An-Najah National University

Faculty of Graduate Studies

Identification of Rhizobacterial Strains by Genotyping

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Dedication

I dedicate my thesis to my family and many friends. Firstly I dedicate this success to my mom, God rest her soul. A special feeling of gratitude to my loving dad Ahmad whose words of encouragement and push for tenacity ring in my ears. My sisters Maram, Manal and Malak, brothers Mohammad, Mohanad, Yasser and Fahed have never left my side. Aunt Rana for her love and attention. My nieces Reema and Almas for the positive energy all the time.

I also dedicate this work to my precious friends Hussam, Rana, Dalya, Mays, Muna, Ebtihaj, Bushra, Abdullah, Loay, Mousa, Ismail, Abedullraziq who have supported me through this process. I will always appreciate all they have done.

I also dedicate this thesis and give special thanks to my friends Basima and Caesar for being there for me and for helping me develop my skills.

To the taste of the most beautiful moments with my lovely people I guide this research.

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Because of their unconditional love and prayers, I have the chance to complete this thesis.

Finally, I thank all those who did not stand by me, who stood in my way and disturbed my work, without their presence I did not feel the pleasure of the research and the sweetness of the positive competition.

الإقرار

أنا الموقع أدناه، مقدم الرسالة التي تحمل العنوان:

Identification of Rhizobacterial Strains by Genotyping

أقر بأن ما شملت عليه هذه الرسالة إنما هو نتاج جهدي الخاص، باستثناء ما تم الإشارة إليه حيثما ورد، وأن هذه الرسالة ككل، وأي جزء منها لم يقدم من قبل لنيل أي درجه أو لقب علمي لدى أي مؤسسة تعليمية أو بحثية أخرى

Declaration

The work provided in this thesis, unless otherwise referenced, is the researchers own work, and has not been submitted elsewhere for any other degree or qualification.

Student's name: **اسم الطالب:**

Signature: **التوقيع:**

Date: **التاريخ:**

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List of Abbreviations

AFLP	Amplified fragment length polymorphism
Bp	Base pair
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate.
EDTA	Ethylenediaminetetraacetic acid
G	Gram
H_2O_2	Hydrogen peroxide
$H_2PO_4^{-1}$	Dihydrogen phosphate
HPO_4^{-2}	Hydrogen phosphate
L	Liter
M	Molar
$MgCl_2$	Magnesium chloride
µl	Micro liter
Ml	Mille liter
µM	Micro molar
mM	Mille molar
NaCl	Sodium Chloride
PCR	Polymerase chain reaction
RAPD	Random Amplification of Polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	Ribonucleic acid
Rpm	Round per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulfate
SDW	Sterile distilled water
˚C	Degree Celsius

Identification of Rhizobacterial Strains by Genotyping

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Abstract

Identification and classification of plant growth promoting rhizobacteria (PGPR) is needed because theses bacteria have very important and significant role in agriculture. PGPRs are considered as the best substitution for chemical fertilizers. By their different mechanisms, PGPR can increase the plant growth rate and crop yield. There were different methods used in bacterial characterization, mainly based on histochemical properties. Molecular tools were also used recently including phenotyping and genotyping methods. One of the most useful genotyping techniques is polymerase chain reaction (PCR). Based on 16S ribosomal RNA gene analysis bacterial strains could be identified and classified. About 21 samples were collected from different sites in Nablus district. After serial dilution 7 different colonies were subjected for histochemical characterization. Four different defined strains of *Brevibacillus formosus, Brevibacillus agri, Staphylococcus aureus* and *Escherichia coli*, in addition to two unknown isolates (numbered as Clone 3 and Clone 5) were chosen for molecular identification and genotyping.

DNA extraction was done by simple boiling method; which was tested for its efficacy and simplicity. Two pairs of primers (27F and 1492R) were

chosen after several trials to be able to amplify the 16S ribosomal RNA genes of most rhizobacteria. The results were showed that the sequenced portion of the rRNA gene were able to identify the bacterial genus and putatively species using the available Bioinformatics tools such as BLAST and phylogenetic tree. By this method we were able to identify the two clones that were isolated from rhizospheric zones of plant reeds in Nablus district. These clones were identified as *Planomicrobium sp.* and *Pseudomonas sp.* for Clone 3 and Clone 5 respectively. These two isolates are indeed has PGPR's activities. This would be considered as the first Palestinian isolates to be nominated and has PGPRs activity. The study recommend to continue screening the other isolates and do further molecular identification of these isolates. It was also worth to recommend testing these isolates and to measure their capability in promoting plant growth as well as their antagonist activity against soil borne pathogens.

Key words: plant growth promoting rhizobacteria, phenotyping, genotyping, 16S rRNA gene, PCR.

CHAPTER ONE
INTRODUCTION

1.1 Introduction

Intensive farming practices with high yield demand continuous implementation of chemical fertilizers. During recent years the price of these fertilizers jumped up several folds which makes them limiting factors for crop production, in addition of their negative effects on agro-ecosystem (Zahid *et al*., 2015).

Plant growth promoting rhizobacteria (PGPR) were introduced to be biofertilizers as a sustainable option to increase nutrient availability, plant growth, and yield, and inhibit phytopathogens activity (Zahid *et al*., 2015; Islam *et al*., 2016).

During last years introduction of micro organisms in agriculture has increased due to successful results in using PGPR species on different crops both in laboratory and in the field under variable conditions (Zahid *et al*., 2015).

Strains of *Pseudomonas*, *Bacillus*, *Azospirillum*, *Enterobacteria* and *Stenotrophomonas* have been successfully used to increase plant growth rate and control phytopathogens (Islam *et al*., 2016).

Plant Growth Promoting Rhizobacteria (PGPR) are considered as the most vital microorganisms that interact with many plant species in the rhizosphere, a region referred to as a biological active zone of soil where microorganisms and plant roots interact (Bumunang and Babalola, 2014; Kaur and Sharma, 2013).

In general, PGPR can be classified according to their inhabiting site into extracellular plant growth promoting rhizobacteria (ePGPR) and intracellular plant growth promoting rhizobacteria (iPGPR) (Bumunang and Babalola, 2014; Shailendra Singh, 2015; Ahemad and Kibret, 2014). The ePGPR may exist in the rhizosphere, or in the spaces between cells of the root cortex while the iPGPR locates inside root cells, generally inside specialized nodular structures. The bacterial genera such as *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Micrococcus*, *Pseudomonas* and *Serratia* belong to ePGPR. Some examples of iPGPR are *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium* of the family Rhizobiaceae (Bhattacharyya and Jha, 2011).

Identification and classification of microorganisms are important in the field of environmental, industrial, medical and agricultural microbiology. Before the appearance and development of molecular science, microorganisms were characterized by the biochemical methods according to their morphological, physiological and cultural characteristics. There are several methods to identified the different types of bacteria: isolation in pure form, staining reaction, morphology of bacterial colony, cultural characteristics, metabolism and biochemical properties. These traditional methods encounter challenges and shortcomings which led to the development of genotyping methods based on DNA (Fakruddin *et al*., 2013).

Pulsed field gel electrophoresis is a molecular fingerprinting technique that is used to establish the degree of genetic relatedness between isolates of the

same species. It can be classified as a form of RFLP in which restriction enzymes are used to digest the genomic DNA into smaller fragments with wide range of sizes. These fragments are separated using specialized gel electrophoresis alternate polarity electrical field (Félix *et al*., 2012).

There are other types of molecular typing techniques which considered as PCR-based typing methods such as AFLP, RFLP, RAPD and many others. Amplified Fragment Length Polymorphism (AFLP) is a PCR-based technique based on the selective amplification of restriction fragments from the total genomic DNA digest (Lin, 1996).

The most efficient method commonly used in molecular characterization of PGPR is polymerase chain reaction (PCR) and it's subtypes methods. PCR is a rapid and inexpensive tool depends on the amplification of specific segments of DNA using synthetic oligonucleotides primers with suitable program.

In this study we decided to use PCR with specific primers to amplify rRNA gene which is a conserved gene in bacteria, that may help us to distinguish between different strains of rhizobacteria.

1.2 Aims of the Thesis

The main aim of this research was to study the applicability of using sequencing technique (genotyping) for quick differentiation among strains of different *Rhizobacterial* isolates. This would be very helpful for quick determination of the isolates from soil, with less labor and little costs.

CHAPTER TWO

LITRATURE REVIEW

2.1 Plant Growth Promoting Rhizobacteria

Plant growth promoting rhizobacteria are the rhizospheric bacteria that stimulate plant growth and suppress plant diseases. Rhizosphere is a zone between root surface and soil adjacent to the root where very important and intensive microbe-plant interactions take place (Rahman and Nasrin, 2009; Dardanelli *et al.*, 2010). PGPR may remain in the soil that adheres to the roots or live freely in the soil. In recent years great attention has been paid to PGPR to replace agrochemicals for the plant growth promotion by different mechanisms to produce various substances either directly or indirectly (Aryal, 2015).

2.2 The Benefits of Plant Growth Promoting Rhizobacteria

Rhizobacteria benefit plants by several ways including the stimulation of growth suppression plant diseases, and excretion antibiotic substances, thereby protecting the roots from phytopathogens by the toxic effects (Rahman and Nasrin, 2009). In general, PGPR promote plant growth directly by either facilitating resource acquisition (nitrogen fixation, phosphate solubilization, siderophore production and iron solubilization), or modulating phytohormones levels (Ahemad and Kibret, 2014). The indirect promotion of plant growth occurs when the bacteria are acting as biocontrol agents by decreasing or preventing the inhibitory effects of phytopathogens by one or more mechanism (Penrose and Glick, 2003).

2.2.1 Biological Nitrogen Fixation

Nitrogen is one of the most essential element in plant growth and development. It represents about 80% of the atmosphere as N_2 gas form. Nevertheless plants cannot fix the atmospheric N_2 which lead farmers to use N-fertilizers. These fertilizers are expensive and cause contamination for soil and ground water. Some micro-organisms are capable to convert atmospheric N_2 to stable NH_3 through a process called biological nitrogen fixation (BNF). These free-living and symbiotic bacteria encode nitrogenase enzyme complex that catalyze the fixation of N_2 gas to ammonia in the rhizosphere of plants. They are involved in endosymbiotic interactions and nodules formation (Santi, Bogusz and Franche, 2013).

Nitrogenase complex is a two-component metallo enzyme consists of:

(1) Dinitrogenase reductase (iron protein) provides high reducing power electrons which be used to reduce N_2 to NH_3, and

(2) Dinitrogenase has metal cofactor (Kuan *et al.*, 2016).

2.2.2 Phosphorus Solubilization

Phosphorus (P) is one of the most essential nutrients for plants which influence various metabolic processes in plants such as cell division, signal transduction, photosynthesis and respiration.

The availability of soluble form of P in soil is low relative to the total soil P because it is almost found in insoluble forms (organic and inorganic forms)

which usually associated with other minerals such as aluminum (Al), iron (Fe) and calcium (Ca), and it vary from soil to soil depending on soil pH. Of the several forms of P in soil, plants absorb only negatively charged primary and secondary ions ($H2PO4^{-1}$ and $HPO4^{-2}$) as nutrients. Therefore, P deficiency shows deferent symptoms on plants such as inhibition of flowering and root system development, dwarf plant and dark leaves. To overcome P deficiency in soil and to provide plants with their P requirement phosphatic fertilizers are used, which contain several forms of P (soluble, organic and inorganic P). these frequent fertilizers are costly and environmentally undesirable because they react strongly with soil contents, hence, we need cheap and ecologically safe solution using micro-organisms. Microbes can convert the inaccessible forms of P into soluble forms through immobilization reaction. Mineralization is the reverse reaction of immobilization and they occur at the same time in soil affected with soil composition and microbes characteristics.

Solubilization depends on some organic acids secreted by rhizosphere micro flora in the soil that chelating P minerals or lowering soil pH and then releasing P ions in the solution (Khan, Zaidi and Ahmad, 2014; Shahab, Ahmed and S. Khan, 2009).

2.2.3 Production of Phytohormones

In general, plants and PGPR produce some compounds which can be categorized according to their chemical structure and their role in rhizosphere for five categories: (1) auxins, (2) gibberellins, (3) cytokinins,

(4) ethylene and (5) inhibitors including abscisic acid (ABA), phenolics and alkaloids.

Indol acetic acid (IAA) is the main auxin which considered as the most active hormone. Rhizospheric microbes synthesize and secrete auxins in the rhizosphere as secondary metabolites that control several processes in plants such as cell enlargement, tissue differentiation, root initiation and development, nitrogen fixation and plant response to light and gravity.

Microbes synthesize IAA in one of three pathways:

(1) Using indole-3-pyruvic acid and indole-3-acetic aldehyde as precursors.

(2) Via conversion of tryptophan into indole-3-acetic aldehyde.

(3) Via indole-3-acetamide formation.

Indole acetic acid is produced by rhizobacteria affects nodule formation process and help in N fixation and absorption (Khan, Zaidi and Ahmad, 2014).

2.2.4 Rhizobacteria as Biocontrol Agents

Bacteria that reduce the severity of plant diseases are defined as biocontrol agents while those who show antagonistic activity toward pathogens are called antagonists. These bacterial antagonistic activities can be categorized for four sections:

(1) hydrolytic enzymes synthesis includes lipases, proteases, chitinases and glucanases.

(2) competition with phytopathogens for their ecological niche at the root surface.

(3) production of ACC-deaminase enzyme, which can regulate plant ethylene levels.

(4) siderophores and iron chelating and antibiotics production (Beneduzi, Ambrosini and Passaglia, 2012).

2.3 Biochemical Identification

The first step is to isolate the bacteria from the soil and roots in broth media using serial dilution and culturing on selective media to get different single colonies and record their morphology. After that, each single colony needs to be sub-cultured and identified through many biochemical tests.

Simple staining methods bring out the best morphology, size and arrangement. In Simple stains divide into two groups: (1) basic stains which color bacterial cells themselves such as crystal violet, methylene blue and safranin. (2) acidic stains which color the background surrounding the bacterial cells include congo red, india ink and nigrosin. Differential stains are used to bring out much more characteristics such as cell membrane components, flagella, capsule and others, include gram stain and acid-fast stain (Acharya, 2013).

For complete identification of bacterial isolates we need to combine the cultural tests with biochemical tests. Biochemical tests evaluate the metabolic properties of bacteria. These tests include Catalase test, lactose

utilization, coagulase test, oxidase test, urease test, citrate utilization and SIM test (production of hydrogen sulfide, indole and motility).

2.4 Identification of PGPR Using Molecular Fingerprinting

Polymerase chain reaction (PCR) is one of the most effective molecular techniques used in PGPR identification depending on ribosomal RNA genes amplification. Ribosomal RNA genes are used in classification and identification of bacteria because they are universal genes in all bacteria, they have highly conserved and variable regions and develop slowly with random mutations can be correlated with evolutionary distances between species. In general, rRNA genes are more difficult to align than other genes (Clarridge, 2004).

Some researches based on the amplification of 16S rRNA genes, while others based on internal transcribed spacer (ITS), a region located between the small 16S and the large 23S rRNA genes.

Chapter Three

Materials and Methods

3.1 Isolation and Histochemical characterization of Rhizobacteria

3.1.1 Isolation of Bacteria

Firstly, rhizospheric bacteria were collected out of soil surrounding the reeds roots. About 21 samples were collected from different sites in Nablus district to be subjected for bacterial identification.

For isolation of bacteria, small amount of soil and few pieces of root were aseptically transferred into 5 ml Trypticase Soy Broth (TSB) tubes, mixed well to suspend any organisms from the soil and root into the broth, then tubes were let at 25ºC (room temperature) for about 30 minutes until the most of the soil particles and the root pieces have settled. After that, upper liquid broth was serially diluted four times, and 50 µl aliquots from the last dilution were spread on Trypticase Soy Agar (TSA) plates and incubated for 48 hours at 37ºC. Secondary cultures were prepared based on morphology. Different colonies were selected and cultured on new TSA plates and incubated at 37ºC for 48 hours. Bacterial colonies were took from secondary culture plates by sterile loops and cultured in Trypticase Soy Broth (TSB) tubes before tubes were incubated at 37ºC for 48hrs .

3.1.2 Identification of Bacterial Isolates by Staining

Morphology, size and arrangement of bacterial cells were determined by simple staining. A drop of normal saline was placed in center of slide. By sterile inoculating loop a very small amount of culture was picked up,

mixed into the saline drop and spread out to about 1/2 inch area. After that the slide was passed over the flame of bunsen burner for heat-fixation and killing the bacteria. Then smear was covered with crystal violet for 20 to 60 seconds. After that; it was washed by distilled water to remove excess stain. Before washing, smear was blotted with paper towel and let dry, then, stained smear was examined microscopically.

To determine if our bacteria were gram positive or gram negative, Gram stain was tested. Briefly, bacterial smears were prepared and flooded with crystal violet for one minute. Then washed off with tap water and decolorized with acetone-alcohol until no more color washes off. After that it was washed off with tap water, before safranin was applied for one minute. Then it was washed off with tap water. Smears were blotted with a paper towel and let it dry to be examined microscopically.

3.1.3 Physiological Characteristics of Bacterial Isolates

Growth on selective and differential media, bacterial isolates were cultured on nutrient agar, mannitol salt agar and high- salt (7.5%) agar (which were prepared by dissolving 20g of agar powder and 30g of Trypticase Soy Broth powder and 75g of NaCl in 1L of sterile distilled water (SDW) plates, and incubated at 37˚C for 48 hours.

To determine the optimum temperature, bacterial isolates were cultured on TSA plates and incubated at 25˚C, 37˚C and 55˚C for 48 hours.

To determine osmotic effects, isolates were cultured on TSA media with different concentrations of NaCl (0.5%, 5% and 20% NaCl) and incubated at 30˚C for 48 hours.

To determine oxidase activity, small amount of bacteria was took by sterile loop and smeared on oxidase card, color was checked after 20 seconds. For Catalase activity detection, small amount of bacterial colony was transferred to a surface of clean glass slide with a drop of H_2O_2 and mixed, then observed if there are bubbles or not.

3.2 Optimization of DNA Isolation Techniques

Five different defined strains of *Brevibacillus formosus, Brevibacillus agri, Staphylococcus aureus, Pseudomonas putida* and *Escherichia coli*, in addition to two unknown isolates (numbered 3 and 5) were chosen for molecular identification and genotyping.

Two methods of extraction were tested and compared for the optimization and recommendation of the best results obtained. These: DNA extraction methods were by using either Phenol/Chloroform or boiling methods

3.2.1 DNA Extraction Using Phenol/Chloroform Method

1.5 ml of bacterial fresh culture were transferred to 1.5 ml eppendorf tube and centrifuged at max speed for 1 minute then the broth was discarded. The pellet was resuspended in 600 µl of lysis buffer (0.05 M Tris, 0.01 M EDTA, 4% SDS, pH 12.45) and vortexed until completely resuspending. 5 µl of 0.05 M RNase were added to each tube and mixed by inverting, then

kept in 37˚C incubator for 1 hour. Equal volume of phenol\chloroform was added and mixed well by inverting the tube without vortex and centrifuged at max speed for 10 minutes, the upper aqueous phase was transferred to a new tube. This step was repeated until the white protein layer disappears. To remove phenol equal volume of chloroform was added to the aqueous layer, mixed well by inverting and then centrifuged at max speed for 5 minutes and again the aqueous was removed. 2.5 volumes of cold absolute ethanol were added to the aqueous layer and mixed gently then incubated at -20˚C for 30 minutes (the longer the better), after incubation tubes centrifuged at max speed for 15 minutes, the supernatant was discarded. The pellet was washed with 1ml of 70% ethanol at room temperature and centrifuged at max speed for 2 minutes, the supernatant was discarded and the pellet was air-dried by inverting on a tissue paper for 15 minutes. The DNA pellet was resuspended in 50 µl TE buffer (0.05 M Tris, 0.01 M EDTA, pH 8.0).

3.2.2 DNA Extraction by Boiling Method

Pure colonies from fresh culture species were suspended in 1 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the mixture was briefly mixed on a vortex mixer. The suspension was centrifuge at 11,500 Xg for 5 min then discards supernatant. 400 µl of sterile distilled H_2O was added into precipitant, and was boiled for 10-15 min. the cells then immediately were incubated on ice for 10 min. the debris was pelleted by centrifugation at 11,500 Xg for 5 min. DNA which extracted from the supernatant was transferred to sterile eppendorf tube and stored at -20˚C until PCR testing.

3.3 Ribosomal RNA Gene Amplification

Primers from literatures were selected and proved on their ability to discriminate the bacterial strains. The following primers were applied for amplifying the rRNA gene of the bacteria: 27 F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R(5'-TACGGTTACCTTGTTACGACTT-3') primers (Frank *et al*., 2008).

Because they are universal genes in all bacteria; rRNA gene was chosen for genotyping of the selected bacteria. For achieving that, DNA fragments were amplified in a PCR thermocycler using the previously published primers.

DNA fragments were amplified in a PCR thermocycler using 27 F and 1492 R primers using 5X Green GoTaq reaction mixture with final volume of 50 µl containing 0.2 mM of dNTPs, 0.625 U\µl GoTaq polymerase, 1 µl DNA, 4 mM $MgCl_2$, 0.5 µM of each primer.

PCR reaction was performed as initial denaturation at 94 ˚C/3 min, then 35 cycles of at 94˚C/45 sec, 51˚C/50 sec, 72˚C/1 min and final extension was at 72˚C for 7 min. Amplified fragments were visualized with 1.5% agarose gel electrophoresis using TBE buffer.

Another trial carried with different buffer using 10X PeQLab reaction mixture with final volume of 25 µl containing 0.4 mM of dNTPs, 1.5 mM $MgCl_2$, 0.625 U\µl polymerase, 5 µl DNA, 0.2 µM of each primer.

3.4 Sequencing and Data Analysis

DNA PCR products were sequenced by dideoxynucleotide chain termination method using 3130 Genetic Analyzer (Applied Biosystem®, Bethlehem University, Palestine). The sequencing PCR reaction was performed with primers used singly in forward and reverse reaction and BigDye® Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems®, UAS).

The rRNA sequences of two isolates (3 and 5) were compared with available rRNA sequences of the control samples in NCBI (National Center for Biotechnology Information) using BLASTn and multiple alignments were done by using Clustal Omega.

For identification of unknown sequences (e < 90%), phylogenetic trees were constructed using MEGA 7.0.26. with expected known rhizobacteria.

CHAPTER FOUR

RESULTS

4.1 Isolation and Histochemical Identification of Bacteria from Soil

Soil samples from reeds rhizospheric areas were subjected to serial dilutions, and screened on TSB media. Seven different colonies were chosen based on their morphological characteristics for more histochemical tests as shown in table 4-1

Table 4-1 Biochemical characteristics of isolated bacterial colonies and cells.

Test	**Sample**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Morphological characteristics of isolated bacterial colonies								
	Shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular
	Color	Tan	Orange	Cream	White	White	Yellow	Orange
	Size	Large	Small	Small	Large	Small	moderate	Moderate
Identification of Bacterial cells by Staining								
	Morphology and Aggregation	Diplo-cocci	tetrad	Diplo- cocci	Single bacilli	Diplo-bacilli	tetrad	Diplo-bacilli
	Gram Stain	-ve	-ve	+ve	-ve	-ve	+ve	+ve
Bacterial Growth on Differential Media								
	Nutrient Agar	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	High-salt Agar	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	Mannitol- salt Agar	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Detection of Optimum Temperature for Bacterial Growth								
	25˚C	+ve	+ve	+ve	+ve	-ve	-ve	+ve
	37˚C	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	55˚C	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Detection of Osmotic Effect on Bacterial Growth								
	0.5% NaCl	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	5% NaCl	-ve	+ve	+ve	-ve	+ve	+ve	+ve
	20% NaCl	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Oxidase and Catalase Activity Detection								
	Oxidase	-ve	-ve	-ve	+ve	-ve	-ve	-ve
	Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve

4.2 Total DNA Extraction

The best DNA extraction from bacterial isolates was obtained by using phenol\chloroform extraction method (Figure 4-1).

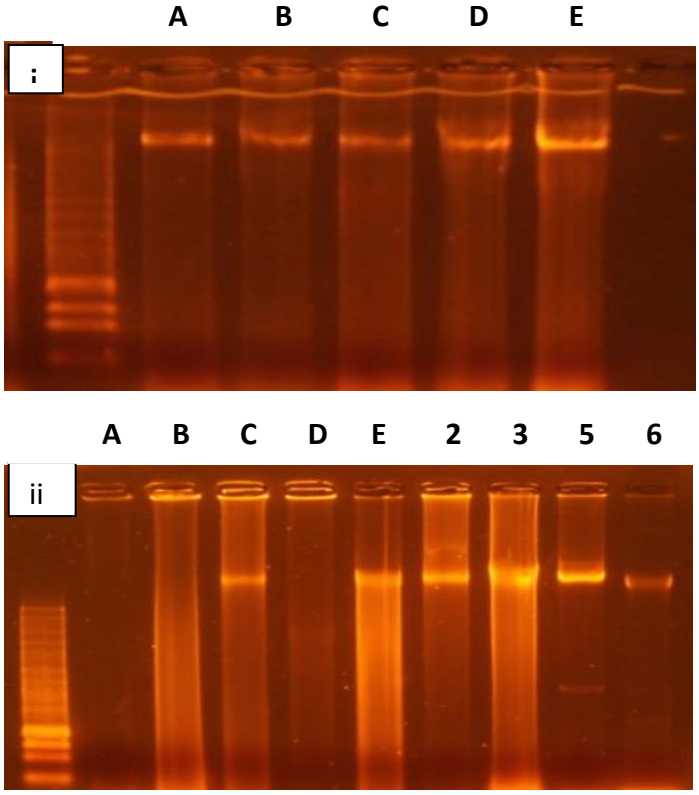

Figure 4-1. The total DNA extractions were gel electrophoresis analysis in 1% agarose and TAE buffer. The DNA was stained by GelRed (Biotium) stain. Lanes from A-E were represented the DNA extracted from known bacterial samples (**A, B, C, D** and **E**) meanwhile samples numbered as **2,3,5,6** represent DNA from soil unknown isolates. **M** referred to 100Bp DNA ladder.

Boiling methods were unable to extract DNA in quantities to be visualized, thus it was recommended to be used for PCR amplification purpose, since it is reliable and quick procedure for that purpose.

4.3 rRNA Gene Amplification

The chosen primers PCR were able to amplify rRNA of the bacteria. The expected size of the PCR product (rRNA gene) was about~1500 bp as shown in Figure 4-2.

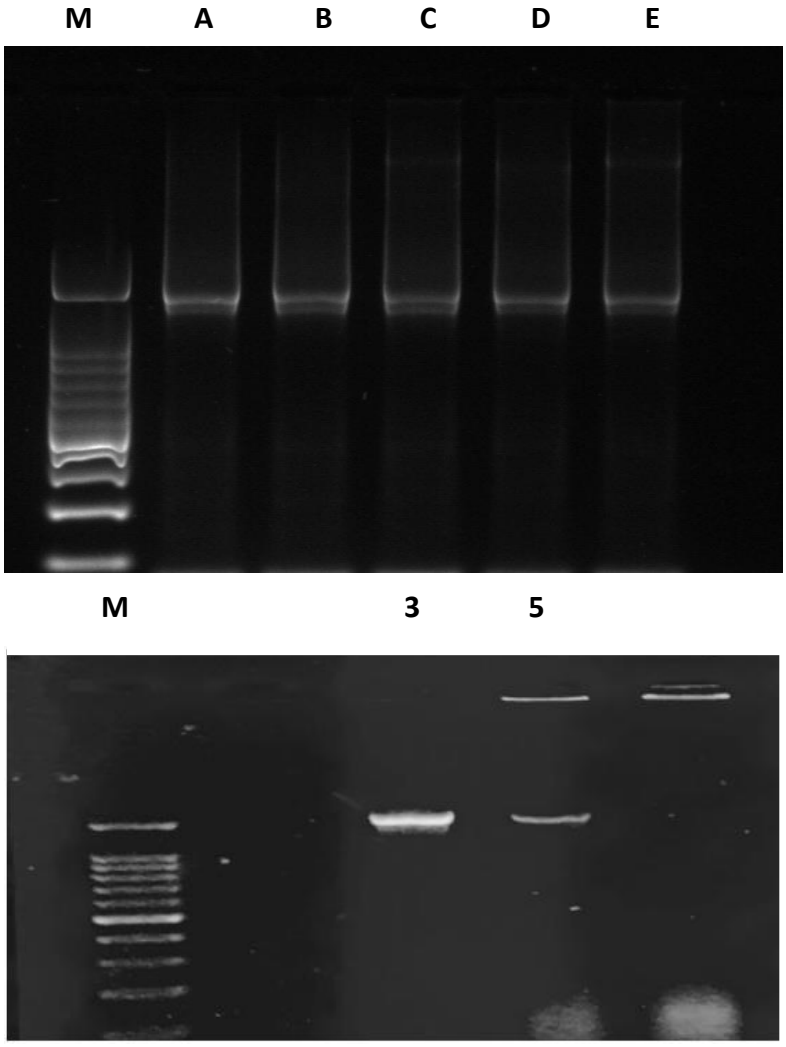

Figure 4-2. Amplified PCR products of rRNA gene for bacterial samples. Lane **M** referred to 100 bp ladder.

4.4 Sequencing and Data Analysis

The rRNA genes of the known and unknown bacteria were sequenced by using 3130 Genetic Analyzer (Applied Biosystem®, Bethlehem University, Palestine). The obtained sequencing results were shown in Figure 4-3:

```
>Clone-A
AGCAAAACGATACGCTAACCCCCGCTTCGGTCGGCTTGGCTCCTTGCGGTTACCTCACC
GACTTCGGGTGTTGCAAACTCCCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAAC
GTATTCACCGCGGCATGCTGATCCGCGTTACTAGCGATTCCGACTTCATGTAGGCGAGT
TGCAGCCTACAATCCGAACTGAGATTGGTTTTAAGAGATTGGCGTCCTCTCGCGAGGTA
GCATCCCGTTGTACCAACCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGAT
GATTTGACTCATCCCCGCCTTCCTCCGTCTTGTCGACGGCAGTCTCTCTAGAGTGCCCA
ACTGAATGCTGGCAACTAAAGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATC
TCACGACACGAGCTGACAAACAACC

>Clone-B
AAAGCTGGACAGCGTGCCTAAACATGCAAGTCGAGCGAGTCTCTTCGGGGGCTAGCGGC
GGACGGGTGAGTAACACGTAGGCAACCTGCCTCTCAGATGGGATAACATAGGGAAACTT
ATGCTAATACCGGATAGGTTTTTGGATCGCATGATCTGAAAAGAAAAGATGGCTTTTCG
CTATCACTGGGAGATGGGCCTGCGGCGCATTAGCTAGTTGGTGGGGTAACGGCCTACCA
AGGCGACGATGCGTAGCCGACCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTAGGGAATTTTCCACAATGGACGAAAGTCTGATG
GAGCAACGCCGCGTGAACGATGAAGGTCTTCGGATTGTAAAGTTCTGTTGTCAGGGACG
AACACGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTGACGAGAAAGCCACGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGG
GCGTAAAGCGCGCGCAGGCGGCTATGTAAGTCTGGTGTTAAAGCCCGGGGCTCAACCCC
GGTTCGCATCGGAAACTGTGTAGCTTGAGTGCAAAGAGGAAAAGCGGTATTCCACGGTG
TAACCGGTGA

>Clone-D
GCTCCTAATATTTGTCCCACGCTTCGGAGGAATACTCTCCTAGGAAGTTACTCCACCGG
CTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGT
ATTCACCGTAGCATGCTGATCTACGATTACTAGCGATTCCAGCTTCATGTAGTCGAGTT
GCAGACTACAATCCGAACTGAGAACAACTTTATGGGATTTGCTTGACCTCGCGGTTTCG
CTGCCCTTTGTATTGTCCATTGTAGCACGTGTGTAGCCCAAATCATAAGGGGCATGATG
ATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCAACTTAGAGTGCCCA
ACTTAATGATGGCTACTAAGCTTAAGGGTTGCGCTCGTTGCGGGACTTACCCAAACATC
ACACGACACGAGCTGACGACAACCATGCACCACCTGTCACTTTGTCCCCCGAAGGGGAA
GGCT

>Clone-E
CGTGTGTAGCTTGCCGCCGCGTTCATCTGACCGGATCAACTCTATACTTCTTTTGCAAC
CCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCCCGTGGCAT
TCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCC
GGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCGCGAGCTCGCTTCTCTTTGTATG
```

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CGCCATTGTAGCACGTGTGTAGCCCTGGTCGTAAGGGCCATGATGACTTGACGTCATCC
CCACCTTCCTCCGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCGCTGGCA
ACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCT
GACGACAGCCATGCAGCACCTGTCTCAGGGTTCCCGAAGGCACATTCTCATCTCTGAGA
AACTTTCCGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCAC
ATGCTCCACCGCTTGTGCGGGCCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCC
GTACTCCCCAGGCGGTCGACTTAACGCGTTAACTCCGGGAACCCACGCCTCAAGGGGCA
CAACCGGCAAA

>Clone-3
AAAAAAATACTGTCCACCTTCGCGGCTGGCTCCACAAGGGTTACCTCACCGACTTCGGG
TGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACC
GTGGCATGCTGATCCACGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCT
GCAATCCGAACTGAGAACGGTTTTCTGGGATTGGCTCCCCCTCGCGGGTTGGCAACCCT
TTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGAC
GTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAAT
GCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGAC
ACGAGCTGACGACAACCATGCACCACCTGTCACCGCTGTCCCCGAAGGGAAAGGCGTAT
CTCTACACCGGGCAGCGGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATT
AAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTG
CGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAA
CCCCCCT

>Clone-5
CCATTAACGGAAATACCTCCGTGGTAACCCGTCCCCCCGAAGTTAGACTAGCTACTTCT
GGAGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCA
CCGTGACATTCTGATTCACGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGA
CTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACC
CTTTGTACCGACCATTGTAGCACGTGTGTAGCCCAGGCCGTAAGGGCCATGATGACTTG
ACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCTTA
ACGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCA
CGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCAGAGTTCCCGAAGGCACCAATC
CATCTCTGGAAAGTTCTCTGCATGTCAAGGCCTGGTAAGGTTCTTCGCGTTGCTTCGAA
TTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCT
TGCGGCCGTACTCCCCAGGCGGTCGACTTAATGGCGTTAGCTGCGCCCACTAAAAATCT
CAAGGA
```

Figure 4-3: Partial rRNA gene sequences for the tested bacteria.

The sequences were reviewed and checked for their accuracy, before they were subjected to BLAST searching tools available at the NCBI website (Altschul, *et al.*, 1990;Camacho, *et al.*, 2008). The sequences had been searched in NCBI Database of nucleotide collection (ns/nt). The nucleotide collection consists of GenBank, EMBL, DDBJ, PDB and RefSeq

sequences, but excludes EST, STS, GSS, WGS, TSA, patent sequences as well as phase 0, 1, and 2 HTGS sequences. The database is non-redundant. The identical sequences were merged into one entry, while preserving the accession, GI, title and taxonomy information for each entry.

The sequences similarity search was done using (BLASTn) searching tool in order to the bacterial genus and species that had more than 95% homology score with e-value close to zero. All matches were revised and reported in the table 4-2.

Table 4-2: The predicted bacterial genus and putative species based on the BLAST similarity searching with e-value close to zero.

Clone	**Predicted Genus**	**putative species**	**Identity**	**e-value**
Clone A	***Brevibacillus***	*Formosus*	98%	0.0
Clone B	***Brevibacillus***	*Agri*	98%	0.0
Clone D	***Staphylococcus***	*Aureus*	97%	0.0
Clone E	***Escherichia***	*Coli*	98%	0.0
Clone 3	***Planomicrobium***	*Sp*	99%	0.0
Clone 5	***Pseudomonas***	*Stutzeri*	99%	0.0

Clone A; B; D; and E were found matching the expected previously identified bacteria. Clon3 and Clone5 were found belonging to *Planomicrobium* and *Pseudomonas* species; both were known to have promoting growth activities (PGPRs).

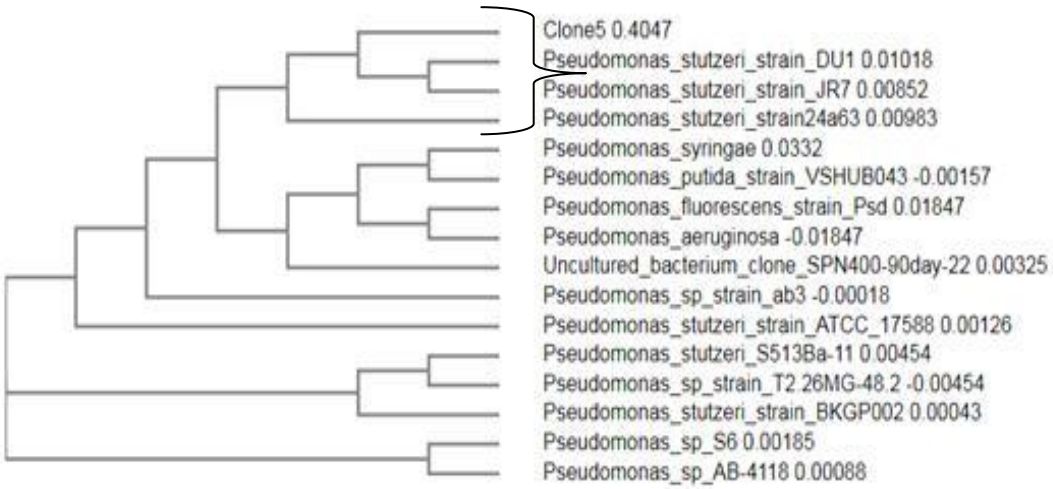

Figure 4-4. Phylogenetic tree constructed for Clone 5 on partial sequences of 16S ribosomal RNA genes of Pseudomonas strains (*P. aeruginosa, P. fluorescens, P. syringae, P. putida* strain VSHUB043, *P.stutzeri* S513Ba-11, *P. sp* AB-4118, *P. stutzeri* strain DU1, *P. stutzeri* strain 24a63, *P. stutzeri* strain JR7, *P.stutzeri strain* ATCC17588, *P. stutzeri* strain BKGP002, *P. sp* S6, P. sp strain ab3 and *P. sp* strain T2.26MG-48.2; in addition to Uncultured bacterium clone SPN400). The tree revealed that Clone 5 matches with *Pseudomonas* strains of species *stutzeri.*

Chapter Five
Discussion, Conclusion and Recommendations

5.1 Discussion

In the last decades, microorganisms were investigated intensively to be applied in agriculture, based on their capability to improve plant's growth and production (Fitriani Wangsa Putrie *et al.*, 2013). These were referred to as Plant growth promoting rhizobacteria (PGPR). Plants treated with one or more of these rhizobacteria were introduced to many crops in vitro and proved to be as biofertilizers as well as having biological control against many soil borne pathogens (Fitriani Wangsa Putrie *et al.*, 2013).

Identification and classification of microorganisms were essential in the field of environmental and agricultural applied microbiology. Biochemical method was based on bacterial morphology, physiology and cultural characteristics. This classical methods of isolation and identification of rhizospheric bacteria were laborious and time consuming. Considering the huge quantity of colonies produced in artificial culture make these methods impracticable. Looking for efficient and quick methods of identification was inevitable.

This research was built on the assumption of the availability of database for many microbes sequences that deposited in the gene banks. In fact the databases were increased exponentially in the last decades that were also available freely for researcher use (Land *et al.*, 2015).

The small subunit of ribosomal RNA was a useful phylogenetic marker that has been used extensively for evolutionary analyses. The available databases of 16S ribosomal RNA sequences that correspond to bacteria type materials can be utilized for taxonomy and identification of species

(Land *et al*., 2015). This thesis were verified that bacterial species can be identified based on the sequences of their rRNA genes. Primers from published researches (Frank *et al*., 2008) capable to amplify the bacterial rRNA were tested and the pairs of the best results were selected as the tool for amplifying the gene. These primers were 27 F and 1492 R primers. These primers were able to amplify ~ 1500bp of the bacterial rRNA gene.

Bacterial genome was extracted using home-made protocols; and the best extraction was reported. It was found that boiling extraction method was the easiest and cheapest method, besides its efficacy in PCR amplification. This was in accordance of many researches mentioned the reliability in amplifying bacterial genes (Queipo-Ortuno *et al*., 2007).

Based on the restricted capacities in sequencing technologies in Palestine; the rRNA genes were partially sequenced. This was quite enough to reveal the identity of the bacterial species as it was proved on previously identified bacterial clones (Clone D and Clone E) since they were known as *Staphylococcus aureus* and *Escherichia coli*, respectively). These were used as control of the analysis procedure. In fact the results obtained were in accordance of what was expected. This was encouraging for using the proposed method of identification of the rhizospheric bacterial isolates (Clone A; Clone B; Clone 3 and Clone 5). The last two isolates were chosen based on screening of bacterial colonies out of reeds rhizospheric area in Nablus district. This was considered as the first national attempts to identify rhizospheric isolates. By using the bioinformatics tools, it was able to identify the bacterial isolates based on the analysis of rRNA gene

analysis. The partial sequences of the rRNA gene were searched by using BLASTn and the bacterial genus was identified as sequence homology was more than 95% with e-value close to zero. This method of identification of species was commonly used for many microbes and it was efficient to determine the genus and species of the organisms (Rajendhran and Gunasekaran, 2011).

Clone 3 was identified as *Planomicrobium* sp.; which was the first encouraging result revealing the first Palestinian rhizospheric isolate that was identified in the country. *Planococcus* bacteria is included some of its member as PGPRs (Rajput *et al*., 2013).

Clone 5 was found to be homologous with *Pseudomonas* sp. which was known as a genus that had been reported in several researches as plant growth promoting genus. Even though, Pseudomonas has also pathogenic side for other organisms including animal, human and even plants.

Further investigations were built to construct phylogenetic tree to reveal the evolutionary relation with known rhizospheric *Pseudomonas* species (Clone 5) matches with strains of *P.stutzeri*; suggesting that it might belong to them.

5.2 Recommendations

This research study was recommended to use the boiling method for bacterial DNA extraction that would be used for PCR amplification. It was found to be quick; cheap; simple and safe method compared with other available ones. Meanwhile phenol /chloroform extraction method is recommended for genomic extraction of the bacteria. It could be also used for application of other genotyping technologies.

This research study was also recommended to use rRNA for identification of bacterial isolates, since the availability of sequence databases and PCR technology. Further investigations are recommended to design universal primers on other conserved genes or regions on eubacteria domain. In addition to that all; studies on the growth promoting activities and biological control capability of these two newly discovered Palestinian isolates should be conducted.

References

- Acharya, T. (2013). **Overview of Biochemical tests used to identify bacteria in Microbiology laboratory**. microbe online.

- Ahemad, M. and Kibret, M. (2014). ***Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective***. **Journal of King Saud University - Science**, 26(1), pp.1-20.

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990). ***Basic local alignment search tool*. J. Mol. Biol**. 215:403-410

- Aryal, S. (2015).**Methyl Red (MR) test-principle, procedure and result interpretation.** Online Microbiology Notes.

- Beneduzi, A., Ambrosini, A. and Passaglia, L. (2012). **Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents**. Genetics and Molecular Biology, 35(4), pp.1044–1051.

- Bhattacharyya, P. and Jha, D. (2011). ***Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture*. World Journal of Microbiology and Biotechnology**, 28(4), pp.1327-1350.

- Bumunang, E. and Babalola, O. (2014).**Characterization of Rhizobacteria from field grown Genetically Modified (GM) and non-GM maizes**. Brazilian Archives of Biology and Technology, 57(1), pp. 1-8.

- Camacho C., Coulouris G., Avagyan V., Ma N., Papadopoulos J., Bealer K., & Madden T.L. (2008) **BLAST+: architecture and applications**. BMC Bioinformatics 10:421

- Clarridge, J. (2004). **Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases**. Clinical Microbiology Reviews, 17(4), pp.840-862.

- Compant, S., Duffy, B., Nowak, J., Clement, C. and Barka, E. (2005). **Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action, and Future Prospects.** Applied and Environmental Microbiology, 71(9), pp.4951-4959.

- Dardanelli, M., Carletti, s., Paulucci, n., Medeot, d., Rodriguez Cáceres, e., Vita, f., Bueno, m., Fumero, m. and Garcia, m. (2010).**Benefits of Plant Growth-Promoting Rhizobacteria and Rhizobia in Agriculture**. springerlink, 18, pp.1-20.

- Fakruddin, M., bin mannan, K., mazumdar, R., chowdhury, A. and hossain, M. (2013).***Identification and characterization of microorganisms: DNA fingerprinting methods***. **Songklanakarin J. Sci. Technol**., 35 (4), pp. 397-404.

- Félix, b., Dao, T., Lombard, b., Brisabois, a. and Roussel, s. (2012).**The Use of Pulsed Field Gel Electrophoresis in Listeria monocytogenes Sub-Typing – Harmonization at the European Union Level**. InTech.

- Fitriani Wangsa Putrie, R., Tri Wahyudi, A., Asih Nawangsih, A. and Husen, E. (2013). **Screening of Rhizobacteria for Plant Growth Promotion and Their Tolerance to Drought Stress**. Microbiology Indonesia, 7(3), pp.94-104.

- Frank, J., Reich, C., Sharma, S., Weisbaum, J., Wilson, B. and Olsen, G. (2008). **Critical Evaluation of Two Primers Commonly Used for Amplification of Bacterial 16S rRNA Genes**. Applied and Environmental Microbiology, 74(8), pp.2461-2470.

- Islam, S., Akanda, A., Prova, A., Islam, M. and Hossain, M. (2016).**Isolation and Identification of Plant Growth Promoting Rhizobacteria from Cucumber Rhizosphere and Their Effect on Plant Growth Promotion and Disease Suppression.** Frontiers in Microbiology, 6.

- Kaur, N. and Sharma, P. (2013).***Screening and characterization of native Pseudomonas sp. as plant growth promoting rhizobacteria in chickpea (Cicerarietinum L.) rhizosphere***. **African Journal of Microbiology Research,** 7(16)(1996-0808), pp.1465-1474.

- Khan, M., Zaidi, A. and Ahmad, E. (2014).**Mechanism of Phosphate Solubilization and Physiological Functions of Phosphate-Solubilizing Microorganisms. Springer International Publishing Switzerland**, pp.31-62.

- Kuan, K., Othman, R., Abdul Rahim, K. and Shamsuddin, Z. (2016).**Plant Growth-Promoting Rhizobacteria Inoculation to Enhance Vegetative Growth, Nitrogen Fixation and Nitrogen Remobilization of Maize under Greenhouse Conditions.** PLOS ONE, 11(3), p.e0152478.

- Land, M., Hauser, L., Jun, S., Nookaew, I., Leuze, M., Ahn, T., Karpinets, T., Lund, O., Kora, G., Wassenaar, T., Poudel, S. and Ussery, D. (2015). **Insights from 20 years of bacterial genome sequencing. Functional & Integrative Genomics**. 15(2), pp.141-161.

- Lin, J. (1996). **A PCR-based DNA fingerprinting technique: AFLP for molecular typing of bacteria**. Nucleic Acids Research, 24(18), pp. 3649-3650.

- Lucas García, J., Probanza, A., Ramos, B., Barriuso, J. and Gutierrez Mañero, F. (2004). **Effects of inoculation with plant growth promoting rhizobacteria (PGPRs) and Sino Rhizobium fredii on biological nitrogen fixation, nodulation and growth of Glycine max cv**. Osumi. Plant and Soil, 267(1-2), pp.143-153.

- M., Abbasi, M., Hameed, S. and Rahim, N. (2015).**Isolation and identification of indigenous plant growth promoting rhizobacteria from Himalayan region of Kashmir and their effect on improving growth and nutrient contents of maize.** (Zea mays L.).Frontiers in Microbiology, 6.

- Penrose, D. and Glick, B. (2003).**Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria**. Physiologia Plantarum, 118(1), pp.10-15.

- Queipo-Ortuno, M., De Dios Colmenero, J., Macias, M., Bravo, M. and Morata, P. (2007). **Preparation of Bacterial DNA Template by Boiling and Effect of Immunoglobulin G as an Inhibitor in Real-Time PCR for Serum Samples from Patients with Brucellosis**. Clinical and Vaccine Immunology, 15(2), pp.293-296.

- Rahman, M. and Nasrin, S. (2009). ***Isolation and Characterization of Rhizosphere Bacteria and their Effect on Germination of Rice Seeds and Growth of Seedlings.*** **Journal of Bio-Science**, 15(0).

- Rajendhran, J. and Gunasekaran, P. (2011). **Microbial phylogeny and diversity: Small subunit ribosomal RNA sequence analysis and beyond**. Microbiological Research, 166(2), pp.99-110.

- Rajput, L., Imran, A., Mubeen, F., and Hafeez, F. Y. (2013). **Salt-tolerant PGPR strain Planococcus rifietoensis promotes the growth and yield of wheat**. (Triticumaestivum L.) cultivated in saline soil. Pak. J. Bot. 45, 1955–1962

- Santi, C., Bogusz, D. and Franche, C. (2013).**Biological nitrogen fixation in non-legume plants**. Annals of Botany, 111(5), pp.743-767.

- Shahab, S., Ahmed, N. and S. Khan, N. (2009).***Indole acetic acid production and enhanced plant growth promotion by indigenous PSBs***. **African Journal of Agricultural Research**, 4 (11)(1991-637X), pp. 1312-1316

- Shailendra Singh, G. (2015). ***Plant Growth Promoting Rhizobacteria (PGPR): Current and Future Prospects for Development of Sustainable Agriculture.*** **Journal of Microbial & Biochemical Technology**, 07(02).

- Sulieman, S. and Tran, L. (2014). ***Symbiotic Nitrogen Fixation in Legume Nodules: Metabolism and Regulatory Mechanisms***. **International Journal of Molecular Sciences,** 15(11), pp.19389-19393.

- Vessey, J. (2003). **Plant growth promoting rhizobacteria as biofertilizers**. Plant and Soil, 255(2), pp.571-586.

- Wahyudi, A. (2013). **Screening of Rhizobacteria for Plant Growth Promotion and Their Tolerance to Drought Stress**. Microbiology Indonesia. 7. 2087-8587. 10.5454/mi.7.3.2.

جامعة النجاح الوطنية

كلية الدراسات العليا

تحديد سُلالات البكتيريا الجذرية باستخدام الطرز الجينية

إعداد

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قُدّمت هذه الأطروحة استكمالاً لمتطلبات الحصول على درجة الماجستير في برنامج العلوم الحياتية، بكليّة الدراسات العليا، في جامعة النجاح الوطنية، نابلس–فلسطين

2018

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الملخّص

إن تعريف و تصنيف سلالات البكتيريا الجذرية المحفّزة لنموّ النباتات أمرٌ مهم بسبب الدور المهم و البليغ لهذه البكتيريا في الزراعة و تأثيرها على النباتات. تُعتبر البكتيريا الجذرية البديل الأفضل للأسمدة الكيماوية، حيث أنّها باستخدام آليّاتها المختلفة تستطيع زيادة و تحفيز معدّل نموّ النباتات و زيادة إنتاج المحاصيل الزراعية.هناك عدّة طرق تُستخدم لتعريف وتصنيف البكتيريا، بعضها يعتمد على الخصائص البيوكيميائية للعزلات فيما يعتمد البعض الآخر على دراسة وتحليل الطرز الجينية والتي تُعتبر من الطرق المعتمدة حديثاً. يُعتبر تفاعل البلمرة المتسلسل من أهم و أفضل التقنيات المُستعملة في دراسة الطرز الجينية للبكتيريا الجذرية و تعريفها، و يتمّ ذلك غالباً بالاعتماد على دراسة و تحليل الحمض النووي الرايبوسومي لها. تمّ جمع 21عينة من تراب و جذور النباتات من مناطق مختلفة في محافظة نابلس، و بعد التخفيف المتسلسل لها تمّ التوصّل إلى سبع عينات بكتيريّة مختلفة تمّ تعريفها و وصفها اعتماداً على الطرق التقليدية حسب خصائصها البيوكيميائية. تمّ اختيار أربع سلالات بكتيريا مختلفة معرّفة جينيّاً: *Brevibacillus formosus, Brevibacillus agri, Staphylococcus aureus* and *Escherichia coli* بالإضافة إلى عيّنتين غير معرّفتين (العيّنات 3 و 5) و تمّ تعريفها جُزيئياً و تحليل الطراز الجيني لكلٍ منها . تمّ عزل الحمض النووي من البكتيريا بطريقة الغلي والتي أثبتت فعاليّتها رغم بساطتها. بعد عدّة تجارب لتضخيم و تكثير الحمض النووي الرايبوسومي لمعظم العينات تم اعتماد زوج من البادئات المتخصصة. أظهرت النتائج أن تسلسل جين الحمض الرايبوسومي له القدرة على تعريف الأجناس والأنواع البكتيريّة المختلفة باستخدام النُظُم

المعلوماتية الحيوية المختلفة. باستخدام هذه التقنيّات تمكّننا من تعريف العينات التي تمّ عزلها من جذور النباتات في محافظة نابلس. تُعتبر هذه العيّنات من أوائل العيّنات المعزولة والمعرّفة فلسطينياً والتي تمتلك خصائص البكتيريا الجذرية. توصي هذه الدراسة بمواصلة فحص باقي العينات المعزولة من التربة الفلسطينية و تعريفها جينيّاً. و توصي بدراسة إمكانية هذه العينات وقدرتها على تحفيز نموّ النباتات و مقاومة الآفات الزراعية.

الكلمات الرئيسيّة: البكتيريا الجذرية المُحفّزة لنموّ النباتات، الأنماط الظاهرية، الطرز الجينيّة، الحمض النووي الرايبوسومي، تفاعل البلمرة المتسلسل.