REPUBLIC OF TURKEY
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF AGRICULTURAL SCIENCE AND TECHNOLOGIES FACULTY OF AGRICULTURE

PLANT GROWTH PROMOTING PROPERTIES OF *BACILLUS* SPECIES FROM SOIL SAMPLES

Prepared By
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Thesis Supervisor
Assoc. Prof. Dr. Semih YILMAZ

M. Sc. Thesis

December 2017
KAYSERİ

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SCIENTIFIC ETHICS SUITABILITY

I declare that all information in this work was obtained in accordance with academic and ethical rules. All results and material that not been at the essence of this work are also transferred and expressed by giving reference as required by these rules and behavior.

Khalid Abdullah MAMOORI

SUITABILITY FOR GUIDE

The MSc thesis entitled "**Plant Growth Promoting Properties of *Bacillus* Species from Soil Samples"** has been prepared in accordance with Erciyes University Graduate Education and Teaching Institute Thesis Preparation and Writing Guide.

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ACCEPTANCE AND APPROVAL PAGE

This study entitled "**Plant Growth Promoting Properties of *Bacillus* Species from Soil Samples"** prepared by Khalid MAMOORI under the supervision of Assoc. Prof. Dr. Semih YILMAZ was accepted by the jury as MSc. Thesis in Department of Head of Department of Agricultural Science and Technologies, Faculty of Agriculture.

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That the acceptance of this thesis has been approved by the decision of the Institute's Board of Directors with the 26/12/2017 date and 2017/55-15 numbered decision

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Director of the Institute

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PLANT GROWTH PROMOTING PROPERTIES OF BACILLUS SPECIES FROM SOIL SAMPLES

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Erciyes University, Graduate School of Natural and Applied Sciences
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ABSTRACT

Plant growth promoting bacteria (PGPR) are free living microorganisms and can colonise on roots and have enhancing effect on plant growth through direct or indirect mechanisms. The interaction between bacteria and plants are reciprocal so as to benefit from each other. In the present study, *Bacillus* spp. were aimed to isolate from soil samples, screen in terms of genes encoding the enzymes related with growth promotion, and apply on corn plant. The promoting effect of 19 isolates carrying one or more of ACC deaminase, siderofore, and AcPho genes were studied on plants both as seed and seedlings inoculations in greenhouse conditions. All bacterial isolates indicated significant growth promotion in seed and seedling inoculation trials of corn plant. However the results in seed inoculation trials were more prominent in terms of important parameters as shoot length (F=2,507; p=0,002), stem fresh weight (F=6.091; p=0,000), shoot dry weight (F=5.244; p=0,000), shoot diameter (F=4.812; p=0,000), root volume (F=7.275; p=0.000), root diameter (F=3.639; p=0.000), root length (F=3.119; p=0.001), root fresh weight (F=4.378; p=0.000), and root dry weight (F=4.432; p=0.000). For example, isolates SY29.1, KH6.2, and KH18.2 resulted in 59, 58, and 56% increase in stem diameter, respectively. Isolates SY29.1, KH28.1, and KH6.2 caused an increasing in stem fresh weight at a level of 156%, 136%, and 130%, respectively. Most notably, SY29.1 and KH13.3 caused 267, and 236% increase in shoot dry weight compared to control. Among 150 isolates, phylogenetic analysis of 16S rDNA region of best-acting six were performed and characterised at species level as *Bacillus subtilis* KH28.1, *Bacillus subtilis* KH18.2 *Bacillus* sp. KH16.2, *Bacillus cereus* KH14.2, *Bacillus cereus* KH21.4 *and Bacillus simplex* SY29.1 by using NCBI's (National Centre for Biological Information) Basic Local Alignment Search Tool (BLAST) and comparing with known sequences of bacteria in NCBI database. In conclusion, results obtained from seed inoculation tests were better compared to

seedling inoculation tests on corn plant. Pot trials in greenhouse conditions indicated that KH28.1, KH13.3, KH14.3, KH6.2 isolates seem to be promising strains as biofertilising inoculants for growth promotion, but the results remain to be verified in field conditions.

Keywords: PGPR, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus simples*, Corn, ACC deaminase, siderofore, AcPho

TOPRAK ÖRNEKLERİNDEN İZOLE EDİLEN BACİLLUS TÜRLERİNİN BİTKİ BÜYÜMESİNİ TEŞVİK EDİCİ ÖZELLİKLERİ

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ÖZET

Bitki büyümesini teşvik eden bakteriler (PGPR) serbest yaşayan mikroorganizmalar olup kökte kolinize olabilir ve doğrudan veya dolaylı mekanizmalarla bitki büyümesi üzerinde teşvik edici etki göstertilirler. Bakteriler ve bitkiler arasındaki ilişki birbirinden fayda sağlayacak şekilde karşılıklıdır. Bu çalışmada toprak örneklerinden *Bacillus* türlerinin izolasyonu, büyüme teşvikiyle ilgili enzim kodlayan genlerin taranması ve mısır bitkisi üzerinde denenmesi hedeflenmiştir. ACC deaminaz, siderofor ve asit fosfataz genlerinden bir veya daha fazlasını taşıyan 19 izolatın teşvik edici özelliği serada bitkiler üzerinde hem tohum hem de fide inokülasyonu şeklinde denenmiştir. Mısırda tüm izolatlar hem tohum hem de fide inokülasyonunda büyümeyi anlamlı düzeyde teşvik etmiştir. Fakat, gövde uzunluğu (F=2,507; p=0,002), gövde yaş ağırlığı (F=6.091; p=0,000), gövde kuru ağırlığı (F=5.244; p=0,000), gövde çapı (F=4.812; p=0,000), kök hacmi (F=7.275; p=0.000), kök çapı (F=3.639; p=0.000), kök uzunluğu (F=3.119; p=0.001), kök yaş ağırlığı (F=4.378; p=0.000) ve kök kuru ağırlığı (F=4.432; p=0.000) gibi önemli parametrelerde tohum inokülasyonu sonuçları daha iyi bulunmuştur. Örneğin SY29.1, KH6.2 ve KH18.2 izolatları kök çapında sırasıyla %59, %58 ve %56 oranlarında artışa neden olmuştur. SY29.1, KH28.1 ve KH6.2 izolatları gövde yaş ağırlığında sırasıyla 156%, 136% ve 130% oranında artış sağlamıştır. En belirgin olarak ise SY29.1 ve KH13.3 izolatları gövde kuru ağırlığında kontrole göre sırasıyla %267 ve %236 oranlarında artış sağlamıştır. Elde edilen 150 izolat içerisinde en iyi etki gösteren 6 tanesinin 16S rDNA bölgesi NCBI'da (National Centre for Biological Information) BLAST (Basic Local Alignment Search Tool) program ile bilinen bakterilerle kıyaslanmış ve filogenetik analizi yapılarak tür seviyesinde *Bacillus subtilis* KH28.1, *Bacillus subtilis* KH18.2 *Bacillus* sp. KH16.2, *Bacillus cereus* KH14.2, *Bacillus cereus* KH21.4 *ve Bacillus simplex* SY29.1 olarak belirlenmiştir. Sonuç olarak tohum inokülasyon denemeleri fide inokülasyon denemelerine göre daha etkili

bulunmuştur. Serada yapılan saksı denemelerine göre KH28.1, KH13.3, SY29.1 ve KH6.2 izolatları büyümeyi teşvik etme yönüyle önemli biyofertilizer inokülantlar olarak ortaya çıkmasına rağmen sonuçların arazi çalışmalarıyla teyit edilmesi gerekmektedir.

Anahtar Kelimeler: PGPR, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus simples*,Mısır, ACC deaminaz, siderofor, AcPho

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ABBREVIATIONS

ACC **:** 1-aminocyclopropane-1-carboxylic acid

ATP **:** Adonintri-phosphat

C° **:** Degree celsius

CUF **:** Colony Forming Units

dNTP **:** Deoxyribonucleotid triphosphate

EC **:** Eictrical Conductivity

EDTA **:** Ethylenediaminetetraacetic acid

F **:** Forward

FLPs **:** Flavodoxin-Like Proteins

GPS **:** The Global Positioning System

gr **:** Gram

h **:** Hours

IAA **:** Indole-3- acetic acid

KH **:** Khalid

LB **:** Luria Bertani

ml **:** Milliliter

PGPB **:** Plant growth promoting bacteria

PGPR **:** Plant growth promoting rhizobacteria

PSB **:** Phosphate solubilising bacteria

R **:** Reverse

Rpm **:** Rotations per minute

SY **:** Semih YILMAZ

µl **:** Micro liter

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INTRODUCTION

The world around us suffer from environmental damage resulting from human intervention in the work of nature, misuse of natural resources and large population pressure. All these will unfortunately result in the unintended consequence that the global food of the world may not be enough in the coming years to feed the whole world. There are many reports confirming that the population of the world is currently 7 billion and could grow to 8 billion in 2020. Food and Agriculture organization is confronting a difficult challenge, population growth and rising incomes in many developing countries that have increased in food and other agricultural yield demand to unprecedented levels [1]. It is therefore essential that agricultural production should increase significantly in the coming decades without harming the nature. In order to put an end to these risks, agricultural applications are moving toward a more sustainable and environmentally friendly ways. This involves both plant growth promoting rhizobacteria and the increased use of genetically modified plants. Plant growth promoting rhizobacteria (PGPR) are normally soil dwelling bacteria that forcefully colonize plant roots and advantages plants by providing growth promotion (PGPB) as an important part of the prevailing agricultural practices. The utilization of plant growth promoting rhizobacteria is steadily expanding in agriculture and offers an appealing approach to replace chemical fertilizers, pesticides, and nutrients. An assortment of bacterial qualities components and particular genes contribute to this process, however, just a few of them have been distinguished. These involve motility, chemotaxis to seed and root secretions, creation of pili and fimbriae, creation of particular cell components, capacity to utilize particular components of root secretions, protein secretion, and quorum sensing. An amazing natural environment where various microorganisms colonize on, and around the growing plants' roots is the rhizosphere. Root systems in all higher plants effectively affect the diversity of bacterial strains [2]. These bacterial groups are considered as efficient microbial rivals in the root zone, and the net impact of plant-microorganism associations on plant development could be positive, neutral, or

negative. Such microorganisms (especially bacteria) in close relationship with roots, which are able to stimulate the plant growth by any mechanism(s) of activity, are regarded as plant growth promoting rhizobacteria. Excessive use of chemical fertilizers adversely affect the living organisms in rhizosphere region and consequently reduce productivity of the soil and the efficient use of nutrients. Plant growth promoting rhizobacteria are alternative to excessive use of chemical fertilizers due to their ability to solubilize a lot of non-soluble elements as phosphorus, and production of necessary hormones that help in the growth of plants as well as their efficiency in biological control. PGPB are an important alternative for soil amendments that enhance the growth of plants and yield in several mechanism(s) for instant phytohormone production, supply of nitrogen through nitrogen fixation, free phosphorus by solubilization, sequestering iron by sidrophores [3]. PGPR additionally prevent plants from pathogens by direct hostile interactions between the pathogen and the biocontrol agent, and also by stimulation of host resistance. Phosphate dissolving bacteria can advance plant growth by production of phytohormones indole-3-acetic acid (IAA), gibberellins and cytokinins and different other plant growth promoting materials [4]. Phosphorus is the second most imperative supplement after nitrogen because it plays a vital role in root structure and root system architecture, stalk, crop ripeness, blooming and seed development, and biological nitrogen fixation. When the plant and the other part is transformed into insoluble forms such as iron and aluminium phosphate and in acidic conditions and the calcium phosphate in basal conditions or natural soil [5]. Bacteria can confer inorganic and organic phosphorus from the soil and make it free to plant for development and growth. Various types of bacteria like *Pseudomonas* and *Bacillus* spp are prominent soil bacteria in the root zone and non rhizosphere soil that called PSB. Insoluble inorganic phosphate is solubilized by organic acid secreting bacteria. The carboxyl and hydroxyl groups of these acids chelate the cations as Ca, Al and Fetied to phosphate and reduce the pH of soil. In addition, ethylene in plants has a wide range of biological activities, and especially be more effective at a concentration of 0.05 μL/L. Ethylene able to influence plant growth and development in a substantial number of ways, including promotion of fruit ripening, flower wilting and root initiation as well as inhibiting root elongation. Stress is the main reason for the production of ethylene. Stress ethylene includes different stresses like biotic and abiotic stresses.

Some rhizobial bacteria can produce ACC deaminase (1-aminocyclopropane-1-carboxylate) that able to remove portion of the ACC (the immediate precursor of ethylene in plants) before its is convertion to ethylene. *Bacillus* type of bacteria are among the most important types of PGPB with several species. *Bacillus* bacteria contain a variety of gram-positive, rod-shaped, obligate or facultative aerob species with the ability to form endospores. *Bacillus* species have a lot of properties that assist and promote plant growth in both direct and indirect ways. But unfortunately most of these properties remains to be clarified. In the present study, it was aimed to find some of the features and benefits of *Bacillus* species, obtained from soil samples in Iraq, in terms of growth promotion on corn plants. In the present study, we planned to find potential *Bacillus* type of PGPB species in soil samples obtained from different ecological places Iraq. The candidate organisms were screened in terms of genes coding for growth promoting chemicals.

CHAPTER 1

LITERATURE REVIEW

1.1. Plant Growth Promoting Rhizobacteria (PGPR)

The significance of root zone (rhizosphere) microbial communities for the maintanance of plant root health, supplement uptake, and resistance to environmental stress is well recognized [6]. These gainful microorganisms can be an important segment of management practices to obtain sufficient crop yields under limited physical conditions and natural hereditary potential. Plant growth-promoting rhizobacteria (PGPR) were initially characterized by Kloepper and Schroth (1978) to depict soil microscopic organisms that colonize plant roots following inoculation onto seeds, and promote plant growth. Colonization processes of PGPB strains involve the following steps [7].

1. Replication in the zone surrounding the seed (spermosphere) using the exudates of seed.

2. Attachment to root surfaces.

3. Inoculation onto the seed.

4. Colonize the growing root systems.

The main reason for the ineffectiveness of PGPB has often been attributed to their disability to colonize plant roots [8-9].

1.2. The Relation Between PGPB Colonization Roots in Rhizosphere

The expression "rhizosphere" was initially specified by Hiltner (1904) to portray the zone of soil occupied by plant roots. The rhizosphere is the region where microbial

diversity and activity increased. From around 0 to 2 mm from the root surfaces, the soil is fundamentally affected by living roots. The rhizosphere can in this way be depicted as the longitudinal and outspread inclinations where the extending growth of root, supplement and water uptake, exudation, and subsequent microbial growth takes place [10]. The rhizosphere is significant in expression of exudates product, root development, and community growth of both macro and microorganisms. The rhizospheric effect is known as the release of different organic products by the roots because of stimulation of microbial multiplication around the root. One of the most wonderful metabolic traits of plant roots is the capability to excrete an enormous array of products into the rhizosphere. Root products released into the soil (rhizosphere) from plants have been normally classified into low and high molecular weight compounds. Proteins, polysaccharides, and mucilage are grouped into high molecular weight compounds. The root cap, epidermal cells (including root hairs), and the primary cell wall between epidermal and sloughed root cap are all responsible from the release of plant mucilages. Additionally, the roots release lysates during autolysis. Microorganisms in rhizosphere are also able to release microbial mucilages. Mucilages from plant and microorganisms, microbial cells and secretions of living organisms together with related mineral and organic matter are called mucigel [11]. Ethylene, polysaccharides, vitamins, amino acids, sugars, and enzymes released from roots are altogether known as low-molecular weight organic compounds (Table 1.1). Colonization, competition, and population structure are affected by the nutritional resources. Living organisms in and around the roots contain fungi, protozoa, yeast, and bacteria. The bacterial community is one of the most important groups in the rhizosphere, which contain different species in *Bacillus*, *Flavobacterium*, *Pseudomonas*, and *Alcaligenes*, etc. Bacteria are generally classified into two groups as free-living and symbiotic with plants. The association amongst microorganisms and roots might be useful, destructive, or natural for the plant and sometimes the impact of microorganisms may change as an outcome of soil conditions [12-13]. The activities of living organisms in rhizosphere are the main determinant whether these root colonizers are symbiotic, pathogenic, or plant growth promoting microorganisms. The plant beneficial microorganisms can be grouped as phytostimulators, rhizoremediators, biofertilizers, and biopesticides. In spite of their significance to plant development, the molecular basis of plant-bacteria interaction is not perfectly known. This is the main reason for the

limited efficiency of PGPR in field conditions. Bacterial colonization of plant roots can be considered as an advancement of the best acclimatized living organisms to a specific ecological niche. The rhizosphere colonization is defined as the colonization of the part of soil affected by the root [7-14] Several benefits can be obtained from the colonization of the roots. It is not only a first step in microorganism-derived plant disease, but also critical for the use of microorganisms for advantageous applications. A certain set of bacterial genes contributes to the process of colonization, but few have been characterized [8-15]. PGPR, in general, promotes plant growth by colonizing the root zone and start the establishment of or repressing unhealthy rhizosphere bacteria. The most important step is the colonization of roots by inoculated bacteria and represents the relationship between bacteria and host plant roots. The first step of the colonization of roots is seed colonization. Microorganisms established on sprouting seed can multiply and colonize the root as it rises and develops through the soil.

Table 1.1.Compounds of rhizosphere

Class of compounds	Type of compounds
Amino acids	Alanine, a-amino adipic acid, g-amino butyric acid, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, histidine, homoserine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, therionine, tryptophan, tyrosine, valine
Organic acids	Acetic, aconitic, aldonic, butyric, citric, erythronic, formic, fumaric, glutaric, glycolic, lactic, malic, malonic, oxalic, piscidic, propionic, pyruvic, succinic, tartaric, tertronic, and valericacid
Sugare	Arabinose, deoxyribose, fructose, galactose, glucose, maltose, Oligosaccharides, raffinose, rhamnose, ribose, sucrose, xylose
Vitamins	p-Amino benzoic acid, biotin, choline, n-methionylnicotinic acid,niacin, panthothenate, pyridoxine, riboflavin, thiamine
Fatty acids and sterols	Palmitic, stearic, oleic, linoleic, and linoleic acids; cholesterol, campestrol, stigmasterol, sitosterol
Nucleotides	Adenine, guanine, uridine, cytidine
Enzymes	Amylase, invertase, phosphatase, polygalactouranase, proteases
Miscellaneous	HCO3−, OH−, H+, CO2, H2; auxins, flavonones, glycosides, saponin,scopolotin

[16,17,18,19,20].

1.3. Root colonization factors and efficiency of Rhizobacteria:

Bacterial root colonization is basically affected by particular bacterial qualities required for connection and resulting establishment; in any case, other abiotic and biotic components take a critical part in colonization. Whenever a living organism colonizes a root, the process must be affirmed with a variety of outer parameters including temperature, pH, water content, soil property (structure of soil, texture, organic matter, availability of nutrients like N, P, K, and Fe), content of root exudates, and existence of other living organisms. Another major determinant of general microbial diversity is a plant type [16-17]. Bacterial root colonization is negatively influenced by predation (protozoa) and parasitism (bacteriophages). Inoculated bacteria must compete with common residents of the soil for nutrients.The biosynthesis of antagonistic materials by rhizobacteria could be influenced by increasing the competence. Antimicrobial secretion additionally takes a critical part in the establishment of bacteria in the rhizosphere [18-19-20]. The process of root colonization is difficult; a few features related with tolerance, competence with native rhizospheric organisms, and term of root colonizing traits are significant. A good understanding of the colonization steps is demanded the improvement of strains possessing great growth enhancement and biocontrol activity in field.

1.4. The Root Colonization Ability of PGPR is in influenced by Flagella and Pili

The exudates of root and soil supplementation determine the destiny of establishment of microorganisms in the rhizosphere. Characteristics of the bacterial partner, containing the biochemical and morphological properties, are similarly vital in deciding their survival on roots. Bacteria are probably going to find roots through signals oozed from the root, for example, sugars and amino acids activating chemotaxis on the surfaces of the root [21]. Motility of bacteria is recognized as a crucial part in root colonization. Bacterial motility can possibly improve rhizoplane efficiency of competence for both movement towards the roots and along the roots [22]. It was established that motility is very important for beneficial and pathogenic micoroorganisms in terms of competence in rhizosphere [15-23]. Participation of motility in damage of a few plant pathogenic microbes, as *Ralstonia solanacearum*, *Agrobacterium tumefaciens*, *Pseudomonas syringae pv*. *tabaci*, and *Erwinia carotovora subsp*. *atroseptica* was reported [24-25].

Likewise dynamic bacterial motility towards the root hair zone is vital to the start of root colonization by *Azospirillum brasilense* at these locales [26]. Subsequently, effective motility of beneficial and pathogenic microorganisms encourages competition and additionally colonization in rhizosphere/rhizoplane. Motility by flagella has been thought to be profitable to the bacteria to look for good condition or to escape from negative conditions and furthermore for effective rivalry with different living organisms [27]. *Short-term in vitro* studies with *A. brasilense* revealed that there were two steps for attachment mechanism [28]. The first step, called the adsorption step, involves fast and weak binding to the surface of root via the polar flagellum. The second step, named the anchoring step, happens in high ratio C/N containing medium and performed by a bacterial polysaccharide. Polysaccharides are used by bacteria to adhere themselves to the roots to form large clusters on the surface. Polar flagellum of *Azospirillum* and /or vital components situated on the polar flagellum account for root adhesion [29]. Bacteria use flagellins to distinguish the host and non-host plants. Plants have the potential to be sensitive to the presence of a wide range of rhizobacteria using transmembrane receptor-like kinases that reacts with a part of flagellin peptide [30]. Another important determinant Type IV pili plays a vital role in plant colonization process by endophytic bacteria [31-32].

1.5. Rhizoplane Colonization

It was stated that population densities in rhizoplane range from 10^5-10^7 CFU g^{-1} of fresh weight [8]. With the help of microscopic tools it was elucidated that the bacterial cells colonize in rhizospher it has been elucidated that the bacteria initially colonize the rhizospher immediately after soil inoculation [32]. Subsequently, entire root surface is colonized and microcolonies or biofilms are formed [8]. Rhizoplane colonization was characterized not only by *in vitro* plant growth but also with plants grown in natural soil with sufficient microbial diversity.

1.6. Endophyte Behavior and Its Genes

In light of genomic investigation, a few scientists have attempted to answer the question: Why bacterium becomes an endophyte? Right now, the mechanisms are not completely comprehended, due to fact that the ability to enter and survive inside plant

tissues is multifactorial. Furthermore, rhizospheric bacteria able to colonize internal plant tissues, so that both forms of life share an assortment of mechanisms.

Notwithstanding screening individual biochemical/hereditary mechanisms that may be included in the communication of a bacterial endophyte with a plant, it is conceivable to utilize a bioinformatics to deal with a portion of the key traits that differentiate endophytic from rhizospheric PGPB [33]. Genes encoding for proteins as transporters, plant polymer degradation or modification, transcriptional regulation, redox potential maintenance, detoxification, unknown functions, secretion and delivery systems, and functions like 2-isopropylmalatesynthase and diaminopimelate decarboxylase are required in endophytes. In all analyzed genomes of endophytes the resistance nodulation cell division (RND) family efflux transporter Membrane Fusion Protein (MFP) subunits, ABC transporter proteins of internal membrane, and branched-chain amino acid ABC transporter ATPase were observed. The vast majority of the genes distinguished by this method encode functions already proposed by separate biochemical/hereditary reviews to be required in endophytic behaviour. Plant and endophytic colonization are complicated procedure that requires microscopic organisms to contend in the rhizosphere soil to discover a place to contact and interface with the plant roots.

In order to confirm their role in endophytic colonization, several genes were modified, including the genes encoding a minor pilinPilX, a serine–threonine kinase as a putative component of the type IV secretion system (T6SS) and signal transduction proteins. Considering all the above, bacteria can colonize the roots in several ways (rhizospher, planespher, and endophytic colonization) depending on the factors surrounding them (soil factors), the nature of competing species of microorganisms, and the species of plant host.

1.7. PGBR Mechanisms

The mechanisms utilized by PGPR to promote plant development are sensibly understood and well known [34-35]. PGPB may influence plant development either directly or indirectly. Direct promoting of PGPR can be accomplished in two ways; (i) they facilitate the uptake of nutrients as nitrogen, phosphorous and iron from the surroundings medium; or (ii) promotes the growth by supplying different plant

hormones including ethylene, auxin, and cytokinin. Contrarily, indirect enhancement of plant growth by PGPB takes place when a bacterium reduces or inhibits the harm on plants that may caused by pathogenic organisms as bacteria, fungi, and nematodes. There are many indirect ways for PGPB to promote development, including synthesis of cell wall-degrading enzymes, pathogen-inhibiting unstable components, antimicrobials, and also induced systemic resistance, reducing ethylene levels, and diminishing the amount of iron accessible to pathogens [36]. There are two general types of soil microorganisms acting as PGPB; rhizospheric bacteria which are normally found around plant roots; and endophytic bacteria [37]. Which are found inside the tissues of the plant itself (despite the fact that endophytic microbes may likewise be discovered free-living in the soil). In general, rhizospheric and endophytic PGPB use comparative, if not indistinguishable, mechanisms to promote plant development. The major variation being that endophytic PGPB, once established inside the host tissues, are no longer vulnerable to the changing status of soil. The conditions may hinder multiplication of rhizospheric PGPB, change soil pH and water content, and incorporate varieties in temperature, and microbes may compete for adhesion sites on the surface of host plant root [36]. Any logical reason doesn't appear for differentiating the utilization ofrhizospheric PGPB from endophytic PGPB given that it is obvious for these organisms to use basically similar mechanisms to promote plant growth, it would be beneficial to use intentionally the endophytic PGPB to promote growth in horticulture, agriculture, and silviculture.

1.7.1. Direct Mechanism

1.7.1.1. Nitrogen Fixation

In addition to *Rhizobia spp*, there are many numbers of free- living bacteria like *Azospirillum spp*. Also can fix nitrogen and provide to plant [38]. However, it is generally believed that free-living bacteria provide only a small amount of fixed nitrogen that the bacteria-associated host plant requires [39]. Nitrogenase (*nif*) genes involve structural genes that code for activation of the F protein, iron molybdenum cofactor bio formation, electron donation, and regulatory genes wanted for formation and function of the enzymes. In di-azotrophic bacteria, *nif* genes are normally existed in a cluster around 20-40 kb and seven operons encoding 20 different proteins. The

complexity this system leads genetic strategies to increase nitrogen fixation have been elusive. At the same time, some researchers supposed that once the *nif* gene is characterised, it would be tolerable to genetically engineer perfection in nitrogen fixation, and, also it may be conceivable to hereditarily engineer plants to fix their own particular nitrogen. Since the procedure of nitrogen fixation requires high energy use, it would be profitable if bacterial carbon resources were coordinated toward oxidative phosphorylation, which brings about the synthesis of ATP, instead of glycogen synthesis, which brings about the capacity of vitality as glycogen.

In a study, a strain of *Rhizobium tropici* was developed with an erasure in the quality for glycogen synthase. Treatment of bean plants with this bacterium acquired about a significant increase in both the quantity of nodules and dry weight when compared with that of wild-type strain. This is one of the rare cases that researchers hereditarily adjusted the nitrogen fixation process of a bacterium and obtained high levels of fixed nitrogen. Regrettably, when this mutant organism increased nodule amount and plant biomass in the field, it does not survive well in the soil environment. Oxygen is both inhibitory to the enzyme nitrogenase and is additionally a negative regulator of *nif* gene expression.

1.7.1.2. Siderophores

Regardless of the way that iron is the fourth most plentiful component on earth, iron is not promptly acclimatized by either bacteria or plants because ferric ion or Fe^{+3} is just sparingly dissolvable so that the measure of iron accessible for digestion by living organisms is extremely low [40]. Bacteria and plants need a high of level of iron, and acquiring adequate iron is considerably more problematic in the rhizosphere where plant, bacteria and fungi compete for iron [41, 42]. To survive with such a constrained supply of iron, bacteria synthesize low-molecular mass siderophores (~400–1500 Da), molecules with an incredibly high affinity with Fe^{+3} ($KKaa$ ranging from 1023 to 1052). Membrane receptors can tie the Fe-siderophore complex to facilitate iron uptake by bacteria [43, 45]. Right now, there are more than 500 known siderophores; the chemical structures of 270 of these mixes have been resolved [43]. The immediate advantages of bacterial siderophores on the development of plants have been shown in several different sorts of examinations. For instance, (i) a few reviews utilizing

radiolabeled ferric-siderophores as a unique source of iron demonstrated that plants can take up the labelled iron [46,47]. Mung bean plants, inoculated with the siderophore delivering *Pseudomonas* strain GRP3 and developed under iron constraining conditions, demonstrated decreasing chlorotic side effects and an upgraded chlorophyll level compared to uninoculated plants [48]; (iii) the Fe-pyoverdine complex formed by *Pseudomonas fluorescens C7* was absorbed by *Arabidopsis thaliana*, prompting to an increase of iron in tissues and enhancing plant development [49]. The conservation of iron for plants by soil microscopic organisms is more obligatory when the plants are subjected to a natural stress like overwhelming metal contamination. In such a situation, siderophores ease to alleviate the stress imposed on plants by high soil levels of overwhelming metals [50–51]. Plant iron nutrition can influence the structure of bacterial groups in the rhizosphere. For instance, transgenic tobacco that over expresses ferritin and collects more iron than nontransformed tobacco has less bioavailable iron in the rhizosphere [52].

1.7.1.3. Production of Plant Growth Regulators by PGPR

One of the immediate components by which PGPR promote plant development is the production of growth regulators or phytohormones [53]. Plant hormones are organic compounds that are produced naturally within the plant and act as chemical messengers. They are synthesized in one part of the plant and transferred to another, where they stimulate physiological responses as plant growth. Plant hormones are effective at low concentrations [54]. Plant hormones are vital for many processes. Strikingly certain microorganisms can biologically manufacture compounds that are comparable or indistinguishable from the hormones synthesized by plant cells [55]. Plant growth-promoting rhizobacteria can synthesize plant hormones like auxin, cytokinin, gibberillin and decrease ethylene levels by 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase [56]; [57] [58] [59]. Plant hormones, particularly auxins and cytokinins regulate many phases of plant development and improvement, including cell division, tissue differentiation, specialization, and cell elongation [60]. Auxins which are derived from tryptophan are delivered essentially in meristems and plays an essential role in many developmental processes. Few microorganisms synthesize comparable or indistinguishable compounds similar to these. In the mid-1930s, indole-3-acetic acid (IAA) was identified as the most abundant and most physiologically applicable auxin in

higher plants [61]. Around 74-80% of the bacteria isolated from plant rhizosphere produce IAA [62]. Tryptophan is the essential precursor for IAA biosynthesis. Bacteria synthesize IAA in five pathways by utilizing the tryptophan[63]. Indole- 3-acetamide (IAM) pathway is the best-described pathway in bacteria. Initially tryptophan is converted into IAM by enzyme tryptophan-2-monooxygenase (IaaM) and then the IAM is converted into IAA by IAM hydrolase (IaaH) [62]. The most familiar tryptophan-dependent pathway for IAA biosynthesis in plants is the idol-3-pyruvate (IPyA) pathway. The pathway includes the change of tryptophan into IPyA by aminotransferase, followed by convertion into indole-3-acetaldehyde (IAAld) and then indole-3-acetaldehyde is oxidized to IAA by a dehydrogenase [60]. Formation of indole-3-acidic acid via the IPyA pathway has been observed in microscopic organisms, for example, *Azospirillum*, *Cyanobacteria*, *Rhizobium*, and *Bradyrhizobium* [62].The tryptamine (TAM) pathway is like the IPyA pathway in plants, the distinction is that the reaction of the deamination and the decarboxylation is carried out through various proteins [60]. As opposed to the bacterial pathway, the last step of this pathway includes an amine oxidase that modify TAM specifically into IAAld. The TAM pathway has been recognized in *Bacillus cereus* and *Azospirillum brasilense* [64]. One particular tryptophan-subordinate IAA pathway is the tryptophan side-chain oxidase (TSO) which has just been shown in *Pseudomonas fluorescens* CHA0. In this pathway, tryptophan is simply converted to IAAld [65]. There is no evidence of such a pathway in plants [62]. In indole-3-acetonitrile (IAN) pathway tryptophan is converted to indole-3-acetaldoxime and then to indole-3-acetonitrile [60]. However, two diverse pathways are suggested for main tools required in the synthesis of IAN from tryptophan. In one of the pathways indolicglucosinolates (glucobrassicin) is used, while in other indole-3acetaldoxime is utilized [64-65]. Transformation of IAN to IAA has been found in *Bacillus amyloliquefaciens* FZB42 and Azospirillum brasilense [66]. Numerous PGPR deliver the auxin, indole-3-acidic acid (IAA), and inoculation with auxin-creating rhizobacteria was appeared to expand plant development [67]. It was revealed that certain PGPR as Pseudomonas fluorescens deliver cytokinins. However, more research is required for precisely elucidating the part that cytokinins delivered by bacteria promotes plant growth [59].

1.7.1.4. Phosphate Solubilizing Bacteria

Phosphorus is one of the fundamental mineral supplements that regularly limits crop growth. It is fundamental for plant development and improvement, constituting approximately 0.2 % of the plant's dry weight.

Plants get phosphorus from soil as phosphate anions; however phosphorus is normally not accessible to plants as it might be insoluble through precipitation with ions, for example, Ca^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+} [53]. Rodriguez and Fraga (1999) stated that under reasonable conditions the insoluble compounds can be solubilized so that phosphorus become accessible for both microorganisms and plants [68]. Some PGPR provide a system that can solubilize the inorganic phosphorus of soil and produce accessible phosphorus to the plants [69]. Phosphate solubilizing microorganisms (PSB) create phosphatases and natural acids that solubilize inorganic phosphate and converts the insoluble phosphates into solvent phosphate ions, hence making soil phosphorus accessible to plants. Microorganisms belonging to *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aereobacter*, *Flavobacterium* and *Erwinia* have the capacity to solubilize phosphate [70]. Katznelson, Peterson, and Rouatt (1962), and Raghu and MacRae (1966) noticed that more prominent groupings of phosphate solubilizing microscopic organisms are available in rhizosphere when compared with non-rhizosphere soil [71-64]. In any case, quantities of phosphate solubilizing microorganisms in soil are low due to competition. Thus, the amount of phosphate freed by them is too low for impressive plant development, hence for increasing soluble phosphate available to plants PSB inoculation is required [68]. As indicated by Elkoca, Kantar and Sahin (2008) simultaneous inoculations of *B. subtilis* (OSU-142) and P-solubilizing *B. megaterium* (M-3) expanded plant height, shoot, nitrogen content, chlorophyll content, root and nodule dry weight, total biomass yield, pod number, seed yield, and seed protein content in chickpea compared with the control treatments[72]. Peix et al. (2001) demonstrated that phosphorous was activated by *Mesorhizobium mediterraneum* strain PECA21in grain and chickpea when tricalcium phosphate was added to the soil medium [73]. Besides, the phosphorous substance, calcium, dry matter, nitrogen, magnesium, and potassium content in both plants were extensively expanded upon inoculation with strain PECA21 and expansion of insoluble phosphates.

1.7.1.5. ACC Deaminase Activity

Ethylene is an endogenously created gaseous phytohormone that works at low concentrations, taking an interest in the control of all processes of plant development, improvement and senescence [68-72]. Ethylene has likewise been recognized as a stress phytohormone. Under abiotic and biotic stress conditions (e.g. Infection, salt, flooding, drought, and organic and inorganic contaminants), endogenous ethylene synthesis is considerably eased and have a negative effect root development and hence on plant development as a whole. Various mechanisms have been explored for decreasing the levels of ethylene in plants. Bacterial enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase is one of them [74-75-76]. It controls the formation of plant ethylene by metabolizing ACC (the quick precursor of ethylene biosynthesis in higher plants) into α-ketobutyric acid and ammonia. An important amount of plant ACC may be discharged from roots and consequently taken up by soil-living organisms and hydrolyzed by the enzyme ACC deaminase, therefore diminishing the amount of ACC in the environment. At the point related with plant roots, soil microorganisms with ACC deaminase can utilize ACC as nitrogen source and may have a superior developmental effect compared to other free microorganisms [74]. Bacterial ACC deaminase activity can conceptually be classified into two groups depending on low or high enzymatic action. High ACC deaminase-expressing bacteria nonspecifically attach to plant surfaces, and included in rhizosphere and phyllosphere bacteria and endophytes. Nonetheless, low ACC deaminase-expressing bacteria just attach to particular plants or are just present in specific tissues, and because these organisms couldn’t bring down the general level of ethylene delivered by the plant, they may keep a limited increase in ethylene levels. Plant development and efficiency are negatively influenced by abiotic stresses. Bal et al. (2013) showed the viability of microscopic organisms displaying ACC deaminase action, for example, *Alcaligenes sp*., *Bacillus sp*., and *Ochrobactrum sp*., in prompting salt resistance and thus enhancing the development of rice under salt stress [77]. In addition, the choice of endophytes with ACC deaminase activity could likewise be a helpful approach for promoting an effective phytoremediation technique, given the capability of these microorganisms to decrease plant stress [78].

1.7.2. Indirect Mechanisms

1.7.2.1. PGPR as Biocontrol Agents

PGPR are natural to the plant rhizosphere and soil and exhibit an important role in bio-control of plant pathogens. They can suppress a broad range of bacterial, nematode and fungal diseases. PGPR can likewise provide protection against viral diseases. The utilization of PGPR has turned into a typical practice in the world. Although significant biocontrol of plant pathogens via PGPR has experienced in laboratory and greenhouse conditions, the results were inconsistent in the field. Recent advances in our understanding of their assorted qualities, mechanism of action, and colonizing capacity, formulation and application ought to encourage their improvement as dependable biocontrol agents against plant pathogens. Some of these rhizobacteria may likewise be utilized as part of integrated pest management programs. Further utilization of PGPR is conceivable in agriculture for biocontrol of plant pathogens and biofertilization [79]. The bacterial strains isolated from Lolium perene rhizosphere are able to act as plant growth promoting microorganisms and as biocontrol agents as they exhibit different plant promoting activities. A noteworthy group of rhizobacteria with biocontrol potential is the *Pseudomonades*. *Pseudomonas* has numerous characteristics that make them appropriate for biocontrol and growth promoting agents [80]. These include the capacity to (i) develop quickly *in vitro* and to be mass delivered; (ii) quickly use seed and root exudates; (iii) colonize and duplicate in the rhizosphere and spermosphere conditions and inside the plant; (iv) create a wide range of bioactive metabolites i.e., anti-toxins, siderophores, volatiles, and growth promoting materials; (v) compete forcefully with different microorganisms; and (vi) adapt to stress conditions. Cyanide generation is one of the ways by which rhizobacteria may supress plant development in soil. Rudrappa et al., (2008) explained the role of cyanide production in *pseudomonas* virulence influencing plant root development and other rhizospheric processes [81].

1.7.2.2. Antifungal Activity

PGPR enhance plant development by supressing the multiplication of phytopathogens and thereby promote plant growth. Some PGPR produce antifungal, and anti-microbials,

e.g. *Pseudomonas fluorescens* provides 2,4-diacetylphloroglucinol which hinders the development of phytopathogens [82].

A concern has appeared on the utilization of FLPs in crop plants as the antifungal agents released by the bacterium, especially 2,4-diaacetylphloroglucinol (DAPG) could influence the arbuscular mycoorhizal fungi.

1.7.2.3. Induced Systemic Resistance

Plant growth advancing bacteria can trigger a phenomenon in plants identified as induced systemic resistance (ISR) that is phenotypically considered as the systemic acquired resistance (SAR) that happens when plants initiate their protection mechanisms in response to the disease by pathogens [83]. ISR-positive plants are said to be "primed" so they respond quicker and more firmly to pathogenic invasion through inducing protection mechanisms. ISR does not target particular pathogens or it might be effective at controlling infections caused by various pathogens. ISR includes jasmonate and ethylene signalling inside the plant and these hormones stimulate the host plant's resistance responses to a scope of pathogens. Other than ethylene and jasmonate, O-antigenic side chain of the bacterial external membrane protein-lipopolysaccharide, pyoverdine, flagellar proteins, chitin, and salicyclic acid was reported to act as signals for the stimulation of systemic resistance .

1.8. *Bacillus* as PGPR in Crop Ecosystem

Most types of *Bacillus* and *Paenibacillus* are disseminated globally and the widespread existence of subspecies of *B. cereus* and *B. subtilis* with their capacity to suppress the plant pathogens has been broadly perceived.

1.8.1. Phtyostimulation and Biofertilization Effects

Improvement of plant growth by root colonizing types of *Bacillus* and *Paenibacillus* is famous. It is additionally likely that growth promoting impacts of different PGPRs are due to stimulatory effect of plants on bacterial proliferation with the effect of indole-3-acidic acid (IAA), gibberellins, and cytokinins. A vast majority (80%) of microscopic organisms colonizing the rhizosphere have been considered as positive for IAA

synthesis, but reports portraying IAA production by Gram-positive soil microorganisms are very rare [84]. Nonetheless, Idris et al. (2004) indicated the production of substances with auxin (IAA) such as bioactivity from strains of *B. subtilis* and *B. amyloliquefaciens* including strain FZB42 [85]. Also, gibberellin production was confirmed in *B. pumilus* and *B. licheniformis* [86].

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Locations of soil samples from Central, North and South regions of Iraq were given in Table 2.1.

Table 2.1. Location and salinity of soil samples

Number	Soil sample	EC (/ms/cm)	GPS location
1	Hilla-Iraq	11,74	454822N,3595863E
2	Hilla-Iraq	23,40	454813N,3595857E
3	Hilla-Iraq	10,84	454840N,3595871E
4	Hilla-Iraq	13,53	454786N,3595924E
5	Hilla-Iraq	12,91	454816N,3595939E
6	Najef-Iraq	3,26	46207N,28011E
7	Najef-Iraq	14,70	33634N,71852E
8	Najef-Iraq	11,80	31227N,59287E
9	Najef-Iraq	95,50	40725N,53348E
10	Duhook-Iraq	20,30	392771N, 61652E
11	Duhook-Iraq	43,70	364751N,55809E
12	Duhook-Iraq	16,58	862561N, 67157E
13	Duhook-Iraq	3,39	382275N,54355E
14	Diala-Iraq	28,80	334058N,444618E
15	Diala-Iraq	22,10	334057N,444616E
16	Diala-Iraq	33.00	33410N,444618E
17	Diala-Iraq	24,90	33410N,444621E
18	Diala-Iraq	22.00	334058N,444620E
19	Diala-Iraq	19,74	334819N,444222E
20	Diala-Iraq	26,90	334818N,444222E
21	Diala-Iraq	11,45	334818N,444219E
22	Diala-Iraq	16,67	334819N,444218E
23	Diala-Iraq	25,80	334819N,444220E
24	Diala-Iraq	32,30	334911N,443112E
25	Diala-Iraq	32,40	33498N,443112E
26	Diala-Iraq	36,20	33495N,443111E
27	Diala-Iraq	22,70	33495N,443114E
28	Diala-Iraq	5,76	334911N,443116E

2.1.1. Materials used in bacterial growth

The following media were used for the activation and cultivation of bacteria:

• Nutrient Broth (NB) liquid medium (pH 6.8±2): Prepared by solubilizing 8 g of solid media in 1L distilled water and sterilizing at 121 C° for 15 minutes in autoclave.

- Nutrient Agar (NA) medium (pH 6.8±0,2): Prepared by suspending 20 g of solid media in 1 liter distilled water. The medium was sterilized by autoclaving at 121 C° for 15 minutes.

• Tryptic soy agar (pH 7,0±0,2): Prepared by dissolving 40 g of solid media in 1 liter of distilled water. The medium was sterilized by autoclaving at 121 C° for 15 minutes.

2.1.2. Oligonucleotide primers used in PCR reactions

Properties of primer pairs are given in Table 2.2

Table 2.2. Properties of primer pairs

Primer pairs		Sequences	Product size (bp)	Tm (°C)	Reference
ACC Deaminase	F	5'-GTGAACCACCTGAATGTA-3'	750	53.9	[87]
	R	5'-AAACGAGATGATTTACTTGG-3'		58.6	
AC Phosphatase	F	5'-AAGAGGGGCATTACCACTTTATTA-3'	734	53.9	[87]
	R	5'-CGCCTTCCCAATCRCCATACAT-3'		58.7	
Siderofore	F	5'-GAGAATGGATTACAGAGGAT-3'	750	48.6	[87]
	R	5'-TTATGAACGAACAGCCACTT-3'		52.0	

2.1.3. Materials used in plant-bacterial inoculation experiment

- Soil and organic compost (Suli Flor, SF1)
- Corn seed (Kindly supplied by Prof. Dr. Mehmet ARSLAN)
- Pots (2kg)
- Device (Win Rhizo)

2.2. Methods

2.2.1. Collection of Soil Samples

Soil samples (28) with various salinity were collected from root regions of plants growing in Central, South and North regions of Iraq. The surface of the soil was scraped with a spatula and samples (~15g) were taken into sterile falcon tubes from locations up to 10 cm deep and stored at +4°C until use. Salinity rates of all soil samples were analyzed in laboratory of Soil Science at Seyrani Faculty of Agriculture.

2.2.2. Bacterial Isolation

Bacterial species were isolated from soil samples by using the method of Travers et al [88]. After preparation of liquid broth medium (Nutrient broth and Luria Bertani broth) 1 g of soil samples was added into 20 ml medium in 150 ml Erlenmeyer flasks and incubated at 200 rpm for 4-5 h at 30°C in rotary shaker. A small amount of medium was spread on to solid broth medium in petri plates and incubated at 30°C for overnight.

2.2.3. Obtaining pure colonies

Morhologically, different colonies in every petri plate were monitored and transferred by streak plate technique to a new solid medium and incubated at 30°C for overnight to obtain pure cultures. A loopfull of pure colonies was transferred to labeled sterile microfuge tubes containing 800µl of LB broth and 200µl of 50% glyserin for preserving them in stock cultures at -80°C

2.2.4. Activation of bacterial species from stock culture

Bacterial species from the stock culture of microbial biotechnology laboratory from the Department of Agricultural Biotechnology were activated by inoculating the cultures in appropriate nutrient medium at 30°C for 24 hour through continuous shaking at 200 rpm and then were incubated in solid medium for overnight to obtain fresh cultures.

2.2.5. DNA isolation

Bacterial species were grown in LB medium for overnight, and then a loop of cells was transferred into a microfuge tube containing 300-400 µl sterile dH_2O. After mixing well, they were kept at -80°C for half an hour and immediately immersed into boiling

water for 10 minutes to fracture cells and release their content into medium. Subsequently the solution was centrifuged at 10.000 xg for 5 minutes and the supernatant was transferred to a new tube. The supernatant was used as DNA templates.

2.2.6. Polymerase chain reaction

PCR analysis is going to be performed using the 16S rDNA, siderofore and ACC deaminase specific primer pairs in ABI veriti device.PCR program was set up as follows: A single denaturation step of 5 min at 95°C, a step cycle program set for 34 cycles with a cycle consisting of denaturation at 95 °C for 1 min, extension time at 72 °C for 1 min, and final extension at 72 °C for 10 min. The annealing temperatures were adjusted separately for every primer pairs. Each reaction contained the reagents at a final concentration as 2.3 mM $MgCl_2$, 1x taq buffer, 0.2 mM dNTPmix, 0.2 µmol primers (each), 0.5 U taq DNA polymerase, and 30-100 ng of template DNA in 25µl of final volume . Following amplification, the PCR products were electrophoresed (at 100 V for 2 h) on a 1x Tris-acetate-EDTA (TAE with ethidium bromide) buffer in 1% agarose gel and the specific PCR products were excised from the gel and purified for sequencing analysis by Easy Pure Quick Gel Extraction Kit (EG101) according to the following protocol;

1. The DNA fragment was excised and weighed.
2. 3 volume of gel solubilization buffer were added to 1 volume of gel. After that, the gel incubated at 55°C for 6-10 minutes. The solution was mixed by vortexing the tube every 2-3 minutes to help dissolve the gel during the incubation.
3. At room temperature, the solution was transferred to spin column and centrifuged at 10000×g for 1 minute. The flow-through was discarded.
4. 650µl of washing buffer were added and centrifuged at 10000 ×g for 1 minute. The flow-through was discarded.
5. The empty column was centrifuged at 10000×g for 1-2 minutes to remove the residual WB.
6. The spin column was placed in a clean microcentrifuge tube and 30-50 µl of elution buffer were added directly to the center of the column matrix. Then, centrifuged at 10000×g for 1 min to elute the DNA.

2.2.7. Gel electrophoresis

The gel was prepared by dissolving and homogenizing 1g of agarose in 100 ml of Tris acetate EDTA (TAE) buffer. 15µl of PCR product w mixed with 3 µl of loading dye and loaded into gel and run at 100V for 2 h. After that the bands are going to be visualized under UV light using BioRad ChemiDoc MP.

2.2.8. Inoculation of seeds with bacterial isolates

Corn seeds were surface sterilized through keeping in 5% sodium hypochlorite for 8 minutes and washing thoroughly with sterile water. After drying the seeds for a while, they were inoculated with bacterial isolates at a concentration of 10^8 cfu. 10 seeds for corn were inoculated with bacterial isolates separately and incubated for 30-50 min for continuous shaking at 180 rpm so that the bacterial cells can be attached to the surface of the seeds.

2.2.9. Preparing the pots for planting

Small pots were immersed into a plastic bag containing 5% hypo chloride for sterilizing them. After that pots were washed properly with water for removing the residual hypochloride and filled with sterile soil and compost (Suli Flor) mixture at a rate of 1/2.

2.2.10. Inoculation of seedlings with bacterial isolates

Approximately 180 seeds of corn were seeded into pots, and after 20 days 6 seedlings of approximately the same size were transferred into 60 pots (2k g) separately. Plants were inoculated with 2 ml of bacterial suspension at the concentration of 10^8 cfu.

Figure 1. Inoculation of seedlings with isolates

2.2.11. Germination test

Seeds of corn were used for testing the effect of isolates on germination tests. Surface sterilization of seeds was carried out by exposing them to 5% hypochlorite (NaOCl). Bacterial inoculums were prepared as 10^8cfu/ml concentration and seeds were inoculated for half an hour. Seeds placed in a humid medium in petri plates at 25°C and number of germinated seeds were recorded daily. Control seeds were immersed in the sterile distilled water for one hour. Trials were carried out in petri plates by wrapping plastic film with three replications and 10 seeds for replication.

Figure 2. Corn seed on Petri dish

2.2.12. Harvest and Measurements

The plants were harvested 20 days after transfer to pots (with 2 kg of growth media) and shoot length, root length, shoot diameter, fresh weight, dry weight and the number of leaves per plant were determined. Shoots and roots dried in an oven at 70°C for 48 h measuring the dry weights. Root volume was determined by immersing into water using a graded cylinder after cleaning from growth mixture.

Root diameter and lengths were estimated with a software using WinRhizo device in Plant Physiology Laboratory of Agricultural Faculty.

Figure 3. Washing of the roots and volume determination

Figure 4. Measurement of stem dry weight of corn

2.2.13. Preparation of bacterial inoculants

The isolates were incubated in liquid nutrient mediums at 30°C and 200 rpm for overnight using a rotary shaker incubator. Then, 20 µl from the culture were diluted in 2 ml of water and optical densities were measured at 600 nm using a spectrophotometer (Shimadzu UV-1800). One OD was regarded as approximately $5x10^8$ cells/ml in liquid medium [89]. The obtained OD_{600} value for each bacterium was estimated by proportionating. The cultures were diluted to obtain 10^8 cfu value used in inoculation experiments.

2.2.14. Statistical analysis

Data were subjected to analysis of variance (ANOVA). Post-hoc analyses were performed with Duncan and Dunnett's T3 tests. Analyses were conducted using SPSS 13.0 [90]. $P<0.05$ was considered as statistically significant

CHAPTER 3

RESULTS AND DISCUSSION

The plant growth promoting effect of PGPR are very complex process and take place in a reciprocal interaction. They are free living microorganisms and can colonise on roots and have an enhanced effect on plant growth through direct (as phosphate solubilisation, hormone production, nitrogen fixation) or indirect (as competence with plant pathogens, increasing mineral uptake) mechanisms. The compounds produced by plants can be used as energy source by rhizobaceria, however from the other side bacteria promotes growth through producing chemicals as ACC deaminase, IAA, acid phosphatase and facilitates the mineral uptake by plants [91- 92]. In the current study the local isolates of *Bacillus* spp. were aimed to isolate, screen in terms of genes encoding the enzymes related with growth promotion, and apply to corn plants for observing their growth promoting effects.

3.1. Screening of Bacterial Isolates

Prior to seed and plant inoculation tests total DNA of isolates were screened for genes that code for ACC deaminase, Acid phosphatase (ACP), and siderofore. According to the results 19 isolates were selected for germination tests and searched on registered corn cultuar (DKC5741, FAO550). The study was carried out as both seedling inoculation and seed inoculation tests. 19 bacterial isolates were positive for ACC deaminase gene (Figure 5). It is known that many species of PGPR have the potential to produce ACC deaminase which has stimulatory effect through hydrolysing ACC and hence preventing the ethylene production in plants [93-94]. It can be released from seeds and roots. Bacteria with deaminase activity can decrease ACC concentration and hence ethylene biosynthesis in roots and promote growth [95].

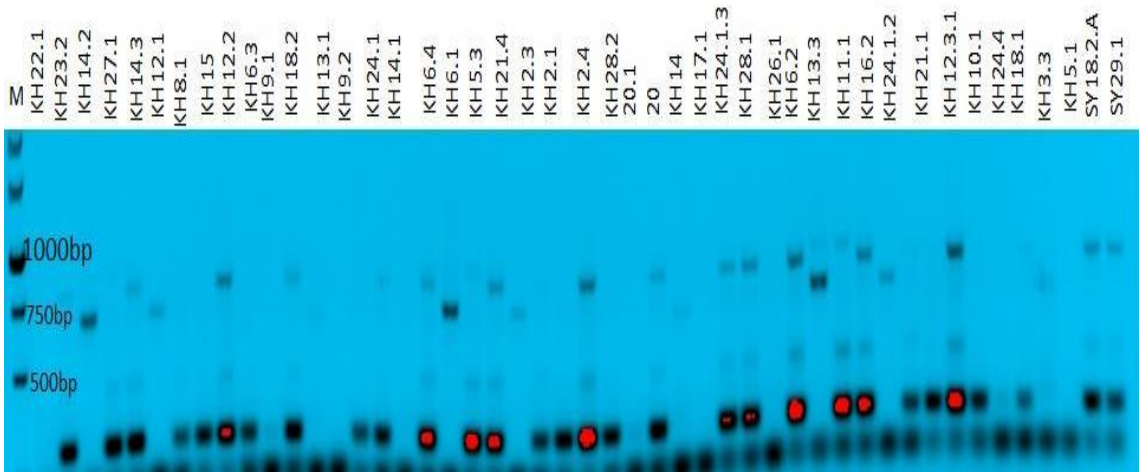


Figure 5. PCR amplification of ACC deaminase gene in isolates

Out of all isolates 17 were yielded expected length of bands with ACP primers (Figure 6). It is clear that P is the major element in nucleic acid biosynthesis and also in energy metabolism, and hence has a critical importance for plant growth [96]. *Bacillus* species are among the microbes expressing significant amount of acid phosphatases/phytases and help in growth and development of plants [96-97].

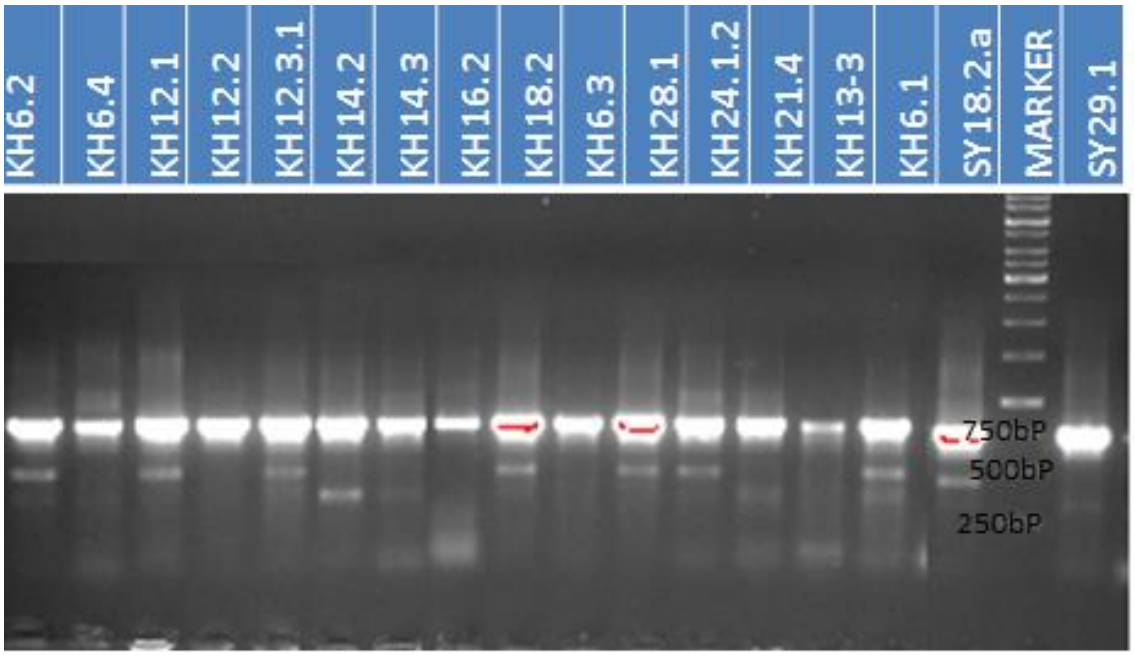


Figure 6. PCR amplification of AcPho genes in bacterial isolates

Out of all isolates screened for siderofore gene, only 5 were positive (Figure7). Siderophore is commonly produced by *B. cereus* group [98]. Bacteria can use siderofore especially for biocontrol of phytopathogenic fungi by competition effects for iron, and also providing the plant with iron [49-99]. The isolates carrying both siderofore and AcPho genes are good candidates for growth promotion and can have the ability to provide plants with iron and phosphate. However, the presence of genes are not enough for considering the bacteria as growth promoting. Also, the bacteria promoting growth under controlled condition remain to be demonstrated in field conditions. Because some strains effective *in vivo* may not retain their capacity under field conditions because of changing physical and chemical factors as temperature, pH, and salinity [100].

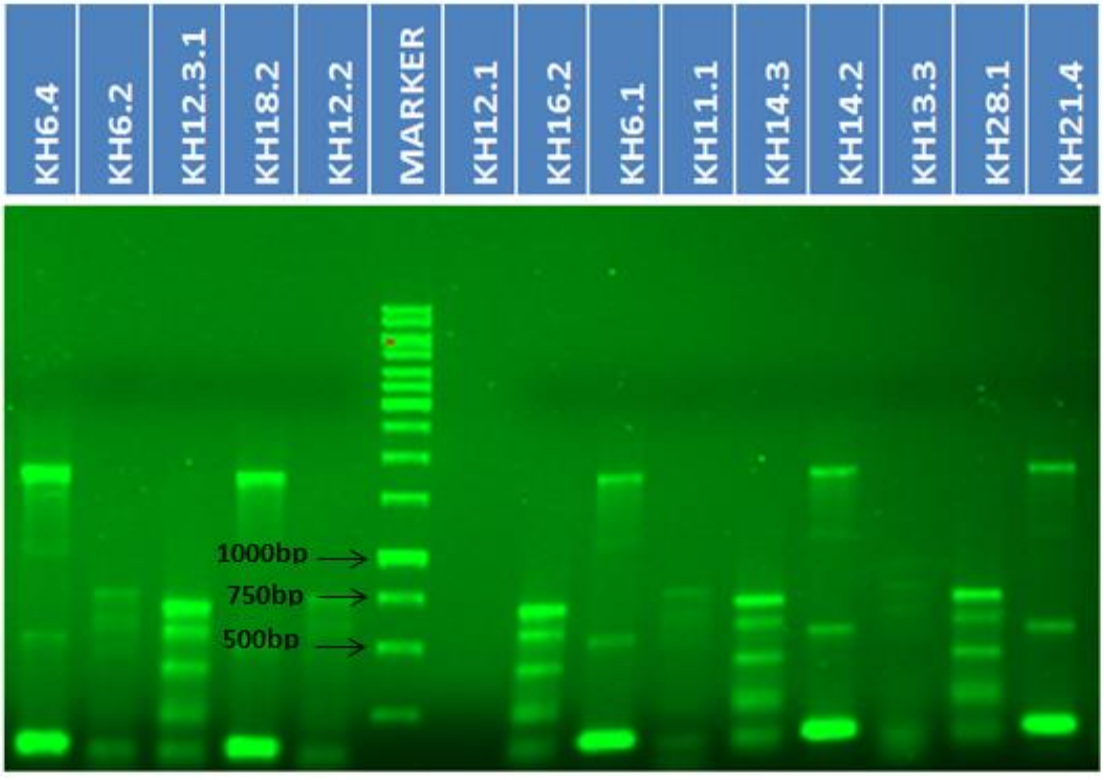


Figure 7. PCR amplification of siderofore gene in isolates

PCR amplification of 16S rDNA region of promising isolates yielded approximately 1500 bp (Figure 8). Partial sequencing analysis of isolates were performed in Genom and Stem Cell Center at Erciyes University. Alignment analysis of the sequences was carried out via NCBI's (National Centre for Biological Information) Basic Local Alignment Search Tool (BLAST) to compare with known sequences of bacteria in

NCBI database. Phylogenetic trees were constructed using the Neighbour Joining (NJ) and Bootstrap Tree methods.

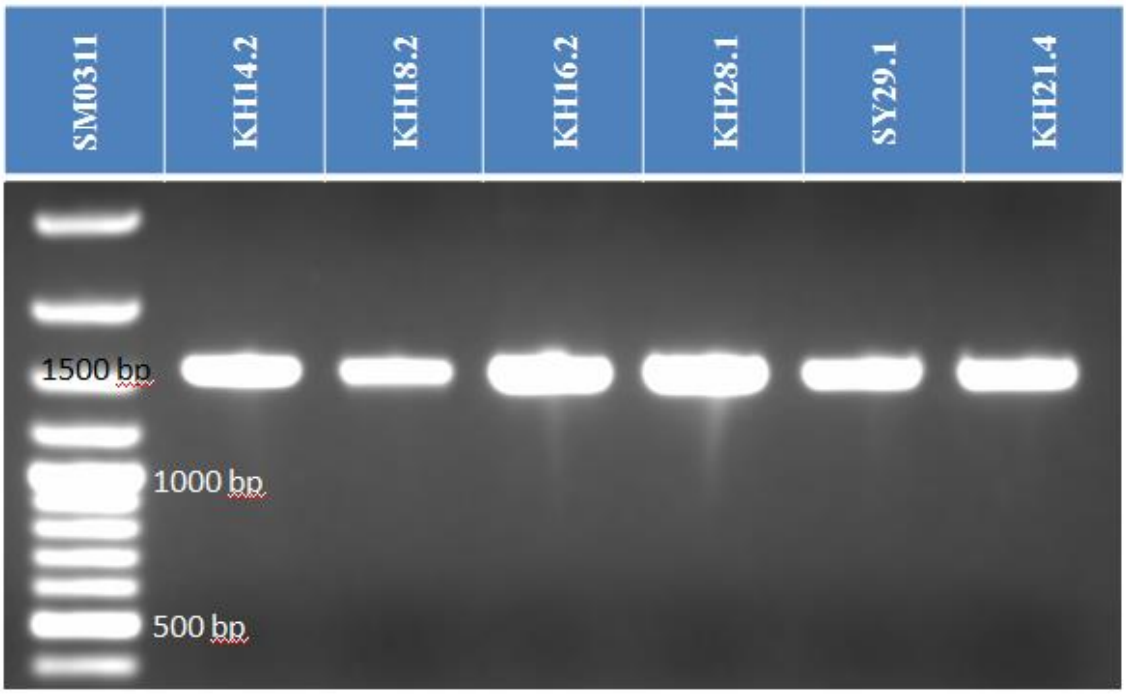


Figure 8. 16S rRNA gene amplification of isolates

Phylogenetic analysis of 16S rRNA regions from 6 isolates KH28.1, KH16.2, KH14.2, KH18.2, KH21.4, SY29.1 were performed by obtaining PCR products of 1160, 1175, 1129, 1143, 1031, and 1139 bp, respectively and sequenced. BLASTN homology search of 16S rDNA of isolates indicated that isolate KH28.1 resembled to *Bacillus subtilis* at a level of 99%, KH16.2 resembled to *Bacillus* sp. strain at a level of 97%, KH14.2 resembled to *Bacillus cereus* at a level of 98%, KH18.2 isolate resembled to *Bacillus subtilis* at a level of 99%, KH21.4 isolate resembled to *Bacillus cereus* at a level of 96%, and SY29.1 isolate resembled to *Bacillus simplex* at a level of 98%. The isolates were clustered with all members of Bacillacea family recorded in NCBI database (Figures 9,10,11,12,13,14,15,16,17,18,19,20)

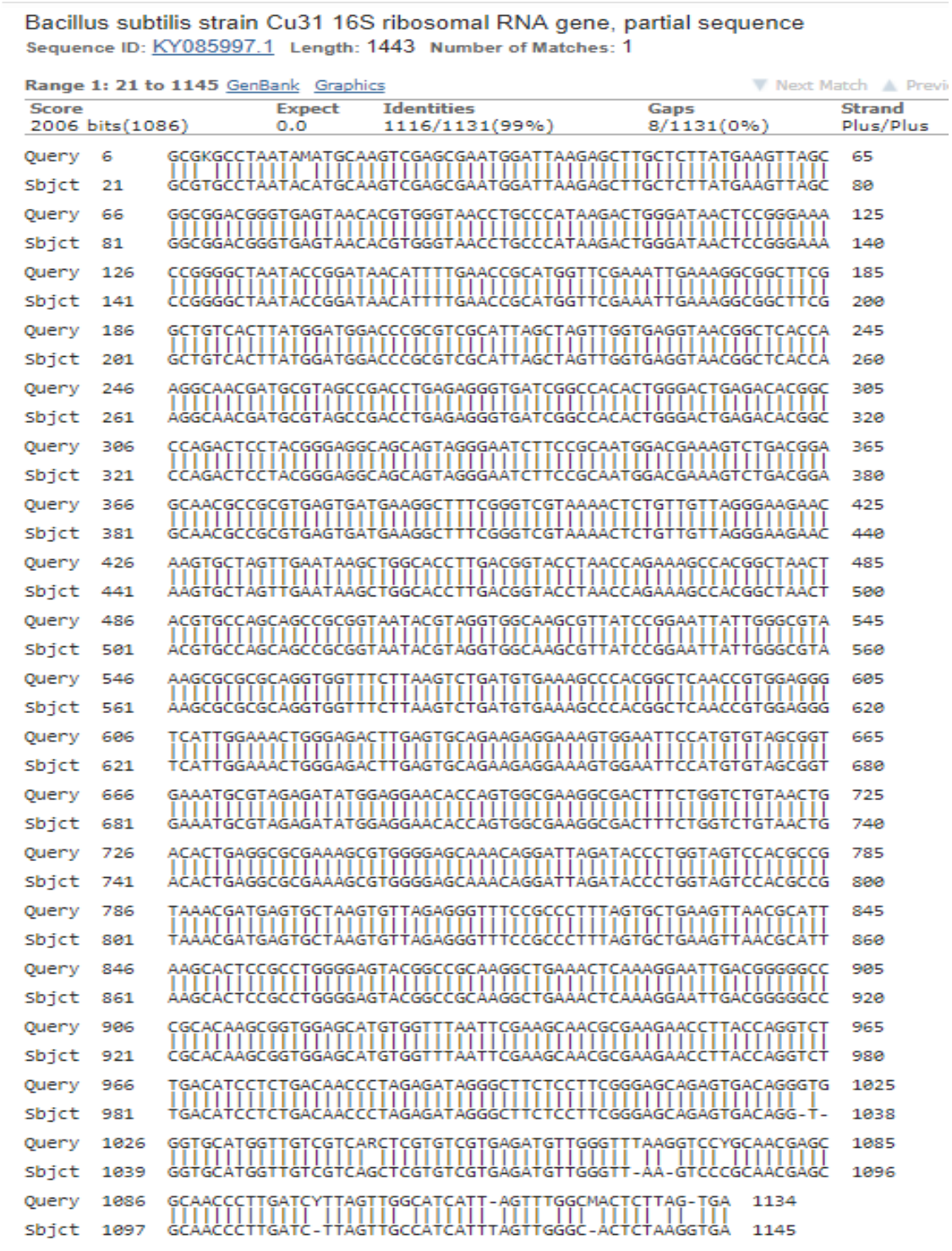


Figure 9. Alignment result of KH28.1 with *Bacillus subtilis* .

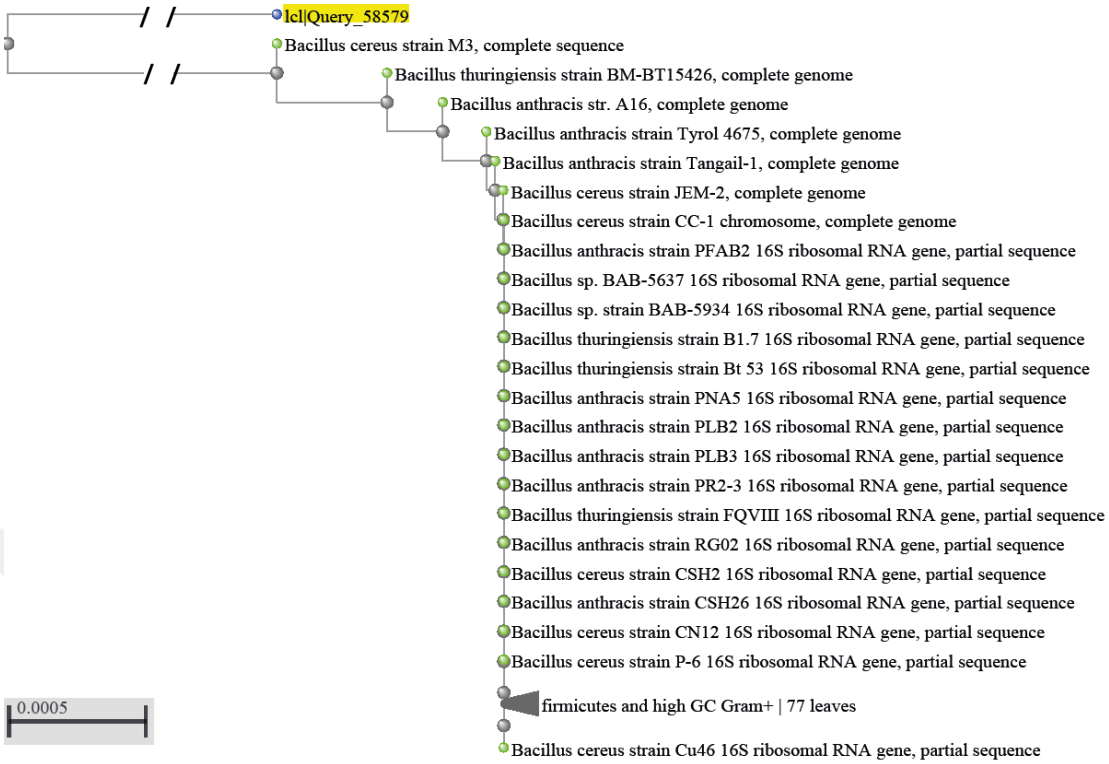


Figure 10. Phylogenetic relationships of KH28.1 obtained from the alignment of the 1160 bp of 16S rDNA region.

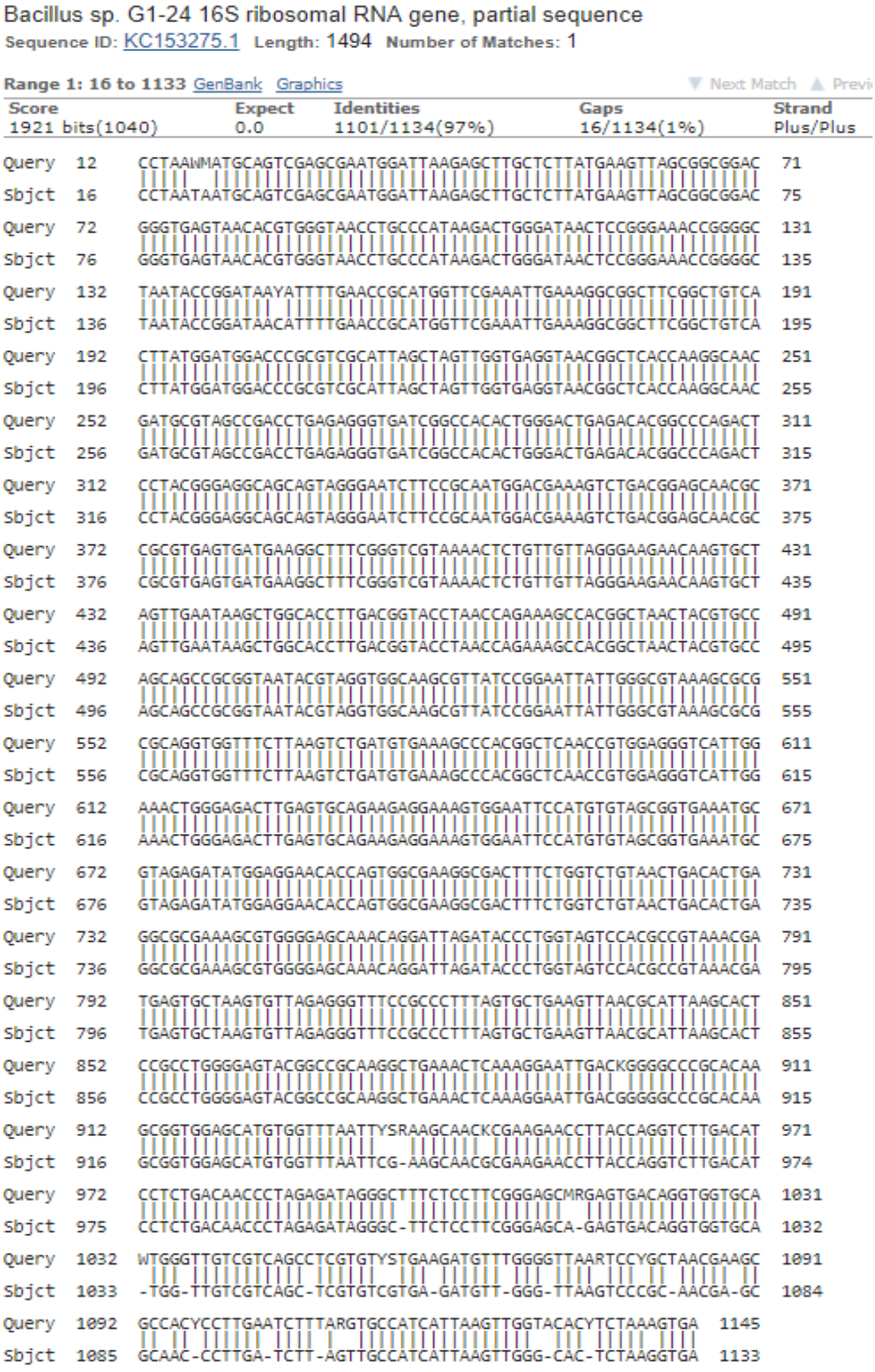


Figure 11. Alignment result of KH16.2 with *Bacillus* sp.

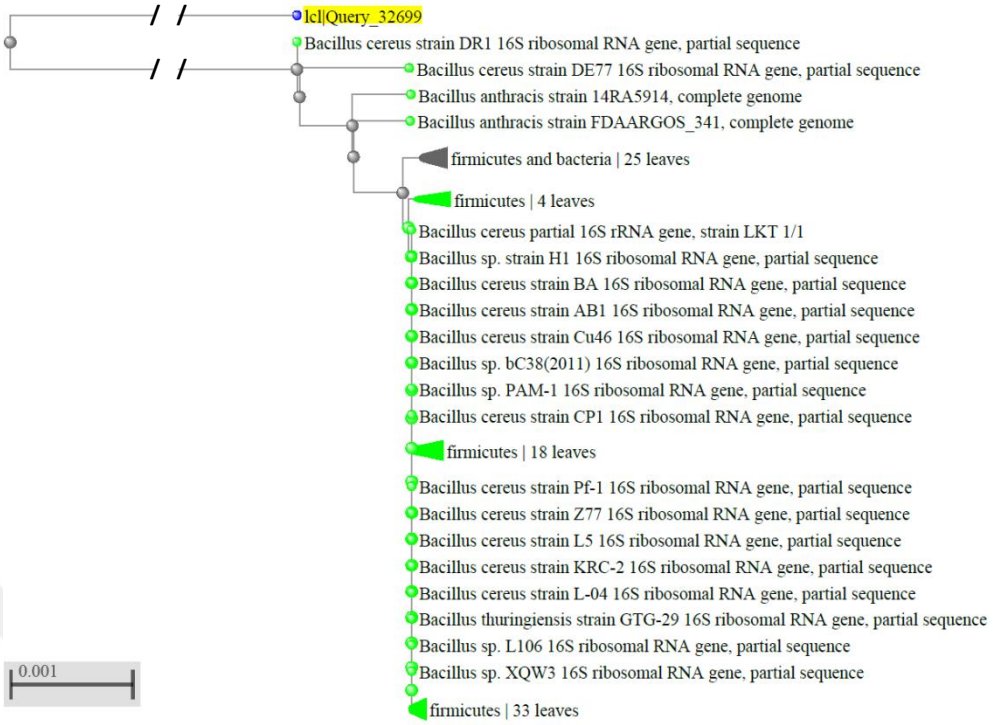


Figure 12. Phylogenetic relationships of KH16.2 obtained from the alignment of the 1175 bp of 16S rDNA region.

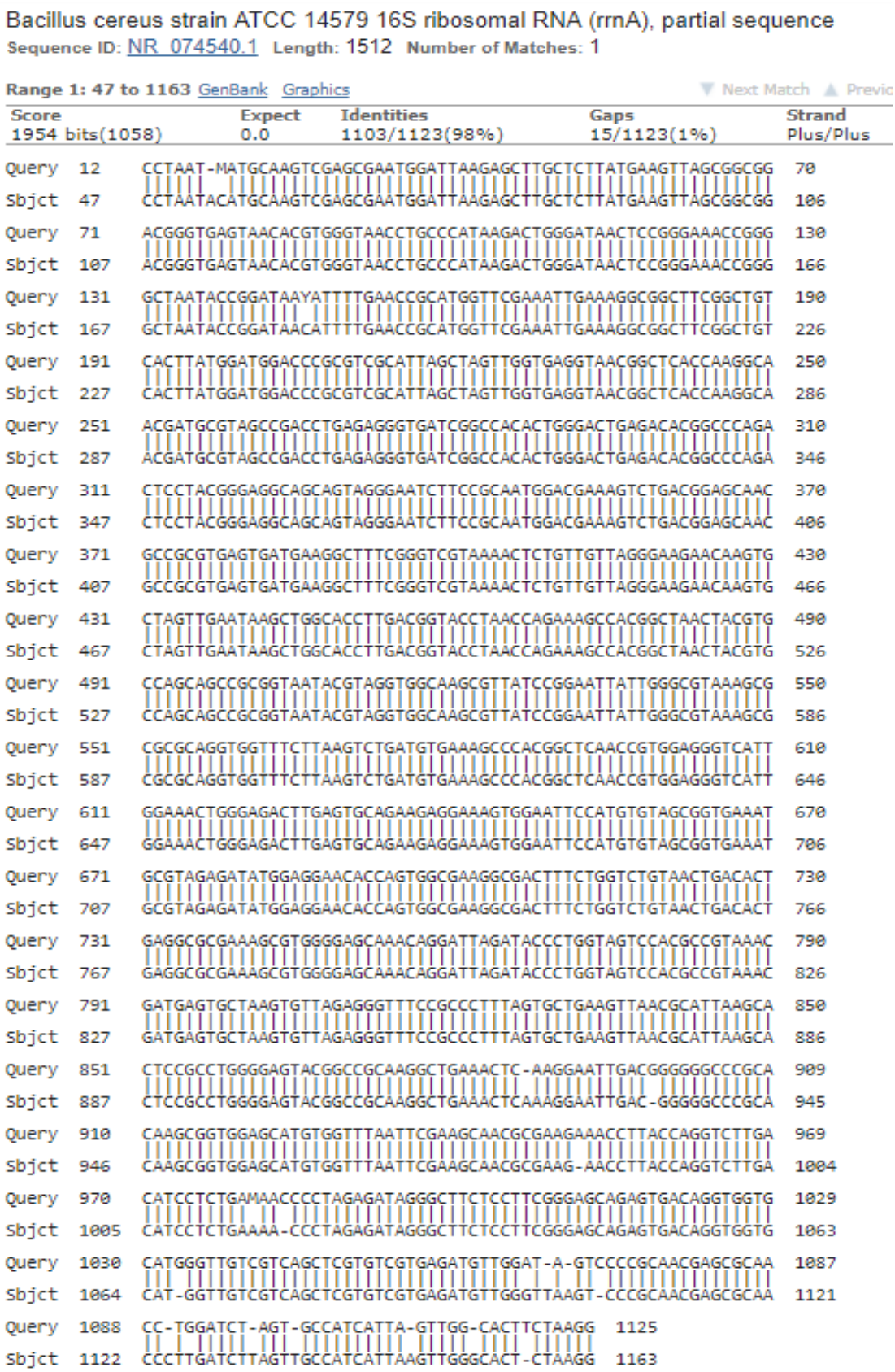


Figure 13. Alignment result of KH14.2 with *Bacillus cereus* strain .

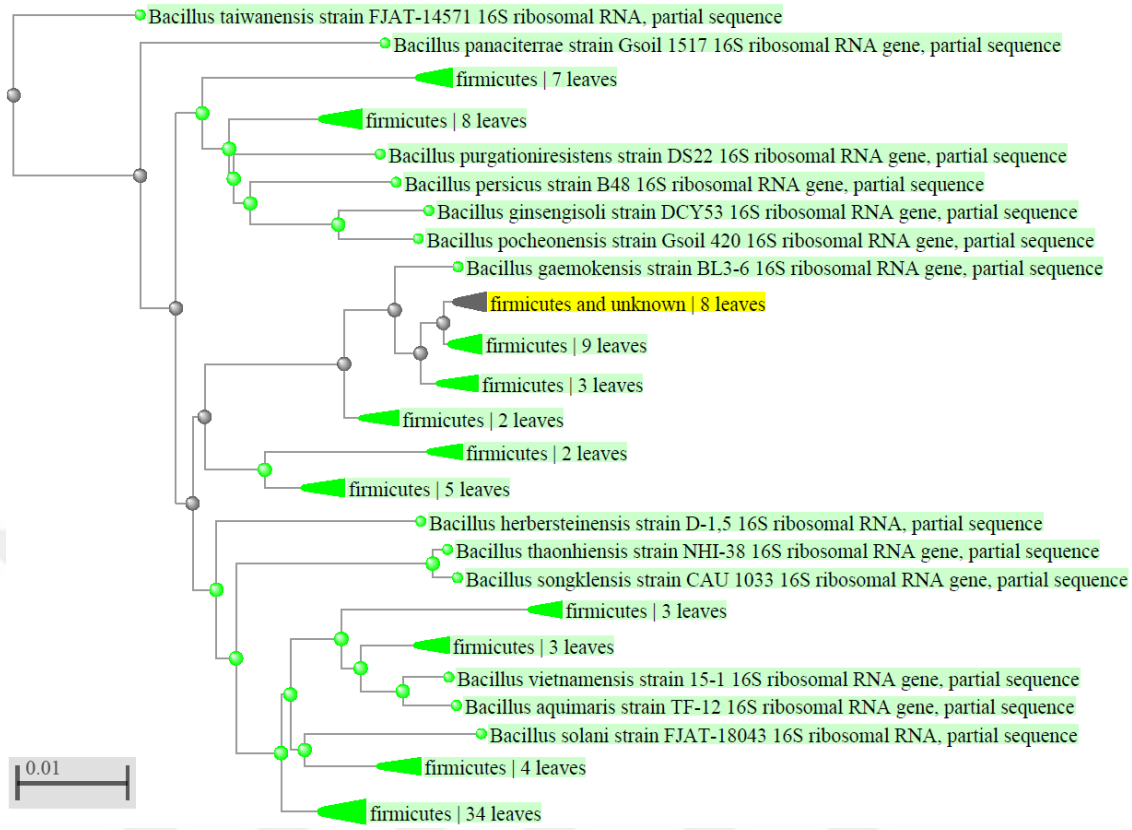
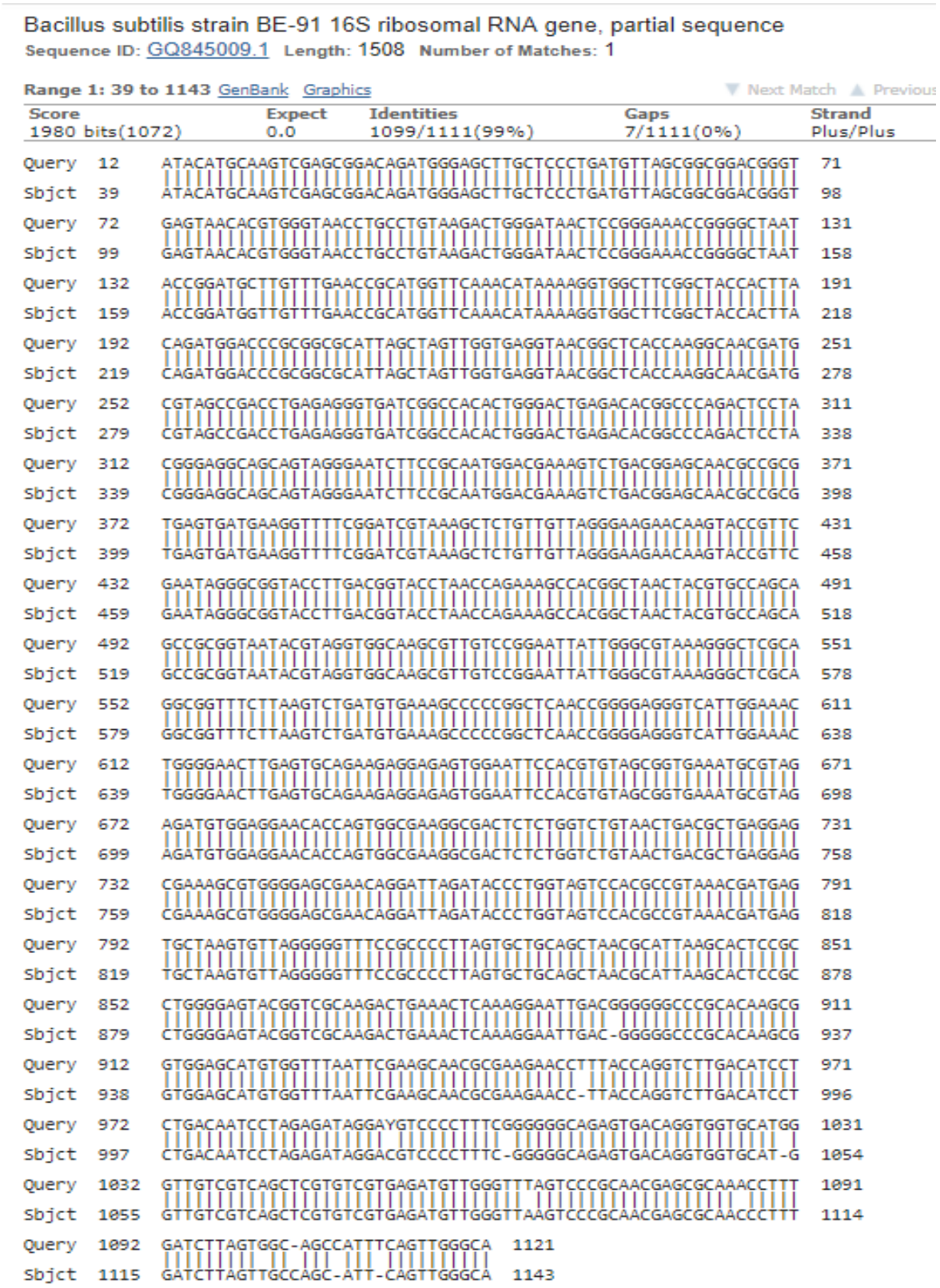


Figure 14. Phylogenetic relationships of KH14.2 obtained from the alignment of the 1129 bp of 16S rDNA region.



Bacillus subtilis strain BE-91 16S ribosomal RNA gene, partial sequence

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Range 1: 39 to 1143 GenBank Graphics Next Match Previous

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Figure 15. Alignment result of KH18.2 with *Bacillus subtilis* .

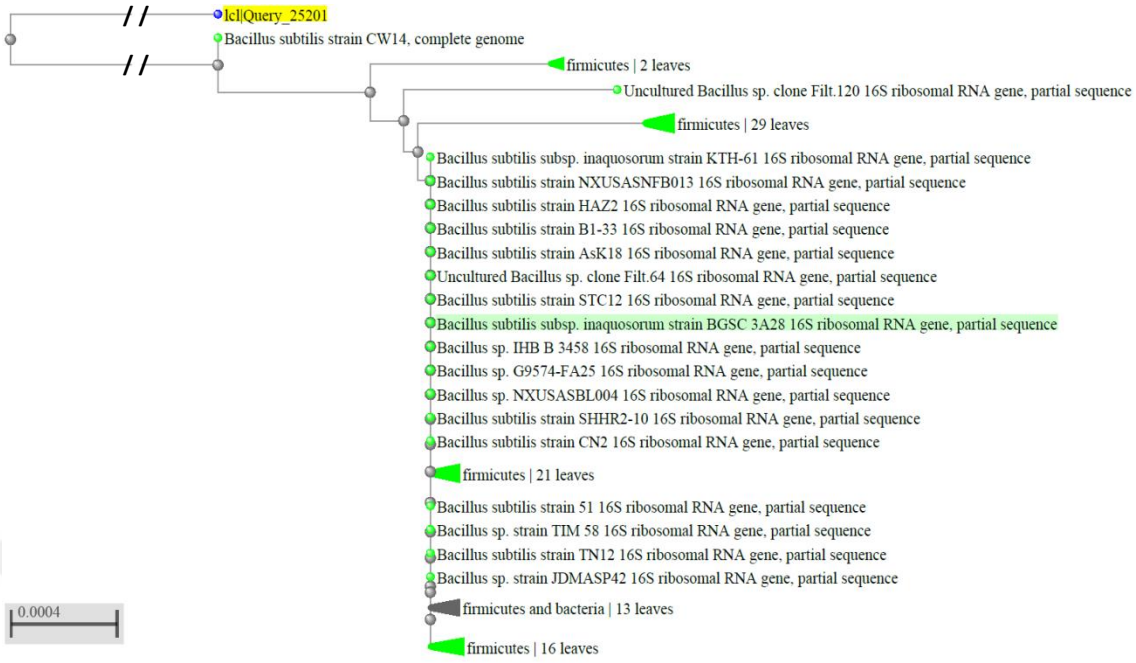

Figure 16. Phylogenetic relationships of KH18.2 obtained from the alignment of the 1143 bp of 16S rDNA region.

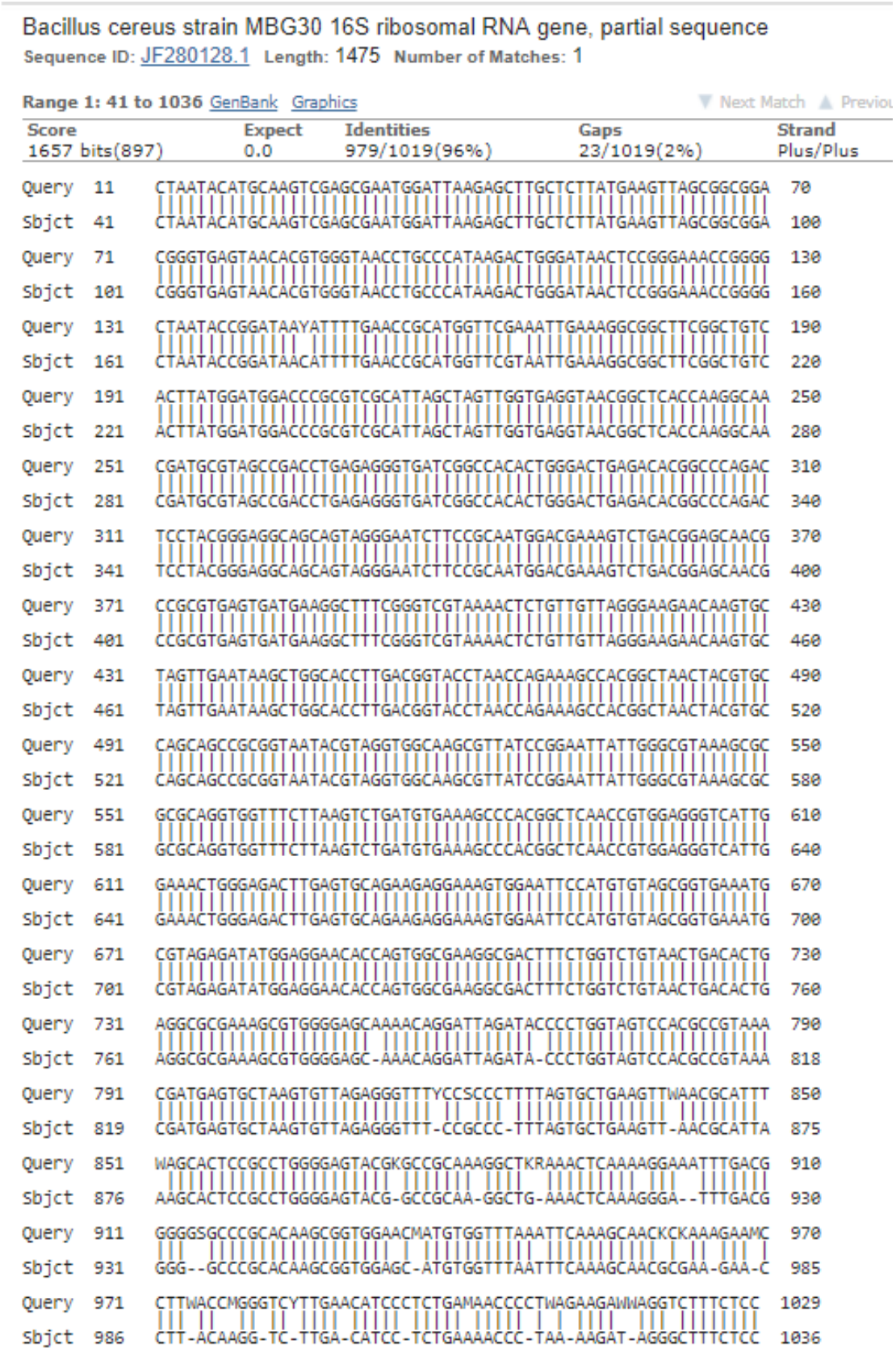

Figure 17. Alignment result of KH21.4 with *Bacillus cereus* .

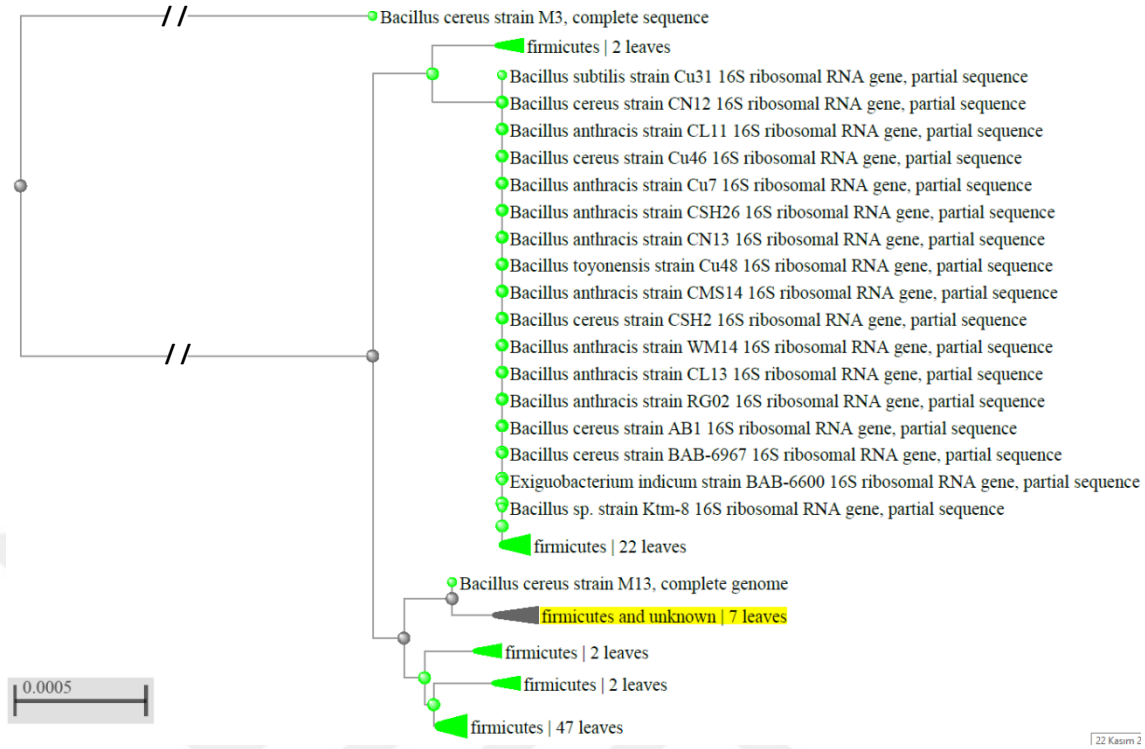

Figure 18. Phylogenetic relationships of KH21.4 obtained from the alignment of the 1031 bp of 16S rDNA region.

Bacillus simplex strain Qtx-12 16S ribosomal RNA gene, partial sequence

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Range 1: 12 to 1121 GenBank Graphics Next Match Previo

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Figure 19. Alignment result of SY29.1 with *Bacillus simplex* .

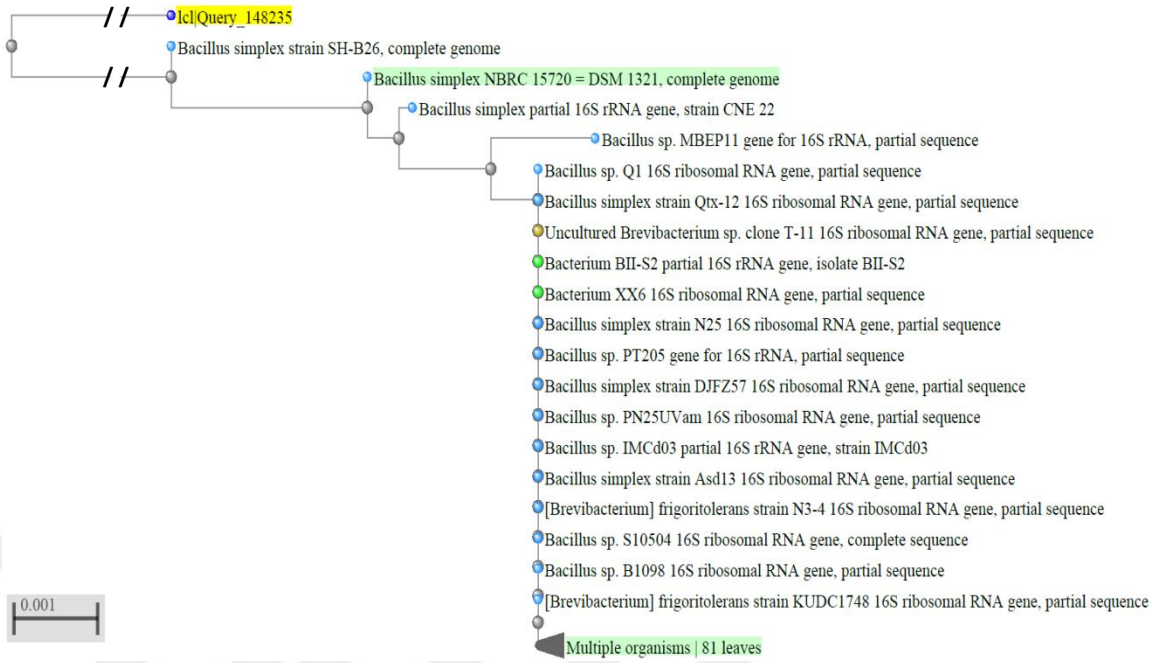

Figure 20. Phylogenetic relationships of SY29.1 obtained from the alignment of the 1139 bp of 16S rDNA region.

3.2. Pot Trials

3.2.1. Seedling inoculation tests

All bacterial isolates indicated significant growth promotion in plant inoculation trials of corn plant in terms of important parameters as stem diameter (F=4.812; p=0,000), length (F=2,507; p=0,002), shoot fresh weight (F=6.091; p=0,000) and dry weight (F=5.248; p=0,000) (Table 3.1,3.2). Also the results obtained from root measurements were significant when considering root diameter (F=3.639; p=0.000), root length (F=3.119; p=0.001), root volume (F=7.275; p=0.000), root fresh weight (F=4.378; p=0.000), and root dry weight (F=4.432; p=0.000) (Table 3.2, 3.3). For example our isolates SY29.1, KH6.2, and KH18.2 resulted in 59%, 58%, and 56% increase in stem diameter just above the soil surface, respectively. Isolates SY29.1, KH28.1, and KH6.2 seem to be promising for increasing shoot fresh weight at a level of 156%, 136%, and 130%, respectively. On the other hand, SY29.1 and KH13.3 caused 267%, and 236% in dry weight of shoot parts. Likewise, in a study carried out by Mohamed, and Gomaa (2012) [101]. *Bacillus subtilis* caused a significant increase in fresh and dry masses of roots and leaves in radish plant through increasing photosynthetic pigments, proline,

total free amino acids and crude protein contents. All other values for promoting effect of bacteria are given in Table (3.1-3.3).

Table 3.1. Shoot Characteristics of Seedling Inoculation Experiment

Isolates	Diameter1 (mm)	Diameter2 (mm)	Number of leaves	Length (cm)	Fresh weight (g)	Dry weight (g)	Water contents(%)
KH2.4	7.96±0.3	8.61±0.2	6.83±0.2	77.83±1.6	28.95±1.8	6.70±0.8	76.86
SY18.2.a	7.97±0.4	9.22±0.3	7.00±0.0	80.67±2.0	34.84±2.1	6.52±0.2	81.29
KH6.1	8.19±0.4	9.24±0.4	7.00±0.3	82.75±2.4	35.45±2.4	5.27±0.4	85.13
KH6.2	8.50±0.3	9.63±0.3	7.67±0.3	78.08±1.8	37.90±1.5	6.36±0.3	83.22
KH 6.4	7.61±0.3	8.39±0.3	7.00±2.3	80.58±3.2	29.57±3.2	3.72±1.3	87.42
KH 11.1	8.31±0.4	9.34±0.4	7.00±0.0	68.95±2.3	34.60±2.9	5.47±0.7	84.19
KH12.1	7.42±0.1	8.06±0.1	7.00±0.3	79.00±1.0	30.83±0.9	4.72±0.4	84.69
KH 12.2	7.73±0.4	8.98±0.4	6.83±0.2	81.33±1.4	33.61±3.0	6.42±0.9	80.90
KH 12.3.1	7.04±0.3	8.76±0.3	6.67±0.2	79.17±2.1	30.04±2.0	4.05±0.3	86.52
KH 13.3	8.10±0.6	8.54±0.9	7.00±0.3	77.83±3.2	32.92±4.7	6.50±0.4	80.26
KH 14.2	6.68±0.4	8.04±0.2	6.83±0.3	74.83±1.3	25.06±1.2	4.21±0.3	83.20
KH 14.3	7.69±0.4	8.99±0.4	6.67±0.2	81.08±2.4	35.20±2.9	7.64±0.4	78.30
KH 16.2	7.22±0.3	8.31±0.4	6.83±0.2	77.25±0.8	28.40±1.0	4.37±0.3	84.61
KH 18.2	8.42±0.2	9.16±0.3	7.17±0.4	80.25±1.5	36.79±0.9	6.36±0.2	82.71
KH 21.4	7.92±0.3	8.81±0.4	7.17±0.2	82.50±2.6	31.94±3.6	5.42±0.1	83.03
KH 24.1.2	7.77±0.4	8.79±0.4	6.50±0.2	83.17±2.4	33.97±3.4	5.90±0.7	82.63
KH 24.1.3	7.70±0.3	8.48±0.3	6.67±0.2	81.33±1.5	31.75±2.2	4.92±0.2	84.50
KH 28.1	8.36±0.3	9.60±0.2	6.83±0.3	78.33±1.6	38.90±2.1	5.96±0.1	84.68
SY29.1	8.57±0.4	9.67±0.2	7.17±0.3	86.17±1.6	42.27±2.2	8.36±0.5	80.22
CONTROL	5.39±0.3	7.17±0.2	6.17±0.3	61.42±1.5	16.47±1.0	2.27±0.1	86.22

Table 3.2. ANOVA Table of Parameter Measured in seedling inoculation trials

ANOVA			df	Mean Square	F	Sig.
Plant inoculation	Stem Diameter	Between Groups	19	3.300	4.812	0.000
	Stem length	Between Groups	19	173.355	2.507	0.002
	Stem fresh weight	Between Groups	19	182.952	6.091	0.000
	Stem dry weight	Between Groups	19	7.184	5.248	0.000
	Root diameter	Between Groups	19	0.007	3.639	0.000
	Root length	Between Groups	19	67095231.748	3.119	0.001
	Root volume	Between Groups	19	256.081	7.275	0.000
	Root fresh weight	Between Groups	19	206.731	4.378	0.000
	Root dry weight	Between	19	0.627	4.432	0.000

Table 3.3. Root Characteristics of Seedling Inoculation Experiment

Isolates	Diameter (mm)	Length (cm)	Volume (mm^3)	Fresh weight (g)	Dry weight (g)	Water contents(%)
KH2.4	0.38±0.03	15514.36±3697	35.00±4.60	36.73±3.62	1.68±0.16	95.43
SY18.2.a	0.33±0.02	20973.63±1725	46.67±0.33	35.27±2.40	1.84±0.12	94.78
KH6.1	0.34±0.03	23908.50±1176	49.00±4.71	43.23±4.73	1.81±0.25	95.81
KH6.2	0.35±0.03	24538.90±3126	49.00±1.47	48.27±2.96	2.60±0.12	94.61
KH6.4	0.34±0.14	20364.99±8314	37.33±2.25	36.60±4.35	1.79±0.21	95.11
KH 11.1	0.27±0.01	24724.85±4651	38.33±4.25	37.20±4.33	1.53±0.17	95.89
KH12.1	0.33±0.02	18056.71±712	35.00±2.04	35.50±2.11	1.44±0.12	95.94
KH12.2	0.33±2.46	16568.02±3152	41.67±4.25	33.40±3.61	1.65±0.26	95.06
KH12.3.1	0.30±0.01	19506.13±1516	38.00±0.82	34.07±0.78	1.42±0.07	95.83
KH13.3	0.33±0.03	15292.60±1559	37.33±1.03	40.00±3.59	1.69±0.17	95.78
KH14.2	0.32±0.02	19697.93±1712	31.00±0.41	29.17±0.88	1.50±0.08	94.86
KH14.3	0.37±0.03	19687.51±1741	48.00±3.34	47.10±3.86	2.32±0.26	95.07
KH16.2	0.30±0.002	19655.07±780	30.67±0.47	32.73±1.00	1.40±0.04	95.72
KH 18.2	0.33±0.01	20622.39±1483	37.67±2.39	38.13±3.21	1.91±0.21	94.99
KH21.4	0.29±0.002	24394.99±1677	38.00±1.08	36.23±1.51	1.93±0.07	94.67
KH24.1.2	0.31±0.02	21589.03±1171	39.00±1.63	35.03±3.93	1.69±0.23	95.18
KH 24.1.3	0.34±0.01	20151.72±1165	45.00±3.54	38.77±2.29	2.01±0.05	94.82
KH28.1	0.33±0.01	18227.73±1482	39.67±2.62	41.20±1.70	2.17±0.07	94.73
SY29.1	0.32±0.02	20246.36±1526	45.33±1.84	43.70±1.82	2.17±0.15	95.03
CONTROL	0.31±0.01	15163.57±1306	26.33±0.47	22.53±0.98	1.14±0.08	94.94

3.2.2. Seed Inoculation Tests

Our bacterial isolates exhibited significant increase in seed inoculation trials of corn plant in terms of diameter (F=5.558; p=0.000), stem length (F=8.097;p=0.000), shoot fresh weight (F=5.736;p=0.000), shoot dry weight, root diameter, root length, root volume, root fresh weight (F=3.463;p=0.000), and root dry weight (Table 3.4, 3.6). For example the most promising isolates KH28.1, KH14.3 resulted in 88%, and 69% increase in shoot fresh weight compared to control, respectively. The same isolates caused approximately 85%, and 61% increase in root dry weight, respectively (Table 3.4, 3.6). All other isolates enhanced the growth at varying degrees compared to control (Table 3.4, 3.6). In general, the results of the germination test were almost identical to the results indicated as shown in the Table (3.7).

Table 3.4. Shoot Characteristics in seed inoculation Experiment

Isolates	Diameter1 (cm)	Diameter2 (cm)	Number of leaf	Length (cm)	Fresh weight (cm)	Dry weight (cm)	Water contents(%)
KH2.4	8.43±0.41	10.14±0.39	6.83±0.17	95.33±2.96	45.83±4.79	5.42±0.61	88.17
SY18.2.A	7.34±0.19	8.45±0.24	7.00±0.00	80.67±1.17	28.33±0.06	3.81±0.06	86.55
KH6.1	8.03±0.19	9.09±0.36	7.00±0.00	90.67±1.47	36.97±2.06	4.43±0.22	88.02
KH6.2	7.82±0.15	9.31±0.22	7.00±0.00	90.92±0.78	38.37±1.07	4.75±0.11	87.62
KH6.4	8.29±0.31	10.22±0.28	6.83±0.17	85.17±2.80	45.04±2.95	5.66±0.41	87.43
KH11.1	7.70±0.27	8.96±0.21	7.17±0.17	87.33±1.48	34.47±0.12	4.27±0.05	87.61
KH12.1	6.78±0.44	7.90±0.39	7.00±0.26	75.33±3.71	25.70±0.82	3.22±0.08	87.47
KH12.2	7.45±0.17	8.59±0.19	7.17±0.31	89.67±0.83	32.30±1.34	4.44±0.22	86.25
KH12.3.1	7.00±0.43	9.34±0.45	7.17±0.65	82.67±2.69	34.59±3.51	4.37±0.56	87.37
KH13.3	7.56±0.25	8.99±0.28	7.17±0.17	92.67±2.04	34.35±0.76	4.44±0.06	87.07
KH14.2	7.98±0.24	9.46±0.21	6.50±0.22	86.33±1.02	33.56±0.91	4.57±0.13	86.38
KH14.3	9.18±0.29	10.53±0.31	7.50±0.34	93.50±1.09	47.94±2.80	6.56±0.52	86.32
KH16.2	7.79±0.20	8.95±0.29	7.33±0.21	81.00±1.37	32.75±2.68	4.65±0.39	85.80
KH18.2	8.25±0.14	10.17±0.38	6.67±0.33	88.33±4.17	20.05±1.55	2.34±0.08	88.33
KH21.4	7.49±0.48	8.83±0.42	6.40±0.24	86.60±2.44	27.27±4.87	3.26±0.81	88.05
KH24.1.2	6.69±0.54	8.63±0.33	6.67±0.49	76.83±4.25	29.69±3.84	3.47±0.53	88.31
KH24.1.3	7.50±0.36	8.89±0.44	6.83±0.31	90.33±1.82	33.28±1.59	3.90±0.34	88.28
KH28.1	9.16±0.47	10.82±0.27	7.33±0.21	95.83±2.96	53.05±2.31	6.63±0.38	87.50
SY29.1	7.40±0.22	9.06±0.27	7.50±0.22	84.00±1.57	31.34±1.00	4.27±0.19	86.38
CONTROL	6.75±0.21	8.81±0.18	6.50±0.22	75.67±1.48	28.32±1.10	3.55±0.10	87.46

Table 3.5. ANOVA Table of seed inoculation Experiment

ANOVA			df	Mean Square	F	Sig.
Seed inoculation	Stem Diameter	Between Groups	19	3.184	5.558	0.000
	Stem length	Between Groups	19	232.831	8.097	0.000
	Stem fresh weight	Between Groups	19	196.268	5.736	0.000
	Stem dry weight	Between Groups	19	3.413	4.553	0.000
	Root diameter	Between Groups	19	0.006	1.776	0.063
	Root length	Between Groups	19	69218041.245	1.918	0.041
	Root volume	Between Groups	19	364.382	9.148	0.000
	Root fresh weight	Between Groups	19	182.819	3.463	0.000
	Root dry weight	Between Groups	19	0,846	2,611	0,005

Table 3.6. Root Characteristics of in seed inoculation Experiment

Isolates	Diameter (mm)	Length (cm)	water.vol (mm^3)	Fresh weight (g)	Dry weight (g)	Water contents (%)
KH2.4	0.33±0.02	21753.66±1254	50.67±3.32	44.20±2.12	2.57±0.27	94.19
SY18.2.A	0.29±0.01	26402.96±2519	52.00±2.16	48.70±2.33	1.94±0.06	96.02
KH6.1	0.29±0.01	21974.53±797	31.67±1.18	35.40±1.13	1.80±0.07	94.92
KH6.2	0.30±0.02	22990.88±2335	40.00±3.67	41.03±1.72	2.20±0.13	94.64
KH6.4	0.30±0.01	30808.16±2487	61.33±2.90	55.03±3.07	3.43±0.35	93.77
KH11.1	0.32±0.01	20747.70±1161	41.67±2.25	40.23±0.21	2.34±0.21	94.18
KH12.1	0.32±0.01	18546.69±1776	27.67±0.24	34.40±0.54	1.78±0.06	94.83
KH12.2	0.30±0.00	26451.56±1686	48.67±0.24	46.07±0.67	2.24±0.21	95.14
KH12.3.1	0.32±0.01	21264.17±2863	47.33±3.17	43.13±4.47	2.45±0.37	94.32
KH13.3	0.28±0.01	26788.57±2980	46.00±1.47	40.03±0.81	2.36±0.09	94.10
KH14.2	0.31±0.01	23239.62±2073	48.67±0.47	40.03±0.80	2.34±0.08	94.15
KH14.3	0.49±0.09	20544.20±4574	49.67±0.62	62.07±1.77	3.50±0.05	94.36
KH16.2	0.30±0.00	30339.08±2321	57.00±2.55	51.10±4.06	2.63±0.36	94.85
KH18.2	0.31±0.00	10638.76±1041	22.00±3.54	19.40±1.96	0.87±0.13	95.52
KH21.4	0.29±0.02	16460.04±3987	25.00±4.26	30.00±7.84	1.89±0.38	93.70
KH24.1.2	0.32±0.01	17318.35±2092	41.00±3.89	35.67±5.45	1.79±0.32	94.98
KH24.1.3	0.90±0.02	66441.12±4190	41.00±3.94	39.63±4.42	1.96±0.28	95.05
KH28.1	0.38±0.02	21629.46±3181	44.00±0.82	51.97±2.25	2.96±0.05	94.30
Sy29.1	0.31±0.01	22198.02±3013	54.67±2.62	42.43±2.22	2.44±0.00	94.25
CONTROL	0.32±0.01	16655.29±2936	28.33±0.62	31.40±1.77	2.14±0.25	93.18

Table 3.7. Results of corn seed in germination test

Isolates	Root length (cm)	Shoot length (cm)	Number of lateral roots	Weight (g)
KH2.4	5.65	5.18	11.97	3.39
SY18.2.a	8.50	5.62	10.67	3.66
KH6.1	11.68	7.68	18.73	4.11
KH6.2	9.73	5.78	8.20	3.77
KH 6.4	8.06	4.46	8.00	3.17
KH 11.1	6.73	4.42	9.57	3.09
KH12.1	8.52	4.20	10.03	3.38
KH 12.2	8.99	5.18	6.03	3.69
KH 12.3.1	3.73	3.17	7.73	3.11
KH 13.3	9.80	5.71	9.17	3.91
KH 14.2	8.02	3.90	7.47	3.38
KH 14.3	10.23	6.00	11.33	3.72
KH 16.2	6.27	4.87	10.77	3.29
KH 18.2	4.00	3.86	7.00	2.96
KH 21.4	7.22	4.95	7.37	3.29
KH 24.1.2	6.73	4.81	9.59	3.63
KH 24.1.3	4.58	5.17	8.17	3.85
KH 28.1	6.93	4.42	9.48	2.95
SY29.1	7.65	3.32	7.93	3.22
CONTROL	8.70	4.20	7.53	3.48

3.3. Bacterial cell concentration Calculation

Bacterial isolates were grown in liquid LB medium at 30°C and 200 rpm for overnight and their concentrations were calculated through measuring the OD_{600} values. 1 OD value was considered as $5x10^8$ to $1x10^9$ cells/ml [102]. The average number of cells/ml for all bacterial isolates were estimated to be 10^9. The cultures were diluted 10 times for obtaining 10^8 cells/ml for plant inoculation tests.

Table 3.8. Calculation of bacterial cell concentration in nutrient medium

Isolates	OD_{600}	Stock (Cells/ml)	Diluted conc. (Cells/ml)
KH2.4	0.091	3.64×10^9	7.28×10^8
SY18.2.a	0.085	3.40×10^9	6.80×10^8
KH6.1	0.081	3.24×10^9	6.48×10^8
KH6.2	0.085	3.40×10^9	6.80×10^8
KH 6.4	0.080	3.20×10^9	6.40×10^8
KH 11.1	0.080	3.20×10^9	6.40×10^8
KH12.1	0.082	3.28×10^9	6.56×10^8
KH 12.2	0.077	3.08×10^9	6.16×10^8
KH 12.3.1	0.085	3.40×10^9	6.80×10^8
KH 13.3	0.076	3.07×10^9	6.08×10^8
KH 14.2	0.083	3.32×10^9	6.64×10^8
KH 14.3	0.085	3.36×10^9	6.72×10^8
KH 16.2	0.075	3.00×10^9	6.00×10^8
KH 18.2	0.087	3.48×10^9	6.96×10^8
KH 21.4	0.083	3.32×10^9	6.64×10^8
KH 24.1.2	0.084	3.36×10^9	6.72×10^8
KH 24.1.3	0.080	3.20×10^9	6.40×10^8
KH 28.1	0.084	3.36×10^9	6.72×10^8
SY29.1	0.088	3.52×10^{90}	7.04×10^8

CONCLUSION

Species in *Bacillus*, *Azospirillum*, *Enterobacter*, *Serratia*, *Azotobacter*, *Pseudomonas*, *Arthrobacter*, *Clostridium*, and *Gluconacetobacter* were reported to have PGPR properties [103]. In the present study it was demonstrated that out of 150 *Bacillus* isolates obtained from soil samples in Iraq, 19 species carry ACC deaminase, 8 carry siderofore and 17 carry ACP genes and seem to be desirable microorganism for growth promotion in corn plant. Out of all bacterial isolates, 16S rDNA region of best-acting six were sequenced and characterised at species level as *Bacillus subtilis*, *Bacillus* sp., *Bacillus cereus*, and *Bacillus simplex*. Plant growth was promoted all applied bacterial isolates both seed and seedling inoculation test as compared to control plant. Results obtained from seed inoculation tests were better compared to seedling inoculation tests for corn plant. Pot trials in greenhouse conditions indicated that KH28.1, KH13.3, KH14.3, KH6.2 seemed to be promising strains as biofertilising inoculants for growth promotion, but the results remain to be verified in field conditions.

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