People's Democratic Republic of Algeria

Ministry of Higher Education and Scientific Research

Amar Telidji University - Laghouat

FACULTY OF SCIENCES

DEPARTMENT OF AGRONOMIC SCIENCES

MASTER'S THESIS

Presented by

Khelifi iman

FIELD OF NATURAL AND LIFE SCIENCES (SNV)

AGRONOMIC SCIENCES PROGRAM

PLANT PROTECTION OPTION

Theme

Contribution to the study of rhizobacterial biocontrol of the vascular fusarium of the pea *Pisum sativum*

Committee Members

Mrs. MARFOUA Meriem	M CA	Pesident
Mrs. ZAZA Messouda	M AA	Reviewer
Mrs. AMEUR Djamila	M AA	Reporter

Academic Year: **2023-2024**

ACKNOWLEDGMENTS

I would like to express my gratitude to the Almighty ALLAH above all, for guiding me throughout my years of study and giving me the will, patience, and courage to complete this work.

I extend my respect and gratitude to:
Madame MARFOUA Meriem for presiding over the jury.
Madame ZAZA MESSOUDA for examining and evaluating this work.

I also want to thank:
My supervisor, Miss AMEUR Djamila, for her insight, support, assistance, and invaluable advice that significantly contributed to my approach to the subject and its treatment. I can never thank you enough, Madame.

All the teachers in the Department of Agronomic Sciences for dedicating their time and expertise to provide us with the best education Mrs. RANAN Zahra, Mrs. AZOUAOU Karima, Mrs. MARFOUA Meriem, Mrs. MEALEM Hamida, Mr. SERIDI Abdelkader, Mr. MECHRAOUI Choaib, Mr. AMARA Yacine.

A special thanks goes to Madame HOUITI Fatiha and her research team, especially Anfal, for their collaboration, assistance, and companionship during the research phase.

I would also like to thank the engineers from the laboratory for their assistance, Mrs. Halima TAIBI, Mrs. Fatima AOUISSI, and Mrs. Rokaia OTHMANI. I also would like to thank my friends M. KHADIDJA, Berreh HIZIA, Bensaad MANEL, kerkabi AICHA, Benbouglimina ABDELKADER DJILALI

To all those who have assisted us directly or indirectly in the completion of this work.

Dedications

I dedicate this work to my dear parents for their patience, love and encouragement.

To my two sisters and my little brother.

To my dear friends: M.khadidja, B hizia , K aicha

This work is dedicated to all the people who believed in me and supported me throughout my studies.

Contribution to the study of rhizobacterial biocontrol of the vascular fusarium of the pea *Pisum sativum*

Abstract

This study examined the use of biological approaches to control vascular *Fusarium* in plants through the interaction of root and fungal diseases. One of the main forces controlling plants by biological methods is the antifungal bacteria found in rhizosphere pathogens. Twenty isolates were identified based on bacterial as well as biochemical and biophysical tests. During our biostimulation experiments, we have enzyme activity to discover how bacteria respond to substrate sources and how environmental factors affect enzymatic reactions. As part of our biostimulation experiments, we studied the activity of enzymes to understand how bacteria access various sources of substrates and the influence of conditions on these enzymatic reactions. This study was carried out on using 8 different bacteria, and led to the discovery of a catalytic enzyme in these processes. In addition, the activity of rhizobacteria promoting the growth of was studied and in this context, most bacterial isolates showed their ability to improve phosphates. We also observed the production of compounds such as hydrocyanide and Indole-3-acetic acid (IAA), as well as the production of ammonia. These results improve our understanding of the impact of bacteria on enzymatic processes and their ability to stimulate plant growth, and indicate the importance of PGPR bacteria in promoting plant health and environmental sustainability.

Keywords: Rhizobacteria, PGPR, vascular fusarium, biocontrol, *Pisum sativum.*

Contribution à l'étude du biocontrôle rhizobactérien du fusarium vasculaire du petit pois (*Pisum sativum*)

Résumé

Cette étude a examiné l'utilisation d'approches biologiques pour contrôler le Fusarium vasculaire dans les plantes par l'interaction des maladies des racines et des maladies fongiques. Vingt isolats ont été identifiés sur la base de tests bactériens, biochimiques et biophysiques. Dans le cadre de nos expériences de biostimulation, nous avons étudié l'activité des enzymes pour comprendre comment les bactéries accèdent à diverses sources de substrats et l'influence des conditions sur ces réactions enzymatiques. Cette étude a été réalisée à l'aide de 8 bactéries différentes et a conduit à la découverte d'une enzyme catalytique dans ces processus. De plus, l'activité des rhizobactéries favorisant la croissance des plantes a été étudiée et dans ce contexte, la plupart des isolats bactériens ont montré leur capacité à améliorer les phosphates. Nous avons également observé la production de composés tels que l'hydrocyanide et l'acide indole-acétique, ainsi que la production d'ammoniac. Ces résultats améliorent notre compréhension de l'impact des bactéries sur les processus enzymatiques et leur capacité à stimuler la croissance des plantes, et indiquent l'importance des bactéries PGPR dans la promotion de la santé des plantes et durabilité environnementale.

Mots clé : Rhizobactéries, PGPR, Fusarium vasculaire, Biocontrôle, *Pisum sativum*.

المساهمة في دراسة المكافحة البيولوجية بواسطة البكتيريا الجذرية لفيوزاريوم الأوعية في البازلاء *Pisum sativum*

الملخص

تهدف هذه الدراسة الئ البحث عن استخدام الأساليب البيولوجية لمكافحة آفة الفيوزاريوم في النباتات من خلال التفاعل بين الأمراض الجذرية والفطرية. إحدى القوى الرئيسية التي تتحكم في النباتات بالطرق البيولوجية هي البكتيريا المضادة للفطريات الموجودة في مسببات الأمراض الجذرية.

تم تحديد عشرين عزلة بناءً على الاختبارات البكتيرية وكذلك الاختبارات البيوكيميائية والبيوفيزيائية الحيوية. خلال تجارب التحفيز الحيوي لدينا، قمنا بدراسة نشاط الإنزيمات لاكتشاف كيفية استجابة البكتيريا لمصادر الركائز وكيفية تأثير العوامل البيئية على التفاعلات الإنزيمية، وكجزء من تجارب التحفيز الحيوي لدينا، قمنا بدراسة نشاط الإنزيمات لفهم كيفية وصول البكتيريا إلى مصادر الركائز المختلفة وتأثير الظروف على هذه التفاعلات الإنزيمية، وقد أجريت هذه الدراسة على استخدام 8 بكتيريا مختلفة، وأدت إلى اكتشاف الإنزيم المحفز في هذه العمليات. بالإضافة إلى ذلك، تمت دراسة نشاط البكتيريا الجذرية المعززة لنمو النباتات وفي هذا السياق، أظهرت معظم العزلات البكتيرية قدرتها على تحسين الفوسفات، كما لاحظنا إنتاج مركبات مثل الهيدروسيانيد وحمض الإندول أسيتيك وكذلك إنتاج الأمونيا، هذه النتائج تحسن فهمنا لتأثير البكتيريا على العمليات الأنزيمية وقدرتها على تحفيز نمو النباتات، وتشير إلى أهمية بكتيريا PGPR في تعزيز صحة النباتات والاستدامة البيئية.

الكلمات الرئيسية: بكتيريا الجذور، بكتيريا محفزة لنمو النباتات(PGPR) ، فيوزاريوم الأوعية، المكافحة الحيوية، البازلاء (*Pisum sativum*)

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INTRODUCTION

Introduction

In the field of plant protection, our goal is to explore simple and effective methods that are environmentally friendly and safeguard human health, while protecting plant species that benefit the ecosystem from pests and diseases. Ensuring plant health requires care throughout their life cycle, including the application of fertilizers and protection against diseases. Plant protection practices have evolved from traditional methods to chemical approaches. While chemical controls have been effective for decades, the shift towards using microorganisms for disease management aims to minimize the impact of chemicals on human health and the environment (Parker, 2017).

Biocontrol has emerged as a solution in response to changes caused by the accumulation of pollutants in various environments, such as the rhizosphere and atmosphere. The excessive use of pesticides has been identified as a major contributor to this pollution. Researchers have demonstrated that using microorganisms to combat pathogens indirectly is an innovative and effective approach to disease management (Gurr and Wechsler, 2010).

Plant growth-promoting rhizobacteria (PGPR) represent a particularly promising strategy. They are eco-friendly and cost-effective, contributing to agricultural profitability and improving the livelihoods of small farmers. The increasing utilization of PGPR in agriculture offers a viable alternative to chemical fertilizers, pesticides, and other harmful supplements (Ansari *et al.,* 2017; Ansari and Mahmood, 2019a). These soil microorganisms produce substantial quantities of growth-promoting substances that directly or indirectly influence the morphology and physiology of crops. Recent advancements in sustainable development rely on the use and diversity of PGPR, their colonization capabilities, and their mechanisms of action, which can be harnessed to manage sustainable agricultural systems (Bhattacharyya and Jha, 2012; Di Benedetto *et al.*, 2017; Ansari and Mahmood, 2019).

Rhizobacteria offer a promising approach for the biocontrol of *Fusarium* vascular, providing an ecological alternative to chemical pesticides. Their ability to colonize plant roots, produce antimicrobial metabolites, and induce systemic resistance in host plants makes them effective biocontrol agents against this formidable pathogen. Recent studies have highlighted the importance of understanding the complex interactions between rhizobacteria, plants, and *Fusarium* to optimize their use in sustainable agriculture (Li *et al.,* 2023; Khan *et al.*, 2022).

Introduction

Humans maintain a close relationship with plants, particularly cultivated species of nutritional and economic value, such as green vegetables like peas, which are vulnerable to Fusarium vascular contamination. This is why our study for our master's thesis will focus on: "Contribution to the Study of Biocontrol of *Fusarium* Vascular in Peas with Rhizobacteria."

In our research, we aim to assess the effectiveness of biocontrol against *Fusarium* vascular using rhizobacteria. Our work will involve isolating, purifying, and identifying bacterial strains, and testing them on *Fusarium* in peas to evaluate their interaction. Thus, our central research question will be: Do rhizobacteria contribute to the biocontrol of peas against *Fusarium* vascular?

The following hypotheses are suggested:

- the results will be positive: rhizobacteria will be effective against vascular *Fusarium*
- the results will be negative: rhizobacteria will not interact with vascular *Fusarium*.

Our work will therefore follow a series of experiments carried out in the laboratory explained in this thesis which is divided into:

- ✓ Chapter 1: Background and literature review
- ✓ This section will focus on defining the key concepts on which the research is based (*Fusarium* Vascular, rhizobacteria, peas).
- ✓ Chapter 2: Materials and methods
  In this second part we will describe the material and explain the protocol followed in the experiments carried out.
- ✓ Chapter 3: Results and discussion
  This final chapter is devoted to the presentation of the results obtained, together with a discussion and interpretation.
- ✓ To conclude, we will provide a general summary of the conclusions drawn from the research.

CHAPTER ONE

Theoretical Framework

I.1 Vascular *Fusarium*

Vascular *Fusarium* is a plant disease caused by various fungi of the *Fusarium* genus, principally *Fusarium oxysporum*. This fungal pathogen infects the vascular systems of plants, obstructing the vessels that conduct water and nutrients. This leads to symptoms such as leaf wilting, yellowing and premature death of the plant (Fravel, *et al.,* 2003).

Vascular wilt is a significant threat to many important agricultural and horticultural crops, such as peas, date palms, flax, onions, tomatoes, cotton and many others. It is caused by different pathotypes of *Fusarium oxysporum*, such as *Fusarium oxysporum* f.sp. *pisi* (Fop), which specifically affects peas.

Mac Hardy and Beckman (1981) highlighted the extent of this disease in various agricultural settings, while Kraft and Pleger (2001) reported its widespread severity in pea-growing regions around the world. *Fusarium* head blight mainly causes plant wilting and can cause significant yield losses if not managed effectively.

I.1.2 *Fusarium oxysporum* f.sp. *pisi*

Fusarium oxysporum f. sp. *pisi* lives in the soil in the form of spores and mycelium. It infects roots through wounds or apexes, and then colonizes the plant's vascular system. Contamination and development of the disease are mainly observed when soil temperatures exceed 20°C.

Different races of *Fusarium oxysporum* f. sp. *pisi* are distinguished by their specific targets among pea varieties, their optimal aggressiveness at certain temperatures, as well as the types of symptoms they cause.(https://ephytia.inra.fr/fr/C/22555/Vigi-Semences-*Fusarium-oxysporum*-f-sp-*pisi*-Fusariose-vasculaire-du-pois)

I.1.3 Taxonomy

The taxonomy of *Fusarium oxysporum* f.sp. *Pisi* according to "The Fungal Kingdom" by Joseph Spatafora and his collaborators is as follows:

- Kingdom: Fungi (1FUNGK)
- Phylum: Ascomycota (1ASCOP)
- Subphylum: Pezizomycotina (1PEZIQ)
- Class: Sordariomycetes (1SORDC)
- Subclass: Hypocreomycetidae (1HYPRL)
- Order: Hypocreales (1HYPRO)
- Family: Nectriaceae (1NECTF)
- Genus: *Fusarium* (1FUSAG)
- Species: *Fusarium oxysporum* f. sp. *pisi* (FUSAPI)

It is important to quote the taxonomic classification in order to accurately identify and classify the fungi in the genus *Fusarium*, also specifying the special form (f. sp.) which indicates the particular adaptation of *Fusarium oxysporum* to infect peas (*Pisum sativum*).

I.1.4 Symptoms

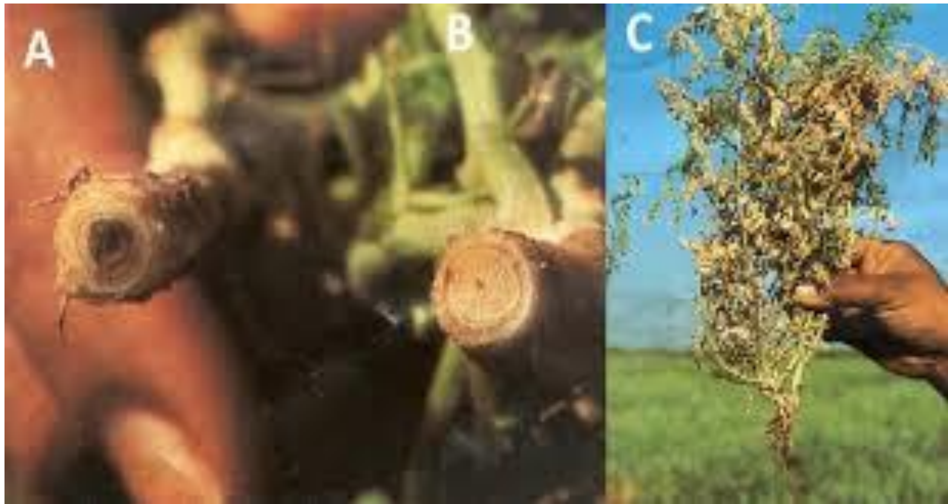
Symptoms of infection by *Fusarium oxysporum* f. sp. *pisi* include rapid stunting of pea plants, followed by wilting and eventual death. Old leaves turn yellow and wilt first, while the disease generally progresses from the base to the top of the plant. Newly infected shoots show a crook-shaped curvature. The wilting may be partial and temporary, with the stems regaining their turgidity after abundant irrigation, but it can also become permanent, leading to the death of the entire plant. A cross-section at the base of affected stems reveals browning of the vessels, eventually forming a completely brown ring. Small pinkish filamentous masses can be seen on dead tissue. This disease can cause significant losses, especially when soil temperatures are high (https://www.agrireseau.net/documents/Document_99534.pdf.

Not to be confused with symptoms of gummy canker (Didymella bryoniae), bacterial wilt (Erwinia tracheiphila) or water stress.

Figure 01:Pea wilt (*Fusarium oxysporum*) dying in pea plant cultivation

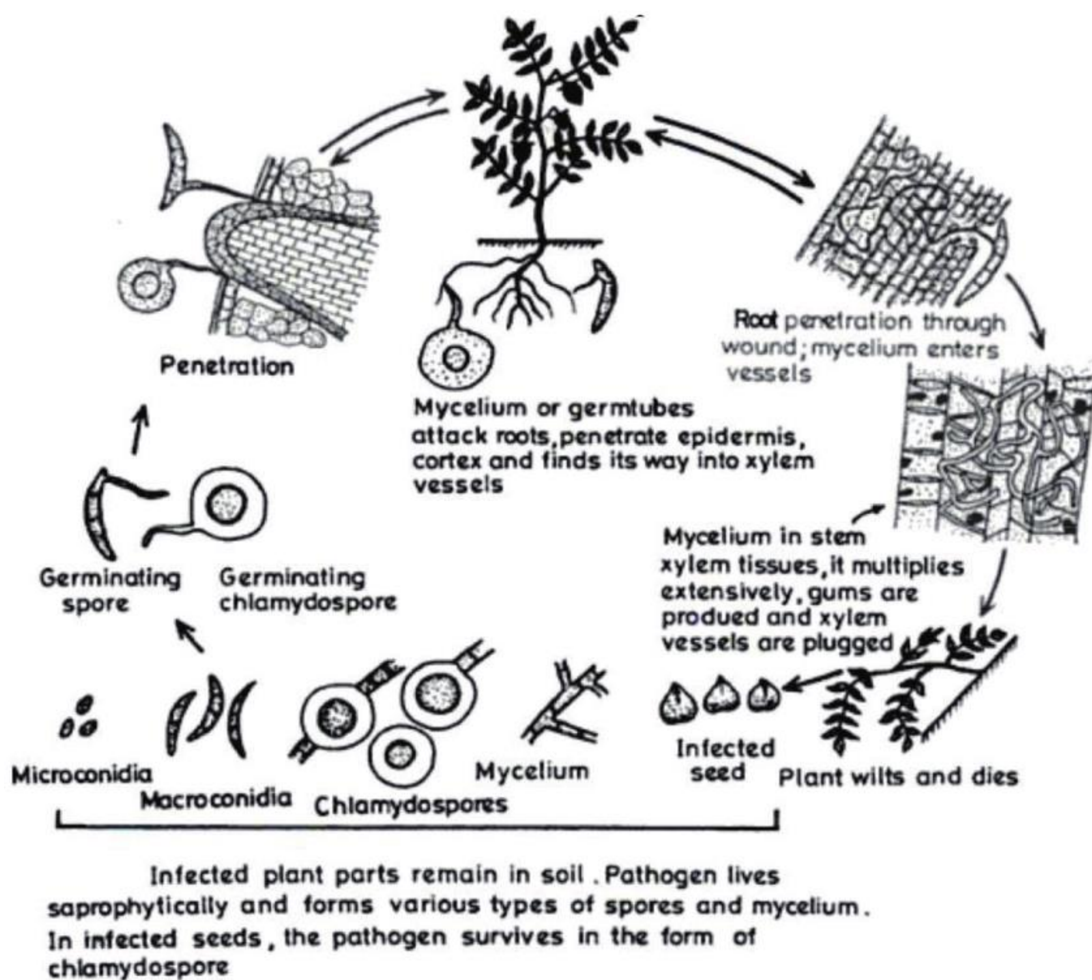
Figure 02 Vascular fusariosis (*Fusarium oxysporum f. sp. lycopersici* (FOL)) - Tomato

Figure 03 Root and *crown fusarium (Fusarium oxysporum f. sp. radicis-lycopersici* (FORL)) - Tomato



A: infected plant B: healthy plant C: on whole plant (Souad, 2016)

Figure 05: *Fusarium* head blight on chickpea (Cunninton *et al.,*2009)



Disease cycle of *F. oxysporum f. sp. ciceris* (Jalali and Chand, 1992)

I.1.5 Control methods

Controlling specific forms of *Fusarium oxysporum* remains particularly difficult as there are currently no chemical products that are effective in both prevention and treatment. As a result, crop protection relies mainly on cultivation methods and preventive chemical control.

For optimum effectiveness, an integrated approach is recommended, combining the optimization of cultivation practices with a reasoned strategy of chemical control and possibly biological control (Cyclamen - Plant diseases).

I.1.5.1 Cultural control

To prevent the introduction of *Fusarium* fungus into glasshouses, a number of preventive measures are essential. Firstly, it is essential to maintain rigorous hygiene in the greenhouse, and to control the pH of the soil so that it remains unfavorable to the development of the pathogen, ideally around 5.8. We recommend using well-aerated potting soils and avoiding black peat or substrates that are too compact. Using new substrates and planting equipment helps to reduce the risk of introducing contaminated spores. It is vital to prevent plants coming into direct contact with the soil outside or in the greenhouse, and to monitor crops regularly for early detection of contamination. At the end of each growing cycle, it is essential to thoroughly disinfect all equipment, including pots, shelves and irrigation systems, taking care to maintain sufficient distance between the pump suction pipe and the bottom of the recycled water tank to reduce the risk of spores being sucked in. In summer, it is advisable to keep greenhouse temperatures below 23°C to avoid conditions conducive to infection. An appropriate balance in nitrogen, potassium and calcium inputs is also crucial to minimize the risk of infection, as is adjusting watering patterns to prevent splashing of spores or contaminated particles that could lead to root contamination through leaching.

These combined practices provide an effective integrated approach to protecting crops against *Fusarium oxysporum*, in line with recommendations for integrated plant disease management. (Cyclamen - Plant diseases)

I.1.5.2 Chemical control

It is ineffective to treat plants already affected by *Fusarium* with curative treatments. Preventive methods using systemic products show limited results against this disease caused by *Fusarium* spp. However, options such as biofungicides and conventional fungicides are registered for the control of *Fusarium*. It is recommended that priority be given to the use of products that pose a low risk to human health and the environment, in order to effectively manage this disease while minimizing harmful impacts. (https://www.agrireseau.net/documents/Document_99534.pdf)

I.1.5.3 Biological control

For biological control of *Fusarium* head blight, here are some documented approaches

a. Utilization antagonistic micro-organisms: Studies have shown the effectiveness of Pseudomonas and Bacillus strains against *Fusarium oxysporum*. For example, research has shown that certain strains of Pseudomonas fluoresces can suppress the growth of *Fusarium oxysporum* through competition and the production of inhibitory metabolites (Haas and Défago, 2005).

b. Biofungicides based on micro-organisms: Commercial products containing fungi such as *Trichoderma* spp. are used for their ability to colonize the rhizosphere and inhibit *Fusarium oxysporum*. Studies have confirmed their effectiveness in suppressing soil-borne pathogens (Hermosa *et al.,* 2012).

c. inducing plant resistance: The induction of systemic resistance in plants by beneficial microorganisms has been studied. For example, certain biocontrol agents can activate plant defense mechanisms against fungal infections, thereby reducing the incidence of disease (Pieterse *et al.*, 2014).

d. promoting soil health: Promoting good soil health with beneficial microbial activity helps to reduce pathogen pressure. Practices such as compost application and rational fertilizer management are recommended to maintain a favorable microbiological balance (Bonanomi *et al.,* 2010).

e. Pratiques integrated cropping: Integrating organic approaches with other cropping practices such as crop rotation and the choice of resistant varieties is essential. Research shows that combining different strategies can maximize the effectiveness of fungal disease control (Weller *et al.,* 2002).

I.2. Rhizobacteria

Rhizobacteria, often referred to as rhizosphere bacteria, are a diverse group of microorganisms found in the zone surrounding plant roots. They play a crucial role in the beneficial interactions between plants and their underground environment. These bacteria can benefit plants in a number of ways, by promoting growth, improving nutrition and boosting disease resistance (Lugtenberg and Kamilova, 2009).

I.2.1 The role of rhizobacteria

The positive effects of rhizobacteria on plants include the biological fixation of atmospheric nitrogen, which improves the availability of essential nutrients for plant growth (Hartmann *et al.*, 2008). In addition, some rhizobacteria are able to solubilise nutrients such as phosphorus, making these elements more accessible to plants (Richardson and Simpson, 2011). These beneficial activities contribute to better plant nutrition and can reduce dependence on chemical fertilizers.

In addition, rhizobacteria produce plant growth compounds such as hormones and enzymes that promote root development and plant resistance to environmental stresses (Glick, 2012). These plant growth promotion mechanisms are crucial for optimizing agricultural yields while minimizing environmental impact.

I.2.2.1 Rhizobacteria as a means of control

Studies have also shown that rhizobacteria can act as biocontrol agents by suppressing plant pathogens through competition for nutrients and production of antimicrobial metabolites (Berendsen *et al.,* 2012). This ability to reduce disease pressure in the soil contributes to more sustainable agriculture by reducing the need for synthetic chemical agents.

Beneficial rhizobacteria, often referred to as PGPR (Plant Growth-Promoting Rhizobacteria), are distinguished by their beneficial properties for plants. Here are some common types of rhizobacteria and their main PGPR properties:

Rhizobacteria, or root-associated bacteria, are beneficial micro-organisms that live in the rhizosphere, the zone of the soil influenced by plant roots. They are recognized for their ability to promote plant growth and improve plant health in a number of ways. These beneficial interactions include the fixation of atmospheric nitrogen, the solubilisation of nutrients such as phosphorus, the production of plant growth substances, and the protection of plants against pathogens via the induction of systemic resistance (ISR) (Philippot *et al.,* 2013; Lugtenberg and Kamilova, 2009).

I.2.2.2 Nitrogen fixers

Nitrogen-fixing rhizobacteria, such as the genera Rhizobium and Bradyrhizobium, establish symbioses with leguminous plants to convert atmospheric nitrogen into ammonium, an essential nutrient for plant growth (Hartmann *et al.,* 2008). By solubilising inorganic phosphorus, bacteria such as *Bacillus* and *Pseudomonas* improve the availability of phosphorus to plants, thereby facilitating their nutrition (Richardson and Simpson, 2011).

I.2.2.3 Producers of plant growth substances

Rhizobacteria also produce plant hormones such as auxins and cytokinins, which stimulate root growth and plant development (Glick, 2012). In addition, studies have shown that certain rhizobacteria can induce disease resistance in plants by activating natural defense mechanisms, thereby reducing their susceptibility to fungal and bacterial infections (Berendsen *et al.,* 2012).

These complex interactions between rhizobacteria and plants are crucial for soil health and sustainable agricultural productivity, by reducing dependence on chemical fertilizers and minimizing the environmental impact of agricultural practices (Philippot *et al.,* 2013).

CHAPTER TWO

Materiel and methods

In this study, we are iterterested in the screning for new isoltates isolted for varieuse rhizosphere of spentaniouse and crop plants to be used in agreculter as biofertilsant. In the same time we have used same of the isolates wich was isolated from peas (*Pisum sativum* L.) and common bean (*Phaseolus vulgaris* L.) rhizosphere in Amar Telidj university laboratories – Laghouat.

II. 1 Biological Materials

II.1.1 Bacterial isolaes

In this stusy we have used 20 isoltes, 11 of them was isolated in 2023 from peas (*Pisum sativum* L.) and common bean (*Phaseolus vulgaris* L.) rhizosphere from Aflou, in Amar Telidj university laboratories (Laghouat). They were reacteveted in GN medium. The others were isolated this year.

II.1.2 Target fungus isolats

Fusarium oxysporum is a fungus commonly found in soil, typically functioning as a saprophyte. However, certain strains or specialized forms of this fungus, such as *Fusarium oxysporum* f.sp. *pisi*, exhibit pathogenic characteristics. This specific strain was utilized in screening assays to identify antagonist bacterial isolates.

II.1.3 Target bacterial reference strains

Three reference strains (*Bacillus cereus*, *Escherichia coli*, *Micrococcus luteus*) were provided to us for antibiotic testing.

Tabl 1 : Target bacterial reference strains

Gram	strains	Code
Gram +	*Bacillus cereus*	ATCC 25991
	Micrococus luteus	ATCC 9341
Gram -	*Escherichia coli*	ATCC 8739
	klebsiella pneumonia	

II. 2 Soil sampling

Soil samples were collected from the rhizosphere of four crop plants: *Allium cepa* (onion), *Vicia faba* (fava bean), *Medicago sativa* (alfalfa), *Brassica oleracea* L. (cauliflower), and a spontaneous plant, *Stipa tenacissima* (Halfa) colected repectvly from Kaf mokrane ,El-Assafia; and daya . After removing several centimetres of surface soil, including plant debris, samples weighing 500 g each were taken at a depth of 15 to 20 cm. Each sample was carefully labeled with the date, the name of the region and the respective plant species before being transported to the laboratory for further analysis.

II.3 Isolation of bacteria

a. Preparation of the stock solution

Isolation of. rhizospheric bacteria was performed by the suspension-dilution method described by (Vidhyasekaran *et al.,* 1997). From each soil sample of the five plants, 1 g was placed in a test tube containing 9 ml of sterile distal water and the tubes are vortex [the contents represent the stock solution].

After that, we have taken one (1) ml of the stock solution and put it in a tube containing 9ml of sterile distilled water making the concentration to 1/10., a series of dilution was carried out until the dilution of 10^{-3}

b. Preparation of Dilutions Plating Techniques

After vortexing the microbial mixture, 0.1 ml of each dilution was pipetted onto two types of culture media: Ashby and NBRIP . The inoculated samples were evenly spread on agar plates using a sterile Pasteur pipette for uniform distribution. Each dilution was replicated three times. Subsequently, the plates were incubated inverted at 28ºC untel the apparence of colinies on each plates.

After the incubation period, the plates are inspected for the presence of individual colonies exhibiting the desired characteristics. Following this, we begin the purification process by serially subculturing the isolates to obtain pure bacterial isolates, which are ready for further identification procedures.

II.4 Identification of bacterial Strains

For an identification of bacterial isolates are based on biochemical and physiological tests The identification of purified isolats follows a step by step approach, based on analysis and determination of various characters.

The identification of bacterial isolates involves assessing fluorescent pigment production along with conducting biochemical and physiological tests. The identification of purified isolates are done systematically by analyzing and determining different characteristics. we used methods based on morpho-physiological and phenotypic characteristics.

II.4.1 Gram Staining

Gram staining is a laboratory method used to categorize bacteria as either Gram-positive or Gram-negative. This procedure involves staining bacterial cells with crystal violet dye, followed by iodine treatment, decolonization using alcohol or acetone, and counterstaining with a red dye like safranin.

Gram-positive bacteria retain the purple color of the crystal violet stain due to their thick peptidoglycan layer in the cell wall, while Gram-negative bacteria lose the stain and appear pink or red after counterstaining. Gram staining serves the purpose of swiftly providing initial insights into bacterial cell wall structure, aiding in bacterial identification and influencing treatment strategies. It's an essential technique in microbiology, widely applied in clinical diagnostics and research

To prepare the fixed smear, we placed a drop of sterile distilled water on a microscope slide.microscope slide. Then, using a Pasteur pipette, we picked and spread a well-isolated colonyon the slide in a regular circular motion. Then we passedthe slide through the flame of a Banzen burner to heat-set the sample.For the first staining step, we applied a drop of gentian violet solution to the slide.gentian violet solution onto the slide to impregnate the bacterial cells, and left to act for one minute.minute. We then rinsed the slide briefly to remove excess dye, using waterwater over the preparation. Next, we applied a few drops of lugol solutionlugol solution and left to stand for 1 minute, then rinsed off with water. After gentle rinsing with an alcohol bleaching agent for 30 seconds (this step removes the dye from the bacteria), we rinsed the preparation.step removes the dye from Gram-negative bacteria, making them more permeable); we applied a few drops of fuchsin solution to the smear and left for one minute.Then rinsed with water.After drying in the air or with a gentle movement. at the end of the microscopic observation was carried out using the optical microscope at magnification 100. Purple coulure indicates Gram-positive but pink coulure indicates Gram-negative bacteria.

II.4.2 KOH Test

In our stady this test is a recommended test to confirm the previous test, the staining coloration, the principle of this test is to put a loupe of the bacteria with a drop of 3% KOH solution, the results are an immediate, after gentle agitation Gram-positive bacteria release lipids from their cell wall, forming a creamy or viscous emulsion. In contrast, Gram-negative bacteria do not release lipids, and the suspension remains watery without forming an emulsion.

II.4.3 Biochemical Tests

Biochemical tests are methods used to identify and characterize bacteria based on their chemical and metabolic reactions. In our approach, we've chosen several biochemical tests, which we'll be mentioned below.

II.4.3.1 Anaerobic growth

n the Hugh and Leifson medium, which includes 1% glucose, we observe bacterial metabolism under conditions where the environment is replenished. This setup naturally creates a gradient of oxygen concentration. Bacteria that use glucose produce acids. Acid production is visualized by the change in the bromothymol blue pH indicator in the medium. At the end of incubation, the color change of the medium observed to determine whether it is oxidizing or fermentative (Schaad *et al.,* 2001).

We have inoculated the bacteria by central pricking into two test tubes containing Hugh and Leifson medium. In one tube, we added approximately 1 cm of sterile vaseline oil, while leaving the other tube without oil. The tubes were then incubated at 28±2°C for 24 to 48 hours.

II.4.3.2 Mannitol Motility Test

Is designed to differentiate bacteria on the basis of their motility and ability to ferment mannitol, a positive reaction to fermentation shows a change of color to the media and causing turbidity away from the stab line concerning the motility results

Using a Pasteur pipette, we inocult the semi-solid culture medium about 2/3 of the way down the tube. After incubationat the appropriate temperature (30°C) for 24 to 48 hours; if the bacteria are mobile, we'll see diffusion of growth diffusion from the point of inoculation. If the bacteria are not mobilemobile, growth will remain limited to the inoculation site.

II.4.3.3 Simmons Citrate Test

This is a defined medium used to determine if an organism can use citrate as its sole carbon source. The alkaline pH turns the pH indicator (bromothymol blue)

In culture tubes containing simmons citrate medium, we used a sterile pasteur pipette to remove a small quantity of pure bacterial culture. and gently inoculated the surface of the citrate medium.citrate medium. After incubation at the appropriate temperature of 30°C for 24 to 48 hours, if the bacteria is able to use citrate as a carbon source, the culture medium will turn blue due to the increase in pH due to the conversion of citrate to bicarbonate. the result is positive , it is negative for citrate use , if the bacteria is not able to use citrate, the culture medium will remain green.

II.4.3.4 Cystine Test

Cystine is a sulfur-containing amino acid that can be detected and quantified using various test methods

We inoculated a few drops of bacterial suspension using a Micropipette. In culture tubes containing liquid medium. After incubation at the Appropriate temperature at 30°C for 24 to 48 hours, the observation of a yellow or brown coloration indicates a positive result for the presence of Cystine. The absence of color change Color change would indicate a negative result.

II.4.3.5 TSI triple sugar iron

In 1940, Sulkin, Willet and Hajna described a three-sugar ferrous sulfate medium for the identification of enteric bacilli. TSI agar is used for presumptive identification of enterobacteria based on fermentation of glucose, lactose and sucrose, and the production of gas and H_2S.

In culture tubes containing Triple Sugar Iron (TSI) agar culture medium using a sterile pasteur pipette, we picked up a small quantity of pure bacterial culture and gently inoculated the

surface of the medium (TSI). After incubation at the temperature of 30°C for 24 to 48 hours. The results are read by examining several characteristics:

- Red: indicates fermentation of sugars with acid production.
- All yellow tube: indicates fermentation of the three sugars (glucose, lactose, sucrose).
- Red at the top and yellow at the bottom: indicates glucose fermentation only.
- Cracks or detachment of the medium: indicate gas production.
- Yellow with medium cracking: indicates high gas production.

II.4.3.6. API 20 E gallery (bio Mérieux)

This API 20 E gallery contains 20 microcupules containing dehydrated substrates eight coventional test and 12 assimilation test.We have used it to be read it as ivdudividel biochimic test, We applied the API 20 E gallery to 20 bacterial isolates from our collection, following the protocol and instituions of bio Mérieux .

A bacterial suspension is used to rehydrate each and the strips are incubated. During incubation, the metabolism produces spontaneous color changes, revealed by the addition of reagents.

For example, when carbohydrates are fermented, the pH within the well decreases and that is indicated by a change in the color of the pH indicator.

a. Preparation of inoculums

On a test tube containing 5 ml of sterile physiological water, we picked young, well isolated young bacterial colonies on agar medium (GN), then carefully homogenized with a vortex to produce the bacterial suspension.

b. Gallery inoculation

First, sterile distilled water was poured into the cells to create a humid atmosphere. Next, we filled the micrtubes (not the cups) with the bacterial suspension, from NO3 tests to PNPG, For tests: ADH, GLU, URE, we created anaerobiosis by filling their cups with sterile kerosene oil. With the bacterial suspension we filled the micr-tubes and the cups of the GLU tests to PAC. At the end, we resealed the plates and incubated them at temperature 29 ± 2^0C for 24 h to 48 h.

These reactions are read by referring to the reading table (Appendix). After incubation, the NO3 and TRP tests should be developed just before reading the results by adding the corresponding reagents for each test. For the TRP test, a drop of JAMES reagent is added to the micr-tube, along with the two reagents NIT 1 +NIT 2 and/or Zn, and for the TDA test, the TDA reagent is added.

II.5 Enzymatic activity

We examined the enzyme activity of 20 isolates; phosphatase; amylase; lipase; protease, gelatinase, cellulase, and the esterase; The quantifiable alteration of the reaction or the reaction media, such as a change in color, the formation of gas, a modification in or precipitation of a substance, often indicates the presence of enzymatic activity. This alteration is frequently the outcome of the enzyme's response.

II.5.1 Phosphatases

Phosphatases catalyze the dephosphorylation process, which involves removing a phosphate group from a molecule. The ability of isolated bacteria to solubilize phosphates is tested using the method described by Nautiyal (1999) on NBRIP medium (Appendix 1) containing 0.5% tricalcium phosphate ($Ca_3(PO_4)_2$) as an insoluble phosphate source (Pikovskaya, 1948).

Bacterial isolates are inoculted by spot inoculation on the surface of solid medium. Solubilization capacity is assessed by observing the formation of a transparent halo around the colony. After 7 days of incubation, the diameter of the solubilization halo is determined by subtracting the colony diameter from the total diameter.

II.5.2 Cellulase test

This study aims to produce and assay cellulolytic enzyme activity Whether the bacteria produce the enzyme Bacterial isolates were inoculated onto the culture medium by the spot method. medium containing 1% Na-carboxymethylcellulose.

After incubating the plates at an optimal temperature for cellulose for 48 h and observing whether the material had the appearance of a halo around the bacterial spots after Congo Red, and the addition of Congo Red Congo and M NaCl.

II.5.3 Protease test

In order to preserve homeostasis, proteases break down misfolded and damaged proteins and cleave them, supplying vital acids for protein synthesis. Additionally, proteases function as agents and signaling molecules and have a wide range of uses in biotechnology.

The procedure was used to inoculate the bacterial isolates on the protease culture medium. We saw that the protein substance broke down into simpler components after 48 hours of incubation at the temperature. The halo-like appearance of the bacterial spots with the addition of copper

II.5.4 Gelatinase test

Certain bacteria secrete extracellular proteases called gelatinases, which hydrolyze or digest gelatin. There are two successive reactions that occur during this procedure. Inject bacteria into a gelatin meduim culture For 24 hours, place the agar in an incubator set to 30 °C.

II.5.5 Lipase test

The spot method was used to inoculate bacterial isolates onto the culture medium. The culture medium's composition (g l-1) is as follows: peptone 10.0, pH 7.4, NaCl 5.0, CaCl2 2H2O 0.1, agar 18.0, and after incubating the plates at a temperature ideal for lipase activity for 24 to 48 hours, supplemented with tween 801% (v/v) (Sierra , 1957). It was then evaluated whether the lipid was broken down to glycerol and fatty acids by looking for the formation of a halo around the bacterial spots.

II.5.6 Esterase test

The esterase test is a microbiology procedure used to identify esterase enzymes in bacterial cultures. For the esterase test, the medium we used for the lipase test remains the same, but Tweem 80 has been substituted for Tweem 20 (Sierra , 1957).

II.5.7 Amylase test

Complex carbohydrates such starch are hydrolyzed into simple sugars by amylases. Basic carbohydrates like glucose. Isolated bacteria have been planted on GN culture media containing GN medium with a 2% soluble starch content. The plates were flooded with Lugol after being incubated for 24 to 48 hours at a temperature that is ideal for amylases activity; the presence of the enzyme is shown by the formation of a clear zone surrounding the colony.

II.5.8 Catalase test

Catalase is an enzyme that destroys peroxides toxic to bacteria. It catalyzes the transformation of hydrogen peroxide into water with the release of O2. O2 we put a bit of the bacteria into a slide and we drope littel bit of hydrogen peroxid H_2O_2 . the results are a immediate reaction by releasing bubbles (2 H2O2 $\longrightarrow$ 2 H2O +).

II.6 PGPR activity

Rhizobacteria play an important role in maintaining soil balance. Among these bacteria are those which promote plant growth. These are known as plant growth-promoting rhizobacteria. Among the plant growth-promoting rhizobacteria (PGPR) activities, assessing their abilities to produce hydrogen cyanide (HCN), indole-3-acetic acid (IAA), ammonia (NH4+) production, nitrogenase activity

II.6. 1 Activity Indole acetic acid (IAA)

This test was performed on 20 strains, in enriched LB broth with 400 micrograms per milliliter of tryptophan inoculated with bacterial suspensions according to Khalid et al. (2004). After 96 hours of incubation at 28°C and stirring 150rpm/min. the medium was centrifuged for 20 minutes at 4500 rpm. Then 1ml of the supernatant was moved to a different tube, clean one using a micropipette. Next, add 2 ml of Salkowski reagent (1ml of $FeCl_3$ (0.5M) in 50 ml of perchloric acid (35 %). After about half an hour at room temperature in the dark. A pink to purple color indicates the presence of acid. Use a UV-Vis spectrophotometer with a wavelength of approximately 530 nm to measure absorbance. Use the extraction solvent as a reference.

II.6. 2 Production of Hydrocyanic Acid (HCN)

The capacity of the bacterial strains to produce cyanide is determined according to the method of Bakker and Schippers.Each strain is inoculated on GN medium using GN medium supplemented with glycine. A Filter paper disc saturated with alkaline picrate (0.5% picric acid and 2% sodium carbonate) is placed in the lids of the boxes, which are sealed with parafilm and inverted during incubation at 28°C ± 2 for 5 days (Ahmad et al., 2008). They are checked daily to identify HCN+ strains to make the color of the paper change from yellow to orange.

II.6. 3 Ammonia activity ($NH4+$)

We inoculated 100 µl of the bacterial suspension test into a test tube containing peptone medium. Containing peptone-water medium. This was incubated at 30°C for 48 hours. of 0.5 ml of Nessler reagent, which gives yellow to brown color, indicates the production of $NH3$.

II.6. 4 Fixation of azote

In this test, bacterial isolates were inoculated onto Winogradsky medium (Appendix1) and incubated at 28°C for 24 hours. After incubation, the presence of bacterial colonies was recorded as a positive result, while the absence of bacterial colonies was recorded as a negative result.

II.7. Screening assays to identify antagonist bacterial isolates

II.7.1 Anti-fungal activity by direct confrontation *in vitro*

We used a variety of culture media, such as KB, GN, MH, and PDA, to investigate the antifungal activity of the bacteria. The purified isolates were plated 2 cm away from pure cultures of Fusarium oxysporum, which were centrally plated in Petri dishes. After 48 hours of incubation at 28°C,.Likewise in control plates with just the disk of *Fusarium oxysporium*. We used the same protocol as in 1, due to the fact that the fungus and the isolates were applied on the same day. observations were made A caliper is used to assess the fungal growth following a 5 day incubation period. The percentage inhibition rate of mycelial growth was evaluated using the following formula (Hamouni *et al.,* 1996):

$$\mathbf{I(\%)=(1-Dt/Dpa)\times 100}$$

Where :

- Dt: radial growth of the control.
- Dpa: radial mycelial growth of the pathogen in the presence of bacteria.

II.7.2 Antibiotic production

II.7.2.1Antibiotic extraction

In this experiment, we inoculated each strain into 100 ml of liquid LB medium and allowed it to incubate for 5 days at 28°C with agitation at 180 rpm. After centrifuging the samples at 4500 rpm for 15 minutes, we separated the supernatant into flasks and added an equal volume of ethyl acetate. The resulting mixtures were transferred to ampoules, utilizing the organic phase, and the solvent was evaporated using a rotovapor at 40°C. Subsequently, we added 1 ml of methanol to recover the dry residue. These samples were stored at 4°C until conducting an antibiogram against 7 bacterial strains and *Fusarium oxysporum* f. sp. *pisi*.

II.7.2.2 Antimicrobien activity

Rhizobacteria play an important role in maintaining soil balance. Among these bacteria are those which promote plant growth. These are known as plant growth-promoting rhizobacteria. To assess antimicrobial activity using the disk diffusion protocol (Kirby-Bauer method), follow these detailed steps:

Starting with a bacterial suspension adjusted to the standard turbidity scale, which usually correlates with a specific optical density measured at 625 nm, indicating a bacterial concentration of 1-2 x 10^8 cell/mL, is the first step in evaluating antimicrobial activity using the Kirby-Bauer disk diffusion method. This suspension is evenly distributed onto Petri dishes that have been sanitized and filled with the proper solid culture medium, Mueller-Hinton agar. Next, be ready to test the antimicrobial agent by preparing sterile paper disks and impregnating them with standardized concentrations. As certain adequate contact by placing these disks uniformly spaced on the agar surface that has previously been injected with bacteria. For 18 to 24 hours, incubate the plates at the ideal temperature (usually 37°C) to promote bacterial growth and antibiotic diffusion from the disks. After incubation, measure the diameter of inhibition zones around each disk using calipers or a suitable measuring device.

II.8. Statistical analysis

The data were analyzed statistically for ANOVA.) was performed using the STATBOX VEGETAL essay version7.6. Differences between treatment mean values were determined following LSD test at 0.05 probability levels. Newman-Keuls test ($\alpha = 5\%$) grouped. The mean values were compared test at $p < 0.05$.

CHAPTER THREE
Results and discussion

II.1 Results

We isolated bacterial cultures from the rhizospheres of five plants: onion (*Allium cepa*), fava bean (*Vicia faba*), alfalfa (*Medicago sativa*), cauliflower (*Brassica oleracea* L.), and halfa (*Stipa tenacissima*). These plants were collected from different regions in Laghouat. We obtained hundreds of distinct isolates on various growth media (GN, KB, and Ashby).

Initially, we selected isolates based on their visible morphological characteristics. In addition, we included 11 isolates previously obtained from pea (*Pisum sativum* L.) and common bean (*Phaseolus vulgaris* L.) rhizospheres in 2023.

II.1.1 Identification of the bacterial isolates

After purifying the isolates, we retained only about 20 following isolates: B4; B5; B6; B8; B9; B10; B12; B15; B16; B18; F1; F2; B; C; H; F; O; E11; E10 and R. To identify them, we performed morphological features description a range of biochemical and physiological tests, including the Gram stain and the Hugh-Leifson test.

II.1.1.1 morphological features

The initial important characteristic for describing colonies is their general shape (Table 2). While many bacterial species form round colonies, others produce a range of shapes. We identified 10 strains with circular forms (B4, B6, B9, B11, B12, B15, B16, C, R, B5), 9 strains with punctiform shapes (B8, B10, B17, F1, F2, E11, E10, B, O), and one with an irregular shape (H).

The surface texture of a bacterial colony can vary between subcultures and is a crucial criterion linked to other characteristics, including pathogenicity. Colonies can be categorized as smooth (S) or rough (R). The isolates were classified as follows: smooth and shiny (B9, B10, B12, B15, B16, B18, F1, F2, B, C, O, R), mucoid or sticky (B4, B5, B6, B8, E11, E10), and rough (R).

The colony margin or edge can be classified into four distinct types. An entire margin is observed in isolates B4, B6, B9, B10, B11, B12, B15, B16, B, and H. isolates B5, B18, E11, and E10 exhibit an undulated margin, while B8, F1, and F2 have a lobate margin. The filamentous margin is characteristic of isolate H.

Regarding the elevation of colonies, which can be visualized like a cross-section, several types of relief are noted. Convex colonies are represented by isolates B4, B5, B6, B8, B15, and R. Flat colonies are observed in isolates B9, B10, B12, B18, F1, F2, B, C, O, and H. Finally, isolates B11, E11, and E10 show a raised colony elevation.

Colonies can be opaque, translucent, or transparent. We observed 7 opaque isolates (B4, B5, B6, B, R, E11, E10) and 13 transparent isolates (B8, B9, B10, B11, B12, B15, B16, B1, F1, F2, H, C).

Table 2: Morphological features

Isolates	shape	Texture	margin	Elevation	pigmentation	optical properties
B4	Circular	smooth and shiny	entire	convex	cream	opaque
B5	circular	smooth and shiny mucous	undulate	Convex	cream	opaque
B6	circular	smooth and shiny sticky	entire	convex	cream	opaque
B8	punctiforme	smooth and shiny	Lobate	Convex	white	transparent
B9	circular	smooth and shiny	entire	Flat	white	transparent
B10	puctiforme	smooth and shiny	entire	flat	white	transparent
B11	circular	smooth and shiny	entire	Raised	white	transparent
B12	circular	smooth and shiny	entire	flat	white	transparent
B15	circular	smooth and shiny	entire	convex	white	transparent
B16	circular	smooth and shiny	entire	Raised	white	transparent
B18	punctiforme	smooth and shiny	undulate	Flat	white	transparent
F1	punctiforme	smooth and shiny	Lobate	Flat	white	transparent
F2	Punctiforme	smooth and shiny	lobate	Flat	white	transparent
E11	punctiforme	smooth and shiny sticky	undulate	Raised	cream	opaque
E10	Punctiforme	smooth and shiny sticky	undulate	Raised	cream	opaque
B	Punctiforme	smooth and shiny sticky	entire	Flat	cream	opaque
C	circular	smooth and shiny	entire	Flat	white	transparent
O	Punctiforme	smooth and shiny	entire	Flat	white	transparent
H	irregular	Rough	filament ous	flat	cream	transparent
R	circular	smooth and shiny	entire	convex	cream	opaque

(Orginal,2024)

Figure 06. Morphological features of three bacterial isolates.

II.1.1.2 Biochemical tests Results

II.1.1.2.1 Gram coloration

The Gram stain, a fundamental microbiological staining technique, significantly aids in the identification and characterization of bacteria. After completing all the steps of the staining protocol, our results categorized the bacteria into two groups(table 3):

- Gram-negative bacteria: B4, B5, B6, B8, B9, B10, B11, B12, B15, E11, E10, B
- Gram-positive bacteria: B16, B18, F1, F2, H, C, O, R

The Gram staining method, developed in 1884 by Danish bacteriologist Hans Christian Gram, divides bacteria into two categories based on their staining properties. Gram-positive bacteria appear purple, while Gram-negative bacteria appear pink. This classification is a crucial systematic criterion for bacterial taxonomy. It provides insights into the bacteria's shape, size, and staining characteristics.

The difference in staining properties between Gram-positive and Gram-negative bacteria is due to the thickness of the peptidoglycan layer and the presence or absence of an outer lipid membrane. Gram-positive bacteria have a thick peptidoglycan layer and lack an external lipid membrane, making them monodermal. In contrast, Gram-negative bacteria have a thin peptidoglycan layer and an external lipid membrane, making them didermal. The structural differences influence the retention of crystal violet dye during the Gram staining procedure, which is observable under a light microscope.

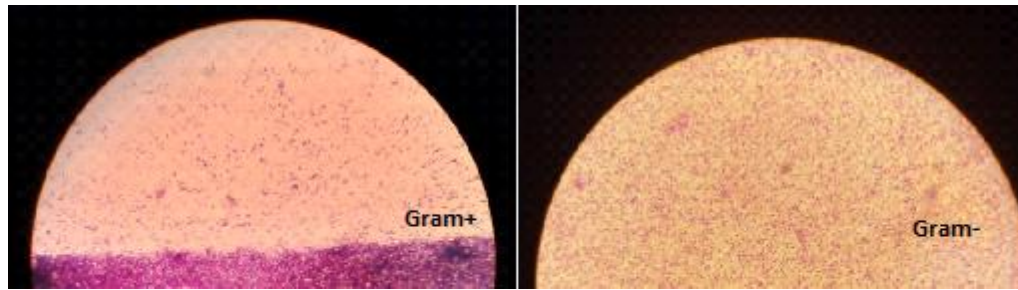


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Figure 07.: staining gram's results positive gram and negative gram bacteria

II.1.1.2.2 KOH Test

The KOH test (table3).is positive for all isolates except B16, B18, F1, F2, H, C, O, and R, which aligns with the Gram staining results. A positive KOH reaction is indicated by a viscous solution or the formation of a mucoid mass, suggesting that the bacteria are Gram-negative. This occurs due to their thinner peptidoglycan layer, which allows KOH to lyse the cells and release DNA, leading to increased viscosity. In contrast, a negative KOH reaction, where no significant viscosity change is observed, indicates Gram-positive bacteria. Their thicker peptidoglycan layer protects them from KOH's lytic action, preventing cell lysis and DNA release.

II.1.1.2.3 Growth in Anaerobic

Table 3 shows that strains R, H, F2, B15, B9, and F1 grow in aerobic conditions, whereas the other strains (B4, B5, B6, B8, B10, B11, B12, B16, B18, E11, E10, B, C, and O) thrive in anaerobic conditions.

The results indicate that when an organism is inoculated into Hugh and Leifson medium tubes containing a carbohydrate source, the medium in one tube is covered with melted Vaseline oil before incubation. This setup distinguishes between different metabolic patterns. Oxidative organisms will only produce an acidic reaction in the open tube, showing little or no growth and acid formation in the coated tube. In contrast, fermentative organisms will produce an acidic reaction in both types of tubes. Changes in the coated tube are attributed to true fermentation, while changes in the open tube result from oxidative metabolism of the carbohydrate. If the carbohydrates are not utilized by either method, no acid production occurs in either tube.

II.1.1.2.4 Mannitol motility test

This test provides two key results: bacterial motility and mannitol fermentation (table 3). For motility, Motile bacteria exhibit diffuse growth throughout the medium, while non-motile bacteria grow only along the inoculation line. All isolates tested show positive motility except F1, F2, B and R, suggesting that motility is a common trait among most isolates.

For mannitol fermentation, which produces acidity in the medium, phenol red is used as a pH indicator. The color change from red to yellow indicates acidity and positive reaction

Table 02 shows that isolates B8, B9, B10, B15, B16, H, C and O tested positive for mannitol fermentation, Conversely, for those isolates that showed no color change and remained red, it indicates that they did not ferment mannitol.

The Mannitol Motility Test Medium is designed to differentiate bacteria based on their ability to ferment mannitol as a carbon source. Fermentation of mannitol results in the production of acids, which lowers the pH of the medium and leads to a color change.

II.1.1.2.5 Simmons Citrate test

Table 3 indicates that isolates B5, B9, B16, and E10 tested positive for citrate utilization, as evidenced by a color change from green to blue in the medium indicating that these bacteria can use citrate as a sole carbon source. Conversely, isolates B4, B6, B8, B10, B11, B12, B15, B18, F1, F2, H, O, C, E11, R, and B did not show this color change, indicating a negative result.

II.1.1.2.6 Cystine test

Most isolates demonstrated a positive reaction in the Cystine test, as indicated in Table 2. The exceptions are isolates C, O, and B.

A positive result in the Cystine utilization test indicates that the bacteria possess specific enzymes capable of cleaving the disulfide bonds in cystine, allowing them to utilize cystine as a sulfur source for growth which is typical in some Enterobacteriaceae

II.1.1.2.7 Triple sugar iron TSI

The Triple Sugar Iron (TSI) test is used to assess the ability of certain intestinal bacteria to produce gas, reduce sulfur compounds, and ferment sugars such as glucose, lactose, and/or sucrose. The results of this test are summarized in Table 3.

A positive reaction was observed in the following strains: B4, B5, B6, B8, B9, B10, B12, B15, B18, F1, F2, E11, E10, B, and R. The negative reaction was observed in strains B11, B16, H, C, and O.

The interpretation of TSI test results is as follows:

- **Red Slant/Yellow Butt (K/A)**: Indicates fermentation of glucose only.
- **Yellow Slant/Yellow Butt (A/A)**: Indicates fermentation of glucose and lactose/sucrose.
- **No Color Change (K/K)**: Indicates no fermentation of sugars.
- **Gas Production**: Presence of gas bubbles in the medium.

Additional details include:

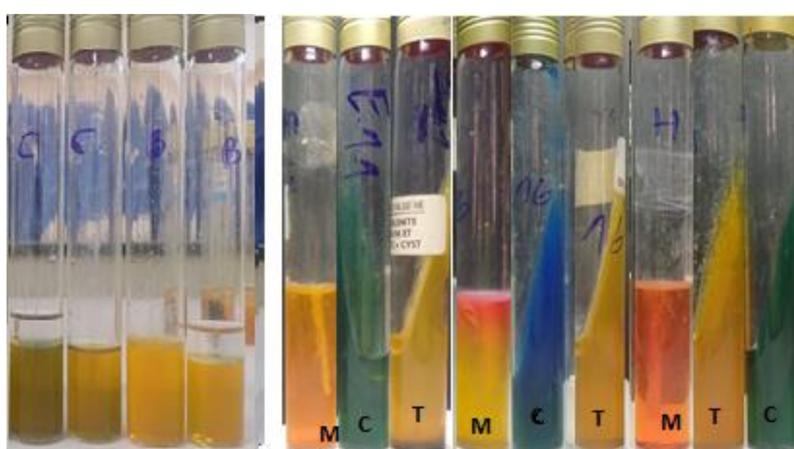
- **Alkaline/Acid (Red Slant/Yellow Butt; K/A)**: Only glucose (dextrose) is fermented.
- **Acid/Acid (Yellow Slant/Yellow Butt; A/A)**: Dextrose, lactose, and/or sucrose are fermented.
- **Alkaline/Alkaline (Red Slant/Red Butt; K/K)**: No carbohydrate fermentation.
- **Blackening of the Medium**: Indicates the production of hydrogen sulfide (H2S).
- **Bubbles or Cracks in the Agar**: Indicates gas production (G).

Note that large amounts of hydrogen sulfide may obscure acid production in the butt of the medium. Since hydrogen sulfide production requires an acidic environment, any butt portion completely covered by black precipitate should be considered acidic (A

Table 3: shows the results of the biochemical tests

isolates	Gram coloration	KOH test	motility test	Anaerobic Growth	Mannitol test	Simmons citrate	Cystine test	TSI triple sugar iron
B4	-	+	+	+	-	-	+	+
B5	-	+	+	+	-	+	+	+
B6	-	+	+	+	-	-	+	+
B8	-	+	+	+	+	-	+	+
B9	-	+	+	-	+	+	+	+
B10	-	+	+	+	+	-	+	+
B11	-	+	+	+	-	-	+	-
B12	-	+	+	+	-	-	+	+
B15	-	+	+	-	+	-	+	+
B16	+	-	+	+	+	+	+	-
B18	+	-	+	+	-	-	+	+
F1	+	-	-	-	-	-	+	+
F2	+	-	-	-	-	-	+	+
E11	-	+	+	+	-	-	+	+
E10	-	+	+	+	-	+	+	+
B	-	+	-	+	-	-	-	+
H	+	-	+	-	+	-	+	-
C	+	-	+	-	+	-	-	-
O	+	-	+	ND	+	-	-	-
R	+	-	-	-	-	-	+	+

(-): Negative response (+): Positive response



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Left/ Hugh and Leifson test

right /M: Mannitol motility test; C: Simmons Citrate test; T: Triple Sugar Iron (TSI) test

Figure 08. Effect of bacterial factor and medium factor interaction on *Fusarium oxysporum* f. sp. p*isi* inhibition rate performance in Medium GN

II.1.1.2.8 API 20 E gallery (bio Mérieux)

The API 20 E gallery was utilized for biochemical identification, incorporating 8 conventional tests and 12 assimilation tests. The results are detailed in Table 4. Each test is tailored to detect specific enzymatic activities or metabolic characteristics of the bacteria.

All isolates, exhibit positive results for key tests such as OPNG, ADH, LDC, and ODC, suggesting similar metabolic pathways, particularly for amino acid and carbohydrate utilization. They also demonstrate the ability to utilize citrate (CIT) and hydrolyze urea (URE), indicating versatility in their metabolic capabilities. In contrast, isolates B9, B10, B11, B12 and B15 show negative results for OPNG, which may imply distinct metabolic traits or adaptations.

The **GEL** test shows variability, with some isolates like B4 and B5 negative, suggesting they do not possess gelatinase, while others show positive results. All isolates, except B9 and B10, demonstrate positive fermentation of **GLU** (glucose), with a strong positive trend among many isolates, indicating their ability to ferment glucose for energy. The fermentation tests for other sugars like **MAN**, **INO**, and **SOR** also show variability. Notably, B9 and B10 lack fermentation ability for certain sugars, which could indicate their limitations in utilizing various carbon sources.

Variability in hydrogen sulfide (H2S) production and sugar fermentation further highlights the diversity among the isolates. Only isolates B6, B18, F1and B show positive results for (H2S) production While all can ferment glucose except B9 and B12's limited fermentation abilities may restrict their ecological roles. Isolates F1 and E11, with broader positive results across various tests, may possess enhanced adaptability. The presence of indole (IND) and Voges-Proskauer (VP) production in isolates such as B4, B5, B6, B15 and B16 suggests their capacity for tryptophan utilization and B8, B9, B10, B18, F2, E11, B, and H indicting an acetoin fermentation. All Isolates except B5, B9, B10, B12, B18, F1 and E10 show positive TDA results, indicating their ability to deaminate tryptophan.

Table 4: indicate the results of the gallery API 20 E

isolates	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
B4	+	+	+	+	+	-	+	+	+	-	-	+	+	+	-	+	+	+	+	+
B5	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+
B6	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
B8	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+	+	+	+	+	+
B9	-	+	+	+	+	-	+	-	-	+	+	-	+	-	+	-	+	-	+	-
B10	-	+	+	+	+	-	+	-	-	+	+	+	+	-	+	-	+	-	+	-
B11	-	+	+	+	+	-	+	+	-	-	+	+	+	-	-	-	+	-	-	-
B12	-	+	+	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
B15	-	+	+	+	+	-	+	+	+	-	+	+	+	-	-	+	+	-	+	-
B16	+	+	+	+	+	-	+	+	+	-	+	+	+	-	+	-	+	-	+	-
B18	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+
F1	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+
F2	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+	-	+	-	-	-
E11	+	+	+	+	+	-	+	+	-	+	+	+	+	-	-	-	+	+	+	+
E10	+	+	+	+	+	-	+	-	-	-	+	+	+	+	+	-	-	-	-	-
B	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-
O	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	-	-	-
H	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	-	+	-
R	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	-	-	+

(-) : Negative response (+): Positive response

(Orginal,2024)

Figure 09. Results of two API 20 E galleries.

III.1.2 Enzymatic Activity

We evaluated the enzymatic activity of 20 isolates, testing for phosphatase, amylase, lipase, protease, gelatinase, cellulase, and esterase. The presence of enzymatic activity is often indicated by observable changes in the reaction media, such as color shifts, gas formation, or precipitation of substances. These changes are typically the result of the enzyme's interaction with the substrates (table 5).

- **Catalase (Cat)**: All isolates except H exhibit catalase activity, indicating a majority of these isolates can break down hydrogen peroxide, a common oxidative stress marker.
- **Protease (Pro)**: Most isolates show protease activity (+), except forB6, E11, B, and H. This suggests that these isolates can degrade proteins, potentially aiding in nutrient acquisition.
- **Cellulase (Cel)**: Only a few isolates (B9, E10, E11, C,B and H) show cellulase activity, which indicates their potential to degrade cellulose. This may be relevant for isolates that play a role in breaking down plant materials.
- **Amylase (Amy)**: Several isolates (B6, B8, B9, B10, B11, B12, F1, F2, E11,E10, C, H, O and R) demonstrate amylase activity, suggesting their capability to hydrolyze starch into sugars.
- **Lipid Degradation (Lipa)**: Most isolates have lipase activity (+), indicating a capacity to break down fats, which can be crucial for energy sources.
- **Phosphatase (Phos)**: Nearly all isolates exhibit phosphatase activity, except F2 and R indicating their ability to hydrolyze phosphate esters, which is important for phosphorus acquisition.
- **Gelatinase (Gel)**: Gelatinase activity is present in many isolates, suggesting a common ability to degrade gelatin, a protein that can provide nutrients.
- **Esterase (Est)**: The presence of esterase activity in most isolates except B, C, H, and R) suggests a widespread ability to hydrolyze esters, which is relevant for various metabolic processes.

Table 5: shows the results of the enzymatic activity

Isolates	Cat	Pro	Cel	Amy	Lipa	Phos	Gel	Este
B4	+	+	-	-	+	+	+	+
B5	+	+	-	-	+	+	+	+
B6	+	-	-	+	+	+	+	+
B8	+	+	-	+	+	+	+	+
B9	+	+	+	+	+	+	+	+
B10	+	+	-	+	+	+	+	+
B11	+	+	-	+	+	+	+	+
B12	+	+	-	+	+	+	+	+
B15	+	+	-	-	+	+	+	+
B16	+	+	-	-	+	+	+	+
B18	+	+	-	-	+	+	+	+
F1	+	+	-	+	+	+	+	+
F2	+	+	-	+	+	-	+	+
E11	+	-	+	+	+	+	+	+
E10	+	+	+	+	+	+	+	+
B	+	-	+	-	+	+	-	-
C	+	+	+	+	+	+	+	-
H	-	+	+	+	+	+	+	-
O	+	+	-	+	+	+	+	+
R	+	+	-	+	+	-	+	-

Cat: Catalase; **Pro:** Protease **Amy**: Amylase; **Gel:** Gelatinase**; Est :** Esterase;
Phos: Phosphatase; **cel:** cellulase (-): Negative response (+): Positive response

(Orginal,2024)

Left/ cel: cellulase activity
right / Gel: Gelatinase activity

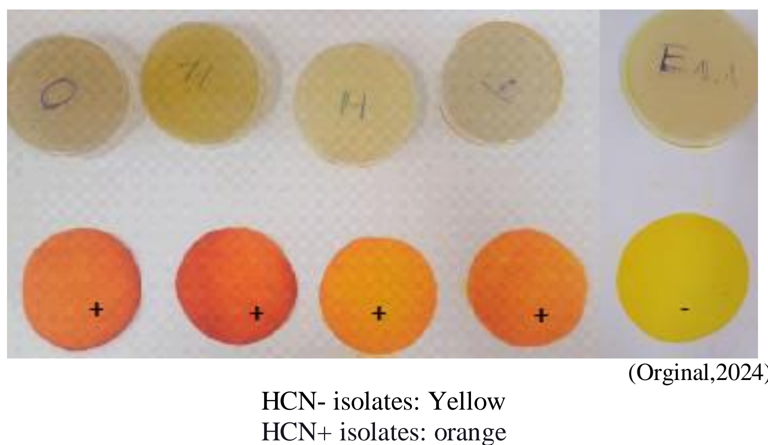
Figure 10. shows the results of some enzymatic activity

III.1.3 PGPR ACTIVITY

Rhizobacteria are crucial for maintaining soil balance, with certain types known as plant growth-promoting rhizobacteria (PGPR) that enhance plant growth. the table5 below summarizes the results of four tests performed on various isolates: hydrogen cyanide (HCN) production, indole acetic acid (IAA) production, ammonia (NH4+) production, and nitrogen fixation.

III.1.3.1 Hydrogen Cyanide (HCN) Activity

We tested all 20 isolates for their ability to produce hydrogen cyanide (HCN). There was significant variability in HCN production among the Isolates. HCN production is observed in some isolates (B6, B8, B10, B11, B12, E10- , B18, F1, F2, C, H, O, and R), as indicated by a color change in the filter paper from yellow to dark orange. In contrast, the other isolates did not produce HCN, as no color change was observed (table6, Figure 11).



(Orginal,2024)

HCN- isolates: Yellow
HCN+ isolates: orange

Figure 11. Hydrogen Cyanide (HCN) Activity of some bacteria

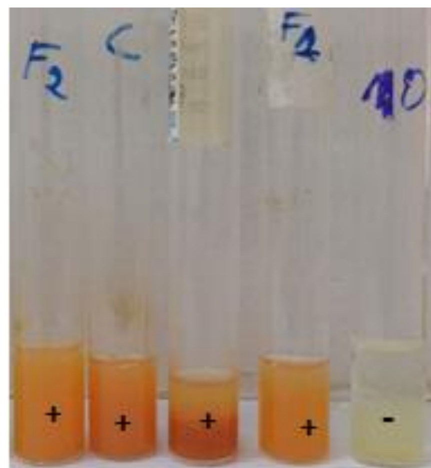
III.1.3.2 Indole Acetic Acid (IAA) Activity

We evaluated the ability of 20 isolates to produce indole-3-acetic acid (IAA) on LB medium supplemented with tryptophan, as summarized in Table 4. Most isolates demonstrated IAA production, which is essential for promoting plant growth by enhancing root development and overall plant health. Notably, isolates B4, B5, B6, and B8 exhibited high levels of IAA

activity (++). In contrast, isolates F2, B, C, H, O, and R showed no IAA production. Additionally, isolates B9, B11, B12, B15, B16, B18, F1, and E11 displayed lower levels of IAA activity (table6).

III.1.3.3 Ammonia (NH4+) production

Ammonia production is limited among the isolates, with only ten isolates (B9, B11, B12, B15, B18, F1, F2, C, H, and R) showing positive activity. which indicates their capability to convert nitrogen into ammonia (table6, Figure 12).



(Orginal,2024)

(-) : Negative response
(+) : Positive response

Figure 12. Ammonia production

III.1.3.4 Nitrogen fixation

The *in vitro* nitrogen fixation performance tested on Winogradsky medium, which is nitrogen-free Every isolate tested demonstrates some level of nitrogen fixation (indicated by +). This means that all isolates have the capability to convert atmospheric nitrogen (N_2) into ammonia (NH_3) or related compounds (table6).

Table 6: PGPR activity

isolates	HCN	IAA	Ammonia (NH4+) production	Nitrogen fixation
B4	-	++	-	+
B5	-	++	-	+
B6	+	++	-	+
B8	+	++	-	+
B9	-	+	+	+
B10	+	+	-	+
B11	+	+	+	+
B12	-	+	+	+
B15	-	+	+	+
B16	-	+	-	+
B18	+	+	+	+
F1	+	+	+	+
F2	+	-	+	+
E11	-	+	+	+
E10	+	+	-	+
B	-	-	-	+
C	+	-	+	+
H	+	-	+	+
O	+	-	-	+
R	+	-	+	+

HCN: hydrogen cyanide; IAA: indole-3-acetic acid, (-): Negative response, (+): Positive response

III.1.4. Screening assays to identify antagonist bacterial isolates

III.1.4.1 Anti-fungal activity by direct confrontation *in vitro*

The results of the *in vitro* antagonism test for the 20 bacterial isolates (B4, B5, B6, B8, B9, B10, B12, B15, B16, B18, F1, F2, B, C, H, F, O, E11, E10, and R). against *Fusarium oxysporum* f. sp. *Pisi* (FOP), was carried out on five different culture media MALT (Figure 13, Figure 14), PDA (Figure 15), GN (Figure 16), King B (Figure 17), and MH (Figure 18).

Across the five mediums (KB, PDA, GN, MH, and MALT), several modalities consistently demonstrate top performance. The (B 17 48 h) modality stands out as the most consistently high performer, achieving top rankings in all five mediums. It holds the highest average scores in Medium PDA (94.64 %, Group A) and Medium MH (95.00 %, Group A), and performs well in Medium GN (91.86%, Group E) and Medium KB (91.43%, Group B).

Another notable performer is (B12 48 h), which shows strong results in Medium GN (94.19 %, Group D) and Medium MH (92.50 %, Group D), and maintains high scores in

Medium KB (92.14 %, Group B) and Medium PDA (92.86 %, Group C). Despite its lower performance in Medium MALT (87.50 %, Group B), it consistently ranks among the top in other mediums. (F1 48 h) is also a significant performer, achieving high scores in Medium KB (99.29%, Group A) and Medium MH (96.67%, Group C). However, it experiences a drop in performance in Medium MALT (57.50%, Group H), showing some variability. Lastly, (E11 48 h) shows strong results in Medium MH (95 %, Group A) but lower scores in other mediums like Medium MALT (42.50 %, Group I).

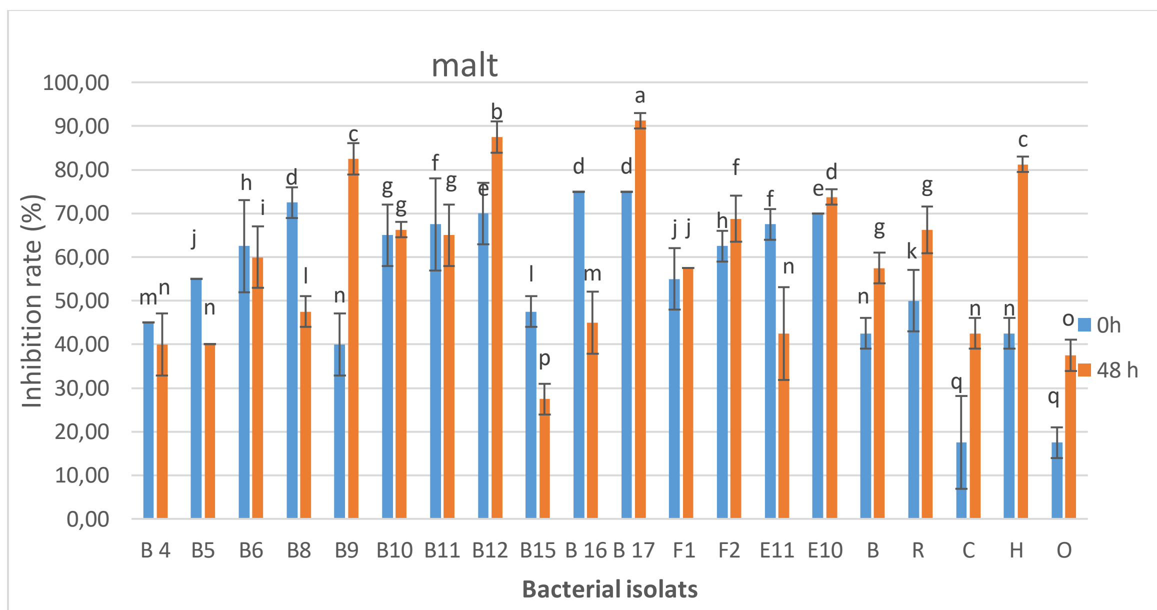
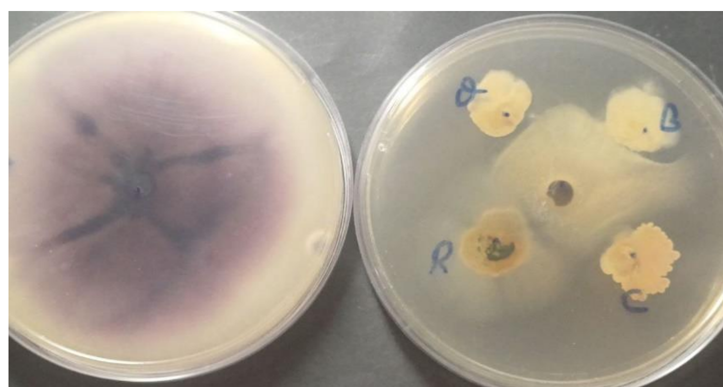


Figure 13. Effect of bacterial factor and medium factor interaction on *Fusarium oxysporum* f. sp. p*isi* inhibition rate performance on Medium MALT



(Orginal,2024)

Left/ **FOP:** *Fusarium oxysporum* f.sp. *pisi*

right / **FOP:** *Fusarium oxysporum* f.sp. *pisi* in middle surrounded by bacterial isolates B, C, R, O.

Figure 14. Anti-fungal activity of bacterial isolates on *Fusarium oxysporum* f. sp. p*isi* by direct confrontation performance.

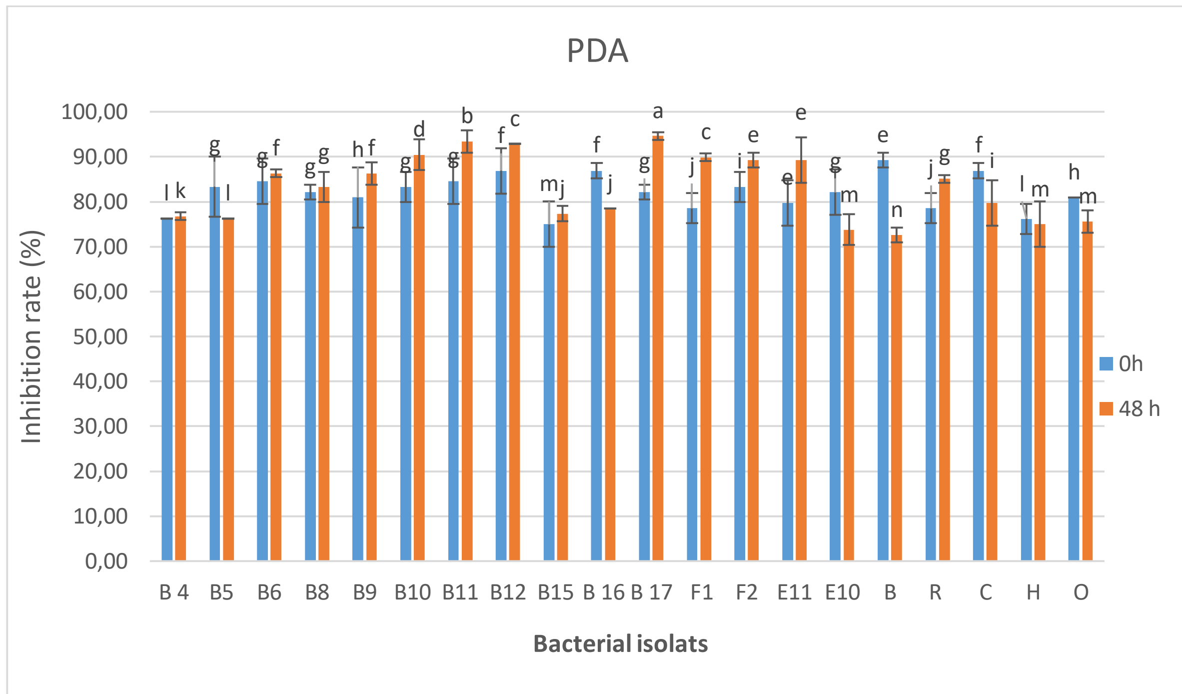


Figure 15. Effect of bacterial factor and medium factor interaction on *Fusarium oxysporum* f. sp. p*isi* inhibition rate performance on Medium PDA

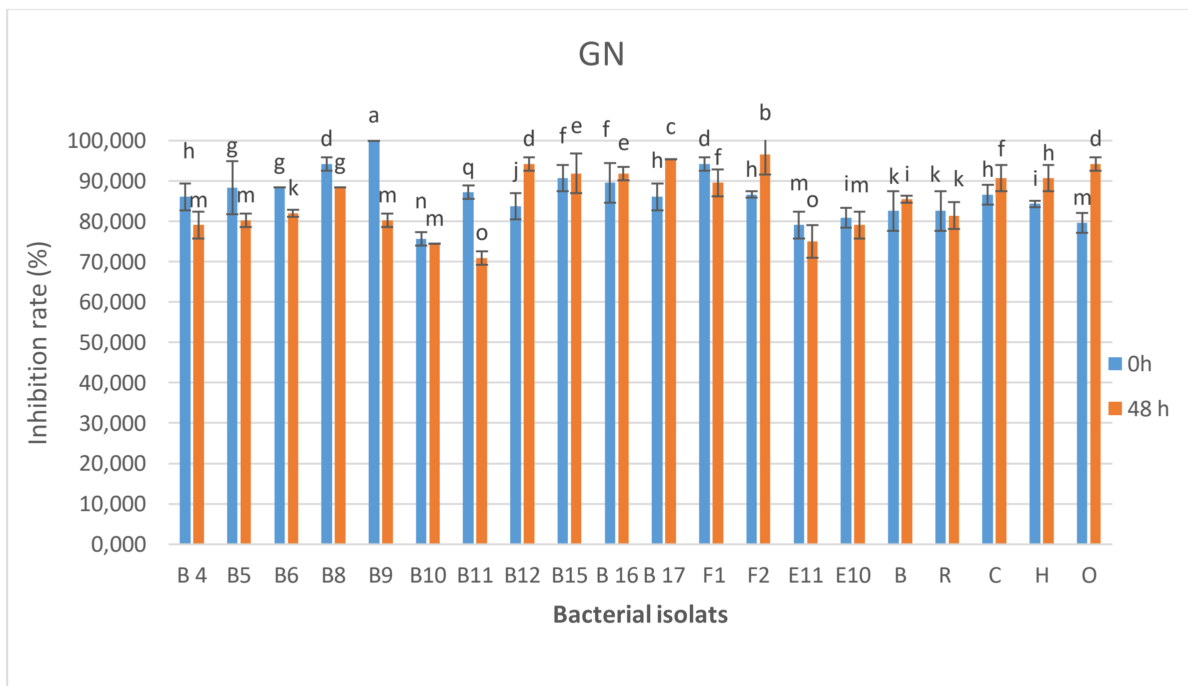


Figure 16. Effect of bacterial factor and medium factor interaction on *Fusarium oxysporum* f. sp. p*isi* inhibition rate performance on Medium GN

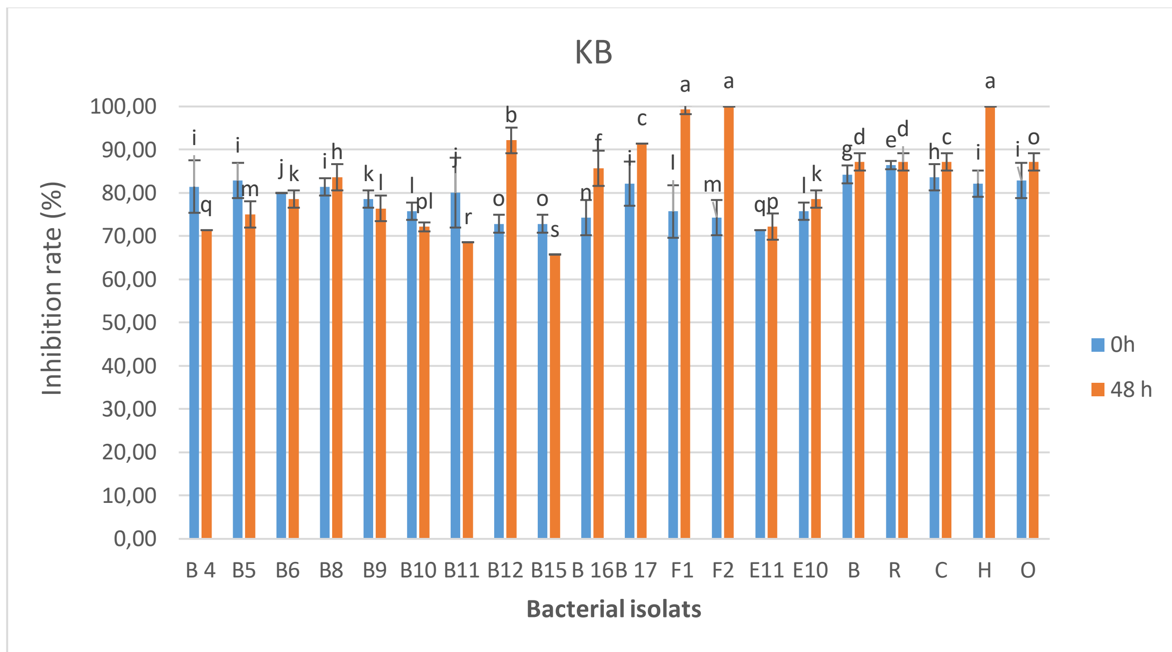


Figure 17. Effect of bacterial factor and medium factor interaction on *Fusarium oxysporum* f. sp. p*isi* inhibition rate performance on Medium KB

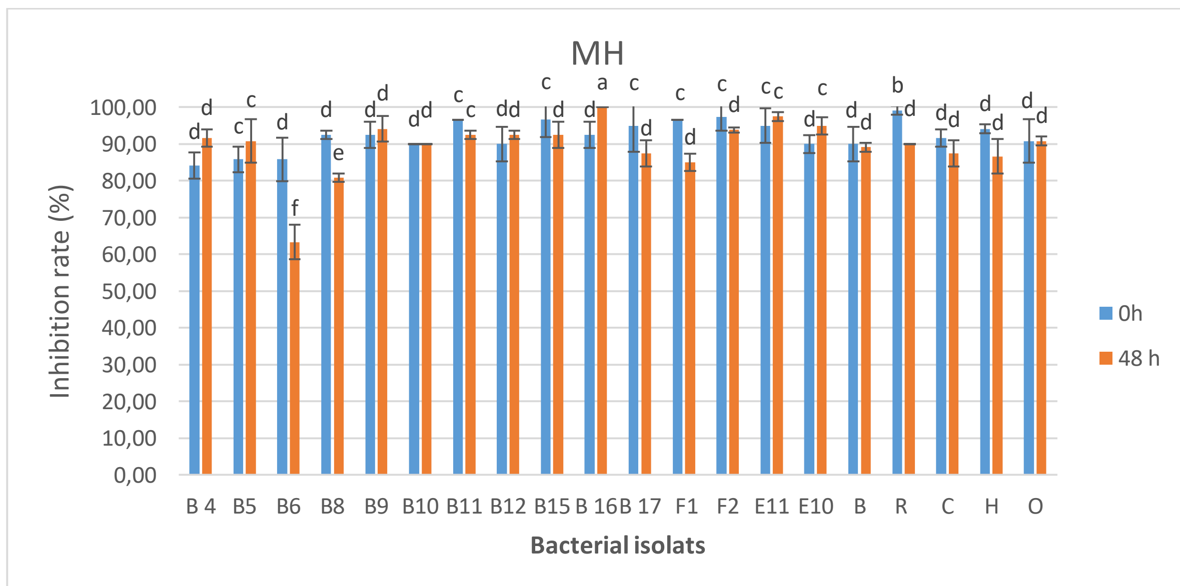


Figure 18. Effect of bacterial factor and medium factor interaction on *Fusarium oxysporum* f. sp. p*isi* inhibition rate performance on Medium MH

III.1.4.2 Antibiotic production

The results of antibiotic extraction and antimicrobial activity testing against specified bacterial and fungal strains, each isolate (B4, E1, and B6) demonstrates varying levels of effectiveness (table7, Figure 19).

Isolate B4 shows strong antimicrobial activity, particularly against *Escherichia coli* (15.00 mm) and *Bacillus cereus* (14.50 mm), suggesting it has broad-spectrum potential against both Gram-positive and Gram-negative bacteria. However, its moderate effectiveness against *Micrococcus luteus* (10.50 mm) and *Fusarium oxysporum* f.sp. *pisi* (12.50 mm). In contrast, isolate E1 stands out with the highest overall activity, notably against *Bacillus cereus* (15.50 mm) and maintaining good performance across all tested strains, including a strong response to *Fusarium oxysporum* f.sp. *pisi* (14.00 mm). On the other hand, isolate B6 exhibits the weakest performance, especially against *Escherichia coli* (10.50 mm).

Table 7: The results of antimicrobial activity

ISOLATS	BC*	EC*	ML*	KP*	FOP**
B4	14,50	15,00	10,50	14,00	12,50
E1	15,50	14,50	12,50	14,50	14,00
B6	12,00	10,50	11,00	12,50	12,00

***Bacterial strains**/ BC : *Bacillus cereus* **EC** : *Escherichia coli* **ML** : *Micrococcus luteu*
KP : *klebsiella pneumonia*
****Fungal strain**/ **FOP:** *Fusarium oxysporum* f.sp. *pisi*

(Orginal,2024)

Left/ **EC** : *Escherichia coli*
right / BC : *Bacillus cereus*

Figure 19. Effect of antimicrobial activity on MH medium

III.2. Discussion

For our study, we selected twenty isolates based on their morphological characteristics. These isolates underwent initial biochemical and physiological characterization. Subsequently, we assessed their ability to utilize various sugars and acids, as well as their enzymatic activity and plant growth-promoting rhizobacteria (PGPR) activity.

Isolates demonstrating promising results were then tested for their antifungal properties against the plant pathogen *Fusarium oxysporum* f. sp. *pisi*. Additionally, these isolates were screened for antibiotic production.

The isolates exhibit a diverse range of morphological characteristics that provide insight into their classification. Most of them feature a smooth and shiny texture, with either circular or punctiform shapes. Margins vary from entire to lobate and undulate, while elevation is categorized as either flat or convex, with a few raised forms. Pigmentation primarily ranges from cream to white, with certain isolates displaying opaque qualities, whereas others are transparent. Notably, sticky and mucous textures are present in some isolates, adding to the complexity of their physical attributes. This variety in morphology suggests a rich ecological diversity, warranting further investigation to understand the potential biological implications of these traits.

After staining, Gram+ bacteria turn purple while Gram- bacteria appear pink. The division of bacteria into Gram+ and Gram- is an important systematic criterion for the classification of bacteria (Kwon and Kim, 2016).

The isolates exhibit a diverse array of biochemical and physiological characteristics. Most are Gram-negative and show positive KOH test results, suggesting distinct cellular properties. Twelve (12) of the isolates are classified as Gram-negative, confirmed by both Gram staining and positive KOH test results, which indicate the presence of a thinner peptidoglycan layer and an outer membrane. This structural feature typically makes Gram-negative bacteria more resistant to certain antibiotics and highlights their ecological significance, particularly in the rhizosphere. In contrast, eight (8) isolates are Gram-positive exhibit a negative KOH test, suggesting a thicker peptidoglycan layer. These bacteria may have different metabolic capabilities and interactions with plants and soil. According to Ghafir (2007), the cell walls of Gram-negative bacteria have a thinner layer of peptidoglycan with a thin and elastic wall. This

characteristic of the wall allows the penetration of alcohol, which will decolorize the cell and subsequently be recolored by fuchsine.

The 3% KOH solution has a destructive effect on the cell walls of Gram-negative bacteria, while it has no effect on the cell walls of Gram-positive bacteria. When a bacterial sample is mixed with a small volume of 3% KOH solution for no more than 60 seconds, it causes damage to the cell wall, followed by the release of DNA, resulting in a viscous appearance of the mixture. This reaction is a positive result, indicating that the bacteria are Gram-negative (Bejot, 2015).

Most isolates being motile indicates a potential advantage in the rhizosphere, allowing them to move toward plant roots and utilize exudates.

Results shows that when an organism is inoculated into Hugh and Leifson medium tubes containing a carbohydrate source that the medium in one of the tubes is coated with melted Vaseline oil before incubation, Metabolism patterns have different meaning. Oxidizing organisms will only produce an acidic reaction in an open tube with little or no growth and acid formation in the coated tube. Fermentative organisms will produce an acidic reaction in the tube types. Changes in coated agar are considered to be due to true fermentation, while changes in open tubes are due to oxidative use of the present. If the carbohydrates are not used by either method, there no acid production in either tube (Lee et Yoon, 2013).

The ability to grow anaerobically in several isolates may suggest adaptability to varying oxygen levels in the soil environment, beneficial for surviving in the rhizosphere. The variability in mannitol fermentation suggests some isolates may contribute to nutrient cycling or possess specific metabolic capabilities that could benefit plant growth. The ability to utilize citrate in some isolates indicates potential for nutrient utilization in the rhizosphere, where organic acids may be present. This trait is often seen in beneficial bacteria that promote plant health. Positive cystine utilization can be indicative of the ability to contribute to nitrogen cycling, which is advantageous in plant-soil interactions

A positive result in the citrate utilization test suggests that the bacteria possess citrate-permease enzymes, enabling them to transport and utilize citrate (Forbes *et al.,* 2007).

The Simmons citrate test is a biochemical assay used to assess whether bacteria can use citrate as their sole carbon source under specific conditions, aiding in bacterial differentiation based on metabolic capabilities (Murray *et al.,* 2015).

Among the microbial groups in the soil that influence plant growth, we can mention *Rhizobium*, *Pseudomonas*, *Alcaligenes*, *Azospirillum*, *Klebsiella*, *Burkholderia*, *Enterobacter*, *Azotobacter*, *Serratia* (Kumar et al., 2012; Osman et al., 2017). *Arthrobacter*, *Bacillus*, *Acinetobacter*, *Flavobacterium*, *Micrococcus*, *Agrobacterium*, *Clostridium*, and *Xanthomonas* (Mitter et al., 2002; Tsakelova et al., 2006; Joo et al., 2009). Plant growth-promoting rhizobacteria (PGPR) are soil bacteria that colonize the roots of plants, enhancing their growth either directly or indirectly. In response, plant roots secrete metabolites that serve as nutrients (Bhattacharyya and Jha, 2012; Arora, 2015).

The effects of rhizobacteria can be categorized into direct and indirect mechanisms. While the distinction between these effects may not always be clear, direct mechanisms influence the plants' internal processes and metabolism, whereas indirect mechanisms typically occur externally to the plants (Gopalakrishnan et al., 2012; Huang et al., 2014; Osman et al., 2017).

Among the plant growth-promoting rhizobacteria (PGPR) activities of various isolates, assessing their abilities to produce hydrogen cyanide (HCN), indole-3-acetic acid (IAA), ammonia ($NH4+$) production, nitrogenase activity, enzymatic activity and antibiotic production.

The hydrolytic enzymes accumulate at the site of penetration of *Fusarium oxysporum* f.sp. *pisi* and, consequently, may be directly involved in the degradation of fungal cell walls (Benhamou et al., 1996). Proteases are extracellular enzymes secreted by bacteria (Rajmohan et al., 2002). They play a role in the degradation of fungal cell walls (Stanier et al., 1966). Chitinases are important hydrolytic enzymes, as chitin is a major component of the cell walls of most phytopathogenic fungi (Kishore et al., 2005).

Several bacterial strains such as *Pseudomonas*, *Bacillus*, *Burkholderia*, Flavobacterium, *Azotobacter chroococcum*, *Pseudomonas putida*, and *Rhizobium* spp. as well as *Bradyrhizobium* spp. have the ability to solubilize inorganic phosphate. The use of these bacteria as bioinoculants increases phosphate availability (Idris et al., 2016; Sáenz-Mata et al.,

2016). These microorganisms produce organic acids and release protons, which, through their carboxylic groups, chelate cations bound to insoluble phosphates, converting them into soluble forms (Taktek, 2015).

The isolates exhibit a range of activities related to plant growth promotion and biocontrol. HCN production is observed in many isolates, which may enhance their biocontrol potential. IAA production, essential for plant growth, is common among the isolates. Ammonia production is less widespread but still present in several isolates, indicating their ability to contribute to nitrogen nutrition. Nitrogenase activity is prevalent across most isolates, highlighting their potential role in nitrogen fixation. The variability among isolates suggests differing capabilities in promoting plant growth and biocontrol.

Nitrogen (N) is the most vital nutrient for plant growth and productivity. Although approximately 78% of the atmosphere is composed of N_2, it is not available for growing plants (Muness, 2013). There are several types of nitrogen-fixing bacteria (associative, symbiotic, and free-living bacteria found in the rhizosphere); however, all nitrogen-fixing bacteria such as Rhizobium spp., Azospirillum, Burkholderia, Gluconacetobacter, and Pseudomonas form an enzymatic complex called nitrogenase (Pereg and McMillan, 2015). Thus, nitrogen-fixing rhizobacteria are important for proper soil fertilization and sustainable agricultural systems (Wdowiak-Wróbel et al., 2017). Nitrogen-fixing PGPR can enhance plant growth and maintain high levels of nitrogen in agricultural soils (Damam et al., 2016).

Phytohormones are among the most important growth regulators; they are known to have a significant impact on plant metabolism and growth, and additionally, they play a crucial role in stimulating plant defense response mechanisms against stress (Egamberdieva et al., 2017). Plant growth regulators are also referred to as exogenous plant hormones because they can be applied externally by certain PGPR bacteria. The group of phytohormones includes gibberellins, cytokinins, abscisic acid, ethylene, steroids, and auxins (Damam et al., 2016).

We assessed the ability of 20 isolates to produce indole acetic acid (IAA). All isolates, except for B and F2, produced IAA in LB medium supplemented with tryptophan, IAA, the most widespread natural auxin, is a hormone produced by plants, fungi, and bacteria (Li et al., 2018). Bacterial IAA can loosen the cell walls of plant tissues and, consequently, promote increased root exudation, providing additional nutrients to support the growth of rhizosphere bacteria. It can attract more bacteria to the rhizosphere by increasing the amount of root

exudation. Since bacterial IAA stimulates the development of the host plant's root system, IAA-producing isolates can enhance the suitability of plant-microbe interactions (Hassan et al., 2015).

Hydrogen cyanide (HCN) can be a toxic compound that some PGPR use as a defense mechanism against pathogens, but excessive production may negatively impact plant health. Isolates B4, B5, B9, B12, B15, B16, and B are negative for HCN production, suggesting they may be safer options for promoting plant growth without the risk of cyanide toxicity.

According to Blumer et al. (2000) Volatile compounds such as ammonia and hydrogen cyanide (HCN) are produced by a large number of rhizobacteria and play a significant role in biocontrol *in vitro*. The production of HCN can inhibit the growth of several phytopathogenic agents. HCN inhibits the cytochrome oxidase of many organisms. The producing strains possess an alternative cytochrome oxidase that is resistant to HCN, making them relatively insensitive to its effects (Voisard et al., 1989; Blumer and Haas, 2000; Ahmad et al., 2008)).

According to several authors, antagonistic activities can be attributed to the synthesis of antibiotics and other types of secondary metabolites with antibiotic effects (Lemanceau et al., 1988; Défago, 1993; Digat, 1992; Keel et al., 1992; Whipps and Lymsen, 2001), as well as to competition for nutrients and space (Bloemerg and Lugtenberg, 2001; Jetyanon and Kloepper, 2002; Persello-Cartieaux et al., 2003).

In assessing bacterial performance across various media and incubation times, it is evident that a 48-hour incubation period generally yields better results compared to a 0-hour period, indicating improved bacterial growth and interaction over time. Isolates such as B9, B 17, F2, and F1 consistently perform well, especially at 48 hours, across multiple media, suggesting they are robust and interact positively with the media or fungi. In contrast, isolates like B15, B4, and C often show lower performance, particularly at 0 hours, indicating less favorable interactions or conditions. Among the different media, KB and MH are generally effective, supporting high bacterial performance, while PDA and MALT show greater variability and may be less consistent.

Isolate E1 antibiotic stands out with the highest overall antimicrobial activity, notably against *Bacillus cereus* with 15.50 mm and maintaining good performance across all tested strains, including a strong response to *Fusarium oxysporum* f.sp. *pisi* with 14.00 mm.

Rhizobacteria can inhibit *Fusarium oxysporum* by utilizing the same nutrients or by altering the nutritional environment. Some metabolites produced by rhizobacteria may induce physiological changes in *Fusarium oxysporum*, affecting its growth and virulence *(*García et al. 2014). also Rhizobacteria can produce antibiotics, enzymes, or other metabolites that directly inhibit the growth of *Fusarium oxysporum (*Baker and Cook ,1983).

The antibacterial activity of rhizobacteria refers to their ability to produce substances or carry out processes that inhibit the growth or kill other bacteria present in their environment, particularly in the rhizosphere, the zone around plant roots where these micro-organisms actively interact (Maldonado-González *et al.,* 2015).

CONCLUSION

Conclusion

Our study focuses on the potential of isolated rhizobacterial strains to promote plant growth while providing biocontrol against fungal pathogens. The findings suggest that these strains can be used simultaneously as biofertilizers and biocontrol agents.

The isolates exhibit a variety of physical traits, reflecting their ecological roles and adaptations to environmental pressures. Most isolates are circular and punctiform, with smooth and shiny textures that indicate typical growth patterns on solid media. Notably, isolates B4 and B5 show cream pigmentation and opaque properties, suggesting possible classification under specific bacterial genera. In contrast, isolates B8 and B9 are white and transparent, indicating different physiological characteristics. This morphological analysis is useful for understanding ecological dynamics within the sampled environment.

The biochemical analysis revealed that most isolates utilize mannitol as a carbon and energy source, with others also capable of metabolizing glucose, lactose, and sucrose. Gram staining results indicated that most isolates (B4 to B12, B15) are Gram-negative, while B16, B18, H, R, and C are Gram-positive, showcasing diversity in cell wall structure. All isolates were motile, enhancing their ecological adaptability for effective rhizosphere colonization. Positive anaerobic growth results further confirm their ability to thrive in low-oxygen environments.

The enzymatic activity tests highlighted the biochemical diversity among the isolates. All tested positive for catalase, indicating resilience against oxidative stress. Protease activity suggests their capability to degrade proteins, aiding nutrient cycling in the rhizosphere. Many isolates exhibited positive phosphatase activity, crucial for phosphorus solubilization, while nitrogenase activity indicated potential nitrogen-fixing capabilities, enhancing their role as plant growth-promoting rhizobacteria (PGPR).

The assessment of PGPR activity yielded several important findings. Isolates B6 and B8 were identified as hydrogen cyanide (HCN) producers, which may exert biocontrol effects against specific plant pathogens. Most isolates produced auxin, promoting root development and overall plant health. Positive ammonia production results indicate the potential for nitrogen enrichment in the rhizosphere, significantly contributing to plant nutrition.

Conclusion

Isolates B6, B10, and B18 stand out due to their combination of auxin production and nitrogenase activity, making them strong candidates for agricultural applications. The varying assimilation of sugars among different isolates may also indicate their adaptability to different environmental conditions. Notably, isolate B17 performed exceptionally well across all media, consistently achieving high average scores. Isolate E1 showed significant activity against all tested organisms, while B4 and B6 displayed potential but require improvement in specific areas.

The interaction between bacteria and fungi can involve competition for resources or direct antagonistic interactions, where bacteria produce substances inhibiting fungal growth. Isolates well-adapted to specific media may be more effective in these interactions. Therefore, KB and MH media are recommended for reliable experimental results, with a 48-hour incubation period for optimal bacterial activity.

The combined results indicate that the isolates possess significant metabolic versatility and biochemical activities beneficial for plant growth. The presence of nitrogen-fixing, phosphatase-producing, and auxin-producing bacteria suggests their potential as effective PGPR. Identifying these isolates can contribute to the development of biofertilizers or biocontrol agents, enhancing agricultural productivity sustainably. Future studies should explore their interactions with specific plant species and further investigate the mechanisms behind their beneficial effects on plant health. Molecular characterization could also provide insights into their phylogenetic relationships, aiding in classification and understanding of their ecological roles.

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