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Bacillus safensis strain YA8 linked with *Opuntia* ficus-indica leaves: Molecular identification and impact on *Cucumis sativus* growth under salt stress

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Abstract: This study aimed to identify the bacterial endophytic strain linked to the leaves of the *Opuntia ficus-indica* plant that grows close to the Makkah Desert in the Kingdom of Saudi Arabia. Bacterial endophytes can considerably penetrate plant leaves. For the sake of plant survival, living in demanding (salinity and drought) ecosystems seems to be especially crucial. Several endophytic bacteria were isolated from the leaves of *Opuntia ficus-indica*. For phylogenetic analysis, the 16s rRNA region was sequenced. To study phylogeny, the ten species were divided into three different operational taxonomic groups and classified as Operational taxonomic unit (OTUs). Four isolates were examined, all having plant growth-promoting abilities among them. *Bacillus safensis* strain YA8 was assigned as one of the isolates that stood out for its ability to stimulate plant growth by producing the Indole-3-acitic acid (IAA) enzyme. *Bacillus safensis* strains have shown strong antimicrobial effects, successfully suppressing growth of *Staphylococcus aureus* with a 9 mm inhibition zone, *Sarcoptes scabiei* with a 12 mm zone, and *Erwinia carotovora* with a 19 mm zone. The results indicate that these strains could be effective in treating infections from these pathogens. The measurements of zone of inhibition in the well diffusion test demonstrate the potent antibacterial activity of *Bacillus safensis* strains YA8. These strains specifically show inhibitory zones that measure 9, 12, and 19 mm against *Erwinia carotovora*, *Streptomyces scabiei*, and *Staphylococcus aureus*, respectively. The study found that the creation of IAA has increased the length of the roots and shoots and helped in biomass production as well as total chlorophyll contents in different NaCl concentrations (100 mM, 150 mM, and 200 mM). Although *Opuntia ficus-indica plants* have an unusually wide variety of endophytic associations, the bacillus genera alone, which are frequently reported as endophytes in plant leaves, predominated in the bacterial community. This study demonstrates that a variety of endophytic bacteria are present in *Opuntia ficus-indica leaf* tissue. These bacterial endophytes' potential contributions to plant hosts' ability to withstand harsh situations are examined.
Key words: *Opuntia ficus- indica*, Salinity, *Bacillus safensis*, Endophytes

Introduction

Excess salt of any variety is hazardous to plant health. High salinity soils alter the physiological processes of plants. The high salt concentration has a negative impact on vital soil processes such as respiration, residual degradation, nitrification, denitrification, soil biodiversity, and microbial activity[1]. When excessive fertilizer is applied to the soil, crop production is lost, and the soil

becomes highly salinized [2]. Furthermore, farming practices affect how well crops thrive in salt soil. In these conditions, deeper ploughing or tilling results in more water evaporating from the surface of the ground and salts being deposited.

Furthermore, the salt content of irrigation water may increase soil salinity, which lowers productivity.[3]. The method of removing salt from salty soil is labor-intensive and expensive [4]. However, physical and chemical methods have been the mainstays of saline soil restoration for a very long time. In a physical procedure, scraping, flushing, and leaching techniques are used to remove soluble salts from the root zone [5]. But when the salt content is too high, these solutions are deemed ineffective and not sustainable.

Endophytic Bacteria colonize the cell walls and xylem vessels of plant roots, stems, and leaves. The bacteria are also present in the tissues of flowers [6], fruits [7], and seeds [8]. Endophytes can promote plant growth by making nutrients like nitrogen and phosphorus more accessible or by producing substances that include plant growth hormones and are mediated by the microbes and the host cells [9] .Endophytes also aid in the availability and uptake of nutrients, improve stress tolerance, and provide disease resistance for plants [10].

Endophytes can be employed as bioinoculants in agriculture due to their ability to promote plant growth and fight disease, which will help the development of sustainable agricultural production methods [11].

The dicotyledonous angiosperm *Opuntia ficus-indica* (L.) Mill. is a plant commonly referred to as the prickly pear or nopal cactus. It belongs to the Cactaceae family, which contains over 1500 species and 130 genera. This plant can grow anywhere in the world in dry or semi-arid conditions and has a large economic potential [13]. This plant is indigenous to Mexico and can be found widely throughout the country, all over the American continent, Africa, and the region that comprises the Mediterranean, since it has the ability to heal a variety of illnesses and ailments, particularly those with anti-inflammatory capabilities, it has been utilized as conventional local medicine. effects of hypoglycemia that reduce stomach ulcers and have neuroprotective properties in many nations throughout the world, it is also used to cure indigestion, burns, bronchial asthma, diabetes, and other conditions through its antioxidant properties.

The aim of this study is to investigate the endophytic bacteria *Bacillus safensis* YA8 linked to leaves of *Opuntia ficus- indica* growing in the desert of Makkah, Saudi Arabia. Through the amplification of 16s rRNA region of bacterial genomic DNA, we were able to isolate and identify the endophytic bacteria *Bacillus safensis* YA8 that related to the leaves of the *Opuntia ficus- indica* plant. This investigation provides information on the interaction between *Opuntia ficus- indica* and its endosymbiotic microorganisms, particularly bacterial endophytes. This is the first research done on Cucumis sativus.

Material and Methods

Isolation of bacterial endophytes

The leaves sample of *Opuntia ficus- indica* were collected from the desert of Bahra region Makkah on 14 February 2022. Plants without visible damage were collected in a polythene bag and stored in the ice box. In the lab, the healthy leaves of *Opuntia ficus- indica were* cut off from the plant for further research. The leaves were then surface sterilized with ethanol (70%) for 4 min, and sodium hypochlorite (1%) for 1 min, followed by three rinses in sterile distilled water for 3 min each, the leaves were then taken to laminar to grind theirs in mortar and pestle. After grinding, the leaves were added to falcon tubes having nutrient broth and were kept in a shaker incubator for 24h at 37°C. Next

bacteria colony growths were absorbed, and bacteria colonies were cultured on tagged and separate sterile Petri plates and kept for incubation for 24h.

The success of colonization of endophytic bacteria were confirmed after screening on NA Plates and were then incubated at room temperature for the next 24h. After that time, emerging colonies were subcultures to obtain Pure isolates. Four Pure isolates named as AB0017, ABS YA8 (*Bacillus safensis* strain YA8), AB0019 and AB0020 were selected and considered to examination for salt tolerance assay, IAA assay. After IAA and salt tolerance assay the best isolate, ABS YA8 (Bacillus safensis strain YA8) were considered for DNA extraction and for plant promotion application on cucumber plant.

Halotolerance Assay

Bacterial isolates were screened for halotolerance using 10 milli Nutrient agar (NA) media supplemented with various levels of NaCl 1)100 mmol (0.584 g) 2)150mmol (0.876) and 3)200 mmol (1.0968 in falcon tubes. The tubes were inoculated in a shaker with fixed volumes of starter inoculum (OD600 = 0.05) and the cultures were incubated for 3 days at 28◦C [12].

Indole Acetic Acid Production Assay

A modified quantification technique created by [13] was used to assess the production of indole acetic acid by bacterial strains Briefly, the four selected bacterial isolates were inoculated with 100 µl of Nutrient broth (NB) medium (OD600 = 0.5) and transferred into 5 ml of NB medium supplemented with 1% L-tryptophan after being cultured for 24 hours in 10 ml of NB (Sigma Aldrich, Germany). 2 ml of the bacterial cultures were centrifuged at 6,000 rpm and 4◦ C for 4 days while being continuously shaken at 150 rpm. The supernatant from this process was then placed into a well of a microtiter plate. The supernatant was then mixed with the Salkowski reagent (88.5 ml of $HClO_4$ + 1.2g $FeCl_3$), and the combination was left at room temperature for 25 minutes. Using an Epoch plate reader from Biotech (USA), the absorbance at 530 nm was calculated As a negative control, the Salkowski reagent was combined with the tryptophan-containing media that had not been infected. The appearance of pink in the well-indicated Indole-3-acitic acid (IAA) output [14].

Molecular characterization of endophytic bacteria.

The selected bacterial isolates were partially identified via 16S rDNA sequencing and analyzed through phylogenetic analysis. To achieve this, total genomic DNA isolation was performed on 5 ml of bacterial cultures cultured in liquid NA medium using the Wizard R Genomic DNA Purification Kit (Promega, Madison, WI, United States) in accordance with the manufacturer's instructions. The 16S rDNA bacterial universal primers, forward (50 - CTGACGGAGCAACGCCGCCT-30) and Reverse (50 TGCAACTCGACTGATGAATTG-30), were used in PCR experiments to amplify the 16S rDNA region [15] utilizing the microorganisms' and non-redundant (nr) databases. The obtained bacterial strains' 16S rDNA sequences were compared to GenBank sequences using the BLASTn tool. the phylogenetic analysis of the 16S rDNA sequences of the bacterial isolates with the reference bacterial sequences found in the BLAST search was performed by Using the MEGA11software package [16] The sequences were aligned with the help of the ClustalW, and the results were used to create a phylogenetic tree by calculating distance matrices for neighbor joining (NJ) and a bootstrapping analysis with 10,000 replicates to check the stability of internal branches. The phylogenetic tree included several 16S rDNA sequences of previously discovered PGP endophytic bacteria as references.

Anti-bacterial activity

Four isolated endophytic bacteria were used to examined against three common human and plant pathogen *Staphylococcus aureus*, *streptomyces scabiei* and *Erwinia carotovora* respectively. The pathogenic strains used for this study were provided by the agriculture department and microbiology lab at King Abdulaziz University. In this method Mueller Hinton Agar plates well diffusion method was used for the antibacterial activity of plant different endophytic t bacteria with the help of a sterile swab, Mueller Hinton Agar (MHA) agar plates were swabbed with pathogen organism and were left for about 5 minutes to let surface dry. Using sterile well borer 5 well were made on agar plates. 100µl isolated bacteria were loaded and for control only broth was loaded in each different well and left at 37°C for 24 hours. After overnight incubation, inhibition zones were measured. The zone of inhibition was considered the point at which no growth was visible to the naked eye. The zone was measured using normal mm scale.

Pot experiment

To analyses plant growth under different NaCl concentration pot experiment was conducted. For this purpose, 36 pots were arranged in randomized complete block design for three different replications of NaCl, 12 pots each selected for 100, 150, and 200 m/mole of salt and control.

Cucumber seeds were transported from Jeddah. The seeds were treated with sodium hypochlorite (1.5% solution) for 3 minutes after being surface sterilized with 70% ethanol for 5 minutes. The seeds were washed four times with sterilized distilled water. Moreover, soil is obtained from Jeddah's agricultural center. Salt stress was applied to plants 72 hours after germination. Endophytic bacteria were added to the soil 24 hours after salt stress, and plants were observed every 24 hours.

Used of Different parameters to examine plant growth after endophyte application.

For calculation of Plant's Morphological Characteristics, the electronic balance was used, fresh weights (FW) of roots and shoots were measured. Samples were dried in an oven at 70 °C (C) for 48 hours to determine dry weight (DW), after which they were weighed. Grams per plant were used to indicate G and Plant length was determined in centimeters using a metric scale and indicate by using letters CM. Calculating the total amount of chlorophyll present to determine the quantities of chlorophyll-a, b, freshly crushed leaves (0.5 g) from each treatment were crushed using a pestle and mortar. The mixture was added to 10 mL of acetone (80%), which was then centrifuged for 5 minutes at 12000 rpm. Using a spectrophotometer (UV-1900), the absorbance at 663 and 645 nm for the pertinent pigments was measured [17].

Data analysis

When it came to the initial characterization of endophytic bacteria and the inhibitory effects of endophytic bacterial isolates, the results were presented in figures and analyzed descriptively and qualitatively. On the other hand, the GraphPad Prism Program was used to analyze the data related to plant growth and the assessment of antibacterial activity.

Results

Based on different morphological and biochemical characteristics, four different bacterial isolates were obtained and maintained on agar media. All the isolates were circular in shape and opaque in nature with smooth surface. Potent one isolate was selected for molecular identification. Based on 16s rRNA and was identified as *Bacillus safensis* YA8 and Phylogenetic tree for these strains is shown in Fig 1C. The sequences of the selected strains were submitted to NCBI for accession number. The phylogenetic tree shows Ten different species representing the same genera.

IAA production assessment

For the indole acetic acid assay, four distinct endophytic species were investigated. The findings demonstrate that all isolates generate IAA. *Bacillus safensis* YA8 strains derived from *Opuntia ficus-indica* (A8) named as ABS YA8 produced IAA 0.12 µg/ml showed maximum IAA production in the presence of 1 % (w/v) l-tryptophan after 25 min in dark at room temperature , while other endophyte bacteria strains AB0017,AB 0019 and AB0020 produced 0.170 µg/ml, 0.095 µg/ml and 0.1086 µg/ml respectively that indicates less auxin production as shown in Fig 1A.

Anti-bacterial activity

Four different bacterial isolates have been checked for antimicrobial activity against human pathogen named *Staphylococcus aureus* and two plant pathogens *streptomyces scabiei* and *Erwinia carotovora*. Some isolates showed good antimicrobial activity as compared to others (Fig 1B). Out of four isolates, *Bacillus safensis* strain YA8 was found effective against the tested pathogen showing good zone of inhibition 9mm, 12mm, and 19mm respectively. The other isolates also show a promising inhibition zone.

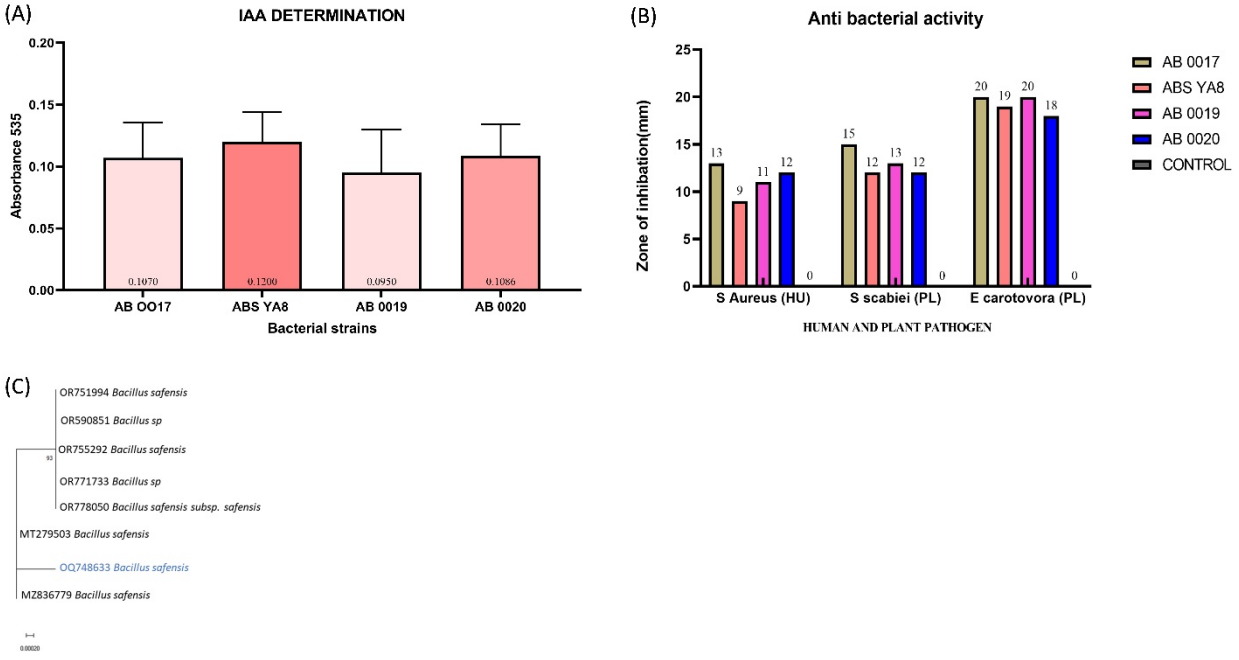

Fig.1 A) Presenting production of IAA by different endophyte bacterial isolates. X-axis indicates bacterial isolates and Y-axis indicates IAA production in µg/L. **B)** anti bacterial activity of different endophytic bacteria stains along with AB YA8 strains aginst human and plant phatogen.**C)** phylogenetic relationship of *Bacillus safiansis* strain YA8with other closesly related species .

Plant growth assessment analysis.

Plants when treated with different concentrations of NaCl and inoculated with bacterial isolates showed satisfactory results as shown in Figure 2. Increasing concentration of NaCl treatment i.e., 100 Mm, 150 Mm and 200 Mm) had slightly reduced the root and shoot lengths, however no minimum of damage was reported in any plant due to the effective action of bacterial isolates. Although the control(A) only treated with salt, showed plant death. the plant provided only bacteria showed significant amount of increase in root and shoot length as compared to control(B) which were provided only with water. the plants treated with both salt and bacteria show satisfactory growth results at 150 and 200 while at 100 no growth was examined as shown in fig 2C.

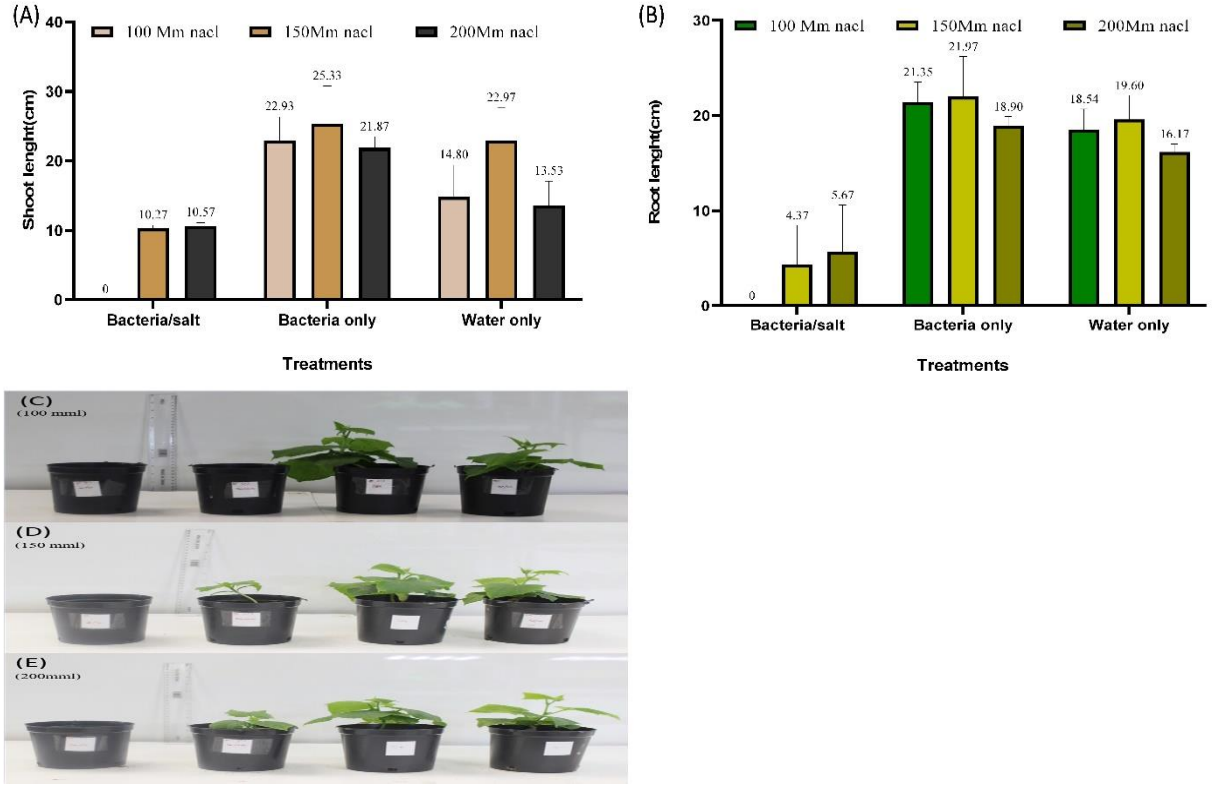

Fig 2 *Bacillus safensis* effects on growth of *Cucumis sativus* **A)** Shoot length of cucumber treated with different concentrations of NaCl + bacteria isolate and also plants inoculated with bacterial isolate *Bacillus safensis* YA8 only along with control (water only) .**B)** Root length of cucumber treated with different concentrations of NaCl stress + bacteria isolate and also plants inoculated with bacterial isolate *Bacillus safensis* YA8 only along with control (water only) .**C**) Pics show plant growth at 100 Mm, **D**) Plant growth150 Mm and **E**) Plant growth at 200 Mm of NaCl.

Plant Biomass

The cucumber plants Inoculated with only *Bacillus safensis* YA8 showed best increase in the fresh and dry biomass of plant as compared to the control which were only provided water, while the plant which were treated with both salt and bacteria shows slightly decreased biomass under the increasing concentrations of NaCl from 100Mm, 150Mm and 200Mm respectively. Showing in Fig 3 A and B. At 100Mm of NaCl concentration plants do not survive while at 150Mm plants show significant

increase in fresh and dry weight, at 200 of NaCl concentration plants show good increase in fresh and dry weight as shown in Fig 3 A and B.

Total chlorophyll content

It has been observed that the cucumber plants provided with bacteria isolates *Bacillus safensis* YA8 show significant increase in total chlorophyll count while plant provided with only salt indicate wilting and plant death shown above in Fig 2C. The plants provided with both bacteria and salt stress also show good results at 150 and 200Mm compared to 100Mm. At 100 Mm of NaCl plant not grow, at 150Mm plant show satisfactory increase in chlorophyll content while at 200Mm plant indicate good increase in chlorophyll content. as shown in the Fig 3C.

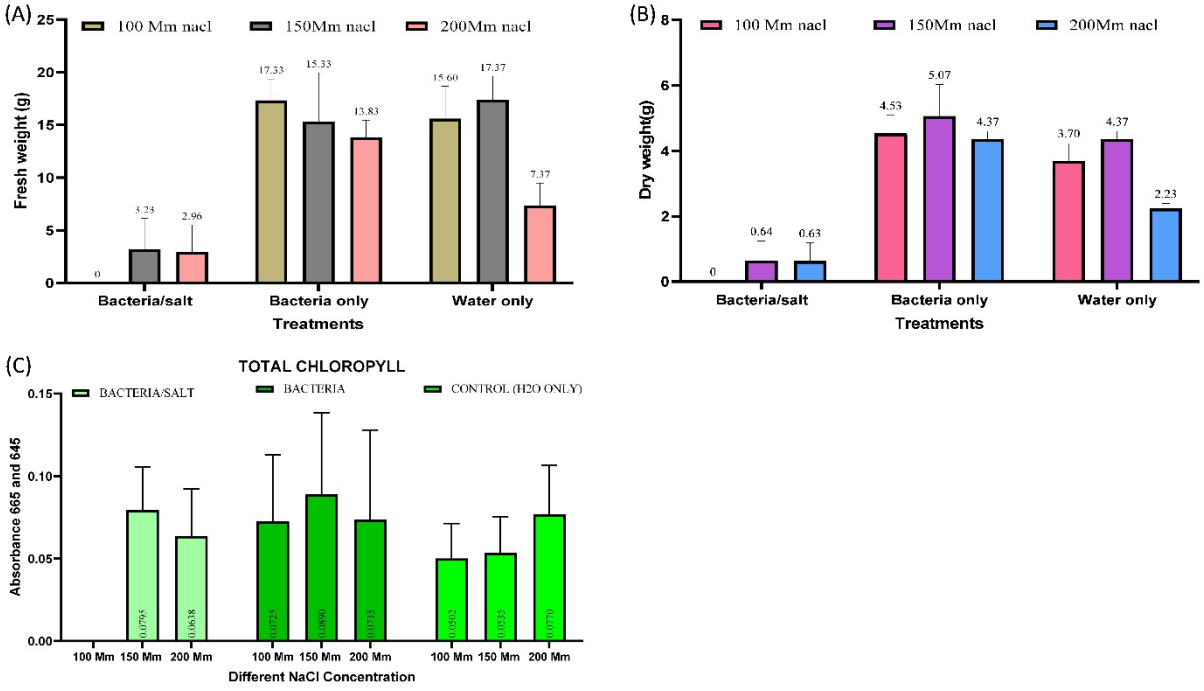

Fig 3 biomass under different salt stress **A and B)** Fresh and dry weight of cucumber treated with different concentrations of NaCl stress + bacteria and plant inoculated with bacterial isolate *Bacillus safensis* YA8 only along with control (water only) **C)** Total chlorophyll content of cucumber plant treated with different concentrations of NaCl stress + bacteria isolate and also of plants inoculated with bacterial isolate *Bacillus safensis* YA8 only along with control (water only).

Discussion

Bacterial endophyte populations in desert conditions typically seem to be diverse. The enormous diversity of bacterial communities in desert ecosystems has been attributed to the ecosystems' varied circumstances. All of the isolates could be identified down to the genus level, thanks to the sequencing of the amplified endophyte and its areas, and in some cases, this allowed us to classify the isolates as different species based on nucleotide conservation.

In our study total four endophytic bacteria were isolated from the leaves of Opuntia *ficus-indica* a plant from Cactaceae family [18]. According to [19, 20] endophytes isolates were identified on the basis of morphological characteristics. In our study for identification Consensus primers were used to successfully amplify the 16S rDNA gene. By using agarose gel electrophoresis, the 1,500 bp

fragment was amplified. Sequencing of nucleotides verified the size of the purified PCR result. Using the BLAST program, the nucleotide sequence was compared for homology with the nucleotide data that was accessible on the NCBI GenBank. When the nucleotide sequence was aligned with the recovered data, *Bacillus safensis* (GenBank accession no. 0Q748633) was found to have 99% identity. The strain recognized as *Bacillus safensis* and given the ABS-YA8 designation based on homology and alignment results.

Our data analysis that *Bacillus safensis strain* YA8 isolates from *Opuntia ficus- indica* were able to create IAA in our investigation, which suggests that bacteria isolate's may one day be used in agriculture to fully address crop growth issues. It has been observed that the synthesis of IAA by bacteria may enhance the legume-rhizobium symbiosis and nutrient intake, hence increasing root and shoot size and, ultimately, the growth of the plant [21].Other papers discuss that The primary auxin in plants, IAA, maintains the growth and developmental phases of plants, including responses to light, gravity, and pathogens, as well as tissue elongation and cell division [22]. Previous Studies revealed that increased salt stress had an impact on plants' xylem and phloem IAA concentrations [23]. Additionally, IAA plays a significant role in plant-microbe interactions, which may become unstable under various abiotic conditions (such as salt stress) [24].

It has been shown that endophytic bacteria have antimicrobial activity against a range of plant diseases [25, 26].In our study endophytic bacteria was tested against human pathogen S. aureus and 2 plant pathogen *S scebei*, and *E carotovora*. The results reveled that *Bacillus safensis strain* YA8 show good activity against S. aureus with 9mm zone of inhibition and promising activity against *S scebiei* and *E carotovora* with inhibition of 12mm and 19 mm respectively. Numerous papers discuss the use of endophytic Bacillus species in the biological management of wheat scab. For instance, *F. graminearum's* fungal growth and spore germination were severely inhibited by endophytic Bacillus megaterium and *Bacillus subtilis* isolated from wheat grain [27]. Because the antimicrobial chemicals produced by Bacillus species, such as hydrolases and secondary metabolites, are so powerful at preventing plant pathogens. Additionally, according [28] by increasing the availability or supply of important nutrients, endophytic bacteria enhance plant growth. [29] Concluded that endophytic bacteria can be used as a biofertilizer. This process involves turning atmospheric nitrogen into ammonia, or nitrogen fixation. Certain endophytic bacteria that support plant growth might raise the amount of phosphorus that is available to the plant by solubilizing it [30].

Conclusion

Our research focuses on studying endophytic bacteria, specifically a strain called *Bacillus safensis* YA8 that is found in *Opuntia ficus-indica*. This strain of bacteria has shown immense potential as bioinoculants in combating salt stress. One of the remarkable characteristics of these bacteria is their ability to promote plant growth even under saline conditions. They are also salt-tolerant themselves, which makes them excellent candidates for bioinoculation. Additionally, they produce a phytohormone called IAA, which further enhances plant biomass in salt-stressed environments. Therefore, these findings have suggested that *Bacillus safensis* YA8 could be a valuable tool in improving agricultural productivity in areas affected by high salinity. Further experiments are needed to test the efficacy of these bacteria in real-world agricultural settings, but their potential benefits are evident. By harnessing this, we can provide much-needed assistance to regions grappling with salinity issues and strengthen the resilience of our agricultural systems in the face of climate change.

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