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Field: BIOLOGICAL SCIENCES

Option: BASIC AND APPLIED MICROBIOLOGY

Theme:

Effects of bacterial and fungal endophytes on the growth of the model plants Medicago sativa and Arabidopsis thaliana under salt stress

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Abstract :

This study investigates the role of endophytic microorganisms, two bacterial strains (M17 and M67.2) and two fungal strains (KM405 and DA413) from Algerian soil, in improving salt stress tolerance in *Medicago sativa* and *Arabidopsis thaliana*. Under non-saline conditions, microbial inoculation sometimes reduced root growth, likely due to early competition or the activation of plant defense responses. However, under high salinity, the tested microorganisms significantly enhanced plant growth and stress tolerance. In *Medicago sativa*, bacterial strains M17 and M67.2 mitigated the inhibitory effects of 150 mM NaCl, suggesting the activation of salt-tolerance mechanisms such as ion homeostasis, phytohormone synthesis, and Osmo protectant accumulation. In *Arabidopsis thaliana*, fungal strains DA413 and KM405 substantially improved root development, leaf number, and lateral root formation under 100 mM NaCl, demonstrating their ability to enhance plant performance under severe stress.

Overall, the results highlight the promising potential of endophytic bacteria and fungi as sustainable biological agents capable of enhancing plant resilience to salinity, providing an eco-friendly alternative for agriculture in salt-affected regions.

Keywords: *Medicago sativa*, *Arabidopsis thaliana*, endophytes, salt stress

Résumé

Cette étude évalue le rôle de microorganismes endophytes, deux souches bactériennes (M17 et M67.2) et deux souches fongiques (KM405 et DA413) isolées de sols algériens, dans l'amélioration de la tolérance au stress salin chez *Medicago sativa* et *Arabidopsis thaliana*.

Les tests réalisés sous différentes concentrations de NaCl ont montré que, en conditions non salines, les inoculations bactériennes et fongiques peuvent réduire la croissance racinaire en raison d'une compétition initiale ou d'une activation précoce des défenses de la plante. Toutefois, sous forte salinité, les microorganismes testés ont significativement amélioré la croissance et les paramètres morphologiques des plantes. Chez *Medicago sativa*, les souches M17 et M67.2 ont atténué l'inhibition racinaire à 150 mM NaCl, suggérant une activation de mécanismes de tolérance tels que la régulation ionique, la production de phytohormones et la stimulation d'osmoprotecteurs.

Chez *Arabidopsis thaliana*, les souches fongiques DA413 et KM405 ont permis une meilleure croissance sous 100 mM NaCl, en améliorant la longueur des racines, le nombre de feuilles et de racines secondaires.

L'ensemble des résultats souligne le potentiel des endophytes comme agents biologiques naturels capables d'améliorer la résistance des plantes au stress salin, offrant une alternative durable pour l'agriculture dans les zones affectées par la salinité.

Mots-clés : *Medicago sativa*, *Arabidopsis taliana*, endophytes, stress salin

ملخص :

تهدف هذه الدراسة إلى تقييم دور الكائنات الحية الدقيقة الداخلية في تعزيز قدرة النباتات على تحمل الإجهاد وسلالتين فطريتين معزولتين من التربة الجزائرية. أُجريت التجارب àالملحي، وذلك باستخدام سلالتين بكتيريتين على نباتي الفصفصة ونبات أرابيدوبسيس، حيث أظهرت النتائج أنّ التلقيح الميكروبي قد يُضعف نمو الجذور في غياب الملوحة بسبب المنافسة أو تنشيط الدفاعات النباتية في المرحلة الأولى. ومع ذلك، أظهر التلقيح تأثيراً إيجابياً واضحاً تحت الملوحة العالية.

في نبات الفصفصة، ساهمت السلالات البكتيرية في تخفيف التأثيرات السلبية للملوحة العالية من خلال تحسين النمو وتنشيط آليات التحمّل مثل تنظيم توازن الأيونات وإنتاج الهرمونات النباتية والمركّبات الواقية. أمّا في نبات أرابيدوبسيس، فقد أدّت السلالات الفطرية إلى تحسين نمو الجذور وزيادة عدد الأوراق والجذور الثانوية تحت مستويات مرتفعة من الملوحة، مما يدل على قدرتها على تعزيز مقاومة النبات للإجهاد الملحي.

تؤكد هذه النتائج الدور الواعد للكائنات الدقيقة الداخلية كعوامل حيوية طبيعية يمكن أن تسهم في تحسين نمو النباتات وزيادة إنتاجيتها في البيئات المالحة، مما يجعلها بديلاً مستداماً للممارسات الزراعية التقليدية في المناطق المتضررة من تملّح التربة.

الكلمات المفتاحية: نبات البرسيم الحجازي ، نبات ارابيدوبسيس تاليانا،بكتيريا الداخلية، الإجهاد الملحي

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Dedication

(وَآخِرُ دَعْوَاهُمْ أَنِ الْحَمْدُ لِلَّهِ رَبِّ الْعَالَمِينَ)

اللهم لك الحمد حتى ترضى ولك الحمد إذا رضيت ولك الحمد بعد الرضى.

أولاً وقبل كل شيء، أتقدم بخالص الشكر والامتنان لله تعالى، مصدر القوة والنور والحكمة، الذي منحني الصبر والعزيمة، ومكنني من إتمام هذا العمل المتواضع، ووفقني في كل خطوة إلى النجاح والتوفيق

إلى القلوب التي أحبتني بصدق، إلى العيون التي رأت فيّ الأمل، إلى الأيدي التي رفعتني عند كل عثرة، أهدي هذا العمل المتواضع إلى عائلتي الكريمة التي كانت سندي وعوني طوال رحلتي الدراسية، وبدعمهم اللامحدود وإيمانهم بقدراتي وصلت إلى هذا اليوم المميز

إلى روح والدي الحبيب

كنتَ السند والنور الذي أنار طريقي، وكم تمنّيت أن تشهد يوم تخرّجي غيابك وجع لا يُنسى، لكن عزائي أنك في دارٍ أرحم رحمك الله وجعل مثواك الجنة يا أغلى ما فقدت

إلى نبع المحبة والإيثار والكرم أمي الحبيبة

إلى من وضع المولى - سبحانه وتعالى - الجنة تحت قدميها، ووقَّرها في كتابه العزيز...، إلى ملاكي الطاهر، رمز الحنان والعطاء، أمي الغالية التي كانت لي الأم والأب، والسند الذي لم يخذلني يومًا، إلى صبرك الذي لا يعرف حدودًا، وإلى حنانك الذي كان دوائي في لحظات الضعف، أهديك هذا العمل تقديرًا لعطائك العظيم ودعائك الصادق الذي رافقني في كل مرحلة من رحلتي عرفاناً بجميلك وتضحياتك التي لا تقدر بثمن.

إلى إخوتي

شكرًا لكم على تشجيعكم الدائم، ووقوفكم بجانبي مهما كلف الأمر هذا الإنجاز لنا بقدر ما هو لي

الى أصدقائي

اشكر صديقتي **زينب بن رزاق** الذي رزقني الله بها والتي تعرفت عليها في اصعب ايامي كانت سندي حفظها الله واشكر كل من صديقات طفولتي **امال وخولة ورقية وبشرى ونورة وحنان** الذين شاركوني الأفراح والأحزان، وكانوا خير رفيق في هذه الرحلة الطويلة، أشكركم على تشجيعكم الذي جعل الطريق أسهل وأجمل, كما أتقدم بالشكر الجزيل لمجموعتي الذين رافقونا طيلة فترة العمل ورحلة تخرجي الذين قدّموا لي الدعم، سواءً بالنصيحة أو التشجيع أو المساعدة العملية إلى من جعلوا الطريق أجمل بخطواتهم، والأيام أهون بوجودهم شكرًا لدعمكم، وذكرياتكم، وضحكاتكم فلولاكم، ما كانت الرحلة بهذا الجمال

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شكراً لكم على تعليمكم وإرشادكم ودعمكم المتواصل، فقد كان لجهودكم الأثر الكبير في تطوير مهاراتي وتحقيق هذا الإنجاز.

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Iman

Dedication

Before I say anything, I would like to begin by thanking the One who taught mankind all that it knows. Nothing can truly describe Your greatness, Your mercy, and Your endless grace. Thank You, Allah, for all Your blessings upon me, for the light You have always placed in my darkest times. When the stars faded and the moon disappeared, You were my constant guidance. Oh Allah, You are the Greatest.

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zineb

Summery

- **General Introduction**

Chapter 1: Fundamentals of plant and microbial interactions

Chapter 2: Plants in front of biotic and abiotic stresse

Chapter 3: Endophytic microbes

Part III : Materials and Methods

List of abbreviations :

- **PGPR :** Plant Gowth-Promoting Rhizobacteria
- **AMF :** Arbuscular Mycorrhizal Fungi
- **ROS :** Reactive Oxygen Species .
- **Ncrs :** Nodule-specific Cysteine Rich peptides .
- **Nres :** Non-Rhizobial Endophytes .
- **Nacl :** Chlorure de Sodium .
- **NSCCs :** nonselective cation channels .
- **CNGCs:** yclic nucleotide gated channels .
- **SOD :** Superoxide Dismutase
- **SOS :** Salt Overly Sensitive .
- **SDS :** Sodium dodecyl sulfate
- **ABA :** Acide Abscissique .
- **GA :** Gibbérelline .
- **SA :** Acide salicylique
- **JA :** Jasmonate
- **IAA :** Indole-Acetic Acid
- **HGT :** hosting stress-tolerant genes

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General Introduction

Introduction

Climate change is one of the most significant environmental challenges facing the modern world. It exacerbates natural pressures that threaten global food security and negatively impacts plant growth and the growth of associated microorganisms. Plants are among the most vulnerable organisms to climate fluctuations, given their inability to escape harsh environmental conditions. Their growth and productivity depend on the stability of essential factors such as water availability, temperature, and soil quality.[1] With the accelerating pace of climate change and rising temperatures, evaporation and transpiration rates increase, leading to water loss, soil desiccation, and increased salinity. These factors collectively contribute to soil degradation and reduced crop productivity. Salinity and drought are among the most significant challenges facing agriculture in arid and semi-arid regions, as their effects on plants are similar in that they reduce soil water availability. Salts lower water potential, hindering water absorption by roots and causing physiological stress that negatively impacts plant growth and productivity.[2] [3]. This leads to a decline in the productivity and quality of crops like alfalfa (*Medicago sativa*) (*Arabidopsis thaliana*), weakening their nutritional value and their ability to withstand volatile climatic conditions .Faced with these challenges, meeting the growing global demand for food is becoming increasingly difficult, especially with the world's population projected to reach nearly 10 billion by 2050. This reality necessitates the adoption of sustainable and innovative agricultural practices and strategies that enhance plants' ability to adapt to environmental stresses, while simultaneously ensuring the continuity of agricultural production in a balanced and efficient manner that guarantees future food security.[4], [5]

Both alfalfa (*Medicago sativa*) and *Arabidopsis thaliana* are considered essential plant models used by researchers to understand the mechanisms of plant tolerance to environmental stresses, including salinity and drought. Alfalfa cultivation is highly susceptible to a range of environmental factors, including biotic and abiotic stressors, which are responsible for reduced crop yields.[6]

Legume plants, members of the *Fabaceae* family, include a wide variety of species such as beans, lentils, peas, chickpeas, soybeans, and alfalfa (*Medicago sativa*). These plants are cultivated worldwide because they are nutritionally rich, economically affordable, and environmentally sustainable [21]. Legumes are considered the second most important food source after cereals and play a crucial role in both human diets and animal feeding systems [22].

For humans, legumes are an essential source of plant-based protein, usually containing between 20–40% protein, depending on the species [22][23]This high protein content makes legumes an important alternative to meat, especially in regions where animal protein is expensive or limited. Legume proteins are rich in lysine, an amino acid that is often lacking in cereals, which is why combining cereals with legumes improves the overall protein quality of the diet[24]. In addition to proteins, legumes are also an excellent source of complex carbohydrates**,** dietary fiber, and resistant starch. These components help improve digestive health, stabilize blood sugar levels, and promote a longer feeling of fullness, which supports weight management and reduces the risk of type 2 diabetes [25], [26]

Legumes are also rich in micronutrients such as iron, magnesium, potassium, calcium, zinc, and folate. For example, iron is essential for the formation of hemoglobin, magnesium plays a role in muscle and nerve function, and folate is crucial for DNA synthesis and fetal development [27], [28]. In many low-income regions, legumes help prevent nutrient deficiencies, particularly iron-deficiency anemia and folate deficiency. Furthermore, legumes are low in saturated fat and contain mostly unsaturated fatty acids, which are beneficial for cardiovascular health **.**[28]

Several studies have shown that regular legume consumption is associated with numerous health benefits. These include a reduction in cardiovascular disease risk, better blood lipid profiles, lower blood pressure, improved glycemic control, and lower rates of obesity[29], [30]. Legumes also contain bioactive compounds such as polyphenols, flavonoids, tannins, and saponins, which possess antioxidant and anti-inflammatory properties [41]. These compounds help protect cells from oxidative damage and may lower the risk of chronic diseases like cancer and metabolic syndrome.For animals, legumes are equally valuable. Forage legumes like alfalfa, clover, and vetch are widely used in livestock production because of their high nutritional value**.** Alfalfa, in particular, is known as the “queen of forages” because it contains high levels of protein, vitamins (especially vitamin A and E), and minerals**,** as well as excellent digestibility [31]. These qualities make it an ideal feed for dairy cattle, sheep, goats, and horses, improving milk yield, growth rates, and reproductive performance. Legume seeds are also used in poultry and aquaculture feeds because they improve feed conversion efficiency and support healthy growth [140].

In addition to their direct nutritional benefits, legumes contribute to sustainable animal production through biological nitrogen fixation. Their root nodules host symbiotic bacteria (such as *Rhizobium* species) that fix atmospheric nitrogen into forms plants can use, enriching the soil with nitrogen and reducing the need for synthetic fertilizers .This not only improves pasture quality but also lowers production costs and environmental impacts[27].

Alfalfa (*Medicago sativa* L.) is a perennial legume used for forage and belongs to the subfamily Papilionoideae. Cultivated alfalfa is cross-pollinated and tetraploid. It is believed to be native to southwest Asia, with Iran being its main geographic center of distribution. In Europe and some other countries, it is also known as Lucerne.[6] Alfalfa, nicknamed the "queen of forages," is a perennial legume cultivated worldwide that is known for its high productivity, nutritional value, environmental resilience, delicious taste, and nitrogen fixation.. [7] Alfalfa possesses a unique ability to adapt to adverse environmental conditions, such as drought, salinity, and fluctuating high and low temperatures [8]. It is also a perennial plant capable of continuous growth and regeneration after mowing. It also enters a dormant phase in the fall, allowing it to regrow multiple times during a single season, making it a highly valuable crop in terms of yield and productivity. [9], [10]

However, alfalfa, like other crops, faces increasing challenges due to the combined effects of global climate change and human activities.[11] These challenges include biotic stresses, and abiotic stresses, these factors pose a direct threat to alfalfa growth, productivity, and quality, negatively impacting its role in agricultural production and various applications [12] Plants adapt to environmental stresses through physiological, chemical, and molecular mechanisms that ensure their growth and survival. In the case of alfalfa, the response begins with the perception of environmental cues, followed by the activation of cellular signaling networks, and finally modulation of gene expression and physiological and biochemical changes. Several key genes play important roles in these processes..[13] Available research findings indicate that water stress may alter the concentrations of feed nutrient components such as crude protein (CP), water-soluble carbohydrates (WSC), and fiber (NDF and ADF), to varying degrees depending on the response pattern and plant species.[14]

Arabidopsis thaliana, or mouse cress, is a small plant in the Brassicaceae family [15], It was adopted as a research model in the 1980s due to its characteristics that made it ideal for studying plant physiology and genetics. Thanks to its small size, simple five-chromosome genome, short life cycle, high seed yield, ease of cultivation and manipulation in laboratories, and potential for genetic modification, this plant became the primary reference model in plant research during the genome era.[16], [17]

This small annual herbaceous plant from the mustard family facilitated gene identification and fostered collaboration among laboratories, thus accelerating scientific progress. Its importance lies in the possibility of applying the knowledge gained from it to crop plants to improve genetic traits. Its five-chromosome genome was the first plant genome to be sequenced in 2000 making it ideal for studying genetic and physiological processes in plants. [16]

Endophytes (bacteria and fungi) are among the most prominent modern solutions for achieving sustainable agriculture, offering safe and environmentally friendly biological alternatives that eliminate the excessive reliance on chemical fertilizers and pesticides[18]. The symbiotic relationship between legumes and bacteria is a prime example of this sustainable approach. Research has shown that root nodules not only contain rhizobia bacteria but also other types of non-rhizobia bacteria (NRBs) capable of secreting growth hormones and enzymes such as ACC deaminase, which stimulate plant growth and mitigate the negative effects of ethylene. Furthermore, these bacteria contribute to the solubility and absorption of essential mineral elements by the plant. Horizontal gene transfer (HGT) also plays a crucial role in the exchange of beneficial traits between bacterial strains, enhancing their adaptability to different environments. Experiments have demonstrated that double inoculation between rhizobia and NRBs leads to improved legume growth and increased yields, making it a promising approach for reducing the use of chemical fertilizers and promoting agricultural sustainability..[19], [20]

This study investigates the growth-promoting potential of two bacterial and fungi isolated from Algerian soil on Medicago sativa plants, by exploring the following aspects under different salinity conditions:

- The effect of the fungi on the length of the main root, the number of secondary roots and leaves, and the fresh weight of the plant.

Parte I

Bibliographic Synthesis

Parte I. Bibliographic Synthesis

Chapter I. Fundamentals of plant and microbial interactions

1. Plant interacting microbes :

The soil is rich in numerous microorganisms .and the biodiversity of soil microbes is fundamental for supporting life on earth .the interactions plant-associated microorganisms play crucial role in terrestrial ecosystems, particularly plant growth, development and function. furthermore an increase in nutrition, stress resistance and pathogenicity in plants.[32] this interaction can happened by several ways including beneficial , pathogenic or commensal .In these interactions, plants provide sheltered habitats for the microorganisms that can colonize their surfaces and the surrounding rhizosphere soil. [33]Roots harbor a high density of microorganisms. The association between plant root and microorganisms improves growth and plant productivity [34] . Depending on their location, microorganisms can be classified as epiphytic or endophytic. Epiphytic microbes colonize the external surfaces of the aerial parts of the plant, while endophytic microbes reside within the internal tissues, including both aerial and root tissues.[35].the assembly of plant microbiome is depends on plants emission signaling molecules . (sugars , amino acids, organic acids ,phenolics flavonoids , proteins, etc.) known as rhizodeposits Microorganisms respond by colonizing different plant parts, both epiphytically and endophytically.

These compounds widely attract beneficial bacteria that support nutrient metabolism and disease control .(For example, *Pseudomonas spp.* can reduce the growth of plant pathogens through competition and antibiosis) via simple mechanisms that enhance the efficacy of disease-suppressive soils . include the production antimicrobial metabolite and volatile in antagonistic bacteria . [36]

2. Bacteria associated with plants :

bacteria are the most prevalent microbial in plant are generally recruited from the surrounding root zone (rhizosphere).bacteria -plant interact occur in various ways . in mutualistic interaction. beneficial, harmful, or neutral[139] . where the plant benefit from bacterial production of phytohormonest and antibiotics. nutrient uptake . controlling phytpathogenic. enhance stress resistance .while the plant provides safe shelter and nutrient for bacteria [35]

3. Plant response to pathogense :

Root exudates attract beneficial bacteria but can invite pathogens inadvertently [37]Pathogens diffuse to plants by various modes such as water, air, and transmission by insects, animals, and humans. The strategies they applicate to infect plants are: reprogram host physiology, involve immune suppression,and the secretion of toxins and degradative enzymes that help nutrient release and favor pathogen colonization .[38], [39]Plant defense response is activated when pathogens are detected, using a multi-layered immune system. It responds by cell wall strengthening, production of reactive oxygen species (ROS), antimicrobial compounds, and callose deposition [40] In addition, phytopathogens exhibit host specificity via different plant species, which is determined by multiple factors within the plant–pathogen interaction system [41]

4. Plant grwoth promoting Rhizobacteria Plant :

Growth-Promoting Rhizobacteria (PGPR) are a diverse group of beneficial bacteria that colonize plant roots (rhizosphere)[42].they have the capacity of stimulating plant growth by several mechanisms .including the production of antibiotic, siderophore, hydrolytic enzymes[43], high uptake of minerals and growth-promoting metabolites such as vitamins and phytohormones sush as indole-acetic acid (IAA) that have capacity to formation of pigments and biosynthesis of metabolites, controls responses to gravity, light, and fluorescence, affects photosynthesis and resistance to extreme conditions which is boost longer roots , increase root biomass and decrease stomata size and density .In addition PGPR are beneficial in enhancing plant tolerance to drought and salinity pathogens on acting as biocontrol agent [37], [44]

5. Molecular mechanisms underlying the regulation of plant microbe interactions the :

rhizosphere are the hot spot for microbial colonization . plants can efficiently communicate they're microbes by signals plant roots exudates compound [139] Plants detect microbes via cell surface receptors by recongnize molecular signals of microbes .the receptors activates intracellular signaling pathways. for activating Defense gene expression, or symbiosis Genes for nodule formation or arbuscule development this depending microbes are benfical or pathgenic [45], [46].

6. symbiotic associations :

Leguminous plants form a symbiotic relationship with rhizobia bacteria.[47] resulting in the formation of root nodules capable of fixing atmospheric nitrogen and converting it to ammonia thanks to the enzyme nitrogenase and makes it available to the plant [48]. This process begins when the plant secretes flavonoids, which stimulate the bacteria to produce Nod factors. This exchange of signals between the two parties stimulates the division of root cortex cells and the formation of nodules [49] Rhizobia enter through infection threads in root hairs or through intercellular spaces. Within the nodules, they transform into specialized bacteroids surrounded by a plant membrane. Several genes are involved in this process, such as nod, nol, and noe, responsible for nod formation, and nif and fix, which are involved in nitrogen fixation. [50] Surface sugars and bacterial secretion systems also help evade plant defenses, while NCR peptides contribute to bacterial differentiation and the stability of the symbiotic relationship. [51]

The symbiotic relationship between rhizobia and legumes is based on the exchange of complex molecular signals that lead to the production of diverse hormones and metabolites, such as auxins, cytokinins , Nod factors, and siderophores. These compounds contribute to improving nutrient uptake, reducing the toxic effects of ethylene, and enhancing plant resistance to environmental stresses and diseases.[52]

The symbiosis of rhizobia with legumes contributes to enhancing plant growth and reducing the need for fertilizers. However, its results may vary, ranging from improving the diet of herbivores to strengthening plant defenses. Its impact also extends to natural enemies through defensive mechanisms or via nectar outside the flower.[53]

7 . **Symbiotic nitrogen fixation :**

Nitrogen is an essential element for plant growth and development, as it participates in the formation of proteins, nucleic acids, phospholipids, chlorophyll, hormones, vitamins, and alkaloid.[54] When nitrogen levels in the soil are low, plants show signs of poor growth, reduced photosynthesis, and accelerated leaf aging, forcing the plant to break down its proteins for reuse. This element also plays an important role in enhancing the plant's ability to withstand drought and regulating its growth by activating certain enzymes and hormones. It also contributes to microbial symbiosis, which supports its biological efficiency.[55]

Legumes form an endosymbiotic relationship with soil bacteria known as rhizobia.[47] It results in the formation of specialized root nodules that fix atmospheric nitrogen and convert it into a form available to the plant by the enzyme nitrogenase. This process begins when the plant secretes flavonoids that act as signals that stimulate the bacteria to produce lipopolysaccharides called nodulation factors, leading to the formation of root nodules that fix atmospheric nitrogen as ammonia.[48][56]. Nitrogenase consumes a large amount of energy to produce ammonia according to the following equation:

$$N_2 + 8\,e^- + 8\,H^+ + 16\,MgATP \rightarrow 2\,NH_3 + H_2 + 16\,MgADP + 16P_i$$

This gives plants the ability to grow in nitrogen-poor soils. Mature bacteroids fix atmospheric nitrogen using the enzyme nitrogenase.[57]

Leghemoglobin protects nitrogenase from oxygen damage by maintaining low oxygen levels inside root nodules. The pink color of mature nodules is due to the presence of this protein[57] Mature root nodules turn pink due to the presence of leghemoglobin, an indicator of efficient nitrogen fixation, while white indicates weak or failed symbiotic interaction.[58]

During nodular aging, the nitrogen-fixing zone turns from pink to green due to the oxidation of the heme group in leghemoglobin (LHb). The decrease in LHb content is a direct indicator of advanced aging and declining nitrogen-fixing efficiency.[59]

8 . Non- rhizobial endophytes (NREs) :

The legume-rhizobia symbiosis is one of the oldest and most well-researched models of plant-microbe interactions, playing a key role in nitrogen fixation and promoting plant growth. However, advances in molecular microbiology and next-generation sequencing techniques have revealed that root nodules contain not only rhizobia but also non-rhizobial endophytes (NREs). This discovery underscores the complexity of the microbial architecture of root nodules and highlights the importance of the triadic interactions between legumes, rhizobia, and NREs[60].[61]

Non-rhizobial endophytes (NREs) colonize root nodules through various mechanisms, most notably by entering with rhizobia via infection threads or infiltrating through natural wounds in the root caused by lateral root growth. This process is believed to not occur randomly, but rather is controlled by the host plant through specific molecular signals and bacterial surface components such as exopolysaccharides, in addition to the role of certain plant genes such as Cyclopes, Cerberus, and Nep1.

Metagenomic studies have also shown that nodules contain a wide microbial diversity, including bacteria such as Bacillus, Pseudomonas, and Enterobacter. Although they do not fix nitrogen, these bacteria contribute to enhanced plant growth and nutrient uptake.[62]

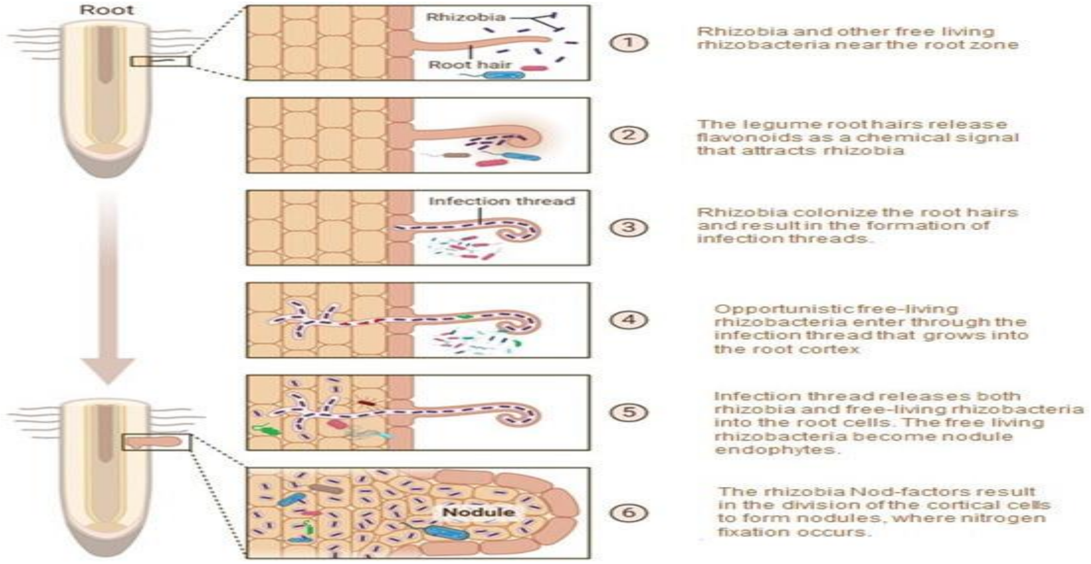


Figure 1: Mechanisms of entry of non-rhizobial endophytes into root nodules and their association with the nodule microbiome, which includes both symbiotic rhizobia and rhizobacteria [62].

9. Epiphytes : Phyllobacteria include all bacteria associated with leaves, whether on their surfaces or within their tissues. Epiphytes rely on two survival strategies: tolerating harsh conditions and migrating to protected areas within the plant. Epiphytes and endophytes represent two complementary forms of bacterial symbiosis. Studies have shown that epiphytes and endophytes represent two complementary forms of bacterial symbiosis, as these organisms can colonize both the outer and inner surfaces of leaves, giving some of them the potential to cause plant diseases [63]

10. Fungal Epiphytes : Epiphytes include fungi, bacteria, yeasts, and algae, Epiphytes mostly belong to the phylum Ascomycota and perform a variety of functions; they may be plant pathogens, act as natural antidotes to pathogenic fungi, or contribute to stimulating plant growth and improving plant health.[64]

11. Epiphytic bacteria : are microorganisms that live on the surfaces of various plant parts, such as roots, stems, leaves, and fruits, without causing any harm. They contribute to enhancing plant growth and increasing resistance to biotic stresses by producing secondary compounds with antimicrobial and antipathogenic activity. These compounds also have diverse therapeutic and preventive properties.[65]

which contribute to stimulating plant growth and pathogen resistance through the production of antimicrobial compounds. Epiphytes also secrete plant hormones, (auxins, cytokinins, gibberellins), solubilization of nutrients such as phosphorus, potassium, and zinc, and fixation of atmospheric nitrogen., making them promising candidates for the development of natural biopesticides that support sustainable agriculture. .[66]

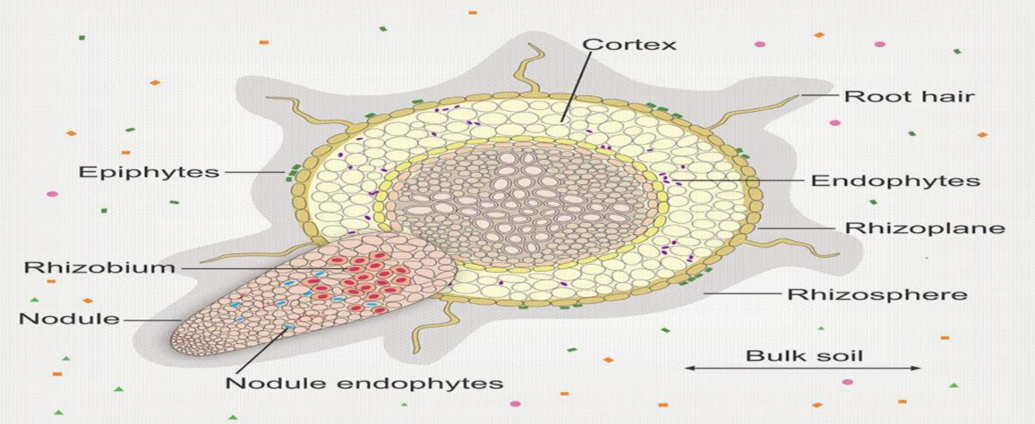


Figure 2. The complexity of bacterial root microbiota [67]

Chapter 2: Plants in front of biotic and abiotic stress

Plants, as living organisms, are exposed to a wide range of harmful environmental stresses, both biotic and abiotic, which can hinder various stages of their development, from germination to seed formation (14)that are clearly influenced by environmental stresses. These stresses fall into two main categories: .[69], [70]

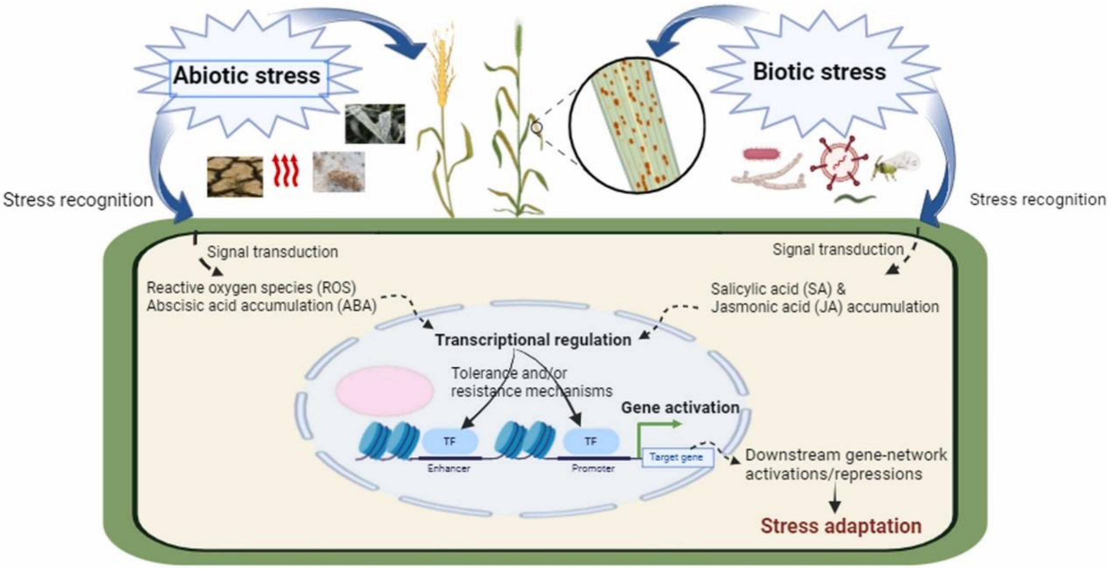


Figure 3 : abiotic and biotic stress in plants [71]

1. Plants in biotic stress :

Biotic stress is one of the most significant challenges facing plants. It results from harmful microorganisms and living agents such as bacteria, fungi, viruses, weeds, insects, and nematodes. These factors negatively impact plant physiological and molecular processes and lead to increased production of reactive oxygen species, resulting in reduced crop yields worldwide. .[72] Therefore, controlling plant pathogens and pests is a commercially important issue and a major focus of scientific research.[73]

2. Plants in abiotic stress :

Abiotic stresses, such as drought, salinity, extreme temperatures, nutrient deficiencies, and environmental pollution, negatively affect plant growth, productivity, [74] These factors affect plant physiological processes, impair nutrient uptake, cause cell damage, and reduce photosynthesis, leading to reduced yields. [75]. Abiotic factors such as soil type, pH, structure, moisture content, organic matter content, root exudates, and salinity play an important role in shaping plant microbiomes. Different environmental conditions, along with pathogens, human interventions, and climate change, also affect both epiphytes and endophytes. Abiotic stress is a natural factor that directly impacts living organisms, sometimes helping them adapt and survive in harsh conditions by promoting a more complex and stable environment.[76] Also, just one stressor can cause multiple forms of stress; for example, salt stress can lead to three types of stress: osmotic stress, ionic stress, and secondary stress such as oxidative stress.[77]

3. The most important types of abiotic stresses that affect alfalfa plants:

3.1 Effect of drought stress on alfalfa :

Water scarcity is one of the most significant abiotic factors negatively impacting crop productivity. Drought causes physiological and cellular disturbances such as membrane shrinkage, cell damage, and decreased nutrient uptake efficiency. It also inhibits photosynthesis due to stomatal closure, chlorophyll damage, and the accumulation of reactive oxygen species (ROS), exacerbating oxidative stress. [78] Plants adapt to drought through osmotic adjustment and increased root growth, while the severity of drought, soil characteristics, and plant type lead to leaf wilting, leaf fall, senescence, and sometimes death of branches or the entire plant, while excess water causes leaf rot and fungal spread. [79]inhibits photosynthesis through reduced cytokinin levels and increased abscisic acid accumulation, ultimately leading to stomatal closure. Although this defense mechanism reduces water loss, it also hinders gaseous exchange (CO_2 and O_2) and limits transpiration, increasing the accumulation of highly harmful reactive oxygen species such as superoxide dismutase and hydrogen peroxide.[80] Drought affects alfalfa morphologically, physiologically, and molecularly. Morphologically, it reduces root length, biomass, and leaf area, and alters the shape and number of root shoots [81]. Physiologically, it reduces gas exchange efficiency, lowers chlorophyll (Chl a and Chl b) content and mineral concentrations [82], and causes the accumulation of reactive oxygen species (ROS), which impede growth and metabolism. Plants respond by increasing the activity of antioxidant enzymes such as SOD, POD, and CAT. Molecularly, genes such as UGTs and MsTERT play a role in regulating metabolic pathways, reducing toxicity, and increasing the production of stress-resistant secondary compounds. Studies also show significant changes in root morphology, microbial communities, and biomass compared to well-irrigated plants. [83]

3.2 Effect of Salinity stress on alfalfa :

Salt stress reduces water uptake, inhibits stomatal opening, and disrupts ion balance, impairing photosynthesis and negatively affecting plant growth. [84]Salt accumulation in the soil causes osmotic stress [85], reducing water availability to the plant, negatively impacting root and surface tissue growth, and accelerating leaf aging and flowering. .[86]. The effect of salt on the plant occurs in two stages: first, osmotic stress, then the direct toxic effects of ions such as sodium and chloride. Seedlings are more sensitive to these effects, which reduces their growth and productivity. .[87]Plants respond to salinity by sensing changes in salt and extracellular osmotic pressure, resulting in increased sodium in the apoplast, a rapid rise in cytoplasmic calcium, ROS accumulation, and cGMP production. Sodium enters through NSCCs and CNGCs, while proteins such as GIPC, BON, and OSCA1 contribute to osmotic sensing and primary calcium signaling. Calcium signaling coordinates with ROS to regulate ionic balance and gene expression, and cGMP promotes potassium uptake and sodium inhibition. Signaling peptides and their receptors (RLKs and FER) also coordinate the cellular response and cell wall protection under salt stress.[88]

3.3 Effect of Temperature stress on alfalfa :

Global warming raises temperatures, causing abiotic stress that severely impacts photosynthesis in plants, particularly photosystem II (PSII) and the more sensitive enzyme Rubisco compared to photosystem I (PSI). This stress leads to increased production of ROS, heat shock proteins, and secondary metabolites. [89]

This stress impairs the plant's vital functions, including photosynthesis, carbohydrate metabolism, protein synthesis, antioxidant defense mechanisms, and hormone regulation.[90] Chloroplasts play a key role in photosynthesis and are responsible for sensing high temperatures and regulating appropriate plant responses. When exposed to heat stress, several essential processes, such as photophosphorylation, the Calvin cycle, and electron transport, are affected. Chloroplast components are also damaged, leading to reduced photosynthetic efficiency, redox imbalance, and, in severe cases, cell death.[91]

3.4 Effect of Heavy metal stress on alfalfa :

Heavy metals accumulate in soil as a result of industrial and agricultural activities.[92]Soil contamination with heavy metals poses a significant risk to plants, human health, and food production, as it also affects soil properties, microorganisms, and the environment. [92]Among the most dangerous heavy metals arearsenic (As), copper (Cu), cadmium (Cd), chromium (Cr), lead (Pb), mercury (Hg), nickel (Ni), and zinc (Zn).[93] Toxic metals disrupt plant physiological processes, including damage to cell membranes, increased oxidative damage, impaired nutrient uptake, decreased photosynthetic activity, and altered plant morphology and function [94] .The environment contains these metals to varying degrees, including soil, food, water, and even the air. These substances harm plants and alter soil properties, reducing crop yields.[95]Plant growth and production depend on the availability of essential minerals such as magnesium, copper, manganese, zinc, iron, calcium, molybdenum, and nickel[96]. These minerals are involved in vital functions such as pigment synthesis, photosynthesis, respiration, and enzyme activation. However, concentrations above the optimal level can cause toxicity that hinders growth and reproduction, while low levels can lead to clear deficiency symptoms that reflect disruption of plant processes.[97]

4. Plant response to salt stress

Plants possess cross-tolerance, where exposure to one type of stress increases their resistance to another.[98] Because plants are fixed in place, they are forced to develop effective adaptive mechanisms to deal with highly saline environments. In response to salt stress signals, plants adopt a variety of strategies [99] , including :

4.1 **Production of osmolytes :**Osmotic stress reduces water uptake and leads to stomata closure,.[100] Proline contributes to plants' ability to tolerate environmental stresses through osmotic adjustments and is produced through the glutamate and ornithine[101] Under drought, proline and soluble sugars rise due to increased P5CS activity and reduced proline oxidase, enhancing water retention, sustaining vital processes, and reducing stress damage.[102]Proline treatment also increases chlorophyll content in leaves under stress conditions, improving photosynthetic efficiency and enhancing plant tolerance. Proline is transported between cells by

specialized transporters, such as AAAP/PA (ProT1, ProT2, AAP6) and Prots in Arabidopsis thaliana, to facilitate its distribution to various plant tissues.[100] [103]
Glycine betaine (GB) can be synthesized endogenously in chloroplasts, cytoplasm, and peroxisomes via the choline metabolism pathway.[104]In addition, glycine betaine (GB) enhances plant stress tolerance as an osmotic buffer, interacting with hormones and cellular signaling pathways, reducing oxidative stress (ROS), protecting membranes, and supporting photosynthesis [105].

4.2 Ion homeostasis : Plants adapt to salinity by regulating the uptake and transport of sodium and potassium to maintain ionic balance . In barley , the transporter HvHKT2;1 transports Na^+ and K^+ ions , and its activity increases when potassium concentrations are low or sodium concentrations are high, promoting sodium uptake and movement into the leaves . [106] During salt stress , plants activate Na^+/H^+ antitransporters to store sodium within vacuoles , under the regulation of the calcium signaling-dependent SOS pathway. The transporters AKT1, TPK1, and HKT1 also contribute to maintaining Na^+/K^+ balance , while aquaporins and circadian clock proteins help regulate water uptake and ion transprt .

Dysregulation of these systems increases plant sensitivity to salt stress. [107]
Melatonin (MT) and calcium (Ca^{2+}) contribute to enhancing plant resistance to salt stresss . Melatonin (MT) regulates vital plant processes such as stomatal opening, seed germination , delayed senescence , defense genes , and hormonal signaling , while calcium (Ca^{2+}) activates physiological signaling pathways that aid in adapting to salinity, suggesting a complementary interaction between them in improving plant tolerance to stress[108]

4.3 ROS detoxification : Reactive oxygen species (ROS) are part of the signaling process in higher plants at low concentrations , but when they accumulate at high concentrations, they cause oxidative stresss. [109] Plants have a comprehensive system of enzymatic and non-enzymatic antioxidants that work directly and indirectly to maintain redox balance, eliminate reactive oxygen species (ROS) , and regenerate antioxidants, thereby protecting cell membranes and organelles under environmental stress .[110]

Most antioxidants are concentrated in the cytosol , chloroplasts, and mitochondria, whereas their oxidized forms tend to accumulate in vacuoles and cell membranes , which have limited efficiency in redox recycling .[109], [111]Recent research suggests that DUF proteins, such as At DUF569 in Arabidopsis , play an important role in the plant's response to oxidative and nitro-oxidative stress and enhance its primarydefense .[112]

4.4 Phytohormones regulation : Plant hormones such as Abscisic acid (ABA), gibberellins (GAs), auxin, cytokinins, salicylic acid (SA), jasmonic acid (JA)ABA, JA, SSA , ethylene, auxin, cytokinins and gibberellins and also play a key role in improving plants' ability to adapt to water and salt stress control plant responses to stress through complex networks of interactions. These pathways integrate defense and drought signals and regulate gene expression and stomatal behavior, helping the plant balance growth and adaptation[89], [113]

4.5 Activation of SOS signaling pathway : The SOS pathway is a major mechanism for salinity tolerance in plants[114]. The proteins SOS3 and SCaBP8 sense calcium signals and activat the kinase SOS2 , which in turn activates Na^+/H^+ transporters such as SOS1 in the plasma membrane and regulates the activity of similar transporters in vacuoles. SCaBP8's membrane binding differs from SOS3 and is phosphorylated by SOS2 and PKS5, enhancing its stability and function and suggesting a role in salinity and alkalinity tolerance. SOS2 interacts with SOS3 and SCaBP8 via the FISL catalyst at the carboxy-terminus, with amino-terminal input also contributing, reflecting a complex regulatory mechanism that enhances stress[115], [116]

Chapter III. Endophytic microbes

1. Introduction to endophytic microbes :

Plants live in complex environments where they interact closely with their surrounding microbes, forming an integrated adaptive unit known as the holobiont.[117] Among these microbes, endophytes stand out: microorganisms capable of colonizing plant tissues and residing in plant roots without causing any apparent harm to the host plant[118]. [119].These organisms are widespread in various plant species and can be found in leaves, stems, roots, and even seeds. Bacteria and fungi are the most common [120] performing vital roles such as promoting plant growth and performance, fixing nitrogen, and contributing to disease protection, in addition to their role as biological control agents by naturally protecting plants from pests. They also contribute to increasing the plant's ability to withstand various biotic and abiotic stresses. Based on their transmission mechanism, [121] endophytes are classified into two main categories: ordinary endophytes, which are inherited through seeds and establish a stable relationship with the plant; and irregular, which colonize tissues temporarily and are not transmitted vertically across generations [122]

2.<u>Endophytes:</u> Endophytes are aboundantly presence in root nodules . and help protecting plants from harmful conditions of the environment such as heavy metal presence and drought. produce biocontrol agents, antimicrobial agents, increase nutrient uptake, bioactive metabolites like produce hormones stimulate (PGPR)[120],[123]The mechanisms by which endophytic microorganisms contribute to promoting plant growth and increasing their resistance can be classified into two main types: direct and indirect mechanisms. Direct mechanisms rely on the secretion of active compounds by the endophyte, such as antibiotics, lytic enzymes, plant hormones, and indole compounds, in addition to their ability to compete with pathogenic organisms within the plant environment. Indirect mechanisms, on the other hand, involve the plant's response to the presence of these microorganisms by stimulating induced systemic resistance (ISR) and acquired systemic resistance (SAR), and activating the production of secondary metabolites that contribute to strengthening its natural defenses and improving its tolerance to various environmental stresses.[124]

3.Endophytic fungi : Endophytic fungi are a diverse class of heterotrophic fungi that colonize plant tissues without causing significant damage. These fungi typically remain dormant after colonizing a host, becoming active only when favorable environmental conditions are present or the plant reaches a specific growth stage. At this point, they begin their metabolic processes and contribute to multiple functions within the host.[125]

4.Mycorrhizal symbiosis : Mycorrhizal fungi are fungi that live in a symbiotic relationship with plants, obtaining carbon from photosynthesis and, in return, providing them with water and essential nutrients, especially phosphorus and nitrogen. This symbiosis helps plants grow better, strengthens their resistance to environmental and biotic stresses, improves soil fertility, and enables them to communicate with each other through fungal networks. Arbuscular mycorrhizal fungi (AMFs) are the most common, as they can penetrate roots and form specialized structures for nutrient exchange. The success of this association depends on chemical signals and hormones, along with the influence of genetic and environmental factors [126], [127], It is particularly important in poor soils, where it contributes to improving plant growth and reproductive capacity, making them more competitive and tolerant of harsh environmental conditions compared to non-mycorrhizal plants.[128]making them a key element in achieving sustainable agriculture.[127] Mycorrhizal fungi are classified into three main types :Ectomycorrhiza which form a sheath around the root Endomycorrhizae They are fungi that penetrate the cells of the root cortex, forming distinctive structures known as arbuscules and vesicles. They are the most common form of mycorrhizae, found in about 80% of green plants .ectendomycorrhizae which display characteristics of both. [129]

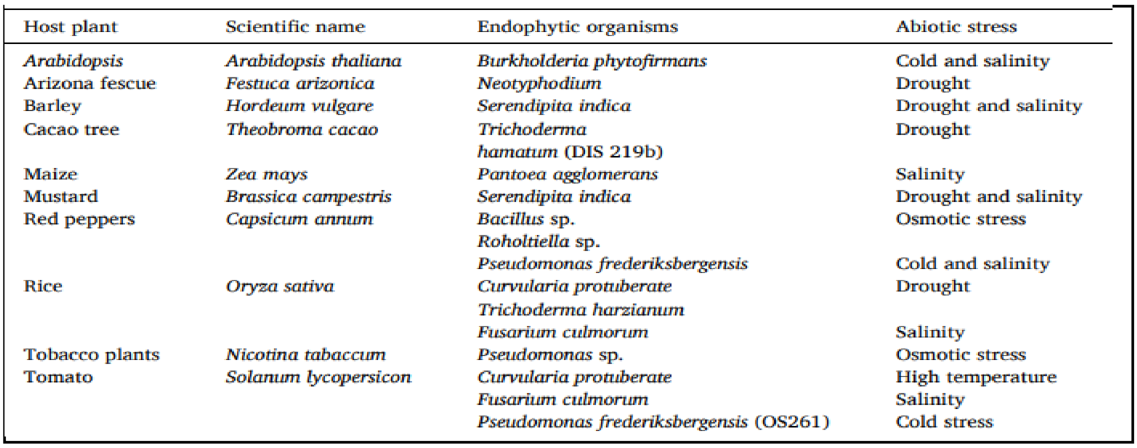
5 . Microorganisms improve salt resistance of plant :

Plant roots secrete signals into the soil that act as a call to action to attract beneficial microbes, which interact with the plant to mitigate the effects of various environmental stresses. These interactions contribute to enhancing the plant's resilience and overall health, especially when faced with challenging environmental conditions. The plant microbiome, composed of bacteria, fungi, and other microbes, enhances plant resilience and health under abiotic stresses such as drought and salinity. Microbes reside in the roots, phyllosphere, and endosphere.[130]

Plant Growth-Promoting Rhizobacteria (PGPR), Such As *Bacillus Amyloliquefaciens* Qst713, Improve Alfalfa's Drought Tolerance By Promoting Root Growth, Producing plant hormones, and increasing water and nutrient availability via eps and serophores . These bacteria also support the uptake of essential minerals and contribute to plant Biocontrol.[131]endophytic bacteria play an important role in promoting plant growth. They live near or within plants, providing better protection and facilitating symbiotic Relationships with the host plant . These bacteria contribute to strengthening plant Defenses against salt stress by producing antioxidants and regulating ionic balance via The sos1 Na^+/k^+ transporter. Endophytic bacteria known for their ability To improve plant growth. [132]*pseudomonas* and *bacillus* proteobacteria (PGPR) are Effective in promoting plant growth, due to their ability to colonize roots, improve the Availability of nutrients such as nitrogen , phosphorus, and iron , and produce growth-Promoting plant hormones such as indole-3-acetic acid, making them promising in Agricultural applications.[133]

Endophytes and fungi enhance plants' ability to tolerate abiotic stresses such as drought, salinity, and heat by activating defense genes, regulating antioxidants, and producing protective plant hormones. They also improve osmotic balance and proline accumulation, and increase the efficiency of photosynthesis, water transport, and nutrient uptake, making them an important element in supporting plant resistance and achieving sustainable agriculture.[134]

Table 1 : Endophytes importance in alleviating Abiotic Stress in Plant [135]



Host plant	Scientific name	Endophytic organisms	Abiotic stress
Arabidopsis	*Arabidopsis thaliana*	*Burkholderia phytofirmans*	Cold and salinity
Arizona fescue	*Festuca arizonica*	*Neotyphodium*	Drought
Barley	*Hordeum vulgare*	*Serendipita indica*	Drought and salinity
Cacao tree	*Theobroma cacao*	*Trichoderma hamatum* (DIS 219b)	Drought
Maize	*Zea mays*	*Pantoea agglomerans*	Salinity
Mustard	*Brassica campestris*	*Serendipita indica*	Drought and salinity
Red peppers	*Capsicum annum*	*Bacillus* sp.	Osmotic stress
		Roholtiella sp.	
		Pseudomonas frederiksbergensis	Cold and salinity
Rice	*Oryza sativa*	*Curvularia protuberate*	Drought
		Trichoderma harzianum	
		Fusarium culmorum	Salinity
Tobacco plants	*Nicotina tabaccum*	*Pseudomonas* sp.	Osmotic stress
Tomato	*Solanum lycopersicon*	*Curvularia protuberate*	High temperature
		Fusarium culmorum	Salinity
		Pseudomonas frederiksbergensis (OS261)	Cold stress

Table 2: Some agricultural benefits of PGPMs (PGPR, PGPFs, and AM) [135].

PGPMs Biostimulant	Crop	Benefit	Mechanism of action
Methylobacterium exotorquens	*Arabidopsis thaliana* Barley (*Hordeum vulgare*) Soyabean (*Glycine* max)	Aids in bio-stimulation	Cytokinin output
Azospirillum brasilense and *Bacillus cereus*	Bean (*Phaseolus vulgaris* L.)	Bio-fertilisation and Boosts bio-protection	Phosphate and Lipopeptides solubilisation
Azospirillum brasilense	Barley (*Hordeum vulgare*) Legumes (*Pisum sativum*) Maize (*Zea mays* L.)	Bio-stimulated growth and production	Synthesis of auxin (IAA), carotenoids, nitric oxide (NO), and several cell-surface components
Azospirillum brasilense (Sp7b and Sp245b)	Cucumber (*Cucumis sativus*) Lettuce (*Lactuca sativa*) Tomato (*Solanum lycopersicum*)	Enhanced germination, root length, and weight; vigor index of germinating seeds	Synthesis phytohormones like IAA
Azotobacter chroococcum	Cereals	Aids in bio-stimulation	synthesis of gibberellin (GA)
Bacillus sp.	Lettuce (*Lactuca sativa*)	Growth, biomass, and yield	Raised synthesis of phytohormones and nutrients availability
Mesorhizobium loti	Lotus (*Nelumbo nucifera*)	Bio-fertilisation	Nitrogen fixation
Bacillus amyloliquefaciens (RaSh1)	Pepper (Capsicum annuum L.)	Fungicidal resistance	Decreased electrolyte leakage and malondialdehyde (MDA) content
Serratia marcescens	Pumpkin (*Poa pratensis*)	Bio-protection	Producing protease, chitinase, and siderophore
Pseudomonas aeruginosa	Pea (*Pisum sativa*) Rice (*Oryza sativa*)	Aids in bioremediation	Cellulase synthesis
Pseudomonas aeruginosa	Wheat (*Triticum aestivum* L.)	Nutrient uptake	N_2-fixation including several reactions and organic acids formation

Perspectives and future prospects :

Recent research has shown that plant-microbe interactions are a promising area for achieving more sustainable and productive agriculture. Advances in understanding the molecular mechanisms and genes responsible for plant responses to environmental stresses, such as drought and salinity, have helped improve their ability to adapt to challenging conditions. Modern technologies, such as Multi-Omics and NGS, have also contributed to elucidating the intricate relationship between roots and microbes [136].Indoor plants, whether fungi or bacteria, are a cornerstone of sustainable agriculture, playing a vital role in promoting plant growth and increasing their resistance to diseases and environmental stresses in safe and environmentally friendly ways. These microorganisms produce a variety of bioactive compounds, such as antibiotics, plant hormones, and cerophores, which help suppress pathogens, improve nutrient uptake, and fix nitrogen, thus reducing reliance on chemical fertilizers and pesticides. Species like Trichoderma, Bacillus subtilis, and Pseudomonas fluoresce are prime examples used in biological control programs within integrated pest management systems. However, the complex interactions between these microbes, plants, and the environment remain a challenge, necessitating further research to discover new strains and enhance their ability to penetrate plant tissues. Therefore, indoor plants represent a strategic option for achieving more productive and sustainable agriculture, contributing to food security, and minimizing the environmental impact of agricultural activities.[137], [138]

Despite the high potential of these organisms to increase agricultural productivity in a sustainable manner, expanding their use requires the following:

- Expanding their application and developing effective techniques for their isolation and characterization.
- Improving their large-scale industrial production.
- Finding suitable bio-carriers that increase their shelf life.
- Promoting their field application within integrated agricultural practices. [135]

Part 18

Materials and Methods

Part 19 : Materials and Methods

1 Plant Materials, Growth Condition and Stress Treatment

1.1. *Medicago Sativa* growth and inoculation

*Medicago **s**ativa* seeds (America - California) are cleaned with liquid soap solution for 5 minutes and rinsed four times for 5 minutes with distilled water to remove the chemicals on the surface ,seeds are then sterilized with a 5° chlorine treatment
For 20 minutes and rinsed four times with sterile distilled water for 15-20 minutes[172]. The sterilized seeds deposited in round petri dishes (140mm) containing a sterile solution of 1.2%(W/V) agar and distilled water. The seeds were stratified at 4°C for 48h in the dark[173]. then the seeds were germinated in dark growth chamber for 24h .the seedling transferred in round petri dishes of (140mm) containing buffered nodulation medium (BNM) with 1.2%(W/V) bactoagar and supplemented with potassium nitrate ($KNO3+$) 5g/L each of these dishes contains eight seedlings[174]. Plants were cultivated in suspensions growth chamber with a photoperiod of 16h light /8h dark in 26° respectively . were prepared bacterial suspensions and added 1ml of suspensions at OD600 = 0.1 to plants by rinsing the roots and removed excess inoculum[174].

The suspension previously prepared from solid bacteria in petri dishes containing 25ml 1/2MS cultivated for 72h at 30°C in yeast extract broth agar medium(YEB**)** The inhibition of root growth its done by NaCl we measuring the growth and assessed after 7 days at 26°C of cultivation.
The following strains were used in this study: M17(***P**seudomonas **a**eruginosa*),
M67.2 (***B**acillus **a**myloliquefaciens*).

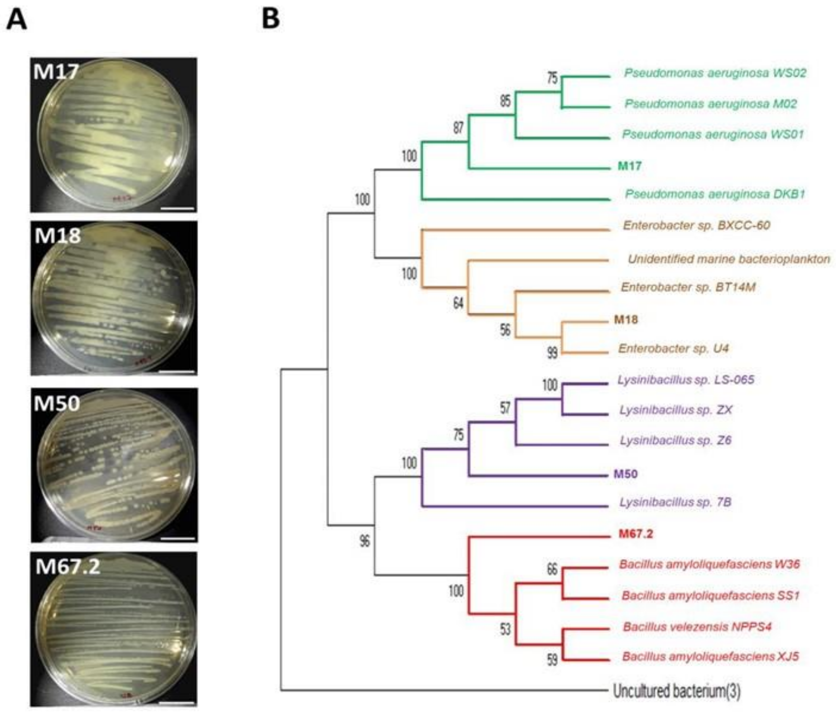


Figure 04. Identification of bacterial strain isolated from roots of Medicago

1.2. Traitement for salt stress:

For salt stress test were prepared different concentration of NaCl :0 mM (control) .100 mM.150 mM by mixed NaCl with the (BNM) medium to impose salt stress treatments in the culture medium[175].

1.3. salt stress responses analysis:

In purpose to determining plant tolerance to salt stress after germination and transferred the seedlings to (BNM) media containing NaCl 0mM. 100mM. 150mM. the seedlings incubated at 26°C for 7days in growth chamber.

Were taken the pictures after 7-14 days .the images were analyzed by using Image J software[176].for one replicate were analyzed for every condition. For every plant, the root length was determined, and a graphic shows the results.

1.4. Arabidopsis thaliana growth and inoculation

Arabidopsis thaliana seeds (France) are sterilized using 0.02% (W/V) of SDS (Sodium dodecyl sulfate) with agitated for 12 minutes then rinsed 3 times using ethanol 70% (V/V) and the fourth one by using ethanol pur 100%. The washed seeds are then dried in sterile conditions[172].

The sterilized seeds were deposited in 25 ml 1/2MS growth medium (Murashige and Skoog medium) with 1.2 % (W/V) of bacto agar[174]. The seeds are stratified for 48h at 4°C. the seeds putted in petri dishes (140mm) in growth chamber at 26°C for 5 days .A fungal spore suspension was prepared at OD600 = 0.1uisng spores harvested from grown on petri dishes containing 25ml 1/2MS previously cultivated for 72h[173] at 30°C in (MC**)** medium .

The following strains were used in this study: DA413 KM405

1.5. Traitement for salt stress:

to evaluate the effect of salt stress on plant growth, three concentrations of sodium chloride (NaCl) were applied:0mM(control) ,75mM ,100mM. The treatement was carried out by supplementing the culture medium with 25ml of respective NaCl solution. For the control group ,25ml of physiological water was used instead[175]. This procedure was designed to impose varying degrees of salt stress in a standardized manner across all experimental units.

1.6. salt stress responses analysis:

In object to analyze the plant response to the salt stress,after germination.

Transferred the plants to a 1/2 MS growth medium:were added three concentration of sodium chloride (NaCl) were applied:0mM(control) ,75mM ,100mM mM NaCl. The plants were then incubated for 15 days at 26 °C in the growth chamber. Images were taken after 15 days, and subsequently analyzed using ImageJ software[176]. Three replicates were evaluated for each experimental condition. For every plant, root length was measured, and the results were represented graphical.

Results

Results

1. Impact of bacterial inoculation on root growth of Medicago truncatula under non-saline conditions

In the absence of salt stress (figure 01), control plants produced longer roots (median ~10 cm). Plants inoculated with M17 displayed significantly shorter roots, approximately 6 cm long, indicating a strong inhibitory effect. M67.2 again resulted in intermediate values (~8 cm), closer to the control but still reduced compared to non-inoculated plants. The overall distribution indicated that bacterial inoculation under non-stressful conditions consistently suppressed root elongation, with M17 having the strongest negative effect. In the absence of salinity, root elongation was maximal in control plants, with median values around 20 cm. Inoculation with M17 strongly suppressed root growth (~8 cm), while M67.2 also reduced elongation (~10–12 cm), although to a lesser extent than M17. This confirmed that in non-stress conditions, bacterial inoculation—particularly with M17—was detrimental to root development.

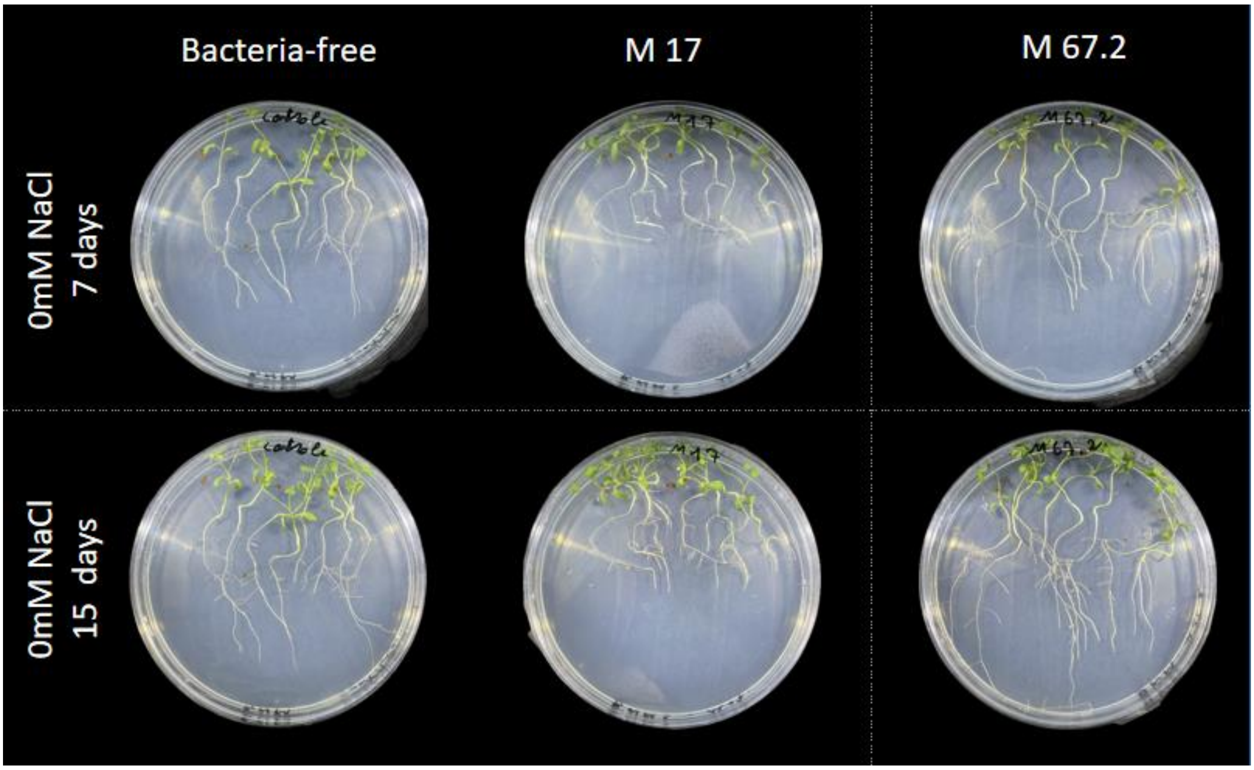


Figure 05. M17 and M67.2 reduce stress effects and enhance *Medicago sativa* growth under 0 mM NaCl . Plants were cultivated *in vitro* for 7 and 15 days.
Inoculated plants showed better root and shoot growth than the control.Original

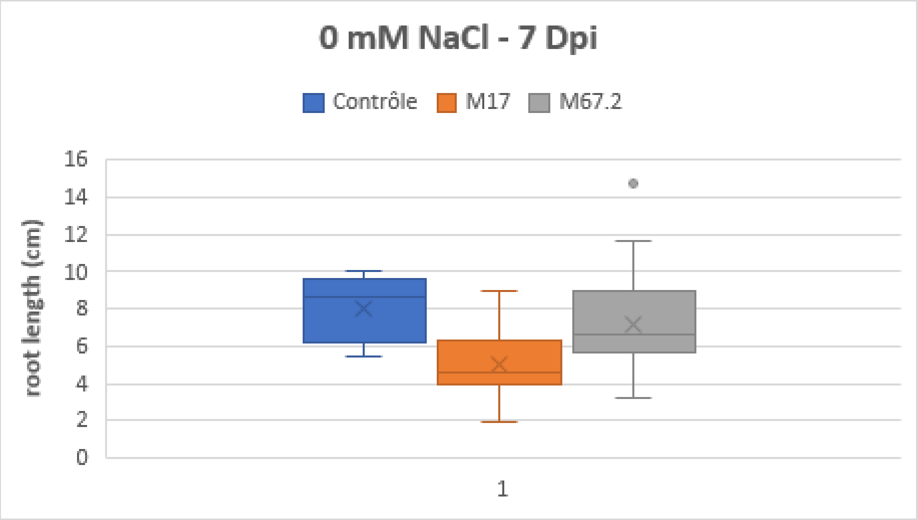


Figure 06. Effect of 0 mM NaCl at 7 dpi on root length of *Medicago sativa*.

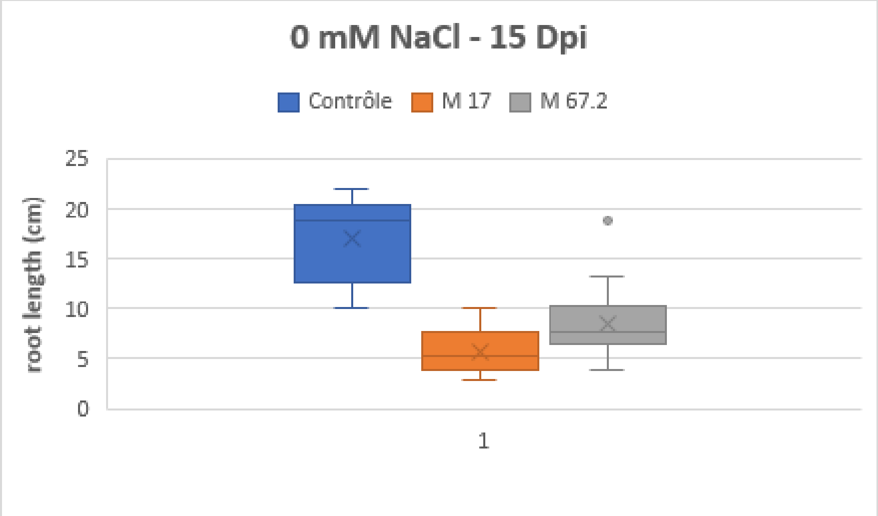


Figure 07. Effect of 0 mM NaCl at 15 dpi on root length of *Medicago sativa*.

2. Effect of M17 and M67.2 on root elongation under moderate salt stress (100 mM NaCl)

At 7 dpi under moderate salinity (figure 2) (100 mM NaCl), the control plants exhibited the longest roots, with median values around 11–12 cm. Inoculation with M17 reduced root length substantially, with a median closer to 8 cm. M67.2 inoculation resulted in intermediate values, around 9–10 cm, but still lower than the control. Variability was higher in the control compared to inoculated treatments, suggesting that inoculation limited root elongation under this condition. At moderate salinity (100 mM NaCl) and later sampling (15 dpi), all treatments displayed relatively short roots compared to non-stressed conditions. Control plants had root lengths around 7–8 cm. M17 inoculation further reduced root growth (~5–6 cm), while M67.2 plants showed values close to the control (~7 cm). The differences between treatments were smaller than those observed at 7 dpi, suggesting a progressive convergence of root growth responses over time.

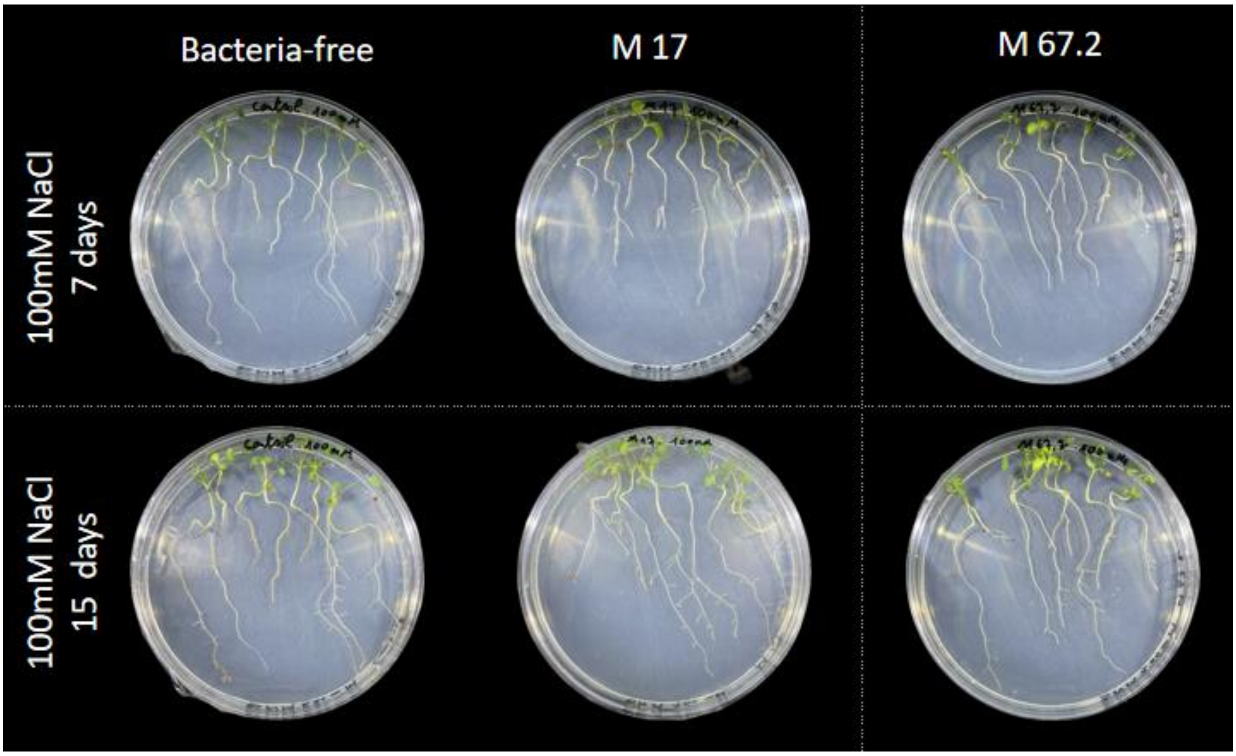


Figure 08. M17 and M67.2 reduce stress effects and enhance *Medicago sativa* growth under 100mM NaCl .Plants were cultivated *in vitro* for 7 and 15 days.
Inoculated plants showed better root and shoot growth than the control.Original

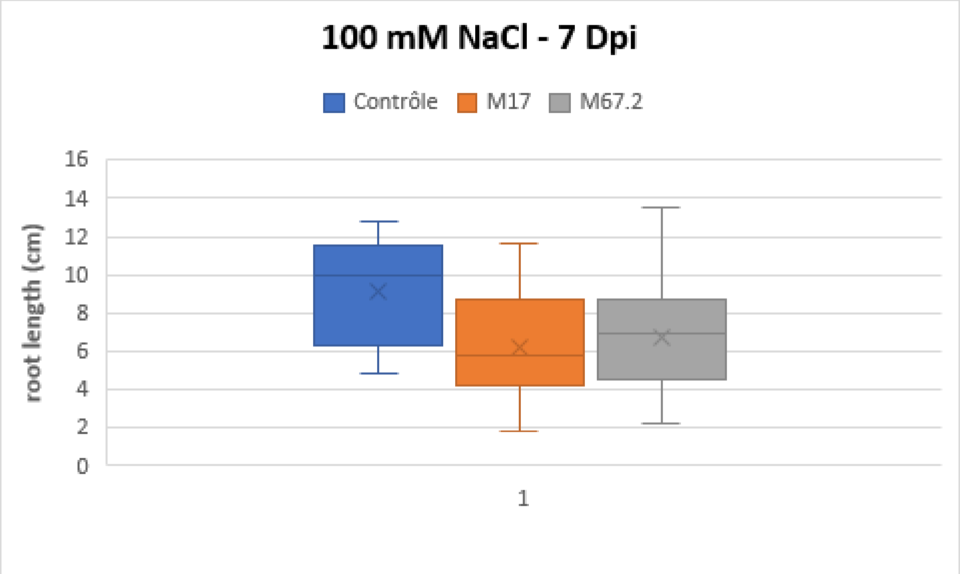


Figure 09 Effect of 100 mM NaCl at 7 dpi on root length of *Medicago sativa*.

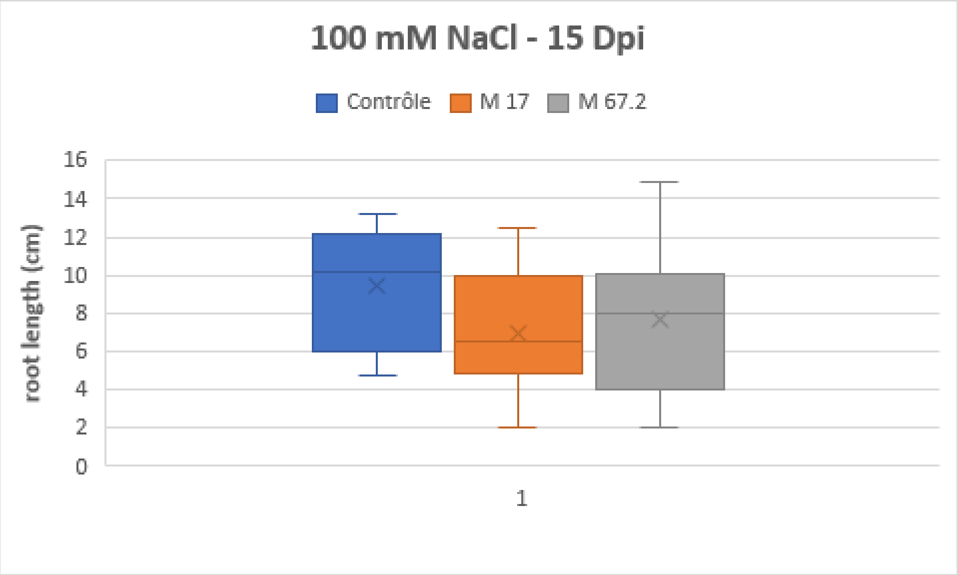


Figure 10. Effect of 100 mM NaCl at 15 dpi on root length of *Medicago sativa*.

3. Bacterial inoculation mitigates root growth inhibition under severe salt stress (150 mM NaCl)

High salinity (150 mM NaCl) strongly inhibited control plants, which displayed very short roots (median ~3–4 cm). In contrast, plants inoculated with M17 and M67.2 showed enhanced growth, with root lengths around 6–7 cm for M17 and slightly higher (~7–8 cm) for M67.2. Both bacterial strains clearly mitigated the inhibitory effect of salt stress, with M67.2 performing slightly better than M17. At 15 dpi, control plants under 150 mM NaCl had roots of about 5–6 cm in median length. Inoculated plants showed similar lengths, with M17 roots slightly shorter (~4–5 cm) and M67.2 maintaining a moderate improvement (~6 cm). However, the strong beneficial effect observed at 7 dpi was reduced at this later stage, and overall root elongation remained low for all treatments under high salinity.

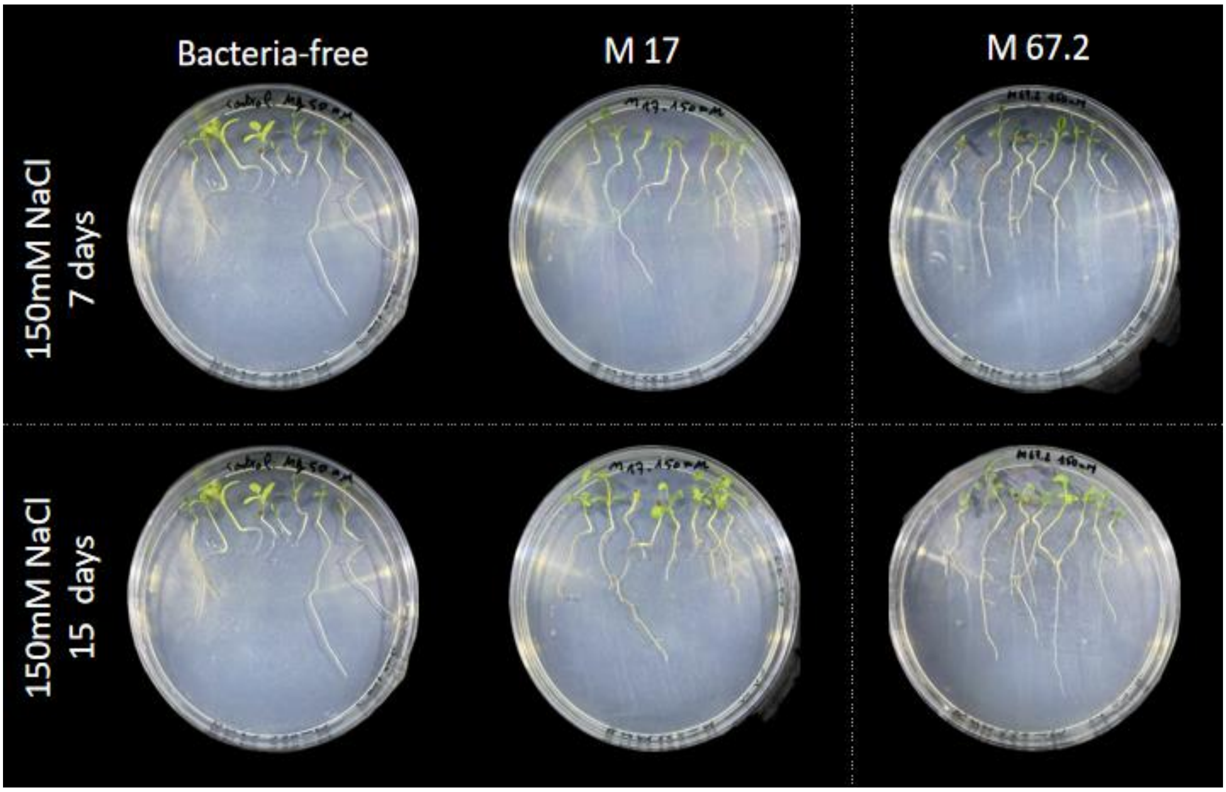


Figure 11. M17 and M67.2 reduce stress effects and enhance *Medicago sativa* growth under 150 mM NaCl .Plants were cultivated *in vitro* for 7 and 15 days.

Inoculated plants showed better root and shoot growth than the control.Original

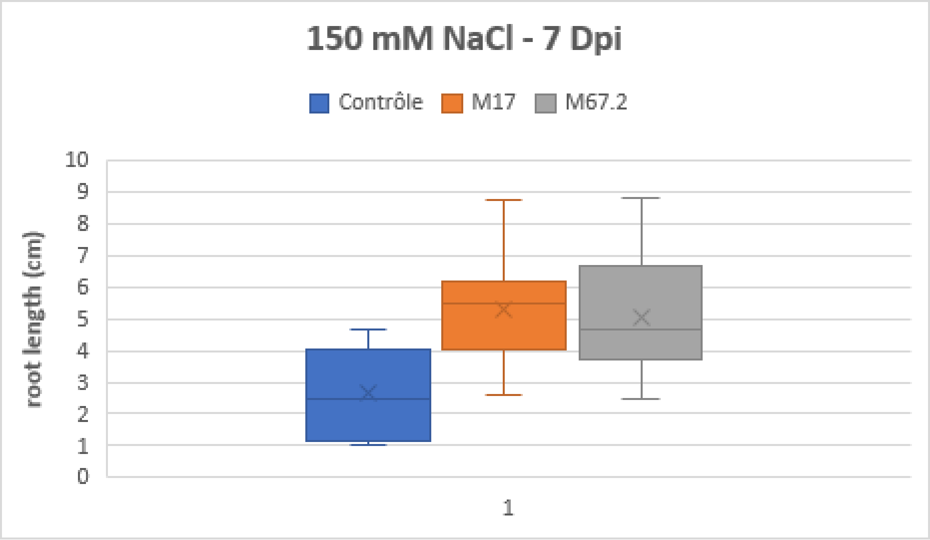


Figure 12. Effect of 150 mM NaCl at 7 dpi on root length of *Medicago sativa*.

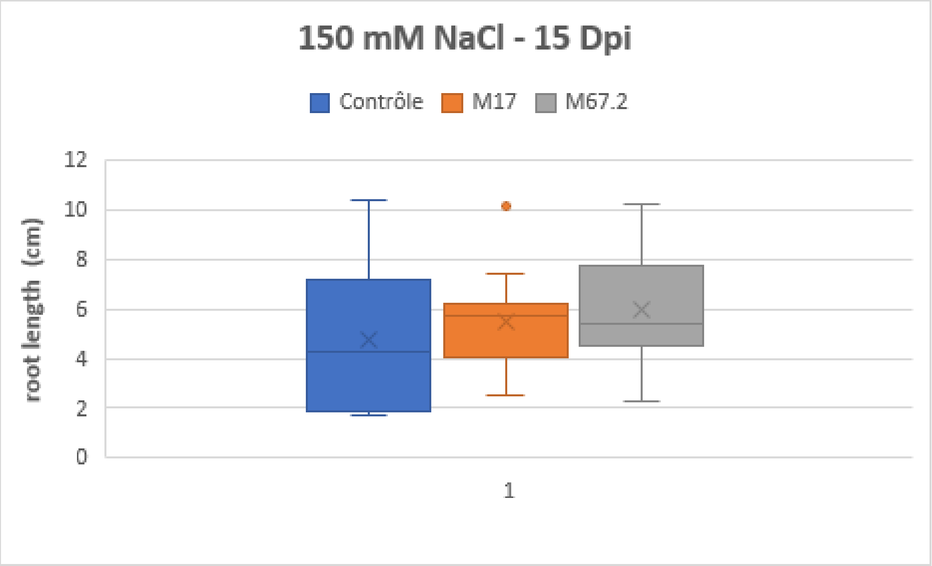


Figure 13. Effect of 150 mM NaCl at 15 dpi on root length of *Medicago sativa*.

Discussion

Discussion

These results indicate that bacterial inoculation with strains M17 and M67.2 differentially affects the root growth of *Medicago sativa* under saline and non-saline conditions.

At 0 mM NaCl, both M17 and M67.2 reduced root elongation compared to controls at both 7 and 15 dpi. This suggests that under optimal conditions, the bacterial strains may exert a cost on the plant, likely due to competition for resources or stress from initial colonization [145] Similar negative effects have been reported when plants interact with non-optimized or non-native rhizobacteria [156 .

At moderate salinity (100 mM NaCl), the control generally maintained better root growth than inoculated plants, especially at 7 dpi. This indicates that at this salt level, the tested bacteria did not confer a clear advantage and may have even acted as additional stressors [158]

At high salinity (150 mM NaCl), the beneficial effect of bacterial inoculation became apparent, especially at 7 dpi, where both M17 and M67.2 significantly improved root length relative to the control. This supports the hypothesis that certain rhizobacteria enhance plant salt tolerance by promoting root system development, osmolyte accumulation, or ion homeostasis [148] In particular, endophytic and rhizosphere-associated bacteria have been shown to mitigate the deleterious effects of NaCl stress by producing phytohormones such as indole-3-acetic acid (IAA) or by inducing antioxidant systems [146].

Interestingly, the beneficial effect of inoculation at 150 mM NaCl was stronger at 7 dpi than at 15 dpi, suggesting that bacterial enhancement is more relevant in the early stages of stress adaptation. This aligns with previous findings that plant–microbe interactions often trigger early protective responses, but their long-term effectiveness depends on the bacterial strain and host compatibility [166]

Taken together, these data suggest that strains M17 and M67.2 may not benefit *M. truncatula* under non-saline conditions, but they provide a growth-promoting effect under high salt stress, highlighting their potential as bio-inoculants for improving plant tolerance to salinity. Future studies should investigate the underlying mechanisms (e.g., IAA production, ACC deaminase activity, osmoprotectant synthesis) and whether these bacteria can also improve biomass and nodulation efficiency under salinity.

2. Effect of fungal inoculation on root growth of *Arabidopsis thaliana* under different NaCl concentrations

The results presented in the boxplots illustrate the effect of two microscopic fungal strains, KM405 and DA413, on the root growth of *Arabidopsis thaliana* under three salinity conditions: 0, 75, and 100 mM NaCl.

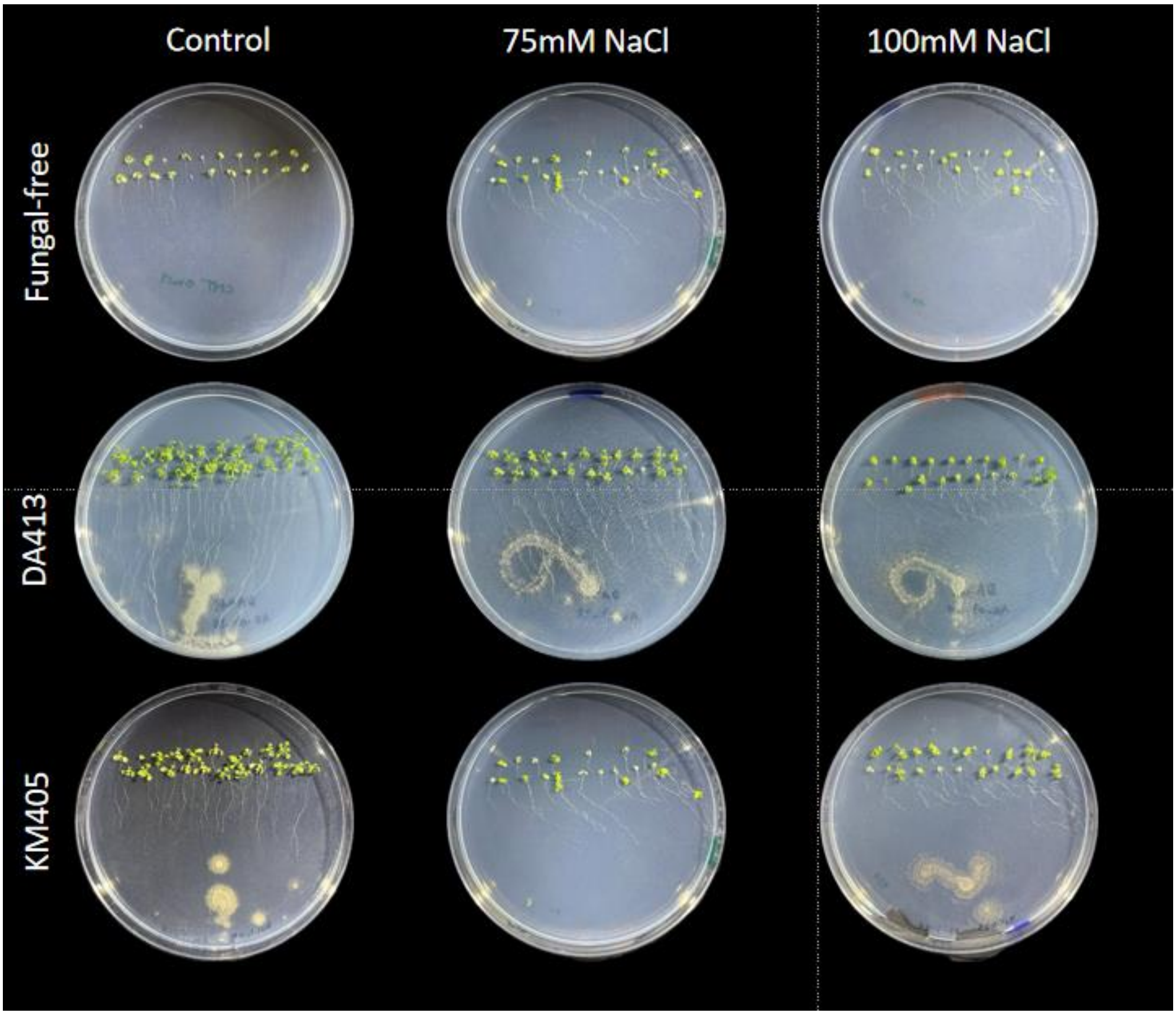


Figure 14. Fungal endophytes DA413 and KM405 enhance *Arabidopsis thaliana* growth under salt stress.Plants were cultivated *in vitro* under 0, 75, and 100 mM NaCl conditions, with or without fungal inoculation. Inoculated plants showed better root and shoot growth compared to the fungal-free treatment.Original

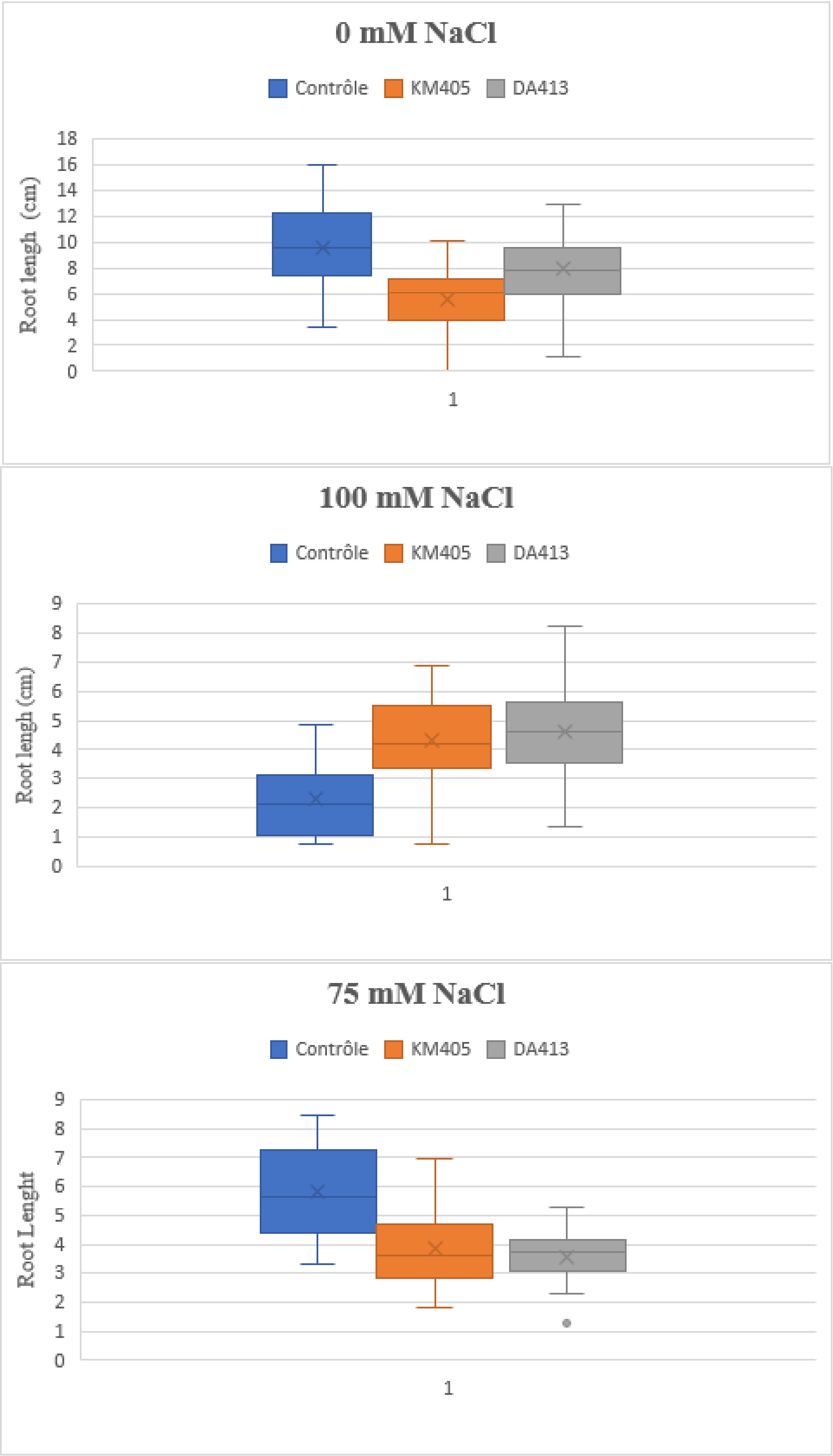


Figure 15: Effect of endophytic fungal inoculation (KM405 and DA413) on root growth of *Arabidopsis thaliana* under different NaCl concentrations (0, 75, and 100 mM).

2.1 0 mM NaCl (non-saline condition)

Under non-saline conditions, the control plants (non-inoculated) exhibited the highest root growth, with median values around 11–12 cm. Plants inoculated with KM405 showed a clear reduction in root length (median ≈ 6 cm), while those inoculated with DA413 displayed slightly higher root lengths (≈ 8 cm) but still lower than the control.

This initial reduction in root growth may be attributed to competition for nutrients or transient activation of plant defense mechanisms upon fungal colonization. Early plant–microbe interactions often trigger defense signaling before mutualistic compatibility is established [164] ; [163] Such early negative or neutral effects have also been reported in *Arabidopsis* inoculated with endophytic fungi that later become beneficial under stress conditions [152]

2.2 75 mM NaCl (moderate salt stress)

At 75 mM NaCl, root growth was significantly reduced in all treatments, indicating that moderate salinity imposes a strong inhibitory effect on *Arabidopsis* root development. Control plants showed median root lengths around 6 cm, while KM405 and DA413 further reduced growth (≈ 4 cm and 3–3.5 cm, respectively).

Under moderate stress, the fungal inoculations did not improve growth performance. This could be due to an intermediate stress level where neither the plant nor the fungus is fully able to activate tolerance mechanisms. Similar observations have been reported where the beneficial effects of endophytes are only visible under high-stress intensity, once the symbiosis is firmly established [161] ; [159]

2.3 100 mM NaCl (severe salt stress)

Under high salinity (100 mM NaCl), root growth in the control plants was the most severely inhibited (median ≈ 2 cm). In contrast, plants inoculated with KM405 and DA413 showed a marked improvement in root elongation, with median values of approximately 4–5 cm and 5–6 cm, respectively. The DA413 treatment displayed the greatest increase in root length, suggesting that this strain has a strong capacity to mitigate salt stress effects.

Endophytic fungi are known to enhance salt tolerance in host plants through several physiological and molecular mechanisms. These include:

- improved ionic homeostasis by regulating Na^+/K^+ balance [170] ,
- accumulation of osmolytes such as proline and soluble sugars [142] ,

- increased antioxidant enzyme activities (SOD, CAT, POD) to limit oxidative damage [154]
- and modulation of gene expression involved in water transport and stress signaling [160]

The observed improvement in *Arabidopsis* root growth under 100 mM NaCl, particularly in association with DA413, suggests the activation of similar protective mechanisms. These results are consistent with previous reports demonstrating that fungal endophytes such as *Piriformospora indica*, *Trichoderma spp.*, and *Serendipita vermifera* enhance root development and salt stress tolerance in *Arabidopsis* and other plants [168] [151]

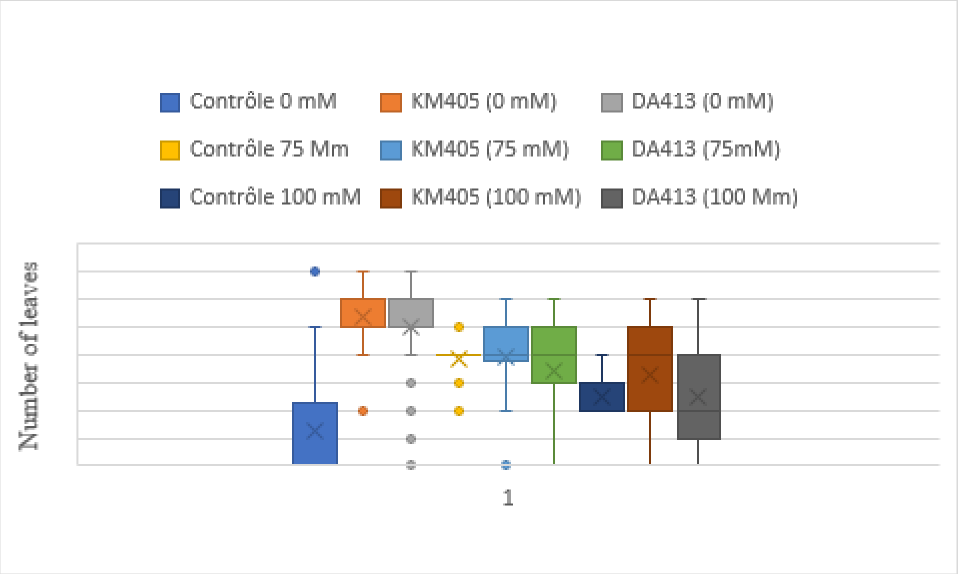


Figure 16. Effect of salt stress and bacterial inoculation on the number of leaves in *Arabidopsis thaliana*

This box plot illustrates the number of leaves produced by *Arabidopsis thaliana* grown under different salt concentrations (0, 75, and 100 mM NaCl) and inoculated with bacterial strains KM405 and DA413, either alone or in association with fungal symbionts.

Under non-saline conditions (0 mM), control plants show a moderate number of leaves, comparable to those inoculated with the bacterial strains.

At 75 mM NaCl, leaf number slightly decreases in the non-inoculated control, whereas plants inoculated with DA413 (75 mM) maintain higher leaf numbers, suggesting a protective effect.

At 100 mM NaCl, leaf production is markedly reduced across treatments, yet plants associated with KM405 and DA413 still retain more leaves than the uninoculated control.

Variability increases under higher salt stress, indicating heterogeneous plant responses to combined bacterial and fungal interactions.

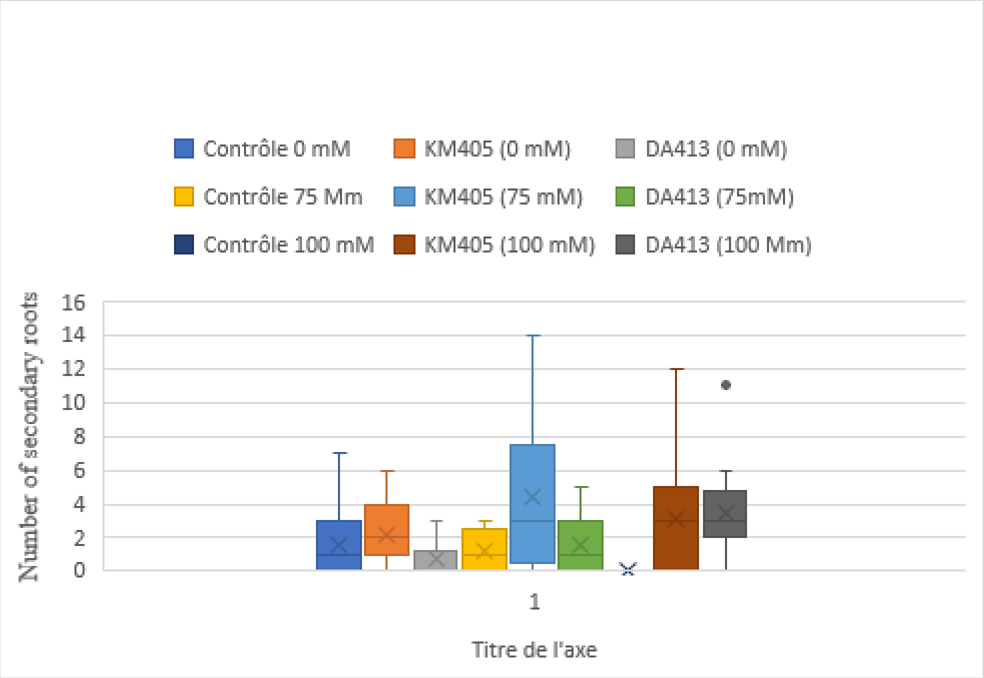


Figure 17. Effect of salt stress and microbial inoculation on the number of secondary roots

This figure represents the distribution of the number of secondary roots of *A. thaliana* subjected to the same experimental conditions.

Under control conditions (0 mM), plants produce few lateral roots (1–3 on average), with minimal differences between treatments.

At 75 mM NaCl, a clear stimulation of lateral root formation is observed, particularly in plants inoculated with KM405 (75 mM), highlighting a strong induction of root branching.

At 100 mM NaCl, root number tends to decline in all treatments, but inoculated plants still display higher root proliferation than the control, indicating a partial mitigation of salt stress.

The wide distribution in the KM405 (75 mM) group suggests an individual variability in root plasticity under combined biotic and abiotic stresses.

The obtained results demonstrate that *Arabidopsis thaliana* plants inoculated with bacterial strains KM405 and DA413, in association with fungal symbionts, exhibit improved growth performance under salt stress. Both microbial partners appear to confer enhanced tolerance by promoting leaf development and stimulating the formation of secondary roots.

❖ Impact of salinity on *Arabidopsis thaliana* growth

Salt stress adversely affects plant growth by disrupting osmotic balance, ion homeostasis, and photosynthetic capacity [157]; [171]. In this experiment, the progressive increase in salinity (75 and 100 mM) led to a reduction in leaf and root development in control plants, consistent with typical salt-induced growth inhibition observed in *Arabidopsis*.

❖ Beneficial effects of bacterial–fungal co-inoculation

Co-inoculated plants maintained higher leaf and root numbers under salt stress, suggesting synergistic effects between bacterial and fungal partners.

Such synergism has been reported to enhance plant performance through multiple mechanisms:

- Improved nutrient and water uptake via increased root branching and mycelial networks [165]
- Reduction of ethylene-mediated stress through bacterial ACC deaminase activity [149]
- Accumulation of osmoprotectants such as proline and glycine [144]
- Induction of antioxidant enzymes and defense-related genes [169]
- Enhanced ion homeostasis and modulation of Na^+/K^+ balance [147]
- The increased number of secondary roots observed with KM405 under 75 mM NaCl suggests that this strain, possibly in concert with fungal hyphae, stimulates root morphogenesis and surface expansion, thereby improving resource acquisition.

  Conversely, DA413 seems to better maintain shoot growth and leaf development, indicating that it may act more effectively on physiological or molecular pathways that sustain photosynthetic activity and hormonal balance.

❖ Tripartite interactions and stress resilience

Tripartite interactions involving plants, bacteria, and fungi are increasingly recognized as key components of plant stress resilience [153] ; [167] .

The combination of bacterial and fungal endophytes can activate complementary stress responses, leading to enhanced tolerance compared to single inoculations. In *A. thaliana*, such microbial consortia may modulate root system architecture, ROS scavenging, and gene expression involved in salinity response [143] ; [150]

Biological implications

These findings support the hypothesis that microbial consortia (bacteria + fungi) play a significant role in mitigating salt-induced damage by reinforcing both structural (root branching) and functional (photosynthetic efficiency) aspects of plant physiology.

Moreover, the *Arabidopsis* model provides a valuable framework for understanding similar interactions in leguminous species such as *Medicago sativa*, where KM405- and DA413-like endophytes could enhance nodulation efficiency and stress adaptation [143]

Conclusion

This study investigated the potential of both bacterial endophytes and fungal strains as natural bioagents to enhance plant tolerance to salt stress. By microbial inoculation and stress induction experiments, we evaluated their effectiveness in mitigating the negative effects of salinity on two model plants: *Medicago sativa* and *Arabidopsis thaliana*. The results revealed that the bacterial endophytes M17 and M67.2 had a remarkable positive effect on *Medicago sativa* growth under saline conditions, while the fungal strains DM413 and KM405 significantly improved the salt resilience of *Arabidopsis thaliana*.

Genomic analyses confirmed the identities of the bacterial strains, with M17 classified under the *Bacillus* genus and M67.2 under *Pseudomonas*.

The fungal strains DM413 and KM405 exhibited functional genes related to osmotic adjustment, ROS detoxification, and secondary metabolite synthesis, which together may explain their beneficial effects on *Arabidopsis thaliana* under salt stress. Both bacterial and fungal inoculations enhanced plant physiological and biochemical responses, improved growth parameters, and reduced oxidative damage compared to non-inoculated controls. These results suggest that microbial symbiosis can activate complex plant defence networks that improve resilience to abiotic stress.

Overall, this research demonstrates the promising role of beneficial microorganisms both bacterial and fungal in improving plant tolerance to salinity. The combined findings highlight the importance of microbial partnerships as eco-friendly, sustainable tools for enhancing crop performance under harsh environmental conditions. By harnessing these natural interactions, future agricultural systems may better sustain productivity, especially in salt-affected soils.

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