ÇUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

MSc THESIS

Zahraddeen Kabir SANI

1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID DEAMINASE SYNTHESIZING RHIZOBACTERIA ISOLATION AND EVALUATION OF ITS EFFECT ON ALLEVIATING SALINITY AND DROUGHT STRESS IN PLANTS

BIOLOGY DEPARTMENT

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1-AMINOSİKLOPROPAN-1-KARBOKSİLİK ASİT DEAMİNAZ ÜRETEN RİZOBAKTERİLERİN İZOLASYONU VE İZOLATLARIN TUZ VE KURAKLIK STRESİNE KARŞI BİTKİ ETKİLERİNİN ARAŞTIRILMASI

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ABSTRACT

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: Prof. Dr. Osman GULNAZ
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Soil salinization, the second leading cause of desertification and land degradation, is one of the major threats being faced by the crop production sector. With about 20% of Agricultural fields worldwide being prone to osmotic as well as ionic stress, the need to salvage lost agricultural fields due to salinity becomes germane. An eco-friendly means of achieving the aforesaid aim is through the utilisation of Plant-Growth Promoting Rhizobacteria (PGPR). Through the synthesis and release of various compounds, PGPR regulate soil physicochemical characteristics and plant growth. This research is aimed at the isolation of multi-trait halotolerant PGPR from a hypersaline environment as well as evaluating the ability of the PGPR in mitigating the effect of salinity on *Triticum aestivum.* From the rhizosphere of halotolerant plants, 10 bacterial isolates were isolated. A randomized block design bioassay comprising of 11 treatments (10 bacterial isolates and control) and 4 salt (0 mM, 100mM, 200 mM and 300 mM) concentrations was conducted to assess salinity stress mitigating ability of the isolates. Though the isolates exhibited an array of plant-growth promoting traits, the effect of salinity stress on *T. aestivum* seedlings had been partially alleviated via inoculating the seedlings with the isolates.

Keywords: PGPR, salinity, *Triticum aestivum*, eco-friendly, halotolerance

ÖZ

YÜKSEK LİSANS TEZİ

1-AMINOSİKLOPROPAN-1-KARBOKSİLİK ASİT DEAMİNAZ ÜRETEN RİZOBAKTERİLERİN İZOLASYONU VE İZOLATLARIN TUZ VE KURAKLIK STRESİNE KARŞI BİTKİ ETKİLERİNİN ARAŞTIRILMASI

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: Prof. Dr. Osman GÜLNAZ
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Çölleşme ve arazi bozulmasının ikinci önde gelen nedeni olan toprak tuzlanması, bitkisel üretim sektörünün karşı karşıya olduğu en büyük tehditlerden biridir. Dünya çapındaki Tarım alanlarının yaklaşık %20'sinin ozmotik ve iyonik strese yatkın olması nedeniyle, tuzluluk nedeniyle kaybedilen tarım alanlarının kurtarılması ihtiyacı çok önemli hale geliyor. Yukarıdaki amaca ulaşmanın çevre dostu bir yolu, Bitki Büyümesini Destekleyen Rhizobacteria'nın (PGPR) kullanılmasıdır. PGPR, çeşitli bileşiklerin sentezi ve salınımı yoluyla toprak fizikokimyasal özelliklerini ve bitki büyümesini düzenler. Bu araştırma, çok özellikli halotolerant PGPR'nin hipersalin ortamından izole edilmesini ve ayrıca PGPR'nin tuzluluğun *Triticum aestivum* üzerindeki etkisini azaltmadaki kabiliyetini değerlendirmeyi amaçlamaktadır. Halotolerant bitkilerin rizosferinden on bakteri izolatı izole edildi. İzolatların tuzluluğun etkisini azaltmadaki yeteneklerini değerlendirmek için 11 tedaviden (10 bakteri izolatı ve kontrol) ve 4 tuz (0 mM, 100mM, 200 mM and 300 mM) konsantrasyonundan oluşan randomize blok tasarımlı bir biyoanaliz yapıldı. İzolatların bir dizi bitki büyümesini teşvik edici özellik sergilediği bulunmasına rağmen, tuzluluğun *T. aestivum* fideleri üzerindeki etkisi, fidelerin izolatlarla aşılanması yoluyla kısmen hafifletilmiştir.

Anahtar kelimeler: PGPR, tuzluluk, *Triticum aestivum*, çevre dostu, halotolerance

EXTENDED ABSTRACT

A significant role is played by soil salinization in land degradation and desertification. About 20% of Agricultural fields worldwide are prone to osmotic as well as ionic stress, and this makes the recovery of agricultural fields lost due to salinity crucial. Different strategies, involving Plant growth promoting rhizobacteria (PGPR) utilization, have been designed with the objective of achieving the aforementioned goal. PGPR regulate plant growth and soil physicochemical properties through the synthesis and discharge of various compounds such as 1-Aminocyclopropane-1-carboxylate deaminase, biofilm, cellulase, indole acetic acid, phytase, phytohormone, siderophore. Other mechanisms utilized PGPR in enhancing plant growth include nitrogen fixation, phosphate, potassium, and zinc solubilization.

This work has been aimed at the isolation of multi-trait halotolerant PGPR from a hypersaline environment as well as evaluating the ability of the PGPR in mitigating the effect of salinity on *Triticum aestivum*. From the rhizosphere of native plants growing around Tuzla lagoon of Karataş district, Adana, soil samples were obtained. To obtain the bacterial isolates, the samples were serial diluted and spread plated onto Nutrient agar plates supplemented with 4% NaCl and were incubated for 7 days at 28 ± 2ºC. Distinct bacterial colonies identified were collected and preserved on Nutrient agar slants. The ability of the isolates to resist higher salinity level was assessed. Moreover, the isolates that were able to tolerate salinity level of 15% were screened for plant growth promoting attributes, the attributes screened for are: 1-Aminocyclopropane-1-carboxylate deaminase synthesis, Biofilm formation, cellulase synthesis, hydrogen cyanide production, siderophore synthesis, solubilization of phosphate, phytase activity, and indole acetic acid synthesis.

A randomized block design bioassay comprising of 11 treatments (10 bacterial isolates and control) and 4 salt concentrations (0, 100, 200 and 300 mM)

was conducted to assess the ability of the isolates in mitigating the effect of salinity on *T. aestivum.* For the bacterial treatment, seeds of *T. aestivum* were coated with the bacterial isolates (10^8 CFU mL^{-1}), while for the control, 0.03 M $MgSO_4$ was used to coat the seeds. The seeds were sown and grown in the green house for 27 days. For the first 7 days, they were watered with normal sterilized distilled water, while for the remaining 20 days the seedlings were watered with sterilized saline water. The seedlings were harvested and rinsed thoroughly with water. Subsequently the shoot and root length, and fresh and dry weight of the seedlings were evaluated.

All the isolates were found to be siderophore synthesizers. Isolate B22, B30, C23, D11 and E4 did not solubilized phosphate, while the rest were able to solubilise it. Phytase activity was exhibited by isolate D15, E2 and H9. Except for isolate B15, B22, B30 and C10 all the isolates synthesized IAA. However, none of the isolates was able to synthesize ACC deaminase. Excluding isolate D15 and H9, all the isolates produced HCN. Moreover, isolate E2 was unable to synthesize cellulase. All but isolate C23 were able to form biofilm *in-vitro*.

The isolates were able to partially alleviate the effect of salinity stress on the *T. aestivum* seedlings. Hence, this could be attributed to the inability of the isolates to synthesize ACC deaminase. Despite the inability of the isolates to effectively alleviate the effect of salinity stress on the seedlings, their ability to augment plant growth under different environmental conditions should investigated in the future.

GENİŞLETİLMİŞ ÖZETİ

Toprak tuzlanmasının çölleşmeye ve arazi bozulmasına neden olmada önemli bir rol oynadığı gösterilmiştir. Dünya çapındaki tarım alanlarının yaklaşık %20'si ozmotik ve iyonik strese yatkındır ve bu, tuzluluk nedeniyle kaybedilen tarım alanlarının geri kazanılmasını çok önemli hale getirir. Yukarıda belirtilen hedefe ulaşmak için farklı stratejiler tasarlanmıştır. Araştırmacılar tarafından speküle edilen stratejiler arasında, bitki büyümesini teşvik eden rizobakterilerin (PGPR) kullanılması yer almaktadır. Toprak fizikokimyasal özellikleri ve bitki büyümesi, siderofor, indol asetik asit, Biyofilm, fitohormon, 1-Aminosiklopropan-1-karboksilat deaminaz, selülaz, fitaz gibi çeşitli bileşiklerin sentezi ve salınımı yoluyla PGPR tarafından düzenlenir.

Bu çalışma, çok özellikli halotolerant PGPR'nin hipersalin ortamından izolasyonunu ve ayrıca PGPR'nin tuzluluğun *Triticum aestivum* üzerindeki etkisini azaltmadaki kabiliyetini değerlendirmeyi amaçlamıştır. Adana ili Karataş ilçesi Tuzla lagünü çevresinde yetişen yerli bitkilerin rizosferinden toprak örnekleri toplanmıştır.

Bakteri izolatlarını elde etmek için örnekler seri olarak seyreltildi ve %4 NaCl ile takviye edilmiş Nutrient agar plakalarına yayıldı ve 28±2ºC'de 7 gün inkübe edildi. Nutrient agar slantları üzerinde farklı koloniler toplandı ve korundu. İzolatların daha yüksek tuzluluk seviyelerine dayanma yetenekleri değerlendirildi. Ayrıca, %15'lik bir tuzluluk seviyesini tolere edebilen izolatlar, bitki büyümesini teşvik edici özellikler açısından tarandı, taranan özellikler arasında siderofor sentezi, Biyofilm oluşumu, fosfat çözünürlüğü, fitaz üretimi, indol asetik asit, 1-Aminosiklopropan-1- karboksilat deaminaz, hidrojen siyanür ve selülaz sentezi.

On bir tedaviden (kontrol ve 10 bakteri izolatı) ve 4 tuz konsantrasyonundan (0, 100, 200 ve 300 mM) oluşan randomize blok tasarımlı bir biyoanaliz, izolatların tuzluluğun *T. aestivum* üzerindeki etkisini azaltma yeteneğini değerlendirmek için yürütülmüştür. Bakteri muamelesi için *T. aestivum*

tohumları bakteri izolatları (10^8 CFU mL^{-1}) ile kaplanırken, kontrol muamelesi için tohumlar 0.03 M $MgSO_4$ ile kaplanmıştır. Tohumlar ekilmiş ve 27 gün boyunca serada büyütülmüştür. İlk 7 gün normal steril saf su ile sulanmış, kalan 20 gün ise steril tuzlu su ile sulanmıştır. Fideler hasat edildi ve büyüme parametreleri, yani kök ve sürgün uzunluğu, yaş ve kuru ağırlık değerlendirildi.

Tüm izolatların siderofor sentezleyicileri olduğu bulundu. B22, B30, C23, D11 ve E4 izolatı fosfatı çözmezken, geri kalanı onu çözebildi. Fitaz aktivitesi, izolatlar D15, E2 ve H9 tarafından sergilendi. B15, B22, B30 ve C10 izolatları dışında tüm izolatlar IAA sentezlemiştir. Ancak izolatların hiçbiri ACC deaminaz sentezleyemedi. D15 ve H9 izolatları hariç tüm izolatlar HCN üretmiştir. Ayrıca, E2 izolatı selülaz sentezleyemedi. Tüm izolatlar biyofilm oluşturabildi, ancak izolat C23 bunu üretemedi.

Sera denemesi sonucu, izolatlar, tuzluluk stresinin *T. aestivum* fideler üzerindeki etkisini kısmen hafifletebilmiştir. Bu nedenle, bu, izolatların ACC deaminaz sentezleyememesiyle ilişkilendirilebilir. İzolatların tuzluluğun fideler üzerindeki etkisini azaltamamasına rağmen, gelecekte farklı çevre koşullarında bitki büyümesini arttırma yetenekleri araştırılmalıdır.

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SYMBOLS AND ABBREVIATIONS

$\mu g\ mL^{-1}$	: Microgram/millilitre
μg	: Microgram
μL	: Microlitre
ACC	: 1-Aminocyclopropane-1-carboxylate
B15	: The alphabet refers to the ID of the plant sample while the number refers to the number of the bacterial strain obtained from that particular sample
$CFU\ mL^{-1}$	: Colony forming units/millilitre
EPS	: Extracellular polymeric substance
HDTMA	: Hexadecyltrimethylammonium chloride
IAA	: Indole acetic acid
$Kg\ ha^{-1}$	: Kilogram/hectare
M	: Molar
Mg	: Milligram
mm	: Millimetre
mM	: Millimolar
nm	: Nanometre
ºC	: Degree Celsius
OD	: Optical density
PGPR	: Plant growth-promoting rhizobacteria
pmol	: Picomole
rpm	: Revolution per minute
U/mL	: Unit/millilitre
w/v	: Weight/volume

1. INTRODUCTION

The world population has been projected to reach 10 billion people by the year 2050, this upsurge in human population may lead to increase in food demand (Rani and Kumar, 2019). With the intensification of agricultural activities, crop production has increased, and this has translated into more demand for pesticides and fertilizers (Bhat et al., 2019). In agriculture, the utilization of artificial/chemical fertilizer has been associated with greenhouse gas emissions (Antonella Di Benedetto et al., 2017). As agricultural systems expand, the emission level of these gases also increases, thus triggering the need for more environmentally friendly methods of crop production (Hungria et al., 2013).

Also, Salinity is another problem being faced by the crop production sector. The second leading cause of desertification and land degradation is soil salinization (Orhan, 2016). Up to 20% of cultivated agricultural land worldwide has been estimated to be susceptible to osmotic stress as well as ionic stress (Orozco-Mosqueda et al., 2020).

The two main forms of salinity are Primary and secondary salinity, the former occurs naturally while human activities such as agriculture and land development trigger the latter (Barnawal et al., 2012). By interfering with a variety of biochemical, physiological and metabolic activities, salinity stress inhibits plant growth (Sapre et al., 2018). Excess accumulated salts of CO_3^{2-}, Cl^-, Mg^{2+}, Ca^{2+}, Na^+, SO_4^{2-} and electrical conductivity (EC_e) greater than 4 dS/m at 25ºC characterized saline soils (Shahid et al., 2018).

Ion toxicity, hormonal, and physiological disorders, as well as nutritional imbalance are some of the various factors that greatly impede plant growth in saline environments (Zhu et al., 2020). The nutrient status and physical structure of the soil are adversely influenced by salinity (Fatima et al., 2020). Due to sodication, salination and waterlogging, about 10 million hectares of irrigated agricultural fields are vacated annually worldwide (Szabolcs, 1989). Turkey, whose

1.5 million hectares of agricultural land is negatively impacted by high salt concentration, is among the countries currently experiencing salinity problems (Dinc et al., 1993; Szabolcs, 1989).

To mitigate the aforesaid problems, an alternative technique that utilizes biofertilizer becomes pertinent. Any organic product encompassing substantial microbial population that augments soil fertility or enhances plant growth is referred to as a biofertilizer (Simarmata et al., 2016). Alternatively, biofertilizer is any active microbial agent capable of promoting plant growth by improving the availability of nutrients in the rhizosphere (Singh et al., 2019). Bioformulations, microbial inoculants, microbial cultures, bioinoculants, and bacterial fertilizers are used interchangeably to refer to biofertilizers (Arora et al., 2011).

Rhizosphere, the region surrounding plant roots, is inhabited by a substantial quantity of microbes (Lugtenberg and Kamilova, 2009). Tailor & Joshi (2014) defined rhizosphere as a living environment that accommodates various groups of microbes. The rhizosphere serves as a rich source of microbes; hence it is referred to as the repository of microbes (Kundan et al., 2015).

Compared to other parts of the soil, microbes colonized both the rhizoplane and rhizosphere of plants more intensely. Among these microbes, those found to to exert beneficial influence on the plant are referred to as plant growth promoting rhizobacteria "PGPR", based on their association with the plant root, these rhizobacteria are categorized into free living and symbiotic (Verma et al., 2019). These organisms feed on the exudates secreted by the plants (Lugtenberg and Kamilova, 2009). Inhibition of phytopathogens, ability to colonize plant roots, and ability to thrive and reproduce in the rhizosphere environment are the three main features of PGPRs.

Some of the bacterial strains that have been utilized as biofertilizers include *Acinetobacter, Agrobacterium, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Bradyrhizobium, Frankia, Pantoea, Rhizobium, Serratia, Stenotrophomonas, Streptomyces,* and *Thiobacillus.*

Although PGPR do proliferate in the soil, their population is normally not sufficient enough to vie with other bacteria found in the rhizosphere, hence the need to inoculate plants with a much higher concentration of the microbes (Subba Rao, 1999).

At the molecular level, plants leak in nature, meaning they constantly exude nutrients, cells, moisture, carbohydrates, and other components. Therefore, the proximal zone surrounding the root surface is occupied by a wide variety of organisms. With an allocation of more than 40% photosynthesis, the root surface and the surrounding rhizosphere proves to be a significant carbon sink, thereby providing a variety of suitable nutrient rich niches, hence enticing a wide variety of microbes (phytopathogens inclusive) to proliferate (Richardson, 2001). PGPR protect plants against phytopathogens primarily through competition for nutrients and niches (Dong et al., 1997).

Amino acids, special sugars and organic acids are some of the acknowledged chemical attractants found in plant root exudates (Volkmar and Bremer, 1998). Some exudates tend to be antimicrobial in nature, so organisms that are in possession of sufficient enzymatic mechanism to detoxify them have an ecological niche advantage over those who can't. The composition and quantity of antimicrobials and chemo-attractants leached from plant roots are influenced by genetics and environment (Azcon, 1993). Likewise, plant root exudate composition is influenced by plant genotype and plant developmental stage, thereby interfering with the composition of the Rhizomicrobiome (Bouffaud et al., 2012). Efficient root colonization in certain PGPR is associated with their secretion of site-specific recombinase.

1.1. Plant growth promoting mechanism of PGPR

PGPR enhances plant growth through direct and indirect mechanisms that (Zakry et al., 2012). Both the direct and indirect mechanisms are elaborated below:

1.1.1. Biological Nitrogen fixation

About two-thirds of globally fixed nitrogen are accounted for by biological nitrogen fixation, while the rest is synthesized industrially via the Haber Bosch process (Rubio and Ludden, 2008). Nitrogen fixers, widely distributed in nature, execute biological nitrogen fixation (Raymond et al., 2004). Biological nitrogen fixation proves to be environmentally sound and economically viable, thus, chemical/synthetic fertilizers can be substituted via the utilization of biological nitrogen fixers (Ladha et al., 1997). Some microbes utilize the nitrogenase enzyme complex in fixing atmospheric nitrogen, the fixed atmospheric nitrogen becomes readily available for plant utilization (Ahemad and Kibret, 2014). Inoculating plants with nitrogen fixers have been shown to enhance plant growth under controlled conditions (Aloo et al., 2020). Non-symbiotic and symbiotic nitrogen-fixing bacteria are the categories of nitrogen fixing bacteria.

The plant host of nitrogen fixing bacteria received a very little amount of fixed nitrogen needed by the plant from the bacteria (Glick, 2012). Nodule formation in leguminous plants arises due to a complex symbiotic relationship that the symbiotic nitrogen fixing bacteria established with its host plant (Giordano and Hirsch, 2004). Through the inoculation of crops with biological nitrogen fixing PGPR, the required nitrogen level of plants can be achieved (Damam et al., 2016).

1.1.2. Phosphate solubilization

The second crucial macronutrient that plants need in sufficient quantity to achieve optimum growth is phosphorous (Anand et al., 2016a). Energy transfers, respiration, signal transduction, photosynthesis are some of the important roles that phosphorus plays in plant metabolic processes. Although phosphorus is present in relatively large amount (400 to 1,200 mg kg^{-1}) in soil, it is virtually unavailable for plant use due to its presence in inert/insoluble form. It occurs both as an inorganic mineral and as one of various organic forms such as phosphomonoester and inositol phosphate (Khan et al., 2009). On applying soluble phosphorus to soil as

fertilizer, a significant amount of it becomes immobilized immediately, hence becoming inaccessible for plant utilization (Glick, 2012). pH and soil type significantly affect fixation and precipitation of phosphorus in soil; in acidic soils, phosphorus is fixed by free hydroxides and oxides, while in alkaline soil, it is fixed by calcium (Rodríguez and Fraga, 1999).

In soil, the second major repository of phosphorus is organic matter (Rodríguez and Fraga, 1999). In most soils, organic phosphorus constitutes around 30 to 50% of total phosphorus (Paul and Clark, 1988). Besides, the most stable form organic phosphorus in soil is phytate (Inositol phosphate). Both plants and microbes produce phytate. About of 50% of total organic phosphate in soil is made of phytate (Anderson, 2015; Dalai, 1977; Harley and Smith, 1983). Phosphotriesters, phosphodiesters and phosphomonoesters are other forms of soil organic phosphorus (Rodríguez and Fraga, 1999).

Phosphate is absorbed by plants only as dibasic (HPO_4^{2-}) and monobasic ($H_2PO_4^-$) ions (Gouda et al., 2018). The bacteria that, through the dissolution and mineralization of phosphate, can make insoluble phosphorus suitable for plant use is called phosphate-solubilizing bacteria. Phosphate solubilizers solubilize phosphate through the discharge of organic acids and phosphatases (Kundan et al., 2015).

Even though phosphate solubilizers are common in soil, their performance and growth are severely hampered by adverse environmental factors. In addition to supplying soluble phosphorus to plants, Phosphate-solubilizing bacteria enhance plant growth through efficient stimulating of nitrogen fixation and synthesis of plant growth enhancing compounds (Ahemad and Kibret, 2014).

Numerous reports show that inoculation with phosphate solubilizers enhances growth in plants. Despite the availability of significant body of literature, studies on the utilization of phosphate solubilizers as bacteria as biofertilizer are still sketchy (Gouda et al., 2018).

1.1.3. Potassium solubilization

The third largest plant macromolecule after phosphorus and Nitrogen is Potassium (Anand et al., 2016b). Reduced crop yield arising due to stunted growth is caused by Potassium deficiency in plants (White and Karley, 2010). Likewise, plants susceptibility to diseases is increased by potassium deficiency (Armengaud et al., 2010; Troufflard et al., 2010). More than 90% of potassium in soil occur in insoluble complexes, besides the amount readily accessible is inadequate for plant growth (Parmar and Sindhu, 2013).

Feldspar, muscovite, mica, biotite, orthoclase, smectite and vermiculite are the main potassium-containing minerals in the soil. These minerals need to be solubilized with potassium solubilizing bacteria to make the Potassium in them suitable for plant use (Ahmad et al., 2016). Solubilization by PGPR of Potassium from potassium-containing minerals by has been widely investigated (Gouda et al., 2018). Similarly, the bacteria have been found to protect plants against phytopathogens and stress conditions. Potassium solubilizers release potassium from minerals through production and release of organic acid (Ahmad et al., 2016). Utilization of potassium solubilizers as can help reduce our over-reliance on chemicals and thus support crop production in an environmentally friendly way (Gouda et al., 2018).

1.1.4. Zinc solubilization

Zinc is among the essential micronutrients required for plant optimum growth and development. Zinc deficiency leads to decrease in membrane integrity. Likewise, it causes decline in auxin, carbohydrate, chlorophyll, cytochrome and nucleotide (Goteti et al., 2013).

Worldwide, 25kg ha^{-1} of $ZnSO_4$ heptahydrate synthetic zinc fertilizers are used to address zinc deficiency in soil. One disadvantage of this attempt is that synthetic fertilizers are expensive and they become easily converted into insoluble forms thereby becoming unavailable for plant utilization (Aloo et al., 2020).

About 30% of agricultural soil worldwide is Zinc deficient, causing huge losses in agricultural yields (Ahmad et al., 2012). The prevalent occurrence of zinc deficiency is ascribed to the low solubility of zinc (Cakmak, 2002). Various factors regulate the availability of zinc from insoluble sources, one of which is the biochemical reactions carried out by rhizospheric microorganisms that convert zinc from a form not available for plant utilization to one readily available through the organic acids discharge (Sindhu et al., 2019).

1.1.5. Iron sequestration

Even though iron, an important micronutrient needed by plants, occurs in the soil as the 4th most abundant element, it cannot benefit the plant due to its availability in insoluble ferric form (Fe^{3+}) (Kundan et al., 2015). Several microbial genera have developed mechanisms for obtaining iron through the synthesis of siderophores. Siderophores exhibit high affinity for iron in a low iron environment, hence they act as iron chelators (Kundan et al., 2015). By supplying stressed plants with iron, bacteria synthesizing siderophores can help in alleviating the effect of stress on the plants (Glick, 2012).

Siderophores occur mostly as water-soluble and are classified as extracellular and intracellular siderophores. A few Rhizobacteria have the capability of utilizing siderophores synthesized by rhizobacteria of the same genus whereas others utilize the siderophores synthesized by rhizobacteria of dissimilar genera (Khan et al., 2008).

On the bacterial membrane, Fe^{3+} is reduced to Fe^{2+} in the Fe^{3+}-siderophore complex and is similarly discharged from the siderophore via a passage membrane into the cell. The siderophore becomes recycled or destroyed though the course of this process (Rajkumar et al., 2010). In addition to iron, stable complexes are formed by siderophores containing the environmentally important heavy metals: aluminium, cadmium, copper, gallium, indium, lead and zinc (Kiss and Farkas, 1998; Neubauer et al., 2000).

1.1.6. Phytohormone production

A significant role is played by phytohormones in the growth and development of plant. Plants are often predisposed to an array of non-fatal stresses that may hamper growth till the plant overcome the effect of the stress by adjusting its metabolism or the stress becomes eliminated (Bernard R. Glick, 2012). While the above tends to be successful, rhizospheric microbes may tend to control or synthesize phytohormones under in vitro conditions, thereby influencing plant hormonal balance. According to their reaction towards physiological processes and structural composition, phytohormones are categorized into: Abscisic acid, Auxins, Cytokinins, Ethylene and Gibberellins - according to their structural composition and their response to physiological processes occurring within plants (Khatoon et al., 2020).

1.1.7. 1-aminocyclopropnae-1-carboxylate (ACC) deaminase production

1-aminocyclopropane-1-carboxylate deaminase is among the specific enzymes that both the direct and indirect plant growth promotion work in collaboration with, these enzymes cause physiological reactions at the molecular level in plants. ACC deaminase has been shown to play a crucial role in the regulation of plant hormone and ethylene (Turan et al., 2014). ACC deaminase utilizes a broad array of mechanisms to regulate ethylene production (Honma and Smmomura, 1978; Sun et al., 2009).

The optimum pH and temperature for the activity of ACC deaminase is 8.5 and 25 – 35ºC respectively (Honma and Smmomura, 1978; Jacobson et al., 1994; Jia et al., 1999). Though the major function is ACC deaminase is the cleavage of ACC, D-cysteine and D-serine can substitute ACC as substrates, even though less efficiently than ACC (Brigado et al., 2015). IAA can be produced and discharged by PGPR bounded to the seeds and roots of a growing plant in reaction to the presence of tryptophan in plant root exudate (Bayliss et al., 1997; Patten and Glick, 1996; Penrose et al., 2001). The IAA synthesized by PGPR in collaboration with

that synthesized by the plant can stimulate plant growth. Likewise, IAA induces the transcription of ACC synthase encoding gene, and the gene transforms S-adenosyl-methionine (SAM) to ACC. Subsequently, ACC oxidase converts the ACC to ethylene. ACC synthesizing bacteria cleaves the secreted ACC to ammonia and α-ketobutyrate (Fig 1).

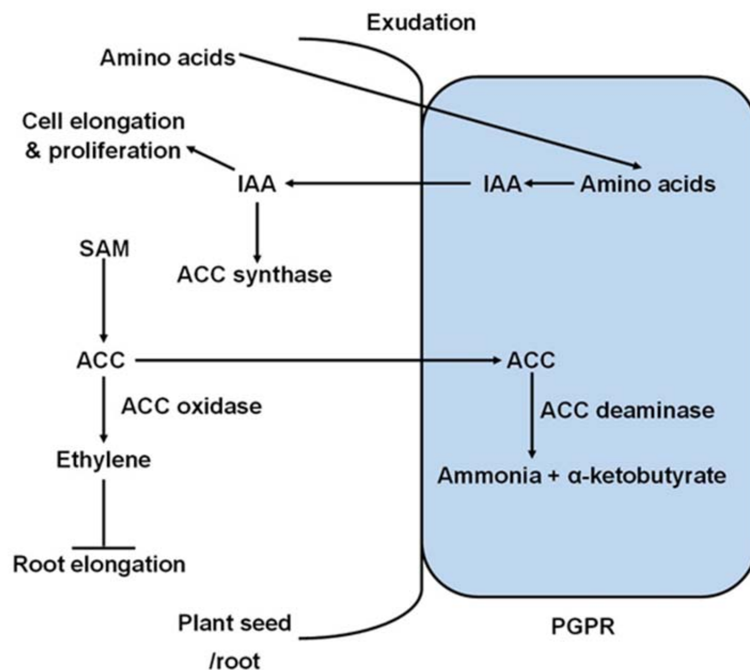

Figure 1.1. Schematic representation of ethylene concentration reduction with PGPR. Key: ACC, 1-aminocyclopropane-1-carboxylic acid; IAA, Indole acetic acid; SAM, S-adenosyl-methionine (Burd et al., 1998)

Plants treated with ACC deaminase synthesizing bacteria tend to exhibit luxuriant root growth owing to less ethylene level, likewise the plants were found to withstand stress better, hence culminating into increase survivability of seedlings (Burd et al., 2000).

In managing and regulating the growth and development of plants, ethylene has been shown to play a significant role. Likewise it controls plants response to environmental stresses (Eric Schaller and Voesenek, 2015). It was first assumed to be a hormone that triggers maturation, later it was discovered that its importance parallel that of other plant hormones (Arshad & Frankenberger, 1998). Under salinity stress, ethylene production is induced instantly (Yang et al., 2009). Elevated ethylene level leads to diminished seed germination and root growth (Belimov et al., 2001; Saravanakumar and Samiyappan, 2007).

When the ethylene content in plants is examined, two levels of ethylene peaks will be observed; the smaller initial peak that occurs when the plant is exposed to the stress early – which triggers protective defence responses - and a second peak - tends to be larger than the first - occurring a few hours to a few days after the stress application (Stearns and Glick, 2003). Enhancing the growth of plants under normal and stress conditions using ACC deaminase synthesizing PGPR has appealed to the interest of researchers (Sergeeva et al., 2006).

2. PREVIOUS STUDIES

Turan et al. (2014) conducted a greenhouse experiment to evaluate the effect of inoculating cabbage seedlings with *Bacillus megatarium* strain TV-91C, *Pantoea agglomerans* strain RK-92 and *B. subtilis* strain TV-17C and found that the seedlings growth and quality were greatly promoted by the aforesaid bacteria.

Albdaiwi et al. (2019) isolated halotolerant bacterial isolates from the rhizosphere and endosphere of *Triticum turgidum* subsp. *durum* plants been cultivated in saline environments, six of the isolates proved to enhance the survivability of plants growing under high salinity stress and three out of the six isolates were also found to enhance plant tolerance towards water deficit stress.

Gupta et al. (2021) evaluated plant growth promotion by two halotolerant bacterial isolates, *Bacillus marisflavi* (CHR JH 203) and *B. cereus* (BSTY51_42), exhibiting high 1-aminocyclopropane-1-carboxylic acid deaminase activity on pea (*Pisum sativum*) plants grown under saline induced soil condition. Their finding reveals that, inoculating the plants with the acds^{+} strains alleviated the deleterious effect of salinity on the inoculated plants.

Mayak et al. (2004) isolated *Achromobacter piechaudii*, an ACC deaminase synthesizing rhizobacteria, from the dry riverbeds of Arava region of southern Israel. The bacterium was found to significantly stimulate growth of tomato seedlings exposed to high salt concentration. Moreover, the production of ethylene by the tomato seedlings was lowered by the bacterium. The uptake of potassium and phosphorus was slightly increased by the isolate, however.

Halotolerant endospore forming *Bacillus pumilus, B. subtilis, B. licheniformis, B. safensis* and *B. cereus* were isolated by Sharma et al. (2018) from the rhizosphere of Chickpea plant. The isolates were found to exhibit inhibitory effect on *Alternaria alternata*, *Colletotrichum capsica*, *Fusarium oxysporum* f.sp. *ciceri*, *F. solani*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Sclerotium rolfsii* and *Sclerotinia sclerotiorum* which are plant pathogenic fungi.

Egamberdieva et al. (2022) isolated 10 halotolerant endophytic bacteria belonging to *Agrobacterium, Bacillus, Brevibacterium, Curtobacterium, Pantoea, Pseudarthrobacter, Pseudomonas, Raoultella, Stenotrophomonas* and *Streptomyces* from *Tetragonia tetragonoides* (Pall.) Kutze. The isolates were found to exhibit plant growth promoting traits and antifungal activity against the phytopathogenes; *Fusarium oxysporum*, F. *solani* and *Verticillium dahlia.*Moreover, half of the isolates prove to be ACC deaminase synthesizers. Among the isolates, *Stenotrophomonas maltophilia* Tetr 2, *Bacillus amyloliquefaciens* Tetr 11, *Pseudomonas moraviensis* Tetr 18, and *Curtobacterium plantarum* Tetr 19 promoted the growth of *Tetragonia tetragonioides* (Pall.) Kuntze seedlings grown under 200mM NaCl induced salinity.

Vishnupradeep et al. (2022) investigated the effect of inoculating *Zea mays* with the multi stress tolerant PGPB; *Providencia* sp. and *Proteus mirabilis* have on the plant's ability to withstand chromium and drought induced stress. They arrived at the conclusion that inoculating the plant with the aforesaid inoculants significantly ameliorate the effect of the induced stress.

An endophytic *Pseudomonas aeruginosa* FG106 with multiple plant growth promoting traits has been isolated from the roots of tomato plant by Ghadamgahi et al. (2022). The isolate was found to enhance tomato seedlings growth under greenhouse conditions. Likewise, it exhibited antagonistic effect against the certain phytopathogens.

From the rhizosphere of Plum (*Prunus domestica*), Essalimi et al. (2022) isolated *Pseudomonas stutzeri* Pr 7 and *Bacillus toyonensis* Pr 8 that exhibit multiple plant growth promoting traits and antifungal activity against *Fusarium oxysporum* f.Sp. *melonis* and *Verticillium dahliae*. The isolates significantly enhanced the growth, *Verticillium* wilt resistance and salinity tolerance of tomato seedlings in-vitro. Moreover, the acclimatization of *Vitis vinifera* cv. Pinot noir and peach root stock GF 305 has been enhanced by the isolates.

Barman et al. (2022) isolated from the coal dumping area of Dudhnoi, Goalpara, Assam, India, three heavy metals tolerant PGPR that can synthesize ACC deaminase. Moreover, the isolates were found to exhibit other plant growth promoting traits such as phosphate solubilization and production indole-3-acetic acid and siderophore.

The plant growth promoting potential of 6 bacterial isolates has been evaluated by Giannelli et al. (2022). Two of the isolates – *Beijerinckia fluminensis* and *Pseudomonas protegens* - tend to significantly enhance the growth of Arabidopsis thaliana as well as exhibit high antifungal activity against *Aspergillus niger*, *Fusarium proliferatum* and *F. verticilliodes*.

Danish et al. (2020) evaluated the role of played by ACC deaminase synthesizing PGPR singly and in combination with timber waste biochar in promoting maize seedling growth under drought stress. They arrived at the conclusion that the deleterious effect of drought is ameliorated through the application of biochar along with the PGPR.

Chandra et al. (2019) reported 42.2 and 31.9% overall growth enhancement of wheat plants growing under drought and irrigated conditions inoculated with ACC deaminase *Variovorax paradoxus* RAA3. Likewise, a consortia inoculation comprising of *Ochrobactrum anthropic* DPC9, *Pseudomonas palleroniana* DPB13, *Pseudomonas fluorescens* DPB15, *Pseudomonas palleroniana* DPB16 enhanced the overall growth of wheat plants by 40.4 and 32% under drought and irrigated conditions respectively.

Leclercia adecarboxylata MO1, a halotolerant PGPR capable of synthesizing ACC deaminase has been isolated from the rhizosphere of tomato (*Solanum lycopersicum* L.) by Kang et al. (2019). Moreover, the isolate significantly promotes the growth of tomato seedlings, thereby qualifying as a potential candidate for biofertilizer.

Safronova et al. (2006) studied the effect of inoculation with *Pseudomonas brassicacearum* Am3, *Pseudomonas marginalis* Dp1 and *Rhodococcus* sp. Fp2

(ACCD synthesizers) on growth and uptake of Nitrogen, Phosphorus, Potassium, Calcium, Sulphur, and Cadmium by *Pisium sativum* (pea) plant in Cadmium contaminated soil. They found *Rhodococcus* sp. Fp2 to be the only inoculant that did not exhibit ACCD activity in the presence of Cd and did not enhance the plant's growth in the presence of Cd. While the other inoculants ameliorated the Cd-induced inhibition of nutrient uptake by the plant.

Grichko & Glick (2001) evaluated the effect of inoculating tomato seedlings with ACC deaminase synthesizing and non-ACC deaminase synthesizing bacterial inoculants on the ability of tomato seedlings to withstand flooding stress. The seedlings inoculated with the ACC deaminase synthesizing inoculants were observed to significantly withstand the stress.

Research on ameliorating the apparent toxicity of the heavy metal, Nickel to Canola seedlings using the ACC deaminase synthesizing bacteria, *Kluyvera ascorbata* SUD165 has been conducted by Burd et al. (1998). Despite the ability of the bacteria to decrease the toxicity of Nickel to canola, it did not influence the amount of Nickel accumulated by the plant. Moreover, the amount of ethylene evolved from the plant has been lowered from 590 ± 182 nmol mg^{-1} (in the absence of the bacteria) to 275 ± 90 nmol mg^{-1} (in the presence of the bacteria).

Enterobacter cloacae PM23, a halotolerant ACC deaminase synthesizing PGPR has been reported by Ali et al. (2022) to have significantly enhanced the resistivity of maize plants to elevated salt concentrations. Inoculating the maize seedlings with the halobiont bacterium leads to significant reduction of oxidative stress markers under salinity stress.

Seed germination and seedling growth of Foxtail millet (*Setaria italica* L.) has been stimulated by *Pseudomonas fluorescens* DR11, *P. migulae* DR35, *P. fluorescens* DR7 and *Enterobacter hormaechei* DR16. The production of Extracellular polymeric substances (EPS) by these isolates boosts their effectiveness as PGP bacteria in drought stressed environment (Niu et al., 2018).

Sergeeva et al. (2006) transformed canola (*Brassica napus* cv Weslar) to express the bacterial ACC deaminase gene. Following growth of the transformed and the non-transformed canola plants in the presence of 0 – 200 mM NaCl, the transformed canola plants were found to exhibit tolerance to the inhibitory effect of salt stress.

ACC deaminase synthesizing *Bacillus megatarium* NRCB001, *B. subtilis* subs. *subtilis* NRCB002 and *B. subtilis* NRCB003 isolated from the rhizosphere of rice seedlings significantly promoted the growth and development of Alfalfa (*Medicago sativa* L.) grown under normal conditions. Moreover, on inoculating the isolates to alfalfa seedlings growing under 130 mM NaCl condition, *B. subtilis* subs. *subtilis* NRCB002 and *B. subtilis* NRCB003 significantly boosted the plant's growth, thereby qualifying as potential candidates for bioinoculants to be utilized in alleviating salinity stress (Zhu et al., 2020).

An ACC deaminase synthesizing PGPR *Achromobacter xylosoxidans* SQU-1 isolated from the rhizosphere of date palm growing under salinity significantly enhanced the germination of date palm seeds as well as the growth of Arabidopsis under normal and saline conditions (Jana and Yaish, 2021).

Three ACC deaminase synthesizing PGPR, that is *Pseudomonas fluorescens* (ACC-5), *P. fluorescens* (ACC-14) and *P. putida* (Q-7), significantly ameliorated the effect of drought stress on peas plants. However, at the lowest moisture level *P. putida* (Q-7) proved to significantly enhanced the growth of the plant (Zahir et al., 2008).

From the rhizosphere of *Cajanus cajan* (Pigeon pea), a multi-trait ACC deaminase synthesizing *Enterobacter* has been isolated by (Anand et al., 2021). The isolate was found to mitigate the effect of salinity stress in *Cajanus cajan* under greenhouse as well as field conditions.

A study conducted by Kruasuwan & Thamchaipenet (2018) demonstrated that ACC deaminase producing endophytic diazotrophic Enterobacter sp. colonizes and facilitates the growth of *Saccharum* sp. (sugarcane) under salinity stress. Their

findings evidently shows that the bacteria play a significant role in plant growth promotion. Moreover, the ACC deaminase synthesized by the bacteria conferred the plant with salinity stress tolerance.

Achromobacter xylosoxidans, a halotolerant ACC deaminase synthesizing endophytic bacteria isolated from *Catharanthus roseus* (Madagascar periwinkle), under gnotobiotic and pot conditions ameliorated salt stress in *C. roseus*. At 150 mM NaCl level, seedlings inoculated with the isolate recorded an ethylene level of 394.1 pmol ethylene g^{-1} FW h^{-1} while the uninoculated ones recorded an ethylene level of 516 pmol ethylene g^{-1} FW h^{-1}. Moreover, the inoculated seedlings show a maximum germination percentage of 98.3, vigour index of 2231.4, plant height of 120.4 cm, root dry weight of 53.24 g $plant^{-1}$ and ajmalicine content of 1.60 mg g^{-1}, compared with germination index of 91.6%, vigour index of 1511.5, plant height of 105.8 cm, root dry weight of 47.2 g $plant^{-1}$ and ajmalicine content of 1.23 mg g^{-1} in uninoculated control grown under normal conditions (Karthikeyan et al., 2012).

Belimov et al. (2009) studied the effect of inoculating *Pisum sativum* (pea) plant growing under drought stress condition with the ACC deaminase synthesizing rhizobacteria *Variovora paradoxus* 5C-2. Inoculation of the plants with bacteria massively improved growth and water use efficiency of the plants.

Inoculating *Capsicum annuum* L. (red pepper) seedlings exposed to 150 mM salt stress with – *Bacillus licheniformis*, *Brevibacterium iodinum* and *Zhihengliuela alba* leads to the suppression stress ethylene production (Siddikee et al., 2011). Moreover, the salt tolerance index of the plant was significantly increased via inoculation with isolates.

Inoculation with *Enterobacter* sp. leads to significant mitigation of salinity induced stress in wheat seedlings and enhanced growth. Likewise, ionic balance in the seedlings was maintained via less accumulation of toxic Na^+ and increased uptake of K^+ (Singh & Jha, 2016). Palaniyandi et al. (2014) isolated a halotolerant ACC deaminase synthesizing *Streptomyces* sp. from agricultural soil. The isolate promoted the growth of Arabidopsis seedlings in-vitro and mitigated the effect of salinity stress in tomato plants exposed to 180 mM NaCl stress growing under gnotobiotic condition.

3. MATERIAL AND METHOD

3.1. Material

3.1.1 Media composition

3.1.1.1. Carboxymethyl cellulose (CMC) agar

To determine the ability of bacteria in synthesizing cellulase, CMC agar was used, the media was prepared as described by Kim et al. (2005).

Table 3.1. Carboxymethyl cellulose (CMC) agar composition

Ingredient	L^{-1}
Carboxymethyl cellulose	10 g
Peptone	5 g
Yeast extract	5 g
Potassium dihydogen phosphate (KH_2PO_4)	1 g
Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)	0.2 g
Sodium chloride (NaCl)	10 g
Agar	15 g

3.1.1.2. Chrome azurol S (CAS) agar

In determining the siderophore synthesizing ability of the isolates, CAS media was utilized. The media was prepared according to the description of Schwyn & Neilands (1987) with slight modification.

Table 3.2. Chrome azurol S (Agar) composition

Ingredient	L^{-1}
Iron (III) chloride ($FeCl_3$)	3.244 mg
Chrome azurol S	60.5 mg
Hexadecyltrimethylammonium chloride (HDTMA)	72.9 mg
10mM Hydrochloric acid (HCl)	20 mL
Nutrient agar	20 g

The various components of the medium were prepared as follows:

CAS indicator preparation

Iron (III) chloride solution: 3.244mg of $FeCl_3$ was dissolved in 20mL 10mM HCl.

CAS solution: 60.5mg CAS solution was dissolved in 50 mL distilled water, 10mL $FeCl_3$ was dispensed upon it.

HDTMA solution: 72.0mg HDTMA was dissolved in 40mL distilled water.

All the above prepared solutions were mixed, and their final volume was made to 100mL.

CAS agar preparation

Twenty-gram nutrient agar was dissolved in 900mL distilled water. The dissolved nutrient agar and 100mL of the CAS solution whose final pH were 6.8ºC were separately incubated at 121ºC for 15 minutes. The nutrient agar was cooled to below 50ºC upon which the CAS solution was dispensed onto it. The emergent CAS agar was dispensed unto sterile petri plates.

3.1.1.3. Dworkin-Foster Minimal salts (DF salts) media

This media was utilized in determining the ability of the rhizobacteria to synthesize ACC deaminase, it was prepared according to the description of Penrose & Glick (2003).

Table 3.3. Dworkin-Foster Minimal salts media composition

Ingredient	L^{-1}
Potassium dihydrogen phosphate (KH_2PO_4)	4 g
Sodium hydrogen phosphate (Na_2HPO_4)	6 g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	0.2 g
Glucose	2 g
Gluconic acid	2 g
Citric acid	2 g
Ammonium sulphate ($(NH_4)_2SO_4$)	2 g
Essential elements	
Iron (II) sulfate ($FeSO_4 \cdot 7H_2O$)	1 mg
Boric acid (H_3BO_3)	10 µg
Manganese (II) sulfate ($MnSO_4 \cdot H_2O$)	11.19 µg
Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)	124.6 µg
Copper (II) sulfate ($CuSO_4 \cdot 5H_2O$)	78.22 µg
Molybdenum trioxide (MoO_3)	10 mg

The trace elements were prepared by dissolving 10mg H_3BO_3, 11.19 mg $MnSO_4.7H_2O$, 124.6 mg $ZnSO_4$.7H2O, 78.22 mg $CuSO_4.5H_2O$, and 10mg MoO_3 in 100mL sterile distilled water and stored in the refrigerator. $FeSO_4.7H_2O$ (100mg) was dissolved in 10mL sterile distilled water. The media was prepared by dissolving all the ingredients including 0.1 mL each of $FeSO_4$ and trace elements in 1 L distilled water and autoclaved at 121ºC for 15 min.

3.1.1.5. Phytase screening media

This media was used in determining phytase production potential of the isolates, the media was prepared according to the description of Kerovuo et al. (1998).

Table 3.4. Phytase screening media composition

Ingredient	**L^{-1}**
D-Glucose	20 g
Sodium phytate	4 g
Calcium chloride ($CaCl_2$)	2 g
Ammonium nitrate (NH_4NO_3)	5 g
Potassium chloride	5 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5 g
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	0.01 g
Manganese sulfate ($MnSO_4 \cdot H_2O$)	0.01 g
Agar	15 g

3.1.1.6 Phytase production media

This media was used to quantitatively determine phytase activity by the isolates. It was prepared according to the description of Choi et al. (2001).

Table 3.5. Phytase production media composition

Ingredient	**L^{-1}**
Glucose	5 g
Peptone	5 g
Magnesium sulfate ($MgSO_4$)	0.5 g
Calcium chloride ($CaCl_2$)	0.5 g
Phytic acid	0.5 g
Beef extract	2.5 g

3.1.1.7. Pikovskaya's media

This media was used in phosphate solubilization assay, the media was prepared according to the description of Pikovskaya (1948).

Table 3.6. Pikovskaya's media composition

Ingredient	**L^{-1}**
Yeast extract	0.5 g
Calcium phosphate ($Ca_3(PO_4)_2$)	5 g
Ammonium sulfate ($(NH_4)_2SO_4$)	0.5 g
Potassium chloride (KCl)	0.2 g
Magnesium sulfate ($MgSO_4$)	0.1 g
Manganese sulfate ($MnSO_4$)	0.1 mg
Ferrous sulfate ($FeSO_4$)	0.1 mg
Dextrose	10 g
Agar	15 g

3.1.2. Reagents

3.1.2.1. Phytase screening reagents

The following reagents were utilized in phytase activity evaluation, the reagents were prepared as described by (Choi et al., 2001; De Angelis et al., 2003; Yanke et al., 1998).

Substrate

The substrate was prepared by:

1. Making 0.1M Tris-HCl solution: 1.2114g Trizma base was dissolved in 100 mL distilled water and its pH was adjusted with HCl to 7.

2. 16.48mg Sodium phytate was dissolved in 100mL 0.1M Tris-HCl solution.

Reaction termination solution

5% trichloroacetic acid was utilized as reaction termination solution.

Colouring reagent

The colouring solution comprises of:

Solution I: 5.73mL H_2SO_4 was dispensed into 93.7mL distilled water to give 5.5% H_2SO_4.

Solution II: 2.5g Ammonium molybdate was dissolved in appropriate amount of solution I, the solution was vortexed, and the final volume was brought to 100 mL.

Solution III: 2.5g $FeSO_4$ was dissolved in appropriate volume of distilled water, the final volume was brought to 100 mL and 1 to 2 drops of 96% H_2SO_4.

To obtain the colouring solution, solution II and III were mixed in 4:1 ratio.

Note: The colour reagent is freshly prepared daily.

3.1.2.2. Salkowsky reagent

Salkowsky reagent was prepared according to Gang et al. (2019). Into a beaker containing 49 mL distilled water, 49 mL 60% Perchloric acid was gently dispensed, the beaker and its contents was vortexed, and 2 mL 0.5 $FeCl_3$ was added. The emergent solution was stored in a duran bottle.

3.1.2.3. Picric acid solution

The picric acid solution was prepared by dissolving 1 g Picric acid in an appropriate amount of distilled water, the final volume was made to 200 mL (0.5% picric acid). 4 g of Sodium carbonate (Na_2CO_3).

3.2. Method

3.2.1. Sampling

Samples were collected from the area surrounding Tuzla lagoon of Karataş district, Adana province, Republic of Turkey. Sampling was done on October 14, 2021. The region has an annual average temperature of 19.4°C and an average annual precipitation of 1019 mm. The samples were collected by gently uprooting the plants with the soil attached to their roots undisturbed, the samples were placed into sterilized polythene bags, and were transported to the laboratory.

Table 3.7. Rhizosphere soil samples collected

ID	Plant	Coordinates		Elevation (m)
A	*Juncus sp.*	36.68° N	35.08° E	-15.7
B	*Juncus sp.*	36.68° N	35.08° E	-16.6
C	*Salicornia sp.*	36.68° N	35.08° E	-8.2
D	*Halopeplis amplexicaule*	36.68° N	35.08° E	-6.2
E	*Limonium sp.*	36.68° N	35.08° E	-10.9
F	*Halimione portulacoides*	36.68° N	35.08° E	-7.1
G	*Arythrocnemum sp.*	36.68° N	35.08° E	-13.3
H	*Halopeplis amplexicaule*	36.68° N	35.08° E	-5.6

3.2.2. Isolation of halotolerant Rhizobacteria

Rhizosphere soil adhering to the plant roots were gently washed into 50 mL sterilized phosphate buffered saline (Composition per L: 8 g NaCl; 0.2 g KCl; 1.44 g Na_2HPO_4; 0.24 g KH_2PO_4; 2 mL tween 20; pH 7.2) contained in 250 mL Erlenmeyer flasks. The flasks with their contents were agitated for 30 mins at 150 rpm on a Lab companion (SKF2025) orbital shaker. The stock preparations were serially diluted (10^{-1} – 10^{-5}), 1 mL aliquot from the last dilution was plated on Nutrient agar plates (Merck 1.05450) supplemented with 4, 6, 8 and 10% sodium chloride (Sigma-Aldrich 13423) with the aim of isolating halotolerant Rhizobacterial strains. The inoculated plates were incubated at 28 ± 2 ºC for 7 days. Distinctive bacterial colonies were selected, purified, and maintained on Nutrient agar slants supplemented with 4% sodium chloride at 4 ºC.

3.2.3. Halotolerance assay

Nutrient broth supplemented with 6, 8, 10, 12, 15, 25 and 30% sodium chloride added was used in evaluating the halotolerance of the isolates. Test tubes containing 5 mL of the medium were inoculated with 10^8 cfu/mL of freshly

prepared inoculum of the isolates. Inoculated tubes were incubated at 200 rpm in a shaker incubator for 24 hours at 28 ± 2ºC.

3.2.4. Screening for siderophore production

The ability of the isolates to synthesize siderophores was determined according to Schwyn and Neilands (1987). For this purpose, modified Chromium Azurol S (CAS) blue agar was used. Isolates that were able to withstand salinity of 15% were selected. Seven µL of the isolates were spot inoculated on CAS agar plates. Inoculated plates were incubated at 28 ± 2ºC for 72 hours. A change in the colour of the medium, indicated by the appearance of orange-yellow colour around the colonies, indicates siderophore production (Yasmin et al., 2020).

3.2.5. Phosphate solubilization assay

3.2.5.1 Qualitative phosphate solubilization assay

The ability of isolates to dissolve phosphate in solid medium was performed using Pikovskaya's agar supplemented with 4% NaCl. To inoculate the isolates onto the medium, 7 µL of the bacterial isolates were inoculated onto the centre of the agar plates. The plates were then incubated at 28 ± 2ºC for 10 days. The diameter of the halo region and that of the bacterial colony were measured, and the formula reported by Hariprasad & Niranjana (2008) was used to determine the solubilization index.

$$\text{Phosphate solubilization index (SI)} = \frac{\textbf{\textit{Total diameter (colony + halo zone)}}}{\textbf{\textit{Colony diameter}}}$$

3.2.5.2. Quantitative phosphate solubilization assay

Isolates that were to solubilized phosphate on agar were further screened in Pikovskaya's broth to quantitatively determined the amount of phosphate they solubilized. The isolates were cultured in Nutrient broth overnight. The final

bacterial cell suspension was adjusted to 10^8 CFU mL^{-1} (OD595 = 0.3) as described by Thompson et al. (1996). Erlenmeyer flasks containing 50 mL of Pikovskaya's broth were inoculated with 100 µL of the bacterial inoculum and were incubated at 200 rpm for 3 days at 28 ± 2ºC.

After each 24-hour interval, 1 mL aliquots were taken aseptically and centrifuged at 10,000 rpm for 15 minutes. To determine the amount of solubilized inorganic phosphate in the supernatant, the Molybdenum blue method as described by Chaihan & Lumyang (2009), (Gull et al, 2004) and Zhu et al (2020) was used. Five hundred µL of the supernatant was introduced into a test tube containing 500 µL of 10% Trichloroacetic acid, the tube was vortexed, and 4 mL of colouring reagent was added. After incubating the tube for 15 minutes at 28 ± 2ºC, absorbance was recorded at 700 nm using a Shimadzu UV-spectrophotometer (UV-1800), an uninoculated medium served as blank. The amount of dissolved phosphorus was determined from a standard phosphate solubilization curve (Fig. 3.1). Moreover, the pH of the culture was determined.

3.2.5.3. Standard phosphate solubilization curve

To obtain 50µgmL^{-1} KH_2PO_4 stock solution, 12 mg KH_2PO_4 was dissolved in 240 mL distilled water. The solution was diluted to obtain concentrations ranging from 0 to 10 µg mL^{-1} (Table 3.9).

Upon 1 mL of various concentrations prepared from the stock solution 1.5 mL of colouring reagent was added, and absorbance at 700 nm was recorded. The standard curve was generated by plotting absorbance against concentration (µg/mL) using the IBM SPSS statistics (v26.0) application (Figure 3.1).

Table 3.8. Standard phosphate solubilization curve

Concentration (µg mL^{-1})	0	1	2.5	5	7.5	9.5	10
Distilled water (mL)	5	4.90	4.75	4.50	4.25	4.05	4.00
Stock KH_2PO_4 (µL)	0	100	250	500	750	950	1000

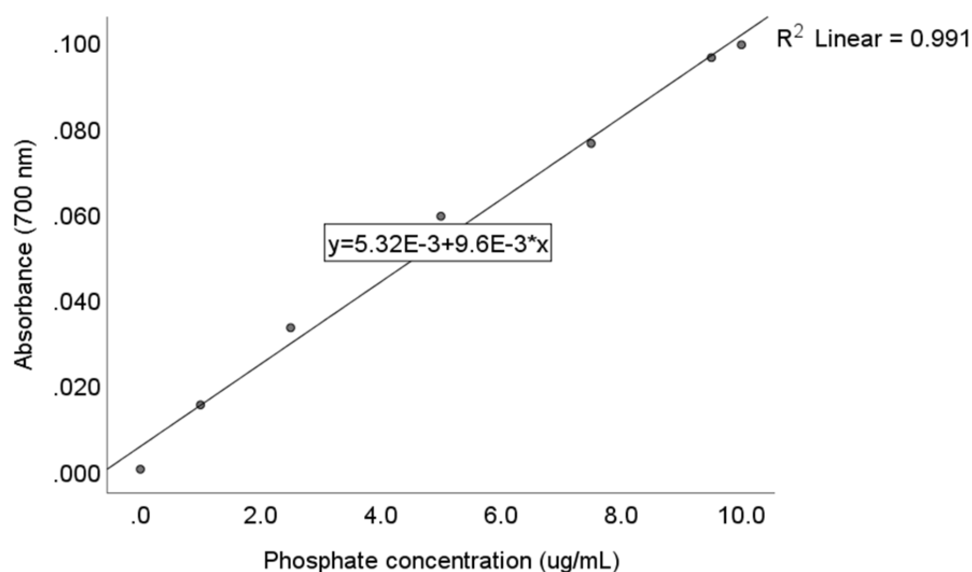

Figure 3.1. Standard phosphate solubilization curve

3.2.6. Phytase activity determination

3.2.6.1. Qualitative determination of phytase activity

Phytase activity was qualitatively determined on solid medium following the description of Yanke et al. (1998). The isolates were inoculated onto phytase screening agar and incubated at 28 ± 2ºC for 48 hours. The plates were flooded with 2% cobalt chloride solution. The cobalt chloride solution was replaced after 5 minutes incubation at room temperature with a freshly prepared solution containing equal volumes of a 6.25% (w/v) ammonium molybdate and a 0.42% (w/v) ammonium vanadate solution. After 5 minutes incubation at room temperature, the

solution was discarded, and the plates were visually examined for halo zones that indicate phytase activity.

3.2.6.2. Quantitative determination of phytase activity

The phytase activity of the isolates was quantitatively evaluated according to the method described by Choi et al. (2001) and De Angelis et al. (2003). Hundred µL of the bacterial culture was inoculated into Duran bottles containing 30 mL Phytase production medium (PPM) and were incubated at 200 rpm for 48 hours at 28 ± 2ºC. One mL aliquot was taken and centrifuged at 10,000 rpm for 10 minutes. One hundred µL of the supernatant was transferred to a test tube, and 900 µL of substrate was added. The final solution was vortexed and incubated on a water bath for 15 minutes at 37ºC. The reaction was terminated adding 750 µL of 5% Trichloroacetic. The tubes were removed from the water bath, 1.5 mL colouring solution was added, and the tubes were allowed to stand for 10 minutes. Subsequently, 2 mL from the tubes were transferred to Eppendorf tubes. The tubes were centrifuged at 10,000 rpm for 5 minutes and absorbance was recorded at 700 nm using Shimadzu UV-spectrophotometer (UV-1800). The phytase activity was graphically calculated based on standard phosphate calibration curve. One unit (U) of phytase activity is the amount of enzyme required to liberate 1 nmol of inorganic phosphate (P_i) per minute (Choi et al., 2001; De Angelis et al., 2003).

3.2.7. Indole acetic acid production

The ability of the isolates to synthesize indole acetic acid in the presence of L-tryptophan was determined following the description of Gang et al. (2019). The isolates were grown overnight in test tubes containing 5 mL nutrient broth at 28 ± 2ºC. Subsequently, the bacterial cells were harvested and centrifuged at 10,000 rpm for 15 mins. The concentration of the bacterial inoculum was adjusted to approximately 10^8 CFU mL^{-1} (OD_{595}=0.3) with the aid of Shimadzu UV-spectrophotometer (UV-1800) (Thompson et al., 1996). Test tubes containing 5 mL

0.1% L-Tryptophan amended nutrient broth were inoculated with 100 µL of the bacterial inocula. The tubes were subsequently incubated at 150 rpm for 48 hours at 28 ± 2ºC. From each tube 1.5 mL was harvested and centrifuged at 10,000 rpm for 15 minutes. To a test tube containing 4 mL Salkowsky reagent, 1 mL of the supernatant was added. The resultant solution was vortexed and incubated for 30 minutes at 30ºC. Absorbance was taken at 536 nm using Thermo scientific Multitaskan Go (v100.40) spectrophotometer. Uninoculated broth to which the reagent has been added served as blank.

The amount of indole acetic produced was determined from a standard indole acetic acid curve (10 – 100 µg mL^{-1}) (Figure 3.2).

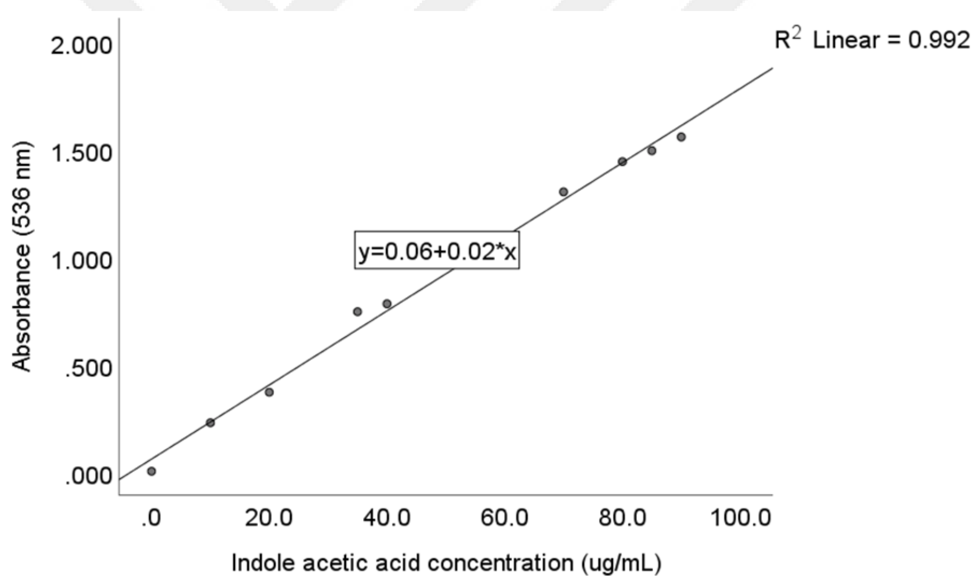

Figure 3.2. Standard indole acetic acid curve

To obtain 100 µg mL^{-1} indole acetic acid stock solution, 0.01 g Indole-3-acetic acid was dissolved in 100 mL distilled water. The solution was diluted to obtain concentrations ranging from 0 to 100 µg ml^{-1} (Table 3.10.).

One mL of Salkowsky reagent was added to 1 mL of the various concentrations of the stock solution prepared above and absorbance was recorded at 536 nm. The standard curve was generated by plotting absorbance against concentration (µg mL^{-1}) using the IBM SPSS statistics (v26.0) application.

Table 3.9. Standard indole acetic acid curve

Concentration (µg mL^{-1})	0	10	20	35	40	55	60	70	75	80	85	90
Distilled water (mL)	5	4.5	4	3.25	3	2.25	2	1.5	1.25	1	0.75	0.5
Stock IAA (mL)	0	0.5	1	1.75	2	2.75	3	3.5	3.75	4	4.25	4.5

3.2.8. ACC deaminase activity determination

The ACC deaminase activity of the isolates was determined following the protocol of Penrose & Glick (2003). LB broth was inoculated with the isolates and incubated for 24 hours at 28 ± 2ºC. The culture was harvested at 8,000 g for 10 min at 4ºC. The supernatant was discarded, and the cell pellets were washed with sterile DF salts media. The washed pellets were resuspended in DF salts media. Hundred microliters of the suspended bacterial cells were inoculated into tubes containing 5 mL of DF media supplemented with 3 mM ACC as a sole Nitrogen source. The inoculated tubes were incubated at 28 ± 2ºC for 48 hours.

The culture was harvested at 16,000 g for 5 minutes. Subsequently, the cell pellets were washed twice in 0.1 M Tris HCl (pH 7.6). The pellets were re-suspended in 600 µL of 0.1 M Tris HCl (pH 8.5) and 30 µL of toluene was added and the tubes were vortexed for 30 seconds.

Two hundred microlitre of the toluenized cells were transferred into Eppendorf tube and 20 µL of 0.5 M ACC was added. The suspension was vortexed and incubated at 30ºC for 15 minutes. One millilitre of 0.56 M HCl was added, the

tubes were vortexed and centrifuged at 16,000g for 5 minutes at room temperature. One millilitre of the supernatant was vortexed in a glass tube with 800 µL of 0.56 M HCl. Thereupon, 300 µL of 2,4-dinitrophenylhydrazine reagent was added. The content of the tube was vortexed and incubated for 30 minutes at 30ºC. The absorbance of the mixture was recorded at 540 nm after the addition of 1 mL 2 N NaOH.

The concentration of the α-ketobutyrate generated was determined through comparison with a standard α-ketobutyrate curve. Five hundred µL aliquot of different α-ketobutyrate solution (Table 3.11.) was mixed with 400 µL of 0.56 M HCl and 150 µL of 2,4-dinitrophenylhydrazine. One millilitre of 2 N NaOH was added to the mixture and the absorbance at 540 nm was recorded. A graph of absorbance against α-ketobutyrate concentration was plotted (Figure 3.3).

Table 3.10. Different concentrations of α-Ketobutyrate

Concentration (mM)	0	0.01	0.05	0.1	0.2	0.75	0.85	0.95
α-ketobutyrate (µL)	0	5	25	50	100	375	425	475
Distilled water (µL)	5000	4995	4975	4950	4900	4625	4575	4525

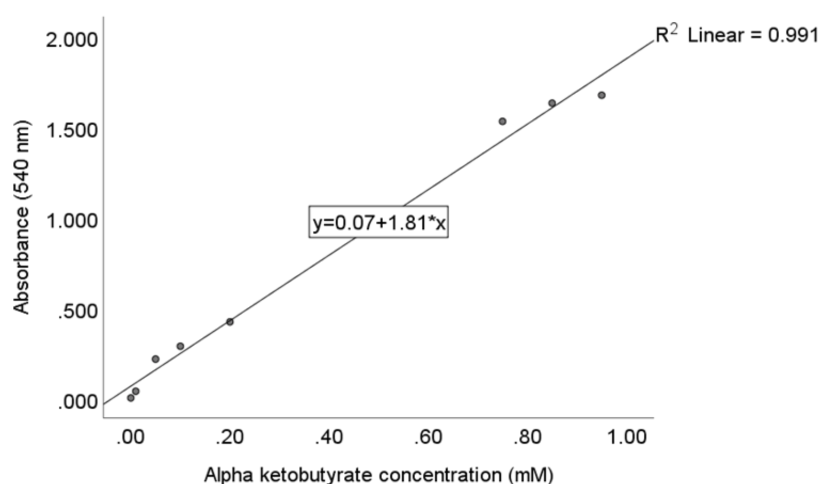

Figure 3.3. Standard α-Ketobutyrate concentration curve

3.2.9. Screening for Hydrogen cyanide production

Nutrient agar amended with 4.4 g L^{-1} glycine was inoculated with the isolates. A sterilized Whatman No. 1 filter paper was soaked in Picric acid solution. The soaked filter paper was placed onto the inoculated petri plates. The plates were incubated at 28 ± 2ºC for 4 days. HCN production was indicated by the development of orange to red colour (Lorck, 1948).

3.2.10. Cellulase activity detection

Carboxymethyl cellulose agar plates were inoculated with the bacterial isolates and incubated for 48 hours at 28 ± 2ºC. After incubation, the plates were flooded with 1% (w/v) Congo red solution. After 15 minutes, the solution was discarded, and the plates were de-stained with 1 M NaCl solution for 15 minutes. Degradation of cellulose by the isolates was indicated by the appearance of clear zone of hydrolysis around the bacterial colonies (Baharuddin et al., 2010).

3.2.11. Screening for biofilm formation

The ability of the isolates to form biofilm in-vitro was determined according to the protocol reported by (Stepanovic et al., 2000). Into the wells of a sterile microtiter plate, 180 µL of sterile Trypticase soy broth supplemented with 1% glucose was dispensed. The wells were then inoculated with 20 µL of overnight grown culture of the bacterial isolates. The plates were subsequently incubated aerobically at 28 ± 2ºC for 48 hours.

The content of the plate was discarded, and the plate was rinsed thrice with sterile distilled water. The bacteria adhering to the wells were fixed with 200 µL of 99% Methanol. The microplate was emptied after 15 minutes and was air dried. The microplate was stained with 200 µL of 2% crystal violet, the stain was rinsed off after 5 minutes with distilled water. The plate was air dried, and the cells were resolubilized with 160 µL of 33% glacial acetic acid. Absorbance was determined at 570 nm. Isolates were classified into the following categories: non-adherent (0),

weakly adherent (+), moderately adherent (++) and strongly adherent (+++) based on ODs of the bacterial biofilms. The cut-off OD (OD_c) for the microtiter plate assay is defined as:

$OD_c = OD_{blank} + (3 \times SD_{blank})$

$OD \leq OD_c$	Non-adherent (0)
$OD_c < OD \leq 2 \times OD_c$	Weakly adherent (+)
$2 \times OD_c < OD \leq 4 \times OD_c$	Moderately adherent (++)
$4 \times OD_c < OD$	Strongly adherent (+++)

3.2.12. *In-Vitro* Plant growth promotion test

3.2.12.1. Seed bacterization and soil preparation

The bacterial isolates were inoculated into Erlenmeyer flasks containing 50 mL Trypticase soy broth and where incubated at 28 ± 2ºC for 48 hours at 150 rpm. The culture was harvested by centrifugation at 10,000 rpm for 15 minutes and the cell pellets were washed twice with sterile distilled water. The cell pellets were resuspended in 0.03 M $MgSO_4$ solution, and the absorbance was adjusted to 0.3 at OD_{595}.

Prior to bacterization of the seeds, the seeds were sterilized by been soaked in 70% ethanol for 5 minutes, after which they were rinsed three times with sterile distilled water. The seeds were soaked further in 1.5% sodium hypochlorite solution for 10 minutes and were rinsed thoroughly with distilled water. The sterilized seeds were blotted dry on sterile paper in a biosafety cabinet.

The seeds were bacterized by been soaked in the bacterial suspension prepared above for 1 hour on an orbital shaker (150 rpm). Likewise, control seeds were soaked in 0.03 M $MgSO_4$ solution under the same conditions (Singh & Jha, 2016).

3.2.12.2. Green house trial

The experimental setup consists of 4 levels of salt treatment and 11 levels of bacterial treatment (Table 3.12). In each well of seedling trays containing commercially prepared potting soil, 6 seeds were planted. The trays were irrigated daily with sterile distilled water. Salinity stress was induced after 7 days by irrigating the trays with different concentrations of saline water for 20 days. On harvesting the seedlings on the 27th day, the shoot and root length of seedlings were measured. Likewise, the fresh weight of the seedlings was determined. While the dry weight of the seedlings was determined after they were dried in a hot air oven at 70ºC for 48 hours.

Table 3.11. Green house trial experimental setup

Salt concentration	Bacterial isolates										
0 mM	C	B15	B22	B30	C10	C23	D11	D15	E2	E4	H9
100 mM	C	B15	B22	B30	C10	C23	D11	D15	E2	E4	H9
200 mM	C	B15	B22	B30	C10	C23	D11	D15	E2	E4	H9
300 mM	C	B15	B22	B30	C10	C23	D11	D15	E2	E4	H9

Note: C stands for control, for the coding used to refer to the bacterial isolates refer to the symbols and abbreviations sections

3.2.13. Molecular identification of the bacterial isolates

3.2.13.1. Gram staining

Prior to the isolation of the genomic DNA of the isolates, gram status of the isolates was determined according to the description of Cappuccino ve Sherman (2010). A smear was made from a freshly prepared bacterial culture on a clean, grease-free glass slide, the smear was air-dried and heat-fixed. The smear was covered with crystal violet, the dye was gently washed with distilled water after 1 minute. The smear was coated with Lugol's iodine, the dye was gently washed with distilled water after 1 minute. The smear was decolorized with 95%

ethanol, washed with distilled water and counter stained with safranin for 45 seconds. The slide was washed with distilled water and blotted dry. The dried slide was observed under oil immersion lens (1000×).

3.2.13.2. Genomic DNA isolation

Genomic DNA of the isolates was isolated using the GeneJET genomic DNA purification kit. For Gram-negative isolates, 1 mL of bacterial culture grown in Nutrient broth for 24 hours at 28 ± 2°C was centrifuged in a microcentrifuge tube for 10 minutes at 5,000 g. After discarding the supernatant, the pellet was resuspended in 180 µL of Digestion solution. Twenty µL of Proteinase K solution was added and the tube was vortexed. The tube with its contents was incubated at 56°C in a shaker incubator. Twenty µL of RNase A solution was added, the tube was vortexed and incubated at room temperature. Two hundred µL of lysis solution was added to the tube and mixed thoroughly by vortexing for 15 seconds until a homogeneous mixture was obtained, and 400 µL of 50% ethanol was added.

The prepared lysate was transferred to a GeneJET genomic DNA purification column placed in a collection tube. The column was centrifuged at 6,000 g for 1 minute. The collection tube containing the flow solution was discarded. The GeneJET genomic DNA purification column was inserted into a new 2 mL collection tube, and 500 µL of wash buffer was added. The column + collection tube was centrifuged at 8,000 g for 1 minute. The flow solution was discarded, the column was placed back in the collection tube, and 500 µL of wash buffer II was added to the column. The column + tube was centrifuged at 12,000 g for 3 minutes and the collection tube was discarded. The column was transferred to a sterile 1.5 mL microcentrifuge tube, and 200 µL of elution buffer was added to the centre of the column membrane to separate the genomic DNA. The column + tube was incubated for 2 minutes at room temperature and was subsequently centrifuged at 8,000 g for 1 minute. The purification column was discarded, and the purified DNA was stored at -20°C.

All genomic DNA purification steps are the same as for gram-negative bacteria, except for resuspending the pellets of gram-positive bacteria in 180 µL of gram-positive bacteria lysis buffer and incubating at 37°C for 30 minutes.

Extracted genomic DNA was amplified by PCR using the following universal primer: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). PCR products were electrophoresed on a 1% agarose gel. The obtained PCR sequence products were purified and sequenced by Macrogen (Madrid, Spain). The Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to analyse the 16S rDNA sequences of the isolates.

3.2.14. Data analysis

All experiments were performed in triplicate, the IBM SPSS (v26.0) software package was used to perform ANOVA on the dataset, and multiple pair-wise comparisons were made using the Tukey test ($p<0.05$).

4. RESULTS AND DISCUSSION

Salinity stress interferes with the physiological and biochemical mechanisms of plants, causes imbalance in nutrient uptake, alters growth-inducing regulators, and inhibits protein synthesis as well as photosynthesis (Ali et al., 2022). Halotolerant microbes are of great agricultural importance as they can be utilized in increasing crop productivity in both arid and semi-arid regions.

4.1. Halotolerance of the isolates

On evaluating the ability of the isolates to tolerate high salt concentration, none of the isolates was able to tolerate salt concentration of up 30%. (Table 4.1).

Table 4.1. Salt tolerance of the isolates

Isolate	Salt concentration						
	6%	8%	10%	12%	15%	20%	25%
B15*	+	+	+	+	+	-	-
B22	+	+	+	+	+	-	-
B30	+	+	+	+	+	+	+
C10	+	+	+	+	+	+	+
C23	+	+	+	+	+	+	+
D11	+	+	+	+	+	-	-
D15	+	+	+	+	+	+	-
E2	+	+	+	+	+	+	-
E4	+	+	+	+	+	+	-
H9	+	+	+	+	+	-	-

*For the coding used to refer to the bacterial isolates refer to the symbols and abbreviations sections

Since bacteria that tolerate 1-5% NaCl, 6-18% NaCl and 9-30% NaCl are classified as low, medium and high halotolerant (Hindersah et al., 2019), respectively, 3 of the isolates were found to be medium halotolerant while the rest were high halotolerant. Halotolerant PGPR proliferate around plant roots, thereby forming a rhizosphere microbiome (Fitriatin et al., 2018; Larsen, 1986). Halotolerant PGPR balance their osmotic pressure to prevent denaturation due to salt present in their environment (Khumairah et al., 2022). Thus, they can thrive and provide more benefits to plants than their non-halotolerant counterparts (Etesami and Glick, 2020). Halotolerant bacteria limit the entry of large amount of salt into their cells through their cell membranes or walls. Cell membranes or walls of halophilic bacteria have a special composition that is strictly resistant to high salt concentration (Khumairah et al., 2022)

The osmotic adaptation in these bacteria assists them in regulating the intracellular ionic concentration, and regulation of the intracellular ionic concentration is achieved by pumping out Na^+/K^+ using antiporter or K^+/Na^+ transporters (Khumairah et al., 2022). in addition, bacteria accumulate compatible solutes via endogenous biosynthesis and up-regulation of the synthesis of essential amino acids, enzymes and proteins (Noori et al., 2018)

4.2. Siderophore production

Siderophore synthesizing ability of the selected isolates was evaluated. Isolates with colonies surrounded by orange-yellow colour were considered siderophore synthesizers. (Figure 4.1).

Siderophores are small, high-affinity, iron-chelating compounds secreted by microbes that help them overcome iron limitation (Baek et al., 2022). Where they form a complex with Fe^{3+} they act as solubilizing agents for iron, thus making it available to both plants and microbes (Indiragandhi et al., 2008). Also, siderophore production by microbes is beneficial for plants as it can inhibit the growth and reproduction of phytopathogens (Sharma & Johri, 2003).

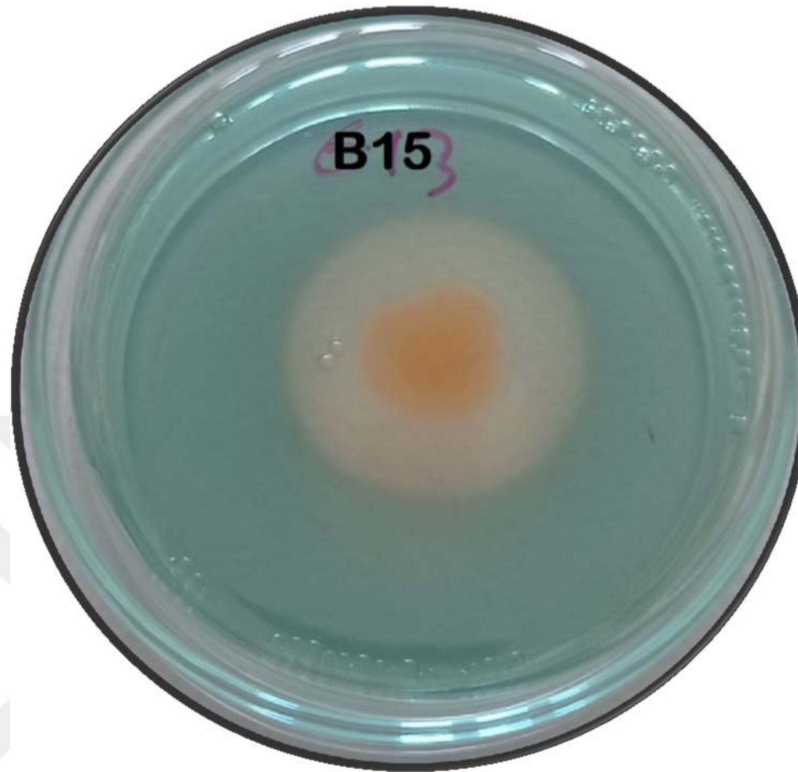

Figure 4.1. Siderophore synthesizing isolate

4.3. Phosphate solubilization

The Selected isolates were also evaluated for their ability to solubilize phosphate on solid medium, phosphate solubilization is indicated by a clear halo region around bacterial colony (Figure. 4.2). *Paenibacillus sinopodophylli* sp. nov. - a siderophore producing endophytic bacteria- isolated by Chen et al. (2016). Similarly, *Bacillus aryabhattai* MS3 was found to synthesize siderophore under iron-limiting conditions (Tamariz-Angeles et al., 2021). Other bacteria reported to be siderophore producers include *Acinetobacter baumannii* (Penwell and Actis, 2019), *Bacillus flexus*, *B. mycoides*, *B. toyonensis*, *B. Velezensis* (Barman et al 2022), *Burkholderi*a sp. (B. Ali et al, 2022), *Methylobacterium* spp. (Lacava et al 2008).

Figure 4.2. Phosphate solubilizing isolate

On evaluating the phosphate solubilization index of the isolates, it was found that isolate B15 showed the lowest phosphate solubilization index of 1.02, while isolate E2 with phosphate solubilization index of 2.09 was found to exhibit the highest solubilization index (Table 4.3).

To obtain more reliable results regarding phosphate solubilization, the phosphate solubilizing isolates were evaluated for their ability to solubilize tricalcium phosphate in Pikovskaya's broth. A significant increase in solubilized phosphate was found in the culture filtrate, resulting in a decrease in pH. Isolate E2 with a phosphate solubilization of 83.90 ± 1.32 µg mL^{-1} was found to exhibit the highest solubilization rate among the isolates, while isolate B15 with a solubilization rate of 38.00 ± 0.09 µg mL^{-1} had the lowest solubilization rate

(Figure 4.3). After incubation, the pH of the medium was observed to dropped significantly from 7 to 4.26 - 5.17. The decrease in pH clearly indicates the production of organic acid and phosphatase (Chaihan and Lumyang, 2009). By affecting the environmental condition as well as the enzyme activity of a microbe, pH significantly interferes with microbial (Bhojiya and Joshi, 2016; Sahu et al., 2022).

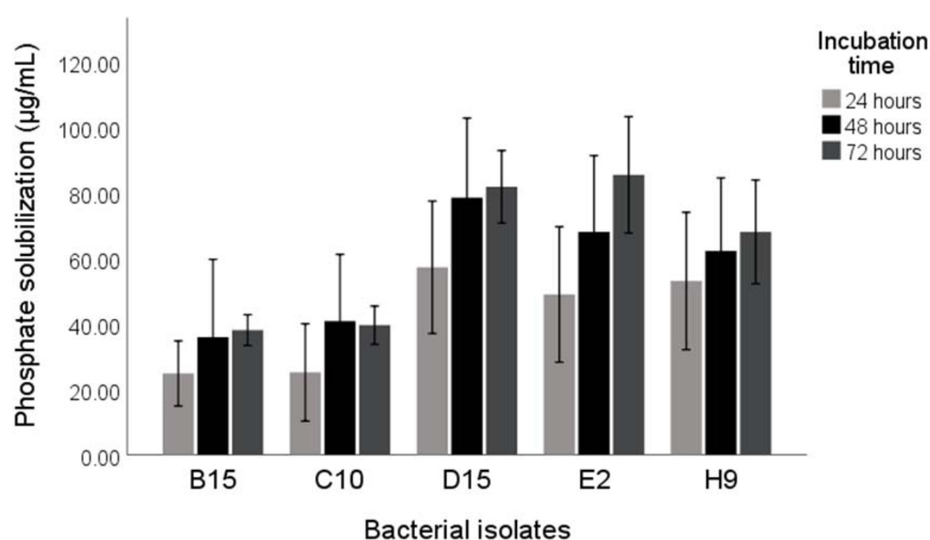

Figure 4.3. Phosphate solubilization activity of the isolates. Error bars indicate standard error (n = 3)

Fluctuations in concentration of solubilized phosphorus concentration can be attributed to precipitation of organic metabolites with phosphorus and/or formation of organo-phosphoric compounds (Babenko et al., 1984; Illmer and Schinner, 1992; Khan and Bhatnagar, 1977). Otherwise, when the phosphorus uptake rate exceeds its solubilization, a decrease in phosphorus concentration in the medium may occur, similarly, when the uptake rate decreases, the phosphorus concentration in the medium increases (Rodríguez and Fraga, 1999).

Phosphate solubilization by microbes is generally attributed to acidification, chelation of ligands, and redox processes (Wei et al., 2018). Tricalcium phosphate in the medium was converted to a soluble form that manifests itself in bacterial culture supernatant or microbial biomass (Sahu et al., 2022). Insoluble phosphorus can be converted into soluble form by microbes through acidification, chelation, polymeric substance formation and exchange reactions (Wei et al., 2018). However, the main strategy employed by solubilizing phosphate is acidification (Pérez et al., 2007). There seems to be a clear relationship between dissolution of phosphate from $Ca_3(PO_4)_2$, acidification, and bacterial growth. A common strategy developed by microbes to scavenge for phosphorus from insoluble sources is the synthesis and exudation of organic acids (Rodríguez and Fraga, 1999).

4.4. Phytase activity

Only three of the selected isolates were able to produce phytase. Phytase production is indicated by the appearance of a halo zone around bacterial colony (Figure 4.4).

Figure 4.5 presents the phytase activity of the isolates determined quantitatively. Various studies have been carried out on phytase producing bacteria isolated from the environment. For example, Kumar et al. (2013) isolated from Himalayan soil *Acromobacter* sp., *Bacillus* sp. and *Tetrathiobacter* sp. that hydrolyze phytate. Other bacteria proven to be phytase producers include *Bacillus amyloliquefaciens* (Idriss et al., 2002), *Bacillus laevolacticus* (Gulati et al., 2007), *Bacillus subtilis* (Kerovuo et al., 1998), *Burkholderia* sp. (Unno et al., 2005), *Klebsiella terrigena* (Ralf Greiner et al., 1997), *Pseudomonas* sp. (Richardson & Hadobas, 1997), *Staphylococcus lentus* (Hussin et al., 2007).

Figure 4.4. Qualitative assessment of phytase production by the isolates

About 20 - 80% of phosphate found in soil occurs in organic form as phytate, and plants may not easily obtain phosphorus directly from soil (Greiner and Alminger, 2001; Alan E Richardson et al., 2001). Microbes reduce phytate to lower phosphate esters using phytase. Phytases (Myo-inositol hexakisphosphate phosphohydrolase) gradually hydrolyse phytate to Myo-inositol and phosphate to form myo-inositol phosphate mediator (Konietzny and Greiner, 2002). Dephosphorylation of phytate by phytase is thought to play a vital role in plant nutrition (Unno et al., 2005). Due to the lack of extracellular phytase activity, many plant species do not utilize soil phytate as a source of phosphorus (Unno et al., 2005). However, plants were able to utilize phytate when the phytase gene from

Aspergillus niger was expressed or exogenous phytase was added in the transgenic plant (Hayes et al., 2000; Idriss et al., 2002; Richardson et al., 2001).

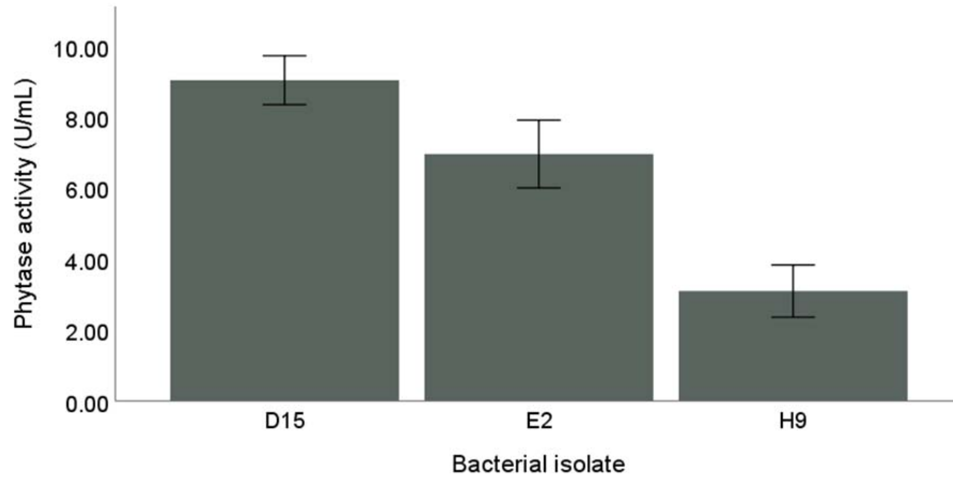

Figure 4.5. Phytase activity of the isolates. Error bars indicate standard error (n = 3)

4.5. Indole acetic acid production

Four of the isolates were found to be synthesizers of indole acetic acid (Table 4.2). IAA released by rhizobacteria interferes with plant development processes because the endogenous pool of plant IAA can be altered through uptake of IAA secreted by soil bacteria (Uzma et al., 2022). IAA is synthesized through several independent biosynthetic pathways. In growing stems, IAA causes a rapid increase in cell wall extensibility and promotes the growth of auxiliary bud. It also aids apical dominance in plants, likewise it stimulates lateral and adventitious root development and growth (Mohanty et al., 2021). Furthermore, IAA plays an important role in stimulating plant growth by being involved in DNA synthesis (Glick, 1995).

4.6. ACC deaminase synthesis

None of the isolates was found to significantly express ACC deaminase (Table 4.2).

4.7. Hydrogen cyanide production

The hydrogen cyanide synthesizing ability of the isolates was evaluated and, as noted in the materials and methods section, hydrogen cyanide production is manifested by the evolution of brown colour on the picric acid paper (Figure 4.6).

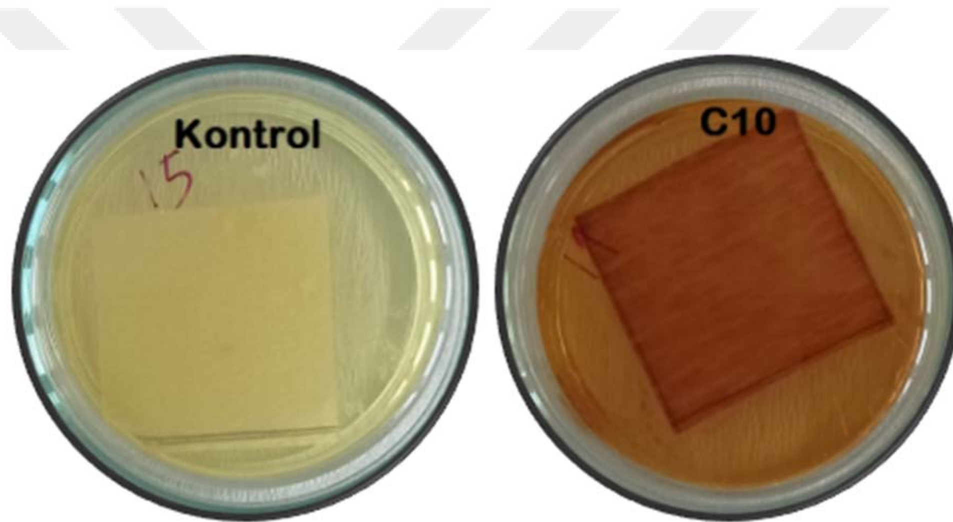

Figure 4.6. Hydrogen cyanide producing isolate

In acidic soil, HCN increases the sequestration of iron, resulting in increased availability of phosphate (Rijavec and Lapanje, 2016). In addition, HCN interrupts the activity of metalloenzymes and reacts with keto compounds and Schiff-base intermediates thereby inhibiting the functioning of many enzymes or protein transporters, and in some special cases may possibly inhibit the growth of certain organisms (Cooper and Brown, 2008).

Control of nematodes by some bacterial strains has been attributed to their ability to produce HCN (Abd El-Rahman et al., 2019). However, in order to

effectively control nematodes, a direct contact between HCN producing bacteria and nematodes is required (Lanteigne et al., 2012). Nevertheless, HCN is not a universal biocontrol agent (Rijavec and Lapanje, 2016). B*acillus megaterium*, *Pseudomonas* sp. strain Gamma-81, *P. chlororaphis*, *P. japonica*, *P. mosselii* and *P. tolaasii* - bacteria synthesizing HCN - inhibited the growth of *Agrobacterium tumefaciens* and affected the in vitro viability of *Meloidogyne incognita* (Abd El-Rahman et al., 2019).

Table 4.2. Plant growth promoting properties of the isolates

Isolate	SD	PSI	IAA (µg mL^{-1})	ACC	HCN	Cellulase	BF
B15	+++	1.02	-	-	++	+	+++
B22	++	-	-	-	++	+	+++
B30	+++	-	-	-	+	+	+
C10	++	1.42	-	-	+++	+	++
C23	+++	-	0.47 ± 0.20*acd	-	+++	+	-
D11	++	-	2.04 ± 0.68bce	-	++	+	+
D15	++	1.45	1.63 ± 0.30abcde	-	-	+	+++
E2	++	2.09	1.80 ± 0.27abcde	-	+	-	+++
E4	++	-	0.33 ± 0.03ad	-	+	+	+
H9	++	1.58	3.22 ± 0.31be	-	-	+	+++

SD: siderophore; PSI: Phosphate solubilization index; IAA: Indole acetic acid; ACC: 1 aminocyclopropan-1-carboxylate deaminase; HCN: Hydrogen cyanide; BF: Biyofilm; Negative (-), low (+), medium (++), high (+++). * Values represent the mean ± standard error of three replicates, Different letters represent significant difference ($p<0.05$)

4.8. Cellulase activity

All but one isolate were found to synthesize cellulase enzyme (Table 4.2). About 20 - 30% of the plant biomass is made up of cellulose, thus making it the most abundant biopolymer (Berg and Laskowski, 2006). Decomposition of cellulose is mainly due to the activity of soil microorganisms that assimilate simple

sugars resulting from the reduction of complex polysaccharides (Baldrian and Valásková, 2008; Lynd et al., 2002).

4.9. Biofilm formation

All the isolates except one were found to form biofilm (Table 4.2). A biofilm is a population of microbial cells growing on a surface and enclosed in an amorphous extracellular matrix (Stepanović et al., 2004). Biofilm formation under variable stress conditions is an important strategy adopted by bacterial strains for their successful survival in plant rhizosphere (Kasim et al., 2016). Because of successful plant-microbe interaction, PGPR are effective in colonizing plant roots and proliferate in micro-colonies and produce biofilm. These plant-associated biofilms can protect against external stress, reduce microbial competition, and confer beneficial effects on the host plant (Lugtenberg and Kamilova, 2009; Ramey et al., 2004; Saleh-Lakha and Glick, 2006).

4.10. Promotion of plant growth under salinity stress

Plant growth is adversely influenced by salinity. Water uptake by plants is hindered by high osmotic pressure, and toxicity from specific ions occurs due to the presence of large amount of soluble ions and molecules (Bensidhoum et al., 2019). Owing to ionic and osmotic stress caused by salinity, plants experience fluctuations in their biochemical, morphological and physiological properties (Yañez-Yazlle et al., 2021). Biochemical and physiological changes include changes in antioxidant enzymatic activities, drop in chlorophyll content, increase in reactive oxygen species (ROS), and osmolyte production (Bensidhoum et al., 2019; Kibria and Hoque, 2019).

From the results obtained (Figure 4.7 - 4.10), it was observed that none of the bacterial inoculants was able to significantly mitigate the effect of salinity on the seedlings. This could be attributed to the inability of the isolates to synthesize ACC deaminase. ACC deaminase hydrolyses ACC to ammonia and α-ketobutyrate,

thereby lessening the level of stress-induced ethylene (Glick et al., 1999; Kusale et al., 2021). Decreased ethylene levels help roots to grow well and increase the absorption of plant nutrients (Kusale et al., 2021). Since this research is based solely on mitigation of salinity stress by the isolates, the ability of the isolates to promote plant growth cannot be ignored. However, to authoritatively determine the level at which the isolates can enhance plant growth, independent research examining different parameters should be conducted in the future.

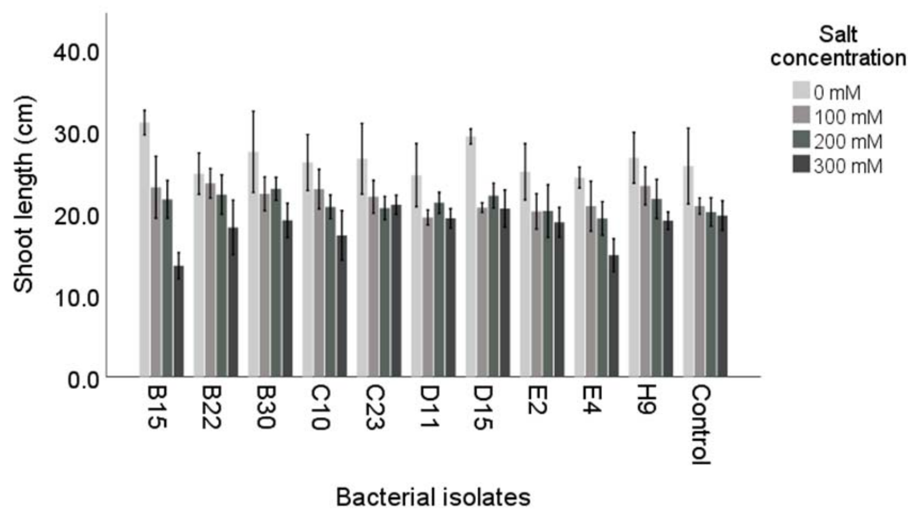

Figure 4.7. Shoot length of PGPR inoculated *T. aestivum* seedlings grown under salinity stress. Error bars indicate standard error (n=7)

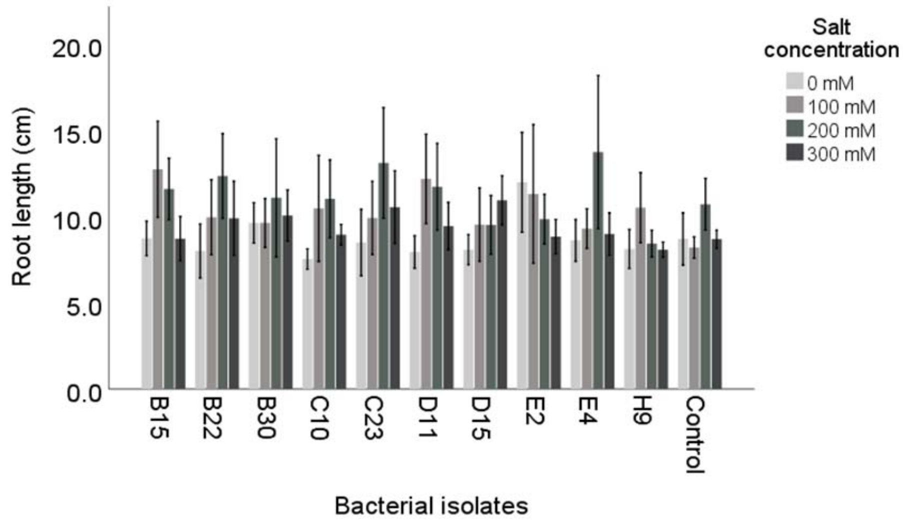

Figure 4.8. Root length of PGPR inoculated *T. aestivum* seedlings grown under salinity stress. Error bars indicate standard error (n=7)

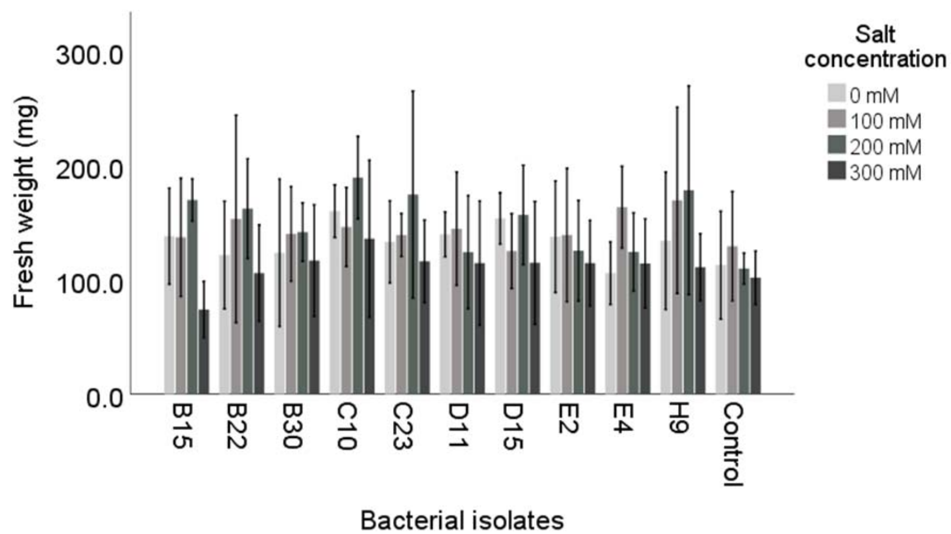

Figure 4.9. Fresh weight of PGPR inoculated *T. aestivum* seedlings grown under salinity stress. Error bars indicate standard error (n=7)

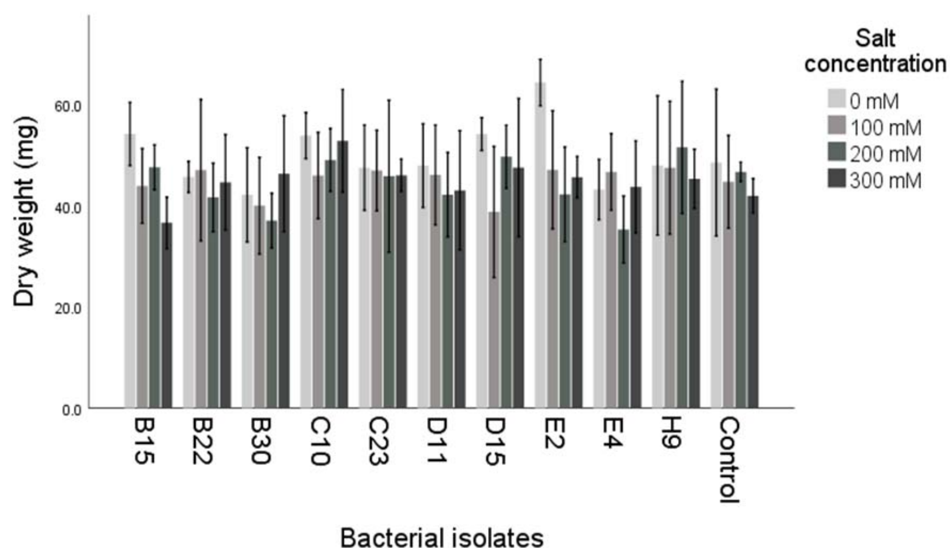

Figure 4.10. Dry weight of PGPR inoculated *T. aestivum* seedlings grown under salinity stress. Error bars indicate standard error (n=7)

Table 4.3. Plant growth parameters of *T. aestivum* irrigated with 0 mM salt water

	0 mM salt concentration			
Isolate	Shoot length	Root length	Fresh weight	Dry weight
B15	31.06±0.76*a	8.74±0.50a	137.73±13.15a	54.25±3.12a
B22	24.81±1.27a	8.01±0.79ab	121.25±14.75a	45.75±1.55a
B30	27.47±2.48a	9.64±0.59a	123.25±20.21a	42.2±4.66ab
C10	26.17±1.71a	7.54±0.3ad	159.5±7.22a	53.93±2.28a
C23	26.63±2.16a	8.5±0.97ae	132.75±11.24a	47.58±4.22a
D11	24.63±1.93a	7.96±0.48af	139.25±6.14a	47.98±4.14a
D15	29.34±0.47a	8.09±0.44ag	153.25±7.04a	54.23±1.63a
E2	25.04±1.72a	12±1.45ac	137.25±15.29a	64.43±2.3ac
E4	24.33±0.64a	8.63±0.61a	105.5±8.57a	43.25±2.98ad
H9	26.74±1.56a	8.13±0.57ah	133.75±18.83a	48.03±6.9a
Control	25.74±2.32a	8.71±0.76a	112.5±14.78a	48.6±7.26a

Values represent Means ± Standard error from seven replicates. Control refers to seedlings without bacterial inoculation. *Data of columns indexed by the same letter are not significantly different according to Tukey post hoc test ($p<0.05$)

Table 4.4. Plant growth parameters of *T. aestivum* irrigated with 100 mM salt water

	100 mM salt concentration			
Isolate	Shoot length	Root length	Fresh weight	Dry weight
B15	23.14±1.89*a	12.76±1.4a	136.75±16.22a	43.98±3.69a
B22	23.63±0.9a	9.97±1.09a	152.75±28.44a	47.1±6.99a
B30	22.34±1.03a	9.64±0.71a	139.75±12.97a	40.08±4.79a
C10	22.91±1.21a	10.49±1.54a	145.75±10.83a	46.03±4.27a
C23	21.99±1.01a	9.93±1.07a	138.75±5.91a	47.03±3.98a
D11	19.46±0.46a	12.2±1.3a	144.25±15.53a	46.15±4.94a
D15	20.67±0.29a	9.54±1.07a	124.75±10.27a	38.83±6.48a
E2	20.2±1.08a	11.33±2.02a	138.75±18.28a	47.15±5.85a
E4	20.84±1.53a	9.31±0.57a	163±11.23a	46.75±3.78a
H9	23.31±1.16a	10.53±1.02a	169±25.51a	47.58±6.57a
Control	20.86±0.49a	8.21±0.31a	129±14.97a	44.8±4.59a

Values represent Means ± Standard error from seven replicates. Control refers to seedlings without bacterial inoculation. *Data of columns indexed by the same letter are not significantly different according to Tukey post hoc test ($p<0.05$)

Table 4.5. Plant growth parameters of *T. aestivum* irrigated with 200 mM salt water

	200 mM salt concentration			
Isolate	Shoot length	Root length	Fresh weight	Dry weight
B15	21.67±1.16*a	11.61±0.89a	169.25±5.79a	47.65±2.22a
B22	22.26±1.21a	12.37±1.23a	161.75±13.67a	41.7±3.4a
B30	22.96±0.71a	11.1±1.72a	141.25±8.04a	37.1±2.71a
C10	20.76±0.73a	11.04±1.13a	188.75±11.32ab	49.1±3.12a
C23	20.61±0.71a	13.13±1.61a	174±28.36a	45.9±7.52a
D11	21.26±0.65a	11.74±1.26a	123.75±15.52a	42.25±4.18a
D15	22.13±0.76a	9.53±0.85a	156.25±13.63a	49.75±312a
E2	20.26±1.61a	9.86±0.72a	125±13.83a	42.3±4.69a
E4	19.34±1.03a	13.75±2.23a	124±10.73a	35.35±3.32a
H9	21.73±1.19a	8.44±0.39a	177.75±28.62a	51.6±6.54a
Control	20.13±0.87a	10.73±0.75a	109.5±4.25ac	46.75±0.96a

Values represent Means ± Standard error from seven replicates. Control refers to seedlings without bacterial inoculation. *Data of columns indexed by the same letter are not significantly different according to Tukey post hoc test ($p<0.05$)

Table 4.6. Plant growth parameters of *T. aestivum* irrigated with 300 mM salt water

	300 mM salt concentration			
Isolate	Shoot length	Root length	Fresh weight	Dry weight
B15	13.57±0.8*ab	8.73±0.64a	73.75±7.72a	36.68±2.55a
B22	18.24±1.66a	9.91±1.08a	105.5±13.23a	44.7±4.72a
B30	19.11±1.05ac	10.07±0.75a	116.5±15.3a	46.4±5.74a
C10	17.27±1.51a	8.96±0.3a	135.5±21.53a	52.9±5.07a
C23	21.01±0.59ad	10.56±1.05a	115.75±11.33a	46.08±1.6a
D11	19.36±0.6ae	9.46±0.69a	114.25±16.99a	43.1±5.91a
D15	20.56±1.13af	10.96±0.71a	114.5±16.81a	47.6±6.84a
E2	18.87±0.91ag	8.84±0.5a	114.25±11.74a	45.7±2.05a
E4	14.86±1.02a	9±0.62a	114±12.21a	43.78±4.55a
H9	19.07±0.54ah	8.09±0.22a	110.75±9.19a	45.38±2.93a
Control	19.7±0.9ai	8.7±0.26a	101.5±7.33a	42.03±1.7a

Values represent Means ± Standard error from seven replicates. Control refers to seedlings without bacterial inoculation. *Data of columns indexed by the same letter are not significantly different according to Tukey post hoc test ($p<0.05$)

Soil salinity in arid and semi-arid regions leads to increased evapotranspiration than precipitation, and this results to water stress conditions, likewise in the plant root zone soil minerals are constantly leached (Zörb et al., 2019). Plant growth and productivity deteriorates due to limitation of gas exchange, cessation of stomatal opening and closing, and reduced carbon assimilation (Lisar et al., 2012).

Huang et al. (2022) reported that under salt-free conditions, salt-tolerant PGPR did not significantly promoted wheat growth, but under increased salt concentration, the PGPR was able to promote growth, moreover the effect of PGPR on growth significantly decreased when the salt concentration exceeded 300 mM. Similarly, *Bacillus megaterium* PN89 has been shown to reduce the effect of salinity stress on wheat plants (Lee et al., 2015). *Bacillus sp. HX11* with multiple plant growth promoting traits and salinity tolerance has also been reported to show great potential as a bioinoculant in lessening the effect of salinity stress on plants (Yañez-Yazlle et al., 2021).

Kapadia et al. (2021) reported a significant reduction in root length by 32.1% and shoot height by 19.7% in wheat seedlings after 45 days of growth under salinity. However, on inoculating the seedlings with halotolerant *Klebsiella variicola*, the effect of salinity on the seedlings was lessened significantly and the development of the seedlings was enhanced. Similarly, Redondo-Gómez et al. (2022)reported that inoculating Swiss chard (*Beta vulgaris* L.) with halotolerant PGPR consortium significantly alleviated the effect of salinity stress.

4.11. Morphological and molecular identity of the isolates

Both the morphological and molecular identity and the bacterial isolates are presented in Table 4.3. All the isolates but *Staphylococcus epidermidis* have been isolated from saline environments and where classified as halotolerant (Bangash et al., 2015; Quesada et al., 1984; Sripo et al., 2002; Vreeland et al.,

1980; Wu et al., 2008) The emergence of *S. epidermidis* amongst the isolates can be attributed to contamination during sampling.

Table 4.7. Morphological and molecular identity of the isolates

Code	**Morphology**	**Bacterial strain**
B15*	Gram-negative Bacilli	*Halomonas elongata*
B22	Gram-negative Bacilli	*Halomonas elongata*
B30	Gram-negative Coccobacilli	*Halomonas caseinilytica*
C10	Gram-negative Bacilli	*Halomonas elongata*
C23	Gram-negative Bacilli	*Halomonas halophila*
D11	Gram-negative Bacilli	*Halomonas salina*
D15	Gram-positive Cocci	*Staphylococcus epidermidis*
E2	Gram-negative Bacilli	*Kushneria pakistanensis*
E4	Gram-negative Coccobacilli	*Halomonas caseinilytica*
H9	Gram-negative Bacilli	*Halomonas elongata*

*For the coding used to refer to the bacterial isolates refer to the symbols and abbreviations sections

5. CONCLUSION AND RECOMMENDATIONS

In both arid and semi-arid regions, one of the foremost abiotic stresses hampering crop production is salinity stress. One of the promising strategies of mitigating the effect of salinity on plants and enhancing plant growth is the inoculation of plants with PGPR. In this study, potential halotolerant PGPR were isolated from the rhizosphere of halotolerant shrubs growing around Tuzla lagoon of Karataş district, Adana province, Republic of Turkey. The isolates proved to exhibit multiple plant growth promoting traits such as siderophore production, indole acetic acid synthesis, cellulase production, Biofilm formation and hydrogen cyanide production. However, ACC deaminase, a critical enzyme in reducing salinity stress in plants, was not produced by the isolates. Hence, when the effect of inoculating wheat seedlings - growing under salinity stress - with the isolates was evaluated, none of the isolates was able to alleviate the effect of salinity on the seedlings.

Even though the isolates were able to partially mitigate the effect of salinity on the seedlings, their ability to enhance plant growth under different environmental conditions - such as drought stress, potassium, phosphate and iron deficiency conditions - should be explored in the future.

This work has demonstrated the potential for the isolating halotolerant PGPR from the rhizosphere of halophytes. The PGPR isolated have been found to exhibit desirable plant growth promoting traits. Thus, the findings of this study clearly indicate that the rhizosphere of halophytes can serve as a useful reservoire of halotolerant rhizobacteria with multiple plant growth promoting traits. Moreover, the growth and yield of both non-halophytic and halophytic crops can be enhanced via inoculation with halotolerant PGPR isolated from the rhizosphere of halophytes.

This approach of utilizing halotolerant PGPR in crop production has the potential to boast the productivity of unproductive lands. Furthermore, as the field of microbial bioremediation expands exponentially, research can be conducted to evaluate the potential of these isolates in the bioremediation of heavy metal contaminated soils.

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RESUME

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