Using one-way ANOVA.

In planta assays further validated these findings. Visual inspection at 0-, 10-, and 20-days post-infection (dpi) showed that plants treated with the phage–REO combination remained largely asymptomatic, while those receiving single treatments or the positive control displayed progressive chlorosis, wilting, and necrosis (Figure 21). Disease Severity Index (DSI%) scores confirmed this trend: positive control – 90%, phage alone – 60.0%, REO alone – 75%, and phage + REO – 40%. (Figure 22)

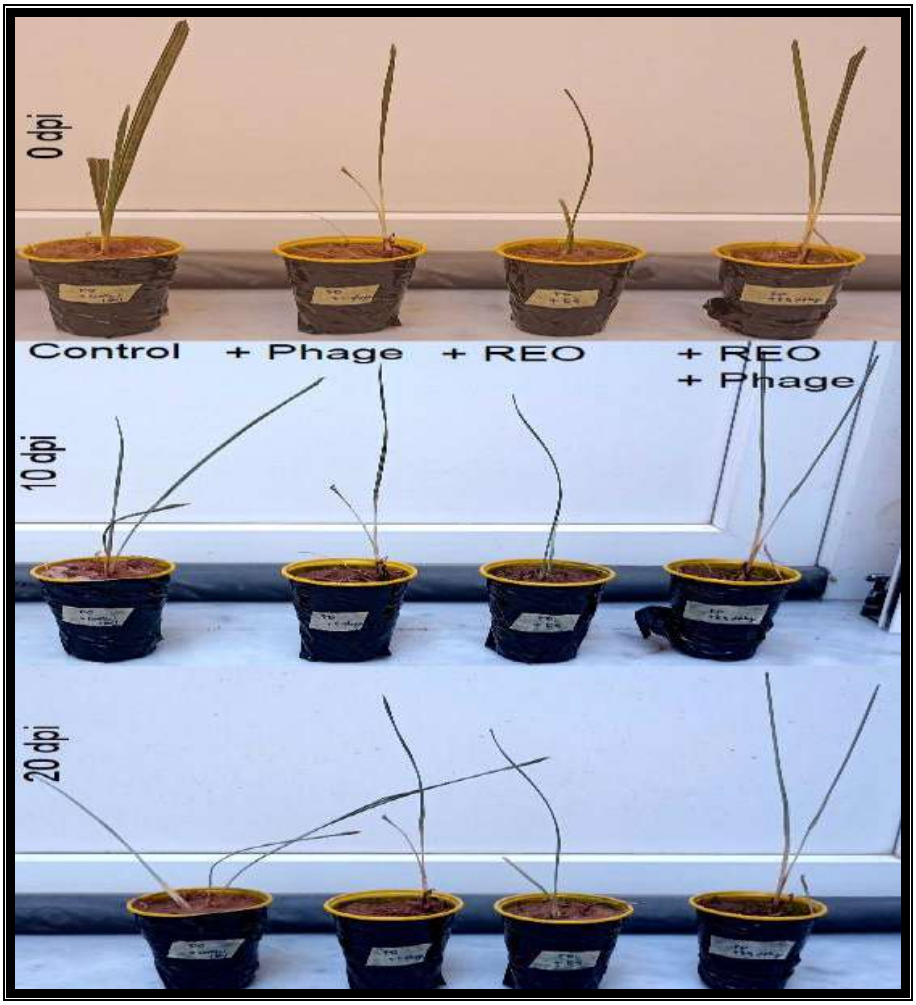

Figure 21*: In Planta* assay showing the progression of disease symptoms in date palm seedlings inoculated with *Dickeya sp*. under different treatments: from **left** to **right**; positive control (bacterial suspension only); Treatment with lytic phage cocktail (MOI = 1); Treatment with rosemary essential oil (REO, 10% v/v), and combined treatment with phage cocktail and REO. Observations recorded at 0-, 10-, and 20-days post-inoculation (dpi).

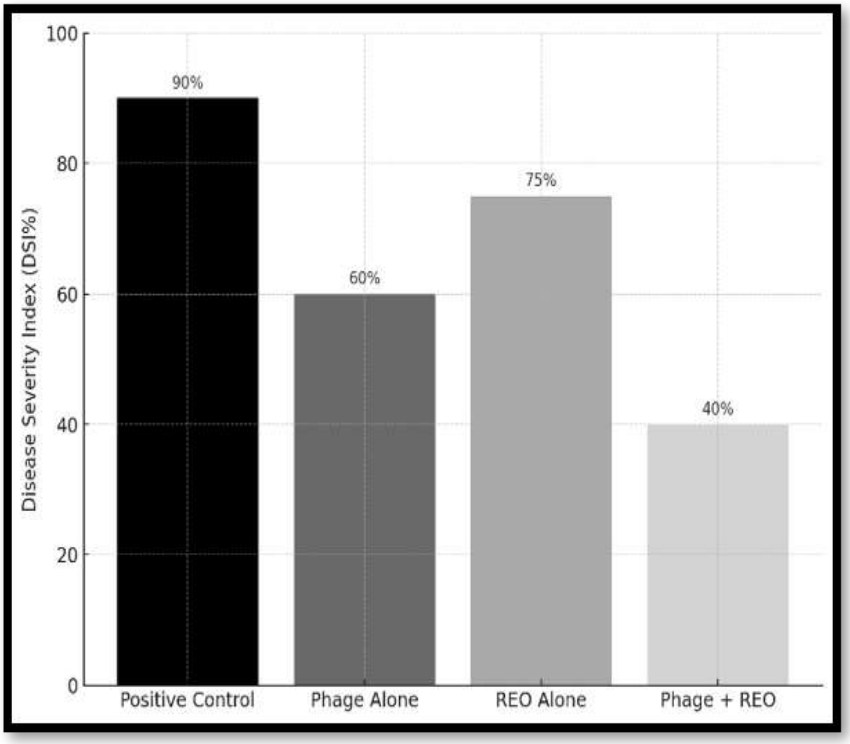

Figure 22: Effect of Phage alone, REO alone, and combined treatments on Disease Severity Index (DSI%) in *P. dactylifera* Seedlings at 20 dpi

These results support a synergistic biocontrol potential of combining bacteriophages and REO against *Dickeya* -induced sudden decline in date palms. The enhanced antimicrobial performance of the combined treatment likely results from complementary mechanisms of action. Bacteriophages, with their high specificity and lytic activity, directly target and lyse *Dickeya* cells (Sabri *et al.,* 2022), while REO exerts broad-spectrum antimicrobial effects, including membrane disruption, enzyme inhibition, and interference with bacterial respiration (Soliman *et al.,* 2024). The synergism may be attributed to REO-induced sub-lethal membrane damage that enhances phage adsorption and penetration or to the metabolic stress imposed by REO, which sensitizes bacteria to phage-mediated lysis (Elafify *et al.,* 2025). The significantly lower DSI observed in the combined treatment group underlines the potential of integrating phage therapy with essential oil application as a sustainable and effective biocontrol strategy in agroecosystems.

CONCLUSION

CONCLUSION

This research represents an initial step toward the development of sustainable biocontrol strategies against Sudden Decline (SD) disease affecting *Phoenix dactylifera* L. in the arid agroecosystems of Ouargla, Algeria. Through a multidisciplinary approach, a presumptive bacterial pathogen was successfully isolated and phenotypically characterized, with biochemical profiling suggesting a likely affiliation with the genus *Dickeya*. Although molecular identification via Sanger sequencing was hindered likely due mixed DNA pattern the pathogenicity of the isolate was clearly demonstrated through both *in vitro* and *in planta* assays, confirming its virulence under controlled conditions.

To propose environmentally friendly alternatives to chemical treatments, two complementary biocontrol strategies were investigated: a lytic bacteriophage cocktail targeting the pathogen, and the evaluation of the antimicrobial activity of *Rosmarinus officinalis* essential oil (REO). Both agents exhibited significant antibacterial effects, and their combined application demonstrated a synergistic interaction, effectively reducing bacterial load and disease symptoms. These findings underscore the potential of integrating phage therapy and plant-derived antimicrobials as eco-friendly solutions for managing bacterial diseases in date palm cultivation.

However, several limitations must be addressed. The molecular identification of the pathogen remains inconclusive and warrants validation using more robust methods such as high-throughput or next-generation sequencing (NGS) to achieve precise taxonomic and phylogenetic resolution. Additionally, phage characterization is incomplete: the isolated bacteriophages have not been fully described at the genomic level and do not yet meet the classification standards set by the International Committee on Taxonomy of Viruses (ICTV). Comprehensive genomic and functional analyses are essential for future applications.

Regarding REO, its high volatility and the absence of minimum inhibitory concentration (MIC) data limit its reproducibility and stability. Future studies should aim to determine the MIC and develop stable formulations such as encapsulated forms to enhance efficacy and shelf life. Moreover, the interaction mechanisms between the two biocontrol agents remain poorly understood. Further research is needed to clarify the sequence of action, and how environmental factors influence their synergy. Optimizing delivery methods such as spraying, injection, or alternative systems under field conditions is also crucial.

In terms of disease epidemiology, this study constitutes the first report of Sudden Decline in the region, and its distribution remains uncharted. The pathogen-host interaction is likewise insufficiently understood. To fill these gaps, metagenomic approaches should be employed to detect and map the occurrence of Sudden Decline across various regions of Algeria.

From an experimental standpoint, future research should incorporate a larger number of replicates, particularly for *in planta* assays, preferably conducted under greenhouse conditions. Long-term experiments extending beyond one month and covering a range of environmental conditions (e.g., elevated temperatures,) are strongly recommended to yield more ecologically valid and statistically robust data.

CONCLUSION

In summary, this research lays a promising foundation for the development of integrated, scalable, and environmentally sound biocontrol strategies to manage date palm diseases in arid ecosystems. By addressing the current limitations and pursuing the proposed directions, a transition from laboratory findings to practical field applications can be realized, contributing significantly to sustainable agriculture in arid regions.

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ANNEXES

ANNEXES

Annex 1: Composition of media and buffers used in this study

Nutrient Agar (NA) Composition (per liter): Peptone: 5.0 g Yeast extract: 1.5 g Beef extract: 1.5 g Sodium chloride (NaCl): 5.0 g Agar: 15.0 g Final pH: 7.4 ± 0.2 at 25°C	**Modified-Crystal Violet Pectate Medium (CVP)** Composition (per liter): Sodium polypectate: 18.0 g Sodium hydroxide: 0.36 g Calcium chloride dihydrate (CaCl2·2H2O): 0.6 g Crystal violet: 0.0015 g Agar: 4.0 g Final pH: 7.2 ± 0.2 at 25°C
Mueller-Hinton Agar (MH) Composition (per liter): Beef extract: 2.0 g Casein hydrolysate (acid hydrolysate of casein): 17.5 g Starch: 1.5 g Agar: 17.0 g Final pH: 7.3 at 25°C	**Gelatin Liquefaction Medium** Composition (per liter): Gelatin: 120.0 g Peptone: 5.0 g Beef extract: 3.0 g Final pH: 6.8 ± 0.2 at 25°C
SM Buffer Composition (per liter): Sodium chloride (NaCl): 5.8 g Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$): 2.0 g Tris-HCl (1 M, pH 7.5): 50 mL Distilled water (H_2O): qs to 1,000 mL	

Annex 2.1: Interpretation Table of the API 20 E Haddani, H. (2023)

Test	Active component	Enzymatic reaction	Negative Result	Positive Result
ONPG	2-nitrophenyl-βD-galactopyranoside	β-galactosidase (Ortho NitroPhenyl-βDGalactopyranosidase)	Colorless	Yellow
ADH	L-arginine	Arginine Dihydrolase	Yellow	Red – Orange
LDC	L-lysine	Lysine Decarboxylase	Yellow	Red – Orange

Test	Active component	Enzymatic reaction	Negative Result	Positive Result
ODC	L-ornithine	Ornithine Decarboxylase	Yellow	Red – Orange
CIT	Citrate	Citrate Utilization	Pale green – Yellow	Blue green – Blue
H2S	Sodium thiosulfate	H2S Production	Colorless – Grayish	Black deposit – Fine line
URE	Urea	Urease	Yellow	Red – Orange
TDA	L-tryptophan	Tryptophan Deaminase	Yellow	Reddish brown
IND	L-tryptophan	Indole Production	Colorless – Pale green – Yellow	Pink
VP	Sodium pyruvate	Acetoin Production	Colorless – Pale pink	Pink – Red
GEL	Gelatin	Gelatinase	No diffusion	Black pigment diffusion
GLU	D-glucose	Fermentation – Oxidation	Blue – Blue-green	Yellow – Yellow gray
MAN	D-mannitol	Fermentation – Oxidation	Blue – Blue-green	Yellow
INO	Inositol	Fermentation – Oxidation	Blue – Blue-green	Yellow
SOR	D-sorbitol	Fermentation – Oxidation	Blue – Blue-green	Yellow
RHA	L-rhamnose	Fermentation – Oxidation	Blue – Blue-green	Yellow
SAC	D-sucrose	Fermentation – Oxidation	Blue – Blue-green	Yellow
MEL	D-melibiose	Fermentation – Oxidation	Blue – Blue-green	Yellow
AMY	Amygdalin	Fermentation – Oxidation	Blue – Blue-green	Yellow
ARA	L-arabinose	Fermentation – Oxidation	Blue – Blue-green	Yellow

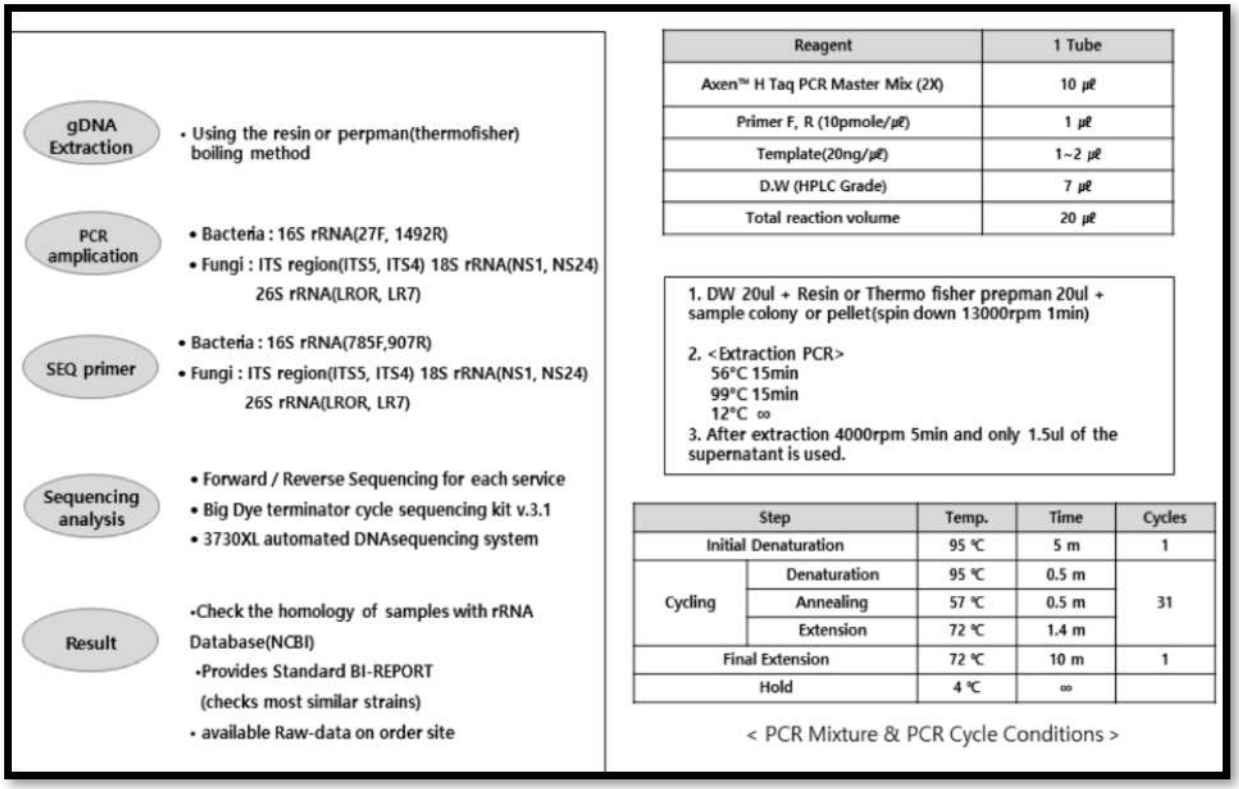
Annex 2.2: API 20 E identification profile of a bacterial isolate

Annex 3: Test panel and composition of the VITEK GN card Haddani, H. (2023)

Well	Test Name	Mnemonic	Dosage / well
2	Ala–Phe–Pro–ARYLAMIDASE	APPA	0.0384 mg
3	ADONITOL	ADO	0.1875 mg
4	L-Pyrrolidonyl–ARYLAMIDASE	PyrA	0.018 mg
5	L-ARABITOL	IARL	0.3 mg
7	D-CELLBIOSE	dCEL	0.3 mg
8	BETA–GALACTOSIDASE	BGAL	0.036 mg
10	H2S PRODUCTION	H2S	0.0024 mg
11	BETA-N-ACETYL-GLUCOSAMINIDASE	BNAG	0.0408 mg
12	Glutamyl ArylamidasepNA	AGLTp	0.0324 mg
13	D-GLUCOSE	dGLU	0.3 mg
14	GAMMA–GLUTAMYL–TRANSFERASE	GGT	0.0228 mg
15	FERMENTATION/GLUCOSE	OFF	0.45 mg
17	BETA–GLUCOSIDASE	BGLU	0.036 mg
18	D-MALTOSE	dMAL	0.3 mg
19	D-MANNITOL	dMAN	0.1875 mg
20	D-MANNOSE	dMNE	0.3 mg
21	BETA–XYLOSIDASE	BXYL	0.0324 mg
22	BETA–Alanine arylamidasepNA	BALap	0.0174 mg
23	L-Proline ARYLAMIDASE	ProA	0.0234 mg
24	LIPASE	LIP	0.0192 mg
26	PALATINOSE	PLE	0.3 mg
27	Tyrosine–ARYLAMIDASE	TyrA	0.0276 mg
31	UREASE	URE	0.15 mg
32	D-SORBITOL	dSOR	0.1875 mg
33	SUCROSE	SAC	0.3 mg

Well	Test Name	Mnemonic	Dosage / well
34	D-TAGATOSE	dTAG	0.3 mg
35	D-TREHALOSE	dTRE	0.3 mg
36	CITRATE (SODIUM)	CIT	0.054 mg
37	MALONATE	MNT	0.15 mg
39	5-KETO-D-GLUCONATE	5KG	0.3 mg
40	Alkalinization of L-LACTATE	ILATk	0.15 mg
41	ALPHA–GLUCOSIDASE	AGLU	0.036 mg
42	Alkalinization of SUCCINATE	SUCT	0.15 mg
43	Beta-N-ACETYL-GALACTOSAMINIDASE	NAGA	0.0306 mg
44	ALPHA–GALACTOSIDASE	AGAL	0.036 mg
45	PHOSPHATASE	PHOS	0.0504 mg
46	Glycine ARYLAMIDASE	GlyA	0.012 mg
47	ORNITHINE DECARBOXYLASE	ODC	0.3 mg
48	LYSINE DECARBOXYLASE	LDC	0.15 mg
52	BASE OF DECARBOXYLASE	ODEC	N/A
53	Assimilation of L-HISTIDINE	IHISt	0.087 mg
56	COUMARATE	CMT	0.126 mg
57	BETA–GLUCURONIDASE	BGUR	0.0378 mg
58	RESISTANCE O/129 (comp. vibrio)	O129R	0.0105 mg
59	Glu–Gly–Arg–ARYLAMIDASE	GGAA	0.0576 mg
61	Assimilation of L-MALATE	IMLTa	0.042 mg
62	ELLMAN	ELLM	0.03 mg
64	Assimilation of L-LACTATE	ILATa	0.186 mg

Annex 4: Experimental protocol for molecular identification of isolates via Sanger Sequencing (Macrogen®)



Reagent	1 Tube
Axen™ H Taq PCR Master Mix (2X)	10 ㎕
Primer F, R (10pmole/㎕)	1 ㎕
Template(20ng/㎕)	1~2 ㎕
D.W (HPLC Grade)	7 ㎕
Total reaction volume	20 ㎕

1. DW 20ul + Resin or Thermo fisher prepman 20ul + sample colony or pellet(spin down 13000rpm 1min)

2. <Extraction PCR>
56°C 15min
99°C 15min
12°C ∞
3. After extraction 4000rpm 5min and only 1.5ul of the supernatant is used.

Step		Temp.	Time	Cycles
Initial Denaturation		95 ℃	5 m	1
Cycling	Denaturation	95 ℃	0.5 m	31
	Annealing	57 ℃	0.5 m	
	Extension	72 ℃	1.4 m	
Final Extension		72 ℃	10 m	1
Hold		4 ℃	∞	

< PCR Mixture & PCR Cycle Conditions >

Annex 5: Preparation of different concentrations of Rosemary Essential Oil (REO) solutions using Tween 20 and Sterile distilled water (SDW)

Components	**1.5% REO**	**2.5% REO**	**5% REO**	**10% REO**
REO	15 µL	25 µL	50 µL	100 µL
Tween	7.5 µL	12.5 µL	25 µL	50 µL
SDW	977.5 µL	962.5 µL	925 µL	850 µL

Annex 6: Effect of Phage, Rosemary Essential Oil (REO), and combined treatment on bacterial growth in microplate wells.