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Title

Evaluation of the plant growth promoting potential of some bacterial and fungal isolates under saline stress

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Praise be to Almighty God, who has allowed me to see this long-awaited day.

In memory of:

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My paternal and maternal grand-parents

My maternal Uncle Djillali

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May God welcome them into His vast paradise and may they rest in peace.

I dedicate this work:

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To this source of tenderness, patience, and generosity,

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Abstract

Plant growth-promoting microorganisms (PGPM) represent a promising strategy for enhancing plant resilience and yields under salt-stress conditions.

The present study aims to test the plant growth promoting capacity of selected microbial species, isolated in a previous study, under saline stress both in vitro via the measure of halotolerance, auxin production and siderophore production and in vivo by testing the growth promoting potential of the isolates on Maize seeds.

Results showed that the all the tested isolates have the ability to grow under saline concentration, at least up to 600 mM of NaCl. However, *Bacillus rugosus* demonstrated the higher tolerance to salt (900 mM NaCl) compared to the other isolates. In addition, all the isolates showed their ability to produce auxin under the different salt concentrations tested except for *Pseudomonas granadensis* that showed lower value above 600 Mm. Moreover, all the tested isolates produce siderophores at all the tested NaCl concentrations.

Furthermore, in vivo plant growth parameters such as root length and shoot length showed an increase when treated with all the isolates but limited to a concentration of 50 mM NaCl.

These results reflected the positive impact of the tested microbial isolates on plant growth. Our data highlight the ability of the isolated microorganism strains we used to have a high plant growth-promoting (PGP) capabilities. However, these strains need to be tested under field conditions and with more crops before being considered biofertilizer candidates.

Keywords: Salt stress, plant growth promoting microorganisms (PGPMs), halotolerance, auxin, siderophores.

Résumé

Les micro-organismes favorisant la croissance des plantes (PGPM) représentent une stratégie prometteuse pour améliorer la résilience et les rendements des plantes dans des conditions de stress lié à la salinité.

La présente étude vise à tester la capacité de promotion de la croissance végétale sous stress salin à l'aide de quelques espèces microbiennes isolées dans une étude précédente, à la fois in vitro via la mesure de l'halotolérance, de la production d'auxine et de la production de sidérophores et in vivo en testant les capacités PGP des isolats microbiens sur la croissance des graines de maïs.

Les résultats ont montré que tous les isolats testés ont la capacité de croître sous salinité, au moins jusqu'à 600 mM de NaCl. Cependant, *Bacillus rugosus* a démontré une tolérance plus élevée au sel (jusqu' à 900 mM) par rapport aux autres isolats. De plus, tous les isolats ont montré leur capacité à produire de l'auxine sous les différentes concentrations de sel testées, à l'exception de *Pseudomonas granadensis* qui a montré une valeur plus faible au-dessus de 600 Mm. De plus, tous les isolats testés produisent des sidérophores à toutes les concentrations de NaCl testées.

En revanche, les paramètres de croissance des plantes in vivo, tels que la longueur des racines et la longueur des pousses, ont montré une augmentation lorsqu'ils étaient traités avec tous les isolats, mais limités à une concentration de 50 mM de NaCl.

Ces résultats reflètent l'impact positif de l'utilisation des isolats microbiens testés sur la croissance des plantes. Nos données mettent en évidence la capacité des micro-organismes isolées à promouvoir la croissance des plantes. Cependant, ces souches doivent être testées dans des conditions de terrain et avec davantage de cultures avant d'être considérées comme candidates aux biofertilisants.

Mots clés : Stress salin, microorganismes promouvant la croissance des plantes (PGPM), halotolérance, auxine, sidérophores.

ملخص

تمثل الكائنات الحية الدقيقة المعززة لنمو النبات (PGPM) استراتيجية واعدة لتعزيز مرونة النبات وإنتاجيته في ظل ظروف الإجهاد الملحي. تهدف الدراسة الحالية إلى اختبار قدرة تعزيز نمو النبات لأنواع ميكروبية مختارة، معزولة في دراسة سابقة، تحت ضغط ملحي سواء في المختبر (اختبار تحمل الملوحة، إنتاج الأوكسين وإنتاج حامل الحديد) وفي الجسم الحي (السمات المعززة لنمو النبات).

أظهرت النتائج أن جميع العزلات المختبرة لديها القدرة على النمو تحت تركيز ملحي يصل على الأقل إلى 600 ملي مولار من كلوريد الصوديوم. ومع ذلك، أظهرت *Bacillus rugosus* قدرة أعلى على تحمل الملح (900 ملي مولار كلوريد الصوديوم) مقارنة بالعزلات الأخرى. بالإضافة إلى ذلك، أظهرت جميع العزلات قدرتها على إنتاج الأوكسين تحت التراكيز الملحية المختلفة التي تم اختبارها باستثناء *Pseudomonas granadensis* التي أظهرت قيمة أقل فوق 600 ملي مولار.

علاوة على ذلك، فإن جميع العزلات التي تم اختبارها تنتج حوامل الحديد عند جميع تراكيز كلوريد الصوديوم المختبرة.

بالإضافة أظهرت معاملات نمو النبات في الجسم الحي مثل طول الجذر وطول الساق زيادة عند معاملتها بجميع العزلات ولكنها اقتصرت على تركيز 50 ملي مول كلوريد الصوديوم

وعكست هذه النتائج التأثير الإيجابي للعزلات الميكروبية المختبرة على نمو النبات. تسلط بياناتنا الضوء على قدرة سلالات الكائنات الحية الدقيقة المعزولة التي استخدمناها على التمتع بقدرات عالية على تعزيز نمو النبات. ومع ذلك، يجب اختبار هذه السلالات في الظروف الميدانية ومع المزيد من المحاصيل قبل اعتبارها مرشحة للأسمدة الحيوية

الكلمات المفتاحية: الإجهاد الملحي، الكائنات الحية الدقيقة المعززة لنمو النبات، تحمل الملوحة، الأوكسين، حامل الحديد.

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List of abbreviations

PGPMs: Plant growth promoting microorganisms.

PGPR: Plant growth promoting rhizobacteria.

PGPF: Plant growth promoting fungi.

CAS: Chrome azurol S

HCN: Hydrogen cyanide

LB: Luria-Bertani medium

NB: Nutrient broth

Introduction

Introduction

Along with population growth, there is a major increase in the world's food needs, which presents a number of nutrition-related challenges as well as the need for novel strategies and initiatives (Godoy et al., 2018).

In the upcoming decades, climate change will undoubtedly bring a variety of challenges, adding to the uncertainty about how to provide food and address these issues (Duchenne-Moutien and Neetoo, 2021). To combat and adapt to rising temperatures, diminishing freshwater resources, and an increase in the frequency of extreme events like fires, floods, and droughts, a comprehensive strategy, teamwork, and thorough study are needed (Bolan et al., 2024).

Soil salinity, one of the most significant variables, is present everywhere and has a variety of effects on the soil biota and soil characteristics (Shilev, 2020). Over 20 % of arable land globally is damaged by salt, and by 2050, that percentage is predicted to rise to 50 % (Stanković et al., 2015). This edaphic problem is mostly seen in arid and semiarid countries, including Algeria, where about 3.2 million hectares of agricultural lands are shown as being salt affected (Hadjadj et al., 2022).

Meanwhile, soil salinization poses a serious threat to the health of regional ecosystems and the production of biomass since it coexists with soil (land) degradation, desertification, soil erosion, and other ecological issues (Wang et al., 2023). Most plant species are adversely affected by saline soils that inhibit a number of physiological and biochemical processes in salt-sensitive plant species (and even in some salt-tolerant or halophyte species), including respiration, protein metabolism, DNA replication, photosynthetic processes, and water and nutrient absorption (Bessai et al., 2023). As a result, high salinity has detrimental effects on crop yields. Highly sensitive crops like beans, citrus, and stone fruits can experience 50-100 % yield losses, while moderately tolerant crops like wheat and barley can experience 10-25 % losses (Flowers and Yeo, 1989).

Recently, numerous studies have revealed that certain microorganisms have the ability to improve plant growth under variable stresses and can boost plant tolerance to salt in a variety of crops, including wheat, maize, mung beans, potatoes, and tomatoes. These are called plant growth promoting microorganisms ‘PGPM’ and belong to various bacterial and fungal genera, including *Bacillus*, *Pseudomonas*, *Rhizobium*, and *Streptomyces* (Bhise and Dandge, 2019). PGPM can improve crop production with multiple modes of action such as the synthesis of growth-promoting substances including phytohormones, improve plant nutrition, and resistance to biotic and abiotic stresses (Maryum et al., 2022).

The present study aims to test the plant growth promoting capacity of selected microbial species, isolated in a previous study, under saline stress both *in vitro* and *in vivo*.

Literature review

Literature review

1. Salt stress

Salt stress is a major abiotic stress influencing plant growth, development, and crop yield (Dzinyela et al., 2023). The occurrence of soil salinization is mainly due to the accumulation of water-soluble salts, including sodium (Na^+), potassium (K^+), chloride (Cl^-), and sulfate (SO_4^{2-}) in the root zone (Balasubramaniam et al., 2023). Growth inhibition, rapid development, senescence, and death after extended exposure are common signs of salt stress damage (Bressan et al., 2001). Plants under salinity stress have higher concentrations of sodium and chloride ions, which upsets ion homeostasis and hinders the absorption of nutrients. Plant mortality results from the buildup of Na^+ and Cl^- ions, which also cause ion toxicity, membrane disintegration, inhibition of photosynthesis, and impaired nutrient absorption (Hasanuzzaman et al., 2012). Indeed, salt stress can induce osmotic stress, ionic and nutritional imbalances, and disruption of several physiological and biochemical pathways in plants. Moreover, salinity inhibits transpiration, stomatal conductance, germination, and photosynthesis, which lowers leaf water potential, turgor pressure, and osmotic stress (kumar et al., 2021). Severe salt shock can also cause programmed cell death (Bressan et al., 2001).

Salt stress signals in plants (Fig. 1) trigger various transduction pathways, including reactive oxygen species buildup, excess Na^+ and intracellular Ca^{2+} levels. These signals trigger sodium stress reactions, which are quickly sensed. Ca^{2+} channels are constantly activated due to salt stress and variations in osmotic pressure, raising Ca^{2+} levels in the cytosol that serves as an essential messenger, binding to and activating Ca^{2+} sensors, which trigger a calcium signal cascade (Xiao and Zhou, 2023).

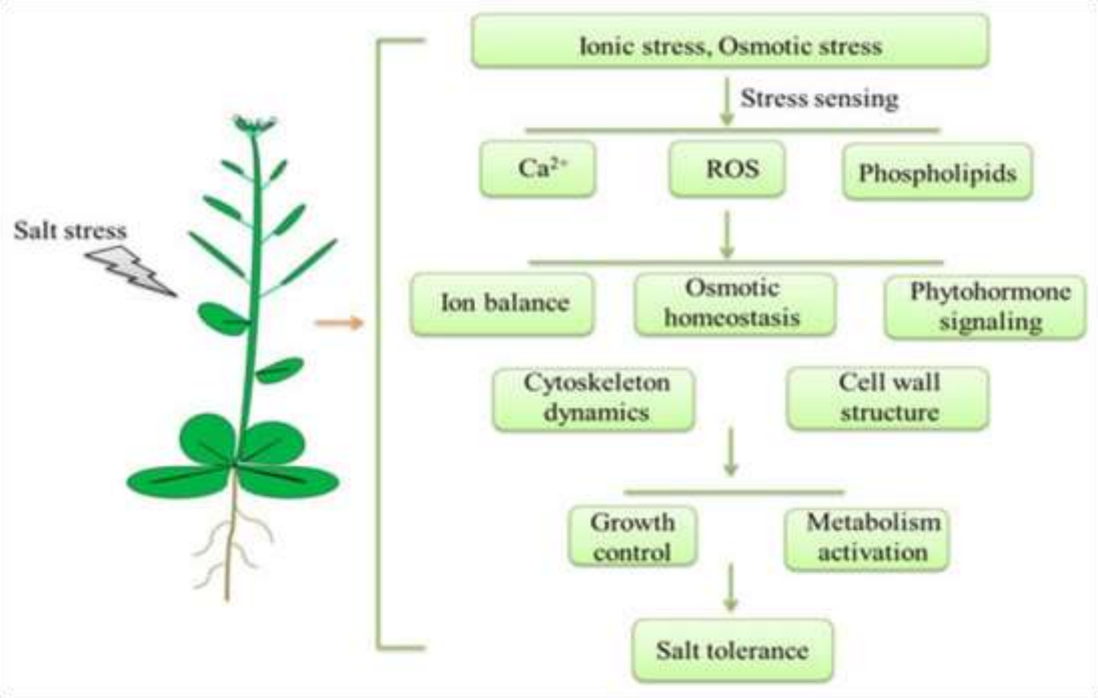


Figure 1. A simplified model of the plant salt stress response. Salt stress primarily causes ionic stress and osmotic stress. After sensing Na^{+} and hyperosmolality, plants accumulate Ca^{2+}, activate ROS signaling, and alter their phospholipid composition. These signals activate adaptive processes to alleviate salt stress, including maintaining an ion balance and osmotic homeostasis, inducing phytohormone signaling and regulating cytoskeleton dynamics and the cell wall structure. Subsequently, through an array of signal transduction pathways, plant growth is slowed and metabolism is activated to increase salt tolerance (Zhao et al., 2021).

2. Plant response to salinity

A complex interaction of physiological, biochemical, and molecular pathways drives plants' response to salt stress. Osmotic stress is the first reaction to salinity, it causes a reduction in the amount of water that roots can absorb and the growth of leaves. In an effort to preserve osmotic equilibrium, plants accumulate suitable solutes such as sugars, polyols, and betaines in an attempt to adapt (Zhao et al., 2020). High cytoplasmic concentrations of the sodium and chloride ions might be harmful for plants. In order to counteract this, plants use H^{+}-ATPase and Na^{+}/H^{+} antiporters to separate Na^{+} into vacuoles or keep it out of the cytoplasm (Shams and Khadivi, 2023). Salinity is perceived by cell surface receptors, triggering signaling cascades involving Ca^{2+}, reactive oxygen species (ROS), and phytohormones. This leads to altered gene expression and activation of stress response pathways (Chourasia et al., 2022).

In addition, plants may alter the production of some hormones as a response to external stresses, which promotes the synthesis of stress-related proteins. For example, in many crop plants, ethylene is the most common phytohormone that is produced in response to stress. However, high levels of ethylene affect negatively plant performance (Shahid et al., 2023).

3. Strategies to combat salt stress in plants

Plant growth and development are severely hampered by salt stress, which has an effect on global agricultural production. Developing efficient ways to counteract the detrimental effects of salt stress requires an understanding of the physiological and biochemical responses of plants to this stressor. Deciphering these reactions can offer important information on improving plant resistance to salt stress (Sumbul et al., 2020). Practical methods for preventing salt stress in plants exists such as:

The use of plant extracts as biostimulants instead of chemical fertilizers to reduce the effects of salt stress. This tactic reduces the amount of water used and provides a sustainable way to help plants that are stressed by salinity (Zhao et al., 2021). Plants can withstand ionic, oxidative, and osmotic stress brought on by salt stress thanks to the enzymes, proteins, osmoprotectants, co-factors, and other substances found in natural plant extracts (Zhao et al., 2021).

Physiological adaptations in response to salt stress, plants alter their pace of development, turn on ion transporters to maintain ion balance, use osmotic and oxidative stress-reduction techniques, and store osmotic adjustment materials like nitrogen molecules and carbohydrates (Zhao et al., 2021; Sumbul et al., 2020).

Genetic methods increasing agricultural yield in salty settings requires the development of salt-tolerant crop varieties using genetic methods such as interspecific hybridization, genetic alteration, and breeding programs (Zhao et al., 2021).

Using microorganisms to help plants resist salt stress using the potential of microorganisms, especially those that are suited to the salt, can be a promising way to help plants cope with salt stress and become more resilient. These microorganisms are called plant growth promoting microorganisms ‘PGPMs’.

4. Plant growth promoting microorganisms (PGPMs)

PGPMs are a class of beneficial bacteria and fungi that, via a variety of processes, both promote and shield plants against disease and abiotic stressors. These microbes can settle in rhizospheres and plant roots, where they engage in interactions with the plant and its surroundings (Cherif-Silini et al., 2021; Lopes et al., 2021).

4.1. Plant growth promoting rhizobacteria (PGPR)

A class of bacteria known as plant PGPR is present in the rhizosphere or the area around plant roots and promote plant growth. The rhizosphere, or accessible soil zone for plant roots, is a zone of high microbial activity that creates a small nutrient pool from which vital macro- and micronutrients are derived. Since root exudates serve as a source of nutrients for microbial growth, the microbial population in the rhizosphere differs somewhat from that of its surrounds. PGPR can be classified as symbiotic bacteria based on their interactions with plants; these bacteria reside inside plants and exchange metabolites with them (Vejan et al., 2016; Ghadamgahi et al., 2022).

4.2. Plant growth promoting fungi (PGPFs)

PGPFs are a diverse collection of non-pathogenic fungi that can be found in the plant's roots, on their root surfaces, or in the rhizosphere. They help plant growth development (seed germination, seedling vigor, and photosynthetic efficiency), protect from diverse phyto-pathogens, permit root expansion and soil improvement. Though their precise mechanism of action is unknown, it is believed that fungi that promote plant growth could aid in the high yield production of plants, agricultural crops, herbal remedies, and uncommon medicinal plants. (Kumari et al., 2021). Their mechanisms of action involve solubilizing and mineralizing nutrients for easy uptake by plants, regulating hormonal balance, producing volatile organic compounds and microbial enzyme, suppressing plant pathogens and ameliorating abiotic stresses. Moreover, PGPFs are expected to have positive effects on human health and the ecosystem. The interaction between PGPFs and plants requires a certain level of specificity for root colonization and growth promoting effects (Kumar Das, 2020).

5. Mechanisms of action of PGPMs

PGPMs can act in a direct and indirect manner (Table 1). The direct mechanisms are biofertilization, stimulation of root growth, rhizoremediation, and plant stress control. For example; the PGPMs that possess 1-aminocyclopropane-1-carboxylate deaminase ACCD activity regulate growth and development of plants under harsh environmental conditions by limiting ethylene levels in plants; this enzyme is, therefore, often called a “stress modulator” (Shahid et al., 2023). On the other hand, the mechanism of biological control by which rhizobacteria are involved as plant growth promotion indirectly is by reducing the impact of diseases, induction of systemic resistance and competition for nutrients and niches (Vejan et al., 2016).

Table 1. List of PGPMs and their mechanisms of action.

PGPRs	PGPR mechanisms	Crops	References
Azoarcus	- Nitrogen fixation - 1-aminocyclopropane-1-carboxylate (ACC) deaminase - Production of indole-3-acetic acid (IAA)	Rice	(Fernández et al., 2014; Fernández-Llamosas et al., 2020).
Azobacter	- Cytokinin synthesis - Nitrogen fixation - IAA - ACC deaminase - Solubilization of nutrients phosphorus, potassium, and zinc	Cucumber, wheat, barley, oats, rice, sunflowers, maize, line, beetroot, tobacco, tea, coffee and coconuts	(Stanković et al., 2015; Aasfar et al., 2021)
Azorhizobium	- Nitrogen fixation - IAA	Wheat	(Jalal et al., 2023)
Azospirillum	Nitrogen fixation	Sugar cane	(Vejan et al., 2016)
Bacillus	- Cytokinin synthesis - Gibberelin synthesis - Potassium solubilization - Induction of plant stress resistance - Antibiotic - Siderophore - IAA	Potato, cucumber, pepper, peanuts, maize, alfalfa	(Vejan et al., 2016; Bessai et al., 2023)
Paenibacillus	- IAA - Potassium solubilization	Lodgepole pine, black pepper	(Vejan et al., 2016)
Phyllobacterium	- Phosphate solubilization - Siderophore	Strawberries	(Vejan et al., 2016; Backer et al., 2018)

Pseudomonas	- Chitinase and β-glucanases production - ACC deaminase - Induction of plant stress resistance - Antibiotic - Siderophore	Mung beans, wheat, Cotton, Maize, Potato	(Vejan et al., 2016; Ghadamgahi et al., 2022)
Rhizobia	- Nitrogen fixation - Induction of plant stress resistance - Hydrogen cyanide production	Legumes, Peanuts	(Vejan et al., 2016)
Rhizobium	- Nitrogen fixation - IAA - ACC deaminase - Siderophore production	Rice, pepper, tomato, lettuce, carrot, mung, beans	(Vejan et al., 2016)
Penicillium	- IAA - Phosphate solubilization - Inhibition of phytopathogens - Mitigation of abiotic stress	Rice, maize	(Adedayo and Babalola, 2023)
Trichoderma	- Phosphate solubilization - IAA - Inhibition of phytopathogens - Volatile organic compounds (VOCs) - Production of antifungal and antibacterial agents such as xylanase, cellulase, pectinase, protease, and chitinase		(Adedayo and Babalola, 2023)
Arbuscular mycorrhizal fungi	- Carry out an important role in alleviating abiotic stresses		(Galeano et al., 2021).

Aspergillus niger	- IAA - Siderophores - Phosphate solubilization, - ACC deaminase - Enzymes such as proteases, phosphatases, and other hydrolases	Forage grass	(Galeano et al., 2021).
Purpureocillium	- IAA Phosphate solubilization		(Adedayo and Babalola, 2023)
Piriformospora	- Volatile organic compounds (VOCs) - IAA - Phosphate solubilization - ACC deaminase		(Hossain and Sultana, 2020; Sreedevi et al., 2022)

5.1. Biofertilizers

PGPMs can function as biofertilizers by enhancing nutrient availability and balancing hormonal regulation in plants, thereby promoting plant growth and development (Vejan et al., 2016). They accomplish this through a number of ways, such as:

- **Nitrogen fixation**

In agricultural production, nitrogen is the major nutrient that has a remarkable effect on plant growth. The free-living and symbiotic bacteria in nature can fix atmospheric N_2 by means of the enzyme nitrogenase (Shultana et al., 2022). Nitrogen-fixing organisms are generally categorized as: a) symbiotic nitrogen fixing bacteria including members of the family rhizobiaceae which forms symbiosis with leguminous plants (e.g. Rhizobia) and non-leguminous trees (e.g. Frankia) and b) non-symbiotic nitrogen fixing forms such as cyanobacteria (anabaena, nostoc), azospirillum, azotobacter, gluconoacetobacter diazotrophicus and azocarus … etc (Gangopadhyay and Ghosh, 2019).

- **Phosphate solubilization**

Phosphorus is an essential component of biological molecules and one of the primary factors limiting biomass output (Tian et al., 2021). One important process that promotes plant development and nutrient absorption is phosphate solubilization PGPMs. By using a variety of strategies, phosphate-solubilizing microorganisms solubilize phosphate molecules making it available to plant. The primary methods via which phosphate is solubilized are mineral dissolving compounds including siderophores, organic acids (OAs), carbon dioxide (CO_2), and hydroxyl ions (OH^-) (Tariq and Ahmed, 2023). Phosphate-solubilizing microorganisms interact with insoluble phosphate substances such as hydroxyapatite and tricalcium phosphate through chelation and exchange processes (Gupta et al., 2021). Phosphate solubilization is facilitated by enzymes, which disassemble complex organic and inorganic phosphate molecules into simpler forms that are easily absorbed by plants (Gupta et al., 2022).

- **Phytohormones**

Apart from the phytohormones generated by the plant, soil microbes are a source of exogenous phytohormones. These include compounds produced and expelled by microorganisms, which are advantageous to plants such as auxins, cytokinins, ethylene, gibberellins, abscisic acid (ABA) (Egamberdieva et al., 2017; Kumar et al., 2022) jasmonates, strigolactones, brassinosteroids (Kumar et al., 2022).

5.2. Plant stress control

PGPMs can encourage root growth by lengthening and densening the roots. This increases agricultural output, enables plants to absorb water and nutrients, and preserves their osmotic balance. Plants can absorb more water and nutrients because to their improved root system, which helps them withstand stress (Chieb and Gachomo, 2023).

Plant defense mechanisms can be triggered by PGPMs, which increases resistance to a variety of stressors, including drought. This systemic resistance aids in a plant's ability to withstand harsh environments and continue growing when stressed (Grover et al., 2021; Chieb and Gachomo, 2023).

Essential plant hormones involved in defense and stress responses, such as salicylic acid (SA), ethylene and abscisic acid (ABA), can have their levels influenced by PGPMs. Plant stress tolerance is enhanced by PGPMs through the regulation of these hormone levels (Chieb and Gachomo, 2023; Jalal et al., 2023).

5.3. Rhizoremediation

Rhizoremediation—where "rhizo" means "root" and "remediation" means "degradation of organic pollutants"—is a potentially effective method for eliminating toxins from soil (Saravanan et al., 2020). This process uses interactions between plants and microbes to break down or immobilize pollutants in the soil, especially organic pollutants like pesticides, oils, volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), persistent free radicals (PFRs), and petroleum hydrocarbons (Chojnacka et al., 2023). PGPR are essential for rhizoremediation because they break down or immobilize pollutants in the soil through a variety of processes. In rhizoremediation, a few of the main PGPR processes include rhizodegradation and rhizoattenuation in addition to the use of functional materials, such as carbon materials and nanomaterials, which increases the effectiveness of rhizoremediation (Saeed et al., 2021).

5.4. PGPMs in competition for nutrients and niches

Through a variety of processes, particularly in the fight for resources and habitats, PGPM***s*** significantly contribute to the enhancement of plant growth and health. Few of the main processes played in nutrient competition are iron sequestration and phosphate solubilization. Furthermore, PGPMs often produce antimicrobial compounds such as antibiotics and antifungal substances. These compounds can inhibit the growth of potential pathogens in the rhizosphere, reducing competition for nutrients (Tahir et al., 2017; Bessai et al., 2023).

Regarding competition for niches, PGPMs are effective in colonizing the plant root and further multiplying into microcolonies and/or producing biofilm as a result of a successful plant-microbe interaction and these plant-associated biofilms are highly capable of providing protection from external stress, decreasing microbial competition, and giving beneficial effects to the host plant supporting growth, yield and crop quality as reported (Kasim et al., 2016). PGPMs can efficiently utilize root exudates produced by plants, creating a competitive advantage for colonization. This utilization of plant-derived compounds helps in establishing a strong association between PGPMs and plant roots (Parrow et al., 2013)

Methodology

Methodology

1. Objective of the study

This study aims to test the plant growth promoting potential of selected microbial species, isolated in a previous study, under saline stress both *in vitro* and *in vivo* on Maize seeds.

2. Material and methods

2.1. Material

2.1.1. Microbial species

In this study 5 bacteria and 1 fungus isolated in previous works have been tested for their potential to promote plant growth. These isolates have been identified as bacteria; *Bacillus rugosus*, *Cytobacillus oceanisediminis*, *Paenibacillus lautus*, *Pseudomonas granadensis* and a fungus *Penicillium tardochrysogenum* and were isolated from Essebkha in Skhouna region. The fifth bacteria *Enterococcus gallinarum* has been isolated from petroleum contaminated soil.

2.1.2. Plant material

Maize seeds were used in this study to determine the effect of the microbial isolates in promoting its growth.

2.2. Methods

2.2.1. In vitro test

2.2.1.1. Halotolerance test

The tolerance to salinity of the bacterial isolates and the fungus (i.e., halotolerance) was tested with increasing NaCl concentrations (0, 300, 600 and 900 mM) using nutrient-broth medium (NB). For each NaCl concentration, the bacterial and fungal strains were inoculated with an initial OD_{600} = 0.1 and incubated under shaking (150 rpm) at 30°C during 24 h for the bacteria and at room temperature for the fungus during 7 days. Then, bacterial growth was measured as cell density determined using spectrophotometric readings at 600 nm. Each NaCl concentration was tested in triplicate (Sharma et al., 2016).

2.2.1.2. Production of indole-3-acetic acid (IAA) test

The Production of indole-3-acetic acid (IAA) was tested utilizing the following steps. The bacterial strains were grown for 24 h and the fungus for 7 days, following that 100 µl of freshly grown bacterial and fungal culture were taken separately at a density of approximately 1.5 x10^8 CFU/ml, and inoculated in nutrient-broth (NB) supplemented with 1% of L-Tryptophan. After inoculation in the NB medium, the bacterial and the fungal strains were incubated on a rotary shaker at 180 rpm for 4 days at 30 ± 2°C for bacteria and for 7 days at room temperature for the fungus. After centrifugation for 10 min at 10,000× g, 1 ml of supernatants were collected and mixed with 4 ml of Salkowski's reagent. The mixtures were incubated for 30 min in the dark at 25 ± 2°C, then the absorbance was read using a spectrophotometer at 530 nm. The IAA quantification was performed based on standard curves prepared with pure IAA (Lebrazi et al., 2020). All experiments were performed in triplicate. Furthermore, we tested the effect of NaCl on IAA production by adding increasing NaCl concentrations (0, 300, 600 and 900 mM) to the media. After inoculation and incubation, in the same conditions as previously described, the amount of IAA produced was estimated spectrophotometrically at 530 nm.

2.2.1.3. Production of siderophores

Quantitative estimation of siderophore was done by taking supernatant of bacterial cultures grown in Luria Bertani broth medium. For this, 1 ml broth was taken in 1.5 ml centrifuge tube and after sterilization inoculated with 100 µl of freshly grown bacterial culture (10^8 CFU/ ml). Three replicates (tubes) were taken for each strain. Apart from this, control tube (uninoculated broth) was also maintained. After incubation at 28°C for 48 h, bacterial cultures were centrifuged at 10,000 rpm for 10 min, cell pellets were discarded, and supernatant was used to estimate siderophore. Supernatant (0.5 ml) of each bacterial culture was mixed with 0.5 ml Chrome Azurol S (CAS) reagent and after 20 min optical density was measured at 630 nm. We tested the effect of NaCl on siderophore production by preparing LB media (as previously described) with increasing NaCl concentrations (0, 300, 600 and 900 mM). After inoculation and incubation (in the same conditions as previously described), the amount of siderophore produced was estimated spectrophotometrically at 630 nm. Siderophore produced by strains was measured in percent siderophore unit (psu) which was calculated according to the following formula (Arora & Verma, 2017):

Siderophore production (psu) $= \frac{(\mathrm{Ar}-\mathrm{As})\times 100}{\mathrm{Ar}}$

where Ar = absorbance of reference (CAS solution and un-inoculated broth), and

As = absorbance of sample (CAS solution and cell-free supernatant of sample)

2.2.2. In vivo test

2.2.2.1. Plant growth-promoting potential of the isolates

Maize (*Zea mays*) seeds were surface sterilized with bleach for 5 min. The seeds were washed two times with sterilized distilled water for 5 min. To prepare the inoculum (10^8 CFU/ ml), the microorganisms were previously grown in Petri dishes containing nutrient agar medium for 24 h at 35°C for the bacteria and at 30 ± 2°C during 7 days for the fungi.

For seed treatment, 10 ml of the microbial suspension was added to 120 seeds and homogenized to contact the seeds with the microorganisms. 10 maize seeds were sown in Petri dish containing filter paper. The experiment was designed to have three treatments and three repetitions: (i) Control without microorganisms and normal irrigation; (ii) inoculated with microorganisms and without salt stress; (iii) irrigated with 50, 300, 600, 900 mM NaCl and inoculated with microorganisms. The pots were maintained at 25 °C ± 2. After three days, thinning was performed, keeping one per Petri dish. After that we seed and we put them in sterilized plastic pots. To simulate normal irrigation with tap water, after every 24 hours, the plants were collected to measure roots and stems lengths.

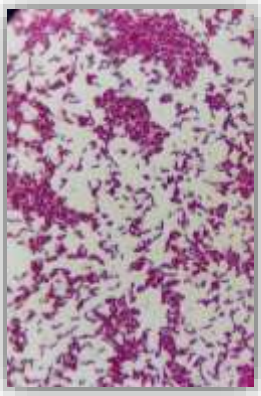
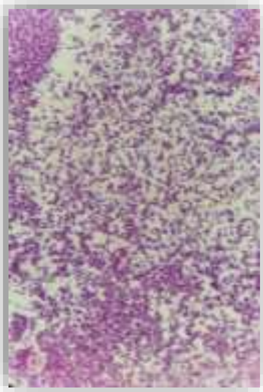
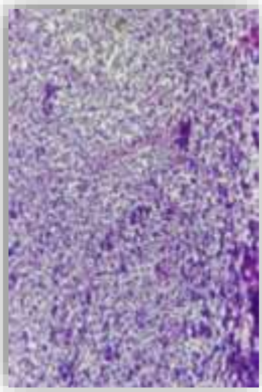
Results

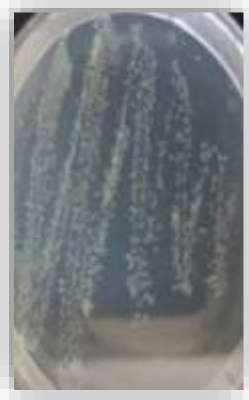
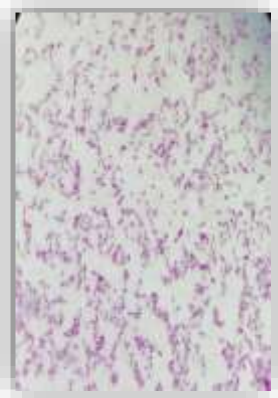
Results

1. Macroscopic and microscopic observations of the tested microbial isolates

The tested microorganisms are represented in Table 2.

Table 2. Macroscopic and microscopic observations of the tested microorganisms.

Microbial isolates	Code	Macroscopic observation	Microscopic observation
Bacillus rugosus	Ba		
Enterococcus gallinarum	C50		
Cytobacillus oceanisediminis	B8		

Paenibacillus lautus	B11(2)		
Pseudomonas granadensis	Ps		
Penicillium tardochrysogenum	PN		

2. In vitro test

2.1. Halotolerance test

We notice that the bacteria *Bacillus rugosus*, *Enterococcus gallinarum*, *Cytobacillus oceanisediminis* and *Pseudomonas granadensis* and the fungus *Penicillium tardochrysogenum* showed growth in salinity up to a concentration of 600 mM of NaCl. However, above that concentration (at 900 mM NaCl) we notice a decrease in growth rate (Fig. 2). *Bacillus rugosus* demonstrated the higher growth rate compared to the other microorganisms tested and it has shown the ability to form a biofilm on the surface of the medium (Fig. 3). In another side *Paenibacillus lautus and Pseudomonas granadensis* were found to tolerate salinity from 0 to 300 mM and a decrease in growth is seen at a concentration of 600 mM.

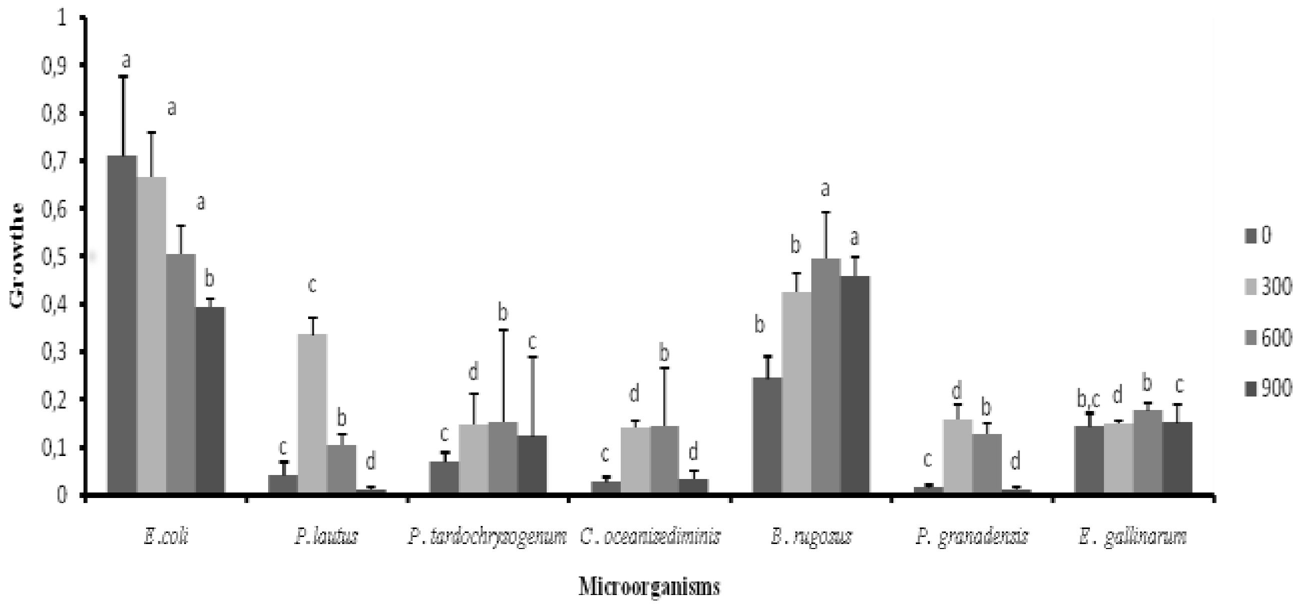


Figure 2. Halotolerance of the tested microbial isolates.

Figure 3. A biofilm formed by *Bacillus rugosus***.**

2.2. Production of indole-3-acetic acid (IAA)

We notice in this study that the highest production of auxin in normal condition (0 mM of NaCl) was shown from the bacteria *Paenibacillus lautus* 8.57 µg/ml and the lowest production was shown from the fungus *Penicillium tardochrysogenum* 2.11 µg/ml. The

highest production under 300 mM NaCl is recorded in the bacteria *Pseudomonas granadensis* 9.48 µg/ml and the lowest one in the fungus *Penicillium tardochrysogenum* 7.07 µg/ml.
The highest production in 600 mM of NaCl was seen in the bacteria *Bacillus rugosus* 8.68 µg/ml and the lowest production was seen the bacteria *Pseudomonas granadensis* 6.17 µg/ml. Moreover, the highest production in 900 mM of NaCl was shown by the bacteria *Bacillus rugosus* 8.09 µg/ml and the lowest production by the bacteria *Pseudomonas granadensis* 2.82 µg/ml (Fig. 4). In general, *B. rugosus* demonstrated the higher production rates of auxin under all the tested concentration of NaCl.

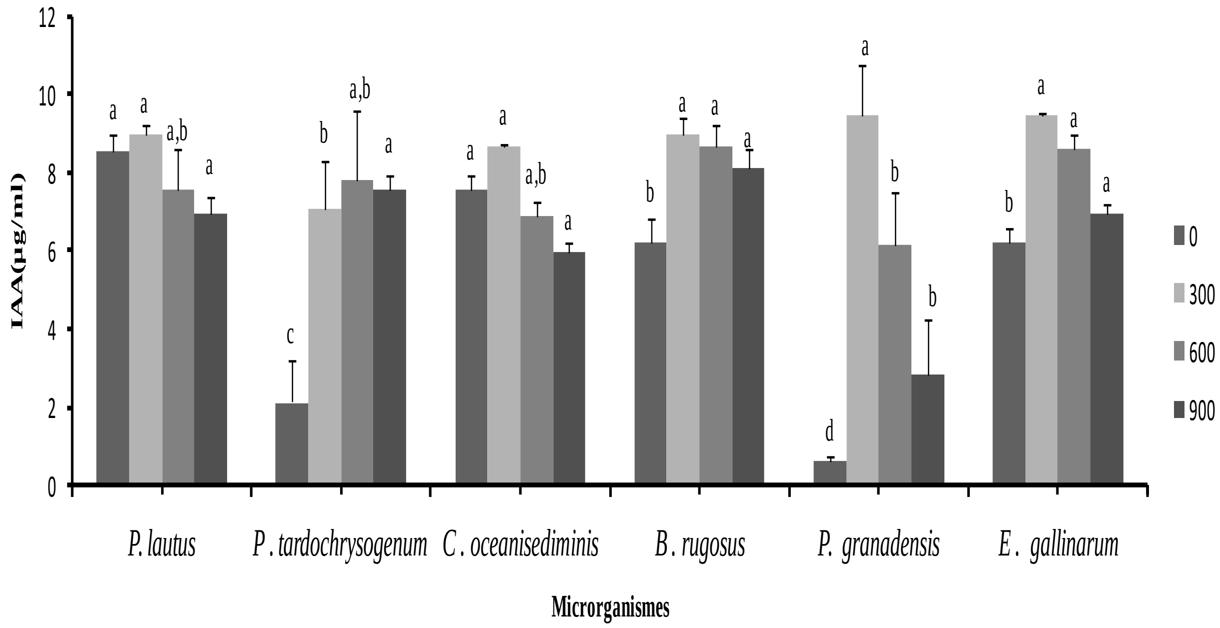


Figure 4. Production of IAA by the tested microbial isolates.

2.3. Production of siderophores

We notice in this study that the highest production of siderophores in normal condition (0 mm of NaCl) was seen in *Paenibacillus lautus* 72.44 % and the lowest production was seen in *Pseudomonas granadensis* 39,73 %. *Pseudomonas granadensis* showed the highest production in 300 mM of NaCl (81,79 %) and *Bacillus rugosus* the lowest production (42,48 %). The highest production in 600 mm of NaCl was recorded from *Paenibacillus lautus* 89,68 % and the lowest production from *Bacillus rugosus* 38.065 %. *Paenibacillus lautus* showed the highest production (95,46 %) in 900 mM of NaCl and *Bacillus rugosus* the lowest production 37.95 % (Fig. 5).

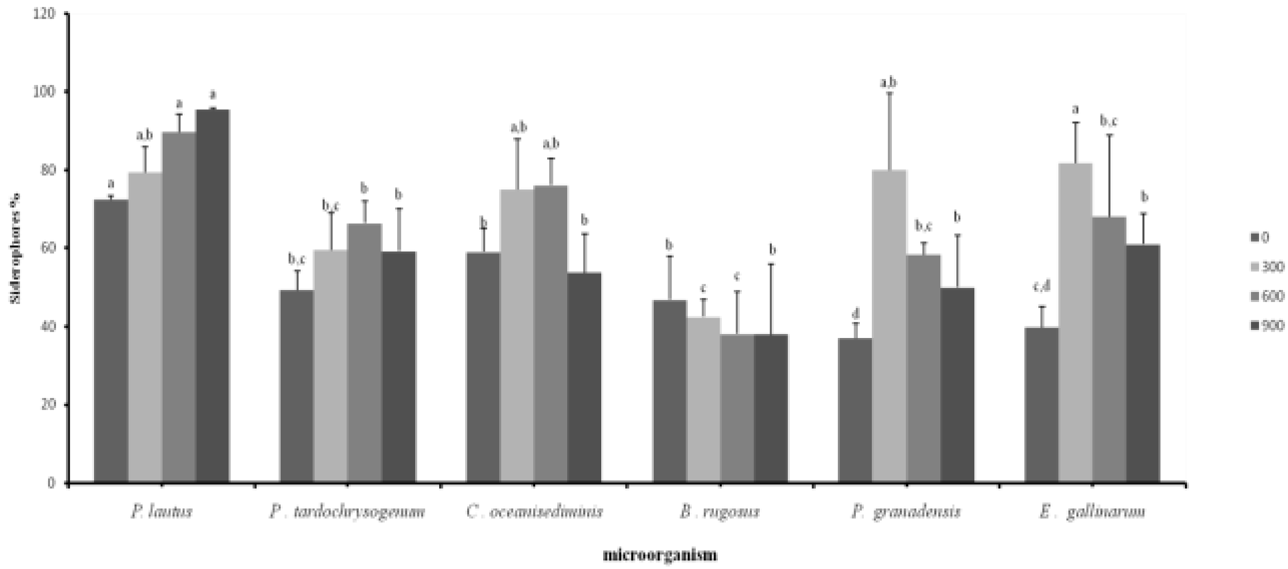


Figure 5. Production of siderophores in the tested microbial isolates

3. In vivo test

It should be noted that the seed of Maize that were tested at concentration of 300, 600, and 900 mM of NaCl did not germinate and even got altered. However, the seed treated with 50 mM NaCl showed different responses depending on the microbial isolate being treated with (Fig. 6).

Figure 6. Maize (*Zea mays*) growth after treatment with the microbial isolates.

3.1. Root growth

For root growth, significant increase after 48 hours in normal condition was exhibited by seeds treated with *Cytobacillus oceanisediminis*, 31.6 mm and *Pseudomonas granadensis* 34.7 mm. the other tested isolates showed relatively similar root lengths (Fig. 7).

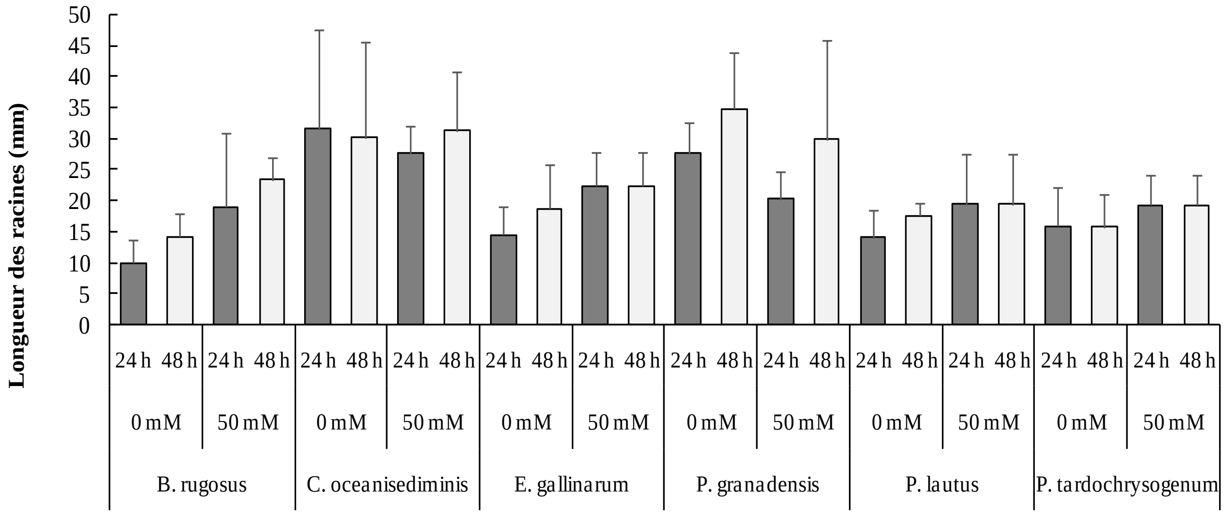


Figure 7. Root length of Maize treated with the microbial isolates.

3.2. Shoot growth

The results revealed a range of reactions to growth conditions. After 48 hours in normal conditions, there was a noticeable increase in shoot length demonstrated by *Bacillus rugosus* 21.6 mm and *Cytobacillus oceanisediminis* 27.3 mm. The seeds treated with the rest of microbial isolates showed relatively similar results (Fig. 8).

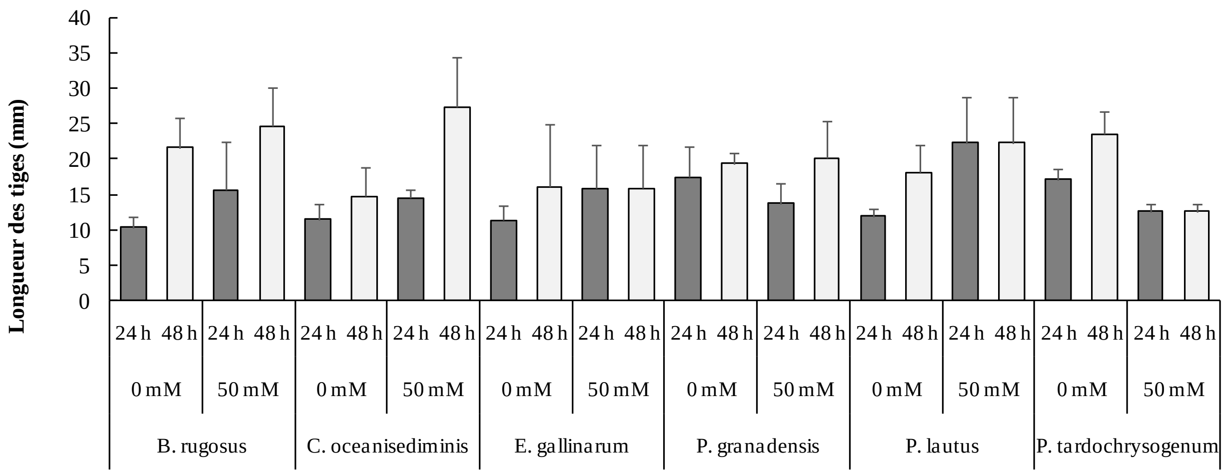


Figure 8. Shoot length of Maize treated with the microbial isolates.

Discussion

Discussion

Soil salinity, one of the most significant variables that affect crop, is present everywhere and has a variety of effects on the soil characteristics (Shilev, 2020). Over 20 % of arable land globally is damaged by salt, and by 2050, that percentage is predicted to rise to 50 % (Stanković et al., 2015). PGPM have been found effective in increasing the drought tolerance of plants that can be used for sustainable agriculture practices. This can be achieved by inducing many mechanisms, including alteration of root architecture, osmoregulation, production of phytohormones and extracellular polysaccharides, and regulation of antioxidants (Vejan et al., 2016; Galeano et al., 2021; Bessai et al., 2023).

In this study, 5 bacteria and 1 fungus were isolated from the Sabkha and petroleum contaminated soil to study their plant growth promoting (PGP) potential under saline stress both in vitro and in vivo.

Results demonstrated that all tested microbial strains had PGP traits, including halotolerance, production of siderophores, and IAA production. Bacterial strains demonstrated excellent resistant to salinity. Maximum salinity was showed by *Bacillus rugosus, Enterococcus gallinarum* and *Cytobacillus oceanisediminis*.

B. rugosus demonstrated salt tolerance up to 900 mM of NaCl which is in agreement with other studies that showed that *Bacillus rugosus* is a halotolerant bacterium that grows well in soil and plants with high salt levels. They showed that proline levels and plant growth metrics are markedly elevated by a strain NOK85 which is a good option for bioinoculants for salt soil phytoremediation and reducing salt stress in plants, as it can also thrive at 55°C and 10 % NaCl (Chebotar et al., 2022). Under salinity, the halotolerant bacterium *B. rugosus* may synthesize significant amounts of exopolysaccharide (EPS), which surrounds plant roots in a nutrient-rich sheath and serves as a physical barrier against salt stress. Additionally, EPS possesses antioxidant qualities that provide resistance to oxidative damage brought on by salinity (Ramasamy and Mahawar, 2023). Under conditions of high salt, *Bacillus rugosus* can accumulate suitable osmolytes such as trehalose, ectoine, and betaine to preserve osmotic balance and cellular integrity (Sagar et al., 2022; Ramasamy and Mahawar, 2023). Their ability to control the absorption, movement, and segregation of ions such as Na^+ and K^+ helps prevent ion toxicity and preserve healthy cellular processes (Ji et al., 2022; Ramasamy and Mahawar, 2023).

In order to scavenge reactive oxygen species (ROS) produced under salt stress conditions, halotolerant bacteria can upregulate their antioxidant enzymes and metabolites (Ramasamy and Mahawar, 2023). This protects bacterial cells from oxidative damage and improves their halotolerance. Another method that microorganisms use to live and prosper in salty conditions is the creation of biofilms. To promote halotolerance, the biofilm matrix retains nutrients, acts as a physical barrier, and aids in quorum sensing (Ramasamy and Mahawar, 2023).

Based on our investigation, we showed that the bacteria *Enterococcus gallinarum* has a tolerance of 600 mM of NaCl. Many studies validated our findings and showed that *E. gallinarum* is a halotolerant microbe that thrives in high salinity soil and plants. Research on the isolation of the halotolerant *Egallinarum* bacterium from saline settings has demonstrated that this bacterium thrives in conditions with elevated salt concentrations. It is resistant to vancomycin and can withstand up to 6.5 % NaCl (Maryum et al., 2022). This bacteria, thrives in salt-rich environments due to its regulation of ion homeostasis, osmotic tolerance, antioxidant defense, genetic adaptations, physiological adaptations, signal transduction pathways, compartmentalization, and hormone regulation (Maryum et al., 2022).

Our inquiry uncovered that the bacteria *Cytobacillus oceanisediminis* has a tolerance at 600 mM of NaCl. Studies have demonstrated that *C. oceanisediminis* is a halotolerant bacterium that grows well in soil and plants with high salt levels. Its ideal development temperature is 30 °C, and it can withstand up to 8 % NaCl (w/v) (Gao et al., 2023). This bacterium regulates halotolerance by controlling exopolysaccharides, siderophores, ion homeostasis, osmotic adjustment, antioxidant processes, and genetic adaptations (Zhou et al., 2023).

Excellent potential was shown by bacterial strains for auxin production in vitro. Maximum production of auxin was demonstrated by *Bacillus rugosus*, *Paenibacillus lautus* and *Pseudomonas granadensis*. We found in our research that the IAA production of *Bacillus rugosus* has been reported to be around 6.22 – 9.011 mg/ml and the maximum IAA production by *B. rugosus* was under 600 mM of NaCl. Previous researches showed that the maximum IAA production by B. *rugosus* under optimal conditions is 0.067.18 mg/ml (Dasri et al., 2014). Different concentrations may be explained by the different experimental conditions i.e. they employed an alternative medium (LB) that contained a different concentration of salt, a various concentration of L-tryptophan, a different temperature 32°C, and a different incubation period (4 days). The ideal circumstances for IAA production by *B. rugosus* are King-B medium supplemented with 2.5 mM L-tryptophan (0.5 g/l), pH 7.0, at 37°C in static condition for 6 days (Dasri et al., 2014). *Pseudomonas granadensis* has been reported to produce between 3.49 – 9.48 mg/mL of IAA, with its maximal production occurring in a salinity environment of 300 mM NaCl. Studies indicated that *Pseudomonas granadensis* may yield a maximum of 0.318 mg mL-1 of IAA after 24 hours of incubation under different circumstances (Sasirekha and Shivakumar, 2012). The ideal conditions for *Pseudomonas granadensis* to create IAA are basic yeast extract mineral medium supplemented with 0.1 mg mL-1 L-tryptophan, pH 7.0, at 37°C for six days.

Our research showed that *Paenibacillus lautus* produces between 6.92 -8.99 mg/mL of IAA, with its maximal production occurring in a salinity environment of 300 mM NaCl. We noticed additional data demonstrating that *Paenibacillus lautus* may produce up to 5.3-5.0 µg/mL of IAA during different circumstances of incubation (Xue et al., 2023).

Outstanding potential was shown by bacterial strains for the in vitro manufacture of siderophores. Maximum synthesis of siderophores was demonstrated by *Paenibacillus lautus, Pseudomonas granadensis* and *Cytobacillus oceanisediminis*.

We reported that *P. lautus* can create siderophores at a rate of roughly 95.46 % when exposed to high salinity (900 mM NaCl). Xue et al. (2023), showed that two species belonging to the same genus; *Paenibacillus radicibacter* sp. and *Paenibacillus radicis* sp. have the capacity for siderophores production.

Our investigation demonstrated *Pseudomonas granadensis* produces siderophores at a rate of 81.79 % when exposed to 300 mM NaCl. Barbaccia et al. (2022), found that all *Pseudomonas* strains tested positive for siderophores production and one of them is *Pseudomonas granadensis.*

Cytobacillus oceanisediminis may produce siderophores at a rate of approximately 76.06 % at 600 mM NaCl. Results found by Bano et al. (2022) support our findings and showed that *C. oceanisediminis* could produce siderophores.

Besides, the evaluation of wheat's response to salt stress following inoculation with the microbial isolates has provided critical insights into their potential to enhance plant stress tolerance. *Cytobacillus oceanisediminis* and *Pseudomonas granadensis* showed the higher increase in root length while *Cytobacillus oceanisediminis* and *Bacillus rugosus* exhibited higher rise in stem length. Research conducted in 2020 by Pereira et al. on water-stressed maize (*Zea mays* L.) reveals that treating seeds with two PGPR strains: *Cupriavidus necator* and *Pseudomonas fluorescens* increases yield. In comparison to the control, it results in an increase in the length of the plant's roots and shoots.

Plant growth is known to be impacted by PGPR. It has been discovered that PGPRs increase crop productivity by lengthening and multiplying their roots. Salem et al. (2024) found that the application of *B. rugosus* increased the levels of proline and chlorophyll while decreasing those of malondialdehyde. The results show how well PGPR inoculation works to improve wheat's morphology, physiology, and resistance to drought stress. Isolated strains of PGPR show potential as biofertilizers to increase grain output in water-scarce environments.

Conclusion and perspectives

Conclusion

Through this study we could conclude that the microbial strains tested in this study harbour beneficial characteristics for plant growth promotion.

Bacterial strains demonstrated excellent resistant to salinity. Maximum halotolerance was showed by *Bacillus rugosus, Enterococcus gallinarum, Cytobacillus oceanisediminis,* and the fungus *Penicillium tardochrysogenum.* Excellent potential was shown by bacterial strains for auxin production in vitro. Maximum production of auxin was demonstrated by *Bacillus rugosus*, *E. gallinarum* and *Pseudomonas granadensis*. In addition, bacterial strains also demonstrated outstanding promise for the in vitro synthesis of siderophores where maximum production was shown by *Paenibacillus lautus, P. granadensis*, and *E. gallinarum*.

Moreover, in vivo plant growth indicators such as root length and shoot length have been shown to increase when microbial isolates were added to Maize seeds. Strains that showed better increase in root length and shoot length, are *Cytobacillus oceanisediminis*, *Pseudomonas granadensis* and *Bacillus rugosus*.

These results reflect the positive impact of the used microbial strains especially bacteria for plant growth enhancement. Hence, the tested isolates can be used for crop inoculations and plant growth enhancement. However, these strains need to be tested under field conditions and with more crops before being considered biofertilizer candidates.

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