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Protection of *Alfalfa* from salt stress using endophytic bacteria

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Abstract

Endophytes are microorganisms mostly bacteria present asymptomatically in plants and can exercise several beneficial activities for their hosts. There are many reports currently that microorganisms isolated from the extreme environments have great biotechnological applications in medicine, agriculture, and industry. Alfalfa is a fodder legume of great S116) were studied for their ability to reduce *Medicago sativa* salt stress. Results show different levels in responses of *M. sativa* alfalfa. Genomic analyses were performed using selectedeconomic importance, but its productivity is often affected by salt stress in arid and semi-arid regions. Endophytic bacteria, which colonize the internal tissues of plants in a symbiotic manner, represent a promising solution for improving the salt tolerance of alfalfa. To improve alfalfa salt stress resistance, four endophytic bacteria (M17, M50, S113 and strains M17 that show a good protection from salt stress. M17 is identified as *Pseudomonas aeruginosa* strain, Comparative genomic for both strains is illustrated. For Pseudomonas strains (M17): two strains associated with plants and two non-associated with plant, one phytopathogens and Human pathogen). The results shows 521 common genes and synteny analyses revelas a high conservation between the bacteria associated with plants. Pseudomonas which indicate a mechanism to protect plants from salt and other abiotic and biotic stresses.

Key words: Plant microbiome, salt stress, endophytic bacteria, PGPR, genes.

Résumé

Les endophytes sont des micro-organismes pour la plupart des bactéries présents de manière asymptomatique dans les plantes et pouvant exercer plusieurs activités bénéfiques pour leurs hôtes. De nombreux rapports indiquent actuellement que les micro-organismes isolés des environnements extrêmes ont de grandes applications biotechnologiques en médecine, en agriculture et dans l'industrie. La luzerne est une légumineuse fourragère du grand S116) ont été étudiées pour leur capacité à réduire le stress salin de Medicago sativa. Les résultats montrent différents niveaux de réponse de la luzerne M. sativa. Des analyses génomiques ont été réalisées en utilisant une importance économique sélectionnée, mais sa productivité est souvent affectée par le stress salin dans les régions arides et semi-arides. Les bactéries endophytes, qui colonisent les tissus internes des plantes de manière symbiotique, représentent une solution prometteuse pour améliorer la tolérance au sel de la luzerne. Pour améliorer la résistance au stress salin de la luzerne, quatre bactéries endophytes (M17, M50, S113 et souches M17 qui présentent une bonne protection contre le stress salin. M17 est identifiée comme étant la souche Pseudomonas aeruginosa. La génomique comparative des deux souches est illustrée. Pour les souches Pseudomonas (M17) : deux souches associées aux plantes et deux non associées aux plantes, une phytopathogène et une pathogène humaine). Les résultats montrent 521 gènes communs et les analyses de synthèse révèlent une conservation élevée entre les bactéries associées aux plantes. Pseudomonas qui indiquent un mécanisme pour protéger les plantes du sel et d'autres stress abiotiques et biotiques.

Mots clés : Microbiome végétal, stress salin, bactéries Endophytes, PGPR, gènes.

ملخص:

النابتات الداخلية هي كائنات حية دقيقة معظمها بكتيريا تتواجد بدون أعراض في النباتات ويمكنها ممارسة العديد من الأنشطة المفيدة لمضيفيها. هناك العديد من التقارير حاليًا تفيد بأن الكائنات الحية الدقيقة المعزولة من البيئات القاسية لها تطبيقات تكنولوجية حيوية عظيمة في الطب والزراعة والصناعة. البرسيم هو من البقوليات العلفية S116 العظيمة) تمت دراسته لقدرته على تقليل إجهاد ملح Medicago sativa. أظهرت النتائج مستويات مختلفة في استجابات M. sativa alfalfa. تم إجراء التحليلات الجينية باستخدام أهمية اقتصادية مختارة، ولكن إنتاجيتها غالبا ما تتأثر بالإجهاد الملحي في المناطق القاحلة وشبه القاحلة. تمثل البكتيريا الداخلية، التي تستعمر الأنسجة الداخلية للنباتات بطريقة تكافلية، حلاً واعداً لتحسين قدرة البرسيم على تحمل الملح. لتحسين مقاومة إجهاد ملح البرسيم الحجازي، تم استخدام أربع بكتيريا نابتة داخلية (S113، M50، M17 والسلالات M17 التي تظهر حماية جيدة من إجهاد الملح. تم تحديد M17 على أنها سلالة Pseudomonas aeruginosa، وتم توضيح الجينوم المقارن لكلا السلالتين. بالنسبة لسلالات Pseudomonas (M17) : سلالتان مرتبطتان بالنباتات وسلالتان غير مرتبطتين بالنبات، إحداهما ممرضة نباتية وممرضة بشرية). أظهرت النتائج وجود 521 جينًا مشتركًا، كما كشفت التحليلات الجينية عن وجود نسبة عالية من الحفظ بين البكتيريا المرتبطة بالنباتات. الزائفة والتي تشير إلى آلية حماية النباتات من الملح والضغوط الحيوية وغير الحيوية الأخرى.

الكلمات المفتاحية: الميكروبيوم النباتي، الإجهاد الملحي، البكتيريا الداخلية، الجينات، البكتيريا الجذرية المعززة لنموالنباتات

List of Abbreviations

MAMP: molecular signals associated with pathogen

PAMPS: microbe-associated molecular patterns

PRRS: Porcine Reproductive and Respiratory Syndrome

SAR: systemic acquired resistance

SA : salicylic Acid

JA : jasmonate Acid

ET : ethylene

PGPBS: Plant growth-promoting bacteria

IAA: acid indole-3-acétique

ACC: 1-aminocyclopropane-1-carboxylate

PGPR: Plant growth-promoting rhizobacteria

PGBF: Plant growth-promoting fungi

PGBEBS: Numerous plant growth-promoting endophytic bacteria

ABA: acid abcssique

ROS: Reactive oxygen species

BNM: Buffer nodulation medium

CFU: Colony –forming weight

NCBI: National Center for Biotechnology Information (NCBI)

BLAST: Basic Local Alignment Search Tool

YEB: extract broth agar medium

NACL: sodium chloride

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Part I. Literature review

Remerciements

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Dédicace

As a preamble to this project, I thank ALLAH who helps me and gave me patience and courage during these years of biology study.

Affable, honorable, kind, you represent for me the symbol of goodness par excellence, the source of tenderness and the example of devotion. You have done more than a mother can do for her children, guiding me on the right path in my life and studies. I dedicate this work to you as a testimony of my deep love. At my faithful side, to the one whose prayers were the reason for my success and the balm for my wounds, to my angel in life, my mother. May God prolong your life and grant your heart everything it desires.

I thank my family and my cousins who were by my side during my academic journey

Special thanks go to my friend and companion in good times and bad, Nour, to my favorite character who encourages me in my studies and supports me thank youand I extend my sincere thanks to my group for standing with me throughout my graduation journey and who did not spare me anything Toufiq, Marwa, Hanan.

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finally, to all those I love and who love me I dedicate this work.

1 Introduction

The phyllo sphere, the rhizosphere and the interior of plant tissues constitute the habitat of a great diversity of microorganisms (14). When microorganisms colonize a host plant and the host tissue is apparently healthy, the relationship between the microorganism and its host can range from latent pathogenesis to mutualistic symbiosis. These microorganisms can be epiphytes or endophytes (39). The term endophyte was first introduced by De Bary (1866), defined as any organism that grows in plant tissues (40). These endophytes belong to various taxa such as bacteria, fungi, protists, archaea (41). Endophytes are called microorganisms that inhabit the internal parts of a plant. They penetrate the seed, leaf, stem and root of a plant and are not harmful to the host plant (42). These endophytes have been identified in many varieties of plants, some of which are rice, wheat, tomato, corn, strawberry, and citrus (43). The advent of microbial biotechnology has helped establish the fact that endophytic microorganisms play an important role in industry, agriculture and medicine (44).

Most of the time, when endophytes are inoculated into the plant, they produce a significant increase in biomass and also help boost commercial agriculture (45). Plant pathogens are generally considered for their negative effect on the germination and growth of surrounding crop plants, measured as reductions in crop yields (46). These endophytes enhance plant growth by secreting phytohormones and hence contribute to improved plant health by protecting them against plant pathogens and pests (47). Plant-endophyte interaction triggers the protection of plants against adverse environmental conditions such as the presence of heavy metals, drought, salinity and extreme temperatures (48).

Endophytic microorganisms help improve plant fitness through several mechanisms of action. The mechanisms generally employed by endophytic bacteria and fungi are mainly the production of siderophores, the production of enzymes, the synthesis of bioactive molecules, and the induction of plant resistance and competition (49).

Part I. Literature review

1.1 Introduction to plant microbe's interactions

The beneficial, commensal, and pathogenic microorganisms that comprise the plant microbiota coexist with plants in their natural habitat. Because of the intimate relationship that exists between plants and their microbiotas, there are now important concerns regarding how plants react to these microorganisms as well as the primary drivers of microbiota structure, diversity, and function in bulk soil, the rhizosphere, and plant organs. Long employed as crop inoculants, beneficial microorganisms provide the foundation for the rational engineering of the plant microbiota to enhance sustainable agriculture. This is made possible by the current creation of synthetic microbial communities and the discovery of plant characteristics that are responsive to the microbiota. Although microbes are essential to the survival of life on Earth, our knowledge of the vast majority of these organisms in habitats including soils, oceans, the atmosphere, and even our own bodies are limited. Bacteria that have been isolated may now be thoroughly investigated thanks to culture-based techniques, and molecular methods like metagenomics are making it possible to identify bacteria in real time more and more**. (5)**

Microbes are present in almost every plant tissue, including the rhizosphere (root surface), phyllo sphere (aerial plant surfaces), and endosphere (internal tissues). The rhizosphere is enriched with microbial diversity due to the presence of plant mucilage and root exudates, while the phyllo sphere is comparatively nutrient-deficient and experiences extreme environmental conditions. Microbes living inside plant tissues (endosphere) are classified as endophytes, while those living on or near plant surfaces (rhizosphere and phyllo sphere) are called epiphytes. These microbes can form beneficial, neutral, or harmful relationships with their host plants, depending on their proximity to the plant. Well-known symbiotic relationships, such as the Rhizobium-legume symbiosis, exist between certain bacteria and model plants. However, there is limited understanding of the vast majority of the plant microbiome and how it influences the extended phenotypic traits of the host plant. (5)

Photosynthetic energy is a major source of energy for a plant's microbiome. Plants use this process to transform carbon dioxide, water, and sunshine into food and oxygen in the form of sugars, which are partially carbon-containing molecules. Through their roots, plants release these sugars as well as other substances. In the microbiome, fungi and other bacteria aid in the breakdown of organic matter into basic chemical compounds that are useful to plants and other microbiome creatures. Plants can occasionally produce substances that draw specific microorganisms. Additionally, the short- and long-term relationships between plants and their microbiota are influenced by a variety of biotic and abiotic variables **(6)**. In addition to the availability of resources including nutrients, oxygen, temperature, water, presence of contaminating metals or metalloids, salinity, and soil type, abiotic variables also include soil pH, which is purportedly important for the microbiome-plant interactions in the rhizosphere (**6)** .

Numerous microorganisms, such as fungus and bacteria, interact with plants and can have either neutral or beneficial effects on them (**5)**. Positive outcomes result from the symbiotic relationships that exist between plants and microbes. In these relationships, plants give microbes photo synthetically fixed carbon, and microbes in turn enable plants to better withstand abiotic stress, acquire nutrients, grow, produce hormones, and defend against pathogens (**5)(5**). Microbes can have detrimental consequences on plants, such as parasitism, disease, and resource competition, which can lower plant performance. **(5)**

Plant performance can be enhanced by manipulating the interactions between microbes and plants in a variety of contexts, from agricultural output to mitigating climate change. Numerous plant physiological measures, such as productivity, survival, stress tolerance, uptake of CO2 and nutrients, and water use efficiency, can all be improved by these manipulations. The bacteria known as the plant microbiome, which reside in and on plants, are essential to their health. They do this by assisting in the uptake of nutrients, controlling plant hormone levels, and defending against pathogen invasion. As meta-organisms, plants are connected to intricate microbiomes.

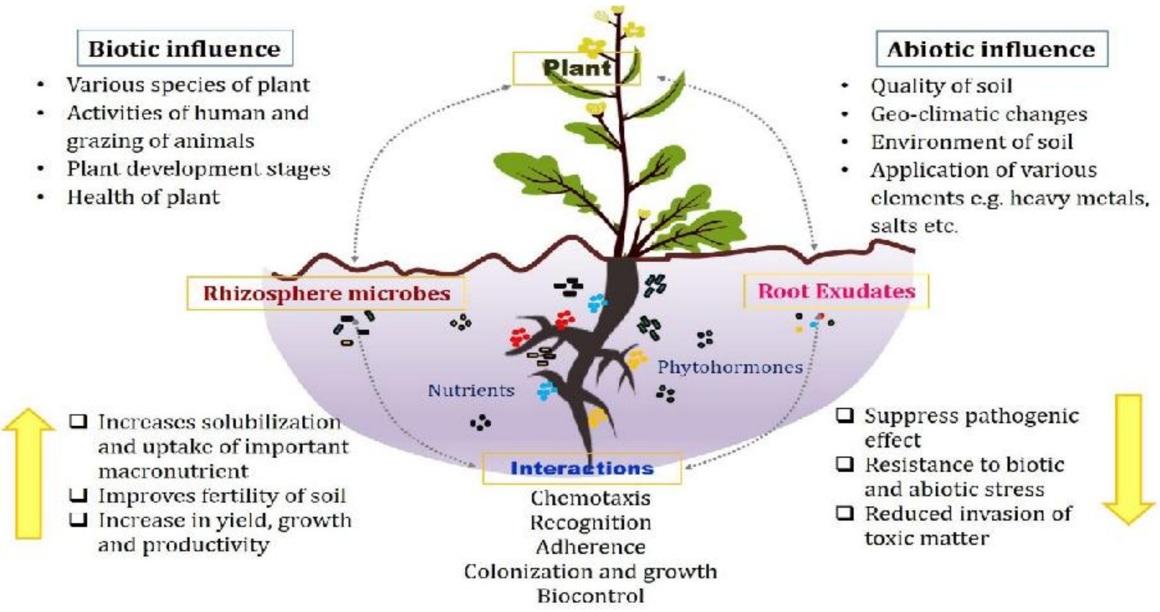


Figure 1 : Associations in the rhizosphere between plant roots, microbes, and root exudates under biotic and abiotic influences.

1.2 Colonizing the endosphere:

Microbiomes from below-ground or above-ground locations can be recruited by plants. As was already established, rhizodeposits have a fundamental impact on the Rhizospheric microbiome's slow recruitment **(6).** The microbial composition that lived in the three micro ecosystems connected to the roots, rhizosphere, rhizoplane, and endosphere showed progressive alterations, according to the data. **(7)** , in which plant rhizodeposits and soil abiotic characteristics operate as filters of specific microbial diversity from the rhizosphere **(7).**

1.3 Endophytic microbes

Any microorganism that lives inside the tissues of plants without harming them is called an endophyte. These organisms are usually fungi or bacteria. Endophytes are found in all or most plants. They are primarily transmitted by seeds and start to support plant growth and health as soon as the seeds germinate. Although they can be obtained from the soil, other endophytes also help plants. Endophytic bacteria are crucial parts of plants that serve the following purposes: improve the amount of nutrients that plants take in; protect plants from insects and pathogens; promote plant stress tolerance; control plant development; and inhibit the growth of weeds. The specific processes by which endophytic bacteria carry out the different roles in plants probably vary from microorganism to plant. **(16)**

The term endophyte refers to a heterogeneous group of microorganisms living in the host plant, consisting mainly of bacteria and fungi. (2). The etymology of this word comes from ancient Greek: endo means “in or interior”, and phyte means “vegetable or plant” (50). The most widely used definition is that of Petrini (1991), which describes endophytes as "all microorganisms living in internal plant organs at some time in their lives and can colonize internal plant tissues without causing apparent damage to the plant .(51). Endophytic bacteria and fungi live in plants in an inter/or intracellular manner by interacting biochemically and genetically with the host; this expanded definition relates the main functions of these microorganisms, including growth promotion and defense by synthesis of phytohormones, enzymes or precursors of plant secondary metabolites as well as to provide a new hidden repertoire of bioactive natural products with uses in the fields of pharmaceutical agro chemistry and other biotechnology applications .The notion of the «plant microbiome» has emerged, which has fundamentally altered the situation. As a result, it is now necessary to monitor the benefits of the relationship and identify the elements that contribute to the coexistence of both parties by observing the co-evolution of plants and their symbionts **(1)** . Endophytic bacteria and fungi live in plants in an inter/or intracellular manner by interacting biochemically and genetically with the host; this expanded definition relates the main functions of these microorganisms, including growth promotion and defense by synthesis of phytohormones, enzymes or precursors of plant secondary metabolites, as well as to provide a new hidden repertoire of bioactive natural products with uses in the fields of pharmaceutical agro chemistry and other biotechnology applications .

1.4 Bacteria endophytic

Endophytic bacteria are prokaryotes associated with plants and living inside tissues, which live there without harming their host (52). Endophytic bacteria can colonize the internal tissues of the host plant, including above-ground and underground parts and seeds (53). They include a significant range of Gram-positive and Gram-negative bacteria belonging to the Proteobacteria section of Actinobacteria and Firmicutes (54). Bacterial endophytes have been found in all plant species, so a plant without an endophyte is a rare exception in the natural environment. In fact, a plant without the associated beneficial bacteria would be less able to cope with plant pathogens and more susceptible to stressful conditions (55). Research regarding endophytic bacteria is one of the most promising aspects of microbiological studies (56).

They can directly benefit host plants by improving plant nutrient uptake and modulating growth- and stress-related phytohormones. Indirectly, endophytic bacteria can improve plant health by targeting pests and pathogens with antibiotics, hydrolytic enzymes, nutrient limitation, and inducing plant defenses. Some endophytic bacteria can have a wide host range and can be used as bio-inoculants to develop a sustainable agricultural system. Diversity of endophytic bacteria Endophytic bacteria of a particular plant host are not classified into a single species but include several genera and species. More than 129 species of endophytic bacteria have been isolated from different crop plants, including Gram-negative and Gram-positive species representing more than 54 genera (57).

1.5 The Science of Endophytic Bacteria: Natural Protector The Potential of Endophytic Bacteria as Bio fertilizers for Saline Soils

Endophytic bacteria have a promising future as bio fertilizers for saline soils. These bacteria are a good substitute for contemporary agriculture and sustainable development since they can increase plant production, health, and growth in saline soils. Examples of the possible applications of endophytic bacteria as bio fertilizers for salty soils include their capacity to mitigate drought and salinity stress in wheat and to promote alfalfa growth in saline environments. Isolation and Characterization of Alfalfa Plant-Originated Salt-Tolerant Endophytic Bacteria: An investigation was carried out to identify and describe salt-tolerant endophytic bacteria. The endophytic bacterial strains that the researchers isolated and identified from the root tissues of alfalfa plants that demonstrated tolerance to salt stress were found. It was discovered that these endophytic bacteria had a variety of defense mechanisms for alfalfa. from salt stress, including improving the intake of nutrients, generating chemicals that respond to stress, altering the regulation of osmotic pressure, and stimulating the creation of plant growth hormone. Endophytic bacteria have shown promise in increasing plant resistance to abiotic stresses, such as salinity. By using salt-tolerant endophytic bacteria as bio-fertilizers, farmers may be able to improve the growth and production of alfalfa crops in saline soils, even under salt stress conditions. These endophytic bacteria can colonize the root tissues of alfalfa and help mitigate salt stress by promoting the synthesis of growth-promoting hormones, enhancing osmotic adjustment, and improving nutrient availability. The identification and isolation of salt-tolerant endophytic bacteria from alfalfa grown in saline soils have demonstrated significant potential for improving plant growth, health, and productivity. The use of endophytic bacteria as bio-fertilizers to protect alfalfa from salt stress and enhance its development and productivity in saline soils is a promising strategy. Host Plant Factors and Endophytic Lifestyle (35)

1.6 Plant-Endophyte Interaction

Defines a «endophyte» as organisms that live inside plant tissues, such as roots, leaves, stems, flowers, and seeds (4). These organisms might be either bacteria or fungi. Although an endophyte cannot strictly harm its host plant, (4) the existence of the symbiont benefits the plant's ecology. (4) Better germination, (4) nutrient mobilization, (4) and antagonistic effects toward phytopathogens (4) are the majority of these benefits. Microorganisms known as endophytes infest the internal plant tissues, such as the roots, stems, leaves, and reproductive organs. The close relationship between the plant and the endophyte facilitates complex metabolic exchanges and metabolite exchanges. A wide range of substances, including phytohormones, siderophores, and enzymes that aid in the uptake of nutrients, can be produced by many endophytes. Furthermore, endophytes can provide improved resistance to biotic and abiotic stressors like heavy metals, pests, drought, and salinity. This protective impact is frequently achieved by altering the physiological responses of the plant, inducing systemic resistance pathways, or producing antimicrobial chemicals. In exchange, the plant gives the endophytic microbes safety and a nutrient-rich habitat. Plant-microbe interactions that are compatible are necessary for successful endophyte colonization. When the endophyte penetrates the host plant, the plant recognizes it and begins to exchange signal molecules with other plants. Numerous investigations have demonstrated endophytes' chemotactic reaction to host plant root exudates (3). Rich in biomolecules, root exudates draw in or are recognized by endophytes and other beneficial microorganisms. Additionally, nutrient- and water-rich, exudates draw a wide variety of microorganisms. One such metabolite, flavonoids, is classified as a chemoattractant and is released by a variety of plants. It is essential to the connection between the endophyte and the root hair.

1.7 Modulation of Plant’s Immune System by Endophytes

To penetrate the host plant, endophytes must first overcome the first line of defense of the immune system this implies that plants recognize conserved molecules typical of many micro-organisms called molecular signals associated with the MAMPS pathogen. beta _glucan chitin lipopolysaccharid. MAMPS are recognized as specific receptors on the surface of plant cells that trigger defense responses in the case of fungal endophytes the receptors are the chitin oligomers of the fungal wall Additionally, there is evidence that endophytic bacteria make their own MAMPs, which either the plants' PRRs do not identify or cause a relatively mild and fleeting defense response in comparison to pathogenic encounters. (34)

1.8 Role of plant defense responses and phytohormones in controlling endophyte

One of the primary methods for identifying invasive infections is the recognition of pathogen-associated molecular patterns by pattern recognition receptors, Other «non-pathogenic» microbes, such as bacteria, can also be identified by plants thanks to microbe-associated molecular patterns(PAMPs), which control the innate immune system's reaction to plant defense. The intracellular immune receptors, or NOD-like receptor type, are the second recognition filter after these PRR (7). Effector-triggered immunity is induced by these receptors when they directly or indirectly recognize virulence effectors released by pathogens within host cells (7). Only a small number of PRRs have been explored in Arabidopsis and rice (7), despite the fact that research on these microbial recognition systems by plants has advanced significantly. Additional plant models still require investigation and compared.

Systems that individually identify and detect colonizing microorganisms—which try to establish mutually advantageous relationships with their plant hosts—include MAMPs. Phytohormones, which may be connected to defense functions, are other active participants. Examples of these include salicylic acid, jasmonate acid, abscisic acid, and ethylene (7), Generally speaking, SA reacts to interactions between biotrophic and hemobiotrophic organisms and abiotic stimuli, causing plants to mount a defense mechanism known as systemic acquired resistance, or SAR (7). On the other hand, PGPBs interact with other beneficial microbes to trigger the response of JA/ET, a different plant pathway (7). Nevertheless, some research (7) purportedly found variations in the concentrations of SA and JA in plants of maize treated with the entomopathogenic fungus Metarhizium, which correlates to plant Physiological reactions according to the established JA/ET and SA pathways.

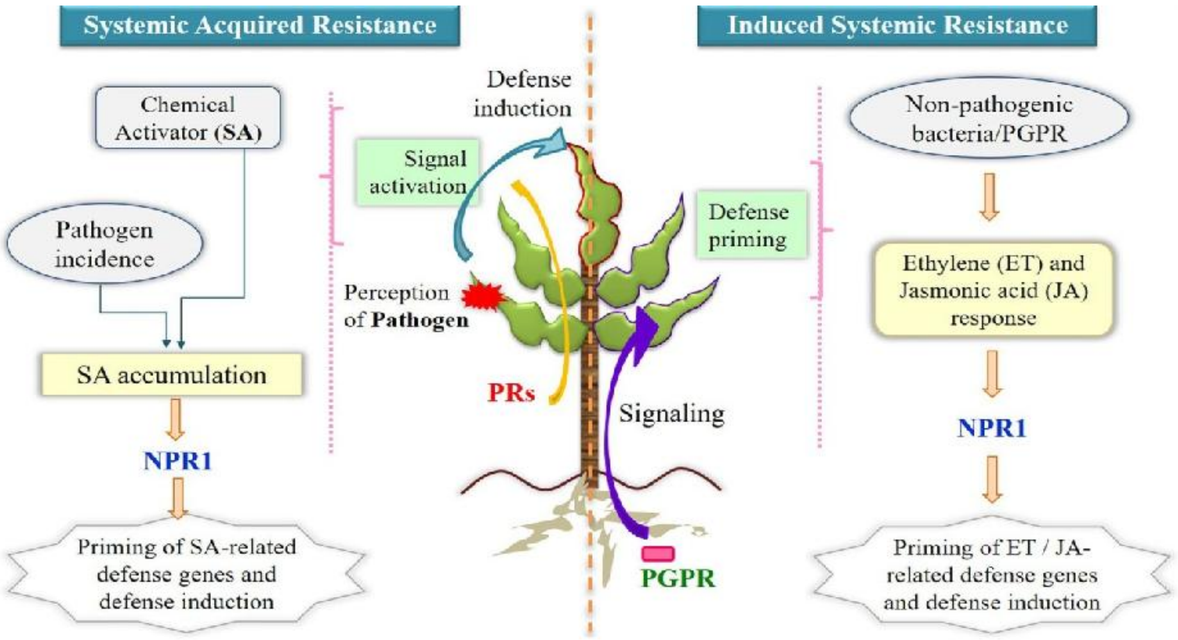


Figure 2 : Mechanism of SAR and ISR utilizing phytohormones for inducing defense responses upon microbial incidence.

1.9 Overview of plant root-colonizing endophytic bacteria and their mechanism of action

It is possible to isolate endophytic bacteria from sterilized surfaces and use them to colonize plant roots endophytic bacteria promote plant growth via several mechanisms 1_ nitrogen fixation growth stimulation through phytohormone synthesis and hormonal modulation. for the ability of endophytes to produce auxin IAA helps regulate plant growth and developmental processes such as tissue differentiation, division, cell elongation, the apical domain, responses to pathogens, light and gravityACC deaminase synthesis by endophytes is crucial to reduce ethylene levels in the plant and stimulate its growth Highlights from the different PGPEBs that reside in the such as fimbriae or pili, might improve the bacteria's adhesion to the root matrix and, as a result, the soil nutrients that plants can absorb (8). Because they are in frequent contact with potentially dangerous bacteria, plants have developed a highly developed defense mechanism to prevent bacterial infections.

1.10 Molecular mechanisms of resistance in plants

Plants have a sophisticated defense signaling network that includes a two-tiered innate immune system against bacterial **diseases. MAMP-Triggered Immunity**:

Microbe-associated molecular patterns, which are bacterial elicitors capable of impairing cellular, metabolic, and physiological activities, are recognized by plant receptors, triggering this first response. **Effector-Triggered Immunity**: At this second stage, plants identify and produce a strong defense against particular bacterial effectors, which are proteins released by bacteria**. Elicitor-Receptor Recognition:** Upon detection of MAMPs, signal transduction pathways are initiated. (21)

Nutrient depletion zones can develop around roots due to rapid nutrient uptake, low soil moisture, etc., making it difficult for plants to acquire sufficient nutrients. Mycorrhizal fungi form symbiotic associations with plant roots, colonizing the living root tissue while the plant is actively growing. The thin fungal hyphae can extend beyond the depletion zone, accessing nutrients that the roots cannot reach alone, especially phosphorus. This symbiosis is particularly beneficial in nutrient-poor soils, where the fungi can help plants acquire up to 20% more carbon. Mycorrhizal fungi also provide physical defense against pathogens, induce broader plant defense mechanisms, and offer protection to plants growing in metal-contaminated or acidic soils. There are two main types of mycorrhizae: ectomycorrhizal (forming a dense sheath around roots, common in trees) and endomycorrhizae/Arbuscular mycorrhizae (embedded within root tissues, present in over 80% of terrestrial plants). In the symbiosis, the plant provides carbon/sugars to the fungi, while the fungi aid in the uptake of phosphates, zinc, copper, and other minerals, ultimately increasing the plant's surface area for nutrient absorption. (28)

Definition of Rhizobia Symbiosis: Rhizobia are bacteria that fix nitrogen and grow as nodules on the roots of legume plants. Nitrogen Fixation: Rhizobia transform atmospheric nitrogen (N_2) into a form that plants can utilize, ammonium (NH_3^+). Rhizobia produce two types of nodules in general: determinate and indeterminate. The indeterminate nodules are elongated and have a persistent meristem that continuously produces new nodule cells, which are then infected by rhizobia present in the nodule. The determinate nodules are usually round, lack a persistent meristem, and do not show an obvious developmental gradient. Historically, the production of indeterminate nodules has been studied using models such as Medicago sativa (alfalfa), Medicago truncatula). The rhizobia inside the nodules reproduce; receive nourishment and protection from the plant. They, absorb N_2 from the air, and transform it into amino acids that the plant needs for growth (29). Plant growth-promoting rhizobacteria (PGPR) provide a wide range of beneficial effects to plants, including producing metabolites, enzymes, nutrients, biological pest control, inducing disease resistance, and detoxifying pollutants, heavy metals, and pesticides. Currently, most major field crops heavily rely on agrochemicals and synthetic pesticides, which can negatively impact human and animal health as well as pose threats to ecosystems. Beneficial microorganisms like PGPR hold great promise as alternatives to harmful chemicals and pesticides in organic farming, sustainable agriculture practices, and bioremediation of heavy metal contaminated sites. PGPR have the potential to complement and replace the use of hazardous chemicals and pesticides in agriculture. However, only a small number of bio-formulation products containing PGPR strains with desirable traits such as metabolite production, enzyme activity, nutrient mobilization, and biocontrol abilities have been developed for field crops so far. (30)

PGPF are a group of non-pathogenic fungi belonging to various genera that provide benefits to their host plants. They play a useful role in sustainable agriculture by helping increase crop production without harming the environment. Using PGPF is considered an environmentally responsible approach to boost agricultural productivity to meet growing food demand. PGPF can enhance various aspects of plant growth and development through direct or indirect mechanisms, including improving germination, seedling vigor, plant growth, root morphogenesis, photosynthesis, and flowering, ultimately leading to higher crop yields. The mechanisms by which PGPF promote plant growth include production of volatile organic compounds and microbial enzymes, solubilization and mineralization of nutrients for easy plant uptake, regulation of hormonal balance, suppression of plant pathogens, and amelioration of abiotic stress factors. (31)

1.11 Plant endophytic interactions in promoting plant growth

Numerous bacterial communities coexist in plants, and their collaboration supports the physiological processes of the host plant (8). Nutritional bioavailability and plant tissue colonization capacity are prerequisites for the mutually beneficial cooperation of endophytic bacteria and host plants in their natural habitats. Pseudomonas brenneri, Pseudomonas aeruginosa, and Pseudomonas otitidis are a few genera of recognisable endophytic bacteria. (8)

Numerous plant growth-promoting endophytic bacteria (PGPEBs) have been identified that can be utilized to increase crop yields and promote agricultural sustainability. In agricultural management practices, species of rhizobacteria and other nitrogen-fixing bacteria have been employed as PGPEBs.PGPEBs can provide direct benefits to plants by colonizing the root endosphere and carrying out functions like nitrogen fixation, regulating plant hormone levels, solubilizing nutrients like phosphate, iron and potassium, producing secondary metabolites and antibiotics. Indirectly, they can combat plant diseases, induce plant tolerance to abiotic stresses, and enhance systemic resistance. Root endophytes have been successfully developed as probiotics, showing promise for sustainable farming approaches. Various microorganisms that stimulate plant growth have been studied for potential use in boosting agricultural output through organic farming to prevent future food crises. PGPEBs are also documented to play roles in bioremediation of heavy metals, xenobiotic and environmental pollutants, as well as in enzyme, antibiotic, and siderophore production. Further research is needed to optimize the potential of endophytic bacteria for promoting sustainable plant health and increasing crop productivity, given their complex contributions to agricultural sustainability (8).

the direct and indirect mechanisms employed by plant growth-promoting endophytic
bacteria (PGPEB):

Direct Mechanisms: Nitrogen fixation, Synthesis of phytohormones like auxins, cytokinin, gibberellins, and abscisic acid, Enhancing plant survival under abiotic stresses like soil salinity and drought by reducing ethylene levels via the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, Solubilization of minerals. Indirect Mechanisms: Production of siderophores, organic acids, enzymes, and antibacterial substances like cyanides and antibiotics, The ACC deaminase enzyme helps promote plant growth in the presence of high levels of toxic metals, Some nitrogen-fixing endophytic bacteria can regulate rhizobacteria and bacteria-bacteria interactions, Endophytic bacteria producing ACC deaminase can lower plant ethylene levels, favoring nodulation by rhizobia, Inducing plant defense responses or producing secondary metabolites that protect against plant diseases, Triggering systemic resistance pathways in plants, such as the jasmonic acid pathway (33)

1.12 Plant root endophytes and bio inoculant synthesis

Specialized organs called roots help plants absorb nutrients from the soil by providing mechanical support (8). Water and nutrients can be directly absorbed and moved from the soil to the stem and other parts of the plant with the help of the plant supporting tissue (xylem and phloem) in the roots (8). the capacity of root-associated endophytic bacteria to generate IAA can improve root development, hence aiding in plant nutrition through the uptake of nutrients from the soil. damage to the roots of plants permits the free release of root exudates into the soil environment that include fixed carbon in the form of polysaccharide mucilage. These substances can operate as a chemoattractant and a source of energy for the microorganisms that surround the root. Similar to soils, the endosphere compartments of plant roots are home to a community of bacteria. The activities of root endophytic bacteria in the endo-rhizosphere regions can be modulated by the root architectural system and rhizodeposition of nutrients. Endophytes may have comparable roles in crop breeding, regardless of the plant areas they colonize. Furthermore, choosing the right carrier and bacterial strains is necessary for the efficient commercial production of bio inoculants. Furthermore, because of their diverse surroundings, bacteria in the plant endosphere might be challenging to separate (9).

1.13 Genomic insights into root endophytic bacteria communities

Genomic insights into endosphere biology have greatly enhanced the understanding of root endophytic bacteria and their roles in promoting plant growth and protecting crops. There are several methods available for studying endophytic bacterial communities, and it is crucial to employ the appropriate techniques when investigating the bacterial endophytes residing in the root endosphere. The two main approaches are culture-dependent and culture-independent techniques. Culture-dependent techniques involve the direct culturing of relevant bacteria. (10) Endophytes have a wide range of interactions with their host plants, ranging from parasitism to mutualism. Many endophytes are able to produce various compounds, including antioxidants and osmoprotectants, which could potentially help plants cope with salt stress, making them particularly interesting for salt stress mitigation. Some endophytes have been found to increase stress tolerance and promote plant growth through mechanisms that are not affected by stress conditions. (11)

1.14 Introduction of Medicago Sativa (alfalfa)

Because of its great nutritional content and capacity to flourish in a variety of environmental circumstances, alfalfa is a feed crop that is widely grown across the world. Alfalfa's vulnerability to salt stress, which can restrict its development and output, is one of major challenges. High salt content in the soil can cause harmful ions to build up in plant cells and interfere with regular physiological processes, which is known as salt stress. It has been established that endophytic bacteria, which live within plant tissues without harming them, may help plants become more resilient to abiotic conditions like salinity. These bacteria can help shield alfalfa from salt stress through a variety of methods. Increasing nutrient intake, creating chemicals that respond to stress, adjusting osmotic regulation, and encouraging.

Endophytic bacteria can colonize alfalfa root tissues and enhance nutrient availability, osmotic adjustment, and growth-promoting hormone production, thereby alleviating the effects of salt stress. These bacteria can also release bioactive substances that can inhibit the growth of salt-sensitive pathogens, increasing alfalfa's disease resistance under salt stress conditions. The presence of endophytic bacteria is essential for protecting alfalfa plants against salt stress while supporting overall plant growth and development. Utilizing endophytic bacteria as bio-inoculants presents a sustainable and environmentally friendly approach to reducing salt stress and increasing crop yields in alfalfa farming. Researching endophytic bacteria and their role in conferring salt stress tolerance to alfalfa is crucial for developing novel strategies for crop improvement and agricultural sustainability in salt-affected areas. (12)

Plants go through various life processes like growth, respiration, and reproduction, which require energy sources and involve cellular activities. Salt stress causes ionic and osmotic imbalances, interfering with normal plant metabolic activities, growth, and gas exchange. Endophytic bacteria are non-pathogenic bacteria that colonize and inhabit plants, and they are found in various plant species, from simple to complex. Endophytes are vertically transmitted across plant generations and have been shown to be beneficial in stress-prone environments, such as drought conditions. Endophytes can enhance host plant growth, aid in overcoming physical stresses, pesticides, salt stress, and nutrient deficiencies. Alfalfa, an important crop grown in diverse climates, harbors endophytes. With global warming, sea-level rise, and increasing soil and water salinity, salt stress is becoming a significant threat to alfalfa cultivation. The research aims to investigate whether endophytes can mitigate salt stress in alfalfa. Alfalfa, a protein-rich legume from the Fabaceae family, is known as the "Queen of forage" and is cultivated for its high yield, quality feed, and soil fertility benefits. Different alfalfa cultivars exhibit varying degrees of salt tolerance, and research is ongoing to develop new salt-tolerant cultivars. (17)

1.15 Overview of Salt Stress in Alfalfa

1.15.1 Abiotic stress

The term "abiotic stress" refers to environmental factors that affect plant growth and productivity, such as salinity, dryness, and extreme temperatures (either positive or negative). In contrast to animals, plants are unable to move in order to escape unfavorable situations. As a result, they have developed adaption strategies to deal with environmental changes. Plants' responses to stress are influenced by the type and degree of stress, as well as by their genetic history and ability to adapt to certain environmental conditions. The stress response mechanisms may be common to multiple stressors or specific to one particular type of stress

1.15.2 Effects of Salinity on Plant Microbiomes

Plants' Rhizospheric and resident endophytic bacteria are shaped by salt stress. As a result, a large body of research has focused on the glycophyte and halophyte microbiomes, exposing a distinct microbiome population that is chosen by plants that are cultivated in salt stress. It has also been shown that salinity is one of the primary elements controlling the bacterial community connected to halophytes' roots. It is thought that the microbiomes of plants under salt stress can offer potential allies in the fight against the detrimental effects of salt stress on farmed crops. (17)

One of the primary environmental stressors that lowers plant growth and productivity is salt stress. Plant cells experience a water deficit due to hyper ionic and hyperosmotic stress brought on by the presence of salt in the soil solution. In addition, an ionic imbalance, physiological and metabolic alterations, and even oxidative stress may result from this. Plant development and agricultural output significantly decrease when salt stress compromises a plant's basic function. Since 20% of irrigated lands and 6% of the world's land area are high salinity, it is critical to identify solutions to boost main crops' salt tolerance as a result of rising salinization. (15) One of the main reasons affecting crop productivity is salinity, which raises the possibility of decreased food security. The availability of adequate and nourishing food for humans is one of the variables that determines food security. Medicago sativa is considered the queen of fodder crops due to its significant role as a feed crop and as a source of forage for livestock, including sheep and cattle. Nonetheless, excessive soil salinity negatively impacts alfalfa, which means that high NACl soils significantly hinder the plant's ability to grow and yield. (16)

1.15.3 Bacterial Adaptation to High Salt Levels

Numerous acclimation strategies are available to bacteria for hypersaline environments. To maintain an analogous ion outflow across the cellular membrane, for instance, bacteria experience osmotic pressure, a phenomenon that occurs between their internal and external mediums. Na+ ions are inhibited in the extracellular media of bacteria that absorb excess salt until an osmotic balance is reached. If not, a number of bacteria could gather and/or produce suitable solutes in large quantities, such as sugars, polyols, and amino acids and their derivatives. These suitable solutes don't interfere with osmosis and don't carry a particular charge. Rather, they work by expanding the amount of water and cytoplasm in bacteria, which enables them to proliferate and withstand harsh environments. certain bacterial groupings create exopolysaccharides to build biofilms that, in the event of elevated NACl concentrations, are in charge of preserving bacterial moisture. Furthermore, Gram-negative bacteria have improved salt tolerance thanks to the lipid layer in their membrane. The synergistic interactions within a bacterial consortium have also been shown by numerous scientists to be an effective means of mitigating the effects of salt stress. In halotolerant bacteria, the secondary metabolites that allow for the tolerance of salt stress have undergone genetic improvement or alteration, making the bacteria more adaptable. Because endophytic metabolites fluctuate based on both internal and external factors, several molecular routes of bacterial salt resistance remain incompletely know (19)

1.16 Selection of salt tolerant endophytic bacteria

1.16.1 Soil Salinity and Its Impact

Soil salinity is a severe problem that affects over 45 million hectares of agricultural land worldwide, with 1.5 million hectares being nearly unusable. Traditional methods for developing salt tolerance in plants are time-consuming and resource-intensive. Endophytic bacteria, microbes residing within plant tissues, have gained attention for their ability to protect plants from environmental stressors, including salinity. In saline areas, endophytic bacteria offer an inexpensive and environmentally friendly approach to increasing crop productivity. Endophytic bacteria enhance plant salt tolerance through various mechanisms, such as reducing ionic and osmotic stress, increasing antioxidant defenses, adjusting phytohormonal signaling, and improving nutrient uptake efficiency. Research has demonstrated the beneficial roles of endophytic bacteria in promoting plant health, resilience, nutrient availability, stress response, and growth promotion. Screening techniques for salt tolerance include evaluating germination rate, growth parameters (root and shoot length, fresh weight), ion content and discrimination (K^+/Na^+ ratio, Cl^- exclusion), and leaf traits (stomatal density, yellowing, and damage). These screening parameters provide insights into a plant's ability to tolerate salt stress during early growth stages and aid in identifying salt-tolerant genotypes. (21)

1.16.2 Responses to Osmotic Stress

plant cells usually adjust their osmotic potential to meet the requirement of the whole plant in balancing its water budget, Significant changes in water potentials in the environment can impose osmotic stress to plants, which disrupts normal cellular activities, or even causes plant death, Under natural conditions, excessive salinity and drought are the primary causes of osmotic stress in plants.(61) Here, we examine the molecular pathways involved in plant responses to osmotic stress, Plant responses to osmotic stress involve a complex interplay of molecular, cellular, and whole-plant mechanisms to maintain cellular homeostasis and restore growth under conditions of water deficit or high salinity. Key components of these responses include: Phytohormone Regulation like (ABA), (ethylene), and (reactive oxygen) species (ROS) play important roles in regulating plant responses to osmotic stress (62)

1.16.3 Plants–Endophytes–Salt Interactions

Plants can improve their resistance to salt stress by forming symbiotic relationships with endophytic and Rhizospheric bacterial populations. Soil salinity exerts selective pressure on rhizosphere populations, favoring the growth of halotolerant bacteria. The rhizosphere, where plant exudates attract competing bacteria, is the primary source of endophytes. Endophytic bacteria gain entry into the plant's interior tissues by secreting enzymes like cellulase and pectinase to break down the root cortex, or by exploiting wounds caused by natural processes (e.g., secondary root formation) or phytopathogens. Endophytic bacteria are defined as those that penetrate and colonize the internal tissues of plants for some or all of their life cycle without causing disease, and can potentially benefit their host plant. (20)

1.16.4 Role of Endophytic Bacteria in Salt Stress Tolerance:

High salt (NACl) concentrations are believed to affect osmotic metabolism in plants. Excess accumulation of $Na+$ may damage the normal physiological and biochemical processes of the cell (60), the functionality of endophytic bacteria and their modes of action to enhance salinity tolerance in plants, with special reference to osmotic and ionic stress management. There is concrete evidence that endophytic bacteria serve host functions, such as improving osmolytes, anti-oxidant and phytohormonal signaling and enhancing plant nutrient uptake efficiency. (14)

1.16.5 Plant hormone regulation of abiotic stress responses

Abiotic stresses, such as drought, salinity, heat, cold and flooding, have profound effects on plant growth and survival (21) , Agricultural crops normally undergo series of physiological processes (photosynthesis, respiration, stomatal functions and nutrition)during developmental stages of their life cycles that are sensitive to environmental conditions(57) ,Plant hormones are signaling compounds that regulate crucial aspects of growth, development and environmental stress responses (21) , the major plant hormones in regulating abiotic and biotic stress responses with special focus on the significance of crosstalk between different hormones in generating a sophisticated inefficient stress response , the two key hormones as ABA and GA in regulating plant responses to environmental signals (57), The effects of abiotic stress in plants range from the qualitative and quantitative changes in the synthesis of type of proteins in crops to the disruption of the flower bud formation and pollination process in plan(3), the role phytohormones in abiotic stress in the aforementioned papers, hence this review wilconcentrate on primary and secondary metabolites (59)

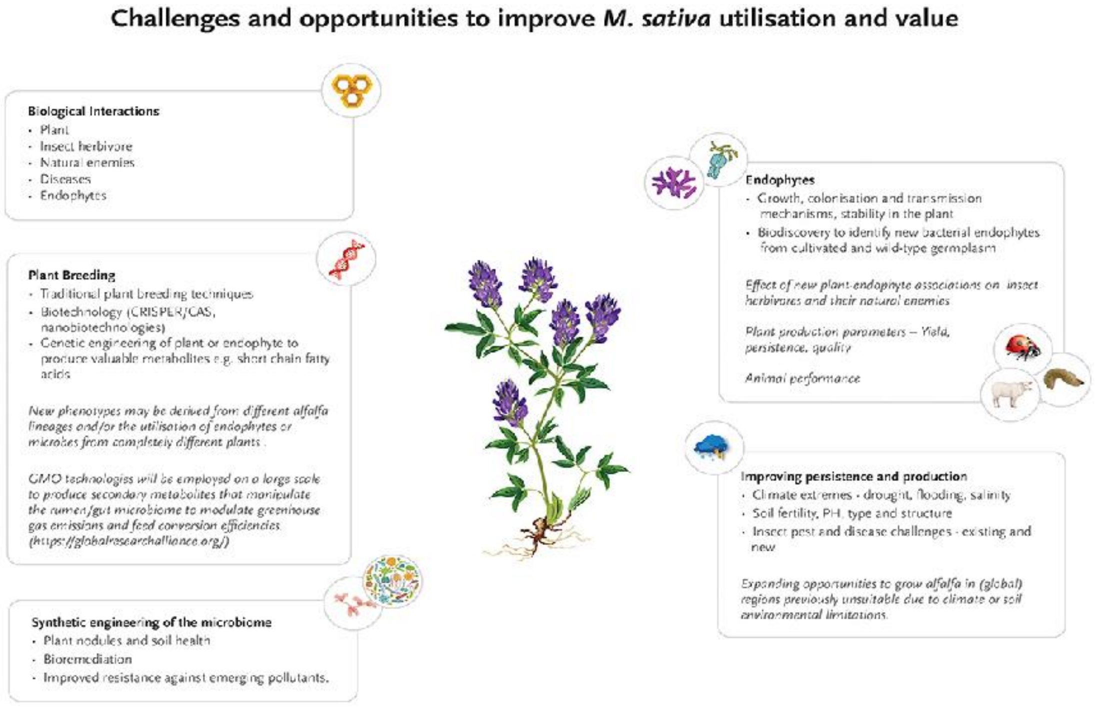


Figure 3 : :Current and future research opportunities to improve the production, persistence, quality and geographic range of Medicago sativa as forage crop

1.17 Introduction to the problematic of the study

Due to its high nutritional content and palatability, alfalfa (Medicago sativa L.) is referred to as the "queen" of fodder crops and is a crucial feed crop for animals. But because alfalfa is a somewhat salt-sensitive plant, salt stress may significantly reduce the crop's production and quality, particularly in arid and semiarid areas where salinization is posing a serious challenge to the sustainability of agriculture. One of the most dangerous abiotic stressors is salinity; Salt stress has been shown in the past to induce nutritional imbalance, metabolic disorders, and oxidative damage in plants, which can lead to reduced biomass, slowed development, and even plant mortality. Different Many strategies, including the exogenous delivery of osmotic regulators, the use of genetically resistant strains, the enhancement of irrigation systems to reduce salt buildup, and the selection of salt-tolerant plants, have been developed to mitigate the effects of salt stress on plants. (18)

Four hypotheses will be investigated in this study: One can isolate salt-tolerant endophytic bacteria from alfalfa; two, certain endophytic bacteria can colonize alfalfa and shield the plants from salt stress; three, the dynamics of the selected endophytic bacterial population in alfalfa cells is linked to salt stress; and four, there is a notable variation in the salt stress tolerance of the endophytic bacteria used in this study.

1.18 Objective

The main objective of this study is to assess the ability of previously described PGPR endophytic bacteria to protect Alfalfa plants from salt stress. To illustrate this study, four strains of endophytic bacteria were studied for their ability to protect Alfalfa from salt stress. Genomic comparative and functional analyses were performed to are used to identify the genes and the metabolic pathways by which endophytic bacteria can potentially improve salt resistance in M. sativa (alfalfa).

Part. II. Materials and Methods

1.19 Plant Materials, Growth Condition and Stress Treatment

1.19.1 Medicago sativa growth and inoculation

Alfalfa seeds are cleaned with a liquid soap solution (pec) and rinsed four times with distilled water to remove traces of soap. Seeds are then sterilized with a 5° chlorine treatment for 12 minutes and rinsed four times with sterile distilled water. The sterilized seeds are deposited in round petri dishes (150mm) containing a sterile solution of 1.2% agar and distilled water. The seeds are then placed at 4°C in the dark for 48 hours for stratification. Germination is then carried out by placing the seeds for 24 hours in a climatic chamber at 26°C in the dark. The seedlings are then transferred to a modified Buffer Nodulation Medium (BNM) medium containing 1.2% bactoagar and containing or not 150 mM NaCl. The plants are then grown in climate 26°C with 16h of light and 8h of darkness. Freshly prepared bacterial suspensions of solution are added to plants by rinsing the roots and excess inoculum is removed. Inhibition of root growth by NaCl is assessed by measuring growth 5 days after cultivation.

1.20 Inoculation With Bacteria

Available bacteria in the lab were used during this study: S113, M50, S116, and M17. These strains were cultured on yeast extract broth agar medium (YEB) for 48 hours at 30°C. After incubation, the cells were re-suspended in sterile saline water at 0.9 %. The bacterial suspensions were then diluted to an optical density of OD_{600}=0.1 to prepare the inoculum. Using a pipette, 1 cc of the inoculum solution was applied to each alfalfa seedling and excess liquid was removed.

1.21 Treatment for Salt Stress

For the salinity test, solutions were prepared to achieve different concentrations of 0 mM, 150 mM and 200 mM. These salt solutions were then applied to seedlings to impose salt stress treatments in the culture medium.

1.22 Data Collection

To monitor plant development, photos of the seedlings are taken periodically. Additionally, to evaluate plant growth, seedlings are harvested and the fresh and dried weights of their roots are recorded.

1.23 Data Analyses

To evaluate the influence of endophytic bacteria on alfalfa development, the growth characteristics (including root fresh and dry weights) of inoculated plants are compared against those of non-inoculated control plants using statistical analysis. This comparison is conducted with two technical and two biological replications to ensure robustness of the results.

Comparative genomic analyses

To identify putative key genes in the strain M17 (Pseudomonas aeruginosa) required for plant infection. Genomic analyses were performed on selected strains of Pseudomonas associated with plants: plant growth promoting bacteria (PGPB): P. koreensis 16610, P. atacamensis M7D1, P. fluorescence F113 ; plant pathogens: P. syringae DC3000 and human pathogens P. aeruginosa 138244. The genomic analyses were performed in the MicroScop platform (https://mage.genoscope.cns.fr/microscope/compgenomics/pancoreTool.php?).

Genomic structural biology

Structural genomics, is a field of study that focuses on the three-dimensional structures of genomes and their components. It involves the investigation of how genetic information is organized, packaged, and functions within the cell at a molecular level. This area of research delves into understanding the spatial arrangement of DNA, proteins, RNA, and other molecules that make up the genome.

Annotation aims to identify and mark the locations of genes and their various features in a genome sequence. The primary target of gene annotation is to accurately predict the location of protein-coding genes, non-coding RNA genes, regulatory elements, and other functional elements within the genome. This process involves predicting gene structures, including exon-intron boundaries, transcription start sites, and regulatory regions. Additionally, gene annotation targets the identification of specific functional elements within genes, such as protein domains, motifs, and regulatory sequences that control gene expression

Annotation process for coding gene: is known as the Targeted stage which is followed by the Similarity stage in which proteins from closely related species are used to build transcript structure in regions were a Targeted transcript structure is absent

Annotation for non-coding RNAs (ncRNAs) are involved in many biological processes and are increasingly seen as important. As is the case with proteins, it is the overall structure of the molecule which imparts function: tRNA transfer RNA, rRNA ribosomal RNA).

Part III. Results

1. Root growth effect on seedling length

In the first repetition, we observed the roots of Medicago sativa alfalfa plants compared to the control under three NACl concentrations: 0 mM, 150 mM, and 200 mM. In the control groups at 150 mM and 200 mM, there was no significant effect on plant growth (Figure 4). Without saline stress, we observed a high growth rate, with root lengths varying across the three control concentrations. Some of these control plants even progressed to a more advanced growth stage. For plants inoculated with the M17 bacteria, at 0 mM NACl, there was a noticeable positive effect on root growth. However, at 200 mM NACl, there was no growth effect observed, and at 150 mM NACl, there was also no growth effect, with notable differences in root length.

For the M50 bacteria, at 0 mM NACl, there is a noticeable effect on root growth. However, at 200 mM NACl, there is no growth effect, and at 150 mM NACl, there is a slight growth effect with varying root lengths. The S113 bacteria, at 0 mM NACl, there is a positive effect on root growth. At 150 mM NACl, there is an increase in root length, while at 200 mM NACl, there is no growth effect on the roots. The S116 bacteria, at 0 mM NACl, there is tolerance and growth in the roots. At 150 mM NACl, there is a reduction in root length, and at 200 mM NACl, there is an inhibition of root growth.

In the second repetition, there is a significant difference compared to the results of the first repetition regarding root growth effects. For the control without bacteria (0 mM, 150 mM, 200 mM NACl), there is an increase in root growth. For the M17 bacteria, there is a wide variety of root growth effects. For the M50 bacteria at 0 mM NACl, there is an increase in root regrowth. For the S113 bacteria, there is a strong effect on root growth, and for the S116 bacteria, there is a notable effect on root growth.

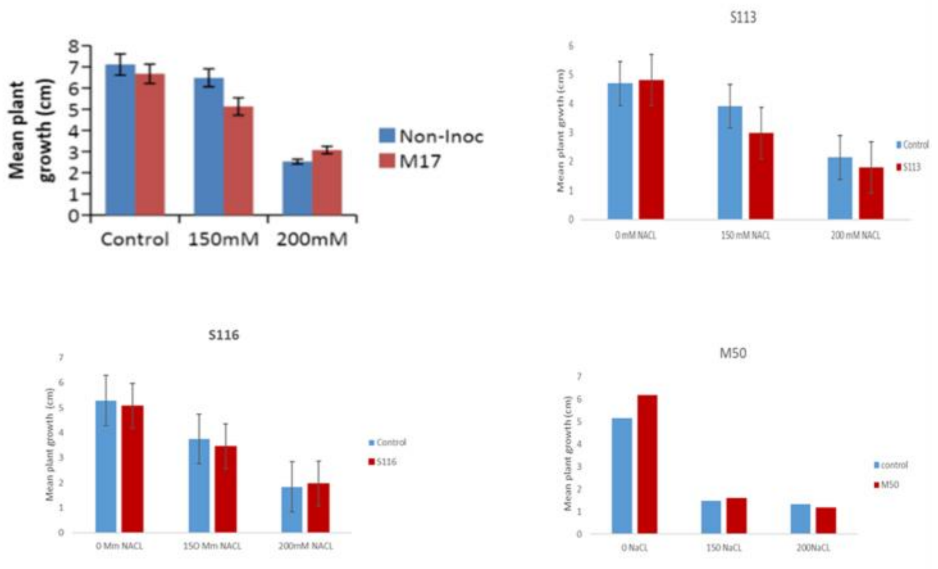


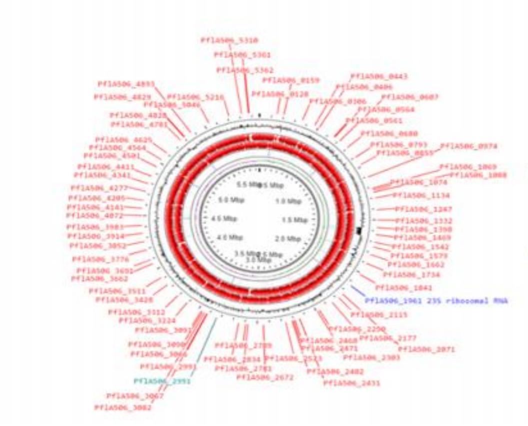
Figure 4 The impact of M17, S113 and S116 on Medicago sativa (alfalfa) the stress response Two biological repetitions of 8 plants each were analyzed. The error bares show the Standard Error (SE)

2. Comparative genomics

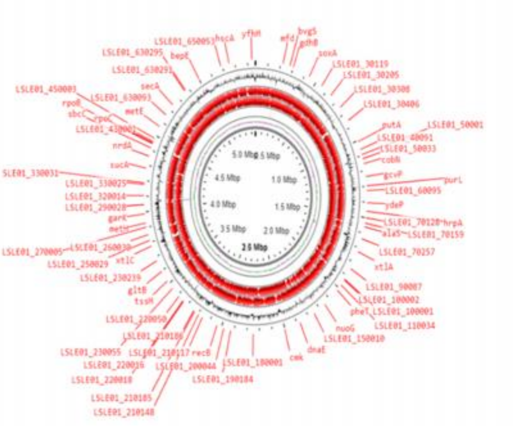
2.1. Genomes of studied Pseudomonas genome

Molecular identification was previously performed by the lab on the strain the M17 and reveals that this strain is a *Pseudomonas aeruginosa*. In the aim study the molecular mechanisms involved in the protection of the plant from salt stress by M17. To identify candidate genes required for the plant bacteria interaction comparative analysis of the genomes of five *Pseudomonas* species was performed, three Pseudomonas showing plant growth promotion effect: *Pseudomonas fluorescens A506; Pseudomonas putida BIRD-1* .Two not associated with the plant *Halopseudomonas sp. RR6; Pseudomonas mosselii BW11M1* .And a plant pathogen *Pseudomonas syringae pv. phaseolicola 1448A.*

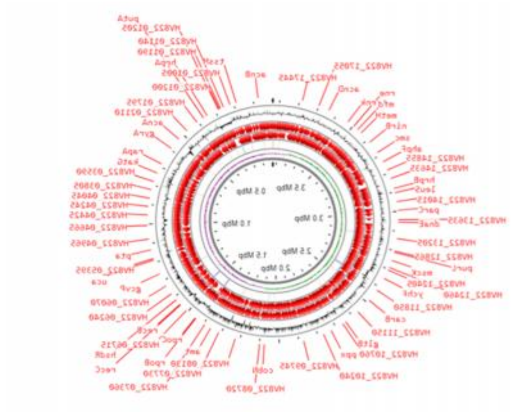
The overview of the genome reveals that the genome of the studied strain shows low seize variability from 5,7 to 6,1 Mpb (Figure), with ***Pseudomonas fluorescens A506; Pseudomonas putida BIRD-1*** showing respectively the lowest and the highest genome size.



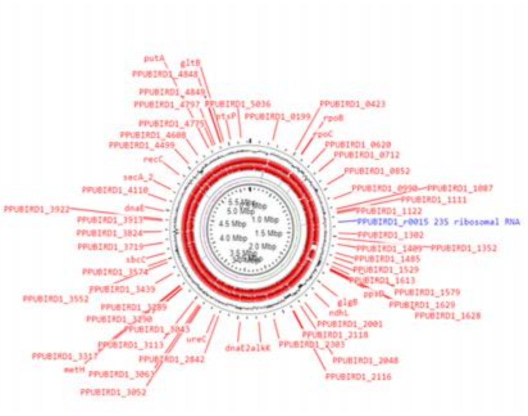
6 Mpb



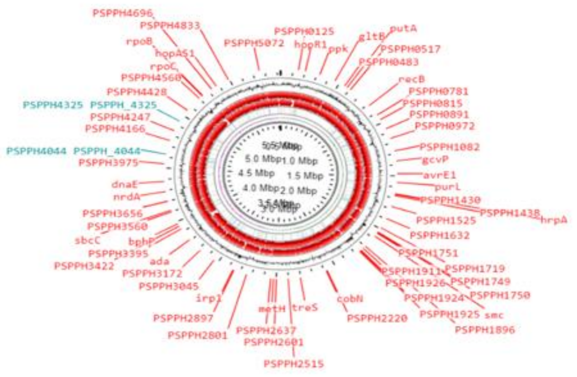
5,4 Mpb



3,9 Mpb



5,7 Mpb



6,1Mpb

Figure 5 :Genomes Pseudomonas strains studied for the genomic comparison. Circular genome viewers of two Pseudomonas showing plant growth promotion effect: Pseudomonas fluorescens A506, Pseudomonas putida BIRD-1 one plant pathogen; Pseudomonas syringae pv. phase

Pseudomonas fluorescens A506 show the highest number of genes with 5688 genes, followed by *Pseudomonas putida BIRD-1, Pseudomonas mosselii BW11M1, Halopseudomonas sp. RR6, Pseudomonas syringae pv. phaseolicola 1448A.* showing respectively 5379 ,5199, 3651, and 5857 genes.

2.2. Selection of strains

We select *Pseudomonas aeruginosa strain PaLo34* wish is M17 based on NCBI data (nucleotide) and found the complete genome of this strain.

2.3. Genomic description of the strains used

The rod-shaped, gram-negative bacteria *Pseudomonas aeruginosa* is well-known for its adaptable metabolism and capacity to flourish in a variety of settings. *Pseudomonas aeruginosa* strain PaLo34 is one in particular that displays possible uses in agriculture, especially in relation to plant growth stimulation. The closest strains from a genomic point of view *Pseudomonas fluorescens; Pseudomonas aeruginosa; Pseudomonas putida. The opportunistic pathogen Pseudomonas aeruginosa* is a common environmental inhabitant, which is also capable of causing both acute and chronic infections in a range of hosts from amoeba and plants to humans

2.4. Genome annotations by RAST

The activities and chemical characteristics of an organism may be highlighted through an examination of the gene composition of its genome. We employed the RAST server (quick annotation using subsystem technology) to acquire quick genome annotation in order to confirm the divergence and conservation of metabolic pathways amongst the various strains. This enables us to verify the genome annotations and get gene distribution based on important metabolic pathways. RAST is a fully automated server that is used to identify bacteria and arches. The service predicts which subsystems are represented in the genome, identifies the genes encoding proteins, both ARNr and ARNt, and assigns functions to the genes (25).

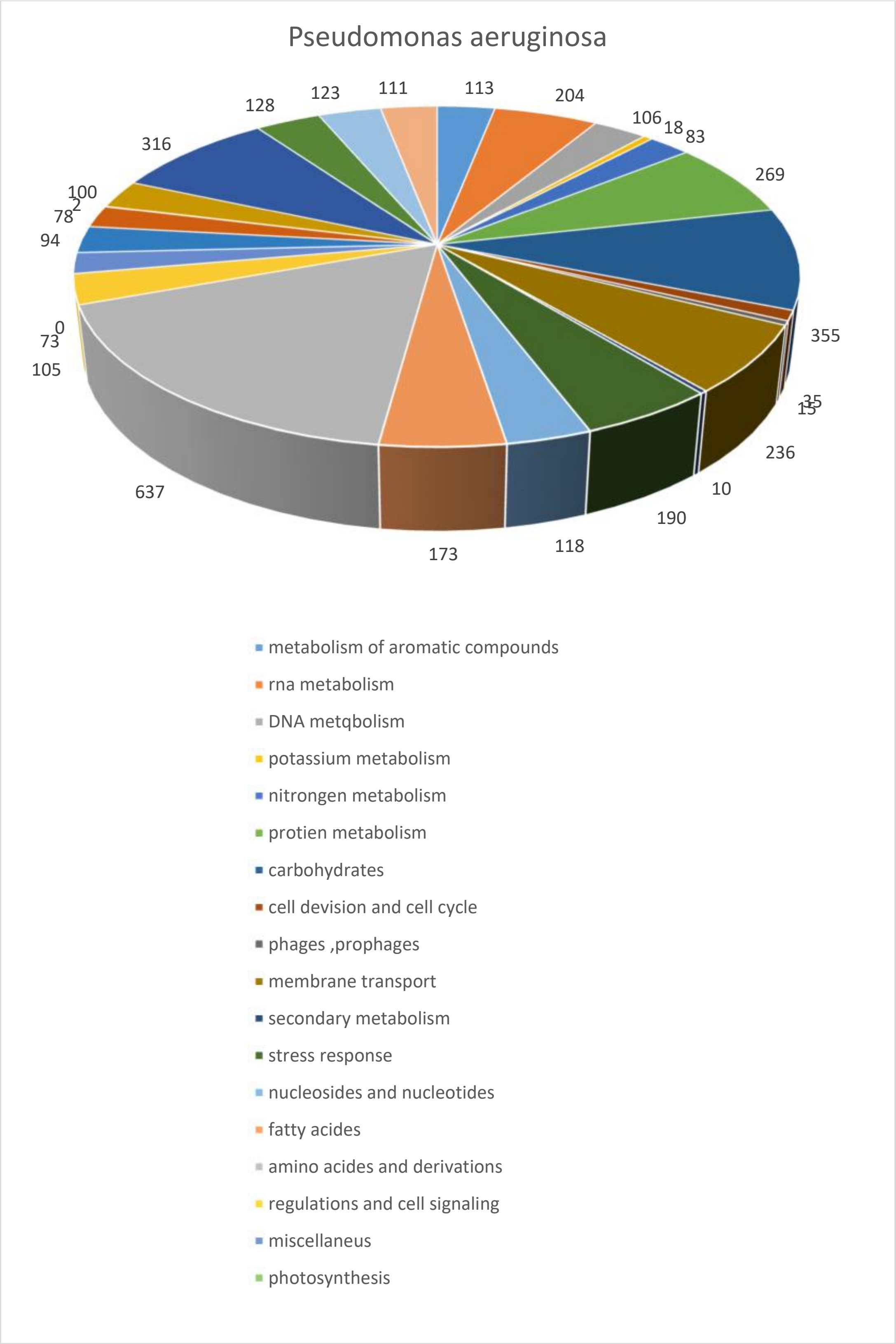


Figure 6: Representation of the percentage of plasmid genes of each metabolic pathway for the different strains studied.

- Since genes are arranged in subsystems based on their biochemical functions, each subsystem represents a metabolic pathway with a proportion of genes. Participants in each function or pathway. The obtained data indicate that, in terms of the number of genes per metabolic pathway. since the results of rast_*Pseudomonas aeruginosa strain PALO34* we have noticed Aromatic amino acids are essential building blocks for proteins and play crucial roles in various metabolic pathways in plants. These aromatic amino acids can be further metabolized into a wide range of secondary metabolites, including flavonoids, alkaloids, lignin, and phytoalexins. Flavonoids are known for their antioxidant properties and their role in plant defense mechanisms against environmental stresses. The biosynthesis of these secondary metabolites from aromatic amino acids is tightly regulated and can be influenced by various stress factors that the plant encounters. Periplasmic stress refers to the stress conditions that affect the periplasmic space of bacterial cells or the extra cytoplasmic space of plant cells. molecular responses aimed at maintaining cellular homeostasis and protecting the plant from damage. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the ability of the plant to detoxify them.

2.5. The server Microscope (Microbial Genome Annotation Analysis Platform)

It makes it possible to calculate the pan and core genome sizes, their evolutions for set of genomes, while determining the set of variants in common between them.

- Strains comparison result, Number of genes in common and different between the strains studied. The result presents the number of common or divergent gene families obtained, a family may contain one or more homologous genes between different strains. (22)
- Plant-associated bacterial communities caught the attention of several investigators, which study the relationships between plants and soil and the potential application of selected bacterial species in crop improvement and protection. Medicago sativa L. is a legume crop of high economic importance as forage in temperate areas and one of the most popular model plants for investigations on the symbiosis. (24)
- Several similar strains in comparison with other similar strains (from a genomic and functional point of view interacted with the plant).

2.6. Comparative genomic analysis reveals high conservation of genes between studied *Pseudomonas* strains

In the aim to determine the Pan/Core genome between *Pseudomonas fluorescens A506, Pseudomonas putida BIRD-1* and *Pseudomonas mosselii BW11M1*, *Halopseudomonas sp. RR6* a comparative genomes analysis was performed on the platform Microscope. (ttps://mage.genoscope.cns.fr/microscope/).

The analysis reveals an important conservation of the gene between the studied strains. *Pseudomonas fluorescens A506, Pseudomonas putida BIRD-1 and Pseudomonas mosselii BW11M1*, *Halopseudomonas sp. RR6* shows respectively 1754 ;3039 ;1510 and 3077 specific genes, by contrast the genome shared between all strain include 521 genes. These results indicate that the core genome remain important compared to the specific genome.

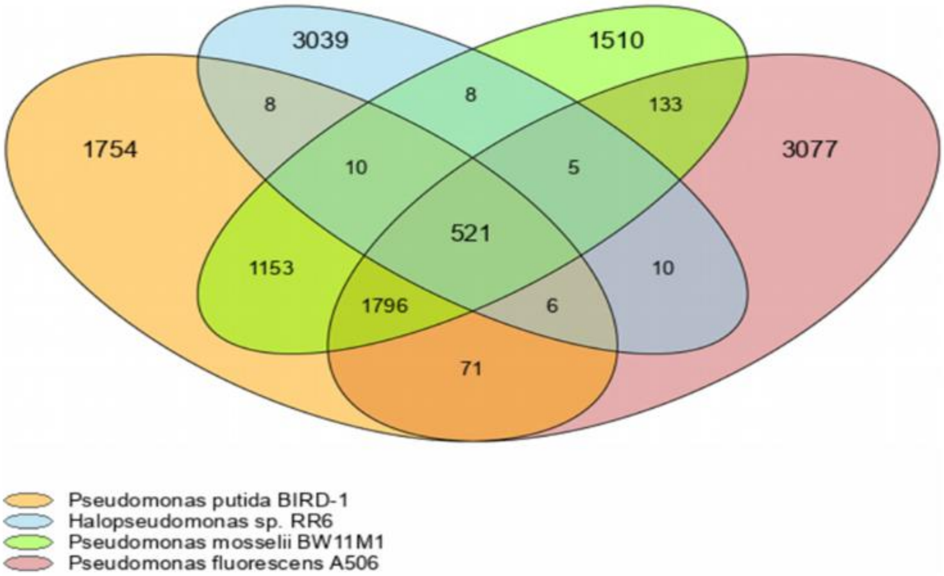


Figure 7: PAN/CORE genome analysis between Pseudomonas strains. Vann diagram show the common (CORE) and specific (PAN) genes between Pseudomonas fluorescens A506, Pseudomonas putida BIRD-1 and Pseudomonas mosselii BW11M1, Halopseudomonas sp. RR6

2.7. Pseudomonas showing PGPR effect display the higher level of genome conservation

To further characterize the genomic conservation between the studied strains, a Synteny analysis was performed using the genome of *Pseudomonas putida BIRD-1* which show the PGPR. The genome of *Pseudomonas fluorescens A506* was compared to the genome of the other strains.

The analysis reveals that *Pseudomonas putida BIRD-1* show the highest level of conservation of the genome sequence and order with *Pseudomonas fluorescens A506; Halopseudomonas sp. RR6* and *Pseudomonas mosselii BW11M1*. contrast low level of conservation is observed for comparisons with *Pseudomonas syringae pv. phaseolicola 1448A* and *Halopseudomonas sp. RR6*

Comparing the PGPR-displaying Pseudomonas species to other Pseudomonas species (plant or human diseases), the results demonstrate that the former has a high degree of genomic conservation.

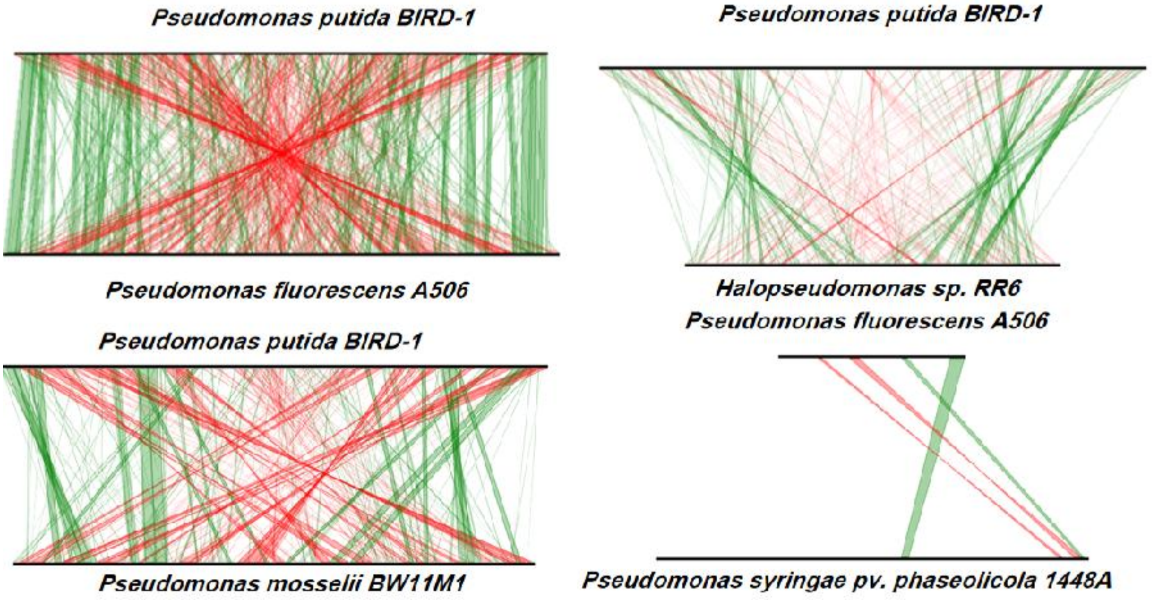


Figure 8: Synteny comparisons between Pseudomonas putida BIRD-1 and the other Pseudomonas species. Comparison of the genome Synteny between Pseudomonas fluorescens A506, and Pseudomonas mosselii BW11M1, Halopseudomonas sp. RR6 and Pseudomonas syringae pv. Phase

2.8. Genes between PGPB are associated with various genes Conserved

The conserved genes between the strains under study and those important to the infection process and bacterial resistance were found in order to identify prospective candidate genes involved in the interaction between Pseudomonas strains and the plants under study.

The table 1 shows the conserved genes associated to bacteria resistance and plant-bacterial infection. We observed a conservation of numerous genes involved in the followed process: resistance to antibiotics, secretion systems and effector involved in the short-circuit of the plant immunity and other enzymes and protein preventing oxidative damage and detoxification of antimicrobial compounds.

The various genes conserved among the strains indicate the recruitment of complexes network by Pseudomonas strain associated to the plants.

The genes discovered in this study provide a foundation for future research on the M17 strain. These identified genes can be used to verify and explore the genetic networks activated during the strain's interactions with Medicago sativa. This validation process will help elucidate the molecular mechanisms underlying the relationships between the M17 strain and these plant species.

Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	2,4-dienoyl-CoA reductase
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	3-oxoacyl-[acyl-carrier-protein] synthase II
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	5,10-methylenetetrahydrofolate reductase
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	ABC transporter ATP-binding protein
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	Acetolactate synthase isozyme 3 large subunit
Pseudomonas fluorescens A506	aconitate hydratase 2

Pseudomonas putida BIRD-1	
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	acyl-CoA dehydrogenase family protein
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	aldehyde dehydrogenase
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	Alpha-L-glutamate ligase-like protein
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	Anthranilate synthase component 1
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	AtpC
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	ATP-dependent protease HslVU, peptidase subunit
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	bifunctional protein HldE
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	branched-chain amino acid ABC transporter, permease protein BraE
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	carbamoyl transferase
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	chaperonin GroL
Pseudomonas fluorescens A506	coenzyme PQQ biosynthesis protein C

Pseudomonas putida BIRD-1	
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	cysteine synthase A
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	deoxyguanosinetriphosphate triphosphohydrolase
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	dihydrodipicolinate reductase
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	endonuclease III
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	fatty oxidation complex subunit alpha
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	Isochorismatase superfamily hydrolase
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	LepA
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	LysR family transcriptional regulator
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	Metallophosphoesterase
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	nucleoside diphosphate kinase
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	PhoH family protein

Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	phosphoribosylaminoimidazole carboxylase catalytic subunit
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	polyribonucleotide nucleotidyltransferase
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	recombination factor protein RarA
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	regulator of ribonuclease activity A
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	ribonuclease T
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	rod shape-determining protein MreB
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	Short chain dehydrogenase
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	TetR family transcriptional regulator
Pseudomonas fluorescens A506 Pseudomonas putida BIRD-1	Valyl-tRNA synthetase

Table 1. Gene conserved between species

Table 1 : Some Genes potentially involved in the plant-microbe interaction and conserved between studied Pseudomonas.

Part 4. Discussion

This study aims to investigate the relationship between Medicago plants and endophytic bacteria. The purpose of this study is to examine how endophytic bacteria shield alfalfa from salt stress and to determine whether or not endophytic bacteria may be used as a long-term solution to increase alfalfa yield in soils impacted by salt, this cultivation method puts the plants under more stress than either *in vitro*. Through gene identification, we want to characterize the processes by which plant Alfalfa respond to salt stress in the presence of endophytic bacteria.

Medicago sativa L (alfalfa) is plant model that we choose to examine in this work about the significance of endophytic bacteria in reducing salt stress. First, we employed four bacteria that are well-known and not known for their capacity to colonize plant roots. When comparing various NACl concentrations to the control, a variable potential for reducing salt stress was seen. Plants may benefit greatly from the protection offered by one endophytic bacterium (M17) against salt stress. These are a part of Pseudomonas (Gram -). Similar results are observed in other studies showing significant promoting growth under 200 mM and 150 Mm of NACL because of the beneficial impacts of the implanted bacteria during the early stages of Medicago sativa seedling development (63). M17 has shown positive favorability in the host plant at varying NACl concentrations. Numerous studies show that the PGPR regulate stress in multiple ways when certain conditions are met.

development of endophyte strain library. Four strains (M17, M50, S113, S116) identification indicates that (M17, M50, S113, S116) are respectively related to *Pseudomonas aeruginosa strain PaLo34*, *Lysinibacillus sp.7B* and S113, S116 are not yet sequenced. As a result, we obtained three beneficial bacteria against salt stress which they are M17; S113 and S116, depending on the result, the figure 5 .M17 is *Pseudomonas aeruginosa strain PaLo34*.

There are magazines that have the same results regarding this study and found it to have a positive impact on beneficial bacteria and according to the following source, it confirms When a key strain of Pseudomonas that is salt and alkali-tolerant colonizes the roots of alfalfa, it can significantly enhance the physiological characteristics and growth of the plant. This colonization by Pseudomonas plays a crucial role in improving the plant’s resistance to salt and alkali stress. The composition and function of endophytic bacterial communities in the roots likely contribute to this enhanced tolerance. Pseudomonas is known for its ability to form beneficial relationships with plants as endophytes, residing within plant tissues without causing harm. In the case of alfalfa, the presence of this specific strain of Pseudomonas on the roots leads to several mechanisms that help the plant cope with salt and alkali stress. (32)

And therefore the strain pseudomonas aeruginosa strain PALO34 it has a growth promoting effect PGPR and in all salinity conditions.

Secondly, in order to be able to delve deeper into the study, to comprehend the manner in which our bacterial strains support Alfalfa in the face of salt stress as well as other related stressors (osmotic, ionic, and oxidative stress). We chose to do out genetic analysis, beginning with genetic comparison within each genus. For M17, we selected more particular genes linked to the observed behavior using two bacteria connected with plants, one that is not, and one human disease. 521common genes were discovered (core-genome). But our goal is to identify the conserved genes that may be linked to fundamental functions in the course of the evolutionary relationships between these soils. Therefore, we conduct Synteny analyses (figure9).

Conclusion

This work has shown that endophytic bacteria are a viable way to reduce salt stress in Medicago alfalfa, an important fodder crop. Our research adds to the increasing amount of data that suggests using plant-microbe interactions to improve crop resilience in difficult settings.

Identification of several endophytic bacterial strains that significantly improved alfalfa growth and productivity under saline conditions. Elucidation of multiple mechanisms by which these endophytes alleviate salt stress, including osmolytes production, antioxidant enzyme activation, and phytohormone modulation.

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